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THE INFLUENCE OF TEMPERATURE ON THE FLOCCULATION RATE OF RENNETED CASEIN MICELLES

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The flocculation rate constant of completely renneted casein micelles in milk ultrafiltrate was measured by Rayleigh light scattering between 20 and 35°C. In this temperature range an apparent energy of activation of 103 kJ/mol (±11 kJ/mol: $n = 50$) was measured. At 15°C clotting was not longer perceptible. The activation of the flocculation between 20 and 35°C is explained not so much by the height of the energy barrier separating the clotting micelles, as by the very negative temperature coefficient of that barrier. In line with this conclusion it is suggested that renneted micelles adhere through hydrophobic bonding. The flocculation rate constant of renneted casein micelles is independent of micelle size at the four temperature levels studied.

1. Introduction

The process of the curdling of milk is a well known example of an enzyme-triggered flocculation reaction. Its significance to the dairy industry is obvious and technologically important aspects of the process have been discussed in recent reviews [1,2]. This article will deal with some mechanistic aspects of the reaction.

In milk the clotting species proper is the casein micelle, a globular, proteinaceous particle with an average radius of about 100 nm [3–5]. Its destabilization sets in with the limited proteolysis of the κ-casein component of the micelle by the clotting enzyme [6,7]. In this study we have used diluted, commercial rennets as the enzyme source.

From the proportion of the κ-casein in the micelle (about 12%), its molecular weight (19000), as well as the molecular weight of the peptide split off by the rennet (6700), it is readily calculated that the decrease in micelle weight as a consequence of proteolysis is only about 3–4%. Such changes are at the detection limit of the light-scattering technique, which we have used to measure the progress of the flocculation.

In a number of recent articles on the kinetics of enzyme-triggered flocculation reactions [8–10], the origin of the lag in the clotting was explained on the basis of sequential Michaelis-Menten and Von Smoluchowski kinetics. It was also shown [10,11] that after complete conversion of κ-casein by the enzyme, one is left with a purely 'smoluchowskian' system, in which the time dependence of the weight-average particle weight, $\bar{M}_w$, is given by

$$\bar{M}_w(t) = M_o(1 + k_s c t). \quad (1)$$

where M_o is the particle weight at the start of the reaction, k_s a universal flocculation rate constant, c the micelle concentration and t the reaction time elapsed since the substrate became exhausted by the enzyme.

From the linear stage in the clotting process, Dalgleish et al. [12] deduced a flocculation rate constant of about 10^7 l mol^{-1} s^{-1} at room temper-

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ature, i.e., about 600-times smaller than the diffusion-controlled limit. Since with completely stripped micelles an orientational constraint on the kinetics appears unlikely *, this value would suggest the occurrence of a moderate energy barrier opposing the adhesion of the renneted micelles. The nature of this energy barrier is not well understood, mainly because the conventional analysis in terms of the interplay of long-range electrostatic repulsion and London-Van der Waals attraction appears to break down [9,14]. In the present study we have re-approached the problem by measuring the temperature coefficient of the flocculation rate between 20 and 35°C.

2. Experimental procedures and results

The isolation and fractionation of casein micelles according to size and the preparation of milk ultrafiltrate ('MUF') have been described in detail in a previous paper [12]. For the present investigation the milk of an individual cow was aseptically drawn and skimmed at 2500 × *g* for 30 min. The definition of the various size fractions can be observed in fig. 1. Their light-scattering averaged molecular weights and radii of gyration, derived from Zimm plots such as that shown in fig. 2, are collected in table 1. As in the previous study light scattering was measured with a CENCO-TNO photometer at 546 nm in a thermostatted room at the temperature desired.

For kinetic experiments the micelles were diluted between 500- and 1000-fold with MUF [12]. Rennet was diluted to the extent that the times needed for mixing of enzyme and substrate (about 10 s) and that necessary for measurement of the angular dependence of the scattering (about 20 s) were negligibly short compared to the total duration of the experiment. The intensity data at different angles and reaction times were plotted in a Zimm diagram, in which the usual concentration variable was replaced by the reaction time (cf. fig.

