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HEAT-INDUCED WHEY PROTEIN REACTIONS IN MILK

KINETICS OF DENATURATION AND AGGREGATION AS RELATED TO MILK POWDER MANUFACTURE

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN FOOD TECHNOLOGY

BY

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ABSTRACT

The objective of this study was to gain a better understanding of the heat-induced whey protein reactions that occur during the manufacture of milk powders. Attention was focused on the preheating step, because most of the whey protein reactions that affect powder properties occur during this step.

Skim milk was heated at a range of temperatures (70 to 130°C) and times (5 s to 1800 s), normally used in powder manufacture, using a pilot-scale UHT plant equipped with direct steam injection. The temperature and time conditions were characterized by residence time distribution analysis. After heating the milk samples were analyzed by quantitative polyacrylamide gel electrophoresis under non-dissociating and dissociating conditions.

Using reaction progress data (reactant concentration versus time) determined over a range of temperatures, apparent reaction orders, reaction rates and Arrhenius parameters were determined by non-linear regression. This one-step approach gave Arrhenius parameters of considerably higher precision than the commonly used alternative of first determining the rate constants and then the Arrhenius parameters from the temperature dependence of those constants. Kinetic parameters were calculated for β-lactoglobulin A, β-lactoglobulin B, α-lactalbumin, immunoglobulin G and bovine serum albumin. Reaction orders for β-lactoglobulin varied from 1.0 to 1.6, while values for α-lactalbumin were in the range 0.9 to 1.1. The denaturation of Immunoglobulin G could be described by a 2nd order reaction, whereas bovine serum albumin followed a reaction order of 2.8. There was a marked change in activation energy for β-lactoglobulin at 90°C (51.18 to 301.73 kJ mol⁻¹) and α-lactalbumin at 80°C (52.87 to 203.26 kJ mol⁻¹). No such change was observed for Immunoglobulin G and bovine serum albumin over the temperature range 70-90°C. At temperatures <80°C the rates for β-lactoglobulin and α-lactalbumin denaturation were similar, but at higher temperatures α-lactalbumin denatured at a slower rate than β-lactoglobulin.
The aggregation of \( \beta \)-lactoglobulin mainly involved the formation of disulphide-linkages, whereas \( \alpha \)-lactalbumin aggregates were formed through both hydrophobic interactions and disulphide-linkages. The kinetics of \( \beta \)-lactoglobulin aggregate formation followed an Arrhenius relationship similar to \( \beta \)-lactoglobulin denaturation, with comparable values of reaction orders, activation energies, and reaction rates.

The rates of \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin association with the casein micelles were slower than the corresponding rates for denaturation and aggregation. At temperatures >80°C \( \beta \)-lactoglobulin associated at a faster rate than \( \alpha \)-lactalbumin, but <80°C the rates of association were similar. Under all heating conditions only a portion (=55%) of the denatured \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin associated with the casein micelles; the rest remained in the serum as aggregates.

Based on the interrelationships between denaturation, aggregation and association behaviour of \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin a novel mechanism was proposed and a mathematical model was developed. This model could accurately predict the formation of \( \beta \)-lactoglobulin aggregates and their subsequent association with the casein micelles.

The extent of whey protein denaturation, aggregation and association in milk was affected by compositional factors, such as pH, whey protein concentration and total solids content. Increasing the pH of milk from 6.48 to 6.83 prior to heating had little effect on \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin denaturation and aggregation, but greatly decreased their association with the casein micelles. When the whey protein concentration in milk was increased from 0.52 to 1.24 g/100 g there was a marked increase in the extent of denaturation, aggregation and association of \( \alpha \)-lactalbumin with the casein micelles, the effect being less marked on \( \beta \)-lactoglobulin. As the total solids content increased from 6% to 13% the extent of \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin reactions increased. Examination of whey protein reactions in milks obtained during the New Zealand dairying season, showed that the extent of denaturation, aggregation and association was greater in late season milk. This increase was possibly caused by the increased whey protein and \( \kappa \)-casein concentrations.
Preliminary studies were carried out on the evaporation and spray drying processing steps. Little further denaturation and aggregation of whey proteins occurred during the evaporation and spray drying steps, while the association of whey proteins with the casein micelles increased slightly during evaporation. However both these processing steps caused considerable changes in soluble minerals and calcium ion activities.
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CHAPTER 1: INTRODUCTION

Most of the milk produced in New Zealand is manufactured into dairy products destined for export (e.g. milk powders, cheese, butter). The export of milk powders from New Zealand amounted to 378,000 tonnes in 1992 (IDF, 1993), and of the total industry returns, approximately half were from milk powder sales. Thus, milk powder forms an important part of the industry revenues in New Zealand.

The manufacturing process for skim milk powder consists of four distinct stages; (a) separation of raw milk and pasteurization (72°C for 15 s) of the skim milk, (b) heat treatment of skim milk, (c) concentration of preheated skim milk to about 45% total solids content by evaporation and (d) spray drying of the concentrate to remove most of the remaining water.

Heat treatment of skim milk prior to concentration, usually known as preheating, is the most important processing step in the manufacture of milk powders. Many of the functional properties of powders, are determined by the preheating conditions used. These conditions vary widely from normal pasteurization (72°C for 15 s), through low temperature long time (e.g. 85°C for 30 min) to high temperature short time (e.g. 120°C for 2 min) treatments. The commonly used preheating conditions for the manufacture of milk powders cause denaturation of whey proteins and complex formation between β-lactoglobulin and κ-casein. Although denaturation reactions have been reported extensively, relatively little is known about the kinetics and reaction mechanisms involved in β-lactoglobulin/κ-casein complex formation in heated milk. Preheating also causes change in calcium phosphate equilibria and a shift in the ionic composition of the milk serum.
In addition to concentration, evaporation causes other changes in the milk system. These include a decrease in the pH of milk, and an increase in micelle size due to an increase in colloidal calcium phosphate or coalescence of the micelles. Further more heat sensitivity of whey proteins and casein micelles may possibly change with increasing concentration of milk solids. Unfortunately, little information is available on the effects of heat on protein and minerals during evaporation of milks, concentrated up to 45% total solids content.

During spray drying the changes in protein denaturation and aggregation are likely to be dependent on the temperature of the air into which the milk is sprayed (inlet air temperature), degree of concentration, temperature of concentrate prior to drying, size of drying droplets and the temperature of the air/powder mixture exiting the drier (outlet air temperature). The effects of these parameters on the changes in proteins and minerals are not well understood.

In New Zealand milk production is seasonal and this causes marked changes in the composition of milk and the functional properties of powders. For example, the absolute concentration of protein is high at the beginning and end of the season and this causes difficulties with the production of a high quality product. It is probable that some of the difficulties result from the high levels of whey proteins in the milk and their altered sensitivity to heat treatment. Little is known about the effect of seasonal changes in milk composition on whey protein interactions and subsequent milk powder functionality.

Artificial modification of various milk components has been shown in some instances to influence the functional and physico-chemical properties of milk, e.g. heat stability and rennet clotting times. However further knowledge is required before this relationship can be fully exploited by the milk powder industry to improve the functionality of milk powders.

Industry uses an empirical approach, based on experience and tradition, to combat problems associated with variations in milk composition and processing conditions,
because knowledge is incomplete. Thus, the objectives of this study were to obtain a better understanding of the protein and mineral changes that occur during the heating of milk and to investigate the effect of seasonal and artificial modification of milk composition on these changes. This knowledge should provide answers to some of the problems currently encountered in skim milk powder manufacture.
CHAPTER 2: LITERATURE REVIEW

2.1 COMPOSITION OF MILK

The main components of milk are fat, lactose, proteins and minerals. These contribute to the ~14% total solids content in milk. The general composition of raw milk is shown in Table 2.1. The fat component of milk is mainly triglycerides (98%), present as an emulsion of fat globules stabilised by a phospholipid and glycoprotein membrane. The minerals of milk occur either in solution or are associated with the proteins, as either undissolved salts or bound ions. Lactose is a soluble carbohydrate molecule, a disaccharide of glucose and galactose.

Table 2.1 Typical raw milk composition.

<table>
<thead>
<tr>
<th>Component</th>
<th>Level in milk % (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87.3</td>
</tr>
<tr>
<td>Fat</td>
<td>3.9</td>
</tr>
<tr>
<td>Protein</td>
<td>3.25</td>
</tr>
<tr>
<td>Casein protein</td>
<td>2.6</td>
</tr>
<tr>
<td>Whey protein</td>
<td>0.75</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.6</td>
</tr>
<tr>
<td>Minerals</td>
<td>0.65</td>
</tr>
<tr>
<td>Organic acids</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*From Walstra and Jenness (1984)*

The protein content of milk, ~3.5% (w/w), can be divided into whey and casein. Protein precipitated at 20°C from milk adjusted to pH 4.6 is called casein, and comprises ~80% of the total protein. Casein exists mainly as spherical colloidal structures known as
micelles. The remaining proteins which remain soluble at pH 4.6 are called the whey or serum proteins.

2.1.1 Caseins

Casein proteins can be fractionated into four distinct groups; $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$- and $\kappa$-casein. The properties of casein proteins have been reviewed by Whitney (1988), Rollema (1992) and Swaisgood (1992) (Table 2.2). The casein proteins are amphipathic as they contain both polar and non-polar regions. Casein proteins appear to have some secondary structure as determined by spectral methods and various predictive methods, and a definite unordered tertiary structure (Swaisgood, 1992). $\kappa$-Casein is estimated to contain 10-20% $\alpha$-helix, 20-30% $\beta$-structure and 15-25% $\beta$-turns (Swaisgood, 1992). $\alpha_{s1}$-, $\alpha_{s2}$- and $\beta$-caseins appear to have more $\alpha$-helix and less $\beta$-structure, than $\kappa$-casein, particularly $\alpha_{s1}$- and $\alpha_{s2}$-caseins (Swaisgood, 1992). Holt and Sawyer (1988) showed that the conserved predicted helix content of $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$- and $\kappa$-casein are 24%, 17% and 13% and 3%, respectively. Approximately 5-17% of casein protein residues are proline, and this has a marked influence on the protein structure, as proline is often found in the bends of folded protein chains. Proline residues are occasionally found in both $\alpha$-helix and $\beta$-structures, but are generally not favourable to either (Richardson et al., 1992). The tertiary structure of the caseins has been investigated by three-dimensional modelling, based on the secondary structure developed from spectroscopic data (Richardson et al., 1992).

Caseins undergo post-translational phosphorylation, glycosylation and proteolysis. The seryl and occasionally threonyl residues of all caseins are phosphorylated to varying degrees. Glycosylation of $\kappa$-casein occurs mainly in the polar domain and the availability of threonyl and seryl glycosylation sites are determined by the secondary and/or tertiary structures, as none of the threonyl residues in $\alpha_{s1}$-, $\alpha_{s2}$- and $\beta$-casein are modified (Swaisgood, 1992). $\beta$-Casein is particularly susceptible to proteolysis by plasmin, releasing $\gamma$-caseins and proteose peptones. Chymosin is noted for its specific hydrolysis of the Phe-Met bond which splits $\kappa$-casein into its hydrophobic and hydrophilic domains (Swaisgood, 1992).
Table 2.2 Properties of casein proteins.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Casein protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_1$-casein</td>
</tr>
<tr>
<td>Concentration in milk (g.l$^{-1}$)</td>
<td>12-15</td>
</tr>
<tr>
<td>Molecular weight (Da)</td>
<td>23,614</td>
</tr>
<tr>
<td>Genetic variants</td>
<td>A,B,C,D</td>
</tr>
<tr>
<td>Phosphoseryl residues</td>
<td>8,9</td>
</tr>
<tr>
<td>Pro</td>
<td>17</td>
</tr>
<tr>
<td>Cys</td>
<td>0</td>
</tr>
<tr>
<td>Hydrophobicity (kJ/residue)</td>
<td>4.9</td>
</tr>
<tr>
<td>Distribution of protein in the micellar phase (%)</td>
<td>94</td>
</tr>
<tr>
<td>Net charge at pH 6.6</td>
<td>-21.0 to -23.5</td>
</tr>
</tbody>
</table>


The binding of Ca$^{2+}$ to caseins is mainly through the phosphoseryl residues. Binding capacity follows the order $\alpha_2$- > $\alpha_1$- > $\beta$-casein, where Ca$^{2+}$-induced precipitation starts in the range of 2 mM to 15 mM of Ca$^{2+}$. Even at Ca$^{2+}$ concentrations less than 2 mM $\alpha_2$-casein begins to precipitate (Toma & Nakai, 1973), and at concentrations of 3-8 mM $\alpha_1$-casein precipitates. At 1°C, $\beta$-casein remains soluble in Ca$^{2+}$ concentrations up to 400 mM, but precipitates in the range of 8-15 mM Ca$^{2+}$ at 37°C (Farrell et al., 1988). $\kappa$-Casein remains relatively soluble due to the low number of phosphoseryl residues (Toma & Nakai, 1973).
Self-association of caseins is mainly through electrostatic and hydrophobic interactions. \( \alpha_{\text{s1}} \)-Casein association is initiated by increasing ionic strength; at low ionic strength (0.003-0.01 M) and neutral pH the protein exists as a monomer. The association decreases as the pH increases, and temperature has little effect (Rollema, 1992). The extent of \( \alpha_{\text{s2}} \)-casein association is dependent on ionic strength, with self-association increasing with increasing ionic strength, but decreasing above 0.2 M. The polymerisation of both \( \alpha_{\text{s1}} \)- and \( \alpha_{\text{s2}} \)-caseins proceeds by a series of consecutive steps (Figure 2.1) (Payens & Schmidt, 1966; Snoeren et al., 1980).

\[
(\alpha_2)_1 + \alpha_2 \rightleftharpoons (\alpha_2)_{i+1}
\]

Figure 2.1 Polymerisation of \( \alpha_{\text{s1}} \) or \( \alpha_{\text{s2}} \)-caseins, where \( \alpha_2 \) represents either of the caseins.

The self-association mechanism of \( \beta \)- and \( \kappa \)-caseins, with their amphiphilic behaviour is micellar-like in its nature, and can be described as a monomer-polymer equilibrium (Figure 2.2).

\[
n\beta \rightleftharpoons \beta_n
\]

Figure 2.2 Association of \( \beta \)- or \( \kappa \)-casein as a monomer-polymer equilibrium.

Association is driven by hydrophobic interactions between the C-terminal segments, and opposed by repulsion between the charged N-terminal segments. Hydrophobic interactions cause the temperature effect, and the ionic strength effects are a result of the charge repulsion (Rollema, 1992). \( \beta \)-Casein association is strongly dependent on ionic strength and temperature. As the temperature or ionic strength is increased \( \beta \)-casein association increases (Schmidt & Payens, 1972). The degree of \( \beta \)-casein polymerisation \( (n) \) depends on temperature, ionic strength and probably pH, and ranges between 12-59 (Rollema, 1992). Polymerisation of \( \kappa \)-casein is relatively independent
of temperature (4 and 20°C) and ionic strength (0.1 to 1.0 M), and  \( n \) is approximately 30 (Vreeman et al., 1981).

2.1.1.1 Casein Micelle Structure

Due to their high hydrophobicity, \(~95\%\) of the caseins associate together to form micelles. The micelle is composed of casein protein (92%) and inorganic salts (8%) largely in the form of calcium phosphate (Swaisgood, 1992). The size distribution of the casein micelles is relatively wide, 15-600 nm with an average diameter of 200 nm (McGann et al., 1980). The micelle structure is rather porous, indicated by the high values for voluminosity and hydration that have been found (Rollema, 1992). Compositional studies based on micellar size show that the fractional content of \( \alpha_s \) and \( \beta \)-casein decreases and that of \( \kappa \)-casein increases with decreasing micelle size (Donnelly et al., 1984). The outer surface layer of the micelle is thought to be composed of equimolar amounts of \( \alpha_s \) and \( \kappa \)-caseins with a small amount of \( \beta \)-casein, while the interior contains \( \beta \)- and \( \alpha_s \)-caseins in equimolar amounts and only a minor amount of \( \kappa \)-casein (Dalgleish et al., 1989). \( \kappa \)-Casein which is found predominantly at the surface is responsible for micelle stabilisation. The hydrophilic C-terminal of \( \kappa \)-casein protrudes into the surrounding solution, reducing surface hydrophobicity and providing electrostatic and steric stabilisation (Walstra, 1990; Swaisgood, 1992).

Although the structure of the casein micelle is not entirely clear, it is believed to be composed of a number of sub-micelles or sub-units linked together by colloidal calcium phosphate (CCP), with hydrophobic and hydrogen bonds contributing to the relatively stable structure. Electron micrographs have been the basis for the sub-micelle theory, as discreet subunits \(~10\) nm in diameter have been observed in casein micelles (Schmidt & Buchheim, 1970; Knoop et al., 1973; Kalab et al., 1982). A number of models based on the sub-micelle structure have been put forward by Slattery and Evard (1973), Schmidt (1982) and Walstra and Jenness (1984). The structural model proposed by Schmidt (1982) is shown in Figure 2.3. Sub-micelles of varying casein composition are linked by CCP. Sub-micelles enriched in \( \kappa \)-casein are located at the surface while \( \kappa \)-casein depleted sub-micelles are buried inside the micelle. Walstra and Jenness (1984) developed the model to include the concept of steric stabilisation of the micelle by
κ-casein (Figure 2.4). The C-terminal end of κ-casein is proposed to stick out into the surrounding solution giving the micelle a hairy surface. A recent model proposed by Holt (1992) suggests that the micelle is composed of macrogranules of calcium phosphate incorporated in a protein matrix, with the micelle surface having a hairy layer to provide steric stabilisation. This model is without sub-micelles, and there have been recent objections to a sub-micelle model (Visser, 1992). Visser (1992) claims that changes in the micelle caused by calcium sequestration, cooling and the lowering of pH of skim milk are not effectively explained by the sub-micelle model. Therefore, the casein micelle structure is not fully established, and further work is required to determine the true nature of the protein/mineral complex.

Figure 2.3 Sub-micelle model proposed by Schmidt (1982) showing A. sub-micelle and B. Casein micelle.
Figure 2.4 Model proposed by Walstra and Jenness (1984) showing the protruding C-terminal portion of κ-casein.

Dissociation of casein from the micelle is caused by a number of treatments, such as heating, cooling, pH adjustment, calcium chelating agents. Heating (120°C) and cooling milk (from 30 to 5°C) both causes dissociation of casein proteins from the micelle, in particular κ-casein on heating (Singh & Creamer, 1991b) and β-casein on cooling (Downey & Murphy, 1970). Acidification results in the loss of the micellar calcium phosphate, which is accompanied by a concomitant release of casein proteins from the micelle (Rose, 1968; Roefs et al., 1985; Dalgleish & Law, 1988). At constant pH, gradual removal of calcium from the micelles by chelation with EDTA, initially causes a release of caseins without any major effect on the micelle size, but ultimately leads to complete micellar disintegration (Lin et al., 1972).
2.1.2 Whey proteins

Raw bovine milk contains ~0.7% whey protein. The whey proteins are globular to ellipsoidal in structure, relatively soluble and heat labile, with the exception of the proteose peptones. The five major groups of whey proteins are, β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA), immunoglobulins (Ig) and proteose peptones (PP). The structure and properties of various whey proteins have been reviewed by Swaisgood (1982), Eigel et al. (1984), Mulvihill and Donovan (1987) and Whitney (1988) (Table 2.3).

Table 2.3 General characteristics of whey proteins in milk.

<table>
<thead>
<tr>
<th>Whey protein</th>
<th>Concentration in milk (g kg⁻¹)</th>
<th>MW (Da)</th>
<th>Isoelectric point</th>
<th>Disulphide bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactoglobulin</td>
<td>3.3</td>
<td>18,363</td>
<td>5.13</td>
<td>2</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>1.2</td>
<td>14,147</td>
<td>4.2-4.5</td>
<td>4</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.4</td>
<td>66,267</td>
<td>4.7-4.9</td>
<td>17</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>0.7</td>
<td>(1.5-10) x 10⁵</td>
<td>5.5-8.3</td>
<td>21</td>
</tr>
<tr>
<td>PP and minor whey proteins</td>
<td>0.8</td>
<td>(4.1-40.8) x 10³</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

Adapted from Mulvihill and Donovan (1987) & Walstra and Jenness (1984)

2.1.2.1 β-Lactoglobulin (β-lg)

The most abundant whey protein is β-lg, which represents 50% of the total whey proteins in bovine milk. There are seven known genetic variants of β-lg; A, B, C, D, E, F and G (Eigel et al., 1984). The A and B genetic variants (β-lg A and β-lg B) are the most common and exist in almost the same frequency. These two variants differ at positions 64 and 118, where Asp and Val in β-lg A are replaced by Gly and Ala in β-lg B (Braunitzer et al., 1972).

In its natural state, β-lg is a dimer of two non-covalently linked monomeric subunits. The dimer is stable between pH 5.5 and 7.5, but dissociates due to strong electrostatic
repulsions below pH 3.5. In the pH range 3.5 to 5.2, the dimers tetramerize to form octomers. Above pH 7.0 β-lg undergoes reversible conformational changes, and above pH 8.0, the protein is unstable and forms aggregates of denatured protein (Lyster, 1972).

There are five cysteine residues per molecule of β-lg, but only four of these are able to form disulphide linkages. The first disulphide bridge occurs between positions 66 and 160, while the second forms between position 106 and one of two positions, 119 and 121. This leaves one free thiol group distributed equally between positions 119 and 121 (McKenzie, 1971; McKenzie et al., 1972). The thiol group is important in the interactions that occur between β-lg and the other proteins on heating, mainly κ-casein and α-lactalbumin (Walstra & Jenness, 1984).

The secondary structure of β-lg, determined by circular dichroism, contains 10% α-helix, 43% anti-parallel β-sheet and 47% unordered structure (Townend et al., 1967). The monomer is roughly spherical with a diameter of 3 nm (Green et al., 1979). Four distinct crystal forms of β-lg (lattices K, X, Y & Z) have been obtained by X-ray crystallography (Green et al., 1979). Papiz et al. (1986) identified the orthorhombic lattice Y as consisting of nine strands of anti-parallel β-sheet, eight of which wrap around to form a flattened conical barrel. Around this barrel is a three-turn α-helix.

The biological function of β-lg still remains unknown, although from its amino acid composition the protein is high in nutritional value. Stability to acidic conditions and gastric proteolysis have suggested that β-lg serves a transport function in the intestine of neonate calves where specific receptor sites have been found (Papiz et al., 1986). There is some indication that retinol and fatty acids are bound to β-lg in milk (Garrick, 1986; Puyol et al., 1991), and that their uptake in the intestinal tract is enhanced by this association (Said et al., 1989).

2.1.2.2 α-Lactalbumin (α-la)
The second most abundant whey protein, α-la, has three known genetic variants, and accounts for 20% of the whey proteins (Eigel et al., 1984). There are four interchain
disulphide bonds, but no sulphhydryl groups are present. The disulphide bridges are located between amino acids 6 and 120, 28 and 111, 61 and 77 and between 73 and 91.

The secondary structure of $\alpha$-la contains 26% $\alpha$-helix, 20% $\beta$-sheet, and 60% unordered structure (Robbins & Holmes, 1970). The similarity in the primary structure of $\alpha$-la and lysozyme has led to the development of a three dimensional structure for $\alpha$-la based on the main chain conformation of lysozyme (Browne et al., 1969).

The physiological role of $\alpha$-la is to form part of the enzyme galactosyltransferase, which produces galactose in lactose synthesis. $\alpha$-La binds two atoms of calcium very closely, and is rendered susceptible to denaturation when these atoms are removed (Walstra & Jenness, 1984).

2.1.2.3 Bovine serum albumin (BSA)

BSA is identical to the serum albumin found in the blood stream, and represents ~5% of the total whey proteins (Eigel et al., 1984). The protein is synthesised in the liver and gains entry into the milk through the secretory cells. The secondary structure consist of 55% $\alpha$-helix, 16% $\beta$-sheet and 25% unordered structure (Reed et al., 1975). There is one free thiol group and 17 disulphide bonds which act to form the protein into a multiloop structure. The structure is thought to be ellipsoid in shape with three domains, two large double loops and one small loop (Brown, 1977). BSA appears to function as a carrier of small molecules, such as fatty acids, but any specific role that BSA may play is unknown (Walstra & Jenness, 1984).

2.1.2.4 Immunoglobulins (Ig)

Immunoglobulins which make up 10% of the whey proteins are antibodies, that are polymers of two kinds of polypeptide chains, light chains (MW of 22,400 Da) and heavy chains. There are two types of light chains ($\kappa$ and $\lambda$) which differ in amino acid chain structure but have homologous sequences (Larson, 1992). The heavy chains can be of several different types, $\gamma$ (MW of 52,000 Da), $\alpha$ (MW of 52,000-56,000 Da) or $\mu$ (MW of 69,000 Da). Both light and heavy chains have a constant region and a variable region, which is associated with immunological specificity. Two light and two
heavy chains are joined by disulphide linkages to form a Y shaped structure (Silverton et al., 1977). A disulphide bond connects each of the light chains to a separate heavy chain, and the heavy chains are held together by disulphide bonds (Larson, 1992). Immunoglobulins contain 21 disulphide bonds per molecule (Kumar & Mikolajcik, 1973). The majority of Ig in milk are transported there from the blood, and these blood Ig are overwhelmingly comprised of Immunoglobulin G (IgG). IgG consist of two identical heavy (γ) and two identical light (κ or λ) chains. Four types of immunoglobulins have been found in bovine milk, IgM, IgA, IgE, IgG (Eigel et al., 1984).

2.1.2.5 Proteose peptones (PP)
The whey proteins which remain soluble at pH 4.6 after heating at 95-100°C for 30 min, but which are insoluble in 8-12% trichloroacetic acid are called proteose peptones. There are four groups, named after their electrophoretic mobility; PP-3, PP-5, PP-8-fast and PP-8-slow. PP-3 is probably derived from a fat globule membrane constituent, and components PP-5, PP-8-fast, and PP-8-slow are fragments of β-casein produced from proteolysis by alkaline milk proteinase (Walstra & Jenness, 1984; Mulvihill & Donovan, 1987).

2.1.2.6 Minor whey proteins
β₂-Microglobulin is a single polypeptide chain consisting of approximately 100 amino acid residues (MW of 11,800 Da). Transferrin is a common blood plasma protein. Lactoferrin and transferrin are both iron binding proteins that exist as large single chain polypeptides of 600-700 residues. Lactoferrin is a inhibitor of bacteria because it deprives them of iron, but the antibacterial effect is not significant because of its very low concentration (Walstra & Jenness, 1984).

2.1.3 Minerals
The salts in milk are divided mainly between the colloidal and soluble phases, with a limited amount bound to the fat globules (Walstra & Jenness, 1984). Milk is supersaturated with calcium and phosphorus in the form of phosphate. This allows the formation of insoluble colloidal calcium phosphate (CCP) complexes which stabilise the
micelle. X-ray absorption and infrared spectroscopy indicate that CCP resembles brushite, CaHPO₄·2H₂O (Holt et al., 1982, 1989). The structure of CCP has been shown to be amorphous by X-ray and electron diffraction (Knoop et al., 1979). Holt (1995) suggests that CCP can be viewed as hydrated clusters of calcium and phosphate ions surrounded by casein phosphate clusters. The ion cluster is approximately 2.5 nm in diameter (McGann et al., 1983), indicating a composition of approximately 66 calcium ions, 132 water molecules and 66 phosphate moieties (Holt, 1995). An alternative model for the structure of CCP have been proposed by van Dijk (1990a, b, 1991). Two organic phosphate groups are linked to a structure combining 4 inorganic phosphates and 8 divalent ions (mostly calcium). The micellar system also contains magnesium and citrate which interact and form part of the CCP structure. The salt balance between the colloidal and soluble phases largely determines the physico-chemical state of milk and hence the thermal behaviour of the proteins.

The distribution of the main salts between the two phases is shown in Table 2.4.

Table 2.4 Distribution of major salts between the colloidal and soluble phases.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Concentration in milk (mg per 100 g)</th>
<th>Present in serum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>48</td>
<td>95</td>
</tr>
<tr>
<td>K</td>
<td>143</td>
<td>94</td>
</tr>
<tr>
<td>Mg</td>
<td>11</td>
<td>66</td>
</tr>
<tr>
<td>Ca</td>
<td>117</td>
<td>32</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>203</td>
<td>53</td>
</tr>
<tr>
<td>Citrate</td>
<td>175</td>
<td>92</td>
</tr>
</tbody>
</table>

From Walstra and Jenness (1984)

Milk salts fall into three families of correlations. The first involves lactose, K, Na and Cl. Lactose is negatively correlated with K in order for the milk to be osmotically balanced with blood, and Na is positively correlated with Cl. The second correlation involves; (i) diffusible Ca, Mg and citrate and; (ii) Ca²⁺, HPO₄²⁻ and pH. At constant
pH, diffusible Ca and Mg are closely and directly correlated with citrate, and there is a negative correlation between Ca\(^{2+}\) and HPO\(_4^{2-}\) (Holt, 1985). The third involves colloidal Ca, Mg, P and citrate (constituents of CCP), which are closely related to the casein content.

In the serum phase, the monovalent ions Na\(^+\), K\(^+\) and Cl\(^-\) exist in milk mainly as free ions, whereas the multivalent ions such as Ca\(^{2+}\), Mg\(^{2+}\), PO\(_4^{3-}\) and Cit\(^{3-}\) form complexes such as HPO\(_4^{2-}\), CaCit\(^-\) and MgHPO\(_4\) (Holt, 1985). Free ion concentrations in the serum phase determined colorimetrically, by ion-selective electrode, by ion exchange methods and by calculation indicate that 20-30% of Ca and Mg are free ions (Holt, 1985).

2.1.4 Variations in milk composition

Milk composition is highly complex and affected by breed, condition of the cow, feed, seasonal changes and stage of lactation. The difference in breeds can be seen between Friesian and Jersey, with the later having a higher fat and protein content (Walstra & Jenness, 1984).

Seasonal variations affect the fat, protein and lactose levels in milk. In New Zealand, milk produced in April (late-season) has higher fat and protein content and lower lactose than milk produced during November (mid-season) (McDowell, 1972a; Singh & Newstead, 1992). Protein nitrogen of Friesian milk destined for town supply (calving in late winter or autumn) is low over January and February (summer) rising in March and April to a peak value before gradually declining to a second minimum in July and August (McDowell, 1972a). The fat content of northern-hemisphere milks (England & Wales) is high during winter and low in summer (Harding & Royal, 1974). The total protein, casein and whey protein content is highest in October, decreasing gradually during winter till an April minimum before a sharp increase and then a gradual decline over summer (Harding & Royal, 1974). Similar trends were also observed in the total protein, casein and whey protein seasonal composition of milks from the south-west of Scotland, the Netherlands, the United States and Ireland (Dellamonica et al., 1965; de Koning et al., 1974; Davies & Law, 1980; Phelan et al., 1982). The seasonal variation may be caused by the feed, as a considerable increase in milk protein content occurs
shortly after the cows are put out to grass in spring (Davies & Law, 1980). White and Davies (1958) showed that the nitrogen content of total milk, casein and α-la and β-lg combined were high at the beginning of lactation, fell away rapidly and then increased slowly during mid-lactation, then increased rapidly towards the end of lactation.

The change in the individual proteins, αs1-, β-, κ-caseins and β-lg, of northern-hemisphere milk follows the same trend as the total protein (Davies & Law, 1980). Sanderson (1970b) showed that in New Zealand milk there was an apparent change in the ratio of αs2-, β- and κ-casein from 1.0:0.52:0.28, respectively in October (mid-season), to 1.0:0.45:0.36 in May (late-season). The level of β-lg increases in the later part of lactation, at a faster rate than α-la and BSA (Sanderson, 1970b). Regester and Smithers (1991) showed that the level of β-lg in whey protein concentrate (WPC) increased during the final three months of lactation. Other authors have reported the changes in α-la are minimal (Dellamonica et al., 1965; de Koning et al., 1974). Davies and Law (1980) reported α-la had a peak value in May (spring) followed by a gradual decline to a minimum in November (winter); αs1-casein also follows a similar trend.

Early lactation milk contains on average higher levels of total and soluble Ca and Mg, total and soluble citrate and total Na and Cl than mid-lactation milk (White & Davies, 1958). The concentration of sodium and chloride gradually increases during mid-lactation, while K decreases (White & Davies, 1958). During late lactation significant increases in Na, and in the colloidal concentrations of P, Ca and Mg, and decreases in K and soluble P were reported by Keogh et al. (1982). Pouliot and Boulet (1995) observed only minor changes in phosphate, Mg, Citrate and K in both colloidal and soluble phases in bulk raw milk, but colloidal calcium increased significantly between December and February in bulk milk (northern-hemisphere's winter). The lactose level in milk decreases sharply towards the end of lactation to compensate for the increase in sodium and chloride to maintain an osmotic pressure similar to blood (Phelan et al., 1982). White and Davies (1958) showed the pH of milk is low in early lactation (6.55) increasing rapidly to a mid-lactation value of 6.73, and at the end of lactation rises to 6.98.
2.2 WHEY PROTEIN DENATURATION

2.2.1 Mechanism of whey protein denaturation

Protein denaturation has been defined by many authors as the unfolding of the tertiary and secondary structure without breakage of the peptide bonds (de Wit, 1981; Walstra & Jenness, 1984; de Wit & Klarenbeek, 1984; Mulvihill & Donovan, 1987). The globular structure of native whey protein is maintained by hydrogen bonding, van der Waals forces, hydrophobic and electrostatic interactions. Once these forces are ruptured by physical or chemical means the protein unfolds into a random configuration which exposes reactive side chain groups previously buried in the interior. This process often called unfolding is considered reversible, but the unfolded protein in its high state of disorder and entropy is prone to further interactions (Walstra & Jenness, 1984; Mulvihill & Donovan, 1987). The unfolded protein can then proceed by a separate irreversible step through to an aggregated form. The process of aggregation is primarily through the formation of disulphide linkages by sulphydryl-disulphide interchange reaction. Thus the overall denaturation process of whey proteins can be viewed as a two step process (Figure 2.5) (Mulvihill & Donovan, 1987; de Wit, 1990; Paris et al., 1991).

\[ P_n \overset{\text{unfolding or }}{\Rightarrow} P_u \overset{\text{aggregation or irreversible denaturation}}{\rightarrow} P_x \]

Figure 2.5 Whey protein denaturation pathway, where \( P_n \) represents whey protein in its native state, \( P_u \) in its unfolded state and \( P_x \) in an aggregated form.

2.2.2 Methodology of the measurement of protein denaturation

The range of methods available to measure protein denaturation are varied and each relies on measuring a different aspect of the physico-chemical changes the protein undergoes. The methods used in the study of thermal denaturation of whey proteins have included differential scanning calorimetry, and various other techniques to analyse the solubility, spectral and immunological characteristics. Most of the methods rely on
taking measurements after cooling the heat treated sample, which allows for renaturation and other temperature related interactions to occur.

2.2.2.1 Differential scanning calorimetry

One of the established methods for studying protein unfolding is differential scanning calorimetry (DSC). This method has been used extensively in characterising the thermal denaturation of β-lg, and to a lesser extent the other whey proteins (de Wit & Swinkels, 1980; Park & Lund 1984; Relkin & Launay 1990). The method involves heating a sample of native protein and a reference sample, which may consist of solvent in the absence of protein or an identical protein solution previously heat denatured under the same conditions (Jelen & Rattray, 1995). The endothermic heat flow caused by the protein unfolding is measured. The method is useful in studying protein denaturation as the technique monitors the reaction directly. A disadvantage is the relatively high protein concentration and media such as distilled water and buffers which are required to obtain a measurable endothermic change. Thus the method is not necessarily suitable for research of denaturation of whey proteins in skim milk (Jelen & Rattray, 1995).

2.2.2.2 Protein solubility

Interactions between proteins when unfolded are enhanced through exposed reactive side groups. The loss of solubility which these intermolecular interactions leads to, can be used to assess whey protein denaturation. The loss of solubility is usually carried out by adjusting the heat treated sample to pH 4.6, and centrifuging for a set combination of centrifugal force (1,500-2000 g), time (15-20 min) and temperature (≈20°C). Filtration of the pH adjusted sample can also be used instead of centrifugation. The protein in the supernatant is assumed to be native and is determined by a number of methods including, polyacrylamide gel electrophoresis, HPLC and Kjeldahl nitrogen. Maximum sensitivity is obtained when the protein solubility is measured at the isoelectric point, where the electrostatic repulsive forces are at their lowest (Jelen & Rattray, 1995).

Protein solutions after heating are rapidly cooled before analysis, thereby permitting reversal of unfolding and association-dissociation reactions (Sawyer, 1969). This
explains why the high heat sensitivity of α-la observed by DSC is not seen in solubility studies because the unfolded protein renatures on cooling (Jelen & Rattray, 1995).

Polyacrylamide gel electrophoresis (PAGE) is used for qualitative (e.g. phenotyping) and quantitative protein analysis. Separation is based on protein mobility across a voltage gradient and the molecular sieving action of the porous gel. Scanning densitometry is commonly used to quantify individual protein bands on a gel. Densitometers capable of integrating the absorbance of a band rather than measuring a single line through the band are more accurate. Lateral spreading and distortion of the band produces errors in single line measurements. Comparison between gels should only be made with a standard run on both gels, as significant differences can result from staining/destaining methods, gel composition/manufacture and electrophoretic running conditions (Andrews, 1988).

HPLC methods are used extensively in quantitative protein determinations. The basis of protein separation is that different proteins have different velocities in the mobile phase, depending upon their distribution between the mobile and stationary phases. The stationary phase in HPLC has very small particles (<10 μm) and hence a high pressure is essential for forcing the mobile phase through the column (Meyer, 1994). Some of the techniques available to separate proteins are; reversed-phase, ion exchange, size exclusion and affinity chromatography. Reversed-phase chromatography uses a very non-polar stationary phase, silica with covalently attached non-polar groups (such as C8 or C18 alkyl chains), and a relatively polar mobile phase, so that separation is based on hydrophobicity. Ion exchange has charged groups incorporated into the stationary phase, and separation is by charge. The stationary phase in affinity chromatography has covalently bound biospecific ligands, and separation is achieved by the interaction, biochemical in nature (e.g. antigen-antibody), between the ligand and the substance of interest. Size exclusion chromatography is basically different from the other methods as the protein size rather than any interactions with the porous stationary phase forms the basis of separation.
Comparison of methods

The results of fast protein liquid chromatography (FPLC) using an MONO-Q anion exchange column, and PAGE for measuring $\alpha$-la, $\beta$-lg A and $\beta$-lg B denaturation in milk, correlate highly with each other. Overall FPLC offers the advantages of better accuracy over PAGE, speed with an automated sampler, minimal labour in preparing samples and good resolution of the main whey proteins (Manji et al., 1985). Manji and Kakuda (1987) compared four methods for measuring protein denaturation in heated skim milk. DSC results were too erratic to be accurately quantified. The other methods used were FPLC (MONO-Q anion exchange column), Kjeldahl nitrogen, and Whey protein nitrogen index (WPNI). All the methods were comparable, except results between FPLC and WPNI, and between Kjeldahl nitrogen and WPNI. Total protein in raw milk measured by sodium dodecyl sulphate (SDS) PAGE is a suitable method for determining the ratio of casein to whey protein (Basch et al., 1985). WPNI is unable to measure accurately whey protein in blends of whey concentrate and skim milk powder (Basch et al., 1985). Results obtained from reverse phase HPLC and ultrathin layer isoelectric focusing for measuring whey protein denaturation in skim milk were in good agreement (Kessler & Beyer, 1991). Most of the analytical techniques are in agreement with each other, though the lack of good correlation with WPNI is of concern (Manji & Kakuda, 1987), as this methods is used to differentiate skim milk powders on the basis of undenatured whey protein.

2.2.2.3 Spectral methods

Structural changes in whey proteins caused by unfolding can be followed by the change in their optical activity. Thermal unfolding of $\beta$-lg increases the number of asymmetric centres exposed to the solvent, which increases the specific levorotation of the sample (Harwalker 1980a, b). The two spectral methods used to measure whey protein unfolding are optical rotary dispersion (ORD) and circular dichroism (CD). The ORD method measures changes in the angle of rotation of polarised light plane while CD is concerned with the ability of molecules to absorb circularly polarised light (Jelen & Rattray, 1995).
Unfolding of the whey protein exposes tryptophan residues which are normally buried in the interior of the protein (Jelen & Rattray, 1995). The exposed residues alter the fluorogenic behaviour of the protein, causing a shift in the excitation wavelength from 289 nm to a maximum of 350 nm (Lametti et al., 1995). The disadvantages of this method are the necessity for high dilution, the avoidance of contamination and the slope of the standard curve is so small that errors in measuring fluorescence result in large errors in protein concentration (Walstra & Jenness, 1984).

2.2.2.4 Immunological methods
Whey proteins are highly antigenic and have the ability to bind specific antibodies. Denaturation causes a decrease in immunological activity. Micro compliment fixation was used by Baer et al. (1976) to measure the conformational changes (unfolding) of α-la heated at temperatures up to 90°C. The method of immunodiffusion was used by Lyster (1970) to measure native β-Ig and α-la in heated skim milk. Diluted samples were placed in agar containing antiserum, and after incubating at 37°C a distinct front of complexed protein and antibody was obtained. The migration distance of the front was compared against a standard curve to obtain the native protein concentration.

2.2.3 Denaturation behaviour of individual whey proteins
The resistance of the whey proteins to heat denaturation in milk (Larson & Rolleri, 1955; Dannenberg & Kessler, 1988a; Singh & Creamer, 1991a) and rennet whey (Donovan & Mulvihill, 1987) follows the order; α-la > β-Ig > BSA > Ig. However, de Wit and Klarenbeek (1984) using DSC on individual proteins (8-10% concentration) in 0.7 M phosphate buffer (pH 6.0), showed a different order; β-Ig > Ig > BSA > α-la. Though having a low denaturation temperature as measured by DSC, α-la dissolved in simulated milk ultrafiltrate (SMUF) is the most heat stable of the whey proteins due to its renaturation (>90%) on cooling. This reversibility does not occur with β-Ig and BSA (Rüegg et al., 1977). Neither does it occur in WPC and milk, possibly because of irreversible heat-induced interactions between α-la and the other whey proteins. Therefore, the thermal behaviour of purified individual proteins cannot be directly compared with their behaviour in milk and processed milk systems.
Denaturation of β-lg and α-la (78°C and 117°C) in whole milk is not significantly different from that in skim milk, and is unaffected by consecutive heat treatments at 78°C and 117°C. Preheating to 60% denaturation at either temperature has no effect on the subsequent denaturation at the second temperature (Lyster, 1970).

There is no clear agreement on which of the two main genetic variants of β-lg is the most heat labile. Dannenberg and Kessler (1988a) have shown that in skim milk heated in the range 70-150°C, the B variant (β-lg B) is less stable than the A variant (β-lg A). This is in agreement with the studies of Gough and Jenness (1962) and Parnell-Clunies et al. (1988), in skim milk and whole milk, respectively. In buffered solutions, purified β-lg B is also less stable (Sawyer, 1968; McKenzie et al., 1971). This is not the case in cheese whey, where at temperatures above 90°C β-lg A is less stable than β-lg B (Hillier et al., 1979). Using DSC, Imafidon et al. (1991) observed that 10% solutions of β-lg A were more heat sensitive than β-lg B (both variants dissolved in SMUF). The protein concentration could be important in determining the relative stabilities of the variants, as McSwiney et al. (1994) reported a 10% solution of β-lg B to be more heat stable than β-lg A (in 20 mM imidazole buffer, 0.1 M NaCl, pH 7.0, heated at 75-85°C). Varunsatian et al. (1983) found that in a 1% solution of WPC β-lg A is more heat sensitive than β-lg B. Genetic variant C (β-lg C) is the most heat labile of the three variants at 97.5°C and 75°C (Sawyer, 1968). The thiol group reactivity of β-lg A and β-lg B in a potassium phosphate buffer are the same, while the thiol group of β-lg C is less reactive (Phillips et al., 1967). β-Lg C forms an aggregated product with less thiol/disulphide interactions than the A and B variants (Sawyer, 1968). The differences in reported heat stabilities of the variants is perhaps not surprising because of the wide range of heating media and analytical techniques employed. The rate of denaturation of β-lg (combined A and B variants) increases with concentration (Relkin & Launay, 1990). Neilsen et al. (1996) showed that below a concentration of 5% (w/v) β-lg B, heated at 75°C in an imidazole-HCl buffer, aggregated faster than β-lg A but above 5% (w/v) β-lg A was more sensitive to aggregation. The pH and media composition also affect the denaturation of the variants (Imafidon et al., 1991).
2.2.4 Factors influencing whey protein denaturation

2.2.4.1 Effect of temperature

Temperatures of up to 60°C cause only small changes in protein structure and solubility. These reactions are reversible and are governed mainly by hydrophobic interactions, which are enhanced as the temperature increases up to 60°C (Scheraga et al., 1962; de Wit & Klarenbeek, 1984). At temperatures higher than 70°C, hydrogen, and hydrophobic bonds are ruptured or weakened, resulting in the destruction of the secondary and tertiary structure. Increased kinetic energy at higher temperatures causes an increase in the frequency of molecular collisions and enhances thiol-disulphide interactions (Li-Chan, 1983). Also the development of thiol groups begins at 72°C reaching a maximum at 95°C (Kirchmeier et al., 1984; Donovan & Mulvihill, 1987). This results in irreversible changes as the whey proteins interact through disulphide linkages, or thiol-disulphide interchange. In DSC studies, de Wit (1981) and de Wit and Klarenbeek (1981) observed two endothermic peaks near 80°C and 140°C. The peak at 80°C is caused by protein unfolding, and the peak at 140°C is due to the disruption of the disulphide bonds. The critical temperature above which irreversible denaturation (aggregation) of whey proteins begins to occur is 70°C (de Wit & Swinkels, 1980).

2.2.4.2 Effect of pH

The denaturation of whey proteins shows a strong pH-dependence. Whey proteins in milk carry charges at their surface due to ionization of surface groups. At physiological pH (~6.7) the charge is negative and this causes electrostatic repulsion between proteins.

Donovan and Mulvihill (1987) studied the solubility (at pH 4.5, 2000 g for 20 min) of heat treated (60-90°C) whey proteins in whey adjusted to pH 4.5-7.0. Individual whey proteins exhibit different responses to pH which is probably related to differences in content and distribution of the polar residues in the different polypeptides (Donovan & Mulvihill, 1987). β-Lg was least stable at pH 6.7, while BSA was most stable at this pH. DSC studies by de Wit and Klarenbeek (1984) demonstrated that at pH 3.0 both α-la and BSA were already unfolded prior to heat treatment. Donovan and Mulvihill (1987) found denaturation of α-la to be relatively independent of pH (4.5-7.0), although Hillier et al. (1979) reported that α-la heated at pH 4.0 had a slower rate of denaturation.
than at 6.0. β-Lg denaturation decreases with a decrease in pH from 7.0 to 4.5 (Donovan & Mulvihill, 1987). The relatively high net negative charge of β-lg at neutral pH results in strong intra-molecular forces, thus facilitating opening up and denaturation of the β-lg molecule. The thermal stability of β-lg at the isoelectric point (5.13) occurs from the absence of any net charge, hence intra-molecular repulsion is very weak and the protein conformation is tight and more resistant to unfolding (Donovan & Mulvihill, 1987). The effect of pH on the thiol group activity is particularly important in β-lg, which undergoes a conformational transition at pH 6.9 with an increase in thiol group activity (pKa of thiol group ~8) (Dunnill & Green, 1966). The decrease in thermal stability at pHs above 7.0 may be in part due to an increase in the sulphydryl-disulphide interactions.

Aggregation of β-lg is also pH dependent, but heating at pH favouring denaturation has the opposite effect on aggregation. Formation of insoluble aggregates (at heating pH, 3000 g for 30 min) was the greatest at pH 4.5 to 5.5 (Donovan & Mulvihill, 1987). Similar results have been reported by Varunsatian et al. (1983), de Rham and Chanton (1984) and Xiong et al. (1993). These results suggest insoluble aggregates form near the isoelectric point of β-lg where inter-molecular electrostatic charge repulsion is minimal.

