Effect of Succinylation on the Rennet Coagulation of Milk

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ABSTRACT

The effects of succinylation on the rennet coagulation of milk were investigated to find a relationship between the level of chemical modification and the ability of milk protein to coagulate. The strengthening rate and the final firmness of the gel decreased as the extent of succinylation increased, which could be related to the microstructural differences caused by milk succinylation. The kinetics of nonprotein N release were not affected by milk succinylation, but the rennet clotting time increased as the extent of chemical modification increased. These results suggest that milk succinylation affected the secondary or aggregation phase of enzymatic coagulation by increasing the electrostatic repulsions between paracasein micelles and, more indirectly, by dissociating casein from micelles and preventing them from participating in network formation of the gel.
(**Key words**: succinylation, rennet milk gelation, rheology, electron microscopy)

Abbreviation key: **SEM** = scanning electron microscopy.

INTRODUCTION

Many investigations (1, 13, 15, 16) have been conducted on food proteins using specific chemical modifications in order to study the relationships between the structure and function of these proteins and to improve the functional properties. At this time, the functional property of casein coagulability in renneted milk is incompletely understood and characterized. The chemical modification of milk proteins constitutes one approach to probe the mechanisms involved in the enzymatic coagulation of milk. However, most of the studies dealing with the effect of succinylation on the physico-chemical properties of proteins have been conducted on isolated casein or artificial reconstituted micelles (10, 11, 14).

By modifying amino groups of protein with acyl groups, acylation allowed us to assess the contribution of the electrostatic charges implied by different mechanisms of protein interactions. Depending on the nature of the modifying agent, the mechanism of acylation can affect the net charge on the protein, either by partially suppressing the positive charge or by replacing one original positive charge by a negative charge. Acylation by means of negative charge addition includes reactions of proteins with anhydrides of succinates, methyl succinates, glutarates, and others (1, 3, 4, 8, 17). Compared with other acylation processes of this class, succinylation of food proteins has generally resulted in the most desirable functional qualities (5).

The purpose of this research was to investigate the effect of succinylation on milk proteins in their natural environment to find a relationship between coagulation ability and the level of chemical modification.

MATERIALS AND METHODS

Milk Preparation

Reconstituted skim milk was made by dissolving a commercial powder that had been processed using low heat (Laiterie Matines-SILI, Plouvien, France) at 12% (wt/vol) in deionized water. The milk was stored at 4°C for 12 h before use to allow components to equilibrate. To prevent bacterial growth, 0.02% (wt/vol) sodium azide was added.

Renneting and Rennet Clotting Time

Commercial rennet (520 mg of chymosin/L, EC 34234; Chr. Hansen's Arpajon, France) was used at 25×10^{-3}% (vol/vol) at 30°C; a fresh batch of rennet was used when activity loss reached 10%. Rennet clotting time was recorded from rennet addition to the

69

TABLE 1. The extent of protein succinylation as related to the succinic anhydride concentration in milk.

Succinic anhydride concentration	Protein succinylation[1] (%)	
(mM)	$\overline{X}$	SE
0	0[e]	. . .
1.5	4.2[d]	0.3
7.0	7.7[c]	0.7
20.0	18.8[b]	0.8
250.0	89.8[a]	4.0

[a,b,c,d,e]Means without a common superscripts differ ($P < 0.05$).

[1]Number of succinylated amino groups divided by the total number of reactive amino groups.

onset of aggregation by the method of Sommer and Matsen (18).

Procedure of Protein Succinylation

Milk protein succinylation was performed at room temperature (25°C) according to the method of Hoagland (4). Defined amounts of succinic anhydride (Merck, Darmstadt, Germany) were progressively added to reconstituted skim milk (Table 1) with vigorous stirring. During succinylation, the pH was kept constant at 6.7 by the addition of 10 N NaOH. Succinylated milk was used when its pH had stabilized. To determine the effect of the chemical modifications from succinylation on the rennet coagulation of milk, two types of reference milk samples were used: an untreated milk sample and a milk sample that had undergone the same pH variations as the succinylated samples using 0.5 N lactic acid.

