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Microstructural Changes in Casein Micelles during Acidification of Skim Milk

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MICROSTRUCTURAL CHANGES IN CASEIN MICELLES DURING ACIDIFICATION OF SKIM MILK

by

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Hongwen Du

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ABBREVIATIONS

ANOVA	Analysis of variance
CCP	Colloidal calcium phosphate
df	Degrees of freedom
EDTA	Ethylenediamine tetraacetate
GDL	Glucono-delta-lactone
MS	Mean square
MW	Molecular weight
PCS	Photon correlation spectroscopy
SC	Starter culture
SDS	Sodium dodecyl sulphate
SV	Source of variation

ABSTRACT

Microstructural Changes in Casein Micelles
during Acidification of Skim Milk

by

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Pasteurized skim milk was acidified using glucono-δ-lactone (GDL) at 10, 20, 30, and 40°C or with 1.2% freeze-dried yogurt starter culture at 40°C. Milk coagulation was followed by measuring turbidity, curd firmness, particle size, and casein micelle microstructural changes using transmission electron microscopy.

The pH of milk was gradually lowered during acidification with GDL or starter culture. Acidification rate showed greater influence on turbidity change at 10°C than at 20, 30, or 40°C.

Average casein micelle size increased with decreasing temperature. The patterns of average micelle size versus pH were not affected by temperature. No great variation of average micelle size was observed above pH 5.2. Below pH 5.0 the size increased exponentially as the milk gelled. Acidification rate did not influence average micelle size at 10°C. Acidification rate, types of acidifying agents, and temperature had no effect on the Formagraph gelation pH and the rate at which curd firmness developed.

Casein micelles became less compact and less distinct with decreasing temperature before acidification. As pH was lowered, protein was dissociated from and

then reassociated with casein micelles. Acidification rate had no effect on microstructure change of casein micelles at 10°C.

(80 pages)

INTRODUCTION

Coagulation of milk is an association reaction of casein micelles involving the separation of dispersed casein particles from the continuous phase (7). Acid coagulation can be induced by lactic culture, hydrolysis of glucono-delta-lactone (GDL), or direct addition of acids such as hydrochloric and sulfuric acid. Direct addition of acids can initiate fast coagulation, while GDL and lactic cultures acidify milk gradually by forming gluconic acid and lactic acid.

Acid coagulation of milk is the foundation of many dairy products such as yogurt, cottage cheese, cream cheese, sour cream, and other cultured products. It is known that 30% of USA cottage cheese is produced using GDL as the acidifying agent (3). Some research papers about milk acid coagulation have been published; however, our knowledge about the physical and chemical changes during this process is still limited. A better understanding concerning acid coagulation of milk would lead to the development of a wide range of new dairy products.

LITERATURE REVIEW

Composition of Milk

Under physiological conditions milk contains about 87% water, 4.9% lactose, 3.5% protein, 3.6% fatty acids, and .7% minerals, but the composition varies among species and under different physical and physiological conditions. Casein micelles are the functional units in a milk system. The micelles consist of about 63% water, 7% inorganic compounds (mainly calcium and phosphate), and 30% proteins (40). About 93% of the dry matter of casein micelles is casein. The remainder is mainly inorganic calcium and phosphate, which is called colloidal calcium phosphate (54).

Caseins make up about 80% of bovine milk proteins. Most of them are found in micelles (6). The four main groups of caseins found in micelles are α_{S1}-, α_{S2}-, β-, and κ-casein. Their weight ratio is about 3:.8:3:1 (12, 54). A fifth group, commonly called γ-caseins (but more correctly referred to as β-casein fragments) comes from hydrolysis of β-casein. Almost all κ-casein is on the surface of the casein micelle, but α_{S1}- and β-casein can be found throughout the whole micelle (54). There is more κ-casein in smaller casein micelles (17, 22, 23), whereas, Davies and Law (22) observed that the amounts of α_{S2}- and β-casein decreased with decreasing micelle size, but α_{S1}- and γ-casein had little systematic change. Dalgleish et al. (17) found that both α_{S1}- and α_{S2}-casein contents were independent of micelle size.

Electron microscopy observation reveals that calcium is not homogeneously distributed through the casein micelle (37). Calcium and other ions can exist in many forms in milk (6). Soluble inorganic compounds of Ca can exist as free hydrated ions, or as complexes with citrate, phosphate, or various serum proteins. Colloidal Ca may be complexed with phosphate esters and carboxyl groups of micellar caseins, or the phosphate (and perhaps citrate) can be associated with casein micelles. Skim milk

contains about 32 m*M* Ca, of which 22 m*M* is colloidal, and 10 m*M* is soluble. The soluble calcium is approximately 3 m*M* free ionic Ca and 7 m*M* bound to citrate and phosphate. Of the 30 m*M* phosphate anions found in skim milk, 19 m*M* is colloidal, 6 m*M* is bound to calcium, and 5 m*M* is free inorganic phosphate. The Ca to P ratio in colloidal calcium phosphate (CCP) of casein micelles is 3:2. There is also 8.4 m*M* citrate in skim milk, of which .4 m*M* is in colloidal particles and the rest is in solution (6).

Changes Occurring during Acidification of Milk

It is known that acidification of milk causes micellar calcium phosphate and caseins to dissociate from the micelle (18, 51, 59). Acidification also causes changes in surface charge (ζ-potential) and particle size of casein micelles (7, 51, 64). The mechanism of these occurrences is unclear, and other factors are involved in this process.

Using electron microscopy, casein micelles have been observed to retain their integrity, shape, and dimensions when pH is decreased from 6.6 to 5.5 (28, 60). It is believed that the composition and structure of the micelles differ although their size and shape do not change (28).

Calcium and Phosphate. Studies of micelle dissociation in acidified milk at low temperature (30), and complexing of calcium by a chelating agent (30, 53) have verified that micellar calcium is essential for the structure and stability of casein micelles. Removal of CCP reduces the molecular weight of casein micelles from $>10^8$ to $2x10^6$ Dalton, and further to $2x10^5$ Dalton if the soluble milk salts are also removed (24), indicating dissociation of caseins from the micelle. Removing a subcritical amount of Ca^{2+} from the micelle by EDTA causes dissociation of β- and κ-casein, but a size-determining micellar framework of mainly α_s-casein remains intact (38). Munyua and Larsson-Raznikiewicz (42) found that removal of a considerable amount of Ca^{2+} by 3 m*M* EDTA did not change micellar size significantly, but only caused dissociation of some caseins. Complete dissociation of micelles occurs if the amount of Ca^{2+} removed is

above the critical level (38). The linear relationship between colloidal calcium and inorganic phosphate concentration resulting from the addition of EDTA (29) is statistically similar to the results obtained through acidifying milk (13). This suggests that the dissolution of micellar calcium is directly related to that of micellar phosphate.

The importance of colloidal inorganic phosphate in maintaining intact casein micelles is manifested when the phosphate depletion causes dissociation of caseins even when the free calcium ion concentration is equal to or greater than that in normal milk (30). In artificial casein micelles, Aoki et al. (1) observed that there was no intermolecular cross-linkage in a calcium caseinate micelle system, but CCP cross-linkage was present if phosphate were added. By dialysis, Holt et al. (30) found that removal of about 30% inorganic phosphate produced no significant casein dissociation. However, removal of a higher percentage of phosphate produced significant dissociation.

The amount of micellar calcium and phosphate is decreased as the pH of skim milk is lowered. A linear relationship exists between the decrease of calcium and inorganic phosphate in the pH range of 6.7 to 5.3, i.e., the decline of calcium is directly proportional to that of inorganic phosphate (59). Dalgleish and Law (19) observed a similar relationship between these two components at pH above 5.5. It is further observed that micellar calcium and phosphate ratios do not change over the pH range of 4.0 to 8.0, indicating that a linear relationship exists between the two components over this pH range (13). At 30°C all colloidal inorganic phosphate was solubilized after about 2 h at about pH 5.0 using 2% GDL as acidifying agent (28). All CCP is dissociated from casein micelles below pH 5 (44). At pH 5.0, most of the calcium is released from micelles, and almost all of it is removed between pH 6.0 and 5.0 (49). Visser et al. (60) reported that all calcium and almost all phosphate were solubilized at pH 5.2. Dalgleish and Law (19) observed, however, that 1 m*M* Ca was still sedimentable and nondiffusible at pH 4.9.

Caseins. Acidification of milk can result in the dissociation of caseins from casein micelles. As the pH of milk is lowered, the amounts of dissociated caseins increase initially, but decrease after reaching a maximum (18). Roefs et al. (51) reported a distinct maximum solubility of all casein fractions near pH 5.4 at 4°C. This dissociation (solubility) decreased to zero around pH 4.6. However, Dalgleish and Law (18) found that at 20°C the maximum dissociation was reached at pH 5.4, but maximum dissociation at 4°C occurred at pH 5.1. At 30°C, the maximum dissociation was at pH 5.6 (59).

Of the caseins, β-casein shows the greatest degree of dissociation (18, 51, 59). Between pH 5.5 and 5.2, most of the β-casein is in the serum rather than in the casein micelles. As pH is decreased further (from 5.2 to 4.8), the β-casein reabsorbs onto the relaxed framework of α_S-caseins (28).

van Hooydonk et al. (59) suggested that the dissociation of caseins probably occurs in the form of submicelles with outer-layer submicelles being more easily dissociated. Dalgleish and Law (18) had hypothesized that the dissociation of different caseins might be correlated, i.e., the dissociation of one kind of casein accompanies the dissociation of another, if the dissociation is via submicelles. It was found, however, that little correlation existed between the dissociation of α_{S1}-, β- and κ-caseins at 4°C. These three types of caseins dissociate independently. This might be due to their dissociation occurring independently of the formation of small complexes of constant composition. The same phenomenon was also observed by Downey (24).

Particle Size of Casein Micelles. Unlike other proteins, casein micelles have a large range in size distribution (5). The reason postulated is that casein micelles are primarily concentrated stores of minerals and proteins, and presumably no evolutionary pressure has been put on them to remain a constant size during solubilization and in the absence of flocculation.

The size of casein micelles ranges from 20 to 600 nm (54). Donnelly et al. (23) obtained a similar range (from about 30 to greater than 600 nm). It is reported that, under normal conditions, 80% of casein micelles have radii between 50 to 100 nm, 95% between 40 to 220 nm, with the most probable average being 80 nm (6). These results were obtained using inelastic light scattering, and the results are greater than those reported by other workers using electron microscopy. Griffin and Anderson (27) observed that the hydrodynamic diameters obtained by dynamic light scattering were 100 nm larger than those from electron microscopy. They attributed the difference to 1) micelle shrinkage during the dehydration process of sample preparation for electron microscopy producing lower than the true value; and 2) probable shifting to higher values through light scattering.

Roefs et al. (51) used light scattering and found that particle size did not change much with pH. As pH is lowered from 6.7 to 5.2, the average hydrodynamic diameter (315 nm) decreases gradually to the minimum (280 nm), then increases slightly to 285 nm if the pH is lowered to 4.8. Using a Malvern Autosizer III, Banon and Hardy (4) observed that average micelle size decreased slightly as pH was dropped to 5.7 at 42°C and to pH 5.5 at 30°C. The micelle size increased as pH was further lowered. However, they also found that average particle size remained constant above pH 5.75 at 15°C and pH 5.55 at 20°C, then decreased significantly. As pH was lowered to pH 5.4 at all temperatures, average micelle size increased as gelation approached. Roefs (50) and Vliet et al. (61) reported the particle size of casein micelles did not change much during acidification.

Casein micelles above pH 6 and below pH 5 are different particles and are held together by different bonds (51). Visser et al. (60) suggested that a size-determining micellar framework of α_{s1}-casein remained during acidification, even when all micellar calcium phosphate was depleted from the micelle at pH 5.1. Thus the size distribution of

casein micelles is relativly constant. Lin et al. (38) proposed that a size-determining micellar framework of mainly α_s-casein existed when a subcritical amount of Ca^{2+} was removed by EDTA. On the other hand, Vreeman et al. (62) considered that the reduction of pH from 6.7 to 5.6 decreased the number of smallest micelles with an increase in micelle porosity due to the increasing dissociation of caseins. This would result in light scattering measurements of the average size of particles remaining practically constant.

Zeta-Potential. The ζ-potential of native casein micelles is about -15 mV (28). It increases with increasing temperature (4). It is known that the minimum ζ-potential of casein micelles occurs as the pH of milk is lowered to pH 5.2 (8, 28). If pH is further reduced, ζ-potential increases until about pH 5.0, then decreases again. A similar change in ζ-potential was observed by Schmidt and Poll (55). They consider that the minimum ζ-potential occurring at pH 5.4 might be due to the specific adsorption of calcium ions to caseins. But Heertje et al. (28) suggested that ζ-potential change is related to the behavior of β-casein. Most β-casein is dissociated from micelles into serum between pH 5.5 and 5.2 and precipitates at its isoelectric point, pH 5.2. This causes the decrease of ζ-potential to the lowest point. Between pH 5.2 and 4.8, the β-casein with positive charge reabsorbs to the negatively charged α_s-casein framework; the new particles then start to aggregate and contract. This process leads to the increase and final decrease of ζ-potential. However, Darling and Dickson (21) observed that ζ-potential of casein micelles curvilinearly decreased directly with pH over the range of 6.9 to 5.4. Similar results were also obtained by Banon and Hardy (4).

Turbidity Change. Turbidity change as a function of time or pH during milk acidification by GDL at lower temperature (15 and 20°C) occurs in three phases: a lag phase followed by a decrease, and a final increase (3, 4). The lag phase is due to the fact that no changes occur to the size, concentration, or optical properties of micelles. The dissociation of β-casein and Ca causes the decrease of turbidity. The reincorporation of

dissociated caseins and gel network formation contribute to the final increase phase. However, at higher temperatures (30 and 42°C), only a lag period and a final rise are observed (4). The absence of the decreasing phase at 30 and 42°C is that there is no solubilization of micellar protein at temperatures greater than 25°C. When Bringe (7, 9) acidified diluted raw skim milk with GDL, he observed that turbidity at 400 nm (25°C) increases slowly at the beginning, and sharply in the final phase, without any decrease phase. From studies on sedimentation behavior of micelles after pH adjustment with HCl (49), no association or dissociation occurred between pH 6.6 and 5.5 because the turbidity of the supernatant did not change. A sharp decrease was observed between pH 5.5 and 5.0 due to precipitation of casein, and the decrease was less pronounced between pH 5.0 to 4.5. At pH 4.5, turbidity was negligible, indicating the completion of casein precipitation.

Factors Affecting Acid Coagulation of Milk

Acid coagulation of milk is affected by temperature, pH, preheat treatment, different types of anions and cations, rennet, and acidification rate. The combination of rennet and acidification, instead of acidification alone, results in a much wider pH and temperature range over which gels can be made (52).

Temperature is an important factor affecting milk acid coagulation. At higher temperatures during acidification, milk starts to coagulate at higher pH (7, 11, 28, 34). At lower temperatures more caseins are dissociated during acidification, and the maximum value of dissociation occurs at a lower pH value (18). The aggregation rate of casein micelles and the pH of maximum coagulation increase with increasing temperature (35).

Not only caseins, but also calcium and phosphorus dissociate from casein micelles upon cooling of milk (45, 47). Micellar calcium and phosphate decrease proportionally with decreasing temperature in the range of 4 to 90°C (46). Among the caseins, β-casein is especially temperature dependent with only monomers exiting at 4°C, but at higher

temperature it associates because of increased hydrophobic interactions. Its dissociation from casein micelles is considerably temperature dependent (22, 24, 43). At 0 or 4°C, β-casein in the serum is exchangeable with that in the micelle, and as temperature is raised to 37°C, the serum β-casein can stick on the micelle surface and move into the micelle interior (14). About 80% of the total casein released from casein micelles is β-casein. About 50% of β-casein, 5% of α_s-casein, and 20% of κ-casein are removed from micelles at 4°C (24). This results in the decrease of the negative charge on the micelle at natural pH of milk. On the other hand, Qvist (47) considered that casein micelles had increased charge at low temperature because the ratio of Ca to P dissociated during cooling was larger than that in micelles of uncooled milk. α_s-Casein is least sensitive to temperature (26, 43, 54), where only about 4% is solubilized from the micelle at 5°C (26). Green and Crutchfield (26) summarized three effects of cooling on casein micelles: 1) solubilizing caseins from micelles results in reduced micelle negative charge, 2) increasing pK values of ionizing groups (imidazole and phosphate) slightly decreases micelle negative charge, 3) inducing conformational change of β-casein increases micelle negative charge due to the exposure of previously masked aromatic residues. Some researchers (4, 16, 26) observed that ζ-potential of casein micelles decreases as temperature is lowered, while others (21, 25) showed the opposite.

Colloidal calcium phosphate is very important in maintaining the integrity of casein micelles. It is known that CCP is dissolved by cooling milk, but if temperature change is not severe, the original CCP level can be recovered on warming (2). Between 4°C and 64°C, the amount of colloidal calcium increases with increasing temperature (63). Dalgleish and Law (19), however, found that temperature has little effect on the composition of micellar calcium phosphate and its dissociation.

Temperature functions via hydrophobic interactions (9), and average particle size of casein micelles increases as temperature is lowered (4). As temperature decreases,

hydrophobic interactions are weakened, and coagulation tendency of casein micelles is diminished. Steric repulsion between proteins may also be affected by temperature (34) because at low temperatures, steric repulsion between proteins may increase as a result of protrusion of β-casein from micelles. On the other hand, higher temperature also increases collisions between particles with an increasing thermal (Brownian) motion. This weakens steric repulsion enough to result in micelle aggregation (4). Decreasing temperature also enhances electrostatic interactions (8).

Interactions Involved in Milk Acid Coagulation

Interactions occurring between casein molecules include hydrophobic and electrostatic interaction, hydrogen bonding, disulfide bonding, and calcium bonding (54). Schmidt (54) also proposed that the interaction between CCP and casein was electrostatic, CCP being positively charged and casein negatively charged. Casein micelles are stable under physiological conditions. This is due to the surface hydration, steric repulsion, a negative surface charge (28), as well as attractive Van der Waals forces (54). It is not clear whether steric repulsion of κ-casein or surface charge is more important in stabilizing the micelle (15). Changes of physical and chemical environment can alter the tendency of casein micelles to aggregate, precipitate, coagulate, or remain dispersed. Acidification of milk weakens repulsive forces and, consequently, facilitates hydrophobic interactions between casein micelles, resulting in their coagulation (9).

The different association behavior of the four caseins is due to their primary structure variation. Schmidt (54) suggested that the association of α_{s1}-casein is governed by electrostatic repulsion and attraction due to hydrophobic and hydrogen bonding. Electrostatic interaction between the positive tail of one molecule and the negative part of another plays the key role in the association of α_{s2}-casein. The character of β-casein association is similar to that of anionic detergents such as sodium dodecyl sulphate (SDS) due to its one negatively charged end and the other very hydrophobic end. Thus, the

critical micelle concentration of β-casein decreases with increasing temperature. This is the reason that β-casein is strongly temperature dependent. Electrostatic interaction is minor in the association of κ-casein because low charge is involved, whereas, steric repulsion or entropic repulsion of the macropeptide part is most important (54).

Interactions of κ-caseins and probably β-caseins on surfaces of adjacent casein micelles produce steric repulsion (31). The interpenetration of these protein molecules causes (a) osmotic effect due to the high concentration of molecular chains, and (b) volume restriction due to the loss of possible conformations. It is believed that this hairy layer (protruding κ-caseins) results in casein micelles having a high voluminosity. The voluminosity of casein micelles decreases as pH is lowered from 6.5 to 4.5. This indicates that the reduced steric repulsion may contribute to the coagulation of casein micelles at low pH. Roefs et al. (52) suggested that κ-casein exhibited a stabilizing effect at low pH by observing that gels could be made over a much wider pH (4.4 to 5.8) and temperature (as low as 2°C) range than by acidification alone. Banon and Hardy (4) proposed that the elimination of steric repulsion due to the collapse of the outer hairy layer was greatly responsible for the micelle aggregation during acidification. This phenomenon is also observed when milk is heat treated, or when rennet or ethanol is added.

