HEAT STABILITY OF CONCENTRATED SKIM MILK

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HEAT STABILITY OF CONCENTRATED SKIM MILK

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Abstract

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Heat-induced coagulation of concentrated skim milk was studied. Heat-induced changes in partition between serum and colloidal particles of calcium and phosphate and of the various proteins were investigated as a function of heating time and conditions like pH, addition of phosphate and preheating. Aggregation of casein micelles upon heating was assessed by various methods, both at 120°C and after cooling to 20°C. A qualitative model for heat-induced coagulation of concentrated skim milk was proposed, which largely explained phenomena like the effects of pH, addition of phosphate and preheating. For concentrated skim milk produced from preheated milk, this model was also discussed semi-quantitatively. It is shown that the geometry of the emerging aggregates influences time of visible coagulation as much as the rate of aggregation of casein particles.

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Stellingen

1. Elke melk is moedermelk.

2. Een goed inzicht in de aggregatie van deformeerbare kolloïdale deeltjes is alleen te verkrijgen door tenminste twee meetmethoden te gebruiken, waarbij de invloed van de ruimtelijke structuur op het meetresultaat verschillend dient te zijn.
   Dit proefschrift

3. Bij het bestuderen van de hittecoagulatie van zuivelprodukten met een hoge volumefractie deeltjes is het vaststellen van de aard van het coagulum evenzeer van belang als het vaststellen van de coagulatietijd.
   Dit proefschrift

4. Kengetallen, zoals de WPN-index, hebben weinig waarde voor het karakteriseren van melkpoeders die gebruikt worden in produkten waarvoor een hoog verhit poeder vereist is, omdat het kengetal alleen aangeeft hoeveel serumeiwit niet-geassocieerd is, terwijl het aannemelijk is dat de hoeveelheid geassocieerd serumeiwit en de mate van associatie relevant zijn voor de eigenschappen van de uiteindelijke produkten.

5. De nu voor humane melk in gebruik zijnde Kjeldahlfactoren van 6,38 en 6,25 zijn, gezien de eiwitsamenstelling van deze melk, beide te hoog.

6. Het gebruik van emulgatoren bij de bereiding van gerecombineerde lang houdbare melk, zoals noodzakelijk geacht door Newstead, is alleen zinvol bij een slechte homogenisatie van het produkt.
   D.F. Newstead, p. 209-218 in: Recombination of milk and milk products. *IDF special issue* no. 9110 (1988).

7. Elektronenmicroscopie is slechts beperkt bruikbaar bij het bestuderen van nadikking en gelering van geconcentreerde melk, zoals blijkt uit het grote structuurverschil tussen gegeleerde UHT en in blik gesteriliseerde geconcentreerde melk, terwijl de microscopische structuur van de gelen vergelijkbaar is.
   O.F. Hunziker, Condensed milk and milk powder. 6th edition, Author, La Grange (1946), p. 251.
   P.J. de Koning, J. Kaper, H.S. Rollema and F.M. Driessen, Age-thinning and gelation in unconcentrated and concentrated UHT-sterilized skim milk. Effect of native milk proteinase. *Neth. Milk Dairy J.* 39 (1985) 71-87.
   P.J. de Koning, J.N. de Wit and F.M. Driessen. Process conditions affecting age-thickening and gelation of sterilized canned evaporated milk. *Neth. Milk Dairy J.* 46 (1992) 3-19.

8. Een manager met een lege agenda heeft niets te doen en een onderzoeker met een volle doet niets. Gegeven dit grote verschil in aard van het werk is het verwonderlijk dat vele mensen na het afronden van een onderzoeksgerichte opleiding, waarvoor ze min of meer bewust gekozen hebben, zeer snel een managementfunctie gaan vervullen en dit ook lijken te ambiëren.

9. Survivaltochten zijn gewild omdat overleven gegarandeerd is.

Stellingen bij het proefschrift "Heat stability of concentrated skim milk" van Hans Nieuwenhuijse, te verdedigen op 3 juni 1992.

Contents

Introduction

1 Milk

Milk is a liquid containing a great many components. It consists mainly of water (about 87% by weight); milk also contains lactose (about 4.6%), fat (about 3.5%), protein (about 3.3%) and minerals (about 0.7%). It is a system of emulsified fat and dispersed proteinaceous particles in a continuous phase containing dissolved proteins, lactose and salts. About 80% of milk protein is casein, which is, at room temperature, almost totally present in the proteinaceous particles, called casein micelles. The main dissolved or serum proteins are β-lactoglobulin and α-lactalbumin.

Casein micelles are roughly spherical, with a diameter ranging between 20 and 300 nm. Besides casein, they contain a large part of the calcium and phosphate in milk. Figure 1 shows a model for a cross-section of a casein micelle (adapted from Ref. 1). A micelle is, supposedly, built up of relatively dense small particles, the submicelles, which are held together partly by small areas of calcium phosphate. A submicelle contains about 25 casein molecules. Presumably, submicelles are not uniform in composition, all submicelles containing three of the major types of casein, α_{s1}, α_{s2}, and β-casein. By contrast, the fourth type, κ-casein, is mostly present in the outer submicelles. Casein

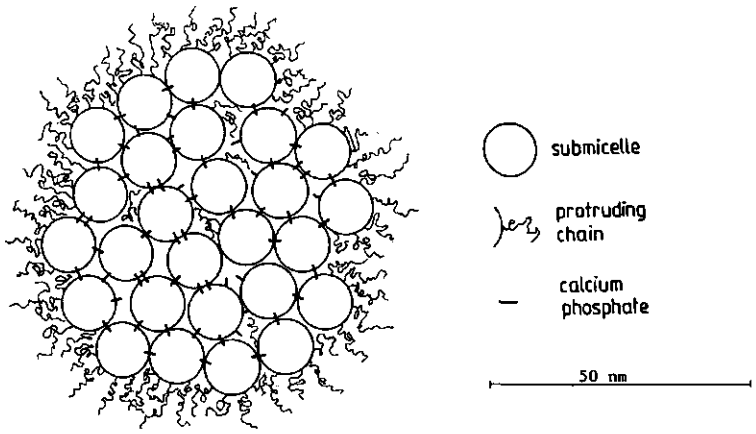

Fig. 1. Section through a casein micelle.

molecules in the casein micelles have a low mobility, except for the C-terminal part (of about 70 amino acids) of the κ-casein, which sticks out into the serum (the 'hairs' in Fig. 1). These flexible protruding chains make the micelles colloidally stable by steric repulsion. Electrostatic repulsion will also contribute to stability. How the calcium phosphate is incorporated in the micelle is still subject of some debate. Calcium phosphate is certainly essential to keep the submicelles together, but is also largely responsible for the low mobility of caseins within the submicelles. Consequently, as an alternative to the model of Figure 1, a model in which many small areas of calcium phosphate are present within the submicelles has also been proposed (Ref. 2).
Whatever their exact structure, casein micelles have a high voluminosity of about 4 ml/g casein. Thus, a mass fraction of casein of about 0.07, as in concentrated skim milk, gives a volume fraction of casein micelles of about 0.3, which is rather high for a dispersion.

2 Some effects of heating

Heating, particularly for several minutes above 100°C, induces many changes in the structure and composition of milk. A main one is a decrease in stability of casein micelles, resulting in aggregation of these particles at a measurable rate, and finally in coagulation of the milk. The resistance of milk to coagulation during heating is mostly called its heat stability. Three other heat-induced changes in milk affect the decrease in stability of casein micelles. One is association of additional calcium and phosphate with the micelles, the more so at a higher temperature. This additional calcium phosphate may have a structure different from that of the original micellar calcium phosphate whose structure may, moreover, change (Ref. 3). A second is reaction of lactose to form organic acids and other substances. This and the association of calcium and phosphate cause a pH decrease. A third change is denaturation of serum proteins and their subsequent association, either among themselves or with the casein micelles.

One last aspect when studying heat-induced changes in milk is that this is mostly done by comparing unheated and heat-treated samples at room temperature. Heating, however, may cause both reversible and irreversible changes, of which only the latter can be accurately observed at room temperature. Consequently, the conditions under which irreversible changes like heat coagulation occur are not well known, so that studies on the mechanisms of heat-induced changes are problematic.

3 Concentrated milk and its heat stability

This thesis concerns concentrated milk, i.e. milk from which about two-thirds of the water has been removed. Papers 2, 3 and 4 concern concentrated skim milk, i.e. milk from which almost all fat has been removed and which has subsequently been concentrated.

Sterilized concentrated milk has a history of just a hundred years. In 1885, it was produced for the first time by the Helvetia Milk Condensing Company, using an in-can sterilization process invented by J.B. Meyenberg. Before that time, concentrated milk was either preserved by addition of sugar or sold fresh. The sterilized product became one of the major dairy products after the 1920s; presumably, easy transport and long shelf-life presumably contributed to this commercial success.

In the early years, sterilized concentrated milk was produced simply by evaporating water from milk at reduced pressure and sterlizing the concentrate. Because creaming and coalescence of fat globules impaired the quality of such a product upon storage, homogenization of the concentrated milk before sterilization became common practice soon after the invention of the homogenizer. Sterilization of homogenized concentrated milk, however, easily yields a coagulated product, and two additional steps in manufacture are needed to control the production process, one being preheating of the milk before concentrating, for instance for 3 min at 120°C, and the other adding a stabilizer, commonly some salt of phosphoric acid.

Heat stability of concentrated milk has been the subject of numerous studies. Between 1920 and 1960, research concentrated on processing and compositional factors in the time needed to initiate visible coagulation of concentrated milk. The effects of preheating and adding certain salts became better known. The relevance of pH was revealed (Ref. 4), time of coagulation as a function of pH showing a maximum at a certain pH, mostly about 6.5. The significance of that maximum was not, however, widely recognized. From about 1960, studies on heat stability shifted from concentrated to unconcentrated milk. A working hypothesis was postulated by Rose (Ref. 5), in which he presumed that aggregation of casein micelles plays a pivotal role in heat stability of (unconcentrated) milk, and that the pH dependence of heat stability is largely due to 'changes of the micellar surface', including complex formation with β-lactoglobulin. Heat-induced changes of the casein micelles were extensively studied in the 1980s, in particular by Kudo (Ref. 6), Singh and Fox (e.g. Ref. 7) and Dalgleish at al. (Ref. 8). Especially the observation, first by Kudo (Ref. 6), that heat stability of milk was related to the strongly pH dependent association of β-lactoglobulin with and dissociation of

κ-casein from the casein micelles, served as a starting point for van Boekel et al. (Refs. 9 & 10) in proposing a semi-quantitative model for heat coagulation of skim milk. Most published observations on heat stability could be explained by this model (11).

The objective of the present study was to extend the model for unconcentrated skim milk to concentrated skim milk. Only after incorporating the effect of homogenized fat globules on heat stability in the model would it result in a viable tool for heat stability control in industrial practice. Although most observations on heat stability of homogenized concentrated milk, like those in Ref. 12, fit qualitatively into the model presented in Papers 3 and 4, however, a complete and quantitative explanation of heat stability of full cream evaporated milk cannot yet be given. Still, the model for concentrated skim milk may contribute to a better understanding of heat stability in industrial practice.

4 Outline of the thesis

The first part of this thesis concerns heat-induced changes of casein micelles and of the liquid in which they are suspended, i.e. serum of concentrated milk, each as a function of heating time. Paper 1 describes the changes induced by concentrating whole milk and by heating concentrated milk on calcium ion activity, pH, and on partition of calcium and of phosphate between serum and particles. Paper 2 describes the changes induced by concentrating skim milk and by heating concentrated milk on the partition of the various proteins. The experiments revealed the emerging of particles of different composition, according to conditions and heat-treatment. Subsequently, aggregation of casein micelles in concentrated skim milk was studied (Paper 3). Seven analytical methods were used, some at 120°C, to minimize the probability of misinterpretation of the results by any specific effect of the various composition of the particles, or of the high volume fraction of particles in concentrated skim milk, or of cooling before measurement. The results of these experiments and of those published in Papers 1 and 2 were used to extend the model for unconcentrated skim milk (Refs. 9 & 10) to concentrated skim milk. Paper 4 describes the effect of protein content on heat coagulation time of concentrated skim milk at various pH. The results of this experiment were used to discuss kinetic aspects of the proposed model semiquantitatively.

All papers were published in the Netherlands Milk and Dairy Journal.

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Calcium and phosphate partitions during the manufacture of sterilized concentrated milk and their relations to the heat stability

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Summary

The calcium and phosphate partitions during the manufacture of full cream evaporated milk were examined. Calcium and phosphate became colloidal during preheating and concentration of the milk, but scarcely during sterilization, unless sodium phosphate had been added to the concentrated milk. More phosphate than calcium became colloidal, and the Ca/P ratio in the CCP thus decreased. The results on the Ca/P ratio and its changes during processing, support the ideas of Holt that the colloidal calcium phosphate of the casein micelles has a brushite-like composition and includes the greater part of the esterified phosphate. The Ca^{2+} activity in concentrated milk was slightly lower than in milk. Sterilization resulted in a further decrease.

In addition, the Ca^{2+} activity and pH in concentrated milk after heating it for various times at 120 °C were studied. Both decreased; the pH linear except for the initial minutes, the Ca^{2+} activity for the greater part during the initial minutes. The Ca^{2+} activity remained almost constant after the initial heating period. If phosphate had been added to the concentrated milk, the Ca^{2+} activity kept decreasing for about 15 minutes at 120 °C before reaching an almost constant value.

Possible relations between the changes in the calcium and phosphate partitions and the heat stability of concentrated milk are discussed. The Ca^{2+} activity appears to be an essential factor at pH values below the optimum.

1 Introduction

Many factors influence the heat coagulation of concentrated milk. The calcium and phosphate partitions and the changes therein during the manufacture of concentrated milk are often mentioned as being important (1, 2). Although research on this subject has been extensive for milk (3, 4, 5, 6), studies on concentrated milk have been limited.

Addition of calcium accelerates coagulation of concentrated milk during sterilization (7), and part of the seasonal differences in the heat stability of concentrated milk can be related to variations in the amount of calcium in the milk (8). Also the use of phosphates as stabilizers in the manufacture of sterilized evaporated milk is often explained by the influence of these salts on the calcium partition in the milk (7, 9).

Actual determinations of calcium and phosphate have been scarce. In 1955, Van Kreveld & Van Minnen (10) determined the changes in Ca^{2+} and Mg^{2+} concentrations during the manufacture of concentrated milk. They showed that Ca^{2+} and Mg^{2+} concentrations in concentrated milk were higher than in milk, but addition of an unspecified amount of phosphate to the concentrated milk resulted in a Ca^{2+} concentration slightly lower, and a Mg^{2+} concentration slightly higher than in milk. After sterilization the Ca^{2+} and Mg^{2+} concentrations were lower than in unconcentrated milk. Rose & Tessier (3) and Vujicic & De Man (11) gave values for soluble Ca, Mg, PO_4 and citrate in milk and concentrated milk, and Belec & Jenness (12) determined dephosphorylation rates. These authors did not pay particular attention to the manufacturing process of concentrated milk. The amounts of ultrafilterable calcium and phosphate in the different stages of the manufacturing process of concentrated milk were investigated by Hardy et al. (9). They found a decrease during every processing step, while for both calcium and phosphate the largest transfer from plasma to the colloidal state took place during concentrating. The overall transfer of calcium and phosphate was about 4 mmol/l and 5 mmol/l per unit original protein, which is approximately 0.12 mol/kg protein and 0.15 mol/kg protein. This gives, rather surprisingly, a ratio of 0.8 for Ca/P in the salt that did become colloidal. In their investigation no attention was paid to dephosphorylation of casein, which may occur during heating and thereby influence the heat stability of milk (6). Neither Van Kreveld & Van Minnen nor Hardy et al. considered the influence of the pH. Some authors (13) claimed that in unconcentrated milk part of the HCT curve profile is caused by a sudden decrease of the solubility of phosphate after heating, although others (5, 6) were unable to confirm this.

We made an attempt to investigate the possible changes in the state of calcium and phosphate during the manufacturing of concentrated milk. Besides changes during processing we also investigated changes after heating samples in an oil bath at 120 °C. In this way we not only obtained information about the effects of a relatively long heating time, with a slow heating up and cooling of the concentrate, but also about the effects of heating for shorter times, with a rapid heating up and cooling.

2 Materials and methods

2.1 Processing

Raw milk and thermized skim milk were obtained from a dairy plant. After overnight storage at 4 °C, the standardized milk was heated in a tube heat exchanger to 120 °C, held at this temperature for 3 minutes and then cooled to 74 °C in a plate heat exchanger prior to evaporation. In other experiments the milk was only heated to 74 °C in a plate heat exchanger immediately before evaporation. Evaporation was carried out in a pilot-scale (900 l/h) three-stage falling film evaporator, with vapour temperatures in the first, second and third stage of 70 °C, 60 °C and 50 °C, respectively. The approximate temperature profile during the whole process is given in Fig. 1.

The concentrated milk was homogenized at 18 MPa and 50 °C. Phosphate (NaH_2PO_4/Na_2HPO_4 40/60 on a molar basis) was added to subsamples, the pH of the concentrated milk was adjusted with 1 N HCl or 1 N NaOH, and the concentration was adjusted to 31.3 % total solids by adding water. In some experiments the concentrated milk was filled into lacquered tin cans (0.17 kg) and sterilized; the approximate temperature profile above 110 °C is given in

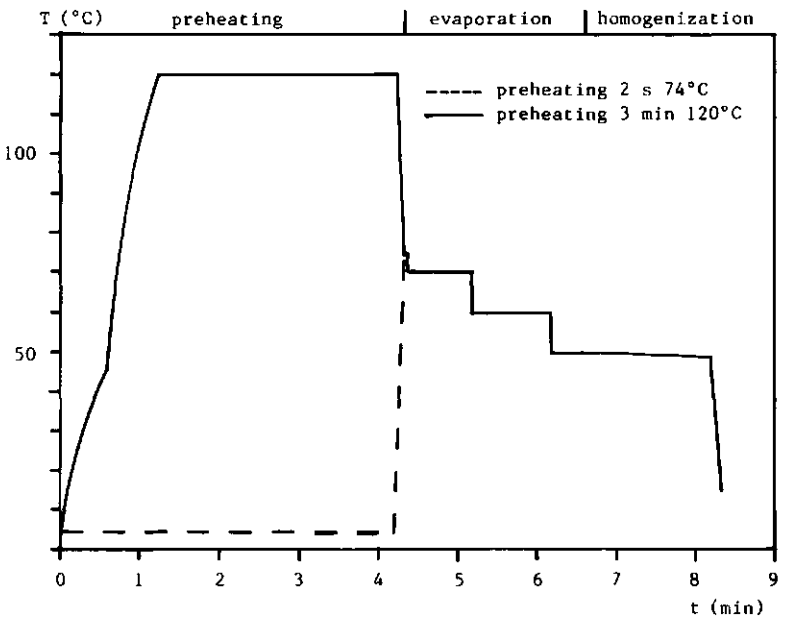


Fig. 1. Temperature of the milk during the concentration process.

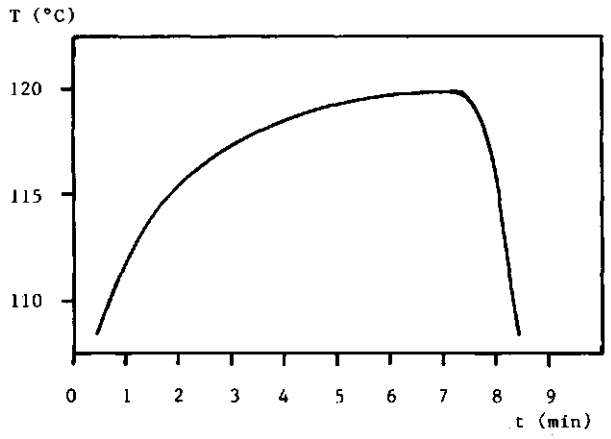


Fig. 2. Temperature of concentrated milk during in-can sterilization.

Fig. 2; the heating up and cooling rates were about 8 °C/min. In other experiments the concentrate was heated in glass and stainless steel tubes in an oil bath.

2.2 Experiments on changes during processing: sampling procedure

To investigate the changes during processing, four experiments were done. To keep the analytical work at a manageable level, sampling and analyses were varied. This is outlined in Table 1. The raw milk had been stored at 5 °C for at least 12 h before sampling, while the preheated and concentrated milks were cooled to about 15 °C and were analyzed immediately afterwards. Storage for one night or two days took place at 4 °C.

2.3 Oil bath experiments

Two experiments were done, in April (E) and in September (F). After overnight storage, standardized concentrated milk was filled into glass tubes for determination of the heat coagulation time (HCT) by the method of Davies & White (15), and into stainless steel tubes for determination of pH and Ca^{2+} activity. The content of the glass tubes was about 2.5 ml with 1.5 ml headspace and of the stainless steel tubes about 5 ml with a headspace of about 1.0 ml. After a certain heating time in a 120 °C oil bath, the Ca^{2+} activity and the pH of the concentrated milk were determined. The contents of six tubes were needed for these determinations, and they all could be removed from the oil within 20 seconds. Cooling was done with running tap water.

Table 1. Sampling scheme.

	Experiments A and B (September, October)	Experiments C and D (March, June)
Samples taken from		
Raw milk	– immediately	– immediately
	– after 48 hours	– after 48 hours
Heated milk	– immediately	– immediately
	– after 48 hours	– after 48 hours
Concentrated milk	– immediately	– immediately
	– after 48 hours	– after 48 hours
Concentrate after concentration adjustment	– after 6 hours	– after overnight storage
	– after 48 hours	
Sterilized concentrate	– immediately	– immediately
	– after 48 hours	
Analyses	– Ca^{2+}	– Ca^{2+}
	– pH	– pH
	– ultrafilterable Ca	– total Ca
	– inorganic ultrafilterable P	– ultrafilterable Ca
		– total P
		– inorganic P
		– total ultrafilterable P
		– inorganic ultrafilterable P

2.4 Terminology

To describe the partition of calcium and phosphate we will use the following terms:

– Colloidal calcium. This fraction is mainly present in the colloidal calcium phosphate (CCP) of the casein micelles, and furthermore as counterions of the casein. In heated milk, some calcium is also associated with the denaturated whey proteins.

– Diffusible or ultrafilterable calcium. This is mainly associated with some anion of low molecular weight, but also includes the Ca^{2+} ions.

– Ionic calcium: Ca^{2+}.

– Colllidal phosphate. This fraction consists mainly of the (inorganic) phosphate present in the colloidal calcium phosphate, and of the phosphate groups esterified to casein.

– Diffusible or ultrafilterable phosphate. This is mainly in an ionic state, or associated with some low molecular weight cation; the non-protein organic phosphate is also ultrafilterable.

Phosphate can also be divided into an organic and an inorganic fraction. The organic fraction consists of the phosphoric esters in the casein molecules,

some low molecular weight esters, and the phospholipids. The inorganic fraction comprises phosphate in the CCP, ionic phosphate and diffusible phosphate salts.

2.5 Analyses

Before analyzing, the samples of concentrated milk that were coagulated during sterilization were homogenized with an Ultraturrax.

– The Ca^{2+} activities were determined with an Orion 701 A digital ion meter equipped with an Orion 93-20 ion-selective electrode, and an Orion 90-01 single junction reference electrode with a filling solution of 4 mol/l KCl saturated with Ag^{+}. We used the procedure given by Geerts et al. (14).

– The pH values were determined with a WTW pH 521 meter.

An Amicon MMC-A No. 875 ultrafiltration unit, with Nuclepore PC 0.1 μm filters, was used to separate the milk and the concentrated milk. The pressure difference was 1 bar, and the permeate volume never exceeded 5 % of the total volume. The first few drops of permeate were discarded. For milk 30 min were needed to obtain a sufficient quantity of permeate, for concentrated milk 1 to 1.5 hour. The partition coefficient (ratio of concentration in the feed over that in the permeate) for the Nuclepore 0.1 μm membrane was about 2 for whey proteins in milk and about 1 for glucose-1-phosphate.

– Calcium in milk and permeate was determined by atomic absorption spectrometry at 422.7 nm. For this determination 20 μl permeate of milk or 10 μl permeate of concentrated milk was diluted with 5 ml of a $LaCl_3$ solution (1.82 g $LaCl_3 \cdot 7H_2O$ and 0.08 g NaCl per 1).

– Phosphorus was determined as molybdano-vanado-phosphoric acid by a colorimetric method (16). With this method only inorganic phosphorus can be detected. Total P was determined after digestion with HNO_3 and $HClO_4$ at 200 °C, and inorganic P after precipitation of the protein using 12 % TCA. Total ultrafilterable P was determined after digestion of the permeate, and inorganic ultrafilterable phosphate in the permeate as such. For these analyses 25 μl permeate of milk and 10 μl permeate of concentrated milk were needed.

Total P minus inorganic P gives the concentration of organic P. Total ultrafilterable P minus inorganic ultrafilterable P gives the amount of ester phosphate. The colloidal organic phosphate can then be calculated by subtraction of ester phosphate from organic P. Finally the amount of inorganic colloidal P can be calculated from the difference between inorganic P and ultrafilterable inorganic P.

The amounts of Ca and P determined in permeate were recalculated to values in milk and concentrated milk by applying the corrections for non-sol-

vent water and the volume occupied by colloidal particles. We adapted a formula given by Walstra & Jenness (17, p. 381):

$$C_{milk} = C_{permeate}\,(1 - 1.01\,f - 1.08\,c - h{\cdot}p)\,(\varrho_{milk}/\varrho_{permeate}) \qquad (1)$$

where C = concentration (mmol/l), f = fat content (kg/kg), c = casein content (kg/kg), p = protein content (kg/kg), h = factor for non-solvent water for a small molecule = 0.2 (kg water/kg protein), ϱ = density (kg/m^3).

An estimation of the accuracy of the calcium and phosphate determinations is given in Appendix 1.

3 Results

3.1 Changes during the manufacture of sterilized concentrated milk

3.1.1 The influence of preheating and concentration on the calcium and phosphate partitions. The results obtained for pH, Ca^{2+} activity and the Ca and P partition in raw milk, preheated milk and concentrated milk (32-33 per cent TS) are given in Figs. 3 and 4 and Table 2. In Figs. 3 and 4 the results are given as such, and for Table 2 the Ca and P contents of the concentrated milk were recalculated to values in milk. Average values, and average values after storage of the samples for two days are given in Table 3.

The pH decrease in the manufacturing process of concentrated milk occurred mainly during the concentration step. The average decrease after 2 s 74 °C preheating was 0.04 units and after 3 min 120 °C preheating 0.09 units. Concentration induced a decrease of 0.25 units and 0.24 units for milk after a low and a high intensity preheating, respectively. After 48 hours storage at 4 °C the pH differences caused by the difference in preheating had virtually disappeared.

Our results for the Ca^{2+} activity in milk and preheated milk are in good agreement with the values found by Van Kreveld & Van Minnen (10), who found 0.88 mmol/l for raw milk and 0.64 mmol/l after 3 min 120 °C preheating. In agreement with the results of Rose & Tessier (3) and Geerts et al. (14) the Ca^{2+} activity in heated milk did not return completely to its value in raw milk, at least not within 2 days. In order to compare the Ca^{2+} activities in concentrated milk with the free calcium concentrations as measured by Van Kreveld & Van Minnen, the molar free ion activity coefficient of Ca^{2+} in concentrated milk must be known. Using the method outlined in Appendix 2 we estimate a value of 0.30. This gives a Ca^{2+} concentration of 2.7 mmol/l at a Ca^{2+} activity of 0.80, which is close to the 2.55 mmol/l found by Van Kreveld &

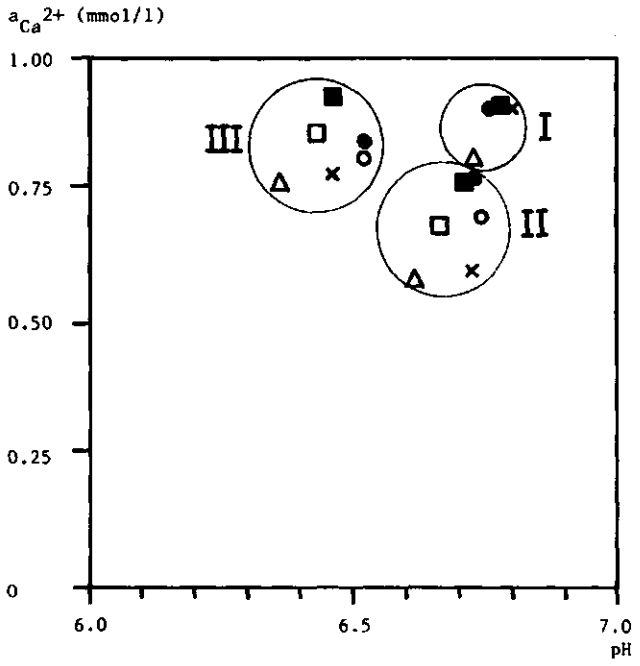


Fig. 3. Ca^{2+} activity in raw milk and after preheating and concentration.
Preheating 2 s 74 °C: ● Exp. B, ■ Exp. D.
Preheating 3 min 120 °C: × Exp. A, ○ Exp. B, △ Exp. C, □ Exp. D.
I: milk; II: preheated milk; III: concentrated milk.

Van Minnen. The Ca^{2+} activity in concentrated milk did not change greatly upon storage, but the values for milk concentrated after a low preheating and after a high preheating became equal.

Our results for P in its different states in raw milk agree well with those obtained by Belec & Jenness (12) and Dalgleish et al. (6), except that we found significantly less ultrafilterable ester P. Processing induced only small changes in total P; the slight differences may even be due to experimental errors in the P determination and in the concentration factor. The fraction inorganic P remained virtually constant. The values we found for ultrafilterable organic P increased upon preheating and concentrating. Probably the separation characteristics of the ultrafiltration membrane and/or the treatment of the milk have much influence. Micellar Ca and inorganic P agree well with the results of Holt (20).

Our results for ultrafilterable Ca and inorganic P in milk and concentrated

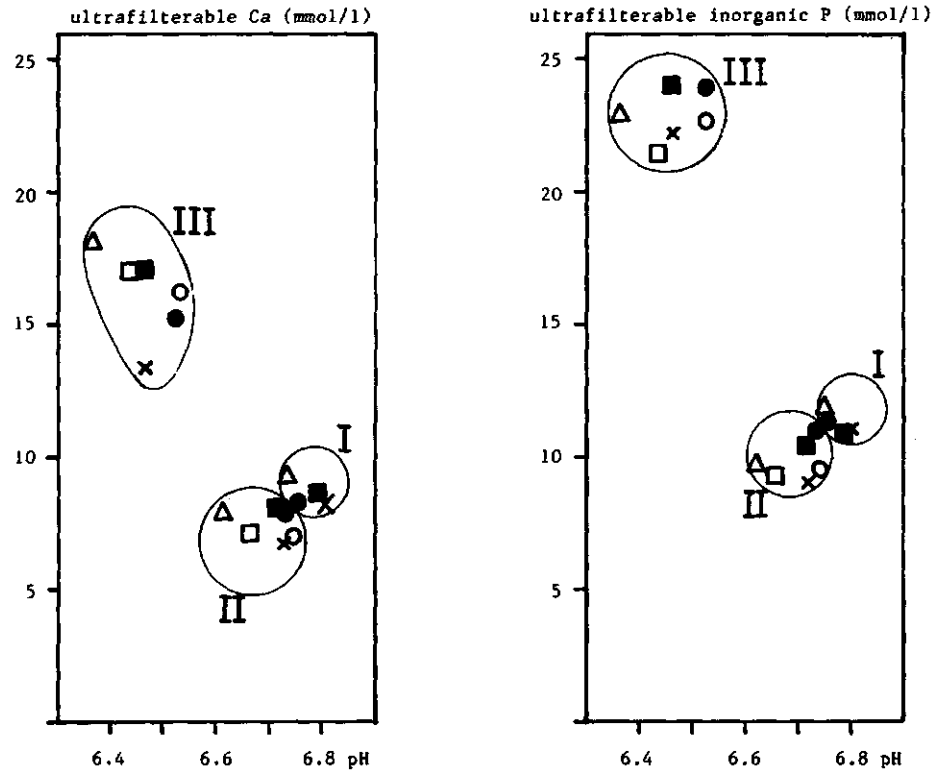


Fig. 4. Ultrafilterable Ca and ultrafilterable inorganic P in raw milk and after preheating and concentration. For symbols see Fig. 3.

milk are about 15 % higher than those of Hardy et al. (9). However, this comparison is complicated by the fact that Hardy et al. used an empirical correction factor, based on the partition of the chloride ion, to recalculate values in permeate to values in milk. In evaporated milk this factor varied from 0.85 to 0.90. In milk that had been preheated at 74 °C, the values for ultrafilterable Ca and inorganic P became about equal to those in raw milk within two days storage, but after a preheating at 120 °C the recovery was far more limited. The values found for ultrafilterable Ca and inorganic P in concentrated milk did not change significantly during two days storage.

The results in Table 3 show that a significant amount of ultrafilterable Ca became associated with the colloidal particles during preheating and concentration. Average values for ultrafilterable Ca were 8.7 mmol/l, 7.4 mmol/l and 6.0 mmol/l in raw, high preheated and concentrated milk, respectively;

Table 2. Partition of Ca and of P in raw milk, preheated milk and concentrated milk. The values for concentrated milk have been recalculated to mmol/l unconcentrated milk.
a. Calcium.

		Total	Ultrafilterable	Colloidal
Milk	(Exp. C)	30.0	9.3	20.7
	(Exp. D)	28.5	8.8	19.7
milk 2 s 74 °C	(Exp. D)	28.3	8.2	20.1
Milk 3 min 120 °C	(Exp. C)	30.0	7.9	22.1
	(Exp. D)	28.0	7.2	20.8
Conc. milk 2 s 74 °C	(Exp. D)	27.7	6.2	21.5
Conc. milk 3 min 120 °C	(Exp. C)	30.0	6.8	23.5
	(Exp. D)	28.7	6.4	22.3

b. Phosphorus.

		1 Total	2 Inorganic	3 Total UF	4 Inorganic UF	1-2 Organic	2-4 Inorganic colloidal	3-4 Ultrafilterable esters	1-2-(3-4) Organic colloidal
Milk	(Exp. C)	31.1	21.3	13.8	12.0	9.8	9.3	1.8	8.0
	(Exp. D)	29.5	19.9	12.9	11.2	9.6	8.7	1.7	7.9
Milk 2 s 74 °C	(Exp. D)	29.5	20.2	11.8	10.4	9.3	9.8	1.4	7.9
Milk 3 min 120 °C	(Exp. C)	31.3	21.5	12.6	9.9	9.7	11.6	2.7	7.1
	(Exp. D)	29.5	19.6	11.3	9.3	9.9	10.3	2.0	8.3
Conc. milk 2 s 74 °C	(Exp. D)	30.3	20.5	11.2	8.8	9.8	11.7	2.4	7.4
Conc. milk 3 min 120 °C	(Exp. C)	31.8	22.4	12.0	8.7	9.4	13.7	3.3	6.1
	(Exp. D)	31.3	20.9	11.3	8.0	10.4	12.9	3.3	7.1

Table 3. Average values for pH, Ca^{2+} activity, ultrafilterable Ca and ultrafilterable inorganic P in milk, preheated milk and concentrated milk.
a. If a 2 s 74 °C preheating was used; average of two experiments.