2.2.4.3 Effect of other milk components

The milk salts have a significant effect on heat-induced protein interactions. Varunsatian et al. (1983) showed that the effects of Ca²⁺, Mg²⁺ and Na⁺ were to promote denaturation and aggregation, but only on the alkaline side of the isoelectric point (>pH 5.5). The effect of Ca²⁺ was greater than Mg²⁺ but both the divalent ions affect whey protein denaturation and aggregation to a much greater extent than Na⁺. Varunsatian et al. (1983) suggested that the Ca²⁺ and Mg²⁺ bind specifically to the heat denatured whey proteins to form aggregates. The effect of Na⁺ may be to mask some of the exposed ionic groups thus altering the electric double layer to facilitate protein-protein interactions (Xiong et al., 1993). Xiong et al. (1993) using DSC observed broader transition peaks of β-lg in CaCl₂ than in NaCl. This suggest that the binding of Ca²⁺ to different structural domains of β-lg differs considerably, where as Na⁺
interacts with all parts of β-lg similarly so that simultaneously unfolding occurs (Xiong et al., 1993). Parris et al. (1993) showed that the extent of soluble aggregate formation (non-sedimentable at 12,000 g for 30 min at 4°C) increased with a concomitant decrease in large insoluble aggregates, as the calcium concentration of sweet whey was reduced. This indicated that calcium ions are involved in the formation of large insoluble aggregates.

The effect of calcium on β-lg denaturation is dependent on the pH. As the pH is reduced from 7.0 to 6.4 less calcium is required to induce the same degree of aggregation, measured by turbidity (transmission at 600 nm) (de Wit & Klarenbeek, 1984). Addition of Ca²⁺ does not increase aggregation of rennet whey in the pH range 4.5-6.0, but does so above pH 6.0 (Donovan & Mulvihill, 1987).

Addition of CaCl₂ to WPC promotes the denaturation of β-lg but has no significant affect on the other whey proteins (Li-Chan, 1983). Calcium binding is essential for the heat stability and renaturation of α-la (Kronman et al., 1981; Bernal & Jelen, 1984; Kuwajima et al., 1986; Relkin et al., 1993). In its native state, α-la binds Ca²⁺ and this binding stabilises protein conformation upon heating (Relkin et al., 1993). DSC analysis of α-la dissolved in SMUF showed that by adding 0.1 M ethylenediaminetetraacetate (EDTA) as a calcium chelating agent, α-la is unable to renature (Bernal & Jelen, 1984).

Fatty acids appear to stabilise the structure of BSA. Removal of naturally bound fatty acids from BSA, without altering the native structure, decreases the denaturation temperature by 7-12°C in DSC studies carried out by Bernal and Jelen (1985).

Lactose has a protective effect on the thermal denaturation of whey proteins (Hillier et al., 1979; de Wit, 1981; de Wit & Klarenbeek, 1981). The denaturation temperature measured by DSC increases in the presence of lactose, with a further slight increase obtained when this sugar is replaced by glucose or galactose (Bernal & Jelen, 1985). Lactose maintains or increases the hydration of the protein molecule, thus contributing to its stability (Bernal & Jelen, 1985). Pappas (1992) showed that addition of lactose to β-lg/casein mixtures (30 mM sodium barbital buffer, pH 7.00) reduced the calcium
binding ability of the proteins; this suggests that the number of intermolecular electrostatic linkages that can be formed by calcium is reduced.

2.2.4.4 Effect of concentration
McKenna and O’Sullivan (1971) showed that increasing the total solids content of skim milk from 9 to 28 to 44% reduced the whey protein denaturation (80°C for 20 min) from 80 to 59 to 39%, respectively. The individual whey proteins show different responses to total solids concentration. Increasing the total solids in cheese whey retarded the denaturation of β-Ig A and B, but hastened the denaturation of α-la (Hillier et al., 1979). Increasing the total solids also has a protective effect on BSA and Ig in heated whey (Nielsen et al., 1973).

2.2.5 Kinetics of Whey Protein Denaturation
Reaction kinetics is a mathematical tool which can be used to model the denaturation of whey proteins. Studies on denaturation kinetics show a wide variation in both the reaction order and the kinetic constants. The different media used and conditions of pH, protein concentration and heating are highly significant. The method of native protein determination is also important, as each method measures a different chemical or physical effect resulting from denaturation. Studies on denaturation have been carried out in a broad range of media, and have used a variety of analytical techniques, including solubility at pH 4.6, quantitative electrophoresis, DSC and HPLC. The two major whey proteins, β-Ig and α-la, have been extensively researched, while Ig and BSA have received only minor attention. The denaturation kinetics of β-Ig have been studied extensively in skim milk (Lyster 1970; Hillier & Lyster, 1979; Manji & Kakuda, 1986; Dannenberg & Kessler, 1988a; Dalgleish, 1990), cheese whey (Hillier & Lyster, 1979), SMUF (Park & Lund, 1984), buffered solutions (Gough & Jenness, 1962; El-Shazly et al., 1978; Harwalkar, 1980b) and in distilled water (de Wit & Swinkels, 1980; Relkin & Launay, 1990).
2.2.5.1 Order of denaturation

The reaction order can be determined by the integral method, using the general rate equation (Equation 2.1). Kinetic constants for β-lg and α-la denaturation obtained by various workers are shown in Tables 2.5 and 2.6, respectively.

\[-\frac{dC}{dt} = k_nC^n\]  \hspace{1cm} (2.1)

\[C = \text{concentration of native protein (mol} \, \text{L}^{-1}).\]
\[t = \text{time (s)}.\]
\[k_n = \text{rate constant ((mol} \, \text{L}^{-1})^{(1-n)} \, \text{s}^{-1}).\]
\[n = \text{reaction order}.\]

There is no clear agreement on the reaction order for β-lg denaturation in skim milk. Reaction orders \(n\) of 1.5 (Dannenberg & Kessler, 1988a), pseudo first (Dalgleish, 1990), and second-orders have been obtained (Hillier & Lyster, 1979; Manji & Kakuda, 1986). In other media, \(n\) also varies. For example, the reaction order of β-lg dissolved in SMUF is 2 (Park & Lund, 1984). In distilled water, pseudo first-order (Harwalker, 1980b), first-order (de Wit & Swinkels, 1980), and second-order (Relkin & Launay, 1990) have all been found. There is greater agreement on the denaturation of α-la which follows first-order reaction kinetics in skim milk (Hillier & Lyster, 1979; Manji & Kakuda, 1986; Dannenberg & Kessler, 1988a) and cheese whey (Baer et al., 1976). Of the other whey proteins, the denaturation of Ig follows second-order reaction in whole and skim milk (Resmini et al., 1989), but BSA is unable to be described by either simple first or second-order kinetics (Hillier & Lyster, 1979).

Protein concentration could be important in determining the reaction order. In distilled water (Harwalker, 1980b) and phosphate buffer (Gough & Jenness, 1962; de Wit & Swinkel, 1980) at concentrations below 1% the order is 1. At 3.5% β-lg denaturation
Table 2.5 Summary of Kinetic Constants for β-lactoglobulin (β-lg) Denaturation.

<table>
<thead>
<tr>
<th>Whey protein</th>
<th>Medium</th>
<th>Kinetic parameters</th>
<th>Experimental conditions</th>
<th>Analysis Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reaction order $n$</td>
<td>Activation energy (kJ mol$^{-1}$)</td>
<td>Heating conditions</td>
</tr>
<tr>
<td>β-lg A $^A$</td>
<td>Skim milk</td>
<td>1.5</td>
<td>265.21 54.07</td>
<td>70-90°C 95-150°C</td>
</tr>
<tr>
<td>β-lg B $^A$</td>
<td></td>
<td>1.5</td>
<td>279.96 47.75</td>
<td>70-90°C 95-150°C</td>
</tr>
<tr>
<td>β-lg A $^B$</td>
<td>Skim milk</td>
<td>2</td>
<td>275.9 46.0</td>
<td>70-90°C 90-140°C</td>
</tr>
<tr>
<td>β-lg B $^B$</td>
<td></td>
<td>2</td>
<td>259.2 46.0</td>
<td>70-90°C 90-140°C</td>
</tr>
<tr>
<td>β-lg A $^C$</td>
<td>Skim milk</td>
<td>2</td>
<td>36.4 100-150°C</td>
<td>6.67-6.62</td>
</tr>
<tr>
<td>β-lg B $^C$</td>
<td></td>
<td>2</td>
<td>32.0 100-150°C</td>
<td></td>
</tr>
<tr>
<td>β-lg A $^D$</td>
<td>Skim milk</td>
<td>pseudo 1st</td>
<td>250 46.0</td>
<td>75-90°C 90-140°C</td>
</tr>
<tr>
<td>β-lg B $^D$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.5 Continued.

<table>
<thead>
<tr>
<th>Whey protein</th>
<th>Medium</th>
<th>Kinetic parameters</th>
<th>Experimental conditions</th>
<th>Analysis Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reaction order $n$</td>
<td>Activation energy (kJ mol$^{-1}$)</td>
<td>Heating conditions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-lg $^E$</td>
<td>SMUF, 10% β-lg</td>
<td>2.5</td>
<td>521</td>
<td>NA</td>
</tr>
<tr>
<td>β-lg $^F$</td>
<td>SMUF, 0.5% β-lg</td>
<td>2</td>
<td>329.11</td>
<td>60-100°C</td>
</tr>
<tr>
<td>β-lg $^G$</td>
<td>Phosphate buffer, 0.29% β-lg</td>
<td>1</td>
<td>341</td>
<td>NA</td>
</tr>
<tr>
<td>β-lg $^H$</td>
<td>Phosphate buffer, 1% β-lg</td>
<td>1</td>
<td>299.3</td>
<td>67-75°C</td>
</tr>
<tr>
<td>β-lg $^I$</td>
<td>Distilled water, 1% β-lg</td>
<td>pseudo 1st</td>
<td>180</td>
<td>60-130°C</td>
</tr>
<tr>
<td>β-lg $^J$</td>
<td>Distilled water, 3.5% β-lg</td>
<td>2</td>
<td>409</td>
<td>NA</td>
</tr>
</tbody>
</table>

β-lg A. Genetic variant A  β-lg B. Genetic variant B  NA. Not available or not applicable
SMUF. Simulated milk ultrafiltrate  FPLC. Fast Protein Liquid Chromatography  DSC. Differential scanning calorimetry
PAGE. Polyacrylamide gel electrophoresis

<table>
<thead>
<tr>
<th>Whey protein</th>
<th>Medium</th>
<th>Kinetic parameters</th>
<th>Experimental conditions</th>
<th>Analysis Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reaction order</td>
<td>Activation energy</td>
<td>Heating conditions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>(kJ mol⁻¹)</td>
<td></td>
</tr>
<tr>
<td>α-la A</td>
<td>Skim milk</td>
<td>1</td>
<td>268.56</td>
<td>70-80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>69.01</td>
<td>85-150°C</td>
</tr>
<tr>
<td>α-la B</td>
<td>Skim milk</td>
<td>1</td>
<td>164</td>
<td>70-95°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>62.0</td>
<td>100-150°C</td>
</tr>
<tr>
<td>α-la C</td>
<td>Skim milk</td>
<td>1</td>
<td>177</td>
<td>70-90°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72</td>
<td>90-140°C</td>
</tr>
<tr>
<td>α-la D</td>
<td>MUF, 0.1% α-la</td>
<td>1</td>
<td>NA</td>
<td>80°C</td>
</tr>
</tbody>
</table>

NA. Not available or not applicable
MUF. Milk ultrafiltrate
FPLC. Fast Protein Liquid Chromatography
PAGE. Polyacrylamide gel electrophoresis

C. Manji & Kakuda (1986).  
D. Baer et al. (1976).
in distilled water follows 2nd order, further increases up to 24% has no effect on the order (Relkin & Launay, 1990). Park and Lund (1984) also found a reaction order of 2 for 10% β-lg in phosphate buffer. In the pH range 6.0 to 9.0, β-lg denaturation can be described as 2nd order and below pH 5.0 as 3rd order (Park & Lund, 1984). Addition of 5% lactose to SMUF has no significant effect on the reaction order (Park & Lund, 1984). Dannenberg and Kessler (1988a) found that the ratio of whey protein to casein affects the reaction order. Adjusting the casein/whey ratio from 0/100 (whey solution) to 83/17 (skim milk) decreases the order from 2.0 to 1.5.

From the varied reaction orders of β-lg it is apparent that the mechanism of denaturation is complex. The reaction order and denaturation mechanism in distilled water and phosphate buffer systems may be significantly different from what is occurring in milk. Harwalker (1980b) and Sawyer et al. (1971) have suggested that denaturation in distilled water and phosphate media are pseudo first-order involving two consecutive first-order reactions. From the evidence of the Arrhenius plots it seems more plausible to treat denaturation as two consecutive reactions (Hillier & Lyster, 1979). It should be noted that the kinetic equations are only a means of describing a highly complex reaction. Though the reaction orders are reasonably constant, a greater variation is seen in the kinetic parameters, such as the activation energy and rate constant.

2.2.5.2 Temperature dependency of denaturation

Once the order is established the kinetic parameters can be determined so that the reaction can be modelled. The Arrhenius equation is the most commonly used way of defining the temperature dependence of the rate constant (Equation 2.2).

\[
k_n = k_0 \exp\left(-\frac{E_a}{RT}\right)
\]

(2.2)

\[k_0\] = frequency factor \((\text{mol} \cdot 1^{-1})^{(1-n)} \text{s}^{-1}\).

\[E_a\] = Activation energy \((\text{kJ} \cdot \text{mol}^{-1})\).

\[R\] = Universal gas constant \((8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1})\).

\[T\] = Absolute temperature \((\text{K})\).
The natural log of \( k_n \) plotted against the reciprocal of the absolute heating temperature yields a straight line, i.e. the Arrhenius plot (Figure 2.6). From the slope of the line the activation energy can be calculated. In skim milk and cheese whey there is a break in the line for \( \beta \)-lg and \( \alpha \)-la (Lyster, 1970; Hillier & Lyster, 1979; Hillier et al., 1979; Manji & Kakuda, 1986; Dannenberg & Kessler, 1988a). This results in two distinct temperature ranges which have different activation energies. The break occurs at 90-95°C for \( \beta \)-lg in cheese whey and skim milk (Lyster, 1970; Hillier et al., 1979; Hillier & Lyster, 1979; Manji & Kakuda, 1986; Dannenberg & Kessler, 1988a). Both genetic variants of \( \beta \)-lg, \( \beta \)-lg A and \( \beta \)-lg B, exhibit a break in the line at 90°C in skim milk (Manji & Kakuda, 1986; Dannenberg & Kessler, 1988a).

![Arrhenius plot for the denaturation of \( \beta \)-lg A and B, and \( \alpha \)-la in skim milk](Dannenberg & Kessler, 1988a).

Dannenberg and Kessler (1988a) have shown that for \( \alpha \)-la in skim milk the break occurs at 80°C. Other authors have found this break at higher temperatures, 90°C (Hillier et al., 1979; Manji & Kakuda, 1986) and 95°C (Hillier & Lyster, 1979) in both skim milk...
and cheese whey. Resmini et al. (1989) reported no break in the Arrhenius plot for Ig denaturation in skim milk.

Two-fold dilution or up to three-fold concentration of the total solids content in milk and cheese whey has no effect on the temperature at which the reaction mechanism changes, though the reaction rates change (Hillier et al., 1979).

Activation energies at lower temperatures <90°C are typical for a denaturation reaction (Dannenberg & Kessler, 1988a). In skim milk, Dannenberg and Kessler (1988a) obtained activation energies of 265.21, 279.96 and 268.56 kJ mol⁻¹ for β-lg A, β-lg B and α-la, respectively. The corresponding entropies of activation (ΔS*) ranged from 0.45 to 0.5 kJ mol⁻¹ K⁻¹ indicating an increase in the degree of freedom of translation and rotation. At the higher temperatures (>80°C) the activation energies are 54.07, 47.75 and 69.01 kJ mol⁻¹ for β-lg A, β-lg B and α-la, respectively. These are in the range for chemical reactions. The negative ΔS* (-0.115 to -0.150) shows that the transitional state is in a higher state of order than the reactants, possibly through an aggregation mechanism (Dannenberg & Kessler, 1988a). Dannenberg and Kessler (1988a) suggested that the break in the Arrhenius plot is caused by two reactions; an unfolding of the compact native structure dominant at temperatures <90°C, and irreversible aggregation which is the rate determining step above 80°C. The break in the curve could be related to sulphhydryl (SH) group formation, which reaches a maximum at 95°C. The decrease in SH group formation at temperatures greater than 95°C is due to the shielding of these groups as a result of intermolecular protein aggregation (Kirchmeier et al., 1984).

Activation energies obtained by other authors for β-lg and α-la are shown in Tables 2.5 and 2.6, respectively. In whole milk and skim milk Ig has an activation energy of 345 kJ mol⁻¹ (Resmini et al., 1989). The activation energy of BSA denaturation has not been reported.
2.3 HEAT-INDUCED INTERACTIONS OF MILK PROTEINS

2.3.1 Interaction of whey proteins and \( \kappa \)-casein

An important irreversible protein complex formed in heated milk is between \( \beta \)-lactoglobulin and \( \kappa \)-casein. The complex is formed via sulphhydryl-disulphide interchange reactions and hydrophobic interactions (Purkayastha et al., 1967; Haque & Kinsella, 1988; Jang & Swaisgood, 1990). Numerous studies have reported interactions between \( \beta \)-lg and \( \kappa \)-casein in milk (Snoeren & Spek, 1977; Noh et al., 1989a; Law et al., 1994), model micelle systems (Smits & Brouwershaven, 1980; Noh et al., 1989b; Reddy & Kinsella, 1990) and \( \beta \)-lg/\( \kappa \)-casein mixtures (McKenzie et al., 1971; Euber & Brunner, 1982; Haque et al., 1987; Haque & Kinsella, 1988).

2.3.1.1 Interactions in \( \beta \)-lg/\( \kappa \)-casein mixtures

Investigations into complex formation between whey proteins and caseins were initially carried out with purified proteins in buffers, particularly \( \beta \)-lg and \( \kappa \)-casein, after Tobias et al. (1952) and Slatter and van Winkle (1952) suggested a possible interaction between \( \beta \)-lg and \( \alpha \)-casein.

The interaction between \( \beta \)-lg and \( \kappa \)-casein is affected by the proportion of the two proteins in the mixture. Increasing the proportion of \( \beta \)-lg causes more of the \( \beta \)-lg to interact, and the ratio of \( \beta \)-lg to \( \kappa \)-casein in the complex increases (Long et al., 1963). When there is a large excess of \( \beta \)-lg in solution the \( \beta \)-lg: \( \kappa \)-casein ratio in the complexes increases to a maximum of 3:1 on heating at 80°C for 10 min (Tessier et al., 1969). After heating at 85°C for 20 min the maximum interaction ratio in the complex is 2.2 \( \beta \)-lg: 1 \( \kappa \)-casein (Long et al., 1963). Temperature also has an affect, with the ratio of \( \beta \)-lg to \( \kappa \)-casein in the complex decreasing from 2.2 to 1.4, when the heating temperature is raised from 85 to 99°C in a 1:1 mixture of \( \beta \)-lg and \( \kappa \)-casein, heated for 20 min at pH 6.5 (Long et al., 1963). The degree of \( \beta \)-lg interaction with \( \kappa \)-casein is at a maximum of 82% when heated at 85°C, decreasing to 76% at 99°C (Long et al., 1963). This follows the pattern of titratable thiol groups of \( \beta \)-lg which are at their greatest at 85°C (Sedmerová et al., 1972). Doi et al. (1981) using mixtures of \( \beta \)-lg and \( \kappa \)-casein heated at 90°C for 10 min, in imidazole-HCl buffer, pH 7.1, containing 70 mM KCl, examined the effect of carbohydrate moiety of \( \kappa \)-casein on complex formation with
β-lg. The resistance of β-lg to heat precipitation increased as the sugar content of κ-casein increased (Doi et al., 1981).

Haque and Kinsella (1987) used HPLC to identify three different states of molecular association in an equimolar solution of β-lg/κ-casein heated at 70°C; homo-dimers of β-lg and κ-casein, a trimer of β-lg and a tetramer of β-lg/κ-casein in a 3:1 ratio. During heating (70°C) the β-lg trimer dominates the early stages up to 720 s, after which the β-lg/κ-casein tetramer increases in concentration until it equals the β-lg trimer (Haque & Kinsella, 1987). Earlier investigations (Long et al., 1963; Tessier et al., 1969) using electrophoretic methods, though able to determine the overall composition of the heat-induced associations, may have been unable to differentiate between β-lg aggregates and β-lg/κ-casein complexes. The effect of temperature and protein concentration could well affect the relative proportions of the different associations observed by Haque et al. (1987), rather than affect the ratio of β-lg to κ-casein in the complex.

Disulphide bonds have been shown to be involved in the complex formation between β-lg and κ-casein. Complex formation is inhibited by N-ethylmaleimide, and disrupted by the reducing agent, 2-mercaptoethanol (Purkayastha et al., 1967). Para-hydroxymercuribenzoate inhibits complex formation (Kresheck et al., 1964), as does alkylated (S-carboxyamidomethyl) κ-casein (Purkayastha et al., 1967). Euber and Brunner (1982) using β-lg immobilised on Sepharose gel, which is then heated at 80°C for 40 min, demonstrated that a β-lg/κ-casein complex was formed by intermolecular disulphide bonds. Urea (6-8 M) released only ~50% of the bound κ-casein, while 2-mercaptoethanol (2 M) removed all the κ-casein. The minimal self-interaction of the immobilised β-lg suggests that aggregation of β-lg is not essential for the interaction of κ-casein (Euber & Brunner, 1982).

Complex formation, though inhibited in the presence of N-ethylmaleimide, is not entirely prevented, suggesting other interactions having a role in stabilising the complex (McKenzie et al., 1971). A 1:1 mixture of β-lg and κ-casein heated at 70°C in 20 mmol L⁻¹ imidazole buffer (pH 6.8) showed that the initial stages of the complex
formation are driven by mainly hydrophobic forces (Sedmerová et al., 1972; Haque & Kinsella, 1988). The β-lg/κ-casein complex formation can resist chymosin digestion, indicating that the Phe-Met (105-106) bond of κ-casein is buried in the interior of the hydrophobic complex (Haque & Kinsella, 1988). At low temperatures (=70°C) where SH group development is very slow, hydrophobic interactions are important in stabilising the complex, but as the temperature increases hydrophobic interactions are weakened and sulphydryl-disulphide interactions become more dominant. Formation of hydrophobic complexes, observed in media where κ-casein is an individual protein rather than as part of the micelle, may not occur to the same extent in milk. In milk the predominantly polar C-terminal of κ-casein protrudes into solution (Rollema, 1992), and it seems more likely that disulphide interactions would play a more important role. The structure and composition of such a complex formed in a milk system may differ significantly from that formed in mixtures of β-lg and κ-casein (Snoeren & Spek, 1977).

Hartman and Swanson (1965) reported no complex between κ-casein and α-la occurred after heating at 85°C for 30 min. At room temperature a complex does form, but is disrupted on heating at 60-90°C (Sedmerová et al., 1972). α-La is thought to associate indirectly with κ-casein, by complexing with β-lg (Baer et al., 1976). Doi et al. (1983) showed that on heating at 90°C for 30 min, an α-la/κ-casein complex was formed in 35 mM phosphate buffer, pH 7.6, containing 0.4 M NaCl. This complex was not observed under the same heating conditions in 10 mM imidazole-HCl buffer, pH 7.1, containing 0.07 M KCl, suggesting that a high pH is necessary for complex formation (Doi et al., 1983). Complex formation between BSA and κ-casein has not be observed (Hartman & Swanson, 1965).

2.3.1.2 Interactions between whey proteins and the casein micelle
The heat-induced association of β-lg with the casein micelle is primarily through κ-casein located on the micelle surface. Intermolecular disulphide bonds and hydrophobic interactions play a role in bond formation (Smits & Brouwershaven, 1980); at temperatures <75°C the complexes form primarily through noncovalent interactions (Jang & Swaisgood, 1990). Direct evidence for interactions between β-lg and κ-casein was shown by Jang and Swaisgood (1990). Casein micelles, separated from skim milk
by ultracentrifugation and then redispersed in SMUF, when heated interacted with β-lg immobilized on glass beads. At temperatures below 65°C disulphide bond formation did not occur. Increasing the temperature from 75°C to 85°C resulted in a greater amount of κ-casein forming disulphide bonds with β-lg. Of the caseins only κ-casein was observed to react covalently with β-lg (Jang & Swaisgood, 1990).

The association of β-lg with casein micelles is dependent upon several factors, such as heating conditions, protein concentration and salt concentration, in particular calcium. Smits and Brouwershaven (1980) showed that at 70°C the association was slow, only 10-20% of the β-lg sedimented (78,000 g for 1 h at 20°C) with the micelles after 20 min heating, but at 90°C the association was 75-80%. Increasing the β-lg concentration from 4 to 8 g L⁻¹ doubled the amount of association in a modified milk salts buffer medium (Ca²⁺, Mg²⁺ and citrate replaced by Na⁺ and Cl⁻, to prevent gross aggregation of β-lg) heated at 90°C, a further increase to 12 g L⁻¹ resulted in more association. Lowering the ionic strength reduced interactions between β-lg and κ-casein, and as pH increased from 5.8 to 7.3 the amount of sedimentable β-lg decreased (Smits & Brouwershaven, 1980). Addition of calcium (5 mM) to milk (pH 6.7) heated at 90°C for 30 min increased the association of whey proteins to the casein micelle (~35%→~70%), and EDTA reduced the association (~35%→~10%) (Visser et al., 1986).

The extent of interaction between the whey proteins and the casein micelle can be measured by the reduction in the total amount of peptides released by chymosin action. β-Lg interacts with κ-casein in such a way as to shield the Phe-Met bond from cleavage by chymosin. Shalabi and Wheelock (1976) found that both α-la and β-lg inhibited chymosin action, and chymosin action was the same whether α-la and β-lg or just β-lg were heated with the micelle in SMUF. The addition of either heat treated β-lg or α-la to unheated casein micelle dispersion caused a reduction in peptide release.

As to interactions involving other whey protein or caseins there is some dispute. The presence of β-lg is required for α-la and casein micelles to interact, this is probably through a heat induced β-lg/α-la complex which then interacts with the micelle (Elfagm & Wheelock, 1978a, b). No evidence of a covalently linked β-lg/α-la complex
independent of the casein micelle has been found by gel filtration chromatography in skim milk (Noh et al., 1989a). \( \alpha \)-La appears to have no influence on the complex formation between \( \beta \)-lg and \( \kappa \)-casein (Smits & Brouwershaven, 1980). Snoeren & van der Spek (1977) isolated a complex of \( \beta \)-lg/\( \kappa \)- and \( \alpha_\text{s2} \)-caseins from UHT milk. Noh et al. (1989b) showed that although a mixture of \( \alpha_\text{s2} \)-casein and \( \beta \)-lg complexes readily, \( \alpha_\text{s2} \)-casein is relatively unreactive in milk because of its positioning within the micelle.

2.3.2 Heat-induced interactions of whey proteins

The heat labile whey proteins unfold on heating and are able to interact with each other, mainly through thiol-disulphide interchange reactions, to produce aggregates. Heat-induced interactions among \( \beta \)-lg, \( \alpha \)-la and BSA have been studied in model systems and in milk.

2.3.2.1 Interactions between \( \beta \)-lactoglobulin and \( \alpha \)-lactalbumin

Besides self-aggregation, \( \beta \)-lg can form a heat-induced complex with \( \alpha \)-la. A soluble complex is formed mainly through thiol-disulphide interchange between \( \beta \)-lg and \( \alpha \)-la (Matsudomi et al., 1992; Calvo et al., 1993). Aggregation of \( \alpha \)-la is affected by the presence of \( \beta \)-lg in sodium phosphate buffer (pH 6.7) heated at 75°C for 30 min. The amount of native \( \alpha \)-la decreases by 14% when heated alone, but decreases by 84% when heated with \( \beta \)-lg, and when heated in whey decreases by 36% (Hunziker & Tarassuk, 1965). Melo and Hansen (1978) have also shown that when heated alone, \( \alpha \)-la is more heat resistant than when heated with \( \beta \)-lg. Conversely, the aggregation of \( \beta \)-lg is unaffected by \( \alpha \)-la in skim milk and whey (Elfagm & Wheelock, 1978a, b).

2.3.2.2 Interactions between \( \beta \)-lactoglobulin and bovine serum albumin

Hines and Foegeding (1993), using a 1% (w/v) solution containing equimolar amounts of \( \beta \)-lg and BSA heated at 80°C, showed that although BSA aggregation appears unaffected by \( \beta \)-lg, the rate of \( \beta \)-lg aggregation is enhanced by the presence of BSA. The formation of soluble \( \beta \)-lg/BSA aggregates occurs through disulphide crosslinks (Matsudomi et al., 1994).
2.3.2.3 Interactions between α-lactalbumin and bovine serum albumin

Baer et al. (1976) showed that a mixture of immunoglobulins, bovine serum albumin, lactoferrin and whole casein had no affect on α-la (1 mg ml⁻¹) heated at 80°C for 30 min in milk ultrafiltrate. The amount of BSA present may be significant, as Calvo et al. (1993) reported a good correlation between BSA concentration and formation of α-la/BSA aggregates in milk ultrafiltrate at 90°C (α-la 1.5 mg ml⁻¹, BSA 0.3 to 10.0 mg ml⁻¹). Matsudomi et al. (1993) showed thiol-disulphide interchange to be involved in aggregate formation between 0.2% α-la and 0.2% BSA heated at 80°C for 30 min.

2.3.2.4 Interactions involving Immunoglobulins

Using radiolabelled IgG, Oh and Richardson (1991) studied heat-induced interactions in skim milk at 95°C. Gel filtration chromatography of samples dissolved in 6 M guanidine HCl showed all the radioactivity eluted in the same volume as untreated IgG. Elution profiles of IgG heated alone indicated the formation of large complexes through covalent interactions, possibly thiol-disulphide interchange. The milk system appears to inhibit covalent complex formation, possibly BSA is involved as Ig complexes are suppressed in the presence of BSA (Soltis et al., 1979).

2.4 MANUFACTURE OF MILK POWDER

The basic plant layout for milk powder manufacture is shown in Figure 2.7. Raw milk is first pasteurised and then separated into skim milk and cream. Some of the cream is returned to the skim milk to achieve a product with a standardized fat content in whole milk powder manufacture. Both skim and standardized whole milks are preheated for a set temperature/time combination prior to evaporation. Besides ensuring that milk will boil at the lower pressure on entering the evaporator and meet microbiological requirements, preheating is used to impart specific functional properties to the powder.
Multistage falling film evaporators remove $\approx80\%$ of the water from milk. The concentrate leaves the evaporator at 46-50% total solids and 50°C. Free fat levels in whole milk powder are minimised by homogenising the concentrate before drying. Concentrate is atomised as it enters the spray dryer. The small droplets ensure a large surface area over which drying can take place. To improve the droplet size distribution, concentrate viscosity is reduced by heating the concentrate to $\approx70°C$ before drying. The powder particles leaving the spray drier are dried to their final moisture content in a vibrating fluid bed. The two stages of drying offers efficient energy usage and improved powder quality. By the end of the vibrating fluid bed, the powder is sufficiently cooled so that it can be packed or stored without fear of caking or its solubility being reduced (Singh & Newstead, 1992).
2.5 PROCESS INDUCED CHANGES

Heating is invariably used in order to preserve milk by killing pathogenic or spoilage organisms, inactivating enzymes or by altering its physico-chemical state. In milk powder manufacture, heat is applied to produce a dried product with improved storage and desired functional properties. In general the thermal energy in the evaporation and drying stages is independent of the type of powder, as the overriding consideration in these steps is efficient energy usage in the removal of water. The amount of heat applied in the preheat is dependent on the desired end use of the powder. Though all of the stages contribute to the overall thermal effect, the functional properties of the milk powder are largely determined by the preheat treatment. Table 2.7 shows the changes that occur at each step of milk powder manufacture.

Table 2.7 Effect of individual processing steps on milk.

<table>
<thead>
<tr>
<th>Process</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preheat treatment</td>
<td>Destruction of bacteria; inactivation of enzymes; denaturation of whey proteins; aggregation of whey proteins; formation of a complex between κ-casein and β-lactoglobulin; shift of the soluble salts to the colloidal phase; Changes in micelle structure; Maillard reaction between protein and lactose; pH decrease.</td>
</tr>
<tr>
<td>Evaporation</td>
<td>Concentration of milk solids; increase in colloidal salts; increase in micelle size; decrease in pH; limited denaturation of whey proteins.</td>
</tr>
<tr>
<td>Concentrate heating</td>
<td>Reduction in concentrate viscosity; increase in colloidal salts; protein interactions.</td>
</tr>
<tr>
<td>Homogenization</td>
<td>Increase in number of fat globules; adsorption of casein onto fat surface; decrease in protein stability.</td>
</tr>
<tr>
<td>Spray drying</td>
<td>Further removal of water; relatively minor changes in protein and salts.</td>
</tr>
</tbody>
</table>

Adapted from Singh and Newstead (1992)
2.5.1 Preheating

A major consequence of preheating is the denaturation of the heat labile whey proteins and their interactions with the casein micelle. These heat-induced changes have been discussed in Sections 2.2 and 2.3. Preheating by direct steam injection at 120°C for 2 min denatures ≈85% of the β-lg and ≈35% of the α-la. For low preheat treatment (72°C for 15 s) denaturation is slight (≈6%) (Singh & Creamer, 1991a). Many of the properties of milk powders stem from the behaviour of the whey proteins.

The whey protein nitrogen index (WPNI) is a commonly used measure for classifying powders on the basis of undenatured whey protein nitrogen. Casein protein and denatured whey protein are precipitated from reconstituted milk powder using sodium chloride. The undenatured whey protein is then precipitated using amido black dye, and the excess dye determined spectrophotometrically (Sanderson, 1970a). The WPNI is an absolute measure and does not take into consideration the original amount of whey protein in the milk. A powder that is substantially denatured may be classified as being low heat because of a high protein content in the original milk. This may lead to powders with the same WPNI, but different levels of denaturation. Also the method only measures native whey proteins, and there is no indication of what has happened to the protein after denaturation. Formation of aggregates and interactions with the micelle are important factors in determining the functional properties of the powder.

Changes also occur in the mineral distribution in milk. Calcium and phosphate are transferred from soluble to the colloidal phase as heating proceeds (Walstra & Jenness, 1984). Nieuwenhuijse et al. (1988) reported a pH drop of 0.04 and 0.09 units for preheats of 72°C for 2 s and 120°C for 180 s, respectively. The casein proteins are relatively heat insensitive, but changes in the micelle structure occur during heating.

2.5.1.1 Changes in micelle structure

In skim milk heated at 100°C for 30 min (pH 6.65) thread like particles at the micelle surface are observed by electron microscopy. Micelle appearance in heated milk is dependent on pH. At pH 6.8 the micelles look similar to those in unheated milk. At pH 6.5 the micelles acquire amorphous appendages (Creamer et al., 1978). The
appearance perhaps relates to the dissociation of κ-casein/β-lactoglobulin complex at high pH >6.9, and the association of this complex with the casein micelles at pH <6.9 (Singh & Fox, 1987a).

During preheating a number of other changes occur to the casein micelle structure besides association with whey proteins at the micelle surface. Below 90°C changes in micelle size are minimal, but at higher temperatures micelle size increases. Dalgleish et al. (1987) using photon correlation spectroscopy observed the changes in average micelle diameter during heating at 130°C. Initially the size increased slowly, but just prior to visible coagulation the diameter increased rapidly. The pH at heating affects micelle size. Micelle diameter increases as the pH is increased from 6.7 to 6.9, but decreases with a further increase in the pH to 7.2 (Dalgleish et al., 1987). The increase in micellar size and an increase in the number of protein particles smaller than micelles after heating has been observed by electron microscopy. Disintegration of the micelle is thought to be responsible for some of the small particles (Morr, 1969; Creamer & Matheson, 1980; McMahon & Yousif, 1993).

Dissociation of the casein proteins from the micelle increases with the severity of the heat treatment and follows the order κ-, β-, α-caseins (in order of decreasing extent) (Singh & Creamer, 1991b). The dissociation is also dependent on the pH at heating. At pH <6.9, κ-casein and any associated β-lg or α-la co-sediment (100,000 g for 1 h at 20°C) after heating at 140°C for 1 min (Singh & Fox, 1985). Above pH 6.9 κ-casein, β-lg and α-la dissociate from the casein micelle.

2.5.1.2 Mineral balance between soluble and colloidal phases

Heating milk causes a shift in the mineral balance from the soluble to the colloidal phase, in particular Ca and P, which are important in determining the stability of the casein micelles. Research into the area of heat induced changes in minerals is complicated by separation of the soluble/colloidal phases and reversibility of heat induced changes. The behaviour of minerals during processing and their role in determining the functional properties of the powder is not well understood. Reversibility of reactions is important in mineral changes that occur upon heating milk. The mineral
balance between the soluble and colloidal phases is in equilibrium and any shift away from this state during heating is reversible upon cooling (Pouliot et al., 1989b). Therefore the time at which measurements are taken after cooling is critical. The use of an ultrafiltration membrane by Pouliot et al. (1989a, b) allowed for the almost immediate separation of soluble and colloidal phases at the temperature of heating. Advantages of ultrafiltration are that the minerals (Ca, P and Mg) are not concentrated in the permeate, and the mineral compounds bound to the protein in the retentate increases proportionally with the concentration ratio (Brule et al., 1974). At present only low temperature conditions have been used (≤90°C).

Ultrafiltration of milk at the heating temperature used by Pouliot et al. (1989a) allows for the measurement of reversible changes. Increasing heating temperature causes more of the soluble minerals to shift to the colloidal phase. The shift in soluble salts is mainly due to Ca and P, and to a lesser extent Mg and citrate. The decrease in soluble Ca, P and citrate was shown to involve two steps. During the first minute of heating the majority of the decrease takes place, after which a small decrease occurs over an extended period of time (up to 120 min). After the initial decrease, Ca, P and citrate show some reversibility before further heating results in the second step proceeding. The reversibility of Ca and P in milk heated at 85°C for 40 min increases as the cooling temperature decreases (60 → 4°C). During the 1st minute of cooling there is an initial increase in the soluble Ca and P and then a slow increase over the following 120 min. The reversibility ranges from 90-99% after 120 min of cooling at 4 to 60°C (Pouliot et al., 1989b).

The effect of heating and reversibility can also be observed in the Ca$^{2+}$ activity. Recovery of the Ca$^{2+}$ activity on cooling follows a logarithmic relationship with time (Greets et al., 1983). Thus Ca$^{2+}$ activities can be determined at the point of sampling with reasonable accuracy, and past discrepancies caused by different measuring times can be eliminated. Augustin and Clark (1991) observed that the effect of preheating conditions on the Ca$^{2+}$ activity in reconstituted and recombined milks was small, though reversible changes while the milk was held at 4°C overnight may have masked any differences caused by preheating.
2.5.1.3 Lactose

The Maillard reaction is an important consequence of heating lactose with amino compounds as it leads to browning, off flavours, and loss of digestible lysine. The main reaction in Maillard browning is condensation of an amino compound with the carbonyl group of a sugar (Walstra & Jenness, 1984). Some of the products of the Maillard reaction give heated milk products a caramelized flavour.

2.5.2 Concentration related changes

After preheating the solids in milk are concentrated by evaporation and spray drying. Most of the water is removed during evaporation, typically 80%, in a multistage falling film evaporator. Evaporation temperatures are kept low (70 to 50°C) by vacuum, and the residence time in each stage is about 1 min (Singh & Newstead, 1992).

2.5.2.1 Evaporation

While numerous studies have been carried out on the physico-chemical behaviour of milk, research into concentrates has been hampered by difficulties in concentrate handling, in particular age thickening and separation of the colloidal and soluble phases.

Whey protein denaturation during evaporation is probably minimal as the temperatures are low (≤70°C). At low heat treatment (72°C for 15 s) a small decrease in the amount of undenatured β-lg and α-la (1-2%) after evaporation was observed by Singh and Creamer (1991a). Increasing the total solids also reduces denaturation of whey proteins in skim milk (McKenna & O’Sullivan, 1971), and β-lg in cheese whey (Nielsen et al., 1973; Hillier et al., 1979), though α-la denaturation increases (Hillier et al., 1979). The immunoglobulins and BSA which are more heat sensitive than β-lg may denature during evaporation, especially in the first effect which is at about 70°C. The heat load from the evaporator may come into consideration in the manufacture of low heat powders.

Casein micelles increase in size mainly due to coalescence of the micelles. If milk is preheated so that most of the whey proteins are associated with the micelle the increase in size is less (Walstra & Jenness, 1984).
Soluble minerals continue to move into the colloidal phase and Ca\textsuperscript{2+} activity increases slightly during evaporation (Walstra & Jenness, 1984). Nieuwenhuijse \textit{et al.} (1988) suggest that changes during the evaporation process are fast enough to overcome most differences induced by preheating.

The concentrate viscosity restricts the maximum concentration that can be achieved without adversely affecting the properties of the spray dried powder (Bloore & Boag, 1981). Factors which affect concentrate viscosity are preheating temperature and holding time, concentrate total solids, and temperature and holding time of the concentrate. Bloore and Boag (1981) observed that preheating at a high temperature, short holding time (113°C for 10 s) produced concentrates with a lower viscosity than concentrates preheated at a low temperature, long holding time (80°C for 120 s), even though the extent of denaturation was similar. The viscosity increases with the total solids content, markedly so above 45% total solids. It becomes necessary to heat the concentrate prior to spray drying, from 50°C to 70°C to reduce the viscosity (Muir, 1980; Bloore & Boag, 1981). The combination of high temperature (>60°C) and holding time can lead to aggregation and gelation, producing powders with poor solubility characteristics (Muir, 1980).

2.5.2.2 Spray drying

Atomization of the concentrate gives a large surface area over which drying can take place. The droplets are brought into immediate contact with a stream of dry heated air (200°C). As the droplet passes through the dryer the moisture evaporates and the temperature of the droplet remains relatively stable (=70°C). The outlet air temperature of the dryer is important as by the time the droplet has reached that point, most of the moisture has been removed and the semi-dried droplet is susceptible to heat damage (Singh & Newstead, 1992).

Little is know about the protein and mineral changes during spray drying. The design and operation of the drier are important factors as they affect the amount of thermal treatment that the concentrate is exposed to. For the most part the properties of reconstituted milk powder preheated at low temperatures are similar to that of the raw
milk. Singh & Creamer (1991a) observed that spray drying had no significant effect on
the denaturation of α-la and β-lg A and B in the manufacture of low heat (72°C for
15 s) and high heat (110-120°C for 2-3 min) powders. The calcium activity of milk
powders immediately after being reconstituted is lower than in raw milk, but increases
linearly with the logarithm of time (Greets et al., 1983). The spray drying step is
thought to have an important effect of Ca²⁺ activity (Greets et al., 1983) though further
work is required to determine what the exact relationship is.
OBJECTIVES

The research work was carried out using skim milk in order to achieve the following objectives.

- Obtain a better understanding of the reactions of milk proteins occurring during preheating, evaporation and spray drying steps of milk powder manufacture.

- Develop mathematical models to describe protein reactions during heat treatment of milk.

- Investigate the effects of artificial and seasonal modifications to milk composition on protein reactions.

As preheating has a large impact on the properties of the milk powder, the whey protein interactions occurring in this step were chosen as the main focus for investigation in this study.
CHAPTER 3: TIME AND TEMPERATURE DETERMINATIONS IN A PILOT SCALE ULTRA-HIGH-TEMPERATURE PLANT

3.1 INTRODUCTION

The temperature/time combination is important in determining the extent of thermal treatment the milk receives during heat processing. When milk flows through a holding tube the individual milk particles move at different velocities. Those particles close to a boundary wall flow more slowly than those at the centre of the holding tube. There are also random variations in the forward flow velocity caused by turbulence. The result is a distribution of flow times, with small proportions of the total flow taking either significantly more or less time than the mean time. The largest proportion of the product will have an intermediate flow time.

The residence time distribution (RTD) shows the distribution of residence times for particles in the system. It is normally represented by the function $E(t)$ (Levenspiel, 1972). The distribution can be normalized so that the area under the curve is unity (Equation 3.1).

$$\int_0^\infty E(t) \cdot dt = 1$$  \hspace{1cm} (3.1)

Two experimental techniques can be used to determine the RTD of a system. A step change can be brought about by suddenly replacing the liquid flowing in the system by another liquid of different chemical composition. Sampling of the output allows the change from the first to the second liquid to be determined. The second method
involves injecting a small volume of a high concentration tracer at the beginning of the flow system. Successive small samples of the output can be quantitatively analyzed for the tracer, or an in line detector can be used. Typically a dye (Heppel, 1985), NaCl tracer (Edgerton & Jones, 1970; O’Callaghan & McKenna, 1974; Sancho & Rao, 1992) or radioactive tracer (Dickerson et al., 1968) is used. Despite the high natural background salt concentration of milk, RTD determinations have been carried out successfully in whole milk using NaCl (Janssen, 1994).

The objectives of the work carried out on the ultra-high-temperature (UHT) plant were to determine the suitability of the RTD method for calculating the holding time and to characterise the temperature changes through the UHT plant, and its affect on the whey protein denaturation in milk.

3.2 MATERIALS AND METHODS

3.2.1 Raw materials

The liquids tested were water and skim milk. Raw whole milk obtained from the No. 1 Dairy farm, Massey University, was separated at 40°C, without pasteurisation and the skim milk stored at 5-8°C. The tracer used was a 20% salt solution.

3.2.2 Equipment

3.2.2.1 Ultra-high-temperature plant

Figure 3.1 Shows a schematic diagram of the UHT plant (type D, Alfa-Laval, Sweden) in which the test liquids were processed. Indirect heating to 65°C prior to the direct steam inject unit (DSI) was used to reduce the temperature step required in the DSI, hence reduce steam input and dilution. The DSI unit was used to provide an instantaneous temperature rise to the required temperature. The heated milk then flowed through the holding tube and into the flash vessel, which was under vacuum. This caused the milk to boil, creating steam, which caused an instantaneous step reduction in the temperature of the milk.
Figure 3.1 Schematic diagram of the pilot-scale UHT plant.
The UHT plant was set-up with a range of different length holding tubes in order to achieve temperature/time combinations suitable for kinetic studies of heat-induced protein reactions. The holding tubes were constructed from stainless steel, outside diameter 12.7 mm, wall thickness 1.2 mm, and were housed in an insulated steel box. The temperature drop across the holding tube was measured by a thermocouple placed in a 90° bend before the RTD conductivity cell. A West 2075 PDI controller (West Instruments, Brighton, England) was used to control the exit temperature from the DSI unit.

3.2.2.2 Residence time distribution equipment

A solenoid driven diaphragm pump with variable stroke (gamma/ 4 1002 ProMinent, Heidelberg, Germany) was used to inject the salt tracer into a 90° bend just prior to the DSI unit. The pump was triggered by an output signal from a personal computer. Two orifice plates were positioned before the DSI unit to ensure good mixing. The test liquid was then passed through the holding tube. Once through the holding tube, two orifice plates provided further mixing before the conductivity cell. The conductivity signal was converted by a transmitter (Model 697-C1A1, Great Lakes Instruments, Milwaukee, USA) into a 4-20 mA signal, and then to the personal computer where it was recorded.

3.2.3 Determination of RTD

The RTD method used was a stimuli response method, but instead of a pulse input, a random sequence was used as the stimuli. The method used was that of Janssen (1994) who used a cross-correlation technique, described by Isermann et al. (1974), to measure the RTD of whole milk in a holding tube. The technique assumes that the process is a linear dynamic system with constant coefficients, and that the process input and outputs are stationary random variables with constant means and variances (Janssen, 1994).
The cross covariance function \( r_{xy} \) is given by Equation 3.2.

\[
r_{xy}(\tau) = \lim_{N \to \infty} \frac{1}{N+1} \sum_{k=0}^{N} (x(k-\tau) - \mu_x)(y(k) - \mu_y)
\]

where

- \( x(k) \) = the process input at time \( k \times \Delta t \).
- \( y(k) \) = the process output at time \( k \times \Delta t \).
- \( \Delta t \) = the sampling interval (s).
- \( \mu_x \) = the mean of the process input.
- \( \mu_y \) = the mean of the process output.
- \( N \) = total number of data points.
- \( \tau \) = range over which Equation 3.2 is calculated.

The autocovariance function \( r_{xx} \) is defined by Equation 3.3.

\[
r_{xx}(\tau) = \lim_{N \to \infty} \frac{1}{N+1} \sum_{k=0}^{N} (x(k-\tau) - \mu_x)(x(k) - \mu_x)
\]

A convolution equation can be used to relate the cross covariance function to the impulse response function \( h(\lambda) \) and the autocovariance function (Equation 3.4).

\[
r_{xy}(\tau) = \sum_{\lambda=0}^{\infty} h(\lambda) r_{xx}(\tau-\lambda)
\]

If the input signal is white noise then the autocovariance function can be given as Equation 3.5. A pseudo random binary sequence (PRBS) can be used as the input
signal. These signals switch randomly between two levels at each sampling interval, and repeat themselves after a set period of time called the sequence length $L$, defined by the shift register length $n$ (Equation 3.6) (Korn 1966). The autocovariance function of a PRBS is similar to that of white noise, but repeats itself at integer values of the shift sequence length.

$$r_{xx}(\tau) \bigg|_{\tau = 0}^{\tau = \pm 1, \pm 2, \ldots}$$

(3.5)

$$L = 2^n - 1$$

(3.6)

The convolution integral (Equation 3.4) simplifies to Equation 3.7 when a PRBS input signal is used. This allows the impulse response function to be calculated directly without the need for a deconvolution procedure.

$$h(\tau) = \frac{1}{\sigma_y^2} r_{xy}(\tau)$$

(3.7)

The PRBS and relative conductivity response from the conductivity cell were analyzed by Equation 3.2 and 3.7. The resulting RTD curve or impulse response function was obtained. The $E(t)$ curve could then be obtained by scaling the RTD curve so that the area under the curve equalled 1.

