Comparison of Casein Micelles in Raw and Reconstituted Skim Milk

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ABSTRACT

During the manufacture of skim milk powder, many important alterations to the casein micelles occur. This study investigates the nature and cause of these alterations and their reversibility upon reconstitution of the powders in water. Samples of skim milk and powder were taken at different stages of commercial production of low-, medium-, and high-heat powders. The nature and composition of the casein micelles were analyzed using a variety of analytical techniques including photon correlation spectroscopy, transmission electron microscopy, turbidity, and protein electrophoresis. It was found that during heat treatment, whey proteins are denatured and become attached to the casein micelles, resulting in larger micelles and more turbid milk. The extent of whey protein attachment to the micelles is directly related to the severity of the heat treatment. It also appeared that whey proteins denatured during heat treatment may continue to attach to casein micelles during water removal (evaporation and spray-drying). The process of water removal causes casein and Ca in the serum to become increasingly associated with the micelles. This results in much larger, denser micelles, increasing the turbidity while decreasing the viscosity of the milk. During reconstitution, the native equilibrium between colloidal Ca and serum Ca is slowly reestablished. The reequilibration of the caseins and detachment of the whey proteins occur even more slowly. The rate of reequilibration does not appear to be influenced by shear or temperature in the range of 4 to 40°C.

Key words: casein micelle, skim milk powder, reconstitution

INTRODUCTION

Skim milk powder is among the most abundantly produced and important dairy products. It is produced by water removal in a process that usually includes

steps of heat treatment, evaporation, and spray-drying. The removal of water confers extended shelf-life and facilitates economical transportation. It is used in a multitude of food applications, many of which require the powder to be dissolved back into an aqueous solution.

It is the proteins that confer much of the desirable functional properties of skim milk (flavor, color, gelling, foaming, etc.). Of particular importance are the so-called CN micelles, which represent over half of the protein in milk. These large (ca. 100 to 300 nm), approximately spherical protein aggregates consist of 4 different hydrophobic proteins (the CN) that interact with small colloidal centers of Ca phosphate (Walstra and Jenness, 1984). Many different models of the internal structure of CN micelles have been put forward, including one in which the micelles are composed of discrete subunits (Walstra, 1999) and another which proposes a more uniform and continuous internal structure (Holt and Horne, 1996; Holt et al., 2003). Despite many points of departure between these 2 models, there is much overlap. The interpretations presented in this work are essentially compatible with both models.

Much research has focused on the effects of processing conditions such as temperature and pH on CN micelles. However, although there is partial knowledge of the effects of the powder manufacturing process on CN micelles (Singh and Creamer, 1991; Singh and Newstead, 1997) and whey proteins (Oldfield et al., 2005), how these effects are reversed upon rehydration has not been properly investigated. This study makes the first direct comparison between CN micelles in fresh raw skim milk with those in milk reconstituted from skim milk powder. By using a variety of analytical techniques including photon correlation spectroscopy (PCS), transmission electron microscopy (TEM), turbidity, and protein electrophoresis, we were able to confirm many alterations to the CN micelles. The causes of these alterations were investigated by examining milk samples taken from different stages throughout the powder manufacturing process for 3 different skim milk powders made under different processing conditions. Reversibility of these alterations upon reconstitu-

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Table 1. Composition and heat-treatment conditions of skim milk powder (SMP) samples¹

Powder type	Preheat temperature (°C)	Preheat time (s)	UWPNI ² (mg/g)	Composition (% wt/wt)				
				Protein	Lactose	Fat	Ash	Moisture
Low-heat SMP	79 ± 1	<5 ± 1	6.0 ± 0.2	34.0 ± 0.5	53.9 ± 0.5	0.7 ± 0.1	7.8 ± 0.2	3.6 ± 0.1
Medium-heat SMP	90 ± 1	30 ± 1	3.1 ± 0.2	33.2 ± 0.5	54.6 ± 0.5	0.6 ± 0.1	8.0 ± 0.2	3.6 ± 0.1
High-heat SMP	120 ± 1	240 ± 1	0.4 ± 0.2	36.0 ± 0.5	51.5 ± 0.5	0.7 ± 0.1	8.2 ± 0.2	3.6 ± 0.1

¹Errors represent the estimated uncertainty in the measurements.

