5-1983

Enzymic Milk Coagulation: Casein Micelle Aggregation and Curd Formation

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ENZYMIC MILK COAGULATION: CASEIN MICELLE AGGREGATION AND CURD FORMATION

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1983

ACKNOWLEDGEMENTS

Thanks are extended to Dr. Tony Ernstrom for giving me the opportunity to study at Utah State University; Dr. Rod Brown for guidance and encouragement during my studies; to my fellow students for their friendship; to Drs. Gary Richardson, Art Mahoney and Betty Boeker for being members of my advisory committee; and especially to my wife Penelope for her understanding and support while I completed this endeavor.

This work is dedicated to Buckwheat, who couldn't come.

Donald J. McMahon

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ABSTRACT

Enzymic Milk Coagulation: Casein Micelle Aggregation and Curd Formation

by

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Utah State University, 1983

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Enzymic milk coagulation was monitored by measuring changes in curd firmness and apparent absorbance of undiluted milk. Detection of coagulation, visually or rheologically, occurred after the milk changes from a system of aggregating particles to an extended space network. This change was observed as a shoulder in apparent absorbance plots and coagulation time was defined as the critical point in the aggregation process analogously to non-linear condensation polymerization reactions. It corresponds to the inflexion point during the period when apparent absorbance was rapidly increasing and can be calculated by fitting curd firmness data to an exponential equation.

Addition of calcium chloride to milk reduced coagulation time with a minimum occurring at .05M calcium. Also, curd firmness increased with a maximum at .02M calcium. It appears that calcium affects all stages of coagulation: proteolysis, micelle aggregation, and gelation. When bulk culture media was added to milk, the pH of the media had a greater effect on coagulation time than did presence of phosphate in the media.

Non-specific proteolytic activity of milk coagulants affects the initial rate of curd firming but not the maximum firmness. The more proteolytic the enzyme the slower the curd firming rate. This can be used to rapidly assay for pepsin content of bovine rennets.

(216 pages)

GENERAL INTRODUCTION

Casein micelles are stabilized in milk solutions by a combination of hydrophobic and electrostatic forces, and surface protein interactions. Removal of the macropeptide portion of κ-casein by enzymic proteolysis renders casein micelles unstable in the presence of calcium ions and induces milk coagulation. Such coagulation is a complex process and is affected by numerous factors including: milk composition, temperature, pH, calcium concentration, enzyme activity, and enzyme proteolytic specificity. Understanding the effect of changing any of these variables is essential for consistency in cheese manufacture and to optimize cheese yields.

Enzymic coagulation is generally thought to involve two processes: the limited proteolysis of κ-casein and the aggregation of para-casein micelles. Many techniques have been devised to measure milk coagulation time and curd firmness. Unfortunately, such measurements enjoy only empirical relationships with the coagulating milk system. An exact definition of coagulation time in terms of a specific point in the aggregation process would aid in the complete mathematical modelling of milk coagulation.

The aims of this study were:

1. To develop and characterize some new methods of studying milk coagulation.
2. To determine the effects of varying enzyme activity, enzyme type, calcium content, and temperature on milk coagulation.
3. To define enzymic coagulation time using mathematical modelling and observations of changes occurring in curd firmness and molecular weight during the coagulation process.

PART 1A. COMPOSITION, STRUCTURE, AND INTEGRITY OF CASEIN MICELLES: A REVIEW

PHYSICAL PROPERTIES OF CASEIN MICELLES

The biological function of bovine casein micelles is to provide efficient nutrition to the young calf. It does not inherently require a high degree of ordered structure but rather an effective mechanism for the secretion of a highly concentrated solution of protein, calcium and phosphate. During the past twenty years extensive research has been conducted to determine the composition and structure of casein micelles, and to identify the forces that maintain its integrity. The approximate composition of bovine casein micelles is given in Table 1. In cows' milk, casein micelles occur as a colloidal

Table 1: Approximate composition of bovine casein micelles (77, 79).

Milk Component	%
α_{s1}-casein	35.6
α_{s2}-casein	9.9
β-casein	33.6
κ-casein	11.9
Minor caseins	2.3
Calcium	2.9
Phosphate	2.9
Magnesium	0.1
Sodium	0.1
Potassium	0.3
Citrate	0.4
Sialic acid	0.3
Galactose	0.2
Galactosamide	0.2

dispersion. Structure and properties of the casein group of proteins, which comprise over 90% of casein micelles, have been extensively reviewed (11, 13, 76, 87, 88, 95). Casein micelles are highly hydrated and spongelike colloidal particles containing about 3.7g H_2O/g protein

(6,47,48). Relatively little of this water (.5g H_2O/g protein) is bound to the protein. The remainder is occluded within the micelle and moves with the micelle during hydrodynamic experiments. Numerous models describing casein micelles have been proposed and reviewed (7, 21, 22, 28, 76, 83, 87, 89, 93). A casein micelle consisting of numerous subunits is the most widely accepted model. Some calculated parameters of casein micelles are summarized in Table 2.

Table 2: Average parameters of casein micelles (6, 43, 60, 81).

Parameter	Value
Diameter	130-160 nm
Surface	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g/cm^3
Mass	2.2×10^{-15} g
Voluminosity	4.4 cm^3/g
Hydration	3.7 g H_2O/g protein
Water content	63%
Molecular weight (hydrated)	1.3×10^9 Daltons
Molecular weight (dehydrated)	5×10^8 Daltons
Number of peptide chains (MW: 30000)	10^4
Number particles/ml milk	$10^{14} - 10^{16}$
Whole surface of particle	5×10^4 cm^2/ml milk
Distance of closest approach	10 nm

Casein micelles exhibit a broad statistical distribution of sizes governed by physical chemical principles (5). Researchers using inelastic light scattering have observed that 80% of micelles by weight have a diameter in the range 100-200nm, with 95% between 80 and 440nm (51), and a most probable diameter of 160nm. Laser light scattering has showed a more broad molecular weight (MW) distribution than did either electron microscopy measurements or quasi-elastic light scattering (39),

with a long tail extending up to 680nm diameter (53). Particles less than 20nm diameter account for nearly 80% by number of all casein particles, but their volume comprises less than 3% of the total micellar volume (81). If particles less than 20nm diameter are excluded, then significant differences in estimated parameters are obtained (Table 3).

Table 3. Size distribution parameters of casein particles (81).

Parameter Estimated	Distribution Exclusive of Particles < 20 nm Diameter	Total Distribution
Concentration of micelles	1.2×10^{14}/mL milk	6×10^{14}/mL milk
Number mean diameter	65 nm	25 nm
Volume mean diameter	104 nm	86 nm
Volume median diameter	134 nm	129 nm

THERMODYNAMIC FORCES IN CASEIN MICELLES

Hydrophobic Interactions

Caseins are among the most hydrophobic of all proteins, and it is not unexpected that casein micelle stabilization should involve hydrophobic interactions (23). All casein associations are promoted by an increase in temperature, indicating involvement of hydrophobic interactions (66). Proline reduces hydrophobicity when it is bound to non-polar regions of protein, and it causes partial dissociation of casein micelles (57). Urea is more effective than oxalate in dissociating micelles, again suggesting the importance of hydrophobic interactions in stabilizing micelles (58).

Thermodynamic properties of a solution are the composite result of all components and are not solely due to the nature of the solute (5). Changes in water structure surrounding non-polar solutes play an important role in the formation of hydrophobic interactions and in determining solution free energy (26, 62). When a non-polar solute dissolves in water it modifies water structure towards greater "crystallinity" -- i.e. the water builds an "iceberg" around it. Hydrogen bonding of water in the immediate neighborhood of the solute is thus increased over its average value in pure water.

Interaction of non-polar groups with water is unfavorable, and there is a thermodynamic tendency for non-polar groups to contact each other rather than to remain surrounded by water molecules. A hydrophobic interaction occurs when two or more non-polar groups come into contact (62). A property unique to hydrophobic interactions is their dependency on solvent medium for existence.

In an aqueous solution, proteins occur in a solvent whose intermolecular arrangements are strongly influenced by interactions with protein residues. Folding of a protein molecule and stability of its various conformations are the combined results of all its interactions. The position of equilibrium of intermolecular forces is governed by Gibbs free energy change and, at room temperature, unfavorable enthalpy of formation is more than counterbalanced by positive entropy. Entropy due to changes in water structure is the most important factor stabilizing hydrophobic interactions. For each nm^2 of protein surface removed from contact with water, approximately 10kJ of free energy is obtained (5). The endothermic nature of hydrophobic interactions causes them to be stronger with increasing temperature (62).

If noncovalent associations modify conformational freedom of flexible regions of a protein chain, an appreciable contribution to entropy results (5). For loosely packed proteins such as caseins, this is a distinct possibility. Few side chains may be brought into contact simultaneously but they are important because hydrogen bonds do not exist in significant numbers.

Hydrophobic interactions, taken individually, are weaker than most other side chain interactions in proteins. They become important because of their high frequency of occurence in proteins with non-polar side chains (62). They exhibit less specificity than other interactions both with respect to steric requirements of side chain orientation, and number and kind of side chains that can participate in their formation. Neither bond angle nor length is uniquely fixed, and relative orientation of non-polar groups in contact is unimportant, also, the degree to which groups overlap varies (62). Hydrophobic interactions

would lead to incorrect associations in the absence of complimentary pairing surfaces (5). When two hydrophobic protein groups establish contact the total number of water molecules in contact, with them is decreased. Removal from aqueous environment is not complete since they still retain some water neighbors (62). Hydrophobic interactions of various strengths are possible, and several side chains belonging to different backbone portions may associate to form a hydrophobic region from which water is completely excluded (62).

Distribution of protein side chains between interior and surface affects character and behavior of the macromolecule (44). Ionic side chains are generally fully exposed to water. A combination of high hydrophobicity and casein molecular weight prevents the formation of a globular structure in which nonpolar groups are completely buried in the protein interior (79). Some casein side chains are still exposed to water and non-polar areas on the protein surface are available for interactions with other protein molecules (23, 62). Such residues find it favorable, in terms of free energy, to cluster with similar residues from other molecules. This endows caseins with pronounced tendencies to associate and form complexes, even in the absence of Ca^{2+} ions (6).

Electrostatic Interactions

Ionic interactions contribute little to stability of a monomeric protein except when an ion pair can be formed in a hydrophobic enviornment (23). They are critical for close packing and proper formation of specific aggregates. When specific ion pairs such as phosphate-calcium-phosphate bridges in casein are formed, they stablize protein quaternary structure (5). Such interactions play a

greater role in caseins than in most other proteins and the high content of acidic groups increases calcium binding capacity and enhances crosslinking.

Native casein micelles can approach each other unhindered down to atomic distances and experience a considerable energy barrier in their approach at interparticle distances of 0.1nm (67). Short range interactions such as hydrophobic interactions, calcium bridging, and electrostatic interactions between positively charged para-K-casein and negatively charged regions on other micelles, then become predomonant.

The magnitude of London-van der Waals forces, which depends on differences in polarizability of colloidal particles and surrounding medium, is relatively low for casein micelles due to their porosity. A low level of electrical repulsion between micelles is further suggested by electrokinetic measurements. However, the fact that casein particles can be flocculated at their isoelectric point demonstrates charge is important for their stabilization (66).

The region surrounding a particle surface can be divided into two major parts. The first consists of ions adsorbed at the surface which form an inner, compact double layer and the second is a diffuse Gouy layer (1). The inner layer can be subdivided into a layer of desolvated, chemically absorbed and potential determining ions, and a Stern layer containing counterions that are partially electrostatically adsorbed and partially solvated. Thickness of the electrical double layer on micelle surfaces in milk is approximately 1nm (66). Ions and surrounding medium in the Stern layer are rigidly held and immobile in the sense of resisting shear, while the outer layer is populated by a diffuse distribution of anions and cations. Calcium, magnesium,

phosphate, and citrate adsorbed on casein micelles can be removed by gel filtration (8) and shows three distinct zones: adsorbed ions, bound minerals, and casein counterions. All of the citrate, 22% of the Ca, 50% of the Mg and 47% of the phosphate of whole micelles can be removed by gel filtration.

The potential at the shear region in the diffuse Gouy layer is known as the electrokinetic (or ζ-) potential. The ζ-potentials of casein micelles immersed in a medium of the same ionic strength as milk depend only upon the surface charge density of the micelles and not on micelle size. Fixed charges within the micelle are neutralized by ions present in the serum and do not contribute to electrokinetic potential. All micelles have similar electrokinetic mobilities and therefore have similar surface charge densities and surface chemical composition regardless of size (69).

Calculated ζ-potentials of casein micelles vary with both temperature and pH as shown in Table 4 but heat treatment itself has no significant effect. Milk can be heated to within a few minutes of

Table 4. Variation of electrokinetic potential of casein micelles with temperature and pH (16).

pH	Potential (mV) 20°C	30°C	45°C
5.7	-8	-10	-14
6.8	-13	-17	-22

coagulation without exhibiting any consistent change in ζ-potential (16). Calculations of casein micelle ζ-potentials are complicated

because the micelle surface is irregular very diffuse, and its double layer has an unknown structure. Ionic composition of the serum phase also affects electrophoretic mobility (17). Casein micelles have lower mobilities electrophoresed in milk ultrafiltrate than in milk centrifugate (-10.2mV compared with -15.5mV, 20°C, pH6.85).

MICELLE STABILITY

Effect of Micelle Charge

Hydrophobic colloids are generally stabilized by surface charge, and the repulsion between double layers prevents their aggregation (1). Stability of casein micelles, however, is attributed only in part to surface charge because hydrophobic colloids with ζ-potentials less than 20 - 30mV, such as native casein micelles, are generally unstable (17). The exact contribution of surface charge to casein micelle stability has not been determined. It is an oversimplification to view casein micelle coagulation as just a reversal in the balance of repulsive and attractive electrostatic forces. Other more specific interactions are involved.

Cationic materials are rapidly and extensively absorbed by micelles, but subsequent decrease in micellar charge is insufficient to allow coagulation (33). Lysozyme is unable to induce clotting of casein micelles even though it carries the same number of positive charges (approx. 3×10^{17} mg^{-1}) as negative charges on casein micelles (32). Also, micelle stability decreases with increases in temperature, yet surface charge increases (Table 4), demonstrating the involvement of hydrophobic interactions in micelle aggregation (16). Cationic materials reduce rennet clotting time by increasing both affinity of rennet for kappa-casein and aggregation rate.

Specific interactions between positively charged para-κ-casein on one micelle and negatively charged groups of α_{s1}- and β-casein on another micelle may also be involved in aggregation. Calcium binding to casein is a function of Ca^{2+} activity and on cooling, Ca^{2+} activity

increases due to dissociation of colloidal calcium phosphate (CCP) from micelles (73). Therefore, as temperature decreases, increase in casein-bound calcium increases and causes a decrease in net negative charge of the micelle. Micelle charge is also reduced as pH is lowered toward the isoelectric point of the micelle. Casein micelles do not coagulate at pH 4.6, however, below 5°C even though net charge is negligible (17). This suggests electrostatic forces contribute to, but do not determine, overall micelle stability.

Surface Protein Interactions

Casein micelle stabilization also involves steric interactions of surface proteins (18, 35, 61). When macromolecule layers interpenetrate, the increased polymer segment concentration in the interaction zone between the two particles leads to a local osmotic pressure effect. Macromolecules in the interaction zone may also compress without interpenetration, which may be more applicable to casein micelles because of the highly hydrated and concentrated protein surface. When compression occurs the protein molecules in the interaction region lose configurational entropy on collision and provide repulsive energy to prevent aggregation.

MICELLE STRUCTURE

A casein micelle subunit model was proposed as a result of observing internal structure of casein micelles using electron microscopy (EM) (82). An approximately spherical α,β,κ-casein complex of molecular weight 3×10^5 Daltons, and 10nm diameter was described as the building unit (submicelle). Such a model agrees with disintegration studies of micelles in which three stages were observed (58):

1. Dissolution of intercomplex CCP linkages producing calcium-casein complexes less than 30nm diam.
2. Removal of intracomplex calcium forming soluble casein complex units.
3. Disaggregation to monomers occuring by disruption of hydrogen bonds, hydrophobic interactions and salt linkages.

Early models implied that the submicelles contain different proportions of κ-casein, but that their composition did not change rapidly with time (86). Those containing a high proportion of κ-casein combine together to constitute a small micelle, while large numbers of subunits, each containing less κ-casein, constitute larger micelles. From size exclusion experiments it has been observed that added κ-casein elutes with micelles, suggesting casein components can randomly rearrange and that they exist in a dynamic system (12). The size, composition and molecular weight of submicelles is determined by casein concentration, pH and temperature. When κ-casein is added, a new equilibrium position is reached in which added κ-casein is interchangeable with κ-casein present in the complex.

Dimensions of Casein Submicelles

Casein submicelles are not of fixed dimensions but vary in size in an equilibrium system, with a continual interchange operating between serum casein monomers and submicelles (12). Restrictions on interchange by calcium-phosphate bridges between submicelles tend to lock casein molecules into position once a micelle is formed.

Ion beam sputtering of casein micelles enables details of the underlying structure of submicelle spheres to be observed (38). At high energy, submicelles are sputtered off, presumably by disintegration of calcium-phosphate bonds. Etching of submicelles reveals an infrastructure of curved rod-like components about 2nm diameter and 7nm in length, which corresponds to casein complex units produced by treatment with oxalate (58).

Nature of Casein Submicelles

Not only κ-casein but also α_{s1}-, α_{s2}- and β-caseins contain highly charged regions expected to be located on the submicelle surface (76). It has been postulated that submicelles have the non-polar portion of each protein chain oriented radially inward while the charged acidic residues of the calcium-sensitive caseins, and the hydrophilic carbohydrate portion of κ-casein are located on the submicelle surface. This results in a hydrophobic core surrounded by a polar layer. The κ-casein is located primarily in one area of the submicelle (54) and, therefore, there are phosphate-rich and phosphate-depleted areas on the surface as well as hydrophilic and hydrophobic areas (64). Segregation of highly charged regions from hydrophobic regions produces an

amphipolar structure enabling formation of small aggregates.

Using size exclusion chromatography to separate submicelles from micellar casein, it has been observed that submicelles consist of complexes of α_{s1}-casein with β-casein or κ-casein, and polymers of α_{s1}- and β-casein (63). Submicelles of α_{s1}- and β-casein, which are sensitive to Ca^{2+} ions would form the core region of the micelle. Some submicelles contain only α_{s1}-casein and presumably represent a type of submicelle situated in the micellar core region (63). Submicelles containing α_{s1}- and κ-casein complexes would preferentially be located on the micelle surface.

Submicellar aggregation

A major difference between surface areas composed of α_{s1}- or β-caseins, and those of κ-casein is that the phosphate groups are potential calcium binding sites (97). Ionic bonds between adjacent submicelles may be produced by calcium binding and the resultant decrease in net negative charge would enhance hydrophobic interactions.

A possible first step in micelle formation involves tetrahedral arrangement of submicelles (83). In a system containing less than 15% κ-casein, further association would then occur. This would produce a minimum micelle of 14 submicelles (MW ca. 3.5×10^6) if enough κ-casein is on the surface to hinder further growth.

Experimentally observed micelle size distribution has been mathematically produced (84). Submicelles with maximum concentration when κ-casein content is 15%, contain 4 κ-casein molecules and 26 α_s- or β-caseins. Such submicelles are most likely to form the micelle interior.

An alternate scheme for building casein micelles from subunits is to assign different functionalities to subunits of different compositions (14). Micelle size and size distribution are then governed by interactions of functionalities rather than geometrically.

Micelle Size Regulation

Micelle growth is limited by concentration at the micelle surface of submicelles rich in κ-casein. As submicelles that are poor in κ-casein aggregate, average composition of remaining submicelles changes towards a larger fraction of κ-casein and approach the fraction of κ-casein in milk serum, i.e. 0.29 (75, 84). Submicelles with low κ-casein contents are buried in the interior of the micelle. Potential hydrophobic interactions inwards and sideways at the interacting surfaces tend to align entering submicelles so their hydrophilic areas extend radially outward. As the radius of curvature of the micelle gets larger, steric hindrance causes less of the surface of a given micelle to be available for further interaction (86). A surface rich in κ-casein is reached at a smaller radius as submicelle κ-casein content is increased. Submicelles with a low percentage of hydrophobic surface area (50%) are unable to aggregate together and are found on the micelle surface (86). Addition and incorporation of κ-casein into submicelles would decrease surface hydrophobic interactions and favor smaller micelles. In contrast, addition of α_{s1}-casein would create more hydrophobic areas resulting in increased average micelle size (86).

Accumulated strain has also been proposed as a means of micelle size regulation. Each submicelle experiences distortion in order to maximize its free energy of bonding to the growing micelle (5).

Submicelles added late in the aggregation process are distorted more than those added early, until finally the ascumulated-strain free energy is greater than bonding energy and aggregation ceases.

Micelle Porosity

The porous nature of casein micelles is consistent with high water content and shrinkage upon vacuum drying, partial dissociation of casein components without alteration of hydrodynamic radii, and complete access of the C-terminal of all casein molecules to carboxypeptidase A (CPA) (28). CPA has molecular dimensions of 5.4 x 4.4 x 4.0nm and can penetrate channels or cavities in the micelle. Its diffusion time through a micelle of 100nm diameter is reported as 10^{-4} to 10^{-5} s (74). Some restrictions on availability of casein molecules to proteolysis exist because proteolysis by chymosin is dependent on the physical state of the casein.

Component proteins of a casein system become progressively less susceptible to proteolysis by chymosin as degree of aggregation increases (54). Considerable interaction occurs between α_{s1}- and β-caseins in a "soluble" casein system. Susceptibility of α_{s1}- and β-casein in such a system is reduced 10 - 15 times compared to isolated solutions. In colloidal phosphate free (CPF) milk, susceptibility of β-casein to proteolysis is reduced further. In going from CPF milk to native skim milk a 50 fold increase in particle size occurs, but β-casein susceptibility to proteolysis is only slightly decreased while α_{s1}-casein shows a significant decrease in susceptibility.

There are three rennin-susceptible bonds in β-casein (all near the C-terminal), and α_{s1}-casein has six of its seven such bonds in a similar

area. Simple aggregation is unlikely to account for this reduced susceptibility, because if the C-terminal is free for reaction with CPA then the bonds susceptible to chymosin would be expected to be free as well. Removal of CCP renders micellar α_{s1}-casein more accessible to chymosin, suggesting submicelles are linked by calcium-phosphate bridges predominantly between α_{s1}-casein molecules, which would restrict micelle porosity.

Micelle Surface Structure

The initial effect of renneting casein micelle solutions is a decrease in viscosity and volumnosity (92). If this were a result of molecular weight reduction only, these changes would be less than observed. They therefore indicate that the micelle surface is nonspherical. Submicellar structure of casein micelle results in a surface that is neither smooth nor spherical (42, 78) but has an unevenness equivalent to submicelle radius (i.e. 5-10nm). Molecular chains (or "hairs") protruding from the micelle surface would help to explain the high voluminosity of casein micelles (92). Reduction in viscosity could be explained by their removal as part of the macropeptide portion of κ-casein. In heat-treated milk most micelles observed using EM exhibit small appendages and it has been proposed that heat treatment causes deposition of calcium and phosphate onto these "hairs" of κ-casein/β-lactoglobulin complex (27, 42).

Micelle Structure and κ-Casein

The dichotomous function of κ-casein, to interact hydrophobically with α_{s1}-casein and at the same time provide a hydrophilic surface on

the micelle, is due to its primary structure (37). The N-terminal two-thirds of κ-casein is hydrophobic and the C-terminal third (the macropeptide) is hydrophilic. A physical model of κ-casein consists of a fairly rigid globular structure, comprising two-thirds of the molecule, to which a flexible, highly solvated tail is attached (87). The hydrophobic para-κ-casein portion of the molecule could serve to anchor the outwardly directed hydrophilic macropeptide portion to the casein micelle and endows it with a negatively charged, solvated surface envelope (37).

Considerable heterogeniety exists for κ-casein as a consequence of genetic variability of the polypeptide chain, and varying amounts of carbohydrate attached to the macropeptide (2). Variations in carbohydrate content do not affect the ability of κ-casein to stabilize α_{s1}-casein (19) because all κ-casein fractions separated by ion-exchange chromatography on the basis of carbohydrate content are able to stabilize α_{s1}-casein against precipitation by calcium (Table 5).

Table 5. Variations in carbohydrate and phosphate content of κ-casein (19). Each fraction contains one phosphoserine residue per mole.

Fraction	moles/mole protein		
	Sialic Acid	Hexose	Hexosamine
P2	0	0	0
P3	0	1	1
P4	1	2	1
P5	2	3	2
P6	3	4	3

S-carboxymethyl-κ-casein possesses micelle stabilizing ability and rennin sensitivity yet does not contain any carbohydrate (56). Casein micelles that have had 99% of the total sialic acid removed by neuraminidase also remain stable (58,90). As long as the macropeptide portion is attached κ-casein retains its stabilizing ability. This does not imply that the carbohydrate is devoid of function, because there is no indication how stable such micelles are to heat or pH (37).

The fact that rennetted micelles carry a net negative charge while para-κ-casein is positively charged shows that the exterior micelle surface is not composed solely of κ-casein. In whole casein the ratio of (α_{s1}-casein plus β-casein) to κ-casein has been calculated as 5:1 (36). By covalently immobilizing native micelles onto glass beads and then releasing them after dissociation of the micelle by urea, it has been observed that the ratio on the micelle surface is approximately 1:1 (36). A surface position is thus favored for κ-casein in the micelle and, in small micelles, the proportion of κ-casein on the surface is even higher.

Seventy-five percent of micellar sialic acid is on the micelle surface and accessible to immobilized neuraminidase (58). Investigations using stains specific for glycosylated κ-casein indicate the glycosylated κ-casein is located mainly in the outer layers of large micelles while for small micelles it is evenly distributed throughout (49). This implies that aggregation or interaction of κ-casein with other casein components may prevent addition of carbohydrate to all molecules (56).

Degree of aggregation does not affect access of enzyme to the chymosin sensitive bond in κ-casein. Skim milk and CPF milk

(dissociated micelles) show equivalent proteolysis by chymosin (9). Investigations using pepsin-dextran complexes (average Stokes radii 5, 8.5 and 11.5 nm) show κ-casein is more accessible for proteolysis in casein micelles than when micelles are dissociated or solubilized (10). It is perhaps in a more readily accessible conformation and relatively little α_{s1}- or β-casein is available for binding to the enzyme. In comparison, α_{s1}- and β-caseins are almost inaccessible to proteolysis in native micelles but are rendered accessible upon removal of CCP (24).

Micelle Structure and β-Casein

Relatively high susceptibility of some of the micellar β-casein is due to its dissociability from the micelle. Average hydrophobicity of β-casein is greatest of all the caseins so it shows the largest effect of temperature on solubility (3). Part of the β-casein is only loosely associated with the micelle (by hydrophobic interactions) and reversibly dissociates from it at low temperatures (7, 70). The remainder (50%) is relatively firmly "fixed" within the micelle and not free to dissociate.

An intermediate role in binding of β-casein to the micelle is played by CCP (68). The amount of colloidal calcium removed from the micelle by cooling, affects release of β-casein (70). Solubilization of CCP may result in calcium-phosphate bridges between β-casein and the micelle being broken. This increases the amount of β-casein bound solely by hydrophobic interactions and shifts equilibrium towards dissociation. When mineral migration is prevented, the β-casein released during cooling is that bound to the micelle by hydrophobic interactions only (70).

CASEIN ASSOCIATIONS

Caseins are strongly interacting proteins and do not exist as monomers at physiological conditions (88). Different mechanisms of association between casein molecules occur. For SH-κ-casein either electrostatic or hydrophobic interaction can predominate under various conditions (86). When α_{s1}- and β-casein are present, intermolecular hydrophobic interactions predominate.

Oxidation of SH-κ-casein in native micelles leads to covalently bound polymers of κ-casein (86) suggesting κ-caseins are adjacent to each other in micelles. Disulfide bonds may contribute to, but are not essential for, κ-casein self-association (91) or α_{s1}-casein stabilization (96). Electrostatic attraction between κ-casein molecules results in discrete hydrophillic surface areas on submicelles. The remainder of the surface consists mainly of α_{s1}- and β-caseins which would be available for hydrophobic bonding.

Complex formation between α_{s1}- and κ-casein is important in micelle formation. Growth of micelles from α_{s1},κ-casein complexes in the presence of Ca^{2+} depends on interaction between Ca^{2+} ions and phosphoserine groups of α_{s1}-casein (71). Hydrodynamic radii for casein polymers and their hydrophobic surface area have been calculated (Table 6). Upon complex formation the exposed hydrophobic surfaces of α_{s1}-casein polymers are buried in the complex interior (20). This shows that α_{s1}, κ-casein complexes are formed as a result of both hydrophobic and electrostatic interactions.

Table 6. Molecular weight and size of casein polymers (20).

Complex	Molecular Weight	Spherical Radii nm	Nonspherical Radii nm	Hydrophobic Surface Area nm^2
α_{s1}-polymers	10×10^4	3.0	---	198
κ-polymers	78×10^4	6.1	6.6	357
α_{s1}:κ-complex	45×10^4	5.1	5.6	299

Stabilization of α_{s1}- and β-Caseins

Gelatin is only five percent as effective as κ-casein in stabilizing α_{s1}- and β-casein and more gelatin than casein is required indicating stabilization occurs by a different mechanism compared to κ-casein. Stabilization by κ-casein is a cooperative phenomenon and the protection of a proportion of α_{s1}- or β-casein facilitates protection of the rest (31). It involves specific interactions between κ-casein and α_{s1}- or β-casein, primarily at hydrophobic regions and probably involves definite sites in each molecule (30).

Complex formation between κ-casein and α_{s1}- or β-casein does not occur in the absence of Ca^{2+} under the same conditions as in the presence of Ca^{2+} (30). Interactions can occur directly by Ca-links, and Ca^{2+} binding can neutralize casein negative charges enabling non-ionic interactions to occur. Such ionic interactions are important in maintaining maximum stabilization but are not essential for stabilization to occur (30). In the absence of Ca^{2+}, hydrophobic interactions are perhaps not strong enough to prevent solvation of the casein molecules, but when added, hydrophobicity is enhanced and the

caseins associate. Interaction of nonpolar side groups and strong hydrogen bonds are major factors influencing complex formation, but growth of micelles from α_{s1},^{K}-casein complexes in the presence of Ca^{2+}, depends on interaction between Ca^{2+} and esterified phosphate residues between α_{s1}-casein molecules (71).

Calcium Induced Association of α_{s1}-Casein

Self association of α_{s1}-casein is mainly electrostatic in nature (15, 54). Binding of Ca^{2+} to casein is important in micelle synthesis because it renders α_{s1}- and β-caseins liable to precipitation (15). A critical level of Ca^{2+} ion binding exists at which precipitation begins (13). At Ca^{2+} concentrations lower than the critical value α_{s1}-casein is fully soluble, while above it solubility decreases sharply (14).

In the absence of phosphate, α_{s1}-casein is precipitated between 6-7mM Ca^{2+}. At low phosphate levels, aggregation is induced at 2mM Ca^{2+} while at intermediate levels the system can be stabilized as a colloid. (40, 41). Precipitation occurs at higher levels because there is insufficient α_{s1}-casein present to maintain stability of the colloidal co-precipitate.

Binding of Ca^{2+} ions to casein creates units analogous to monomers in a polyfunctional condensation polymerization (13, 14). The first two Ca^{2+} ions bind to phosphoserine groups, and subsequent Ca^{2+} ions bind to carboxylate groups (13). The number average functionality of the particles is close to two, corresponding to between 4 to 10 Ca^{2+} ions bound per α_{s1}-casein molecule (14).

Role of Casein Phosphate Esters

There are eight phosphoserine residues in α_{s1}-casein. The consequences of dephosphorylation are:

1. Ca^{2+} induced precipitated occurs via non-phosphate Ca^{2+} binding sites (22).
2. Ability to be stabilized by κ-casein in the presence of Ca^{2+} is impaired but not eliminated (22,71).
3. Incorporation into micelles is decreased (94).
4. Micelle formation and stability are reduced (4).
5. α_{s1},κ-casein micelles are disrupted, indicating a minimum number of phosphate groups is necessary for micelles to remain dispersed in their medium (94).
6. α_{s1}-casein is shifted from the micelle phase to an insoluble phases, i.e. it precipitates (94).

Chemical phosphorylation of κ-casein impairs its stabilizing ability (71). Low phosphate content for κ-casein is thus a requisite for formation of stable micelles and explains why specific phosphorylation of casein components in the mammary gland is required. Native β-casein has five phosphoserine groups and like κ-casein is amphipolar but it does not stabilize α_{s1}-casein. Conversely, dephosphorylated β-casein is not only soluble in the presence of Ca^{2+} but also stabilizes α_{s1}-casein. Thus, three properties of a protein required to stabilize α_{s1}-casein are (97):

1. A hydrophobic region as site for interaction with α_{s1}-casein.
2. A hydrophilic region as site for interaction with water.

3. A low phosphate content.

Dephosphorylation of β-casein reduces the rate at which β-casein self-associates but does not affect the final level of association (98).

COLLOIDAL CALCIUM PHOSPHATE

Calcium is present in milk in a number of forms (6). Of the 32 mM calcium in skimmilk: 22 mM is in the colloidal state and 10 mM is soluble, of which only 3 mM is free ionic Ca^{2+} (7). In the serum phase it can exist as the free hydrated ion or complexed with citrate, phosphate or serum proteins. In the colloidal state it can be complexed with phosphate ester and carboxyl groups of micellar casein, or it can be complexed with phosphate (and perhaps citrate) associated with the casein micelle (55, 65, 79). Skimmmilk contains 30 mM phosphate anions dispersed as: 19 mM colloidal, 5 mM free, and 6 mM bound to calcium. Of the 8.4 mM citrate 0.4 mM is incorporated into the casein micelle (7).

Calcium is only slowly removed by dialysis of skimmilk (25) suggesting that nearly all the calcium, including "ionized" calcium, is at least loosely associated with non-dialysable constituents. Integrity and structure of micelles remain essentially constant after dialysis for 24 h with little change in CCP. This suggests that its rate of solution is slow and not in true equilibrium with the serum environment (25).

Calorimetric studies of casein micelle systems suggest that CCP more closely resembles OH-apatite than amorphous calcium phosphate or any other crystalline form (52). It can be represented by an empirical formula of $3Ca_3(PO_4)_2.CaHcitrate^-$ (72). Numerous types of linkages are possible between CCP and casein and within the CCP network (Table 7).

Serum casein content can be increased by addition of phosphate or acid or by removal of CCP (75). Removal of calcium from micelles up to a critical Ca^{2+} level causes no reduction in hydrodynamic radius (7).

Table 7. Types of linkages possible in colloidal calcium phosphate (59). XX = carboxylate or phosphoserine group.