Acid can reduce net negative charge of proteins to initiate protein-protein interactions (8). At high pH values most proteins have net negative charge. This causes long-range electrostatic repulsion and short-range hydration repulsion between protein molecules. As pH is lowered, amine groups of protein are protonated to their cationic form, and carboxyl groups to their nonionic form; thus, the strength and range of electrostatic repulsion are minimized.

Coagulation occurs when the pH of milk is lowered to the isoelectric point of casein micelles (about pH 4.6) at an appropriate temperature. This indicates that

electrostatic repulsion takes part in the stabilization of casein micelles in milk. At 5°C, milk does not coagulate at pH 4.6 (8, 10). This implies that other types of interactions are involved in the stability of casein micelles. Darling and Dickson (20) suggested that the stability of casein micelles was only partly due to electrostatic interactions and that other interactions, such as hydration, are involved.

Molecular interactions resulting from the entropy-driven removal of nonpolar side-chains from an aqueous environment are referred to as hydrophobic interactions. These are important in maintaining protein stability (12). Furthermore, Bloomfield (5) considered that these interactions were the primary cause of protein-protein interactions in aqueous solutions. Increase in temperature facilitates hydrophobic interactions. As temperature decreases, hydrophobic interactions are inhibited, and the tendency of protein association is diminished. Temperature affects hydrophobic interactions by altering the structure of water. At lower temperature, because water molecules are more ordered around protein molecules, this would decrease the entropy of water and lead to the increase of free energy; thus, protein molecules are more stable, and no coagulation occurs.

Although hydrophobic interactions play an important role in protein interactions, hydrogen bonding and ionic bonding are also involved. This is confirmed by the fact that the strength of the gel network, once formed, is greater at lower temperature because hydrogen and ionic bonds are facilitated (8).

Roefs (50) concluded that the relative importance of these interactions had not been understood, but the internal structure of casein micelles played an important role in determining the number of bonds between casein micelles. In contrast, Bloomfield (5) suggested that the role of electrostatic and hydrogen bonds in the stability of protein structure is minor, and that hydrophobic interactions contribute significantly. As for milk acidification by GDL, the elimination of steric repulsion is the most important

destabilizing factor, but the relative importance of charge neutralization, mineral and protein solubilization, and thermal motion cannot be determined (4).

OBJECTIVES

The objectives of the proposed study were

1) to determine the effect of temperature and acidification rate on the coagulation process of milk during acidification;

2) to determine microstructural change of casein micelles during milk acidification;

3) to develop a descriptive mechanism of acid coagulation of milk.

MATERIALS AND METHODS

A schematic diagram of this research is shown in Figure 1. Three replicates were performed under all experimental conditions.

Preparation of Milk

Raw whole milk was obtained from the Dairy Products Laboratory of Utah State University, centrifuged in a Sorvall® RC-5C centrifuge at 3000xg for 60 min at 4°C, and filtered with S/P glass fiber filter paper under suction. The milk was pasteurized in a water bath at 63°C for 30 min. If the milk was to be acidified with GDL, .02% (w/w) sodium azide (Mallinckrodt, Paris, KY), .01% chloramphenicol (Sigma Chemical Co., Louis, MO), and .01% benzylpenicillin potassium salt (Fluka Biochemika, Buchs, Switzerland) were added to inhibit bacteria growth. The milk was refrigerated before further measurement.

Acidification of Milk

GDL (Sigma Chemical Co., Louis, MO) was used as the acidifying agent to perform acid coagulation of milk at concentrations (w/w) of 2.0, 3.0, and 4.0% at 10°C; 2.0, 2.5, and 3.0% at 20 and 30°C; 1.5, 2.0, and 2.5% at 40°C. A 1.2% (w/w) mixed freeze-dried yogurt starter culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*, Marschall Products, Madison, WI) was added to some milk samples at 40°C to compare its acidifying behavior with that of GDL.

Turbidity

A Beckman DU-8B UV/Vis single beam spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) was used to measure turbidity change of milk during acidification at 600 nm (41).

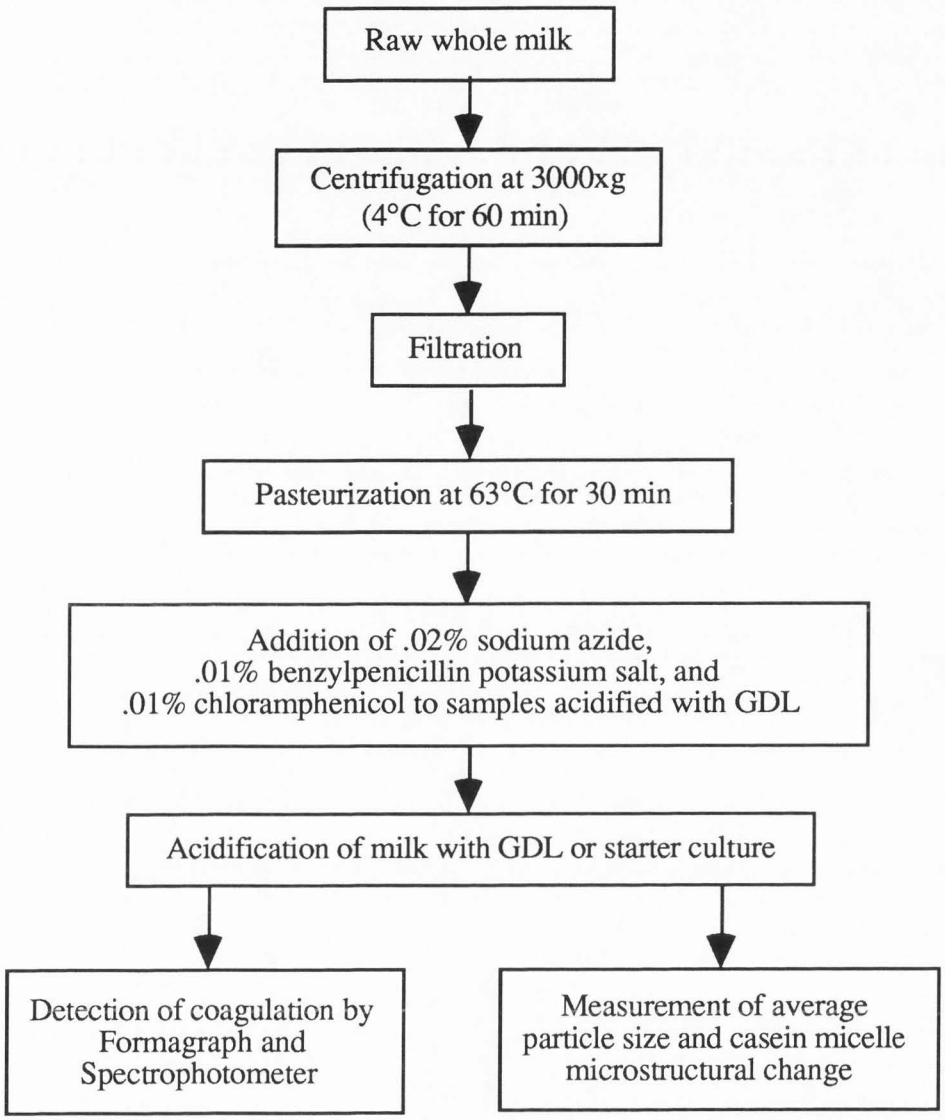

Figure 1. Schematic diagram of experiment design.

The refrigerated milk was put into a beaker and warmed to the corresponding temperature (10, 20, 30, or 40°C) for 30 min in a constant temperature water bath (Blue M Electric Company, Blue Island, IL) before GDL or starter culture was added. The spectrophotometer with cuvettes was also set to the same temperature as the milk sample for 30 min before measurement. The appropriate amount of GDL or starter culture was added to the milk in the beaker and mixed rapidly.

A 1-cm pathlength cuvette with untreated milk was placed in the spectrophotometer, and the absorbance reading was set to zero. A slit width of 5 nm was used. About 3 ml of the mixture of milk and GDL or starter culture was placed in two cuvettes in the spectrophotometer. One cuvette was used to measure changes of absorbance at 600 nm. Changes were recorded and transmitted via an RS 232 connection to a Tektronix 4052 microcomputer for derivatization and analysis. A microelectrode (Microelectrodes, Inc., Londonderry, NH) connected to a Beckman ϕ 34 pH meter (Beckman Instruments, INC., Fullerton, CA) was placed in the other cuvette to monitor pH changes of the acidified milk.

Formagraph

A Formagraph (Dicky-John Corp., Fishkill, NY) was used to detect gelation of milk during acidification (39). The refrigerated milk was put into a beaker and warmed to the corresponding temperature (10, 20, 30, or 40°C) for 30 min in the water bath before adding GDL or starter culture. The Formagraph with a cuvette was also set to the same temperature as the milk sample for 30 min before measurement. An appropriate amount of GDL or starter culture was added to the beaker of milk sample and mixed rapidly. The recorder module of the Formagraph was started when GDL or starter culture was added to the milk. About 10 ml of the milk was deposited in each sample well, and the cuvette was transferred to the recorder module. The milk coagulation process was monitored by the pendulum loops suspended in the milk, and light flashes reflected from the pendulum

mirrors were recorded on photographic paper. The coagulation point of the Formagraph was defined as the point where the baseline of firmness versus time diagram began to increase in width. pH changes of the milk samples were recorded using the microelectrode connected to the Beckman ϕ 34 pH meter in the water bath.

Measurement of Average Particle Size of Casein Micelles

Particle size of casein micelles was determined on selected samples (Table 1) (based on observations from the turbidity studies) using photon correlation spectroscopy (Malvern Instruments Inc., Southborough, MA). Modified Jenness-Koops buffer solutions (32, 59) with fortified calcium and phosphate concentrations (Table 2) and the same pH values as the acidified milk samples were used to dilute the milk sample 15 to 20 times when conducting particle size measurement.

TABLE 1. pH values at which samples were drawn for measurement of average particle size of casein micelles in milk acidified with glucono-δ-lactone (GDL) and freeze-dried yogurt starter culture (SC) at various temperatures.

10°C 2.0% GDL	10°C 3.0% GDL	10°C 4.0% GDL	20°C 2.5% GDL	30°C 2.0% GDL	40°C 1.5% GDL	40°C 1.2% SC.
Native pH	Native pH	Native pH	Native pH	Native pH	Native pH	Native pH
6.6	6.6	6.4	6.4	6.2	6.2	6.2
6.4	6.4	6.2	6.2	6.0	6.0	6.0
6.2	6.2	6.0	6.0	5.8	5.8	5.8
6.0	6.0	5.8	5.8	5.6	5.6	5.6
5.9	5.9	5.6	5.6	5.4	5.4	5.4
5.8	5.8	5.4	5.4	5.2	5.2	5.2
5.6	5.6	5.2	5.2	5.0	5.0	5.0
5.4	5.4	5.0	5.0			
5.3	5.3					
5.2	5.2					
5.0	5.0					

TABLE 2. Calcium and phosphate concentrations in the modified Jenness-Koops buffer solutions (32, 59) at individual pH values.

pH	Calcium (m*M*)	Phosphate (m*M*)
6.7	10.1	12.8
6.4	13.9	15.8
6.2	17.9	18.0
6.0	22.9	20.6
5.8	29.5	24.8
5.6	34.9	28.2
5.4	39.7	30.2
5.2	42.9	31.2
5.0	44.7	

Preparation of Milk Samples for Transmission Electron Microscopy

Milk samples were acidified with 2.0, 3.0, and 4.0% GDL at 10°C; 2.5% at 20°C; 2.0% at 30°C; 1.5% at 40°C; and 1.2% freeze-dried yogurt starter culture (SC) at 40°C. Samples with different pH values (Table 3) were taken, fixed with 50% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in the ratio of .2 ml glutaraldehyde to 5 ml milk for five min, then mixed with 3% Bacto-Agar (DIFCO Laboratories, Detroit, MI) in the ratio of 1:1. After the mixture solidified, the samples were cut into small strips and preserved in 2% glutaraldehyde solution, using .01 *M* phosphate buffer solution of pH 6.8. The fixed samples were refrigerated until further use.

The strips were cut into 1 mm^3 cubes, rinsed with .1 M phosphate buffer, and post-fixed in 2% osmium tetroxide. The cubes were dehydrated using an increasing concentration of graded ethanol (30%, 50%, 70%, 95%, and 100%). After dehydration, the samples were transitioned into propylene oxide and mixed in varying proportions ranging from 25% ethanol in propylene oxide to 100% propylene oxide alone in four steps. The cubes were infiltrated with a solution containing propylene oxide and epoxy resin (Electron Microscopy Sciences, Fort Washington, PA) stepwise in various

proportions ranging from 25% propylene oxide in epoxy resin to 100% epoxy resin. The infiltrated samples were embedded in Beem capsule (Electron Microscopy Sciences, Fort Washington, PA) and incubated at 45°C followed by 60°C for 24 h each. The hardened epoxy resin was removed from the Beem capsule, and excess epoxy was trimmed to expose the sample. Ultrathin sections were cut using an Ultracat E ultramicrotome (Leica, Inc., Gilroy, CA) and collected on 300 hex grids. Sections were post-stained with uranyl acetate and lead citrate and examined using a Zeiss CEM 902 transmission electron microscope (Zeiss Inc., Thornwood, NY) at 80 KV. Images were recorded on Kodak electron image film SO-163 (Eastman Kodak Co., Rochester, NY).

TABLE 3. pH values at which samples were taken for transmission electron microscopy in milk acidified with glucono-δ-lactone (GDL) and freeze-dried yogurt starter culture (SC) at various temperatures.

10°C 2.0% GDL	10°C 3.0% GDL	10°C 4.0% GDL	20°C 2.5% GDL	30°C 2.0% GDL	40°C 1.5% GDL	40°C 1.2% SC
6.94	6.94	6.94	6.68	6.64	6.58	6.58
5.90	5.90	5.90	5.70	5.60	5.50	5.40
5.30	5.30	5.30	5.20	5.30	5.20	5.00
5.00	5.00	5.00	5.00	5.00	4.95	4.75
4.80	4.80	4.80	4.80	4.80	4.80	4.65

Experimental Design

A split-plot experimental design was used in this study. Three replicates were performed under all experimental conditions. Analysis of variance (ANOVA) on average casein micelle size data was performed using Minitab.

RESULTS

Turbidity and pH

The pH of milk was gradually lowered upon addition of GDL due to its slow hydrolysis into gluconic acid. The acidifying behavior of GDL is shown in Figures 2, 4, 6, 8, and 10, along with the respective changes in turbidity in Figures 3, 5, 7, 9, and 11. The rate of pH drop slows down over time. As hydrolysis continues, the concentration of residual GDL decreases, and less gluconic acid is formed per unit time. At constant temperature, by increasing GDL concentration, pH is lowered more quickly. However, to compare the effect of temperature while maintaining comparable acidification rates, more GDL must be added at lower temperature because the hydrolysis rate of GDL is slower (Figure 2).

The relative turbidity changes of milk as a function of pH at similar acidification rates at 10, 20, 30, and 40°C are shown in Figure 3. At 10 and 20°C, as pH was lowered, turbidity increased slightly, then decreased, and finally increased sharply. The decrease was more pronounced at 10 than at 20°C. However, at 30 and 40°C, there was no decrease between the slight increase and the sharp increase, but I observed a slight increase below pH 4.9 at 30°C, and a decrease and a final stationary period following the sharp increase at 40°C. Table 4 shows that when the inflection point of the turbidity curve is taken as a measure of the point of gelation, gelation pH (pH_g) increased with increasing temperature, but acidification rates had no effect on pH_g.

The three curves of relative turbidity change in Figure 5 correspond to the acidification with 2.0, 3.0, and 4.0% GDL at 10°C shown in Figure 4. At all three acidification rates, the turbidity decreased as pH dropped below 6.0. This decline continued until about pH 5.35, at which point the turbidity was less than its initial value. The turbidity then increased abruptly below pH 5.35. There was a greater decrease at a

TABLE 4. Gelation pH (pH_g) as inflection point calculated from turbidity change at different acidification rates and temperatures.

Temperature (°C)	10	20	30	40
1.2% SC				5.01
1.5% GDL				5.20
2.0% GDL	4.99	4.99	5.09	5.18
2.5% GDL		4.98	5.02	5.21
3.0% GDL	4.94	4.96	5.03	
4.0% GDL	4.95			

lower acidification rate. At 20°C (Figure 7), the decrease was only slightly perceptible compared to that at 10°C (Figure 5). The turbidity declined slightly between pH 5.52 and 5.24, then increased greatly as pH was further lowered. Acidification rate had no significant effect on turbidity change at 20 and 30°C, as shown in Figures 7 and 9, respectively. The sharp increase in turbidity started at pH 5.27 followed by a stationary period at pH 4.79 at 30°C (Figure 9). At 30 and 40°C, there was no turbidity decrease between the slight and the sharp increase. At 40°C, the difference among the three turbidity curves of different acidification rates occurred below pH 5.0 (Figure 11). Unlike the turbidity curves at lower temperatures, there was a decrease in turbidity below pH 4.94 following the sharp increase. At higher GDL level, turbidity decreased more, and the stationary phase (below pH 4.8) was at a lower level.

The pH decreased to a similar value within the same time after addition of 1.5% GDL or 1.2% freeze-dried yogurt starter culture at 40°C (Figure 12). However, GDL caused a much faster pH drop above pH 5.0, and a slightly slower drop below pH 5.0 than SC. Similar relative turbidity change with pH was observed on GDL and starter culture (Figure 13). The sharp increase, the decrease, and the final stationary period all occurred at lower pH values with SC (pH 5.35, 4.75, and 4.65, respectively) than with GDL (pH 5.43, 4.94, and 4.78, respectively). Similar observation on pH_g was also obtained: 5.01 with SC and 5.20 with 1.5% GDL.

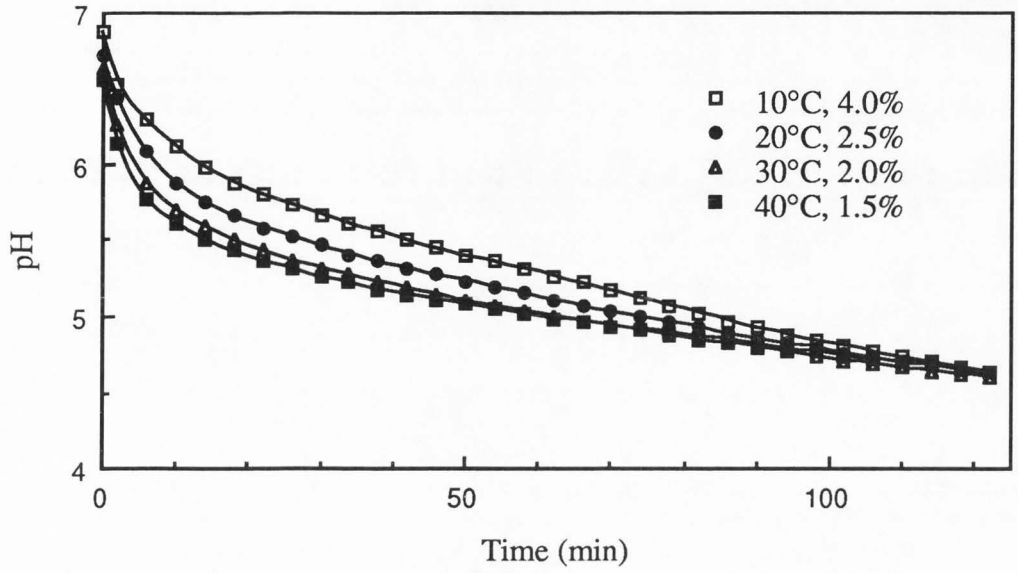

Figure 2. Acidification of milk after addition of 4.0%, 2.5%, 2.0%, and 1.5% glucono-δ-lactone at 10, 20, 30, and 40°C, respectively.

