

# Gelation of Concentrated Skimmilk: Electron Microscopic Study

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## Abstract

Concentrated skimmilks (26% solids), high-temperature, short-time sterilized and aseptically canned, with or without the addition of polyphosphate, were examined at weekly intervals by electron microscopy. In the control concentrated skimmilk, incipient gelation (aggregation of casein particles) was observed after nine weeks of storage and six weeks before gelation was observed visually. Micelles from concentrated skimmilks were smooth textured and twice the diameter of those of skim-milk. The addition of sodium tetrapolyphosphate retarded gelation, for which a number of possible explanations are presented.

## Introduction

The problem of gelation of evaporated milk sterilized by the high-temperature, short-time method has been recognized since 1944 (1, 4). A number of explanations have been given for the mechanisms of gelling. Anoptral contrast microscopy by King (13) and phase contrast microscopy by Wilson et al. (25, 26) have indicated a change in size of the casein micelle as contributing to gelation. Electron microscopy has been employed by several investigators to show changes in the casein micelle system. Hostettler and Imhoff (8) demonstrated changes in the micelle resulting from milk processing. Huth (10) in 1957 reported that casein micelles in evaporated milk were approximately 3,900 Å in diameter as compared to 1,000 Å for micelles from untreated skim-milk. Hostettler, Imhoff, and Stein (9) correlated the effect of heating under various conditions with changes in the micelle. They suggested that an increase in micelle size arises from extensive heating and also reported an

increase in the number of micelles <500 Å in diameter which may have arisen from larger micelles. Morr et al. (15) and Josephson et al. (12) related changes in the electron microscopic appearance of the micelle and whey protein systems to off-flavors in processed milks. Schmidt and Buchheim (19) with metal shadowing and ultra-thin sectioning techniques, investigated the casein micelles in sterile concentrated milks containing orthophosphates and polyphosphates. They suggested that the confluence (or joining of the casein micelles to form a gel) involved the outer layers of the micelles and subsequently the "nuclei" of the micelles to form a three-dimensional network.

In a more recent publication, Schmidt (20) investigated the gelation of ultra high-temperature short-time sterilized concentrated skimmilk, and showed the casein micelles to be two times larger than fresh milk micelles. He demonstrated that both fine particles (<400 Å) and large micelles were involved in gelation and assumed that the fine particles were expelled from the larger aggregates.

All of these investigators used formalin fixation for electron microscopic observations except Morr et al. (15) and Josephson et al. (12), who simply diluted the micelle suspension in 0.01 M CaCl<sub>2</sub> for stabilization. Recently we used short-time fixation of casein micelles with the glutaraldehyde method of Carroll et al. (3), a procedure which, in addition to saving time, preserves the relationship of the protein-whey fraction of skimmilk.

This report is concerned with changes involving the casein micelle and the relationship between the whey proteins and the casein micelles as gelation proceeds.

## Experimental Procedures

*Specimen preparation.* Concentrated skimmilks (26% solids) were prepared in the Carnation pilot plant at Van Nuys, California, as follows: Concentrated milks were fore-

warmed at 132 C for 35 sec, sterilized at 140 C for 5 sec and aseptically canned. Quadrafos<sup>1,2</sup> was added at 0.56% of the total solids following forewarming. The cans were stored at room temperature and samples removed at weekly intervals. The concentrated skimmilks were fixed in 1% glutaraldehyde (0.1 ml of skim-milk to 1 ml of fixative) for 15 min, diluted 1:50 with distilled water, dispersed on a glass slide by dipping and air-dried. The specimen was shadowed with platinum-carbon pellet at a 3:1 angle, floated onto a clean water surface and picked up on a copper grid for electron microscopic examination (3).