Tanks-in-series was the one parameter model used to represent the RTD of the holding tubes (Levenspiel, 1972). The model treats the fluid as flowing through a series of equal sized tanks. The number of tanks ($N$) was calculated from the reciprocal of $\delta^2_\theta$ (Equation 3.8). The dimensionless variance $\delta^2_\theta$ was obtained by Equation 3.9.
\[ N = \frac{1}{\delta^2} \]  

(3.8)

Where
\[ \delta^2 = \text{Relative variance} \]

\[ \delta^2 = \frac{\delta^2}{c^2} \]  

(3.9)

Where
\[ \delta^2 = \text{Variance of the RTD curve (s}^2\text{).} \]
\[ c = \text{Mean residence time of E(t) curve (s).} \]

The mean residence time \( (\bar{c}) \) was calculated by Equation 3.10, over an interval \( (k = a \text{ to } k = b) \) encompassing the RTD peak, so that noise from the data was kept to a minimum in the calculations.

\[ \bar{c} = \frac{\sum_{k=a}^{b} t_k C(t_k)}{\sum_{k=a}^{b} C(t_k)} \]  

(3.10)

Where
\[ t_k = \text{Time at } k \times \Delta t \text{ (s).} \]
\[ C(t_k) = \text{Value of the impulse response function at time } t_k. \]
The $E(t)$ curve for comparing water and milk was plotted using dimensionless time units ($\theta$) defined in Equation 3.11.

$$\theta = \frac{t}{\tau}$$

3.2.3.1 Experimental procedure

The test liquid was heated to 65°C by indirect heating in a plate heat exchanger. Various flow rates, DSI temperatures and holding tubes were tested. Once through the holding tube, the fluid passed through the conductivity cell and into the flash vessel where the temperature dropped to~70°C. The flow rate measured at the outlet after the cooling heat exchanger was adjusted to typically ~115 kg h$^{-1}$, ~78 kg h$^{-1}$ or ~140 kg h$^{-1}$. DSI temperatures were varied from 80 to 130°C. The plant was allowed to reach steady state before the RTD measurements began. A PRBS was used to trigger the pump. The pulse volume was set at 0.3 ml per stroke, and the sampling interval ($\Delta t$) was set at 0.5 s with an appropriate shift register so as to give a sequence length longer than the nominal holding time. A shift register of 10 was used for holding times greater than 120 s, while $n = 9$ was used for 30 s to 120 s and $n = 7$ at holding times less than 30 s. Each RTD measurement lasted for 15 to 20 mins, so that enough data could be collected to calculate the RTD.

Milk was passed through a holding tube (nominally 30 s at 115 kg h$^{-1}$) without heating and the RTD recorded. The mean residence time was then compared with the nominal holding time estimated from the holding tube volume and the milk flow rate.

3.2.4 Effect of heating prior to the DSI and DSI heating on native whey proteins

The only significant heat-induced whey protein reactions were assumed to occur solely in the holding tube. To test this, temperature effects prior to the DSI unit and in the DSI unit were investigated.
To determine if indirect heating prior to the DSI unit caused denaturation of whey proteins, skim milk was heated by indirect heating to 60, 65 and 70°C, and held for 21.4 s, after which the milk was cooled by the flash vessel and cooling heat exchanger, and then collected in an ice bucket. To determine the effect of DSI, skim milk at approximately 10°C was heated to 65°C by DSI, without prior indirect heating. The milk was collected in an ice bucket, after passing through the flash vessel and cooling heat exchanger. Sub-samples were ultracentrifuged (175,000 g for 1 h at 20°C), and the supernatants analyzed for the amount of native whey protein using quantitative PAGE as outlined in Section 4.2.3. The total solids of the heated milks and raw milk was determined (Section 3.2.5) and used to correct for any dilution or concentration that may have occurred.

3.2.5 Dilution of the milk by DSI
Skim milk was heated by DSI to achieve a range of temperature rises from 25 to 70°C. The dilution was determined by the change in total solids (A.O.A.C., 1984) of milk collected after DSI compared with the total solids of the raw milk.

3.3 RESULTS AND DISCUSSION
3.3.1 RTD determination in a UHT plant
The injection volume of the salt pump was 0.3 ml, and operating on the PRBS signal injected on average once every 1 s. Thus the extra dilution caused by salt injection amounted to ~1 kg for every hour. The level of dilution and its affect on the residence time was considered insignificant when compared to the flow rates, which were typically 78-140 kg h⁻¹.

Reynolds numbers for milk and water heated at 80°C, with a flow rate of 115 kg h⁻¹, were 7,080 and 11,128, respectively. This indicated that the conditions in the holding tube were turbulent or in the transitional region. The critical Reynolds number is in the range 2000 to 3000, below which the flow is laminar (Sakiadis, 1984). At the slowest flow rate for milk (78 kg h⁻¹) the Reynolds number of 4800 is still above the critical region, therefore the flow within the tubes was not at anytime laminar.
A comparison between the holding time calculated by flow rate and volumetric considerations of the pipe, and the holding time calculated by RTD was undertaken. When unheated skim milk was passed through a holding tube with a nominal holding time of 30 s at 115 kg h⁻¹, the calculated RTD curve had a mean residence time of 30.3 s and variance of 3.85 (Figure 3.2).

![RTD curve of unheated skim milk (10°C) in a 30 s nominal holding tube](image)

**Figure 3.2** RTD curve of unheated skim milk (10°C) in a 30 s nominal holding tube

Some typical RTD curves for water and skim milk are shown in Figures 3.3a and 3.3b, respectively. Dimensionless time units (θ) were used to remove variations in the mean residence time from the E(t) curve, caused by differences in the flow rate. Thus, Figure 3.4 shows the corresponding E(θ) curves for water and skim milk derived from Figures 3.3a and 3.3b. Skim milk displayed a wider distribution than water, and the slight offset of the skim milk peak from 1 was caused by the tail evident in the RTD curve (Figure 3.3b). A comparison of the RTD parameters given in Table 3.1 shows milk to have a larger variance (δ²) than water. These results are essentially in agreement with Heppell (1985) who studied the RTD of water and milk in an infusion UHT plant, and found the variance for milk to be about twice that of water. Heppell (1985)
suggested that the difference in RTD between the two fluids may result from their different viscosities which could affect type of flow behaviour shown.

Figure 3.3a RTD curve of water in a 30 s holding tube heated by DSI to 80°C.

Figure 3.3b RTD curve of skim milk in a 30 s holding tube heated by DSI to 80°C.
3.3.2 Temperature effects in the UHT plant

3.3.2.1 Temperature control and effect of heating equipment

The average DSI exit temperature controlled by the PDI controller was very accurate, variation from the set point was ± 0.14°C (95% C.I.). The effect of preheating
temperature prior to the DSI unit on the denaturation of whey proteins is shown in Figure 3.5. Preheating skim milk in the plate heat-exchanger up to 70°C, and then holding for 21.4 s before cooling, had no effect on the levels of native β-lg A, β-lg B, α-la and BSA. This was not surprising since the critical temperature at which irreversible denaturation starts to occur has been reported to be about 70°C (de Wit & Swinkels, 1980). The actual holding time from the plate heat-exchanger to the DSI unit is 6.4 s, and is therefore not long enough to have any significant effect on the whey proteins.

![Figure 3.5](image-url)  
**Figure 3.5** Effect of heating temperature prior to the DSI unit on native whey proteins in skim milk. Whey proteins; β-lg A (○), β-lg B (●), α-la (△) and BSA (●).
Figure 3.6 shows the effect of steam condensation in the DSI unit on the denaturation of whey proteins. It is clear that DSI had no significant effect on the level of native whey proteins. Because the milk also passed through the flash vessel and cooling heat exchanger before being collected, the rate of cooling after the holding tube did not have a significant effect either.

![Graph showing integrated intensity for whey proteins](image)

**Figure 3.6** Effect of DSI on native whey proteins in skim milk. Whey protein level of original milk (raw) compared with those after the DSI (DSI). Error bars show the 95% confidence intervals.

3.3.2.2 Temperature drop across the holding tube

A temperature drop was observed across the holding tube, and typically increased with holding time and heating temperature of the DSI unit. Examples of temperature drops are shown in Table 3.2. The temperature drops were recorded and used in the analysis of heat-induced whey protein denaturation as will be described in Chapter 4.
Table 3.2 Typical temperature drops for different holding times and DSI exit temperatures.

<table>
<thead>
<tr>
<th>Holding time (s)</th>
<th>DSI Temperature (°C)</th>
<th>Temperature drop (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.7</td>
<td>110</td>
<td>1.43</td>
</tr>
<tr>
<td>12.7</td>
<td>90</td>
<td>1.18</td>
</tr>
<tr>
<td>12.7</td>
<td>70</td>
<td>0.97</td>
</tr>
<tr>
<td>32.6</td>
<td>130</td>
<td>2.75</td>
</tr>
<tr>
<td>33.1</td>
<td>90</td>
<td>1.36</td>
</tr>
<tr>
<td>32.6</td>
<td>70</td>
<td>0.89</td>
</tr>
<tr>
<td>108.8</td>
<td>120</td>
<td>3.87</td>
</tr>
<tr>
<td>108.8</td>
<td>90</td>
<td>2.13</td>
</tr>
</tbody>
</table>

3.3.3 Effect of dilution by DSI on heat-induced whey protein reactions

The dilution of milk by condensing steam in the DSI unit was 4.8% for a temperature step of 25°C. The maximum possible temperature step of 70°C diluted the milk by 11.0%. The maximum step normally used in the DSI unit was 65°C, i.e. 65°C indirect heating up to 130°C DSI temperature. The effect of dilution will be discussed in Chapter 6, where the milk was diluted by 35%, approximately a three fold increase in the maximum dilution observed on the UHT plant. There was no major affect on the rates of denaturation and interactions of the whey proteins in skim milk at 35% dilution. So dilution of 11% caused by DSI is assumed to have no significant affect on whey protein denaturation and interactions in skim milk.

3.4 CONCLUSIONS

The RTD method of Janssen (1994) was found to be suitable for determining the residence time of skim milk in the UHT holding tubes.
Temperature conditions and their effects on whey protein denaturation in the UHT plant were characterised. Indirect heating to 65°C, prior to DSI, had no significant effect on the whey proteins in skim milk. The effect of DSI on the whey proteins, either by condensation of steam or dilution of the product stream, was insignificant. Therefore, it can be concluded that all the heat-induced whey protein reactions are occurring in the holding tube.
CHAPTER 4: KINETICS OF HEAT-INDUCED DENATURATION AND INTERACTIONS OF PROTEINS IN SKIM MILK

4.1 INTRODUCTION

Heat treatment of milk in the manufacture of dairy products causes significant physico-chemical changes in the milk constituents. Heat-induced changes in the milk proteins, in particular denaturation of the whey proteins, are important in determining the functional properties of the final product. Depending on the type of product, the extent of whey protein denaturation can be varied. For example milk is preheated to produce milk powders with high levels of whey protein denaturation, to be used in yoghurt manufacture, bakery, confectionary and meat products where protein interactions are important in developing the textural properties required. Excessive heating of milk can also be detrimental, such as in the manufacture of cheese.

Denaturation of whey proteins has been measured by numerous authors in a number of model and real systems, using a wide range of methods; DSC (Park & Lund, 1984), serological activity (Baer et al., 1976), solubility at pH 4.5, specific optical rotation (Harwalker, 1980b), electrophoretic techniques (Hillier & Lyster, 1979; Dannenberg & Kessler, 1988a) and FPLC (Manji & Kakuda, 1986). Each method measures a different aspect of protein denaturation, so it is not surprising that the results differ, considering the complexity of the reaction and the influence of other milk components on denaturation.

Denatured whey proteins can interact with each other to form aggregates. The formation of whey protein aggregates is primarily through thiol-disulphide interchange reactions. An important interaction in milk is the association between the principal whey protein, β-lg, and the casein micelle, specifically κ-casein (Smits & Brouwershaven, 1980). Previous studies have mainly investigated denaturation, aggregation and association steps
in isolation, although in milk it is not clear how these individual steps interact. Denaturation kinetics of whey proteins have been studied extensively (Donovan & Mulvihill, 1987; Dannenberg & Kessler, 1988a), but the reaction kinetics of whey protein interaction and whey protein/casein micelle interaction have not been as thoroughly reported.

This chapter describes the heat-induced changes of milk proteins in skim milk, processed by direct steam injection on a UHT plant. Using quantitative polyacrylamide gel electrophoresis the heat-induced interactions, denaturation, aggregation and association with the casein micelle, of individual whey proteins were determined. These reactions were then modelled using reaction kinetics.

4.2 MATERIALS AND METHODS

4.2.1 Milk supply
Raw whole milk (Friesian herd, 225 cows) was obtained from the No. 1 dairy farm, Massey University on five separate occasions over a two week period in July 1993 (autumn calving; feed content, half grass and the other half maize and silage supplement). Bulk whole milk was also obtained from the Tui Dairy Company, Longburn, in March 1994. The milk obtained from the No. 1 dairy farm was separated at the Food Technology pilot plant, Massey University (Alfa-Laval Hermetic milk separator s2181 m), at 40°C. Bulk whole milk from the Tui Dairy Company was separated at the New Zealand Dairy Research Institute’s (NZDRI) processing hall, at 55°C. Both milks were separated without pasteurisation and stored at 5°C.

4.2.2 UHT processing

4.2.2.1 Holding tubes
Skim milk obtained during July 1993 was processed on a pilot scale UHT plant, as described in Section 3.2.2. For each run, the skim milk was first heated to approximately 65°C by a plate heat exchanger. Direct steam injection (DSI) was then used to achieve the desired temperature. DSI allowed for extremely short come-up times. A range of temperatures 70-130°C and holding times (3-160 s) were investigated. After the holding tube, a flash vessel was used to reduce the temperature to
approximately 65°C. The skim milk was further cooled by a shell and tube heat exchanger to 50-40°C. The skim milk was then collected and cooled in an ice bucket. The residence time distribution was computed for each run as described in Chapter 3.

4.2.2.2 Water bath
The combination of holding tubes and flow rates in the UHT plant could give holding times from 5 to 160 s. For longer holding times a water bath (Grant Instruments Ltd., Cambridge, England) was used to heat the skim milk. The skim milk obtained in March 1994 was heated to approximately 65°C, after which DSI was used to obtain the required temperature (70-90°C). Immediately after the DSI unit, the milk was passed through a valve used to maintain back-pressure and was then collected in a screw cap glass test tube (125 mm length, 20 mm diameter, 1 mm wall thickness). The test tubes were immediately placed into the water bath at the required temperature and a stop watch was started. Prior to their use the test tubes were kept in the water bath at the heating temperature. After the required heating time the tubes were cooled in an ice bucket.

4.2.3 Milk protein analysis
Skim milk samples were held overnight at 5°C, then warmed to 20°C and held for at least one hour before ultracentrifugation. The samples were sealed in disposable plastic centrifuge tubes (13.5 ml, part No. 344322, Beckman, Palo Alto, California) and placed in a Type 80 Ti rotor (Beckman, Palo Alto). Ultracentrifugation was carried out in a Beckman L8-80M centrifuge at 50,000 RPM (average 175,000 g) for 1 h at 20°C. After centrifugation the top of each tube was cut open and the all the supernatant was carefully removed. The supernatants were then analyzed for individual whey proteins by polyacrylamide gel electrophoresis (PAGE) under non-dissociating and dissociating conditions and IgG was determined by affinity chromatography as described below. Some of the casein pellets were also analyzed by PAGE under dissociating conditions.
4.2.3.1 Concentration and dilution effects

So that the concentration of whey proteins in the supernatant could be converted to their relative concentration in skim milk, the effect of casein removal by ultracentrifugation and the gain or loss of water by DSI and flash cooling were calculated.

The whey protein concentration in the supernatant differs from that in skim milk. The removal, by ultracentrifugation, of casein and the small amount of fat that remains in skim milk alters the volume, as does water bound to the protein and also water not available as solvent through steric exclusion at the micelle surface (Walstra & Jenness, 1984; van Boekel & Walstra, 1989). Correction factors for converting whey protein concentration in the supernatant to that in the original skim milk were determined. The correction factor $F^*$ was calculated from the weight of skim milk and the casein pellet after centrifugation (Equation 4.1). The correction factor $F$ was calculated using Equation 4.2. The coefficient 1.01 is needed to convert the gravimetric fat content to content of fat globules, the coefficient 1.08 is needed to correct for the micellar colloidal calcium phosphate content and $h$ is the steric exclusion factor for serum proteins (van Boekel & Walstra, 1989). Once the $F$ value was calculated, the concentration of whey protein in the milk can be calculated from the concentration in the supernatant using Equation 4.3.

\[
F^* = \frac{M - Cas}{M} \tag{4.1}
\]

Where

- $F^*$ = correction factor based on casein pellet weight.
- $M$ = weight of milk in centrifuge tube (g).
- $Cas$ = weight of casein pellet in centrifuge tube (g).
\[ F = 1 - 1.01 f - 1.08 c - h c \]  
\[ (4.2) \]

Where

- \( F \) = correction factor.
- \( f \) = fat content of milk (kg kg\(^{-1}\)).
- \( c \) = casein content of milk (kg kg\(^{-1}\)).
- \( h \) = exclusion factor (kg water kg\(^{-1}\) casein).

\[ C_{\text{milk}} = C_{\text{super}} F \]  
\[ (4.3) \]

Where

- \( C_{\text{milk}} \) = concentration of individual whey proteins in milk (g kg\(^{-1}\)).
- \( C_{\text{super}} \) = concentration of individual whey proteins in supernatant (g kg\(^{-1}\)).

The changes in the concentration of protein components in heated milk, due to steam injection and water removal in the flash vessel, were determined by comparing the protein nitrogen (PN) content of the heated milk sample with that of raw milk. Protein nitrogen was calculated by subtracting non-protein nitrogen (NPN) values from the total nitrogen (TN) values. Both heated and raw milks were analyzed for nitrogen by the Kjeldhal method (Rowland, 1955; IDF, 1986) using a Kjel-Foss automatic 16200 (Foss Electric, Hillerød, Denmark) at the Analytical Chemistry Section, New Zealand Dairy Research Institute.

4.2.3.2 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was performed on a mini-gel electrophoresis unit (Biorad Laboratories, Richmond, CA, USA), under non-dissociating (native-PAGE), dissociating but non-reducing (SDSNR-PAGE) and dissociating and reducing (SDSR-PAGE) conditions as described by Singh and Creamer (1991a). All the
reagents were made from Analar grade chemicals (Biorad Laboratories, Richmond, California; BDH Chemical Ltd., Poole, England; United States Biochemical Corp., Cleveland, Ohio), and deionised water was used in their preparation (MilliQ reagent water system, Millipore Corporation, Bedford, MA, USA).

**Sample preparation**

The supernatant samples were diluted 1:4 in sample buffer. Native-PAGE buffer was composed of 50 ml of a 0.5 M Tris(hydroxymethyl)aminomethane (Tris/HCl) buffer, 2 ml glycerol and 5 ml of a 0.1% (w/v) bromophenol blue solution (tracking dye) made up to 250 ml with deionised water, pH 6.8. It was assumed that the absence of dissociating agent in the sample buffer meant that aggregates stayed intact and were unable to enter the resolving gel. The native protein is assumed to remain as a monomer under the electrophoretic running conditions used and separate according to its native charge and molecular weight. The SDSNR sample buffer contained 12.5% (v/v) 0.5 M Tris/HCl, 10% glycerol, 20% sodium dodecyl sulphate (SDS) stock (10% (w/v) SDS), 2.5% bromophenol blue stock (0.05% w/v) and made up to the required volume with MilliQ water, pH 6.8. It is assumed that SDS dissociates all aggregates into monomeric protein except disulphide-linked aggregates. These aggregates are presumably dissociated in the SDSR buffer, which is the same as the SDSNR but contains 5% 2-mercaptoethanol (2-ME). After the SDSNR samples were diluted with buffer they were heated for 15 min at 40°C. Similarly the SDSR samples were heated at 95°C for 10 min.

Casein pellets were dissolved in 20 ml of SDSNR sample buffer (bromophenol blue dye was not added). The mixture was stirred for 48 h at 5°C, after which 0.5 ml of bromophenol blue stock (0.05% w/v) was added. A portion of the solution (1.5 ml) was taken and designated the SDSNR sample, and this sample was heated at 40°C for 15 min. To 1.425 ml of the solution 0.075 ml of 2-ME was added. This sample was heated at 95°C for 10 min, and designated the SDSR sample.
Native-PAGE

Native (unaggregated) whey protein was separated by native-PAGE in the absence of dissociating agent. A 30% (w/v) stock solution of acrylamide was prepared from an acrylamide/N,N'-methylene-bis-acrylamide mixture, in a ratio of 37.5:1 (2.6% cross-linking). The resolving gel buffer was 3 M Tris/HCl adjusted to pH 8.8. The stacking gel buffer was a 0.5 M solution of Tris/HCl at pH 6.8. A stock solution of electrode buffer was prepared from, 7.5 g of Tris and 36 g of glycine, made up to 500 ml with deionised water, pH 8.3. All reagents were stored at 4°C.

The resolving gel was composed of 10 ml of 30% acrylamide stock, 2.5 ml of resolving gel buffer and 7.5 ml of deionised water. The gel solution was degassed for 15 min using a magnetic stirrer and a water aspirator. Once degassed, 10 μl of N,N,N',N'-tetramethylenediamine (TEMED) and 100 μl of a freshly made ammonium persulphate solution (10% w/w) were added and gently mixed. The resolving gel (3.6 ml) was poured between two glass plates and overlaid with deionised water to ensure a level gel surface. The resolving gel was left at room temperature for 45 min to set, after which the water was removed from the top of the resolving gel prior to application of the stacking gel. For the preparation of the stacking gel the following solutions were mixed; 1.25 ml of acrylamide stock, 2.5 ml of resolving gel buffer and 6.25 ml of deionised water. After degassing, 10 μl of TEMED and 50 μl of ammonium persulphate (10% w/w) were added and gently mixed. The stacking gel was poured on top of the set resolving gel and a 10-slot comb inserted. Before the gel was run, the comb was removed and the wells, formed by the comb, were rinsed with deionised water.

SDSNR- and SDSR-PAGE

The resolving gel buffer was 1.5 M Tris/HCl buffer, pH 8.8. The stacking gel buffer and stock acrylamide were the same as described for native-PAGE under Section 4.2.3.2. A stock solution of electrode buffer was prepared from 9 g of Tris-base, 43.2 g glycine and 3 g of SDS, made up to 600 ml with deionised water, pH 8.3.

The resolving gel was prepared by adding 2.02 ml of deionised water to 2.5 ml of 1.5 M Tris/HCl, 5.3 ml acrylamide stock and 100 μl of a 10% (w/v) SDS stock. The resolving
gel was degassed and 5 µl of TEMED and 50 µl of freshly made ammonium persulphate (10% w/w) were added and mixed gently. The resolving gel (3.6 ml) was poured between two glass plates and overlaid with deionised water. The resolving gel was left at room temperature for 45 min to set and the water was removed from the top of the resolving gel prior to application of the stacking gel. The stacking gel was prepared from 1.3 ml of acrylamide stock, 2.5 ml of stacking gel buffer, 100 µl of the SDS stock and 6.1 ml of deionised water. After degassing, 10 µl of TEMED and 50 µl of ammonium persulphate were added and gently mixed. The stacking gel was poured on top of the set resolving gel and a 10-slot comb inserted. Before running the gel, the comb was removed and the wells, formed by the comb, were rinsed with deionised water to remove unpolymerised gel solution.

Electrophoretic running of the gel
Two gels were placed into an electrode buffer chamber. The stock electrode buffer solution was diluted 1:4 with deionised water and used to fill the inner buffer chamber. A Hamilton syringe (Hamilton company, Reno, Nevada, USA) was used to inject 10 µl of sample into a well. Both native and SDS gels were run at 200 V until the tracking dye (bromophenol blue) had run off the bottom of the resolving gel.

Staining and destaining
The gels were removed from the casting assembly and put into plastic containers containing 100 ml of staining solution (1 g of coomassie blue (R) in 500 ml of isopropanol and 200 ml of acetic acid made up to 2 L with distilled water). The gels were then gently rocked for 1 h to provide uniform staining. The staining solution was then poured out and replaced by 100 ml of destaining solution composed of 1:1:8 acetic acid/isopropanol/distilled water. After rocking for 1 h the destaining solution was replaced with 100 ml of fresh destaining solution and left to rock for a further 19 hours. The gels were then scanned using a densitometer.

Quantitative determination of individual whey proteins by densitometry
The gels were scanned on a densitometer (Computing Densitometer, Molecular Dynamics, Sunnyvale, California 94086) and the integrated intensities of the
\(\beta\)-lactoglobulin, \(\alpha\)-lactalbumin and BSA protein bands were calculated by a software program, ImageQuant (Molecular Dynamics).

Normally, supernatants of heated skim milk samples together with a supernatant of the unheated skim milk were run on separate lanes. In order to determine the concentration of individual whey proteins in the heated skim milk samples, the supernatant of the unheated skim milk was run on a separate gel together with protein standards of different concentrations. Standard curves were used to determine individual whey protein concentrations in the unheated sample, so that the band intensities in all the gels could be converted to concentration units (g kg\(^{-1}\)). The following purified proteins were used to prepare the protein standards; \(\beta\)-lg A (Sigma No. L-7880 lot 13H7020, Sigma Corp, St. Louis, MO, USA), \(\beta\)-lg B (Sigma No. L-8005, lot 13H7150), \(\beta\)-lg (Sigma No. L-0130, lot 51H7210), \(\alpha\)-la (Sigma No. L-6010, lot 128F8140) and BSA (Sigma No. A-3803, Lot 113H0584). The concentration of individual whey proteins determined in the supernatant were converted to their equivalent concentration in milk by using the correction factors for ultracentrifugation, DSI and flash cooling (Section 4.2.3.1).

**Interpretation of PAGE results**

The PAGE methods used enabled the following to be determined by subtracting the relative band intensities shown in Figure 4.1 (the unheated sample on each gel was taken as the 100% reference point):

(i) the amount of native whey protein in the supernatant;

native-PAGE; the loss of native protein from this PAGE was considered as "denaturation"

(ii) the amount of non-covalently linked aggregated (e.g. hydrophobic) whey protein in the supernatant;

SDSNR-PAGE less native-PAGE.

(iii) the amount of disulphide-linked aggregated whey protein in the supernatant;

SDSR-PAGE less SDSNR-PAGE.

(iv) the amount of whey protein associated with the casein micelles;

Total whey protein less SDSR-PAGE.
Figure 4.1 Diagrammatic representation of the loss of electrophoretic band intensities from the different gel types, and their interpreted results.

4.2.3.3 Affinity Chromatography

Immunoglobulin G analysis was performed on an affinity column (HiTrap affinity column. Protein G, Pharmacia, Uppsala, Sweden) according to the method of Kinghorn et al. (1995). The affinity column was attached to a HPLC system, consisting of a Waters 490 programable multiwavelength detector, Waters associates chromatography pumps and Waters WISP710B (Millipore Corp., Waters Chromatography Division, Milford, Massachusetts, USA). Supernatant samples were left undiluted or were diluted up to five times in a 0.05 M NaH₂PO₄.2H₂O buffer, pH 6.5. A 100-200 µl volume of the sample was run through the column in the same buffer, at a flow rate of 2 ml min⁻¹. The protein bound to the column and then eluted off using 0.05 M glycine buffer at pH 2.5. The protein in the eluent was determined by measuring the absorbance at 280 nm. The IgG samples and standards (Sigma I-5506, lot 103H-8812) eluted off the column as a distinct peak, and the sample concentrations were determined from the standards calibration graph. A computer program (Millennium 2010 chromatography manager, Millipore Corp.) computer program was used to control the running of the column and analyse the peaks.
4.2.4 Statistical analysis of kinetic data

The reactions which occur are complex in nature and the interactions between individual whey proteins are not fully understood. Despite this complexity, the general rate Equation (4.4) and the Arrhenius Equation (4.5) were used to model the loss of native whey protein in heated skim milk. Five different methods of data analysis were used (Levenspiel, 1972; Wilkinson, 1981) and their precision analyzed by a statistical software package (SPSS version 4.0.1, SPSS Inc., Chicago).

\[-\frac{dC}{dt} = k_n C^n\]  \hspace{1cm} (4.4)

Where

\[
\begin{align*}
C & = \text{protein concentration (g kg}^{-1}\text{).} \\
t & = \text{time (s).} \\
k_n & = \text{rate constant ((g kg}^{-1})^{(1-n)} \text{ s}^{-1}). \\
n & = \text{reaction order.}
\end{align*}
\]

\[
k_n = k_o e^{-\frac{E_a}{RT}}\]  \hspace{1cm} (4.5)

Where

\[
\begin{align*}
k_o & = \text{pre-exponential term ((g kg}^{-1})^{(1-n)} \text{ s}^{-1}). \\
E_a & = \text{activation energy (kJ mol}^{-1}). \\
R & = \text{universal gas constant (8.314 J mol}^{-1} \text{ K}^{-1}). \\
T & = \text{temperature (K)}.
\end{align*}
\]

The activation energy \((E_a)\) is the kinetic energy of reactant molecules, that is necessary to achieve sufficiently close contact between them for the reaction to occur (van Boekel
The pre-exponential term \( k_0 \) represents the value of the rate constant in the absence of the activation energy constraint (if \( E_a = 0 \), or \( T = \infty \)). (van Boekel & Walstra, 1995).

4.2.4.1 Two-Step method for obtaining Arrhenius parameters \( E_a \) and \( k_0 \) (linear regression)

The two-step method is used frequently for calculating a reaction rate expression (Hill & Grieger-Block, 1980). The first step was to determine the rate constant from the integrated general rate equation (Equation 4.4). This equation for \( n \neq 1 \) and \( n = 1 \) is given by Equations 4.6 and 4.7, respectively.

When \( n \neq 1 \).

\[
\left( \frac{C_t}{C_0} \right)^{1-n} = 1 + (n-1) k_n C_0^{n-1} t
\]  

(4.6)

Where

\( C_t \) = concentration of undenatured protein at \( t = t \) (g kg\(^{-1}\)).

\( C_0 \) = concentration of undenatured protein at \( t = 0 \) (g kg\(^{-1}\)).

When \( n = 1 \) (first order reaction).

\[
\ln \left( \frac{C_0}{C_t} \right) = k_n t
\]  

(4.7)

First, the left hand side of the equation was plotted against time and from the slope \( k_n \) was calculated. The second step was to plot \( \ln(k_n) \) against \( 1/T \) so that the Arrhenius
equation (Equation 4.5) could be used to calculate $E_a$ and $k_0$ from the slope and intercept respectively.

4.2.4.2 Two-Step method with adjusted intercept (linear regression)
In this method the Arrhenius equation was modified so that a reference temperature was used in the exponential term (Equation 4.8). This modification created a new pre-exponential term, $k_{ref}$, which is related to $k_0$ by Equation 4.9.

$$k_n = k_{ref} e^{-\frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T_{ref}} \right)} \tag{4.8}$$

Where
- $k_{ref} = \text{reference pre-exponential term (g kg}^{-1}(1-n) s^{-1})$
- $T_{ref} = \text{reference temperature (K)}$

$$\ln k_0 = \frac{E_a}{R T_{ref}} + \ln k_{ref} \tag{4.9}$$

Calculation of $k_0$ from Equation 4.5, involved finding the intercept at $1/T=0$ or when $T\rightarrow\infty$. This point is a long way from where the data points lie on the Arrhenius plot, so a small change in $E_a$ causes a large change in $k_0$. Linear regression assumes the parameters can be determined independently. By placing the intercept in the middle of the data set, $k_{ref}$ becomes independent of the slope ($E_a$), and the estimated error in the pre-exponential term will be reduced (Equation 4.8). Thus for a given temperature range, the average temperature was designated as $T_{ref}$.

4.2.4.3 Scale transformation (linear regression)
Scale transformation of the concentration/time data was carried out by substituting Equation 4.5 into Equation 4.6 and rearranging (Equation 4.10). Plotting the left hand
side versus 1/T yields $E_a$ and $k_0$ from the slope and intercept respectively (Wilkinson, 1981, Arabshahi & Lund, 1985).

\[
\ln\left(\frac{\left(\frac{C_t}{C_0}\right)^{\frac{1-n}{n-1}}}{C_0^{n-1}t}\right) = -\frac{E_a}{R} \frac{1}{T} + \ln k_0
\]  

(4.10)

4.2.4.4 Nonlinear Regression (NLR)

Equation 4.5 was substituted into Equation 4.6 and then rearranged to make $C_t$ the subject (Equation 4.11).

When $n \neq 1$.

\[
C_t = C_0 \left[1 + (n-1) k_0 C_0^{n-1} t e^{-\frac{E_a}{RT}(\frac{1}{T^\frac{1}{1-n}})}\right]
\]  

(4.11)

NLR was used to fit the concentration/time data at a range of temperatures selected, directly into Equation 4.11. This treats all the data as having equal absolute precision. The statistical package SPSS uses a Levenberg-Marquardt algorithm (Bates & Watts, 1988). The equation allows for the program to fit the following parameters; $E_a$, $k_0$, reaction order ($n$) and initial concentration ($C_0$). Suitable starting estimates of these parameters, determined by the two-step method and native-PAGE analysis, were put into the program.

4.2.4.5 NLR with adjusted intercept

As in the two-step method with an adjusted intercept, the Arrhenius term in the NLR equation (Equation 4.11) can be modified using a reference temperature (Equations 4.12
and 4.13). This reduces the possibility for uncertainty in $E_a$ to influence the estimate of the intercept (See section 4.2.4.2).

When $n \neq 1$. 

$$C_t = C_0 \left[ 1 + (n-1) k_{ref} C_0^{n-1} t \ e^{-\frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T_{ref}} \right)} \right]^{\frac{1}{1-n}} \quad (4.12)$$

When $n = 1$ (first order reaction).

$$C_t = C_0 \ e^{k_{ref} \ t \ e^{-\frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T_{ref}} \right)}} \quad (4.13)$$

4.2.5 Statistical analysis of the rate constant used in the two-step method

In the first step of the two-step method, the rate constant is determined from the slope obtained by Equation 4.6. The standard error of the slope was calculated by Equation 4.14.

$$\sigma_{B1} = \frac{S}{\sqrt{(N - 1) S_x^2}} \quad (4.14)$$

Where

$\sigma_{B1}$ = standard error of the slope.

$S$ = standard deviation of the residuals.

$N$ = total number of data points.

$S_x$ = standard deviation of the dependent variable ($t$).
The standard deviation of the residuals ($S$) is given by Equation 4.15.

$$S = \sqrt{\frac{\sum_{i=1}^{N} (Y_i - B_0 - B_1 X_i)^2}{(N - 2)}}$$ (4.15)

Where

$Y_i$ = dependent variable \((C/C_0)^{1-s}\).

$B_0$ = estimate of the intercept.

$B_1$ = estimate of the slope.

$X_i$ = independent variable \((t)\).

The standard deviation of the independent variable ($S_x^2$) is given by Equation 4.16.

$$S_x^2 = \frac{\sum_{i=1}^{N} (X_i - \bar{x})^2}{(N - 1)}$$ (4.16)

Where

$\bar{x}$ = mean of the independent variable \((t)\).

A rate constant \((k)\) can be defined as Equation 4.17.

$$k = k_n C_0^{(n-1)}$$ (4.17)

Where

$k$ = concentration dependent rate constant \((s^{-1})\).
This rate constant was calculated from the slope and its standard error is given by Equation 4.18.

\[ F_{ek} = \sqrt{F_{en}^2 + F_{Bl}^2} \]  

(4.18)

Where

- \( F_{ek} \) = standard error of \( k \) expressed as a fraction \( k \).
- \( F_{en} \) = standard error of \( n \) expressed as a fraction \( (n - 1) \).
- \( F_{Bl} \) = standard error of \( \sigma_{Bl} \) expressed as a fraction \( \sigma_{Bl} \).

Values of \( n \) at each temperature were determined by linear regression of Equation 4.6. The standard deviation of these values over the square root of the number of temperatures, was used to calculate the standard error of \( n \).

### 4.2.6 Statistical analysis used in the mathematical model

A mathematical model for \( \beta \)-lg association with the casein micelle has been proposed and discussed later (see Section 4.3.5). The statistical analysis was carried out using the program derived from the model (Appendix A1). Predicted and experimental data were compared using a weighted least squares analysis (Equation 4.19).

\[ SSE = \sum_{n=1}^{N} \left( Y_n - \hat{Y}_n \right)^2 \frac{1}{y e_n^2} \]  

(4.19)

Where

- \( SSE \) = Sum of squares of residuals.
- \( Y_n \) = Experimental value (mol \( \times \) 10\(^6\) kg\(^{-1}\)).
- \( \hat{Y}_n \) = Predicted value (mol \( \times \) 10\(^6\) kg\(^{-1}\)).
- \( y e_n \) = Relative error of the \( n \) th experimental data point.
The total sum of squares ($SST$) was calculated by Equation 4.20, and the regression sum of squares ($SSR$) was calculated by Equation 4.21.

$$SST = \sum_{n=1}^{N} (Y_n - \overline{Y})^2$$ \hspace{1cm} (4.20)

Where

$\overline{Y}$ = mean of the predicted values (mol x $10^6$ kg$^{-1}$).

$$SSR = SST - SSE$$ \hspace{1cm} (4.21)

Accordingly the mean sum of regression ($MSSR$) and residuals ($MSSE$) were calculated by Equation 4.22 and 4.23, respectively. The associated degrees of freedom of $SSR$ are the number of terms in the model ($p$), 2 in this case, minus 1.

$$MSSR = \frac{SSR}{(p - 1)}$$ \hspace{1cm} (4.22)

The degrees of freedom for $SSE$ is the total number of data points ($N$) minus $p$.

$$MSSE = \frac{SSE}{(N - p)}$$ \hspace{1cm} (4.23)

The F-statistic was used to test the significance of the model to the experimental data (Equation 4.24).
The mean total sum of squares (MSST) (Equation 4.25) was used in calculating the adjusted $R^2$ (Equation 4.26).

\[
F = \frac{MSSR}{MSSE} \tag{4.24}
\]

\[
MSST = \frac{SST}{(N - 1)} \tag{4.25}
\]

\[
R^2 = 1 - \frac{MSSE}{MSST} \tag{4.26}
\]

4.3 RESULTS AND DISCUSSION

4.3.1 PAGE analysis

Typical electrophoretic patterns for native-, SDSNR- and SDSR-PAGE are shown in Figures 4.2, 4.3 and 4.4, respectively. The intensities of native $\beta$-lg A, $\beta$-lg B, $\alpha$-la and BSA bands decreased as heating time at 75 and 80°C increased (Figure 4.2). In heated samples, some aggregated protein material was excluded from the resolving gel and remained at the top of the stacking gel. SDSNR-PAGE showed that the intensities of $\beta$-lg, $\alpha$-la and BSA bands decreased with an increase in heating time (Figure 4.3), but at a slower rate than for the native-PAGE (Figure 4.2). In the SDSNR system non-covalently linked protein aggregates were dispersed and migrated into the resolving gel, but disulphide-linked aggregates remained at the top of the stacking and resolving gels. In the SDSR-PAGE, all the aggregates were dissociated and their constituent proteins migrated into the resolving gel. The decrease in the intensities of $\alpha$-la, $\beta$-lg and BSA bands on SDSR-PAGE, which was much less than the native- and SDSNR-PAGE patterns, was due to sedimentation of denatured whey protein along with the casein
pellet. The casein proteins in the supernatant were resolved as three discrete bands on SDS-PAGE; \(\alpha_\text{r}-\text{casein}\), a combination of \(\alpha_{1\text{-}}\text{-casein}\) and \(\alpha_{2\text{-}}\text{-casein}\), \(\beta\)-casein and \(\kappa\)-casein.

From the densitometer readings, the whey protein concentrations were determined in the supernatant and converted to their equivalent concentrations in milk. The correction factor \(F\) was calculated from Equation 4.2, using an \(h\) value of 2.6 (van Boekel & Walstra, 1989) and data obtained for the fat and casein content of the skim milk, 0.0004 (kg/kg) and 0.0224 (kg/kg), respectively. The fat content in skim milk is negligible and was not considered in the calculation. The casein content was calculated as being 80% of the protein content in raw milk. The \(F\) value of 0.917 obtained using Equation 4.2 compared well with the \(F^*\) value calculated from the weights of the casein pellets after centrifugation, 0.912 ± 0.024 (95% confidence interval). The \(F^*\) value of 0.912 was used to convert the concentration of whey proteins in the supernatant to their equivalent concentrations in milk. The average protein nitrogen of the original skim milks was 0.439 %(w/w). The protein nitrogen of the skim milk samples after DSI and flash cooling averaged approximately 98% of the original skim milk value.

**Figure 4.2** Native-PAGE electrophoretic patterns of the ultracentrifugal supernatants obtained from heated skim milk samples. Lane description: (1 & 2) standard whey solution; skim milk samples heated at 75°C for, (3) 600 s, (4) 900 s, (5) 1200 s and (6) 1800 s; samples heated at 80°C for, (7) 300 s and (8) 600 s; lane (9) unheated skim milk sample.
**Figure 4.3** SDSNR-PAGE electrophoretic pattern of the ultracentrifugal supernatants obtained from heated skim milk. For lane description see Figure 4.2

**Figure 4.4** SDSR-PAGE electrophoretic patterns of ultracentrifugal supernatants obtained from heated skim milk samples. For lane description see Figure 4.2.
4.3.2 Kinetics of whey protein denaturation in skim milk

The changes in native protein level with time, of β-lg A, β-lg B, α-la, BSA and IgG were determined at temperatures in the range 70-130°C; data obtained at 100°C and 120°C by native-PAGE are shown in Figure 4.5.

**Figure 4.5** Loss of individual whey proteins from native-PAGE gels of ultracentrifugal supernatants obtained from skim milk heated at 100°C, β-lg A (○), β-lg B (▲), α-la(□), and 120°C, β-lg A (●), β-lg B (▲), α-la(■).

The temperature/time profiles showed a decrease in the amounts of native whey proteins with increasing holding time and/or temperature. β-Lg B was found to denature faster than β-lg A at all temperatures (70-130°C). The denaturation of α-la was significantly slower than the two genetic variants of β-lg. The densitometer results of BSA were too variable at temperatures above 80°C to obtain reliable results, and above 90°C IgG was totally denatured (>90%) after only a few seconds. The concentration/time data at the various temperatures were then used to determine the kinetics of whey protein denaturation.
4.3.2.1 Comparison of statistical methods of data analysis

Several different methods of statistical data analysis were investigated in order to determine their precision in determining the kinetic constants. The concentration/time data of native β-lg A, determined by native-PAGE, was used to test the different methods. As a break in the Arrhenius plot is observed at 90°C (Dannenberg & Kessler, 1988a; Manji & Kakuda 1986), a temperature region of 100-130°C was chosen to avoid this break. In the adjusted intercept methods (see Section 4.2.4.2 & 4.2.4.5) the reference temperature chosen was 115°C, being in the middle of the temperature range 100-130°C. Thus, $k_{ref}$ and $T_{ref}$ were renamed $k_{115}$ and $T_{115}$, respectively. The data used were unweighted so that all the data points were treated equally by the program. The apparent reaction order ($n$) for the denaturation of β-lg A was found to be 1.5, using least squares regression analysis on Equation 4.6, and this value was subsequently used in all the linear regression methods.

Using the two-step method, Equation 4.6 was plotted (Figure 4.6a). From the slopes the rate constants were determined along with their 95% confidence intervals (Table 4.1). The 95% confidence intervals (C.I.), degrees of freedom (d.f.) and standard errors were different at each temperature. A plot of ln($k_n$) versus $1/T$ yielded a straight line (Figure 4.6b), and least squares linear regression was used to calculate $E_a$ and ln($k_0$). The calculated values of $E_a$ and ln($k_0$) were 48.36 (kJ mol$^{-1}$) and 12.29, respectively which are in general agreement with earlier studies on β-lg A denaturation in skim milk (Hillier & Lyster, 1979; Manji & Kakuda, 1986; Dannenberg & Kessler, 1988a).
Figure 4.6a Denaturation of β-lactoglobulin A in skim milk as a 1.5 order reaction. Heating temperature; 90°C (O), 95°C (●), 100°C (▲), 110°C (●) and 130°C (□).

Table 4.1 Rate constants obtained from the two-step method for β-lg A denaturation in skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Rate constant (k) ( \times 10^3 ) ( \text{s}^{-1} )</th>
<th>95% C.I.</th>
<th>d.f.</th>
<th>Standard error(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>7.54</td>
<td>0.95</td>
<td>6</td>
<td>0.37</td>
</tr>
<tr>
<td>95</td>
<td>27.72</td>
<td>3.70</td>
<td>9</td>
<td>1.60</td>
</tr>
<tr>
<td>100</td>
<td>43.37</td>
<td>7.94</td>
<td>6</td>
<td>3.09</td>
</tr>
<tr>
<td>110</td>
<td>77.75</td>
<td>3.21</td>
<td>7</td>
<td>1.31</td>
</tr>
<tr>
<td>120</td>
<td>96.02</td>
<td>6.31</td>
<td>5</td>
<td>2.27</td>
</tr>
<tr>
<td>130</td>
<td>143.22</td>
<td>16.29</td>
<td>5</td>
<td>5.86</td>
</tr>
</tbody>
</table>

\(^a\)Standard error is obtained from the standard error of the slope (Equation 4.18).
Figure 4.6b Arrhenius plot of β-lg A denaturation in skim milk as a 1.5 order reaction, where \( k_n \) has units of \( \left( \text{g kg}^{-1}\right)^{(1-n)} \text{s}^{-1} \).

Table 4.2 shows the kinetic parameters derived from the different methods of data analysis and their corresponding 95% C.I. The values of \( E_a \) and \( k_0 \) were slightly different between the methods, but all were within the 95% confidence bounds calculated. Statistically, the two-step method produced the least precise results with only 2 d.f., and NLR the most precise as shown by the lowest 95% C.I.

The predicted levels of native β-lg A calculated from the kinetic parameters are given in Figure 4.7. Both the NLR, and NLR with adjusted intercept methods, estimated the same numerical values for the parameters (Table 4.2), so the NLR curve in Figure 4.7 represents both methods. When compared with the experimental data, the two-step method and the two-step method with adjusted intercept did not predict the native protein levels as closely as the NLR and scale transformation methods.
Table 4.2 Kinetic parameters $E_a$ and $k_0$ and their respective 95% C.I. derived from different methods of data analysis for $\beta$-lg A denaturation in skim milk heated at 100-130°C.

<table>
<thead>
<tr>
<th>Method of Analysis</th>
<th>Kinetic parameters ± 95% C.I.</th>
<th>d.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_a$ (kJ mol$^{-1}$)</td>
<td>$\ln(k_0)$</td>
</tr>
<tr>
<td>Two-Step</td>
<td>48.36 ± 26.36</td>
<td>12.29 ± 8.14</td>
</tr>
<tr>
<td>Two-Step with adjusted intercept</td>
<td>48.40 ± 26.38</td>
<td>13.89 ± 8.17</td>
</tr>
<tr>
<td>Scale transformation</td>
<td>49.65 ± 11.08</td>
<td>12.55 ± 3.44</td>
</tr>
<tr>
<td>NLR</td>
<td>54.23 ± 7.07</td>
<td>13.89 ± 2.19</td>
</tr>
<tr>
<td>NLR with adjusted intercept</td>
<td>54.23 ± 7.07</td>
<td>13.89 ± 2.19</td>
</tr>
</tbody>
</table>
Figure 4.7 Comparison of predicted and experimental levels of β-lg A denaturation in skim milk heated at 100°C. Experimental data (O), two-step (—), two-step with adjusted intercept (— —), scale transformation (----) and NLR (-----).