$\text{Casein - XX - (Ca-PO}_4\text{}^{\text{Ca}}_{\text{Ca}}\text{PO}_4\text{)}_n\text{ - Ca - XX - Casein}$
- H
- CaOH
$\text{- Ca - PO}_4\text{ = Ca}$
$\text{Casein - XX - Ca - PO}_4\text{ - Ca - XX - Casein}$
Ca - Citrate = Ca
$\text{Casein - XX - Ca - PO}_4\text{ - Ca - XX - Casein}$
CaOH

As Ca^{2+} activity is reduced below the critical value, the molecular weight of the micelles decreases as a result of dissociation of caseins, predominantly β- and κ-caseins (7). Further removal of Ca^{2+} causes dissociation of the micelle. Addition of Ca^{2+} initially causes transfer of soluble casein to the micelle without changing hydrodynamic radius. Further addition causes formation of increasingly larger casein micelles (7).

Two distinct forms of ions are associated with casein micelles, an outer system readily removed and an inner system resistant to removal (22). Removal of adsorbed ions does not destroy integrity of the micelle. Forty percent of micellar calcium is "hard-to-exchange" and is present as part of the CCP (65). Substitution of calcium with Ba, Pb or Ur, and EM observations have shown that Ca is not homogenously distributed within the casein micelle but is located in a network system (45). Dye penetration studies have also shown that the micelle is rather loosely constructed and this calcium network corresponds to

connecting interstites between submicelles (45). At the pH of milk CCP is insoluble and casein prevents it from being precipitated. Early stages of micelle formation may be promoted by CCP spatially arranging submicelles (29) or by acting as a nucleating agent (78).

Rapid mineral exchange occurs in micelles formed without phosphate and leads to micelle instability (13). Mineral exchanges are controlled by salt solubility (70). Calcium phosphates are in saturated solution in the soluble phase of milk and phosphate solubility in normal milk at 37°C is limited by the saturation equilibrium (70). An important function of calcium phosphate may be to slow down exchange of calcium between the micellar phase and serum. Calcium phosphates are slow to equilibrate, and both Mg^{2+} and pyrophosphates inhibit the crystallization of OH-apatite, indicating milk may not be in true equilibrium with the solid phase (52).

MICELLE SYNTHESIS

Micelle Bio-assembly

Casein polypeptide synthesis occurs in the rough endoplasmic reticulum (ER) of epithelial cells. Addition of prosthetic groups and bioassembly takes place in the golgi apparatus (GA) in preparation for cell export (50). There appears to be a specific chronological order in which micellar components enter the GA; and in which phosphorylation and aggregation occur (41). Globular particles 10-20nm in diameter initially appear in newly formed golgi vesicles (GV). These globular particles eventually aggregate to form individual casein micelles and only one micelle appears to be synthesized per GV. Size of casein micelles may ultimately be limited by GV size (57). The GV containing milk constituents bud off from the GA and move toward the cell apical portion and discharge their contents into the glandular lumen.

The GA exhibits its highest specific activity toward dephosphorylated casein, and a pool of unphosphorylated casein observed in mammary cells suggests phosphorylation of serine and threonine side chains occurs following polypeptide synthesis but before micelle assembly (50).

Micelle formation could begin under conditions in which a colloidally stable casein-phosphate-calcium co-precipitate that is formed in the GA as phosphorylation is completed (41). Bio-assembly of casein micelles can be summarized as (22, 50):

1. After protein synthesis on ribosomes, casein polypeptides interact to form subunit particles composed of several monomers.

2. Upon reaching the GA, these subunits are phosphorylated and aggregate to form particles of about 10-20nm diameter.
3. Addition of Ca^{2+} (which is incorporated into milk in the GA) initiates polymerization of these particles into partially condensed micelles.
4. Deposition of CCP yields the mature casein micelle.

Some newly formed GV contain long stringy, protein filaments (50) and some are completely filled by loosely arranged submicelles (34). More mature vesicles contain casein micelles similar to those observed in secreted milk in the lumen.

Artificial Casein Micelle Synthesis

Artificial synthesis of casein micelles requires presence of calcium, phosphate and citrate as well as the major caseins (α_{s1}, β and κ). Properties of artificial micelles depend on the way constituents are allowed to react (78,80). One method starts with a salt solution of calcium, phosphate and citrate at milk serum concentrations (46). Casein, and additional calcium, phosphate, and citrate are then added at 15 min intervals. All the casein forms micelles within the range 30-400 nm diameter with typical submicelle structure. Another method starts with sodium caseinate (pH 6.7, 37°C) and inorganic constituents are added simultaneously in three separate solutions: calcium and magnesium chlorides, sodium and potassium phosphates, and potassium citrate. Volume distribution closest to native micelles is obtained with ratios of α_{s1}:β:κ-casein of 6:2:1 (78).

In the presence of inorganic phosphate, Ca^{2+} addition initially causes casein polymer formation and growth to submicelle size and then

promotes micelle formation (85). Once submicellar size is reached, additional Ca^{2+} disrupts intra-submicellar salt bridges, and aided by inter-submicellar hydrophobic interactions, forms longer chains or bridges between submicelles. This occurs at the point where calcium-sensitive caseins precipitate and argues for their interaction in micelle formation, with κ-casein providing stabilization by limiting the precipitation process (78).

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PART 1B. ENZYMIC COAGULATION OF CASEIN MICELLES: A REVIEW

INTRODUCTION

Three separate, but overlapping, stages occur during the enzymic coagulation of milk: enzymic proteolysis, aggregation and gelation. The first two stages of milk coagulation have been described (63) as:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P_1 + M$$

$$iP_1 \xrightarrow{k_s} P_i \quad (i = 1,2,3,..)$$

where P_1 stands for unaggregated para-casein micelles, M for macropeptide split off by the enzyme and k_s is the aggregation rate constant for the production of micelle aggregates, P_i. The first equation accounts for enzymic production of P_1 by Michaelis-Menten kinetics (6) and the second represents disappearence of P_1 by bimolecular aggregation reaction.

Temperature coefficients (Q_{10}) for these two phases of milk coagulation are quite different and proteolysis of milk can be conducted at a low temperature without coagulation occurring. Most enzymic reactions have temperature coefficients between 2 and 4 (1). The Q_{10} for the enzymic phase of milk coagulation at pH 6.7 between 1° and 30°C is 1.8 to 2.0 (57). Aggregation is more sensitive to temperature and has a Q_{10} of approximately 11 to 12 (9, 10). Following aggregation of micelles coagulation is observed and a gel structure is formed which undergoes a period of curd firming then syneresis.

PROTEOLYSIS OF K-CASEIN

A physical model of κ-casein consists of a fairly rigid, globular structure comprising two thirds of the molecule to which is attached a flexible, highly solvated tail (84). The chymosin sensitive peptide bond of κ-casein (Phe_{105}-Met_{106}) occurs between these two domains and its proteolysis results in formation of a carbohydrate-containing macropeptide and para-κ-casein. When studying kinetics of the enzymic phase of milk coagulation the integrated form of the Michaelis-Menten equation is used because dilution of milk may affect the stability of the casein micelle and alter the concentration of other milk constituents (6).

For individual milk samples, K_m is in the range .67 to 5.4 x 10^{-5} M κ-casein and K_{cat} is equal to 2 to 13 s^{-1} (6). Such differences in K_m and K_{cat} for various milks may be due to differences in ionic environment, the structure and composition of the casein micelles or the composition of κ-casein. The carbohydrate content of κ-casein affects K_m and κ-casein devoid of carbohydrate has lower K_m, and thus a higher affinity for the enzyme (7).

Effect of Extent of Proteolysis

In milk κ-casein exists distributed throughout casein micelles, predominantly on the surface, and proteolysis of one κ-casein molecule within the micelle is not sufficient to bring about aggregation (14). By the time coagulation is physically observed at least 90% of the κ-casein in milk has been proteolysed and proteolysis of κ-casein by chymosin is essentially complete at 80% of the visually observed coagulation time (CT) (8, 23). There are two schools of thought with

regard to the extent of proteolysis of κ-casein required before coagulation occurs.

According to Cheryan, et al (10) not all the κ-casein need be enzymically proteolysed for coagulation to occur but only sufficient to initiate aggregation of micelles. This conclusion is based on coagulation of skimmilk treated with immobilized pepsin and diluted with either water, acid whey or untreated skimmilk. Addition of fresh skimmilk not only dilutes the reaction mixture but also increases the number of unreacted micelles, yet the entire mixture of fresh and reacted skimmilk still produces a typical firm curd. The CT of such a mixture is greater than that of skimmilk diluted with water or whey and suggests interference in the clotting process by unreacted micelles. Recovery of casein in a 1:1 mixture of treated skimmilk diluted with fresh skimmilk was the same as for treated milk, i.e. 95.5% (50). Similar results were obtained when ultracentrifuged skimmilk supernatant was treated with immobilized pepsin and combined with 2X concentrated, washed casein micelles. No limiting nonprotein nitrogren release was observed at which coagulation would not occur (39). Studies involving immobilized pepsin require milk substrate adjusted to pH 5.6 to pH 5.9 and this may affect coagulation rate because it not only reduces the negative charge on the micelles but also increases Ca^{2+} activity.

It has been suggested that the relationship between extent of proteolysis and subsequent aggregation should be included in mathematical models describing milk coagulation (14). Dalgleish (12) found that aggregation rate of skimmilk casein micelles treated with immobilized chymosin (diluted by a factor of 60) was not significant until 88% of available κ-casein had been proteolysed. He proposed that

micelles remain uncoagulable until very extensive conversion of surface κ-casein. Part of the lag phase is produced by slow aggregation of casein micelles but a major part of the delay before observable coagulation is thought to be caused by time required for the enzyme to produce the extensive proteolysis of the κ-casein required to produce aggregable particles (14).

Payens et al. (67), however, postulate that lag time is merely a consequence of the second order of the aggregation reaction which will not begin as long as concentrations of aggregating species remains low. Accordingly, both phases start simultaneously and casein micelles do not need to be completely modified before aggregation begins.

COAGULATION TIME

In the simplest case, coagulation reactions can be considered bimolecular and aggregation rate diffusion controlled (67). The lag time between enzyme addition and observable coagulation can then be considered to arise as a consequence of the second order of the aggregation reaction which will not start as long as the concentrations of the flocculating species, para-casein micelles remains low (66). If, as according to Dalgleish, a micelle cannot participate in coagulation until a certain proportion of its κ-casein has been proteolysed then CT is the sum of the time required for this critical proteolysis and the time required for this amount of material to aggregate.

From equations derived to explain changes in particle size Payens (64, 65) defined actual coagulation time as:

$$\tau = (k_s V/_2)^{-\frac{1}{2}}$$

where τ is the enzymic coagulation time and can be visualized as the time needed to produce a limiting number of particles in the absence of any flocculation, k_s is the aggregation rate constant, and V is the maximum enzymic velocity. This shows that lag is determined by k_s to the same extent as enzymic velocity. However, k_s is a measure of the repulsion between aggregating particles, and consequently a measure of the amount of κ-casein proteolyzed. If not every collision between two micelle particles leads to permanent contact, but only those encounters in which both interaction areas contain enzyme-modified κ-casein, the fraction of sites available for permanent contact increases with enzyme activity (67). Aggregation rate therefore increases as enzyme concentration increases, and enzymic clotting time is be proportional to the inverse of enzyme activity (56).

Measurements of CT using different methods give values which represent particular molecular weights or degrees of coagulation depending on the method used (67). Various methods have been used to measure milk coagulation time (4, 28). The most commonly used method involves observaton of graininess, or the appearence of flecks, in a moving milk film (2, 3, 54, 78). A decrease in the rate at which milk runs through a capillary tube can also be used as a measure of CT (73, 74) and was found to approximate a "cheese makers' test" in which a drop of water is dropped onto the milk and the first sign of a "crater" being formed is taken as CT. These two methods give a later end point than the moving milk film method.

To measure viscosity, or the appearence of graininess, the milk must be flowing, if only very slowly. Milk at rest may have a very small but definite rigidity, whereas the sample under test may only show

a rise in viscosity and not be at its CT endpoint. The very soft gel structure as it is first formed is easily destroyed by shearing and may well be thixotropic (73).

Coagulation time can also be measured using a rotary viscometry (46, 47). Turbidity has also been used to measure CT of diluted milk as either the intersection of the baseline and an extrapolation of the steeply increasing turbidity that occurs on coagulation or the inflexion point in the turbidity curve (80). Methods based on measurement of curd firmness give a longer CT than is observed visually since the milk gel must become sufficiently rigid to, for example, transmit oscillations between diaphragms (49) or to restrict movement of an immersed wire loop (55).

CHANGES OCCURRING DURING COAGULATION

After enzymic addition a decrease in viscosity is observed (74) as a consequence of release of macropeptide from κ-casein molecules. This decrease cannot be explained simply by reduction in molecular weight but a reduction in volume and/or change in shape of the casein micelles is also required (88). At the sites of proteolysis of κ-casein the micelle surface is changed from a net negatively charged area to a positively charged area which could affect viscosity (75). The drop in viscosity could also be explained if the intact macropeptide is present as molecular chains protruding from the micelle (88). Removal of such "hairs" would then reduce hydrodynamic radius of the micelles, lower their viscosity, and decrease entropic repulsion.

Before rigidity increases are observed, milk ceases to behave as a true fluid and viscosity rises due to formation of aggregates (74).

FACTORS AFFECTING COAGULATION

Effect of pH

When pH is decreased from 6.7 to 5.6 CT decreases 30 fold (10). Effect of pH on the enzymic phase of milk coagulation is minor compared to its effect on aggregation (10). Rate of increase in curd firmness following coagulation increases as pH is decreased from 6.8 to 6.3 (48) even when CT is kept constant, showing different factors (or their magnitude) affect initial aggregation and formation of the casein gel network. Reduction in CT caused by acidification with HCl is a result of changes in Ca^{2+} activity (20). Direct acidification causes a reduction in colloidal calcium phosphate (CCP) concentration and a concomitant increase in Ca^{2+} activity (76). If milk is acidified with citric acid Ca^{2+} activity remains constant, CCP concentration is reduced and CT is increased (76). Intact state of the CCP is thus important in affecting CT (68).

Effect of Temperature

Decreasing temperature by 10°C reduces the enzymic phase by a factor of 2 and the aggregation phase by a factor of 11 to 12 (9). Low temperature (less than 8°C) has been used to allow proteolysis of κ-casein, in the absence of coagulation, using immobilized enzyme systems. Coagulation occurs on subsequent warming. It has been observed, however, that after extensive proteolysis using immobilized chymosin even low temperature is not sufficient to prevent aggregation (12). The effect of temperature on aggregation suggests that hydrophobic interactions play an important role in aggregation (48).

Effect of Substrate Concentration

Coagulation time remains fairly constant as milk concentration is increased by ultrafiltration (15), but the amount of casein not incorporated into the curd at CT increases. In unconcentrated milk 11% of the casein is not incorporated into the curd at CT, while 50% is not incorporated in 4X concentrated milk (15). The subsequent behavior of these "free" casein particles may determine the final properties of the curd. The proportion of casein that forms the initial curd decreases as CT increases (15). Enzyme concentration required to produce the same proportion of casein incorporated into the coagulum at CT, compared to unconcentrated milk, increases in proportion to the concentration factor of the milk (15). This, however, reduces CT.

Coagulation time increases upon dilution of milk and is highly dependent on dilution factor (6.231). Observations of turbidity changes at very high dilutions (1:10000) with no visual coagulation led to the suggestion that para-casein micelles undergo a cooperative transition of quaternary structure (80). Only slight variations occur in the inflexion point of turbidity-time curves for milk diluted 10^{-1} to 10^{-4}. Rapid changes in absorbance near the point of coagulation have been assigned to this intramicellar cooperative transition as a rate determining step.

As concentration of milk increases, extent of κ-casein proteolysis at CT, decreases (26). Volume of aqueous phase decreases and shortens the mean free distance between micelles (24). This results in an increased number of effective collisions of para-casein micelles and increased aggregation velocity. At low substrate concentration, aggregation rate is slow compared to proteolysis while at high

concentration overall rate is dominated by rate of enzymic action (13).

Effect of Calcium

Addition of calcium chloride to milk decreases CT. Federal Definitions and Standards of Identity (85) permit addition of 0.02% (w/w) anhydrous $CaCl_2$ to milk for cheesemaking. This allows the amount of rennet required for optimum curd formation to be reduced (22). Addition of $CaCl_2$ up to .05M causes a reduction in CT to a minimum. At very high $CaCl_2$ levels (.4M) CT is severely retarded and only a very weak curd is obtained (McMahon, unpublished data).

Effect of Enzyme

Milk-clotting enzymes of both animal and microbial origin are used in cheese making. The prime action of milk-clotting enzymes is the proteolysis of a specific bond in κ-casein. However, beyond that, the different enzymes vary in their proteolytic activity on κ-casein and other proteins (21). A low proteolytic activity to milk clotting activity ratio is an essential requirement for any calf rennet substitute (5, 87). Animal proteases used include: calf rennet, adult bovine rennet (containing approximately 50% bovine pepsin), porcine pepsin, and chicken pepsin. Proteases of microbial origin include those extracted from Mucor miehei, Mucor pusillus v. lindt and Endothia parasitica. Most calf rennet substitutes are more proteolytic relative to their milk clotting activity than calf rennet. If proteolytic activity is excessive, cheese yield and fat retention by the curd may be diminished and during cheese ripening this can have undesirable effects

on cheese body and texture (31). Curd firming rates vary from enzyme to enzyme with the more specific enzymes producing a firm gel more quickly (49).

GENERAL FLOCCULATION PRINCIPLES

Aggregation of colloid suspensions such as milk can be considered to occur by the mechanism described by von Smoluchowski (60). The rate of disappearence of P_1 particles is determined by diffusion rates as a result of Brownian movement, interactions between the particle when they are close together and their collision efficiency. The simplest case is when repulsion is completely negated and remaining attraction is represented by a sphere of action surrounding each particle (11). If a second particle enters this sphere of action, the two particles coalesce irreversibly. In this form of coagulation every encounter between two particles leads to permanent contact. The rate of such "rapid coagulation" is completely determined by Brownian motion. Smoluchowski also derived a theory for "slow coagulation" in which only a fraction of encounters is successful (60).

The surface of para-casein micelles can be described as chemically heterogeneous. A successful encounter, not only requires sufficiently close approach of the two particles but also their specific mutual orientation (77). If their positions are such that at least one molecule is completely unreactive at the place of contact, no reaction can occur and the spheres may diffuse away from each other. On the other hand, only if contact is made between strongly reactive sites on both spheres is success assured. If the repulsive energy is other than zero only a fraction of collisions leads to aggregation (69). Chemical

heterogeneity of the molecular surface of micelles thus produces factors smaller than unity by which Smoluchowski's ideal diffusion controlled rate constant terms must be multiplied in order to describe the effects of finite reactivity and finite rate of rotational diffusion (77). The aggregation rate constant for skimmilk is of the order 5×10^5 mL moles^{-1} sec^{-1} compared to 6×10^{12} for rapid diffusion-controlled aggregation (65). This suggests that only a fraction (10^{-6}) of the particle collisions leads to permanent contact. A restricted number of aggregation sites also tends to produce a gel-like structure rather than a dense precipitate (66).

In a flocculating system containing particles of different sizes it is observed that smaller particles disappear more quickly than large ones (60). As the complex of small and large particles is still large, collisions between small and large particles do not change the number of large particles but they do decrease that of the small ones giving the impression that small particles are caught by the larger ones. Collisions are random and there is no preference for the formation or reaction of aggregates of any particle size (38).

When particles of colloidal size aggregate, chain formation is frequently observed and geometric models proposed to explain carbon black aggregates (29, 81, 82, 83) can be applied to milk coagulation. Such models consider the floc to be formed by collisions of clusters of particles rather than separate units, yielding a very porous structure. The process occurring between clusters containing various numbers of primary particles can be explained by von Smoluchowski coagulation theories (19, 51), in which a collision between an i-fold cluster and a j-fold cluster yields an (i+j)-fold cluster. Growth kinetics which

favor cluster addition accentuate chain formation while any link flexibility reduces it.

Size and shape of an aggregate is the important unit affecting behavior of flocculating systems rather than those of primary particles. Particle chain flexibility and inter-particle fusion during the course of aggregate growth cause rearrangement of particles in the final floc. In a real system, with all particles under random Brownian motion, clusters of many different sizes may co-exist during the aggregation process. The possibility of forming a large floc entirely by the addition of single particle is very remote; the growth of such aggregates is almost entirely by the addition of clusters. Theoretically, the process continues until there is only one floc present, but in practice, gravitational and fluid shear forces eventually outweigh effects of Brownian diffusion.

Aggregates are of ragged and ill-defined shape and the floc is made up of long, haphazard strings of particles joining regions of somewhat denser structure. Growth by cluster addition leads to an open network because in only the rarest cases can clusters interpenetrate, and large holes are left in the particle matrix. Very expanded floc structures are likely to form irrespective of the nature of the flocculating system and if a rigid bond is formed then compression, such as applied during cheese cheddaring, will be needed to obtain a denser structure.

As floc size increases, internal rearrangements occur to permit transfer of increasing stresses. If particle size and solids concentration are such that cluster-addition growth fills the whole of the suspension space before breakdown can occur by disruptive forces a gel is formed. The dispersed phase does not settle out but the system

gradually becomes more viscous and solidifies forming a coherent system (19). Flocs rely on rigid bonds between particles to maintain their open structures. In the final floc a relatively large number of primary particles will have lost their original identity due to structural rearrangements and/or interparticle fusion.

For molecules with functionality greater than two there is a build up of polymer aggregates with many unreacted sites. This leads to a high probability of larger molecules growing faster, with the result that weight average molecular weight, $\overline{M}_w$, rises very rapidly (62). A point is reached where $\overline{M}_w$ becomes so large the system separates into an insoluble and soluble fraction: the critical point in polymer chemistry and the coagulation point in milk systems. With such a system the difference between precipitation and gelation often only depends on the dilution involved. In concentrated solutions, gel formation occurs at the critical point whereas in dilute solutions a precipitate forms. Although the weight average degree of polymerization becomes very large its number average is still low, ca. 4, and gelation does not occur at an infinite molecular weight (61). A distribution exists with a very few large particles in a system in which most of the molecules are still of very low molecular weight.

MODELS OF MILK COAGULATION

Numerous models have been proposed to describe the events that occur during the initial stages of milk coagulation. Dalgleish (12, 14, 15, 16) proposed a model based on aggregation rate of diluted skimmilk treated with immobilized chymosin being insignificant until 88% of available κ-casein had been proteolysed. It includes:

1. Action of chymosin upon κ-casein of the casein micelles.

2. Probability that a micelle with a given proportion κ-casein proteolysed will aggregate.

3. Aggregation of casein micelles by Smoluchowski mechanism. He suggested that a micelle will not aggregate until 97% of its available κ-casein has been proteolysed and effects of aggregation will be manifest at 88% overall proteolysis of κ-casein.

The growth of $(\bar{M}_w)$ in the aggregating system is given by:

$$\bar{M}_w(t) = \bar{M}_w(o)\{1 + [\frac{2k_s}{\int(\frac{dw}{dt})dt} \int\{\int\frac{dw}{dt}\,dt\}^2\,dt]\}$$

where dw/dt is the rate at which aggregable material is produced as described by the integrated Michaelis-Menten equation.

Payens et al. (63, 64, 65, 67) developed equations describing changes in the number of P_i particles existing during the aggregation reaction. In initial stages of coagulation and when the substrate concentration is greater than K_m, the aggregation reaction is bimolecular and k_s is diffusion controlled and independent of particle size. Initially, total particle number is determined only by enzymic production of P_1.

Payens developed an equation describing the time dependence of weight average molecular weight of a coagulating milk system:

$$\frac{\bar{M}_w}{\bar{M}_o} = 1 - M_o(1-f)(\frac{8V}{k_s})^{1/2}\{f(\frac{t}{\tau}) - (1-f)(\frac{t}{\tau})^3/3\}/C_o$$

where M_o is average molecular weight of casein micelles in milk, f is the ratio of the molecular weight of released peptide to M_o and C_o is the substrate concentration.

Darling and van Hooydonk (17) derived a model in which the stability factor, W, in Smoluchowski's theory is considered as a variable and determined by the concentration of unhydrolysed κ-casein. During the enzymic reaction κ-casein molecules on micelles are gradually hydrolysed leading to a decrease in micelle stability and consequently to a gradual increase in the aggregation rate constant. The stability factor is exponentially dependent on substrate concentration and approaches a constant value before the enzymic reaction is complete. Coagulation time can then be expressed as:

$$t_c = \frac{1}{v}\left[S_o + \frac{1}{C_m}(\exp(-C_m.S_o)-1)\right] + \frac{W_o.\exp(-C_m.S_o)}{k_s}\left[\frac{1}{n_c} - \frac{1}{n_o}\right]$$

where S_o is the initial κ-casein concentration, W_o is the initial stability factor, C_m is a proportionality constant, V is the enzymic velocity, and n_o and n_c are number of micelle particles at time t=0 and $t=t_c$, the coagulation time. The coagulation time can be considered as the time required to reduce the number of micelle particles from n_o to n_c. By rearranging this equation the relationship between particle concentration and time can be obtained and particle size calculated from the concentration.

GELATION

Curd Firmness

It is important in cheesemaking to cut curd at the correct firmness so that whey drains properly while loss of milk solids is minimized (31). When using milk-clotting enzymes other than calf rennet, curd firmness and syneresis should ideally be similar to those when calf

rennet is used and there should be no significant losses of fat or protein. Actual rate of curd firming may not be important in determining properties of the curd (31), but its control is important in cheese making (90). Variations in curd firmness at time of cutting may result in greater losses of milk components and reduced cheese yield (59). Monitoring curd firmness during cheese making offers the potential for reducing such losses by cutting at consistent curd firmness to optimize cheese manufacturing (59). A slow set means the curd must be cut when it is more fragile than desired, or cutting must be delayed. When cutting is delayed, time is lost and the cheesemaking schedule upset (16).

Rate of increase of curd firmness, decreases as the extent of proteolysis of casein increases (89). Curd firming rate for calf rennet> pepsin > M. miehei and M. pusillus v. lindt proteases > papain > trypsin (89). Excess proteolysis apparently inhibits increase in curd firmness by degrading the protein molecules involved in curd formation and shrinkage (90). Excessive yield loss due to use of porcine pepsin has been related to cutting curd before it was sufficiently firm (20). Curd produced using pepsin has a more fibrous structure than calf rennet-treated milk and this looser structure may be the reason for a softer curd (18).

Some methods of monitoring curd firmness include: vibrating reed viscometer (53), pressure transmission systems (PTS) (27, 53, 86), Formagraph (55, 56, 70), and resistance to penetration or cutting of the curd (71, 79). The response of a particular instrument to the curd-firming process depends on the method of measurement and the general term, curd firmness, describes a complex of two elastic and two

viscosity moduli (73). No instrument measures any moduli specifically (53). The vibrating reed viscometer is more sensitive to changes in viscosity than PTS and indicates an earlier maximum rate of curd firming than the PTS (53). Penetration studies (79) do not show any curd firming until about 140% CT and indicate occurrence of two processes: formation of the gel network as a result of micelle aggregation, followed by incorporation of "free" casein aggregates into the existing coagulum (79).

Stages of Gelation

Transmission electron microscopy (TEM) has been used to monitor changes occurring in milk during the process of gel assembly (34, 35) and manufacture of Cheddar cheese (45). Initally, micelle distribution suggests the existence of repulsion between micelles. After 80% CT, micelles are clumped together. Sum of proportions of micelles not touching or bridged decreases dramatically between 60% and 100-130% CT. Micelles are linked by wide bridges consisting of several strands or by direct contact involving large areas of their surface. As micelles group together, a gel network is built up in which the bridges between micelles contract forcing micelles into contact and causing partial fusion. At the same time, chains link together to form an extended irregular network. As links contract, the micellar chains become concentrated into loose strands about 5 micelles thick and separated by wide spaces. In the final gel (300% CT) micelles are only loosely packed and entrap much serum.

At the soft gel stage of Cheddar cheese making, after cutting and before the onset of syneresis, micelles are clumped together and some

are deformed. Fusion is widespread and involves up to 50% total volume of each micelle. After cooking of the curd, the casein network has formed strands 1-2 μm in diameter (44, 45) and fusion has progressed to the extent that the individual nature of micelles has been lost (43). The mesh structure is still retained and crosslinks observed between strands are possibly a result of their being pulled out as the strands shrink and move apart. Syneresis is brought about by contraction of the three dimensional gel network of linked casein micelles (25,72).

Protein Side Chain Interactions

Photooxidation of histidine residues causes κ-casein and whole casein to lose the ability to clot when renneted (42). It alters κ-casein so that chymosin is unable to split off the macropeptide, and by interfering with aggregation in the presence of Ca^{2+} ions. Effect on chymosin attack may be a result of a specific modification to histidine residues or a general change in charge. Modification of tryptophan or methionine residues has little affect on coagulation (42). Blocking of lysine residues on κ-casein by dansyl chloride has no effect on κ-casein proteolysis (41) but causes complete inhibition of coagulation when 2-3 lysine residues per molecule are blocked, indicating that lysine side chains of κ-casein play some role in enzymic coagulation (41). Blocking of lysine on α_s- or β-casein appears not to be involved in stabilization by κ-casein or coagulation (41)

Modification of arginine residues also results in inhibition of coagulation of rennet-treated casein, suggesting that arginine forms part of a positively charged region important in coagulation that also includes side chain groups of lysine and histidine (40). The

critical lysine residues are not readily accessible unless in micelle dissociating conditions, suggesting they are covered by the macropeptide portion of κ-casein (41).

Role of Casein Phosphoester Groups

Dephosphorylation of milk (60% phosphoryl groups removed) results in formation of a soft curd upon coagulation by chymosin (91). Coagulation, therefore, proceeds in part by formation of salt bridges between casein phosphoserine groups. Milk fortified with β-casein produces a 50% firmer curd than native milk (90) or milk fortified with α_s-casein (58). If β-casein is proteolysed by trypsin to remove the C-terminal hydrophilic moiety, including the phosphoserine groups, a fragile curd is formed (90). Proteolysis of β-casein by chymosin removes a hydrophobic moeity near the N-terminus and addition of such modified β-casein still produces an increase in curd firmness (91). Addition of dephosphorylated β-casein produces lower curd firmness than native β-casein. Trypsin-modification of α_s-caseins has a negligible effect on curd firmness when used to fortify milk (91). Consolidation of coagulum, therefore, involves interactions between Ca^{2+} ions and phosphate groups bound primarily to β-casein molecules (90). One role of β-casein is thus to keep calcium in the micellar network (19).

IONIC INTERACTIONS

The role of ionic interactions in milk coagulation has been studied by adding ionic materials and detergents to milk. Those which are bound most strongly to the micelle, and have the greatest effect on

coagulation, are those that bind more strongly to caseinate than hydroxyapatite (33). They are highly cationic or contain both charged and hydrophobic moieties (37). Adsorption occurs at areas containing both charged, particularly anionic, and hydrophobic sites. Binding apparently occurs at sites inside the micelle and not on the surface where they would shield the macropeptide portion of κ-casein (32). By shielding charged groups and increasing the hydrophobicity of areas within the micelle (37) cationic materials reduce CT by increasing affinity of chymosin for the micelles and by accelerating the aggregation phase (36). Rennet action is still necessary for coagulation to occur. The main factors affecting CT are the amount, charge, and molecular weight of the additive (52).

Anionic detergents increase CT while cationic detergents decrease CT, can cause coagulation in absence of rennet at detergent concentrations greater than 0.01M and can promote syneresis of rennet coagulum (10). The interactions involved in milk coagulation are facilitated either by reduction in energy barrier, caused by shielding of charged areas, or by reduction in steric effect by increasing the area of sites on para-casein micelles available for intermicelle interactions (37). Any inhibitory effect of anionic areas might be masked by the binding of cations increased by anionic materials or counteracted by materials which enhance hydrophobicity of the micelles (52).

Coagulation cannot be viewed solely as a reversal in the balance of repulsive and attractive forces between micelles. Lysozyme which carries an equal number of positive charges as the net negative charges on micelles, does not cause coagulation (30). Some specific

interactions such as between positively charged residues of para-κ-casein on one micelle and negatively charged groups of α_s- and β-caseins or another, may be involved. Coagulation should be considered as the sum of all thermodynamic forces including non specific hydrophobic and electrostatic interactions, and specific interactions between enzyme modified micelles.

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PART 2A. EVALUATION OF FORMAGRAPH FOR COMPARING RENNET SOLUTIONS

INTRODUCTION

Numerous methods have been used to determine rennet activity (4). The most common method depends upon visual observation of the formation of a clot, or rather the sudden fracture of a film of milk on the wall of a bottle or test tube (2, 8). An ideal way to measure milk-clotting activity of an enzyme, however, has yet to be devised. Unlike usual procedures in enzyme chemistry, milk clotting activity may be determined only by observing the rapidity with which the enzyme clots milk under a set of specified conditions.

The Formagraph is an instrument designed to record the coagulation properties of cheese milk. The technique by which the Formagraph determines coagulation is based upon movement of small, stainless steel, loop pendulums immersed in linearly oscillating samples of coagulating milk (Figure 1).

Minute forces are applied to the pendulums as a consequence of the formation of a gel in the moving milk sample. A light flash at each end of the sample oscillation records the pendulum position on self-developing photographic paper. The result is a diagram of firmness versus time. The light flashes are timed at 4/min to coincide with the limits of the oscillation strokes.

The Formagraph and the rolling bottle method described by Sommer and Matsen (8) were compared for measuring milk-clotting enzyme activity. The viscosity of milk at which coagulation is detected by the two methods was compared with the method of Kopelman and Cogan (6).

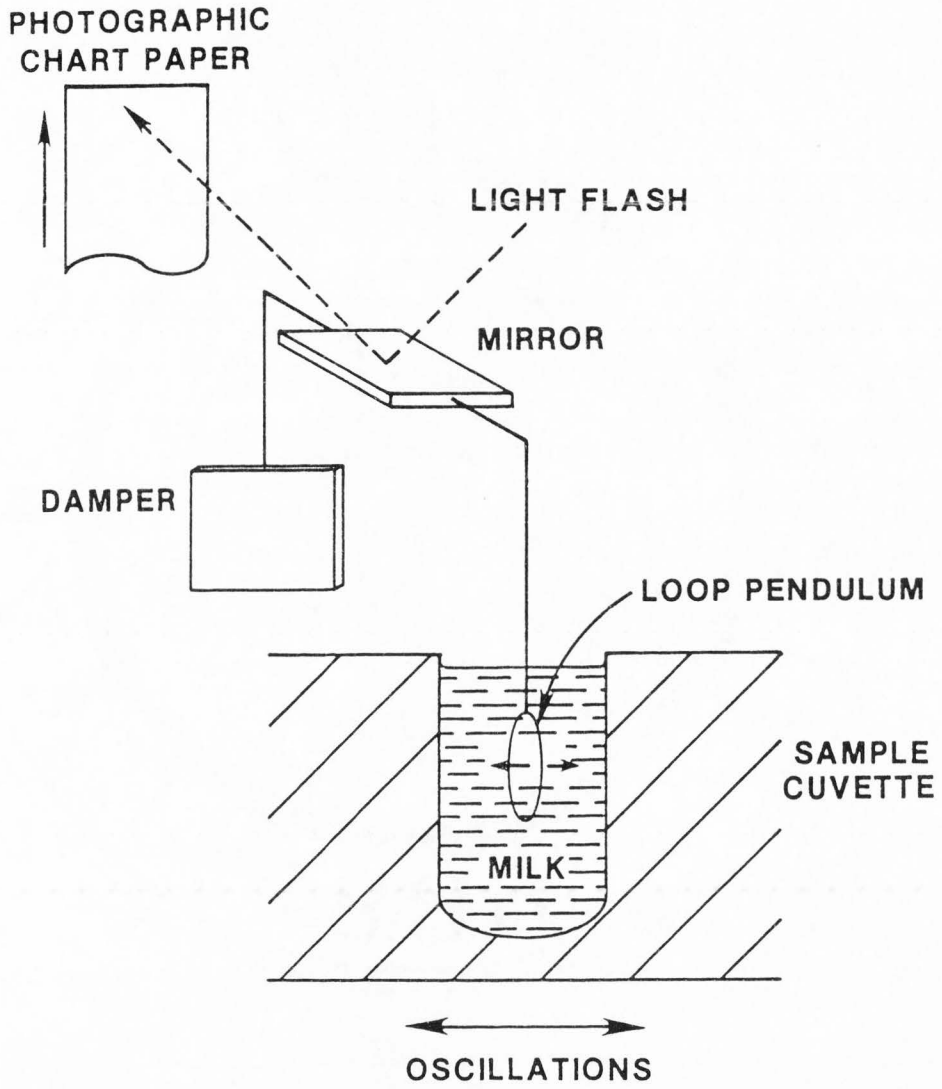

Figure 1. Schematic diagram of the Formagraph instrument for measuring and recording coagulation of milk.

