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EFFECT OF HEATING TO 200°C ON CASEIN MICELLES IN MILK: A METAL SHADOWING AND NEGATIVE STAINING ELECTRON MICROSCOPE STUDY

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Abstract

Milk was heated to 200°C for 3 min in sealed inverted-Y-shaped glass vials and reacted with a glutaraldehyde solution at that temperature. Electron microscopy of the metal-shadowed and negatively stained samples revealed that casein micelles in the milk did not disintegrate extensively at the high-temperature used but, rather, became enlarged. Some of them were found to be either clustered or distorted.

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Introduction

Ultra-high temperature (UHT) treatment of milk has been developed to significantly increase its shelf life. The treatment, which destroys bacteria and their spores, consists of heating milk to a high temperature for a short period of time (*e.g.*, 141°C for 15 s [29]). The effects of UHT and high-temperature short-time pasteurization (HTST, 92°C for 20 s) on casein micelles in milk were studied by several authors. A slight increase in the mean dimensions of casein micelles was found in unconcentrated milk [1, 7, 10, 11, 18, 24, 29] but in UHT-sterilized concentrated skim milk the mean dimensions of the casein micelles increased twice to three-fold [5, 12]. This increase has been reported [2, 6, 24, 29, 35] to be accompanied by an increase in the number of particles smaller than casein micelles. Several authors [5, 7, 24, 29] suggested that these small particles are composed of heat-denatured whey proteins which were not adsorbed on the casein micelles. An increased concentration of soluble casein was found following heating of a system consisting of whey protein-free casein micelles [2]. Heating of milk was also reported to lead to the release of macropeptides [15] and to shifts in the mineral constituents from the colloidal to the soluble state [2, 10, 11]. Hostettler and Imhof [18] hypothesized that casein micelles disintegrate into submicellar particles at high temperature and, upon cooling, the latter particles reaggregate into particles larger than the original casein micelles.

The composition, structure, and dimensions of casein micelles in UHT-sterilized milk may play a role in its gelation during prolonged storage [3, 12, 13]. It is now generally accepted on the basis of current knowledge that age-gelation of UHT-sterilized milk is preceded by changes taking place at the surface of casein micelles. Questions concerning whether such changes are caused by enzymatic action of heat-stable proteases or are the result of purely physico-chemical alterations have not yet been unequivocally answered [12]. However, clarification of even some facets of the problems may contribute to the understanding of the age-gelation of UHT-sterilized milk.

Changes in the casein micelle dimensions in milk and their distribution have been studied by a variety of techniques and the results were

reviewed [3, 16]. The techniques used include light scattering [17], size exclusion chromatography [24], chromatography on controlled pore glass [1, 21], light microscopy [34], and electron microscopy [4, 6, 7, 11, 13, 22, 28-33]. In the electron microscopic studies, the casein micelles were examined either unfixed or fixed after the heated milk was cooled. No attempts to study the micelles at the high temperature were reported. For example, Rüegg and Blanc [29] examined UHT-treated milk samples by electron microscopy following their cooling to room temperature. Glycerol added to the milk was used as a cryoprotective agent. The samples were freeze-fractured and then replicated with platinum and carbon [32]. In the milk that had been heated, the number of submicellar particles was increased and there was also a slight increase in the number of large casein particles at the expense of medium-sized micelles. The authors hypothesized that the large particles originated as a result of the reaggregation of submicellar casein and that the submicelles preferentially associated with large micelles although they recognized that non-specific precipitation of heat-denatured serum proteins on casein micelle surfaces may also have contributed to the micelle enlargement.

The objective of this study was to subject milk to extreme conditions, *i.e.*, heating to 200°C for up to 3 min, and to examine the integrity of the casein micelles by electron microscopy. Such high temperatures may not be used in dairy processing but may be attained in other areas such as the manufacture of confectioneries. In order to visualize the casein micelles in the state as they existed at the high temperature, a simple aid was developed which made it possible to interact the casein micelles with glutaraldehyde at the high temperature.

