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RHEOLOGY AND MICROSTRUCTURE OF ACID MILK GELS : The Role of Fat Globule Membrane

A THESIS
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ABSTRACT

The objectives of this study were to investigate the effects of different compositions of fat globule membranes as well as heat treatment of reconstituted skim milk, on the properties of acid milk gels. Rheological and microstructural properties of acid milk gels were determined.

Recombined milks were made by mixing reconstituted skim milk with homogenised (20.7 and 3.5MPa, first and second stage pressures, respectively) emulsions which were stabilised by low-heat, medium-heat or high-heat skim milk powder, sodium caseinate, whey protein concentrate, heated (80 °C for 30 min) whey protein concentrate, Tween 60 or the native fat globule membrane of whole milk. To study the effect of milk heat treatment, unheated reconstituted skim milk or milk heated at 80 °C for 30 min was used for making recombined milk. Gels were formed by acidification of recombined milk with glucono-δ-lactone by incubating at 30 °C for 16 h.

Each type of emulsion was characterised by determining the size distribution of fat globules, protein load and composition of fat globule membrane. The average fat globule size in the emulsion systems varied from 0.66 to 0.48 μm. Emulsions made with low-heat skim milk powder had the highest protein load (7.05 mg/m^2), because of the adsorption of large particles (casein micelles) on to fat globules. In contrast, emulsion systems made with whey protein concentrate had the lowest protein load (1.13 mg/m^2). The membrane of emulsions stabilised by skim milk powder solutions contained both caseins and whey proteins while whey protein concentrate stabilised emulsions had both β–lactoglobulin than α-lactalbumin

adsorbed onto the fat globule surface. The membrane of Na caseinate stabilised emulsions contained all caseins at the surface.

The rheological properties of recombined milk during acidification were determined by low amplitude oscillation using a Bohlin Rheometer and a penetration test using the Instron. The storage modulus (G') of acid gels made from recombined milk that was made from heated skim milk were in the range ~ 180 to 530 Pa, whereas acid gels made from recombined milk made from unheated skim milk systems produced gels with G' values in the range ~ 20 to 90 Pa. The G' of acid gels made from recombined milk containing fat globules stabilised by different materials was in the order: heated whey protein concentrate > sodium caseinate > skim milk powders > Tween 60, unheated whey protein concentrate or natural membrane material (fresh cream). The results of the penetration test were variable and did not fully agree with the trends from the oscillation tests.

For all recombined milk systems both the pH of gelation and the gelation time were influenced by the heat treatment of reconstituted skim milk, i.e. heating increased the pH of gelation and decreased the gelation time.

The microstructure of the acid gel network was determined by confocal scanning laser microscopy. Acid gels made from unheated reconstituted skim milk appeared to be formed from irregular clusters and strands, interspersed with fat globules whereas more crowded structure was observed from unheated systems. Recombined fat globules appeared to be embedded in the matrix. Differences in microstructures between gels containing different types of fat globule membranes were not very clear. Acid gels made from Tween 60 and whole milk were different from the other fat systems; the fat globules in the Tween 60 stabilised

milk appeared to be very small while in contrast those in whole milk were much larger probably because whole milk was not homogenised.

Possible mechanisms have been proposed which explain the effects of heat treatment on gel properties and the role of fat globule membrane material in gel structure and stiffness.

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CHAPTER 1.
INTRODUCTION

Fermented milk products are very popular food products and are consumed throughout the world. There has been increased consumption of fermented milks, especially yoghurt, due to various health claims and benefits associated with some of the bacterial cultures. The increased interest in fermented milk has led to more research into the improvement of product quality. Additional information is needed on the physico-chemical and rheological changes that milk proteins undergo during acidification and gel formation. These changes are important in determining the structural, textural and sensory quality of fermented milk products. The ability of milk to form acid-induced gels has been widely studied to improve product texture and functionality, to develop new products and to examine new and existing manufacturing processes (Humphrey and Plunkett, 1969; Robinson and Tamime, 1975; Tamime and Deeth, 1980; Mulvihill and Grufferty, 1995).

Acid milk gels can be formed by slowly acidifying the milk to pH values close to the isoelectric point of caseins (pH ~ 4.6). During acidification, casein micelles aggregate due to the reduction in charge repulsion and form a continuous network of chains, strands or clusters. The exact mechanisms involved in the formation of acid-induced milk gels are not fully understand but have been extensively studied (Harwalkar and Kalab, 1973, 1980; Heertje et al., 1985; Roefs et al., 1990; Roefs and van Vliet, 1990; Lucey et al., 1997a). Several workers have reported on the role of fat globules and their membranes in the formation and structure of acid

milk gels and suggested some possible mechanisms of fat globule-casein particle interactions in acid gels (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988; Xiong and Kinsella, 1991a, b; Aguilera et al., 1993). However, the mechanisms by which different types of fat globule membranes affect the fundamental rheological properties of acid gels are poorly understood.

Depending on the nature of fat globule membrane and the gel matrix two extreme cases can be distinguished: (a) no interaction occurs between the gel matrix and the emulsified fat globules, and (b) a strong interaction between both. For acid-induced milk gels cross-links between fat globules and the gel matrix may be expected if casein is present on the fat globule surface. However, there is little information available on the effects of fat globules stabilised by other materials (e.g. whey proteins, different classifications of skim milk powders and non-protein materials) on the rheological properties of acid milk gels (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988; Xiong and Kinsella, 1991b).

Heat treatment of milk is one of the most important processing parameters affecting texture and the formation of acid gels (Robinson and Tamime, 1986; Mulvihill and Grufferty, 1995). It is generally considered that unheated milk forms weak gels that have strong tendency to synerese (Kalab et al., 1976). Although there have been many studies on the effects of heat treatment on the properties of acid gels (Parnell-Clunies et al., 1987; Dannenberg and Kessler, 1988b; van Vliet and Keetels, 1995; Lucey et al., 1997a), little is known about the effects of heat treatment on the properties of acid milk gels containing fat and the role of different types of fat globule membranes in the formation of acid-induced gels (i.e. the effects of heat treatment of reconstituted skim milk on the gels made from recombined milk).

OBJECTIVES

1. To investigate the effects of heat treatment of reconstituted skim milk on the rheological properties, at large and small deformations, of acid milk gels made from recombined milk containing different types of fat globule membrane materials.

2. To investigate the influence of different types of fat globule membranes on the rheological properties.

3. To investigate the influence of different types of fat globule membranes combined with heat treatment on the microstructure of acid milk gels.

CHAPTER 2.
LITERATURE REVIEW

2.1 Properties of milk proteins

2.1.1 Caseins

The proteins of milk are a heterogeneous mixture and include two main groups, casein and whey (or serum) proteins. Other protein fractions are also present but at very low levels, including bovine serum albumin, immunoglobulins, lactoferrin and proteose-peptones (Fox and Mulvihill, 1982). Caseins represent the major protein group in milk (~ 80% of total) and have been defined as phospho proteins precipitated from raw milk by acidification to pH 4.6 at 20 °C. The residual proteins in the serum or whey after the removal of caseins are referred to as whey proteins.

The casein composition of milk varies with season, stage of lactation, from cow to cow and even with time of day. However, the overall casein composition of bulk milk, produced by a large number of cows, is fairly constant (Walstra and Jenness, 1984).

Four main groups of caseins can be distinguished α_{s1}- (mol wt. 23,600 daltons), α_{s2}- (mol wt. 25,200 daltons), β- (mol wt. 24,000 daltons) and κ-caseins (mol wt. 19,000 daltons) and these occur in casein micelles in the approximate proportions 4:1:4:1 (Davies and Law, 1980). Each of the caseins exhibits genetic polymorphism and none of them has a highly organised secondary structure. Their conformation appears to be somewhat like that of denatured globular proteins, which is partly due to the rather high proline content (~ 9 wt %), an amino acid

that normally inhibits the formation of ordered helical structures. The complete amino acid sequence has been determined for the primary structure of all the major caseins. From the amino acid sequence their average hydrophobicity can be calculated. Caseins contain many hydrophobic amino acid residues; by the method of Bigelow (1967), the hydrophobicities of the α_{s1}-, β- and κ-caseins are 4.9, 5.6 and 5.4kJ/mol, respectively, which gives them all a strong tendency to self-associate, under appropriate conditions. A minor component, γ-casein, which usually makes up about 3 wt% of casein, has a primary structure which corresponds to a fragment of β-casein resulting from proteolytic break-down (Walstra and Jenness, 1984).

Casein represents 75-88% of the total protein in cow's milk. Casein is present in milk as macromolecular aggregates called "casein micelles" ranging in size from 20 to 300 nm in diameter, and each micelle is comprised of some 20 - 150,000 casein molecules. In addition, micelles also contain small ions such as calcium, phosphate, magnesium and citrate (Walstra and Jenness, 1984).

Micelles are usually considered to be composed of discrete submicelles (10-15 nm), which give it a porous structure (Walstra and Jenness, 1984). Submicelles are formed due to interactions among the hydrophobic domains of the individual caseins, and these submicelles probably have a variable composition with respect to the individual caseins. The nature of insoluble calcium phosphate in micelles is a matter of controversy, although many investigators have suggested that it exists as amorphous tertiary calcium phosphate interspersed throughout the micelle. The presence of other metal ions, particularly Mg^{2+}, and of the caseins, is believed to prevent transformation of amorphous calcium phosphate in milk to more stable forms (Swaisgood, 1992).

Casein micelles are remarkably stable. They survive high shear-rates (homogenisation) and high temperatures; and they are easily redispersed after drying to produce milk powders. At least part of this stability is electrostatic since (a) they can be aggregated at the isoelectric point and (b) the rate of aggregation at neutral pH is sensitive to the calcium-ion concentration (Dickinson and Stainsby, 1988).

Numerous models for the structure of casein micelle have been proposed; two models appeared to satisfy most of the requirements. Both the models of Slattery (1973) and Schmidt (1980) view the submicelle as a protein complex of variable composition, held together predominantly by interactions between the hydrophobic domains of the individual caseins. These models also suggest a non-uniform distribution of κ-casein in the submicelle. This non-uniform distribution could result from specific intermolecular interaction between β-sheet structures in the hydrophobic domains of κ-casein. The major difference between the two models is the relative importance of hydrophobic interactions and tertiary calcium phosphate in binding the submicelles together. In the model of Schmidt (sub-unit model), it is suggested that casein micelles consist of a larger number (10^2 -10^4) of 'sub-micelles' (diameter 10-20 nm) held together ("cemented") by colloidal calcium phosphate (CCP) in an open and disordered structure. According to the model of Slattery (network model), the sub-micelles contain 25-30 molecules (~ 6 X 10^5 daltons) of α_{s1}-, α_{s2}-, β- and κ-caseins held together by hydrophobic interactions (although it was suggested that some ionic bonding probably occurs between α_{s1}- and κ-caseins). Submicelles would resemble conventional (soap) micelles in so far as hydrophobic groups concentrate at the center and polar groups at the surface. In the "coat-core" representation of the model of Slattery, a 'coat' of κ-casein envelopes a 'core' of α_{s1}-, α_{s2}- and β-caseins. It is not clear what stabilises the discrete submicelle structure.

A modified model (from the model of Schmidt) of the structure of casein micelle is shown in Fig. 2.1 (Walstra and Jenness, 1984). In this model most of the κ-casein is situated on the outside of the micelle, and the protruding chains of its C-terminal end give the micelle a "hairy" surface. These hairs are flexible and exhibit Brownian motion. The effective thickness of the hairy layer is ~ 5 nm. Some κ-casein also occurs in the interior of the micelle.

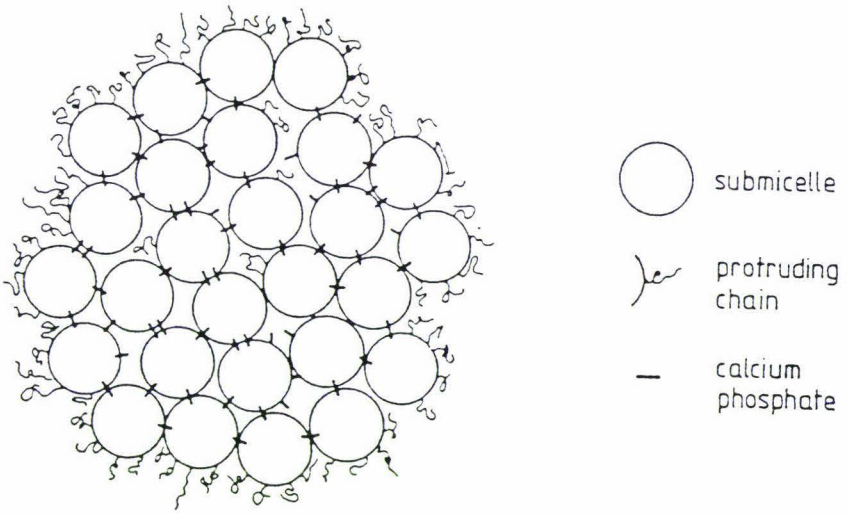

Fig. 2.1. Model of casein micelle; highly schematic (from Walstra and Jenness, 1984).

Visser et al. (1986) suggested that the formation of casein micelles involves a "skeleton" of α_{s1}-casein with β-casein "loosely" bound with amorphous calcium phosphate, surrounded by a stabilising coat of κ-casein. The skeleton is retained even after the removal of a considerable amount of amorphous calcium phosphate via calcium complexion (e.g. EDTA) or by acidification.

At the natural pH of milk (~ 6.7), casein micelles, although they are very numerous and in constant Brownian motion, never appear to make lasting contact. Stability is due mainly to electrostatic and steric repulsion (Walstra and Jenness, 1984; Walstra, 1990).

2.1.2 Whey proteins

Whey (or serum) proteins represent 15-20 % of the total protein in milk. Whey proteins are globular proteins and are soluble at pH 4.6. The major whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins. The whey proteins are more readily denatured than caseins and in many cases optimum functionality in an application is achieved when they are in a denatured state.

β-Lactoglobulin is the most abundant of the whey proteins and has a molecular weight of 36,000. It is rich in lysine, leucine, glutamic acid and aspartic acid. The functionality of β-lactoglobulin is influenced greatly by the presence of both a sulfhydryl group and disulfide bonds. The relative importance of the sulfhydryl group is influenced by conformational changes since this determines the availability of the sulfhydryl group for reactions. α-Lactalbumin has a molecular weight of 16,000. It is high in tryptophan. It has a very compact, nearly spherical overall shape.

2.2 Gel formation on acidification of milk and microstructure of acid gels

2.2.1 Gel formation by acid addition

During acidification, solubilization of colloidal calcium phosphate and a reduction of the net negative charge on casein occur. These changes diminish the stability of casein particles against aggregation and lead to gel formation (Roefs et

al., 1990). Acid-induced gels derived from casein are formed as a result of a reduction in the net negative charge on casein, which facilitates aggregation and fusion of casein micelles in the form of chains and clusters (Kalab et al., 1983).

The formation of acid gels during acidification of heated milk has been studied by freeze-fracture electron microscopy (Heertje et al., 1985; Visser et al., 1986). These authors suggested that network formation appeared to be a much more complex process than just an aggregation of the original casein micelles; they suggested that aggregation was also accompanied by subtle dissociation and association phenomenon of the caseins. The observed microstructures of heated milk were explained from the changes in the zeta potential of caseins, the association of the β-casein, the release of CCP from the micelle, and from some observations on the internal structure of the casein micelle. No major role for denatured whey proteins in the formation of acid gels made from heated milk was proposed by these authors.

Some properties of acid gels can also be explained with the fractal aggregation theory, which describes how flocculation leads to the formation of a gel (Bremer et al., 1989, 1990; Bremer, 1992). In particle gels a fractal scaling regime may occur only over small length scales, which are of the order of the aggregating clusters. At longer length scales the microstructure appears homogeneous. Fractal behaviour is not usually expected in gels made from very high volume fraction systems. Fractal aggregation assumes that spherical particles of radius a can move by Brownian motion and that they can aggregate when they encounter each other. The aggregates (clusters) formed then also aggregate with each other. These fractal clusters may be considered as the building blocks of the gel. If no further changes occur among the particles in an aggregate, once they are incorporated,

this cluster-cluster aggregation process leads to aggregates obeying the scaling relation:

$$N_p = (R / a)^D$$

where N_p is the number of particles in an aggregate of radius R, and D is a constant called the fractal dimensionality, which is always smaller than 3. This implies that the density of the aggregate will become smaller with increasing R.

The number of particles that could be present in an aggregate (N_a) is:

$$N_a = (R / a)^3$$

This implies that the average volume fraction of particles in an aggregate is given by:

$$\varphi = N_p / N_a = (R / a)^{(D - 3)}$$

The average volume fraction of an aggregate will decrease during flocculation. A gel is formed when the aggregate fill the total space available. For casein gels (both rennet and acid), $D \simeq 2.3$ has been observed generally (Bremer, 1992).

The formation of an acid milk gel network is influenced by many factors such as heat treatment and total solids content of the milk, pH, type of bacterial culture and the presence of thickening agents. An understanding of the mechanisms involved in structure formation is important in order to influence the final texture of acid milk gels (Kalab et al., 1983).

2.2.2 Microstructure of acid gels

Scanning and transmission electron microscopy are important tools in elucidating the microstructure of dairy products. Acid casein gels have been characterised as an open network of branched, casein particle chains arranged like a string of beads or as thick aggregates, leaving large cavities (pores) filled with liquid (Harwalkar and Kalab, 1973, 1980; Heertje et al., 1985; Roefs et al., 1990). The network structure is very inhomogeneous, consisting of strands of casein particles alternating with thicker nodes. These strands and nodes form large clusters which aggregate to form the gel network. Consequently, the liquid phase is immobilised in the interstitial spaces in the protein network, the size of these pores depends on the casein concentration of milk; an increase in the protein concentration causes smaller pores (Harwalkar and Kalab, 1986). The structure of gel network is one of the most important factors which affect the consistency of the product and its susceptibility to syneresis (Harwalkar and Kalab, 1986). van Vliet and Walstra (1994) suggest that the dynamics of the gel network is the main factor controlling the case of water removal from gels.

A relatively new technique, confocal scanning laser microscopy (CSLM), which can also be used to observe the gel formation process (Brooker, 1995). Although electron microscopy is a useful technique to study the microstructure of milk gel because of its high resolution and ability to characterise surfaces as well as the internal structure (Brooker, 1985), dehydration of a high water food system and other sample preparation steps may alter the natural structure and lead to the creation of artefacts. CSLM has the advantage of needing little preparation procedures. The possibility of examining fully hydrated samples, to produce optical sections (3-D) of these samples, and to utilise the digital information for further data analysis makes CLSM a useful technique for observing the microstructure of milk gels (Hassan et al., 1995). Fluorescent dyes are available

for both fat and protein as well as other substances, which allows visualisation of both of these components in milk gels.

Acidification of milk, which contains fat globules encapsulated in an artificial protein membrane, results in the formation of gel where the fat globules are integrated (embedded) into the protein matrix (Kalab et al., 1976; Tamime et al., 1984; Harwalkar and Kalab, 1986; Tamime et al., 1995). The protein membrane on the surface of fat globules adds extra bonding units to reinforce a protein-based matrix, leading to a higher resistance to compression (i.e. gel strength) (Aguilera et al., 1993).

2.3 Role of fat globule membranes in acid gels

The structural properties of milk gels are achieved through interactions between the major milk components: casein micelles, whey proteins and fat globules. Both of major protein fractions in milk can form gels (Green, 1980): casein, by acid and/or enzyme hydrolysis; and whey proteins due to denaturation by heat and/or cooling.

Incorporation of a dispersed lipid phase is usually desirable in fabricated foods, since it improves and modifies several sensory and textural attributes and lipids are also used as a carrier of fat soluble nutrients, flavours and colours (Darling and Butcher, 1978).

Important structural properties of fat globules are determined by the properties of the membrane, which can be altered for technological advantages by processing. During homogenisation the surface area of fat globules in whole milk increases by a factor between 4 and 10 (Walstra and Jenness, 1984). This leads to the

formation of new membranes at the fat/serum interface, containing adsorbed casein and serum proteins, in addition to the original membrane (Darling and Butcher, 1978). The adsorption of casein micelles to fat/serum interface after homogenisation has been confirmed by a number of studies (Walstra and Jenness, 1984; Sharma and Dalgleish, 1994).

Emulsified fat globules can be incorporated into the protein gel matrix (van Vliet and Dentener-Kikkert, 1982). However, the effect of fat globules in a gel can be constructive or destructive, depending upon the fat globule size (de Wit and Klarenbeek, 1984) and whether or not the native membrane is present (Aguilera and Kessler, 1988, 1989). Protein gels can be reinforced by fat globules that contain a protein membrane and the composition of the membrane is important in this regard. In milk gels, fat globules may act as "copolymers" forming links within and between strands and clusters of a casein-based particle network (Xiong and Kinsella, 1991a).

van Vliet (1988) suggested that two types of interactions between the dispersed particles and the gel matrix could be distinguished: (1) no interaction between the gel matrix and the dispersed particles; at small deformations this results in a decrease in the gel modulus with increasing volume fraction of filler, (2) a strong interaction between filler and matrix; at small deformations this results in an increase in the modulus of gel with increasing volume fraction of filler material, if the filler material is stiffer than the gel matrix. The extent of the increase in gel modulus (in systems where the filler interacts with the matrix) depends on the modulus of the filler material.

