The Development of an Improved Milk Substrate for Rennet Coagulation Assay on an Automatic Clot-Timer

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THE DEVELOPMENT OF AN IMPROVED MILK SUBSTRATE
FOR RENNET COAGULATION ASSAY ON AN
AUTOMATIC CLOT-TIMER

by

Mark Brimhall Stevens

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

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1973
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ABSTRACT

The Development of an Improved Milk Substrate for Rennet Coagulation Assay on an Automatic Clot-timer

by

Mark Brimhall Stevens, Master of Science

Utah State University, 1973

Major Professor: Dr. Gary H. Richardson
Department: Nutrition and Food Sciences

A substrate was developed for measuring the milk clotting strength of rennet preparations on an automatic clot-timer. The substrate contained 8 percent pasteurized skimmilk solids, 1 percent chloroform, 0.3 percent 200 bloom gelatin, 0.03 M added CaCl₂ and was buffered to pH 6.6 with 0.057 M cacodylic acid and 0.042 M triethanolamine. The substrate was shelf-stable for 18 days at room temperatures. It was found that rennet preparations could be standardized to within 1 percent of each other, in terms of milk clotting strength, by use of the substrate on the automatic clot-timer. The method appears to have advantages over conventional rennet standardizing procedures. The research included studies on the effect of chloroform, nonfat dry milk and CaCl₂ concentrations; heat and ionizing radiation on the substrate coagulation time.

(65 pages)
INTRODUCTION

For many years there has been a need for a simple test for determining the milk clotting strength of rennet and other enzyme preparations. Many methods have been devised to measure the activity of milk coagulating enzymes. Most are based on the time necessary to coagulate a casein-base substrate. These methods thus measure the enzymatic and non-enzymatic reactions in milk coagulation.

The most widely used method in the rennet industry today for standardizing enzyme preparations involves using a Berridge type substrate (13) on a Sommer-Matsen apparatus (95). There are several disadvantages to this method: (A) The endpoint is somewhat subjective since it is based on visual observation of small flakes of coagulated substrate on the wall of a glass bottle; (B) The substrate is not shelf-stable. It must be kept refrigerated and be replaced every few days; (C) The substrate must be tempered about 30 minutes before it can be used; and (D) It is not as sensitive as needed in some current coagulation studies.

The principal objectives of this investigation were to: (A) Develop a simple technique using a modified automatic blood clot-timer that would enable it to be used for standardizing rennet preparations and (B) Develop a shelf-stable substrate suitable for use with the clot-timer.
Achievement of these objectives would allow the advantages of a method having a mechanical endpoint; using a shelf-stable substrate, requiring less time, reagents and space.

Perhaps eventually a shelf-stable substrate can be made available for shipment all over the world. This will allow for one standard test of evaluating rennin activity.
REVIEW OF LITERATURE

In cheesemaking, the enzyme rennin is utilized in the form of rennet, defined as extract obtained from the abomasum (fourth stomach) of milk-fed calves (11). Rennet preparations also contain other protein matter besides rennin.

In young calves the enzyme appears as a precursor called prorennin (23). Below pH 5.0 prorennin is autocatalyzed to rennin and is catalyzed to rennin at pH 5.5 to 6.0 in the presence of pepsin.

Commercial rennet is extracted from dried shredded or flat salted calf abomasums in sodium chloride solutions. The extracted rennin and prorennin is then kept at pH 4.6 to 5.0 until most of the prorennin is changed to rennin and then adjusted to pH 5.7 for stability (62). Sodium chloride solutions (14-20%), sodium benzoate, methyl and propylparabanoaic acids, propylene glycol, ethanol, glycerol and boric acid may be used to preserve and solubilize rennet extracts (26).

Milk Clotting Enzymes

It appears that all proteolytic enzymes can clot milk (12). Historically, rennin has been the most commonly used milk clotting enzyme. In recent years, however, many dairymen have found it more profitable to breed their dairy cows to beef type bulls and grow the calves for sale as
beef, rather than slaughter the calves for milk-fed veal to recover the abomasums for rennet. This trend has driven the price for rennet to un­anticipated highs. The relatively high cost of rennet in recent times has spurred intensive research into the suitability of other proteolytic enzymes for cheesemaking.

Pepsin has been quite successful as a substitute for rennin in cheese­making (25, 61). It is obtained primarily from the swine gastric mucosa (26). Bovine pepsin is also present in small amounts in commercial rennet extracts (49). Adult borine extracts contain predominantly borine pepsin which has received increased acceptance in recent years.

There are several drawbacks to the use of swine pepsin, however. Pepsin is not as active or stable at higher pH values (6.5-6.8), as is rennet (51, 61). Because of this, high fat losses often result with pepsin-made­cheese, since relatively soft cheese curds are formed that tend to shatter when cut, allowing the fat to escape (51, 61). Thus for most cheeses, where the curd is formed at relatively high pH values, pepsin is not as suitable.

Since pepsin is destroyed during cooking, while it is in an unstable pH range, it does not contribute to proteolysis, as can rennet, during curing. However, mixtures for example of 75 percent pepsin and 25 percent rennet or 50/50 rennet/pepsin have been used to make excellent quality Cheddar cheese (96).
Other proteolytic enzymes that have been tested for cheese manufacture include some from microbial and plant sources. Many of them, however, have been found to produce cheese with undesirable characteristics, especially bitter flavors (6, 41, 68). Proteases from the plants *Withania coagulans* (68) and *Ficus carica* (46) produce bitter cheese. Some workers said that a protease from *Mucor pusillus* made Cheddar cheese taste rancid and appeared to have a coarse mealy body (6). However, with the *Mucor pusillus* var. *lindt* protease Richardson et al. (82) produced good quality mild Cheddar, Brick, Parmesan and Pasta Filata cheeses; but in aged Cheddar cheese the enzyme produced a bitter flavor.

A protease from a mutated strain of *Bacillus subtilis* has been reported to produce excellent Cheddar cheese (41). This has since been abandoned due to bitterness problems. A fungal enzyme from *Endothia parasitica* (85) produces bitter aged Cheddar cheese (24), but has been used to make high quality Swiss, Monterey, Colby, Muenster, Limberger, Mozzarella, Provolone, Romano and Asiago cheeses (91). It is thought that this enzyme, like that from *Mucor pusillus*, produces bitter flavors in the form of peptides during proteolysis in aged Cheddar cheese (24). It has been proposed that these enzymes are destroyed during the cooking of cheeses like Swiss and Italian varieties, but are not at the lower cooking temperatures used for Cheddar cheese manufacture (18). The newest successful coagulant for cheesemaking is an extract from *Mucor meihei*. This is
successfully marketed by several U.S. firms (personal communications with Randy West, 1972).

**Rennin Stability**

According to Foltmann (28), rennin is most stable between pH 5.5 and 6.2. From 6.2 to 7.0 he found that the enzyme was destroyed at an increased rate. He also found a region of instability near pH 3.5. He suggested this latter loss of activity may be due to autolysis.

Mickelson and Ernstrom (62) found that rennin activity losses were quite temperature dependent above pH 6.0. At pH 3.8 they found chloride ions to have a destructive effect on rennin.

It has been reported that rennin loses activity by shaking (89). Destruction of rennin activity by ultrasonic vibration has not been found to exceed rennin activity lost as a result of vigorous shaking (26). Some proteases have been found to digest rennin (100). There is some evidence that light waves inactivate rennin (26).

