Effects of Acidification and Storage of Milk on Dissociation of Bovine Casein Micelles

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The effects of acidification and storage of skim-milk on the rate and extent of solubilization of colloidal calcium phosphate and dissociation of caseins from the micelles were examined. As the pH was reduced, calcium phosphate was progressively removed from the micelles, and after each pH adjustment there was an initial rapid increase in serum concentrations of Ca and inorganic phosphate, but only a slight further increase on storage. The extent of disssociation of the caseins from the micelles on acidification was temperature-dependent. At 30 °C, concentrations of serum casein remained low, but at 20 °C, and especially at 4 °C, the levels of all caseins increased in the serum as the pH was reduced to about pH 5.5 and 5.2, respectively. After each pH adjustment, there was an initial rapid increase in the serum concentrations of the caseins and a more gradual increase on storage up to 24 h.

Keywords: *Calcium phosphate; casein; dissociation; pH*

INTRODUCTION

Above 30 °C, most of the casein in milk is in micellar form, closely associated with calcium phosphate and smaller amounts of Mg and citrate. The stability of the casein micelles and the processing characteristics of milk are affected by changes in temperature and pH. When milk is refrigerated and stored at 4 °C, a small amount of the colloidal calcium phosphate dissolves (Davies and White, 1960; Pierre and Brulé, 1981), and up to about half of the β-casein dissociates from the micelles due to reduced hydrophobic interaction at the low temperature (Rose, 1968; Downey and Murphy, 1970). The remainder of the β-casein is more tightly bound within the micelles together with the κ-, α_{s1}-, and α_{s2}-caseins and most of the calcium phosphate, and if the milk is rewarmed the changes in the composition and distribution of micellar and serum caseins can be reversed (Davies and Law, 1983).

On reducing the pH of milk during the manufacture of products such as cheese, fermented products, or acid casein, the colloidal calcium phosphate and small amounts of Mg and citrate are dissolved, and below pH 5.0 removal of the Ca and inorganic phosphate (P_i) from the micelles is almost complete (Van Hooydonk et al., 1986; Dalgleish and Law, 1989; Le Graet and Brulé, 1993). The caseins also dissociate from the micelles on acidification (Snoeren et al., 1984; Van Hooydonk et al., 1986), but the extent of dissociation is temperature-dependent (Rose, 1968). Dalgleish and Law (1988) found that at 30 °C, even when most of the calcium phosphate is removed from the micelles, only a small amount of casein dissociates into the serum. At 20 °C, and even more so at 4 °C, the amount of serum casein increases considerably as the pH is reduced, reaching maximum levels at about pH 5.4 and 5.2, respectively. The combined effect of low temperature and low pH is more than additive in causing dissociation of caseins from the micelles. As the pH is further reduced the amount of casein in the serum decreases due to isoelectric precipitation.

Although the changes in the micellar−serum equilibria of the minerals and caseins on acidification of milk at various temperatures are well-documented, there is comparatively little information about the relative rates of solubilization of the colloidal calcium phosphate and dissociation of the caseins from the micelles. In most of the above studies milks were acidified, and changes in the serum concentrations of minerals and proteins determined after a fixed time interval. In this study we have measured the changes in the levels of Ca, P_i, and individual caseins in the serum immediately after acidification, and at intervals up to 24 h after pH adjustment. The effects of pH on the rates of solubilization of the colloidal calcium phosphate and dissociation of the caseins were examined at temperatures commonly used for storage or processing (4, 20, and 30 °C), and this also allowed comparison with previous studies of acid-induced dissociation of casein micelles by Dalgleish and Law (1988, 1989).

MATERIALS AND METHODS

Milk Samples. Bulk milk was collected from Friesian cows in the Institute herd. The milk was skimmed by centrifugation at 1000*g* for 30 min, and sodium azide was added to a final concentration of 0.01% to inhibit bacterial growth. The skim-milk was equilibrated at 4, 20 or 30 °C for 1 h before acidification.

Colloidal Phosphate-Free Milk. Skim-milk was cooled to 0 °C and adjusted to pH 4.8 by the addition of HCl with vigorous stirring. On acidification, the colloidal calcium phosphate was completely solubilized without precipitation of the caseins. The levels of serum Ca and P_i were restored by dialyzing against a large excess of the original skim-milk (Pyne and McGann, 1960).

Acidification of Milk. The pH of milk at 4, 20 or 30 °C was reduced by the dropwise addition of 5.0 M HCl with

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vigorous stirring over a period of 1 min, giving a range of samples between pH 6.7 and 4.6. The acidified milk was stored for up to 24 h at 4, 20, or 30 °C, and samples were taken at regular intervals to study changes in the serum concentrations of Ca, P_i, and individual caseins as described below.

In one experiment to compare the effects of different rates of acidification, glucono-δ-lactone was added to skim-milk at a concentration of 2% w/v. The milk was kept at 20 °C for 3 h, during which time the glucono-δ-lactone hydrolyzed slowly to gluconic acid, reducing the pH gradually to 4.6.

Serum Ca and P_i. Solutions for determination of concentrations of serum Ca and P_i in acidified skim-milks were prepared by ultrafiltration or, when more rapid separation was required, by filtration (0.2 μm). Ultrafiltration was carried out at the appropriate storage temperature using a stirred cell with a polysulfone membrane having a 10K molecular weight cutoff (Sartorius Limited, Epsom, Surrey, U.K.). Samples of ultrafiltrate were collected at hourly intervals up to 16 h. Filtrate from each of the acidified skim-milks was prepared at the appropriate storage temperature using a 1.0 mL syringe and 0.2 μm Minisart RC15 syringe filter (Sartorius Limited, Epsom, Surrey, U.K.) clamped vertically with the filter at the top. A steady force of 1500*g* was applied, and a sample of filtrate (10 μL) was collected within 2 min using a micropipet. Samples were prepared immediately after the pH had been adjusted and, in experiments to determine the change with time, after 2, 5, 10, 15, 30, 60, 120, and 180 min.

Ca and Inorganic Phosphate (P_i) Analyses. To determine the concentrations of total Ca and P_i in milks, protein was precipitated by mixing with equal volumes of TCA (24% w/v) with continuous stirring for 20 min. The supernatants were filtered (0.2 μm) and diluted 1:100. The Ca and P_i contents of ultrafiltrates or filtrates were determined in the same way, without the final filtration step.