The structure of gels, which have non-interacting particles, is shown schematically in Fig. 2.2a. For the non-interacting particles a decrease in storage

modulus (G') with increasing fat content is found in acid milk gels (van Vliet and Dentener-Kikkert, 1982). The dispersed emulsion droplets are situated in pores in the gel and are not connected to the matrix. In fact, there is a thin layer of water between dispersed particles and gel matrix. Mechanical forces exerted on the gel matrix can only be passed on to the emulsion droplets by this thin layer of water. Therefore, gels filled with non-interacting particles behave at small deformations as if it were filled with particles with the rheological properties of water. The G' of water is much smaller than that of the acid gel matrix (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988). For the interacting fat particles (e.g. recombined milk), G' increases with increasing fat content for acid milk gels (van Vliet and Dentener-Kikkert, 1982).

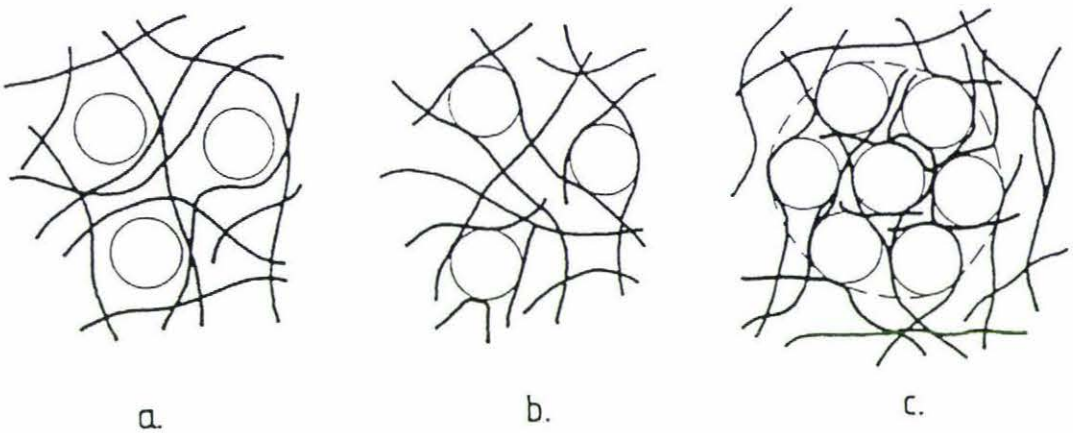

Fig. 2.2. Schematic representation of different possible situations in filled gels (not to scale). (a) No interaction between fat globule membrane and gel matrix; (b) interaction between gel matrix and individual droplets and (c) interaction between gel matrix and aggregates denoted by dashed line (from van Vliet and Dentener-Kikkert, 1982).

It is well known that the nature of interactions in gel systems, containing fat globules can be changed by altering the nature of the fat globule membrane (i.e.

the surface of the dispersed particles). Native fat globules (or modified fat globules with non-protein material on the surface) are inert and do not interact with a milk protein-based gel system. On the other hand, modified fat globules, which contain protein materials on the fat surface, interact with the casein matrix of milk gels. The types of interactions can be summarised as follows:

Dispersed particles	Nature of interaction in milk protein-based gel systems
Native fat globules	Inert
Fat globules with modified membrane:	
Protein materials	Interact with matrix
Non-protein materials	Inert

Aguilera and Kessler (1989) studied the mechanical, physical and microstructural properties of composite milk gels made from two milk protein products (SMP and WPC) and fat globules that had modified membranes. They suggest that if the protein concentration per unit volume in pure, mixed and filled gels is the same, the matrix immobilises the same amount of fluid, hence, any differences in firmness are probably due to interactions of the matrix with modified membranes. The composition of the protein fraction also influences the firmness of milk gels. Several possible outcomes from interactions between protein and fat, leading to various degrees of structural compatibility, are shown in Fig. 2.3. They reported that mixing SMP and WPC at 10% total solids content showed synergistic effects on gel firmness and the addition of fat provided further reinforcement. Syneresis

of gels and their appearance changed from high and clear to low and turbid, respectively, as the WPC content increased.

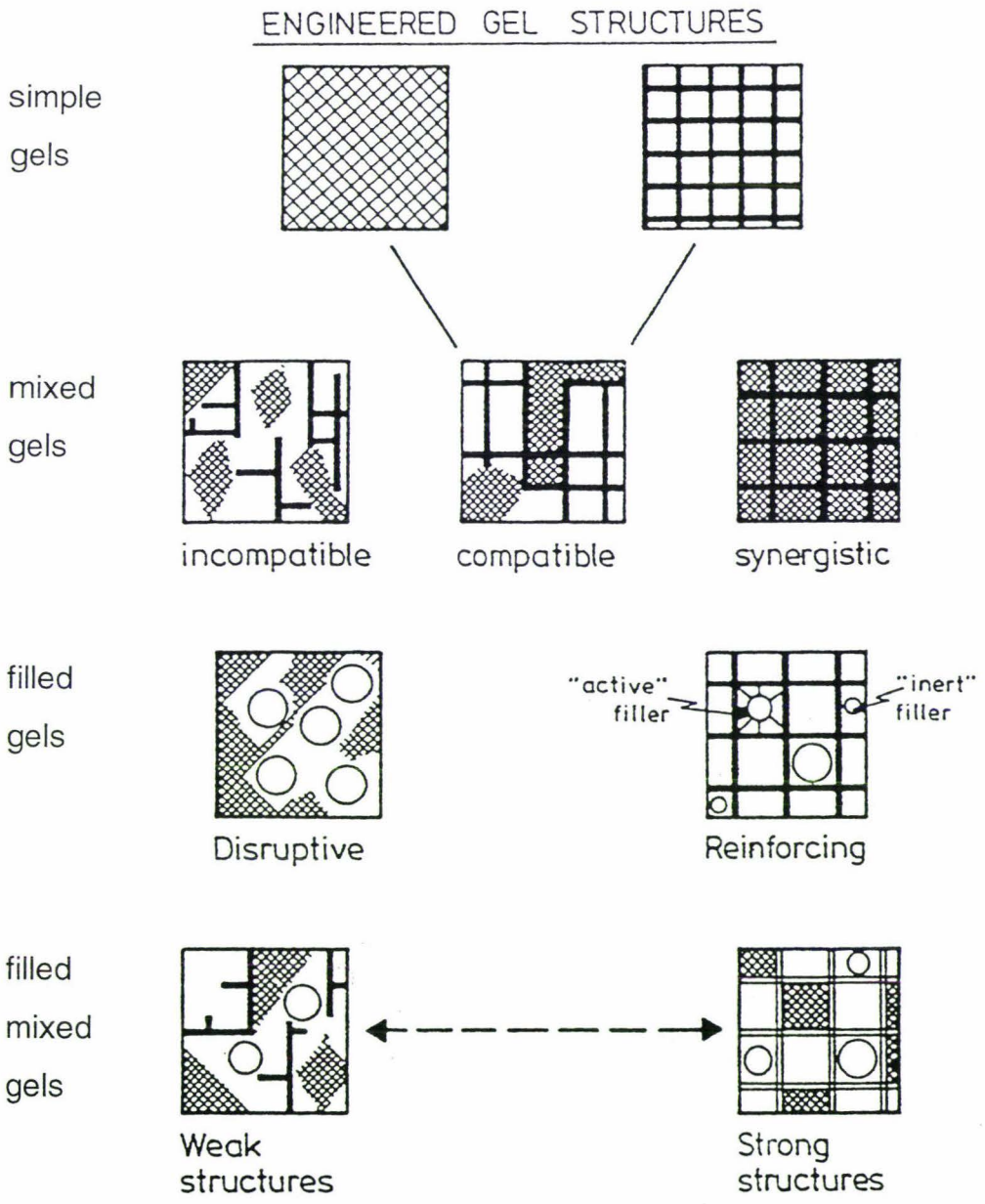

Fig. 2.3. Schematic diagram representing some structural possibilities for simple, mixed, filled and filled-mixed gels (from Aguilera and Kessler, 1989).

Xiong and Kinsella (1991a, b) studied the effects of different fat globule membranes and fat/oil types on the properties of acid milk gels. Some possible mechanisms for fat globule-casein particle interactions in milk gels are given in Fig. 2.4. Fat globules can either act as "copolymers" and become integrated into the matrix of the casein-based gel network, or act as a "fillers" to sterically reinforce the matrix. Interactions were possible via cross-linking between fat globule membrane proteins and proteins (casein particles) in the bulk phase.

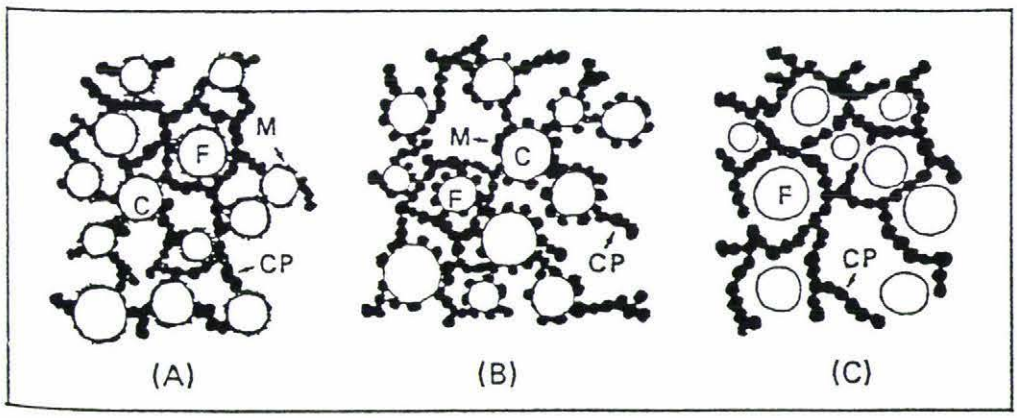

Fig. 2.4. Schematic representation of possible interactions between casein particles and fat globules in milk gels. The fat globules can serve as either copolymers (C) or fillers (F) in the casein particle (CP)-fat globule networks. The fat globules were coated with milk fat globule membrane proteins, whey protein isolate or sodium caseinate (A); or skim milk powder (B); or Tween 80 (C). M: fat globule membrane (from Xiong and Kinsella, 1991b).

2.4 Rheological properties of acid milk gels

2.4.1 Measurement of rheological properties

Rheology is defined as the study of the flow and deformation of materials. It is quantitatively concerned with the relationships between the variables, stress and strain, and their relationship with time. Stress is the force applied per unit area and strain the deformation or response to the force with time. In a viscoelastic substance that response is not instantaneous, but occurs over a period. A viscoelastic material is a substance that has some of the properties of a liquid (viscous) as well as those of a solid (elastic) (Ferry, 1980; Horne, 1993).

Dynamic oscillatory techniques are useful especially in studying flocculated dispersions, emulsions and gelling materials. By applying small strains, the oscillating viscometer gives information on what is close to the condition of zero shear (Ferry, 1980). In dynamic rheology, using constant strain rheometers (e.g. Bohlin VOR Rheometer), a small sinusoidally varying strain is applied to the test sample and the response of the material to the varying strain is measured. The amplitude of the oscillation is kept sufficiently small so that the structure of the material is not damaged (i.e. within the linear viscoelastic range). A number of studies of the fundamental rheological properties of milk gels have been reported using dynamic low amplitude oscillation (Bohlin et al., 1984; Dejmek, 1987; Roefs et al., 1990).

For an elastic material, the stress response to an oscillatory strain shows no phase shift or angle (i.e. $\delta = 0^{\circ}$). In a viscous material that is subjected to a sinusoidally varying strain, the stress response shows a phase shift of 90°. Viscoelastic materials, which have both elastic and viscous components shows intermediate phase angles between 0 and 90°. From the amplitude of the sinusoidal strain and

the phase angle, the rheological properties of the gel can be characterised in terms of a storage modulus (G') and a loss or viscous modulus (G"). The storage (or elastic) modulus is the ratio of in-phase stress to strain and a measure of the energy stored per oscillation cycle. On the other hand, the loss (or viscous) modulus is a measure of the energy dissipated (released) per oscillation cycle. Phase angle is related to the storage and loss moduli through the equation:

$$\tan \delta = G''/G'$$

The larger the value of tan δ the more liquid-like the material is behaving and *vice versa* (Ferry, 1980; Horne, 1993).

The relationship between G', G", G* (complex modulus) and δ can be expressed vectorially (Fig. 2.5).

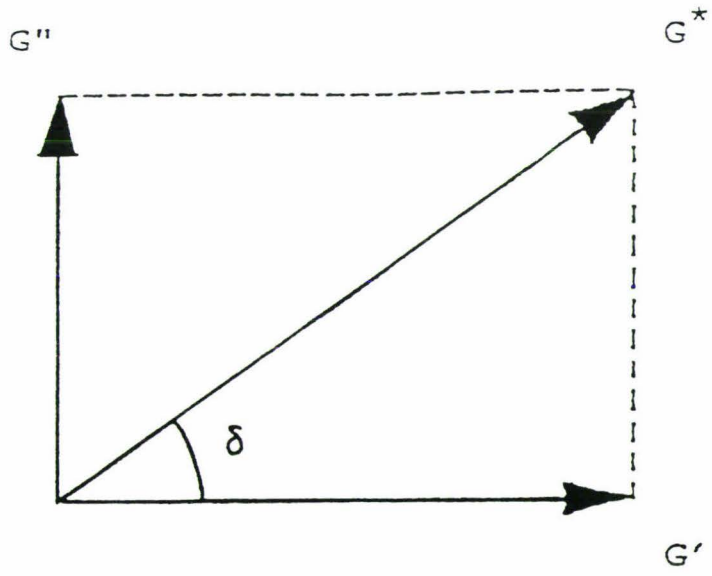

Fig. 2.5. The vectorial relationship between G', G", G* and δ (from Ferry, 1980).

For a perfectly elastic material, all the energy is stored and the stress and strain will be in-phase and G" will be zero. For a liquid processing no elastic character, all the energy is dissipated as heat and G' will be zero and the stress and strain are completely out-of-phase. When milk gels are subjected to an applied (shear) stress, the energy of deformation is not completely conserved as in an ideal elastic solid nor is it dissipated as in a liquid. Stress applied for a short time results in the elastic character of the gel predominating but if applied for a long time, the viscous character of the gel predominates. When viscoelastic protein gels are subjected to a sinusoidally oscillating strain, the stress is neither completely in-phase nor completely out-of-phase (Fig. 2.6).

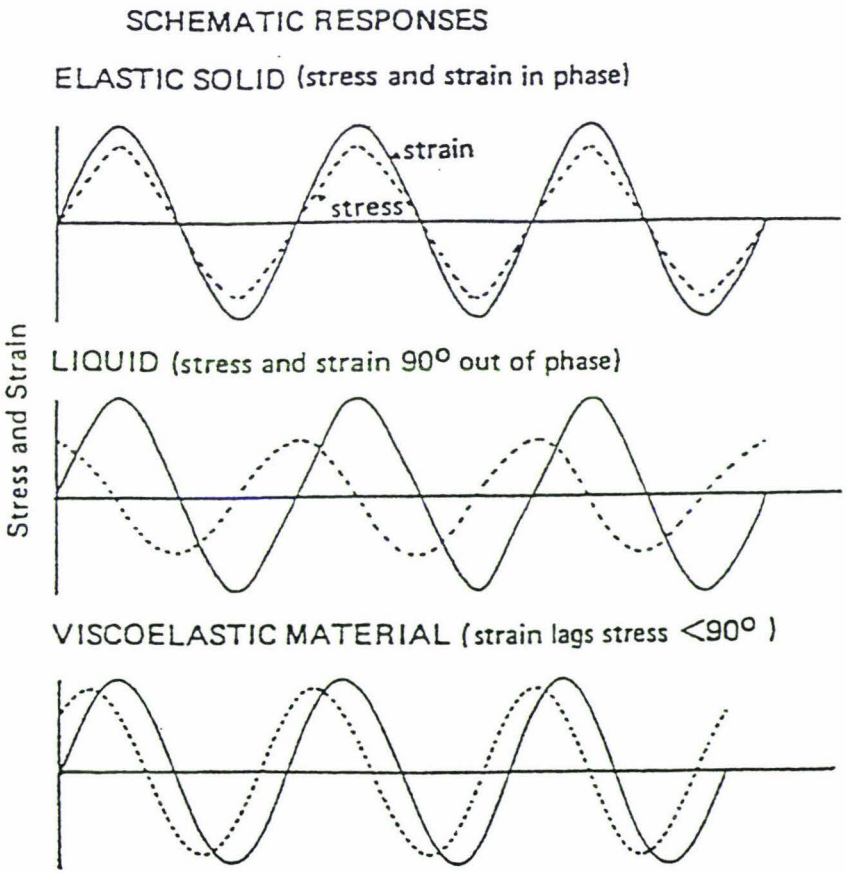

Fig. 2.6. Dynamic experiment showing response of elastic solid, liquid and viscoelastic material (from Ferry, 1980).

The viscoelastic properties of acid gels have been evaluated using low amplitude oscillation by a number of workers (e.g. Roefs et al., 1990; Xiong and Kinsella, 1991a, b; Lucey et al., 1997a) on the formation of acid milk gels.

According to Bourne (1982), penetrometers constitute one of the simplest and most widely used types of texture measuring instruments. The penetration test measures the force required to push a probe into a food product. The test can be characterised by (1) a force measuring instrument; (2) penetration of the probe, which irreversibly breaks or crushes the food; and (3) a depth of penetration, which is usually held constant. For most cases, the initial breaking force or the maximum penetration force are used as the relevant parameters for assessing the influence of technological factors on the texture of yoghurt (Benezech and Maingonnat, 1994). The depth of penetration of the probe can be used as a significant parameter (Becker and Puhan, 1989). Because a large variety of different probes, penetration rates, penetration depths, and measuring temperatures have been used in different studies, it is not possible to compare the results from this type of empirical method.

2.4.2 Effects of processing on physical/rheological properties of acid milk gels

2.4.2.1 Heat treatment

Heat treatment of milk is one of the most important factors affecting the rheological properties of milk gels. Heat treatment before acidification causes interactions between β–lactoglobulin and casein (mainly κ-casein) which alter the formation of acid-induced gels (Lucey et al., 1997a). These interactions affect the rate of network formation and alter the gel structure.

Dannenberg and Kessler (1988b) reported that when the denaturation of β–lactoglobulin increased from 10 to 60%, there was a linear increase in firmness (i.e. resistance to penetration) of acid milk gels. van Vliet and Keetels (1995) showed that the dynamic moduli of acid milk gels made from low-heat (milk pasteurised at 72 °C for 15 sec) skim milk powder were much lower, their dependence on casein concentration was stronger, and their fracture strain was higher than gels made from high-heat (the same milk after additional heating at 85 °C for 5 min) skim milk powder. However, the permeability and the overall structure (i.e. fractal dimensionality) of the large casein aggregates forming the gel was about the same for these two different types of gels. The rheological differences were considered to arise from the way the large aggregates are linked together, namely by straight or by bent strands.

Lucey et al. (1997a) investigated the effects of a range of heat treatments on the rheological properties of acid skim milk gels. G' of acid gels increased with an increase in severity of heat treatment. However, the strain at yielding decreased markedly with increasing heat treatment of reconstituted milk making these gels brittle and easier to fracture. They proposed that denatured whey proteins aggregated with casein particles during the acidification of heated milk and were responsible for most of the effects of heat treatment. Lucey et al. (1997a) also suggested a mechanism by which heating affects the properties of acid milk gels. They suggested that denatured whey proteins (associated with casein micelles) aggregate with casein during the acidification of heated milk and this is responsible for the increase in the pH of gelation since whey proteins have a higher isoelectric point and would start to aggregate earlier than casein particles. The large increase in the G' value of acid gels made from heated milks was explained as denatured whey proteins actively contributing to the rigidity of the gel, i.e. forming additional cross-linkages.

2.4.2.2 Effect of protein composition

The addition of small amounts of whey proteins to milk and heat treatment resulted in an increase in gel strength probably through inter-chain bridging by denatured whey proteins (Modler and Kalab, 1983). Aguilera and Kinsella (1991) reported that the addition of small quantities of skim milk powder to whey protein isolate resulted in weaker gels and this effect was only overcame when the skim milk powder concentration was increased to > 4-5 %. Casein-based acid gels are formed by interactions of casein particles forming a "string-of-beads" type of arrangement (Kalab et al., 1976).

Aguilera and Kinsella (1991) studied the microstructures involved in the formation of milk protein gels and proposed several different types of microstructures for mixed and filled gels (Fig. 2.7). The basic microstructure of these milk protein gels involved: casein micelles with denatured whey proteins attached to the surface as well as recombined fat globules with artificial membranes. Heat treatment of milk and acidification resulted in the formation of several complex types of gel structures with three main models; (a) skim milk based gels, where the addition of small amounts of whey proteins and heat treatment reinforced the structure by causing bridging between casein chains; (b) whey protein based gels, where skim milk weakened the gel due to small amounts of casein micelles and short casein chains interfering with the whey protein network and; (c) skim milk powder/whey protein gels that were reinforced by recombined milk fat globules containing artificial protein membranes, which actively participated in the gel network.