*Heat treatment of skim milk and whey proteins.* Fresh skim milk was concentrated in a rotary evaporator at 40 C and heated in a water bath at 100 C for 15 min. Aliquot samples were taken for electron microscopic examination following concentration and heat treatment. Supernatant proteins (including some residual nonmicellar casein, the amount of which was not calculated) were obtained from unheated concentrated skim milk by centrifugation at  $96,000 \times g$  for 30 min. The pellet containing micellar casein was discarded. An aliquot of the supernatant whey protein fraction was heated in a water bath at 100 C for 15 min and prepared for the electron microscope as described.

*Isolation of gel protein.* The gel protein was prepared from concentrated milk by adding urea to 5.0 M and citrate to 0.1 N. The solution was clarified by centrifugation for 30 min at  $40,000 \times g$  and diluted with two parts of 0.1 N potassium citrate. The gelatinous pellet was recovered and purified by washing three times with 1.7 M urea - 0.1 N potassium citrate. The precipitate was subsequently washed three times with distilled water and lyophilized.

*Electron microscopy.* An RCA EMU 3-G electron microscope operating at 100 kv with a 25  $\mu$  objective aperture was employed. Micelle size distribution measurements of the 2:1 concentrated milk were obtained from photographic enlargements of electron micrographs of the weekly samples. Size distribution measurements on micelles observed in fresh milk are from the data of Carroll et al. (3).

<sup>1</sup> Sodium tetrapolyphosphate.

<sup>2</sup> Reference to brand or firm name does not constitute recommendation by the U.S. Department of Agriculture over any other similar products not mentioned.

## Results

Casein micelles<sup>3</sup> from concentrated skim milk are much larger than micelles from fresh skim milk. Figures 1a and 1b depict casein micelles from concentrated skim milk; 1b, polyphosphate added; 1a, no polyphosphate. The micelles are spherical with a smooth surface; particulate material is associated with the individual micelles and at times forms chain-like aggregates which are especially noticeable in Figure 1a. Similar particulate material, casein micelle associated, has been reported by Schmidt and Buehheim (19, 20) in concentrated skim milk.

The graph, Figure 2, indicates that the average size of casein micelles in fresh sterilized concentrated skim milk are about twice as large as micelles in fresh untreated skim milk.

<sup>3</sup> It is recognized that particles which do not possess micellar characteristics, also exist in heated milk systems. This particulate material is not synonymous with the large aggregates which we term "casein micelles."

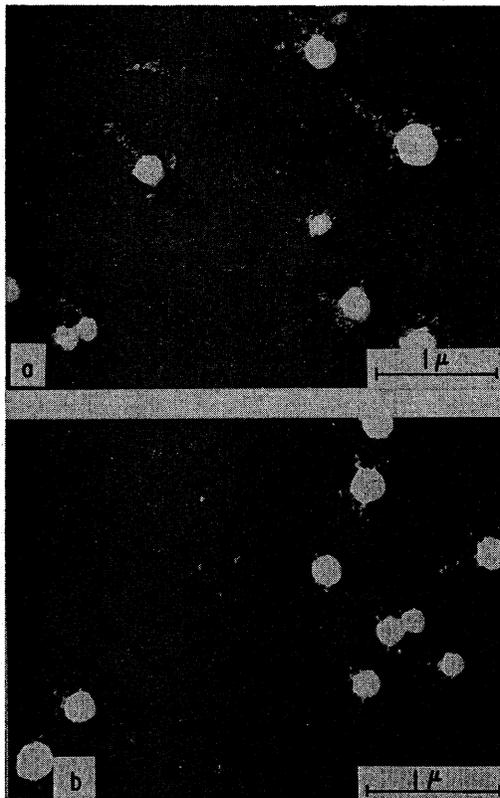


FIG. 1. a. High-temperature short-time 3:1 sterile concentrated skim milk (1 week). b. High-temperature short-time 3:1 sterile concentrated skim milk with polyphosphate added (1 week).