Ca was determined by a colorimetric method based on the formation of a complex with *o*-cresolphthalein complexone, in the presence of 8-hydroxyquinoline to prevent interference by Mg (Connerty and Briggs, 1966). Colloidal Ca was calculated as the difference between the total in skim-milk and the Ca in the corresponding ultrafiltrate. The coefficient of variation for the analysis was 0.9% (Law, 1996).

The concentrations of total inorganic phosphate (P_i) were determined by the colorimetric method of Fiske and Subbarow (1925). Colloidal P_i was calculated as the difference between the total in skim-milk and the P_i in the corresponding ultrafiltrate. The coefficient of variation for the analysis was 2.4% (Law, 1996).

Separation of Serum Caseins. The serum and micellar caseins were separated by ultracentrifugation immediately after acidification of the skim-milks and after storage at 4, 20 or 30 °C. Samples were prepared at hourly intervals for the first 5 h of storage, and additional samples were prepared after about 18 and 24 h. Each control (3.0 mL) and pH-adjusted skim-milk was centrifuged at 32000*g* for 1 h in a Sorvall SS-34 rotor fitted with adapters to take 4-mL centrifuge tubes. Centrifugation was carried out at the appropriate storage temperature (4, 20, or 30 °C). The supernatant and interphase layer were poured off together and adjusted to room temperature for analysis of the caseins by reverse-phase HPLC as described below.

Reverse-Phase HPLC. Representative samples (0.1 mL) of the control skim-milks and supernatants obtained by ultracentrifugation were mixed with 0.4 mL of denaturing buffer at pH 7.5, consisting of 17.5 mM bis-tris-propane containing 7 M urea (BioRad electrophoresis grade) and 5 μL mL^{-1} of 2-mercaptoethanol. After incubating with shaking for a minimum of 1 h at room temperature, the samples were passed through 0.2 μm filters. Protein composition was determined by reverse-phase HPLC on an APEX WP ODS column (25 cm × 4.6 mm i.d.; 7 μm bead size; Jones Chromatography, Hengoed, Mid-Glamorgan, U.K.). This was an adaptation of the method of Visser et al. (1991). Samples (20 μL) were loaded onto the column and proteins eluted at a flow rate of 1 mL min^{-1} using a linear gradient of 33 to 55% acetonitrile in 0.1% TFA over 30 min. Column temperature was 46 °C and detection was at 214 nm. The amount of each casein in the supernatants was expressed as a percentage of the corresponding amount in skim-milk.

Table 1. Comparison of Mean Values for Concentrations of Ca and P_i in Ultrafiltrate (10 K Molecular Weight Cutoff) and in Filtrate (0.2 μm) from Control and Acidified Skim-Milks at 20 and 4 °C

milk (no. of determinations)	Ca (mM)		P_i (mM)	
	ultrafiltrate	filtrate	ultrafiltrate	filtrate
20 °C, pH 6.7 (2)	8.6	8.3	11.7	11.8
20 °C, pH 5.5 (4)	23.5	22.3	18.9	18.9
20 °C, pH 5.0 (2)	29.4	29.4	22.1	22.2
4 °C, pH 6.7 (4)	10.7	10.4	11.7	11.6
4 °C, pH 5.5 (4)	22.0	22.6	18.1	18.1

RESULTS

Determination of Serum Ca and P_i. Serum concentrations of Ca and P_i are usually determined in the supernatant obtained by ultracentrifugation or in ultrafiltrate, prepared with membranes typically of 10K molecular weight cutoff. However, ultracentrifugation is a lengthy procedure, and preparation of ultrafiltrate tends to be slow due to the low flow rates through membranes and the substantial void volumes of most types of equipment. To study the rate of solubilization of colloidal calcium phosphate on acidification of skim-milk, trials were carried out in which skim-milk and acidified skim-milk were filtered rapidly using a larger pore size (0.2 μm). Small samples of filtrate were obtained within 2 min, and analysis of the protein in the filtrate by reverse-phase HPLC indicated that the pore size of the 0.2 μm filters was effectively reduced, so that micellar caseins were unable to pass through. At pH 6.7, the concentration of casein in the filtrate at 30, 20, and 4 °C was less than 5% of that in skim-milk. As milk was acidified to pH 5.5, close to the point of maximum dissociation of the casein micelles (Dalgleish and Law, 1988), the concentration of casein in the filtrate at 20 and 30 °C was 3.9 and 2.8% respectively of that in skim-milk. At 4 °C, as the milk was acidified to pH 6.0, the concentration of casein in the filtrate increased to about 10%, and at pH 5.5, to about 30% of the concentration in skim-milk. However, the presence of caseins in the filtrate obtained at low temperature and pH had no effect on values for the concentrations of Ca and P_i in the serum (Table 1). Statistical analysis using a paired *t*-test showed that there were no significant differences between corresponding values for concentrations of Ca and P_i obtained in filtrates and ultrafiltrates from skim-milk and acidified skim-milk, even near the pH of maximum dissociation of the caseins from the micelles. Filtration, therefore, was a suitable, rapid alternative for studying changes in the levels of serum Ca and P_i.

Effects of Acidification and Storage on Colloidal Calcium Phosphate. When the pH of skim-milk at 20 °C was reduced by the addition of HCl, the colloidal calcium phosphate was solubilized and the concentrations of Ca and P_i in the serum obtained by filtration (0.2 μm) increased (Figure 1). Below pH 5.0 nearly all of the Ca and P_i was present in the serum.