Materials and Methods

Heating of milk

The skim milk used was either of commercial origin or was prepared from fresh pooled whole milk obtained from the Central Experimental Farm dairy cattle herd. Fat was separated from the fresh milk by low-speed centrifugation (415 g for 30 min). Inverted-Y-shaped tubes (Fig. 1) made from Pyrex glass were used to heat both the milk and a 2.8% aqueous glutaraldehyde solution separately at the same time. The milk (0.2 mL) was placed in one arm of the tube and the glutaraldehyde solution (0.2 mL) was placed in the other arm. The tube was flame-sealed and the contents were heated in a sand bath for varying periods of time. The temperature of the sand bath was maintained at 150°±2°C or 200°±3°C. The glass tubes were completely immersed in the sand bath, otherwise water vapour would have condensed on the cooler wall and the condensate would have flowed down in either compartment. When the heating of the milk was completed, the tubes were removed from the sand bath and immediately inverted, which allowed the milk to react with the glutaraldehyde solution at the high temperature. Then the mixtures were cooled to 25°C. In parallel experiments, the milk and the glutaraldehyde solution were allowed to cool to 25°C before they were combined.

The tubes were opened and the reacted milk was prepared for electron microscopic examination. Unheated milk fixed with glutaraldehyde at 25°C was used as the control.

Electron microscopy

Two electron microscopic methods were used to examine the integrity of the casein micelles: (a) shadowing with platinum and carbon (replica technique) and (b) negative staining.

In the replica technique, a droplet of the treated milk was spread on a freshly cleaved mica sheet (5 x 8 mm) pretreated with an aqueous 0.1% poly-L-lysine hydrobromide solution (60,000-120,000 daltons; Polysciences, Inc., Warrington, PA, USA) and after 1 min, the excess milk was washed off with distilled water. Corpuscular milk proteins (casein micelles and submicellar particles) adhering to the mica sheet [23, 25] were then dehydrated in a graded ethanol series and critical point-dried from carbon dioxide. Following shadowing at a fixed angle of 27° or rotary shadowing by evaporating platinum and carbon *in vacuo* [20], the replicas thus prepared were separated from the mica support by floating on a 6% sodium hypochlorite solution. Milk proteins adhering to the replicas were digested within 30 min. The replicas were then washed with water, picked up on 400-mesh grids, and examined in a Philips EM-300 transmission electron microscope operated at 60 kV. Micrographs were taken on 35-mm film (Eastman Fine Grain Release Positive Film 5302) and intermediate negatives were made on Kodak Plus-X 35 mm film [20].

For negative staining, the fixed milk was diluted with a 0.01 M $CaCl_2$ solution in the ratio of 1:50 [28, 33]. A droplet of this mixture was applied to a 200-mesh (hexagonal) grid which had been coated with Formvar and carbon films and then treated by the glow-discharge method in order to make the carbon surface hydrophilic [9]. Then, a

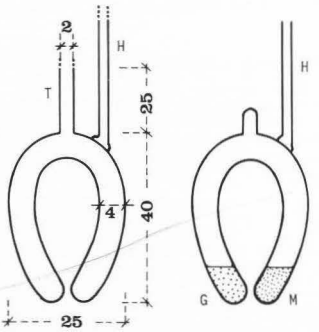


Fig. 1. A Pyrex glass apparatus is injected with milk (M) and glutaraldehyde solution (G) through filling tube (T). The tube is sealed (right figure) and immersed into a bath using handle (H). Internal volume of the sealed tube is approximately 1.5 mL. Measures are in millimetres.

droplet of an aqueous 2% sodium phosphotungstate solution, pH 7.2, was added to the milk; after 15 s, surplus liquid was drained off with a piece of filter paper. The grids were air-dried and subsequently examined in the Philips EM-300 electron microscope.

Results and Discussion

Visualization of casein particles, as they exist in milk while it is heated to temperatures above the boiling point, is difficult to achieve. Such heating can be accomplished only under elevated pressure, and fixation should be done at the high temperature in order to preserve all changes in the integrity of the micelles which may have been caused by the high-temperature treatment.

This objective could be approached from two directions. One approach was to physically fix the hot milk by freezing it rapidly in Freon 12 at -150°C, freeze-fracture it, and to examine platinum-and-carbon replicas by transmission electron microscopy (TEM). Such experiments were carried out, but it was impossible to freeze the hot milk rapidly enough to prevent the development of ice crystals and the distortion of the casein micelles.

