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ON ENZYMATIC CLOTTING PROCESSES II. THE COLLOIDAL INSTABILITY OF CHYMOSIN-TREATED CASEIN MICELLES

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The enzymatic clotting of casein micelles dispersed in 0.01 M $CaCl_2$ was monitored by turbidimetry and electrophoresis. The relation between the duration of the lag phase and the enzyme concentration, (e), can be represented by $t = K(e)^{-\gamma}$, where K is a constant and the exponent γ is found to vary between 0.92 and 1.00. This result is interpreted in terms of a flocculation rate constant increasing with the concentration of the enzyme. It is shown that the colloidal instability of chymosin-treated casein micelles cannot be explained on the basis of the well-known theory of the stability of lyophobic colloids, but that clotting is achieved through short-range interactions. The short-range effects that most probably account for the clotting are: hydrophobic bond formation, Ca-bridges and electrostatic interactions. Under typical experimental conditions (33°C; maximum rate of enzymatic product formation about 1.8×10^{-10} mol ml^{-1} s^{-1}) the flocculation rate constant of clotting micelles was found to be 5×10^5 ml mol^{-1} s^{-1}. Various factors, which could be responsible for this low value, are discussed. In the initial stages of the clotting process the turbidity of the system passes through a shallow minimum, which is ascribed to the cleavage of a macropeptide from κ-casein by the clotting enzyme. The condition for the minimum has been derived.

1. Introduction

The coagulation of the casein micelles from milk by milk-clotting enzymes is interesting both from a theoretical [1,2] and a practical [3] point of view. In this paper I shall restrict myself to the theoretical aspect of the process.

In the preceding article [2] it was shown that the lag period observed with the clotting of blood or milk is determined by the so-called enzymatic clotting time $\tau = 1/\sqrt{k_s V/2}$, where k_s is the bimolecular flocculation rate constant of the product formed [4] and V the Michaelis–Menten maximum rate of product formation. The theory predicts that as long as the coagulation is diffusion-controlled, the lag period should be inversely proportional to the square root of the enzyme concentration. With milk or micellar casein as a substrate this proved, however, not be the case. The lag period proved rather to be inversely proportional to the enzyme concentration proper, though many exceptions to this simple rule have been reported during the years [1–3]. It was presumed that the deviations from the theoretical $\sqrt{e}$-rule should be explained in terms of a flocculation rate constant increasing with the concentration of the enzyme. In line with von Smoluchowski's original explanation of the slow coagulation of colloidal particles [4], it was supposed that in the clotting process only a fraction of the particle-encounters leads to permanent contact. The "hot" sites on the particle surface which do lead to clot-formation were identified with those κ-casein molecules from which a peptide was split off by the enzyme. Since, especially with micellar casein each particle contains a large number of such potential sites [5], their surface concentration, and therefore the flocculation rate constant is seen to increase with the concentration of the enzyme.

This explanation is essentially different from that, which was put forward by Green and Crutchfield [6] to account for the colloidal instability of chymosin-treated casein micelles. These authors presumed that the clot-formation can be explained on the basis of the theory of the stability of lyophobic colloids [4], because the ζ-potential of the micelles is nearly halved by the clotting enzyme. In the following I shall de-

monstrate that such an explanation is not likely to hold true. Stability computations to be presented below, show that casein micelles do not experience the long-range electrostatic repulsion and London–van der Waals attraction commonly found with lyophobic colloids. On the contrary, the micelles can approach each other unhindered down to atomic distances, at which all kinds of short-range interactions become predominant. This forms the rationale for the use of von Smoluchowski's early flocculation theory.

The structure and size-distribution of casein micelles are fairly well understood nowadays [5]. In brief: the radius of an average micelle is about 50 nm. It is composed of several hundreds of submicelles, which are held together by calcium phosphate linkages. The submicelles proper are spongy protein particles with a molecular weight of about 250 000 [5]. They consist of 10 to 12 casein subunits, mainly α_s-, β- and κ-casein held together by hydrophobic interactions. Kappa-casein is the clotting enzyme's true substrate. It protects the other casein components against flocculation by Ca-ions and its limited proteolysis triggers the flocculation of the micelle as a whole. It is now generally agreed [5] that κ-casein is evenly distributed throughout the micelle and thus differs in this respect from normal protective colloids, which are restricted to the particle interface [4].