²Undenatured whey protein N index.

tion of the powders in water was then investigated at different temperatures and mixing intensities.

MATERIALS AND METHODS

Skim Milk and Skim Milk Powder Samples

All skim milk and skim milk powder (SMP) samples were obtained from commercial plants in Victoria, Australia. Sodium azide was added as a preservative at 0.2 g/L. All fresh milk samples (raw, heat-treated, and evaporated) were refrigerated at 4°C. All fresh milk samples were analyzed within 1 wk of collection and were reequilibrated to 20°C for 1 h before measurement.

Sets of samples were obtained for each of the 3 standard classifications of SMP: low heat, medium heat, and high heat. Each set included samples taken across a single production batch of raw skim milk, heat-treated milk, evaporated milk, and powder. Raw skim milk is milk taken before pasteurization. Heat-treated milk is milk preheated at controlled conditions (Table 1) but yet to undergo evaporation. Evaporated skim milk samples were taken between the evaporator and spray-drying process steps and were approximately 45% solids. Compositional analysis of the powders was performed by the manufacturer using the methods described in Australian Standard 2300 (Standards Australia, 1995; Table 1).

Reconstitution

Skim milk powder was reconstituted in distilled water at 10% (wt/wt) to yield reconstituted skim milk of the same overall composition as the raw skim milk. Sodium azide was added as a preservative at 0.2 g/L. Temperature was controlled by immersing the reconstitution vessels (100-mL glass bottles, Schott AG, Mainz, Germany) in a temperature-controlled water bath. Rapid powder dissolution was achieved by vigorously shaking the reconstitution vessel for 20 s. Subsequent gentle mixing was provided by a magnetic stirrer bar. High shear was applied using an IKA model RW20 overhead stirrer run at approximately 1200 rpm (IKA, Staufen, Germany).

Particle Size Analysis

Particle size analysis was performed by PCS using a Brookhaven BI-9000AT autocorrelator with a BI-200SM goniometer (Brookhaven Instruments Corporation, Holtsville, NY) fitted with a 632-nm laser. The scattered light was measured at an angle of 90°. The temperature of the samples was maintained at 25°C. The method of cumulants was used to determine an intensity-weighted average hydrodynamic diameter and a measure of the polydispersity of the particles.

Samples were diluted approximately 1,000-fold in a buffer containing Tris base (0.02 mol/L), NaCl (0.05 mol/L), and CaCl₂ (0.003 mol/L), which was adjusted to a pH of 6.7 using 0.1 M HCl. This buffer is a simplified simulated milk ultrafiltrate that was used instead of actual milk ultrafiltrate due to its more convenient preparation. Indistinguishable particle sizes were obtained for milk diluted in this buffer as for milk diluted in skim milk ultrafiltrate.

TEM

Milk samples were mixed with molten 5% agar to a final agar concentration of 2.5% and immediately refrigerated until set. The solidified agar was then cut into cubes of approximately 1 mm³. The agar-encapsulated samples were immersed in 5% glutaraldehyde for 2 h for cross-linking of the proteins. The samples were then immersed in 1% osmium tetroxide for 1 h before dehydration in a graded series of ethanol solutions. Finally, the samples were embedded in LR white resin, sectioned (100-nm thickness), and viewed using a Philips CM120 BioTwin transmission electron microscope (Eindhoven, the Netherlands) operated at 120 kV at 65,000× magnification.