MATERIALS AND METHODS

Reagents

Low heat nonfat dry milk was reconstituted in 0.01M $CaCl_2$ (1) and allowed to equilibrate at 5°C for 20 h (3). Prior to each analysis the milk substrate was warmed to 30°C and maintained at that temperature for 30 min.

A purified calf rennet solution of nominal clotting activity of 100 rennet units (3) per ml (RU/mL) was obtained from The New Zealand Cooperative Rennet Co., Ltd., Eltham, New Zealand. All dilutions were made using distilled water and maintained at below 2°C throughout the experiment.

Formagraph Method

The Formagraph (5) consists of two modules. A service module heats the sample cuvettes and milk, controls the temperature of the instrument and houses the on/off controls for up to five recorder modules. The recorder module consists of a ten-channel recording system. Each channel comprises a pendulum with counterbalanced damper and an adjacent optical system. Common to all channels are the sample oscillator system, light flashing unit and strip chart recorder.

Sample cuvettes are placed on the service module heating plate and 10 mL of substrate is deposited in each sample well. Enzyme solution (200 µL for each sample) is pipetted into a multiple spoon apparatus to allow simultaneous dispensing of enzyme into all samples. The recorder module is started the instant the enzyme is added to the

milk. Samples are stirred with the spoons, then the cuvette is transferred to the recorder module.

Samples are brought into contact with the pendulum loops. While the milk remains uncoagulated, insufficient force is transmitted to the pendulum from the linearly oscillating milk to cause the pendulum to move. When coagulation occurs, the resultant increase in viscosity and formation of a curd causes synchronous motion of the pendulum and light flashes reflected from the pendulum mirrors are recorded at different lateral positions on the chart.

In a typical firmness versus time diagram (Figure 2), r represents time until formation of a gel. The value of r was determined by measuring distance from the origin to the point where the baseline begins to increase in width. The time from the start of gel development until a width of 20 mm is reached on the chart is shown as k_{20}. This equates with a curd firmness adequate for cutting of cheese curd. It has been suggested that by keeping enzyme concentration constant, the measurement a_{30} can be used for comparing cheese milk samples (5). This is the width of the graph 30 min after enzyme is added.

Milk clotting is influenced by numerous factors including temperature, substrate composition and enzyme concentration. The effect of each of these parameters can be examined by holding all of the others constant.

Rolling Bottle Method

The apparatus described by Sommer and Matsen (8) was used. One half-milliliter of rennet solution was added to each 25-mL portion of

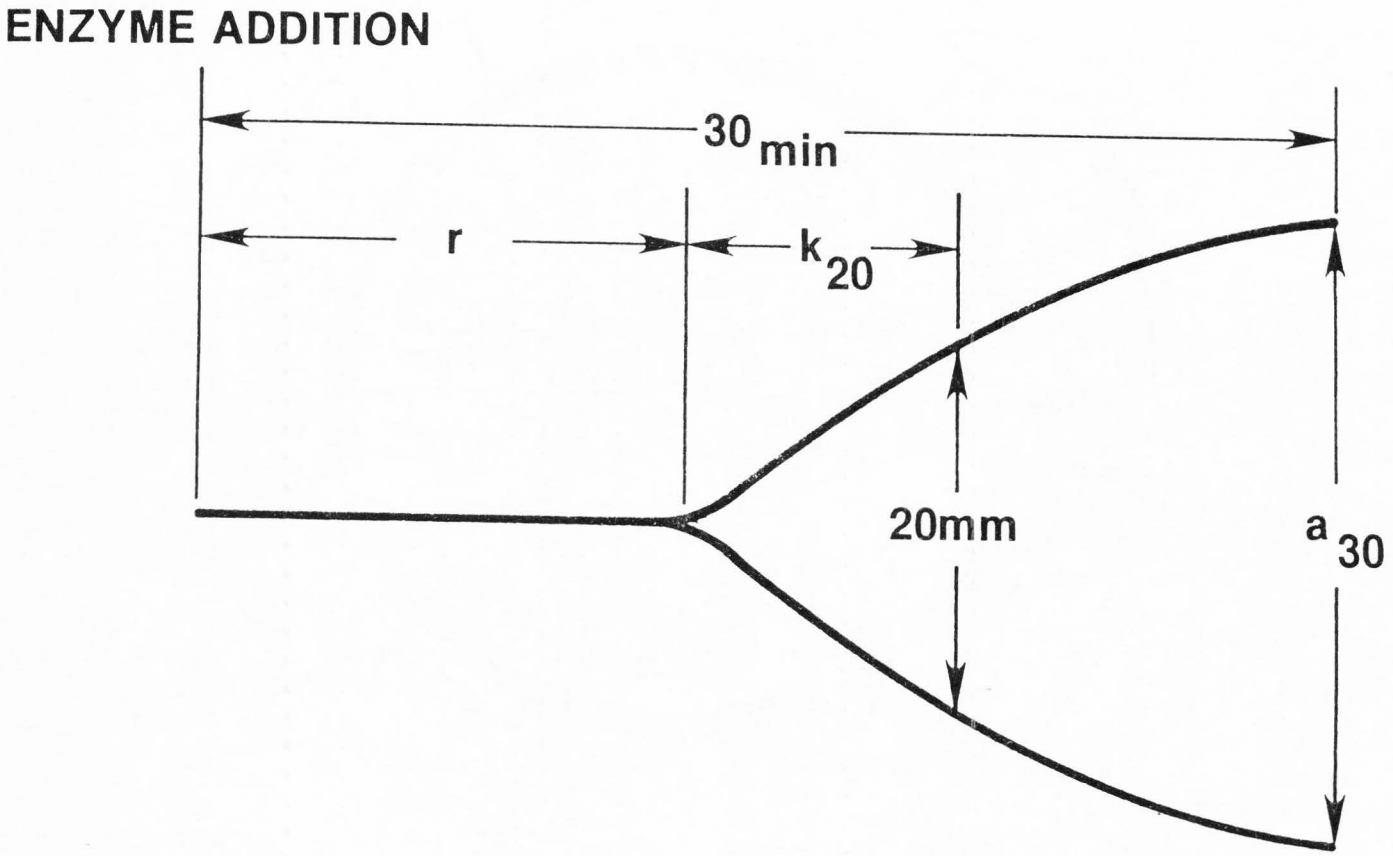

Figure 2. Diagram of coagulation and curd firmness as a function of time as recorded with the Formagraph.

Berridge substrate in 125 ml wide mouth bottles. The analysis was carried out at 30°C.

Viscosity Measurement

Viscosity was measured using the procedure of Kopelman and Cogan (6).

Statistical Design and Analysis

A randomized block design was used to eliminate the effects of possible changes in standard enzyme activity during the experiment. Both instruments accommodated 10 samples simultaneously, which permitted duplicates of five enzyme concentrations within each block.

Measurements of coagulation time were carried out simultaneously on the two instruments. If a mistake occurred in preparing or recording a sample, then all measurements at that rennet concentration in that block were discarded to maintain balance between the two methods. Analysis of variance and linear regressions were done using the SAS statistical package (7).

RESULTS AND DISCUSSION

Comparison of Methods

There was a difference ($P \leq .0001$) in coagulation time measured by the two methods (Table 8). Separate analyses of variance at each level of rennet concentration also showed significant differences in coagulation time for the two methods. The interaction between method and rennet concentration was also significant. At lower rennet concentrations, the rate of curd formation (once coagulation has

started) was slower (Figure 3). As the coagulation time was extended, the Formagraph endpoint fell further behind that of the rolling bottle method.

Table 8. Analysis of variance of Formagraph method versus rolling bottle measurements of milk coagulation at various rennet concentrations (blocking over time).

Source	DF	MS	F	Significant α- level
Block	4	1.68	-----	-----
Rennet	4	2072.16	12228	.0001
Method	1	82.70	488	.0001
Rennet*Method	4	5.22	31	.0001
Residual	67	.17		

Linear regressions of the inverse of actual rennet concentrations versus measured clotting times for both methods (Figure 4) yielded R^2 values >0.995. Both R^2 were essentially the same, which indicates that the two methods are equally useful for measuring coagulation time as a function of enzyme activity in comparison to a standard enzyme solution. Coefficients of variance (CV) of mean coagulation times for each method are shown in Figure 4, indicating more variance in Formagraph measurements than in rolling bottle measurements.

Viscosities of milk at the point of coagulation as measured by the two methods were compared (Figure 5). The coagulation point for the Sommer and Matsen rolling bottle method, which is defined as the breaking of a milk film on the inner surface of the bottle (8),

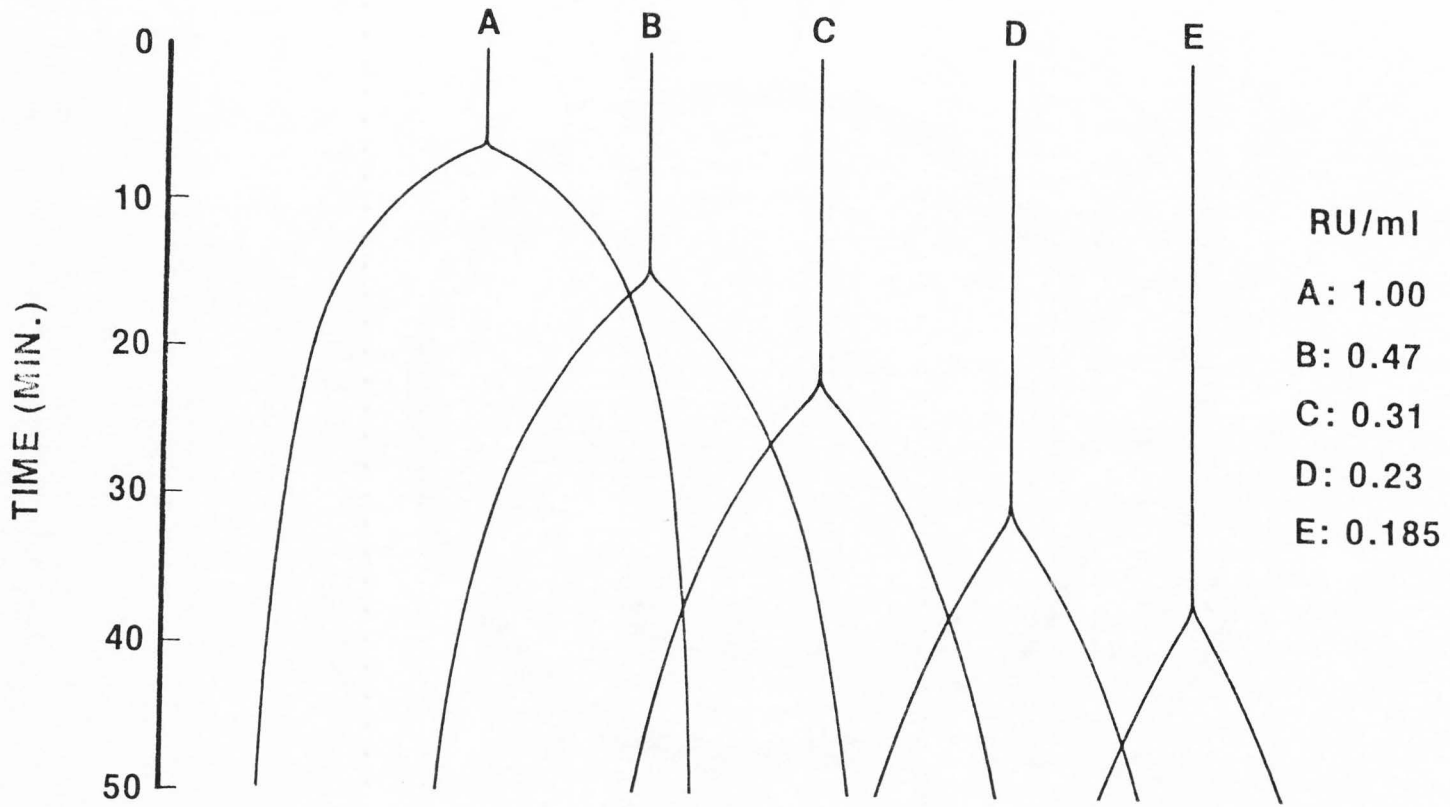

Figure 3. Coagulation and curd firmness as a function of time at various rennet concentrations.

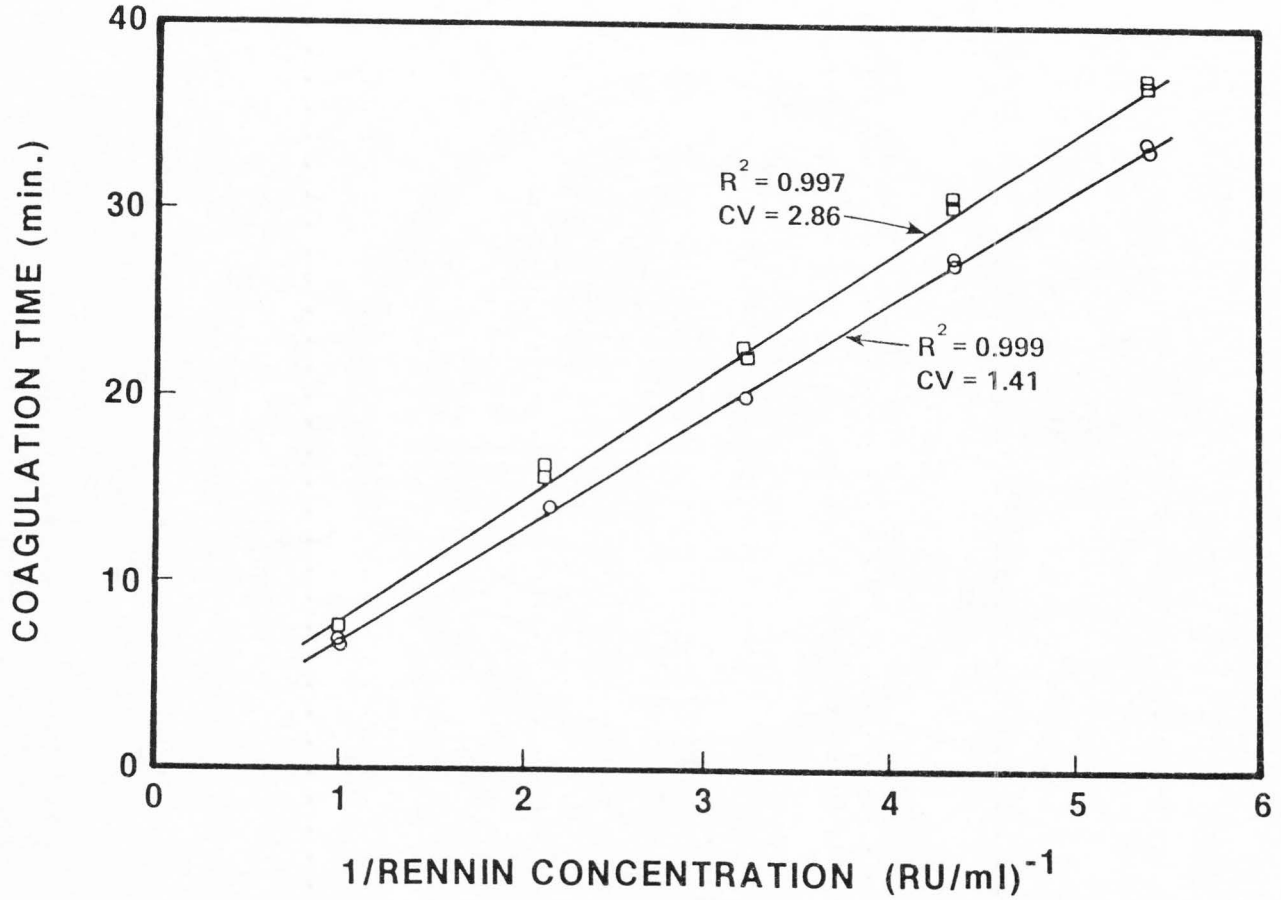

Figure 4. Linear regressions of coagulation time versus inverse of rennet concentration. Both methods were used simultaneously with duplicate samples at each concentration. □ Formagraph; o rolling bottles.

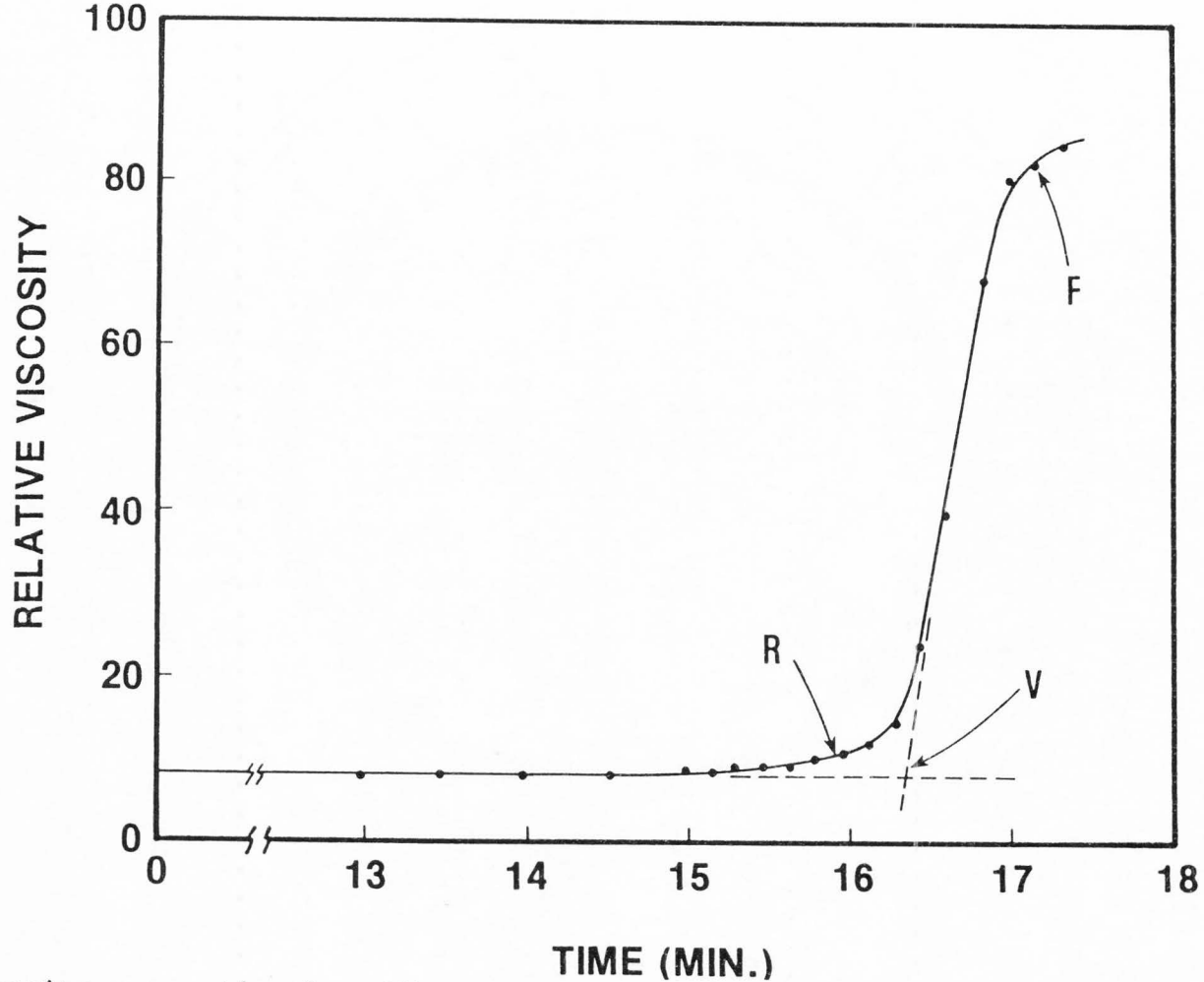

Figure 5. Viscosity versus time for milk-rennet system (2% of .40 RU/mL in Berridge substrate) showing measurement of coagulation by three different methods: Formagraph (F); rolling bottles (R); and viscosity (V).

occurred prior to the coagulation point of the Formagraph. The latter requires minimal gel formation to induce pendulum movement before coagulation can be detected. The coagulation point in the Kopelman and Cogan viscosity procedure is defined as the extrapolated intersection of the two straight line portions of a plot of viscosity versus time (6). The Formagraph shows the progress of curd formation, whereas the rolling bottle method gives only an endpoint.

CONCLUSIONS

Coagulation time as measured by the Formagraph is linear with the inverse of rennet concentration within the five fold range of 0.2 to 1.0 RU/mL. Milk-clotting enzyme activity can be measured equally well by Formagraph or rolling bottle method for enzyme activity compared to a standard sample.

SUMMARY

A new instrument for measuring milk clotting, the Formagraph, was evaluated. Measurement is based upon the movement of small pendulums immersed in linearly oscillating samples of coagulating milk. Pendulum movements are recorded on photographic paper, producing a diagram of firmness versus time . The instrument can be used for measuring milk-clotting enzyme activity in comparison to a standard, and in contrast to the rolling bottle method, it does not require continual observation. Coagulation times measured by the Formagraph are longer than those measured by the rolling bottle method, but both methods give linear standard curves between 0.2 and 1.0 rennet units/mL enzyme concentrations.

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PART 2B. MILK COAGULATION TIME: LINEAR RELATIONSHIP WITH INVERSE OF ENZYME ACTIVITY

INTRODUCTION

Milk-clotting enzyme activity commonly is calculated by comparing coagulation time of an unknown relative to a standard of known activity. Because of reported deviations from linearity of the relationship between coagulation time and the inverse of enzyme activity, it has been suggested that unknown and standard samples should have approximately the same enzyme concentration (5). In (12) this relationship was linear over coagulation time ranging from 5 to 30 min when clotting time is measured with the Formagraph or the Sommer and Matsen apparatus (20).

The linear relationship between milk coagulation time and the inverse of enzyme concentration first was postulated by Storch and Segelcke (21), who stated that the product of enzyme concentration and coagulation time is constant:

$$t_c E = k \qquad \text{(I)}$$

where t_c is coagulation time, E is enzyme activity, and k is a constant.

Holter (9) observed that this equation is only valid for a narrow range of enzyme concentration (variations of 1:4) and concluded that deviations from the Storch and Segelcke relationship are due to a time lag in the aggregation phase during the coagulation process. He introduced a correction factor into the equation:

$$E(t_c - x) = k \qquad \text{(II)}$$

where x represents the time required to allow enzymically converted casein to aggregate.

To facilitate statistical analysis, Foltmann (6) rearranged the Holter equation into the form:

$$t_c = \frac{k}{E} + x \qquad \text{(III)}$$

Observing that k remained constant whereas x varied for several fresh milk samples, he concluded that differences in coagulability were a consequence of the nonenzymic aggregation phase of the overall coagulation process.

Various mathematical models have been proposed to describe enzymic coagulation of milk. Payens (14,17) derived an expression for changes in weight average molecular weight of coagulating milk by von Smoluchowski's rate theory (13) for the production of multiple particles in bimolecular flocculation:

$$\frac{M_w}{M_o} = 1 - M_o(1-f)\left(\frac{8V}{k_s}\right)^{1/2}\left\{f\left(\frac{t}{\tau}\right) - (1-f)\left(\frac{t}{\tau}\right)^3/3\right\}/C_o \qquad \text{(IV)}$$

where M_o is the average molecular weight of casein micelles in milk, k_s is the aggregation rate constant, V is the enzymic velocity, f is the ratio of the molecular weight of released peptide to M_o, C_o is the substrate concentration and τ, is the parameter governing the kinetics of the clotting process, is defined as:

$$\tau = \left(\frac{k_s V}{2}\right)^{-1/2} \qquad \text{(V)}$$

in which V is proportional to enzyme concentration and τ is assumed to approximate t_c (18,19). The τ can be visualized as the time needed for

the enzyme to produce a minimum number of hydrolysed particles in the absence of any flocculation.

Measured t_c depends on the technique chosen to monitor coagulation and according to Foltmann (6) is only an empirical approximation, because the aggregation stage begins as soon as the first casein is hydrolysed. The lag time is merely the result of different orders of enzymic and aggregation reactions (19).

Equation V has an advantage over the Storch and Segelcke equation in that it states explicitly that the lag period is dependent on aggregation rate to the same extent as on enzymic velocity (10). If k_s is constant, equation V predicts a plot of log t_c versus log E^{-1} to be a straight line with a slope of .5.

Payens et al. (15,19) postulated that not every collision between para-casein micelles leads to permanent contact but only those in which para-κ-casein sites are involved. Because k_s is a measure of repulsion between flocculating particles, it can be considered a function of the amount of macropeptide split from κ-casein (15). Clotting starts before the maximal number of peptides are proteolysed by the enzyme, and the fraction of sites available for permanent contact (and, hence k_s) increases in proportion to the enzyme concentration (19). At a first approximation, equation V reduces to the linear form of equation III.

Another mathematical model for milk coagulation was described by Darling and Van Hooydonk (2) from which an equation expressing t_c was derived:

$$t_c = \frac{1}{V}\{S_o + \frac{1}{C_m}\exp(-C_m S_o)^{-1}\} + \frac{Wo}{K_s}\exp(-C_m S_o)\{\frac{1}{n_c} - \frac{1}{n_o}\} \qquad \text{(VI)}$$

where S_o is the micellar surface κ-casein concentration at time zero, W_o is the stability factor at time zero, n_o is the particle concentration at time zero, n_c is the particle concentration at time t_c and C_m is a proportionality constant. For this model, coagulation time can be visualized as the time required to reduce the number of micelles from n_o to n_c.

If k_s is a function of enzyme concentration or a constant, this equation also reduces to equation III.

From either of these models of milk coagulation, it appears that the inverse linear relationship between coagulation time and enzyme activity is valid for use in measuring enzyme activity. A study was made of the extent to which it remains valid when coagulation time is measured using the Formagraph. Linearity between coagulation time and the inverse of enzyme activity over an extensive range would indicate that it is not neccessary to dilute samples to approximately the same coagulation times when comparing enzyme activities. The ability to work with the Formagraph at low enzyme concentrations also may provide new possibilities for studying mechanism of milk coagulation.

EXPERIMENTAL PROCEDURES

The Formagraph was used to measure coagulation time as in (12) at a temperature of 35°C. A purified rennet solution[1] of known activity (80RU/mL)[2] was used to prepare dilutions ranging from .055RU/mL to 8RU/mL. Aliquots of 200uL were added to 10mL of substrate giving a final activity of enzyme in the milk of .0011RU/mL to .16RU/mL. Three replicates were prepared at each activity.

By Tektronix 4052 microcomputer (22), data were fitted to plots of t_c versus E^{-1} and $\log(t_c)$ versus $\log(E)^{-1}$ by linear least squares regression. Marquardt's nonlinear least squares regression (11) was used to fit the data to equation III without making any transformation.

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1. From New Zealand Cooperative Rennet Co., Eltham, New Zealand
2. Rennin activity units as described by Ernstrom (4).

RESULTS

Measured coagulation times covered the range from .7 to 66 min corresponding to a relative variation in enzyme activity of 1:140. For the simple ratio estimation of the Storch and Segelcke equation to be valid, the linear regression of t_c versus E^{-1} must cross the origin. This was not observed. The data were, instead, described by Foltmann's equation, in which the intercept approximates the minimum time required for the enzyme to produce coagulatable material (6) under the finite constraints of the experimental conditions. The linear transformation (Figure 6) and the nonlinear method (Figure 7) produced identical k and x:

$$\text{i.e.} \qquad t_c = .685 + .0754 \; / \; E \qquad \text{(VII)}$$

If this relationship is linear, then the slope of a double logarithmic plot should equal unity. An experimental slope was .94 (Figure 8). This agrees with other slopes (10) and shows that a marginally better fit ($R^2 = .999$) is obtained compared with equation VII ($R^2 = .996$). This difference is so small, however, that in determining enzyme activity equation III gives results comparable to those of a logarithmic plot and is easier to use routinely.

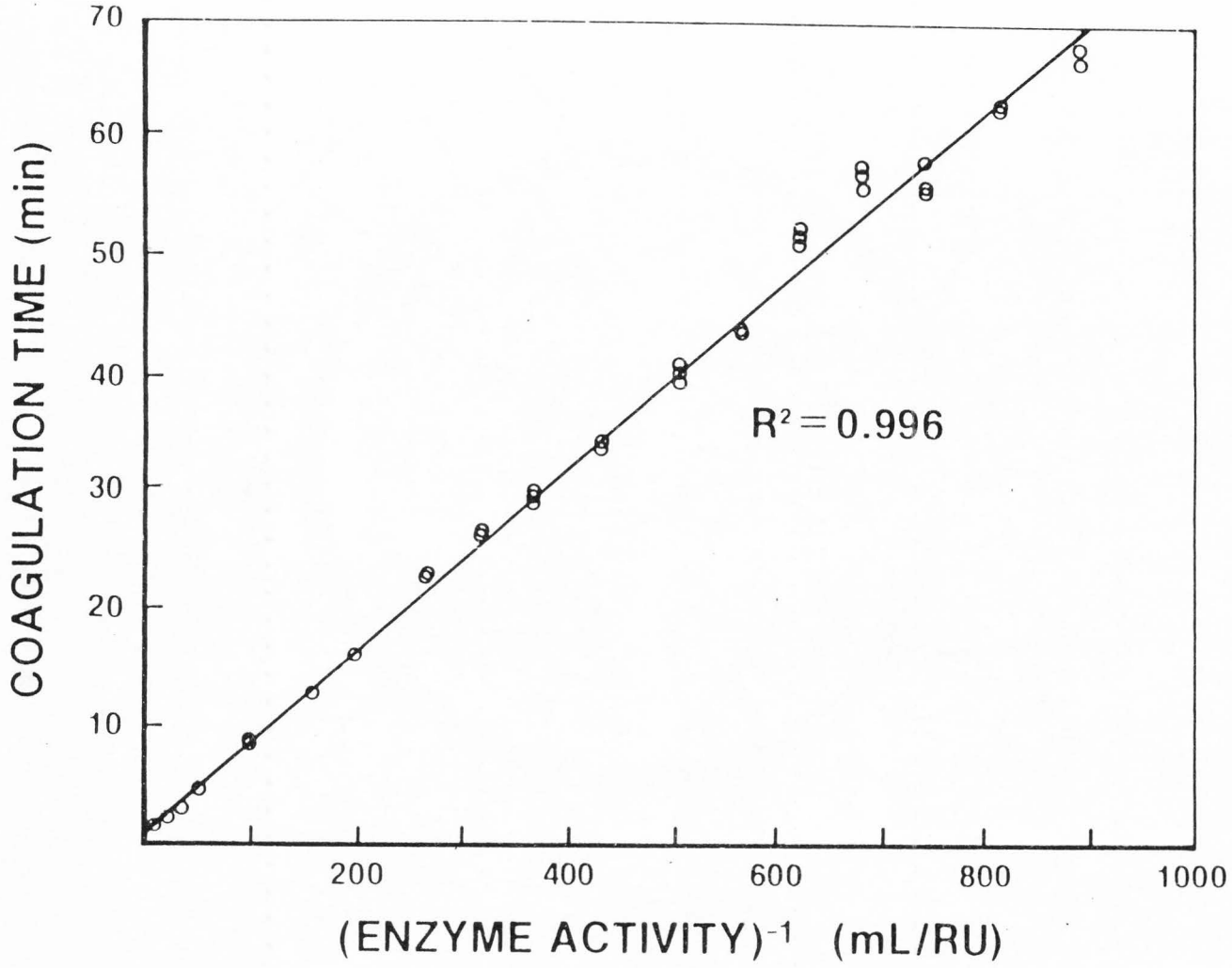

Figure 6. Linear regression of coagulation time on inverse rennet activity. Triplicate samples at each activity.

