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The Role of Lactose in the Age Gelation of Ultra-High-Temperature Processed Concentrated Skim Milk

Venkatachalam Narayanaswamy

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THE ROLE OF LACTOSE IN THE AGE GELATION OF ULTRA-HIGH-TEMPERATURE PROCESSED CONCENTRATED SKIM MILK

by

Venkatachalam Narayanaswamy

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

1992
This work is dedicated to my father, Mr. S. Narayanaswamy; mother, Mrs. Rajam Narayanaswamy; sister, Mrs. Usha Ramakrishnan; brother-in-law, Mr. S. Ramakrishnan; and nephew, baby Raunak.
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Venkatachalam Narayanaswamy
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ABSTRACT

The Role of Lactose in the Age Gelation of Ultra-high Temperature Processed Concentrated Skim Milk

by

Venkatachalam Narayanaswamy, Master of Science
Utah State University, 1992

Major Professor: Dr. Donald J. McMahon
Department: Nutrition and Food Sciences

The purpose of this research was to relate lactose reactivity and age gelation of UHT processed concentrated milk. Skim milk was pasteurized, diafiltered batchwise to reduce lactose concentration to less than 0.05%, and UF concentrated to 3X (one-third volume reduction). Lactose and sucrose were then each added at 3% or 6% w/v to part of the concentrate. The five samples, control (<0.05% lactose), 3% w/v lactose, 6% w/v lactose, 3% w/v sucrose, and 6% w/v sucrose, were UHT processed at 140°C for 4 s using the indirect heating method. Samples were collected aseptically in presterilized plastic containers and stored at 4°C, 20°C, and 35°C for periodic analysis.

All samples stored at 4°C and 20°C gelled after 21 weeks of storage. The viscosity changed slightly during the first 19 weeks of storage but increased suddenly (> 100 cPs) just before gelation. Samples stored at 35°C did not gel but showed sedimentation. Samples stored at 4°C or 20°C underwent little browning, but samples containing 3% and 6% lactose, stored at 35°C, browned considerably. The SDS-PAGE patterns of gelled samples showed new bands because of proteolysis whereas samples stored at 35°C showed bands due to
proteolysis and protein crosslinking and a streaking pattern. Electron micrographs of gelled samples showed various casein particles connected together by hairy appendages protruding from the surface of casein particles, to form a continuous three-dimensional network. In non-gelled samples, the micelles were not joined into a continuous network and few hairy appendages protruded from their surfaces. Hairy appendages were not a result of Maillard reaction occurring during storage.

Maillard reaction neither provided protection against nor promoted age gelation. Proteolysis was not the only cause for gelation. Protein modifications prevented gelation in samples stored at 35°C. Age gelation was probably a two-step process involving dissociation of proteins from the casein micelles that reformed onto the micelle surface as hairy appendages. Aggregation of the protein particles occurred through these appendages rather than through the original micelle surface.
INTRODUCTION

Raw milk is a highly perishable product and is potentially unsafe for consumption without further treatment. It is a source of pathogenic bacteria and is susceptible to rapid spoilage by naturally-occurring enzymes and contaminating microorganisms. These undesirable attributes can be controlled by the age-old practice of boiling milk before consumption. This is still prevalent in many parts of the world, but it imparts an undesirable cooked flavor to milk. Pasteurization, which is a milder heat treatment, destroys pathogenic microorganisms and inactivates some of the undesirable enzymes. This is more suitable for handling and transportation of milk over wide areas, but refrigeration is essential for milk to be stored safely for up to three weeks (48).

In industrialized countries, milk is processed in a limited number of centralized dairy plants. Distribution of milk to wider and more remote areas requires prolongation of shelf life of milk beyond the limits afforded by pasteurization. Sterilization of milk is an excellent means of extending shelf life (48). Ultra-high temperature (UHT) treatment causes only slight changes in chemical, nutritional, and organoleptic qualities of milk (14) because at ultra-high temperatures (ca. 140°C), the rate of bacterial spore destruction is faster than the rate of most detrimental chemical changes (13).

Milk Concentrates

Concentrating milk increases its total solids content and reduces the amount of material to be preserved. Evaporation is the most commonly used concentration process. Its limitations are the undesirable effects of high temperature or losses of volatile components when evaporating under vacuum. Membrane processes are now also being used in the food industry for
concentration (74). Ultrafiltration (UF) and reverse osmosis (RO) offer attractive prospects of concentrating milk at ambient temperature and avoiding chemical damage and flavor changes caused by heating (40). Economic advantages of membrane processing have resulted in its widespread usage. The change in composition of milk using membrane concentration may affect the storage stability of such concentrates sterilized by UHT.

**Age Gelation of UHT milk**

Age gelation of UHT processed milk is an unsolved problem since it signifies the final limit of useful shelf life (13). It has been a hindrance to widespread commercial exploitation of UHT processed concentrated milk (36, 48). Gelation is characterized by loss of fluidity of the product occurring during storage and has been described by different terms such as coagulation, sweet curd formation, thixotropic gel formation, age thickening, partial gelation, or lumpiness (48). Gelation is always preceded by a sharp rise in viscosity (36). Initially there is thinning of product, followed by a long period during which very little change is observed. This is terminated by a sudden rise in viscosity culminating in gel formation within a short period (1–3 weeks). When gelation occurs, the product exhibits a custard-like consistency that is irreversible (49).

**Factors Affecting Age Gelation**

Onset of age gelation is affected by heat treatment, homogenization and sequence of operation, milk solids content, composition of milk, quality of milk, and storage temperature (48). Commercial sterilization of concentrated milk at higher temperature and longer holding times retards gelation (72, 95, 115). However, at equivalent sterilizing effectiveness, higher sterilization temperatures with shorter exposure times result in reduced resistance to gelation (36, 48). Gelation is also influenced by the UHT processing method.
Commercial sterilization by direct heating methods offers less protection against gelation during storage than indirect heating methods (13, 45, 48, 55, 69, 90). The UHT indirect process gives a more intense heat treatment than the direct process (98). An increased severity of heat treatment increases the useful shelf life of UHT milk probably by making the micelles more resistant to changes that promote their aggregation during storage.

There is no unanimous agreement regarding the effect of storage temperature on the gelation of unconcentrated and concentrated UHT processed milk. Some believe that lower temperatures accelerate gelation (5, 94), while others believe that higher storage temperatures accelerate gelation (36, 45, 67, 72, 93). However, there is some indication that very low (<6°C) and very high (>35°C) storage temperatures retard gelation (59, 73, 115).

**Proposed Mechanisms for Age Gelation**

Various explanations have been offered to explain age gelation based on changes observed in UHT milk during storage and on conditions that alter the gelation time. In general, gelation of stored sterilized milk is brought about by loss of colloidal stability of the casein micelles in conjunction with direct interaction between casein micelles leading to the formation of a three-dimensional gel network (48, 97). Assuming that the stability of the casein micelles in milk is maintained by a combination of presence of κ-casein, colloidal calcium phosphate, a high negative zeta potential (-18 mV), and steric stabilization (97), gelation is preceded by changes at the surface of casein micelles enhancing the interaction between the micelles (48, 97). In general, the changes that trigger loss of stability of casein micelles fall into two categories (48):

1. Changes that arise from proteinase activity and
2. Changes that arise from nonenzymic reactions.

*Proteinase hypothesis*

Enzymes in milk are of two kinds: enzymes secreted by the mammary glands and enzymes produced by bacterial growth (15). Some proteolytic enzymes of native and microbial origin can survive UHT treatment or be reactivated during storage and may cause gelation of UHT milk (1, 11, 12, 17, 19, 21, 28, 37, 38, 45, 47, 54, 55, 64, 70, 82, 83, 93, 95, 98, 102, 112, 113, 115) or development of bitterness (28, 30, 93) during storage.

*Nonenzymic basis for gelation*

Gelation of UHT processed concentrated milk has been observed to occur without any proteolysis (25, 54, 72). The absence of a quantitative relationship between gelation time and proteolytic activity has prompted some authors (22, 72, 94) to propose that some physico-chemical process is responsible for gelation. This includes the involvement of whey proteins, chemical modification of casein micelles by Maillard reactions, milk salts, modification of κ-casein during storage, sulfide-disulfide interchange reactions, changes in casein micelle surface potential, or casein micelle dissociation (48).

Andrews and Cheeseman (2, 4, 5) suggested that gelation is caused by polymerization of casein and whey proteins by Maillard-type reactions that are promoted as temperature of storage is increased. However, failure to observe gelation during storage of sterilized milk above 35°C is not consistent with their suggestion (94). Turner et al. (107) suggested that the reaction between lactose and amino groups (predominantly ε-NH₂ groups of lysine) involves Schiff's base formation, which then undergoes Amadori rearrangement to give keto and enol structures. Continuation of Maillard-type reactions during storage of UHT milk would eventually lead to gelation by crosslinking of protein chains.
into very large complexes. This implies that lactose, and not its degradation products, reacts with proteins. This reaction is greater with casein fractions (κ > α, β) than with whey proteins (107). Any reaction of lactose with κ-casein would have profound effects on casein micelle stability during storage. However, Maillard browning of UHT concentrated milk was reported to be similar regardless of rate of gelation (49). Some workers (22, 23) could not correlate Maillard reaction with age gelation since UHT casein micelle dispersions with lactose or sorbitol gelled around the same time. Others (66) observed an increase in shelf life when the nonreducing sugars, sucrose or sorbitol, and reducing sugars, lactose and dextrose, were added to milk before UHT processing. Also, changes on the surface of casein micelles, as seen by electron microscopy, could not be explained by Maillard reaction occurring during storage (6).

An alternative view is that blocking lysine groups in κ-casein results in a loss of sensitivity of casein micelles to aggregate in the presence of rennet. Apart from Ca++ bridging and hydrophobic interactions, ionic interactions between casein micelles through oppositely charged regions of suitable configuration may also be involved in their aggregation. Lysine residues may contribute to the configurations of such regions (51, 52) and could affect gelation of sterile milk because the blockage of their ε-NH₂ groups by interaction with lactose could prevent casein micelles from interacting and gelling.
REVIEW OF LITERATURE

Introduction

The importance of milk in human nutrition has long been recognized. Raw milk is a highly perishable product and is potentially unsafe for consumption without further treatment. It is a source of pathogenic bacteria and is susceptible to rapid spoilage by naturally-occurring enzymes and contaminating microorganisms. These undesirable attributes can be controlled by the age-old practice of boiling milk before consumption. This is still prevalent in many parts of the world but it imparts an undesirable cooked flavor to milk. Pasteurization, which is a milder heat treatment, destroys pathogenic microorganisms and inactivates some of the undesirable enzymes. This is more suitable for handling and transportation of milk over wide areas, but refrigeration is essential for milk to be stored safely for up to three weeks (48).

In industrialized countries, milk is processed in a limited number of centralized dairy plants. Distribution of milk to wider and more remote areas requires prolongation of shelf life of milk beyond the limits afforded by pasteurization. Sterilization of milk is an excellent means of extending shelf life. When selecting heating conditions for sterilization, the following parameters must be considered: optimal destruction of microorganisms (and their heat-resistant spores); inactivation of deleterious enzymes; and retention of desirable attributes of quality in terms of nutrients, color, and flavor, and their stability during storage (48).

Ultra-High Temperature Sterilization

In practice heat sterilization of fluid milk falls into two categories:

1. Retort sterilization (110–120°C for 5–20 min)
2. Ultra High Temperature (UHT) processing (135–145°C for 4–8 s) (13, 14, 48, 76).

UHT processing is applied to milk in continuous flow through heat exchangers. This causes minimal chemical and physical change. UHT milk is a product that has been UHT processed and then packaged into a sterile container under aseptic conditions. There are two advantages of UHT processing over retort sterilization:

1. A UHT sterilizer/aseptic filling system is more economical in energy consumption. In a retorting system much heat is lost through the physical size of the installation and the rejection of low grade heat in waste water.

2. UHT sterilization occurs with only slight changes in chemical, nutritional, and organoleptic qualities of milk (14) because at higher temperatures the rate of spore destruction is faster than the rate of most chemical changes (13).

**Milk Concentrates**

Concentrating milk increases its total solids content and reduces the amount of material to be preserved. Evaporation is the most commonly used concentration process. Its limitations are the undesirable effects of high temperature or losses of volatile components when evaporating under vacuum. An alternative is freeze-concentration that is conducted at low temperature and results in a high quality product. Mechanical separation of ice, however, is a difficult and expensive process if losses of solids are to be avoided (74).

