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Acid Gelation in Heated and Unheated Milks: Interactions between Serum Protein Complexes and the Surfaces of Casein Micelles

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The acid-induced interactions between different protein particles in milk (casein micelles and serum protein/κ-casein complexes) were studied in a series of different mixtures of heated and unheated proteins using diffusing wave spectroscopy (DWS) and small deformation rheology. The measurements were made as functions of pH during acidification by addition of glucono-δ-lactone (GDL). Heat treatment (85 °C, 10 min) affected the composition of the serum and the reactivity of casein micellar surface based on the pH at which the casein micelles aggregated during acidification. It was observed that the gel points as defined by DWS and rheology did not always coincide. The experiments showed that all systems containing heated serum proteins gelled at a higher pH than those containing unheated serum proteins. For systems containing heated micelles, an intermediate network can be formed between heat-induced aggregates of serum proteins and κ-casein formed at the surfaces of the micelles and dispersed as soluble complexes in the serum. This can explain the observation that DWS measurements detected aggregation of casein micelles at an earlier stage than did rheology. For systems containing unheated micelles and soluble complexes from heated milk, the results appear to be explained only by a direct interaction between soluble serum protein complexes and the casein micelles themselves, once the pH has decreased to below about 5.5. Comparison of the different systems studied gives a more complete description of the possible mechanism of interaction of the different protein materials during the acid-induced coagulation of milk-based systems.

KEYWORDS: Casein micelles; acidified milk; milk gelation; whey protein aggregates

INTRODUCTION

Milk is a colloidal suspension of highly hydrated protein particles, the casein micelles, dispersed in a serum that contains whey proteins (WP), lactose, and salts (*1*), and many of the uses of milk rely on the stability or controlled instability of this casein micellar system (*2*). Specifically, destabilization of the casein micelles by acid, using lactic acid bacteria or acidulants such as glucono-δ-lactone (GDL) to give a coagulum, is important in a number of milk products, such as yogurts, fresh cheeses, and cottage cheese (*3*). The acidification of milk results in several structural and compositional changes in the casein micelles, which lead to their aggregation and gelation at pH $\approx$ 4.9 (*4*, *5*).

In their native state, casein micelles are particles with an average diameter of about 200 nm containing several thousand individual casein molecules organized around nanodomains of inorganic calcium phosphate (*6*). The particle surface is dominated by one of the caseins, κ-casein, that appears to form an extended hairy layer that provides steric (*7*) and electrostatic stabilization to the particle (*8*). As the pH of milk is decreased from its natural value of 6.7, the inorganic micellar calcium phosphate is gradually dissolved until it becomes fully soluble at a pH near 5.2 (*9*, *10*). However, little dissociation of caseins from the micelle occurs if the acidification is performed at temperatures above 25 °C (*10*−*12*). As the pH decreases, the surface charges of the casein micelle are titrated, resulting in collapse of the κ-casein hairy layer, so that steric and electrostatic stabilization are diminished and at a pH of around 4.9 the casein micelles coagulate to form a gel (*13*, *14*). Several models of the mechanism of formation and structure of this particulate gel have been proposed, but none are completely satisfactory (*4*).

Heat treatment of milk at temperatures above about 70 °C causes changes in the structure and functional properties of the casein micelles as a result of the denaturation of the WP, which form complexes with themselves and with κ-casein. These complexes are located partly at the surfaces of the casein micelles and partly in the serum as small dispersed particles, the so-called "soluble complexes" (*15*−*19*). Acid gels obtained from heated milk have a higher pH of gelation and a higher gel strength and undergo less syneresis than do gels from unheated milk (*20*, *21*). In addition, an increased concentration of soluble complexes in the milk gives a greater gel strength (*22*−*24*).

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The presence of soluble complexes in milk modifies the aggregation of casein micelles, whether or not the latter have been heated (*20*, *25*). Therefore, any heat treatment that induces the denaturation of whey protein and formation of WP/κ-casein aggregates affects the acid gelation of casein micelles, and it has been shown that different types of WP aggregates produce different gel networks when they are acidified in the presence of casein micelles (*26*, *27*). Thus, it is clear that denatured and aggregated whey proteins have an effect on the acid gelation of casein micelles, but the mechanism of the effect is not well defined.

In a preliminary restricted study we used diffusing wave spectroscopy (DWS) and small deformation rheology to try to describe some features of the acid-induced interaction of the different protein components present in unheated and heated milks (*28*). The combination of the two techniques was used to obtain information on the early stages of gelation in the systems and formation of structure in the milk gels at different scales of observation. The results of that study suggested that in unheated milk the casein micelles mainly govern the gelation behavior of the systems whereas in heated milk both soluble complexes and casein micelles interact in specific ways to form the gels.

The present study is intended to confirm and expand on the earlier work and separate and better understand the contributions of casein micelles and serum protein complexes during the acid gelation process. This involved production of a number of reconstituted systems by mixing casein micellar and serum components separated from heated or unheated milks. The gelation processes in these systems were then studied using DWS and rheology. The main objective of the research is to understand which of the protein components (micellar casein or WP/κ-casein complexes) and which conditions (heated or unheated) affects the early stage of gelation (pH of onset of gelation) and how the different protein particles interact in these model systems. In addition to the presence of the WP/κ-casein complexes, the effect of increasing their concentration was studied to define whether it was simply their presence or their amount that was significant in increasing the gelling properties of the milk samples containing heated components.

MATERIALS AND METHODS

Milk. Fresh milk was obtained from the University of Guelph dairy farm and skimmed by centrifugation (6300 *g* for 20 min at 4 °C), followed by filtration through glass-fiber filters (Whatman GF-C, Fisher Scientific, Mississauga, Canada). Sodium azide (0.2 g kg^{-1}) was then added to prevent bacterial growth, and the milk was stored at 4 °C until required. Prior to each experiment, the milk was tempered at ambient temperature until it reached approximately 24−25 °C.