	Raw milk		Preheated milk		Concentrated milk	
	immediately	after 48 h	immediately	after 48 h	immediately	after 48 h
pH	6.77	6.66	6.73	6.71	6.48	6.48
Ca^{2+} activity	0.90	0.88	0.78	0.83	0.89	0.84
Assumed activity coefficient	0.40	0.40	0.40	0.40	0.30	0.30
Ca^{2+} concentration	2.3	2.2	2.0	2.1	3.0	2.7
Ultrafilterable Ca	8.6	9.0	8.0	8.8	6.0[a]	6.0[a]
Ultrafilterable inorganic P	11.3	11.7	10.7	11.3	9.0[a]	9.0[a]

b. If a 3 min 120 °C preheating was used, average of four experiments

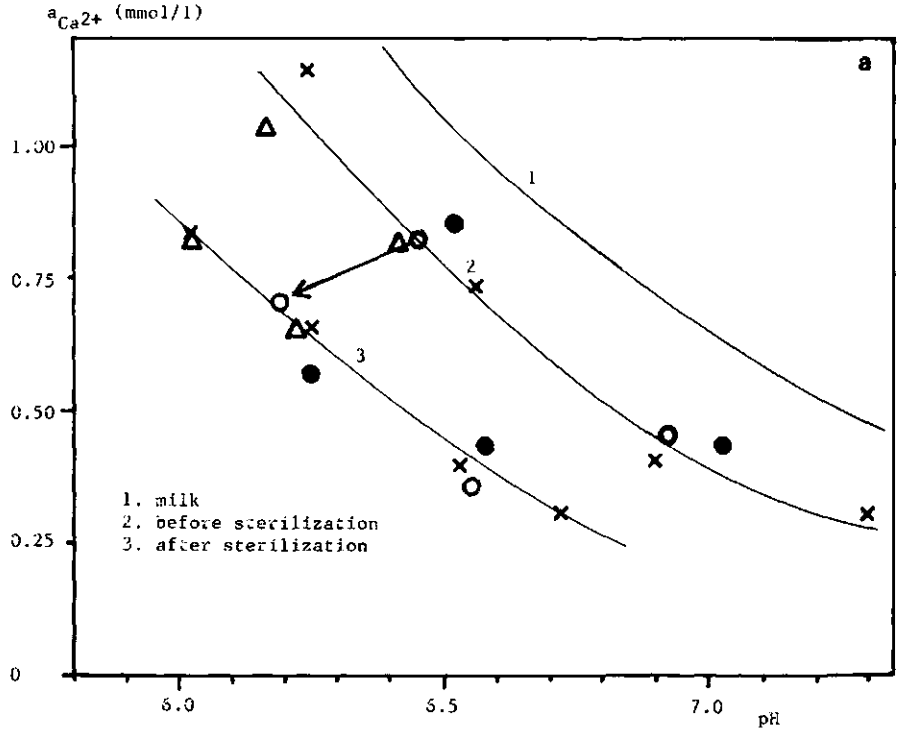
	Raw milk		Preheated milk		Concentrated milk	
	immediately	after 48 h	immediately	after 48 h	immediately	after 48 h
pH	6.77	6.70	6.68	6.72	6.44	6.47
Ca^{2+} activity	0.87	0.90	0.64	0.78	0.80	0.84
Assumed activity coefficient	0.40	0.40	0.40	0.40	0.30	0.30
Ca^{2+} concentration	2.2	2.3	1.6	2.0	2.7	2.9
Ultrafilterable Ca	8.7	8.4	7.4	7.9	6.0[a]	5.8[a]
Ultrafilterable inorganic P	11.5	11.5	9.4	9.8	8.4[a]	8.1[a]

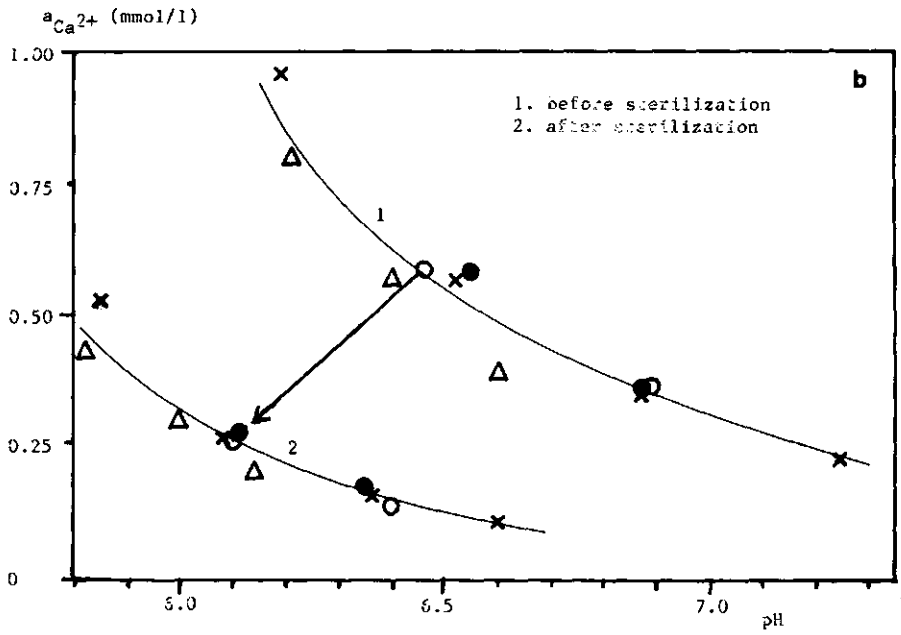
[a] Recalculated to concentrations in milk.

and 8.6 mmol/l, 8.0 mmol/l and 6.0 mmol/l for raw, low preheated and concentrated milk, respectively. The values for concentrated milk have been recalculated to milk. Hence the total amount that had become associated was independent of the preheating intensity but the stage at which most of this association occurred was determined by this factor.

The decrease of ultrafilterable inorganic P was 2.3 mmol/l milk for the process with a 2 s 74 °C preheating and 3.1 mmol/l milk for the process with a 3 min 120 °C preheating. This difference persisted during two days storage.

Ultrafilterable citrate was determined in one experiment; the amount did not change by more than 0.3 mmol/l unconcentrated milk upon preheating or concentrating.


$a_{Ca^{2+}}$ (mmol/l)
a
1.00
0.75
0.50
0.25
0
1
2
3
1. milk
2. before sterilization
3. after sterilization
6.0
6.5
7.0
pH


$a_{Ca^{2+}}$ (mmol/l)
b
1.00
0.75
0.50
0.25
0
1
2
1. before sterilization
2. after sterilization
6.0
6.5
7.0
pH

3.1.2 The influence of pH differences, phosphate addition and sterilization on the calcium and phosphate partitions. The Ca^{2+} activity in unsterilized concentrated milk (Fig. 5) showed a pH-dependency that is comparable with that in milk, but the values were about 0.3 mmol/l lower. Addition of 18.1 mmol PO_4/l lowered the Ca^{2+} activity by an additional 0.2 mmol/l at pH 6.2 and by 0.05 mmol/l at pH 7.2. In particular if phosphate had been added, the values decreased somewhat during storage (Table 4).

Data on the pH-dependency of changes caused by heating can be handled in various ways. In most of the literature on this subject the values of, for instance, the ultrafilterable inorganic P before and after heating are plotted against the pH before heating. If one looks at the Ca^{2+} activity in this way, the concentrated milk with added phosphate attains a much lower Ca^{2+} activity due to sterilization. In concentrated milk without additive, however, this decrease is limited at a low pH and nil at a high pH. For instance, in a sample with an initial pH of about 6.5 the Ca^{2+} activity decreased from 0.60 mmol/l to 0.27 mmol/l if phosphate had been added, and from 0.83 mmol/l to 0.71 mmol/l if it had not. These changes are indicated by arrows in Fig. 5. On the other hand, the pH decreases due to heating and this must influence the Ca and P partition at 120 °C and after cooling. Looking at it at a constant pH, the

Table 4. The change of pH, Ca^{2+} activity, ultrafilterable Ca and ultrafilterable inorganic P during two days storage of standardized concentrated milk before and after sterilization (value after 2 days minus initial value).

Phosphate added	pH before steril.	Δ P mmol/l		Δ Ca mmol/l		Δ Ca^{2+} mmol/l		Δ pH	
		Exp. A	Exp. B	Exp. A	Exp. B	Exp. A	Exp. B	Exp. A	Exp. B
Standardized concentrated milk									
–	~6.5	+0.2	−0.7	−1.2	−1.6	+0.01	−0.06	−0.04	−0.03
–	~6.9	−0.4	−0.1	−2.4	−0.8	+0.01	−0.05	−0.02	−0.04
+	~6.5	−2.1	−1.6	−2.4	−1.6	−0.07	−0.08	0.00	−0.02
+	~6.9	−2.8	–	−3.6	−1.2	−0.02	−0.05	+0.06	−0.04
Sterilized concentrated milk									
–	~6.5	−0.3	+0.2	−4.4	−0.2	+0.15	+0.11	+0.10	+0.07
–	~6.9	+0.7	+0.7	−2.0	0.0	+0.16	+0.12	+0.12	+0.05
+	~6.5	−0.6	−0.6	−1.2	+0.4	+0.08	+0.09	+0.16	−0.02
+	~6.9	+0.8	−1.3	−1.0	+0.4	+0.04	+0.04	+0.16	−0.03

←

Fig. 5. The Ca^{2+} activity in milk (from Ref. 14) and concentrated milk before and after sterilization. a) no phosphate added; b) 18.1 mmol PO_4/l added. For symbols see Fig. 3.

decrease of the Ca^{2+} activity due to sterilization is considerable, both with and without added phosphate, as is seen in Fig. 5.

Both Ca^{2+} activity and pH increased during storage for two days after sterilization (Table 4), although for the pH the extent was different in the two experiments. Without added phosphate the Ca^{2+} activity almost returned to its value before sterilization. But the pH decrease is partly irreversible, and the Ca^{2+} activity at a certain pH two days after sterilization was still lower than the Ca^{2+} activity at the same pH in a non-sterilized concentrate. If phosphate had been added both Ca^{2+} activity and pH remained much lower than before sterilization.

The results for the amounts of ultrafilterable Ca and inorganic P before and after sterilization are plotted in Figs. 6 and 7. Again, if one does not take the pH decrease into account, the changes were very small in concentrated milk without added phosphate: for ultrafilterable Ca either a slight increase or a slight decrease occurred for all initial pH values, and for ultrafilterable inorganic phosphate a decrease was mainly found, except at the lower pH values. If phosphate had been added, sterilization caused a significant amount of Ca and P to become colloidal. However, at a constant pH both ultrafilterable Ca and ultrafilterable inorganic P were about 2 mmol/l lower if no phosphate had been added, and more if it had.

The results on the Ca and P partitions in Experiment D are given in Table 5. All values were recalculated to milk concentration. Values for total P in sterilized concentrate with pH 6.23 and 6.37 were not determined. The recovery of the added P (7.2 mmol/l unconcentrated milk) is within experimental error.

Dephosphorylation turned out to be limited during sterilization of concentrated milk. In experiments with a 2 s 74 °C preheating, sterilization gave a decrease in total organic P by 6 per cent and after 3 min 120 °C no significant change. By using the rate constants as given by Belec & Jenness (12) we calculate, for twofold concentrated milk, a decrease in casein P by 7 per cent if no preheating was used and by 5 per cent after a 10 min 90 °C preheating.

The values for ultrafilterable ester P decreased slightly during sterilization of high preheated concentrated milk, and increased during sterilization of 2 s. 74 °C preheated concentrated milk; the values for all samples were about equal after sterilization. Belec & Jenness (12) reported only a slight decrease of TCA soluble ester P during heating of milk at 121 °C.

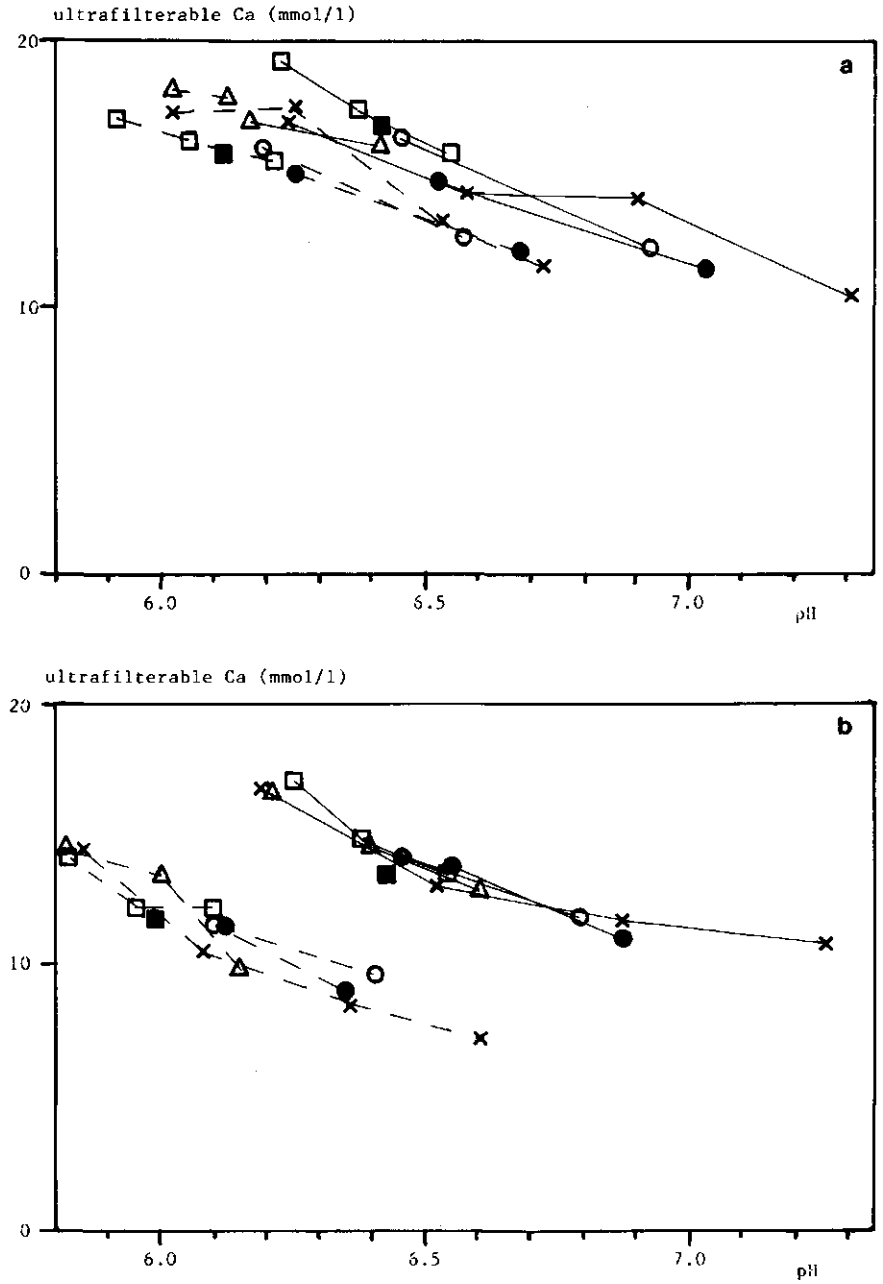


Fig. 6. Ultrafilterable Ca in concentrated milk before (——) and after (– – –) sterilization. a) without added phosphate; b) 18.1 mmol PO_4/l concentrated milk added. For symbols see Fig. 3.

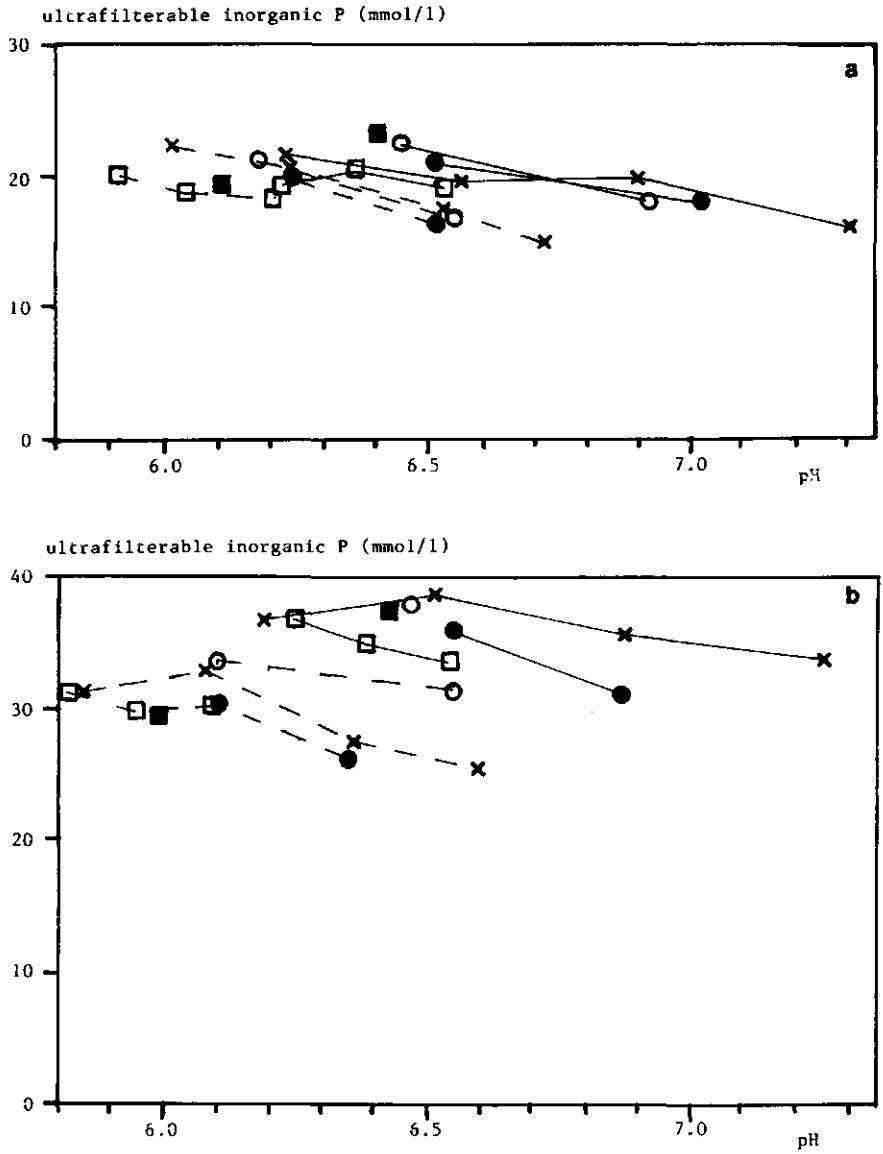


Fig. 7. Ultrafilterable inorganic P in concentrated milk before (——) and after (– – –) sterilization. a) without added phosphate; b) 18.1 mmol PO_4/l concentrated milk. For symbols see Fig. 3.

3.2 Heat stability

Although we did not determine the HCT-pH plots of the concentrated milks in Exps. A-D, we checked all sterilized samples for the presence of coagulate. All low preheated samples were coagulated, although in Exp. B the extent of coagulation was limited in the samples with added phosphate.

In high preheated samples without added phosphate and a pH of about 6.5 no coagulation could be detected in Exps. A and D and a slight coagulation in Exps. B and C. Samples both with a higher or lower pH were coagulated. With added phosphate the samples with a pH of 6.25 were coagulated, and also the sample with a pH of 7.3. The sample with a pH of 6.7 in Exp. C showed a slight coagulation, while samples at this pH were stable in the other experiments. Generally speaking, the March milk, and also the October milk were less stable than the September and June milks.

3.3 Oil bath experiments

To gain more insight into the role of Ca and (added) P in the heat coagulation process, we determined Ca^{2+} activity and pH in evaporated milk after different heating times at 120°. First the HCT-pH curve was determined to get an idea of the relevant sampling times. Results for Exp. E with and without 18.1 mmol PO_4/l concentrated milk added are in Fig. 8. In this Fig. also the Ca^{2+} activities at 20 °C of samples heated in stainless steel tubes until the HCT are given.

In agreement with the results of Sweetsur & Muir (7) for concentrated skim milk, addition of phosphate gave a higher stability at the acid side of the maximum. The results for experiment F were similar for milk without added phosphate. With added phosphate, the increase of the HCT was larger, and the coagulation on the alkali side of the maximum was difficult to estimate, the first coagulation being visible after about 10 minutes, but little further coagulation occurring until about 40 minutes heating. With low preheated milk we found a slight improvement of the heat stability on the acid side of the maximum. Again the coagulation on the alkaline side was difficult to estimate. In this respect our results do not agree with those of Sweetsur & Muir (7), which showed that phosphate addition only gave an improvement of the heat stability if the concentrated skim milk had been preheated. Maybe full cream evaporated milk behaves differently in this respect.

The Ca^{2+} activity determined at 20 °C after different heating times is given in Fig. 9, the pH in Fig. 10. Both Ca^{2+} activity and pH in concentrated milk without added phosphate change in a way that is very different from concen-

Table 5. Partition of Ca and P in concentrated milk at various pH, before and after sterilization (Exp. D). All values have been recalculated to mmol/l unconcentrated milk.
a. Calcium, preheating 3 min 120 °C.

pH	PO_4 added	Conditions	Total	Ultra-filterable	Colloidal
6.23	–	not sterilized	27.6	7.8	19.8
5.9	–	sterilized	27.7	7.0	20.7
6.37	–	not sterilized	27.7	7.0	20.7
6.1	–	sterilized	27.8	6.6	21.2
6.54	–	not sterilized	27.5	6.4	21.1
6.2	–	sterilized	27.6	6.3	21.3
6.25	+	not sterilized	27.7	6.9	20.8
5.8	+	sterilized	27.8	5.6	22.2
6.39	+	not sterilized	27.8	6.0	21.8
6.0	+	sterilized	27.5	5.0	22.5
6.54	+	not sterilized	27.8	5.4	22.4
6.1	+	sterilized	27.5	5.0	22.5

b. Calcium, preheating 2 s 74 °C.

pH	PO_4 added	Conditions	Total	Ultra-filterable	Colloidal
6.41	–	not sterilized	27.7	6.8	20.9
6.1	–	sterilized	27.8	6.4	21.4
6.42	+	not sterilized	27.8	5.9	21.9
6.0	+	sterilized	27.6	4.8	22.8

c. Phosphorus, preheating 3 min 120 °C.

pH	PO_4 added	Conditions	1 total	2 Inorganic	3 Total UF	4 Inorganic UF	1-2 Organic	2-4 Inorganic colloidal	3-4 Ultrafilterable esters	1-2-(3-4) Organic colloidal
6.23	–	not sterilized	30.6	20.7	12.2	8.3	9.9	12.4	3.9	6.0
5.9	–	sterilized	–	21.2	11.3	8.1	–	13.1	3.2	–
6.37	–	not sterilized	30.8	20.8	11.6	8.4	10.0	12.4	3.2	6.8
6.1	–	sterilized	–	21.4	10.7	7.6	–	13.8	3.1	–
6.54	–	not sterilized	30.8	20.5	10.9	7.5	10.3	13.0	3.4	6.9
6.2	–	sterilized	31.2	21.0	10.5	7.5	10.2	13.5	3.0	7.2
6.25	+	not sterilized	37.8	27.7	18.1	14.8	10.1	12.9	3.3	6.8
5.8	+	sterilized	38.8	29.0	15.6	12.5	9.8	16.5	3.1	6.7
6.39	+	not sterilized	37.5	27.7	17.3	14.0	9.8	13.7	3.3	6.5
6.0	+	sterilized	38.5	28.2	15.2	12.0	10.3	16.2	3.2	7.1
6.54	+	not sterilized	38.0	27.9	17.1	13.5	10.1	14.4	3.6	6.5
6.1	+	sterilized	38.1	28.2	15.2	12.1	9.9	16.1	3.1	6.8

d. Phosphorus, preheating 2 s 74 °C.

pH	PO_4 added	Conditions	1 total	2 Inorganic	3 Total UF	4 Inorganic UF	1-2 Organic	2-4 Inorganic colloidal	3-4 Ultrafilterable esters	1-2-(3-4) Organic colloidal
6.41	–	non-sterilized	31.4	20.7	11.4	9.3	10.7	11.4	2.1	8.6
6.1	–	sterilized	31.2	21.2	10.7	7.8	10.0	13.4	2.9	7.1
6.42	+	non-sterilized	37.8	27.9	15.1	15.1	9.9	12.8	3.2	7.6
6.0	+	sterilized	38.1	28.7	14.5	11.8	9.4	16.9	2.7	6.7

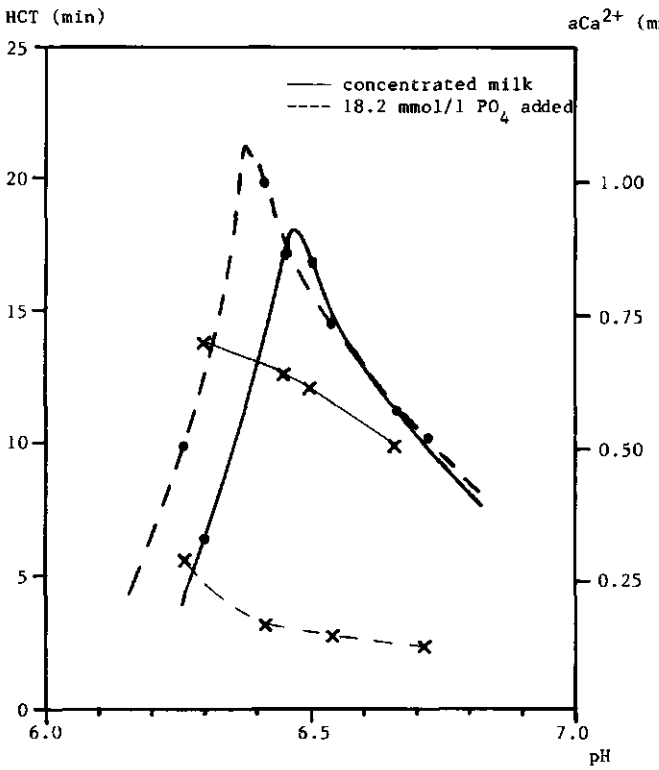


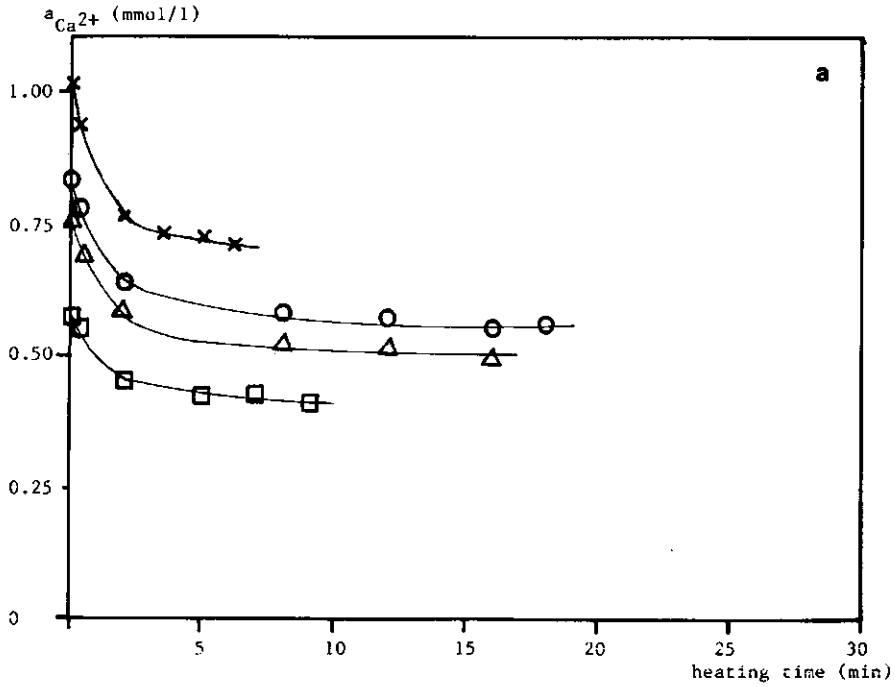
Fig. 8. HCT (●) and Ca^{2+} (×) activity in the just coagulated samples for Experiment E.

trated milk with added phosphate.

Without added phosphate the Ca^{2+} activity decreased fast during the time needed to bring the sample to 120 °C, while afterwards the decrease was slight. In some cases we even observed a slight increase. With added phosphate the Ca^{2+} activity decreased continually for about 10-15 minutes and then remained roughly constant at a very low level. Rose & Tessier (3) found that the Ca^{2+}, total Ca and PO_4 concentrations in an ultrafiltrate produced at 82 °C after different times at that temperature decreased to an equilibrium value within the first 5 min. The equilibrium value was lower for a higher ultrafiltration temperature.

→

Fig. 9. The Ca^{2+} activity of concentrated milk at 20 °C as a function of the heating time of the milk at 120 °C. a) no phosphate added; b) 18.1 mmol/l phosphate added. ×: pH 6.32; ○: 6.46; △: 6.54; □: 6.73.


$a_{Ca^{2+}}$ (mmol/l)
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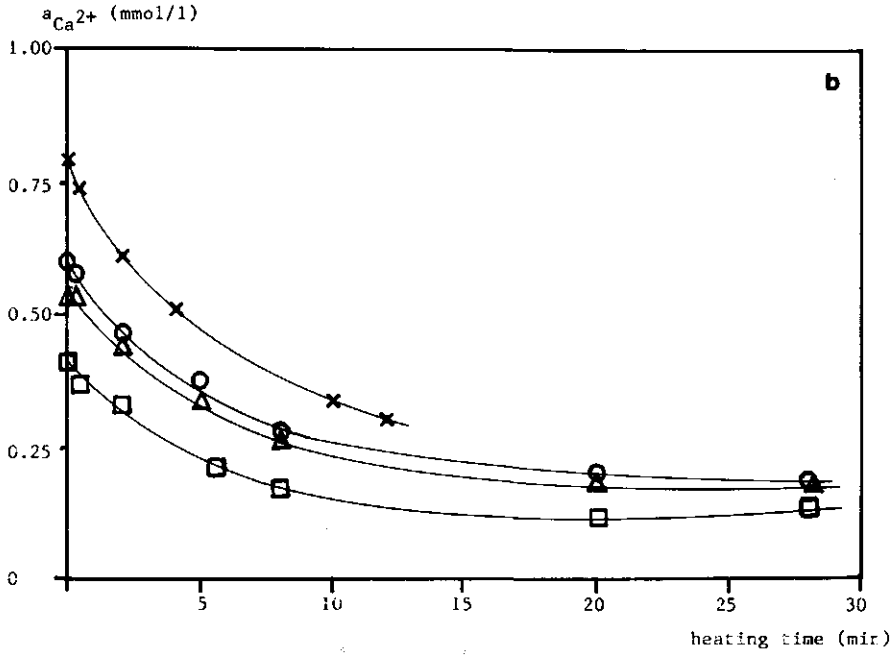
0.50
0.25
0
5
10
15
20
25
30
heating time (min)


$a_{Ca^{2+}}$ (mmol/l)
b
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0.75
0.50
0.25
0
5
10
15
20
25
30
heating time (mir)

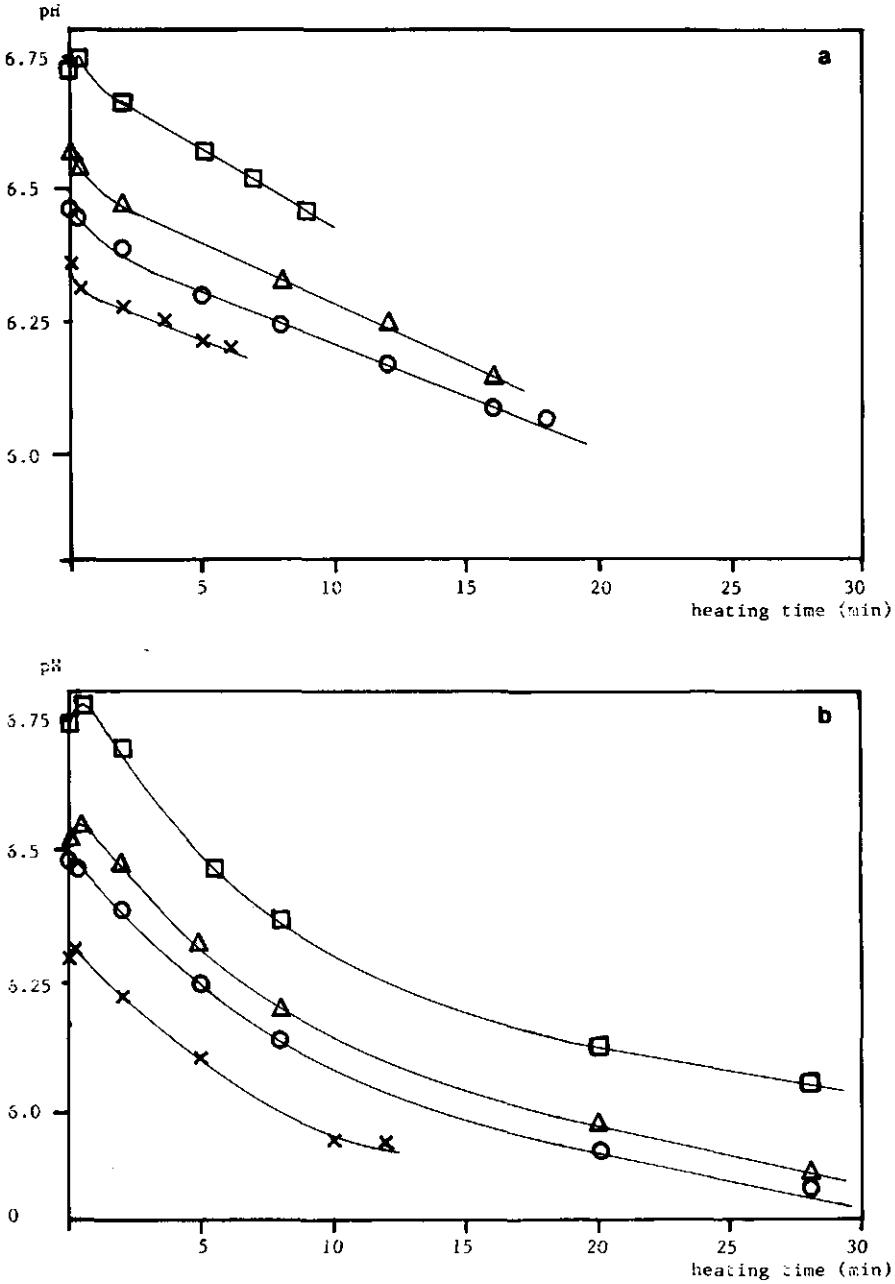

Fig. 10. The pH of concentrated milk at 20 °C, as a function of the heating time of the milk at 120 °C. a) no phosphate added; b) 18.1 mmol/l phosphate added. For symbols see Fig. 9.

The increase of the Ca^{2+} activity after cooling to 20 °C was similar to the increase in milk as found by Geerts et al. (14); we obtained a $_{Ca}2+ = 0.62 + 0.055$ ln t for concentrated milk with a pH of 6.3, where t = time (min).

The pH decrease in concentrated milk was linear during the first 10 to 15 minutes (except for the initial two minutes), as has been reported by some authors for milk (e.g. 18, 25). Our results show that the pH of concentrated milk after a certain heating time did not become independent of the initial pH as was reported for milk (23). With added phosphate, the initial decrease was much faster, but after about 12 minutes at 120 °C the rate of pH decrease became about the same, or even a little less, than that in concentrated milk without added phosphate.

4 Discussion

4.1 Changes in the calcium and phosphate partitions

Some care should be taken with the interpretation of the results presented here, since several dynamic 'equilibria' exist between the salts in milk, which change upon heating, cooling and concentrating the milk (17). To be sure, true equilibria are rarely reached, since changes may continue for a long time after conditions have been altered. Different changes in the salt composition may occur at different rates, e.g. changes that take place in the salt solution will proceed much faster than those in the colloidal calcium phosphate. However, since all equilibria are interrelated the rate of change of the former will also depend on that of the latter. It should also be noted that all determinations were done at 20 °C, some time after a processing step, and the keeping at 20 °C as such may have caused some changes.

As a consequence of the above, a different process or a different method of sampling can easily give somewhat different results. According to Rose & Tessier (3), especially the amount of colloidal calcium but also that of colloidal phosphate is higher in milk at a high temperature than in the heated milk after cooling. Thus the values given for the preheated milk are not equal to the values in the milk as it enters the evaporator. During the concentration process, water is continuously withdrawn, but the temperature decreases in three steps. Only in the buffering vat before the homogenizer can some sort of 'equilibrium' establish, at 50 °C. This will shift to an 'equilibrium' at 20 °C before the determinations can be carried out. Furthermore, upon heating different samples of milk quite large variations can occur in the extent of transfer of calcium and phosphate to the colloidal particles as well as in the reversal to solution upon cooling (28).

However, the average values for concentrated milk after a 2 s 74 °C or a 3

min 120 °C preheating differed only with respect to pH and ultrafilterable inorganic and organic phosphorus. The pH difference disappeared after 48 h storage at 4 °C and all other values did not change. Apparently the changes during the concentration process, at temperatures between 70 and 50 °C, were fast enough to overcome most differences induced by preheating and to give an apparent equilibrium shortly after.

Sterilization, or more generally heating at 120 °C, of the concentrate resulted in a decrease of the pH and the Ca^{2+} activity, but hardly in a change of the (absolute) amounts of ultrafilterable calcium and phosphate.

The pH decrease during sterilization consists of an irreversible part and a reversible part. The former is mainly caused by breakdown of lactose, the latter mainly by association of calcium and phosphate. The pH as determined after cooling to 20 °C is affected by only a part of the latter effect, since already during cooling the association is partly reversed. The extent of this effect can be roughly calculated from data of Rose & Tessier (3). These authors determined an association of about 5 mmol PO_4/l upon heating twofold concentrated milk to 110 °C. Extrapolation to 2.5 times concentrated milk and 120 °C would give approximately 8.4 mmol PO_4/l. If this were all to associate as PO_4 (e.g. $Ca_3(PO_4)_2$) it would cause a release of 14 mmol/l of H^+ (see p. 412 for an example of the calculations involved). At 20 °C this amount would cause the pH to decrease from 6.5 to 6.2. During 48 h storage at 20 °C the pH of sterilized concentrated milk increased by about 0.1 unit. Assuming the association of calcium and phosphate to be completely reversible this would imply that about two-thirds of the reversion had already occurred before the first determination.