Membrane processes are now also being used widely in the food industry for concentration of fluids (74). Ultrafiltration (UF) and reverse osmosis (RO) offer attractive prospects of concentrating milk at ambient temperature and
avoiding chemical damage and flavor changes caused by heating (40). Economic advantages of membrane processing have resulted in its widespread usage. UF uses membranes that allow passage of molecules of sizes up to the minimum pore size of the membrane. It separates solutes based on their molecular size (40). RO allows passage of water and some salts through the membranes. The change in composition of milk occurring when using membrane concentration may affect the storage stability of such UHT processed concentrates.

**Age Gelation of UHT Milk**

Gelation of UHT processed milk is an unsolved problem since it signifies the final limit of useful shelf life (13). It is a hindrance to widespread commercial exploitation of UHT processing of concentrated milk (36, 48). Sensitivity to gelation is greater with UHT processing than with retort sterilizing (76).

Gelation is characterized by loss of fluidity of the product due to the changes occurring during storage and has been described by different terms such as coagulation, sweet curd formation, thixotropic gel formation, age thickening, partial gelation, or lumpiness (48).

Gelation is always preceded by a sharp rise in viscosity (36). The viscosity of UHT milk concentrate undergoes changes during storage. Initially there is thinning of product, followed by a long period during which very little change is observed. This is terminated by a sudden rise in viscosity culminating in gel formation within a short period (1–3 weeks). When gelation occurs the product exhibits a custard-like consistency that is irreversible (49).

**Factors Affecting Age Gelation**

Onset of age gelation is affected by heat treatment, homogenization and sequence of operation, milk solids content, composition of milk, quality of milk,
and storage temperature (48).

Quality of milk

Factors that influence the composition of milk may indirectly affect the gelation behavior of sterilized milk. Seasonal variation affects composition and influences gelation (42). Summer milk gives more stable products than winter milk (115), and mastitis milk subjected to UHT treatment is more susceptible to gelation than normal milk (48). Early lactation milk is more susceptible to storage gelation of UHT products (42, 115).

Microbial quality of raw milk is also important in gelation. Raw milk with a high microbial load particularly of psychrotrophs, gives a UHT product that is very susceptible to storage gelation (64, 102, 103, 115). Higher psychrotrophic counts before sterilization results in faster gelation during storage (64). A high psychrotrophic count, in excess of $10^7$/ml, results in proteinase production (28). Samples with a high bacterial count before UHT treatment show a sharp decrease in casein N and gel rapidly during storage. Para-κ-casein-like material appears progressively, as identified by electrophoresis. No breakdown of caseins is observed during storage of UHT milk from low microbial count raw milk, and it has a longer shelf life (98). Thus a high count before sterilization can influence the quality of the sterilized product.

Incubation of raw milk for 4 h at 30°C, to increase the bacterial load before UHT treatment, does not influence gelation time (115). Other studies have shown that the time of gelation does not appear to be correlated with microbial quality of raw milk (59). However, even in these cases, proteolysis during storage is observed in samples with initially high bacterial counts so that even in the absence of gelation, shelf life may be limited by bitter flavor. Therefore, to maximize shelf life, milk of high bacterial quality is desirable (61).
Low-temperature inactivation treatment

Refrigerated storage of bulk raw milk at the farm for 2 or 3 days and the subsequent storage of bulk raw milk at the dairy factory for another day at low temperatures favors the development of psychrotrophic bacterial flora in milk (105, 106). Some of these psychrotrophs produce heat-stable proteinases that survive UHT treatment and cause deterioration of the product during storage (37).

Low temperature inactivation (LTI) treatment at 55°C for 60 min inactivates heat-stable proteinases from psychrotrophic bacteria (10, 61) without altering the flavor or protein content of milk (10). LTI treatment significantly inhibits proteolysis during storage of UHT milk (28, 61), because the amount of proteinase surviving LTI treatment is 8-22 times less than expected (103).

However, LTI treatment does not appear to retard the onset of age gelation, and its effects vary from batch to batch. Therefore, it may be of limited value in controlling and preventing age gelation (61). This may be due to the type of microbial flora and the proteinase present in milk in the various batches. Others observe a three-fold increase in shelf life (110) and an improvement in the keeping quality (28) of UHT processed milk. LTI is effective over a wide range of proteinase concentrations, and it may be effective at low proteinase concentrations expected in raw milk (103). It also may partly inactivate milk proteinase (28). A thermization treatment of 63-65°C for 12-15 s before storage in bulk tanks may be used to minimize the growth of psychrotrophs (30).

LTI treatment can significantly add to the product quality by inhibiting proteolysis, thereby preventing bitterness and other proteolytic defects from occurring during storage.
Initial pH before heat treatment

pH of raw milk defines the ionization state of the amino acid side chains and the ionic equilibria related to free ionic calcium and phosphate. This in turn may influence the kind of reactions taking place during UHT treatment and subsequent storage.

When pH of raw milk (6.7) is varied between 6.6 and 7.2 by addition of NaOH or HCl, gelation time is not affected (115). Thus, initial pH may not affect gelation time.

Preheat treatment

Preheat treatment or forewarming plays a very important role in the gelation of UHT processed concentrated milk (36, 48, 65, 66, 67, 111). Forewarming treatments at higher temperature and longer holding times delay gelation, but sedimentation and flavor defects, due to excessive heat treatment, limit its use (48). Preheating followed by pH adjustment back to control pH gives a longer gel time (115). Thus, preheat treatment can influence the gelation time.

Severity of heat treatment

The gelation-free period depends upon the time and temperature of heat treatment either before or during sterilization (13, 19, 36, 42, 48, 95, 115). Sterilization of concentrated milk at higher temperature and longer holding times retards gelation. However, at equivalent sterilizing effectiveness, higher sterilization temperatures with shorter exposure times result in reduced resistance to gelation (36, 48). As severity of heat treatment increases, gelation time increases. An increase in the holding time or temperature of sterilization results in a product more resistant to gelation (72, 95, 115). Longer holding times during sterilization improves keeping quality but also results in a more frequent occurrence of cooked flavor defect; whereas, shorter holding times
result in more proteolysis, bitterness, and transparency of milk during storage (28). A holding time of 0.7-4.4 s at 142°C results in a product showing extensive proteolysis during storage which decreases when the holding time is increased to 8.4-18.0 s (32). No proteolysis is observed during storage when a holding time of 18 s or more is employed (32). It has been suggested that if sterilization conditions are used alone to achieve stability against gelation, a heat treatment of 120 s at 132.2°C (270°F) is required to provide a gelation-free storage life of one year. This heat treatment is about four times that needed to achieve sterility (36).

Gelation is also influenced by the UHT sterilizing method. Sterilization by direct heating methods offers less protection against gelation during storage than sterilization by indirect methods (13, 45, 48, 55, 69, 90). This may be because the indirect process gives a more intense heat treatment than the direct process for the same time and temperature conditions (98).

Thus, increased severity of heat treatment increases the useful shelf life of UHT milk probably by making the micelles more resistant to changes that promote their aggregation during storage.

Additives

Studies have been carried out to determine the effect of various additives on the storage stability of UHT milk. These studies may help to understand the underlying mechanism of age gelation since various additives have different effects on different components of milk. Disodium phosphate (DSP) (49, 67, 102) and orthophosphates (66) accelerate gelation, but polyphosphates delay the onset of age gelation (49, 50, 66, 67, 84, 102). Addition of sodium hexametaphosphate (SHMP) at levels of .05-.1% results in a six-fold extension of shelf life (60) while addition at a level of .4% results in a ten-fold increase in
shelf life (67). SHMP at a level of .05% in samples with high psychrotrophic count does not prolong the shelf life (98). Sodium polyphosphate at a level of 1% accelerated gelation in UHT concentrates (66). Cyclic polyphosphates delay age gelation (61). The antigelation activity of polyphosphates increases with increasing concentration and chain length. The cyclic tetrametaphosphate and adenosine triplypolyphosphate are more effective than the corresponding linear polymer and sodium tripolyphosphate (66). A mixture of monophosphate and polyphosphate induces gelation (42, 66). Polyphosphates delay but do not prevent gelation because they undergo hydrolysis with time during storage and lose their ability to stabilize the micelles against aggregation (50).

DSP and SHMP do not affect the rate of proteolysis of casein (49, 98), yet DSP accelerates gelation, whereas SHMP delays gelation. The stabilizing effect of SHMP is independent of proteolysis (49) or its calcium-complexing role (61).

Trisodium citrate (61, 95) and disodium EDTA (61, 115) addition accelerates the onset of age gelation, similar to DSP. Thus, complexing calcium from micelles appears to have a deleterious effect on storage stability. On the other hand, manganous sulfate addition at a level of .05% results in a three-to-six-fold increase in the storage life of UHT concentrates (66).

Addition of polyhydric compounds like lactose, sucrose, dextrose, or sorbitol to concentrated milk at a level of 9.6% results in a two-fold increase in storage life. This may be due to an interaction between these compounds and micellar proteins (66). On the other hand, samples with lactose and sorbitol do not affect the gelation time of UHT processed concentrated casein micelle dispersions (22, 23).

Sulfhydryl blocking agents such as p-mercuribenzoate (PMB), N-ethylmaleimide, and iodoacetamide retard gelation of α-caseins (a mixture of
\( \alpha_s \) and k-caseins), but not sterile concentrates. Disulfide reducing agents, such as mercaptoethanol, thioglycolate, and glutathione, promote gelation of both \( \alpha \)-caseins and sterile concentrates (85).

Addition of hydrogen peroxide to UHT processed skim milk concentrate hastens gelation (50). Di-isopropyl fluorophosphate (DFP) and aprotinin inhibit proteolysis during storage of UHT milk (22, 23, 28) and improve the quality of UHT milk by retarding bitter flavor and other defects due to proteolysis.

Addition of calcium at levels of 2 to 14% increase over the original content has no effect on gelation during storage at 37°C. However, different calcium levels show different effects during storage at room temperature. There is no effect on gelation up to 6% increase in calcium content, but higher levels increase gelation rate (36).

Storage temperature

Gelation time of UHT processed milk is greatly influenced by the storage temperature (48, 115) although results are not consistent. There is no unanimous agreement regarding the effect of temperature on the gelation of unconcentrated and concentrated UHT processed milk. UHT processed unconcentrated milk gelled when stored at 25°C and 30°C, but did not gel when stored at 2°C and 40°C (115). The order of resistance to the onset of age gelation is 50 = 40 > 2 > 10 > 15 > 20 > 25 > 30°C (59). Some believe that lower storage temperatures (ca. 4°C) accelerate gelation (5, 94), while others believe that higher storage temperatures (ca. 35-37°C) accelerate gelation (36, 45, 67, 72, 93). Some researchers have observed that UHT processed milk gelled after 34 months of storage at 4, 20, 30, and 37°C (6). It is possible that the milk protein system may become inherently unstable at the end of such a long storage period leading to gelation. However, there is some indication that
very low (< 6°C) and very high (> 35°C) storage temperatures retard gelation (59, 73, 115).

**Changes occurring during storage**

Some of the changes occurring during storage are outlined below.

**Protein breakdown**

Many workers have observed protein breakdown (12, 49, 71, 84) and changes in the electrophoretic pattern of casein (12, 19) in UHT milk during storage. Protein cleavage or breakdown may occur due to the action of heat stable proteolytic enzymes of natural or microbial origin (12, 19, 49, 84).

*Native proteinases.* Milk, as secreted, contains several proteinases that probably originate from the blood. The major proteinase is plasmin, alkaline milk proteinase, or native milk proteinase (as will be referred to in the future). Thrombin, an acid proteinase, and an aminopeptidase are also present (15). Native milk proteinase in milk is very similar or probably even identical to the blood enzyme, plasmin (35, 46, 55, 57, 58, 91). It originates from blood (55) and occurs in milk mainly as plasminogen, the zymogen of plasmin. Plasminogen is activated by urokinase (30, 57).

Acid proteinase and aminopeptidase are heat labile whereas native milk proteinase is highly heat stable (30, 55). It can survive UHT treatment (15, 21, 25, 28, 31, 98, 101, 102). Native milk proteinase shows maximum activity at 37°C (55). It is active over a wide pH range of 4 to 9 (55) but has a maximum activity at a slightly alkaline pH (55, 58).