Heat Treatments and Acidification. Heated milks were prepared by placing 15 mL tubes of milk in a water bath at 85 °C, allowing 2−3 min for the samples to reach the final temperature, and then leaving for 10 min before removing and cooling rapidly to room temperature in an ice bath. The samples were stored for 1 h at ambient temperature after heat treatment before any further analysis. Acidification of the milk was by addition of glucono-δ-lactone (GDL, at concentrations of 1.0% and 1.5% w/w). GDL was added to the milk (or mixture, see below), which had been warmed to 30 °C, and agitated for 1 min to allow total dissolution. Samples for DWS or rheological measurements were taken and placed in the measuring equipment. The rest of the sample was used for monitoring of the pH, which was followed continuously until the pH decreased to below 4.8.

Isolation of Serum and Casein Micellar Fractions from Milk. Samples of milk (50 mL) were centrifuged at 48 000 *g* for 1 h at 20 °C in a Beckman Coulter Optima LE-80K ultracentrifuge with rotor type 45.1 Ti (Beckman Coulter Canada Inc., Mississauga, ON, Canada).

Table 1. Different Casein Micelle/Serum Mixtures Used in the Experiments

mixture	casein micelles from	serum from
UPUS	unheated milk	unheated milk
$UPUS_{conc}$	unheated milk	unheated milk and concentrated
UPHS	unheated milk	heated milk
$UPUS_{conc}$	unheated milk	heated milk and concentrated
HPUS	heated milk	unheated milk
$HPUS_{conc}$	heated milk	unheated milk and concentrated
HPHS	heated milk	heated milk
$HPHS_{conc}$	heated milk	heated milk and concentrated
UPUF	unheated milk	ultrafiltrate from UH milk
HPUF	heated milk	ultrafiltrate from UH milk

The serum material (supernatant) (40 mL) was carefully removed from the casein micelle material (pellet) and filtered through a 0.45 μm filter to remove any trace of fat. This contains the soluble WP/κ-casein complexes. To concentrate these components by a factor of 2, a centrifugal filtration device was used (Macrosep 10K Omega, Pall Corp.).

Reconstitution of Milks from Different Micellar and Serum Samples. Different mixtures of casein micelles and sera were reconstituted from the separated fractions (heated (H) or unheated (U) pellet (P) and H or U serum (S) concentrated ($_{conc}$) or not) by mixing the appropriate serum and micellar fractions using a high-shear mixer (Power Gen 125, Fisher Scientific) for 3 min at medium speed. This procedure had been shown to redisperse casein micelles that had been sedimented so as to give the original particle size. Tests had also shown that the treatment did not cause any denaturation of serum proteins. The reconstituted mixtures were then left overnight at 4 °C before any measurements were made. In total eight different reconstituted systems, as defined in **Table 1**, were created using the components of unheated and heated milks. Two other systems containing casein micelles were also reconstituted by resuspending the micelles from unheated or heated milks in ultrafiltrate. The latter was prepared by ultrafiltration of milk using a Prep-scale cartridge (Millipore CDUF001LG; Fisher Scientific, Mississauga, Ontario) and contains no serum proteins.

Analysis of Sera of Reconstituted Milks by Size-Exclusion Chromatography (SEC). To isolate the soluble complexes of casein and serum protein, the different reconstituted milk samples were centrifuged at 25 000*g* for 1 h at 20 °C in a Beckman Coulter Optima LE-80K ultracentrifuge with rotor type 70.1 Ti (Beckman Coulter Canada Inc., Mississauga, ON, Canada). The supernatants were removed from each centrifuge tube with a syringe and then filtered through a 0.45 μm cellulose nitrate filter (Millipore Corp., Bedford). They were stored at 4 °C and used for analysis within 3 days after preparation.

Samples (1 mL) of each supernatant were analyzed by size-exclusion chromatography (SEC) as described previously (*18*). The observed elution profiles were fitted and normalized with a routine of the Sigmaplot program (version 8.0, SPSS Inc., Chicago, IL) to create profiles based on a standard relative elution time scale (T_R) from 0 to 2.7, where $T_R = 1$ corresponded to the dead volume of the column. The composition of the proteins in the fractions was determined using a method described previously (*18*) using SDS-PAGE.

Diffusing Wave Spectroscopy (DWS). The DWS equipment used has been described elsewhere (*25*). Light from a laser (532 nm, 100 mW) was passed through a rectangular flat-faced silica cuvette with a path length $L = 5$ mm immersed in a tank of water maintained at a temperature of 30 °C. The transmitted light was detected and analyzed by photomultipliers and a correlator (FLEX2K-12x2, Bridgewater, NJ), which performed a cross-correlation analysis. With this geometry, the correlation function $g_1(t)$ can be described by

$$g_{(1)}(t) \approx \frac{\left(\frac{L}{l^*}+\frac{4}{3}\right)\sqrt{\frac{6t}{\tau}}}{\left(1+\frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l^*}\sqrt{\frac{6t}{\tau}}\right]+\frac{4}{3}\sqrt{\frac{6t}{\tau}}\cosh\left[\frac{L}{l^*}\sqrt{\frac{6t}{\tau}}\right]} \quad (1)$$

where $L \gg l^*$ (the photon transport mean free path) and $t \ll \tau$ (s) (the decay time) (*29*).