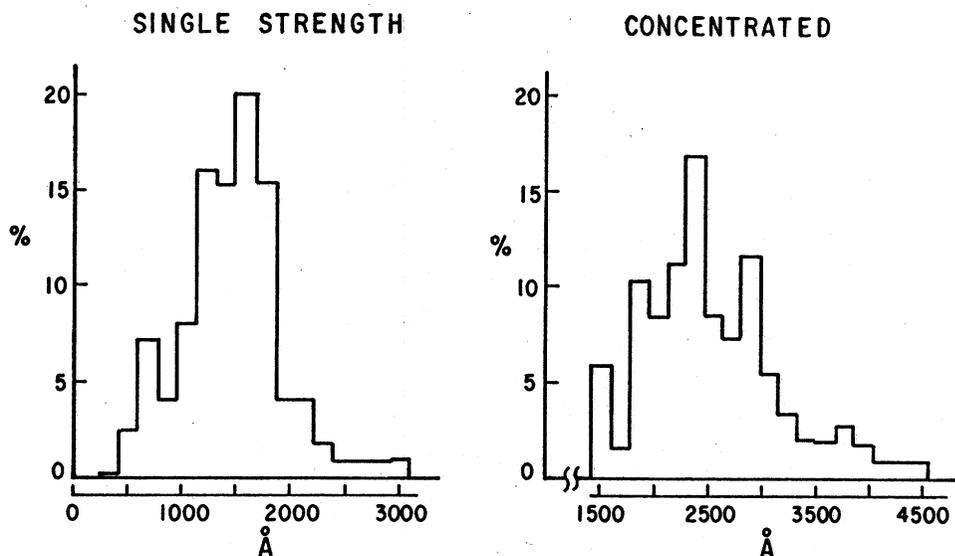


FIG. 2. Size distribution of casein micelles from fresh single strength skimmilk compared with casein micelles from 3:1 HTST sterilized concentrated skimmilk.

Ordinary skimmilk casein micelles range from 500 to 2,500 Å in diameter with 75% in the 1,000 to 1,700 Å range. Micelle diameters for concentrated skimmilk range from 1,500 to 4,000 Å, with 80% between 1,800 and 3,200 Å. Schmidt (20) reported 2,000 to 2,500 Å for casein micelles in concentrated milk processed by HTST, a range that coincides with ours.

The concentrated skimmilk with added Quadrafos was examined by electron microscopy at weekly intervals for 17 weeks; no sign of gelling was observed. In the concentrated skimmilk without added Quadrafos, initiation of the gelling phenomenon was observed after nine weeks Figure 3a; at 13 weeks, Figure 3b, increased micelle aggregation was evident. A prominent feature of this aggregation is the presence of linkages or bridges between the micelles. Bridging of micelles from HTST milks, but not from conventionally sterilized milks, has also been reported by Schmidt and Buchheim (19).

After 15 weeks signs of gelling were visible in the container and at 17 weeks gelation was complete; the concentrated skimmilk would not pour, but had to be spooned from the can. Sampling was stopped at this point. Figure 3c shows the tight packing of the casein micelles in the gel and the areas of bridging between the micelles. At somewhat higher magnification, Figure 3d, the bridging of micelles is depicted more vividly. It appears that the particulate material also present in ungelled samples (Fig.

1a and 1b) may comprise the bridging material in gelled samples. It is important to note that the micelles have not lost their individuality, but their surfaces are much more textured than those observed at the beginning of the storage period.

What is the nature of the bridging and particulate material found associated with the individual micelles? To attempt to answer this question, fresh skimmilk was concentrated to 26% solids at 40 C in a rotary evaporator. In Figure 4a the micelles from this skimmilk appear normal, with a wide size distribution and a small amount of particulate material scattered throughout the background. When this concentrated skimmilk was heated at 100 C for 15 min, a marked change in the appearance of the background was observed (Fig. 4b). An increase of the particulate material is evident and by close inspection chain-like aggregates of this material are seen. In some instances these chains are connected to the micelles in a manner similar to the chain-like aggregates in Figures 1a and 1b. An increase in both average size and electron density of micelles resulted from heat treatment.