Turbidity Measurements

The turbidity of the milk samples was measured by transmission of light of 860-nm wavelength using a Carey 3E UV-Visible Spectrophotometer (Varian, Palo Alto, CA). Measurements were made, without dilution, using a 2-mm pathlength quartz cuvette.

Protein Electrophoresis

Micelles were separated from the milk serum in a Beckman bench-top centrifuge (model Avanti30, F0630 rotor, Beckman Instruments Inc., Palo Alto, CA) at 21,000 rpm ($25,000 \times g$ average) for 1 h at 20°C. This divides the milk samples into 2 fractions: the pellet, assumed to consist solely of the micelles, and the supernatant, which is assumed to contain only nonmicellar components (whey proteins, soluble nonmicellar CN, water, lactose, and salts). The small amount of remaining fat accumulates above the supernatant. Although the relatively small force used in this method should minimize the chance of including whey protein aggregates in the pellet, it has been shown that this method adequately removes all CN micelles from the supernatant (Rodriguez del Angel and Dalgleish, 2006).

The protein composition of the supernatants and corresponding complete (i.e., not centrifuged) milk samples were determined by SDS-PAGE using a BioRad Criterion Cell electrophoresis unit (BioRad Laboratories, Richmond, CA). The difference between the complete milk and the supernatant represents the amount of protein in the pellet, and by inference, the composition of the micelles.

The SDS-PAGE was performed by first diluting samples 10-fold in 1 mM EDTA (pH 6.74). Aliquots (0.05 mL) of diluted samples were added to 0.1 mL of BioRad Laemmli buffer containing 5% mercaptoethanol and placed in a boiling water bath for 5 min. Aliquots (10 μ L) of samples were then loaded into 10.5 to 14% linear gradient precast Tris-HCl Criterion 18-well gels (BioRad) and run at 100 V for 2 h. Gels were stained with Coomassie Brilliant Blue and destained until the bands could be clearly distinguished. The gels were digitally scanned using an Epson GT-7000 desk scanner (Epson America, Long Beach, CA). The band intensity was then quantified against lane distance by analyzing the scanned images using ImageJ software. Approximate quantification was achieved by integrating the peaks of the β -LG and α -LA.

Viscosity

Relative viscosity measurements were performed using a capillary viscometer (type 531-03/0c, Schott AG). Samples were maintained at $20^\circ\text{C} \pm 0.1^\circ\text{C}$ using a temperature-controlled water bath.

RESULTS AND DISCUSSION

Alterations to CN Micelles During SMP Manufacture

In this laboratory, it was observed that the turbidity of milk reconstituted from SMP is in every case (regard-

Table 2. Intensity-weighted average hydrodynamic diameter (nm) of CN micelles in raw, heat-treated, evaporated, and reconstituted skim milk as measured by photon correlation spectroscopy using the method of cumulants¹

Samples	Low heat	Medium heat	High heat
Raw skim milk	200.7 $\pm$ 4.1	186.7 $\pm$ 2.2	190.7 $\pm$ 1.1
Heat-treated skim milk	203.4 $\pm$ 0.6	192.8 $\pm$ 0.4	229.6 $\pm$ 1.7
Evaporated skim milk	263.1 $\pm$ 8.8	270.5 $\pm$ 4.4	313.3 $\pm$ 2.3
Reconstituted SMP			
10 min	229.1 $\pm$ 1.3	250.2 $\pm$ 0.9	297.6 $\pm$ 2.8
35 min	231.0 $\pm$ 1.6	245.6 $\pm$ 3.4	286.1 $\pm$ 3.7
2 h	229.8 $\pm$ 1.6	244.7 $\pm$ 2.9	276.0 $\pm$ 2.0
5 h	226.1 $\pm$ 1.4	248.4 $\pm$ 1.4	285.8 $\pm$ 3.4
19 h	226.1 $\pm$ 1.2	254.3 $\pm$ 4.4	284.9 $\pm$ 3.0
24 h	215.3 $\pm$ 0.1	248.4 $\pm$ 3.7	278.4 $\pm$ 4.2

