On the Stability of Casein Micelles

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ABSTRACT

A view of the structure of the casein micelle is given. It is built of submicelles, roughly spherical aggregates of several casein molecules held together by hydrophobic bonds and salt bridges. Regions of amorphous calcium phosphate link the submicelles to each other; the ester phosphate groups form part of this colloidal phosphate. In this way, almost all regions of the casein molecules are severely restricted in mobility. The C-terminal part of the κ-casein is however predominantly present as flexible “hairs” located at the outside of the micelles. There are essentially two types of submicelles, with and without (much) κ-casein. The casein micelles greatly change in properties upon lowering the pH, mostly due to dissolution of colloidal phosphate; at still lower pH, increased formation of salt bridges predominates. Temperature also has pronounced effects: upon lowering it, the micelles become more voluminous, presumably due to protruding hairs of (mainly) β-casein. Also at high temperature (>70°C), parts of the casein molecules become more flexible.

Casein micelles are very stable. If conditions are changed they may disintegrate or aggregate. Aggregation mostly leads to formation of a gel. The application of the theory of fractal floc formation to gelation is briefly discussed: it serves to explain the very strong dependence of gelation times on volume fraction of aggregating particles.

The stability against aggregation is primarily due to steric repulsion, caused by the hairs of κ-casein, and at low temperature presumably also β-casein. The hairs on different casein micelles may however touch, and this may lead to lasting contact of the micelles, i.e., aggregation. The bonds formed can be salt bridges or, at high temperature, covalent bonds (chemical crosslinks). Hydrophobic bonds are probably not involved. The probability that casein molecules in different micelles may touch each other for a sufficient time for bonds to be formed appears to depend on electrostatic as well as steric repulsion, which thereby affect aggregation rate.

(Key words: casein micelle structure, stability, heat coagulation)

INTRODUCTION

In 1981, the late Theo Payens was the first recipient of the Miles-Marschall Award, and on that occasion he reviewed the subject of stable and unstable casein micelles (44). Since then, new work has been published and ideas have matured. Some of these developments and their technological significance will be briefly reviewed in this article.

Before the discussion on stability, the structure and some properties of the casein micelles will be considered, but detailed information is elsewhere (35, 52). Micelles are roughly spherical aggregates, mostly between 40 and 300 nm in diameter, and they are fairly voluminous, containing much water or, more precisely, a solution similar to milk serum. They contain essentially four kinds of casein molecules, at a molar ratio of about \( \alpha_{s_1}:\alpha_{s_2}:\beta:\gamma:\kappa = 4:1:4:1.3 \), and about 7% of the DM of micelles consists of inorganic material, predominantly calcium and phosphate. The micelles show considerable variation in composition, structure, and size distribution; milk serum also varies, especially in salt composition. The effects of variation will not be systematically considered here.
A MODEL OF THE CASEIN MICELLE

Figure 1 illustrates the structure of the casein micelle (79). The model has evolved over the years, and several authors have contributed to it (37, 56, 58), especially Schmidt (52) and Schmidt and Payens (54).

The reality of submicelles is now fairly clear. All electron microscopic methods show them, and the not-quite-spherical shape of the micelles fits well with the assembly from discrete units. Such an assembly is also strongly suggested by electron micrographs of lactating cells (3, 17), which show casein micelles in various stages of development. Neutron diffraction (60) and X-ray diffraction (46) also show the existence of discrete subunits in the micelle. Full agreement has not been reached about their size, but the diameter is probably between 10 and 15 nm, and they probably contain between 15 and 25 casein molecules. The bonds between the molecules in a submicelle are both hydrophobic and electrostatic (salt bridges) (52).

The submicelles are not all the same. Essentially, there are two major types, with and without κ-casein (40, 41, 79). This is not surprising, since κ-casein exists in milk as an oligomer, on average consisting of six molecules (61). κ-Casein is predominantly at the outside of the micelles, as follows for instance from the electron microscopy of micelles with labeled κ-casein (53) and from the proportionality between κ-casein content and specific surface area of casein micelles (12, 15). The very hydrophilic C-terminal part of most κ-casein molecules is sticking out from the micelle core into the solvent as flexible “hairs.” Evidence for this comes from hydrodynamic studies (24, 28, 29, 77) and from proton nuclear magnetic resonance (NMR) in D₂O (20, 49), which shows that part of the κ-casein has considerable freedom of motion. This concerns the C-terminal end, starting at a point between residues 86 and 96 (49); the phenylalanine-methionine (Phe-Met) bond cleaved by chymosin is at residues 105-106. These hairs are essential in providing stability against flocculation of the micelles. The hydrodynamic thickness of the hairy layer is about 7 nm.

