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INSTITUTE OF FOOD NUTRITION AND HUMAN HEALTH
MASSEY UNIVERSITY
PALMERSTON NORTH



**Effect of Processing and Storage on the
Reconstitution Properties of Whole Milk and
Ultrafiltered Skim Milk Powders**

THESIS
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ANTHONY B MCKENNA

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SUMMARY

Concentrated and dry milk products have a longer storage stability than fresh milk because of a lower water activity and they are therefore desirable in regions with unsuitable climates for fresh milk production and distribution.

During the manufacture of spray dried milk products, there are processing steps that cause interactions between the various components in milk that influence powder functionality. These processes include pasteurisation, homogenisation, concentration, heating, atomisation and the spray drying conditions.

Whole milk powders (WMP), particularly those sold directly to the consumer, are required to disperse rapidly in water and to be quickly and completely soluble *i.e.* form a stable colloidal suspension of fat and protein leaving little or no visible residue suspended in the water or coated to the inside surface of the container. Spray dried ultrafiltered skim milk containing 85% protein (UFSMP85) is used for a wide range of applications including protein fortification of liquid milks, nutritional foods and cheese milks. The UFSMP85 should have good solubility in water and milk for it to be used successfully in these applications.

The main objective of this work was to further our knowledge about the influence of processing factors on component interactions during the manufacture of WMP and UFSMP85, using microscopy as a major investigative tool. Evaluation of the influence of milk component interactions on the functional characteristics of the powders was an integral part of the work and provided important insights towards improving the reconstitution properties of these powders.

The development of confocal laser scanning microscopy methodology for WMP proved valuable for the localisation of fat globules, lactose crystals and a phospholipid-based surface wetting agent.

Major structural changes in the fat globules, casein micelles and whey proteins occurred during the manufacture of WMP. Preheating resulted in the formation of hair-like structures on and between casein micelles onto the fat globule surface. Upon further heating the adsorbed protein on the fat aggregated with other micelles to form a chain-like network. Reduction in the whey protein concentration of the concentrated milk appeared to reduce the number of hair-like structures, aggregation and the extent to which this network formed upon heating.

A study of instant-WMP products, obtained from the marketplace, indicated that WMP with poor functional properties (solubility in coffee and hot and cold water, and dispersibility) generally had been manufactured using high preheating conditions, had a high fat globule protein load (excessive shear during processing) and exhibited a high degree of protein-protein interactions. The instant-WMP sample with the most favourable functional properties (good solubility in hot and cold water and coffee, excellent dispersibility and good agglomerate structure) was manufactured using low preheating conditions and exhibited fewer protein-protein interactions as observed by transmission electron microscopy (TEM).

In the manufacture of UFSMP85 there were changes that occurred during membrane

concentration and evaporation that predisposed the concentrated milk to protein-protein and protein-mineral interactions upon drying. The extent of these protein interactions increased with an increase in protein content of the UFSMP. It was considered that these interactions gave UFSMP85 a solubility of only 40% when it was reconstituted in water at 20°C. The solubility was approximately 98% when UFSMP85 was reconstituted in water at 60°C. Determination of the location of β -lactoglobulin and κ -casein by immuno-gold labelling and TEM showed that these components may be associated with the formation of an aggregated matrix that 'sets' upon drying thus influencing particle solubility.

Storage of UFSMP85 at temperatures $\geq 20^\circ\text{C}$ caused a "skin" to develop at the particle surface that reduced water penetration. This skin eventually (after 6 months of storage) decreased the powder solubility even in water at 60°C. The application of shear at approximately 14.50 MPa was required to break down these poorly soluble reconstituted UFSMP85 particles.

Changes in the pH of milk prior to ultrafiltration and a reduction of temperature during drying and storage of the powder may result in some incremental improvements in the solubility of the UFSMP85.

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CHAPTER 1

INTRODUCTION

The climates of many areas in Asia, Central America, the Middle East and Africa make it difficult to support a dairy industry based on fresh milk. In these areas the shelf life of fresh, undried milk products is short. Concentrated and dry milk products have increased storage stability because of a lower water activity and are therefore desirable in regions with such climatic conditions.

Concentrated milk products are obtained from milk by the application of one or a combination of water removal techniques. Drying of these products can reduce the water content to below 3%. The reduction in water content of milk will increase shelf life and reduce transportation costs. This is particularly important for manufacturers that export products to distant markets. These products may be recombined (water and a combination of ingredients added) or reconstituted (just water added) and then packed and sold by a food retailer, or sold directly to the consumer as a dry or concentrated product, *e.g.* whole milk powder (WMP) or canned evaporated milk. The range of concentrated and spray-dried dairy powders commercially produced today is wide and includes ultra-high-temperature (UHT) treated concentrated milks, in-can sterilised milks, skim milk powder, WMP, formulated infant food powders, high protein powders and high fat powders, whey powders, buttermilk powders, cream powders, casein and caseinates. Lately, membrane technology, *e.g.* ultrafiltration, has improved and applications have been developed for many processes leading to the production of a new range of concentrated and dried products, *e.g.* milk protein and whey protein concentrates.

With an increasing desire to satisfy consumer requirements for high quality dairy products, manufacturers are required to produce powders that maintain a fresh flavour and are highly nutritious. These powders are required to disperse rapidly in water and to be quickly and completely soluble, *i.e.* form a stable colloidal suspension of fat and casein micelles leaving no visible residue suspended in the water or coated to the surface of the container. The most typical complaint confronted by manufacturers of WMP relates to aspects of solubility in both cold and hot water. It is likely that these defects arise from either the raw milk quality or the processing steps during the manufacture of the milk powder. Pasteurisation, homogenisation, concentration, heating and drying will influence the interaction of various components of the milk, specifically the milk proteins and the fat. To avoid these solubility problems, it is important to have a good understanding of the component interactions within the concentrated milk during manufacture and in the resulting powder. Very little work on the effect of component interactions on the structure and functional properties of dried milk products has been published in the literature. Further knowledge about the effect of these interactions on functional properties may lead to improved processes and better performing milk powder products.

Component interactions during the manufacture of dried milk powder products may be studied by a range of physical and chemical techniques. However, many techniques are difficult to apply to concentrated milks, particularly in the presence of fat. Visualisation of the components

in milk by microscopy can be very powerful, enabling the spatial distribution and interaction of fat, protein and crystals within the milk or on the surface of the powder to be assessed.

The goal of this thesis was to further our knowledge about the influence of processing factors on component interactions during the manufacture of WMP and high protein powders, using microscopy as a major investigative tool. Evaluation of the influence of milk component interactions on the functional characteristics of the powders was an integral part of the work. The knowledge obtained will probably provide important insight towards improving the reconstitution properties of milk powder products. The following studies each contributed to this work.

1.1 MICROSTRUCTURAL CHANGES DURING THE MANUFACTURE AND RECONSTITUTION OF WMP

This section of the thesis focuses on the structural changes that occur during the manufacture of WMP and the performance of this product upon reconstitution.

The chapters in this section cover the following topics.

- | | |
|-----------|--|
| Chapter 3 | The examination of whole milk powder by confocal laser scanning microscopy. |
| Chapter 4 | Microstructural changes in milk during the manufacture of whole milk powder. |
| Chapter 5 | Microstructure of instant whole milk powder and of insoluble material detected by powder functional testing. |
| Chapter 6 | Microstructural observations in a range of commercial instant whole milk powder samples. |

In Chapter 3, the use of a new light microscopy technique, confocal laser scanning microscopy (CLSM), for the evaluation of fat, lactose crystals and a surface wetting agent in WMPs is reported. CLSM allows optically thin sections of relatively intact foods to be made without long sample preparation processes. CLSM has proved most advantageous in the examination of high fat foods, which are difficult to prepare for conventional microscopy without loss or migration of the fat. There has been little reported on the examination of milk powder using this technique.

In Chapter 4, the structural changes during the manufacture of a standard WMP are evaluated. The various processes applied during the manufacture of WMP *i.e.*, preheating, evaporation, homogenisation, concentrate heating and spray drying, all have some influence on the interaction of components within milk. These interactions may then have a major influence on the reconstitution properties of WMP.

The functional properties of instant whole milk powder (IWMP) are routinely measured to determine powder performance and quality. Little or no work has been carried out examining the characteristics of the insoluble material collected after reconstitution of milk powder. In Chapter 5, the microstructure of milk powder and the insoluble material collected after

reconstitution is evaluated in an attempt to understand how the material is formed during reconstitution.

The functional properties of IWMPs from around the world are likely to vary enormously. In Chapter 6, the chemical, physical and functional properties and the microstructures of a range of commercial IWMPs obtained from South East Asia and the Middle East are evaluated and discussed.

1.2 MICROSTRUCTURAL CHANGES DURING THE MANUFACTURE AND STORAGE OF 85% PROTEIN ULTRAFILTERED SKIM MILK POWDER (UFSMP85)

This section of the thesis focuses on the structural changes that occur during the manufacture of spray dried ultrafiltered skim milk with a protein concentration of 85%.

The chapters in this section cover the following topics.

- Chapter 7 Chemical and microstructural changes in milk during ultrafiltration and spray drying.
- Chapter 8 Microstructural changes during the storage of spray-dried ultrafiltered skim milks and their affect on dissolution properties in water.
- Chapter 9 The influence of skim milk pH on micelle structure and protein distribution before and after ultrafiltration.

Little work on the microstructural changes that occur during the ultrafiltration of skim milk has been reported. In Chapter 7 the structural and chemical changes that occur during the ultrafiltration, diafiltration, evaporation and spray drying of skim milk are evaluated and discussed.

In Chapter 8 the information obtained from the previous chapters is combined with studies on changes during the storage of UFSMP85 to determine the structural elements that influence the powder dissolution properties before and after storage of the dried powder.

The microstructural changes caused by altering the initial pH of skim milk prior to concentration by ultrafiltration have not been thoroughly investigated. In Chapter 9 the influence of these changes on the structure of the casein micelle and the distribution of κ -casein and β -lactoglobulin is studied.

CHAPTER 2

REVIEW OF THE LITERATURE

This literature review covers chemical and microstructural changes that occur during the manufacture of standard milk powders and powders produced after the ultrafiltration of milk. The influence of these changes on the functional properties of the dried powders is reviewed as are the common microstructural techniques that have been applied to these food systems.

Major reviews of relevance on these subjects have been written. Powder manufacture: Kalab (1981), Green *et al.* (1984), Pisecky (1985), Caric & Kalab (1987), Masters (1991), Mistry & Hassan (1991a), Caric (1994), Barbosa-Canovas & Vega-Mercado (1996). Chemical changes during powder manufacture: Srilaorkul *et al.* (1991), Singh & Newstead (1992), Schuck *et al.* (1994), Oldfield (1997). Food microstructure: Buma (1971), Glauert & Reid (1974), Brooker (1979, 1993, 1995), Buchheim (1981), Alleyne (1993), Heertje (1993a, 1993b), Kalab (1993), Heertje & Paques (1995), Kalab *et al.* (1995).

2.1 TYPICAL POWDER MANUFACTURING PROCESSES

The manufacturing procedures for milk powder and ultrafiltered skim milk powder are described.

2.1.1 Milk Powder

A typical manufacturing process for milk powder is shown schematically in Figure 2.1. Raw milk is first pasteurised and then separated into skim milk and cream. Some of the cream is returned to the skim milk to achieve a product with a standardised fat content in whole milk powder (WMP) manufacture. Milk is preheated for a set temperature/time combination prior to evaporation. Preheating is an important operation used by manufacturers to impart specific functional properties and storage stability to the powder. Concentration of the milk is used to improve the efficiency of spray drying. Evaporators, typically multistage falling film versions, are used to remove approximately 80% of the water from the milk. The milk concentrate leaves the evaporator at 46-50% total solids and at approximately 50°C. The fat globules are protected from coalescence by homogenising the concentrate before drying, thus reducing the extent of free fat formation on drying. The viscosity of the concentrate prior to drying is reduced by heating it to 80-85°C. The concentrate is then atomised by pumping either through a number of small orifices in a nozzle or on to a rapidly rotating disc as it enters the top of the drying chamber. The small droplets produced ensure a large surface area for rapid removal of moisture in the top of the drying chamber where the inlet air temperature is typically between 180 and 220°C. The powder particles leaving the spray drier (at temperatures between 70 and 90°C) are further dried in an internal or external fluidised bed. Instantisation is used by many manufacturers of consumer milk powders to improve the speed of reconstitution. Instantised powders are manufactured by

agglomerating smaller particles with the primary milk powder particles by recirculating the smaller particles ('fines') from the cyclones to some point near the atomiser. Improved powder wetting speeds can be achieved by spraying a layer of phospholipid, *e.g.* soy lecithin, on the surface of the powder usually at the start of the external fluid bed.

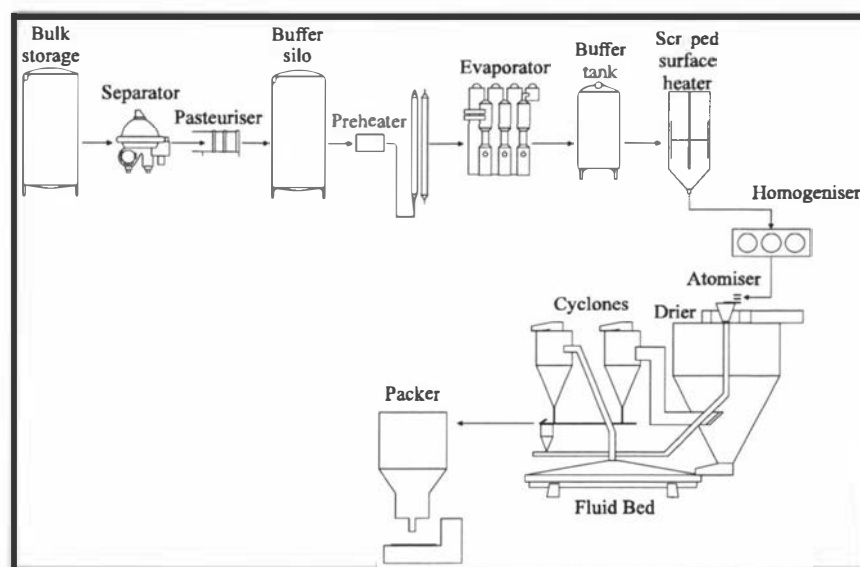


FIGURE 2.1 Schematic showing a typical milk powder manufacturing process (adapted from Singh & Newstead, 1992).

2.1.2 Milk Protein Concentrate Powder from Ultrafiltered Skim Milk

Another type of process for the concentration of milk is membrane filtration, *e.g.* ultrafiltration (UF), which produces a product of quite different properties. The principles of UF, its application and the manufacture of ultrafiltered products have been adequately covered in the literature (Green *et al.*, 1984; Srilaorkul *et al.*, 1991). A typical manufacturing process for high protein milk powders is shown in Figure 2.2. UF is a process in which milk components are fractionated according to molecular size. The pore size of the membrane determines the molecular size cut-off, *i.e.* differentiates between the low molecular weight components (water, ions and lactose) and the high molecular weight components (fat and protein). The membranes allow water, lactose, the non-protein nitrogen fraction of the milk and the soluble salts to pass through while retaining protein (Muir & Banks, 1985). The pore size of the membranes available can vary considerably, thus resulting in the possibility of producing a wide range of concentrated products. Generally the protein and fat fractions will be retained within the retentate. Some lactose, minerals and vitamins will be associated with components in the retentate (Zadow, 1984). Calcium, magnesium, phosphate and citrate are present

partly bound to protein in the milk and partly in solution. The nitrogen losses through the membrane are mainly urea and free amino acids. The retention of lactose may be up to 10%.

In the manufacture of higher protein powders (normally above 70% protein on a dry powder basis) the flux during UF is reduced to almost zero because of the high protein concentration. To achieve these higher protein concentrations, diafiltration is normally applied (Peri *et al.*, 1973). In this case, water is added to the retentate to dilute the aqueous phase; after concentration to the same total solids level, the concentrate has a lower level of soluble low molecular weight components such as minerals but a higher protein concentration in the dry matter (Muir *et al.*, 1984; Renner & Abd El-Salam, 1992). For instance, the protein:lactose ratio is increased from 0.61:1 to 1.22:1 when skim milk is ultrafiltered to a concentration factor of 2; however, this ratio is raised to 9.17:1 by a subsequent three step diafiltration (Thompson & de Man, 1975). After ultrafiltering and diafiltering skim milk, the liquid milk protein concentrate may be evaporated and spray dried in order to obtain a powdered milk protein concentrate (Mistry & Hassan, 1991a; Getler *et al.*, 1996).

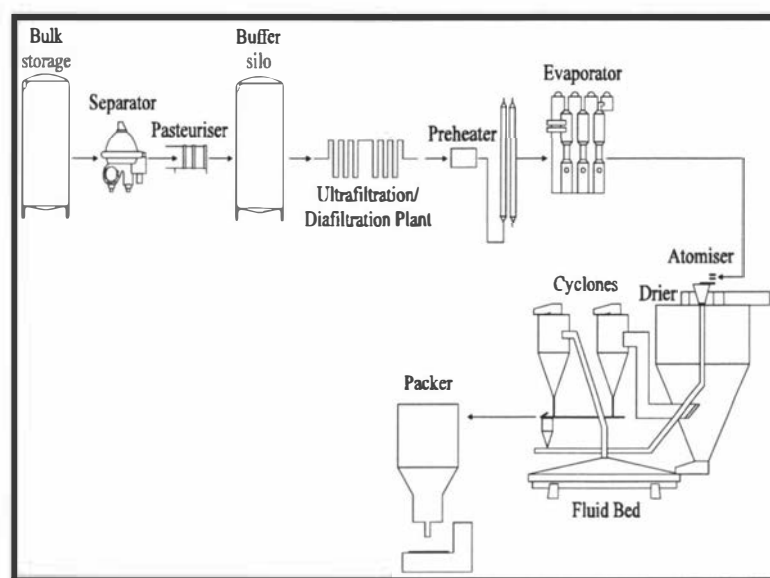


FIGURE 2.2 Schematic showing a typical high protein milk powder manufacturing process.

Typical compositions of WMP and high protein (85%) powder are given in Table 2.1.

TABLE 2.1 Typical compositions of WMP and milk protein concentrate powder

Product		
Component	WMP	Ultrafiltered Skim Milk Powder
Protein (% w/w)	27.9	85
Lactose (% w/w)	36.7	2.8
Minerals (% w/w)	5.8	6.4
Fat (% w/w)	26.8	1.8
Moisture (% w/w)	2.8	4

2.2 PROCESS-INDUCED CHANGES DURING POWDER MANUFACTURE

This area has been previously reviewed by Buma (1971), Caric & Kalab (1987), Singh & Newstead (1992), Singh *et al.* (1995) and Oldfield (1997).

The process-induced changes that occur during milk powder manufacture are summarised in Table 2.2.

TABLE 2.2 Effect of individual processing steps on milk (from Singh & Newstead, 1992)

Process	Effect
Preheating	Destruction of bacteria; inactivation of enzymes; denaturation of whey proteins; formation of aggregates of whey proteins; formation of a complex between κ -casein and β -lactoglobulin; shift of the soluble salts to the colloidal phase; changes in micelle structure; pH decrease.
Evaporation	Concentration of milk solids; increase in colloidal salts; increase in micelle size; decrease in pH; limited denaturation of whey proteins.
Concentrate heating	Reduction in concentrate viscosity; increase in colloidal salts; protein interactions.
Homogenisation	Increase in number of fat globules; adsorption of casein on to fat surface; decrease in protein stability.
Spray drying	Rapid removal of water; relatively minor changes in protein and salts.

The heating applied to milk prior to evaporation during milk powder manufacture (preheating or forewarming) aims to produce powders with improved flavour stability and specific functional properties. The amount of heat applied in the preheat is dependent on the desired end use of the powder. The latter stages of heating during powder manufacture, *i.e.* evaporation and concentrate heating, generally have less influence on flavour and powder functional properties.

The impact of key process operations on the properties of the milk is now discussed.

2.2.1 Preheating

The influence of heat treatment on milk has been thoroughly reviewed in an International Dairy Federation (IDF) publication (Fox, 1995) and in a number of papers (Singh & Fox, 1987a, 1987b; Singh & Creamer, 1991b).

Preheating may be achieved by direct steam injection followed by vacuum flash cooling or by the use of a plate heat exchanger. A major effect of preheating is the denaturation of whey proteins. Many of the functional properties of milk powder are influenced by the whey proteins and their interaction with other components in the milk particularly during heat processing. Heat, prior to evaporation at temperatures of 80-120°C for 20-

120 s, is normally applied by manufacturers to improve the storage stability of WMP (van Mil & Jans, 1991). It is thought that the denaturation of β -lactoglobulin during preheating exposes sulphhydryl groups which then react with any free radicals of unsaturated fatty acids formed during storage thus inhibiting autooxidation. However, the denaturation of β -lactoglobulin and the subsequent activation of the sulphhydryl groups allow the protein to participate in a number of other reactions that may influence the milk during further processing. These reactions include: interactions with other denatured whey protein molecules to form a cross-linked network (Mulvihill & Kinsella, 1987); interactions with κ -casein on the surface of the casein micelle (Dalgleish, 1990); interactions with non-micellar or dissociated κ -casein in heated concentrated systems (Singh & Creamer, 1991a, 1991b, 1991c); interactions with the milkfat globule membrane (Fink & Kessler, 1985; Dalgleish & Banks, 1991; Dalgleish & Sharma, 1992; Sharma & Dalgleish, 1993). The changes to the milkfat globule membrane on heating were studied (Fink & Kessler, 1985; Dalgleish & Banks, 1991) and it was shown that the amount of protein bound to fat increased on heating to a maximum of approximately twice the original concentration. β -Lactoglobulin and α -lactalbumin made up the majority of the newly adsorbed material.

The whey protein nitrogen index (WPNI) is a commonly used measure for classifying powders on the basis of undenatured whey protein nitrogen (Sanderson, 1970). The preheating temperatures used during processing will be dependent on the particular application of the powder. Manufacturers can apply pasteurisation (72°C for 15 s), low temperature long time (85°C for 30 min) or high temperature short time (120°C for 2 min) treatments.

Nieuwenhuijse *et al.* (1988) reported pH drops of 0.04 and 0.09 units after preheating at 72°C for 2 s and 120°C for 180 s, respectively. The casein proteins are relatively heat insensitive, but changes to the micelle structure during heating, *i.e.* attachment of whey protein, may influence how the micelle interacts with other serum proteins or neighbouring micelles during further processing. Hair-like structures have been observed on the surfaces of casein micelles in heated milks (Davies *et al.*, 1978). In skim milk heated at 120°C for 30 min (pH 6.65), thread-like particles at the micelle surface were observed by electron microscopy (Creamer *et al.*, 1978). The micelle appearance in heated milk is dependent on pH. At pH 6.8 the micelles look similar to those in unheated milk. At pH 6.5 the micelles acquire amorphous appendages (Creamer *et al.*, 1978). The appearance perhaps relates to the dissociation of κ -casein/ β -lactoglobulin complex at pH > 6.9, and to the association of this complex with the casein micelles at pH < 6.9 (Singh & Fox, 1987a). During preheating a number of other changes occur to the casein micelle structure besides association with whey proteins at the micelle surface. Below 90°C changes in micelle size are minimal, but at higher temperatures the micelle size increases. Dalgleish *et al.* (1987) observed, by photon correlation spectroscopy, changes in the average micelle diameter during heating at 130°C. Initially the size increased slowly, but the diameter increased rapidly just prior to visible coagulation. An increase in micellar size and an increase in the number of small protein particles after heating has been observed by electron microscopy. Disintegration of the micelle is

thought to be responsible for some of the small particles (Morr, 1969; Creamer & Matheson, 1980; McMahon *et al.*, 1993).

The mineral distribution in milk is altered to some extent during preheating. Calcium and phosphate are transferred from the soluble to the colloidal (micellar) state as heating proceeds (Walstra & Jenness, 1984). This is important as the calcium and phosphate distribution determines the stability of the casein micelles. Research into the area of heat-induced changes in minerals is complicated by separation of the soluble and colloidal phases and the reversibility of heat-induced changes. The behaviour of minerals during processing and their role in determining the functional properties of the powder are not well understood. Reversibility of reactions is important in mineral changes that occur upon heating milk. The mineral balance between the soluble and colloidal phases is in equilibrium and any shift away from this state during heating is reversible upon cooling (Pouliot *et al.*, 1989). Increasing the heating temperature causes more of the soluble minerals to shift to the colloidal state. The decrease in soluble calcium, phosphate and citrate has been shown to involve two steps. The majority of the decrease takes place during the first minute of heating, after which a small decrease occurs over an extended period of time (up to 120 min). After the initial decrease, calcium, phosphate and citrate show some reversibility before further heating results in the second step proceeding. Augustin & Clark (1991) observed that the effect of preheating conditions on the calcium activity in reconstituted and recombined milks was small, though reversible changes while the milk was held at 4°C overnight may have masked any differences caused by preheating.

2.2.2 Evaporation

The evaporation of milk is normally carried out at temperatures between 50 and 70°C with a residence time in each stage of about 1 min (Singh & Newstead, 1992). Under these conditions, whey protein denaturation is minimal. At low heat treatment (72°C for 15 s) a small decrease in the amount of undenatured β -lactoglobulin and α -lactalbumin (1-2%) after evaporation was observed by Singh & Creamer (1991a). Oldfield (1997) found that there was little or no decrease in β -lactoglobulin or immunoglobulin G during evaporation whereas some denaturation of α -lactalbumin was observed. Increasing the total solids also reduced the denaturation of whey proteins in skim milk (McKenna & O'Sullivan, 1971) although the denaturation of α -lactalbumin increased (Hillier *et al.*, 1979). Although the influence of evaporation on whey protein denaturation is small, the total heat applied to the milk during evaporation may become important when attempting to manufacture low heat powders.

Casein micelles increase in size during evaporation mainly due to aggregation of some of the micelles (Walstra & Jenness, 1984). During evaporation there is an increase in the amount of β -lactoglobulin and α -lactalbumin associated with the micelle (Oldfield, 1997). If milk is preheated so that most of the whey proteins are associated with the micelle, the increase in size during evaporation is less (Walstra & Jenness, 1984). Whereas numerous studies have been carried out on the physico-chemical behaviour of standard milk, research into concentrated milks is difficult due to age thickening and changes between the colloidal and soluble phases.

Soluble minerals continue to move into the colloidal phase and calcium ion activity increases slightly during evaporation (Walstra & Jenness, 1984). Nieuwenhuijse *et al.* (1988) suggest that changes during the evaporation process are fast enough to overcome most differences induced by preheating. The concentrate viscosity restricts the maximum concentration that can be achieved without adversely affecting the properties of the spray-dried powder (Bloore & Boag, 1981). Factors that affect concentrate viscosity are preheating temperature and holding time, concentrate total solids, and temperature and holding time of the concentrate. Bloore & Boag (1981) observed that preheating at a high temperature, short holding time (113°C for 10 s) produced concentrates with a lower viscosity than preheating at a low temperature, long holding time (80°C for 120 s), even though the extent of denaturation was similar. The viscosity of milk increases with increasing total solids concentration particularly above 45%. It becomes necessary to heat the concentrate prior to spray drying, from 50 to 70°C, to reduce the viscosity (Muir, 1980; Bloore & Boag, 1981). The combination of high temperature (>60°C) and holding time can lead to aggregation and gelation, producing powders with poor solubility characteristics (Muir, 1980). In concentrated milk the dissociation of the casein proteins from the micelle increases with the severity of the heat treatment and increases in the order κ -, β -, α_s -caseins (Singh & Creamer, 1991c).

2.2.3 Ultrafiltration

Concentration (reduction in the volume of milk by 80%) of skim milk by UF resulted in an increase in fat (from 0.11 to 0.60%), protein (from 3.42 to 17.1%) and most minerals (calcium 4.3-fold, magnesium 3-fold, zinc 4.9-fold, iron 4.9-fold, copper 4.7-fold and manganese 3-fold increase). Lactose was reduced from 5.06 to 4.06% and the B vitamins were reduced by 85, 71, 87, 82 and 84% for thiamine, riboflavin, niacin, pantothenic acid and biotin, respectively (Premaratne & Cousin, 1991). It has been shown that the UF temperature can have an impact on the composition of the retentate. UF of skim milk at 5 and 50°C (Pompei *et al.*, 1973) showed that higher temperatures resulted in higher permeation rates and lower retention of lower molecular weight components such as some of the whey proteins.

Examination of casein micelles in UF concentrates by electron microscopy shows that significant differences, compared with the micelles in unconcentrated milk, occur. The size of the highest proportion of casein micelles (at 80-100 nm in skim milk) was reduced (to 60-80 nm) in skim milk at a volume concentration factor of 5. The volume distribution and the average diameter of the casein particles were decreased. Changes in the mineral levels were claimed to effect changes in the size of the micelles (Sriakul *et al.*, 1991). Milk protein concentrates are more susceptible to the denaturation of whey protein. For instance, when heat treatment at 75°C for 5 min was applied the denaturation degree increased from 31% in skim milk to 64% in UF retentate with a concentration factor of 4.4:1 (McMahon *et al.*, 1993). Increasing the processing temperature of 3x concentrated skim milk resulted in an increase in casein micelle size due to the attachment of additional protein material (Harwalkar *et al.*, 1989). McMahon *et al.* (1993) found that whey protein denaturation was greater in UF-concentrated milk

than in unconcentrated milk whereas Waungana *et al.* (1996) found no noticeable difference in β -lactoglobulin denaturation.

Concentrate produced by UF was found to be more heat stable than conventional skim milk concentrate (Sweetsur & Muir, 1980). Heat inactivation of phosphatase is more rapid in a UF retentate containing 16.6% protein than in non-ultrafiltered skim milk or in a concentrate containing 10.0% protein (Mistry, 1988). Milk proteins and insoluble salts of calcium and phosphates are concentrated by UF and therefore exert an increased buffering effect (Brule *et al.*, 1979; Covacevich & Kosikowski, 1979). This increased buffer capacity of the milk may slow the rate of pH reduction in cheese made using ultrafiltered milk. The buffering effect of skim milk ultrafiltrate was reduced significantly with the removal of more of the minerals when UF was carried out cold (4°C) and at pH 5.3 (St-Gelias *et al.*, 1992). Changes in heat treatment (65-85°C for 30 s) and pH (pH 6.4-7.0) of skim milk retentate had only small effects on the chemical and physical properties of the spray dried powder. Changes in pH affected the ash content and the solution viscosity whereas heat treatment influenced the powder solubility (El-Samragy *et al.*, 1993).

2.2.4 Homogenisation

In homogenised liquid whole milk, the milkfat globules acquire a protein surface load of 10 mg/m² of which 95% is casein (Buchheim & Dejmek, 1988). The homogenisation pressure and temperature used, the concentration of the milk and the previous heating history of the milk will influence this protein load and its composition, *i.e.* the ratio of casein to whey protein. These factors will in turn influence the type of protein and fat interactions that will occur on further processing and, of course, the reconstitution properties of the resulting powder. It was shown in the work by Mol (1975) that increasing the homogenisation pressure of concentrated milk into which casein-free cream had been added increased the number of insoluble particles in the resulting WMP. This suggests that the new fat globule membrane formed after homogenisation contained casein micelles that were subsequently involved in protein-protein interactions. Homogenisation results in the adsorption of protein, in particular casein micelles (Walstra & Oortwijn, 1982), to the fat globule interface and therefore promotes interactions between these fat-bound proteins and other proteins probably mediated by denatured whey proteins. As a result, the homogenised milk concentrate forms aggregates more readily, particularly at temperatures above the denaturation temperature of whey protein, *i.e.* approximately 70°C. It has been suggested that increased adsorption of protein on the fat globule interface results in localised regions of high protein concentration and that this reduces heat stability (Sweetsur & Muir, 1983).

Sweetsur & Muir (1983) have shown that the heat stability of fresh homogenised milk gradually decreases as the homogenisation pressure is increased from 3.5 to 43.5 MPa. Heat coagulation is the result of casein micelle aggregation (McCrae & Muir, 1991).

2.2.5 Spray drying

There has been little work examining the specific effects of spray drying on the interaction of milk components.

Atomisation of the concentrate gives a large surface area over which drying can take place. The droplets are sprayed into the top of the main drying chamber and are intimately mixed with dry heated air (180-220°C). As the droplet passes through the drier, the moisture evaporates and the temperature of the droplet remains relatively stable (approximately 70°C). Most of the moisture is removed from the droplet when it reaches the wet bulb temperature. After this stage the droplet becomes susceptible to heat damage (Singh & Newstead, 1992) and any prolonged exposure may be detrimental to powder quality. Little is known about the protein and mineral changes that occur during spray drying. It was found that the properties of reconstituted milk powder made from low temperature preheated milk were similar to those of the raw milk (Singh & Creamer, 1991b). These authors noted that spray drying had no significant effect on the denaturation of the major whey proteins in the manufacture of low heat (72°C for 15 s) and high heat (110-120°C for 2-3 min) powders. The denaturation of whey proteins during spray drying was minimal with no apparent loss of immunoglobulin G and only a small loss of bovine serum albumin (3-7%) (Oldfield, 1997). The calcium activity of milk powders immediately after being reconstituted is lower than in raw milk, but increases linearly with the logarithm of time (Greets *et al.*, 1983). The spray drying step is thought to have an effect on calcium ion activity (Greets *et al.*, 1983). The influence of shear during the atomisation of whole milk during WMP manufacture was investigated by Ohba *et al.* (1989). It was concluded that insoluble material in the WMP was formed by shear during atomisation. Insoluble material increased with increasing fat content, higher total solids and higher atomisation pressure and decreased with the addition of sodium polyphosphate and with the complexing of calcium ions.