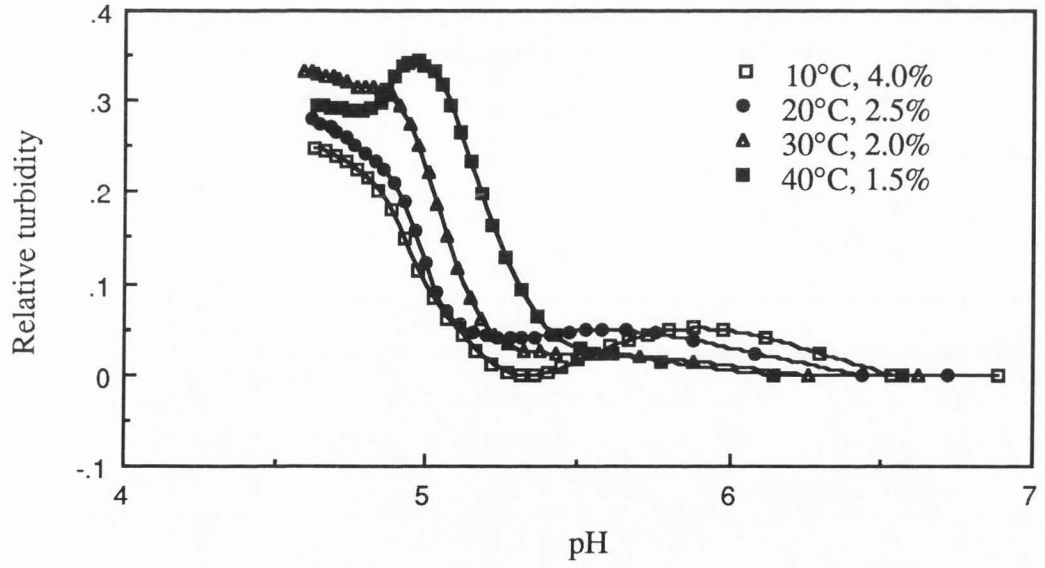

Figure 3. Relative turbidity change at 600 nm as a function of pH with the initial turbidity of milk as a reference after addition of 4.0%, 2.5%, 2.0%, and 1.5% glucono-δ-lactone at 10, 20, 30, and 40°C, respectively. Each line represents the average of three replicates.

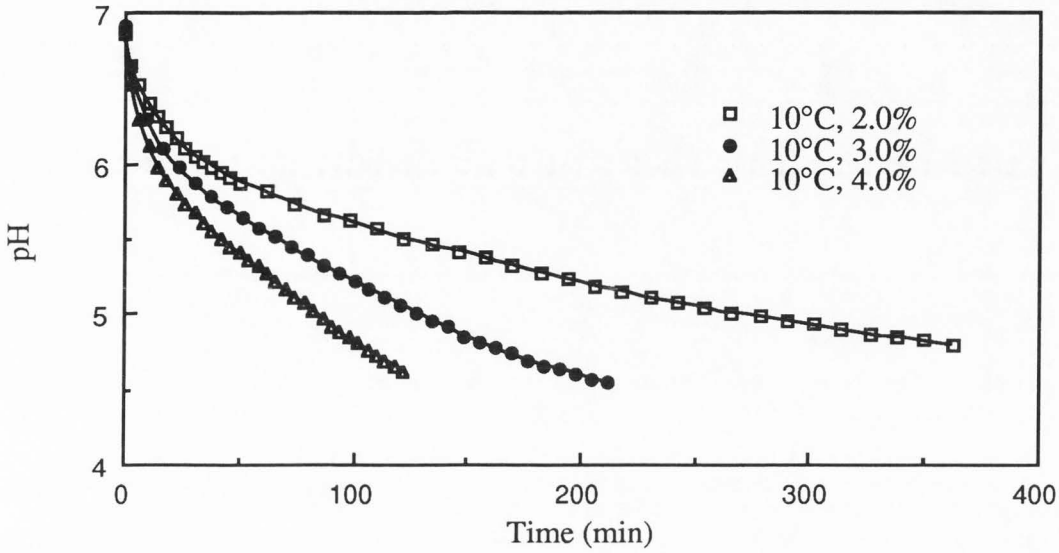

Figure 4. Acidification of milk after addition of 2.0%, 3.0%, and 4.0% glucono-δ-lactone at 10°C.

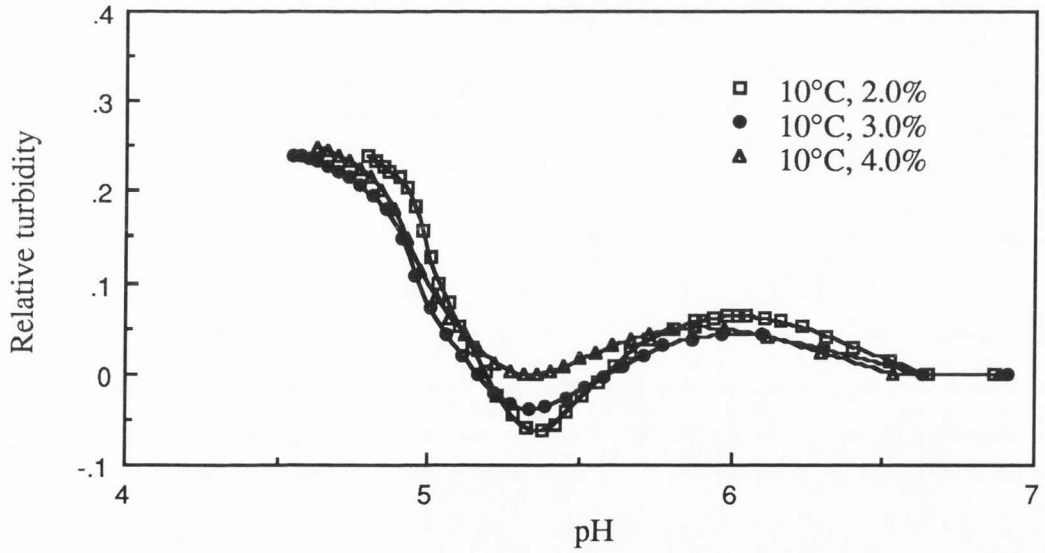

Figure 5. Relative turbidity change at 600 nm as a function of pH with the initial turbidity of milk as a reference after addition of 2.0%, 3.0%, and 4.0% glucono-δ-lactone at 10°C. Each line represents the average of three replicates.

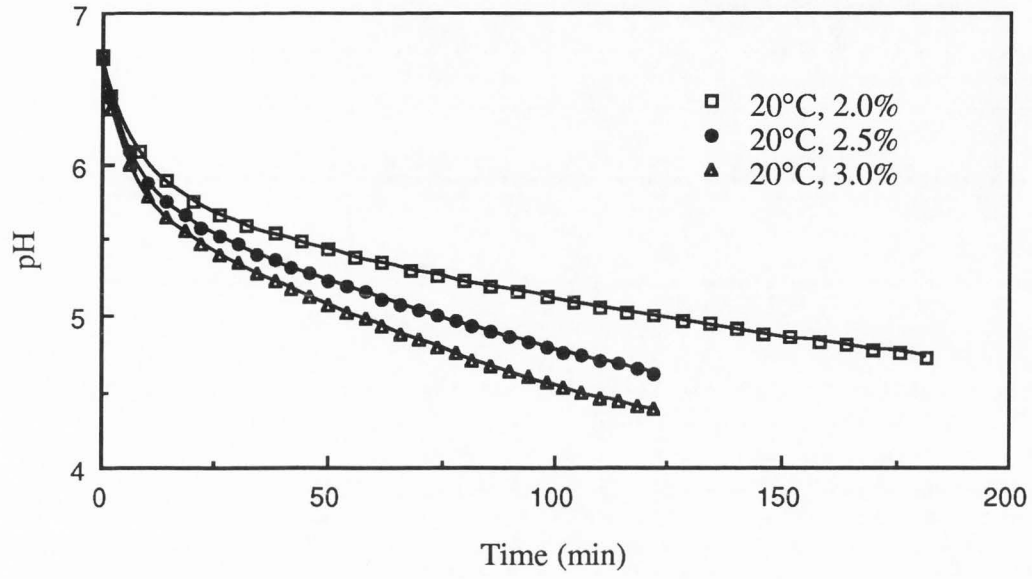

Figure 6. Acidification of milk after addition of 2.0%, 2.5%, and 3.0% glucono-δ-lactone at 20°C.

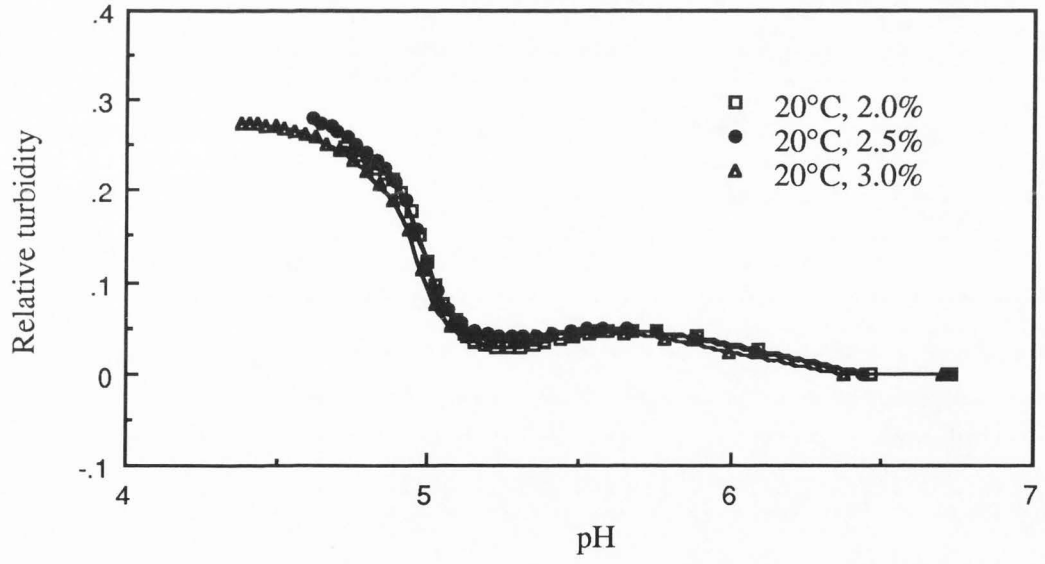

Figure 7. Relative turbidity change at 600 nm as a function of pH with the initial turbidity of milk as a reference after addition of 2.0%, 2.5%, and 3.0% glucono-δ-lactone at 20°C. Each line represents the average of three replicates.

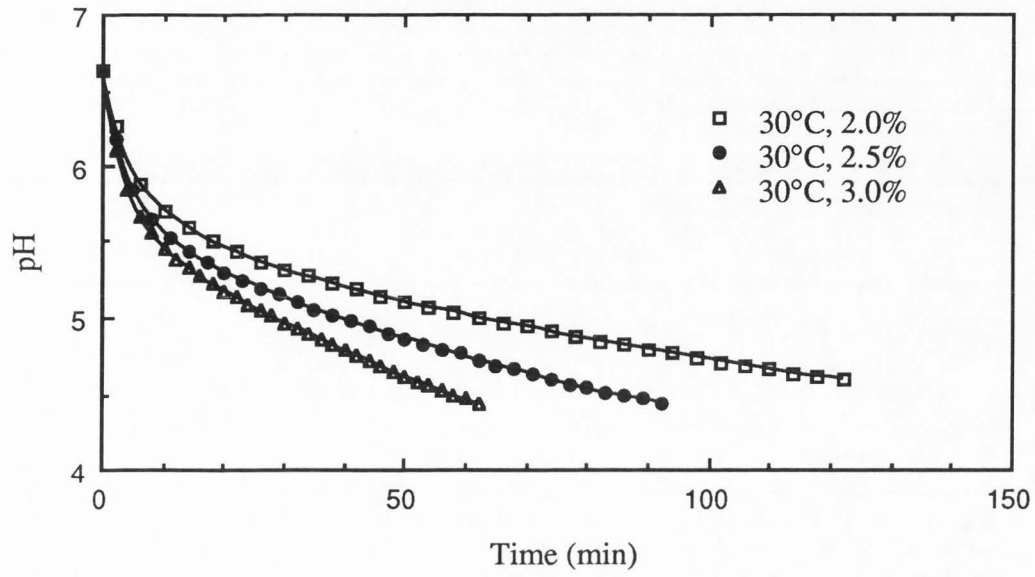

Figure 8. Acidification of milk after addition of 2.0%, 2.5%, and 3.0% glucono-δ-lactone at 30°C.

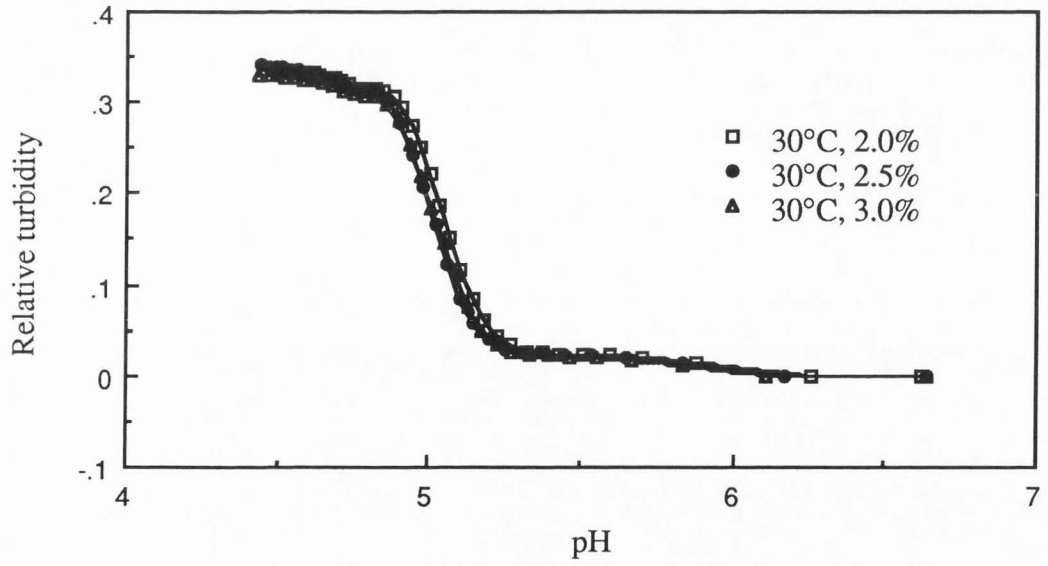

Figure 9. Relative turbidity change at 600 nm as a function of pH with the initial turbidity of milk as a reference after addition of 2.0%, 2.5%, and 3.0% glucono-δ-lactone at 30°C. Each line represents the average of three replicates.

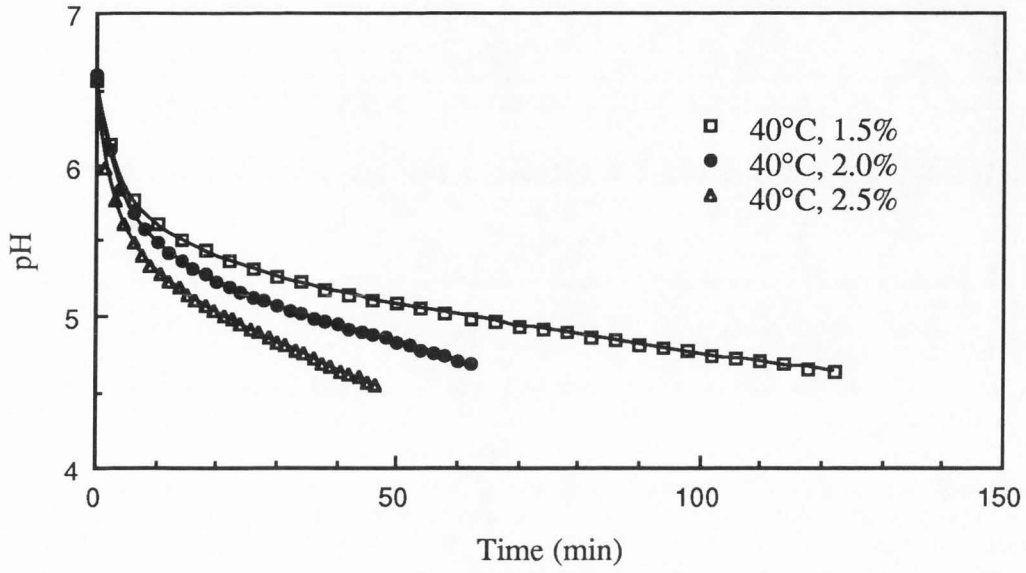

Figure 10. Acidification of milk after addition of 1.5%, 2.0%, and 2.5% glucono-δ-lactone at 40 °C.

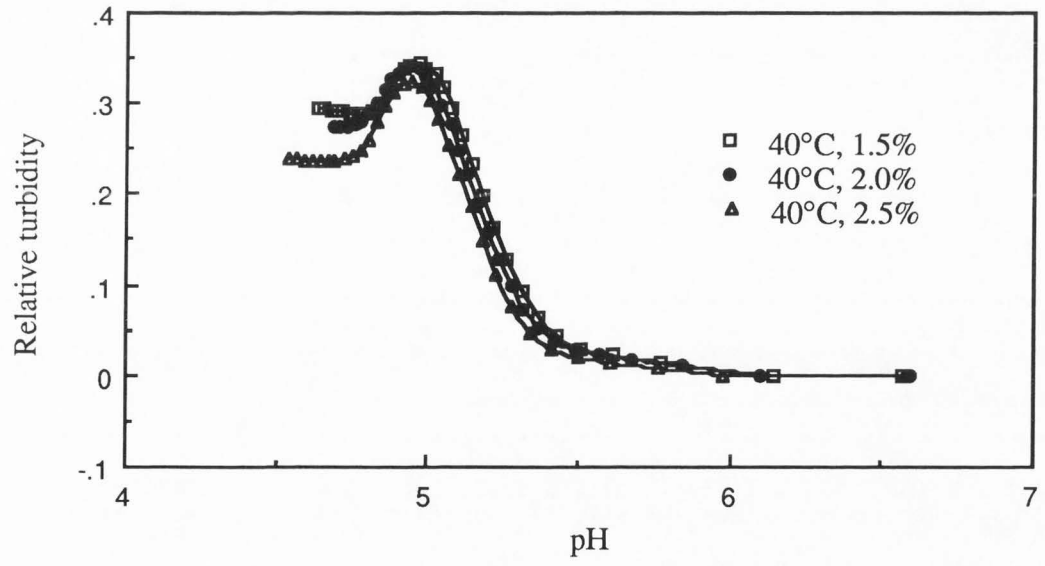

Figure 11. Relative turbidity change at 600 nm as a function of pH with the initial turbidity of milk as a reference after addition of 1.5%, 2.0%, and 2.5% glucono-δ-lactone at 40°C. Each line represents the average of three replicates.