The low pH, about 5.9, of an ultrafiltrate obtained at 110 °C is caused by a different phenomenon. Presumably, the dissociation of the buffering groups of the proteins is on average higher at 110 °C than at 20 °C, as is the case for the dissociation of water molecules. Hence, relatively many H^+ ions remain free at 110 °C, while upon cooling after ultrafiltration these buffering groups are no longer present. Of course, the pH of concentrated milk at 120 °C is much lower than at 20 °C, but as the neutral pH is also lower at high temperature this need not have consequences for the stability of the milk proteins.

The results on the Ca^{2+} activity after heating in an oil bath indicate that the calcium and phosphate partitions rapidly come to a new dynamic equilibrium if no phosphate had been added. For concentrated milk to which phosphate had been added before heating the situation is very different. The changes induced by 48 h storage at 4 °C after phosphate addition were small, but sterilization had a considerable effect. Apparently a high temperature is needed to quickly establish the change in dynamic equilibria due to the phosphate addi-

tion. The changes proceeded for a longer time than in the concentrate without phosphate addition, and were for a large part very slow or irreversible.

The fact that the Ca^{2+} activities in sterilized concentrate were quite a bit lower than before sterilization (in particular at low pH), and that they increased during storage, indicates that the reversion of the decrease of the Ca^{2+} activity is rather slow. In comparison, it is rather remarkable that the amounts of ultrafilterable calcium and inorganic phosphorus had hardly decreased due to sterilization. Presumably, during sterilization some calcium and phosphate become associated with the colloidal particles (3), which pertain to the concentrate after cooling, but our results show only small differences in the amounts in the permeate before and after sterilization. This was also observed for milk by Dalgleish et al. (6). As is often the case with calcium and phosphate partitions the observations are the resultant of several processes: the high temperature causes association; the cooling already gives a reversion of this effect; the pH decrease induces dissociation; and probably some irreversible change also occurs at 120 °C.

4.2 Some calculations concerning the colloidal calcium phosphate

To gain more insight into the possible effects of the additional calcium phosphate on the casein micelles, we made some calculations about the Ca/P ratio and the protonation of the phosphate.

First we calculated the Ca/P ratio in the salt that had become associated with the colloidal particles during preheating and concentration from the depletion in the serum. The salt solution of the 2 s 74 °C preheated milk contained on average 9.2 mmol/l Ca and 12.2 mmol/l inorganic phosphate. (These are the values determined in the permeate; they can be calculated from the values in Table 3.a divided by 0.93, which is the factor resulting from Equation 1.) The concentration factor of the salt solution is 3.05, being the concentration factor of the milk (2.66), multiplied by the ratio of the volume fraction of salt solution in the milk to that in the concentrated milk (0.93/0.81). This gives 28.1 mmol/l Ca and 37.2 mmol/l inorganic phosphate in the salt solution of the concentrate. In the permeate of low-preheated concentrate we determined on average 19.7 mmol Ca and 29.6 mmol/l inorganic phosphate, hence 8.4 mmol/l and 7.6 mmol/l, respectively, became associated with the colloidal particles. This yields a Ca/P ratio of 1.1. A similar calculation for the process with a 3 min 120 °C preheating yields a ratio of 0.9. This is higher than the 0.8 that can be calculated from the results of Hardy et al. (9), but we did not include the changes caused by sterilization in our calculations, and during that process mostly more phosphate than calcium became colloidal, which would result in a lower Ca/P ratio.

Secondly, from the pH decrease and the decrease of the phosphate in the permeate caused by concentrating the milk one may calculate the average protonation of the phosphate that became associated with the colloidal particles. We used the values for the process with a 2 s 74 °C preheating and assumed that changes in the calcium and phosphate partitions were the only source of acid production (at high-preheating additional acid may have been formed). The salt solution of a milk with 12.2 mmol/l inorganic phosphate contains at pH 6.77, according to the model by Holt et al. (22), the following species of phosphate: 1.0 mmol/l Ca and Mg phosphates, 1.2 mmol/l Na and K phosphates, 7.0 mmol/l $H_2PO_4^-$, 3.0 mmol/l HPO_4^{2-}.

As shown before, the permeate of concentrated milk contained 29.6 mmol/l inorganic phosphate, while 7.6 mmol/l inorganic phosphate had become associated with the colloidal particles. The $H_2PO_4^-$ and HPO_4^{2-} concentrations at a given pH can be calculated from ultrafilterable inorganic P through the relation:

$$\frac{[HPO_4^{2-}]}{[H_2PO_4^-]} = \frac{K \cdot \gamma_{univalent}}{10^{-pH} \cdot \gamma_{divalent}} \qquad (2)$$

where K = intrinsic dissociation constant of $H_2PO_4^- = 3.52 \cdot 10^{-8}$ mol/l (ref. 22), and γ = free ion activity coefficient.

In addition we assume that, upon concentration, the concentrations of the Ca and Mg phosphates in the salt solution remain constant, while the concentrations of the Na and K phosphates increase proportionally to the concentration factor. These assumptions, the amount of inorganic phosphate in the permeate of concentrated milk, and equation 2 yield for the salt solution of concentrated milk at pH 6.48: 1.0 mmol/l Ca and Mg phosphates, 3.6 mmol/l Na and K phosphates, 19.8 mmol/l $H_2PO_4^-$, 5.2 mmol/l HPO_4^{2-}.

Upon titration of concentrated milk from pH 6.48 to 6.77, about 9.0 mmol OH^-/l had to be added. Using Equation 2 it is calculated that during this titration 3.3 mmol OH^-/l is needed to convert $H_2PO_4^-$ into HPO_4^{2-}. During concentrating, the reversal of this process consumes only 2.3 mmol/l H^+ because of the concomittant changes in γ; hence, the total amount of H^+ ions produced by association would have been 8.0 mmol/l. The associating species were: 2.1 mmol/l Ca and Mg HPO_4, 3.8 mmol/l H_2PO_4, 1.7 mmol/l HPO_4. If all these species were to have been converted into $Ca_3(PO_4)_2$, 11.4 mmol/l H^+ would have been released. Since only 8.0 mmol/l was to be explained, 3.4 mmol/l remains; this is 45 % of the PO_4 that became associated with the micelles. Consequently 45 % may have become present as HPO_4 rather than

PO_4. Although this calculation is, admittedly, a rough one, it is clear that a fairly acidic phosphate becomes associated with the colloidal particles on concentrating.

These fairly straightforward calculations reveal that not only the amount, but also the composition of the CCP might change during the manufacturing of sterilized concentrated milk. Since we feel that it cannot readily be justified that the already existing and the newly formed calcium phosphate are separate entities, we will also calculate the Ca/P ratios in the total CCP.

This calculation is somewhat tentative, since we have to estimate the amount of calcium present as a counterion to the colloidal particles. We assume that this is equal to the amount of counterions of casein only; the total value may be about 10 % higher (17). For raw milk, Holt (20) found a value of 4.6 mmol/l of Ca as a counterion to casein, but this is likely to change upon preheating and concentration, since the crowding of the different cations around a colloidal particle depends among other things on their activity (17). Consequently, if the Ca^{2+} activity changes due to heating or phosphate addition, the amount of calcium present as a counterion will change. Concentration will also have an influence since the Ca^{2+} activity remains almost constant, but the Na^+ and K^+ activities will be roughly proportional to the concentration factor. The calculations are given in Appendix 3.

Two Ca/P ratios can be calculated from the data in Tables 2 and 5 and Appendix 3: the $Ca/P_{inorganic}$ ratio and the $Ca/P_{organic + inorganic}$ ratio. We calculated both. The latter ratio was calculated either with the determined values for ultrafilterable organic phosphorus, or assuming this amount to have a constant value of 3.0 mmol/l, since we do not know whether the values have been influenced by the separation characteristics of the microfiltration membrane. The results are in Table 6.

These Ca/P ratios should be interpreted with some reserve. On the one hand, the ratios given in Table 6 may be overestimates because of two factors. The first is the above mentioned ten per cent difference in counterions between casein and total colloidal particles. Taking this into account gives, for instance, a $Ca/P_{inorganic}$ ratio of 1.68 instead of 1.74 in raw milk and 1.47 instead of 1.50 in high-preheated concentrated milk. The second is that the colloidal particles probably have more counterions in a solution with a higher ionic strength. Some sort of maximum for this effect is given by the amount of counterions calculated with the assumption of an increase proportional to the ion activities. This gives for concentrated milk after a high preheating a $Ca/P_{inorganic}$ ratio of 1.41, instead of 1.50.

On the other hand, the ratios in Table 6 may be underestimates because of competition of the additional CCP with Ca^{2+} for binding sites on the casein.

Table 6. Calculated values of $Ca/P_{inorganic}$ and $Ca/P_{organic + inorganic}$ for the calcium phosphate in the colloidal particles. All values are expressed in mmol/l unconcentrated milk; the amount of Ca present as counterions to casein has been calculated (Appendix 3). Values for the process with a 3 min 120 °C preheating, Exp. D.

Sample	pH	PO_4 added	$Ca_{colloidal}$	Ca_{ci}	$P_{inorganic}$ colloidal	$P_{organic}$ colloidal	$Ca/P_{inorganic}$	$Ca/P_{colloidal}$ [1]	$Ca/P_{colloidal}$ [2]
– milk	6.75	–	19.7	4.6	8.7	7.9	1.74	0.91	0.99
– preheated milk	6.6	–	20.8	4.3	10.3	8.3	1.60	0.89	0.96
– concentrated milk	6.4	–	22.3	3.0	12.9	7.1	1.50	0.97	0.95
– concentrate after 1 night at 4 °C	6.4	–	20.7	3.3	12.4	6.8	1.40	0.91	0.90
– concentrate, immediately after sterilization	6.1	–	21.2	3.0	13.8	–	1.32	–	–
– concentrate after 1 night at 4 °C	6.4	+	21.8	2.5	13.7	6.5	1.41	0.96	0.94
– concentrate, immediately after sterilization	6.0	+	22.5	1.6	16.2	6.7	1.29	0.91	0.89

[1] Calculated with determined values of ultrafilterable organic phosphate.
[2] Calculated with 3.0 mmol/l ultrafilterable organic phosphate.

This would give less Ca^{2+} as a counterion and thus a higher ratio. Also the incorporation of some Mg in the CCP will give slightly higher ratios. Although in milk little Mg is part of the CCP (17), this amount is likely to rise upon concentrating since the solubility product of $MgHPO_4$ becomes exceeded (17). If it is assumed that the amount of ultrafilterable $MgHPO_4$ remains constant upon concentration, about 0.2 mmol Mg/l unconcentrated milk would become associated with the colloidal particles. Furthermore, the assumption that the decrease of Mg^{2+} found by Van Kreveld & Van Minnen (10) is solely due to association with the colloidal particles, gives an additional 0.3 mmol Mg/l unconcentrated milk. This would give a $(Ca + Mg)/P_{inorganic}$ ratio in high-preheated concentrated milk of 1.53 instead of a $Ca/P_{inorganic}$ ratio of 1.50.

The net effect of these over and underestimates is likely to be small, since they partly compensate. Hence, the decrease of the Ca/P ratio may not proceed exactly according to the values in Table 6, but the differences will be fairly small. In any case, some change of the CCP is to be expected since it has ion-exchanging properties and any change in the environment will change its composition (32).

If one assumes that the CCP in milk is an amorphous calcium phosphate (ACP) with an hydroxy apatite-like composition ($HA = Ca_5OH(PO_4)_3$) the $Ca/P_{inorganic}$ ratio decreased from 1.7 in milk to 1.3 in sterilized concentrated, hence a change to a more acidic composition. About 3 mmol/l unconcentrated milk of OH would then be released, which seems very unlikely in the light of the observed pH decrease.

The $Ca/P_{colloidal}$ ratios calculated with the assumption that the CCP in milk is an amorphous calcium phosphate with a brushite-like composition ($DCPD = CaHPO_4 \cdot 2H_2O$) (19) are fairly constant. The relatively small changes are partly due to the calculation. For instance, with 17 mmol/l of Ca, 7 mmol/l of organic P and 10 mmol/l of inorganic P, the $Ca/P_{inorganic}$ ratio is 1.7 and the $Ca/P_{organic + inorganic}$ 1.0. With 12 mmol/l of inorganic P the ratios become 1.4 and 0.9, i.e. 0.3 and 0.1 lower, respectively. From Table 6 it is also clear that the increase in the amount of ultrafilterable organic phosphorus during the process has a large influence on the calculated ratios. Not all organic phosphate groups appear to be incorporated in the CCP, since the $Ca/P_{organic + inorganic}$ ratios are mostly lower than 1.0. This does not seem unreasonable. About 40 % of the inorganic phosphate in milk is present as HPO_4^{2-} or $H_2PO_4^-$, while the other 60 % is associated, mainly with calcium, both in the CCP and in the salt solution. There seems to be no reason why the organic phosphate groups would not have any tendency to dissociate.

The $Ca/P_{colloidal}$ ratios given in Table 6 indicate that the CCP in milk may well have a DCPD-like composition which supports the ideas proposed by

Holt et al. (19). To provide the amount of protons that are necessary to give the determined pH decrease from milk to concentrate, a salt with a lower protonation has to associate during concentration. After sterilization, more phosphate than calcium became colloidal, which may indicate that the newly formed salt changes into one with a composition closer to DCPD. This may be not unreasonable, since Van Kemenade & De Bruijn (24) found that the precipitating sequence from supersaturated calcium phosphate solutions in the presence of casein at pH 6.0 and 26 °C was OCP → DCPD.

One can only speculate about the consequences of the results of these calculations for the structure of the casein micelles. All changes may be the resultant of a change in the inorganic calcium phosphate and a change in the bonding to phosphate and/or lysine residues, which changes may have a different sign.

4.3 Relations with the heat stability

As discussed in Section 4.1 preheating had a fairly small effect on the calcium and phosphate partitions in the concentrated milk, both with and without added phosphate, and the differences became even smaller during storage. Consequently, any differences in heat stability between preheated and non-preheated concentrate are not likely to be due to the calcium and phosphate partitions. This conclusion also implies that some other explanation has to be found for the much stronger stabilizing effect of phosphate addition as a concentrate of preheated as compared to non-preheated milk (7).

As can be seen from Figs. 5, 6 and 7, the Ca^{2+} activity and the amounts of ultrafilterable Ca and inorganic phosphate in concentrated milk all showed a monotonic change with pH, both before and after sterilization. Thus the typical HCT-pH profile cannot be related to variation of Ca^{2+} activity, ultrafilterable Ca, or ultrafilterable inorganic P with pH.

Whether or not dephosphorylation is an important factor in the heat stability of milk has been debated over many years. In 1936, Howat & Wright (21) suggested that a relationship between dephosphorylation and heat stability is likely. In 1962, Belec & Jenness (12) stated that dephosphorylation does not appear to play a key role in the heat stability of milk, since the amount of P liberated at the coagulation time in different milks could vary by a factor of 2. In 1987, Dalgleish et al. (6) performed similar experiments, and in addition they determined the dissociation of protein from the casein micelles. They concluded that dephosphorylation almost certainly will have consequences for the micellar structure, but did not relate this to heat stability. Our results show that the extent of dephosphorylation at the coagulation time of concentrated milk is far smaller than in milk; this must be due to the lower tempera-

ture, the shorter coagulation times, and possibly also to the relatively high-intensity preheating applied. Dephosphorylation cannot be an important factor in the heat stability of concentrated milk.

During preheating and concentration considerable amounts of calcium and phosphate become colloidal. Since CCP is an essential component for the integrity of the casein micelles, it has been assumed that association of calcium phosphate with the casein micelles upon concentration has a destabilizing effect (e.g. 30). Several possible mechanisms of destabilization of casein micelles by additional CCP can be given. Shielding of charge may occur, although there is no clear experimental evidence for this theory yet. Also the micelle core may become more compact, which will give an increased Van der Waals attraction. An increase of the micelle size during the concentration process is a third possibility, giving larger particles at the start of the sterilization and thus a lower stability. The additional calcium phosphate may act as a cementing agent between two micelles, thus inducing fusion. Further research would be needed to elucidate these phenomena; for the time being we can only compare our results with investigations on milk with an increased amount of CCP.

Doubling the CCP content of milk by the method of Pyne and McGann resulted in a slightly lower heat stability over the pH range 6.5-7.3 (34). In another study it was found that a twofold increase of the serum concentrations of calcium and phosphate in milk gave a slightly lower heat stability below pH 6.7, and almost immediate heat coagulation at higher pH values (35). Both methods can be questioned with respect to changing only the CCP content of the micelles, however (33). Our results show that in concentrated milk the amount of calcium in the CCP was about 20-25 % higher than in milk, and of inorganic phosphate about 35-45 %. If phosphate had been added these amounts were 30-35 % and 45-60 %, respectively, and sterilization gave a further increase.

We believe that additional CCP may have some destabilizing effect, in particular at a high pH, but that in most cases concomittant changes in the Ca^{2+} activity are more important. Phosphate addition to concentrated milk lowers the Ca^{2+} activity thereby enhancing the heat stability, while the additional CCP would have an opposite effect. Apparently the influence of the former is greater than that of the latter. A similar theory has already been proposed by Ter Horst (29), and also the theory of Horne (31) for the ethanol stability of milk contains a comparable line of thought. In conclusion, the Ca^{2+} activity appears to be an essential factor for the heat stability of concentrated milk at pH values below the optimum.

Appendix 1. Estimation of the accuracy

Standard deviations of the results were estimated from the difference between duplicate analyses of one sample. The standard deviation of the concentration factor is about 0.02, and is neglected in the calculations. Since all results were recalculated to milk concentration, the standard deviation of the results for concentrated milk were also divided by the concentration factor. If a standard deviation found for milk was different from that for concentrated milk divided by the concentration factor we have taken the higher one. This gives:

σ (total Ca)	= 0.19 mmol/l
σ (UF Ca)	= 0.11 mmol/l
σ (total P)	= 0.33 mmol/l
σ (inorganic P)	= 0.34 mmol/l
σ (total UF P)	= 0.28 mmol/l
σ (inorganic UF P)	= 0.25 mmol/l

Assuming a normal distribution of the deviations and no correlation between the deviations of the results of different analyses, the following formulae apply:

$$\sigma^2 (x \pm y) = \sigma^2 (x) + \sigma^2 (y) \text{ and } \quad \frac{\sigma^2(\frac{x}{y})}{(\frac{x}{y})^2} \simeq \frac{\sigma^2 (x)}{\bar{x}^2} + \frac{\sigma^2 (x)}{\bar{y}^2}$$

We thus obtain:

σ (colloidal Ca)	= 0.22 mmol/l
σ (organic P)	= 0.47 mmol/l
σ (UF esters P)	= 0.38 mmol/l
σ (colloidal P)	= 0.43 mmol/l
σ (colloidal organic P)	= 0.60 mmol/l
σ (colloidal inorganic P)	= 0.42 mmol/l
σ ($Ca/P_{inorganic}$ in CCP)	= 0.08
σ ($Ca/P_{inorganic + organic in CCP}$)	= 0.03

These are probably overestimates, since the deviations of the results of different analyses will often be positively correlated.

Appendix 2. Calculation of an activity coefficient in concentrated milk

For calculation of an activity coefficient several approximations can be used: for instance the Debeije-Hückel, the pH and the MacInnes approximations (26). It was shown (26) that the estimation of the $\gamma_{Ca^{2+}}$ with the MacInnes approximation gives values that are in accordance with determined activities up to I = 1 M. A calculation of the $\gamma_{Ca^{2+}}$ for increasing ionic strength gives almost identical results for the pH and the McInnes approximation (27). We used the pH approximation:

$$\log \gamma = \frac{-0.5 \cdot 2^2 \cdot \sqrt{I}}{1 + 1.5 \sqrt{I}}$$

To calculate the ionic strength we used a computer model developed by Holt et al. (22) for milk. Since not all association constants used in this model will be exact, any subsequent errors will be magnified in concentrated milk, but it was the most accurate method to calculate the ionic strength available to us. If milk is concentrated 2.5 times, the non-colloidal phase is concentrated by a factor of 2.5·0.93/0.82 = 2.83; for concentrated milk before concentration adjustment this is 2.66·0.93/0.81 = 3.05.

The values of all components were entered into the model as those in milk times 2.83 or 3.05, except for Ca and PO_4 for which we used the determined values. Perhaps this gives a slight overestimation for Mg; determination of citrate in milk and concentrated milk showed that this component increased nearly in proportion to the concentration factor. In concentrated milk with added phosphate an additional 35 mmol/l Na^+ is present.

Sample	Ionic strength (mmol/l)	Free ion activity coefficient (molar)	
		univalent ions	divalent ions
Milk	80	0.80	0.40
Concentrated milk	180	0.74	0.30
	190	0.74	0.30
	200	0.73	0.29

Appendix 3. Tentative calculation of the amount of calcium that is present as a counterion to casein

For these calculations we used the values for Ca and Mg bound directly to casein in milk that were determined by Holt (20), and for K and Na values given by Walstra & Jenness (17). We assumed that the fraction of an ion that is directly bound to casein is proportional to its activity. We used values for the Ca^{2+} activity that were found in this investigation and values for the Mg^{2+} activity that were found by Van Kreveld & Van Minnen (10). For the Na^+ and K^+ activities in milk the values given by Walstra & Jenness (17) were used. We assumed that the concentrations of these ions increase in proportion to the concentration factor. The concentration factors and activity coefficients used were the same as in Appendix 2.

Two values for each ion were calculated: one assuming the number of moles of counterions to be proportional to the ion activities (mM_{ci} proportional) and one assuming that the number of ionic charges of counterions to be constant (mEq_{ci} equal). The latter was used for the calculation of Ca in CCP. We give three examples of the results of the calculations, and the values for milk.

Milk	a (mM)	$mM_{counterion}$	Proportionality factor	$mEq_{counterion}$
Na^+	17	1.1	0.065	1.1
K^+	29	2.3	0.079	2.3
Mg^{2+}	0.32	1.1	3.4	2.2
Ca^{2+}	0.87	4.6	5.3	9.2

	a (mM)	mM_{ci} proportional	mEq_{ci} equal
3 min 120 °C preheated milk			
Na^+	17	1.1	1.4
K^+	29	2.3	2.9
Mg^{2+}	0.24	0.8	1.0
Ca^{2+}	0.64	3.4	4.3
Concentrated milk (3 min 120 °C preheated)			
Na^+	48	3.1	2.2
K^+	82	6.5	4.7
Mg^{2+}	0.38	1.3	0.9
Ca^{2+}	0.80	4.2	3.0
Sterilized concentrate with added phosphate, pH ~ 6.0			
Na^+	71	4.6	4.5
K^+	76	6.0	5.8
Mg^{2+}	0.17	0.6	0.6
Ca^{2+}	0.32	1.7	1.6

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Samenvatting

J. A. Nieuwenhuijse, W. Timmermans en P. Walstra, *De verdeling van calcium en fosfaat tijdens het bereiden van gesteriliseerde geconcentreerde melk*

In dit artikel worden de veranderingen van de calcium- en fosfaatverdeling tijdens het bereiden van gesteriliseerde geconcentreerde melk (Engelse standaard) beschreven. Calcium en fosfaat associeerden vooral tijdens de vóórverhitting en het indampen met de colloïdale deeltjes, maar nauwelijks tijdens het steriliseren, behalve als fosfaat was toegevoegd aan de geconcentreerde melk. Meer fosfaat dan calcium associeerde; de Ca/P verhouding in het colloïdale calciumfosfaat daalde dus. De waarden gevonden voor de Ca/P verhouding na de verschillende processtappen ondersteunen de theorie van Holt dat het colloïdale calciumfosfaat van de caseïnemicellen een op brushite lijkende samenstelling heeft en dat het grootste deel van de esterfosfaatgroepen deel uitmaakt van het colloïdale calciumfosfaat.

De Ca^{2+} activiteit van geconcentreerde melk was iets lager dan die van melk, bij een gelijke pH. Na sterilisatie was de activiteit verder gedaald.

Ook worden de resultaten van metingen van de Ca^{2+} activiteit en de pH van geconcentreerde

melk die verhit is geweest op 120 °C voor verschillende tijdsduren gegeven. Zowel de Ca^{2+} activiteit als de pH daalden. De daling van de Ca^{2+} activiteit vond vooral plaats tijdens het opwarmen en de eerste minuten bij 120 °C, terwijl een langduriger verhitting vrijwel geen verdere verandering gaf. De daling van de pH verliep lineair, behalve gedurende de eerste minuten. Wanneer echter fosfaat was toegevoegd aan de geconcentreerde melk bleef de Ca^{2+} activiteit dalen gedurende 15 minuten bij 120 °C. De eindwaarde was in dat geval zeer laag.

Een discussie wordt gegeven van mogelijke relaties tussen de calcium- en fosfaatverdeling en de hittestabiliteit van geconcentreerde melk. Bij een pH lager dan die van het stabiliteitsmaximum lijkt de Ca^{2+} activiteit een belangrijke rol te spelen.

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On the heat-induced association and dissociation of proteins in concentrated skim milk

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Summary

The heat-induced association and dissociation of the proteins in concentrated skim milk was studied; effects of heating time, preheating, pH, and phosphate addition were determined. At high initial pH, dissociation of κ-casein from the casein micelles occurred largely during the heating up period, but a low initial pH dissociation proceeded more gradually. During holding at 120 °C α_{s1}- and β-casein dissociated gradually. The initial dissociation of κ-casein was strongly pH-dependent; dissociation of all caseins during holding at 120 °C depended on pH to a lesser extent. The heat-induced association of α-lactalbumin and β-lactoglobulin depended strongly on pH. Preheating of the milk before concentrating resulted in a somewhat slower initial dissociation of κ-casein at high initial pH, but the (apparently final) extent after about 10 min was hardly influenced by preheating. Dissociation of α_{s1}- and β-casein proceeded somewhat faster in preheated concentrate. Addition of phosphate to preheated concentrated milk induced more dissociation of all caseins, and less association of α-lactalbumin and β-lactoglobulin, at the same pH.

Factors affecting dissociation and association are discussed: an attempt is made to distinguish the changes during heating from those occurring between the onset of cooling and the moment of separation of serum from particles. The principal effect of the various heat-induced irreversible changes in the structure of the casein particles is presumably a reduction of the tendency of all caseins to associate into large particles. The extent to which this actually results in a change in the partition of the proteins between serum and particles, both during heating and during ultracentrifugation at 20 °C, is presumably determined by a combination of pH, αCa^{2+}, and hydrophobic interactions.

Introduction

Heating (concentrated) milk causes dissociation of caseins from the casein micelles (e.g. Ref. 1). Also, complexes containing κ-casein and β-lactoglobulin (and presumably other serum proteins as well) are formed; these complexes appear to be largely associated with the casein micelles after heat-

ing at low initial pH, and largely present as small particles after heating at high initial pH (2, 3). The heat-induced dissociation of casein in unconcentrated milk, serum protein-free unconcentrated milk and serum protein-free concentrated milk has been observed to be pH dependent: almost no dissociation occurs at pH 6.4 and extensive dissociation at pH 7.0 (4, 5). A large part of the dissociated casein is κ-casein, both in milk (6) and in serum protein-free concentrated milk (7). In unconcentrated milk, the heat-induced aggregation of serum proteins and their association with the casein micelles has also been observed to be pH dependent (2, 9), most serum proteins being sedimentable by centrifugation at 78 000 *g* for 1 h after heating for 30 min at 90 °C at pH 6.4, but not after heating at pH 7.0.

A possible relation between these association/dissociation phenomena and the heat coagulation of (concentrated) milk was suggested by Aoki et al. (7) and Kudo (4), but generally recognized only recently (e.g. 8, 10). In our recent model for the heat coagulation of unconcentrated milk, the heat-induced dissociation of κ-casein plays a key role in the explanation of a pessimum pH (i.e. the pH at which a local minimum occurs) in the heat coagulation time (HCT) versus pH plot (10). The heat stability of concentrated milk is likely to be influenced by this phenomenon in a similar way.

To fully understand the relation between the association and dissociation of proteins and the heat stability, the partition of the proteins between serum and particles at 120 °C, and the kinetics of its change, need to be known. No data are available on the former, and only a few on the latter. These mostly concern the total amount of non-sedimentable protein nitrogen, which has been found to pass through a maximum after some time of heating (e.g. 1,4,11). The amount of sedimentable β-lactoglobulin, however, was found to reach a constant value, after an initial increase (2). All changes proceeded faster at a higher temperature (e.g. Ref. 1, 2).

In this paper, we present the results of a study on the dissociation of caseins and the association of serum proteins after various heating times in concentrated milk at various pH, with or without phosphate addition, and with or without preheating of the milk before concentration. In a subsequent paper we will discuss the effects of dissociation and association of the proteins on the heat stability of concentrated skim milk.

Materials and methods

Production of the concentrate

Skim milk was obtained from a local dairy. The milk had been skimmed at 50 °C and thermalized at 67 °C. It was preheated, either for 2 s at 74 °C for

3 min at 120 °C, and concentrated as in our previous experiments (12). In this paper, we will use 'preheated' for the latter preheating intensity, and 'not-preheated' for the former. The pH was altered by slowly adding 1 N HCl or NaOH, any phosphate was added as a mixture of NaH_2PO_4 and Na_2HPO_4 (40/60 on a molar basis), and the concentration was adjusted to 24.5 % total solids, i.e. to the same ratio of solids-not-fat to water as in English standard full-cream evaporated milk. NaN_3 (0.02 %) was added as preservative, and Aprotinin (0.01 %, strength 22.8 TIU/ml, Sigma) was added to the raw milk samples to slow down plasmin activity. The HCT of the concentrates was determined by the method of Davies & White (13).

Heating and centrifugation

Subsamples were heated for various times at 120 °C in stainless steel tubes in an oil bath; the reported heating times include the heating-up period of about 2 min. All samples were held overnight at 20 °C, before centrifugation. The definition of 'soluble' casein given by Dalgleish & Law (14) was adopted: protein contents were determined in the supernatant obtained after centrifugation at 70 000 *g* for 2 h at 20 °C. The supernatant was withdrawn with a syringe. A Beckman L-8 centrifuge was used with a Ti-75 8 × 13.5 ml fixed-angle rotor at a speed of 33 000 rev./min.

HPLC

The κ-casein content of the (concentrated) milks and their supernatants was estimated by determining their caseino-marcopeptide (CMP) contents using the method of van Hooydonk & Olieman (15). The concentrates and their supernatants were diluted with demineralized water to milk concentration and equilibrated for at least 45 min at 30 °C. Liquid rennet (0.5 %, 10 800 Soxhlet Units; CSK Leeuwarden, the Netherlands) was added; after 1 h the reaction was stopped by adding a 12 % TCA solution to a final concentration of 8 %. HPLC conditions were as in (15), except that only one TSK G2000SW (300 × 7.5 mm) column (LKB) was used. Peak area was taken as a measure of the CMP concentration; no correction for a decrease in peak area during storage of the samples in the automatic injector was applied, since a sample of concentrate and its supernatant were injected in succession. The correction for the peak area obtained with the TCA-filtrate of a sample before renneting was also omitted, because it is not clear whether this peak is caused by CMP or not. If it is not, the reported values for dissociated κ-casein as obtained by this method are somewhat too high, by about 0.5-1 % of the total κ-casein for unheated samples, and by about 2-3 % for samples which were heat-treated for 5-10 min.

The protein composition of raw milk and of the supernatants (those of concentrated milk diluted to milk concentration with demineralized water) was determined by reversed-phase HPLC on an RP 318 (250 × 4.6 mm) column (BioRad). The procedure described by Visser et al. (16) was used. The proteins in the samples were dissolved by mixing a sample 1:1 with a solution containing 0.04 M 1,3-Bis[tris(hydroxymethyl)-methylamino]propane, 8 M urea and 0.96 % dithiothreitol, and, after 1 h, by diluting this mixture 1:3 with 8 M urea. Peak area was taken as a measure of the protein concentration.

Recalculation

The fraction of non-micellar protein can be calculated from the peak areas obtained for the ultracentrifugal (UC) supernatants and for the corresponding (concentrated) milks. In this calculation, one has to account for the non-micellar protein content in milk or concentrate being lower than in its UC supernatant due to the presence of colloidal particles and of non-solvent water around or within the colloidal particles (17). We used the equation:

$$C_{milk} = C_{sup}\,(1 - 1.01 \cdot f - 1.08 \cdot p_s - h \cdot p_s) = C_{sup} \cdot F \qquad [1]$$

in which C_{milk} and C_{sup} are the concentrations in milk (or concentrate) and in supernatant; f is the fat content (mass fraction), p_s is the sedimentable ($\approx$ colloidal) protein content (mass fraction), h is the exclusion factor, and F the recalculation factor. The factor 1.01 is used to convert the gravimetric fat content to the content of fat globules (17). The factor 1.08 is used to account for the non-protein part of the casein micelles (17); it may be slightly different in (heat-treated) concentrate, but we used 1.08 for all samples. In unheated milk, p_s is the total protein content minus the serum protein and the non-sedimentable casein content. After heating, part of the serum proteins are sedimentable, and the amount of non-sedimentable casein is different. To estimate p_s, the sum of the area of the protein peaks in the RP-chromatograms of unheated milk and that in the (diluted) supernatants was used, applying the following equation:

$$p_s = \frac{A_m - A_s \cdot F}{A_m}\, p \qquad [2]$$

in which A_m and A_s are the sums of the peak areas in raw milk and in (diluted) supernatant, respectively: F is the recalculation factor, which has to be found by an iterative procedure (because p_s is used to calculate F with Equation 1); and p is the total protein content of the milk or concentrate.

From the results in Ref. 17, the average exclusion factor h for α-

lactalbumin (α-LA) and β-lactoglobulin (β-LG), which are the serum proteins that could be determined by our RP-HPLC method, was estimated to be 3.1 g/g. We used the same value for the dissociated caseins in unheated (concentrated) milk, which is, of course, somewhat arbitrary. In heat-treated (concentrated) milk h is likely to be higher, because most non-sedimentable proteins are associated, and thus present in particles which are larger than undenatured serum proteins. We assumed that casein micelles are virtually inaccessible for such particles, and used a value h of 3.4, which can be calculated from the voluminosity of the casein micelles and the above assumption (17).
The recalculation factors thus found are given with the results.

In case of the CMP determination, the amounts of CMP found in the 8 % TCA filtrates have to be recalculated in a similar manner. This correction factor is difficult to estimate, because the exclusion factor for CMP with respect to flocculated proteins in 8 % TCA is unknown. If there were no exclusion, F would be about 0.99 for milk and diluted concentrate. Arbitrarily, we assumed the radius of a CMP-molecule to be 1.7 nm, and the surface area of the flocculated protein to be 1000 m^2/g casein, yielding $h = 1.7$ g/g. This gives $F = 0.97$ for milk and diluted concentrate, and 1.00 or 0.99 for the supernatants, depending on the protein content.

Results

After 1 h renneting, the amount of CMP present in diluted preheated concentrate that had been heated for 2 min at 120 °C was found to be 20 % lower than that in thermalized milk, if 0.02 % rennet was used. However, if 0.5 % or 1 % rennet was added to the same diluted concentrate, the amount of CMP produced after 1 h incubation was about equal to that in unheated milk. Adding more than 0.02 % rennet to unheated milk had virtually no effect. Thus, a high rennet concentration appears to almost eliminate the effects of heating on the results of the enzymatic reaction, and it was decided to use 0.5 % rennet in all experiments. It was also found that the relative amount of supernatant CMP was not affected by the rennet concentration. Hence the effect of heating on CMP production appears to be the same for micellar and serum κ-casein. Determination of CMP in concentrate and in its supernatant thus appears to be a sufficiently accurate method to determine the percentage of dissociated κ-casein in heat-treated (concentrated) milk. In Tables 1A, 2A and 3A the amounts of CMP in (heat-treated) diluted concentrate relative to milk are given; heating the milk, concentrating, and heating the concentrate all resulted in slightly different detected quantities. The difference between thermalized and preheated milk is comparable to that found between reconstitut-

Table 1. A) Relative quantity of CMP (after renneting) in milk and diluted concentrate; the quantity in thermalized milk was set at 100. B) Percentages of dissociated κ-casein, and the recalculation factors (between brackets). Not-preheated concentrate and the milk it was prepared from.

