Food Structure

Volume 7 | Number 2 Article 7

1988

The Role of B-Lactoglobulin in the Development of the Core-and-Lining Structure of Casein Particles in Acid-Heat-Induced Milk Gels

V. R. Harwalkar

Miloslav Kalab

FOOD MICROSTRUCTURE, Vol. 7 (1988), pp. 173-179
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0730-5419/88$3.00+.00

THE ROLE OF β-LACTOGLOBULIN IN THE DEVELOPMENT OF THE CORE-AND-LINING STRUCTURE OF CASEIN PARTICLES IN ACID-HEAT-INDUCED MILK GELS

V. R. Harwalkar and Miloslav Kaláb

Food Research Centre, Research Branch, Agriculture Canada
Ottawa, Ontario, Canada K1A 0C6

Abstract

Acid-heat-induced gels were obtained by coagulating casein micelle dispersions at 90°C using glucono-δ-lactone. The casein micelles used were isolated from raw skim milk by centrifugation, washed free of whey proteins and soluble salts, and dispersed in water or a milk dialyzate. The pH values of the gels varied from 4.7 to 6.3. A core-and-lining ultrastructure developed in casein particles coagulated at pH 5.2 to 5.5 from casein micelle dispersions in the milk dialyzate provided that β-lactoglobulin or whey proteins (10 mg/mL) were added to them prior to coagulation. Addition of β-lactoglobulin to aqueous casein micelle dispersions led to the development of a considerably less distinct core-and-lining ultrastructure of the resulting gels. Coagulated casein particles obtained from casein micelle dispersions in water or in the milk dialyzate to which neither β-lactoglobulin nor whey proteins were added, did not show the core-and-lining ultrastructure but contained void spaces inside and were covered with loosely aggregated protein on the surface.

It was concluded that both β-lactoglobulin or whey proteins and the milk salt system are essential for the formation of the core-and-lining ultrastructure in the casein micelle dispersions gelled by heating at 90°C at pH 5.2 to 5.5.

Initial paper received April 02, 1988
Manuscript received August 15, 1988
Direct inquiries to V.R. Harwalkar
Telephone number: 613 995 3722

Key Words: Acid-heat-induced milk gels, Casein micelles, Core-and-lining structure, Electron microscopy, Gelation, Glucono-δ-lactone, β-Lactoglobulin, Milk.

Introduction

Variations in the ultrastructure of casein micelles have been observed in gels obtained by coagulating milk using various acidulants. A unique 'core-and-lining' structure was observed in gels obtained by coagulating milk heated to 90°C at pH 5.5 [3, 4, 6]. This structure, observed in gels produced with many acidulants used in the study (*i.e.*, glucono-δ-lactone and citric, hydrochloric, and oxalic acids) is characterized by an outer membrane-like lining surrounding a solid core which is separated from the lining by an annular space, 50 to 80 nm wide. The core-and-lining structure is characteristic of some acid-heat milk gels, such as the Latin-American White cheese (Queso Blanco) [9] and Indian paneer [8]. It is very stable and has been observed in process cheese which contained White cheese as an ingredient [1]. The existence of this structure was demonstrated using various electron microscopy techniques such as thin-sectioning of samples embedded in a resin, replication of frozen hydrated samples, and replication of dried samples [6]. The findings were confirmed by others [M. A. Christman, personal communication].

The mechanism of the formation of the core-and-lining structure is not fully understood. However, it was observed to develop only at temperatures exceeding 70°C [4], *i.e.*, at temperatures at which the formation of a complex composed of β-lactoglobulin and κ-casein is promoted.

The objective of this study is to examine the role of protein interactions in the development of the core-and-lining structure. For this purpose, model systems involving isolated casein micelles, β-lactoglobulin, and milk dialyzate were studied.

Materials and Methods

Casein micelle dispersions

Fresh skim milk used in this study was prepared by separating cream from pooled milk obtained from the herd of dairy cows at the Central Experimental Farm of Agriculture Canada in Ottawa.