The other approach involved chemical fixation [14] of casein present in the milk, in either the micellar or submicellar forms, at the high temperature using a glutaraldehyde solution and then cooling the mixture as described in the Methods section. Although it is not common to fix biological materials with glutaraldehyde at 200°C [27], it is believed that this reaction followed by cooling of the mixture would not result in any severe changes in the state of the protein particles present.

Metal-shadowing

Replication of casein micelles in fresh unheated milk using a metal-shadowing technique showed that they were mostly spherical (Fig. 2). At a high magnification, they appeared to be composed of submicelles (Fig. 3). A low concentration of submicellar particles in the milk was also noticeable. Intermediate negatives were made from the original negatives in order to show areas coated with platinum as light in colour.

In milk that had been heated, some casein micelles were aggregated and the incidence of submicellar casein was somewhat increased. Also the shapes of some of the casein micelles were found to have been altered. The severity of the changes depended on the intensity of the heat treatment. In milk heated at 100°C for 3 min, the casein micelles acquired a ragged surface topography (Fig. 4). Aggregation of protein and the formation of so-called "spikes" or "hair" were reported earlier also by other authors [8, 11, 13, 19, 35]. Changes were also found in the casein micelle ultrastructure: the submicelles were more clearly visible (Fig. 4) than in casein micelles in unheated milk (Fig. 3). This was probably the result of some loosening of the bonds between the submicelles that took place due to the effect of the heat treatment and this loosening may also be responsible for the enlargement of the micelles. In milk heated at 200°C for 3 min, casein micelles with a tight packing density (Fig. 5) as well as the looser packing density (Fig. 6) were found. Aggregated casein micelles with spiked surfaces (Fig. 7) were also present but the incidence of the particle clusters was low as evident from Fig. 8. Severely distorted casein micelles (Fig. 9) were occasionally noticeable even in the vicinity of undistorted micelles in milk heated at 200°C. Also occasionally, clusters of two or three casein micelles connected by fibre-like material (Figs. 10 and 11) were seen in the heated milk.

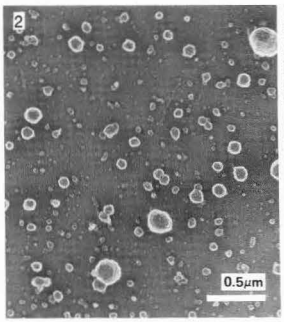
The images of casein micelles present in milk which had been heated to 200°C and then cooled to 25°C prior to fixation with glutaraldehyde resembled the images of casein micelles interacted with the glutaraldehyde solution at 200°C.

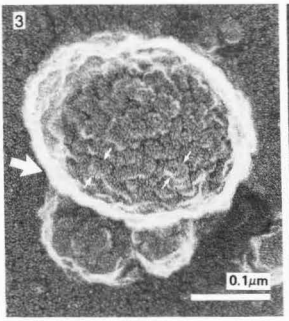
Casein micelles shadowed with platinum and carbon using either the stationary (unidirectional) or rotary techniques reveal their three-dimensional structure in great detail. Tilting the replicas in the electron microscope and taking pairs of micrographs each at a different angle makes it possible to view the three-dimensional structure as was shown earlier [20]. However, the technique used poses the risk that the attachment of the casein micelles to the mica sheet treated with polylysine may have been selective. It is possible that only unchanged casein micelles would be attached to the support whereas altered casein micelles would not be attached. This would mean that even if most of the casein micelles had disintegrated during heating of the milk, only the remaining intact casein micelles would become attached to the polylysine-treated support, and thus give an impression that there were no changes in the integrity of the micelles. In the replication technique, milk is applied to the polylysine-treated mica sheet and a few minutes later the milk is washed off with water. Thus, only a small proportion of the protein particles (casein micelles and submicelles) present in the milk remain attached to the mica support. Subsequently, the mica sheet with the protein particles attached is treated with increasing concentrations of ethanol in order to dehydrate the protein. Finally, the material adhering to the mica support is exposed to liquid carbon dioxide during critical-point drying. Because of the preparation steps involving the use of liquids such as ethanol and carbon dioxide, it is possible that some protein particles are washed off. For these reasons, it was necessary to use a method which prevents the loss of protein particles during the preparative steps and allows the observation of the protein particles in their initial distribution ratio.