2.3 POWDER PROPERTIES DURING RECONSTITUTION

The functional requirements of instant WMPs and tests used to measure these have been well reviewed by Pisecky (1985, 1990). Typical functional requirements of instant WMPs include the following: wettability (time for a specified quantity of powder to fall below the surface of still water) (International Dairy Federation, 1979); dispersibility (amount of undispersed particles collected on a filter after reconstitution under set conditions (International Dairy Federation, 1979); slowly dispersing (or dissolving) particles (flecks seen in the milk after reconstitution in hot or cold water) (Pisecky, 1990); various tests for sediment and sludge after dispersion in hot water.

The wettability of milk powder is a function of the surface properties of the powder particles. Skim milk powder contains very little fat and is wettable without the addition of a surfactant. Agglomerated WMP shows poor wetting characteristics, particularly in cold water. To overcome this surface hydrophobicity, soy lecithin (a surfactant) is sprayed on to the surface of the agglomerated powder. The rate of sinking and the rate and extent of dispersion are functions largely of the size, density and structure of the agglomerated powder particles. High levels of fine (non-agglomerated) particles result in poor dispersion and particles with low density may not sink. In most plants, fine powder particles are returned to the drier after separation in fluid beds and cyclones. The fine powder is sprayed into semi-dried concentrate droplets, resulting in the formation of agglomerates. The ideal agglomerate has a coarse open structure in which the small particles are still identifiable.

The process-induced chemical interactions between the proteins themselves and/or the proteins and the fat globules probably have a major influence on the reconstitution properties of the powder.

2.3.1 Solubility of WMP

Prior to the application of any processing to whole milk, there are almost no interactions of the caseins and whey proteins with the milkfat. The milkfat is enclosed in its own membrane of protein, lipids and phospholipid (Walstra, 1983). Milk containing naturally secreted milkfat globules has much greater heat stability than milk in which the milkfat globules have been modified by adsorption of micellar casein to the interface (Sweetsur & Muir, 1983). Milkfat globules that have a large proportion of their surface composed of adsorbed casein micelles behave chemically very much like a large casein micelle, *i.e.* very active in protein-protein interactions. It was found that the incorporation of a casein-free cream into skim milk concentrate followed by low pressure homogenisation resulted in a WMP with improved solubility in water at 20°C (Mol, 1975). The electron micrographs of this WMP showed fewer casein micelles adsorbed to the fat globule interface and less fat globule clustering than the micrograph of a WMP manufactured using a standard process (Mol, 1975).

The processing steps required for the manufacture of milk powder will influence the interaction of various components of the milk, specifically the milk proteins and the fat. Little is known about the interaction of these components with lactose. In the dried milk, lactose acts as a continuous matrix in which the casein micelles and fat are dispersed. Increasing the ratio of protein to lactose provides a greater opportunity for the proteins to interact. It has been shown that β -lactoglobulin denaturation is reduced with increasing lactose concentration (Plock & Kessler, 1993). Protein and fat interactions have been shown to influence the reconstitution properties of the dried powder in water (Buma, 1971; Buma & Henstra, 1971; Mol, 1975; Caric & Kalab, 1987; Ohba *et al.*, 1989; de Ruyck, 1991). Apart from flavour, the most typical complaint confronted by manufacturers of WMP relates to aspects of solubility in both cold and hot water. These defects have been previously reported (Mol, 1975; Ohba *et al.*, 1989; Pisecky, 1990; de Ruyck, 1991). Strictly speaking, only the lactose, native whey proteins and some of the inorganic salts in WMP completely solubilise; the fat and casein protein, with associated denatured whey protein, will disperse to form a colloidal suspension. It is the degree to which this colloidal suspension forms upon reconstitution that will reflect the dissolution properties (referred to here as solubility) of the WMP.

Stability in Hot Coffee

An important aspect of poor solubility is occasionally observed when WMP is dispersed into hot water or hot coffee. The insoluble material can be observed as flecks floating in solution or as material that settles at the bottom of the container. The composition of this insoluble material has been evaluated to be 50% fat with the remaining material (approximately 40%) being protein (Ohba *et al.*, 1989).

Casein proteins may flocculate in coffee because the pH of coffee is near their isoelectric point (approximately pH 4.8 for black coffee and pH 5.9 after milk addition at 80°C). The electrostatic repulsion between the negatively charged proteins normally maintains them in colloidal suspension; however, near their isoelectric point, the negative charge is reduced allowing flocculation to occur. Temperature (60-80°C) has also been shown to decrease the colloidal stability of the milk proteins in coffee cream (Geyer & Kessler, 1989).

It has been found (Sweetsur, 1976) that the insoluble material from instant skim milk powder and coffee is casein and some denatured whey protein. High ionic calcium and surface fat coverage were associated with high coffee instability in instant WMPs (Teehan *et al.*, 1997).

Another form of instability observed in fat-containing systems is feathering which is defined as the formation of fine white flecks after the addition of whiteners *e.g.*, creamers, to hot water or coffee (Geyer & Kessler, 1989). Generally, unhomogenised coffee cream does not feather. Homogenisation increases the protein load on the fat globule. It has been suggested (Geyer & Kessler, 1989) that whey proteins are the main participants in feathering and that it is the whey protein's attachment to the fat globule that makes feathering visible.

Slowly Dissolving Particles

Slowly dissolving particles formed on the reconstitution of milk powder into water, commonly referred to as white flecks (Mol, 1975; Ohba *et al.*, 1989; Pisecky, 1990; de Ruyck, 1991), produce a milk with an appearance generally unacceptable to the consumer. These particles consist mainly of fat (often greater than 50%) and casein (Ohba *et al.*, 1989). Increasing the fat content of the milk powder above 26% has been shown to result in a sharp increase in insoluble material (Ohba *et al.*, 1989). It was concluded from the work of Ohba *et al.* (1989) that the shear applied during atomisation was a major factor influencing the formation of the insoluble particles. The work of Mol (1975) suggested that protein-fat interactions, particularly those of casein micelles adsorbed to the fat globule surface, are the most important factor influencing the formation of these particles.

2.3.2 Solubility of Ultrafiltered Skim Milk Powders

The poor solubility of high protein powders (> 80% protein) has been observed by a number of workers (Jiminez-Florez & Kosikowski, 1986; Panfil-Kunciewicz *et al.*, 1992; Schuck *et al.*, 1994).

Mistry & Hassan (1991a, 1991b) detailed the manufacture of a high protein powder containing 84% protein by UF, diafiltration and spray drying. The solubility of the powder in water was not complete at room temperature but improved at higher temperatures. The solubility of native micellar casein powders (90% protein), produced

by microfiltration, was evaluated (Schuck *et al.*, 1994). The solubility index as measured by the IDF solubility test (International Dairy Federation, 1988) showed that the powder without modifications had a solubility index of 73.7% in water at 24°C. Modifications during powder manufacture (decreasing pH, increasing ionic strength, addition of lactose and a decrease in particle size) improved the solubility index up to 92.6%. The study showed that, as the volume concentration factor increased, the solubility and the dispersibility of the powder in water decreased. The poor solubility of the powder was related to the poor water transfer into the high protein particle rather than to a thermal effect. The solubility index (representing the volume of insoluble protein in 50 mL of reconstituted powder) of ultrafiltered skim milk powders increased with an increase in protein concentration, storage time and storage temperature (Jiminez-Florez & Kosikowski, 1986; Mistry & Hassan, 1991b; Mistry & Pulgar, 1996). The improvement in the dispersibility and solubility properties of the micellar-casein-rich milk powders cannot result from anything other than a modification of the biochemical composition of the products introduced into the drying tower (Schuck *et al.*, 1994).

2.4 MICROSCOPIC TECHNIQUES FOR THE EVALUATION OF STRUCTURAL COMPONENTS IN DAIRY FOODS

The microstructure of milk, concentrated milk and dried milk has been studied in detail and reviewed by a number of authors (Buma, 1971; Buma & Henstra, 1971; Davies *et al.*, 1978; Harwalkar & Vreeman, 1978; Brooker, 1979, 1995; Buchheim, 1982a, 1982b, 1986; Carroll & Farrel, 1983; Caric & Kalab, 1987; Heertje *et al.*, 1987a; Buchheim & Dejmek, 1988; Sargent, 1988, Kalab, 1993; Heerje & Paques, 1995; Kalab *et al.*, 1995).

Examination of the structure of food using microscopy is fraught with difficulty. The foodstuff will contain fat and protein and volatile components such as water that, if not stabilised, deteriorate significantly under the high vacuum conditions within the electron microscope (Buchheim, 1982a). Therefore specific sample preparation techniques have been developed to stabilise a wide range of foods for microscopy. However, most preparation techniques used will alter the structure of the food sample to some extent. Awareness of how these changes are exhibited within the food is essential to ensure that incorrect conclusions are not made.

The range of microscopy techniques used for the examination of milk and milk product structure includes various forms of light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and a range of emerging techniques including confocal laser scanning microscopy (CLSM) and near-field microscopy, *i.e.* atomic force and scanning probe microscopy.

2.4.1 Light Microscopy

The most common forms of light microscope are the stereo and compound microscopes. Stereo microscopes give a good first impression of the overall structure of the foodstuff. The sample is illuminated by a light source above the specimen. The quality of the light source is critical for good viewing (Flint, 1994). The compound microscope requires a

specimen that is thin enough to transmit light. The specimen must be either squashed or cut into a thin section by microtomy.

Bright-field, phase contrast, differential-interference contrast, polarised light and fluorescence microscopy techniques are the most frequently used modes for a compound microscope. These techniques are described comprehensively in Slayter & Slayter (1992). In conventional bright-field microscopy, light is transmitted through a condenser, the specimen and the objective, producing a real image that is upside down and reversed, and magnified within the microscope tube. The real image is then further magnified by the eye piece lens, which produces either a virtual image or a real image on photographic film placed above the microscope tube. Polarised light microscopy can be achieved by the insertion of two polarisers in the light path: one between the light source and the specimen, and the other between the objective and the viewer. Plane-polarised light, which is produced by the first polariser, has rays that vibrate only in one plane, perpendicular to the direction of travel. If the second polariser, the analyser, is rotated such that the transmitted vibration is perpendicular to the vibration of the incident light (crossed polars), amorphous regions within the specimen will appear dark, whereas crystalline or ordered regions will appear as bright structures. In fluorescence microscopy, light of a specific wavelength illuminates the sample and is absorbed by specific molecules that are present in the specimen, and the energy is re-emitted as light of a longer wavelength and lower intensity (fluorescence) (Slayter & Slayter, 1992). A barrier filter removes all but the fluorescent radiation which then forms the image. There is an ever-increasing selection of fluorescent probes to choose from, designed to impart fluorescence to the component of interest. Many of these new probes are listed in catalogues put out by companies such as Molecular Probes (Molecular Probes, Eugene, Oregon, USA).

A recent development in light microscopy has been the CLSM. The main difference between a confocal microscope and a conventional microscope is in the placement of a pinhole at the focal plane of the image. The sample is illuminated by a scanning laser and the resulting image reaches the photomultiplier through the pinhole point by point. This allows the removal of light that is not from within the very narrow plane of focus, thus resulting in sharp images from within food samples (Pawley, 1990; Blonk & van Aalst, 1993; Brooker, 1995). Computer technology now allows sequential optical sections of the specimen to be collected, stored and viewed as a rotation or a three-dimensional image. Such processes can result in excellent visualisation of the spatial distribution of specifically stained food components, such as protein and fat, within a food. To date, the area of food analysis in which CLSM has proved most advantageous is the examination of high fat foods, which are difficult to prepare for conventional microscopy without loss or migration of the fat (Heertje *et al.*, 1987b; Brooker, 1995). CLSM has been used to evaluate salad dressing, butter and margarine. Several different components can be identified and localised at once with CLSM by using specific fluorescent dyes, depending upon the number of laser lines available on the instrument. Commonly used fat dyes are Nile Red for water-in-oil emulsions and Nile Blue for oil-in-water emulsions (Brooker, 1991). Typical protein stains include rhodamine, Texas Red and Fast Green FCF.

2.4.2 Electron Microscopy

Image formation in electron microscopy is similar to that in light microscopy, but the illumination source is electrons focussed with magnetic lenses rather than photons focussed with glass lenses. Electron microscopy is carried out under vacuum (typically 1×10^{-6} mbar). Food samples contain large quantities of volatile substances such as water and fat that, when not stabilised, will be released when placed in the high vacuum of the microscope. The volatile components can be stabilised by drying or freezing and then coating the material with electron conducting metals such as gold, platinum and carbon. Two major types of electron microscopy are used *i.e.* SEM and TEM.

Scanning Electron Microscopy

SEM has been used for food samples (Buma, 1971; Kalab, 1979). SEM is mainly used for the examination of surfaces. The sample is either dried (conventional SEM) or frozen below -80°C (cryo-SEM). A 5-20 nm-thick metal (gold) coating provides electrical conductivity. The sample is scanned by a focussed electron beam, secondary or back-scattered electrons are detected and this information is passed on to a photomultiplier to produce the image. SEM images have a great depth of focus and are relatively easy to understand. Samples that contain large quantities of water and/or fat must first be dehydrated or frozen to stabilise these components. Cryo-SEM involves the sample being rapidly frozen in a cryogen such as liquid nitrogen, freon or propane to retain the water in a near vitreous form with only very tiny ice crystals present. The frozen food is then coated with gold or carbon and observed on a low temperature stage within the microscope (Sargent, 1988; Echlin, 1992). Optional freeze etching (sublimation of the ice) exposes the product matrix for examination. Cryo-SEM is very useful for observing aerated samples (Sargent, 1988; Brooker, 1990; Caldwell *et al.*, 1993), and high fat samples, that are difficult to stabilise using conventional preparation methods. The formation of ice crystals which distort the structure is a common artifact. These can be reduced in size by optimising the freezing process, *i.e.* minimising the sample size, increasing the freezing rate and using a lower temperature freezing medium.

Transmission Electron Microscopy

TEM allows for the visualisation of the internal structure of food samples, allowing complex interactions between the food components to be studied. Either thin resin-embedded sections (15-90 nm) or platinum-carbon replicas of the sample are placed in the path of the electron beam, and the enlarged transmitted image is observed and captured on film. The electrons are transmitted through the sample with varying degrees of energy loss. Contrast in the images is achieved by the use of heavy metal stains (*e.g.* osmium, uranium or lead) (Glauert & Reid, 1974) or by differences in the thickness of the metal replica (Echlin, 1992). Negative staining and metal shadowing are other TEM techniques suitable for visualising suspensions and emulsions. The type of preparation technique used will depend on the composition and state of the food sample.

For thin section TEM, primary fixation is normally achieved by a glutaraldehyde solution (Sabatini *et al.*, 1963) that stabilises the protein matrix by cross-linking some of the amino acid groups of the protein. Osmium tetroxide solution is used as a heavy-metal oxidative fixative to stabilise unsaturated lipids. Protein-based foods (*e.g.* meat and some milk products) are relatively easy to fix, but high fat foods are more difficult as the saturated fats cannot be chemically fixed and therefore require longer periods (up to 2 weeks) in buffered osmium (Lewis, 1979). Following fixation the samples must be dehydrated, typically in a graded series of ethanol or acetone, and then embedded in a suitable resin. The samples are then sectioned in a microtome to typically 70-90 nm prior to staining in uranyl acetate (if not used earlier during preparation) and lead citrate.

Rapid freezing followed by freeze fracturing and metal shadowing has been shown to be a very useful alternative to chemical fixation and thin sectioning. Freeze fracturing has been used by Buchheim and co-workers with great success in many dairy food systems (Buchheim, 1982a, 1982b; Buchheim & Dejmek, 1988). The process has been well described by Echlin (1992). When a final product is to be examined, it is held for some hours at the temperature of interest prior to rapid quenching in a coolant. If, however, the sample is obtained from within a process or it is an unstable preparation, then instant quenching will be required. The coolants typically used include liquid nitrogen, propane and freon (Echlin, 1992). Many workers infiltrate high moisture samples in glycerol prior to freezing to minimise ice crystal formation (Buchheim, 1982a). These samples require only simple plunging into the coolant whereas products that cannot have glycerol added may require more rapid freezing such as propane jet freezing (Muller *et al.*, 1993).

2.4.3 Immunolabelling

Conventional microscopy (light microscopy, TEM and SEM) has been used in food structure studies to obtain a qualitative description of the structure of samples. Localisation techniques can be used in conjunction with the conventional microscopies, to determine the distribution of structural elements, macromolecules and elements of interest in the samples. The specific identification of many individual proteins, glycoproteins and polysaccharides is possible by using appropriately labelled antibodies and lectins. Little work identifying the structural location of proteins in complex food systems has been reported. Immunogold labelling in conjunction with electron microscopy is a technique that enables the specific location of α_s -, β - and κ -caseins, α -lactalbumin and β -lactoglobulin, membrane material, lipoprotein, cellulose and other carbohydrate stabilisers to be determined in foods. Immunogold labelling has been found to be very valuable for studying the fundamental relationships between structure and texture in foods (Armbruster & Desai, 1993; Alleyne, 1993).

Colloidal gold particles are formed by the reduction of an aqueous solution of tetrachloroauric acid by condensation (Handley, 1989; Leunissen & de Mey, 1989). The gold particles can be manufactured in very precise size ranges. For immunolocalisation,

an antibody specific to the desired antigen (such as α_s , β - or κ -casein) is applied to the sample. Colloidal gold probes coated with a secondary antibody then interact with the bound primary antibodies to label the component of interest.

2.5 MICROSCOPY OF MILK AND MILK PRODUCTS

A combination of various microscopy techniques provides a more accurate view of food structure than any one technique.

2.5.1 Microscopy of the Effects of Heat on Milk

The appearance of hair-like appendages on the surface of casein micelles in heated milk has been observed by TEM (Creamer *et al.*, 1978; Davies *et al.*, 1978; Mohammed & Fox, 1987). These hair-like structures, considered to be β -lactoglobulin/ κ -casein complex as determined by Haque *et al.* (1987), influence the properties of subsequent products made from the heated milk. It has been proposed by McMahon (1995) that this complex is involved in the formation of a three-dimensional network of cross-linked protein that causes age gelation in UHT milk during storage.

In a study by Mohammed & Fox (1987) the formation of β -lactoglobulin/ κ -casein complexes in milk (pH 6.7-7.4) was evaluated after heating at 130 or 140°C. Complexes appeared to be attached to the casein micelle surface after heating in milks with pH values below 6.7. Complexes were not observed in heated milks with pH values above 7.0. Singh *et al.* (1995) studied the structure and composition of the fat globule surface layers after heating at 130°C for periods up to 75 min. Initially whey proteins interacted with casein micelles and fat globules with a rapid increase in micelle size and protein loading on the fat globule surface. With increasing heating time, protein cross-linking by non-disulphide bonds increased followed by an increase in protein particle and fat globule (by way of surface protein) aggregation. The aggregated network was made up of chains of protein particles and fat globules. The fat globules were linked through adsorbed casein micelles.

The effect of processing on protein-protein and protein-fat interactions in infant formula was investigated by Guo *et al.* (1999). Analyses carried out included microstructure by TEM, nitrogen, mineral distribution on centrifugation and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Fat globules and casein micelles in the infant formula blend appeared as well-defined discrete spheres before processing. After pasteurisation, the casein micelles displayed rough irregular surfaces, presumably due to casein and whey protein interactions. After homogenisation, TEM revealed the presence of casein-whey protein aggregates that appeared to be attached to the surfaces of fat globules. SDS-PAGE and nitrogen analysis confirmed that high levels of caseins and whey proteins were present in the fat fraction. TEM analysis of the pellet fraction after homogenisation showed the presence of fat globules. Protein-fat interactions were confirmed by both the presence of protein in the fat fraction and the presence of fat

globules in the pellet on centrifugation. There was little change in microstructure, nitrogen and mineral distribution after evaporation and spray drying.

2.5.2 Microscopy of Concentrated Milk Systems

Mol (1975) showed that homogenisation of milk in the manufacture of WMP can have a detrimental effect on powder solubility. Electron microscopy observations showed that concentrated skim milk standardised with homogenised butterfat emulsion in skim milk showed fat globules with adsorbed casein micelles forming aggregates whereas concentrated skim milk with butterfat homogenised in casein-free cream had no such aggregates.

The structure of fat-protein complexes in evaporated milk was investigated using freeze-fracture replication by Schmidt *et al.* (1971). The complexes appeared as small fat globules embedded in numerous small casein particles that had formed on the dissociation of the casein micelles during sterilisation of the concentrated milk. The complexes rose or sedimented during storage depending on their bouyancy. An analysis of the layers formed in the product after storage showed: the top layer had a high fat content and an increased concentration of protein; the middle layer had reduced protein and fat contents; the bottom layer was rich in protein and inorganic matter and contained some fat.

The changes in casein micelle structure during the storage of UHT-processed concentrated skim milk was examined by TEM (Harwalkar & Vreeman, 1978). Viscosity increases leading to gelation during storage were accompanied by changes in the structure of the micelles: firstly the appearance of thread-like structures on the casein micelles followed by aggregation of some casein micelles and then, at the time of gelation, chains of micelle aggregates. The addition of sodium hexametaphosphate delayed the onset of gelation by reducing the changes to the casein micelles during storage.

The structural changes in sweetened condensed milk during storage were analysed by De Filipe *et al.* (1991). Rheological changes were related to structural changes using TEM. Micellar aggregation and the association of micelles with the fat globules increased during storage as did the viscosity of the sweetened condensed milk.

Immunogold labelling was used with TEM to examine the influence of UHT processing on the proteins in ultrafiltered concentrated milk (Alleyne, 1993). UHT sterilisation resulted in denaturation of the whey proteins and their interaction with κ -casein on the casein micelle. The formation of β -lactoglobulin/ κ -casein complexes weakened the association of κ -casein with the casein micelle, resulting in its migration into the serum. It was concluded that the loss of κ -casein to the serum was a critical factor in the age gelation of UHT milk.

2.5.3 Microscopy of Milk Powders

The structure of spray-dried products has been described by Buma (1971), Buma & Henstra (1971), Buchheim (1982a, 1982b), Caric & Kalab (1987) and Barbosa-Canovas & Vega-Mercado (1996).

SEM has been the most popular technique used for examination of the surface structure of milk powder particles (Buma, 1971; Buma & Henstra, 1971; Mistry & Hassan, 1991b) whereas TEM techniques such as thin sectioning or freeze fracturing (Buchheim, 1982b) have allowed the identification of specific milk powder components such as casein micelles and fat particles.

Atomisation of fluid milk into small droplets usually causes the spray-dried milk powder particles to be spherical with diameters in the range from 20 to 300 μm . Milk powders generally have vacuoles (from air entrapped during processing or the expansion and removal of moisture during drying) within the body of the particles (Buma, 1971). The external appearance and the internal structure of milk powders were studied by Buma & Henstra (1971). These authors used a nozzle atomisation pressure of 7.85-9.80 MPa (80-100 kg/cm^2). They found that even particles of the same powder, *e.g.* skim milk powder, may have different structures resulting from the conditions applied during drying, *i.e.* more wrinkles were formed as the inlet air temperature was increased. De Vilder *et al.* (1976) studied the effect of the spray drying conditions on the physical characteristics of WMP using disc atomisation. Bulk density and the mean powder particle density decreased with increasing inlet air temperature because of an increase in the vacuole size. It was also found that the bulk and mean powder particle densities decreased as the number of atomiser revolutions was increased. It is well reported that the outlet temperature has the greatest effect on the overall milk powder quality (Knipschildt, 1986; Masters 1991). Muller (1964) studied the distribution of casein micelles in spray-dried milk powders and found that they retained their globular nature. This finding was confirmed for skim milk powder by Kalab & Emmons (1974) who examined spray-, roller- and freeze-dried skim milk powders by TEM. They found that the powder microstructure did not reflect the differences in the preheat treatment of the milk during powder manufacture.

Spray-dried ultrafiltered milk retentates resembled other milk powders when examined by SEM (Caric & Kalab, 1987).

Buchheim (1981) examined the replicas of freeze-fractured milk powders. Micrographs of low heat and high heat skim milk powders and micrographs of WMP showed casein micelles and fat globules dispersed in an amorphous lactose and mineral phase. The presence of lactose crystals in milk powder indicates that there has been sufficient moisture available during milk powder storage for the lactose to crystallise. However, Saito (1985) found no lactose crystals in freshly produced milk powders examined by SEM and X-ray diffraction.

The morphology of fat in WMP has been found to be in globular (spherical) form within the particle, in coalesced pools at the surface and inside the particles and spread within pores and cracks leading to the surface of the particle (Buma, 1971).

The surface composition of a range of spray dried food powders has been measured using electron spectroscopy for chemical analysis (ESCA). This technique allows for the surface coverage by a particular component to be estimated (Faldt *et al.*, 1993). It has been used to estimate the surface coverage in spray-dried protein-lactose powders (Faldt & Bergenstahl, 1994) and fat-encapsulated powders (Faldt & Bergenstahl, 1995), and the surface composition of WMPs (Faldt & Sjöholm, 1996; Teehan *et al.*, 1997).

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CHAPTER 3

THE EXAMINATION OF WHOLE MILK POWDER BY CONFOCAL LASER SCANNING MICROSCOPY

3.1 INTRODUCTION

Milk powders sold to the consumer for domestic use are required to disperse rapidly in water without leaving visible traces of particulate material on the side or bottom of the container. These requirements have led to a greater need for the determination of ingredient interactions and the localisation of ingredients within or on the powder particle by a range of physical and chemical techniques. Visualisation of these components by microscopy can be a very powerful tool for enabling the spatial distribution and interaction of fat, protein and crystals within or on the surface of the powder to be assessed. The techniques which have been used for powders include light microscopy (King & Shimmin, 1961; Saito, 1985) scanning electron microscopy (Buma, 1971; Buma & Henstra, 1971; Kalab, 1979; Kerr *et al*, 1983) and various forms of transmission electron microscopy) namely freeze fracturing (Buchheim, 1982a, 1982b;) and thin sectioning of embedded powders (Muller, 1964). These techniques have generally been either laborious and/or have produced limited useful information.

A relatively new form of light microscopy, confocal laser scanning microscopy (CLSM), has yet to be applied to milk powders. The problems normally associated with observing thick food specimens by light microscopy *i.e.* a blurred image produced from a large depth of focus, can be overcome in CLSM as image formation does not depend on transmitting light through the specimen. In CLSM, a scanning laser illuminates a layer within the specimen at a specific focal plane and removes out-of-focus information by the use of a confocal pinhole. For a more detailed description of CLSM, the reader is referred to the literature (Blonck & van Aalst, 1993; Brooker, 1991, 1993, 1995; Heertje *et al*, 1990; Heertje *et al*, 1987). In CLSM, images of the components such as proteins and lipids are produced by using the laser light to excite a selective fluorescent dye that has been introduced to the food system either during manufacture or to the surface of the food after manufacture (Brooker, 1995). More specific localisation of components can be achieved using fluorescently labelled antibodies, enzymes and ligands. Modern CLSM instruments, many of which contain combined krypton/argon lasers, produce light at 488, 568 and 614 nm allowing the use of multiple dyes which excite at these differing wavelengths. Therefore dyes specific to fat and protein can be examined within the same sample. As well as fluorescence, CLSM allows images of the sample to be produced using the reflection and transmission modes.

The advances in microscopy provided by CLSM may provide advantages in the examination of whole milk powder (WMP). Sample preparation that has been difficult may be simplified by the addition of specific dyes to an appropriate mounting medium. This work describes the sample preparation and use of CLSM for the examination of a number of components in WMP.

3.2 MATERIALS & METHODS

Three different types of WMP samples were examined by CLSM: standard 28 % fat WMP for fat localisation, WMP containing high concentrations of crystalline lactose, and a WMP instantised by spraying with a fluorescently labelled phospholipid (Table 1).

TABLE 3.1 Summary of materials and methods for WMP evaluation

STUDY	POWDER	STAIN/FILTER
Fat localisation	28 % fat WMP's (experimental, New Zealand Dairy Research Institute)	Nile blue /fluorescein isothiocyanate filter (488nm)
Lactose crystal localisation	28 % fat WMP containing 50% of the lactose in crystalline form (experimental, New Zealand Dairy Research Institute)	Nile blue/fluorescein isothiocyanate filter (488nm) or reflection
Surface phospholipid localisation	28 % fat WMP (experimental, New Zealand Dairy Research Institute)	Fluorescently labelled phosphatidylcholine and fat blend sprayed onto powder surface/ rhodamine filter (568nm)

3.2.1 Fat Localisation

The study of fat in aqueous emulsions by CLSM is best achieved using the stain Nile blue (Brooker, 1991). This contains trace amounts of fluorescent Nile Red (Nile blue A Oxazone) dye which diffuses into the oil phase and then becomes strongly fluorescent when excited in the range 450-500 nm.

A number of solutions for mounting WMP in preparation for CLSM were examined. The requirements were that it must allow the solubilisation of the aqueous dye Nile blue and its diffusion to the fat on the surface and within the WMP particle, that it must inhibit or allow only very slow dissolution of the WMP, and that its use must result in little or no background fluorescence.

Three commercially available fluorescent mounting media were evaluated for their suitability: Citifluor (UKC, Chemical Laboratory, Canterbury), Dako (Dako Corporation, Carpinteria, CA), and Fluoprep (BioMerieux, Marcy-l'Etoile). These fluids were all

glycerol based and contained anti-fading agents. The Fluoprep and Dako media also contained a chemical agent that caused the fluid to set into a gel within minutes of attaching the cover slip thus inhibiting movement of the powder particles. The powders were also evaluated in propylene glycol and glycerol.

The 28% fat WMP was mixed with the mounting medium containing the Nile blue dye (added as a powder at 10mg/L) and placed on a microscope slide. The fat stained with Nile blue in the WMP was fluoresced using the fluorescein isothiocyanate filter block (excitation at 488nm) of a Leica confocal laser scanning microscope (model TCS 4D, Leica, Heidelberg).

WMP was prepared for transmission electron microscopy (TEM) as follows. The WMP was dispersed in propylene glycol at a 1:1 ratio and then mixed with warm melted 3% low-temperature gelling agarose in a 1:1 ratio. This solution was poured on to a microscope slide, allowed to set, and chopped into 1 mm³ cubes which were transferred into a 3% glutaraldehyde/sodium cacodylate buffer solution. The samples were fixed in glutaraldehyde solution for a minimum of 3 hours before being replaced with cacodylate buffer. The buffer was changed after 30 min and 1 h and then left for a further 1 h prior to replacement with 1 mL of a solution containing 50% osmium tetroxide (2% solution) and 50% cacodylate buffer. This was left to stand for 2 h before being replaced with 1 mL of 1% uranium acetate for 30 min after which the product cubes were washed with water before dehydration. Dehydration consisted of washing with 50, 70, 90 and 100% ethanol for 5, 30, 30 and 180 min respectively. The 100% ethanol was changed after 30 and 60 min. The ethanol was poured off and the bottle was filled with incomplete resin (20 mL epoxy resin, 20 mL dodecylsuccinic anhydride and 2 mL dibutyl phthalate) and placed on a rotator overnight before replacement with complete resin (incomplete formulation with the addition of 0.6 mL of the plasticiser benzyldimethylamine) and further rotation for 4 h. One cube of sample was added to each of three moulds containing fresh complete resin which were then baked overnight at 60°C. The samples were microtomed using a Reichert Ultracut microtome, mounted on 3 mm copper grids and stained using lead citrate before examination in a Philips 201 transmission electron microscope (Philips, NL-5600 MD Eindhoven, The Netherlands) at an accelerating voltage of 60 kV

3.2.2 Lactose Crystal Localisation

The WMP evaluated for this work contained approximately 50% of the lactose in crystalline form.

The WMP was simply dispersed into immersion oil (for examining crystalline lactose alone) or into Fluoprep mounting medium with added Nile blue (for dual imaging of fat and lactose), mounted on a slide and presented to the microscope.

3.2.3 Surface Phospholipid Localisation

A fluorescently labelled phospholipid (Bopidy 3806, phosphatidylcholine with excitation and emission maximums at 581 and 591 nm respectively; Molecular Probes Inc., Eugene, Oregon) was added at 1mg/kg to a 1:1 blend of anhydrous milk fat and a commercially available soya lecithin. This blend (1g) was heated to 60°C and sprayed

(using a small pressurised air gun) onto 100g of prewarmed (60°C) standard 28% fat WMP, while being mixed in a Kenwood cake mixer. The resulting instantised WMP and the unmodified standard WMP were evaluated 2 days and 6 months (storage was at 20°C for 2 months, 37°C for 1 month and 20°C for 3 months to mimic conditions while shipping the powder to a consumer) after addition of the phosphatidylcholine.