2. Experimental and results

Following Berridge's suggestion [7], the milks used in this study were reconstituted from low-heat skim milk powder to ensure constant colloidal properties. For turbidity measurements these milks were diluted 10 to 22 times with 0.01 M $CaCl_2$, which solvent practically prevents the dissociation of the micelle [8,9]. Before turbidimetry the diluted milks were held for several hours at 33°C to equilibrate the micelles with respect to the temperature-dependent association of β-casein [5,10].

The enzyme preparation was a commercial rennet (CSKF, Leeuwarden), diluted with 0.01 M $CaCl_2$ as indicated with the experimental results. Its original clotting strength amounted to 10 000 S.U. [11]. Fresh dilutions were made for each experiment.

The clotting process was monitored in 0.5 cm cuvettes in the Cary 14 spectrophotometer at a wavelength of 500 nm and a constant (±0.1°) temperature of 33°C. Rapid mixing (mixing times < 1 s) of enzyme and substrate solutions was achieved with a hollow, piston-like mixer, which contained the enzyme solution. A spring-loaded switch moved the piston down and upward through the substrate solution in the cuvette at zero time.

Clotting times were measured by linear extrapolation of the steep part of the absorbance curve to zero absorbance increase. This method has been criticized in the preceding paper [2], because the turbidity is not a linear function of the reaction time. The estimated standard error involved is, however, relatively small: 4%, $\varphi = 9$.

The electrophoretic mobilities, μ, of native and chymosin-treated micelles were obtained from the velocities of the descending boundary in free electrophoresis [12]. Since for the average micelle the ratio of particle radius to double-layer thickness is about 50, and since the ζ-potentials turn out to be < 25 mV, the latter were calculated with the Smoluchowski–Henry equation [4]:

$$\zeta = 4\,\pi\eta\mu/D, \qquad (1)$$

where η is the viscosity of milk dialysate at 2° (i.e. 1.87 mPa.s) and D the dielectric constant. Linear extrapolation of the results obtained at various dilutions (cf. fig. 1) yields ζ-potentials of −12.8 and −4.7 mV for native and renneted micelles respectively.

Fig. 2 presents typical clotting curves measured at different enzyme concentrations. For the present study we are only interested in the lower half of the sigmoidal curves, the leveling off at the end of the reaction being due to exhaustion of the substrate. One should notice that the slope of the plots increases with enzyme-concentration, as is to be expected from the analysis given in this and the preceding paper [2]. Also it is seen that before the steep increase of the turbidity sets in, the absorbance passes through a shallow minimum, the possible occurrence of which was discussed earlier [2].

Fig. 3 and table 1 show the double-logarithmic relation between enzyme dilution and clotting time for different diluted milks. The clotting times are found to be nearly inversely proportional to the enzyme concentration. All computations have been carried out on the Hewlett-Packard 9830A calculator, equipped with the 9862A calculator plotter.

