The Two-Stage Coagulation of Milk Proteins in the Minimum of the Heat Coagulation Time-pH Profile of Milk: Effect of Casein Micelle Size

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ABSTRACT

Milks with casein micelles larger or smaller than control milk were prepared by differential centrifugation. The heat stability of these modified milks increased markedly throughout the pH range 6.4 to 7.1 with decreasing casein micelle size. Within the region of the minimum in the heat coagulation time-pH profile, the control milk coagulated by a two-stage process, but the modified milks, because of their narrower casein micelle size distribution, coagulated by a single-stage process at the pH of minimum stability. The content of κ-CN and protein hydration increased as the size of the casein micelles decreased, and the level of glycosylation of κ-CN and protein surface hydrophobicity increased as a function of micelle size. The effect of casein micelle size on the heat stability of milk is likely to be related to changes in the above physico-chemical properties.
(**Key words:** milk, heat stability, casein micelle size)

Abbreviation key: HCT = heat coagulation time, **SMUF** = synthetic milk ultrafiltrate.

INTRODUCTION

Since the pioneering work of Rose (24), the heat stability of milk is normally studied in relation to pH. The method used most commonly to study the heat stability of milk is the 'subjective heat stability assay' initially developed by Miller and Sommer (21) and modified (8) to take cognizance of the pH dependence of the heat stability of milk. This method involves submerging samples of milk ranging in pH from ~6.3 to 7.3, contained in sealed tubes, in a thermostatically controlled oil bath (usually at 140°C for unconcentrated milk or 120°C for concentrated milk) and measuring the time required to cause coagulation, usually indicated by flocculation of the milk protein. Two typical heat coagulation time (**HCT**)-pH profiles occur: type A milks, the predominant form, which exhibit a marked maximum at pH ~6.7 and a minimum at pH ~6.9, and type B milks, the stability of which increases as a function of pH. A more informative method for measuring heat stability, the 'objective heat stability assay', is available (8) in which the percentage of total protein sedimentable by low centrifugal forces (~400 × g) is measured as a function of heating time at a constant temperature. The resulting protein depletion curve shows a sharp break at the onset of coagulation; the inflexion point in the curve indicates the heat coagulation time (10).

Sweetsur and White (34) used the objective heat stability assay to study the mechanism of coagulation within the minimum of the subjective HCT-pH profile. Protein depletion curves within the minimum have two distinct inflexion points, suggesting that the milk protein undergoes two distinct and mutually exclusive coagulation processes (34). The first inflexion point corresponds to the HCT as determined by the subjective heat stability assay, while the second inflexion point occurs at a time that would be expected if the minimum did not exist (34, 23). It has been suggested (34) that the two-stage coagulation process is due to the premature coagulation of large casein micelles caused by adsorption of precipitated calcium phosphate, followed by a second coagulation involving smaller casein micelles, which also adsorb precipitated calcium phosphate but are inherently more stable than the larger micelles.

This communication reports on the effect of altering the size distribution of casein micelles and the protein profile on the heat stability of milk with particular reference to pH values within the region of the minimum. The theory proposed by Sweetsur and White (34) (i.e., the existence of two distinct coagulating species) is also considered in relation to more recent research on the mechanism of the heat-induced coagulation of milk.

MATERIALS AND METHODS

Milk Supply

Raw milk, obtained from a local dairy, was defatted by centrifugation at 2,000 × g for 20 min at 20°C, followed by filtration through glass wool to remove fat particles. Toluene, which does not affect heat stability

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(10), was added at 0.02%, vol/vol, to inhibit microbial growth.

Heat Stability

Subjective heat stability assay. Samples of skimmed milk were adjusted to pH values in the range 6.3 to 7.1 with 1.0 M NaOH or HCl and heat stability determined by the method of Davies and White (8).

Objective heat stability assay. Samples of skimmed milk were adjusted to pH values in the range of 6.6 to 7.0 with 1.0 M NaOH or HCl and heated for periods ranging from 0 to 65 min. The heated milks were cooled to ambient temperature (~21°C), centrifuged at 500 × *g* for 10 min, filtered through Whatman No. 1 filter paper and the protein content of the filtrate determined by the method of Lowry (18).

Because very small differences in pH can have a very large effect on the heat stability of milk and the pH-dependence of heat stability can vary considerably, statistical analysis was not possible; all experiments were repeated at least three times and were reproducible to the extent that a similar pattern was found in repeat experiments.

Ethanol Stability

The ethanol stability of milk was determined as described by O'Connell et al. (22).