After each pH adjustment, there was an initial rapid increase in the serum concentrations of Ca and P_i (Figure 1). The overall time for acidification of each milk was 1 min, and filtration required a further 2 min. Most of the Ca and P_i release into the serum at a particular pH, therefore, occurred during this initial period. There

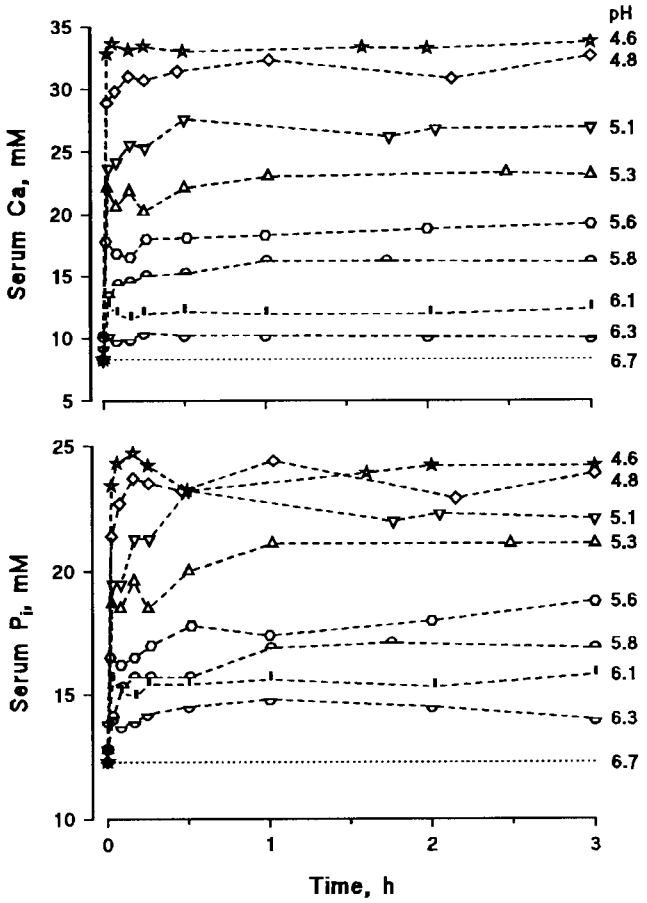

Figure 1. Effect of acidification and storage time on the concentrations of Ca and P_i in the serum of skim-milk at 20 °C. Concentrations of Ca and P_i were determined by filtration (0.2 μm), as described in the text.

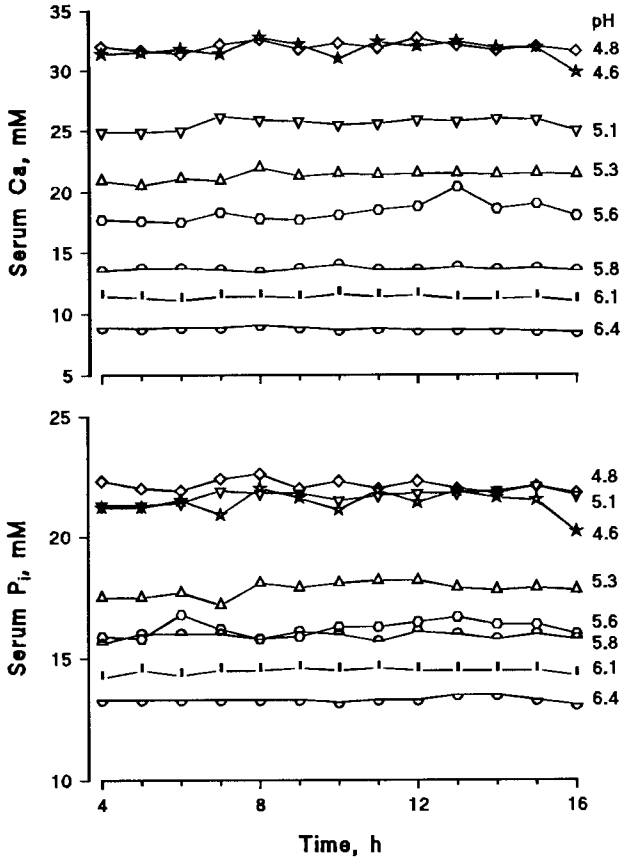

Figure 2. Effect of acidification and prolonged storage on the concentrations of Ca and P_i in the serum of skim-milk at 20 °C. Concentrations of Ca and P_i were determined in ultrafiltrate obtained using a membrane with a 10K molecular weight cutoff, as described in the text.

was a small, slower increase in serum concentrations of Ca and P_i over the next 20 min, which may have been due to redispersal of small amounts of casein precipitate formed on adding the HCl. Between 30 min and 3 h after acidification, however, there was no significant change in serum concentrations of Ca and P_i.

The effect of prolonged storage at 20 °C on the levels of serum Ca and P_i after acidification of milk was examined using ultrafiltration (Figure 2). Results from two-way analysis of variance showed that levels of serum Ca and P_i increased only slightly by 0.4 ($P < 0.001$) and 0.3 mM ($P < 0.010$), respectively.

Rate of Acidification. When milk was acidified at 20 °C by the addition of HCl, the serum concentrations of Ca and P_i determined in filtrate (0.2 μm) immediately after acidification, increased with decreasing pH as shown in Figure 3. The serum concentrations of Ca and P_i increased in a sigmoidal way, and below pH 5.0 most of the Ca and P_i was in the serum. The changes in serum concentrations of Ca and P_i with pH were similar to those described by other workers who stored the milk for several hours after acidification (Van Hooydonk et al., 1986; Dalgleish and Law, 1989).

When milk at 20 °C was acidified by the addition of glucono-δ-lactone, the pH decreased from 6.7 to 4.6 over a period of 3 h. There was a gradual increase in the levels of Ca and P_i in the serum, and below pH 5.0 most of the colloidal calcium phosphate was solubilized (Figure 3). Results from two-way analysis of variance showed that over most of the pH range, the amounts of

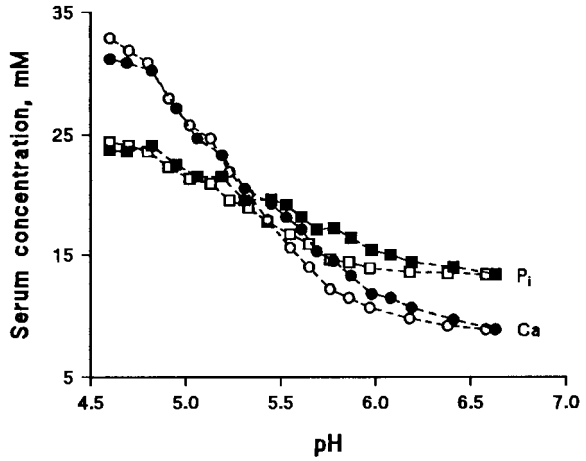

Figure 3. Changes in the levels of serum Ca and P_i in skim-milk at 20 °C with (a) rapid acidification by the addition of HCl (filled symbols) and (b) slow acidification by the hydrolysis of glucono-δ-lactone (open symbols). Concentrations of Ca and P_i were determined by filtration (0.2 μm), as described in the text.