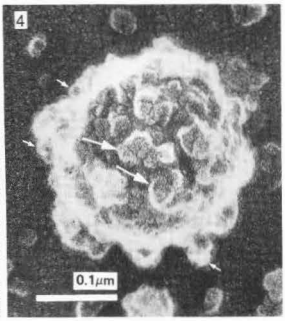
Negative staining

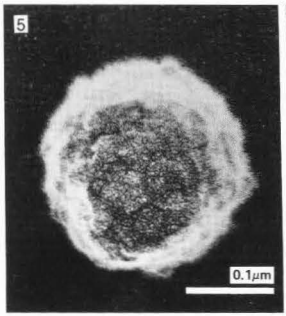
To overcome problems associated with shadowing, negative staining was carried out. This method was used by several authors [21, 33] to study casein micelles in milk. It was necessary, however, to establish the optimal conditions for this study. Of the several stains tested, a 3% phosphotungstic acid solution at pH 7.2 gave the best results. Uranyl acetate applied at various concentrations and pH values reacted with the micelles and stained them positively, whereas ammonium molybdate caused an increase in background staining.

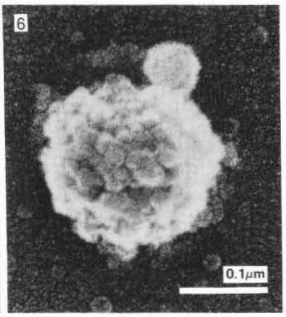
The milk was diluted in order to obtain a suitable distribution of the casein micelles on

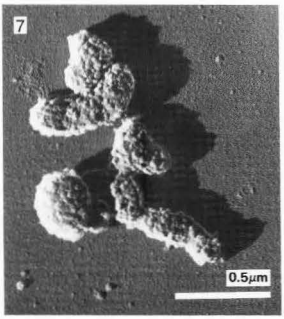












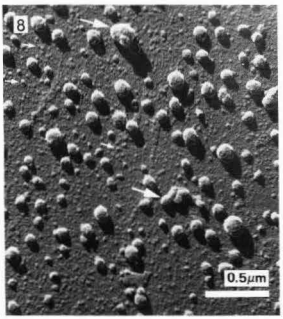


Fig. 2. Rotary shadowing of casein micelles in unheated milk with platinum and carbon reveals their spherical shapes and size distribution.

Fig. 3. Detail of the structure of a casein micelle in unheated milk. Rotary shadowing shows the submicelles (small arrows). The perimeter of the casein micelle is excessively light (large arrow) and lacks detail because the electron beam was absorbed by passing through the platinum coating on the vertical wall; this phenomenon is characteristic of the rotary coating technique.

Fig. 4. Detail of the structure of a casein micelles in milk heated at 100°C for 3 min. Casein submicelles (large arrows) and aggregated protein particles (small arrows) may be seen.

Figs. 5 and 6. In milk heated at 200°C for 3 min, some casein micelles show a tight packing density (Fig. 5) and other micelles show a looser packing density (Fig. 6).

Fig. 7. Aggregated casein micelles were present in milk heated at 200°C for 3 min. Stationary shadowing reveals *spiked* micellar surfaces.

Fig. 8. The incidence of aggregated casein micelles (large arrows) is relatively low in milk heated at 200°C. Small arrow points to casein submicelles.