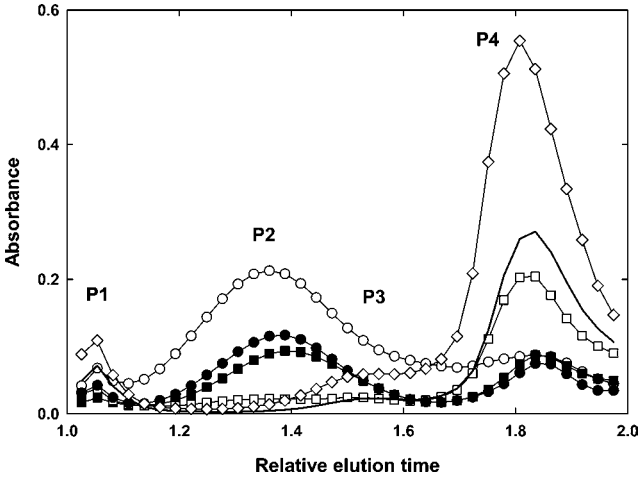


Figure 1. Elution profiles of sera of milk samples: $HPHS_{conc}$ (○), HPHS (●), HPUS (□), UPHS (■), UPUS (line), $UPUS_{conc}$ (◇). Peaks P1, P2, and P3 are described in the text.

The value of l^* was calculated from the total intensity of the scattered light from the sample and previous determination of the laser intensity using 269 nm diameter latex spheres (cat no. 3269A, Portland Duke Scientific, Palo Alto, CA) dispersed in water (*30*). This was used to give a best fit for τ from the experimentally measured correlation functions. Since $\tau = (Dk^2)^{-1}$, where $k = 2\pi n/\lambda$, the diffusion coefficient D ($m^2 \cdot s^{-1}$) was calculated using a value of 1.34 for n, the refractive index of milk serum. Apparent particle radii were calculated using the Stokes−Einstein relationship with the viscosity of milk serum taken to be 1.130 Pa·s (*31*). The mean square displacement (MSD) was calculated from the correlation functions according to eq 1, where $\langle \Delta r^2(\tau) \rangle$ (m^2) is calculated point by point in time using the relation

$$\langle \Delta r^2(\tau) \rangle = \frac{6}{k^2} \frac{t}{\tau} \quad (2)$$

Dynamic Rheological Measurements. Time sweep oscillatory measurements were performed at a frequency of 0.1 Hz with a stress of 0.02 Pa by use of a controlled-stress rheometer (AR 2000, TA instruments) equipped with a Peltier temperature controller with a Couette device consisting of two concentric cylinders of diameters 30 and 28 mm. After addition of GDL as described above, 10 mL of sample was then transferred in the geometry. A few milliliters of paraffin oil were poured on top of the sample to prevent evaporation. Experiments were run for 12 h at 30 °C, which allowed the sample to reach a stable pH.

Monitoring of pH. The pH of the mixtures was measured as a function of time with a pH meter AR15 (Fisher Scientific, Mississauga, Ontario). Different master curves of pH versus time were then calculated for the different reconstituted systems. These master curves were then used to plot DWS and rheological data as a function of pH. Error bars on the graphs show 2× the standard deviation from the average.

Replication of Experiments. In all of the description that follows, the results are the averages of at least three experiments.

RESULTS

Compositions of the Reconstituted Milks. A set of analyses was performed to confirm that the different reconstituted milks contained the appropriate materials and that no re-equilibration of material between serum and casein micelles had occurred. **Figure 1** shows the SEC chromatograms of sera of the milk samples UPUS and HPHS and sera of the reconstituted samples UPHS, HPUS, $UPUS_{conc}$, and $HPHS_{conc}$. The profiles of $UPHS_{conc}$ and $HPUS_{conc}$ were close to $HPHS_{conc}$ and $UPUS_{conc}$, respectively, and are not shown; the similarity shows, however, that the separation and reconstitution of the casein micellar particles did not cause loss of casein into the serum nor, conversely, was there loss of serum components by binding to the casein micelles during the reconstitution process. A dynamic light scattering experiment was performed to confirm that there was no significant change in the average micellar sizes between the original and reconstituted systems.

The chromatograms showed four peaks identified in the figure as P1−P4, of which only three contain protein (*18*). Peak P2, eluting at a relative elution time (T_R) of 1.3−1.5, contains the soluble complexes composed of whey proteins (WP) and κ-casein, created during the heating of the milk. Peak P3, eluting at $T_R = 1.6$ in some of the profiles, contains small quantities of caseins, significant amounts of β-lactoglobulin (β-lg), and traces of α-lactalbumin (α-lac); peak P4, eluting at $T_R = 1.8$, contains the native WP.

In the samples containing US, the SEC profile contained only P3 and P4. The sample made with HS, however, showed a decrease in the area of P4 because heating causes denaturation of the WP (loss of P4) and formation of soluble complexes between denatured WP and κ-casein (*16*, *18*) (increase in P2). In the mixed samples based on UPHS and HPUS, the protein material present in the serum was close to that present in the sera of heated and unheated milk, respectively, indicating that little material was exchanged between serum and micelles in the course of the different mixing experiments. In the sera of reconstituted milk samples derived from concentrated sera, the amounts of native whey protein for systems containing US_{conc} and the amounts of soluble complex for systems containing HS_{conc} were approximately doubled, as seen from the increased areas of P3 and P2, respectively.

The concentration of total protein in each sample was affected by production of these different mixed systems but from the known average composition of milk varied from approximately 2.5% (UPUS) to 4% (HPHSconc). In all of the mixtures, the concentration of casein micellar material was approximately constant because the total micellar fraction from milk was used in all mixtures; however, the WP concentrations were varied intentionally to evaluate the effect of the different protein fractions on the gelation process.

