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ABSTRACT

AN ASSESSMENT OF THE EFFECTS OF AGING OF MILK ON CHEESE YIELD

By

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The effects of aging milk at 4 C on cheese yield and hydrolysis of β-casein were examined. With the partial hydrolysis of β-casein, proteose-peptone components and γ-caseins are expected to increase after 48 h of milk storage.

No significant differences in the nitrogen distribution of casein and proteose-peptone fractions were observed at 0, 24, 48 and 72 h ($P<0.05$). Curd tension decreased from 0 h to 48 h then increased slightly, indicating the possibility of partial hydrolysis of β-casein. Casein yield decreased slightly over 72 h. Electrophoretic assays showed an increase of components 3 and 5 in proteose-peptone. Components 8-fast and 8-slow, and proteins of the casein fraction did not change significantly. The percentage of protein in experimental cheeses decreased at 48 h and increased slightly at 72 h of milk storage.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

Recent work done by Ali et al.(1980a) indicated that milk held at refrigerated temperatures for 48 h yields less cheese than the milk supply held for shorter or longer periods of time. The low yield was coincident with the highest apparent levels of soluble casein. After 48 h, there was a period of time in which the level of soluble casein, especially β-casein, decreased. This indicated a possibility for increasing cheese yields if high quality milk were kept under proper storage conditions for a period of time greater than 48 h.

Because the dissociation of β-casein from the micellar state during milk storage appears to be a key factor in the differences of this phenomenon, the following hypothesis is suggested. When β-casein dissociates from the casein micelle during cold storage, it is partially proteolyzed by plasmin, an endogenous casein-associated proteinase. Possibly, during the initial 48-hour storage period, the plasmin does not have enough time or the inactive zymogen, plasminogen, is not sufficiently activated to proteolyze the casein significantly. Then, as the cheese milk is warmed to the setting temperature, the caseins migrate back into the micellar state. Possibly,

β-casein moves back onto the surface of the micelle in a different arrangement from that of the fresh milk. This configuration could cause previously available cleavage sites on κ-casein to be obscured from the action of chymosin. A reduction in cheese yield could result. However, after 48 h of storage, plasmin might have enough time to proteolyze a portion of the dissociated β-casein. An increase in proteose-peptones and γ-caseins would result. When the milk is heated for cheesemaking after 48 h of cold storage, there are fewer β-casein molecules to migrate back onto the micelle, leaving more cleavage sites available to the action of chymosin. This rationale might explain the subsequent increase in cheese yield that results when milk has been stored for longer than 48 h.

To evaluate the aforementioned hypothesis, a study was initiated to elucidate the components and properties of milk stored at 4 C for up to 72 h as compared to fresh milk. The action of plasmin on β-casein yields γ-caseins and proteose-peptones which are the C-terminal and N-terminal fragments of β-casein, respectively. This study followed the levels of proteose-peptone over time with the idea that after approximately 48 h of milk storage, dissociated β-casein would be partially proteolyzed by plasmin, resulting in an increase in proteose-peptone levels. Other observations which reflect the partial hydrolysis of β-casein hypothesis, i.e., curd tension and casein yields, were also pursued.

LITERATURE REVIEW

Raw milk is a heterogeneous system which contains emulsified fat globules, colloidally dispersed casein micelles and dissolved proteins, lactose, and salts. There are several principal casein components which are phosphoglycoproteins that comprise about 80% of the total protein content of milk (Brunner,1977). The American Dairy Science Association and the Committee on the Nomenclature and Methodology of Milk Proteins grouped the caseins into the following families: α_{s1}-, α_{s2}-, β- and κ-caseins, and suggested the respective abbreviations: α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN (Eigel et al., 1984) There are also some minor caseins present in milk frequently referred to as γ- and λ-caseins.

The α_{s}-caseins consist of one major and one minor component both with the same amino acid sequence (Eigel et al., 1984). The minor component, previously classified as α_{s0}-casein (Whitney et al., 1976), has the same amino acid sequence as α_{s1}-CN but contains one additional phosphorylated serine residue. The α_{s1}-caseins are insoluble in the presence of calcium(II) at concentration levels occurring in milk (Swaisgood, 1973).

Milk protein components previously classified as α_{s3}-,

α_{s4}-, and α_{s5}-casein by the Committee on the Nomenclature and Methodology of Milk Proteins (Rose et al., 1970; Whitney et al., 1976), and α_{s6}-casein by Annan and Manson (1969), appear to be components of the α_{s2}-CN family. Evidence strongly suggests that all have the same amino acid sequence but differ in phosphate content (Brignon et al., 1976; Brignon et al., 1977). α_{s2}-caseins are also sensitive to the calcium ion (Brunner, 1981).

β-CN has two characteristics which clearly distinguish it from the other caseins. First, it exhibits a strong temperature dependent association, and second, its solubility in the presence of calcium(II) is also temperature dependent. The temperature at which β-casein precipitates is decreased as the concentration of calcium(II) increases (Swaisgood,1973).

The κ-CN molecule has several unique features. It remains soluble in calcium(II) solutions under conditions that precipitate all other casein components. It has the capacity to stabilize other caseins in the presence of calcium(II) through formation of colloidal micelles. In addition, it is specifically hydrolyzed by several enzymes (particularly chymosin) causing destabilization of the micelle and subsequent curd formation. Finally, it is the only casein in which some species are variably glycosylated (Swaisgood, 1973).

A critical concentration of calcium(II) is required to form micelles in model mixtures of κ- and α_{s1}-caseins,

hence, these three components represent a minimum requirement for micelle formation. The extent to which properties of these model systems represent those of native micelles is somewhat uncertain. Removal of calcium(II) from the micelle does not change the hydrodynamic radius until a critical level is reached at which complete dissociation occurs. Above the critical level, removal of calcium(II) results in the appearance of soluble κ- and β-caseins (Swaisgood, 1973). Addition of calcium(II) to native micelle suspensions causes an incorporation of the soluble caseins into the micelle without increasing the hydrodynamic radius (Lin et al., 1972).

The γ- and λ-caseins arise as a result of proteolysis of the β- and α_{s_1}-caseins, respectively.

The liquid remaining after casein has been removed from milk is designated as whey or milk serum. Whey proteins represent about 20% of the total milk proteins. The two principal components are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). The other principal proteins of whey include blood serum albumin, immunoglobulins, and the proteose-peptones. Numerous enzyme proteins and proteins with specific metabolic functions have also been identified in whey (Brunner,1977).

The proteose-peptone fraction of bovine milk has been characterized as a mixture of heat-stable, acid soluble (pH 4.6) phosphoglycoproteins which are insoluble in 12% trichloroacetic acid (Whitney et al., 1976). The major

fractions of proteose-peptone were first designated as components 3, 5, and 8 based upon their ascending mobilities in free-boundary electrophoresis (Larson and Rolleri, 1955). The latter fraction was subsequently separated into components 8-fast and 8-slow (Kolar and Brunner, 1970). Components 5 and 8 were reported to exist in equilibrium between micellar casein and milk serum. Component 3 could only be found in milk serum and was thought to be of blood origin (Kolar and Brunner, 1969). More recent data suggest that component 3 is of milk fat globule membrane origin (Kester and Brunner, 1982). Along with the γ-caseins, the proteose-peptones have also been found to be the products of proteolysis of the β-CN molecule (Andrews 1978a,b; Eigel, 1981).

All the principal milk proteins exhibit genetic polymorphism, usually due to substitution of one or two amino acids, and less frequently to a deletion of up to eight residues. The frequency of occurrence of genetic polymorphs is genus and breed dependent (Aschaffenburg, 1968; Bell et al., 1981; Swaisgood, 1973).

In normal milk, approximately 95% of the casein exists as coarse colloidal particles or micelles. While the detailed structure of the casein micelle is not known with certainty, it is generally accepted that it has a porous structure being composed of spherical submicelles (Fox and Mulvihill, 1982). Numerous models of the casein micelle structure have been proposed. Schmidt (1982) suggested a

subunit structure linked by colloidal calcium phosphate (CCP) in which the subunits contain variable levels of surface κ-CN. κ-CN deficient submicelles are located within the micelle while κ-CN rich submicelles are concentrated at the surface. This gives the intact casein micelle a surface rich in κ-CN, thus, stabilizing the other caseins against precipitation in the presence of calcium. There are, however, other caseins on the surface of the micelle as well.

Caseins, along with the entire milk system, can be easily destabilized by improper handling. Cold storage of bulk milk is one of the most suitable procedures used to avoid undesireable quality changes by inhibiting the growth of contaminating microorganisms. In modern practice of milk processing, raw bulk milk is stored at refrigerated temperatures before and during its transport to the dairy and also at the dairy before processing. The introduction of cold storage of bulk milk was necessary for economic and practical reasons related to centralization in the dairy industry. However, it has resulted in several unexpected problems. Some physical and chemical changes occur, including dissociation of casein from the micelle, proteolysis of the caseins, changes in the rennet coagulation time (RCT) and curd firmness, and reduced cheese yields (Reimerdes, 1982).

The effects of cold storage on the processing parameters of raw milk are related to the micellar character of

the casein fraction of milk proteins. Many authors have described the dissociation of micellar components, especially of the highly hydrophobic β-CN, during cooling of raw milk (Reimerdes, 1982). In 1955, Sullivan et al. suggested that β-CN is removed from casein micelles when skim milk is chilled. Rose (1968) later demonstrated that α_s-, β-, and κ-caseins dissolved in the serum when milk was cooled to 4 C, with β-CN accounting for 55% of the total increase in serum casein.

The temperature dependent dissociation of β-CN from the casein micelle was investigated by Downey and Murphy (1970) using high speed centrifugation and gel filtration. The percentage of the total casein in supernatants prepared by centrifugation of mid-lactation milks was found to increase from approximately 6 to 15% on cooling the milks from 30 to 5 C. β-CN accounted for about 46% of this increase, while α_s- and κ-caseins constituted 30 and 23% respecitvely. On gel filtration, maximum amounts of free β-CN (60% of the total) were obtained at 5 C. The remainder of the β-CN appeared to be more strongly bound to the α_s- and κ-caseins, and they thought that it might be involved in the internal cohesion of casein micelles. The free β-CN appeared to be in equilibrium with the bound β-CN.

Creamer et al. (1977) later undertook to determine the effect of temperature on the extent and rate of this dissociation of β-CN from the micelle into the serum, and to

determine whether some of the micellar β-CN is fixed and cannot exchange with serum β-CN. Ultracentrifugation and gel filtration chromatography were used to separate micellar and serum caseins from milk which had been subjected to various temperature treatments. When milk was held at low temperatures, the concentration of β-CN in the serum increased with time. The amount of β-CN that dissociated from the micelle on standing for 18 hours increased with a decrease in holding temperature.

To follow the movement of β-CN in the milk system, Creamer and his co-workers utilized the properties of ^{14}C-labelled β-CN and the A and B genetic variants of β-CN. These experiments showed that when milk was cooled to 0-4 C, the β-CN in the serum was able to interchange with the β-CN in the micelle. However, complete equilibration between the serum and micellar components was not attained. The interpretation of these data resulted in the following model of casein behavior: β-CN in the interior of the micelle can exchange with that on the surface, which in turn can exchange with β-CN in the serum. Thus, when milk is cooled, β-CN dissociates from the surface sites on the micelle and there follows a redistribution of β-CN within the micelle. The investigators felt this indicated a continual flow of β-CN into and out of the micelle. Warming the casein micelles in the presence of serum that contained soluble β-CN results in a transfer of the β-CN from the serum to the outside of the

micelle. The β-CN can then redistribute itself within the micelle at any temperature in the range 0-37 C.

Creamer and his co-workers proposed this model of casein behavior would predict that, in cold milk, micellar and serum β-CN would slowly interchange as the β-CN on the micelle surface associates and dissociates, and as the β-CN monomers move within the micelle from its surface. The model would also predict that whenever milk is cooled, the β-CN initially released into the serum comes from the micelle surface. The subsequent change in the protein composition of milk serum reflects the distribution of β-CN between the micelle surface and the micelle interior.

Ali et al. (1980a) also studied the overall pattern changes followed by soluble casein concentration in cold-stored milk. Bulk milks and samples from individual cows, with Na benzylpenicillin added to prevent bacterial growth, were stored for up to 72 h at 4, 7, 10, 13 and 15 C. Samples were taken every 24 h and centrifuged at 38,000 g for 2 h at the temperature of storage. Storage temperature was shown to play an important role in the distribution of casein between the micellar and soluble phases in milk. Dissociation into the soluble phase resulted from storage at 4 and 7 C to give soluble casein percentages as high as 42% of the total casein. β-CN was responsible for much of this increase; 30-60% could be found in the soluble phase. Up to 30 and 40% of α_{s1}- and κ-casein, respectively, could

also occur in the soluble phase. Association of the caseins predominated at higher temperatures (13 to 15 C).

The initial dissociation of micellar casein into the soluble phase at low temperatures (4 and 7 C) reached a maximum after 48 h, and was followed by a reversal of this change so that a minimum was seen after storage for 72 h. These results were seen in all individual animal milk samples, regardless of the stage of lactation, and for all bulk milk samples examined. After storage at 4 C for 3 d, losses in all casein components due to proteinase activity were very small (1.0-2.1%). The whey proteins were not affected under these storage conditions.

In addition to cold storage of milk, several other factors have been observed to affect micelle stability and the protein equilibrium in milk. Rose (1968) found that at a fixed level of calcium caseinate, the calcium phosphate content of the micelles and the degree of polymerization of the temperature-sensitive casein components (especially β-CN) are the major factors controlling the proportion of casein present in micellar form. Ali et al. (1980b) assessed the influence of a number of factors, in conjunction with cold storage time and temperature of milk, on the equilibria between soluble and micellar phases. After storage at 4 C both decreasing and increasing pH caused increases in soluble casein. Both soluble calcium and phosphate showed a steady pH dependence, being lowest at the highest pH used. Increasing calcium levels in milk

reduced soluble casein concentrations while treatment with EDTA caused increases in soluble casein. In most cases the pattern of casein dissociation from the micellar phase was followed by a partial reassociation at 4 C over a 3d period (Ali et al., 1980a). Addition of low levels of urea led to some dissociation of all casein components, calcium and phosphate into the soluble phase while treatment of milk with urease resulted in small decreases.

During storage milk proteins may be subject to proteolysis, either by endogenous enzymes (Andrews and Alichanidis, 1983; Reimerdes, 1982) or enzymes produced by microorganisms present in the milk system (Visser, 1981; Adams et al., 1976; Skean and Overcast, 1960; Cousin and Marth, 1977). The presence of a natural protease in bovine milk was first documented by Babcock and Russell in 1897. It is now generally accepted that the milk protease, previously identified as alkaline milk proteinase (Kaminogawa et al., 1972), and known to be transferred from blood into milk (Eigel et al., 1979), is actually blood plasmin or its zymogen plasminogen (Kaminogawa et al., 1972; Eigel et al., 1979; de Rham and Andrews, 1982; Andrews, 1983). This protease is a trypsin-like enzyme which belongs to the group of serine proteinases (Reimerdes, 1982).

Transformation of plasminogen (the inactive form of the enzyme) to active plasmin requires a specific peptide bond cleavage by plasminogen activators, serine proteinases, that are present in the mammary gland and in milk

(Christman et al., 1977; Okamoto et al., 1981; Ossowski et al., 1979). Results of a study by Korycka-Dahl et al. (1983) pointed out that most of the proteolytic activity in freshly drawn milk was in the form of inactive plasminogen. Rollema et al. (1983) drew similar conclusions from their own work. They also suggested that large amounts of plasminogen could influence the storage life of milk products. The activation of a fraction of this zymogen could lead to a significant increase in proteolytic activity.

The final plasmin activity of milk, and subsequent casein hydrolysis would depend not only on the amount of plasminogen and plasminogen activators but also on the quantity of inhibitors. The occurrence of blood serum trypsin inhibitors in milk has been documented (Horkanen-Buzalski and Sandholm, 1981; Lindberg, 1979; Reimerdes et al., 1976). Presumably these inhibitors would interfere with the function of serine proteinases, and therefore, with plasmin and plasminogen activator activity (Korycka-Dahl et al., 1983).

Plasmin activity is variable; the more significant variations are found between individual animals, accounting for up to 82% of the total variance. Stage of lactation has also been observed to have a significant effect on plasmin activity. Enzymic activity decreases as stage of lactation increases (Humbert et al., 1983). Rollema et al. (1983) observed a strong variation in the levels of plasmin and plasminogen in samples of bulk milk and particularly in

samples of milk from individual cows.

Eigel (1977a) studied the proteolytic effect of bovine plasmin on α_{s_1}-CN B and κ-CN. The extent of proteolysis was monitored by disc gel electrophoresis. No significant changes could be detected in the electrophoretic pattern of κ-CN. The electrophoretic band corresponding to α_{s_1}-CN B practically disappeared from the gel after 30 min incubation with plasmin at 37 C. Eigel (1977b) also reported that the γ_1 -, γ_2 -, and γ_3 -caseins, which are identical to the C-terminal amino acid residues 29-209, 106-209, and 108-209, respectively, of the β-CN molecule (Gordon et al., 1972; Ribadeau Dumas et al., 1972), could be produced in vitro by degradation of bovine β-CN with plasmin. Whether actual proteolysis of β-CN took place in the mammary gland or after milking remained to be determined. Reimerdes (1982) surmised that the transfer of milk proteinases and β-CN into milk serum during cooling provides a mechanism for γ-casein formation.