Determination of the Extent of Chemical Modification

The ninhydrin assay, adapted from the procedure of Moore and Stein (9), was used to quantify the extent of chemical modification. The ninhydrin reagent was prepared by mixing 1.6 g of ninhydrin (Sigma Chemical Co., St. Louis, MO) and 0.24 g of hydrindantin with 20 ml of acetate buffer (6.5 M, pH 5.51) and 60 ml of methyl Cellosolve (Merck, Darmstadt, Germany). The reagent solution, which was unstable when stored, was prepared as and when required.

The ninhydrin assay was performed by mixing 2 ml of ninhydrin reagent with 2 ml of succinylated milk diluted 200 times with deionized water. The mixture was heated at 90°C for 15 min precisely, cooled to room temperature (25°C), and diluted with 3 ml of ethanol and water solution (50:50 vol/vol). The absorbance of the mixture was measured at 570 nm against a blank solution of distilled water and ninhydrin. A calibration curve was prepared by reaction of ninhydrin with standard solutions of N-α-carbobenzen-L-lysine p-nitrophenyl ester (Sigma Chemical Co.). The absorbance indicated the number of free amino groups that were available for reaction with ninhydrin reagent. The ninhydrin assay that was performed on untreated and succinylated milk allowed estimation, respectively, of the total number of reactive amino groups (TAG) and the number of amino groups that had not reacted with succinic anhydride (unmodified amino groups; UAG). The number of succinylated amino groups (SAG) was determined as SAG = TAG – UAG. The extent of milk succinylation was calculated using the ratio SAG/TAG, which was expressed as a percentage.

Rheological Measurements

Stiffness was followed as a function of time by dynamic measurements at 10 Hz using a rheometer (Viscoprocess; Metravib, Ecully, France) as described by Lagoueyte et al. (7). Stiffness, as the elasticity modulus (12), represents the ratio of the stress (Newtons per square meter) applied on the product to the strain (dimensionless number). All rheological measurements were started 1 min after rennet addition and were carried out in situ in the rheometer measuring cup at 30°C. Changes in the slope of the curve of the stiffness versus time permitted us to determine the gel point that corresponds to solution-gel transition.

Scanning Electron Microscopy

The microstructure of milk samples before and after rennet addition was examined with a JEOL JSM-6400F field emission scanning electron microscope (**SEM**; JEOL Europe S.A., Croissy/Seine, France) operated at 15 kV using the procedure described by Gastaldi et al. (2).

Kinetics of NPN Release

The kinetics of enzymatic hydrolysis of κ-CN in normal and succinylated milks were studied by measuring the release of NPN as a function of time. At appropriate intervals after rennet addition, 10 ml of a 24% (wt/vol) TCA solution were mixed with 10 ml of renneted milk samples. After filtration of the precipitated renneted samples through Whatman number 42
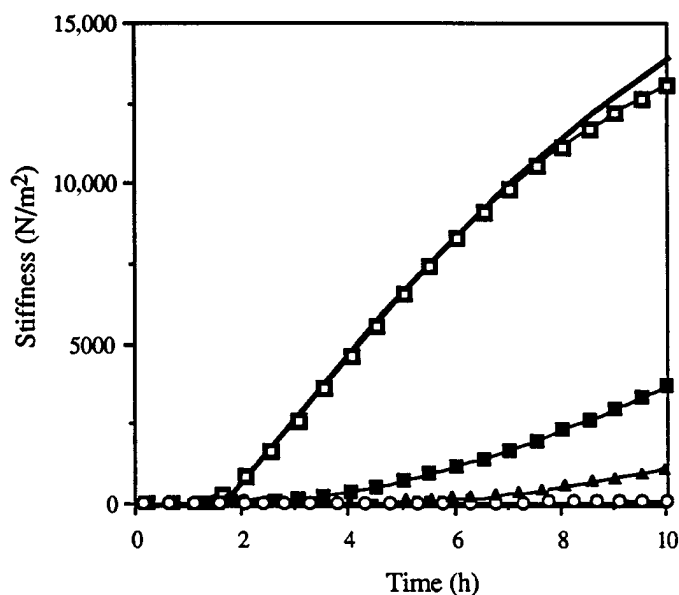
Figure 1. Effect of milk succinylation on stiffness during enzymatic coagulation: untreated milk (□), reference sample (———), and milk succinylated at 4.2% (■), 7.7% (▲), and 18.8% (○).

