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STRUCTURE FORMATION IN ACID MILK GELS

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Abstract

The structure formation in acid milk gels is influenced by many factors such as heat, salt system, pH, culture and thickening agents. Understanding of the mechanism of structure formation is important in order to be able to influence the final texture of these products.

In the present study the network formation in acid milk gels during acidification is monitored by freeze-fracture electron microscopy. Network formation appears to be a much more complex process than just an aggregation of the original milk casein micelles; it is accompanied by subtle dissociation and association phenomena of the milk caseins. The observed sequence of events can be explained from the course of the zeta potential, the association of the beta casein, the release of colloidal calcium phosphate from the micelle, the influence of heat treatment and from some observations on the internal structure of the casein micelle.

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Introduction

Cultured dairy products, e.g. yoghurt and fresh cheese, are produced using very subtle microbiological and physicochemical processes. Without careful process control and due to seasonal variations in milk composition they are therefore subject to large variations in properties such as gel strength and texture, whey separation, smoothness or granularity, ropiness, etc. Factors responsible for these variations are heat, salt system, milk composition, pH, type of culture, added thickening agents, etc.

A well known example of an acid milk gel is yoghurt, resulting from the acidification of skim milk by a symbiotic culture of *Lactobacillus bulgaricus* and *Streptococcus thermopilus*. It appears as a three dimensional network of caseins and culture bacteria (10, 15-17) (Fig. 1).

The casein micelle which is considered to be the building element in this network is a macromolecular assembly of α_S-, β- and κ-caseins held together by an amorphous calcium phosphate citrate complex and occurs as a separate entity in milk.

Acidification and network formation is induced by the formation of lactic acid from lactose in the milk by the bacterial culture. The current view is that upon acidification the casein micelles become aggregated by charge neutralization under the formation of a network of chains and clusters (10, 15-17). In this view inorganic constituents present have no separate role.

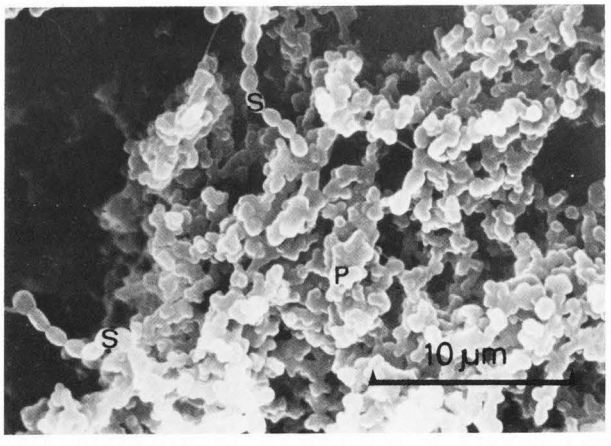


Fig. 1. Structure of yoghurt. p: protein network; s: *Streptococcus thermophilus*.

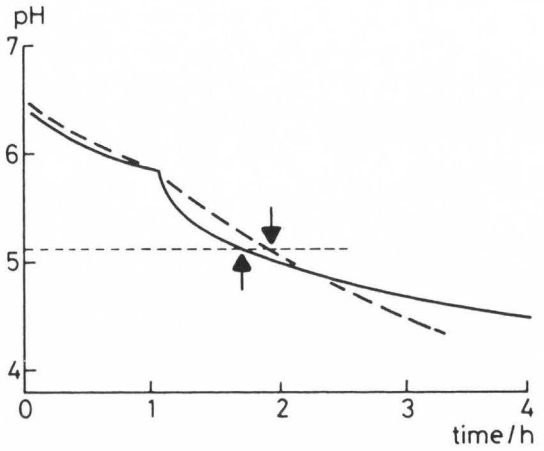


Fig. 2. pH-time profile of the acidification of skim milk. Comparison between glucono-δ-lactone (GDL) and a yoghurt culture. --- yoghurt culture; ——— GDL. Arrows indicate onset of gelation (pH 5.1).

However, the inorganic constituents, in particular calcium phosphate, perform an important function in that they maintain the integrity of the casein micelle (26, 34). Removal of calcium by calcium sequestering agents results in the disintegration of the micelles into separate protein subunits (18, 19, 28).

It is known that upon acidification of milk, micelle bound calcium phosphate is solubilized. At a pH of 5.1, calcium is almost completely removed from the micelle (11) and there are reasons to believe that at this pH the casein micelle also is disrupted. Since this pH is very close to the pH where gelation starts, one might wonder whether indeed the acid induced gelation of milk should be described as an aggregation process of the original individual micelles, or whether first a (partial) disintegration in casein subunits is taking place before an aggregation sets in.

For this reason, an in-depth study into the aggregation process during the acidification of milk has been carried out. Insight into the mechanism of aggregation is important for understanding the factors that influence the final texture of acid milk gels. In the present study, freeze-fracture electron microscopy was used to investigate the processes taking place during acidification and gel formation. The acidification process, as it occurs in the preparation of yoghurt, was imitated by the use of glucono-δ-lactone (GDL), which on hydrolysis gradually produces H_3O^+ ions. In order to better understand the mechanism of gelation, in this study also the internal structure of casein micelles was examined.

To support a proposed sequence of events, physico-chemical data, including zeta potential, the voluminosity of the casein micelles and changes in the milk salt system will be included.

Materials and Methods

Three kinds of milk samples were used. Sample I consisted of commercial pasteurized skim milk (9% dry matter) to which spray-dried medium heat skim milk powder was added to a final concentration of 12% solids and which was heated for 15 min at 90°C as is normal practice in yoghurt making. This sample was used to obtain a complete set of measurements on the microstructure during the acidification of milk. The other two samples were used to show the influence of pre-heating the milk on the microstructure, in particular in the region where gelation starts.

Samples II and III were made by dissolving freeze-dried milk powder, obtained from pasteurized skim milk in water to a final concentration of 12% solids. To study the influence of heating, sample II was pre-heated for 15 min at 90°C before use. Sample III was used directly.

Upon addition of 1% GDL (purum, ex Fluka A.G. Switzerland) at 20°C to sample I the pH dropped from 6.6 to 5.8; hereafter the mixture was heated to 30°C and an additional amount of 1% GDL was added. This procedure was chosen to obtain an acidification regime which conforms as close as possible to a normal yoghurt culture acidification. A comparison between GDL and a yoghurt culture is given in Fig. 2. To samples II and III, 1.2% GDL was added at 43°C. pH-time curves similar to those given in Fig. 2 were obtained.