On the basis of advances in knowledge of the origin of the γ-caseins the Committee on the Nomenclature and Methodology of Milk Proteins (Eigel et al., 1984) recommended that fragments resulting from proteolytic cleavage be named as derivatives of the parent polypeptide from which they were derived. Thus, γ_1 -, γ_2 -, and γ_3 -caseins would be called β-CN-1P (f29-209), β-CN (f106-209), and β-CN (f108-209) respectively (for convenience the trivial names will be used throughout this paper). For γ_1 -casein,

1P determines the number of phosphates in the molecule, and (f29-209) refers to the amino acid residues of the β-CN molecule of which the fragment consists.

Several investigators (Chen and Ledford, 1971; Kaminogawa et al., 1969) have reported that rates of proteolysis of the major caseins by alkaline milk protease, or plasmin, occur in the order: β- > α_s - > κ-caseins. These observations, seem to be confirmed by the results obtained by Eigel (1977a), using isolated plasmin.

In the course of studies on proteolysis in milk, Andrews (1978a) found the N-terminal segments of the β-CN molecule located in the proteose-peptone fraction. The proteose-peptone fractions denoted as 5, 8-fast and 8-slow were subsequently identified as β-CN amino acid residues 1-105/107, 1-28, and 29-105/107 respectively (Andrews, 1978 a,b, 1979; Jenness, 1979; Eigel and Keenan, 1979; Brignon et al., 1977). The demonstration by Andrews (1978a) that components 5 and 8-fast represent the N-terminal portions of the β-CN molecule while γ_1 -, γ_2 - and γ_3 -caseins represent the corresponding C-terminal portions provides very strong evidence that both groups of minor proteins are formed by a proteolytic breakdown mechanism from β-CN. The Committee on the Nomenclature and Methodology of Milk Proteins recommended proteose-peptone fractions 5, 8-fast and 8-slow be renamed β-CN-5P (f1-105/107), β-CN-4P (f1-28), and β-CN-1P (f29-105/107), respectively, corresponding to the fragments of β-CN from which they were derived (for

convenience the trivial names will be used throughout this paper). Whether proteolysis of β-CN by plasmin occurs primarily in the mammary epithelial cells, during temporary storage in the alveolar and glandular lumina, during refrigerated storage, or during protein isolation and purification remains yet unknown (Eigel et al., 1984).

Schaar (1985) analyzed the variation in plasmin activity and proteose-peptone content. Proteose-peptone samples were prepared from fresh milk and from the same milk samples after 72 h cold storage (5 C). Plasmin activity increased with parity and stage of lactation and differed between breeds. Breed differences were found to be mainly due to differences in milk casein content with plasmin activity decreasing with increasing casein content. Stage of lactation was found to be the most important factor influencing plasmin activity.

Proteose-peptone from both fresh and stored milks were significantly higher in milks containing the BB genotype of β-LG (which contain a higher proportion of casein) than in milks with the AA genotype. The reason for the effect of β-LG genotype on proteose-peptone samples is not clear. It is possible that the β-LG genotype, through its effect on the relative amounts of the casein and whey protein fractions, influences the distribution of plasmin between casein and whey and thus the access of plasmin to its casein substrate (Schaar, 1985).

In the study by Schaar (1985) it was concluded that

only a part of the casein-derived proteose-peptone can be accounted for by post-secretory plasmin activity. Donnelly and Barry (1983) made a similar observation when they concluded that the proteinase activity of freshly drawn milk was insufficient to account for the γ-casein content at milking. The amount of proteose-peptone formed will increase with time but in direct proportion to the amount of plasmin present. Therefore, Schaar felt that some of the casein breakdown products do not arise from post secretory proteolysis but are actually secreted into the alveolar lumen.

Andrews and Alichanidis (1983) presented evidence that the fragment comprising β-CN amino acid residues 29-105 had been incorrectly identified (Eigel and Keenan, 1979) as proteose-peptone component 8-slow. They felt it was improbable that a small molecule such as component 5 could lose the small, fast-moving acidic N-terminal portion, identified as proteose-peptone fragment 8-fast, and still leave another small fast-moving acidic fragment as the residue. Treatment of β-CN with plasmin, using single dimension 12.5% polyacrylamide gel electrophoresis separation, led to formation of the γ-caseins, component 5, component 8-fast, and a number of other bands. When purified component 5 was treated with plasmin component 8-fast was formed along with a few other bands, all having mobilities less than component 5. In either case, there was no component having a mobility appropriate for component 8-slow.

Since component 5 represents residues 1-105 and 1-107 of β-CN, Andrews and Alichanidis felt this was strong evidence that component 8-slow is derived neither from β-CN nor component 5 and it cannot be equated with residues 29-105 or 29-107 of β-CN. Andrews and Alichanidis (1983) also showed that many of the peptides which arise from caseins by the action of plasmin are subsequently recovered in the proteose-peptone fraction, and since many of these products are transient in nature the composition of the fraction as a whole is both time and temperature dependent.

Available immunological evidence indicates that proteose-peptone component 3 is identical with a soluble glycoprotein prepared from the milk fat globule membrane (Kanno and Yamauchi, 1979). Plasmin has been shown to be associated with the milk fat globule membrane, and to be capable of degrading associated polypeptides (Hofmann et al., 1979). Eigel (1981) reported the possibility that component 3 is a plasmin derived fragment of a milk fat globule membrane (MFGM) polypeptide. In a study by Kester and Brunner (1982) the proteose-peptone glycoprotein (component 3) was found to contain a component antigenically similar to one in the saline-soluble MFGM glycoprotein fraction 1. Component 3 was established as a principal constituent of the proteose-peptone glycoprotein fraction. It appeared that this glycoprotein was also a component of the MFGM. Thus component 3 was thought to originate in one of three ways: it might be a product of endogenous proteo-

lytic degradation of a principal membrane glycoprotein. Or, it could represent a loosely bound membrane component which is desorbed partially into the aqueous phase following secretion. Lastly, it could be a serum component that is adsorbed partially to the membrane complex. Kester and Brunner felt the second possibililty was the most acceptable, however further study was deemed necessary.

λ-casein appears to consist primarily of components which are actually fragments of α_{s1}-casein. It appears that λ-casein also occurs as a result of proteolysis of proteins in milk. Whether these peptides are actually generated intracellularly or result from proteolysis during storage after milking, or during preparation of various milk fractions is not known (Aimutis and Eigel, 1982).

Plasmin does not affect the major whey proteins α-LA (Yamauchi and Kaminogawa, 1972) and β-LG (Chen and Ledford, 1971; Yamauchi and Kaminogawa, 1972). In fact, the latter has been found to act as an inhibitor for the enzyme (Chen and Ledford, 1971; Kaminogawa et al., 1972; Snoeren et al., 1980).

The proteins in milk may also be proteolyzed by enzymes produced from a bacterial source. Psychrotrophic bacteria which are in most raw milk supplies (Foster et al., 1957) can grow readily at refrigeration temperatures. These orgamisms produce proteolytic enzymes which can attack milk proteins (Adams et al., 1976). There have been reports suggesting that proteolytic psychrotrophs may de-

crease cheese yields by breaking down the milk proteins and causing increased N losses into the whey (Olson, 1977; Yates and Elliot, 1977; Cousin and Marth, 1977; Nelson and Marshall, 1977; Aylward et al., 1980; Hicks et al., 1982).

Hicks et al. (1982) found that when pasteurized grade A raw milk was inoculated with psychrotrophic Bacillus and Pseudomonas strains, cheese yield decreased as inoculum increased. All milk samples were stored for at least 6 days at 10 C before processing into cheese. Nelson and Marshall (1977) reported that test psychrotrophic species either had no effect or decreased cheese yield.

Yan et al. (1983) stored two different supplies of grade A raw milk at 4 C and 7 C. After 0, 2, 4, 6 and 8 days of storage, aliquots from each storage temperature were pasteurized and used for the manufacture of direct-acid-set experimental cheese curd. Both aerobic and psychrotrophic counts of the raw milk increased from less than 10^4 cfu/ml at day 0 to greater than 10^8 cfu/ml after 8 days of storage at 4 or 7 C. Proteolysis increased with an increase in storage time.

No apparent decreases in cheese yield were observed for milk stored up to 4 days at 7 C and up to 6 days at 4 C. In fact, slight increases in yield were observed for cheese made from these milks. Cousin and Marth (1977) suggested that psychrotrophs can decrease pH of milk during low temperature storage, thus decreasing the soluble casein. As soluble casein decreased, micellar casein would

increase, which may account for the slight increases observed in yield (Yan et al., 1983). A decrease in yield was shown in cheese made from milk stored 6 days at 7 C and 8 days at 4 C. Rapid decreases in cheese yield occurred only when counts of raw milk were greater than 10^8 cfu/ml.

Partridge (1983), in a study of the effects of lactic culture seeding of raw milk on the yield and quality of Cheddar cheese, found yields of control and seeded cheeses to show a trend toward higher levels on the third and fifth days of manufacture when milk was stored at 3.3 C.

Joshi et al. (1983) investigated the possibility of a correlation between proteose-peptone content of stored pasteurized milks with their bacteriological contents since this fraction might be released from major milk proteins due to proteolytic action of bacteria. Before storage, milk samples were analyzed for their proteose-peptone and bacteriological contents. Subsequently, the milk samples were stored at 8-10, 22, and 37 C for 7 days, 42 h, and 18 hrs respectively. At the end of storage the milk samples were further analyzed for their proteose-peptone and bacteriological contents. The proteose-peptone content of the samples stored at 8-10 C for 7 d did not reveal any increase in proteose-peptone level, whereas there was a slight increase in coliform and enterococcal counts. The samples stored at 22 C for 42 h and 37 C for 18 h both exhibited a considerable increase in the level of proteose-peptone with an increase in total bacterial counts. An

increase in the level of proteose-peptone was mainly attributed to an increase in the coliform counts.

Adams et al. (1976) studied the effects of psychrotrophic growth in raw milk on proteins of milk. Pseudomonas spp. isolates were inoculated into raw skim milk samples to initial populations of 10^6/ml or 10^3/ml. The standard plate count of the uninoculated milk was always less than 100/ml. The caseins of raw skim milk were attacked readily by the psychrotroph isolates during storage at 5 C. κ-casein appeared most susceptible to proteolysis by the psychrotrophic enzymes. The β-caseins were also proteolyzed rather rapidly. α_s-Caseins appeared to under go some proteolysis, but it was not as significant as the other two caseins. Adams' results were similar to those reported by Skean and Overcast (1960). However, Adams and his coworkers felt that in both studies the psychrotrophic populations were much higher than would occur in normal raw milk. Colony counts of uninoculated milk remained below 100/ml, and no proteolysis was detected. Detectable proteolysis of β- and α_s-caseins required much higher populations, at least with the isolates used by Adams and and his co-workers. They concluded that the effect of proteolysis on raw milk might depend on the composition of the psychrotrophic flora of the milk since this could influence the extent of proteolysis and the proteins that are attacked.

Law et al. (1979) felt that the reports concerning

Cheddar cheese yield loss due to proteolytic bacteria involved experiments in which either the numbers of proteolytic psychrotrophs in milk had reached 10^8/ml before pasteurization, or proteolytic enzymes had been added in amounts which could not be related to an equivalent bacterial population. In practice, total psychrotrophic counts do not normally exceed 10^7 cfu/ml in stored milk and only a proportion of these are caseinolytic (Chapman et al., 1976; Law et al., 1979). Law and his co-workers then experimented with Cheddar cheeses made from milks in which proteolytic, psychrotrophic bacteria had grown to numbers likely to be encountered in practice. Casein breakdown in the milks, stored at 7.5 C for up to 72 h, and the cheeses was measured and yield and qualilty of cheese was compared with results obtained using low-count, stored milk. Despite the growth of the proteolytic psychrotrophs in the cold stored milks to approximately 10^6 and 10^7 cfu/ml in 24 and 72 h respectively, very little casein breakdown was evident. Differences between inoculated and control milk samples were either undetectable or only very slight in all cases. Law et al. concluded that excessive proteolysis due to psychrotrophs in stored raw milk is unlikely to be a problem in practice.

Ali et al. (1980a), in studying the effect of cold storage of milk on cheesemaking parameters, determined that the growth of psychrotrophs to levels of 5×10^5 cfu/ml (milk stored up to 72 h at 4 C) had very little apparent

effect on cheese yield or quality.

The changes in the physical and chemical properties of milk due to cold storage are known to prolong coagulation times (Fox, 1969) and to lower curd firmness (Klostermeyer and Reimerdes, 1976). Significant variations in coagulation time and curd firmness have been observed in relation to individual animals, their stage of lactation, and milk pH (Okigbo et al., 1985). Milk pH was the most significant factor affecting coagulation time and curd firmness. Reducing pH shortened coagulation time and increased the curd firming rate. Variation in curd firmness has also been attributed to mastitis (Ali et al, 1980b), feed (Hill, 1931), milk composition (Garnot et al., 1982), temperature history of the milk, and season (McDowell et al., 1969). Curd firmness at cutting influences the texture (Lyal, 1969) and yield (Bynum and Olson, 1982) of cheese.

The process of milk clotting involves a primary and a secondary phase. The secondary phase of the overall coagulation process is the phase in which the coagulum actually forms. This phase is known to involve interactions between calcium ions and α_{s1}-, β-, and para-κ-casein to form an insoluble coagulum (Mehaia and Cheryan, 1983).

Milks stored at 4 C were found by Ali et al (1980a) to prolong rennet coagulation time (RCT) and processing times. The extent of this increase reached a maximum after 48 h of storage. After 72 h the increase in RCT was slightly less than after 48 h. Ali and his co-workers felt that the

increase in RCT could be explained either by the changes occurring in the calcium phosphate equilibrium (Fox, 1969), or by the changes occurring in the casein micelle, or by both. Yan et al. (1983) also found the time required for rennet to develop a firm coagulum was increased as storage time of milk at 4 and 7 C was increased. It was concluded that extensive proteolysis during extended storage apparently caused damage to the casein micelles, causing the delayed coagulation time and preventing the coagulum from reaching the desired firmness.

The many factors discussed above must all be considered when studying the effect of certain parameters on cheese yield and quality. Ali et al. (1980a) found changes in cheese quality and yield correlated well with the changes in casein distribution on cold storage. In laboratory as well as larger-scale experiments, milk stored at 4 C for 24, 48, and 72 h was used to make cheese curds with the standard procedure for Cheddar cheesemaking (Chapman and Burnett, 1972). Over a storage period of up to 48 h the percentage of moisture in the curd increased while cheese yields decreased. On further storage there was a reversal of these trends and all these changes reflected the pattern of changes occurring in serum casein concentrations of stored milks.

Dzurec and Zall (1985) found that cottage cheese yields increased as a result of heating (74 C, 10 s), cooling (3 C), and storing (7 d) milk prior to cheese-

making. Heating prior to storage apparently caused part of the β-CN to be trapped physically in the micelle, leading to increased yields since less casein would be lost in the whey.

There is evidence that cheesemaking properties of milk are also affected by genetic variation of milk proteins (Mariani, 1983). The linkage between α_{s1}-, β-, and κ-casein loci, and variations in gene frequencies between cattle breeds must be considered. Cow's milk containing κ-CN BB has better coagulation properties than milk containing κ-CN AA. Variation at the β-CN locus also affects cheesemaking properties; the β-CN B variant is superior to the β-CN A variant. Polymorphism at the β-LG locus has an indirect effect on cheesemaking properties of milk; cows with the BB variant yield more casein than cows with the AA variant (Mariani, 1983). However, evidence indicates that while β-LG genotype AA is associated with higher casein, and perhaps milk yield, milk from the BB genotype is better for cheesemaking (Sartore and Stasio, 1984).

MATERIALS AND METHODS

The experiments conducted in this study involved the analysis of raw whole milk subjected to four different treatments. Fresh raw milk was taken immediately after milking, cooled to 20 C and divided equally into four containers. The first portion, representing the control, was analyzed immediately. The remaining portions were stored at 4 C for 24, 48, and 72 hours and subjected to the same analyses as the control.

All milk was obtained from Holstein cows of the Michigan State University dairy herd.

The bulk of the fat in the milk used for protein fractionations and polyacrylamide gel electrophoresis was removed by centrifugation.

Preparation of Milk Fractions for Analysis

Protein and non-protein fractions of raw whole milk were prepared according to the procedure used by Shahani and Sommer (1951a). The fractions prepared included total nitrogen (TN), non-casein nitrogen (NCN), proteose-peptone plus non-protein nitrogen(PP+NPN), and non-protein nitrogen (NPN). The nitrogen content of these fractions

was determined by a semi-micro Kjeldahl method.

Nitrogen Analysis by Kjeldahl

Nitrogen analyses were performed using a micro-Kjeldahl apparatus. The digestion mixture consisted of concentrated H_2SO_4 and employed $CuSO_4$ and SeO_2 as catalysts. Samples were digested for one hour in the digestion mixture, H_2O_2 was added and the samples digested for a second hour. The digestion mixture was neutralized with 40% NaOH and the ammonia released was steam distilled into 4% boric acid. The ammonium borate complex was titrated with .02N HCl using an indicator consisting of 0.1% methyl red and 0.03% methylene blue in 60% ethanol.

Tryptophan was used as a standard with an average recovery of 102.51%.