DWS and Rheology of Heated and Unheated Milks. In interpreting the results from DWS it must be remembered that in all of the systems it is the properties of the casein micelles that are being directly measured. This is because they are larger (diameter 220−230 nm in most of our experiments) than the whey proteins (<5 nm) or their complexes (∼30−50 nm; *17*, *18*, *22*) that are present in the different systems. They also constitute a volume fraction of about 10% in all of the mixtures. The volume fraction of the WP/κ-casein complexes can only be estimated because their extent of hydration is not known but is not likely to exceed 1.5% since their hydration will be smaller than that of casein micelles. In milk, the caseins constitute more than 80% of the protein. Therefore, the scattering of the casein micelles will dominate the behavior of the DWS. Even if the WP complexes aggregate together, their contribution to the overall light scattering will be smaller than that of the casein micelles. Thus, the behavior of the WP/κ-casein complexes can only be inferred from the way that they influence the behavior of the casein micelles. On the other hand, the rheology measurements depend on all of the particles present in the suspensions.

In the rheological data, we limit our discussion to the first step of aggregation. The final pH of the systems differs because it depends on the different protein concentration (and buffering effect). The gel strengths obtained at the end of the acidification

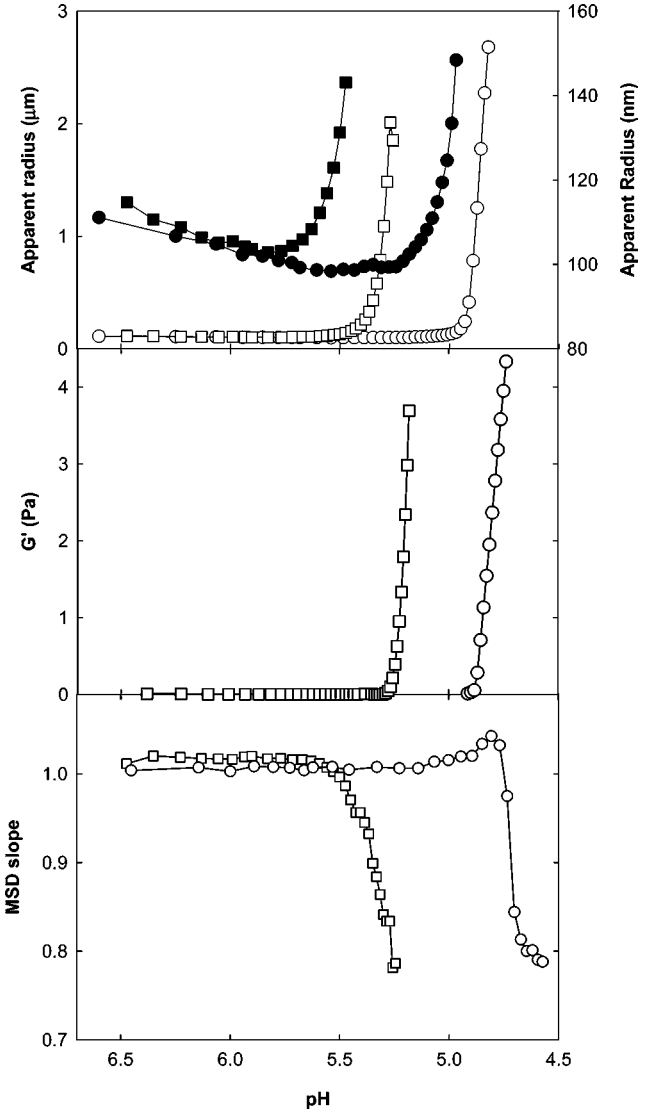


Figure 2. Changes in DWS parameters and rheology during acidification of unheated (○, ●) and heated (□, ■) milks: (top) apparent radius, plotted on large scale (○, □, and left-hand axis) and small scale (●, ■, and right-hand axis); (middle) elastic modulus G'; (bottom) slope of the MSD against time.

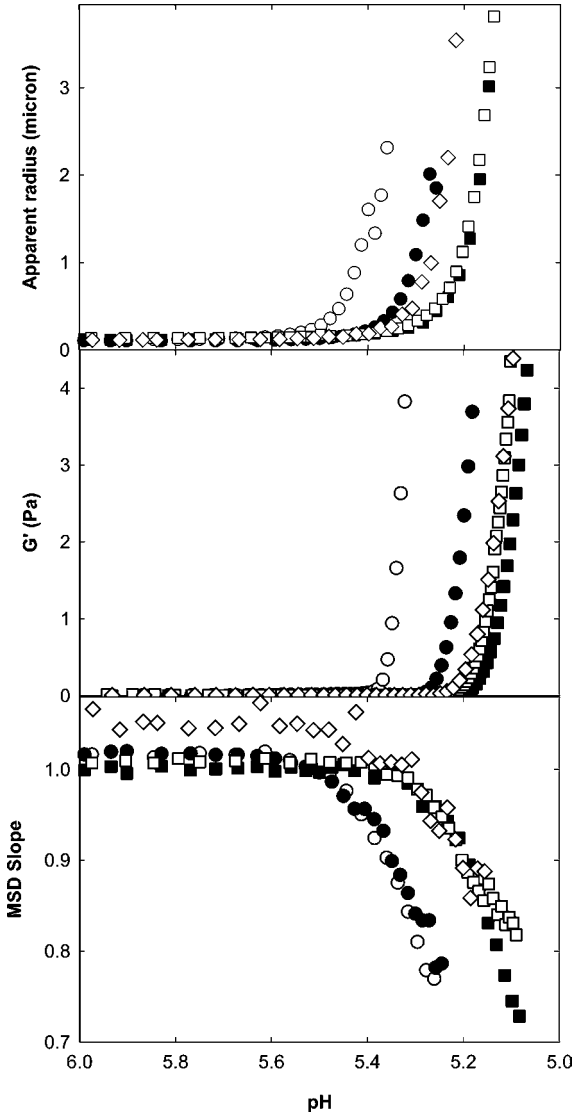


Figure 3. Changes in DWS parameters and rheology during acidification of reconstituted milks containing heated casein micelles (HP): (top) apparent radius; (middle) elastic modulus G'; (bottom) slope of the MSD against time. Symbols are as follows: (○) $HPHS_{conc}$, (●) HPHS, (□) $HPUS_{conc}$, (■) HPUS, (◇) HPUF.

process are therefore not directly comparable since some of the systems had not reached a stable value.