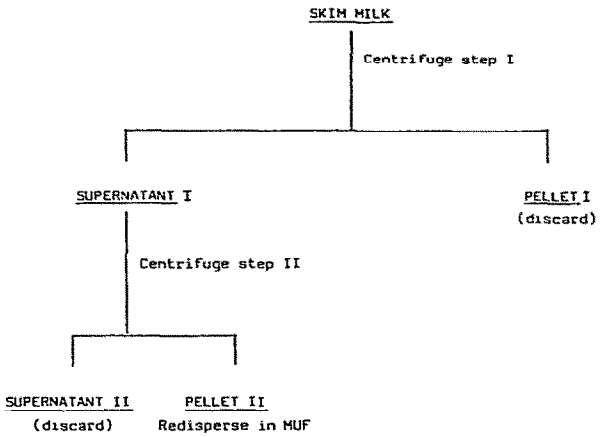

Fig. 1. Fractionation of casein micelles according to size. Small micelles: step I, 30 min at 554000 × *g*; step II, 30 min at 274000 × *g*; Medium micelles: step I, 15 min at 246000 × *g*; step II, 15 min at 385000 × *g*; Large micelles: step I, 15 min at 10950 × *g*; step II, 15 min at 17105 × *g*.

3). Since in these highly diluted systems nonideality correction may be neglected [12], the extrapolation to zero scattering angle directly yields the growth of the weight-average particle weight (fig. 4). These growth curves occasionally show a shallow minimum during the lag phase, which is likely to be explained by proteolysis of the κ-casein [9].

The lag phase is followed by a linear increase in weight-average particle weight, in agreement with eq. 1. It has previously been demonstrated [12] that the beginning of this linear phase indeed coincides with the completed conversion of κ-casein by the rennet.

Flocculation rate constants were estimated from the linear part of the $\bar{M}_w(t)$ vs. t plot. At this stage the flocculation rate constant should be independent of the casein and rennet concentrations. This was confirmed in a number of duplicate experiments at 20°C with medium-sized micelles (fig. 5). Note that the resulting average rate constant (0.82 × 10^7 l mol^{-1} s^{-1}; table 1) does not differ significantly from the previous [12] estimate at this temperature. Rate constants at 25, 30 and 35°C have likewise listed in table 1. At 15°C clotting is so slow that it is no longer measurable.

* The occurrence of an orientational constraint on the rate of clotting was demonstrated, though, during the lag phase, provided that not all the κ-casein had been converted into para-κ-casein by the enzyme [13].