Comparison of Rennet Curd Tension

Rennet curd was prepared using the method of Ashworth and Nebe (1970). Four replicate 225 ml samples of raw whole milk were placed into 250 ml beakers, and warmed in a water bath to 35 C. Each sample was mixed with 4.5 ml of a 1/50 dilution of commercial rennet extract (Dairyland Food Laboratories Inc.) in water, and returned to the water bath at 35 C for 30 min. Curd tension was measured on a Raytheon curd tensiometer.

Comparison of Casein Yield

Rennet curd was again prepared using the method of Ashworth and Nebe (1970). Four replicate 80 ml samples of raw whole milk were placed in centrifuge tubes and warmed to 35 C. Diluted commercial rennet extract (1.6 ml of 1/50 dilution) was mixed with each sample which was held in a water bath at 35 C for one-half hour. The curd was cut and the samples centrifuged at 1000 g for 15 min to pelletize the casein matrix. The final weight of the entire pellet was determined using the procedure for total solids on the Mojonnier Milk Tester. Dried samples were placed under vacuum and reweighed until the weight of the dish was stabilized.

Kjeldahl nitrogen determination of protein fractions, polyacrylamide gel electrophoresis of the proteose-peptone fraction, and microbiological analysis for mesophilic and psychrotrophic bacteria were performed on several milk samples from individual cows.

Sample Preparation for Polyacrylamide Gel Electrophoresis

Proteose-peptone was prepared from 100 ml of raw milk by heating in a 95 C water bath for 40 min, cooling promptly to 20 C, and adjusting to pH 4.6 with 1N HCl. The serum was filtered through Whatman No. 1 filter paper. Thirty milliliters of the proteose-peptone solution designated for electrophoresis was dialyzed overnight in a solu-

tion of polyvinylpyrrolidone to remove most of the water. The contents of the dialysis tubing was rinsed with a small amount of distilled water into a small vial. This sample was then evaporated under nitrogen and the open vial placed in a dessicator containing anhydrous $CaSO_4$ to remove residual moisture. Prior to electrophoresis the sample was rehydrated with stacking gel buffer.

Casein samples were prepared by adjusting a 100 ml sample of milk at 20 C to pH 4.6 with 1N HCl and filtering with Whatman No. 1 filter paper. The precipitated casein was washed three times with acetate buffer (pH 4.6) and blotted dry. Samples used for electrophoresis were solubilized with stacking gel buffer.

The protein content of the proteose-peptone and casein samples was determined using the colorimetric Pierce BCA protein assay. In this assay proteins react with alkaline copper II to produce copper I. BCA protein assay reagent reacts to form an intense purple color at 562 nm with copper I.

The BCA protein assay reagent consists of:

- Reagent A - sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1N NaOH

- Reagent B - 4% $CuSO_4 \cdot 5H_2O$

The BCA working reagent is a 50:1 mix of BCA reagents A and B. 2 ml of the BCA working reagent was added to a 100 sample containing 1-120 μg protein. The sample and reagent mixture was incubated at 37 C for 30 min. The absorbance

of the samples was then read against a reagent blank at 562 nm. Standard curves were prepared using lyophilized samples of proteose-peptone and Na caseinate (obtained from J.R. Brunner). Kjeldahl nitrogen determinations were performed on these standards to determine protein content. From the results of the BCA protein determinations of the proteose-peptone and casein samples, the size of each sample to be used for electrophoresis was calculated to provide the same amount of protein in each assay. Thus apparent differences in the electropherograms would be reflected in the protein distribution.

Polyacrylamide Gel Electrophoresis in a Discontinuous Buffer System

Polyacrylamide gel electrophoresis was performed according to the method of Ornstein (1964) and Davis (1964). All electrophoretic studies were performed in glass gel tubes measuring 0.5 x 7.5 cm. All gels were allowed to age at least 18 h before use to assure complete and consistent polymerization. Two different gel concentrations (T=8.0%, C=2.5% and T=12%, C=2.5%) were employed for proteose-peptone samples and T=10%, C=2.5%, 7M urea gels were used for casein samples. Protein staining was by Coomassie Brilliant Blue G-250, according to Reisner et al. (1975). All gels for each milk sample, consisting of the control and milk stored for 24, 48, and 72 h at 4 C, were stained and destained for the same amount of time, such that the staining/destaining procedure would be uni-

form and not contribute to any differences in dye retention.

Staining for Glycoproteins in the Proteose-Peptone Samples in Acrylamide Gels

Glycoproteins were visualized by the technique described by Zacharius et al. (1969). Following electrophoresis, acrylamide gels were washed thoroughly with distilled water to remove the buffer ions before beginning the staining procedure. Table 1 shows the steps in the staining procedure. The trichloroacetic acid fixes the protein, and complete removal of the periodic acid is imperative to obtain a stained gel with a clear background. The fuchsin-sulfite (Schiff's reagent) was prepared as follows: 2 g basic fuchsin were dissolved in 400 ml water with warming and then cooled and filtered. 10 ml 2N HCl and 4 g $K_2S_2O_5$ were added, and the solution was kept cool and dark overnight in a stoppered bottle. 1 g activated charcoal was stirred in and the solution was filtered, and sufficient 2N HCl (10 ml or more) was added until a drop dried on a glass slide did not turn red.

Densitometric Scanning of Stained Polyacrylamide Gels

A Shimadzu dual-wavelength, thin-layer chromato scanner, model CS-930, was used to assess the polyacrylamide gels stained with Coomassie Brilliant Blue G-250 at 580 nm.

Table 1. Procedure for staining of glycoproteins in acrylamide gels

	Staining Procedure	Time (min)
1)	Immerse gels in 12.5% trichloroacetic acid	30
2)	Rinse with distilled water	0.25
3)	Immerse in 1% periodic acid in 3% acetic acid	50
4)	Wash 6X for 10 min in distilled water or wash overnight with a few changes of distilled water	60 or overnight
5)	Immerse in fuchsin-sulfite stain in dark	50
6)	Wash 3X for 10 min with freshly prepared 0.5% meta-bisulfite solution	30
7)	Wash with frequent changes of distilled water until excess stain is removed	overnight
8)	Store in 5% acetic acid	

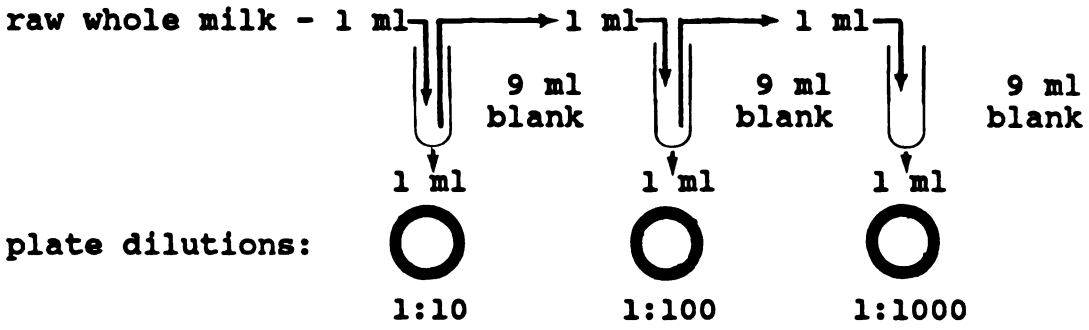
Microbiological Analysis of Milk for Mesophilic and Psychrotrophic Bacteria

The Standard Plate Count method as described by Di Liello (1982) was used to enumerate mesophiles and psychrotrophs in the milk samples. Plate count agar was the medium used. Sample dilutions for mesophilic and psychrotrophic bacteria are shown in Figure 1.

Cheesemaking Process

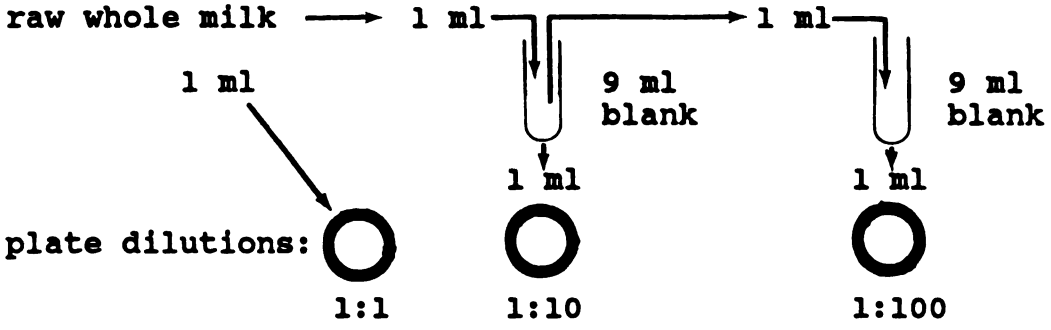
The cheesemaking process was performed according to the method used by Chapman and Burnett (1972). However, it was modified to an experimental, bench-scale procedure in which six replicate 1000 ml beakers containing 900 g of raw whole milk were used. The beakers of unpasteurized milk were heated in a warm water bath. Pre-cheese treatments included milk that had been stored at 4 C for 0, 48, and 72 h. A direct vat-set starter culture, obtained from Hansen's Lab., Inc. via the Michigan State University Dairy Plant, was used. Starter (1.4 ml) was diluted in 8.6 ml milk for a total volume of 10 ml. One milliliter of this mixture was added to each of the six beakers to give .0164% starter in each sample. One milliliter of a 1/50 dilution of commercial rennet extract in water was added when the samples had reached 30 C and pH 6.58. Temperature and pH were followed over time rather than the development of titratable acidity. In this way curd "fines" present in the whey, which would contribute to the final weight of the cheese, were not lost. When the cooking stage was

Mesophilic Bacteria



Plates were incubated at 32 C for 48 h.

Psychrotrophic Bacteria



Plates were incubated at 7 C for ten days.

Figure 1. Dilution diagrams for mesophilic and psychrotrophic bacterial analysis of milk samples stored at 4 C for up to 72 h.

finished, at 39 C and pH 6.15, whey was drained and filtered to catch the fines. The curds and fines from each batch of cheese were combined and wrapped in cheese cloth and held under pressure for 15 h. The final cheeses were weighed and a sample was taken from each for a Kjeldahl nitrogen determination from which the percentage of protein of each cheese sample was calculated. A sample of the cheese milk from each treatment was retained for proteose-peptone analysis by gel electrophoresis. A similar assay was performed on the whey produced in the cheesemaking process. The nitrogen content was determined by Kjeldahl. The milk used for cheesemaking was also analyzed microbiologically for the presence of psychrotrophic bacteria.

Experimental Design

Statistical analysis of the data accumulated in this study was performed using a randomized complete block design (Steel and Torrie, 1980). Milk samples from individual cows were identified as blocks, and the treatments involved storage of milk at 4 C for 0, 24, 48 and 72 h. The data were analyzed by the following statistical model: $Y_{ij} = \mu + \beta_i + \tau_j + \epsilon_{ij}$

where: μ = overall mean,

τ_j = fixed effects of the jth treatment (t=4),

β_i = Random effects of the ith block trial,

ϵ_{ij} = residual error.

Data were analyzed using the factor procedure of the MSTAT program from Michigan State University.

RESULTS

Kjeldahl Analysis of Protein Fractions of Milk

TN, NCN, PP+NPN, and NPN were the fractions analyzed. Casein nitrogen was calculated by subtracting NCN from TN. Proteose-peptone nitrogen was determined by subtracting NPN from PP+NPN. Table 2 shows the nitrogen distribution of 10 individual milk samples subjected to the four treatments used in this study. Tables 3, 4,and 5 demonstrate the trends followed over time by the TN, casein nitrogen, and proteose-peptone nitrogen fractions, respectively, of each individual milk sample.

Comparison of Rennet Curd Tension

Rennet curd tension values for individual milk samples stored at 4 C are represented in Figure 2.

Comparison of Casein Yield

The final weights of casein curd obtained from rennet-treated individual milk samples stored at 4 C are reported in Figure 3.

Discontinuous Polyacrylamide Gel Electrophoresis of Proteose-Peptone Samples

Polyacrylamide gel electrophoresis of a preparation of proteose-peptone, utilizing T:8%, C:2.5% and T:12%, C:2.5% gels, showed a number of protein bands. Figure 4 depicts electropherograms and corresponding densitometric traces of a proteose-peptone sample on gels of T:8%, C:2.5% acrylamide concentration. Figure 4 A, B, C, and D represent proteose-peptone samples isolated from raw milk stored at 4 C for 0, 24, 48 and 72 h, respectively. Figures 5, 6, 7, 8 and 9 show graphic representations of changes in the area percentages over time on the densitometric traces for each major peak.

Figure 10 represents the electropherograms and corresponding densitometric traces of the same proteose-peptone sample on T:12%, C:2.5% acrylamide gels. The densitometric traces in Figure 10 A, B, C, and D show changes in the dye retention of each protein band, which is proportional to changes in concentration of the proteins over time.

Staining of Glycoproteins of Proteose-Peptone Following Electrophoresis

Carbohydrate-containing proteins were detected in acrylamide gels by the technique described by Zacharius et al. (1969). In this method, protein bands which contain glycoproteins are identified by a distinctive fuchsia colored zone. Figure 11 A shows one principal band as well as several minor bands on the gel.

Discontinuous Polyacrylamide Gel Electrophoresis of Casein Samples

PAGE of the casein preparation on T:10%, C:2.5% acrylamide , 7M urea gels produced electropherograms and densitometric traces such as those shown in Figure 12. Changes over time in protein concentration, determined by changes in the area percentage of the peaks on the densitometric traces of three major bands are depicted graphically in Figures 13, 14 and 15.

Microbiological Analysis for Mesophilic and Psychrotrophic Bacteria

Individual Standard Plate Counts are reported in Table 6.

Laboratory Scale Cheesemaking Process

The final protein content, expressed as a percentage of the total cheese weight, of the cheeses made with milk stored at 4 C for 0, 48, and 72 h is represented in Figure 16. Microbial analysis of the cheese milk samples for psychrotrophic bacteria yielded no more than 10 cfu/ml at up to 72 h of storage at 4 C.

PAGE of proteose-peptone samples made from the milk used for cheese produced the electropherograms and corresponding densitometric traces depicted in Figure 17. Changes in the area percentages of the peaks are reported in Table 7.

The electrophoretic assay performed on cheese whey

resulted in the electropherograms and corresponding densitometric traces shown in Figure 18. Corresponding changes in peak areas are reported in Table 8.

Table 2. Nitrogen distribution of ten individual milk samples

Storage Time (h)	Component	(mg/100ml milk) Low°	High	Average
0	Total N	494.0	635.3	555.9 ± 46.1
	Casein N	364.3	489.1	429.0 ± 35.1
	P-P N	13.5	29.5	20.5 ± 4.7
24	Total N	489.5	638.7	560.1 ± 47.6
	Casein N	375.9	497.8	433.7 ± 34.9
	P-P N	16.0	35.8	22.5 ± 5.8
48	Total N	499.6	625.9	557.3 ± 44.5
	Casein N	371.2	486.1	432.2 ± 32.2
	P-P N	15.0	35.2	22.2 ± 5.4
72	Total N	497.0	645.4	570.3 ± 48.4
	Casein N	358.7	502.4	440.0 ± 38.6
	P-P N	13.5	39.0	23.4 ± 6.4

° Mean of two determinations.

Table 3. Comparison of total nitrogen distribution in mg/100ml of ten milk samples stored at 4 C

Sample	Hours of Storage 0	24	48	72
1	546.5[a](100)[b]	527.5(96.5)	512.0(93.7)	549.0(100.5)
2	529.5(100)	544.5(102.7)	529.5(100)	551.0(104.1)
3	540.0(100)	545.0(100.9)	536.5(99.4)	584.5(108.2)
4	494.0(100)	489.5(99.1)	515.0(104.3)	497.0(100.6)
5	567.5(100)	593.0(104.5)	579.5(102.1)	580.0(102.2)
6	578.9(100)	569.6(98.4)	580.1(100.2)	579.5(100.1)
7	615.7(100)	615.1(99.9)	613.1(99.6)	625.2(101.5)
8	494.9(100)	503.0(101.6)	499.6(100.9)	497.6(100.5)
9	635.3(100)	638.7(100.5)	625.9(98.5)	645.4(101.6)
10	556.3(100)	575.4(103.4)	582.0(104.6)	593.8(106.7)

[a]Mean of two determinations.
[b]Values in parentheses reported as percent of 0 h N.

Table 4. Comparison of casein nitrogen distribution in mg/100 ml of ten milk samples stored at 4 C

Sample	Hours of Storage 0	24	48	72
1	421.0[a](100)[b]	411.5(97.7)	404.0(96.0)	424.0(100.7)
2	416.5(100)	424.5(101.9)	419.0(100.6)	426.0(102.3)
3	422.5(100)	424.0(100.4)	417.0(98.7)	455.0(107.7)
4	402.5(100)	402.0(99.9)	425.5(105.7)	411.0(102.1)
5	434.0(100)	459.5(105.9)	445.3(102.6)	447.6(103.1)
6	445.6(100)	437.1(98.1)	448.4(100.6)	447.6(100.4)
7	473.2(100)	467.9(98.9)	463.0(97.8)	473.4(100.1)
8	364.3(100)	375.9(103.2)	371.2(101.9)	358.9(98.5)
9	489.1(100)	497.8(101.8)	486.1(99.4)	502.4(102.7)
10	421.0(100)	436.3(103.6)	442.3(105.1)	452.9(107.6)

[a]Mean of two determinations.
[b]Values in parentheses reported as percent of 0 h N.