Previous studies have described the general behavior of UPUS and HPHS by DWS (*25*, *27*, *30*, *32*) and rheology (*5*, *20*, *21*). By combining the two methods, it is possible to observe the interactions between particles in the systems at different scales of observation. The behavior of the different parameters as a function of pH during acidification of UPUS and HPHS is shown in **Figure 2**. Although we published a preliminary description of the two systems (*28*), it is necessary to describe some aspects of these systems to allow comparison with the other mixtures of micelles and WP/κ-casein complexes.

As shown previously (*25*, *27*), in the early stages of acidification the radii of the casein micelles decrease in both heated and in unheated milks (**Figure 2**, top). This has been ascribed to collapse of the κ-casein "hairs" on the micellar surface (*13*, *14*). At a pH of about 5.5 for UPUS and 5.8 for HPHS, the radii show a minimum, after which they increase again, but relatively slowly, until pH about 4.9 for UPUS and 5.4 for HPHS, below which pH values there is a rapid increase in apparent particle size (*25*, *27*).

For UPUS, between pH 5.5 and 5.0 the apparent radii of the casein micelles increased only slowly with pH and, because the MSD slope remained close to a value of 1, the particles were freely diffusing. It is possible that this stage in the reaction may represent establishment of equilibrium between slightly aggregated and nonaggregated micelles (*13*, *28*). At a pH of 4.9 $\pm$ 0.07, the MSD slope began to decrease and the apparent radius increased rapidly; this can be taken as an indication that network formation is starting. It is possible to define a different critical pH for aggregation by extrapolating the plot of apparent radius against pH, which was 4.9 $\pm$ 0.07 for the UPUS mixture. At a different scale of observation, rheological measurements allowed definition of a third point of gelation, at the point where tan $\delta = 1$ and a sharp increase of G' was seen (**Figure 2**), i.e., the elastic behavior of the systems became dominant. In UPUS, this point was found to be at pH 4.9 $\pm$ 0.07. Thus, for the unheated system, all of the gel points, as defined by DWS and rheology, coincided.

In heated milk (HPHS), this was not the case. The rapid increase of the apparent particle radius and the decrease in the MSD slope occurred at higher pH values than in unheated milk

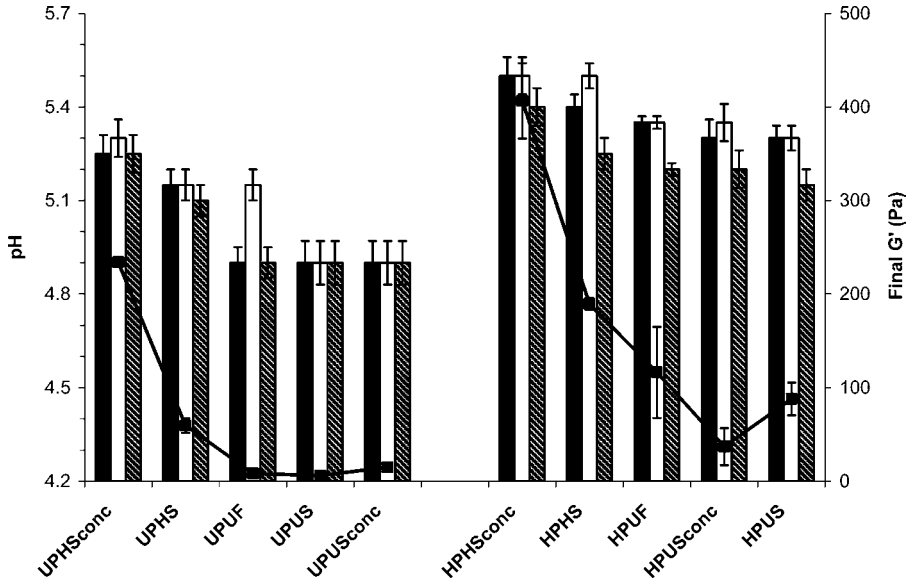


Figure 4. pH of aggregation defined by DWS and rheological measurements: divergence of the apparent radius (filled bars), onset of the decrease of MSD slope from 1 (open bars), and significant increase of G' (hatched bars). Details on the determination of the pH are given in the text. Error bars show 2× standard deviation of pH master curves from average. The points and connecting line and RH scale show the final G' values for the different gels formed.

and approximately coincided. The MSD slope began to decrease at pH 5.5 ± 0.06, and the rapid increase in apparent radius was about 0.1 pH unit lower. However, the gel point as defined by rheology was significantly lower than either (pH 5.25 ± 0.05).

The results confirm the well-known shift toward higher pH of gelation when milk is heated (*25*−*27*, *33*). We suggested that the coincidence of the aggregation point defined by DWS and rheology measurements in UPUS and their noncoincidence in HPHS can be explained on the basis of the behavior of the soluble whey protein complexes (*28*). In UPUS, the casein micelles are the only particles that can interact to form the gel, but in HPHS the presence of soluble complexes in the serum allows formation of "chains" of complexes linking the casein micelles, resulting in an intermediate network of aggregated soluble particles and casein micelles that is detectable by DWS as a decrease in the mobility of the casein micelles but too weak to be measured with the experimental set up of rheological measurements. Interaction between soluble complexes and HP would be possible via the whey protein complexes present on the surfaces of the modified casein micelles. In milk heated at its natural pH, it has been estimated that 20−40% of the whey proteins are bound to the casein micelles in this way (*15*, *18*, *19*, *34*, *35*). Once the preliminary network has been formed, further decrease in the pH of the milk allows aggregation of the micelles themselves and formation of a more rigid gel network is then detectable by rheology. A similar approach has been suggested on a model system of casein micelles mixed with complexes composed only of whey protein (*36*).