Casein micelles were prepared from the skim milk by centrifugation at 8×10^4 *g* in a Beckman Model L4 ultracentrifuge operated at 4°C for 2 h. The whey and residual fat layers were removed by suction using a Pasteur pipet. The casein micelle pellet was washed twice by dispersing it in

glass-distilled water or in a milk dialyzate at the original volume of the milk and recentrifuging it under the same conditions as mentioned above. The washed casein micelles were finally dispersed in distilled water or in a milk dialyzate for use in this study.

Milk dialyzate was obtained by dialyzing sterile distilled water closed in dialyzing tubing, 20 mm in diameter, that was suspended in a bulk milk tank at 4° to 6°C for 24 h.

Whey proteins were prepared by thoroughly dialyzing acid whey against distilled water and by freeze-drying the dialyzate. β-Lactoglobulin was of commercial origin as '3x crystallized β-lactoglobulin' (Sigma Co., St. Louis, MO, USA).

Gelation

Washed casein micelles were dispersed in distilled water or in a milk dialyzate to obtain 2% solutions. Aliquots (3 mL) of these dispersions were used either plain or following the addition of crystalline β-lactoglobulin or whey proteins (10 mg/mL). The dispersions were placed in small test tubes, heated at 90°C in a water bath, and solid glucono-δ-lactone (GDL, 7 to 30 mg) was added to them to form 0.25% to 1.0% solutions. The mixtures were stirred and held at 90°C until the protein formed gels as the result of acidulation by gluconic acid developing from hydrolysis of GDL. The protein gels were then rapidly cooled to 22°C using cold water and sampled for electron microscopy.

Electron microscopy

Small cubes (approximately 1 mm^3) of the gels under study were fixed at 22°C in an aqueous 2.8% glutaraldehyde solution for 2 h and were postfixed under similar conditions in a buffered (0.05 M veronal-acetate buffer, pH 6.75) 2% osmium tetroxide solution. Then the samples were washed with the veronal-acetate buffer, dehydrated in a graded (20%, 40%, 60%, 80%, 96%, and 100%) ethanol series, and embedded in Spurr's low-viscosity resin (J. B. EM Service, Inc., Pointe-Claire, Dorval, Quebec). Sections, 90 nm thin, were stained with uranyl acetate and lead citrate solutions and examined in a Philips EM-300 electron microscope operated at 60 kV [9].

Results and Discussion

Addition of solid GDL (0.25 to 1.0%) to casein micelle dispersions heated at 90°C produced gels having pH values between 5.0 and 6.3 and varying in characteristics (Table 1).

Plain casein micelle gels

The microstructures of the acid-heat-induced protein gels obtained from casein micelle dispersions in distilled water are shown in Figs. 1 - 3 and gels obtained from casein micelle dispersions in a milk dialyzate are presented in Figs. 4 - 6. All these figures show large, loosely aggregated-to-fused particles of casein. In some samples, there are large pores or void spaces which presumably result from the solubilization of colloidal calcium phosphate at low pH [11]. In the absence of colloidal calcium phosphate, the submicellar structures of the casein micelles collapse and fuse by hydrophobic interaction which is promoted by low pH and high temperature. However, the typical core-and-lining structures were not evident in any of these gels obtained solely from washed casein micelles (Figs. 1 to 6). Although the gels made at pH 5.5 using casein micelles dispersed in the milk dialyzate showed a compact layer at the surface of the aggregated casein particles (Fig. 5), there was no annular space that would separate the core from the lining. The formation of the compact protein layer at the casein particle

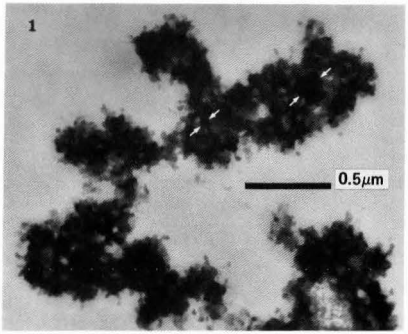
Table 1. Characterization of casein micelle gels