The enzyme specificity of native milk proteinase is trypsin-like. It cleaves peptide bonds at the C-terminal side of Arg and Lys residues (108) and Lys-X peptide bonds are cleaved faster than Arg-X bonds (46, 57). The enzyme lacks rennin-like activity. Plasmin causes a reduction in major casein components,
namely $\alpha_s^\beta$, $\beta$- and $\kappa$-caseins, in milk (79, 114). Addition of the enzyme precursor plasminogen results in gradual breakdown of protein; whereas, control samples without added plasminogen show little casein breakdown during storage (62) indicating that plasminogen can be activated to plasmin in milk. The milk proteins most susceptible to alkaline milk proteinase/plasmin are $\beta$- and $\alpha_s^{2\beta}$-caseins, which in UHT treated milk seem to be degraded at about the same rate (23, 25, 32, 55, 62, 100, 101, 102, 108), although some studies have shown that $\beta$-casein is degraded 2 to 3 times faster than $\alpha_s^\beta$-casein (72, 86), or, conversely, degradation of $\alpha_s^\beta$-caseins is faster (49). The $\alpha_s^{2\beta}$- and $\beta$-caseins may be degraded with (28, 32) or without the formation of $\gamma$-caseins (49). $\kappa$-Casein is relatively resistant to the cleavage by plasmin (16, 32, 34, 62, 72, 101, 108) since plasmin causes no increase in $\kappa$-casein macropeptides (49) or formation of para-$\kappa$-casein (23, 32). Plasmin hydrolyzes $\beta$-casein by cleaving the Lys$^{28}$-Lys$^{29}$, Lys$^{105}$-Lys$^{106}$ and Lys$^{107}$-Glu$^{108}$ peptide bonds to yield three large C-terminal fragments, $\gamma_1^\beta$, $\gamma_2^\beta$ and $\gamma_3^\beta$-caseins (3, 17, 21, 41, 43, 44, 79, 82, 108). On incubation with $\alpha_s^{2\beta}$-casein B, it produces three early degradation products with relative molecular weights of 20 500, 12 300, and 10 300 daltons. Specificity on $\alpha_s^{2\beta}$-casein is due to its high lysine content (34). Alkaline milk proteinase causes an increase in noncasein nitrogen (NCN) and formation of $\gamma$-caseins (28).

Microbial proteinases. Milk and milk products may contain a variety of microorganisms capable of secreting lipases and proteinases which subsequently may alter these products (15). Refrigerated storage of bulk raw milk at the farm for 2 or 3 days and the subsequent storage of bulk raw milk at the dairy factory for another day at low temperatures favors the development of psychrotrophic bacterial flora in milk. Thus, spoilage of milk by gram-negative
Dominant psychrotrophic flora in bulk-tank milk is usually *Pseudomonas* with lower proportions of Acetinobacter (Achromobacter / Alcaligenes), Flavobacterium, and Coliform organisms (105). *Pseudomonas fluorescens* AR11 has been found to grow in cold stored milk (64).

Thermoresistance of the gram-negative psychrotrophic microflora is generally low. HTST treatment will kill all the organisms (18, 63), but these psychrotrophs produce extracellular proteinases (11, 64, 93) which are very thermoresistant and can have substantial activity in UHT processed milk (11, 19, 30, 37, 64, 88, 93). The proteinases show considerable activity at normal milk pH (1, 30), at room and elevated temperatures between 35 and 45°C (1, 78, 93), and do not lose their activity during storage (1). Conditions in skim milk appear to be favorable for activity of any proteinase that survives UHT treatment. Optimum activity for proteinases that survive UHT is in the temperature range of 37-45°C and near neutral pH (104). Hence, casein concentration and pH of normal milk are close to optimal for proteinase activity (1).

The effect of microbial proteinases on milk proteins appears to depend on the specific organisms present. In one study extracellular proteinases of eight psychrotrophic bacteria from raw milk initially hydrolyzes β- and κ-caseins followed by nonspecific hydrolysis (26, 64). Other studies have shown that some microbial proteinases predominantly attack κ-casein to form para-κ-casein-like material (1, 11, 32, 64, 77, 98, 103) with considerable degradation (71), slight degradation (1, 64), or no degradation (98) of αs1- and β-caseins. κ-casein is degraded between 50-100% while β- and αs-caseins are degraded between 10-68% and 2-33% respectively (77). Action of proteinases from *Pseudomonas* cultures on UHT milk result in an increase in sialic acid indicating attack on κ-casein (11). Some workers report wide substrate
indicating attack on κ-casein (11). Some workers report wide substrate specificities for proteinases from different *Pseudomonas fluorescens* strains with caseins being degraded more than whey proteins (11, 78). In most cases proteolysis caused by bacterial enzymes is accompanied by an increase in nonprotein nitrogen (NPN) and formation of para-κ-casein (28).

Protein breakdown with some loss of β-casein and κ-casein to para-κ-casein is observed after 34 months of storage but not after 14 months of storage (6). Intensity of αs1−, αs2− (19, 22), κ-casein (19), and β-casein electrophoretic bands decreases during storage (12, 19, 22) and this coincides with the appearance of γ-caseins (breakdown product of β-casein) (12, 19, 22). When a proteinase inhibitor, soya bean trypsin inhibitor (STI), is added, no γ-casein bands are observed. This suggests that the formation of γ-caseins may be due to the result of action of enzymes inhibited by STI, chiefly plasmin. Little proteolysis that is observed may be due to other proteinases not inhibited by STI (22).

Changes in the distribution of N during storage, either NPN or NCN, may indicate protein breakdown. Nonprotein nitrogen (NPN) and NCN increased during storage in UHT milk after the addition of microbial proteinase after sterilization (77). NPN content increases with storage time (11, 19) and with storage temperature (11). Similarly, an increase in NPN and 2% TCA insoluble N parallel a decrease in casein + whey protein N (17, 19, 49, 94). Although there is no change in 2% TCA soluble but 12% TCA insoluble N and proteose-peptones (49), there is an increase in pH 4.6 soluble and 12% TCA insoluble N, proteose peptones + native whey proteins (94). In samples that had a high psychrotrophic count before sterilization, casein N decreases sharply and soluble N increases during storage (71). However, there is no correlation between onset of age gelation and changes in N distribution during storage.
the samples gelled (25).

Whey proteins, β-lactoglobulin and α-lactalbumin, are insensitive to native milk proteinase (16, 55, 114) and microbial (Pseudomonas) proteinases (11, 32, 64, 77). Intact native β-lactoglobulin (16, 55, 57) and denatured β-lactoglobulin (101) inhibit the cleavage of αs2- and β-casein by plasmin.

Indications of casein micelle cleavage by proteolytic enzymes are the decrease of casein N, increase of NPN, and appearance of new bands with smaller molecular weight in SDS-PAGE. These chemical changes are linked with microstructural changes in casein micelles and an increase in sediment formation and viscosity (93). Thus, it is possible to determine the kind of proteinase involved from the pattern of protein breakdown. It is thought that proteolysis, which is accompanied by an increase in NPN and the formation of para-κ-casein, is caused by bacterial proteinases while the native milk proteinase induces an increase of NCN and the formation of γ-caseins (29).

Other kinds of protein breakdown, not related to proteolysis, can also take place. During heat treatment, some protein dissociates from the casein micelles. Heating skim milk and whey protein-free milk at sterilization temperatures causes a substantial increase in the level of soluble casein, consisting of 40% of κ-casein (97). κ-Casein breakdown during storage can be followed by measuring the release of sialic acid from casein, indicative of glycomacropeptide (GMP) formation. An increase in the amount of non-TCA precipitable sialic acid released indicates a breakdown of κ-casein. Dissociation of κ-casein from the micelle surface during storage of UHT processed milk may trigger the loss of stability, and this may be caused by changes in salt equilibrium, especially Ca, Mg, and phosphate, during storage (97). UHT treatment results in a slight increase in sialic acid content of nondialyzable, 12% TCA soluble fraction, suggesting protein breakdown. Sialic
acid is also released during storage of UHT processed samples. Greater release of sialic acid corresponds with higher storage temperature, indicating a more rapid breakdown of \( \kappa \)-casein (94). The amount of carbohydrate attached to \( \kappa \)-casein decreases during storage of UHT processed milk (111). However, some workers reported no increase in sialic acid content during storage (49) or after gelation had occurred (23, 25, 49), indicating that \( \kappa \)-casein is not broken down. Hence, there seems to be no general agreement on the breakdown of \( \kappa \)-casein in sterilized milk and its effects on gelation.

\( pH \)

\( pH \) of UHT processed milk samples decreases steadily during storage (6, 60, 62, 115), the decrease being faster at higher storage temperatures (6, 60, 73, 115). No correlation is found between the rate of decrease of \( pH \) (60) and the level and extent of decrease of \( pH \) (6, 49) with age gelation.

Andrews et al. (6) suggest that the decrease in \( pH \) may be due to the loss of positive charges on protein molecules caused by reaction of free \( \epsilon \)-NH\(_2\) groups of lysine with lactose in Maillard type of reaction. The changes in mineral balance during storage may also contribute to a decrease in \( pH \).

*Changes in micelle structure observed by electron microscopy*

Electron microscopy has been used by many workers (6, 25, 50) to study the changes occurring on the surface of casein micelles during storage of UHT milk.

Electron micrographs of UHT processed concentrated skim milk at various periods during storage reveal changes in the structure of casein micelles which coincide with the observed changes in viscosity. During the initial period, when there was no increase in viscosity, the micelles are well separated but had a changed surface appearance. Filamentous appendages on the micelle surface
that may be attributed to denatured β-lactoglobulin become less prominent over time. When viscosity begins to rise, casein micelles show slight thread-like tails on the surface. After the milk has become viscous, pairs or triplets of casein micelles join either by fusion or by thin, fiber-like material. At this stage the micelles retain their identity, and many remain as separate individual micelles. After gelation occurs, there is severe distortion and aggregation of casein micelles into chains connected through the fiber-like material to form a continuous network (50).

A different study (25) shows the gel in unconcentrated milk is composed of fully disintegrated casein micelles connected by thread-like structures. Aggregates are not made from casein micelles themselves but are breakdown products of casein. In concentrated milk, gels have been observed arising from aggregation of partly deformed casein micelles into a three-dimensional network and hair-like structures or tendrils protruding from the surface of casein micelles (25).

Andrews et al. (6) observed the changes on the casein micelles during storage using electron microscopy. The changes during storage at a lower temperature (4°C) are different from those occurring at higher temperatures. Tendril formation is more marked at 4°C than at either 20°C or 30°C. At 4°C, micelles are bridged together into a network by spiky tendrils, 200 nm to 300 nm long, and there is no coalescence of micelles. At 20°C there is no evidence of extensive network of casein micelles. At 30°C, micelles are linked by tendrilar bridges, but there is very little tendril formation. At both these temperatures the micelle surface appears smooth and regular in shape, and aggregates are formed by coalescence of two to three micelles. At 37°C, micelles are much larger than the micelles in UHT milk stored at 4°C, 20°C, or 30°C and often exceed 400 nm in diameter. Large micelles are linked to smaller particles by
protein bridges suggesting that large particles are formed by accretion of smaller particles. The bonds involve both in the formation of tendrils or bridges and in the integrity of casein aggregates or micelles in long-term stored milks are not susceptible to disruption by removal of calcium ions by EDTA treatment alone (6). This suggests that the nature of these bonds involves forces other than ionic interaction.

At 4°C, formation of tendrils, bridges, and open network structure are stabilized by the presence of calcium ions. At higher temperatures, a chemical mechanism of crosslinking between polypeptide chains such as Maillard reaction may be involved, as considerable browning is observed in such samples. No changes on the surface of casein micelles (no tendril formation) is seen by electron microscopy after six months of storage at 37°C though there is a lot of crosslinking due to Maillard reaction. Very little change by chemical crosslinking due to Maillard reaction is expected within 34 months of storage at 4°C (very little Maillard browning takes place at 4°C), and yet striking changes (appearance of tendrils on the surface of casein micelles) are observed in the micelle structure by electron microscopy. Thus changes in micelle structure as seen using electron microscopy cannot be explained from extent of polymer formation due to Maillard reaction (6).

Casein micelles appear unchanged, spherical, and well separated from one another in micrographs of non-gelled samples (25, 50).

The above observations suggest that gelation is preceded by changes on the surface of casein micelles that promote either aggregation of casein micelles by fusion or the formation of hairs that later form bridges between casein micelles into a continuous three-dimensional network leading to gelation.
Maillard reaction

UHT milk, when stored for prolonged periods, can become brown in color (2, 4, 5, 49) because of participation of lactose in Maillard browning. The Maillard reaction is a complex series of reactions that culminates in the formation of brown colored polymers, melanoidins. Extent of polymer formation due to Maillard reaction depends both on storage time and temperature (2). After 12 months of storage, samples at 30°C brown considerably whereas those at 4°C do not (4). When stored at 30 and 37°C, extent of polymerization of casein and whey proteins is several times greater than the heat induced changes resulting from UHT processing itself (2).