The submicelles are linked together by colloidal calcium phosphate (CCP) (52). Much
TABLE 1. Approximate relaxation times\(^1\) for changes occurring with casein micelles when changing the temperature or some other change at constant temperature.

<table>
<thead>
<tr>
<th>Phenomenon observed</th>
<th>Temperature</th>
<th>Relax. time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolution of (\beta)-casein</td>
<td>37→5</td>
<td>.5</td>
<td>(6)</td>
</tr>
<tr>
<td>Exchange of (\beta)-casein</td>
<td>4</td>
<td>-.6</td>
<td>(6)</td>
</tr>
<tr>
<td>Exchange of Ca</td>
<td>18</td>
<td>1.5</td>
<td>(81)</td>
</tr>
<tr>
<td>Ca goes to micelles</td>
<td>78→20</td>
<td>18</td>
<td>(79)</td>
</tr>
<tr>
<td>Specific viscosity</td>
<td>30→20</td>
<td>.5 – 1</td>
<td>(68)</td>
</tr>
<tr>
<td>Gel permeability(^2)</td>
<td>20→30</td>
<td>2</td>
<td>(83)</td>
</tr>
<tr>
<td>Firming of rennet curd(^2)</td>
<td>20→30</td>
<td>1.5</td>
<td>(83)</td>
</tr>
<tr>
<td>Dynamic shear modulus(^2)</td>
<td>20→30</td>
<td>.1</td>
<td>(83)</td>
</tr>
</tbody>
</table>

\(^1\)Time needed for a change to proceed to \(1/e\) of its final value.

\(^2\)Of renneted skim milk.

The debate has focused on the composition and lattice structure of the CCP. It has no clear crystalline structure — the regions of CCP are too small for that — but Holt (23) and Holt et al. (25) have shown that CCP probably has a structure somewhat resembling brushite. This fact may seem surprising, since brushite has a Ca:P ratio of 1, whereas in the micelles the ratio of calcium to inorganic phosphate (Ca:P\(_{in}\)) is much higher, but is explained by the ester phosphate groups of the casein being part of the CCP (23). This fits with the observation that the very small regions of CCP nevertheless show no Ostwald ripening: these regions are thus stabilized by bonds with the casein. The CCP does not necessarily represent a state of thermodynamic equilibrium: that state would probably be precipitated hydroxyapatite, separate from the micelles. Additional CCP formed in the casein micelles by heat or concentration also has a Ca:P ratio of about 1 (39), but this does not necessarily have the same properties as the "natural" CCP.

The latter observation leads to the question: how dynamic are casein micelles? Exchange of radiolabeled casein (6) and Ca (80, 81) has been observed. However, most dynamic equilibria between substances in the serum and the micelles are far to the micelle side. Several kinds of small changes in the serum cause changes in the micelles, but these usually take some time. Table 1 summarizes some of the relaxation times observed, which are very long compared with those of most changes occurring at the molecular level (say, nanoseconds). Even after acid is added to milk, several minutes may be required before the pH becomes more or less constant (Geurts, unpublished). Presumably, most changes in equilibrium are more complicated, more than one relaxation phenomenon occurring. Moreover, the dissociation of a protein molecule requires that several bonds are broken simultaneously.

Casein micelles change considerably when pH is lowered (Figure 2). The CCP goes into solution, and although micelle-like particles remain, they have very different properties (48). Figure 2 suggests that the loss of CCP is primarily responsible for the changes observed; this is especially clear for the electrokinetic potential. The variation in voluminosity in the pH range 6 to 6.6 is not quite certain; moreover, some slight change in the size distribution of the micelles may occur when lowering the pH (76). The change in voluminosity parallels that of the spin-spin relaxation time \(T_2\) of the water protons in the solution; in the case of a caseinate solution, \(T_2\) is similar at low pH, but
it keeps increasing when the pH is raised. The very sharp transitions near pH 5.2 are also manifest in some properties of rennet skim milk gels (Figure 2). The loss tangent of the gel is a measure of the extent to which viscous relative to elastic behavior is present. (Note that the peak in viscous-like behavior near pH 5.2 corresponds to the optimum for “meltability” or “stretchability” of curd.) We may tentatively conclude that the bonds keeping the casein micelles together are weakest or fewest at pH 5.2 or 5.3. At lower pH, increasing electrostatic attraction between casein molecules keeps the “micelles” more tightly together; at higher pH an increasing quantity of CCP does the same (74).