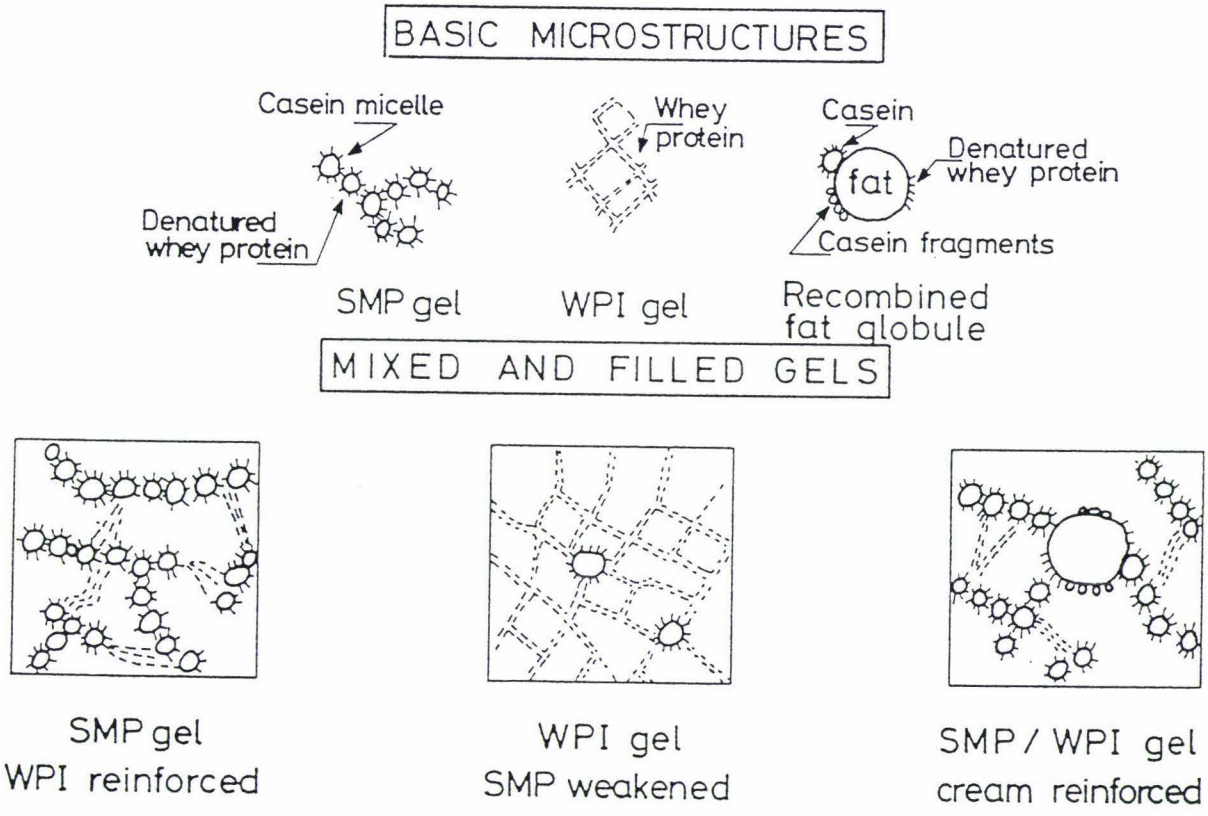

Fig. 2.7. Proposed interactions between whey proteins and recombined fat globules in the formation of mixed and filled gels (from Aguilera and Kinsella, 1991).

2.4.2.3. pH and rate of acidification

When the pH of milk is reduced, CCP is solubilized from casein micelles, resulting in partial micellar disintegration, especially at low temperatures. At low pH values, casein particles associate to form a gel network (Heertje et al., 1985; Roefs et al., 1990). The rate of acidification affects this process and the properties of the gel. Rapid acidification results in aggregation and precipitation of casein from solution, which is the basis of acid casein production (Jablonka and Munro,

1985). Slow acidification generally leads to the formation of a gel network that has a good water holding capacity, which is the principle for the production of yoghurt and other acid gels (Harwalkar et al., 1977).

Cobos et al. (1995a) reported that the gelation time (i.e., the point when G' and G" started to increase) was decreased by a low level of total solids, high heat treatments of milk, high incubation temperatures and high concentrations of glucono-δ-lactone (GDL). The use of higher levels of fat, milk fat instead of olive oil, high heat treatment of milk, and high incubation temperatures increased the gelation pH (i.e., the pH of the sample at the point of gelation). They also concluded that the rheological properties (i.e., G', G" and δ) were only influenced by level of total solids, heat treatment, incubation temperature, and concentration of GDL. High heat treatment of milk and high levels of total solids increased both G' and G", but only heat treatment influenced δ. The conditions of homogenisation (temperature, pressure and order of heat treatment) had no significant influence on gelation or the rheological properties (Cobos et al., 1995a, b).

2.4.2.4 Syneresis or whey separation

Changes in the structure of rennet-induced milk gels can occur even after the network has been formed (Walstra et al., 1985). Rearrangements of the network are caused by attractive forces between individual casein particles or clusters which can lead to additional intermolecular bonds and to contraction of the gel, which results in expulsion of water (syneresis). Spontaneous whey separation is accompanied by a reduction in the height of the gel and is not produced by external forces (e.g. centrifugation). It is favoured by an increase in temperature and by pH values away from isoelectric point of casein, and by mechanical factors such as vibration. Dannenberg and Kessler (1988a) reported that the susceptibility

to syneresis, as measured by a drainage (whey expulsion) test, decreased with increasing denaturation of β–lactoglobulin in milk.

2.5 Manufacture of yoghurt

2.5.1 Set- and stirred-type yoghurt

Yoghurt is an well known example of an acid milk gel. There are two main types of yoghurt; stirred- and set-style. Stirred-type yoghurt is made by bulk incubation in large vats; after fermentation the gel is broken by stirring and fruit is generally mixed and the "liquid" is pumped and filled into containers. Set-type yoghurt is made by inoculating milk and immediately filling into the retail pot and a firm gel is formed as opposed to the weaker more liquid-like system of the stirred product (Humphrey and Plunkett, 1969; Horne, 1993; Benezech and Maingonnat, 1994). A flow diagram for the manufacture of plain set and stirred-type yoghurt is given in Fig. 2.8.

Stirred-type yoghurt is a non-Newtonian fluid which exhibits thixotropic behaviour (Horne, 1993). Thixotropic fluids are characterised by a decrease in apparent viscosity both under increasing shear rate and duration of shearing, followed by gradual, often only partial, recovery when shearing is stopped (Horne, 1993; Benezech and Maingonnat, 1994).

In rheological terms, set yoghurt is essentially a viscoelastic solid, i.e. the elastic component is predominant, although a viscous component is also present. In set gel products the principal method used in rheological studies is a measurement or assessment of the resistance of the gel to a small applied strain (Horne, 1993).

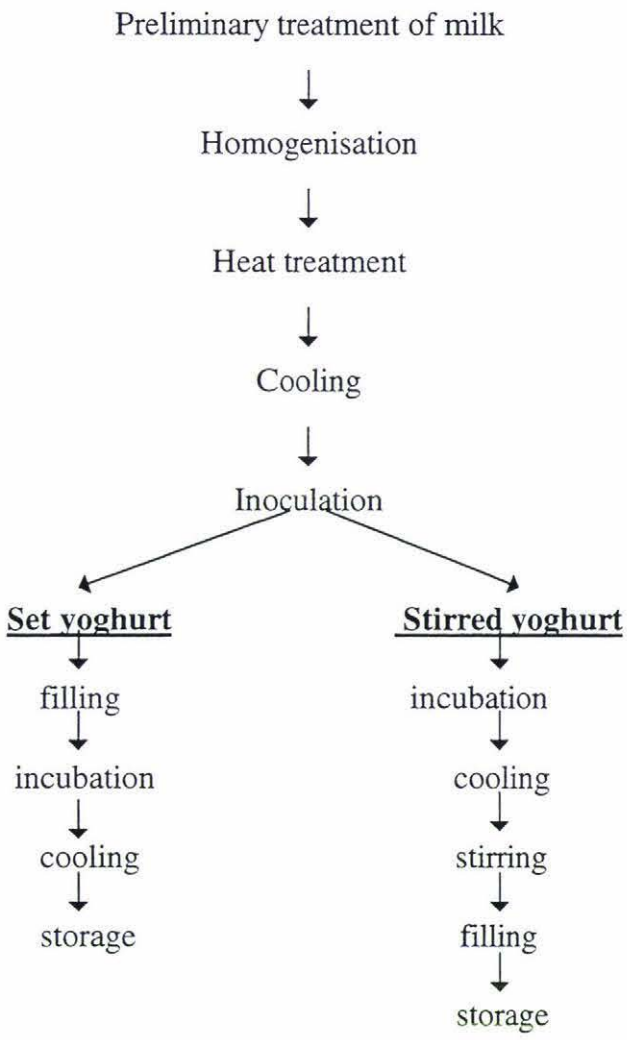

Fig. 2.8. Flow diagram for the manufacture of set and stirred yoghurt (from Tamime and Greig, 1979).

2.5.2 Effects of homogenisation

Homogenisation is an important step in yoghurt manufacture. In commercial practice, homogenisation of yoghurt milk is performed usually at 55-65°C and at pressures between 10-20 MPa (Robinson and Tamime, 1986). It is usually carried out before heat treatment of milk. It is performed to disperse all the dry ingredients and to increase the viscosity of the product. Homogenisation also

improves the mouth-feel of the product and thus increases the organoleptic quality. Some of physico-chemical effects of homogenisation of milk and their relevance in yoghurt manufacture are given in Table 1.

Table 1. Physical and chemical changes caused by homogenisation of milk during yoghurt manufacture (from Tamime and Deeth, 1980).

A. Components that are increased by homogenisation
Viscosity due to a reduction in fat globule size and an increased adsorption of casein micelles on fat globule surface, which increases the effective total volume of 'suspended' particles
Xanthine oxidase activity due to the disruption of the fat globule membrane, which contains about half of the total enzyme concentration of this enzyme in milk
Colour (whiteness) due to the increase in number of fat globules, which increases the reflectance and scattering of light
Lipolysis due to the increase in total fat surface area available to lipases and the destruction of natural fat globule membrane
Efficiency of mixing, especially if milk has been fortified with dry ingredients, e.g. skim milk powder
Phospholipids in skim increases as a result of physical action, more fat globule membrane material is transferred to the skim milk phase
Foaming increases as a result of the increased content of phospholipids in the skim milk phase

(continued)

B. Components that are decreased by homogenisation

Fat globule size reduction prevents the formation of a 'cream line' in yoghurt, especially in high fat products during incubation and storage

Oxidised flavour due to migration of phospholipids to skim milk and formation of sulphydryl compounds which act as antioxidants

Protein stability due to changes in protein-protein interactions as a result of some denaturation and a shift in the salt equilibrium

Agglutination decreases due to adsorption of casein micelles and submicelles on fat globules

Syneresis due to increased structural rigidity due to casein-fat globule membrane interactions and other protein-protein interactions

Homogenisation is particularly important in milk containing fat, i.e. either low fat (1-2%) or especially whole milk (~3.5%). Homogenisation of milk for yoghurt manufacture is considered to prevent fat separation during storage, improve consistency, increase whiteness and reduce whey separation (Tamime and Deeth, 1980; Robinson and Tamime, 1986; Puhan, 1988).

Homogenisation effectively reduces the size of fat globules, which reduces their tendency to cream or rise. Simultaneously, the surface area of fat globules is increased between 4 to 7 times and fat globules become coated with a layer of casein and other milk proteins creating a new surface membrane. In that way, the fat becomes evenly dispersed throughout milk and does not separate out during incubation or on storage (Tamime and Deeth, 1980; Walstra and Jenness, 1984).

2.5.3 Effects of heat treatment

Heating is one of the most fundamental steps in the manufacture of various types of fermented milk products. It is general practice to preheat milk for yoghurt manufacture at high temperatures such as 80 °C for 30 min or 90-95 °C for 5-30 min (Tamime and Deeth, 1980; Robinson and Tamime, 1986).

Milk undergoes several important changes during heat treatment. The heat treatment applied to yoghurt milk is sufficient to inactivate most of the vegetative cells of micro-organisms present in milk, but spore formers and heat stable enzymes will remain, which are not considered to be a significant problem in yoghurt. Heat treatment also improves the nutritive properties of the product since denatured whey proteins are more easily digested by intestinal enzymes; however it may destroy some heat labile vitamins (Mulvihill and Grufferty, 1995). The most important change, in relation to yoghurt manufacture, is change in the physico-chemical properties of the proteins. It is also widely recognised that the heat treatment results in a reduction of gelation time and an increase of pH at gelation during the acidification of milk. This is mainly due to denaturation of whey proteins and association with casein micelles (Horne and Davidson, 1993; Lucey et al., 1997a).

A vast literature exists on the denaturation of whey proteins by heat and heat-induced interactions between whey proteins and caseins (Smit and van Brouwershaven, 1980; Walstra and Jenness, 1984; Dalgleish and Banks, 1991; Kim and Jimenez-Flores, 1995; Singh, 1995). It has been recognised that β-lactoglobulin interacts mainly with κ-casein, thus becomes associated with the casein micelles or partly with nonmicellar casein if the pH during heating is > 6.6 (Singh and Creamer, 1991; Singh, 1995).

In yoghurt manufacture, most of the whey proteins are denatured as a result of the high heat treatment of milk, in particular, β–lactoglobulin is almost completely denatured. The interaction between the denatured β–lactoglobulin and casein is very important as this facilitates the formation of a firm coagulum (Grigorov, 1966; Labropoulos et al., 1984; Mulvihill and Grufferty, 1995). It is generally considered that unheated milk forms weak yoghurt gels that have a strong tendency to synerese (Kalab et al., 1976).

Mottar et al. (1989) investigated the role of the nature of the casein micelle surface, as affected by adsorption of heat-denatured whey proteins, on yoghurt texture. They suggested that during heating initially β-lactoglobulin aggregated with casein and that later during heating α-lactalbumin also interacted. Mottar et al. (1989) suggested that the precipitation of α-lactalbumin apparently filled up the gaps on the micellar surface left by β-lactoglobulin.

The viscosity and firmness of yoghurt has been related to the degree of whey protein denaturation (Grigorov, 1966; Labropoulos et al., 1984; Parnell-Clunies et al., 1986; Dannenberg and Kessler, 1988a, b).

Several researchers have observed differences in microstructure of acid milk gels as a result of heat treatment of milk. Davies et al. (1978) reported that heated milk (95 °C for 10 min) had filamentous appendages attached to casein micelles, which appeared to consist of denatured β–lactoglobulin. Kalab et al. (1983) and Parnell-Clunies et al. (1987) also observed the formation of 'spikes' on the micellar surfaces of heated milk. The interaction between whey proteins and casein micelles appeared to involve disulfide linkages, probably κ-casein. These appendages appeared to inhibit micellar contact and fusion, which prevented the

formation of very large aggregates that would have poor water holding capacity (Davies et al., 1978). In gels made from unheated milk, casein micelles form aggregates or clusters in which the protein is irregularly or unevenly distributed, resulting in the formation of a 'coarse network' (Harwalkar and Kalab, 1981, 1983). Gels made from unheated milk are considerably weaker and more susceptible to whey separation compared with gels made from heated milk (Kalab et al., 1983). It is probable that the firmer gels made from heated milk is a result of bridging or cross-linking between denatured whey proteins and caseins (Lucey et al., 1997a).

CHAPTER 3.
MATERIALS AND METHODS

3.1 Materials

Fresh, unpasteurised whole milk was obtained from the Massey University, Dairy Farm Herd No. 4, Palmerston North, New Zealand.

Low-, medium- and high-heat skim milk powders (SMP) were obtained from the New Zealand Dairy Research Institute, Palmerston North. The whey protein nitrogen index of these powders were 8.0, 2.7 and 1.5 mg/g powder, respectively.

Spray-dried whey protein concentrate (WPC, Alacen 392) and sodium caseinate (Alanate 180) powders were obtained from the New Zealand Dairy Board, Wellington.

Tween 60 was purchased from BDH Chemicals (BDH Ltd., Poole, England).

All the chemicals used were of analytical grade obtained from either BDH Chemicals (BDH Ltd., Poole, England) or Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise.

3.2 Preparation of protein solutions

Appropriate quantities of low-, medium- or high-heat SMP, Na caseinate or WPC were dissolved in Milli-Q water to give protein concentration of ~2.0% (w/w). In some instances, WPC solutions (2.3%) are heated at 80 °C for 30 min to denature the whey proteins using a temperature-controlled water bath.

3.3 Preparation of emulsion systems

The protein solution was mixed with anhydrous milk fat, the mixture heated to 55°C, and then passed through a two-stage valve homogeniser (Rannie, model LAB, type 12.50 H, capacity 100 L/h, Rannie a/s, Roholmsvej 8, DK - 2620, Albertslind, Denmark) without applying any pressure. This produced a temporary oil-in-water emulsion. The mixture was then homogenised at the desired pressure, 20.7/3.5 MPa for the first and second stages, respectively. The resulting emulsion was cooled to room temperature and then used for the preparation of recombined milk. Typically, these emulsions contained ~ 2.0% protein and 10% (w/w) milk fat. In one case, emulsion was prepared using 0.5% Tween 60. The flow diagram for the process used is shown in Fig. 3.1.

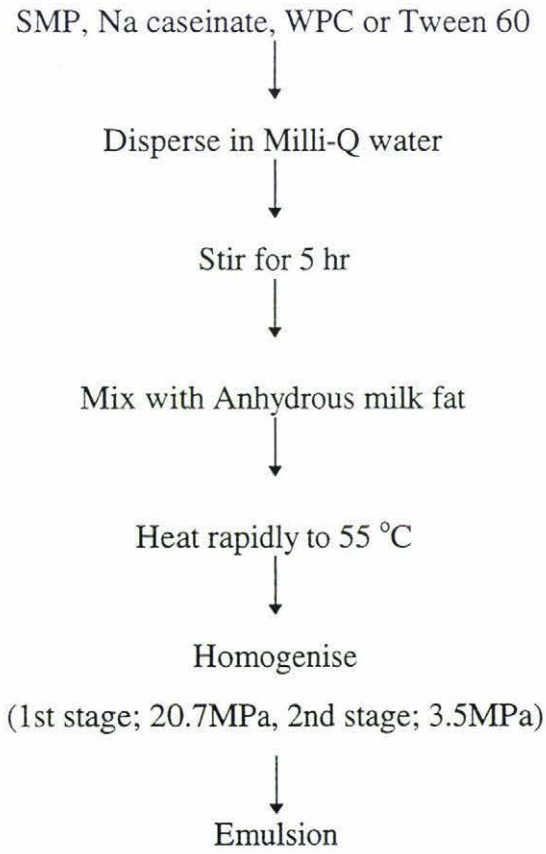

Fig. 3.1. Preparation of emulsion system

3.4 Preparation of recombined milks containing modified fat globules

Recombined milk was prepared by mixing 364g of low heat-SMP solution (~ 12% TS) with 196g of emulsion. The mixture was gently stirred for 5 h. In some cases, low heat-SMP (12% TS) solutions are first heated at 80°C for 30 min, cooled to room temperature, and then mixed with the emulsion. The final recombined milks contained 3.54 $\pm$ 0.07% fat and 5.26 $\pm$ 0.14% protein. A flow diagram of the process is shown in Fig 3.2.

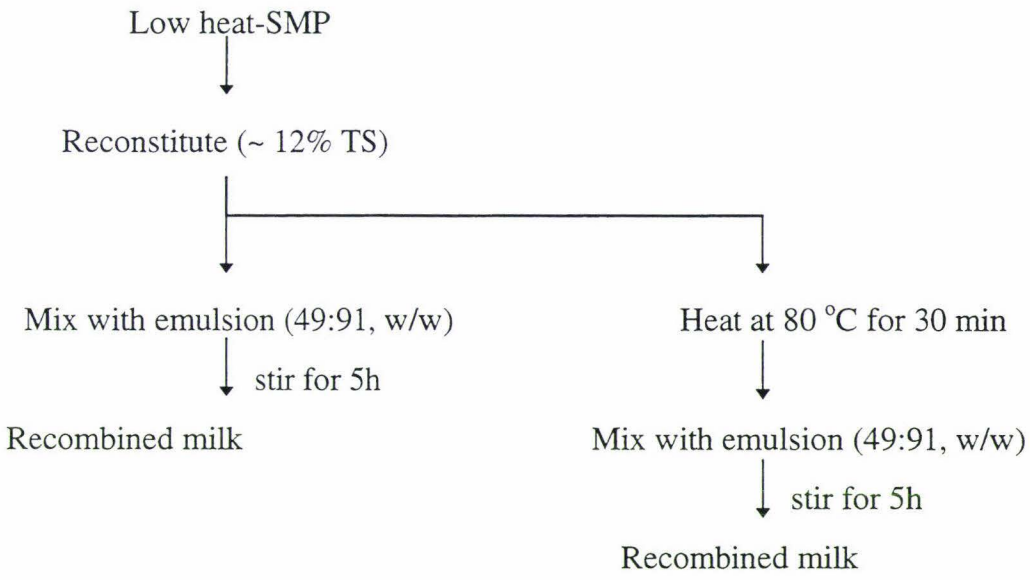

Fig. 3.2. Experimental procedure for the preparation of recombined milks.