Polymerization and association lead to alterations in electrophoretic mobility and loss of definition in the bands attributable to various protein components. None of the individual protein bands are distinguishable when UHT milk is stored at 37°C for a prolonged period, indicating protein modification occurring at that temperature (2, 4). Changes in electrophoretic patterns of caseins and the appearance of streaks on starch gels qualitatively demonstrate the early stages of Maillard reaction, namely the modification of charge on the protein molecules by interaction of ε-NH₂ groups of lysine with lactose, which depends both on the length of storage and temperature (2, 4, 5). Streaky patterns have also been observed by other workers in UHT processed concentrated casein micelle dispersions containing lactose but not when lactose was replaced by sorbitol (22, 23). Pure caseins when heated with lactose at 100°C give streaky patterns on electrophoresis and become brown in appearance (4) suggesting the involvement of lactose.

Changes in molecular weight distribution of pH 4.6 insoluble casein, as implied by gel permeation chromatography, have been shown to occur during storage of UHT milk. The changes are more pronounced at higher storage
temperatures of 37°C and 30°C (37°C > 30°C) than at 4°C (5) and indicated that polymerization occurs during storage. SDS-PAGE shows some bands at the site of loading of samples (wells) (5), indicating that the polymers are so large that they do not enter the gel.

The bonds formed between polypeptide chains are covalent because they are not cleaved by dissociating solvents like SDS, urea, and guanidium hydrochloride. Neither periodate oxidation or borohydride reduction are effective. Therefore, the polymers do not possess a structure with two adjacent hydroxyl or amino groups. However, some polymer aggregates are linked together through disulfide linkages between whey proteins and κ-casein (5).

Streaky patterns in PAGE are retarded in the presence of sodium metabisulfite which removes carbonyl intermediates. Hence, alteration in electrophoretic properties may be due to the action of carbonyl compounds produced by the Maillard-type reaction, leading to changes in the charge of the protein and to some degree of polymerization. Participation of charged groups in various stages of Maillard reaction might be expected to lead to a somewhat random number of modifications to the molecules of a single protein component resulting in a population of molecules with differing net charge, leading to a streaky gel pattern. Polymerization, leading to brown color formation, occurs at a later stage of Maillard reaction (4).

Residual oxygen levels are relatively unimportant to progress of chemical reactions, charge modification, and polymerization (2).

Andrews and Cheeseman (5) propose that at least two processes take place during storage of UHT milk. One process leads to the formation of gel which is disrupted by solvents containing dissociating agents such as urea, guanidium chloride, or SDS and is held together by physical forces of association like hydrophobic interaction. The second process is Maillard reaction which leads
to browning and sediment formation by production of covalently bonded polymers. During early stages of Maillard reaction, before polymerization is extensive, modification of net charge on polypeptide chains by interaction with specific groups may lead to instability of milk colloid with consequent gel formation (5).

*Stability to alcohol, calcium ions, and rennet*

The stability of proteins to ethanol decreases with time in UHT milk (94). Samples that finally gelled show progressive decrease in stability towards alcohol (49). UHT milk stored at 4°C shows greater instability to ethanol than the samples stored at 30°C or 37°C at the end of 12 months of storage. However, gelation is observed at all three storage temperatures (6). Milks that have a tendency to gel show a progressive decrease in stability to alcohol during storage.

Stability of sterile UHT milk (83) and casein in UHT milk (94) to precipitation by added calcium ions decreases progressively during storage. Calcium sensitivity increases when milk is stored at 30°C but does not change when stored at 4°C (4), and a casein complex isolated from sterile concentrated milk shows reduced stability to calcium ions after reduction of disulfide linkages. This suggests that the structure that maintains stability to calcium ions is destroyed by disulfide reduction. However, the stability was restored by air oxidation (85). Reduced stability towards calcium ions reflects a progressively decreasing stability of the milk protein colloidal system. Thus disulfide linkages may have a role to play in maintaining the stability of the colloidal milk protein system during storage. However, autoclaved milk which did not gel, showed no change in stability to calcium ions when stored at 20°C but showed decreased stability when stored at 4°C (94). In general, the tendency of samples to gel
coincides with the decrease in stability to calcium ions.

Rennet coagulation time (RCT) of UHT milk samples during storage can yield information regarding the accessibility of $\kappa$-casein to rennet. The RCT of samples stored at 20°C decreases during storage, while it increases for the samples stored at 37°C. The samples stored at 20°C gel, whereas those stored at 37°C do not. The RCT of autoclaved samples do not decrease during storage. Autoclaved samples do not coagulate with rennet addition, after autoclaving, and through storage (94). The samples which have a tendency to gel show a decrease in RCT during storage.

Decreased stability to calcium ions, rennet, and alcohol, which induce or promote protein-protein interaction of samples that have a tendency to gel, indicates that the proteins on the surface of casein micelles are being modified during storage such that protein-protein interaction between micelles is favored resulting in their linkage to form a gel.

*Changes in mineral balance*

Colloidal calcium phosphate is necessary for the stability of casein micelles, and, therefore, changes in the mineral balance of milk may affect its stability during heat treatment and storage. At elevated temperatures, the concentrations of ionic calcium and magnesium decrease as precipitates of their citrates and phosphates. During storage, some of the calcium phosphate may dissolve, but on prolonged storage there is precipitation of some forms of calcium phosphate (48). Removal of calcium causes dissociation of the micelles and if this occurs during storage of UHT milk, micellar dissociation may lead to an increase in non-sedimentable casein, and the partially dissociated micelles may aggregate during storage causing gelation. However, Ca/N and P/N ratios of the casein fraction sedimentable by ultracentrifugation (>100 000
g) increase during storage of UHT skim milk. The destabilization of the casein complex during storage is attributed to the incorporation of calcium and phosphate into the micelles (8, 9). However, the deposition of calcium and phosphorus is also observed in autoclaved milk which does not gel so rapidly (48). These inconsistent observations and the effect of EDTA and citrate on calcium show that mineral balance is more complex than simple deposition.

Other changes

The viscosity of samples increases suddenly over a period of 2-3 weeks, just before gelation (49). Changes in micelle structure leading to gelation are gradual and correlate with observed changes in viscosity and state of gelation. The micelles do not coalesce abruptly just before gelation. Even at early stages of storage, distortion of micelles and development of thread-like tails are observed. This indicates a continuous and gradual change in the surface of the casein micelles (50).

Storage of UHT milk results in changes that affect its stability towards heat and low pH, and these changes precede the onset of age gelation (94). This again indicates a gradual change on the surface of casein micelles that favors protein-protein interaction between micelles leading to aggregation and the increased instability of the colloidal milk protein system during storage.

Age-thinning, which is manifested as a decrease in viscosity during storage preceding gelation, has been observed by many workers (22, 23, 25, 45), while others do not (73). Age-thinning is thought to be due to the action of heat stable proteinases (45, 88, 89) and as a clear diagnosis of proteolysis (88, 89), while others (22, 23) believe that it is not due to any proteolytic effect but is due to structural changes in the micelles initiated by heat treatment.

Thus the nature of changes occurring during storage may provide a clue
regarding the various steps involved in the mechanism of age gelation.

**Proposed Mechanisms of Age Gelation**

Various explanations have been offered to explain age gelation based on changes observed in UHT milk during storage and on conditions that alter the gelation time. Gelation of stored sterilized milk is due to loss of colloidal stability of the casein micelles, and a direct interaction between casein micelles leading to the formation of a three-dimensional network (48, 97). Assuming that the stability of the casein micelles in milk is maintained by κ-casein, colloidal calcium phosphate, a high negative zeta potential (-18 mV), and steric stabilization (97), gelation is preceded by changes at the surface of casein micelles as a result of which interaction between the micelles is enhanced (48, 97). In general, the changes that are believed to trigger the loss of stability of casein micelles fall into two categories (48):

1. Changes that arise from proteinase activity and
2. Changes that arise from nonenzymic reactions.

**Proteinase Hypothesis**

Enzymes in milk are of two kinds: enzymes naturally present in milk and enzymes which appear in milk as a result of bacterial growth (92). Proteolytic enzymes of native and microbial origin either survive UHT treatment or reactivate during storage and may cause the formation of coagulum in UHT milk (1, 11, 12, 17, 19, 21, 28, 37, 38, 45, 47, 54, 55, 64, 70, 82, 83, 93, 95, 98, 102, 112, 113, 115) and cause flavor changes like bitterness (28, 30, 93) during storage.

A number of workers suggest that the phenomenon of age gelation and the process of renneting in cheese manufacture are similar (1, 11, 19, 42, 54, 64,
The rennet clotting of milk and the phenomenon of age-thinning and age thickening of UHT processed, concentrated milk show similar kinetics. Both are characterized by a lag phase during which viscosity decreases, followed by an explosive increase in viscosity. If microstructural changes have a non-enzymic origin, the kinetics will yield a linear increase in viscosity with no lag phase and no rapid increase shortly before gelation (88, 89). From kinetic considerations, Payens (88, 89) concludes that gelation of unconcentrated and evaporated UHT-milk during storage is due to the action of proteolytic enzyme as no other theory can account for age thinning followed by explosive growth of average particle weight. Guthy et al. (45) measured the volume of aggregating particles during storage of UHT milk. After a distinct lag phase, they report an exponential growth of particles shortly before aggregation, as calculated by Payens (88, 89) in all milk samples. This may indicate activity of one or more enzymes which survived UHT treatment (45).

Native proteinases

Milk as secreted contains several proteinases which probably originate from the blood. The major proteinase has been identified as plasmin, native milk proteinase. Thrombin, an acid proteinase, and an aminopeptidase are also present (15).

Acid proteinase and aminopeptidase are heat labile. Native milk proteinase (plasmin) is highly heat stable (30, 55). It survives 30 min at 60°C (33) and pasteurization heat treatments (86). Its high heat stability is indicated by its low Q_{10} and high Z and D-values (30, 31). It can survive UHT treatment (15, 21, 25, 28, 31, 98, 101, 102) and in milks sterilized by friction (21). It also survives 16 s at 140°C, both direct and indirect heat treatments (28), and 18 s at 142°C (32). Some researchers have observed that both plasmin and plasminogen survive
direct UHT steam injection treatment (21, 73, 101), but only plasminogen survives indirect UHT heat treatment (73). Others report that plasmin survives both direct and indirect UHT sterilization treatment (20). A number of HTST pasteurization treatments (up to three) before sterilization does not influence the activity of the enzyme during storage (20). This enzyme has been isolated from milk sterilized by direct steam injection at 140°C, 4 s using affinity chromatography (101) suggesting its thermoresistance. A heat treatment of 135°C, 15 s reduces plasmin/plasminogen derived activity by 80% (23). It is inactivated by in-container sterilization process of 120°C, 15 min (30, 31). Considering the thermoresistance of this enzyme, Driessen and Vanderwalls (31) recommend a heat treatment of 16 s at 142°C to completely inactivate the enzyme.

Plasminogen activity decreases and plasmin activity increases during storage due to the transformation of plasminogen to plasmin (25, 62, 73). Directly and indirectly processed milk may contain heat stable activators that convert plasminogen to plasmin (62). Milk with added plasminogen (addition before UHT) showed plasmin activity after UHT heating and storage and caused gelation (62). Native milk proteinase present in aseptically drawn milk plays an important role in proteolysis and possibly gelation of raw milk preserved using antimicrobial agents (25) and UHT treated milk (16, 21, 25, 28, 29, 32, 55, 62, 88, 96, 98).

**Microbial proteinases**

Milk and milk products may contain a variety of microorganisms capable of secreting lipases and proteinases which subsequently may alter these products (15). Refrigerated storage of bulk raw milk at the farm for 2 or 3 days and the subsequent storage of bulk raw milk at the dairy factory for another day at low
temperatures favors the development of psychrotrophic bacterial flora in milk (3, 105, 106).

Thermoresistance of the gram-negative psychrotrophic microflora is generally low. HTST treatment will kill all the organisms (18, 63), but these psychrotrophs produce extracellular proteinases (11, 64, 93) which are very thermoresistant and can have substantial activity in pasteurized milk (78), UHT processed milk (11, 19, 30, 64, 88, 93), and can be expected even in milk autoclaved for 15 min at 120°C (30). Strains of *Pseudomonas* and *Acinetobacter* spp. growing to approximately $10^7$ CFU/ml and above produce sufficient proteinases to degrade caseins to an extent detectable by PAGE and starch gel electrophoresis (64). Counts of at least $5 \times 10^6$ CFU/ml are necessary before proteolysis is detectable in milk (63). A psychrotroph count of $< 1 \times 10^4$/ml may be sufficient to produce $> 10$ units of heat stable enzyme/ml of milk (81, 103); and therefore, some proteolytic activity can still be expected at such low counts. A high psychrotrophic count in milk before sterilization results in a sharp decrease of casein N during storage of UHT milk, indicating proteolytic activity in sterilized milk. When milk is inoculated with cultures and then UHT processed, an increase in NPN is observed during storage while the uninoculated ones do not show any increase in NPN (11). Thus these proteinases can degrade caseins during storage (63, 98).