¹Results are the average of 3 measurements of separate aliquots taken from a single milk sample $\pm$ standard deviation. Skim milk powder (SMP) was reconstituted at 20°C with gentle mixing.

less of sample or type) greater than that of fresh, raw milk. It was quickly established that the difference in turbidity is not due simply to dissolving powder particles, sample variation, or the presence of air bubbles during mixing. It seemed that the difference must be due to alterations in the micelles (the primary source of turbidity in skim milk) caused during the powder manufacturing process. A review of the current literature, while providing some insights, could not fully identify or explain what these alterations are. It was therefore decided to do a systematic investigation of these observed differences.

The primary distinction that is made among SMP is the extent of heat treatment they have undergone. As such, SMP are categorized as low heat, medium heat, or high heat. It was decided to test commercially manufactured samples from each of these categories. For true comparisons to be made (unaffected by seasonal or batch-to-batch variation), samples were sourced from the batch of raw skim milk from which the tested powders were made. To test at which point(s) in the process any identified changes were occurring, samples of the heat-treated milks and postevaporator concentrates (evaporated milks) were obtained. The initial investigation performed was a PCS analysis of the samples comparing the relative hydrodynamic size of the micelles (Table 2). Polydispersity of all samples was approximately 0.15 ± 0.05 , with no noticeable trends.

Before interpreting these results, it should be remembered that the intensity-weighted average diameter is an attempt to describe, with a single numerical value, the average size of particles in a polydisperse distribution. An increase in the intensity-weighted average diameter could represent many different changes to the particle size distribution (e.g., smaller particles could be removed, some or all of the particles could increase

in size by the same or different amounts). What can be said is that somewhere in the population of particles, there has been an increase in size. As such, the absolute value does not have much meaning on its own. However, as in the following discussion, it is very useful for monitoring relative particle size changes. Full particle size distribution data were not required for the following discussions, and have not been presented, because they could not be reliably obtained for this complex polydisperse system.

It is also worth noting at this point the natural variation in micelle size present in the samples of raw milk. The samples were sourced within a few months of each other from different plant locations. The magnitude of the difference is sufficient to have adversely affected the results of this study, confirming the need to have traced samples sourced from the same batch of milk.

With these points in mind, the results show that many significant alterations to the micelles occur at different stages in the manufacturing process. First, an increase in micelle size occurs during heat treatment, in line with the severity of the applied heat. According to current knowledge, this size increase is attributable to attachment of denatured whey proteins onto the outside of the CN micelles. It has long been known that denatured whey proteins, in particular β -LG and α -LA, become associated with the CN micelles during heat treatment (Singh and Creamer, 1991; Oldfield, et al., 2005). This association of denatured whey proteins has been shown to occur on the outside κ -CN layer of the micelles, increasing their hydrodynamic size (Oldfield, et al., 1998; Corredig and Dalgleish, 1999; Anema and Li, 2003b). The extent and nature of whey protein association has been shown to be highly dependent on the pH of the heated milk (Donato and Dalgleish, 2006).

To test this theory, micelles were separated from the serum by centrifugation, and the protein in the supernatant was analyzed by SDS-PAGE. Comparing the milk and supernatants of the raw milk samples (low, medium, and high-heat), all of the whey protein was recoverable in the supernatant. This confirms the native state of the raw milk containing soluble whey proteins and micelles consisting solely of CN. Analysis of the heat-treated samples confirmed that whey proteins had been pelleted along with the micelles in line with the severity of heat treatment (approximately 5, 20, and 75% for low-heat, medium-heat, and high-heat treatments, respectively). Although not providing conclusive evidence that these whey proteins are attached to the micelles (there is also the possibility of the formation of large whey protein aggregates), the results accord with current opinion that they do become directly attached (Oldfield et al., 1998; Corredig and Dalgleish, 1999; Anema and Li, 2003b).

