The renneting of milk

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The renneting of milk

A kinetic study of the enzymic and aggregation reactions

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Abstract

Van Hooydonk, A. C. M. (1987). The renneting of milk. A kinetic study of the enzymic and aggregation reactions. Doctoral thesis, Agricultural University Wageningen (164 pp., English and Dutch summaries).

The rennet-induced clotting of milk was studied under various conditions. The kinetics of the enzymic and aggregation reactions was analysed separately and, where possible, related to the physico-chemical properties of the casein micelle and its environment.

The effects of important variables, such as temperature, pH, divalent cations, ionic strength, casein concentration and pre-heat treatment of milk, are given and discussed in relation to the reaction kinetics and to the implications for the control of the renneting process.

The kinetics of the enzymic reaction in milk differs substantially from that observed in pure κ-casein solutions. This discrepancy was ascribed to the immobilised state of the substrate in the relatively large casein micelles. The calculated upper limit of the enzymic reaction was close to the experimentally obtained reaction rate, suggesting that the reaction may be diffusion-controlled. Interactions between the enzyme molecule and the micelle surface are discussed.

The relation between the rate of aggregation and the degree of κ-casein conversion was deduced from the change in viscosity during renneting. The correct interdependence between clotting time and enzyme concentration could be predicted with a kinetic model combining the enzymic and aggregation reactions.

De artikelen 2 t/m 6 en 8 in dit proefschrift zijn ook verschenen als Verslagen (resp. V246, V263, V264, V266, V270 en V271) van het NIZO te Ede.

STELLINGEN

1. De door chymosine gekatalyseerde specifieke hydrolyse van κ-caseïne in melk kan niet beschreven worden met de Michaelis-Menten-parameters die gelden voor oplossingen waarbij het κ-caseïne als monomeer aanwezig is.
Dit proefschrift.

2. De versnelling van de stremreactie door toevoeging van calciumchloride aan kaasmelk bij constante pH is voornamelijk een gevolg van de verhoging van het gehalte aan micellair calciumfosfaat en niet, zoals vaak wordt aangenomen, van de toename van de calciumionactiviteit.
Dit proefschrift.

3. Voor een goede beheersing van het kaasbereidingsproces verdient het aanbeveling de stremeigenschappen van kaasmelk te standaardiseren.
Dit proefschrift.

4. Mede gezien de snelle wijzigingen in fokmethoden dient bij de selectie van koeien die bestemd zijn voor melkproduktie niet alleen gelet te worden op het totale eiwitgehalte van de melk maar ook op de verhouding tussen de diverse eiwitfracties en de genetische varianten van de melkeiwitten.
o.a. J. Schaar, Scandinavian Journal of Dairy Technology and Know-how 1 (1984) 43.

5. Bij de bereiding van grondstoffen via enzymatische processen wordt onvoldoende aandacht geschonken aan geringe restactiviteiten van thermoresistente enzymen. Deze grondstoffen kunnen bij toepassing in langhoudbare UHT-gesteriliseerde produkten aanleiding geven tot bederf.

6. De door Baust et al. geconstateerde beperkte gebruiksduur van een kationwisselaar in de Ca^{2+} vorm voor de analyse van suikers en de noodzaak deze wisselaar periodiek om te keren is niet representatief en berust op een onderzoek met een inferieure kolom.
J.G. Baust, R.E. Lee, R.R. Rojas, D.L. Hendrix, D. Friday & H. James, Journal of Chromatography 261 (1983) 65.

7. De dimensies van de Posthumustrechter zijn niet optimaal voor de doelstelling van de meting.
A.C.M. van Hooydonk, H.G. Hagedoorn & T. van Vliet, Zuivelzicht nr. 20 en 30/31 (1984) 432 en 632.

8. Het is onwaarschijnlijk dat via genetische manipulatie van melkzuurbacteriën voldoende lysozymproduktie bereikt kan worden om het gebrek laat-los in kaas tegen te gaan.

9. De gelering van UHT-gesteriliseerde geconcentreerde melk wijst erop dat de caseïnemicellen bij verouderen hun wilde haren kwijtraken.

P.J. de Koning, J. Kaper, H.S. Rollema & F.M. Driessen, Netherlands Milk and Dairy Journal 39 (1985) 71.

10. De door Matthiasson gevonden verschillen in eiwitadsorptie aan polysulfonmembranen met verschillende poriegrootten moeten niet worden toegeschreven aan een mogelijk verschil in oppervlakte-eigenschappen van deze membranen maar aan het verschil in adsorberend oppervlak.

E. Matthiasson, Journal of Membrane Science 16 (1983) 23.

11. De stremeigenschappen van gereconstitueerde melk worden in belangrijke mate bepaald door de temperatuur waarbij het melkpoeder wordt opgelost.

12. Hoewel men het vermogen om over water te lopen toeschrijft aan bovennatuurlijke krachten is het juister om over bovennatuurlijke snelheden te spreken.

13. Wanneer de door produktiebeperkingen vrijkomende landbouwgronden gebruikt gaan worden voor scharrelen worden de vastgestelde quota waarschijnlijk niet gehaald.

A.C.M. van Hooydonk
THE RENNETING OF MILK.
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Voorwoord

Dit proefschrift is de afronding van een onderzoek dat alleen tot stand kon komen door de samenwerking van vele personen. Hierbij wil ik allen bedanken die direct of indirect aan de voltooiing ervan hebben bijgedragen. Ik denk dan in de eerste plaats aan mijn ouders, die mij alle vrijheid hebben gegeven om te gaan studeren.

In het bijzonder gaat mijn dank uit naar mijn promotor Prof. Dr. Ir. P. Walstra. Beste Pieter, een doctoraal vak van drie maanden onder jouw stimulerende begeleiding is voor mij bepalend geweest voor mijn verdere loopbaan. Tijdens onze veelvuldige gesprekken in de afgelopen jaren heb ik mijn ideeën steeds kunnen toetsen aan jouw bewonderenswaardige kennis op dit vakgebied.

Het bestuur en de directie van het NIZO ben ik zeer erkentelijk voor de gelegenheid die mij geboden is deze promotie te realiseren.

De stimulerende discussies met mijn collega's van het NIZO hebben in belangrijke mate bijgedragen tot de vormgeving van het onderzoek.

Henk Vreeman en Thom Snoeren waren voor mij steeds een vraagbaak bij vele praktische en theoretische problemen op het gebied van de fysische chemie van melk.

De besprekingen met Gerard van den Berg zijn onmisbaar geweest voor de vertaling van de onderzoeksresultaten naar de praktijk van de kaasbereiding.

Veel dank ben ik verschuldigd aan Cees Olieman voor zijn bijdrage aan de ontwikkeling van een analysemethode voor het meten van de enzymatische reactie tijdens het stremmen van de melk.

Wout Heinen wil ik bedanken voor de zeer plezierige samenwerking en voor de ruimte die hij mij bood om dit onderzoek te verrichten.

Mijn dank gaat verder uit naar Hennie Hagedoorn en Ingrid Boerrigter voor de kundige wijze waarop zij de experimenten hebben uitgevoerd.

Gosse Stel en Jacques Maessen bedank ik voor de hulp bij het persklaar maken van de manuscripten, Henk van Brakel en Joop Mondria voor het vervaardigen van de figuren en Yvonne Munter en haar medewerksters voor het typewerk.

De collega's in mijn nieuwe werkkring bij DMV Campina BV ben ik erkentelijk voor de gelegenheid die zij mij hebben geboden dit proefschrift af te ronden.

Annelies, jouw bijdrage, hoewel niet direct zichtbaar in dit boekje was onmisbaar voor de totstandkoming ervan. Aan jou draag ik dit proefschrift op.

Contents

1 Introduction

1.1 Objectives and motivation

About 40 % of the Dutch farm milk is processed into cheese. More than 30 cheese varieties are already produced in the Netherlands, but in terms of volume the Gouda and Edam types are by far the most important. In the past decades the manufacture of these two types has developed into a large-scale, fully automated process. Optimal production relies on proper control of the raw materials and the various process steps. The development of new cheese types and new manufacturing technologies is a continuing activity. Control of automated plants as well as new developments require a basic understanding of the principles that underlie cheesemaking. The rennet-induced clotting of milk is an essential step in the manufacture of cheese. Cheese yield and cheese quality are, among other factors, determined by this process step. A detailed knowledge of the reactions taking place during renneting is needed to optimize cheese production.

The objective of this study was to investigate the effect of important variables on the renneting process and to gain a more fundamental understanding of the mechanisms involved. Several studies have already been made in model systems, and these have certainly improved our knowledge of the renneting reaction. But the situation may be rather different in milk, and that is why this study was intentionally restricted to the total system of skim milk.

1.2 The renneting of milk

The clotting of milk is initiated by the specific enzymic hydrolysis of κ-casein. This alters the properties of casein micelles in such a way that they become unstable and start to aggregate. As aggregation proceeds, a three-dimensional network of casein micelles forms eventually. At a well-defined firmness the casein gel, usually called curd, is cut into small pieces to promote the expulsion of whey. This study deals with the enzymic and aggregation stages of the renneting process and not with the factors involved in the syneresis of the curd.

1.2.1 Enzyme and substrate. Proteolytic enzymes, extracted from calves' stomachs, have been in use for a long time to initiate the clotting of milk for cheesemaking. The extract, commonly referred to as rennet, contains two acid proteinases designated chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1). The major part of the proteolytic activity during renneting is due to

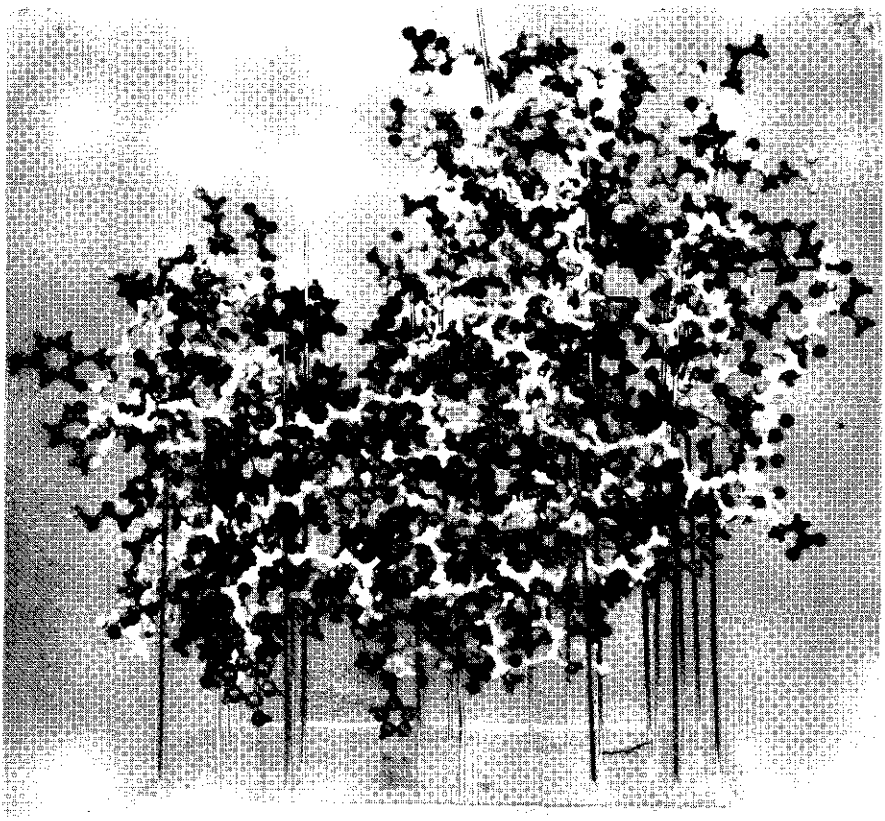

Fig. 1. Model of chymosin as developed in cooperation with the University of London (Department of Crystallography) and the University of Groningen (Department of Chemical Physics). By courtesy of Dr. S. Visser, NIZO.

chymosin (1). The activity is based on the presence of two aspartic acid residues. The molecule consists of two domains, separated by a deep cleft (see Fig. 1). The active site of the molecule is in the cleft where the two aspartic acid residues are located (2, 3). The other parts of the cleft serve as an extended site which may accommodate a peptide chain of some seven amino acid residues (3). The pH optimum lies in the acid region (i.e. < 5.0), as for all acid proteinases. The remarkably high activity for a specific peptide bond of κ-casein at neutral pH has not yet been satisfactorily explained.

During the evolution of mammals, an adaptation between the primary structure of the substrate and the binding sites of chymosin has occurred (3). Calf chymosin, for instance, clots bovine milk faster than does pig chymosin, whereas the opposite is true if both enzymes are tested against porcine milk. The physiological significance of the great milk-clotting activity and the weak

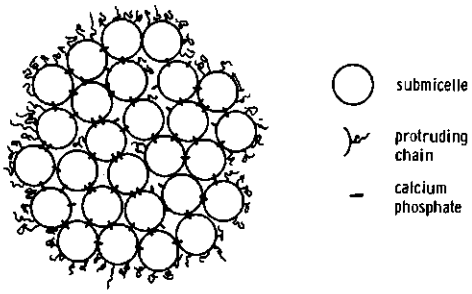

Fig. 2. Model of a casein micelle; highly schematic (From: Walstra and Jenness (1), by courtesy of Wiley).

general proteolytic activity is, according to Foltmann (3), to prevent extensive damage to the immunoglobulins. The very young calf needs these substances – that are abundant in colostral milk – and pepsin, unlike chymosin, can easily attack them. Hence the absence of pepsin and the prevalence of chymosin in the new-born calf.

The knowledge of the renneting reaction has increased considerably over the past few decades, mainly because of a better understanding of the physico-chemical properties of the milk proteins. The main protein fractions in milk are the caseins (80 % of total protein) and the whey or serum proteins (1). The latter fraction is expelled with the whey during the last stage of the renneting process. The caseins occur in milk as nearly spherical colloidal particles (see Fig. 2) with sizes ranging from 20 to 300 nm. These casein micelles are thought to be composed of a number of small aggregates (submicelles). These subunits, having a size of 10 to 20 nm, contain the major casein molecules, i.e. α_s-, β- and $\varkappa$-casein. Their composition, however, cannot be identical because $\varkappa$-casein is almost exclusively located at the surface of the casein micelle (4). Casein micelles are voluminous particles, holding a considerable amount of water ($\approx$ 3 g/g casein) (5). They also contain inorganic matter, particularly calcium phosphate, which plays an essential role in maintaining the integrity of the micelles (6, 9). Removal of calcium phosphate disintegrates a casein micelle into smaller aggregates. The nature of the interaction forces between calcium phosphate complexes and casein within the micelle has not yet been resolved. The most relevant feature of the structure of the micelle for the renneting reaction is the presence of $\varkappa$-casein at the surface.

1.2.2 The enzymic reaction. The peptide bond specifically attacked by chymosin is located in the sequence of $\varkappa$-casein between residues 105 and 106 (phenylalanine and methionine). Studies with isolated $\varkappa$-casein and simulated parts thereof have considerably improved the understanding of the catalytic mechanism of enzyme-substrate binding and of the reaction kinetics (7, 8). Despite the extensive literature on the renneting of milk, quantitative data on

the enzymic reaction in this more complicated system are scarce, mainly because of the lack of suitable methods of following the degree of proteolysis. One of the main questions still is whether or not the kinetic results obtained with model substrates may be applied to the total system of milk. The immobilisation of the substrate in the relatively large casein micelles must have implications for the reaction. The number of encounters between enzyme and substrate is solely determined by the diffusion of the enzyme. The collision frequency is therefore reduced by orders of magnitude compared to the situation in which the substrate consists of free molecules. Furthermore, the presence of other proteins in the vicinity of the active site of the substrate may influence the reaction rate. Other complications, such as adsorption and surface inactivation of enzyme molecules, may occur.

No pH optimum has been reported for the action of chymosin in milk, nor is any direct information available about the effect of ionic strength. Inconsistent results are published on the effect of preheating of milk and addition of calcium. The aim of this study was to investigate the effects of these important variables on the kinetics of the enzymic reaction in milk, in relation to physico-chemical changes induced by these variables.

1.2.3 The aggregation reaction. The C-terminal part of κ-casein is hydrophylic with an excess of negative charges (1). The remarkable stability of the micelle depends on this protein. If there were no κ-casein, the micelles would flocculate. The protective action is thought to originate from the hydrophylic moiety protruding into the surrounding serum as a flexible 'hair' (Fig. 2). The mechanistic background of the stability has not been established with certainty, but the generally held view is that the 'hairs' impart steric repulsion, presumably with an electrostatic contribution (see Paper 8).

This hydrophylic part of κ-casein is split off by the enzyme and, after sufficient κ-casein has been cleaved, aggregation starts. This process can be described by Smoluchowski kinetics (9). The reaction proceeds many times more slowly than predicted by a diffusion-controlled process, indicating the presence of an energy barrier between approaching micelles. Repulsion between rennet-converted micelles increases with decreasing temperature, and below 15 °C the rate of aggregation is virtually zero (10, 11). Addition of calcium promotes the reaction but the mechanistic explanation is still obscure (12, 13). Below a certain level of calcium, no aggregation takes place (1). Model experiments with fully converted micelles in special buffers showed a decreasing rate of aggregation with ionic strength (13). On the other hand, addition of NaCl to milk initially decreased the clotting time before it started to rise again (14), but inconsistent results exist (15). Lowering of the pH accelerates the reaction and below a critical pH (5.0 at 30 °C) rennet is no longer necessary to start aggregation (10).

1.3 Outline of the thesis

This thesis consists of eight papers which have already been published or have been submitted for publication. The first paper deals with a new method of following the action of chymosin in milk. In the Papers 2 to 6 the results of the effect of important variables, such as temperature, pH, casein concentration specific ions, ionic strength and pre-heat treatment of milk, are presented. The implications for the control of the renneting process during traditional cheesemaking are discussed in Paper 7. In the last paper a more theoretical and mechanistic interpretation of the combined results is given.

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Neth. Milk Dairy J. 36 (1982) 153-158 SHORT COMMUNICATION

A rapid and sensitive high-performance liquid chromatography method of following the action of chymosin in milk

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Introduction

The clotting of milk by chymosin is a complex process, involving an enzyme-catalysed proteolysis of κ-casein and a subsequent aggregation of the unstable casein micelles.

A detailed understanding of the kinetics of the process requires a separate analysis of both individual reactions.

Several methods have been proposed for measuring the extent of the enzymic reaction, either by estimation of the production of glycomacropeptide (GMP) or that of para-κ-casein.

A frequently used method is based on the increase in the amount of non-protein nitrogen (NPN) during proteolysis of κ-casein. Alais et al.(1) and later on, in more detail, Nitschmann & Bohren (2) have shown that the increase in NPN was caused by the formation of GMP. In our opinion the sensitivity of this simple and rapid method is insufficient to determine the initial reaction rate.

A specific and sensitive, but time-consuming method is based on the determination of N-acetyl neuraminic acid present in GMP (3). Beeby (4) used a fluorescence technique to study the release of GMP in milk. The method involves the reaction of fluorescamine with α-amino groups at pH 6.0. Variations of the order of 5% in the final level of κ-casein in milk could be detected in this way.

Para-κ-casein can be estimated by electrophoretic separation on cellulose acetate strips (5) or on polyacrylamide gels (6). Electrophoretic techniques, however, are laborious and not very suitable for quantitative analysis.

Indirect methods, based on the determination of the initial decrease in viscosity (7) and turbidity (8), give a quick indication of the initial reaction rate, but cannot be used to describe the complete enzymic reaction.

This paper reports the use of high-performance liquid chromatography (HPLC) to determine the GMP content of milk. HPLC of peptides requires, in many cases, little sample pre-treatment, the method is sensitive and can be easily automated.

Materials and methods

Renneting

A solution (11 % m/m) of low-heat, spray-dried skim milk powder in distilled water and a 0.35 % (m/m) solution of κ-casein in a Jenness-Koops buffer (9) were used as substrates.

Before performing the kinetic measurements, both solutions were stored overnight at 7 °C and subsequently equilibrated for 30 min at the reaction temperature (31 °C).

10 mg of a purified chymosin (strength 5.1×10^6 Soxhlet units), prepared by the method of de Koning (10), were diluted with 50 g distilled water, and 50 μl of this solution was added to 35 g of the substrate solution. After stirring for 30 s, 2-ml aliquots were quickly pipetted into tubes and the reaction was allowed to proceed. At appropriate time intervals 4 ml of a 12 % (m/m) trichloracetic acid (TCA) solution were mixed with the sample in a tube. A sample without chymosin, which had been treated in the same way with TCA, was used as a blank. TCA immediately inactivates chymosin and precipitates selectively the casein and the whey proteins. After 1 h storage at 31 °C the precipitated samples were filtered (Whatman 42) and the filtrates analysed by HPLC.

HPLC analysis

A Waters Model 6000A pump with a Wisp 710B automatic sample injector and an M 450 variable-wavelength UV detector were used in combination with a Waters Data Module M 730 and a Kipp Analytica 9222 column oven. Two TSK 2000 SW columns (30 cm × 0.75 cm i.d.) were maintained at 35 °C.

The eluent reservoir was kept at 85 °C to prevent bacterial growth and to keep the eluent degassed. The eluent consisted of a solution of potassium hydrogen phosphate (1.74 g), potassium dihydrogen phosphate (12.37 g) and sodium sulphate (21.41 g) in 1000 ml double distilled water. The eluent was filtered through a 0.45 μm Millipore filter.

15 µl of the samples were injected automatically. The flow was set at 1.0 ml/min, and the UV detector operated at 205 nm, 0.2 A. The Data Module was programmed to allocate the baseline at the beginning and the end of the chromatogram. The response factor was calculated on the basis of the complete conversion of pure κ-casein into para-κ-casein and GMP. Peak areas were corrected by subtraction of the peak area obtained with the TCA filtrate of the substrate before renneting, and by taking into account the degradation of GMP during storage of the samples in the automatic sample injector (about 0.17 %/h at 30 °C).

Results and discussion

The average molecular weight of GMP is about 8000 (11). Therefore gel permeation chromatography on high-performance columns can be used to measure the GMP, provided that interfering whey proteins can be removed selectively. Preliminary experiments with GMP derived from κ-casein and with whey indicated that 8 % of TCA is the optimum final concentration. At this concentration the whey proteins are virtually absent. Lower TCA concentrations resulted in insufficient removal of the whey proteins, as indicated by monitoring at 280 nm. Higher TCA concentrations caused precipitation of increasing amounts of GMP.

Identification of the GMP peak is possible by comparing its retention time with that of purified GMP and by checking the absence of UV absorption at 280 nm (GMP contains no aromatic amino acids).

A small peak remained at the GMP position in the chromatogram of the blank. This peak is probably caused by proteose peptone or by small amounts of GMP. Peak areas of GMP were corrected for this contribution and, as already mentioned, for a decrease in peak area (0.17 %/h) during storage of the samples in the automatic injector.

The HPLC separation of GMP, NPN and TCA together with the increase in peak height of GMP after different renneting times is given in Fig. 1. The resolution of the two main fractions, NPN and GMP, is excellent. There is no indication that other TCA-soluble degradation products with a molecular size differing from that of GMP are produced during the first 70 min of renneting.

A run time of approximately 40 min suffices to produce a complete chromatogram. Fig. 2 shows the release of GMP (nmol/ml) during renneting of a solution of low-heat milk powder and of κ-casein.

Although the initial concentration of κ-casein is apparently higher in the milk powder substrate, the initial rate of proteolysis is lower. This sug-

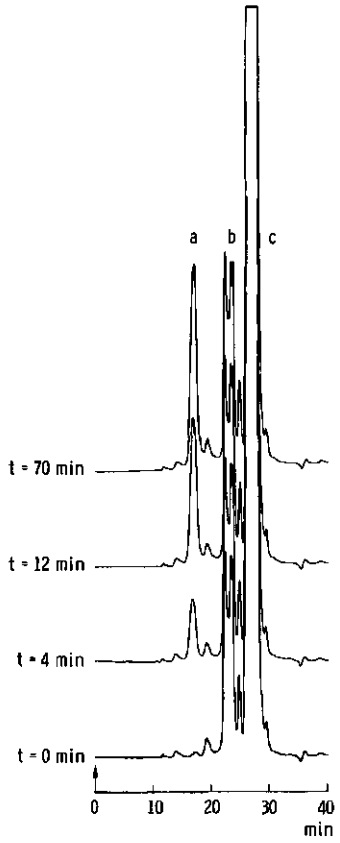

Fig. 1. HPLC determination of GMP after renneting times of 0, 4, 12 and 70 min respectively at 31 °C. 0.28 μg chymosin was added per g of an 11 % (m/m) solution of milk powder.
a: GMP; b: NPN; c: TCA; flow rate 1.0 ml/min; detection: UV 205 nm, 0.2 A; injection: 15 μl.

gests that the κ-casein molecules are more difficult to attack if they are present in the native casein micelles of milk powder.

The repeatability of the method is very good, mainly due to the little sample pre-treatment involved. The pooled standard deviation of three identical experiments, using milk powder as a substrate, was 3.0 nmol GMP/ml. This value was found to be independent of the level of GMP. This means that the amount of κ-casein in milk may be estimated with a variation coefficient of less than 2 %, assuming that chymosin converts all the κ-casein into para-κ-casein and GMP.

The method described in this paper might be very useful for assessing the proteolytic activity of commercial rennets and rennet substitutes. It might also be used to estimate the amount of cheese whey powder present in milk powder.

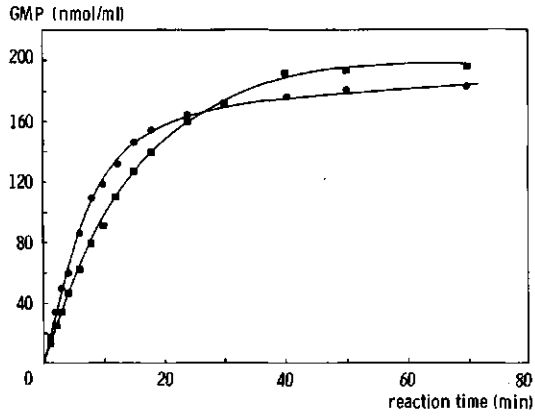

Fig. 2. Production of GMP (nmol/ml) during renneting of a solution of low-heat milk powder (■, 11 % m/m) and of κ-casein (• 0.35 % m/m) with 0.28 μg chymosin per g substrate at 31 °C.

Kinetic studies of enzymic reactions are easily performed, especially with automated HPLC equipment. The Michaelis-Menten parameters may be evaluated directly from the curves, as shown in Fig. 2, if a proper computer programme is available to fit the data.

Acknowledgement

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Samenvatting

A. C. M. van Hooydonk en C. Oliemans, *Een snelle en gevoelige HPLC-methode voor het volgen van de werking van chymosine in melk*

Een snelle en gevoelige methode om de proteolyse van κ-caseïne door chymosine tijdens het stremmen van melk te volgen, is beschreven. De methode berust op een kwantitatieve bepaling van het afgesplitste glycomacropeptide met behulp van gelpermeatiechromatografie in HPLC-uitvoering. De monstervoorbereiding is eenvoudig en beperkt zich tot het neerslaan van de storende caseïne- en wei-eiwitten met trichloorazijnzuur (eindconcentratie 8 %).

De methode is uitermate geschikt voor het vaststellen van de proteolytische activiteit van stremselpreparaten, maar ook voor het bepalen van de hoeveelheid κ-caseïne in melk en de hoeveelheid kaas-weipoeder in melkpoeder.

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Kinetics of the chymosin-catalysed proteolysis of κ-casein in milk[1]

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Summary

The proteolysis of κ-casein in milk by chymosin followed first-order kinetics with respect to the substrate and enzyme concentration.

The apparent energy of activation of the enzymic reaction was approximately 26 kJ/mol.

Both pasteurization (5 s, 74 °C) and cooling (4 °C) of milk prior to renneting decreased the rate of proteolysis.

Increasing the casein concentration by ultrafiltration decreased the reaction rate constant. This is probably due to a decrease in the effective diffusion rate of the enzyme.

A mechanistic model to explain the observed kinetics is given by assuming that the adsorption of the enzyme on the micelle surface is negligible. The reaction rate is then controlled by the number of effective collisions between substrate and enzyme, and thus by the diffusion rate. The diffusion rate of the enzyme determines the reaction velocity, because the size of the enzyme is much smaller than that of the casein micelle. The number of effective collisions decreases proportionally with the degree of hydrolysis, resulting in a first-order rate expression.

1 Introduction

The first step in the renneting process is the specific proteolysis of κ-casein by chymosin. The subsequent coagulation of unstable casein micelles occurs partly during the enzymic reaction (1, 2, 3). The rennet clotting time of milk depends on the rate of both individual reactions. The response to changes in, for instance, temperature, pH and calcium ion activity is expected to be different for the two reactions. A detailed understanding of the intricate renneting process therefore requires a separate study of the enzymic and coagulation reaction. Quantitative data on the enzymic reaction in milk are, howev-

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er, scarce mainly because of the lack of suitable methods to quantify the degree of proteolysis.

One of the earliest investigations of the kinetics of the enzymic reaction was performed by Nitschman & Bohren (4). They used sodium caseinate solutions as a substrate and measured the increase in non-protein nitrogen (NPN). The reaction could adequately be described by first order kinetics. The energy of activation was calculated to be 42 kJ/mol, equivalent to a Q_{10} value of 1.78 at 30 °C. Scott-Blair & Oosthuizen (5) used a viscometric technique to establish the reaction rate. They found zero-order kinetics at low rennet concentrations and short incubation times. With higher rennet concentrations and longer incubation times the reaction changed to first-order kinetics.

Dalgleish (6) and Chaplin & Green (7) applied electrophoretic techniques to determine the production of para-κ-casein in milk. They both fitted their data into the integrated form of the Michaelis-Menten equation and found similar values for the Michaelis-Menten constant ($K_m \simeq 5 \times 10^{-4}$ mol/l). However, these values are about 10 to 100 times higher than those given by Castle & Wheelock (8), who used a modified NPN-method to determine the reaction rate. The results of Garnot & Corre (9) suggest first-order kinetics at pH 6.7 and a more complex behaviour at pH 6.2, but they did not attempt to estimate the kinetic parameters.

It is clear that the kinetics of the enzymic reaction are still not well established, necessitating further investigations.

The present paper reports the first results of a kinetic study on the action of chymosin in milk. The effect of the enzyme and protein concentration and of the reaction temperature on the reaction rate were studied in order to establish the reaction constants and the energy of activation. Prolonged cooling and pasteurization of milk are important steps in the processing of milk for cheese production and are known to affect the rennetability. The effect of these treatments on the kinetics of the enzymic reaction were also studied.

2 Materials and methods

2.1 Materials

– Whey-protein-free milk was prepared by ultracentrifugation (88 000 *g*, 90 min) of heat-treated (10 s, 65 °C) skim milk at 30 °C. The protein pellet was twice washed by suspending it in the UF-permeate of the same skim milk and re-centrifuging. After the last wash the pellet was re-suspended in UF permeate to a normal casein level of about 2.5 % and stored overnight at

7 °C. Ultrafiltration of the skim milk was carried out at 30 °C by using a stirred dead-end cell from Amicon and a PM 10 membrane.
– Low-heat, spray-dried skim milk powder was dissolved in distilled water (11 % m/m) at 40 °C and stored for 24 h at 7 °C before use.
– Cheesemilk. Bulk-collected raw whole milk was heat-treated (10 s, 65 °C) on arrival at the NIZO experimental dairy, cooled to 4 °C, standardized to a fat/protein ratio of 0.96 and stored for 20 h at 4 °C. The milk was subsequently pasteurized for 4.5 s at 75 °C, cooled and stored overnight at 7 °C.
– Concentrated skim milk was prepared by ultrafiltration of heat-treated (10 s, 65 °C) skim milk at 30 °C (i.e. renneting temperature) with a Wafilin pilot plant ultrafiltration unit equipped with a WFA-500 membrane (Wafilin, Hardenberg, Netherlands). The skim milk was concentrated to a protein content of approximately 10 % and diluted with the permeate to give milk samples with various protein concentrations. The milks were stored for 24 h at 7 °C before use.
– Raw uncooled milk collected from a local farm was used to study the effect of cooling and pasteurization on the rennetability.
– Purified chymosin was prepared by the method of De Koning (10); it had a strength of 5.1×10^6 Soxhlet Units and a chymosin content of 45 % (m/m) while pepsin was absent (11). Just before use the purified chymosin sample was dissolved in distilled water to give a strength of 10 800 Soxhlet Units. This is equivalent to a chymosin concentration of 0.09 % (m/m) and comparable with the concentration of chymosin + pepsin in commercial rennets (12).
– Commercial liquid rennet with a strength of 10 800 Soxhlet Units was purchased from CSK (Leeuwarden, The Netherlands).

2.2 Methods

2.2.1 Determination of casein macropeptide (CMP)

The release of CMP during the enzymic reaction was followed quantitatively by gel-permeation chromatography on high-performance columns as described in a previous paper (13).

Each sample was equilibrated for 30 min at the reaction temperature prior to renneting. After adding the enzyme to the substrate solution the mixture was stirred for 30 s, after which 2-ml aliquots were pipetted into a sufficient number of tubes in which the reaction was allowed to proceed. At appropriate, pre-selected time intervals, 4 ml of a 12 % (m/v) trichloroacetic acid (TCA) solution was added to a tube to inactivate the chymosin and to selectively precipitate the casein and whey protein. Lower TCA concentrations

were used in whey-protein-free milks. After 1 h storage at 25 °C the precipitated samples were filtered (Whatman 42), and the filtrates analysed by HPLC.

2.2.2. Calculation of reaction constants

The experimental data were fitted into the integrated Michaelis-Menten equation by using a computer program developed by Luyben et al. (14). The 'best' parameter values (V_{max}, K_m) were calculated by a non-linear optimization procedure. It is also possible to perform a numerical differentiation of the relation between concentration of CMP and time in order to obtain the conversion rate as a function of the substrate concentration.

Because all results obtained by fitting the parameters into the Michaelis-Menten equation were compatible with first-order kinetics, a new program was developed to fit the integrated first-order equation by linear regression. Because of the logarithmic transformation of the data, such a procedure is very sensitive to small errors in the experimentally obtained final product concentration. An iterative method was therefore incorporated in the regression analysis, aimed at minimizing the sum of the squares of differences between the experimental and calculated values by changing the magnitude of the final product concentration. This concentration was also used as the starting value for the non-linear optimization with the Michaelis-Menten equation. It should be noted that the last-mentioned procedure is less sensitive to small errors in the final product concentration, but it was always checked whether the quality of the fit could be improved by changing this value or not.

3 Results

3.1 The effect of TCA concentration on CMP recovery

Bovine κ-casein is a heterogeneous molecule of which two genetic variants are known. Each may vary in *N*-acetyl-neuraminic acid (NANA) content and the presence of a second phosphate ester group in the peptide chain (15). The CMP part of the κ-casein contains all the carbohydrate as well as the genetic substitutions. The solubility of CMP in TCA was found to depend on the charge and carbohydrate content (16, 17). The more negatively-charged and/or carbohydrate-rich components are better soluble in TCA. Because of the interference of whey proteins in the HPLC measurements of CMP, a final concentration of 8 % TCA had to be used (13). To check the effect of TCA concentraction on CMP recovery a series of kinetic experiments was performed with whey-protein-free milk. The results are given in Fig. 1a and Table 1. From Fig. 1a it shows that at the end of the renneting process the

Table 1. The effect of TCA concentration on the CMP recovery after different reaction times (three experiments). The CMP values are expressed as a percentage of the value obtained in 2 % TCA. The value at $t = \infty$ has been derived by extrapolation (section 2.2.2). Renneting temperature 30.5 °C; rennet concentration 0.02 %.

TCA concentration (%)	Experiment A, reaction time (min)			Experiment B, reaction time (min)			Experiment C, reaction time (min)		
	5	10	40	3	12	∞	5	12	∞
2	100	100	100	100	100	100	100	100	100
5	78	80	92	–	–	–	–	–	–
8	54	57	61	50	60	74	58	68	74
12	24	26	30	–	–	–	–	–	–

CMP recovery in 8 % TCA is about 75 % of the recovery in 2 % TCA. In Table 1 the values for several TCA concentrations at different reaction times are given. The CMP concentration at each reaction time is expressed as a percentage of the concentration in 2 % TCA, assuming that all CMP fractions are soluble in 2 % TCA (17).

The CMP solubility decreases substantially with increasing TCA concentrations, which is in agreement with reported results of experiments with pure κ-casein solutions (16, 17). The CMP concentration relative to the value in 2 % TCA, increases during renneting (Table 1). This indicates that the κ-casein with the lowest charge and NANA content in the CMP part is more rapidly attacked by rennet than are the more hydrophilic and charged molecules. These results are in agreement with the observations on pure κ-casein solutions (17, 18).

3.2 The kinetics of the enzymic reaction

The release of CMP during the renneting process of a skim milk powder solution (11 %) and of normal cheesemilk is depicted in Fig. 1b. Each curve, including those of the whey-protein-free milks with various TCA concentrations (Fig. 1a), was fitted into the integrated Michaelis-Menten equation (solid curves). As can be seen in the figure, this equation adequately describes the experimental data. In all cases the calculated value of K_m was much greater than the initial κ-casein concentration, which means that the Michaelis-Menten equation reduces to a first-order rate expression. The velocity of the reaction should thus decrease proportionally with the substrate concentration during the course of the reaction. A numerical differentiation of the experimental data of Fig. 1b indeed resulted in such a relationship (Fig. 2).