Ca and P_i released into the serum were not significantly different from those when the pH was adjusted rapidly by the addition of HCl (Figure 3). The extent of solubilization of colloidal calcium phosphate at a given time, therefore, depended on the pH of the milk and was not much affected by the length of time required to reach the pH. The slow reduction in pH during lactic acid fermentation of milk is similar to that obtained by the hydrolysis of glucono-δ-lactone, and results indicate that

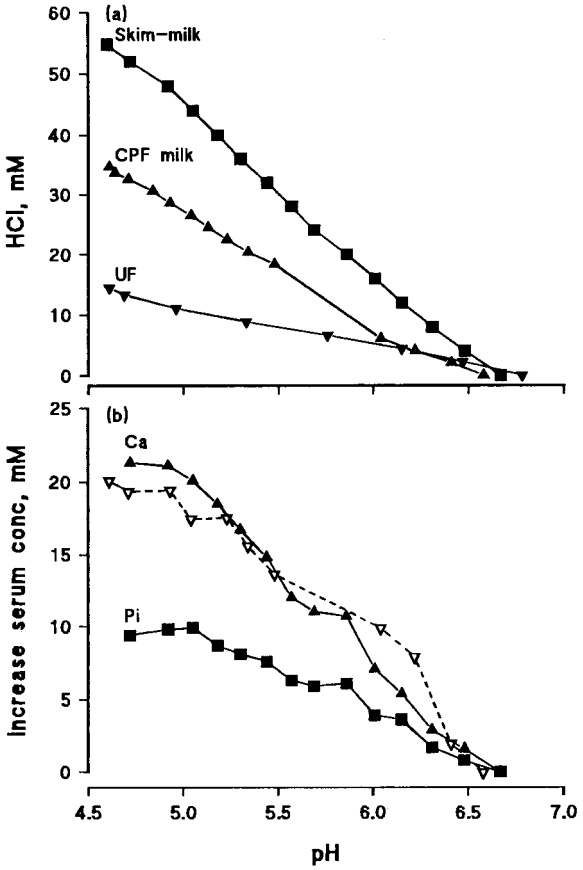

Figure 4. (a) The titration of skim-milk, colloidal phosphate-free skim-milk (CPF), and ultrafiltrate with HCl (UF) and (b) the increase in levels of serum Ca and P_i on acidification of skim-milk at 20 °C, as determined by filtration (0.2 μm). (▽) The concentration of HCl (calculated from Figure 4a) required to titrate the micellar calcium phosphate in skim-milk.

provided there is no extensive breakdown of citrate during fermentation, the extent of solubilization of the colloidal calcium phosphate would similarly be determined by the final pH, with the total fermentation time having little effect.

Titration of Colloidal Calcium Phosphate-Free Skim-Milk. When milk was acidified with HCl, various components including the colloidal calcium phosphate, caseins and whey proteins and other nonprotein materials, were titrated. In this study, the amount of HCl required to titrate ultrafiltrate was considerably less than for skim-milk (Figure 4a), the difference being due to the removal of caseins, colloidal calcium phosphate, and whey proteins during ultrafiltration. The amount of HCl required to titrate colloidal calcium phosphate-free skim-milk was also considerably less than for skim-milk (Figure 4a). As serum Ca and P_i concentrations and the amount of Ca^{2+} bound to casein were restored to the levels in skim-milk during the preparation of colloidal phosphate-free skim-milk (Pyne and McGann, 1960), the difference in the amount of HCl required was due mainly to titration of micellar calcium phosphate. In the present study, the amount of HCl required to titrate the micellar calcium phosphate was similar, on a molar basis, to the increase in serum Ca on acidification of skim-milk (Figure 4b).

On plotting the increase in serum Ca against that for P_i on acidification of skim-milk with HCl (Figure 5), a curvilinear relation was found and, as the pH was reduced, the ratio of the Ca/P_i released from the micelles

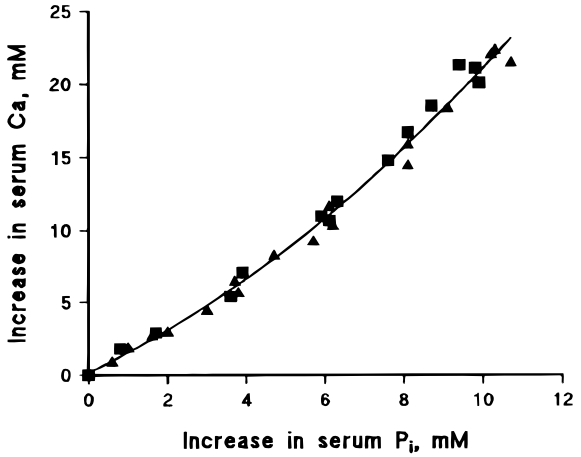

Figure 5. Relation between the increase in the serum concentrations of Ca and P_i on acidification of skim-milk at 20 °C by the addition of HCl, as shown in Figure 3. Different symbols represent values for two herd bulk milks.

into the serum increased from about 1.5 at pH 6.7 to 2.0 at pH 4.6. In previous studies on the pH-induced dissociation of caseins micelles, a linear relationship was found between the Ca and P_i remaining in the micelles on the reduction of the pH from 6.7 to about 5.3, and the slope of the line indicated that the ratio of Ca/P_i in micellar calcium phosphate was about 1.7−1.9 (Van Hooydonk et al., 1986; Dalgleish and Law, 1989). There is considerable evidence that the structure of micellar calcium phosphate resembles brushite ($CaHPO_4 \cdot 2H_2O$) and, to account for the higher Ca/P_i ratio found in dissociation studies, it has been proposed that some of the HPO_4^{2-} ions in the brushite lattice are replaced by SerP residues of the caseins (Holt, 1992). In the above acid dissociation studies, the intercept of the line on the axis representing calcium concentration was taken as a measure of the amount of Ca^{2+} remaining tightly bound to negatively charged groups on the caseins, and was between 1.8 and 4.6 mM (Van Hooydonk et al., 1986; Dalgleish and Law, 1989). The results presented here, and the marked deviation from linearity below pH 5.3 in the above studies, indicate that the increase in the ratio of Ca/P_i in the serum may be due to the release of this more tightly bound Ca^{2+} as the Asp, Glu and SerP residues become protonated at lower pH.