Figure 1. Shift of CN and whey proteins from the serum to the micellar phase during heat treatment and water removal. Profiles represent the intensity of stained protein bands along lanes of a single SDS-PAGE gel for samples of noncentrifuged raw milk (○) and supernatants of centrifuged raw milk (✱), medium-heat-treated milk (solid line) and medium-heat skim milk protein reconstituted for <1 h at 20°C with gentle mixing (●). Due to the high protein concentration required for detailed investigation of the serum CN, the intensity of the most concentrated bands is saturated. The nonlinear response may distort the apparent ratio of total CN to whey and proportion of CN in the serum phase.

The next alteration to the micelles occurs during evaporation. The PCS results show that a dramatic increase in micelle size occurs during evaporation seemingly regardless of the intensity of the previous heat treatment. This result has not been previously reported, and what happens to micelles during evaporation and spray-drying is not yet fully understood. To investigate the possible cause of the increased size, the composition of the micelles was again examined using SDS-PAGE analysis of supernatants from centrifugation. Although not quantified, it was evident that both whey protein and CN are transferred from the serum (supernatant) to the micellar phase (pellet) during evaporation and spray-drying (Figure 1). Although it is questionable whether temperatures in the evaporator are high enough to denature whey proteins, it is possible that proteins already denatured during heat treatment may continue to associate with the micelles during water removal. It is not unexpected that the equilibrium between serum and micellar CN would be shifted toward the micellar phase as the temperature is raised and water removed. Considerable variability in the data (gel-to-gel variation) prevented accurate quantification of this shift; however, the trend was consistently observed for all samples.

It is interesting to compare these results with those reported by Oldfield et al. (2005). In their study, they also reported an increase in the amount of whey protein associated with micelles during evaporation, although it was very minor. The less noticeable increase in whey protein association in their study may be a result of using a much greater centrifugation force for micelle separation ($175,000 \times g$ compared with $25,000 \times g$). This could have resulted in the pelleting of small aggregates of whey proteins denatured during heat treatment that had not yet (but would during evaporation) become associated with the micelles.

Because the focus of their study was on whey proteins, Oldfield et al. (2005) did not report any change to the serum CN or to the CN micelle size. They did, however, observe that the Ca activity was altered significantly during evaporation. Micellar Ca (colloidal Ca phosphate) is in dynamic equilibrium with soluble ions in the milk serum, and the state of this equilibrium is dependent on pH, temperature, and ionic concentration (Augustin and Clarke, 1991; Udabage et al., 2000). During evaporation, milk is exposed for long periods to changes in all of these variables, resulting in a shift in the Ca phosphate balance toward the micellar phase (Oldfield et al., 2005). Although not fully understood, it is well known that Ca and CN are interrelated in the structure of micelles and that both exist in equilibrium between the micellar and serum phases. It is therefore unsurprising that CN would accompany a shift in Ca to the micellar phase and that larger micelles may result.

Having uncovered some important alterations to the CN micelles brought about during powder manufacture, it was decided to directly examine the samples using TEM. The method selected has been widely used (Lucey et al., 1996; Parris et al., 1997; Beaulieu et al., 1999) and involves setting the milk in agar, cross-linking the proteins with glutaraldehyde, and then setting the samples in resin. Detailed images of CN micelles were obtained at $65,000\times$ magnification (Figure 2). These images show clearly the attachment of whey proteins to the micelle caused by heat treatment (viewed as a fuzzy layer surrounding the micelles clearly visible for reconstituted high-heat SMP, somewhat apparent for medium-heat samples, and not present in the raw skim sample). However, the alterations caused by dehydration are less clearly revealed, the medium-heat SMP sample being perhaps more closely packed but otherwise similar to the medium-heat-treated sample (the incomplete coverage of the medium-heat SMP image is a result of a smaller milk droplet in the agar). Despite the potential problems arising from the inevitable alterations caused by sample preparation, these images provide supporting evidence that whey proteins attach to micelles during heat treatment. However, they do less

in helping to determine the nature of changes incurred during water removal.