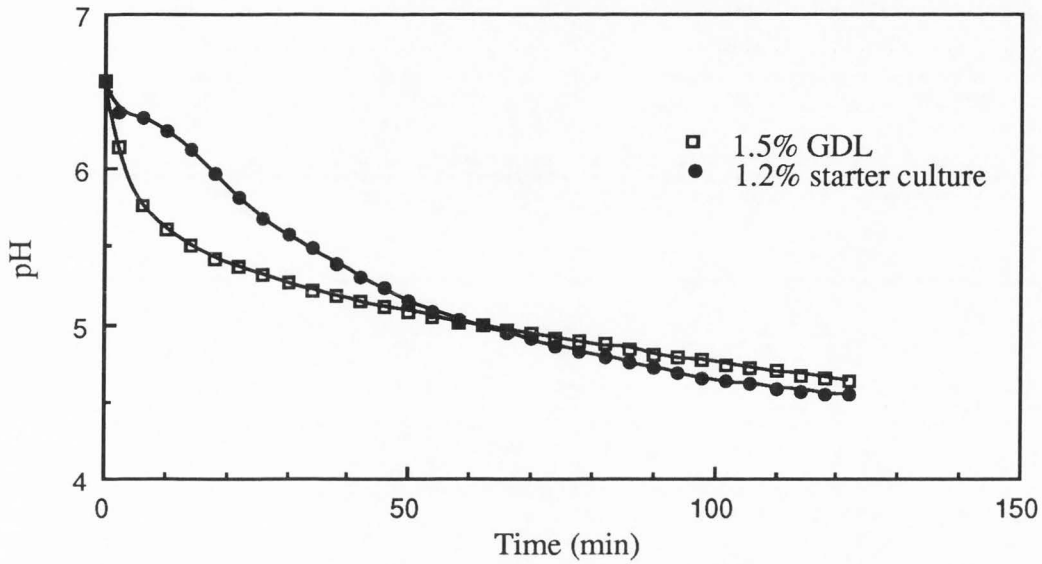

Figure 12. Acidification of milk after addition of 1.5% glucono-δ-lactone (GDL) and 1.2% freeze-dried yogurt starter culture at 40°C.

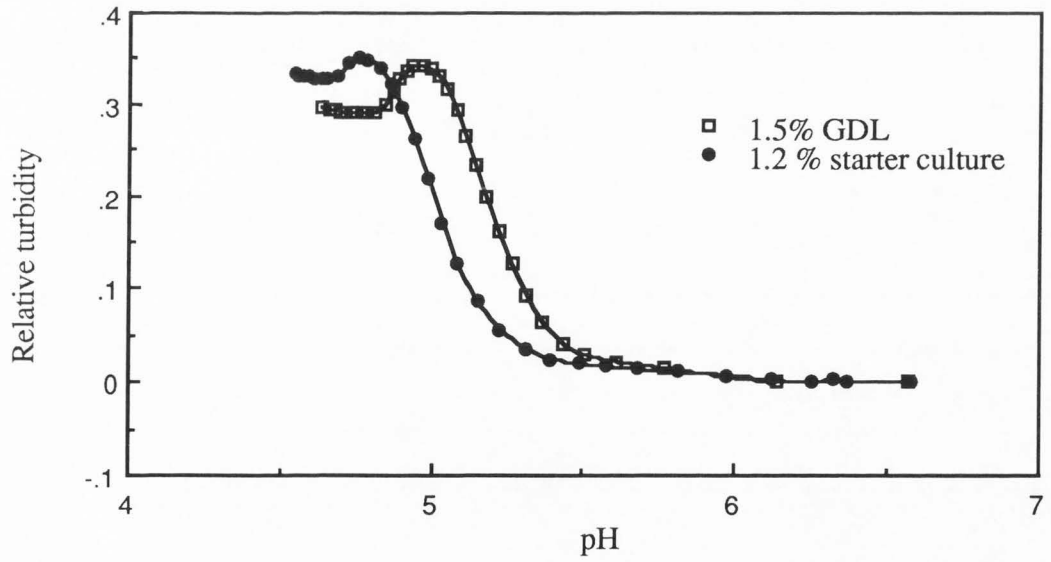

Figure 13. Relative turbidity change at 600 nm as a function of pH with the initial turbidity of milk as a reference after addition of 1.5% glucono-δ-lactone (GDL) and 1.2% freeze-dried yogurt starter culture at 40°C. Each line represents the average of three replicates.

Average Particle Size of Casein Micelles

Temperature. The average particle sizes of casein micelles before acidification were 285, 202, 152, and 117 nm at 10, 20, 30, and 40°C, respectively. They increased significantly with decreasing temperature. The dependence of average casein micelle size on temperature before acidification is shown in Figure 14. Their relationship for temperatures of 10 to 40°C fits the equation:

$S = 327 - 5.53T$ ($r^2 = .963$)

where S is the average particle diameter (nm) of casein micelles and T is temperature in the range of 10 to 40°C.

pH. Average diameters of casein micelles as a function of pH are shown in Figures 15, 16, and 17. These data were analyzed using ANOVA. At similar acidification rates, the temperature, pH, and interaction between temperature and pH all had significant effect on the average diameter of casein micelles (Table 5).

TABLE 5. Split-plot design ANOVA for average particle size of casein micelles after addition of 4.0, 2.5, 2.0, and 1.5% glucono-δ-lactone at 10, 20, 30, and 40°C, respectively.

SV	df	MS	F	P
Rep	2	461.6		
Temperature	3	118417.3	1273.30	<.001
Error (a)[1]	6	93.0		
pH	7	1333.9	31.51	<.001
Temperature x pH	21	105.5	2.49	.003
Error (b)[2]	56	42.3		
Total	95			

[1]Error term of the whole plot (a).
[2]Error term of the subplot (b).

Temperature had little influence on the changing pattern of average particle size as a function of pH under similar acidification condition (Figure 15). No variation in

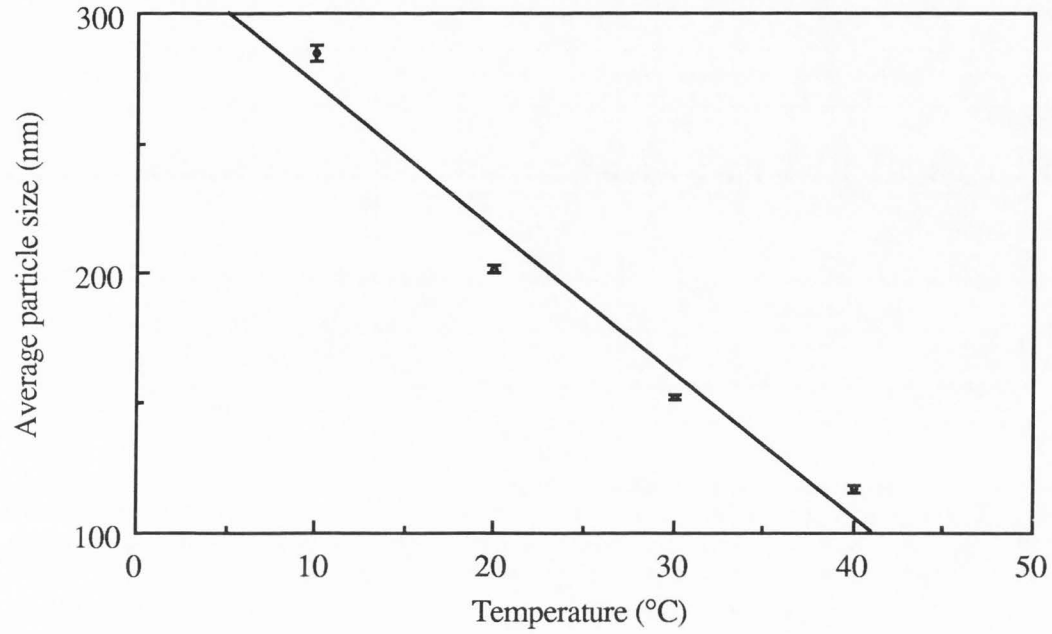

Figure 14. Average particle size of casein micelles in diameter as a function of temperature before acidification of milk. Error bars are standard error of means.

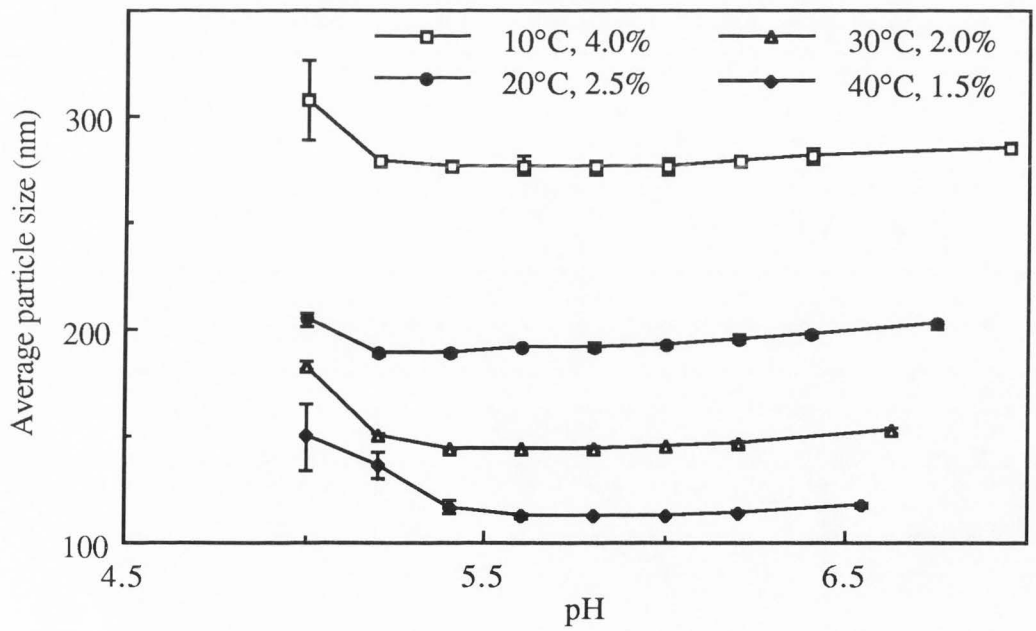

Figure 15. Average particle size change in diameter of casein micelles with pH after addition of 4.0% (10°C), 2.5% (20°C), 2.0% (30°C), and 1.5% (40°C) glucono-δ-lactone. Error bars are standard error of means.

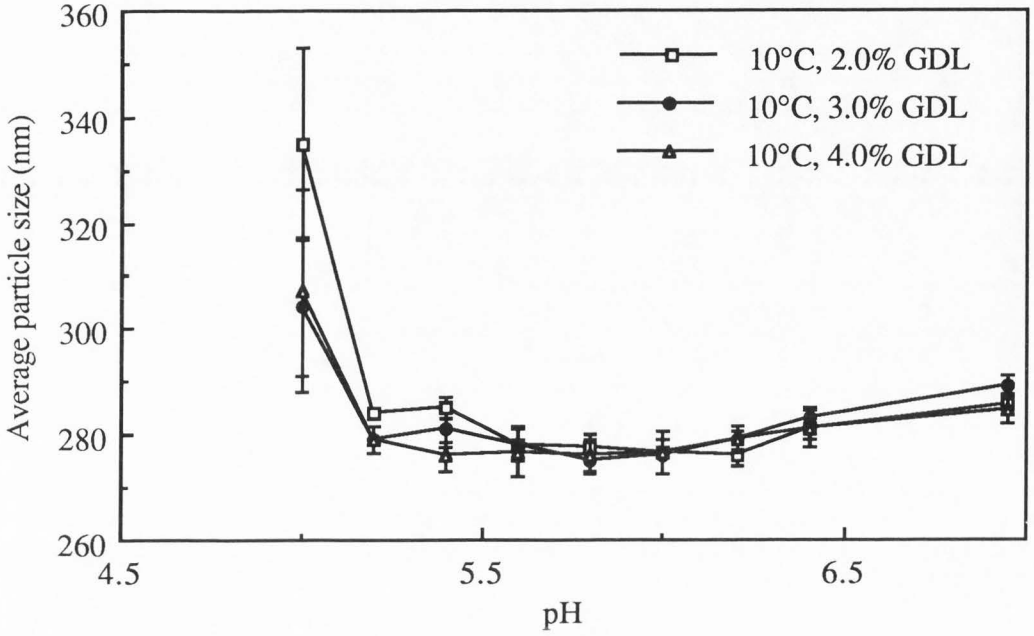

Figure 16. Average particle size change in diameter of casein micelles with pH after addition of 2.0, 3.0, and 4.0% glucono-δ-lactone (GDL) at 10°C. Error bars are standard error of means.

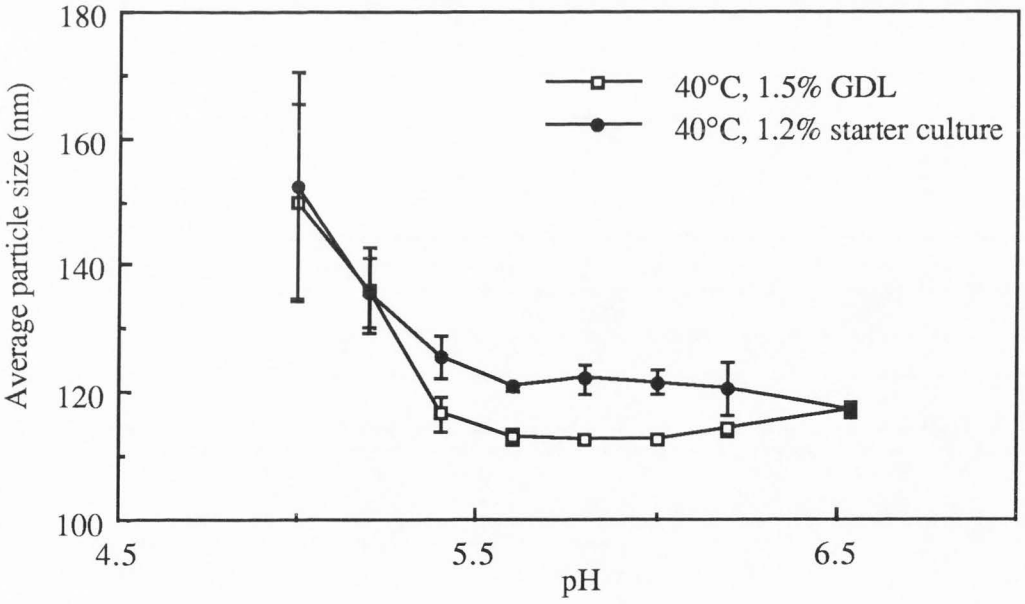

Figure 17. Average particle size change in diameter of casein micelles with pH after addition of 1.5% glucono-δ-lactone (GDL) and 1.2% freeze-dried yogurt starter culture at 40°C. Error bars are standard error of means.

average micelle size was observed above pH 5.2. Average particle size increased rapidly below pH 5.0 as the milk gelled. Statistical analysis indicated that the average particle size at 20 and 30°C showed more variability than at 10 and 40°C. All values at 10°C with 4% GDL showed no significant difference at $\alpha = .05$ confidence level. At 40°C, the average particle size at pH 5.2 and 5.0 was significantly higher than those above pH 5.4. As pH dropped to 5.2 at 20°C, the average particle size of casein micelles decreased significantly, but at pH 5.0 it recovered to the similar size of the original micelles before acidification. At 30°C, significant decrease occurred in the average particle size of casein micelles as pH was lowered to 5.4. At pH 5.2, average micelle size came back to the similar value of the original particles. As pH was further lowered, the average particle size increased significantly.

There was no significant effect of acidification rate on average particle size of casein micelles at 10°C (Table 6). Even though there was an overall pH effect, there was no significant difference ($\alpha = .05$) at the fastest rate (4.0% GDL) while at the slower rates (2.0 and 3.0% GDL) only the size at pH 5.0 was significantly greater than at other pH values.

TABLE 6. Split-plot design ANOVA for average particle size of casein micelles after addition of 4.0, 3.0, and 2.0% glucono-δ-lactone at 10°C, respectively.

SV	df	MS	F	P
Rep	2	457.60		
Acidification Rate	2	174.78	.54	.128
Error (a)[1]	4	326.88		
pH	8	1371.04	16.81	<.001
Rate x pH	16	101.19	1.24	.247
Error (b)[2]	48	81.55		
Total	80			

[1]Error term of the whole plot (a).
[2]Error term of the subplot (b).

GDL vs Culture. At 40°C, the average particle size values at pH 5.2 and 5.0 with 1.2% freeze-dried yogurt starter culture and 1.5% GDL were identical in all samples (Figure 17). Prior to coagulation (pH > 5.2), the average particle size values with 1.2% freeze-dried yogurt starter culture were a little higher than those with 1.5% GDL, probably due to the presence of bacteria. Acidifying methods only slightly affected average particle size (Table 7), but the interaction between method and pH did not significantly affect average particle size of casein micelles.

TABLE 7. Split-plot design ANOVA of average particle size of casein micelles after addition of 1.5% glucono-δ-lactone or 1.2% freeze dried yogurt starter culture at 40°C, respectively.

SV	df	MS	F	P
Rep	2	549.9		
Acidification Method	1	364.7	4.78	.069
Error (a)[1]	2	76.3		
pH	7	965.9	9.50	<.001
Method x pH	7	25.0	.25	.969
Error (b)[2]	28	101.7		
Total	47			

[1]Error term of the whole plot (a).
[2]Error term of the subplot (b).

Formagraph

Curd firmness of milk as a function of pH at different temperatures but similar acidification rates (Figure 2) is shown in Figure 18. I found that by using the Formagraph, temperature had no consistent effect on the rate at which curd firmness developed, and the milk coagulated at pH 4.80 ± .03.

Acidification rates also had no apparent influence on the rate at which curd firmness developed as a function of pH at all experimental temperatures, as shown in Figures 19, 20, 21, 22, and 23. Table 8 shows the pH and times at which coagulation was measured with the Formagraph.

TABLE 8. Coagulation time and pH of milk acidified with glucono-δ-lactone (GDL) or starter culture (SC).

Temperature	10°C		20°C		30°C		40°C	
	pH	Time (min)	pH	Time (min)	pH	Time (min)	pH	Time (min)
1.2% SC							4.77	84
1.5% GDL							4.78	82
2.0% GDL	4.78	331	4.77	160	4.78	89	4.82	47
2.5% GDL			4.83	100	4.79	57	4.77	32
3.0% GDL	4.81	162	4.80	72	4.79	41		
4.0% GDL	4.79	107						

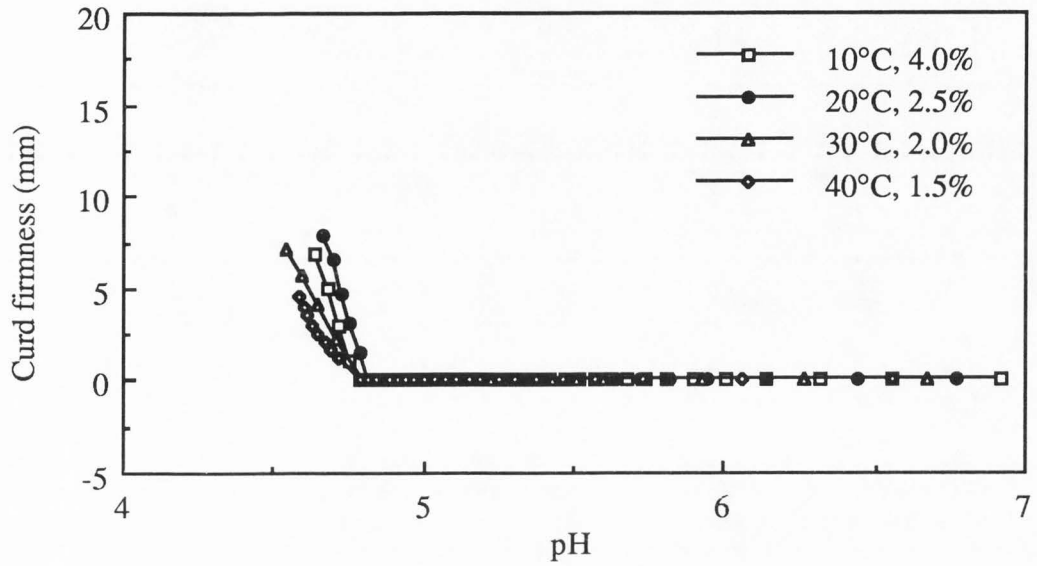

Figure 18. Formagraph curd firmness of milk as a function of pH during acidification with 4.0, 2.5, 2.0, or 1.5% glucono-δ-lactone at 10, 20, 30, and 40°C, respectively. Average of three replicates.