Milks with a Modified Casein Micelle Size Distribution

Casein micelles were fractionated by differential centrifugation of skimmed milk at 50,000 × *g*, 15 min; 60,000 × *g*, 15 min; 70,000 × *g*, 15 min; and 90,000 × *g*, 45 min at 20°C. The final supernatant was essentially milk serum containing ~0.30%, wt/vol, nonsedimentable casein [10% of total casein, a value within the range 3.4 to 14.95%, reported by Zbikowska et al. (38)].

The pelleted micelles were dispersed in the ultracentrifugal supernatant from the fourth centrifugation step to a protein concentration similar to that in the original skimmed milk, and the four resulting milks are referred to as large, intermediate-1, intermediate-2, and small, corresponding to the micelle size in the pellets obtained by centrifugation at 50,000, 60,000, 70,000 and 90,000 × *g*, respectively.

Serum Protein-Free Casein Micelle Dispersions with Modified Casein Micelle Size Distributions

Casein micelles of different sizes were prepared as previously described. The pellets were dispersed in lactose-free synthetic milk ultrafiltrate (**SMUF**; 15). The protein content was adjusted to 2.5%, wt/vol, and the solution was exhaustively dialyzed against bulk milk (60 ml dialyzed against 2 × 1 L of bulk milk for 48 h at 4°C). Control serum protein-free milk was prepared by centrifuging skimmed milk at 100,000 × *g* for 1 h at 20°C; the pellet was dispersed in SMUF and treated as with the serum protein-free milks with modified micelle size distributions.

Casein Micelle Size Distribution

The size distribution of the casein micelles in the control and experimental milks and serum protein-free casein micelle dispersion, diluted with SMUF (3.4 μl/ml), was determined, as a function of micelle number, in triplicate with a Malvern Zetamaster (Malvern Instruments Ltd, Malvern, Worcs, UK). All size distribution data are expressed in terms of number of micelles rather than on a weight or volume basis.

Zeta Potential of Casein Micelles

The zeta potential of micelles in control and experimental milks, pH 6.7, diluted 1:500 with SMUF, was determined with a Malvern Zetamaster (Malvern Instrument Ltd, Malvern, UK) at an applied potential of 120 V and a modulation frequency of 250 Hz. The instrument was calibrated with a standard carboxyl modified polystyrene latex solution, with a surface potential of –50 mV, provided by Malvern Instruments.

Hydration of Sedimentable Protein in Heated Milks

Milks with different casein micelle size distributions were heated at 120°C for 10 min at pH values in the range 6.5 to 7.1, cooled to ambient temperature, and centrifuged at 50,000 × *g* for 2 h at 20°C. The supernatant was removed, and the centrifuge tube containing the pellet was weighed and lyophilized. After initial drying, the tube was weighed and then repeatedly lyophilized until a constant weight was achieved; the amount of hydration was determined as described by Singh and Fox (32).

Hydrophobicity of Casein Micelles

The hydrophobicity of unheated casein micelles (2.7%, wt/vol, in SMUF) was determined by the method of Bonomi et al. (3). One milliliter of milk was diluted with 9 ml of 0.05 M potassium phosphate buffer, pH 6.8, containing 250 μmol of anilino naphthalene sulfonic acid/L; the fluorescence of the mixture was measured at an excitation and emission wavelength of 390 and