Reversibility of Alterations to CN Micelles During Reconstitution

Now with some understanding of the alterations to the proteins brought about during powder manufacture, it was decided to investigate the reversibility of these alterations by rehydration during reconstitution. Turbidity was selected as the method to monitor changes during reconstitution. It was found to be an extremely sensitive, repeatable, and noninvasive way of monitoring changes to the colloidal CN micelles. Before doing a detailed study of powder reconstitution, it was necessary to determine what changes in turbidity actually correspond to in terms of the previously described micelle alterations. To do this, turbidity of all samples was measured and compared with PCS data (Table 3). The close correspondence of the trends in the turbidity and intensity-weighted average diameter data for all samples demonstrates that the alterations incurred during heat treatment can be well monitored using turbidity.

However, at this point, it is not completely clear what the differences in turbidity actually represent. A greater turbidity could be caused by an increase in one or more of the following: the number, relative size, or refractive index of the particles (CN micelles). The observed turbidity increase caused by heat treatment has been adequately explained by the attachment of whey proteins to the micelles surface. This would increase the relative size of the micelles (as evidenced by PCS) and therefore the turbidity. According to the equations of Einstein and Eiler, this should also result in an increase in the viscosity of the milk due to an increase in the volume of particles (Anema et al., 2004). Accordingly, as measured by capillary viscometry, the viscosity of high-heat-treated milk was approximately 10% higher than that of the raw milk (Table 3).

More difficult to explain, however, is the even greater increase in turbidity that results during evaporation and dehydration. In the previous section, it was shown that during evaporation, CN and Ca shift from the serum phase to the micellar phase, resulting in micelles with a much increased relative size. From these observations, it would be expected that reconstituted milk would have a higher turbidity than heat-treated milk, which was indeed found to be the case (Table 4). It would also seem to follow that the viscosity of reconstituted milk would be considerably greater than heat-treated milk, owing to an increased volume of micelles. However, the viscosity of reconstituted high-heat milk was found to be less than that of the heat-treated milk.

Figure 2. Transmission electron microscopy images of CN micelles from raw milk, medium-heat-treated milk, reconstituted medium-heat skim milk protein (SMP; <10 min, gentle mixing at 20°C), and reconstituted high-heat SMP (<10 min, gentle mixing at 20°C) viewed at 65,000 $\times$ magnification. Image exposure was adjusted using Adobe Photoshop (Adobe, San Jose, CA) to remove background noise.

Reconstituted medium-heat milk was in fact found to have a lower viscosity even than that of the original raw milk (Table 3). For the total amount of CN in the

Table 3. Relative viscosity of skim milk samples¹

Sample	Medium heat	High heat
Raw skim milk	1.00	1.00
Heat-treated skim milk	NA ²	1.09
Reconstituted skim milk powder <1 h	0.94	1.02

¹The data presented are ratios of the flow time (through the capillary viscometer) of the sample divided by the flow time of the corresponding raw skim milk. Negligible variation resulted from triplicate measurements of each sample.

²Not available.

micellar phase to have increased (as measured by SDS-PAGE analysis of centrifuged supernatants), but for the viscosity to decrease, there must be a change in the density of the particles. It is possible that in raw milk, the serum CN is in fact present as small submicelles (Walstra, 1999) or loose aggregates that are too small to be pelleted by centrifugation but still large enough to contribute to the viscosity. The observed shift of CN and Ca from the serum to the micelles may in fact be the assembly or incorporation of these submicelles into larger and denser proper CN micelles.