Table 5. Comparison of proteose-peptone nitrogen distribution in mg/100 ml of ten milk samples stored at 4 C

Sample	Hours of Storage 0	24	48	72
1	17.0[a](100)[b]	23.0(135.3)	18.0(105.9)	23.0(135.5)
2	22.5(100)	27.0(120.0)	22.0(97.8)	25.0(111.1)
3	24.0(100)	20.2(84.2)	25.5(106.3)	20.5(85.4)
4	13.5(100)	16.0(118.5)	15.0(111.1)	13.5(110)
5	19.1(100)	21.0(109.9)	22.9(119.9)	21.0(109.9)
6	16.7(100)	16.8(100.6)	21.0(125.7)	22.4(134.1)
7	29.5(100)	35.8(121.4)	35.2(119.3)	39.0(132.2)
8	16.8(100)	18.3(108.9)	18.8(111.9)	21.5(128.0)
9	22.3(100)	25.4(113.9)	21.9(98.2)	24.9(111.7)
10	23.7(100)	21.6(91.1)	21.8(92.0)	22.8(96.2)

[a]Mean of two determinations.
[b]Values in parentheses reported as percent of 0 h N.

Table 6. Mesophilic and psychrotrophic Standard Plate Counts of three individual milk samples stored at 4 C

	Mesophilic Count (cfu/ml)				Psychrotrophic Count (cfu/ml)			
	Hours of Storage				Hours of Storage			
Sample	0	24	48	72	0	24	48	72
1	1000	1200	1300	1700	20	15	26	50
2	1400	1600	1700	2900	110	320	150	450
3	18000	20000	19000	18000	730	680	970	1000

Table 7. Changes over time in PAGE densitometric trace area percentages° of the protein-stained zones of proteose-peptone isolated from cheese milk

Protein Zone	Storage Time (hours) 0	48	72
	Area under peak (%)		
Component 3	20.3	15.8	19.7
Intermediate between "3" and "5"	13.7	25.3	18.4
Component 5	45.0	47.9	49.3
Intermediate between "5" and "8-fast & 8-slow"	3.9	4.4	4.1
Components "8-fast and 8-slow"	6.1	6.6	6.3

°Area reported is average of three determinations.

Table 8. Changes over time in PAGE densitometric trace area percentages[a] of the protein zones of whey samples

Protein Zone	Storage Time (hours) 0	48	72
	Area Under Peak (%)		
BSA	11.2	11.2	12.1
Unknown protein[b] zone between BSA and α-Lactalbumin	4.9	4.2	4.2
α-Lactalbumin	43.7	43.8	44.3
β-Lactoglobulin	40.2	40.8	39.4

[a]Area reported is average of three determinations.
[b]Unknown protein zone did not compare to proteose-peptone zones.

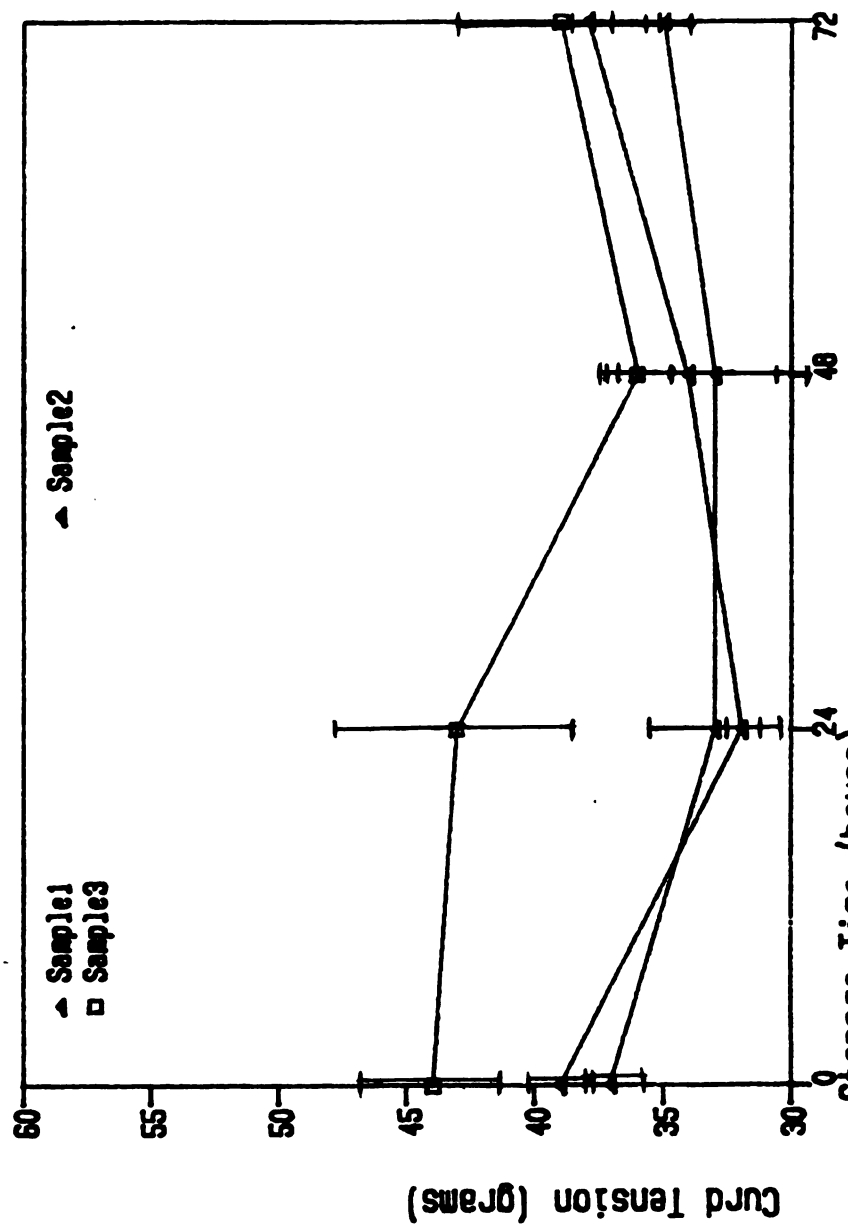


Figure 2. Comparison of rennet curd tension of individual milk samples stored at 4°C.

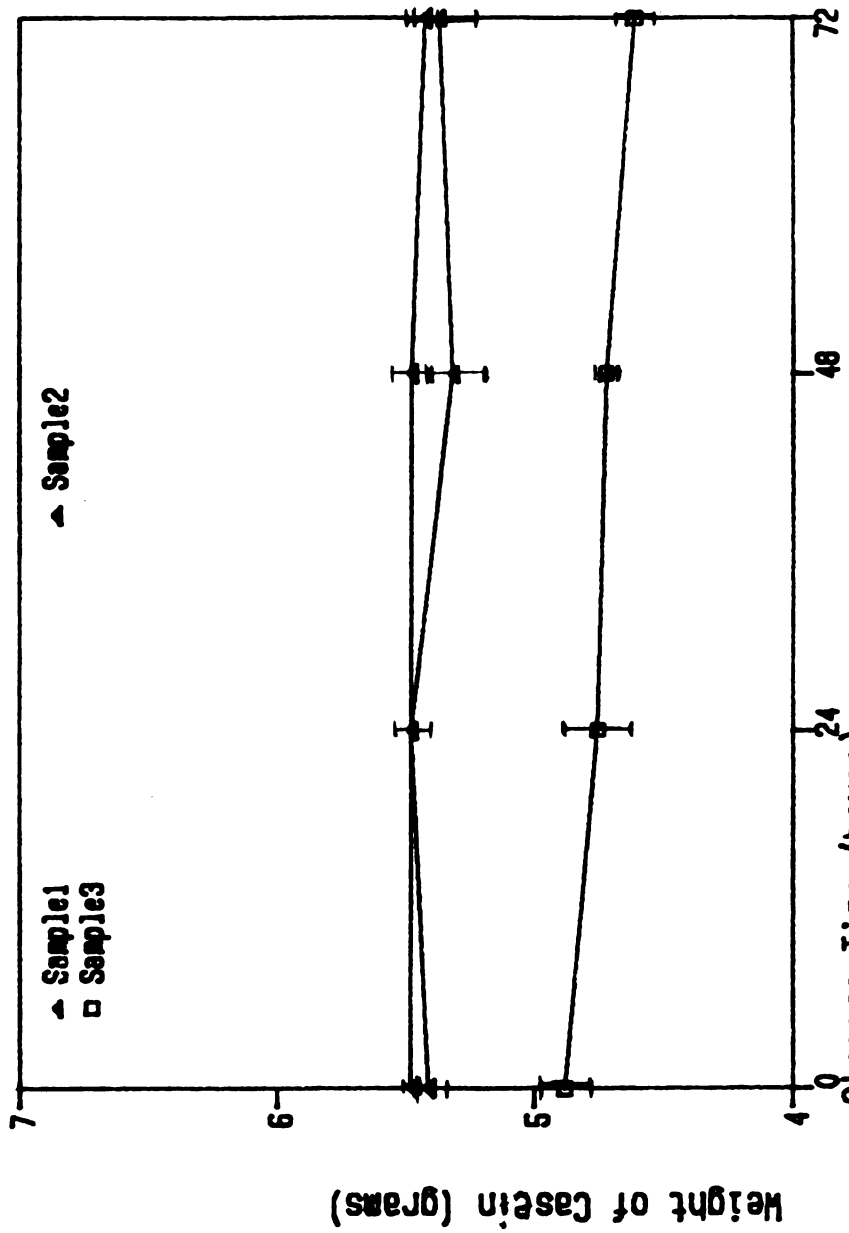
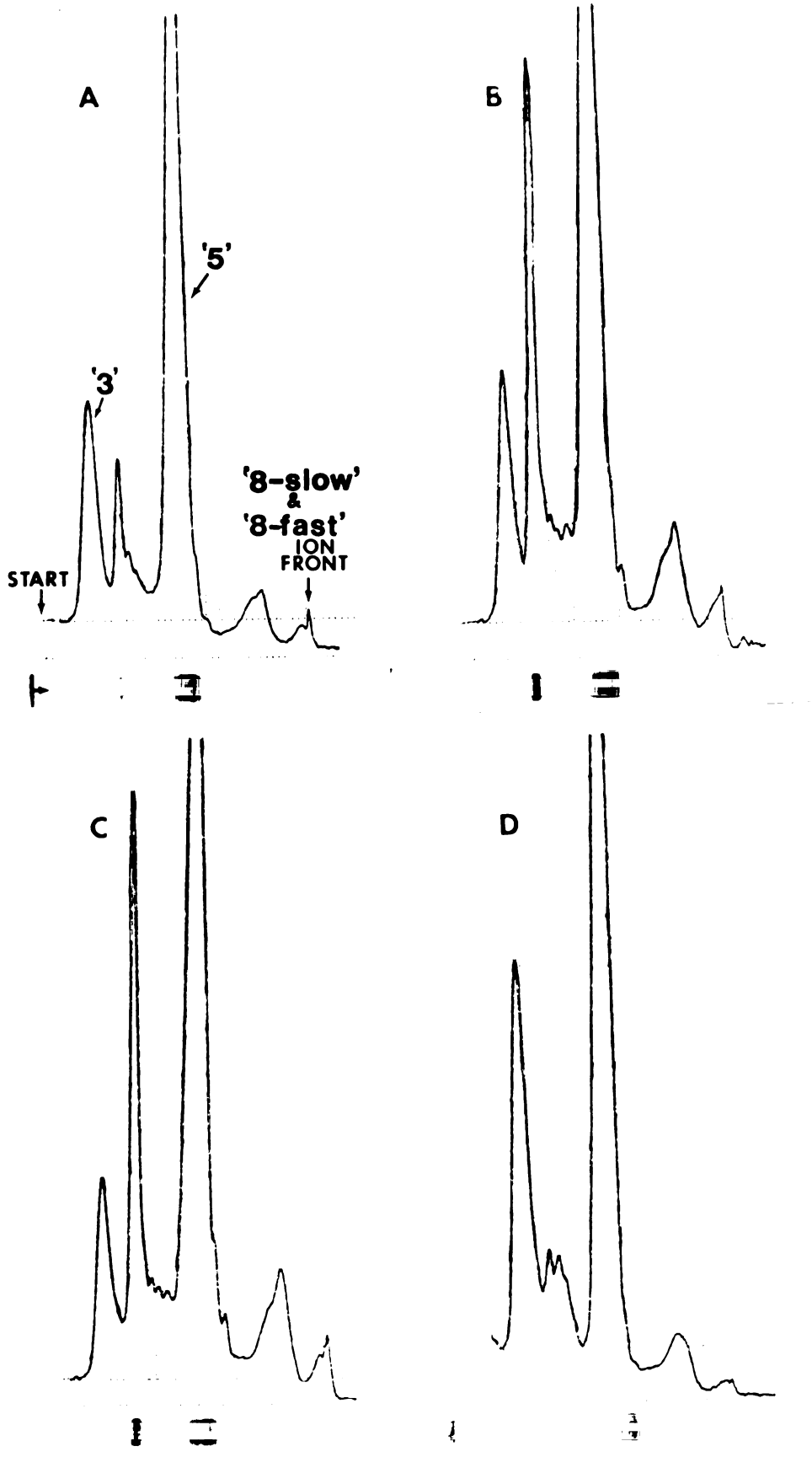


Figure 3. Comparison of casein yield from rennet curd made with individual milk samples stored at 4°C.

Figure 4. Electropherograms and corresponding densitometric traces of proteose-peptone samples made from milk stored at 4 C for A) 0 h, B) 24 h, C) 48 h and D) 72 h on acrylamide gels of T:8%, C:2.5% concentration.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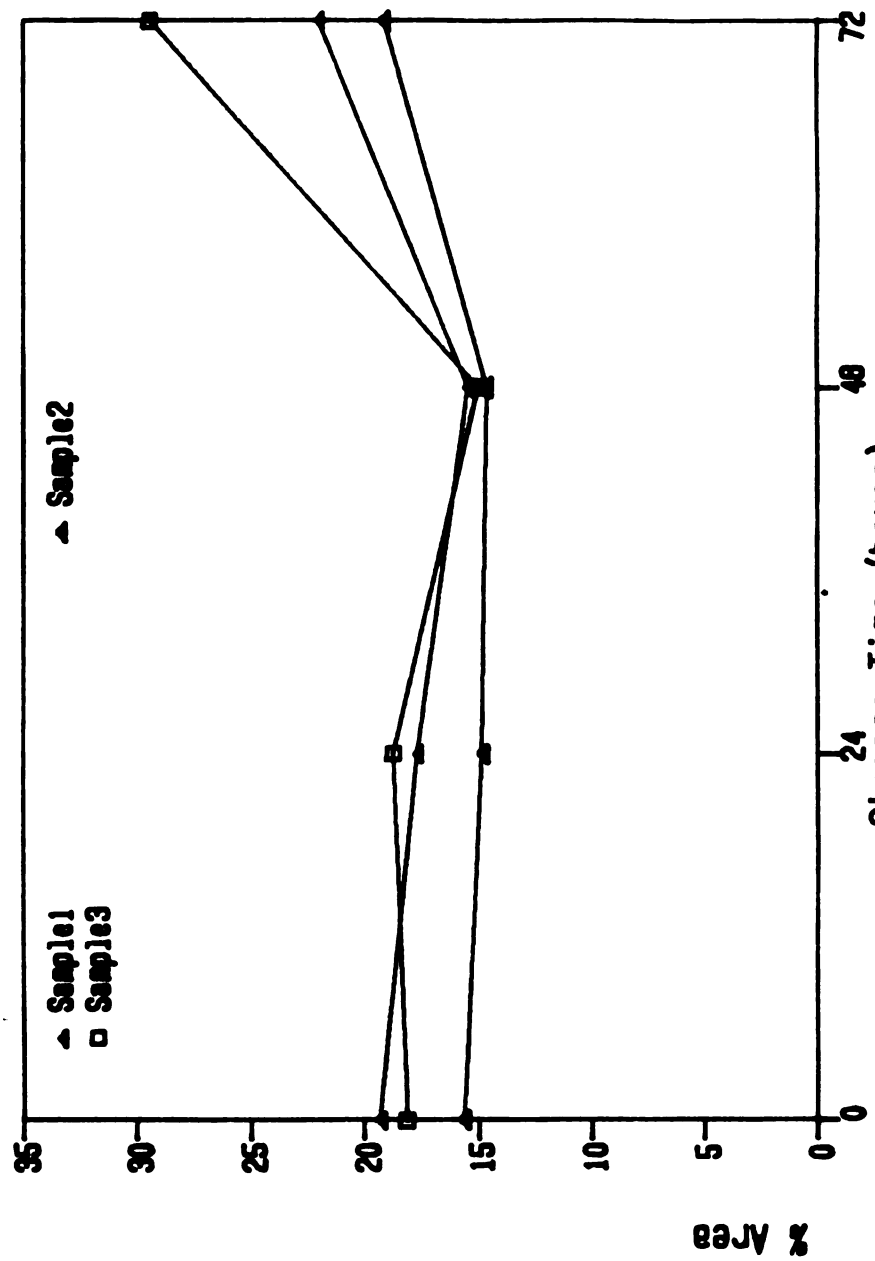


Figure 5. Change in percent area over time of peaks representing protein concentration of proteose-peptone component 3 from densitometric traces of three samples of proteose-peptone made from individual milks stored up to 72 h at 4 C.