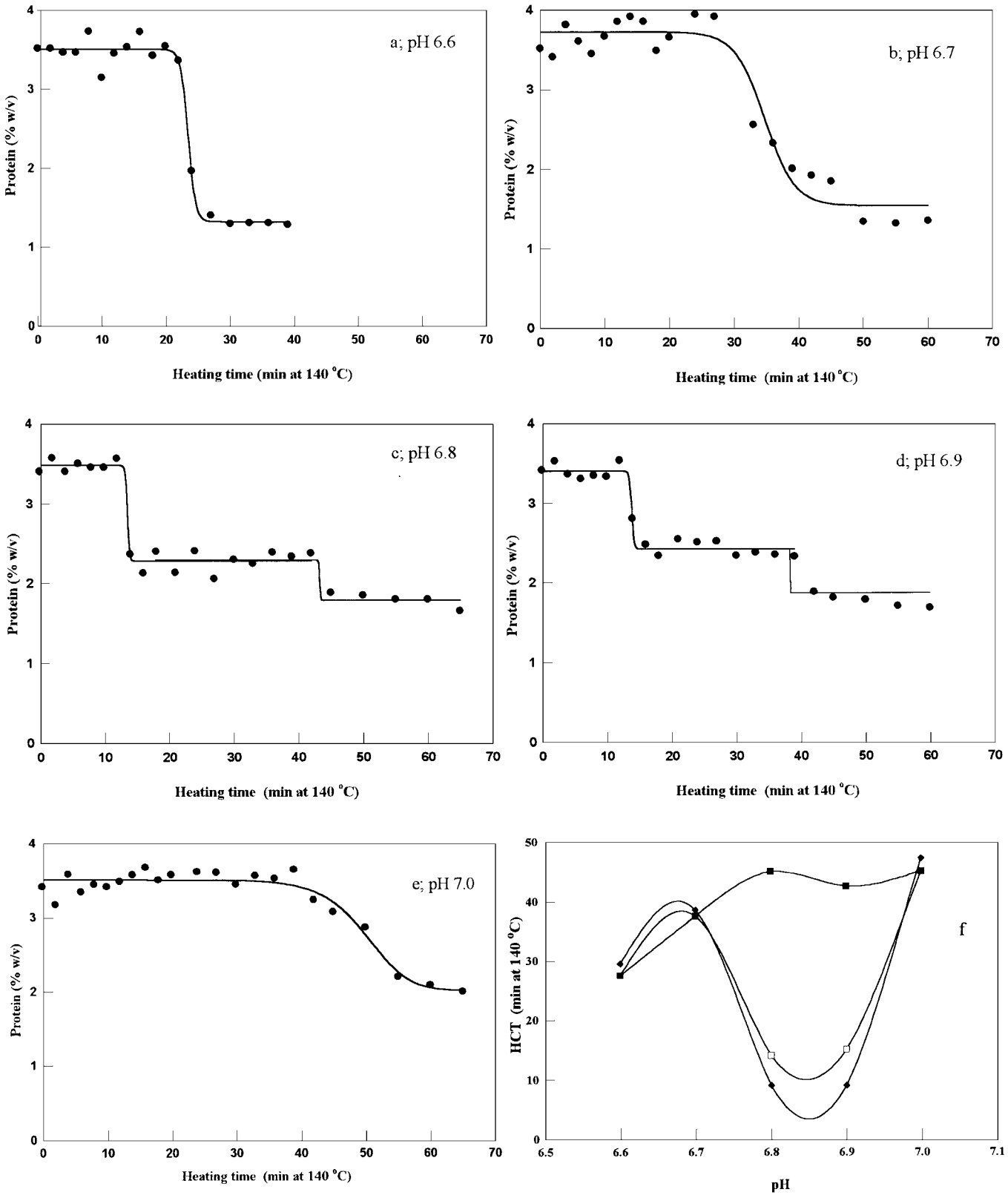

Figure 1. (a to e), Protein depletion curves for skimmed milk at pH values 6.6 to 7.0 on heating at 140°C and (f) the heat coagulation time-pH profile of skimmed milk as determined by the subjective heat stability assay (◆), the objective heat stability assay taking the first inflexion points within the minimum (□), the objective heat stability assay taking the second inflexion points within the minimum (■).

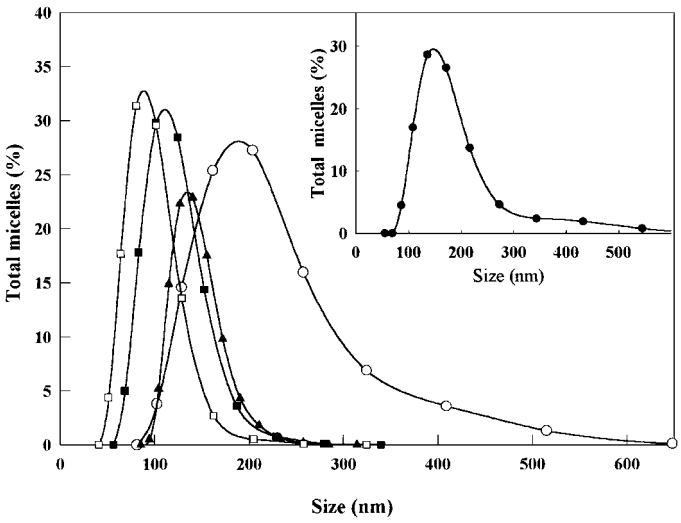

Figure 2. Casein micelle size distribution of modified milks, large (○), intermediate-1 (▲), intermediate-2 (■), small (□). (Inset: casein micelle distribution of control milk).

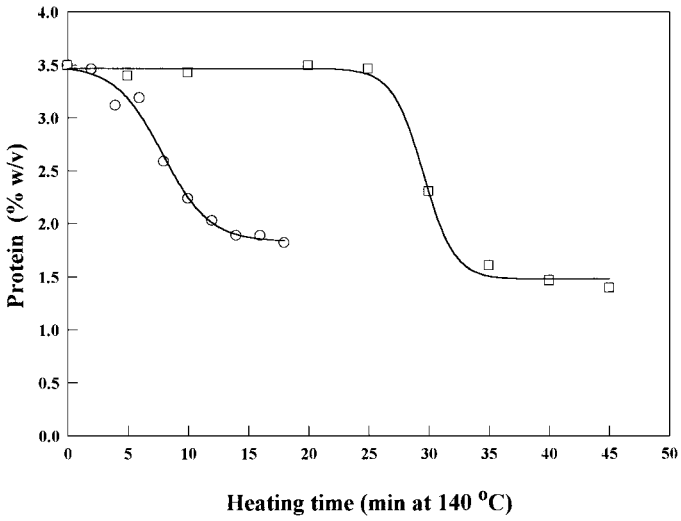

Figure 4. Protein depletion curves for milks, large (○) or small (□) casein micelles at pH 6.9.