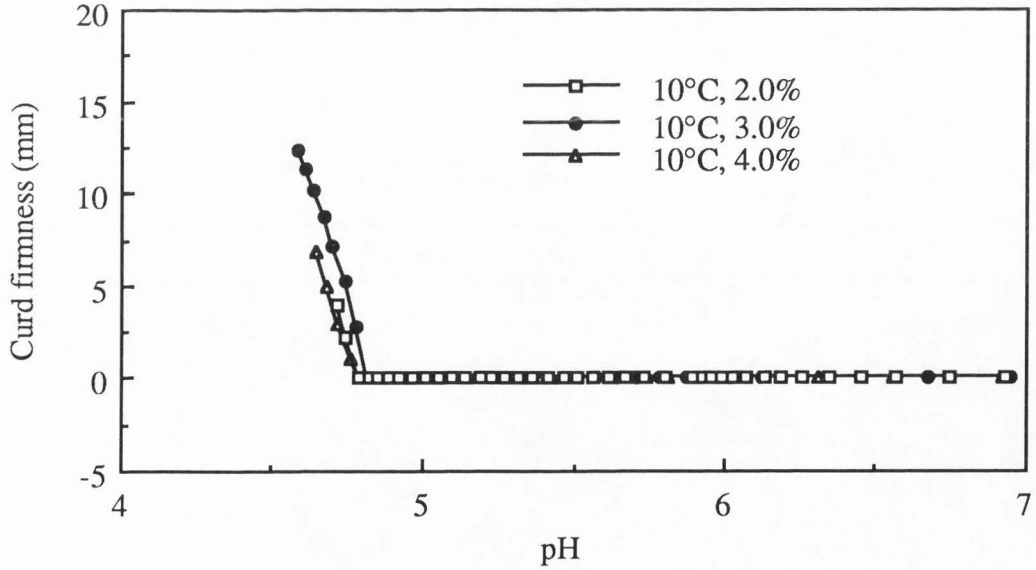

Figure 19. Formagraph curd firmness of milk as a function of pH during acidification with 2.0, 3.0, or 4.0% glucono-δ-lactone at 10°C. Average of three replicates.

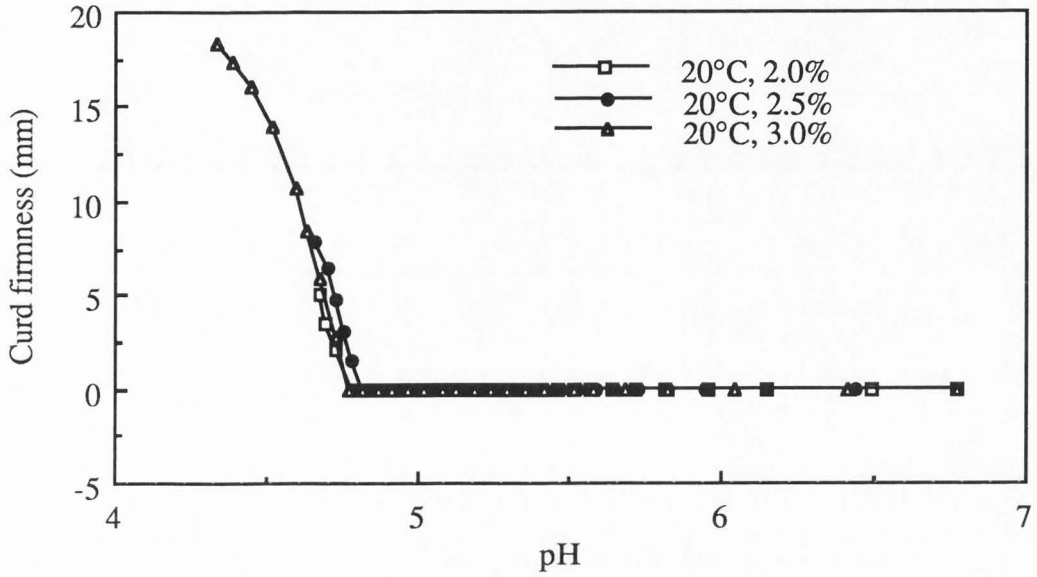

Figure 20. Formagraph curd firmness of milk as a function of pH during acidification with 2.0, 2.5, or 3.0% glucono-δ-lactone at 20°C. Average of three replicates.

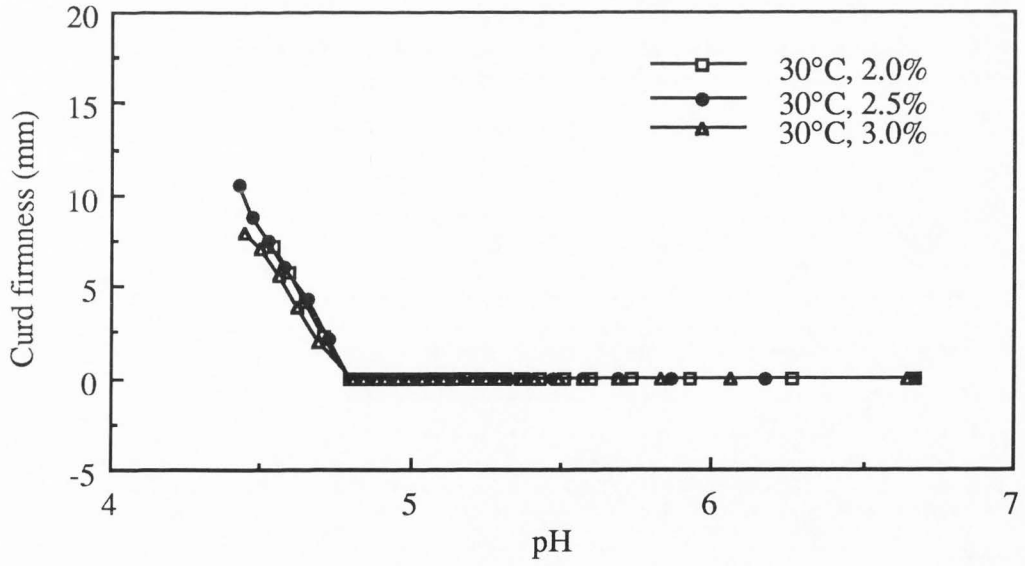

Figure 21. Formagraph curd firmness of milk as a function of pH during acidification with 2.0, 2.5, or 3.0% glucono-δ-lactone at 30°C. Average of three replicates.

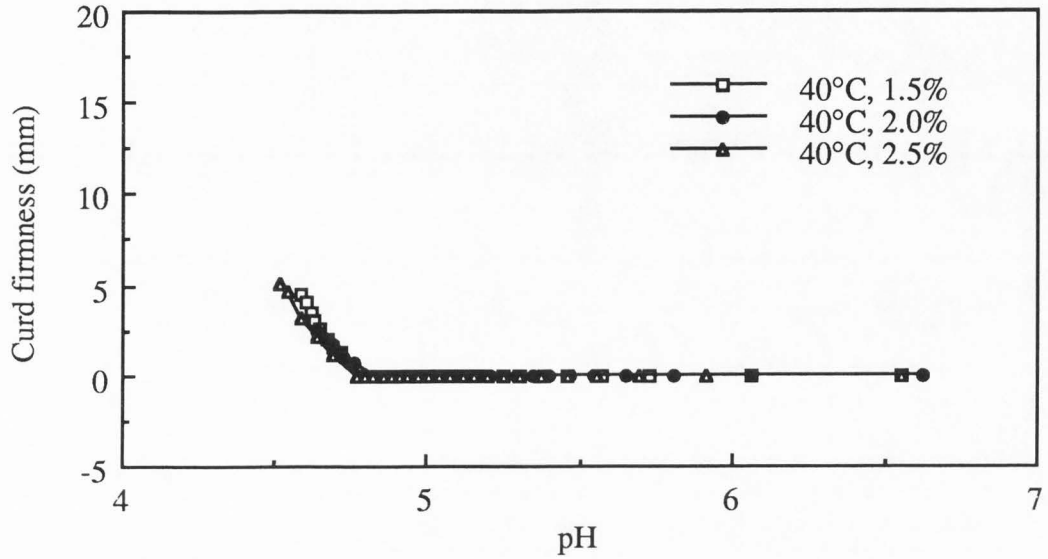

Figure 22. Formagraph curd firmness of milk as a function of pH during acidification with 1.5, 2.0, or 2.5% glucono-δ-lactone at 40°C. Average of three replicates.

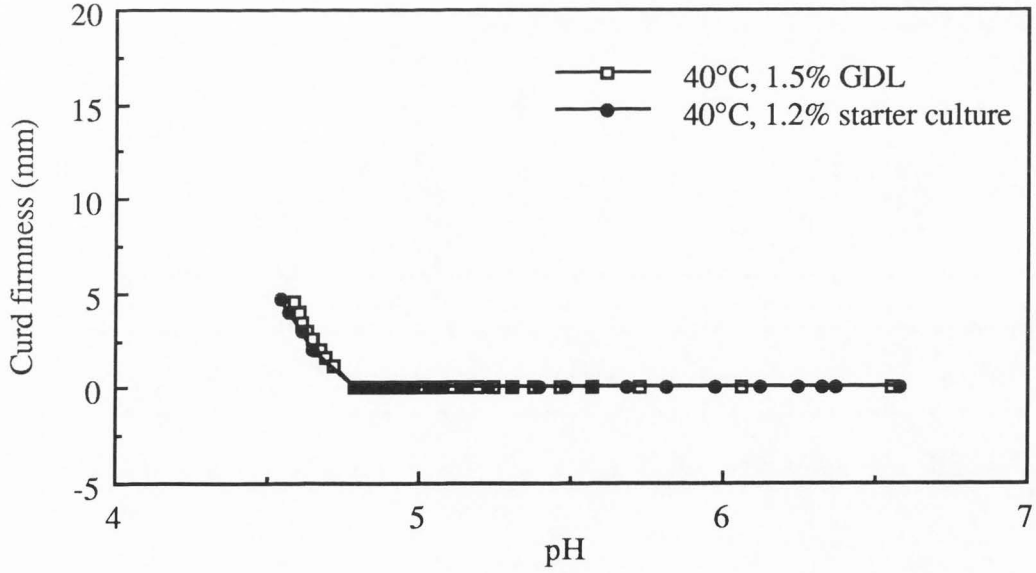

Figure 23. Formagraph curd firmness of milk as a function of pH during acidification with 1.5% glucono-δ-lactone (GDL) or 1.2% freeze-dried yogurt starter culture at 40°C. Average of three replicates.

Microstructure of Casein Micelles

At the native pH of milk, the microstructure of casein micelles was relatively compact. At 30 and 40°C, the casein micelles had generally smooth spherical surfaces (Figure 24). At 10°C, they became less electron-dense, and their surfaces became less distinct with a relatively open microstructure and protein material dispersed among casein micelles (Figure 24a). Figure 24d shows very compact micelles at 40°C. Casein micelles at 20 and 30°C (Figure 24b and c) had an intermediate microstructure between 10 and 40°C.

As pH was decreased, protein was dissociated from casein micelles initially, and then reassociated with the micelles during further acidification. At 10°C, acidification rates showed no effect on microstructure change of casein micelles during acidification (Figure 25, 26, and 27). As pH was lowered to 5.9, considerable protein was dissociated from micelles (Figures 25a, 26a, and 27a). At pH 5.3, reassociation among the proteins, as well as between the protein and casein micelles, occurred (Figures 25b, 26b, and 27b). This type of association was in a relatively uncompact state. At pH 5.0, further association among these components was observed (Figures 25c, 26c, and 27c). At this stage, there were some less compact and much more compact structures. As pH was decreased to 4.8, a gel network formed (Figures 25d, 26d, and 27d).

Microstructure change of casein micelles during acidification at 20°C (Figure 28) was similar to that at 10°C. At pH 5.0, the gel network started to form. The gel network that formed at pH 4.8 was less pronounced at 20°C than at 10°C. In fact, the gel network became less and less pronounced as the temperature increased in the range of 10 to 40°C (Figure 32a-d). This indicates that there was a finer aggregation of casein micelles (i.e., smaller cells between protein strands) and protein with decreasing temperature. As the temperature increased, the cells became sufficiently large such that the gel structure was not readily apparent in the approximately 70 nm sections cut from the embedded samples.

The microstructure of casein micelles after addition of 2.0% GDL at 30°C is shown in Figure 29. Some dissociated protein was observed at pH 5.6 and 5.3. There was some reassociation between protein and casein micelles as pH dropped to 5.0. Further association was perceptible at pH 4.8.

At 40°C, slight dissociation of protein was observed as pH dropped to 5.5 after addition of 1.5% GDL, but casein micelles were still quite compact in structure (Figure 30). More protein was released from, and dispersed among, casein micelles at pH 5.2. The casein micelles were slightly less compact than at higher pH values. As pH was lowered to 4.95, reassociation among the micelles, the protein, as well as between the micelles and protein, occurred. The average size of these reassociated micelles was larger than at lower temperatures, and some protein protruded from the surface of casein micelles. Some dissociated protein united into a type of larger incompact structure. At pH 4.8, extensive association was observed. Most of the micelles came together, although some incompact protein was perceptible.

Compared to the microstructure change of casein micelles during acidification with 1.5% GDL at 40°C, milk acidified by SC had more protein dissociation at pH 5.4 and 5.0 (Figure 31a and b). At pH 4.75 (Figure 31c), less association was observed, compared to that at pH 4.8 with 1.5% GDL (Figure 32d and e). The extent of association at pH 4.65 with 1.2% SC was similar to that at pH 4.8 with 1.5% GDL, except that there was more loose protein at pH 4.65 with SC (Figure 32d and f).

As shown in Table 9, pH of both protein dissociation from and reassociation with casein micelles decreased with increasing temperature, and was lower with 1.2% SC than 1.5% GDL.

TABLE 9. pH values at which protein dissociation from and reassociation with casein micelles were observed.

	10°C 4.0% GDL	20°C 2.5% GDL	30°C 2.0% GDL	40°C 1.5% GDL	40°C 1.2% SC
Protein dissociation	5.90	5.70	5.60	5.50	5.40
Protein reassociation	5.30	5.20	5.00	4.95	4.75

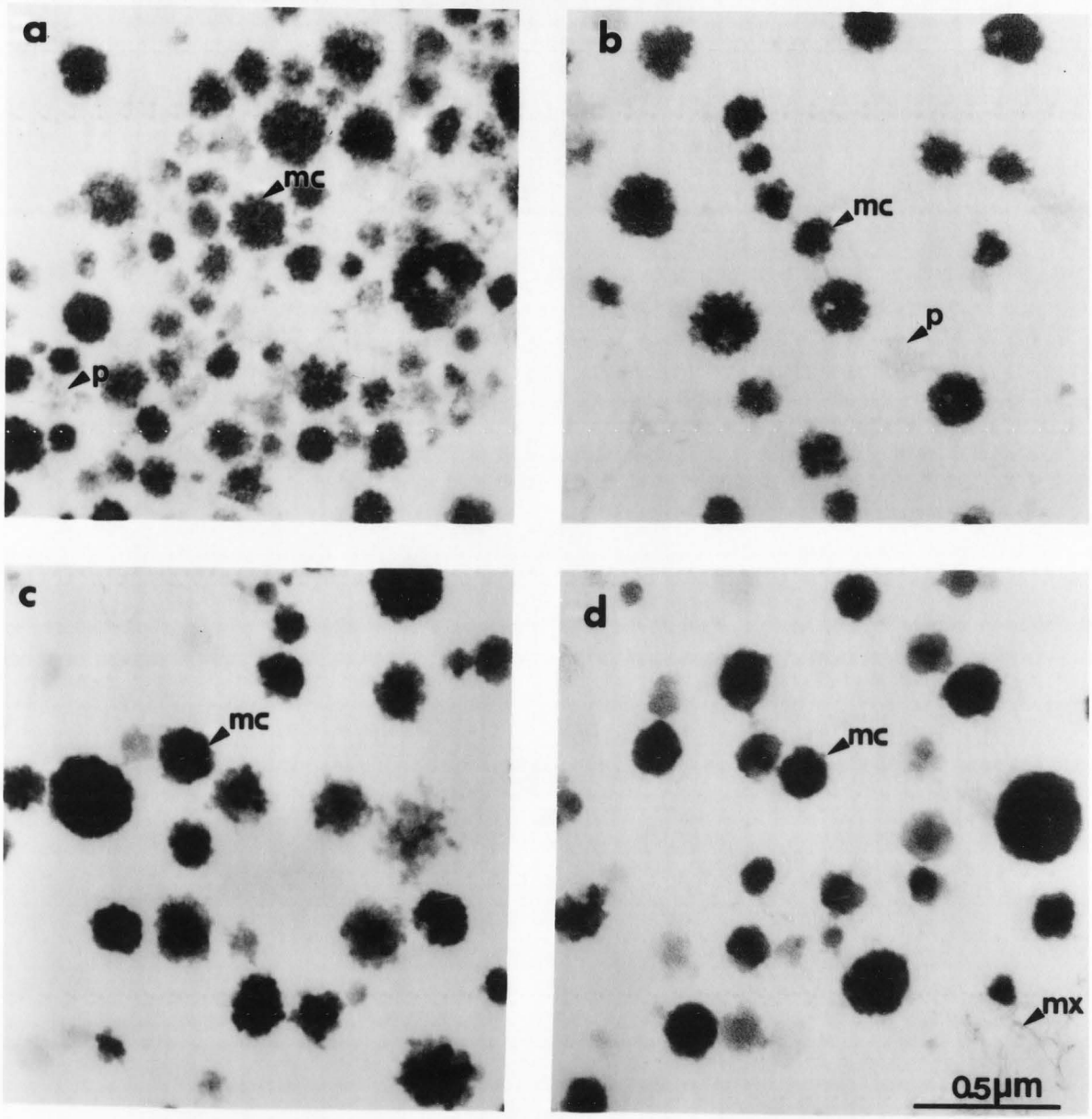

Figure 24. Transmission electron micrographs of casein micelles before acidification of skim milk. a) 10°C, pH 6.94, b) 20°C, pH 6.68, c) 30°C, pH 6.64, d) 40°C, pH 6.58. mc = micelles, mx = matrix, p = protein.