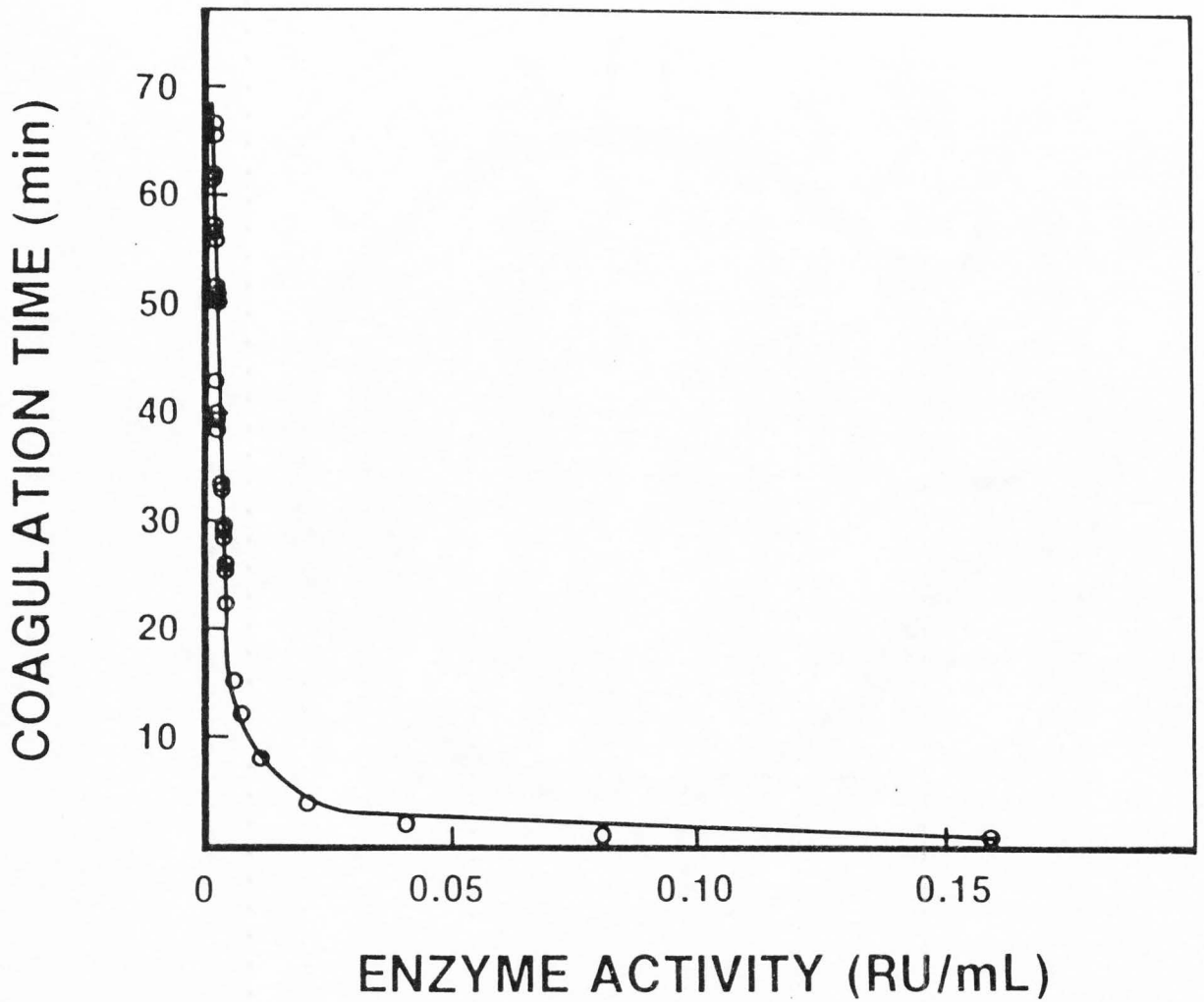

Figure 7. Nonlinear regression of coagulation time on rennet activity. Triplicate samples at each activity.

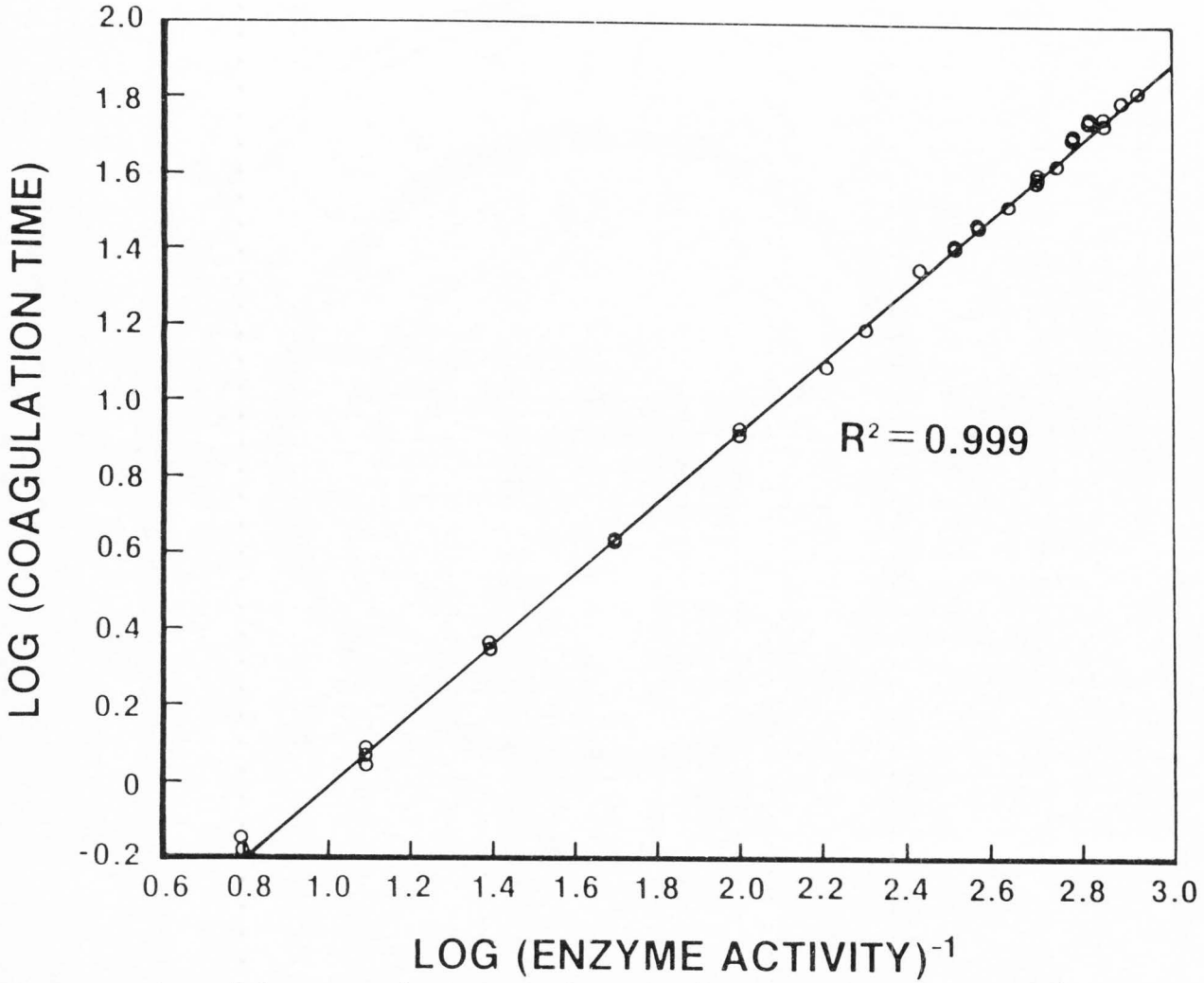

Figure 8. Linear regression of log(coagulation time) on log(inverse rennet activity). Triplicate samples at each activity.

DISCUSSION AND CONCLUSIONS

Enzymically induced coagulation of milk is a continuous process. Coagulation time, therefore, is not an integral part of the coagulating system. Its determination by turbidity or rheological methods results in t_c that correspond to attainment of a particular degree of coagulation (14). Some arbitary point in the overall coagulation process is chosen as the endpoint. For the Formagraph, this point occurs when the substrate gel is of sufficient viscosity to inhibit movement of an immersed pendulum. Consequently, k and x vary according to the method used to measure coagulation time (12).

Linearity between coagulation time and inverse enzyme concentration is affected by substrate composition. Such variations are largely due to changes in the calcium phosphate equilibrium between the ionic and colloidal states and can be eliminated by using nonfat dry milk reconstituted in .01M $CaCl_2$ (1). The time that reconstituted milk is held at the experimental temperature can also cause variation in coagulation time (1,7). It is recommended that the substrate be aged for 20h at $2^{o}C$ then equilibrated at the experimental temperature for 30 min (3).

Provided that conditions under which measurements are made (substrate composition, temperature and enzyme composition) can be controlled, then rennet activity can be accurately calculated from Formagraph milk coagulation data. The procedures described in this paper for preparing the substrate and measuring coagulation time allow enzyme activity to be measured accurately in comparision to a standard curve, without having to dilute the unknown to the same concentration as the reference.

The standard curve can cover a range that extends at least from .0011RU/mL to .16RU/mL. By modifying the procedure to allow the addition of a larger amount of enzyme, this method can measure milk-clotting enzyme activity within the range of the gel diffussion test of Holmes et al. (8).

SUMMARY

The extent to which time for milk coagulation is linear with the inverse of enzyme activity was investigated. The Formagraph can be used to measure time for coagulation of milk over a range of rennet activity. Linearity of coagulation time versus inverse of enzyme activity exists within the range of concentration .0011RU/mL to .16RU/mL, which represents relative variation in rennet activity of 1:140.

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PART 3. ENZYMIC COAGULATION OF MILK CASEIN MICELLES

ENZYMIC COAGULATION OF MILK CASEIN MICELLES

Induction of milk coagulation by chymosin (E.C. 3.4.23.4) is considered to consist of two phases. During the primary phase the enzyme proteolyses κ-casein (1) resulting in destabilization of the colloidal casein micelle system. Destabilized micelles participate in the secondary phase of micelle aggregation. The delay between initiation of the enzyme reaction and visually observable coagulation is believed to be a result of the time required for the enzyme to produce appreciable concentrations of aggregable material, and the time required for this material to aggregate by a diffusion controlled Smoluchowski mechanism (17,18).

Dalgleish (2) postulated that for a micelle to participate in the aggregation process, 96% of its κ-casein must be hydrolysed. Payens et al. (19), however, concluded that both phases start simultaneously and that lag time is merely the result of the different orders of the enzymic and aggregation reactions. As κ-casein is hydrolyzed the micelles gradually become less stable leading to an increase in aggregation rate (4).

Turbidity measurements of milk are usually made with milk diluted by a factor of 60 - 200 to remove multiple light scattering effects (2). Dilution however, can affect aggregation rates of systems in which particles diffuse freely until they touch (18). Changes in turbidity of undiluted milk were monitored to determine whether observations made on dilute solutions hold true at normal milk concentrations.

Nonfat dry milk was dispersed in 0.01M $CaCl_2$ (12g + 100mL) and equilibrated overnight at 4°C. Thirty minutes before enzyme addition 3 mL of the substrate (35°C) were placed in a 1cm pathlength cuvette in

a Beckman DU-8B UV/Vis spectrophotometer. An ion exchange purified chymosin solution of sufficient activity to give a visually observable coagulation time (CT) of approximately 6 min was added (10 μL) and the reaction mixture was rapidly stirred (<10 sec). Every four seconds, apparent absorbance measurements (at 600nm) were collected, stored, derivitized, and plotted, using a Tektronix 4052 microcomputer.

Monitoring of apparent absorbance of undiluted milk enabled the coagulation process to be followed through its various phases (Fig. 9). Although measurements were made in absorbance units, they are a consequence of light scattering changes caused by changes in molecular weight, size, and number of the colloidal casein micelle aggregates.

There is an initial decrease in apparent absorbance (Fig. 9 inset), which has been ascribed to a reduction in weight average molecular weight ($\bar{M}_w$) of the micelles as the macropeptide portions of κ-casein molecules are released (18). Apparent absorbance begins to increase at approximately 12% CT. Observations of coagulating milk using transmission electron microscopy (TEM) do not show any effects of aggregation until 60% CT (9), at which time enzymic action is almost complete. TEM micrographs, however, underestimate degree of aggregation because chains of casein micelles can appear as individual micelles or chains depending on the angle at which they cross the sectioned sample (13) and a tendency for chain formation does exist for milk micelle systems (8).

Payens et al. (19) derived mathematical equations describing changes over time in numbers of particles at various degrees of aggregation. The term governing these equations was the "actual" enzymic coagulation time, τ, and theoretically, aggregates consisting of two casein micelles

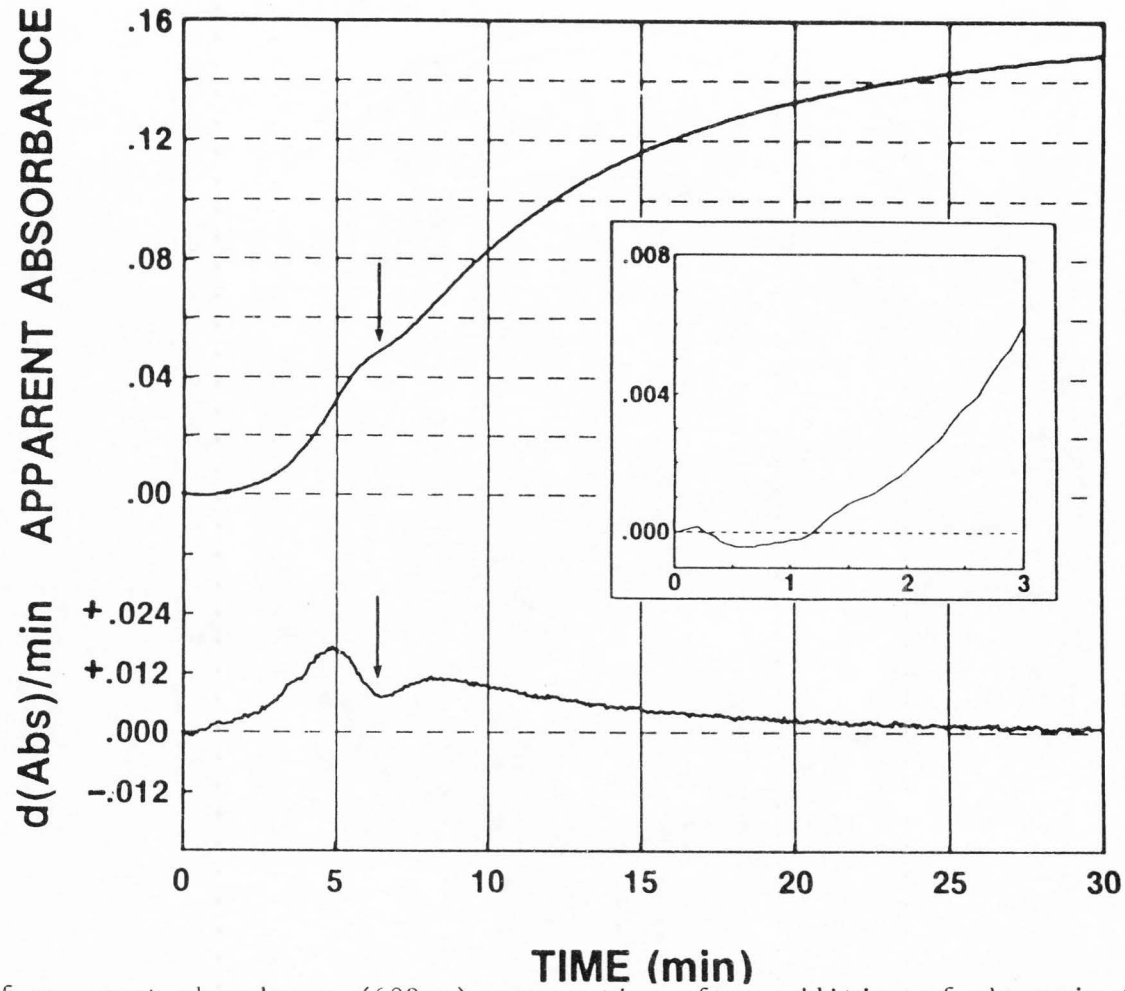

Figure 9. Plot of apparent absorbance (600nm) versus time after addition of chymosin to reconstituted nonfat dry milk (12g + 100mL .01M $CaCl_2$). Blank is substrate before enzyme addition. Arrows represent the time at which coagulation is observed using Formagraph method (15). Inset shows expanded view of the first 3 min of coagulation.

(P_2) show appreciable concentrations beginning at 25%τ, P_3 particles at 50%τ, P_4 particles at 80%τ, and P_5 particles not until around 100%τ.

The counteracting effects of enzymic production of aggregatable particles and decrease of total particle number by aggregation result in a limiting particle number. The value of τ can be visualized as the time needed to produce this limiting particle number in the absence of any flocculation. Coagulation time found by visual inspection of turbidity or by a rheological technique is a measure of a t/τ-value corresponding to a particular molecular weight. From the time of incipient rise in turbidity (4), τ was estimated as approximately 3.6 min, or 56% CT.

A geometric model of floc formation (21) can be applied to casein micelles. Aggregates are formed by collisions of clusters of particles rather than of separate units. Growth by addition of clusters leads to an open network because only rarely can clusters interpenetrate the growing floc. A gel is formed if particle size and solids concentration allow cluster growth to fill the whole suspension space before breakdown by disruptive forces can occur .

The rate at which apparent absorbance increases reaches a maximum at 80% CT or $t/\tau = 1.3$. It has been observed by TEM that at this stage micelles are grouped together in aggregates containing up to four micelles within a sectioned sample (8). When considered three dimensionally, these aggregates would be much larger. From such studies it has also been observed that aggregation proceeds steadily with no sudden changes in type or extent of aggregation at any point. This agrees with turbidity measurements of milk diluted by a factor of five or more. Using undiluted substrate, however, a shoulder was observed in

the apparent absorbance versus time curve (Fig. 9), indicative of some change in the aggregation process before visual coagulation is observed. This suggests that a point is reached at which the aggregating particle clusters occupy a critical volume and their mean free distance is so reduced that an explosive growth rate in particle size occurs. After this critical volume has been reached the majority of particle collisions result in addition to the main floc. The particle clusters have a considerable degree of chain flexibility and the chains of particles can bend until they come into contact with other chains and eventually rearrange themselves into more stable positions (7). The coagulating system, thus changes from one of essentially independent aggregating particles to an extended network composed of interconnected casein micelle chains, and the milk ceases to behave as a true fluid of constant viscosity (23). A similar shoulder in the coagulation curve has been observed by reflection photometry (10).

Lag time has been defined as the time needed to reduce the number of particles to half the initial value which occurs at approximately 60% CT (9) or $t = \tau$. Coagulation is not observed visually until this critical volume (or number) is passed (9). Determination of coagulation visually merely corresponds to attainment of a particular degree of coagulation (15).

The aggregation that occurs during gel formation is less complete than in flocculation and the loose, ramified aggregates encompass the solvent to form a coherent system (16). Initially, only part of the colloidal particles are linked together into the network and some particles are relatively free. As a result of Brownian movement they eventually collide and cling together.

Light scattering measurements of macromolecules are usually made in very dilute solutions so that the solute molecules behave as independent scatterers (11). If particles are of the same size as, or larger than, the wavelength used a more complex problem exists that is further compounded at high concentration. Scattering centers within individual particles cannot then be considered independent, and interference between them occurs. Scattering intensity is always less for an extended particle than for a compact particle of the same weight. Turbidity is thus not only related to $\overline{M}_w$ but also to the z-average particle scattering factor (Q_z) (12).

As molecular size increases, Q_z decreases so that an increase in size can eventually offset an increase in light scattering caused by $\overline{M}_w$ changes. Reduction of the rate of increase in apparent absorbance measurement observed after the critical volume is passed corresponds to such an effect. Formation of a loose, extended network structure causes a marked decrease in Q_z in comparison with that of individual aggregates. The large increase in $\overline{M}_w$ (approaching infinity) does not compensate for the large decrease in both the number of particles and a reduced Q_z.

After formation of the gel network, the apparent absorbance continues to increase as coagulum firming (gelation) takes place (6, 15). It has been calculated that at initial coagulation, 11% of the casein is not incorporated into the coagulum network (3). The incorporation of this "free" casein into the gel network, as well as changes that occur in the structure of the network chains, should be considered in any explanations of the gelation process (20). By 200% CT

TEM shows that chains of micelles appear to be grouped together into loose, convoluted strands (8).

Once the gel network has been formed, further aggregation is no longer a strictly random process controlled solely by diffusion. Specific interactions occur between micelles (and micelle chains) that cause the network chains to cluster together into strands of about five micelles diameter (8). Flexibility and rearrangements of the links between casein micelles can lead to more points of contact and a denser structure (22). At 300% CT, observations on TEM micrographs show a gel network of relatively large mesh. Within the strands that form this network, the chains of micelles are loosely packed and linked together forming an inner network of smaller mesh. Partial fusion of micelles has occurred and much of the casein micelle individual character has been lost (14). The forces and specific interactions that cause these phenomena to occur are as yet undetermined.

Absorbance measurements asymptote to a final value dependent on test conditions such as protein concentration, calcium concentration, and nature of substrate. Decreasing enzyme activity decreases the rate of all phases of the coagulation process but does not affect their sequence under specified conditions (Figure 10). If final apparent absorbance corresponds to extent of gel crosslinking, then it appears that changing enzyme activity (and thus coagulation time) does not affect the final apparent absorbance but only the rate at which it is attained.

If the coagulation reaction is followed for a sufficiently long time, the apparent absorbance decreases. This is thought to be a consequence of syneresis. No exuded liquid was observed, but

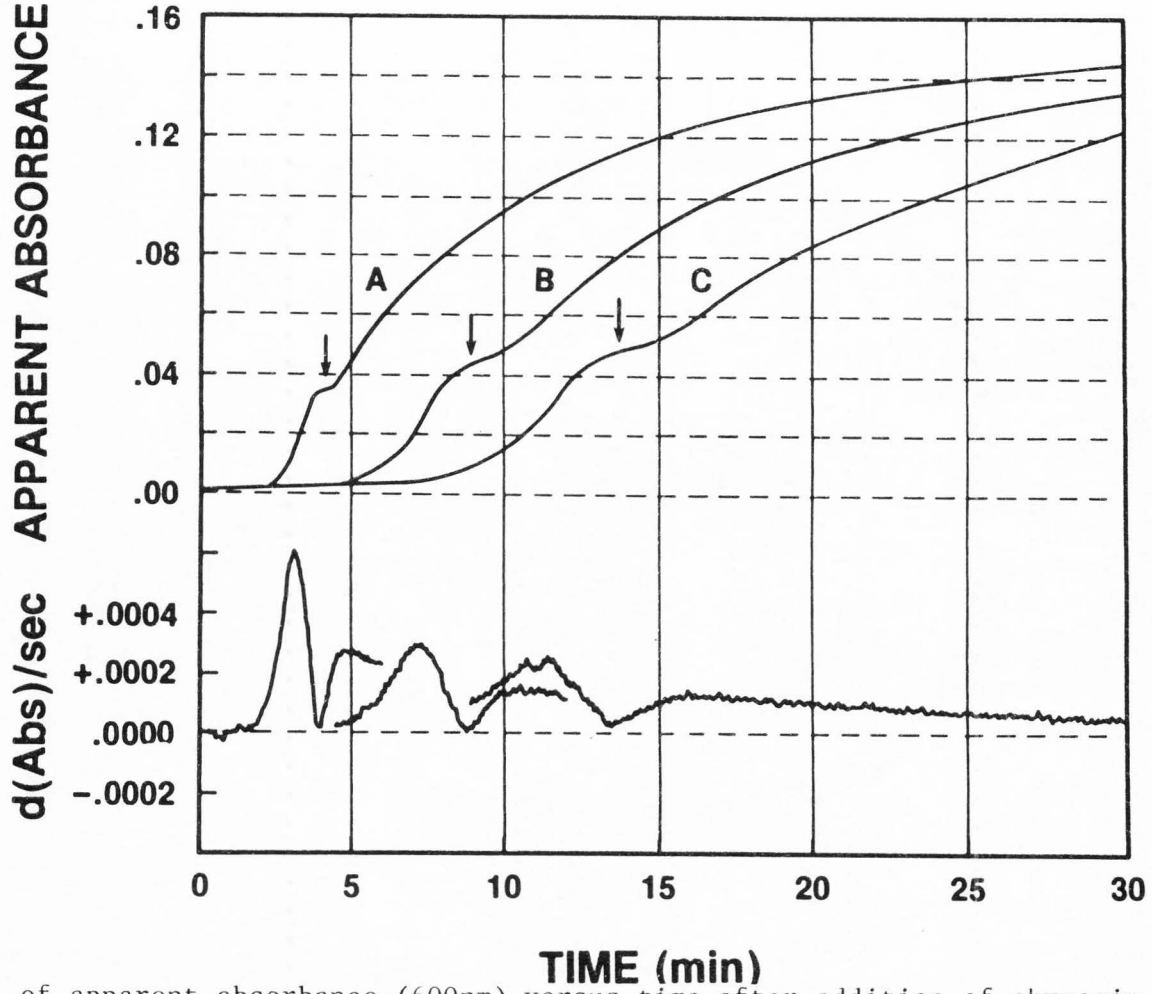

Figure 10. Plot of apparent absorbance (600nm) versus time after addition of chymosin to reconstituted nonfat dry milk (12g + 100mL .01M $CaCl_2$) at three levels of enzyme activity. A: .027RU/mL, B: .011RU/mL, C: .0053RU/mL (5). Blank is substrate before enzyme addition. Arrows represent time at which coagulation is observed using Formagraph method (15).

consolidation of individual chains of micelles into strains would cause formation of micro-droplets of whey within the gel network.

SUMMARY

Enzymic milk coagulation consists of several phases. Initial hydrolysis of κ-casein reduces colloidal stability of casein micelles, allowing aggregation by random diffusion. Aggregation rate is initially slow but can be observed well before visual coagulation occurs. Enzymic coagulation actually occurs when a critical aggregate volume is reached and the system changes from aggregating particles to an extended gel network.

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PART 4A. EFFECTS OF CALCIUM, PHOSPHATE, AND BULK CULTURE MEDIA ON MILK COAGULATION

INTRODUCTION

Enzymic milk coagulation is a consequence of the proteolysis of a chymosin-sensitive bond in κ-casein causing destabilization of casein micelles. In the presence of calcium ions these micelles aggregate and a gel forms. Addition of calcium chloride to milk decreases the lag time between enzyme addition and observed coagulation. Many studies on the influence of added salts on milk coagulating properties have been reported in the literature, but little has been published on effects of calcium addition at levels higher than 0.2% (w/w). Federal Definitions and Standards of Identity (35) permit addition of 0.02% (w/w) anhydrous calcium chloride to milk used for cheesemaking. The quantity of rennet required can thus be reduced by 50% (11). Cost effectiveness of such action is determined by the relative costs of rennet and calcium chloride.

Milk coagulation is a complex process and although a considerable amount is known about it, the actual mechanisms involved in aggregation and the exact role of calcium have not been completely elucidated. Milk coagulation is affected not only by calcium addition but also by phosphate addition, pH, ionic strength, and temperature (34). Anything that alters the ionic environment surrounding the casein micelles influences coagulation.

Calcium in milk is present in a variety of forms (3). Of the 32 mM calcium in skimmilk, 22 mM is in the colloidal state and 10 mM is soluble. Only 3 mM (less than 10%) of this soluble calcium is actually free ionic Ca^{2+}. The remainder is complexed with citrate, phosphate or serum proteins (4). In the colloidal state, calcium can be complexed

with phosphoester or carboxyl groups of micellar casein, or with colloidal phosphate (and citrate) associated with the casein micelle. The 30 mM of phosphate anions in skimmilk is distributed as follows: 19 mM is in a colloidal state, 5 mM is free inorganic phosphate, and 6 mM is bound to calcium in the milk serum. Of the 8.4 mM citrate in skimmilk about 0.4 mM is incorporated into the casein micelles (4).

Enzymic coagulation of milk is inversely related to pH and is sensitive to pH changes in the range pH 6.5 - 7.0 (10). This effect is compounded, however, by alterations in Ca^{2+} activity caused by changes in pH (26). Direct acidification with HCl causes a reduction in colloidal calcium phosphate (CCP) concentration and a reduction in enzymic coagulation time (CT). This is probably due to the concomitant increase in Ca^{2+} activity (31). If Ca^{2+} activity is held constant then CT increases both above and below the natural pH of milk.

Acidification of milk causes serum casein levels to increase, possibly as a result of loss of calcium from the micelle (30). Addition of phosphate has a similar effect, while addition of calcium moves casein from serum into the micelle. If milk is acidified with citric acid, Ca^{2+} activity remains constant while [CCP] is reduced. Citrate acts as a calcium chelator and the $CaCit^{-}$ complex has a greater stability constant than $CaHPO_4$ (15). When $[citrate]_{total}$ is less than $[Ca^{2+}]$, more than 95% of the citrate is bound in a 1:1 complex with Ca^{2+}, reducing Ca^{2+} activity by that amount (16). In milk, however, a large reserve of calcium exists in the form of micellar CCP in equilibrium with free Ca^{2+}. As Ca^{2+} ions are complexed with citrate, CCP dissolves to re-equilibrate the system and maintain Ca^{2+} activity.

Acidification with citric acid, thus causes a retardation of

coagulation. No coagulation has been observed at pH 6.4 in such a system (31). Reducing CCP by acidification and dialysis also retards CT. Coagulation has not been observed after removal of 30% of the CCP (31). Rennet-treated whole casein in the presence of Ca^{2+} but in the absence of phosphate, forms only a flocculent precipitate and not the firm gel typical in its presence (37).

Phage-inhibitory media are used extensively in preparation of bulk cultures for cheese making. Bacteriophage require Ca^{2+} ions to function (6) and by addition of phosphates to the media, Ca^{2+} activity is reduced and phage activity inhibited (38). The question arises as to whether such addition of phosphates affects coagulation properties of milk.

EXPERIMENTAL

Enzyme

An ion-exchange purified sample of chymosin (E.C. 3.4.23.4) was obtained from New Zealand Cooperative Rennet Co., Ltd., Eltham, New Zealand. Dilutions were made each day from a stock solution of 80 Rennet Units (RU)/mL (9) with distilled water and stored at 4°C between use.

Substrate

Raw whole milk was obtained from the Utah State University Dairy Products Laboratory. Spray dried nonfat dry milk (NDM) was reconstituted at a level of 12 g + 100 mL.

Bulk Culture Media

Whey-based media (WB) was prepared in the laboratory using 5% whey solids and .4% yeast extract. To one sample of whey-based media was added 0.75% (w/w) phoshate salts (WBP). IN-SURE™ internal pH-controlled bulk media (IC) was obtained from Stauffer Chemical Co. MSM™ milk-based culture media (MC) was obtained from Marschall Div., Miles Laboratories and made to 11% solids.

Four methods were used to monitor coagulation and curd formation.

Formagraph Method (22)

Ten milliliter aliquots of milk substrate were placed into sample wells in the cuvette and equilibrated at 35°C for 30 min. Enzyme

solution was added simultaneously to each sample and coagulation was detected by induction of movement of small wire-loop pendulums in the linearly oscillating samples. A light flash at the end of each oscillation stroke was registered on self-developing photographic paper and coagulation time was measured as the point of divergence of the lines on the firmness versus time diagram.

Moving Film Method

Twenty five milliter aliquots of milk substrate were added into 125 mL wide mouth bottles and placed in the apparatus described by Sommer and Matsen (33) and maintained at 35°C. Coagulation time was defined as the first appearance of graininess in the moving milk film inside the revolving bottle.

Rotary Viscometry Method (20)

Five hundred milliliters of milk substrate in a 600 mL glass beaker was placed inside a water jacket maintained at 35°C. Changes in viscosity were monitored using a Brookfield Model LVT viscometer with No. 1 spindle at 30 rpm. Near the coagulation time viscosity readings were recorded every 5 sec. Coagulation time has been defined as the intersection with the baseline of an extrapolation from the rapidly increasing portion of the viscosity curve.

Turbidity Method (24)

Three milliliters of the milk substrate was added to a 3mL disposable cuvette maintained at 35°C in a Beckman DU-8B UV/Vis spectrophotometer. An appropriate volume of enzyme solution (10-60 μL)

was added and changes in turbidity were measured at a wavelength of 600 nm. Substrate before enzyme addition was used as the reference and set to zero absorbance. A slit width of 2 nm was used and the average of two turbidity measurements was output via an RS232 connection to a Tektronix 4052 microcomputer every 4 sec. A computer program was written to collect and store the data, calculate and smooth the first derivitive, and plot the data on a Tektronix 4662 plotter. Over a 30 min period 450 data points were collected.

Calcium Studies

Milk substrates were prepared by reconstituting 12 g NDM in 100 mL of .5, 1, 5, 10, 50 or 100 mM $CaCl_2$ (distilled water was used as a control). The pH of each substrate solution was then adjusted to 6.3 and stored at 4°C for 18 h. An alternate method of preparation was to disperse 13.2g NDM in 100 mL of distilled water and then added 10 mL of a $CaCl_2$ solution of eleven times the desired final molarity.

Changes in coagulation time were measured using the Formagraph method in which 250 µL of a 0.8 RU/mL chymosin solution was added to 10 mL of milk substrate. The enzyme activity required to give a Formagraph CT = 15 min was determined from regression equations of CT versus inverse of enzyme activity for each level of calcium addition. The effect of calcium on coagulation and gelation was studied using the Formagraph and turbidity methods.

Comparison of the four methods was carried out by warming 620 g of milk substrate to 35°C, maintaining 35°C for 30 min and then adding 15 mL of the appropriate enzyme solution (CT = ca. 15 min). Subsamples

were used to monitor coagulation simultaneously by the turbidity, rotary viscometry, moving film and Formagraph methods.

Phosphate Studies

Raw whole milk was used as substrate. To each 10mL aliquot at 35°C in a Formagraph sample cuvette was added 100 µL (1%) of phosphate solutions of 0.04, 0.08, 0.1, 0.2, 0.4, 0.8, 1 or 2 M concentration. Distilled water was used as a blank. The phosphate solutions were prepared from K_2HPO_4/KH_2PO_4 mixtures so that their pH was 6.3 ± .03. To one of the samples was added 200 µL of 2M phosphate solution to give a final addition of 40mM in the milk. The samples were equilibrated at 35°C for 30 min after which 200 µL of an 0.8 RU/mL chymosin solution was added. Triplicate analyses were performed.

Bulk Culture Media Studies

Samples of the media were adjusted to pH 6.3 ± .02 with lactic acid/NaOH. Samples of IC and MC were also prepared at the pH typical after culture growth, and when added to milk in the cheesemaking process, ie. pH 5.4 and pH 4.9 respectively. Raw whole milk was heated to 35°C and 10mL aliquots were pipetted into sample wells in a Formagraph sample curvette. To these were then added 200 µL of a 0.8 RU/mL chymosin solution and coagulation was monitored using the Formagraph. Duplicate analyses were performed.

RESULTS

Coagulation time initially decreases with addition of calcium and reaches a minimum CT at 0.05 M calcium. At higher calcium levels CT increases and at very high levels (0.4 M Ca) it is severely retarded (Figure 11). By increasing the enzyme activity CT can be restored to its original value, but this requires 14 times as much enzyme activity in 0.4 M Ca milk substrate than if no calcium were added. Regression equations of CT versus the inverse of enzyme activity yielded correlation values of $R^2 \geqslant 0.99$ at all levels of calcium addition. The enzyme concentrations calculated to produce coagulation in 15 min are shown in Table 9. The actual CTs using these estimated enzyme concentrations were in the range 15 ± .6 min. Enzyme activity was reduced to a minimum for Ca addition at 0.01 M in order to produce coagulation in the specified time. The slope of the regression equations showed similar effects.

Table 9. Estimated chymosin activity required to produce visible coagulation in 15 ± .6 min. 12 g NDM + 100 mL, pH 6.45, 35°C, 2.5% (v/v) enzyme solution added.