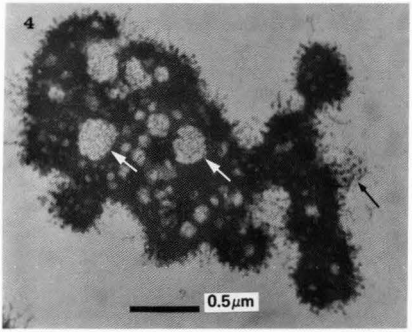
Dispersion in:	Protein added:	pH:	C-&-L*:	Fig.:	Note on structure:
Water	None	6.3	No	1	Corpuscular
Water	None	5.6	No	2	Void spaces
Water	None	5.3	No	3	Void spaces
Dial.**	None	6.3	No	4	Void spaces
Dial.	None	5.5	No	5	Void spaces
Dial.	None	5.0	No	6	Void spaces
Dial.	β-LG†	4.7	No	7	Like yoghurt
Dial.	β-LG	5.5	Yes	8	-------
Dial.	β-LG	5.3	Yes	9	-------
Water	β-LG	5.5	Weak	10	Corpuscular
Water	β-LG	5.3	Weak	11	Corpuscular
Dial.	WP††	5.3	Strong	12	Like in milk gels

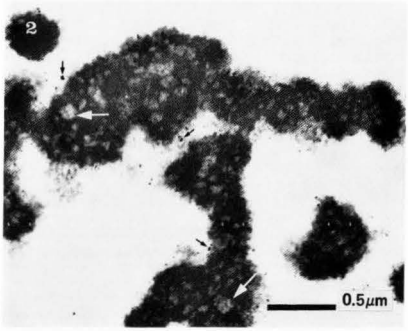
* Core-and-lining ultrastructure
** Milk dialyzate
† β-Lactoglobulin, 10 mg/mL
†† Whey proteins, 10 mg/mL

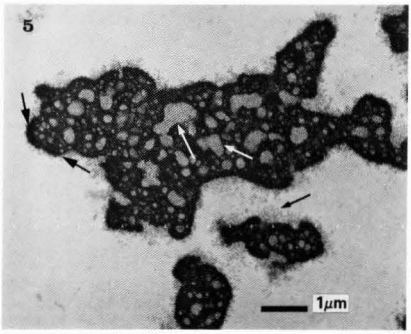
Figs. 1 - 3. Heat-induced gels obtained from aqueous dispersions of casein micelles at 90°C at pH 6.3 (Fig. 1), pH 5.6 (Fig. 2), and pH 5.3 (Fig. 3). Casein micelle entities have vanished in all gels. Corpuscular ultrastructure composed of particles varying in dimensions (pairs of small light arrows in Fig. 1) was observed in all 3 gels. Large pores or void spaces (large light arrows) developed as pH was decreased (Figs. 2 and 3). Minute dark particles in Fig. 2 (small dark arrows) are an artefact (probably a glutaraldehyde-osmium tetroxide complex [10]).

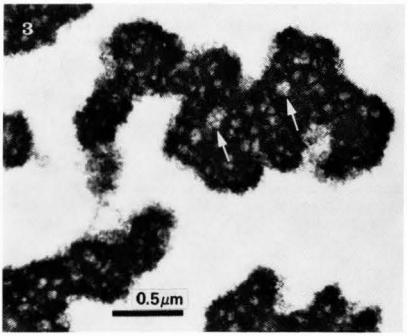
Figs. 4 - 6. Heat-induced gels obtained from dispersions of casein micelles in a milk dialyzate at pH 6.3 (Fig. 4), pH 5.5 (Fig. 5), and pH 5.0 (Fig. 6). Large void spaces (light arrows) in compact casein particles are filled with loosely aggregated protein. Similar protein may be observed on the particle surface (small dark arrows). Casein gels obtained at pH 5.5 (Fig. 5) have a compact protein layer (large dark arrows) at their surface. Corpuscular ultrastructure composed of particles smaller than 0.1 μm in diameter is particularly evident in gels made at pH 5.0 (pairs of small light arrows in Fig. 6).

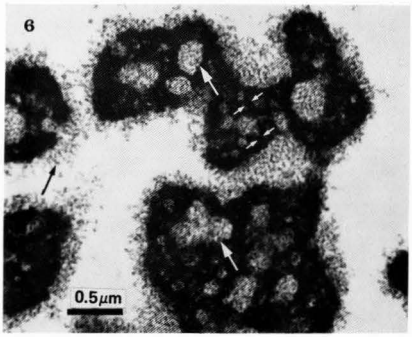

1
0.5μm


4
0.5μm


2
0.5μm


5
1μm


3
0.5μm


6
0.5μm

surface may be linked to the dissociation and reassociation of casein micelles observed by Heertje et al. [5]. As pH is further lowered to 5.5, β-casein is released from the micelles [13]. An increase in non-sedimentable caseins and a decrease in colloidal calcium phosphate are also observed at pH 5.5 [11].