Effect of Temperature of Acidification. On acidifying skim-milk at 4, 20 or 30 °C to pH 5.9, the levels of serum Ca (Figure 6) and P_i (Figure 7) showed a moderate increase, but were not affected by the temperature at which acidification and storage were carried out. At each temperature, as described above for acidification at 20 °C, serum Ca and P_i initially increased rapidly but showed little change on storage for 3 h.

At pH 5.5, and especially at pH 5.1, levels of serum Ca (Figure 6) and P_i (Figure 7) increased markedly on acidification, and the corresponding serum concentrations at 20 and 30 °C were similar. However, at pH 5.5 and 5.1, serum concentrations of Ca and P_i were significantly greater ($P < 0.001$) at 4 °C than at 20 or 30 °C, the increase being more marked at pH 5.1. At each pH and temperature, there was an initial rapid increase in the serum concentrations of Ca and P_i but little significant change on storage for 3 h.

On acidifying milk at 4 °C to pH 5.5 and storing for a longer period of 16 h, there was only a slight increase in serum levels of Ca and P_i (Figure 8).

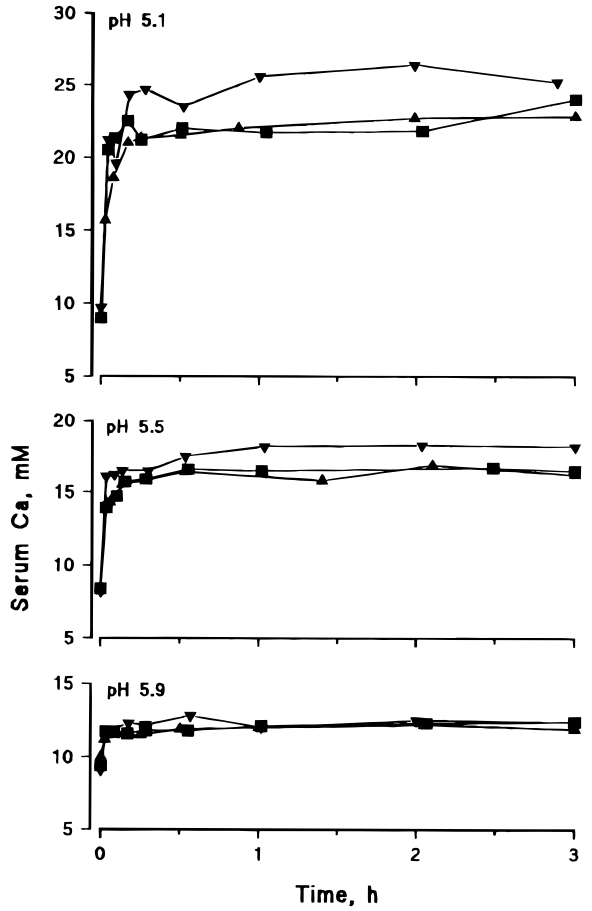

Figure 6. The effect of temperature and storage time on concentrations of Ca in the serum of acidified skim-milk, determined by filtration (0.2 μm): (▼) 4 °C, (■) 20 °C, (▲) 30 °C.

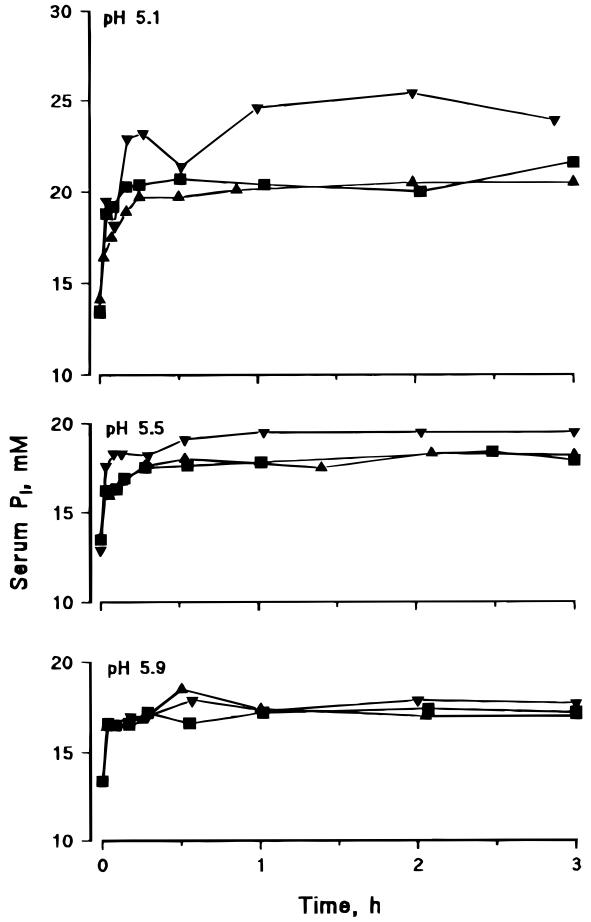

Figure 7. The effect of temperature and storage time on concentrations of P_i in the serum of acidified skim-milk, determined by filtration (0.2 μm): (▼) 4 °C, (■) 20 °C, (▲) 30 °C. Samples as in Figure 6.

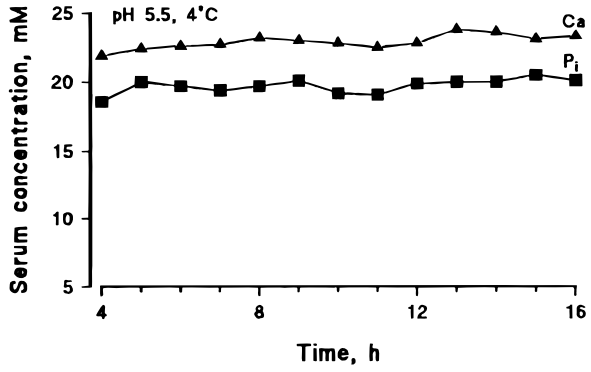

Figure 8. Effect of prolonged storage on the concentrations of Ca and P_i in the serum of skim-milk at pH 5.5 and 4 °C. Concentrations of Ca and P_i were determined in ultrafiltrate prepared using a membrane with a 10K molecular weight cutoff, as described in the text.