Systems Containing Casein Micelles from Heated Milk (HP). To further study the effects of the soluble whey protein complexes on the aggregation of casein micelles, different systems were prepared with HP as described previously. The micellar fraction from heated milk contains particles that have quantities of denatured serum protein adhering to their surfaces. It has already been suggested that these modified casein micelles have an increased isoelectric point so that they tend to precipitate at higher pH (*31*).

The change in apparent radius and MSD slopes and changes in elastic modulus during acidification of systems containing micelles from heated milk and different serum preparations are shown in **Figure 3**. Aggregation, as defined by both DWS and rheology, was always seen at a higher pH than for unheated milk and ranged from pH 5.5 to 5.1 depending on the particular mixtures. Average results for all of the pH of aggregation of all systems are given in **Figure 4**. In all cases, the pH values for aggregation measured by DWS were higher than those defined by rheology, although the difference depended on the system. The presence of increasing amounts of soluble complex ($HPHS_{conc}$) in the serum clearly induced gelation at higher pH.

The pH values of aggregation of the HP systems were lowest in the presence of US or US_{conc}, being about 5.3 ± 0.04 as measured by the decrease of MSD slope and the increase in apparent radius and pH 5.2 ± 0.05 by rheology. Thus, the casein micelles were more susceptible to acid gelation than those from unheated milk, where the pH of aggregation was around 4.9 (**Figure 2**). A possible reason for this shift could be that the casein micelles have denatured whey protein on their surfaces and could begin to interact at a higher pH than did native casein micelles. The unheated whey proteins in US and US_{conc} are not believed to interact with casein micelles, and it may be significant that in these systems the difference between DWS and rheology is the smallest. Also, increasing the concentration of undenatured whey protein by almost two times (**Figure 1**) was not significant in defining the gel point. Interestingly, the HPUF system, in which there were no free serum proteins, did not show exactly the same behavior as HPUS and $HPUS_{conc}$ but seemed to begin to gel at a slightly higher pH (**Figure 4**), and this result is still not explained. However, in this sample, the DWS and rheology still gave a significantly different pH of gelation.

In $HPHS_{conc}$, the MSD slope began to decrease and the apparent radius to increase at the same pH, 5.5 ± 0.6. These values are almost identical to the values shown by HPHS given the errors in the measurement of the pH shown in **Figure 4**. However, in this system the rheometer shows aggregation at pH 5.4 ± 0.6. The amount of soluble complexes present in the serum is approximately doubled in this system (**Figure 1**), and the possibility of interactions between casein micelles and soluble complexes will increase, so that any intermediate gel of soluble complexes linked with the micelles is likely to be

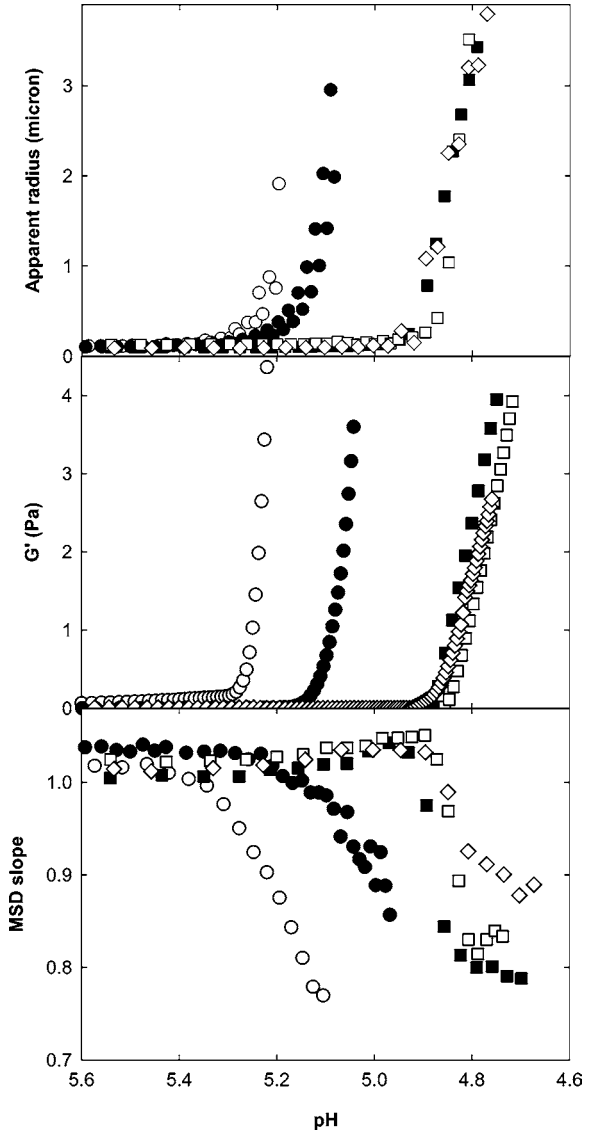


Figure 5. Changes in DWS parameters and rheology during acidification of reconstituted milks containing unheated casein micelles (UP): (top) apparent radius; (middle) elastic modulus *G*′; (bottom) slope of the MSD against time. Symbols are as follows: (○) UPHS$_{conc}$, (●) UPHS, (□) UPUS$_{conc}$, (■) UPUS, (◇) UPUF.

stronger than in the HPHS milk, and therefore, the differences between pH of aggregation defined by the two methods is reduced. In addition, the final gel strength of HPHS$_{conc}$ was higher than HPHS (**Figure 4**), which confirms results from the literature where the effect of concentration of soluble complex on mechanical properties on acid gel has been shown by varying the concentration of added whey proteins or changing the pH of heating (*23*, *26*, *34*).