All the samples, I–III, were observed by freeze-fracture electron microscopy at properly selected intervals during the acidification process. Special attention was paid to samples with a pH close to the onset of gelation. Freezing is considered to be the most suitable fixation technique in studies of the aggregation and disaggregation processes of caseins, because in such a sensitive system chemical fixation techniques may induce artefacts. To avoid ice crystal artefacts and phase separation during freezing, either rapid freezing or the addition of a cryoprotectant such as glycerol is essential. In our acidification experiments, rapid freezing has been applied, since addition of a cryoprotectant may influence the aggregation and disaggregation behavior. To attain complete vitrification in systems containing much water, like the present one, very high freezing velocities are required (10,000°C/s or higher). These conditions were obtained by propane jet freezing in a Balzers propane jet freezing system. The samples were placed between two copper plates and fixed between two nozzles through which liquid propane (−180°C) was blown from opposite directions onto the sample. The samples were subsequently fractured in a Balzers freeze-fracture unit (BAF 400D) at a low temperature (−100°C), etched for 15 s to expose the structure, and shadowed with platinum and carbon at an angle of 45°. The resulting replicas were viewed in a JEOL 100C transmission electron microscope operated at 80 kV.

Sample I was observed at pH 6.6, 5.9, 5.5, 5.2, 4.8 and 4.5, sample II was observed at pH 5.5, 5.4, 5.2 and 5.1 and sample III was observed at pH 5.4, 5.2, 5.1 and 5.0.

The structure of casein micelles was studied by the freeze-fracture methodology described by Schmidt and Buchheim (28, 29). Milk samples were mixed with glycerol to a final concentration of 30% and rapidly frozen in melting nitrogen (nitrogen slush). These experiments were performed at the normal pH of milk (about 6.7). The addition of a glycerol was considered to be permitted as there is no question of subtle aggregation and disaggregation phenomena as expected during acidification. Freeze fracturing was again carried out in a Balzers (BAF 400D) freeze-fracture unit. Apart from unidirectional shadowing, also rotary shadowing was applied. The latter procedure is particularly suitable for recognition of elongated structures.

Sodium citrate (0.02 M) was used to study the influence of calcium removal on the internal structure of the casein micelle.

Negative staining was applied in the study of the heat induced association of whey proteins and casein micelles as a function of pH. The heated samples were fixed in a 1% solution of glutaraldehyde in milk salt buffer during 1 h at room temperature.

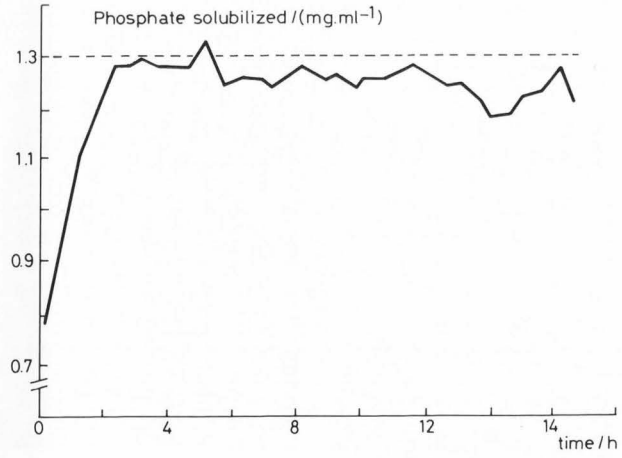


Fig. 3. Solubilization of phosphate upon acidification of skim milk at 30°C by GDL (2%) as determined by ^{31}P NMR. (---) level of soluble phosphate in milk.

After fixation the solution was dialysed to remove glutaraldehyde and any interfering inorganic ions. A drop of the protein solution was brought onto a freshly prepared parlodion carbon-coated grid. For good adhesion of the protein particles to the carbon film it is essential to use the carbon film within a few hours after preparation. Then, a drop of a 1% solution of uranyl acetate at pH 4.0 was applied to the grid and dried with filter paper.

^{31}P-NMR spectra were measured on a Bruker EP-200 WB NMR spectrometer operating at 81.0 MHz. Total soluble phosphate was estimated by comparing the resonance intensity of total soluble phosphate with the resonance intensity of an external standard consisting of a saturated solution of pyrophosphate in D_2O. The skim milk was brought into an NMR tube with an outer diameter of 20 mm and the saturated pyrophosphate solution into a tube with an outer diameter of 5 mm which was placed in the center of the first tube. The saturated pyrophosphate solution was calibrated against a known concentration of inorganic phosphate.

The viscosities of the acidified milk up to the point of gelation were measured with a Deer Rheometer. The milk was sheared between a thermostatic plate and a perspex cone of 1.5°. The shear rate was monitored during acidification while a constant shear stress of 0.24 Pa was applied. The pH at which the shear rate was reduced to zero was taken as the pH of onset of gelation.

Results and Discussion

Acidification/gelation

The pH region where gelation starts depends on the temperature of gelation (30°C and 43°C in this study) and on the heat pretreatment of the milk. Table 1 presents pH values at the onset of gelation. It appears that both pre-heating of milk and a higher temperature during acidification increase the pH of onset of gelation.

Another aspect of the structure formation is the milk salt system, especially the behavior of colloidal calcium phosphate. The solubilization of phosphate upon acidification of skim milk is shown in Fig. 3. By using 2% GDL as the acidification agent at 30°C all inorganic (colloidal) phosphate present in milk is solubilized after approximately 2 h at a pH of about 5.0. These observations substantiate earlier findings (11) that at pH 5.0 calcium is completely removed from the casein micelle.

The results of the microstructural observations during acidification are given in Figs. 4–16. In interpreting these figures it should be realized that they represent arbitrary cross-sections through a three-dimensional structure, which may cause chains of particles to appear as single particles, depending on the angle of cross-section.

The micrographs in Figs. 4, 5 and 6, taken from sample I show that the casein micelles keep their integrity, shape and dimensions during the initial drop in pH from 6.6 till 5.5. Some small particles are noticeable, however, at pH 5.5.