3.5 Preparation of acid gels

GDL was used as an acid precursor. About 1.4% (wt/vol) GDL was added to recombined milk and incubated at 30°C for 16 h in an incubator.

The pH of the samples was continuously measured with an N61 combination electrode (Schott Gerate, Hofheimia Ts, Germany) attached to a Radiometer pH meter (PHM 84, Copenhagen, Denmark). The pH of recombined milks was between 4.55 and 4.65 after 16 h.

3.6 Characterisation of emulsions

3.6.1 Size distribution of fat globules

Samples of emulsion were diluted with Milli-Q water or 2% (w/w) SDS buffer in the ratio of 1:3 sample to buffer. After 30 min mixing, the size distribution of fat globules in each sample was measured by MasterSizer E (Ver. 1.1, Malvern Instruments Ltd., Malvern, U.K.) at the New Zealand Dairy Research Institute, Palmerston North. For analysis of particle size, the presentation mode 2NAD polydispere model was used.

In this method, a low power laser beam is diffracted by the fat globules in the solution and the diffracted light is collected over a range of scattering angles by series of semicircular photo-electric diodes. The sizes of fat globules are divided into 22 classes across the sub-micron range, 0.1 - 1.0 microns. The volume size distribution is calculated from the intensity of light diffracted at each angle using Lorenz-Mie theory. For the calculation of size distribution, the refractive index of the medium in which the particles are dispersed is required.

3.6.2 Determination of protein load

The amount of protein adsorbed onto the fat surface was determined by measuring the protein content of the washed cream layer after separation of the dispersed and aqueous phases by centrifugation. The emulsion samples were centrifuged at 20,000 *g* for 20 min at 20 $\pm$ 2°C in a temperature controlled

centrifuge (Sorvall RC5C, DuPont Company, USA). The cream layer was removed from the centrifuge tube using a spatula and then suspended in 10 volumes of deionised water and left at room temperature for 1 h. This mixture was recentrifuged at 20,000 *g* for 20 min at 20 ± 2°C and the cream layer was collected. The specific surface area was obtained from the MasterSizer results and the protein load calculated as follows:.

$$\text{Protein load } (mg/m^2) = \frac{\text{Total g protein adsorbed}}{\text{Total g fat in cream layer}} \times \frac{1}{\text{Specific surface area } (m^2/g \text{ fat})} \times 1000$$

3.6.3 Determination of fat globule membrane composition

The washed cream was dispersed in SDS buffer (10% SDS and 0.5% β-mercaptoethanol) and the amounts of individual proteins in the washed top layer were determined by SDS-polyacrylamide gel electrophoresis.

3.7 Protein, fat and total solids analysis

Total protein was measured by determining total nitrogen by the macro-Kjeldahl method (AOAC, 1974) and multiplying it by a factor of 6.38. The samples were digested using a Kjetec 1007 digester (Tecator, Sweden) and distilled using a Kjetec 1026 Distilling Unit (Tecator, Sweden).

Total fat was determined using the Rose-Gottlieb gravimetric method for milk (International Dairy Federation, IDF 1C: 1987) and cream (IDF 16C: 1987), respectively.

Total solids content was obtained using air-oven method (AOAC, 1974). The temperature of oven was kept at approximately 105°C for overnight during drying of samples.

3.8 Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli (1970), as described by Singh and Creamer (1991).

Preparation of stock solutions

Acrylamide/Bis (30 % T, 2.67 % C)

Acrylamide (29.2 g) and N, N-bis acrylamide (0.8 g) were dissolved in deionised water (Milli Q reagent water system, Millipore Coperation, Bedford, MA, USA), made up to 100 mL, filtered and stored at 4°C in the dark.

1.5 M TRIS-HCL buffer, pH 8.8

TRIS (tris hydroxylmethyl aminoethane, 18.15 g), obtained from the United States Biochemicals Corporation (Cleveland, OH, USA), was dissolved in approximately 60 mL of deionised water, the pH adjusted to 8.8 with 1 M HCl and the volume made to 100 mL with deionised water. This buffer was stored at 4°C.

0.5 M TRIS-HCl buffer, pH 6.8

Tris (6 g) was dissolved in approximately 60 mL of deionised water. The pH was adjusted to 6.8 with 1 M HCl, the volume made to 100 mL with deionised water, and the buffer stored at 4°C.

10 % SDS

10 g of sodium dodecyl sulfate (SDS) was dissolved with gentle stirring in deionized water and the volume made to 100 mL. This was stored at room temperature.

SDS-sample buffer

The following solutions were added to 55 mL of deionized water: 0.5 M TRIS-HCl buffer (12.5 mL), glycerol (10 mL), 10 % (w/v) SDS (20 mL), and 0.1 % (w/v) bromophenol blue (2.5 mL). For reducing conditions, β–mercaptoethnol (5 mL) was added and the volume of deionised water was reduced to 50 mL. This solution was made up fresh each day to prevent the oxidation of β–mercaptoethnol.

5X electrode buffer, pH 8.3

Electrode buffer (5X) was made by dissolving Tris (15 g), glycine (72 g) and SDS(5 g) in deionised water. The pH was adjusted to 8.3, the volume to 1 L and the buffer stored at 4 °C. For each electrophoresis run, 60 mL of 5X electrode was diluted to 300 mL with deionised water.

Preparation of resolving gel (16%, w/v, 0.375 M TRIS, pH 8.8)

For preparation of 10 mL of resolving gel buffer, the following solutions were mixed: deionised water (2.02 mL), 1.5 M TRIS-HCl (2.50 mL), 10 % (w/v) SDS solution (100 µL), and acrylamide/bis mixture (5.30 mL). The mixture was degassed for 15 min. Immediately after that, 50 µL of 10% (w/v) ammonium persulphate (prepared earlier the same day) and 5 µL of TEMED (tetramethylethylenediamine) were added. After gentle mixing, the contents were poured between electrophoresis casting plates (Bio-Rad Protean, Bio-Rad, Richmond, CA, USA). A small quantity of deionised water was added to form an

upper layer and the acrylamide solutions allowed to polymerise at the room temperature for 35 min. The water was poured off carefully and removed with pieces of filter paper before pouring the stacking gel.

Preparation of stacking gel (4.0%, w/v, 0.125 M TRIS, pH 6.8)

For preparation of 5 mL of stacking gel buffer, the following solutions were mixed: deionised water (3.05 mL), 0.5 M TRIS-HCl (1.25 mL), 10 % (w/v) SDS solution (50 µL), and acrylamide/bis mixture (0.65 mL). The mixture was degassed for 15 minutes. Immediately after that, 25 µL of 10% (w/v) ammonium persulphate (prepared earlier the same day) and 5 µL of TEMED were poured and gently mixed. The stacking gel was poured on the top of the set resolving gel and a slot former (10-slot plastic comb) was inserted between the plates to form appropriate slots for the samples. Polymerisation was carried out at the room temperature for 35 min. Before running the gel, the comb was removed and the wells were rinsed with deionised water to get rid of unpolymerised gel solution. Water was removed using pieces of filter paper.

Sample Preparation

Samples of washed top layers were diluted in sample buffer. It is assumed that under reducing conditions (with β–mercaptoethnol), all protein aggregates are presumably dissociated. The samples were heated for 5 min at 95 °C.

Electrophoretic running of the gel

Two gels were placed in an electrode buffer chamber. The electrode buffer stock solution was diluted 1:4 with deionised water and used to fill the inner buffer chamber. The samples (12 µL) were injected into the slots of the gel. The gels were run on a Mini-Protean system (Bio-Rad, Richmond, CA, USA) at 200 V using a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Richmond, CA,

USA), until the tracking dye moved out of the gel. The approximate running time was 1 h.

Staining and Destaining

The gels were removed from the casting assembly and placed in plastic containers containing about 50 mL staining solution (1 g brilliant blue R dissolved in 500 mL isopropyl alcohol and 200 mL acetic acid and made up to 2 L with distilled water) was added. The gel containers were put on rocking table for 1h for uniform staining. The staining solution was then poured off and replaced with 100 mL destaining solution (100 mL isopropyl alcohol and 100 mL acetic acid made up to 1 L with distilled water). After rocking for 1 h, the destaining solution was replaced with 100 mL of fresh destaining solution and the container was rocked for a further 19 h.

Densitometry

Quantitative determination of the protein components of the sample separated by SDS-PAGE was performed by densitometry using a laser densitometer (LKB Utrascan XL, LKB Produkter, AB, Sweden). The protein bands on the stained gel are scanned with a narrow beam of light and the absorbance at 522 nm is plotted as a function of track distance. The output from the densitometer was quantified by measuring the area under each individual peak.

3.9 Measurement of rheological properties

3.9.1 Penetration test

The recombined milk was incubated for 16 h at 30°C in a 50 mL glass beaker (58 mm i.d. and 54 mm height). The height of samples was 36 mm. To prevent evaporation, the beaker was covered with tin foil. The strength of the gel was

measured using a penetration test using Instron (Model No. 4502, S. No. H3096). A 10 N load cell and the persplex probe (20 mm diameter) was used to penetrate gel to a depth of 30 mm at a rate of 20 mm/min and the penetrating force was continuously monitored and recorded using a computer. Three replicates of each sample were tested and the average of the three was taken to represent the strength of each acid gel.

3.9.2 Dynamic low amplitude oscillation

A VOR Bohlin Rheometer (Bohlin Rheologi, Lund, Sweden) was used to measure the rheological properties of recombined milk during acid gelation. The Bohlin VOR Rheometer is a computer controlled instrument working in three different modes performing oscillation, viscosity and relaxation measurements (Fig. 3.3). The oscillatory mode was used in the present experiments. The instrument consists of a Couette type cup and a fixed bob system. The bob is suspended in a torsion bar and a torque shaft is suspended on an air bearing. The sensitivity of the instrument is determined by the choice of torque bar and the bob and cup size. The properties of the gel, the elastic (storage) modulus G', the viscous (loss) modulus G" and loss tangent (tan δ = G"/G') are measured at a predetermined strain and frequencies as functions of time. The C25 measuring system consists of a fixed bob (diam. inner cylinder 25 mm) and a rotating cup (diam. outer cylinder 27.5 mm) with a sample being contained in the annular gap between them.

On addition of GDL to the recombined milk the mixture was stirred for 2 min, and then 13 mL of the mixture was transferred into the cup of the rheometer. Vegetable oil was added on the top of the surface to prevent evaporation of water and subsequent surface drying. For unheated samples, the 0.46 mN/m torsion torsion bar was used while for the heated samples the 4.29 mN/m bar was used.

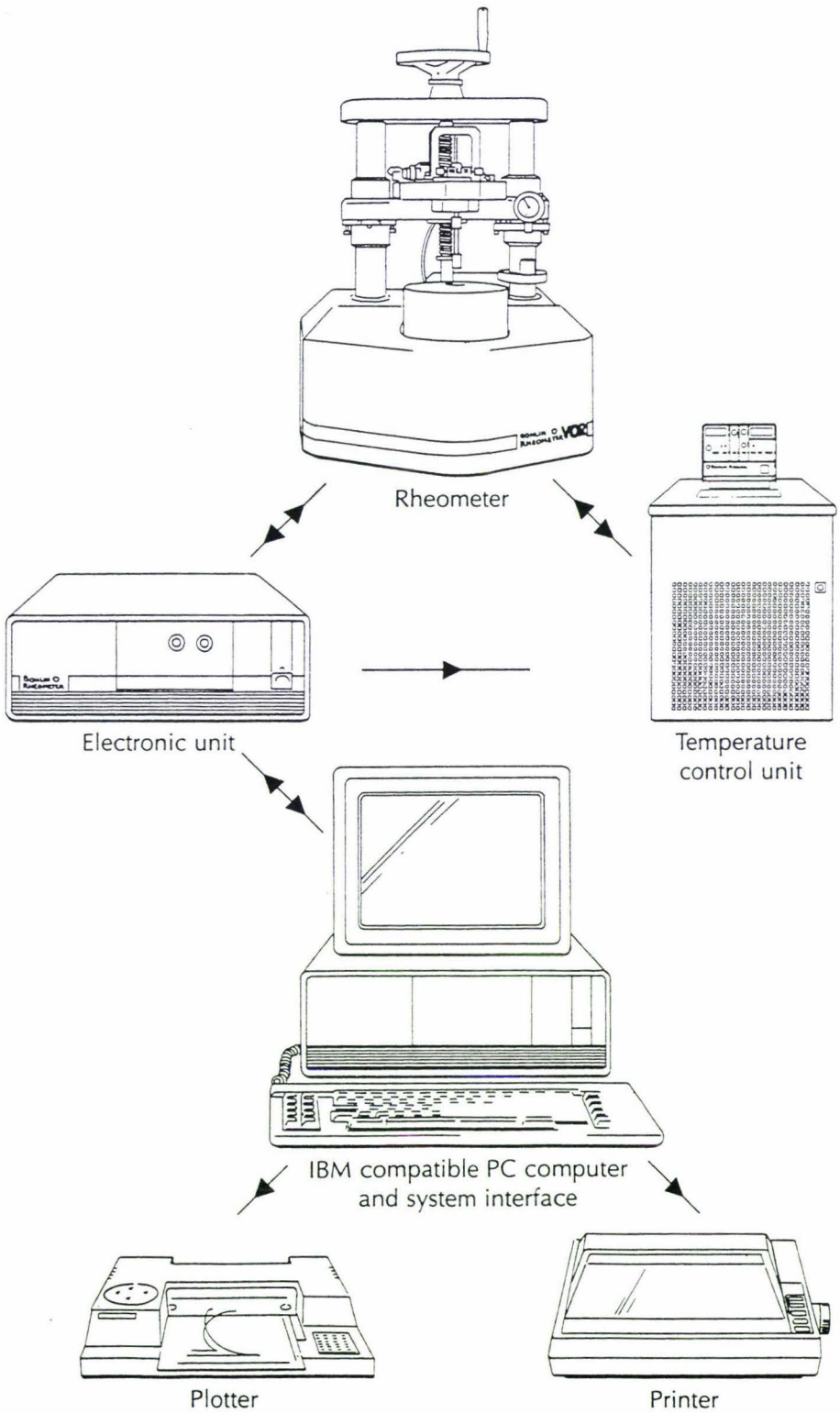

Fig. 3.3 The Bohlin Rheometer set-up with associated equipment.

During measurement in the oscillatory mode, the cup oscillates applying a sinusoidal deformation of defined amplitude and frequency (0.1 Hz for this particular test) for 16 hr. The amplitude of oscillation was 5% and the maximum strain applied was < 0.0103.

The effect of the time scale of deformation on the rheological properties was determined by frequency sweep (up and down) approximately 16 h after addition of GDL; frequency was varied from 0.001 to 0.1 Hz.

3.10 Incorporation of fat in acid gels

The samples of recombined milk (before and after acidification) were ultracentrifuged at 90,000 *g* for 1 h at 20 $\pm$ 2°C in a temperature controlled ultracentrifuge (Beckman, Model L2-65B ultracentrifuge, USA). After centrifugation, three fractions were obtained, i.e., cream (top) layer, middle layer and sediment. The top layer was removed from the centrifuge tube using spatula. The middle layer was then decanted off leaving behind the sediment. For one set of experiments, the three layers were weighed and sediment analysed for total fat content in order to determine how much fat was incorporated in the sediment or gel.

3.11 Microstructure (Confocal Scanning Laser Microscopy)

A Leica TCS 4D confocal scanning laser microscope (Leica Laser technik mbH, Heldelbars, Germany) with a 100 x 1.4 oil immersion objective and was used to monitor the gel structure in acid-induced milk gels. After addition of GDL to recombined milk, either Fast Green FCF (Merck, Damstradt, Germany) or Nile blue (hydrogen sulphate, BDH Chemicals Ltd, England) was added to acidified milk. Then this mixture was incubated for 16 hr at 30°C in a slide glass (with a cavity in the centre) covered with cover glass. The confocal microscope had an air cooled Ar/Kr laser that was used with an excitation wavelength of 568 nm.

CHAPTER 4.
RESULTS AND DISCUSSION

Recombined milks containing fat globules with modified membranes were prepared by mixing reconstituted skim milk and emulsion systems, as described in Chapter 3 (Fig. 3.2). The emulsion systems were selected to have different membrane material adsorbed at the fat globule surface, namely casein micelles, soluble caseins, native whey proteins, denatured whey proteins, Tween 60 and various combinations.

4.1 Characterisation of emulsion systems

The composition and size of fat globules as well as the protein load of the emulsions were determined prior to measuring the effects of different type of fat globule membranes on the properties of acid milk gels. A standard emulsion system was necessary to minimise other factors which could also influence the properties of gels. Emulsions were characterised by (a) volume-surface droplet diameter, d_{32}, (b) protein load and (c) proportions of individual proteins at the milk fat globule surface (using SDS-PAGE).

4.1.1 Size distribution of fat globules

Droplet size is the most important fundamental property used to characterise emulsions because it influences properties such as stability, viscosity and mouth-feel (Dickinson and Stainsby, 1988).

The effect of homogenisation pressure on volume-surface average droplet diameter, d_{32} (μm), for various emulsion systems is shown in Fig. 4.1.

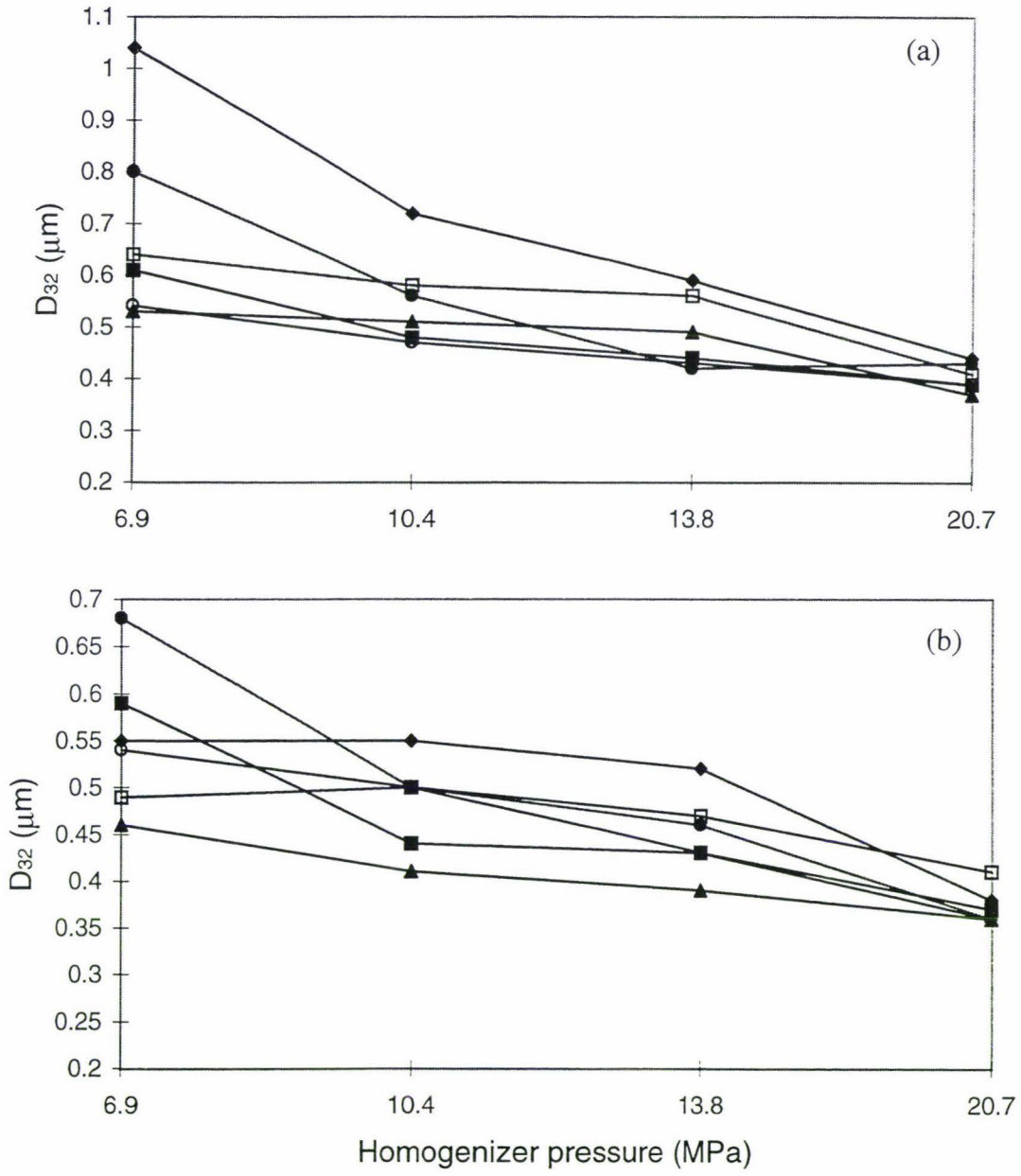

Fig. 4.1. Effect of homogenisation pressure (first stage) on the average droplet diameter (d_{32}) in emulsions containing 10% milk fat and stabilised by low-heat (-◆-), medium-heat (-■-), high-heat skim milk powder (-▲-), sodium caseinate (-⊟-), whey protein concentrate (-●-) or Tween 60 (-⊖-). Emulsions were diluted with (a) Milli-Q water or (b) 2% SDS buffer prior to particle size measurement.