Some changes with temperature are depicted in Figure 3. The increase in the electrokinetic potential with temperature seems to conflict with the increase in bound Ca, since more bound Ca should correspond to a smaller negative charge, hence, a smaller potential. The voluminosity of the micelles markedly increases at low temperature, although the data below 20°C are not quite certain because of the partial dissolution of β-casein. Because β-casein is primarily held in the submicelles by hydrophobic bonds, its dissolution at low temperature, where hydrophobic bonds are weak, is no surprise (52). In addition to some β-casein molecules going into solution, others may only
Figure 3. Properties of casein micelles at physiological pH as a function of temperature. Electrokinetic or zeta potential (various sources). Percentage of lactose found in the supernatant after high-speed centrifugation (6). Voluminosity of the casein micelles, calculated from specific viscosity data (79). Amount of Ca bound to $\alpha_s$-casein (13).

be loosened, thereby constituting another category of flexible hairs at low temperature. If the increase in voluminosity (Figure 3) resulted only from this effect, the hairy layer would become quite thick. Presumably, the core of the micelle would also swell to some extent and a limited disintegration of micelles into smaller ones might occur, which would also cause the voluminosity to increase (77).

At high temperatures, starting above about 70°C, another change occurs. Proton NMR in D$_2$O shows that considerable parts of the casein molecules become much more flexible (49), as if part of the submicelle structure melts. This may be important in the changes that occur upon heating.

**TYPES OF INSTABILITY**

Casein micelles can be disintegrated by removing (dissolving) CCP, thereby leaving free submicelles, or by adding agents that break hydrogen bonds or hydrophobic bonds, thereby disintegrating the submicelles (62). This aspect will not be considered herein.

Casein micelles, or rather the particles derived from them by changing their environment, can aggregate. They are generally much more prone to aggregation than is dissolved casein under comparable conditions. This is presumably due to the far greater loss of conformational entropy on aggregation of casein molecules, which compensates for the decrease in enthalpy when the bonds causing aggregation are formed.

Some types of aggregation can be distinguished. 1) Simple flocculation, as occurs with hard lyophobic particles; undisturbed, eventually leads to formation of a gel (2). 2) Flocculated micelles may fuse into bigger micelles of roughly spherical shape. If this type predominates, visible particles appear and possibly a sediment. 3) Micelles may aggregate by means of adsorbing (macromolecular) material connecting them, also leading to a gel.

The first is the most common type of aggregation, although casein micelles are certainly not hard lyophobic particles. Fusion, as noted under the second type, may occur after renneting, albeit slowly, and at high temperature if the pH is not too low. In these situations, the micelles are more or less hairless.

Table 2 lists various causes for coagulation. Cause 3, 5, and 6 will be considered further on in some detail; 1 will not be discussed, since its explanation is unclear (21); the gelation may be comparable with type 3. Cause 2, i.e., the irreversible aggregation of casein adsorbed onto air bubbles, even after the air itself has dissolved, has received little attention, but is quite clear (38). Cause 4 is rather trivial: the casein becomes insoluble near its isoelectric pH. At low temperature, where it still is soluble, the molecules form micelle-like particles that aggregate on increasing the temperature. Cause 7 is comparable to salting out, but not quite: a high concentration of CCP leads to a kind of growing or fusion of micelles. Apparently, the stabilizing factors are overcome if much CCP can be deposited. In the rare "Utrecht milk
**TABLE 2. Various causes for the aggregation of casein micelles.**

<table>
<thead>
<tr>
<th>Cause</th>
<th>Micelles changed?</th>
<th>Aggregation reversible?</th>
<th>Aggregation at low temperatures?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Long time (age gelation)</td>
<td>Presumably</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2. At air-water interface</td>
<td>Spreading</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3. High temperature (heat coagulation)</td>
<td>Chemically</td>
<td>No</td>
<td>. . .</td>
</tr>
<tr>
<td>4. Acid to pH = 4.6</td>
<td>No CCP left</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5. Ethanol</td>
<td>Presumably</td>
<td>No</td>
<td>?</td>
</tr>
<tr>
<td>6. Renneting</td>
<td>k-casein split</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>7. Excess Ca²⁺ etc.</td>
<td>More CCP</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>9. Some polymers</td>
<td>No</td>
<td>Mostly</td>
<td>Yes</td>
</tr>
</tbody>
</table>

abnormality,” i.e., milk with a low citrate content and a relatively high Ca²⁺ activity, this seems to occur spontaneously (51). Cause 8 is comparable, but here there also is true salting out because of the very high ionic strength resulting from freezing most of the water. An irreversible change appears to occur in the CCP, causing the aggregated micelles to be not fully redispersible after thawing (5). The material causing aggregation meant in Cause 9 may be various polysaccharides (59, 63).