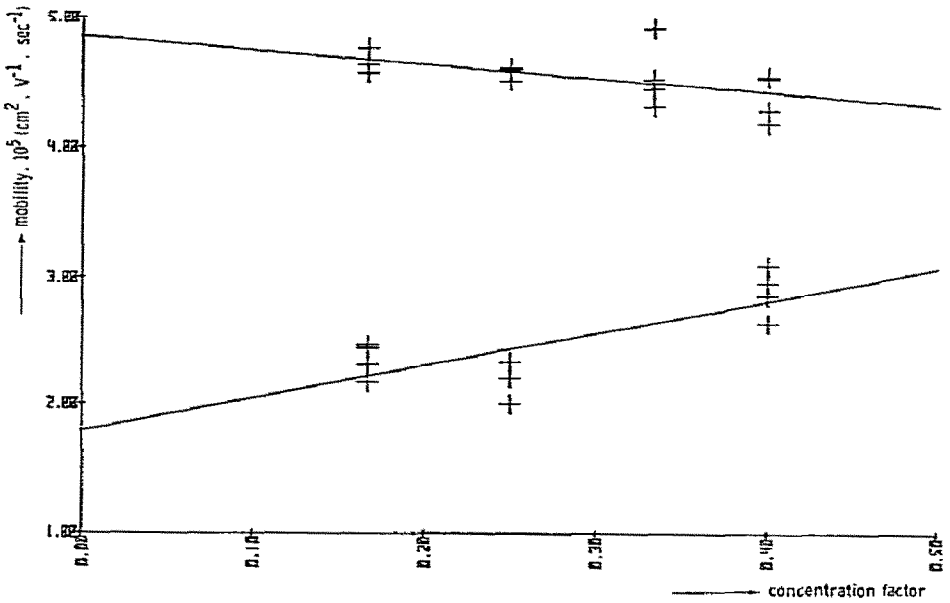

Fig. 1. Descending electrophoretic mobilities of casein micelles before and after the action of milk-clotting chymosin. Milks diluted with milk dialysate.

3. Discussion

A ζ-potential of -12.8 mV for the native micelles compares favourably with the values reported by Hankinson and Briggs (-13.1 mV; ref. [13]) and by Pearce (-14.3 mV at 6°; ref. [14]). A somewhat lower value (-17.4 mV) was measured by Green and Crutchfield [6] in density-gradient electrophoresis at 26°. These authors reported also that the ζ-potential decreased to -26.8 mV at 5°, but Pearce demonstrated that this effect was due to the sucrose added. Pearce himself observed a reversed temperature-effect

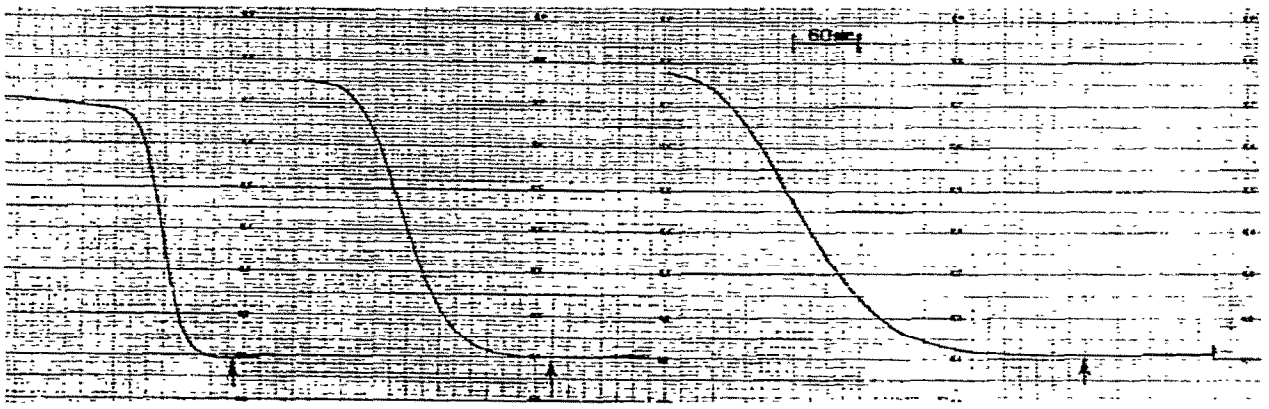

Fig. 2. Absorbance-time plots of the enzymatic clotting of a 10-fold diluted, reconstituted milk at 33°C. Rennet dilutions from left to right: 20, 50 and 100-fold. Note the shallow minimum (↑) in the initial stage of the clotting process.