filter paper (Whatman, Clifton, NJ), the NPN content of the 12% (wt/vol) TCA filtrates was determined using the standard Kjeldahl method.

Protein Solubilization Analysis

Separation of the soluble phase of both the milk and rennet gels was achieved by centrifugation at 149,000 × g for 50 min at 30°C (Beckman ultracentrifuge, rotor Ti 70; Beckman Instrument France S.A., Gagny, France). After centrifugation, the supernatant was carefully removed, and the nonsedimentable N was determined by the standard Kjeldahl method. The casein content of supernatant was analyzed by SDS-PAGE according to the method of Laemmli (6) using a 5 to 20% separating gradient gel and a 4.5% stacking gel. Protein bands were fixed in 12% (wt/vol) TCA for 30 min, stained overnight in 0.05% (wt/vol) R-250 Coomassie blue, and destained in a solution of 5% (vol/vol) methanol and 7.5% (vol/vol) acetic acid.

Statistical Analysis

Experimental data were presented as the means of triplicate measurements from five experiments and subjected to ANOVA (Stat View; Abacus Concepts, Inc., Berkeley, CA). Fisher's protected least significant difference test was used to compare paired means, and differences between means were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Extent of Chemical Modification

As shown in Table 1, milk samples that were treated with increasing amounts of succinic anhydride to give a final concentration in milk of 1.5 to 250 mM, progressively succinylated 4.2 to 89.8% of the reactive amino groups in the milk proteins. At the pH of milk, succinic anhydride reacts mainly with ε-amino groups of lysine residues because of its relatively high pKa (≈10.6) and steric availability for reaction (1). At succinic anhydride concentrations higher than 20 mM, skim milk became slowly as transparent as whey and could be compared with an alkalinized skim milk (19). Accordingly, studies were conducted on samples of succinylated amino groups between 0 and 18.8% (Table 1) in order to avoid excessive protein denaturation.

Rheological Behavior of Succinylated Milk During Rennet Coagulation

The changes in the stiffness versus time for milk succinylated to various extent are reported in Figure 1. Results indicated that both the onset of gelation and the rate of gel strengthening were affected ($P < 0.05$) by the succinylation treatment applied to milk. Moreover, the observed changes were assumed to have been only caused by chemical modifications from succinylation because no significant differences were noticed between the samples of untreated and reference milks that had undergone the same pH variations as the 4.2% succinylated sample. The slope of the curves for stiffness versus time and the final firmness of the gel decreased as the extent of succinylation increased. As shown in Table 2, the solution-gel transition (i.e., gel point) appeared later as the level of succinylation increased.

TABLE 2. The effect of protein succinylation on the point of gelation determined by the change in the stiffness versus time at 30°C.

Milk succinylation[1]	Gel point	
(%)	(min)	
	X̄	SE
0	45.6[d]	0.5
4.2	210.6[c]	0.9
7.7	300.7[b]	1.2
18.8	421.2[a]	1.5

[a,b,c,d]Means with no common superscripts differ ($P < 0.05$).

[1]Number of succinylated amino groups divided by the total number of reactive amino groups.