The wetting time (the time required for all the particles of an instantised WMP to become wetted *i.e.*, sink below the surface or assume a wet appearance in cold water (International Dairy Federation standard 87 (1979) Annex B)) and location of the phospholipid/fat blend were determined. The latter was examined by dispersing the WMP into immersion oil, mounting on a slide and presenting this to the confocal microscope. A combined image was produced by examining firstly, the Bopidy-stained phospholipid using the rhodamine filter block (568nm) and then the whole particle using the reflection mode of the confocal microscope. The quantity of phospholipid in the powders was measured as described in Chapter 6 (6.2.5).

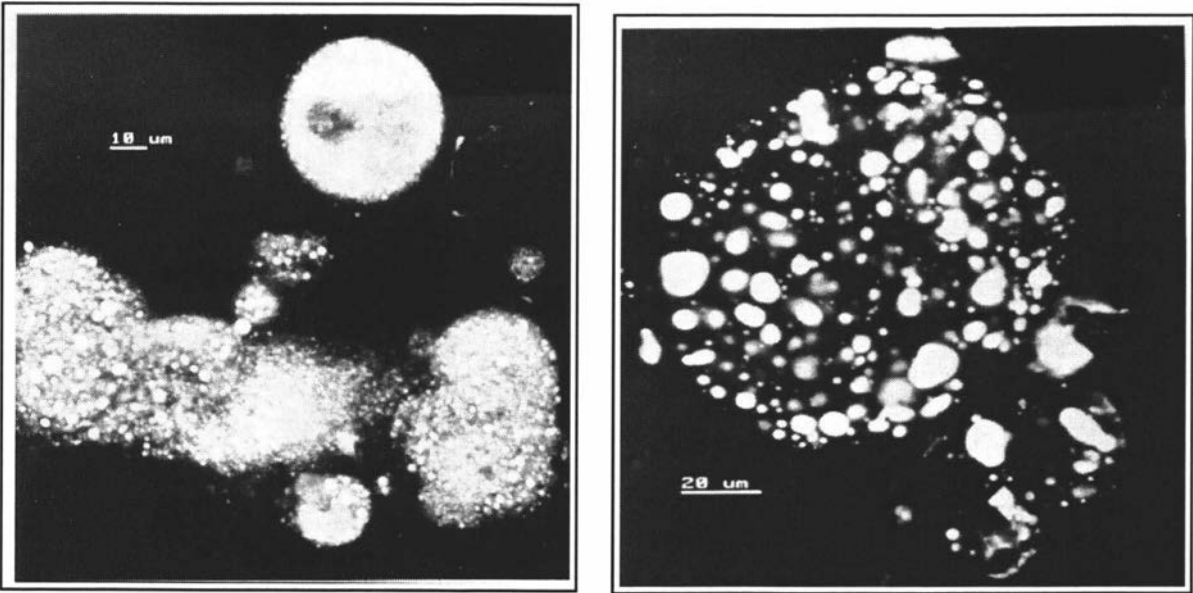
3.3 RESULTS & DISCUSSION

The images presented here (as with all the images presented in this thesis) are representative of each specific sample and the features discussed were consistent throughout the sample.

3.3.1 Fat Localisation

Use of all the commercial mounting media gave images with an excellent signal from the fat and little background signal from the mounting media itself. The use of Dako and Fluoprep media (gel-setting) gave the added benefit of minimising streaming of the WMP particles during observation. However the agent used for gel-setting may have been responsible for some disruption of the fat globules prior to gel formation. The use of glycerol resulted in the gradual dissolution of powder and gave some background fluorescence in the presence of the dye Nile blue. Propylene glycol was by far the least viscous of the mounting media and as a result the powder streamed extensively although dissolution was slow.

CLSM of WMP was clearly able to show the location and size of individual fat globules distributed through an optical section of the powder particle (Figure 3.1). Powders that contained different fat globule size distributions could easily be compared (Figures 3.1a and 3.1b). Fat globule size may be a factor in influencing the functional properties of the WMP in applications such as milk chocolate. Large fat globules may provide more easily extractable fat for reducing plastic viscosity during chocolate manufacture (Haylock, 1995).



(a)

(b)

FIGURE 3.1 Confocal micrograph of whole milk powder particles stained with Nile blue. (a) Powder with fat globules (fg) no larger than 5 μm in size. (b) Powder with most fat globules in the 5-15 μm size range.

The occasional presence of relatively large regions of fat on the surface and within some powder particles was identified using CLSM (Figure 3.2a). These regions of coalesced fat globules were also observed within the same samples using TEM (Figure 3.2b). However the coalesced fat globules were not as easily distinguished from air bubbles by TEM as they were by CLSM.

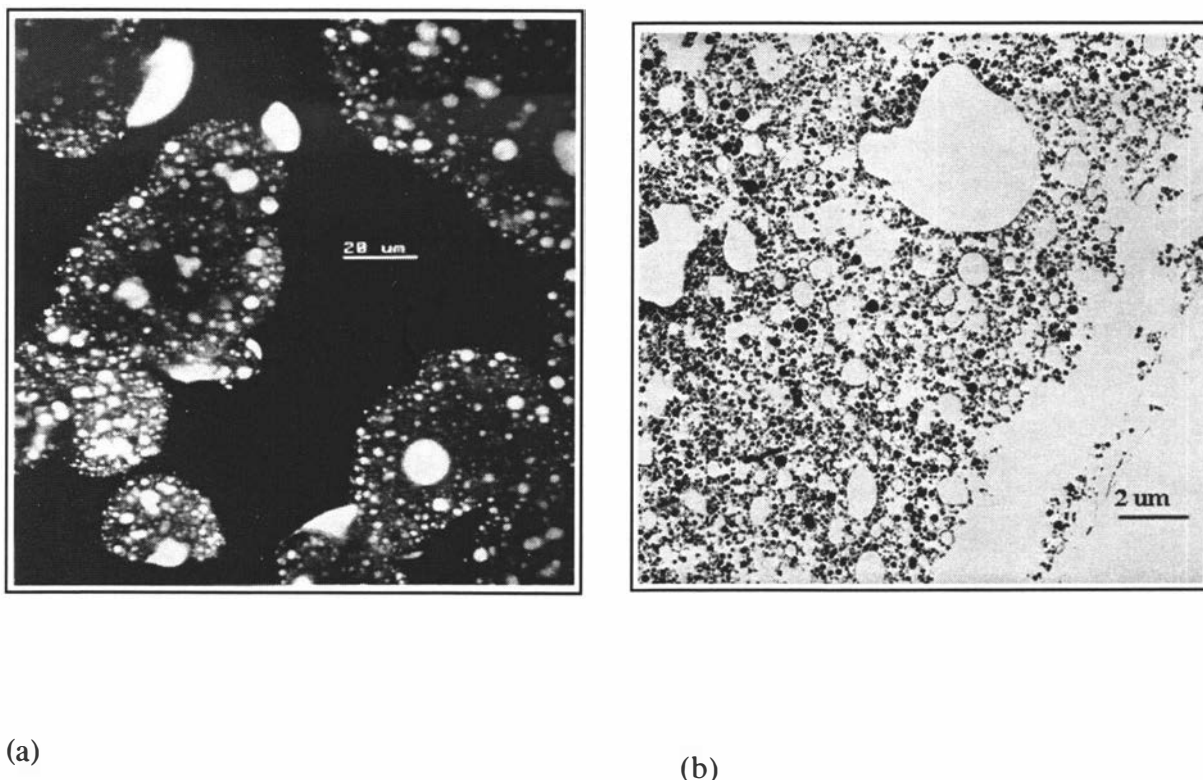
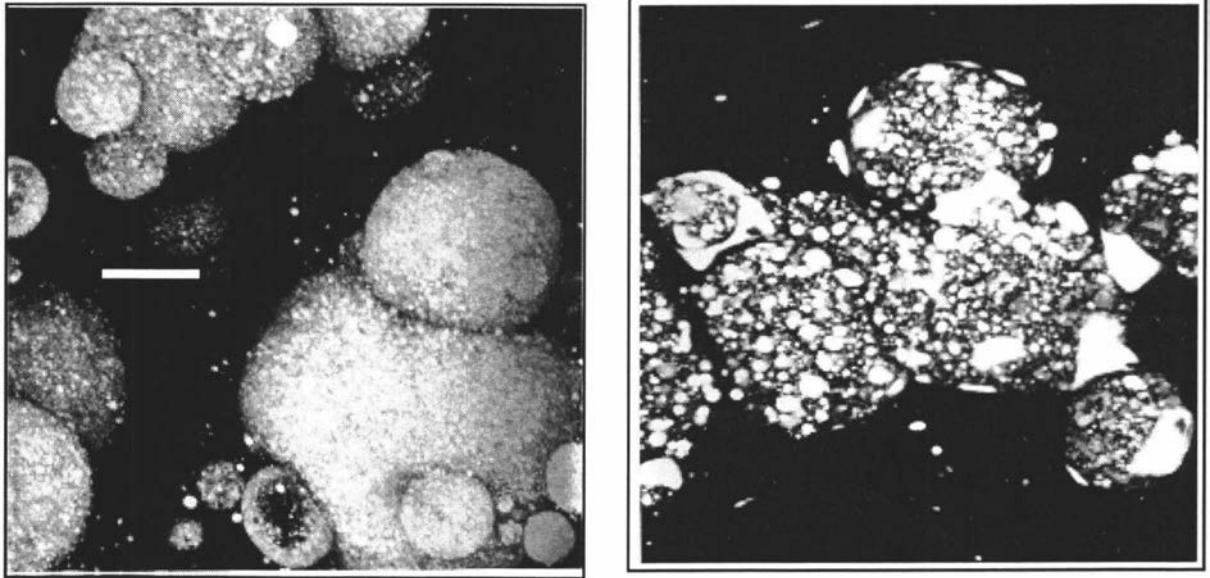


FIGURE 3.2 Regions of coalesced fat within whole milk powder particles (a) as examined using confocal microscopy, (b) as examined by transmission electron microscopy.

Regions of surface fat could be clearly observed using CLSM. It is evident that they appeared to pool at the joining points of agglomerated powder particles. These regions of coalesced fat are likely to influence the flow properties and solubility of the powder particles (Buma, 1971). Reconstructions by merging a series of optical sections from two agglomerated powders with completely different powder properties showed the presence or absence of surface fat (Figure 3.3).



(a)

(b)

FIGURE 3.3 Reconstruction from a series of optical sections, from confocal microscopy, showing (a) no surface free fat on particles of whole milk powder, (b) regions of surface fat on particles of whole milk powder. Bar = 20 μm (both images).

3.3.2 Lactose Crystal Localisation

Lactose crystals in WMP have been examined previously by the use of Heinz solution which disperses the powder and leaves the lactose crystals intact for microscopic examination (Saito, 1985), although there is a possibility that some larger lactose crystals were formed by the mounting procedure. The use of CLSM for the examination of lactose crystals is very simple and non-invasive (no change to the structure of the WMP). When stained for fat, the entire powder particle can be seen with the lactose crystals in diamond-like lactose α -hydrate form clearly evident by negative contrast (Figure 3.4a). When operating the microscope in the reflection mode (Pawley, 1990) the crystals were also clearly visible (Figure 3.4b). It was notable that the majority of the reflecting signal in the powder at this magnification was from the fat globules and air bubbles and that the lactose crystals were only visible by negative contrast. This is explained by the thickness of these crystals and that only scanning at the crystal surface produced a reflection of the laser light.

It has been previously reported that crystallisation of lactose in the milk concentrate prior to drying results in the tomahawk (or diamond) lactose α -hydrate crystals whereas crystallisation of the lactose in the dry milk powder results in the needle-like lactose β -anhydride crystals (Caric & Kalab, 1987).

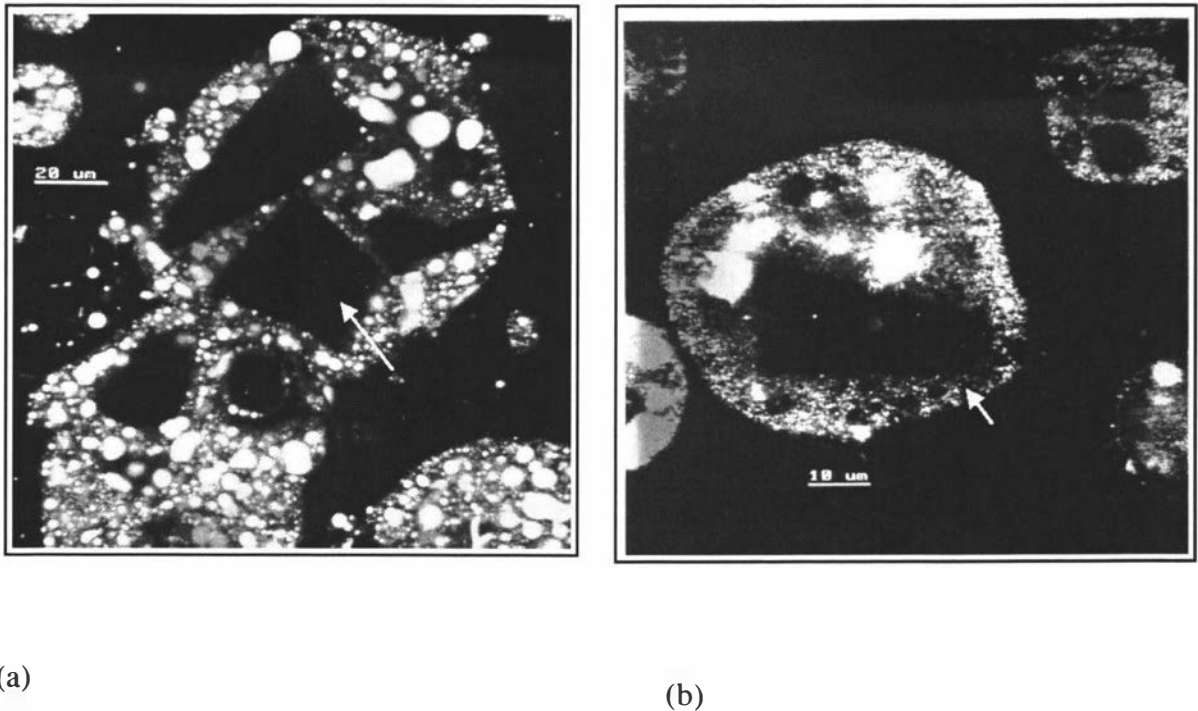
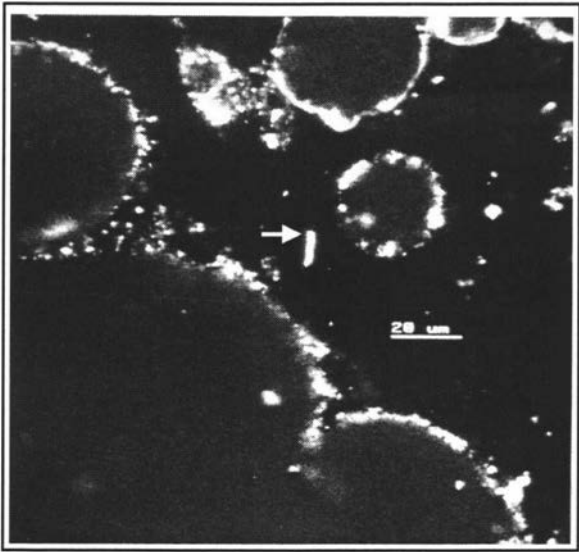


FIGURE 3.4 Lactose crystals (arrows) within whole milk powder particles using confocal laser scanning microscopy, (a) as observed by negative contrast when the fat was examined in the fluorescent mode, (b) as examined in the reflection mode.

3.3.3 Surface Phospholipid Localisation

The quantity of phospholipid in the powders was measured. The addition of the phospholipid-fat blend had resulted in 0.15% phospholipid in the experimental powder whereas only 0.01% phospholipid was present in the control powder.

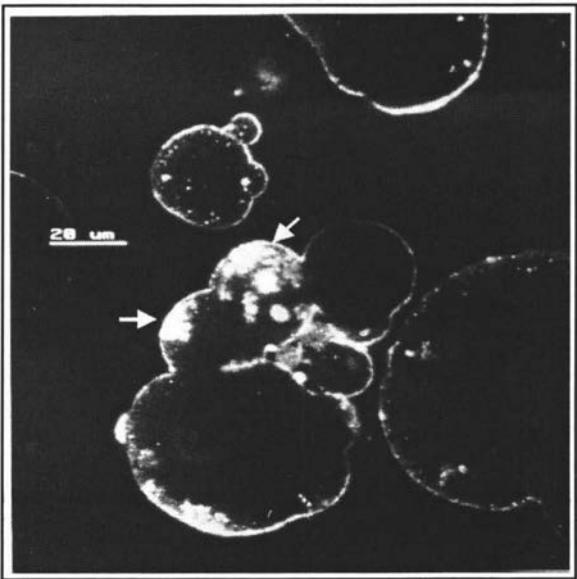
Initially, the WMP containing the added phospholipid wetted faster (16s) than the same powder without the added phospholipid (64s). After 6 months storage, the wetting times were similar for both powders (>90s).



(a)



(b)



(c)

FIGURE 3.5 Whole milk powder particles with fluorescent phospholipid layer (a) examined in a glycerol-based mounting medium, (b) examined in immersion oil (note good coverage of the fluorescent layer on the powder particle) and (c) after 6 months storage ; the arrow shows a region where the phospholipid appears to have entered into the powder particle.

The use of a glycerol-based mounting medium for the examination of the surface phospholipid layer resulted in this layer being partially stripped off the surface of the WMP particle (Figure 3.5a). The use of immersion oil resulted in a clear image of the fluorescent phospholipid around the surface of the powder particle (Figure 3.5b), showing that a completely non-aqueous mounting medium was necessary for this layer to be retained. Storage of treated powder for 6 months, including 1 month at 37°C, resulted in the pooling of this surface layer at the junction of agglomerated particles (Figure 3.5c). The fluorescent phospholipid also appeared to become absorbed into the WMP, probably through cracks during high temperature storage. It is likely that storage of milk powder at temperatures that allow the wetting agent to partly melt and migrate around the surface of the WMP particle may explain the reduction in powder wettability. At 37°C, nearly all (>99%) of the fat in anhydrous milkfat is in the liquid state (MacGibbon & McLennan, 1987) and is therefore able to migrate to regions of lower surface tension *i.e.*, the junction of agglomerated particles or into cracks on the particle surface. This may reduce the amount of surface phospholipid (which is presumably carried with the fat) that is available to act as an amphiphilic wetting agent. Storage at lower temperatures would reduce the amount of liquid fat, thus reducing its mobility, and may improve the wettability of the powder over longer storage periods.

3.4 CONCLUSIONS

The application of CLSM to WMP overcomes many difficulties associated with the microscopic examination of milk powder, such as the inability to focus on a thin focal plane (light microscopy), inability to locate some of the components (scanning electron microscopy) and long preparatory techniques (TEM). CLSM requires only simple sample preparation and then allows non-invasive preparation of optical sections from below the surface of the WMP sample. However, for examining detailed protein-protein and protein-fat interactions in WMP only TEM will suffice.

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CHAPTER 4

MICROSTRUCTURAL CHANGES IN MILK DURING THE MANUFACTURE OF WHOLE MILK POWDER

4.1 INTRODUCTION

The formation of insoluble material on the reconstitution of whole milk powder (WMP) arises from the aggregation of milk components: casein, whey protein, fat globules and minerals (Ohba *et al.*, 1989). This aggregation is probably controlled by the heat denaturation and aggregation of whey protein during powder manufacture and reconstitution.

It is widely understood in the milk powder industry that control and manipulation of concentrate viscosity are important in the manufacture of milk powder with good solubility (Snoeren *et al.*, 1984).

In order to understand the structural interactions that influence the functional properties of instant WMP, it may be valuable to determine the changes that occur at specific processing stages during manufacture. No previous work has been carried out evaluating the structural changes that occur during the major processing steps of WMP manufacture.

In this study, samples of milk at various stages throughout the WMP manufacturing process were obtained and examined by transmission electron microscopy.

4.2 MATERIALS AND METHODS

4.2.1 Milk, Milk Concentrate and Milk Powder Samples

Whole milk was pasteurised (72°C/15 s), standardised and then preheated to 90°C for 20 s prior to evaporation. The milk was concentrated to 54% total solids through a Wiegand evaporator (three-effect, falling film), heated to 80°C and homogenised at 2000 psi (single stage) before being spray dried through a DeLaval pilot spray drier (disk atomisation).

Samples for microstructure evaluation were collected after standardisation, preheating, evaporation and concentrate homogenisation. The milk concentrate collected after homogenisation was held at 80°C and further samples were taken after 5 and 10 min. Upon sampling, the milks were fixed in glutaraldehyde (25%) in a 9:1 ratio.

4.2.2 Transmission Electron Microscopy

Each glutaraldehyde-fixed solution was mixed and then added warm to melted 3% low-temperature gelling agarose in a 1:1 ratio. This solution was poured on to a microscope slide, allowed to set, and chopped into 1 mm³ cubes which were transferred into 0.2 M sodium cacodylate/HCl buffer in a bijou bottle. The buffer was replaced after 30 min and 1 h and then left for a further 1 h prior to replacement with 1 mL of a solution containing 50% osmium tetroxide (2% solution) and 50% cacodylate/HCl buffer. This was left to stand for 2 h before being replaced with 1 mL of 1% uranium acetate for 30 min after which the product cubes were washed with water before dehydration. Dehydration consisted of washing with 50, 70, 90 and 100% ethanol for 5, 30, 30 and 180 min respectively. The 100% ethanol was changed after 30 and 60 min. The ethanol was poured off and the bottle was filled with incomplete resin (20 mL epoxy resin, 20 mL dodecylsuccinic anhydride and 2 mL dibutyl phthalate) and placed on a rotator overnight before replacement with complete resin (incomplete formulation with the addition of 0.6 mL of the plasticiser benzyldimethylamine) and further rotation for 4 h. One cube of sample was added to each of three moulds containing fresh complete resin which were then baked overnight at 60°C.

The samples were microtomed using a Reichert Ultracut microtome, mounted on 3 mm copper grids and stained using lead citrate before examination in a Philips 201 transmission electron microscope (Philips, NL-5600 MD Eindhoven, The Netherlands) at an accelerating voltage of 60 kV.

4.3 RESULTS

4.3.1 Preheating

The most obvious change in the structure of the milk during preheating occurred in the casein micelle. There appeared to be a change from a rather irregular, rough surface structure (Figure 4.1) to a smooth surface structure (Figure 4.2).

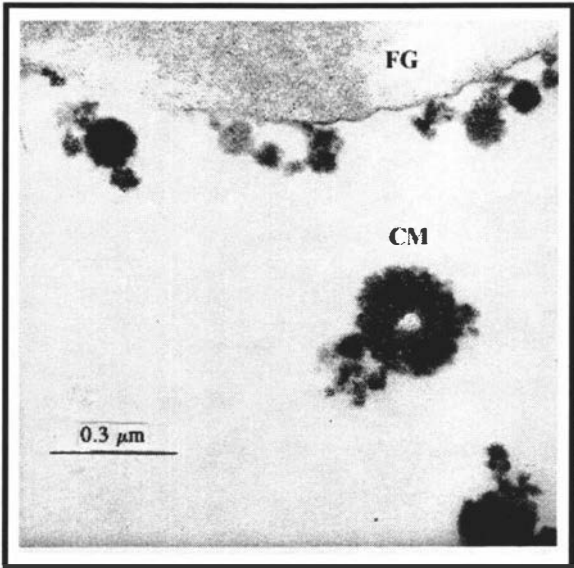


FIGURE 4.1 Casein micelles (CM) and a fat globule (FG) in standardised pasteurised milk (72°C/15 s).

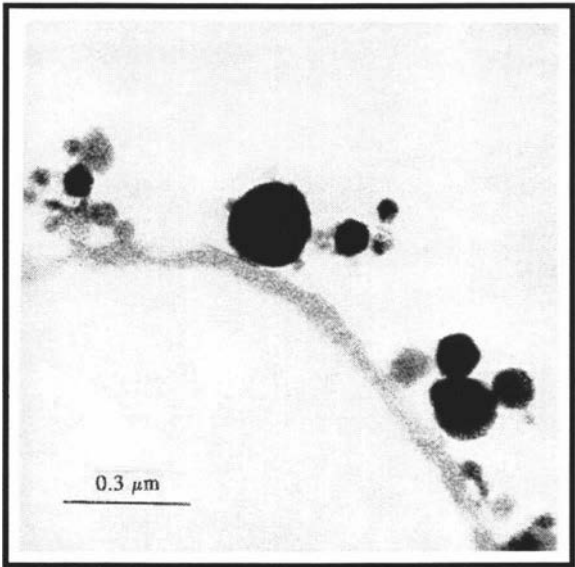


FIGURE 4.2 Casein micelles and a fat globule in preheated milk (90°C/20 s).

4.3.2 Evaporation

During evaporation, the protein, mineral and fat components became more concentrated as the water was removed. After evaporation (Figure 4.3), there appeared to be an increase in the number of casein micelles associating and forming chains (as indicated by arrows). There appeared to be a greater number of micelles associated with the fat globules after evaporation (Figure 4.3).

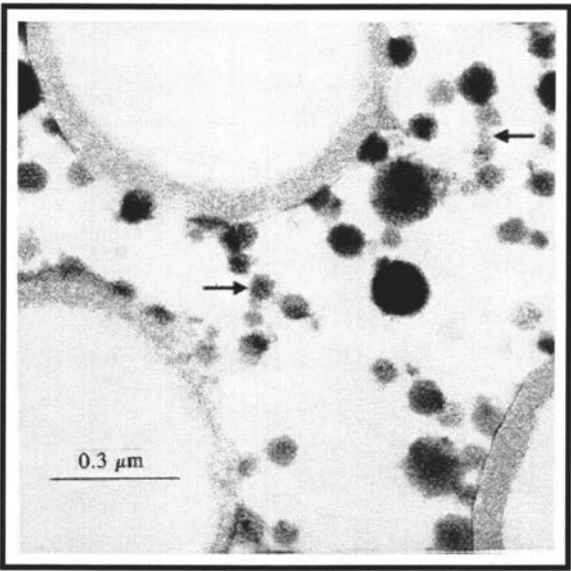


FIGURE 4.3 Concentrated milk (54% solids) after the evaporator.

4.3.3 Homogenisation

Homogenisation had a marked effect on the association of the casein micelles with the fat globules. Prior to homogenisation, there appeared to be some association of micelles with the fat globule surface; after homogenisation, a large proportion of the new fat globule surface was made up of casein micelles (Figure 4.4). Homogenisation creates smaller fat globules with a much increased surface area. The observed incorporation of the micelles onto the fat globule surface after homogenisation in preference to the non-micellar protein is in agreement with the theory of isotropic turbulence for adsorption during homogenisation as proposed by Walstra & Oortwijn (1982). Therefore, the micelles, although less surface active than non-micellar protein, become adsorbed onto the fat globule surface (as indicated by arrows).

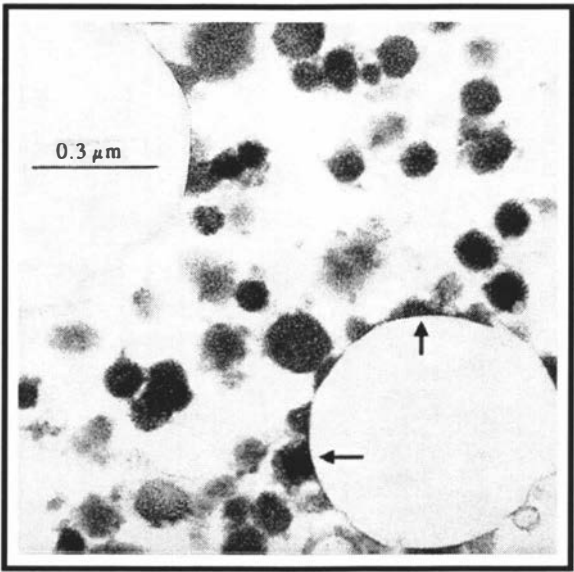


FIGURE 4.4 Homogenised concentrate showing adsorption of the casein micelles onto the fat globule surface.

4.3.4 Aging of Concentrate At 80°C

The major structural change observed after aging of the concentrate at 80°C was the aggregation or fusion of the casein micelles that were attached to the fat globules (Figures 4.5 and 4.6). After 5 min at 80°C, the micelles on the fat globules were no longer discrete but had spread over the fat globule surface and in doing so had fused with other micelles (FC, Figure 4.5). There was more contact between the micelles adsorbed at the fat globule surface and micelles in the serum. After 10 min at 80°C, the micelles around the fat globule continued to spread around the interface. In some instances, the entire surface of the fat globule appeared to be covered by an almost continuous protein interface composed of fused casein micelles (Figure 4.6). In the matrix between fat globules, there was an increase in the amount of aggregated material between the casein micelles as the concentrate was aged. Micelles in the matrix between fat globules were joined together by thread-like material into a chain-like network. The non-micellar material between the casein micelles may be involved in the formation of this casein micelle network.

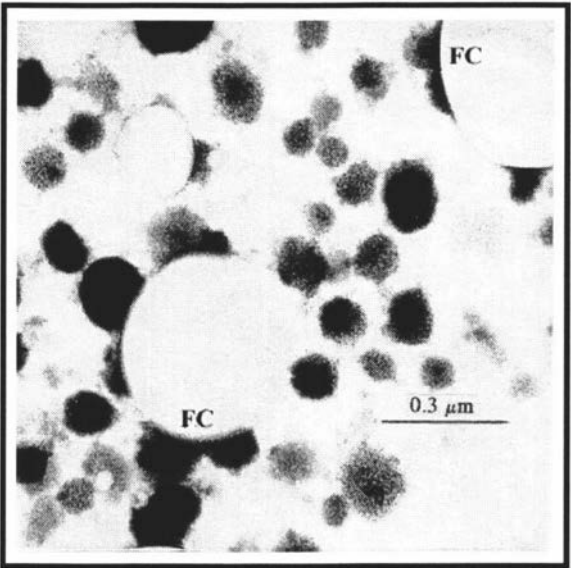


FIGURE 4.5 Homogenised concentrate after 5 min at 80°C showing fused micelles (FC) at the fat globule surface.

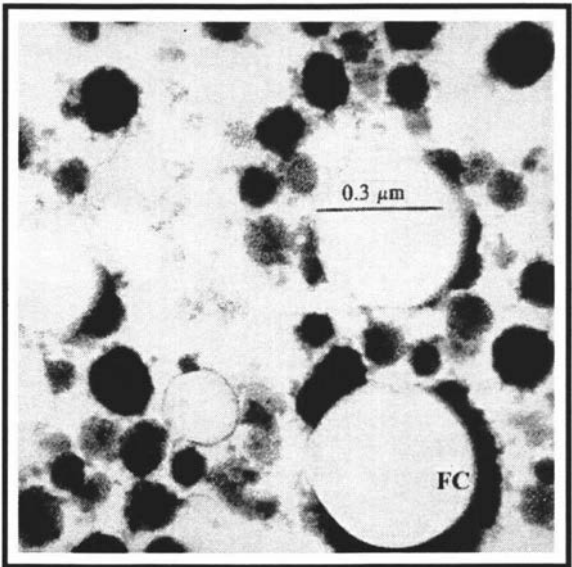


FIGURE 4.6 Homogenised concentrate after 10 min at 80°C showing fused micelles (FC) at the fat globule surface.

4.3.5 Influence of Whey Protein Concentration on the Structure of Concentrated Milk

The results above suggest that whey proteins may have played a major role in the formation of this chain-like network during the concentrate heating stage of WMP processing. Jeurnink (1995) compared whey-protein-depleted milks produced by microfiltration with standard milk for its fouling properties in heat exchangers at 80°C. The whey-protein-depleted milks were found to produce approximately a quarter of the fouled deposit formed by the standard milk.

For this study whey-protein-depleted skim milks (depleted by microfiltration by 16, 32 and 40%, of the original whey protein) and a control skim milk were standardised (30% fat, dry basis) and preheated to either 95°C for 20s or 100° for 60s. The milk was then concentrated through a Wiegand evaporator (three-effect, falling film), heated to 80°C. Samples for microstructure evaluation were collected immediately after concentrate homogenisation and then after holding at 80°C for 10 min.

Micrographs of the milk concentrates (low and high preheat treatments) before and after aging at 80°C for 10 min are shown for the control milk (Figure 4.7), and for the whey-protein-depleted milks: 16% (Figure 4.8), 32% (Figure 4.9) and 40% (Figure 4.10).