Systems Containing Casein Micelles from Unheated Milk (UP). The results on the systems containing UP are presented in **Figure 5**, and different pH values of aggregation defined by the methods used are summarized in **Figure 4**. As the nature of materials present in the systems changed, so did the pH of aggregation. Casein micelles dispersed in ultrafiltrate, US, and US$_{conc}$ behaved similarly and did not differ significantly from the original UPUS system, showing that the undenatured whey proteins did not play a part in the aggregation. The results from DWS and rheology coincided for all of these mixtures at pH 4.9 ± 0.07. However, in the presence of normal or concentrated HS, the pH of gelation was increased to 5.1 ± 0.05 with HS and 5.2 ± 0.06 with HS$_{conc}$. That is, the increasing quantities of soluble aggregates of whey protein caused early gelation of the native casein micelles, although not as early as for heated casein micelles. In all of the systems based on UP there appeared to be no difference between the gelation pH values as defined by DWS and rheology (**Figure 4**).

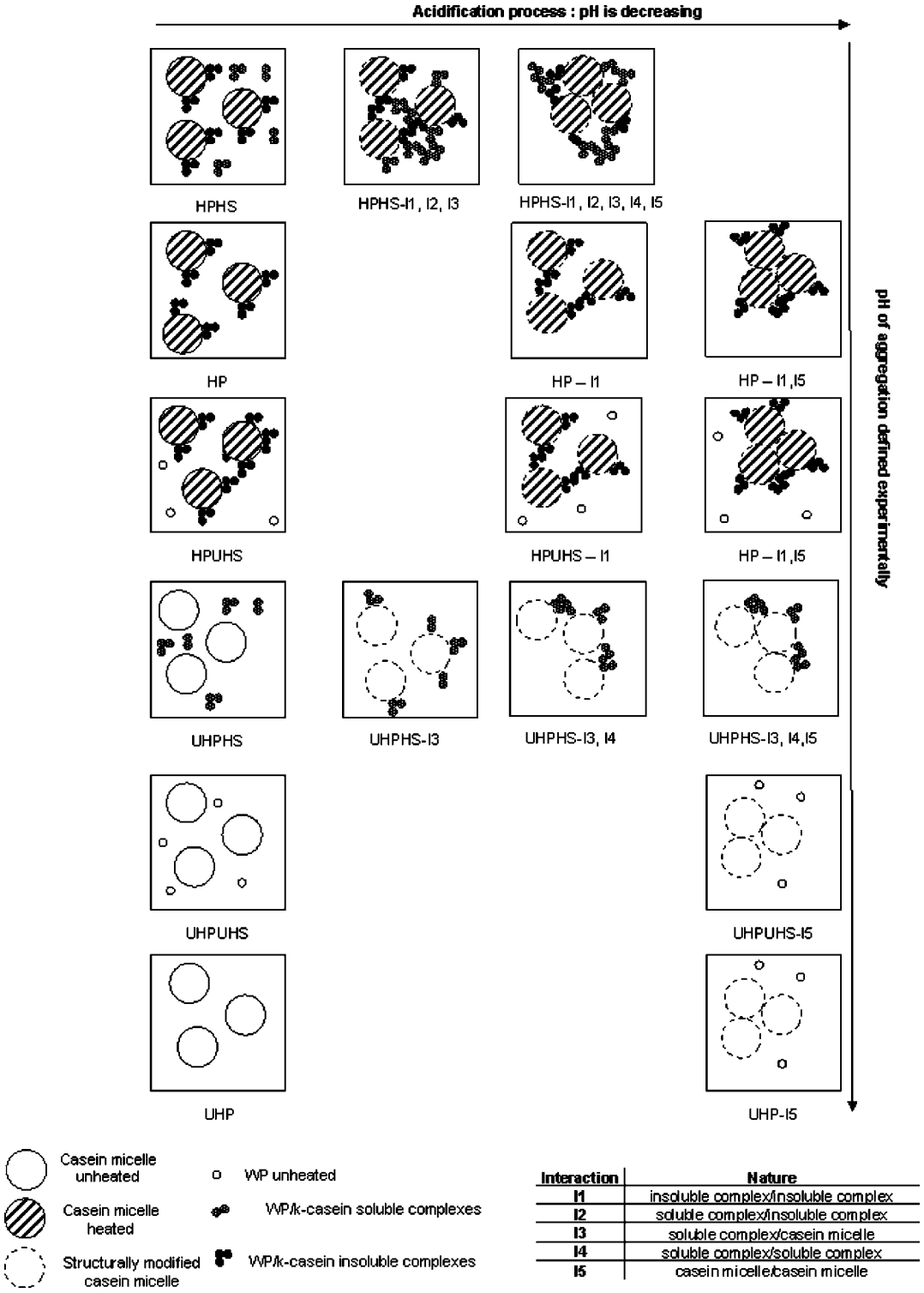
DISCUSSION

Our results (**Figure 4**) lead to a number of conclusions about the onset of gelation in different model systems where micellar casein or serum protein has been heated or not. Results from two different techniques provide a view at different scales of observation of the possible mechanisms of interaction that can occur within the protein materials during the earliest stage of gelation.