At pH 5.2 (Figs. 7, 8) the individual casein micelles appear to lose their integrity. The freeze-etch replica (in particular Fig. 8) shows separate particles. However, this structure should be carefully interpreted; it may be indicative of an aggregated network structure as has been observed before (1, 35). An observation supporting this view is the inhomogeneity of the apparent particulate distribution. Under the electron microscope, fields of these particles are observed, separated by empty spaces. This also points to an aggregated structure.

At pH 4.8 various structures can be distinguished. Fig. 9 shows a continuous field of particles, Fig. 10 shows more empty spaces, which are even more apparent in Fig. 11. These observations suggest that after a stage of aggregation, a stage of contraction and rearrangement occurs under formation of a particulate network. This view is supported by a micrograph of the final gel structure at pH 4.5 (Fig. 12).

These results show that at a certain pH different stages of protein aggregation and disaggregation can be distinguished.

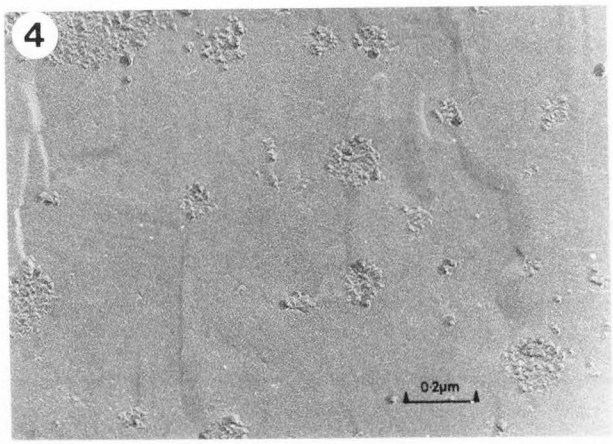
An analogous behavior is observed for mixtures II and III, which differ in their detailed gelation pattern, in particular with respect to the pH of the onset of gelation (see Table 1). This is reflected in the microstructure. The unheated milk still shows a homogeneous distribution of micelles at pH 5.2 (Fig. 13) whereas at pH 5.1 an inhomogeneous distribution, apparently of aggregated caseins is observed (Fig. 14). On the other hand the heated milk already shows this inhomogeneous aggregated structure at pH 5.5 (Fig. 15). The same type of structure is observed at pH 5.2 (Fig. 16).

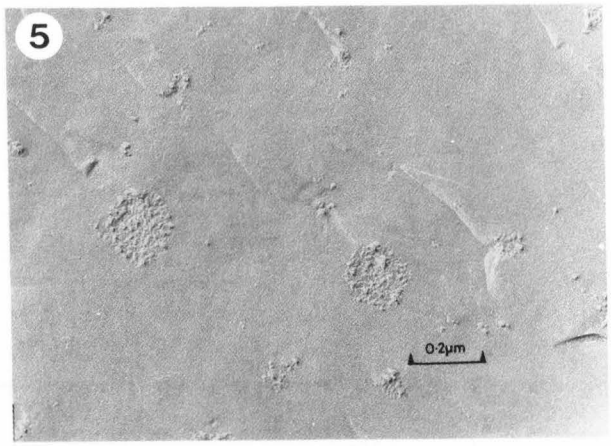
Role of the casein micelle

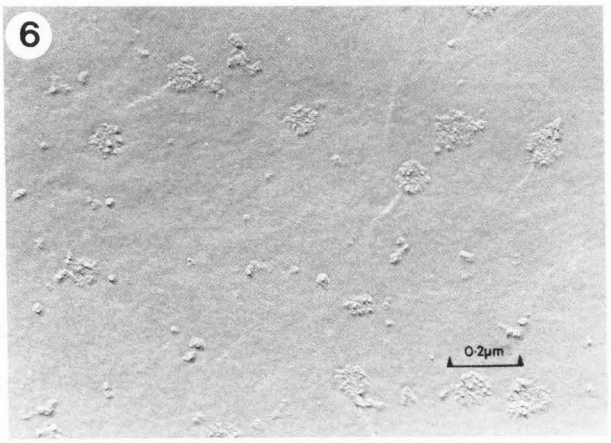
The current view is that the formation of yoghurt is achieved by the aggregation of the individual casein micelles into a 3-dimensional network of chains and clusters (10, 14, 16).

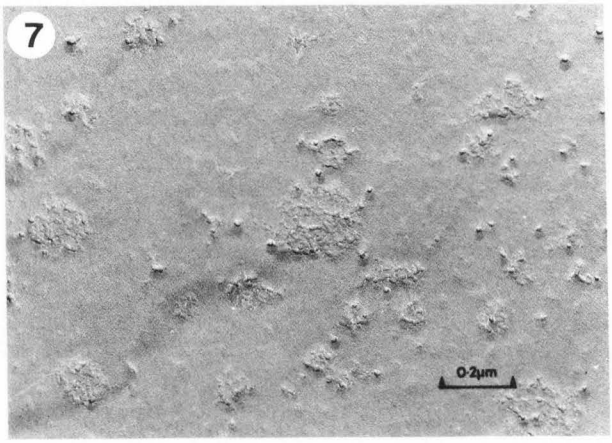
Table 1. pH at onset of gelation determined by viscosity measurements (Deer Rheometer)

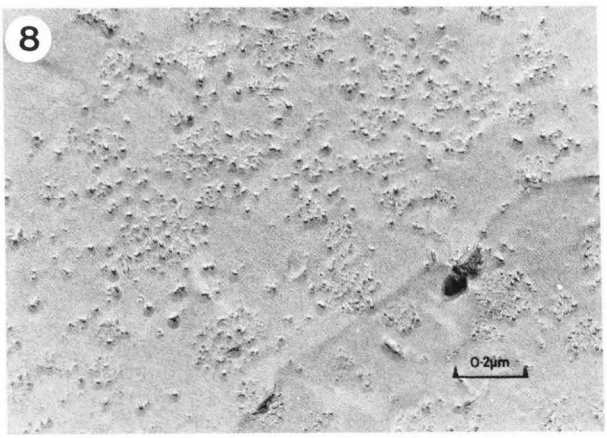
	Sample		
	I heated milk 30°C	II heated milk 43°C	III unheated milk 43°C
pH	5.1	5.5	5.1











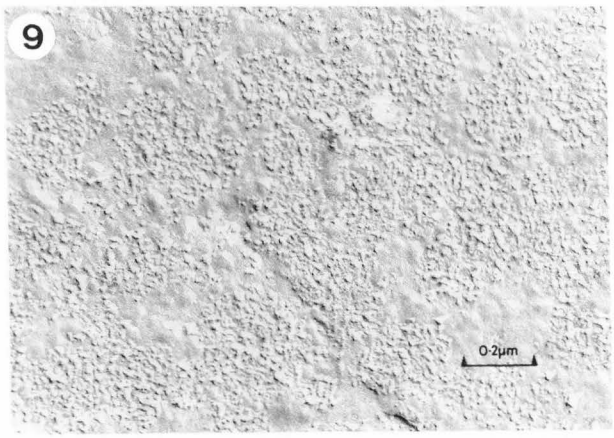


Fig. 4. Intact micelles at pH 6.6 (sample I).