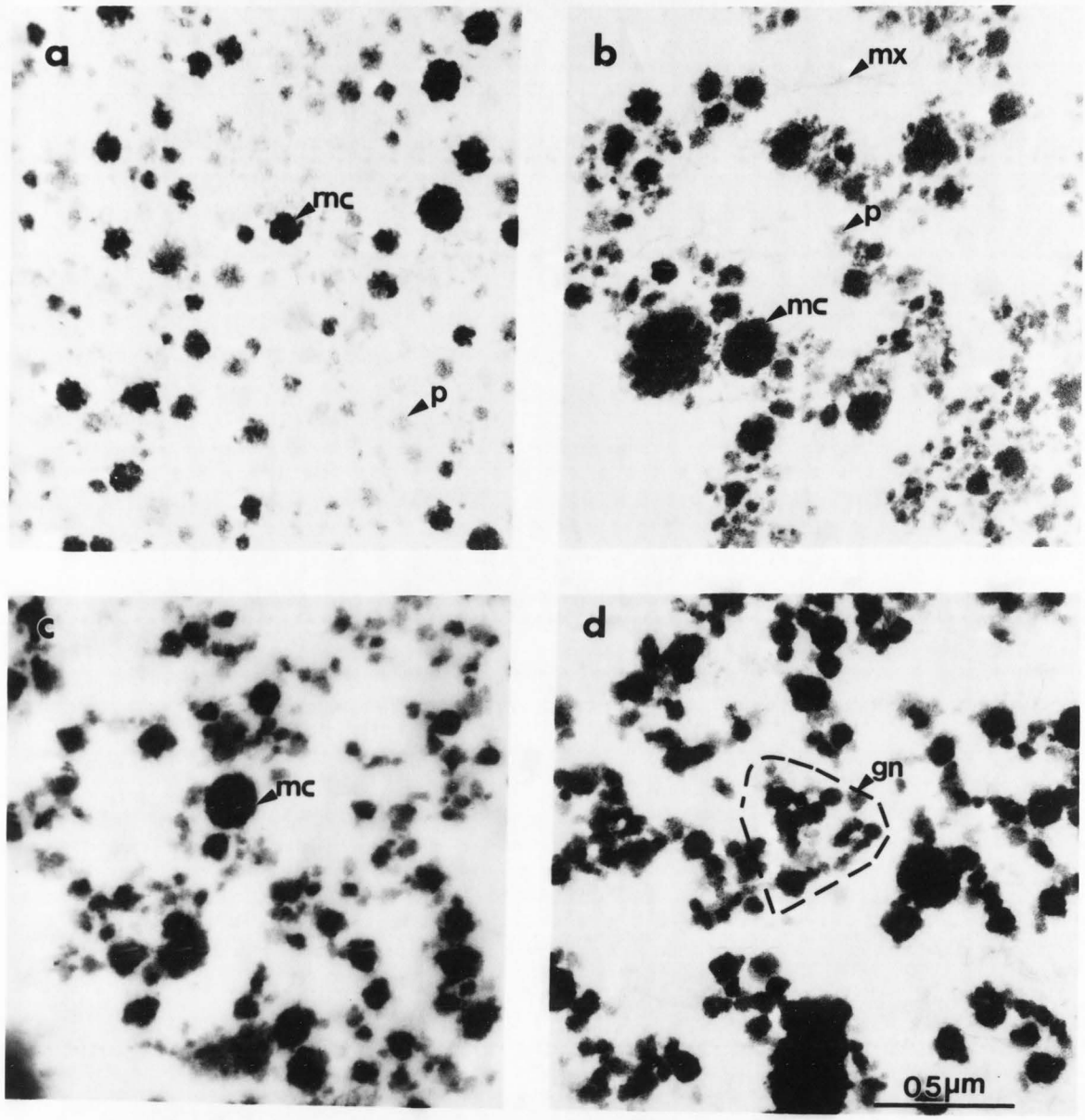

Figure 25. Transmission electron micrographs of casein micelles during acidification of skim milk after addition of 2.0% glucono-δ-lactone (GDL) at 10°C. a) pH 5.9, b) pH 5.3, c) pH 5.0, d) pH 4.8. gn = gel network, mc = micelles, mx = matrix, p = protein.

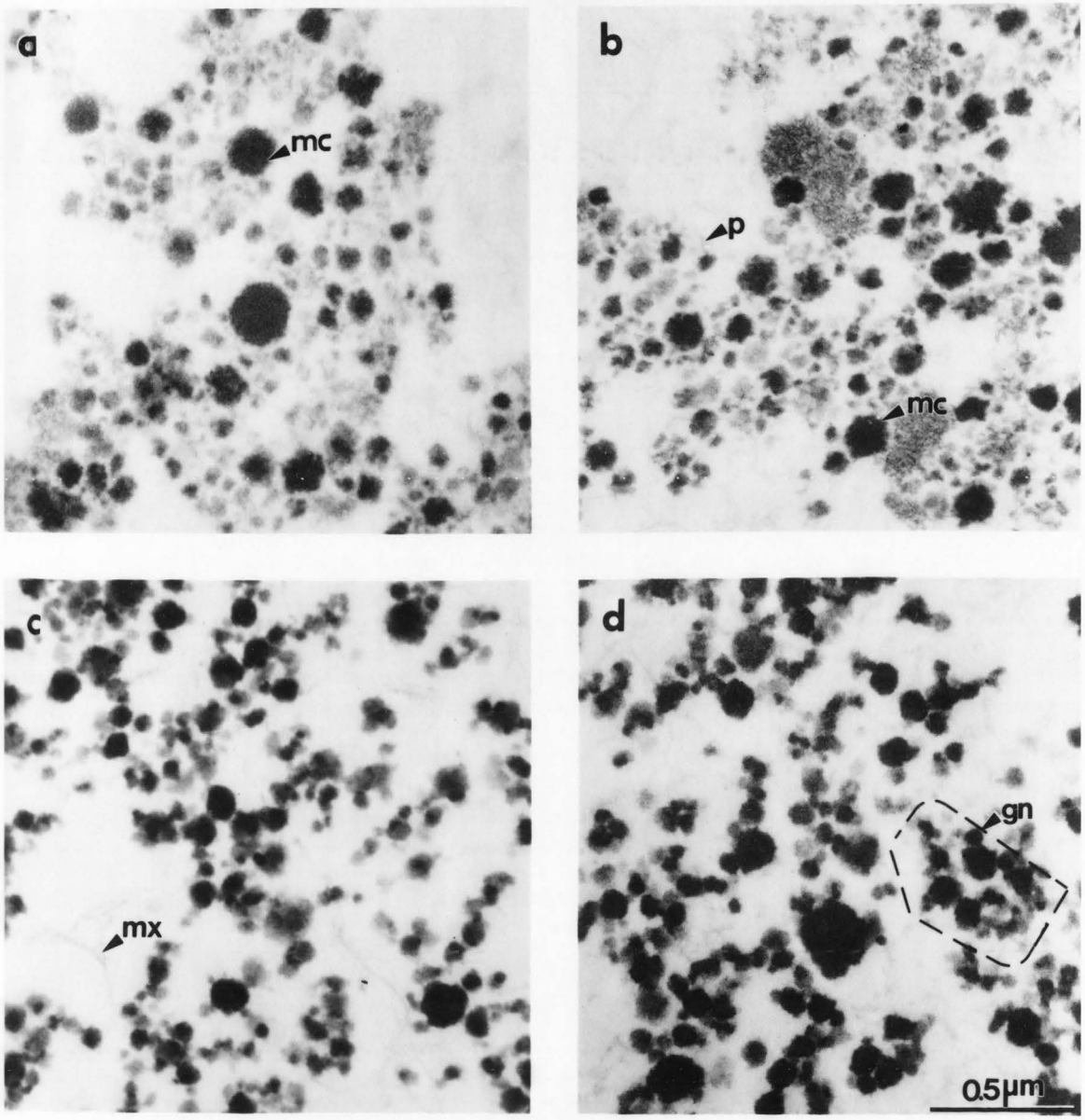

Figure 26. Transmission electron micrographs of casein micelles during acidification of skim milk after addition of 3.0% glucono-δ-lactone (GDL) at 10°C. a) pH 5.9, b) pH 5.3, c) pH 5.0, d) pH 4.8. gn = gel network, mc = micelles, mx = matrix, p = protein.

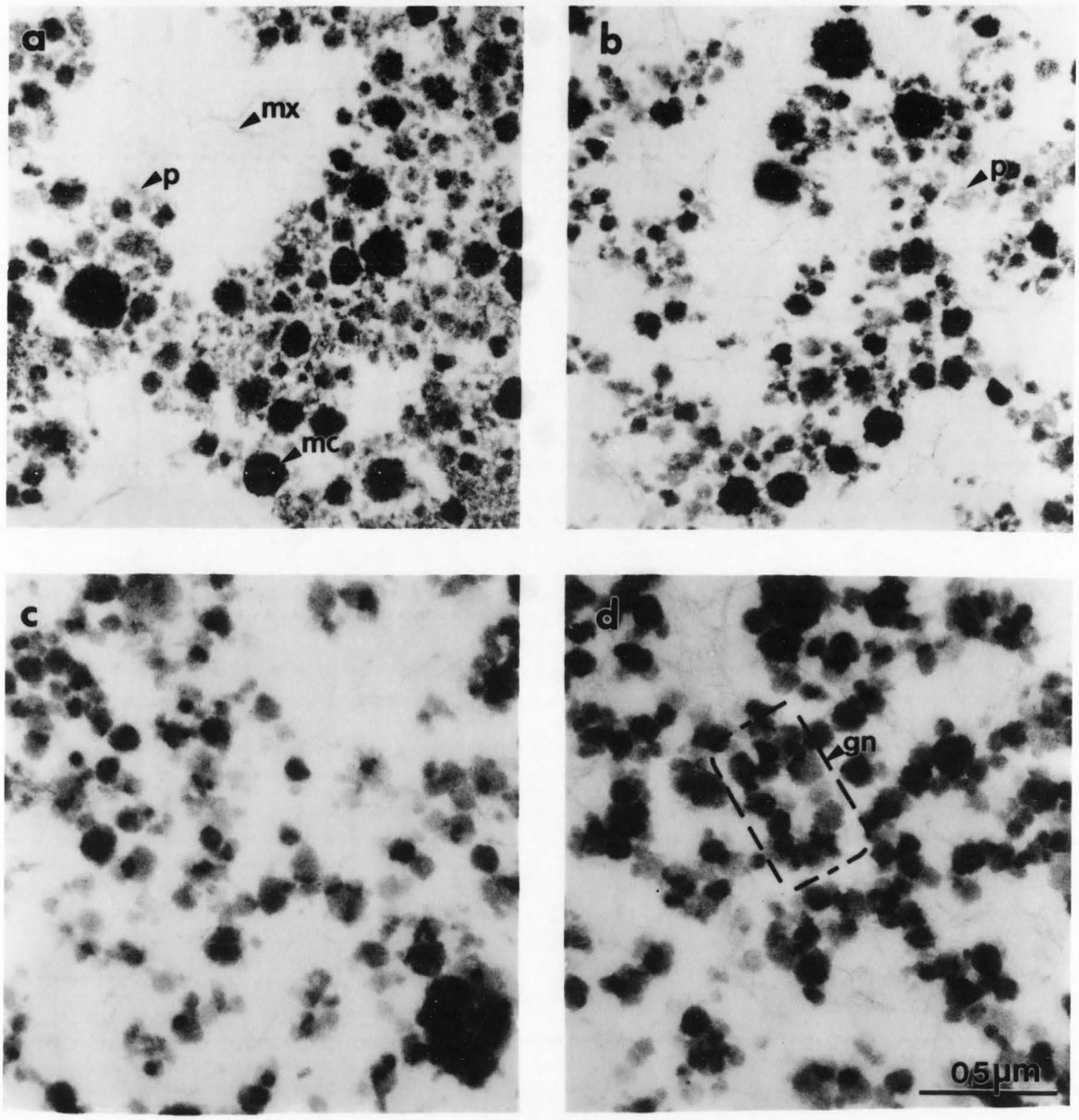

Figure 27. Transmission electron micrographs of casein micelles during acidification of skim milk after addition of 4.0% glucono-δ-lactone (GDL) at 10°C. a) pH 5.9, b) pH 5.3, c) pH 5.0, d) pH 4.8. gn = gel network, mc = micelles, mx = matrix, p = protein.

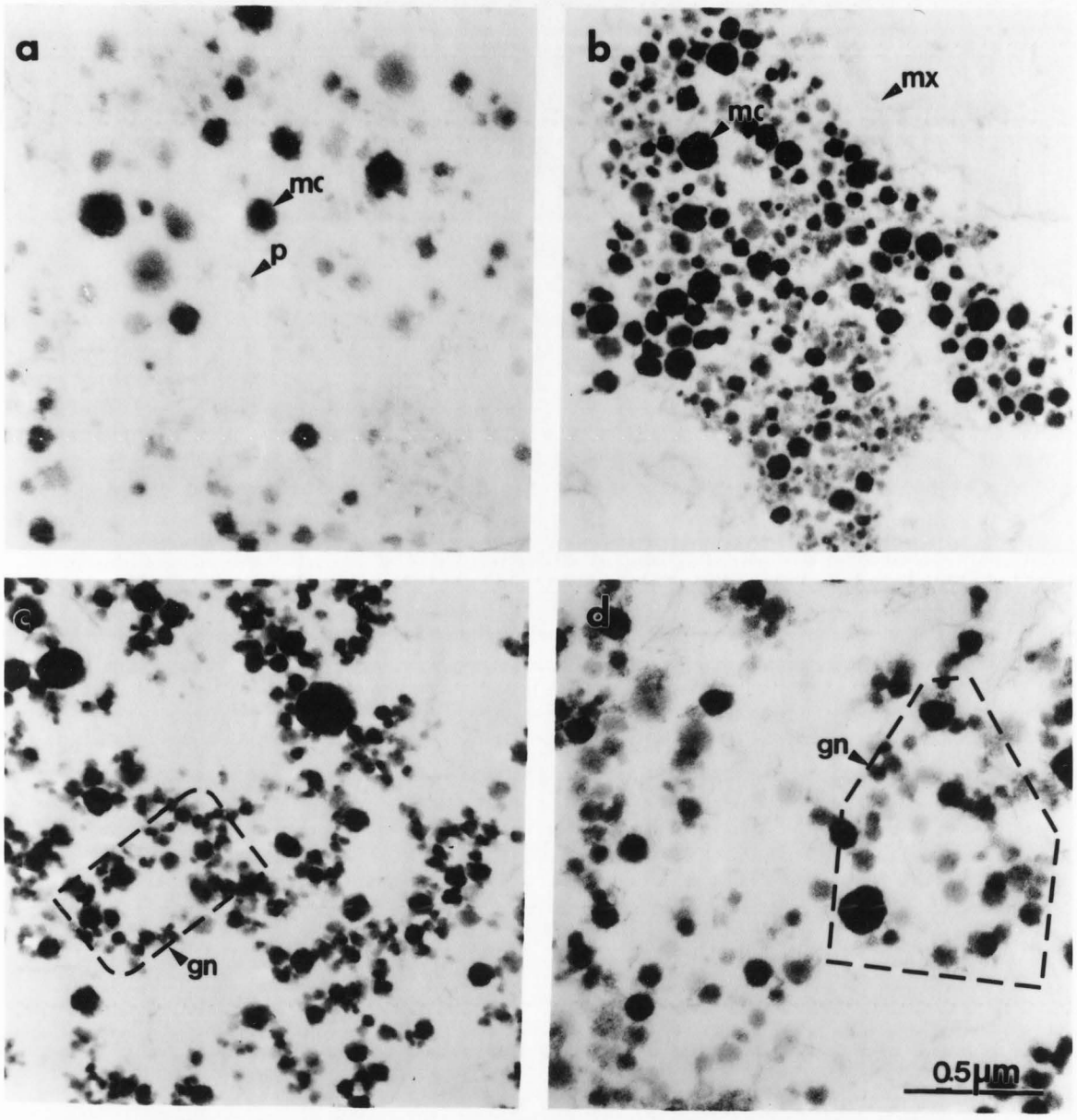

Figure 28. Transmission electron micrographs of casein micelles during acidification of skim milk after addition of 2.5% glucono-δ-lactone (GDL) at 20°C. a) pH 5.7, b) pH 5.2, c) pH 5.0, d) pH 4.8. gn = gel network, mc = micelles, mx = matrix, p = protein.

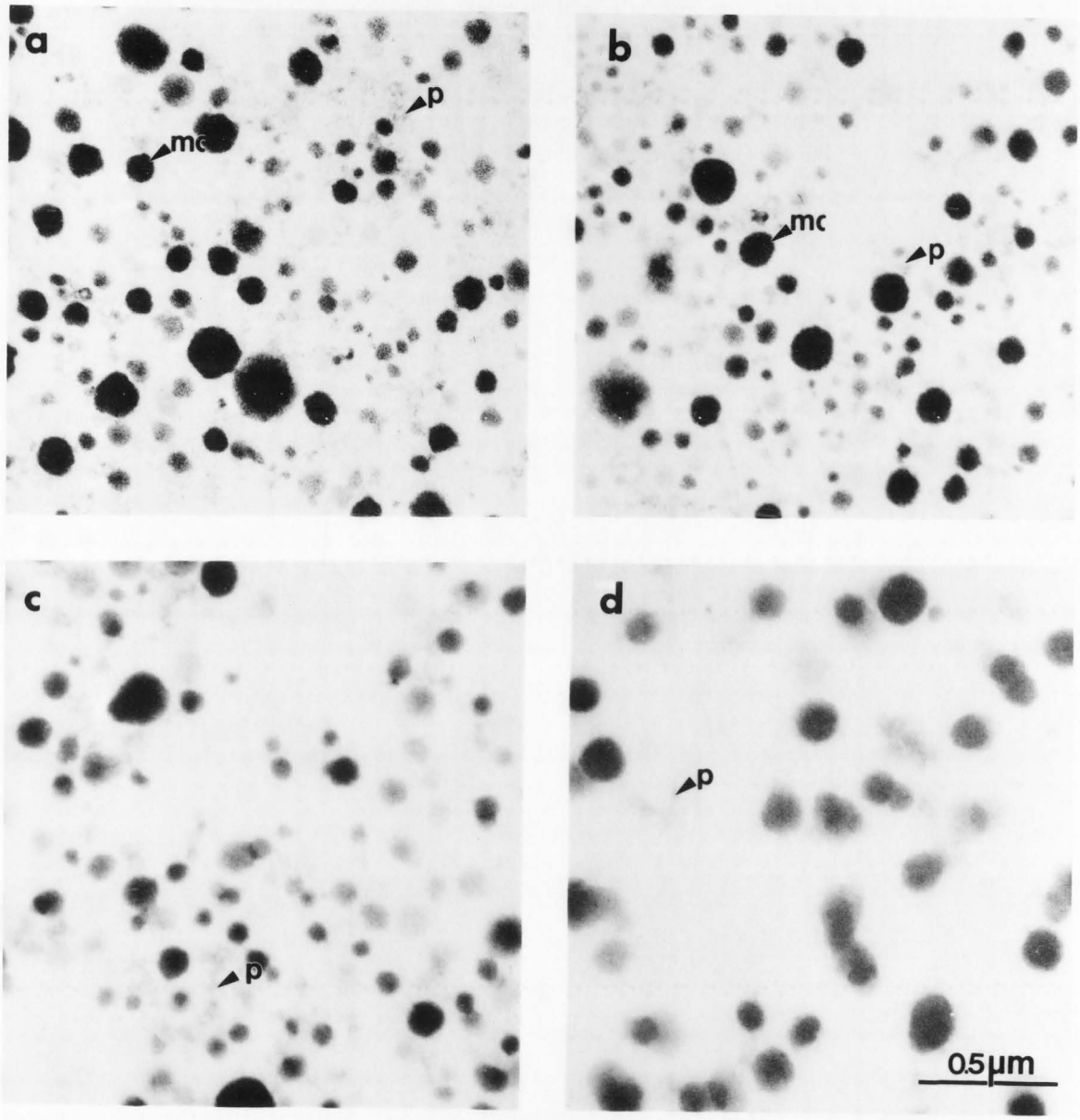

Figure 29. Transmission electron micrographs of casein micelles during acidification of skim milk after addition of 2.0% glucono-δ-lactone (GDL) at 30°C. a) pH 5.6, b) pH 5.3, c) pH 5.0, d) pH 4.8. mc = micelles, mx = matrix, p = protein.