Sample	A (heating time at 120 °C (min))				B (heating time at 120 °C (min))			
	0	2	5	10	0	2	5	10
thermalized milk	100				3(0.89)			
not-preheat. milk	97				3(0.89)			
conc. pH 6.40	109	102			3(0.70)	15(0.63)	33(0.63)	≈48(0.66)
conc. pH 6.48		101	103	110		19(0.63)	43(0.64)	51(0.67)
conc. pH 6.56	107	101	103	115	3(0.70)	24(0.63)	43(0.65)	55(0.68)
conc. pH 6.69		101	107			37(0.65)	58(0.67)	
conc. pH 6.95	106	99	108		4(0.70)	59(0.67)	66(0.68)	≈71(0.70)

Table 2. A) Relative quantity of CMP (after renetting) in milk and diluted concentrate; the quantity in thermalized milk was set at 100. B) Percentages of dissociated κ-casein, and the recalculation factors (between brackets). Preheated concentrate and the milk it was prepared from.

Sample	A (heating time at 120 °C (min))					B (heating time at 120 °C (min))				
	0	2	5	10	15	0	2	5	10	15
thermalized milk	100					4(0.89)				
preheated milk	95					10(0.86)				
conc. pH 6.29	104	103	105	106		14(0.64)	11(0.63)	21(0.64)	33(0.67)	
conc. pH 6.41		102	103		106		16(0.64)	27(0.65)		46(0.69)
conc. pH 6.47	104	103		106		17(0.64)	21(0.64)		49(0.69)	
conc. pH 6.59		102	104		108		25(0.64)	43(0.67)		59(0.72)
conc. pH 6.71		101	103	107			29(0.65)	50(0.68)	61(0.70)	
conc. pH 6.97	99	100	104	108		28(0.65)	38(0.66)	64(0.70)	71(0.74)	

Table 3. A) Relative quantity of CMP (after renetting) in milk and diluted concentrate; the quantity in thermalized milk was set at 100. B) Percentages of dissociated κ-casein, and the recalculation factors (between brackets). Preheated concentrate. + with added phosphate (18 mmol/l), − without.

Sample	A (heating time at 120 °C (min))			B (heating time at 120 °C (min))		
	0	2	5	0	2	5
thermalized milk	100			3(0.89)		
conc. pH 6.25 −		98			12(0.62)	
conc. pH 6.29 +	96	99	102	16(0.62)	22(0.63)	45(0.67)
conc. pH 6.36 −		98			11(0.62)	
conc. pH 6.38 +	97	97	101	13(0.62)	21(0.63)	51(0.68)
conc. pH 6.52 −		96			19(0.62)	
conc. pH 6.52 +	96	98	100	17(0.62)	35(0.64)	68(0.72)

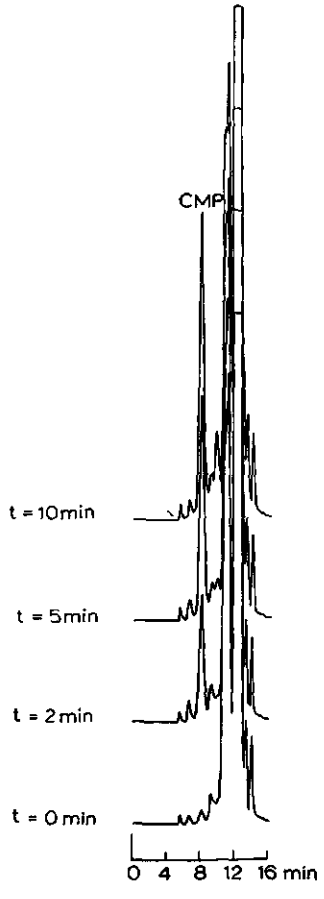

Fig. 1. Chromatograms of the renneted supernatants of not-preheated concentrate, pH 6.6, heat-treated at 120 °C for various time. CMP is indicated.

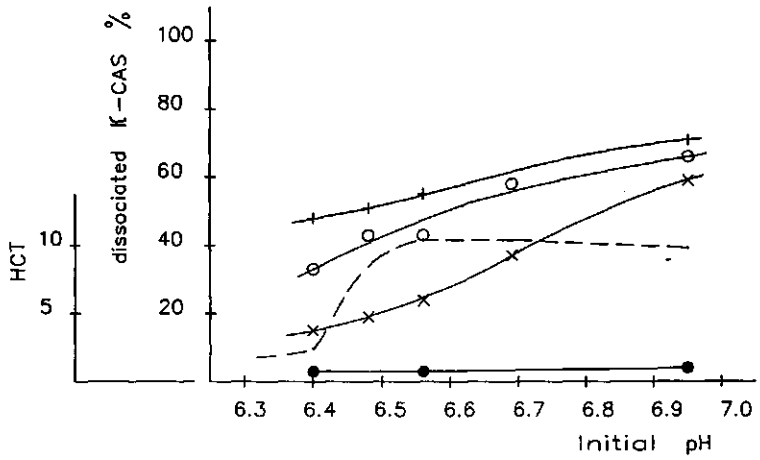

Fig. 2. Dissociated κ-casein, as calculated from the amounts of CMP in concentrate and corresponding supernatant, in not-preheated concentrate at various pH, after various heating times. • not-heat-treated concentrate; × heat-treated for 2 min; ○ heat-treated for 5 min; + heat-treated for 10 min. The HCT is indicated by – – –.

ed unheated and heat-treated (5 min at 120 °C) milk (18).

Fig. 1 shows, as an example, the chromatograms of the renneted supernatants of not-preheated concentrate having a pH of 6.56 and heat-treated for various times. As expected, the CMP peak is larger after longer heating. The amounts of CMP in the supernatants relative to the corresponding concentrates are given in Tables 1B, 2B, and 3B. The results for concentrates without added phosphate are also plotted in Figs 2 and 3; in addition, the HCT of the samples is given. The results of duplicate experiments agreed rather well: for two-thirds of the samples, the difference in CMP content of the supernatants was less than 3 % of the total CMP. The largest difference found was 11 %, for concentrate heated for 2 min at a pH of about 6.75, i.e. for a heating time and pH at which rather large variations may be expected as a consequence of small variations in sample treatment. Fig. 2 shows that in unheated not preheated concentrate most κ-casein was sedimented at all pH values, while after heating this concentrate for 2 min, i.e. heating it up to 120 °C, the amount of not-sedimented κ-casein was strongly pH-dependent, ranging from 15 % at pH 6.4 to 60 % at pH 7.0. After longer heating, relatively more κ-casein was not-sedimented, especially at low pH values (even in coagulated samples): hence, the pH-dependency became less upon longer heating. Not-heated preheated concentrate contained more non-sedimentable κ-casein than did the preheated milk, the amount being larger at a higher pH of the concentrate.

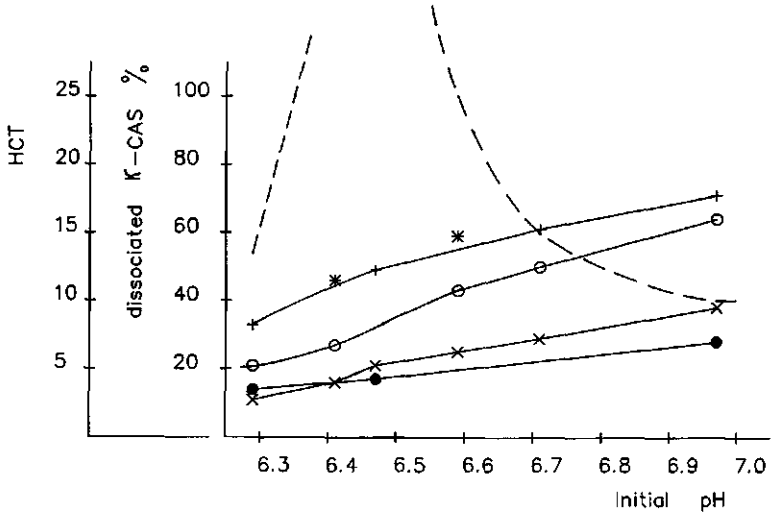

Fig. 3. Dissociated κ-casein, as calculated from the amounts of CMP in concentrate and corresponding supernatant, in preheated concentrate at various pH, after various heating times. • not-heat-treated concentrate; × heat-treated for 2 min; o heat-treated for 5 min; + heat-treated by 10 min; * heat-treated for 15 min. The HCT is indicated by – – –.

Heating-up preheated concentrate resulted in more not-sedimented κ-casein at high initial pH, but in a little less at low initial pH (Fig. 3). The dissociated amounts above pH 6.6 were considerably lower than in not preheated concentrate. After longer heating, gradually more not-sedimented κ-casein was found at all pH values; after 10 min heating the amounts found were about equal to those in not-preheated concentrate, and heating for 15 min did not result in any more not-sedimented κ-casein. Addition of phosphate to preheated concentrate resulted in more κ-casein in the supernatants in the investigated pH and heating time ranges (Table 3).

In preliminary experiments with the RP-HPLC method, about 20 % of the α_{s2}-casein seemed to be present in the supernatant of thermalized milk, and about 7 % of the β-casein. However, this was for a large part due to some carry-over occurring; therefore in later experiments the supernatant of milk was analysed before the milk itself and a blank was put between the milk and the other supernatants. We did not account for carry-over between the different supernatants, since these samples contained little or no α_{s2}-casein, and they were analysed in order of increasing protein concentration. A more serious drawback of the RP-method was that less protein was detected in heated milk than in thermalized milk: in milk-heat-treated for 3 min at 120 °C the detected amounts of α_{s1}-, β-, and κ-casein, and serum proteins were 90, 95,

85, and 75 % of the amounts detected in thermalized milk, respectively. Apparently, heating changes part of the proteins to such an extent that they no longer elute under the conditions used. Denaturation of the (serum-)proteins, or polymerization due to S-S bond formation, cannot be the cause of this change, because all proteins are denatured and S-S bonds are broken during sample pretreatment. Neither can hydrolysis of proteins be an important cause, since it affects at most 2 % of the total protein, as can be calculated from data given in Ref. 19, p. 174. Whatever its cause, the decrease in detectability may well be different for proteins in particles than for proteins in the serum, and we think that it may be higher for proteins in particles, which are relatively close together, than for proteins in the serum, in particular at 120 °C in the absence of hydrophobic bonds. Thus, it was decided to express not-sedimentable protein in the (heat-treated) concentrates as a percentage of the amount detected in thermalized milk, although this may underestimate the amounts actually dissociated.

The RP-HPLC chromatogram of milk and those of the supernatants from not preheated concentrate at pH 6.56 heat-treated for different times are given in Figs 4a and b. In the chromatogram of the supernatant of not-heat-treated concentrate, only very small (α-, and β-)casein peaks can be observed, and large serum protein peaks. The chromatogram of the supernatant of 2 min heat-treated concentrate shows fairly distinct κ-casein peaks, and the serum protein peaks are smaller. After longer heating, all the casein peaks are larger and less distinct. It is noteworthy that in the supernatant of not-heat-treated concentrate (or milk) mainly the later-eluting κ-casein fractions, which are the carbohydrate- and/or phosphate-lacking proteins (16), were present, while in the supernatants of heat-treated concentrate (or milk) the earlier-eluting fractions were dominant, as in unheated milk itself. The percentages of not-sedimented protein are tabulated in Tables 4, 5 and 6.

With RP-HPLC, more κ-casein was detected in the supernants of milk and not-heat-treated concentrate than with the CMP-method. However, the amount in milk was about the same as that found by Dalgleish & Law in raw milk (14). This may be due to the main κ-casein peak on the RP-chromatogram of not-heat-treated supernatant being that of a not-glycosylated κ-casein, whereas the CMP of this protein is not soluble in 8 % TCA (20), and is thus not detected by the CMP-method. In supernatants of heat-treated concentrate the amounts detected by RP-HPLC were less than those detected with the CMP-method, the more so for a longer heating time. This may well be largely due to the decrease in detectability of κ-casein by RP-HPLC in heat-treated samples, while the fractions of the various κ-caseins in supernatants of heat-treated samples were about the same as those in milk.

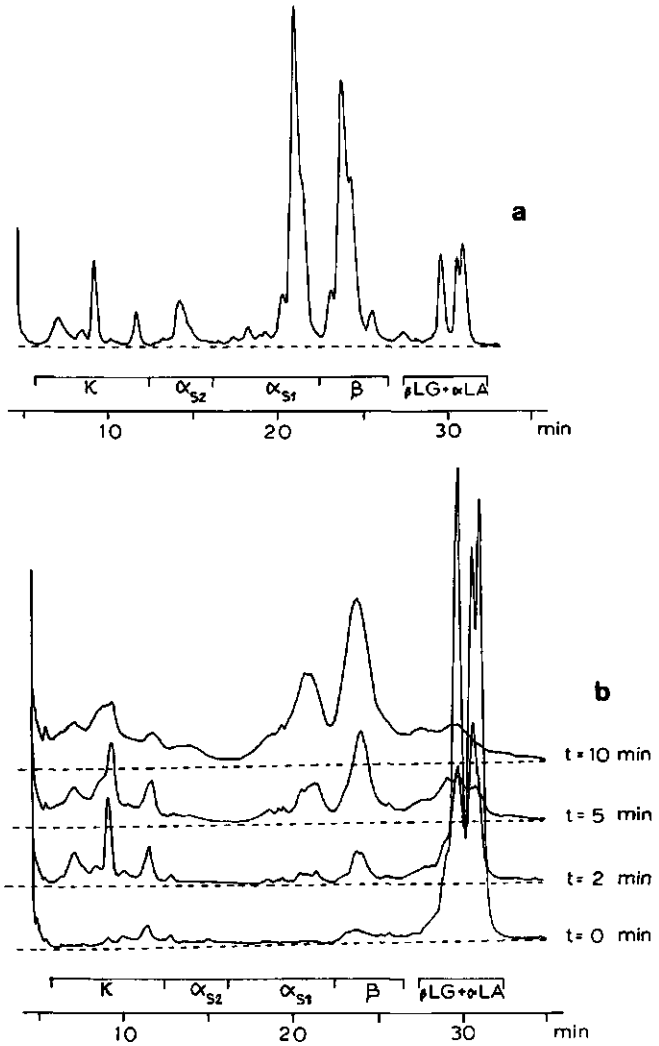

Fig. 4. a) Reversed-phase-chromatogram of thermalized milk, attenuation 10^9. b) Reversed-phase-chromatograms of the supernatants of not-preheated concentrate, pH 6.6, heat-treated for various times, attenuation 10^{11}.

The amount of not-sedimented α-LA + β-LG in not preheated concentrate after heating-up to 120 °C showed a pH-dependency similar to the amount of not-sedimented κ-casein (Table 4). Longer heating resulted in less α-LA + β-LG in the supernatants at all pH-values investigated. However, since the

Table 4. Proteins in (heat-treated) serum, as a percentage of their total quantity as detected in the thermalized milk (P); fractions of the individual caseins of the total serum casein (F). Not-preheated concentrate and the milk it was prepared from.

Sample	Protein	Heating time at 120 °C (min)							
		0		2		5		10	
		(P)	(F)	(P)	(F)	(P)	(F)	(P)	(F)
thermalized milk	$\varkappa$-cas	7	31						
	α_{s1}-cas	2	17						
	β-cas	5	42						
	αLA + βLG	93							
not-preheat. milk	$\varkappa$-cas	9	35						
	α_{s1}-cas	≤2	15						
	β-cas	4	36						
	αLA + βLG	91							
conc. pH 6.40	$\varkappa$-cas	9	39	14	54	24	44	34	32
	α_{s1}-cas	≤2	16	2	7	4	22	10	27
	β-cas	3	32	2	19	6	30	15	37
	αLA + βLG	98		28		8		8	
conc. pH 6.48	$\varkappa$-cas			16	58	31	43	36	29
	α_{s1}-cas			≤2	15	5	21	12	28
	β-cas			2	18	9	31	18	39
	αLA + βLG			26		13		11	
conc. pH 6.56	$\varkappa$-cas	9	40	21	59	33	45	43	31
	α_{s1}-cas	≤2	18	2	15	5	21	13	27
	β-cas	2	27	3	19	9	30	20	37
	αLA + βLG	99		28		15		17	
conc. pH 6.69	$\varkappa$-cas			32	62	47	45		
	α_{s1}-cas			2	13	7	20		
	β-cas			4	20	12	30		
	αLA + βLG			36		26			
conc. pH 6.95	$\varkappa$-cas	9	42	51	60	56	47	52	31
	α_{s1}-cas	≤2	15	4	14	8	19	16	27
	β-cas	2	29	8	23	14	30	23	36
	αLA + βLG	100		51		39		32	

amount of sedimented (^{3}H-labelled) β-LG in heat-treated milk was found to remain constant after 4 or more minutes heating (2), this decrease is likely to be (partly) due to the decrease in detectability of α-LA + β-LG by RP-HPLC. In preheated concentrate, most α-LA + β-LG was sedimented. Heating at high initial pH resulted in less sedimented α-LA + β-LG, and after 5 min heating the partition of these proteins in preheated concentrate was not significantly different from that in not-preheated concentrate. In concentrate with added phosphate, serum-protein association was reduced.

For supernatants of unheated samples, no accurate results could be ob-

tained for α_{s1} and α_{s2}-casein, because the corresponding peaks were of small height and large width. Furthermore, the small peak eluting at 13 min (Fig. 4b, t = 0 min) may be a $\varkappa$-casein, but it coelutes in milk with α_{s2}-casein (Fig. 4a). On the chromatograms of the supernatants of heat-treated concentrate (Fig. 4b) a fairly distinct α_{s1}-casein peak can be seen, but for α_{s2}-casein this was not so. Thus, results are given for α_{s1}-casein, if present in appreciable quantities, but not for α_{s2}-casein. The amount of not-sedimented β-casein in thermalized milk was smaller than is reported for raw milk in Ref. 14. The difference may well be due to the pretreatment of the milk — cold storage, thermalizing, cold storage, and equilibration for one night at 20 °C in this investigation, and equilibration at 20 °C after milking in Ref. 14. Upon heating the concentrates at 120 °C the not-sedimented amounts of α_{s1}- and β-casein increased approximately linearly with time, the rate increasing with increasing pH. Even heating-up to 120 °C resulted in some dissociation, in particular at high initial pH, and in samples with added phosphate. In preheated concentrate somewhat more not-sedimented α_{s1}- and β-casein was found. In preheated concentrate with added phosphate dissociation of these proteins was far more extensive than in concentrate without added phosphate: after heating concentrate with added phosphate at pH 6.52 for 5 min the supernatant contained about the same amounts as a concentrate without after 10 min at pH 6.97 (Tables 5 and 6).

The amounts of the different caseins as a percentage of the total supernatant casein were also calculated. The total peak area in a supernatant between a retention time of 4.5 and 26.5 min was taken as 100 %; the area in the α_{s2}-range was not allocated because it may contain some α_{s1}- and $\varkappa$-casein. From the values tabulated in Tables 4, 5 and 6, it can be concluded that the proportions of the serum caseins depended little on pH, but strongly on the heating time. For serum protein-free concentrated milk, Aoki et al. (7) reported about 55 % $\varkappa$-casein, 20 % β-casein, and 20 % α_s-casein in the 'soluble' casein obtained after heating to 140 °C, which is somewhere between our results after 2 min and 5 min at 120 °C (including the heating-up time) for a concentrate at any pH.

Discussion

The results presented above, as well as those in other investigations on this subject, should be interpreted with great care, since they only show the effects of irreversible, or at most slowly reversible, heat-induced changes of the proteinaceous particles on the subsequent partition of the proteins at 20 °C. For the heat stability of (concentrated) milk, however, only changes occuring during heating are important. Hence an attempt must be made to separate

Table 5. Proteins in (heat-treated) serum, as a percentage of their total quantity as detected in the thermalized milk (P); fractions of the individual caseins of the total serum casein (F). Preheated concentrate and the milk it was prepared from.

Sample	Protein	Heating time at 120 °C (min)									
		0		2		5		10		15	
		(P)	(F)	(P)	(F)	(P)	(F)	(P)	(F)	(P)	(F)
thermalized milk	κ-cas	7	30								
	α_{s1}-cas	2	19								
	β-cas	4	36								
	αLA + βLG	101									
preheated milk	κ-cas	8	31								
	α_{s1}-cas	2	22								
	β-cas	4	31								
	αLA + βLG	13									
conc. pH 6.29	κ-cas	12	65	9	55	15	37	24	25		
	α_{s1}-cas	≤2	8	≤2	12	3	21	9	28		
	β-cas	≤2	13	≤2	19	6	36	16	42		
	αLA + βLG	13		7		4		4			
conc. pH 6.41	κ-cas			14	58	20	38			29	19
	α_{s1}-cas			≤2	13	4	22			14	28
	β-cas			2	20	7	34			27	47
	αLA + βLG			10		8				4	
conc. pH 6.47	κ-cas	14	64	15	56			34	24		
	α_{s1}-cas	≤2	9	≤2	13			13	28		
	β-cas	≤2	15	2	23			22	41		
	αLA + βLG	17		11				11			
conc. pH 6.59	κ-cas			19	57	35	40			42	22
	α_{s1}-cas			≤2	13	6	22			18	29
	β-cas			3	24	11	34			32	43
	αLA + βLG			16		19				12	
conc. pH 6.71	κ-cas			22	54	38	37	45	26		
	α_{s1}-cas			2	14	8	24	17	28		
	β-cas			4	25	14	35	27	40		
	αLA + βLG			21		26		23			
conc. pH 6.97	κ-cas	21	54	31	49	49	37	52	25		
	α_{s1}-cas	2	24	4	18	11	24	20	28		
	β-cas	4	24	7	28	19	36	33	40		
	αLA + βLG	25		28		36		27			

changes during heating from those occuring afterwards.

1 Effect of a heat-treatment on the association of the proteins after cooling
In unheated milk, the casein micelles have a fairly rigid structure, mainly because of the presence of micellar calcium phosphate and, in the case of κ-casein, the existence of S-S-linked oligomers. Hence, small changes in environmental conditions have only a small effect on the partition of the caseins

Table 6. Proteins in (heat-treated) serum, as a percentage of their total quantity as detected in the thermalized milk (P); fractions of the individual caseins of the total serum casein (F). Preheated concentrate and the milk it was prepared from, + with added phospate (18 mmol/l), – without.

Sample	Protein	Heating time at 120 °C (min)					
		0		2		5	
		(P)	(F)	(P)	(F)	(P)	(F)
conc. pH 6.25 –	$\varkappa$-cas			10	34		
	α_{s1}-cas			2	18		
	β-cas			4	34		
	αLA + βLG			6			
conc. pH 6.29 +	$\varkappa$-cas	14	46	17	43	33	28
	α_{s1}-cas	2	15	3	20	12	29
	β-cas	4	29	5	28	20	38
	αLA + βLG	13		11		13	
conc. pH 6.36 –	$\varkappa$-cas			10	42		
	α_{s1}-cas			≤ 2	16		
	β-cas			3	30		
	αLA + βLG			5			
conc. pH 6.38 +	$\varkappa$-cas	12	46	17	42	36	26
	α_{s1}-cas	2	16	3	20	15	30
	β-cas	3	26	5	30	24	39
	αLA + βLG	10		9		16	
conc. pH 6.52 –	$\varkappa$-cas			13	46		
	α_{s1}-cas			2	15		
	β-cas			4	30		
	αLA + βLG			10			
conc. pH 6.52 +	$\varkappa$-cas	13	47	24	40	51	25
	α_{s1}-cas	2	17	4	21	22	31
	β-cas	3	24	9	33	34	39
	αLA + βLG	13		20		32	

between casein micelles and serum. As is clearly shown by several observations reported in the literature, as well as by some of our own results, a similar variation of the pH or the temperature of heat-treated (concentrated) milk results in a much larger change in the partition of the caseins. One observation is that if milk had been heat-treated at high initial pH and subsequently centrifuged at the pH of heating or after readjustment to pH 6.7, the amount of not-sedimented $\varkappa$-casein was larger if the milk was centrifuged at the pH of heating than if it was centrifuged at pH 6.7 (21); i.e. in the (presumably short) period between pH-adjustment and centrifugation, part (about 40 %) of the dissociated $\varkappa$-casein reassociated, either with the casein micelles, or perhaps to form different sedimentable particles. Our results show another, but similar, phenomenon. The amount of $\varkappa$-casein not sedimenting at 20 °C was larg-

er in the concentrate prepared from preheated milk than in the preheated milk itself, although the heat treatment during concentrating was slight, and the pH of concentrated milk is lower than that of milk. In addition, adjustment of the pH of the concentrate to higher values, followed by overnight storage at 20 °C, resulted in still more not-sedimented κ-casein. In not preheated concentrate these phenomena did not occur. A third interesting observation in this respect is that, upon cooling from 20 to 4 °C, the increase of not-sedimentable κ-casein in milk (18), and that of α_s-, β-, and κ-casein in serum protein-depleted concentrate (22), was found to be much larger for heat-treated for the corresponding not-heat-treated samples. These observations indicate that the integrity of the casein micelles is weakened by heating, i.e. that heating results in some irreversible change of the structure of the micelles (see Part 3 for the possible nature of such a change), as was also concluded in Ref. 22. Consequently, a change of temperature (4-30 °C) or pH, which affect e.g. hydrophobic and ionic interactions, has a larger effect in heat-treated than in unheated milk. The tendency of the various caseins to associate may be affected differently by different types of irreversible changes of the casein micelles; hence, a certain heat-treatment may have a different effect on the partitions of the various caseins after heating (see Part 3). For κ-casein, a heat-treatment of 5 min at 70 °C is, apparently, sufficient to induce such a change, since ref. 18 reports an appreciably larger amount of non-sedimentable κ-casein in milk heat-treated at its natural pH for 5 min at 70 °C than in unheated milk, if both were stored for 24 h at 5 °C before centrifugation.

The association of the denatured serum proteins may be considered as a similar process: denaturation and S-S-interactions result in a greatly changed aggregation behaviour, while conditions like pH and temperature determine the partition of these proteins between serum and sedimentable particles after heating.

2 Partition of the proteins at high temperatures

As for changes in partition actually occurring at the high temperature, this depends on the rate at which the (irreversible) change of the structure of the micelles proceeds, as well as on the strength of hydrophobic interactions and on the extent to which ionic bonds (whether or not mediated by some positive ion) are present at the high temperature. It can be estimated from indirect evidence only. The mobility of part of the casein molecules in a casein micelle dispersion in simulated milk ultrafiltrate at pH 6.7 was found to increase progressively above 65 °C (up to 95 °C, Ref. 23). This increase was reversible upon cooling to 20 °C; both heating and cooling were done slowly. A higher

mobility of the caseins at high than at low temperature does not necessarily imply dissociation, but it at least indicates that the total interaction between the caseins is less at high than at low temperature. Presumably, hydrophobic interactions are weak at 80-90 °C and virtually absent above 100 °C, and, moreover, the effect of changes in conformational entropy becomes larger at a higher temperature. The higher mobility at a higher temperature shows that any change in ionic bonding at most partly compensates for these effects. All in all, it is quite likely that dissociation of caseins actually occurs during heating. Micelle-like particles, however, still exist at 120 °C (10). This may well be attributed to the reduced solubility of calcium phosphates at high temperature, which may result in precipitation of calcium phosphate of a composition different from and more stable than the native micellar calcium phosphate, but still able to link casein molecules to the micelles (25); see, however, Part 3 for the possible effect of a longer heat-treatment. The observation that the increased mobility at high temperature is reversed upon cooling implies that either the heat treatment during the determination of the mobility was insufficient to induce an irreversible change in the structure of the casein micelles, or that such a change of structure does not result in a higher mobility of the caseins at 20 °C. After all, heating milk for 10 min at 90 °C at pH 6.7 did not result in more non-sedimentable $\varkappa$-casein at 20 °C than before heating (e.g. 21), but heating for 5 min at 90 °C did, at 5 °C (18). Also, $\varkappa$-casein molecules in (not-sedimentable) $\varkappa$-casein aggregates (micelles) were found to have a low mobility at 20 °C (24).

In conclusion, the total interaction forces between the protein molecules are likely to be smaller at 120 °C than at 20 °C, and thus at 120 °C more proteins may be present in the serum than after cooling to 20 °C. Hence, changes in the partition of the proteins which appear to be small as judged from experimental results obtained after cooling to 20 °C may still be considerable at 120 °C and thereby have a distinct effect on heat stability.

3 Effects of heating time, pH and phosphate addition on the association and dissociation of the proteins

Our results show that the association and dissociation of each protein is affected in a different way by the variables investigated. To explain the observed differences it is useful to distinguish the effects of a heat-treatment on the integrity of the micelles from the effect of factors that also depend on conditions after heating. Of course, from data on association and dissociation only indirect indications of possible mechanisms can be derived, but at the moment this is the best possible.

For α_{s1}- and β-casein we found an almost linear increase of not-

sedimented protein with heating time. This indicates that the main factor inducing dissociation is a slow change in the integrity of the casein micelles, since any dissociation induced by a change in e.g. hydrophobic interactions or ionic bonds is likely to depend almost instantaneously on temperature rather than on heating time. To be sure, an instantaneous change of some interaction with temperature may result in a slow change in dissociated casein, but it seems quite unlikely that, after cooling, this would result in a linear increase for more than 10 min. A change in the integrity of the casein micelles that affects the association of α_{s1}- and β-casein may well be a change of part of the native micellar calcium phosphate into a different, more stable, form, resulting in a reduced binding to casein. This idea has been put forward by Van Dijk (25), and is in agreement with recent results of Aoki et al. (30). It is not unlikely that such a change proceeds faster in concentrate at a higher pH, or with added phosphate, because of the higher supersaturation of calcium phosphates in such concentrates, thereby explaining the faster dissociation in such concentrates. In addition, the virtual absence of hydrophobic interactions at 120 °C appears to play a part, considering the preponderance of β- over α_{s1}-casein in the supernatants of heat-treated concentrates. Apparently, cooling does not result in a complete reassociation of (especially β-)casein with the depleted casein micelles, or in the formation of different sedimentable particles. The tendency of these proteins to associate, however, will almost certainly be stronger at 20 than at 120 °C, even in heat-treated concentrates, in which the integrity of the casein micelles is presumably smaller.

The principal heat-induced change of the α-LA + β-LG molecules is, of course, the denaturation of these proteins, and this results in a great change in aggregation behaviour (e.g. Ref. 26). Denaturation proceeds rapidly upon heating at 120 °C, and so did the change in sedimentable β-LG in heat-treated milk, before reaching a constant value (2). Thus, exept for denaturation, heat-induced changes of these proteins appear to have little effect on their aggregation behaviour. This may explain the almost equal amounts of not-sedimented α-LA + β-LG in not-preheated and in preheated concentrate after 5 or 10 min heating. In Ref. (2), an S-S-mediated complex of β-LG and κ-casein was shown to be present in a heat-treated suspension of casein micelles and [^{3}H]-β-LG in a milk salt buffer. The sedimentability of this complex was shown to depend strongly on both $a\mathrm{Ca}^{2+}$ and pH. In agreement with these observations, we found a strong dependence of the sedimentability of α-LA + β-LG on pH, and a rather large effect of phosphate addition. Thus, ionic (Ca^{2+}-mediated) interactions appear to be of great importance for the aggregation of these proteins. Considering that, in (concentrated) milk, a lower initial pH always goes along with a higher $a\mathrm{Ca}^{2+}$ (12), the $a\mathrm{Ca}^{2+}$ may even be the prin-

cipal factor for the association of α-LA + β-LG.

As argued in ref. 10, the dissociation of κ-casein upon a short heating, and its dependence on pH and $a\mathrm{Ca}^{2+}$, may well be explained by the heat-induced association of β-LG and κ-casein. Our results show that the dissociation in not-preheated concentrate after 2 min heating is similar to that in milk, although apparently more κ-casein was dissociated in concentrate than in milk at a certain pH (compare Fig. 2 with Ref. 27, Fig. 2), in agreement with Ref. 28. Apart from an effect of heating time and temperature, this may be due to the lower $a\mathrm{Ca}^{2+}$ in concentrate than in milk at a certain pH (12), or to the lower $\{a^2(\mathrm{Mg}^{2+}) + a^2(\mathrm{Ca}^{2+})\}/\{a\mathrm{Na}^+ + a\mathrm{K}^+\}$ in concentrated milk. In preheated concentrate, the changed partition of κ-casein after heating-up may also be due to the behaviour of the β-LG-κ-casein complex. Longer heating of preheated and not-preheated concentrates resulted in more dissociated κ-casein, but less not-sedimented α-LA + β-LG. To explain this observation, disruption of the complexes, and in particular of the S-S-bonds in them, is needed. Even if it is assumed that β-LG-κ-casein complexes are only present in briefly heat-treated concentrates above a pH of about 6.5 (which may be considered as the minimum needed to explain the dissociation of κ-casein upon a short heating) disruption of S-S-bonds upon longer heating must occur below a pH of about 6.5, since κ-casein is present in the casein micelles as S-S-linked oligomers (29), and dissociation of these oligomers is not likely to proceed at a rate higher than that of e.g. β-casein. Thus, it looks as if S-S-bonds are not very stable at 120 °C. Reactions like the formation of cysteic acid or dehydroalanine residues, as well as disulphide interchange, from a micellar disulphide-linked complex to a not-sedimentable disulphide-linked complex may play a part. Assuming that S-S-bonds are not stable, dissociation of κ-casein from the micelles at 120 °C may be expected, considering the great importance of hydrophobic bonds for the association of κ-casein. At 120 °C, the dissociated κ-casein may be present as a monomer or as oligomers, and after cooling to 20 °C the tendency for κ-casein to associate will be stronger than at 120 °C, but this apparently induces only a limited reassociation with the depleted casein micelles.

In conclusion, the amounts of not-sedimentable proteins in concentrates during heating at 120 °C are likely to depend qualitatively on conditions like pH as the amounts detected in heat-treated concentrate at 20 °C. Quantitatively, less rather than more protein is likely to be part of the casein micelles at 120 °C. Since heat stability depends on the stability of the casein micelles as existing at 120 °C, the large differences in association and dissociation of the proteins in concentrated milk are likely to exert a significant effect on heat stability. We will return to this in a subsequent article.

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The heat stability of concentrated skim milk

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Summary

The heat-induced coagulation of concentrated skim milk was studied using seven different methods. These were: determination of the heat coagulation time (HCT), electron microscopy, determination of the amount of non-sedimentable nitrogen after cooling to 20 °C, of the apparent viscosity after cooling to 20 °C, of the rolling time in a Höppler-type viscometer at 120 °C, of the optical density after cooling to 20 °C, and of that at 120 °C, all as a function of heating time. The results show that coagulation of concentrate prepared from not-preheated milk below a pH of about 6.5 occurs within the heating-up period, and is not induced by aggregation of the casein micelles. At all other investigated conditions, coagulation is due to aggregation of casein micelles; below pH 6.5 these particles flocculate into irregularly shaped clusters, whereas above pH 6.5 the aggregates tend to fuse into roughly spherical particles.

A qualitative model for the heat-induced coagulation of concentrated skim milk is proposed. Coagulation of concentrates prepared from not-preheated milk below about pH 6.5 appears to be induced by aggregation of denatured serum proteins, and to depend mainly on hydrogen and calcium ion activities. If casein micelles are the aggregating material, the aggregation rate is determined by a combination of the colloidal stability of these particles and the rate constant(s) of the bond-forming reaction(s). Colloidal stability is supposed to be greatly affected by the dissociation of κ-casein from the micelles, which proceeds faster at high initial pH. The activation free energy of bond formation depends strongly on temperature and on the type of bonds formed. Salt bridges (ionic, Ca^{2+}-mediated or micellar calcium phosphate-like) are likely to be the predominant type of bonds involved in heat coagulation. The latter type appears to be important only if the casein micelles are κ-casein depleted, i.e. at high initial pH. Covalent cross-linking of proteins may also be involved in heat coagulation, but only if specific protein residues can come into close contact. A large heat-induced pH-decrease, or depletion of κ-casein from the micelles, presumably promote such contacts. In addition to the rate of aggregation of the particles, the HCT is supposed to depend strongly on the geometry of the emerging aggregates. Dissociation of κ-casein and the high supersaturation of calcium phosphates in concentrated milk are presumed to be the principal factors determining this geometry.

A combination of these factors is used to qualitatively explain the shape of the HCT-pH plot, the effects of preheating, and those of phosphate and formaldehyde addition.

1 Introduction

Concentrated milk must be able to withstand sterilisation. Regulating heat stability, however, is not a problem 'of producing maximum heat stability but it is a problem of achieving optimum viscosity' as was stated by Hunziker (1). To our knowledge, only one investigation, by Deysher et al. (2), has dealt with the relationship between the heat stability of concentrated milk and the viscosity it attained during heating. Other authors were aware of the duality of the heat stability problem, if only for its practical importance, but focused their attention on the effects of compositional and processing factors on the time necessary to initiate visible coagulation. In our opinion, the limited attention paid to the way in which coagulation proceeds has been the principal reason for the limited advance in understanding heat stability, although much factual information has been gathered (see e.g. Rose (3), Muir (4), and Singh et al. (5) for reviews). Most studies were done on unconcentrated milk since it was generally felt that the heat stability of concentrated milk is even more complicated than that of milk. We agree, however, with Pyne (6) in that 'coagulation of concentrated milk is a simpler type of phenomenon to study, in the sense of involving the intervention of fewer factors'.