The D-values for such enzymes have been calculated as 8-11 min at 130°C (30), 5-6 min at 140°C, and 30 s to several min at 150°C (93). At a temperature of 145°C, these proteinases are 400 times more heat resistant than *PA3679* and 4000 times more resistant than *Bacillus stearothermophilus* spores (1). Such a heat treatment, necessary for the complete inactivation of the proteinases, cannot be applied to milk as it would cause considerable damage to the product (93, 103). At a lower temperature, typically 6 h at 72°C, it is
equally damaging and uneconomical (103). Psychrotrophs capable of producing heat resistant proteinases have been found in 70-90% of raw milk samples. These proteinases can survive 10 s at 149°C (1) and 110 s at 132°C (71). Such a heat treatment destroys only 10% of the proteinase activity (1). The enzymes have a low temperature coefficient of inactivation.

Mitchell and Ewings (77) isolated proteolytic psychrotrophic bacteria, six strains of *Pseudomonas fluorescens*, and two strains of *Serratia marcescens* from raw milk. When extracellular proteinases produced by these microorganisms are filter-sterilized and added to sterilized milk, they cause gelation during storage (1, 77). Therefore, bacterial proteinases can cause gelation of UHT milk (27, 64, 77). Heat-resistant proteinases produced by psychrotrophs may be a serious problem in the production of sterile milk and other foods, and their destruction by heat is impractical. Long-term storage expected of sterile milk not only allows time for surviving proteinase to act but enhances its susceptibility to proteinase (1).

Heat resistance, $Q_{10}$, and $Z$ values suggest that these enzymes may remain active or partly active after UHT heat treatment is applied in the manufacture of dairy products and cause bitter off flavor, gelation, and physical changes in milk and milk products (1, 15, 28, 30, 93, 103) thus limiting their shelf life (93). The keeping quality of UHT milk may be reduced by the residual activity of native milk proteinase (28, 30, 93) and proteinase due to the growth of psychrotrophs (15, 28). Milk proteinase is important for UHT processed products to have a long shelf life at ambient temperature (30).

Though proteinase activity seems to be important in the gelation process, certain observations suggest that proteolysis may not be the only cause of age gelation (97). Native milk proteinase does not survive UHT or reactivate during storage of either UHT processed concentrated milk (84) or 10% cream (17), and
these researchers (17, 84) suggest that native milk proteinase is of no significance in age gelation. These researchers (17, 84) used an increase in NPN and hemoglobin as the substrate to measure proteolytic activity. It may be difficult to measure milk proteinase activity from an increase of NPN in milk during storage because proteose-peptones and γ-caseins formed as a result of proteolysis are precipitated by 12% TCA, and native milk proteinase caused only a small increase in NPN (30, 31). Moreover, milk proteinases have specific substrates, and, therefore, hemoglobin may not be a good substrate (31). UHT milk with and without proteolysis (proteolysis inhibited by the addition of plasmin inhibitors like STI, DFP, and aprotinin) gelled after identical storage periods. The researchers suggest that proteolysis plays a minor role in the process of age-thinning and gelation (22, 23). In contrast, when proteolysis is inhibited by adding DFP and aprotinin, no gelation is observed in unconcentrated samples, but the control samples without DFP and aprotinin gel (28). Some workers observe that unconcentrated sterilized milk that gelled shows proteolysis whereas concentrated sterilized milk shows no proteolysis and yet gel (25, 54). This prompted de Koning et al. (25) to propose that gelation in unconcentrated milk is preceded by a proteolytic effect caused by native milk proteinase while in concentrated milk, it is due to a nonenzymic physicochemical phenomenon.

Protein decomposition precedes gelation and may cause gelation because smaller extent of protein decomposition in autoclaved milk correlates with greater resistance to gelation. Protein decomposition occurring in samples during storage at 30°C and 37°C is similar. But gelation is observed at 30°C and not at 37°C. Protein decomposition also occurs in autoclaved UHT samples, but these samples do not gel. The authors (94) concluded that proteolytic enzymes or proteolysis may not be the primary cause of gelation on
storage. Hence, searching for surviving or reactivated proteinase may be irrelevant to the problem of gelation (94). Other studies have also shown a lack of relationship between gelation and extent of proteolysis (22, 23, 49, 59, 60, 61, 62, 72, 73, 75, 77, 94).

It has been shown that storage at various temperatures resulted in different rates and extents of sialic acid release, indicating different levels of κ-casein breakdown. However, all the samples gel at the same time, indicating a lack of relation between gelation and extent of protein breakdown (94). Samuelsson and Holm (95) observed an inverse relationship between degree of protein decomposition and time of onset of gelation. A correlation between proteinase activity and proteolysis (73) and between proteinase activity and gelation time (77) has been observed. Gelation does not occur in samples with extensive proteolysis (59, 62, 75) because a rapid breakdown of caseins may not allow the enzyme-modified proteins to associate into a gel network. Continued attack during storage may result in disaggregation of gel protein structure and finally, in a reduced apparent viscosity. Slow or limited proteolysis, on the other hand, will result in a gentle casein breakdown and lead to aggregation of enzyme-modified micelles to give a gel consisting of casein fragments detectable by SDS-PAGE (62). A lack of correlation between rate or extent of proteolysis and the onset of age gelation may be due to the type of psychrotrophic-proteolytic flora; the specificity, activity, and the number of heat resistant proteolytic enzymes of microbial origin; specificity, activity, and number or heat resistant milk proteinases; rate of physicochemical reactions; or a combination of these factors (59).

Proteinases may not be detected in UHT milk or cream because the amount is too low to be detected by current assay methods but high enough to modify the caseins sufficiently to cause coagulation after prolonged storage (39).
Hence, proteinases may be involved in age gelation of unconcentrated and concentrated milk, and in the instances where their presence is not detected, the level may be too low to be detected by current methods of analysis.

Nonenzymic Basis for Gelation

Gelation has been observed to occur without any proteolysis in UHT processed concentrated milk (25, 54, 72). The absence of a quantitative relationship between gelation time and proteolytic activity has prompted some authors (22, 72, 94) to propose that the triggering action consists of some physico-chemical process and is responsible for gelation.

Whey proteins

Whey proteins denature during heat treatment in the range of 70-140°C. At lower temperatures (ca. 70-90°C) a complex is formed between denatured serum proteins and casein, but at higher temperatures (ca. 120-140°C) as in UHT processing, much of the denatured serum protein remains uncomplexed with casein. The amount of whey proteins attached to the casein decreases as heat processes at higher temperatures and shorter times are used. α-Lactalbumin and β-lactoglobulin not only complex with each other, but also complex with caseins (15). A complex consisting of αs2- and κ-caseins with β-lactoglobulin has been isolated from UHT milk (99). After heat treatment some of the denatured serum proteins are attached to the casein and precipitate with casein in the turbidity test, while the serum contains the undenatured and denatured serum proteins which are not attached to casein (15). Many workers suggest that the β-lactoglobulin-casein complex may be important in the stability of UHT milk (55). Some workers suggest that this complex, between β-lactoglobulin and caseins, may have a tendency to form aggregates that sediment and bring about coagulation (95). Others believe that the dissociation
of this complex from the micelles during storage, formed during UHT treatment, may lead to the destabilization of casein micelles causing gelation (19, 54). If heat treatment is severe, as in retort-sterilized milk, this complex is irreversible and protects the micelles from further changes during storage (19, 54). This is supported by the fact that as whey protein denaturation increases, gelation is also delayed (72). This suggests that complexing of denatured whey proteins with caseins may play a role in gelation. The amount of native β-lactoglobulin has been found to decrease after UHT treatment, then increase initially during storage but later on to decrease. This may be due to restoration of denatured whey protein to the native state and consequently may show that the complex formation is reversible (19). On the other hand, other studies have shown that there is no renaturation of denatured β-lactoglobulin during storage (49).

Soluble whey protein, which indicates the degree of polymerization, decreases from 18.8% in raw milk to 11.9% (direct) and 7.2% (indirect) after UHT. This indicates crosslinking between whey proteins and κ-casein. The soluble β-lactoglobulin content varies during storage, but this could not be correlated to gelation time (53). Added whey proteins, in the form of dialyzed acid whey, to raw milk before processing increases the rate of gelation (36). β-lactoglobulin accelerates gelation of α-casein and whole casein in a model system study (85). The role of whey proteins in age gelation is still not clear.

Modification of κ-casein during storage

κ-Casein breakdown during storage may result in κ-casein losing its ability to stabilize the casein micelles leading to gelation (95). Dissociation of κ-casein from the micelle surface during storage of UHT processed milk may trigger the loss of stability, and this may be caused by changes in salt equilibrium, especially Ca, Mg, and phosphate, during storage (97). During
heat treatment, some protein dissociates from the casein micelles. Heating skim milk and whey protein-free milk at sterilization temperatures causes a substantial increase in the level of soluble casein, consisting of 40% \( \kappa \)-casein (97). UHT treatment results in a slight increase in sialic acid content of nondialyzable, 12% TCA soluble fraction, suggesting protein breakdown. Sialic acid is released during storage of UHT processed samples (94). The amount of carbohydrate attached to \( \kappa \)-casein decreases during storage of UHT processed milk (111). Greater release of sialic acid corresponds with higher storage temperature, indicating a higher \( \kappa \)-casein breakdown at higher storage temperatures (94), but gelation is not observed at higher storage temperatures. But some workers report no increase in sialic acid content during storage (49), indicating no \( \kappa \)-casein breakdown during storage (49) or after gelation has occurred (23, 25, 49). Hence, there seems to be no correlation between \( \kappa \)-casein breakdown and gelation, unless breakdown is considerable.

**Milk salts**

Changes in mineral equilibria during storage may initiate some changes in the micelle structure or modify the micelles so that they become more reactive and conducive to aggregation. Some workers suggest that calcium plays a role in the stability of UHT product during storage (19). Added calcium up to 6% over the original content has no effect on the storage stability of UHT processed concentrates at storage temperatures of 37°C and room temperature. However, higher levels up to 14% over the original content increase gelation rate when milk is stored at room temperature but has no effect when stored at 37°C (36). Thus the role of calcium in gelation at room temperature may be attributed to calcium ion-mediated aggregation of casein micelles, but this cannot explain the retardation of gelation at higher temperature where the amount of added
calcium is the same. The addition of DSP, which binds to ionic calcium, accelerates gelation. Polyphosphates, which bind to similar amounts of calcium, and cyclic polyphosphates, which do not bind to any calcium, retard gelation. The level of ionic calcium and the changes in ionic calcium during storage do not correlate with the onset of age gelation (60, 61). The addition of calcium or the removal of calcium ions accelerates gelation while cyclic polyphosphates, which do not change the state of calcium, retard gelation. Thus the role of ions like calcium is still obscure in the gelation of UHT milk.

**Maillard reaction**

Chemical modification of casein micelles by Maillard-type reactions has also been implicated in gelation of UHT milk. Andrews and Cheeseman (2, 4, 5) suggest that gelation is caused by polymerization of casein and whey proteins by Maillard-type reactions that are promoted as temperature of storage is increased. However, failure to observe gelation during storage of sterilized milk above 35°C is not consistent with their suggestion (94). Turner et al. (107) suggest that the reaction between lactose and amino groups (predominantly ε-NH₂ groups of lysine) involves Schiff's base formation, which then undergoes Amadori rearrangement to give keto and enol structures. Continuation of Maillard-type reactions during storage of UHT milk would eventually lead to gelation by crosslinking of protein chains into very large complexes. This implies that lactose, and not its degradation products, reacts with proteins. This reaction is greater with casein fractions (κ > α, β) than with whey proteins (107). Any reaction of lactose with κ-casein could have profound effects on casein micelle stability during storage. Browning, due to Maillard reaction, was similar in all samples regardless of rate of gelation in gelled and non-gelled samples (49). Some workers (22, 23) could not correlate Maillard reaction with age
gelation since samples with lactose and with sorbitol gelled around the same time. Others (66) observe an increase in shelf life when non-reducing sugars, like sucrose and sorbitol, and reducing sugars, like lactose and dextrose, are added to milk and sterilized. Reducing sugars take part in Maillard reaction while non-reducing sugars do not. Also, some changes as seen by electron microscopy, could not be explained with Maillard reaction during storage (6).