Changes in the mineral balance caused by heating may also contribute to the deposition of casein on the surfaces of the altered casein micelles. As the temperature is increased, soluble calcium phosphate precipitates. This precipitation lowers pH possibly to the point of minimum Zeta-potential of β-casein to approximately pH 5.2 and causes the precipitation of β-casein on the periphery of the caseinate particles. The membrane-like lining is less evident in aqueous dispersions than in the milk dialyzate dispersions. There is very little soluble calcium phosphate in the aqueous dispersions and pH changes due to heating are presumably less extensive.

Casein micelle gels containing β-lactoglobulin or whey proteins

The microstructures of gels obtained by heating dispersions of casein micelles to which 1% of β-lactoglobulin was added, are shown in Figs. 7 to 11. Gels obtained from casein micelles dispersed in milk dialyzate are featured in Figs. 7 to 9 and gels obtained from casein micelles dispersed in distilled water are shown in Figs. 10 and 11. The microstructure varied depending on the final pH value of the gels after heating and on the medium in which the casein micelles had been dispersed. At pH 4.7, the gel network of fused casein micelles is similar to that of skim milk gels heated at that pH as observed earlier. At pH 5.2 or 5.5, the typical core-and-lining structure is evident (Figs. 8 and 9) though not as clearly as in the skim milk gels [3, 4]. The typical core-and-lining structure was also evident when the dispersions of casein micelles in milk dialyzate were heated in the presence of whey proteins at pH 5.3. Fig. 12 shows the structure to be well developed. However, micrographs of gels obtained by heating aqueous dispersions of casein micelles with β-lactoglobulin at pH 5.3 and 5.5 (Figs. 10 and 11) showed the core-and-lining structure to be developed less distinctly. It may be assumed, therefore, that the presence of either β-lactoglobulin or whey proteins in conjunction with the milk salt system is essential for the development of the typical core-and-lining structure. It was observed that heating of casein micelle dispersions with the milk salt system in the absence of β-lactoglobulin or whey proteins did not lead to the development of the core-and-lining structure (Figs. 4 to 6).

Earlier work [4] has shown that the heating of skim milk, which contained β-lactoglobulin and the milk salt system, at pH 5.5 and at temperatures higher than 70°C was essential for the formation of the core-and-lining structure to take place. It is well known that heating at temperatures above 70°C promotes the formation of a complex between β-lactoglobulin and κ-casein. The formation of the core-and-lining structure is thus associated with the existence of that complex and the presence of the milk salt system.

Since pH of 5.2 to 5.5 is critical to the development of the core-and-lining structure, it may be assumed that the properties of casein micelles at this pH are very important. In this pH range, casein micelles have the optimal voluminosity or hydrodynamic volume, high percentage of non-sedimentable casein, and a reduced concentration of colloidal calcium phosphate. These phenomena have been linked to the removal of the colloidal calcium phosphate and some casein and swelling of the residual micelles [11]. The heat-induced interaction between β-lactoglobulin and κ-casein results in the development of filamentous appendages [2, 7]. Calcium ions enhance this interaction [12] which partly explains the importance of the milk salt system for the formation of the core-and-lining structure. The caseins, particularly β-casein, dissociated from the micelle during the heat treatment precipitate on the protruding filamentous appendages to form a lining and leave an annular space between the casein core and the lining formed. These considerations based on the data presented in this paper are consistent with the model proposed earlier [4] to illustrate the mechanism of the formation of the core-and-lining structure. The hypothesis that the core-and-lining structure may be the result of differences in contraction of the different proteinaceous materials during redeposition of dissociated caseins [5] cannot be supported by the present work. The micrographs in Figs. 1 to 6 produced in the absence of β-lactoglobulin do not show the contraction behaviour mentioned above to result in the formation of the core-and-lining structure.