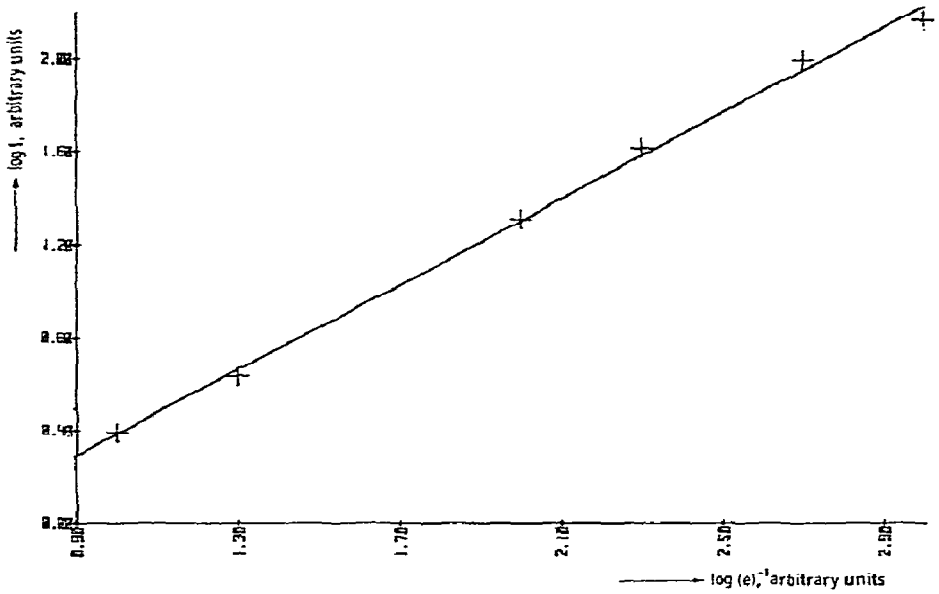

Fig. 3. Double-logarithmic regression of clotting time on enzyme dilution for a 20-fold diluted, reconstituted milk in 0.01 M $CaCl_2$ at 33°C.

(−19.6 mV at 30°). Both Pearce and Green mention that the ζ-potential is not altered by addition of $CaCl_2$ upto 0.01 M. Much lower ζ-potentials were reported by Kirchmeier (−47.6 mV; ref. [15]) and by Puri and Toteja (−51 mV; ref. [16]). The low value arrived at by Kirchmeier can be explained by improbable premises in the computation of ζ. As regard the work of Puri and Toteja, their results are difficult to judge, because no experimental and theoretical details were given.

Table 1
Double-logarithmic regression of clotting time versus enzyme dilution observed with the clotting of reconstituted, diluted milks by chymosin at 33°C

Substrate dilution	Slope	R^2 a)	Number of exps.
10 ×	0.9957	0.9888	5
10 ×	0.9856	0.9756	6
20 ×	0.9167	0.9970	5
22 ×	0.9608	0.9990	4

a) Data digested according to J.H. Nairn, Plotter Pack Program for Polynomial Regression, Hewlett-Packard Co., ed., Loveland, Colorado.

All authors agree that the ζ-potential is about halved by the action of chymosin. It is now known that this is caused by the cleavage of a highly acidic peptide from κ-casein by the enzyme [17,18], the remaining para-κ-casein being positively charged.

The existence of a lag period in the enzymatic clotting of casein micelles is convincingly demonstrated by the turbidimetric experiments shown in fig. 2. The statistical analysis of the log t versus log $(e)^{-1}$ plots (cf. table 1) suggests that for the clotting of casein micelles in 0.01 M $CaCl_2$ the lag period is about proportional to the reciprocal of the enzyme concentration. As is well known, this result is widely used for the assay of clotting enzymes [1,3,19], though the frequent exceptions to this simple rule have caused much confusion [2].

Since the condition for the lag period is [2]:

$$t\sqrt{k_s V/2} = C, \qquad (2)$$

and since V is proportional to the enzyme concentration, the results of table 1 seem to indicate that also the flocculation rate constant under the experimental conditions is nearly proportional to the concentration of the enzyme.

Green and Crutchfield [6] estimated the potential energy of a pair of casein micelles in milk serum assuming the surface potential of the particles to be constant, and accepting a Hamaker constant of 10^{-21} J. These assumptions are disputable.

Firstly, casein micelles are spongy particles, containing about 3.5 g water per g of dry protein [5]. The Hamaker constant might therefore be considerably lower than 10^{-21} J [5,20].

Secondly, the charge of the micelle is due to the ionization of the protein's acidic and basic groups instead of to adsorption of potential-determining ions. As long as the pH and the ionic strength of the medium remain unchanged, the assumption of a constant surface charge therefore appears to be a more realistic representation of the actual situation. It is true that when two micelles of constant charge approach each other, the dissociation of the protein's functional groups may change as soon as the electrical potential on the surface starts to increase [21]. Strictly speaking, this implies that for protein particles both charge and potential are variables. The electrochemistry of such double layers has been treated by the author [22] in connection with the surface pressure of ionizing monolayers, but application of this theory to the casein micelle is impeded by the fact that the numbers and kinds of the ionizing groups creating the surface charge are not known. Since moreover, the potentials involved are rather low and affect the ionization of both acidic and basic groups, I have restricted myself to a computation of the potential energy of a pair of casein micelles of constant charge.