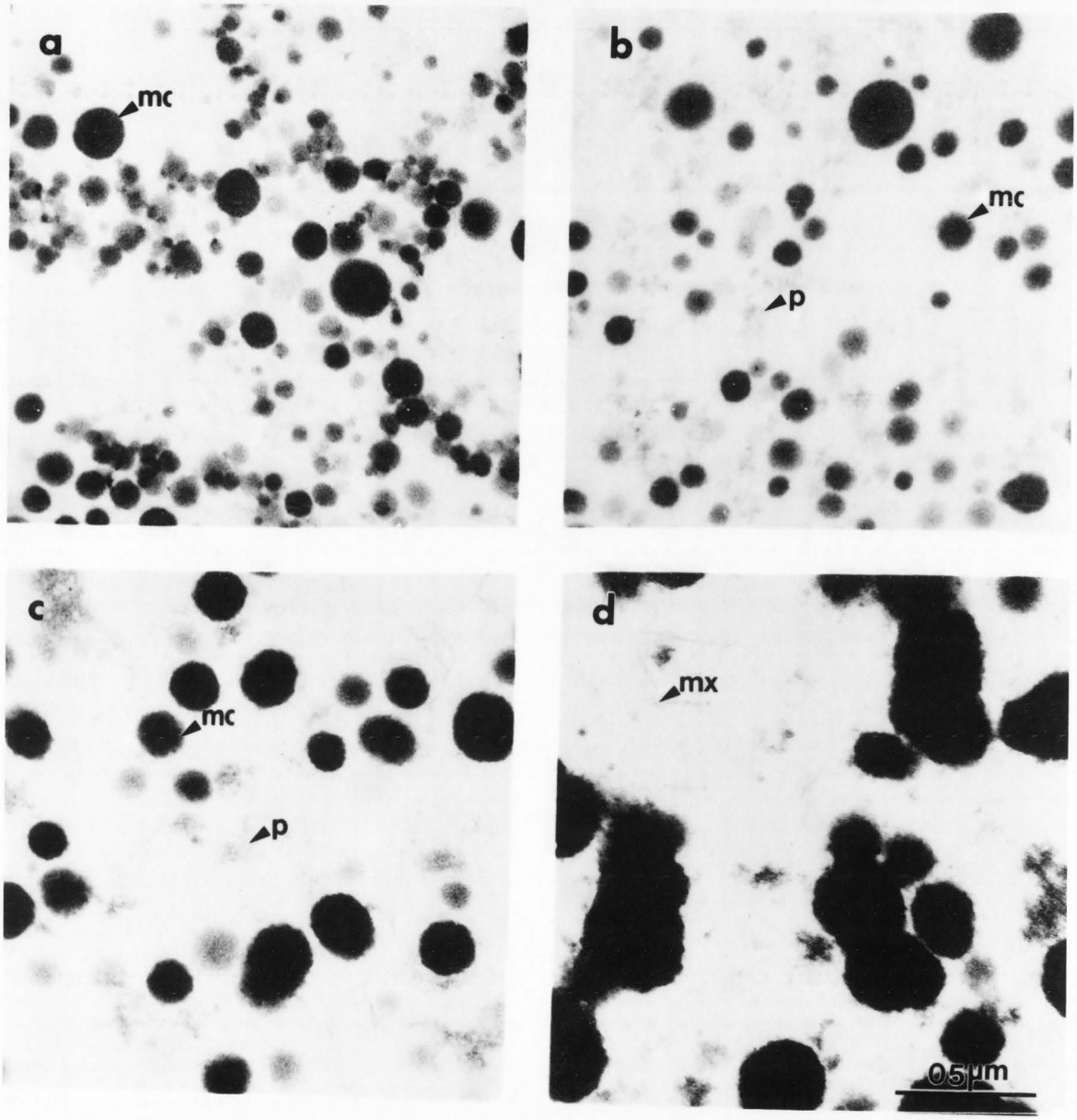

Figure 30. Transmission electron micrographs of casein micelles during acidification of skim milk after addition of 1.5% glucono-δ-lactone (GDL) at 40°C. a) pH 5.5, b) pH 5.2, c) pH 4.95, d) pH 4.8. mc = micelles, mx = matrix, p = protein.

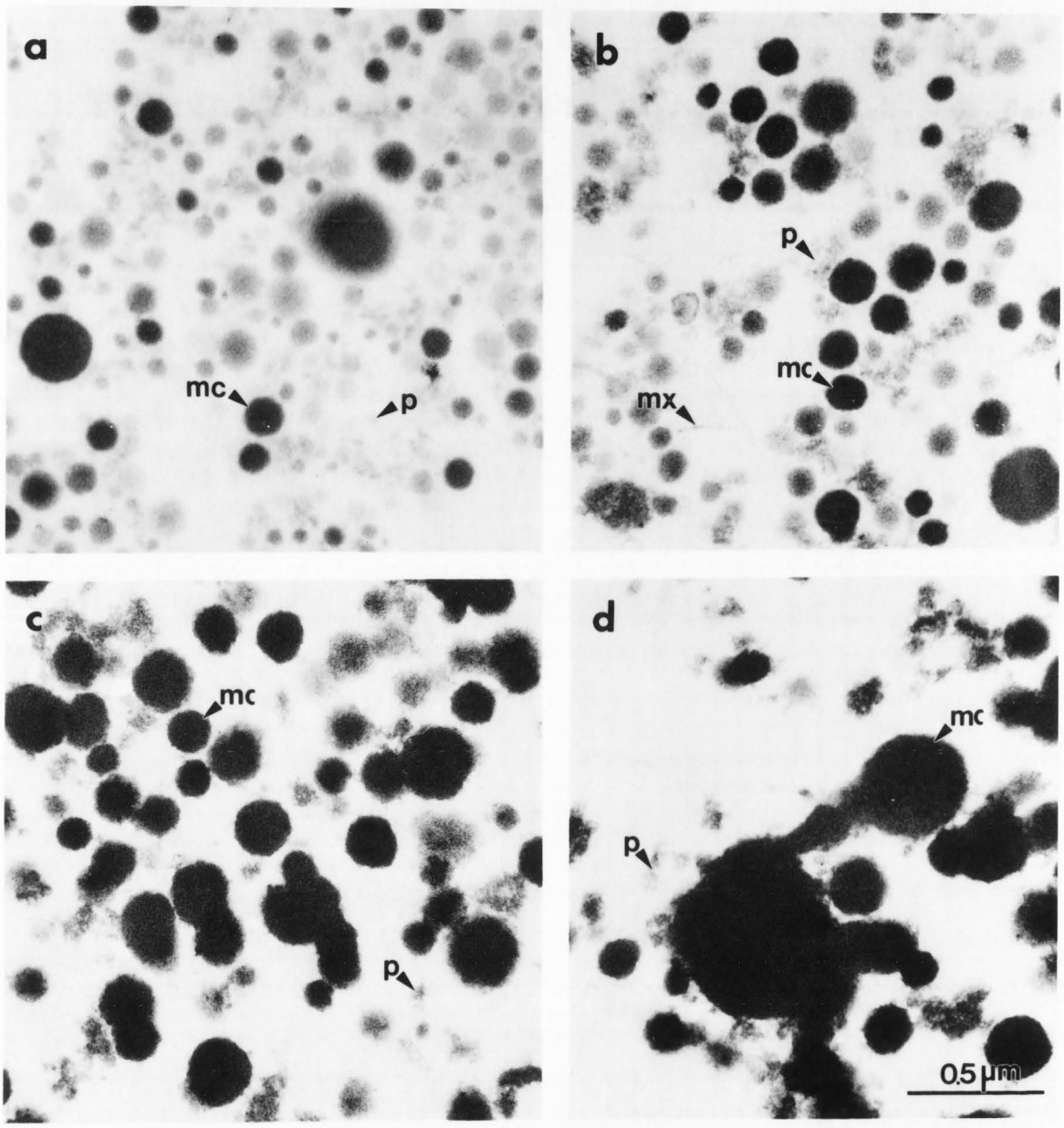

Figure 31. Transmission electron micrographs of casein micelles during acidification of skim milk after addition of 1.2% freeze dried yogurt starter culture at 40°C. a) pH 5.4, b) pH 5.0, c) pH 4.75, d) pH 4.65. mc = micelles, mx = matrix, p = protein.

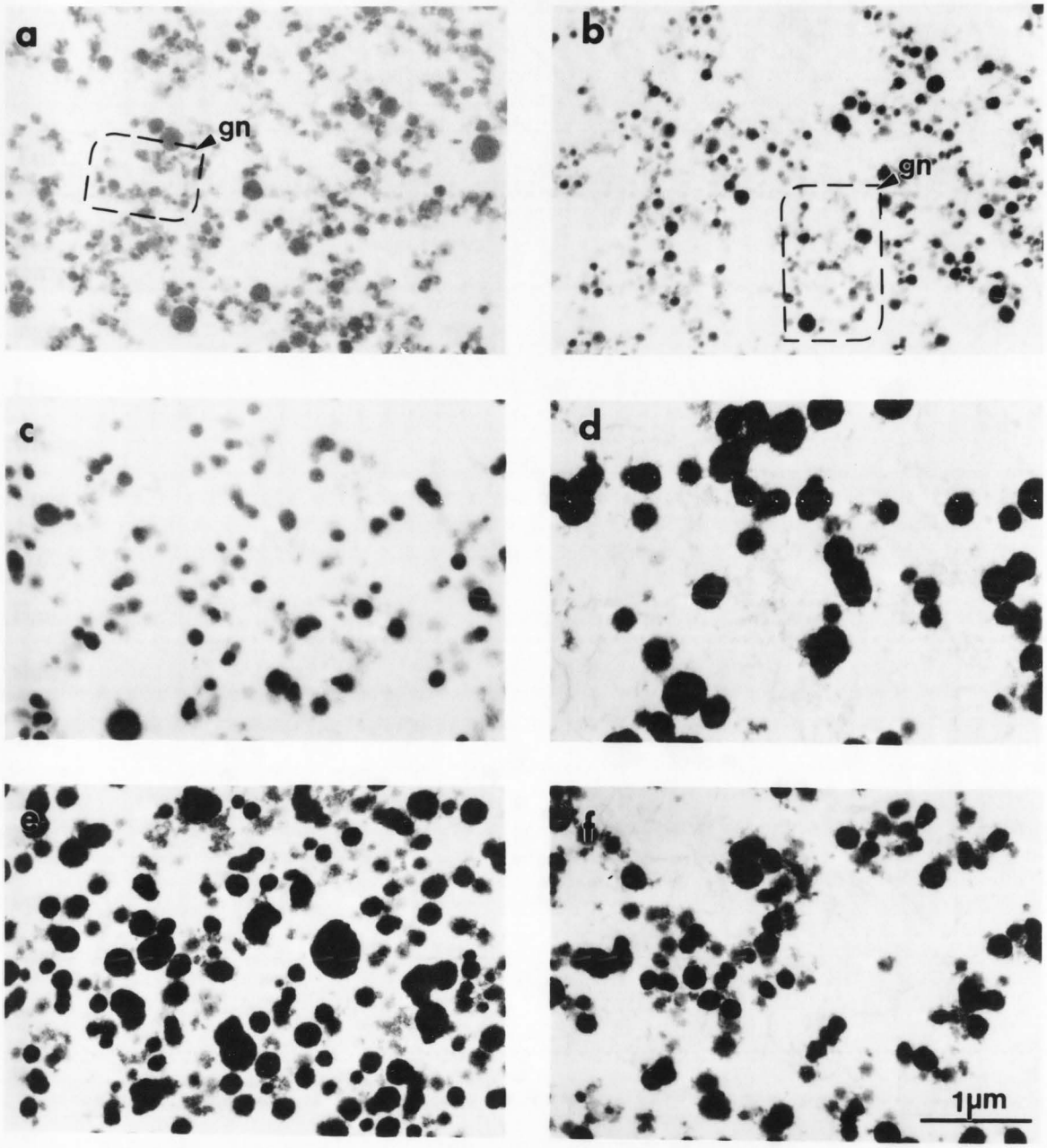

Figure 32. Transmission electron micrographs of casein micelles during acidification of skim milk comparing gel network formation after addition of a) 4.0% glucono-δ-lactone (GDL), pH 4.8 at 10°C, b) 2.5% GDL, pH 4.8 at 20°C, c) 2.0% GDL, pH 4.8 at 30°C, d) 1.5% GDL, pH 4.8 at 40°C, e) 1.2% freeze dried yogurt starter culture, pH 4.75 at 40°C, f) 1.2% freeze dried yogurt starter culture, pH 4.65 at 40°C. gn = gel network.

DISCUSSION

Turbidity

The observation on turbidity change during acidification of milk at different temperatures is in agreement with that of Banon and Hardy (3, 4). Temperature significantly affected the shape of turbidity change curves during acidification (Figure 3). I found that the slight increase and decrease were more pronounced at 10°C than at 20°C, while the final increase was greater at 20 than at 10°C. Banon and Hardy (3, 4) obtained similar results. At 30 and 40°C, there was no decrease following the slight increase. This is consistent with the observation of Bringe and Kinsella at 25°C (9), and Banon and Hardy at 30 and 42°C (4). They did not observe any other change in turbidity after the sharp increase because the lowest experimental pH of Bringe and Kinsella was 5.05 (9), and different equipment was used by Banon and Hardy (4).

At all experimental temperatures, as pH was lowered from the initial value of milk, protein was gradually dissociated from casein micelles. Before the protein was completely released into the serum, the dissociating protein molecules were protruding from the surface of casein micelles, giving them a greater hydrodynamic size. This would account for the slight increase in turbidity observed as the pH of milk was lowered from its original value to about 6.0.

At lower temperatures, and especially when milk coagulation proceeded slowly, protein dissociation from the micelles occurred well before reassociation began. Thus a decrease in turbidity occurred. At 10°C, protein dissociation was predominant between pH 5.8 and 5.27, leading to the decrease in turbidity. At slower acidification rates, this became even more evident. At 20°C, the decrease in turbidity between pH 5.52 and 5.24 was less pronounced than at 10°C. The reason may be that 1) there was less protein (presumably β-casein) dissociation at 20°C; and 2) some protein molecules also

reassociated with casein micelles at the same time as protein dissociation was occurring. Overall there was slightly more dissociation than reassociation, so a decrease was observed. At 30 and 40°C, reassociation of protein with casein micelles was predominant over dissociation, as observed in electron micrographs. No decrease was observed before the sharp increase. The sharp increase in turbidity at all experimental temperatures was evidence of aggregation of casein micelles occurring well before coagulation was detected using the Formagraph.

At 40°C, the decrease following the sharp increase could be explained by a decrease in particle number due to the aggregation of casein micelles. Similar results have been observed in rennet coagulation of milk (41). When equilibrium was reached, turbidity appeared to be constant.

There was no difference in turbidity change at different levels of GDL at 30°C (Figure 9). This implies that acidification proceeded quickly enough so that the separation between protein dissociation and reassociation was diminished, and, as observed in the micrographs, there was much less dissociated protein at 30°C than at 10°C.

At 40°C, there was no decrease in turbidity observed at any of the acidification rates. There was more dissociated protein observed at pH 4.8, i.e., after gelation, than at the higher pH's. At the fastest acidification rate the aggregation of the micelles occurs at the same time that some protein is also being released from the micelles. This effect is observed by the decrease in turbidity after gelation. At the slower acidification rates the dissociation would have greater time to occur during aggregation; hence, the "hump" in the turbidity curve was not observed. It is suggested that because the turbidity curves all were at approximately the same level at the end of coagulation, the final gel structures were all similar in spite of differences in acidification rates.

Compared to freeze-dried yogurt starter culture, GDL caused a much faster pH drop above pH 5.0 because of the rapid hydrolysis of GDL compared to the culture, which must first multiply before significant acid production occurs. Below pH 5.0, acidification by GDL was slightly slower than SC because of its diminishing concentration. Similar turbidity change was observed with GDL or SC although the SC curve was slightly offset to lower pH. Aggregation of casein micelles and protein occurred at lower pH with SC, as observed by electron microscopy. The reason might be that the proteolytic activity of the bacteria in addition to the pH drop causes more protein liberation from casein micelles than pH decrease alone as happens when GDL is used. It would take the milk with SC longer to reach a similar extent of micelle and protein association when pH is dropping. The last decrease and stationary period in turbidity change were at greater values with SC because there was less decrease in particle number. Thus, turbidity decreased less.

Average Particle Size of Casein Micelles

Figure 15 indicates that temperature had little influence on the shape of average particle size curves as a function of pH. No great variation was observed above pH 5.2. This result is basically consistent with the observations made by other workers (3, 4, 50, 51, 61). Below pH 5.0, average particle size increased rapidly due to the extensive aggregation of casein micelles and protein as the milk gelled.

According to the result of Dalgleish and Law (18), as more caseins are dissociated from casein micelles during acidification at lower temperature, casein micelle size should decrease more at lower temperature. However, my results showed little change at 10°C, indicating that a size-determining micellar framework of caseins remained during acidification, as proposed by Visser et al. (60). Another reason might be the increase in number of small particles resulting from the dissociated protein. Average casein micelle

size remained relatively constant, before milk gelation, independent of protein dissociation or reassociation with casein micelles.

Average casein micelle size before acidification increased significantly with decreasing temperature. Banon and Hardy (4) also found a similar change of average casein micelle size with temperature, although their measured micelle size is generally smaller than ours due to the different equipment used. The fact that average casein micelle size before acidification was inversely related to temperature is consistent with the hypothesis that hydrophobic interactions play an important role in determining micelle size since hydrophobic interactions are also diminished by decreasing temperature.

As reported, more caseins (18, 45, 47) and calcium and phosphate (45, 47) are dissociated from the micelle at low temperature. Therefore, casein micelles should be smaller at lower temperature if their size at least partly depends on the dissociable caseins or calcium and phosphate. My results, however, imply that average casein micelle size before acidification is not determined by the dissociable caseins, calcium, or phosphate.

As temperature is reduced, the attractive forces between various casein components of the micelles are weakened. The protein molecules are less strongly bound to each other (bond lengths increase), and the micelle expands. In addition, the number of molecular sites at which proteins on the micelle surface are tethered to the micelle could be reduced, allowing some molecules to "float" away from the other proteins and thus extend the "hairy" nature of the micelle surface.

This shows that there is a framework structure with the micelles (and probably the micelle subunits) that is formed through electrostatic interactions. Through hydrophobic interactions, the proteins, mainly β-casein and some κ-casein, are "pulled" closer together into the relatively compact structure of native casein micelles. As these hydrophobic interactions are diminished by lowering temperature, the proteins are separated more

(inversely related to the extent of hydrophobic interactions). Thus, the micelles are larger at lower temperature.

Although average micelle size measured using photon correlation spectroscopy (PCS) increased significantly with decreasing temperature, a similar phenomenon was not observed on electron micrographs. At 10°C, casein micelles were less compact than at higher temperatures. There were lots of diffused materials around the surfaces of casein micelles at lower temperature. PCS may identify the diffused materials as part of the micelle. Thus, larger micelle size was obtained using PCS at lower temperature. On the other hand, larger micelles may appear smaller if they were not cut exactly through the largest section.

Formagraph

Under the experimental conditions, temperature had no effect on the pH (~4.8) at which milk coagulation was detected using the Formagraph. However, calculated pH_g (based on turbidity change) increased with increasing temperature, and all pH_g values were greater than 4.8. The difference was that the Formagraph is not as accurate as the turbidity method in monitoring the coagulation point. In the Formagraph, insufficient force would be transmitted to the pendulum from the linearly oscillating milk to cause the pendulum to move until formation of a curd. Kim and Kinsella (34) confirmed that pasteurized skim milk samples started to coagulate at very similar pH (5.1 to 5.2) in the range of 35 to 50°C. However, at 55°C, the coagulation pH increased to 5.6. Other workers also found that increasing temperature elevated the pH at which milk gelled (7, 11, 28).

Although coagulation of all milk samples was observed at about pH 4.8 using the Formagraph, a longer time was required for the initiation of milk coagulation with decreasing temperature if the GDL level was the same (Table 8). This means that the tendency of casein micelles to coagulate was greater at higher temperature. When the

temperature was reduced, the milk took longer to coagulate. This was a function of slower acidification rates. When this was corrected by using pH rather than time after GDL addition, no difference in coagulation point was observed as all samples coagulated at pH 4.80 ± .03.

Microstructure of Casein Micelles

The observation on microstructure of casein micelles before acidification at different temperatures can be correlated to the results on average micelle size measurement. Casein micelles became more compact with increasing temperature. At higher temperature, hydrophobic interactions of protein are facilitated; protein molecules are more strongly bound to each other, and casein micelles are more compact and smaller.