With this understanding, turbidity was used to monitor changes in the micelles of reconstituting skim milk. The 2 variables most likely to affect reconstitution are temperature and shear (or mixing). It was decided to

Table 4. Comparison of relative turbidity and intensity-weighted average diameter of skim milk samples normalized to the corresponding raw skim milk

Sample	Turbidity			Intensity-weighted average diameter		
	Low heat	Medium heat	High heat	Low heat	Medium heat	High heat
Raw skim milk	1.00	1.00	1.00	1.00	1.00	1.00
Heat-treated skim milk	1.07	1.21	1.39	1.01	1.03	1.20
Evaporated skim milk	NA ¹	NA	NA	1.31	1.45	1.64
Reconstituted SMP ²						
<10 min	1.54	1.84	2.99	1.14	1.34	1.56
5 h	1.31	1.49	2.38	1.13	1.33	1.50
24 h	1.23	1.42	2.31	1.07	1.33	1.46

¹Not available.²Skim milk powder.

examine all 3 powders (low heat, medium heat, and high heat) at 3 commercially relevant temperatures (4, 20, and 40°C) and to investigate shear by using 2 degrees of mixing (gentle mixing and high shear) at 20°C. To eliminate the effect of minor variations in CN content between samples, results are presented in terms of turbidity (cm^{-1}) per gram of CN per liter ($\text{L} \cdot \text{g}_{\text{casein}}^{-1}$). For all 3 powder types, there is a slow, almost linear decrease of turbidity with respect to the logarithm of time (Figure 3). The slight deviation from linearity be-

fore about 5 min may be attributable to the effects of dissolving powder particles and air bubbles. Interestingly, the relationship is independent of shear and temperature from 20 to 40°C.

The cause of the decrease in turbidity of reconstituting milk toward that of the original raw milk can now be related to reversal of the micelle alterations incurred during powder manufacture. The first indication of what is happening is provided by monitoring the pH during reconstitution. For all powders, it was found to increase concomitantly with the decrease in turbidity. The increase of pH during SMP reconstitution has been previously observed (Anema and Li, 2003a; Oldfield et al., 2005) along with an effectively parallel increase in ionic Ca. This shows that the shift of Ca from the serum to the colloidal phase is reversed, albeit slowly, during reconstitution.

Because the colloidal Ca and CN are interdependent, it would be expected that some of the CN from the colloidal phase would be returned to the serum phase along with the Ca. Results of an SDS-PAGE analysis showed that a small amount of CN was very slowly returned to the serum phase over 24 h of reconstitution (Figure 4). Additionally, for the high-heat SMP, there was a noticeable return of whey proteins to the serum. These results suggest that at least some of the alterations caused by heat treatment (whey protein attachment) and evaporation and dehydration (concentration of CN and Ca in the colloidal phase) are very slowly reversible.

The final data to be considered is the PCS analysis of the reconstituting milks (Tables 2 and 4). This data shows that, despite a considerable drop in turbidity, only a small decrease in particle size occurs. This could be explained if the return of Ca to the serum takes place more rapidly than the corresponding return of CN and whey proteins. As previously mentioned, turbidity depends not only on the number and size of the particles but also on their refractive index. The colloidal Ca phos-

Figure 3. Turbidity and pH of reconstituted skim milk with reconstitution time. Turbidity of low-heat skim milk protein (SMP) was reconstituted at the following temperatures: 20°C with gentle mixing ($\diamond$), 40°C with gentle mixing ($\square$), and 20°C with high-shear mixing ($\circ$). Turbidity of medium-heat SMP was reconstituted at the following temperatures: 20°C with gentle mixing ($\diamond$), 40°C with gentle mixing ($\square$), and 20°C with high-shear mixing ($\circ$). Turbidity of high-heat SMP was reconstituted at the following temperatures: 20°C with gentle mixing ($\diamond$), 40°C with gentle mixing ($\square$), and 20°C with high-shear mixing ($\circ$). Turbidity of raw skim milk was reconstituted at 20°C for low-heat (thin solid line), medium-heat (short-dash line), and high-heat (long-dash line) powders. The pH of low-heat ($\ast$), medium-heat ($\ast$), and high-heat ($+$) SMP was reconstituted at 20°C with gentle mixing. The thick solid line indicates the pH of raw skim milk.