Adjusting the method of finding the intercept greatly reduced the error in the pre-exponential term (Table 4.2). The 95% C.I. for ln($k_0$) obtained by the two-step method was ± 8.14. By placing the intercept in the middle of the data set, the 95% C.I. of ln($k_{115}$) was greatly reduced to ± 0.24, similarly there was an improvement in the NLR model, ± 0.12. Correlation between $E_a$ and $k_0$ was high (0.9984) in the NLR model without an intercept adjustment, and non existent (-0.0612) in the adjusted intercept model. Because the correlation between $E_a$ and $k_0$ was reduced, errors in $E_a$ had less effect on $k_0$. This was observed as a reduction in the 95% C.I. of the pre-exponential term, while those of $E_a$ remained constant at ± 7.07. It should be noted that when ln($k_{115}$) was converted to ln($k_0$) by Equation 4.9, there was an increase in the 95% C.I. This occurred because $E_a$ which was used in Equation 4.9, has a much larger relative error than ln($k_{115}$).
NLR and scale transformation can be considered as one-step methods, which process the raw data into the kinetic parameters without going through the intermediary rate constant calculations. The result of this was that the one-step methods had smaller errors in $E_a$ in comparison with the two-step method (Table 4.2). The two-step method though commonly employed has some limitations with regards to statistical analysis of the data. The variability in the rate constants are generally ignored when calculating $E_a$ and $k_0$, and there is less utilisation of the raw data (Arabshahi & Lund, 1985).

When using linear regression to calculate the rate constants for the loss of whey proteins from heated skim milk, the method assumes the variance is evenly distributed over the raw data, which means that each data point is assumed to have the same variance or absolute error. However, normally it is the relative error which is constant. The result is that data points furthest away from the intercept have the largest influence, when they are often the least accurate. These points may have larger errors than the rest of the data for two possible reasons; first the samples are usually taken at longer holding times where other reactions may interfere with the reaction under study, second the measured concentrations are relatively low and may be at the limits of detection and accuracy of the analytical method employed. The rate constants from the two-step method all have different standard errors (Table 4.1) so the assumption of the least squares regression does not hold true. A disadvantage of the two-step method is that a lot of information about the raw data is lost when it is transformed into a rate constant, so that only a few data points are used in the Arrhenius plot, from all the concentration/time data collected. In contrast, scale transformation or NLR methods use all the raw data to determine $E_a$ and $k_0$, and consequently provide greater precision.

From the NLR method $n = 1.1 \pm 0.3$, and $C_0 = 1.51 \pm 0.11 \text{ (g kg}^{-1})$ were calculated together with their respective 95% C.I. for $\beta$-lg A denaturation in skim milk. The apparent reaction order determined by linear regression was $1.5 \pm 0.4$. The difference in the reaction orders was caused by the two methods treating the data differently. NLR gives all the data the same weighting, where as linear regression is influenced by the larger numerical values, which lie at the longer holding times. The initial concentration
of β-lg A determined from an unheated sample by native-PAGE, 1.53 ± 0.25 (g kg⁻¹), agrees with the calculated value from NLR.

Of the methods tested, NLR with adjusted intercept appears to be the most suitable for providing estimates of the kinetic parameters. The method utilises all the raw data, and considers all the kinetic parameters at the same time when trying to obtain the best fit. This method, now referred to as NLR, was used in the following kinetic calculations.

4.3.2.2 Effect of time and temperature variations on the kinetic calculations

In kinetic calculations either the mean residence time or the integrated $E(t)$ curve could be used to define the holding time (see Chapter 3). Predicted levels of native β-lg A in skim milk heated at 110°C, were calculated, using both the mean residence time and the $E(t)$ curve integrated by Simpson’s rule, with the kinetic parameters calculated by NLR (Table 4.2). Both methods predicted the same level of residual native protein (Figure 4.8). Therefore the easier method of mean residence time was used for kinetic calculations.

![Figure 4.8](image-url) Comparison of predicted β-lg A denaturation in skim milk at 110°C. Mean residence time (-), integrated residence time (---) and raw data points (O).
NLR required temperature as one of the variables. To see what affect the temperature drop (Table 3.2) would have on the kinetic parameters, the temperature was adjusted for each temperature/time combination. In one case the full temperature drop was subtracted from the DSI temperature, in the second an arithmetic mean of the temperature drop was subtracted from the DSI temperature, and in the last case isothermal conditions were assumed to exist and the DSI temperature was left unaltered.

The kinetic parameters obtained using NLR are shown in Table 4.3, and the predicted levels of native β-lg A heated at 100°C are shown in Figure 4.9. The difference between the three temperatures is small, approximately 3% between the extreme conditions, as shown in Figure 4.9. The kinetic parameters of the three temperature regimes all lie within their respective 95% C.I. (Table 4.3).

Table 4.3 Kinetic parameters for β-lg A denaturation in skim milk calculated from different temperature conditions.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Isothermal conditions</th>
<th>Arithmetic temperature</th>
<th>Full Temperature drop</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>1.2 ± 0.3</td>
<td>1.16 ± 0.21</td>
<td>1.13 ± 0.21</td>
</tr>
<tr>
<td>$E_a$ (kJ mol⁻¹)</td>
<td>59.70 ± 7.21</td>
<td>58.75 ± 5.68</td>
<td>57.82 ± 5.51</td>
</tr>
<tr>
<td>$\ln(k_{ref})$</td>
<td>-3.10 ± 0.14</td>
<td>-3.06 ± 0.11</td>
<td>-3.02 ± 0.11</td>
</tr>
<tr>
<td>$\ln(k_0)$</td>
<td>15.50 ± 2.40</td>
<td>15.26 ± 1.77</td>
<td>15.01 ± 1.72</td>
</tr>
<tr>
<td>$C_0$ (g kg⁻¹)</td>
<td>1.55 ± 0.10</td>
<td>1.56 ± 0.08</td>
<td>1.57 ± 0.08</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.971</td>
<td>0.971</td>
<td>0.971</td>
</tr>
</tbody>
</table>

*Temperature range from which the kinetic parameters were calculated was 95-130°C.*
Figure 4.9 Comparison of β-lg A denaturation at 100°C from differently derived Arrhenius parameters. Method of temperature determination; no drop (−), mean drop (---), full drop (···) and raw data points (O).

Clearly isothermal conditions are not true as a temperature drop was observed, and the other extreme of a full temperature drop is false as the temperature drop occurred gradually over the holding tube and not instantaneously at the beginning (see Chapter 3). The arithmetic temperature drop seems the closest to an equivalent temperature for the entire holding tube. An equivalent-point method for processing kinetic data in non-isothermal continuous flow systems has been developed by Swartzel (1986), and Nunes and Swartzel (1990), but as the difference between the extreme conditions is small in this study, it is assumed that the arithmetic mean is accurate enough for kinetic studies on the UHT plant.

4.3.2.3 Activation energy and thermodynamic parameters

The experimental data was found to fit two temperature regions in which the activation energies were markedly different (Figure 4.10). The break in the Arrhenius plot was found at 90°C for β-lg A and B; there was a break at 80°C for α-la, while no break was
observed for IgG over the temperature range 70-90°C. The kinetic parameters are shown in Table 4.4. The kinetic parameters for β-lg A in the 95-130°C range are slightly different from those determined in the statistical analysis section (Table 4.2), as the temperature range used was 100-130°C. The initial concentrations of the whey proteins in the milks heated at 70-90°C were slightly higher than the milks heated at 95-130°C. This was probably due to the two different milks (July and March) used for these experiments.

![Arrhenius plot of the denaturation of β-lg A (○), β-lg B (○), α-la (●) and IgG (●) in skim milk, where $k_n$ has units of (g kg$^{-1}$)($^{1-n}$) s$^{-1}$).](image)

**Figure 4.10** Arrhenius plot of the denaturation of β-lg A (○), β-lg B (●), α-la (●) and IgG (●) in skim milk, where $k_n$ has units of (g kg$^{-1}$)($^{1-n}$) s$^{-1}$).

The calculated activation energies, along with their 95% C.I., were compared with the values reported in the literature (Table 4.5). In the temperature range 70-90°C, the activation energies of β-lg A and B were slightly higher than those reported by Dannenberg and Kessler (1988a) and Manji and Kakuda (1986). In the higher temperature range 95-130°C, the activation energies of β-lg A and B were similar to those of Dannenberg and Kessler (1988a) and Manji and Kakuda (1986), but higher than Hillier and Lyster (1979). The activation energy of α-la in the 70-80°C range was lower than Dannenberg and Kessler’s reported value, but higher than those reported by Hillier and Lyster (1979) and Manji and Kakuda (1986). For α-la in the 85-130°C range the
Table 4.4 Kinetic parameters for denaturation of individual whey proteins in skim milk

<table>
<thead>
<tr>
<th>Whey protein</th>
<th>Temperature range (°C)</th>
<th>Parameters from NLR with 95% confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$n$</td>
</tr>
<tr>
<td>(\beta)-lg A</td>
<td>95-130(^a)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>70-90(^b)</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>(\beta)-lg B</td>
<td>95-130(^a)</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>70-90(^b)</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>(\beta)-lg (^c)</td>
<td>95-130(^a)</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>70-90(^b)</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>(\alpha)-la</td>
<td>85-130(^c)</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>70-80(^d)</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>IgG</td>
<td>70-85(^e)</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>BSA</td>
<td>70-75(^f)</td>
<td>2.8 ± 0.8</td>
</tr>
</tbody>
</table>

\(^a\) combined \(\beta\)-lg A and \(\beta\)-lg B concentrations. \(^b\) Adjusted $R^2$, see Equation 4.26. \(^c\) $E_a$ is the average of the temperature range quoted in the table.

\(T_{ref}\) is the average of the temperature range quoted in the table.

The number of data points in each temperature range; \(^*\)43, \(^*\)25, \(^*\)61, \(^*\)15, \(^*\)28 and \(^*\)8.
Table 4.5 Comparison between experiment and literature activation energies.

<table>
<thead>
<tr>
<th>Source of $\textit{E}_a$ data</th>
<th>Activation energy ($\textit{E}_a$) of individual whey protein denaturation of individual whey protein denaturation ± 95% C.I. (kJ mol$^{-1}$)</th>
<th>Lower temperature region (70-90°C)</th>
<th>Upper temperature region (80-140°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\beta$-lg A</td>
<td>$\beta$-lg B</td>
</tr>
<tr>
<td>Table 4.4</td>
<td></td>
<td>285.50 ± 30.78</td>
<td>296.72 ± 42.16</td>
</tr>
<tr>
<td>Dannenberg &amp; Kessler (1988a)</td>
<td></td>
<td>265.21</td>
<td>279.96</td>
</tr>
<tr>
<td>Manji &amp; Kakuda (1986)</td>
<td></td>
<td>275.9</td>
<td>259.2</td>
</tr>
<tr>
<td>Hillier &amp; Lyster (1979)$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li-Chan et al. (1995)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resmini et al. (1989)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Calculated from kinetic equations reported by Hillier & Lyster (1979).
activation energy was lower than the values reported by Dannenberg and Kessler (1988a), Manji and Kakuda (1986) and Hillier and Lyster (1979). The activation energy of IgG over 70-85°C was in reasonable agreement with Resmini et al. (1989) and Li-Chan et al. (1995), but differed from Luf et al. (1993) (70-75°C; 674.42 kJ mol⁻¹, 76-79°C; 89.22 kJ mol⁻¹) who found a break in the curve at 76°C.

Using the kinetic parameters presented in Table 4.4, levels of remaining native β-lg A, β-lg B, α-la and IgG after a given heat treatment were determined. The levels were compared with those from the literature and the results are shown in Table 4.6. Native β-lg A, β-lg B and α-la calculated from the kinetic parameters were generally in good agreement with the results of Dannenberg and Kessler (1988a). However, the kinetic parameters of Manji and Kakuda (1989) predicted higher levels of remaining native protein (Table 4.6). IgG values were compared with the results of Resmini et al. (1989) and Luf et al. (1993); the levels of native protein remaining after heating at 70 or 80°C were higher than those of Resmini et al. (1989), but were much lower than those reported by Luf et al. (1993).

These differences in the levels of denaturation could be caused by different heating methods, analytical methods and compositional variations in the milk samples (e.g. pH, protein and mineral composition). Errors in the activation energies have not been reported in the literature, so it is not known whether the differences are significant. Hillier and Lyster (1979) and Manji and Kakuda (1986) used glass capillary tubes in thermostatically controlled baths to heat the samples. Dannenberg and Kessler (1988a) used a tubular heat exchanger to achieve short come-up times (<0.5 s), and a thermostatically controlled stainless steel holding tube section, in which the milk was held at the desired temperature and time by control valves. The heating systems above differ significantly from the direct heating of the DSI system used in this present study. The pilot scale UHT plant with the DSI system, should be more representative of industrial conditions than static oil or waterbath heating. The DSI and flash vessel operate as an instantaneous step up and step down in temperature, respectively. In contrast, heating in a waterbath may result in appreciable heating up and cooling down times, which would affect the calculation of denaturation kinetics.
Table 4.6 Comparison between experimental and literature results for native protein levels of $\beta$-lg A, $\beta$-lg B, $\alpha$-la and IgG in skim milk after specified heat treatments.

<table>
<thead>
<tr>
<th>Whey protein/Heating condition</th>
<th>Predicted levels of native protein after heat treatment (% of initial whey protein)</th>
<th>Kinetic parameters</th>
<th>Dannenberg &amp; Kessler (1988a)*</th>
<th>Manji &amp; Kakuda (1986)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Table 4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-lg A 80°C, 600 s</td>
<td>34.3</td>
<td>38.8</td>
<td>62.4</td>
<td></td>
</tr>
<tr>
<td>$\beta$-lg A 100°C, 20 s</td>
<td>59.4</td>
<td>45.3</td>
<td>69.0</td>
<td></td>
</tr>
<tr>
<td>$\beta$-lg B 80°C, 600 s</td>
<td>23.0</td>
<td>31.8</td>
<td>64.4</td>
<td></td>
</tr>
<tr>
<td>$\beta$-lg B 100°C, 20 s</td>
<td>43.0</td>
<td>36.9</td>
<td>66.4</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-la 80°C, 600 s</td>
<td>43.6</td>
<td>43.8</td>
<td>87.1</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-la 100°C, 60 s</td>
<td>71.3</td>
<td>74.3</td>
<td>91.1</td>
<td></td>
</tr>
<tr>
<td>IgG 70°C, 180 s</td>
<td>64.0</td>
<td>39</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>IgG 80°C, 15 s</td>
<td>59.5</td>
<td>42</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

* Predicted values determined from the kinetic parameters of the respective authors.
Although PAGE methods were used by Dannenberg and Kessler (1988a) and Hillier and Lyster (1979), the samples were acidified to pH 4.6 to precipitate casein and denatured protein. This method of casein separation may produce different levels of denatured and aggregated whey protein than the present study. All the methods cooled the heat treated sample, which allows for possible renaturation and other temperature related interactions to occur. A number of different methods have been used to measure protein denaturation, and each relies on measuring a different aspect of the physico-chemical changes that the protein undergoes. The differences could also be caused by changes in milk composition; protein concentration, pH and mineral levels, have all been shown to affect the level of protein denaturation (see Chapter 2).

**Thermodynamics of denaturation**

The effect of temperature on denaturation rates can be studied using the activated complex theory. The basis of this theory is that reactants (A and B) form an unstable intermediate (activated complex, $X^*$), which then immediately decomposes to form products (P and Q) (Equation 4.27). A quasi-equilibrium is assumed to exist between the reactants and the activated complex. The thermodynamic properties of the activated complex can then be determined by use of the Eyring equations (Equations 4.28-4.30) (Moore, 1972).

$$A + B \rightleftharpoons X^* \rightarrow P + Q$$  \hspace{1cm} (4.27)

$$k_f = \frac{k_B T}{h} e^{-\Delta H^f / RT} e^{\Delta S^f / R}$$  \hspace{1cm} (4.28)

Where

$k_f$ = reaction rate constant for the breakdown of the activated complex to product ($s^*$).
\( k_b = \) Boltzmann's constant \((1.38062 \times 10^{-23} \text{ J K}^{-1})\).
\( h = \) Planck's constant \((6.6262 \times 10^{-34} \text{ J s})\).

\[
E_a = \Delta H^\ddagger + RT
\]  
\((4.29)\)

\[
\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger
\]  
\((4.30)\)

The enthalpy \((\Delta H^\ddagger)\), entropy \((\Delta S^\ddagger)\) and free energy \((\Delta G^\ddagger)\) of formation of the activated complex for \(\beta\)-lg A and B and \(\alpha\)-la are shown in Table 4.7.

In the lower temperature range, \(<90^\circ\text{C}\), the \(\Delta H^\ddagger\) values varied from 280-290 kJ mol\(^{-1}\) for \(\beta\)-lg A and B, and were about 200 kJ mol\(^{-1}\) for \(\alpha\)-la. These values were typical for heat-induced protein denaturation (Labuza, 1980). Above 90°C the \(\Delta H^\ddagger\) values were similar to those of chemical reactions (40-56 kJ mol\(^{-1}\)). At temperatures below 90°C, these \(\Delta H^\ddagger\) values were slightly higher than those reported by Dannenberg and Kessler (1988a) for \(\beta\)-lg A and B (260-280 kJ mol\(^{-1}\)) and lower for \(\alpha\)-la (260 kJ mol\(^{-1}\)). At temperatures above 90°C the \(\Delta H^\ddagger\) values were similar (45-65 kJ mol\(^{-1}\)).

The entropy of activation \((\Delta S^\ddagger)\) indicates the loss or gain of translational and rotational entropy. Below 90°C, the change in entropy was positive (0.27-0.53 kJ mol\(^{-1}\)K\(^{-1}\)) indicating a gain in translation and rotational entropy, which could be interpreted as the protein changing into a more open conformation, i.e. denaturation. In the higher temperature range (\(>90^\circ\text{C}\) \(\Delta S^\ddagger\) was negative (-0.17 to -0.13 kJ mol\(^{-1}\)K\(^{-1}\)). This decrease in disorder may be a result of association reactions becoming the rate determining step (Dannenberg & Kessler, 1988a).
Table 4.7 Enthalpy ($\Delta H^\ddagger$), entropy ($\Delta S^\ddagger$) and free energy ($\Delta G^\ddagger$) of formation of the activated complex.

<table>
<thead>
<tr>
<th>Whey protein</th>
<th>Temperature (°C)</th>
<th>$\Delta H^\ddagger$ (kJ mol$^{-1}$)</th>
<th>$\Delta S^\ddagger$ (kJ mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G^\ddagger$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lg A</td>
<td>70</td>
<td>282.65</td>
<td>0.502</td>
<td>110.50</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>282.56</td>
<td>0.501</td>
<td>105.51</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>282.48</td>
<td>0.501</td>
<td>100.52</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>55.65</td>
<td>-0.128</td>
<td>103.37</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>55.56</td>
<td>-0.128</td>
<td>104.62</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>55.48</td>
<td>-0.128</td>
<td>105.88</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>55.40</td>
<td>-0.128</td>
<td>107.14</td>
</tr>
<tr>
<td>β-lg B</td>
<td>75</td>
<td>293.83</td>
<td>0.536</td>
<td>107.06</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>293.78</td>
<td>0.536</td>
<td>104.55</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>293.70</td>
<td>0.536</td>
<td>99.53</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>40.88</td>
<td>-0.162</td>
<td>101.15</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>40.79</td>
<td>-0.162</td>
<td>102.75</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>40.71</td>
<td>-0.162</td>
<td>104.35</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>40.63</td>
<td>-0.162</td>
<td>105.96</td>
</tr>
<tr>
<td>α-la</td>
<td>70</td>
<td>200.41</td>
<td>0.266</td>
<td>109.11</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>200.32</td>
<td>0.266</td>
<td>106.45</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>49.85</td>
<td>-0.156</td>
<td>106.61</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>49.77</td>
<td>-0.157</td>
<td>108.33</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>49.68</td>
<td>-0.158</td>
<td>110.07</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>49.60</td>
<td>-0.158</td>
<td>111.78</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>49.52</td>
<td>-0.158</td>
<td>113.51</td>
</tr>
</tbody>
</table>
The free energy of formation ($\Delta G^\circ$) indicates whether the reaction occurs spontaneously or not. The $\Delta G^\circ$ values are positive so the reaction is not spontaneous and therefore requires energy to proceed. For $\beta$-lg A, $\beta$-lg B and $\alpha$-la, the value of $\Delta G^\circ$ remained relatively constant at approximately 100 kJ mol$^{-1}$, over the temperature range studied (70-130°C). Similar values for $\Delta G^\circ$ have been reported for $\beta$-lg A and B, and $\alpha$-la denaturation in milks (Gough & Jenness, 1962; Dannenberg & Kessler, 1988a; Anema & McKenna, 1996).

The downward concave shape of the Arrhenius plot (high slope at low temperature and vice versa) (Figure 4.8) is consistent with the two reactions in series with different temperature dependent rate constants (Bunnett, 1974). At low temperatures ($<90°C$) the high activation energy of denaturation caused this to be the rate limiting step. As the temperature was increased the rate of denaturation increased faster than that of the aggregation step and consequently aggregation became the rate limiting step.

4.3.2.4 Order of denaturation reaction

The apparent order of denaturation reactions varied greatly among the whey proteins ($n = 0.9 - 2.8$, Table 4.4). The break in the Arrhenius plot did not cause the reaction orders to change markedly between the two temperature regions for $\beta$-lg A, $\beta$-lg B and $\alpha$-la (Table 4.3). However in the lower temperature region ($<95°C$) the reaction orders were slightly lower.

Because of the complexity of the reactions occurring in milk, the order of denaturation reaction may not be significant in defining a reaction mechanism. Thus the order should be treated as an empirical constant, fitting the experimental data to a relationship between the reaction rate and a number of the reactants. Therefore fitting the data to first and second order reactions was not done, even though the results and errors indicated this was possible for a number of the whey proteins.

$\alpha$-lactalbumin

The denaturation of $\alpha$-la had a reaction order of $1.1 \pm 0.3$ in the temperature range 85-130°C, dropping slightly, to $0.9 \pm 0.4$, in the temperature range 70-80°C. This is in
general agreement with other researchers who observed first-order kinetics (Baer et al., 1976; Hillier & Lyster, 1979; Manji & Kakuda, 1986; Dannenberg & Kessler, 1988a) and it appears that the reaction order of α-la denaturation in skim milk is first order. A reaction order of one would indicate a unimolecular unfolding process consistent with denaturation. However, the native-PAGE method only differentiates between monomeric and aggregated proteins and presumably does not distinguish between the unfolded and native conformations of α-la. Furthermore, there is also the possibility for renaturation after cooling and storage of the sample, although this is probably limited due to irreversible interactions between α-la and β-lg and BSA (Rüegg et al., 1977). The native-PAGE method therefore only measures the extent of irreversible denaturation (aggregation). In view of these, the reaction order should be treated as an apparent reaction order, as interactions between α-la and other whey proteins, in particular β-lg, have been shown to influence denaturation (Elfagm & Wheelock, 1978b; Melo & Hansen, 1978). Thus the observed denaturation is unlikely to be a simple unfolding mechanism.

β-Lactoglobulin A and B
The denaturation order of β-lg B was found to be higher than β-lg A; 1.6-1.4 compared with 1.2-1.0, respectively (Table 4.4). The reaction orders for β-lg denaturation reported in the literature vary considerably. Most researchers have found 1st or 2nd-order reactions (Hillier & Lyster, 1979; de Wit & Swinkels, 1980; Manji & Kakuda, 1986; Relkin & Launay, 1990), but Dannenberg and Kessler (1988a) have reported a non-integer order, 1.4 and 1.5 for β-lg A and β-lg B, respectively. The difference in the reaction orders of the variants could be due to the slight conformational changes in the native protein structure induced by the genetic substitutions of different amino acids at sites 64 and 118. These substitution positions are close to the thiol group and a disulphide bond, so could possibly affect their denaturation behaviour.

Immunoglobulin G
A denaturation order of 2.0 was found for IgG. Resmini et al. (1989) found a reaction order of 2, Luf et al. (1993) reported a best fit of 1.16, and Li-Chan et al. (1995) assumed a first-order reaction. These different reaction orders could be due to different
methods of analysis or different heating techniques. Both Resmini et al. (1989) and Luf et al. (1993) used reversed phase HPLC, although different heating systems were used. Li-Chan et al. (1995) quantified IgG by enzyme-linked immunosorbant assay and radial immunodiffusion. Resmini et al. (1989) used both HTST and batch heating in a waterbath, while Luf et al. (1993) and Li-Chan et al. (1995) used a waterbath to heat the milk samples. The HTST heating used by Resmini et al. (1989) and the UHT plant heating used in this study should be more comparable than the static heating in a water bath used by Luf et al. (1993).

**Bovine serum albumin**

The high reaction order of 2.8 (Table 4.4) suggests BSA undergoes complex interactions with other whey proteins or self-aggregation during denaturation. Few studies have investigated the denaturation kinetics of BSA. Hillier & Lyster (1979) found that BSA denaturation follows a complicated mechanism and that its denaturation in skim milk can be described by either 1st or 2nd-order kinetics. The low thermal stability of BSA and presence of a thiol group may allow it to react readily with the other whey proteins during heating to form aggregates. For example, BSA is known to influence the heat stability of β-lg, forming aggregates through disulphide cross-linkages (Matsudomi et al., 1994). Interactions with α-la via thiol-disulphide interchange have also been reported by Matsudomi et al. (1993).

**4.3.3 Formation of whey protein aggregates**

After heat-induced denaturation (unfolding) the whey proteins aggregate through complex and poorly understood reactions. Quantitative PAGE analysis was used to follow the changes of aggregated protein species, as defined in Section 4.2.3.2 (see Figure 4.1). Figure 4.1 identifies four different protein species, which are outlined below.

(i) **Native protein.** Unaggregated, non-sedimentable whey protein. This may also include denatured whey protein, containing intermolecular bonds that break under the alkaline electrophoretic running conditions (e.g. ionic bonds).
(ii) *Hydrophobic aggregates.* Non-sedimentable whey protein aggregates linked via intermolecular hydrophobic interactions, while being devoid of intermolecular disulphide bonds.

(iii) *Disulphide-linked aggregates.* Non-sedimentable whey protein and whey protein/κ-casein aggregates containing intermolecular disulphide bonds.

(iv) Associated with the casein micelle. Whey proteins that sediment with the casein micelle, as either a whey protein/κ-casein complex at the micelle surface, or as large insoluble aggregates.

SDSNR-PAGE follows the change in native whey protein and hydrophobic whey protein aggregates. The protein that is resolved by SDSNR-PAGE (native protein + hydrophobic aggregates) can be described as SDS-monomeric. The level of SDS-monomeric protein decreased with an increase in heating time and/or temperature. This decrease was evident in both β-lg and α-la, Figures 4.11a and 4.11b, respectively.

![Figure 4.11a](image-url) Loss of SDS-monomeric β-lg from SDSNR-PAGE gels (non-reducing). Heating temperature; 85°C (○), 90°C (●), 95°C (△), 100°C (★), 110°C (□) and 130°C (■).
4.3.3.1 Whey protein interactions in the casein pellet

The casein pellets of milks heated at 75-90°C were analyzed by SDSNR- and SDSR-PAGE. The total concentration of the whey protein in the pellet was determined by SDSR-PAGE, and hydrophobically-aggregated whey protein was determined by SDSNR-PAGE. The concentration of disulphide-linked aggregates were determined by the difference between the concentrations of the two gels (SDSR less SDSNR).

β-Lg present in the pellet was almost entirely in a disulphide aggregated form (results not shown); presumably these aggregates were β-Lg/K-casein complexes. However, some of the β-Lg that co-sedimented with the micelles may be in the form of insoluble β-Lg aggregates as the PAGE method does not differentiate between the two types. In contrast to β-Lg, the percentage of α-la in the pellet that was hydrophobically aggregated was variable and averaged 40%. A possible explanation is that prior to associating with the micelle, β-Lg interacted with α-la either by sulphydryl-disulphide interchange or hydrophobic interactions to form an aggregate. The β-Lg of this aggregate then interacted with κ-casein on the micelle surface via thiol-disulphide interchange reactions,
while the α-la remained unchanged. Previous workers have reported that α-la does not interact with the micelle or polymerise on its own, but associates with the micelle as a β-lg/α-la complex (Elfagm & Wheelock, 1978b; Calvo et al., 1993). Therefore, hydrophobic or disulphide linkages between α-la and κ-casein were probably not occurring. Hence, the loss of β-lg from SDSNR-PAGE of the supernatant could be entirely due to the formation of disulphide bonds. The loss of α-la from SDSNR-PAGE of the supernatants followed a different mechanism, and involved both the formation of disulphide aggregates and aggregates that contained only hydrophobically-bonded β-lg/α-la. Because of the complex nature of α-la aggregation the kinetics of disulphide aggregate formation were considered for β-lg only.

4.3.3.2 Kinetics of β-lg aggregation via disulphide bond formation (disulphide aggregation) The kinetics of β-lg aggregation due to intermolecular disulphide bond formation in insoluble aggregates and complexes associated with the micelle (Figure 4.11a) were determined using NLR. A break in the Arrhenius plot was observed at 90°C, which is the same break-point that was observed in β-lg denaturation. The kinetic parameters for the two temperature regions are shown in Table 4.8. The reaction order was found to be 1.6-1.7 which is higher than the denaturation order (1.4 and 1.3, respectively, Table 4.4). The numerical values of the parameters, $E_a$, ($ln_{ref}$) and $ln(k_0)$ are similar to those of β-lg denaturation (Table 4.4).

The rates of β-lg denaturation and disulphide aggregation ($k$), calculated by equation 4.17, are shown in Table 4.9. The rate of disulphide aggregation was not significantly different from the rate of denaturation, on both sides of the Arrhenius break (75-130°C). The similar rates show that β-lg denaturation and disulphide aggregation are not affected by the formation of hydrophobic aggregates. Thus hydrophobic aggregation may be a step in the denaturation mechanism, which is not rate limiting. As discussed is Section 4.3.2.3, $\Delta S^f$ was negative in the upper temperature region (>90°C), and consequently the rate limiting step in this region was protein aggregation. This aggregation step appears to be determined mainly by disulphide aggregation.
<table>
<thead>
<tr>
<th>Temperature range (°C)</th>
<th>Parameters from NLR with 95% confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
</tr>
<tr>
<td>95-130</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>75-90</td>
<td>1.7 ± 0.4</td>
</tr>
</tbody>
</table>

$^a T_{ref}$ is the average of the temperature range quoted in the table.

$^\dagger$ adjusted $R^2$, see Equation 4.26.
Table 4.9 Comparison of the rates of $\beta$-lg disulphide aggregation and denaturation in skim milk.

<table>
<thead>
<tr>
<th>Temperature ($^\circ$C)</th>
<th>Reaction rate ($k$) $(s^{-1}) \times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>denaturation</td>
</tr>
<tr>
<td>130</td>
<td>127.2</td>
</tr>
<tr>
<td>120</td>
<td>85.8</td>
</tr>
<tr>
<td>110</td>
<td>56.7</td>
</tr>
<tr>
<td>100</td>
<td>36.6</td>
</tr>
<tr>
<td>95</td>
<td>29.2</td>
</tr>
<tr>
<td>90</td>
<td>23.1</td>
</tr>
<tr>
<td>85</td>
<td>11.5</td>
</tr>
<tr>
<td>80</td>
<td>2.8</td>
</tr>
<tr>
<td>75</td>
<td>0.7</td>
</tr>
</tbody>
</table>

4.3.3.3 Hydrophobic and disulphide aggregation

The changes in different $\beta$-lg aggregate species derived from the different PAGE results (see Figure 4.1) with time is shown in Figure 4.12. $\beta$-Lg associated with the micelle was found to be predominantly disulphide-linked (see Section 4.3.3.1), and is therefore grouped with the disulphide aggregates. In the total milk system, $\beta$-lg denatured to form aggregates, and these were mainly disulphide-linked in nature (Figure 4.12). The level of $\beta$-lg hydrophobic aggregates in the milk was minor and remained relatively constant at both 75 and 120°C, and was possibly part of an intermediate step in the aggregation mechanism.

Figure 4.13 shows the change in $\alpha$-la aggregates with time in heated skim milk. The quantity of hydrophobic aggregates that formed in the supernatant at 75°C appeared to increase as heating progressed, while there was negligible amounts of disulphide-linked
aggregates in the supernatant. In contrast, in skim milk heated at 110°C the level of disulphide-linked aggregates in the supernatant increased with time, but the level of hydrophobic aggregates remained small and relatively constant.

The quantities of disulphide-linked β-lg aggregates in relation to denatured β-lg in the supernatant is shown in Figure 4.14. Over the temperature range 75-130°C the increase in denatured β-lg was mirrored by an increase in disulphide-linked β-lg aggregates. The slope of almost 1 (0.86) suggests that upon unfolding β-lg aggregates rapidly through disulphide bonds. The α-la data was more scattered, but also showed a similar trend at temperatures above 80°C. Dalgleish (1990) observed that there was a close correlation between the amount of disulphide-linked aggregates formed and the amount of serum protein lost. It appears that both β-lg and α-la contributed to the formation of disulphide-linked aggregates.

From the above results, it appears that the aggregation mechanisms of β-lg and α-la are probably different, at least below 85°C where hydrophobic interactions played a greater role in α-la aggregation (Figure 4.13). The absence of a thiol group in the native α-la structure could hinder the ability of α-la to form covalent aggregates. The development of reactive thiol-groups by β-lg is possibly a prerequisite for sulphhydryl-disulphide interchange reactions between α-la and β-lg (Calvo et al., 1993). In this connection Kirchmeier et al. (1984) showed that development of reactive thiol groups in milk is slow at 75°C, but rises rapidly around 80°C. This slow development of reactive thiol-groups at 75°C could result in α-la forming hydrophobic aggregates with β-lg through a lack of available thiol-groups. In contrast, at temperatures above 80°C the aggregates are predominantly disulphide-linked, presumably the higher temperatures weaken the hydrophobic bonds and the faster rates of denaturation (unfolding) allow for more sulphhydryl-disulphide interchange reactions. Furthermore, the higher concentration of β-lg (3.495 g kg⁻¹) compared to α-la (0.672 g kg⁻¹) and the faster rate of denaturation should present more opportunities for β-lg to aggregate with other β-lg molecules rather than with α-la. It should be noted that thiol blocking agents do not prevent β-lg aggregation from occurring, which indicates that bonds other than disulphide linkages are also involved in the formation of aggregates (Sawyer, 1968).
Figure 4.12  β-Lg aggregates in skim milk heated at 75°C (A) and 120°C (B). β-Lg species; native (□), hydrophobic aggregates (///) and disulphide-linked aggregates (XXX).
Figure 4.13 \(\alpha\)-La aggregates in skim milk heated at 75°C (A) and 110°C (B). \(\alpha\)-La species; native (☐), hydrophobic aggregates (///), disulphide-linked aggregates in the supernatant (XXX) and aggregates (hydrophobic and disulphide-linked) associated with the micelle (■).
Figure 4.14 Formation of disulphide-linked β- lg aggregates in relation to denatured β- lg in heated skim milk. Heating temperature; 95-130°C (O) and 75-90°C (●).

Figure 4.15 Formation of disulphide-linked α-l a aggregates in relation to denatured α-l a in heated skim milk. Heating temperature; 85-130°C (O) and 70-80°C (●).
From the results presented above it is not clear what effect heating has on the exact composition of the aggregates formed in skim milk. The aggregates are probably a mixture of β-lg (β-lg/β-lg) and a β-lg/α-la complex. The β-lg/β-lg aggregates may either be disulphide-bonded or hydrophobically-bonded, the distribution between the two primarily being dependent on the availability of reactive thiol groups. Disulphides are probably the predominant bonds in the formation of β-lg/α-la aggregates, although at low heating temperatures, a lack of reactive thiol groups may allow β-lg/α-la aggregates to form by hydrophobic interactions. The formation of various aggregates is shown in the following reaction pathways for β-lg and α-la, Equation 4.31 and 4.32, respectively.

\[
\begin{align*}
\beta-N & \Leftrightarrow \beta-U \rightarrow (\beta-A)_{ss} \\
\downarrow & \uparrow \\
(\beta-A)_{s} 
\end{align*}
\]  

(4.31)

Where

- $\beta-N$ = native β-lg.
- $\beta-U$ = unfolded β-lg.
- $(\beta-A)_{s}$ = hydrophobic aggregates of β-lg.
- $(\beta-A)_{ss}$ = disulphide-linked aggregates of β-lg.

\[
\begin{align*}
\alpha-N & \Leftrightarrow \alpha-U + \beta-D \rightarrow (\alpha\beta-A)_{ss} \\
\downarrow & \uparrow \\
(\alpha\beta-A)_{s} & + \beta-D 
\end{align*}
\]  

(4.32)

Where

- $\alpha-N$ = native α-la.
- $\alpha-U$ = unfolded α-la.
(αβ-A)$_s$ = hydrophobic aggregates of α-la and β-lg.
β-D = denatured β-lg (β-U, β-A$_{cr}$, β-A$_s$).
(αβ-A)$_{ss}$ = disulphide-linked α-la and β-lg aggregate.

In both proteins the native structure (β-N, α-N) unfolds on heating to expose hydrophobic groups, and in the case of β-lg a thiol group is also exposed. At low temperatures i.e. up to 70-75°C the low activity of the thiol groups allows for the formation of hydrophobic aggregates (β-A$_s$, αβ-A$_s$). At higher temperatures the hydrophobic bonds are weakened and thiol-group development increases, thus driving denatured β-lg (β-U) to aggregate by sulphydryl-disulphide interchange, forming disulphide-linked polymers, (β-A)$_{ss}$. The absence of a thiol group in α-la and its high levels of renaturation (Rüegg et al., 1977), means that α-la does not readily form aggregates with itself. Instead aggregates are formed with β-lg, either by hydrophobic interactions (αβ-A)$_s$ or sulphydryl-disulphide interchange (αβ-A)$_{ss}$.

4.3.4 Heat-induced interactions between whey proteins and the casein micelle

The total amount of whey proteins in the supernatant of heated skim milk were determined by SDS-PAGE (dissociating and reducing conditions). Total whey protein in the supernatant contained non-sedimentable native protein, hydrophobic aggregates and disulphide-linked aggregates (see Figure 4.1).

The loss of total β-lg from the supernatant is shown in Figure 4.16. The level of β-lg in the supernatant decreased as heating time and/or temperature increased. This decrease was presumably due to β-lg associating with the micelle and co-sedimenting upon centrifugation. As expected the loss of β-lg from the supernatant in this system was smaller than the observed loss of either native or SDS-monomeric β-lg (Figures 4.5, 4.11a). The loss of total α-la from the supernatant followed a trend similar to that for β-lg (Figure 4.17), although the loss of α-la was slower.
Figure 4.16  Loss of β-lg from SDS-PAGE gels in heated skim milk. Heating temperature; 90°C (○), 95°C (●), 100°C (△), 110°C (▲) and 130°C (□).

Figure 4.17  Loss of α-la from SDS-PAGE gels in heated skim milk. Heating temperature; 90°C (○), 95°C (●), 100°C (△), 110°C (▲) and 130°C (□).
4.3.4.1 Relationship between native whey protein and whey protein associated with the casein micelle

The relationship between the quantities of native β-lg and the levels of β-lg associated with the casein micelle (difference between total and β-lg in the ultracentrifugal supernatant) is shown in Figure 4.18. At any given level of denaturation, only a fraction of the denatured β-lg associated with the micelle. The rest of the denatured β-lg remained in the serum as predominantly disulphide-linked aggregates (Figure 4.14). The level of association of β-lg with the micelle reached a maximum of approximately 55% when most of the β-lg was denatured (<20% native remaining). In the temperature region 95-130°C, β-lg association occurred to a slightly lesser extent than at 75-90°C (Figure 4.18).

![Figure 4.18](image)

**Figure 4.18** Relationship between native β-lg and β-lg associated with the casein micelle. Heating temperature; 95-130°C (○) and 75-90°C (●).

The relationship between the amounts of native α-la and the α-la associated with the micelle followed a similar pattern to that of β-lg (Figure 4.19). The level of association of α-la with the micelle varied with heating temperature; for the same level of denaturation, it was greater when heated at 75-90°C, than at 95-130°C. The maximum
association of α-la with the micelle was 50% in the 75-90°C range and 40% in the 95-130°C range.

![Figure 4.19](image)

**Figure 4.19** Relationship between native α-la and α-la associated with the casein micelle. Heating temperature, 95-130°C (○) and 75-90°C (●).

### 4.3.4.2 Relationship between β-lg and α-la associated with the casein micelle

The relationship between β-lg and α-la associations with the micelle after heating at 80-130°C and at 75°C is shown in Figure 4.20. Heating at 80-130°C resulted in an initial lag phase where there was little association of α-la with the micelle (<40 x 10⁻⁶ mol kg⁻¹ associated β-lg). Further heating resulted in both β-lg and α-la associating with the micelle. In contrast, at 75°C the association behaviour of β-lg and α-la was different. There was little or no lag phase at the start of heating, and the ratio of associated α-la to associated β-lg was greater than the corresponding ratio at higher temperatures. The ratio of α-la to β-lg associated with the micelle was approximately 1 mole of α-la to 2 moles of β-lg, up to 40 x 10⁻⁶ mol kg⁻¹ of β-lg.

The lag phase suggests that a β-lg/α-la complex was not required for β-lg to associate with the micelle. These results suggest that there were different association mechanism at 75°C and at 80-130°C. The association behaviour in the two different temperature
regions could be explained by the rates of denaturation. In this study the rates of denaturation of β-lg and α-la were similar at 75°C, but above 80°C β-lg denaturation occurred at a much faster rate (see Figure 4.10). Thus heating at 80-130°C caused significant amounts of the β-lg to denature and associate with the micelle, before α-la began to denature. α-La does not associate with micelle on its own, but is thought to form an aggregate with β-lg which then associates with the micelle (Elfagm & Wheelock, 1978b; Calvo et al., 1993). It is possible that β-lg, which had aggregated or associated with the micelle, was less accessible for sulphydryl-disulphide interchange reactions with α-la than denatured β-lg in an unfolded and monomeric state. This would explain the slower rate of α-la association observed at temperature above 80°C.

![Figure 4.20](image_url)

**Figure 4.20** Association of β-lg and α-la with the casein micelle in heated skim milk. Heating temperatures; 80-130°C (○) and 75°C (●).

The maximum level of β-lg that associated with the micelle in heated skim milk was found to be approximately 55% of the total β-lg, i.e. 80.5 mg β-lg/g casein. Singh and Creamer (1991a) using pilot scale DSI heating (120°C for 3 min) also reported that approximately 50% of the β-lg sedimented with the micelles. However, higher levels of association (75-80%) have been reported by Smits and Brouwershaven (1980), who
heated mixtures of casein micelles and β-lg (milk salts buffer) in sealed tubes in a thermostatically controlled waterbath. Both temperature and time of heating have been known to affect the extent of association of β-lg with casein micelles. Richardson and Noh (1988) showed that increasing the heating time from 30 s to 20 min at 95°C, caused the association of β-lg with κ-casein in skim milk to rise from 60 to 90%. Furthermore, the system of heating used may have an effect on the maximum level of association. Comparison of the results of this study with those of Smits and Brouwershaven (1980) indicates that heating in a pilot scale DSI system appears to result in less association than heating in a waterbath. The time taken for samples to reach the required temperature would be slower in a waterbath and indirect heating systems, than a DSI system. This suggests that reactions which occur while the sample is heating up may affect the final level of association. In addition, the pH of milk at heating has been shown to influence the association of β-lg with the casein micelle; at pHs 6.5 to 6.7 β-lg remains associated with the casein micelles, but at higher pH β-lg/κ-casein complex dissociates from the micelle and remains in the serum (Singh & Fox, 1987a).

It is conceivable that the amount of β-lg associating with the micelles depends on the amount of available κ-casein. The molar concentrations of both β-lg and κ-casein in milk are typically 0.18 mmol L⁻¹ (Walstra & Jenness, 1984). If the reaction proceeded in a 1 to 1 ratio, then all the β-lg that denatured should react with the κ-casein, and thus be associated with the micelle. From this study, it is clear that β-lg/κ-casein complex formation is not as simple, as a 1 to 1 sulphydryl-disulphide interchange reaction between β-lg and κ-casein. Under an electron microscope, the casein micelle surface in heated milk has thread like particles protruding from it; these particles are whey protein/κ-casein complexes (Creamer & Matheson, 1980). These attached whey proteins may increase the steric hinderance at the micelle surface, and thus limit further β-lg association. In addition, the dissociation of κ-casein, either as individual proteins or as a whey protein/κ-casein complex could also limit the extent of association. In heated milk samples the average ratio of β-lg/κ-casein (ratio of band intensities) in the supernatant was 2.0. In contrast, the ratios of β-lg/κ-casein in the micelle varied between 0.5-0.7 with an average value of 0.6. These ratios suggest that it is possible
a portion of the whey protein did not associate with the micelle and remained in the supernatant as aggregates. During the formation of \( \beta \)-lg aggregates the reactive thiol group may have been buried within the interior of the aggregate, and therefore unavailable for sulphydryl-disulphide interchange reactions with \( \kappa \)-casein. Kirchmeier et al. (1984) observed that the level of available thiol groups reached a maximum at 95°C, and decreased as the temperature increased further. The lack of available thiol groups may result in less sulphydryl-disulphide interchange reactions between \( \beta \)-lg and \( \kappa \)-casein, and thus less association.

A possible reaction pathway for whey protein association with the micelle is shown in Equation 4.33, where the principal reaction is between denatured or aggregated whey proteins and \( \kappa \)-casein on the micelle surface.

\[
\begin{align*}
\beta-U & \rightarrow \text{Association} \rightarrow \text{Dissociation} \\
(\beta-A)_{s,s} & \rightarrow \text{with the} \rightarrow \text{of whey protein/\( \kappa \)-casein} \quad (4.33) \\
(\alpha/\beta-A)_{s,s} & \rightarrow \text{Casein complex and } \kappa \text{-casein} \\
(\alpha/\beta-A)_{x} & \rightarrow \text{Micelle}
\end{align*}
\]

Interactions between the whey proteins and the casein micelle have been shown to occur principally between \( \beta \)-lg and \( \kappa \)-casein via thiol-disulphide interchange and hydrophobic interactions (Smits & Brouwershaven, 1980; Haque & Kinsella, 1988; Jang & Swaisgood, 1990), while \( \alpha \)-la can only complex with the micelle indirectly through a \( \beta \)-lg/\( \alpha \)-la aggregate (Baer et al., 1976). Though dissociation of a whey protein/\( \kappa \)-casein complex occurs (Singh & Fox, 1987a), it is not known how this complex behaves. After dissociation of \( \kappa \)-casein it is probable that interactions with the whey proteins can occur, as studies have shown purified \( \beta \)-lg/\( \kappa \)-casein interactions in buffered solutions (Long et al., 1963; Tesier et al., 1969; Haque & Kinsella, 1987). Whether whey protein/\( \kappa \)-casein complexes in the serum can reassociate is unknown. Van Boekel et al.
(1989) found reassociation of κ-casein, and association of the whey proteins occurring, when milk (pH 7.05) was heated at 140°C, but this may have been a result of the pH decreasing during heating, as below pH 6.6 reassociation increased markedly. The pH affecting the charge on the micelle surface in combination with the hydrophobic interactions among the caseins is probably the major influence in determining the extent of association and reassociation.

4.3.5 Mathematical model for β-lg association with the casein micelle

4.3.5.1 Model concept

A reaction pathway for β-lg denaturation, aggregation and association with the micelles is proposed in Equation 4.34.

\[ \beta-N \rightarrow \beta-D \rightarrow \text{Association with the micelle} \]  

(4.34)

Where

\[ \beta-D = \text{aggregated } \beta\text{-lg (} \beta-A_{s,s}, \beta-A_s, \alpha/\beta-A_{s,s}, \text{and } \alpha/\beta-A_s) \]

The model has been kept relatively simple, i.e. two irreversible reactions in series, and is treated as an overview of the complex reactions occurring in heated skim milk. The mechanism of whey protein denaturation has been previously described as a two-step process (de Wit, 1990; Mulvihill & Donovan, 1987) (see Section 2.2.1). Recently an intermediate in native protein unfolding was identified as the molten globular state. While the secondary structure is retained, a molten globular protein increases in size slightly from the native state, and has a more fluid tertiary structure, which exhibits greater hydrophobicity (Kronman et al., 1967; Dolgikh et al., 1981; Kuwajima et al., 1989). The native-PAGE method used in this study, is assumed to measure irreversible denaturation, or the conversion of native β-lg to aggregated β-lg. Thus an unfolded or a molten globular state was not included in the model. Most of the β-lg aggregates in the supernatant were disulphide-linked (Figure 4.13), so a possible separate pathway for hydrophobic aggregates was ignored and both disulphide-linked and hydrophobic aggregates were assumed to form, and associate with the micelle following the same
pathway. Interactions with other whey proteins were not considered in the model. β-Lg comprises the largest portion of the whey proteins (~50%) (Eigel et al., 1984) and although α-la constitutes 20% of the whey proteins, it has been shown not to affect β-lg denaturation, and association with the casein micelle (Elfagm & Wheelock, 1978b; Smits & Brouwershaven, 1980).