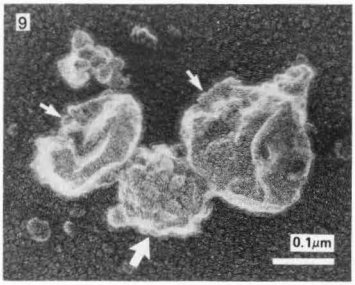
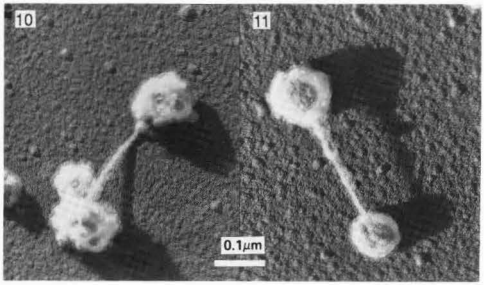


Fig. 9. Severely distorted casein micelles (small arrows, rotary shadowing) in milk heated at 200°C for 3 min. Another casein micelle (large arrow) has a regular appearance and shows the submicellar ultrastructure.



Figs. 10 and 11. Occasionally, protein strands connecting two or more micelles were noticeable in milk heated to 200°C for 3 min.

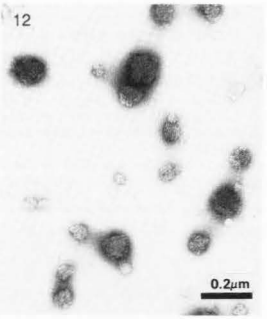


Fig. 12. Negative staining of casein micelles in unheated milk.

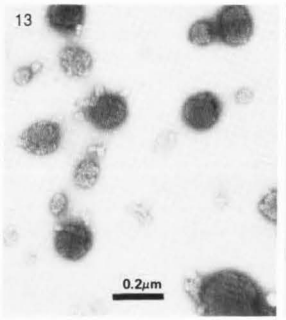


Fig. 13. Casein micelles in milk heated at 200°C for 3 min and mixed with a glutaraldehyde solution at the high temperature.

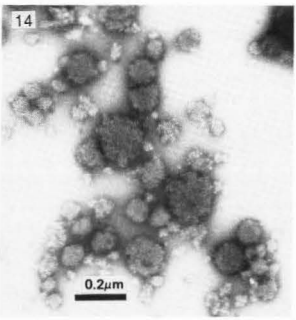


Fig. 14. Aggregated casein micelles in milk heated at 200°C for 3 min and mixed with a glutaraldehyde solution at the high temperature.

the grids for electron microscopic examination so that it could be observed whether or not the casein micelles were connected to each other. The best results were obtained by diluting the milk with a 0.01 M $CaCl_2$ solution in the ratio of 1:50 as initially suggested by Nitschmann [26]. Casein micelles disintegrated in milk that had been diluted with distilled water.

With the optimal conditions determined, casein micelles were observed both in unheated milk (Fig. 12) and in milk heated to 200°C for 3 min, irrespective of whether the milk had been mixed with the glutaraldehyde solution at the high temperature (Fig. 13) or after both the milk and the glutaraldehyde solution had been cooled to 25°C. Similar to metal shadowing, casein micelle clusters (Fig. 14) were found in heated milk by negative staining. In all milk samples, submicellar casein was also noticeable.

Electron microscopy of milk which had been heated at 200°C for 3 min and reacted with glutaraldehyde at that temperature revealed that casein micelles in that milk were not disintegrated, at least not to any noticeable extent. Metal shadowing using the stationary or rotary technique [20] showed the casein micelles as three-dimensional entities, the shapes of which were slightly distorted and the submicellar structure somewhat loosened as the result of heating. The question of whether samples prepared by the shadowing technique would give an accurate representation of the effect of heat on the integrity of the micelles has been answered by the results of the negative staining method, which produced images of casein micelles similar to those found in the control (unheated) milk. Thus, the results presented do

not support the hypothesis [18] that upon severe heat treatment casein micelles disintegrate into submicelles which reassociate upon cooling.

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Discussion with Reviewers

Y. Kakuda: Does all the liquid (water from the skim milk and glutaraldehyde and glutaraldehyde itself) vaporize under these conditions? If most of the water vaporizes, then dry caseins would be left on the bottom of the tube. Is it possible for unhydrated casein micelles to be less prone to disintegration, hence the results you see?
Authors: The water in the sample and the glutaraldehyde solution vaporizes only if the vial is not completely immersed in the sand bath; the water then condenses on the inside wall at the cooler top of the vial. No water condensation was observed provided that the vial was fully immersed. In earlier experiments, where this defect occurred, the *evaporated* milk was not examined but was discarded.