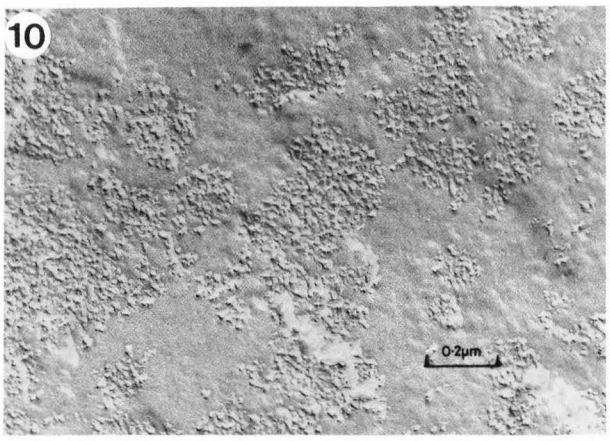
Fig. 5. Intact micelles at pH 5.9 (sample I).

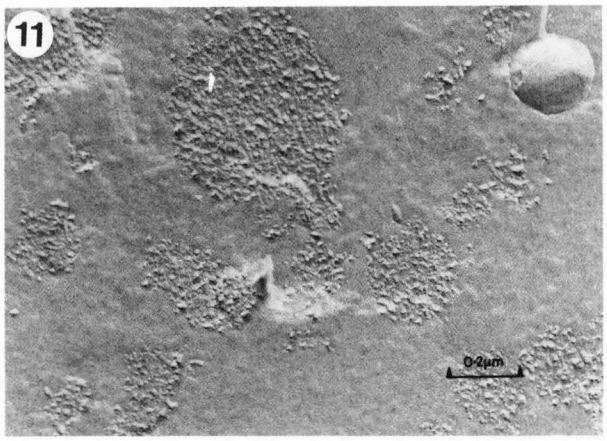
Fig. 6. Intact micelles and some small particles at pH 5.5 (sample I).

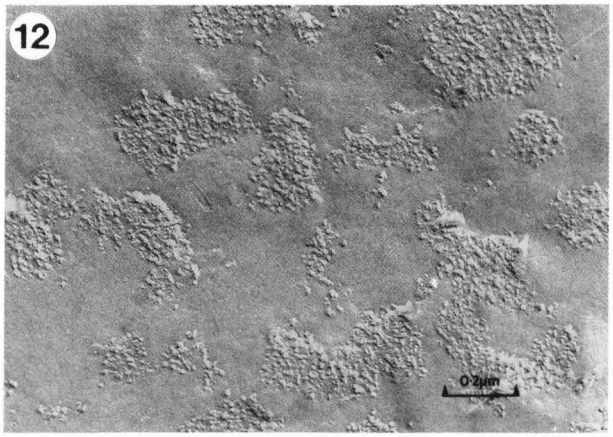
Fig. 7. Small particles and intact micelles at pH 5.2 (sample I).

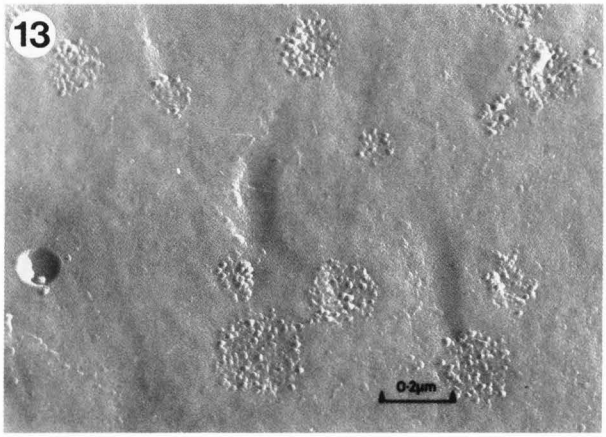
Fig. 8. Fields of aggregated particles at pH 5.2 (sample I). Aggregation stage.

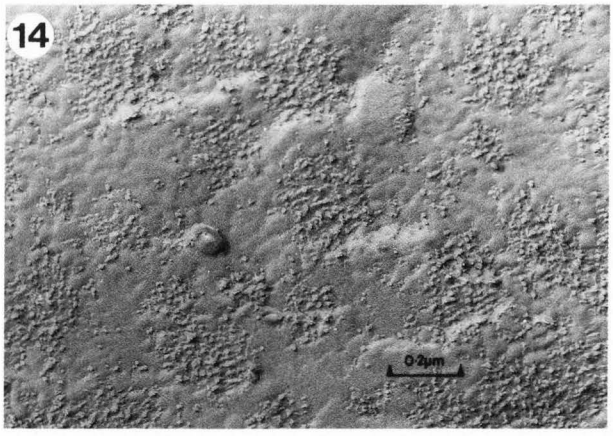
Fig. 9. Fields of aggregated particles at pH 4.8 (sample I).











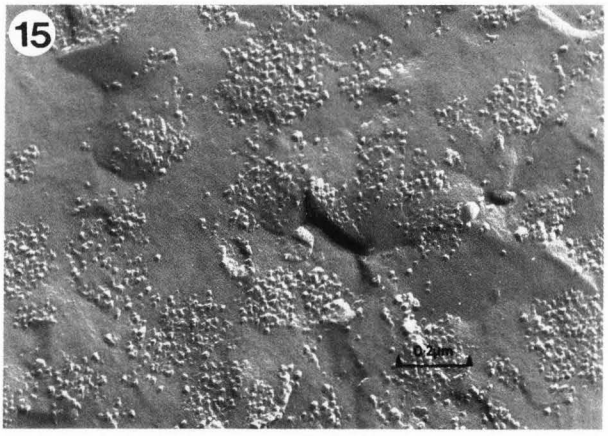


Fig. 10. Aggregates of particles at pH 4.8 (sample I). Start of contraction stage.