**The Milk Clotting Action of Rennin**

The action of rennin on milk and the formation of a clot is a complex process that has only recently been understood. In 1877 Hammarsten (33) proposed that casein existed as a pure protein that was split by rennin into two forms, a soluble whey albumin portion and paracasein. He considered that paracasein clotted in the presence of calcium ions.
In 1914 Van Slyke and Bosworth (102) stated that in their opinion casein was a pure protein of M. W. 8888. They believed it was split by rennin into halves, of M. W. 4444, called paracasein, which clotted in the presence of calcium ions. They thought soluble whey albumin was the result of additional proteolytic action of rennin on casein.

With the recognition of the heterogeneity of casein (48, 60) and the idea of Linderström-Lang (48) that one of the components of casein was a "protective colloid" and stabilized the other casein fractions, a much clearer view of the action of rennin on milk became possible. Linderström-Lang (48) believed that once this stabilizing fraction was attacked, the remaining casein proteins coagulated in the presence of calcium ions.

Cherbuliez and his associates (15) discovered that one of the casein fractions was more sensitive to rennin attack than were the others. Waugh and Von Hippel (110) found a part of alpha-casein they called kappa-casein, that was not precipitated in the presence of calcium ions under most circumstances. They felt that kappa-casein was the stabilizing fraction specifically attacked by rennin.

The work of Wake (104) and McKenzie (59) showed that the nonprotein nitrogen soluble in 12 percent trichloroacetic acid after treatment of casein with rennin, came from the kappa-casein portion. They found that kappa-casein coagulated with or without calcium ions present. Waugh (108) and
others (109, 113) found that the remainder of alpha-casein minus kappa-casein was precipitated in the presence of calcium ions. This protein fraction is commonly referred to as alpha-casein.

Zittle (111) showed that the presence of kappa-casein could prevent alpha-casein from being precipitated by calcium ions. To Ernstrom (26) this suggested that kappa-casein stabilized the casein micelle, but lost this ability after attack by rennin. The calcium sensitive casein fractions then clotted and joined the already insoluble para-kappa-casein.

Nitschmann et al. (67) studied the nonprotein nitrogen soluble in 12 percent trichloroacetic acid and reported that it was a hydrophilic glycomacropeptide (GMP). They found it contained 30 percent carbohydrate as galactose (15.2%), glucose amine (4.3%) and neuraminic acid (11.4%).

Nitschmann and Beeby (65) found that the GMP came from kappa-casein. In their opinion the hydrophilic nature of the GMP could explain the stabilizing ability of kappa-casein with the GMP attached and loss of the ability with its removal by rennin. Sinkinson and Wheelock (92) found that the carbohydrate content of glycopeptides produced from raw milk by the action of rennin was the same as that found from the action of rennin on kappa-casein. They concluded that the initial action of rennin in clotting milk is to split kappa-casein.
The Nonenzymatic Phase of Rennin Coagulation

With the loss of stabilization as kappa-casein is attacked by rennet, the casein micelle becomes more sensitive to calcium ions. The rate of paracasein coagulation is dependent on the type of casein fractions and the amount of calcium ions present (26). A number of workers have found that the nonenzymatic phase of rennin coagulation is quite sensitive to changes in calcium ion concentration (10, 14, 19, 20, 57, 94). Pyne (74) reported that a difference of 0.2 millimole per liter in a total concentration of 3 to 4 millimoles per liter was significant at temperatures where paracasein coagulation would occur. Hostettler and Imhoff (38) found that in higher calcium concentrations, the micellar size of suspended calcium caseinate particles is manifestly increased.

Pyne and McGann (75) found that native caseinate micelles in milk are joined in a complex with colloidal calcium-phosphate-citrate. It has been reported that these micelles are extremely stable to calcium ions whereas artificial caseinate solutions made from acid precipitated casein are not (58). Ernstrom (26) suggested that colloidal phosphate lends stability to large caseinate micelles along with kappa-casein. McGann and Pyne (58) found that the colloidal calcium phosphate content of a casein micelle is related to its size and thus may determine micelle size.

Zittle (112) found that firm gels were obtained with the action of rennin on solutions of whole casein (0.5%) with 0.010M calcium chloride and
0.0030 M sodium phosphate. Without phosphate present, only flocculent precipitates were obtained. He concluded that the phosphate-calcium bond is no weaker than the calcium-casein bond.

DeMann and Batra (19) found that only about one-fourth of calcium added to skimmilk remained in the ionic form. They reported that the addition of citrate increased rennet coagulation time, presumably by forming complex calcium-citrate ions. The addition of orthophosphates appeared to give a slight decrease in casein micelle stability. They concluded that orthophosphates cannot form complex ions with calcium. They reported polyphosphates to have a calcium complexing ability similar to citrate.

It has been found that the nonenzymatic reaction in milk clotting has a high temperature coefficient. Berridge (10) reported that the temperature coefficient for the non-enzymatic phase to be 1.3-1.6 per degree C. He and Pyne (74) found the enzymatic phase coefficient to be about 0.2 per degree C. They used the difference in the temperature coefficients for the two phases to help estimate the length of the nonenzymatic phase.

The action of rennin on milk was studied under the electron microscope by Hostettler and Imhoff (38). They observed spherical calcium-caseinate particles cross-linking to form a network that trapped whey and butterfat within. Scott-Blair and Oosthuizen (88) found that when rennin acts upon skimmilk, the viscosity drops and then rapidly increases with clotting.
They also found that the initial decrease in viscosity was not associated with calcium ions.

**Conditions Which May Affect Both Phases of Milk Clotting**

Lundsteen (53, 54) determined that the optimum pH for the enzymatic phase of rennin coagulation was 5.35. Rotini (83) found it to be 5.7. The concentration of calcium ions and sensitivity of paracasein to calcium ions seems to be affected by pH so that little soluble calcium is needed at pH values below 5.4 to cause coagulation (53). Kelly (44) reported that rennin coagulation time was shortened with higher temperatures up to 40°C. Hamdy and Edelsten (32) found the time decreased up to 42°C.

Ernstrom (26) reported that the literature evidence regarding the influence of some colloidal materials on the clotting of milk by rennin was confusing. Alexander (2) reported that gelatin and gum arabic stopped rennin coagulation. This claim was opposed by Palmer and Richardson (69) and Bendixen (9). The latter found that gelatin enhanced coagulation and curd development. He did not find this effect with albumin. According to Bang (7), ovalbumin retards rennin clotting of calcium caseinate solutions.

Maze (56) reported that adding NaCl to milk reduces clotting action. It has also been reported to weaken the curd strength (93). Zittle (112) reported the viscosity of whole casein solutions was reduced in the presence...
of 0.033 M NaCl. He found that the addition of 0.016 or 0.033 M CaCl₂ with the above NaCl reduced viscosity even more. Hamdy and Edelsten (32) found rennet milk coagulation time decreased by 13 percent with 0.3 percent NaCl added. Above 0.6 percent NaCl added, coagulation time was prolonged. Rüdiger and Wurster (84) found that calcium chloride had its maximum effectiveness at a concentration of 0.142 percent (0.014 M). They found coagulation was slower above and below that concentration. Chloride ions have been reported to be without effect on rennet milk clotting (50).

Ernstrom (26) has reported that the effect of inorganic salts on milk is confusing since the added salts may have altered the very nature of the colloidal calcium-phosphate-casein complex.