Reviewer 1: Why was size distribution not established?
Authors: Extensive experiments would be required to correctly establish the size distribution, but the data would not be important in this particular study, the objective of which was to only examine whether casein micelles would disintegrate when exposed to 200°C for 3 min.

N. A. Carrell: Can you quantitate the aggregation and incidence of submicellar casein? This is not evident on the fields shown.
Authors: Quantitation of aggregated casein micelles could be based on electron micrographs provided that a sufficiently high number of casein micelles is examined. The incidence of submicellar casein could be established by examining electron micrographs of negatively stained samples.

N. A. Carrell: Is it possible that the ragged surface of heated casein micelles is due not to attached protein particles but to voids created due to released protein particles?
Authors: Although this may be possible, protein aggregation rather than release has been reported by other authors to be the cause of the ragged casein micelle surface.

N. A. Carrell: The structures shown in Figs. 10 and 11 are intriguing. Did you find many of these structures? What do you think they are?
Authors: Their incidence was a fraction of a percentage point of all casein micelles present; we, too, find them intriguing.

N. A. Carrell: Can you estimate what proportion of the casein micelles in the heated milk were aggregated or were altered in shape? Did you find a similar proportion in the milk which was fixed after it was cooled?
Authors: There were approximately 5% of casein micelles clusters in heated milk irrespective of whether it was mixed with glutaraldehyde while hot or cooled. Less than 2% of the micelles were distorted.

N. A. Carrell: Could the smaller structures which you refer to as submicelles, or the roughness on the micelle surface, actually be due to aggregates of whey proteins? If you were to heat washed micelles, not milk, would you expect any differences in structure?
Authors: The surface of washed casein micelles showed no protein aggregates (roughness) due to heating to 100°C.

Y. Kakuda: Could the glutaraldehyde vapour fix the unhydrated casein micelles during the heating process and prevent disintegration as well?
N. A. Carrell: The authors state that the milk was *reacted* with glutaraldehyde at 200°C. However, the milk was *mixed* with glutaraldehyde at that temperature and there are no data to prove that the *reaction* did not occur at a lower temperature due to vaporization of glutaraldehyde. Nor is there any evidence that glutaraldehyde, that had been subjected to high heat and pressure, functions in the same manner as glutaraldehyde that had not been treated in this manner. Since the conclusion is based entirely on the premise that the micelles were *fixed* at high temperature, and since the effect of high heat treatment on milk is such an important issue, I feel that the premise must be substantiated. Two simple experiments would provide this proof:

To test the effect of heat and pressure on glutaraldehyde functionality, milk (at room temperature) could be fixed with glutaraldehyde which has not been heated and, separately, with glutaraldehyde which has been heated in the sealed tube

to 200°C and then cooled. Then, the resulting structures could be compared.

To test whether the fixation did indeed occur *after mixing*, a simple biochemical experiment could be carried out. Milk could be (1) not mixed with glutaraldehyde, (2) mixed with glutaraldehyde at 200°C as described in the manuscript, and (3) heated to 200°C in the special vial containing glutaraldehyde in the other arm but not mixed with the fixative after cooling. The samples could then be analyzed by gel electrophoresis or by HPLC. Either technique would indicate whether the caseins in the milk from Treatment 3 were crosslinked by glutaraldehyde as those in Treatment 2 would be, or were not crosslinked, as those in Treatment 1 would be.

Secondly, all the experiments were done with milk. The whey proteins in the milk can, and do, attach to casein micelles as a result of heating. This could contribute to an increase in micellar dimensions. Additionally, both heat and glutaraldehyde fixation cause aggregation of whey proteins. Is it possible that these aggregates, and not submicelles, are what we see on the surface of the micelles in Figs. 4 and 6? If washed micelles were heated and fixed as the milk was and the same structures were observed, then the effect of whey proteins could be ruled out.

Authors: Whey protein aggregates as well as casein submicelles may be seen in Figs. 4 and 6.

We agree that the suggestions for additional experiments outlined above are excellent and should be followed. However, we cannot extend the study at this time and will return to this subject at a later date.