The fat globule size distributions of emulsions were measured after dilution with Milli-Q water or dilution with 2 % SDS buffer. The d_{32} decreased with increasing homogenisation pressure, for both types of buffer conditions. When the emulsions were diluted with distilled water, the d_{32} for various emulsions varied from 1.04 to 0.54 μm when homogenised at 6.9 MPa. However, at 20.7 MPa the d_{32} for various emulsions varied to a smaller extent, i.e., from 0.66 to 0.48 μm. A smaller range of droplet diameters were observed when emulsions were diluted with SDS buffer, especially when using low homogenisation pressures. This may indicate some flocculation of droplets at low pressure.

de Wit and Klarenbeek (1984) reported that the size of fat globules affects various properties of protein gels. Xiong et al. (1991) reported that emulsions containing small-sized fat globule (i.e., also more globules) reinforced the gels more than emulsions comprised of large globules at an equal fat concentration, reflecting the importance of the size of globules for the preparation of gels. Therefore, a pressure of 20.7 MPa, which gave a relatively small range of fat globule size distributions for all samples, was chosen as this would minimise the effect of a variation in the sizes of fat globules on any gel properties.

4.1.2 Protein load

Since most adsorbed proteins form an interfacial film around oil droplets, it is important to know how much protein is present at the interface. The amount of protein present at the interface per unit surface of dispersed phase is termed as protein load and is usually expressed as milligrams of protein per unit area of the dispersed phase. The determination of protein load requires information on the total surface area of the emulsion (Walstra, 1987).

The protein content of various emulsions was relatively constant and it only varied from 1.82 % to 1.85 % (w/w) for all the emulsions. The protein load (mg/m^2) of emulsions made with different types of protein materials is shown in Table 4.1. Emulsions containing SMP had higher protein loads than other types of emulsions. Emulsions made with low-heat SMP had the highest protein load (7.05 mg/m^2), while those made with WPC had the lowest load (1.13 mg/m^2). The large differences in protein load observed between different emulsions could be due to adsorption of large particles (casein micelles) on to the fat globules in emulsions made with SMP. In contrast, Na caseinate and WPC solutions contain "soluble" proteins which are smaller protein particles and they form more compact surface layers than casein micelles. This would result in lower protein loads.

Table 4.1 Protein load (mg/m^2) in emulsions containing 10% milk fat and different types of protein material.

Type of protein	Protein load (mg/m^2)
Low-heat SMP	7.05
Medium-heat SMP	4.64
High-heat SMP	5.09
Na caseinate	1.26
WPC	1.13
Heated WPC	1.99
Whole milk	2.00

If the protein load on fat globules was ~ 1 mg/m^2, it would suggest that protein molecules are fully unfolded or that there was adsorption of an extended polypeptide chain. If the protein load was ~ 2 mg/m^2, this would suggest that there may be adsorption of a monolayer of globular proteins or unfolded molecules that are adsorbed in the conformation of "trains", "loops", and "tails". A protein load > 3 mg/m^2 suggests that adsorption of aggregates of proteins or multilayers of proteins was occurring, although some higher molecular weight proteins may also give high protein loads (Mulvihill and Murphy, 1991). The protein loads obtained for WPC and Na caseinate stabilised emulsions are in agreement with earlier studies (Mulvihill and Murphy, 1991; Srinivasan, 1995) and suggest that these proteins are adsorbed in the form of a monolayer with some unfolding. The protein load of emulsions made with heated WPC was higher than the those made with unheated WPC. This is probably due to presence of denatured and aggregated whey proteins in the heated WPC, which subsequently adsorb on the fat globule surface.

The differences in protein loads between various types of SMP can be explained as follows: when emulsions are made using SMP, the fat globule surface is covered with some apparently intact micelles and some disaggregated casein micelles (Sharma et al., 1996). This disruption of micelles could occur either during homogenisation or due to spreading at the fat globule surface. The lower protein loads in medium-heat and high-heat SMPs stabilised emulsions as compared to low-heat SMP could be due to greater disintegration or spreading of the micelles at the fat globule surface. This may be due to some structural changes in the micelle structure during relatively high heat treatments used in the manufacture of medium-heat and high-heat SMPs, which makes them susceptible to greater disintegration or spreading during homogenisation.

4.1.3 The composition of proteins of fat globule membranes

The differences in individual proteins between washed cream layers and emulsions are shown in Fig. 4.2.

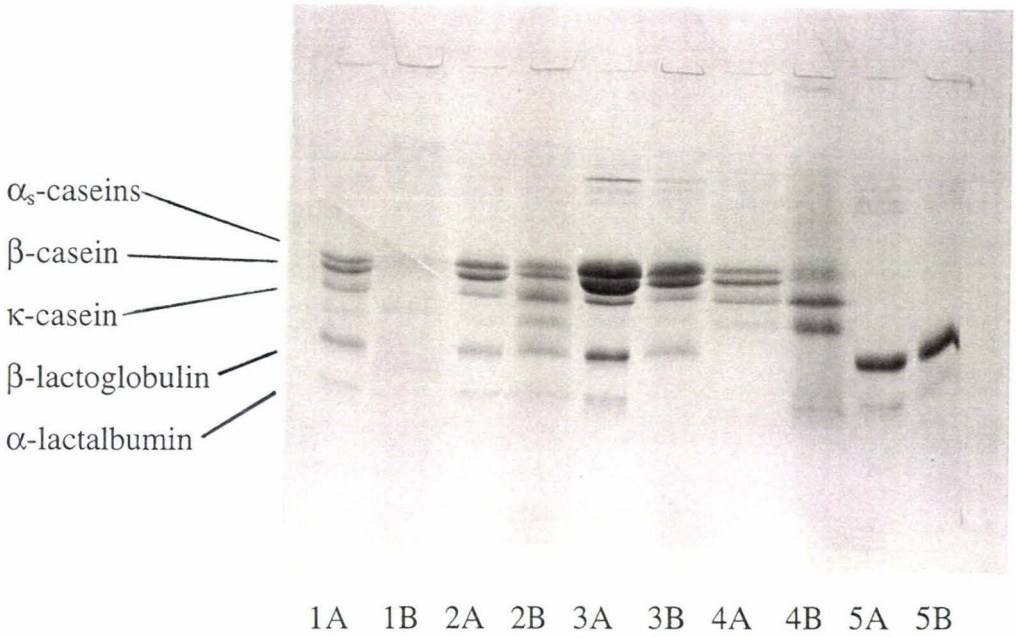

Fig. 4.2. SDS-PAGE of emulsions (A) and washed cream layers (B) made with low-heat skim milk powder (1), medium-heat skim milk powder (2), high-heat skim milk powder (3), sodium caseinate (4), whey protein concentrate (5), respectively.

The proportions of individual proteins in the cream layers (i.e. adsorbed protein) were quite similar to those in emulsions. Emulsions stabilised by SMP solutions contained both caseins and whey proteins adsorbed onto fat globule surface. Emulsions stabilised by high-heat SMP had a slightly higher proportion of whey proteins than the other emulsions stabilised by low- or medium-heat SMP. High-heat SMP contains a lower content of undenatured whey proteins than the others with the result that more denatured β–lactoglobulin (in particular) would

associate with casein micelles, via complexes with κ–casein, and have complexes would adsorb onto the fat globule surface during homogenisation.

Emulsion stabilised by WPC had both β–lactoglobulin than α-lactalbumin adsorbed onto globules. Na caseinate stabilised emulsions contained all caseins at the fat globule surface, but no whey proteins.

4.2 Composition of recombined milks

The methods of making recombined milk and acid gels are described in Chapter 3. The protein, fat and total solids contents of recombined milks containing fat globules stabilised by different types of materials are shown in Table 4.2.

The protein content was adjusted to values in the range 5.1 to 5.4% to minimise differences between samples; except for the recombined milk containing fat globules stabilised by Tween 60 in which the protein content was 4.16% (since only a small concentration of Tween 60 was needed as an emulsifier in this system).

The fat content was adjusted to values in the range 3.3 to 3.5%. The total solids contents varied from 13.3 to 16.5% between different samples as a result of constant protein and fat contents. The average fat globule size (d_{32}) in these system varied from 0.66 to 0.48 μm.

Table 4.2 Protein, fat and total solids contents of recombined milks prepared by mixing unheated or heated (80 °C for 30 min) reconstituted skim milk and emulsions stabilised by various materials.

Type of emulsion used	Reconstituted skim milk	Protein (%)	Fat (%)	Total solids (%)
Low-heat SMP	Unheated	5.33	3.39	16.00
	Heated	5.20	3.45	15.89
Medium-heat SMP	Unheated	5.22	3.32	16.42
	Heated	5.17	3.43	16.46
High-heat SMP	Unheated	5.27	3.47	15.69
	Heated	5.06	3.31	15.78
Sodium caseinate	Unheated	5.23	3.52	14.47
	Heated	5.30	3.44	14.63
WPC	Unheated	5.23	3.54	13.78
	Heated	5.12	3.47	13.91
Heated WPC	Unheated	5.28	3.43	14.55
	Heated	5.54	3.44	14.89
Tween 60	Unheated	4.16	3.51	13.34
	Heated	4.18	3.55	13.45
Fresh cream	Unheated	5.12	3.53	15.28
	Heated	5.13	3.52	15.51

4.3 Rheological properties of acid milk gels made from recombined milks

4.3.1 Penetration test

To evaluate the effects of heat treatment of reconstituted skim milk and type of fat globule membrane on gel firmness, a penetration method was used. The results of the penetration test are shown in Table 4.3. The load at the first breaking point was recorded. All experiments were done at least in triplicate and means and standard deviations are presented.

Table 4.3 Load at fracture of acid milk gels made from recombined milks containing fat globules stabilised by various materials.

	Load at fracture (N)	
Type of emulsion used	Unheated reconstituted skim milk	Heated (80 °C for 30 min) reconstituted skim milk
Low-heat SMP	0.261 ± 0.039	0.353 ± 0.007
Medium-heat SMP	0.179 ± 0.009	0.419 ± 0.053
High-heat SMP	0.153 ± 0.018	0.433 ± 0.023
Sodium caseinate	0.338 ± 0.015	0.732 ± 0.091
WPC	0.251 ± 0.004	0.353 ± 0.020
Heated WPC	0.268 ± 0.019	0.538 ± 0.066
Tween 60	0.151 ± 0.003	0.255 ± 0.027
Fresh cream	0.088 ± 0.085	0.300 ± 0.013

In general the gels made from heated reconstituted skim milk had a higher firmness compared to unheated gels, essentially in agreement with earlier studies (Kalab et al., 1976; Parnell-Clunies et al., 1986; Mottar et al., 1989), who observed that heating increased the firmness of acid (yoghurt) gels.

In the unheated systems, gels containing fat globules stabilised by high heat-SMP had lower firmness than those stabilised by low heat-SMP. However, in heated skim systems, the opposite trend was observed. For both unheated and heated skim systems, the firmness of gels containing fat globules stabilised by Na caseinate was the highest.

In the present study, the gel firmness in acid gel system followed a slightly different pattern: Na caseinate > WPC $\simeq$ low heat-SMP > medium heat-SMP > high heat-SMP > Tween 60.

Acid gels containing fat globules stabilised by WPC had lower firmness in both unheated and heated systems than gels stabilised by heated WPC, the effect being much more pronounced in the heated system. Acid gels made from recombined milk containing fat globules stabilised by Tween 60 or natural membrane material (fresh cream) had very low firmness, suggesting that these fat globules did not interact with the gel matrix as they contained non-protein membranes.

Xiong and Kinsella (1991a, b) reported that milk fat emulsions stabilised by whey protein isolate (WPI) resulted in the strongest gels in acid skim gels formed by heating cold acidified milk. Emulsions stabilised by Na caseinate and low heat-SMP had the next highest firmness, using compressive test, while Tween 80 emulsified milk fat only slightly increased the gel firmness. They calculated

compressive stress from the peak force divided by the initial area of the cross section of the gel. Xiong and Kinsella (1991a, b) used WPI (> 95% protein) instead of WPC (81% protein) used in this study. It appears, therefore, that the strength of acid gels is determined by the type of fat globule membrane, and fat globules play a structural role in acid gels.

Aguilera et al. (1993) reported that fat globules stabilised by WPI reinforced the gel structure, leading to higher compression strength in mixed WPI/SMP gels, while fat globules stabilised by Tween 20 did not interact with the protein-based gel network leading to weaker gels filled with isolated fat globules.

4.3.2 Dynamic low amplitude oscillatory rheology

The process of gel formation during the acidification of milk was monitored by dynamic rheological measurements. Most rheological parameters characterising casein gels (e.g., the dynamic moduli) depend on the number and strength of bonds between the casein particles and the spatial distribution of the strands making up these particles (Roef et al., 1990). In all cases, G' increased rapidly initially after gel formation and tended to plateau only after very long ageing periods (Figs. 4.3 and 4.4).

The G' values of heated skim gel systems, 16 hr after addition of GDL, were in the range 180 to 540 Pa, whereas unheated skim systems produced gels with G' values in the range of ~ 20 to 90 Pa (Figs. 4.3, 4.4 and Table 4.4). Lucey et al. (1997a) reported on the effects of heat treatment of milk on the rheological properties, at small and large deformations, of acid skim milk gels. They also found that heating milks at temperatures $\geq$ 80 °C increased the G' value compared to unheated milk (~ 15 Pa) and produced gels with G' values in the range 350 to 450 Pa (lower G' values than present study due to lower total solids content).

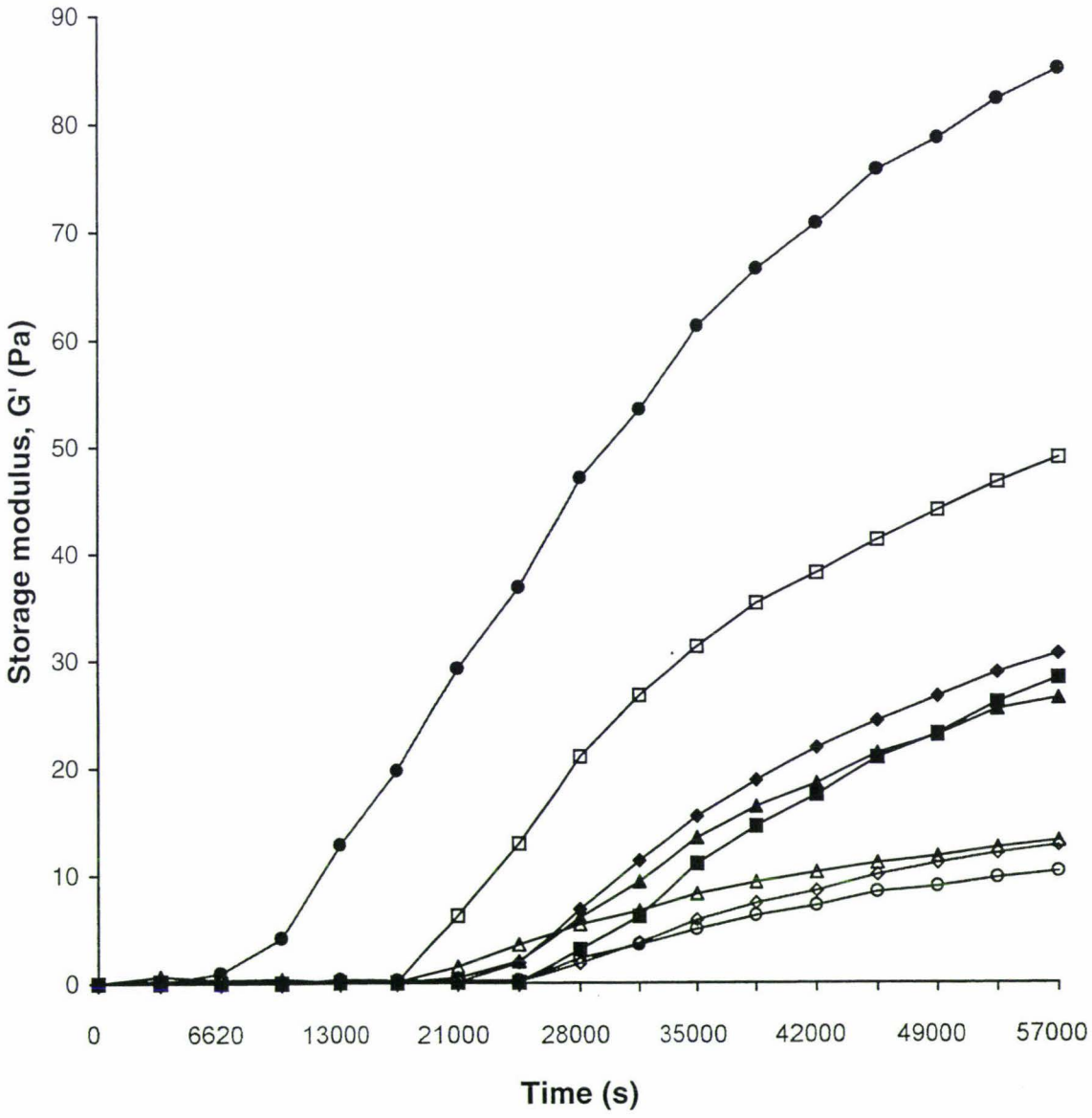

Fig. 4.3 Storage modulus (G') as a function of time for acid milk gels containing fat globules stabilised by low-heat (-◆-), medium-heat (-■-), high-heat skim milk powder (-▲-), sodium caseinate (-□-), whey protein concentrate (-◇-), heated whey protein concentrate (-●-), Tween 60 (-△-) or fresh cream (-○-) in unheated reconstituted skim milk system. Gels were made at 30°C with glucono-δ-lactone (GDL).

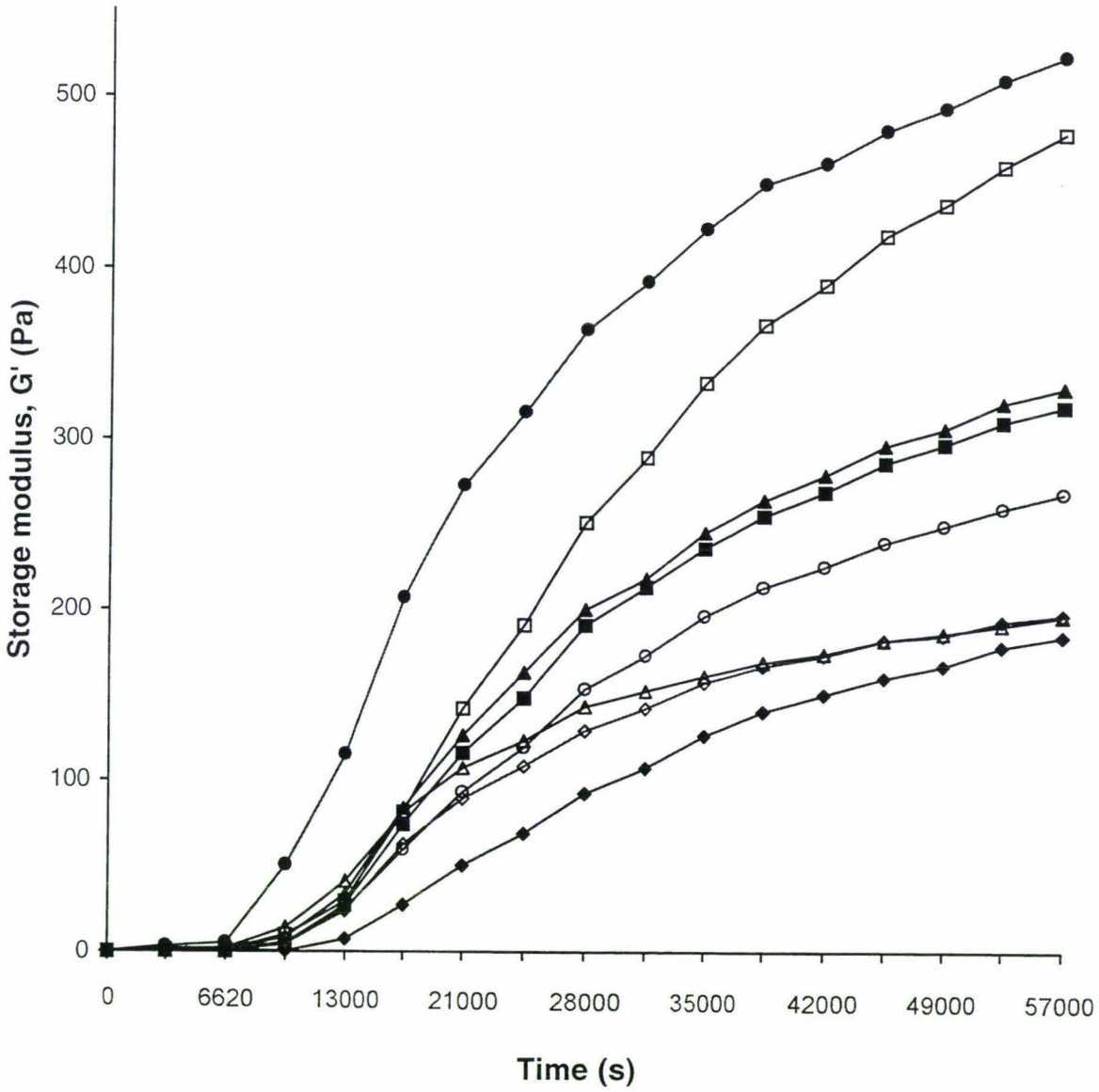

Fig. 4.4 Storage modulus (G') as a function of time for acid milk gels containing fat globules stabilised by low-heat (◆), medium-heat (■), high-heat skim milk powder (▲), sodium caseinate (⊟), whey protein concentrate (◇), heated whey protein concentrate (●), Tween 60 (△) or fresh cream (○) in heated reconstituted skim milk system. Gels were made at 30°C with glucono-δ-lactone (GDL).