Hostettler and Imhoff (39) found that homogenization accelerates rennet clotting. This may be caused by an increased aggregation of casein micelles. Sasaki and Miyasawa (86) reported that homogenization accelerated rennet clotting, but also decreased curd tension. Beck and Rouyer (8) found in 1951 that ultrasonic treatment of milk does not affect its ability to clot. Kelly (44) reported that beyond 0.07 percent calcium chloride added to milk gave no further increase in curd tension. He and others (78) found pasteurized milk to form a softer curd than raw milk. Scott-Blair and Burnett (87) said that once setting began, homogenized and separated became firm faster and pasteurized milk slower than did raw milk.
Measuring the Milk Clotting Activity of Rennin and Other Enzymes

Many methods have been devised to measure the degree of milk clotting ability in enzyme solutions, but they all leave much to be desired. The usual way enzyme activity is measured is by the rate the substrate disappears or products of the reaction appear. Since milk clotting involves enzymatic and nonenzymatic reactions, it can only approximate rennin activity. When the specific reaction site of rennin on kappa-casein is identified, it may be possible to develop a peptide upon which the action of rennin can be accurately measured. The release of nonprotein nitrogen from casein has been suggested as a measure of rennin activity, but has not yet been proven practical (66).

Many workers have studied changes in viscosity as a way of determining rennin activity (37, 45, 52, 88). The initial decrease in viscosity has been found to follow zero order kinetics (26). Scott-Blair and Oosthuizen (88) produced the same slope with different sources of casein, but unequal slopes were produced when rennet from different sources was used. It was suggested by them that proteolytic enzymes other than rennin may have been present.

Some researchers have investigated changes in light transmission in order to define a point of coagulation (64, 106). Others have investigated light reflected by means of a thrombelastograph (99). Lundsteen (54)
suggested using the differences in solubility of casein and paracasein. A light scattering method was described by Claesson and Nitschmann (16) to measure the clotting action of rennin on skim milk.

Lawrence and Sanderson (47) have described a method of quantifying the proteolytic activity of rennin and other milk clotting enzymes. They used a micro-method assay with calcium caseinate on agar slides. The number and width of the enzyme precipitated zones was used to measure the concentration of enzyme used. Some workers have found this technique difficult to use and feel that proteolytic methods are not suitable for measuring the milk clotting activity of enzymes (79). Everson and Winder (27) have described an ultrasonic determined end point for rennet milk coagulating measurement.

Most of the milk clotting enzyme tests used today involve observation of a visual end point. In 1911 Graber (31) described a method of determining rennet strength. He put about two quarts of milk in a container with an amount of rennet. Then he tipped the container to one side periodically to test for coagulation.

A more precise method was developed by Sommer and Matsen (95). They invented a machine consisting of a water bath and apparatus to support and rotate 125 ml wide-mouth test bottles. A test is begun by adding enzyme to 50 ml of milk substrate in each bottle and ends when the coagulation
becomes visible on the bottle wall. Coagulation time is determined by a counter which measures 16 counts per minute.

Because of the differences in milk from different sources, Berridge (13) suggested a substrate consisting of 60 g nonfat dry milk (NDM) reconstituted in 500 ml 0.01 M CaCl₂. Ernstrom (23) found that storing Berridge's substrate for 20 hours at 2 °C gave greater reproducibility. He described an exact procedure for testing the strength of a rennet extract using Berridge's substrate on the Sommer-Matsen apparatus. This coagulation time would then be compared to that obtained using a standard rennet exact. For quantitative expression of the milk clotting strength of a rennet solution, he suggested the formula:

\[ RU/ml = 100 \times \frac{Ts}{Tu} \times \frac{Cs}{Cu} \]

where:  
- \( RU/ml \) = rennin units per ml of unknown  
- \( Ts \) = coagulation time of the standard  
- \( Tu \) = coagulation time of the unknown  
- \( Cs \) = concentration of the standard  
- \( Cu \) = concentration of the unknown

Recently, deMan and Batra (19, 20) described the use of an automatic blood-clot timer for measuring the effect of mineral constituents on rennet clot times. They felt precision with the clot timer was satisfactory and suggested further studies would prove the instrument to be a valuable tool. A commercial manufacturer of rennet extracts has reported successful use of
the blood-clot timer for standardizing rennet preparations (personal communications with John Sharers, 1970).

Storrs (98) designed and tested an automatic tester with NDM substrate for measuring the activity of rennet. The end point was reached when the pressure of coagulation in a rotating cylinder forced a paddle to break an electric circuit. He felt the error of individual tests to be about one percent.

Many modifications upon the substrate prepared by Berridge have been reported (18, 27, 30, 70, 80, 81, 100). Usually NDM is used for the substrate, but evaporated milk, skimmilk and whole milk have also been utilized. Milk solids content in these tests ranged from 1 to 20 percent. From 0 to around 0.01 M calcium was added. Some substrates were buffered, but most were not. pH values ranged from 5.1 to 6.3. Pretempering times ranged from 0 to 48 hours and at temperatures ranging from 2 to 30 C. The substrate was used in volumes ranging from 0.2 to 50 ml.

The tests included other wide ranging controlled conditions. Water bath tempering times ranged from 5 to 30 minutes. Test temperatures went from 30 to 37.6 C. The desired clot times ranged from 0.5 to 30 minutes. Added enzyme volumes extended from 0.1 to 1 ml. The enzyme solutions were buffered in some cases. Enzyme to substrate ratios varied from 1:2 to 1:100. These ranges point out how buffering the enzyme-substrate mixture is necessary where pH and salt concentration differences may exist.
It is obvious from the above, that rennet milk clotting strengths cannot be meaningfully defined under such wide ranging test conditions.

**Effect of Cold and Heat on Milk**

Raw milk that is cooled for several hours, then tested with rennin at 20 to 30°C is found to have a progressively longer coagulation time with length of cooling. This effect, known as "hysteresis," is reversed when the cool milk is held at 30°C before adding rennin (77). It was also reported by Rapp (76) that this hysteresis effect could be negated by pasteurization for 30 minutes at 62°C providing the milk was not recooled. Earlier research showed that milk given heat treatments to 40.6-60.0°C for 30 minutes had a shorter rennin coagulation time than did unheated milk. After storage though, the heated milk gained a progressively longer clotting time (55).

Ernstom (26) reported that the effect of temperatures to 62°C on milk coagulability is mainly reversible. It was reported by Pyne (73) that soluble calcium and phosphate were transferred to the colloidal calcium-phosphate-casein complex in milk heated at lower temperatures. He concluded that paracaseinate clotted faster in the presence of more calcium ions. Davies and White (17) reported that upon cooling, calcium and phosphate return to the soluble state. Thus the paracaseinate has less colloidal calcium-phosphate available to sensitize it to calcium ions.
The change in state of calcium and phosphate during the heating of milk, causes the pH to decrease. Albrecht (1) considered the pH change more important than the increase in colloidal calcium phosphate. Heating milk to 65 - 100 °C for 30 minutes was found by many workers to increase rennin coagulation time with an additional increase in clotting time upon cooling the milk (1, 71, 72). Ernstrom (26) interprets this effect as an alteration of the milk proteins to an extent that overshadows the effect of the decrease in pH and increase in colloidal calcium-phosphate.

Kannan and Jenness (42) reported that beta-lactoglobulin appeared to interfere with the action of rennin on casein when casein was heated to 85 °C for 30 minutes in the presence of beta-lactoglobulin. Hindle and Wheelock (36), however, found that the primary phase of rennin action on milk was unaffected by heat sterilization. Their work indicated that the action of rennin on sterilized milk is only retarded.