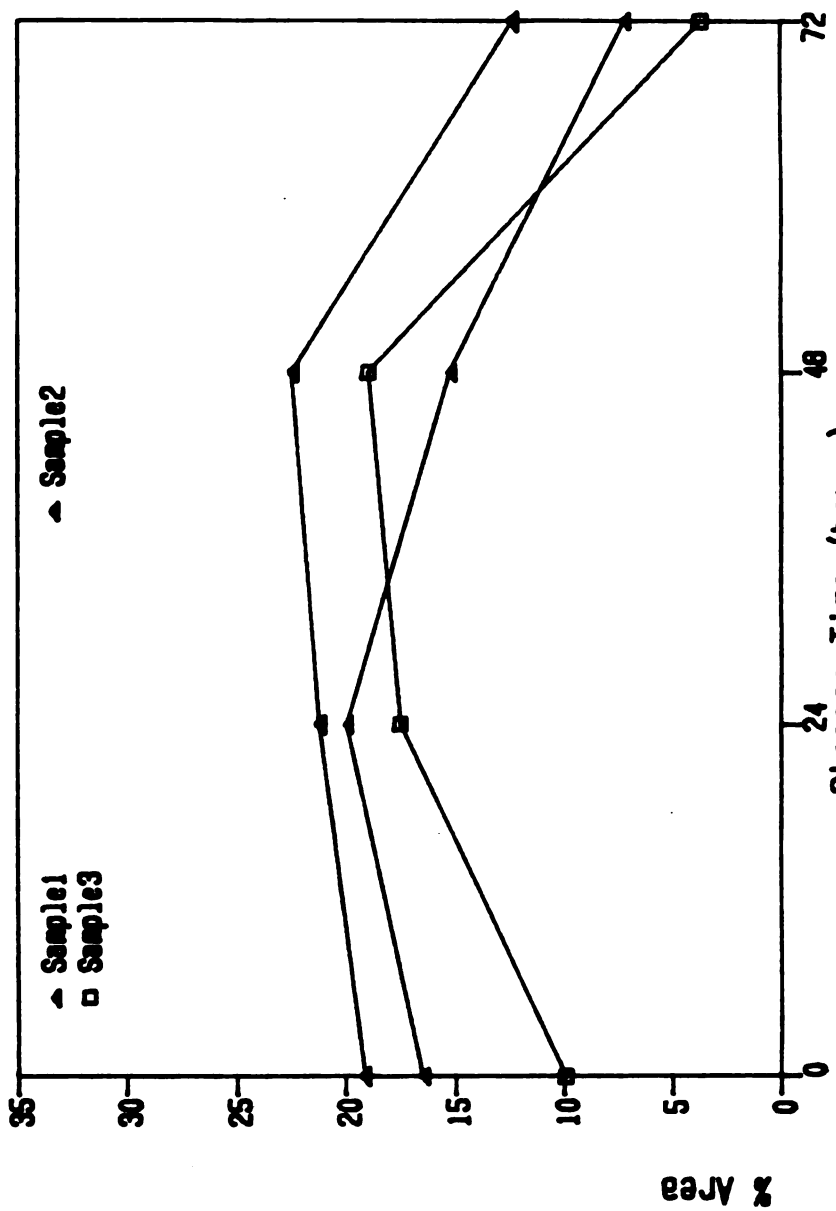


Figure 6. Change in percent area over time of peaks representing protein concentration of an unknown intermediate component between components 3 and 5 from densitometric traces of three samples of proteose-peptone made from individual milks stored up to 72 h at 4 C.

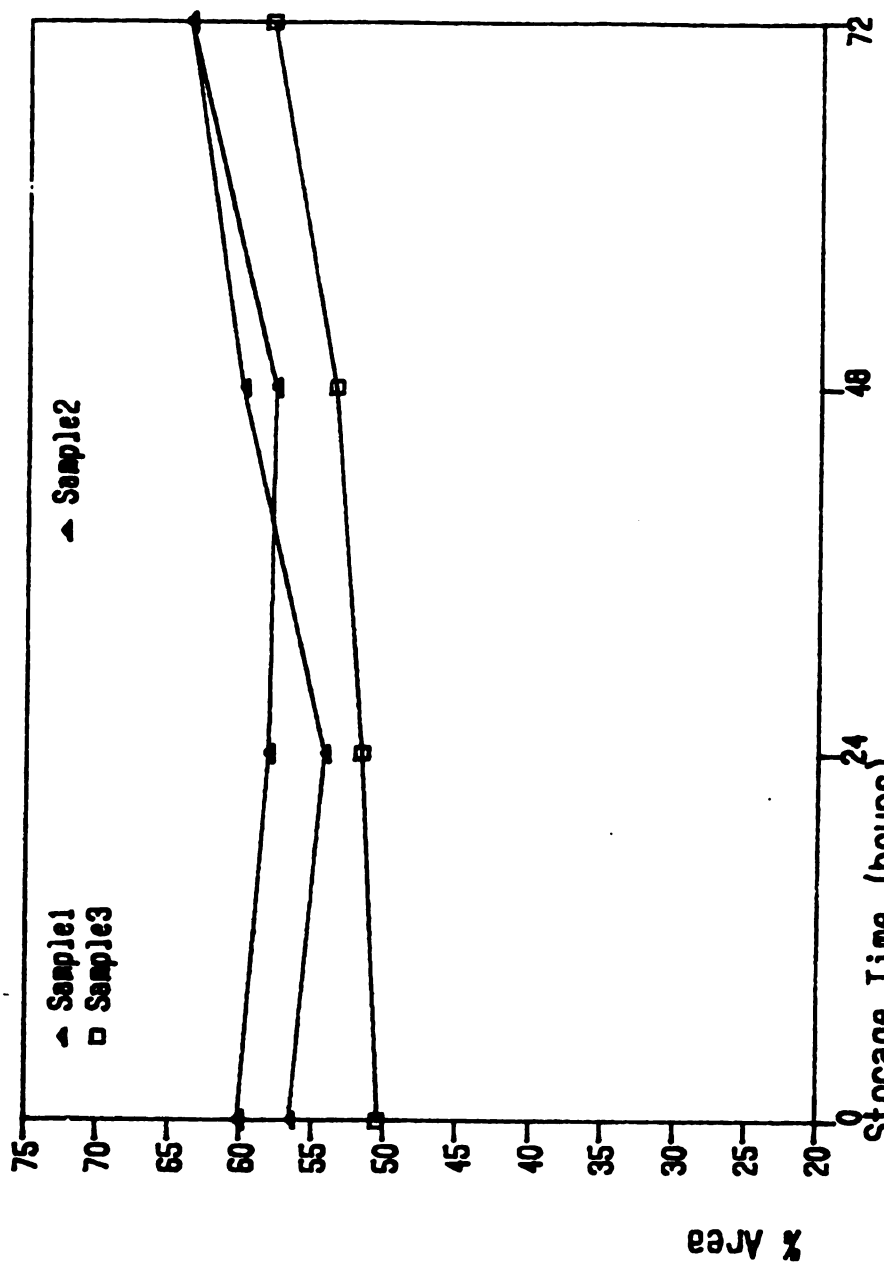


Figure 7. Change in percent area over time of peaks representing protein concentration of component 5 from densitometric traces of three samples of proteose-peptone made from individual milks stored up to 72 h at 4°C.

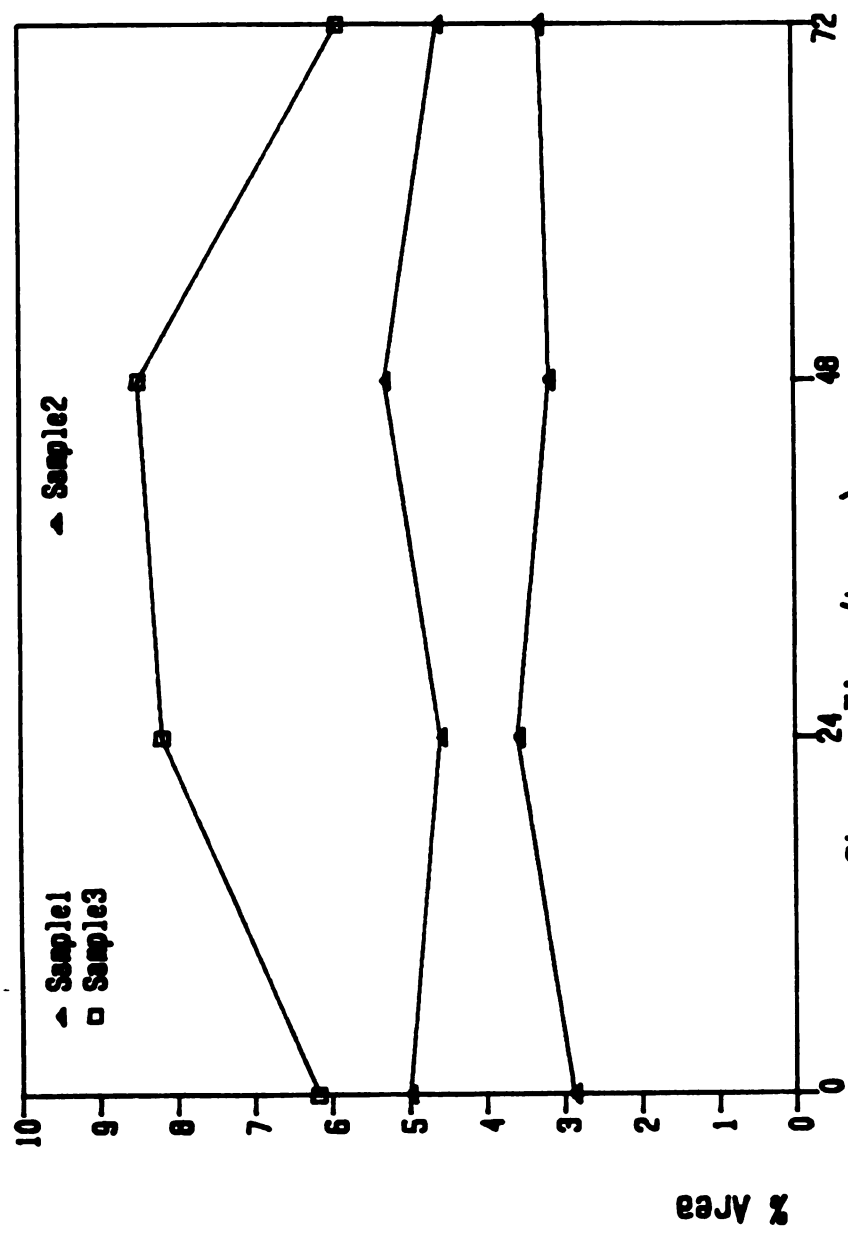


Figure 8. Change in percent area over time of peaks representing protein concentration of the intermediate zone between components 5 and 8-fast & 8-slow from densitometric traces of three samples of proteose-peptone made from individual milks stored up to 72 h at 4 C.

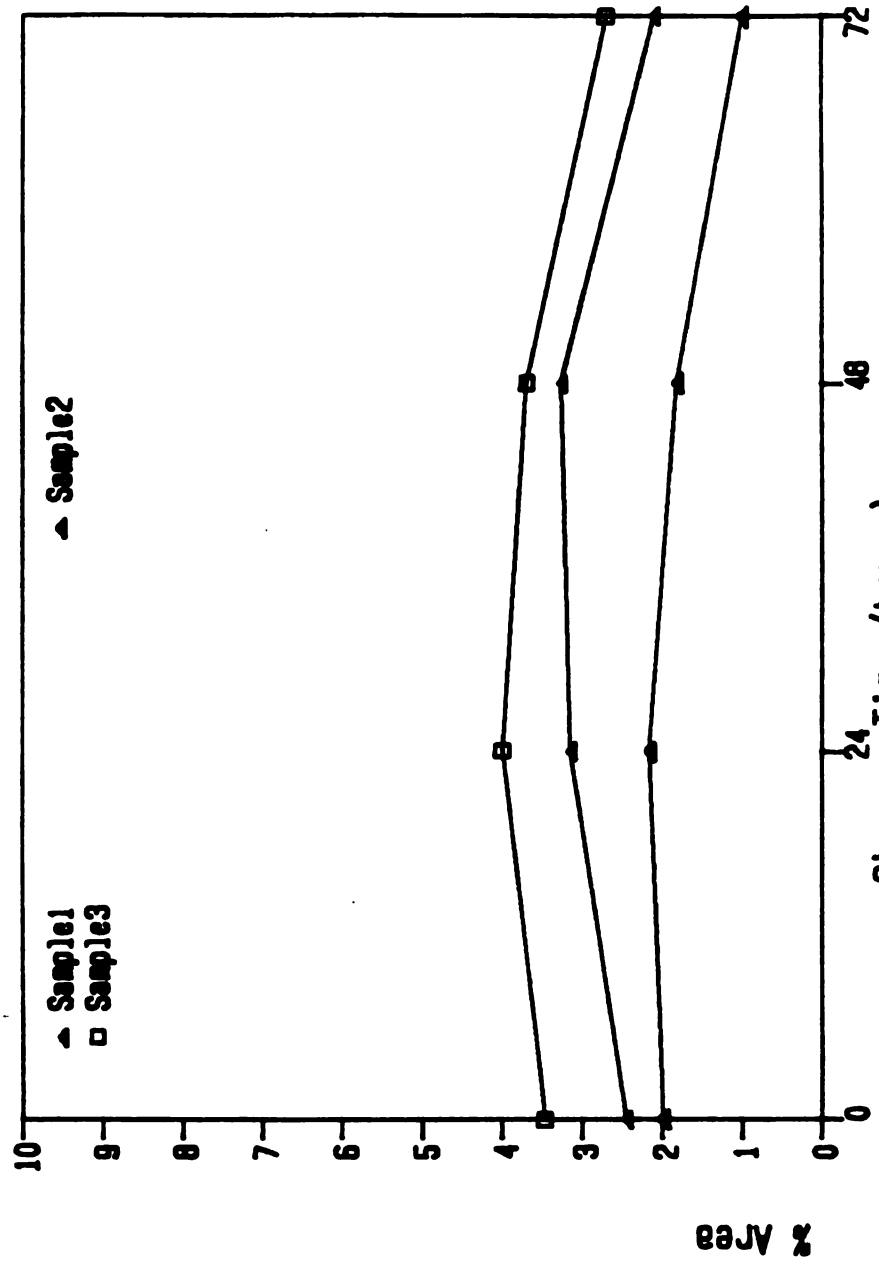
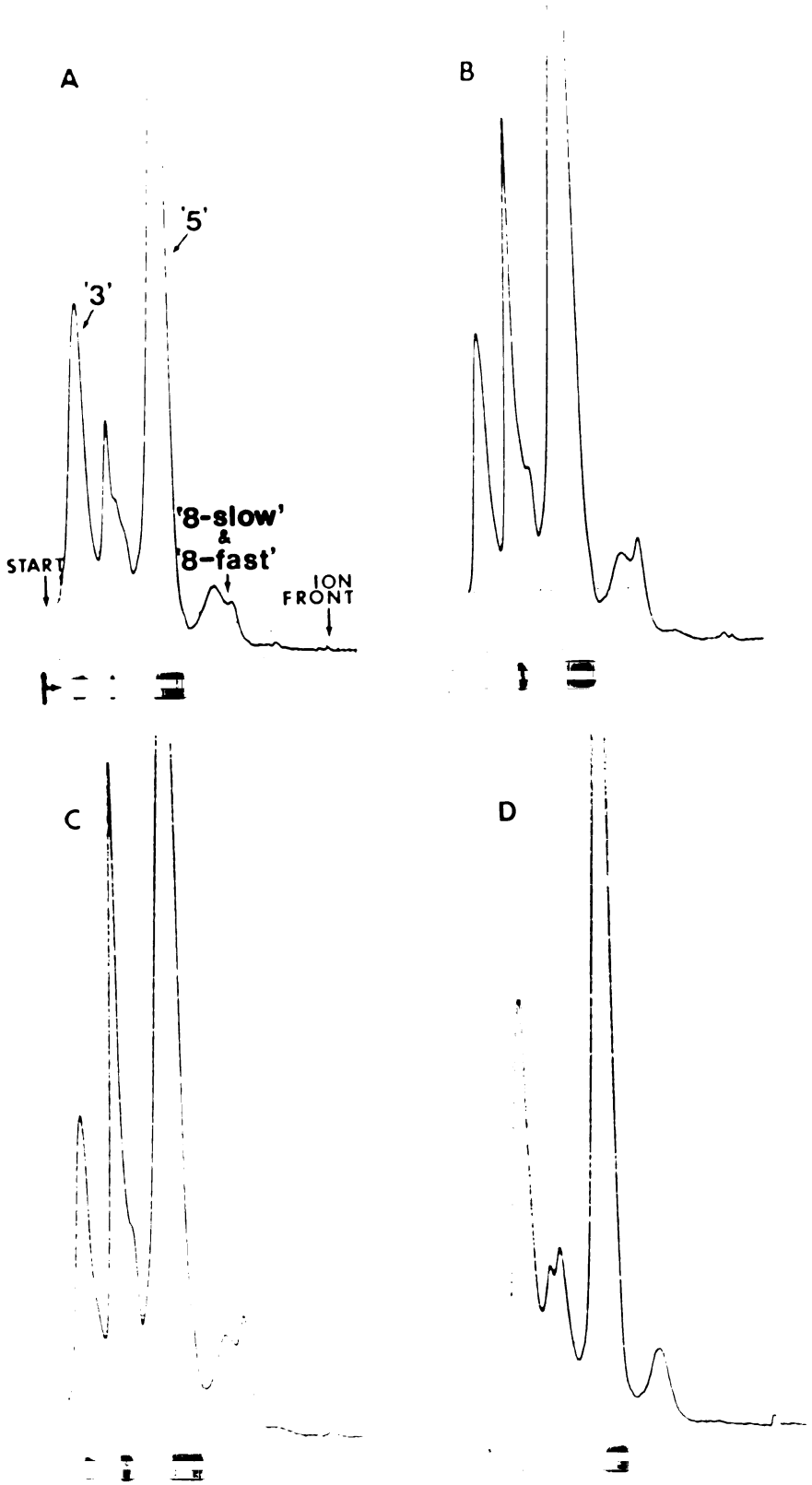


Figure 9. Change in percent area over time of peaks representing protein concentration of components 8-fast and 8-slow from densitometric traces of three samples of proteose-peptone made from individual milks stored up to 72 h at 4 C.