Table 4.4 Storage modulus values (16 h after addition of GDL) of acid milk gels made from recombined milks containing fat globules stabilised by various materials.

	Storage modulus, G' (Pa)	
Type of emulsion used	Unheated reconstituted skim milk	Heated (80 °C for 30 min) reconstituted skim milk
Low-heat SMP	30	184
Medium-heat SMP	28	319
High-heat SMP	26	330
Sodium caseinate	49	478
WPC	13	197
Heated WPC	85	523
Tween 60	13	196
Fresh cream	10	268

A mechanism by which heating affects the properties of acid milk gels was recently proposed (Lucey et al., 1997a). They suggested denatured whey proteins in heated milk become susceptible to aggregation during acidification, as the isoelectric points of whey proteins are approached. Denatured whey proteins associated with casein micelle (or in the serum) after heat treatment could act as bridging material by interacting with other denatured whey proteins in acid skim milk gels. It was proposed that cross-linking, by denatured whey proteins, within gels made from heated milk should lead to an increase in the rigidity of the network; indeed a large increase in G' values was reported in heated milk (van Vliet and Keetels, 1995; Lucey et al., 1997a). van Vliet and Keetels (1995) also reported that acid skim milk gels made from reconstituted low heat-SMP had much lower dynamic moduli, higher fracture strain than gels made from high heat-SMP, in agreement with the results of Lucey et al. (1997a).

In recombined milk made from heated skim system, G' values (at 16 hr) of acid milk gels, containing fat globules stabilised by medium or high-heat SMP, were higher than gels containing fat globules stabilised by low heat-SMP (Fig. 4.4 and Table 4.4). This can be explained by differences in the composition of fat globule membranes, i.e. gels formed from high- and medium-heat SMP had higher concentration of denatured whey proteins on the fat globule membrane than those formed from low-heat SMP (Section 4.1.2). Possibly, the presence of denatured whey proteins on the fat globule membrane provided some additional cross-links in the gel. On the other hand, there were no significant differences among these gels in unheated skim milk systems (Fig. 4.3 and Table 4.4).

Gels made from recombined milk that had SMP-stabilised fat globules had lower G' values than gels containing Na caseinate-stabilised fat globules in either

unheated or heated skim milk systems (Fig. 4.3 and 4.4). In SMP casein is present as micelles, whereas the protein particles Na caseinate exists as small "soluble" casein aggregates. Differences in the state of aggregation could cause the different interactions between serum proteins and fat globules. Dickinson and Hong (1995) reported that one obvious effect of the dispersed fat phase is to concentrate the structure-forming protein molecules more effectively within the space available in heat-set whey protein gels. The higher the local protein concentration, the higher is the expected gel strength due to the greater density of structurally important cross-links (Mulvihill and Kinsella, 1987). In addition, Dickinson and Hong (1995) suggested that for strongly interacting adsorbed and nonadsorbed molecules, the viscoelastic protein layer around the emulsion droplets itself becomes an important load-bearing component of the total aggregated protein network structure. Therefore, smaller Na caseinate aggregates would have a greater total surface area and would be closer than larger casein micelle in SMP and so the number of interactions between fat globule membrane and gel network would be increased, which leads to an increased gel strength. By providing more contact or cross-linking zones than SMP stabilised fat globules, Na caseinate stabilised fat globules would have more potential protein interacting materials in acid gel system.

Acid gels made from recombined milk containing fat globules stabilised by heated WPC had the highest G' values for both heated and unheated milk systems, followed by acid gels containing fat globules stabilised by Na caseinate and then SMP. Heated WPC was considered to act as an interacting fat globule membranes during the formation of acid gels. Typically, it has been found experimentally that the incorporation of whey protein coated droplets does lead to a substantial increase in gel strength at constant overall protein content in whey protein gel (Yost and Kinsella, 1993; Dickinson and Hong, 1995). Higher G'

values of acid gels made from milk containing fat globules stabilised with heated WPC than those made form unheated WPC was probably due to denatured whey protein in heated WPC interacting with casein micelles and the gel matrix during gel formation while unheated WPC act as a inert filler and can even decrease the G' of acid gels. Lucey et al. (1997c) suggested that native (undenatured) whey proteins do not contribute to the gel matrix and may act as an inert filler in acid milk gels, however, addition of WPC to milk followed by heat treatment (WPC would denature which subsequently could interact with casein micelles or original whey proteins in milk) greatly increased G'.

Acid gels containing fat globules stabilised by WPC, Tween 60 or fresh cream showed a low G' value in unheated skim milk systems. It has been reported that a small molecule surfactant, e.g. Tween 60, does not cross-link with a milk protein gel network instead; it may even weaken the network (Yost and Kinsella, 1992, 1993; Dickinson and Hong, 1995). However, Xiong and Kinsella (1991b) reported that commercial light cream emulsified milk fat markedly reinforced milk gels. They studied commercial light cream since it contained natural fat globule membrane material, but during emulsion preparation the cream was homogenised with skim milk and the new fat globule membrane would be formed. Walstra and Jenness (1984) suggested that newly formed fat globule membranes by homogenisation contains primarily casein and some serum protein, which is different from natural fat globule membrane. In this study, acid gels made from fresh cream containing natural fat globule membrane did not appear to interact with casein matrix in unheated system. But, when fat globules from fresh cream were suspended in a heated skim milk system, the G' of gels increased considerably, indicating the importance of the heat-induced changes in milk proteins for gel properties.

Xiong and Kinsella (1991b) reported that low-heat SMP stabilised fat globules had lower G' values than either WPI or Na caseinate-stabilised fat globules, in gels formed by heating cold acidified milk. Milk fat emulsified with Tween 80 only had a small effect on the G' of acid milk gels in contrast to protein stabilised emulsions. They suggested that fat globules stabilised with Tween 80 were loosely entrapped in the gel matrix and act as an inert filler. This result supported the observations made by van Vliet and Dentener-Kikkert (1988) who showed that polyprovinyl alcohol-stabilised milk fat decreased G' of milk gels with increasing fat content.

Emulsified fat globules can fill the spaces between the matrix of whey protein milk gels (Aguilera and Kessler, 1988; Jost et al., 1986), but the effects of fat are determined by the composition of fat globule membrane. van Kleef et al. (1978) reported that the number of cross links in protein gels at equilibrium was proportional to the magnitude of G'. The increase in G' with time observed in this study could be attributed to increase in fusion and contact of casein particle which would increase the number of cross-links in the network. The presence of fat globules may increase the G' value via increasing the number of cross-links between the casein particles and the membrane around fat globules.

In experiments in which the time-scale of the applied deformation (frequency sweep) was varied, log G' versus log frequency gave linear curves with a slope of $\simeq$ 0.15, for various type of acid casein gels (Roef and van Vliet, 1990; Lucey et al., 1997a). In this experiment, log G' versus log frequency gave straight lines with slope of ~ 0.16 (Table 4.5 and Figs. 4.5 and 4.6). The slopes obtained from gels made from unheated skim milk systems had slightly higher values than gels from heated skim milk systems. At low frequency (i.e. over long time scale) G' decreased probably due to relaxation of some bonds that contributed to the G'

measurement over shorter time scale. Physical gels (including milk protein particle gels) usually have a frequency for their viscoelastic component (e.g. G') whereas chemical polymer gels are independent of frequency (Ferry, 1980).

Table 4.5 The slope of log G' versus log frequency curves of acid milk gels containing fat globules stabilised by various materials.

	Slope	
Type of emulsion used	Unheated reconstituted skim milk	Heated reconstituted skim milk
Low-heat SMP	0.16	0.15
Medium-heat SMP	0.16	0.14
High-heated SMP	0.16	0.14
Sodium caseinate	0.16	0.15
WPC	0.14	0.14
Heated WPC	0.13	0.13
Tween 60	0.17	0.14
Fresh cream	0.17	0.14

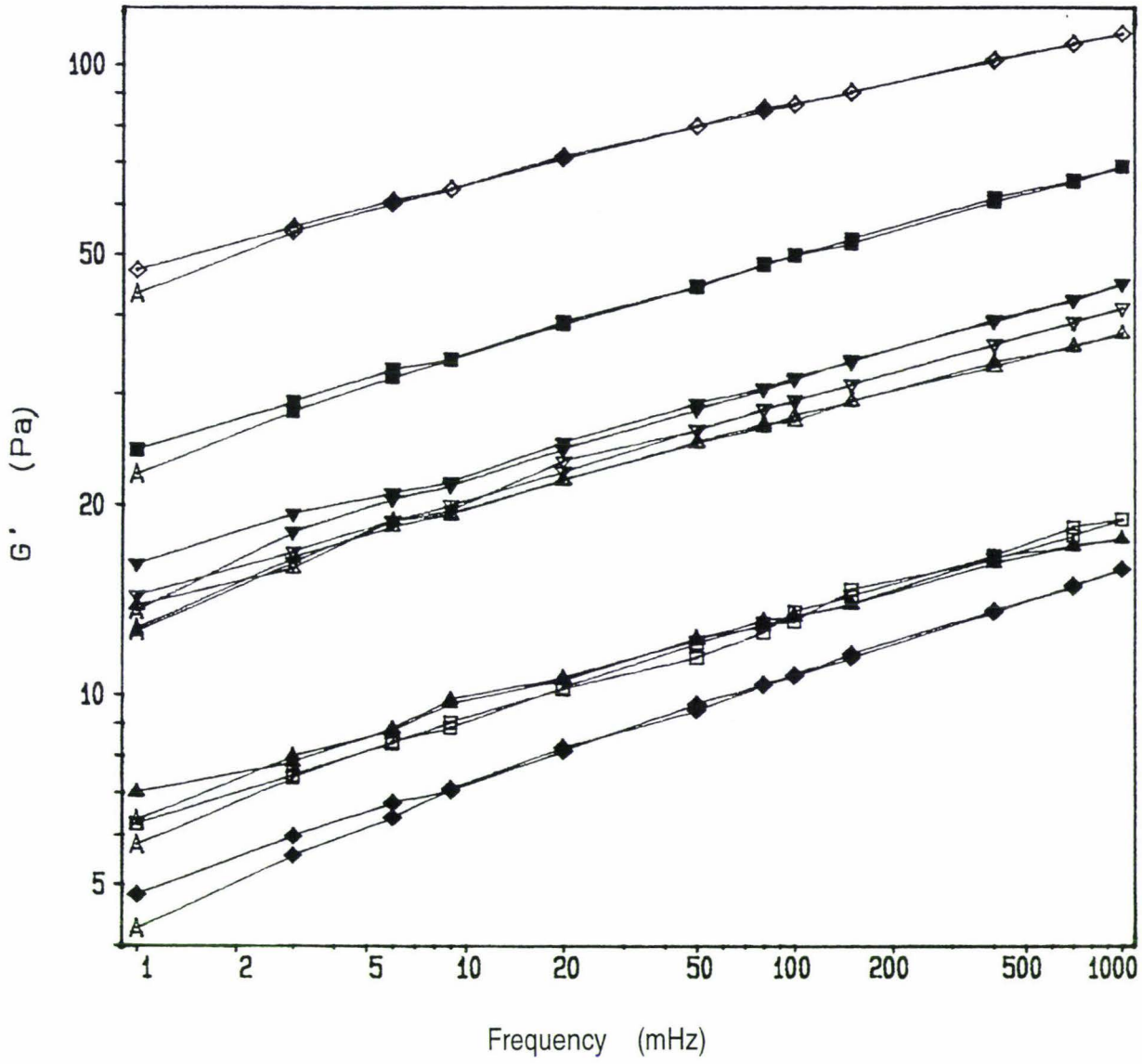

Fig. 4.5 The slope of log G' versus log frequency curves of acid milk gels containing fat globules stabilised by low-heat (▲), medium-heat (▽), high-heat skim milk powder (△), sodium caseinate (■), whey protein concentrate (▲), heated whey protein concentrate (◇), Tween 60 (□) or fresh cream (◆) in unheated reconstituted skim milk. Gels were made at 30°C with glucono-δ-lactone (GDL) and frequency sweep performed 16 h after addition of GDL.

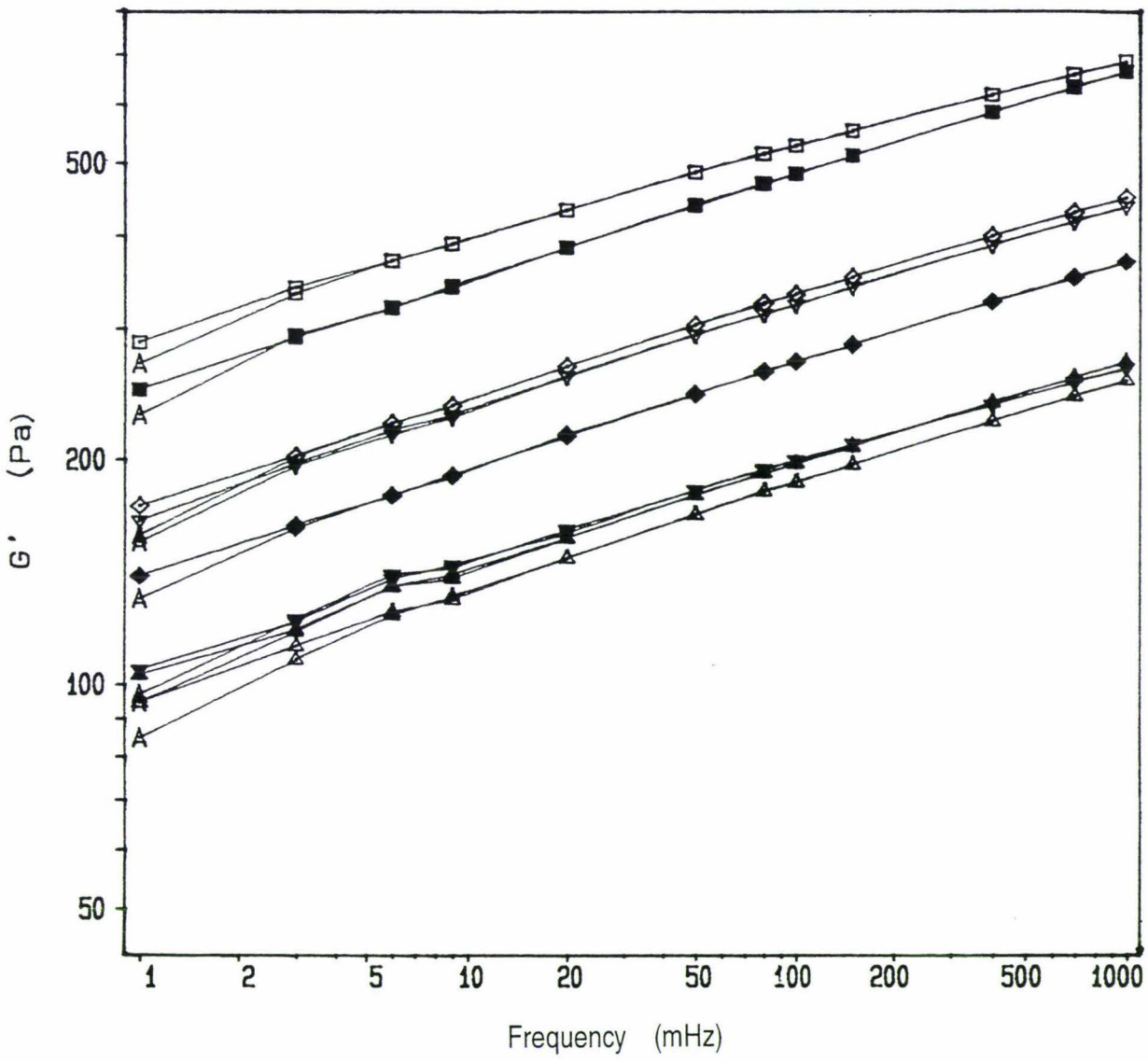

Fig. 4.6 The slope of log G' versus log frequency curves of acid milk gels containing fat globules stabilised by low-heat (△), medium-heat (▽), high-heat skim milk powder (□), sodium caseinate (■), whey protein concentrate (▼), heated whey protein concentrate (◇), Tween 60 (▲) or fresh cream (◆) in heated reconstituted skim milk. Gels were made at 30°C with glucono-δ-lactone (GDL) and frequency sweep performed 16 h after addition of GDL.

The results of the penetration test are in agreement with those of oscillation tests about the effect of heat treatment on the firmness of acid milk gels. In general the gels made from heated reconstituted skim milk had a higher firmness compared to unheated gels. However, the results of the penetration test were variable and did not show same trends from the oscillation tests about the effect of different type of fat globule membrane materials on the firmness. Several workers (Paulson and Tung, 1989; Tang et al., 1995) reported that the relationship between destructive penetration tests and non-destructive dynamic shear measurements was poorly correlated in various types of gels. Even though penetration test is easy to perform and quickly provides information, it is empirical so provides no information on fundamental rheological properties (Tang et al., 1995).

4.4 Gelation time and pH

The effects of the type of fat globule membrane material and heat treatment of skim milk on the gelation time and pH at gelation are shown in Figs. 4.7 and 4.8, respectively. Both the pH of gelation and gelation time were influenced by heat treatment of reconstituted skim milk with an increase in the pH at gelation and a reduction in the gelation time. These findings are in agreement with those reported by other authors (Heertje et al.; 1985; Horne and Davidson; 1993; Cobos et al., 1995 a, b; Lucey et al., 1997a).

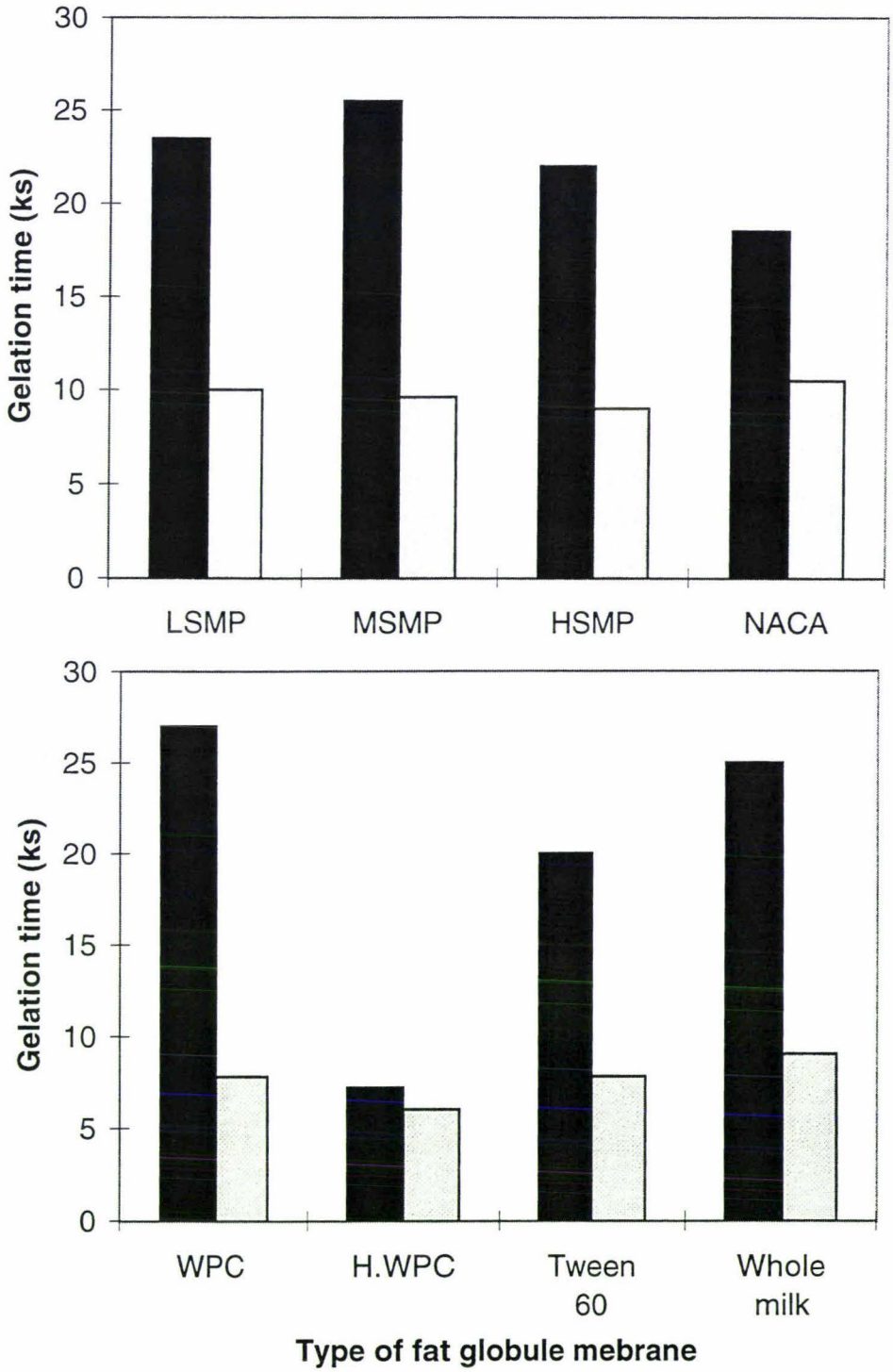

Fig. 4.7 Gelation time of acid milk gels containing fat globule stabilised by various materials, unheated (■) and heated (□) reconstituted skim milk during acidification at 30 °C by GDL.