The first attempt to explain variation in the heat stability of milk and concentrated milk was the so-called 'salt balance theory' of Sommer & Hart (7). Although the heat stability control in industrial practice is still based on the results of the experiments done by these authors, the 'salt balance theory' merely describes the effects of, on the one hand calcium and magnesium and, on the other, phosphate and citrate on the HCT of (concentrated) milk, and it can hardly be called a theory for the heat-induced coagulation of (concentrated) milk. The 'working hypothesis' advanced by Rose (3) in 1963 was much more complete, being based on the stability of the 'colloidal caseinate-phosphate' in milk, and the presumed heat-induced changes therein. Of these, changes of the micellar surface, including complexation with β-lactoglobulin, were considered important. This qualitative working hypothesis has since been improved, not least because of a better understanding of the structure of the casein particles in milk. Only recently, results on the change in particle size upon heating milk were published (8, 9, 10), and a quantitative model for the heat-induced coagulation of unconcentrated milk based on these data was proposed (10, 11).

For concentrated milk, a quantitative interpretation of experiments on the aggregation of particles is more difficult than for unconcentrated milk: optical methods are hampered by considerable dependent and multiple scattering, viscosity measurements by non-Newtonian behaviour, and methods based on

sedimentation by mutual hindrance of particles. To overcome the above-mentioned problem, it was decided to study the heat-induced coagulation of concentrated skim milk by several analytical methods. The methods used were: the subjective (13) and objective (14) tests of Davies & White, determination of an apparent viscosity after cooling to 20 °C, use of the Klaro-graph (15), determination of the turbidity at 120 °C and after cooling to 20 °C, and electron microscopy. The use of the Klaro-graph, which is a Höppler-type viscometer operating at high temperatures, and measurement of the change of the optical density at 120 °C, were included to minimize the probability of misinterpretation of the results by any specific effect of cooling to 20 °C after heating.

The results obtained in this, and in previous investigations on concentrated milk (16, 17) will be used to extend the model for unconcentrated milk (10, 11) to concentrated skim milk.

2 Materials and methods

2.1 Analytical methods

2.1.1 Subjective heat stability test. We used the procedure as given by Davies and White (13), at 120 °C, except for the observation of the coagulation, which was done by the naked eye. The time required for gelation of the sample or for particles to become visible throughout the sample (whichever came about first) was taken as the HCT.

2.1.2 Objective heat stability test. The procedure we used was similar to that of White & Davies (14). Samples of concentrate (5 ml) were heat-treated for various times at 120 °C in rocking stainless steel tubes of 6.5 ml capacity. After cooling, the contents of two tubes were quantitatively transferred to a centrifuge tube. Also, 10 ml unheated concentrate was centrifuged. White & Davies centrifuged for 15 min at 300 × g, but preliminary trials showed that the centrifugal force needs to be higher for concentrated milk, particularly at low initial pH. This is to be expected, since the viscosity of the continuous phase is higher and the effect of mutual hindrance is larger in concentrated milk. To yield a similar extent of sedimentation, the product of centrifugal acceleration and time should be about 6-19 times higher for concentrated milk than for milk, as can be calculated by the various relations given by Walstra & Oortwijn (18). To obtain a firm sediment and a more or less homogeneous supernatant, we used 30 min at 2000 × g. Sediment and supernatant were separated by turning the centrifugation tubes upside down for about 10 s. The volume of the sediment was measured. Total and non-protein nitrogen in the

supernatants and the nitrogen content of a not centrifuged concentrate were determined as ammonia on a Technicon autoanalyser (19).

2.1.3 Determination of the apparent viscosity at 20 °C. The concentrate was heat-treated as in the objective heat stability test. After cooling to 20 °C, the apparent viscosity was determined with a capillary tube viscometer, operating at a constant average shear rate of about 1100 s^{-1}.

2.1.4 The Klaro-graph. The Klaro-graph is a Höppler type viscometer operating at high temperatures (15). We used the version with tubes of an inner diameter of 9.30 mm and a glass ball of 9.0 mm, and a temperature of 120 °C.

2.1.5 Turbidity at 20 °C. The concentrate was heat-treated as in the objective heat stability test. After cooling to 20 °C the turbidity was measured in a Zeiss PMQ2 spectrophotometer with an attachment for turbidity measurements (20). The absorbance was determined at a wavelength of 1120 nm in a 1 mm cuvette. At such a long wavelength, scattering by casein particles can be described by the relatively simple Rayleigh-Gans-Debije theory up to a diameter of about 4 μm (21), which facilitates interpretation of the results. The wavelength of 1120 nm was choosen because the absorption by water molecules shows a minimum at this wavelength. Water was used as a blank.

2.1.6 Turbidity at 120 °C. A cuvette with an optical path of 0.5 mm that could be heated with silicon oil was constructed for use in the Zeiss spectrophotometer. The housing was made of polypropylene and held 4 parallel quartz windows, at net distances of 5, 0.5, and 5 mm. The milk was in the middle cell, while silicon oil of the desired temperature was pumped through the outer ones. The joint optical density of concentrate and oil layers was determined. About once per hour a measurement was done with water as a blank. This was needed, since the response of the photocell was slightly influenced by the slow warming of the spectrophotometer which occurred upon prolonged use of the heated cuvette.

2.1.7 Electron microscopy. Samples of concentrate heat-treated in stainless steel tubes were prepared for electron microscopy with the microencapsulation technique of Henstra & Schmidt (22). The preparation of the samples and the electron microscopy were done at the Netherlands Institute for Dairy Research (NIZO), using a Jeol 1200 EX microscope.

2.1.8 Solubility of the coagula. Samples of concentrate were heat-treated as in the objective heat stability test. The (coagulated) concentrate was diluted with the following liquids: water 1:10; 0.25 N NaOH to pH 8; 8 M urea 1:4; 0.96% dithiotreitol (DTT) in 8 M urea 1:2; or 0.4 M disodium ethylenediaminetetra-acetate (EDTA) plus 5% Tween 20 1:1. Solubility was checked visually.

2.1.9 Heating profiles. The heating profiles of the concentrate in the different heating vessels are given in Fig. 1. For the heated cuvette it was assumed that the temperature of the milk in the cuvette was equal to the temperature of the circulating silicon oil leaving the housing, which may slightly overestimate the heating-up rate.

2.2 Preparation of the samples

Skim milk was obtained from a local dairy. The milk had been skimmed at 50 °C, and thermalized at 67 °C. It was preheated, either for 2 s at 74 °C or for 3 min at 120 °C, and concentrated as described before (16). We will use 'not-preheated' for the former preheating intensity, and 'preheated' for the latter. Serum-protein-depleted concentrate was prepared by suspending the ultracentrifugal (2 h 70 000 × *g*) pellet of not-preheated concentrate in its own ultrafiltrate.

NaN_3 (0.02%) was added as a preservative. The pH was altered with HCl or NaOH and the concentration was adjusted to 24.5% total solids, i.e. the

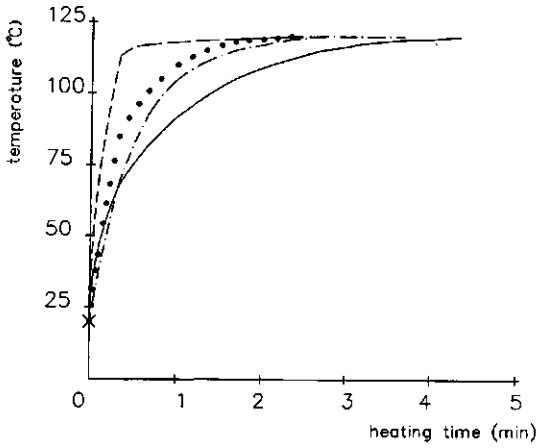

Fig. 1. Heating profiles of the concentrate in the various heating vessels. – – – heated cuvette, ... glass tubes, –·–·– stainless steel tubes, —— Klaro-graph.

same ratio of solids-not-fat to water as in English-standard full-cream evaporated milk. This was done by slowly adding a mixture of the necessary amounts of 1 N NaOH or 1 N HCl and demineralized water, while stirring vigorously. Any phosphate was added as a 10% solution of a mixture of NaH_2PO_4 and Na_2HPO_4 (40/60 on a molar basis), immediately before NaOH or HCl addition. After these additions, the concentrate was stored for at least one night at 4 °C. Formaldehyde (12 mmol/kg) was added to the concentrate as a 9% solution, about 2 h before heating. All experiments were carried out within three days.

The pH of the unheated concentrated milks was determined after slowly warming the samples to 20 °C, and the viscosity after slowly warming to 55-60 °C and then cooling to 20 °C. Split samples were used for measurement of viscosity and optical density, and for the electron micrographs; all tubes were removed from the oil bath and put in water (10-15 °C) within 30 s. The viscosity of the heat-treated samples was determined within 1 h after cooling to 20 °C. Centrifugation was done after completing a series of heatings; the samples had been at room temperature for at most 3 h. The optical density at 20 °C was determined in heat-treated concentrate that had been stored overnight at 15 °C, the unheated samples had been stored at 20 °C for at least 3 h.

3 Results

3.1 The course of the aggregation as followed by different techniques

The results obtained by all the analytical methods varied slightly among experiments with different batches of milk. Both the values found and the pH at which a certain change occurred showed some variation. The differences were, presumably, partly due to the slightly varying composition of milk, but also to small differences in experimental conditions, since measurements with the Klaro-graph, and of the optical density at 120 °C, which involved only filling of the heating vessels, showed the least variation. Unless otherwise stated, the results given below are from the experiments carried out in September 1988.

3.1.1 Heat coagulation times. The HCT-pH plots obtained in 3 experiments with each preheating intensity (hence of 6 different milks) are given in Fig. 2A. In Fig. 2B the HCTs of the same concentrates are plotted against the HCl/NaOH additions to the concentrates. A larger variation exists in the position on the abscissa for the different HCT-pH plots than for the HCT-addition plots. This must at least partly be due to inaccuracy in the determination of the (absolute value) of the pH, for the same reasons as mentioned by

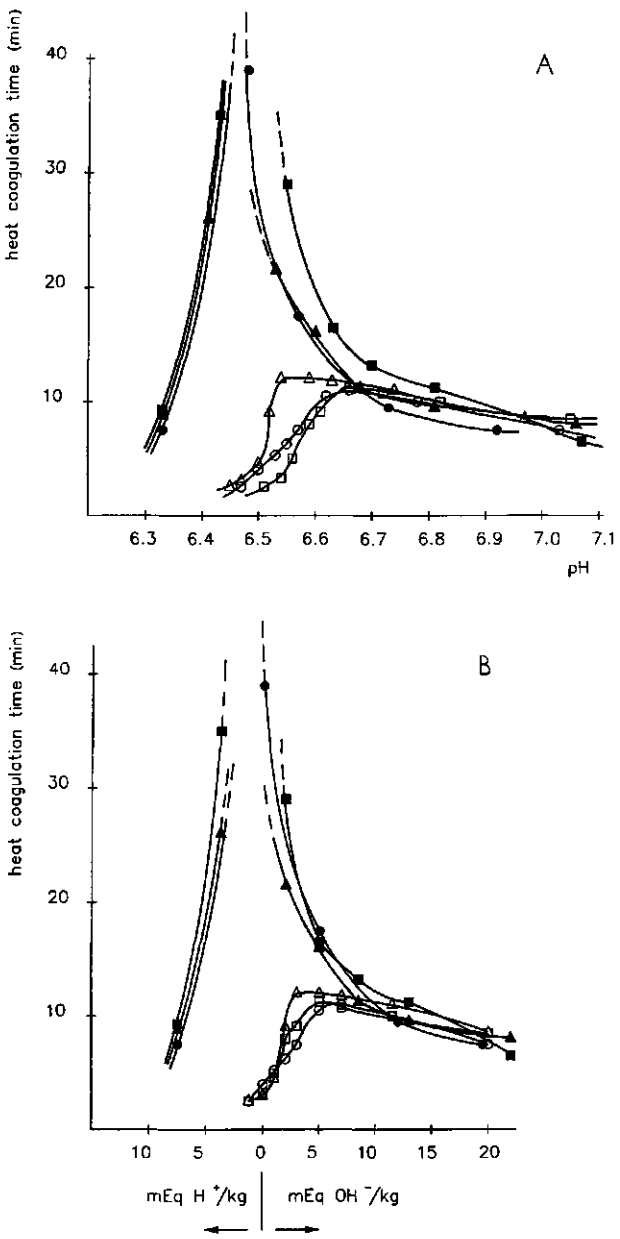

Fig. 2a. HCT-pH plots of various concentrates. Not-preheated concentrate: open symbols, preheated concentrate: closed symbols. Experiments in January/February: ○, ●; experiments in July: △,▲; experiments in September: □,■.

Fig. 2b. HCT plotted against the H^+/OH^- addition to the concentrate before heating. For symbols see Fig. 2a. The natural pH of the concentrates was: not-preheated: 6.50, 6.47, 6.54, in the February, July and September experiments, respectively; preheated: 6.48, 6.49, 6.51, for the January, July and September experiments, respectively.

Darling (23): it was observed that pH differences in one series of concentrate could be determined to 0.02 pH-unit, but if a series was measured on two subsequent days, the level could differ by 0.04 or 0.05 units.

Coagulation at an initial pH below about 6.5 showed in not-preheated concentrate as formation of a gel, and in preheated concentrate as a gradual retardation of the air bubble in the tube, followed by fouling and finally a precipitate that was intermediate between a gel and particles (the shorter the HCT, the more gel-like). At high initial pH, a change in the flow of the liquid was observed some 15-30 s before a sudden appearance of particles.

Above about pH 6.7, preheating had almost no stabilizing effect, below pH 6.6 a very strong one. This is more or less in accordance with the results of Sweetsur & Muir (12), who observed that preheating as such slightly improved the heat stability above pH 6.7, irrespective of the preheating temperature (80, 90, or 100 °C for 10 min), while below pH 6.7 a larger improvement was found, depending on the preheating temperature. In our experiments, the HCT of not-preheated concentrate at pH values above the optimum was only slightly shorter than at the optimum, as compared to results published by other authors (e.g. 24). Small variations in processing or in the total solids content may have caused this difference.

3.1.2 Electron microscopy. Examples of the electron micrographs are shown in Fig. 3 (experiments in July 1988). Large differences, particularly in the size of the particles, can be seen between the particles that emerged at different heating conditions. Particles in not-preheated concentrate heat-treated for 3 min at pH 6.47 were hardly larger than those in unheated concentrate. In preheated concentrate at pH 6.33, heating-up mainly resulted in larger particles: the average diameter of the cross-sections of 100 casein particles increased from 75 nm in unheated concentrate to 150 after 2 min, and 170 nm after 9 min heating. Some particles had developed a regular core-and-lining ultrastructure after heating, which was similar to the structure observed by Harwalkar & Kalab (25) in heat-treated milk gels at pH 5.5. Also the particles on the micrographs made by Harwalkar & Vreeman (26) of UHT-heated concentrated skim milk had a similar, though less developed structure. After heating at pH 6.52, fairly large particles, having a smooth surface, were present in not-preheated concentrate; in preheated concentrate at pH 6.49, however, flocs of relatively small particles could be observed. After heating either concentrate at a pH of about 6.8, the casein particles had a smooth surface and they had become much larger: after 10 min heating the average diameter of the particles was about 1 μm in not-preheated concentrate, and about 0.7 μm in preheated.

3.1.3 Sedimentation experiments. The N-depletion curves are given in Figs. 4A and B, for the not-preheated and the preheated concentrated skim milks, respectively. None of the samples showed a clear two-step coagulation, as was found for concentrated skim milk at its natural pH by Muir & Sweetsur (24). This may well be due to the considerably more intensive centrifugation conditions used in our experiments. Only for some concentrates did the time at which the first material became sedimentable coincide with the HCT. This is not too surprising, considering the different modes of coagulation at various pH values, as observed at the subjective HCT test. Only if aggregation of micelles results in large, more or less globular particles, are sedimentation methods suitable for determining the progress of aggregation. If, however, particles flocculate into voluminous aggregates and finally a gel, sedimentation of the aggregates will be slow and the gel will be merely compressed during centrifugation. Furthermore, transferring a gel-like coagulate to a centrifuge tube may disperse it.

We found a great difference between the sedimentation behaviour at low initial pH and at that high initial pH. At low initial pH the decrease in non-sedimentable protein upon heating was slow, while all supernatants were turbid. At high initial pH, clear supernatants were obtained after the rapid decrease in non-sedimentable protein around or before the HCT. This indicates that different types of particles emerged at varying pH. The protein nitrogen content of the supernatants was remarkably high: values less than about half of the content in the concentrates were not found. The amount of non-protein-nitrogen increased from 0.9 to at most 1.3 g/kg (after 70 min heating, pH 6.5, preheated concentrate, results not shown).

We also tried to calculate the voluminosity of the protein in the pellet (v_p, ml/g) from the volume of the pellet (V_{pellet}, ml) and the decrease of supernatant nitrogen using the equation:

$$v_p = \frac{V_{pellet}}{(W_{conc} \cdot N_{conc} - W_{sup} \cdot N_{sup})6.4}$$

in which W_{conc} and W_{sup} are the weights (g) of the concentrated milk and of the supernatant, respectively, N_{conc} and N_{sup} the nitrogen contents (g/g) of concentrate and supernatant, respectively, and 6.4 is the Kjeldahl conversion factor. The calculations indicated that v_p at a pH below 6.4 was about twice that at a pH about 6.8 (7-8 and about 4 ml/g, respectively), but the method was not accurate enough for more detailed conclusions to be drawn.

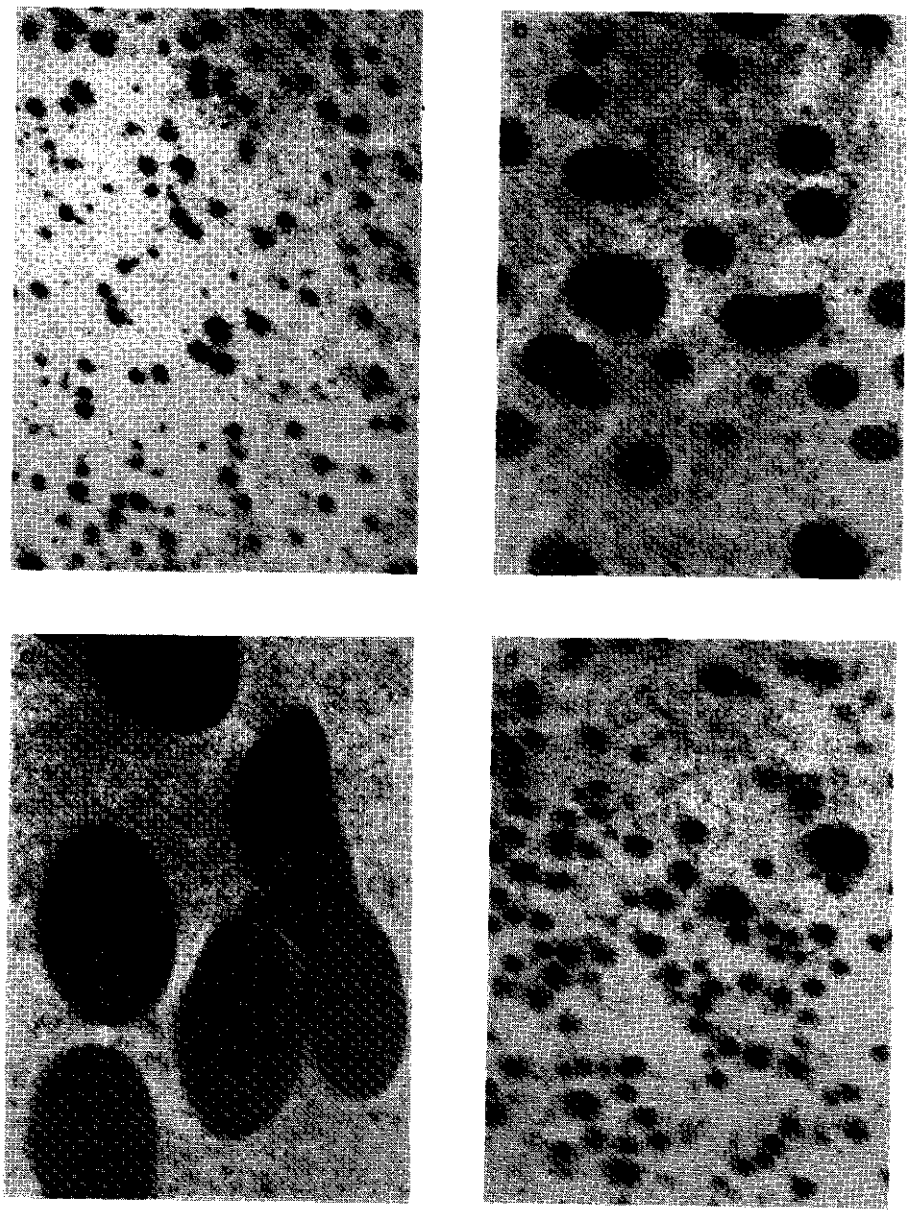

Fig. 3. Electron micrographs (×12 000) of heat-treated concentrate shortly before coagulation. Experiments in July. Not-preheated concentrate, a: pH 6.47 heat-treated for 3 min, b: pH 6.52 heat-treated for 6 min, c: pH 6.74 heat-treated for 11 min. Preheated concentrate, d: pH 6.33 heat-treated for 8 min 45 s, e: pH 6.49 heat-treated for 35 min, f: pH 6.81 heat-treated for 11 min.

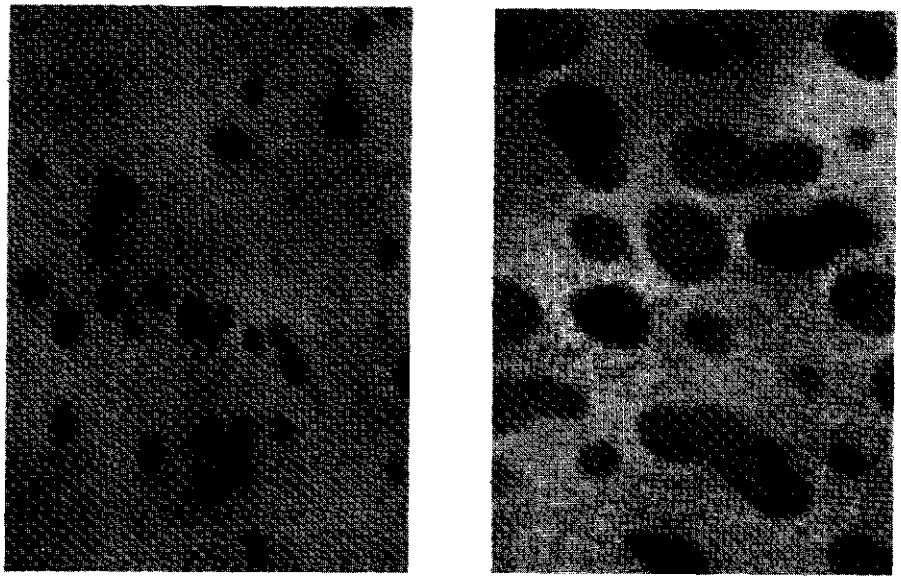

Fig. 3. (Continued).

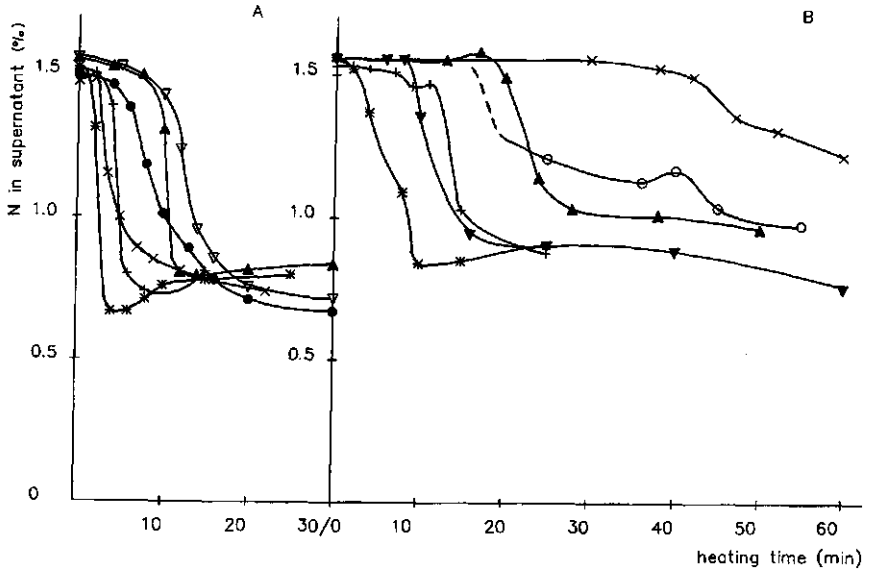

Fig. 4. N-depletion curves for experiments in September. A: not-preheated; × pH 6.54, ● pH 6.57, ▽ pH 6.61, ▲ pH 6.66, + pH 6.82, • pH 7.05. B: preheated; ▼ pH 6.33, ○ pH 6.43, × pH 6.51, ▲ pH 6.63, + pH 6.81, • pH 7.07.

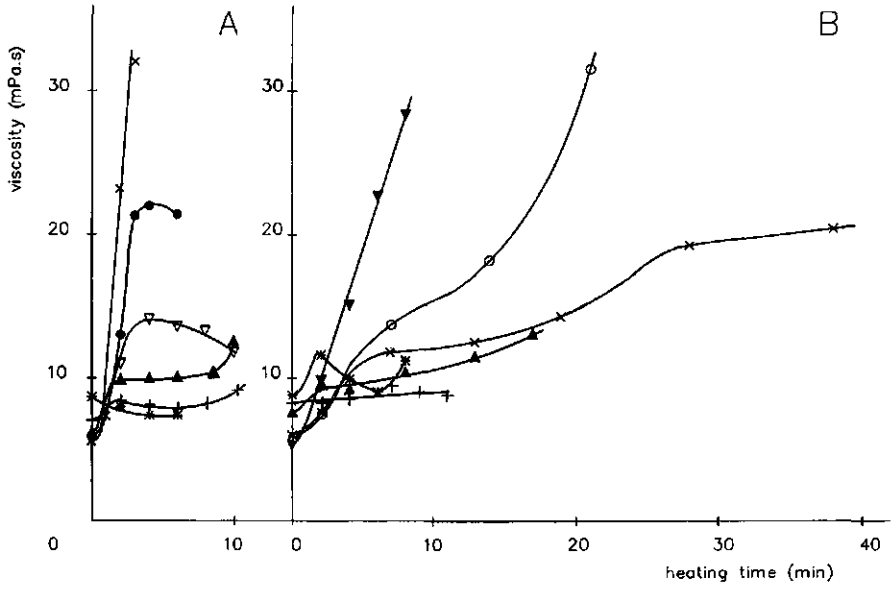

Fig. 5. Apparent viscosity of (heat-treated) concentrates after cooling to 20 °C for experiments in September. A: not-preheated concentrate. B: preheated concentrate. Symbols as in Fig. 4.

3.1.4 Viscosity. The change upon heating of the apparent viscosity at 20 °C depended greatly on the pH of the concentrate (Figs. 5A and B). In both preheated and not-preheated concentrate the viscosity increased continually upon heating at a pH below the optimum. At a pH clearly above the optimum, the viscosity decreased for most of the heating time; an increase was detected only shortly before the HCT. At the optimum pH in not-preheated concentrate, the viscosity first increased, then decreased and in some cases increased again shortly before the HCT. In preheated concentrate, an initial increase was followed by a plateau. The shape of the plots in Fig. 5B for concentrates at intermediate and low pH is quite similar to those given in Ref. (2) for concentrate at its natural pH and after lactic acid addition, respectively.

The time for the ball in the Klaro-graph to roll over 10 cm is plotted against the heating time in Figs. 6A and B. The heating time at which the ball came to a standstill was fairly close to the HCT, considering the difference in heating-up profile, the absence of air in the tube, and the different detection method. The change of the rolling time with heating time was not very different from the change of the apparent viscosity after cooling to 20 °C. This indicates that the change of the relative extent of aggregation was similar at 120 °C and after cooling to 20 °C, at least for as far as could be detected at a high shear

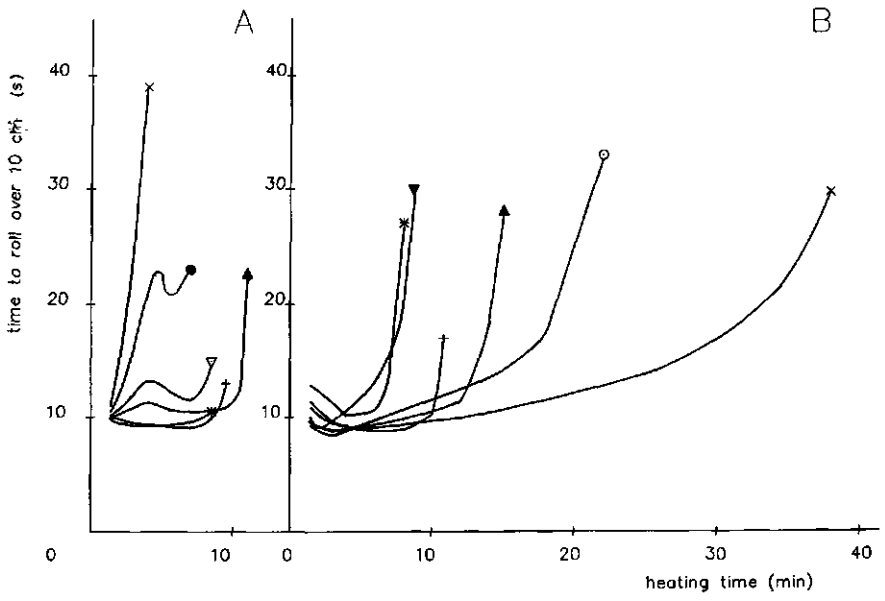

Fig. 6. Rolling time of the glass ball in the Klaro-graph for experiments in September. A: not-preheated concentrate. B: preheated concentrate. Symbols as in Fig. 4.

rate. Small changes in viscosity could be detected less well by the Klaro-graph, while near the HCT changes became magnified, but this is only to be expected, considering that coagulating concentrate is a non-Newtonian liquid, and that the Klaro-graph is essentially a constant-stress viscometer.

3.1.5 Turbidity. The optical densities (E) determined after storing the (heat-treated) concentrates overnight at 20 °C are given in Figs. 7A and B. Quantitative interpretation of the results in terms of number and size of particles is almost impossible for concentrated skim milk, because of the large influence of dependent scattering on the optical density. However, in combination with the results of our other determinations, the change of E can provide part of the picture of the processes occurring during heat coagulation. Apart from effects of aggregation or fusion of particles, the optical density of a heat-treated concentrated skim milk may differ somewhat from that of an unheated sample due to a change of the total dry mass present in particles. This mass will change by association and dissociation of proteins, which may involve up to 25% of the total protein (17). The heat-induced association of calcium phosphate with the particles is only of minor importance if E is determined some time after cooling to 20 °C, due to the net reversibility of this association (16).

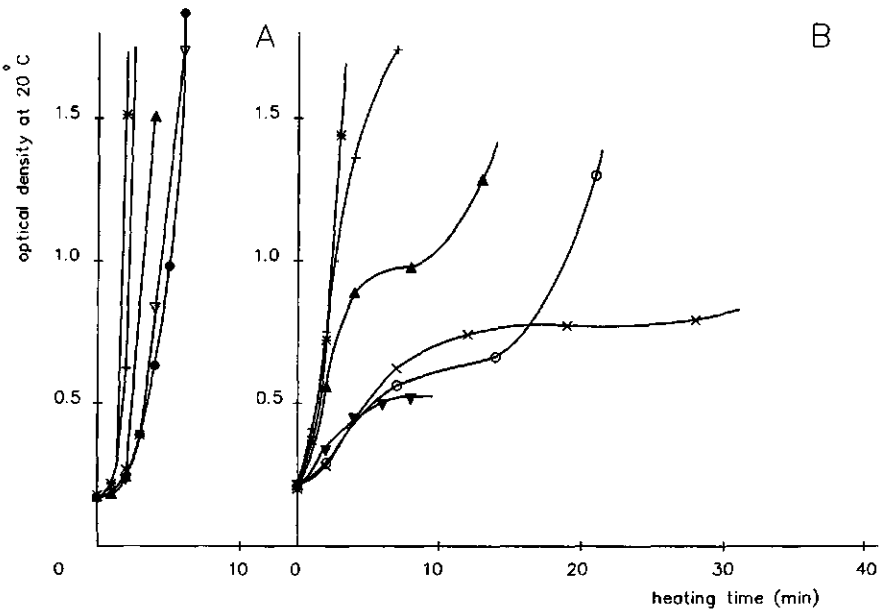

Fig. 7. Optical density of the concentrates after cooling to 20 °C for experiments in September. A: not-preheated concentrate. B: preheated concentrate. Symbols as in Fig. 4.

A somewhat lower E after heating at high initial pH, and a somewhat higher E after heating at low initial pH, is likely to be the result of such a change in mass. However, if the aggregation of particles proceeds fast, the above effects will be overshadowed, as, apparently, is the case in our results. Flocculation and fusion of particles will both cause a higher E. The extent depends on three factors, as long as the scattering can be described by the Rayleigh-Gans-Debije theory (27):

- Larger particles, if of the same shape and polarisability (which for casein micelles roughly comes down to equal voluminosity), scatter more light per unit mass,
- Less dense particles (higher voluminosity), if of the same dry mass, scatter less light,
- If particles are closer to each other, and/or if they have a more anisometric shape, the optical density is smaller due to more dependent scattering.

Thus, fusion of casein particles will result in a marked increase of E. Flocculation without much fusion will not greatly increase E, since the effect of an increase in particle diameter will be partly compensated by the increase of dependent scattering (in particular if the aggregates become large).

The increase in optical density proceeded fairly gradually with heating time, the higher the pH the faster (Figs. 7A and B). In not-preheated concentrate, the optical density of a sample just before coagulation was still low (about 0.3) at pH 6.54 (HCT ≈ 3 min), but high (about 1.8) at pH 6.57 (HCT ≈ 6 min). At high initial pH only the initial particle growth was detected by our turbidity measurements, because E became higher than 1.5 long before visible coagulation occurred. For preheated concentrate, the increase of the optical density with heating time was slower than for not-preheated concentrate, in the investigated pH-range. Furthermore, a kind of plateau was observed in preheated concentrate at low and intermediate pH. Since the viscosity measurements showed a similar plateau, it is likely that in this pH range neither particle size nor state of aggregation changed substantially for some time. In the reported experiment, no further increase was observed after the plateau in preheated concentrate at pH 6.33 and 6.51, but an increase was seen at pH 6.43 and 6.63. This must have been due to the precise moment of sampling, since in some of the other experiments a sudden increase of E shortly before the HCT was also found for concentrates having a pH of about 6.3 or 6.5.

In an additional experiment, we also determined the change of the optical density upon storing heat-treated preheated concentrate at 20 °C. For concentrate with an initial pH of 6.45, the value found after 2 h at 20 °C was about 85% of that determined 10 min after heating, and after about 24 h this was about 80%. Longer storage had no further effect. For concentrate with an initial pH of 6.8, the optical density determined after 2 h was about 90% of that determined 10 min after heating; afterwards no significant change was found. For either concentrate, the heating time (5 or 10, and 2 or 4 min, respectively) had no influence on the change after cooling. In our opinion, the change at high initial pH is likely to be largely due to solubilization of calcium phosphate, and/or swelling of the particles, but at low initial pH deflocculation may also play a part, since casein particles once fused are not likely to become dispersed again, whereas aggregated particles may do so.