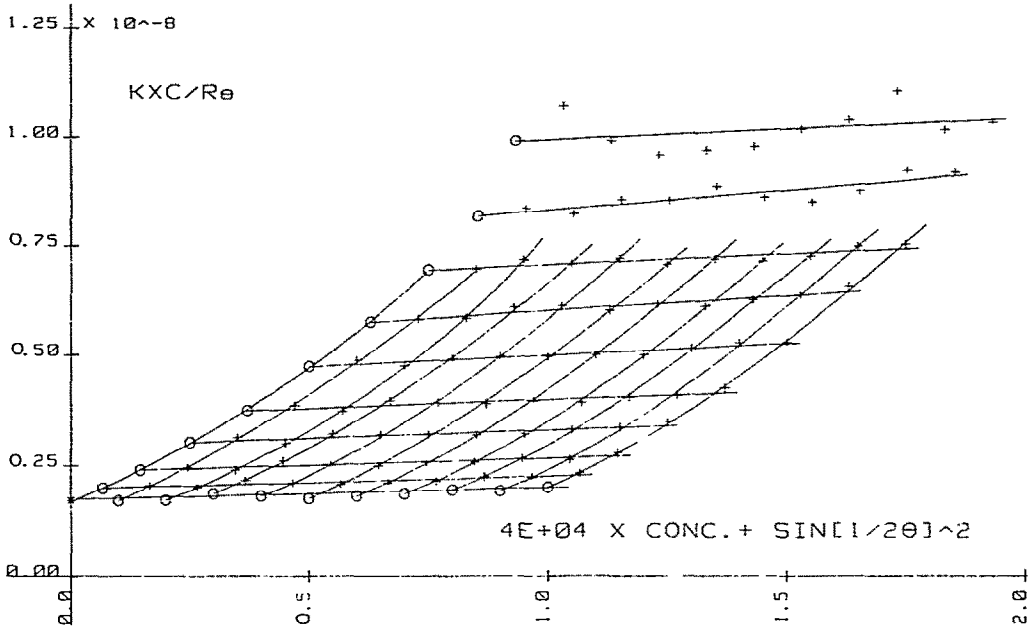

Fig. 2. Zimm diagram for the light scattering of native, medium-sized casein micelles in milk ultrafiltrate at 25°C. (+) Measured values of Kc/R_θ, where K is the Rayleigh-Debye constant, c the concentration and R_θ the excess scattering at observation angle θ. (○) Extrapolated data at $c = 0$ and $\theta = 0$.

Table 1

Summary of initial molecular weights, radii of gyration and flocculation rate constants of fractionated, renneted casein micelles in milk ultrafiltrate

Temperature (°C)	Initial molecular weight ($\times 10^{-8}$)	Radius of gyration (nm)	Flocculation rate constant ($\times 10^{-7}$) ($l\ mol^{-1}\ s^{-1}$)
35	7.68 (0.34; 5) *	69.8 (9.2; 5)	10.65 (1.30; 5)
	5.82 (0.24; 4)	80.0 (2.4; 4)	10.69 (1.56; 4)
	1.83 (0.09; 3)	55.2 (7.8; 3)	16.02 (1.76; 4)
30	8.65 (1.24; 3)	91.3 (17.9; 3)	6.07 (0.82; 4)
	7.36 (0.17; 3)	93.5 (4.6; 3)	5.89 (2.51; 3)
	1.88 (0.03; 3)	73.3 (9.0; 3)	3.81 (1.19; 4)
25	8.61 (0.49; 5)	105.1 (2.5; 5)	2.30 (0.37; 4)
	5.86 (0.01; 2)	89.5 (2.3; 2)	2.82 (0.39; 4)
	1.62 (0.03; 4)	72.9 (2.2; 4)	5.75 (0.25; 4)
20	9.2 **	–	3.24 (0.89; 3)
	4.43 (0.18; 10)	90.2 (0.6; 10)	0.82 (0.09; 10)
	2.7 **	–	1.07 (0.34; 4)

* Standard error of the mean and sample size, respectively.