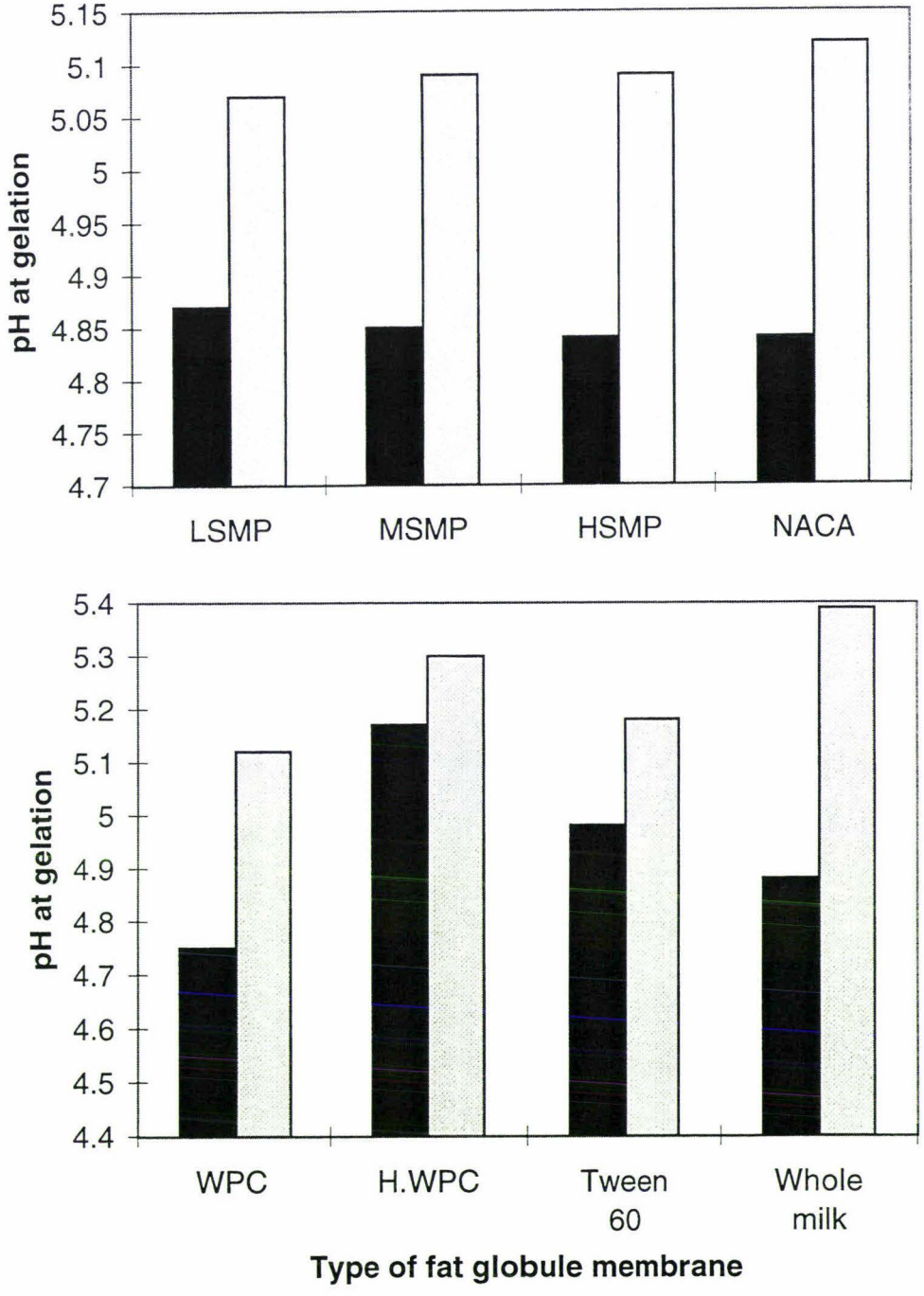

Fig. 4.8 pH at gelation of acid milk gels containing fat globule stabilised by various materials, unheated (■) and heated (□) reconstituted skim milk during acidification with GDL at 30 °C.

Horne and Davidson (1993) proposed that heating denatures the whey proteins and these denatured whey proteins diminishes the capacity of κ-casein to stabilise casein micelles, allowing these particles to coagulate at a higher pH and consequently a shorter time is required to reach this pH. However, Lucey et al. (1997a) proposed that in heated milk denatured whey proteins in the serum, as well as those associated with the casein micelles, may become susceptible to aggregation during acidification, as the isoelectric points of whey proteins are approached. Because the isoelectric points of whey proteins are higher than those of casein (e.g. β-lactoglobulin pH $\simeq$ 5.2), gelation occurs at a higher pH value and in a shorter time.

No significant differences were observed in the gelation between acid milk gels containing fat globules stabilised by low-heat, medium-heat or high-heat SMP. Whereas acid milk gels containing fat globules stabilised by heated WPC had very short gelation time especially in heated systems.

The pH at gelation showed similar trends, i.e. higher pH at gelation in heated systems compared to unheated ones. Acid milk gels containing fat globules stabilised by heated WPC had a very high pH at gelation compared to the other systems, suggesting that denatured whey proteins do contribute to the increased pH of gelation of acidified milk.

4.5 Incorporation of fat globules in acid gels

The fat contents of sediment, obtained after ultracentrifugation of recombined milks (before and after acidification) at 90,000 *g* for 1 h at 20 ± 2°C, were measured (Table 4.6). Generally, the fat content of sediments increased rapidly around the pH at gelation, after that it remained relatively constant (data not presented). A mass balance for the three fractions (i.e., sediment, middle and cream layers) was also determined as a function of pH and this showed quite similar results for all types of emulsified samples (data not presented).

During ultracentrifugation, casein micelles should sediment along with any associated fat globules. Only very small amounts of fat were sedimented at high pH values (i.e., before gelation occurred ≃ pH 6.7). All samples showed similar trends, i.e. no increase in the amount of sedimented fat was observed until near the pH at gelation. All recombined milks that were made from heat-treated skim milk system had a higher pH of gelation and an earlier increase in the sedimentable fat content than unheated emulsions. This means fat globules associated with casein matrix during acidification even though not much change observed for non-interacting fat globules (i.e. containing WPC, Tween 60 or fresh cream).

Table 4.6 Fat content of sediments from recombined milk containing different types of fat globule membrane before and after acidification with GDL at 30 °C. Sediments were produced as a result of ultracentrifugation at 90,000 *g* for 1h at 20°C.

Type of emulsion used	Type of skim milk	Fat content of the sediment (%)	
		Prior to gelation	After gelation
Low-heat SMP	Unheated	0.69	16.03
	Heated	0.09	18.89
Medium-heat SMP	Unheated	0.80	20.70
	Heated	0.52	24.57
High-heat SMP	Unheated	0.75	17.33
	Heated	0.59	25.27
Sodium caseinate	Unheated	0.42	9.46
	Heated	0.34	14.53
WPC	Unheated	0.63	12.91
	Heated	0.76	15.49
Heated WPC	Unheated	0.66	14.55
	Heated	0.61	17.89
Tween 60	Unheated	0.67	10.12
	Heated	0.62	18.27
Fresh cream	Unheated	0.12	12.53
	Heated	0.34	14.75

4.6 Microstructure of acid milk gels

Confocal scanning micrographs of acid milk gels containing different types of fat globule membrane material are shown in Figs 4.9 to 4.12.

Acid gels made from recombined milk with unheated reconstituted skim milk appeared to have larger protein clusters as compared to gels made from those made with heated reconstituted skim milk (Kalab et al., 1976; Parnell-Clunies et al., 1987; and Mottar et al., 1989). Especially in gels containing high-heat SMP (Fig. 4.10) or WPC (Fig. 4.11), there appeared to be a greater difference between unheated and heated skim milk systems than in gels made from milk containing low-heat or medium-heat SMP. These observations were in agreement with the results of Kalab et al. (1976), Parnell-Clunies et al. (1987) and Mottar et al. (1989). Differences between gels containing other types of fat globule membranes were not obvious, which suggests the heat treatment is more important factor in the structure of acid gels than fat globule membrane. The presence of both fat and protein in milk gels probably observed the details of the gel network, as the strands and clusters were very obvious in skim milk gels.

Lucey et al. (1997b) using CSLM found that heating milks at $\geq$ 80 °C resulted in a microstructure of acid skim milk gels that appeared 'branched' and had a higher 'apparent interconnectivity' (in the thin optical sections of the x-y plane) of aggregates compared to unheated or less severely heated milks, which had tortuous, bent or irregular clusters and strands making up the gel network and less 'apparent interconnectivity' of strands and clusters. There did not appear to be major differences in the microstructure of acid milk gels formed from milk heated in the range 80 to 90°C. van Vliet and Keetels (1995) suggested (from rheological and permeability data) that heat treatment of milk resulted in 'straightening' of the

strands in the gel network compared to unheated milk, which were reported to have tortuous strands.

Various electron microscopy studies (Aguilera and Kessler, 1988; Xiong et al., 1991; Aguilera et al., 1993) have reported on the interaction between fat globule membrane and gel matrix during acidification. Xiong et al. (1991) observed that the proteins (i.e., low-heat SMP) stabilised fat globules interacted with the casein particles in the gel to form a cross-linked matrix. They suggested that the effect of milk fat globules on milk gels was somewhat similar to that of casein particles, i.e. they could serve as basic units of the matrix during the formation of a network. Aguilera et al. (1993) observed that fat globules having a protein-based membrane became an integral part of the protein strands of a mixed WPI/SMP gels, but fat globules stabilised with Tween 20 did not interact with the network. However, the results of the present study cannot be compared directly with previous studies due to the different gelation temperatures and modes of acidification.

Confocal images can only be cautiously compared with those obtained by electron microscopy because CLSM shows the overall gel structure but not the details of the individual casein aggregates. Electron microscopy studies of acid casein gels have demonstrated that these gels consist of a coarse particle network. The network has pores or void spaces where the aqueous phase is confined. The diameter of these pores can vary from 1 -30 μm, with larger pores in gels made at high gelation temperatures and from milk with low protein content (Kalab et al., 1976, 1983; Davies et al., 1978; Parnell-Clunies et al., 1987; Mottar et al., 1989). Many of the preparation steps used in elecrton microscopy remove or alter fat globules, making it difficult to directly compare with CSLM.

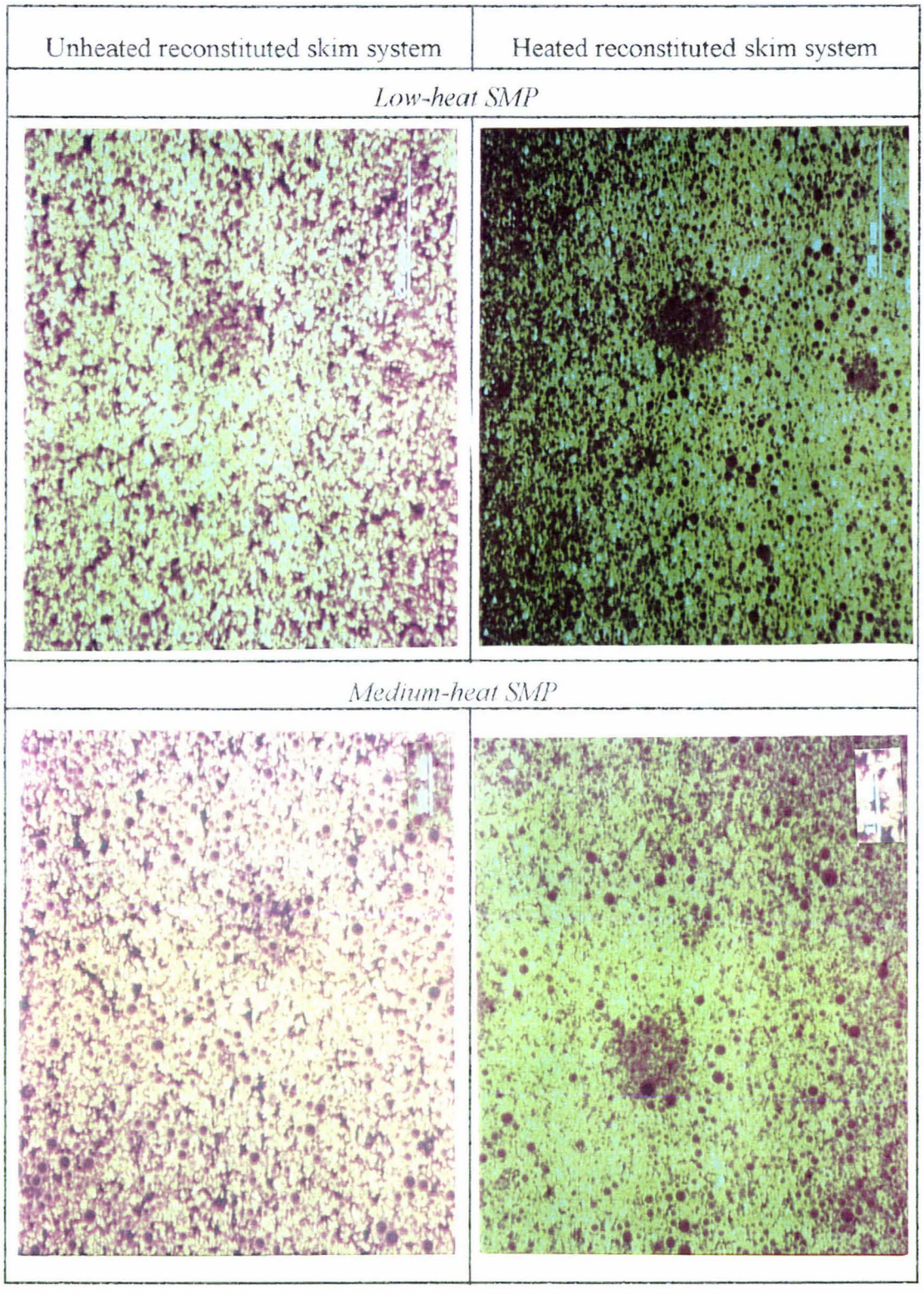

Fig. 4.9 Confocal scanning laser micrographs of acid milk gels containing fat globules stabilised by low-heat or medium-heat skim milk powder. Bar = 20μm (low-heat skim milk powder) and 10μm (medium-heat skim milk powder), protein is dyed and is the fluorescent material. Fat globules appear as dark spherical droplets.

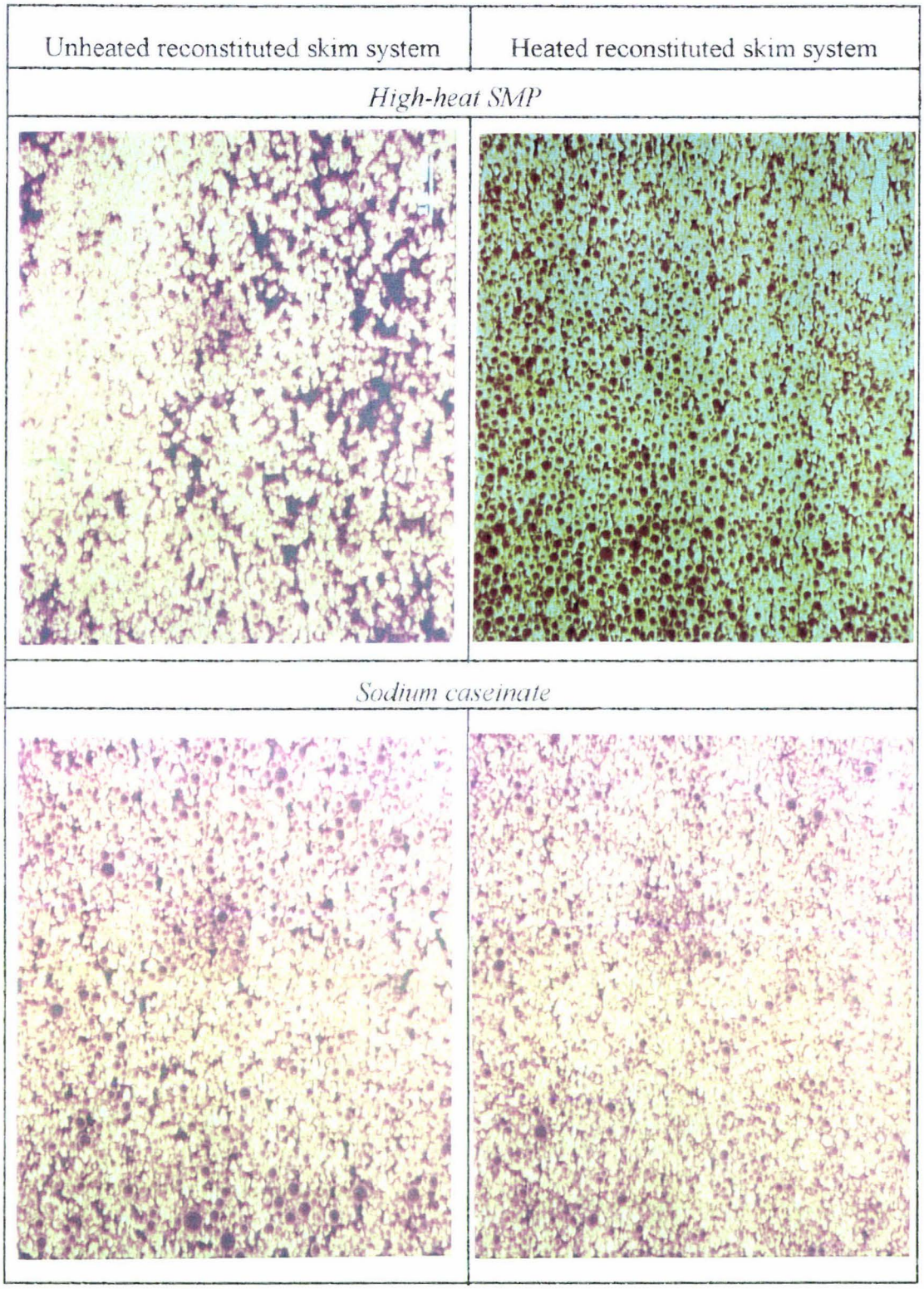

Fig. 4.10 Confocal scanning laser micrographs of acid milk gels containing fat globules stabilised by high-heat skim milk powder or sodium caseinate. Bar = 10μm, protein is dyed and is the fluorescent material. Fat globules appear as dark spherical droplets.