It may be noted from Table 2 that in nearly all cases, the aggregating particles are no longer the native casein micelles. The latter are presumably perfectly stable, and they have to undergo some essential change before aggregation can occur. This fits with the irreversibility of the aggregation mostly observed. Also, even the altered micelles in many cases stable at very low temperature, but this may be a kinetic and not necessarily a thermodynamic stability. Once the “micelles” are aggregated, they do not disperse again on lowering the temperature. Renneted micelles do aggregate at 5°C at pH 4.6 (47).

**KINETIC ASPECTS**

For particles to aggregate, they must encounter each other. The encounter frequency can in principle be calculated from Smoluchowski’s theory (42). We will first consider aggregation type 2, where aggregating particles fuse, assuming the fusion to be rapid compared with the time between encounters. The coagulation time then is the time needed for visible particles to appear, say .2 mm large.

For diffusion controlled encounters it can be derived that

\[ t_{\text{coag}} = \left(\frac{\pi a^3 \eta}{8 kT}\right) \left(\frac{q^3}{\phi} W\right) \]

where \( k \) = Boltzmann’s constant, \( T \) = absolute temperature, \( a \) = original particle radius, \( \eta \) = viscosity, \( q_a \) = radius of the particle when visible (or \( q^3 \) the number of original particles making up a visible particle), \( \phi \) = the volume fraction of the particles, and \( W \) = the stability factor, i.e. the ratio of the number of encounters to the number leading to lasting contact.

For simple flocculation, it is now well established that the flocs formed are of a fractal nature (16, 28, 36), i.e., their structure is scale invariant at scales larger than \( a \). Considering the radius \( R \) of a growing floc containing \( N_p \) particles, we have:

\[ N_p = (R/a)^D \]  

where \( D \) is the fractal dimensionality, which is always <3. Equation [2] is found to hold remarkably well in a wide variety of situations and over a very wide range of floc sizes. The magnitude of \( D \) depends on some conditions, such as the colloidal interactions between the particles (16). The number of particles a floc of the same radius could contain if they were close packed \( N_a = (R/a)^3 \). Consequently, the volume fraction of particles in the floc is given by:

\[ \phi_{\text{floc}} = N_p/N_a = (R/a)^{D-3} \]

Thus, \( \phi_{\text{floc}} \) becomes ever smaller as the floc
Applying again Smoluchowski's theory, the gelation time turns out to be:

\[ R_{\text{crit}} = a \phi^{1/(D-3)} \]  \hspace{1cm} [4]

Applying again Smoluchowski's theory, the gelation time turns out to be:

\[ t_{\text{gel}} = (\pi a^3 \gamma / kT) \phi^{3/(D-3)} W \]  \hspace{1cm} [5]

For various types of casein gels (2) and for heat coagulation (Nieuwenhuijse et al., to be published), \( D = 2.3 \pm .1 \).

Results calculated according to Equations [1] and [5] are in Figure 4. The theory outlined here is simplified, not taking into account polydispersity (which may cause somewhat quicker aggregation), any effects of velocity gradients (quicker), or increased viscous resistance when particles approach closely (slower). Nevertheless, Figure 4 serves to illustrate important points, such as the enormous effect of \( \phi \) on gelation time. For example, homogenization of milk and especially cream may greatly speed up any aggregation process of the casein micelles. Homogenization causes casein micelles or fragments thereof to become attached to the fat globules, which now react as if they were casein micelles (38). Thus, homogenization greatly increases the effective volume fraction of casein, the more so if homogenization clusters have formed (38). This, in combination with Figure 4, serves to explain the great detrimental effect of homogenization on heat stability, especially of cream (33).

What is the magnitude of \( W \) or what magnitude is needed to provide stability? Figure 4 shows that milk at room temperature (\( \phi = .09 \)) would have a \( t_{\text{gel}} \) of about 10 s if \( W = 1 \). Because the milk may be stable (if sterile and without proteolytic enzymes) for say 3 yr, this implies that \( W \) is at least about \( 10^7 \). With concentrated (evaporated) milk at 120°C, \( W = 1 \) would lead to a coagulation time of .01 s, taking into account that the encounter frequency at 120°C is about 10 times that at 20°C. Since the coagulation time mostly is at least \( 10^{-3} \) s, \( W \) must be about \( 10^5 \) or more under these conditions.