4.3.5.2 Model development

An empirical relationship (Equation 4.35) was developed from the model for β-lg denaturation, aggregation and association (Equation 4.34). The following rate equations, Equation 4.36, 4.37 and 4.38, were formulated from Equation 4.35.

\[
\begin{align*}
C_n &\rightarrow C_a + (C_k - C_m) & \rightarrow C_m
\end{align*}
\]

(4.35)

Where

- \(C_n\) = concentration of native β-lg (mol × 10^6 kg\(^{-1}\)).
- \(k_1\) = denaturation rate constant for β-lg (mol × 10^6 kg\(^{-1}\))\(^{(1-n)}\) s\(^{-1}\).
- \(C_a\) = concentration of aggregated β-lg in the supernatant (disulphide-linked and hydrophobically-bonded) (mol × 10^6 kg\(^{-1}\)).
- \(C_k\) = maximum concentration of β-lg that can associate with the micelle (mol × 10^6 kg\(^{-1}\)).
- \(k_2\) = association rate constant (mol × 10^6 kg\(^{-1}\))\(^{-1}\) s\(^{-1}\).
- \(C_m\) = concentration of β-lg associated with the micelle (mol × 10^6 kg\(^{-1}\)).

The loss of native β-lg is defined by Equation 4.36.

\[
-\frac{dC_n}{dt} = k_1 C_n^n
\]

(4.36)

Where

- \(n\) = reaction order
The formation of aggregated β-lg (disulphide-linked and hydrophobically-bonded) is defined by Equation 4.37.

\[ \frac{dC_a}{dt} = k_1 C_n^n - k_2 C_a (C_k - C_m) \]  \hspace{1cm} (4.37)

The formation of β-lg associated with the micelles is defined by Equation 4.38.

\[ \frac{dC_m}{dt} = k_2 C_a (C_k - C_m) \]  \hspace{1cm} (4.38)

The rate equations (Equation 4.37 & 4.38) treated the association of β-lg with the casein micelle as a bimolecular reaction between aggregated β-lg \((C_a)\) and the term \((C_k - C_m)\). This term is effectively the remaining capacity of the micelles to associate with β-lg.

It was assumed that \(C_k\) is not significantly affected by temperature effects such as dissociation of κ-casein from the micelle and development of reactive thiol groups. The relationship between native and associated β-lg (Figure 4.18) was similar in both the 75-90°C and the 95-130°C regions, and the maximum extent of association was the same (about 55%). Although β-lg aggregation is not necessary for interaction with κ-casein (Euber & Brunner, 1982) it appears that the main form of association involves aggregated β-lg (Haque & Kinsella, 1987). The model was simplified by the exclusion of any dissociation of a β-lg/κ-casein complex from the micelle. It should be noted that the term \(C_k\) is an empirical approach to fitting a curve which does not approach zero, even after prolonged heating (Figure 4.16).

The rate equations were numerically integrated using the Runga-Kutta fourth-order method (Bajpai et al., 1987). A program was written in Turbo Pascal (version 6.0, Borland International, Inc., 100 Borland Way, Scotts Valley, California, USA) to calculate the numerical solutions (Appendices A1 & A2). The denaturation rate
constant, $k_n$, was calculated from the kinetic parameters already obtained by NLR (Table 4.4). Different values of $k_2$ were used in the program to obtain predicted values of β-lg associating with the micelles. The predicted levels of β-lg association with the micelle were compared with the experimental results (e.g. Figure 4.16) using weighted least squares analysis (Equation 4.14). The results of fitting the model to the experimental data are shown in Table 4.10. The adjusted $R^2$ values were calculated using Equation 4.26 and the F values were determined by Equation 4.24. All the F values comparing the model variance against the error variance were greater than their corresponding F values for $\alpha = 0.05$ and their respective degrees of freedom, so the model was adequate in predicting β-lg association with the micelle. An example of the predicted curves and experimental data points, shows a reasonable level of fit (Figure 4.21).

The accuracy of the model is highly dependent on the value of $C_k$. This term has no mechanistic basis, and is only an empirical constant. Therefore the model is satisfactory for predicting β-lg association, as long as the value of $C_k$ is accurately known. Experimental conditions, such as heating method (DSI compared to waterbath heating) and different methods of protein analysis, may affect β-lg association with the micelle, and hence affect the value of $C_k$. Thus the values of $C_k$ need to be determined for the different experimental conditions. Variations in milk composition could also affect the predictability of the model. Further research on the mechanism of β-lg/κ-casein interaction in milk will need to be undertaken to improve the accuracy of the model.
Table 4.10 Statistical analysis of the model against the experimental data for β-lg association with the micelle.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Total number of data points (N)</th>
<th>$R^2$</th>
<th>F</th>
<th>$F_{0.05,P:1,N-P}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td>7</td>
<td>0.82</td>
<td>28.3</td>
<td>6.61</td>
</tr>
<tr>
<td>120</td>
<td>8</td>
<td>0.64</td>
<td>15.2</td>
<td>5.99</td>
</tr>
<tr>
<td>110</td>
<td>8</td>
<td>0.92</td>
<td>78.3</td>
<td>5.99</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>0.82</td>
<td>18.7</td>
<td>10.1</td>
</tr>
<tr>
<td>95</td>
<td>9</td>
<td>0.97</td>
<td>275.8</td>
<td>5.59</td>
</tr>
<tr>
<td>90</td>
<td>5</td>
<td>0.97</td>
<td>150.0</td>
<td>10.1</td>
</tr>
<tr>
<td>85</td>
<td>6</td>
<td>0.87</td>
<td>35.5</td>
<td>7.71</td>
</tr>
<tr>
<td>80</td>
<td>5</td>
<td>0.75</td>
<td>13.1</td>
<td>10.1</td>
</tr>
<tr>
<td>75</td>
<td>5</td>
<td>0.96</td>
<td>69.3</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Figure 4.21 Loss of β-lg from SDSR-PAGE gels, (-) predicted from the model and experimental data 75°C (○), 80°C (●) and 85°C (△).
4.3.5.3 Kinetics of β-lg association with the micelle

The rate of β-lg association with the micelles ($k_2$) predicted from the model followed an Arrhenius relationship (Figure 4.22). A break in the curve was found at 100°C, which corresponds closely to the break found at 90-95°C for β-lg denaturation. The rate at 75°C was very slow compared with the other rates in the 100-80°C range, and appears to break away from the Arrhenius relationship in this temperature region (Figure 4.19). The lack of reactive thiol-groups at this temperature (Kirchmeier et al., 1984) probably slows down the association reaction. Association was found to occur at a much slower rate than denaturation; at 130°C the rate of denaturation was 0.0160 (mol × 10^6 kg^{-1})^{-0.4} s^{-1} compared with 0.00039 (mol × 10^6 kg^{-1})^{-1} s^{-1} for association. Using the Arrhenius equation, the activation energies and pre-exponential terms were calculated (Table 4.11).

![Figure 4.22](image-url) Arrhenius plot of β-lg association with the micelle predicted by the model. Rate constants; $k_2$ - association (○) and $k_1$ - denaturation (●).
Table 4.11 Kinetic parameters for β-lg association with the casein micelle in skim milk.

<table>
<thead>
<tr>
<th>Temperature range (°C)</th>
<th>Kinetic parameters with 95% confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_a$ (kJ mol$^{-1}$)</td>
</tr>
<tr>
<td>80-100</td>
<td>112.14 ± 37.14</td>
</tr>
<tr>
<td>100-130</td>
<td>7.29 ± 8.10</td>
</tr>
</tbody>
</table>

An explanation for the break in the Arrhenius plot could be outlined as follows. The association reaction involves two steps; the first is the development of reactive thiol-groups by β-lg so that sulphhydryl-disulphide interchange can occur with κ-casein, and the second is the actual association where β-lg has to orientate itself to the disulphide bond of κ-casein in order for association to occur. The availability of reactive thiol groups would depend on the rate of denaturation (unfolding) and the rate of β-lg/β-lg disulphide-linked aggregation. The activation energy predicted by the model was very low in the 100-130°C range. Thiol group availability at the higher temperatures may have contributed to this low activation energy. At 95°C the level of reactive thiol groups in milk reaches a maximum, and as the temperature increases the level drops (Kirchmeier et al., 1984) Presumably this is caused by disulphide-linked aggregation. Lack of available thiol groups above 95°C, would slow the sulphhydryl-disulphide interchange reactions between β-lg and κ-casein. As this effect was not considered by the model, the actual activation energy may be higher.

Thermodynamic parameters, as previously outlined in Section 4.3.2.3, were calculated for β-lg association with the micelle (Table 4.12). The negative entropy of activation ($\Delta S^o$) above 100°C indicates that the activated complex is in a higher state of order than the reactants, probably through the association of β-lg with the micelle. The values of $\Delta S^o$ in the 80-95°C range are only slightly above zero. A possible explanation is that the activated complex is formed by both the loss of native structure (unfolding), and the formation of aggregates, which correspond to a gain and loss, respectively, of degrees...
of freedom of translational and rotational entropy. The negative values of $\Delta S^\ddagger$ at temperatures above 100°C indicates an increase in the order of the system, possibly caused by the association of $\beta$-lg with $\kappa$-casein via thiol-disulphide interchange reactions. As discussed before with denaturation (section 4.3.2.3), there appears to be two steps involved in the association of $\beta$-lg with the micelle. Denaturation and aggregation of $\beta$-lg to form available thiol-groups appears to be the rate limiting step in the 80-95°C range, while above 100°C the rate limiting step is actual reaction of association.

Table 4.12 Thermodynamic parameters; $\Delta H^\ddagger$, $\Delta S^\ddagger$ and $\Delta G^\ddagger$ for $\beta$-lg association with the micelle.

<table>
<thead>
<tr>
<th>Temperature range (°C)</th>
<th>$\Delta H^\ddagger$ (kJ mol$^{-1}$)</th>
<th>$\Delta S^\ddagger$ (kJ mol$^{-1}$K$^{-1}$)</th>
<th>$\Delta G^\ddagger$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>109.06</td>
<td>0.024</td>
<td>100.70</td>
</tr>
<tr>
<td>85</td>
<td>109.02</td>
<td>0.022</td>
<td>101.31</td>
</tr>
<tr>
<td>90</td>
<td>108.98</td>
<td>0.020</td>
<td>101.54</td>
</tr>
<tr>
<td>95</td>
<td>108.94</td>
<td>0.021</td>
<td>101.18</td>
</tr>
<tr>
<td>100</td>
<td>4.19</td>
<td>-0.259</td>
<td>100.88</td>
</tr>
<tr>
<td>110</td>
<td>4.10</td>
<td>-0.260</td>
<td>103.67</td>
</tr>
<tr>
<td>120</td>
<td>4.02</td>
<td>-0.260</td>
<td>106.17</td>
</tr>
<tr>
<td>130</td>
<td>3.94</td>
<td>-0.260</td>
<td>108.69</td>
</tr>
</tbody>
</table>

4.4 CONCLUSIONS
NLR was an effective method for the determination of denaturation kinetic parameters, and produced values for these parameters with a higher level of precision than the
frequently employed two-step method. Further advantages over the two-step method were the greater utilisation of the raw data and better treatment of the raw data variance than linear regression. Though more researchers in different fields are starting to use NLR, the use of NLR in milk protein denaturation studies has yet to be reported.

In decreasing order of sensitivity to heat denaturation, the whey proteins followed the sequence IgG $> \beta$-Ig B $> \beta$-Ig A $> \alpha$-la. Breaks in the Arrhenius plot for $\beta$-Ig A and B (90°C) and $\alpha$-la (80°C) were found, though no break occurred for IgG over the temperature range 70-90°C.

Aggregation of $\beta$-Ig occurred mainly by disulphide-linkages, though hydrophobic aggregation occurred to a minor extent. At temperatures 75-80°C $\alpha$-la aggregated principally through hydrophobic interactions, while at higher temperatures it probably aggregated mainly by thiol-disulphide interchange with $\beta$-Ig.

Both $\beta$-Ig and $\alpha$-la sedimented with the micelles on ultracentrifugation, presumably associated with the micelle. Mathematical modelling showed that $\beta$-Ig association followed a similar Arrhenius relationship to that of denaturation, with a break in the curve at approximately 100°C.
CHAPTER 5: EFFECT OF SEASONAL VARIATIONS ON HEAT-INDUCED WHEY PROTEIN DENATURATION AND INTERACTIONS

5.1 INTRODUCTION
The production of milk in New Zealand is predominantly seasonal (= 90% of the herds) and occurs typically from August to May (Holmes, 1981; IDF, 1993). In New Zealand, where cows are fed pasture throughout the year, the shape of the lactation curve (change in milk composition with stage of lactation) is dominated by the way climatic factors affect grass growth and hence availability of feed (Holmes & Wilson, 1987). There are marked variations in milk composition during the season which ultimately affect the properties of the manufactured dairy products. Changes in total protein (McDowell, 1972a; Harding & Royal, 1974; Davies & Law, 1980), individual milk proteins (Dellamonica et al., 1965; Sanderson, 1970b; de Koning et al., 1974) and minerals (White & Davies, 1958; Pouliot & Boulet, 1995) during the season have been observed.

One of the major problems caused by seasonal variation is that the heat stability of milk tends to be low at the beginning and end of the dairying season (Newstead et al., 1975). The solubility of some types of milk powders is reduced towards the end of the season (Singh & Newstead, 1992). The effects of seasonal variation on the heat stability of normal milk, concentrated milk and recombined milks have been reported from New Zealand (Newstead et al., 1975; Singh & Tokely, 1990), Ireland (Kelly et al., 1982), the Netherlands (de Koning et al., 1974) Scotland (Holt et al., 1978) and Australia (Augustin et al., 1990). No information is available on the denaturation and aggregation behaviour of whey proteins in milk during the dairying season.
The objective of the work presented in this chapter was to compare the extent and rates of heat-induced reactions of whey proteins in early, mid and late season skim milk.

5.2 MATERIALS AND METHODS

5.2.1 UHT processing

Bulk whole milk (spring calving) was obtained on three occasions, i.e. early season (August, 1994), mid season (December, 1993) and late season (April, 1994), from the Tui Dairy company, Longburn, and separated without pasteurisation, at the NZDRI processing hall. Heating was carried out on a pilot scale UHT plant at a range of temperatures (70-130°C) and holding times (5-1800 s) as described in Sections 4.2.2.1 and 4.2.2.2.

5.2.2 Skim milk analysis

The skim milk samples were centrifuged and the supernatants were analyzed by PAGE as previously outlined in Section 4.2.3. The pH of the unheated milks was measured at 20°C using an Orion pH meter (Model 720 pH/ISE meter, Orion Research Incorporated, Boston, MA) and probe (Model 91-55/56, Orion Research Incorporated).

5.2.3 Kinetic analysis

Analysis of the raw data from each trial was carried out by NLR as shown in Section 4.2.4. As there were not enough temperature points in the WPNI results to determine the Arrhenius parameters, the NLR equation (Equation 4.12) was modified to exclude the $E_a$ and $k_{ref}$ terms (Equation 5.1). Instead of the concentration, the proportion of native whey protein remaining after a particular heat treatment was used as the dependent variable in the NLR equation. The rate constant ($k$) and reaction order ($n$) were calculated for each temperature. The holding tube temperature drop was not considered significant, and isothermal conditions across the tube were assumed.
Where

\[ P_t = P_0 \left[ 1 + (n-1) k t \right]^{\frac{1}{1-n}} \]  

(5.1)

\[ t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(n_1 - 1) s_1^2 + (n_2 - 1) s_2^2}{N_1 + N_2 - 2} \left( \frac{1}{N_1} + \frac{1}{N_2} \right)}} \]  

(5.2)

The means of the kinetic parameters for different seasonal milks were compared to see if they were significantly different, using the \( t \) statistic (Equation 5.2).

The subscripts, 1 and 2, denote the two milk types being compared.

**5.3 RESULTS AND DISCUSSION**

**5.3.1 Composition of seasonal skim milks**

The composition of the seasonal skim milks is shown in Table 5.1. The total solids and pH did not change from early to mid season in skim milk, although the protein concentration was slightly higher in the early season skim milk. The late season skim
milk had a higher total solids content, protein concentration and pH when compared with the early and mid season skim milks. The concentrations of β-lg A, β-lg B and IgG were higher in early and late season skim milks (Table 5.1). The concentration of α-la was highest in the early season skim milk and followed a downward trend reaching a low in the late season skim milk. Climatic conditions during August 1994 (early season) and December 1993 (mid season) were typical for those months, but dry Autumn conditions in April 1994 (late season) may have affected milk production and composition.

Table 5.1 Composition of seasonal skim milks (this study).

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Early season</th>
<th>Mid season</th>
<th>Late season</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Solids (%)</td>
<td>9.00</td>
<td>9.02</td>
<td>9.49</td>
</tr>
<tr>
<td>Total Protein (g/100 g)</td>
<td>3.34</td>
<td>3.29</td>
<td>4.10</td>
</tr>
<tr>
<td>pH</td>
<td>6.66</td>
<td>6.67</td>
<td>6.69</td>
</tr>
<tr>
<td>Individual whey proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-lg A (g kg⁻¹)</td>
<td>1.69</td>
<td>1.54</td>
<td>1.90</td>
</tr>
<tr>
<td>β-lg B (g kg⁻¹)</td>
<td>1.88</td>
<td>1.72</td>
<td>2.13</td>
</tr>
<tr>
<td>α-la (g kg⁻¹)</td>
<td>0.81</td>
<td>0.76</td>
<td>0.60</td>
</tr>
<tr>
<td>IgG (g kg⁻¹)</td>
<td>0.58</td>
<td>0.51</td>
<td>1.16</td>
</tr>
</tbody>
</table>

The changes in protein concentration observed in this study were similar to that observed by McDowell (1972a) for the nitrogen content of milk, where the level was high at the beginning of the season, fell away during the middle of the season and then increased rapidly towards the end of the season. The pH has been shown to increase towards the end of the season/lactation (White & Davies, 1958; Salam et al., 1983). Sanderson (1970b), and Regester and Smithers (1991) found that towards the end of the season β-lg concentrations increased. The decrease in immunoglobulins during lactation has been reported by a number of authors (Dellamonica et al., 1965; Davies and Law,
1980; Walstra & Jenness, 1984). In contrast, Newstead et al. (1977) reported that the concentration of immunoglobulins in bulk New Zealand skim milk was lowest during the middle of the season (November), and was greater in early (September) and late (April) season skim milks. The high concentration of immunoglobulins that were observed in the late season skim milk could have been caused by milking cows that were at the end of lactation. Seasonal changes in $\alpha$-la have been reported as either minimal (Dellamonica et al., 1965; de Koning et al., 1974) or as a gradual decline during the season (Davies & Law, 1980; Regester & Smithers, 1991).

The range in seasonal/lactation variation of total solids, protein, whey protein concentration and pH based on previous studies is shown in Table 5.2.

Table 5.2: Range of seasonal/lactational variation in total solids, total protein, whey protein concentration and pH of skim milk (literature values).

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Seasonal/lactation variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids concentration (% w/w)$^a$</td>
<td>8.63 - 9.72</td>
</tr>
<tr>
<td>Total protein concentration (g/100 g)$^b$</td>
<td>3.42 - 3.99</td>
</tr>
<tr>
<td>Whey protein concentration (g/100 g)$^c$</td>
<td>0.53 - 0.82</td>
</tr>
<tr>
<td>pH$^d$</td>
<td>6.64 - 6.95</td>
</tr>
</tbody>
</table>

References:


$^d$Newstead et al. (1977).

5.3.2 Kinetics of whey protein denaturation

The loss of native $\beta$-lg A from skim milk heated at 75-130°C was determined. The results obtained at 80 and 90°C are shown in Figure 5.1. At heating temperatures $\leq 80$°C
the loss of β-lg A was least in early season skim milk, slightly greater in mid season skim milk, and greater still in late season skim milk. A similar trend was observed at heating temperatures ≥90°C, but the difference between the skim milks was much smaller. A similar trend was observed for β-lg B (Figure 5.2), α-la (Figure 5.3) and IgG (Figure 5.4).

Denaturation kinetic parameters for β-lg A, β-lg B and α-la in early, mid and late season skim milks are shown in Tables 5.3, 5.4 and 5.5, respectively. In some cases the initial concentration, as determined by native-PAGE (Table 5.1), had to be set as a constant in NLR analysis so that a solution could be found. Kinetic parameters for β-lg A and β-lg B in the 100-130°C region of late season skim milk could not be determined (Table 5.3 & 5.4), because denaturation proceeded faster in late season skim milk and the levels of protein measured were very low. The rates of IgG denaturation at 80°C \( (n = 2) \) were 0.055, 0.101 and 0.160 s\(^{-1}\) (for a definition of the rate see Chapter 4, Equation 4.17), in early, mid and late season skim milks, respectively.

The reaction orders for β-lg A and β-lg B varied from 0.7 to 1.9, with β-lg B having a higher reaction order than β-lg A. This agrees with the trend observed in skim milk from Autumn calving (Chapter 4, Table 4.4). In the 85-130°C temperature range, the denaturation of α-la was approximately 1st order. Lower reaction orders for α-la, \( n = 0.5-0.9 \), were calculated in the 70-80°C range, but their 95% confidence intervals covered 1.0. As the change in concentration of α-la with time in the 70-80°C range was not as great as in 85-130°C range, the precision with which the reaction order could be determined was less (van Boekel & Walstra, 1995), hence the larger confidence intervals.

The activation energies of β-lg A, β-lg B and α-la in the late season skim milk were significantly different \( (P < 0.01) \) from those in the early and mid season skim milks (Table 5.3-5.5). In general, the activation energies of β-lg A, β-lg B and α-la were slightly lower in late season skim milk compared to the early and mid season skim milks. The activation energies and \( \ln(k_0) \) for β-lg A and β-lg B in mid season skim milk were in good agreement with the values observed in Autumn calving milk (Chapter 4,
Figure 5.1 Loss of β-lg A from native-PAGE gels of skim milk heated at 80°C (A) and 90°C (B). Early (O), mid (●) and late (△) season skim milk.
Figure 5.2 Loss of β-lg B from native-PAGE gels of skim milk heated at 80°C (A) and 90°C (B). Early (O), mid (●) and late (Δ) season skim milk.
Figure 5.3 Loss of α-la from native-PAGE gels of skim milk heated at 80°C (A) and 90°C (B). Early (○), mid (●) and late (△) season skim milk.
Figure 5.4 Loss of native IgG from skim milk heated at 80°C. Early (○), mid (●) and late (△) season skim milk.

Table 4.4, although the values in late and early season (in the 110-130°C range) skim milks differed. In contrast, the kinetic parameters for α-la in late season skim milk were similar to the values reported in Chapter 4 (Table 4.4), while the values in early and mid season skim milk were different.

An Arrhenius plot for β-lg A (Figure 5.5) showed that below 90°C the value for the rate constant was lowest for the early season skim milk and highest for the late season skim milk, and the mid season skim milk was intermediate. In contrast, above 90°C there did not appear to be any seasonal trend. A similar seasonal trend was observed for β-lg B (Figure 5.6). The rate constants for α-la (Figure 5.7) in the temperature range 70-130°C were lowest in early season skim milk, highest for the late season skim milk and intermediate for mid season skim milk. Increasing the temperature, above 80°C, reduced the difference between the rate constants for different skim milks.
Table 5.3 NLR kinetic parameters and 95% confidence intervals for β-lg A denaturation in heated early, mid and late season skim milk.

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Season (Temperature range)</th>
<th>Values from Chapter 4 (Table 4.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early (75-90°C)</td>
<td>Mid (70-90°C)</td>
</tr>
<tr>
<td>No. data points</td>
<td>45</td>
<td>24</td>
</tr>
<tr>
<td>( n )</td>
<td>1.1 ± 0.3</td>
<td>1.3 ± 0.7′</td>
</tr>
<tr>
<td>( E_a ) (kJ mol(^{-1}))</td>
<td>274.49 ± 22.82′</td>
<td>274.94 ± 35.92</td>
</tr>
<tr>
<td>( \ln(k_{o_d}) )</td>
<td>-6.71 ± 0.14</td>
<td>-6.53 ± 0.16</td>
</tr>
<tr>
<td>( \ln(k_0) )</td>
<td>86.12 ± 7.72′</td>
<td>87.11 ± 12.23</td>
</tr>
<tr>
<td>( C_o ) (g kg(^{-1}))</td>
<td>1.57 ± 0.07</td>
<td>1.54b</td>
</tr>
<tr>
<td>( R^2 )</td>
<td>0.969</td>
<td>0.949</td>
</tr>
<tr>
<td></td>
<td>Early (110-130°C)</td>
<td>Mid (100-130°C)</td>
</tr>
<tr>
<td>No. data points</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>( n )</td>
<td>1.6 ± 0.4′</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>( E_a ) (kJ mol(^{-1}))</td>
<td>36.75 ± 11.00′</td>
<td>57.64 ± 5.29</td>
</tr>
<tr>
<td>( \ln(k_{o_d}) )</td>
<td>-2.42 ± 0.40</td>
<td>-2.52 ± 0.08</td>
</tr>
<tr>
<td>( \ln(k_0) )</td>
<td>8.82 ± 3.39′</td>
<td>15.34 ± 1.64</td>
</tr>
<tr>
<td>( C_o ) (g kg(^{-1}))</td>
<td>1.76 ± 0.92</td>
<td>1.54b</td>
</tr>
<tr>
<td>( R^2 )</td>
<td>0.981</td>
<td>0.997</td>
</tr>
</tbody>
</table>

\(^a\)Kinetic parameters defined in Section 4.2.4.
\(^b\)\( C_o \) was treated as a constant and set equal to the value found by native-PAGE (Table 5.1).
\(^*\)Values differ significantly from those of early and mid season skim milks (\(P < 0.01\)).
\(^†\)Values differ significantly from those reported in Autumn calving milk (Chapter 4, Table 4.4) (\(P < 0.01\)).
Table 5.4 NLR kinetic parameters and 95% confidence intervals for β-lg B denaturation in heated early, mid and late season skim milk.

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Season (Temperature range)</th>
<th>Values form Chapter 4 (Table 4.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early (75-90°C)</td>
<td>Mid (70-90°C)</td>
</tr>
<tr>
<td>No. data points</td>
<td>45</td>
<td>24</td>
</tr>
<tr>
<td>$n$</td>
<td>1.3 ± 0.3</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td>$E_a$ (kJ mol$^{-1}$)</td>
<td>295.31 ± 17.32</td>
<td>291.10 ± 36.80</td>
</tr>
<tr>
<td>$\ln(k_{o0})$</td>
<td>-5.93 ± 0.10</td>
<td>-6.29 ± 0.25</td>
</tr>
<tr>
<td>$\ln(k_0)$</td>
<td>93.94 ± 5.86</td>
<td>92.86 ± 12.53</td>
</tr>
<tr>
<td>$C_0$ (g kg$^{-1}$)</td>
<td>1.73 ± 0.06</td>
<td>1.62 ± 0.12</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.983</td>
<td>0.960</td>
</tr>
</tbody>
</table>

|                   | Early (110-130°C) | Mid (100-130°C) | Values from Chapter 4 (95-130°C) |
| No. data points   | 15 | 8 | 43 |
| $n$               | 1.9 ± 0.3" | 1.6 ± 0.2 | 1.6 ± 0.2 |
| $E_a$ (kJ mol$^{-1}$) | 35.14 ± 14.30" | 41.99 ± 8.83 | 43.98 ± 5.76 |
| $\ln(k_{o0})$     | -2.20 ± 0.14 | -2.26 ± 0.13 | -2.78 ± 0.11 |
| $\ln(k_0)$        | 8.55 ± 4.38 | 10.75 ± 2.74 | 10.94 ± 1.80 |
| $C_0$ (g kg$^{-1}$) | 1.88" | 1.72 | 1.76 ± 0.12 |
| $R^2$             | 0.959 | 0.992 | 0.980 |

*Kinetic parameters defined in Section 4.2.4.

*C$_0$ was treated as a constant and set equal to the value found by native-PAGE (Table 5.1).

*Values differ significantly from those of early and mid season skim milks (P < 0.01).

"Values differ significantly from those reported in Autumn calving milk (Chapter 4, Table 4.4) (P < 0.01).
Table 5.5  NLR kinetic parameters and 95% confidence intervals for α-la denaturation in heated early, mid and late season skim milk.

<table>
<thead>
<tr>
<th>Kinetic parametera</th>
<th>Season (Temperature range)</th>
<th>Values form Chapter 4 (Table 4.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early (75-80°C)</td>
<td>Mid (70-80°C)</td>
</tr>
<tr>
<td>No. data points</td>
<td>31</td>
<td>19</td>
</tr>
<tr>
<td>n</td>
<td>0.5 ± 0.7t</td>
<td>0.9 ± 2.0</td>
</tr>
<tr>
<td>$E_a$ (kJ mol$^{-1}$)</td>
<td>220.49 ± 61.66</td>
<td>258.10 ± 132.74</td>
</tr>
<tr>
<td>$\ln(k_{eq})$</td>
<td>-8.23 ± 0.33</td>
<td>-8.28 ± 1.03</td>
</tr>
<tr>
<td>$\ln(k_0)$</td>
<td>67.40 ± 21.15</td>
<td>80.89 ± 45.87*</td>
</tr>
<tr>
<td>$C_0$ (g kg$^{-1}$)</td>
<td>0.81b</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.980</td>
<td>0.859</td>
</tr>
</tbody>
</table>

|                    | Early (90-130°C)          | Mid (90-130°C)                    | Late (85-130°C)                  |
| No. data points    | 29                        | 13                                | 17                               |
| n                  | 1.3 ± 0.4t                | 1.0 ± 0.5                         | 1.0 ± 0.4                        |
| $E_a$ (kJ mol$^{-1}$) | 78.84 ± 5.41t             | 65.82 ± 5.90t                     | 52.32 ± 5.79*                    |
| $\ln(k_{eq})$      | -4.94 ± 0.34              | -4.65 ± 0.44                      | -4.61 ± 0.53                     |
| $\ln(k_0)$         | 19.81 ± 1.74              | 16.01 ± 1.90t                     | 11.92 ± 1.91*                    |
| $C_0$ (g kg$^{-1}$) | 0.78 ± 0.04               | 0.81 ± 0.07                       | 0.65 ± 0.08                      |
| $R^2$              | 0.960                     | 0.960                             | 0.979                            |

*Kinetic parameters defined in Section 4.2.4.

b$C_0$ was treated as a constant and set equal to the value found by native-PAGE (Table 5.1).

Values differ significantly from those of early and mid season skim milks (∆P < 0.01).

Values differ significantly from those reported in Autumn calving milk (Chapter 4, Table 4.4) (∆P < 0.01).
Figure 5.5 Arrhenius plot of β-lg A denaturation in seasonal skim milks heated at 75-130°C. Early (O), mid (●) and late (△) season skim milks. $k_n$ in (g kg$^{-1}$)(1-n)s$^{-1}$.

Figure 5.6 Arrhenius plot of β-lg B denaturation in seasonal skim milks heated at 75-130°C. Early (O), mid (●) and late (△) season skim milks. $k_n$ in (g kg$^{-1}$)(1-n)s$^{-1}$.
5.3.3 Effect of seasonal changes in milk composition on whey protein denaturation

In this study when skim milk was heated at 70-130°C, the extent of whey protein denaturation was generally similar in the early and mid season skim milks, but the late season skim milk had a higher level of denaturation. A number of factors could have contributed to this. Dry Autumn (late season) conditions may have affected milk production and composition beyond the typical variations expected in late season milk. The seasonal changes in concentration of protein, lactose, retinol, fatty acids and milk salts, reported for late season milk may have contributed to the increased denaturation. The increased concentration of β-lg (thiol groups) in the late season milk (Table 7.1) may have resulted in more irreversible aggregation reactions occurring and thus increased the extent of denaturation (Elfagm & Wheelock, 1978b; Calvo et al., 1993). Lactose has a protective effect on the thermal denaturation of whey proteins; increasing the lactose concentration in acid whey increases the thermal stability of whey proteins (Ibrahim et al., 1995). The increased level of denaturation in late season skim milk observed in this study may have resulted from a decrease in the concentration of lactose.
which occurs in late season milk (Phelan et al., 1982; Singh & Newstead, 1992). The binding of retinol to β-lg increases its denaturation temperature but decreases the denaturation enthalpy, as measured in a DSC study of β-lg saturated with retinol (Puyol et al., 1994). The concentration of retinol in whole milk powder from New Zealand Friesian-cross herds is at a minimum during December but increases during the latter stages of the season. The effect of retinol may not be very significant as the naturally occurring concentrations are small, and saturation of β-lg with retinol may be required before there is any observable change. In addition, most of the retinol in milk is esterified with fatty acids (Pérez & Calvo, 1995). The denaturation temperature and enthalpy of β-lg increase when it binds palmitic acid (Puyol et al., 1994). In milk the amount of fatty acids bound is about 1.0 mol per mol of β-lg dimer (Pérez & Calvo, 1995). The binding of fatty acids, rather than retinol, to β-lg may be more important in determining its thermal stability. The concentration of fat increases during the latter part of the season in New Zealand milk (Holmes & Wilson, 1984), although it is not known how this affects the free fatty acid concentration and its binding to β-lg in skim milks. The increase in Na and total Ca content in late season milk may have caused the increase in denaturation, although the concentration of soluble Ca decreases (Pearce & Newstead, 1970; Keogh et al., 1982). Varunsation et al. (1983) showed that the effect of Ca and Na were to promote denaturation and aggregation reactions in WPC solutions.

The increased denaturation of both β-lg and α-la in late season milk were approximately proportionate. The increase in α-la denaturation could have been caused by the increase in β-lg denaturation, resulting in more β-lg/α-la aggregates forming. Increasing the whey protein concentration in skim milk (0.52 to 1.24 g/100 g) increased the extent of α-la denaturation but the increase in β-lg denaturation was comparatively small (results to be discussed in Chapter 6, Section 6.3.2.2). Thus, the increased whey protein concentration in late season skim milk may be responsible for the increased α-la denaturation, but was probably not solely responsible for the increased β-lg denaturation. The lactose and milk salt concentrations, discussed above, may have had a significant role in determining the heat denaturation of β-lg. The influence of β-lg on the rate of IgG denaturation may be small. IgG denaturation was faster than β-lg, therefore most of the immunoglobulins would have denatured prior to β-lg denaturation. Little
information is available in the literature on the effect of milk composition on immunoglobulin denaturation.

5.3.4 Whey protein aggregation and association with the micelle

The changes in β-lg and α-la aggregate species with time, derived from different PAGE results (see Figure, 4.1), are shown in Figures 5.8 and 5.9, respectively. The majority of β-lg aggregates in the supernatant were disulphide-linked, while the α-la aggregates contained proportionally more hydrophobic aggregates.

In all the seasonal skim milks the relationship between denatured β-lg and disulphide-linked aggregates in the supernatant followed a linear relationship with a slope close to 1 (results not shown). This is in agreement with the results for Autumn calving skim milk reported in Chapter 4 (Figure 4.14). In contrast, the formation of disulphide-linked α-la aggregates was less for a given level of denaturation. This indicated that hydrophobic interactions were important in the formation of α-la aggregates. After α-la denatures (unfolding), the lack of a thiol group may limit the opportunity for α-la to form intermolecular disulphide bonds (see Section 4.3.3.3).

The association of β-lg and α-la with the micelle tended to be greatest in the late season skim milk (Figures 5.8 & 5.9). The maximum extent of β-lg association with the micelle was approximately 70, 50 and 75% for early, mid and late season skim milk, respectively. It was possible that the greater extent of association in the early and late season skim milks was caused by the increase in the whey protein concentration (Table 5.1). The amounts of κ-casein in the early and late season skim milks were also greater than in the mid season skim milk (analyzed by SDSR-PAGE bands intensities, data not shown). The combination of increased β-lg and κ-casein, either at early or mid season, may promote increased association between the two proteins. Smits and Brouwershaven (1980), using β-lg and micelles dispersed in a milk salts buffer (without Ca$^{2+}$ or Mg$^{2+}$), showed that increasing the concentration of β-lg from 4 g L$^{-1}$ up to 12 g L$^{-1}$ increased the extent of β-lg associating with the micelles, after heating at 80°C.
Figure 5.8 β-Lg aggregates in early (A), mid (B) and late (C) season skim milk heated at 90°C. β-Lg species; native (□), hydrophobic aggregates (///), disulphide-linked aggregates (XXX) and aggregates associated with the micelle (■).
Figure 5.9 α-La aggregates in early (A), mid (B) and late (C) season skim milk heated at 130°C. α-La species; native (□), hydrophobic aggregates (///), disulphide-linked aggregates (XXX) and aggregates associated with the micelle (■).
The increased association in the late season skim milk may have been caused by the increase in Na concentration, although the effect is likely to be small. The change in skim milk pH is very small, 6.66 to 6.69, and may not have a noticeable effect on whey protein association with the micelle.

The relationship between β-lg and α-la associated with the micelle in Autumn calving skim milk is shown in Figure 4.20. Similar results were observed for each of the seasonal skim milks. Below 80°C the ratio of α-la to β-lg associating with the micelle was much greater than at higher temperatures (> 80°C).

5.4 CONCLUSIONS

The effect of seasonal variation on whey protein denaturation was small during the early (August) and mid parts (December) of the season, but may have become significant during the latter (April) stages of the season. There was a small increase in the extent of heat-induced β-lg and α-la denaturation in late season skim milk. It is possible that the increase in protein concentration coupled with the decrease in lactose in late season skim milk increases the susceptibility of β-lg to heat-induced denaturation. In general the late season denaturation kinetic parameters for β-lg A, β-lg B and α-la were different (P < 0.01) from the corresponding early and mid season parameters.

The maximum extent of association of β-lg with the micelle was greatest in early- and late-season skim milks and lowest in mid-season skim milk. The increased concentration of β-lg and κ-casein in the early and late season skim milks may have resulted in the greater association.
CHAPTER 6: EFFECT OF pH, WHEY PROTEIN
CONCENTRATION AND TOTAL SOLIDS
CONCENTRATION ON HEAT-INDUCED PROTEIN
INTERACTIONS IN SKIM MILK

6.1 INTRODUCTION

Changes in milk composition are known to influence the thermal stability and heat-induced interactions of whey proteins. The effects of whey protein concentration, pH, total solids concentration (total solids) and minerals on whey protein denaturation have been reported in the literature (Elfagm & Wheelock, 1978b; Hillier et al., 1979; Smits & Brouwershaven, 1980; Donovan & Mulvihill, 1987).

Hillier et al. (1979) showed that increasing the total solids, by reconstituting cheese whey powder to different total solids, reduced the rate of thermal denaturation of β-lg A and β-lg B, but increased the denaturation of α-la. The effect of pH on whey protein denaturation has been studied in whey and purified proteins in buffer systems, with pH ranging from 2.5 to 9.0 (Hillier et al., 1979; de Wit & Klarenbeek, 1981, 1984; Bemal & Jelen, 1985). Donovan and Mulvihill (1987) studied the heat-induced denaturation of whey proteins in pH adjusted whey (pH 4.5-7.0). β-Lg is less stable at the higher pH, while α-la stability is relatively independent of pH effects.

It has been well established that pH of milks when heated affects casein dissociation from the micelle, and the extent of whey protein association with the micelle (Smits & Brouwershaven, 1980; Singh & Fox, 1985; Visser et al., 1986; Singh & Creamer, 1991b). At pH >6.9 κ-casein/β-lg complexes dissociate from the micelle on heating, and in the pH range 6.5-6.7 the κ-casein/β-lg complex appears to stabilise the micelle and reduce the dissociation of caseins from the micelle (Singh & Fox, 1987a).
The objective of the work presented in this chapter was to determine the effects of relatively small changes in the pH, whey protein concentration and total solids of skim milk, on whey protein denaturation, aggregation and association with the micelles, in order to ascertain the likely influence of natural variations in milk.

6.2 MATERIALS AND METHODS

Three different trials were carried out to determine the effects of pH, whey protein concentration and total solids, as described below.

6.2.1 Effect of pH

Bulk whole milk was obtained from the Tui Dairy Company, Longburn, in June 1994 (Autumn calving). The milk was separated at 55°C, without pasteurisation, at the NZDRI processing hall. The skim milk (pH 6.67) was split into three 500 L batches and the pH of each batch was adjusted with either 1 M NaOH or 1 M HCl. After stirring overnight at 5°C, the pH of each batch was measured at 20°C (see Section 5.2.2) and the skim milks were then processed on a pilot scale UHT plant, as described in Section 4.2.2.2. Four heating temperatures were used: 75, 80, 85 and 90°C. The pH of the three batches were 6.48, 6.60 and 6.83. The heated skim milk samples, taken after different heating times, were centrifuged and their supernatants were analyzed by quantitative PAGE according to the methods outlined in Section 4.2.3.

6.2.2 Effect of whey protein concentration

Bulk whole milk (Spring calving) obtained from the Tui Dairy Company, was pasteurised and separated at the NZDRI processing hall. Two trials were carried out, one in September 1994 and the other in December 1994, corresponding to early and mid season milk, respectively.

Figure 6.1 shows a schematic diagram of the process used to manufacture the whey-protein adjusted skim milks described below. The skim milk was separated by microfiltration (MF) into a "casein" retentate and a "whey" permeate stream (MFS19 dairy applications membrane, Tetra Laval Filtration Systems, Denmark). The "whey" permeate was then separated by ultrafiltration (UF) to produce a concentrated whey
protein retentate stream and a permeate stream containing minerals and lactose (HFK131 membrane, Koch Membrane Systems, Wilmington, MA 01887, USA). Three 800 L batches of skim milk with varying whey protein concentrations were made up in the NZDRI processing hall. The first batch was untreated skim milk used as the control. The second batch, a whey-protein depleted (WPD) milk, was blended from skim milk, MF retentate ("casein") and UF permeate (minerals and lactose). The whey-protein enriched (WPE) milk was blended from skim milk and UF retentate. The batches of skim milks were stored overnight in stirred tanks at 5°C, and then heat treated on a pilot scale UHT plant for a number of different temperature/time combinations. Heating at 120 and 90°C was carried out in holding tubes, as described in Section 4.2.2.1. Heating at 80°C was carried out using a waterbath, as described in Section 4.2.2.2.

The milks heated on the UHT plant were centrifuged and the supernatants were analyzed by quantitative PAGE, as described in Section 4.2.3. Milk samples were analyzed for NPN and TN as described in Section 4.2.3.1. Total solids and non casein nitrogen (NCN) were determined at the Analytical Chemistry Section of NZDRI. The total solids were determined by drying in a 102°C oven for at least 5 hours. Samples for NCN analysis were prepared according to the method of Aschaffenberg and Dewry (1959) and the N content of the samples was determined using a Kjel-Foss Automatic Titrator 16200 (Foss Electric, Hillerød, Denmark). The whey protein nitrogen index (WPNI) was measured at the Milk Powder Section, NZDRI, in early season skim milk, using the method of Sanderson (1970a). The casein and denatured whey proteins were precipitated from the sample by NaCl, and the undenatured whey protein was then precipitated by amido black dye, and excess dye was determined spectrophotometrically. Unheated milk samples from each batch were analyzed for calcium, total phosphorus, inorganic phosphorus and magnesium at the Analytical Chemistry Section of NZDRI. Calcium and inorganic phosphorus were determined on an autoanalyser system (Chem. Laboratory Instruments Ltd, Hornchurch, England) according to the Chemical Laboratory Instruments Methods (1979). Total phosphorus was determined by digesting the milk using nitric, perchloric acid and sulphuric acid, and then measuring the phosphate colorimetrically as molybdenum blue at 820 nm (Watanabe & Olsen, 1965). Magnesium was measured by atomic absorption spectrometry at 422.7 nm (Boston & Gray, 1986).
Some of the ultracentrifugal supernatant samples were digested in nitric acid and analyzed for calcium, phosphorus and magnesium at AgResearch (Palmerston North) using plasma emission spectrometry. The pH of the unheated skim milks was measured at 20°C (see Section 5.2.2).

Figure 6.1 Schematic diagram of the manufacture of whey protein-adjusted skim milks, blended using the retentates and permeates from MF and UF membrane processes.
6.2.3 Effect of total solids

Bulk whole milk (mid season, December 1994, spring calving) was obtained from the Tui Dairy Company and separated at the NZDRI processing hall, without pasteurisation. The milk was split into three batches; the first batch was the control, the second the diluted milk and the third batch was the concentrated milk. The diluted milk was made by mixing 530 L of skim milk with 270 L of demineralised water. The concentrated milk was manufactured by passing skim milk through the first effect of a three-effect pilot scale (1600 L h$^{-1}$) falling film evaporator (Wiegand, Karlsruhe, Germany). The first effect temperature was set at 70°C and the product temperature was 58-62°C. The concentrate was cooled to 5°C, and both the concentrated and diluted milks were left overnight in a stirred tank at 5°C.

For each batch a number of different temperature/time combinations were used. Heating at 110°C was carried out in holding tubes, as outlined in Section 4.2.2.1, and a waterbath was used to heat the milk at 85 and 90°C, as described in Section 4.2.2.2. Analysis of the supernatants from the centrifuged milks was carried out as previously described in section 4.2.3. The analysis of calcium and phosphorus was carried out at the Analytical Chemistry Section of NZDRI, as described above. The pH of the unheated skim milks was measured at 20°C (see Section 5.2.2).

6.2.4 Kinetic analysis

Analysis of the raw data from the pH trial was carried out by NLR as described in Section 4.2.4 (Equations 4.12 and 4.13). There were not enough temperature points in the whey protein concentration and total solids results to determine the Arrhenius parameters using the NLR equations. The NLR equations were modified to exclude the $E_a$ and $k_{ref}$ (Equation 6.1). The rate constant ($k_n$) and reaction order ($n$) were calculated for each temperature and batch combination. The holding tube temperature drop was not considered significant and isothermal conditions were assumed across the tube.

$$C_t = C_0 [1 + (n-1) k_n C_0^{n-1} T^{(\frac{-1}{1-n})}]$$  \hspace{1cm} (6.1)
6.3 RESULTS AND DISCUSSION

6.3.1 Effect of pH

6.3.1.1 Kinetics of whey protein denaturation

The effect of pH adjustment prior to heat treatment of skim milk at 75 and 80°C on the denaturation of β-lg A and α-la is shown in Figures 6.2 and 6.3, respectively. β-Lg B showed the same trends as β-lg A, and heating at 85 and 90°C showed the same trends as heating at 80°C (data not shown). At 80-90°C the pH had no noticeable effect on the extent of β-lg A, β-lg B or α-la denaturation (Figures 6.2B and 6.3B). However, at 75°C the denaturation of β-lg A, β-lg B and α-la in pH 6.60 milk appeared to occur to a slightly greater extent, than at pH 6.48 and 6.83.

The kinetic parameters for β-lg A, β-lg B and α-la denaturation in pH adjusted and heated skim milks were determined by NLR, and the results are shown in Table 6.1. The break in the Arrhenius plot was found at 90°C for β-lg A and β-lg B, and at 80°C for α-la (see Section 4.3.2.3), and the kinetic parameters were determined over the temperature range 75-85°C. The activation energies of β-lg A and β-lg B were highest at pH 6.48. There was no significant difference between the activation energies at pH 6.60 and 6.83 (Table 6.1).

At the higher pH the protein structure is weakened by the electrostatic repulsion of ionised groups within the protein molecule (Donovan & Mulvihill, 1987). This could cause the reduction in the activation energy observed in this present study, as less energy would be required to break the internal protein bonds. De Wit and Klarenbeek (1981, 1984), using DSC, also found that the enthalpy of β-lg denaturation decreases as the pH increases from 6.0 to 8.0. The same pH trend was not apparent with α-la, where the activation energies showed no downward trend with increased pH (Table 6.1). Little change was observed in the denaturation temperatures and enthalpy of α-la over the range 4.5 to 7.5 by de Wit and Klarenbeek (1984), and Bernal and Jelen (1985). Calcium binding may have a more important role in stabilising the native conformation of α-la than pH (Bernal & Jelen, 1985) which could possibly explain the observed results.
Figure 6.2 Loss of β-lg A from native-PAGE gels of skim milk heated at 75°C (A) and 80°C (B). Milk pH; 6.48 (○), 6.60 (●) and 6.83 (▲).
Figure 6.3 Loss of α-la from native-PAGE gels of skim milk heated at 75°C (A) and 80°C (B). Milk pH: 6.48 (○), 6.60 (●) and 6.83 (△).
Table 6.1 Kinetic parameters for the denaturation of β-lg A, β-lg B and α-la in pH adjusted skim milks. Temperature range 75-85°C.