480 nm, respectively. The average fluorescence of the control milk was assigned a value of one and the fluorescence of the modified milks are reported relative to the control.

Fast Protein Liquid Chromatography Analysis of Caseins

The protein profile of casein micelle fractions was determined by fast protein liquid chromatography, as described by Hollar et al. (12), except that after the sample had been incubated with β-mercaptoethanol at pH ~7.0, it was readjusted to pH 5.0 by dialysis against buffer A (6 M urea, 0.2 M acetate, pH 5.0) for ~24 h.

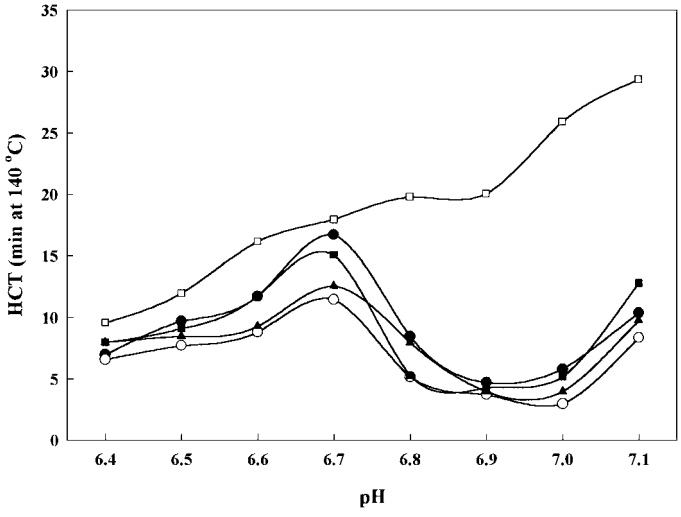

Figure 3. Effect of casein micelle size on the heat coagulation time (HCT)-pH profile of milk, control (●), large (○), intermediate-1 (▲), intermediate-2 (■) and small (□) micelles.

N-Acetyl Neuraminic Acid Content

The 12% trichloroacetic acid-insoluble N-acetyl neuraminic acid content of control and experimental milks was determined as an index of the degree of κ-CN glycosylation, by the method of Warren (37).

RESULTS AND DISCUSSION

In agreement with the results of Sweetsur and White (34), it was found that the coagulation of milk within the region of the objective heat stability minimum is a two-stage process (Figure 1 a–e). Figure 1f shows the subjective heat stability HCT-pH profile (closed diamonds), if coagulation times derived from the protein depletion curves (objective heat stability assay) were expressed as a HCT-pH profile (Figure 1f), it is found that coagulation times based on the first inflexion point (open squares), which denotes 'premature' coagulation, occurred at times corresponding to HCT as determined by the subjective heat stability assay, while coagulation times based on the second inflexion point (closed squares) coincided with coagulation times which would be expected if the minimum did not exist (Figure 1f).

As shown in Figure 2, differential centrifugation permitted the preparation of milks with four discrete micelle size distributions. The effect of altering the casein micelle size distribution on the HCT-pH profile, as determined by the subjective heat stability assay, is