According to Deryagin [4] the electrical repulsion of two spherical particles a distance H_0 apart, is given by:

$$V_R = \pi a \int_{H_0}^{\infty} V_R(H)\, dH, \qquad (3)$$

where a is particle radius and $V_R(H)$ the electrical repulsion of two flat double layers a distance H apart.

For the case of constant charge Frens [23] derived:

$$V_R(H) = V_R^z + \frac{8nkT}{\kappa}\left[(z_H - z_\infty)\cosh(z_\infty/2) - 2\{\cosh(z_H/2) - \cosh(z_\infty/2)\}\right], \qquad (4)$$

where z is the dimensionless ratio $e\psi/kT$, e being the elementary charge and kT the energy of brownian movement. Further $1/\kappa$ is the thickness of the electrical double layer, n the number of ions per ml and V_R^z the repulsion of two imaginary particles of constant potential, ψ_H, calculated according to Verwey and Overbeek [4]. ψ_H is computed with the Gouy–Chapman theory of the electrical double layer and the surface potential, ψ_∞, of a single particle, in the manner indicated by Frens [23]. It is assumed here that ψ_∞ equals the extrapolated ζ-potential at infinite dilution.

The London–van der Waals attraction was computed in the familiar way [4] with a Hamaker constant of 10^{-21} J.

Some representative potential energy curves for a typical micelle (a = 50 nm; $1/\kappa$ = 1 nm) are collected in fig. 4. It is seen that even with the relatively large Hamaker constant of 10^{-21} J, the native micelles, in contrast to the renneted ones, experience a considerable energy barrier in their approach. However, this barrier develops at interparticle distances of the order of 0.1 nm, at which short-range effects certainly are predominant. This conclusion is not altered with a lower Hamaker constant or by keeping the surface potential constant as my own computation as well as those of Green and Crutchfield [6] show. It appears therefore unrealistic to explain the stability and clotting of casein micelles exclusively by the classical DLVO theory, which is based on long-range effects. Clotting casein micelles should rather be treated according to von Smoluchowski's original flocculation theory [4], in which the particles are supposed to diffise freely untill they touch.

It is not so difficult to indicate a number of short-range effects that could be important in the clotting. Thus para-κ-casein has a much higher hydrophobicity index [5,24] than the original κ-casein. This fact, together with the reported large temperature-coefficient ($Q_1 \approx 1.5$; ref. [25]) suggests that hydrophobic bond formation plays a role in the clotting. Further, it is well known that the clotting can be slackened by decreasing the Ca-ion concentration, which points to the formation of Ca-bridges. Finally, it was mentioned above that the residue of κ-casein that is left after the macropeptide is split off by the enzyme, is positively charged [17,18]. It is thus well conceivable that electrostatic interactions between positively and negatively charged patches on the micellar surface play also a role in the clotting process. A similar explanation was