As first suggested by Tarassuk and Tamsma (22), gelling of evaporated skimmilk is by direct interaction between denatured protein molecules. To investigate the appearance of denatured whey proteins, unheated concentrated skimmilk, prepared as aforementioned, was centrifuged at  $96,000 \times g$  for 30 min to separate the majority of casein micelles from

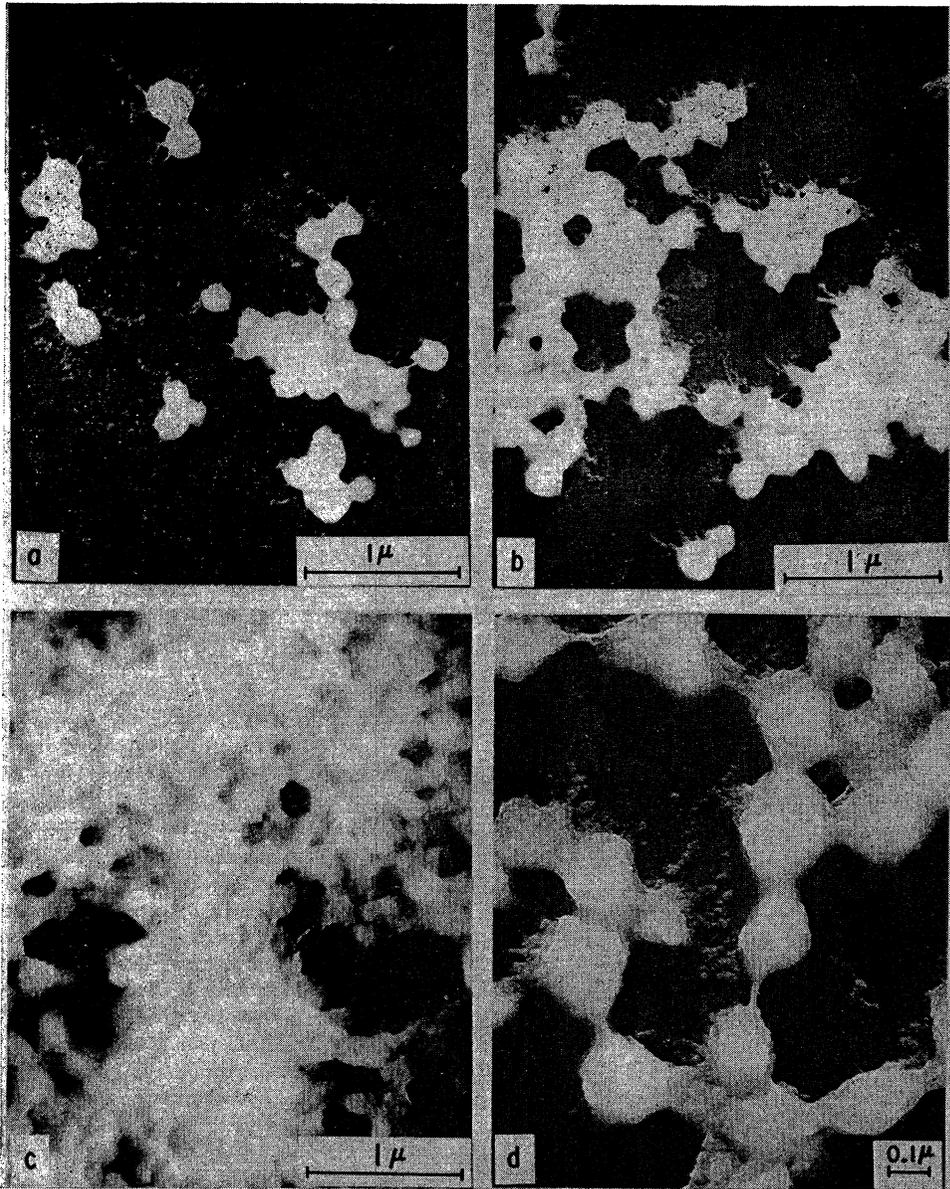


FIG. 3. High-temperature short-time 3:1 sterile concentrated skimmilk at: a. 9 Weeks; b. 13 weeks; c. 17 weeks; d. 15 weeks, at higher magnification, showing bridging of the micelles.