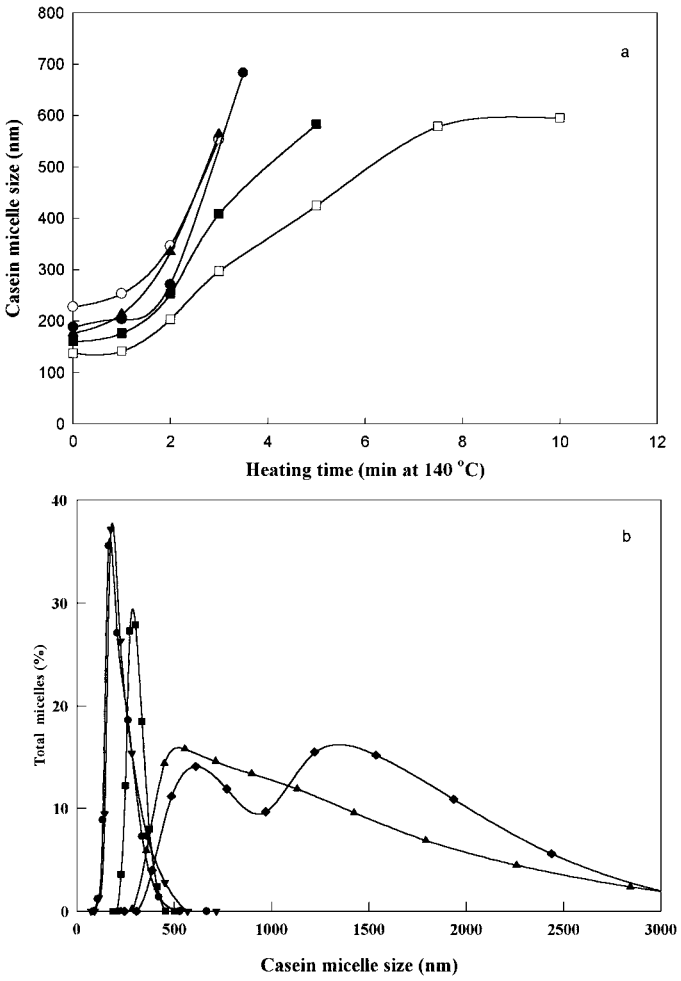

Figure 5. (a) Increase in the size of casein micelles in milks, with modified casein micelle size, as a function of heating; control (●), large (○), intermediate-1 (▲), intermediate-2 (■), small (□) micelles. (b) Casein micelle size distribution in control milk heated at 140°C at pH 6.9 for 0 (●), 1 (▼), 2 (■),3.5 (◆) or 4.5 min (▲). Note: the milk coagulated after 4.4 min.

shown in Figure 3. In agreement with results by Zbikowska et al. (38), alteration of casein micelle size distribution markedly affected heat stability at and around the pH of maximum stability (natural pH of milk). Tziboula et al. (35), who studied the effect of microfiltration with ceramic membranes on the heat stability of milk, found that the permeate which contained smaller casein micelles than the retentate, was more heat stable than the latter in the region of maximum stability.

The heat stability of milk within the region of the minimum was also affected by casein micelle size. Milks with an average casein micelle size larger than the control milk exhibited maximum/minimum behavior, while reducing the mean casein micelle size progressively increased HCT_{min}, the minimum was eliminated in the milk containing the smallest micelles (i.e., heat stability increased as a function of pH), and its HCT-pH profile was very similar to the HCT-pH profile of milk when determined by the objective heat stability assay and taking the second inflexion point within the region of the minimum (Figure 1f). Coagulation of milks with a more homogeneous micelle size distribution at the pH of the minimum, pH 6.9, was a single-stage process (Figure 4), which supports the theory proposed by Sweetsur and White (34).

Further support for the theory that the existence of the heat stability minimum is due to the premature coagulation of larger casein micelles was obtained by examining the increase in size of casein micelles that occurred with heating. Regardless of the initial value, the size of casein micelles increased on heating (Figure 5a). In the control milk at pH 6.9, casein micelle size increased on heating up to the coagulation point, ~4.4 min, and two distinct peaks in the size distribution profile were evident just before coagulation (Figure 5b). After coagulation, the micelles present in the supernatant were of a size similar to the micelles just prior to coagulation. When interpreted in conjunction with the data in Figure 4a, it appears that the larger casein micelles coagulated at ~4.4 min, while the size of the smaller micelles increased with heating.

Sweetsur and White (34) suggested that the smaller casein micelles coagulated at a time that would be expected if the minimum did not exist. This view was extended by Parker et al. (23), who proposed that there was little interaction between the coagulating species (large and small micelles). To test this hypothesis, milk at pH 6.9 was heated to coagulation (~6 min, as determined by the subjective heat stability assay). The coagulated milk was cooled to ambient temperature, centrifuged at 600 × *g* for 10 min at 20°C, and filtered through Whatman No. 1 filter paper. The filtrate was concentrated by ultrafiltration to 3.5%, wt/vol, protein and its HCT-pH profile determined by the subjective heat stability assay [$HCT_{total} = HCT_{(for\ second\ heating)}$ + duration of initial heating; this presumption is justified by the results of Darling (5)]. The heat stability of the concentrated supernatant did not differ greatly from that of the control milk up to the pH of the maximum (~pH 6.6) but was markedly more stable in the region of the minimum (pH 6.7 to 6.8; Figure 6). The HCT-pH profile of the concentrated supernatant also closely resembled the HCT-pH profile as determined by the objective heat stability assay and taking the second inflexion point within the region of the minimum (Figure 1f). However, it should be noted that the stability of the concentrated supernatant throughout the HCT-pH profile was enhanced because of alkalization of the sample by 0.3 pH units (i.e., upon initial heating, the pH of the milk had