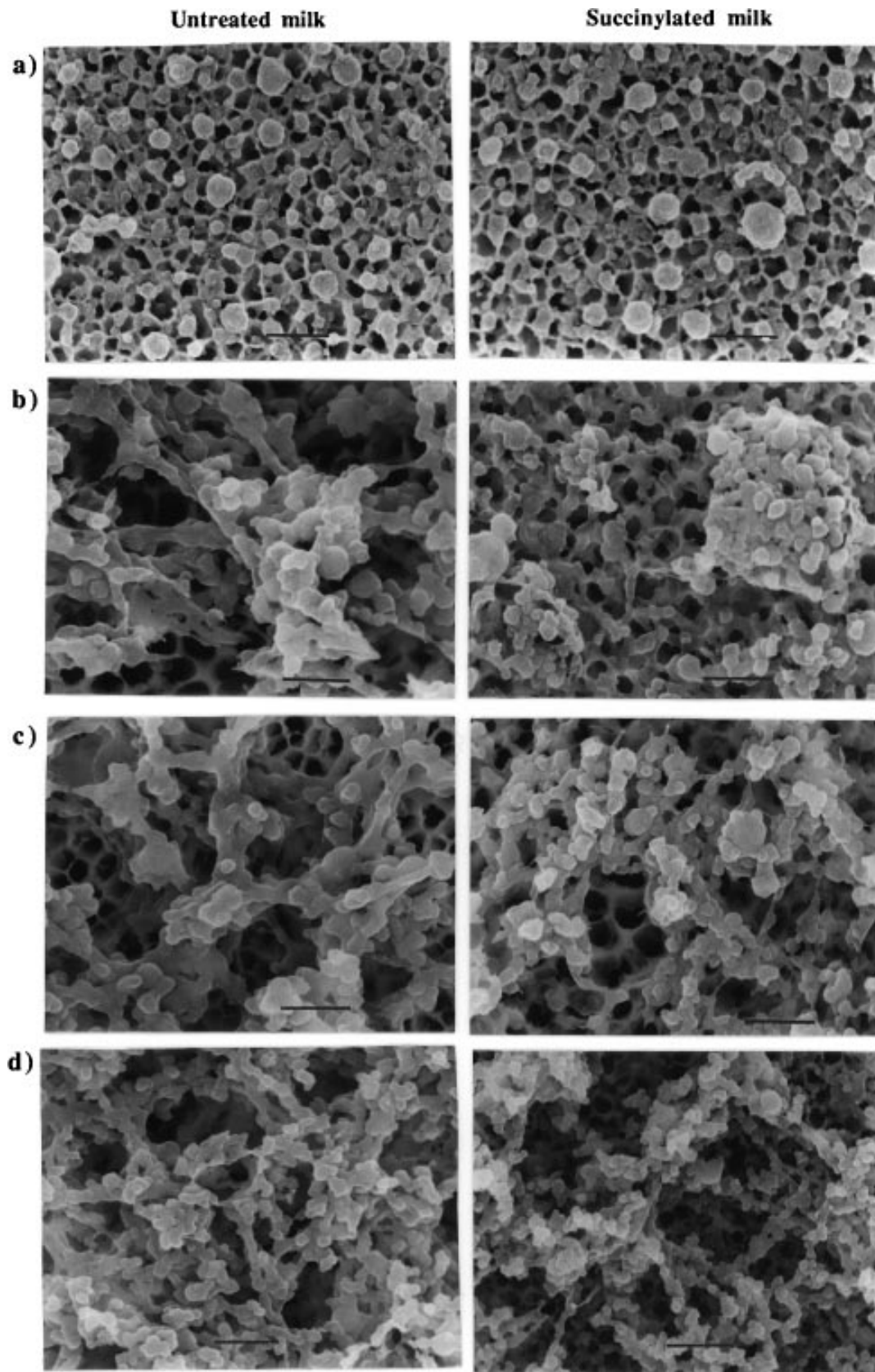**Untreated milk**   **Succinylated milk**