the whey proteins. Figure 4c shows that the supernatant consists of discrete particulate material 150 to 200 Å in diameter. This supernatant protein material has previously been observed (2) and is similar to the supernatant protein particles observed by Hostettler et al. (8). Doubtless they represent small micellar units. When the supernatant was heated at 100 C, a flocculant precipitate formed and in the electron microscope appeared as large

masses (Fig. 4d). This material appeared strikingly similar to the bridging material connecting the micelles in the gelled concentrated skimmilk, Figure 3d, but was more concentrated.

#### Discussion

Electron microscopy of sterilized concentrated skimmilks has disclosed the following: a) an increase of two times the diameter of unconcentrated milk micelles results whether or

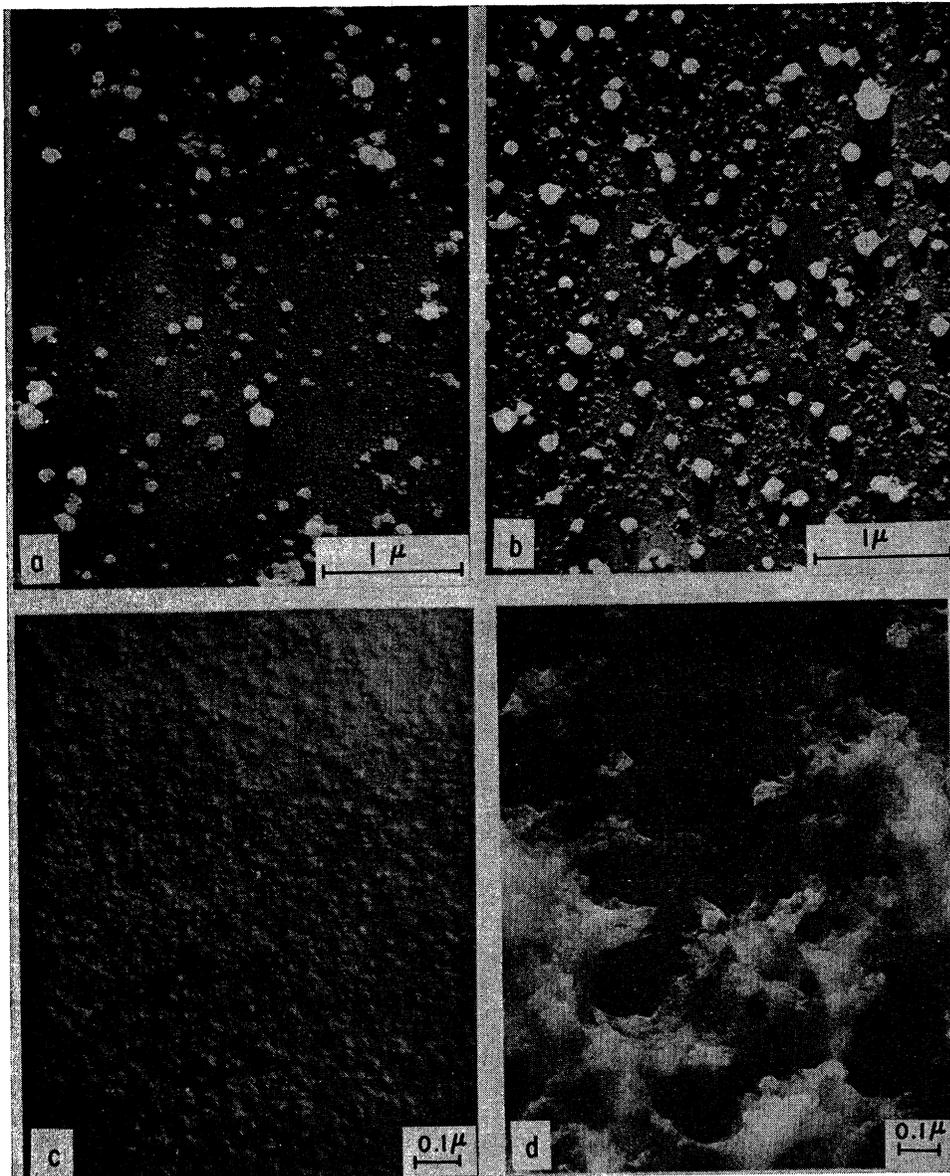


FIG. 4. a. 2:1 Concentrated skim milk (prepared in laboratory). b. (a) Heated at 100 C for 15 min. c. Supernatant from (a) casein micelles were removed by centrifugation. d. (c) Heated at 100 C for 15 min.

not polyphosphate has been added to the concentrate; b) a time dependent change in the appearance of micelle surfaces accompanied by the formation of bridging material holding the micelles in a three-dimensional network ensues; and c) added polyphosphate minimized alteration of micelle surfaces, the appearance of bridging material and subsequent gelling.

*Increase in micelle diameter.* Three explanations for this phenomenon appear plausible.

The first involves the interaction of heat denatured whey protein and other serum constituents with the micelle surface. This could occur at either stage of processing, forewarming or sterilization. Admittedly, only 20% of the total protein is whey protein, but the entire micelle surface need not be coated with whey protein to cause an increase in volume. Two assumptions must be made: that  $\kappa$ -casein, with its -S-S- bond, and the -S-S- of the whey