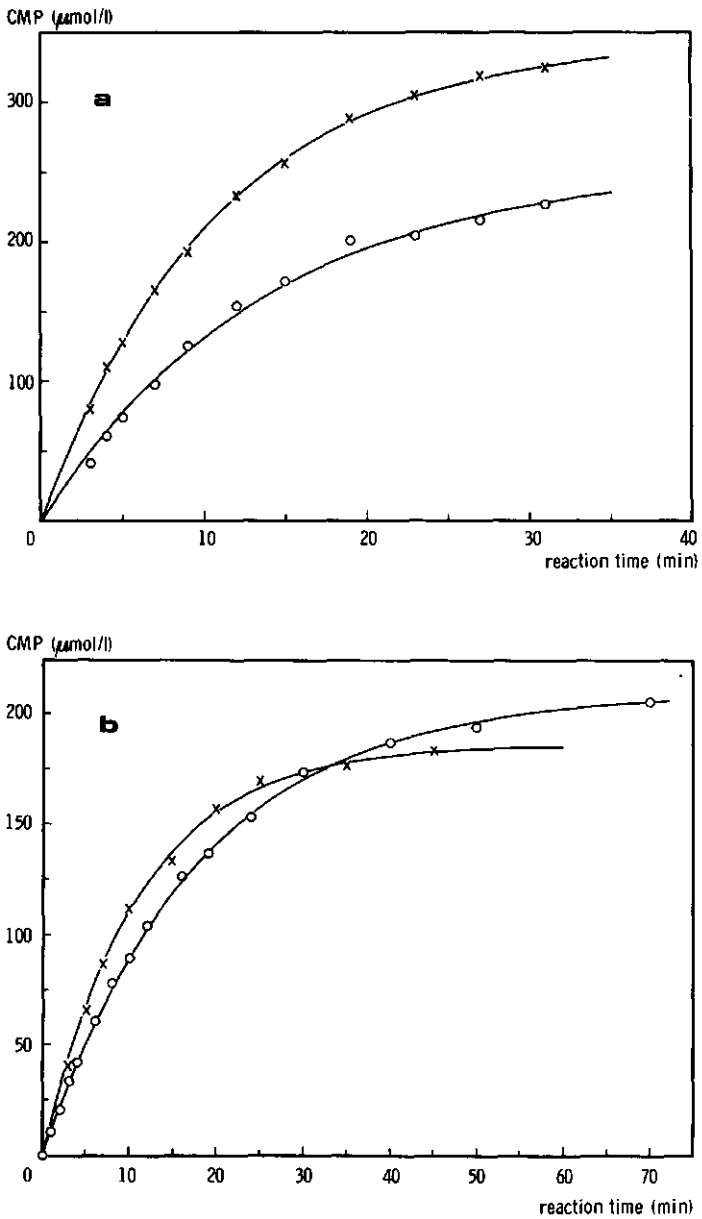

Fig. 1. Production of CMP (μmol/l) during renneting of different milk samples at 30.5 °C. a. Whey-protein-free milk; 2 % (×) and 8 % (○) TCA to precipitate the casein; enzyme: 0.02 % commercial rennet. b. Cheesemilk and 0.02 % commercial rennet (×); a solution of 11 % (m/m) low-heat skim milk powder in distilled water and 0.02 % of a purified chymosin solution (○); TCA concentration 8 %.

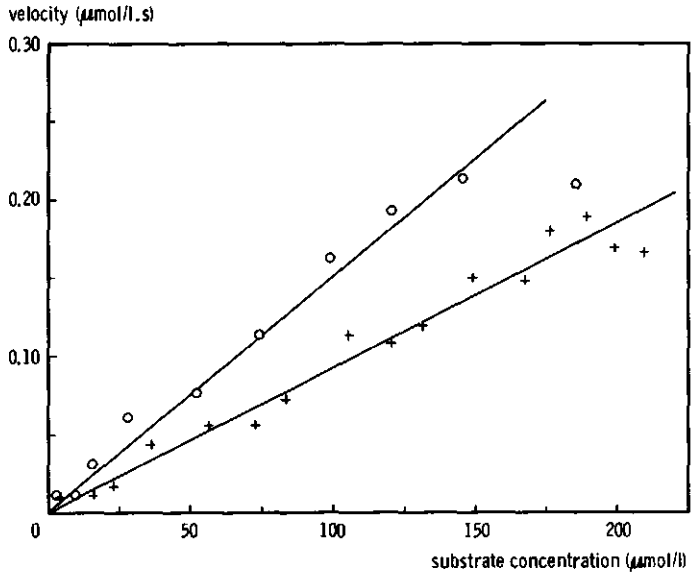

Fig. 2. The relation between the reaction velocity derived by numerical differentiation of the experimental results of Fig. 1b, and the substrate concentration. + = milk powder; ○ = cheesemilk.

The calculated values of the reaction rate constant of whey-protein-free milk were 1.54×10^{-3} and 1.20×10^{-3} s^{-1}, with 2 and 8 % TCA respectively.

For normal cheesemilk a value of 1.51×10^{-3} s^{-1} was calculated, and for the skim milk powder solution treated with purified chymosins a value of 0.93×10^{-3} s^{-1}. All constants were obtained with 0.02 % rennet or chymosin solution, both having a strength of 10 800 Soxhlet Units.

As will be shown below, the reaction rate constant $K = K_{enz} \times e$, where e is the enzyme concentration and K_{enz} the reaction rate constant per unit quantity of enzyme solution.

The concentration of chymosin in the experiment with skim milk powder as substrate amounted to 0.13 mg/l or 4.3×10^{-6} mmol/l, assuming a molecular weight of the enzyme of 30 000. K_{enz} is then calculated to be 216 (s^{-1} $mmol^{-1}$ l). The calculation of K_{enz} for the experiments with commercial rennets is more difficult because the amount of chymosin (and pepsin) is unknown. In this case it might be more appropriate to express K_{enz} per % liquid rennet added to the milk.

3.3 The effect of the rennet concentration

To check the validity of the assumption that $K = K_{enz} \times e$, the enzyme concentration was varied and the values of K werd calculated according to the Michaelis-Menten and the first-order reaction model. Cheesemilk was used as a substrate and commercial rennet as an enzyme source. Again the reaction kinetics were apparently first-order for all rennet concentrations and both calculation procedures resulted in nearly equal values for the reaction rate constant.

The results of two experiments carried out on different milk samples are shown in Fig. 3. A good proportionality is found between the reaction rate constant and the enzyme concentration. From the slope of the curve, K_{enz} was calculated to be 0.078 (s^{-1} $\%_{rennet}^{-1}$).

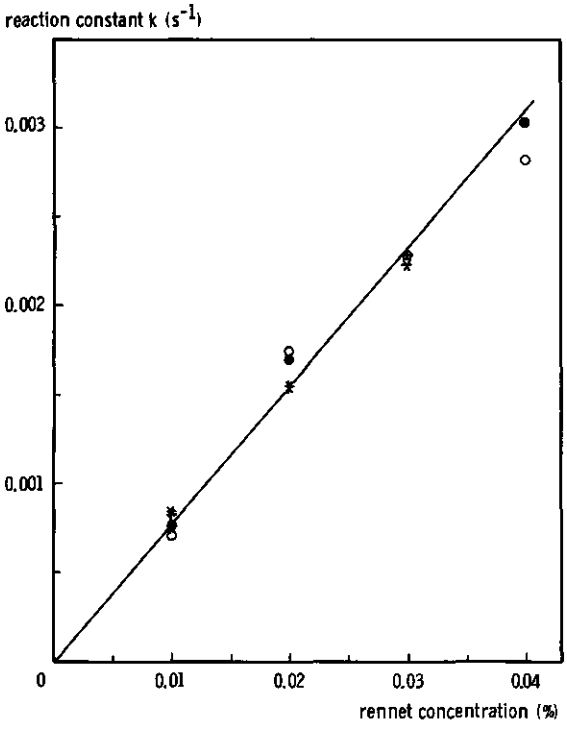

Fig. 3. The reaction rate constant K (s^{-1}) as a function of the rennet concentration. Results of two different milks. Renneting temperature 30.5 °C. Milk A: * and +, milk B: ● and ○; * and ● calculated with Michaelis-Menten model, + and ○ calculated with first-order rate equation (Section 2.2.2).

3.4 The effect of the protein concentration

The influence of the protein concentration on renneting was studied with skim milk concentrated by ultrafiltration. In order to prevent a shift in the salt equilibrium, the skim milk was concentrated at the renneting temperature and the concentrated milk was diluted with the permeate to give various protein concentrations. The milk was renneted with 0.02 % (v/m) commercial rennet at 30.5 °C.

The results of two experiments are shown in Fig. 4. Irrespective of the protein concentration the release of CMP was again best described by a first-order reaction. Both calculation procedures (see Section 2.2) resulted in similar values for the reaction rate constant, the value of which decreased with increasing protein concentration.

The relationship is approximately linear, suggesting that the drop in the reaction rate constant might be due to the retardation of the effective diffusion rate of the enzyme. Muhr & Blanshard (19) showed that the diffusion rate of a solute in a polymer solution depends on the polymer concentration. They

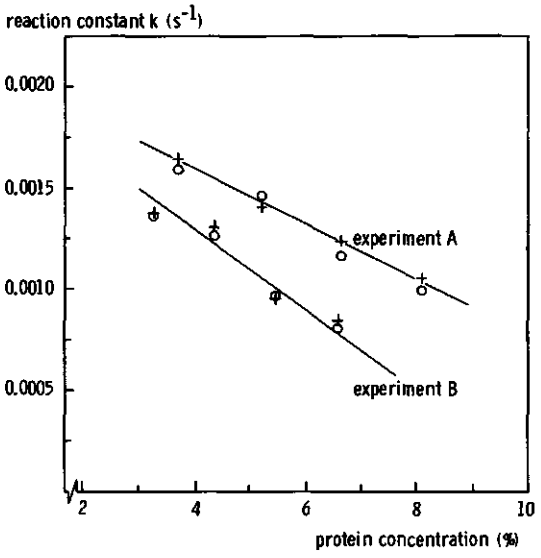

Fig. 4. The reaction rate constant K (s^{-1}) as a function of the protein concentration. Two experiments with UF-concentrates of different milk. Renneting temperature 30.5 °C, rennet concentration 0.02 %. ○ calculated with Michaelis-Menten equation; + calculated with first-order rate equation.

found that the fractional decrease in the diffusion rate (D_{eff}/D_0 can often be written as:

$$D_{eff}/D_0 = (1-B\phi)$$

where ϕ denotes the polymer volume fraction and B a constant. With a similar approach to our system it can be shown that the fractional decrease in the rate constant follows the relation

$$K_{eff}/K_0 = (1-B'\phi_p)$$

where ϕ_p is the volume fraction of the milk proteins (~ 0.1 in milk with a protein content of 3.4 g/l). The value of B' was 2.1 and 3.1 for Experiment A and B respectively in Fig. 4. These values for B′ are very similar to those which can be calculated from the results given by Muhr & Blanshard (19) for the diffusion of different molecules in polymer gels and solutions. The extrapolated value for maximal velocity (K_0) (reached when ϕ approaches zero) was 2.15×10^{-3} and 1.96×10^{-3} s^{-1} in Experiment A and B respectively.

3.5 The effect of the renneting temperature

The temperature dependency of the reaction was studied in the temperature range from 23 °C to 36 °C. No decrease in enzyme activity due to degradation is expected to occur in this region. Normal cheesemilk was used as a substrate and commercial rennet (0.02 % v/m) as the enzyme source. The reaction rate constant was determined by fitting the data into the first-order reaction.

Fig. 5 shows the Arrhenius-plots of the calculated values together with the least squares linear fits of two experiments with different milk samples. According to the Arrhenius equation, the energy of activation (ΔE) may be derived from the slope of the curves. Values of 21 kJ/mol and 32 kJ/mol were found for ΔE, which correspond to Q_{10} values of 1.31 and 1.50 respectively at 30 °C. These values are slightly lower than those quoted by Nitschmann & Bohren (4). It should be noted, however, that their measurements were performed with sodium caseinate solutions and not with normal milk.

3.6 The effect of cooling and pasteurization of milk

Cooling and heating of milk cause time-dependent shifts in the distribution of proteins and salts over the colloidal and soluble phases of the milk. Owing to these changes, the rennet coagulation properties of milk alter and show hysteresis. It is well established that cooling of milk before renneting results in a prolonged rennet coagulation time (20, 21). Ageing of cold-stored milk at the

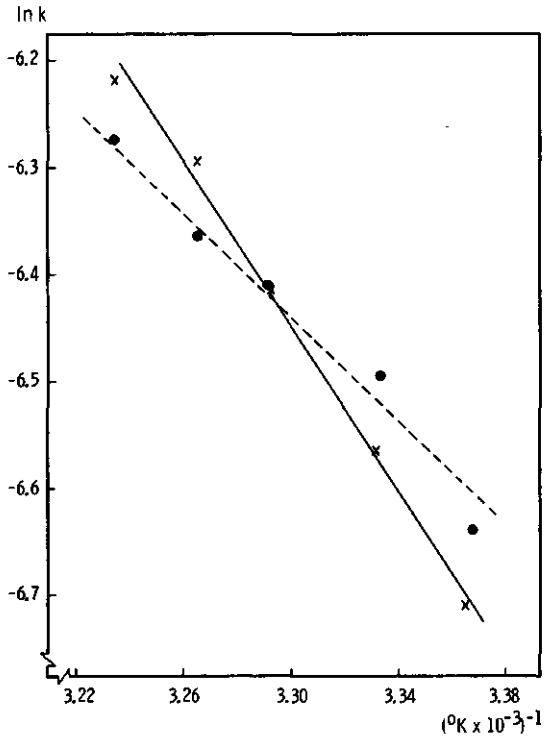

Fig. 5. Arrhenius plot of the reaction constant together with a least-squares linear fit through the experimental points. Cheesemilk with 0.02 % rennet. x = milk A; • = milk B.

temperature of renneting slowly restored the rennetability (22, 23).

Low-temperatue short-time pasteurization (e.g. 5 s, 75 °C) of uncooled milk normally retards the coagulation (20, 24, 25). The effect of pasteurization on the rennetability of cooled milk is less clear. Reimerdes et al. (22) found a complete recovery of the original rennetability when cooled milk was pasteurized, whereas a further retardation was observed by Schütz & Puhan (21). The results of Quist (20) were variable with respect to the pasteurization effect.

In our experiments, pasteurization of uncooled milk caused a significant decrease in the rate of κ-casein hydrolysis (Table 2). Cooling of milk also retarded the reaction. The longer the milk was cold-stored, the more slowly did the reaction proceed.

Pasteurization of cooled milk scarcely improved the velocity of the enzymic reaction.

Table 2. The effect of cooling and pasteurizing on the enzymic reaction rate constant. The milks were renneted with 0.02 % rennet at 30.5 °C.

Treatment of the milk	Reaction rate constant (s^{-1})
uncooled milk	1.70×10^{-3}
pasteurized (5 s, 74 °C) uncooled milk	1.39×10^{-3}
cooled milk, 5 h at 4 °C	1.46×10^{-3}
cooled milk, 24 h at 4 °C	1.34×10^{-3}
cooled milk, 48 h at 4 °C	1.21×10^{-3}
pasteurized (5 s, 74 °C) cooled milk, 48 h at 4 °C	1.31×10^{-3}

4 Discussion

The results of the experiments with different TCA concentrations not only demonstrated that the solubility of CMP decreased with the TCA concentration, but also indicated that the rate of release declined with the NANA and/or phosphate content. The accessibility of the active centre in κ-casein for chymosin is probably reduced by the steric or elektrostatic repulsion of the carbohydrate side chains and the presence of an extra phosphate group. This might also implicate a slower rate of CMP production in cow's milk containing only the κ-casein A variant.

It was found that the release of CMP during the renneting of milk could adequately be described by first-order rate kinetics. This was also true for the experiments with different TCA concentrations, despite the fact that the different CMP fractions are hydrolysed at different rates. This might be explained as follows: assuming that each κ-casein fraction splits according to a first-order reaction of which the reaction constants are not too far apart, it can be shown that the total CMP production (2 % TCA) may also be described by a first-order rate expression of which the reaction constant has an intermediate value.

Normally a homogeneous enzyme-catalysed reaction is, under steady-state conditions, often completely described by the Michaelis-Menten equation:

$$-ds/dt = (V_{max}.s)/(K_m + s)$$

V_{max} denotes the maximal velocity ($V_{max} = K_{cat} \times e$), K_m the Michaelis-Menten constant, s the substrate concentration and t the reaction time.

This equation reduces to a first-order rate expression if $K_m >> s$. The K_m values for the reaction between chymosin and κ-casein fragments (residue 98-112) are within the range of 0.01-0.03 mmol/l (26), which means that the con-

dition of $K_m >> s$ is not met in our experiments because milk contains about 0.15 mmol κ-casein per liter. Based on these values it is more reasonable to assume that the reaction would be nearer to zero order since $s >> K_m$.

It should be realized, however, that κ-casein does not exist as single molecules but as aggregates in the casein micelles. Each micelle contains approximately a thousand κ-casein molecules. The enzyme kinetics of a heterogeneous system, consisting of soluble enzyme and insoluble substrate, cannot normally be treated with the classical Michaelis-Menten theory (27, 28). The reaction may be complicated by several factors, such as the two-dimensional state of the substrate involved, the adsorption and desorption of the enzyme at the interface and the possibility of activation or inactivation of the adsorbed enzymes. An extensive review of interfacial enzyme kinetics is given by Verges & De Haas (29).

A model to describe kinetically the action of soluble enzymes at interfaces was proposed by Verges et al. (28). This model basically consists of a combination of the reversible adsorption of the enzyme onto an interface and the subsequent formation of the interfacial enzyme-substrate complex. The resultant kinetic treatment is a rather complicated expression for the reaction velocity as a function of the substrate concentration. But it was also demonstrated that the velocity is linearly dependent on the substrate concentration, in the particular case that the amount of the enzyme at the interface is very small compared to the amount in the bulk:

$$-ds/dt = (V_{max}.s)/(K_d/K_p)K_m^*$$

Here K_d denotes the desorption rate constant, K_p the penetration (adsorption) rate constant and K_m^* the interfacial Michaelis-Menten constant.

Under practical conditions (0.02 % rennet) each enzyme splits about 30 micelles, assuming that milk contains 10^{14} micelles per ml. This means that the available surface area for a single enzyme to adsorb on is roughly 10^5 times the area occupied by the enzyme itself. If chymosin had a high affinity for the (para)-casein micelle, virtually all the chymosin would be retained in the cheese. Stadhouders & Hup (30) found, however, only a small proportion of the added rennet in the cheese. Also Geurts (31) found hardly any adsorption of chymosin onto para-casein micelles. The above model might thus indeed be the explanation of the observed kinetics, although it is still unknown whether the adsorption of chymosin on unhydrolysed casein micelles is also negligible.

An alternative approach could be based on the assumption that the enzymic reaction is controlled by the diffusion rate. A low energy of activation

and a linear relationship between the reaction rate constant and the reciprocal of the volume fraction of the dispersed material are typical for diffusion-controlled reactions (19, 32). The rate of such a reaction depends only on the number of effective collisions between the reactants and is normally of the second order. For the casein micelle and chymosin we have the special case that the diffusion rate of the micelle is negligible compared to the diffusion rate of the enzyme. The system may be considered as one where a fast moving enzyme collides with an immobile surface containing the substrate. Assuming that this process is completely non-selective, the velocity of the reaction then depends on the number of effective collisions between the enzyme and the unreacted substrate and may be written as follows:

$$-\mathrm{d}s/\mathrm{d}t = K \cdot e \cdot c_m = p \cdot A \cdot \exp(-\Delta e/RT) \cdot \text{constant}$$

where e and c_m denote the enzyme and micelle concentration respectively. c_m will be constant if the aggregation of micelles is limited during the reaction. A is called the frequency factor, and p the steric factor accounting for the fraction of collisions not leading to a reaction because the active centres of the enzyme molecule and the substrate molecule did not meet. Such an ineffective collision occurs for instance if the molecules are not in the proper orientation and if the enzyme collides with the micelle at a position where the κ-casein has already been cleaved.

The effective fraction of the surface of the micelle therefore decreases during proteolysis. It seems reasonable to assume that p is directly proportional to the concentration of unhydrolysed κ-casein ($p = K's$). The latter equation then changes to a first-order rate equation.

Retardation of the rate of hydrolysis after cooling and pasteurization may be viewed from the decrease of the value of p. The mechanistic explanation could, for instance, be that the electrostatic or steric repulsion between the enzyme and micelle increases because of alterations at the micelle surface.

Additional experimental evidence for a diffusion-controlled enzymic reaction has been given by Chaplin & Green (33). They found that the rate of hydrolysis in milk declined rapidly with increasing size of dextrane-pepsin conjugates.

Our model also leads to the conclusion that insoluble enzymes are very ineffective for clotting milk, despite some conflicting results from other workers (34). Another consequence of the observed result is that the existing mechanistic models on the combined kinetics of hydrolysis and coagulation should be adapted to a first-order rate equation for the enzymic reaction, instead of the Michaelis-Menten or zero-order rate expression normally used.

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Samenvatting

A. C. M. van Hooydonk, C. Olieman en H. G. Hagedoorn, *De kinetiek van de door chymosine gekatalyseerde splitsing van κ-caseïne in melk*[1].

De splitsing van κ-caseïne in melk door het enzym chymosine bleek goed beschreven te kunnen worden door een reactie van de eerste orde met betrekking tot de substraat- en enzymconcentratie. De schijnbare activeringsenergie van de omzetting was ongeveer 26 kJ/mol.

Zowel het pasteuriseren (5 s, 74 °C) als het koelen (4 °C) van melk voorafgaand aan het stremproces vertraagde de snelheid van de proteolyse. Verhoging van de caseïneconcentratie door middel van ultrafiltratie resulteerde in een afname van de enzymatische reactiesnelheidsconstante. Deze vertraging moet waarschijnlijk worden toegeschreven aan een afname van de effectieve diffusiesnelheid van het enzym.

Een mechanistisch model ter verklaring van de waargenomen kinetiek is gegeven door uit te gaan van een proces waarbij de adsorptie van het enzym aan de caseïnemicel te verwaarlozen is. De reactiesnelheid is dan afhankelijk van het aantal botsingen tussen substraat en enzym, dus van de diffusiesnelheid. Omdat het enzym vele malen kleiner is dan de caseïnemicellen is de diffusiesnelheid van het enzym bepalend. Het aantal effectieve botsingen neemt evenredig af met de splitsingsgraad, hetgeen resulteert in een reactie van de eerste orde.

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pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 1. Effect of acidification on physico-chemical properties[1]

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Summary

A linear relation was found between micellar calcium + magnesium and micellar inorganic phosphate + citrate in the pH range 5.6-6.7. The amount of calcium and magnesium directly bound to casein appears to be independent of pH in this region. It is postulated that the carboxyl groups significantly contribute to the binding.

The dissociation of casein from the micelle at 30 °C showed a peak value around pH 5.6. All major caseins are liable to dissociate at this pH, probably in the form of submicelles. It is suggested that mainly the outer-layer submicelles dissociate. No dissociation was observed in rennet-treated milk.

The voluminosity of casein in normal skim milk was at a maximum around pH 5.3. After renneting the voluminosity decreased and the maximum, though less pronounced, was shifting towards pH 5.6.

The observed pH-dependence of the dissociation and voluminosity is explained on the basis of MCP content of the micelles and the electrostatic repulsion and attraction between charged groups.

1 Introduction

The fermentation of milk by lactic acid bacteria is an essential step in the manufacture of many dairy products. The physico-chemical changes of the casein micelles brought about by acidification are reflected in the rheological properties of these products.

The understanding of these changes and how they are affected by various factors is still limited. The most important effects of lowering the pH of milk

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are the solubilization of micellar calcium phosphate (MCP) and the decrease of the net negative charge of casein molecules. As a result the interaction between the different caseins in the micelle and the adsorption of ions onto the micellar aggregate are changed.

Previous work on this subject also revealed that some important colloidal properties of the casein micelles, such as voluminosity (1, 2, 3), ζ-potential (4) and the dissociation of certain caseins (3, 5), show a peculiar pH-dependence.

This study was mainly conducted to investigate the difference in pH-induced changes between normal casein micelles and para-κ-casein micelles. In a second paper we shall attempt to relate these findings to the pH-dependence of the chymosin-catalysed proteolysis of κ-casein and the subsequent aggregation of micelles.

2 Materials and methods

2.1 Skim milk

Bulk-collected milk (4 °C) was stored for 24 h at this temperature and subsequently heat-treated (10 s, 65 °C), cooled to 47 °C, skimmed and the skim milk cooled further to 4 °C.

2.2 pH adjustment

Prior to pH adjustment 0.02 % sodium azide was added to prevent bacterial growth and also 0.01 % aprotinin (Sigma Chemical Company, St. Louis, USA; strength 26 TIU/ml) to prevent plasmin action. No effect of these additions was found on the clotting time of milk when rennet was added.

All pH values were adjusted at 4 °C by very slow addition of 4 mol/l HCl and vigorous stirring. After overnight storage at 7 °C the samples were heated to 30 °C and the pH was measured after 30 min equilibration. Small pH corrections were sometimes necessary to achieve the desired value.

2.3 Centrifugation

A Beckman L8-30M ultracentrifuge was set at 30 °C and the milk samples were put into the prewarmed tubes and rotor. After 1.5 h centrifugation at 88 000 *g* at 30 °C the serum was carefully withdrawn by means of a syringe and the tubes were drained.

2.4 Renneting

0.02 % rennet (10 800 SU, from CSK, Leeuwarden, Netherlands) was added to the samples after 30 min equilibration at 30 °C. The samples were incu-

bated for 1 h at this temperature prior to centrifugation.

2.5 Solvation

The pellets formed during centrifugation were removed from the tubes and freeze-dried. The loss in weight after freeze-drying was determined. The nitrogen content of the dry material was analysed by a rapid colorimetric procedure (6).

2.6 Viscosity

The pH-adjusted samples were equilibrated for 30 min at 30 °C and the viscosity was measured at this temperature using an Ubbelohde-type capillary viscometer. The corresponding permeates, obtained by ultrafiltration (Amicon cell with pm 10 membrane) at 30 °C were also measured.

2.7 Chemical analysis

The calcium and phosphate concentration in milk and in the supernatants were determined by standard methods (7, 8) and the nitrogen content by a rapid colorimetric procedure (6).

2.8 Electrophoretic analysis

Starch gel electrophoresis and SDS polyacrylamide gel electrophoresis of the supernatants were carried out as described in Refs. 9 and 10 respectively.

3 Results and discussion

3.1 Solubilization of micellar calcium phosphate (MCP)

The partition of calcium and inorganic phosphate between the micelles and the serum was determined on the basis of the total concentration of the constituents in the original milk and that in the serum. Separation was achieved by ultracentrifugation at 30 °C.

MCP, i.e. the Ca-phosphate in the sedimented micelles, was calculated after applying the necessary correction for non-solvent water (11).

The results for three milk samples (renneted and non-renneted) are given in Fig. 1. No significant differences were found between normal skim milk and the same skim milk treated with rennet. At pH 5.3 virtually all of the inorganic micellar phosphate was transferred to the serum phase, whereas still about 14 % of the calcium (≈3.3 mol Ca/mol casein) was present in the micelles.

The relation between micellar calcium and inorganic phosphate is shown in Fig. 2. A linear relation between the two components was found in the pH

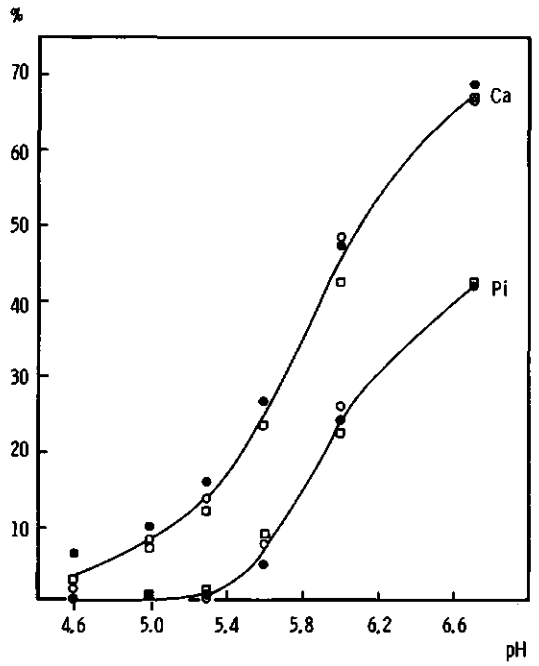


Fig. 1. Micellar calcium and inorganic phosphate at 30 °C. Three experiments; each symbol represents the average of skim milk and the same skim milk after rennet-treatment. Percentage of total concentration in milk.

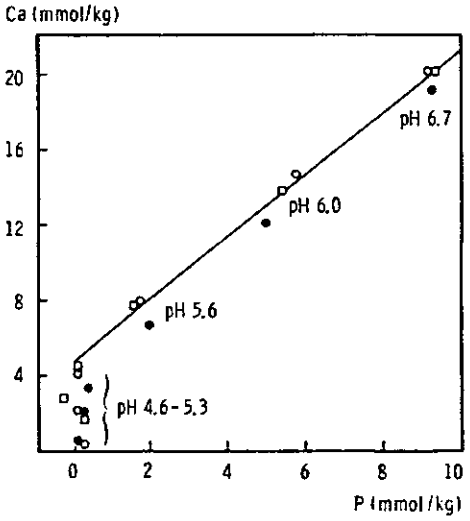


Fig. 2. Relation between micellar calcium and inorganic phosphate in the pH range 4.6-6.7 at 30 °C. Results taken from Fig. 1; equal symbols refer to the same milk sample.

range 5.6-6.7. The calculated slope was 1.66 ± 0.08 mol Ca/mol P_i and the intercept 4.64 ± 0.05 mmol Ca/kg. These results are not significantly different from those of Holt (12), who used EDTA depletion and those of Chaplin (13), who also used acid depletion. According to Chaplin (13) this linear relation is valid in the pH range 4.8-8.4. This seems unlikely because of the almost complete removal of inorganic phosphate from the micelle at pH values below 5.3. Our results are in better agreement with the titration experiments of Eilers (14), who concluded that inorganic phosphate was entirely dissolved at pH 5.2.

The similarity between the EDTA experiments at constant pH and experiments with pH variation is striking. Holt (12) interpreted the intercept as being the adsorption of Ca directly to casein and the slope as the real ratio between Ca and P_i.

In our opinion the contribution of citrate and Mg should also be taken into account because it is well established (15, 16) that the ratio of Ca/P_i in MCP depends on the presence of these constituents as well. We therefore recalculated our data. The concentration of citrate and Mg were taken from relations given by Holt (12) and incorporated in the statistical linear regression procedure. For comparison the data provided by Davies & White (11) are also given (see Table 1).

The following relation was found:

$$Ca + Mg = 5.84 + 1.53\,(P + Cit) \text{ in mmol/kg } (r = 0.999)$$

This suggests a tricalcic relation between both micellar P_i and Cit, whereas 4.9 mol Ca + Mg is directly bound to 1 mol casein (1 kg milk contains about 1.2 mmol casein). It is noteworthy that Mg mainly increased the intercept, suggesting that it may occur in combination with casein rather than with micellar P_i. This is in agreement with the expectation on basis of solubility (16, 17). If the above concept is valid then the adsorption of Ca + Mg seems to be

Table 1. Effect of pH on the micellar concentration (mmol/kg milk) of calcium, inorganic phosphate, magnesium and citrate.

pH	Ca	P	Mg		Cit	
			Holt (12)	Davies & White (11)	Holt (12)	Davies & White (11)
6.7	19.9	9.2	1.5	1.7	1.0	0.6
6.0	13.5	5.3	1.3	-	0.6	-
5.6	7.5	1.5	1.2	1.0	0.2	0.2

independent of pH in the range 5.3-6.7. This is surprising because one would expect an important decrease in this region because of protonation of the ester phosphate of casein (pK 1.5 and 6.5). Model studies (18, 19) indicate that Ca binding depends mainly on the double charge of these groups. Therefore hardly any adsorption is expected below pH 5.5.

An explanation for the apparent pH-independence of Ca binding is not easily at hand. It could be that the ester-phosphate groups are not the main Ca binders but that the carboxyl groups are also involved (pK 4.5). The slight decrease in negative charge in the pH range would also affect the Ca binding but the increase of Ca^{2+} activity might compensate for this effect. Furthermore, a competition between MCP and counter-ions for charged groups of the caseins may not be excluded. The pH independence of Ca binding may, in fact, be just fortuitous.

3.2 Dissociation of casein

In a previous paper (3) we showed that a significant amount of casein dissociated from the micelle in the pH range 5.4 to 6.0 at 20 °C. From starch gel electrophoresis (SGE) it was concluded that β-casein was the major dissociating component.

The aim of the present experiments was mainly to compare the pH dependence of the dissociation in normal skim milk and rennet-treated skim milk at 30 °C. After separating the casein and para-κ-casein micelles by ultracentrifugation, the supernatants were analysed for total nitrogen content and the presence of different casein fractions by SGE and SDS polyacrylamide gel electrophoresis (PAGE). The change in serum nitrogen concentration with pH is plotted in Fig. 3 for the three milk samples also used for MCP determinations. Equal symbols refer to the same milk.

It is clear that the amount of dissolved protein varies considerably between the samples, despite the almost identical results for MCP solubilization. Below pH 6.0 the trend in dissociation was similar but the level varied. In all three samples a peak value was observed around pH 5.6 followed by a gradual decrease until the isoelectric pH of casein is reached.

Rose (5) showed that the amount of dissociated casein increases at pH values above the natural pH of milk which suggests that the minimum of one of our samples (●) is at a higher pH value.

In order to calculate the fraction of dissociated casein from the results given in Fig. 3 it is necessary to correct for the non-casein nitrogen. Casein normally precipitates completely at pH 4.6 and the non-casein part remains in solution. In one of the samples (○) the latter fraction was higher than normally encountered in milk. PAGE analyses of the sample (results not shown)

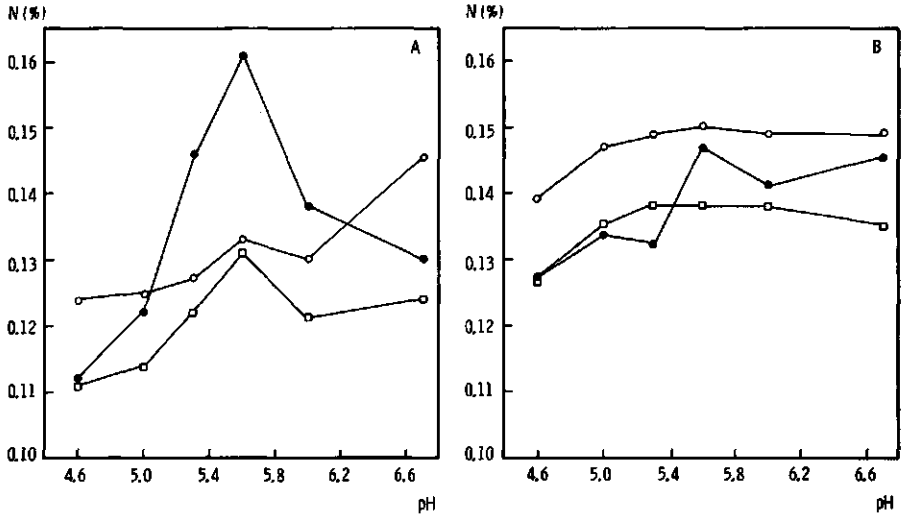


Fig. 3. Nitrogen concentration in serum after ultracentrifugation (88 000 *g*, 1.5 h) at 30 °C. Three milk samples. A: skim milk; B: same milk after rennet-treatment. % N in original milk: 0.537 (●); 0.513 (□); 0.528 (○).

revealed that an extra protein band appeared around the position of β-casein irrespective of pH. No explanation can be offered for this somewhat abnormal behaviour. The best guess is probably that proteolytic enzymes produced some soluble casein during cold storage.

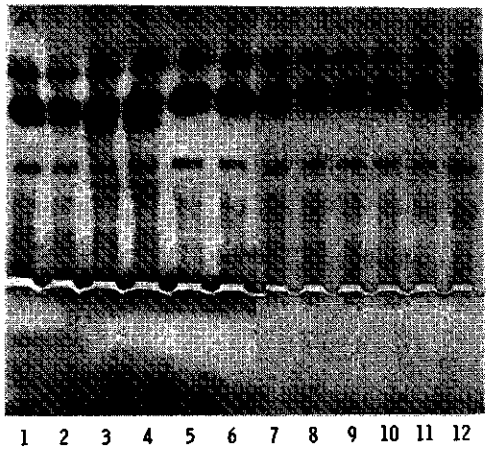
Taking 0.112 % N (the most probable value) for the non-casein part it can be calculated that the dissociated fraction at pH 5.6 varied between 5 % and 12 % of the total concentration of casein in the milk.

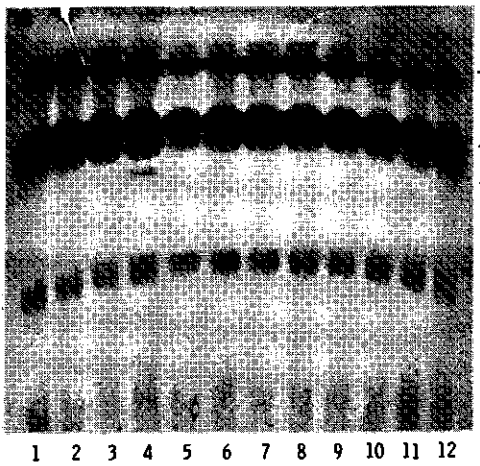
The pH-dependent dissociation in rennet-treated milk is different (Fig. 3B). After substraction of the same amount for the non-casein fraction and an additional 0.016 % N for the casein macropeptide (CMP) released by the action of rennet, the dissociated casein was less than 5 % of the total.

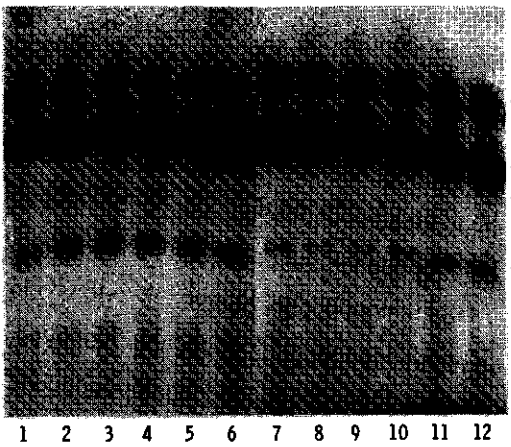
The results of the SGE analysis (Fig. 4) confirmed the features of the protein determinations. β-casein was detected in all three samples at pH 5.6 if no rennet was added. Only the sample with the highest soluble casein content (sample ●) contained in addition a detectable amount of $\varkappa$-casein.

One of the milk samples (○) showed also a significant amount of dissociated β-casein at the normal pH of milk.