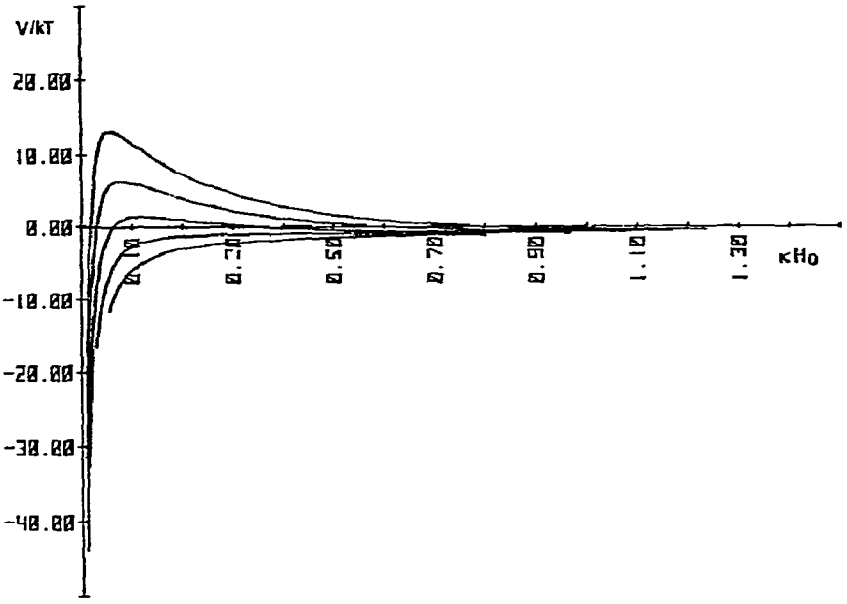

Fig. 4. Potential energy curves for a pair of casein micelles of constant charge in milk serum at different stages of enzymatic discharge. The plots were computed for ζ-potentials of 15, 12.5, 10, 7.5 and 5 mV respectively. Other computational parameters: particle radius 50 nm; thickness of the electrical double layer 1 nm; Hamaker constant 10^{-21} J.

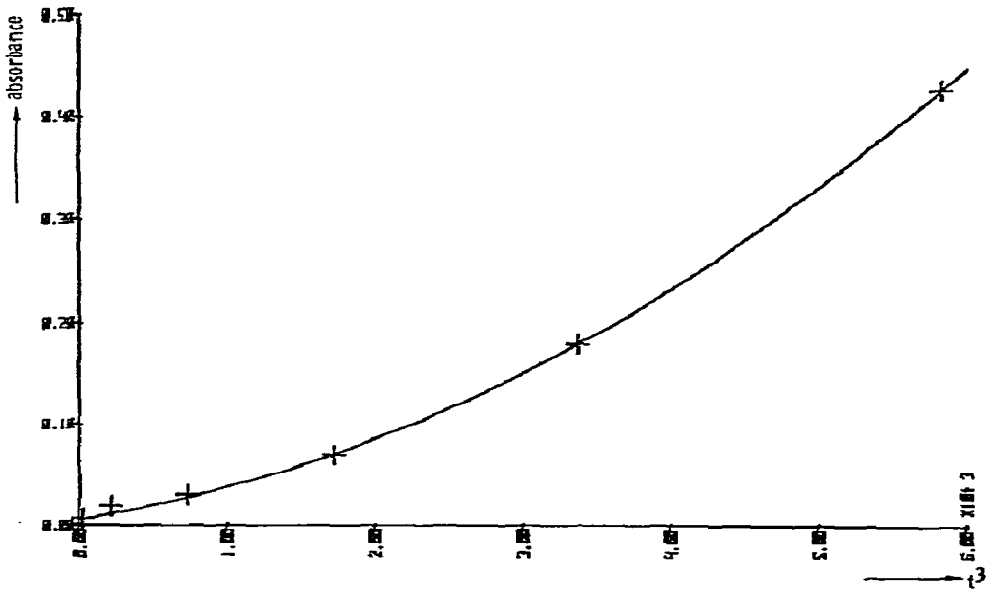

Fig. 5. The plot of the absorbance versus t^3, showing the disturbing effect of multiple scattering in the used experimental set-up. Milk and rennet were diluted 10 and 100 times respectively with 0.01 M $CaCl_2$ – 33°C.

put forward with the thrombin-induced polymerization of fibrin [26]. The relative importance of these three factors is investigated at present.

The theory presented in the Appendix to this article predicts that the plot of the weight-average molecular weight versus t^3 should be linear with a slope proportional to the flocculation rate constant. For a determination of k_s the plot of the absorbance versus t^3 thus seems more promising than any conventional practice to measure the lag time [2]. This expectation is, however, not fulfilled with the present experimental set-up as the example of fig. 5 demonstrates. The plot shows a strong, upward curvature from which the initial slope is difficult to establish with some confidence. No doubt, the curvature is to be explained by multiple scattering and internal interference of the scattered light [27].

A rough estimate of k_s can, however, also be made from the measured lag times. Thus in a typical experiment (0.01 ml of a 100-fold diluted rennet added to 1.4 ml of a 10-fold diluted milk) the lag period amounted to 150 s. Eq. (16) of the preceding article and eq. (A.7) of the Appendix to this article suggest that the constant in eq. (2) is of the order unity and the enzymatic clotting time, τ, therefore about 150 s. With a catalytic constant of 84 s^{-1} [28] and remembering that the clotting strength of highly purified chymosin is about 10^7 S.U. and its molecular weight 34000 [29] it is readily calculated that $V \approx 1.8 \times 10^{-10}$ mol $ml^{-1}s^{-1}$. This result, together with the above estimate of τ yields $k_s \approx 5 \times 10^5$ ml $mol^{-1}s^{-1}$, to be compared with the theoretical value of 6×10^{12} for rapid, diffusion-controlled flocculation [4].