proteins (17, 18, 23) may reduce to -SH by heating and indiscriminately reoxidize after cooling, resulting in  $\beta$ -Lg- $\kappa$ -casein complex formation. (Whereas the  $\beta$ -Lg- $\kappa$ -casein complex can be demonstrated by heating of individual components, no evidence exists that a similar complex forms when milk is similarly heated.) Thus a minimum number of whey protein molecules would be necessary to bridge micelles into substantially larger particles.

A second explanation for the increase in micelle size is the effect of heat on the state of ionic calcium in milks. It is generally agreed that the amount of serum calcium is decreased by heating milk. Serum calcium precipitates on the surface of the casein micelle either as  $\text{Ca}^{++}$  or in a less soluble form such as  $\text{Ca}_3(\text{PO}_4)_2$ . An increase in calcium content would certainly lead to calcium bridging among micelles with a concomitant increase in micelle size and the eventual precipitation of the colloid (24). Thirdly, Hostettler et al. (9) found that heating milk increased the number of casein micelles in both the diameter ranges 1,250 to 1,750 Å and <500 Å. He suggested that the smaller particles resulted from splitting of the micelle surface. However, our micrographs show only very large micelles (1,500 to 3,500 Å) plus chain-like aggregates that are not identical to those reported by Hostettler et al. Others (6, 9) have proposed that micelles disintegrate upon heating; upon cooling they form aggregates larger than the original micelles. Perhaps the discrepancy in results between other laboratories and ours can be explained in part by the method of sterilization, retort versus HTST.

Without any absolute data we hypothesize that the increase in micelle size, subsequent to heating, results from the combined effects of heat denaturation of whey protein and their deposition into micellar surfaces and to increases in micellar calcium.

The argument has been advanced that collapse of micellar structure results from heating. While we do not accept this thesis, we believe that a reorganization of structure occurs in such a way as to accommodate the loss of serum calcium. A reorganization of structure would account for the formation of a "gel protein" high in calcium and phosphate.