Figure 4. Shift of CN and whey proteins from the micellar phase back to the serum during reconstitution of (A) high-heat skim milk protein (SMP) and (B) and medium-heat SMP. Profiles represent the intensity of stained protein bands along lanes of SDS-PAGE gels for samples of noncentrifuged raw milk (○) and supernatants of SMP reconstituted at 20°C with gentle mixing for <1 h (●), 7 h (*), and 24 h (solid line).

phate particles in the micelles are dense light scatterers and must contribute significantly to the refractive index of the particles (Griffin and Griffin, 1985). A decrease in the concentration of colloidal calcium phosphate would be enough to explain the proposed decrease in refractive index of the micelles and how the turbidity could decrease more rapidly than the particle size.

In addition to the data shown in Figure 3, all 3 powders were reconstituted at 4°C with gentle mixing. It has been observed that refrigeration of milk at 4°C results in the dissociation of Ca and CN from the micelles into the serum phase. Because the fresh milk samples were stored in a refrigerated state, it was necessary to reequilibrate the milk to 20°C to make valid comparisons with milk reconstituted at 20°C. Reequilibration was monitored by turbidity and found to be

Figure 5. Turbidity of medium-heat skim milk powder reconstituted at the following temperatures: 20°C with gentle mixing (◇) and 4°C with gentle mixing (△). Turbidity of samples was taken during reconstitution at 4°C and was remeasured after being stabilized at 20°C for 15 min (▲). Turbidity of raw skim milk was reconstituted at 20°C (dashed line) and 4°C (solid line). Similar trends were found for low-heat and high-heat powders reconstituted at 4°C.

achieved in approximately 15 min. When comparing reconstitution at 4°C to that at 20°C, the turbidity drops more quickly, suggesting that reequilibration may occur more rapidly (Figure 5). However, when allowed to reequilibrate to 20°C, the turbidity returns almost exactly to that of powder reconstituted at 20°C, meaning that refrigeration in fact has no real effect on the rate of reequilibration.

From the results presented here, it appears that within the tested limits, neither temperature nor shear significantly affects the rate of reequilibration during reconstitution. It is not surprising that the extent of mixing does not seem to affect the above-described changes. These changes would involve a reequilibration of the inside of the micelles with their external environment. Increased mass transfer on the outside surface of the micelles would not be expected to significantly affect the mass transfer rates inside the micelles.

The rate at which protein alterations are reversed during reconstitution appears to be much slower than the rate at which the alterations take place during powder production. If milk is thought of as a dynamic system, it seems that for some reason, the establishment of a new equilibrium during concentration takes place more rapidly than reversal during dilution.

Also of interest is that the alterations occurring during dehydration and rehydration appear independent of the heat-treatment stage. The slope of turbidity decrease and the pH of the low-heat, medium-heat, and high-heat powders are indistinguishable. It seems that

effect of heat treatment on the whey proteins operates independently to the effects of evaporation and dehydration on the CN and salts.

CONCLUSIONS

During the manufacture of SMP, many important alterations to the CN micelles occur, none of which are readily reversed during reconstitution.

Depending on the severity of heat treatment, varying portions of the whey proteins are denatured and become attached to the CN micelles. Additional attachment of denatured but free whey proteins may occur during evaporation and dehydration.

As water is removed, initially in the evaporator and eventually in the spray drier, Ca and soluble CN become increasingly associated with the micelles. This results in larger, possibly more optically dense micelles and an increased turbidity. During reconstitution, the native equilibrium between colloidal Ca and serum Ca is slowly reestablished. The reequilibration of the CN and detachment of the whey proteins occur even more slowly. The rate of reequilibration does not appear to be influenced by shear and is only temporarily influenced by refrigeration.

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