The essential question is whether the stability factor can be predicted: \( W \) can be >1 because only part of the surface of the particle is reactive, i.e., lasting contact between two particles depends on their orientation when they meet. An example may be casein micelles during renneting. The other cause for slowing down aggregation is that the particles do not always come close enough to make lasting contact, because of a net repulsion between them caused by colloidal interaction forces.

**COLLOIDAL INTERACTION**

In the classical Deryagin Landau Verwey Overbeek (DLVO) theory (22) electrostatic repulsion and Van der Waals attraction are taken into account to calculate the free energy needed to bring two particles from infinite to some close distance from each other. If this interaction free energy is negative at all distances, the particles can come to touch each other, i.e., flocculate. If the interaction free energy is positive at some (small) distance, its maximum value may roughly be taken as an activation free energy for aggregation, thus permitting the calculation of \( W \). The DLVO theory has often been applied to casein micelles.
cannot explain their stability (43): the maximum is too low. Moreover, the presuppositions of the theory are not fully met; the particles are not perfect homogeneous spheres, and so-called hydration repulsion, which is manifest up to distances from the particle surface of about 1 nm (32), is not taken into account. Nevertheless, some variables that affect micelle stability seem to correlate at least qualitatively with predictions from the DLVO theory. Some important variables in this theory are:

1. For large particles, the maximum interaction free energy is higher, and the particles would thus be more stable: This does not fit with the observations (10), but such a size dependence has rarely been found at all.

2. A higher surface potential, hence, a higher charge of the particles, causes better stability: This may well fit, and the negative potential of the micelles is particularly decreased by decreasing the pH and by increasing the Ca\(^{2+}\) activity (71).

3. A higher ionic strength causes less stability, because the electrostatic repulsion works over a shorter distance: This does not always fit, but addition of salt also dissolves some micellar Ca and somewhat increases micelle voluminosity (see variable 5).

4. A lower dielectric constant gives less stability, because it decreases electrostatic repulsion: This would fit with the destabilizing action of alcohols.

5. A higher Hamaker constant would cause lower stability. The Hamaker constant is a material property and is a measure of the intensity of the attractive London-van der Waals forces acting between the particles. In the DLVO theory, it is the difference in properties between the particles and the continuous phase (solvent) that matters. If casein micelles have a higher voluminosity, i.e., contain more solvent, the difference between them and the solvent becomes less, hence the Hamaker constant lower. This appears to fit with the general observation that a higher voluminosity of the micelles goes along with a higher stability (79).

All the same, an additional mechanism for providing colloidal stability must be present, and it is now fairly clear that steric repulsion caused by the micellar hairs is responsible (14, 24, 26, 27, 71, 73, 77, 78). In steric repulsion, two mechanisms can be distinguished (75). If the presence of a second particle restricts the freedom of motion of the flexible hairs on the surface of a particle, this always causes repulsion. This is called the volume restriction term, and its magnitude is proportional to the hair density. If the hairy layers of two particles overlap (interpenetrate), the solvent quality of the hairs determines what will happen: if it is good, the so-called mixing term is repulsive (osmotic repulsion); if it is poor, the term may be attractive. Often, the mixing term is predominant; its magnitude is proportional to the hair density squared. Theories for the calculation of steric repulsion are now available (50, 75), but too many uncertainties remain to apply them to

Figure 5. Hypothetical picture of interactions between two casein micelles, (a) illustrating the configuration of hairs and charges (oversimplified). In (b), the average segment density of the hairs of one micelle as a function of the distance from the core of that micelle is illustrated, as well as the relative probability of finding a segment of a hair of the other micelle at the same place, for a high and a low negative charge of the micelles.
the casein micelles. Nevertheless, the repulsion must be very considerable, because of the hydrophilic nature of the macropetide part of \( \kappa \)-casein. This is borne out by the observation that casein micelles in a pellet obtained by high speed centrifugation, where they are so closely packed as to materially deform one another, can nevertheless be resuspended. How then can casein micelles be made to aggregate in some conditions, and how can the effects of the variables listed above be explained?