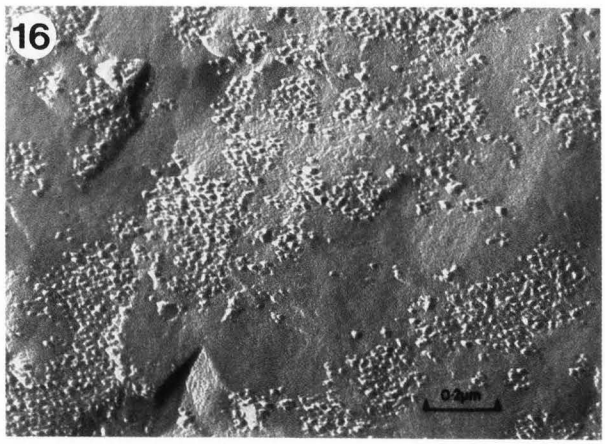
Fig. 11. Aggregates of particles at pH 4.8 (sample I). Contraction stage.

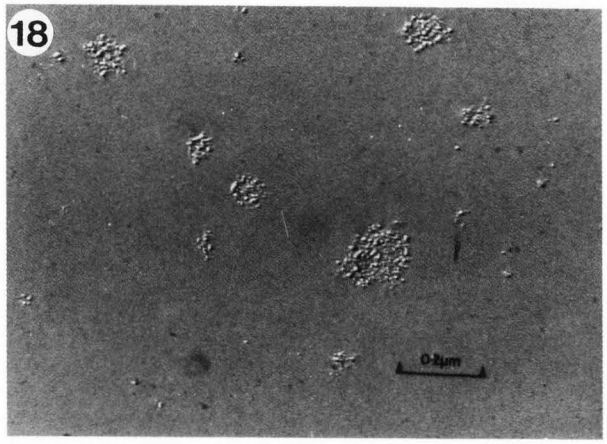
Fig. 12. Aggregates of particles at pH 4.5 (sample I). Final network.

Fig. 13. Intact micelles at pH 5.2 (sample III).

Fig. 14. Aggregates of particles at pH 5.1 (sample III).

Fig. 15. Aggregates of particles at pH 5.5 (sample II).





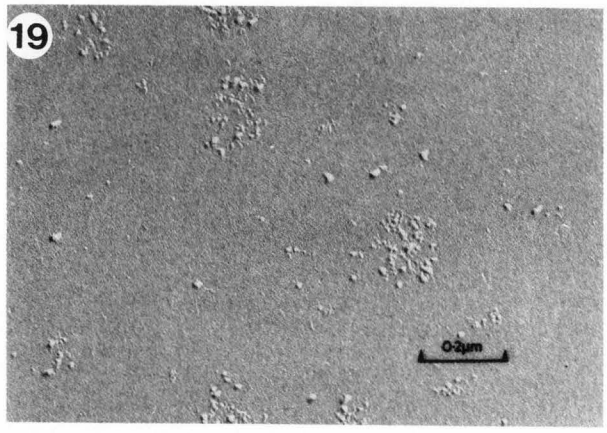


Fig. 16. Aggregates of particles at pH 5.2 (sample II).

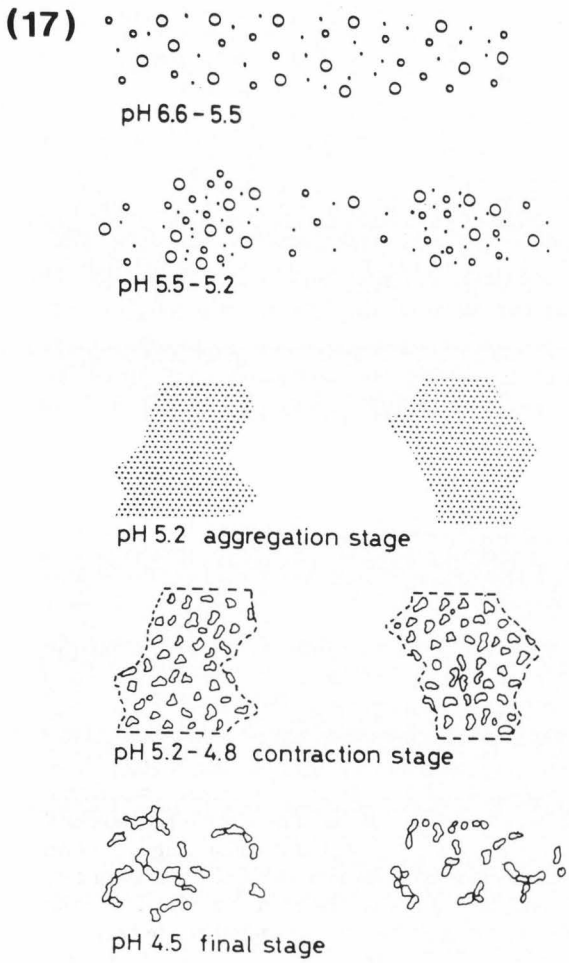


Fig. 17. Schematic drawing of the structure formation in acid milk gel.

Fig. 18. Casein micelles in milk, showing a closely packed structure.

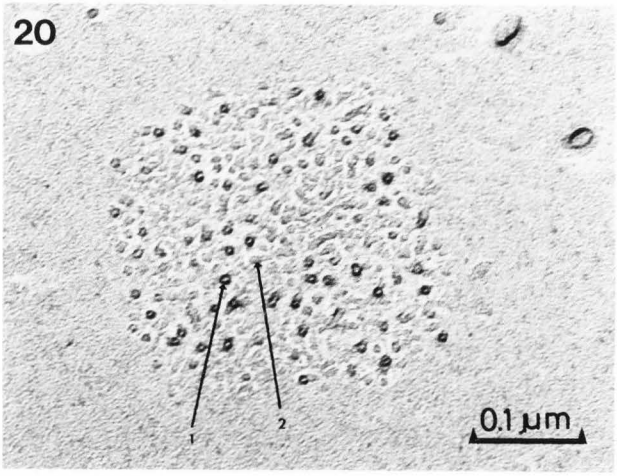


Fig. 19. Casein micelles treated with 0.02 M sodium citrate, showing an open coherent structure.