The optical densities determined at 120 °C are given in Figs. 8A and B. For most samples, the values determined at 120 °C were roughly the same as those determined after cooling to 20 °C (after recalculation to an equal optical path). A precise comparison is difficult, however, due to the different heating up periods. At the high temperature, more calcium phosphate is likely to be part of the micelles (28), but this results, apparently, in only a small change in E. However, in samples of preheated concentrate at low and intermediate pH a continuous increase of E was found if measured during quiescent heating at 120 °C, instead of the plateau that was found if E was deter-

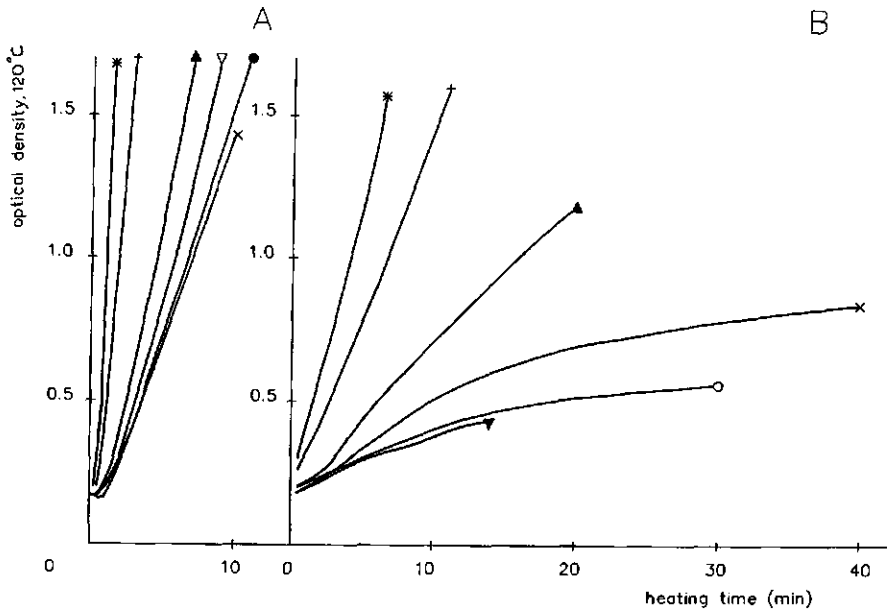

Fig. 8. Optical density of the concentrates at 120 °C for experiments in September. A: not-preheated concentrate. B: preheated concentrate. Symbols as in Fig. 4.

mined after cooling of agitated samples. Also continuing the measurements for times longer than the HCT did not give any irregularity in the plots. In our opinion both phenomena are largely due to the liquid being undisturbed in the cuvette, which results in an undisturbed gelation of the samples, without any influence of shear on the aggregation rate, or of breaking-up or syneresis of the flocs.

3.1.6 Combined results. As a summary of the results presented above, the HCT, the time needed for the volume fraction of the protein at time t (ϕ_t) to become $1.25 \times \phi_0$ (ϕ_t and ϕ_0 were calculated from the viscosities at 20 °C with the Eilers equation (29)), the time needed for E to become $4 \times E_0$, and the time needed for the N% in the supernatant to become $0.75 \times N_0$ are plotted against the pH in Figs. 9A and B. It is clear that at low initial pH flocculation of particles prevails, and at high initial pH fusion, in both types of concentrate.

3.2 Solubility of the coagula

The results of the (additional) dissolution experiments for preheated concen-

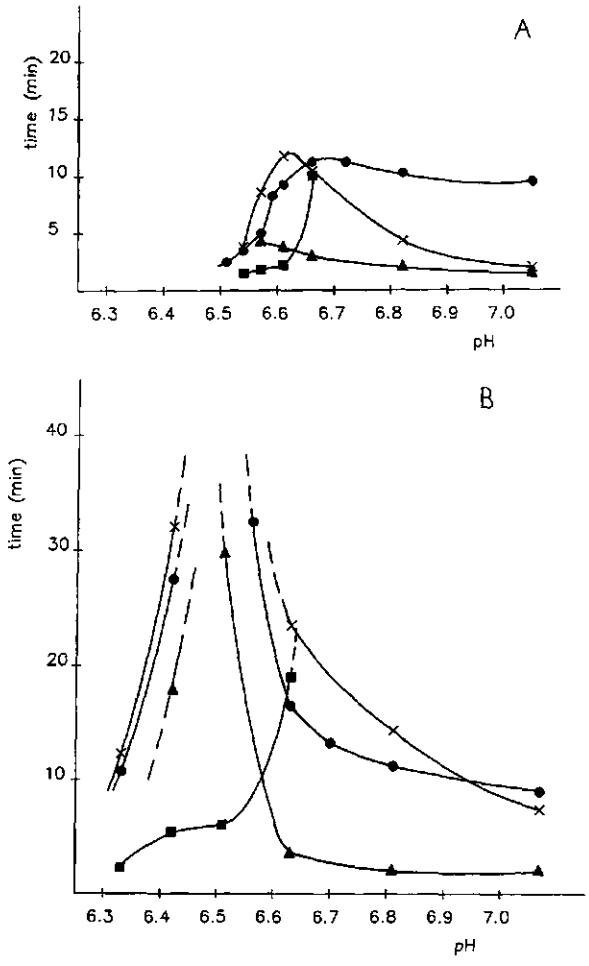

Fig. 9. HCT (●), time needed for the N percentage in the supernatant to become 0.75 × N_0 (×), time needed for $\phi_{protein}$ to become 1.25 × ϕ_0 (■), and time needed for E to become 4 × E_0 (▲) plotted against the pH. A: not-preheated concentrate, B: preheated concentrate.

trate are in Table 1. The coagulum formed at pH 6.19 did not dissolve in water and dissolved only partly at pH 8, but the other three solutions were completely clear, also after heat-treatment of 3.5 min, when a firm gel had formed. The coagulum formed at pH 6.45 was neither soluble in water nor at pH 8; in EDTA at pH 8 a soft gel was formed, and in urea (with or without DTT) a slightly turbid liquid remained. The coagulum formed at pH 6.93 was neither soluble in water nor at pH 8, and in the other media a soft gel was ob-

Table 1. Appearance of the coagula diluted in different solvents; preheated concentrate.

Initial pH 6.19 (HCT 2.5 min) Solvent	Heating time		
	2.5 min	3 min	3.5 min
water	settled flocs	id.	id.
NaOH	flakes	id.	id.
Urea	clear liquid	id.	id.
DTT + urea	clear liquid	id.	id.
EDTA pH 8	clear liquid	id.	id.
Initial pH 6.45 (HCT ≈ 45 min) Solvent	**Heating time**		
	45 min	53 min	60 min
water	settled flocs	id.	id.
NaOH	settled particles	id.	id.
Urea	turbid liquid	id.	id.
DTT + urea	turbid liquid	id.	id.
EDTA pH 8	soft gel	firm gel	id.
Initial pH 6.93 (HCT 10 min) Solvent	**Heating time**		
	10 min	11 min	12 min
water	settled flocs	id.	id.
NaOH	small particles	settled particles	id.
Urea	clear liquid	soft gel	id.
DTT + urea	clear liquid	soft gel	id.
EDTA pH 8	clear liquid	firm gel	id.

served. Only after 10 min heating, when a coagulum was hardly visible, was it soluble in these liquids.

3.3 Effects of added formaldehyde and phosphate

In addition to all the determinations on concentrated skim milk without additives, we also determined the HCT-pH plots of not-preheated, preheated, and serum-protein-free concentrate, and of the same concentrates to which 12 mmol/l formaldehyde had been added (all prepared from the same milk). The results are shown in Fig. 10. In accordance with the results of Muir et al.(30), addition of formaldehyde gave a very large increase of the HCT, for all concentrates. Interestingly, the pH above which the HCT became longer than the time needed for heating up was hardly affected by addition of formaldehyde. At slightly higher pH values, coagulation in concentrate with added formaldehyde was very slow, and was preceded by a viscosity increase

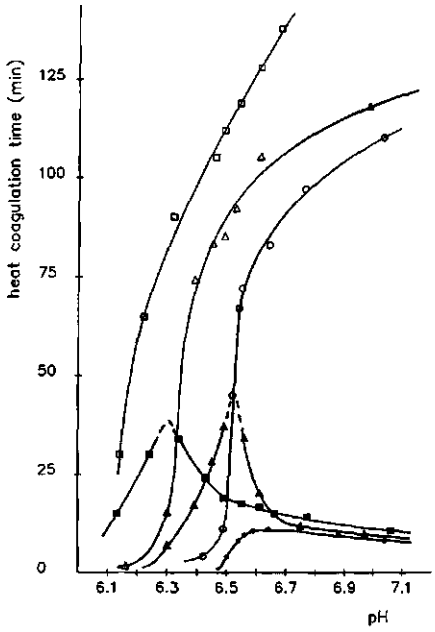

Fig. 10. HCT-pH plots of not-preheated (●,○), preheated (▲,△) and serum-protein-free (■,□) concentrates, without and with added formaldehyde respectively. Experiment in February 1989.

of the concentrate and heavy fouling of the heating tubes. The detrimental effect of serum proteins on the HCT at low initial pH is striking, in agreement with results in Ref. 31. However, a quantitative comparison between the HCT of serum-protein-depleted concentrate and the other concentrates can not be made, since not all the casein in the ultracentrifugal pellet was resuspendable, and we will show in a forthcoming paper that the casein concentration has a large influence on the coagulation time.

The HCT-pH plots of preheated concentrate with and without added phosphate (18.1 mmol PO_4/l) were also determined (Fig. 11). In accordance with the results obtained by Sweetsur & Muir (12), phosphate addition enhanced stability at a low initial pH, and destabilized the concentrate at its original optimum pH and above. Sweetsur & Muir (12) found a higher maximum at the new optimum pH; our results are inconclusive in this respect, but any difference appears to be small.

We also determined the viscosity and optical density of samples of different pH heated for different times, with and without added phosphate (after cooling to 20 °C). For concentrate with added phosphate, similarly shaped curves were found as for concentrate without, only at a lower pH.

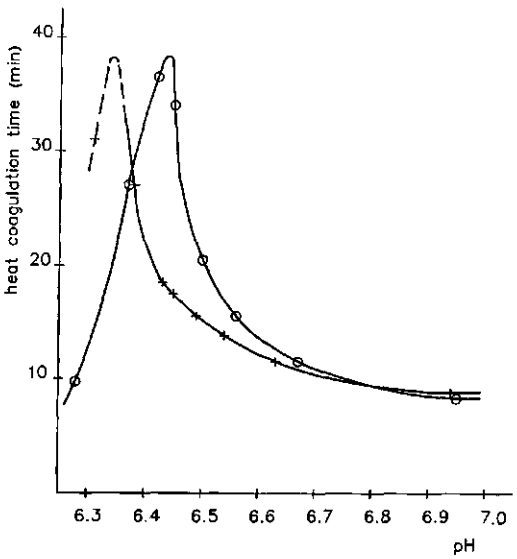

Fig. 11. HCT-pH plots of preheated concentrate (O), and of the same concentrate with 18.1 mmol PO_4/l added (+). Experiments in May 1989.

4 Discussion: a model for the heat-induced coagulation of concentrated skim milk

An overview of the most important phenomena observed during the heat coagulation of concentrated skim milk is given in Table 2 and Fig. 12. The data were obtained from Refs. 16 and 17 and this article.

The basic cause of the heat coagulation of concentrated skim milk is irreversible aggregation of proteins in the liquid. The proteins involved are either caseins, serum proteins, or both. Aggregation is due to heat-induced chemical or physical changes in the solvent or of the proteinaceous particles themselves. Aggregation may be depicted as a process of simple flocculation of particles, followed by fusion, each with its own characteristic time scale, τ_1 and τ_2, respectively. If $\tau_1 \ll \tau_2$, gel formation is the ultimate result, while if $\tau_1 \gg \tau_2$, large particles are formed. To explain the various HCT-pH plots of differently processed concentrated skim milk, and the effect of some additives on the HCT-pH plot, we will extend to concentrated skim milk the model given by van Boekel et al. (11) for unconcentrated skim milk. An essential feature of this model is that upon heating, two types of casein particles may emerge, according to the initial pH. One type - casein particles onto which serum proteins have deposited - emerges at a pH lower than about 6.7, and an-

Table 2. Compilation of some characteristic data on the heat-induced coagulation of concentrated skim milk.

	Not-preheated			Preheated		
	pH ≈ 6.3	pH ≈ 6.5	pH ≈ 6.8	pH ≈ 6.3	pH ≈ 6.5	pH ≈ 6.8
HCT (min)	1	6	10	12	40	10
Conditions at HCT						
pH	6.3	6.3	6.4	6.1	5.6	6.4
αCa^{2+}	0.85	0.55	0.40	0.70	0.55	0.40
T (°C)	100	120	120	120	120	120
κ-casein in the micelles (%)						
t = 0 min	91	91	91	88	86	82
t = 2 min	85	80	55	90	80	70
t = 5 min	70	60	45	80	70	50
Approximate amount of serum proteins on the micelles (%)						
t = 0 min	0	0	0	85	80	75
t = 2 min	70	70	55	90	90	70
t = 5 min	90	85	65	95	90	65

other type - casein particles from which most κ-casein has dissociated - emerges at a pH higher than about 7.0. Both kinds of particles are subject to at least two reactions, each of which can lead to coagulation, according to conditions: if salt-induced coagulation is fast, it determines the (relatively short) coagulation time, and if it is slow, coagulation is caused by covalent cross-linking of protein.

4.1 Coagulation of not-preheated concentrate at a pH below 6.5

If not-preheated concentrated milk was heated at an initial pH below about 6.5, coagulation occurred within the heating-up period, i.e. before the temperature of the concentrate reached 120 °C (Table 2). It resulted in the formation of a gel. This gel cannot be the result of flocculation of the casein micelles, or of casein micelles with denatured serum proteins associated with them, since both serum-protein-free and preheated concentrate were quite heat stable between pH 6.2 and 6.5 (Fig. 10). From the electron micrographs, and from the virtually constant optical density up to the HCT (Fig. 12), it can also be concluded that fusion of the casein particles hardly occurred under these conditions. On the other hand, heat denaturation and association of serum proteins must be involved, since Q_{10} was found to be larger than 5 between 70 and 90 °C (results not shown), which is typical for aggregation due to heat denaturation of proteins (29). If the assay temperature was higher

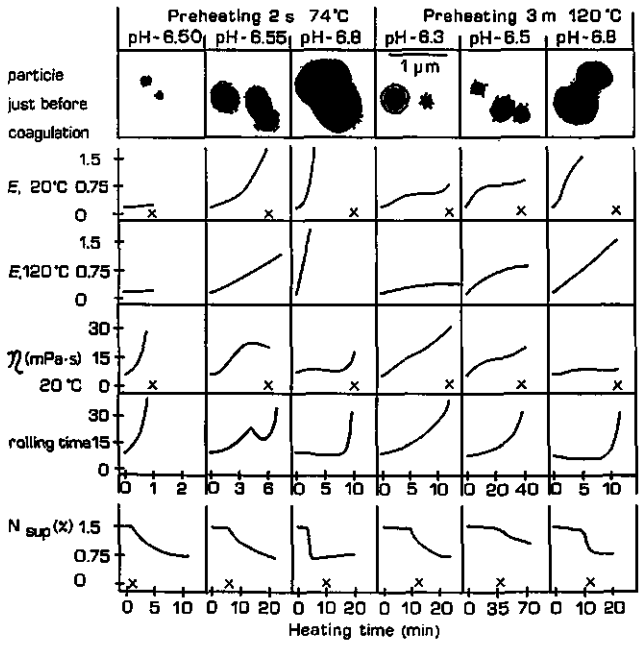

Fig. 12. Summary of the changes in concentrated skim milk upon heating under different conditions. × = HCT.

than 100 °C, coagulation occurred within the heating-up period.

These observations indicate that the heat-induced association of serum proteins is the rate-determining process in the heat coagulation of not-preheated concentrated milk below about pH 6.5. The result is a network (a gel) in which the casein micelles may also take part, since heat-induced interactions between casein and serum proteins were reported to occur in milk in this pH-range (32). The rate of formation and the strength of the gel are determined by a combination of the serum protein concentration, the rate of denaturation and the tendency of the denatured serum proteins to associate; and, in addition, by conditions during the test. Hence, the conditions under which this type of coagulation may occur will vary. When comparing various investigations, heating-up rate, final heating temperature and the intensity of agitation will have been more or less the same if the method of Davies & White (13) had been used. However, conditions during model experiments on serum protein denaturation and aggregation (e.g. 33, 34, 35) are quite different, and using these results in explaining heat stability should be done with great care. For instance, formation of a weak gel in a quiescent sample does

not necessarily go along with visible coagulation in a rocking sample, even if other conditions are the same. Investigations like those reported in Refs. 33 and 34 do indicate, however, that formation of β-LG gels may occur at a concentration as low as 1%, and that the rheological properties of such gels are affected by conditions like pH. In concentrated skim milk, the β-LG concentration is about 0.9%. In addition, other serum proteins are present, and the volume fraction of casein micelles is about 0.3, hence the effective β-LG concentration is likely to be large enough to induce gelation. Presumably due to the same mechanism, gelation of quiescent dispersions of casein micelles in synthetic milk ultrafiltrate occurred upon heating at 90 °C in the presence of 20 g/kg β-LG at pH 6.8 (32), or 12 g/kg β-LG at pH 7.3 (36).

It is not yet clear which types of interaction determine the serum protein-induced gelation. The large effects of pH and of addition of calcium (10.2 mM), which widened by about 0.2 units the pH range over which coagulation occurred within the heating-up period (12), indicate that electrostatic interactions are important. Addition of phosphate (14.1 mM) or citrate (7.1 mM), however, had little effect on the pH range in which this type of coagulation occurred (12). Thiol interactions appear not to be very important: addition of N-ethylmaleimide to not-preheated concentrate had little effect on the pH range in which immediate coagulation occurred (24).

4.2 Coagulation under other conditions

If a not-preheated concentrate having an initial pH above 6.5 was heated it did not gel, and neither did unconcentrated milk at its natural pH during preheating. Thus, the heat-induced denaturation and aggregation of the serum proteins in these samples did not lead to immediate gelation. Presumably, this is due to too high a pH or too low a $a\mathrm{Ca}^{2+}$, while during preheating a combination of the relatively low serum protein concentration in the unconcentrated milk and the intens agitation are also likely to play a part. This implies that heat-induced aggregation of partly serum-protein-coated, partly κ-casein-depleted casein micelles determines the heat stability of not-preheated concentrate at a pH above 6.5, and of preheated concentrate at any pH. In the following, we will primarily discuss preheated concentrate; the phenomena in not-preheated concentrate (above pH 6.5) will, in our opinion, show merely quantitative differences.

Upon heating, the aggregation rate of the casein micelles is determined by: (*i*) the encounter frequency between the particles (s^{-1}); (*ii*) the probability that during an encounter two reactive sites come in close contact (which is likely to be correlated with the duration of an encounter); and (*iii*) the activation free energy of bond formation. The HCT is, moreover, determined by

(*iv*) the volume fraction of aggregating particles and (*v*) some phenomena resulting from aggregation, especially the ratio between the rates of flocculation and fusion, which ratio determines whether a gel is formed or separate particles become visible (37). To be sure, the situation may be more complicated, since (a) the said ratio may change during heating, (b) aggregates may undergo rearrangements and (c) an initially formed weak gel may be broken up again in certain conditions. In this article, we will qualitatively discuss the factors mentioned above.

4.2.1 Changes with temperature and upon heating. An obvious change with temperature (20 to 120 °C) is the increase in the encounter frequency between the particles by a factor of about 6, according to Smoluchowski (38). A second effect of temperature is the association of calcium phosphate with the casein particles, which appears to depend little on heating time: from results reported in Ref. 28 it can be deduced that, in a heat stability test, the net association is completed when the temperature has reached 120 °C. The association of calcium phosphate with the particles results in less Ca^{2+} in the serum, but this does not necessarily imply that the aCa^{2+} at high temperature is lower, as one might expect from the aCa^{2+} determined after cooling (Table 2). Both the presence of additional charged material in the particles, and a possibly reduced aCa^{2+} may affect the frequency and the duration of the encounters between particles (37), although these effects are likely to be small in a solution having a high ionic strength like concentrated milk. In addition, bond formation may be affected by these two factors: see the section on bond formation.

In Ref. 11, it was argued that the slow heat-induced decrease of the pH strongly affects the heat coagulation of milk: if the pH has become sufficiently low, encounters between particles last sufficiently long (or the hairy layers interpenetrate sufficiently far) for cross-linking of proteins to become important (37). In concentrated milk, however, the pH-decrease is limited. Only around the optimum pH in preheated concentrate is the pH at the HCT lower than about 6.0 (Table 2); in this pH-range the rate of the pH-decrease may thus affect heat stability, although the effect may be smaller in concentrated milk than in milk because of the higher ionic strength in the former.

The particles also change by heating: casein, especially κ-casein (17), dissociates and serum proteins associate with the micelles (Table 2). Dissociation of even a limited amount of κ-casein may have a large destabilizing effect: it significantly increases the probability that a segment of a κ-casein hair of one micelle is found within the hairy layer of another, and thus may increase the probability of bond formation (37). Dissociation of larger amounts

of κ-casein from the micelles increases the probability of contact between the micellar cores, which presumably is a prerequisite for fusion of micelles to occur. Association of serum proteins with the casein particles probably increases the charge of the particles (36), somewhat suppresses the initial dissociation of κ-casein (17, 36), and may result in a stronger steric repulsion (which also depends on the charge of the serum proteins, Ref. 11). A second change of the particles may be a change of the native micellar calcium phosphate (MCP), which may affect bond formation (39).

Considering the large difference in stability between casein micelles at 20 and 120 °C, which is at least by a factor of 10^4, the reaction constant(s) of the bond-forming reaction(s) must increase with temperature by several orders of magnitude. This is not unrealistic: it can be calculated from the Arrhenius equation that the activation energy of such a reaction would only be about 70 $kJ.mol^{-1}$, assuming that the increase of the frequency factor is about 6-fold. Of course, the activation Gibbs energy of bond formation is also affected by the type of bonds formed, and these are not necessarily the same at 20 and at 120 °C.

4.2.2 Types of bonds involved in heat coagulation. The results of the dissolution experiment (Table 1) show that, at pH 6.3, polymerization of protein can hardly be the cause of heat coagulation of preheated concentrated milk. At a higher pH, covalent bonds appear to be of some importance: in concentrate of pH 6.5, the coagulum at the HCT (= 45 min) did not dissolve in urea, DTT + urea, or EDTA, while at pH 6.9 the coagulum did dissolve in these solvents at the HCT, but not 1 min later. It thus appears that for concentrated milk at low and intermediate pH the same reasoning as for unconcentrated milk applies (11): if salt-induced coagulation is fast it determines the coagulation time, and if it is slow, cross-linking of proteins becomes important. Still, the salt-induced coagulation appears to be far more important for concentrated milk than for milk: the initial aggregation at intermediate pH, which is quite extensive (Fig. 12), must be salt-induced, at least for as long as the pH is fairly high (say above 6.0), i.e. during the initial 20-25 min heating (16). Also, the observed viscosity decrease of sterilized concentrated milk upon stirring (40) indicates that at least part of the bonds in the aggregates must be weak, and thus non-covalent. As follows from the solubility in EDTA of the coagulum formed at the HCT (= 10 min), salt bridging is far more important in concentrate at pH 6.9 than in unconcentrated milk at the same pH (12). Still, the results of the dissolution experiment also show that covalent bonds are at least as important for the integrity of the coagula at pH 6.9 as at pH 6.5, although the heating time was much shorter.

Salt bridges between negatively and positively charged groups, or salt bridges mediated by Ca^{2+}, or even MCP-like bridges may be involved in the salt-induced coagulation. Except at low pH, involvement of Ca^{2+}, or some other positive ion, is likely, considering the domination of negatively charged residues on the caseino-macropeptide part of κ-casein. MCP bridges, native micellar calcium phosphate-like or consisting of some other type of calcium phosphate, are not likely to be formed upon the first contact of two particles, except if calcium phosphate crystals emerge at the surface of the micelles upon heating (39). However, simple ionic or Ca^{2+}-mediated bonds, once formed, may be made more permanent by changing into an MCP bridge, which will occur faster at a higher supersaturation of a concentrate with respect to calcium phosphates. Such a change may well affect heat stability, because the lifetime of an MCP-bridge is, presumably, much longer than that of the other two types of salt bridges. Consequently, formation of Ca^{2+}-mediated bonds will be slower at a low $a Ca^{2+}$, but if the low $a Ca^{2+}$ is the result of a change in the serum which increases the supersaturation of calcium phosphates (e.g. an increase of the pH or addition of phosphate), salt bridges will last longer, i.e. fewer salt bridges are needed to form 'permanent' aggregates.

Cross-linking by covalent bonds presumably becomes important at intermediate pH when the pH has decreased to such an extent that the hairy layers can interpenetrate sufficiently far. This is needed, since formation of all bonds possibly involved in polymerization (see Ref. 11) requires considerable interpenetration of the hairy layers of the particles: the flexible C-terminal end of κ-casein starts between residues 86 and 96 (41), while side-chains of cysteine, present at position 88, and lysine, present at positions 86, 111, 112, and 116 can take part in the reactions that are most likely involved in cross-linking, namely formation of lanthionine, lysinoalanine and the Maillard reaction (11). Only if the micelles are partly depleted of κ-casein, e.g. at high initial pH, such a pH decrease would not be needed, and cross-linking may be important even if the coagulation time is short, as is indeed observed.

4.2.3 Effects of pH. A main factor requiring explanation is the HCT-pH plot. Changing the pH of a concentrate results in a change of the charge on the protein residues and of the solubility of all calcium phosphates. Presumably, pH has a relatively small effect on the net charge of the protein particles: the negative ζ-potential of the casein micelles in milk increases by about 15% upon a pH increase from 6.2 to 7.0 (29, 42). This results in an increase of the electrostatic repulsion by a factor of about $1.15^2 = 1.32$. For concentrated milk, this variation may even be smaller, considering the high ionic strength in concen-

trated milk. As follows from Section 4.2.2, changing the pH must considerably affect the rate of formation of salt bridges. On the one hand, the rate of ionic bond formation becomes lower at higher pH because of the lower $a\mathrm{Ca}^{2+}$: the $a\mathrm{Ca}^{2+}$ in concentrated milk is about 1.1 mM at pH 6.2, 0.8 mM at pH 6.5, and 0.4 mM at pH 7.0 (16). On the other hand, a higher pH goes along with a higher supersaturation of all calcium phosphates, which may increase the rate of formation of calcium phosphate bridges. The pH also affects the dissociation of (especially κ-)casein: at a high pH it proceeds much faster than at a low one (see Table 2). Thus, with increasing pH, the aggregation rate of the casein micelles would decrease because of the lower rate of ionic bond formation by Ca^{2+}, and increase by the faster dissociation of κ-casein and the higher rate of formation of calcium phosphate bridges.

The extent of aggregation needed for visual coagulation is strongly affected by the type of aggregates that emerge (37), and this type, presumably, depends largely on the rate of dissociation of κ-casein. Below pH 6.5-6.6, the coagulating particles exhibit strong steric repulsion, but contact of the hairs is frequent, since electrostatic repulsion is relatively small. Because of this frequent contact, and of the high $a\mathrm{Ca}^{2+}$, the probability of bond formation is relatively high. Furthermore, the strong steric repulsion effectuates a low probability of contact of the micellar cores for particles once flocculated. Thus the characteristic time of aggregation (τ_1) is short relative to the characteristic time of fusion (τ_2), i.e. irregularly shaped flocs emerge. This results in e.g. a rapidly increasing viscosity, and a slow increase of the optical density (Fig. 12). Above pH 6.5-6.6, electrostatic repulsion may be somewhat higher, but steric repulsion is relatively weaker. Also, formation of calcium phosphate bonds may proceed at an appreciable rate. In addition, no considerable pH-decrease is needed for covalent cross-linking of proteins to be possible and, presumably, one covalent bond is sufficient for aggregation of two particles, whereas several salt bridges would be needed. Contact of the micellar cores becomes more likely, thus τ_2 now is about the same or shorter than τ_1, i.e. any aggregates rapidly attain a roughly spherical shape. This results among other things in a rapid increase of the optical density, and little change in viscosity (Fig. 12).

We conclude that the shape of the HCT-pH plot is determined by a combination of the effect of pH on the geometry of the aggregates, and of the effect of pH on the rate of aggregation. The continuously increasing HCT in the pH-range 6.2-6.5 is a result of the decreasing rate of ionic bond formation, while the geometry of the aggregates is relatively constant. Upon a further increase of the pH, the geometry of the aggregates greatly changes, and the rate of bond formation becomes higher, partly because the particles change, and

partly because the types of bonding change; the result is a decreasing HCT with increasing pH.

4.2.4 Phosphate addition. Phosphate addition to concentrated milk resulted in a shift of the HCT-pH plot to lower pH values (Refs. 12, 43, Fig. 11). Also, a higher HCT at the new optimum pH was observed (12,16) and, at high levels of added phosphate (12 or 39 mmol/kg concentrate), a stabilization at pH values above the original optimum pH (43). In concentrate with added phosphate, the amount of calcium phosphate associated with the protein particles is larger at any pH, and the $a\mathrm{Ca}^{2+}$ in the serum is lower (16, 43). Thus, the effects of addition of phosphate on the heat stability of concentrated milk are similar to those of a pH increase, which explains the pH-shift of the HCT-pH plot induced by phosphate addition. By the same reasoning, the effect of calcium addition comes down to a pH decrease.

However, the analogy explains only part of the observations, as can be seen from the effect of high levels of phosphate addition. Clearly, part of the additional effect must be due to a pH-change always resulting in a change of the amounts of both calcium and phosphate that are associated with the micelles, while after addition of only one of these the ratio of the serum concentrations is greatly changed. This may affect the dissociation of (especially κ-)-casein, formation of Ca^{2+} mediated ionic bonds and formation of MCP-bridges differently. Also, differences between the rates of the different processes may play a part. After phosphate addition, a new 'equilibrium' between phosphate, calcium, and other ions is attained very slowly at 20 °C, which upon heating to 120 °C results in a continuing association of calcium phosphates with the micelles during a period of at least 10 min, while in concentrate without added phosphate this association is almost completed after heating-up (16). This may affect the rates of serum protein association, κ-casein dissociation, and bond formation during heating-up and holding. Thus, although the basic effect of added phosphate is easy to explain, further research would be needed to establish the importance of the various factors involved.

4.2.5 Preheating. Preheating resulted in a large increase of the HCT between a pH of about 6.2 and 6.6. The main difference between preheated and not-preheated concentrate is that the serum-proteins in preheated concentrate are denatured and aggregated; hence, serum-protein-induced gelation does not occur in preheated concentrate. As discussed in the previous section, the heat-induced aggregation of partly serum-protein coated, partly κ-casein-depleted casein micelles proceeds rather sluggishly in this pH-range, resulting in

a relatively high heat stability. In Fig. 10 and Ref. 31 it can be seen that depletion of serum-proteins from the concentrate has a similar effect: at a pH above about 6.0 the HCT of the concentrates was longer than 2 min. However, Fig. 10 and Ref. 31, however, also show that the presence of serum-proteins in preheated concentrate, which presumably associate with the casein micelles in the pH-range 6.0-6.6 (17), does not simply result in a higher heat stability than that found for serum-protein-depleted concentrate, as one might expect from the effect of β-LG on the heat stability of casein micelles in synthetic milk ultrafiltrate (36). Also, time and temperature of preheating, and in particular variation in heat-treatment above the intensity needed to denature 90% of the β-LG (as can be estimated from Ref. 45), have a large effect on the heat stability of the subsequently produced concentrate (44). Thus, the manner and extent of aggregation of the serum proteins after denaturation, rather than the denaturation itself (as characterized by insolubility at pH 4.6), appear to determine the effect of preheating on the HCT.

A second effect of preheating is that, in concentrate having a pH higher than about 6.6, the dissociation of κ-casein upon heating proceeds more slowly than in concentrate prepared from not-preheated milk (Table 2). This may well be the cause of the slower increase of optical density and sedimentable protein at pH $\geq$ 6.6, but it apparently has no effect on the final HCT. Thus the coagulate at high pH formed in not-preheated concentrate is composed of relatively larger particles than that in preheated concentrate. We will return to this in a subsequent article.

4.2.6 Addition of formaldehyde. Formaldehyde addition to concentrated milk greatly increased the HCT if the HCT of the sample without formaldehyde had a coagulation time longer than the heating-up period (Fig. 10). According to Refs. 30, 46 and 47, formaldehyde may affect heat stability by cross-linking of casein within the micelles, and by causing an increase of the charge of the casein particles. Singh & Fox (46) reported a 70% reduction in available lysine after heating in the presence of formaldehyde. If this is interpreted as a 70% reduction in positively charged lysine residues of the casein molecules, it can be calculated from the concentrations and amino acid composition of the caseins given in (29) that heating in the presence of formaldehyde results in an increase of the net negative charge on the casein particles by a factor of about 1.6 (and the electrostatic repulsion by 1.6^2). The continuously increasing HCT-pH plot after formaldehyde addition is likely to be a result of the cross-linking of casein in the micelles, since the increase of the negative charge of the particles due to heating in the presence of formaldehyde is not likely to result in a different shape of the HCT-pH plot, but rather in a longer

HCT at all pH values. Even below the optimum pH of concentrate without additive a significant influence of a reduced dissociation of κ-casein is to be expected since any dissociation of κ-casein presumably results in further interpenetration of the hairy layers of two particles. Presuming that formaldehyde has little effect on the rate of formation of MCP-like bonds, it can also be concluded from the continuously increasing HCT-pH plot that such bonds are apparently of little importance for as long as the casein micelles are not depleted of κ-casein. Still, these bonds are likely to affect the aggregation rate of depleted casein micelles, because depleted casein micelles aggregate only slowly in unconcentrated milk, but fast in concentrated milk, while the supersaturation of calcium phosphates in concentrate is far higher than in milk.

Because the former two mentioned effects will result in long coagulation times, acid production may become an important factor for the final coagulation of concentrate with added formaldehyde (similar as in unconcentrated milk). Then, the reduction in available lysine may have another important effect, namely a reduction of the amount of lysinoalanine formed upon heating. Lysinoalanine may be one of the covalent bonds that are important for heat-induced coagulation under conditions where salt-induced coagulation is slow (11), and hence blocking of lysine residues may result in an even more stable concentrate. Finally, it can be noted that formaldehyde should have no effect if coagulation times are only a few minutes, as indeed is observed, because no significant dissociation of κ-casein during heating-up occurs in concentrate at low initial pH (17), and a reduction of available lysine requires a certain heating intensity (46).

4.2.7 Conclusion. In conclusion, a combination of our experimental results, which show the emerging of two types of aggregates upon heating concentrated skim milk, according to conditions, and the mechanisms described in our previously published model (10, 11) for the heat stability of milk, yields a rather promising model for the heat stability of concentrated milk. The fact that similar mechanisms can be used to describe the heat coagulation of concentrated and of unconcentrated milk does not, however, imply that the HCT of a concentrate should correlate with that of the milk it is prepared from. Both Refs. 10 & 11, as well as this article, show that heat coagulation is affected by several heat-induced changes, proceeding at various rates, according to conditions; hence the rate-determining reaction(s) will under most conditions be different for milk and for its concentrate. Quantitative aspects of the heat coagulation of concentrated skim milk, including the effect of the ratio between the rates of aggregation and fusion of particles on the HCT, will be treated in a subsequent article.

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Kinetic aspects of the heat-induced coagulation of concentrated skim milk

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Summary

The effect of protein content on the heat stability of concentrated skim milk was found to be very large for samples having an initial pH below 6.6, but almost negligible for samples having a higher initial pH. For concentrated milk having a pH of 6.27 or 6.38, no detectable difference was found between the heat coagulation time (HCT) of samples that were rocked during heating and samples that were heated quiescently. For samples having a pH of 6.8-6.9, the latter was about 15-20 s longer, i.e. about 4%. Quiescent heating resulted in gelation of all samples. These results are used to extend our previously published study on the heat coagulation of concentrated skim milk, in which it was established that voluminous flocs emerge in concentrated milk below pH 6.5, whereas above pH 6.7 large compact particles are formed which grow into anisometric particles only a few minutes before the HCT.

The dependency of the HCT on the protein content of concentrated milk having a pH below 6.5 could be described rather well by a model in which coagulation of the samples is presumed to be due to gel formation via aggregation of casein micelles into flocs of a fractal nature. The fractal dimensionality would then be about 2.1. The model predicts that fractal flocs need not become large to cause gelation in a concentrated dispersion. In this case, only aggregation due to encounters caused by Brownian motion can be important, in agreement with our observations on the effect of rocking the samples. Describing the rate of aggregation by Smoluchowski's equation for slow perikinetic aggregation resulted in stability factors of about 0.5-2.0 $\times$ 10^6, the factor increasing with higher pH of the concentrated milk (up to the pH of maximum heat stabilty). However, these values may well be too low, perhaps by a factor of about 50, because Smoluchowski's equation considerably underestimates the rate of aggregation in concentrated dispersions.

The almost total absence of an effect of protein content on the HCT of concentrated milk having a pH higher than 6.7 could not be simply explained by Smoluchowski's theory for aggregation – even when combining the effects of Brownian motion and shear flow – of the casein micelles into compact spherical particles. Moreover, aggregation into spherical particles would not yield a gel at the HCT, as observed for quiescently heated samples. It is therefore proposed that the HCT above pH 6.7 is largely determined by the time after which the aggregates start to become anisometric and thus voluminous, which is supposed to be determined by the ratio of the time it takes for two particles to fuse to the time elapsing between subsequent aggregation steps. Because this ratio was estimated to depend on the initial volume fraction of particles only during the second half of the HCT, and because the stability factor may well be somewhat lower for samples

having a high initial volume fraction of particles, this model can semi-quantitatively explain our observations.