**Effect of Ionizing Radiation on Milk**

Proteins may be denaturated as a result of ionizing radiation (29, 35, 40). The protein molecules are split and the fractions are then polymerized (29, 34). Desrosier et al. (21) found that ionizing radiations on milk casein produce changes that cause an increase in the rennin clotting time and a loss of heat stability. They felt that radiation affects milk two ways. First the enzymatic phase is inhibited, possibly by calcium combining with activated
protein molecules in a way that the reactive sites on kappa-casein are not available for rennin action. The evidence for this is based on studies of the effect of cathode rays on purified casein in solutions. The changes in viscosity of the solutions after radiation suggest the association of casein molecules and fractions thereof into complexes (21). The nonenzymatic phase is probably affected by radiation precipitating calcium as tricalcium phosphate. In this manner calcium ions become unavailable for coagulation of paracasein (21).

The radiation effect on microorganisms is felt to be due to the direct hit of a radiation particle through a cell wall or near to it (35, 43). The immediate result of this hit or near hit may be alteration of the cell nucleus, cytoplasm or perhaps with a near hit, changes in the media adjunct to the cell that make the media nonnutrative (34, 35, 43). The final result of microbial irradiation may be lethality, gene mutation, inhibition of growth and alterations in nutrient requirements (34, 43). The microbial environment may modify the lethal effect of irradiation. Anaerobic cultures of yeast have been found to be more resistant to radiation than the same cultures under aerobic conditions (43). Organisms may become viable on new media even though they appear to be dead (43). Bacterial spores are much more resistant to ionizing radiations than are vegetative cells (63).

In food with a pH greater than 4.5, sterilizing doses of radiation must be based on the absence of Clostridium botulinum type A (40). Doses of 4.5
Mrad have been found to be sterilizing for this organism in canned meat (22). Enzymes usually require five to ten times higher radiation doses for inactivation than are required for microorganism destruction. Destruction of Clostridium botulinum type A toxin requires greater than 7 Mrad (40). Certain microbes possess very high radiation resistance. Micrococcus radiodurans survives radiation doses that eliminate Clostridium botulinum heat sensitive types (40).

Effect of Chemical Preservatives on Rennet and Milk

Davis (18) reported that chloroform and similar antiseptics tend to inactivate rennin. He stated that milk containing chloroform can be acted upon by rennet, but milk with 0.3 percent formaldehyde will be unchanged by the enzyme. According to him, rennet is best sterilized with iodine, which will not reduce its stability.

In 1897 Babcock and Russell (3) used chloroform, ether, benzol, thymol, sodium fluoride, salicylic acid and other agents to control microbial growth in samples of milk they were investigating for the presence of natural proteases. They found that 12 percent ether preserved skimmilk, but that whole milks required more ether. They reported that after being exposed to the anaesthetics for a time, the milks contained only bacteria in spore bearing forms. After some months they said even these spores were destroyed. They found that in these aseptic milks, regardless of the
anaesthetic used, that the casein was gradually precipitated and converted into a soft gelatinous material. Considering the aseptic conditions present, they could only suggest that there was a proteolytic enzyme (s) naturally present in the milk. They named the protease "galactase."

In 1898 they and Vivian (4, 5) investigated the antiseptic value of certain chemicals in milk. Of the sixteen chemicals they studied, they considered chloroform to be the best antiseptic to use with milk. Van Slyke et al. (103) in 1901 studied enzymes in cheese. They found that milk with 2.5 percent chloroform contained 13 "germs" per ml and milk with 20 percent chloroform contained 6 "germs" per ml after 192 days. In another study, milk with 4 percent chloroform had no germs after 152 days. In their work they carefully mixed the samples each day to insure complete contact of chloroform with the milk, since chloroform separates out and produces conditions favoring the germination of spores in parts of the milk. They concluded that chloroform was more effective as an antiseptic than ether or a mixture of chloroform and ether. Cheese that contained 12-15 percent chloroform was made from milk containing 4-5 percent chloroform. They felt that the settling of chloroform on the bottom of the milk containers was useful in preventing considerable evaporation losses.

A few years later, Thatcher and Dahlberg (101) reported in their study of enzymes in milk and butter, that 0.5 percent chloroform inhibited bacterial action and that 1.0 percent chloroform completely inhibited proteolysis.
in skim milk. Warner and Polis (107) in 1945 reported on the presence of a proteolytic enzyme in casein. They found the soluble nitrogen content greatest at pH 8.5 indicating the greatest proteolytic activity at this pH. They could destroy the activity by heating casein to 80°C for ten minutes. Chloroform was found to have an inhibitory effect on the enzyme. They presumed that the enzyme they studied was the same (galactase) as that studied by Babcock et al. (4) and Van Slyke et al. (103). They eliminated bacterial activity by sterilizing casein solutions with filtration through a Seitz filter. Washing casein with ethanol reduced enzyme activity to very low levels.
METHODS AND PROCEDURE

Technique with Clot-timer

A Mechrolab Dual Channel Clot-timer - Model 202A, modified with a 30 C water bath (see Figure 1) was purchased from Heller Laboratories, Santa Rosa, California. The Mechrolab Clot-timer was developed to increase accuracy and automate blood coagulation tests. In blood coagulation tests the method of measurement began with simultaneous plasma-reagent mixing and timer startup. Controlled and systematic stirring continued with stainless steel needles until a fibrin-gel of sufficient strength was picked up by a needle and short-circuited two electrodes to stop the timer.

The clot-timer contained a thermostat which maintained water bath temperatures at 30 ± 0.2 C. The dual channel feature enables the operator to make two different sample coagulation time determinations at the same time or compare the sample under test with a control or duplicate sample. It also allowed the operator to make a timer cross-check measurement. The timers were digital counters which measure to the nearest 0.1 second. However, since the rotor made just one complete revolution each second (thus a needle passes the electrodes every one-half second) deviations in reproduction times were in one-half second increments. The rotors and reaction cups were disposable. The water bath and prewarm rack held 20 samples at one time. Output receptacles were provided for data processing.
Figure 1. Mechrolab Dual Channel Clot-timer - Model 202A.
systems. The clot-timer was equipped with a 30 C water bath so that preparations could be evaluated at a temperature approximating their use in cheese manufacture.

When the clot-timer was delivered, it was tested and installed according to the instruction manual. All procedures were done in accordance with this manual except where not applicable to blood testing methods. The water bath temperature was constantly monitored. The instrument was left on at all times, so no warm up time was required. Periodic time cross-checks of the timer modules resulted in values of $60 \pm 0.1$ seconds. Time checks of both modules with a stop watch resulted in differences of less than two seconds in ten minutes.

Before use, the reaction cups were warmed in the water bath and rotors on the instrument surface. Reaction cups, rotors, test tubes and pipette tips were discarded after one use. The Biopette automatic pipette (0.1 to 1.0 ml) was used for all tests. A check of the automatic pipette was made to see if variations in discharge were significant. Twenty samples of 0.2 ml of pasteurized whole milk discharged from the automatic pipette differed in weight from each other by 0.8 percent or less. Inner surfaces of the pipette tips were wetted before discharge.

A test using the clot-timer was begun by filling reaction cups with 0.2 ml of substrate. After one minute the rotors were loaded with 0.1 ml of 30 C tempered enzyme preparation and the module was immediately
started. As with coagulation tests, the rotor needles continuously stirred the mixture until a mass formed on one of the needles, which was of sufficient size to short out the two electrodes and stop the timer.