Fig. 20. Structure of casein micelles in milk. Rotary shadowed. 1: corpuscular structure; 2: thread-like elements.

Scanning electron microscopy in particular showed (14), however, that the observed casein particles must have undergone considerable changes in their dimensions and shapes, considering that casein micelles in milk are spherical and approximately 0.1 μm in diameter.

Based on the information obtained in the present study, we have come to the conclusion that the aggregation of milk proteins into a network is more complex than has been considered so far. A summarizing schematic drawing of the findings on mixture I is given in Fig. 17. At pH 6.6 and 5.9 casein micelles of an approximate size of 0.1 μm are present, homogeneously distributed in space. Micellar disintegration takes place at lower pH. Apparently the release of calcium phosphate upon acidification has progressed so far (Fig. 3) that its cementing role in keeping the individual casein molecules together in the form of a micelle has been reduced and part of the casein is released from the micelle. It has been shown (18) that calcium removal from native micelles initially dissociates the weakly bound β- and κ-caseins from the micelle, while the size-determining micellar framework of α_s-caseins remains intact.

The existence of such a size-determining frame work on which β- and κ-casein become reversibly attached is more in accordance with a model considering the micelle as a continuum of linear and branched polymers of molecular casein subunits (12, 13) than with a submicellar model (2, 3, 22, 26–28, 30, 31). This view is also in accordance with a number of microstructural observations on the internal structure of the casein micelle.

The internal structure of the casein micelle

The findings on the structure formation were supported by a study of the structure of casein micelles containing calcium and casein micelles depleted of calcium. To avoid any additional pH effect, use was made of the effect of sodium citrate, known as a calcium complexing agent at neutral pH, on the structure of the casein micelle. Micrographs of casein micelles in milk and in milk treated with 0.02 M sodium citrate are presented in Figs. 18 and 19. The addition of sodium citrate results in the partial disintegration of the micelle while forming smaller particles, which is in agreement with the observations of Lin et al. (18) on the removal of calcium from casein micelles. In addition to the large number of small particles formed, large particles are still present which show a more open structure than the original micelles. The most likely explanation is that the small particles (most likely β- and κ-casein) have been released from the micelles, leaving behind a micellar framework of α_s-caseins, without affecting the structural integrity of the original particle.

Fig. 20 shows a micrograph of the casein micelle structure obtained by rotary shadowing. Apart from a corpuscular structure apparently identical with the particulate structure shown in Figs. 18 and 19, also thread-like structures are observed. The corpuscular structure appears to be connected to the thread-like structure and is most likely formed by stretching linear protein aggregates out of the plane of fracture by plastic deformation.

These observations suggest that the casein micelle is a continuous network structure of protein molecules rather than a structure based on separate spherical sub-micelles. One may expect that such a network structure indeed can release particles without affecting the overall structure. Moreover, such a model representing a continuity in protein aggregation accounts better for the aggregation behavior of proteins in general (4) and caseins in particular, because of the known readily formed polymers of α_s- and β-caseins and their mixtures (8, 20, 21, 23, 24), whereby κ-casein known as a protective colloid has a limiting effect on the aggregation of these proteins (37).

On the basis of these observations, we consider the casein micelle to be a continuous structure of thread-like protein strands. In our view, a discontinuity in the aggregation of the constituting proteinaceous material, which would have to be assumed if the casein micelles were composed of spherical submicelles, does not occur. In this particular respect we strongly support the model as proposed by Garnier (12, 13), in so far as the casein micelle is considered as a continuum of protein strands. The length of the polymeric filaments and consequently the size of the micelles is limited by their interaction with the κ-casein.

This model of the casein micelle structure is indeed much better able to explain the events occurring during the acidification of milk. After the stage of partial micellar disintegration between pH 5.5 and 5.2, leaving a micellar framework of α_s-caseins, extensive fields of aggregated structures, apparently indicating interaction of caseins, with a size of a few μm are formed (at pH 5.2) with empty space in between. This aggregation stage is followed by a contraction stage, in which again identifiable particles appear to be formed, although these particles are considerably larger than the original micelles. In this stage and in particular in the final stage, rearrangement and aggregation of particles take place under formation of the ultimate milk protein network.

The zeta potential and the role of β-casein

The complex behavior of disintegration and integration phenomena may well be connected with the change in the zeta potential of the caseins as a function of pH. Micelles at normal pH (6.6) owe their stability to surface hydration and an overall negative potential of about 15mV. Hydration, steric repulsion and a negative surface charge together provide a barrier towards close approach and aggregation of the micelles (7, 22, 25, 36). Normally on lowering the pH the zeta potential of colloidal protein particles drops continuously till it becomes zero at the isoelectric point (IEP).

In the case of caseins (36) the pH dependence of the zeta potential shows an anomalous behavior (Fig. 21) (36). The exact course of the curve depends on temperature (9) and milk pretreatment. The minimum in zeta potential shown at pH 5.2, coincides with the start of the aggregation phase (for mixture I). On further lowering the pH the potential starts to increase again accompanied by contraction and separation of particles. The newly formed particles start to aggregate, forming a network as soon as their potential is sufficiently reduced. An explanation for the observed phenomena and the anomalous behavior of the zeta potential may be found in the behavior of β-casein. In the pH region between 5.5 and 5.2, β-casein is predominantly found in the serum phase (33) till it starts to precipitate at its IEP at pH 5.2, where the zeta potential has its minimum (Fig. 21) and the reaggregation process starts. As may be observed in Fig. 8, the structure differs from that of the original micelles as well as from the final structure, in the sense that a more loose type of aggregation is apparent. This may well be connected with the fact that after release of β-caseins at higher pH, leaving a relaxed frame work of α_s-caseins reabsorption of β-caseins on this framework starts to occur at its IEP.