Calcium Added (M)	Slope of Regression equation ($min.RU.mL^{-1}$)	Estimated Chymosin Activity ($RU.mL^{-1}$)	Percent Of Control
0 (control)	6.59	.454	100
.0005	6.63	.447	98
.001	5.75	.398	88
.005	4.11	.278	61
.01	3.04	.209	46
.05	2.27	.154	34
.1	4.83	.337	74

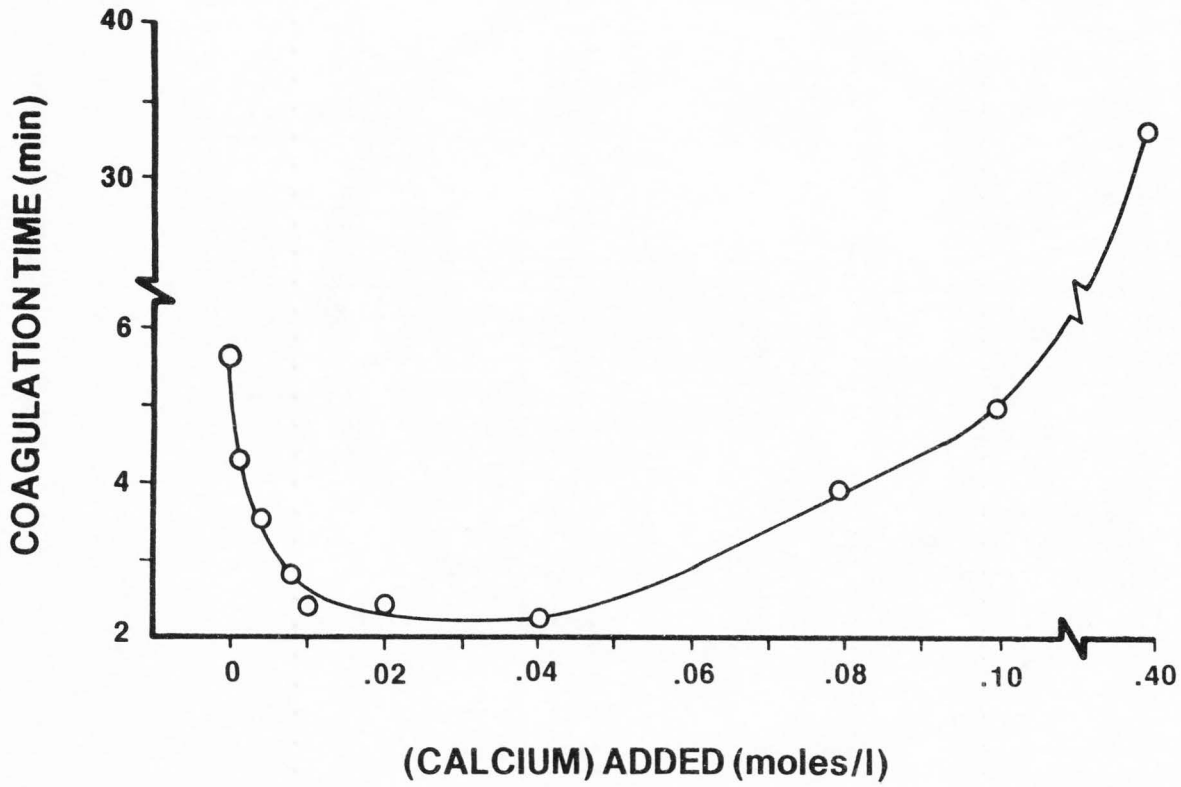

Figure 11. Plot of Formagraph coagulation time versus added calcium content of reconstituted NDM, 12g + 100mL, 35°C, pH 6.5, 2.5% of .8 RU/mL chymosin solution.

Rate of curd firming also changed with calcium addition, as did the final curd firmness (Figure 12). Curd firmness initially increased and then decreased after addition of 0.05 M calcium. Addition of 0.1 M calcium gave a CT approximately equal to the original value but curd firmness had been reduced considerably. If enzyme concentrations were adjusted to give equal CTs the curd firming rate decreased after addition of 0.01 M calcium (Figure 13).

When monitoring turbidity of undiluted milk substrate, a shoulder is observed in the turbidity versus time curve prior to visual observation of coagulation. As calcium was added this became more pronounced and was observed as a separate peak at a concentration of 0.01 M added calcium (Figure 14). Position of the shoulder relative to CT, was not altered. The final absorbance value decreases slightly on addition of up to 0.02 M calcium and decreases rapidly after that. At short coagulation times this peak was more pronounced (Figure 15), but again its relative position was not changed. The rate of increase in turbidity is greatest at higher enzyme activity but the turbidity approachs the same maximum value.

Addition of phosphate reduces coagulation time with a minimum occurring at 0.01 M (Figure 16). Addition of 0.04 M phophate increases CT over its original value .

Addition of WB, WBP or MC medias adjusted to pH 6.3 did not cause any change in coagulation time (Figure 17). IC media at pH 6.3 caused retardation in CT but when adjusted to pH 5.4, CT decreased. A similar decrease was observed with MC at pH 4.9.

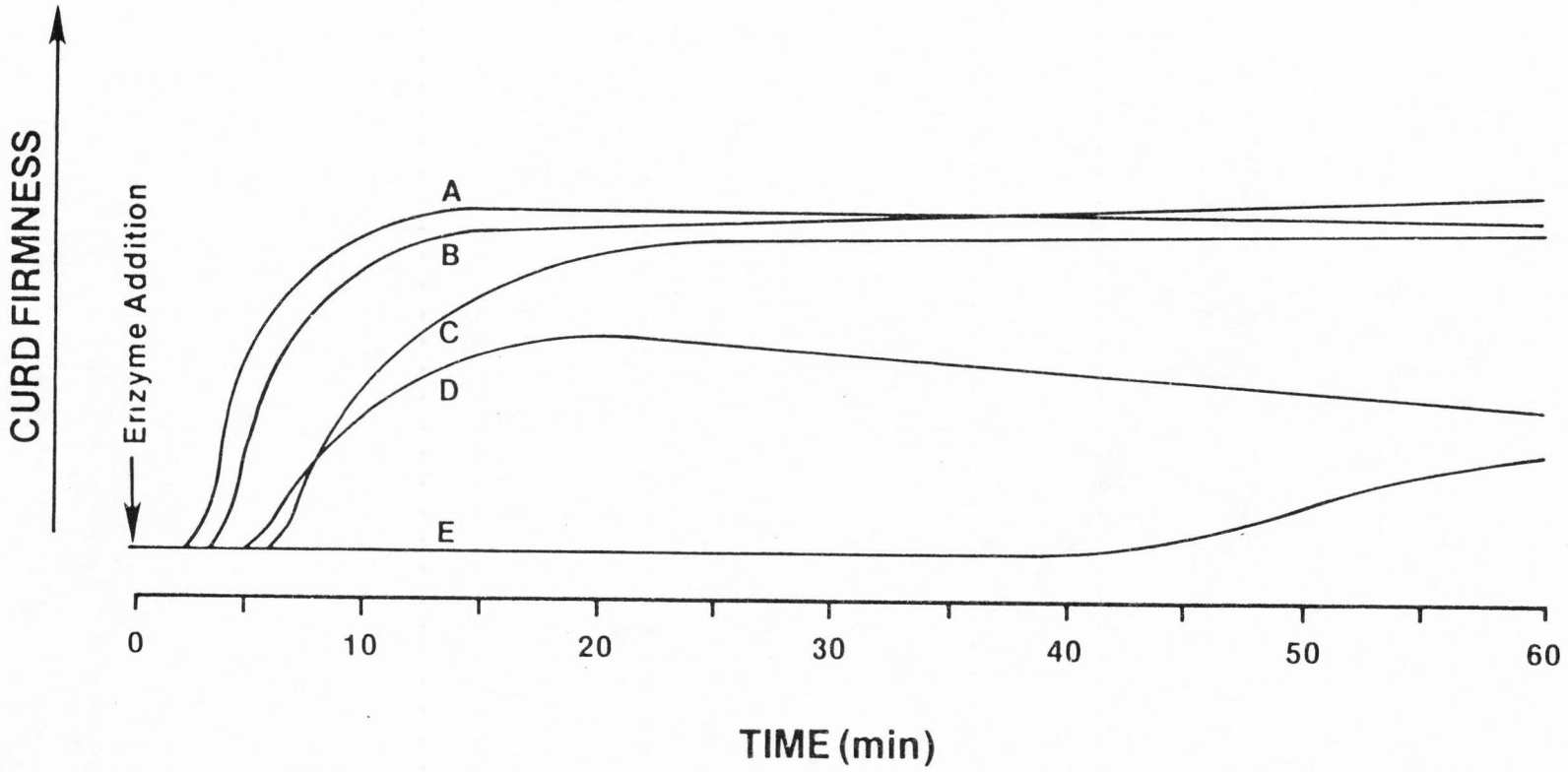

Figure 12. Formagraph firmness versus time diagrams of reconstituted NDM, 12g + 100mL of various $CaCl_2$ solutions, 35°C, pH 6.5, 1.67% of 1.6 RU/mL chymosin solution. A: .004M, B: .01M, C: H_2O, D: .1M, E: .4M $CaCl_2$.

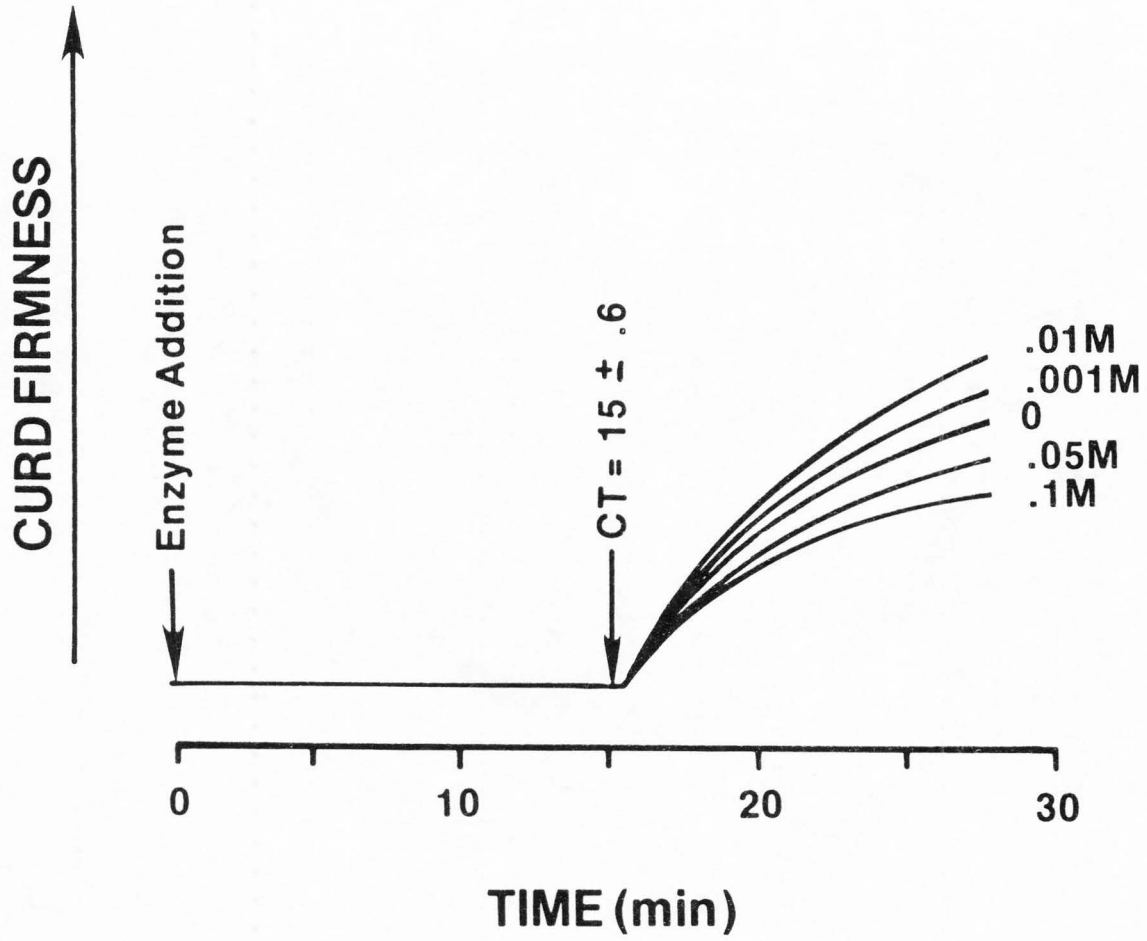

Figure 13. Formagraph firmness versus time diagrams of reconstituted NDM, 12g + 100mL, in various $CaCl_2$ solutions, 35°C, pH 6.45, CT = 15 ± .6 min.

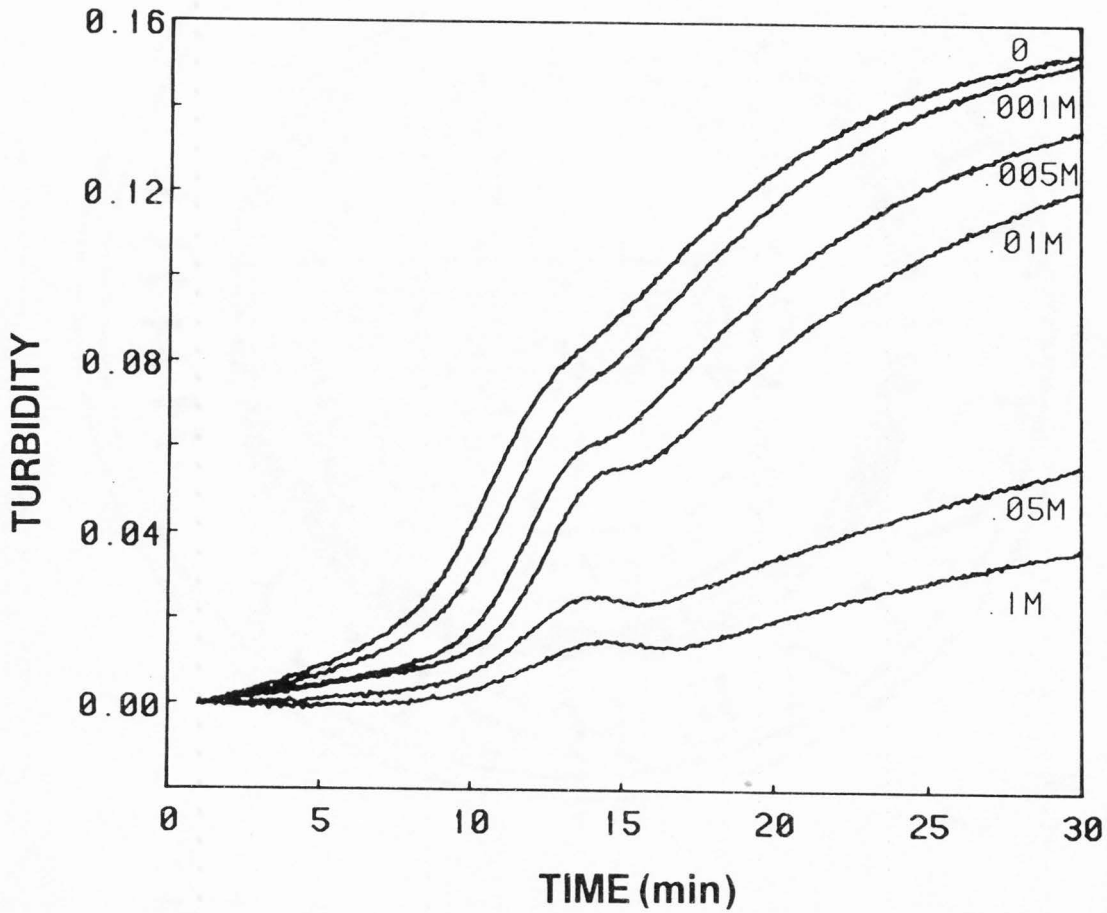

Figure 14. Turbidity at 600nm of reconstituted NDM, 12g + 100mL in various $CaCl_2$ solutions, 35°C, pH 6.45, CT = 15 ± .6 min. Turbidity of blank = 3.2.

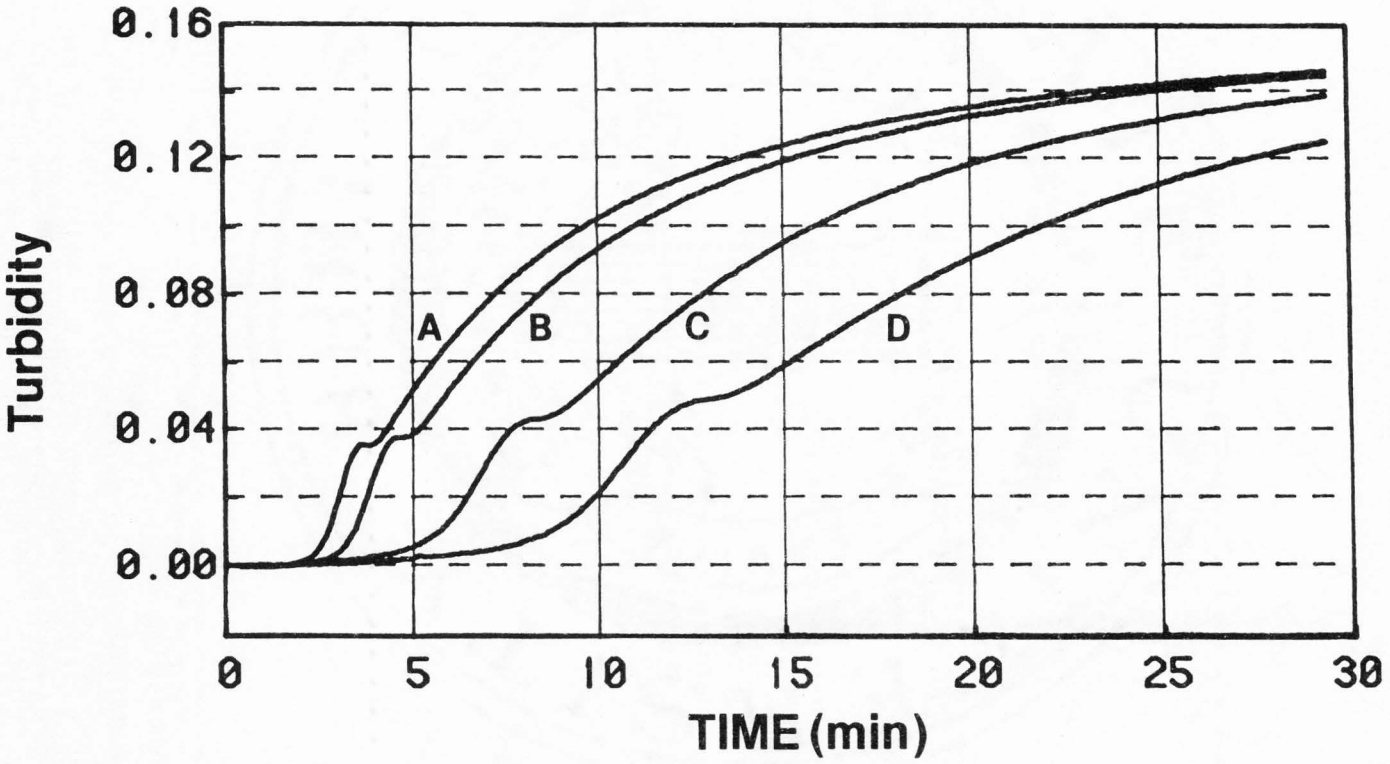

Figure 15. Turbidity at 600nm of reconstituted NDM, 12g + 100mL 7.2mM $CaCl_2$, 35°C, pH 6.45. Chymosin concentration in milk was A: .0266, B: .0213, C: .0107, D: .0053 RU/mL. Turbidity of blank = 3.2.

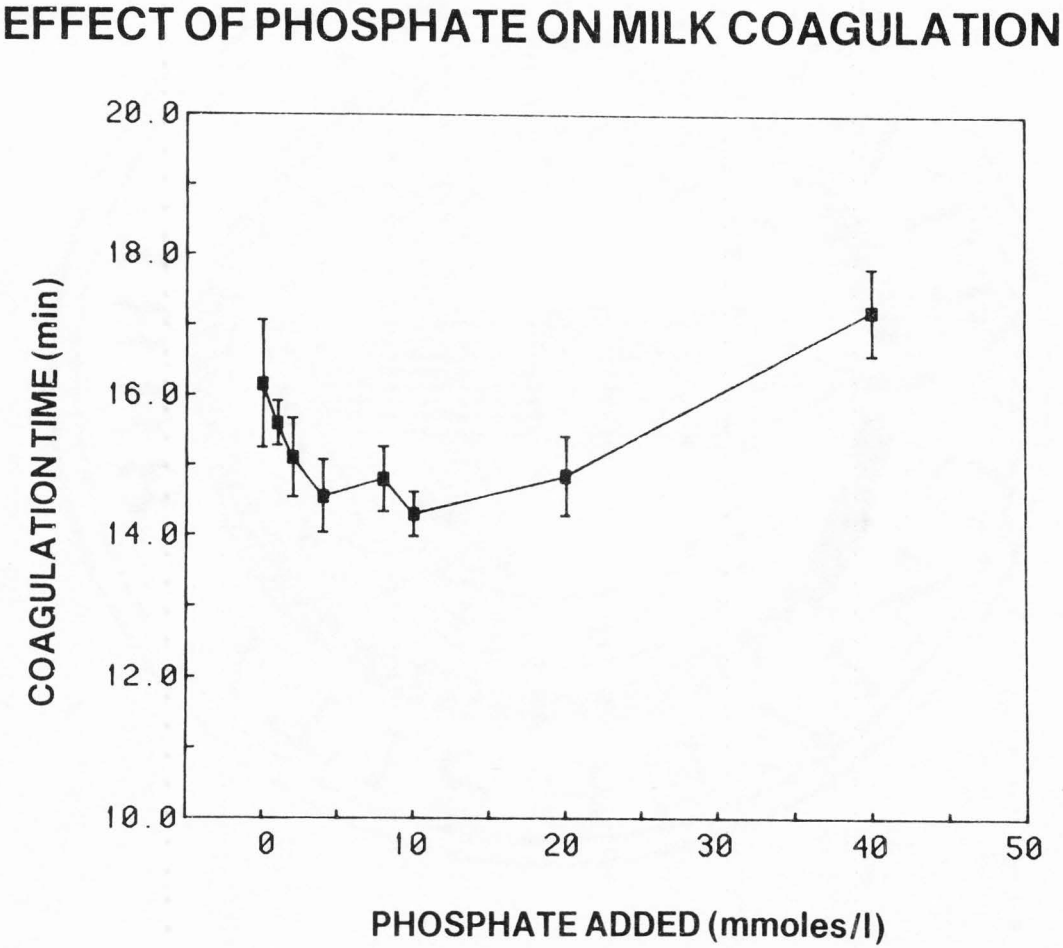

Figure 16. Effect of phosphate addition on Formagraph coagulation time of raw whole milk, 35°C, pH 6.67, 2% of .8 RU/mL chymosin solution added. Error bars represent 95% confidence intervals from triplicate analyses.

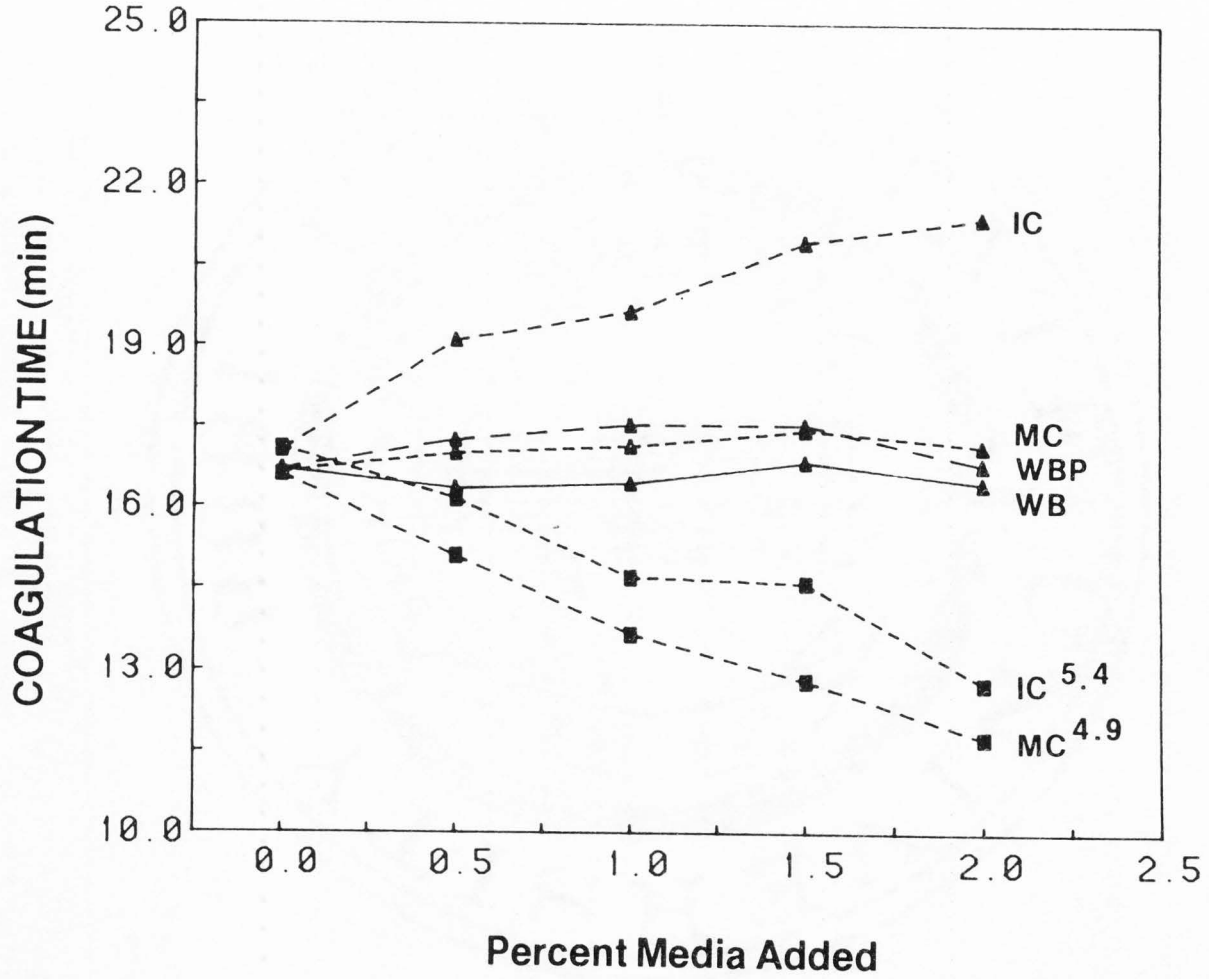

Figure 17. Effect of bulk culture media on Formagraph coagulation time of raw whole milk, 35°C, pH 6.67, 2% of .8 RU/mL chymosin solution added. Points represent average of duplicate analyses. ——— whey-based media (WB), — — — whey-based media plus phosphates (WBP), - - - - milk culture media (MC), - - - - - internal pH-controlled media (IC). ▲ pH 6.3, ■ pH 5.4 - IC, pH 4.9 - MC.

DISCUSSION

High turbidity of undiluted milk normally prevents measurement of changes in turbidity that occur during coagulation. At a wavelength of 600 nm, milk substrates prepared in this study yielded absorbance of approximately 3.2 absorbance units. The single beam nature of the Beckman DU-8B spectrophotometer and a slit width of 5 nm allows sufficient light to reach the photomultiplier even with such high background absorbance. Prior to enzyme addition, the instrument was set to read zero absorbance and changes in absorbance typically in the range 0 to .1 absorbance units recorded.

Monitoring enzymic milk coagulation by turbidity changes of undiluted milk is complimentary to measurement of changes in curd firmness. Turbidity coagulation curves cannot be related directly to molecular weight changes because of the non-ideal light scattering effects of such a concentrated colloidal dispersion but this method is sensitive to the initial stages of micelle aggregation and demonstrates that the process of aggregation begins well before any visual observation of coagulation is possible (Figure 18).

An increase in turbidity was observed two minutes after enzyme addition even though coagulation was not observed for approximately 15 min. Occurrence of two peaks in the first derivitive of the turbidity curve indicates that two separate phases of micelle aggregation are occurring. It has been postulated that the first peak represents formation of micelle aggregate clusters and that the second peak represents formation and consolidation of the gel network (24). Detection of coagulation by the other methods does not occur until formation of the gel network structure has begun and the milk system has

EFFECT OF METHOD ON MILK COAGULATION TIME

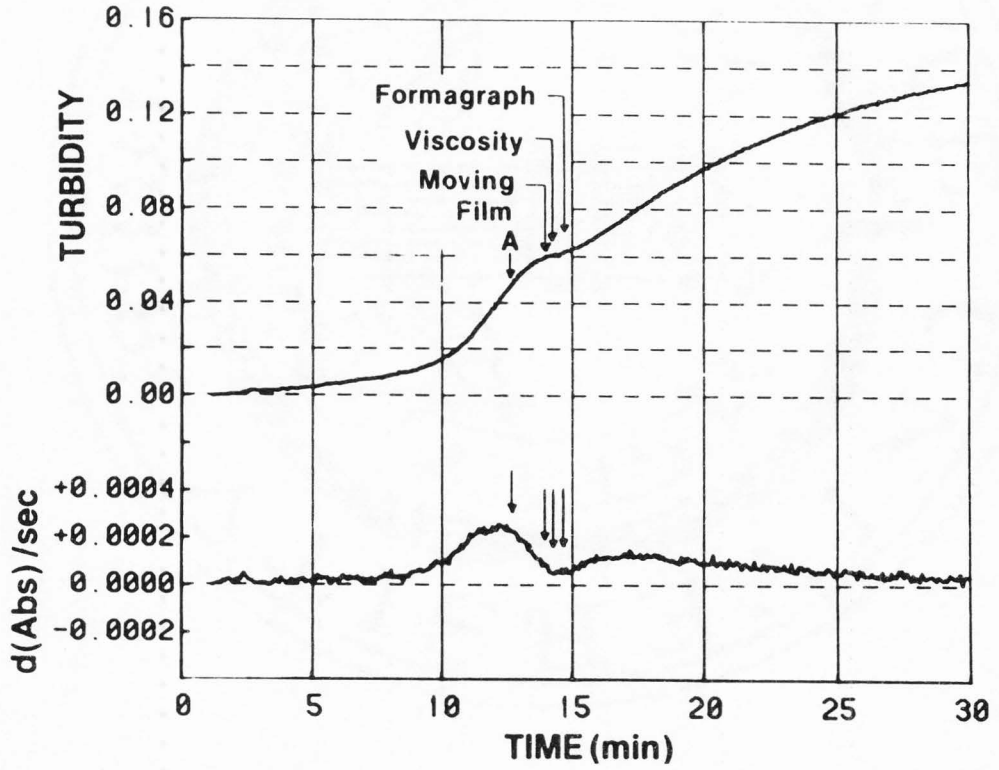

Figure 18. Turbidity at 600 nm of reconstituted NDM, 12g + 100mL .005M $CaCl_2$, pH 6.45, 35°C. Arrows represent coagulation time measured by Formagraph, rotary viscometry and moving film methods, and A represents a slight increase in observed viscosity.

ceased to function as a true fluid of constant viscosity (34). As the gel network becomes more extensive the turbidity gradually increases to a maximum, after which the effects of syneresis cause a reduction in turbidity. At each calcium concentration the relative order of coagulation detection is: turbidity, moving film, rotary viscosity, and then the Formagraph method. Measurement of coagulation time by any of these methods corresponds to attainment of a particular degree of coagulation and therefore, should not be considered an integral part of the coagulating system (23). A slight increase in viscosity occurred prior to the phase of rapid viscosity increase at the rotary viscometry CT. Such an increase in viscosity was also observed using the moving film method. The film of milk on the inner surface of the bottles not only had a thicker appearence but moved more slowly down the bottle wall. It occurs at approximately the same time as the most rapid rate of turbidity increase during the phase of coagulation when aggregate clusters are rapidly growing. As the clusters increase in size they reach a critical volume when any further collisions produce a three dimensionally crosslinked network of casein micelle chains. When this occurs the viscosity and elasticity of the milk increases rapidly and prevents the milk from behaving as a true fluid when subject to shear: such as that caused by gravitational forces as it flows down a moving surface, or rotation of an immersed cylinder. The milk also gains sufficient firmness to inhibit movement of the Formagraph wire loop pendulum through it and causing a divergence of the reflected light flashs at each end of the oscillation stroke.

The NDM substrates were allowed to stand for 18 h after being reconstituted to give time for the protein and salt molecules to become

completely hydrated. Before enzyme addition substrates were maintained at 35° C for 30 min to allow time for the colloidal casein micelle system to re-equilibrate. This is a necessary procedure for consistent measurements of CT to be obtained (1,8). Any pH adjustments of milk systems must take this into consideration, pH rises approximately .15 pH units during 18 h equilibration. Cold storage reportedly causes calcium, magnesium, and phosphate to dissociate from the micelle and consequently increases [Ca^{2+}] and pH (29). No further change in pH was observed upon heating the milk to 35°C. Calcium was added before equilibration because the calcium - phosphate system is notoriously slow and CCP is perhaps not in a true equilibrium with the serum environment (12). Addition of Ca^{2+} also causes transfer of soluble casein to the micelles (4). Mineral exchanges are controlled by solubility of the salts involved and an important function of calcium phosphate may be to slow down exchange of calcium between the micellar and serum phases (27).

Addition of $CaCl_2$ up to .0025 M results in a linear increase in Ca^{2+} activity as measured by a Ca^{2+} sensitive electrode (7) and two thirds of the added Ca^{2+} converts to complexed forms of calcium. Above 0.005 M essentially all added Ca^{2+} remains in the free ionic state.

Calcium activity varies among samples of reconstituted NDM as a result of changes in the calcium equilibrium due to heat treatment. Such properties have been used as a measure of heat treatment of NDM (25). Calcium activity of high heat NDM is only of the order .8 mM while in low heat NDM it approximates that of raw milk, i.e. 3 mM. Heat treatment of skimmilk at 85°C for 30 min causes an average transfer of 6 mg Ca and 4 mg P per 100 mL from the soluble to the colloidal state (18). This is reversible on cooling. Decreased Ca^{2+} activity from high

heat treatment during drying, however, is irreversible and results in formation of a softer curd upon coagulation (25). Calcium addition after heat treatment partially restores coagulability (18). Such effects have been observed in this laboratory using spray dried, low heat NDM to prepare 0.01 M $CaCl_2$ Berridge substrate (2). When coagulation was monitored using the turbidity method it produced a coagulation curve corresponding to previously used NDM reconstituted in 0.03 M $CaCl_2$. To avoid any ambiguous results due to variations in Ca^{2+} activity (or CCP) between NDM samples all experiments in this study were carried out using the same batch of NDM.

The maximum permissible level of $CaCl_2$ added to cheese milk of 0.02% (w/w) or 0.0018 M results in a firmer curd and a shorter clot-to-cut time (Figures 12 and 13). However, from turbidity studies there was a lowering of final absorbance value as calcium is added indicating that the gel network is less extensive (Figure 14). Increasing calcium concentration in milk may in fact enhance aggregation while inhibiting gelation as is evidenced by separation of these phases in the turbidity curves when calcium is added. After coagulation has occurred it has been reported that the chains of casein micelles group together into strands of about five micelles in thickness (13). A retardation of this process by excess calcium could account for curdiness in cheese produced using .02% $CaCl_2$ added to the milk (11).