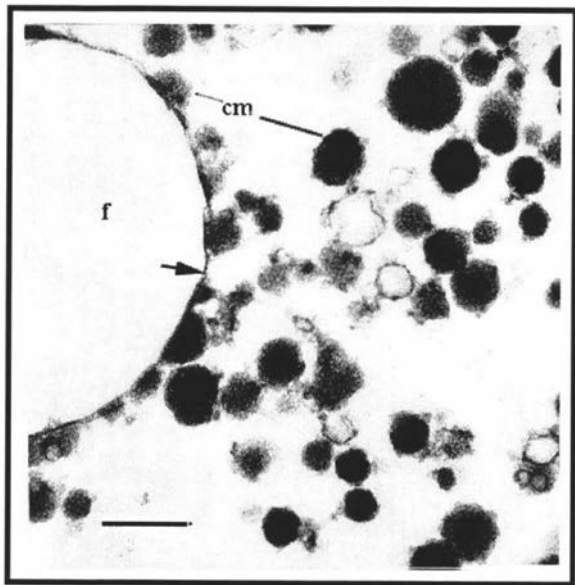
The micrographs of the milk concentrates showed a large amount of structural information. The details of these structural components lay the foundation for the major theme of discussion in the remainder of this chapter. The micrographs, *e.g.* Figure 4.7(a), showed fat globules (f), which contain a membrane (arrow), composed of non-micellar casein and whey proteins (Brooker, 1985), and also include adsorbed casein micelles (cm). Casein micelles were also seen in the serum between fat globules. The interaction of the casein micelles has been shown to be by hairy attachments on the casein micelles (Creamer & Matheson, 1980) which are thought to be denatured whey protein complexed (disulphide linked) to κ -casein on the micelle surface (Mohammad & Fox, 1987; Singh & Fox, 1985, 1986). There is evidence of these structures in the micrographs shown here, *e.g.* h in Figure 4.7(c). The differences between various concentrated milks as shown by the micrographs relate mainly to the quantity of these hairy attachments present on and between the casein micelles.

Control Whole Milk Concentrate

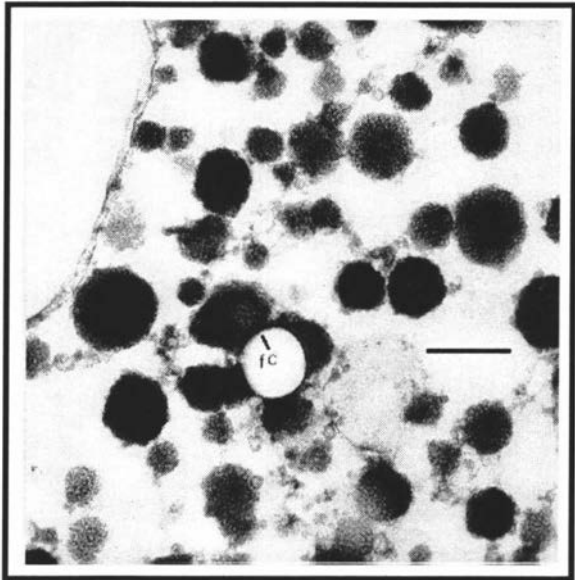
The influence of holding the concentrate at 80°C for 10 min can be clearly seen when comparing Figures 4.7(a) and 4.7(c) (before holding) with Figures 4.7(b) and 4.7(d) (after holding). The phenomenon of casein micelle fusion or spreading around the surface of the fat globule can be seen after 10 min at 80°C (fc in Figures 4.7(b) and 4.7(d)).

There appeared to be more hair-like attachments on the casein micelles in the milk preheated at 100°C for 60 s (Figure 4.7(c)) than in the milk given the lower preheat treatment (Figure 4.7(a)). The aggregation of casein micelles by way of these hair-like attachments appeared to increase significantly after holding the concentrated milk at 80°C for 10 min (h, Figure 4.7(d)). This result is consistent

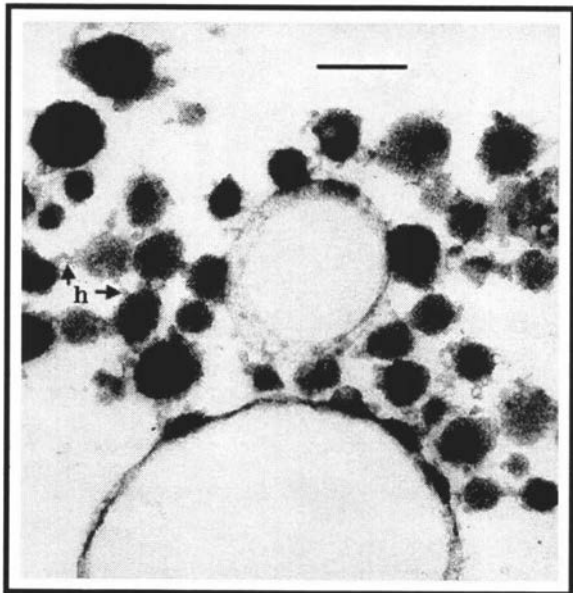
with earlier work on unconcentrated milks, suggesting that heating leads to denaturation of whey proteins and their interaction with the casein micelle by complexing with κ -casein (Singh & Fox, 1985, 1986). These interactions were evident in the milk preheated at 95°C for 20 s (Figure 4.7(b)) but were more marked in the milk preheated for 100°C for 60s.



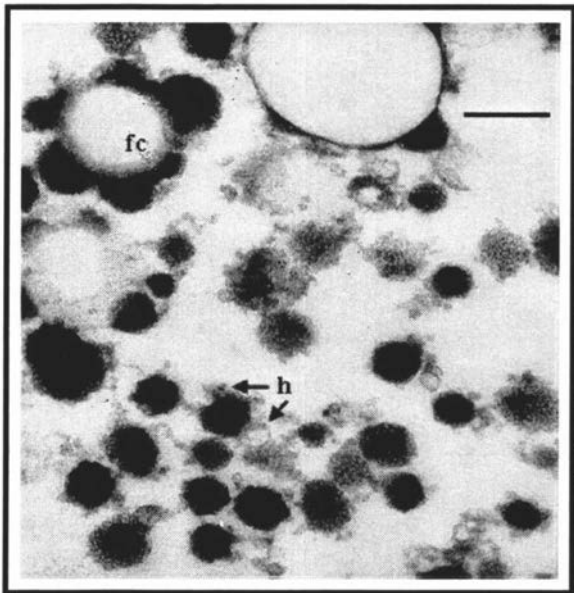
(a) Low preheat, 0 min at 80°C
(95°C for 20s)



(b) Low preheat, 10 min at 80°C



(c) High preheat, 0 min at 80°C
(100°C for 60s)



(d) High preheat, 10 min at 80°C

FIGURE 4.7 Transmission electron micrograph of control whole milk concentrate. f = fat globule, cm = casein micelle, h = hair-like attachment, fc = fused casein micelle (Bar = 0.2 μ m).

Whole Milk Concentrate (16% Whey Protein Depleted)

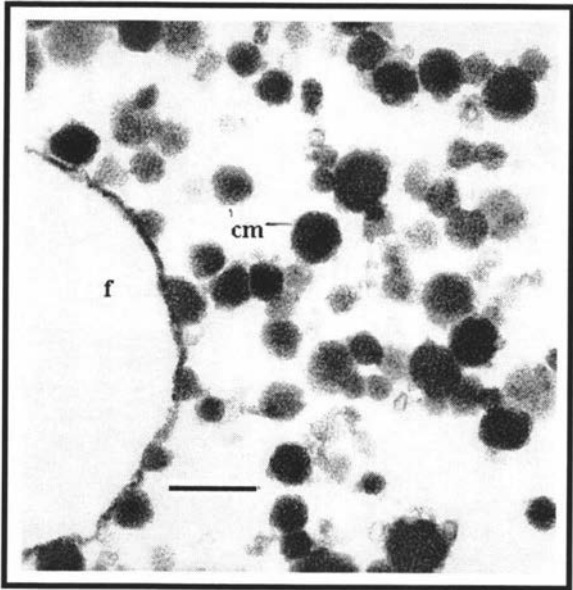
The micrographs of these concentrates (Figure 4.8) were similar to those of the control concentrates. However, there appeared to be fewer hair-like structures attached to the casein micelles, particularly in the concentrate produced from the low heat (95°C for 20 s) milk. The milk concentrate produced from the high heat (100°C for 60 s) milk held at 80°C for 10 min showed a large number of these hairs linking numerous casein micelles (Figure 4.8(d)).

Whole Milk Concentrate (32% Whey Protein Depleted)

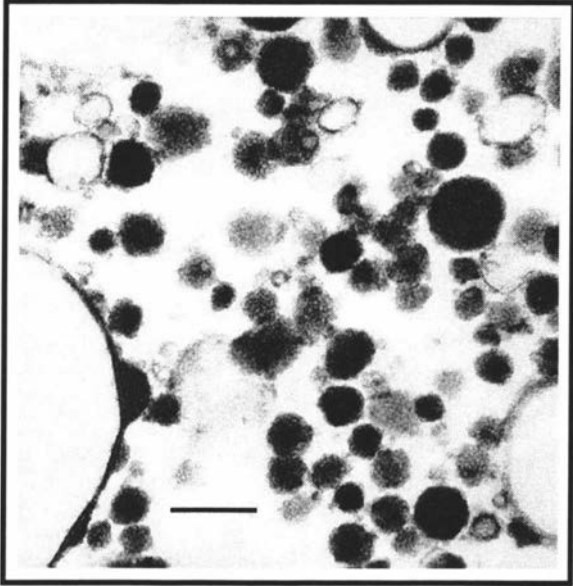
Reduction of the whey protein content by 32% of the original concentration resulted in a further decrease in the number of hair-like attachments on the casein micelles (Figure 4.9). There was visibly less material on the outside of the casein micelles, resulting in more discrete, clearly defined micelles within the serum phase between the fat globules. There was also less interaction between the casein micelles in this concentrated milk than in the previous two milks (compare Figure 4.9(d) with Figures 4.7(d) and 4.8(d)).

Whole Milk Concentrate (40% Whey Protein Depleted)

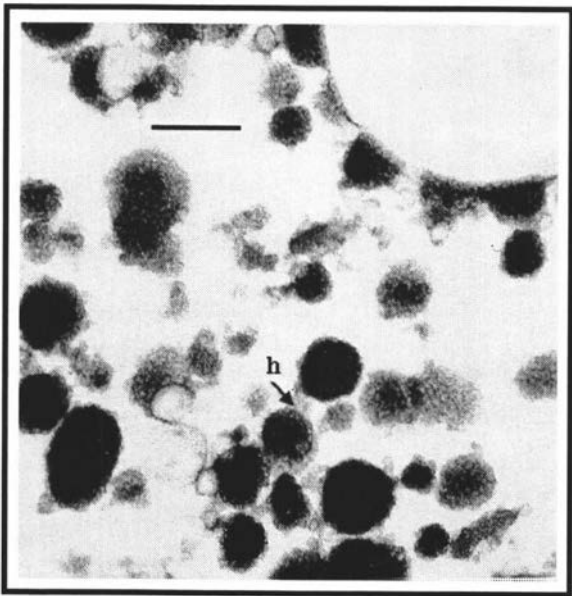
The micrographs of the 40% whey-protein-depleted concentrates (Figure 4.10) were similar to those of the 32% whey-protein-depleted concentrates. They showed that holding the concentrated milk at 80°C for 10 min did not result in the casein micelles being pulled together by hair-like attachments. However, there was some evidence of micelles beginning to come together (arrows, Figure 4.10(d)), *i.e.* micelle-micelle contact. The casein micelles in this concentrated milk appeared to be almost free of the surface material seen on the micelles in the control milk concentrate (Figure 4.7(d)). In addition there appeared to be a decrease in the extent of protein material spreading at the fat globule surface as the concentration of whey protein decreased.



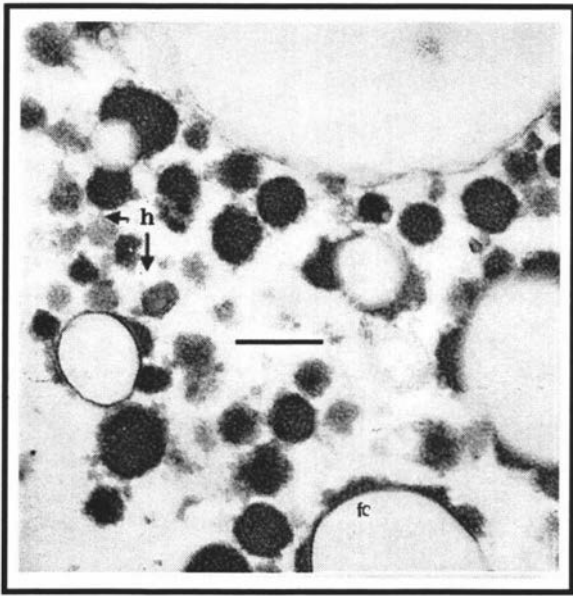
(a) Low preheat, 0 min at 80°C



(b) Low preheat, 10 min at 80°C

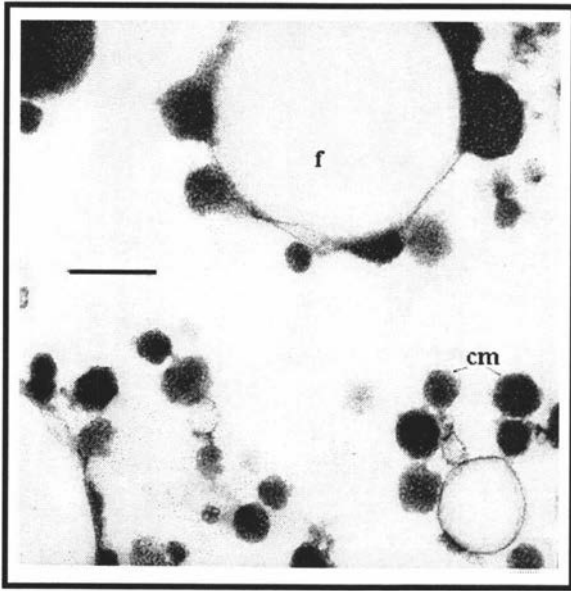


(c) High preheat, 0 min at 80°C

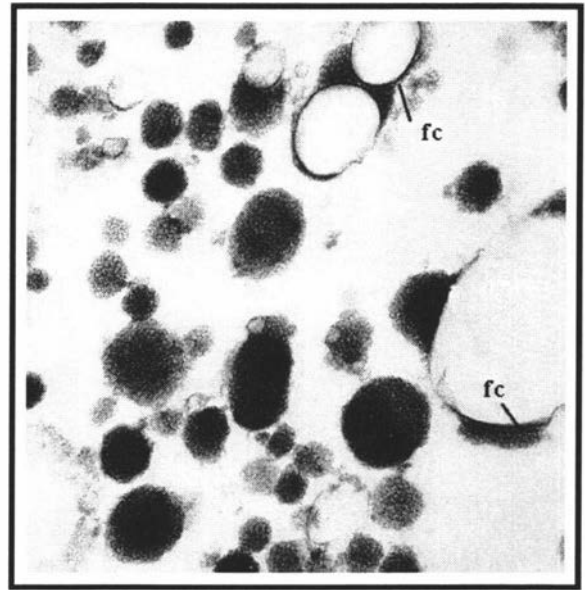


(d) High preheat, 10 min at 80°C

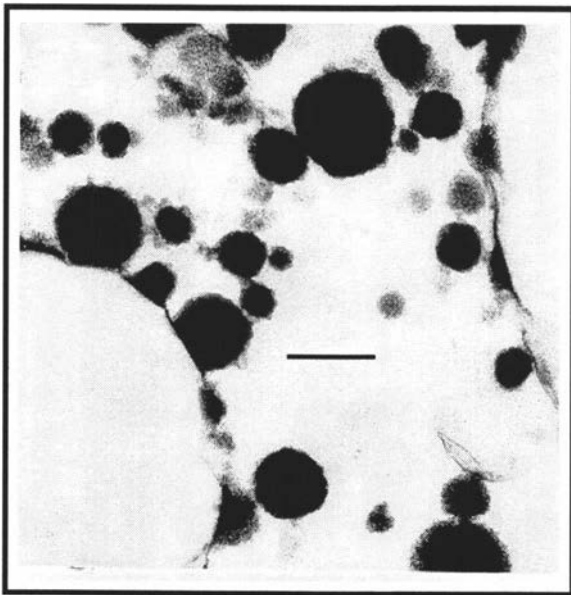
FIGURE 4.8 Transmission electron micrograph of 16% whey-protein-depleted whole milk concentrate. f = fat globule, cm = casein micelle, h = hair-like attachment, fc = fused casein micelle (Bar = 0.2 μ m).



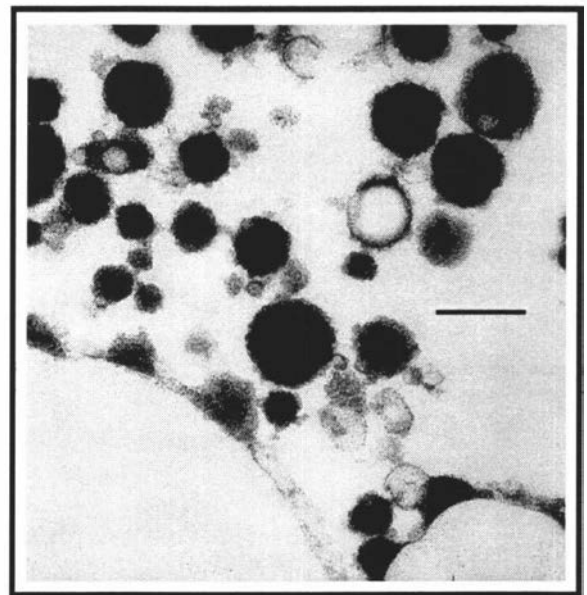
(a) Low preheat, 0 min at 80°C



(b) Low preheat, 10 min at 80°C

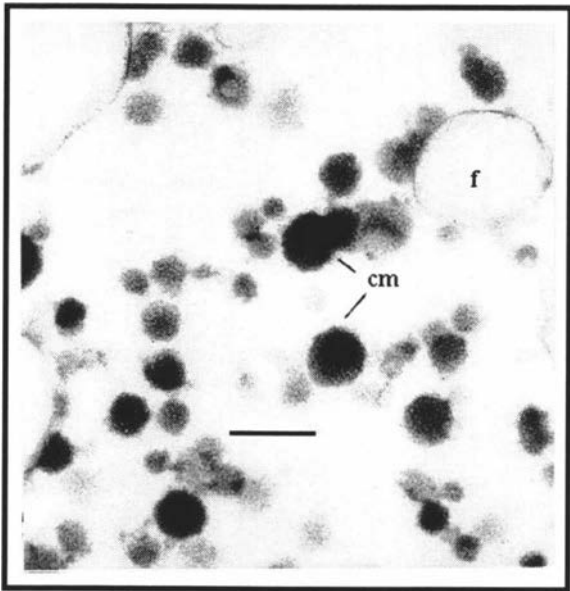


(c) High preheat, 0 min at 80°C

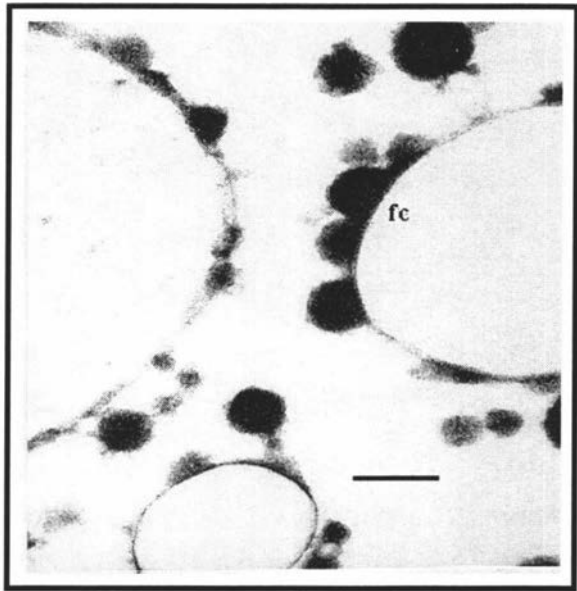


(d) High preheat, 10 min at 80°C

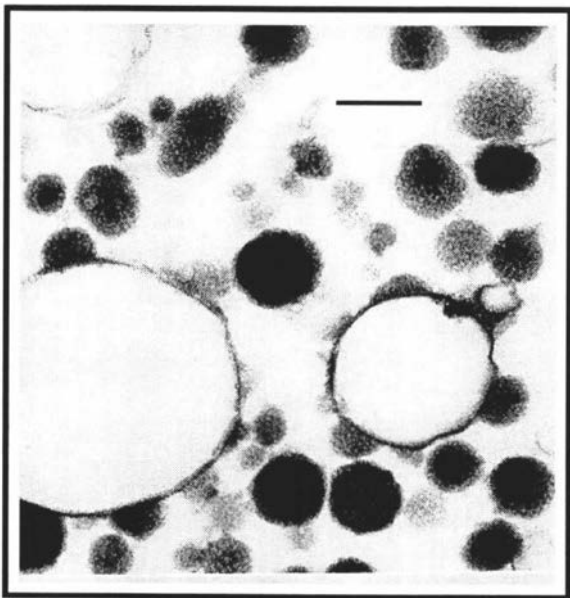
FIGURE 4.9 Transmission electron micrograph of 32% whey-protein-depleted whole milk concentrate. f = fat globule, cm = casein micelle, fc = fused casein micelle (Bar = 0.2 μm).



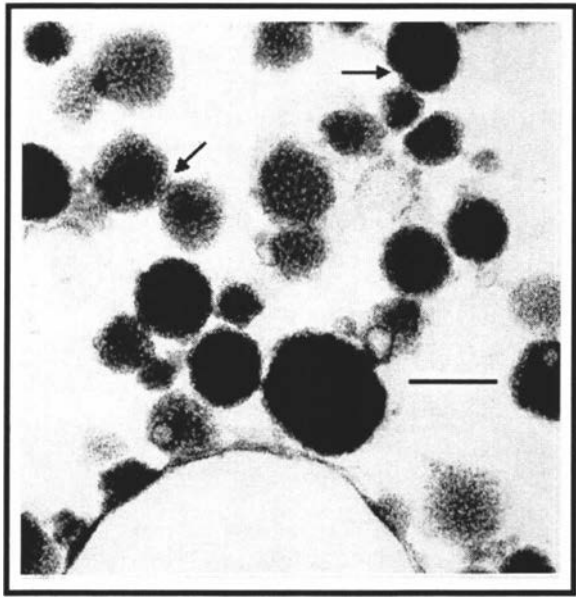
(a) Low preheat, 0 min at 80°C



(b) Low preheat, 10 min at 80°C



(c) High preheat, 0 min at 80°C



(d) High preheat, 10 min at 80°C

FIGURE 4.10 Transmission electron micrograph of 40% whey-protein-depleted whole milk concentrate. f = fat globule, cm = casein micelle, fc = fused casein micelle (Bar = 0.2 μ m).

4.4 DISCUSSION

In some recent work (Anema, 1998), it was noticed that κ -casein dissociation from the micelle increased as the concentration of skim milk components was increased by evaporation. Increasing the solids concentration from 20 to 60% resulted in an increase in dissociated κ -casein from approximately 10 to 20%. As κ -casein is associated with β -lactoglobulin after preheating, the dissociation of this charged complex will reduce the negative charge on the micelles. The reduction in micellar charge will probably cause an increase in casein micelle aggregation. This aggregation is evident where the micelles are in close contact with each other, *i.e.* at the fat globule surface (Figure 4.5) and probably at the air interface on the surface of the powder particle after it has been spray dried. Away from these interfaces, the micelles are in less intimate contact and therefore micelle fusion rarely occurs (within the time frame examined here) but the micelles did appear to aggregate to some degree. The material promoting aggregation appeared to be either non-micellar material or 'hairs' on the surface of the micelle, which could clearly be seen in Figures 4.5 and 4.6 to link many of the casein micelles in the serum. Hair-like structures have been observed on the surfaces of casein micelles in heated milks (Davies *et al.*, 1978; Mohammed & Fox, 1987). In skim milk heated at 120°C for 30 min (pH 6.65), thread-like particles at the micelle surface were observed by electron microscopy (Creamer *et al.*, 1978).

Not all of the fat globule surface was composed of casein micelles after homogenisation. However, the micelles adsorbed at the fat globule surface appeared to aggregate with adjacent micelles, and appeared to become more electron dense. This suggested that there was some change in the structure of the micelles as they adsorbed to the fat globule surface and/or as the milk was heated. There appeared to be an increase in the quantity of protein material on the interface, suggesting that protein from the serum (probably β -lactoglobulin) was also depositing on the fat globule interface. Eventually during aging of the concentrate it appeared, in some instances, that the casein micelles spread and covered the fat globule surface completely to produce a very thick protein layer around the fat globule (Figure 4.6). It is likely that fat globules of this nature linked by protein-protein bonds via a network of casein micelles to other fat globules will result in the formation of dense aggregates that will contribute to the sediment material in reconstituted WMP. When Mol (1975) examined the composition of the sediment from a reconstituted WMP of poor solubility, the main protein component was casein. He concluded that the adsorption of casein on the fat globule membrane strongly influenced powder solubility. The removal of casein from the serum prior to homogenisation resulted in no casein being present at the fat globule interface and an improvement in powder solubility (Mol, 1975).

After examining the effect of whey proteins on fouling, Jeurnink (1995) concluded that, if whey proteins were present in their denatured state, they were able to interact with each other, with casein micelles and with the stainless steel surface of a heat exchanger to form a deposit on this surface. It was stated that the serum proteins acted like a 'sticking agent' between the casein micelles.

The micrographs from this work clearly showed a reduction in the number of hair-like attachments on the casein micelles as the whey protein concentration in the milk was reduced, see Figure 4.7 (control) versus Figure 4.10 (40% whey protein depleted). This resulted in a

reduction in the extent of casein micelle aggregation in the whole milk concentrate. The formation of large aggregates of micelles linked by the hair-like attachments after heating (10 min at 80°C) was evident only in the milk concentrates made from the control milk preheated at both 95°C for 20s and 100°C for 60 s and in those made from the 16% whey-protein-depleted milk preheated at 100°C for 60 s.

The formation of a protein chain between the micelles adsorbed on adjacent fat globules is likely to produce a network that will influence the rheological characteristics of the milk concentrate, *i.e.* age thickening.

The structure that exists in the powder particle after drying could dictate the rate of dissolution in water and the extent of ongoing protein interaction when the powder is reconstituted in hot water and coffee. The state of the whey proteins, the concentration of ionic calcium and the pH of the powder would also have an influence on the reconstitution properties of the milk powder in hot water and coffee (Ohba *et al.*, 1989).

4.5 CONCLUSIONS

This work showed that major structural changes in the fat globules, casein micelles and whey proteins occurred during the manufacture of WMP. Heating and homogenisation resulted in the greatest structural changes. Heating resulted in the formation of hair-like structures on and between casein micelles. Homogenisation resulted in the adsorption of casein micelles onto the fat globule surface. Upon further heating the adsorbed protein on the fat aggregated with other micelles to form a chain-like network. The material promoting aggregation appeared to be either non-micellar material or 'hairs' on the surface of the micelles. Reduction in the whey protein concentration of the concentrated milk appeared to reduce the number of hair-like structures, aggregation and the extent to which this network formed upon heating.

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CHAPTER 5

MICROSTRUCTURE OF INSTANT WHOLE MILK POWDER AND OF INSOLUBLE MATERIAL DETECTED BY POWDER FUNCTIONAL TESTING

5.1 INTRODUCTION

Milk powders manufactured for consumer use must perform within a number of harsh reconstitution environments without noticeable defect. They must disperse rapidly in water (hot and cold) or hot coffee and be quickly and completely dissolved, *i.e.* form a stable colloidal suspension of fat and casein micelles without leaving a visible residue on the surface of the milk or on the sides and the bottom of the container. Protein-protein and protein-fat interactions have been shown to influence the reconstitution properties of dried milk in water (Mol, 1975; Caric & Kalab, 1987; Ohba *et al.*, 1989; de Ruyck, 1991).

A number of functional tests for instant whole milk powder (IWMP) were developed during the 1970s to test for potential defects when the IWMP was reconstituted under conditions generally used by consumers. The functional requirements of IWMP and the tests used to measure these requirements have been reviewed by Pisecky (1990). Typical functional tests for IWMP include the following: wettability (time for a specified quantity of powder to fall below the surface of still water) (International Dairy Federation, 1979), dispersibility (amount of undispersed particles collected on a filter after reconstitution under set conditions (International Dairy Federation, 1979), slowly dispersing (or dissolving) particles (flecks seen in the milk after reconstitution in hot or cold water) (Pisecky, 1990), various tests for sediment and sludge after dispersion in hot water. Generally milk powder is not completely insoluble but in this chapter the material collected after functional tests will be referred to as “insoluble material”.

Little or no work examining the characteristics of the insoluble material collected after reconstitution of milk powder has been carried out. An examination of the microstructure of milk powder and of the insoluble material collected after applying a series of functional tests may provide insight into why the insoluble material does not dissolve.

5.2 MATERIALS & METHODS

A pair of commercial IWMP samples was obtained from each of two milk powder manufacturing companies. Each pair consisted of powders with different functional properties manufactured on the same powder plant within 24 h of each other. These powders are referred

to as X1 and X2 (Factory X) and Y1 and Y2 (Factory Y). The processing conditions for the manufacture of the powders within each pair were identical apart from a change in shear, increase in homogenisation pressure, during powder X2 manufacture and a change in heating conditions, increase in preheating temperature and time, during powder Y2 manufacture.

5.2.1 Determination of Cold (25°C) Slowly Dissolving Particles (SDP), Sludge and Sediment (25°C)

A method similar to that described by Pisecky (1990) was used. Milk powder was mixed into water at 25°C using a prescribed mixing procedure. After 2 min hydration time the milk was poured through a stainless steel mesh. The residue (sludge) deposited on the mesh was weighed. The reconstituted milk was then poured into a glass test tube, inverted and drained. After 2 min, the residue, SDP, deposited on the inner surface of the test tube was compared with a standard chart and graded from A (few SDPs) to E. The remaining milk was poured into centrifuge tubes. The sediment (25°C) was measured as millilitres of sediment remaining in the calibrated sample tube after centrifugation at 160g for 5 min.

5.2.2 Determination of Hot (85°C) Slowly Dissolving Particles (SDP), Sludge and Sediment (85°C)

The same procedure as described in Section 2.1 was used except that the test temperature was 85°C and the powder was hydrated for 15 min.

5.2.3 Determination of Hot Coffee Sediment

A method similar to that described by Teehan *et al.* (1997) was used to measure the stability of milk powder in hot coffee. Milk powder was mixed into a standard coffee solution (80°C). After a 10 min standing time, the sample was re-mixed, poured into a centrifuge tube and centrifuged at 160 g for 5 min. The coffee sediment was measured in millilitres.

5.2.4 Measurement of Dispersibility

A method based on the International Dairy Federation method (International Dairy Federation, 1979) was used to measure dispersibility. Powder was mixed into water (45°C). The reconstituted milk was immediately poured through a mesh under vacuum. The residue remaining on the mesh was compared with a standard chart and assigned a grade from 1 (low) to 7.

5.2.5 Microstructural Analysis

The milk powders were evaluated by confocal microscopy and transmission electron microscopy (TEM). Sludge samples were prepared for microscopy by mixing with glutaraldehyde (final concentration = 3%) in a bijou bottle. The cold, hot and coffee sediments were resuspended in water (20°C) and centrifuged (5 min at 160 g), and the sediment was mixed with glutaraldehyde (final concentration = 3%) in a bijou bottle.

Confocal Microscopy

The powders and insoluble material collected after testing (coffee sediment, hot sediment, hot sludge and cold slowly dissolving particles) were prepared for microscopy by dispersing the samples into a glycerol-based solution (Citifluor, UKC, Chem. Lab., Canterbury, UK) containing 10 mg/L of Nile Blue (fat stain) and placing this solution on a microscope slide for viewing using a Leica confocal microscope (Leica TCS 4D, Leica, Heidelberg, Germany). A 63 X oil immersion lens with a fluorescein isothiocyanate filter block in the scanner was used for excitation of the dye in the fat phase at a wavelength of 488 nm.

Transmission Electron Microscopy

Refer to Chapter 4 for methodology.

Scanning Electron Microscopy

The powder samples were mounted on an aluminium stub and sputter coated with 20 nm of gold then transferred into a Cambridge 250 Stereoscan scanning electron microscope (Cambridge Ltd, Cambridge, England).

5.2.6 Particle Size Analysis of Reconstituted IWMP

The particle size distribution of the samples was measured using a Malvern Mastersizer E (Malvern Instruments, Malvern, UK), with a presentation code that assumed the following optical parameters: a differential refractive index between the dispersed and continuous phases of 1.095, a particle absorbance of 0.00, a dispersed refractive index of 1.456 and a continuous phase refractive index of 1.33. This corresponded to a presentation code of 2NAD in the Malvern Mastersizer E software. The samples were analysed with and without the incorporation of a micellar protein dissociating buffer (a solution containing 0.79% w/v EDTA and 0.25% w/v Tween 20 adjusted to pH 10) (Walstra, 1965) for determination of the degree of protein-induced fat globule aggregation.