In addition, it is found (unpublished results; 33) that at this pH (5.2) a relative maximum in the "micelle" voluminosity occurs, also indicating the presence of a reassociation mechanism without contraction. On further lowering the pH (5.2–4.8), the

β-casein acquires a positive charge and may then act as a center for aggregation with the α_s-casein framework which is still negatively charged. This leads to the formation of new particles and a corresponding lowering of "micelle" voluminosity. Due to the described disaggregation and aggregation phenomena, induced by the solubilization of calcium phosphate, these particles are completely different in structure from the original micelles. The total evidence for the proposed sequence of events has been included in Fig. 21.

Finally, the higher pH at the onset of gelation in pre-heated milk (sample II) in comparison to the same milk without heating (sample III) may be related to changes which occur during heating of the milk at 90°C as described below.

Heat induced association of whey proteins and casein micelles

Model studies have shown that upon heating skim milk the whey proteins present interact with κ-casein in two ways. At low pH (6.4), the whey proteins adhere to the casein micelle surface via reaction with κ-casein, whereas at high pH (7.0), whey proteins and the κ-casein form separate aggregates, which are present in the serum phase (5, 6, 32). This influence of pH is illustrated by electron micrographs, obtained by negative staining, showing the interaction between beta-lactoglobulin (the major whey protein) and casein micelles (Figs. 22–24). It is clear that the adherence of whey proteins to casein micelles as a consequence of heating critically depends on pH. In the present acidification model experiments, the milk has been preheated at pH 6.6. This means that a part of the whey proteins become associated with the casein micelles. It is assumed that such an association increases the hydrophobicity of the micellar surface. The hydration barrier preventing the aggregation of the casein micelles thus is lowered and gelation takes place already at pH 5.5. It may be speculated that if this is the case, disintegration of the micelles will proceed to a lesser extent and the network formation should rather be described as an aggregation of micelles that have maintained their integrity.

Another explanation for a higher pH value at the onset of gelation may be found in the effect of heating on the distribution of the caseins over micellar and serum phase. This effect is accompanied by a decrease in serum α_{s_1}-casein and by an increase in serum β-casein (unpublished results). The resulting change in micellar composition (i.e., the increase in the α_{s_1}-casein content) will lead to a higher calcium sensitivity and thus to a more rapid development of a framework of aggregated α_s-casein and consequently to an earlier start of gelation. In this case, micellar integrity is lost and the network formation with newly formed particles occurs along the lines described earlier (Fig. 17). The experimental evidence contained in the micrographs on structure formation of preheated milk supports this view.

In conclusion, the aggregation of caseins into a network during the acidification of milk is a much more complex process than just the aggregation of the casein micelles. It involves the disaggregation of the micelles and formation of a loosely aggregated α_{s_1}-casein accompanied by release of β-casein and bound calcium phosphate; followed by reabsorption of β-casein on the α_s-casein framework under formation of new particles completely different in structure and composition from the original micelles. This hypothesis is supported by the dependence of the zeta potential and the micelle voluminosity on pH and by an internal structure of the casein micelle composed of continuous protein aggregates rather than a discontinuous structure of submicelles.

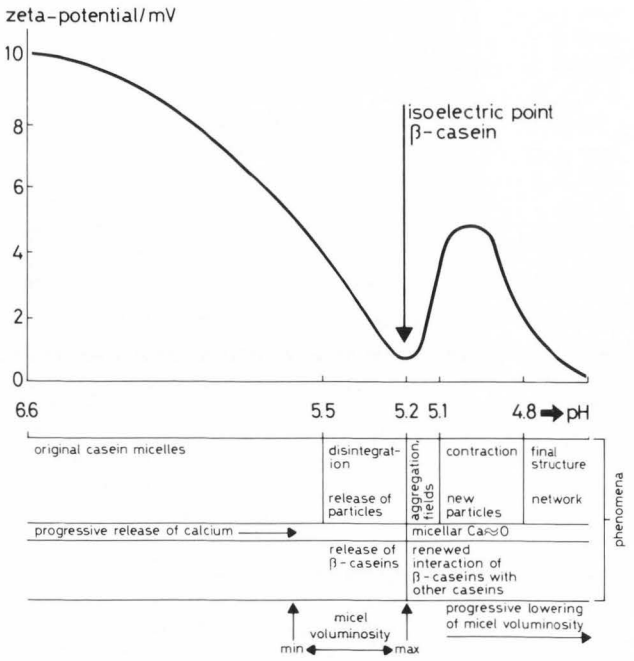


Fig. 21. Zeta potential as measured on the acidification of casein micelles in skim milk (schematic) and further evidence for the proposed structure formation.

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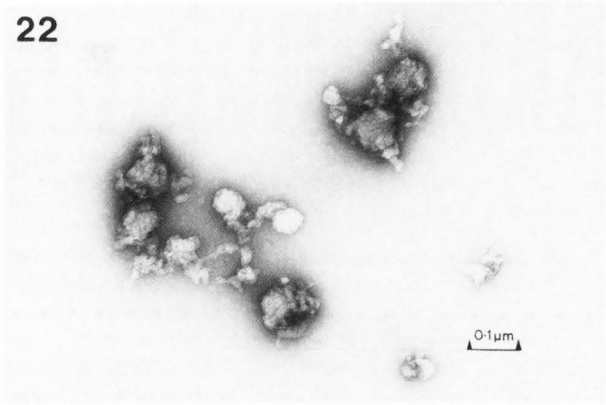

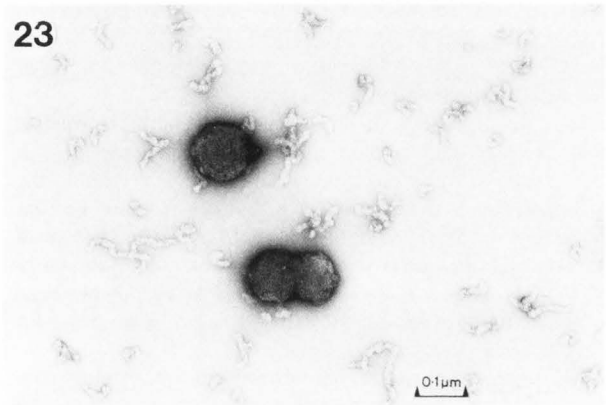

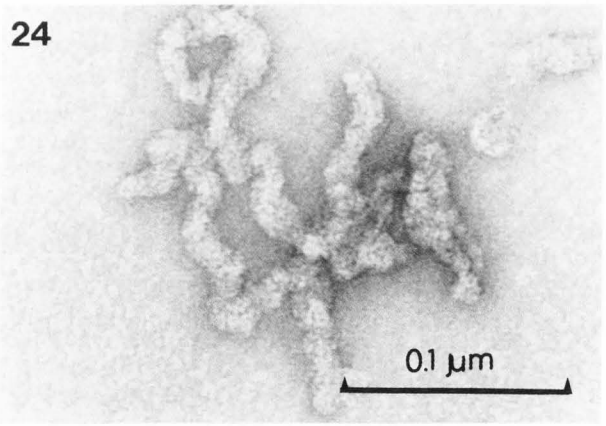

Fig. 22. Structure of a heated mixture of β-lactoglobulin and casein micelles at pH 6.5, showing the adherence of β-lactoglobulin to casein micelles.