Blockage of lysine side chain residues in κ-casein results in a loss of sensitivity to rennet. Hence, apart from Ca++ bridging and hydrophobic interaction, interaction between separate casein molecules through oppositely charged regions of suitable configuration may also be involved. The charged regions owe their configuration to specific groups. Lysine residues may contribute to the configurations of the regions that interact (51, 52). This may also apply to gelation of sterile milk since the blockage of ε-NH₂ groups of lysine residues by interaction with lactose may prevent casein micelles from interacting and gelling. The changes due to Maillard browning, involving lysine residues, may either prevent aggregation or block regions that are responsible for protein-protein interaction. Gelation of UHT processed milk may occur partly due to slow conformational changes in molecules on the surface of casein micelles exposing regions which subsequently interact to form casein aggregates (94).

**Sulfide-disulfide reactions**

Reactive sulfhydryl groups are formed during UHT, which then may participate in a variety of reactions: may be lost by volatilization, may oxidize to disulfides, or may be reburied in the protein structure. Only a small fraction seems to be volatilized and the fate of the remaining groups depends on the storage temperature. Reactive and total sulfhydryl concentrations decrease
with time, more rapidly at room temperature than at 4°C. Storage at room
temperature results in a greater oxidation of heat-exposed sulfhydryl groups.
Until these groups are oxidized completely, such groups can participate in
sulfhydryl-disulfide interchange reactions with various proteins, and such
interchange reactions may be involved in age gelation (87).

Sulfhydryl blocking agents such as PCMB, N-ethylmaleimide and
iodoacetamide, retard gelation of α-caseins (a mixture of αs- and κ-caseins)
(85), but have little effect on retarding gelation of concentrated milk (80, 85).
Disulfide reducing agents, such as mercaptoethanol, thioglycolate, and
 glutathione, promote gelation of both α-caseins and sterile concentrates (85).
This suggests that free sulfhydryl groups created by the action of the reducing
agent may have a role to play in the gelation of UHT milk.

Directly heated UHT milk tends to gel sooner than indirectly heated milks,
though both heat treatments are adjusted to give a similar severity of treatment.
The difference has been attributed to different levels of residual oxygen at the
time of heat treatment. The direct treatment removes the residual oxygen,
whereas the indirect treatment does not. The presence of oxygen promotes
disulfide linkages and results in more complexation of whey proteins with
 caseins. This results in the prolongation of shelf life for indirectly heat treated
UHT milk. Hence, disulfide linkages formed during storage may contribute to
stability of the product during storage (53).

Whole casein, κ-casein, and casein-complex isolated from sterile
concentrated milk show decreased stability to Ca++ in the presence of mild
reducing agents like thioglycolate and mercaptoethanol which reduce their
disulfide linkages. Air oxidation of reduced disulfides (with thioglycolate)
restores the stabilities of κ- and whole caseins. Blocking of sulfhydryl groups
prevents restoration of stability. The formation of disulfide linkages may
contribute to stability to calcium while their reduction may contribute to a
decrease in stability. Therefore, the structure of casein that maintains stability to
calcium ions may be destroyed by disulfide reduction, which could be restored
by oxidation. Casein solutions, isolated from sterile concentrates, do not gel
without the addition of calcium ions even in presence of disulfide reducing
agents such as mercaptoethanol and glutathione, but gel only in the presence
of calcium ions (85). Also the gelled samples have a stability of 75% (original
stability taken as 100%) which decreased to 60% by reduction, but air oxidation
restores stability to near original value. This indicates that the disulfide
interchange reaction alone may not be responsible for the gel structure. The
decreased stability due to long storage could not be explained by disulfide
reduction or disulfide interchange reaction alone (85).

_Micelle surface potential_

Another hypothesis (42) based on nonenzymic modification of casein
micelles considers age gelation in terms of free energy. Casein micelles are
perceived as being in a meta-stable state with a high surface potential. During
storage, a progressive and spontaneous transformation of the micelle from a
high potential to a more stable micelle with lower surface potential occurs by
random-potential jumps. This decrease in surface energy of some micelles
creates an electrostatic difference between them which promotes aggregation
of micelles depending upon probability of contacts and number of low-potential
micelles. Both of these factors increase with time. Initially, aggregation is
insignificant, but when average surface potential of micelles reaches a critical
level, aggregation becomes evident and is observed as an increase in viscosity.

According to Payens (88), even with a large surface potential of -15 mV, the
coagulation time of casein micelles in milk would still be less than or equal to 1
Apparently the relatively large stability of casein micelles, and therefore also its clotting during prolonged storage, cannot be explained by electrostatic repulsion alone.

**Dissociation of casein micelles during storage**

There is an increase in non-sedimentable casein (at > 100 000 g) which may be due to partial disaggregation of casein micelles. This is seen by electron microscopy as fine particles or subunits of casein. This may modify surface properties of casein micelles and would expose regions on their surfaces that promote interaction between micelles (48).

**Other Mechanisms Proposed for Age Gelation**

None of the mechanisms proposed above completely explain the phenomenon of age gelation suggesting a two-stage/two-step process as the gelation mechanism. Since proteolysis is observed in gelled and non-gelled samples, it is proposed that: (i) some proteolysis of milk proteins by heat stable enzymes is needed for gelation to occur, and (ii) this is followed by storage induced physico-chemical changes that affect aggregation of destabilized micelles (49, 60, 61).

This has been observed when phosphate salts like DSP are added to concentrated milk. DSP accelerated gelation whereas addition of sodium hexametaphosphate delayed gelation even though equal proteolysis occurred. Possibly, these additives modify the surface of casein micelles such that the secondary stage of coagulation taking place is affected, or alternatively, slow changes occurring at the surface of micelles are influenced by the additives during storage.

Electron microscopy has revealed the appearance of many tendrils
protruding from the surface of casein micelles when UHT milk is stored at 4°C. There is some tendril formation when stored at 37°C. At higher temperatures, a chemical mechanism of crosslinking between polypeptide chains such as Maillard reaction may be involved. No changes in micellar structure are seen by electron microscopy at 6 months of storage at 37°C, even though there is a lot of crosslinking due to Maillard reaction. Very little change by chemical crosslinking is expected within 34 months of storage at 4°C, yet striking changes were observed in the micelle structure by electron microscopy. This lack of correlation between electron microscopic changes and Maillard reaction and the observation of some protein breakdown prompted some workers (6) to suggest a combination of enzymic and purely chemical reaction pathways to be involved in the gelation of UHT milk.

Some workers (19) have measured N distribution during storage. β-Lactoglobulin N decreases after UHT processing then increases initially during storage and then finally decreases prior to gelation. They suggest a two-step mechanism based on the change in N distribution occurring during storage. The first step may be the dissociation of the reversible complex into two former fractions and may be purely chemical-physical in nature. The heat supplied for UHT treatment may be insufficient to form a stable physical entanglement or for sufficient intermolecular non-covalent bonds. The second step may be the slow coagulation of casein which is no longer protected. The second step may be viewed as a gentle enzymatic process due to the action of residual proteinases which survives the heat treatment or were reactivated during storage.

Recent research reveals that the gelation process is more likely to involve at least a two-step mechanism involving proteolysis and one or more of the above listed physico-chemical mechanisms.
OBJECTIVES

Conflicting reports have been reported about the role of lactose in the age gelation of UHT concentrated milk. To better understand the causes of age gelation, the experiments described in this paper were conducted. The objective was to determine whether lactose actively participated in age gelation of UHT milk concentrate. The storage stability of UHT processed concentrated milk was related to the chemical reactivity, lactose in comparison to non-reducing sugar, sucrose by measuring browning, electrophoretic patterns in SDS-PAGE and observing changes in microstructure using electron microscopy.
MATERIALS AND METHODS

Milk

Skim milk was obtained from the Utah State University Dairy Products Laboratory and batch-pasteurized at 62.5°C for 30 min before further processing.

Ultrafiltration and Diafiltration

The scheme for ultrafiltration and diafiltration is shown in Figure 1. The skim milk was concentrated at 50°C, using a three-module in-series UF system (Osmonics Co., Minnetonka, MN.) with spiral wound membranes (5000 daltons nominal molecular weight cutoff, 15 m² membrane area). Inlet and outlet pressures of 483 kPa and 345 kPa, respectively, were maintained during concentration. Batch diafiltration, using simulated milk ultrafiltrate (SMUF) (56), was used to reduce lactose concentration. All the chemicals used for the preparation of the buffer were analytical grade. The pH of the buffer was adjusted to that of pasteurized milk using 6N KOH. SMUF, at 20°C, was added to the warm concentrated milk (50°C) and stirred for 45 min each time before diafiltration. The lactose levels at various stages are given in Figure 1.

Preparation of Samples

The 3X concentrate (volume reduction) containing < .05% lactose was divided into five parts. One part was used as the control. Lactose (regular 80 mesh, spray dried, Foremost Whey Products, Baraboo, WI) or sucrose (commercial food grade) were each added at 3 and 6% w/v levels to the other samples. These were refrigerated overnight before UHT processing.
Figure 1. Schematic representation of milk diafiltration and ultrafiltration, [L] = Lactose concentration.
Sterilization Treatment

The five concentrated milks were UHT processed in a four-stage pilot plant UHT system (Alfa-Laval Sterilab™, Lund, Sweden) with indirect heat exchange. Milk was preheated to 77.5°C over 58 s with 8 s holding time and then heated to 140°C over 97 s in the second plate heat exchanger with a holding time of 4 s. The milk was cooled to 55°C in a third heat exchanger and passed through a homogenizer using two-stage homogenization with 13.79 MPa first stage and 3.45 MPa second stage pressures. The homogenizer was used to provide a constant flow rate of 100 L/h. The milk was cooled to 20°C, packaged in presterilized 120 mL plastic containers (Fischer Scientific Co., Pittsburg, PA) under aseptic conditions inside a Stericab™ cabinet (Alfa-Laval, Lund, Sweden) kept under positive pressure with microfiltered air.

Storage of Samples

Samples were stored in constant temperature rooms at 4°C, 20°C and 35°C and analyzed after 7, 13, 19 and 22 weeks.

Viscosity

Viscosity was measured using a Brookfield LVT viscometer (Brookfield Engineering Laboratories, Stoughton, MA) fitted with a UL adapter to follow the onset of age gelation. A sample was considered as gelled when the viscosity exceeded 100 cPs. The viscosity was measured at 21±1°C using 16 mL of the sample at a spindle speed of 30 rpm. The scale reading after 30 s was taken and viscosity reported as an average of 4 readings.

pH

pH was measured at 21±1°C using an Orion pH meter (Orion Research Incorporated, Cambridge, MA).
Lactose Determination

Lactose concentration at various stages of diafiltration was measured using an enzymic assay (catalog # 176 303, Boehringer Mannheim, Indianapolis, IN).

Maillard Browning During Storage

Browning was monitored using a reflectance colorimeter (Omnispec 4000, Wescor Inc., Logan, UT). These measurements were made at 35°C by placing 200 µL of the sample in a microtiter plate and measuring the b* value.

SDS-PAGE

SDS-PAGE of fresh skim milk and samples after 22 weeks of storage was performed using a Phast System (Pharmacia, Uppsala, Sweden) with a PhastGel homogeneous 20 gel (Pharmacia, Uppsala, Sweden).

Portions of the milk concentrate samples were diluted with Tris (10 mM)-EDTA (1 mM) buffer, pH 8.0, to thrice their original volume. Then 100 µL of each sample solution was further diluted to 1 mL with the above buffer. Raw milk was used as a standard. To these were added 300 µL of 10% SDS and 50 µL of 2-mercaptoethanol, and the samples were heated in a boiling water bath for 5 min and then cooled and refrigerated until further use, when 0.3 µL was loaded on to the gel.

The following separation conditions were employed. The samples were automatically loaded at the anodic end of the gel at 250 V, 1.0 mA, 3.0 W, and 15°C at 0 Vh. The gel was run till 95 Vh with the final conditions being 250 V, 10.0 mA, 3.0 W and 15°C.

The gels were stained using a solution containing 0.1% Coomassie Blue and 10% acetic acid and then destained using a solution containing 9% methanol and 1% acetic acid in the development unit of the Phast system (Pharmacia, Uppsala, Sweden). Further destaining overnight was done in a
The gels were then fixed in a solution consisting of 10% glycerol and 10% acetic acid, air dried at room temperature, and then photographed.