An attempt at an explanation is illustrated in Figure 5. Figure 5a is a greatly oversimplified picture of the micellar surface. The hairs themselves are charged, in addition to the charge on the core surface, and this has two effects. First, the electrostatic repulsion extends over a distance much farther from the core surface than would be the case if the hairs were uncharged; this is because the thickness of the electrical double layer (the distance over which an electrostatic potential decreases – due to shielding by counterions in the solution – to 1/e of its value at the surface) is in milk only about 1.1 nm (79), whereas the hairy layer is much thicker. A first attempt at deriving the electric potential of the micelles as a function of distance from the core has been made (24) and appears to fit with this idea. Second, the charge affects the conformation of the hairs themselves. At high pH the charges on the hairs are predominantly negative, and this causes them to “stretch” somewhat, thus extending farther from the particle surface. At lower pH, fewer negative and more positive charges are on the hairs, which would cause them to move or less “curl up”. At the isoelectric pH, no hairy layer is probably left, except at low temperature.

Figure 5b shows a presumption about the probability that a segment of a hair at one micelle is found within the hairy layer of another micelle. This probability must be determined by the steric repulsion exerted by the hairy layers themselves, but also by the electrostatic repulsion. At present, these probabilities cannot be calculated, but in a qualitative sense the picture may be correct. The importance is that the hairs, which show continuous Brownian motion, may touch one another. If that occurs at positions of reactive side groups, crosslinking may occur, thereby linking the micelles together. The stability factor \( W \) may now be interpreted as the inverse of the probability of touching of reactive sites of hairs from two micelles encountering each other, multiplied by the inverse of the rate constant of the crosslinking reaction. It appears very unlikely that the cores of the micelles can come to touch, unless the hairy layer is somehow removed.

Figure 4 reveals that at low temperature the voluminosity of the micelles is higher, and this is presumably due to a (further) extension of hairs of \( \beta \)-casein (77), thereby providing additional steric repulsion. The higher voluminosity may also go along with a lower Hamaker constant, as mentioned. The absolute value of the electrokinetic potential is lower at a lower temperature, which fits neither with the increased stability (although variation in the potential is of questionable importance in these conditions) nor with the decreased adsorption of Ca ions, which would have an opposite effect on the potential. The explanation probably lies in the fact that a thicker hairy layer causes the slipping plane, at which the potential is sensed, to be farther away from the micelle core, thus at a distance where the potential has further decayed.

The considerable effect of temperature on the aggregation rate of micelles under various conditions (Table 2) may not be taken as an indication that hydrophobic bonds are the cause for keeping the micelles together after they have touched. Undoubtedly, any stronger steric repulsion at low temperature due to hairs of \( \beta \)-casein is ultimately caused by the hydrophobic bonds responsible for keeping \( \beta \)-casein in the micelles to be much weaker at a lower temperature. But this causes only a higher activation free energy for aggregation, not a lower bond energy between micelles. This is borne out by the observation that the shear moduli of acid and rennet milk gels considerably increase after lowering the temperature (47, 83). If hydrophobic bonds were to keep the micelles aggregated, the modulus should become lower or the gel should even dissolve again on lowering the temperature.

What then may be the kind of bonds causing lasting contact between closely approached micelles? The first type of bond is salt bridges, either between negatively and positively charged groups on either peptide chain, or mediated by Ca ions, or even by a CCP-like linkage. Presumably, several bonds must be
formed between two micelles for the contact to be lasting, considering the short lifetime of many ion pair bonds. The tendency to form salt bridges presumably increases with increasing supersaturation of milk salts (predominantly phosphates), thus with increasing concentration and increasing pH, and for the same pH at increasing Ca²⁺ activity and temperature. A parallel may be seen with the effect of pH in the range 5.3 to 6.7 on micelle properties (Figure 2). A second type of bond is the covalent linkages between groups on the peptide chains, of which various types can be possibly formed (79), but only at high temperatures at a reasonable rate. Presumably, one bond would suffice to ensure lasting contact.

After the micelles have been linked, further bonds, also of another nature, can be formed, the micelles more or less fusing. Complete or partial fusion has been observed after renneting (19), at high temperature at high pH (7), and at the air/water interface at room temperature (38).

**ETHANOL STABILITY**

This aspect has especially been studied by Horne et al. (26, 27, 29, 30, 31). Their work confirms that steric stabilization by a hairy layer is essential. But electrostatic effects are also present. Ethanol stability decreases with decreasing pH, hence decreasing charge; Ca²⁺ activity and ionic strength also play a part. Addition of ethanol lowers the dielectric constant, and according to the DLVO theory markedly reduces electrostatic repulsion. Ethanol also lowers the solvent quality for the macropeptide part of κ-casein. This factor and the decreased electrostatic repulsion between negative groups on one hair may cause the hairs to curl up and finally collapse. Correlation was good between the hydrodynamic thickness of the hairy layer and the ethanol concentration needed to cause aggregation.