Various explanations can be put forward to account for such a low rate constant. Firstly, Deryagin [30] has suggested that two approaching particles experience an enhanced hydrodynamic drag as a consequence of the proximity of the particles. This effect could easily reduce the rate constant by one order of magnitude [31]. Secondly, the Q_1-value mentioned above, corresponds to a formidable activation energy, which would more than account for the observed k_s. It should be kept in mind, however, that this Q_1 refers to the enzymatic clotting process as a whole, and that it is not known what part of it is due to the flocculation proper. Thirdly, in terms of von Smoluchowski's theory the low experimental value of k_s should be interpreted as a fraction 10^{-6} of the particle collisions leading to permanent contact, or about one thousandth of the particle surface being able to clot. This last explanation, in whole or in part, is attractive since it lies at hand to identify the "hot" sites on the particle surface with κ-casein molecules from which a peptide has been split off by the enzyme. It is also compatible with the idea proposed above of a rate constant increasing with the concentration of the enzyme.

The present treatment shows that the clotting process can be used to determine the flocculation rate constant. The importance of the study of k_s lies in the fact that this quantity can afford insight into the surface properties of the clotting species, and aspect hitherto neglected in the clotting of milk or blood. It is obvious, however, that the measurement of the angular dependence of light scattering is to be preferred to the present turbidity technique.

On close inspection of the absorbance plots it is found that the turbidity passes through a shallow minimum, the occurrence of which was discussed in the preceding paper [2]. In the Appendix it is shown that the condition for the minimum leads to

$$t = \sqrt{f/(1-f)}\,\tau, \tag{5}$$

where f is the ratio of the molecular weights of the peptide split off and the substrate.

In principle therefore, the minimum can also be used to measure the enzymatic clotting time. The straightforward application of eq. (5) is, however, obstructed by the heterogeneity of casein micelles with respect to size [5].

Appendix: The time-dependence of the weight-average molecular weight of the whole solute

The masses of para-casein and macropeptide per ml are given by respectively

$$M_1 \sum_j jP_j \quad \text{and} \quad M_2 \sum_j jP_j,$$

where M_1 and M_2 are the molecular weights of unpolymerized para-casein and macropeptide and P_j is the concentration of the para-casein species of degree of polymerization j, in moles/ml.

If the original casein concentration was C_0 g/ml, the concentration of intact casein during clotting is

given by $C_0 - M_0 \Sigma_j j P_j$, M_0 being the molecular weight of the supposedly monodisperse casein micelles

The weight-average molecular weight during clotting now becomes:

$$\bar{M}_w = M_0 - \left\{(M_0^2 - M_2^2) \sum_j j P_j - M_1^2 \sum_j j^2 P_j\right\} \Big/ C_0. \tag{A.1}$$

Let the ratio of the molecular weights of the macropeptide and the casein micelle be represented by

$$f = M_2/M_0. \tag{A.2}$$

From the analysis given in Appendix II of the preceding paper [2] we have

$$\sum_j j P_j = \sqrt{2V/k_s}\, x \tag{A.3}$$

and

$$\sum_j j^2 P_j = \sqrt{2V/k_s}\,(x + 2x^3/3), \tag{A.4}$$

where x is defined as t/τ and V and k_s are now in mol $ml^{-1}\,s^{-1}$ and ml $mol^{-1}\,s^{-1}$ respectively. The time-dependent weight-average particle weight thus becomes

$$\bar{M}_w = M_0 - M_0^2(1-f)\sqrt{8V/k_s}\{fx - (1-f)x^3/3\}/C_0, \tag{A.5}$$

from which the minimum is easily found at

$$x = \sqrt{f/(1-f)}. \tag{A.6}$$

For vanishing small values of f eq. (A.5) reduces to

$$\bar{M}_w = M_0 + (M_0^2\sqrt{8V/k_s}/3\,C_0)\,x^3, \tag{A.7}$$

which shows the weight-average molecular weight of the whole solute to be cubic in the reaction time with a slope proportional to k_s.

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