**Electron Microscopy**

Liquid samples were warmed to 45°C and mixed with an equal volume of 3% agar solution (Bitek™, BIFCO Laboratories, Detroit, MI) maintained at 45°C. The mixture was immediately poured in a petri dish and allowed to set. The milk-agar gel was then cut into strips approximately 10 mm x 2 mm x 2 mm. These strips were immersed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 4-5 h and stored refrigerated in vials with 2.5% glutaraldehyde until further use. Samples that had gelled during storage were cut into strips approximately 10 mm x 2 mm x 2 mm and fixed as described above.

The strips were cut into 1 mm³, rinsed in 0.1 M phosphate buffer, and postfixed in 2% osmium tetroxide. The cubes were dehydrated using increasing concentration of graded ethanol (30%-50%-70%-95%-100%). Further, the dehydrated cubes were treated 4 times with a mixture of 100% ethanol and propylene oxide (transition fluid) mixed in varying proportions ranging from 3:1 ethanol:propylene oxide to 100% propylene oxide alone. The cubes were then infiltrated 5 times with a solution containing propylene oxide and epoxy resin (Epon-Araldite®, Electron Microscopy Sciences, Fort Washington, PA) in various proportions ranging from 3:1 propylene oxide:epoxy resin to 100% epoxy resin. The infiltrated samples were embedded in Beem® capsule (Electron Microscopy Sciences, Fort Washington, PA) and incubated at 45°C followed by 60°C for 24 h each. The hardened epoxy resin was removed from the Beem capsule, and excess epoxy was trimmed to expose the sample.
Ultrathin sections were cut using a MT2B ultramicrotome (Research and Manufacturing Co. Inc., Tucson, AZ) and collected on 600 hex grids. Sections were post-stained with uranyl acetate and lead citrate and examined using a Zeiss CEM 902 transmission electron microscope (Zeiss Inc., Thornwood, NY) at 80 KV. Images were recorded on Kodak electron image film 50-163.

**Total plate count**

Total plate count of samples at the end of 22 weeks was measured using Petrifilm™ 6400 (Aerobic count plates, 3M, St. Paul, MN).
RESULTS

The results reported are those from a single trial. Similar trials were conducted, but the samples behaved differently during storage. One batch of samples showed a very high viscosity when the unsterilized concentrate was stored overnight at 4°C. The samples showed a similar increase in viscosity even during storage at 4°C after UHT processing. The viscosity of the samples decreased when warmed, but they regained the original viscosity when cooled back to 4°C. The samples stored at other temperatures behaved in a manner similar to those reported in this experiment.

Two batches, after UHT processing, showed sedimentation after 4 weeks of storage at all three temperatures. The five samples; control (<.05% lactose), 3% and 6% w/v lactose, 3% and 6% w/v sucrose stored at 4°C and 20°C gelled after 20 weeks. The UHT milk samples stored at 35°C showed increased sediment formation during storage and did not gel. The microbial load in all these samples before UHT treatment were very high because the milk samples could not be UHT processed immediately after membrane concentration due to problems encountered during processing of the milk. The sediment formation may be related to the high microbial load or modification occurring due to microbial growth before sterilization though no microorganisms survived the UHT treatment.

In yet another trial antibiotics (penicillin and chloramphenicol) were added to arrest microbial growth during the initial processing stages. The samples had < 100 cfu/mL before UHT processing. All the samples (control with < .05% lactose and samples with added lactose and sucrose) gelled after 40 weeks of storage at 4 and 20°C, but did not gel when stored at 35°C. The high microbial quality of the samples before UHT treatment may perhaps have resulted in a
storage life of 40 weeks.

**Viscosity Changes During Storage**

The viscosity changes of all samples during storage at the three storage temperatures are shown in Figure 2. The viscosity of the gelled samples changed slightly during the first 19 weeks of storage and suddenly increased to a value greater than 100 cPs just before gelation. The viscosity of samples stored at 35°C initially increased and then decreased to their original value during further storage.

For the samples stored at 4°C, the general trend was a slight initial increase followed by a slight decrease followed by a sudden increase just before gelation (Figure 2a). Samples stored at 20°C (Figure 2b) behaved differently. Their viscosity decreased (in samples with 3% and 6% lactose) and increased in others, initially. This change in initial period was followed by a decrease in viscosity in the 3% sucrose sample and an increase in the rest. This second change was followed by another period during which the viscosity increased in samples with 3% sucrose whereas it decreased in other samples. This was followed by a sudden increase in viscosity in all samples just before gelation.

All samples when stored at 4°C and 20°C gelled after 21 weeks. This was observed by the sudden rise in viscosity of the control sample as well as those containing lactose or sucrose (Figure 2a and 2b). None of the samples stored at 35°C gelled (Figure 2c), but after 32 weeks of storage they started showing slight sedimentation which increased with storage. After 40 weeks of storage, two separate layers (with sediment as the bottom layer and clear serum as the top layer) formed. The sediment was compact and did not redisperse when shaken.
Figure 2. Viscosity changes in control + 3% lactose, control + 6% lactose, control + 3% sucrose and control + 6% sucrose samples during storage at (a) 4°C, (b) 20°C, and (c) 35°C. The viscosity of all samples in figures (a) and (b) exceeded 100 cPs after 22 weeks of storage.
(a) 4°C

(b) 20°C

(c) 35°C
pH Changes During Storage

Raw milk pH was 6.83 and decreased to 6.80 after pasteurization. After diafiltration and concentration to 3X, the pH further dropped to 6.77. The pH of control and samples with 3% and 6% added sucrose after sterilization was 6.73, while that of sample with 3% added lactose was 6.72 and 6% added lactose was 6.71. Figure 3 shows the pH changes during storage at all three storage temperatures.

The pH of all samples decreased during storage with the greatest decrease occurring in samples with 3% or 6% lactose. The pH also decreased at a faster rate as a function of storage temperature. The extent of pH decrease during storage was highest at storage temperature 35°C (Figure 3c) followed by 20°C (Figure 3b) and then 4°C (Figure 3a). At 35°C, the pH decreased to approximately 6.45 for samples containing sucrose and to 6.25 and 6.05 for those containing 3% and 6% lactose. Thus the pH of gelled samples was in the range of 6.54 to 6.68 and the non-gelled samples were in the range of 6.08 to 6.41. This change in pH was observed to be related to the extent of browning that occurred during storage.

Browning During Storage

The extent of browning, as measured using b* values, in different samples stored at different temperatures is shown in Figure 4. The b* value measures blue and yellow colors on a scale of -60 to +60 respectively. Browning (or an increase in yellow coloration) results in b* values above zero.

Samples stored at 4°C or 20°C did not visually appear brown (Figures 4a and 4b). The extent of browning in samples stored at 35°C increased gradually during storage (Figure 4c). The samples containing 3% and 6% lactose underwent considerably more browning than samples with sucrose, which did
Figure 3. pH changes in control, control + 3% lactose, control + 6% lactose, control + 3% sucrose and control + 6% sucrose samples during storage at (a) 4°C, (b) 20°C, and (c) 35°C.
(a) 4°C

(b) 20°C

(c) 35°C
Figure 4. Maillard browning in control, control + 3% lactose, control + 6% lactose, control + 3% sucrose and control + 6% sucrose samples occurring during storage at (a) 4°C, (b) 20°C, and (c) 35°C.
not show any visual browning (Figures 4b and 4c). Samples with 6% lactose underwent more browning than samples with 3% lactose (Figures 4b and 4c).

Proteolysis During Storage

SDS-PAGE electrophoreograms of samples after 22 weeks of storage are shown in Figure 5. Raw skim milk was used as the standard. The electrophoretic patterns of both the gelled and non-gelled samples were different from that of the standard. New set of bands (P) appeared in the gels of samples stored at 4°C, 20°C, and 35°C. A streaking pattern (S) was prevalent in the gel of samples stored at 35°C, in addition to a new set of bands (P and M). The new set of bands could be differentiated into two types. The type P set of bands was seen in front (ahead) of γ-caseins and between the κ-casein and β-lactoglobulin. The type M set of bands appeared above bovine serum albumin (BSA). Samples stored at 4°C and 20°C showed type P bands whereas the samples stored at 35°C showed type P and type M bands.

The bands corresponding to caseins and whey proteins in gelled and non-gelled samples appeared equally intense as the corresponding bands in the standard. The bands shown by samples stored at 35°C are less distinct. The band corresponding to γ-casein is more intense in all the stored samples than the corresponding band in the standard, especially in the ones stored at 4°C and 20°C. Type P bands are quite clear and distinct in the samples stored at 4°C and 20°C and not so distinct in the samples stored at 35°C.

Type M bands are near the top of the gel in the pattern corresponding to samples stored at 35°C. The position of the bands indicated that some material did not enter the stacking gel, and some did not enter the resolving
Figure 5. SDS-PAGE electrophoreogram of control, control + 3% lactose, control + 6% lactose, control + 3% sucrose and control + 6% sucrose samples stored at 4°C, 20°C, and 35°C after 22 weeks of storage. Std = raw milk was used as standard; 3% Lac = 3% Lactose; 6% Lac = 6% Lactose; 3% Suc = 3% Sucrose; 6% Suc = 6% Sucrose; BSA = Bovine serum albumin; α-Cas = αs-casein; β-Cas = β-casein, κ-Cas = κ-casein; γ-Cas = γ-casein; α-LA = α-lactalbumin; β-LG = β-lactoglobulin. The gel shows bands P because of protein breakdown, M because of Protein crosslinking, and a streaking pattern S.
gel. All the samples stored at 35°C showed a streaky electrophoretic pattern.

**Electron Microscopy**

Irrespective of their sugar composition, the electron micrographs of the gelled samples were all similar. Likewise, all the micrographs of the non-gelled samples were similar.

Electron micrographs taken at 30000x magnification of the gelled samples are shown in Figures 6a and 6b. All the casein particles in gelled samples showed tendril or hairy appendages (t) protruding from their surface. The casein particles seem to be connected (g) together by such appendages to form a continuous three-dimensional network, a gel. The particles appear distorted and are not spherical in shape. Some of the particles appear to be fused (f) with other particles (Figure 6a and 6b).

Figures 6c and 6d show the electron micrographs of some non-gelled samples. The casein particles are still roughly spherical (s) and a few tendril appendages seem to protrude from their surfaces. Most of the micelles are well separated from one another and are not joined into a continuous network. Some of the micelles do appear to be joined together or in close proximity in the section viewed. This is either by aggregation or through appendages (t).

Both control samples without lactose (Figure 6a) and samples with added sucrose (Figure 6b), stored at 20°C and gelled, showed appendages on the surface of casein particles. These samples underwent very little, if any, Maillard browning during storage. Samples with added lactose (Figure 6d) stored at 35°C did not gel nor show hairy appendages protruding from the surface of casein particles, but these samples underwent maximum browning during storage. It appears that these hairy appendages are not a result of the
Figure 6. Scanning electron micrographs (30 000x magnification) of gelled samples (a) control, (b) 6% sucrose and un-gelled samples (c) control, (d) 6% sucrose after 22 weeks of storage. The photographs show tendrillar appendages (t) on the casein particles, casein particles connected to form a gel by these tendrillar appendages (g), fused casein particles (f), spherical casein particles (s), and arrows indicate open casein micelles.
Maillard reaction taking place during storage.

The electron micrographs of gelled samples (Figures 6a and 6b) showed some micelles which appear open (indicated by arrows). The micrographs of the non-gelled samples did not show such micelles.

**Total Plate Count**

There was no microbial growth in the samples at the end of 22 weeks of storage at the three temperatures.
DISCUSSION

Viscosity and Browning

Many researchers (22, 23, 25, 36, 49, 61) have observed the sudden increase in viscosity that occurs just before gelation. This viscosity increase reflects a sudden and explosive growth of particles that occurs during gelation. Thus, changes may initially have taken place slowly on the surface of the micelles without really affecting the stability of the whole system and after exceeding a particular level of surface alteration, the particles aggregate culminating in gelation.

The changes in viscosity before gelation (in the first 19 weeks) do not follow a definite pattern between samples that gelled and those that did not gel. These changes may not reflect the changes occurring on the surface of the micelles. They may suggest different pathways leading to the same result. A change in the voluminosity of the casein micelles may have something to do with these changes.

Browning is observed in samples with 3% and 6% lactose during storage at 35°C as others have also shown (2, 4, 5, 61, 94). This browning is caused by the Maillard reactions that take place between a reducing sugar (e.g., lactose) and ε-amino groups of lysine residues of protein. The extensive polymerization caused during the final stages of this type of reaction leads to the formation of brown-colored compounds in the milk. Sucrose, being a nonreducing sugar, does not participate in Maillard browning.

It is interesting that some samples without significant browning during storage do gel. These samples were stored at 4°C and 20°C. Some samples stored at 35°C did become brown while others did not and yet none of them gelled. Thus, there is no correlation between extent of browning and tendency
of the samples to gel during storage. This confirms the similar observation made by other researchers (22, 23, 49).