<table>
<thead>
<tr>
<th>Whey Protein</th>
<th>pH</th>
<th>$n$</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
<th>ln($k_{90}$)</th>
<th>ln($k_0$)</th>
<th>$C_o$ (g kg$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lg A</td>
<td>6.48</td>
<td>1.0 ± 0.4</td>
<td>293.82 ± 28.59</td>
<td>-6.12 ± 0.13</td>
<td>93.95 ± 9.74</td>
<td>1.86 ± 0.14</td>
<td>0.983</td>
</tr>
<tr>
<td></td>
<td>6.60</td>
<td>1.2 ± 0.4</td>
<td>246.96 ± 38.54</td>
<td>-6.07 ± 0.24</td>
<td>78.04 ± 13.13</td>
<td>2.02 ± 0.38</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>6.83</td>
<td>0.6 ± 0.7</td>
<td>245.28 ± 34.06</td>
<td>-6.40 ± 0.22</td>
<td>77.14 ± 11.60</td>
<td>1.68 ± 0.20</td>
<td>0.940</td>
</tr>
<tr>
<td>β-lg B</td>
<td>6.48</td>
<td>1.1 ± 0.3</td>
<td>312.43 ± 28.20</td>
<td>-5.94 ± 0.13</td>
<td>100.47 ± 9.61</td>
<td>1.77 ± 0.12</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>6.60</td>
<td>1.4 ± 0.4</td>
<td>269.84 ± 44.55</td>
<td>-5.81 ± 0.29</td>
<td>86.09 ± 15.18</td>
<td>1.94 ± 0.51</td>
<td>0.985</td>
</tr>
<tr>
<td></td>
<td>6.83</td>
<td>0.9 ± 0.4</td>
<td>237.60 ± 29.29</td>
<td>-6.03 ± 0.20</td>
<td>74.89 ± 9.98</td>
<td>1.71 ± 0.20</td>
<td>0.964</td>
</tr>
<tr>
<td>β-lg (β-lg A + β-lg B)</td>
<td>6.48</td>
<td>1.1 ± 0.4</td>
<td>304.25 ± 28.83</td>
<td>-6.09 ± 0.23</td>
<td>97.53 ± 9.82</td>
<td>3.64 ± 0.26</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>6.60</td>
<td>1.3 ± 0.4</td>
<td>256.73 ± 40.52</td>
<td>-6.16 ± 0.22</td>
<td>87.43 ± 13.80</td>
<td>3.95 ± 0.87</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>6.83</td>
<td>0.7 ± 0.5</td>
<td>235.58 ± 28.57</td>
<td>-6.02 ± 0.34</td>
<td>74.22 ± 9.74</td>
<td>3.43 ± 0.39</td>
<td>0.961</td>
</tr>
<tr>
<td>α-la</td>
<td>6.48</td>
<td>1.3 ± 0.7</td>
<td>193.97 ± 42.12</td>
<td>-6.82 ± 0.49</td>
<td>59.24 ± 14.35</td>
<td>0.88 ± 0.09</td>
<td>0.941</td>
</tr>
<tr>
<td></td>
<td>6.60</td>
<td>1.2 ± 1.0</td>
<td>222.70 ± 75.82</td>
<td>-7.08 ± 0.79</td>
<td>68.77 ± 25.84</td>
<td>0.82 ± 0.11</td>
<td>0.865</td>
</tr>
<tr>
<td></td>
<td>6.83</td>
<td>1.1 ± 0.9</td>
<td>145.08 ± 39.26</td>
<td>-6.69 ± 0.60</td>
<td>42.72 ± 13.38</td>
<td>0.92 ± 0.12</td>
<td>0.930</td>
</tr>
</tbody>
</table>

*20 degrees of freedom for each set of kinetic parameters.

¹For a definition of kinetic parameters used see Section 4.2.4.
6.3.1.2 Whey protein aggregation

The loss of SDS-monomeric β-lg and α-la, from skim milk heated at 90°C, is shown in Figures 6.4 and 6.5, respectively. Similar trends were observed at the other temperatures studied: 75, 80 and 85°C (results not shown). As expected, the loss was faster at the higher heating temperature (see Figures 4.11A, B). The loss of whey proteins band intensities from SDSNR-PAGE gels is assumed to be caused by sulphhydryl-disulphide interchange reactions (see Section 4.3.3). The loss of SDS-monomeric β-lg was somewhat greater from pH 6.48 milk, while the losses from skim milks with pH of 6.60 and 6.83 were comparable. The loss of SDS-monomeric α-la was similar in pH 6.48 and 6.60 milks but slightly less in pH 6.83 milk. As reported in the previous section, the decrease in activation energy of β-lg at higher pH values was probably caused by the repulsion of ionised groups within the protein molecule. However, these charged groups, while facilitating protein unfolding (denaturation), may hinder intermolecular interactions (aggregation) by their charge repulsion. At the pH of normal milk, β-lg has a net negative charge and therefore decreasing the pH would reduce the charge and promote aggregation. Donovan and Mulvihill (1987) showed that aggregation of whey proteins in heated cheese whey was pH dependent, and heating at a pH that promoted denaturation had the opposite effect on aggregation. The trend observed in this study for β-lg is generally in agreement with other studies reported in the literature (Donovan & Mulvihill, 1987; de Rahm & Chanton, 1984).

The proportion of the different forms of β-lg and α-la species (defined in Figure 4.1) present in pH adjusted skim milks which had been heated at 85°C are shown in Figures 6.4 and 6.5, respectively. Typically, the level of α-la hydrophobic aggregates was much greater than that of β-lg. This is probably caused by the absence of a thiol group in α-la, thus limiting sulphhydryl-disulphide interchange reactions and the formation of disulphide-linked aggregates. The results for skim milks heated at 80 and 90°C were similar. However, the results at 75°C tended to be more variable, but the levels of both β-lg and α-la hydrophobic aggregates showed a general increase with time (data not shown).
Figure 6.4 Loss of SDS-monomeric β-lg from SDSNR-PAGE gels of skim milk heated at 90°C. Milk pH; 6.48 (○), 6.60 (●) and 6.83 (△).

Figure 6.5 Loss of SDS-monomeric α-la from SDSNR-PAGE gels of skim milk heated at 90°C. Milk pH; 6.48 (○), 6.60 (●) and 6.83 (△).
Figure 6.6 β-Lg aggregates in skim milk pH adjusted to 6.48 (A), 6.60 (B) and 6.83 (C), then heated at 85°C. β-Lg species; native (□), hydrophobic aggregates (///), disulphide-linked aggregates (XXX) and aggregates associated with the micelle (■).
Figure 6.7 α-La aggregates in skim milk pH adjusted to 6.48 (A), 6.60 (B) and 6.83 (C), then heated at 85°C. α-La species; native (□), hydrophobic aggregates (///), disulphide-linked aggregates (XXX) and aggregates associated with the micelle (■).
For \( \beta \)-lg at 85°C, there were 10-15% hydrophobic aggregates during the initial stages of heating. Thereafter the level decreased rapidly to almost zero (Figure 6.6). In contrast, for \( \alpha \)-la the level of hydrophobic aggregates was considerably greater and there were significant amounts of these aggregates remaining after lengthy heating (Figure 6.7). Thus hydrophobic aggregates of \( \beta \)-lg appeared to form initially along with disulphide-linked aggregates, but as heating progressed the hydrophobic aggregates were able to undergo thiol-disulphide interchange reactions. Since the hydrophobic interactions between protein molecules are weakened at temperatures above 70°C (Li-Chan, 1983), it is possible that the whey protein aggregates could be broken down into their unfolded monomeric constituents. Subsequently, the monomeric protein could then interact via sulphydryl-disulphide interchange reactions. Alternatively, the hydrophobic interactions in the aggregate may remain intact and that intermolecular disulphide bonds could form within the aggregate or between \( \beta \)-lg aggregates. Thus the aggregates containing disulphide-bonds should not be considered exclusive of hydrophobic interactions.

The amount of disulphide-linked \( \beta \)-lg aggregates in the supernatant increased as the pH increased from 6.48 to 6.83 (Figure 6.6). Singh and Fox (1987a) showed that when milk is heated at pH \( \geq 6.9 \) the \( \beta \)-lg/\( \kappa \)-casein complex dissociates from the micelle surface and remains largely in the serum, while in the pH range 6.5 to 6.7 this complex is attached onto the micelle surface. Therefore, at pH 6.83 the higher level of disulphide-linked aggregates in the supernatant was due to dissociation of \( \beta \)-lg/\( \kappa \)-casein complexes from the micelle. In contrast, at pH 6.48 - 6.60 there were fewer aggregates in the supernatant because \( \beta \)-lg that associated with micelle remained attached at the micelle surface. The amounts of disulphide-linked \( \alpha \)-la aggregates showed similar trend to that of the \( \beta \)-lg aggregates (Figure 6.7), but the denatured \( \alpha \)-la in the supernatant was mainly in the form of hydrophobic aggregates.
Kinetics of β-lg aggregation via disulphide bond formation

The kinetic parameters for the formation of disulphide-linked β-lg aggregates in pH-adjusted milk were determined by NLR and are shown in Table 6.2. The activation energies for β-lg aggregates were lower than the corresponding values for denaturation (Table 6.1).

6.3.1.3 Whey protein association with the micelle

The loss of β-lg and α-la from the supernatant of heated skim milk (analyzed by SDS-PAGE) is shown in Figures 6.8 and 6.9, respectively. SDS-PAGE measured the total non-sedimentable protein in the supernatant (175,000 g, 1 h at 20°C). Thus the loss of whey protein from SDS-PAGE gels shows the extent of association with the micelle (see Section 4.3.3). The level of β-lg and α-la in the supernatant decreased with heating time at all the pHs studied. After heating at 90°C for about 300 s, the level of β-lg in the supernatant remained relatively constant, at approximately 10, 20 and 40% for pH 6.48, 6.60 and 6.83, respectively, even after prolonged heating. Therefore, the maximum extent of β-lg association with the micelle was 90, 80 and 60% for pH 6.48, 6.60 and 6.83, respectively. Smits and Brouwershaven (1980) observed a similar trend when heating casein micelles and β-lg dispersed in a milk salt solution. At 90°C the maximum levels of association (β-lg sedimented at 78,000 g, 1 h at 20°C) were 55 and 80% at pH 6.8 and 6.3, respectively. Singh and Fox (1987a) showed that on heating at pH above 6.9 β-lg/κ-casein complexes dissociate from the micelle, and at pH 6.5-6.7 this dissociation is reduced. At pH 6.83, an average of 20% of the κ-casein dissociated from the micelle, as determined by SDS-PAGE band intensities of the supernatant. The average κ-casein dissociation was 10% and 8% at pH 6.60 and 6.48, respectively. The greater dissociation of κ-casein at pH 6.83 was responsible for less β-lg associating with the micelles than at pH 6.48 and 6.60. κ-Casein dissociation would reduce the number of κ-casein sites on the micelle capable of associating with β-lg. The κ-casein dissociating as a β-lg/κ-casein complex would also reduce the extent of β-lg association with the micelle.
Table 6.2 Kinetic parameters for the disulphide-linked aggregation of β-lg in pH adjusted skim milks. Temperature range 75-85°C.

<table>
<thead>
<tr>
<th>Whey Protein</th>
<th>pH</th>
<th>$n$</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
<th>$\ln(k_{so})$</th>
<th>$\ln(k_{o})$</th>
<th>$C_0$ (g kg$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lg</td>
<td>6.48</td>
<td>1.3 ± 0.3</td>
<td>242.35 ± 17.42</td>
<td>-6.00 ± 0.21</td>
<td>75.96 ± 6.14</td>
<td>3.69 ± 0.27</td>
<td>0.973</td>
</tr>
<tr>
<td></td>
<td>6.60</td>
<td>1.1 ± 0.4</td>
<td>215.44 ± 24.64</td>
<td>-5.95 ± 0.31</td>
<td>66.91 ± 9.32</td>
<td>3.58b</td>
<td>0.936</td>
</tr>
<tr>
<td></td>
<td>6.83</td>
<td>1.6 ± 0.9</td>
<td>166.55 ± 53.04</td>
<td>-6.73 ± 0.80</td>
<td>49.60 ± 19.13</td>
<td>3.69b</td>
<td>0.720</td>
</tr>
</tbody>
</table>

a20 degrees of freedom for each set of kinetic parameters.
bInitial concentration value from native-PAGE gel results (21 degrees of freedom).
† For a definition of the kinetic parameters used see Section 4.2.4.
Figure 6.8 Loss of β-lg from SDSR-PAGE gels of skim milk pH adjusted to 6.48 (A), 6.60 (B) and 6.83 (C), then heated at 75 (♦), 80 (△), 85 (●) and 90°C (○).
Figure 6.9 Loss of α-la from SDSR-PAGE gels of skim milk pH adjusted to 6.48 (A), 6.60 (B) and 6.83 (C) then heated at 75 (▲), 80 (△), 85 (●) and 90°C (○).
α-La associated with the micelle rapidly during heating for the first 300 s, then continued to associate more slowly with further heating (Figure 6.9). Although α-la cannot associate with the micelle in the absence of β-lg, it forms a heat-induced β-lg/α-la complex which then interacts with κ-casein (Elfagm & Wheelock, 1978b). As α-la continued to associate with the micelle even though little or no further β-lg association was observed, sulphydryl-disulphide interchange reactions may occur between α-la and β-lg that is already associated with the micelle surface.

The association of β-lg and α-la with the micelle is shown in Figure 6.10 for pH 6.48, 6.60 and 6.83 milks heated at 80-90°C. At low concentrations of β-lg association (<40 × 10^{-6} mol kg^{-1}) very little α-la association occurred. As more β-lg associated with the micelle, the concentration of associated α-la increased slowly. In the pH 6.83 milk the concentration of associated β-lg and α-la reached a maximum of 120 × 10^{-6} mol kg^{-1} and 22 × 10^{-6} mol kg^{-1}, respectively. At pH 6.48 and 6.60 the concentration of associated β-lg showed little further increase above 160 × 10^{-6} mol kg^{-1}, but the concentration of associated α-la continued to increase. The molar ratios of β-lg/α-la associating above 160 × 10^{-6} mol kg^{-1} of β-lg, were 2.5 and 1.4 for pH 6.48 and 6.60, respectively.

Heating pH adjusted milk (pH 6.60) at 75°C caused more of the α-la to associate with the micelle than at 80-90°C, for a given level of β-lg association (Figure 6.11). Similar results were also observed for pH 6.48 and 6.83 samples. These results are comparable with the β-lg/α-la association with the micelles in Autumn season milk (see Figure 4.20). The difference between heating at 75°C and at temperatures greater than 80°C has already been discussed in Section 4.3.4. It is possible that hydrophobic interactions (Haque & Kinsella, 1988) played a greater part in association of α-la with the micelles at 75°C because of the slower development of thiol groups at 75°C (Kirchmeier et al., 1984).
Figure 6.10 Association of β-lg and α-la with the casein micelle in skim milk heated at 80-90°C. Milk pH; 6.48 (○), 6.60 (●) and 6.83 (△).

Figure 6.11 Association of β-lg and α-la with the casein micelle in pH 6.60 skim milk. Heating temperature 75°C (●) and 80-90°C (○).
6.3.2 Effect of whey protein concentration

6.3.2.1 Skim milk composition

Two trials, one using early (September) and the other using mid season (December) milk, were carried out to investigate the effect of whey protein concentration on the denaturation, aggregation and association of whey proteins. The composition of the early and mid season milks are shown in Tables 6.3 and 6.4, respectively. Both MF and UF processes were used to manufacture milks containing different whey protein concentrations. These processes did not have a large affect on the mineral content of the different milk batches (Table 6.3 & Table 6.4). The mineral distribution between the colloidal and serum (supernatant of milk centrifuged at 175,000 g, 1 h at 20°C) phases was also largely unaffected by the separation and remixing that was performed.

The change in total whey protein content was measured by NCN, native-PAGE and WPNI (Table 6.4). All three methods produced similar results, although there was some variation that may have been caused by the different physical and chemical techniques used for measuring the whey protein content.

The whey protein concentrations of WPD and WPE skim milks were chosen as representing the extremes in seasonal variation (0.52 to 1.24 g/100 g). Seasonal variations in whey protein concentration are shown in Chapter 5, Table 5.2.

6.3.2.2 Kinetics of whey protein denaturation

The loss of native β-lg A and α-la from whey-protein adjusted mid season skim milk heated at 80, 90 and 120°C is shown in Figures 6.12 and 6.13, respectively. The heating time at 90°C (up to 150 s) was not long enough for any significant denaturation of α-la to occur, so the results for 90°C are not shown. The differences in the rate of loss of β-lg between the three types of milks were small, but a greater difference was observed for the loss of α-la. The loss of native α-la was greatest in the WPE milk, least in the WPD milk, and intermediate in the control. A comparison of the early and mid season milks showed the trends for the loss of native β-lg and α-la were similar (data not shown).
Table 6.3 Protein composition of WPD, control and WPE early season skim milks.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Early season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WPD</td>
</tr>
<tr>
<td>Total solids (%) (w/w)</td>
<td>8.94</td>
</tr>
<tr>
<td>Total protein (g/100 g)</td>
<td>3.25</td>
</tr>
<tr>
<td>Casein (g/100 g)</td>
<td>2.73</td>
</tr>
<tr>
<td>Whey protein (g/100 g)</td>
<td>0.52</td>
</tr>
<tr>
<td>Change in whey protein (%)</td>
<td>71</td>
</tr>
<tr>
<td>Individual whey proteins</td>
<td></td>
</tr>
<tr>
<td>β-lg A (g kg⁻¹)</td>
<td>1.32</td>
</tr>
<tr>
<td>β-lg B (g kg⁻¹)</td>
<td>1.26</td>
</tr>
<tr>
<td>α-la (g kg⁻¹)</td>
<td>0.54</td>
</tr>
<tr>
<td>BSA (g kg⁻¹)</td>
<td>0.15</td>
</tr>
<tr>
<td>Change in sum of individual whey proteins (%)</td>
<td>65</td>
</tr>
<tr>
<td>Minerals</td>
<td></td>
</tr>
<tr>
<td>Ca Total (mmol kg⁻¹)</td>
<td>34.1</td>
</tr>
<tr>
<td>Serum</td>
<td>10.2</td>
</tr>
<tr>
<td>P Total (mmol kg⁻¹)</td>
<td>30.0</td>
</tr>
<tr>
<td>Serum</td>
<td>16.5</td>
</tr>
<tr>
<td>Mg Total (mmol kg⁻¹)</td>
<td>4.1</td>
</tr>
<tr>
<td>Serum</td>
<td>2.9</td>
</tr>
<tr>
<td>pH</td>
<td>6.67</td>
</tr>
</tbody>
</table>

*The percentage changes are calculated relative to the control, which is 100%.

*TTotal protein = (TN - NPN) × 6.38.
Table 6.4 Protein composition of WPD, control and WPE mid season skim milks.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Mid season</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WPD</td>
<td>Control</td>
<td>WPE</td>
</tr>
<tr>
<td>Total solids (%(w/w))</td>
<td>8.74</td>
<td>9.19</td>
<td>9.44</td>
</tr>
<tr>
<td>Total Protein (g/100 g)*</td>
<td>3.58</td>
<td>3.76</td>
<td>4.00</td>
</tr>
<tr>
<td>Casein (g/100 g)</td>
<td>3.05</td>
<td>2.94</td>
<td>2.84</td>
</tr>
<tr>
<td>Whey (g/100 g)</td>
<td>0.54</td>
<td>0.82</td>
<td>1.16</td>
</tr>
<tr>
<td>Change in whey protein (%)*</td>
<td>65</td>
<td>100</td>
<td>140</td>
</tr>
<tr>
<td>Individual whey proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-ig A (g kg⁻¹)</td>
<td>0.97</td>
<td>1.86</td>
<td>2.50</td>
</tr>
<tr>
<td>β-ig B (g kg⁻¹)</td>
<td>1.08</td>
<td>2.03</td>
<td>3.66</td>
</tr>
<tr>
<td>α-la (g kg⁻¹)</td>
<td>0.42</td>
<td>0.77</td>
<td>1.44</td>
</tr>
<tr>
<td>BSA (g kg⁻¹)</td>
<td>0.14</td>
<td>0.19</td>
<td>0.28</td>
</tr>
<tr>
<td>Change in sum of individual whey proteins (%)*</td>
<td>54</td>
<td>100</td>
<td>162</td>
</tr>
<tr>
<td>WPNI and (% change)*</td>
<td>4.01 (51)</td>
<td>7.89 (100)</td>
<td>12.17 (154)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minerals</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>Total</td>
<td>34.0</td>
<td>33.8</td>
</tr>
<tr>
<td>(mmol kg⁻¹)</td>
<td>Serum</td>
<td>11.6</td>
<td>9.5</td>
</tr>
<tr>
<td>P</td>
<td>Total</td>
<td>30.0</td>
<td>31.2</td>
</tr>
<tr>
<td>(mmol kg⁻¹)</td>
<td>Serum</td>
<td>12.8</td>
<td>12.1</td>
</tr>
<tr>
<td>Mg</td>
<td>Total</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>(mmol kg⁻¹)</td>
<td>Serum</td>
<td>3.2</td>
<td>3.1</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>6.69</td>
<td>6.67</td>
</tr>
</tbody>
</table>

*The percentage changes are calculated relative to the control, which is 100%.

^Total protein = (TN - NPN) × 6.38.
Figure 6.12 Loss of β-lg A from native-PAGE gels of mid season skim milk heated at 80 (A), 90 (B) and 120°C (C). Milk type; WPD (O), control (●) and WPE (▲).
Figure 6.13 Loss of α-la from native-PAGE gels of mid season skim milk heated at 80 (A) and 120°C (B). Milk type; WPD (○), control (●) and WPE (△).
Calvo et al. (1993) using a mixture of β-lg, α-la and casein micelles redispersed in milk ultrafiltrate showed that the loss of α-la, after heating at 90°C, increased as the concentration of β-lg was increased. Elfagm and Wheelock (1978b) also observed that the addition of β-lg decreased the amount of native α-la recovered after heating, although the amount of β-lg also decreased, which was not apparent in these results. BSA is also known to increase the loss of native α-la during heating, and it is thought that the thermal denaturation of α-la is dependent on the concentration of free thiol groups (Calvo et al., 1993).

The reaction order \((n)\) for β-lg A, β-lg B and α-la at each temperature and for each batch of milk were calculated (Appendix B1). The values of \(n\) were averaged for each milk batch and used in Equation 6.1 to calculate the rate constant \((k_n)\). The reaction orders for the individual whey proteins did not vary greatly between milk batches, but were variable with respect to temperature. For example, β-lg A denaturation in mid-season control milk, had reaction orders of 1.0, 1.7 and 1.4, for 80, 90 and 120°C, respectively. The average reaction orders of the three mid-season milk batches were, 1.5, 1.4 and 1.3 for the WPD, control and WPE milks, respectively. These values compare well with the 1.5 reaction order reported by Dannenberg and Kessler (1988a), but were slightly higher than those given in Table 4.4. The whey protein to casein ratio in milk is known to affect the reaction order. Kessler and Beyer (1991) reported that as the ratio of whey proteins to casein increases the reaction order of β-lg B rises from 1.5 (skim milk) to 2.0 (sweet whey). The reaction orders in this experiment were too variable to observe any concentration trend.

The rate constants for β-lg A were generally similar for the control and WPE milks (Table 6.5). A similar trend was shown by the rate constants for β-lg B. However, the WPD milk had higher rate constants, especially at 120 and 90°C. In contrast, the rate constants for α-la in WPE milk were higher than in the control and WPD milks (Table 6.6). The rate constants of β-lg have been reported to be independent of the initial β-lg concentration in whey protein isolate solutions (Kessler & Beyer, 1991) and reconstituted whole milk powders (Anema & McKenna, 1996); \(n = 1.5\) was used by both researchers. It is possible that the higher ratio of casein to whey protein in the
WPD milk caused the higher rate constants. Denaturation of whey proteins occurs to a greater extent in milk than in whey (Elfagt & Wheelock 1978b). Casein is thought to facilitate whey protein denaturation, possibly through the formation of a β-lg/α-la/κ-casein complex (Elfagt & Wheelock, 1978b).

Table 6.5 Rate constants for β-lg A denaturation in mid season whey-protein adjusted skim milks heated at 120, 90 and 80°C.

<table>
<thead>
<tr>
<th>Temperature°C</th>
<th>Whey protein level</th>
<th>( k_n^b ) ((g \text{ kg}^{-1})(1-n) \text{ s}^{-1} \times 10^3)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 depleted</td>
<td></td>
<td>144 ± 51</td>
<td>0.954</td>
</tr>
<tr>
<td>120 control</td>
<td></td>
<td>86 ± 10</td>
<td>0.987</td>
</tr>
<tr>
<td>120 enriched</td>
<td></td>
<td>78 ± 10</td>
<td>0.980</td>
</tr>
<tr>
<td>90 depleted</td>
<td></td>
<td>26.8 ± 15.9</td>
<td>0.725</td>
</tr>
<tr>
<td>90 control</td>
<td></td>
<td>9.6 ± 1.4</td>
<td>0.967</td>
</tr>
<tr>
<td>90 enriched</td>
<td></td>
<td>11.0 ± 2.8</td>
<td>0.923</td>
</tr>
<tr>
<td>80 depleted</td>
<td></td>
<td>2.57 ± 0.19</td>
<td>0.993</td>
</tr>
<tr>
<td>80 control</td>
<td></td>
<td>1.68 ± 0.51</td>
<td>0.921</td>
</tr>
<tr>
<td>80 enriched</td>
<td></td>
<td>1.63 ± 0.52</td>
<td>0.922</td>
</tr>
</tbody>
</table>

\(^a\)Degrees of freedom were 7, 6 and 7 for 80, 90 and 120°C, respectively.

\(^b\)Reaction orders were 1.5, 1.4 and 1.3 for WPD, control and WPE skim milks, respectively. For a definition of \( k_n \) see Section 4.2.4.

As the reaction order for β-lg denaturation is ≈1.5 the extent of denaturation should be concentration dependent, but this is not readily apparent from the denaturation curves (Figure 6.12). For a 1.5 reaction order and a rate constant of \( 1.7 \times 10^{-1} \text{ (g kg}^{-1})^{0.5}\text{s}^{-1}\), the difference between the rate of β-lg A denaturation in WPE and WPD mid season milks
should be approximately 8 to 13%. The change in whey protein concentration may not have been large enough to show a difference in the extent of denaturation between the whey-protein adjusted samples.

Table 6.6 Rate constants for α-la denaturation in early season whey-protein adjusted skim milk heated at 120, 90 and 80°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>(k_n^* \pm 95% \text{ C.I.} \times 10^3) (g kg(^{-1})) (^{(1-n)}) s(^{-1})</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>depleted</td>
<td>14.3 ± 2.0</td>
<td>0.971</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>12.9 ± 1.8</td>
<td>0.969</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>16.8 ± 2.7</td>
<td>0.956</td>
</tr>
<tr>
<td>80</td>
<td>depleted</td>
<td>0.90 ± 0.10</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>1.10 ± 0.54</td>
<td>0.874</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>1.43 ± 0.20</td>
<td>0.911</td>
</tr>
</tbody>
</table>

\(k_n^*\), reaction orders 1.5, 1.1 and 1.1, WPD, control and WPE skim milks, respectively.

3Degrees of freedom were 7 for both 80, 90 and 120°C.

6.3.2.3 Whey protein aggregation and association with the micelle

The loss of SDS-monomeric β-lg was similar to that observed in the earlier work reported in Chapter 4 (Figure 4.11a). Both the early and mid season milks showed the same trends. The concentration of β-lg (2.05-7.07 g kg\(^{-1}\)) did not appear to affect the extent of formation of disulphide-linked aggregates. The rate constants and reaction orders were calculated and are shown in Appendix C1. The orders ranged from 1.7 to 2.4, and were higher than the denaturation orders of 1.3 to 1.7. The higher orders may indicate the reaction mechanism was more dependent on a bi-molecular reaction, i.e. aggregation. The WPD milks tended to have higher rate constants (Appendix C1), thus following the same trend as the denaturation rate constants (see Table 6.5).
The formation of β-lg disulphide-linked aggregates in relation to denatured β-lg, in autumn calving milk, was shown in Section 4.3.3.3 (see Figure 4.14). The β-lg in whey-protein adjusted milks followed the same trend. The results for α-la were more variable, but showed the same trend as reported in Chapter 4 (Figure 4.15).

The loss of total β-lg from the supernatant of whey-protein adjusted milks was similar to the results shown in Chapter 4 (Figure 4.16). The maximum extent of association of β-lg with the micelle was approximately 55% in the mid season milk and 65% in the early season milk. The association results followed the same trend as shown by early and mid season milks, described in Section 5.3.4. The percentage association of β-lg with the micelle was unaffected by β-lg concentration (2.05-7.07 g kg\(^{-1}\)), although the actual amount of associated β-lg increased with increasing β-lg concentration. The molar ratios of associated β-lg to κ-casein attached to the micelle were approximately 0.3, 0.7 and 1.1 for WPD, control and WPE mid season milks respectively. Association reactions may occur to a greater extent at higher whey protein concentrations due to the increased concentration of β-lg aggregates, even though the concentration of κ-casein remained constant. An increased concentration of β-lg aggregates may present more opportunities for association reactions to occur. Smits and Brouwershaven (1980) reported the percentage β-lg association with the micelle was unaffected by an increase in β-lg concentration from 4 to 8 g kg\(^{-1}\), but a further increase to 12 g kg\(^{-1}\) resulted in a greater percentage of the β-lg co-sedimenting with the micelle. In solutions containing purified β-lg and κ-casein, increasing the proportion of β-lg causes more of the β-lg to interact with κ-casein. Mixtures of β-lg and κ-casein in solution, when heated, form complexes with a maximum ratio of β-lg to κ-casein of 3:1 to 2.2:1 (Long et al., 1963; Tessier et al., 1969). It is not clear whether this is the case for β-lg/κ-casein interactions at the micelle surface. Steric interference from β-lg aggregates already associated with the micelle may prevent high concentration of β-lg associating with κ-casein at the surface, while κ-casein in solution may be able to react more easily with β-lg aggregates.

The loss of total α-la from SDS-PAGE gels in whey-protein adjusted skim milks, heated at 80 and 120°C is shown in Figure 6.14. Both the mid and early season milks
Figure 6.14 Loss of α-la from SDS-PAGE gels of skim milk heated at 80 (A) and (B) 120°C. Milk type; WPD (○), control (●) and WPE (△).
showed the same trends. The loss of α-la was similar from the WPE and control milks, but the loss of α-la appeared to be slower from the WPD milk. The loss of native α-la followed a similar trend (Figure 6.13), thus the rate of association was probably dependent on the rate of denaturation. Figure 6.15 shows the relationship between native α-la and the extent of α-la association. For a given level of native α-la less association occurred in the WPE milk than either the control or WPD milks. β-Lg denatures faster than α-la, thus association reactions may occur initially between denatured β-lg and κ-casein. Increasing the whey protein concentration caused more denatured β-lg to associate with κ-casein. This could reduce the number of κ-casein sites available for further association reactions. Thus, when α-la/β-lg aggregates form fewer association reactions occurred.

Figure 6.15 Relationship between native α-la and α-la associated with the casein micelles in heated milk (80, 90 & 120°C). Milk type; WPD (○), control (●) and WPE (△).
6.3.3 Effect of total solids

6.3.3.1 Skim milk composition

The protein and mineral composition of the total solids adjusted skim milks is shown in Table 6.7.

Table 6.7 Protein and mineral composition of total solids adjusted mid season milk.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Adjusted total solids, mid season milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diluted</td>
</tr>
<tr>
<td>Total Solids (%(w/w))</td>
<td>6.00</td>
</tr>
<tr>
<td>Change in total solids (%)*</td>
<td>65</td>
</tr>
<tr>
<td>Total Protein (g/100 g)</td>
<td>2.31</td>
</tr>
<tr>
<td>Change in total protein (%)*</td>
<td>65</td>
</tr>
<tr>
<td>Individual whey proteins</td>
<td></td>
</tr>
<tr>
<td>β-lg A (g kg(^{-1}))</td>
<td>1.37</td>
</tr>
<tr>
<td>β-lg B (g kg(^{-1}))</td>
<td>1.16</td>
</tr>
<tr>
<td>α-la (g kg(^{-1}))</td>
<td>0.49</td>
</tr>
<tr>
<td>BSA (g kg(^{-1}))</td>
<td>0.13</td>
</tr>
<tr>
<td>Change in sum of individual whey proteins (%)*</td>
<td>65</td>
</tr>
<tr>
<td>Minerals</td>
<td></td>
</tr>
<tr>
<td>Ca Total (mmol kg(^{-1}))</td>
<td>22.4</td>
</tr>
<tr>
<td>Serum</td>
<td>6.9</td>
</tr>
<tr>
<td>P Total (mmol kg(^{-1}))</td>
<td>21.6</td>
</tr>
<tr>
<td>Serum</td>
<td>9.4</td>
</tr>
<tr>
<td>WPNI</td>
<td>4.4</td>
</tr>
<tr>
<td>pH</td>
<td>6.81</td>
</tr>
</tbody>
</table>

*The percentage changes are calculated relative to the control, which is 100%. 
Total protein as measured by Kjeldahl and total whey protein as measured by native-PAGE changed in proportion with the change in total solids. This suggests that the first effect of the evaporator used to concentrate the milk (70°C, residence time approximately 1 min) did not cause any significant denaturation. The total and serum concentrations of Ca and P changed in proportion to the change in total solids. Evaporation of the milk caused a pH drop, and dilution resulted in a pH increase. Seasonal variations in the total solids concentration are shown in Chapter 5, Table 5.2.

6.3.3.2 Kinetics of whey protein denaturation

The change in native concentrations of β-lg B and α-la heated at 110°C are shown in Figures 6.16 and 6.17, respectively. The loss of native β-lg B and α-la was slightly greater in the evaporated milk, intermediate in the control and slightly less in the diluted milk. β-Lg A followed the same trend as β-lg B. Similar trends were observed at 85°C for both β-lg and α-la, and at 90°C for α-la. At 90°C, β-lg denatured too fast to distinguish any difference between the total solids adjusted milks. The reaction rates at each temperature were calculated using Equation 6.1. In general, the rate constants appeared to be similar for each of the different total solid milks; for example the rate constants for β-lg A at 110°C were 45.2 ± 3.4 × 10⁻³, 57.3 ± 4.5 × 10⁻³ and 48.5 ± 11.5 × 10⁻³ (g kg⁻¹)(l⁻¹) s⁻¹, for control, evaporated and diluted milks, respectively. The rate constants for β-lg B and α-la followed a similar trend and are shown in Appendix D1.

In this study the level of whey protein denaturation over the total solids range 6-13% appeared to be concentration dependent; the higher the concentration the greater the extent of denaturation. This is in agreement with Elvebak and Smith (1992) who found that the degree of β-lg and α-la denaturation increased as whole milk was concentrated by UF, up to 3 times concentration. However, the opposite effect has been shown in the studies carried out in evaporated milk (McKenna & O'Sullivan, 1971) and cheese whey (Hillier et al., 1979). McKenna and O'Sullivan (1971) reported a decrease in whey protein denaturation from 80 to 59% when the total solids increased from 9 to 28% (80°C for 20 min). Increasing the total solids in cheese whey, up to 3 times concentration by reconstituting cheese whey powder to different total solids contents,
retarded the denaturation of β-lg A and B, but hastened the denaturation of α-la (Hillier et al., 1979).

**Figure 6.16** Loss of β-lg B from native-PAGE gels of skim milk heated at 110°C. Milk type; diluted (O), control (●) and evaporated (▲).

Increasing the total solids increases the concentration of protein, lactose and milk salts. The pH of milk decreases and the ionic strength increases during evaporation. All these factors could influence the extent and rate of denaturation of whey proteins. Increasing the whey protein concentration increases the extent of denaturation (Figure 6.13; Elfagm & Wheelock, 1978b). Lactose has a protective affect against whey protein denaturation (Bernal & Jelen, 1985), and increasing the lactose concentration enhances this protective effect (Ibrahim et al., 1995). The effect of pH on denaturation has been discussed in Section 6.3.1.1., and over the pH range 6.83 to 6.60 there was no significant change. Therefore, the pH change in the total solids milks (6.63, 6.68 and 6.81) did not have a major affect on denaturation. Varunsation et al. (1983) showed that Ca²⁺ and Mg²⁺ promote denaturation and increasing the concentration of Ca²⁺ increases the extent of denaturation (de Rham & Chanton, 1984; Ibrahim et al., 1995). Increasing the ionic strength also promotes protein aggregation (Xiong et al., 1993). The overall increase in the extent of denaturation with an increase in total solids would be a combination of
the above effects. The increase in total solids from 9 to 13% in the present study was smaller than the 9 to 28% used by McKenna and O’Sullivan (1971). The protective effect of lactose may only begin to exceed the denaturation effects of increased whey protein concentration, and milk salts, above a certain total solids concentration. This solids concentration is likely to be above 13% total solids. Lactose and salts would have been lost in the permeate stream of the UF process used by Elvebak and Smith (1992). This may explain the difference between their results and those of McKenna and O’Sullivan (1971).

6.3.3.3 Whey protein aggregation and association with the micelle
The loss of SDS-monomeric β-lg from total solids adjusted milk heated at 110°C is shown in Figure 6.18. The trend is the same as the native protein results (Figure 6.16); in the diluted milk there was more SDS-monomeric β-lg (not disulphide-linked) than in the evaporated milk. Therefore β-lg denaturation and subsequent disulphide-linked aggregation is affected by the total solids of milk. The rate constants and reaction orders for β-lg disulphide-linked aggregation were calculated and are shown in Appendix E1.
As the milk was concentrated there was an increase in the concentration of milk salts (Ca$^{2+}$) as well as a decrease in the pH (Table 6.7). Protein aggregation reactions are promoted by increased concentrations of Ca$^{2+}$ and Mg$^{2+}$, especially as the pH decreases from 7.0 to 6.4 (Varunsation et al., 1983; de Wit & Klarenbeek, 1984). In addition, at higher pH, Ca$^{2+}$ is bound by phosphate and citrate, and the concentration of free Ca$^{2+}$ may be too low to affect aggregation (Donovan & Mulvihill, 1987). Thus, these two factors may be responsible for the change in extent of whey protein aggregation in different total solid milks.

![Image of Figure 6.18](image)

**Figure 6.18** Loss of SDS-monomeric β-lg from SDSNR-PAGE gels of milk heated at 110°C. Milk type; diluted (O), control (●) and evaporated (▲).

The loss of total β-lg and α-la from SDSR-PAGE gels of total solids adjusted milks heated at 110°C is shown in Figures 6.19 and 6.20, respectively. After an initial decrease the level of whey protein remained relatively constant as heating progressed. The maximum level of β-lg associated with the micelle, 72%, 65% and 52%, in evaporated, control and diluted milks, respectively, was dependent on the milk total solids. The percentage of κ-casein in the supernatant (soluble κ-casein) was approximately 14%, 17% and 22% in evaporated, control and diluted milks, respectively. The difference in the extent of association and level of dissociated κ-casein between the
milks was probably caused by the pH difference. The pH of the control milk (6.68) increased when it was diluted (pH 6.81). The increase in pH probably caused the level of associated whey protein to decrease by increasing dissociation of the κ-casein from the micelle (Section 6.3.1.3). In addition to the lower pH of evaporated milk (pH 6.63), the increase in calcium concentration in the evaporated milk could also have contributed to the increased level of association. Addition of calcium (5 mM) to milk before heating at 90°C for 30 min has been shown to increase the association of whey proteins with the micelle from 35 up to 70% (Visser et al., 1986). Singh and Fox (1987b) showed that the effect of ions, such as Ca²⁺ and Na²⁺, was to shield negatively charge groups on κ-casein (e.g. seryl phosphate), thus reducing dissociation. It is possible that calcium promotes aggregation between denatured β-lg in solution and β-lg already associated with the micelle through the formation of calcium bridges (Smits & Brouwershaven, 1980).

Figure 6.19 Loss of β-lg from SDS-PAGE gels of milk heated at 110°C. Milk type; diluted (O), control (●) and evaporated (△).
6.3.4 Effect of pH, whey protein concentration and total solids on the denaturation, aggregation and association mechanisms

The mechanisms for β-lg and α-la denaturation, aggregation and association with the micelle have been previously outlined in Equations 4.31, 4.32 and 4.33, respectively (Sections 4.3.3.3 & 4.3.4.3). At high pH (6.83) the increased negative charge on the β-lg and α-la protein molecules would promote unfolding. This may push the equilibrium between native whey protein and unfolded whey protein towards the unfolded state (See Equations 4.31 & 4.32). In contrast, aggregation reactions could be inhibited by the increased charge repulsion. The opposite occurs at low pH (6.48), where the charge on the protein molecule is decreased. This results in less intermolecular repulsion, thus aggregation reactions can proceed more readily. In addition, the reduced intramolecular charge causes the protein molecule to remain in a compact native state. Increasing the pH caused a small decrease in the extent of whey protein disulphide-linked aggregate formation, but the effect on denaturation was negligible. Whey-protein enrichment (1.24 g whey protein/100 g of milk; determined
by Kjeldahl) or whey-protein depletion (0.52 g/100 g) did not significantly affect β-lg
denaturation or aggregation. In contrast, α-la denaturation and aggregation due to
disulphide-bonds greatly increased as the whey protein concentration increased. The
increased concentration of denatured β-lg (β-D) may have driven the unfolded α-la (α-
U) towards the aggregated state (α/β-A)$_s$ (Equation 4.32). Increasing the total solids
increased the concentration of whey proteins and milk salts, and reduced the pH. The
effect of whey protein concentration and pH is described above. The milk salts,
specifically Ca and Mg would facilitate aggregation reactions.

The extent of whey protein association with the micelle was influenced by the pH of the
heated milk. As the pH increased from 6.48 to 6.83 the level of whey protein
association decreased, presumably caused by dissociation of whey protein/κ-casein
complexes from the micelle. It was possible that denatured α-la (α-U) was able to
associate with β-lg/κ-casein complexes at the micelle surface. The amount of denatured
β-lg associating with κ-casein at the micelle surface (Equation 4.33) increased with an
increase in whey protein concentration. More denatured β-lg was also formed and this
may have increased the frequency of association reactions, resulting in the formation of
more β-lg/κ-casein complexes. Increasing the total solids increased the extent of
association through the combined effects of a pH decrease and increased concentration
of both whey proteins and casein micelles. The increase in soluble Ca concentration
would have promoted association reactions.

6.3.5 Heat induced mineral changes

The ultracentrifugal supernatants obtained from selected heated skim milks were
analyzed for mineral content by plasma emission spectometry. The changes in calcium,
phosphorus and magnesium in the supernatants of the mid season control skim milks and
early season whey-protein adjusted skim milks were measured. The heated skim milk
samples were cooled, stored at 5°C overnight and centrifuged at 20°C the following day.
The mineral balance between the colloidal and soluble phases is in equilibrium and any
shift away from this state during heating is reversible upon cooling (Pouliot et al.,
1989b). Thus, the observed changes are probably the result of irreversible reactions.
The concentration of Ca in the supernatant of heated milk (80, 90 and 120°C) held
overnight at 4°C was 92-97% of the initial concentration. This is in agreement with Pouliot et al. (1989b) who reported Ca concentrations of heated milks (85°C) held for 24 hours at 4°C were 90-95% of their initial concentrations. In this study the concentrations of P\textsubscript{i} and Mg relative to their initial concentrations were, 96-99% and 92-97%, respectively.

The effect on Ca, P\textsubscript{i} and Mg concentrations upon heating control skim milk at 80, 90 and 120°C, is shown in Figures 6.21, 6.22 and 6.23, respectively. Although the changes were small, a clear trend in the loss of milk salts from the supernatant was observed. The loss of Ca, P\textsubscript{i} and Mg from the supernatant appeared to involve three steps. Initially a sharp decrease occurred over the first 10 s at 90 and 120°C, or over the first 300 s at 80°C. The concentration then increased, after which further heating resulted in a steady decrease. Pouliot et al. (1989a) used ultrafiltration to measure reversible milk salt changes in milk heated up to 90°C. They reported that the loss of Ca, P\textsubscript{i} and Mg from the permeate followed a two-step mechanism. There was an initial fast decrease in concentration followed by a slow decrease with time. Their results also showed a slight increase in the concentration during the first 1-5 min of heating, although no explanation was given for this phenomena. Thus, it appears that there may be a three-step change in soluble milk salts during heating. The reasons for the three-step mechanism are unclear, although the structure and properties of colloidal calcium phosphate generated during heating must play a key role. Further work is required to clarify the mechanisms involved in the three-step change.

From this study the composition of the material transferred to the casein pellet after heating was calculated, and the ratio of divalent cations (Ca + Mg) to P\textsubscript{i} was determined. The ratios averaged over the entire heating time and their corresponding 95% confidence intervals were 0.80 ± 0.40, 1.10 ± 0.24 and 1.32 ± 0.50 for 80, 90 and 120°C, respectively. These compare with 1.38 and 1.01 at 80 and 90°C, respectively, reported by Pouliot et al. (1989a).
Figure 6.21 Changes in the concentration of Ca (O), P₁ (●) and Mg (△) in the supernatant (175,000 g for 1 h at 20°C) of control skim milk (mid season) heated at 80°C.

Figure 6.22 Changes in the concentration of Ca (O), P₁ (●) and Mg (△) in the supernatant of control skim milk (mid season) heated at 90°C.
The loss of Ca, P<sub>i</sub> and Mg from the supernatant of whey-protein adjusted skim milks (early season) heated at 80°C are shown in Figures 6.24, 6.25 and 6.26, respectively. The losses from the early season control skim milk were similar to the losses of milk salts from mid season skim milk reported above. The decrease in Ca, P<sub>i</sub> and Mg was slightly greater in the WPE and WPD skim milks. These changes are the result of irreversible reactions, as mentioned above. Thus modification of the whey protein concentration by MF and UF appears to decrease the reversibility of the heat-induced milk salt changes that occurred. It is not clear why the WPE and WPD milks showed a different trend to the control milk. Care was taken to ensure the only changes to milk composition occurred in the whey protein concentration. The concentration of Ca, P<sub>i</sub> and Mg and their distribution between the colloidal and serum phases remained relatively constant (Table 6.3 and 6.4). It is unlikely that the whey protein concentration caused the differences. Kannan and Jenness (1961) showed that the addition or removal of β-lg (0.2%) did not affect the reversibility of Ca and P<sub>i</sub> changes. Further studies are required to determine how the whey protein concentration in milk affects heat-induced milk salt changes.
**Figure 6.24** Changes in the concentration of Ca in the supernatant of WPD (△), control (○) and WPE (●) milk (early season) heated at 80°C.

**Figure 6.25** Changes in the concentration of $P_i$ in the supernatant of WPD (△), control (○) and WPE (●) milk (early season) heated at 80°C.
Figure 6.26 Changes in the concentration of Mg in the supernatant of WPD (α), control (O) and WPE (●) milk (early season) heated at 80°C.

6.4 CONCLUSIONS
Increasing the milk pH from 6.48 to 6.83 caused the activation energies of β-lg A and β-lg B to decrease, while the activation energy of α-la remained relatively constant (Section 6.3.1.1, Table 6.1). The formation of disulphide-linked aggregates increased as the pH dropped from 6.83 to 6.48. Association of β-lg and α-la with the micelle increased markedly as the pH decreased.

α-La denaturation, disulphide-linked aggregation and association with the micelle increased as the whey protein concentration increased. The change in whey protein concentration did not have a major affect on the heat-induced behaviour of β-lg, although the concentration of associated β-lg increased as the whey protein concentration increased.

Increasing the milk total solids resulted in a greater level of β-lg and α-la denaturation, disulphide-linked aggregation and association with the micelle. A decrease in pH and an increase in the milk salts concentration is considered to contribute to these reactions.
The irreversible loss of Ca, P, and Mg from the supernatant followed a three-step behaviour. The concentration of the milk salts decreased rapidly at first then rose slightly, after which further heating resulted in a slow decrease.

The changes in composition parameters were relatively small, and could be considered as the extremes of seasonal variations. Although the changes were small, significant effects in denaturation, aggregation and association with the micelle were occurring.
CHAPTER 7: PROTEIN AND MINERAL CHANGES DURING THE MANUFACTURE OF SKIM MILK POWDER

7.1 INTRODUCTION

The manufacture of skim milk powder involves heating the milk, usually known as preheating, then concentrating the milk solids by evaporation and spray drying. The effects of the milk powder process on protein and mineral changes and powder functionality are not well understood as there are a large number of chemical and physical reactions that take place.