It was hoped that while the rotor needles stirred the rennet-substrate mixture, particle agglomeration would increase until the strength of the curd would allow a piece to be picked up, large enough to short out the electrodes. In this way the strength of enzyme solutions could be correlated to curd strength, which is a primary economic factor in cheesemaking.

It was felt, however, that with the method used, viscosity was the main principle involved. Close inspection and high speed motion picture studies indicate that layers of coagulated particles build on the rotor needles, surrounded by a layer of increasingly viscous liquid. Then the surrounding droplet touched both electrodes simultaneously, the timer and rotor stopped.

Figure 2, top frame, shows the viscosity build up on a rotor needle. In the middle frame the circuit is shown completed. Note the liquid bridge between needle and lower electrode. The bottom frame shows the stopped rotor, but no lasting droplet bridging both electrodes. Only a liquid residue on the near electrode is visible. This test was with an 8 percent solids NDM solution. Tests with higher solids solutions gave end points where the electrodes remained bridged; however, reproducibility was quite poor.
Figure 2. Endpoint of test on clot-timer.
**Enzyme Preparations**

The standard rennet extract used in this study was DFL Rennet Working Normal manufactured by Dairyland Food Laboratories, Inc.; Waukesha, Wisconsin. It was stored at 5°C at all times. Water used for dilutions was doubled distilled and stored at 5°C. The standard rennet extract contained 100 rennin units (RU) per ml. Enzyme dilutions were made up as needed. They contained from 1 to 0.05 RU/ml, depending upon the substrate used and the timing desired of from 100 to 300 seconds. Preliminary results indicated that reproducibility was best in this time range.

If standard rennet extract contained 20% NaCl, which is above the 18% normally present following extract finishing, there would be 0.01% NaCl in the mixture of extract and enzyme dilution in the test cup.

\[
(100 \text{ RU} \times \frac{1}{333} = 0.3 \text{ RU/ml enzyme dilution.) Therefore 20\% \times \frac{1}{333} = 0.06\% \text{ NaCl/ml enzyme dilution. This was diluted further 1/1 with salt solutions ranging from 0 to 0.2\%. The maximum salt concentration was thus }\]

\[
\frac{0.2}{2} + 0.03 = 0.13\% \text{ in the extract dilution and } \frac{0.13}{3} = 0.043\% \text{ in the substrate-enzyme dilution mixture.}
\]

In the Berridge test 1 ml of diluted extract is added to 25 ml substrate. If the standard enzyme preparation contained 20% NaCl and was diluted \(\frac{1}{250}\) then the NaCl would be \(\frac{1}{250} \times 20 = 0.08\%\) in the dilution and \(\frac{1}{26} \times 0.08 = 0.003\%\) in the milk-enzyme mixture. There is thus from 3 to over 10 times more NaCl in clot timer test than in the Berridge test.
Thus in situations where rennet extracts have high concentrations of salt (10-20%) and low concentrations of enzyme (<10 RU/ml), concentrations of NaCl will be present that will affect the coagulation time on the clot-timer. In these cases the Sommer-Matsen apparatus would be less affected by the NaCl concentration and thus more suitable for testing.

The pH of enzyme dilutions was 5.8. Buffers were not added. When needed for testing, small amounts of the dilutions were tempered in glass test tubes placed in the prewarm rack of the clot-timer, for two or three minutes until they were 30°C.

**Substrate Preparations**

All chemicals used were reagent grade. Water was double distilled. Stock solutions of 1 M CaCl₂, 1 M cacodylic acid and 1 M triethanolamine were prepared. A pH 6.6 buffer was prepared by adding approximately one part 1 M cacodylic acid to 0.7 part 1 M triethanolamine. Two basic types of substrate were prepared. One was based upon instant NDM and the other pasteurized skimmilk.

NDM substrates were prepared in this manner:

1. Mix the amount of NDM needed in 50 ml H₂O.
2. Add 20 ml of the pH 6.6 buffer.
3. Add the amount of 1 M CaCl₂ needed.
4. If used, add needed amount of 200 bloom gelatin.
5. If used, add needed amount of chloroform.

6. Check pH and adjust to 6.6 if necessary, by adding 1 M triethanolamine dropwise.

7. QS to 100 ml volume.

8. Homogenize with hard homogenizer.

Skimmilk substrates were prepared as follows:

1. Pasteurize 500 ml of raw skimmilk at 63°C for 30 minutes.

2. Rapidly cool to room temperature.

3. Add 50 ml of the pH 6.6 buffer.

4. Add the amount of 1 M CaCl₂ needed.

5. If used, add needed amount of 200 bloom gelatin.

6. If used, add needed amount of chloroform.

7. Check pH and adjust to 6.6 if necessary, by adding 1 M triethanolamine dropwise.

8. Homogenize with hand homogenizer.

All substrates were kept in tightly covered small glass bottles. They were stored for 12 hours before use. Substrates containing chloroform were gently swirled for about 15 seconds each day for three days after preparation. Substrates were kept at room temperature, out of direct sunlight.

Instant low heat NDM was used because it was easier to dissolve than non-instantized low-heat NDM. Skimmilk was pasteurized since most
milk for cheesemaking is pasteurized and also to inactivate the proteolytic enzyme in casein described by Warner and Polis (107) and others (3, 4, 101, 103). The substrates were buffered at pH 6.6 to approximate the pH of milk when acted upon by rennet in cheesemaking. The strength of the substrate buffer was found to be sufficient to also buffer the enzyme-substrate mixture to pH 6.6. The use of triethanolamine and cacodylic acid as buffers was suggested by a method used by Gorini and Lanzavecchia (30). This buffer system appears to not contain ions that affect the action of rennin or clotting of paracasein.

CaCl₂ was used to accelerate the clotting of paracasein so the test would measure, as much as possible, the enzymatic phase of milk coagulation. A small amount of gelatin was used in the substrate to increase viscosity as suggested by the results of Bendixen (9), previously discussed.

Chloroform was used to sterilize the substrates; it was also reported to be effective in deactivating the proteolytic enzyme previously described (3, 4, 101, 103, 107). Substrates with chloroform were kept out of direct sunlight since chloroform gradually decomposes in sunlight. Substrates were homogenized and swirled for three days to increase the likelihood of all bacteria coming in contact with the chloroform. The 12-hour storage period was to ensure equilibrium of salts in the substrates. All skimmilk used in these studies was obtained from the Utah State University Dairy Farm.
RESULTS

Effect of Added Chloroform in NDM Substrate upon Coagulation Time

A substrate containing 12 percent NDM and 0.05 M added CaCl₂ was divided into three portions. Chloroform was added so that the portions contained 0, 0.5 and 1.0 percent, respectively. Each sample was tested on the clot-timer with an enzyme dilution of 0.8 RU/ml. Over a three-hour period ten duplicate tests were run for each sample. Statistical analysis of the tests is shown in Table 1. The mean clot-times are shown plotted in Figure 3.

Table 1. Statistical analysis of tests used to determine the effect of added chloroform upon NDM substrate coagulation time.

<table>
<thead>
<tr>
<th>Added chloroform (%)</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean clot-time (x)</td>
<td>101.9</td>
<td>120.9</td>
<td>231.4</td>
</tr>
<tr>
<td>Standard deviation (s)</td>
<td>2.05</td>
<td>3.14</td>
<td>3.49</td>
</tr>
<tr>
<td>Coefficient of variability (C. V.)</td>
<td>2.01</td>
<td>2.60</td>
<td>2.24</td>
</tr>
</tbody>
</table>
Figure 3. Effect of added chloroform upon NDM substrate coagulation time.
The clot-time increased with increases of added chloroform. It appears that chloroform interferes with the action of and/or inactivates rennin in some manner. A study needs to be made to see if rennin is inactivated by chloroform and/or if chloroform interferes with the action of rennin on casein. This test demonstrates that the chloroform content of a standard substrate for measuring rennin activity must be kept constant.