Figure 2. Scanning electron micrographs of samples of untreated and 7.7% succinylated milk before renneting (a), 1 h after renneting (b), 4 h after renneting (c), and 15 h after renneting (d). Scale bars indicate 0.5 μm.
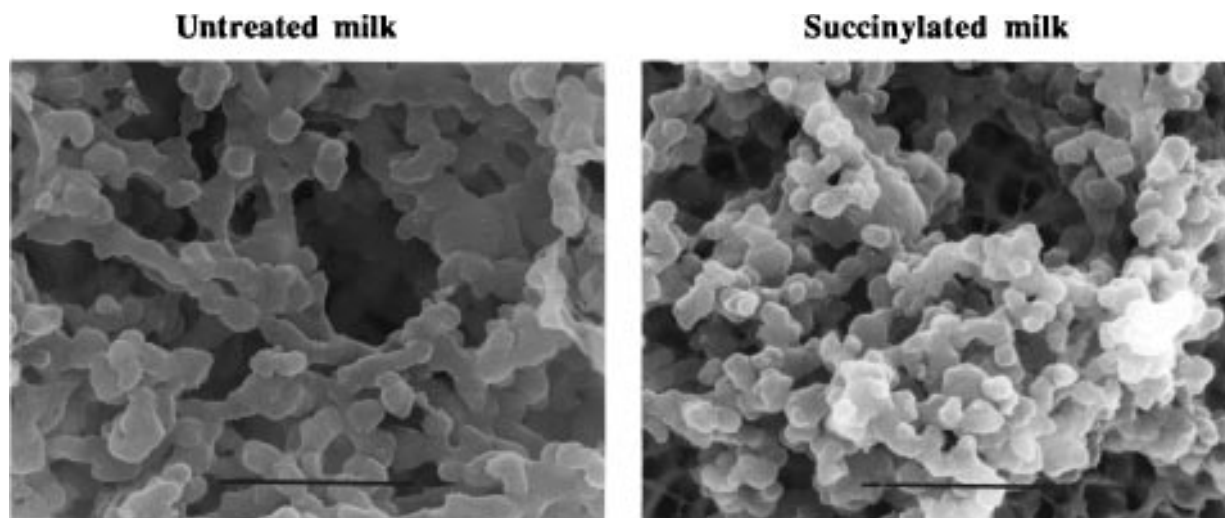
Untreated milk Succinylated milk

Figure 3. Scanning electron micrographs of gels made with samples of untreated and 7.7% succinylated milk 15 h after rennet addition. Scale bars indicate 1 μm.

Effect of Succinylation on the Microstructure of Renneted Milk

Observations using SEM on samples of untreated and 7.7% succinylated milks before and after renneting are shown in Figure 2. Before rennet addition, no differences were noticed between micrographs of untreated or succinylated milk (Figure 2a), indicating that the general aspect of casein micelles seemed to be unaffected by a chemical modification such as succinylation. This result suggested that the structural modifications induced by succinylation at a moderate level could not be observed by the SEM method used in this study.

On the contrary, differences in microstructure became noticeable between untreated and succinylated milk samples taken 1, 4, and 15 h after rennet addition (Figures 2, b, c, d). Micrographs of the succinylated milk showed that casein micelles began to react with one another to form micellar aggregates, which tended to increase as a function of time (Figures 2, b and c). In the untreated milk, casein micelles had lost their individuality and spherical shape and appeared to be connected to each other in a three-dimensional network (Figures 2, b and c). These microstructural differences confirmed the effect of succinylation on the gel point as just described. Indeed, according to the data reported in Table 2, the gel prepared from untreated milk was already formed by 1 h after rennet addition; in the 7.7% succinylated milk sample, gelation occurred later (i.e., 5 h after renneting). Micrographs that were obtained at 15 h after renneting (Figure 2d) showed the effect of milk succinylation on the interactions that were estab-
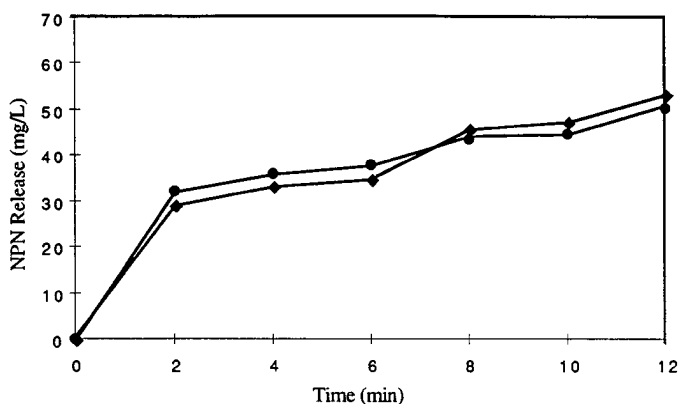