5.2.7 Particle Size Analysis of Powders

Powder particle size was measured using a Malvern 2600c laser diffraction size analyser and a 1000 mm focal length lens. Powder air dispersion was achieved using a dry powder feeder that fed the powder vertically through the laser at low velocity thus preserving the agglomerated particle structure.

5.2.8 Proximate Analyses

The total nitrogen content of samples was determined using the Kjeldahl procedure (International Dairy Federation, 1993). The samples were digested and automatically titrated on a Kjel-Foss automatic 16200, A/S N, Foss Electric, Hillerød, Denmark, to determine the total nitrogen percentage of the original sample. The protein percentage was calculated by multiplying the total nitrogen result by 6.38. The fat content was determined by the Roese Gottlieb gravimetric method. Results are presented as weight percentages of fat in the sample.

Ash content of the powders were measured by ashing at 550°C overnight and then measuring the resulting mass.

Lactose was measured by the reduction of ferricyanide to ferrocyanide that was measured colorimetrically using an autoanalyser at 420 nm according to the Technicon Method (120-71A, Technicon Industrial Systems Tarrytown, NY).

The moisture content of the powders was measured by the Karl Fisher titration moisture method (New Zealand Ministry of Agriculture and Fisheries Dairy Division, 1979).

The free fat content of the milk powders was measured by fat extraction with petroleum ether as described by Buma (1971).

5.2.9 Whey Protein Nitrogen Index (WPNI)

WPNI was evaluated using the method of Sanderson (1970) and undenatured whey protein was expressed as milligram of whey protein per gram of powder.

5.2.10 Fat-bound Protein

This method was used to determine the quantity of protein adsorbed onto the fat globule surface in the whole milk powder samples. The method (D F Newstead, 1998, unpublished work) involved the separation of the cream from the serum by centrifuging at 35 000 g for 30 min in a solution of 7% sucrose (without further rinsing). Samples of both the cream layer and the serum layer were analysed for moisture content and protein content; the fat content of the cream layer was also determined. The fat bound protein value was calculated using the following mass balance equation:

$$\text{Fat bound protein} = \frac{P_c \left(\frac{M_c}{M_s} \times P_s \right)}{(F_c / 1000)} \text{ mg protein/g fat}$$

where

P_c	=	protein content of cream plug (mg/g);
P_s	=	protein content of serum below cream plug (mg/g);
M_c	=	moisture content of cream plug (g/g);
M_s	=	moisture content of serum below cream plug (g/g);
F_c	=	fat content of cream plug (mg/g).

5.3 RESULTS

5.3.1 Composition of Milk Powders

The compositional results obtained by chemical analysis are shown in Table 5.1.

TABLE 5.1 Composition of Whole Milk Powders

POWDER				
	X1	X2	Y1	Y2
Fat (%)	29	29	26	27.5
Protein (%)	27	27	26	26
Lactose (%)	33	34	38	38
Ash (%)	5.1	5.3	5.9	5.8
Moisture (%)	2.7	2.7	2.7	2.7
Titrateable acidity (%)	0.101	0.099	0.155	0.14
pH (10% solution)	6.72	6.7	6.51	6.52
Free fat (%)	1.35	1.07	1.18	1.78
WPNI (mg/g)	1.8	1.8	1.4	1.1

Titrateable acidity and pH measurements generally give an indication of the standard of milk quality. The greater the titrateable acidity, the poorer is the quality of the raw milk. It is commonly recognised that the maximum acidity should not exceed 0.15%; otherwise the solubility of the resulting powder can be reduced (Caric, 1994). The pH of the milk will generally reduce as a result of acid formation in the milk. The results show that the X powders had acceptable titrateable acidity (0.10%) whereas the Y powders were at the upper limit (0.14-0.16%). This suggests that the quality of the milk used for the manufacture of the Y powders was not as good as the milk used for the manufacture of the X powders and may have influenced some of the functional properties of the Y powders. The fat contents of the X powders were slightly higher and the lactose content slightly lower than that of the Y powders.

The powders had whey protein nitrogen index (WPNI) values (Table 5.1) in the medium to high heat range (Sanderson, 1970) with the Y powders having had more severe heating during manufacture.

5.3.2 The Particle Size And Functional Properties of Whole Milk Powders

The particle size, fat-bound protein and milk powder functional results are given in Table 5.2. The powder particle size data show that powders Y1 and Y2 had a greater number of smaller powder particles than powders X1 and X2. The powder particle size distribution is likely to be related to the specific design of the atomiser and the drier and in particular the agglomeration system. The agglomerated particles may break apart to some degree in the fluidised bed or the pneumatic powder transportation system.

TABLE 5.2 Particle Size and Functional Properties of Whole Milk Powders^a

POWDER				
TEST	X1	X2	Y1	Y2
Particle size (powder)				
D(v,0.1) (10% of particles below) (μm)	83	89	74	69
D(v,0.5) (50% of particles below) (μm)	237	233	199	190
D(v,0.9) (90% of particles below) (μm)	557	534	450	408
Particle size (reconstituted milks, 12.5% total solids)				
Fat globules < 0.3 μm (water) (%)	3.8	21.56	13.4	7.7
Fat globules < 0.3 μm (buffer) (%)	3.5	31.52	18.6	9
Fat globules > 2 μm (water) (%)	18	42	20	34
Fat globules > 2 μm (buffer) (%)	10	13	10	13
Fat-bound Protein (mg protein/g fat)	145	175	153	148
Dispersibility	1	3	3	2
Coffee sediment (mL)	0.5	3.1	1.3	1.7
Sediment (85°C) (mL)	0.2	4.2	0.7	2.4
Sludge (85°C) (g)	0	0	0.2	0.4
SDP (85°C)	B	E	C	D
Sediment (25°C) (mL)	1.8	18	4	6.8
Sludge (25°C) (g)	0	0	0	0
SDP (25°C)	C	E	C	D

^a For particle size distributions, percentages are by volume.

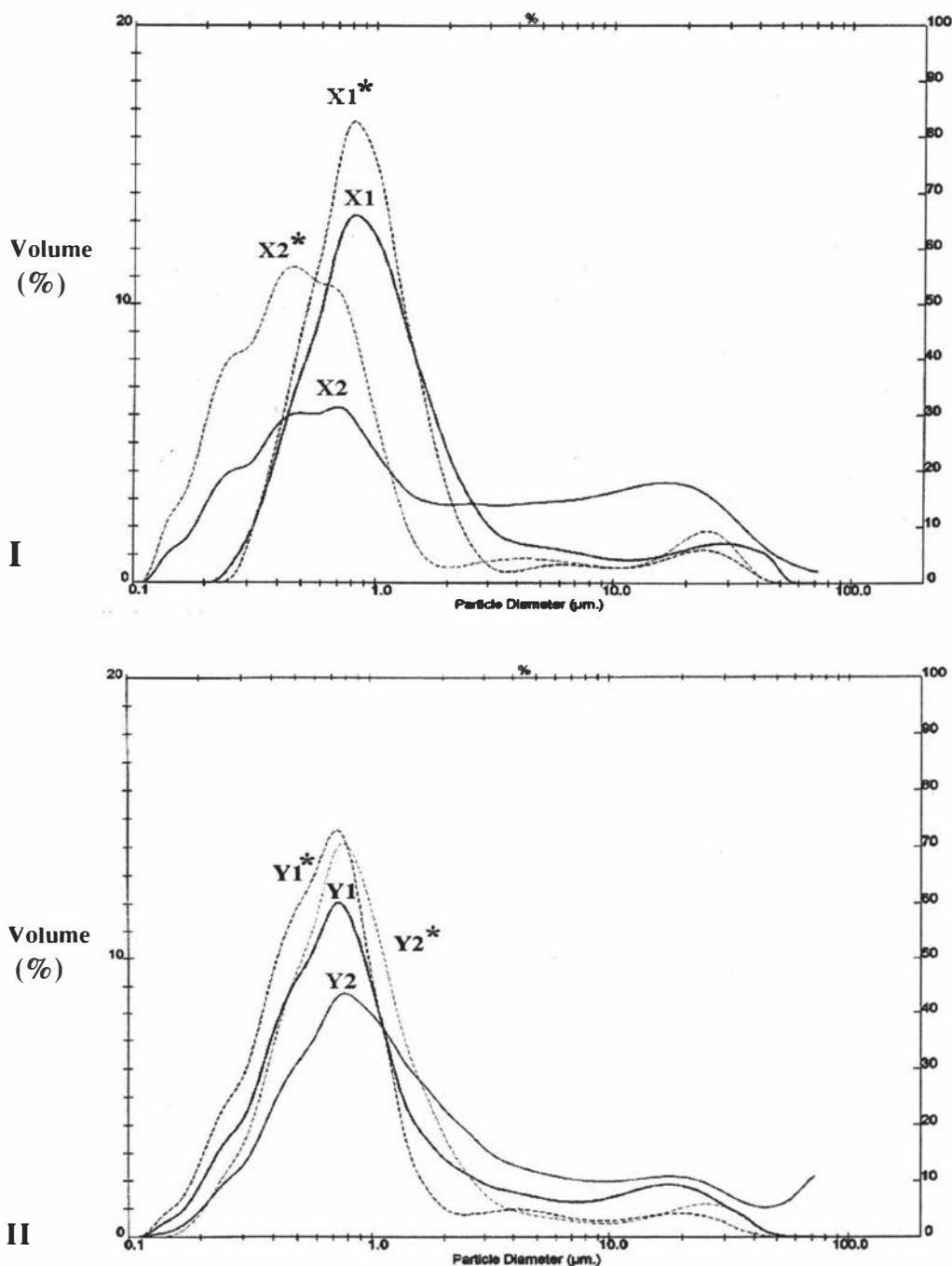


FIGURE 5.1 Particle size distribution of reconstituted whole milk powders, dispersed in water (____) or in a dissociating buffer (-----).

I = X powders ($X1$ = powder $X1$ dispersed in water, $X1^*$ = powder $X1$ dispersed in a dissociating buffer, $X2$ = powder $X2$ dispersed in water, $X2^*$ = powder $X2$ dispersed in a dissociating buffer).

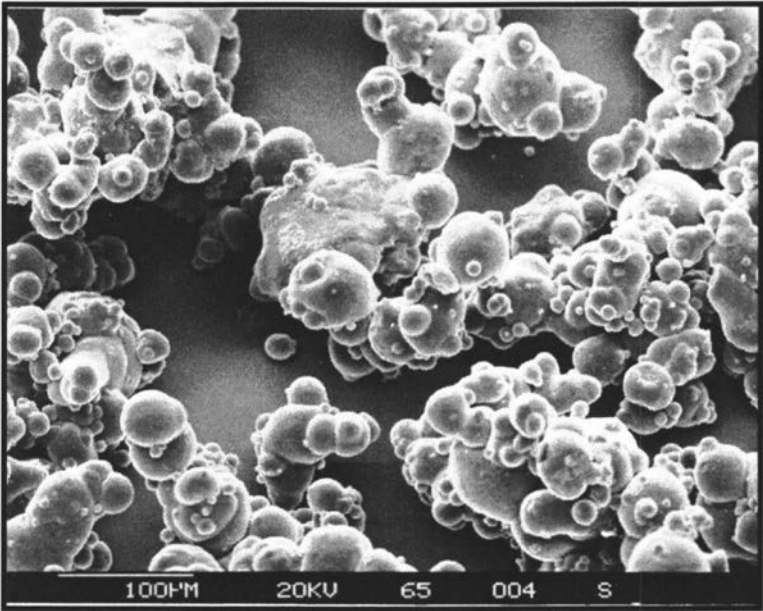
II = Y powders ($Y1$ = powder $Y1$ dispersed in water, $Y1^*$ = powder $Y1$ dispersed in a dissociating buffer, $Y2$ = powder $Y2$ dispersed in water, $Y2^*$ = powder $Y2$ dispersed in dissociating buffer).

The particle size data for the reconstituted powders (12.5% total solids) dispersed in distilled water or in a dissociating buffer (Walstra, 1965) are shown in Figure 5.1 and Table 5.2. The observed particle size distribution is derived from light scattering data from all particles (casein micelles and fat globules) present in the reconstituted milk. However because of their larger size, the fat globules have greater scattering power and thus the measured distribution is predominantly from fat globules. The casein micelle is likely to influence only the low size range of the distribution. After dispersion of the milk in a dissociating buffer, the casein micelles are completely dissociated and the size distribution measured relates only to the fat globule sizes. Since the dispersion of sample into a dissociating buffer would also dissociate the micellar protein bridging between adjacent fat globules (Walstra, 1965). It is evident from these results that there was considerable fat globule clustering in powder X2 (42% of particles greater than 2 μm) and to a lesser extent in powder Y2 (34% of particles greater than 2 μm) as indicated by a reduction in the proportions of particles above 2 μm to only 13% after the addition of dissociating buffer. Powders X1 and Y1 had considerably fewer clustered fat globules, shown by the lower initial proportions of particles above 2 μm . The extent of fat globule clustering appeared to be related to the solubility of the powders. Powder X2 (greatest fat globule clustering) had the poorest scores for all the solubility related functional tests (Table 5.2), *i.e.*, coffee sediment (3.1 mL), hot sediment (4.2 mL), cold sediment (18 mL) and slowly dissolving particles (E for both hot and cold). This was in contrast to powder X1 (lowest clustering) which had the best scores for all the functional tests, *i.e.*, coffee sediment (0.5 mL), hot sediment (0.2 mL), cold sediment (1.8 mL) and slowly dissolving particles (B for hot, C for cold).

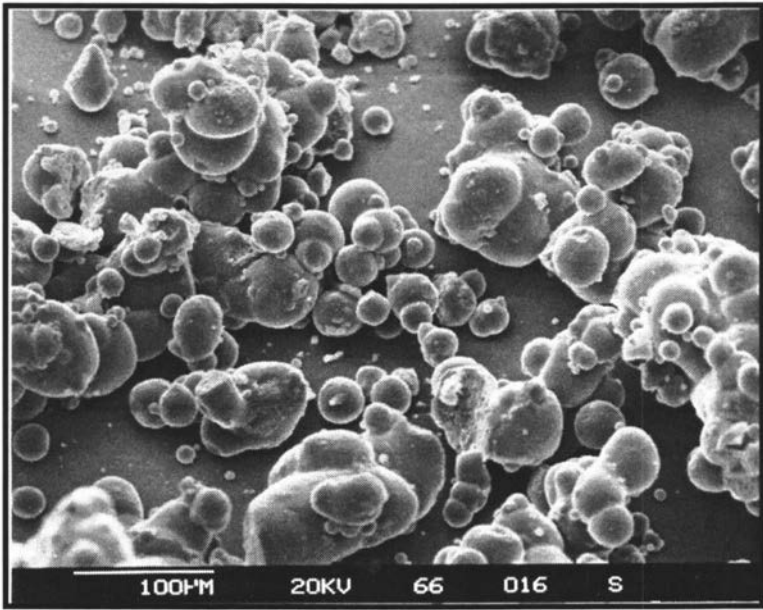
The Y powders had poorer functional properties than powder X1 but were not as poor as powder X2. Powder Y2 (high clustering, Figure 5.2) had poorer functional scores than powder Y1, *i.e.*, coffee sediment (1.7 mL versus 1.3 mL for powders Y2 and Y1 respectively), hot sediment (2.4 mL versus 0.7 mL), cold sediment (6.8 mL versus 4.0 mL) and slowly dissolving particles (D's (hot and cold) versus C's). Powders X1 and X2 had no hot sludge whereas powders Y1 and Y2 had 0.2 and 0.4 g, respectively. This defect could be related to the dry powder particle size results. The Y powders had smaller particles, which may indicate poor powder agglomeration. It has been previously noted that aggregation of very fine particles can occur during reconstitution in water leading to large undispersed clumps of powder (Schubert, 1993).

5.3.3 Milk Powder Microstructure

The size and the surface structure of the powders X1 and Y2 were observed by scanning electron microscopy (Figure 5.2). Scanning electron microscopy can be used to account for functional properties observed in milk powders that are influenced solely by powder particle morphology, *i.e.*, flowability and sludge formation. These images show that there appeared to be more non-agglomerated fine particles in powder Y2 than in powder X1. This result was also obtained from the powder particle size data. The agglomeration process for powder Y2 may have not been as effective as the agglomeration process for powder X1. The fine particles may have been generated within the primary drying chamber, from agglomerate breakdown within the fluidised bed or during packaging and transportation of the powder.

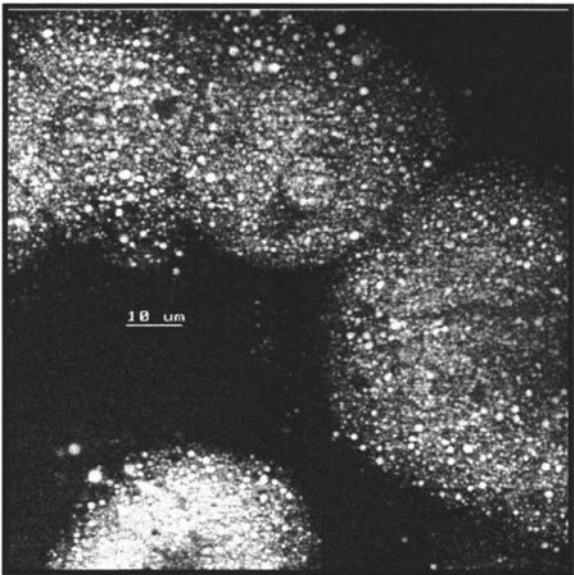


X1

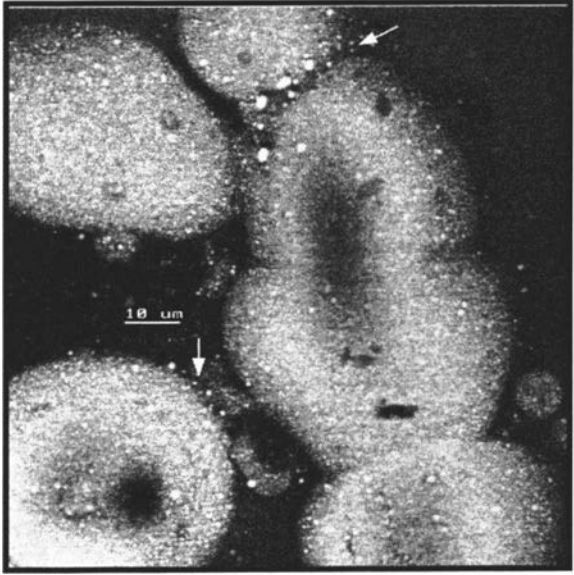


Y2

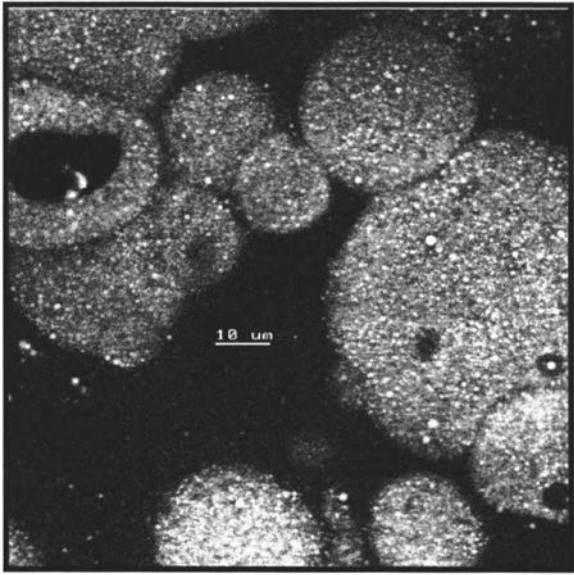
FIGURE 5.2 Scanning electron micrographs of whole milk powders X1 and Y2 showing the morphology of the powder particles.



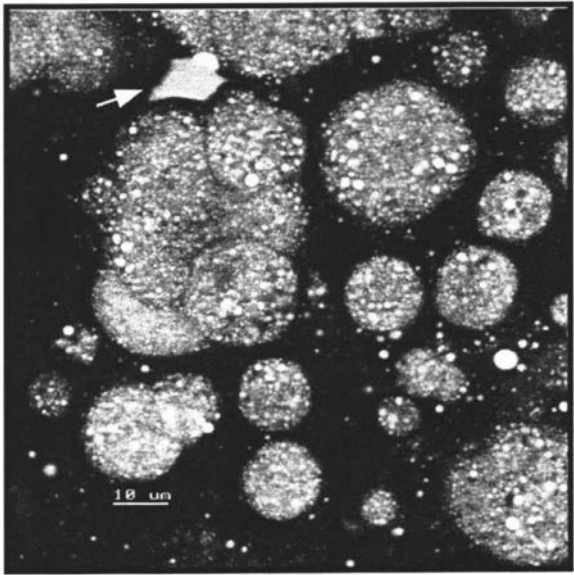
X1



X2



Y1

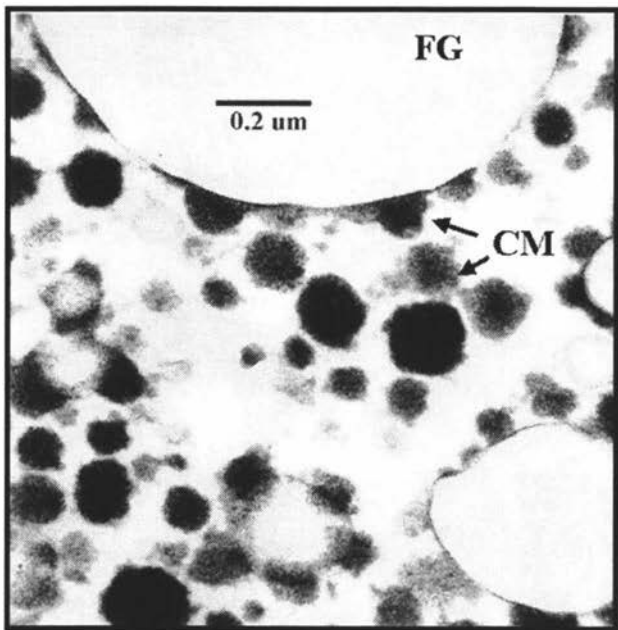


Y2

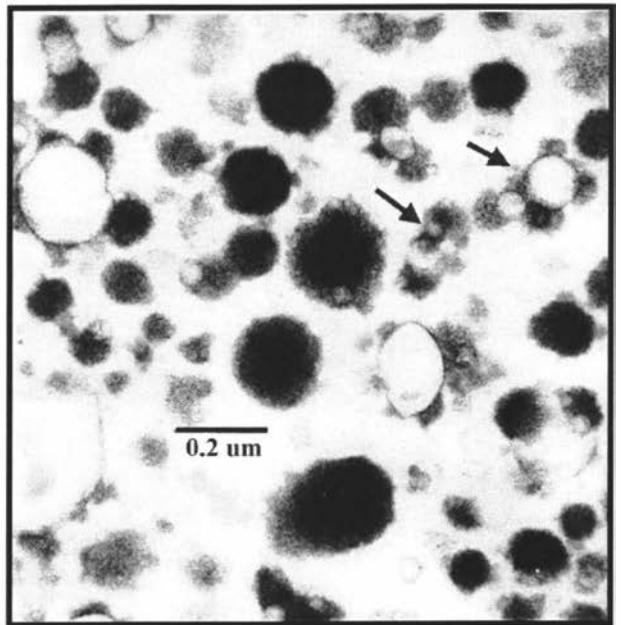
FIGURE 5.3 Confocal micrographs of whole milk powders X1, X2, Y1 and Y2.

Confocal microscopy can provide information about particle morphology and the location of the fat on the surface and within the whole milk powder particle (Chapter 3). Confocal microscopy of the whole milk powders evaluated in this work showed that there was a difference in the size distribution of the fat globules that was not observed by the particle size distribution data. In powder X1, the fat globules were well distributed throughout the particles (Figure 5.3). The fat globules in powder X2 were generally smaller than the globules in powder X1. There also appeared to be a difference in the size of the globules from the outside to the inside of the X2 particles. There were some larger more coalesced fat droplets near the surface of these particles (arrows) that reduced in size towards the centre of the particle. The reason for this is unknown but may be a result of some form of coalescence during rapid moisture removal in the spray drier. The confocal micrographs also showed that there were more fines in the Y powders than in the X powders and showed some free fat in powder Y2 (arrow).

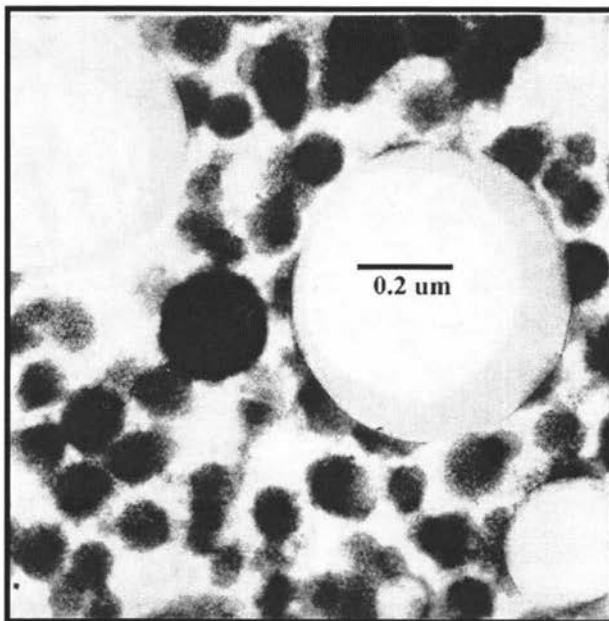
Transmission electron microscopy of powders shows specific details about the protein-protein and protein-fat globule interactions that occur during manufacture (Figure 5.4). The most distinguishing features in these micrographs were the apparent quantity of micellar material associated with the fat globules and the interaction of these micelles with other fat globules. Powder X1 had few very small fat globules and generally had large fat globules with apparently fewer adsorbed casein micelles than X2 (145 mg protein/g fat for X1 compared with 175 mg protein/g fat for X2) as measured by the fat-bound protein test (Table 5.2). Powder X2, with its relatively large number of very small fat globules, had a greater number of protein-associated fat clusters within the powder particle (arrows, Figure 4). Powders Y1 and Y2 appeared to have a greater number of clustered casein micelles in the non-fat phase than powders X1 and X2. Powder Y2 appeared to have more pronounced hair-like structures protruding from the casein micelles than the other powders. These structures, probably denatured whey protein and κ -casein, may have been responsible for the increased aggregation of structures within this powder as seen in the particle size data (Figure 5.1, Table 5.2). This increased aggregation was not related to an increase in fat-bound protein as was seen for powder X2 but may have been the result of the higher acidity in the milk used for the manufacture of the Y powders.



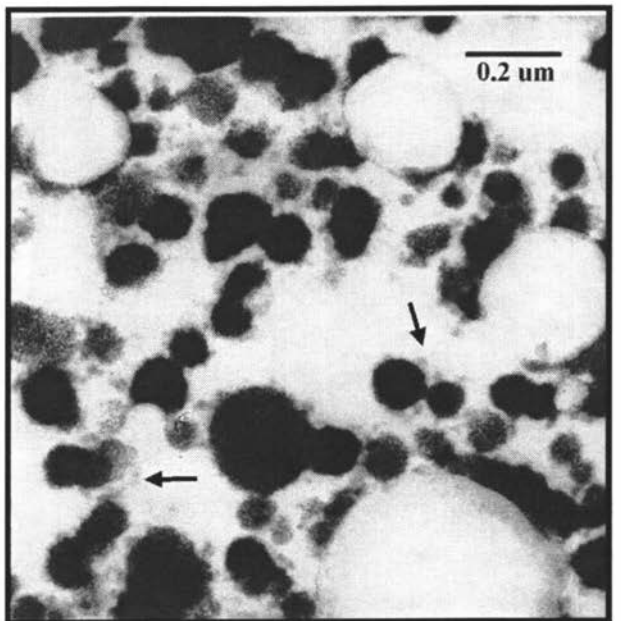
X1



X2



Y1



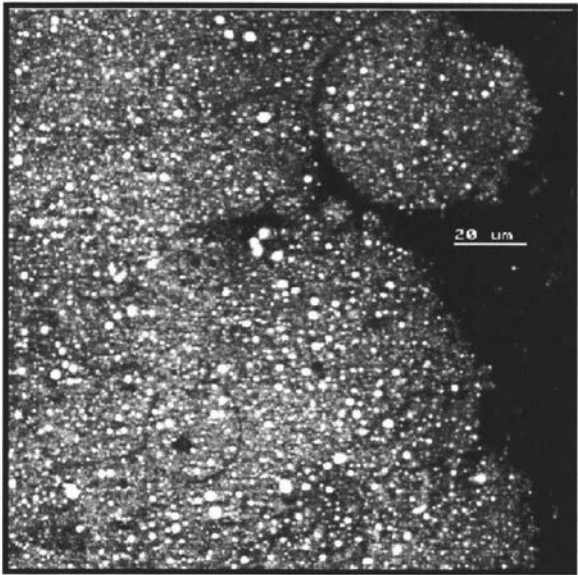
Y2

FIGURE 5.4 Transmission electron micrographs of whole milk powders X1, X2, Y1 and Y2 showing the distribution and aggregation of fat globules (FG) and casein micelles (CM). Arrows in X2 show very small protein-aggregated fat globules. Arrows in Y2 show hair-like structures on the casein micelles.

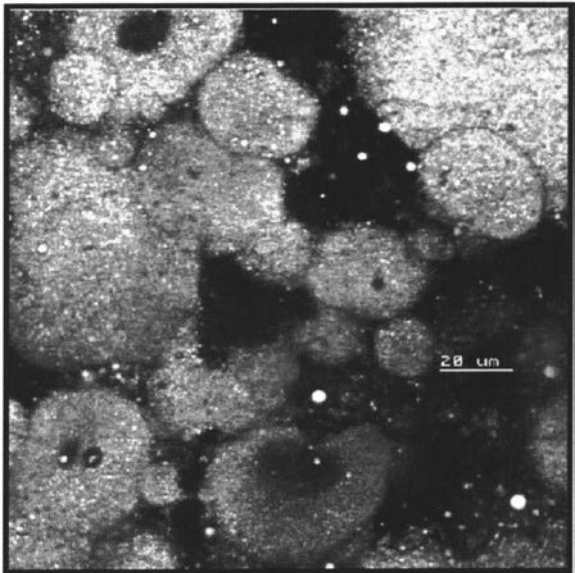
Confocal microscopy of the insoluble material collected after functional testing showed little difference in the structure of the insoluble material between the 4 powders evaluated, *i.e.*, all four powders showed insoluble material with a similar structure at this magnification. Therefore only the micrographs from powder Y2 are shown. Confocal microscopy generally showed that the powder particle structure had been lost when the coffee and hot sediments were analysed (Figure 5.5). The sediment obtained from the coffee and hot sediment tests appeared to be a continuous matrix with little evidence of the intact individual powder particles. The hot sludge appeared to consist of a relatively large number of smaller particles in amongst some larger powder particles. The slowly dissolving particles in cold water appeared as intact milk powder particles.

The microstructures of the coffee sediment obtained by transmission electron microscopy (Figure 5.6), showed different degrees of casein micelle aggregation and protein adsorbed to the fat globules. The coffee sediment from powder X1 (lowest sediment at 0.5 mL) showed some micelle aggregation within the serum and on the fat globules forming cluster-like structures (arrow). The coffee sediment from powder X2 (highest sediment at 3.1 mL) showed extensive micelle aggregation and clustering of fat globules. Most of the casein micelles had aggregated and were associated with protein-fat clusters. These micelles were no longer discrete units but had spread and 'fused' with adjacent micelles to form an almost continuous protein network. The smaller fat globules within the structure were generally clustered together and completely embedded in a continuous protein matrix. There was less extensive aggregation of fat globules in the coffee sediment from the Y powders. The coffee sediment from powder Y2 contained some large aggregated protein structures not seen in the sediment from the other powders.

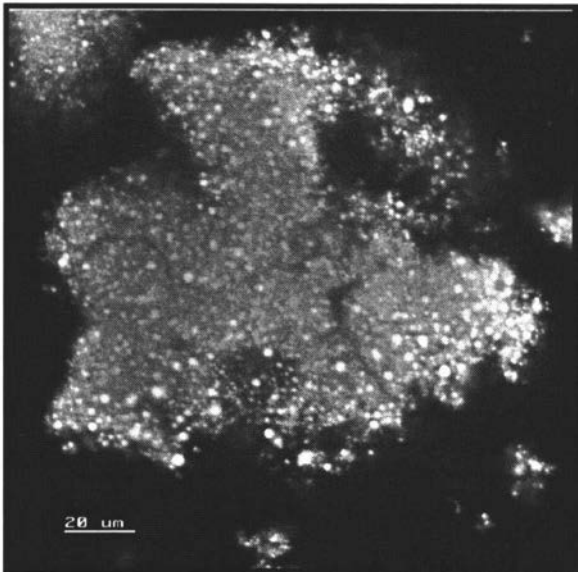
The hot insoluble particles (Figure 5.7) showed less extensive aggregation of fat and protein. The casein micelles appeared to be more intact, especially in the insoluble material produced from powder X1. The hot insoluble material obtained from the Y powders showed a greater number of hair-like attachments on the casein micelles than did the hot insoluble material from the X powders. This difference may be related to the greater heating received by the Y powders during manufacture.