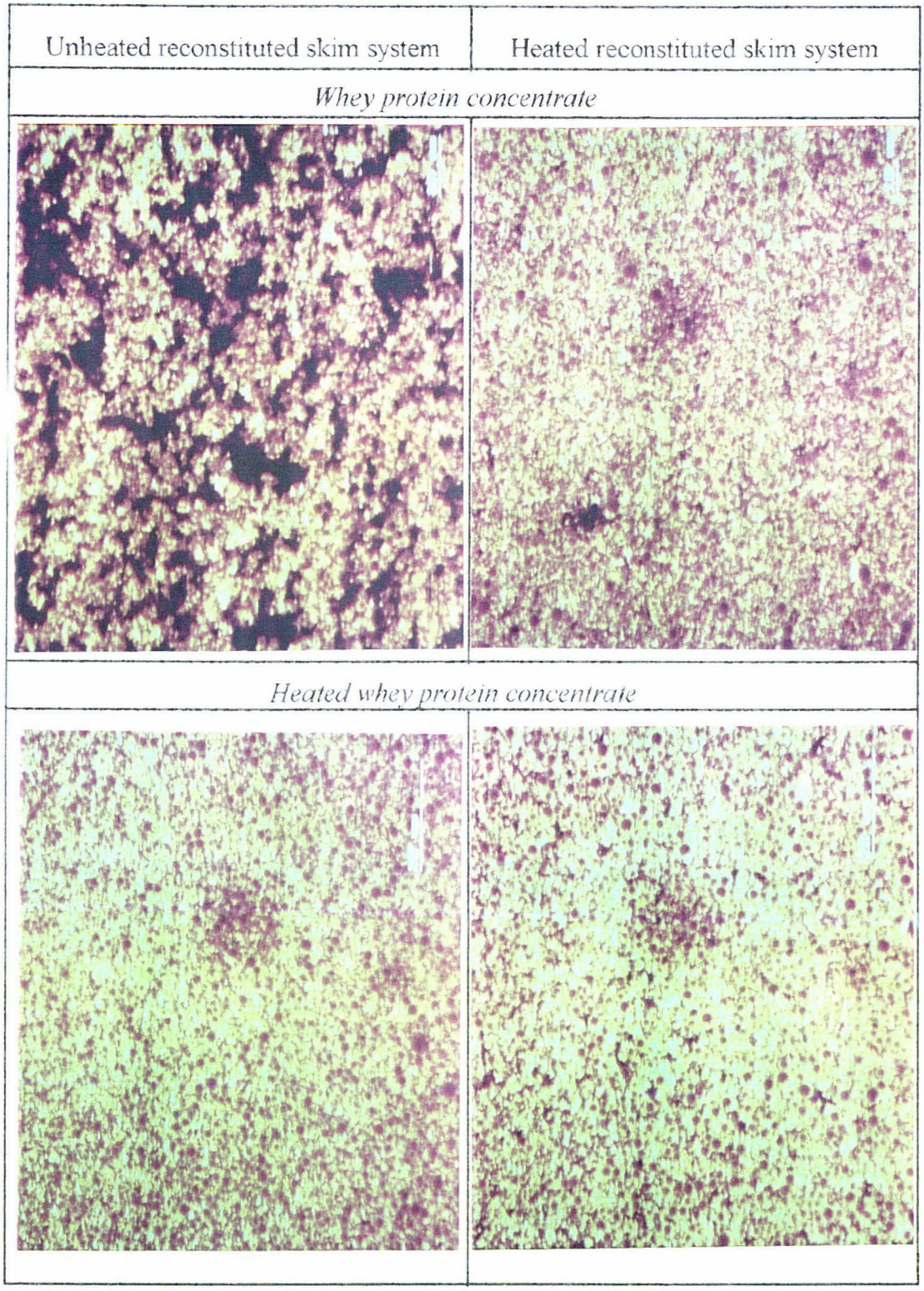

Fig. 4.11 Confocal scanning laser micrographs of acid milk gels containing fat globules stabilised by whey protein concentrate or heated whey protein concentrate. Bar = 10μm (whey protein concentrate) and 20μm (heated whey protein concentrate), protein is dyed and is the fluorescent material. Fat globules appear as dark spherical droplets.

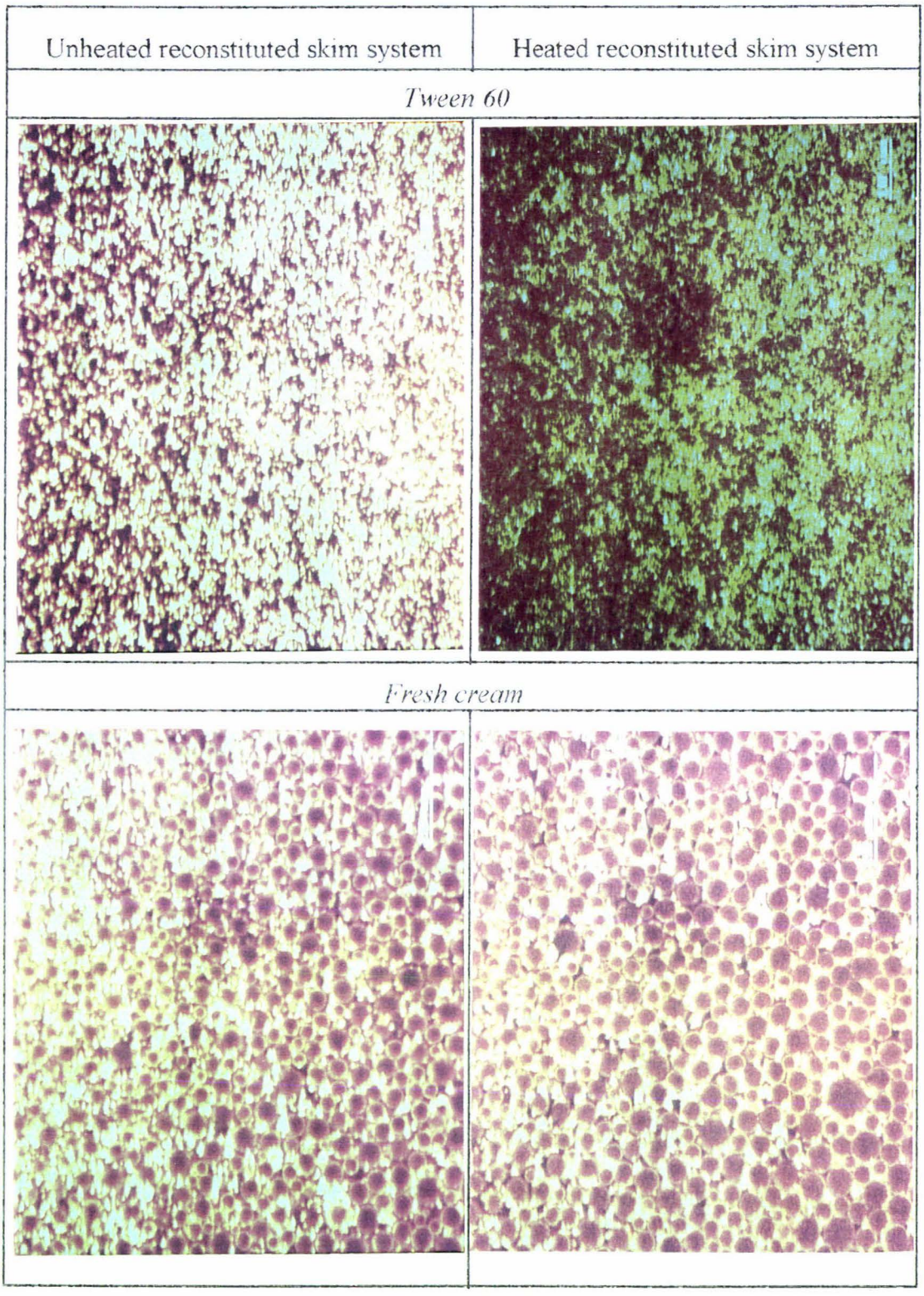

Fig. 4.12 Confocal scanning laser micrographs of acid milk gels containing fat globules stabilised by Tween 60 or fresh cream. Bar = 20μm, protein is dyed and is the fluorescent material. Fat globules appear as dark spherical droplets.

CHAPTER 5.
OVERALL DISCUSSION AND CONCLUSIONS

This study has shown that the artificial protein membrane around the fat globules and the heat treatment of reconstituted skim milk strongly influence the rheological properties and microstructure of acid milk gels.

Acid milk gels were prepared from recombined milks containing fat globules membrane material that should interact with the acid casein-based gel network (low-heat, medium-heat or high-heat SMP, Na caseinate or heated WPC) or material that should not interact with the gel matrix (WPC, Tween 60 or the native fat globule membrane in fresh cream). Since, heat treatment of milk is known to influence the properties of acid milk gels (van Vliet and Keetels, 1995; Lucey et al., 1997a), reconstituted skim milk or skim milk heated at 80 °C for 30 min was used in the preparation of recombined milks. The rheological and microstructural properties of acid gels were determined.

The G' values of recombined milks that were made from heated reconstituted skim milk systems ranged from ~ 180 to 530 Pa, whereas unheated skim systems produced gels with G' values in the range ~ 20 to 90 Pa. Lucey et al. (1997a) reported on the effects of heat treatment of milk on the rheological properties at small and large deformations of acid skim milk gels. Heating milks at temperatures $\geq$ 80 °C increased the G' value compared to unheated milk (~15 Pa) and produced gels with G' values in the range 350 to 450 Pa (lower G' values

than present study due to lower total solids content). The present results confirmed that heat treatment has a strong influence on the rheological properties of acid milk gels.

Acid gels made from recombined milk containing fat globules stabilised by heated WPC had the highest G' values for both heated and unheated milk systems, followed by acid gels containing fat globules stabilised by Na caseinate and then SMP. It was confirmed that these materials acted as interacting fat globule membranes during the formation of acid gels. Higher G' value of acid gels made from milk containing fat globules stabilised with heated WPC than those made form unheated WPC was probably due to denatured whey protein in heated WPC interacting with denatured whey proteins (in serum) or with casein micelles and participating in the gel matrix, while unheated WPC acted as a filler and even caused a slight decrease the G' of acid gels. Lucey et al. (1997c) suggested that in skim milk systems, native (undenatured) whey proteins do not contribute to the gel matrix and may act as an inert filler in acid milk gels. However, addition of WPC to milk followed by heat treatment (WPC would denature which subsequently could interact with casein micelles or original whey proteins in milk) increased G' greatly.

From the results of acid gels containing fat globules stabilised by Na caseinate or SMPs, it seems that the molecular or aggregation state of caseins, whether in the micellar or soluble complex form, was important in determining their functionality in agreement with the results of Mulvihill and Murphy (1991). SMP contains micellar casein and whey proteins, whereas Na caseinate contains soluble casein complexes. In acid gel systems, SMP stabilised fat globules provide less contact or cross-linking zones than Na caseinate stabilised fat globules. Thus, the gel firmness would be higher in Na caseinate than in SMP

systems. Acid gels containing fat globules stabilised by low-heat, medium-heat or high-heat SMP showed similar G' values in unheated skim milk systems. However, acid gels stabilised by low-heat SMP had lower G' values in heated systems. Fat globules stabilised by low-heat SMP had lower level of whey proteins in the membrane compared to the other types of SMP.

Fat globules that had a non-interacting membrane, e.g. Tween 60 or unheated WPC, did not have high G' values suggesting that this type of fat globule membrane material weakened the rigidity of the gel and was not involved in forming contact zones or cross-linkages with the casein-based matrix. Acid gels made from fat globules containing natural membrane material did not appear to interact with casein matrix in unheated system. But, when fat globules from whole milk were suspended in a heated skim milk system the G' of gels increased considerably due to heat-induced interaction between milk proteins.

For all samples both the pH of gelation and gelation time were influenced by heat treatment of reconstituted skim milk, i.e. heating increased the pH of gelation and decreased the gelation time. These findings are in agreement with those reported by other workers (e.g., Horne and Davidson; 1993; Lucey et al., 1997a).

Acid gels made from unheated reconstituted milk appeared to be formed from irregular clusters and strands, interspersed with fat globules. Recombined fat globules appeared to be embedded in the matrix. However, a more crowded structure was observed than in unheated skim systems. Differences between gels containing different types of fat globule membranes were not clear. However, electron microscopy studies have showed that fat globules encapsulated in an artificial protein membrane, resulted in the formation of gel where the fat globules become integrated (embedded) into the protein matrix (Kalab et al.,

1976; Tamime et al., 1984; Harwalkar and Kalab, 1986; Tamime et al., 1995; Aguilera and Kessler, 1988, 1989). Therefore, further work is required to distinguish differences between gels using EM, because information on the finer structure of gels is only possible with EM.

Based on the results of this study and work of others (van Vliet and Dentener-Kikkert, 1982; van Vliet, 1988; Aguilera and Kessler, 1989; Xiong and Kinsella, 1991a, b; Aguilera et al., 1993), possible mechanisms of fat globule interactions in acid milk gel systems can be postulated (see Figs. 5.1 and 5.2). The fat globules can either act as interacting particles and be integrated into the matrix of the casein-fat globule stranded gel networks and contribute to their rigidity of casein-based matrix, or act as inert fillers (non-interacting particles) between the strands in the matrix. A protein membrane of fat globules may provide additional cross-links or contact areas or bonds with other protein particles and thereby increase the effective number of protein particles (pseudo-protein particles). Interactions were possible via cross-linking between fat globule membrane proteins and casein particles. A large effect was observed when the fat globule membrane contained denatured whey protein or soluble casein. In the case of Tween 60 or unheated WPC (Fig. 5.1C and 5.2C), fat globules were non-interacting due to the absence of a interacting protein membrane. Non-interacting particles can fill space between strands and clusters and inhibiting the formation of additional cross-linkages and then produce weaker gels.

In the present study, interactions of fat globules within the gel matrix would depend on the type of reconstituted skim milk system. In the case of unheated reconstituted skim milk system, gels would form by casein-casein interactions as isoelectric pH of casein approaches. Therefore, it would be expected that Na caseinate stabilised fat globules would interact strongly with casein matrix and

SMP stabilised fat globules would also interact to some extent (Fig. 5.1A). When the fat globules stabilised by heated WPC are incorporated into unheated reconstituted milk, it is likely that the these fat globules would interact with other fat globules as the isoelectric point of whey proteins (pH $\simeq$ 5.2) is approached, resulting in an independent gel network, i.e. two separate gel systems could be formed. This would obviously affect the G' of the gel (Fig. 5.1B).

On the other hand, in heated reconstituted skim milk, denatured whey proteins are attached to casein micelles and gel network would be formed *via* interactions between denatured whey protein-coated casein micelles. The preparation of this kind of gel would largely depend on whey protein-whey protein interactions. Heated WPC stabilised fat globules would act as very large denatured whey protein particles and interact strongly within the gel network (Fig. 5.2B). SMP stabilised fat globules could interact since they contain some of denatured whey protein. Na caseinate stabilised fat globules would create a separate structure by casein-casein interaction, resulting in two separate, independent gel networks, i.e. one whey protein-based and the other casein-based (Fig. 5.2A).

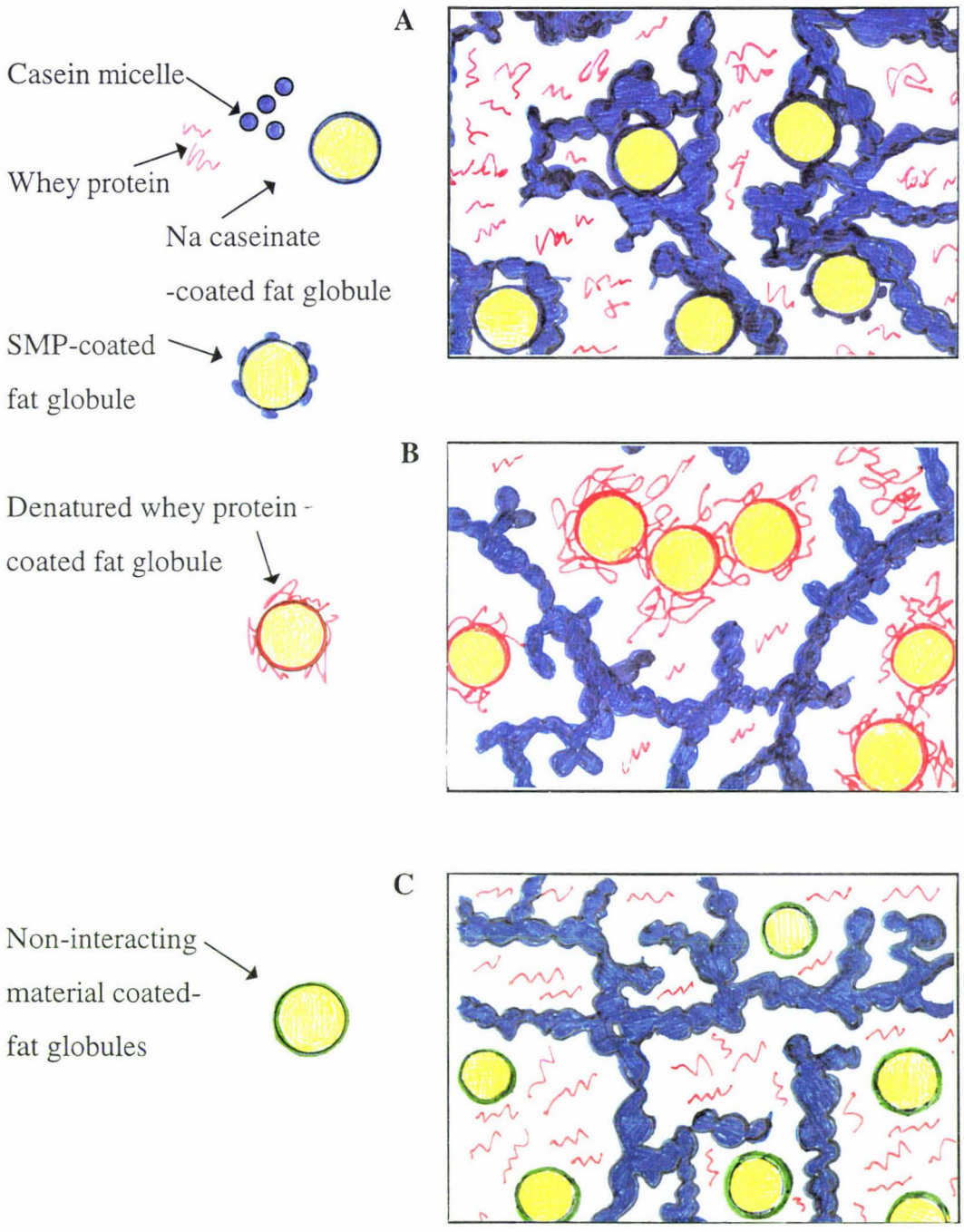

Fig. 5.1. Schematic presentation of the possible interactions between casein matrix and fat globules with modified membranes in acid gels. Unheated reconstituted skim system. (A) Na caseinate or SMP; (B) heated WPC; (C) non-interacting particles.

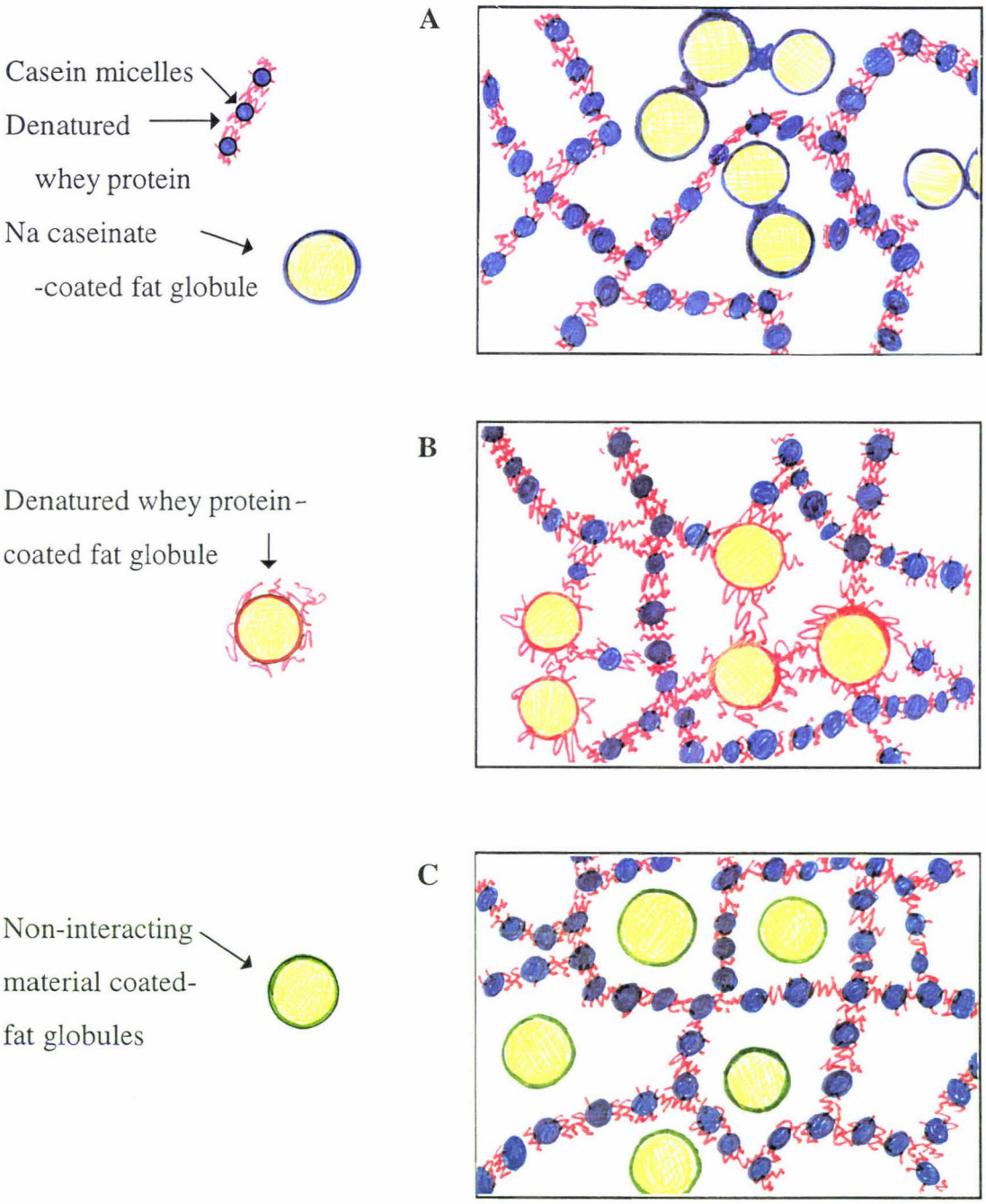

Fig. 5.2. Schematic presentation of the possible interactions between casein matrix and fat globules with modified membranes in acid gels. Heated reconstituted skim system. (A) Na caseinate; (B) heated WPC; (C) non-interacting particles.

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