Figure 4. Effect of milk succinylation at 7.7% (♦) on the kinetics of NPN release compared with untreated milk (●).

TABLE 3. Effect of succinylation on rennet coagulation time (RCT).

Milk succinylation[1]	RCT	
(%)	(min)	
	$\overline{X}$	SE
0	11.34[c]	0.060
4.2	14.61[b]	0.025
7.7	31.27[a]	0.940

[a,b,c]Means with no common superscripts differ ($P < 0.05$).

[1]Number of succinylated amino groups divided by the total number of reactive amino groups.

lished between casein micelles constituting the network of the gel. In rennet gel prepared from succinylated milk, casein micelles appeared to be less deformed and fused than those in the untreated milk (Figure 3). In the gel that was formed from succinylated milk, casein micelles were arranged in large aggregates of particles with few connections. In contrast, the general aspect of the gel network made with untreated milk seemed to be more homogenous and have more connections between particles (Figure 2b). These microstructural differences caused by milk succinylation are coincidental to the differences in the rheological properties of the gels (Figure 1). Therefore, the lower rate of strengthening of the gel made from succinylated milk could be due to a decrease in the number of bonds between particles.

Effect of Succinylation on Rennet Coagulation of Milk

The effect of succinylation on the kinetics of NPN release was investigated to determine whether this chemical modification affected the primary or the secondary phase of rennet coagulation of milk. Figure 4 shows essentially no difference between kinetics, suggesting that succinylation of milk at 7.7% did not affect the primary stage of the enzymatic coagulation of milk. These results indicated that the primary action of the rennet, which involves splitting off the negatively charged macropeptide of κ-CN, was not hindered by the change in the charge of basic amino acid residues, such as lysine, that were located in proximity to the sensitive bond. Table 3 shows that the rennet clotting time increased proportionally with the level of milk succinylation, indicating that the alteration of the rheological properties of succinylated renneted milk gel reported could be due to changes in interactions that occurred during the secondary or aggregation phase of enzymatic coagulation.
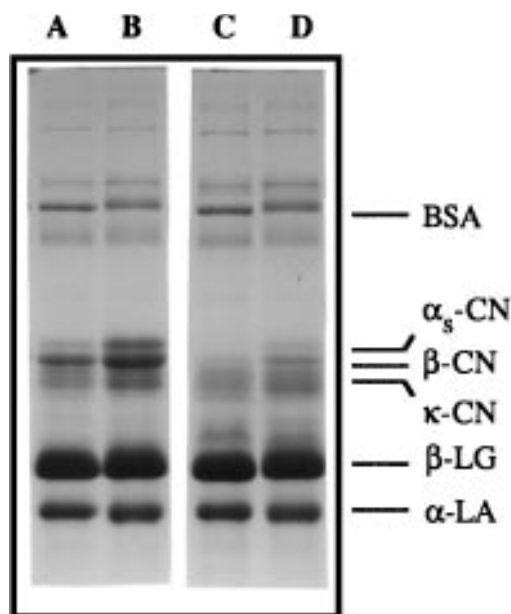

Figure 5. Electrophoresis patterns (SDS-PAGE) of the soluble casein content of milk and rennet gel as affected by milk succinylation. Lane A, untreated milk; lane B, 7.7% succinylated milk; lane C, rennet gel made with untreated milk; and lane D, rennet gel made with 7.7% succinylated milk.