Figure 10. Electropherograms and corresponding densitometric traces of proteose-peptone samples made from milk stored at 4 C for A) 0 h, B) 24 h, C) 48 h and D) 72 h on acrylamide gels of T:12%, C:2.5% concentration.

10



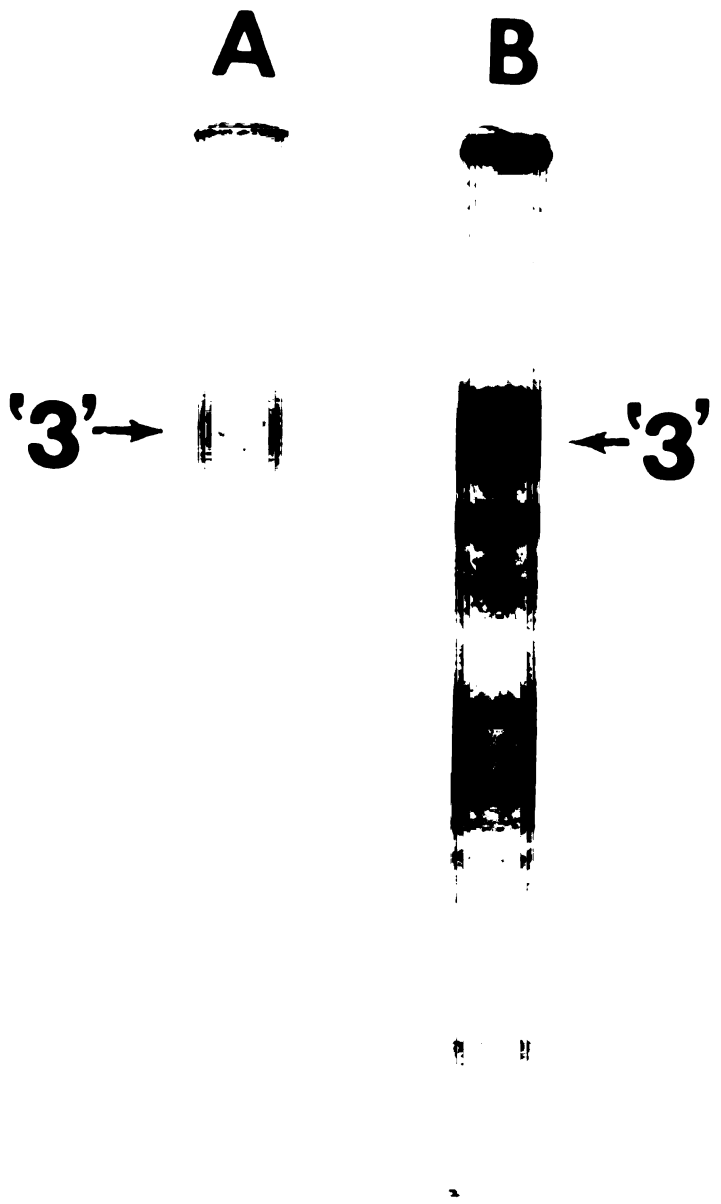


Figure 11. Proteose-peptone samples on polyacrylamide gels stained with A) a fuchsin-sulfite solution to yield fuchsia zones containing glycoprotein and B) Coomassie Brilliant Blue G-250 to yield blue zones containing protein.

Figure 12. Electropherograms and corresponding densitometric traces of casein samples made from milk stored at 4 C for A) 0 h, B) 24 h, C) 48 h and D) 72 h.

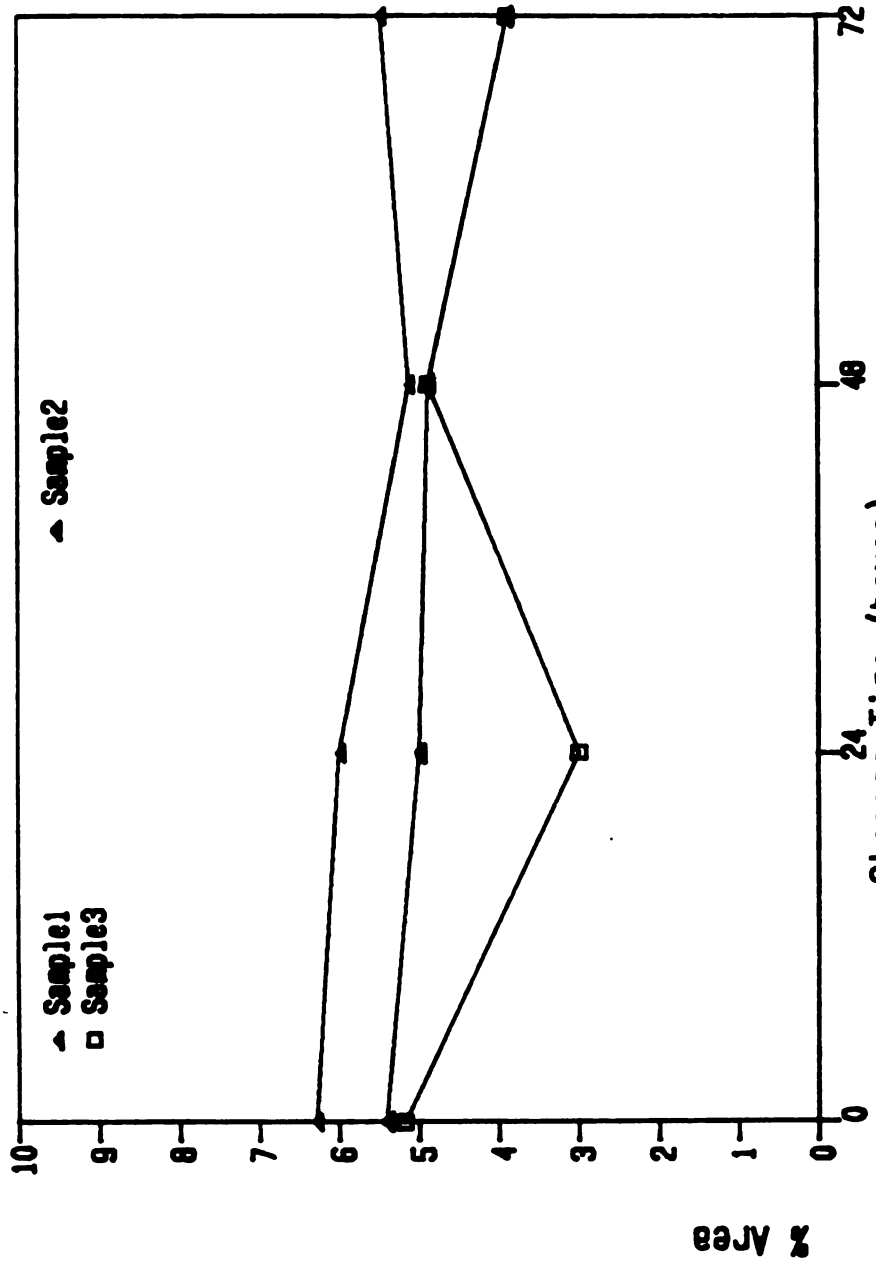


Figure 13. Change in percent area over time of peaks representing protein concentration of γ-CN from densitometric traces of three samples of whole casein made from individual milks stored up to 72 h at 4 C.

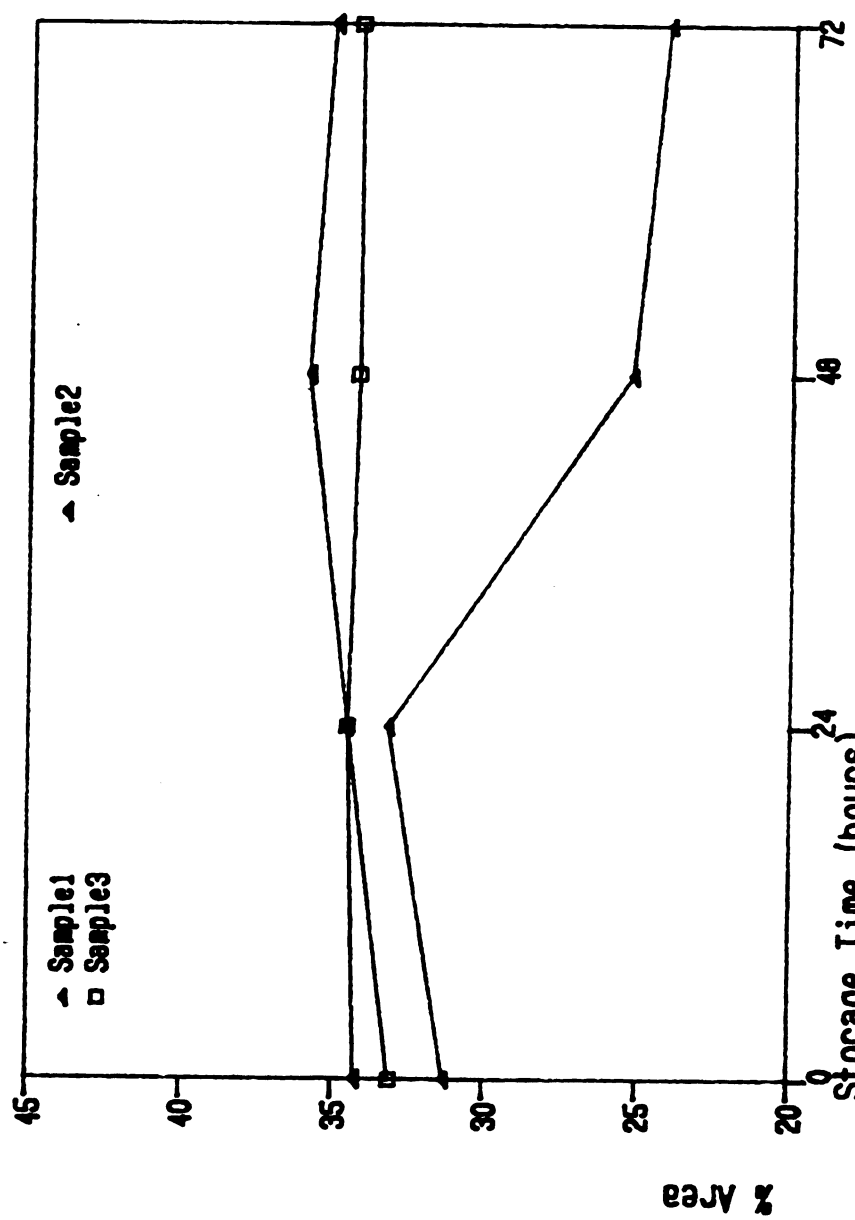


Figure 14. Change in percent area over time of peaks representing protein concentration of β-CN from densitometric traces of the samples of whole casein made from individual milks stored up to 72 h at 4°C.

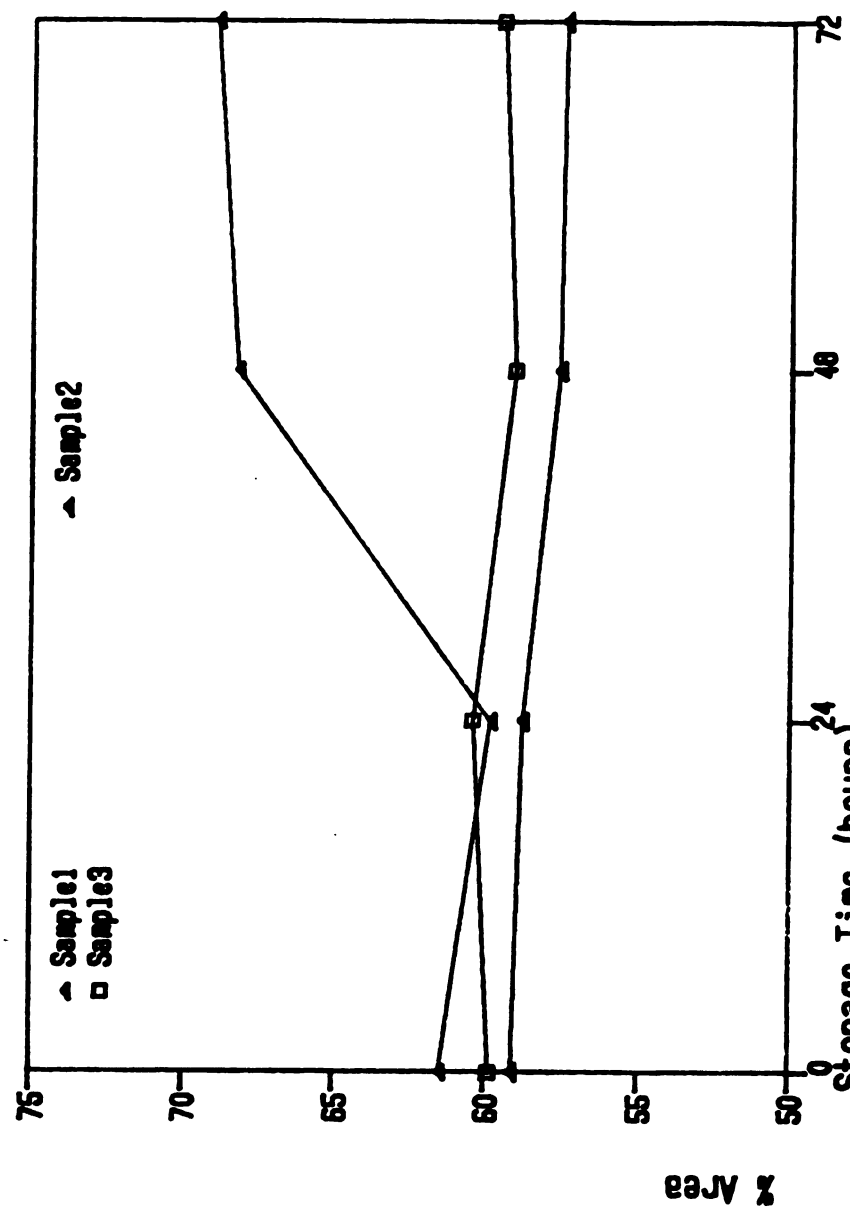


Figure 15. Change in percent area over time of peaks representing protein concentration of α_s-CN from densitometric traces of three samples of whole casein made from individual milks stored up to 72 h at 4°C.