**pH Decrease**

Many other researchers have made similar observations noting that pH decreases during storage of UHT milk (2, 4, 5, 60, 62, 73, 115) with a higher decrease in samples stored at higher temperatures (6, 60, 73, 115). It has been suggested that this decrease in pH may be due to a loss of positive charges on the protein molecule caused by Maillard reaction (2, 4, 5, 6). However, because the pH of the control and sucrose samples decreased when stored at 35°C, the Maillard reaction may not be the only cause because these samples had insignificant browning. The gradual precipitation of calcium phosphate during storage may shift the calcium-phosphate equilibrium towards formation of Ca$^{2+}$ and PO$_4^{3-}$ from other forms of ionic species and release protons leading to a decrease in pH. A change in mineral equilibria may contribute to a decrease in pH.

Another explanation could be that dephosphorylation occurs during storage which would liberate free H$^+$. Alkaline milk phosphatase can cause dephosphorylation of proteins, and many workers report the reactivation of alkaline milk phosphatase during storage of UHT milk (13). The enzyme is absent immediately after heat treatment, but is isolated in an identical form during storage (13). However, Harwalkar and Vreeman (49) find no increase in inorganic phosphorus during storage and suggest that no dephosphorylation occurred.

The pH of gelled samples (6.55 to 6.65) indicates that gelation was not due to isoelectric precipitation of caseins or microbial growth during storage. The samples that did not gel (at 35°C) have a greater decrease in pH (6.05 to 6.4)
than the gelled samples. There is no correlation between the extent and level of pH decrease and age gelation. Other researchers (6, 49, 60, 115) make a similar observation.

**Protein Hydrolysis**

Protein breakdown during storage of UHT milk has been frequently observed (12, 49, 71, 84, 94). The intensity of bands corresponding to the major caseins in gelled samples, as seen using SDS-PAGE (Fig. 5), are similar to the corresponding bands of the standard. This indicates that little proteolysis occurred during storage although the appearance of new bands (Type P) indicates some breakdown of proteins. This proteolytic activity during storage can be due to extracellular proteinases from the growth of psychrophyllic bacteria (11, 19, 30, 64, 88, 93) or native milk proteinase (15, 21, 25, 28, 31, 98, 101, 102) that survive UHT processing.

The increase in the intensity of γ-casein in the gelled samples indicates breakdown of β-casein. The bands in the region between β-lactoglobulin and κ-casein have a molecular weight of about 19 000 daltons, while those in front of γ-casein have a molecular weight of 10 000 daltons. The band corresponding to κ-casein is intact in the electrophoretic pattern of gelled samples, indicating no breakdown of κ-casein during storage.

Native milk proteinase (primarily plasmin) acts mainly on β- and α_{s2}-caseins (23, 25, 32, 55, 62, 100, 101, 102, 108) and not on κ-casein (16, 32, 34, 62, 72, 101, 108). Plasmin hydrolyzes β-casein to yield three large C-terminal fragments, γ₁-, γ₂-, and γ₃- caseins (3, 17, 21, 41, 43, 44, 79, 82, 108). On incubation with α_{s1}-casein B, it produces degradation products with relative molecular weights of 20 500, 12 300 and 10 300 daltons (34).

In contrast, extracellular proteinases of microbial origin predominantly attack
\(\kappa\)-casein with the formation of para-\(\kappa\)-casein-like material (1, 11, 32, 64, 77, 98, 103) followed by extensive nonspecific hydrolysis (26, 64).

The clear and distinct nature of bands shown in Figure 5 indicates a specific action of the proteinase on \(\beta\)-casein leading to the accumulation of certain molecular weight peptides similar in mobility to the \(\gamma\)-caseins. From the observation of this proteolytic behavior it is suggested that plasmin may have survived UHT or been reactivated during storage.

**Protein Modification**

The electrophoretic pattern of samples stored at 35°C (that did not gel) show very little proteolysis. The bands identified as proteolytic products are very faint. These gels also showed a streaking pattern throughout lane (S) and very intense bands (M) near the top of the gel. These bands are in the region where the samples were loaded on the gel and also at the junction between the stacking and resolving gels. This streaking of stored UHT milks has been observed by other workers (2, 4, 5, 22, 23) and has been reported as being a result of Maillard browning caused by reaction of proteins with lactose (2, 4, 5). This does not appear to be the case. In SDS-PAGE with \(\beta\)-mercaptoethanol, all forces involved in protein-protein interactions except covalent linkages (disulfide linkages are broken) are overcome; hence, protein molecules linked covalently show up either as a separate band if really extensive (same extent of crosslinking) or a streak if there is a whole range of crosslinking with a different number of protein molecules. The streaking in the lanes corresponding to 3% and 6% lactose can be attributed to modification by Maillard browning. The streaking in other lanes (control and samples with sucrose) cannot be ascribed to this reaction. Although there is some residual lactose (<.05%) in these samples, this streaking could not have been caused by this because it would
have then been manifested as a brown color in the samples, and this was not observed. It is suggested that the streaking pattern is caused by some protein modification other than Maillard reaction.

Table 1. Activation energies of some chemical reactions (109).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Activation Energy (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Many chemical reaction</td>
<td>80-130</td>
</tr>
<tr>
<td>Most enzyme-catalyzed reactions</td>
<td>40-60</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>60</td>
</tr>
<tr>
<td>Maillard Browning</td>
<td>100-180</td>
</tr>
<tr>
<td>Dephosphorylation of casein</td>
<td>110-120</td>
</tr>
</tbody>
</table>

The UHT concentrated milks with lactose (3% and 6%) stored at 35°C undergo considerable browning (Figure 4c). This browning occurs during extended storage at 35°C rather than as an immediate result of UHT heating. The long storage at 35°C provides the required activation energy for browning to occur. This implies that other chemical reactions with activation energies equal to or less than Maillard browning can take place during storage at 35°C of UHT processed concentrated milk (Table 1).

Heating of the proteins in milk may promote certain reactions involving serine phosphate, thiol, disulfide, lysine, and amide side chains, and may also cleave some peptide bonds. The side chains of amino acids in caseins are readily available for heat-induced or storage-induced reactions, and those of globular whey proteins are made available by the uncoiling and unfolding resulting from denaturation (109). Table 2 shows some of the possible reactions of amino acid side chains of proteins at high temperature.
Table 2. Possible reactions of protein side chain residues at high temperature

<table>
<thead>
<tr>
<th>Side-chain residue</th>
<th>Conversion to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Asparagine + H$_2$O</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>2. Glutamine+ H$_2$O</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>3. Phosphoserine+ H$_2$O</td>
<td>Serine</td>
</tr>
<tr>
<td>4. Phosphoserine</td>
<td>Dehydroalanine</td>
</tr>
<tr>
<td>5. Cysteine</td>
<td>Dehydroalanine</td>
</tr>
<tr>
<td>6. Dehydroalanine + Cysteine</td>
<td>Lanthionine</td>
</tr>
<tr>
<td>7. Lysine + Dehydroalanine + OH$^-$</td>
<td>Lysinoalanine</td>
</tr>
<tr>
<td>8. Lysine + Aspartic acid</td>
<td>$\varepsilon$-$N$-((\beta)-aspartyl)lysine</td>
</tr>
<tr>
<td>9. Lysine + Glutamic acid</td>
<td>$\varepsilon$-$N$-((\gamma)-glutamyl)lysine</td>
</tr>
</tbody>
</table>

The first three reactions of asparagine, glutamine, and phosphoserine may contribute to the decrease in pH by release of protons directly (asparagine and glutamine) or indirectly (phosphoserine). In the Maillard reaction the $\varepsilon$-amino groups of lysyl residues of proteins participate with carbonyls. They may also react with dehydroalanine (formed through reactions 4 and 5) and carboxyl side chains to form cross-links (reactions 7, 8, and 9). The isopeptides $\varepsilon$-$N$-(\(\beta\)-aspartyl)lysine and $\varepsilon$-$N$-(\(\gamma\)-glutamyl)lysine have been detected in dry casein or lactalbumin heated at 112°C, but not in milk products (109). Lysinoalanine is formed during heating (7), especially in alkaline conditions (68). Some authors report the presence of lysinoalanine in conventionally and UHT processed milk (15, 24) and UHT cream (24). Though heating at sterilization temperature may cause the above reactions to take place, it is not known whether such reactions
can occur during storage of milk at 35°C for long periods. However, if activation energies are compared with Maillard browning, such reactions may occur and could cause modification of proteins leading to altered electrophoretic patterns when stored at 35°C.

**Particle Aggregation**

It has been observed before (6, 25, 50) in UHT milk that has gelled; the casein particles have hairy appendages connecting various particles together to form a three-dimensional network. In our study, these tendrils were observed in samples with and without lactose (Figures 6a and 6b). Hence, they cannot be a result of Maillard browning. This is also indirectly confirmed by the appearance of particles of samples with 6% lactose stored at 35°C (Figure 6d). They do not show any tendrils protruding from the surface of micelles. The nature of these hairy appendages, however, is still unknown. If it is proteinaceous in nature, then it is definitely not a single polypeptide chain since some of these tendrils are more than 200 nm long. They are more likely to be a linear aggregate of several polypeptide chains. Because aggregation results from a reactive particle surface, the modification of the milk proteins at 35°C would have occurred at the surface of the casein particles, modifying or blocking the active aggregating sites and thus preventing aggregation. A similar suggestion is made by Samel et al. (94).

Both native (55) and microbial (1, 78, 93) proteinases have a maximum activity at 37°C. Hence, if proteolysis is considered as the only cause for gelation, it should occur faster at 35°C than at 4°C or 20°C. Some proteolysis is observed in the samples (4°C and 20°C) that gel as well as those that do not gel (35°C). Therefore, it cannot be ruled out as a cause of gelation, but it can be concluded that it is not the only cause. After proteolysis, an aggregation
process which is temperature dependent, may be required before UHT concentrated milk ultimately culminates in gelation during storage. This subsequent process must have occurred in the 4°C and 20°C samples but did not occur in the 35°C sample. The modification of protein at a storage temperature of 35°C must therefore have played a role in preventing the necessary aggregation process.

At higher temperatures, hydrophobic interactions, which play a major role in casein micelle integrity, are greater. The samples stored at 35°C maintained a distinct spherical shape. In contrast, the micelles in the gelled samples appear distorted. These had been stored at lower temperatures. It is of considerable interest to determine the origin of the protuberances observed on the particles of age-gelled milks. They could come from within the micelle because the weaker hydrophobic interaction at lower storage temperatures would allow dissociation of protein from the original micelles. This suggestion agrees with the observation of open micelles in gelled samples. In contrast, the increased hydrophobic interactions at a higher storage temperature together with protein modification may act to hold the micelle together by preventing dissociation and thus working against aggregation.

A two-stage mechanism seems appropriate to explain the phenomenon of age gelation in UHT processed milk concentrates. This mechanism comprises an initial stage which makes the surface reactive by the slow accumulation of dissociated protein on the surface of the modified casein micelles that increase surface reactivity. This could be accelerated by slow proteolysis of the caseins in the micelles. A second process then follows which is a temperature dependent aggregation of these activated colloidal particles. Other workers (6, 49, 115) suggest a similar two-stage mechanism for age gelation comprising an
initial proteolytic step followed by aggregation initiated by a physico-chemical process.
CONCLUSIONS

Based on the comparison of stability of UHT processed skim milk concentrates containing lactose or sucrose, it was concluded that

1. Age gelation is not promoted by occurrence of the Maillard reaction. Samples with and without lactose, stored at the same temperature, gelled at the same time even though no browning occurred in the samples without lactose. Similarly, such browning reactions do not provide protection against age gelation over and above that provided by chemical reactions occurring at the storage temperature of 35°C.

2. Proteolysis, as observed by the appearance of low molecular weight protein bands, occurred in the samples that had age-gelled and, hence, may be involved in gelation. Because it was also observed in non-gelled samples, proteolysis alone does not cause gelation.

3. The streaking observed in SDS-PAGE of UHT milk stored at 35°C was determined not to be a function of browning as previously thought. Some other protein modifications occurring at 35°C must be responsible for this streaking as well as acting to prevent age gelation.

4. Temperature of storage is critical in gelation by favoring some changes promoting gelation at one temperature and favoring other changes at other temperatures which do not promote gelation.

5. Age gelation involves dissociation of proteins from the casein micelles and their formation on the micelle surface as protuberances and tendrils. Aggregation of the protein particles occurs through these appendages rather than through the original micelle surface as occurs in rennet or acid coagulation of milk.
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