In none of the rennet-treated samples did a distinct band appear at the position of the major caseins, which suggests that the dissociated casein coprecipitates with the casein micelles after conversion of $\varkappa$-casein. The dissolved N


α-lactalbumin
β-lactoglobulin
β-casein
BSA
κ-casein
1 2 3 4 5 6 7 8 9 10 11 12


α-lactalbumin
β-lactoglobulin
β-casein
BSA
1 2 3 4 5 6 7 8 9 10 11 12


α-lactalbumin
β-lactoglobulin
β-casein
BSA
1 2 3 4 5 6 7 8 9 10 11 12

← Fig. 4. Starch-gel-electrophoretic patterns of supernatants obtained by ultracentrifugation (88 000 *g*, 1.5 h, 30 °C) of skim milk (1-6) and rennet-treated skim milk (7-12). Foto A, B and C refer to milk samples ●, □, ○ respectively. 1 and 7: pH 4.6; 2 and 8: pH 5.0; 3 and 9: pH 5.3; 4 and 10: pH 5.6; 5 and 11: pH 6.0; 6 and 12: pH 6.7.

found in these samples probably consisted of breakdown products to which the non-specific action of rennet (incubation and centrifugation takes about 3 h) may have contributed.

PAGE analysis of the supernatants of one of the milk samples (Fig. 5) revealed that apart from β-casein, α_s-casein is also present at pH 5.6. After addition of rennet to this particular supernatant, the turbidity increased, suggesting that $\varkappa$-casein is also the stabilizing factor for the dissociated casein fraction. This fraction is probably organized in small units (submicelles) because neither of the caseins can exist as monomers under these conditions.

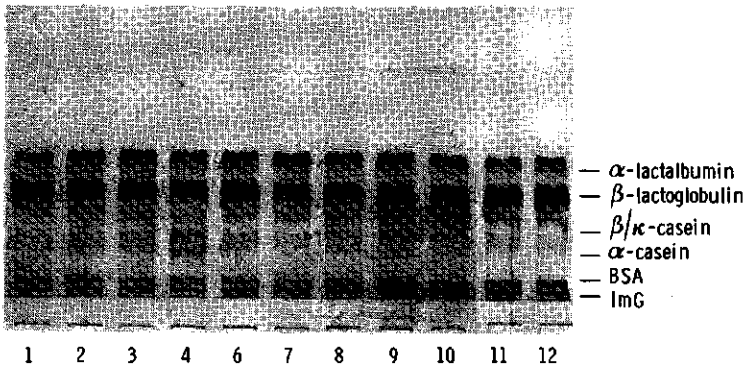


Fig. 5. SDS-PAGE patterns of the supernatants of milk sample (□) obtained by ultracentrifugation (88 000 *g*, 1.5 h, 30 °C) of skim milk (1-6) and rennet-treated skim milk (7-12). Explanation of numbers is given in Fig. 4.

Earlier work (5, 20) on the pH-dependent dissociation of casein in cold milk showed a very extensive solubilization of all caseins indicating the importance of temperature.

3.3 Solvation and voluminosity of casein

Several techniques, such as viscometry, electron microscopy, quasi-elastic

light scattering and ultracentrifugation have been applied to determine the voluminosity of casein micelles. Walstra (21) discussed the different methods and explained the discrepancies between them. For our purpose, sedimentation was the most suitable technique because the solvation of unstable para-κ-casein micelles can be analysed without an extreme dilution of the sample.

Compared to e.g. viscometry, sedimentation underestimates the voluminosity mainly due to a compression of a 'hairy' outer layer of the micelles in the pellet (21).

Solvation and voluminosity can be calculated from the contribution of the different components in the wet pellet. The total sediment volume (V_s) is:

$$V_s = C_p v_p + C_l v_l + C_m v_m + C_w v_w \text{ (ml/g)}$$

where C_p, C_l, C_m and C_w are the concentrations (g/g) of protein, lactose, minerals and water in the pellet. These concentrations are multiplied by the corresponding specific volumes (ml/g). The specific volume of casein is on average 0.70 (22) and of lactose and minerals 0.60 and 0.50 (23) respectively. The average value of 0.55 was used in the calculations for the concentration of other solutes (no protein, no water) in the pellet. It is assumed that the specific volumes are pH-independent.

Solvation is now defined as C_w/C_p (physical entrapped and non-solvent water) and the voluminosity as V_s/C_p. Due to the solubilization of MCP the concentration of minerals changes with pH, making the difference between solvation and voluminosity pH-dependent.

Inter-particle moisture in the pellet is neglected. Casein micelles are easily deformed by the centrifugal forces and form almost a clear gel showing hardly any refractive index modulations. Figure 6 gives the results for the three milk samples. The curves for the native casein micelles in milk at 30 °C showed a similar pattern to that found before at 20 °C (1, 3). The level, however, has decreased over the entire pH range.

The action of rennet considerably decreased solvation but still a weak maximum existed near pH 5.6.

The reduction in solvation caused by rennet is plotted in Fig. 7. At the natural pH of milk the reduction was about 27 % and a maximal value of 37 % was reached at pH 5.3. These results on the solvation of rennet-treated micelles differ considerably from the recently reported observations by Creamer (24). He found a maximum in solvation near pH 5.1 instead of 5.6. This discrepancy is probably due to different experimental conditions. Creamer used a low renneting temperature (6 °C) with relatively short incubation times (30 min). pH adjustments were made after renneting instead of before, as we did.

In order to corroborate that the voluminosities obtained by sedimentation

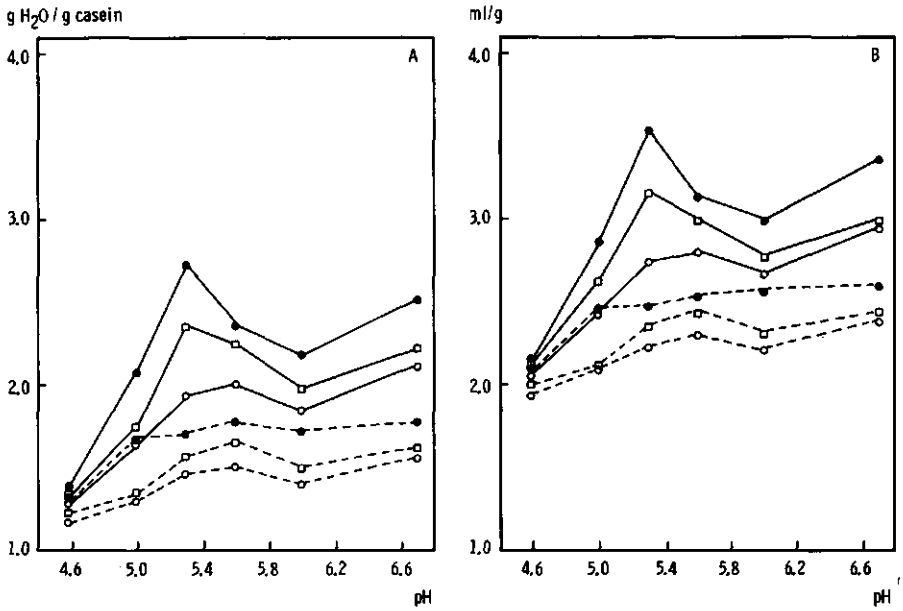


Fig. 6. Solvation (A) and voluminosity (B) of casein in three different skim milk samples (●, □, ○) at 30 °C. Normal skim milk (——); skim milk after rennet treatment (– – –).

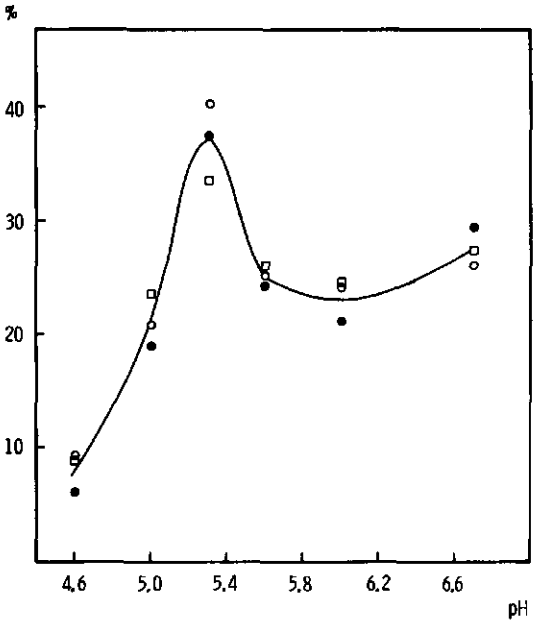


Fig. 7. The reduction (%) in solvation of casein micelles caused by the action of rennet (30 °C). Symbols refer to the same samples as in Fig. 6.

really represent the actual change in micelle volume we also measured the voluminosity of the non-renneted skim milk samples by means of viscometry. Both the viscosities of the milk and the corresponding permeates were determined at 30 °C.

The voluminosity was calculated on the basis of the equation of Eilers (25):

$$\eta_m/\eta_p = [1 + 1.25\ \Phi/(1 - \Phi/\Phi_{max})]^2$$

where η_m and η_p denote the viscosities of the milk and UF permeate respectively. Φ is the volume fraction of the dispersed phase (casein micelles and whey proteins) and Φ_{max} a constant representing the maximal packing.

According to Snoeren et al. (26), $\Phi_{max} = 0.79$ and the voluminosity of whey proteins 1.07 ml/g. For the calculation of the pH-dependent voluminosity of casein we assumed that the contribution of the whey proteins to Φ was constant. On the same milk sample we also determined the voluminosity by sedimentation. Viscosity data and voluminosities are given in Table 2.

It is clear that the voluminosities obtained with the two different methods showed the same pH dependency. According to expectation the values found by viscosimetry were higher than those found by sedimentation. From this we may conclude that the change in voluminosity is predominantly caused by swelling and shrinkage and not by the replacement of the dissociated casein and MCP by water. The latter effect would only change solvation and not the total volume of the dispersed phase.

3.4 Relation between the observed changes

It is well established that the integrity of the casein micelle depends on the amount of MCP present. MCP is thought to be evenly distributed throughout the micelle in the form of small clusters (27). The nature of their interaction with the casein molecules is still a matter of discussion. In several publications (27, 28) it is postulated that the ester-phosphate of casein is in some way linked to MCP, and Holt et al. (29) proposed that these groups may in fact be

Table 2. The pH-dependent voluminosity of casein in milk, determined by viscometry (Vol_v) and sedimentation (Vol_s). Temperature 30 °C. Φ_{cas} = volume fraction of casein (for calculations see text). Total protein 3.80 %, whey protein 0.67 %, NPN 'protein' 0.18 %.

pH	η milk (mPa·s)	η permeate (mPa·s)	ηrel	Φcas	Vol_v (ml/g)	Vol_s (ml/g)
6.79	1.262	0.909	1.387	0.113	3.82	3.18
6.01	1.195	0.914	1.308	0.092	3.14	2.83
5.54	1.231	0.914	1.347	0.102	3.45	3.13

part of a brushite-like structure. Other workers (30, 31) favour the interaction with the carboxyl groups of the protein and even an interaction with the positive amino groups has been suggested (32, 33). In any case, solubilization of MCP will always diminish the interaction between the proteins (according to Schmidt (27) between the submicelles) which may cause swelling and dissociation of casein, probably in the form of submicelles. The outer-layer submicelles, rich in κ-casein and with a minimum of contact points with MCP, are probably more liable to dissociate than the interior ones. A second important factor for the integrity of the micelle is the repulsion and attraction between charged residues, which is not only changed by the direct effect of pH on the ionization of these groups but also by the shielding effect of the ions in the serum. Solubilization of MCP increases the ionic concentration.

In order to explain the typical pH dependence of the dissociation and voluminosity of casein, the relative importance of the two stabilizing factors in the different pH regions has to be considered.

Decreasing the pH from 6.7 to 6.0 causes a slight decrease in voluminosity for both normal casein micelles and para-κ-casein micelles. The swelling effect due to MCP solubilization is, counteracted and in this region apparently dominated, by the increased attraction between charged groups. The ionization of the carboxyl groups (pK 3.6-4.5) does not change significantly in this region. It is uncertain what happens with the ester-phosphate groups (pK 1.5 and 6.5). Some decrease in negative charge is expected, but if these groups are buried in MCP, as is suggested by Holt (29), there might even be an increase in ionization when they are released after solubilization of MCP. The number of positive charges increases due to the protonation of the histidine residues (pK $\approx$ 6.5). The resulting electrostatic attraction with the negative groups is probably the cause of the shrinkage in this region.

In the pH region from 6.0 to 5.6 the micelle swells and dissociation of casein reaches a maximum. All amino groups are almost fully charged and the net negative charge diminishes mainly due to the protonation of carboxyl groups, but there may be some replenishment due to the release of negatively charged ester-phosphate groups. The expansion of the micelle in this region is caused by the dominating effect of MCP solubilization.

In the pH region from 5.6 to 5.3, charge neutralization dominates the effect of MCP solubilization with respect to casein dissociated and the voluminosity of casein in the rennet-treated skim milk. The voluminosity of the micelles in the normal skim milk still increases and reaches a maximum value around pH 5.3. The discrepancy between the pH maximum of voluminosity and casein dissociation is not easily explained.

Electron micrographs made by Kalab et al. (34) during acidification of un-

heated milk showed that micelles have a very ragged surface near to the pH of gelation, indicating that the outer-layer submicelles are loosely bound to the micelle. This might increase the voluminosity more than in the situation (pH 5.6) where these submicelles dissociate from the micelle. The considerable reduction in voluminosity caused by rennet at pH 5.3 is probably due not only to the removal of the relative hydrophylic part of κ-casein but also to the collapse of these loosely bound submicelles.

Below pH 5.3 all MCP is solubilized and further charge neutralization causes a decrease of the voluminosity and a precipitation of not only the dissociated casein fraction but also whole micelles.

4 Conclusions

The results of the pH-dependent solubilization of MCP more or less confirm the results found by others (11, 12, 13, 16). A linear relationship was found between micellar Ca + Mg and micellar P_i + Cit for pH values above 5.3. At and below this pH, virtually all micellar P_i is transferred to the serum whereas still about 14 % of the calcium is bound to the micelle.

The adsorption of Ca + Mg directly to the casein molecules seems to be independent of pH in the range of 5.6 to 6.7. It is suggested that carboxyl groups play an important role in Ca binding.

At pH 5.6 a maximum in the amount of dissociated casein was found with perhaps a small minimum around pH 6.0. All major caseins are liable to dissociate at pH 5.6 in normal skim milk whereas no dissociation could be detected in the rennet-treated samples.

The maximum in voluminosity of normal casein micelles was found around pH 5.3. After renneting the voluminosity decreased and the maximum, though far less pronounced than for non-renneted samples, was shifting towards pH 5.6.

The reduction in solvation caused by rennet was about 27 % at the original pH of milk and maximal, i.e. 37 %, at pH 5.3.

Voluminosities calculated from sedimentation and viscometry showed the same pH dependency. This suggests that the increase in solvation at pH 5.6 is caused by swelling and not by the replacement of the dissociated casein and MCP by 'water'.

The observed pH dependency of the dissociation and voluminosity of casein in milk is controlled by the relative importance of two pH-dependent stabilizing factors (MCP and electrostatic interactions between positively and negatively charged groups) at the different pH values. It is suggested that the outer-layer submicelles are more liable to dissociate than the interior ones.

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We are particularly indebted to Dr H. Vreeman (NIZO) and Prof. P. Walstra (University of Wageningen) for stimulating discussions on this subject. We wish to thank Mr J. Kaper for his contribution in the electrophoretic analysis, and Mr G. G. Mahulette and Mrs S. Makkinga for the determinations of calcium and phosphate.

Samenvatting

A. C. M. van Hooydonk, H. G. Hagedoorn en I. J. Boerrigter, *Fysisch-chemische veranderingen van caseïnemicellen in melk als functie van de pH en het effect daarvan op de stremreactie. 1. Effect van pH-verandering op fysisch-chemische eigenschappen.*[1]

Een lineaire afhankelijkheid tussen micellair calcium + magnesium en micellair anorganisch fosfaat + citraat werd gevonden in het pH-gebied van 5,6 tot 6,7. De hoeveelheid aan het caseïne gebonden calcium en magnesium is in dit gebied blijkbaar onafhankelijk van de pH. Het is waarschijnlijk dat de carboxylgroepen een significante bijdrage leveren aan de binding.

De dissociatie van caseïne bij 30 °C bleek een piek te vertonen bij pH 5,6. De dissociatie vindt vermoedelijk plaats in de vorm van submicellen waarin de drie belangrijkste caseïnes aanwezig zijn. We veronderstellen dat vooral de submicellen uit de buitenste laag van het oorspronkelijke caseïnemicel dissociëren. Na behandeling met stremsel kon geen dissociatie worden aangetoond.

De voluminositeit van het caseïne in normale ondermelk vertoonde een maximale waarde bij pH 5,3. Na stremming nam de voluminositeit sterk af en verschoof het in dit geval minder geprononceerde maximum naar een pH van 5,6.

De gevonden pH-afhankelijkheid van de dissociatie en de voluminositeit van caseïne kan verklaard worden op basis van het calciumfosfaatgehalte in de micellen en de elektrostatische repulsie en attractie tussen geladen groepen van het caseïne.

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Neth. Milk Dairy J. 40 (1986) 297-313

pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. 2. Effect of pH on renneting of milk[1]

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Key-words: acidification, casein micelles, kinetics, chymosin, coagulation.

Summary

Decreasing the pH of milk caused a steep increase in the rate of the enzymic stage of the renneting reaction. The maximum velocity was found around pH 6.0, but the differences in the pH range from 5.6 to 6.4 were small.

Possible reasons for the discrepancy between our results and pH optima for model substrate solutions reported in the literature are discussed. It is suggested that both the accessibility of the Phe-Met bond of κ-casein at the surface of the micelle and the interaction between chymosin and para-κ-casein micelles are affected by pH. The latter effect may lead to a decrease in the amount of effective free enzymes. This might be the explanation for the different kinetics found at lower pH values.

A first-order reaction described the conversion of κ-casein at the original pH of milk very well, but failed to fit the experimental data at pH values above 7.0 due to the inactivation of chymosin. A combined kinetic model for inactivation and proteolysis was derived and verified for these conditions.

The rate of aggregation and gel formation increased with decreasing pH. At pH 5.6 aggregation had already begun before 30 % conversion of κ-casein. At pH 6.7 this was before 70 % conversion, and was most likely with 60 % release of the casein macropeptide.

The elastic modulus of a rennet curd was at a minimum around pH 5.3. This is also the pH where the voluminosity of casein micelles is at a maximum. It is suggested that the minimum in the modulus arises from a weakening of interactions within the micelles rather than between the micelles.

1 Introduction

In the first paper on this subject (1) we dealt mainly with some important physico-chemical changes of micellar casein brought about by acidifying milk.

This present study was conducted to establish the effect of pH on the enzy-

[1] Offprints from this paper distributed as Research Report V264 of the Netherlands Institute for Dairy Research (NIZO), Ede, Netherlands (1986).

mic and coagulation reaction during the renneting process and to relate the effects to the physico-chemical properties of the caseinate complex.

The rennet coagulation time of milk increases with pH in the range from 5.2 to 7.0 (2). Above pH 7.0 para-κ-casein micelles become progressively more stable and do not really aggregate, and below pH 5.2 the measurement of the renneting process in milk is complicated by the isoelectric aggregation of the casein micelles. Only a few studies have been carried out to find the pH dependence of the enzymic reaction in milk. A summary of reported data is given by Carlson (3). A limited pH range was covered in these studies and no optimum has been reported for the action of chymosin.

More attention has been paid to the effect of pH on the rate of proteolysis in model solutions of sodium caseinate, whole κ-casein and certain fragments of κ-casein.

An extensive study of the determination of the optimum pH for the conversion rate of κ-casein fragments by chymosin was performed by Visser et al. (4). The maximum rates of proteolysis of two peptides containing the chymosin-sensitive Phe-Met bond (residues 98-111 and 98-112) were at pH 5.3 and 5.4 respectively. This optimum pH is nearly the same as found for whole κ-casein (5, 6). The optimum for sodium caseinate solutions was also found in this range e.g. between pH 5.1 and 5.3 (6).

Published results of the effect of pH on the rennet-induced aggregation and gelation of casein micelles are scarce. The results of Mehaia & Cheryan (7) indicate that the rate of aggregation increases with pH in the range from 5.5 to 6.6.

2 Materials and methods

2.1 Materials

Skim milk. Bulk collected milk (4 °C) was stored for 24 h at this temperature and subsequently heated for 10 s at 65 °C, cooled to 47 °C, skimmed and the skim milk cooled again to 4 °C.

Commercial liquid rennet with a strength of 10800 Soxhlet Units was purchased from CSK (Leeuwarden, The Netherlands).

2.2 Methods

2.2.1 pH adjustment of milk. All pH values were adjusted at 4 °C by slow addition of 4 mol/l HCl or NaOH and vigorous stirring. After overnight storage at 7 °C the milk samples were heated to 30 °C and the pH measured after 30 min equilibration. It should be noted that a 30-min period is too short to reach complete equilibration. This would take up to 24 h, and even then only a me-

tastable situation is achieved (providing no native proteolytic enzymes are active).

2.2.2 Determination of casein macropeptide (CMP). The release of CMP during the enzymic reaction was followed quantitatively by gel permeation chromatography on high-performance columns as described in previous papers (8, 9).

A 6 % solution of sweet cheese whey powder was used for calibration. This solution, containing 0.21 mmol/l CMP, had previously been calibrated against a known amount of a completely converted sample of pure κ-casein (8).

The calculations of the reaction parameters from the progress curves were made by computer-fitting procedures as described previously (9). The experimental data were fitted into the integrated Michaelis-Menten and first-order equations. Initial velocities were determined by linear regression on the first few CMP values.

Each milk sample was equilibrated for about 30 min at the reaction temperature prior to renneting.

2.2.3 Determination of the aggregation by viscometry. A Deer Rheometer (constant stress instrument) equipped with the MG7103 concentric cylinder geometry was used to monitor the increase in viscosity during renneting. The stress was set to give a shear rate of 142 s^{-1} at the start of the renneting process. During renneting the shear rate drops, due to the increase in viscosity resulting from the aggregation of casein micelles.

The viscosity measurements were run in parallel with, and under the same conditions as the determination of CMP.

2.2.4 Determination of the gel formation. The time course of the gel formation process was followed with an Instron 1122 Universal Testing Instrument. A short description of the method has been given previously (10). A rectangular gauze (85 mm × 60 mm) was moved up and down slowly in a thermostated beaker (internal diameter 80 mm) filled with 550 ml milk.

The mechanical resistance exerted by the gel on the gauze was measured continuously with a 5 N load cell. The amplitude of the oscillatory movement was 0.2 mm, and the applied frequency 0.2 Hz. A schematic representation of the method is given in Fig. 1. These measurements were also run in parallel with the determination of the viscosity and the release of CMP.

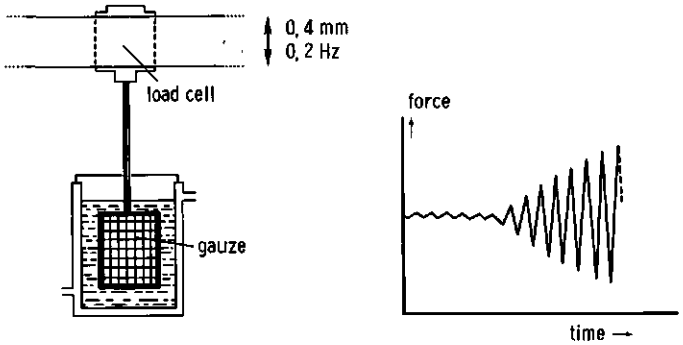

Fig. 1. Schematic representation of the technique applied to monitor the gel formation during renneting.

3 Results and discussion

3.1 Enzymic reaction

3.1.1 Effect of pH on initial velocity. In a previous paper we showed that the release of CMP at the original pH of milk can be described by a first-order reaction (9). At higher pH values however, chymosin was inactivated at a significant rate and the exponential equation failed to fit the conversion of κ-cas-

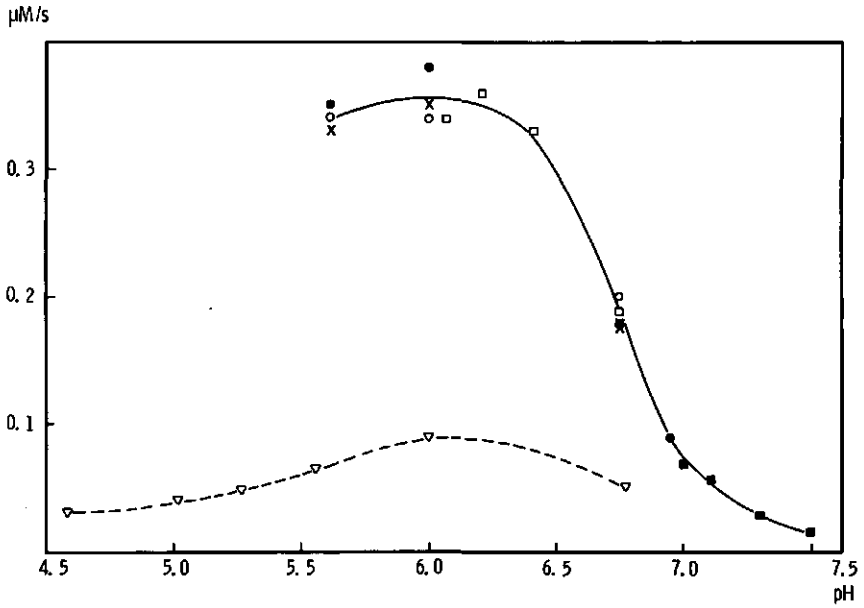

Fig. 2. Effect of pH on the initial rate of the enzymic reaction at 30 °C (——) and 4 °C (– – –). Equal symbols refer to the same skim milk sample. Rennet concentration 0.02 %.

ein. For this reason the initial velocity (V_i) of the reaction was used to analyse the pH dependence of the enzymic reaction.

The effect of pH on V_i is plotted in Fig. 2. In order to extend the experiments to low pH values we also performed measurements at 4 °C. At this temperature isoelectric precipitation of casein does not take place.

Lowering the pH caused a steep increase of V_i with a maximum around pH 6.0, both at 30 °C and 4 °C. Within the pH range of 5.6 to 6.4 velocity differences were small. Increasing the pH up to 7.5 resulted in a gradual drop of V_i.

In Section 3.1.3 these results will be compared with other reported data.

3.1.2 Complete conversion curves at selected pH values. The time course of the release of CMP at five different pH values is given in Figs. 3 and 4. All curves in Fig. 3 were fitted into the integrated first-order and Michaelis-Menten equations. The first-order fit at the original pH of milk was excellent. This is in agreement with previously obtained results (9). The quality of the first-order fit was slightly less at pH 6.2. In this case the Michaelis-Menten equation described the experimental data better (see Fig. 3). The 'best' parameter values, V_{max} and K_m, at pH 6.2 were calculated to be 0.66×10^{-3} mmol $l^{-1}s^{-1}$ and 0.31 mmol l^{-1} respectively.

The rennet concentration used was 0.01 % and the initial substrate concentration amounted to 0.164 mmol/l. From comparative experiments with purified chymosin we estimated that the activity of the rennet solution was equivalent to a chymosin concentration of roughly 2.15×10^{-2} mmol/l. From this it follows that the values for the two kinetic parameters K_{cat} and K_{cat}/K_m, are about 300 s^{-1} and 1000 mmol l^{-1} s^{-1}. The latter value falls within the range reported by Visser et al. (4) for various $\varkappa$-casein fragments and whole $\varkappa$-casein. The value of K_m is however a factor 10 higher than those given in their paper, but in close agreement with K_m values found by Dalgleish (11) and Chaplin & Green (12) at the normal pH of milk. At this pH we never observed Michaelis-Menten kinetics.

As we have already pointed out (9), it is very doubtful that the kinetics of the enzymic reaction in milk can be treated by the classical Michaelis-Menten theory. The usual assignment of the kinetic parameters to any particular process is therefore probably not permissible.

A factor which complicates the interpretation of the kinetic parameters is the presence of two enzymes in rennet, i.e. pepsin and chymosin. According to Visser (13) the clotting activity of rennet is for about 87 % due to chymosin at the normal pH of milk. The activity of bovine pepsin increases more with decreasing pH than that of chymosin (14). Van den Berg & de Vries (15) reported a chymosin activity of 83 % at pH 6.3.

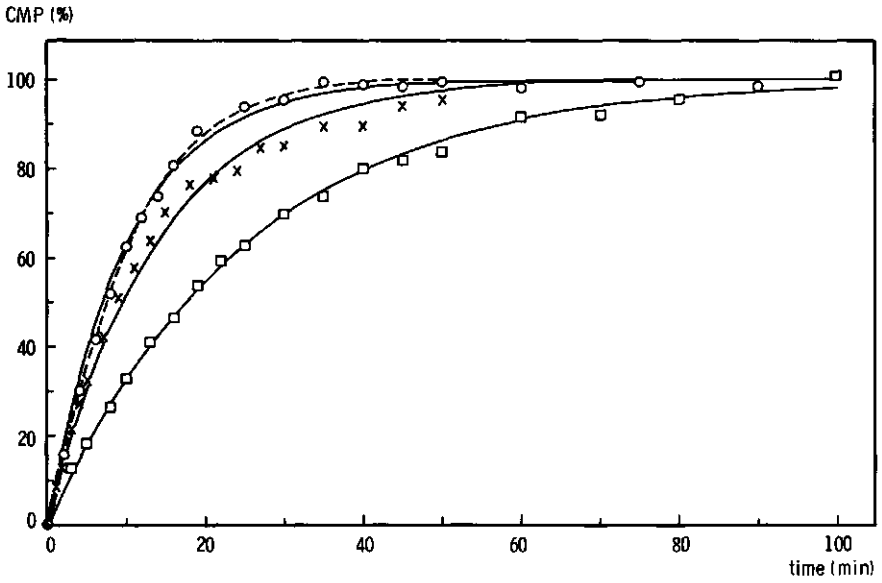

Fig. 3. Progress curves at different pH values. Solid curves are first-order fits, dashed curve is a Michaelis-Menten fit.
pH 6.74 (□) $K = 0.66 \times 10^{-3}\ s^{-1}$ (—);
pH 6.19 (○) $K = 1.64 \times 10^{-3}\ s^{-1}$ (—)
$V_{max} = 0.66 \times 10^{-3}$ mmol $l^{-1}\ s^{-1}$ (– – –)
$K_m = 0.31$ mmol/l;
pH 5.62 (×) $K = 1.23 \times 10^{-3}\ s^{-1}$ (—).
Rennet concentration 0.01 %, temperature 30 °C.

The best fit at pH 5.6 was obtained with the first-order rate equation, but the deviation from the experimental data was considerable, especially after about 80 % conversion.

At pH 7.5 rennet was inactivated at such a rate that only about 40 % conversion could be reached within 5 h (0.02 % rennet). Further breakdown of κ-casein could be achieved by adding extra portions of rennet at different time intervals during incubation (see Fig. 4).

At pH 7.3 the enzymic reaction could almost be completed with the same rennet concentration. No sign of aggregation was detected at this pH.

Carlson (3) combined the kinetics of porcine pepsin denaturation (first-order) with the Michaelis-Menten equation and showed that the resulting model described the experimental data satisfactorily for conditions where both reactions took place simultaneously. In our case it is probably more appropriate to use a first-order reaction for both the splitting of κ-casein and the denaturation of chymosin (the contribution of pepsin is probably negligible at

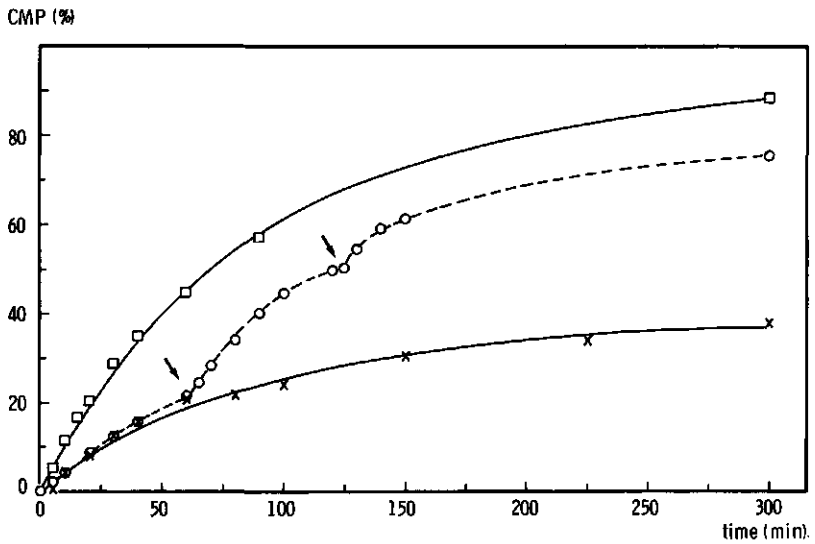

Fig. 4. Progress curves at pH 7.3 (□) and 7.5 (+ and ○). Arrows indicate time at which extra rennet was added. Solid curves are according to Eq. 4.
pH 7.3 (——) $K_{enz} \cdot e_o = 1.8 \times 10^{-4}\ s^{-1}$ and
$K_{den} = 0.5 \times 10^{-4}\ s^{-1}$,
pH 7.5 (——) $K_{enz} \cdot e_o = 0.75 \times 10^{-4}\ s^{-1}$ and
$K_{den} = 1.5 \times 10^{-4}\ s^{-1}$.
Rennet concentration 0.02 %, temperature 30 °C.

pH values above 7.0). The overall reaction can be derived by combination of the two.

– Rate equation for proteolysis:

$$-ds/dt = K_{enz} \cdot e \cdot s \qquad (1)$$

– Rate equation for denaturation of enzyme:

$$-de/dt = K_{den} \cdot e \qquad (2)$$

Where e and s are the enzyme and substrate concentrations respectively, and K_{enz} and K_{den} the reaction constants for the enzymic and the denaturation reactions respectively. Combination and integration for the conditions where $e = e_0$ and $s = s_0$ at $t = 0$ give the following results:

$$-ds/dt = K_{enz} \cdot e_0 \{\exp(-K_{den} \cdot t)\} \cdot s \quad (3)$$

$$\ln s_t/s_0 = \frac{K_{enz} \cdot e_0}{K_{den}} \cdot \exp(-K_{den} \cdot t) - \frac{K_{enz} \cdot e_0}{K_{den}} \quad (4)$$

The experimental data obtained at pH 7.3 and 7.5 were fitted into this equation. As can be seen in Fig. 4 (solid curves) Eq. 4 adequately describes the experimental data, especially at pH 7.3. 'Best' fit values for the two kinetic parameters are also given in Fig. 4.

3.1.3 Comparison with model substrates. Chymosin belongs to the class of aspartic or acid proteinases. The molecule consists of two domains separated by a deep cleft in which the active sites for substrate binding are located. Two aspartic acid residues play a major role in the catalytic functionality of the enzyme. Like all acid proteinases, chymosin has an optimum for general hydrolysis of peptides at low pH, e.g. around pH 4 (5). The optimum for the specific cleavage of the highly susceptible Phe-Met bond (residues 105-106) of model substrates was found in the pH range 5.1 to 5.4 (Section 1).

The discrepancy between the pH optimum in these systems and that in milk is not easily explained. There are of course some important differences with respect to reaction conditions.

– Acidification of milk not only changes the ionization of important catalytic groups of the enzyme and substrate but also solubilizes micellar calcium phosphate. Compared to the situation at the original pH of milk the serum phase at pH 6.0 contains about 6 mmol/kg extra calcium and about 3.5 mmol/kg extra inorganic phosphate (1). The ionic strength and especially the calcium ion activity are therefore increased. The results of Kruger et al. (16) and Kruk (17) indicate that the calcium ion activity at pH 6.0 is increased by about a factor of 2 to 3.

The direct effect of this may be a shielding of charges, which diminishes the electrostatic repulsion between the micelles and the enzyme. Also the ionic bond formation between the negative groups of the enzyme and the positive groups of κ-casein is affected by this shielding influence of the electrolytes. Payens & Visser (18) showed with model calculations that these two opposite effects may result in an optimum ionic strength (about 0.125) for the action of chymosin.

Increasing the ionic strength of milk by addition of NaCl decreased the rate of the enzymic reaction, whilst addition of $CaCl_2$ had no effect on the reaction rate (19). It seems therefore unlikely that the occurrence of the maximum near pH 6.0 instead of around pH 5.3 is caused solely by a difference in ionic strength.

- Another important indirect effect of the changed conditions caused by acidification are conformational and positional changes of the substrate at the micelle surface. In the first paper on this subject it was shown that the voluminosity of casein micelles is at a minimum near pH 6.0 and at a maximum around pH 5.3 (1). As far as we know such behaviour is typical for native casein micelles and is not observed in ($\varkappa$)-caseinate solutions. The accessibility of the Phe-Met bond for chymosin might well be a function of the protrusion of the CMP part of $\varkappa$-casein into the serum. At pH 6.0 the protrusion is probably minimal, allowing a higher probability for an effective collision between enzyme and substrate. Such an explanation would be consistent with a diffusion-controlled reaction as was suggested in a previous paper (9). Below pH 6.0 the voluminosity and the dissociation of casein increases due to the solubilisation of micellar calcium phosphate (1). It is not unlikely that these changes cause alterations at the micelle surface which may reduce the accessibility of the active site of the substrate.
- A third effect which may play an important part in the kinetics could be the adsorption of chymosin onto para-$\varkappa$-casein micelles. Stadhouders et al. (20) and also Holmes et al. (21) showed that the amount of rennet retained in the curd after syneresis increased with decreasing pH. Adsorption of chymosin onto (partly) converted micelles would reduce the number of catalytically active enzyme molecules. This inhibition effect is probably not significant during the early stages of the reaction but would increase with the degree of proteolysis. The shift in the optimum pH (on the basis of initial velocities) is therefore probably not explained by the adsorption effect. The deviation of the progress curves from first-order kinetics in the lower pH range might however be caused by this effect.

According to Geurts & Walstra (22) chymosin appears to interact preferably with β-casein at low pH values, i.e. pH 5.1. It remains uncertain whether these interactions also take place with the intact micelle.

3.2 Aggregation and gelation reaction

3.2.1 Depiction of the renneting process at different pH values. Viscometry is probably the most convenient and reliable method for investigating the initial stages of the aggregation of casein micelles under natural conditions. It does not require dilution of the sample, as is for example necessary in the case of the frequently used light-scattering methods.