** Fractions 4 and 6 of table 2 in ref. 12.

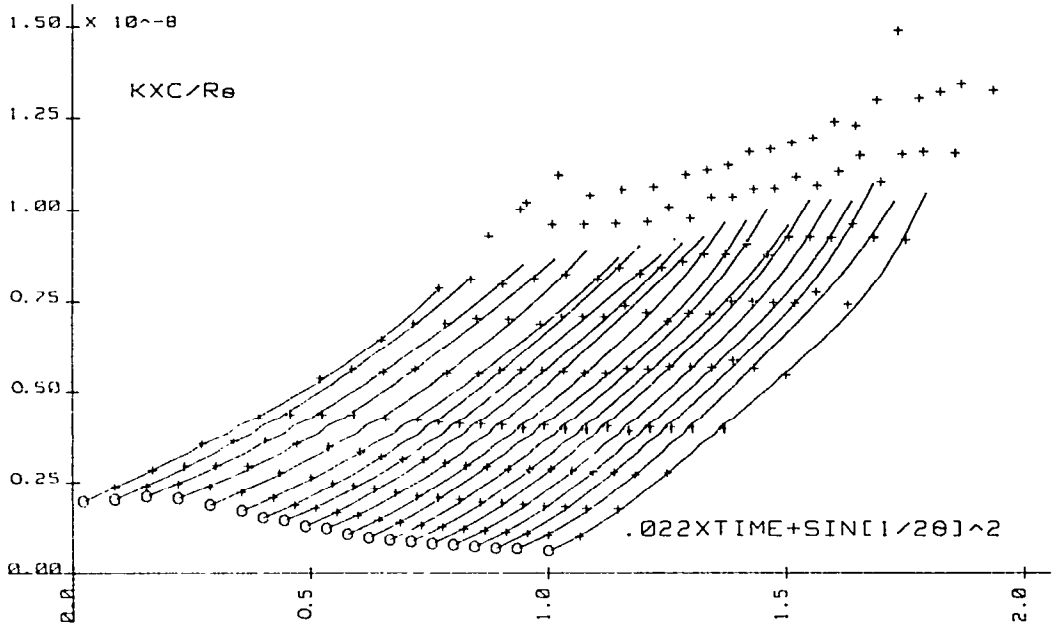

Fig. 3. A modified Zimm plot for the clotting of casein micelles in milk ultrafiltrate at 25°C. Ultimate clotting strength after dilution of the rennet into the reaction medium: 1.03 S.U. (+) Measured values of Kc/R_θ, (○) reciprocal weight-average molecular weight extrapolated to zero scattering angle.

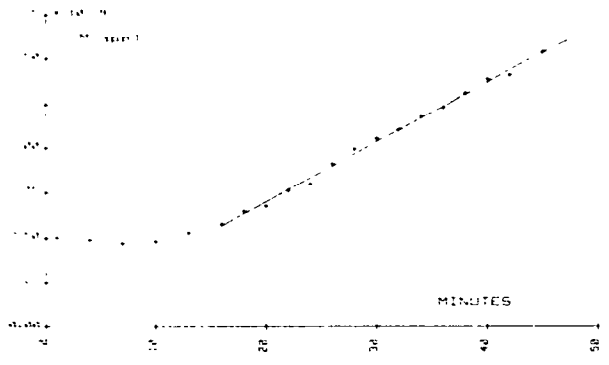

Fig. 4. Increase in apparent molecular weight of renneted casein micelles with time. Experimental conditions: medium-sized micelles in milk ultrafiltrate at 25°C. Ultimate clotting strength after dilution of the rennet into the reaction medium: 1.03 S.U.

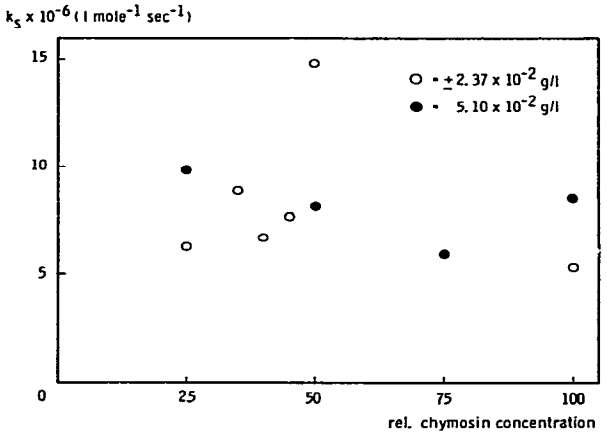

Fig. 5. The flocculation rate constant of completely renneted, medium-sized casein micelles at varying micelle and rennet concentrations. Experimental conditions: micelles resuspended in milk ultrafiltrate at 21°C.

Table 2

Zeta-potentials of native and renneted casein micelles in simulated milk ultrafiltrate at different temperatures.

Temperature (°C)	Zeta-potential (mV)	
	Native micelles	Renneted micelles
4.8	−15.1	−10.0
10.0	−16.0	−7.0
16.5	−14.9	not determined
25.0	not determined	−10.5

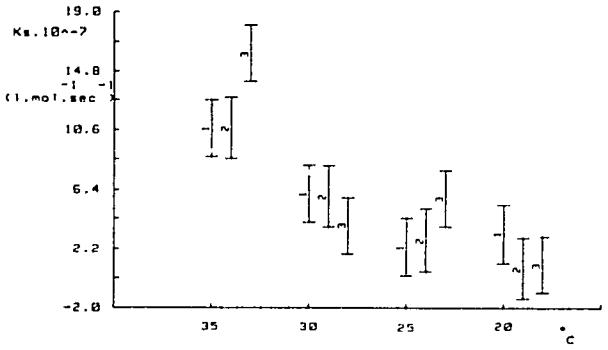

Fig. 6. Tukey's multiple comparison plot [22] for the flocculation rate constants of large (1), medium (2) and small (3) renneted casein micelles at the four temperature levels of table 1. Bars correspond to the 5% significance level.