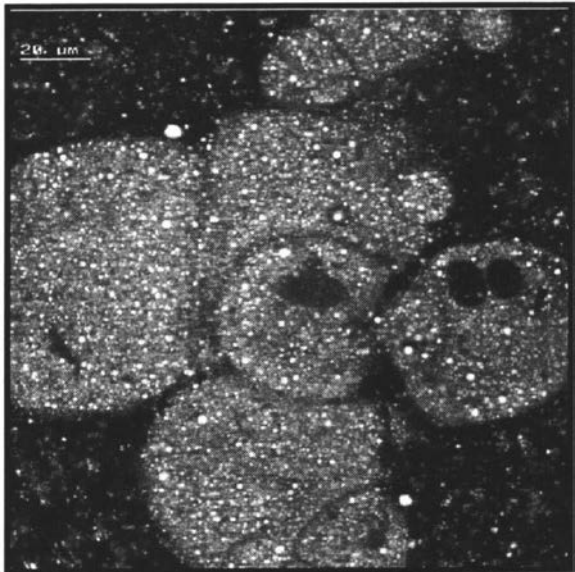
Coffee sediment



Hot sludge

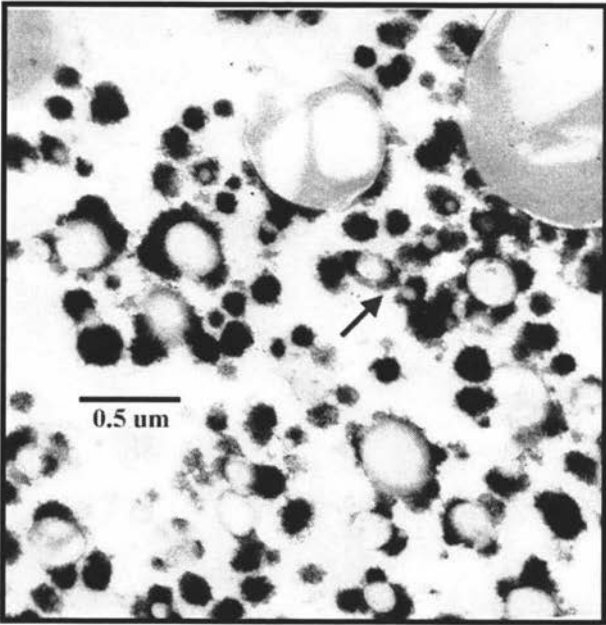


Hot sediment

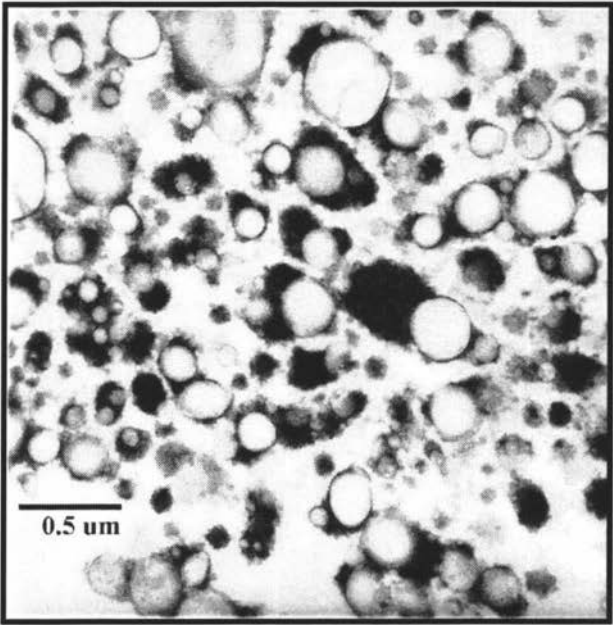


Cold slowly dissolving particles

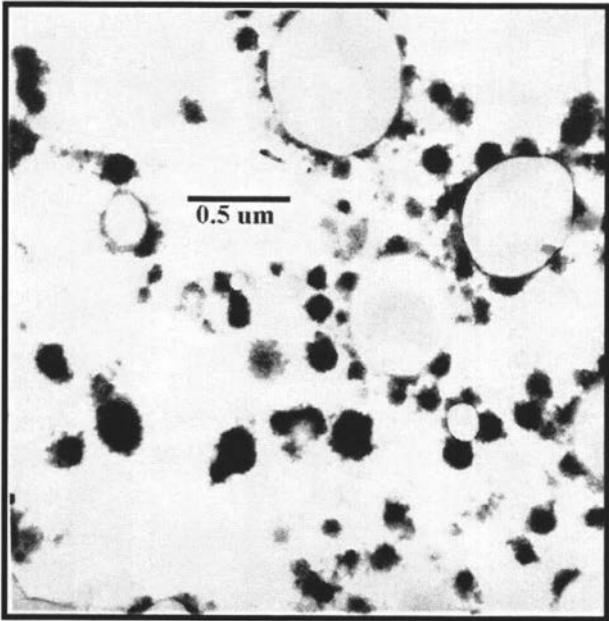
FIGURE 5.5 Confocal micrographs of material produced during functional testing of whole milk powder Y2.



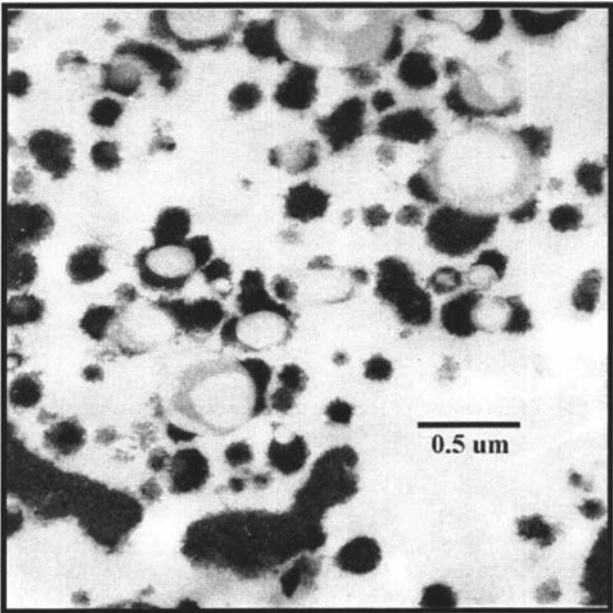
X1



X2



Y1



Y2

FIGURE 5.6 Transmission electron micrographs of coffee sediment produced from whole milk. powders X1, X2, Y1 and Y2.

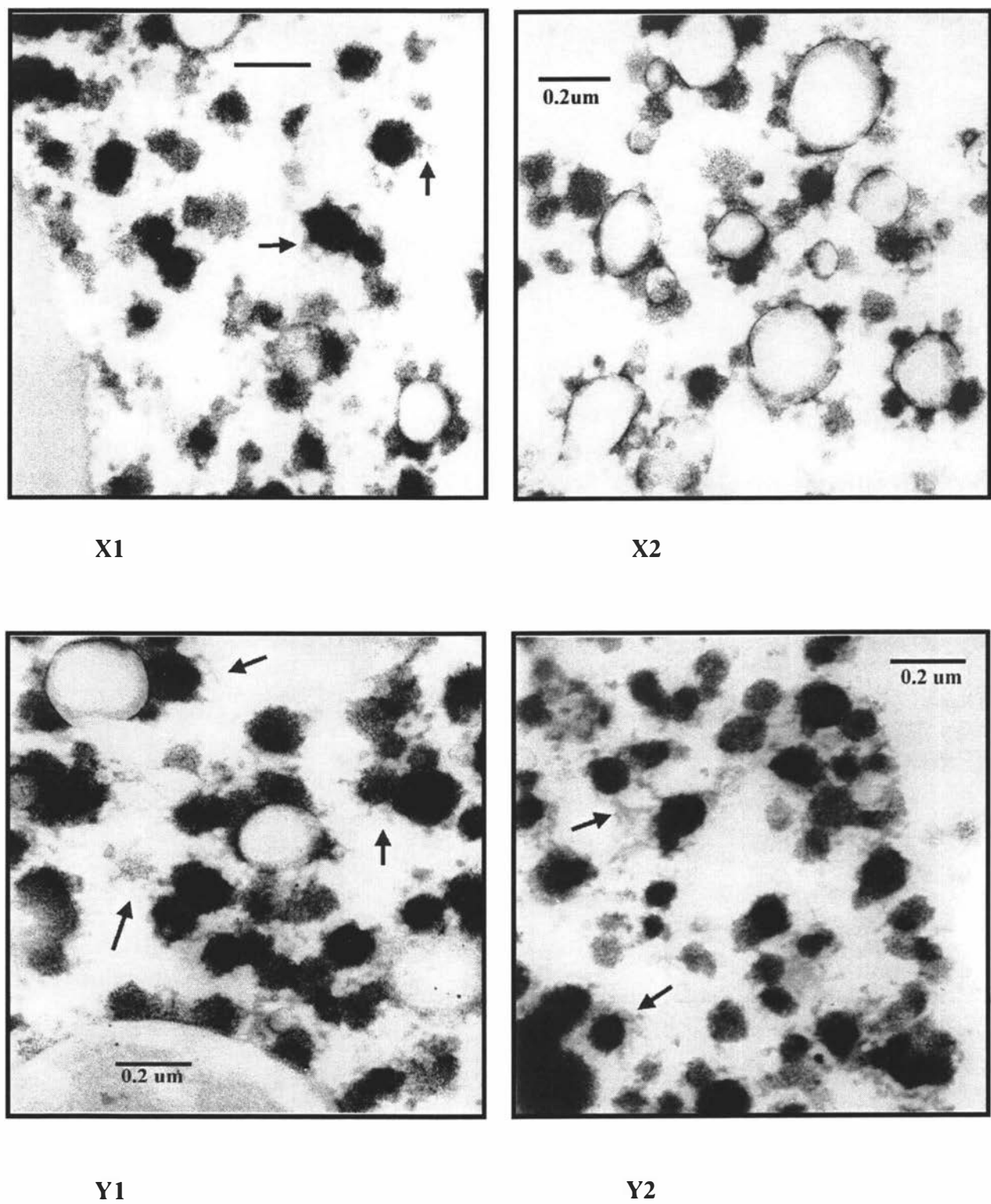


FIGURE 5.7 Transmission electron micrographs of hot sediment produced from whole milk powders X1, X2, Y1 and Y2. Arrows show hair-like attachments on the casein micelles.

5.4 DISCUSSION

There are many variables in milk and milk processing that may contribute to the properties of milk powder when reconstituted in different environments. Differences in casein composition, calcium phosphate concentration, micelle size, calcium ion activity, protein load on the fat globule, pH and heat treatment all contribute to the properties of the powder. It was shown by Mol (1975) that increasing the homogenisation pressure of concentrate prior to spray drying increased the number of insoluble particles in the resulting whole milk powder. It was concluded from this work that the increased adsorption of casein micelles onto the fat globules during homogenisation had a detrimental effect on powder solubility. The influence of heating during the milk powder process has been shown previously to result in the formation of hair-like structures on the casein micelles (Davies *et al.*, 1978; Creamer *et al.*, 1978) and these have been described as κ -casein/ β -lactoglobulin complexes (Singh & Fox, 1987). These two mechanisms were likely to have played an important role in influencing the functional properties of the milk powders examined in this work. The denatured whey protein aggregates, seen as hair-like structures on the micelles in the Y powders and the corresponding hot sediments, and the large number of aggregated micelles may have been the result of the extent of heat processing during manufacture. There was not the same evidence of the hair-like structures in the X powders or in their hot sediments. The WPNI result confirmed that the Y powders had received considerably more heating during manufacture. Previous studies (Visser *et al.*, 1986; Singh & Fox, 1985) showed that the extent of aggregation and association of β -lactoglobulin with casein micelles increased with a decrease in the pH of the milk prior to heating. The lower pH of the Y powders may have contributed to the increased whey protein aggregation and also to the increased casein micelle-casein micelle interactions, observed in these powders (Figure 5.5).

The other major structural change observed during the processing of these milk powders (particularly the X powders) was caused by shear. This resulted in the adsorption of casein micelles onto the fat globules and subsequent clustering of fat globules possibly caused by the sharing of adsorbed casein micelles between two or more fat globules (Mulder & Walstra, 1974). It appeared that this was a major contributing factor towards the poor functional properties of powder X2. Milk powder X2 contained a large number of small clustered fat globules (Figure 5.4); the micelles appeared, in some cases, to have fused and completely enveloped the fat globules. The adsorption of casein micelles on to the surface of small fat globules in this powder appears to be responsible for a decrease in heat stability on further heating *i.e.*, during reconstitution in hot water and coffee. Previous work has shown that homogenisation of whole milk reduces the heat stability of the milk and this effect is enhanced with increasing homogenisation pressure, increasing fat content and concentration (Walstra & Jenness, 1984). Smaller fat globules were found to contain a higher concentration of adsorbed κ -casein and were thus more likely to interact with whey protein during heating forming clusters (Sharma *et al.*, 1996). Dalgleish & Sharma (1992) concluded that when heating homogenised milk the serum proteins interact with the adsorbed micelles by way of the κ -casein on the surface of the fat globule. Therefore it is probable that during powder X2 manufacture the homogenised milk concentrate formed aggregates more readily, particularly when heated above the denaturation temperature of whey protein. The formation of aggregates of fat globules linked by adsorbed casein micelles has been observed in reconstituted milks (at pH 6.7) heated close to the coagulation temperature (Singh *et al.*, 1996). It has been suggested (Sweetsur & Muir, 1983)

that increased adsorption of protein on the fat globule interface results in localised regions of high protein concentration and that this reduces heat stability. High ionic calcium and high concentrations of protein at the fat globule surface were associated with high coffee instability in IWMPs (Teehan *et al.*, 1997). The reduction in heat stability observed in the milk made from powder X2 in hot water and coffee was probably related to the higher protein load on the fat globules.

5.5 CONCLUSIONS

The use of confocal microscopy and transmission electron microscopy in conjunction with particle size analysis and functional tests provided useful information about the influence of heat and shear during powder processing on the formation of insoluble material upon reconstitution.

Heating at temperatures in excess of 70°C throughout the manufacturing process will result in the denaturation of β -lactoglobulin and its interaction with other proteins and fat globule membrane components. The quality of the milk as measured by titratable acidity may also have influenced the extent of protein interactions during heat processing leading to poor functional properties upon reconstitution.

The application of shear during processing resulted in the greatest change to powder solubility in this study. Increasing shear influenced the adsorption of casein micelles onto the fat globule surface and the formation of fat globule clusters caused by the sharing of adsorbed casein micelles between two or more fat globules. The powders containing smaller fat globules showed greater clustering and predisposed the powder to the formation of a greater quantity of insoluble material on reconstitution in water and coffee. Therefore it appears that the reconstitution properties of WMP can be manipulated by the control of shear (homogenisation intensity) during processing.

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CHAPTER 6

MICROSTRUCTURAL OBSERVATIONS IN A RANGE OF COMMERCIAL INSTANT WHOLE MILK POWDER SAMPLES

6.1 INTRODUCTION

The interaction of the specific components in milk, *i.e.*, casein and whey proteins, fat globules and minerals, during milk powder processing plays a major role in determining functional properties when the powder is reconstituted in water. It has been shown (Chapters 4 and 5) that an increase in protein adsorption on to fat globules and an increase in protein aggregation will result in more sediment after powder reconstitution in water and coffee.

A large number of milk powder manufacturers sell their products on the world market. The functional properties of the powders are likely to vary greatly, with the differences probably reflecting differences in milk composition, processing during powder manufacture and storage conditions. It would be valuable to determine whether the use of microscopy reveals details about the interaction of milk components in commercial powders that can be related to their functional properties.

A range of commercial milk powders was obtained and their microstructures and physical, chemical and functional properties were compared.

6.2 MATERIALS AND METHODS

Refer to the methodology sections in Chapters 4 and 5 for electron microscopy, and whey protein nitrogen index (WPNI), moisture, free fat, slowly dissolving particles (SDP), sludge, sediment, coffee sediment, dispersibility and fat globule and powder particle size analyses.

6.2.1 Powder Samples

Eight commercial instant whole milk powder (IWMP) samples were obtained from the Middle East and South East Asian markets (samples A1-A8). The powders were all opened and evaluated within 1 year of their manufacture date.

An initial visual and flavour assessment of seven of the eight powder samples (sample A6 was not available at the time of this initial assessment) was undertaken by a group of seven people all employed at the New Zealand Dairy Research Institute. The properties assessed were lumpiness, flowability, coarseness and flavour.

6.2.2 Flowability

The flowability of the powders was determined by measuring the rate of powder flow through slits in a rotating drum according to the method described by Chen (1994).

6.2.3 Bulk Density

Powder bulk density was measured in grams per millilitre after 100 taps according to method A2a of Niro Atomizer A/S (1978).

6.2.4 Particle Density

Particle density of the powder solids and occluded air was determined using a Beckman air pycnometer according to method A11a of Niro Atomizer A/S (1978).

6.2.5 Added Phospholipid (Lecithin)

The added phospholipid content of the powder was determined by measuring the phosphorus content of the lecithin after soxhlet extraction of the powder with petroleum ether according to the method described by Wewala & Baldwin (1982).

6.2.6 Water Activity (A_w)

The A_w was measured using an EEJA Novasina water activity meter (Novasina AG, Zurich, Switzerland) according to the instrument's normal operating instructions.

6.2.7 Solubility Index (SI)

The SI of the powders was measured according to the American Dry Milk Institute (1965) method.

6.2.8 Titratable Acidity

The powders were reconstituted and titrated with 0.1 M NaOH against phenolphthalein according to the American Dry Milk Institute (1965) method.

6.3 RESULTS

The results of the informal assessment of the commercial milk powder samples are shown in Table 6.1. The chemical, physical and functional property results are shown in Table 6.2.

TABLE 6.1 Initial visual and flavour assessment of IWMP samples

Sample	Lumpiness	Flowability	Coarseness	Flavour
A1	Large lumps (less than 20 mm)	Poor - cohesive	Large particles and coarse	Strong aftertaste of lecithin flavour
A2	Large lumps	Poor - cohesive	Large and coarse	Sweeter than A1
A3	Moderate lumps (less than 10 mm)	Good	Large and coarse	Slow flavour release
A4	No lumps	Very good	Very large and coarse	Gritty, very slow flavour release, bland
A5	Small lumps	Poor	Fine, not as coarse	Dissolved well, good flavour release
A7	Small lumps	Poor - sticky	Large and coarse	Gritty, slow flavour release
A8	No lumps	Poor - cohesive	Large and coarse	Strong lecithin flavour

TABLE 6.2 Chemical, physical and functional property analysis results for IWMP samples

Analyses	IWMP Samples							
	A1	A2	A3	A4	A5	A6	A7	A8
WPNI (mg/g solids-non-fat)	2.8	2.9	3.4	2.2	1.5	5.9	3.5	2
Free fat (%)	2.9	2.9	3.9	2.5	2.1	1.11	1.4	1.7
Sodium (g/100 g)	NM	NM	0.28	0.3	0.41	0.28	0.23	0.25
Phosphate (mmol/kg)	NM	NM	151	147	174	152	152	149
Moisture (%)	3.2	3.1	3.1	3	2.7	NM	2.7	2.9
A _w	0.15	0.16	0.16	0.15	0.11	NM	0.12	0.2
Added phospholipid (%)	0.17	0.17	0.16	0.08	0.16	0.09	0.27	0.13
Powder Particle Size Data - (% volume basis)								
IWMP size distribution ¹ (D(v,0.5)) (μm)	195	192	245	223	148	155	175	174
IWMP size distribution ² (D(v,0.1)) (μm)	81	74	87	85	60	67	76	69
IWMP size distribution ³ (D(4,3)) (μm)	222	224	293	253	180	219	225	197
Reconstituted milk (12.5% total solids) Size Data - (% volume basis)								
Fat globule size ⁴ (D(3,2)) (μm)	0.54	0.53	0.58	0.38	0.44	0.48	0.43	0.47
Fat globule size (D(4,3)) (μm)	1.92	1.95	2.52	1.18	1.1	0.95	0.93	4.07
Fat globule size (D(0.5)) (μm)	0.73	0.71	0.83	0.45	0.54	0.62	0.53	0.54
Fat globule size ⁵ Span	6.12	6.07	6.33	2.02	2.76	2.77	2.13	24.62
Particle density (g/mL)	1.23	1.23	1.23	1.23	1.21	1.21	1.15	1.23
Bulk density 100 taps (g/mL)	0.5	0.49	0.51	0.45	0.48	0.51	0.5	0.48
Flowability (g/s)	0.91	0.82	1.24	1.6	0.64	0.6	0.37	0.58
Solubility index (mL)	0.15	0.2	0.4	0.7	< 0.1	0.3	0.1	0.7
Titrateable acidity (%)	0.11	0.108	0.106	0.113	0.113	0.11	0.104	0.099
pH	6.75	6.72	6.75	6.69	6.76	6.72	6.72	6.73
Wettability (s)	9	14	> 60	> 60	12	48	25	> 60
Dispersibility	3	1	2	3	5	1	6	6
Cold sludge (g)	0.16	0.14	0.05	0.01	0.49	0.01	0.02	0.2
Cold SDP	D	C	C	E	C	C	D	D
Cold sediment (mL)	4.2	5	2.6	6.5	2.4	1.4	2.1	7
Hot sludge (g)	0.03	0.01	0.01	0.02	0.12	0	0.07	0.22
Hot SDP	C	C	C	C	C	A	C	D
Hot sediment (mL)	1.7	1.1	0.8	0.5	0.4	0.2	0.4	3.4
Coffee sediment (mL)	1.2	1.7	1	1.9	0.8	0.2	1	4

¹D(v,0.5) = median diameter; ²D(v,0.1) = the diameter under which 10% of the particles lie; ³D(4,3) = the diameter of a sphere with a volume area equivalent to the sample mean volume area; ⁴D(3,2) = the diameter of a sphere with a surface area equivalent to the sample mean surface area; ⁵Span = measurement of the size distribution range = D(v,0.9) - D(v,0.1) / D(v,0.5).

NM = Not measured.

6.3.1 Transmission Electron Microscopy

The micrographs of the reconstituted milk powders provide information about the interaction of structural components within the powder particles. The nature of these interactions will influence the dissolution properties of the IWMP (see Chapter 5). The micrographs, *e.g.* Figure 6.1b, show fat globules (f), which contain a membrane (arrow) composed of non-micellar casein and whey proteins (Brooker, 1985), and also include adsorbed casein micelles (cm). Casein micelles are also seen in the serum between the fat globules. The interaction of the casein micelles has been seen to be by hairy attachments on the casein micelles (Creamer & Matheson, 1980) which are thought to be denatured whey protein complexed (disulphide linked) to κ -casein on the micelle surface (Singh & Fox, 1985, 1986). There is evidence of these structures in the micrographs shown here, *e.g.* (h) in Figure 6.1b. The structural differences between the IWMPs as shown by the micrographs relate mainly to the size of the milkfat globules and the aggregation of these globules with the casein micelles.

IWMPs A1, A2 and A3

These powders all contained numerous fat globules which were in size range, 1.5-2.0 μm (Figures 6.1a, 6.2a and 6.3a). The micrographs of these powders at higher magnification showed the interaction between casein micelles including micelles on the fat globules (Figures 6.1b, 6.2b and 6.3b). There were no major differences in the structure of the casein micelles and their interaction with other micelles in these powders. However, it appeared that the casein micelles adsorbed onto the fat globules in the A3 powder (Figure 6.3b) were still relatively intact whereas those in the A1 and A2 powders (Figures 6.1b and 6.2b) had dissociated and spread on the fat globule surface.

IWMP A4

The micrographs of this reconstituted IWMP showed a distinct structural feature. The outside edge of the powder particle was composed of a layer/skin of aggregated fat globules and casein micelles. This skin layer is highlighted in Figure 6.4a (arrows). A feature of this skin was the aggregation of protein by way of hair-like structures (likely to be whey protein) on the micelles and on the fat globule membrane (h, Figure 6.4b).

IWMP A5

The fat globules in this reconstituted IWMP (Figure 6.5a) appeared to be similar in size range to those found in the A4 powder. The distinctive feature of this IWMP was the degree to which the casein micelles had interacted with other micelles and the consistently large size and porous nature of these micelles (Figure 6.5b). Large aggregates of casein micelles (such as those seen in Figure 6.5b) were found throughout the sample. These aggregates were unusual in that they were linked mainly by direct micelle-micelle contact (arrow, Figure 6.5b). The casein micelles were typically between 0.1 and 0.3 μm in size, *i.e.* consistently larger than those found in the other IWMP samples.

IWMP A6

The microstructure of this reconstituted IWMP (Figures 6.6a and 6.6b) showed that there was a distinct lack of protein-protein interactions between the casein micelles. This was seen by the relatively clean, smooth surface of the casein micelles whereas the other IWMPs showed a greater number of hair-like protrusions from the micelles.

IWMP A7

There appeared to be a significant quantity of surface fat material in this reconstituted IWMP (f in Figure 6.7a). This fat may have been a commercial mixture of dairy fat and soy lecithin used for instantising the powder to improve the wettability. The cobweb-like stained material in this layer was fat that had different properties from the bulk of the surface fat. The microstructure of the bulk of the IWMP was similar to that seen in powders A1-A3.

IWMP A8

The micrographs of this reconstituted IWMP showed a high degree of protein-protein interactions (Figures 6.8a and 6.8b). There was a high protein load (casein micelles) on the fat globules (arrow, Figure 6.8b) and these micelles were strongly associated with other casein micelles and fat globules, resulting in a highly aggregated network. This high degree of aggregation was particularly apparent when small fat globules were present. These small fat globules were more heavily loaded with casein micelles and appeared to act as nucleation sites for the formation of large aggregates.

6.3.2 Scanning Electron Microscopy

The micrographs shown in Figures 6.9 to 6.16 provided information about the agglomerate structure, *i.e.* the degree of agglomeration, the size of the primary particles and the number and size of fines embedded into these particles, and the surface morphology of the particles. Differences between samples were not obvious although some differences in the smoothness of the particle surface, perhaps indicating surface fat, were noted (see powder A3 (Figure 6.11) and powder A7 (Figure 6.15)).

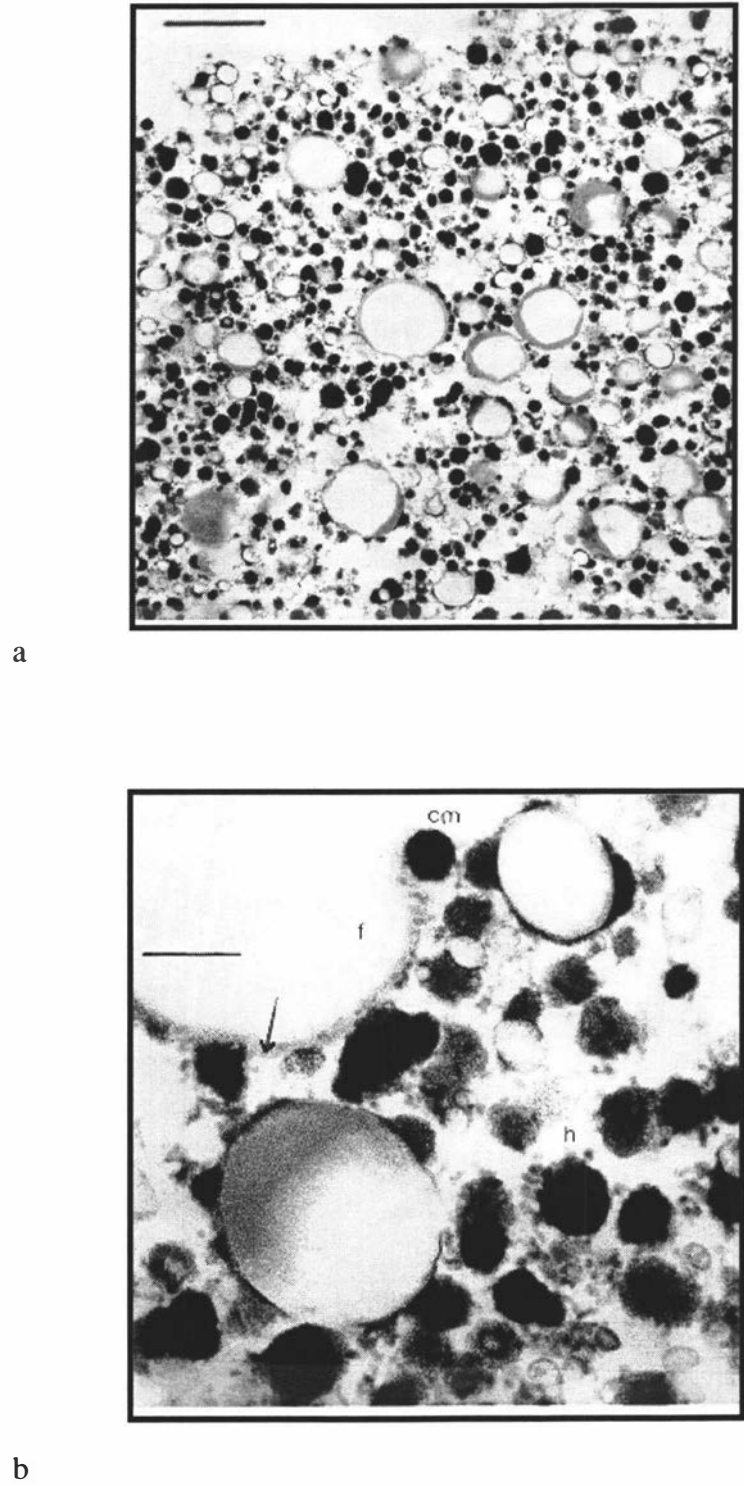
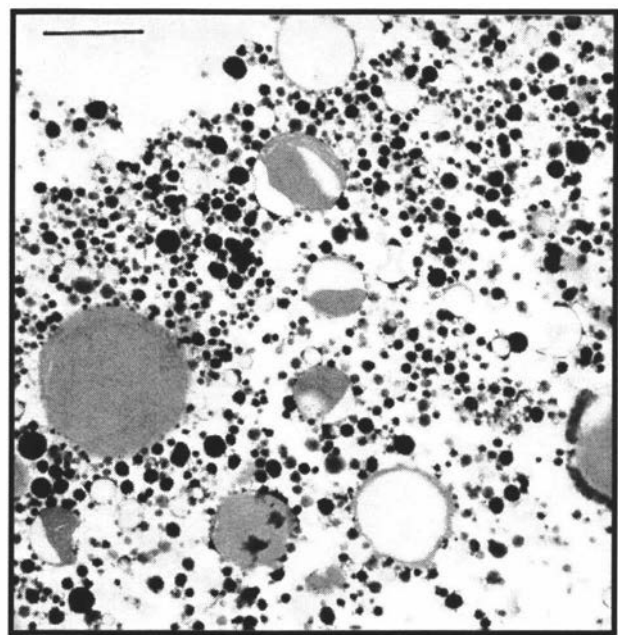
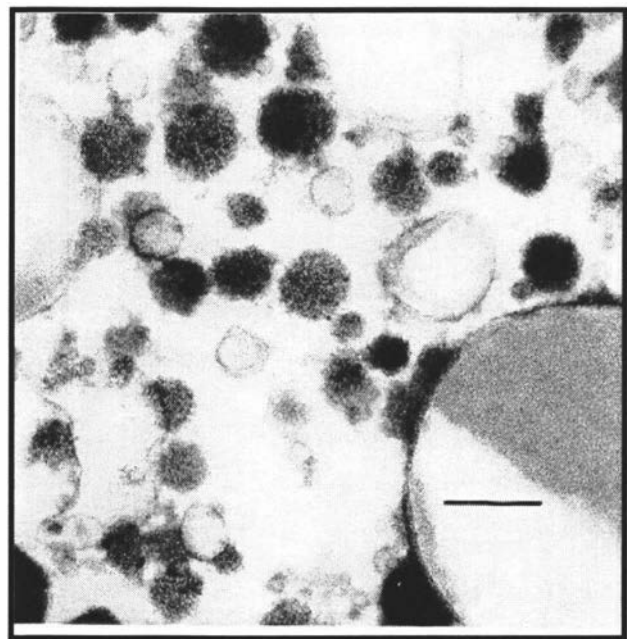


FIGURE 6.1 Transmission electron micrograph of reconstituted AI IWMP; f = fat globule, cm = casein micelle, h = hair-like attachment (a, bar = 1 μm ; b, bar = 0.2 μm).