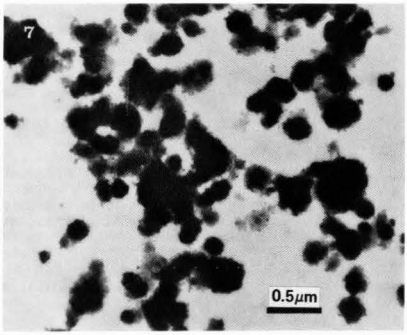
Acknowledgments

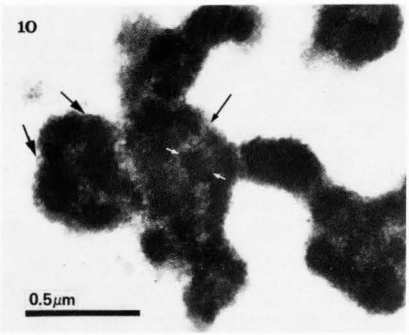
Skillful technical assistance provided by Mrs. Paula Allan-Wojtas and Mrs. Bernadette Boutin-Muma is acknowledged. The authors thank Dr. H. Wayne Modler for useful suggestions. Electron Microscope Unit, Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 772 from the Food Research Centre.

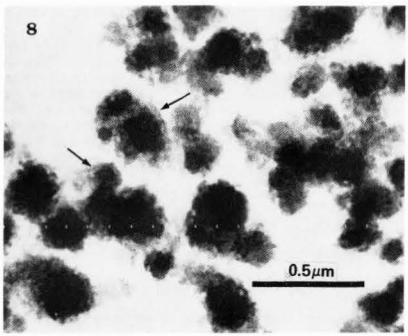
Figs. 7 - 9. Heat-induced gels obtained from casein micelle dispersions in a milk dialyzate in the presence of added β-lactoglobulin (10 mg/mL) at pH 4.7 (Fig. 7), pH 5.5 (Fig. 8), and pH 5.3 (Fig. 9). At pH 4.7, the protein network resembles that of yoghurt [2, 7, 10]. The core-and-lining ultrastructure developed in gels made at pH 5.5 and 5.3 (arrows).

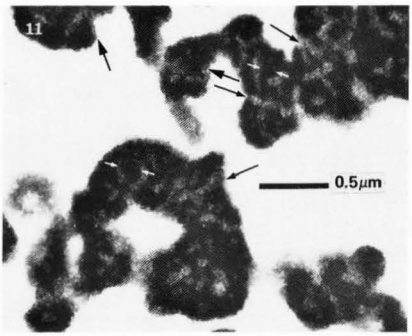
Figs. 10 and 11. Heat-induced gels obtained from aqueous casein micelle dispersions containing β-lactoglobulin (10 mg/mL) at pH 5.5 (Fig. 10) and pH 5.3 (Fig. 11). The core-and-lining structure developed at pH 5.3 is less distinct than at pH 5.5 (large dark arrows). Compact particles approximately 0.1 μm in diameter (pairs of small light arrows) are connected with each other by loosely aggregated protein (small dark arrows).

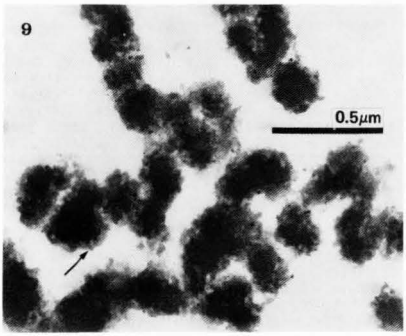
Fig. 12. Heat-induced gel obtained at pH 5.3 from casein micelles dispersed in a milk dialyzate which contained whey proteins. The core-and-lining ultrastructure (arrows) is well developed and similar to that found in milk gels [3, 4].