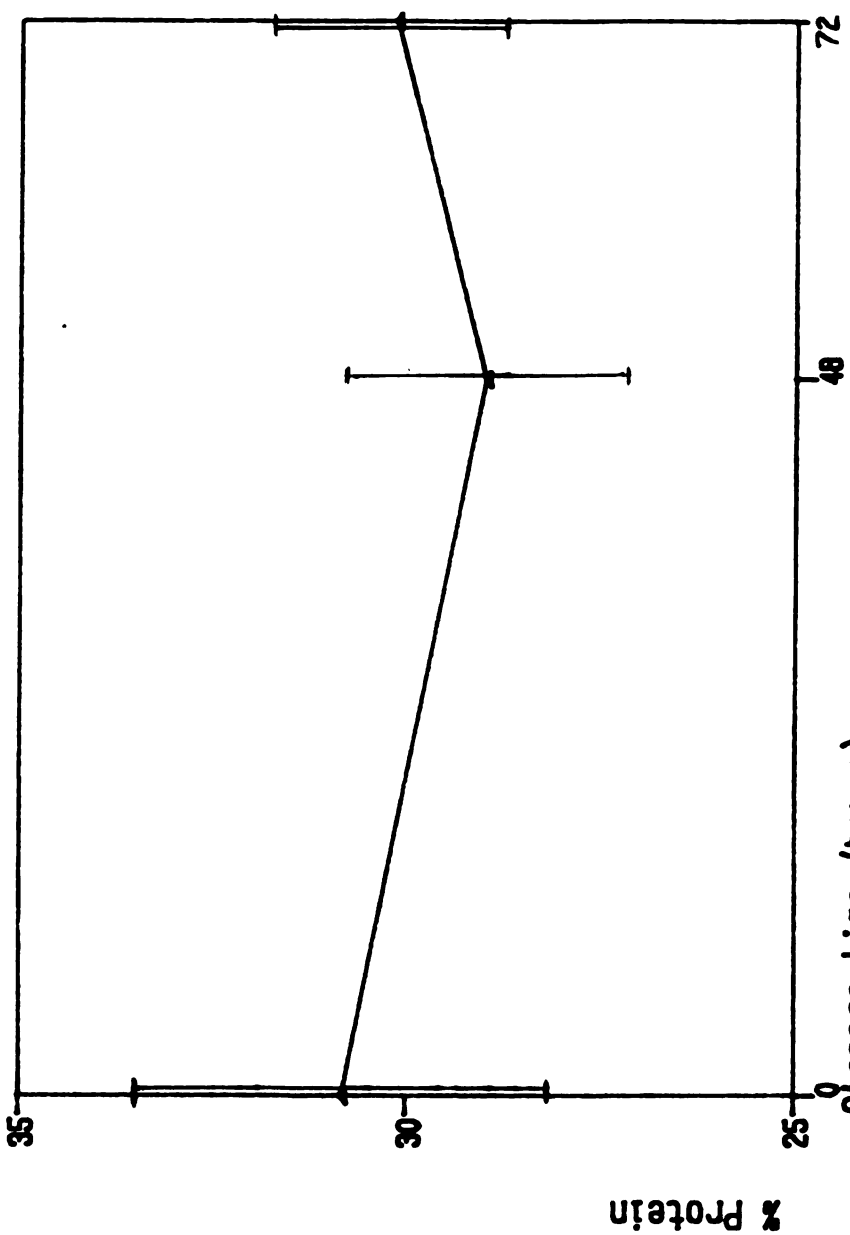
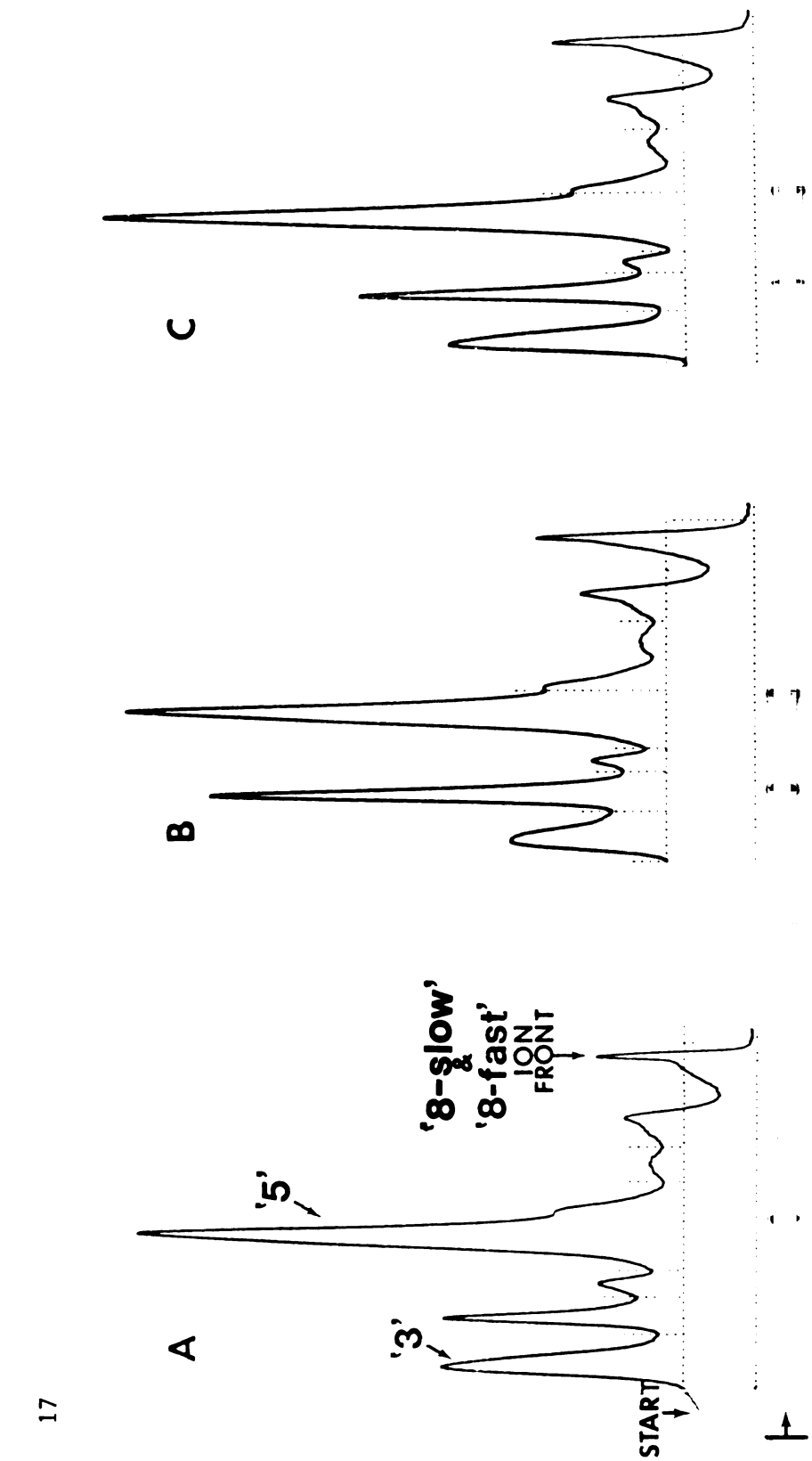


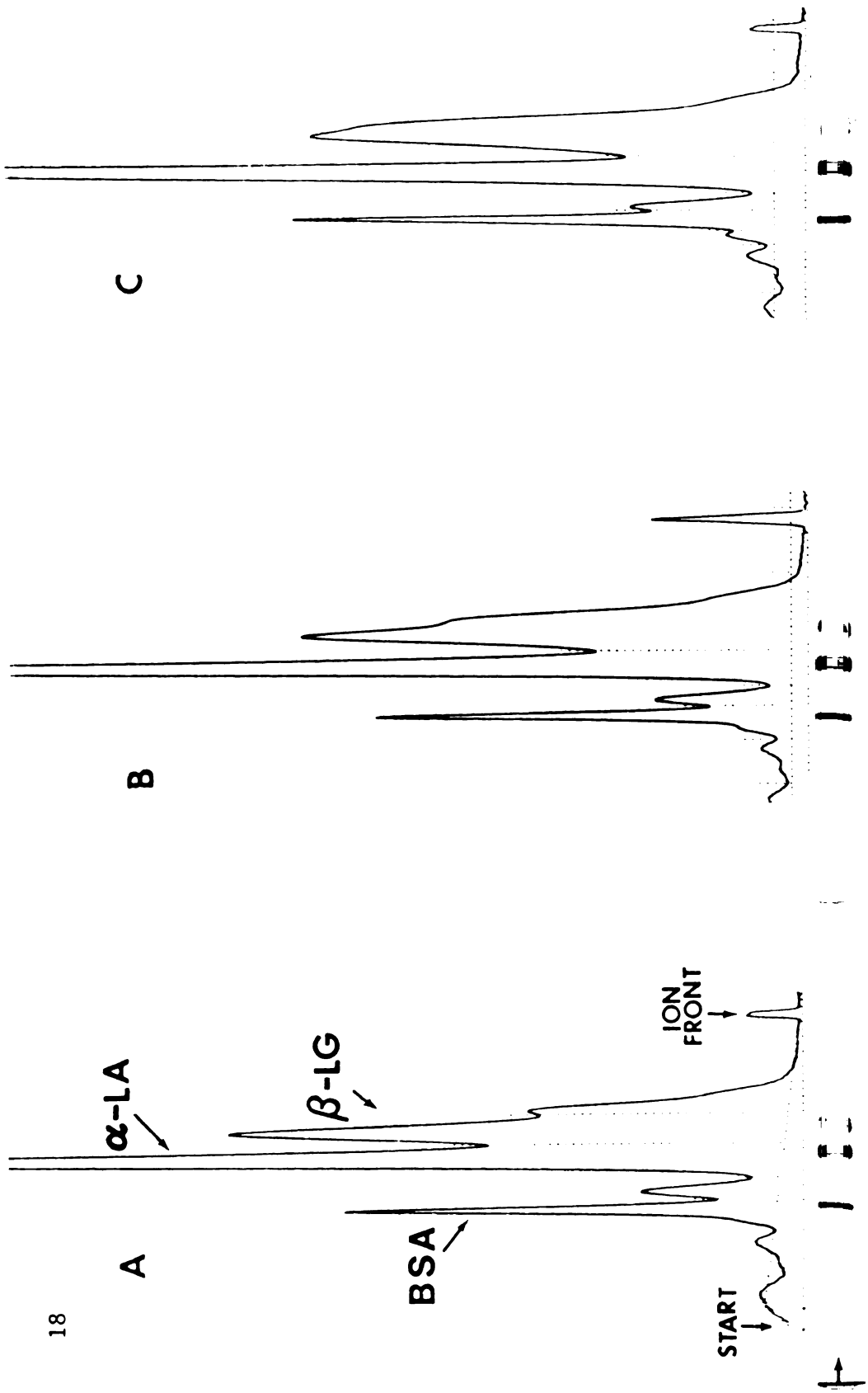
Figure 16. Average percent protein in six experimental cheeses made from milk stored at 4°C for 0, 48 and 72 h.

Figure 17. Electropherograms and corresponding densitometric traces of proteose-peptone samples made from milk used to make experimental cheese, stored at 4 C for A) 0 h, B) 48 h and C) 72 h.



17

Figure 18. Electropherograms and corresponding densitometric traces of whey samples from experimental cheesemaking process using milk stored at 4 C for A) 0 h, B) 48 h and C) 72 h.



18

DISCUSSION

Kjeldahl analysis of the protein fractions in milk showed a rather wide variation in the nitrogen distribution among samples of individual cows. These differences are apparent in Tables 2, 3, and 4 which list the values for total nitrogen, casein nitrogen, and proteose-peptone nitrogen, respectively, of ten milk samples. The data are also expressed as percent of nitrogen value of milk samples stored for 0 h at 4 C to better illustrate the differences in nitrogen distribution. Several authors have reported values for casein nitrogen and non-protein nitrogen as a percentage of total nitrogen (Table 9). Comparable averages of 77.3% for casein nitrogen and 6.1% for non-protein nitrogen were found in this study.

Table 9. Average values of casein nitrogen and non-protein nitrogen reported as a percentage of total nitrogen by several authors.

Casein Nitrogen		Non-Protein Nitrogen		
Individual	Mixed	Individual	Mixed	Source
76.6	78.9	5.5	5.0	Shahani & Sommer (1951b)
76.7	--	--	6.0	a) Davies (1935) b) Davies (1932)
78.7	78.3	--	5.0	Rowland (1938)
76.5	--	--	--	Golding et al. (1932)

The data shown in Tables 2, 3 and 4 indicate no clear trends. Statistical analysis of the nitrogen distributions of each protein fraction showed no significant changes over time ($P<0.05$). This supports the work of Shahani and Sommer (1951a) who analyzed fresh milk samples and samples stored at 0-5 C for 10 d. They found that aging at cold temperatures produced no significant changes in the nitrogen distribution.

Particularly noteworthy is the insignificant change in the proteose-peptone nitrogen fraction. Because the proteose-peptones are formed by limited proteolysis of the β-CN molecule, one would expect an increase in this fraction over time as milk is stored at refrigerated temperatures if proteolytic enzyme activity is high. β-CN dissociates from the casein micelle at low temperatures and becomes accessible to proteolytic enzymes. It appears that storage for up to 72 h at 4 C is not sufficient time for

significant proteolysis to occur.

Statistical analysis of the data collected for curd tension measurements showed no significant changes over time. However, Figure 2 represents graphically the data in which the curd tension generally decreased when milk was stored to 48 h at 4 C, and then increased at 72 h of storage. Ali et al. (1980a) found curd firmness to be reduced as milk was stored up to 72 h at 4 C. The rate of coagulum of chymosin-treated milk has been found to be markedly affected by pH, temperature (Cheryan et al., 1975), the charge on various amino acids (Hill and Craker, 1968), the presence of divalent ions (Ernstrom and Wong, 1974), and interactions of casein with other milk components as a result of processing procedures such as homogenization and heat treatments (Mehaia and Cheryan, 1983).

In their study, Mehaia and Cheryan (1983) concluded that the effect of temperature, pH and calcium concentration on the secondary phase of milk coagulation appeared to favor the concept that the physical aggregation is primarily a charge neutralization process. They also hypothesized that calcium ions do more than merely contribute to the charge neutralization process by actually participating in the coagulation phase during gel formation, perhaps in the form of calcium "bridges" between aggregating micelles. Increasing the concentration of free Ca^{2+} that can participate in the aggregation would hasten the formation of a coagulum.

Ali et al. (1980a) found caseins and calcium in the soluble phase to increase upon storage of milk for 48 h at 4 C after which time a decrease was observed (see Table 10). These workers found cheese yields to follow the same pattern up to 72 h of milk storage at 4 C.

The decrease in soluble caseins and Ca^{2+} after 48 h corresponded to the increase in curd tension reported in Figure 2. Perhaps both the soluble caseins and Ca^{2+} migrate back onto the micelle at this point, and the Ca^{2+} forms "bridges" between aggregating micelles, as hypothesized by Mehaia and Cheryan (1983), to facilitate the formation of a firmer coagulum. After 72 h the firmness might decrease due to an increasing incidence of proteolysis by indigenous or bacterial proteolytic enzymes.

Table 10. Casein and Ca^{2+} concentrations in the soluble phase of bulk milks stored at 4 C for up to 7 d (Ali et al., 1980a)

Storage days	α_{s1}-CN mg/ml	β-CN mg/ml	κ-CN mg/ml	Ca^{2+} ppm
0	1.66	1.32	0.87	430
1	1.73	1.56	0.96	485
2	1.80	1.81	0.98	528
3	1.68	1.51	0.96	506
4	1.71	1.67	0.90	519
5	1.66	1.53	0.88	489
6	1.74	1.59	0.93	492
7	1.72	1.56	0.86	487

Cold storage of milk up to 72 h did not appear to affect the casein matrix of rennet curd such that a significant change in casein yield was observed ($P<0.05$). Differences in yield over the 72 h period were never greater than 5% or 0.3 g. Figure 3 does show, however, that there was a general trend toward a slight decrease in the casein yield up to 72 h of milk storage.

When milk is warmed for the purpose of making a rennet curd, caseins that have dissociated from the micelle during cold storage will migrate back onto the micelle. The decrease in casein yield might be explained by examining the data of Ali et al. (1980a) shown in Table 10. The values of total soluble casein (α_{s1}-CN + β-CN + κ-CN) con-

centration were 3.85 mg/ml, 4.25 mg/ml, 4.59 mg/ml, and 4.15 mg/ml for milk storage at 4 C for 0, 24, 48, and 72 h respectively. The total concentration of soluble casein increased up to 48 h and then decreased slightly, but it did not return to levels observed in fresh milk. Therefore, because more casein remained in the soluble phase after storage, a general decrease in casein yield of a rennet curd might be expected. The data depicted in Figure 3 reflect neither the slight decrease in soluble casein found by Ali et al. (1980a) nor the increase in curd tension of firmness shown in Figure 2 after the 48 h storage period. However, the differences in the final weights of the casein samples are too small to illustrate a relationship. Larger scale production of rennet curd might give casein yields which would show more clearly any differences caused by cold milk storage over time.

Study of the changes in the proteose-peptone fraction should illustrate the hypothesis, stated at the beginning of this study; namely, that when milk, stored at 4 C for up to 48 h, is warmed for cheesemaking, caseins in the soluble phase migrate back onto the micelle in a different configuration obscuring cleavage sites on κ-CN. Significant proteolysis of caseins would not have occurred during this storage period. After 48 h of milk storage, proteolysis of β-CN would result in fewer molecules moving back onto the micelle. Then more cleavage sites on κ-CN would again be available to aid in formation of a firmer curd and give

an increased yield. If this were the case there would also be an increase in the concentration of the γ-caseins and proteose-peptone components in milk after a 48 h storage period.

Electrophoresis, on T:8.0%, C:2.5% acrylamide gels, of the proteose-peptone samples produced several protein bands. The corresponding densitometric traces showed five major peaks. Figure 4 depicts the electropherograms and identifies the major protein components. Staining of glycoproteins of the proteose-peptone sample with a fuchsin-sulfite solution following electrophoresis showed one principal protein band to contain carbohydrate, Figure 11. Comparison of the fuchsin-sulfite stained gel to one stained with Coomassie Brilliant Blue G-250 revealed that the carbohydrate-containing zone corresponded to the slow moving protein zone of the proteose-peptone sample. Kang (1971) identified the slow moving zone of a sample of proteose-peptone on a polyacrylamide gel in a discontinuous system as proteose-peptone component 3. This and the evidence indicating that component 3 is identical with a soluble glycoprotein prepared from the milk fat-globule membrane (Kanno and Yamauchi, 1979) would lead to the identification of the fuchsia stained band in Figure 11 as component 3. Thus, the first peak on the densitometric traces in Figure 4 was identified as component 3.

A graphic representation of changes in the protein concentration over time of component 3 in three milk

samples is shown in Figure 5. There was an increase in this protein fraction following 72 h of storage at 4 C.

Although it has been observed that component 3 is a constituent of both the proteose-peptone fraction and the fat-globule membrane, its origin has only been speculated. If it is a plasmin-derived fragment of an MFGM polypeptide, as suggested by Eigel (1981), then the increase of this component after 72 h of cold milk storage seen in Figure 5 could be attributed to increasing proteolytic activity on the membrane proteins during the storage period. Kester and Brunner (1982) favored the possibility that component 3 represented a loosely bound membrane component which is desorbed partially into the aqueous phase following secretion. This being the case, an increase in the protein component during cold storage of milk might be caused by further desorption of the component from the membrane as storage time increases.