**Effect of NDM Concentration on Substrate Coagulation Time**

Four substrates were prepared with 4.0, 8.0, 12.0 and 16.0 percent NDM, respectively. All contained 0.03 M CaCl$_2$ added and 1.0 percent chloroform. The samples were tested on the clot-timer with a 0.8 RU/ml enzyme dilution. Three duplicate tests were made for each sample over a three hour period. Statistical analysis of the tests is shown in Table 2. The mean clot-times are shown plotted in Figure 4.

The test time increased directly with increasing amounts of NDM. This was as expected. It is the result of lengthening of the enzymatic phase of milk clotting. The speed of the nonenzymatic phase for each sample was not limited by the concentration of CaCl$_2$. Other samples, identical except that the concentration of added CaCl$_2$ was increased to 0.05 and 0.07 M, showed longer clotting times.
Table 2. Statistical analysis of tests used to determine the effect of NDM concentration upon substrate coagulation time.

<table>
<thead>
<tr>
<th>NDM in substrate (%)</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>x (sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.V.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Another purpose of this test was to evaluate which concentration of NDM gave the most reproducible results. The samples with 8 and 12 percent NDM both gave quite consistent test times. The samples with 4 and 16 percent NDM gave relatively poor reproducibility. The clot from the 4 percent sample appeared watery. The 16 percent NDM sample was so viscous that the enzyme dilution was probably not mixed in uniformly. Since the 8 percent NDM sample gave a faster test time, this concentration was used in future testing.

Effect of Exposure to Different Temperatures for 30 Minutes on Skimmilk Coagulation Time

A skimmilk substrate was prepared that contained 0.025 M CaCl$_2$ added and 8 percent milk solids. The substrate was divided into five portions.
Figure 4. Effect of NDM concentration upon substrate coagulation time.
which were heated for 30 minutes at 60, 70, 80, 90 and 120 C, respectively. The samples were then held for 45 hours at 5 C. Each sample was tested on the clot-timer with a 0.2 RU/ml enzyme dilution. Four duplicate tests were conducted with each sample over a four hour period. Statistical analysis of the tests is shown in Table 3. The mean clot-times are shown plotted in Figure 5.

Table 3. Statistical analysis of tests used to determine the effect of exposure to different temperatures for 30 minutes upon skimmilk coagulation time.

<table>
<thead>
<tr>
<th>Treatment temperature (C)</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>215.1</td>
<td>238.6</td>
<td>345.1</td>
<td>428.6</td>
<td>1423</td>
</tr>
<tr>
<td>s</td>
<td>2.95</td>
<td>4.77</td>
<td>15.91</td>
<td>35.55</td>
<td>140</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.37</td>
<td>2.00</td>
<td>4.61</td>
<td>8.30</td>
<td>10</td>
</tr>
</tbody>
</table>

The clot-time increased, roughly logarithmically, to increases in heating temperature. As previously discussed, the increase in clotting time may be due to the effect of heat on beta-lactoglobulin, which slows the action of rennin on casein and also to the hysteresis effect. Another purpose of this test was to see if heat sterilized milk would make a suitable shelf-stable
Figure 5. Effect of exposure to different temperatures for 30 minutes upon skimmilk coagulation time.
LOG COAGULATION TIME (SEC) vs. TEMP (°C)
substrate. Unfortunately, reproducibility became poorer with increased temperatures and was especially poor at 120 C.

**Effect of Cesium 137 Irradiation upon Skimmilk Substrate Coagulation Time**

This test was done with a skimmilk substrate containing 0.025 M CaCl₂ added and 8 percent milk solids. Portions of the substrate were exposed for 0, 10, 20, 30, 40, 50, 60 and 70 hours, respectively, to cesium 137 radiation at 18.9 KRad/hour. The clot-time for each sample was then determined on the clot-timer with a 0.1 RU/ml enzyme dilution. Four duplicate tests were conducted for each sample. Statistical analysis of the tests is shown in Table 4. The mean clot-times are shown plotted in Figure 6.

Table 4. Statistical analysis of tests used to determine the effect of cesium 137 irradiation upon skimmilk substrate coagulation time.

<table>
<thead>
<tr>
<th>Radiation exposure (Hr)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>179.6</td>
<td>195.7</td>
<td>213.1</td>
<td>234.4</td>
<td>267.8</td>
<td>289.7</td>
<td>355.6</td>
<td>369.0</td>
</tr>
<tr>
<td>s</td>
<td>2.72</td>
<td>2.85</td>
<td>2.78</td>
<td>3.65</td>
<td>3.70</td>
<td>3.60</td>
<td>5.40</td>
<td>4.56</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.52</td>
<td>1.46</td>
<td>1.31</td>
<td>1.56</td>
<td>1.38</td>
<td>1.24</td>
<td>1.52</td>
<td>1.24</td>
</tr>
</tbody>
</table>
Figure 6. Effect of cesium 137 irradiation upon skimmilk substrate coagulation time.
The results show a logarithmic increase in loss of coagulating ability with increasing exposure to radiation. This is in agreement with the results reported by Derosier (21) previously discussed. Also with added exposure, the samples became darker brown in color and possessed a stronger peculiar odor.

After the tests were completed, duplicate samples that were unopened after irradiation, were held for two weeks at room temperature. At the end of this period, they were all found to be spoiled by microorganisms. This was not unexpected since about 240 hours of exposure would have been necessary for sterilization. It is noteworthy that reproducibility did not become poorer after 70 hours of exposure. This indicates that a substrate sterilized by irradiation may still have enough reproducibility and clotting ability to make it suitable as a standard shelf-stable substrate.

Effect of CaCl₂ Addition upon Coagulation Time of NDM and Skimmilk Substrates

Ten samples each of NDM and skimmilk substrates with 8 percent milk solids, 1 percent chloroform and 0.3 percent gelatin were prepared. They contained 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08 and 0.09 M CaCl₂ added, respectively. Four duplicate clot-time tests were run with each sample over an eight-hour period. The substrates were evaluated with a 0.1 RU/ml enzyme dilution. Statistical analysis of the tests is shown in Table 5. The mean clot-times are shown plotted in Figure 7.
Table 5. Statistical analysis of tests aged to determine the effect of CaCl$_2$ addition upon coagulation time of NDM and skimmilk substrates.