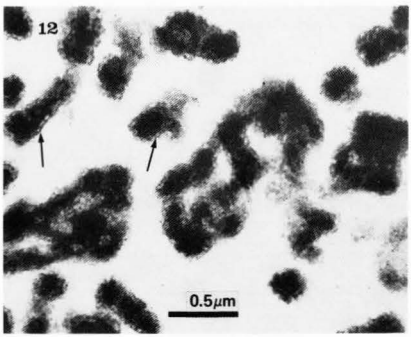

7
0.5μm


10
0.5μm


8
0.5μm


11
0.5μm


9
0.5μm


12
0.5μm

References

1. Carić M, Kaláb M. (1987). Processed cheese products. In: Cheese: Chemistry, Physics and Microbiology. Vol. 2. PF Fox (ed.), Elsevier Applied Science Publishers Ltd., New York, NY, USA, 339-383.
2. Davies FL, Shankar PA, Brooker BE, Hobbs DB. (1978). A heat-induced change in the ultrastructure of milk and its effect on gel formation in yoghurt. J. Dairy Res. 45, 53-58.
3. Harwalkar VR, Kaláb M. (1980). Milk gel structure. XI. Electron microscopy of glucono-δ-lactone-induced skimmilk gels. J. Texture Stud. 11, 35-49.
4. Harwalkar VR, Kaláb M. (1981). Effect of acidulants and temperature on microstructure, firmness and susceptibility to syneresis of skim milk gels. Scanning Electron Microsc. 1981; III:503-513.
5. Heertje I, Visser S, Smits P. (1985). Structure formation in acid milk gels. Food Microstruc. 4(2), 255-267.
6. Kaláb M. (1980). Milk gel structure. XII. Replication of freeze-fractured and dried specimens for electron microscopy. Milchwissenschaft 35(11), 657-662.
7. Kaláb M, Emmons DB, Sargant AG. (1976). Milk gel structure. V. Microstructure of yoghurt as related to heating of milk. Milchwissenschaft 31(7), 402-408.
8. Kaláb M, Gupta SK, Desai HK, Patil GR. (1988). Development of microstructure in raw, fried, and fried and cooked paneer made from buffalo, cow, and mixed milks. Food Microstruc. 7(1), 83-91.
9. Kaláb M, Modler HW. (1985). Development of microstructure in a Cream cheese based on Queso Blanco cheese. Food Microstruc. 4(1), 89-98.
10. Parnell-Clunies EM, Kakuda Y, Humphrey R. (1986). Electron-dense granules in yoghurt: Characterization by x-ray microanalysis. Food Microstruc. 5(2), 295-302.
11. Roefs SPF, Walstra P, Dalgleish DG, Horne DS. (1985). Preliminary note on the changes in casein micelles caused by acidification. Neth. Milk Dairy J. 39, 119-122.
12. Smits P, Van Brouwershaven JH. (1980). Heat-induced association of β-lactoglobulin and casein micelles. J. Dairy Res. 47, 313-325.
13. Snoeren THM, Klok HJ, Van Hooydonk ACM, Damman AJ. (1984). The voluminosity of casein micelles. Milchwissenschaft 39, 461-463.

Discussion with Reviewers

Y. Kakuda and D. G. Schmidt: Were the mixtures heated at 90°C for any appreciable time prior to the addition of glucono-δ-lactone?
Authors: It took approximately 3 to 5 min to reach that temperature and there was no holding before glucono-δ-lactone was added.

Y. Kakuda: Were the initial pH values different for the distilled water samples compared to the dialyzates?
Authors: The initial pH values were nearly the same within 0.02 unit with the micelles suspended in water or the milk dialyzate.

Y. Kakuda: Were the final pH values determined at 90°C or after cooling to 22°C?
Authors: The final pH values were determined after cooling the milk gels to 22°C.

D. G. Schmidt: How much time was required to reach the desired pH?
Authors: We did not determine the time required to reach the final pH. However, it is known that glucono-δ-lactone hydrolyzes very rapidly in water at 90°C. The time required for gelation to take place at 90°C was usually 1 to 2 min at pH values lower than 5.7 and about 3 to 5 min at pH between 5.7 and 6.3. Heating at a higher pH required a longer time to gel the milk although the final pH value may have been reached earlier.

R. Cartwright: Did you assume that β-lactoglobulin and the whey proteins used were totally undenatured prior to gelation? If so, what effects would you expect to see if the β-lactoglobulin or whey proteins had been partially denatured prior to gelation?
Authors: Undenatured β-lactoglobulin or whey proteins were added to the micelle suspension but the heat treatment before the addition of glucono-δ-lactone denatured a considerable portion of these proteins. We have shown previously [4] that the heat treatment insufficient to denature the protein does not give rise to the core-and-lining structure. We have not added previously denatured proteins to the casein micelle suspensions because the solubility would be a problem. It would be interesting to learn how previously denatured proteins will interact with the casein micelles and whether, indeed, they would contribute to the core-and-lining structure.