Dissociation of Micellar Caseins. The effects of temperature and storage time on the dissociation of caseins from the micelles on acidification of skim-milk were examined by ultracentrifugation. The concentrations of the caseins in the serum were determined by reverse-phase HPLC, and each expressed as percentage of the total of each casein in the original skim-milk. The caseins were usually well-resolved from the accompanying whey proteins, but values for α_{s2}-casein were slightly high due to coelution of a minor whey protein. The increase was especially noticeable at 20 and 30 °C, when levels of serum casein were low, but the overall trends in serum α_{s2}-casein were not affected. When milk at 4 °C was acidified between pH 6.7 and 5.2 by the addition of HCl (Figure 9), there was a marked increase in the levels of all caseins in the serum ($P < 0.001$). At each pH there was a substantial increase in the levels of serum caseins soon after the pH was adjusted. In this study, the pH was adjusted and the milk centrifuged, within about 8 min of acidification, for a period of 1 h. The changes in the levels of serum caseins, therefore, occurred within less than 1 h, but it is not possible to say if the changes took place immediately on acidification, as found for the increase in serum concentrations of Ca and P_i. The initial percentage increases in the concentrations of κ- and β-caseins in the serum were greater than those of α_{s1}- and α_{s2}-caseins. Between pH 6.7 and 5.2, there was also a marked increase in the serum concentrations of all the caseins with storage time ($P < 0.001$). Over most of the pH range and storage time, the overall percentage increases in the concentrations of κ- and β-caseins were greater than those of α_{s1}- and α_{s2}-caseins. At 4 °C, concentrations of caseins in the serum were at a maximum at pH 5.2 and, below this pH, decreased due to isoelectric precipitation of the caseins. At pH 5.0, with storage times up to 5 h, the amount of serum casein was not significantly different from that in the original skim-milk, and below pH 5.0 the amount decreased due to isoelectric precipitation. Previous electrophoretic studies have shown that on acidification at low temperature, β-casein is the major component of serum casein, but that κ- and α_s-caseins

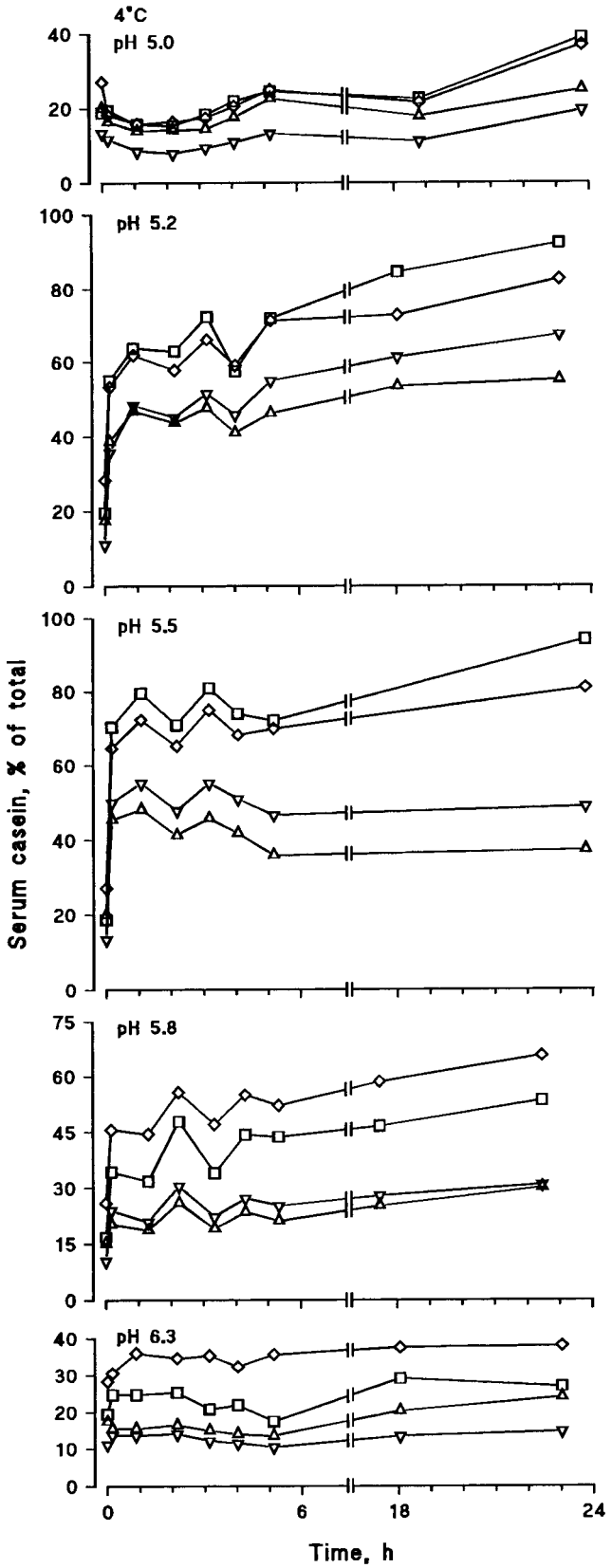

Figure 9. Changes in the serum concentrations of the caseins on acidification and storage of skim-milk at 4 °C. Serum obtained by ultracentrifugation at 4 °C, as described in the text: (□) κ-Casein, (△) α_{s2}-casein, (▽) α_{s1}-casein, (◇) β-casein. Values for the serum caseins are expressed as a percentage of the total of each casein in skim-milk. The storage time indicated does not include the 1-h centrifugation period.

are also present (Rose, 1968; Snoeren et al., 1984). In a quantitative study with cation-exchange FPLC, Roefs et al. (1985) found that at 4 °C and with storage for 20 h, maximum dissociation occurred at pH 5.4, and the percentage dissociation of the caseins was in the order β- > κ- > α_s-casein. The extent of dissociation of the β- and α_{s1}-caseins in the present study was similar to that in the study of Dalgleish and Law (1988) in which milk was acidified at 4 °C and stored for 24 h, but the extent of dissociation of κ-casein was slightly higher in the present study.