The preheating conditions determine, to a large extent, many of the functional properties of the powder (see Sections 2.4 and 2.5). Some of the changes that occur during preheating are whey protein denaturation, association of whey proteins with the casein micelle, a transfer of soluble milk salts to the colloidal phase, destruction of bacteria and enzyme inactivation (Singh & Newstead, 1992). The effects of heat on the denaturation of whey proteins and whey protein/casein micelle association have been reported extensively (see Singh & Newstead, 1992). Although the heat-induced shifts in milk salts have been reported (Pouliot et al., 1989a, b; Holt, 1995) the mechanism is not clearly understood.

Relatively little whey protein denaturation occurs during evaporation as the temperature during evaporation is typically kept below 70°C (Singh and Creamer, 1991a). Concentrating the milk solids reduces the pH (Nieuwenhuijse et al., 1988) and increases the colloidal salts (Le Graet & Brule, 1982; Nieuwenhuijse et al., 1988). The concentrate is heated before drying to reduce the viscosity, which allows for easier atomization during spray drying, and also to reduce microbial numbers. Heating of the concentrate, especially above 60°C and for an extended period, may cause aggregation
of the casein micelles (Muir, 1980). This results in increased viscosity and poor solubility characteristics for skim milk powders (Muir, 1980).

Spray drying is used to remove most of the remaining water from the concentrate. The milk droplet temperature usually does not exceed 70°C, but as the drying process nears completion the droplet temperature approaches that of the outlet air. Little is known of the protein and mineral changes that take place during spray drying, and the effect this has on milk powder properties.

Although preheating is an important operation and the main topic of research for this project, evaporation and spray drying steps should not be overlooked in the overall context of milk powder manufacture. Therefore, this chapter describes the effects of the milk powder manufacturing process on whey protein denaturation, aggregation and association with the micelle. The distribution of Ca and P between the colloidal and soluble phases was also determined.

7.2 MATERIALS AND METHODS
7.2.1 Skim milk powder processing
Bulk whole milk (February, spring calving) from the Tui Dairy Company, Longburn, was separated without pasteurisation at 55°C, at the NZDRI processing plant. The skim milk was then processed into skim milk powder (Figure 7.1). Eight different trials were carried out where a combination of preheating temperature, concentrate heating temperature and drier inlet air temperature were adjusted (Table 7.1).

The milk was preheated by DSI to 70, 80, 100 or 120°C, and then passed through a 52 s holding tube. A flash vessel (defined in Section 3.2.2) was used to reduce the temperature of the heated milk to that of the first effect. The milk was then passed through a three effect falling film evaporator (Weigand, Karlsruhe, Germany), at a feed rate of 1700 L h⁻¹. The temperature of the 1st effect was ≈72°C, and the product temperatures from the 2nd and 3rd effects were 53 and 41°C, respectively. The concentrate exiting the third effect at 48-49% total solids was then heated by a scraped surface heat exchanger (Model VT 422, APV Crepaco Inc., Chicago, Illinois) to either
65, 70 or 75°C. The heated concentrate took 80 s to reach the atomizer of the pilot scale nozzle drier with an integral fluidized bed (IFB drier, NZDRI design, NZDRI, Palmerston North, N.Z.), at a feed rate of 140 L h⁻¹. The inlet and outlet drier temperatures ranged from 160 to 200°C and 90 to 100°C, respectively. The powder was dried to a final moisture content of <5.0%.

Table 7.1 Preheating, concentrate heating and drier conditions used in the skim milk powder manufacture.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Preheating temperature (°C)</th>
<th>Concentrate heating temperature (°C)</th>
<th>Inlet air temperature (°C)</th>
<th>Outlet air temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70.0</td>
<td>69.1</td>
<td>180</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>70.0</td>
<td>65.3</td>
<td>180</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>70.0</td>
<td>73.9</td>
<td>180</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>70.0</td>
<td>69.1</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>70.0</td>
<td>67.9</td>
<td>160</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>120.0</td>
<td>68.5</td>
<td>180</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
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<td>69.2</td>
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<td>90</td>
</tr>
<tr>
<td>8</td>
<td>80.0</td>
<td>69.1</td>
<td>180</td>
<td>90</td>
</tr>
</tbody>
</table>

7.2.2 Analysis of milks, concentrates and powders

7.2.2.1 Milk protein analysis

For each run milk samples from the flash vessel of the first effect, concentrates from the 1st, 2nd and 3rd effects and the concentrate just before the spray drier (concentrate
heating), were collected (Figure 7.1). The flash vessel instantaneously reduced the temperature of the preheated milk to approximately 70°C, effectively stopping further whey protein denaturation. The 3rd effect concentrate and the concentrate before the spray drier were kept in a 50°C waterbath, and ultracentrifuged the same day (175,000 g, 3 h at 45°C). The unheated skim milk and other samples were cooled to 20°C in an ice bucket and ultracentrifuged the same day as outlined in Section 4.2.3. A known weight of each concentrate was diluted using MilliQ water (distilled and deionised water) to the original total solids of the skim milk (approximately 9%). After stirring for 2 hours at room temperature the diluted concentrate was then ultracentrifuged as described in Section 4.2.3. Milk powder samples were reconstituted at 40°C in MilliQ water and stirred for 2 hours at room temperature. Ultracentrifugation was carried out the following day after the reconstituted milk samples were held overnight at 4°C.

The unheated skim milk and the supernatants from ultracentrifugation were analyzed by native-, SDSNR- and SDSR-PAGE and affinity HPLC as described in Section 4.2.3. The concentrations of individual whey proteins in the supernatant of ultracentrifuged milk and milk concentrates were recalculated to values in the skim milk prior to DSI by using the correction factor \(F\) (see Section 4.2.3.1) and the milk total solids (Equation 7.1). The total solids were analyzed at the Analytical Chemistry Section, NZDRI, by drying for 5 hours in a 102°C oven (McDowell, 1972b).

\[
C_{\text{milk}} = C_{\text{super}} F \frac{TS_{\text{milk}}}{TS_{\text{sample}}}
\]  

(7.1)

Where

- \(C_{\text{milk}}\) = concentration in milk (g kg\(^{-1}\)).
- \(C_{\text{super}}\) = concentration in supernatant (g kg\(^{-1}\)).
- \(F\) = correction factor (see Equation 4.2).
- \(TS_{\text{milk}}\) = total solids of the original skim milk (% w/w).
- \(TS_{\text{sample}}\) = total solids of the milk or concentrate sample (% w/w).
Figure 7.1 Schematic diagram of the milk powder plant and sampling points. Plant equipment; milk silo (A), regenerative heating (B), DSI preheater (C), holding tube (D), evaporator 1st effect (E), 2nd effect (F), 3rd effect (G), concentrate heater (H), spray drier (I) and milk powder bag (J). Sampling points; original skim milk (1), Flash vessel 1st effect (2), 1st effect concentrate (3), 2nd effect concentrate (4), 3rd effect concentrate (5), after the concentrate heater (6) and skim milk powder (7).
Ultracentrifugation of concentrate samples taken from the 3rd effect did not form a firm casein pellet. Thus the correction factor may have overestimated the concentration effect of ultracentrifugation on the whey proteins. The true concentration values were probably similar to the samples that were diluted prior to ultracentrifugation. The concentration of whey proteins in these diluted milk samples were similar to the concentrated samples, for the 1st and 2nd effects. Thus the diluted samples were assumed to be indicative of the level of denaturation in the concentrated samples from the 3rd effect and before the spray drier.

7.2.2.2 Mineral analysis

The inorganic phosphorous ($P_i$), and calcium levels in skim milk were determined by methods previously outlined in Section 6.2.2. The concentrations of Ca and $P_i$ were determined in the ultracentrifugal supernatants of skim milk, concentrates and reconstituted skim milks as described below. The protein in the supernatant was precipitated by the addition of 12% (w/w) TCA (1 ml of sample + 11.5 ml of deionised water + 12.5 ml of a 24% TCA solution). The precipitated protein was removed by filtration and the concentration of Ca and $P_i$ was determined in the filtrate.

The calcium was determined using an atomic adsorption method as described by Brule et al. (1974). The absorbance was recorded at 422.7 nm in an atomic adsorption spectrophotometer (Varian AA 175, Varian Techtron, Pty Ltd, Springvale, Australia).

$P_i$ was determined by the IDF method (IDF, 1987). Sodium molybdate solution was prepared by dissolving 2.5 g of sodium molybdate dihydrate in 5 M sulphuric acid and diluting with 5 M sulphuric acid to 100 ml. Ascorbic acid solution was prepared by dissolving 5 g of ascorbic acid in water and diluting to 100 ml. Molybdate-ascorbic solution was prepared by mixing 25 ml of sodium molybdate solution with 10 ml of ascorbic acid solution and diluting to 100 ml. To 1 ml of the filtrate 2 ml of molybdate-ascorbic solution was added; water was added to dilute the mixture to 50 ml. The samples were heated in a boiling water bath for 15 min, and then cooled to room temperature before the absorbance was read at 820 nm.
The concentrations of calcium and phosphorus in the filtrate were recalculated to their concentrations in skim milk by using Equation 7.1. The concentration in the filtrate was assumed to be approximately the same as the concentration in the supernatant. The correction factor \( (F) \) was approximately 1.00 to 0.99 for removal of proteins in the supernatant (Equation 4.2).

### 7.2.2.3 \( \text{Ca}^{2+} \) activity determinations

The \( \text{Ca}^{2+} \) activity in the original skim milk, concentrates and reconstituted skim milk was determined using a calcium ion selective electrode (F2112Ca Radiometer, Copenhagen) attached to a digital readout pH meter (Radiometer). Using the method of Geerts et al. (1983), samples were measured at 20°C in a 100 ml beaker, which was stirred at a moderate speed. Calibrations were performed using \( \text{CaCl}_2/\text{KCl} \) standards with ionic strengths of 0.08 and 0.2 mol L\(^{-1}\) for milk and concentrate readings, respectively. The activity coefficients determined by Nieuwenhuijse et al. (1988) were used to calculate the \( \text{Ca}^{2+} \) activity. An activity coefficient of 0.4 was used for milk. The activity coefficient for concentrated milk was assumed to be 0.3, even though Nieuwenhuijse et al. (1988) calculated the activity coefficient for a concentrate of 31.3% total solids, and the total solids of concentrates used in this trial were approximately 48%. Recovery of \( \text{Ca}^{2+} \) at a constant temperature (20°C) follows a logarithmic relationship, and extrapolation of the logarithmic relationship back to 1 min allows an estimation of the \( \text{Ca}^{2+} \) activity at heating conditions (Geerts et al., 1983). Samples from the flash vessel, 3rd effect and concentrate heating were collected and placed into iced water. After 4 min the temperature had reached 20 ± 3°C; \( \text{Ca}^{2+} \) activity readings after 4 min were used to determine the \( \text{Ca}^{2+} \) activity in the sample at 1 min. Milk powder was reconstituted into 40°C water stirred for 1 hour, and cooled to room temperature (≈20°C). \( \text{Ca}^{2+} \) activity readings were taken once the samples were cooled and used to determine the \( \text{Ca}^{2+} \) activity 1 hour after reconstitution took place.

### 7.3 RESULTS AND DISCUSSION

The composition of skim milk used in the manufacture of skim milk powder is shown in Table 7.2. The concentration of individual whey proteins are generally comparable to values obtained for other milks used in this study (Chapters 4, 5 and 6). The
concentrations of Ca and P are in reasonable agreement with reported values in the literature (e.g. Walstra & Jenness, 1984).

Table 7.2 Protein and mineral composition of skim milk that was used to manufacture the skim milk powder.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lg A (g kg⁻¹)</td>
<td>2.19</td>
</tr>
<tr>
<td>β-lg B (g kg⁻¹)</td>
<td>2.37</td>
</tr>
<tr>
<td>α-la (g kg⁻¹)</td>
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</tr>
<tr>
<td>BSA (g kg⁻¹)</td>
<td>0.25</td>
</tr>
<tr>
<td>IgG (g kg⁻¹)</td>
<td>0.43</td>
</tr>
<tr>
<td>Calcium (total) (mmol kg⁻¹)</td>
<td>31.7</td>
</tr>
<tr>
<td>Calcium (soluble)⁴ (mmol kg⁻¹)</td>
<td>7.9</td>
</tr>
<tr>
<td>Phosphorus (total) (mmol kg⁻¹)</td>
<td>29.2</td>
</tr>
<tr>
<td>Phosphorus (soluble)⁴ (mmol kg⁻¹)</td>
<td>13.7</td>
</tr>
</tbody>
</table>

⁴Soluble mineral concentration determined from supernatant of ultracentrifuged milk.

The pH of skim milk, concentrates and reconstituted milk powder during the skim milk powder process is shown in Table 7.3. The original pH of the skim milk was 6.67. Preheating temperature (70-120°C) and heating of concentrate before the spray drier did not have a significant effect on the pH. Concentrating the milk to 48% total solids by
evaporation reduced the pH from =6.66 (flash vessel of the 1st effect) to 6.17. The pH decrease during evaporation is caused by a number of factors including, liberation of H⁺ in calcium phosphate formation, degradation of lactose and expulsion of CO₂ (Holt, 1985). The total solids of the original skim milk was 8.6%, which increased slightly to 8.9%, in the flash vessel of the 1st effect. The total solid content of the concentrate in the evaporator was 11.5, 19.9 and 47.5% for concentrate exiting the 1st, 2nd and 3rd effects, respectively.

Table 7.3 pH of skim milk, concentrates and reconstituted skim milk powders (measured at 20°C).

<table>
<thead>
<tr>
<th>Processing step⁴</th>
<th>pH after different preheating temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70°C</td>
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<tr>
<td>Fl</td>
<td>6.66</td>
</tr>
<tr>
<td>1E</td>
<td>6.64</td>
</tr>
<tr>
<td>2E</td>
<td>6.44</td>
</tr>
<tr>
<td>3E</td>
<td>6.17</td>
</tr>
<tr>
<td>CH</td>
<td>6.18</td>
</tr>
<tr>
<td>Powder</td>
<td>6.68</td>
</tr>
</tbody>
</table>

⁴Description of processing step abbreviations shown in Figure 7.2.

7.3.1 Whey protein denaturation
The loss of native β-lg A, β-lg B, α-la and BSA at different steps in the milk powder process are shown in Figures 7.2a, 7.2b, 7.3a and 7.3b, respectively. The loss of native IgG is shown in Table 7.4. As the preheat temperature increased, the amount of native individual whey protein in samples from the flash vessel of the 1st effect (Fl) decreased. There was relatively little further decrease of the native whey protein concentration throughout the remainder of the manufacturing process, at all preheating temperatures.
Figure 7.2 Loss of β-lg A (A) and β-lg (B) from native-PAGE gels during skim milk powder manufacture. Preheating temperature; 70 (○), 80 (●), 100 (△) and 120°C (•), for 52 s. Processing step abbreviations; original skim milk (Raw), flash vessel 1st effect (Fl), concentrate from the 1st, 2nd and 3rd evaporator effects, respectively (1E, 2E & 3E), heated concentrate sample just before the spray drier (CH) and reconstituted skim milk powder (Powder).
Figure 7.3 Loss of α-la (A) and BSA (B) from native-PAGE gels during skim milk powder manufacture. Preheating temperature; 70 (○), 80 (●), 100 (△) and 120°C (▲), for 52 s. Processing step abbreviations shown in Figure 7.2.
Table 7.4 Loss of native IgG during skim milk powder manufacture after preheating at 70°C for 52 s.

<table>
<thead>
<tr>
<th>Processing step/Abbreviations</th>
<th>native IgG concentration (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silo (original milk) Raw</td>
<td>0.43</td>
</tr>
<tr>
<td>Flash Fl</td>
<td>0.35</td>
</tr>
<tr>
<td>1st effect 1E</td>
<td>0.38</td>
</tr>
<tr>
<td>2nd effect 2E</td>
<td>0.37</td>
</tr>
<tr>
<td>3rd effect 3E</td>
<td>0.33</td>
</tr>
<tr>
<td>Concentrate heating CH</td>
<td>0.36</td>
</tr>
<tr>
<td>Spray drying Powder</td>
<td>0.37</td>
</tr>
</tbody>
</table>

The concentrations of β-lg A, β-lg B, α-la and IgG after the preheating step were compared with predicted concentrations using the kinetic parameters from Chapter 4 (Table 7.5). The predicted concentrations were generally comparable with the actual concentrations.

After preheating at 70, 80 and 120°C, the level of native α-la appeared to decrease slightly (Figure 7.3) through the three effects of the evaporator. This was also observed for BSA heated at 70°C, although at higher preheating temperatures there was relatively little change during evaporation and drying (Figure 7.3). There was no apparent decrease for β-lg during evaporation and drying at all the preheating temperatures used.

The concentration of native IgG decreased by ~20% after preheating at 70°C, but evaporation and drying had little further effect.
Table 7.5 Comparison of actual and predicted concentrations of whey proteins after preheating at different temperatures for 52 s.

<table>
<thead>
<tr>
<th>Whey protein</th>
<th>Preheating temperature (°C)</th>
<th>Concentration after preheating (g kg⁻¹)</th>
<th>Actual (native-PAGE or HPLC)</th>
<th>Predicted (Table 4.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-lg A</td>
<td>70</td>
<td>2.12</td>
<td></td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>1.97</td>
<td></td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.74</td>
<td></td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.18</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>β-lg B</td>
<td>70</td>
<td>2.25</td>
<td></td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>2.13</td>
<td></td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.55</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.19</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>α-la</td>
<td>70</td>
<td>0.79</td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>0.74</td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.57</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.46</td>
<td></td>
<td>0.40</td>
</tr>
<tr>
<td>IgG</td>
<td>70</td>
<td>0.35</td>
<td></td>
<td>0.37</td>
</tr>
</tbody>
</table>
These results are generally in agreement with those of Singh and Creamer (1991a) who showed that 4-10% denaturation of β-lg A, β-lg B and α-la occurred during evaporation (milk preheated at 110 and 120°C for 2-3 min). In this study the loss of native whey proteins caused by spray drying was minimal. There was no apparent loss of IgG, and only a small loss of BSA (3-7%). Singh and Creamer (1991a) reported the loss of native whey proteins during spray drying was approximately 2%.

In milk the thermal stability of α-la is considered to be greater than β-lg (Chapter 4, Section 4.3.2), although DSC results have shown that α-la has a lower denaturation temperature than β-lg or BSA (de Wit & Klarenbeek, 1984). The thermostability of α-la results from renaturation of α-la upon cooling (Rüegg et al., 1977). Denaturation temperatures of α-la, determined in DSC studies, have been reported as ranging from 61.2 to 68°C (Bernal & Jelen, 1984; de Wit & Klarenbeek, 1984; Paulsson et al., 1985). The loss of α-la during evaporation observed in this study (Figure 7.3) could be explained as follows. It is likely that thermal unfolding of α-la may have occurred in the 1st effect of the evaporator, which was operating at 70-73°C, but renaturation of α-la was retarded by the increased concentration of the whey proteins and the changes in salt balance and pH during the subsequent stages of evaporation. As milk is concentrated calcium moves from the soluble to the colloidal phase (Walstra & Jenness, 1984), and the pH decreases (Nieuwenhuijse et al., 1988; Singh & Newstead, 1992). Since calcium binding is essential for the thermal stability and renaturation of α-la (Kronman et al., 1981; Bernal & Jelen, 1984; Kuwajima et al., 1986; Patocka & Jelen, 1991), the decrease in pH may have caused a weakening of the calcium binding by α-la (Kronman et al., 1981). The ratio of soluble Ca to α-la decreases during evaporation (Le Graet & Brule, 1982), caused by the shift in soluble Ca to the colloidal phase, and this may influence the equilibrium of Ca binding to α-la, such that the amount of Ca binding to α-la could decrease. Hillier et al. (1979) also showed that α-la denaturation was hastened by an increase in the total solids of cheese whey.

The concentrate exiting the 3rd effect was heated to either 65.3, 69.1 or 73.9°C, then took approximately 80 s to reach the drier (see Table 7.1, run No. 1, 2 & 3). The change in concentrate heating temperature had no significant affect on the concentration
of native whey protein in concentrate samples taken before the spray drier (Table 7.6). The drier inlet air temperature was increased from 180°C to 200°C or decreased to 160°C. The corresponding drier outlet air temperature was maintained at 90°C, and increased to 100°C when the inlet air temperature was 200°C (see Table 7.1, run No 1, 4 & 5). Changing the drier inlet and outlet conditions had little affect on the whey protein denaturation in reconstituted skim milk samples (Table 7.6).

Table 7.6 Effect of concentrate heating and drier inlet and outlet air temperatures on the concentration of individual native whey proteins.

<table>
<thead>
<tr>
<th>Processing conditions</th>
<th>Whey protein concentration (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-lg A</td>
</tr>
<tr>
<td>concentrate heating temp. (°C)</td>
<td></td>
</tr>
<tr>
<td>65.3</td>
<td>1.93</td>
</tr>
<tr>
<td>69.1</td>
<td>2.01</td>
</tr>
<tr>
<td>73.9</td>
<td>1.97</td>
</tr>
<tr>
<td>Drier inlet (outlet) air temp. (°C)</td>
<td></td>
</tr>
<tr>
<td>160 (90)</td>
<td>1.75</td>
</tr>
<tr>
<td>180 (90)</td>
<td>1.86</td>
</tr>
<tr>
<td>200 (100)</td>
<td>1.64</td>
</tr>
</tbody>
</table>
7.3.2 Whey protein aggregation and association with the micelle

The distribution of β-lg, α-la and BSA aggregates formed during milk powder manufacture are shown in Figures 7.4, 7.5 and 7.6, respectively. Definitions of the whey protein species have been previously discussed in Section 4.3.3. After preheating at 100 or 120°C, β-lg and BSA aggregates in the supernatant were predominantly disulphide-linked (Figures 7.4 & 7.6) possibly because of availability of thiol groups in these proteins. In contrast, α-la in the supernatant formed mainly hydrophobic aggregates (Figure 7.5) probably because of the lack of a thiol group. The extent of preheating at 70 and 80°C was not great enough to observe any clear trends at these temperatures. The levels of β-lg and BSA aggregates in the supernatant, both hydrophobic and disulphide-linked, remained relatively constant throughout the evaporation and drying process.

The level of β-lg associated with the casein micelle increased from 38 to 50% as the preheating temperature was increased from 100°C to 120°C. Similar increases of 11 to 22% and 49 to 77% were observed for α-la and BSA, respectively. The level of associated β-lg and α-la increased slightly during evaporation and concentrate heating, but the level of associated β-lg decreased during spray drying. In contrast, the level of associated BSA remained relatively constant throughout the process.

It is clear that preheating has the most influence on the extent and nature of whey protein aggregation and association with the micelle. The effects of evaporation and spray drying were small by comparison. β-Lg and BSA denaturation was relatively unaffected by evaporation, because of the low temperatures (<72°C) and the increased heat-resistance in the 2nd and 3rd effects with increasing total solids (Hillier et al., 1979; Ibrahim et al., 1995), although in the 1st effect the concentrate conditions may promote denaturation (see Chapter 6, Section 6.3.3). α-La appeared to be the whey protein most susceptible to denaturation during evaporation, and may possibly interact with β-lg, already denatured by preheating, to form β-lg/α-la aggregates.
Figure 7.4 β-Lg aggregates formed during skim milk powder manufacture. Preheating temperature; 100°C (A) and 120°C (B), for 52 s. β-Lg species; native (○), hydrophobic aggregates (///), disulphide-linked aggregates in the supernatant (XXX) and aggregates (hydrophobic and disulphide-linked) associated with the micelle (■).
Figure 7.5 $\alpha$-La aggregates formed during skim milk powder manufacture. Preheating temperature; 100°C (A) and 120°C (B), for 52 s. $\alpha$-La species; native (O), hydrophobic aggregates (///), disulphide-linked aggregates in the supernatant (XXX) and aggregates (hydrophobic and disulphide-linked) associated with the micelle (■).
Figure 7.6 BSA aggregates formed during skim milk powder manufacture. Preheating temperature; 100°C (A) and 120°C (B), for 52 s. BSA species; native ( ), hydrophobic aggregates (///), disulphide-linked aggregates in the supernatant (XXX) and aggregates (hydrophobic and disulphide-linked) associated with the micelle ( ).
The extent of $\beta$-lg and $\alpha$-la association with the micelle during evaporation was less than the extent of denaturation. This is in agreement with the observations made by Singh and Creamer (1991a). During evaporation the extent of $\beta$-lg and $\alpha$-la associating with the micelle increased. The decrease in pH during evaporation (Table 7.6) would reduce the protein charge and therefore facilitate association reactions. The increase in soluble Ca concentration during evaporation (Le Graet & Brule, 1982) may also promote association reactions between the whey proteins and the micelle (Smits & Brouwershaven, 1980; Visser et al., 1986).

Concentrate heating increased the extent of $\beta$-lg and $\alpha$-la association with the micelle. Heating the concentrate at 70°C for 80 s may have enhanced sulphydryl-disulphide interchange reactions between $\beta$-lg aggregates and $\kappa$-casein at the micelle surface. There was little increase in denaturation of the whey protein observed during the concentrate heating step, therefore association reactions must have occurred between the casein micelle and aggregates that had already formed during preheating.

The extent of $\beta$-lg association with the micelle decreased slightly in the reconstituted skim milk powders compared to the concentrate samples. It is possible that during reconstitution of the skim milk powders some of the $\beta$-lg/$\kappa$-casein complexes may have dissociated from the micelle. $\alpha$-La and BSA association with the micelle showed no clear trend. The effect of spray drying on the association of whey proteins with the casein micelle was small, in comparison with preheating and evaporation.

7.3.3 Changes in the mineral distribution
The concentrations of Ca and P, in the supernatant of milk, milk concentrates and milk concentrates diluted prior to ultracentrifugation were measured. The concentrations were then recalculated to their equivalent concentrations in skim milk using Equation 7.1. The supernatant was assumed to be representative of the soluble phase in milk. It is possible that some reversibility of the milk salt changes occurred due to the time delay before ultracentrifugation (see Chapter 6, Section 6.3.4).
The loss of Ca and P\textsubscript{i} from the soluble phase is shown in Tables 7.7 and 7.8, respectively. Preheating decreased the concentrations of soluble Ca and P\textsubscript{i}. The extent of preheating (30 s at 70°C to 120°C) did not appear to affect the loss of soluble Ca, but the loss of soluble P\textsubscript{i} was slightly less in the concentrates subjected to the lowest preheat treatment (70°C for 52 s). Evaporation decreased the concentrations of Ca and P\textsubscript{i} further and the greatest loss occurred in the 3rd effect (3E). Heating the concentrate at ≈70°C in the concentrate heating (CH) step further reduced the soluble Ca, but had little affect on the P\textsubscript{i}. Upon reconstitution of the milk powder (=9% total solids), the recalculated soluble Ca and P\textsubscript{i} concentrations were greater than in the concentrate, demonstrating the reversibility of the changes caused by evaporation, although the effect of preheating and evaporation was not completely reversible (reconstituted skim milk samples were held overnight at 4°C).

**Table 7.7 Concentration of soluble Ca in skim milks, concentrates and reconstituted skim milk powders during skim milk powder manufacture.**

<table>
<thead>
<tr>
<th>Processing step( ^{a} )</th>
<th>Soluble Ca (mmol kg(^{-1} )) after different preheating temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw (one measurement)</td>
<td>7.9</td>
</tr>
<tr>
<td>Fl</td>
<td>5.0</td>
</tr>
<tr>
<td>1E</td>
<td>3.9</td>
</tr>
<tr>
<td>2E</td>
<td>3.7</td>
</tr>
<tr>
<td>3E</td>
<td>2.8</td>
</tr>
<tr>
<td>CH</td>
<td>3.0</td>
</tr>
<tr>
<td>Powder</td>
<td>4.4</td>
</tr>
</tbody>
</table>

\( ^{a} \)Description of processing step abbreviations shown in Figure 7.2.
Table 7.8 Concentration of soluble $P_i$ in skim milks, concentrates and reconstituted skim milk powders during skim milk powder manufacture.

<table>
<thead>
<tr>
<th>Processing step$^a$</th>
<th>Soluble $P_i$ (mmol kg$^{-1}$) after different preheating temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70°C</td>
</tr>
<tr>
<td>Raw (one measurement)</td>
<td>13.7</td>
</tr>
<tr>
<td>Fl</td>
<td>12.5</td>
</tr>
<tr>
<td>1E</td>
<td>11.7</td>
</tr>
<tr>
<td>2E</td>
<td>10.8</td>
</tr>
<tr>
<td>3E</td>
<td>8.3</td>
</tr>
<tr>
<td>CH</td>
<td>8.3</td>
</tr>
<tr>
<td>Powder</td>
<td>11.9</td>
</tr>
</tbody>
</table>

$^a$Description of processing step abbreviations shown in Figure 7.2.

Nieuwenhuijse et al. (1988) observed that the amount of Ca transferring to the colloidal phase in concentrated milk was independent of the preheating conditions (2 s at 74°C versus 3 min at 120°C), although preheating at 120°C for 3 min resulted in a greater loss of $P_i$ from the soluble phase, and that this difference persisted during storage (48 h at 4°C). Although the temperature and extent of preheating affect the transfer of soluble salts to the colloidal phase in milk (Pouliot et al., 1989a), the differences caused by preheating may become negligible compared to the effect of concentration.

Nieuwenhuijse et al. (1988), using a pilot-scale 3-stage falling film evaporator, reported concentrations of ultrafiltratable Ca from skim milk and concentrated skim milk (31.3% total solids) approximately 1 to 3 mmol kg$^{-1}$ higher than those observed in this study. However, the soluble $P_i$ concentration values determined in this study were similar to
the values reported by Nieuwenhuijse et al. (1988) for skim milk and concentrated skim milk.

The reversibility of the Ca and P shifts to the colloidal phase was investigated by diluting the concentrated skim milk back to the total solid content of the original skim milk (±9% total solids). When the concentrates were diluted there was a shift in the colloidal Ca and P\textsubscript{i} back to the soluble phase (Table 7.9 & 7.10). However, the concentration of soluble Ca and P\textsubscript{i} was below that of the original skim milk. The soluble Ca and P\textsubscript{i} concentrations in the diluted concentrates from the evaporators three effects were approximately 29-35% and 20-24% lower than the original milk, respectively. On average the concentrations of soluble Ca and P\textsubscript{i} in the concentrate heating samples were 38% and 30% lower than in the original milk, respectively. Preheating conditions had no noticeable effect on the extent of reversibility.

Complete reversibility was not apparent when the skim milk powder was reconstituted to the total solids content of the original skim milk. The concentrations of soluble Ca and P\textsubscript{i} were on average 34% and 20% lower than that of skim milk, respectively. This is in agreement with the results of Le Graet and Brule (1982) who reported that the concentrations of soluble Ca and P\textsubscript{i} in reconstituted skim milk were about 20% lower than in the original milk. Since the reconstituted skim milk and diluted concentrates (both ±9% total solids) had similar concentrations of soluble Ca and P\textsubscript{i} (Tables 7.9 & 7.10), it appears that spray drying did not have a noticeable effect on the milk salt balance in reconstituted skim milk powder (held at 4°C overnight). Therefore, preheating and the subsequent evaporation process are likely to have the more influence on the milk salt balance.
Table 7.9 Concentration of soluble Ca in the supernatant of diluted concentrates and reconstituted skim milk powders.

<table>
<thead>
<tr>
<th>Processing step&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Soluble Ca (mmol kg&lt;sup&gt;-1&lt;/sup&gt;) after different preheating temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70°C diluted&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Raw</td>
<td>7.9</td>
</tr>
<tr>
<td>1E</td>
<td>5.0</td>
</tr>
<tr>
<td>2E</td>
<td>5.0</td>
</tr>
<tr>
<td>3E</td>
<td>4.9</td>
</tr>
<tr>
<td>CH</td>
<td>5.0</td>
</tr>
<tr>
<td>Powder</td>
<td>4.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Description of processing step abbreviations shown in Figure 7.2.

<sup>b</sup>These samples correspond to concentrates diluted to ≈9% total solids before ultracentrifugation.

<sup>c</sup>These samples correspond to the concentrated samples (see Table 7.7), averaged from different preheating temperatures (70-120°C).

Concentrating milk by evaporation is known to cause the shift of soluble Ca and P<sub>i</sub> to the colloidal phase (Walstra & Jenness, 1984; Nieuwenhuijse et al., 1988), although the effect this has on the composition and structure of colloidal calcium phosphate (CCP) is poorly understood. Milk is already supersaturated with respect to Ca and P<sub>i</sub> (Walstra & Jenness, 1984). The shift of Ca and P<sub>i</sub> into the colloidal phase during evaporation may result in the formation of colloidal calcium phosphate (CCP) which may have a different composition and structure than the original CCP.
Table 7.10 Concentration of soluble P<sub>i</sub> in the supernatant of diluted concentrates and reconstituted skim milk powders.

<table>
<thead>
<tr>
<th>Processing step&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Soluble P&lt;sub&gt;i&lt;/sub&gt; (mmol kg&lt;sup&gt;-1&lt;/sup&gt;) after different preheating temperatures</th>
<th>70°C diluted&lt;sup&gt;b&lt;/sup&gt;</th>
<th>80°C diluted&lt;sup&gt;b&lt;/sup&gt;</th>
<th>100°C diluted&lt;sup&gt;b&lt;/sup&gt;</th>
<th>120°C diluted&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Average undiluted&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td></td>
<td>13.7</td>
<td>13.7</td>
<td>13.7</td>
<td>13.7</td>
<td>13.7</td>
</tr>
<tr>
<td>1E</td>
<td></td>
<td>10.8</td>
<td>9.6</td>
<td>11.0</td>
<td>10.2</td>
<td>11.2</td>
</tr>
<tr>
<td>2E</td>
<td></td>
<td>11.5</td>
<td>11.4</td>
<td>10.2</td>
<td>10.7</td>
<td>10.5</td>
</tr>
<tr>
<td>3E</td>
<td></td>
<td>8.8</td>
<td>10.0</td>
<td>10.2</td>
<td>10.0</td>
<td>7.2</td>
</tr>
<tr>
<td>CH</td>
<td></td>
<td>10.5</td>
<td>9.4</td>
<td>9.5</td>
<td>9.9</td>
<td>7.1</td>
</tr>
<tr>
<td>Powder</td>
<td></td>
<td>11.9</td>
<td>10.7</td>
<td>9.3</td>
<td>11.6</td>
<td>10.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Description of processing step abbreviations shown in Figure 7.2.

<sup>b</sup> These samples correspond to concentrates diluted to ~9% total solids before ultracentrifugation.

<sup>c</sup> These samples correspond to the concentrated samples (see Table 7.8), averaged from different preheating temperatures (70-120°C).

Holt (1985, 1995) has suggested that the structure of CCP in milk is amorphous and resembles brushite (dicalcium phosphate dihydrate). In contrast, van Dijk (1990a, b) proposed that CCP was a unique (i.e. does not resemble a mineral phosphate) ion cluster of 4 ionic phosphates and 8 calcium ions, linking together two serine phosphate residues. In both models, phosphoserine residues act as sites for the formation of CCP. Approximately half the casein proteins are cross-linked by CCP (Aoki <i>et al.</i>, 1987, 1990), which leaves a large proportion of casein proteins whose phosphoserine residues could act as sites for CCP formation, either by nucleation (Holt, 1995) or by a series of ion-cluster complexes (van Dijk, 1990b). Increasing the total solids increases the concentration of soluble Ca and P (Le Graet & Brule, 1982) and slightly increases the...
calcium activity (Nieuwenhuijse et al., 1988). This may promote CCP formation despite the possible inhibitory effect of the casein proteins at high total solids (van Kemenade & de Bruyn, 1989; Holt, 1995). The phosphoserine residues may also act to stabilise CCP and prevent its dissolution (Holt, 1995). Therefore, the diluted concentrates in this study may have required a longer holding time at 4°C in order for the Ca and P, that had transferred into the colloidal phase to resolubilise.

The changes in CCP and milk salt balance during spray drying are even less well understood than during evaporation. There was no significant difference between reconstituted skim milk powders and diluted concentrate just before the spray drier (both =9% total solids). Thus spray drying did not significantly alter the milk salt balance and probably the CCP structure in reconstituted skim milk powder. Thus preheating and evaporation may be the important steps in determining the properties of the reconstituted skim milk powder.

During spray drying the ionic strength almost certainly increased and the ion activities would have correspondingly decreased (Walstra & Jenness, 1984). The rate of CCP formation in van Dijk's (1990a, b) model is governed by ion activities, thus the rate of CCP formation may have decreased. It is possible that at the total solids concentration in the spray drying droplets, the formation of CCP may be inhibited by the casein proteins (van Kemenade & de Bruyn, 1989; Holt, 1995). In addition, lactose is known to bind Ca²⁺ and Mg²⁺ (e.g. Swartz et al., 1978) and this may have contributed to a possible decrease in CCP formation. Therefore the overall change during spray drying in milk salts and CCP may be small.

7.3.3.1 Ca²⁺ ion activity
The Ca²⁺ activities of skim milk, concentrates and reconstituted skim milk powder after being subjected to different preheating temperatures are shown in Table 7.11. The original skim milk had a Ca²⁺ activity ranging between 0.89 to 0.93 mmol L⁻¹. Preheating caused the Ca²⁺ activity to decrease by 0.17 to 0.40 mmol L⁻¹. The Ca²⁺ activity increased as the skim milk was concentrated (1.00 to 0.81 mmol L⁻¹). At a sampling point just before the spray drier the Ca²⁺ activity decreased by 0.06 to
0.35 mmol L\(^{-1}\). The Ca\(^{2+}\) activities of milk powder 1 hour after reconstitution ranged from 0.50 to 0.61 mmol L\(^{-1}\), which is considerably below the value for the original skim milk. Holding the reconstituted milk at 4°C for a further 24 h resulted in a slight increase in the Ca\(^{2+}\) activity by 0.16-0.23 mmol L\(^{-1}\), but the resulting activities were still lower than the original skim milk.

The Ca\(^{2+}\) activity of the original skim milk agrees with the values of 0.81-0.90 mmol L\(^{-1}\) and 0.87-0.90 mmol L\(^{-1}\) reported by Greets \textit{et al.} (1983) and Nieuwenhuijse \textit{et al.} (1988), respectively.

Table 7.11 Ca\(^{2+}\) activity of skim milks, concentrates and reconstituted skim milk powders (measured at 20°C).

<table>
<thead>
<tr>
<th>Processing step(^a)</th>
<th>Ca(^{2+}) activity (mmol L(^{-1})) from different preheating temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70°C</td>
</tr>
<tr>
<td>Raw</td>
<td>0.91</td>
</tr>
<tr>
<td>Fl</td>
<td>0.74</td>
</tr>
<tr>
<td>3E</td>
<td>1.00</td>
</tr>
<tr>
<td>CH</td>
<td>0.87</td>
</tr>
<tr>
<td>Powder 1 h(^b)</td>
<td>0.61</td>
</tr>
<tr>
<td>Powder 24 h(^c)</td>
<td>0.77</td>
</tr>
</tbody>
</table>

\(^a\) Description of processing step abbreviations shown in Figure 7.2.
\(^b\) Ca\(^{2+}\) activity of reconstituted skim milk powder 1 h after reconstitution.
\(^c\) Ca\(^{2+}\) activity of reconstituted skim milk powder after being held for 24 h at 4°C.

The results showed the effect of preheating appeared to be temperature dependent. The greatest loss of Ca\(^{2+}\) activity from samples taken from the flash vessel occurred after preheating at 120°C, while the least occurred after preheating at 70°C. The effect of
heating milk on Ca²⁺ activity has been reported in the literature, and the decrease in Ca²⁺ occurs to a greater extent when the heating temperature and time are increased (Greets et al., 1983; Nieuwenhuijse et al., 1988).

After preheating the Ca²⁺ increased as the milk was concentrated to 48% total solids. The Ca²⁺ activities of the concentrated skim milk from the 3rd effect and the original skim milk were similar. Nieuwenhuijse et al. (1988) reported the Ca²⁺ activity of concentrated skim milk (31.3% total solids) was slightly lower than the in the original milk. In contrast, Augustin and Clarke (1991) showed that the Ca²⁺ activity of reconstituted concentrated milk (19.6% total solids) was higher compared to normal reconstituted milk (9% total solids). The differences may be due to the different total solids used, the accuracy of the activity coefficient, or natural variations in milk salt composition.

The concentrate heated at 70°C took 80 s to reach the spray drier. The reduction in Ca²⁺ activity of the concentrate, just before the spray drier, may have been caused by a heat-induced shift of Ca²⁺ into the colloidal phase, or possibly the involvement of Ca²⁺ in micellar aggregation.

The effect of spray drying was to decrease the Ca²⁺ activity in the reconstituted skim milk powders (1 h after reconstitution) below the level of the preheated skim milks. The loss of Ca²⁺ was partially reversed after the samples were held for a further 24 h at 4°C. The Ca²⁺ activities of the reconstituted skim milk powders appeared to be independent of the different preheating conditions used, although the powders made using 70 and 80°C preheat treatments had slightly higher Ca²⁺ than those made using 100 and 120°C. Holding the reconstituted skim milk reduced any difference between skim milk powders made using different preheating temperatures. Augustin and Clarke (1991) investigated the Ca²⁺ activities of reconstituted skim milk powders after they were held overnight at 4°C. The skim milk had been subjected to various preheat treatments, ranging from 72°C for 15 s to 120°C for 2 min. They observed that increasing the extent of preheating decreased the Ca²⁺ activity of reconstituted skim milk, but that this effect was small.
7.4 CONCLUSIONS

The major loss of native whey proteins occurred during preheating. The effect of evaporation on β-lg and IgG was negligible, but evaporation caused a decrease in the amount of native α-la and BSA. Spray drying had little or no effect on the native whey proteins.

The β-lg and BSA aggregates in the supernatant were predominantly disulphide-linked, while those of α-la involved mainly hydrophobic interactions. Preheating largely determined the nature of these aggregates, by comparison evaporation and spray drying had little affect.

The association of β-lg and α-la aggregates with the casein micelle increased slightly during evaporation, and increased further during heating of the concentrate.

The soluble Ca and P concentrations, relative to the original milk, decreased during preheating, evaporation and after heating of the concentrate. Diluting the concentrates to the total solids of skim milk did not fully reverse the changes in soluble Ca and P. Spray drying had little effect on the soluble Ca and P concentrations in the reconstituted skim milk. The preheating and evaporation steps appeared to have the greatest effect on the soluble Ca and P concentrations in the reconstituted skim preheating and concentrate heating steps.

The preheating and concentrate heating steps caused a decrease in the Ca\(^{2+}\) activity. After the skim milk powders were reconstituted their Ca\(^{2+}\) activities increased slowly while they were held for 24 h at 4°C.
CHAPTER 8: GENERAL DISCUSSION

8.1 DENATURATION AND AGGREGATION OF WHEY PROTEINS

Denaturation of whey proteins is generally assumed to be a process consisting of at least two steps (Mulvihill & Donovan, 1987; de Wit, 1990; Parris et al., 1991). In the first step, the native protein unfolds cooperatively to expose side chain groups were originally buried within the native structure (Walstra & Jenness, 1984). The second step involves aggregation of the unfolded protein molecules through sulphhydryl-disulphide interchange reactions, hydrophobic interactions and ionic linkages. It is not known to what degree the whey proteins must unfold to allow aggregation to take place. There are distinct differences in the denaturation behaviour of individual whey proteins, although β-lg appears to dominate the overall behaviour of the total whey protein system. The resistance of whey proteins to heat denaturation in milk follows the order; α-la> β-lg B> β-lg A> BSA> Ig as determined using protein precipitation methods (Larson & Rolleri, 1955; Dannenberg & Kessler, 1988a; Singh & Creamer, 1991a). However, α-la has been found to be the least stable protein using DSC (de Wit & Klarenbeek, 1984).

There are a number of reports on the kinetics of denaturation of individual whey proteins in buffer solutions (El-Shazly et al., 1978), whey (Hillier & Lyster, 1979) or skim milks (Dannenberg & Kessler, 1988a). The reaction orders and activation energies are not always in agreement. For example, the denaturation of β-lg in milk has been described as pseudo first order (Dalgleish, 1990), second order (Manji & Kakuda, 1986) or 1.5 order (Dannenberg & Kessler, 1988a), and activation energies have been found to vary between 32 and 54 kJ mol\(^{-1}\) for the 95-150°C temperature range, and 250 and 280 kJ mol\(^{-1}\) for the 70-90°C range.

In most studies, kinetic analysis of the raw data has been carried out using a two-step linear regression method. In this method, the first step is to calculate the rate constant at a given
temperature from the concentration/time data, using the appropriate rate equation and reaction order. Reaction orders of 1 or 2 are typically used, as non-integer values do not fit preconceived unimolecular or bimolecular mechanisms. The second step is to apply the Arrhenius equation to the rate constants obtained at different temperatures, and calculate the activation energy and frequency factor. Ideally, weighted linear regression should be used in both steps although this has not been done in most reported studies. The two-step method requires a lot of raw data to obtain only a few rate constants. Therefore, a lot of information about the precision of the data is lost when the concentration/time data is used to calculate the rate constant.

The present study investigated a number of linear and non-linear regression methods for analysing the denaturation kinetics of individual whey proteins. The NLR procedure (see Section 4.2.4.5) was found to be the preferred method for accurately determining the denaturation kinetics of individual whey proteins. Kinetic parameters were calculated by NLR with greater precision than the commonly used two-step method with linear regression described by Hill and Grieger-Block (1980). In addition, NLR required less data points than the two-step method to achieve the same precision (Section 4.3.2.1). Thus the method is suitable for pilot-scale work which is subject to more time and resource constraints than laboratory-scale research, but which are better able to imitate the commercial practice.

Denaturation kinetic parameters were calculated for β-lg A, β-lg B, α-la, IgG and BSA. Reaction orders for β-lg using NLR ranged from 1.0 to 1.6, and values for α-la were in the range 0.9-1.1. This is in contrast to the 1st or 2nd order reactions typically fitted to denaturation data (El-Shazly et al., 1978; Hillier & Lyster, 1979; Manji & Kakuda, 1986). Although limited information has been published on IgG denaturation, the 2nd order obtained in this study was in agreement with that observed by Resmimi et al. (1989), but disagreed with the 1.16 order reported by Luf et al. (1993). The reaction for BSA has not been reported previously; a reaction order of 2.8 was calculated for BSA in this study. Because the reactions occurring in heated milk are complex, it is suggested that the reaction order should be treated more as an empirical constant used for curve fitting the data, rather than as an indication of the reaction mechanism. The activation energies of
β-lg and α-la denaturation changed markedly at 90°C and 80°C, respectively. For example, the activation energy of β-lg A was 58.75 and 285.50 kJ mol⁻¹, in the temperature ranges 95-130°C and 70-90°C, respectively. At temperatures ≤80°C the rate for β-lg and α-la denaturation were similar, but at higher temperatures (>80°C) the rate for α-la was lower than that of β-lg. It would be expected that the relative rates of denaturation of the two major whey proteins (β-lg and α-la) would have an important effect on aggregate formation in heated milk.

One of the possible reasons for the observed differences in denaturation kinetics between independent studies may be the different heating methods used. These methods include heating samples in glass test tubes or capillary tubes immersed in water/oil baths, and laboratory scale heat exchangers. It is likely that whey proteins respond differently depending on how milk is heated, i.e. the time required to reach the desired temperature, flow conditions and cooling times and rates. Moreover, the time required to reach the set temperature and cooling times have been neglected when interpreting the results or calculating denaturation kinetics.

In the present study, typical commercial preheating regimes were simulated on a pilot-scale UHT plant. The residence time distribution and flow behaviour of milk in the UHT plant was analyzed, and taken into account in the calculations for determining kinetic parameters (Section 4.3.2.2). The results obtained in this study are applicable to commercial plants, and from the kinetic data, it is possible to predict the WPNI for a range of temperature/time conditions.

Various PAGE methods were used to differentiate among the variety of whey protein aggregates formed in heated milk. β-Lg aggregation appeared to be mainly through the formation of disulphide bonds via sulphydryl-disulphide interchange reactions, whereas α-la aggregates were formed through both hydrophobic interactions and disulphide-linked interchange. It is probable that the availability of a thiol group in the β-lg molecule provides more opportunity for sulphydryl-disulphide interactions, whereas the absence of a thiol group in the α-la molecule may limit these reactions. The slow development of reactive thiol groups at heating temperatures ≤75°C (Kirchmeier et al., 1984) presumably
limits thiol-disulphide interchange reactions, thus formation of β-lg and α-la aggregates at these temperatures were predominantly via hydrophobic interactions. Conversely, at temperatures >75°C aggregates were predominantly formed by intermolecular disulphide bonds, possibly due to the weakening of hydrophobic interactions and the increased rate of reactive thiol group development. The kinetics of disulphide-linked β-lg aggregate formation followed an Arrhenius relationship similar to β-lg denaturation, with comparable values of reaction orders, activation energies, reaction rates and break in the Arrhenius plot (90°C) (see Tables 4.4, 4.8 & 4.9). These results suggest that upon unfolding aggregation of β-lg molecules occurs immediately through the formation of intermolecular disulphide bonds. The formation of α-la aggregates involves both disulphide linkages and hydrophobic interactions, therefore kinetic analysis of disulphide-linked α-la aggregation was not possible without a better understanding of the mechanisms taking place.