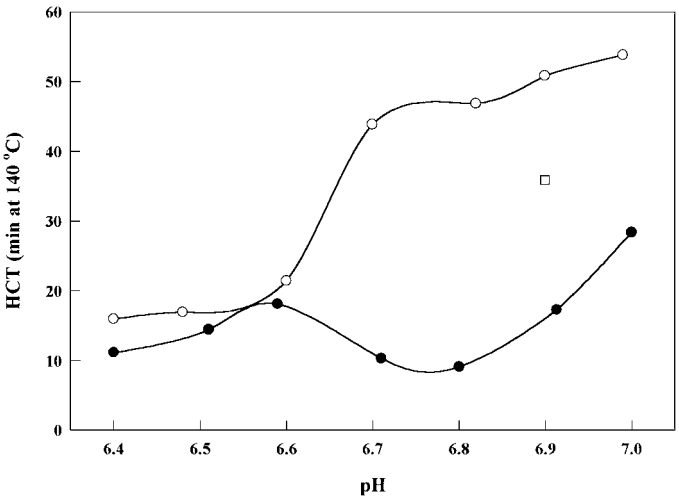

Figure 6. Heat coagulation time (HCT)-pH profile of skimmed milk (●) and concentrated supernatant from milk after coagulation at the HCT-pH minimum with the pH adjusted (○) and without pH adjustment (□) (see text for details).

decreased by 0.3 pH units and the pH of the concentrated supernatant was readjusted to values in the range of 6.3 to 7.2). The coagulation time for the concentrated supernatant at its unadjusted pH was ~35 min, a value consistent with the time for the secondary coagulation as determined from the protein depletion curves. The unconcentrated supernatant was very stable and did not coagulate within 1.5 h (possibly because of the low protein content).

Sweetsur and White (34) proposed that the larger micelles coagulated prematurely due to increased susceptibility to the adsorption of heat-precipitated calcium phosphate and that differences in the protein profile of casein micelles were of less importance. However, the fact that the ethanol stability, which is an index of the intrinsic stability of casein micelles to Ca^{2+}, of milks with differing casein micelle size did not differ (Table 1), suggests that, although precipitation of calcium phosphate is likely to play a role in the actual coagulation mechanism, the low stability of the larger casein micelles is not due directly to increased susceptibility to heat-induced precipitation of calcium phosphate.

The relationship between casein micelle size and protein profile was determined (Table 2) and was consistent with previous studies (19, 7, 38). The κ-CN content increased from 7.8 to 13.7% of total casein as casein micelle size decreased, which is in agreement with previous studies by McGann et al. (19) and Davies and Law (7). Data regarding the relationship between the content of α_s-CN and β-CN and casein micelle size (26, 27, 7, 20) conflict. The results obtained in this study were similar to those reported by McGann et al. (19) and Zbikowska et al. (38), with the α_s/β-CN ratio decreasing as the casein micelle size decreased. Schmidt and Koops (30) studied the heat stability of artificial casein micelles of varying casein profile, dispersed in buffer containing 340 mg of β-LG and 4.0 g of lactose/100 ml. When the ratio of α_{s1}-:β-:κ-CN in the micelles was 3:1:2, 3:2:2, or 2:3:2 there was no minimum in the HCT-pH profile (i.e., a type B HCT-pH profile resulted), while a system containing artificial casein micelles with a low κ-CN content, i.e., an α_{s1}-:β:κ-CN ratio of 1:3:0.25, showed a minimum in the HCT-pH profile (30). When considered in conjunction with the results of Schmidt and Koops (30), it appears that the increased stability of the smaller casein micelles is most likely related to their high κ-CN content.

According to Slattery's model of casein micelle structure, the micelles continue to grow by the assembly of submicelles until the micellar surface is occupied predominantly by κ-CN (28, 29, 31, 33; for a detailed discussion on the location of κ-CN on the casein micelle surface, see 13). Because the caseins remain in colloidal dispersion as micelles on heating at elevated temperatures (11, 36; i.e., the micelles do not disintegrate, as previously suggested, see 11) and since the κ-CN content per unit surface area is approximately similar regardless of micelle size (33), the increased susceptibility of the larger casein micelles to heat-induced coagulation is difficult to explain. On heating within the pH range of minimum stability, dissociation of κ-CN-rich protein from the casein micelles occurs (2, 16, 32) and the κ-CN-

Table 1. Physicochemical properties of casein micelles as a function of casein micelle size.