<table>
<thead>
<tr>
<th>CaCl$_2$ added in substrate (M)</th>
<th>(skimmilk)</th>
<th>(NDM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>540.8</td>
<td>1030.4</td>
</tr>
<tr>
<td>0.02</td>
<td>282.2</td>
<td>387.4</td>
</tr>
<tr>
<td>0.03</td>
<td>238.4</td>
<td>330.7</td>
</tr>
<tr>
<td>0.04</td>
<td>217.0</td>
<td>304.1</td>
</tr>
<tr>
<td>0.05</td>
<td>224.8</td>
<td>331.1</td>
</tr>
<tr>
<td>0.06</td>
<td>245.7</td>
<td>362.0</td>
</tr>
<tr>
<td>0.07</td>
<td>261.2</td>
<td>377.4</td>
</tr>
<tr>
<td>0.08</td>
<td>284.8</td>
<td>410.6</td>
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<table>
<thead>
<tr>
<th>(sec)</th>
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</thead>
<tbody>
<tr>
<td>x</td>
<td>540.8</td>
<td>1030.4</td>
</tr>
<tr>
<td>s</td>
<td>11.88</td>
<td>80.25</td>
</tr>
<tr>
<td>C.V.</td>
<td>2.20</td>
<td>7.79</td>
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</table>

<table>
<thead>
<tr>
<th>(sec)</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>387.4</td>
<td>387.4</td>
</tr>
<tr>
<td>s</td>
<td>10.12</td>
<td>10.12</td>
</tr>
<tr>
<td>C.V.</td>
<td>2.61</td>
<td>1.67</td>
</tr>
</tbody>
</table>
Figure 7. Effect of CaCl$_2$ addition upon coagulation time of NDM and skimmilk substrates.
COAGULATION TIME (SEC)

- SKIMMILK SUBSTRATE
- NDM SUBSTRATE

M CaCl₂ ADDED IN SUBSTRATE
It can be seen that the skimmilk substrates coagulated faster than NDM substrates did and that clot-times were slower when the concentration of CaCl₂ added was greater or less than 0.03 M. It should be noted though, that the addition of enzyme to the substrate in the test, reduced the CaCl₂ added concentration in the mixture by one-third, to about 0.02 M. As previously stated, Rüdiger and Wurster (84) found that CaCl₂ had its maximum effectiveness at a concentration of 0.142 percent = about 0.014 M. They found that above and below that concentration, coagulation was slower. With the clot-timer, perhaps at concentrations in the substrate of greater than 0.03 M CaCl₂ added, the calcium ions interfere with the rennin attack or in some way give increasing stability to the paracasein particles. At any rate the nonenzymatic phase seemed to proceed most rapidly at 0.03 M CaCl₂ added, for an 8 percent nonfat milk solids substrate.

Comparison of the Dilution Factors Reported Using Berridge Substrate on Sommer-Matsen Apparatus (23) to Those Found Using Skimmilk and NDM Substrates on the Clot-timer

The NDM and skimmilk substrates each contained 8 percent milk solids, 1 percent chloroform, 0.3 percent gelatin and 0.03 M added CaCl₂. The NDM and skimmilk substrates were tested on the clot-timer with various enzyme dilutions. Five duplicate tests were made for each dilution used. Statistical analysis of the tests is shown in Table 6. The results in Figure 8 represent (clotting time X enzyme concentration) vs enzyme concentration.
Table 6. Statistical analysis of clot-time values of tests used for Figure 8.

<table>
<thead>
<tr>
<th>Enzyme used with skimmilk substrate (RU/ml)</th>
<th>0.05</th>
<th>0.10</th>
<th>0.15</th>
<th>0.20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>405.1</td>
<td>207.5</td>
<td>145.6</td>
<td>117.5</td>
</tr>
<tr>
<td>s</td>
<td>4.50</td>
<td>2.09</td>
<td>2.31</td>
<td>1.41</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.11</td>
<td>1.01</td>
<td>1.59</td>
<td>1.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme used with NDM substrate (RU/ml)</th>
<th>0.10</th>
<th>0.15</th>
<th>0.20</th>
<th>0.25</th>
<th>0.30</th>
<th>0.40</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>340.2</td>
<td>250.1</td>
<td>189.9</td>
<td>172.2</td>
<td>147.0</td>
<td>125.1</td>
<td>105.8</td>
</tr>
<tr>
<td>s</td>
<td>4.79</td>
<td>4.65</td>
<td>3.81</td>
<td>3.01</td>
<td>3.24</td>
<td>2.95</td>
<td>3.14</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.41</td>
<td>1.86</td>
<td>2.00</td>
<td>1.75</td>
<td>2.20</td>
<td>2.36</td>
<td>2.97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme used with Berridge substrate (23) (RU/ml)</th>
<th>0.4</th>
<th>0.6</th>
<th>0.8</th>
<th>1.0</th>
<th>1.2</th>
<th>1.4</th>
<th>1.6</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>943</td>
<td>644</td>
<td>485</td>
<td>400</td>
<td>328</td>
<td>288</td>
<td>255</td>
<td>212</td>
</tr>
<tr>
<td>s (*)</td>
<td>4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.V.(*)</td>
<td>0.51</td>
<td>0.74</td>
<td>0.99</td>
<td>1.20</td>
<td>1.46</td>
<td>1.66</td>
<td>1.88</td>
<td>2.26</td>
</tr>
</tbody>
</table>

*Estimated based on Ernstrom data (23).
Figure 8. Comparison of the dilution factors reported using Berridge substrate on Sommer-Matsen apparatus (23) to those found using skimmilk and NDM substrates on the clot-timer.
This is based on the relationship proposed by Storch and Segelke (97) (time X concentration) = constant. The results with Berridge substrate shown on the figure are those reported by Ernstrom (23) using Sommer-Matsen apparatus. Ideally for a milk clotting test, the lines would have no slope, indicating agreement with the Storch-Segelke equation. However, all substrates showed a slope or dilution factor per RU change/ml. The greatest slope appeared to be with NDM substrate followed by skim milk substrate. Berridge substrate seemed to have the least slope of all. Because of the increased enzyme to substrate ratio on the clot-timer (12.5 fold higher), the enzyme factor is increased and thus time is decreased.

Holter (37) found that the Storch-Segelke constant decreased with decreasing enzyme concentration. This was also found by Ernstrom (23) and with the NDM and skim milk substrates used in this test. It should be noted that enzymes can be tested at much lower concentrations with the clot-timer, than with Sommer-Matsen apparatus.

**Effect of Rennet Concentration on Coagulation**

**Time of Skimmilk Substrate**

Five enzyme dilutions were tested with a skimmilk substrate containing 8 percent milk solids, 1 percent chloroform, 0.3 percent gelatin and 0.03 M added CaCl$_2$. Five duplicate tests were run on the clot-timer for
each dilution, over a two-hour period. Statistical analysis of the tests is shown in Table 7.

Table 7. Statistical analysis of tests used to determine the effect of rennet concentration upon coagulation time of skimmilk substrate

<table>
<thead>
<tr>
<th>Enzyme used with skimmilk substrate (RU/ml)</th>
<th>0.100</th>
<th>0.101</th>
<th>0.102</th>
<th>0.103</th>
<th>0.104</th>
</tr>
</thead>
<tbody>
<tr>
<td>With both timers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \bar{x} )</td>
<td>207.5</td>
<td>206.4</td>
<td>202.7</td>
<td>202.5</td>
<td>198.3</td>
</tr>
<tr>
<td>( s )</td>
<td>2.09</td>
<td>1.98</td>
<td>2.14</td>
<td>2.56</td>
<td>2.51</td>
</tr>
<tr>
<td>( \text{C.V.} )</td>
<td>1.01</td>
<td>0.96</td>
<td>1.05</td>
<td>1.26</td>
<td>1.27</td>
</tr>
<tr>
<td>With back timer only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \bar{x} )</td>
<td>207.2</td>
<td>206.2</td>
<td>202.7</td>
<td>201.5</td>
<td>197.3</td>
</tr>
<tr>
<td>( s )</td>
<td>1.02</td>
<td>2.04</td>
<td>1.51</td>
<td>2.05</td>
<td>2.92</td>
</tr>
<tr>
<td>( \text{C.V.} )</td>
<td>0.49</td>
<td>0.99</td>
<td>0.74</td>
<td>1.02</td>
<td>1.48</td>
</tr>
</tbody>
</table>

It can be seen that the mean clot-times on the back timer are generally lower than with both timers. Also the standard deviations are less with the back timer and thus also the C.V.'s, than with both timers. The linear correlation coefficient based on the back timer only is 0.87. The calculated linear regression curve and mean clot-times shown in Figure 9 are based on the back timer only. It can be seen that precision is best on the back timer.
Figure 9. Effect of rennet concentration upon coagulation time of skimmilk substrate.
ENZYME CONC (RU/ml) vs. COAGULATION TIME (SEC)
and that when at least five tests are run for each enzyme dilution on the back timer, the skimmilk substrate can be used to standardize the dilutions to within one percent of each other in terms of milk clotting ability with a reasonable degree of confidence. Examination and measurement of the front and back timer electrodes failed to reveal any differences that would account for the difference in precision. Since deviations in reproduction times are in one-half second increments, the limit of test sensitivity is nearly reached.