Fig. 23. Structure of a heated mixture of β-lactoglobulin and casein micelles at pH 7.0, showing micellar structure and loose particles.

Fig. 24. Structure of the loose protein aggregates in a heated mixture of β-lactoglobulin and casein micelles at pH 7.0.

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Discussion with Reviewers

M. Kalab: Freeze-fracturing followed by freeze-etching reveals a corpuscular ultrastructure of the casein micelles in milk. Should not a continuum of protein strands be appearing as a uniform, i.e., non-granular, ultrastructure?
Authors: This is not the case, certainly under conditions of limited etching as has been applied in the present study. An important aspect of the observed freeze-fracture morphology is the non-complementarity of the replicas. This points to the phenomenon of plastic deformation. Networks, strands and even spheres will be stretched out of the plane of fracture and will show up as a corpuscular structure. Typical examples of network structures showing a corpuscular freeze fracture morphology can be found in references 35 and 1.

M. Kalab: It has been mentioned in the section on the internal structure of the casein micelles that a discontinuity in the aggregation would have to be assumed, were the casein micelles composed of spherical submicelles? How would this discontinuity be manifested?
Authors: One important aspect of micellar behavior is the influence of calcium complexing agents (EDTA, citrate) on micellar structure. A "pure" submicellar structure, i.e., a structure of spherical submicelles with colloidal calcium phosphate holding the submicelles together (text reference 26) would show a sudden and total decomposition of micelles into its subcomponents on addition of calcium complexing agents at a given concentration. This is not observed: literature data and the evidence presented in this paper suggest that, up to a certain level, calcium can be removed from the micellar structure, under release of casein-components, without the structural integrity of the casein micelle being affected.

M. Kalab: An interesting core-and-lining structure in casein particles was observed by Harwalkar and Kalab (Effects of acidulants and temperature on microstructure, firmness and susceptibility to syneresis of skim milk gels (Scanning Electron Microsc., 1981; III: 503–513) in milk precipitated at 70–90°C at pH 5.5. Can the development of that structure be explained on the basis of your results?
Authors: The formation of this interesting structure is probably connected with the release of β-casein upon acidification and the dependence of micelle voluminously on the pH of the system as described in this paper. At 20°C and pH 5.5 a considerable release of β-casein takes place and micelle voluminosity is high. At higher temperature these effects are less pronounced and it is very likely that under these circumstances redeposition of β-casein on the micellar periphery occurs.

It may be suggested that the observed "lining" is caused by differences in contraction of the various proteinaceous components of the reconstituted particles during the applied temperature increase.

D.G. Schmidt: The rejection of the submicellar model in favor of a model in which micelles consist of a three-dimensional network of protein filaments is based on the appearance of filamentous structures in Fig. 20. This does not convince me.

Moreover, if chains of polymeric casein do exist, such chains must also be observed when the calcium phosphate has been removed and that is not the case.
Authors: The rejection of the submicellar model is not only based on the filamentous structure observed in Fig. 20, but also on other evidence:

– the morphology of the casein micelles on the addition of 0.02 M sodium citrate (this paper).

– the agreement between this morphology and literature data showing that calcium and proteinaceous components (i.e., β- and κ-caseins) can be removed from the micelle without the structural integrity being affected. In addition it should be considered that the most convincing piece of evidence in favor of a sub-micellar model is the corpuscular structure shown in freeze-fracture replicas of casein micelles. As already has been said in our reply to M. Kalab such a structure is no proof for the existence of corpuscular particles.

Concerning the role of calcium phosphate, we certainly consider calcium and calcium phosphate to be important constituents of the polymeric chains. Without these inorganic components no polymeric structure would exist. In this context the aggregation behavior of α_S-casein under the infuence of calcium, described by a polyfunctional model, should be mentioned (Refs. 23, 20, 21).

D.G. Schmidt: Figs. 18 and 19 would demonstrate that addition of sodium citrate results in loosely structured micelles, consisting of a framework of α_S-casein. If this were the case why did the authors not apply some etching, because that would reveal such a three dimensional structure much better? Is it possible that glycerol causes detrimental effects in this respect?
Authors: In the present experiments etching could not be applied because of the presence of glycerol. An alternative would be to do these experiments by propane jet freezing without the addition of glycerol. First attempts in this direction indeed indicate the presence of a network structure after deep etching (−100°C, 60 s), whereas the familiar corpuscular structure is observed after mild etching (−100°C, 10 s).

D.E. Carpenter: Much of the recent evidence for micelle structure points to more κ-casein on the outside than on the inside of the micelle. Would you care to comment on this, with respect to the Garnier model and your microscopic observations?
Authors: We have clearly stated that we only support the Garnier model in so far as the casein micelle is considered as a continuum of protein strands. We certainly do not support the idea of the detailed location of the κ-casein in nodes of the polymer chains.

We suggest that the length of the polymeric threads and consequently the size of the micelles is limited by the interaction with κ-casein, known as a protective colloid. In this concept it is quite possible and even likely that the hydrophylic κ-casein is located on the outside of the micelle.

D.E. Carpenter: Kalab has seen shell and core and "ragged" micelles due to heat treatment. Do you have any direct microscopic evidence for interaction of β-lactoglobulin with the micelle by the freeze-fracture technique?

Authors: No, we have only looked for the heat induced interaction of β-lactoglobulin with casein micelles by the technique of negative staining. It would be interesting to do this by the freeze fracture technique, certainly when some etching can be applied and keeping in mind the effects of plastic deformation.