**RENNETING**

Renneting involves two reactions, an enzymatic one in which chymosin (EC 3.4.23.4) or another proteolytic enzyme splits a macropeptide off κ-casein, followed by aggregation of the now formed paracasein micelles if temperature and Ca²⁺ activity are high enough. Payens (45) pioneered the quantitative study of the kinetics of both reactions combined. Others have contributed substantially (8, 14, 73). Dalgleish has especially studied the stability of paracasein micelles (9, 11).

A look at Figure 1 makes clear that chymosin thus acts as a “depilatory” (77, 78). Because the position where the enzyme splits the hair is near the micelle core, to realize the splitting by means of immobilized enzyme is virtually impossible, which agrees with the conclusion reached by careful consideration of the experimental evidence (4). Because Brownian motion of the casein micelles is negligible compared with that of the enzyme molecules, it is to be expected that the enzymatic reaction is first order, as is indeed observed (72). This implies and also agrees with other evidence, that the enzyme randomly attacks the κ-casein: the enzyme molecule moves by Brownian motion through the serum and through the hairy layer around a micelle encountered, until it happens to reach the vulnerable site at the κ-casein chain, which it will attack if it is in the correct orientation, subsequently to diffuse away. This

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**Figure 6.** The relative aggregation rate of partly renneted casein micelles, expressed as the logarithm of the stability factor W, as a function of the degree of conversion of the κ-casein. Results (73) derived from the viscosity change in undiluted skim milk (○) and (●) derived from the turbidity change of a diluted micellar suspension (●). Temperature of experiment indicated.
is in agreement with the observation that negligible adsorption of chymosin on paracasein occurs, at least at physiological pH; at lower pH, the enzyme becomes somewhat adsorbed (73; Geurts, unpublished data). The rate of the enzyme reaction and the effect of some variables on it could be fairly precisely calculated from first principles (73).

The stability factor of aggregating micelles for various degree of $\kappa$-casein conversion is illustrated in Figure 6. Measurable aggregation only occurs after most hairs have been removed, in agreement with the concept of steric repulsion. There may be two alternative explanations for the linear relation between log $W$ and degree of conversion. One is that a few remaining hairs cause a weak repulsion. At a low hair density, the volume restriction term of steric repulsion appears likely to remain, the mixing term becoming negligible (73, 75). In that case $kT \ln W$ may be considered to represent an activation free energy for aggregation. Because of the uncertainty about the conformational freedom of the hairs, the effect cannot be calculated, although the slope observed in Figure 6 is not unreasonable. The other explanation is that even one remaining hair causes too much repulsion and that the possibility of lasting contact depends on the orientation that two partly depleted micelles have with respect to each other when meeting. Only if both micelles are "bare" at the area of meeting, would aggregation occur. This yields a similar relation between log $W$ and degree of conversion (11), and the predicted slope is close to that observed. Possibly, the real situation involves both mechanisms. [The line in Figure 6 cannot be extrapolated to lower degrees of conversion, since at, say, 65% conversion, a very slow aggregation could be observed (73); partially converted micelles may be subject to another, very slow, aggregation reaction.]

Another repulsion also exists. It has been observed (9) that only at high temperature (about 60°C) and not too low Ca$^{2+}$ activity does the aggregation rate approximate the Smoluchowski limit; it is then slower by a factor of only two or three. The temperature effect is presumably explained by additional repulsion due to protruding $\beta$-casein hairs at lower temperature. A clear effect of calcium has been found: at a Ca$^{2+}$ concentration below 2 mM and 30°C, fully converted micelles do not or hardly aggregate (9). One may expect this to act via the higher charge on the micelles, because Ca ions lower the charge. However, lowering the pH at constant Ca$^{2+}$ activity (and thus lowering the charge) hardly affects rennet coagulation rate. Although no convincing results have been published on the effect of pH on the aggregation rate of fully renneted micelles, the results on firming rate of rennet gels at a stage where the enzymatic reaction is virtually complete leave little doubt (18, 84). Moreover, at high temperature, variation in the Ca$^{2+}$ activity has far less effect on the aggregation rate (9). Thus, variations in the electric charge are unlikely to have much effect under these conditions. This is in agreement with calculations done according to the DLVO theory that show little effect of variation in electrostatic repulsion for paracasein micelles (18). Another explanation of the effect of calcium may be that deflocculation of paracasein micelles occurs if Ca$^{2+}$ activity is low compared with its activity at saturation. In other words, Ca may be needed in providing lasting bonds, and if insufficient Ca is present, once aggregated micelles may separate again. This
works out as if the stability factor is increased.