Based on the results of Ernstrom (23) shown in Table 6, Berridge substrate on Sommer-Matsen apparatus can standardize enzyme dilutions to within about 0.51, 1.20 and 2.26 percent of each other at 943, 400 and 212 sec., respectively. Therefore, at clot-times less than about 400 seconds, skimmilk substrate on the clot-timer appears to be generally more accurate. For clot-times greater than 400 seconds, Berridge substrate on S-M apparatus appears to be generally more accurate.

**Effect of Storage at Room Temperature on Coagulation Time of Skimmilk Substrate**

Skimmilk substrate containing 8 percent milk solids, 1 percent chloroform, 0.3 percent gelatin and 0.03 M added CaCl₂ was evaluated over an 18-day period. A freshly prepared 0.2 RU/ml dilution was used each test day. Each day of testing consisted of five duplicate tests on the clot-timer.
Figure 10. Effect of storage at room temperature upon coagulation time of skimmilk substrate.
Statistical analysis of the tests is shown in Table 8. The mean clot-time of each period of testing is shown plotted in Figure 10. Bottle #1 was first tested 20 hours after preparation; bottle #2, 8 days after preparation and bottle #3, 17 days after preparation. The substrate was stored at room temperature (about 21-25 C) during the 18 day period.

The results indicate that the substrate is quite shelf-stable for at least 18 days. Unfortunately, lack of time prevented a longer evaluation period; however, there is no reason to believe that the substrate would not remain shelf-stable for an additional length of time. Note that on the seventeenth day, bottle #2 showed a much faster time. This may have been due to contamination with bacteria and evaporation of chloroform. For normal testing, it would probably be best to use a new container each testing day.
Table 8. Statistical analysis of tests used to determine the effect of storage at room temperature upon coagulation time of skim milk substrate.

<table>
<thead>
<tr>
<th>(days)</th>
<th>1</th>
<th>4</th>
<th>5</th>
<th>8</th>
<th>10</th>
<th>17</th>
<th>17</th>
<th>18</th>
<th>All observations except bottle #2 on 17th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottle used</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>(sec)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{x}$</td>
<td>122.6</td>
<td>118.2</td>
<td>117.5</td>
<td>117.9</td>
<td>118.0</td>
<td>106.3</td>
<td>120.1</td>
<td>120.3</td>
<td>119.2</td>
</tr>
<tr>
<td>$s$</td>
<td>1.65</td>
<td>1.64</td>
<td>1.41</td>
<td>1.59</td>
<td>1.44</td>
<td>6.62</td>
<td>1.64</td>
<td>1.11</td>
<td>2.24</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.35</td>
<td>1.39</td>
<td>1.20</td>
<td>1.35</td>
<td>1.22</td>
<td>6.22</td>
<td>1.37</td>
<td>0.92</td>
<td>1.88</td>
</tr>
</tbody>
</table>
COAGULATION TIME (SEC)

AGE OF SUBSTRATE (DAYS)

○ 1
△ 2
□ 3
DISCUSSION

It is believed that the skimmilk substrate developed in this research represents a significant improvement over those in common use. When the substrate is used with the automatic clot-timer, rennet and other proteolytic enzymes can be standardized to within one percent of each other. Under the best of conditions, about 30-40 samples per day can be run on the Sommer-Matsen apparatus. It has been reported that 200 samples per day are currently being run on the clot-timer by a commercial preparer of proteolytic enzymes (personal communications with John Sharers, 1973). Even though many improvements could be made, using the skimmilk substrate on the clot timer might presently offer additional savings for those testing milk-clotting enzymes on a large scale. For others, testing on a smaller scale, it offers the primary advantage of instant availability.

Recommended Procedure for Preparing Skimmilk Substrate

This procedure will make about 570 ml of substrate.

1. Prepare stock solutions of at least 17 ml 1 M CaCl$_2$, 50 ml 1 M cacodylic acid and 35 ml 1 M triethanolamine.

2. Prepare at least 50 ml of pH 6.6 buffer by mixing about 1 part (30 ml) 1 M cacodylic acid with 0.7 part (21 ml) 1 M triethanolamine. Stirring magnetically, adjust to pH 6.6 by adding the needed buffer ingredient dropwise.
3. Pasteurize 500 ml of raw skimmilk at 63 C for 30 minutes. While still hot, slowly add 1.5 g (0.3%) 200 bloom gelatin, stirring magnetically for about 5 minutes in a 600 ml glass beaker. Avoid causing foam. Look at bottom of beaker to make certain all gelatin is dissolved. Rapidly cool mixture to room temperature.

4. While stirring magnetically, first add 50 ml of pH 6.6 buffer, then 17.0 ml of 1 M CaCl₂ (will give 0.03 M CaCl₂ added in substrate). Adjust pH to 6.6 by adding 1 M triethanolamine dropwise.

5. Put mixture in hand homogenizer. Add 5.7 ml (1%) chloroform. Homogenize twice.

6. Immediately fill small (25-50 ml) glass containers and tightly cover.

7. Store out of direct sunlight for 12 hours.

8. Substrate at this point is ready for use.

9. For most reliable bacterial destruction, gently swirl each container for 15 seconds/day for 3 days.

10. Use a new container each testing period (day). Do not leave containers open for more than a few seconds. Gently swirl the container for a few seconds, just before each pipetting.

**Recommended Procedure for Testing Milk-clotting Strength of Unknown Rennet Sample on Automatic Clot-timer**

1. Place unknown and standard rennet extracts in test tubes in the clot-timer prewarm rack. Fill test tubes one-third full. Temper at least 3 minutes or until 30 C.

2. Meanwhile, fill front and back reaction cups, in position, with 0.2 ml of tempered substrate using automatic pipette. Keep substrate container tightly covered, as much as possible.

3. After 1 minute, load both rotors with 0.1 ml of known enzyme preparation and start immediately.
4. Adjust enzyme dilution and repeat steps #1-3 until time range is 100-300 seconds.

5. Repeat steps #1-4 for unknown rennet sample until clot-time is within ± 10 percent of known sample. (This is because of the dilution factor present and to compensate for the action of chloroform on rennin.)

6. Determine rennin activity in unknown sample by the equation proposed by Ernststrom (23):

\[
\text{RU/ml} = 100 \frac{\text{Ts}}{\text{Tu}} \times \frac{\text{Cs}}{\text{Cu}}
\]

where:  \(\text{RU/ml} = \) rennin units per ml of unknown

\(\text{Ts} =\) coagulation time of the standard

\(\text{Tu} =\) coagulation time of the unknown

\(\text{Cs} =\) concentration of the standard

\(\text{Cu} =\) concentration of the unknown
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