Viscosity measurements are of course not suitable for monitoring the process of gel formation because the building of a continuous network of casein micelles is prevented. A dynamic rheological procedure was chosen to follow the assembly of the rennet gel (see Fig. 1). In order not to disturb the network

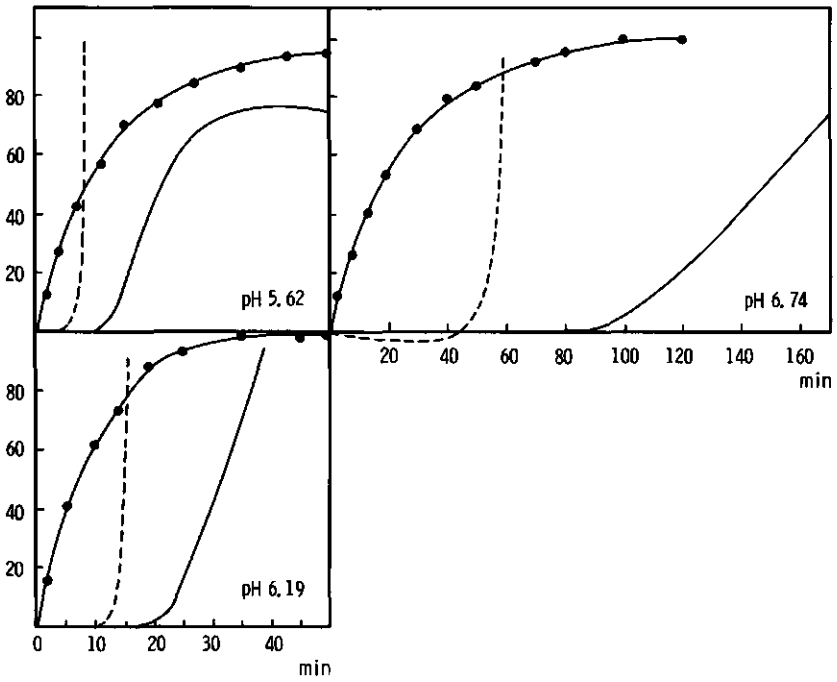

Fig. 5. The renneting process at three different pH values. Rennet concentration 0.01 %, temperature 30 °C.
●—● proteolysis (% of CMP split off on y-axis),
- - - aggregation (% increase of the viscosity on y-axis),
— gel modulus (arbitrary scale readings on y-axis).

the applied deformation should be kept small; typically below 3 % (23). With the described technique the deformation is well below this value. The stress and deformation regimes around the measuring gauze do not allow the transformation of the force signal into well-defined rheological parameters, but a linear relation between the measured force and the elastic shear modulus may be presumed.

The course of the various processes during renneting at three different pH values is depicted in Fig. 5. Table 1 summarizes the features.

At pH 6.7 the viscosity decreased initially. This effect is well established (24) and is due to the decreased voluminosity of (partly) converted micelles. Under the prevailing conditions the reduction in voluminosity is about 30 % after renneting (1). It is noteworthy that this means that CMP has an effective voluminosity of about 25 ml/g at the surface of the micelle. Assuming that the decrease in voluminosity is proportional to the amount of CMP released, the

Table 1. Effect of pH on some characteristic properties of the renneting process (0.01 % rennet, 30 °C).

V_i = initial velocity of enzymic reaction; AT = time at which the viscosity starts to increase; GT = onset of gelation; V_g = maximal rate of increase of gel modulus (arbitrary units).

pH	V_i (μmol l^{-1} s^{-1})	AT (min)	% CMP at AT	GT (min)	% CMP at GT	V_g a·u/min
6.74	0.10	36.5	70	75.0	94	1.08
6.19	0.21	11.0	64	18.2	86	3.54
5.62	0.20	4.5	30	10.0	55	4.20

following relation can be derived for the change in voluminosity of casein during renneting:

$$Vol_t = Vol_0[1-0.30\{1-\exp(-K\cdot t)\}] \quad (5)$$

Vol_t and Vol_0 denote the voluminosity at reaction time t and 0 respectively. K ($=K_{enz}\cdot e_0$) is the first-order rate constant for the enzymic reaction.

The change in the total volume fraction of dispersed particles contributing to the viscosity of the suspension can now be calculated as a function of time, and transformed to a relative viscosity by the equation of Eilers (25).

For the calculation of the total volume fraction, which is the sum of the dispersed components multiplied by their respective voluminosities, it was assumed that casein, whey proteins and the released CMP contribute significantly.

The relevant data for the calculation are given in Table 2. CMP in solution has, arbitrarily, been given the same voluminosity as the average value for caseins in solution (26). The real value is probably somewhat higher but no exact data are available. The predicted change of the relative viscosity is given in Fig. 6 together with the experimentally obtained curve at pH 6.7. The total expected fall in the relative viscosity if no aggregation were to take place is no more than 5 %.

Table 2. Parameter values for the calculation of the reduction of the relative viscosity during the enzymic reaction (Eq. 5).

Casein concentration	26 g/l
CMP concentration	1.2 g/l
Whey protein concentration	7.0 g/l
Voluminosity of casein	3.82 ml/g
Voluminosity of CMP	4.0 ml/g
Voluminosity of whey proteins	1.07 ml/g
Φ_{max} in Eilers equation	0.79

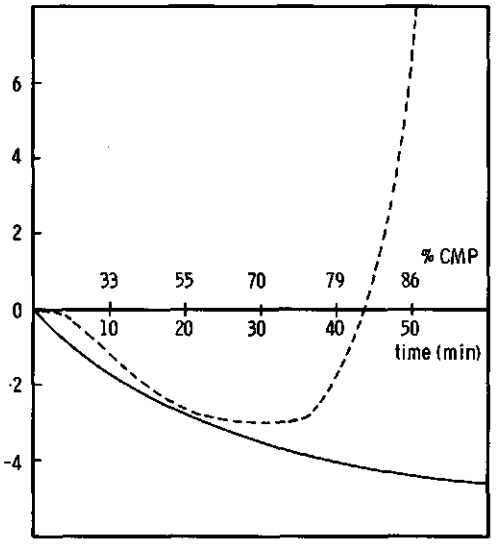

Fig. 6. The change in the relative viscosity (% of starting value) at pH 6.74. Rennet concentration 0.01 %, temperature 30 °C.
- - - experimental curve,
—— according to Eq. 5 and Eilers equation.

After about 70 % conversion the viscosity started to rise again. The relative viscosity is then about 97 % of its original value. This is in close agreement with the results of Green et al. (27). Scott-Blair & Oosthuisen (24) observed a 20 % reduction of the specific viscosity at the minimum. This amounts to about a 6 % reduction on the basis of the relative viscosity.

The actual viscosity at the beginning of the reaction dropped more slowly than the predicted values. This is understandable because the random removal of a few 'hairs' from the surface will hardly affect the amount of trapped water in the CMP shell around the micelle. Walstra et al. (28) observed a similar effect using a light-scattering technique.

The time course of the experimental curve at pH 6.7 suggests that the aggregation has begun around 60 % conversion.

The onset of aggregation (AT = time at which the viscosity starts to increase) at pH 6.2 and 5.6 was found at 64 % and 30 % conversion respectively. No initial fall in viscosity could be detected under these conditions, indicating that aggregation already compensates for the effect of volume reduction on viscosity in the early stages of the reaction.

The degree of proteolysis at gelation time (GT) also increased with pH. This is in agreement with the observations of Pierre (29). The maximum rate

of gelation, calculated from the linear part of the gelation curve, was highest at pH 5.6. The difference between pH 6.2 and 6.7 was however more pronounced than that between pH 5.6 and 6.2.

An interesting phenomenon is the existence of a maximum in the elastic modulus of the gel after a relatively short incubation time at pH 5.6. At the original pH the maximum modulus has a value about three times higher and is only reached after several hours (>4 h) of renneting.

3.2.2 Simultaneous acidification and renneting. In a different way the effect of acidification on the gel formation process in renneted milk was encountered in experiments where acidification with a culture of lactic acid bacteria was combined with a very low addition of rennet. Fig. 7 clearly shows the presence of a minimum in the gel modulus around pH 5.3. This minimum occurs at the pH at which in the voluminosity of casein micelles shows a maximum (1).

Acidification of heated milk (yoghurt making) showed a similar effect, but in this case only a retardation in the development of the modulus was found at this pH, and not a clear minimum (unpublished results).

The question whether the inter or intra-micellar interactions are responsible for the lower modulus is difficult to answer. Micellar calcium phosphate (MCP) plays a key role in maintaining the integrity of the micelle. Solubilisation of MCP at the normal pH of milk desintegrates the micelle into smaller

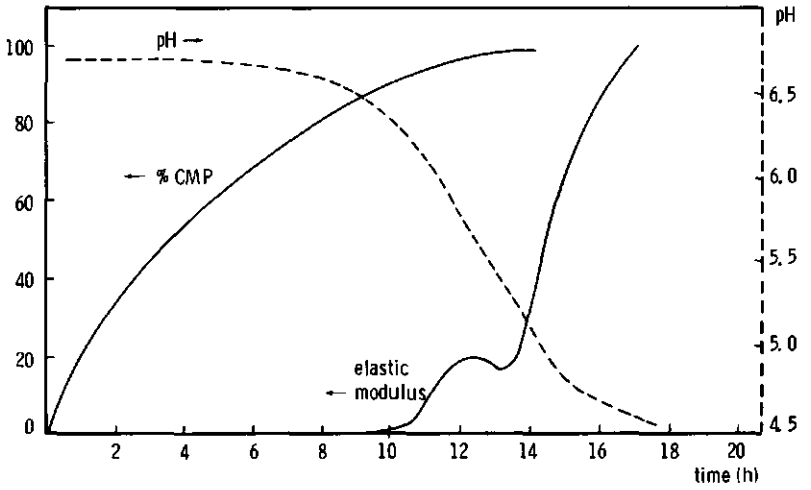

Fig. 7. Acidification, κ-casein conversion and gel formation as a function of time after adding rennet (0.001 %) and culture (NIZO culture of lactic acid bacteria) at 25 °C.

units (30). At pH 5.3 virtually all inorganic phosphate is solubilised, but no dissociation of casein could be detected under these conditions in rennet-treated milk (1). The decrease in the net negative charge of the casein molecules seems to compensate for the effect of MCP solubilisation, but the interaction between the caseins in the micelle is probably reduced which is reflected in the gel modulus. Further charge neutralization below pH 5.3 increases the interaction between the casein molecules again and causes the modulus to rise. Roefs (31) showed that the modulus of renneted milk is at a maximum near its isoelectric pH. At this pH the voluminosity of casein micelles shows a minimum (32). A further demonstration of the relation between intra-micellar interactions and the gel modulus is given by Kelley (33), who showed that the firmness of a rennet curd is at a maximum around pH 6.0; this is the pH value at which the voluminosity shows a second minimum (1).

4 Conclusions

– The optimum pH for the action of rennet in milk was found to be around pH 6.0. This is also the pH where the voluminosity of casein micelles is at a minimum. It is suggested that the accessibility of the Phe-Met bond of κ-casein is increased because of the diminished steric hindrance caused by the CMP part of the molecule.
– The enzymic reaction could adequately be described by a first-order rate equation at the original pH of milk. Above pH 7 the inactivation of chymosin affects the kinetics. This effect can be taken into account by combining the inactivation reaction with the hydrolysis reaction. Also at lower pH values the kinetics of the release of CMP deviated from first-order, especially at pH 5.6. It is assumed that the increased interaction between chymosin and para-κ-casein is the main cause for this change.
– The degree of conversion needed to initiate aggregation depended strongly on pH. At pH 5.6 the time of aggregation, defined as the time the viscosity started to increase, was at 30 % conversion. At the original pH of milk the viscosity initially dropped by about 3 %, and started to rise again after 70 % release of CMP. It could be shown that the aggregation must have started around 60 % conversion. This is at a much lower degree of hydrolysis than the normally assumed value of 80 % (27, 34).
– The rate of aggregation and gel formation increased with decreasing pH. Fig. 8 shows that the gelation time at high pH values is mainly determined by the rate of the enzymic reaction, whereas the increased rate of aggregation is responsible for the shorter gelation times in the lower pH range.

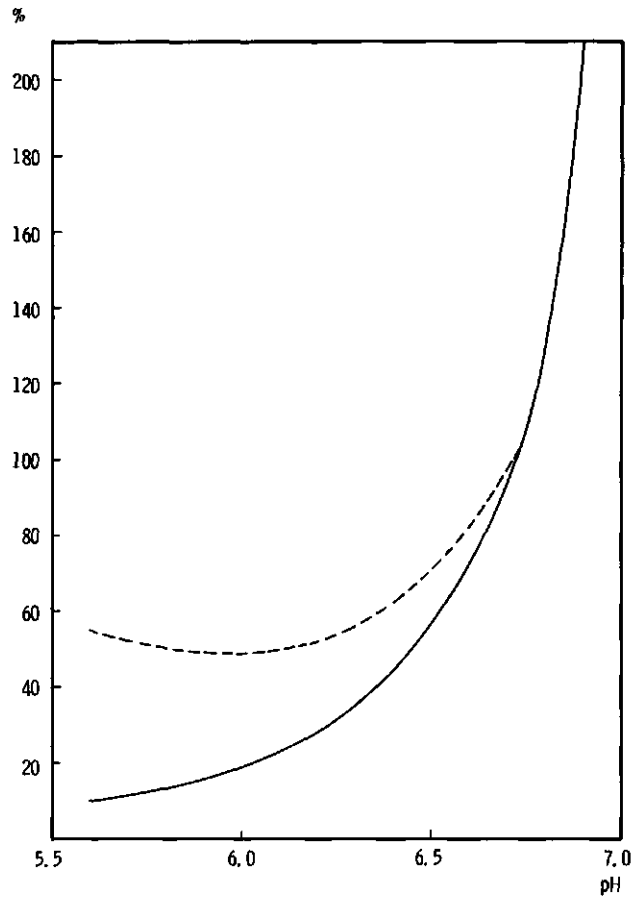

Fig. 8. Gelation time expressed as a % of that at the original pH of milk. Rennet concentration 0.02 %, temperature 30 °C.
(——) experimental results,
(- - -) predicted times if gelation would start at 95 % conversion at all pH values.

– The elastic modulus of a rennet gel was at a minimum around pH 5.3. This coincided with the pH at which the voluminosity of the casein micelles shows a maximum. It is assumed that the solubilization of MCP decreases the interaction between the different caseins in the micelle which results in a low value for the shear modulus at this pH.

Acknowledgement

The authors are much indebted to Mrs R. Holleman for her skilful contribu-

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Samenvatting

A. C. M. van Hooydonk, I. J. Boerrigter en H. G. Hagedoorn, *Fysisch-chemische veranderingen van caseïnemicellen in melk als functie van de pH en het effect daarvan op de stremreactie. 2. Effect van pH op de stremming van melk*[1]

Verlaging van de pH van de melk veroorzaakte een scherpe toename van de snelheid van de enzymatische reactie. De maximale snelheid werd gevonden rond pH 6,0, maar de verschillen waren klein in het pH-traject van 5,6 tot 6,4. Op de discrepantie met de pH-optima zoals gevonden bij model-substraatoplossingen werd nader ingegaan.

We veronderstellen dat zowel de toegankelijkheid van de voor chymosine gevoelige Phe-Met-binding van κ-caseïne, als de interactie tussen chymosine en para-κ-caseïne toeneemt bij pH-verlaging. Het laatstgenoemde effect vermindert de concentratie aan effectieve vrije enzymmolekulen. Dit zou een verklaring kunnen zijn voor de afwijkende kinetiek die bij lage pH werd gevonden.

De omzetting van κ-caseïne bleek bij de oorspronkelijke pH van de melk goed beschreven te kunnen worden door een reactievergelijking van de eerste orde. Bij hogere pH's moet rekening gehouden worden met de inactivering van chymosine. Ook voor deze omstandigheden bleek het mogelijk een kinetisch model af te leiden dat de experimentele resultaten kon beschrijven.

De snelheid van aggregatie en gelvorming namen toe met afnemende pH. Bij pH 5,6 begon de aggregatie van de caseïnedeeltjes reeds voordat 30 % van de κ-caseïne was afgesplitst. Bij pH 6.7 is dit voordat 70 % is gesplitst en vermoedelijk bij 60 %.

De elasticiteitsmodulus van het lebgel was minimaal bij een pH van ongeveer 5,3. Dit is ook de pH waarbij een maximale voluminositeit van caseïnemicellen werd gevonden.

We veronderstellen dat het minimum in de elasticiteitsmodulus een gevolg is van de afgenomen interactie tussen de caseïnemolekulen binnen het micel.

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The effect of various cations on the renneting of milk[1]

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Summary

A considerable amount of divalent cationic additives interacted with the casein micelles. This was in all cases accompanied by a reduction in the amount of dissolved calcium and phosphate, which suggests the formation of mixed complexes rather than a direct binding of the cations with casein. The molar ratio of complexed cation + calcium and phosphate was fairly constant and on average 1.7. Magnesium appeared to be the best soluble cation, whereas virtually none of the added zinc remained dissolved. The results with additions of calcium, barium and manganese were similar with respect to their solubility behaviour.

The voluminosity of the original and the rennet-treated casein micelles was reduced by all divalent additives. A negative correlation existed between the voluminosity and the amount of additive associated with the micelles. Calculations showed that the thickness of the casein-macropeptide layer is also reduced by the various divalent cations. Addition of sodium increased the voluminosity of the original and rennet-treated micelles, probably because of the exchange with casein-bound calcium.

Zinc and copper markedly decreased the rate of the enzymic reaction whereas the other divalent additives did not affect the conversion rate. Calcium and barium were more effective than magnesium and manganese in promoting the aggregation process of the converted micelles. Sodium decreased the rate of the enzymic reaction and accelerated the aggregation in the lower concentration range. It is postulated that the formation of insoluble additive-calcium-phosphate complexes play a key role with respect to the effect of divalent additives and less the direct binding of the cations to casein. These complexes, which are assumed to be positively charged, interact strongly with the negative sites of the casein.

Calcium is probably not directly involved in the slow renneting of cold-stored milk. The difference in the concentration of carbon dioxide between freshly drawn and cold-stored milk was at least partly responsible for the difference in rennetability. The poor renneting properties of milk containing the κ-casein A variant appeared to be connected with the generally high pH and low calcium content of such milk. This mainly affected the aggregation reaction, although the rate of the enzymic reaction was also somewhat decreased.

1 Introduction

A minimum amount of calcium is needed to initiate the gelation of rennet-

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2.2 Methods

2.2.1 Solvation of casein micelles and partition of additives were determined after 1.5 h centrifugation (88 000 *g*) at 30 °C or 4 °C. Additives and sodium

converted casein micelles. Above this minimum level the rennet coagulation time of milk decreases with the amount of calcium (1).

The mechanism behind the effect of calcium on the renneting process has not yet been fully elucidated. Green (2) concluded from experiments with various types of cationic additives that the aggregation was accelerated by the adsorbed cations shielding the negatively charged groups of the casein. It was suggested that cationic material binds to sites in the interior of the micelle and

azide were added to the cold milk and the pH corrected with 1 M NaOH. After overnight storage at 7 °C the samples were heated to 30 °C and the pH, if necessary, corrected again prior to sedimentation. Solvation of para-κ-casein micelles was determined after incubating the same milk samples for 90 minutes with 0.02 % (v/m) rennet at 30 °C. The concentration of the additive, calcium, inorganic phosphate and protein were analysed in the supernatant. Solvation was determined as described previously (13).

2.2.2 Renneting. Additives were added to the cold milk and the pH corrected after heating the samples to renneting temperature (30.5 °C). After about 25 minutes the pH was checked again and, if necessary, adjusted. Renneting was, unless stated otherwise, started 30 minutes after the samples were brought to renneting temperature. The rennet concentration was 0.02 % (v/m) in all experiments.

The rate of the enzymic reaction was determined on the basis of the release of CMP. Experimental and calculation procedures have been reported previously (14,15). A viscometric method was used to monitor the aggregation process and a dynamic test to analyse the curd-firming. Both methods have been described before (16). In several experiments the Formagraph was used to monitor the process of gel formation. This instrument has already been described elsewhere (17). In contrast with the output signal of the dynamic test, the output signal of the Formagraph is not linearly related to the elastic modulus of the rennet gel (18).

2.2.3 Chemical analysis. Ca, Mg, Zn and Ba were determined by atomic absorption spectrometry, and Na and K by flame emission. Inorganic P and N were determined by standard methods (13). Calcium ion activities were obtained with a calcium ion selective electrode (Orion, Cambridge, USA) at 25 °C. Equilibration time at this temperature was 2 h for the experiments with cationic additives (Section 3.1.1) and, for practical reasons, 10 minutes for the analysis of milk samples from individual cows (Section 6). It was found that the calibration curve, based on standard solutions of calcium chloride, shifted to a higher level after the electrode had been in contact with milk. The slope of the calibration curve remained virtually the same. To correct for this shift a standard solution with a calcium activity close to that of normal milk was measured after each milk sample and the obtained value used to calculate the activity in the milk.

3 Results and discussion

3.1 Physico-chemical changes

3.1.1 Partition of additive, calcium and phosphate. The supernatants obtained by ultracentrifugation at 30 °C were analysed for the amount of additive, calcium and phosphate. The change in concentration is given in Table 1, together with the amount of NaOH needed to correct the pH of the milk to its original value. The liberation of H^+ after addition of 100 mM NaCl is probably due mainly to the decreasing effect of ionic strength on the activity coefficients of the various ions in the serum and, consequently, to an increased dissociation of ion pairs. Also some exchange between micellar bound H^+ and Na^+ may take place. The effect of the addition of divalent cations on pH is probably due to the formation of an insoluble complex with phosphate. The results show that, except in the case of Na, only part of the additive remained dissolved. Transfer of the divalent cation from the serum to the micelle was always accompanied by a reduction in the amount of dissolved phosphate and calcium. The ratio between the amount of transferred additive+calcium and phosphate varied between 1.5 and 2.0. About 30 % of the added Mg complexed with the micelles and more than 80 % of the added Ca and Ba. Virtually none of the added Zn was found in the serum. The partition of Mn was not determined. Assuming a ratio of 1.7 between complexed Mn+Ca and P_i, it would follow that about 80 % of Mn became associated with the micelles.

Tessier & Rose (19) observed that addition of calcium to milk not only decreased the serum concentration of phosphate but also that of citrate. Wheth-

Table 1. Amount of NaOH needed to correct the pH to the original value, and amount of added cation, calcium and inorganic phosphate associated with the micelles after addition of various salts (30 °C).

Added		NaOH to correct pH mM	Amount associated with micelles				
cation	mM		additive		Ca mM	P_i mM	ratio additive + Ca/P_i
			mM	%			
Ca	3	1.20	2.6	87	2.6	1.7	1.5
Mg	3	0.80	1.0	33	0.8	0.9	2.0
BA	3	1.44	2.5	83	0.9	2.0	1.7
Mn	3	1.76	nm	–	0.8	2.0	–
Zn	3	2.64	2.9	96	0.5	2.0	1.7
Na	100	1.50	≈0	0	−1.1	≈0	–

nm = not measured.

er the composition and properties of the complexes resembles that of the original MCP or not is uncertain. The ratio between transferred citrate and phosphate found by Tessier & Rose (19) after addition of calcium was higher than that normally found in MCP (20). On the other hand McGann et al. (21) concluded that the composition of precipitates formed after neutralisation of milk ultrafiltrate obtained at low pH were basically similar to that of MCP.

The considerable decrease of the pH of milk after addition of zinc chloride may suggest that it complexes differently with phosphate than do the other additives.

Addition of 100 mM NaCl increased the concentration of calcium in the supernatant by about 1 mM. In agreement with the results of Gufferty & Fox (12), no significant increase in the amount of dissolved phosphate could be detected. This is surprising because solubilisation of MCP would be expected, due to the decreasing effect of ionic strength on the activity coefficients of ions. Also the concentration of dissociated casein was unaltered after addition of NaCl. The change in the distribution of calcium is presumably due to an exchange of casein-bound Ca^{2+} with Na^{+}. The experiments of Parker & Dalgleish (22) have clearly shown that the binding of calcium to casein decreases with the amount of NaCl. The expected loss of 2 mM Na (total concentration 123 mM) is within the standard deviation of the analytical method and could therefore not be detected.

In Table 2 the estimated distribution of some major components and the change after addition of Ca, Mg and Na is given. The calculations are based on the experimentally obtained changes in the phosphate and calcium concentrations and on the assumption that the ratios between Mg, Ca, Cit. and P_i in MCP are constant and equal to the ratios found by Holt (20). Some justification for such an assumption is given by Schipper (27), who showed that the Ca/P and the Ca+Mg/P ratios in calcium-magnesium-phosphate precipitates were only slightly affected if the concentration of Mg was varied in the solution. Also the presence of Na did not significantly change the Ca/P ratio in the precipitates.

The major part of the dissolved Ca and Mg is associated with citrate. The association constants of the two cations with citrate are similar (1) and some exchange may be expected if the concentration of one of the cations is increased. This effect is probably responsible for the increased activity of Ca^{2+} and the formation of undissolved calcium phosphate complexes after addition of Mg. The total divalent cation activity (Ca+Mg) appears to be somewhat higher after addition of Mg than after addition of Ca, which is probably explained by the higher solubility of magnesium phosphate. It is, therefore, to be expected that addition of Mg is at least as effective as addition of Ca if the

Table 2. Estimated distribution (mM) of some major constituents before and after addition of Ca, Mg and Na. Underlined values were determined. The basic assumption is a constant ratio of constituents in MCP as given by Holt (20):
[1] Activity coefficient of 0.4 was taken to convert the measured activity to concentration;
[2] Activity coefficient (≈0.3) corrected for increase in ionic strength;
[3] Average value given by Walstra & Jenness (1).

Constituent	Milk	+3 mM Ca	+3 mM Mg	+100 mM Na
Ca total	28.6	31.6	28.6	28.6
dissolved	9.2	9.6	8.4	10.3
Ca^{2+}	2.0[1]	2.6[1]	2.4[1]	3.6[2]
casein-Ca	5.2	5.1	4.6	4.1
MCP-Ca	14.2	16.9	15.6	14.2
Mg total	4.7	4.7	7.7	4.7
dissolved	3.2	3.1	5.2	3.4
Mg^{2+}	1.0[3]	≈ 1.0	> 1.0	> 1.0
casein-Mg	1.1	1.1	2.1	0.9
MCP-Mg	0.4	0.5	0.4	0.4
P_i total	20.9	20.9	20.9	20.9
dissolved	12.1	10.4	11.2	12.1
MCP	8.8	10.5	9.7	8.8
Cit. total	9.0[3]	9.0	9.0	9.0
dissolved	8.1	7.9	8.0	8.1
MCP	0.9	1.1	1.0	0.9

total cation activity were the determining factor for the renneting of milk. If the renneting is mainly governed by the amount of MCP the opposite is true. Addition of NaCl not only increases the ionic strength but also the concentration and the activity of the divalent cations. This will have a significant influence on the electrostatic repulsion and ion bond formation due to the screening of charges.

Both the enzymic and aggregation reaction may be influenced by these changes but not necessarily in the same direction.

3.1.2 Voluminosity of the micelles. The influence of various cations on the solvation of the casein micelles in the original and in the rennet-treated milk was determined by sedimentation at 30 °C and at a constant pH of 6.68. Voluminosities were calculated from the solvation data as described previously (13).

All divalent cations reduced the voluminosity of casein and para-casein micelles (Table 3). Mg appeared to be less effective than the other cations. This may be related to the relatively high amount of Mg remaining dissolved. The highest reduction in voluminosity of the original micelles was obtained with

Table 3. Effect of various additives on the voluminosity of original and rennet-treated micelles at 30 °C. Thickness of CMP layer was calculated assuming a constant number of micelles and a d_{vs} of 104 nm for the rennet-treated micelles without additive (see ref. 49) $d_{vs} \approx v^{1/3}$.

Added		Voluminosity ml/g			Calculated thickness of CMP layer nm
cation	mM	original	renneted	Δ	
none		3.26	2.40	0.86	5.6
Ca	3	3.06	2.29	0.77	5.2
Mg	3	3.19	2.35	0.84	5.5
Ba	3	3.10	2.31	0.79	5.3
Mn	3	3.11	2.30	0.81	5.4
Zn	3	3.01	2.30	0.71	4.8
Na	100	3.40	2.49	0.91	5.8

Zn, whereas its effect on the converted micelles was comparable to that of Ca, Ba and Mn. Assuming that the micelles may be divided into a core (the part that remains after renneting) and a hairy outer layer containing the highly hydrophilic CMP, it is probable that the voluminosity of both parts is reduced. This does not necessarily imply that the thickness of the outer layer has also decreased. If an additive causes a redistribution of the micelles in such a way that the total number is decreased, the total volume of the outer layer may theoretically be decreased without a reduction of its thickness. The increase of the voluminosity of the original and rennet-converted micelles by sodium ions confirms the results of Gufferty & Fox (12) and Creamer (23) respectively.

The thickness of the outer layer was estimated from the voluminosity data assuming a constant number of micelles and a volume-surface diameter of 104 nm for para-$\varkappa$-casein micelles in the original milk. The calculated thickness of about 5.5 nm for the original micelles is comparable with the value determined by Walstra et al. (24). Vreeman (25) calculated a thickness of 4.5 nm for the CMP part of $\varkappa$-casein, assuming a random coil conformation. Divalent cations seem to slightly reduce the thickness of the CMP layer, whereas sodium seems to increase it. The high reduction found with Zn may be an indication of a strong interaction of this cation or its phosphate complex with CMP.

The suggestion of Bloomfield & Morr (26) that the micelle consists of a size-determining framework which is not affected by calcium is unlikely according to our results. Sood et al. (46) and Snoeren et al. (32) also concluded that the micelle structure must be able to swell and shrink with changes in the calcium content. The almost identical reductions of rennet-converted mi-

celles by Ca, Ba, Mn and Zn may, however, be an indication of a lower limit for contraction of the micelle core.

The distribution of the additive between the serum and the micelle seems to be an important factor. The voluminosity tends to decrease if more of the divalent cation is bound to the micelle. With sodium the increased voluminosity is probably due to the exchange with micellar calcium. This again indicates a special role of calcium for the integrity of the micelle, either by direct association with casein or indirectly via binding with MCP. The finding that addition of divalent cations always leads to an increase of undissolved complexes containing calcium and phosphate (and most likely also citrate and magnesium) suggests that the interaction between these complexes and casein may be of more importance for the observed changes in voluminosity than the direct association of divalent cations with casein. The observations of Schipper (27) that negatively charged polymers such as casein, pectin and carboxy-methylcellulose coprecipitate with calcium phosphate complexes support this. Another indication of the important role of the interaction between these complexes with casein is given by the results obtained with Mg. The calculations presented in Table 2 indicate that addition of Mg increases the total binding of Mg+Ca to casein more than does addition of Ca. The reduction in voluminosity is, however, comparatively small.

3.2 Effect on renneting

3.2.1 Addition and removal of calcium. Cheese milk is normally enriched with calcium chloride to accelerate the renneting process. The acceleration is due to the combined effect of the increased calcium concentration as such and the drop in pH. Figure 1 shows the reduction in gelation time (onset of gelation as measured with the Formagraph) as a function of calcium chloride addition with and without pH correction. The shorter gelation times of the non-corrected samples in this pH range is partly due to the increased rate of the enzymic reaction with decreasing pH (16).

Addition of calcium had no effect on the rate of the enzymic reaction if the pH was kept constant (Fig. 2a). The solid curve is the best fit with a first order rate equation. The calculated rate constant was 1.6×10^{-3} s^{-1}. The shortening of the gelation time (GT) and the renneting time (RT is the time at which the firmness of the curd is sufficient to start cutting) are thus entirely due to an increase in the rate of aggregation. The degree of κ-casein conversion at the time of gelation decreases therefore with an increasing amount of calcium chloride. The amount of CMP released at gelation time was 95 % for the sample without extra calcium and 87 % for the sample enriched with 1.8 mM cal-

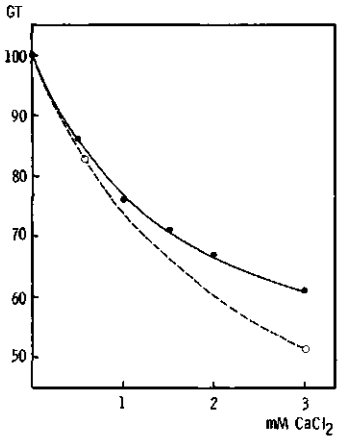

Fig. 1. Effect of $CaCl_2$ addition on the gelation time; with (———) and without (– – –) pH correction. Results obtained with Formagraph (100 = 34 min).

cium. Equilibration of the same sample for 24 h at renneting temperature decreased the rate of the enzymic reaction almost independently of the amount of added calcium (Fig. 2b). The results in Table 4 further show that ageing had an opposite effect on the two reactions. The retardation of the enzymic reaction is more than compensated by the increased rate of aggregation, except for the sample containing the highest amount of calcium. The effect of calcium appeared to be far less in the equilibrated samples. It has been well established that the rennetability of cold-stored milk slowly restores after

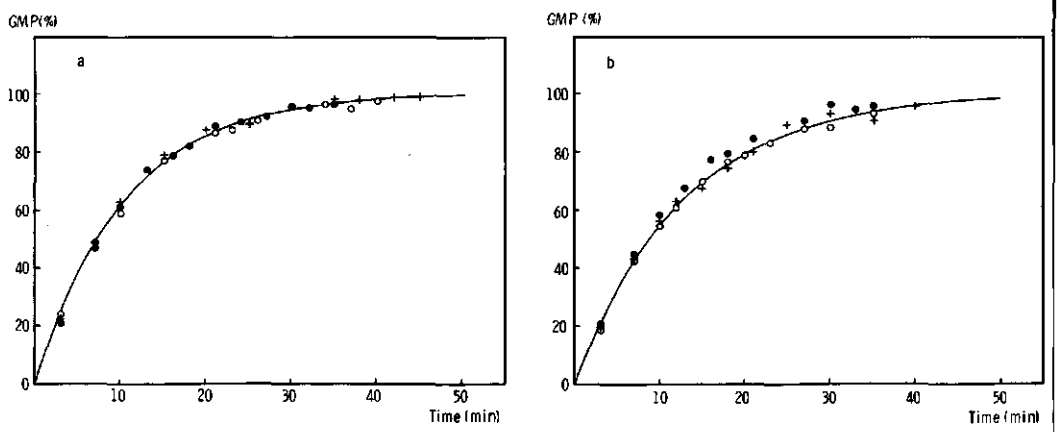

Fig. 2. Effect of $CaCl_2$ addition on the conversion of κ-casein by rennet at constant pH of 6.71. Na_3N was added to prevent bacterial growth. a) rennet added after 0.5 h at 30.5 °C; b) rennet added after 24 h at 30.5 °C. $CaCl_2$: none (+); 0.6 mM (○); 1.8 mM (●).

Table 4. Effect of $CaCl_2$ addition on enzymic and agrregation reactions at constant pH of 6.71. GT and RT obtained with dynamic test.

	Ca added mM		
	0	0.6	1.8
enzymic reaction ($K \times 10^3$ s^{-1}):			
– after 0.5 h at 30.5 °C	1.62	1.62	1.62
– after 24 h at 30.5 °C	1.31	1.31	1.45
aggregation reaction:			
– after 0.5 h at 30.5 °C GT min	30.4	26.2	21.2
RT min	39.8	33.0	26.4
RT-GT min	9.4	6.8	5.2
%CMP at GT	95	92	87
– after 24 h at 30.5 °C GT min	25.2	24.2	22.4
RT min	31.1	29.8	28.0
RT-GT min	5.9	5.6	5.6
%CMP at GT	86	85	86

ageing at elevated temperatures (28,29) but it is surprising that this is only due to an acceleration of the aggregation reaction. Similar time effects were encountered with the renneting of reconstituted milk (30). Most of the reduction in GT takes place within the first 3 hours of ageing (28). This makes it unlikely that proteolysis by native enzymes (plasmin) could be the major cause. Shifts in the salt balance may be involved but also the re-association of the cold soluble caseins. It probably takes considerable time before their most favourable position is attained.

In two experiments the renneting properties of calcium-depleted milk were investigated. Removal of calcium was achieved by exchanging part of the calcium and magnesium by sodium. The ratio of exchanged Ca/Mg was about 6. The renneting experiments were carried out at the pH of the original milk. The time/temperature history of the untreated sample was kept the same as that of the samples treated with the ion-exchange resin. The aggregation process was followed by viscometry. The clotting time (CT) is defined as the time at which the viscosity is twice the initial value. Removal of calcium decreased the rate of the enzymic reaction slightly in both experiments (Table 5). Addition of calcium chloride to the calcium-depleted milk samples had virtually no effect on the conversion rate. It is therefore questionable whether the slight decrease in conversion rate after calcium depletion is due to the calcium as such. Increasing the pH of milk followed by decreasing it again to the original value also impairs the rennetability (31). The increase in pH during the exchange reaction and the correction afterwards may then, in fact, be responsi-

Table 5. Renneting properties of Ca-depleted milk samples (constant pH of 6.76). CT is time at which the viscosity was twice the original value and K the first order rate constant.

Treatment	$K \times 10^3\ s^{-1}$	CT min	% CMP at CT
EXP 1 A; original milk	1.26	32.3	91
B; – 2 mM Ca	1.11	51.8	97
C; sample B + 2 mM $CaCl_2$	1.21	32.6	91
EXP 2 D; original milk	1.25	34.5	93
E; – 4.9 mM Ca	1.12	no clotting	
F; sample E + 4.4 mM $CaCl_2$	1.13	40.4	94

ble for the decrease in the rate of proteolysis. The influence of calcium on the aggregation reaction is much more pronounced. A critical level of calcium is needed to induce aggregation of the casein micelles. The viscosity of sample E, being about 18 % higher than that of the original milk, decreased by 13 % within the first 45 minutes after rennet addition and remained stable afterwards. Using Eilers equation to convert viscosity to voluminosity, and subtracting the contribution of whey proteins and CMP (16), it can be calculated that the voluminosity of the rennet-converted micelles is reduced by about 30 %. This reduction is somewhat more than found from sedimentation (Table 3), but, considering the effect of calcium addition on the difference between original and rennet-treated micelles a greater reduction may indeed be expected.

Removal of calcium causes an increase of the voluminosity and dissociation of casein (mainly β) (32). It is tempting to relate the increased stability of the micelle to protruding chains of the still associated part of the β-casein.

3.2.2 Addition of other divalent cations. The effect of various chloride salts on the gelation time at constant pH is shown in Fig. 3. Ca and Ba appeared to be more effective than Mg and Mn. The effect of Zn is surprising: instead of an expected acceleration of the renneting process a substantial retardation was found. Some preliminary experiments with copper chloride showed a

Table 6. Effect of various cations on the rate of the enzymic reaction.