D. G. Schmidt: At 4°C, a large part of β-casein dissociates from the micelles and the micelles obtained after the second washing, therefore, will have a composition differing from that of the original ones. Dispersing the micelles in distilled water will result in their disintegration, particularly if it takes much time. Will you comment, please?
Authors: We agree that the washing procedure used, particularly using distilled water, may have an effect on the composition of the washed casein micelles. The disintegration of the micelles was not expected in our work since the micelles were not stored for long periods. Prolonged storage of dilute casein micelles is known to cause their dissociation [14]. Despite this limitation, the results obtained by heating aqueous micelle suspensions at various pH values and in the presence or absence of β-lactoglobulin are valid in that they emphasize the need of β-lactoglobulin for the core-and-lining structure to develop.

Y. Kakuda: The model requires conditions where β-casein dissociates into the serum phase and, at the same time, precipitates on the appendages. Does this require a drop in pH from 5.5 (dissociation of β-casein) to 5.2 (minimum charge) or does this signify two different types of interactions

for β-casein - one interaction with the micelle and the other with the appendages?
Authors: It should be remembered that the pH of 5.5 was measured after rapidly cooling the heated samples. During heating, we envisage that the pH at the high temperature may have dropped further to possibly 5.2, *i.e.*, to a point of the minimum charge. This could be the result of the combined effect of high temperature and the precipitation of calcium phosphate. The precipitation of the caseins that dissociated from the micelles as the pH was lowered would take place irrespective of the presence or absence of the appendages on the casein micelle surfaces.

Y. Kakuda: The addition of whey proteins (and skim milk in previous studies) produced a more distinct core-and-lining structure. Does this imply some role for α-lactalbumin?
Authors: The possible contribution of whey proteins other than β-lactoglobulin to the development of the core-and-lining structure was not examined in this report. It may be worthwhile to do so.

B. E. Brooker: How could voids in the casein particles arise by solubilization of calcium phosphate? How would calcium phosphate associate into such large structures in the first place?
Authors: The cause of the void spaces in the casein particles is speculative. These voids presumably result from a number of different effects. As the pH is lowered, colloidal calcium phosphate and some caseins are removed and swelling is observed [11]. The casein particles are closer to their isoelectric point. During the gelation of these particles by heat at the lower pH, aggregation may take place through hydrophobic interactions. Steric hindrance during aggregation by fusion may also play a role.

B. E. Brooker: Why is there greater aggregation of micelles in the heat-induced gels in Figs. 10 and 11 compared with those in Figs. 8 and 9?
Authors: We have no answer for this difference. Possibly, the lack of minerals in aqueous suspensions contributes to a more extensive fusion of the casein particles during heating.

D. G. Schmidt: I have noticed that the 12 micrographs presented have been obtained at 5 various magnifications, which makes their comparison difficult. Is there any reason for such differences in magnification or would it be better to show all the micrographs on the same scale?
Authors: Our intention was to show the casein aggregates as well as the detail of the structures developed. Thus, although the images shown in Figs. 5 and 6 are similar, Fig. 5 shows the entire cluster and Fig. 6 shows the detail of void spaces and submicellar structures. In order to show the development of the core-and-lining structure in some gels (Figs. 8 to 10), a higher magnification had to be used.

D. P. Dylewski: Would the application of scanning electron microscopy (SEM) in conjunction with TEM provide additional information to help interpret the structure of core-and-lining in casein particles, or is this unnecessary?
Authors: TEM is best suited to show the core-and-lining structure, be it by staining thin sections of embedded samples or replication of freeze-fractured samples with platinum and carbon. The need to examine the interior of the casein particles in order to reveal the void annular space around the core and the small dimensions of that void space make it more difficult to study this structure by SEM.

Additional Reference

14. McGann TCJ, Donnelly WJ, Kearney RD, Buchheim W. (1980). Composition and size distribution of bovine casein micelles. Biochim. Biophys. Acta 630, 261-270.