System	Ethanol stability at pH 6.6 (v/v)	Relative hydrophobicity	Zeta potential (mV)	Glycosylation of κ-casein (range μg of NANA mg^{-1} casein)
Control[1]	74.4 ± 6.2[2]	1.000	−17.2 ± 0.8	27.74—32.41
Large	72.5 ± 1.8	1.147	−15.2 ± 2.8	35.45—36.21
Intermediate-1	72.5 ± 2.7	1.058	−15.4 ± 0.2	28.88—30.63
Intermediate-2[1]	72.5 ± 3.5	1.024	−17.4 ± 0.2	26.00—30.87
Small	70.0 ± 3.5	0.965	−15.4 ± 0.6	24.88—27.87

[1]Note similarities between control and Intermediate-2.

[2](Level ± SD, n = 3).

Table 2. Protein profile (% of total casein) as a function of casein micelle size.

Micelle size	α-CN		β-CN		κ-CN		γ-CN		α/κ-CN ratio	α/β-CN ratio
	Average	Range	Average	Range	Average	Range	Average	Range		
Control	53.6 ± 4.1[1]	50.6 to 58.2	36.1 ± 4.6	30.8 to 39.0	9.78 ± 0.3	9.45 to 10.0	0.7 ± 0.3	0.5 to 0.8	5.35	1.41
Large	53.6 ± 3.6	49.7 to 54.3	38.0 ± 3.7	35.44 to 42.3	7.8 ± 0.1	7.6 to 7.86	1.3 ± 0.9	0.4 to 2.1	6.90	1.41
Intermediate-1	52.7 ± 4.6	49.9 to 57.9	36.7 ± 3.2	33.4 to 39.8	9.6 ± 1.0	9.65 to 10.7	1.25 ± 0.4	0.9 to 1.6	5.49	1.43
Intermediate-2	50.2 ± 3.0	47.2 to 53.8	38.2 ± 2.7	35.5 to 41.0	11.3 ± 1.0	10.4 to 12.4	0.7 ± 0.7	0.4 to 1.0	4.44	1.31
Small	47.7 ± 0.7	47 to 48.2	38.2 ± 1.4	37 to 39.8	13.74 ± .5	13.2 to 14.2	0.8 ± 0.5	0.3 to 1.3	3.47	1.25
Soluble	30.7 ± 11.73	22.4 to 39	32.8 ± 3.1	30.6 to 35	21.45 ± 3.6	18.9 to 24	2.8 ± 0.1	2.8 to 2.8	1.43	0.94

[1](Level ± SD, n = 3).

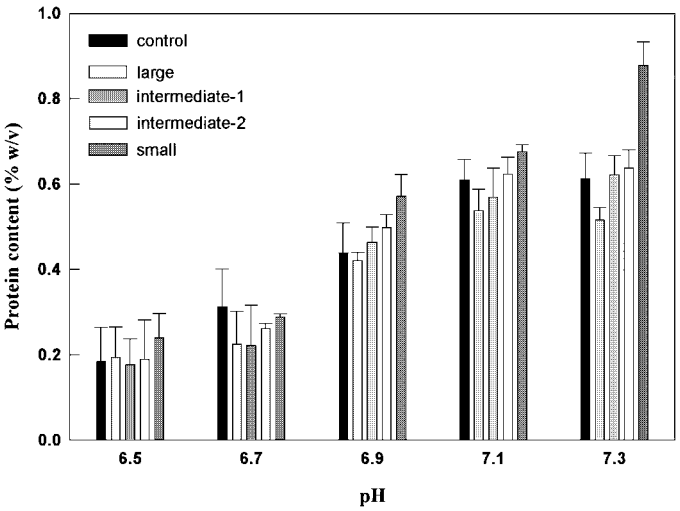

Figure 7. Protein content of ultracentrifugal supernatants from heated milks (120°C for 10 min) with modified casein micelle size distribution.

depleted micelles coagulate because of calcium-induced precipitation (16, 32). The extent of heat-induced dissociation of protein in milks increased with decreasing casein micelle size at pH values >6.7 (Figure 7), which is in agreement with a similar study on concentrated whey protein-free milk by Aoki and Kako (1). However, the results of Aoki and Kako (1) show that although there was an inverse relationship between casein micelle size and heat-induced dissociation of κ-CN, the smaller micelles still had a higher κ-CN content after heating than the larger micelles and, therefore, the former are likely to be more resistant to calcium-induced precipitation.