A number of conclusions can be drawn from these results. First, casein micelles derived from heated milk begin to aggregate at a higher pH than those derived from unheated milk, independently of whether the protein materials in the serum derived from heated milk. Second, the presence of heat-induced whey protein/κ-casein complexes causes casein micelles, from unheated or heated milks, to aggregate at a higher pH than they otherwise would. Finally, in systems containing casein micelles from heated milk, the pH of gelation defined by the rheological changes appears to lag behind that of the light-scattering changes measured by DWS.

Insofar as heated milk is concerned, these results either confirm or extend what has already been demonstrated. It is known that milks heated at different pH values contain different partition of the denatured whey proteins between the micelles and the soluble complexes and that the different composition is correlated with the strength of the gels formed when these milks are acidified (*24*, *37*). It is also known that the pH of gelation of heated milk depends on the extent of denaturation of the whey proteins (*27*), although the types of complexes present in these partially denatured systems have not been defined. Somewhat different complexes made by heating milk serum in the absence of micelles also give broadly similar results (*20*). Our results are in agreement with Lucey et al. (*21*) insofar as they show the importance of the whey proteins bound to the casein micelles (the fact that HPUF, HPUS, and HPUS$_{conc}$ all aggregate at a higher pH than the corresponding mixtures made using UP). However, we differ from these authors in that we find a significant effect of soluble complexes in the pH of aggregation of all systems containing HS, whereas they found only a small effect of the soluble complexes on the aggregation of casein micelles from heated milk.

In casein micelles from heated milk the denatured serum protein bound to the micellar surface is expected to increase the isoelectric point of the particles and cause reduction of the repulsive forces between the particles at pH values around 5.5 (*33*). It is also possible that the bound complexes will tend to reduce the steric stabilization of the κ-casein, partly because it is the site to which the whey proteins bind, but partly also because the whey protein complexes on the micellar surface, if they are as large as the ones in solution, will protrude through the hairy layer. The points of contact between the aggregating casein micelles in HPUF, HPUS, and HPUS$_{conc}$ are probably through the whey protein/κ-casein aggregates on the surface of the casein micelle. In the presence of soluble complexes (in HPHS and HPHS$_{conc}$) the contacts between the casein micelles in heated milk can be envisaged as arising from chains of single or aggregated complexes between the casein micelles. As argued before (*26*, *28*, *36*), these may, in their initial stages, affect the



Interaction	Nature
I1	insoluble complex/insoluble complex
I2	soluble complex/insoluble complex
I3	soluble complex/casein micelle
I4	soluble complex/soluble complex
I5	casein micelle/casein micelle

Figure 6. Schematic diagram of the different interactions occurring as systems are acidified. The different possible mechanisms are as follows: I1, interactions between insoluble complexes and insoluble complexes; I2, interactions between soluble complexes and insoluble complexes; I3, interactions between soluble complexes and casein micelles; I4, interactions between soluble complexes and soluble complexes interactions; I5, interactions between casein micelles and casein micelles.

motion of the casein micelles and an increased concentration ($HPHS_{conc}$) would give a stronger gel; this would explain the smaller lag between DWS and rheology in this system.

The effects of the soluble complexes on the aggregation of micelles from unheated milk are more difficult to explain. Lucey et al. (*21*) found the same trend with an increase in gel point and gel strength shown by rheology measurements but using complexes prepared in a different way. Our results show that soluble complexes provoke early gelation of the casein micelles, detectable by both DWS and rheology. This has been noted in some earlier publications in the rheology of similar mixtures (*33*) and by DWS (*25*), although there no mechanism was defined for the interaction either. Schorsch et al. (*25*) suggested that a filled gel can be formed by denatured whey proteins (not in a state similar to the soluble complexes here), aggregating and trapping the casein micelles. Vasbinder et al.

(*27*) have shown that the pH of first gelation is defined by the extent of denaturation of the whey protein. This is not completely analogous to our system because under their conditions there would have been some bound serum complexes on the micelles (*31*).

Our results suggest that direct interactions between denatured serum protein particles and native casein micelles may occur at pH values about 5.2 or below. At this point the hairy layer on the surfaces of the casein micelles has probably collapsed and the majority, if not all, of the calcium phosphate has been dissolved (*9*, *10*). The casein micelles therefore not only have lost much of their stabilizing influences, but may have some internal flexibility as a result of removing the calcium phosphate. Although caseins do not dissociate from the casein micelle at this pH as long as the temperature is above 25 °C, it is in this pH range that maximum dissociation of caseins occurs at lower temperatures (*11*). Studies of gelation at temperatures around 20 °C have shown that the micelles do appear to change their structures, presumably by dissociation and reassociation of caseins (*38*). The possibility of interaction of the casein micelles with other particles may be enhanced by this flexibility. We consider it probable, therefore, that the early coagulation of the unheated casein micelles is caused by the binding of the serum protein complexes to the acidified structurally modified casein micelles, possibly via electrostatic interactions, at pH around 5.4. The effect of higher surface hydrophobicity as suggested by Jean et al. (*17*) and Famelart et al. (*36*) can also be considered, but as these authors mentioned, measurement of surface hydrophobicity may be affected by the degree of aggregation. We found (Donato et al., unpublished results) that soluble aggregates start to aggregate at pH 5.5 when isolated from casein micelles. Therefore, at this pH the apparent hydrophobicity of soluble aggregates may be significant as a result of the decreasing charge.

As a general summary, a model of the acid gelation of the different systems studied is proposed in **Figure 6**. The components are the soluble complexes of whey protein and κ-casein, the native casein micelles, and the heated casein micelles with bound ("insoluble") whey protein complexes. The nature of the interactions is based on knowledge of the structure of the different protein materials as pH is decreased and the interactions discussed in this paper, although further work needs to be done to clarify the these assumptions more precisely.

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