In practice, renneting occurs much faster at lower pH. This is partly due to the enzymic reaction proceeding faster (70). Moreover, at lower pH chymosin tends to absorb onto paracasein micelles, and once adsorbed, it may thus remove κ-casein hairs over a certain area of one micelle before desorbing and diffusing away. This implies that at an earlier stage bare patches are formed, permitting aggregation of micelles at a lower degree of conversion, as is indeed observed (70).

**HEAT COAGULATION**

The heat stability of milk is an intricate subject. The strong and strange dependence of heat coagulation time (HCT) on pH, with an optimum of about 6.6 and a pessimum (i.e., the pH at which the HCT shows a local minimum) of about 6.85, has long defied explanation. Heating milk profoundly affects the serum composition, the most striking change being a gradual lowering of the pH, but the micelles may also change considerably. In recent work by van Boekel et al. (64, 65, 66), much of the literature is also reviewed, and we will largely follow those studies.

Figure 7 gives information on the rate of aggregation reactions of the casein micelles at high temperature, in this case, without whey proteins being present. Figure 7 shows that two aggregations reactions occur, and these were studied in more detail. Reaction 1 starts immediately after a high temperature is reached, mostly at a slow, constant rate. It does not significantly depend on temperature in the range 120 to 140°C. It is very dependent on the Ca$^{2+}$ activity, and it most probably leads to formation of salt bridges between the micelles. If only this reaction has occurred, the aggregated micelles can be dispersed again by CCP-dissolving agents. The reaction is dependent on pH, proceeding fast at very low initial pH; but it is fairly independent of the rate of lowering of pH during heating, which may not be surprising, because such a lowering does not significantly affect the Ca$^{2+}$ activity. The reaction is second order with respect to time and casein concentration (other conditions being equal). Reaction 2 starts only after the pH has reached a low value, and then proceeds at a rapidly increasing rate (Figure 7). The HCT (if due to reaction 2) is therefore dependent on the initial pH and on the rate of pH decrease, but not on protein concentration, since that hardly affects the rate of acidification. The temperature dependence is high, $Q_{10}$ being about 3, which is somewhat higher than the $Q_{10}$ for acid production. The aggregates formed by this reaction cannot be redispersed any more, neither by CCP dissolving agents, nor by urea, nor by both. The aggregation thus appears to be caused by chemical cross-linking.

At high temperature κ-casein becomes dissociated form the micelles at high pH (1). In the absence of whey proteins, the dissociation increases from about 0 to over 50% in the pH range 6.2 to 7.6 (57). In normal milk, the depletion of κ-casein is less at low pH and higher at high pH, a comparable transition occurring in the range 6.5 to 6.9. This is presumably caused by the association of β-lactoglobulin with κ-casein, which occurs at all pH: at low pH denatured β-lactoglobulin is at the
micelles, at high pH in the serum. The depletion of κ-casein from the micelles also depends on the Ca\(^{2+}\) activity.

From these and some other observations, a model for the heat coagulation was proposed, which is schematically given in Figure 8. Depleted micelles are supposed to be far less stable than nondepleted ones because of the scarcity of κ-casein hairs. In either case, the stability will increase with pH because of increased charge, hence, increased electrostatic repulsion. Near pessimum pH, salt-induced aggregation predominates if the HCT is indeed short; this also depends on the ratio of κ-casein to β-lactoglobulin and the Ca\(^{2+}\) activity. As the pH decreases to values below the pessimum, the κ-casein-β-lactoglobulin complex associates again with the micelles, rendering them far more stable. When starting at a high pH, this may already happen before the micelles have aggregated to a considerable extent via reaction 1; this is reasonable, because during this acidification, the Ca\(^{2+}\) activity does not materially increase and it is thus much lower at, say, pH 6.8 than when starting to heat at that pH.

Which cross-linking reaction is involved is unknown. Several reactions have been reported (79), and they all proceed faster at a higher temperature but also at a higher pH. This seems to disagree with the effect of pH on HCT. However, at lower pH there will be less electrostatic repulsion and as illustrated in Figure 5, this will provide opportunities for the hairy layers to interpenetrate more deeply and more often, thereby providing more possibilities for reactive side groups on the peptide chains to make contact.

The heat stability of concentrated milk is at present under study in the author’s laboratory (Nieuwenhuijse et al., unpublished). The conclusions reached for nonconcentrated milk appear to hold. The theory of fractal floc formation can be usefully applied at low pH. At higher pH, where the micelles are depleted of κ-casein, rapid fusion of flocculated micelles occurs, making the heat coagulation still more intricate.

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