*Changes in micelles during storage.* Electron microscopy of micelles of concentrated skim-milk shortly after sterilization has shown them to be smooth surfaced and electron dense. The micelles lose this appearance during prolonged storage and the micelles in the gel are more

textured than micelles in the fresh concentrate. Three notable features of the concentrated milk system may partly explain this phenomenon as well as the increase in chain-like aggregated material as a function of storage time. First, Fox et al. (7) showed that concentrated milks prepared by conventional evaporation procedures and by HTST developed a sediment,  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , the amount of which increased with time. This phosphate predominated at low temperature storage, whereas calcium citrate predominated at room temperature storage. These authors did not speculate as to the origin (micelles or serum) of the sediment. Secondly, Fox (6) also noted that storage of sterile concentrates resulted in an increase of nonsedimentable nitrogenous constituents which remained following centrifugation of the concentrate at  $106,000 \times g$  for 60 min. Freshly processed concentrated milk contained 30% of the total nitrogen in the nonsedimentable state, increasing to approximately 65% upon prolonged storage (P. M., unpublished results). A third observation made by one of us (P. M.) was the isolation of a "gel" protein from concentrated milks that amounted to 5% of the total protein; the amount depended upon the length of storage. Analysis of the gel protein revealed 6.3% calcium and 3.5% phosphorus. Calcium and phosphorus could be reduced to 2 and 1% by the addition of citric acid and subsequent dialysis of the solution; citric acid destroyed the gel character of the protein. While nothing is known regarding the electron microscopic characteristics or origin of this protein, it can be assumed that this unidentified protein possesses a unique spatial configuration because of its tendency to strongly hydrate.

Three steps lead up to gelation combined with an alteration of micelle surface structure. First, the loss of internal micellar calcium, analyzed as  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , results in a loss of net repulsive charge of the micelle with a concomitant decrease in solvation and a marked tendency for micelles to aggregate. We have observed such aggregation to be true in aseptically canned nonconcentrated HTST sterilized skimmilks stored at 5 C. Aggregation did not occur at room temperature.

Secondly, a marked increase in nonsedimentable nitrogenous material occurs in storage; this may result from the loss of micellar cementing calcium phosphate. Thirdly, the appearance of the gel protein which, coupled with denatured whey protein, may be the bridging material previously noted in Figure 3. The

possibility that heat-denatured whey protein may be vital to gelation was reported by Ellertson and Pearce (5), who noted that a 10% increase in the whey protein content of raw milk before processing resulted in gel formation of an HTST concentrate in half the time necessary for the control to gel. That the characteristics of gelation are not affected by the addition of  $\alpha_{s1}$ ,  $\beta$ - or  $\kappa$ -caseins, although micelle size is altered, has been clearly demonstrated by Schmidt (21).

Lastly, the gel matrix itself (Fig. 3c) is represented by closely packed micelles, with textured surfaces, held together by a bridging material (Fig. 3d) that is similar to heat denatured whey protein (Fig. 4d). Rather surprisingly, concentrated milk gels are poorly solvated, indicating that the water in the three dimensional matrix is not tightly trapped.

*Function of polyphosphate.* Several theories have been postulated pertaining to the role of polyphosphates in delaying gelation of concentrated skim milks. The addition of Quadrafos (.01 to 0.10%) to skim milk (unpublished) showed a rather substantial increase in the amount of nonsedimentable nitrogen over the control; in this study HTST sterilized concentrated skim milk, Quadrafos added, contained 10% more soluble protein than the control. Clearly, the addition of polyphosphate results firstly in the chelation of soluble calcium (16) and secondly by an increase in soluble nitrogen because at least a portion of micellar calcium must be chelated. Therefore, a reduction of sediment analyzed as  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  would be anticipated. Additionally, the role of polyphosphates may be to alter the ionic character of the micelle surface, thus reducing the tendency for whey proteins or gel protein to deposit on the surface with time. In turn, gelation is delayed but not eliminated. Melnychyn and Wolcott (14) have shown that polyphosphates have an affinity for  $\kappa$ -casein. As has already been proposed, polyphosphates could act as effective inhibitors to the action of the proteolytic enzymes on casein components. Such enzymatic activity, for which we have no evidence, could account for an increase in soluble nitrogen with time. Colloidal stability would be impaired as is true when rennin acts on  $\kappa$ -casein during cheese manufacture. Interestingly, the addition of Quadrafos to concentrated skim milk does not alter the physical appearance or average size of micelles when compared to untreated concentrated skim milk.

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