The denaturation and aggregation reactions occurring in heated milk systems depend not only on heating conditions used but also the physico-chemical state of the milk. The effect of artificial modifications in composition (e.g. pH, total solids, soluble milk salts concentration) have been reported in whey and purified protein systems, but there is little reported on the effect these changes have in skim milk. The present results showed that compositional changes, such as pH, whey protein concentration and total solids concentration affected denaturation and disulphide-linked aggregate formation. Increasing the pH (6.48 to 6.83) of milk prior to heating had no significant affect on whey protein denaturation, and only slightly reduced the extent of disulphide-linked aggregation. A greater effect was found by increasing the whey protein concentration, from 5.2 to 12.4 g kg⁻¹. This promoted α-la denaturation and disulphide-linked aggregation, although the effect on β-lg was small. Increasing the total solids content of milk from 6% to 13.2% increased the rate of β-lg and α-la denaturation and disulphide-linked aggregation.

The main source of compositional variation in New Zealand milk is the changes caused by season/lactation. The slight increase in whey protein denaturation observed in late season milk may have been caused by the increase in whey protein and Na⁺ concentrations and the decreases in lactose concentration. This increased denaturation and the subsequent
increase in disulphide-linked aggregate formation may contribute to some of the problems associated with processing of late season milk, e.g. reduced solubility of milk powders. The rates of denaturation and aggregate formation in seasonal milks, described in this study, are likely to be important in explaining the altered functional properties of a number of milk products, e.g. skim milk powder manufactured from the early and late season milks. Further research is required to obtain a thorough understanding of how seasonal variations affect whey protein reactions in heated skim milk.

To understand the mechanisms of whey protein denaturation and aggregation in more detail it would be necessary to isolate and identify the types of β-lg and β-lg/α-la aggregates formed during heating. The different reaction pathways and their rates would need to be investigated in more detail. This may require working with model systems, where the whey protein concentration and other parameters (pH, milk salts concentration, casein micelles) can be more easily manipulated.

8.2 ASSOCIATION OF WHEY PROTEINS WITH THE CASEIN MICELLE

Although there has been extensive research into establishing β-lg/κ-casein interactions in mixtures of purified proteins, information on the heat-induced association of whey proteins with the casein micelle in heated milk is limited. Heating conditions reported in the literature are often limited to a small number of temperatures and heating times. The wide range of temperatures (70-130°C) and times (5 s to 30 min) used in this study, sought to clarify the temperature/time association reactions of β-lg and α-la with the casein micelle in skim milk. Moreover, in this study milks were heated on a pilot-scale UHT plant, and therefore the results obtained should be more applicable to industrial heat treatments.

Ultracentrifugation was used to separate the heated skim milk sample into a casein pellet and a serum supernatant. The whey proteins lost from the supernatant as a result of heat treatment were assumed to be associated (co-sedimented) with the micelle. The amounts of β-lg and α-la associated with the micelle increased with both heating time and temperature. It was possible to explain the rates of β-lg and α-la association with the micelle by comparing the denaturation rates of the two proteins. The rates of β-lg denaturation were greater than the corresponding rates for α-la denaturation at
temperatures above 80°C. The faster rate of β-lg denaturation and subsequent aggregation reactions resulted in a greater proportion of the β-lg associating with the micelle than α-la at any given time. In contrast, the similar rates of β-lg and α-la denaturation below 80°C resulted in similar extents of β-lg and α-la associating with the micelle.

The kinetics of whey protein association with the micelle have not been reported to date. In order to determine the rate of association a mathematical model relating the formation of β-lg aggregates and their association with the micelle was proposed (Section 4.3.5). The rates of association were much slower than the rates of aggregation. The rate constant for association followed a similar Arrhenius relationship as denaturation, with a break in the curve at 100°C.

There have been no studies in the literature relating the extent of association with the extent of denaturation. Earlier studies have showed that most of the whey proteins that denature associate with the micelle (Smits & Brouwershaven, 1980; Corredig & Dalgleish, 1996). In the present study approximately 55% of the denatured whey protein was found to associate with the micelle. The association reactions which occur in commercial plants may differ from those observed in lab scale studies. The nature and composition of the aggregates associating with the micelle were dependent on temperature and extent of heating. The similar rates of β-lg and α-la denaturation below 80°C resulted in the formation of aggregates with a β-lg/α-la ratio of 2:1. In contrast, at temperatures above 80°C the aggregates which associate with the micelle are composed entirely of β-lg in the initial stages of heating essentially because β-lg under these heating conditions denatures and aggregates at a much faster rate than α-la. It is only after prolonged heating that α-la begins to denature and form aggregates with β-lg, these aggregates can then associate with the micelle.

The effect of small changes in pH, whey protein concentration and total solids content were investigated. The effect of pH on association of whey proteins with the micelle was much greater than the observed denaturation and aggregation effects; more association occurred when the pH was decreased (6.83 to 6.48). Increasing the whey protein concentration resulted in a greater association of α-la with the micelle. The concentration of β-lg
associated with the micelle increased, but to a lesser extent when compared with the relative changes in α-la. Increasing the total solids content, from 6% to 13.2%, caused more of the β-lg and α-la to associate with the micelle. The extent of β-lg and α-la association with the micelle was greater in the early and late season skim milks compared with the mid season skim milk, caused by the increased whey protein and κ-casein concentrations.

Further work is required in the area of whey protein association with the micelle. The effect of whey protein association with the micelle needs to be separated into association (sulphydryl-disulphide interchange reactions between denatured β-lg and κ-casein) and the dissociation behaviour of micellar κ-casein, and methods need to be developed where the two-steps can be separately studied.

8.3 OVERALL MODEL FOR β-LG AND α-LA REACTIONS IN HEATED SKIM MILK

Based on the findings of this study, a model for β-lg and α-la denaturation, aggregation and association with the casein micelle has been proposed (see Sections 4.3.3 and 4.3.4). The reactions of β-lg and α-la which occur in heated milk are shown in Figures 8.1 and 8.2, respectively.

The first step (1) involves partial unfolding or conformational rearrangement of free thiol and hydrophobic groups. Consequently, unfolded β-lg molecules aggregate and interact provided the thiol group and hydrophobic groups can be placed in appropriate positions. The present study showed the formation of stable hydrophobic aggregates and disulphide-linked aggregates of β-lg in heated milks cooled to room temperature, as detected by their electrophoretic mobilities when run under dissociating or reducing conditions (Section 4.3.3, Figure 4.1).
Figure 8.1 β-Lg reactions in heated skim milk. Reaction steps; native β-lg unfolding 1, aggregation via disulphide bond formation to produce polymeric products 2, aggregation via hydrophobic interactions to form polymeric products 3, association of aggregates with κ-casein at the micelle surface 4, formation of disulphide bonds within the hydrophobic aggregate 5 and association of unfolded, but unaggregated β-lg with κ-casein at the micelle surface 6. Milk proteins; β-lg (β) and casein micelles (CM). Thiol groups shown as (-SH), inter- and intramolecular disulphide bonds are represented by (-S-S-) and hydrophobic interactions are shown as (-X-). Note that the above disulphide-linked aggregates and hydrophobic aggregates are polymeric products.

The relative proportions of β-lg in the hydrophobic and disulphide-linked aggregates is dependent on the temperature of the reaction. At lower temperatures (e.g. ≤75°C) there is evidence for the formation of some hydrophobic aggregates, whereas at higher temperatures aggregates are formed primarily by disulphide bonds (see Sections 4.3.3.3 & 6.3.1.2). It is reasonable to assume that hydrophobic aggregates are also formed at higher temperatures, but are rapidly converted into disulphide-linked aggregates; hence they can not be detected by the electrophoretic techniques used in this study. Disulphide linked aggregation requires that the -SH group of one molecule comes into close proximity with the disulphide bond of another molecule and that the new arrangement is energetically
more favourable (the energy of the two molecules from the collision is enough to overcome the activation energy of the reaction). If two or more β-lg molecules can first form hydrophobic aggregates, then it is likely that sulphhydril-disulphide interchange reactions can occur more readily within the low polarity environment. Thus, reactions ③ and ④ are likely to be important. Another most likely parallel reaction is ②, which involves intermolecular sulphhydril-disulphide interchange reactions, with the -SH on β-lg becoming exposed, reacting with a disulphide bond on another β-lg molecule and the transferred -SH becoming available for further sulphhydril-disulphide interchange reactions with a new β-lg molecule.

Figure 8.2 α-La reactions in heated skim milk. Reaction steps; native α-la unfolding ①, aggregation of denatured α-la and β-lg via disulphide bond formation to produce polymeric products ②, aggregation of denatured α-la and β-lg via hydrophobic interactions to form polymeric products ③, association of α-la/β-lg aggregates with κ-casein at the micelle surface ④ and formation of disulphide bonds within the hydrophobic aggregate ⑦. Milk proteins; α-la (α), β-lg (β) and casein micelles (CM). Thiol groups shown as (-SH), inter- and intramolecular disulphide bonds are represented by (-S-S-) and hydrophobic interactions are shown as (-X-). Note that the above disulphide-linked aggregates and hydrophobic aggregates are polymeric products.

When α-la reactions are considered, it is likely that α-la undergoes conformational changes at about 62°C (de Wit & Klarenbeek, 1984), but reaction ② (Figure 8.2) can not take place
because of the lack of a thiol group. In addition, there is no evidence to show that \( \alpha \)-la on its own forms hydrophobic aggregates that continue to exist at room temperature (Gezimati, 1995). However, the presence of \( \beta \)-lg in the heated milk system means that hydrophobic aggregates containing both \( \beta \)-lg and \( \alpha \)-la may be formed and at appropriate temperatures the -SH group of \( \beta \)-lg can initiate sulphydryl-disulphide interchange reactions between the two proteins. The present study clearly showed evidence for the formation of hydrophobic aggregates containing \( \alpha \)-la in milks heated below 80°C (see Section 4.3.3.3, Figure 4.13). This would suggest that reactions 3 and 7 are likely to be important in \( \alpha \)-la aggregation (Figure 8.2).

Aggregates of \( \beta \)-lg and/or \( \beta \)-lg and \( \alpha \)-la with reactive -SH groups formed by reactions 2 and 3 then associate with k-casein on the casein micelle (Step 4) via sulphydryl-disulphide interchange. It is apparent from this study that over a wide range of heating conditions (75-130°C, 5-1800 s) not all the aggregated \( \beta \)-lg associated with the micelle (see Chapter 4, Figure 4.18); approximately 55% of the denatured \( \beta \)-lg associated with the micelle. This 55% limit on \( \beta \)-lg association observed in this study is of interest in trying to establish possible reaction mechanisms. From the models (Figures 8.1 & 8.2) it is evident that there are three possible parallel pathways that \( \beta \)-lg can take in order to associate with the micelle. One pathway would be via homologous aggregates as shown in Step 4 (Figure 8.1), another possibility would be association of unfolded monomeric \( \beta \)-lg through Step 5 (Figure 8.1), and thirdly, \( \beta \)-lg/\( \alpha \)-la aggregates could associate via Step 4 (Figure 8.2). The relative rates of these association reactions would be dependent on temperature and heating rate, which in turn affect the relative rates of unfolding, the formation of the various aggregates species and conditions at the micelle surface. Holt and Horne (1996) suggested that the \( \beta \)-lg aggregates, which have been shown to be stiff and rod-like (Griffen et al., 1993), must worm their way through the surface k-casein layer ("hairy layer") in order to react with the disulphide bonds of k-casein. This may become increasingly difficult as \( \beta \)-lg aggregate size increases, due to steric repulsion. In contrast, unfolded monomeric \( \beta \)-lg would be expected to penetrate the hairy layer with greater ease. The formation of unfolded \( \beta \)-lg may be promoted by long heating times at low temperatures, or by heating at a slow rate to the required temperature. DSC studies have shown that denaturation takes place over a longer period of time if the heating rate is
decreased (e.g. de Wit & Swinkels, 1980). Therefore, under these conditions the amount of unfolded β-lg would increase at a slow rate (Step 1, Figure 8.1), thus the rate of self aggregation would be slow (Steps 2 and 3) and more unfolded β-lg may be available for association reactions (Step 6, Figure 8.1). If milk is heated instantaneously (e.g. by DSI) to the required temperature all the whey proteins begin to unfold in a short period of time, thus presenting more opportunity for unfolded monomeric β-lg to self-aggregate, which consequently would associate with the casein micelles less efficiently. It would be expected that indirect heating systems (e.g. plate heat exchangers) would produce milks with different levels of associated whey proteins with the micelles than direct heating systems (DSI, steam infusion).

When heat-denatured β-lg (4.8 g L⁻¹) was mixed with κ-casein (4.8 g L⁻¹), then heated at 75°C for 7.5 h, the level of association between β-lg and κ-casein was 50% compared to 95% for heated (74°C for 10 min) mixtures of native β-lg and κ-casein (McKenzie et al., 1971). As there were no micelles present, steric effects between the two proteins should be minimal. This could be interpreted as a possible shielding effect of available thiol groups within the self-aggregated β-lg or thiol-oxidation reactions. These reactions may possibly limit association reactions in milk systems, in addition to steric affects arising from the hairy layer.

Association of α-la with the casein micelles is dependent on β-lg and can only proceed through the formation of an α-la/β-lg aggregate through Steps 2 and 3 (Figure 8.2). α-La does not react with κ-casein, and probably remains as part of the aggregate, while β-lg links directly with κ-casein through thiol-disulphide interchange reactions. Thus, the rate and extent of α-la association is greatly affected by the denaturation and aggregation behaviour of β-lg.

Because of the complexity of the reactions taking place in a heated skim milk system, it was not possible, at present, to fully resolve the sequence of these above reactions. It was, however possible to indicate pathways and identify reactants which may play a key role in the overall denaturation, aggregation and association mechanism. Further research would be required to establish in greater detail the composition of the various aggregated
and associated species, and their reaction rates and mechanisms. The use of simulated milk systems would be required to elucidate the relationships and links in the overall denaturation, aggregation and association model.

8.4 POSSIBLE INDUSTRY APPLICATIONS

Heat-induced reactions of milk proteins during processing determine, to a large extent, the functional attributes in many milk products. Although extensive research has been carried out on milk protein reactions in a number of heated systems (e.g. milk, whey), relatively little is known about the effect these changes have on functional properties.

Milk powders are used in a wide range of applications. The functional properties of these powders are largely determined by the extent of preheating used during manufacture and hence the rates of whey protein reactions. The kinetic parameters established in this study can be used to predict accurately the extent of denaturation of whey proteins under a wide range of heating conditions. This should allow better control of the processing steps and thus lead to milk powders of a consistent high quality. Additional information obtained from this study on the denaturation behaviour of individual native whey protein concentration would be useful in the production of specialised powders (e.g. powders with high levels of immunoglobulins).

There is a growing trend towards the manufacture of functional powders, made by manipulating milk composition, e.g. powders with modified protein concentrations, casein to whey protein ratio. Information on how variations in composition affects whey protein reactions was obtained in the present study. This knowledge will be valuable in the manipulation of processing conditions to produce powders with different functional properties.

The relationship between the extent of whey protein denaturation, aggregation and association on the physical and functional properties of powders will need to be explored further. Based on such information it would be possible to modify the functional properties (e.g. water binding, emulsification, heat stability and increased viscosity) useful in applications such as yoghurt, confectionery and bakery products.
The effect of heat treatment is important not only in the manufacture of milk powders, but also in a wide range of other dairy products. For example, heating milk is essential in yoghurt manufacture as its physical properties are largely determined by the extent of whey protein reactions (Dannenberg & Kessler, 1988b). The kinetic data and mathematical model from this work can be used to accurately predict the degree of denaturation and association which should enable the production of yoghurt of consistently high quality.

The findings from this study will benefit other areas where whey protein reactions play an important role in determining product quality, e.g. UHT milks, evaporated milks and fresh cheeses.
REFERENCES


APPENDIX A1

PASCAL PROGRAM FOR NUMERICAL INTEGRATION OF \( \beta\)-LG RATE EQUATIONS BY RUNGA KUTTA 4TH ORDER ALGORITHM

\{n+\}

{Declares program variables}

Program blg(Input, Output, Results);

Var
A1,A2,A3,A4,B1,B2,B3,B4,C1,C2,C3,C4: real;
Cn,Ca,Cm,Cno,Cao,Cmo,k1,k2,Ck,n: real;
Tstep,Total,Tprint,Time,Telaps: real;
Results: text;
filename:string [20];

{Procedures for each of the rate equations}

{Rate equation for denaturation}

procedure nat(Cn,Time,k1,n,Tstep: real;var DCn: real);
begin
DCn:= -Tstep * k1 * (exp (n * ln(Cn)));
End;

{Rate equation for aggregation}

procedure agg(Ca,Time,k1,Cn,n,k2,Ck,Cm,Tstep: Real;var Dca: real);
\begin{align*}
\text{DCa} &:= T\text{step} \times (k_1 \times (\exp(n \times \ln(C_n))) - k_2 \times C_a \times (C_k - C_m)); \\
\end{align*}

\textit{(Rate equation for association)}

\textbf{Procedure} asc(Cm, Time, k2, Ck, Ca, Tstep: real; var DCm: real);
\begin{align*}
\text{Begin} \\
DCm &:= T\text{step} \times k_2 \times C_a \times (C_k - C_m); \\
\text{End;}
\end{align*}

\textbf{Begin} (*\text{Modelb}*)

\textit{(Request user inputs for program variables and initial conditions)}

\textbf{Writeln;}
\begin{align*}
\text{Write ('Enter name of results file ');} & \text{ readln(filename);} \\
\text{Assign (results, filename);} & \\
\text{rewrite(results);} & \\
\text{Write('Total simulation time (s) ? ');} & \text{ readln(Total);} \\
\text{Write('Time step (s) ? ');} & \text{ readln(Tstep);} \\
\text{Write ('Time between outputs (s) ? ');} & \text{ readln(Tprint);} \\
\text{Write ('Initial native b-lg (mol x 10^6) ? ');} & \text{ readln(Cno);} \\
\text{Write ('Initial aggregate b-lg (mol x 10^6) ? ');} & \text{ readln(Cao);} \\
\text{Write ('Initial associated b-lg (mol x 10^6) ? ');} & \text{ readln(Cmo);} \\
\text{Write ('denaturation kl ? ');} & \text{ readln(kl);} \\
\text{Write ('association k2 ? ');} & \text{ readln(k2);} \\
\text{Write ('reaction order of b-lg denaturation ? ');} & \text{ readln(n);} \\
\text{Write ('active k-casein sites (mol x 10^6) ? ');} & \text{ readln(ck)};
\end{align*}
\textbf{Writeln;Writeln;
{Writes user inputs to file}

Writeln(results);
Writeln(results,'Initial native b-lg (mol x 10^6) = ', Cno:6:2);
Writeln(results,'Initial aggregates b-lg (mol x 10^6) = ', Cao:6:2);
Writeln(results,'Initial associated b-lg (mol x 10^6) = ', Cmo:6:2);
Writeln(results,'rate constant k1, denaturation = ', k1: 10:6);
Writeln(results,'rate constant k2, association = ',k2:10:6);
Writeln(results,'reaction order of b-lg denaturation = ',n:2:1);
Writeln(results,'active k-casein sites (mol x 10^6) ? ', ck:10:6);
Writeln(results,'Total simulation time (s) = ', total:4:0);
Writeln(results,'Time step (s) = ', tstep:6:2);
Writeln(results,'Time between outputs (s) ? ', tprint:4:0);
Writeln(results);writeln(results);
Writeln(results,' Time (s) native aggregated associated');
Writeln(results);
Writeln(' Time (s) native aggregated associated');
Writeln;

{Sets initial conditions}

Time:=0;
Telaps:=0;
Cn:=Cno;
Ca:=Cao;
Cm:=Cmo;
writeln(results,time:12:2,' ',Cn:14:2,' ',Ca:14:2,' ',Cm:14:2);
writeln(time:12:2,Cn:14:2,Ca:14:2,Cm:14:2);

{Begins numerical integration}

While time + tstep <= total do
begin

if Cn <= 0.001 then
    n := 1
else
    n := n;

\textit{(Performs numerical integration)}

nat(Cn, time, k1, n, tstep, a1);
agg(Ca, time, k1, cn, n, k2, ck, cm, tstep, b1);
asc(Cm, time, k2, ck, ca, tstep, c1);

nat(Cn + 0.5 * a1, time + 0.5 * tstep, k1, n, tstep, a2);
agg(Ca + 0.5 * b1, time + 0.5 * tstep, k1, cn, n, k2, ck, cm, tstep, b2);
asc(Cm + 0.5 * c1, time + 0.5 * tstep, k2, ck, ca, tstep, c2);

nat(Cn + 0.5 * a2, time + 0.5 * tstep, k1, n, tstep, a3);
agg(Ca + 0.5 * b2, time + 0.5 * tstep, k1, cn, n, k2, ck, cm, tstep, b3);
asc(Cm + 0.5 * c2, time + 0.5 * tstep, k2, ck, ca, tstep, c3);

nat(Cn + a3, time + tstep, k1, n, tstep, a4);
agg(Ca + b3, time + tstep, k1, cn, n, k2, ck, cm, tstep, b4);
asc(Cm + c3, time + tstep, k2, ck, ca, tstep, c4);

Cn := Cn + (a1 + 2.0 * a2 + 2.0 * a3 + a4)/6;
Ca := Ca + (b1 + 2.0 * b2 + 2.0 * b3 + b4)/6;
Cm := Cm + (c1 + 2.0 * c2 + 2.0 * c3 + c4)/6;
Time := time + tstep;
Telaps := telaps + tstep;
If telaps >= tprint then
    begin
        telaps := 0.0;
        writeln(results, time: 12:2, ' ', Cn: 14:2, ' ', Ca: 14:2, ' ', Cm: 14:2);
        writeln(time: 12:2, Cn: 14:2, Ca: 14:2, Cm: 14:2);
        end;
    end;

{Final results output}

writeln(results, time: 12:2, ' ', Cn: 14:2, ' ', Ca: 14:2, ' ', Cm: 14:2);
writeln(time: 12:2, Cn: 14:2, Ca: 14:2, Cm: 14:2);
writeln(results); flush(results); close(results);
writeln; writeln;
write('Hit enter to stop program');
readln;
End.
APPENDIX A2

MATHEMATICAL VALIDATION FOR PASCAL PROGRAM
(APPENDIX A1)

A2.1 CHANGING THE TIME STEP

The time step was adjusted from 5-0.001 s in order to determine the error caused by
the time step in the numerical integration procedure.

The following parameters were entered into the program:

\[ t = 1800 \text{ time, (s)} \]
\[ \text{Con} = 178.6 \text{ Concentration of native } \beta\text{-lg at } t = 0 \text{ (mol} \times 10^6 \text{ kg}^{-1}) \]
\[ \text{Coo} = 0 \text{ Concentration of aggregated } \beta\text{-lg at } t = 0 \text{ (mol} \times 10^6 \text{ kg}^{-1}) \]
\[ \text{Com} = 0 \text{ Concentration of } \beta\text{-lg associated with the micelle at } t = 0 \text{ (mol} \times 10^6 \text{ kg}^{-1}) \]
\[ k1 = 0.0160 \text{ Denaturation rate constant (mol} \times 10^6 \text{ kg}^{-1})^{(1-n)} \text{s}^{-1} \]
\[ k2 = 0.00039 \text{ Association rate constant (mol} \times 10^6 \text{ kg}^{-1})^{(1-n)} \text{s}^{-1} \]
\[ n = 1.4 \text{ Order of denaturation} \]
\[ \text{Ck} = 95.6 \text{ Maximum association of } \beta\text{-lg with the micelles (mol} \times 10^6 \text{ kg}^{-1}) \]

The effect of time step was calculated by comparing the sum of \( Cn, Ca \) and \( Cm \) at
0 s and at 1800 s (Table A2.1).
Table A2.1 Effect of time step on the numerical integration error

<table>
<thead>
<tr>
<th>Time step (s)</th>
<th>Concentration of β-lg at 1800s (mol × 10^6 kg⁻¹)</th>
<th>error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cn</td>
<td>Ca</td>
</tr>
<tr>
<td>5</td>
<td>1.95</td>
<td>121.98</td>
</tr>
<tr>
<td>1</td>
<td>1.95</td>
<td>91.42</td>
</tr>
<tr>
<td>0.1</td>
<td>1.95</td>
<td>84.76</td>
</tr>
<tr>
<td>0.01</td>
<td>1.95</td>
<td>84.10</td>
</tr>
<tr>
<td>0.001</td>
<td>1.95</td>
<td>84.04</td>
</tr>
</tbody>
</table>

A2.2 SIMPLIFYING THE MODEL TO CHECK ANALYTICAL SOLUTIONS AGAINST NUMERICAL PREDICTIONS

A2.2.1 Analytical solution for \(k_2 = 0\)

From Equation 4.37

\[
\frac{dC_a}{dt} = k_1 C^n_a - k_2 C_a \left( C_k - C_m \right) \quad \text{(A2.21)}
\]

If \(k_2 = 0\) Equation A2.21 simplifies to Equation A2.22.

\[
\frac{dC_a}{dt} = k_1 C^n_a \quad \text{(A2.22)}
\]
An equation for \( C_n \) (Equation A2.23) can be written by rearranging Equation 4.6 to make \( C_n \) (\( C_1 \) in Equation 4.6) the subject of the equation, and substituting \( C_{0n} \) for \( C_0 \).

\[
C_n = C_{0n} \left[ 1 + (n - 1) k_1 C_{0n}^{(n-1)} t \right]^{\frac{1}{1-n}} \tag{A2.23}
\]

Substituting Equation A2.23 into A2.22 gives Equation A2.24.

\[
\frac{d C_a}{d t} = k_1 C_{0n}^n \left[ 1 + (n - 1) k_1 C_{0n}^{(n-1)} t \right]^{\frac{n}{1-n}} \tag{A2.24}
\]

Integrating between the limits \( t=0 \) to \( t=t \) and putting \( n=1.4 \) gives Equation A2.25.

\[
\int_{C_{0a}}^{C_a} dC_a = k_1 C_{0n}^{1.4} \int_0^t \left[ 1 + 0.4 k_1 C_{0n}^{0.4} t \right]^{-3.5} \tag{A2.25}
\]

The right hand side of Equation A2.25 was integrated by substitution.

\[
u = 1 + 0.4 k_1 C_{0n}^{0.4} t \tag{A2.26}
\]

\[
du = 0.4 k_1 C_{0n}^{0.4} dt \tag{A2.27}
\]
The same conditions were run as in A2.1, except \( k_2 \) was set to zero, the total run time was 100 s and the time step was 0.001 s.

**Table A2.21 Comparison of numerical and analytical results for simplified model \((k_2 = 0)\)**

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Ca, analytical solution</th>
<th>Ca, numerical solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>114.78</td>
<td>114.79</td>
</tr>
<tr>
<td>20</td>
<td>147.75</td>
<td>147.76</td>
</tr>
<tr>
<td>30</td>
<td>161.02</td>
<td>161.03</td>
</tr>
<tr>
<td>40</td>
<td>167.49</td>
<td>167.50</td>
</tr>
<tr>
<td>50</td>
<td>171.06</td>
<td>171.07</td>
</tr>
<tr>
<td>60</td>
<td>173.21</td>
<td>173.22</td>
</tr>
<tr>
<td>70</td>
<td>174.59</td>
<td>174.60</td>
</tr>
<tr>
<td>80</td>
<td>175.52</td>
<td>175.53</td>
</tr>
<tr>
<td>90</td>
<td>176.18</td>
<td>176.19</td>
</tr>
<tr>
<td>100</td>
<td>176.65</td>
<td>176.66</td>
</tr>
</tbody>
</table>
A2.2.2 Analytical solution for $C_m = 0$

By setting $C_{on} = 0$ and $C_m$ always equal to zero, Equation 4.16 simplifies to Equation A2.32.

$$\frac{dA}{dt} = -k_2 C_a C_k$$  \hfill (A2.32)

Integrating between the limits $t = 0$ to $t = t$ gives Equation A2.33.

$$C_a = C_{0a} e^{-k_2 C_k t}$$  \hfill (A2.33)

The following parameters were entered into the program:

- $t = 100$ time, (s)
- $C_{on} = 0$ Concentration of native $\beta$-lg at $t = 0$ (mol $\times 10^6$ kg$^{-1}$)
- $C_{oa} = 178.6$ Concentration of aggregated $\beta$-lg at $t = 0$ (mol $\times 10^6$ kg$^{-1}$)
- $C_{om} = 0$ Concentration of $\beta$-lg associated with the micelle at $t=0$ (mol $\times 10^6$ kg$^{-1}$)
- $k_1 = 0.0160$ Denaturation rate constant (mol $\times 10^6$ kg$^{-1}$)$^{(1-a)}$ s$^{-1}$
- $k_2 = 0.00039$ Association rate constant (mol $\times 10^6$ kg$^{-1}$)$^{(1-a)}$ s$^{-1}$
- $n = 1.4$ Order of denaturation
- $C_k = 178.6$ Maximum association of $\beta$-lg with the micelles (mol $\times 10^6$ kg$^{-1}$)
Table A2.22 Comparison of analytical and numerical results for simplified model (Cm = 0, Con = 0)

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Ca, analytical solution</th>
<th>Ca, numerical solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>123.02</td>
<td>123.02</td>
</tr>
<tr>
<td>20</td>
<td>84.73</td>
<td>84.73</td>
</tr>
<tr>
<td>30</td>
<td>58.36</td>
<td>58.36</td>
</tr>
<tr>
<td>40</td>
<td>40.20</td>
<td>40.20</td>
</tr>
<tr>
<td>50</td>
<td>27.69</td>
<td>27.69</td>
</tr>
<tr>
<td>60</td>
<td>19.07</td>
<td>19.07</td>
</tr>
<tr>
<td>70</td>
<td>13.13</td>
<td>13.13</td>
</tr>
<tr>
<td>80</td>
<td>9.05</td>
<td>9.05</td>
</tr>
<tr>
<td>90</td>
<td>6.23</td>
<td>6.23</td>
</tr>
<tr>
<td>100</td>
<td>4.29</td>
<td>4.29</td>
</tr>
</tbody>
</table>
APPENDIX B1

RATE CONSTANTS AND ORDERS FOR β-LG A, β-LG B AND α-LA
DENATURATION IN WHEY-PROTEIN ADJUSTED SKIM MILKS.

Degrees of freedom at 80, 90 and 120°C were 7, 6 and 7, respectively.

EARLY SEASON

Table B1.1 Rate constants for β-lg A denaturation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>$k_n \pm 95%$ C.I. ((g \text{ kg}^{-1})^{(\text{a})} \text{s}^{-1} \times 10^3)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 depleted</td>
<td></td>
<td>148 ± 15</td>
<td>0.988</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>75 ± 14</td>
<td>0.954</td>
</tr>
<tr>
<td>enriched</td>
<td></td>
<td>72 ± 16</td>
<td>0.925</td>
</tr>
<tr>
<td>90 depleted</td>
<td></td>
<td>18.2 ± 3.9</td>
<td>0.906</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>9.8 ± 1.6</td>
<td>0.990</td>
</tr>
<tr>
<td>enriched</td>
<td></td>
<td>8.1 ± 2.3</td>
<td>0.860</td>
</tr>
<tr>
<td>80 depleted</td>
<td></td>
<td>2.20 ± 4.20</td>
<td>0.966</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>2.10 ± 4.20</td>
<td>0.966</td>
</tr>
<tr>
<td>enriched</td>
<td></td>
<td>1.59 ± 0.48</td>
<td>0.934</td>
</tr>
</tbody>
</table>

*Reaction orders 1.5, 1.1 and 1.3 for WPD, control and WPE skim milks, respectively.*
Table B1.2 Rate constants for β-lg B denaturation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>( k_n \pm 95% ) C.I. ( (g \text{ kg}^{-1})^{0.91} \text{s}^{-1} \times 10^4 )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>depleted</td>
<td>193 ± 20</td>
<td>0.985</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>100 ± 15</td>
<td>0.968</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>70 ± 10</td>
<td>0.971</td>
</tr>
<tr>
<td>90</td>
<td>depleted</td>
<td>37.0 ± 5.7</td>
<td>0.963</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>17.6 ± 3.0</td>
<td>0.955</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>11.0 ± 3.6</td>
<td>0.798</td>
</tr>
<tr>
<td>80</td>
<td>depleted</td>
<td>4.21 ± 0.47</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>2.97 ± 0.60</td>
<td>0.956</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>1.73 ± 0.59</td>
<td>0.910</td>
</tr>
</tbody>
</table>

*Reaction orders 1.6, 1.4 and 1.7, for WPD, control and WPE skim milks, respectively.

Table B1.3 Rate constants for β-lg (β-lg A + β-lg B) denaturation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>( k_n \pm 95% ) C.I. ( (g \text{ kg}^{-1})^{0.91} \text{s}^{-1} \times 10^4 )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>depleted</td>
<td>114 ± 10</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>70 ± 11</td>
<td>0.967</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>44 ± 7</td>
<td>0.967</td>
</tr>
<tr>
<td>90</td>
<td>depleted</td>
<td>17.0 ± 3.8</td>
<td>0.940</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>10.3 ± 1.5</td>
<td>0.966</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>5.7 ± 1.7</td>
<td>0.828</td>
</tr>
<tr>
<td>80</td>
<td>depleted</td>
<td>2.02 ± 0.30</td>
<td>0.977</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>1.99 ± 0.42</td>
<td>0.961</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>0.99 ± 0.34</td>
<td>0.916</td>
</tr>
</tbody>
</table>

*Reaction orders 1.6, 1.3 and 1.6, for WPD, control and WPE skim milks, respectively.
Table B1.4 Rate constants for α-la denaturation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>$k_n^* \pm 95%$ C.I.</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$(g \text{ kg}^{-1})^{(1-n)} \text{ s}^{-1} \times 10^3$</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>depleted</td>
<td>14.3 ± 2.0</td>
<td>0.971</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>12.9 ± 1.8</td>
<td>0.969</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>16.8 ± 2.7</td>
<td>0.956</td>
</tr>
<tr>
<td>80</td>
<td>depleted</td>
<td>0.90 ± 0.10</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>1.10 ± 0.54</td>
<td>0.874</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>1.43 ± 0.20</td>
<td>0.911</td>
</tr>
</tbody>
</table>

*Reaction orders 1.5, 1.1 and 1.1, WPD, control and WPE skim milks, respectively.

MID SEASON

Table B1.5 Rate constants for β-lg A denaturation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>$k_n^* \pm 95%$ C.I.</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$(g \text{ kg}^{-1})^{(1-n)} \text{ s}^{-1} \times 10^3$</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>depleted</td>
<td>144 ± 51</td>
<td>0.954</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>86 ± 10</td>
<td>0.987</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>78 ± 10</td>
<td>0.980</td>
</tr>
<tr>
<td>90</td>
<td>depleted</td>
<td>26.8 ± 15.9</td>
<td>0.725</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>9.6 ± 1.4</td>
<td>0.967</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>11.0 ± 2.8</td>
<td>0.923</td>
</tr>
<tr>
<td>80</td>
<td>depleted</td>
<td>2.57 ± 0.19</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>1.68 ± 0.51</td>
<td>0.921</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>1.63 ± 0.52</td>
<td>0.922</td>
</tr>
</tbody>
</table>

*Reaction orders 1.5, 1.4 and 1.3, for WPD, control and WPE skim milks, respectively.
Table B1.6 Rate constants for β-lg B denaturation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>$k_n \pm 95%$ C.I. (\text{g kg}^{-1} (1-n) \text{s}^{-1} \times 10^3)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 depleted</td>
<td>208 ± 49</td>
<td>0.946</td>
<td></td>
</tr>
<tr>
<td>120 control</td>
<td>92 ± 12</td>
<td>0.983</td>
<td></td>
</tr>
<tr>
<td>120 enriched</td>
<td>79 ± 7</td>
<td>0.980</td>
<td></td>
</tr>
<tr>
<td>90 depleted</td>
<td>49.2 ± 18.1</td>
<td>0.840</td>
<td></td>
</tr>
<tr>
<td>90 control</td>
<td>15.5 ± 2.9</td>
<td>0.957</td>
<td></td>
</tr>
<tr>
<td>90 enriched</td>
<td>15.1 ± 6.6</td>
<td>0.989</td>
<td></td>
</tr>
<tr>
<td>80 depleted</td>
<td>4.87 ± 0.31</td>
<td>0.994</td>
<td></td>
</tr>
<tr>
<td>80 control</td>
<td>2.04 ± 0.40</td>
<td>0.962</td>
<td></td>
</tr>
<tr>
<td>80 enriched</td>
<td>1.83 ± 0.49</td>
<td>0.932</td>
<td></td>
</tr>
</tbody>
</table>

*Reaction orders 1.7, 1.5 and 1.4, for WPD, control and WPE skim milks, respectively.

Table B1.7 Rate constants for β-lg (β-lg A + β-lg B) denaturation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>$k_n \pm 95%$ C.I. (\text{g kg}^{-1} (1-n) \text{s}^{-1} \times 10^3)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 depleted</td>
<td>113 ± 29</td>
<td>0.944</td>
<td></td>
</tr>
<tr>
<td>120 control</td>
<td>67 ± 9</td>
<td>0.982</td>
<td></td>
</tr>
<tr>
<td>120 enriched</td>
<td>66 ± 9</td>
<td>0.975</td>
<td></td>
</tr>
<tr>
<td>90 depleted</td>
<td>22.1 ± 9.8</td>
<td>0.764</td>
<td></td>
</tr>
<tr>
<td>90 control</td>
<td>9.4 ± 1.6</td>
<td>0.958</td>
<td></td>
</tr>
<tr>
<td>90 enriched</td>
<td>10.8 ± 1.2</td>
<td>0.980</td>
<td></td>
</tr>
<tr>
<td>80 depleted</td>
<td>2.28 ± 0.13</td>
<td>0.996</td>
<td></td>
</tr>
<tr>
<td>80 control</td>
<td>1.42 ± 0.32</td>
<td>0.951</td>
<td></td>
</tr>
<tr>
<td>80 enriched</td>
<td>1.08 ± 0.12</td>
<td>0.980</td>
<td></td>
</tr>
</tbody>
</table>

*Reaction orders 1.7, 1.4 and 1.3, for WPD, control and WPE skim milks, respectively.
Table B1.8 Rate constants for α-la denaturation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Whey protein level</th>
<th>$k_s^* \pm 95%$ C.I.</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(g kg$^{-1}$ h$^{-1}$ s$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\times 10^3$</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>control</td>
<td>9.8 ± 3.0</td>
<td>0.881</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>19.7 ± 4.5</td>
<td>0.906</td>
</tr>
<tr>
<td></td>
<td>depleted</td>
<td>1.14 ± 0.39</td>
<td>0.904</td>
</tr>
<tr>
<td>80</td>
<td>control</td>
<td>0.70 ± 0.23</td>
<td>0.887</td>
</tr>
<tr>
<td></td>
<td>enriched</td>
<td>0.23 ± 0.71</td>
<td>0.914</td>
</tr>
</tbody>
</table>

*Reaction orders 0.7, 0.9 and 0.9, for WPD, control and WPE skim milks, respectively.*
APPENDIX C1

RATE CONSTANTS AND ORDERS FOR β-LG DISULPHIDE-LINKED AGGREGATION IN WHEY-PROTEIN ADJUSTED SKIM MILKS.

EARLY SEASON

Table C1.1 Rate constants for β-lg disulphide-linked aggregation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature°C</th>
<th>Whey protein level</th>
<th>$k_n^{b} \pm 95%$ C.I.</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(g kg$^{-1}$)(1-$n$) s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>× 10$^3$</td>
<td></td>
</tr>
<tr>
<td>120 depleted</td>
<td>$40.6 \pm 9.3$</td>
<td>0.957</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>$23.4 \pm 3.4$</td>
<td>0.977</td>
<td></td>
</tr>
<tr>
<td>enriched</td>
<td>$16.5 \pm 4.8$</td>
<td>0.857</td>
<td></td>
</tr>
<tr>
<td>90 depleted</td>
<td>$5.57 \pm 1.91$</td>
<td>0.876</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>$3.17 \pm 1.36$</td>
<td>0.821</td>
<td></td>
</tr>
<tr>
<td>enriched</td>
<td>$4.33 \pm 1.99$</td>
<td>0.855</td>
<td></td>
</tr>
<tr>
<td>80 depleted</td>
<td>$1.20 \pm 0.24$</td>
<td>0.971</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>$0.92 \pm 0.49$</td>
<td>0.743</td>
<td></td>
</tr>
<tr>
<td>enriched</td>
<td>$0.82 \pm 0.08$</td>
<td>0.989</td>
<td></td>
</tr>
</tbody>
</table>

*aDegrees of freedom at 80, 90 and 120°C were 7, 6 and 7, respectively.

*bReaction orders 2.1, 2.0 and 1.8, for WPD, control and WPE skim milks, respectively.
Table C1.2 Rate constants for β-lg disulphide-linked aggregation in whey-protein adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature&lt;sup&gt;a&lt;/sup&gt; (°C)</th>
<th>Whey protein level</th>
<th>$k_n$&lt;sup&gt;b&lt;/sup&gt; ± 95% C.I.</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(g kg&lt;sup&gt;-1&lt;/sup&gt;&lt;sup&gt;(t-n)&lt;/sup&gt;s&lt;sup&gt;-1&lt;/sup&gt; $\times 10^3$)</td>
<td></td>
</tr>
<tr>
<td>120 depleted</td>
<td></td>
<td>58.9 ± 21.4</td>
<td>0.863</td>
</tr>
<tr>
<td>120 control</td>
<td></td>
<td>32.4 ± 9</td>
<td>0.951</td>
</tr>
<tr>
<td>120 enriched</td>
<td></td>
<td>24.2 ± 4</td>
<td>0.976</td>
</tr>
<tr>
<td>90  depleted</td>
<td></td>
<td>8.8 ± 5.1</td>
<td>0.849</td>
</tr>
<tr>
<td>90  control</td>
<td></td>
<td>4.7 ± 0.7</td>
<td>0.962</td>
</tr>
<tr>
<td>90  enriched</td>
<td></td>
<td>4.3 ± 0.6</td>
<td>0.968</td>
</tr>
<tr>
<td>80  depleted</td>
<td></td>
<td>1.40 ± 0.41</td>
<td>0.932</td>
</tr>
<tr>
<td>80  control</td>
<td></td>
<td>0.75 ± 0.15</td>
<td>0.984</td>
</tr>
<tr>
<td>80  enriched</td>
<td></td>
<td>0.75 ± 0.17</td>
<td>0.957</td>
</tr>
</tbody>
</table>

<sup>a</sup>Degrees of freedom at 80, 90 and 120°C were 7, 6 and 7, respectively.

<sup>b</sup>Reaction orders 2.4, 1.7 and 1.7, for WPD, control and WPE skim milks, respectively.
APPENDIX D1

RATE CONSTANTS AND ORDERS FOR β-LG A, β-LG B AND α-LA DENATURATION IN TOTAL SOLIDS ADJUSTED SKIM MILKS.

Degrees of freedom at 85, 90 and 110°C were 7, 6 and 7, respectively.

Table D1.1 Rate constants for β-lg A denaturation in total solids adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Milk batch</th>
<th>$k_n \pm 95%$ C.I.</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(g kg$^{-1}$)(l$^{-n}$) s$^{-1}$ $\times 10^3$</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>diluted</td>
<td>48.5 ± 11.5</td>
<td>0.848</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>45.2 ± 3.4</td>
<td>0.989</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>57.3 ± 4.5</td>
<td>0.989</td>
</tr>
<tr>
<td>85</td>
<td>diluted</td>
<td>6.0 ± 0.2</td>
<td>0.860</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>4.8 ± 0.5</td>
<td>0.981</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>5.7 ± 0.8</td>
<td>0.962</td>
</tr>
</tbody>
</table>

*Reaction orders 1.2, 1.3 and 1.3, for diluted, control and evaporated skim milks, respectively.
Table D1.2 Rate constants for β-lg B denaturation in total solids adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Milk batch</th>
<th>$k_n^* \pm 95%$ C.I.</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(g kg$^{-1}$)(l-n) s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\times 10^3$</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>diluted</td>
<td>70.3 ± 16.4</td>
<td>0.832</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>59.7 ± 7.3</td>
<td>0.968</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>66.3 ± 6.3</td>
<td>0.988</td>
</tr>
<tr>
<td>85</td>
<td>diluted</td>
<td>11.0 ± 3.6</td>
<td>0.882</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>7.9 ± 1.1</td>
<td>0.972</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>8.5 ± 1.6</td>
<td>0.901</td>
</tr>
</tbody>
</table>

*Reaction orders 1.5, 1.6 and 1.7, for diluted, control and evaporated skim milks, respectively.

Table D1.3 Rate constants for β-lg (β-lg A + β-lg B) denaturation in total solids adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Milk batch</th>
<th>$k_n^* \pm 95%$ C.I.</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(g kg$^{-1}$)(l-n) s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\times 10^3$</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>diluted</td>
<td>45.0 ± 10.6</td>
<td>0.835</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>38.2 ± 4.0</td>
<td>0.978</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>56.2 ± 6.4</td>
<td>0.977</td>
</tr>
<tr>
<td>90</td>
<td>diluted</td>
<td>16.8 ± 1.4</td>
<td>0.984</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>16.2 ± 4.3</td>
<td>0.934</td>
</tr>
<tr>
<td>85</td>
<td>diluted</td>
<td>6.10 ± 1.79</td>
<td>0.901</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>4.65 ± 0.60</td>
<td>0.970</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>6.20 ± 0.78</td>
<td>0.951</td>
</tr>
</tbody>
</table>

*Reaction orders 1.4, 1.2 and 1.3, for diluted, control and evaporated skim milks, respectively.
Table D1.4 Rate constants for α-la denaturation in total solids adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Milk batch</th>
<th>$k_n \pm 95% \text{ C.I.}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$(\text{g kg}^{-1})(1-n) \text{ s}^{-1} \times 10^3$</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>diluted</td>
<td>$6.64 \pm 0.52$</td>
<td>0.984</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>$5.83 \pm 0.52$</td>
<td>0.981</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>$9.14 \pm 1.41$</td>
<td>0.940</td>
</tr>
<tr>
<td>90</td>
<td>diluted</td>
<td>$4.56 \pm 1.68$</td>
<td>0.725</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>$2.09 \pm 0.77$</td>
<td>0.784</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>$3.30 \pm 0.66$</td>
<td>0.924</td>
</tr>
<tr>
<td>85</td>
<td>diluted</td>
<td>$1.72 \pm 0.98$</td>
<td>0.821</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>$1.23 \pm 0.15$</td>
<td>0.982</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>$2.08 \pm 0.34$</td>
<td>0.961</td>
</tr>
</tbody>
</table>

*Reaction orders 1.2, 0.7 and 0.7, for diluted, control and evaporated skim milks, respectively.
APPENDIX E1

RATE CONSTANTS AND ORDERS FOR β-LG DISULPHIDE LINKED AGGREGATION IN TOTAL SOLIDS ADJUSTED SKIM MILKS.

Table E1.1 Rate constants for β-lg disulphide-linked aggregation in total solids adjusted skim milk.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Milk batch</th>
<th>$k_n^b \pm 95%$ C.I. ($g$ kg$^{-1}$)(t-n) s$^{-1}$ $\times 10^3$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>diluted</td>
<td>25.4 ± 3.8</td>
<td>0.962</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>36.4 ± 4.3</td>
<td>0.969</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>42.8 ± 3.2</td>
<td>0.989</td>
</tr>
<tr>
<td>90</td>
<td>diluted</td>
<td>9.59 ± 1.06</td>
<td>0.966</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>8.41 ± 1.77</td>
<td>0.890</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>7.16 ± 0.93</td>
<td>0.944</td>
</tr>
<tr>
<td>85</td>
<td>diluted</td>
<td>3.97 ± 0.82</td>
<td>0.949</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>3.17 ± 0.51</td>
<td>0.965</td>
</tr>
<tr>
<td></td>
<td>evaporated</td>
<td>3.41 ± 0.38</td>
<td>0.982</td>
</tr>
</tbody>
</table>

°Degrees of freedom at 85, 90 and 110°C were 7, 6 and 7, respectively.

°°Reaction orders 1.3, 1.5 and 1.2, for diluted, control and evaporated skim milks, respectively.