In addition to the stabilizing role of κ-CN for α_{s1}-, α_{s2}- and β-CN towards Ca^{2+}, a number of other physicochemical properties may be responsible for increasing the heat stability of κ-CN-rich micelles. Hydration, which plays an integral role in the stability of proteins, increased markedly as the size of the casein micelles decreased (Figure 8). In agreement with the level of hydration, there was a direct relationship between casein micelle size and surface hydrophobicity (Table 1). The hydration and hydrophobicity of differently sized casein micelles (or micelles differing in κ-CN content) are presumably related to the hydrophilic C-terminal of κ-CN. [It is likely that there is a delicate balance between casein micelle size and hydrophilicity because, although κ-CN content increases with decreasing micelle size, the extent of κ-CN glycosylation (which is a key determinant of protein hydrophilicity) increases as a function of casein micelle size.] It would be expected that the zeta potential of casein micelles would increase in parallel with κ-CN content. However, in agreement

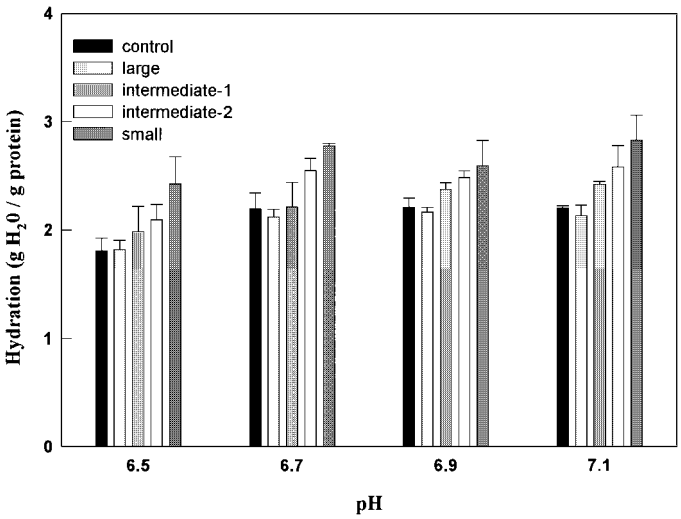

Figure 8. Hydration of sedimentable protein of heated milks (120°C for 10 min) with modified casein micelle size distribution.

with an earlier study (6), the zeta potential of casein micelles was not related to their size (Table 1). However, overall the HCT-pH profile of intermediate-2 is closest to the control (Figure 3) and their overall properties (Table 1) are also closest.

Holt and Horne (14) suggested that the two-stage coagulation of milk may be due to β-LG polymers forming crosslinks between the 'premature coagulating micelles'. Because glycosylation of κ-CN is known to enhance β-LG-κ-CN complex formation (9), the increased susceptibility of the larger casein micelles to β-LG-κ-CN complex formation may be related to the fact that a higher proportion of its κ-CN is glycosylated [Table 1, in agreement with previous studies (4, 17)]. Regardless of the actual coagulation mechanism, the fact that the larger casein micelles react preferentially with β-LG would be expected to make them inherently more susceptible to heat-induced coagulation within the pH range of the minimum (25). However, the heat stability of casein micelles in a serum protein-free casein micelle dispersion also increased as the micelle size was reduced (Figure 9), which suggests that the low heat stability of larger casein micelles is not due exclusively to β-LG-micelle interactions.

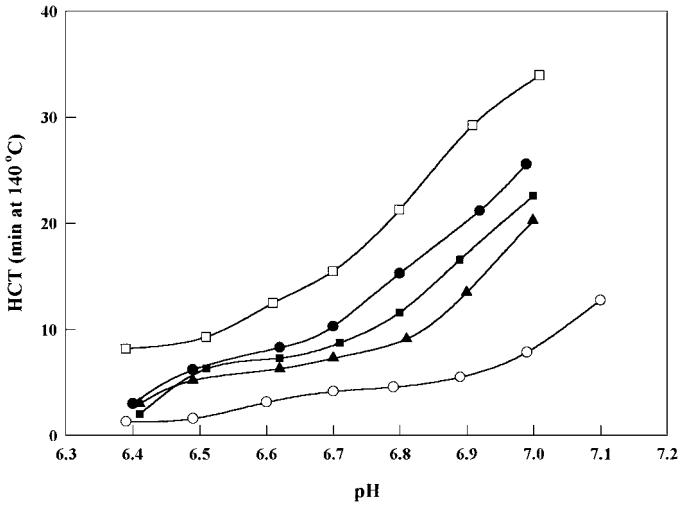

Figure 9. Effect of casein micelle size on the heat coagulation time (HCT)-pH profile of serum protein-free casein micelle dispersions; control (●), large (○), intermediate-1 (▲), intermediate-2 (■) and small (□).

CONCLUSION

From the data presented, it is concluded, in agreement with the theory proposed by Sweetsur and White (34), that the heat-induced coagulation of milk within the region of the minimum is a two-stage process due to the premature coagulation of large casein micelles, followed by the coagulation of smaller casein micelles at a time that would be expected if the minimum did not exist. It is proposed that the lower heat stability of the larger micelles is due to their low content of κ-CN, which makes them more susceptible to Ca^{2+}-induced precipitation. The high degree of κ-CN glycosylation in the larger micelles relative to the smaller micelles is also likely to enhance κ-CN-β-LG complex formation which also reduces stability in the HCT-pH profile minimum.

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