1 Introduction

In a previous article [1], we presented a qualitative model for the heat-induced coagulation of concentrated skim milk. Coagulation was shown to be due to aggregation of casein micelles, except for that of not-preheated concentrated milk having a pH below about 6.5. For preheated concentrated milk having a pH below 6.5 it was established that the casein micelles aggregated into voluminous flocs, but in concentrated milk having a pH above 6.7 compact spherical particles emerged. The large particles present in the latter milks just before the heat coagulation time (HCT) had an anisometric shape, however. The rate of aggregation was presumed to be strongly influenced by pH and $a\mathrm{Ca}^{2+}$, due to the effect of these factors on the colloidal stability of the particles and on the rate of bond formation. The HCT was presumed to be determined by a combination of the geometry of the aggregates and the rate of bond formation.

In this article, we will give kinetic models that describe the aggregation of casein micelles into voluminous flocs or into compact aggregates. Aggregation in quiescent samples as well as the effects of streaming will be treated, since a sample is agitated during (part of) most heat-treatments. The value of the models for estimating the heat coagulation time will be discussed.

2 Materials and methods

2.1 Preparation of the samples.

Concentrated skim milk (25.4% total solids) was produced from preheated (3 min at 120°C) skim milk as described before [2]. NaN_3 (0.02%) was added as a preservative. Part of the concentrated milk was ultrafiltered, using an Amicon TCF 10A unit and PM10 filters, at 30°C. Various amounts of permeate were added back to the retentate or to the concentrated milk itself, thus yielding concentrated milks having various protein contents. About 12 h before ultrafiltration, the pH of the concentrated milk was adjusted to about 6.4 by adding 2.5 ml 1*N* HCL/kg concentrated milk. The pH of the protein-content-adjusted concentrated milks was further adjusted by adding 5.0 or 2.5 ml 1 *N* HCL or 2.5, 10 or 20 ml 1 *N* NaOH/kg concentrated milk. Finally, demineralized water was added to all samples in order to adjust the (salts and lactose) solution in which the particles are suspended to that of a concentrated milk of

24.4% total solids. This procedure was used because it yields samples of protein content-adjusted concentrated milk having virtually the same pH in the pH-range 6.2-6.6. This is essential because, due to the strong pH-dependence of the HCT in this pH-range, the HCT of the various concentrated milks needs to be compared at exactly the same pH, while accurately measuring the pH of concentrated milk is rather difficult [3].

2.2 Analytical methods.

The pH of the samples was determined after keeping the samples for at least 3 h at 20°C, using a Radiometer PHM63 meter equipped with a G 202C pH and a K 401 reference electrode. The viscosity was determined after keeping the samples for at least 1 h at 30°C, using a capillary viscometer operating at a constant average shear rate of 1100^{-1}. The HCT was determined by the method of Davies & White [4], at 120°C. For samples having a pH of 6.27, 6.38 or 6.8-6.9 we also determined the HCT of unrocked samples. This was done by turning off the rocking mechanism 70 s after inserting the samples in the oil bath and turning it on again some time close to the HCT as expected from the standard HCT-test. Only if coagulation was visible during the first movement of the tube after the standstill period, was the sample marked as being coagulated during the stand still period. In this way, any difference in heating-up rate between the standard and the 'quiescent' tests can only be small, and observation of coagulation is quite possible. Two or three trials (in addition to the standard HCT-test) were sufficient to establish the approximate quiescent HCT.

Electron micrographs and results on turbidity were taken from Ref. [1]. The electron micrographs had been made using the technique of Henstra and Schmidt [5], after heating samples of concentrated milk for various times at 120°C. The optical density E at 1120 nm had been recorded while heating a sample at 120°C in an 0.5 mm cuvette [1], in a Zeiss PMQ2 spectrophotometer with an attachment for turbidity measurements [6].

3 Experimental results

3.1 Heat coagulation

In agreement with our previous observations [1], heat coagulation of concentrated milk having a pH below 6.5 became noticeable as a retardation of the air bubble in the test tube and finally as the appearance of a voluminous precipitate, whereas at a pH above 6.7 particles suddenly appeared. The higher the protein content of the concentrated milk, the more gel-like was the preci-

pitate below pH 6.5. Coagulation of quiescent samples resulted in a gel at all investigated pH values. The just perceptible gel in concentrated milk having a pH below 6.5 was very weak, in particular in samples having a low protein concentration, since the air bubble could still move slowly, without immediately disrupting the gel. After cooling, such a sample could be poured out of the heating tube: the gel was too weak to hold its own weight at 20°C. Rocking the sample a few times resulted in disruption of the gel, but syneresis hardly occurred. The gel in samples that had been heated at rest for a short time beyond the HCT mostly disrupted upon the first movement. For concentrated milk having a pH below 6.5, the coagulation times as determined by the quiescent test were roughly the same as those determined by the standard method. Small differences could not be detected because of the heavy fouling of the heating tubes during the standard HCT-test, which made a precise determination of the HCT impossible. Quiescent heating of concentrated milk having a pH above 6.7 resulted in the formation of a rather firm gel, some 15-20 s after the 'rocking HCT' (which could be determined quite precisely for these samples). Repeatedly rocking such a sample, however, resulted in a sudden break-up of the gel and in fast syneresis of the pieces.

The 'rocking' HCT is plotted against pH in Fig. 1. Protein concentration had clearly an enormous effect on the HCT below pH 6.5, but none or only a slight one above pH 6.7. We reported before [7] that ultrafiltration as such had no significant effect on the HCT: retentate reconstituted with permeate to the original concentration yielded virtually the same HCT-pH plot as the original concentrated milk.

Adding the same amount of NaOH or HCl to samples of concentrated milk having various protein contents did not result in a significant variation in pH with protein concentration in the pH-range 6.25-6.60. Presumably, the buffering capacity per unit volume is roughly the same for the proteinaceous particles and for the continuous phase in this pH-range. Above pH 6.60 the contribution of the proteinaceous particles to the buffering capacity predominated: the pH of the samples to which the largest amount of NaOH (17.5 mmol/kg concentrated milk) had been added varied between 6.79 for concentrated milk having the highest, and 6.90 for concentrated milk having the lowest protein concentration.

3.2 Volume fraction of proteinaceous particles

If the viscosity of a liquid is determined by hydrodynamic interactions only, the effective volume fraction of particles φ can be calculated from the viscosity η and the Eilers equation [e.g. Ref. 8]:

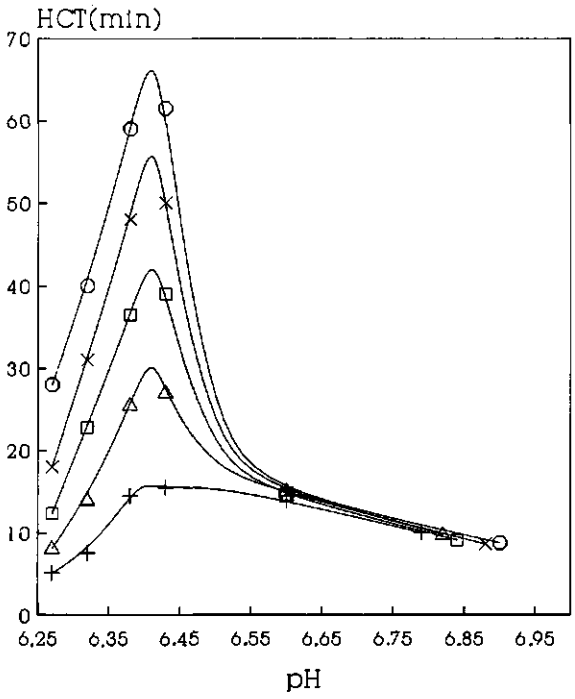

Fig. 1. HCT-pH plots for preheated concentrated milk having various volume fractions of proteinaceous particles. +: $\varphi_0 = 0.46$; △: = 0.41; □: = 0.36; x: = 0.32; o; = 0.29.

$$\eta = \eta_0\left(1 + \frac{1.25\cdot\varphi}{1-\varphi/\varphi_m}\right)^2 \qquad (1)$$

in which η_0 is the viscosity of the continuous phase, and φ_m is φ for the maximum packing density of the dispersed particles. Since concentrated skim milk with 24.4% total solids was found to behave almost as a Newtonian liquid above a shear rate of 10 s^{-1} (results not shown), Eq. (1) can be used to estimate the effective volume fraction of particles in this concentrated skim milk. Some factors deserve attention, however.

In Ref. [8] it was argued that lactose molecules, proteins and fat globules all contribute to hydrodynamic interactions, whereas in Ref. [9] the effect of lactose on the viscosity was accounted for in the viscosity of the solvent. A significant problem in applying the former approach to a dispersion having a high volume fraction of casein micelles is that part of the lactose is present in the micelles, and thus does not contribute to hydrodynamic interactions, and this part has to be estimated. In a system with flocculating particles this prob-

lem becomes increasingly larger, since the continuous phase, containing lactose, becomes entrapped in the flocs. Hence, for practical reasons we followed Ref. [9]. The viscosity of the continuous phase, however, was not assumed to depend linearly on lactose and salt concentrations, but values tabulated in Ref. 10 were used. The concentrations in the continuous phase (x_w, kg solute/kg solution) were calculated with the equation:

$$x_w = x/(1-t+x-h\cdot p) \qquad (2)$$

in which x (kg solute/kg solution) is the concentration of salts or lactose in the concentrated milk, t is the total solids content (kg/kg), p is the protein content (kg/kg), and h is the exclusion factor, which is about 0.2 for small molecules (salts) and 0.55 for lactose [8]. A concentrated milk of 24.4% total solids contains on average about 19 g dissolved salts/kg, about 125 g lactose/kg, and about 92 g protein/kg; the continuous phase thus contains, according to Eq. (2) 25 g salts/kg and 151 g lactose/kg. The viscosity of such a salt solution is, at 20°C, about 1.04 mPa·s and of such a lactose solution 1.65 mPa·s [10]. Since the lactose is dissolved in the salt solution, the viscosities relative to water of both solutions should be multiplied to give the viscosity of the solvent of the particles, yielding 1.71 mPa·s. This value multiplied by the ratio of the viscosity of water at 30°C to that at 20°C yields the viscosity of the continuous phase at 30°C, the temperature at which the measurements were done. Its value is 1.36 mPa·s. φ_m was assumed to be 0.79 [9].

The viscosities (average values of triplicate measurements) of the various concentrated milks having a pH of 6.38, and the calculated volume fractions are given in Table 1. The calculated effective volume fraction depends approximately linearly on the dilution factor, as expected.

Table 1. Viscosities (η, at 30°C) and calculated volume fractions of proteinaceous particles (φ_0) in various concentrated milks. Ret. = retentate, Perm. = permeate, Conc. = original concentrated milk; percentages as (w/w).

Sample	η(mPa·s)	φ_0
100% Ret.	7.70	0.46
88% Ret. + 12% Perm.	5.80	0.41
Conc.	4.50	0.36
90% Conc. + 10% Perm.	3.77	0.32
80% Conc. + 20% Perm.	3.33	0.29

4 Kinetics

Since heat-induced coagulation of (concentrated) skim milk is due to aggregation of casein micelles, encounters between these particles are needed for coagulation to occur. Such encounters may be due to Brownian motion (perikinetic encounters) and to a velocity gradient in the liquid (orthokinetic encounters). Encounters due to diffusion appear to predominate, since we found no detectable difference between the standard and the quiescent HCT-tests for samples having a pH of 6.28 or 6.38, and only a slightly longer quiescent HCT for samples having a pH of 6.8-6.9. Hence, we will focus on perikinetic aggregation, but some attention will also be given to orthokinetic aggregation.

4.1 The rate of perikinetic aggregation

If Brownian motion determines the number of encounters between particles,and only a certain fraction of the encounters results in a permanent contact, Smoluchowski's equation for slow perikinetic aggregation may be used to estimate the change of the number of particles $N(t)$ with time t [11]:

$$\frac{-\mathrm{d}N(t)}{\mathrm{d}t} = \frac{4 \cdot kT}{3 \cdot \eta_o} N^2(t) \frac{1}{W_p} \tag{3}$$

where N_0 is the initial number of particles, kT has its usual meaning, and W_p is the stability factor for perikinetics aggregation, i.e. the inverse of the fraction of encounters leading to aggregation. For convenience, we will just use Eq. (3); many complicating aspects of the Smoluchowski theory will be discussed elsewere [26]. However, the first-order approximation for a correction factor for Eq. (3) that is proposed in the above paper for samples containing a high volume fraction of dispersed particles needs some attention here because of the high volume fraction of primary particles (0.29-0.46) in concentrated skim milk. At such high volume fractions, Smoluchowski's theory is no longer valid, because a stationary state cannot be reached [12], and because the mean distance between the particles becomes progressively smaller with increasing volume fraction of primary particles or aggregates. The aforementioned correction factor (by which the right-hand side of Eq. (3) needs to be multiplied) is given by the square of the ratio of the distance between the centres of two particles over their mean free distance. Using an equation given in Ref. [13] for the mean free distance between spherical primary particles or aggregates, the correction factor may be written as

$\{N(t)^{-1/3}/(1.24\cdot\varphi_{m,f}^{1/3}\cdot N(t)^{-1/3} - 2a(t))\}^2$,
where $a(t)$ is the radius of the aggregate at time t, and $\varphi_{m,f}$ is φ for the maximum packing density of the aggregates. The corrected equation predicts a considerably faster aggregation, the more so for a higher φ_0.

The stability factor W is mostly used to account for colloidal and hydrodynamic interactions between particles, which reduce the frequency of encounters that result in aggregation [12]. In Smoluchowski's theory, an encounter is defined as one particle coming into the sphere of interaction of another, the radius of this sphere being the distance at which Van der Waals attraction always results in permanent aggregation. If electrostatic and hydrodynamic interactions are taken into account, additional energy is needed to bring particles close to each other. Mostly the integral of the product of the hydrodynamic drag and the potential energy with distance from a particle is assumed to slow down the aggregation rate by a factor W. For casein micelles at room temperature the situation is more complicated because steric repulsion is the principal factor retarding the rate of aggregation. Steric repulsion does not greatly reduce the frequency of encounters as predicted by Smoluchowski, if an encounter is defined as a segment of a hair at one particle being within the hairy layer of another [14]. Such encounters, however, do not result in aggregation, but rather in repulsion, unless a (chemical) bond is formed between the protruding hairs of two particles. Thus, a decrease of steric repulsion and/or a high rate of bond formation is needed for aggregation of casein micelles to proceed at an appreciable rate. We can think of three factors that contribute to W for casein micelles at a high temperature:

i) The retarding effect of electrostatic and hydrodynamic interactions on the encounter frequency; this effect, however, is likely to be relatively small for hairy and porous particles like casein micelles.

ii) The probability that during an encounter two reactive sites (in the hairy layers) are in close contact, which is, presumably, positively correlated to the extent and the duration of an interpenetration of the hairy layers of two particles, which will, in turn, depend on the hair density and on the repulsion and attraction between the hairs.

iii) The probability that a contact between reactive sites leads to bond formation, which will depend on the activation Gibbs energy of the reaction involved and on the temperature.

None of these factors is likely to be constant during the course of the aggregation. Effect (*i*) becomes smaller if aggregates emerge that deviate from a smooth spherical shape [26]. Factor (*ii*) is likely to be strongly affected by the dissociation of κ-casein, which, in turn, strongly depends on pH, only about 30% being dissociated after a heat-treatment of 10 min at 120°C in concen-

trated milk at pH 6.3, but about 60% after a heat-treatment of 5 min at 120°C at pH 6.9 [15]. In addition to temperature, heat-induced changes of the continuous phase have, presumably, a large effect on factor (*iii*) [1].

4.2 Geometry of the aggregates

Heat-induced aggregation of casein micelles in preheated concentrated milk was found to result in the formation of voluminous flocs below pH 6.5, and in the formation of compact spherical particles above pH 6.7 [1]. If both processes proceed undisturbed, the former will result in a gel and the latter in visible particles [14]. Below we will apply Smoluchowski's theory to both types of aggregation.

4.2.1 Formation of fractal clusters. If aggregation of particles is simple flocculation only it results in flocs of a fractal nature, as was established both experimentally and by computer simulations [see e.g. various articles in Ref. 16]. A fractal cluster of particles is characterized by its geometric structure being independent of the length scale, for as long as the length scale is between a lower and an upper cut-off length. The (average) size of the primary particle is often taken as the lower, and the (average) cluster size just prior to gelation as the upper cut-off length. The number of primary particles N_a in a growing fractal floc is given by [17]:

$$N_a = \left(\frac{R_a}{a_o}\right)^D \tag{4}$$

where R_a is the radius of the floc, a_0 is the radius of the primary particle, and D is the fractal dimensionality, which is smaller than 3 in 3-dimensional space. In computer simulations of reaction-limited cluster-cluster aggregation D was found to be about 2.1 [e.g Ref. 18]; rearrangement of the clusters may result in a somewhat higher D, however [19]. Acid- and rennet-induced casein gels could be described as a collection of such, on average, fractal clusters; D was found to be about 2.3 [17].

A fractal floc consisting of i primary particles may be considered the same entity as an i-fold particle in the Smoluchowski theory. Hence, at any time $N(t)$ in Eq. (3) equals $N_0/N_a = N_0 \cdot a^D/R^D$ [14]. Substituting $N(t)$, integrating Eq. (3) with respect to R_a and t, and substituting N_0 by $3 \cdot \varphi_0/(4\pi \cdot a^3)$ then yields:

$$\left(\frac{R_a}{a_o}\right)^D = \frac{kT \cdot \varphi_0}{\pi \cdot \eta_0 \cdot a_0^3} \cdot \frac{t}{W_p} + 1 \tag{5}$$

The volume fraction of particles in a growing fractal floc (φ_a) is given by $\varphi_a = (R_a/a_0)^{D-3}$ [16], and consequently φ_a decreases as R_a increases. A 'gel' will be formed when the volume fraction of flocs in the system (φ_0/φ_a) becomes equal to the maximum packing density for the flocs ($\varphi_{m,f}$). At that moment we have $(R_{a,gel}/a_0)^D = (\varphi_0/\varphi_{m,f})^{D/(D-3)}$, and the gelation time t_{gel} is given by:

$$t_{gel} = \frac{(\varphi_0/\varphi_{m,f})^{D/(D-3)} - 1}{\varphi_0} \cdot \frac{\pi \cdot \eta_0 \cdot a_0^3}{k \cdot T} \cdot W_p \qquad (6)$$

A problem in applying Eq. (6) is that $\varphi_{m,f}$ depends on the density of the flocs: for open flocs it may be equal to 1, or even larger, due to interpenetration of the flocs at the moment of gelation, while it is equal to the maximum packing density of spheres if the flocs are rather dense. Arbitrarily, we assumed $\varphi_{m,f}$ to be 1.0 for $\varphi_a = 0.1$ and 0.79 (i.e. the maximum packing density for casein micelles (9)) for $\varphi_a = 0.6$, and a linear dependence of $\varphi_{m,f}$ on φ_a in between these two values. Some results of calculations using Equations (4), (5), and (6) are given in Table 2. The enormous effect of φ_0 on the gelation time is clear, as well as the small number of primary particles in a cluster at gelation. For high volume fractions this number may even be too small for the flocs to have on average a fractal nature: $N_{a,gel} \approx$10-20 appears to be the lower limit [19].

Eq. (6) was applied to the results presented in Fig. 1 and Table 1 by plotting log t_{gel} versus log φ_0 and comparing the experimental plots with plots calculated for various values of D and W_p: see Fig 2. In this way values for D at the moment of gelation and for the average W_p during aggregation were obtained. Drawbacks of this method are that D and W_p cannot be determined independently, and that φ_0 as determined at 30°C was used, whereas t_{gel} depends on φ_0 at 120°C. A better method does not appear to be available, however. A curve calculated for $D = 2.0$ and $W_p = 8.07 \times 10^5$ fits the results obtained for concentrated milks at pH 6.27 reasonably well. For concentrated

Table 2. Some characteristic results for aggregation into fractal clusters. Y is the number of aggregation steps needed for gelation. Calculations for $D = 2.3$; $a_0 = 65$ nm; $\eta_0 = 0.4 \times 10^{-3}$ mPa·s; $kT = 5.42 \times 10^{-21}$

φ_0	0.10	0.25	0.35	0.45	0.60
$\varphi_{m,f}$	1.00	0.93	0.87	0.81	0.79
t_{gel}/W (s)	1.2×10^0	1.9×10^{-2}	3.4×10^{-3}	8.2×10^{-4}	1.6×10^{-4}
$R_{a,gel}/a_0$	27	6.5	3.7	2.3	1.5
$N_{a,gel}$	1931	75	20	6.9	2.5
Y	11	6.2	4.3	2.8	1.3

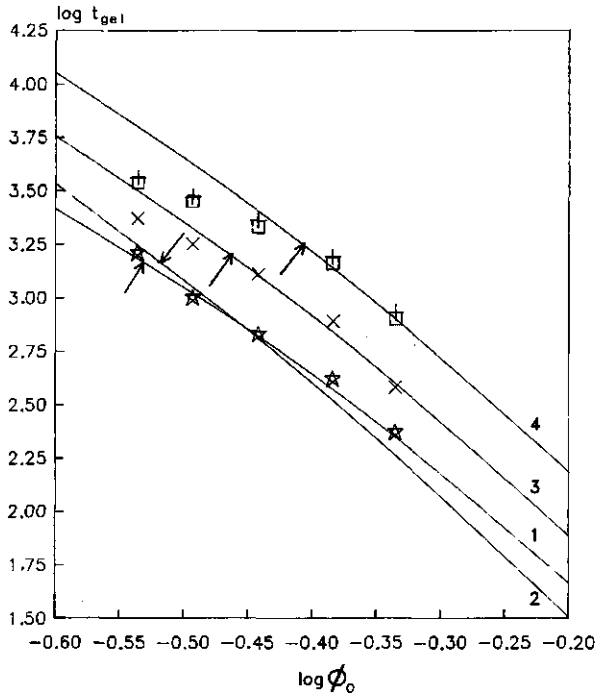

Fig. 2. Log of gelation time (t_{gel}) versus log of initial volume fraction of particles (φ_0) for preheated concentrated milk having various protein contents at various pH values. *: pH = 6.27; x: pH = 6.32; □: pH = 6.38; +: pH = 6.48. Full curves according to Eq. (6) for: $D = 2.0$ and $W \approx 8.07 \times 10^5$ (1); $D = 2.2$ and $W = 3.72 \times 10^5$ (2); $D = 2.1$ and $W = 1.11 \times 10^6$ (3); $D = 2.1$ and $W = 2.20 \times 10^6$ (4). Arrows indicate log t at which the pH of the samples decreased below 5.8.

milks having an initial pH of 6.32, 6.38, or 6.48, a fit according to Eq. (6) cannot be obtained: at the same pH, the dependence of t_{gel} on φ_0 was (much) less at low volume fractions than at high. Only at relatively high φ_0 (thus at relatively short coagulation times) do the curves calculated for $D = 2.1$ fit the experimental data reasonably well. Above the optimum pH (of about 6.45) the concept of fractal geometry no longer applies because roughly spherical aggregates emerge [1].

As for the results presented above, the values of D that fit the experimental points (2.0-2.1) seem reasonable for chemical-limited aggregation of casein micelles at a high temperature. Still, they should be regarded as rough estimates only, due to the approximate nature of the model as well as complications arising from our type of experiments. For instance, using a decreasing $\varphi_{m,f}$ with increasing φ_0 (as in our calculations) yields a steeper slope of the cal-

culated log t_{gel} versus logφ_0 plots than a constant $\varphi_{m,f}$. A lower D is thus needed to fit the experimental results in the former case. In addition, applying the correction factor for concentrated systems given in Section 4.1 would cause the calculated plots to be steeper, and a lower D would fit the experimental results. A factor more specific for heat-coagulation experiments is that D at the moment of gelation may depend on the heating time needed for coagulation (= gelation) to occur. This may be due to a more extensive rearrangment of the flocs with longer heating time, and/or to a change of the voluminosity of the primary particles (similar to the dissociation of caseins from the casein micelles [15]). Furthermore, if φ_0 is higher than about 0.4, the clusters at the moment of gelation may not be on average of a fractal nature, which would come down to a higher apparent D.

The values of W_p reported above are likely to deviate considerably from the 'actual' values. The greatest error is probably introduced by applying Smoluchowski's equation to highly concentrated systems, which may give an underestimation of W_p by a factor of about 50, as was tentatively calculated by applying the correction factor given in Section 4.1. Secondly, a slight variation in the estimated D results in a large variation in W_p (see e.g. the two fits given in Fig. 2 for concentrated milk having a pH of 6.27). A third factor, which is specific for heat coagulation, is that heating may change some of the factors that contribute to W_p (see Section 4.1). If such a change results in a higher rate of aggregation and, moreover, in a relatively large part of the aggregation occurring after some time of heating, this would yield a lower average W_p. This may indeed occur, since the principal reaction involved in the heat coagulation of concentrated milk below the optimum pH presumably changes from salt bridging to covalent cross-linking when the pH of the concentrated milk (at 20°C) decreases to below pH 6.0-5.8 [1], due to heat-induced acid production. The time at which this change may occur was estimated by extrapolating the pH versus heating time plots reported in Ref. [2] to pH 5.8, and is indicated by arrows in Fig. 2. It is clear that the coagulation times left of the arrows are appreciably shorter than expected when extrapolating the values to the right-hand side of the arrows, indicating that covalent cross-linking below a pH of 5.8 may proceed faster than the formation of salt bridges at a pH higher than 5.8, thus affecting W_p.

4.2.2 Formation of spherical particles. In the case of simple flocculation followed by rapid fusion of the particles, one may use Smoluchowski's equation to calculate the time needed for visible particles to appear. Integration of Eq. (3), and substituting N by $3 \cdot \varphi_0/(4\pi \cdot a^3)$, yields:

$$\left(\frac{a(t)}{a_o}\right)^3 = 1 + \frac{k \cdot T \cdot \varphi_0}{\pi \cdot \eta_0 \cdot a_0^3} \cdot \frac{t}{W_p} \tag{7}$$

If the term 1 is neglected (which is allowed if $a(t)/a_0$ is large), and a_c is defined as the average radius of the particles at the moment when the largest particles are just visible by the naked eye, Eq. (7) reduces to:

$$t_c = \left(\frac{\pi \cdot \eta_0 \cdot a_c^3}{k \cdot T \cdot \varphi_0}\right) W_p \tag{8}$$

Eq. (8) applied to concentrated skim milk at a pH of about 6.8 (t_c = 590s, $\varphi_0 = 0.36$, a_0 = 65 nm, $\eta_0 = 0.4 \cdot 10^{-3}$ mPa·s, a_c = 0.1 mm) would yield $W_p \approx 10^{-3}$, which is clearly impossible, even if the correction factor given in Section 4.1 is applied, which would yield $W_p \approx 10^{-2}$. Hence, visual coagulation cannot be the result of perikinetic aggregation of casein micelles into compact spherical particles only.

It is more common to use Eq. (7) to describe the initial increase of the particle diameter. One method is to estimate the increase of the particle diameter with heating time from the change of the optical density E during heating, and calculate W_p from graphical differentiation of the E_t/E_0 versus t plot and the equation:

$$\frac{E(t)}{E_0} \approx \left(\frac{d(t)}{d_0}\right)^3 = \left(\frac{a(t)}{a_0}\right)^3 = 1 + \frac{k \cdot T \cdot \varphi_0}{\pi \cdot \eta_0 \cdot a_0^3} \cdot \frac{t}{W_p} \tag{9}$$

as was done in Ref. [20] for unconcentrated milk. Results reported in Ref.[1] (Fig. 8b) were used. E_0 was obtained by extrapolating the E versus heating time plots to zero time. Because the relation between E and particle radius a derived in Ref. 20 only holds if compact and spherical particles emerge, $E(t)/E_0$ versus heating time plots were only calculated for concentrated milk having a pH of 6.81 or 7.07 (Fig. 3). If the same values for φ_0, a_0, and η_0 as before are used, Eq. (9) yields $W_p = 5.1 \times 10^5$ between 1 and 7 min heating for concentrated milk at pH 6.81, and $W_p = 3.6 \times 10^5$ between 1 and 4 min heating for concentrated milk at pH 7.07. However, Eq. (9), and thereby also the above-given values for W_p, is fairly accurate only for small values of $E(t)/E_0$. Two complicating factors play a part. One is dependent scattering, which gives a lower optical density, and is less for larger particles (φ_0 being constant) [21]. Thus, the slope of an $E(t)/E_0$ versus heating time plot is steeper than it would be if no dependent scattering occurred, the more so for a higher $a(t)/a_0$, which results in an underestimation of W_p after some time of heating.

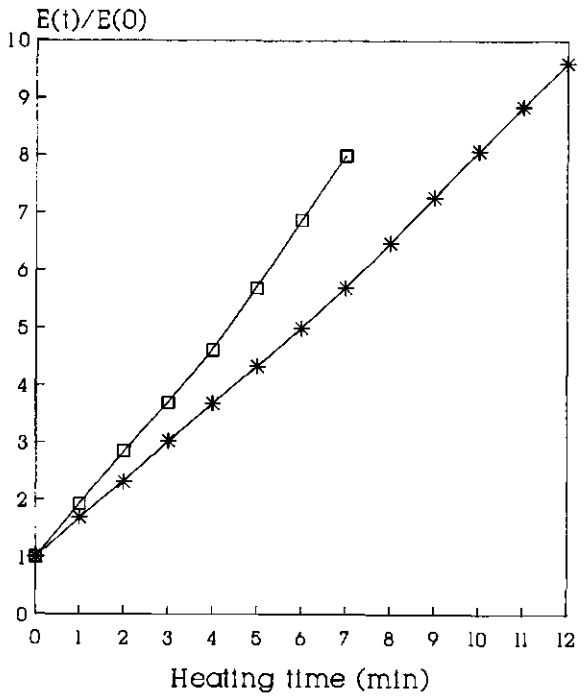

Fig. 3. Optical density $E(t)$ divided by the optical density at zero time (E_0) versus heating time plots for preheated concentrated milk at pH 6.81 (□) and pH 7.07 (*). $E_0 = 0.180$ at pH 6.81, and $E_0 = 0.200$ at pH 7.07. Data taken from Ref. [1].

A second factor, having the opposite effect, is the change in the relation between E and a from $E \propto a^3$ for Rayleigh scatterers to $E \propto a$ for large particles [22].

Another method for calculating W_p is to apply Eq. (7) to the average diameter of the cross-sections of the particles on electron micrographs of concentrated milk heated for various times. We measured 100 particles on Figs. 4a, b, and c (micrographs from the same series as presented in Ref. [1]), and calculated a d_{20} of 86 nm at $t = 0$ min, 152 nm at $t = 2$ min, and 402 nm at $t = 5$ min from the measured (i.e. two-dimensional) moments of the distributions. Fig. 4d was not analysed, since it shows anisometric particles; the diameter of these particles is 0.5-3.0 μm. Only about 100 particles were present on the micrographs used, and the samples had a thickness of about 50 nm, so recalculation of the apparent distributions to the true ones is not allowed. Nevertheless, to get an indication of the magnitude of the errors made, we used Bach's equation [23] to calculate the corrected moments of the diameter distribu-

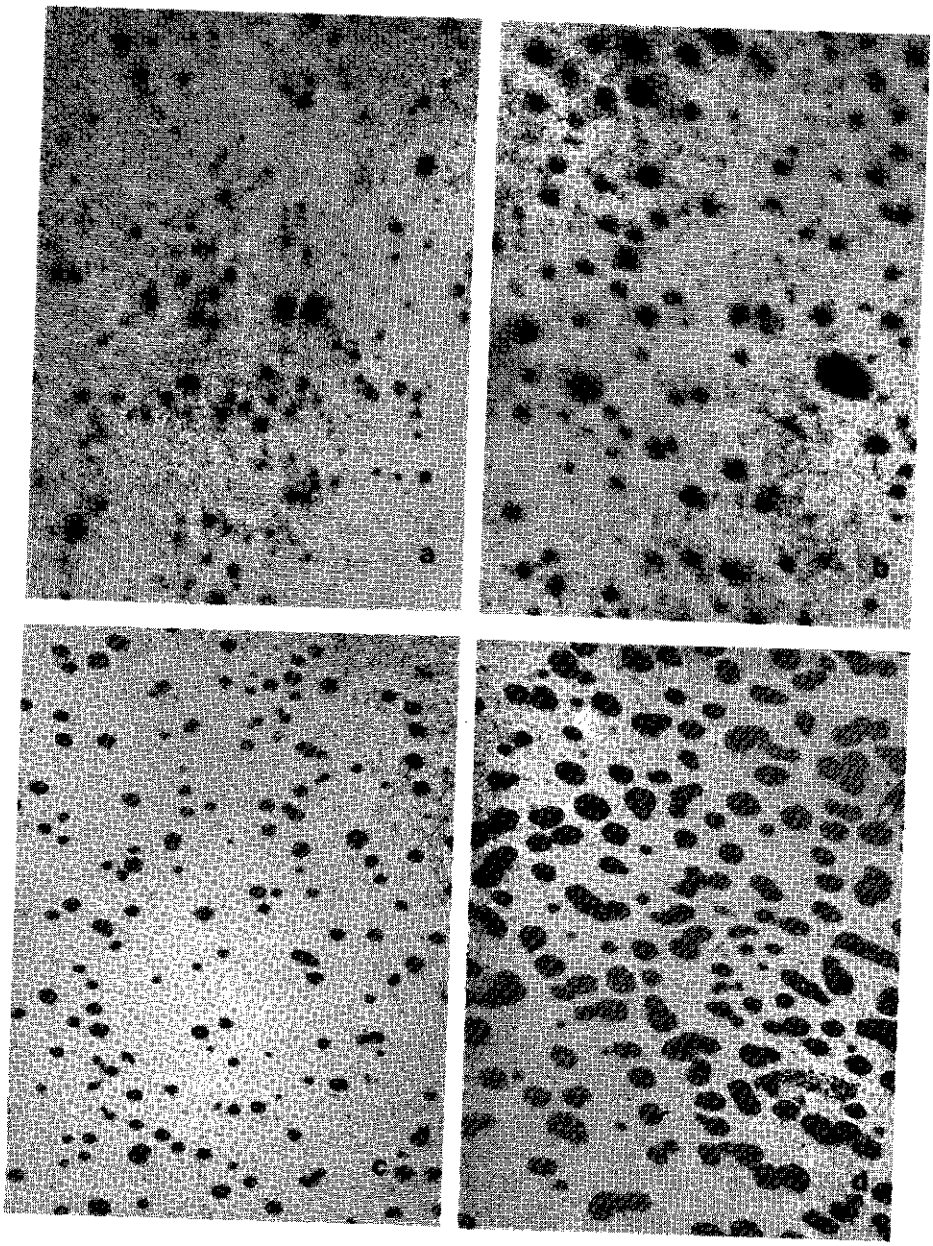

Fig. 4. Electron micrographs of preheated concentrated milk. a: pH 6.45, not heated, ×12 000; b: pH 6.81, heated for 2 min, ×12 000; c: for 5 min, ×4000; d: for 11 min, ×4000. Micrographs made in the same experiment as reported on in Ref. [1].

tions (M_k) from the apparent moments (m_k), where k is the order of the moment:

$$m_k = 1/2 \cdot \sqrt{\pi} \frac{\Gamma\{(k+2)/2\}}{\Gamma\{(k+3)/2\}} \cdot M_k + 1 \qquad (10)$$

where Γ is the gamma function. This yielded a d_{20} of 87 nm at $t = 0$ min, 179 nm at $t = 2$ min, and 505 nm at $t = 5$ min. Using d_{20} calculated from the apparent distributions, and a heating-up time of 70 s, an average W_p of 2.2×10^5 between 70 s and 120 s heating ($a_0 = 86/2$ nm), and of 3.6×10^4 between 120 and 300 s heating ($a_0 = 152/2$ nm) were found. The corrected distributions gave $W_p = 1.2 \times 10^5$ and 1.8×10^4, respectively. Considering that the corrected distribitions overestimate the number of large particles (because Equation (10) is only valid for samples with negligible thickness), values of W_p in between those derived from the apparent and the corrected distributions may considered to be the most appropriate.