The effect of milk succinylation on the nonsedimentable N content (Table 4) showed that increasing amounts of N were solubilized proportionately to the extent of succinylation. Analysis with SDS-PAGE indicated the presence of more α_s-CN, β-CN, and κ-CN in the soluble phase of succinylated milk than in the untreated milk (Figure 5). Succinylation of protein amino groups using succinic anhydride, which proceeds under mildly alkaline conditions, converts cationic amino groups to anionic residues, thus causing a large change in electrostatic relationships. The effect of succinylation on the dissociation of α_s-CN, β-CN, and κ-CN from micelles strongly suggested that electrostatic interactions were involved. However, our results did not confirm those reported by Singh and Fox (17), who observed that an increase in the negative charge on the micelles promoted only the dissociation of micellar κ-CN.

The presence of α_s-CN, β-CN, and κ-CN was also revealed on the SDS-PAGE gel pattern of the soluble phase of rennet gel prepared with succinylated milk (Figure 5), which implied that caseins dissociated from micelle were not reincorporated in the micellar phase and, consequently, could not participate in the formation of the gel network. Accordingly, the effect of

TABLE 4. Effect of succinylation on the level of nonsedimentable (at $149,000 \times g$ for 50 min) N.

Milk succinylation[1]	Nonsedimentable N	
(%)	(g/L)	
	X̄	SE
0	1.58[c]	0.06
4.2	1.82[b]	0.04
7.7	2.02[a]	0.07

[a,b,c]Means with no common superscripts differ ($P < 0.05$).

[1]Number of succinylated amino groups divided by the total number of reactive amino groups.

succinylation on the rennet coagulation time and the strengthening rate of the gel could be related to the decrease in the amount of micellar casein that participates to the formation of the gel network.

Nevertheless, milk protein succinylation resulted in more negatively charged micelles inducing electrostatic repulsions, which would eventually inhibit clotting. The reasons for the aggregation of renneted micelles at the molecular level are not fully understood, but more than one cause is probable because a number of factors are known to affect the rate of coagulation of chymosin-treated milk. The marked effect of temperature on the coagulation of para-casein micelles seemed to support the hypothesis that hydrophobic interactions are the first major driving force for protein-protein interactions and that electrostatic and hydrogen bonds contribute to the specificity and stability of these interactions. However, our results on the effect of succinylation on the secondary phase of milk coagulation appeared to favor the physical aggregation being primarily a process of charge neutralization.

The known change in the rate of enzymatic milk coagulation with the concentration of calcium ions also suggests that their role may be more than merely contributing to the charge neutralization process, but possibly actually participating in the phenomenon, perhaps in the form of a calcium bridge between aggregating micelles. Although no difference was observed (results not shown) in the calcium ion content of the soluble phase of rennet gels made from succinylated and untreated milks, the increase in the rennet coagulation time and the decrease in the strengthening rate of the gel from milk succinylation could be explained by the partial trapping of calcium ions by the carboxylic groups of succinic anhydride.

CONCLUSIONS

Milk succinylation affected the secondary or aggregation phase of enzymatic coagulation directly by increasing electrostatic repulsions between para-casein micelles and, more indirectly, by dissociating casein from micelles, preventing them from participating in gel network formation. As a consequence, the rennet clotting time was increased, and the strengthening rate of the gel was reduced in proportion to the extent of milk succinylation. The effect of succinylation on the formation of rennet milk gel was confirmed by differences in microstructure between gels made from untreated and succinylated milk. These results em-

phasized the importance of electrostatic interactions in the aggregation phase of rennet coagulation of milk.

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