As a rule the linear phase in the clotting is followed by a stage in which the rate of flocculation apparently levels off (not shown in fig. 4; cf. fig. 2 of ref. 9). Such levelling-off has repeatedly been observed with turbidimetry of flocculating colloids. It has been explained by lesser scattering of loose aggregates [15], and by the increasing effect of internal interference of the scattered light as the size of flocs grows [16]. The latter explanation can be rejected in the present case, because the scattering data were extrapolated to zero scattering angle. Another explanation could be that the flocculation rate constant decreases as the aggregation proceeds.

Zeta-potentials of native and renetted micelles have been measured by moving boundary electrophoresis in simulated milk ultrafiltrate ('SMUF' [17]). They were computed from the Von Smoluchowski-Henry equation (cf. ref. 15, ch. V):

$$\zeta = f\eta v/\epsilon_0\epsilon_r E. \qquad (2)$$

where η is the viscosity of the medium, E the field strength, ϵ_r the relative dielectric constant and ϵ_0 the permittivity of a vacuum.

Since micelle radii are of the order of 100 nm (table 1) and the thickness of the electrical double layer in MUF or SMUF about 1 nm [18], the Henry factor f in eq. 2 becomes unity.

Especially at the higher measuring temperatures we have observed a blurring of the moving boundaries comparable to the formation of the 'spikes' described by Pearce [19]. Unlike this author, however, we suspect that this blurring is not completely due to convection, but that it also reflects inherent inhomogeneity of the micelles with respect to mobility. This becomes evident from the partial re-sharpening of the boundary when the electrical field is reversed. At 25°C the effect of convection became prohibitive to mobility measurement.

In table 2 we have listed the ζ-potentials of untreated and renneted micelles at various temperatures. These values are the averages of the ascending and descending mobilities, extrapolated to zero micelle concentration *. Note that they agree well with those reported by Pearce [19], and that an influence of the temperature on the ζ-potential is not clearly apparent.

3. Discussion

The influence of particle size on colloid stability has been an intriguing problem since the development of the DLVO theory [21]. With completely renneted casein micelles one finds that in nearly all cases the flocculation rate constants of the three size classes of micelles are not different at the 5% significance level (cf. fig. 6). This result is in line with general colloid-chemical experience, but contrary to the conclusion reached by Ekstrand

* The extrapolations of the separate ascending and descending velocities do not differ significantly, indicating that Kohlrausch anomalies [20] in computation of the mobility can be disregarded.

et al. [23] in their study of the flocculation of CPG-fractionated casein micelles.

The flocculation rate constant of colloidal particles, experiencing an energy barrier, $V(u)$, during approach, is given by [21]

$$k_s = (4k_BT/3\eta)/\int_0^\infty \{D(\infty)/D(u)\} \exp\{V(u)/k_BT\}\,du/(u+2)^2. \quad (3)$$

where k_B is Boltzmann's constant, T the absolute temperature, η the viscosity of the medium and the ratio $D(\infty)/D(u)$ accounts for the viscous drag, which two particles of radius R meet with when their surfaces are at a distance Ru [24].

Eq. 3 implies that orientational constraints with the clotting of completely renneted casein micelles can be ignored.

From eq. 3, by differentiation, one obtains

$$-k_B\,d\ln(k_s\eta/T)/d(1/T) = \int_0^\infty \left\{V(u) - T\frac{dV(u)}{dT}\right\}\{D(\infty)/D(u)\} \exp\{V(u)/k_BT\}\,du/(u+2)^2 / \int_0^\infty \{D(\infty)/D(u)\}\exp\{V(u)/k_BT\}\,du/(u+2)^2, \quad (4)$$

which may be called the 'apparent energy of activation', E_p [25].

In line with the conclusion reached from fig. 6 we have pooled the data from the three size classes in the Arrhenius plot of the clotting process between 20 and 35°C (fig. 7). From the slope of this plot the apparent energy of activation is between 125 and 82 kJ/mol, within the 95% confidence limits ($n = 50$). Outside this range, however, the apparent activation energy is strongly influenced by the temperature. As we have noted above, at 15°C clotting was so slow that its measurement was no longer feasible. On the other hand, Dalgleish [26] observed that beyond 55°C the flocculation rate constant becomes fixed at about 40% of the diffusion-controlled limit. As the remaining retardation most probably is explained by the viscous interaction [24], the activation energy evidently has disappeared at this temperature.