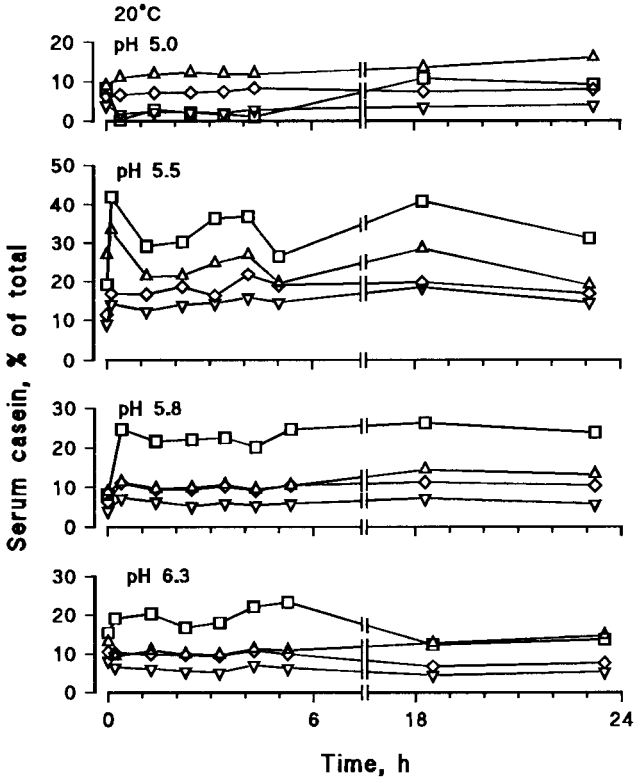

Figure 10. Changes in the serum concentrations of the caseins on acidification and storage of skim-milk at 20 °C. Serum obtained by ultracentrifugation at 20 °C, as described in the text: (□) κ-Casein, (△) α_{s2}-casein, (▽) α_{s1}-casein, (◇) β-casein. Values for the serum caseins are expressed as a percentage of the total of each casein in skim-milk. The storage time indicated does not include the 1-h centrifugation period.

At 20 °C, the extent of dissociation of the caseins, especially κ-casein, increased as milk was acidified between pH 6.7 and about 5.5 (Figure 10), the increases being significant for κ-casein ($P < 0.01$) and α_{s2}-, α_{s1}-, and β-caseins ($P < 0.001$). At each pH, the concentrations of serum casein at 20 °C were lower than at 4 °C. Most of the increase in serum casein occurred soon after acidification of the milk, and levels of serum casein did not increase significantly on storage, in contrast to the increase found at 4 °C. Concentrations of serum caseins were highest near pH 5.5 and, below this pH, decreased due to increasing isoelectric precipitation. Below pH 5.0 serum concentrations of κ-, α_{s1}-, and β-caseins were lower than in the original skim-milk.

When milk was acidified at 30 °C, there was an initial slight increase in the levels of κ- and α_{s2}-caseins at pH 5.8 and 5.5 (Figure 11), but between pH 6.7 and 5.0 and with storage times up to 24 h, changes in the levels of the caseins in the serum were not statistically significant. Dalgleish and Law (1988) storing milks for 24 h, found that as in the present study, concentrations of serum caseins were highest at 4 °C and pH 5.2, whereas at 20 °C they were highest near pH 5.4, and levels of

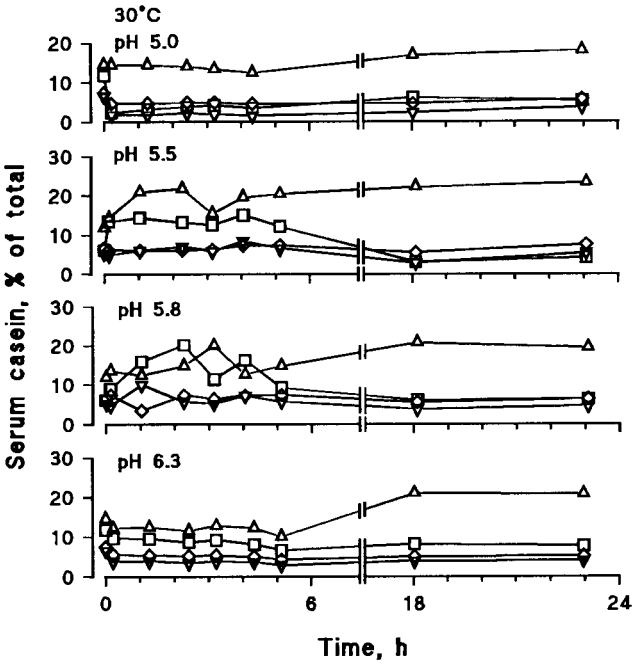

Figure 11. Changes in the serum concentrations of the caseins on acidification and storage of skim-milk at 30 °C. Serum obtained by ultracentrifugation at 30 °C, as described in the text: (□) κ-Casein, (△) α_{s2}-casein, (▽) α_{s1}-casein, (◇) β-casein. Values for the serum caseins are expressed as a percentage of the total of each casein in skim-milk. The storage time indicated does not include the 1-h centrifugation period.

the α_{s1}- and β-caseins in the serum were about half of those at 4 °C. They similarly found that at 20 °C, there was an appreciable increase in the serum concentration of κ-casein reaching a maximum at pH 5.4. At 30 °C, as reported here, levels of dissociation of the caseins were low, but there was a slight increase as the pH was reduced, with a maximum level of serum casein near pH 5.5.

DISCUSSION

When skim-milk was acidified at various temperatures, solubilization of the colloidal calcium phosphate and dissociation of the caseins from the micelles were affected in different ways. As the pH was reduced at 4, 20 or 30 °C, colloidal calcium phosphate was progressively solubilized, and the extent of the calcium phosphate depletion depended on the final pH. The increase in serum Ca and P_i was slightly greater at 4 °C than at 20 or 30 °C, and at each temperature, below pH 5.0 most of the Ca and P_i was in the serum. The concentrations of Ca and P_i in the serum increased rapidly after pH adjustment, with only a slight increase on prolonged storage.