At high calcium concentrations (.4 M) coagulation time is severely retarded. This is in part due to the effect of calcium (or high ionic strength) upon enzyme activity because retardation of CT can be overcome by increasing enzyme activity. To restore CT to its original value, i.e. with no added calcium, the amount of chymosin required must be

increased 14 times. At such high calcium concentrations curd has a very low firmness and is very easily broken. The dual effect of calcium addition on coagulation can be seen in Figure 12 where the same amount of enzyme was added to each sample. Coagulation time decreases upon addition of up to 0.05 M calcium and rate of curd firming increases (mainly as a function of shorter coagulation time). Such effects on rate of curd firming have been attributed to changes induced in the electrostatic attraction operating between negatively and positively charged areas on contiguous enzyme-altered casein micelles (19). Calcium bridging between two negatively charged areas is also believed to be involved in this phenomenum. At 0.05 M levels the final curd firmness had decreased and at 0.1 M CT was approximately equal to the original value. If enzyme concentrations are adjusted to give equal CTs, curd firming rate initially increases then decreases after 0.01M calcium has been added (Figure 13).

Berridge substrate (2) which consists of 12 g NDM dispersed in 100 ml 0.01 M $CaCl_2$ is frequently used in milk coagulation assays for enzyme milk-clotting activity. The amount of decrease in CT upon addition of calcium varies with the milk-clotting enzyme used (14, 28). Standardization of enzymes on the basis of rate of formation of a visible clot in such a substrate may not be a good index of their ability to produce an adequately firm curd at the same rate in milk (21).

The effect of added phosphate in reducing coagulation time was surprising as it had been assumed that phosphates would merely reduce Ca^{2+} activity and therefore retard coagulation. One possible explanation relates to the phosphate being added only 30 min before

enzyme addition. This was done to simulate the effect of adding bulk culture media, containing phosphates, to milk during cheese making. The calcium phosphate system is extremely slow in equilbrating and at low added phosphate levels the phosphate was found to enhance coagulation while at high levels the equilibrium was perhaps forced sufficiently in the direction of complex formation with Ca^{2+} that coagulation was retarded. High ionic strength may be another factor affecting coagulation rate. Without the presence of phosphate, rennet treated whole casein in the presence of Ca^{2+} forms only a flocculent precipitate and not a firm gel as is typical in its presence (37). Further work is required to elucidate the role phosphate plays in coagulation.

Dephosplorylation of casein increases CT of milk (17). Calcium binding to phosphoester groups of β-casein is also important for development of curd firmness (5). Coagulation of dephosphorylated casein is less dependent on Ca^{2+} activity than native casein due to it having less binding sites for calcium (35). Increasing calcium concentration would tend to saturate calcium binding sites of α_s- and β-casein (32) and thus enhance coagulation. When all sites are saturated further additions of calcium are unable to reduce CT and retarding effects of Ca^{2+} on coagulation are able to predominate. In 0.02 M $CaCl_2$, dephosphorylated casein clots more slowly and produces a softer gel although the amount of incorporated casein is the same (36).

It appears from Figure 17 that the pH of culture media has the greatest effect on coagulation time. At pH 6.3 media retards coagulation but when its pH is reduced to a value typical after culture growth (and when added to milk for cheese making) it shortens coagulation time. The whey-based media used in external pH-controlled

systems is typically at pH 6.0 after culture growth and changes CT very little even when phophates are included in the media formulation. Milk-based culture media (MC) also reduces CT when adjusted to a low pH. Further studies are needed to determine the exact effects of culture media on not only coagulation rate but also curd firming properties and cheese yield.

SUMMARY

Monitoring enzymic milk coagulation by turbidity changes of undiluted milk is complimentary to measurements of changes in curd firmness. Turbidity measurements are sensitive to initial stages of micelle aggregation and demonstrate that aggregation begins well before any visual observation of coagulation. A point is reached during aggregation in which a three dimensionally crosslinked network of casein micelles is formed, and following this, coagulation can be observed visually or rheologically.

Coagulation time initially decreased upon calcium addition and reached a minimum at .05M calcium. At high calcium levels (.4M), coagulation time is severely retarded. Curd firming rate increased and reached a maximum at .01M calcium. At higher calcium levels the curd firmness is reduced. Turbidity of milk after coagulation decreased on calcium addition suggesting that calcium affects the extensiveness of the gel network. Addition of phosphate 30 min prior to enzyme addition also reduces coagulation time with a minimum occurring at .01M phosphate. When bulk culture media was added, the pH of the media had a greater effect on coagulation time than did the presence of added phosphates.

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PART 4B. EFFECT OF ENZYME TYPE ON MILK COAGULATION

INTRODUCTION

Limited availability of calf rennet in recent years has resulted in the widespread use of other milk-clotting proteinases for cheese making. The prime action of a milk coagulant is the hydrolysis of a specific bond in κ-casein that subsequently renders casein micelles unstable, in the presence of calcium ions, and induces coagulation. However, beyond such specific proteolysis, proteinases vary in their activity towards κ-casein and other milk proteins (5, 8). To be of use in cheese making, a proteinase must have proteolytic specificity similar to chymosin (EC 3.4.23.4) (26).

Most calf rennet substitutes are more proteolytic than calf rennet in relation to their milk clotting activity (9). If proteolytic activity is excessive, cheese yield and retention of fat in the curd may be diminished. Also, excessive proteolysis during ripening has undesirable effects on the body and texture of the finished cheese (9). Animal proteinases used for cheese making include calf rennet, adult bovine rennet, porcine pepsin (EC 3.4.23.1), and chicken pepsin. Proteinases of microbial origin (EC 3.4.23.6) include those extracted from Mucor miehei, M. pusillus var. lindt and Endothia parasitica.

Calf rennet is extracted from abomasa of milk-fed calves of a range of ages (9). At birth, chymosin is present in gastric mucosa at concentrations of 2 - 3 mg/g but its production declines after one week (6). During the first week of life, pepsin is virtually absent but a rapid increase in its production takes place after about three weeks. Pepsin replaces chymosin as the principal gastric enzyme of the bovine within a relatively short time after birth. Also, the ratio of chymosin to pepsin changes considerable for a calf even from morning until

evening (7). In order to aid the cheese manufacturing industry, it has been suggested that commercial milk coagulants need to be carefully standardized and be of known composition (9).

Different commercial manufacturers have their own standards and specifications for percent composition of each enzyme in a commercial coagulant and for overall percent strength (25). Calf rennet is predominantly chymosin (85 - 95%), with the remainder being bovine pepsin. Adult bovine rennet may contain 55 - 60% bovine pepsin. Mixtures of calf rennet and porcine pepsin may contain 40 - 45% chymosin, 5 - 10% bovine pepsin and 50% porcine pepsin. Mixtures of adult bovine rennet and porcine pepsin typically contain 20 - 25% chymosin, 40 - 45% bovine pepsin and 30 - 40% porcine pepsin. Microbial rennets generally contain 100% of the native milk-clotting proteinase and mixtures with animal coagulants are not widely used (25).

It is important to the cheesemaker to be aware of the pepsin content of calf rennet in order for a consistent product to be manufactured. Proteolytic activity of the coagulant will affect not only flavor and texture of a cured cheese (9), but also curd firming rates during cheese production (27).

Strength of a milk gel at the time that it is cut during cheesemaking is important for maximum recovery of milk components in cheese (19). Variations in curd firmness at cutting can result in reduced cheese yield. A consistent firmness at cutting is thus critical for optimum cheese manufacturing. Conventionally, many cheesemakers cut curd 30 min after adding the coagulant in order to conform to time requirements and factory schedules. A slow set means the curd must be cut when it is more fragile than desired, or cutting must be delayed.

If the curd is too soft when cut, excessive fat losses and a reduced yield may be expected (4). When cutting is delayed time is lost and the cheesemaking schedule upset. Knowing curd firming properties of a coagulant aids in maintaining consistency during cheese manufacture and in optimizing cheese yields.

Numerous methods have been devised to identify components in a coagulant mixture (10) and in particular to measure the content of bovine pepsin in bovine rennets (2). They include gel diffusion (20), anion exchange chromatography (7, 18, 23), proteolytic to milk clotting activity (16), ratio of milk-clotting activity at different pH values (23, 24), immunoelectrophoresis (23, 24, 1), immunodiffusion (3, 13), isoelectric focussing (11, 22) and selective denaturation (17). A simple way of determining pepsin content in bovine rennets, as well as to observe the effects of non-specific proteolytic activity, was developed by measuring differences in curd firming rates of chymosin and bovine pepsin.

EXPERIMENTAL

Milk substrate was prepared by dispersing 12 g low heat nonfat dry milk (NDM) in 100 mL of .005 or .01 $CaCl_2$ solution. Substrate solutions were then refrigerated for 18 h to allow time for complete hydration of the NDM. Thirty minutes prior to analysis the milk substrate was warmed to 35°C and maintained at that temperature throughout the analysis.

An anion exchange purified chymosin solution was obtained from the New Zealand Cooperative Rennet Co. Ltd., Eltham, New Zealand. Trypsin (EC 3.4.21.4) and papain (EC 3.4.22.2) were obtained from Sigma Chemical Co. Bromelain (EC 3.4.22.4) was obtained from Calbiochem-Behring Corp. Calf rennet and adult bovine rennet (BOVIN™) were obtained from Chr. Hansen's Laboratory Inc. M. pusillus proteinase (EMPORASE™) was obtained from Dairyland Food Laboratories, Inc. M. miehei proteinase (MARZYME II™) and CHYMOSET™, a mixture of calf rennet and porcine pepsin, were obtained from Marschall Div., Miles Laboratory Inc. Bovine pepsin was purified using anion exchange chromatography (7) from a sample of adult bovine rennet.

Changes in curd firmness upon addition of coagulant to milk substrate at 35°C were recorded using the Formagraph (14).

RESULTS AND DISCUSSION

Curd Firming Rate

Time after observed coagulation (CT) until a width of 20 mm is obtained by the Formagraph firmness-time graph has been defined as k_{20} (14). It can be considered as a linear estimate of the inverse of initial curd firming rate. The slower the rate of curd firming the longer it will take for the curd to reach a firmness adequate for cutting and hence the larger will be the value of k_{20}.

Firming rate is dependent on CT. At short CT, increase in curd firmness occurs at a more rapid rate compared to milk substrate containing a lower enzyme activity. Valid comparisons of coagulant affect on curd firming require CT to be the same in each case. To avoid the trial and error process of matching CTs by enzyme dilution, measurements of k_{20} were made over a range of enzyme activities and least squares regression analysis used to estimate k_{20} when CT = 10 min. It has been reported that the relationship between CT and cut-to-clot time is not linear when curd firmness is measured by resistance to oscillatory deformation, e.g. for calf rennet $r^2 = .24$ (12). In this study, linear regressions of k_{20} versus CT were obtained with $r^2 \geq .97$ for all of the coagulants used (Figure 19).

Rate of increase of curd firmness decreases as the extent of proteolysis of casein increases and it has been reported that curd firming rate for calf rennet > pepsin > *M. miehei* and *M. pusillus* proteinases > papain > trypsin (27). Similar results were obtained in this study (Table 10) with the exception of pepsin which gave a slower rate of curd firming than the microbial proteinases.

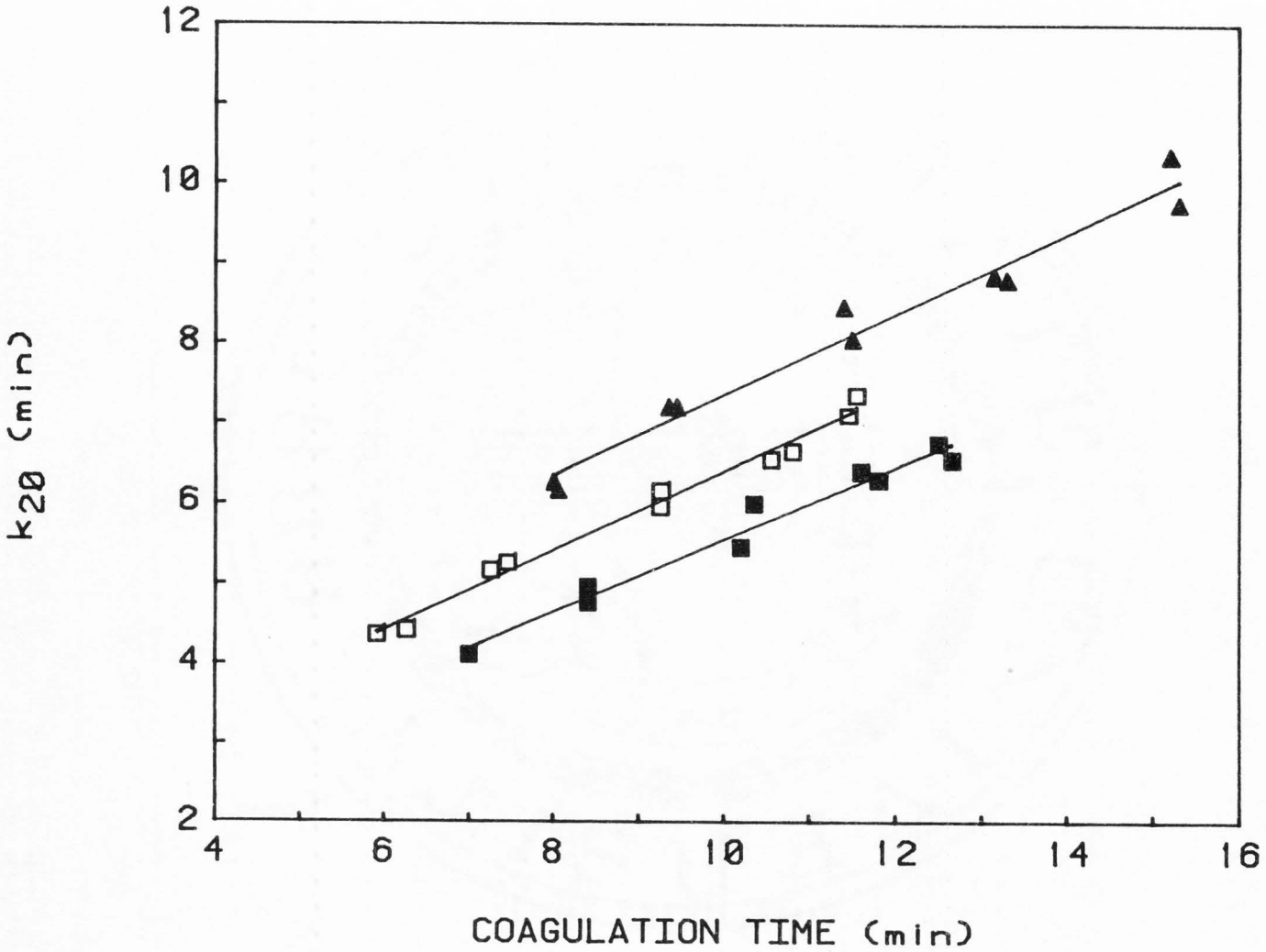

Figure 19. Plot of k_{20} versus coagulation time of three milk coagulants added to reconstituted NDM (12g + 100mL .005M $CaCl_2$), 35°C, pH 6.45. ■ calf rennet, ▲ bovine pepsin, □ M. miehei proteinase.

Table 10. Estimated k_{20} when CT = 10 min for various milk coagulants added to reconstituted NDM (12 g + 100 mL .005M $CaCl_2$), 35°C, pH 6.45.

Coagulant	Estimated k_{20} (min)	95% Confidence Interval (min)	Regression r^2
Chymosin	5.35	.092	.98
Calf rennet	5.55	.14	.97
Adult bovine rennet	6.61	.089	.98
Bovine pepsin	7.36	.22	.97
Calf - porcine pepsin (50:50)	6.66	.13	.99
M. miehei proteinase	6.40	.11	.99
M. pusillus proteinase	6.53	.10	.99

Porcine pepsin gives values of k_{20} similar to bovine pepsin, but because of its instability at the test pH, measurements of curd firming using porcine pepsin are confounded with activity losses. Substantial losses of activity of porcine pepsin occur above pH 6 (5, 15) but no rapid drop in activity occurs with bovine pepsin as pH is increased (6). Milk-clotting activity of bovine pepsin is less pH dependent and at pH 6.35 (30°C) bovine pepsin is inactivated little if at all while porcine pepsin is (5). Assuming that during the test period (< 30 min) no activity of bovine pepsin was lost, bovine pepsin was used as a reference enzyme for comparing affects of non-specific proteolysis of milk coagulants.

All of the commercial coagulants induced curd firming at a slower rate than purified chymosin. When very proteolytic enzymes such as trypsin, bromelain or papain were used, a very weak curd was formed. Trypsin was so proteolytic that the flocculent formed was too weak to cause movement in the immersed pendulums and no value for CT or k_{20}

could be obtained using the Formagraph. When papain or bromelain were added to reconstituted NDM (12 g + 100 mL .01M $CaCl_2$), k_{20} values were three and thirteen times that for chymosin.

Phosphoserine groups of β-casein play an important part in curd firming (28). Proteolysis of β-casein by trypsin removes the C-terminal hydrophilic moeity of β-casein including the phosphoserine groups and this results in a fragile curd being formed (28). Proteolysis by chymosin removes a hydrophobic moeity near the N-terminus and does not appear to affect curd firming (29). Consolidation of the coagulum therefore involves interactions between Ca^{2+} ions and phosphoserine groups principally bound to β-casein molecules (28). One role of β-casein could thus be to keep calcium in the micellar network and enhance curd firming. The effect of high non-specificity of a milk coagulant would thus be a reduction in curd firming rate as a consequence of proteolysis of β-casein near the C-terminus.

The commercial milk coagulants were compared to determine if initial curd firming rate affected the final curd firmness. Each coagulant was diluted to give CT = 10 ± .3 min and tested (in duplicate) simultaneously using the Formagraph. Maximum curd firmness was measured 3 h after enzyme addition. Analysis of variance showed no significant differences between the type of enzyme on maximum curd firmness (α = .05).

Calcium fortified milk substrates such as used in this study are generally used to conduct coagulant testing (21). However, sensitivity of coagulants to calcium and pH varies (12) suggesting that when modifying cheese-make schedules, curd firming and CT data should be obtained from milk samples typically being used for production. On a

laboratory basis, greater consistency of results can be obtained when a calcium fortified milk substrate is used.

Chymosin - Bovine Pepsin Mixtures

The proportion of pepsin in bovine rennet extracts increases as abomasa of calves of increasing age are used (Table 11). Bovine rennets

Table 11. Ratio of chymosin to pepsin in bovine abomasal mucosa, expressed on a weight basis, by age (1).

Age	Chymosin %	Pepsin %
Sucking calves (1 month)	80	20
Milk-fed calves (3 months)	70	30
Concentrate-fed calves (6 months)	12	88
Heifers (12 - 24 months)	2	98
Cows (24 months)	Trace	100

will therefore contain varying amounts of these two enzymes depending on the source of abomasa (24). Assuming a coagulant is a binary mixture of chymosin and bovine pepsin, the curd firming rate would be expected to decrease as the proportion of pepsin increases. Mixtures of chymosin and bovine pepsin were prepared keeping CT constant for each mixture. Five different aliquots of each mixture were then added to milk substrate (12 g NDM + 100 mL .01M $CaCl_2$) and k_{20} estimated for CT = 10 min. A linear relationship was obtained between percent pepsin and estimated k_{20} in which r^2 = .98 (Figure 20). Under specified conditions, curd firming rate could therefore be used to estimate percent pepsin in a bovine rennet.

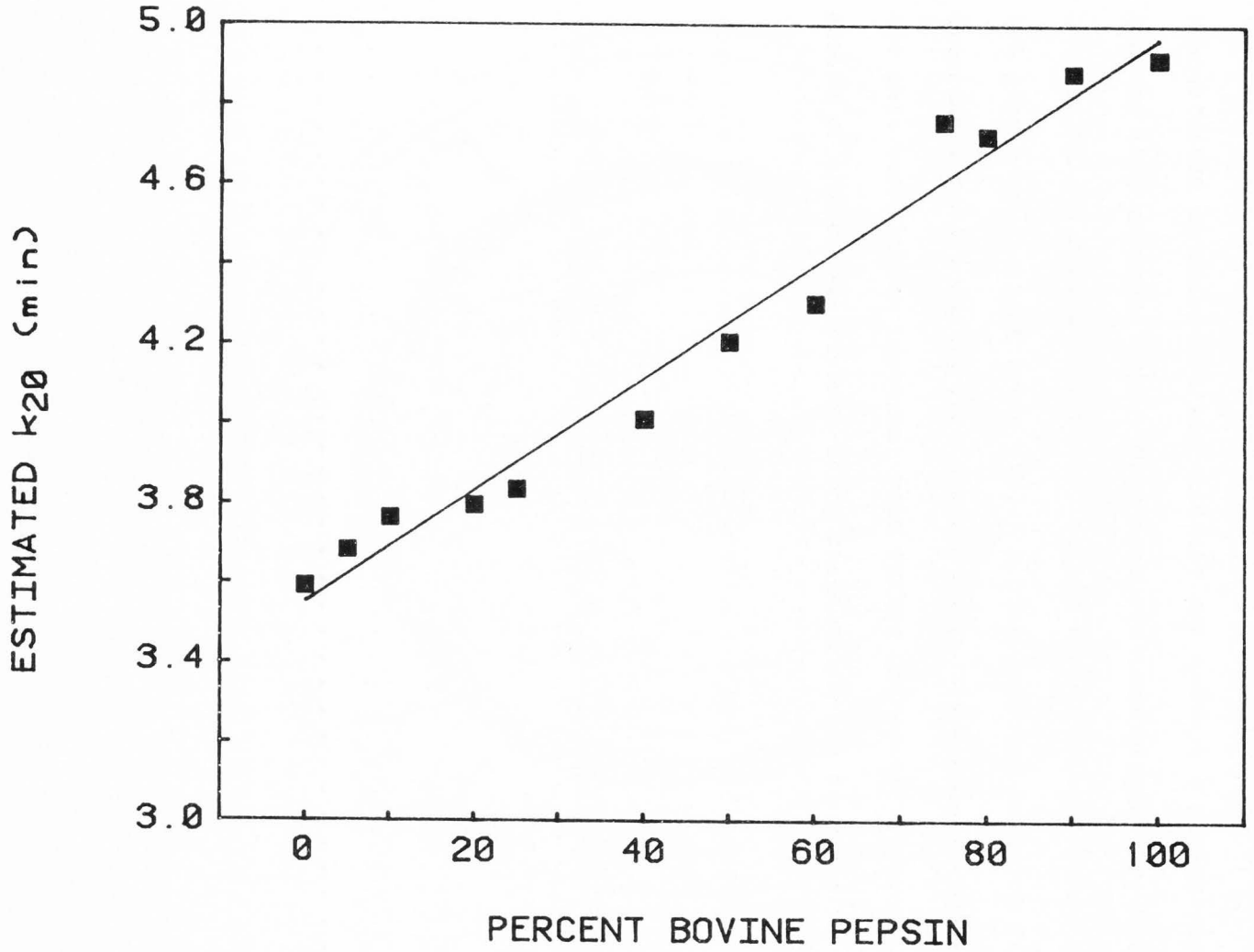

Figure 20. Linear regression of estimated k_{20} (CT = 10 min) versus percent pepsin in bovine pepsin/chymosin mixtures added to reconstituted NDM (12g + 100mL .01M $CaCl_2$), 35°C, pH 6.30. Each point represents a regression estimate from k_{20} values measured at five CTs.

These measurements (Figure 20) were based on percent pepsin according to milk-clotting activity at pH 6.3 and this can vary from enzyme concentration expressed on a weight basis (10). Assuming none of the enzyme has been inactivated, rennets with bovine or porcine pepsin standardized on milk-clotting activity may contain a higher pepsin content than expected (24). When measured immunologically, calf rennet containing 20% pepsin on a milk-clotting basis has been found to contain 44% pepsin on a weight basis (10). A 50:50 mixture based on milk clotting activity may in fact contain up to 77% pepsin on a weight basis and this may play an important role during non-specific proteolytic action of the enzyme mixture after coagulation has occurred.

SUMMARY

Non-specific proteolytic activity of milk coagulants affects the rate of increase in firmness of milk curd produced as a consequence of proteolysis of κ-casein. Cheese yield and fat retention in the curd is also reduced if proteolytic activity is excessive. A consistent firmness at the time of curd cutting is critical for optimum cheese manufacture. The rate at which an enzyme coagulates milk and the subsequent curd firming rate should be known by the cheese maker in order to maintain curd consistency and to optimize cheese yields.

Rate of increase of curd firming decreases as the extent of non-specific proteolsis increases. Relative curd firming rates were chymosin > calf rennet > M. miehei, M. pusillus proteinases > adult bovine rennet > papain > bromelain > trypsin. When porcine pepsin is used the effect of proteolytic specificity on curd firming is confounded with loss of activity of porcine pepsin at the pH of milk. Differences in curd firmness 3 h after enzyme addition were not significant between commercial milk coagulants. Non-specific proteolytic activity of proteinases possibly reduces curd firming rate by removing the C-terminal of β-casein that contains the β-casein phosphoserine groups.

Pepsin content in mixtures of chymosin and bovine pepsin was proportional to curd firming rate and could be used as a rapid assay for bovine rennets.

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PART 5. ENZYMIC MILK COAGULATION: ROLE OF EQUATIONS INVOLVING COAGULATION TIME AND CURD FIRMNESS IN DESCRIBING COAGULATION

INTRODUCTION

Coagulation of milk, induced enzymically by chymosin (EC 3.4.23.4), involves many processes that occur as a consequence of the initial limited proteolysis of κ-casein. Aggregation of resultant para-casein micelles occurs via a diffusion-controlled bimolecular process (3,2) and subsequent to the attainment of a critical point (5) a gel is formed. The processes that occur during curd firming and syneresis are less understood. Rates of the different stages of curd formation are important factors from a cheesemaking point of view since they will directly affect the resultant properties of cheese curd when a fixed make procedure is followed. Optimum cheesemaking requires an understanding of the quantitative effects of all variables and controllable factors on rate of curd production (26). These can be studied by measurements of increase in curd firmness both in the factory and the laboratory.

Numerous methods are available to measure milk coagulation time and to monitor development of curd firmness. Inspite of differences between techniques, the shape of firmness versus time curves are essentially the same. In general, changes in curd firmness due to enzymic action on milk can be described by an exponential equation (9,21). It holds for firmness measurements obtained from transmission of oscillatory motion through milk (9), induction of movement of an immersed wire-loop pendulum (15), or measurement of strain induced as a consequence of coagulation in a torsiometer (26):

$$G = G_{\infty} e^{-\lambda/t} \qquad (I)$$

where G is the pseudo modulus of rigidity, G_{∞} is the theoretical

maximum modulus at infinite time, t is time after coagulation point, and λ is time required after coagulation for the modulus to equal G_∞/e. Actual coagulation time occurs prior to coagulation time (CT) measured by observation of development of curd firmness. A finite amount of firmness must be developed before coagulation can be detected. An estimation of actual coagulation time has to be made before data can be fitted to equation I. To alleviate this problem equation I was modified so that non-linear regression techniques could be applied directly to firmness-time data to solve for all parameters of the prediction equation.

It has been proposed by Payens (18,20) that the lag period between enzyme addition and observed coagulation is the result of different orders of enzymic and aggregation reactions. Measured CT depends on the technique chosen to detect coagulation and consequently there is difficulty in defining CT other than by some empirical relationship. If a specific point in the overall coagulation process could be defined as the precise and actual coagulation time it would be of benefit in any interpretations of enzymic milk coagulation data. Effects of varying system parameters such as enzyme type and concentration, substrate composition and concentration, pH, calcium activity, temperature, micelle composition, etc., on coagulation time could then be more easily explained and would allow the eventual complete modelling of milk coagulation.

A comparison was made between changes in apparent absorbance measurements of undiluted milk as a result of chymosin addition and predictions of actual coagulation time from Formagraph firmness-time data using a modified version of equation I.

EXPERIMENTAL

Reagents

Milk substrate was prepared by dispersing 12 g low heat, nonfat dry milk (NDM) in 100 mL of water or calcium chloride solution of desired molarity. Such substrate solutions were then stored at 4°C for 18 h to allow complete hydration of milk constituents. Thirty minutes prior to enzyme addition the substrate was heated to 35°C (or the desired temperature) and maintained at that temperature for 30 minutes to allow the system to equilibrate.

An ion exchange purified chymosin solution of known activity, 80 rennet units (RU) /mL (7) was obtained from New Zealand Cooperative Rennet Co. Ltd., Eltham, New Zealand. Enzyme dilutions were made with distilled water just prior to use.

Simulated milk ultrafiltrate (SMUF) was prepared according to Jenness and Koops (12) and adjusted to pH 6.55 with HCl.

Coagulation Measurement

Changes in apparent absorbance, at 600 nm, of milk as a consequence of chymosin action were monitored using a Beckman DU-8B UV/Vis spectrophotometer with a temperature controlled cuvette holder and light scattering accessory. Light which passes through the sample cuvette could be collected in three different modes. Normal specular measurements use a 7° light collection cone while scatter mode utilizes a 30° cone minus the 7° specular cone. Light collection was also possible using the total 30° cone. Previous studies showed that for undiluted milk the specular and scatter modes gave identical

readings and that use of the total mode resulted in a higher signal to noise ratio (McMahon, unpublished data). Apparent absorbance measurements on undiluted milk substrate were therefore made in the total mode.

Three milliliters of milk substrate were pipetted into a disposable cuvette (pathlength = 1 cm) and maintained in the cell compartment at the desired temperature. The spectrophotometer was then set to read zero absorbance with the milk substrate in the lightpath. An appropriate aliquot of a dilute chymosin solution was added and rapidly mixed (< 10 sec). Apparent absorbance measurements were recorded every 4 sec. A computer program was written to collect data from the spectrophotometer via an RS232 connection to a Tektronix 4052 microcomputer. Data were stored on a tape, derivitized, and time-average smoothed curves plotted on a Tektronix 4662 interactive plotter.

The Formagraph method was used to measure changes in curd firmness (15). A computer program was written to digitize data from firmness-time curves and store the data on magnetic tape. Gauss-Newton non-linear least squares regression iteration (14) was then performed using the Tektronix 4052 microcomputer to estimate parameters for the prediction equation (24).

RESULTS AND DISCUSSION

Coagulation Critical Point

By combining Michaelis-Menten kinetics and rate theories of coagulation of unstable colloids, Payens et al. (18,20) derived equations describing changes in particle size and particle number during enzymic milk coagulation. The weight average molecular weight, $\overline{M}_w$, could then be described by:

$$\frac{\overline{M}_w}{M_o} = 1 - M_o(1-f)\left(\frac{8V}{k_s}\right)^{\frac{1}{2}}\left\{ f\left(\frac{t}{\tau}\right) - (1-f)\left(\frac{t}{\tau}\right)^3/_3\right\}/C_o \qquad \text{(II)}$$

where M_o is average molecular weight of casein micelles in milk, k_s is the bimolecular aggregation rate constant, V is the maximum enzymic velocity, C_o is substrate concentration, f is the ratio of molecular weight of released macropeptide to M_o, and τ is defined as:

$$\tau = \left(\frac{k_s}{V}\right)^{-\frac{1}{2}} \qquad \text{(III)}$$

It can be seen that τ is the central parameter governing the kinetics of enzymic coagulation as described by equation II. At time $t \ll \tau$, $\overline{M}_w$ will change only slightly and be related to t, while at $t > \tau$ the term $(t/\tau)^3$ dominates and $\overline{M}_w$ increases rapidly. An initial decrease in $\overline{M}_w$ is observed as a result of the predominant influence of proteolysis at the beginning of the coagulation process. Aggregation will not proceed very rapidly while concentration of aggregable particles, remains low, and initially the total particle number is only determined by enzymic production of para-casein micelles and not by disappearance by aggregation.

The relationship of τ to colloidal dispersions can be considered analagous to the critical, or gel, point in organic polymer systems. Gel point is a characteristic of non-linear condensation polymerization reactions in which monomers have a functionality greater than two, and a sharp gel point is observed. Gel point is a well-defined stage in the course of polymerization, and is the point at which the condensate transforms suddenly from a viscous liquid to an elastic gel (8). Condensation of such monomers (functionality > 2) can be expected to yield indefinitely large, polymer structures at sufficiently advanced stages of the process. These will extend throughout the volume of the polymerized material. On a molecular weight scale they can be considered essentially infinite in size, and are referred to as infinite, space network structures. The random nature of polymerization processes makes it improbable that assemblage of units in such a structure would conform to a regular pattern, and space network structures are typically irregular laybrinths. They are often composed of a predominance of linear segments (i.e. chains) which are interupted at intervals by branches.

Formation of an infinite network, concomitant with gelation, is similar to explosion in chain reactions. A build up of polymer aggregates with many potential interaction sites leads to a high probability of larger particles growing faster. The result is that $\overline{M}_w$ rises very rapidly yet the number average molecular weight, $\overline{M}_n$, is still low. At the point of coagulation, $\overline{M}_n \simeq 4\overline{M}_o$ (17). A distribution exists of a few large particles in a system in which most particles are still of very low molecular weight.

Support for a critical point occurring in milk coagulation is given in mathematical modelling by Darling and van Hooydonk (5) in which coagulation time represents a fixed point in the clotting process. It occurs at the point when the number of particles is reduced by aggregation to a critical value. The first visible signs of coagulation occur when particles of the order 10-20 μm are produced and this is presumed to occur when the average particle diameter is approximately 1 μm (5). In measurements of apparent absorbance of undiluted milk a shoulder is observe prior to CT and becomes more pronounced upon calcium addition. A similar should has been observed due to an increase in luminance in studies of milk coagulation using reflection photometry (11).

In a previous paper (16), the appearance of this shoulder in apparent absorbance measurements was ascribed to transformation of the milk from a system comprised of essentially independent micelle aggregates (clusters) to one in which the whole system volume is filled by a space network. Reduction in rate of apparent absorbance increase is thought to be due to combined effects of changes in $\overline{M}_w$ and z-average particle scattering factors as the coagulation critical point is passed. Apparent absorbance then continued to increase as the coagulum consolidates and casein "free" at the coagulation point becomes associated with the space network.

Curd Firming

Equation I was modified to allow prediction of coagulation (gelation) point in milk-enzyme systems from Formagraph curd firmness data. The term, τ, was used to represent calculated gelation time for

reasons that will be discussed later in this paper. The modified equation was defined as:

$$G = 0 \qquad \text{for } t \leq \tau$$
$$G = G_{\infty} e^{-\tau/k(t-\tau)} \qquad \text{for } t > \tau \qquad \text{(IV)}$$

where t is time subsequent to enzyme addition and k is the relative rate of initial curd firming. It expresses time from τ until $G = G_{\infty}/e$ as a function of τ. Compared to equation I, $k = \tau/\lambda$. The relationship of these parameters to Formagraph firmness-time diagrams is illustrated in Figure 21. The term G_{max} is maximum curd firmness recorded by the Formagraph and r is Formagraph CT. Time from r until a width of 20 mm is reached on the recorded firmness graph is defined as k_{20} and r/k_{20} is a similar expression of the rate of curd firming as the parameter k of equation IV. At a fixed coagulation time the greater the value of the faster the initial rate of curd firming.

Using equation IV, the parameters τ, k and G_{∞} were estimated at enzyme concentrations that gave Formagraph coagulation times from 2 to 80 min (Table 12). Equation IV fitted the experimental data during the

Table 12. Effect of varying enzyme activity on curd firming of milk 200 µL chymosin solution added to 10 mL reconstituted NDM (12 g + 100 mL .005M $CaCl_2$), 35°C. G_{max} measured at 3 h.