Summarizing, optical density and micrographs yield a value of about 10^5 for W_p during the first minutes of heating. (The fairly large difference between the values obtained by the two methods is not surprising, considering the differences in sample treatment, and the complications involved in using Eq. (9) and (10).) The results from the electron micrographs indicate that W_p decreased rapidly (by about an order of magnitude) upon longer heating. Still, a much greater decrease of W_p would be needed for perikinetic aggregation to yield particles having a diameter of about 1.5 μm (as observed on Fig. 4d) within 660 s, and even such particles are far too small to be visible. Thus, the time of visual coagulation (about 11 min) cannot be determined by perikinetic aggregation into compact and spherical particles only, and other factors, presumably orthokinetic aggregation and/or the emerging of anisometric particles, must play a part.

4.3 Effects of streaming

During the heat stability test, a sample flows from one side of the test tube to another, and velocity gradients thus occur. An analysis of the flow fields in the tube is given in Appendix 1. The liquid is estimated to be subjected to a shear flow at a rate of on average 50 s^{-1}. In addition, positive and negative elongational flow occurs at the front and the rear of the air bubble, respectively, at a rate of elongation of about 28 s^{-1} during about 1% of the heating time, and at a rate of about -16 s^{-1} during about 2% of the heating time. Quantitative models are available only for aggregation in shear flow; these will be discussed below. Elongational flow will, in our opinion, have little ef-

fect on the rate of aggregation, because the rate and time of elongation are limited as compared to those of shear flow, and because positive elongational and negative elongational flow have opposite effects (a lower encounter frequency, and a higher frequency and longer duration of the encounters, respectively). The calculated rates of shear and of elongational flow are not likely to result in disruption of the aggregates. The flocs that emerge at low initial pH do not become large before the HCT (see Table 2), and the rates of shear and elongation are far too low to induce breakup of the fused particles that emerge at high initial pH. Of course, the gels that emerge at the HCT can be disrupted, as was observed.

The effect of shear flow on the rate of aggregation is commonly calculated by adding the equations for perikinetic and for orthokinetic aggregation [24], i.e. Eq. (3) and $-dN(t)/dt = 16 \cdot a^3_0 \cdot N^2(t) \cdot S/(3 \cdot W_o)$, in which W_o is the stability factor for orthokinetic aggregation, and S is the velocity gradient [24]. This method seems to be the most appropriate for aggregating casein micelles, since hydrodynamic interactions are, presumably, rather weak for these porous and hairy particles. It yields for the net flow of particles into the sphere of interaction of a reference particle J [24]:

$$J = \frac{8 \cdot kT \cdot N}{3 \cdot \eta_0 \cdot W_p} \left\{ 1 + \frac{W_p}{W_0} \left(\frac{4 \cdot a^3 \cdot \eta_0 \cdot S}{kT} \right) \right\} \qquad (11)$$

The frequency of orthokinetic encounters is, according to Eq. (11), equal to that by perikinetic encounters if the right-hand term between the parentheses is equal to 1. In a shear flow at a rate of 50 s^{-1} this is the case for a = 408 nm, if W_p equals W_o. If W_0 is assumed to be smaller than W_p (which is reasonable since orthokinetic encounters last longer than perikinetic ones and therefore provide a higher probability of contact between reactive sites on the particles), the radii are somewhat smaller e.g. a = 324 nm, if S = 50 s^{-1} and W_p = 2 W_o. The largest calculated average radius of a fractal cluster at the moment of gelation was 333 nm (φ_0 = 0.29; $\varphi_{m,f}$ = 0.91; D = 2.3; a_0 = 65 nm), i.e. equal to or smaller than the radius at wich orthokinetic aggregation becomes dominant. This explains why we found no detectable difference (i.e. longer than the period of several minutes between the first observable change in the liquid and the time of clear coagulation upon determination of the rocking HCT) between the quiescent and the rocking HCT for concentrated milks below the optimum pH. On the other hand, the particles on the electron micrograph of a concentrated milk at pH 6.8 heated until shortly before the HCT (Fig. 4d) have a diameter of roughly 1.5 μm; consequently, shear flow should affect aggregation in these samples. (Even during the quiescent test shear

flow, at a rate of about 1 s^{-1}, may occur, however, due to convection and vibrations.)

In Appendix 2, an equation is derived that relates t, a_c/a_0, and W for simultaneous peri- and orthokinetic aggregation of particles that rapidly fuse after flocculation:

$$t = \frac{\pi \cdot W_o}{4 \cdot \varphi_0 \cdot S} \left\{ 3 \ln \frac{a(t)}{a_o} + \ln \left(\frac{kT \cdot \varphi_0}{\pi \cdot a^3(t) \cdot \eta_0 \cdot W_p} + \frac{4 \cdot \varphi_0 \cdot S}{\pi \cdot W_o} \right) - \ln \left(\frac{kT \cdot \varphi_0}{\pi \cdot a_0^3 \cdot \eta_0 \cdot W_p} + \frac{4 \cdot \varphi_0 \cdot S}{\pi \cdot W_o} \right) \right\} \quad (12)$$

Applied to the apparent distributions on the electron micrographs, Eq.(12) yields $W = 2.2 \times 10^5$ between 70 and 120 s heating, and $W = 3.9 \times 10^4$ between 120 and 300 s heating, if S is taken at 50 s^{-1} and W_p equals W_o. Hence, Eq. (12) already predicts a somewhat faster aggregation of particles having a radius of 200 nm (and this results in a somewhat higher W derived from Eq. (12) than from Eq. (7), if both are applied to the same results). The effect of shear becomes progessively greater for larger particles: an increase in particle radius from 201 to say 750 nm would, at a constant $W_p = W_o = 3.9 \times 10^4$, take 10 386 s according to Eq. (7) but, for $S = 50\ s^{-1}$, only 3172 s according to Eq. (12). It also follows from Eq. (12) that W need not decrease greatly during the second half of the HCT to yield particles of the size (but not the shape, and this restricts the applicability of Eq. (12)) observed on Fig. 4d, especially if $W_p > W_o$. However, the particles on Fig. 4d are still far too small to be visible. In addition, quiescent heating resulted in a gel rather than in large particles, indicating that voluminous aggregates rather than spherical particles emerge shortly before the end of the HCT. Consequently, we propose that the HCT of concentrated milk at high pH is largely determined by the time it takes for the aggregates to become voluminous, rather than by the rate of aggregation as such.

For aggregates to become voluminous, it is necessary that the time of fusion of two particles decreases relative to the time elapsing until an additional particle flocculates with the fusing aggregate. Fusion of two flocculated particles proceeded very rapidly during the first half of the HCT of a concentrated milk at high initial pH: the apparent viscosity at 120°C of such samples was constant during most of the HCT [Ref. 1, Fig. 6B]. This implies that two flocculated particles must have become (almost) fused at the moment of a lasting encounter with a third particle. Although at present the actual cause of the fusion of the particles is not known, it is obvious that a rearrangement of the ca-

sein micelles is needed for fusion to occur. Such a rearrangement must occur over longer distances if the particles become larger. Presuming a constant rate of the changes at the submicellar level, fusion of two large particles takes longer than fusion of two small ones. The time needed for fusion (t_f) may well, because it is likely to be diffusion controlled, be proportional to the square of the distance between the centres of two particles at the first contact, i.e. proportional to $N^{-2/3}$. The time needed for collision of another particle with a fusing aggregate (t_a) equals $1/J$ and can be estimated from Eq. (11), for as long as $t_f \leq t_a$. The ratio t_f to t_a is thus proportional to (substituting $3 \cdot \varphi_0/(4\pi \cdot a^3)$ for N:

$$\frac{t_f}{t_a} \propto \frac{1.654 \cdot kT \cdot \varphi_0^{1/3}}{\eta_0 \cdot a(t) \cdot W_p} + \frac{6.617 \cdot S \cdot \varphi_0^{1/3} \cdot a(t)^2}{W_o} \qquad (13)$$

If W_P equals W_o, and neither changes upon heating, this ratio does not change greatly with increasing particle diameter: t_f/t_a is proportional to 4.5×10^{-10}, 1.1×10^{-10} and 1.3×10^{-10} for $a(t)$ equals 100, 500 and 1000 nm, respectively (φ_0 being constant). However, the observed lower W after longer heating time, and the possibility of W_o being smaller than W_p, would both give a considerable increase of the above ratio with heating time. Anisometric particles would be the result, as observed on Fig. 4d. (Eq. (13) no longer holds if the particles become markedly anisometric.)

Using Eqs. (12) and (13), it can be calculated that t_f/t_a is higher for a higher φ_0 only during the second half of the HCT. Thus the HCT will not be as strongly affected by φ_0 as for a coagulation process determined by the rate of encounters between particles; this provides part of the explanation for the (almost) total absence of an effect of φ_0 on the HCT at high initial pH (see Fig. 1). An additional explanation for the smallness of the effect of φ_0 is that W may well be higher for concentrated milks having a high protein content than for those having a low. Concentrated milk having a high content of proteinaceous particles has a lower ratio of serum to particles, which presumably results in less association of additional calcium phosphate with the particles upon heating [2]. This should result in a higher W for concentrated milks having a high protein content if calcium phosphate bridging is one of the bonds involved in heat coagulation at high initial pH, as was supposed in Ref. [1].

5 Conclusions

Although the lack of an established theory for the kinetics of the aggregation of concentrated dispersions and the complexity of the heat-induced coagula-

tion of concentrated milk hamper the description of heat coagulation in kinetic models, the results presented above clearly show that the time of visual coagulation of concentrated milk depends at least as strongly on the geometry of the emerging aggregates as on the (initial) rate of aggregation.

For concentrated milk below the optimum pH, the strong dependence of the coagulation time on the initial volume fraction of particles can be described rather well by fractal aggregation theory. The principal importance of the emerging flocs being of a fractal nature is that it explains why larger flocs are less dense. To our knowledge, this is the only way in which the strong dependency of the gelation (coagulation) time on the volume fraction of primary particles can be explained. Without this property, which comes down to a rapidly increasing swelling factor with increasing number of primary particles in a cluster, only a far weaker dependence of t_{gel} on φ_0 can occur. In addition, the absence of a significant effect of streaming is explained by the small average cluster size at the moment of gelation, if – as is true in the present case – φ_0 is sufficiently large. The calculated values of D and W_p, however, should be regarded as rough estimates, considering the many uncertainties in the applied model (application of fractal aggregation theory to rather small flocs, the use of Smoluchowski's theory for concentrated dispersions, and several physico-chemical factors contributing to W).

In concentrated milk having a pH above 6.7, compact and spherical aggregates emerge during about two-thirds of the HCT. It is shown that, unlike in concentrated milk below pH 6.5, the aggregates become sufficiently large for orthokinetic aggregation to become important. However, it is also shown that aggregation into compact spherical particles cannot yield visible particles within the observed HCT. Furthermore, it cannot yield a gel at the HCT, as observed for quiescently heated samples. Therefore, and to explain the smallness of the effect of φ_0 on the coagulation time, we propose that the HCT of concentrated milk having a pH above 6.7 is largely determined by the time it takes for the geometry of the aggregates to change from compact and spherical into anisometric and thereby more voluminous. These anisometric aggregates form a gel at the HCT. However, the structure of the gel formed above pH 6.7 is rather different from that formed below pH 6.5. This results in two types of visual coagulation in the standard HCT-test, in which the samples areperiodically agitated: generally, a gel is formed and disrupted again, but the fragments tend to form a gel again at low pH, wheras at high pH the fragments synerese, causing visible particles to emerge.

It may finally be mentioned that fractal aggregation theory may also explain the effect of homogenization on the heat stability. Because the fat globules in homogenized dairy products are covered with casein, they partici-

pate in the aggregation reaction, and this implies that the volume fraction of aggregating material is markedly increased by homogenization of a fat-containing dairy product, the more so for a higher fat content. Since coalescence ('fusion') of fat globules will usually not occur, aggregates of fat globules will be fractal. In products having a high fat/protein ratio, aggregates, i.e. homogenization clusters, may already be present after homogenization, thus providing a still higher effective volume fraction. Altogether, homogenization will generally result in a much shorter HCT. We intend to study this quantitatively later.

Acknowledgements

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Appendix 1

Flow fields in a sample during the HCT-test according to Davies & White

In the subjective HCT-test according to Davies & White coagulation isstudied in a tube of 3.8 ml capacity, which is immersed in hot oil, holding 2.5 ml of the sample and 1.3 ml air. The tube length is 10.8 cm, and its diameter 0.68 cm. The tube is rocked over an arc of 36° in a vertical plane at a rate of 8 cycles/min, causing the air bubble to move from one side to the other. An accurate calculation of the flow field around the air bubble is hard to perform. However, we only need a rough estimate of the flow field in order to estimate the importance of orthokinetic as compared to perikinetic aggregation at the relevant particle sizes. Therefore, an order of magnitude calculation will be given.

The most intensive shear flow occurs in the narrow stream of sample, having an estimated with of about 0.5 cm, that flows under the air bubble. From photographs of a tube in the oil bath the total length of the air bubble was estimated to be about 5.3 cm, the front and rear being about 0.4 and 0.7 cm, respectively (Fig. A.1.1). If the cross-section of this stream is supposed to be rectangular, the velocity gradient in the stream can be roughly estimated by using the equation for a velocity gradient in a laminar flow in a narrow slit [25]:

$$\frac{dv_z}{dy} = \frac{3 \cdot Q}{2 \cdot M \cdot H^3} \cdot y \qquad \text{(A.1.1)}$$

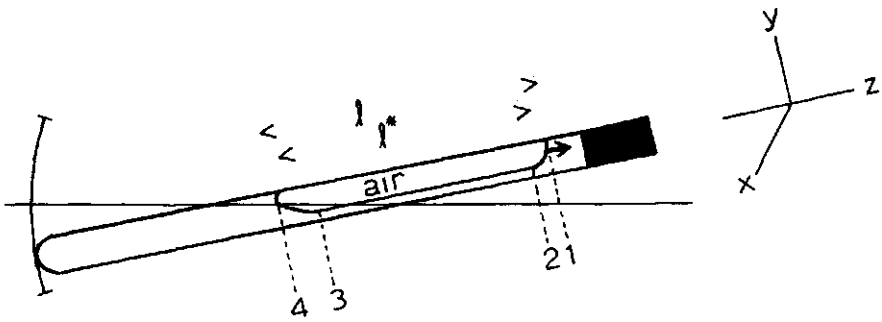

Fig. A.1.1.

in which v_z is the velocity of the liquid along the axis of the tube, y is the distance from the centre of the stream perpendicular to the axis of the tube, $2H$ is the height of the liquid stream, M is the width of the liquid stream, and Q is the volume rate of flow. In this calculation it is assumed that the air-sample interface is immobilized by the presence of a surface tension gradient, and that the velocity of the air bubble is negligible as compared to the average velocity of the liquid in the stream. The volume rate of flow is given by the volume of the sample divided by half the time needed for one movement of the air bubble (half the time because rocking from a horizontal position downwards only causes a fast movement of the air bubble), so $Q = 2.5 \times 10^{-6}/1.9 = 1.32$ cm^3/s. To estimate $2H$, we assumed the air bubble to be of a cylindrical shape, with rectangular front and rear, the equivalent length of this cylinder l^* being 4.8 cm. The volume of liquid under the air bubble is then given by the volume of a 4.8 cm long part of the tube minus the volume of the air bubble (1.5 cm^3), yielding 0.44 cm^3. Hence $2H = 0.18$ cm, $(dv_z/dy)_{max} = 490$ s^{-1}, and $(dv_z/dy)_{av} = 240$ s^{-1}. The liquid is subjected to this shear flow for only a small part of the heating time: the average velocity in the stream is $Q/2{\cdot}M{\cdot}H = 15$ cm/s, hence the liquid is under the air bubble during slighly less than 10% of the heating time. During the remaining 90% of the heating time the liquid is subjected to shear at a much, probably a factor of 10, lower rate. To estimate the effect of shear flow on the rate of aggregation, a shear rate of 50 s^{-1} may considered to be a reasonable average.

In addition to shear flow, positive elongational flow occurs at the front of the air bubble, and negative elongational flow at the rear. The rate of positive elongation can be roughly calculated by dividing the difference between the average velocity of the liquid at position 1 in Fig. A.1.1 and that at position 2 by the length of the front of the air bubble. This yields a rate of 28 s^{-1}, to which the liquid is subjected during 0.04 s; a similar approach yields a negative rate of elongation of 16 s^{-1} during 0.08 s at the rear of the bubble.

Appendix 2

Derivation of an equation relating particle radius to time and stability factor for simultanuous peri- and orthokinetic aggregation

If the frequency of encounters due to Brownian motion and to shear flow are assumed to be additive, the rate of change of the number of particles is given by:

$$-dN(t)/dt = A \cdot N(t)^2 + B \cdot N(t) \qquad \text{(A.2.1)}$$

where:

$$A = \frac{4 \cdot kT}{3 \cdot \eta_0 \cdot W_p} \qquad \text{(A.2.2)}$$

$$B = \frac{8 \cdot \varphi \cdot S}{\pi \cdot W_o} \qquad \text{(A.2.3)}$$

$$\varphi = 4/3 \cdot \pi \cdot a(t)^3 \cdot N(t) \qquad \text{(A.2.4.)}$$

Eq. (A.2.1) can be written as:

$$\frac{-dN(t)}{N(t)\{A \cdot N(t) + B\}} = dt \qquad \text{(A.2.5)}$$

integration of which results in:

$$\frac{B}{1} \ln \left\{ \frac{A \cdot N(t) + B}{N(t)} \right\} = t + c \qquad \text{(A.2.6)}$$

At $t = 0, N(t) = N_o$; after some rearrangement and combining Eq. (A.2.2), (A.2.3), (A.2.4), and (A.2.6) the resulting expression reads:

$$t = \frac{\pi \cdot W_o}{8 \cdot \varphi_0 \cdot S} \left\{ 3 \ln \frac{a(t)}{a_0} + \ln \left(\frac{k \cdot T \cdot \varphi_0}{\pi \cdot a^3(t) \cdot \eta_o \cdot W_p} \frac{8 \cdot \varphi \cdot S}{\pi \cdot W_o} \right) - \right.$$

$$\left. \ln \left(\frac{k \cdot T \cdot \varphi_0}{\pi \cdot a_0^3 \cdot \eta_0 \cdot W_p} + \frac{8 \cdot \varphi_0 \cdot S}{\pi \cdot W_o} \right) \right\}$$

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Summary

Coagulation of concentrated milk during sterilization is one of the problems encountered in its industrial manufacture. Two processing steps are in general use to make this problem manageable, one being preheating of the milk before concentrating, and the other adding a stabilizing salt, mostly a sodium phosphate. This production process, however, depends heavily on trial-and-error procedures.

The objective of this study was to gain a better understanding of the mechanisms of heat coagulation of concentrated milk, in order to improve process control. Attention was focused on stability of casein micelles in concentrated skim milk, because it has long been assumed that instability of casein micelles at high temperature is the main reason for heat coagulation. Casein micelles, however, are very stable in milk at room temperature. So, the first part of this study concerns the effects of the various processing steps in manufacture of sterilized concentrated milk on these particles and the system in which they are dispersed. Milk was treated according to the following scheme:

(skim) milk
- no preheating – concentrating
  - pH adjustment — heat treatment
  - + phosphate – pH adjustment – heat treatment
- preheating – concentrating
  - pH adjustment — heat treatment
  - + phosphate – pH adjustment – heat treatment

Paper 1 describes the change of calcium ion activity, pH, and partition of calcium and of phosphate between serum and particles induced by concentrating whole milk and by heating concentrated milk. In unsterilized concentrate at its natural pH, the amount of calcium associated with the casein particles was about 1.1 times as much as in milk, and that of phosphate about 1.4 times. The amount of calcium associated with the particles was considerably higher at pH 7.0 than at pH 6.3, but the amount of phosphate associated with the particles was almost pH independent. The concentration of calcium and of phosphate in serum of concentrated milk was about twice as high as in milk, while the calcium ion activity was about the same, and the pH lower by 0.3 units. The calcium ion activity of concentrated milk decreased monotonically with pH. Preheating had little effect on these phenomena. Sterilization had little effect on the partition of calcium and of phosphate, despite the pH decrease

induced by sterilization. The calcium ion activity of sterilized concentrated milk was lower than that of unsterilized concentrated milk. Addition of phosphate to concentrated milk before sterilization resulted in more micellar calcium phosphate, and in lower pH and calcium ion activity, in particular after sterilization. Heat stability of portions with added phosphate at a pH lower than 6.5 was also higher. It is concluded that calcium ion activity is an essential factor in heat stability of concentrated milk having a pH below 6.5, and that absolute amounts of calcium and phosphate associated with the micelles or present in the serum have little influence. For concentrated milk having a higher pH, none of the above mentioned factors seems to affect time of visible coagulation, or the various factors counteract one another.

Paper 2 describes changes induced by concentrating milk and by heat-treating concentrated milk on the extent of association of the milk-proteins after cooling to 20°C. Concentrating unpreheated milk did not give any change. Concentrating preheated milk, however, resulted in less caseins in casein micelles, in particular in concentrated milk having a pH of about 7.0. α-Lactalbumin and β-lactoglobulin were largely associated (probably with the casein micelles) in preheated milk and its concentrate. After heating concentrated milk, amounts of micellar casein were less than before, the more so for a longer heat-treatment and for portions having a higher initial pH. Especially the dissociation of $\varkappa$-casein was strongly pH dependent. At pH 6.3, dissociation proceeded slowly and almost rectilinearly with time of heating, but at pH 7.0 about 70% of the $\varkappa$-casein dissociated within a few minutes and afterwards little more. Preheating retarded dissociation of $\varkappa$-casein in concentrated milk having a high initial pH; after a heat-treatment of 5 minutes, however, dissociation in unpreheated and preheated portions was about the same. It is argued that dissociation of casein is likely to occur during heating. Thus, the observed changes in the extent of association of the various proteins, and in particular those of $\varkappa$-casein, are likely to affect heat stability of concentrated milk.

Aggregation of casein micelles in concentrated skim milk was studied by seven analytical methods (Paper 3). Viscosity, turbidity and the amount of protein sedimented (30 min at 2000 *g*) were determined as a function of time of heating for heat-treated concentrated milk. Electron micrographs were made also for some heat-treated portions. Turbidity and falling time in a Höppler-type viscosimeter were determined in concentrates at 120°C, as a function of heating time; lastly the heat coagulation time at 120°C was measured. Since the results for heat-treated portions (at 20°C) were essentially the same as for

concentrated milk at 120°C, cooling did not induce changes that might invalidate results obtained after cooling. The combined results of the various types of measurements showed that aggregation of casein micelles was indeed the cause of coagulation, except for that of unpreheated concentrated milk having a pH lower than about 6.5. In preheated concentrated milk having a pH lower than about 6.5, aggregation of casein micelles yielded voluminous flocs, but in concentrated milk having a higher pH (whether or not preheated), aggregated casein micelles formed compact particles. Shortly before the time of visible coagulation, however, particles in portions having a high pH attained an anisometric shape.

A qualitative model for heat coagulation of concentrated skim milk is proposed. It was based on the above results and those in Papers 1 and 2. Coagulation of unpreheated milk having a pH lower than 6.5 is presumed to be due to aggregation of denatured serum proteins. If coagulation is due to aggregation of casein micelles, the rate of aggregation of these particles and the geometry of the aggregates determine the coagulation time. The rate of aggregation of casein micelles presumably depends on frequency and duration of encounters, and on the probability of bond formation between two micelles during an encounter. Dissociation of κ-casein is supposed to be the principal in the stability of casein micelles. Consequently, stability at high temperature of micelles in concentrated milk is lower at higher pH. The probability of bond formation depends strongly on temperature and on type of bonding. Simple ionic or calcium ion-mediated bonds are, presumably, the principal type of bonding for aggregation of 'hairy' casein particles at a pH below 6.5. At a higher pH, micellar-calcium-phosphate-like bonds may play a part as may covalent bonds. Covalent bonds also seem to be important around pH 6.5, but only if time of heating before coagulation is sufficiently long to allow a large heat-induced decrease in pH (pH must be below about 6.0 or 5.8 at coagulation). The geometry of the aggregates is supposed to depend on dissociation of κ-casein and on extent of supersaturation of calcium phosphate. For compact particles to be formed, a large part of Ú-casein must dissociate from the micelles and in addition supersaturation of calcium phosphate must be high (at least higher than in unconcentrated milk). The above model qualitatively explains the shape of heat coagulation time versus pH plot, and the effects of preheating and addition of phosphate or formaldehyde.

Kinetic aspects of the model are discussed in Paper 4. The effect of protein content of concentrated skim milk on heat coagulation time was studied. In concentrated skim milk below pH 6.5, protein content strongly influenced heat coagulation time, but above pH 6.7 hardly at all. The influence at low pH

can be explained by assuming that the voluminous flocs that emerge at those conditions have a fractal geometry, and that coagulation occurs when the flocs fill the system and thus form a 'gel'. The absence of an effect of protein content on heat coagulation time at high pH can only be explained by assuming that the time elapsing before anisometric particles emerge rather than the rate of aggregation determines time of visible coagulation.

Samenvatting

Het coaguleren van geëvaporeerde melk (evap) tijdens de sterilisatie is een van de problemen die optreden bij de industriële bereiding van dit produkt. Dit probleem is tussen 1920 en 1940 beheersbaar gemaakt door het gaan toepassen van een tweetal additionele processtappen, nl. het verhitten van de melk voor het indampen (de z.g. voorverhitting), en het toevoegen van een variabele, vooraf vast te stellen, hoeveelheid van een stabilisatiezout (meestal een natriumfosfaat). Deze beheersing geschiedt echter via 'trial and error' procedures. In dit onderzoek is geprobeerd meer inzicht te krijgen in de mechanismen van de hittecoagulatie van geëvaporeerde melk, om zodoende een in mindere mate op 'trial and error' procedures gebaseerde procesbeheersing mogelijk te maken.

Hiertoe is vooral de stabiliteit van de caseïnemicellen in geconcentreerde ondermelk bestudeerd, omdat reeds lang verondersteld wordt dat instabiliteit van de caseïnemicellen bij een hoge temperatuur de hoofdoorzaak is van hittecoagulatie. Caseïnemicellen zijn echter zeer stabiel in melk bij kamertemperatuur. Daarom is in eerste instantie onderzocht wat de gevolgen zijn van de verschillende processtappen bij de bereiding van gesteriliseerde geëvaporeerde (onder)melk voor deze deeltjes en het systeem waarin ze zijn gedispergeerd. Het gebruikte proces, met de toegepaste variabelen was als volgt:

(onder)melk
- niet voorverhitten – indampen
  - pH instellen — verhitten
  - + fosfaat – pH instellen – verhitten
- wel voorverhitten – indampen
  - pH instellen — verhitten
  - + fosfaat – pH instellen – verhitten

In Artikel 1 worden de bij de bereiding van gesteriliseerde geconcentreerde volle melk optredende veranderingen in de verdeling van calcium en fosfaat tussen serum en caseïnemicellen beschreven, en ook de veranderingen van de pH en de calciumionaktiviteit. In niet-gesteriliseerde evap was ongeveer 10% meer calcium en 40% meer fosfaat met de caseïnemicellen geassocieerd dan in rauwe melk, terwijl de concentraties (mol/l) calcium en fosfaat in het serum ongeveer verdubbeld waren. De calciumionaktiviteit van evap was ongeveer gelijk aan die van de uitgangsmelk, bij een 0,3 eenheden lagere pH.

Wel of niet voorverhitten had hierop weinig invloed. In niet-gesteriliseerde evap was bij pH 7,0 aanzienlijk meer calcium geassocieerd met de caseïnemicellen dan bij pH 6,3. De hoeveelheid met de caseïnemicellen geassocieerd fosfaat was echter nauwelijks pH afhankelijk. In gesteriliseerde evap was de verdeling van calcium en fosfaat vrijwel gelijk aan die in niet-gesteriliseerde evap, ondanks de lagere pH in gesteriliseerde evap. Het verband tussen de calciumionaktiviteit en de pH was in evap monotoon dalend, zoals in melk, alleen op een lager niveau. Na sterilisatie waren zowel de calciumionaktiviteit als de pH lager dan daarvoor. Het toevoegen van fosfaat aan evap voor sterilisatie resulteerde in meer micellair calciumfosfaat en in een lagere pH en calciumionaktiviteit, vooral na sterilisatie. Bovendien was voor evap met toegevoegd fosfaat de hittestabiliteit (van de volle evap) groter bij een pH lager dan 6,5. Hieruit wordt geconcludeerd dat de calciumionaktiviteit van essentieel belang is voor de hittestabiliteit van evap bij een pH lager dan 6,5, terwijl de absolute hoeveelheden calcium en fosfaat (zowel het met de deeltjes geassocieerde deel als het calcium en fosfaat in het serum) weinig invloed hebben. Bij een hogere pH zijn geen van bovengenoemde factoren blijkbaar van direkt belang voor de verhittingstijd die nodig is voor visuele coagulatie, of de effecten van calciumionaktiviteit en absolute hoeveelheden calcium en fosfaat compenseren elkaar.

Artikel 2 beschrijft de veranderingen die bij de bereiding van gesteriliseerde geconcentreerde ondermelk optreden in de associatietoestand van de eiwitten (bepaald bij 20°C). Het concentreren van niet-voorverhitte melk bleek geen veranderingen te geven; concentreren van voorverhitte melk resulteerde in minder caseïne in de caseïnemicellen, vooral indien de pH van de geconcentreerde melk hoog (7,0) was. α-Lactalbumine en β-lactoglobuline waren in voorverhitte melk en de daaruit bereide evap grotendeels geassocieerd, al dan niet met de caseïnemicellen. Door het verhitten van evap daalde het percentage micellaire caseïne. Deze daling was sterker naarmate de verhittingsduur langer en de pH van de evap hoger was. Vooral de dissociatie van de $\varkappa$-caseïne was zeer sterk pH afhankelijk. Bij pH 6,3 verliep de dissociatie langzaam en ongeveer lineair met de verhittingstijd, maar bij pH 7,0 dissocieerde ongeveer 70% van de totale hoeveelheid $\varkappa$-caseïne in enkele minuten, terwijl daarna weinig meer veranderde. Voorverhitten vertraagde de dissociatie van $\varkappa$-caseïne bij een hoge pH; na een verhitting van ongeveer 5 minuten waren de verschillen tussen voorverhitte en niet-voorverhitte evap echter verwaarloosbaar. Bediscussieerd wordt dat het aannemelijk is dat de dissociatie van de caseïnes tijdens de verhitting plaatsvindt. De waargenomen veranderin-

gen in de associatie van de verschillende eiwitten, en dan vooral de dissociatie van de κ-caseïne, kunnen dus invloed hebben op de hittestabiliteit.

In Artikel 3 worden de resultaten beschreven van metingen waarmee het verloop van de aggregatie van de eiwitdeeltjes in magere geconcentreerde melk werd gevolgd. Hiervoor zijn zeven verschillende onderzoeksmethoden gebruikt. Viscositeit, turbiditeit, en de hoeveelheid gesedimenteerd eiwit (30 min bij 2000 x *g*) zijn, als functie van de verhittingstijd, bepaald voor evap na afkoelen tot 20°C. Ook zijn van een aantal monsters verhitte evap electronenmicroscopische foto's gemaakt. De turbiditeit en de valtijd in een Höppler viscosimeter zijn als functie van de verhittingstijd bepaald voor evap bij 120°C; tenslotte is de hittecoagulatijd bij 120°C bepaald. De meetresultaten bij 120°C waren niet wezenlijk anders dan die na afkoelen tot 20°C. Metingen na afkoelen mogen dus gebruikt worden bij de bestudering van de hittecoagulatie. Uit de gecombineerde resultaten blijkt dat de hittecoagulatie inderdaad het gevolg was van aggregatie van de caseïnemicellen, behalve voor de niet voorverhitte geconcentreerde melk bij een pH lager dan 6,5. Aggregatie van de caseïnemicellen resulteerde in volumineuze vlokken in voorverhitte evap met een pH lager dan 6,5, maar in compacte ronde deeltjes bij een hogere pH (zowel in voorverhitte als in niet voorverhitte evap). Vlak voor de coagulatietijd waren de deeltjes bij een pH hoger dan 6,5 echter anisometrisch. Op grond van bovengenoemde waarnemingen en de resultaten uit artikel 1 en 2 wordt een model voor de hittecoagulatie van geconcentreerde melk voorgesteld. Coagulatie van niet-voorverhitte evap bij een pH lager dan 6,5 wordt verondersteld veroorzaakt te worden door aggregatie, na hitte-denaturatie, van de serumeiwitten onder invloed van pH en calciumionaktiviteit. De snelheid van coagulatie door aggregatie van caseïnemicellen wordt geacht afhankelijk te zijn van de aggregatiesnelheid van deze deeltjes en van de geometrie van de gevormde aggregaten (volumineus dan wel compact). De aggregatiesnelheid van de caseïnemicellen hangt af van de ontmoetingsfrequentie en -duur van de deeltjes en van de kans op de vorming van een binding tussen twee deeltjes tijdens een ontmoeting. Dissociatie van de κ-caseïne tijdens verhitten is de belangrijkste factor die de stabiliteit van de caseïnemicellen beïnvloedt, en verloopt sneller bij een hogere pH. De kans op vorming van een binding hangt sterk af van de temperatuur en van het type binding dat gevormd wordt. Ionbindingen, al dan niet met een calciumion, zijn waarschijnlijk het belangrijkste type bij de aggregatie van de 'harige' caseïnedeeltjes bij een pH lager dan 6,5. Tussen min of meer 'gladde' deeltjes, welke ontstaan door verhitten bij een hogere pH, worden vooral op micellair calciumfosfaat lijkende bindingen en covalente bindingen gevormd. Ook in geconcentreer-

de magere melk met een lagere initiële pH kunnen covalente bindingen ontstaan, maar alleen indien de vorming van ion-bindingen zo traag verloopt, dat de coagulatietijden lang zijn. Door de langdurige verhitting daalt de pH van de evap dan tot beneden pH 5,8-6,0, en verondersteld wordt dat bij deze lage pH de 'harige' κ-caseïne lagen van twee micellen voldoende ver kunnen interpenetreren om covalente bindingen mogelijk te maken. De geometrie van de aggregaten wordt verondersteld afhankelijk te zijn van de dissociatie van de κ-caseïne en van de mate van oververzadiging van calciumfosfaat. Voor de vorming van compacte aggregaten is het nodig dat een groot deel van de κ-caseïne gedissocieerd is; bovendien moet de oververzadiging van calciumfosfaat voldoende groot zijn (in elk geval groter dan in niet geconcentreerde melk). Met dit model kunnen o.a. de invloed van de pH, die van voorverhitting en die van fosfaattoevoeging op de hittestabiliteit van geëvaporeerde ondermelk verklaard worden.

Kinetische aspecten van het bovengenoemde model worden bediscussieerd in Artikel 4. Hiertoe is de invloed van het eiwitgehalte van de magere evap op de hittecoagulatietijd onderzocht. De hittecoagulatietijd van magere evap bij een pH lager dan 6,5 bleek zeer sterk beïnvloed te worden door het eiwitgehalte. Bij een pH hoger dan 6,7 was de invloed van het eiwitgehalte echter verwaarloosbaar. De grote invloed van het eiwitgehalte beneden pH 6,5 kan verklaard worden door aan te nemen dat de volumineuze aggregaten die onder deze omstandigheden gevormd worden een fractale geometrie hebben. Hierbij treedt coagulatie op wanneer de vlokken de gehele vloeistof vullen en zodoende een 'gel' vormen. De afwezigheid van een effect van het eiwitgehalte bij hoge pH kan alleen worden verklaard door aan te nemen dat de aggregatiesnelheid van de deeltjes niet bepalend is voor de tijd van visuele coagulatie, maar veeleer de tijd die nodig is voordat anisometrische deeltjes ontstaan.

Uit de resultaten van dit onderzoek blijkt dat de snelheid van meerdere reactie(s) bepalend kan zijn voor hittecoagulatiesnelheid van geëvaporeerde (onder)melk, afhankelijk van het bereidingsproces en de samenstelling van de evap. Een semi-kwantitatieve beschrijving van de verschillende aggregatieprocessen is echter mogelijk. Voor een nauwkeurige sturing van de hittestabiliteit, zoals meestal gewenst is in de industriële praktijk, blijven 'trial and error' procedures echter (nog) onmisbaar.

Curriculum vitae

De auteur werd op 11 juni 1961 in 's Heer Hendrikskinderen geboren. In 1979 behaalde hij het diploma gymnasium β aan het Christelijk Lyceum voor Zeeland te Goes. Daarna begon hij de studie levensmiddelentechnologie aan de toenmalige Landbouwhogeschool te Wageningen. In november 1985 slaagde hij voor het doctoraalexamen, met als hoofdvak melkkunde en als bijvakken proceskunde gecombineerd met levensmiddelennatuurkunde, en bedrijfskunde. Op 1 december 1985 trad hij als research medewerker in dienst bij de zuivelcoöperatie ccFriesland (sinds oktober 1990 FRIESLAND Frico Domo). In dit dienstverband is het in dit proefschrift beschreven onderzoek uitgevoerd.

Nawoord

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