The results indicate that as found by Pierre and Brulé (1981), the distribution of Ca and P_i between the micellar and serum phases is determined by the solubility of the calcium phosphate salts, which are normally saturated in the serum. Previous studies have shown that micellar calcium phosphate may have a structure similar to brushite ($CaHPO_4 \cdot 2H_2O$), and milk serum contains free Ca^{2+} ions (2 mM) and complexes of Ca^{2+} with HPO_4^{2-} (0.59 mM) and $H_2PO_4^-$ (0.07 mM) together with free ions of HPO_4^{2-} (2.65 mM) and $H_2PO_4^-$ (7.5 mM) (Holt, 1985). When the pH of milk is reduced there is increased conversion of HPO_4^{2-} to $H_2PO_4^-$ and the serum is no longer saturated with respect to $CaHPO_4$. There is also a reduction in the charge of the SerP residues on the caseins which facilitates release of Ca and P_i into the serum. The results presented here show that when the pH was reduced, the changes in the micellar−serum distribution of Ca and P_i occurred rapidly, and the new equilibrium was not much affected by subsequent storage.

In contrast to the behavior of the calcium phosphate, dissociation of the caseins from the micelles on acidification of skim-milk was temperature-dependent, increasing markedly at lower temperatures. As the pH of milk was reduced at 30 °C, most of the calcium phosphate could be removed but only a small amount of dissociation of the caseins occurred. At 20 °C, slightly more casein dissociated from the micelles as the pH was reduced, and the extent of dissociation of κ-casein which contains only one SerP residue, was greater than that of the other caseins. At 4 °C, however, there was a marked increase in dissociation of the caseins, particularly of κ- and β-caseins, after pH adjustment, and a more gradual increase on storage.

Previous studies have shown that dissociation of caseins from the micelles is affected both by the interaction of colloidal calcium phosphate with the caseins and by the hydrophobic interactions between the caseins. On chelation of Ca^{2+} in milk by the addition of EDTA, the colloidal calcium phosphate is depleted, and the micelles dissociate (Pepper, 1972; Lin et al., 1972). At low concentrations of EDTA some colloidal calcium phosphate is removed without disruption of the micelles, but at higher concentrations, as more calcium phosphate is removed, the micelles dissociate into smaller aggregates (Griffin et al., 1988). The importance of calcium phosphate in maintaining micellar stucture has also been shown by selective dialysis experiments (Holt et al., 1986). On dialyzing milk at pH 6.7 and 20 °C against phosphate-free buffer with 3 or 6 mM Ca^{2+}, the levels of both colloidal Ca and P_i are reduced and, when more than 30% is removed from the micelles, the caseins dissociate from the micelles. The ease of dissociation of the caseins is in the order κ-, β- > α_{s1}- > α_{s2}-casein, and occurs through breaking of linkage between the caseins and inorganic constituents. Analysis of amino acids in calcium phosphate-rich material after proteolytic digestion of casein micelles has shown that the phosphate centers of the caseins are involved, and that the SerP-rich α_{s2}- and α_{s1}-caseins are more tightly bound within the micelles than κ- or β-caseins (Holt et al., 1986). Further chemical and physical measurements have shown that the colloidal calcium phosphate is distributed throughout the matrix of the casein micelle in the form of amorphous nanometer-sized clusters (Holt et al., 1996; Holt et al., 1998). Interactions of the caseins are also important in maintaining micellar structure, and McGann and Fox (1974) found that addition of urea to milk causes dissociation of the casein micelles, showing that hydrophobic interactions and hydrogen bonds are involved. Treatment with urea, however, does not affect the binding of calcium phosphate to the caseins, confirming that a different type of linkage is involved.

Acid-induced dissociation of the caseins differs from the types of dissociation described above, in that the decrease in pH leads to a simultaneous solubilization of the colloidal calcium phosphate and decrease in the net negative charge on the caseins. As the pH is

reduced, there is also an increase in the serum concentration of Ca^{2+} ions that can bind to the caseins and further reduce the charge, and at 20 °C the extent of dissociation of caseins on acidification is less than on addition of EDTA. The importance of the reduction in net negative charge in preventing dissociation of caseins from acidified micelles was confirmed by Lucey et al. (1997) who showed that when the pH of micelles that are depleted of colloidal calcium phosphate is again increased, dissociation of caseins into the serum increases.

Results in this study indicate that on acidification at 30 and 20 °C some of the colloidal calcium phosphate was rapidly removed, but most of the casein was held within the micelles by hydrophobic interactions. At 4 °C, however, when hydrophobic interactions were reduced, there was an initial marked increase in dissociation of the caseins when the pH was adjusted and, as found in the selective dialysis experiments described above (Holt et al., 1986), κ- and β-caseins, which usually contain 1 and 5 SerP residues respectively, dissociated more readily than the more highly phosphorylated α_{s1}- and α_{s2}-caseins, containing 8−9 and 10−13 SerP residues, respectively. The changes in the serum concentrations of the caseins on acidifying milk at 4 °C were different from those obtained solely by cooling the milk (Davies and Law, 1983) when most of the increase in the serum casein was due to β- or γ-caseins, with κ-casein showing no increase, and the α_{s1}- and α_{s2}-caseins increasing by just over 1%.

The extent of dissociation of the caseins increased as the pH was reduced, and the concentration of serum casein was at a maximum at pH 5.4. At this pH, about half of the colloidal calcium phosphate was not solubilized, and results indicate that, as found in the selective dialysis experiments discussed above (Holt et al., 1986), the micelles may consist of a framework of calcium phosphate−casein complexes that are enriched in the most highly phosphorylated α_{s1}- and α_{s2}-caseins. On storing the pH-adjusted milk at 4 °C, there was little change in the solubilization of the colloidal calcium phosphate (Figure 8), but there was a slow dissociation of caseins with a proportionately greater release of κ- and β-caseins than of α_{s1}- and α_{s2}-caseins. This dissociation may have been due to reduced hydrophobic interaction at the low temperature, and diffusion of the caseins from the remaining framework of the micelles. As milk was acidified below pH 5.4, more of the colloidal calcium phosphate was solubilized but, as the net negative charge on the caseins was reduced, isoelectric precipitation increased.

Results of this study show that on acidification, solubilization of the colloidal calcium phosphate occurs rapidly and is pH dependent, whereas the extent of dissociation of the caseins from the micelles varies with pH, temperature, and storage time. Acidification of warm milk leads to depletion of colloidal calcium phosphate, whereas reducing the pH of cold milk leads to both solubilization of the colloidal calcium phosphate and dissociation of the caseins, the latter being increased by prolonged cold storage. The differences in the behavior of the colloidal calcium phosphate and micellar caseins therefore provide a means of selectively modifying the mineral and protein composition and resultant processing characteristics of the micelles.

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