The second major peak of the densitometric traces in Figure 4 is an unidentified proteinaceous component. As can be seen from the electropherograms and densitometric traces of Figure 4, the protein concentration of this zone increased significantly up to 48 h of milk storage and then decreased. This trend is illustrated in Figure 6. The gel stained for glycoprotein, as recorded in Figure 11, shows this protein to contain a small amount of carbohydrate. It is apparent that cold storage of milk has an effect on this component, however further investigation is necessary to

characterize the protein.

Figure 7 shows a general increase of component 5 [β-CN-5P (f1-105/107)] in three milk samples over the 72 h cold storage period, which was determined to be statistically significant ($P<0.05$). This increase might be caused by increasing proteolysis of the β-CN molecule over time, which would result in greater concentrations of component 5, component 8-fast [β-CN-4P (f1-28)], component 8-slow [β-CN-1P (f29-105/107)] and γ-caseins. The increasing incidence of component 5 would support the hypothesis proposed. The gel stained for glycoprotein, depicted in Figure 11, reveals the presence of a small amount on carbohydrate in the protein zone.

Components 8-fast and 8-slow (both heterogeneous fractions) are not resolved well by discontinuous polyacrylamide gel electrophoresis. These two components migrate as a single zone with the ion front (Kolar and Brunner, 1970). Figure 8 shows a graphic representation of changes in the fourth major protein component and peak depicted in the electropherograms and densitometric traces of Figure 4. The changes do not appear to follow any clear trends, and the numbers are such that no real significance can be attributed to any differences ($P<0.05$). This protein zone might be component 8-slow, however it is more likely that component 8-slow traveled with component 8-fast at the ion front. Component 8-slow was believed to represent β-CN residue 29-105/107, but Andrews and

Alichanidis (1983) found evidence that this protein consists of components not derived from β-CN. The actual 29-105/107 peptide does not seem to be a prominent proteose-peptone component in fresh milk.

The fifth major peak depicted in the densitometric traces of Figure 4 is most likely components 8-fast and 8-slow because this zone traveled with the ion front of the gels. Figure 9 is a graphic representation of the changes over time in protein concentration of three samples of this fraction stored at 4 C. There was a slight increase up to 24 h and then a decrease at 72 h seen in all three samples, however these changes were non-significant ($P<0.05$). These results do not follow the pattern of increasing concentration of component 5, although one would speculate that they should. The reasons for these results are not clear.

The proteose-peptone samples were also resolved on acrylamide gels of concentration T:12%, C:2.5%. A typical representation of changes in the proteins of the samples over time after milk storage at 4 C are shown in Figure 10. All protein components followed the same patterns as on the T:8%, C:2.5% acrylamide gels discussed earlier. There was, however, no protein zone traveling with the ion front, as seen in Figure 10.

Kasper (1978) found proteose-peptone component 8 to appear in 7.5% polyacrylamide gels in a discontinuous system as an intense, sharp zone moving with the tracking dye, its mobility unimpeded by the gel matrix. When the

gel concentration was increased to 10.0% and 12.5%, zones that were moving unimpeded with the tracking dye in the 7.5% gels, appeared to have been hindered in their migration by the decreased pore size of the gel matrix. The gels depicted in Figure 10 had no protein zone migrating with the ion front. Thus the fourth peak of the corresponding densograms probably represents components 8-fast and 8-slow.

Figure 12 represents changes, illustrated by electropherograms and densitometric traces, in the casein fraction of milk samples stored at 4 C for up to 72 h. Electrophoretic patterns of casein obtained in this study are compared in a schematic representation to those obtained with a discontinuous gel method by Melachouris (1969) and Eigel (1977b) in Figure 19.

Eigel (1977b) identified the γ-caseins using disc PAGE. He found γ_1 - and γ_2-casein to have lower mobilities than β-CN. The first, smaller peak shown in the densograms of Figure 12 corresponds to a very faint protein zone on the acrylamide gels. This zone probably represents one of the γ-caseins. Figure 19A shows a schematic representation of a β-CN sample that had been incubated at 37 C with bovine plasmin for 30 sec (Eigel, 1977b). Upon comparison to gels obtained in this study (schematic representation in Figure 19B) one might conclude that the faint protein zone is one of the γ-caseins. Figure 13 represents graphically the changes in area percentage values of this zone with

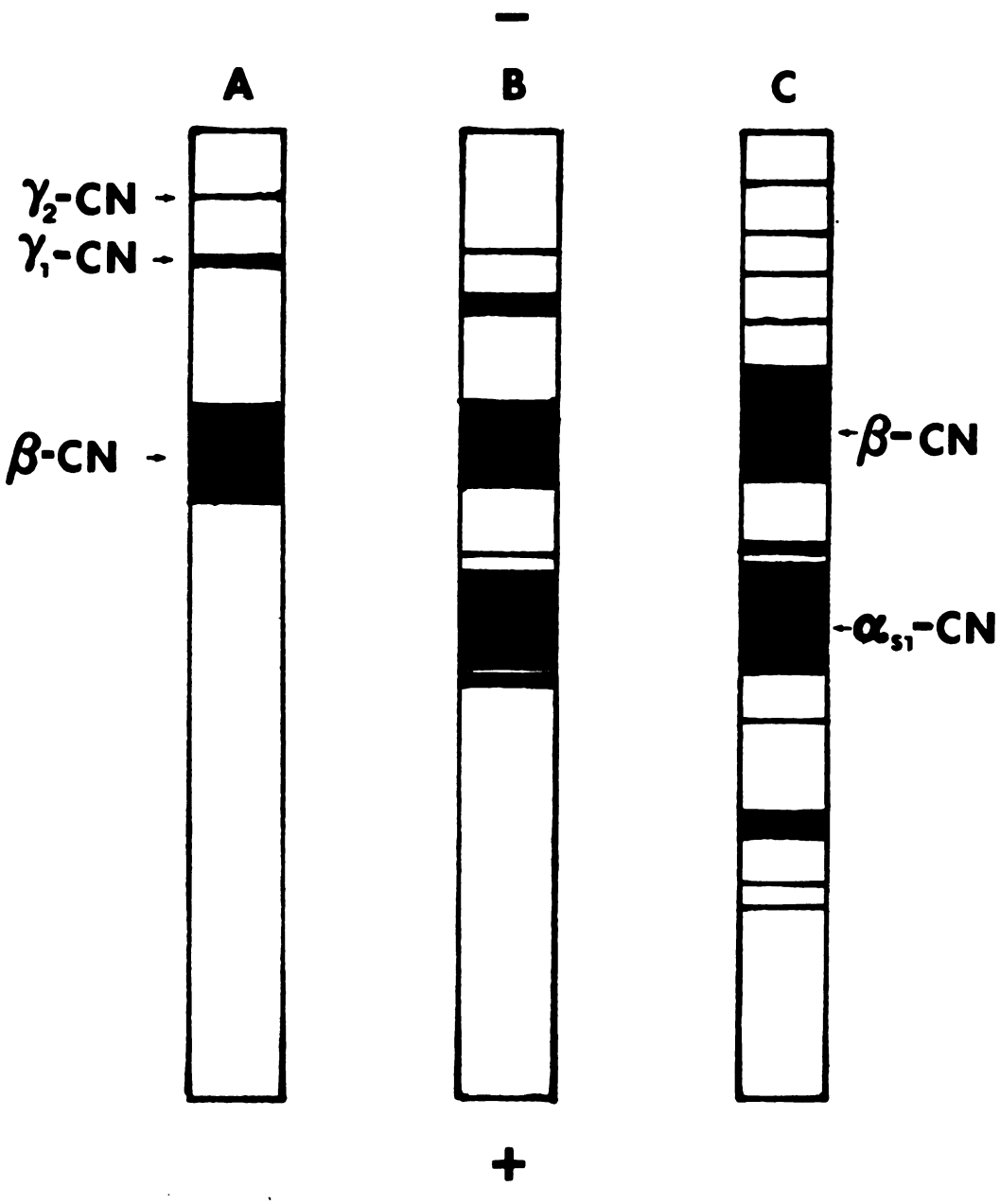


Figure 19. Schematic representations of casein samples on polyacrylamide gels in a discontinuous system obtained A) by Eigel (1977b), B) in this study and C) by Melachouris (1969).

time. There appears to be a general, non-significant ($P<0.05$) decrease in the area under the peaks. These results do not support the hypothesis proposed, in which the γ-caseins would increase over time, particularly after 48 h of cold milk storage. The reason for the decrease is not clear.

The changes in area percentage values of β-CN are represented graphically in Figure 14. Overall, the treatment effect was not significant ($P<0.05$). Samples 2 and 3 changed very little, while sample 1 decreased after 24 h of milk storage. The decrease of β-CN in sample 1 might be due to increased proteolytic activity. The psychrotrophic counts of sample 1 from Table 6 do not reflect high bacterial activity, thus, it is possible that there was increased plasmin activity.

The decrease of β-CN concentration in sample 1 corresponds to an increase in component 5 of sample 1 as shown in Figure 7. Therefore, plasmin activity could very well have been at a higher level in sample 1 than samples 2 and 3. However, the increase in component 5 of sample 1 was approximately equal to that of sample 3.

Figure 15 depicts a graphic representation of the changes in the area percentages of α_s-casein shown in the densitometric traces of Figure 12. Again samples 2 and 3 changed very little while sample 1 increased after 24 h of milk storage. The reason for the change observed in sample 1 was not determined. If the hypothesis upon which this

study is based is valid, one would expect the β-CN fraction to decrease while the proteose-peptone and γ-casein fractions increased. The α_s-CN fraction would also be decreased by proteolysis, but at a slower rate than β-CN. While component 5 was found to increase, there was no significant change in either the β-CN or γ-casein fractions.

The individual standard plate counts for the milk samples used in the electrophoretic assays are listed in Table 6. Mesophilic counts never reached more than 2×10^5 cfu/ml and psychrotrophic counts were never higher than 1000 cfu/ml. Law et al. (1979) found growth of proteolytic psychrotrophs in cold stored milks to approximately 10^6 and 10^7 cfu/ml, in 24 and 72 h respectively, to cause very little casein breakdown. Ali et al. (1980a) determined that the growth of psychrotrophs to levels of 5×10^5 cfu/ml in milk stored up to 72 h at 4 C had very little apparent effect on cheese yield or quality. Hicks et al. (1986) concluded that normally, yield losses have been significant when total plate counts or psychrotrophic count reaches 10^6 cfu/ml prior to pasteurization. Therefore, it is not probable that the growth of proteolytic psychrotrophs to the levels illustrated in Table 6 would have a significant effect on the caseins or any cheese-making parameters. Cold storage of the milk samples would halt the growth of mesophilic organisms, therefore these would not become a problem either.

Figure 16 illustrates the average percent of protein in experimental cheeses based on average final weights. The percentage of protein ranged from 30.8% for fresh milk to 29.0% for 48 h old milk to 30.2% for 72 h old milk. This change in protein percentage could be correlated to a change in cheese yield since the caseins are the proteins which form the curd when milk coagulates. The differences in protein percentage were not significant on an experimental level ($P<0.05$), but on a larger scale they would be enhanced. The slight increase in percent of protein correlates with the findings of Ali et al. (1980a) who found an increased cheese yield for milk stored 72 h and Hicks et al. (1986) who found a slight, non-significant increase in cheese yield for milk stored 24 to 36 h. While Ali and his co-workers felt this phenomenon was due to a temperature influenced change in casein solubility (particularly β-CN) during the initial storage period, Hicks et al. concluded that storage of high quality milk, where initial psychrotrophic counts compose 10 to 20% of the total counts, results in a gradual decrease in pH, overcoming the temperature effect and possibly causing the slight increase in yield.

Psychrotrophic counts in the cheese milk used for this study did not exceed 10 cfu/ml after 72 h of cold milk storage at 4 C. Therefore the reasoning of Hicks and his co-workers would not apply here, but results seen in Figure 16 support the conclusions of Ali et al. (1980a) and the

hypothesis stated in this study.

The protein zones on gels of T:8.0%, C:2.5% acrylamide concentration of the proteose-peptone samples made from the cheese milk follow the same trends as those of the proteose-peptone samples discussed earlier (see Figure 17). Changes in peak area percentages are listed in Table 7. Concentration of component 3 decreased at 48 h and increased at 72 h. The unknown, interstitial protein zone between component 3 and component 5 increased in concentration at 48 h and decreased at 72 h. An increase in concentration of component 5 was seen in all electrophoretic assays. The protein zone between component 5 and the ion front as well as the component traveling with the ion front (most likely components 8-fast and 8-slow) increased slightly at 48 h and then decreased at 72 h.

The overall increase of component 5 seen in all samples would indicate that there was proteolysis of the β-CN molecule, most likely by plasmin. The increase of components 8-fast and 8-slow at 24 to 48 h seen in Figures 9 and 16 supports this conclusion; however, the reason for the decrease at 72 h is not clear. An increase in the concentration of component 5 gives credibility to the hypothesis. The concentration of β-CN did not change significantly, however,the proteose-peptones represent proteolytic fragments of such a small portion of the β-CN fraction that increasing levels of component 5 might not be reflected in small differences in β-CN.

Electrophoretic patterns of milk whey proteins obtained on polyacrylamide gels in a discontinuous system are compared in a schematic representation to those obtained by Melachouris (1969) in Figure 20. The protein zones can thus be identified on the electropherograms and densitometric traces in Figure 17. Area percentages of each peak for the principal whey proteins at milk storage times of 0, 48, and 72 h at 4 C are listed in Table 8. No significant differences were observed ($P<0.05$).

One must realize that there is the possibility of a significant source of error involved in the results reported by the densitometric traces. Error could be introduced when the amount of protein of each sample is being determined, when the sample is applied to the gel, in the staining/destaining procedure of the gel, and in the scanning measurement of dye retention in the gel.

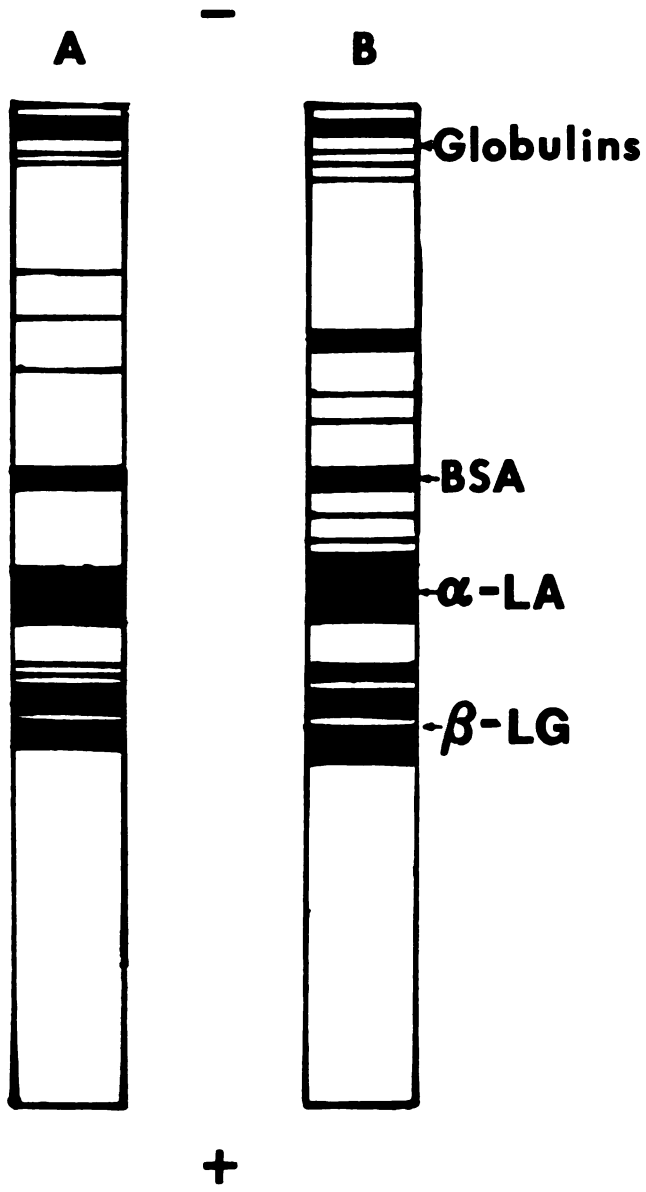


Figure 20. Schematic representations of whey samples on polyacrylamide gels in a discontinuous system obtained A) in this study and B) by Melachouris (1969).

CONCLUSION

Experimental evidence presented in this study shows some support of the partial hydrolysis of β-CN hypothesis. It is not conclusive, however, because the increase of proteose-peptone component 5 was not accompanied by an increase in components 8-fast, 8-slow and γ-caseins, as would be expected. Further studies are necessary to learn more about the changes which occur in the proteins of milk stored at cold temperatures. Monitoring changes in soluble β- and γ-caseins along with the proteose-peptones might give more solid data to support or refute the hypothesis. Centrifugation of milk samples at the temperature of storage to remove fat might provide a means for obtaining less ambiguous results. The unidentified protein in proteose-peptone samples, which appeared on polyacrylamide gels in the region between components 3 and 5, should be studied, as it appears to change significantly during cold milk storage. Finally, in performing a study of this nature, the breed of cow, stage of lactation, and genetic variant of certain protein components should be carefully controlled since these factors have been found to affect plasmin activity and the properties of some milk constituents.

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BIBLIOGRAPHY

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