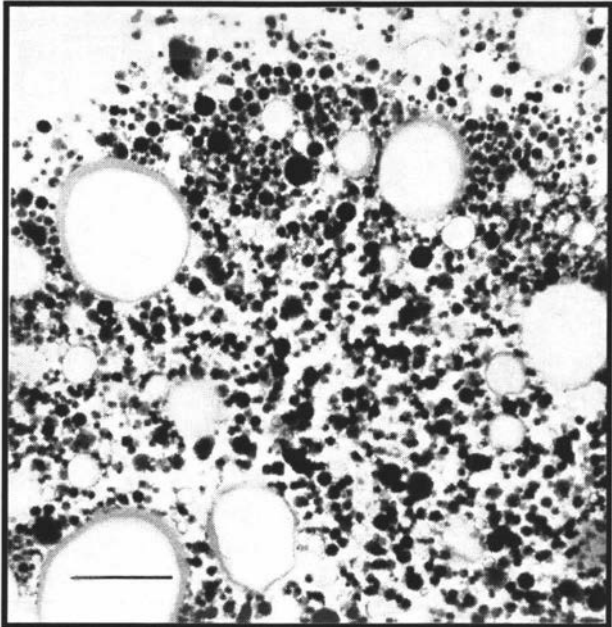


a

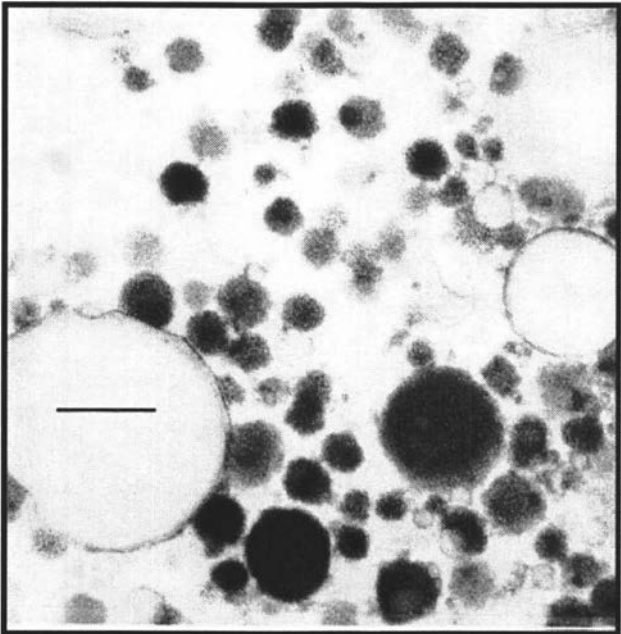


b

FIGURE 6.2 Transmission electron micrograph of reconstituted A2 IWMP (a, bar = 1 μm ; b, bar = 0.2 μm).

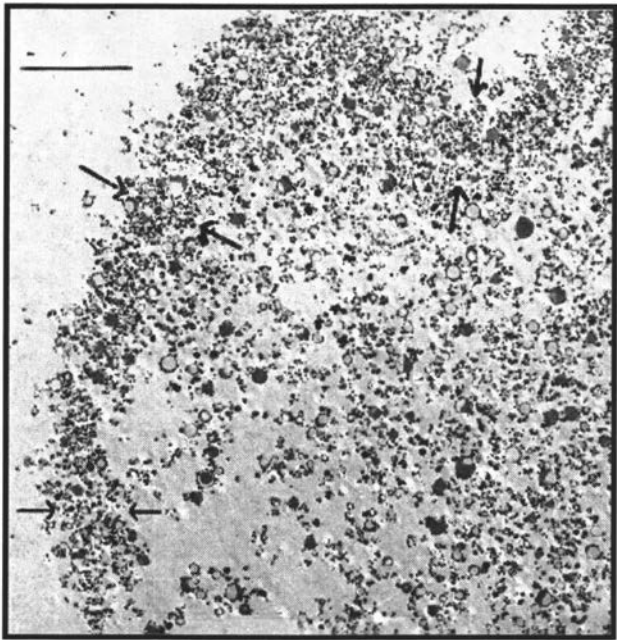


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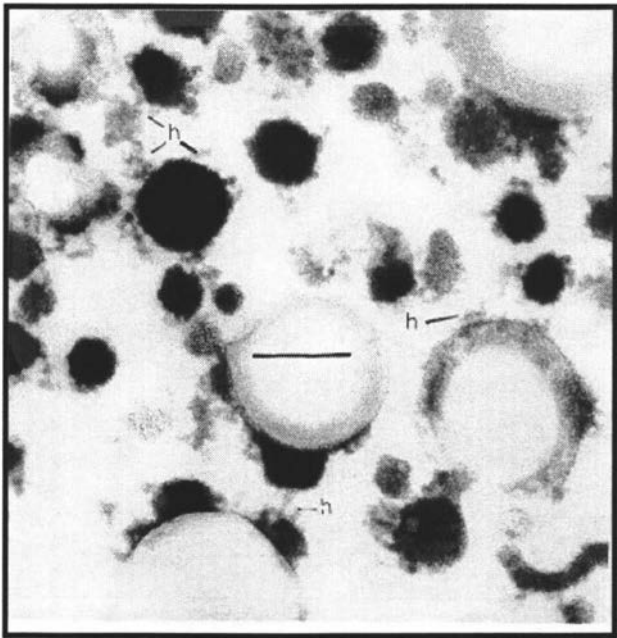


b

FIGURE 6.3 Transmission electron micrograph of reconstituted A3 IWMP (a, bar = 1 μ m; b, bar = 0.2 μ m).

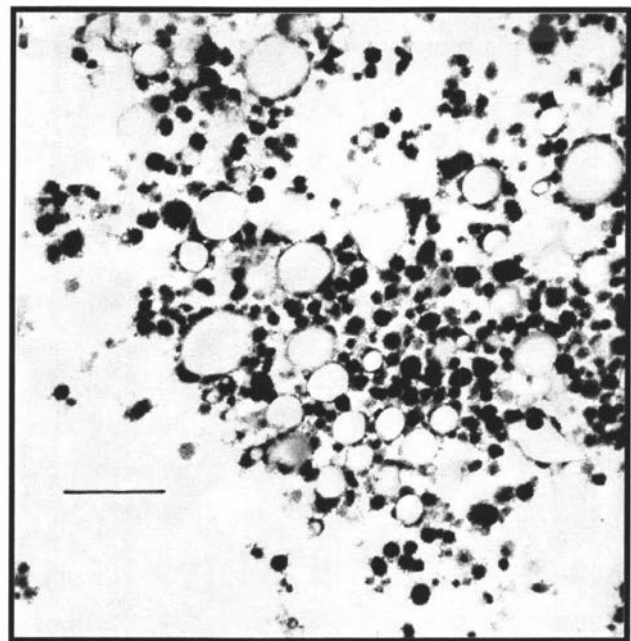


a

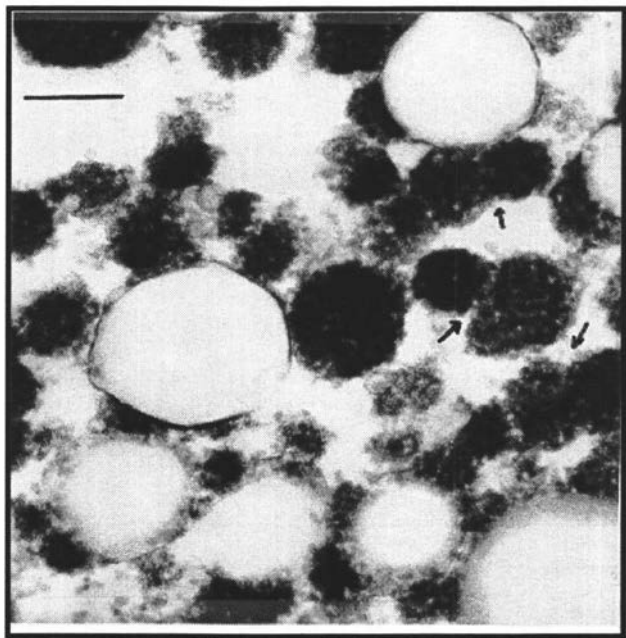


b

FIGURE 6.4 Transmission electron micrograph of reconstituted A4 IWMP; h = hair-like attachment (a, bar = 5 μ m; b, bar = 0.2 μ m).

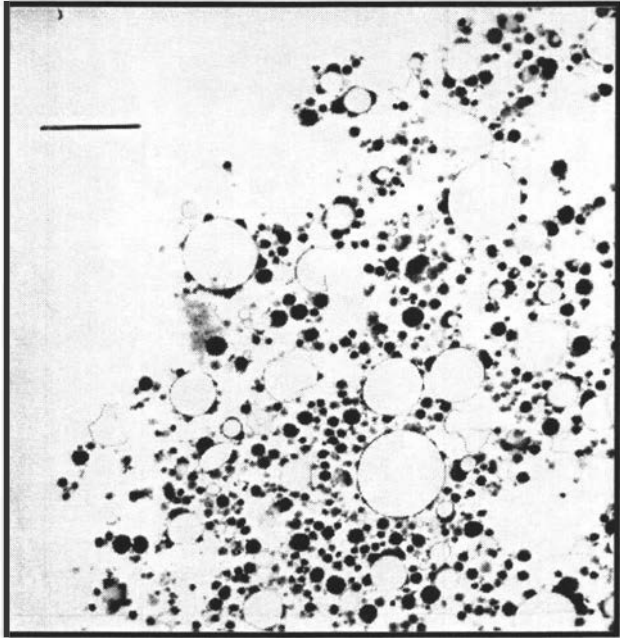


a

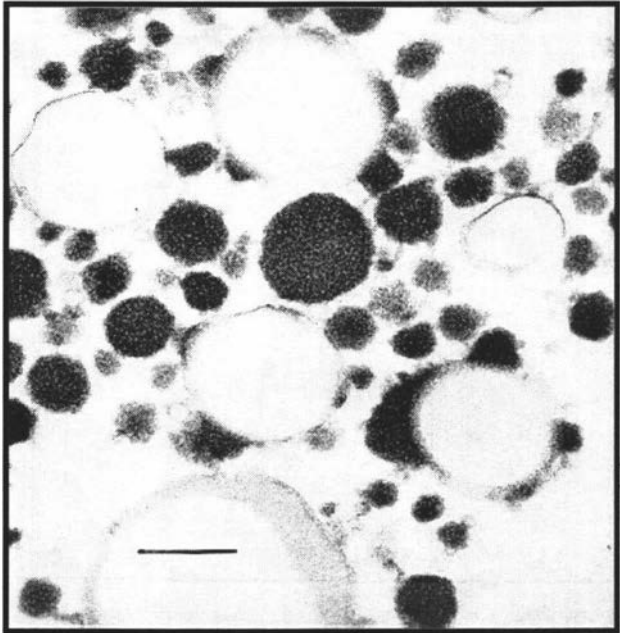


b

FIGURE 6.5 Transmission electron micrograph of reconstituted A5 IWMP (a, bar = 1 μ m; b, bar = 0.2 μ m).

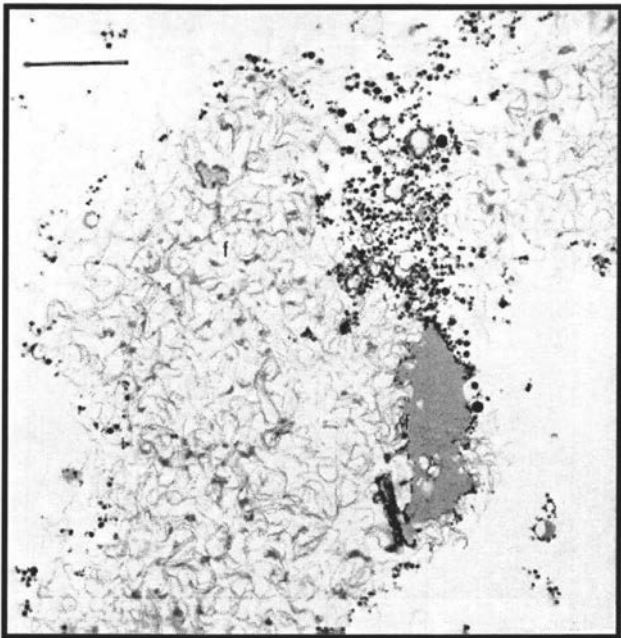


a

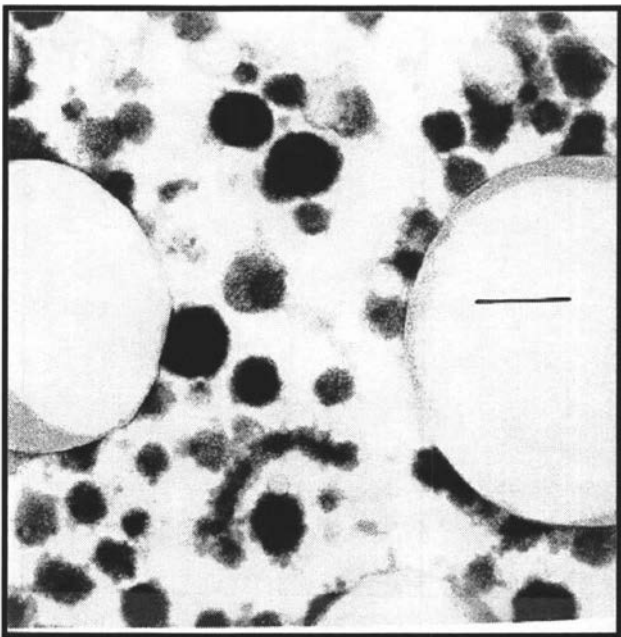


b

FIGURE 6.6 Transmission electron micrograph of reconstituted A6 IWMP (a, bar = 1 μm ; b, bar = 0.2 μm).

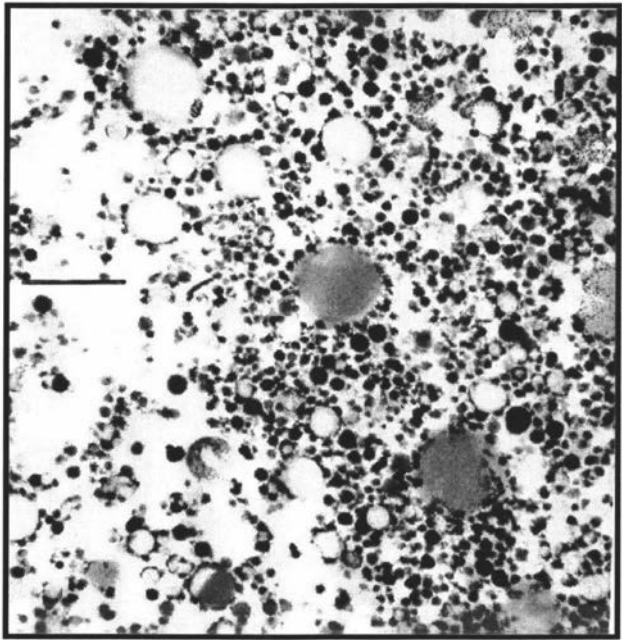


a

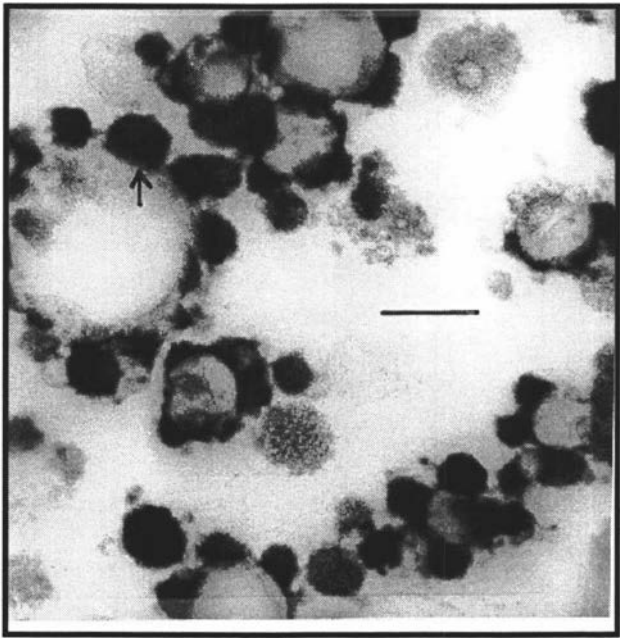


b

FIGURE 6.7 Transmission electron micrograph of reconstituted A7 IWMP (a, bar = 2 μm ; b, bar = 0.2 μm).



a



b

FIGURE 6.8 Transmission electron micrograph of reconstituted A8 IWMP (a, bar = 1 μm ; b, bar = 0.2 μm).

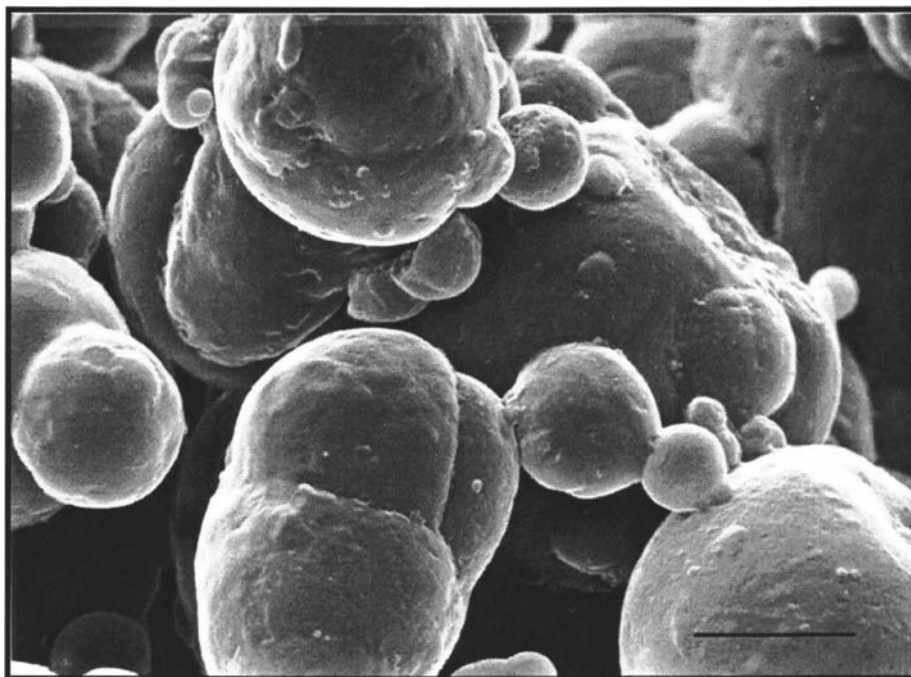


FIGURE 6.9 Scanning electron micrograph of A1 IWMP (bar = 20 μm).

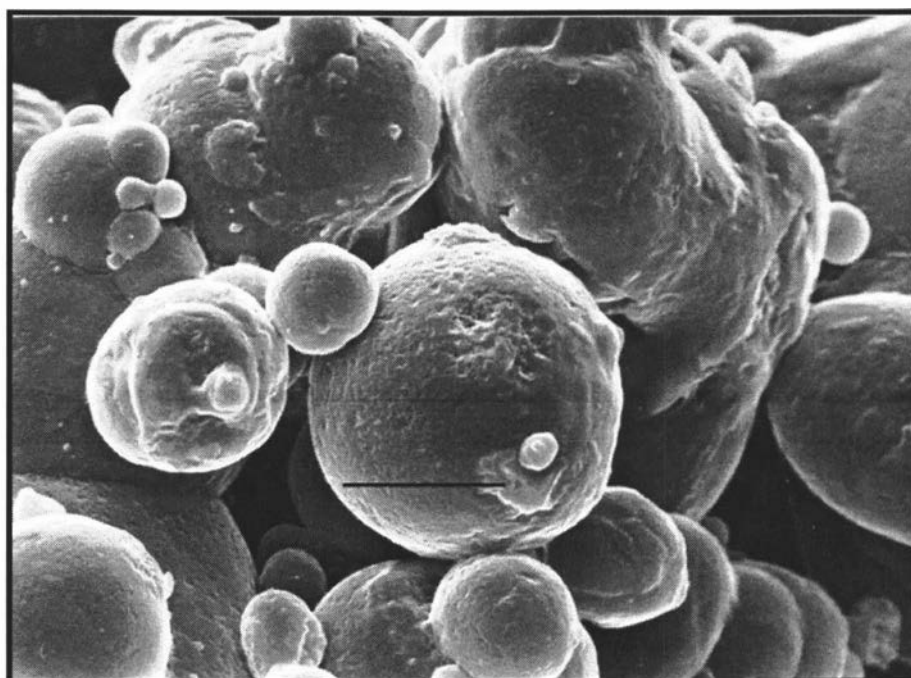


FIGURE 6.10 Scanning electron micrograph of A2 IWMP (bar = 20 μm).

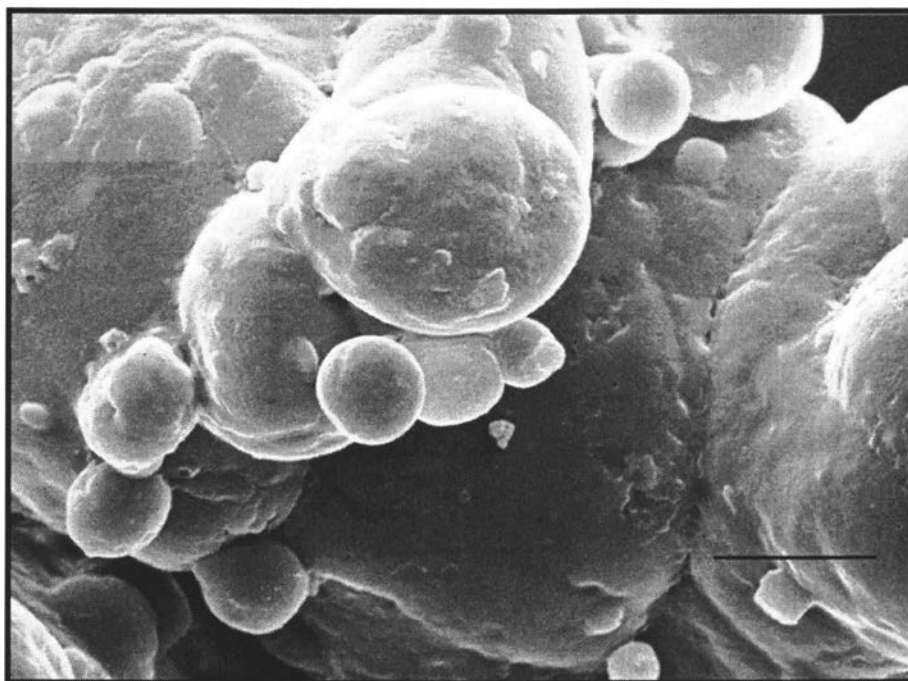


FIGURE 6.11 Scanning electron micrograph of A3 IWMP (bar = 20 μm).

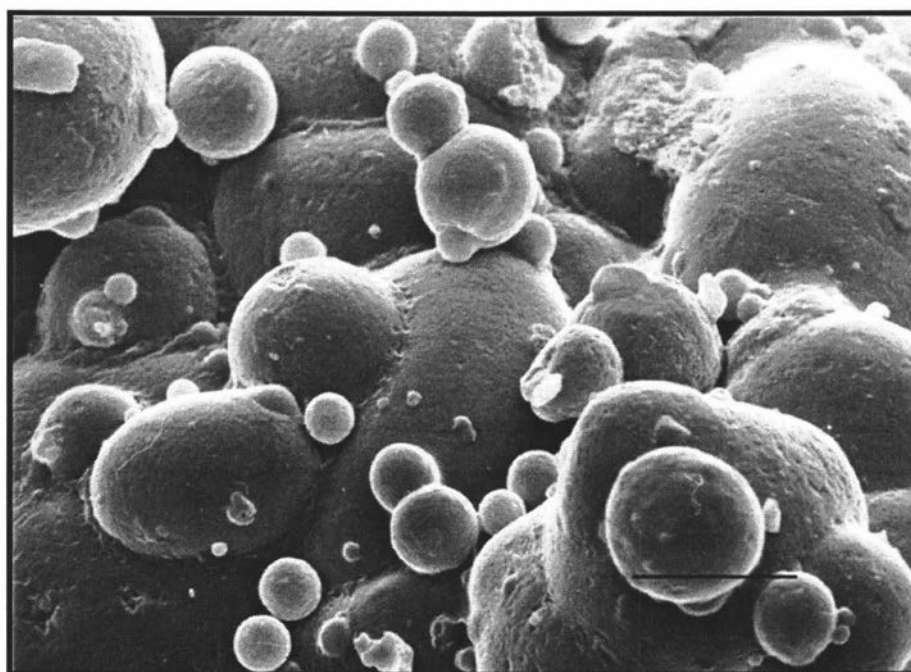


FIGURE 6.12 Scanning electron micrograph of A4 IWMP (bar = 20 μm).

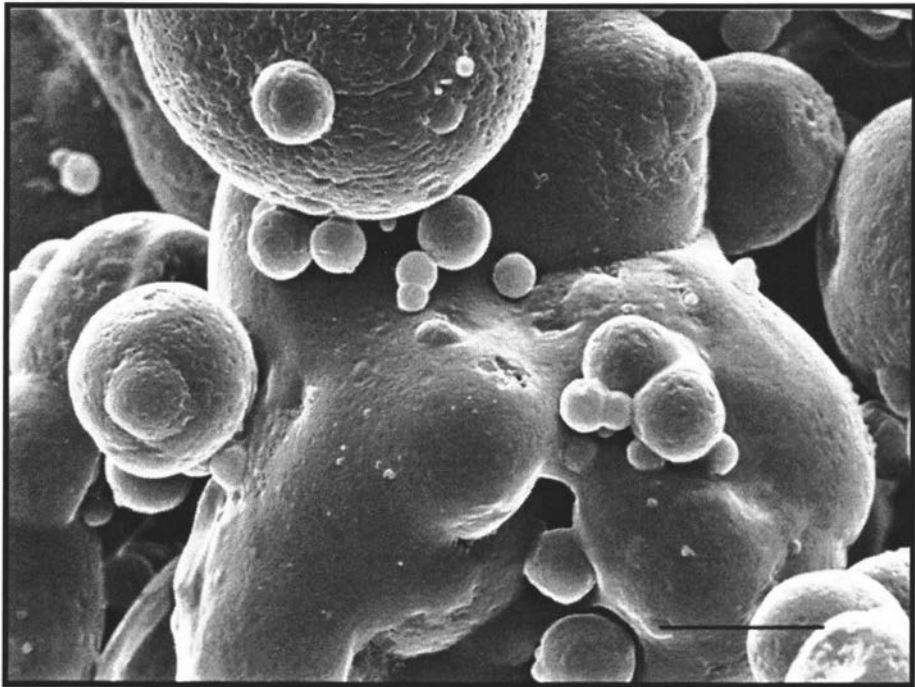


FIGURE 6.13 Scanning electron micrograph of A5 IWMP (bar = 20 μ m).

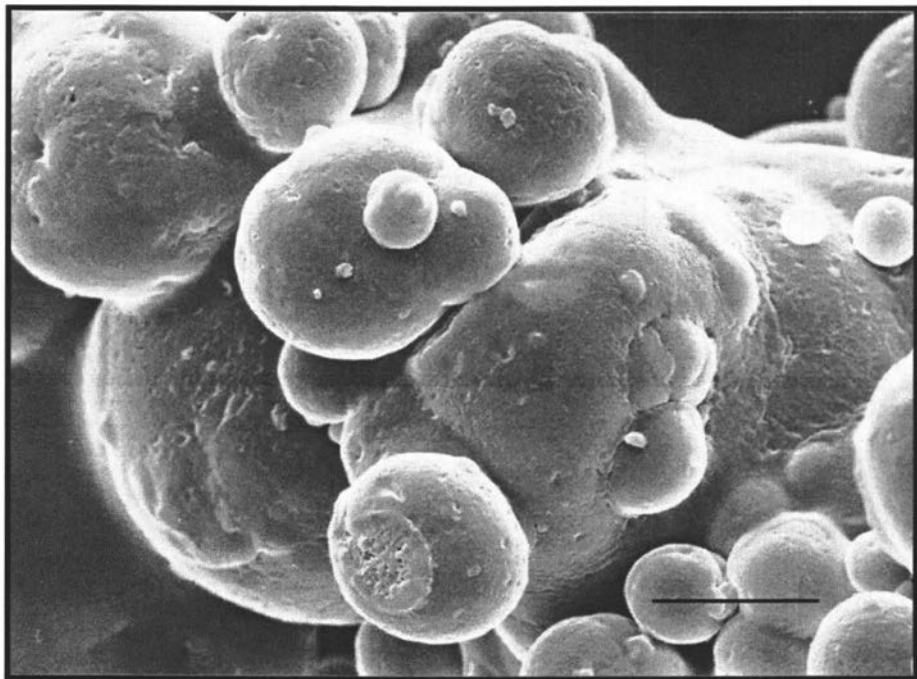


FIGURE 6.14 Scanning electron micrograph of A6 IWMP (bar = 20 μ m).

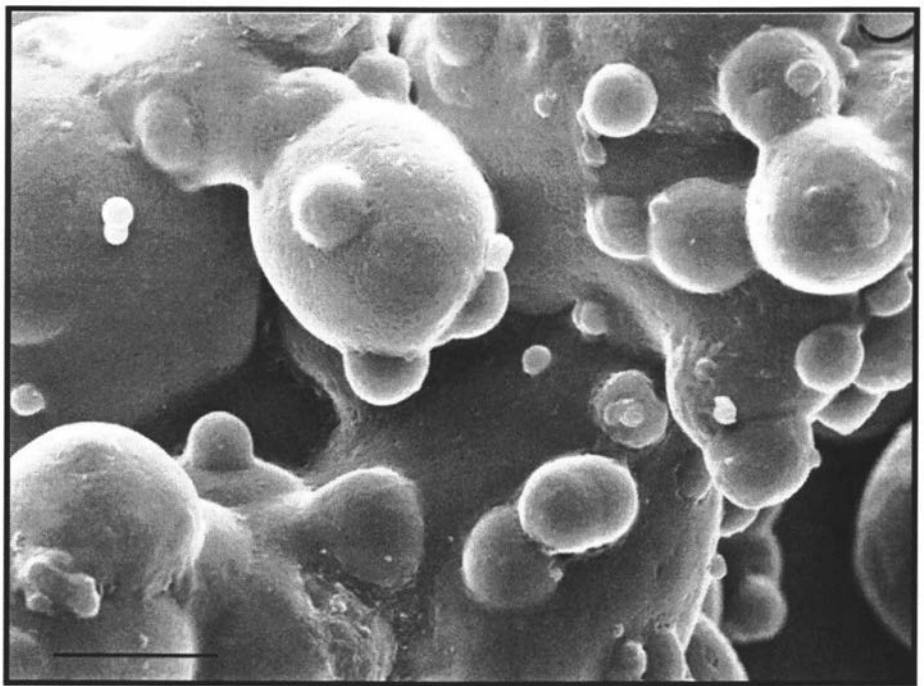


FIGURE 6.15 Scanning electron micrograph of A7 IWMP (bar = 20 μm).

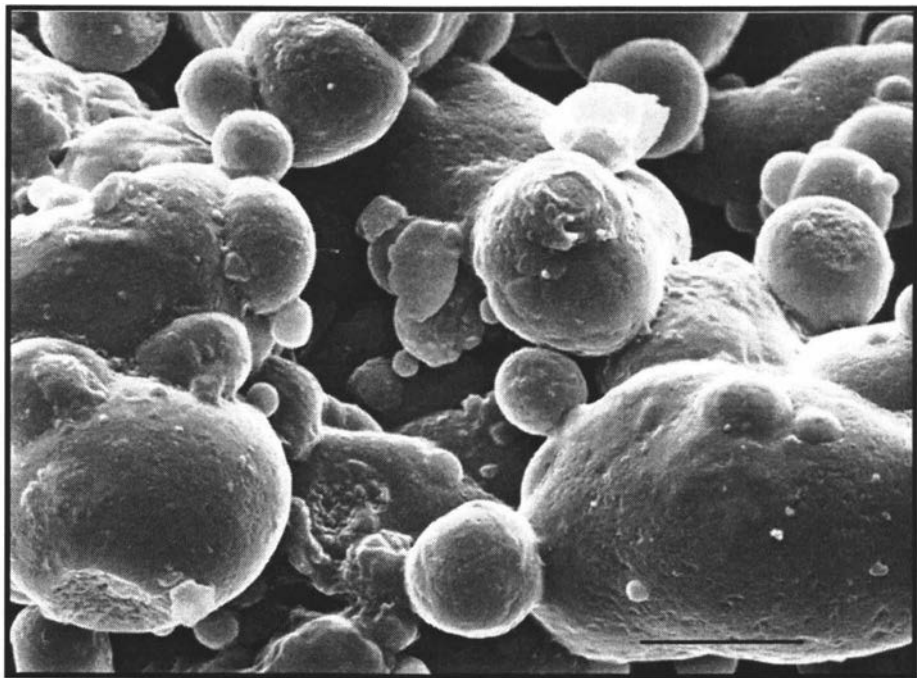


FIGURE 6.16 Scanning electron micrograph of A8 IWMP (bar = 20 μm).

6.3.3 Powder Particle Size Distribution

There was some variation in the powder particle size distribution between the powder samples (Table 6.2). The A5 IWMP sample had a significantly higher level of fines and a smaller mean and median particle size. At the other end of the range, the A3 powder sample had a much larger particle size.