The pH (at which protein dissociation from and reassociation with casein micelles was observed) decreased with increasing temperature. At a lower temperature, hydrophobic interactions are weakened. Those protein molecules bound to the micelle mainly through hydrophobic interactions are more readily dissociated from the micelle. Consequently, the pH is not necessarily so low as to cause protein dissociation from casein micelles, and the following reassociation naturally occurs at higher pH at lower temperature. On the other hand, the gel network became less and less pronounced as temperature increased from 10 to 40°C (Figure 32a-d). This indicates that there was more extensive aggregation of casein micelles and protein with decreasing temperature since there was more time for casein micelles and protein molecules to aggregate at lower temperature. However, this observation cannot be correlated to that of the rate at which curd firmness developed.

Compared to GDL, SC caused more protein dissociation, and the aggregation of casein micelles and protein occurred at lower pH at 40°C. With SC, the proteolytic activity of proteinases and peptidases released by the bacteria (48, 57, 58) contributed to more protein liberation from casein micelles. The proteins hydrolyzed by the bacteria

might not be able to reassociate with casein micelles as efficiently as those released by a lower pH. Therefore, more incompact protein was observed, and it would take the milk with SC longer (i.e., lower pH) to obtain similar aggregation.

Interpreted Mechanism of Milk Acid Coagulation

As pH decreased during acidification, proteins were dissociated from and then reassociated with casein micelles. Average micelle size did not change much to pH 5.2, but increased rapidly below pH 5.0 as the milk gelled. These results indicate that a size-determining framework remained during acidification which was independent of the dissociable protein, calcium, or phosphate. Such a size-determining framework would also apply using the submicelle theory of casein micelle structure.

As the pH of milk decreases from its native value, the ionization states of amino acid side groups are changed. The only affected side groups are imidazole of histidine ($pK_a = 6.04$), γ-COOH of glutamic acid ($pK_a = 4.07$), and β-COOH of aspartic acid ($pK_a = 3.90$); other ionizable groups have pK_a's >> 6.5. Table 10 shows the percentage of each form of these groups at different pH values during milk acidification. Decreasing pH produces more positive charge through the imidazole group of histidine. Part of the negative charge of the micelle is neutralized by the increased $[H^+]$ in milk, which results in the reduction of net charge of casein micelles. Consequently, electrostatic repulsion between casein micelles is diminished. This makes the association among micelles possible and enhances micelle interaction. Another effect of pH decrease is that it causes the release of calcium and phosphate from casein micelles (Table 12). This would result in the dissociation of those protein molecules which are linked to the size-determining micelle framework through colloidal calcium and phosphate. The resulting micelles become less stable due to the minimized steric repulsion and the less hydrophilic surface. By calculation, the results (Tables 11 and 12) show that only a portion of the

TABLE 10. Theoretical percentages of the neutralized and ionized form of three amino acid side groups (imidazole of histidine, pK_a = 6.04; γ-COOH of glutamic acid, pK_a = 4.07; β-COOH of aspartic acid, pK_a = 3.90) at different pH values.

pH	His		Glu		Asp	
	N	NH^+	COOH	COO^-	COOH	COO^-
6.7	82.0	18.0	.2	99.8	.2	99.8
6.6	78.4	21.6	.3	99.7	.2	99.8
6.5	74.3	25.7	.4	99.6	.3	99.7
6.4	69.6	30.4	.5	99.5	.3	99.7
6.3	64.5	35.5	.6	99.4	.4	99.6
6.2	59.1	40.9	.7	99.3	.5	99.5
6.1	53.4	46.6	.9	99.1	.6	99.4
6.0	47.7	52.3	1.2	98.8	.8	99.2
5.9	42.0	58.0	1.5	98.5	1.0	99.0
5.8	36.5	63.5	1.8	98.2	1.2	98.8
5.7	31.4	68.6	2.3	97.7	1.6	98.4
5.6	26.6	73.4	2.9	97.1	2.0	98.0
5.5	22.4	77.6	3.6	96.4	2.5	97.5
5.4	18.6	81.4	4.5	95.5	3.1	96.9
5.3	15.4	84.6	5.6	94.4	3.8	96.2
5.2	12.6	87.4	6.9	93.1	4.8	95.2
5.1	10.3	89.7	8.5	91.5	5.9	94.1
5.0	8.4	91.6	10.5	89.5	7.4	92.6
4.9	6.8	93.2	12.9	87.1	9.1	90.9
4.8	5.4	94.6	15.7	84.3	11.2	88.8

TABLE 11. Some characteristic parameters of casein micelles.

	α_{s1}-casein	α_{s2}-casein	β-casein	κ-casein	Total
Weight proportion (%)	38.46	10.26	38.46	12.82	
MW[1]	23614	25230	23983	19023	
Chain No/micelle	8144	2033	8019	3370	21566
Phosphoserine No/chain	8	11	5	1	
Phosphoserine No/micelle	65152	22363	40095	3370	171075
MW of casein micelle (36)		5.0×10^8 Dalton			
Micelle No/ml milk (56)		6×10^{14}			

[1] MW of α_{s1}-B, α_{s2}-A, β-A[2], and κ-B casein, respectively (65).

TABLE 12. Calculated concentration of colloidal calcium and phosphate, calcium and phosphate atom number per casein micelle or per phosphoserine pair at individual pH values. Based on van Hooydonk et al. (59) and Table 11.

pH	Colloidal Ca (m*M*)	Colloidal P (m*M*)	Ca atoms/ micelle	P atoms/ micelle	Ca atoms/ phosphoserine pair	P atoms/ phosphoserine pair
6.7	19.9	9.2	19973	9234	.234	.108
6.4	18.0	7.7	18066	7728	.211	.090
6.2	16.0	6.6	16059	6624	.188	.077
6.0	13.5	5.3	13550	5320	.158	.062
5.8	10.2	3.2	10238	3212	.120	.038
5.6	7.5	1.5	7528	1506	.088	.018
5.4	5.1	.5	5119	502	.060	.006
5.2	3.5	0	3513	0	.041	0
5.0	2.6	0	2610	0	.031	0
4.8	1.7	0	1706	0	.020	0

phosphoserine groups of caseins is linked through colloidal calcium phosphate even at the native pH of milk. As the pH is lowered, each phosphoserine pair possesses less colloidal calcium and phosphate. Those casein chains bound to the micelle through this linkage would readily or completely leave the micelle.

At a certain pH, the dissociated protein might be stable in the serum through H-bonding with water molecules. As the pH is further lowered, the increased $[H^+]$ compete with the protein for water molecules and make it unstable. This facilitates protein-protein interaction and induces reassociation among the protein molecules and between the protein and micelles. On the other hand, as pH approaches the isoelectric point of a protein, the protein would denature and its solubility would decrease markedly in an aqueous solution (33). Furthermore, some or all of the unsatisfied bonding sites of the denatured protein molecules could reform intermolecular bonds if they come into contact. Therefore, the dissociated protein molecules and casein micelles became reassociated.

At higher temperature, the tendency of casein micelles to coagulate was greater as shown by higher pH_g values. One of the reasons is the increased steric repulsion due to the protrusion of surface protein at lower temperature. Another cause may be the

decreased hydrophobic interactions with decreasing temperature. Hydrophobic interaction is entropy-driven. Entropy is a measure of the order of a system and decreases with increasing order. At lower temperature, water molecules are more ordered around protein molecules, minimizing entropy and free energy. Consequently, casein micelles would be more stable against aggregation with decreasing temperature and would require more acidification before gelation occurs.

CONCLUSIONS

The conclusions from this research can be summarized as follows:

1) Temperature had an influence on turbidity change as a function of pH.

2) Acidification rate had a more pronounced effect on turbidity change at 10°C than at 20, 30 or 40°C.

3) Average casein micelle size increased significantly with decreasing temperature.

4) During acidification, the shape of average micelle size change with pH was not affected by temperature.

5) Average micelle size did not vary much above pH 5.2 and increased greatly below pH 5.0 as the milk gelled.

6) Acidification rate did not influence average micelle size at 10°C.

7) Gelation pH of milk increased with increasing temperature while acidification rate had no effect on it.

8) Acidification rate, types of acidifying agents, and temperature had no effect on the rate at which curd firmness developed.

9) Casein micelles became less compact and their surfaces less distinct with decreasing temperature before acidification.

10) As pH decreased, protein was dissociated from and then reassociated with casein micelles.

11) Acidification rate had no effect on the microstructure change of casein micelles at 10°C.

12) Acidification by starter culture induced more protein dissociation than by GDL at 40°C, but reassociation occurred at lower pH with starter culture.

RECOMMENDATIONS FOR FURTHER STUDY

Recommendations for further research on changes during acidification of milk would include:

1) A study to determine which caseins are dissociated by lowering temperature or by decreasing pH or both so as to understand the role of hydrophobic interactions and electrostatic interactions in forming casein micelles.

2) A study to examine how and by how much phosphoserine groups are actually linked through colloidal calcium phosphate, and how the dissociable protein molecules are linked to casein micelles before dissociation and upon reassociation.

3) A study to determine if a size-determining framework of casein micelles exists, and which interaction(s) is (are) predominant during acidification.

4) A study to determine how temperature induces microstructural change of casein micelles and the average casein micelle size.

REFERENCES

1 Aoki, T., A. Kawahara, and Y. Kako. 1987. Role of individual milk salt constituents in cross-linking by colloidal calcium phosphate in artificial casein micelles. Agric. Biol. Chem. 51:817.

2 Aoki, T., T. Umeda, and Y. Kako. 1990. Cleavage of linkage between colloidal calcium phosphate and casein on heating milk at high temperature. J. Dairy Res. 57:349.

3 Banon, S., and J. Hardy. 1991. Study of acid milk coagulation by an optical method using light reflection. J. Dairy Res. 58:75.

4 Banon, S., and J. Hardy. 1992. A colloidal approach of milk acidification by glucono-delta-lactone. J. Dairy Sci. 75:935.

5 Bloomfield, V. A. 1979. Association of proteins. J. Dairy Res. 46:241.

6 Bloomfield, V. A., and C. V. Morr. 1973. Structure of casein micelles: physical methods. Neth. Milk Dairy J. 27:103.

7 Bringe, N. A. 1988. The mechanism of the acidic coagulation of casein micelles. PhD Dissertation, Cornell University, Ithaca, NY.

8 Bringe, N. A., and J. E. Kinsella. 1987. Forces involved in the enzymatic and acidic coagulation of casein micelles. Chapter 4 *in* Developments in food proteins-5. B. J. Hudson, ed. Elsevier Applied Science, New York.

9 Bringe, N. A., and J. E. Kinsella. 1990. Acidic coagulation of casein micelles: mechanisms inferred from spectrophotometric studies. J. Dairy Res. 57:365.

10 Bringe, N. A., and J. E. Kinsella. 1991. Effects of cations and anions on the rate of the acidic coagulation of casein micelles: the possible roles of different forces. J. Dairy Res. 58:195.

11 Bringe, N. A., and J. E. Kinsella. 1993. Calcium chloride, temperature, preheat treatments and pH affect the rate of acid-induced aggregation of casein. Food Hydrocoll. 7:113.

12 Brown, R. J. 1988. Milk coagulation and protein denaturation. Page 583 *in* Fundamentals of Dairy Chemistry. N. P. Wong, ed.Van Nostrand Reinhold Co. New York.

13 Chaplin, L. C. 1984. Studies on micellar calcium phosphate: composition and apparent solubility product in milk over a wide pH range. J. Dairy Res. 51:251.

14 Creamer, L. K., G. P. Berry, and O. E. Mills. 1977. A study of the dissociation of β-casein from the bovine casein micelle at low temperature. N. Z. J. Dairy Sci. Technol. 12:58.

15 Creamer, L. K. 1991. Some aspects of casein micelle structure. ACS Symposium Series. 454:148.

16 Dalgleish, D. G. 1984. Measurement of electrophoretic mobilities and zeta-potentials of particles from milk using laser Doppler electrophoresis. J. Dairy Res. 51:425.

17 Dalgleish, D. G., D. S. Horne, and A.J.R. Law. 1989. Size-related differences in bovine casein micelles. Biochem. Biophys. Acta. 991:383.

18 Dalgleish, D. G., and A.J.R. Law. 1988. pH-induced dissociation of bovine casein micelles. I. Analysis of liberated caseins. J. Dairy Res. 55:529.

19 Dalgleish, D. G., and A.J.R. Law. 1989. pH-induced dissociation of bovine casein micelles. II. Mineral solubilization and its relation to casein release. J. Dairy Res. 56:727.

20 Darling, D. F., and J. Dickson. 1979a. The determination of the zeta potential of casein micelles. J. Dairy Res. 46:329.

21 Darling, D. F., and J. Dickson. 1979b. Electrophoretic mobility of casein micelles. J. Dairy Res. 46:441.

22 Davies, D T., and A.J.R. Law. 1983. Variation in the protein composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature. J. Dairy Res. 50:67.

23 Donnelly, W. J., G. P. McNeill, W. Buchheim, and T.C.A. McGann. 1984. A comprehensive study of the relationship between size and protein composition in natural bovine casein micelles. Biochem. Biophys. Acta. 789:136.

24 Downey, W. K. 1973. Structure of bovine casein micelles. Neth. Milk Dairy J. 27:218.

25 Ekstrand, B., and M. Larsson-Raznikiewicz. 1984. Free zone electrophoresis of casein micelles. Acta Chemica Scandinavica. 38:351.

26 Green, M. L., and G. Crutchfield. 1971. Density-gradient electrophoresis of native and of rennet-treated casein micelles. J. Dairy Res. 38:151.

27 Griffin, M.C.A., and M. Anderson. 1983. The determination of casein micelle size distribution in skim milk by chromatography and photon correlation spectroscopy. Biochemica et Biophysica Acta. 748:453.

28 Heertje, I., J. Visser, and P. Smits. 1985. Structure formation in acid milk gels. Food Microstructure 4:267.

29 Holt, C. 1982. Inorganic constituents of milk III. The colloidal calcium phosphate of cow's milk. J. Dairy Res. 49:29.

30 Holt, C., D. T. Davies, and A.J.R. Law. 1986. Effect of colloidal calcium phosphate content and free calcium ion concentration in the milk serum on the dissociation of bovine casein micelles. J. Dairy Res. 53:557.

31 Horne, D. S., and C. M. Davidson. 1986. The effect of environmental conditions on the steric stabilization of casein micelles. Colloid and Polymer Sci. 264:727.

32 Jenness, R., and J. Koops. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. Neth. Milk Dairy J. 16:153.

33 Kauzmann, W. 1959. Some factors in the interpretation of protein denaturation. Adv. Protein Chem. 14:1.

34 Kim, B. Y., and J. E. Kinsella. 1989. Rheological changes during slow acid induced gelation of milk by D-glucono-δ-lactone. J. Food Sci. 54:894.

35 Kim B. Y., and J. E. Kinsella. 1990. Effects of calcium anions on the rates of casein aggregation. Food Hydrocolloids. 4:239.

36 Kirchmeier, O. 1973. Arrangement of components, electric charge and interaction energies of casein particles. Neth. Milk Dairy J. 27:191.

37 Knoop, A. M., E. Knoop, and A. Wiechen. 1973. Electron microscopical investigations on the structure of the casein micelles. Neth. Milk Dairy J. 27:121.

38 Lin, S.H.C., S. L. Leong, R. K. Dewan, V. A. Bloomfield, and C. V. Morr. 1972. Effect of Ca ion on the structure of native bovine casein micelles. Biochemistry 11:1818.

39 McMahon, D. J., and R. J. Brown. 1982. Evaluation of formagraph for comparing rennet solutions. J. Dairy Sci. 65:1639.

40 McMahon, D. J., and R. J. Brown. 1984. Composition, structure, and integrity of casein micelles: a review. J. Dairy Sci. 67:499.

41 McMahon, D. J., R. J. Brown., and C. A. Ernstrom. 1984. Enzymic coagulation of milk casein micelles. J. Dairy Sci. 67:745.

42 Munyua, J. K., and M. Larsson-Raznikiewicz. 1980. The influence of Ca^{2+} on the size and light scattering properties of casein micelles. 1. Ca^{2+} removal. Milchwissenschaft 35:604.

43 O'Connor, P., and P. F. Fox. 1973. Temperature-dependent dissociation of casein micelles from the milk of various species. Neth. Milk Dairy J. 27:199.

44 Payens, T.A.J. 1979. Casein micelles: the colloidal-chemical approach. J. Dairy Res. 46:291.

45 Pierre A., and G. Brule. 1981. Mineral and protein equilibria between the colloidal and soluble phases of milk at low temperature. J. Dairy Res. 48:417.

46 Pouliot, Y., M. Boulet, and P. Paquin. 1989. Experiments on the heat-induced salt balance changes in cow's milk. J. Dairy Res. 56:513.

47 Qvist, K. B. 1979. Reestablishment of the original rennetability of milk after cooling. 1. The effect of cooling and LTST pasteurization of milk and renneting. Milchwissenschaft 34:467.

48 Rajagopal, S. N., and W. E. Sandine. 1990. Associative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk. J. Dairy Sci. 73:894.

49 Rajput, Y. S., M. K. Bhavadasan, and N. C. Ganguli. 1983. Changes in the chemical status of calcium in casein micelles with the pH of milk. Milchwissenschaft 38:211.

50 Roefs, S.P.F.M. 1987. Structure of acid casein gels. A study of gels formed after acidification in the cold. Dissertation abstract. Neth. Milk Dairy J. 41:99.

51 Roefs, S.P.F.M., P. Walstra, D. G. Dalgleish, and D. S. Horne. 1985. Preliminary note on the change in casein micelles caused by acidification. Neth. Milk Dairy J. 39:119.

52 Roefs, S.P.F.M., T. van Vliet, H.J.C.M. van den Bijgaart, A. E. A. de Groot-Mostert, and P. Walstra. 1990. Structure of casein gels made by combined acidification and rennet action. Neth. Milk Dairy J. 44:159.

53 Rollema, H. S., and J. A. Brinkhuis. 1989. A 'H-NMR study of bovine casein micelles; influence of pH, temperature and calcium ions on micellar structure. J. Dairy Res. 56:417.

54 Schmidt, D. G. 1982. Association of caseins and casein micelle structure. Chapter 2 *in* Developments in Dairy Chemistry-1. P. F. Fox, ed. Applied Science Publishers. New York.

55 Schmidt, D. G., and J. K. Poll. 1986. Electrokinetic measurements on unheated and heated casein micelle systems. Neth. Milk Dairy J. 40:269.

56 Schmidt, D. G., P. Walstra, and W. Buchheim. 1973. The size distribution of casein micelles in cow's milk. Neth. Milk Dairy J. 27:128.

57 Slocum, S. A., E. M. Jasinski, and A. Kilara. 1988. Processing variables affecting proteolysis in yogurt during incubation. J. Dairy Sci. 71:596.

58 Tamine, A. Y., and H. C. Deeth. 1980. Yogurt: technology and biochemistry. J. Food Prot. 43:939.

59 van Hooydonk, A.C.M., H. G. Hagedoorn, and I. J. Boerrigter. 1986. pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. I. Effect of acidification on physico-chemical properties. Neth. Milk Dairy J. 40:281.

60 Visser, J., A. Minihan, P. Smits, S. B. Tjan, and I. Heertje. 1986. Effects of pH and temperature on the milk salt system. Neth. Milk Dairy J. 40:351.

61 Vliet, T. V., S.P.F.M. Roefs, P. Zoon, and P. Walstra. 1989. Rheological properties of casein gels. J. Dairy Res. 56:529.

62 Vreeman, H. J., B. W. van Markwijk, and P. Both. 1989. The structure of casein micelles between pH 5.5, and 6.7 as determined by light-scattering, electron microscopy and voluminosity experiments. J. Dairy Res. 56:463.

63 Wahlgren, N. M., P. Dejmek, and T. Drakenberg. 1990. A ^{43}Ca and ^{31}P NMR study of the calcium and phosphate equilibria in heated milk solutions. J. Dairy Res. 57:355.

64 Walstra, P. 1990. On the stability of casein micelles. J. Dairy Sci. 73:1965.

65 Walstra, P., and R. Jenness. 1984. Dairy chemistry and physics. John Wiley & Sons, Inc., New York.