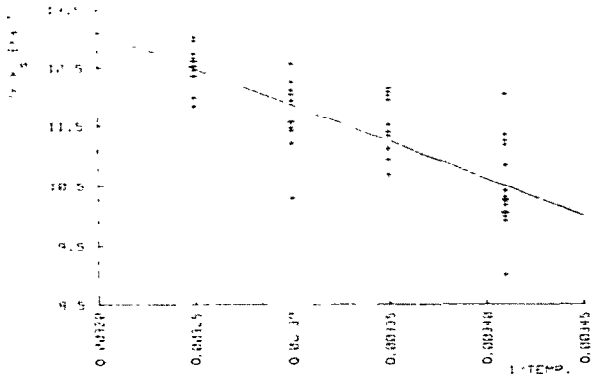

Fig. 7. Arrhenius plot of completely renneted casein micelles in the temperature range 20–35°C. From the slope the activation energy is 103 kJ/mol (±11 kcal/mol ± S.E.; $n = 50$).

The question now arises as to the nature of the apparent energy of activation, as defined by eq. 4. It has been mentioned in section 1 that an explanation of the retardation of clotting solely on the basis of long-range electrostatic repulsion and London-Van der Waals attraction turns out to be unrealistic. With a ζ-potential of the order of 10 mV (cf. table 2) the computed energy barriers in MUF would allow the micelles to approach one another down to atomic distances, without appreciable repulsion taking place (cf. fig. 4 of ref. 9). However, at such small interparticle distances short-range effects due to surface roughness and solvation of the particles might contribute significantly to the interaction. With the classical DLVO approach these effects can be neglected, because the energy barrier separating the particles develops at relatively large interparticle distances. This need no longer be true for the present case of weakly charged casein micelles with a relatively thin electrical double layer. Let us therefore reconsider the chemical events that bring about destabilization of the casein micelle by rennet.

Evidence has been accumulating [3–5,27] that the micelle surface is largely covered by κ-casein, the substrate proper of rennet. The enzyme is known to cleave off a hydrophilic macropeptide from this superficial κ-casein [6,7]. The remaining part, the so-called para-κ-casein, is much more hydrophobic *, and the rennet is thus seen to

* The mean residue hydrophobicities of the macropeptide and para-κ-casein amount to 4.48 and 5.43 kJ/residue, respectively, on the Tanford-Bigelow scale [28].

convert the micelle surface from a largely hydrophilic into a hydrophobic one. This observation, together with the fact that the micelles can approach so closely, then suggests that the adhesion of renneted casein micelles should be explained in terms of hydrophobic bonding rather than by mere London-Van der Waals attraction. The relevant distinction between both concepts is in the recognition of a solvation layer around the apolar 'solute', the breakdown of which accounts for the large temperature coefficient of the hydrophobic bond [29,30]. According to this picture, therefore, the apparent energies of activation are not so much explained by the height of the energy barrier, $V(u)$, as by the strongly negative temperature coefficient, $dV(u)/dT$, originating from the hydrophobic effect.

Bentz and Nir [31], following a suggestion by Spielman [24], have proposed replacing eq. 3 by the approximate expression

$$k_s = (4k_BT/3\eta)(2u^*/R)\exp(-V^*/k_BT), \quad (7)$$

where u^* is the interparticle distance at which the interaction reaches its maximum, V^*. For interacting double layers u^* will be of the order of twice the thickness of the electrical double layer.

With this expression one finds that at room temperature the retardation of clotting can be explained almost exclusively by the viscous drag correction, $2u^*/R$. This result would again point to the relative unimportance of the energy barrier. The argument is questionable insofar as eq. 7 appears to hold only in the case where the interaction energy is sharply peaked about u^* and the interaction profile of renneted micelles is not precisely known. Moreover, at the higher temperatures studied here, eq. 7 seems to overestimate seriously the viscous drag effect [21].

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