Enzyme Activity (RU/mL)	Formagraph Parameters r (min)	k_{20} (min)	$G_{max}/2$ (min)	Prediction Equation Parameters τ (min)	k	G_{∞} (min)
3.2	1.75	2.00	31.0	1.16	.356	31.8
1.6	3.95	2.45	32.8	3.08	.752	33.9
.8	7.80	4.05	33.1	6.65	1.00	35.9
.4	15.1	6.55	32.2	12.5	1.04	38.2
.16	32.7	11.3	32.9	27.4	1.11	44.4
.08	49.9	14.5	32.3	42.2	1.19	48.3
.04	78.5	17.1	31.0	69.8	1.67	51.3

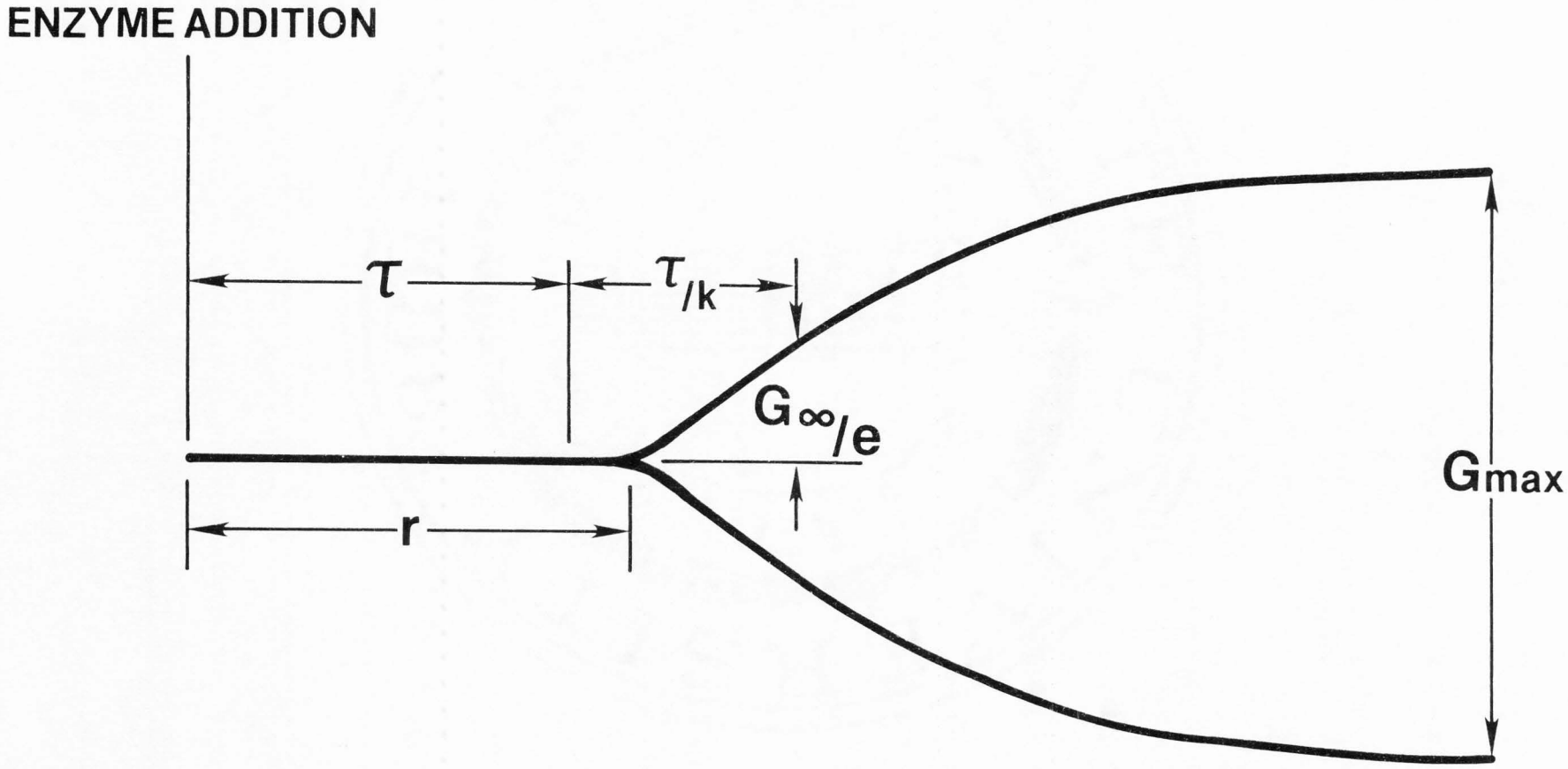

Figure 21. Diagram of coagulation and curd firmness as a function of time as recorded with the Formagraph and showing relation of parameters of equation IV.

time period when curd firmness increased fairly rapidly (Figure 22). As the milk coagulum consolidates, complex changes in the network structure of coagulum occur which limit the application of such predication equations to the early stages of curd firmness development. Coagulum could initally be assumed to be a simple system of crosslinked, casein micelle chains encompassing the whole system volume and containing a large number of individual micelle clusters.

Observations using electron microscopy (10) have revealed that the continuing gelation process involves some specific interactions between casein micelle chains which cause them to become aligned and form strands. Casein micelles lose their individual identity and the coagulum can be considered as a large mesh network of casein strands. These strands are comprised of a network of casein micelle chains and clusters of a smaller mesh size. Small bridging chains occur between the strands.

Tuszynski, et al., found that equation I fitted curd firmness data of renneted milk up to the condition suitable for cutting (26). Attempting to fit equation IV over too long a time period after coagulation results in a sinusoidal pattern being observed in plots of the equation residuals. This is diminshed when curve fitting is restricted to the region of the curve in which development of coagulum network is the predominant process and where the later phases of gelation do not have a major influence on curd firmness. Application of curd firmness data beyond the point at which cutting normally occurs is moot because once the coagulum is cut, syneresis rapidly occurs and the system conditions depart drastically from the laboratory situation in which coagulum is allowed to sit undisturbed.

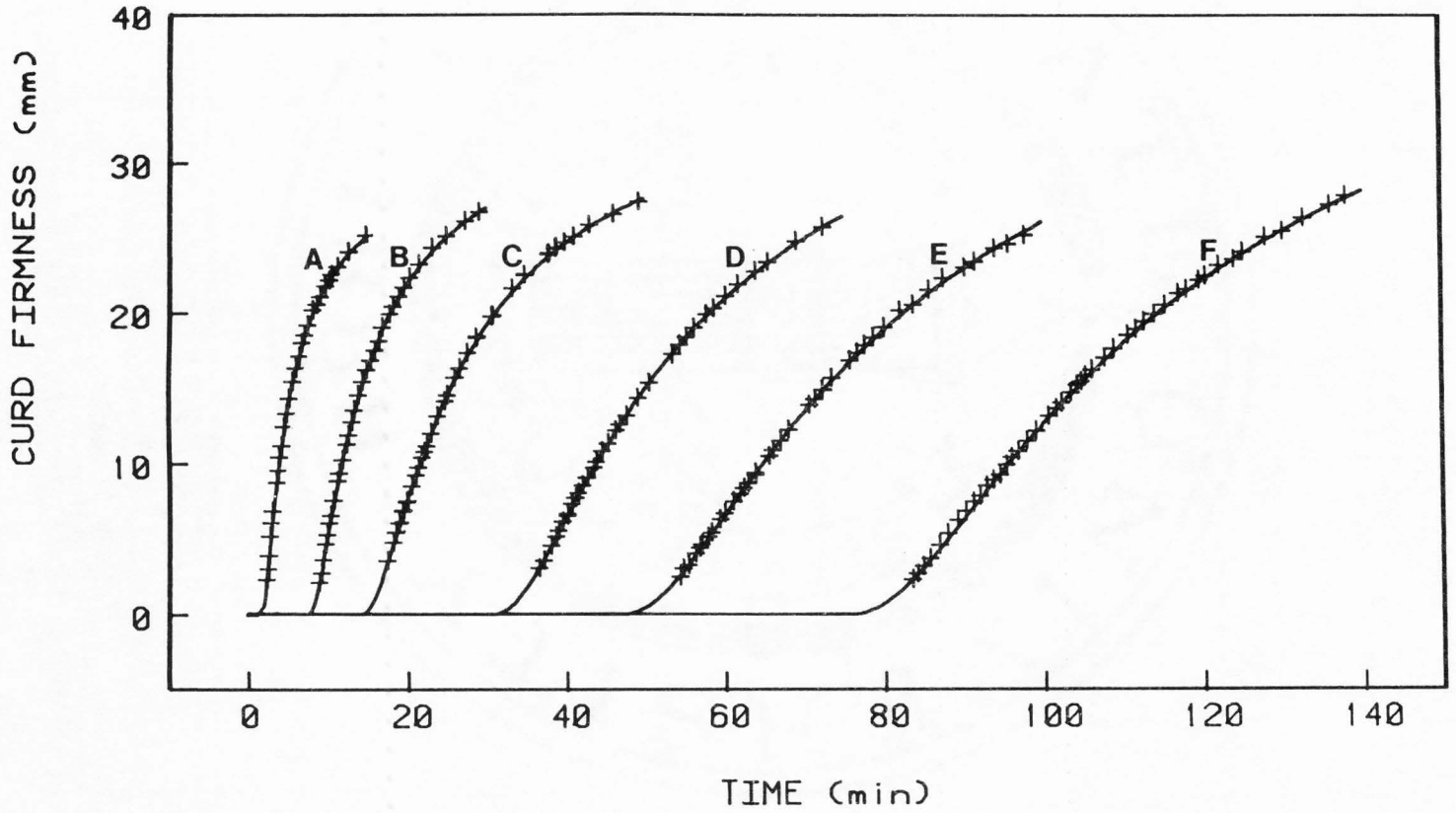

Figure 22. Plot of digitized Formagraph curd firmness data versus time at six enzyme activities and showing estimated curves using equation IV. Reconstituted NDM (12g + 100mL .005M $CaCl_2$), 35°C, 2% enzyme added. A: 3.2, B: 0.8, C: 0.4, D: 0.16, E: 0.08, F: 0.04 RU/mL.

Coagulum consolidation by strand formation would result in milk serum entrapped by the space network being gathered into "micro-droplets" between strands. After two hours at 35°C there was no evidence of exuded serum or the coagulum surface in either the Formagraph or spectrophotometer samples. Gravitational forces are evidently not sufficient to overcome surface tension of the sample to curvette walls and in the absence of any applied forces the coagulum fills the entire sample volume. During this period the curd firmness continued to increase and G_{max} was not obtained until 3 to 4 h after coagulation. Effects of internal "micro-syneresis" were observed as an eventual decrease in apparent absorbance (Figure 23). This reduction occured more rapidly as temperature was increased and demonstrated a major role of hydrophobic interactions in aggregation, gelation and syneresis stages of enzymic milk coagulation (Figure 24).

When curd firmness measurements were extended to long times the actual curd firmness leveled out more quickly than predicted by equation IV (Figure 25). This was presumed to be the result of curd softening as described by Tuszynski (25), and curd fatique caused by applied mechanical forces used to obtain the firmness data. If the milk-enzyme system was such that only a weak curd was produced a decrease in firmness was observed (Figure 26). This was probably due to loss of intimate contact of the wire loop with the coagulum and in extreme cases, actual tearing of the coagulum.

As coagulation time was increased by lowering enzyme activity, predicted values of G_{∞} increased, but G_{max} measured after 3 h remained relatively constant (Table 12). Use of G_{∞} in comparing coagulating properties of milk-enzyme systems may not, therefore, be directly

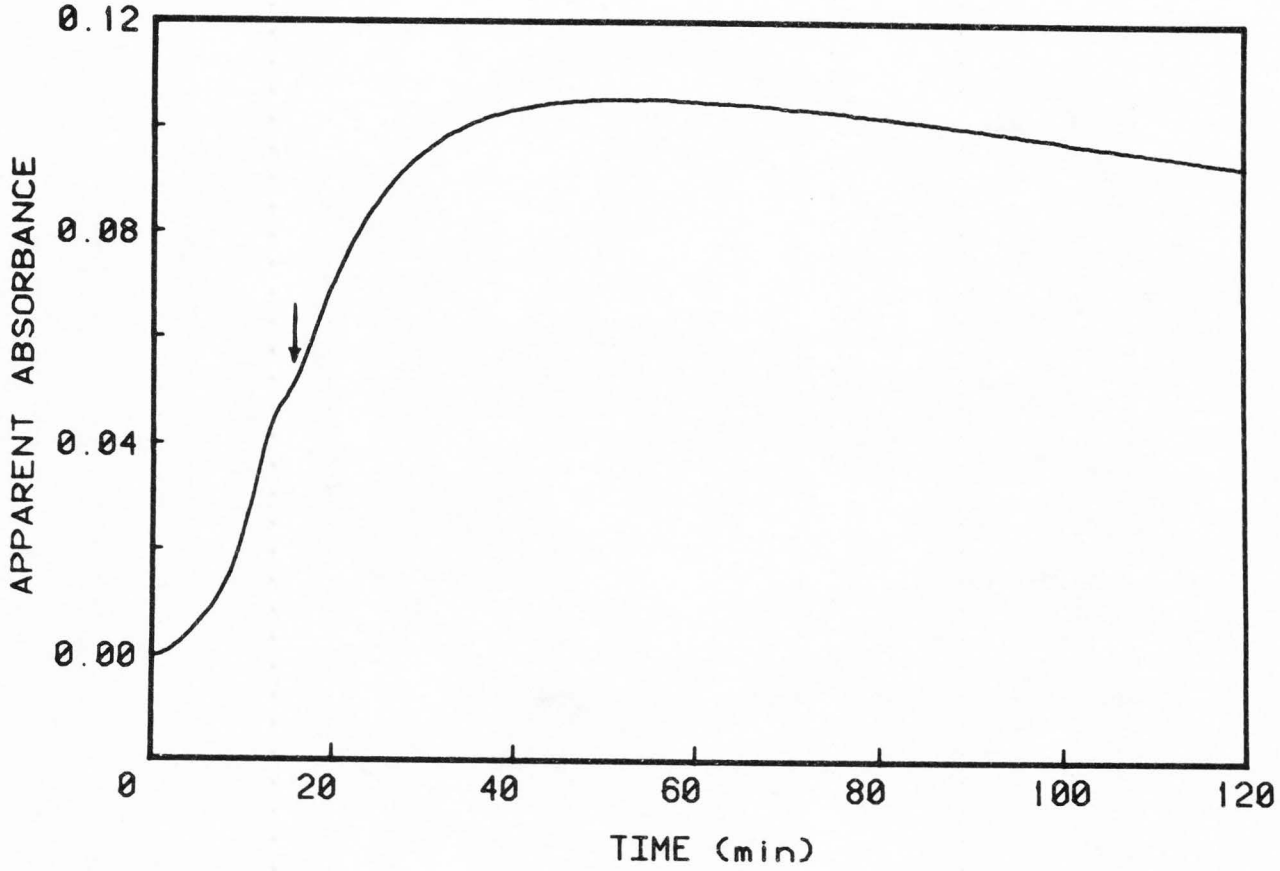

Figure 23. Plot of apparent absorbance (600nm) versus time after chymosin addition to reconstituted NDM (12g +100mL .002M $CaCl_2$), 35°C, 2% .817 RU/mL added.

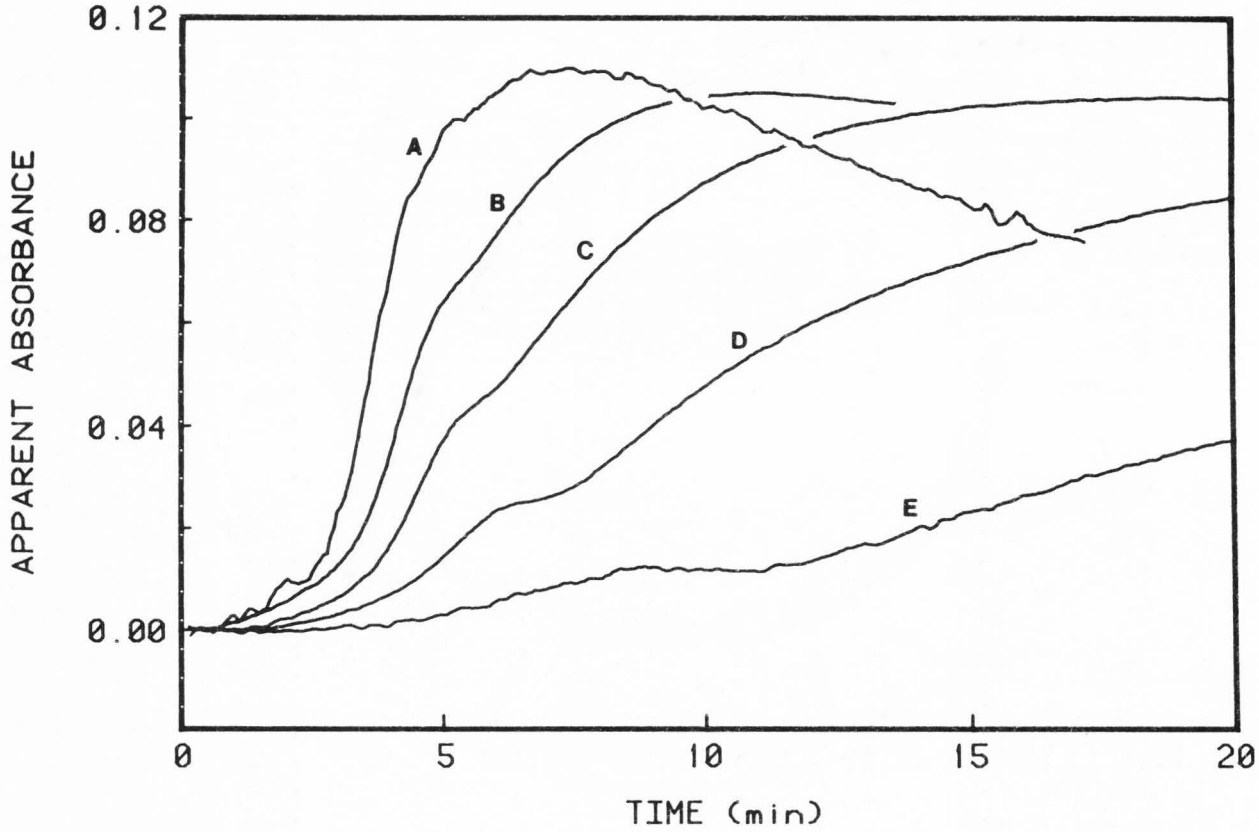

Figure 24. Plot of apparent absorbance (600nm) versus time after chymosin addition to reconstituted NDM (12g + 100mL .002M $CaCl_2$), .33% 8 RU/mL added, at various temperatures. A: 49°C, B: 43.5°C, C:40°C, D: 35°C, E: 29°C.

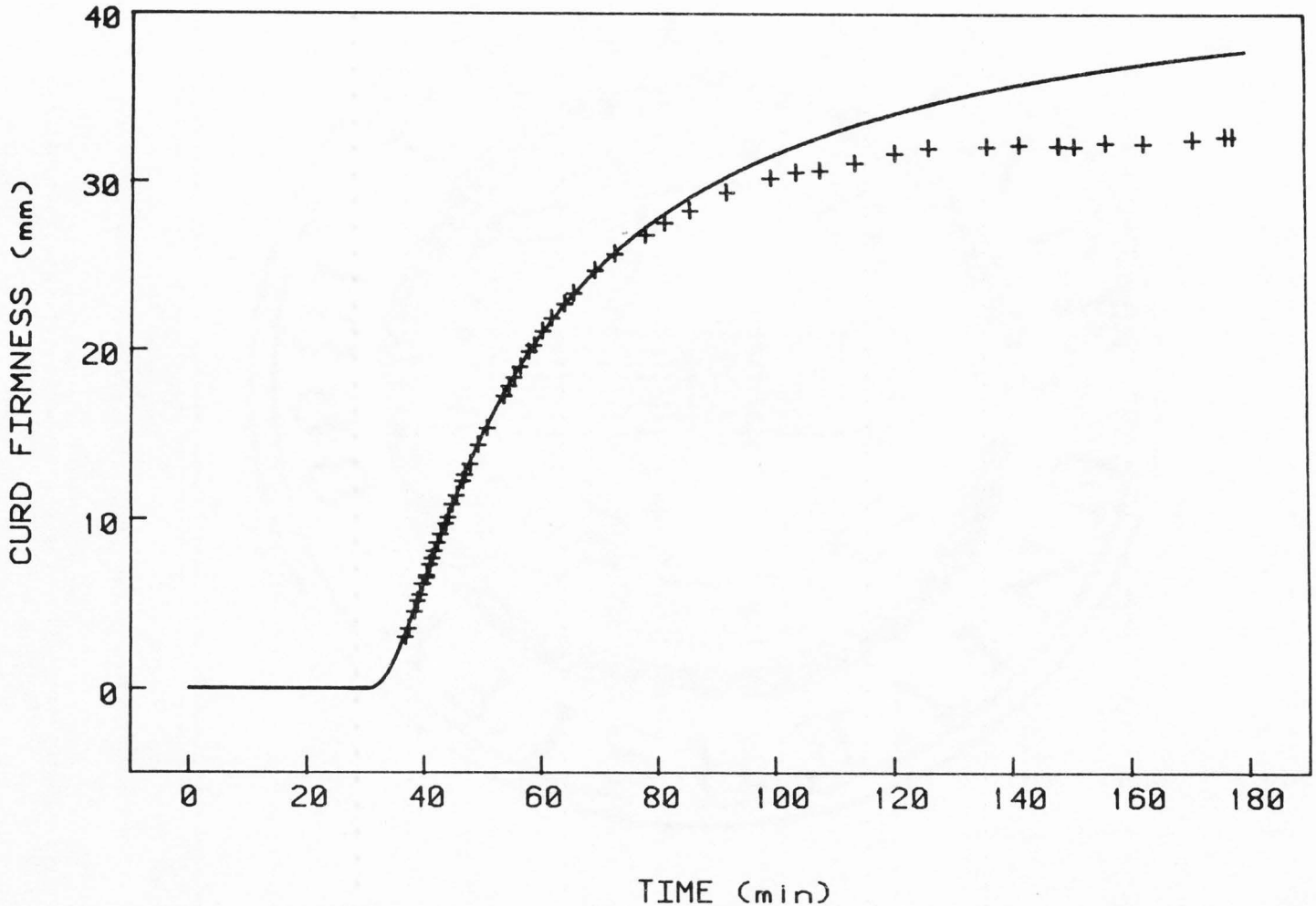

Figure 25. Plot of digitized Formagraph curd firmness data versus time after chymosin addition (2% .16 RU/mL) and estimated curve from equation IV using data points at time t < 75 min for curve fitting, 35°C.

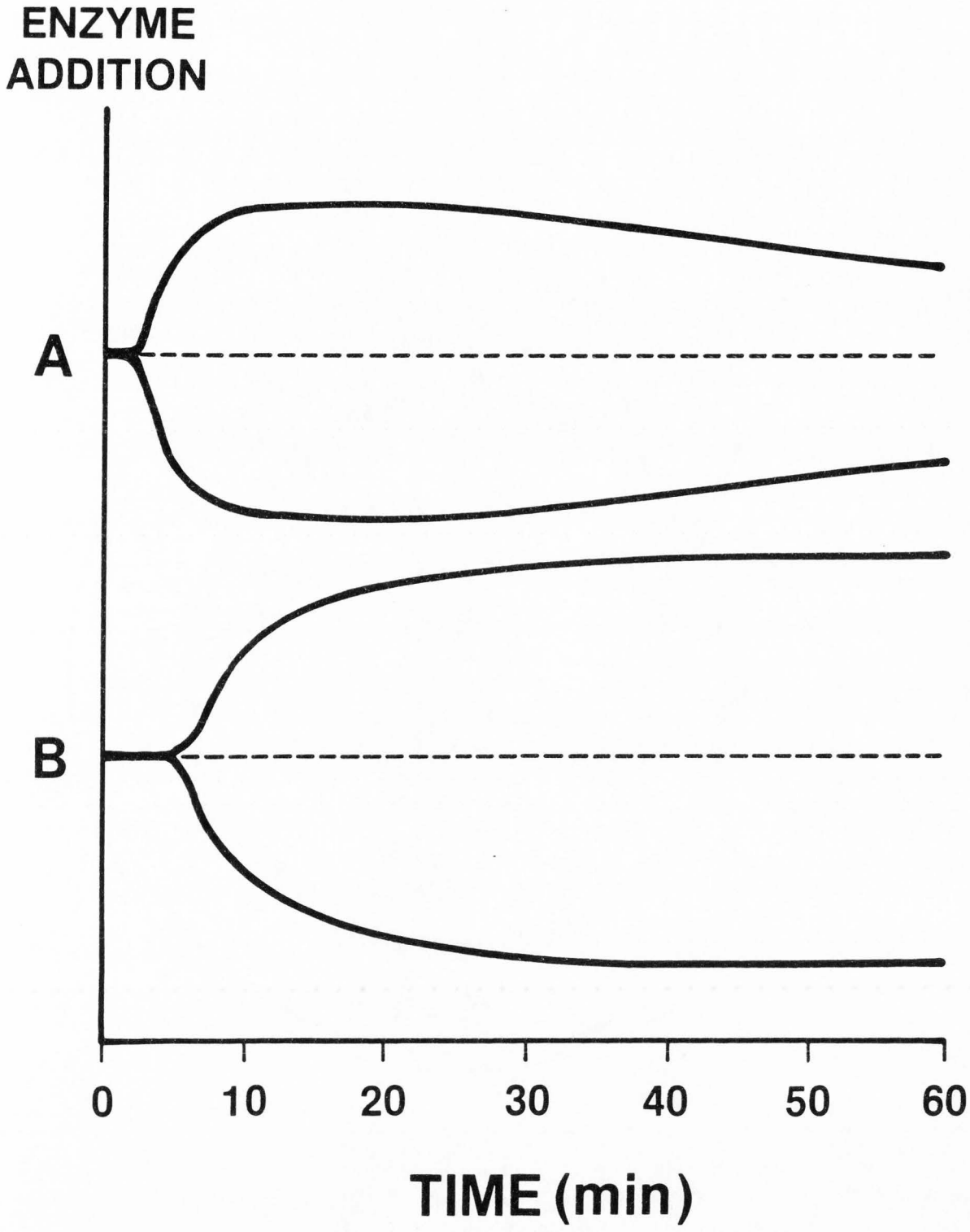

Figure 26. Diagram of curd firmness as a function of time as recorded with the Formagraph of a strong and weak curd milk. Reconstituted NDM (12g + 100mL), pH 6.5, 35°C, 1.76% 1.6 RU/mL added. Reconstituted in A: .04M $CaCl_2$, B: H_2O.

applicable to curd properties during cheesemaking. If a fixed make schedule is followed during cheesemaking, the rate at which curd firmness increases may be of more importance because it will determine curd properties at cutting time and during the period when syneresis is occurring.

When enzyme activity was increased, coagulation time decreased but relative curd firming rate, k, increased. That is, the time required to reach a certain level of curd firmness, when expressed as a proportion of τ, decreases. In practice, therefore, if enzyme activity is increased, time until cutting can be decreased but it should not be assumed that time from cutting to cooking can be decreased in proportion and the same final curd properties obtained. If curd is at the same level of firmness in both cases, differences in relative curd firming rate would not allow the cut-to-cook time to be decreased in the same proportion. Also, laboratory measurement of coagulation time would not allow for arbitrary adjustment of a make schedule based solely on coagulation time.

Curd is formed as a space network of aggregating casein particles but the observation that a coagulum has formed does not imply that all available casein has been incorporated into it (4). As CT increases from 5 to 26 min the proportion of casein incorporated into coagulum at CT decreases from 99% to 75% (4). It is possible that casein incorporated after CT tends to form crosslinks between network chains and builds up curd firmness proportionally more quickly than if virtually all the casein is incorporated into the coagulum by CT. Gels rely on rigid bonds between particles to maintain their open structure and in the final gel a relatively large number of casein

micelles will have lost their original identity as a result of structural rearrangements and interparticle fusion (10,13,23).

Milk Coagulation Time

When calculated values of τ were compared to apparent absorbance curves of the same milk-enzyme system it was observed that τ corresponds to the inflexion point of the rapidly increasing portion of the curves (Figure 27). It appears, therefore, that τ is in fact a measure of the critical gel point in the aggregation process. It was assumed that τ in equation IV corresponds to τ in equations II and III. If this is the case, then experimental values of τ for mathematical modelling using equation II, could be obtained from either curd firmness data or apparent absorbance measurements. Although the space network would be formed at this point it would not possess sufficient rigidity to produce a detectable signal in current curd firmness devices, nor to cause breaking of a moving milk film. It can be considered as the time when the milk ceases to behave as a true fluid (26).

The process of coagulation can be described using a geometric cluster addition model (23). Such cluster aggregation produces a very porous structure and aggregates are of ragged and ill-defined shape. The resultant floc is made up of long hapazard strings of particles joining regions of somewhat denser structure. A gel is formed if particle size and solids concentration are such that cluster-addition growth fills the entire suspension space before breakdown can occur by disruptive forces . The dispersed phase does not settle out but the system gradually becomes more viscous and solidifies (6).

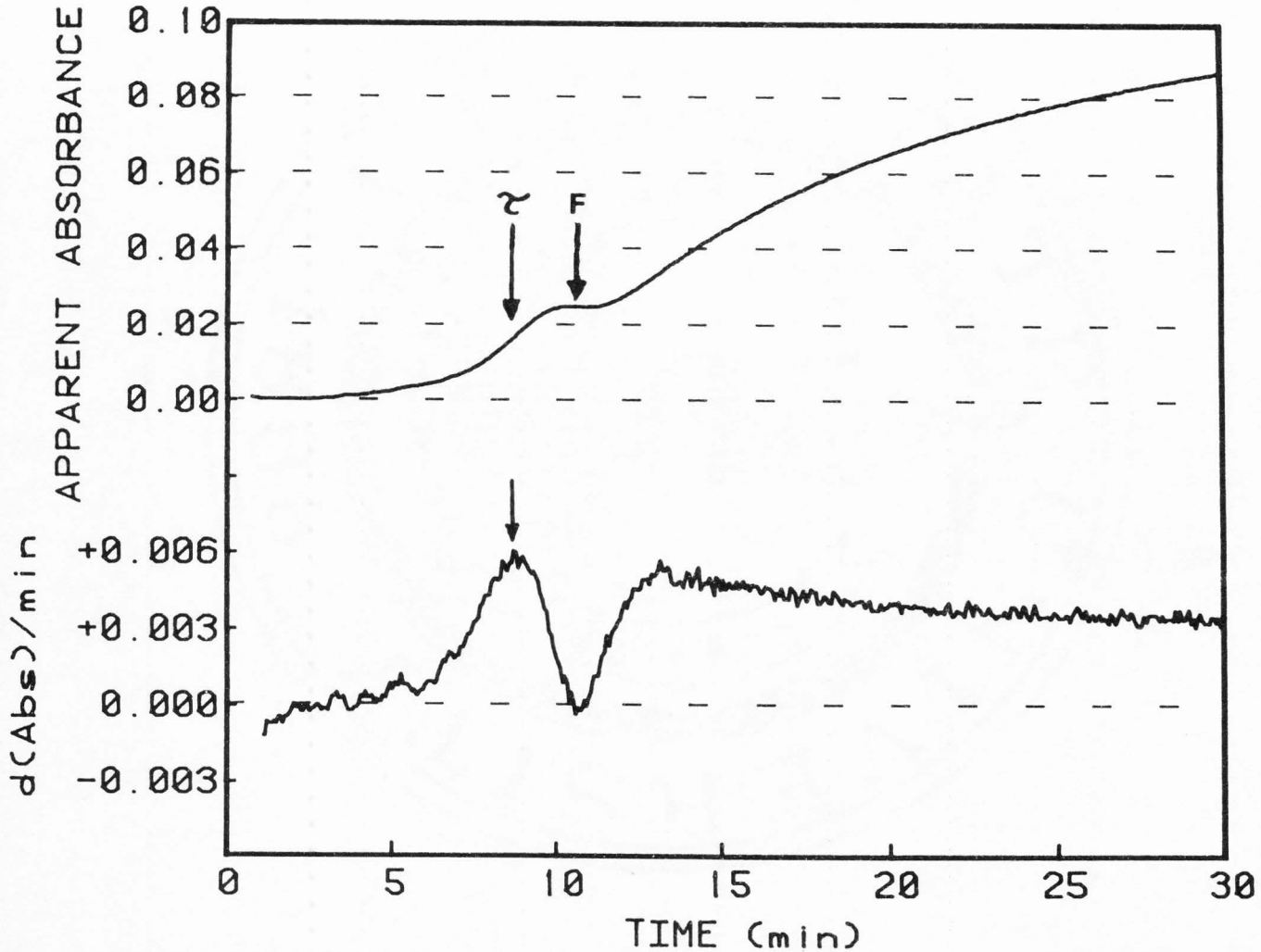

Figure 27. Apparent absorbance (600nm) versus time and first derivitive of reconstituted NDM (12g + 100mL .005M $CaCl_2$), 35°C, showing occurrence of Formagraph coagulation time (F) and coagulation time estimated using equation IV (τ).

Effect of Milk Dilution

A change occurred in light scattering properties of milk upon dilution. Dilution of milk reduces casein micelle concentration and when diluted by a factor of ten or more, no shoulder was observed on apparent absorbance curves (Figures 28, 29). Undiluted milk or .5x milk yielded identical curves in specular or scatter modes. At these concentration levels, multiple light scattering has a large influence on scattering measurements and absorbance measurements cannot be related directly to $\overline{M}_w$. Absorbance measurements are also influenced by the structure of the dispersed particles. When .05x milk was used the change in apparent absorbance was much greater in the specular than scatter mode (Figure 29). The particle concentration at this dilution was such that as coagulation proceeds, proportionally less light enters the 7° cone than the 30°- 7° collection zone. When milk is diluted 1/60, multiple light scattering becomes negligible, and as coagulation occurs light is scattered into the 30 - 7° zone and apparent absorbance decreases. In this case, the scatter mode is an actual measurement of light scattering and is mirrored by the specular measurement (Figure 30). Turbidity measurements of milk are typically performed at high dilutions so that they can be related through light scattering to $\overline{M}_w$ (1). By so doing, however, diffusion-controlled aggregation rates will be decreased because the mean free distance between particles will be increased.