Added cation (3 mM)	$K \times 10^3\ s^{-1}$	Added cation (3 mM)	$K \times 10^3\ s^{-1}$
EXP 1 none	1.55	EXP 2 none	1.33
Ca	1.51	Mg	1.15
Ba	1.55	Zn	0.52
Mn	1.54	Cu	0.59

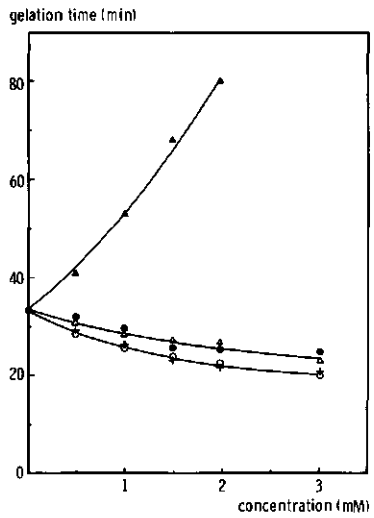

Fig. 3. Effect of various cations on gelation time (Formagraph) at the pH of the original skim milk. Ca (○); Ba (+); Mg (●); Mn (△) and Zn (▲).

similar behaviour. The rate of the enzymic reaction was not much affected by the cations which reduced the gelation time, but markedly decreased after addition of Zn and Cu. Incubation of rennet for 2 h at 30 °C in permeate of milk containing 3 mM Zn did not reduce its activity. Although this experiment does not directly prove that the active site of the enzyme may not be (reversibly) blocked by Zn during the reaction, it seems more likely that the substrate is altered. The fact that most of the added Zn has already interacted with the micelles before the rennet is added suggests that the substrate is less accessible or that the interaction with Zn interferes with the formation of the enzyme-substrate complex.

It is not possible to relate the effect of the various additives on the renneting process to their effect on the physico-chemical properties. The results with Mg suggest that the total activity of divalent cations may be of less importance than the interaction of the additive with the micelle via insoluble complexes. Except in the case of Mg, almost equal amounts of phosphate 'coprecipitated' with the additive. The composition of these 'precipitates' may however be different as is suggested by their varying effect on pH. The observation of Green & Marshall (7), that multivalent cationic material had an effect on renneting comparable to that of divalent cations, suggests that the complexes may in fact also be positively charged, which, as was already proposed by Schmidt (33), may also be true for the original MCP. The difference in composition of the complexes formed with the various cations may determine their charge density and thereby their effect on the renneting pro-

cess. After renneting, the positive clusters presumably become exposed and enhance the interaction between micelles. Not only may the free energy barrier between two approaching micelles be reduced, but also the strength of the ionic bonds formed after contact enhanced and, hence, the aggregation accelerated and the modulus of an equilibrated curd probably increased. Knoop & Peters (34) found indeed a firmer gel after addition of calcium and a decreased tendency of the micelles to fuse together after coagulation.

The observation that addition of phosphate ($\leq$10 mM) to milk accelerates the renneting process (47) may also be explained by the formation of undissolved complexes with calcium and their interaction with micellar casein.

It would be interesting to investigate the effect of Zn after the micelles have been incubated with rennet at low temperature. On the basis of the influence of Zn on the voluminosity it is expected that the rate of aggregation will increase.

3.2.3 Addition of NaCl. Fig. 4 shows the effect of various additions of NaCl on the rate of the enzymic reaction. The conversion rate decreased approximately linearly with the concentration of NaCl. The pH was not corrected in these experiments. With pH correction the conversion rate would decrease even more, considering the effect of pH on the enzymic reaction (16).

A decreased rate of proteolysis with increasing ionic strength was also observed by Visser et al. (35) with a model substrate (residues 98-112). They concluded that the increased screening of both the positive cluster near the Phe-Met bond and the negative charges near the active site of the enzyme was responsible for the decreased velocity. The effect of NaCl on the gelation time and the rate of curd-firming is shown in Fig. 5. The two milk samples differed slightly in their response to NaCl addition. A small minimum in gelation time and a more pronounced maximum in the rate of curd-firming was observed with an addition of 50 mM NaCl in one of the milk samples, whereas no minimum nor maximum were obtained with the other sample (the same

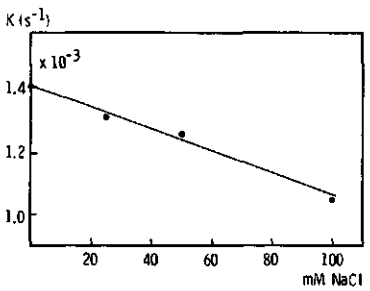

Fig. 4. Effect of NaCl addition on the rate of the enzymic reaction in milk (no pH correction, see Fig. 5). K is first order rate constant.

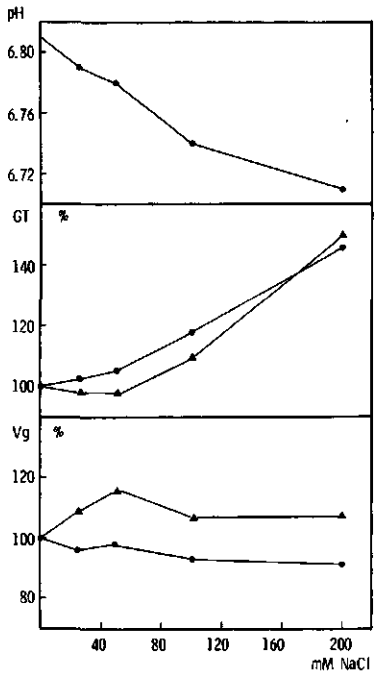

Fig. 5. Effect of NaCl addition on pH, gelation time and rate of curd-firming (Vg=1/RT-GT). Two skim milk samples; ▲ and ●, the latter being the same as in Fig. 4.

milk sample as in Fig. 4). Similar experiments conducted by others showed a very distinct minimum in the rennet coagulation time (8,9,10). Our results are more in line with those of Gufferty & Fox (12). The difference in response to NaCl addition is difficult to explain. The maximum in the rate of curd-firming observed with one of the samples suggests that the rate of aggregation may indeed be increased by a small addition of NaCl. The opposite effect of NaCl on the two reactions may, depending on their relative rates, be responsible for the occurrence of a minimum. At higher concentrations also the aggregation rate decreases, presumably because of the screening influence of the electrolyte on charged groups which impairs the ionic bond formation and thereby decreases the number of effective collisions, despite the reduction of the electrostatic repulsion. An increase of the steric repulsion by protruding chains of casein (addition of NaCl increased the voluminosity) should, however, not be excluded.

3.3 Role of calcium in the renneting of cold-stored milk

Cooling and heating of milk cause time-dependent shifts in the distribution of protein and salt between micelles and serum (1,33). It has been well estab-

lished that cold-stored milk has a prolonged gelation time as compared to the uncooled raw milk (see for example reviews in ref. 36 and 48).

The retardation of the renneting process after cold storage of milk has been attributed to the dissociation of casein (28), to the solubilisation of MCP at low temperature (35) and to the irreversible increase of the pH after cooling (36).

The major physico-chemical changes brought about by cooling milk for 48 h at 4 °C and subsequent heating to renneting temperature are given in Table 7. The serum concentration of Ca, P_i and protein had all been increased after cooling. These results are qualitatively in agreement with the observations of Qvist (35). The ratio between solubilised Ca and P_i (i.e. 2.3) is considerably higher than the ratio in MCP. Part of the solubilised Ca will, however, be directly bound to the dissociated casein. Assuming that this casein fraction (mainly β-casein) binds the usual amount of calcium (i.e. $\approx$4 mol Ca/mol casein, see Table 2) the corrected ratio becomes 1.7. This is close to the ratio normally found in MCP. Heating the cold-stored milk to the renneting temperature, with or without an intermediate pasteurisation, virtually re-established the original partition, but not the renneting process. In agreement with the results of Schmutz & Puhan (36), it was found that the pH of the cold-stored milk increased. The slower rate of the enzymic reaction can be fully explained by this effect. The difference in pH does not seem to be connected to changes in the salt equilibria (reversible); hence, there must be an acid source in the freshly drawn milk which disappears upon cooling. A difference of

Table 7. Effect of cold storage of milk on physico-chemical and renneting properties. All samples, except E, were kept for 0.5 h at renneting temperature before the rennet was added.
A) uncooled centrifuged at 30 °C; B) cooled 48 h at 4 °C and centrifuged at 4 °C; C) B centrifuged at 30 °C; D) B after pasteurization (5 s at 74 °C) centrifuged at 30 °C; E) D kept for 2 h at 30.5 °C prior to renneting; F) D with 0.6 mM $CaCl_2$ added; G) D with HCl added.

	A	B	C	D	E	F	G
P_i sup mM	13.5	14.5	13.9	13.5			
Ca sup mM	10.7	13.0	11.0	11.0			
protein sup % (m/m)	0.85	1.20	0.85	0.86			
voluminosity ml/g	3.2	4.3	3.2	3.2			
pH milk (30.5 °C)	6.66		6.76	6.75	6.75	6.72	6.65
$K \times 10^3\ s^{-1}$	1.7		1.2	1.3	1.3	1.6	1.7
GT[1] min	24.2		29.0	29.3	27.1	25.0	24.5
RT[1] min	30.0		37.7	37.7	34.4	31.9	30.9
RT-GT min	5.8		8.7	8.4	7.3	6.9	6.4

[1] Results obtained with dynamic test.
sup is supernatant

6×10^{-3} °C was observed between the freezing points of the fresh and the cooled milk samples. This must, in our opinion, be related to a difference in the CO_2 content. A study by Van den Berg (38) has clearly shown that the pH is significantly increased (up to 0.16 units) after degassing raw milk. Part of the observed differences in renneting behaviour may therefore be explained by a CO_2 effect. The results obtained with sample E suggest that other factors also play a role, since part of the retardation is recovered despite the pH remaining high. The shortening in gelation time during storage at renneting temperature is typical for cold-stored milk (28,29,30). The re-association of casein and the time needed to achieve the most favourable position in the micelle may be responsible for the effect of equilibration time. Addition of calcium and slight acidification increased the rate of the enzymic reaction and curd-firming to nearly the original values.

3.4 Calcium in relation to abnormal rennetability

The abnormal rennetability of milk from individual cows has received much attention over the past ten years, mainly because of the connection with genetic variants of the different caseins and its implication for cheese yield. All studies on this subject indicate that milk samples containing the κ-casein A variant have a prolonged coagulation time and a lower curd-firmness as compared to those with the B variant (39,40,41,42,43).

Milk containing different κ-casein variants also, on average, differs in casein composition (43), micelle size distribution (44) and salt content; citrate and pH were found to be higher and calcium and phosphate lower in milks containing κ-casein A (39,40).

Occasionally, problems with abnormal rennetability are encountered on cheesemaking farms in the Netherlands. In order to find out whether this is related to the genetic variant κ-casein, the milk of each of 52 cows on a farm with slow renneting cheesemilk was collected and analysed for renneting properties and contents of some major constituents.

The relative frequency distribution of the gelation time is plotted in Fig. 6. More than 60 % of the milk samples may be considered as abnormal in renneting behaviour. Arbitrarily the samples were divided into 3 groups: 'normal' samples having a gelation time of less than 45 minutes, 'abnormal' samples having a gelation time of over 60 minutes and an intermediate group not being considered here. From both groups a limited number of samples were selected for additional analysis (Table 8). The abnormal milk samples appeared to be significantly higher in pH, contained less calcium and ionic calcium. Almost all of these samples contained the κ-casein A variant. These re-

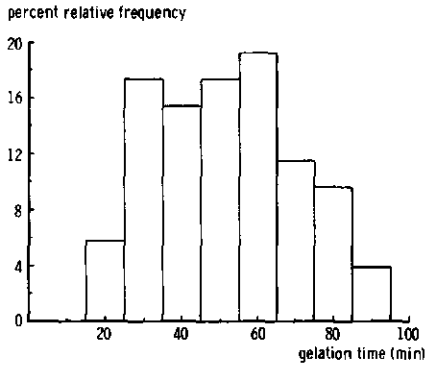

Fig. 6. Gelation time (Formagraph) frequency distribution of 52 individual milk samples.

Table 8. Average values for some major properties of milk with normal and abnormal rennetability. Only properties which differed significantly are given. For details and complete results see ref. 50.

		Normal n = 11		Abnormal n = 13		P
		$\bar{X}$	sd	$\bar{X}$	sd	
gelation time		28.6	7.8	76.5	9.1	
pH		6.69	0.06	6.80	0.06	<0.001
Total Ca	mM	28.9	2.4	26.9	2.1	<0.01
Ca^{2+} activity	mM	0.85[1]	0.12	0.73[2]	0.06	<0.01
total Ca/total protein	mM/g	0.95	0.07	0.84	0.05	<0.001
number of samples containing κ-casein A		2		11		<0.01

[1] n = 4.
[2] n = 12.

sults support the earlier observations that a difference in genotype may go along with important changes in the concentration of other constituents. Two milk samples containing the A variant were examined for rate of proteolysis. One of the samples was specifically selected in order to eliminate the effect of pH. The results, presented in Table 9 and Fig. 7, indicate that the rate of conversion is substantially lower than in the reference milk (bulk collected) and in the mixed sample taken from the storage vat of the farm.

Considering the results of the experiments with addition of Ca on the enzymic reaction, it seems unlikely that the slow rate of proteolysis is due to the difference in Ca concentration. The A variant of κ-casein contains one negatively charged residue more than the B variant (45). The extra negative charge in the CMP part of κ-casein A probably increases the electrostatic or steric repulsion between substrate and enzyme. Although the enzymic reac-

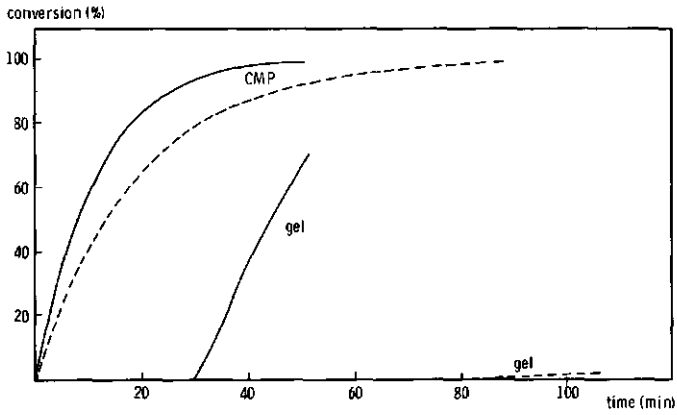

Fig. 7. Conversion of κ-casein (% CMP) and gel formation (arbitrary scale readings) in two milk samples with normal (———) and abnormal (– – – –) rennetability.

Table 9. Rate of enzymic reaction and some physico-chemical properties of two milk samples with abnormal rennetability. Sample A is normal bulk collected milk and sample B is a mixed sample taken from the storage vat at the farm (see text). GT measured with Formagraph.

Sample	κ-cas	pH	Ca mM	Ca/prot mM/g	aCa^{2+} mM	$K \times 10^3$ s^{-1}	GT min
A bulk collected[1]	–	6.7	29	0.88	1.0	1.6	30.1
B mixed	–	6.78	27.7	0.88	0.90	1.3	41.3
C abnormal	A	6.82	22.9	0.77	0.82	0.8	93.4
D abnormal	A	6.77	25.9	0.72	0.71	0.9	81.9

[1] Typical values

tion proceeds more slowly, it does not nearly explain the total change in gelation time. The aggregation of the rennet-converted micelles is markedly retarded, as may be expected on the basis of the low amount of calcium present. The results of Pecorari et al. (40) showed that not only was the total concentration of calcium reduced in milk samples containing the κ-casein A variant, but also the amount of MCP. The latter, may in view of the previous results, be the determining factor.

Addition of $CaCl_2$ to slow renneting milk samples reduced the gelation time considerably (Table 10). The reduction is much higher than that found for normal milk, suggesting that more of the added calcium 'precipitates' with phosphate.

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The renneting properties of heated milk[1]

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Summary

In agreement with previous studies on this subject it was found that the retardation of the enzymic reaction in heated milk could be related to the denaturation of β-lactoglobulin and its interaction with κ-casein. Complete denaturation of β-lactoglobulin caused a reduction of about 20 % in the rate of κ-casein conversion.

The generally held view that the interaction between β-lactoglobulin and κ-casein renders the Phe-Met bond in κ-casein inaccessible to rennet could not be confirmed.

The poor rennetability of heated milk appears to be fully related to the impaired aggregation properties of the rennet-converted casein micelles (partly) covered with denatured whey proteins. The attraction between these protein particles diminishes further if more of the calcium phosphate 'precipitates' during heating. These calcium phosphate complexes probably have different casein-binding properties and solubilise more slowly than does indigenous micellar calcium phosphate. This may explain the phenomenon of rennet hysteresis and the effect of pH cycling (acidification followed by neutralisation) on the renneting behaviour of heated milk.

Heating of milk at elevated pH in order to diminish the interaction between β-lactoglobulin and micellar κ-casein did not improve the rennetability. Lowering the pH is the most effective way to improve the renneting properties of heated milk.

A considerable amount of κ-casein became non-sedimentable after milk had been heated at relatively low temperatures, but no direct correlation with the renneting behaviour was observed.

1. Introduction

The incorporation of whey proteins into cheese via heat treatment of milk may offer an attractive alternative to the use of ultrafiltration. The heating process is not only less costly but also improves the bacteriological status of the milk, especially if ultra-high-temperature (UHT) treatments are employed to reduce the number of spores. Outgrowth of *Clostridium tyrobutyricum* spores leads to excessive hole formation (late blowing) in many types of

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cheese and a preservative has to be added to inhibit this process. Heating of milk also changes the water-holding capacity of the incorporated (denatured) whey proteins and brings it closer to that of casein. The rennetability of heated milk is, however, markedly impaired and texture and flavour defects are encountered in cheese prepared from heated milk (1).

Kannan and Jenness (2) demonstrated that the heat-induced interaction between β-lactoglobulin (β-lg) and κ-casein was responsible for the prolongation of the rennet coagulation time as well as for the further prolongation when the heated sample was stored (rennet hysteresis). Morrissey (3) more or less confirmed these results and concluded in addition that the phenomenon of rennet hysteresis was essentially due to the dissolution of calcium phosphate which had 'precipitated' during heating, because rennet hysteresis was also encountered in heated caseinate systems containing no whey protein. The presence of whey proteins, however, considerably increased hysteresis and Morrissey concluded that the rennet-converted β-lg/caseinate complexes were less sensitive to calcium than were the normal para-κ-casein micelles.

An extensive study on the conversion of κ-casein by rennet in heated milk and in model systems containing casein micelles and β-lg was performed by Wheelock and coworkers (4, 5, 6, 7). The results of these studies implied that the interaction between β-lg and κ-casein inhibited the hydrolysis of κ-casein, because only about 75 % of the substrate appeared to be accessible to rennet after complete denaturation of the whey proteins. Furthermore it was concluded that the reduction in the release of the casein macropeptides (CMP) was for the most part due to incomplete conversion of the carbohydrate-free κ-casein molecules (4).

Damicz and Dziuba (8), who applied milder heat treatments (i.e. 15 s at 85 °C) to the milk, found that not the extent but only the rate of conversion was affected in heated milk. The recent results of Marshall (1) indicate, although they are not fully consistent, that the rate and the extent of conversion are hardly affected by severe heat treatments of milk.

These conflicting results induced us to investigate the renneting behaviour of heated milk in more detail in order to find out which of the two reactions, i.e. the enzymic breakdown of κ-casein or the subsequent aggregation of casein micelles, causes the prolongation of the rennet coagulation time. Some heat-induced physico-chemical changes in milk, such as the denaturation of whey proteins, the dissociation of κ-casein and the partition of salts, were also studied and will be discussed in relation to the renneting properties. The second part of this paper contains the results of several experiments conducted to improve the rennetability of heated milk.

2 Materials and methods

2.1 Materials

– Skim milk. Bulk-collected milk (4 °C) was stored for 24 h at this temperature and subsequently heated for 10 s at 65 °C, cooled to 47 °C, skimmed and the skim milk cooled to 4 °C.
– Reconstituted skim milk was prepared from low-heat skim milk powder by dispersing 100 g in 1 l distilled water at 40 °C. After 5 to 10 minutes stirring at this temperature the reconstituted milk was placed in a cold room (5 °C) and stirred slowly overnight. The same batch of powder was used in all experiments.
– Commercial liquid rennet with a strength of 10800 Soxhlet Units was purchased from CSK (Leeuwarden, Netherlands).

2.2 Methods

2.2.1. Heating procedures. Heat treatments up to 95 °C were carried out in test tubes (70 ml, screw cap) using a water bath. Heating times were 5 or 10 minutes, not including the time needed to reach the desired temperature. A similar procedure using an oil bath was followed for the temperature range above 95 °C. A thermocouple was mounted in the cap to monitor the temperature. UHT heat treatments were carried out on pilot-plant scale using an indirect tubular heat exchanger (Stork Sterideal) or a direct steam injection system (Alfa-Laval). Both systems were equipped with a prewarming section set at 70 °C and a cooler in which the milk was cooled to 20 °C. After heating, the samples were either directly brought to renneting temperature or stored overnight at 5 °C.

2.2.2. Renneting. Unless stated otherwise, renneting was started 30 minutes after the samples had been brought to the renneting temperature of 30.5 °C. The rennet concentration was 0.2 ml/kg in all experiments.

The rate of the enzymic reaction was determined on the basis of the release of CMP soluble in 8% trichloroacetic acid (TCA). Experimental and calculation procedures have been reported previously (9, 10, 11). A viscometric method was used to monitor the aggregation process and a dynamic test to analyse the curd-firming. Both methods have been described previously (11). In several experiments a Formagraph was used to monitor the process of gel formation. This instrument was described and evaluated elsewhere (12, 13).

2.2.3 Dissociation of κ-casein. The heat-induced dissociation of κ-casein in milk was determined by renneting the supernatant obtained after ultracentrifugation for 1.5 h at 88 000 × *g* and 30 °C. The supernatant was carefully withdrawn by means of a syringe, avoiding contamination of proteinaceous material from the diffuse layer on top of the pellet. After incubating the supernatants for 1 h with 1 % rennet at 30 °C the amount of CMP was determined as referred to above.

2.2.4 Denaturation of whey proteins. A direct and specific analysis of the amount of undenatured β-lactoglobulin and α-lactalbumin was obtained by means of gel permeation chromatography on a high-performance column (14). The method is basically similar to the determination of CMP (9). The undenatured whey protein fractions were separated from the casein and the denatured whey fractions by precipitation at pH 4.6 (acetic acid/sodium acetate) followed by filtration (15).

2.2.5 Chemical analysis. The nitrogen content was determined by a rapid colorimetric method (16) and the concentration of inorganic phosphate by the method of Griswold (17).

3 Results

3.1 The renneting properties of heated milk

3.1.1 The enzymic reaction. The changes of the enzymic reaction parameters were determined after heating reconstituted milk for 5 minutes at temperatures ranging from 70 °C to 120 °C. Prior to renneting the heated milk samples were stored overnight at 5 °C and, hence, not only the immediate effect of heat but also the phenomenon of rennet hysteresis is reflected in the results.

Figure 1 shows the effect of heating milk on the initial velocity (V_i) of the conversion of κ-casein and on the final amount of convertible κ-casein (s_0). The latter value was obtained after 3 h of incubation. No additional release of CMP was observed after longer incubation times.

V_i decreased steadily with increasing temperature in the range from 70 °C to 95 °C. The maximum reduction was about 25 % and no further decrease occurred at higher temperatures. The amount of convertible substrate decreased by about 10 % at the highest temperature. Part of the reduction in V_i may be attributed to the decrease in s_0. To correct for this, the relative rate constant for the enzymic reaction was calculated assuming first-order kinetics

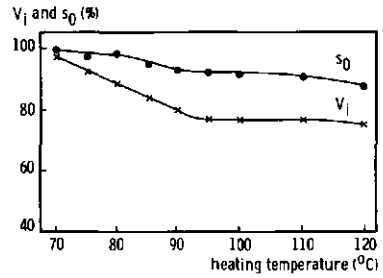

Fig. 1. The initial velocity (V_i) of the enzymic reaction and the amount of convertible κ-casein (s_0) in reconstituted milk heated at various temperatures for 5 minutes. Results for unheated reconstituted milk were set at 100 %.

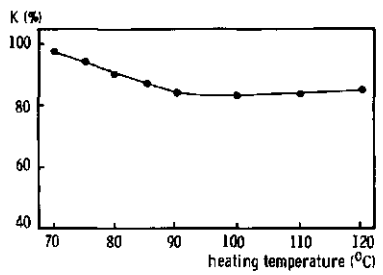

Fig. 2. Decrease of the enzymic reaction rate as a function of heating temperature. Results taken from Figure 1. $K = V_i/s_0$. Value for unheated milk was set at 100 %.

i.e. $K = V_i/s_0$ (see Section 3.1.3). A plot of K versus temperature (Figure 2) indicates that the true reduction in the conversion rate was at most 18 %. A possible cause for the decrease in s_0 may be the thermo-degradation of κ-casein, since the 'zero-time' (no rennet added) gel permeation chromatograms of the heated milk samples showed increased peak sizes at the retention time of CMP. Assuming that the increase of the 'zero-time' peak (always subtracted from the values obtained after addition of rennet) is indeed due to the hydrolysis of the Phe-Met bond of κ-casein by heat, it can be shown that part of the reduction in s_0 at the higher heating temperature may be explained by this effect (Figure 3). After 5 min heating at 120 °C the 'zero-time' peak was

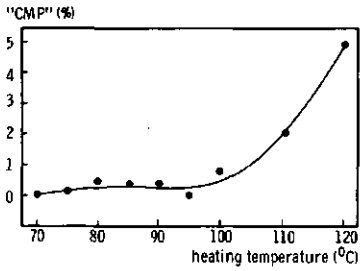

Fig. 3. Effect of heat on the 'zero-time' CMP value. Concentration expressed as a percentage of total CMP release in unheated sample. Heating for 5 min at the indicated temperatures.

found to be equivalent to 5 % (9 μM) of the total substrate concentration in the unheated sample. The results of Marshall (1) and Hindle and Wheelock (18) also suggest some heat-induced degradation of κ-casein after severe heat treatments of milk. From the above results it may be concluded that heating of milk causes a slight reduction in the amount of convertible substrate, but considerably less than found by some others (4, 5). This discrepancy may, however, still be explained by the suggestion of Hindle and Wheelock (4) that mainly the carbohydrate-free κ-casein molecules would become inaccessible to rennet after heating milk. Any reduction in the release of carbohydrate-free CMP moieties would not be observed by our method (only the CMP fractions soluble in 8 % TCA are measured). In a separate experiment we therefore checked whether the concentration of CMP in 2 % TCA (in which all CMP fractions are soluble) was below the expected value. Due to the interference of native β-lg with the CMP determination it was only possible to conduct such an experiment with a heated milk sample in which all the β-lg had been denatured (i.e. 15 minutes at 85 °C). Previous experiments with whey-protein-free milk showed that about 75 % of the CMP is recovered in 8 % TCA (10). The difference between the recovery in 8 % and 2 % TCA of the heated milk sample was 35 %, i.e. somewhat higher than expected. This of course may be explained by the difference in the fraction of carbohydrate-free κ-casein between milk samples but it is also possible that the heating of milk releases carbohydrate groups from the CMP molecule which would decrease the fraction soluble in 8 % TCA. Further research is needed to verify the latter hypothesis. Anyhow, our results do not support the conclusion made by Hindle and Wheelock (4) and it may in fact be questioned whether heating of milk reduces the amount of accessible substrate.

3.1.2 Relation between conversion rate and whey protein denaturation. The gradual reduction of the rate of substrate conversion in the temperature range from 70 °C to 95 °C suggests a correlation with whey protein denaturation. This was checked by analysing the degree of β-lg and α-lactalbumin denaturation. The results are given in Figures 4 and 5. The linear relation between the rate of the enzymic reaction and the degree of β-lg denaturation strongly supports the earlier conclusion that the complex formation between β-lg and κ-casein retards the enzymic reaction.

3.1.3 Aggregation and gel formation. Mild heat treatments leading to about 25 % denaturation of whey proteins (15 s at 95 °C, indirect heating) hardly affected the enzymic reaction but substantially delayed the process of aggregation and even more the rate of curd-firming (Figure 6a). More severe heat

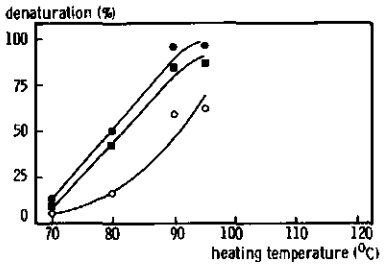

Fig. 4. The denaturation of β-lactoglobulin (●), α-lactalbumin (○) and β-lactoglobulin + α-lactalbumin (■) as a function of heating temperature (5 min).

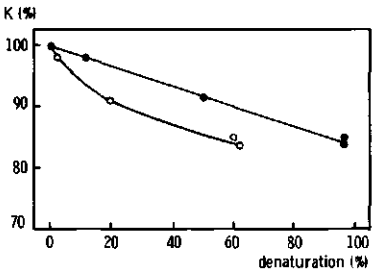

Fig. 5. Relation between the relative rate of the enzymic reaction and the extent of denaturation of β-lactoglobulin (●) and α-lactalbumin (○). Values taken from Figures 3 and 4.

treatments resulting in about 75 % denaturation of whey proteins (5 s at 140 °C, indirect heating) prevented formation of a gel within 3 h of incubation, but aggregation of protein particles could be observed by viscometry (Figure 6b). The conversion of κ-casein in heated milk was adequately described by first-order rate kinetics (solid curves). Unlike the enzymic reaction, the aggregation of the protein particles in heated milk could not directly be related to the degree of β-lg denaturation (Table 1). The 'precipitation' of calcium phosphate probably plays an additional role, but there are insuffi-

Table 1. Effect of various heat treatments on the clotting time (time at which the viscosity is twice the initial value) and the denaturation of β-lg.

Treatment	Clotting time (% of unheated sample)	Denatured β-lg (%)
5 min at 80 °C	169	51
10 s at 140 °C (direct)	156	55
5 min at 85 °C	192	72
30 s at 140 °C (direct)	350	72
10 min at 100 °C[1]	168	100
20 min at 100 °C[1]	262	100
10 min at 110 °C[1]	330	100

[1] 2.4 mM $CaCl_2$ added prior to renneting, no pH correction. Reference is unheated sample with the same addition of $CaCl_2$.

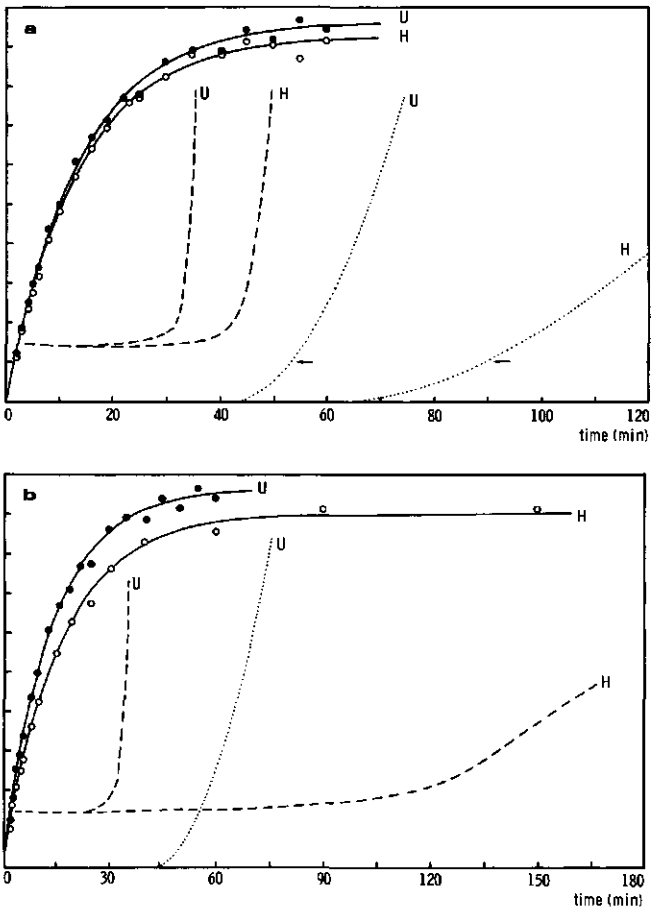

Fig. 6. The release of CMP (——), viscosity (---) and curd-firmness (...) in unheated (U) and heated (H) skim milk. Y-axis: CMP 0–180 μM, viscosity 0–10 mPa.s and curd firmness (arbitrary scale readings). Arrow indicates firmness at the moment cutting normally begins. Heat treatments were carried out with tubular heat exchanger. a) milk heated for 15 s at 95 °C; b) milk heated for 5 s at 140 °C.

cient kinetic data on this subject to treat the results more quantitatively. In Section 4 we will qualitatively discuss the importance of heat-induced 'precipitation' of calcium phosphate.

3.2 Dissociation of κ-casein

The amount of convertible κ-casein present in the supernatants of heated and

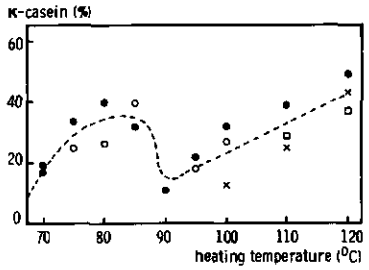

Fig. 7. Amount of non-sedimentable κ-casein (% of total) in reconstituted skim milk heated at various temperatures for 5 minutes. Equal symbols refer to one experiment.

cold-stored (24 h) milk is plotted in Figure 7. A surprisingly high amount of κ-casein dissociated at relatively low heating temperatures. Despite the poor repeatability of the experiment, as is indicated by the variation of duplicate and triplicate values, the combined results suggest a minimum dissociation after heat treatments at about 90 °C. Neither the amount of dissolved phosphate in the supernatants nor the pH of the milk samples were significantly different after the various heat treatments, suggesting that the minimum cannot be explained by differences in the salt equilibria. It should be noted that no discontinuity in the enzymic reaction parameters (V_i and s_0) was observed as a function of heating temperature.

The dissociation of κ-casein in UHT-treated milk increased progressively with increasing holding times at 140 °C (Table 2). A further increase was observed after the heated samples had been cold-stored for 24 h at 5 °C.

3.3 Experiments conducted to improve the rennetability of heated milk

3.3.1 Heating at elevated pH. The aggregation of β-lg diminishes markedly at pH values above 6.8 (19). Also the interaction between β-lg and κ-casein was found to decrease after heating at elevated pH (20). Creamer and Matheson

Table 2. Non-sedimentable κ-casein after heating milk at 140 °C (direct UHT). A) ultracentrifuged immediately after heating; B) ultracentrifuged after heating and cold storage for 24 h at 5 °C.

	κ-casein in supernatant (μM)		Denatured β-lg (%)
	A	B	
unheated	5.0	6.5	0
2 s at 140 °C	8.0	14.7	20
10 s at 140 °C	19.2	27.4	55
30 s at 140 °C	32.2	48.9	72

Table 3. Effect of heating skim milk at pH 7.5 on the renneting properties at pH 6.7. K is enzymic reaction rate constant and GT the gelation time. A) unheated kept at pH 6.7 and 5 °C for 48 h; B) stored at pH 7.5 for 24 h at 5 °C, followed by acidification to pH 6.7 and storage (24 h at 5 °C) prior to renneting; C) as B, but heated for 10 min at 90 °C prior to acidification.

Sample	Exp. 1		Exp. 2		
	$K \times 10^3$ (s^{-1})	s_0 (μM)	$K \times 10^3$ (s^{-1})	s_0 (μM)	GT (min)
A	1.41	166	1.40	157	37.0
B	1.38	162	1.27	154	85
C	0.99	155	1.00	144	no gel

(21) concluded that denatured whey proteins do not attach to the micelles after heating at pH values higher than that of milk, which may suggest that κ-casein is then no longer involved in the SH/SS interchange reactions. In that case an effect on the renneting reaction may be expected. This was checked by heating milk at pH 7.5, followed by acidification to the original value after cooling. The same pH cycle was given to the reference skim milk, however, without heating. The results in Table 3 clearly indicate that heating at elevated pH still had a marked detrimental effect on the renneting process. The rate of the enzymic reaction appeared to be even more reduced compared to heating at the normal pH of milk. Also the aggregation of the rennet-converted micelles in the sample which had been pH cycled without heating was substantially retarded.

3.3.2 Acidification and neutralisation of heated milk. Experiments performed by Banks and Muir (22) showed that sterilised starter milk, which was acidified by lactic acid bacteria and subsequently neutralised by addition to the cheese milk, fully incorporated in the curd. They suggested that this was due to the disruption of micelles at low pH, which would make the hidden κ-casein liable to conversion and causes the casein micelles to participate in the gel formation process. This idea was followed up by giving the milk samples a pH cycle to pH 6.0 and 5.5 prior to renneting.

The results in Table 4 indicate that acidification followed by direct neutralisation had little effect on the renneting properties. Keeping the milk samples, however, for 24 h at the low pH before neutralisation indeed accelerated the renneting process, especially that of the heated sample. The acidification/neutralisation procedure did not release any additional CMP in the heated sample, hence, the improvement of the rennetability cannot be explained by the mechanism suggested by Banks and Muir (22). The transfer of calcium

Table 4. Effect of acidification and neutralisation on the renneting properties of heated milk (5 s at 140 °C, direct UHT) and unheated milk.
pH cycling at 5 °C. A) no pH cycling; B) acidification (HCl) to pH 6.0 followed by neutralisation (NaOH) to pH 6.7; C) as B, but acidified to pH 5.5.

	Direct neutralisation GT (min)	Neutralisation after 24 h storage at 5 °C	
		GT (min)	s_0 (μM)
unheated A	38.1	38.6	
B	40.0	38.3	
C	39.3	35.4	
heated A	53.1	56.0	148 ± 4
B	56.5	47.8	
C	51.5	44.3	146 ± 4

phosphate from the micelle to the serum at low pH and the subsequent 'precipitation' caused by neutralisation may be the most important effect. This suggests that heat-induced calcium phosphate complexes have properties different from those formed by neutralisation in the cold.

3.3.3 Effect of pH and $CaCl_2$ addition. The effect of pH on the rate of the enzymic reaction in heated milk is plotted in Figure 8a. The initial rate is comparable to that of the unheated sample at each pH. Also the aggregation reaction in heated milk appeared to be very sensitive to small pH reductions (Figure 8b), especially if most of the whey proteins were denatured (compare direct with indirect UHT heat-treatments). Addition of $CaCl_2$ (up to 4 mM), keeping the pH constant, decreased the gelation time of heated milk, but the

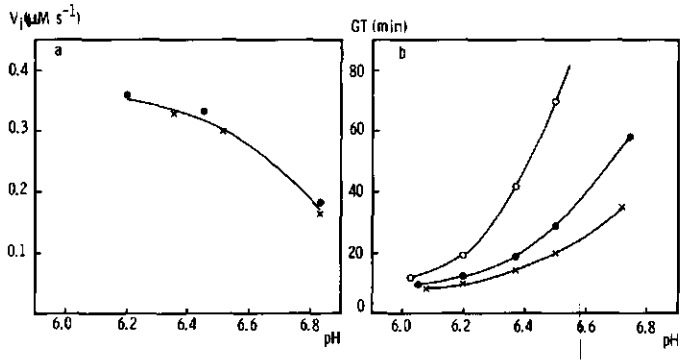

Fig. 8. Effect of pH on the initial velocity of the enzymic reaction (8a) and on the gelation time as obtained by the Formagraph (8b). Unheated skim milk (x), heated skim milk 5 s/140 °C direct UHT (●) and heated skim milk 5 s/140 °C indirect UHT (○).

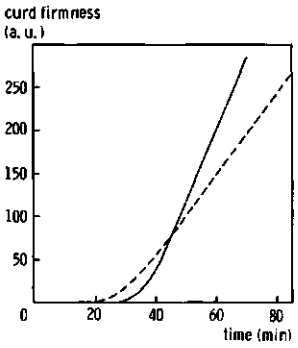

Fig. 9. Curd-firmness of unheated (—) and heated (---) skim milk. The heated milk (5 s/140 °C, direct UHT) was enriched with 1 mM $CaCl_2$ and acidified to pH 6.50; the unheated sample was renneted at the original pH of 6.7.

value of the unheated sample could not be attained. Increasing the Ca concentration prior to heating was found to be less effective. This is probably explained by the stability of calcium phosphate complexes formed at elevated temperatures (see next section). The combined effect of lowering the pH and $CaCl_2$ addition on the rate of curd-firming is shown in Figure 9. This preliminary experiment indicates that it is more or less possible to match the renneting process of heated and unheated milk. Further optimisation of the rennet concentration, pH and $CaCl_2$ addition would be needed.

4 Discussion

Heating of milk causes 'precipitation' of calcium phosphate and denaturation of whey proteins if the temperature is raised above 70 °C. Intermolecular SH/SS interchange reactions are held to be responsible for the association of β-lg (23) and the complex formation with κ-casein (20, 24, 25), although intermolecular hydrophobic bonds may contribute (20). Calcium does not play a primary role in the heat-induced association of β-lg with casein micelles but increases the level of association (20).

The 'precipitation' of calcium phosphate depends on the intensity of the heat treatment and the pH of the milk (26, 27). It may well be that the composition and the properties of calcium phosphate complexes formed at high temperatures differ from those of the original micellar calcium phosphate (MCP) which forms under physiological conditions. Citrate, for instance, is always present in MCP and 'coprecipitates' after addition of calcium to milk (28, 29). At high temperatures only calcium phosphate apparently 'precipitates' (27, 30). The fouling experiments of Hiddink et al. (30) indicate that tricalcium phosphate is formed at high heating temperatures.

Heat-'precipitated' calcium phosphate dissolves much more slowly on cooling than does indigenous MCP (27). The micelles will consequently loose

some MCP after heating to restore equilibrium.

From a previous study (29) we concluded that the concentration of MCP in the micelles is more important for the renneting properties than is ionic calcium. The phenomenon of rennet hysteresis in heated milk supports this, since ionic calcium is enhanced at the expense of MCP, but the rennet coagulation time increases. The observed increase in non-sedimentable $\varkappa$-casein during cold storage of milk may also be explained by the further dissolution of MCP. The unique association properties of MCP for casein are apparently (partly) lacking in the Ca/P complexes formed at high temperatures. The considerable increase in gelation time observed after unheated milk had been subjected to a pH cycle 6.7-7.5-6.7 suggests that rather stable Ca/P complexes are also formed at elevated pH. Cycling in the reversed direction, i.e. pH 6.7-5.5-6.7 will solubilise a substantial amount of both original MCP and heat-induced Ca/P complexes. The subsequent neutralisation in the cold probably leads to the reformation of Ca/P complexes (most likely including citrate) with composition and properties more like the original MCP, which may explain the improved rennetability.

The heat-induced dissociation of $\varkappa$-casein is not directly connected with the interaction between β-lg and $\varkappa$-casein, because it has also been observed in whey-protein-free milk samples at temperatures above 105 °C (31). The presence of whey proteins, however, appears to promote the dissociation of $\varkappa$-casein (32). Some preliminary electrophoretic analysis of the supernatants of heated milk (10 min at 90 °C) indicated that the dissociated casein fraction contains not only $\varkappa$-casein but also some α_s-casein and β-casein. These caseins are presumably associated with denatured whey protein in small non-sedimentable aggregates.

The minimum in the dissociation of $\varkappa$-casein observed around 90 °C coincides with a maximum in the association between β-lg and $\varkappa$-casein (23). It may well be that larger aggregates are formed under these conditions which will sediment during ultracentrifugation.

The reduction in the rate of enzymic reaction was found to be related to the denaturation of β-lg. The increased ζ-potential of the protein particles in heated milk (33, 34) suggests that the complexes of casein and denatured whey proteins are more negatively charged than the original casein micelles. An increased electrostatic repulsion between the enzyme molecule and its substrate is therefore expected, but direct steric hindrance of the β-lg associated with $\varkappa$-casein may cause an additional retarding effect on the enzymic reaction rate. A small reduction in the final amount of CMP (soluble in 8 % TCA) was observed in heated milk. The result obtained with 2 % TCA did not confirm the conclusion of Hindle and Wheelock (4) that mainly the carbo-

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Control and determination of the curd-setting during cheesemaking[1]

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1 Introduction

One of the very important cheesemaking steps is the rennet-induced coagulation of milk. A lot of scientific attention has been devoted to this subject but still a great deal has to be learned, especially with respect to the quantification of the gel formation and the subsequent syneresis of the curd. Prediction of the course of the different reactions taking place during the renneting process is hardly possible because too many milk properties are involved. Accurate on-line determination of the optimum coagulum firmness for cutting is needed to achieve maximum cheese yield and cheese quality. In Section 4 we shall discuss the effect of sub-optimal curd-firmness at the time of cutting in more detail.

Up to now most cheesemakers judge the optimum cutting time by the 'feel' of the curd and, as Olson (1) already stated, with amazing accuracy. A one year comparison between the manual judgement by the cheesemaker of the NIZO experimental dairy and an objective rheological test showed differences of no more than one minute in the optimum time for cutting obtained by both methods.

Many instruments have been proposed and developed in the past to take over this important task of the cheesemaker but none has been widely accepted. The main reason for this is probably that in the traditional way of cheesemaking such an instrument, mostly fragile and difficult to clean, introduces more troubles than advantages. Recent technological developments such as pre-concentration of milk, increased size of cheesemaking plants, introduction of cheesevats which are not suited for manual judgement of the coagulum and the ongoing automation have actuated the interest in objective methods to monitor the process of curd-setting. Several instruments are now commercially available and will be briefly discussed in this paper. No attempt will be made to mention all the instruments developed for this purpose over the years. For this we refer to several review articles on this subject (2, 3).

The main objectives of this paper are to define some criteria to choose an instrument for a specific function and to give suggestions for the actual con-

[1] IDF-document, submitted.

trol of the renneting process. The choice of a particular method and instrument depends of course on the objectives of the measurement. As far as the measurement of the renneting process is concerned, the following distinctions may be made:

a) basic rheological research of the gel formation during renneting of milk,
b) classification of milk samples with respect to renneting behaviour,
c) determination of the optimum cutting time during cheesemaking,
d) standardisation of the renneting process of a batch of milk.

In this paper we shall concentrate on the functions mentioned under c and d.

Large scale operating and automated cheese plants normally have very strict time schedules for the successive batches. The cutting time cannot practically be changed without disturbance of the daily process. In such a situation there is a need to master the curd-setting rather than to measure it and to wait for the moment that the curd has reached its optimum firmness. This raises the question how the renneting process of the delivered milk can be standardised.

Determination of the optimum renneting time (time at which cutting starts) during cheesemaking is more important for smaller factories where the rennetability of the different milk batches may vary considerably and where the time schedules allow variation of the renneting time.

Before discussing the different instruments and their performance, the problem of standardisation will be dealt with.

2 Standardisation of renneting properties

Standardisation of the renneting process of a particular batch of milk in our opinion means that not only the renneting time (start of cutting) should be fixed at a certain time after addition of rennet but also that the rate of firming should be the same in order to be more sure of a constant process of syneresis. A constant rate of firming does not necessarily imply a constant rate of syneresis under all conditions. No experimental evidence is yet available about the relation between curd-firming rate and syneresis, but it seems reasonable to assume a correlation. Secondly, all treatments to achieve a constant renneting behaviour should not have any significant effect on the later stages of the cheesemaking process.

There are several different ways to change the rennetability of milk:

a) changing the rennet concentration,
b) addition of calcium chloride,
c) changing the pH of the milk,
d) adjustment of the renneting temperature,
e) changing the protein (casein) concentration.

These factors have been extensively investigated in the past and it is well es-

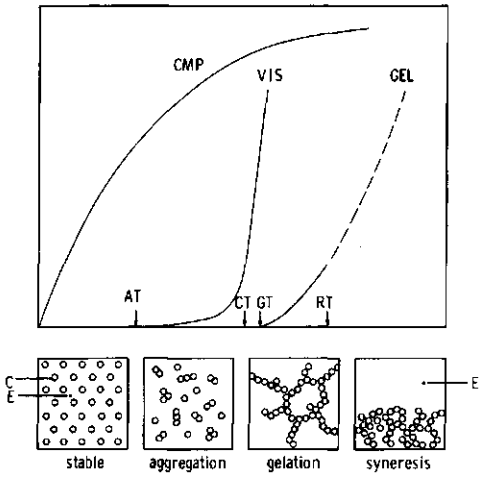

Fig. 1. A typical result of the time course of the different reactions taking place during the renneting of milk and a highly schematic representation showing the number of casein micelles (C) per enzyme molecule (E) and the state of aggregation. CMP = production of casein macropeptide; VIS = increase in viscosity; GEL = gel-firming.
Characteristic times: AT = onset of aggregation; CT = clotting time (i.e. time at which viscosity approaches infinity); GT = onset of gelation; RT = time at which the firmness is sufficient to start cutting.

tablished that they all affect both the renneting time and the rate of firming. The interrelationships between these factors are not always very clear, due to the complex nature of the reactions taking place during renneting. Furthermore the reported results were often obtained under different experimental conditions. In the following part the effect of the five variables on the rennetability of milk will be given for experimental conditions normally encountered with the manufacture of Gouda cheese. Where possible the results will be compared with those of other published work in this area but no complete review will be presented. Several review type articles summarize the state of knowledge on this subject (4, 5, 6, 7, 8, 9).

In order to understand the overall renneting process the effect of the different factors on both the enzymic and coagulation reactions will be given.

The methods applied for the evaluation have been reported previously (10, 11, 12). A typical result of a complete analysis is depicted in Fig. 1 together with some characteristic parameters which are used to describe the renneting behaviour. In Section 5 the instrument used to monitor the gel formation will be treated in more detail, because this method served as a reference for the evaluation of some of the other instruments.

A compilation of the effect of the variables mentioned is presented in Fig. 2. These variables can be changed independently with the exception that addition of calcium chloride will decrease the pH.

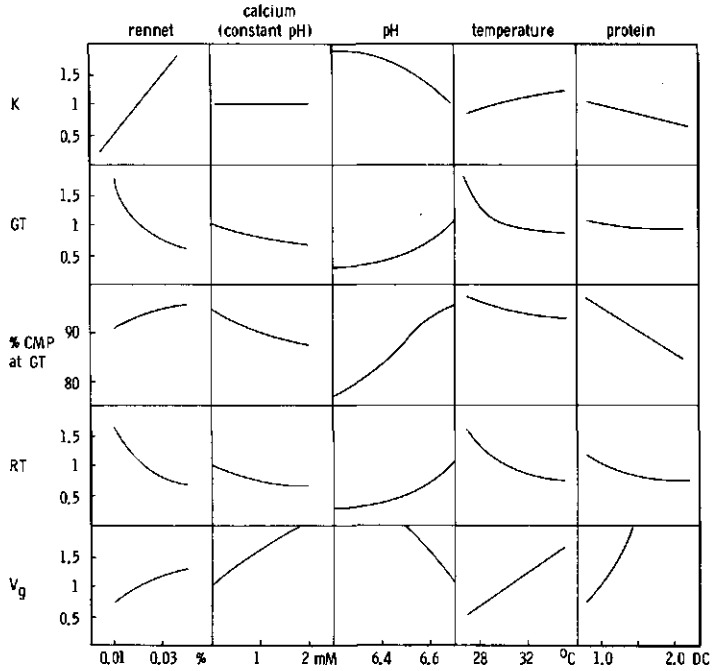

Fig. 2. The effect of some important variables on the renneting properties of milk. Standard conditions are: 30.5 °C, 0.02 % rennet (10800 SU), original pH of the milk, no calcium added and no concentration or dilution. Standard is set at 1. K is first-order rate constant of the enzymic reaction, V_g the rate of curd-firming (the slope of the firming curve at RT) and DC the degree of concentration by ultrafiltration (see Fig. 1 for the other symbols).

2.1 Effect of rennet concentration. Sufficient experimental evidence has now become available showing that the release of the casein macropeptide (CMP) by rennet can be described under normal conditions by a first-order rate expression (11, 13, 14). The rate constant changes linearly with the rennet concentration. The value of the catalytic constant is about 0.075 s^{-1} $\%_{rennet}^{-1}$ for standard conditions.

Holters (15) modification of the well known Segelcke and Storch relation can normally be used to linearize the effect of rennet concentration on a characteristic time (e.g. $GT = a/e + b$, where a and b are constants and e the rennet concentration). The values of the two parameters depend not only on the conditions such as pH (16), temperature (16), temperature history of the milk (17, 18), calcium (19) and protein (20) concentration but also on the characteristic time chosen to evaluate the effect of rennet concentration. Both the slope and the intercept increase with a longer characteristic time (see Fig. 3). The parameter a apparently contains an aggregation term and may not therefore simply be related to the velocity of the enzymic reaction as

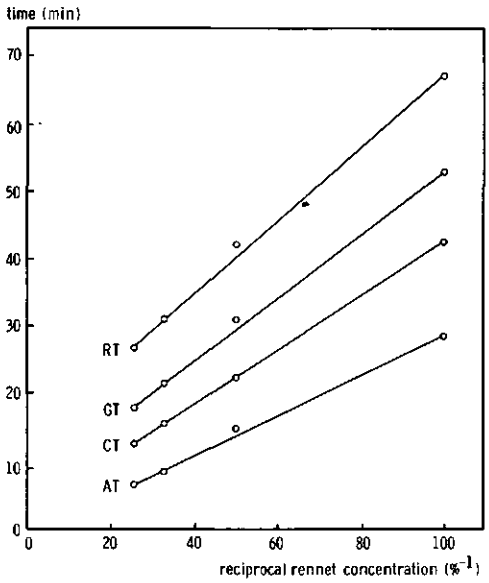

Fig. 3. Effect of rennet concentration on the various characteristic times of the renneting process. Note change of slope and intercept with characteristic time (see Fig. 1) chosen to evaluate the effect of rennet concentration.

is often assumed. Another and probably the most important phenomenon with respect to the problem of standardizing the renneting process of milk is that the rate of curd-firming depends on the rennet concentration (see also Refs. 21, 22, 23, 24). This is presumably partly due to the amount of CMP still to be released after gelation, but not fully. Even after complete conversion of κ-casein the rate of firming increases with the rennet concentration. Also, the ultimate gel strength obtained after ageing the renneted milk gel was found to increase with rennet concentration (25), but conflicting results do exist with respect to this point (26). Nevertheless, it seems that the spatial arrangement of the para-κ-casein micelles or aggregates thereof in the network depends on the rennet concentration or probably more directly on the rate of aggregation during the early stages. Whether the number of junctions in the network or the number of bonds per junction (or per cross section of a strand) is increased with a higher rate of aggregation is still uncertain. Rapid aggregation most likely leads to a coarser network with less junctions but with more bonds involved in the junction and the strand. The latter effect appears to be dominant and the net result is that the elastic modulus increases. The study by Roefs (27) showed that a higher elastic modulus of an acid casein gel went along with an increased permeability, suggesting a coarser network with larger pores.

2.2 Effect of calcium concentration. Addition of calcium chloride to the milk prior to renneting not only increases the calcium concentration but also de-

creases the pH of the milk. Both factors accelerate the renneting process. The pH effect is small, but significant; 1 mM $CaCl_2$ decreases the pH by about 0.03 units and 3 mM by about 0.07 units (28).

It was found that addition of $CaCl_2$ did not affect the rate of the enzymic reaction, providing the pH was corrected to its original value. This result is in agreement with the observations of Mehaia and Cheryan (29) but conflicts with those of Green and Marshall (30) who found an increased rate after addition of calcium.

Sedimentation studies have shown that most of the added calcium binds to the casein (30, 68) either by direct adsorption onto the negatively charged residues of casein or as micellar calcium phosphate because the amount of soluble phosphate was also found to be reduced after addition of $CaCl_2$ (31, 68). The acceleration of the coagulation reaction is probably not so much due to the slight increase in the calcium ion activity but more likely to the amount of calcium (and phosphate) interacting with and perhaps between the casein micelles (30, 68). The nature of these interactions has still to be elucidated. The increase in the rate of firming by small additions of $CaCl_2$ is considerable. Above an addition of 2 mM the curd-firming rate levels off and decreases again around an addition of 10 mM (21) despite the fact that the rate of aggregation during the initial stages of the reaction is still increasing (32). This again stresses the importance of the initial aggregation kinetics for the assembly of the ultimate casein network. Calcium probably not only affects the rate of aggregation and thereby the spatial distribution of the structural elements but also the intra- and interparticle bond strength.

2.3 Effect of pH. The pH-induced physico-chemical changes of casein in milk and their effect on the structure formation in acid and rennet milk gels have recently been extensively studied (33, 34, 35, 36). Drastic changes in the casein micellar system are brought about by acidification of milk, especially in the pH range 5.0 to 6.0. For the standardisation of the renneting process of milk the pH values around 6.3 (mainly soft cheeses) and around 6.6 (mainly hard cheeses) are of interest. The acidity of the curd at drainage determines not only the basic structure of the cheese by controlling the calcium phosphate level but also the amount of rennet retained in the curd (37, 38, 39). The second aspect is important for the proteolytic activity during the ripening of the cheese. For optimum control it is recommended to always start the renneting of the milk around the same pH.

Small changes of the pH in the 6.6 region have a significant effect on both the enzymic and coagulation reactions (Fig. 2). Around pH 6.3 the effect of small changes in acidity on the enzymic reaction is less pronounced but the rate of gelation still increases steeply with decreasing pH. According to Marshall et al. (21) the maximum in curd-firming rate is found around pH 5.8.

2.4 Effect of renneting temperature. The effect of temperature on the enzymic reaction is far less than on the aggregation and gelation reactions. Reported values for the temperature coefficient (Q_{10}) for the splitting of κ-casein by rennet vary between 1.3 and 2.0 (11, 13, 14, 29, 40, 41).

The temperature coefficient of the coagulation reaction is considerably higher and decreases with temperature (32, 42). Below 30 °C this part of the renneting reaction becomes rate-limiting and GT and RT increase steeply with decreasing temperature. The rate of gelation changes almost linearly with temperature (see also 21), implying a non-linear relation between the rate constant for gelation and the reciprocal of the absolute temperature (Arrhenius plot). The slope decreases with temperature, yielding an apparent activation free energy of 170 kJ/mol around 25 °C and of 70 kJ/mol around 35 °C.

2.5 Effect of protein concentration. The renneting behaviour of concentrated milk has recently been reviewed by Garnot (43).

With respect to the standardisation of the renneting process only the casein concentration is relevant.

The rate constant of the enzymic reaction is slightly reduced in concentrated milk, probably because of the decrease in the effective diffusion rate of the enzyme molecules (11). The onset of gelation is not much affected by the degree of concentration, but the renneting time is significantly reduced due to the substantial increase in the rate of firming ($V_g \approx DC^{1.7}$) with the protein concentration (Fig. 2). For this reason it was found necessary to adjust the cutting procedure if pre-concentrated milk is used with the traditional way of cheesemaking (44).

2.6 Standardisation procedure. The ideal situation for cheesemaking and its automation is to have milk supplied with constant renneting properties so that no corrections have to be made during processing. Standardisation of cheesemilk by adjustment of the renneting temperature is not the most practical way because it requires a frequent and accurate resetting of the pasteuriser. Furthermore, temperature changes will also affect the rate of acidification by the lactic acid bacteria. As already mentioned, the acidity of the curd at drainage is important for both the basic structure of the cheese and its ripening. The degree of freedom to alter the pH is therefore restricted and it would in fact be preferable to start the renneting always at the same pH.

Qvist (45) showed that the regulation of the renneting time of a milk sample can be achieved with the remaining variables i.e. rennet, calcium and protein concentration (dilution with water). The change in the rate of firming, which is expected to be of more importance for the process of syneresis than the renneting time, was not incorporated in his standardisation model.

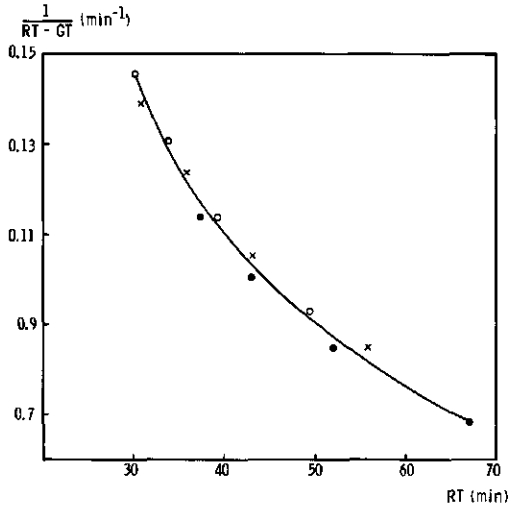

Fig. 4. Relation between renneting time (RT) and rate of firming ($1/[RT\text{-}GT]$) with different combinations of calcium and rennet at 30.5 °C. Results obtained with Formagraph.
$CaCl_2$: 0.9 mM (○), 0.6 mM (×) and none (●). Rennet concentrations: 0.015 %, 0.020 %, 0.025 % and 0.030 % (strength 10800 SU). No pH corrections were made after addition of calcium.

Figure 4 shows the result of an experiment in which both the rennet and the calcium chloride addition were varied. These results were obtained with the Formagraph. The reciprocal of the time difference between RT (time to reach a distance of 10 mm between the two traces on the photographic chart) and GT was used as a measure for the curd-firming rate. A reduction in GT, no matter whether it was achieved by adding more rennet or calcium, went along with a corresponding increase in the rate of gelation (V_g). This means that it is not possible to set GT and V_g independently by different combinations of rennet and calcium. In practice it will probably be sufficient and preferable to standardise RT with calcium chloride additions using a fixed dosage of rennet. If the V_g obtained does not have the desired value then it will be necessary to adapt the renneting temperature at another constant value or to change the protein content by adding water or milk powder. If ultrafiltration is already common practice, adjustment of the protein concentration may be made by adaptation of the degree of concentration. Standardisation by means of the rennet concentration is less attractive because it has an effect on the proteolysis during cheese ripening. Increasing the rennet concentration by more than 20 % might cause bitterness of the cheese.

Standardisation in practice also means that the temperature history of the milk at the time of evaluation should not be significantly different from that at the beginning of cheesemaking. It is well established that cold storage and

pasteurisation affect the rennetability (17, 18). Ageing of cold-stored milk at the temperature of renneting slowly restores the rennetability (28, 46, 47). Furthermore the milk sample used for standardisation should contain the normal amount of starter, because the amount affects the pH.

3 Rheological methods and instruments

3.1 Basic rheological research. In Section 1 it was mentioned that the choice of a particular rheological method or instrument should depend on the objectives of the measurement. Fundamental rheological studies of the structure of the casein network require a sophisticated approach using instruments that yield well defined parameters. In principle three different methods can be applied to analyse the rheological properties of a visco-elastic system such as a renneted milk gel: stress relaxation, creep and dynamic measurements. Dynamic measurements are best suited to monitor the rennet-induced gelation of milk because the magnitude of the moduli changes rapidly with time. Several studies have been carried out with this technique on a setting or equilibrated milk gel (24, 34, 48, 49). The instruments used in these studies are normally multi-purpose complex machines requiring skilled operators. A special commercial version of such an instrument was specifically developed for use in the factory by Gervais et al. (50). It is claimed that this instrument can be used to monitor the curd-setting during cheesemaking.

3.2 Classification of milk samples. The renneting properties of milk from individual cows or farms may vary considerably, and sometimes the milk does not clot at all. Large-scale operating cheese factories do not normally encounter the problem of anomalous rennetability because the system of mixing large quantities of milk ensures average physico-chemical properties of the milk, and relatively small variations in renneting behaviour occur.

Classification of milk samples is for example needed in studies investigating the relation between composition and rennetability. In the Parmigiano-Reggiano cheese area (Italy) a classification system was developed to classify the milk samples according to their renneting behaviour in 9 different groups (51). The total payment to the farmer depends partly on the rennetability of the delivered milk. The classification is based on the traces obtained with the Formagraph. This is the only commercial instrument allowing the simultaneous determination of up to 10 samples and is therefore best suited for this purpose.

3.3 Determination of the optimum cutting time. Determination of the optimum cutting time by means of a rheological technique has received a lot of attention and many instruments were developed for this purpose (2, 3). In- and

off-line constructions are possible. In-line (in the cheesevat) measurement has the advantage that the conditions of the actual process are exactly matched, provided the instrument is placed at a position representative of the whole batch. The main disadvantages are that provisions have to be made to remove the instrument prior to cutting and that proper cleaning of the instrument must be possible without the risk of damaging the inherently fragile measuring bodies. Off-line measurement has the advantage that the instrument can be treated separately from the cheesevat and that one instrument can serve several vats without the necessity to remove the instrument. Sampling of the milk should be easy and preferably automated, and the conditions of measuring should match those in the cheesevat.

Most instruments were developed to operate in-line. Emphasis in the construction has been laid on the measurement and less or none on the problems of removing, cleaning and automation. In a following section the different instruments will be briefly discussed. They all work on the same principle; the resistance exerted by the milk gel on an oscillatory deformation is in some way transformed to a response signal. Frequency, geometry and measuring system can be entirely different. With the exception of the GVT-INRA instrument (50), none of the others allows the transformation of the response signal in well defined rheological parameters. This need not be a serious drawback as long as the cheesemaker has established the experimental relation between signal and renneting time.

3.4 Standardisation of renneting properties. Milk standardisation from just one measurement is not possible, because several calcium, and perhaps protein or rennet concentrations have to be tried to find the desired values of the renneting time and rate of firming. A multi-channel instrument would be needed to restrict the time of analysis. With the Formagraph the standardisation can mostly be achieved in one run.

4 Required repeatability of the method

For basic research it is obvious that the repeatability of the method to evaluate the renneting process should be as good as possible in order to describe the rheological phenomena adequately. For the more practical applications it is not easy to give a guideline. To approach this question it is assumed that the same repeatability would be needed for the classification of the rennetability of milk samples, for the determination of the optimum time for cutting and for the standardisation of the renneting process.

It is claimed that the coagulum firmness at cutting is related to cheese yield (loss of fines and fat) and to the moisture content of the green cheese. The latter is influenced by the curd particle size distribution. Also the cheesevat,

construction of the knives, the curd preparation process and the curd properties in relation to the cheese type are important factors. The cheesemaker will choose the proper coagulum firmness in relation to these factors.

Pilot plant studies revealed that the cheese yield was not significantly influenced if a renneting time was chosen which was considered to be less than optimum (52, 53). But with full-scale processing a 30 % firmer coagulum than normal increased the yield (52). Mayes and Sutherland (54) found, also on a rather small scale, an increased recovery of milk solids at a prolonged renneting time. However, a 16 % increase of the normal renneting time increased the moisture content and decreased the quality of the cheese especially after long ripening. It was concluded that a 10 % deviation from the optimum renneting time could be tolerated. A second conclusion was that deviations below the optimum renneting time were more critical with respect to the overall performance of the process (yield and quality) than deviations above the optimum. A successful application of an objective instrument was reported for the manufacture of Dutch type cheese varieties (55). Practical experience of many trials with different cheesemaking vats at the NIZO (56) has learned that the type of vat has an impact on the control of the curd-firmness at the beginning of cutting. For some types of vats it is necessary to control the renneting time more accurately than for others in order to obtain the desired curd particle size distribution.

Firm conclusions on the tolerable deviation of the curd-firmness from its considered optimum value cannot be made. From our own experience and the reported results in the literature we suggest that an objective method to monitor the renneting time should have a repeatability of one minute.

5 Performance of the different instruments

All instruments developed for monitoring the gel formation give a response signal which is claimed to represent the firmness of the curd. Firmness, in this respect, is not a well defined parameter. The manual judgement of the firmness by the cheesemaker is normally composed of two different gel properties i.e. the feel of the resistance when he moves his finger through the curd and a visual observation of the breaking behaviour. Such a complete evaluation cannot easily be imitated by an objective method. All the newly developed instruments are based on a dynamic measuring system and, consequently, the response signal is expected to be related to the complex elastic modulus to which both elasticity and viscosity contribute. The main requirements are that the applied deformation should be sufficiently low in order not to break the gel and that the gel remains firmly attached to the measuring body.

Most of the results given in Section 2 were obtained by a dynamic method using the Instron Universal Testing Instrument. This method, of which a

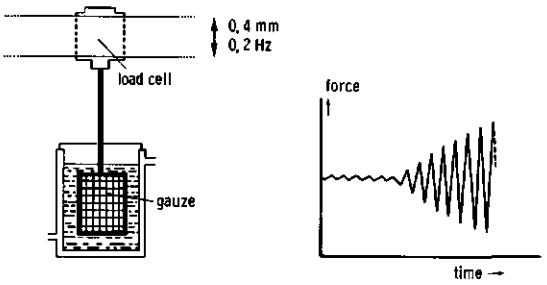

Fig. 5. Schematic depiction of the Instron method to monitor the gel formation and firming during renneting.

short description has been given in a previous paper (47), served also to evaluate the response of some of the other instruments. A schematic representation of the method is depicted in Fig. 5. A similar method but with a different measuring geometry was proposed by Ramet et al. (57). A typical result of the change of the force signal with time during renneting is given in Fig. 6. In order to find the firmness at the beginning of the cutting process a one-year comparison was made between the force signal and the manual judgement of the cheesemaker of the NIZO experimental dairy. It was found that cutting started already in the early stages of gelation. The force signal at renneting time is roughly 2.5 % of the final value which is only obtained after more than 3 hours. A linear relation was found between the force signal and the instantaneous shear modulus obtained with creep measurements. The value of this modulus was about 2 Pa at renneting time. The final value will then be about 80 Pa.

The most recent instruments developed to monitor the course of gelation during renneting are given in Table 1 together with some references, possibilities for use and the coefficient of variation. The latter value is sometimes given with respect to time at a constant value of the firmness and sometimes

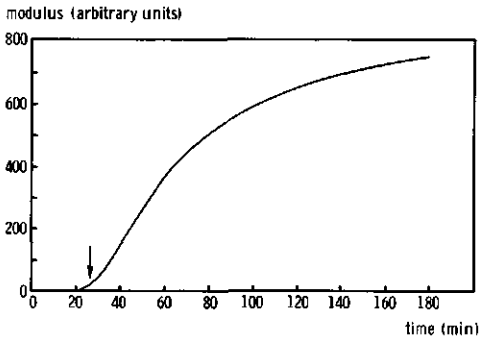

Fig. 6. The gel modulus (arbitrary units) as a function of time after rennet addition. Arrow indicates the renneting time.

Table. 1. Information on dynamic rheological instruments developed to monitor the setting of curd during cheesemaking

Instrument	Ref.	Possible applications			Samples per run	Coefficient of variation (%)			
		in-line	off-line	lab.		a[1]	b[2]	(Ref.)	based on
Instron (reference)	12, 47	–	–	+	1	1.6			time
GVT-INRA	50	?	+	+	1			ni[3]	
PTS: Kowalchyk/Olson	59, 60	+	+	+	1		≤2.5	59	time
Hatfield	21, 61	+	+	+	1		2.6	21	firmness
MKT	62	+	±	±	1		≈2	62	time
Gelograph	63	+	+	+	1	2.4	1.3	63	time
Formagraph	51, 23	–	–	+	10	2.5			time
Formascope	64	–	+	+	1	3.6			time
Ermartic	65	+	±	±	1			ni	
Vatimer	66	+	+	+	1[4]		3.9	66	firmness
Ultrasone: Bendix	21	±	±	+	1		3.5	21	firmness
Unipan	67	±	±	+	1			ni	

[1] Evaluated at NIZO on laboratory scale; renneting times were in the range of 20 to 30 min.
[2] Evaluated elsewhere.
[3] No information available.
[4] A multi-channel system was announced.

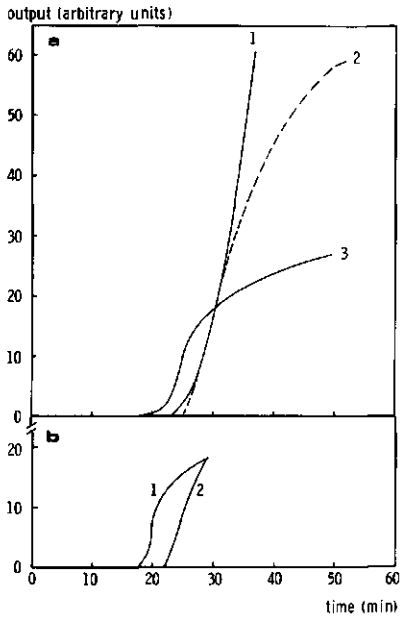

Fig. 7. Typical response curves of several instruments. Output was multiplied by a factor in order to give equal values after 30 minutes (= RT). a) Instron (1), Formagraph (2) and Gelograph (3); b) Gelograph (1) and Ermartic (2).

with respect to firmness after a certain reaction time. Transformation is only possible if the rate of signal increase with time is known.

The response signals of the instruments evaluated at the NIZO are given in Fig. 7. Two of the other instruments were assessed by Marshall et al. (21). The output curves of these instruments are given in Fig. 8. If it is assumed that the curve of the reference method resembles the gel modulus, it is clear that the Formagraph underestimates the final modulus. This need not to be a problem for the determination of the rate of gelation around renneting time but the final firmness cannot be estimated. Kinetic studies based on the Formagraph traces consequently lead to erroneous conclusions (14, 23, 45). The major drawback of the Formagraph is that the output signal is non-electronic so that automated data reduction is not possible. The response of the Gelograph starts around the point of the clotting time (Fig. 1). A three-dimensional network has not then been formed. The signal, being more related to the viscosity than to the rigidity of the gel, rapidly levels off after the point of gelation, which reduces the sensitivity of the instrument to determine the optimum time for cutting. The response of the ultrasonic instruments is similar to that of the Gelograph. The ultrasonic instruments are not specifically developed for monitoring the gel formation during renneting but are normally used to determine the viscosity of newtonian fluids. They operate at a much higher frequency than the Gelograph.

The pressure transmission instruments are modifications of the instrument

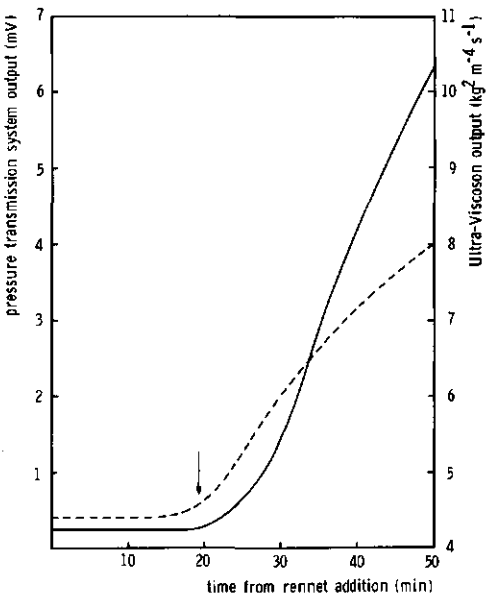

Fig. 8. Typical response curves of pressure transmission system (—) developed by Hatfield and of Bendix Ultra-Viscoson (– – –). Arrow indicates clotting time. Taken from Ref. 21.

originally proposed by Vanderheyden (58) and are, like the Ermartic and the Vatimer, designed to determine the optimum cutting time in the cheesevat. The Ermartic and the MKT instruments are the only instruments equipped with a device to remove the measuring body out of the cheesevat at the time of cutting.

The repeatability of the instruments seems to be sufficient but not all were tested under practical conditions (vibration in the factory, cleaning in place). A non-rheological method to measure the process of curd setting has been proposed by Hori (69). This method is based on the change of heat conduction during renneting. Like the viscometric methods this so called hot-wire method is very sensitive to the viscosity changes in the early stages of aggregation.

6 Concluding remarks

The suitability of an instrument to evaluate the process of curd-firming depends on the objectives of the measurement. The scale enlargement in the manufacturing of cheese has shifted the need for an in-line method towards a procedure to standardize the renneting process, because the very strict time schedules do not allow variation in the renneting time. In Section 2 the possible ways to control the renneting behaviour of a batch of milk were mentioned and it was concluded that adaptation of the amount of calcium chloride added to the milk would normally be sufficient to control the renneting pro-

cess. The standardisation may take place in the laboratory by means of a multi-channel instrument like the Formagraph. In order to avoid the danger of losing the contents of a complete cheesevat due to human errors one may in addition use an instrument in the vat to check if a coagulum has really been formed at the time cutting starts. Provisions have to be made to remove the instrument prior to cutting and to facilitate proper cleaning. Two instruments (Ermartic and MKT) are equipped with a device to remove the measuring body automatically from the vat. For a final check, non-rheological methods based on, for instance, heat conductivity (68) may be even more suitable because they are probably easier to place permanently in the vat and are less fragile.