It has been reported that milk can be diluted to very low particle concentrations (.0001x) and an increase in turbidity still observed as a result of chymosin action (22). The time of inflexion point varies very little over a wide range of milk dilutions (.1x to .0001x). In contrast, CT increases rapidly in the range 1x to .1x (2). The

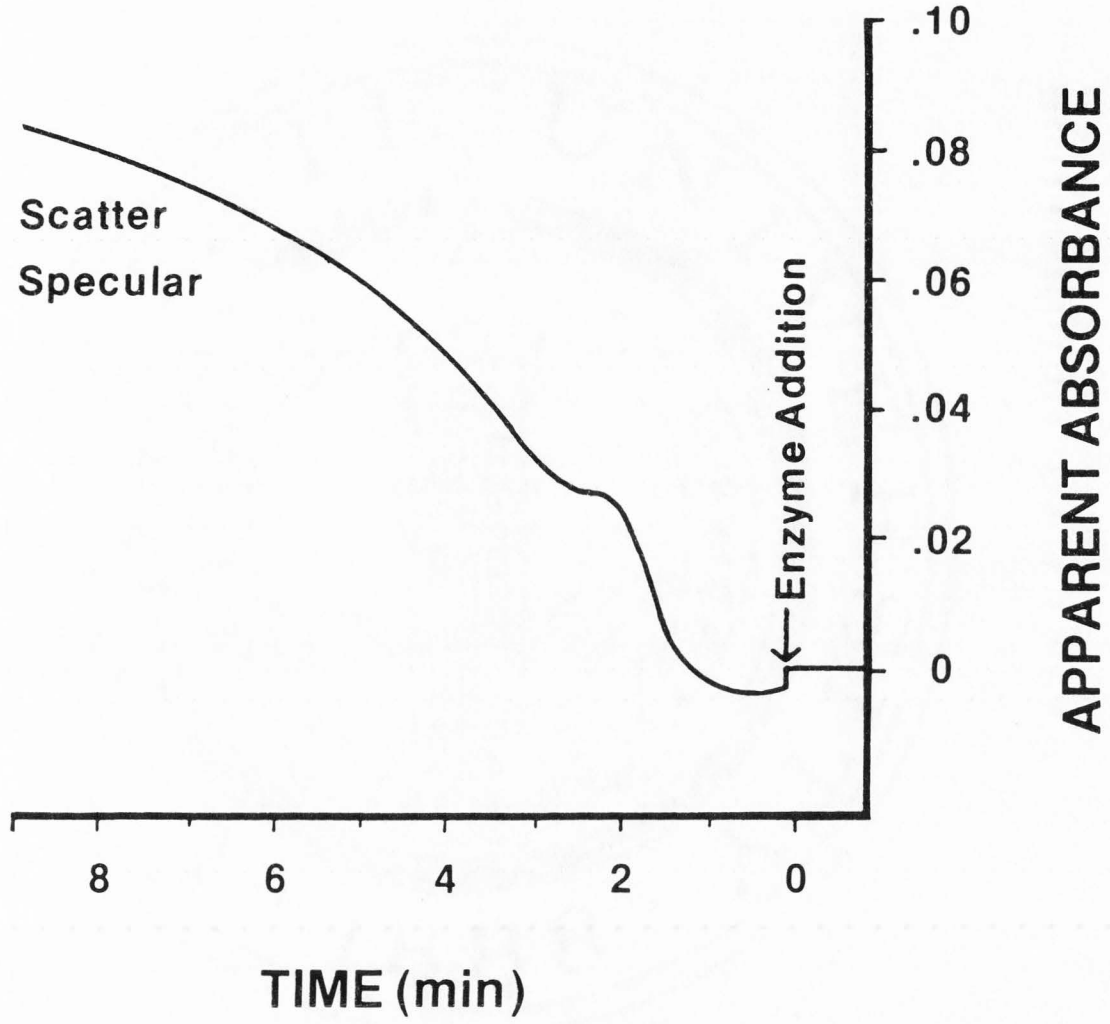

Figure 28. Plot of apparent absorbance (600nm) versus time after chymosin addition (.667% 8 RU/mL) of reconstituted NDM (12g + 100mL .0072M $CaCl_2$) diluted 1/2 with SMUF, 35°C, using specular and scatter modes of light collection.

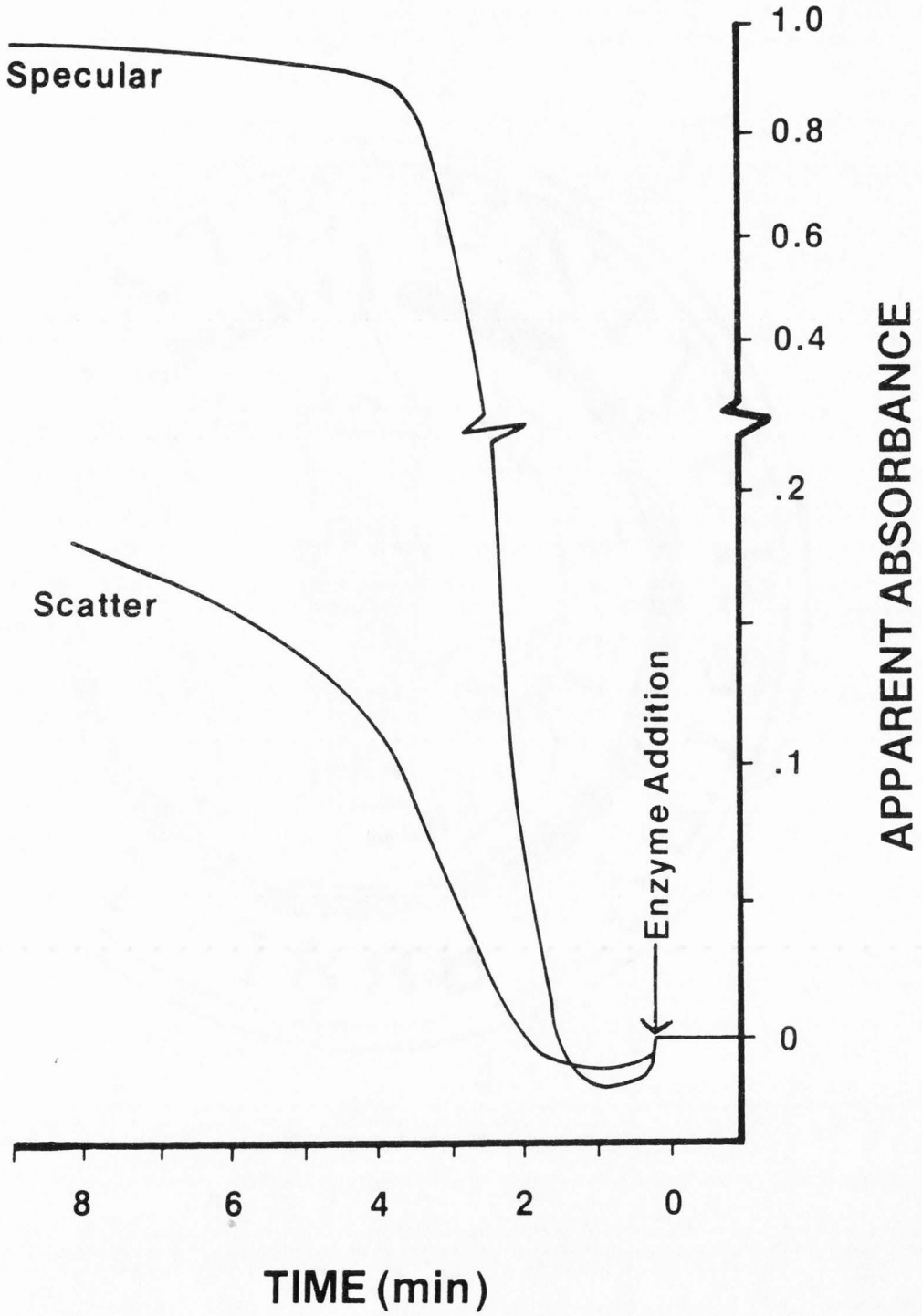

Figure 29. Plot of apparent absorbance (600nm) versus time after chymosin addition (.667% 8 RU/mL) of reconstituted NDM (12g + 100mL .0072M $CaCl_2$) diluted 1/10 with SMUF, 35°C, using specular and scatter modes of light collection.

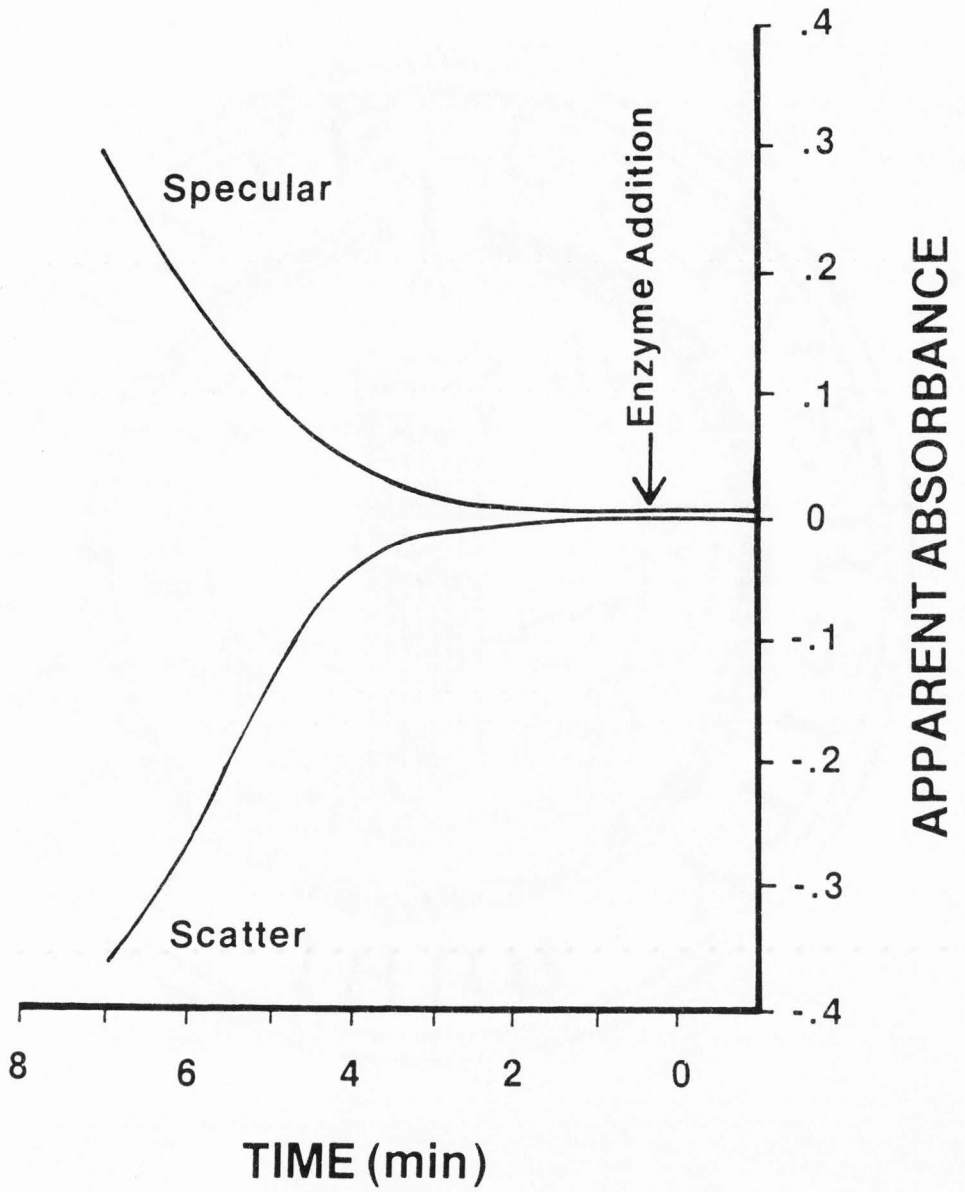

Figure 30. Plot of apparent absorbance (600nm) versus time after chymosin addition (.667% 8 RU/mL) of reconstituted NDM (12g + 100mL .0072M $CaCl_2$) diluted 1/60 with SMUF, 35°C, using specular and scatter modes of light collection.

observation that no visible coagulation occurrs at high milk dilutions has lead to the suggestion that turbidity increases are due to a cooperative transition of quanternary casein structure occuring independent of coagulation (22). This could represent the formation of casein micelle clusters before formation of the space network.

The critical point for milk coagulation can be described as the point at which growing cluster particles occupy a critical volume and virtually any further particle movement by diffusion or rotation results in collision with another cluster and an explosive growth in particle size. After this critical volume is passed, the majority of particle collisions results in addition to the space network. The coagulating system thus changes from essentially independent, aggregating particles to an extended space network comparised of interconnected micelle chains, and the milk ceases to behave as a true fluid. At low particle concentrations, aggregating particles do not obtain this critical volume and no visible coagulation occurs.

Coagulation Time and Surface κ-casein

An estimate of τ can also be made from absorbance data using equation II. At t_i, the time of incipient rise in $\overline{M}_w$, estimated when apparent absorbance equals its initial value, $\overline{M}_w = M_o$ and equation II simplifies to:

$$t_i = \tau \left(\frac{3f}{1-f}\right)^{\frac{1}{2}}$$

The relationship between t_i and τ is then seen to be a function of f, which is dependent on the effect of removing macropeptide molecules from the micelle. If $M_o = 5 \times 10^8$, and casein micelles contain on the

average, 12% κ-casein, f has a value of 0.04 and $t_i = .35\tau$ (19). This assumes all the macropeptide is released from k-casein by chymosin action. If light scattering experiments are used as a measure of $\overline{M}_w$, however, it will only be those macropeptide molecules on the casein micelle surface that will affect hydrodynamic radius of casein micelles and hence cause an alteration in light scattering upon their removal. It has been suggested (27) that intact macropeptide is present as molecular chains protruding from the micelle. Removal of these "hairs" reduces micelle hydrodynamic radius, lowers their viscosity, and by decreasing entropic repulsion between micelles allows for aggregation to occur.

By measuring t_i and τ from apparent absorbance data, f can be calculated and used to estimate the proportion of κ-casein on the micelle surface. For a milk-enzyme system in which τ, measured as the inflexion point equalled 6.65 min, t_i = 2.05 min. Using equation V this yielded a value of f = 0.031. According to this, 77% of the macropeptide portion of κ-casein is so positioned on the micelle surface that its removal reduces casein micelle size.

CONCLUSION

Although determination of coagulation time (CT) visually or rheologically is still the most frequently used method for estimating the actual coagulation time (τ), the closeness of CT to τ is dependent on the method used. In general, high estimates are obtained and $CT > \tau$. Actual coagulation time can be considered analogous to the critical point of non-linear condensation reactions. In milk systems this is the point when aggregating micelle particle clusters occupy a critical volume and the milk changes from a system of independent particles to an extended space network.

The coagulation critical point corresponds to the inflection point of apparent absorbance curves of chymosin-treated undiluted milk. It can also be estimated using prediction equations describing the early stages of curd firmness development. Gelation, or curd formation, can be considered to occur at this critical point. If the casein micelle concentration is reduced sufficiently the critical volume will not be obtained and gelation will not occur.

Rate of curd firming and theoretical maximum firmness are both dependent on coagulation time. When comparing milk-enzyme systems for differences in curd forming properties, measurements of curd firmness should be conducted on samples with equal coagulation time. The application of laboratory experimental data should take into account the effect of cutting the curd as it occurs during the manufacture of cheese.

SUMMARY

Digitized curd firmness data was fitted to an exponential equation using a non-linear, least squares regression, computer program. Solutions were obtained for actual coagulation time, curd firming rate and theoretical maximum firmness. The actual coagulation time, τ, occurred before an increase in firmness was detectable and coincided with the inflection point of apparent absorbance changes of coagulating milk.

Only the initial portion of the firmness versus time curve could be fitted to the exponntial equation. Beyond this region, experimentally observed curd firmness lags behind predicted values. Apparent absorbance measurements suggested this was due to the effects of "micro-syneresis", i.e. the consolidation of casein micelle chains into strands and concomitant formation of micro-droplets of milk serum between the strands of the gel network.

Actual coagulation time corresponds to the instant when an extended space network of casein particles is formed. In order for this network to be formed, aggregating micelle clusters must reach a critical volume subsequent to which any further diffusion results in immediate collision with another cluster. Dilution of milk inhibits attainment of this critical point and a curd is not formed. Determination of coagulation time visually or rheologically (CT) is merely an estimate of τ, and the closeness of CT to τ depends on the method used. In general, CT > τ because a finite amount of rigidity must be produced before coagulation can be detected.

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GENERAL SUMMARY

Two complimentary methods of monitoring milk coagulation were developed and evaluated. Equivalent measurements of actual enzymic coagulation time were obtained from both methods even though observed coagulation occurred at different times. This allowed experimental data from enzymic coagulation of undiluted milk to be related to mathematical models of milk coagulation. The eventual complete modeling of milk coagulation is now one step nearer. Normally when monitoring turbidity during milk coagulation it is necessary to use highly diluted milk to eliminate multiple light scattering effects, and to be able to directly relate turbidity to average molecular weight. However, this prevents formation of a milk curd and measurement of curd firmness. By using undiluted milk, measurements of apparent absorbance could be related to curd firmness in order to develop a unifying theory describing enzymic milk coagulation.

Detection of coagulation using the Formagraph occurred after flaking was observed in a moving milk film, and after viscosity increased when measured using rotary viscometry. The Formagraph can be used for measuring enzyme milk-clotting activity in comparison to a standard of known activity. Coagulation time is linear with the inverse of enzyme activity over a 140 fold range. Under specified conditions, e.g. temperature and substrate composition, it is not necessary to match enzyme activities of the unknown and the reference. Linearity was observed within the enzyme activity range of .0011 to .16 RU/mL milk.

Coagulation of undiluted milk was monitored spectrophotometrically and several phases were observed. Initially, proteolysis of κ-casein reduces the average size of casein micelles and this is observed as a

reduction in apparent absorbance. Aggregation of para-casein micelles occurs by random diffusion and although aggregation rate is initially slow, the effects of micelle aggregation can be observed well before visual coagulation is observed. Coagulation actually occurs when micelle aggregates attain a critical volume and the system changes from aggregating particles to an extended gel network. At this critical volume, any further rotational or translational movement of an aggregate brings it into contact with another aggregate. When milk is diluted the mean free distance between micelles is increased, the critical point is not reached, and gel formation does not occur.

Milk coagulation time can be considered analogous to the time until the critical point is reached in non-linear condensation polymerization reactions. Before the critical point, the predominant reaction is aggregation of casein micelles via a cluster growth mechanism. Once the gel network has been formed, incorporation of micelle clusters into the network predominates and this is the process thought to produce an increase in curd firmness. A multitude of processes are involved in enzymic milk coagulation and predominate at different times throughout coagulation. Modeling of one particular period of coagulation does not allow extrapolation to all other periods.

Formagraph curd firmness data was fitted to an exponential equation using non-linear least squares regression techniques and a solution obtained for coagulation time. Coagulation time calculated by this method corresponded to the inflexion point of the rapidly changing portion of apparent absorbance data. This was assumed to represent the occurrence of the gel critical point. Values for the curd firming rate and the theoretical maximum curd firmness could be obtained using this

equation. Unfortunately, modeling of changes in curd firmness still needs to be extended past the initial development of firmness, including syneresis, for the exponential equation to completely fit Formagraph curd firmness data. Consequently, comparisons of curd firming properties of milk systems should be carried out between samples that have equal coagulation times.

Measurements of coagulation time either by observance of flake formation or development of curd firmness are in fact only estimates of the actual coagulation time, or critical point. They require a small, but finite, amount of curd firmness to be developed and hence yield values of coagulation time higher than the actual critical point.

Addition of calcium chloride to milk caused a reduction in coagulation time with a minimum at .05M calcium. At high calcium levels (.4M), coagulation time is severely retarded. Curd firmness increased with calcium addition and reached a maximum rate of increase at .02M calcium. Addition of phosphate 30 min prior to enzyme addition also reduced coagulation time with a minimum at .01M phosphate. When bulk culture media was added, the pH of the media had a greater effect on coagulation time than did the presence of phosphate in the media.

Non-specific proteolytic activity of milk coagulants also affects milk coagulation. Curd firming rate decreased as the non-specificity of the coagulant increased. Relative curd firming rates were of the order: chymosin > calf rennet > M. miehei, M. pusillus proteinases > adult bovine pepsin > papain > bromelain > trypsin. Differences in curd firmness 3 h after enzyme addition for commercial milk coagulants were not significant. Pepsin content in mixtures of chymosin and bovine pepsin was proportional to curd firming rate.

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APPENDICES

Appendix 1. Computer Program for Collecting and Analyzing Data Generated using a Beckman DU-8B Spectrophotometer

```
4 PAGE
5 P$="N"
6 W=32
7 GO TO 910
8 PAGE
9 GO TO 100
12 W=1
13 P$="n"
14 PAGE
15 GO TO 910
16 GO TO 1210
20 GO TO 1310
24 P$="Y"
25 GO TO 1310
40 GO TO 1780
76 GO TO 460
80 GO TO 2210
100 INIT
110 W=32
120 CALL "RSTRIN","","",""
130 CALL "TSTRIN","","",""
140 CALL "RATE",1200,1,2
150 P$="N"
160 G$=""
170 G=0
180 F$=""
190 P6=0
200 H1=0.1
210 DIM Y(450),Y1(450),Y2(450)
220 U=14.8889
230 Y=0
240 Y1=0
250 Y2=0
260 X4=10
270 GO TO 910
280 IF P$="Y" THEN 900
290 IF W=1 THEN 320
300 VIEWPORT 80,128,30,80
310 GO TO 330
320 VIEWPORT 35,110,20,77
330 WINDOW 0,30,-0.12,0.1
340 AXIS @W:5,0.02,0,-0.12
350 FOR I8=1 TO 3
360 MOVE @W:0+(I8-1)*0.04,-0.12+(I8-1)*2.5E-4
370 DRAW @W:0+(I8-1)*0.04,0.1-(I8-1)*2.5E-4
380 DRAW @W:30-(I8-1)*0.04,0.1-(I8-1)*2.5E-4
390 DRAW @W:30-(I8-1)*0.04,-0.12+(I8-1)*2.5E-4
400 DRAW @W:0+(I8-1)*0.04,-0.12+(I8-1)*2.5E-4
```

```
410 NEXT I8
420 FOR I8=5 TO 25 STEP 5
430 MOVE @W:I8,-0.12
440 RDRAW @W:0,0.004
450 NEXT I8
460 A1=0.15
470 FOR J=0 TO 30 STEP 5
480 IF J<10 THEN 500
490 A1=-0.15
500 MOVE @W:J-1+A1,-0.13
510 IF W<>32 THEN 530
520 PRINT "HH";
530 PRINT @W:J;
540 NEXT J
550 FOR J=0 TO 10 STEP 2
560 MOVE @W:-2.2,J/100-0.0035
570 IF W<>32 THEN 590
580 PRINT @32:"H";
590 PRINT @W: USING "fa,1D.2D":"H",J/100
600 NEXT J
610 REM
620 RESTORE 770
630 FOR J=4 TO 10 STEP 2
640 READ A
650 MOVE @W:-5.1,-J/100-0.0035
660 IF W<>32 THEN 680
670 PRINT @32:"HH";
680 IF A<>0 THEN 710
690 A$="  "
700 GO TO 750
710 IF A>0 THEN 740
720 A$=" "
730 GO TO 750
740 A$=" +"
750 PRINT @W: USING "FA,FD.2D":A$;A;
760 NEXT J
770 DATA 0.04,0.02,0,-0.02
780 MOVE @W:15,-0.143
790 PRINT @W:"HHHHTIME (min)";
800 IF W=32 THEN 880
810 MOVE @W:-3.4,-0.02
820 PRINT @W,25:90
830 PRINT @W:"HAPPARENT ABSORBANCE";
840 REM
850 MOVE @W:-5.7,-0.102
860 PRINT @W:"d(Abs)/min";
870 PRINT @W,25:0
880 MOVE @W:2,0.13
890 PRINT @W:G;": ";G$;
900 RETURN
```

```
910 HOME
920 PRINT "COLLECT DATA OR PLOT STORED DATA ? C/P ";
930 INPUT D$
940 IF D$="C" THEN 1010
950 PRINT "                                    TITLE?";
960 K=1
970 INPUT F$
980 GOSUB 1310
990 IF H1<0 THEN 2140
1000 GO TO 1770
1010 Y=0
1020 Y1=0
1030 PRINT "ENTER FILE # TO STORE DATA ";
1040 INPUT F
1050 G=F
1060 PRINT "ENTER SAMPLE ID ";
1070 INPUT F$
1080 G$=F$
1090 GOSUB 280
1100 I=1
1110 IF I=451 THEN 1180
1120 INPUT @40:X1,X2,Y(I)
1130 IF I>1 THEN 1160
1140 MOVE @W:0,Y(I)
1150 GO TO 1290
1160 DRAW @W:I/U,Y(I)
1170 GO TO 1290
1180 PRINT "GGGGGGGGGGGGGGGGG";
1190 GO TO 1240
1200 HOME
1210 PRINT "JJENTER FILE # TO STORE DATA (0=NOT STORED) ? ";
1220 INPUT F
1230 IF F=0 THEN 1770
1240 FIND F
1250 FOR I=1 TO 450
1260 WRITE @33:Y(I)
1270 NEXT I
1280 GO TO 1770
1290 I=I+1
1300 GO TO 1110
1310 HOME
1320 GOSUB 1340
1330 GO TO 1460
1340 PRINT "JENTER FILE # (0=MEMORY) GG";
1350 INPUT F
1360 IF F=0 THEN 1440
1370 FIND F
1380 FOR I=1 TO 450
1390 READ @33:Y(I)
1400 NEXT I
```

```
1410 G$=F$
1420 G=F
1430 P6=0
1440 U2=0-(Y(1)+Y(2)+Y(3)+Y(4)+Y(5))/5
1450 RETURN
1460 GOSUB 280
1470 MOVE @W:X4/60,Y(1)+U2
1480 HOME
1490 PRINT "JJJ";
1500 PRINT "end time ";
1510 INPUT X6
1520 X6=X6*U
1530 MOVE @W:X4/60,Y(1)+U2
1540 FOR I=2 TO X6
1550 DRAW @W:I/U+X4/60,(Y(I-1)+Y(I+1)+Y(I))/3+U2
1560 NEXT I
1570 IF P6=1 THEN 1590
1580 GOSUB 1610
1590 GOSUB 1670
1600 RETURN
1610 REM
1620 FOR I=2 TO 450
1630 Y1(I)=(Y(I)-Y(I-1))*25*3.75
1640 NEXT I
1650 P6=1
1660 RETURN
1670 REM
1680 FOR I=6 TO 446
1690 Y2(I)=Y1(I-1)+Y1(I)+Y1(I-2)+Y1(I+1)+Y1(I+2)+Y1(I-3)+Y1(I+3)
1700 Y2(I)=(Y2(I)+Y(I-4)+Y(I+4))/9
1710 IF I†6 THEN 1740
1720 MOVE @W:(I-0.5)/U+X4/60,Y2(I)-0.08
1730 GO TO 1750
1740 DRAW @W:(I-0.5)/U+X4/60,Y2(I)-0.08
1750 NEXT I
1760 RETURN
1770 END
1780 PAGE
1790 GOSUB 1340
1800 Y6=(Y(446)+Y(447)+Y(448)+Y(449)+Y(450))/5
1810 GOSUB 2000
1820 PRINT
1830 PRINT "III";G;": ";G$
1840 Y6=(Y(446)+Y(447)+Y(448)+Y(449)+Y(450))/5
1850 K=1
1860 FOR H=H1 TO 25+H1
1870 PRINT USING "4d,6d.2d,6d.3d,s":H,H/U+X4/60,Y(H)+U2
1880 PRINT USING "6d.2d":Y2(H)*100
1890 K=K+1
1900 NEXT H
```

```
1910 INPUT Q$
1920 PAGE
1930 H1=H-4
1940 GO TO 1860
1950 END
1960 STOP
1970 PRINT "press RETURN to continue";
1980 INPUT X$
1990 PAGE
2000 GOSUB 1460
2010 HOME
2020 PRINT "STARTING TIME ";
2030 INPUT H1
2040 H1=H1-X4/60
2050 IF H1<>0 THEN 2080
2060 H=1
2070 GO TO 2100
2080 IF H1<0 THEN 2020
2090 H=INT(H1*U)
2100 H1=H
2110 K=1
2120 PRINT USING "fa,fd.4d":"IIIHHFinal Curd = ";Y6+U2
2130 RETURN
2140 RUN 1780
2150 GO TO 2210
2160 MOVE @W:0,-0.12
2170 DRAW @W:0,0.16
2180 DRAW @W:30,0.16
2190 DRAW @W:30,-0.12
2200 DRAW @W:0,-0.12
2210 FOR J=1 TO 9 STEP 2
2220 MOVE @W:0,(J-1)/100
2230 FOR H=1 TO 15
2240 RDRAW @W:0.5,0
2250 RMOVE @W:1.5,0
2260 NEXT H
2270 NEXT J
2280 GO TO 2330
2290 FOR J=6 TO 31 STEP 5
2300 MOVE @W:J-1,-0.12
2310 RDRAW @W:0,0.28
2320 NEXT J
2330 MOVE @W:0,0-0.08
2340 FOR J=1 TO 15
2350 RDRAW @W:0.5,0
2360 RMOVE @W:1.5,0
2370 NEXT J
2380 END
2390 FOR I=1 TO 300
2400 PRINT I,Y(I);
```

```
2410 IF Y(I)<>0 THEN 2450
2420 PRINT "  ";
2430 INPUT Y(I)
2440 GO TO 2460
2450 PRINT
2460 NEXT I
2470 END
2480 FOR I=350 TO 390
2490 PRINT I,Y(I);
2500 INPUT Y$
2510 IF Y$="" THEN 2530
2520 Y(I)=VAL(Y$)
2530 NEXT I
2540 END
2550 FOR I=1 TO 450
2560 PRINT I,I/15,Y(I)
2570 NEXT I
```

Appendix 2. Computer Program for Digitizing and Storing Data from Formagraph Curd Firmness Graphs

```
1 GO TO 100
4 GO TO 140
8 GO TO 450
24 PAGE
25 GO TO 620
28 PAGE
29 GO TO 810
40 GO TO 1420
80 GO TO 1080
100 INIT
110 W=32
120 DIM X(200),Y(200),Y1(200),X1(200),F$(1)
130 END
140 PRINT "place marker on start and <CR> ";
150 INPUT E$
160 GIN @1:X(1),Y(1)
170 CALL "wait",6
180 PRINT "G";
190 FOR I=2 TO 70
200 CALL "wait",3
210 PRINT "G";
220 IF I<65 THEN 240
230 PRINT "G G G G G";
240 GIN @1:X(I),Y(I)
250 NEXT I
260 PRINT "move marker to other side of curve and <CR> "
270 INPUT E$
280 GIN @1:A,B
290 PRINT "enter final time ";
300 INPUT C1
310 C1=C1/20
320 PRINT "enter maximum width ";
330 INPUT G1
340 U1=X(70)-X(1)
350 U2=(Y(70)-B)/2
360 U3=Y(70)-Y(1)
370 U4=U3-U2
380 G1=G1/20
390 FOR I=1 TO 70
400 U5=(X(I)-X(1))*U4/U1
410 Y1(I)=(Y(I)-Y(1)-U5)*G1/U2
420 X1(I)=(X(I)-X(1))*C1/U1
430 NEXT I
440 PAGE
450 WINDOW 0,200,-10,40
460 VIEWPORT 50,120,40,90
470 AXIS @W:20,10,0,-10
480 FOR I=1 TO 70
490 MOVE X1(I),Y1(I)
500 PRINT "+";
```

```
510 NEXT I
520 MOVE 0,0
530 PRINT "JJ"
540 PRINT "enter file "
550 INPUT F
560 FIND F
570 WRITE @33:69
580 FOR I=2 TO 70
590 WRITE @33:X1(I),Y1(I)
600 NEXT I
610 END
620 PRINT "enter file ";
630 INPUT F
640 FIND F
650 READ @33:N
660 FOR I=1 TO N
670 READ @33:X1(I),Y1(I)
680 NEXT I
690 X0=0
700 X9=200
710 Y0=-10
720 Y9=50
730 X8=20
740 Y8=10
750 PRINT "Jenter calculated CT ";
760 INPUT T1
770 PRINT "Jenter calculated alpha ";
780 INPUT A1
790 PRINT "Jenter calculated G-max ";
800 INPUT G1
810 WINDOW X0,X9,Y0,Y9
820 VIEWPORT 28,135,17,80
830 Q1=1.5/(107/(X9-X0))
840 Q2=1.5/(63/(Y9-Y0))
850 PAGE
860 PRINT "change pen and press any key";
870 INPUT F$
880 FOR J=1 TO 2
890 MOVE @W:0,0
900 DRAW @W:T1,0
910 FOR I=INT(T1+1) TO 200
920 DRAW @W:I,G1*EXP(-(A1*T1/(I-T1)))
930 NEXT I
940 NEXT J
950 HOME
960 PRINT "change pen and press any keyG";
970 INPUT F$
980 FOR I=2 TO N
990 MOVE @W:X1(I),Y1(I)
1000 RMOVE @W:0,0.5*Q2
```

```
1010 RDRAW @W:0,-Q2
1020 RMOVE @W:-Q1/2,Q2/2
1030 RDRAW @W:Q1,0
1040 NEXT I
1050 HOME
1060 PRINT "change pen and press any keyGG";
1070 INPUT F$
1080 FOR I=1 TO 2
1090 E1=Q1/12*(I-1)
1100 E2=Q2/14*(I-1)
1110 MOVE @W:X0+E1,Y0+E2
1120 DRAW @W:X0+E1,Y9-E2
1130 DRAW @W:X9-E1,Y9-E2
1140 DRAW @W:X9-E1,Y0+E2
1150 DRAW @W:X0+E1,Y0+E2
1160 NEXT I
1170 FOR I=Y0+Y8 TO Y9 STEP Y8
1180 MOVE @W:X0,I
1190 RDRAW @W:1*Q1,0
1200 RMOVE @W:-X8*0.2,-Y8*0.1
1210 IF I>0 THEN 1230
1220 PRINT @W:" ";
1230 PRINT @W:"HH";I
1240 NEXT I
1250 FOR I=X0 TO X9 STEP X8
1260 MOVE @W:I,Y0
1270 RDRAW @W:0,1*Q2
1280 RMOVE @W:-Q1*2.2,-3*Q2
1290 IF I>0 THEN 1310
1300 RMOVE @W:Q1*0.5,0
1310 IF I<100 THEN 1330
1320 RMOVE @W:-Q1*0.5,0
1330 PRINT @W:I;
1340 NEXT I
1350 MOVE @W:X0+(X9-X0)/2,Y0-Y8*0.75
1360 PRINT @W:"HHHHTIME (min)";
1370 MOVE @W:X0-X8*0.85,Y0+(Y9-Y0)/2
1380 PRINT @1,25:90
1390 PRINT @W:"HHHHHHHHCURD FIRMNESS (mm)";
1400 PRINT @1,25:0
1410 END
1420 GOSUB 890
1430 END
```

Appendix 3. Partial Derivitives Subroutine for use with Gauss-Newton Non-linear Least Squares Regression Computer Program

```
1100 K=-C(I)/(C(2)*(X(I,1)-C(1)))
1101 Y1=C(3)*EXP(K)
1102 C1(1)=-C(3)*X(I,1)*EXP(K)/(C(2)*(X(I,1)-C(1))↑2)
1103 C1(2)=C(3)*C(1)*EXP(K)/(C(2)↑2*(X(I,1)-C(1)))
1104 C1(3)=EXP(K)
1105 RETURN
```

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