In smaller factories where the rennetability of the milk varies considerably between the different batches and where the time schedules allow variation of the renneting time, several of the instruments mentioned in Section 5 may be used. Except for one, none of the instruments specifically developed to monitor the firming of the curd yields well defined rheological parameters. This makes it difficult to compare the different instruments and restricts the interpretation of the response signals. Furthermore it should be realised that optimal values for the rheological parameters, such as firmness and rate of firming, are probably only valid for particular conditions.

On the basis of reported information in the literature and the experience at the NIZO it was concluded that the required repeatability of an objective instrument should be about one minute. However, when the curd is still very soft at the time of cutting (i.e. some hard cheeses), a better repeatability may be required.

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Interpretation of the kinetics of the renneting reaction in milk[1]

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Summary

The kinetics of the conversion of κ-casein in milk by chymosin differs substantially from that observed in pure κ-casein solutions. This discrepancy was ascribed to the immobilised state of the substrate in the relatively large casein micelles. The upper limit of the reaction was calculated to be close to the experimentally obtained reaction rate, suggesting that the reaction may be diffusion-controlled. Interactions between the enzyme molecule and the micelle surface are discussed. A kinetic model combining proteolysis and surface inactivation by para-casein micelles was developed and verified. The rate of aggregation was calculated from the change in viscosity during renneting. The stability factor decayed exponentially with the degree of κ-casein conversion. It was estimated that the aggregation rate of fully converted micelles was a factor of 2000 lower than the diffusion-controlled limit. The effect of various variables on the stability of para-casein micelles is briefly discussed. A combination of the enzymic and the aggregation reactions resulted in a kinetic model by means of which the correct interdependence between clotting time and enzyme concentration could be predicted.

1 Introduction

The rennet-induced clotting of milk is an essential process in cheese manufacture. Cheese yield and quality depend, among other factors, on proper control of the renneting process.

Clotting of milk involves two reactions: the highly specific cleavage of κ-casein by chymosin and the subsequent aggregation of the para-casein micelles. The rates of and the degree of overlap between these two reactions depend on such conditions as pH, temperature, ionic strength, calcium concentration, casein concentration and the temperature history of the milk. In a series of previous studies (1, 2, 3, 4) the influence of the most important vari-

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ables on the two reactions was systematically investigated. A compilation of the experimental results and their implications for the control of the renneting process during traditional cheesemaking has recently been given in a special document (5). In this paper we try to interpret the enzymic and aggregation reactions from a more theoretical and mechanistic point of view. The two reactions are first treated separately and thereafter combined in a kinetic model which may describe the early stages of the rennet-induced aggregation of casein micelles. The results are compared to literature data, most of which have been obtained in highly simplified or diluted model systems. The kinetics of curd-firming, although of considerable importance, is left out, because of the lack of fundamental theories to describe such a complicated reaction. A qualitative description of the assembly of a rennet gel has been given by Walstra and Van Vliet (6). The effects of the most important variables on the rate of curd-firming were summarized in a previous paper (5).

2 The enzymic reaction

2.1 Characteristics of enzyme and substrate

Commercial rennet contains two enzymes: chymosin (EC 3.4.23.4), which contributes for about 87 % to the specific proteolytic activity under normal conditions (7), and pepsin (EC 3.4.23.1). Both enzymes belong to the group of acid proteinases. Two aspartic acid residues participate in the catalytic mechanism (8). The chymosin molecule has a rod-like shape with dimensions 2.5 and 4.5 nm (7) and consists of two domains separated by a deep cleft running parallel to the smallest diameter. The cleft is the active site of the molecule where the two aspartic residues are located. The enzyme is inactivated if either of these two residues is esterified (8). The pH optimum for general proteolysis of peptides is around 4 (10), whereas the optimum for the specific cleavage of the highly susceptible Phe-Met bond (residues 105-106) was found to be near pH 5.4 for isolated κ-casein and fragments thereof (10, 11) and around pH 6.0 in milk (2).

The predicted secondary structure of κ-casein is shown in Fig. 1 (12). An extended β-plate structure is predicted around the Phe-Met bond. The casein macropeptide (CMP) part of the molecule has an excess of negative charges which probably prevents the folding into a compact structure (12). Visser et al. (13) proposed that the residues 98-111 (His-Pro-His-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys) contribute for the major part to the substrate activity. They assumed that the fragment 103-108 is brought in the correct position via electrostatic bonding (His, Lys) near the entrance of the cleft,

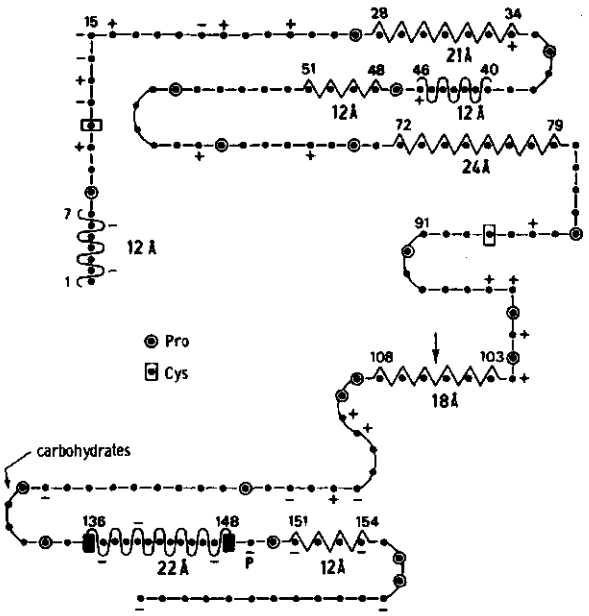

Fig. 1. The predicted secondary structure of κ-casein. After Raap et al. (12). $\bigwedge\!\bigwedge$: α-Helix; $\wedge\!\vee\!\wedge$: extended β-structure. The β-turns are depicted by a reversal of the chain direction with the four constituent residues located in the bend. A β-turn of lower probability is given by a 90° change in chain direction. Arrow indicates the chymosin-sensitive Phe-Met bond. (By courtesy of Academic Press.)

whereas the Pro residues act as stabilizers of the substrate conformation in the enzyme-substrate complex. In milk κ-casein occurs as a very heterogeneous substance. Vreeman et al. (14) identified at least 10 components differing in N-acetylneuraminic acid and/or phosphorus content. Additional heterogeneity is due to amino acid replacements in the two genetic variants (15). The substitutions are exclusively located in the CMP part of the molecule. The residues that contain the substitutions are apparently not directly involved in the formation of the enzyme-substrate complex; nevertheless they influence the rate of the reaction (1, 3, 14, 16, 17), although conflicting results do exist on this point (18, 19).

The κ-casein molecules in milk are thought to be located at the surface of the casein micelles; they are partly polymerised via disulfide bonds (15). The CMP part of κ-casein protrudes into the serum as a flexible 'hair', thereby imparting steric repulsion (probably with an electrostatic contribution) between approaching micelles (6). Vreeman et al. (20) calculated that a random coil

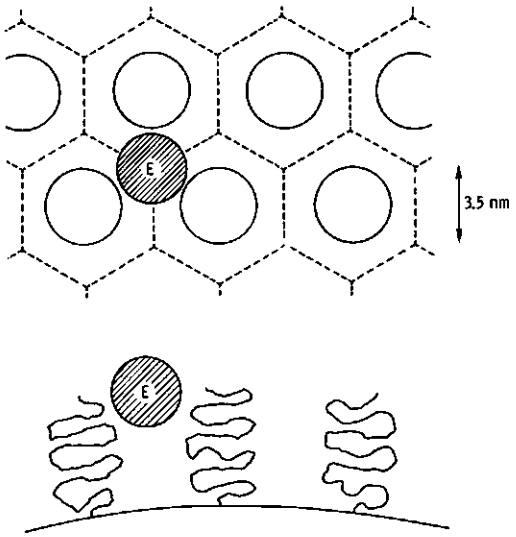

Fig. 2. Schematic representation of the casein micelle surface showing the distribution of the CMP 'hairs' and the enzyme molecule (E). (See also Table 1.)

conformation of the CMP moiety of κ-casein would lead to a thickness of the 'hairy' layer of 4.5 nm. Experimental results yield a slightly higher value, i.e. $\approx$ 5.5 nm (3, 21).

Figure 2 shows an idealized presentation of the surface of the micelle. Numerical values used to scale the drawing are given in Table 1. The surface concentration of κ-casein and the number of micelles were calculated on the basis of a volume-surface diameter of 120 nm for para-casein micelles and a voluminosity of 2.5 ml/g para-casein. The 'hairs' each have an average available surface area of 33 nm^2 and an available volume of 192 nm^3. The average distance between the anchor points is about 6 nm. The CMP molecule appears in a gel permeation chromatogram at the same retention time as does the dimer of β-lactoglobulin (23). The voluminosity of β-lactoglobulin in milk is about 1 ml/g (24) and its molecular weight is 36 000 (dimer). From this it would follow that the voluminosity of CMP is roughly 5 ml/g. About 30 % of the available volume in the 'hairy' layer will then be occupied by CMP, which leaves just about the space for the enzyme molecule to penetrate into the layer and to move towards the active site of the substrate. In reality the total surface area may be somewhat larger due to surface roughness, and the substrate molecules may not be quite evenly distributed over the surface.

Table 1. A compilation of characteristic data for casein micelles and chymosin in milk.

		Reference
Milk:		
casein concentration	28 kg m^{-3}	15
κ-casein concentration (12 %)	3.5 kg m^{-3}	15
molecular weight κ-casein	19500	15
molecular weight CMP	7300	15
number of micelles	8×10^{19} m^{-3}	calculated
Para-casein micelles:		
volume-surface average diameter	120 nm	22
average voluminosity	2.5 ml g^{-1}	3
surface area per ml milk	3.1 m^2	calculated
surface concentration κ-casein	1.0 mg m^{-2}	calculated
surface area per κ molecule	33 nm^2	calculated
Hairy layer:		
thickness	5.5 nm	21
volume per CMP molecule	193 nm^3	calculated
voluminosity of CMP	5 ml/g	deduced
volume occupied by CMP	30 %	calculated
Chymosin:		
concentration (0.02 % rennet)	4.3×10^{-6} mM	1
number of micelles/enzyme molecule	31	calculated
average diameter	3.2 nm	calculated
diffusion coefficient (D)	1.33×10^{-10} m^2 s^{-1}	calculated

2.2 *Kinetics of the enzymic reaction*

2.2.1 Catalytic parameters. The Michaelis-Menten equation can often be used to describe the rate of enzymic reactions in homogeneous systems; i.e. substrate and enzyme are in solution. The substrate in milk, however, is immobilised at the surface of the relatively large casein micelles and this may complicate the kinetics.

A collection of reported kinetic data for the conversion of κ-casein in various systems is given in Table 2. The catalytic constant K_{cat} (turnover number) for the κ(98-112) fragment is comparable to that of non-glycosylated κ-casein monomers. The apparent dissociation constant of the ES complex (K_m) is, however, larger by a factor 2.6, indicating a difference in substrate properties. Furthermore it can be seen that K_{cat} (and thus V_{max}) decreases with increasing N-acetylneuraminic acid content and that polymerisation of the substrate causes a drastic increase in the value of K_m. Reported data on the conversion of κ-casein in milk differ widely; the value of K_m appears to be much larger, which suggests that the degree of polymerisation affects the kinetics.

Table 2. Kinetic parameters for the action of chymosin on κ-casein in various systems.

Substrate	pH	T (°C)	K_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m ($\mu M^{-1} s^{-1}$)	Reference
fragment κ (98-112)	6.6	30	48.2	23	2.1	11
κ_0[1]	6.6	30	43.4	8.9	4.9	14
κ_3[1]	6.6	30	30.9	3.8	8.1	14
κ_6[1]	6.6	30	24.9	3.5	7.1	14
κ_0 polymer (22 S)	6.6	30	59.9	21.5	2.8	14
κ whole	6.6	30	93.3	31.8	2.9	14
κ whole	6.9	35	84	82	1.0	10
whole casein	6.5	23	33	100	0.3	25
milk	6.8	37	2-20	1-50	0.2-2	26
milk[2]	6.5	0	-	500	-	27
milk[2]	6.6	30	-	280	-	28
milk[2]	6.7	30	first-order $K_{enz} = 0.35$			1
milk[2]	6.7	30	first-order $K_{enz} = 0.35$			29

[1] Subscript refers to N-acetylneuraminic acid content.
[2] Based on progress curves; other data obtained by initial rate measurements.

Our analysis of progress curves in milk always showed first-order kinetics at the normal pH of milk (1, 2).

Figure 3 presents the differences between the various substrate systems more clearly. Especially at low substrate concentration the actual conversion rate in milk deviates substantially from that predicted on the basis of the catalytic parameters obtained from initial rate measurements in pure κ-casein solutions. This discrepancy is likely to be related to the immobilisation of the substrate in large particles rather than to such complicating factors as enzyme denaturation and product or substrate inhibition. Incubation of rennet in UF permeate of milk for 1 h at 30 °C did not change its clotting activity. Neither did addition of CMP to milk (dilution of UF concentrated milk with the supernatant of renneted UF concentrated milk) change the clotting time. Vreeman et al. (14) showed that substrate inhibition may also be ruled out, because increasing the substrate concentration under V_{max} conditions did not alter the conversion rate. Inactivation of the enzyme by para-casein micelles is also unlikely because virtually no adsorption of chymosin onto para-κ-casein micelles can be detected at the normal pH of milk (30).

Van Hooydonk et al. (1) suggested two possible explanations for the observed first-order kinetics in milk. One was based on a special solution of the interfacial kinetic model of Verger et al. (31) and one on the assumption of a diffusion-limited reaction rate. Both models will now be discussed in more detail and their predictive capacity to explain the effect of some important variables evaluated.

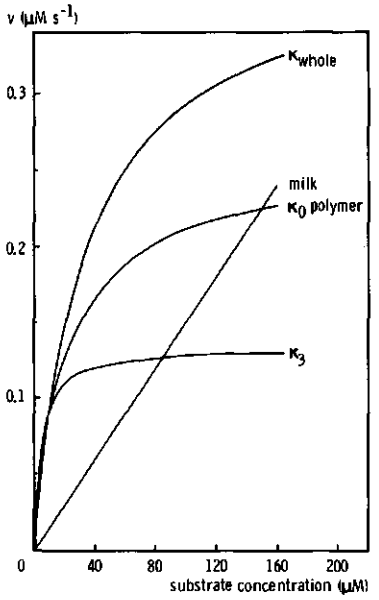

Fig. 3. Conversion rate (V) as a function of substrate concentration at 30 °C. Enzyme concentration is 4.3×10^{-6} μM. The results for κ_3, κ_0-polymer and κ-whole were calculated from kinetic parameters given by Vreeman et al. (14), the result for milk is based on progress curve analysis (first-order).

2.2.2 Diffusion-controlled model. The upper limit of an enzymic reaction is given by the collision frequency of the enzyme and substrate molecules. If the molecules have the proper orientation during collision a reaction will occur. In milk we have the special case that a fast moving enzyme collides with an immobile surface containing the substrate. The collision frequency irrespective of orientation (A) depends on the translational diffusion coefficient of the particles (D), their radius (r) and number concentration (n) (32):

$$A = 4\pi (D_m + D_e)(r_m + r_e)n_m n_e \quad (1)$$

The subscripts m and e refer to micelle and enzyme respectively. Since $D_e >> D_m$ and $r_m >> r_e$, the above equation reduces to

$$A \approx 4\pi D_e r_m n_m n_e \quad (2)$$

Calculation of A using the numerical values given in Table 1 yields 2.25×10^{22} $m^{-3} s^{-1}$ or 8700 encounters per enzyme molecule in one second. This would be the upper limit of the initial reaction rate if each encounter were effective. Long-range repulsion or attraction may, however, exert an appreciable influence on the collision rate. Both enzyme and micelle are negatively charged and some net repulsion may occur.

The magnitude of the electrostatic repulsion energy can be estimated on

the basis of the DVLO theory; for the conditions in milk, it may be approximated by (15):

$$E_R \approx 4.3 \times 10^{-9} r \psi_0^2 \ln(1 + \exp(-\varkappa h)) \quad (3)$$

where r is the radius of the particles, ψ_0 the surface potential, $\varkappa^{-1}$ the double layer thickness or Debije length, and h the distance between the particles. Since enzyme and micelle have widely different diameters, Equation 3 has to be adapted. Instead of r the ratio $2r_m r_e/(r_m + r_e + h)$ should be inserted (33). The ζ-potential of casein micelles is about −16 mV at 30 °C (15), and this value was used for the surface charge. That of the enzyme is not known and we arbitrarily assumed the same value as for the micelle, which is, of course, contestable, but no better estimate can be offered.

The value of $\varkappa^{-1}$ is, for the conditions in milk, about 1.1 nm (15). Likewise an estimation of the long-range attraction energy may be made using the relation (34):

$$-E_A = H r_m r_e/(6h(r_m + r_e)), \qquad h \ll r_e; \quad h \ll r_m \quad (4)$$

where H is the Hamaker coefficient which, for the casein micelles, amounts to about 10^{-21} J (35). Again we assume the same value for the enzyme molecule. The calculated total interaction energy ($E_T = E_R + E_A$) is very low (Table 3) and at maximum about 0.3 kT.

Additional repulsion arises from hydration of the surface of the particles (36). Assuming an effective contact area of 5 nm^2 and an interparticle distance of 0.5 nm the repulsion presumably will be somewhere between 0.3 and 2 kT. In total we would thus have a repulsion energy of the order of kT at a separation of 0.5 nm. Short-range attractive interactions are assumed to be negligible above this distance. The resulting retardation of the collision frequency may be calculated from the following approximated relation between stability factor (W) and interaction energy:

Table 3. Estimation of the interaction energy relative to kT between enzyme and casein micelle. Calculations based on Equations 3 and 4. Hamaker coefficient 10^{-21} J, double layer thickness 1.1 nm and ψ_0 −16 mV.

h (nm)	E_R/kT	$-E_A/kT$	E_T/kT
0.2	0.51	0.32	0.19
0.4	0.44	0.16	0.28
0.6	0.39	0.11	0.28
0.8	0.33	0.08	0.25
1.0	0.29	0.07	0.22

$$W \approx \exp(E_{max}/kT) \tag{5}$$

where E_{max} is the maximum in the potential curve. This implies a value of about 2.7 for W if E_{max} is 1 kT.

Besides overcoming this energy barrier, the enzyme has to diffuse through the CMP layer to reach the active site of the substrate. The diffusion rate in the layer is reduced compared to that in the bulk due to the high concentration of CMP molecules, at least initially. For diffusion of a large particle through a polymer solution the relative reduction of the diffusion rate is in first approximation proportional to the relative increase in the macroscopic viscosity (37), which amounts to 2.57 based on the Eilers equation (59) for $\Phi = 0.30$ and $\Phi_{max} = 0.79$. This is, however, a poor model for diffusion of chymosin through a hairy layer, since it assumes that the 'hairs' themselves can diffuse freely. A model that represents another extreme is that of retardation by hydrodynamic drag in the 'pores' of the layer. Using the relation of Happel and Brenner for cylindrical pores (38) we find for $r_c/R = 0.85$ (where R is the radius of the 'pore'; about 1.9 nm according to Fig. 2) that D_0/D is about 250. This model is not correct either, since it assumes that the 'pores' are inflexible. Taking rather arbitrarily the geometric mean of both results, we arrive at $D = 0.04\ D_0$ in the hairy layer.

To estimate the effect of hindered diffusion on the initial collision frequency, we assume that the enzyme molecule must diffuse over a distance X (= the mean free distance between the micelles) + twice the thickness of the hairy layer. Assuming the latter to be 5 nm we find that the factor by which the collision frequency is diminished as compared to a situation where no hairy layer would exist is given by

$$((X + 10/\sqrt{D/D_0})/(X + 10))^2 \tag{6}$$

where X is in nm. We calculate (see Table 4) $X = 158$ nm, hence the factor becomes 1.53.

A correction for the volume occupied by the micelles ($\Phi \approx 0.11$) should also be made. This causes on the one hand an effective increase of the enzyme concentration by a factor $(1\text{-}\Phi)^{-1}$ and on the other hand a tortuosity factor $\lambda^2 \approx 1.2$ (see Table 4). Combined, this gives a retardation by about 1.1. Finally, the effective enzyme concentration may be lowered because some enzyme molecules stay inside the micelle for longer times, but apart from the hindered diffusion in the hairy layer discussed above, we assume this to be negligible.

The requirement of the enzyme and substrate molecules being in the proper orientation during an encounter must have a considerable effect on the

number of effective collisions. The reduction will roughly be proportional to the area of the active site of the enzyme multiplied by that of the substrate (39). At the beginning of the reaction, the substrate occupies about 30 % of the micelle surface. For the enzyme the active surface is probably no more than 10 % of the molecular surface area. The ratio between rotation time and translation time ($\approx 8/3\ (r_e/\Delta)^2$) is such (i.e. about 30 for a distance Δ of 0.5 nm) that the enzyme must approach the substrate in the proper orientation to be active. The initial effective collision rate would thus be a factor of about 30 ($(0.3 \times 0.1)^{-1}$) lower.

On the basis of the above considerations the number of effective collisions will initially be $1/(2.7 \times 1.1 \times 1.5 \times 30)$ times the collision frequency calculated with Equation 2. The upper limit of the reaction would thus be about 65 cleavages per s for each enzyme molecule. The experimentally obtained initial rate at pH 6.7 amounts to 0.24 μM/s (Fig. 3), or about 55 cleavages per s. Considering the gross approximations used in the foregoing, a diffusion-controlled reaction may indeed explain the results. The weakest point in our calculations is probably the orientation of enzyme with respect to substrate. Undoubtedly, the effect of this will depend on short-distance interactions between both, which may vary, for instance with pH. As already explained in a previous paper (1), a diffusion model predicts the experimentally observed first-order kinetics.

2.2.3 Interface model. Verger et al. (31) proposed a model to describe the action of soluble enzymes at interfaces. The model consists of two successive reversible steps: the penetration of the enzyme into an interface followed by the formation of an interfacial ES-complex.

The general formula for steady-state conditions is:

$$V = \frac{K_{cat}\, e_0\, s^*}{s^* + K_m} \times \frac{s^*\, i/v}{s^*\, i/v + K_i\, K_M{}^*\, s^*/(s^* + K_m{}^*)} \tag{7}$$

where s^* is the surface concentration of the substrate per unit area, i/v the surface-area to total-volume ratio, $K_m{}^*$ the interfacial Michaelis-Menten constant, K_i the ratio of the desorption to penetration rate constants (area/volume), K_{cat} the catalytic rate constant (time^{-1}) and e_0 the enzyme concentration per unit volume. Two solutions for limiting conditions are:

$$V = \frac{K_{cat}\, e_0\, s^*\, i/v}{s^*\, i/v + K_i\, K_m{}^*}\ , \qquad K_m{}^* \ll s^* \tag{8}$$

and

$$V = \frac{K_{cat}\, e_0\, s^* i/v}{K_m^*\, i/v + K_i\, K_m^*}, \qquad K_m^* >> s^* \tag{9}$$

The latter equation is interesting because it predicts first-order kinetics for a given i/v and Michaelis-Menten type kinetics when i/v is increased. Progress curve analysis would therefore lead to first-order kinetics, and initial rate measurements in milk with various degrees of concentration to 'Michaelis-Menten' kinetics. If the interfacial K_m^* value may be compared with the values obtained for the 'free' substrate (Table 2), Equation 8 would, however, be more appropriate, since the substrate concentration in milk is about 180 μM.

An objection to the interface model is the lack of evidence for adsorption of chymosin onto either casein or paracasein micelles at physiological pH; it is even fairly certain that adsorption on para-casein micelles is negligible (30). Nevertheless, in the very beginning of the reaction, an enzyme molecule that has penetrated the hairy layer and splits off a CMP molecule has – due to diffusional hindering by the still dense hairy layer – a somewhat greater probability to at first attack a neighbouring κ-casein molecule than would be the case if no such hindering occurred. This would effectively be like a slight adsorption, at least in the early stages. At lower pH, there is fair evidence for adsorption of chymosin onto para-casein micelles (68, 30, 60) but this cannot directly be fitted into the interface model, because adsorption presumably changes proportionally with the release of CMP, implying that the effective enzyme concentration decreases during the reaction.

2.2.4 Comparison with experimental results. The most important results obtained for the conversion of κ-casein in milk are:

- at pH 6.7 virtually no aggregation of casein micelles occurs before at least 70 % of the κ-casein has been hydrolysed (2, 29),
- the reaction follows first-order kinetics at the normal pH of milk (1),
- the first-order rate constant decreases with milk concentration (1),
- the temperature coefficient is fairly low; $Q_{10} \approx 1.4$ (1),
- the reaction rate constant decreases with ionic strength (3),
- the maximum conversion rate is around pH 6 and, both at low (<6,2) and at high pH (>7.0), the kinetics deviates from first-order (2),
- addition of calcium at constant pH does not affect the reaction rate (3).

The first consequence of a diffusion-controlled model is a non-selective attack of the individual micelles by the enzyme. The existence of a potential dip near the surface of the micelle would cause 'adsorption' or penetration (interface model) of enzymes which necessarily leads to a process favouring the one

by one hydrolysis of the micelle resulting in a mixture of stable and unstable micelles. Experiments of Carlson (29) with milk in which the proteolysis was irreversibly stopped at various time intervals during the reaction showed that no appreciable aggregation took place below a conversion of about 65 %, which is consistent with our own results (see Section 3.3.2). Green et al. (40) concluded from electron-microscopic studies of milk samples taken at different times after addition of rennet that at least 80 % of the κ-casein was split before aggregation could be detected. Similar conclusions were reached by Dalgleish (41). These results strongly support a random attack at the normal pH of milk. The progress curves of the enzymic reaction in milk and in concentrated (ultrafiltered) milk can adequately be described by a first-order equation. The rate constant, however, was found to decrease with increasing concentration of milk.

The diffusion-controlled model in its simplest form (Equation 1) predicts that the initial reaction velocity is proportional to the degree of concentration. Experiments revealed a less than proportional increase. However, concentration of milk also changes the diffusion rate of the enzyme due to the following factors:

a. The viscosity of the serum increases due to increased concentrations of serum proteins and (slightly) of lactose etc. These increases are enhanced by exclusion of solute by the casein (15). We assume the amount of non-solvent water to be 2.5 ml/g casein for serum proteins and 0.55 ml/g for lactose.

b. The effective concentration of enzyme is increased by a factor $(1-\Phi)^{-1}$, where Φ is the micellar volume fraction. We assume the voluminosity of the casein micelles to be 3.99 ml/g casein (47).

c. The enzyme has to go around the micelles. The relative increase in distance is:

$$\lambda = \cos\alpha + \alpha \sin\alpha \tag{10}$$

where,

$$\sin\alpha = (1 + X/2r)^{-1} \tag{11}$$

and X the mean free distance between micelles (66). The relative increase in diffusion time is given by λ^2.

d. Enzyme molecules may be constricted in their motion by the micelles if the condition $2r_c >> X$ is not fulfilled (9). This factor is negligible unless Φ is very high (about 1 % retardation for 2 × concentration).

e. The relative increase in diffusion time through the hairy layer as given by Equation 6 becomes larger.

Some sample calculations are given in Table 4. It is seen that the calculated

Table 4. Calculation of the change in the reaction rate constant K for chymosin action due to concentration of the milk by ultrafiltration (see text).

	Concentration factor			
	1	1.5	2	2.5
casein content (% w/w)	2.70	4.05	5.40	6.75
serum protein (% w/w)	0.67	1.00	1.33	1.67
lactose (% w/w)	4.8	4.9	5.0	5.1
density (kg m^{-3})	1030	1048	1066	1086
Φ (casein micelles)	0.108	0.166	0.224	0.286
X (nm)[a]	158	93	62	43
η/η_0 of serum[b]	1.18	1.21	1.25	1.30
λ^2	1.20	1.35	1.50	1.65
$(X+10)^2/(X+50)^2$	0.65	0.52	0.41	0.32
K/K_0	0.51	0.38	0.28	0.21
K/K_1, calculated	(1)	0.74	0.54	0.41
K/K_1, observed (ref. 2)	(1)	0.78-0.84	0.60-0.69	-

[a] Assuming a micelle diameter of 120 nm.
[b] Calculated from the Eilers equation (59).

retardation is greater than determined. This may be due to an overestimation of the diffusion time in the hairy layer since it is assumed, firstly, that every time an enzyme molecule attacks it has to move to another micelle, which cannot be quite true, and secondly, that the hindering of diffusion is not affected by the removal of CMP, while in fact even a little loss of CMP may have a clear enhancing effect on the effective D. Nevertheless, the calculation shows that a diffusion-controlled model may well be compatible with the observed influence of concentration on the reaction rate.

The interface model according to Equation 9 may explain the experiments equally well – at least in a qualitative way – but no justification can be offered for the assumption that $K_m^* >> s^*$.

The temperature coefficient Q_{10} for the reaction in milk was found to be about 1.4 (1). Since the diffusion rate is inversely related to the viscosity of the solvent the collision frequency decreases with decreasing temperature. The Q_{10} for $1/\eta$ of milk serum $\approx$ 1.37 near 30 °C, which is close to the value for the enzymic reaction and is thus consistent with a diffusion-controlled reaction.

The reaction rate decreases with increasing ionic strength (3). If the reaction were retarded by electrostatic repulsion between enzyme and micelle, an increased rate would be expected due to the compression of the double layer. But, as we have shown, electrostatic repulsion probably plays a very minor role. Other micelle properties are however also changed by addition of NaCl

to milk. For instance the voluminosity of the micelle and its outer layer were increased and some of the micellar calcium was exchanged for sodium (3). These effects may diminish the accessibility of the substrate. The ionic strength may also affect the enzyme/substrate complex formation due to the screening of positive and negative charges involved in the binding. Visser et al. (11) also found a decreased reaction rate with increasing ionic strength in systems containing a soluble fragment of $\varkappa$-casein as substrate. But admittedly, the catalytic parameters obtained for these systems do not nearly agree with the conversion of $\varkappa$-casein in milk, as was shown in Fig. 3.

The rate of conversion is maximal around pH 6.0, being about twice the value found at physiological pH (2). Both at low (<6.2) and at high pH (>7.0) the reaction no longer follows first-order kinetics. The deviation at high pH, as was shown before (2), is caused by a slow denaturation of chymosin. At low pH, the deviation is presumably due to the adsorption of enzyme onto the para-casein micelle surface (2). Accepting initial first-order kinetics at low pH (using either the diffusion-controlled model or the interface model according to Equation 9) the effect of adsorption onto the para-casein surface can easily be incorporated in a kinetic expression describing the release of CMP with time (see Appendix). Lowering the pH causes an initial decrease in the voluminosity with a minimum around pH 6.0 (44), which happens to be the pH of maximal rate of conversion. Again this may be related to a change in the accessibility of the substrate. Another explanation may be the protonation of the histidine residues ($pK \approx 6.5$) involved in the ES-complex formation.

2.2.4 Conclusions. Experimental results have clearly shown that the kinetics of the conversion of $\varkappa$-casein in milk substantially differs from that found in model systems. The discrepancy is presumably related to the immobilized state of the substrate in the relatively large casein micelles. A diffusion-controlled reaction may explain the observed kinetics. Such a model requires a random attack of the micelles, although the chance of attacking a neighbouring substrate molecule on the same micelle will, of course, be higher than attacking one on another micelle, unless the enzyme would release the product (CMP) only after it has moved away from the surface. Experimental results on the aggregation reaction strongly support a random attack by the enzyme at the normal pH of milk. The upper limit of the reaction rate was estimated to be close to the experimentally obtained value, but some crude approximations had to be used in the calculations. Combined with the low temperature coefficient of the reaction a diffusion-controlled model would indeed be a plausible explanation for the observed kinetics. Effects of ionic strength and

especially pH are more difficult to reconcile with a diffusion model and only vague indications about changes in the accessibility of the substrate can be given. The effect of protein concentration can be explained by a diffusion-controlled reaction.

However, an interface model could, under certain conditions, describe the observations equally well, at least qualitatively: a quantitative prediction cannot be made since not all the needed parameters of Equation 7 can be predicted or derived from experiments. This model would also easily explain the decrease of reaction velocity with increasing micelle concentration, which means increasing the surface-to-volume ratio. The conception of adsorbed enzyme molecules in a kind of potential dip near the surface seems unlikely in view of the interaction between micelle and enzyme. The diffusion of the enzyme out of the layer may, however, be hindered if still many hairs are present. This effectively comes down to a weak adsorption which may promote the reaction especially at the beginning.

3 Aggregation reaction

3.1 Interaction forces

Casein micelles are remarkably stable in milk. They possess an overall negative charge resulting in a ζ-potential of about -16 mV at 30 °C (15). Such a value is however insufficient for explaining the stability of the micelles in terms of the DVLO theory (45, 46). Other stabilizing factors must contribute, and it is now recognized that steric stabilisation by protruding chains of protein plays a dominant role in micelle stability (47, 48). The highly hydrophylic CMP part of κ-casein may then be considered as a tail of an adsorbed macromolecule imparting steric stabilisation. Removal of the CMP layer by rennet increases the ζ-potential to about -10 mV (15) and reduces the micelle radius by about 5.5 nm (21, 3). The latter effect causes an initial reduction of the viscosity before aggregation starts. This cannot be explained by a decrease in the average molecular weight of the micelles but must be due to the removal of the voluminous outer layer, as was explained by Walstra (47).

The rate of aggregation is strongly dependent on temperature, i.e. $Q_{10} \approx 30$ at 30 °C. This should not be interpreted as representing an activation energy (15). It is due to the free energy of activation for flocculation strongly increasing with decreasing temperature (41, 45). A temperature-dependent change of the surface charge of the micelle cannot be the explanation. First, the magnitude of the energy barrier calculated on the basis of the DVLO theory cannot account for the retardation of the aggregation of rennet-con-

verted micelles (45) and secondly the ζ-potential appears to increase rather than decrease with increasing temperature (46, 49).

Payens (45) explained the slow coagulation on the assumption that a restricted number of reactive sites on the micelle surface reduces the number of effective collisions. This would imply a gross heterogeneity of the surface of para-casein micelles (stable and unstable patches). This is, of course, a fairly realistic model for the aggregation of partly converted micelles, but to explain the aggregation of fully converted ones by such a mechanism seems to be inconsistent with the assumed homogeneous micelle structure. Furthermore, it is difficult to conceive why the heterogeneity would change with temperature. The peculiar temperature dependency of the rate of aggregation has also been explained on the assumption that hydrophobic bonds are responsible for the permanent contact between para-casein micelles after collision, since such bonds decrease in strength with decreasing temperature (50, 51). This, as such, is not a valid explanation because the strength of the bonds formed after collision (assuming no energy barrier) is irrelevant to the rate of flocculation, unless the formation of enough bonds to yield permanent adherence would depend on the contact time (for instance, due to a required reconformation of surface proteins). The observed increase of the elastic modulus of a rennet gel with decreasing temperature (6) appears to be inconsistent with this conception. Besides, it does not fit with the effects of calcium and ionic strength on the rate of aggregation (3, 50).

In an indirect way hydrophobic bonding may be involved. The partial dissociation of β-casein at low temperature is probably due to the decrease of hydrophobic interactions within the micelle and it is tempting to assume that the hydrophylic part of β-casein causes a temperature-dependent steric repulsion between renneted micelles.

3.2 Kinetic models

More than a century ago Storch and Segelcke (52) published an empirically obtained relation for the clotting of milk by rennet. This relation simply states that the product of the visibly observed clotting time (t_c) and the concentration of rennet (e) was constant for certain conditions. Holter (53) found that this rule was only valid in a narrow temperature range and for limited rennet concentrations. He proposed a modified equation which was rearranged by Foltmann (54) in the currently used form:

$$t_c = a/e + b \tag{12}$$

No exact physical meaning could be given to the parameters a and b, but it

was intuitively assumed that a is related to the rate of the enzymic reaction and b to the aggregation process. The magnitude of both parameters depends not only on reaction conditions but also on the characteristic chosen to evaluate the renneting process, where it should be remembered that various characteristic values are reached at various times after adding rennet (5). Taking for t_c the time of gelation yields higher values for both parameters than for instance the time of visible flocculation.

The first mechanistic model on the rennet-induced aggregation of casein micelles was developed and extended by Payens and coworkers (55, 56, 57, 51). The basic assumption is that clotting may be considered as the result of the production of unstable casein micelles and the subsequent aggregation according to Smoluchowski kinetics. The number of unstable micelles produced by rennet at a given time was arbitrarily assumed to be linearly dependent on the degree of proteolysis and the aggregation rate constant was fixed at a certain value (57). This actually assumes implicitly that the enzyme attacks the micelles one by one rather than at random.

The model of Dalgleish (27) defines more specifically the degree of conversion at which the micelles can be considered aggregatable. The fraction of micelles which achieves this critical degree was calculated on the basis of probability. Similar to the model of Payens the aggregation rate constant was taken not to be affected by proteolysis. Dalgleish (41) simplified his model by introducing a step function at the critical degree in conversion ($\approx$ 97 %). No aggregation would take place below this conversion and above it all micelles should simultaneously become aggregatable. The overall clotting time now is the sum of the time to reach the critical conversion and the time for aggregation to the average molecular weight at which the clotting is observed. In this case the model yields indeed the experimentally obtained relation between clotting time and enzyme concentration (Equation 12).

The model proposed by Darling and van Hooydonk (58) differs from the previous models in that the aggregation rate constant is made a specific function of the degree of conversion. The stability factor representing the ratio of particle collision frequency to the number of successful collisions was assumed to decay exponentially with the degree of conversion. This model implicitly assumes that the micelles are randomly attacked by the enzyme molecule. Using the zero-order approximation of the Michaelis-Menten equation ($s >> K_m$), also this model predicts that the clotting time is linearly dependent on the reciprocal of the enzyme concentration.

In the following sections the relation between the rate of aggregation and the degree of proteolysis will be estimated from the change in viscosity during renneting.

3.3 Relation between κ-casein conversion and rate of aggregation

3.3.1 Theory. Aggregation of dispersed particles will increase the viscosity of the dispersion due to the immobilisation of solvent in the aggregates which increases the effective volume fraction. The volume fraction Φ_0 of a dispersion containing n_0 particles is equal to $n_0 v$, where v is the volume of one primary particle. The effective volume fraction (Φ) of an aggregated dispersion may be written as:

$$\Phi = vn_1 + v\Sigma\, i \cdot n_i \cdot f_i \qquad i = 2, 3, \ldots \tag{13}$$

where n_1 represents the number of primary particles not reacted and n_i that of the i-mer aggregate. The volume of each aggregate is considered to be the product of the volume of the particles in the aggregate and a swelling factor (f), accounting for the amount of 'trapped' solvent and the anisometry of the aggregate. If f is independent of the aggregate size the effective volume fraction becomes:

$$\Phi = vn_1 + fv\,(n_0 - n_1) \tag{14}$$

or, relative to the non-aggregated dispersion:

$$\Phi/\Phi_0 = n_1/n_0 + f(1 - n_1/n_0) \tag{15}$$

The change in the number of primary particles during aggregation follows directly from the Smoluchowski theory (32):

$$n_i/n_0 = (t/t^*)^{i-1}/(1 + t/t^*)^{i+1} \tag{16}$$

in which t is the time since the reaction started and t^* ($= 1/k_s\, n_0$) the time needed to reduce the number of particles to $0.5\, n_0$. k_s is the aggregation rate constant. For a diffusion-controlled process $k_s = k_d = 4\, kT/3\, \eta_0$. If the reaction is retarded by a free energy barrier it is convenient to introduce a stability factor (W) such that $k_s = k_d/W$. Combination of Equation 15 and 16 ($i = 1$) leads to:

$$\Phi/\Phi_0 = (1 + t/t^*)^{-2} + f(1 - (1 + t/t^*)^{-2}) \tag{17}$$

and after some rearrangement:

$$\sqrt{(1-f)/(\Phi/\Phi_0 - f)} = 1 + t/t^* \tag{18}$$

The change of the reaction rate constant with time and thus with the degree of conversion can now be calculated. The effective volume fraction is calculated from the relative viscosity (η/η_0) via the rearranged equation of Eilers (59):

$$1/\Phi = 1.25/(\sqrt{\eta/\eta_0}-1) + 1/\Phi_{max} \quad (19)$$

where Φ_{max} is the maximum obtainable volume fraction. According to Snoeren et al. (24), Φ_{max} would be about 0.79 for skim milk, considering (para-) casein micelles and serum proteins as the dispersed particles.

Using a constant swelling factor limits the application of the foregoing theory to the early stages of aggregation. It is obvious that f must increase during aggregation in order to arrive at the infinitely high viscosity corresponding to gelation. Only for doublets, a reasonable estimate of f can be given. The geometry of a triplet may already vary from rod-like to 'triangular' (i.e. almost disk-like). If we restrict the analysis to time t^*, the majority of the particles will still be singlets and doublets as can be calculated from Equation 16. The swelling factor for a doublet was calculated from data provided by Simha (61) for a low rotary Péclet number (i.e. the ratio of the shear rate to the rotary diffusion coefficient). For casein micelle doublets and the applied shear rate ($\dot{\gamma} \approx 100\ s^{-1}$) $Pe_r \ll 1$. Assuming the doublet to be a prolate ellipsoid of revolution, containing trapped solvent between the constituting particles (which, alone gives $f \approx 1.25$), it follows that the additional swelling factor due to anisometry is about 1.3. Taking both contributions into account f becomes about 1.6, but, since a doublet is not precisely an ellipsoid of revolution, this is probably a slight underestimation. The value is comparable with the experimentally obtained value of 1.7 of Mooney (62).

The viscosity measurement itself may influence the process of aggregation, because streaming affects the meeting frequency of the particles and exerts a viscous stress on the aggregates. The ratio of shear induced (J_s) to Brownian (J_B) collision frequency for particles with diameter d is equal to (15):

$$J_S/J_B = \dot{\gamma}\, d^3\, \eta_0/(2\,kT) \quad (20)$$

For casein micelles in milk this ratio is about 0.02 at the applied shear rate $\dot{\gamma}$ of $100\ s^{-1}$, indicating that the Brownian collision frequency is still dominating. The maximum frictional force experienced by a particle in a doublet will be (63):

$$F_f = (3/2)\,\pi\, d^2\, \eta_0\, \dot{\gamma} \quad (21)$$

which amounts to about 6×10^{-15} N for casein micelles ($\dot{\gamma} \approx 100\ s^{-1}$). The energy needed to separate the particles over a distance of 0.5 nm must be at least a few times kT (say, 10^{-21} J). Hence, the energy needed to pull them apart would be 2×10^{-12} N, i.e. 300 times the force exerted by shear. Consequently the aggregates will not be disrupted.

A straightforward application of Equation 17 is not possible because the

voluminosity of the primary particles changes during the reaction and a correction is necessary. Cohen Stuart et al. (64) developed a model to calculate the hydrodynamic thickness of an adsorbed polymer layer. The calculations are based on streaming in a porous layer using segment density profiles from the Scheutjens-Fleer adsorption theory (65). In this theory the adsorbed layer is divided into layers of lattice sites parallel to the interface and each layer is characterized by a certain volume fraction of polymer segments. The distribution of the segments in the various layers depends on the amount of adsorbed polymer, chain length and two energy parameters representing the segmental adsorption energy (χ_s) and the segment-solvent interaction (the Flory-Huggins χ-parameter). The hydrodynamic thickness can be calculated from the segment distribution by an iterative procedure (64). In applying this theory to casein micelles it was assumed that the CMP part of κ-casein can be considered as an adsorbed polymer chain. Both the number of statistical segments and the χ-parameter are unknown (χ_s may be set to zero because CMP does not adsorb onto the casein micelle surface). The value of χ must be smaller than that of a theta solvent ($\chi = 0.5$) and we arbitrarily assumed a value of 0.3. A reasonable estimate of the number of statistical segments is difficult to make. Several values were tried and a value of 50 corresponded well with the experimentally found hydrodynamic thickness of the intact CMP layer (≈ 5.5 nm). It also fits more or less with the number of amino acid residues in the chain (64, hence at most 126 hinge points) combined with the relative scarceness of secondary structure.

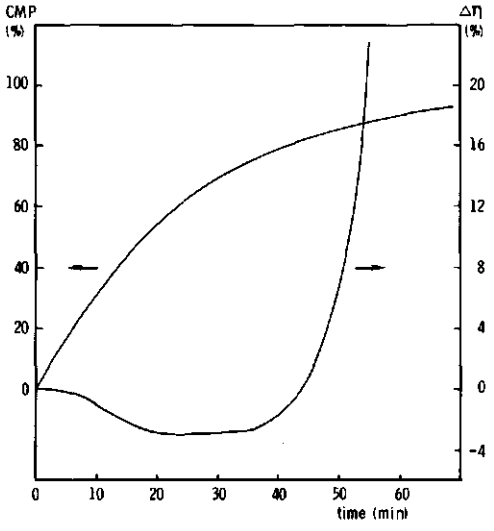

Fig. 4. The relative change in viscosity (% of initial value) and the release of CMP (% of total) during renneting of milk at 30 °C and pH 6.7. Rennet concentration 0.01 %. Results taken from previous paper (2).

3.3.2 Calculation of the aggregation rate constant. The viscosity curve obtained at pH 6.70 (Fig. 4) was used to estimate the reaction rate constant as a function of the degree of conversion. Calculation of the casein volume fraction from the relative viscosity (Equation 19) requires a correction for the whey protein (voluminosity 1.1 ml/g, concentration 0.7 % w/w) and released CMP (voluminosity ≈ 5 ml/g). The hydrodynamic thickness as a function of conversion was calculated using the computer program developed by Cohen Stuart et al. (64). The result is plotted in Fig. 5. Combination of these results with those from Fig. 4 enables the calculation of Φ/Φ_0 during the reaction via

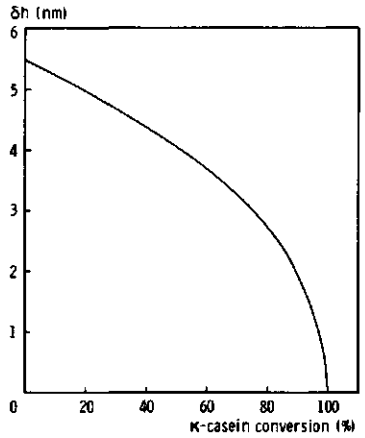

Fig. 5. The calculated relation between hydrodynamic thickness of the CMP layer (δ_h) and the degree of κ-casein conversion.

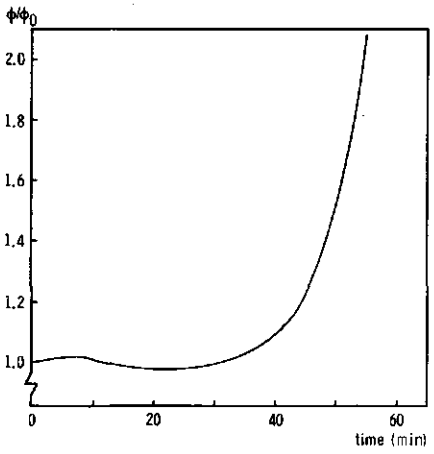

Fig. 6. The relative volume fraction of the casein micelles as a function of reaction time. See text for calculation procedure.

Equation 19 (Fig. 6). The curve initially drops somewhat below 1, indicating that the actual reduction of the voluminosity of the casein micelles does not fully follow the theoretical prediction, but the deviation is small considering the various approximations used in the calculations (For comparison: if the hairs are fully removed and no aggregation occurs, we calculate a reduction of Φ to 0.66 Φ_0 from the micelle size distribution). The results in Fig. 7 are plotted according to Equation 18 for two different swelling factors. The stability factor (W) obtained by graphical differentiation of these curves, is given as a function of the degree of conversion in Figs. 8 and 9. At 80 % conversion

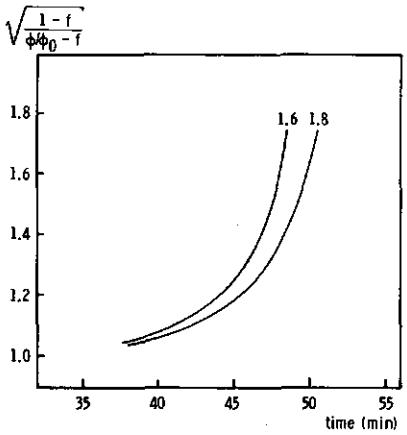

Fig. 7. The transformed relative volume fraction as a function of reaction time for two values of the swelling factor (according to Equation 18).

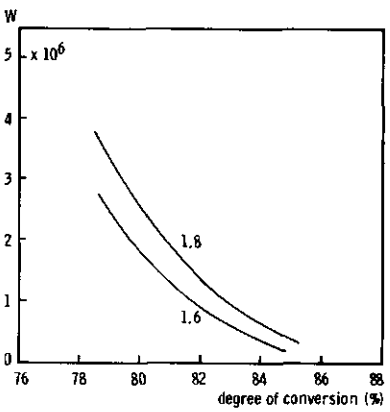

Fig. 8. The stability factor (W) versus degree of conversion. Parameter is the swelling factor f. $k_d = 6.2 \times 10^{-18}$ m^{3}s^{-1} and $n_0 = 10^{20}$ m^{-3}.

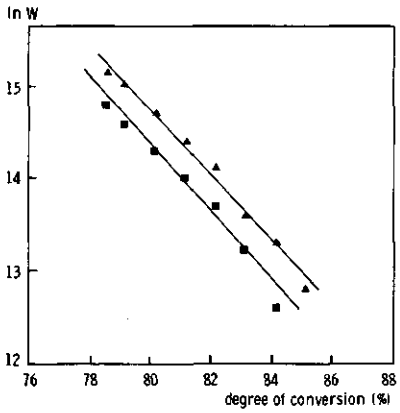

Fig. 9. Logarithmically transformed stability factor (W) versus degree of conversion. Calculated for $f = 1.6$ (■) and 1.8 (▲).

the value of k_s amounts to about 2.4×10^{-24} m^3s^{-1} ($f = 1.8$), which means that it would take roughly 70 min to halve the number of these particles. Assuming that it is allowed to extrapolate the curve in Fig. 9 to fully converted micelles a k_s value of 2.8×10^{-21} m^3s^{-1} ($t^* = 3.5$ s) is obtained for $f = 1.8$ and 5.6×10^{-21} m^3s^{-1} for $f = 1.6$. Dalgleish (50) deduced a rate constant of 17×10^{-21} m^3s^{-1} for fully converted micelles using light scattering. The discrepancy between the rate constants obtained by light scattering in a dilute system and from extrapolation of viscosity measurements in milk (assuming $f = 1.6$) is thus only by a factor 3.

The diffusion-controlled limit is about 6×10^{-18} m^3s^{-1}, hence $1.0\text{-}2.2 \times 10^{-3}$ as large. The discrepancy must be primarily due to repulsion and hydrodynamic hindrance at short interparticle distance. It should also be realised that the Smoluchowski prediction is not quite correct, even if we assume zero interaction energy and zero hydrodynamic hindrance; this is because the volume fraction of the aggregating particles is finite, i.e. about 0.11. If all particles meeting each other would flocculate the finite Φ causes an increased flocculation rate, presumably by a few times 10 %. On the other hand, tortuosity and constriction effects will slow down the flocculation rate, especially of not yet fully converted micelles; unfortunately, these effects cannot be accurately predicted, but it is unlikely that they are very important.

It may be noted that the now experimentally obtained linear relation between ln W and conversion was already assumed in the model of Darling and van Hooydonk (58), on the basis of steric repulsion being responsible for W. This is undoubtedly an important causative factor but electrostatic repulsion and retardation due to hydrodynamic hindrance (67) must also play a part. However, under the experimental conditions, i.e. constant temperature and pH, the steric repulsion must be the main factor that varies. The linearity between ln W and conversion means that W is proportional to exp (hair density). This seems to imply that, at least in the region investigated, the elastic (volume restriction) term rather than the mixing term of macromolecular interactions is responsible for the steric repulsion. Extrapolation to much lower degrees of conversion is probably not allowed. Figure 6 indicates that aggregation must have started between 60 % and 70 % conversion. The curves in Fig. 9, however, predict a t^* of about 1.5 day at 70 % conversion, suggesting that extrapolation overestimates the value of W in that region. It should be noted that the method used by us inevitably gives fairly uncertain results below 78 % and above 84 % conversion.

It is difficult to explain the effect of physico-chemical variables on the aggregation rate. The hydrodynamic hindrance (67) is presumably almost constant. It may explain why only about 40 % of the Smoluchowski limit is ob-

tained as a maximum (high temperature, high pH, high calcium concentration), as was suggested by Brinkhuis and Payens (51). Zeta potentials do not vary greatly with these variables. However, electrostatic repulsion cannot be calculated on the basis of electrokinetic potentials since the distance between slipping plane and particle core surface may vary and, secondly, part of the charge comes from a protruding chain that may vary in conformation as well as in net charge. Conformational changes may be induced by factors such as pH, ionic strength and calcium content. Therefore both steric and electrostatic repulsion are affected by these variables, but there is no way to make a distinction between the two. Generally it was found that a thicker hairy layer or a higher micelle voluminosity goes along with an increased stability to flocculation except for the pH region 5.3 to 6.0 (2, 3).

The strong increase in aggregation rate with increasing temperature must be due to decreased steric repulsion caused by increased hydrophobic interaction between the various casein molecules in the micelle.

4 The enzymic and aggregation reactions combined

The overall kinetic model for the aggregation of casein micelles consists of the combination of the enzymic reaction (first-order) and the aggregation reaction (Smoluchowski):

$$-\mathrm{d}s/\mathrm{d}t = Ks \tag{22}$$

and,

$$-\mathrm{d}n/\mathrm{d}t = (k_\mathrm{d}/W)n^2 \tag{23}$$

where K ($= K_{\mathrm{enz}}e$) represents the first-order rate constant for the enzymic reaction. The relation between the stability factor (W) and the substrate concentration (s) is essentially exponential, at least during the early stages of aggregation. Introducing this relation in Equation 23 yields a differential which can only be solved by a numerical procedure. In order to get some idea about the significance of the various physical parameters, we assume a linear relation between W and s, which permits an analytical solution of the integral. Such an approximation probably does not fully invalidate the conclusions, if the reaction is restricted to, say, about 85 % conversion (see Fig. 9). Because virtually no aggregation takes place up to 60 % proteolysis, the combination of the two basic equations can start from there. We define:

$$W_\tau = Cs_\tau + P \tag{24}$$

and

$$W_t = Cs_t + P \tag{25}$$

where W_τ is the stability factor at 60 % conversion (lag phase), s the substrate concentration ($s_\tau = 0.6\, s_0$) and t the time after conversion to 0.6 s_0. The constant C relates W to the degree of conversion and P is the contribution of other caseins. Subtracting Equation 24 from Equation 25 and inserting the integrated form of Equation 22 yields:

$$W_t = W_\tau - Cs_\tau + Cs_\tau \exp(-Kt) \tag{26}$$

In combination with Equation 23 we get:

$$-\frac{dn}{n^2} = \frac{k_d\, dt}{(W_\tau - Cs_\tau) + Cs_\tau \exp(-Kt)} \tag{27}$$

Integration for the conditions $n = n_0$ at $t = 0$ and $n = n_t$ at $t = t$ results in:

$$t = \frac{W_\tau - Cs_\tau}{k_d}\left(\frac{1}{n_t} - \frac{1}{n_0}\right) - \frac{1}{K}\ln\left[1 - \frac{Cs_\tau}{W_\tau}(1-\exp(-Kt))\right] \tag{28}$$

The equation was intentionally arranged in this form for comparison with the empirical relation of Holter (Equation 12). The term $(1-\exp(-Kt))$ represents the degree of conversion at time t; i.e. $(s_\tau - s_t)/s_\tau$. To relate the total reaction time to the extent of aggregation, we simply have to add the lag time τ that can be written as

$$\tau = -(\ln s_\tau/s_0)/K \tag{29}$$

The total reaction time, which may be taken as the clotting time t_c at which a certain value for n_t is reached, becomes:

$$t_c = \frac{W_\tau - Cs_\tau}{k_d}\left(\frac{1}{n_t} - \frac{1}{n_0}\right) - \frac{1}{K}\ln\left[\frac{s_\tau}{s_0}\left(1 - \frac{Cs_\tau}{W_\tau}(1-\exp(-Kt))\right)\right] \tag{30}$$

Note that t is defined as $(t_c - \tau)$.

This equation predicts a linear relation between the clotting time and the reciprocal of the enzyme concentration (remember that $K = K_{enz}e$) assuming that clotting always starts at the same degree of conversion at each enzyme concentration, i.e. constant (1-exp(-Kt)). This can, of course, be valid only for a narrow range of enzyme concentrations.

The equation also predicts that both the intercept and the slope of a Holter plot must increase if t_c is taken at lower values of n_t (higher degree of aggregation). This is indeed found experimentally (5). It should be realized that the model is probably only valid up to 85 % conversion of κ-casein. Extension to

the time of gelation, which occurs normally at a higher degree of conversion, is not allowed, but at least qualitatively the model predicts the correct features.

The term $(W_\tau\text{-}Cs_\tau)/k_d$ represents the reciprocal of the aggregation rate constant for fully converted micelles. Increasing the temperature or decreasing the pH will therefore decrease the intercept of a Holter plot. This was indeed found in the study by Foltman (54).

It may finally be noted that Equation 30 predicts that a change in Cs_τ/W_τ would lead to a change in slope of t_c against $1/e$. Changing the calcium concentration at constant pH, for instance, can only alter this term (K_{enz} is not affected (3)). The experiments carried out by McMahon et al. (43) clearly showed this change in slope with addition of calcium at constant pH.

Acknowledgements

The authors gratefully acknowledge the invaluable help of Dr J. Scheutjens en Ir B. van Lent (University of Wageningen) in computing the hydrodynamic thickness of the hairy layer around the casein micelle as a function of the conversion.

Appendix

A kinetic model to describe the enzymic reaction in milk if adsorption of the enzyme onto the para-κ-casein micelles occurs

The basic assumption is that the deviation from a first-order reaction is caused solely by adsorption (temporary inactivation) of the enzyme onto the surface of the micelle at a site where the CMP has already been removed. Hence, adsorption would be proportional to the degree of conversion.

First-order equation for proteolysis:

$$-\mathrm{d}s/\mathrm{d}t = K_{enz}\, e_f\, s \qquad (A_1)$$

Adsorption equation for low e_f

$$e_a = C_a\,(s_0\text{-}s)\, e_f \qquad (A_2)$$

K_{enz} denotes the first-order reaction rate constant and e_f the concentration of free enzyme molecules and e_a that of those adsorbed ($e_f = e_0\text{-}e_a$). The constant relating adsorption to degree of conversion is C_a. Combination of the equations yields:

$$-\mathrm{d}s/\mathrm{d}t = \frac{K_{enz}\, e_0\, s}{1 + C_a\,(s_0\text{-}s)} \qquad (A_3)$$

Integrating with $s = s_0$ for $t = 0$ and $s = s_t$ for $t = t$ yields:

$$t = \frac{1 + C_a\, s_0}{K_{enz}\, e_0} \ln \frac{s_0}{s_t} - \frac{C_a}{K_{enz}\, e_0}\,(s_0\text{-}s_t) \qquad (A_4)$$

The substrate concentration cannot be made explicit in this expression. The results obtained at

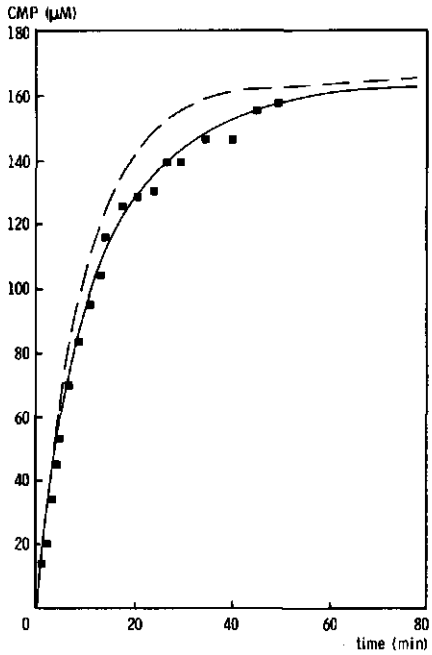

Fig. 10. The release of CMP at pH 5.6 and 30 °C. Experimental results (■) taken from previous paper (2). The dashed curve is without enzyme adsorption on the surface of para-casein micelles, the solid curve with adsorption according to Equation A_4. Initial substrate concentration is 160 μM; $e_0 = 2.15 \times 10^{-3}$ μM; K_{enz} = 0.744 (μM.s)$^{-1}$ and $C_a = 4.6 \times 10^{-3}$ μM^{-1}.

pH 5.6 were fitted into the latter equation. An estimate of K_{enz} was made on the basis of the initial velocity (adsorption then is still negligible) and the value of C_a was derived by trial and error. The resulting fit is shown in Fig. 10. It is predicted that about 42 % of the enzyme molecules is adsorbed at the end of the reaction. Direct adsorption measurements of Geurts and Walstra (30) of chymosin onto para-casein resulted in roughly 50 % adsorption in this pH region if extrapolated to a similar chymosin to para-casein ratio.

Samenvatting

A. C. M. van Hooydonk en P. Walstra, *Interpretatie van de kinetiek van de stremreactie in melk*[1]

De kinetiek van de omzetting van κ-caseïne in melk door het enzym chymosine wijkt in belangrijke mate af van de kinetiek in zuivere oplossingen van κ-caseïne. Deze afwijking werd toegeschreven aan het feit dat het substraat in melk geïmmobiliseerd is in de relatief grote caseïnemicellen. Berekeningen toonden aan dat de experimenteel waargenomen reactiesnelheid ongeveer gelijk is aan de door diffusie bepaalde snelheid. Interacties tussen het enzymmolecuul en het miceloppervlak worden behandeld. Voor het bijzondere geval dat chymosine tijdens de reac-

[1] Overdrukken van dit artikel verspreid als Verslag V271 van het NIZO te Ede (1987).

tie wordt geïnactiveerd door adsorptie aan het oppervlak van para-caseïnemicellen werd een kinetisch model ontwikkeld.

De snelheid waarmee de micellen tijdens de stremreactie aggregeren werd berekend uit de verandering van de viscositeit van de stremmende melk. De stabiliteitsfactor bleek exponentieel af te nemen met de omzettingsgraad van κ-caseïne. Afgeleid kon worden dat de aggregatiesnelheid van volledig omgezette micellen ongeveer 2000 maal zo laag is als voorspeld wordt op grond van diffusie. Het effect van verschillende variabelen op de stabiliteitsfactor wordt in het kort behandeld. Met een kinetisch model waarin de proteolyse en de aggregatie waren gecombineerd, kon de experimenteel genomen afhankelijkheid tussen vloktijd en enzymconcentratie worden voorspeld.

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Summary

The rennet-induced clotting of milk was studied under various conditions, in order to obtain a better understanding of the intricate mechanism of the renneting process. The kinetics of the enzymic and aggregation reactions was analysed separately and, where possible, related to the physico-chemical properties of the casein micelle and its environment. The study was intentionally restricted to the total system of skim milk. The experimental techniques used were chosen or developed to allow measurements in milk to be carried out without the necessity to dilute or simplify the system.

A new method of following the extent of the conversion of κ-casein during the reaction was developed (Paper 1). The method is based on a selective determination of the casein macropeptide, using gel permeation chromatography on a high-performance column. The EEC officially adopted this technique to detect rennet whey solids in skim milk powder for intervention (Official Journal of the European Communities, Regulation EEC No. 718/85).

The second paper is devoted to the kinetics of the enzymic reaction only. The Michaelis-Menten parameters obtained in solutions of isolated κ-casein failed to describe the kinetics of the reaction in milk. A first-order expression with respect to enzyme and substrate concentration adequately described the conversion of κ-casein in milk. The deviation from a Michaelis-Menten mechanism is likely to be related to the immobilisation of the substrate in the relatively large casein micelles. The collision frequency between enzyme and substrate therefore solely depends on the diffusion rate of the enzyme. This, together with the low temperature coefficient ($Q_{10} \approx 1.4$) and the observation that the reaction rate constant decreased with increasing casein micelle concentration, led to the suggestion of a diffusion-controlled reaction.

Papers 3 and 4 deal with important physico-chemical changes brought about by acidification of milk and with their implications for the enzymic and aggregation reactions. The peculiar pH-dependency of the voluminosity of casein micelles and of the dissociation of casein, was explained by the relative contribution of two stabilising factors, i.e. the interaction of casein with micellar calcium phosphate and the inter- and intra-molecular electrostatic interactions between the charged groups of casein molecules. The removal of the hydrophylic part of κ-casein by rennet not only reduces the voluminosity of the casein micelle, but also causes the (re)association of the non-sedimentable casein fraction at each pH. In contrast to normal casein micelles, a distinct maximum in voluminosity at pH 5.3 was absent for para-casein micelles.

The optimum pH for the action of chymosin coincided with the minimum in

the voluminosity curve, i.e. around pH 6.0. Both at low (<6.2) and high pH (>7.0), the reaction no longer followed first-order kinetics. The deviation at high pH is caused by the slow denaturation of chymosin. At low pH the deviation is presumably due to the adsorption of enzyme onto the surface of the para-casein micelle. For both cases a kinetic model was derived and tested (see also Paper 8).

The rate of aggregation increased with decreasing pH. At the normal pH of milk, aggregation started around 65 % conversion, whereas only 30 % release of the casein macropeptide initiated aggregation already at pH 5.6, indicating that the stabilising power of κ-casein decreases markedly with pH.

The effect of various cations on the renneting process is described in Paper 5. All added divalent cations interacted strongly with the micelle and decreased its voluminosity. The formation of insoluble complexes with inorganic phosphate presumably plays a key role, and it was argued that these complexes are positively charged. This would (partly) explain their tendency to interact strongly with casein. The remarkable reduction in the velocity of the enzymic reaction found after addition of zinc and copper ions could be attributed to their interference with the substrate rather than with the enzyme. Other divalent cations such as calcium, magnesium, barium and manganese had no influence on the enzymic reaction rate and accelerated the aggregation process. In promoting aggregation, the direct binding of these cations to negative sites of casein was shown to be less important than the interaction with the inorganic phosphate of the micelles.

The renneting properties of heated milk are described in Paper 6. The generally held view that the interaction between β-lactoglobulin and κ-casein renders the Phe-Met bond in κ-casein inaccessible to rennet could not be confirmed. The poor rennetability of heated milk appeared to be primarily related to the impaired aggregation properties of the rennet-converted micelles (partly) covered with denatured whey protein. The phenomenon of rennet hysteresis was explained on the basis of the different properties of heat-induced calcium phosphate 'precipitates' and original micellar calcium phosphate.

The practical implications of some of the results for the control of the renneting process during traditional cheesemaking are discussed in Paper 7. Some instruments specifically developed to monitor the curd-setting during cheesemaking were evaluated.

A more theoretical and mechanistic interpretation of the experimentally obtained results is given in Paper 8. The upper limit of the enzymic reaction was estimated by taking into account such factors as electrostatic and hydration repulsion, reduction of the diffusion rate in the 'hairy' outer layer, orientational constraints, serum viscosity and tortuosity imparted by the micelles. The resulting effective meeting frequency of enzyme and substrate was close

to the experimentally obtained reaction rate, and a diffusion-controlled reaction model would thus be a plausible explanation for the observed kinetics. Effects of ionic strength and pH were, however, difficult to reconcile with a diffusion model and only a vague indication about alterations in the accessibility of the substrate could be given to explain the effects.

The rate of aggregation was calculated from viscosity changes during renneting. The stability factor decayed exponentionally with the degree of conversion. The aggregation rate of fully converted micelles was estimated to be slower by a factor of 2000 than the diffusion-controlled limit at physiological pH. Steric repulsion is probably the main factor to account for the stability of para-casein micelles, especially at lower temperature. Electrostatic repulsion and retardation due to hydrodynamic hindrance must, however, also play a part.

A mathematical model combining the enzymic and aggregation reactions was developed. The empirically obtained relation between clotting time and enzyme concentration could be explained by means of this model.

Samenvatting

Het doel van het hier beschreven onderzoek was meer inzicht te verwerven in het ingewikkelde mechanisme van de stremming van melk met leb. De kinetiek van de omzetting van κ-caseïne en de aggregatie van para-caseïnemicellen werd daartoe afzonderlijk onderzocht onder verschillende reactieomstandigheden. Voor zover dat mogelijk was, werden de resultaten gerelateerd aan de fysisch-chemische eigenschappen van de caseïnemicel. Met opzet werd het onderzoek beperkt tot melk als zodanig. De toegepaste experimentele technieken werden daarom zodanig gekozen of ontwikkeld dat verdunning of vereenvoudiging van het systeem niet nodig was.

In artikel 1 wordt een nieuwe methode beschreven voor het analyseren van de hydrolyse van κ-caseïne tijdens het stremmen van melk. De methode is gebaseerd op een selectieve bepaling van het caseïnemacropeptide met behulp van gelpermeatiechromatografie onder hoge druk. De EEG heeft deze techniek officieel aangewezen als methode om de aanwezigheid van kaasweipoeder in ondermelkpoeder aan te tonen (Publikatieblad van de Europese Gemeenschap, verordening nr. 718/85).

In het tweede artikel wordt de kinetiek van de enzymatische reactie beschreven. De Michaelis-Menten-parameters die in zuivere oplossingen van geïsoleerde κ-caseïne werden gevonden, bleken ongeschikt te zijn om er de kinetiek van de proteolyse in melk mee te beschrijven. Een eerste-orde-reactie met betrekking tot enzym- en substraatconcentratie beschreef de omzetting van de κ-caseïne in melk het best, althans bij de normale pH van melk. De afwijking ten opzichte van het Michaelis-Menten-mechanisme is waarschijnlijk een gevolg van de substraatimmobilisatie in de relatief grote caseïnemicellen. De botsingsfrequentie tussen enzym en substraat wordt dan ook volledig bepaald door de diffusiesnelheid van het enzym. Dit, gevoegd bij de lage temperatuurcoëfficiënt van de reactie ($Q_{10} \approx 1{,}4$), en het feit dat de reactieconstante afneemt met toenemende concentratie van caseïnemicellen, gaf aanleiding tot de suggestie dat de reactiesnelheid bepaald wordt door diffusie.

De invloed van de pH op de fysisch-chemische eigenschappen van caseïnemicellen in melk en het effect daarvan op de stremreactie wordt behandeld in de artikelen 3 en 4. Het bijzondere verloop van de voluminositeit van de caseïnemicellen en de dissociatie van caseïne met de pH werd verklaard uit de relatieve bijdrage van twee stabiliserende factoren, te weten de interactie tussen caseïne en micellair calciumfosfaat en de inter- en intramolekulaire elektrostatische attractie en repulsie tussen geladen groepen van de caseïnemoleculen. Afsplitsing van het hydrofiele deel van κ-caseïne door stremsel

verminderde niet alleen de voluminositeit van caseïnemicellen, maar veroorzaakte ook een (re)associatie van de niet-sedimenteerbare caseïnefractie. Het kenmerkende maximum in de voluminositeit van normale caseïnemicellen (pH 5,3) was afwezig bij para-caseïnemicellen. De optimale pH voor de omzetting van κ-caseïne in melk was 6,0. Zowel bij lage pH (<6,2) als bij hoge pH (>7,0) kon de reactie niet meer beschreven worden met eerste-orde-kinetiek. De afwijking wordt bij hoge pH veroorzaakt door langzame denaturatie van chymosine, en bij lage pH zeer waarschijnlijk door adsorptie van het enzym aan para-caseïnemicellen. Voor beide gevallen werd een kinetisch model afgeleid en experimenteel getoetst (zie ook artikel 8).

De aggregatiesnelheid was groter bij lagere pH. De kritieke omzettingsgraad van κ-caseïne nodig om het vlokproces in te zetten, was bij de normale pH van melk ongeveer 65 %, terwijl niet meer dan 30 % hydrolyse nodig was bij pH 5,6.

Het effect van verschillende kationen op het stremproces wordt beschreven in artikel 5. Tweewaardige kationen associeerden sterk met de caseïnemicellen en verlaagden de voluminositeit ervan. De complexvorming van het kation met anorganisch fosfaat speelt hierbij een sleutelrol. Deze complexen zijn vermoedelijk positief geladen, hetgeen hun interactie met de caseïnes in de micel mede zou verklaren. De opmerkelijke verlaging van de hydrolysesnelheid die gevonden werd na toevoeging van zink- en koperionen, kon worden toegeschreven aan een interactie met het substraat en niet met het enzym. Andere tweewaardige kationen zoals calcium, barium, magnesium en mangaan hadden geen invloed op de enzymatische reactiesnelheid en versnelden de aggregatie van de caseïnemicellen. Deze toename van de aggregatiesnelheid bleek niet zo zeer veroorzaakt te worden door de directe interactie tussen het kation en de negatieve groepen van de caseïnes, maar meer door de interactie met micellair fosfaat.

Het stremgedrag van verhitte melk wordt beschreven in artikel 6. De algemeen aangenomen opvatting, dat de complexvorming tussen β-lactoglobuline en κ-caseïne de Phe-Met-binding in κ-caseïne ontoegankelijk maakt voor chymosine, kon niet worden bevestigd. De slechte stremming van verhitte melk moet daarom toegeschreven worden aan de verhoogde stabiliteit van de para-caseïnemicellen wanneer deze (gedeeltelijk) bedekt zijn met gedenatureerd wei-eiwit. Het verschijnsel van 'rennet hysteresis' werd verklaard op basis van het verschil in eigenschappen tussen calciumfosfaatcomplexen die gevormd worden bij verhitting en het oorspronkelijke micellair calciumfosfaat.

De praktische implikaties voor de beheersing van het stremproces tijdens het traditionele kaasbereidingsproces worden besproken in artikel 7. Enkele instrumenten die speciaal zijn ontwikkeld om de toename van de gelstevigheid te volgen tijdens de wrongelbereiding werden geëvalueerd.

Een meer theoretische en mechanistische beschouwing van de experimentele waarnemingen wordt in artikel 8 gegeven. De door de diffusie van het enzym bepaalde reaktiesnelheid werd berekend. Hierbij werd rekening gehouden met elektrostatische repulsie, hydratatie, remming van de diffusiesnelheid in de 'harige' buitenlaag van de micel, oriëntatie-effecten, viscositeit van het serum en omwegfactoren. De geschatte ontmoetingsfrequentie tussen enzym en substraat lag dicht bij de experimenteel waargenomen reactiesnelheid, hetgeen suggereert dat de hydrolysesnelheid inderdaad door diffusie bepaald zou kunnen zijn. Effecten van variabelen, zoals ionsterkte en pH, zijn echter moeilijk hiermee te verklaren en slechts vage suggesties over veranderingen van de toegankelijkheid van het substraat kunnen worden gegeven.

De snelheid van vlokking werd berekend uit de verandering van de viscositeit tijdens de stremreactie. De stabiliteitsfactor bleek exponentieel af te nemen met de hydrolysegraad. Para-caseïnemicellen aggregeren bij fysiologische pH ongeveer 2000 maal zo langzaam als op grond van diffusie wordt voorspeld. Deze vertraging wordt waarschijnlijk in belangrijke mate bepaald door sterische afstoting. Elektrostatische afstoting en vertraging door hydrodynamische hindering zullen echter ook een bijdrage leveren.

Met eèn kinetisch model, waarin de hydrolyse en vlokking werden gecombineerd, bleek de experimenteel gevonden afhankelijkheid tussen enzymconcentratie en vloktijd te kunnen worden verklaard.

Curriculum vitae

De auteur werd geboren op 26 januari 1948 te Ulvenhout. Hij behaalde in juni 1969 het diploma van de Hogere Landbouwtechnologische School te 's-Hertogenbosch. Na het vervullen van de militaire dienstplicht van juli 1969 tot januari 1971, begon hij aansluitend hierop met de studie aan de Landbouwhogeschool te Wageningen. In januari 1975 werd het kandidaatsexamen Levensmiddelentechnologie afgelegd en in september 1976 het doctoraalexamen met als verzwaard hoofdvak proceskunde en als bijvakken kolloïdchemie en zuiveltechnologie en melkkunde. Van oktober 1976 tot januari 1977 was de auteur als docent Levensmiddelentechnologie verbonden aan de Rijks Hogere Tuinbouwschool te Utrecht en vanaf maart 1977 tot augustus 1979 was hij als wetenschappelijk medewerker werkzaam bij Unilever Research Laboratories te Sharnbrook, Engeland. Van augustus 1979 tot september 1986 was de auteur als wetenschappelijk medewerker verbonden aan het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede, waar het in dit proefschrift beschreven onderzoek werd verricht. Sinds september 1986 is hij werkzaam bij DMV Campina BV te Veghel.