6.3.4 Particle Size Distribution of Reconstituted Powder

A large variation in particle size distribution was seen when comparing these IWMP samples (Table 6.2). The powders were measured in water at 45°C and therefore clusters of fat globules and clusters of casein micelles were detected as well as fat globules. The A1, A2 and A3 IWMPs all had a greater number of larger fat globules, suggesting that less shear had been applied during their manufacture. The A8 IWMP had a very broad particle size distribution. However, upon examination of the reconstituted samples using a light microscope, it was apparent that, whereas the broad distribution of the reconstituted A1, A2 and A3 IWMP samples was due to large fat globules, the A8 IWMP sample contained a large number of protein-aggregated fat globules and relatively small fat globules. The transmission electron micrographs confirm that this material contained protein-induced aggregated fat globules (Figure 6.8b).

6.3.5 Whey Protein Nitrogen Index

The WPNI values of the IWMP samples ranged from 1.5 to 5.9 mg nitrogen/g solids-non-fat. These results probably represent a wide range of heat treatments applied to the milks and concentrates during their different milk powder manufacturing processes. It is interesting to note that the powder with the best overall functional performance, the A6 IWMP, had the highest WPNI and therefore probably the lowest heat treatment during manufacture. This is also indicated by the absence of protein-protein interactions as shown in the transmission electron micrographs (Figure 6.6b). The A5 IWMP sample, on the other hand, had a low WPNI but maintained good hot water and coffee solubility. However, the micrographs of this powder did show unusually large casein micelles (Figure 6.5b).

6.3.6 Free Fat

The free fat levels in powders A1, A2 and A3 were 2.9, 2.9 and 3.9% respectively whereas the other powders contained no more than 2.5% free fat. This result appeared to be related to a higher number of larger fat globules present in the A1, A2 and A3 powders.

6.3.7 Flowability

The flowability of the powders (Table 6.2) ranged widely from very flowable (A4) to very claggy (A7). The powder with the worst flowability had the highest level of added lecithin and the powder with the best flowability had the lowest level of added lecithin.

The surface structures seen in the scanning electron micrographs of the A4, A7 and A5 IWMP samples appeared to reflect the level of lecithin/milkfat added to the powder. The A7 powder appeared to have a smoother surface (Figure 6.15), probably as a result

of its high level of lecithin addition. However, it is difficult to draw firm conclusions as to the extent of the surface fat coverage in the majority of the scanning electron micrographs.

6.3.8 Solubility Index

The A4 and A8 samples had particularly high SI levels at 0.7 mL. The layer of aggregated casein micelles, incorporating fat globules, on the outside of the A4 IWMP particles (Figure 6.4a) probably influenced this high SI result. The highly aggregated protein and fat structures in the A8 IWMP is likely to have contributed to its high SI value.

6.3.9 Instant Properties

Wettability ranged from 9 to > 60 s. The type and quantity of lecithin, the method of application, the powder size distribution and the degree of agglomerate breakdown all influence powder wettability.

The dispersibility of the IWMPs spanned the range of the test from 1 to 6. It is well known that dispersibility is affected by the level of fine particles within the milk powder sample (Pisecky, 1990). The A8 IWMP agglomerates appeared to contain a large number of smaller particles and the powder had poor dispersibility. The A7 powder, which also had very poor dispersibility, had the highest surface coating of added lecithin probably resulting in powder particle agglomeration and resistance to dispersion into water.

The cold SDP results ranged from C to E. Interestingly, the A4 sample had the most gritty mouthfeel (initial assessment), which may relate to the rate of dissolution of the powder particles, probably caused by the skin on the outside of the particle formed by heat-induced protein-protein interactions (Figure 6.4a).

The hot sediment and coffee sediment test results (Table 6.2) showed that the A8 IWMP rated poorly whereas the A6 powder performed well. The distinct lack of protein-protein interaction seen in the A6 IWMP (Figure 6.6), as discussed previously, contrasts with the high level of casein micelle aggregation and casein-fat globule interaction seen in the A8 IWMP sample (Figure 6.8).

6.4 DISCUSSION

In the studies described in Chapters 4 and 5, transmission electron microscopy revealed that the incorporation of casein micelles into the fat globule membrane after homogenisation promotes the formation of a protein network, with the casein on the fat globules and the whey proteins providing linkages. The protein-coated fat globules act as large protein units active in protein-protein reactions, with the whey proteins becoming actively involved in this protein network upon heating above approximately 72°C. The protein on the surface of homogenised fat globules is less heat stable than that on fat globules prior to homogenisation (Mol, 1975; McCrae & Muir, 1992). It is suggested that, the more developed this network prior to spray

drying, the greater would be the resulting powder's resistance to dissolution during reconstitution. Powder A8 showed a rather aggregated fat and protein network by transmission electron microscopy and had very poor functional properties. It has been reported by Teehan *et al.* (1997) that poor stability in coffee was related to powders having a high degree of protein coverage on the fat globules. Powder A5 also had an aggregated structure that would have suggested poor functional properties. However, a number of features of this particular powder suggested that some modification to the composition of the milk occurred before spray drying. The chemical analysis showed that the powder had an elevated level of sodium and phosphate compared with the other powders (Table 6.2). It has been reported (Harwalkar & Vreeman, 1978; Ohba *et al.*, 1989) that the addition of calcium chelators such as phosphate salts can improve the reconstitution properties of milk powder. The swelling of the casein micelles observed in this powder (Figure 6.5b) may have been the result of micellar calcium moving to the serum phase to set up a new calcium equilibrium after the addition of the chelator. The surprisingly good functional properties of this powder despite the aggregated protein matrix observed by microscopy may be explained by the likely addition of this calcium chelator.

Powder A7 had the highest surface coating of lecithin and milkfat (as seen by the added phospholipid level) and had the worst flow properties by subjective and objective analysis. Powder A4 had very good flowability, low lecithin levels and a very coarse granular appearance. However, this powder also exhibited a coarse, gritty mouthfeel. The transmission electron micrographs of this IWMP showed that the particles were covered in a distinct skin. It is likely that this phenomenon influenced the rate of particle dissolution and led to the gritty mouthfeel.

Free fat results seem to have some bearing on powder flow, cohesiveness and wetting. However, this influence is probably related only to the fat on the surface of the particle, which can be determined using a very short solvent extraction time (Buma, 1971). Buma (1971) also observed that the solubility of spray-dried WMP was better if its free fat content was higher, indicating that more highly homogenised milk would give lower free fat but may reduce powder solubility. As discussed in Chapter 5, the adsorption of casein micelles onto the fat globule surface during homogenisation and the formation of clusters of fat globules predisposes the powder to the formation of insoluble material on reconstitution.

Powders that fully disperse produce homogeneous solutions with a thicker mouthfeel. Powders that have undissolved particulates have less material in the solute (water) phase and thus may result in a watery mouthfeel upon reconstitution. Increased homogenisation of the milk during processing results in the formation of smaller fat globules studded with adsorbed casein micelles which possibly could influence powder dispersion and thus mouthfeel when the reconstituted IWMP is consumed.

6.5 CONCLUSIONS

A number of different milk powder manufacturing approaches and final powder properties were represented by the IWMP samples evaluated in this study. The A8 IWMP stands out as having the worst functional performance (solubility in coffee and hot water, dispersibility and cold water solubility), a low WPNI and a high level of protein-protein interaction, and a poor agglomerate structure composed of small primary particles. The A6 IWMP had the most

favourable functional properties. This powder had by far the best solubility in both hot and cold water and coffee, excellent dispersibility, good agglomerate structure and little protein-protein interaction. The only area in which the A6 IWMP performance was not good was wettability. The manufacturers of the A6 IWMP appeared to have optimised the processing conditions (*i.e.* low temperature processing) to produce this functionality. Of the IWMP samples tested, the A6 sample produced a reconstituted milk with functional properties and microstructure most similar to those of fresh milk.

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CHAPTER 7

CHEMICAL AND MICROSTRUCTURAL CHANGES IN MILK DURING ULTRAFILTRATION AND SPRAY DRYING

7.1 INTRODUCTION

The poor solubility of high protein milk powders (> 80% protein) manufactured by membrane filtration has been observed by a number of workers (Jiminez-Florez & Kosikowski, 1986; Panfil-Kuncewicz *et al.*, 1992; Schuck *et al.*, 1994). The solubility index (a measure of undissolved residue) of ultrafiltered skim milk powders (UFSMPs) increases with an increase in protein concentration, storage time and storage temperature (Jiminez-Florez & Kosikowski, 1986; Mistry & Hassan, 1991; Mistry & Pulgar, 1996).

It has been shown that the major factor influencing the dissolution properties of spray-dried ultrafiltered skim milk containing 85-90% protein is related to the rate of water transfer into the high protein particle, *i.e.* a protein-protein network, rather than to the thermal processes during manufacture (Schuck *et al.*, 1994). The protein:lactose ratio is increased from 0.6:1 to 1.2:1 when skim milk is ultrafiltered to a concentration factor of 2 and is then further increased to 9.2:1 after three-stage diafiltration (Thompson & de Man, 1975). The lactose in skim milk acts like a solvent for the protein particularly when the skim milk is dried. Removal of this lactose during membrane concentration removes this solvent and results in changes in the properties of casein micelles and whey protein *e.g.* greater susceptibility to the thermal denaturation of whey proteins (Harwalkar *et al.*, 1989).

Little work examining the structural and compositional changes that occur during the manufacture of UFSMP has been carried out. It is possible that, by evaluating the compositional and structural changes during the different processing stages, a mechanism for the cause of the poor solubility of UFSMP may be determined. Immunogold labelling in conjunction with electron microscopy is a technique that gives the location of specific proteins within the structure of the milk. In a study by Alleyne (1994) it was shown, using immunogold techniques, that the movement of β -lactoglobulin/ κ -casein complex from the micelle to the serum possibly initiated age gelation in UHT milk.

The objective of this work was to examine, using a range of electron microscopy techniques including immunogold labelling, the influence of the major processing steps during the production of UFSMP with about 85% protein (on a dry powder basis), referred to as UFSMP85, on chemical changes and microstructure of the casein micelle.

7.2 EXPERIMENTAL

7.2.1 In-process Samples

Samples of non-fat milk throughout the UFSMP85 process were obtained as follows.

Skim milk (pasteurised)
Retentate and permeate after ultrafiltration
Retentate and permeate mid way through diafiltration
Retentate and permeate after diafiltration
Concentrate after evaporation
UFSMP85 after spray drying

7.2.2 Chemical Analyses

Total Solids (% w/w)

The total solids content was measured by the method of Mojonier & Troy (1925).

Calcium (mmol/kg)

The sample was mixed with deionised water and lanthanum chloride, and the calcium concentration was measured by atomic absorption spectrometry at 285 nm.

Total Nitrogen (% w/w)

The sample was digested and the total nitrogen was measured by automatic titration using a Kjel-Tec analyser (Foss Electric, Hillerod, Denmark).

Non-casein Nitrogen (% w/w)

The sample pH was adjusted to 4.6 to precipitate the casein and the nitrogen content of the filtrate was measured using a Kjel-Tec analyser.

Ash (% w/w)

The samples were ashed at 550°C overnight and the resulting mass was weighed.

Lactose (% w/w)

Lactose was determined by the reduction of ferricyanide to ferrocyanide which was measured colorimetrically using an autoanalyser at 420 nm according to the Technicon method (120-71A, Technicon Industrial Systems, Tarrytown, New York, USA).

7.2.3 Thin-sectioning Transmission Electron Microscopy

The samples (excluding permeate) were mixed at a 4:1 ratio with 20% formaldehyde and left for 4 h (the UFSMP85 powder was reconstituted in 20°C water (10% w/v) and hydrated for 10 min prior to the addition of formaldehyde). All samples were then

added warm to melted 3% agar at a 1:1 ratio. The warm solution was poured on to a microscope slide and allowed to set. The gelled mixture was then chopped into 1 mm³ cubes which were transferred into cacodylate/HCl buffer in a bijou bottle. The buffer was replaced after 30 min and 1 h and then left for a further 1 h prior to replacement with 1 mL of a solution containing a 1:1 ratio of osmium tetroxide (2% solution) and cacodylate/HCl buffer. This was left to stand for 2 h before being replaced with 1 mL of 1% uranyl acetate for 30 min after which the product cubes were washed with water before dehydration (for immunolabelling, no staining in osmium tetroxide or uranyl acetate was carried out). Dehydration consisted of washing with 50, 70, 90 and 100% ethanol for 5-10, 30, 30 and 60 min respectively. The 100% ethanol was changed after 30 and 60 min. The ethanol was poured off and the bottle was filled with a 1:1 ratio of acrylic resin (LR White) and 100% alcohol and rotated for 4 h prior to rotating overnight in pure LR White. One cube of sample was added to each of three moulds containing fresh LR White resin which were then baked for 24 h at 55°C.

The samples were microtomed using a Reichert OmU2 microtome, mounted on 3 mm copper/rhodium grids and stained using lead citrate before examination in a Philips transmission electron microscope at an accelerating voltage of 60 kV. For immunolabelling, the following procedure was carried out after microtoming.

Immunolabelling Protocol

The grid containing the resin embedded milk section was floated on a drop of 20 mM Tris buffer (pH 6.6) containing blocking agents (0.5% fish gelatin and 0.5% normal goat serum) for 15 min. The grid was then rinsed in 50 mM Tris buffer (pH 7.4) for 5 min (one change at 2.5 min) and then floated on a drop of primary antibody overnight at 7°C. Separate grids were floated on the following two antibody preparations: (1) rabbit anti-bovine β -lactoglobulin (Bethyl Laboratories) at a ratio of 1:1000 in 50 mM Tris buffer with blocking agents; (2) rabbit anti-bovine κ -casein (polyclonal, Utah State University) at a ratio of 1:1000 in 50 mM Tris buffer with blocking agents). The grid was then rinsed in 50 mM Tris for 30 min (six x 5 min washes) and then floated on a drop of secondary antibody (goat anti-rabbit immunoglobulin G conjugated to 10 nm colloidal gold (Amersham) at a ratio of 1:40 in 50 mM Tris buffer) for 3 h at room temperature. The grid was then rinsed in double-distilled water for 30 min (six x 5 min), stained for 20 min in 1% uranyl acetate, rinsed in water, stained for 2 min in lead citrate and finally drip rinsed in water. Control grids containing resin embedded milk sections were floated on 50 mM Tris buffer, rather than the two primary antibody preparations, to determine the level of non-specific colloidal gold binding using this technique.

7.2.4 Freeze Fracturing

The concentrates and powders were mixed with glycerol (1:1) and placed on a copper sample holder that was plunged into liquid nitrogen (-196°C) and transferred on to a Balzers specimen holder. The specimen holder was then transferred into a Balzers freeze etch apparatus (Balzers BAF060, Baltec Ltd, Balzers, Liechtenstein) where the samples were fractured using a knife, and then coated with approximately 2 nm of

platinum followed by 20 nm of carbon. The replicas were removed from the Balzers apparatus and placed into a solution of chromic acid for 2 days. The chromic acid was gradually replaced with water and then left for 1 day. The replicas were finally washed in water, washed in acetone and then mounted on 3 mm copper/rhodium grids and viewed in a Philips transmission electron microscope at an accelerating voltage of 60 kV.

7.2.5 Particle Size Analysis by Photon Correlation Spectroscopy

Size measurements of the particles in the skim milk samples were made by photon correlation spectroscopy using a Malvern Zetasizer 4 instrument and the associated ZET5110 particle sizing cell (Malvern Instruments Ltd, Malvern, UK). The temperature of the cell was maintained at $27 \pm 0.5^\circ\text{C}$ for the duration of the experiments. Measurements of the dynamics of the scattered light were collected at a scattering angle of 90° only. Average diffusion coefficients were determined by the method of cumulants and translated into average particle diameters using the Stokes-Einstein relationship for spheres. UFSMP powder was reconstituted in warm (60°C) water at a concentration of about 10% total solids (w/w). The UFSMP85 samples obtained throughout the manufacturing process were dispersed in either permeate obtained from the diafiltration process or calcium imidazole buffer (5 mM CaCl_2 , 20 mM imidazole, 30 mM NaCl) and allowed to stand for at least 10 min before the particle size was determined. For each sample, no changes in particle size or scattering intensity were observed over a period of several hours, indicating that the casein micelles were stable in this buffer system at all stages of the UFSMP process.

Photon correlation spectroscopy provided the cumulant z-average particle size and the polydispersity of the particles in the milk fractions. The cumulant z-average size is an intensity-weighted average particle diameter (based on the intensity of scattered light) and the polydispersity is a dimensionless measure of the broadness of the distribution and ranges from 0 for a monodisperse sample to 1 for a very polydisperse sample. The z-average size and the polydispersity are derived independently of the properties of the particles (*e.g.* refractive index) and are very sensitive to small changes in size and polydispersity. However, without the knowledge of some key properties of the particles (especially refractive indices of the surface layers and core of the particles), it is difficult to obtain accurate and detailed information on the size distribution.

7.2.6 Ultracentrifugation

Liquid samples obtained during the UFSMP manufacturing process, and reconstituted UFSMP85 (10% total solids (w/w) in warm water (60°C)), were placed in polycarbonate ultracentrifuge tubes of 15 mL nominal volume and heat sealed. The samples were centrifuged for 2 h at 40 000 rev/min (110 000 g) in a Beckman L8-80M ultracentrifuge and the associated Ti80 fixed angle rotor (Beckman Instruments Ltd, Palo Alto, California, USA).

7.2.7 Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories,

Richmond, California, USA) and a method similar to that described by Laemmli (1970). The resolving gel contained 15% acrylamide (2.6% Bis) and 0.1% SDS in 1.5 M Tris/HCl buffer at pH 8.8, and the stacking gel contained 4% acrylamide and 0.1% SDS in 0.5 M Tris/HCl buffer at pH 6.8. Samples were dispersed in 0.5 M Tris/HCl buffer at pH 6.6 containing 2% SDS, 0.05% β-mercaptoethanol and 0.01% bromophenol blue, and heated at 100°C for 4 min. Ultracentrifugal supernatants were diluted 1:12 and the untreated milk samples were diluted 1:40, with the sample buffer. The gels were run at 210 V for 1 h and stained with Coomassie Blue (R) in 25% (v/v) 2-propanol and 10% (v/v) acetic acid for 1 h. After staining, the gels were destained with two changes of a 10% 2-propanol and 10% acetic acid solution for a total period of 24 h.

SDS-PAGE gels were scanned using a Molecular Dynamics Model P.D. computing densitometer (Molecular Dynamics Inc., Sunnyvale, California, USA). The integrated intensities of the major casein bands were determined using the Imagequant software associated with the densitometer. The quantity of each protein in the ultracentrifugal supernatants was determined as a percentage of that in the original milk sample.

7.3 RESULTS

7.3.1 Chemical Analyses

The results of the chemical analyses for the in-process samples are given in Table 7.1

TABLE 7.1 Chemical analysis of samples obtained throughout UFSMP85 manufacture

Sample	Lactose (% w/w)	Ash (% w/w)	Calcium (mmol/kg)	Non-casein Nitrogen (% w/w)	Total Nitrogen (% w/w)	Total Solids (% w/w)
Skim milk	4.58	0.8	36.6	0.15	0.65	9.47
Ultrafiltration midway	4.77	0.91	43.6	0.18	0.84	10.86
Ultrafiltration end	4.76	1.16	58.8	0.26	1.32	14.14
Diafiltration midway	1.25	1.17	82.1	0.35	1.94	15.06
Diafiltration end	0.75	1.25	92	0.39	2.11	16.21
Evaporator	0.99	1.82	131.2	0.5	3.15	24.3
Powder	6.05	7.33	587.9	1.9	12.73	95.5

7.3.2 Microscopy

The results for thin-section and freeze-fracture transmission electron microscopy are presented together, followed by the immunolabelling results which are presented separately.

7.3.2.1 Freeze-fracture and Thin-section Transmission Electron Microscopy

Skim Milk

The thin-section micrographs (Figure 7.1) of the skim milk showed casein micelles of different sizes, generally well separated in the dispersed phase, containing water, lactose, soluble minerals and non-micellar proteins. The freeze-fracture micrographs (Figure 7.2) also showed the casein micelles dispersed in the milk serum. The micelles were composed of subunits which could be seen as small raised structures within the casein micelle. These single subunits were present in skim milk only as part of the casein micelle and were generally not present as single units within the milk serum.

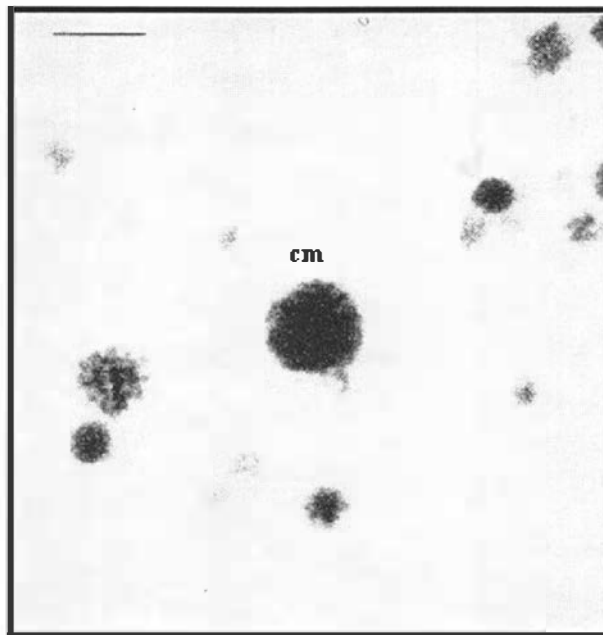


FIGURE 7.1 Skim milk microstructure by thin-section transmission electron microscopy. Bar = 0.2 μm , cm = casein micelle.

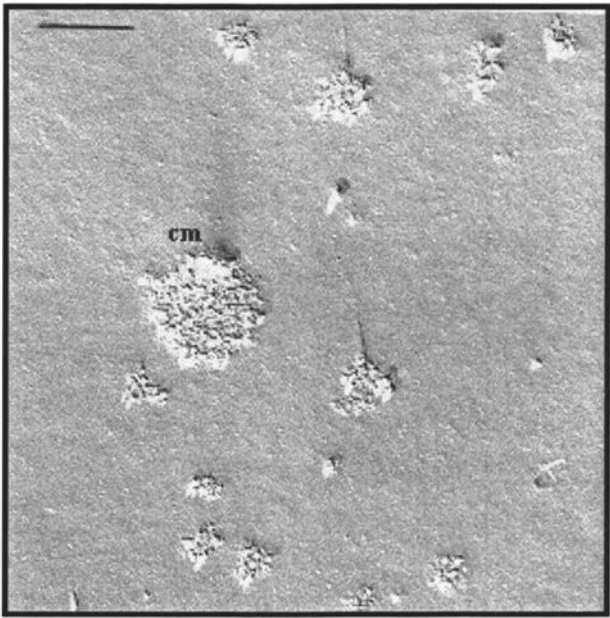


FIGURE 7.2 Skim milk microstructure by freeze-fracture transmission electron microscopy. Bar = 0.2 μ m, cm = casein micelle.

Ultrafiltered Milk

There did not appear to be a major change in the microstructure of the micellar material after ultrafiltration. The thin-section image (Figure 7.3) did indicate that more non-micellar material may have been present after ultrafiltration (arrow). There did appear to be a number of small subunit structures (arrows) present in the serum, shown by freeze fracturing (Figure 7.4).

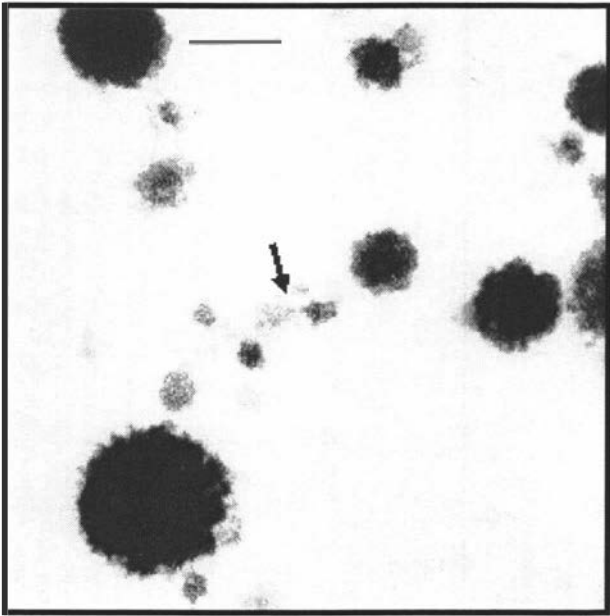


FIGURE 7.3 Ultrafiltered skim milk microstructure by thin section transmission electron microscopy. Bar = 0.2 μm .

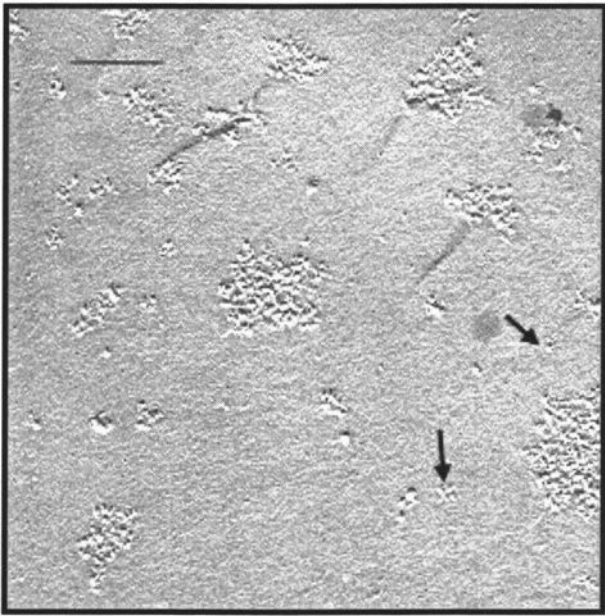


FIGURE 7.4 Ultrafiltered skim milk microstructure by freeze-fracture transmission electron microscopy. Bar = 0.2 μm .

Diafiltered Milk (midway)

There appeared to be a significant change to the structure of the skim milk concentrate approximately midway through diafiltration. There was an increase in the amount of non-micellar material (arrow, Figure 7.5). There was also a noticeable change in the electron density of the casein micelles when compared with the skim milk. The casein micelles were less dense in this sample than in the skim milk or ultrafiltered milk, suggesting that some swelling of the casein micelle had occurred or that material was being lost from the micelle, *i.e.* protein or minerals. This result is supported by the freeze-fracture micrograph (Figure 7.6) which showed an increase in the size of the subunit structures (arrow) within the micelle (particularly when compared with the size of the skim milk subunits). There also appeared to be a greater number of singular subunits in the serum phase, indicating that there was a loss of material from the casein micelle during concentration.

Diafiltered Milk (end)

Diafiltration over the final few membrane columns resulted in further swelling of the micelle structure and the release of more protein material from the micelle (Figures 7.7 and 7.8). The freeze-fracture micrograph showed a further increase in the size of the protein subunits and the presence of more of these units separate from the micelle (arrows).

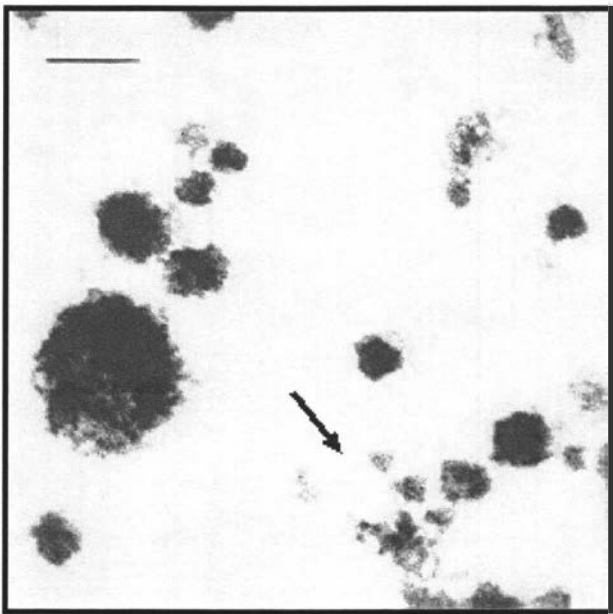


FIGURE 7.5 Diafiltered (midway) skim milk microstructure by thin-section transmission electron microscopy. Bar = 0.2 μm .

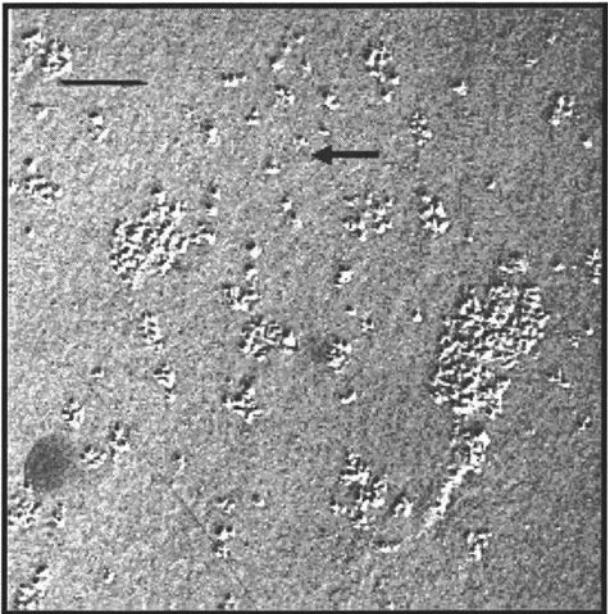


FIGURE 7.6 Diafiltered (midway) skim milk microstructure by freeze-fracture transmission electron microscopy. Bar = 0.2 μm .

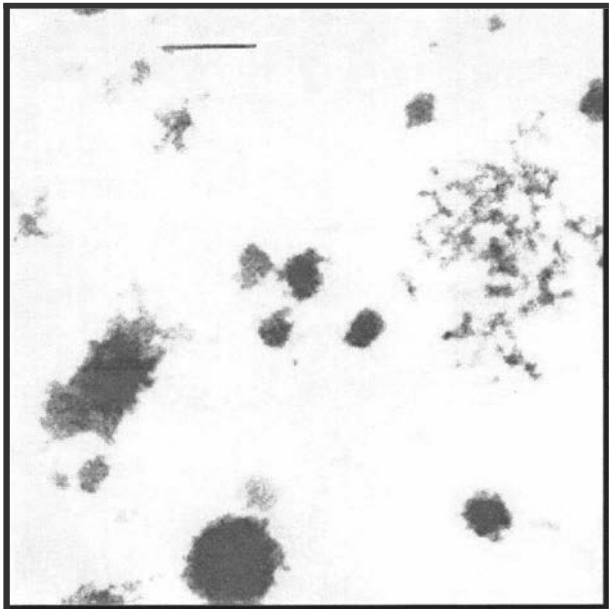


FIGURE 7.7 Diafiltered (end) skim milk microstructure by thin-section transmission electron microscopy. Bar = 0.2 μm .

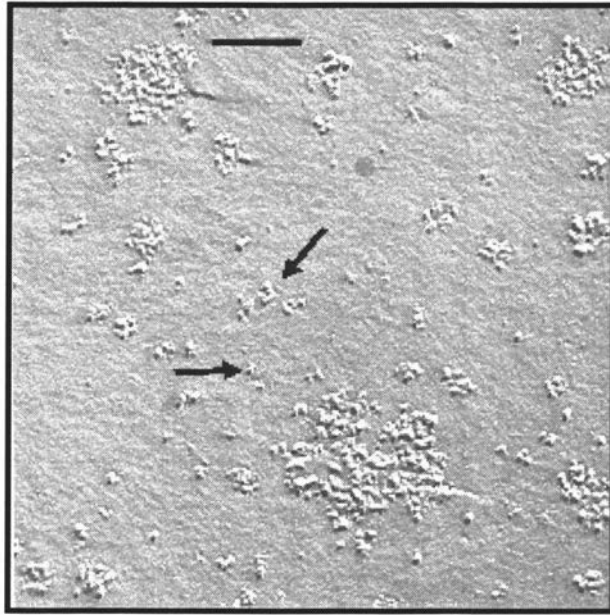


FIGURE 7.8 Diafiltered (end) skim milk microstructure by freeze-fracture transmission electron microscopy. Bar = 0.2 μm .

Evaporated Milk

The casein micelles were packed closer together in the evaporated skim milk retentate, resulting in an increase in micelle aggregation (Figure 7.9). This aggregation appeared to be by way of hair-like structures (arrows) that were more abundant in this sample probably as a result of its higher total solids concentration. The freeze-fracture micrograph (Figure 7.10) showed a large number of fracture ripples across the replica (arrow). It was difficult to determine any increase in the number of protein units away from the micelles or any change to the size of the micelles because of the rippled nature of the replica.

Reconstituted UFSMP85

The effect of drying on the microstructure of the concentrated skim milk was to force the casein micelles into close proximity (Figure 7.11). It is evident that there were a large number of hair-like structures linking these micelles together in this powder (arrows). A number of the micelles appeared to have aggregated. There appeared to be a reformation of micelle-like aggregate structures during evaporation and drying. There were no freeze-fracture micrographs of the dried UFSMP85 as little detail was obtained.

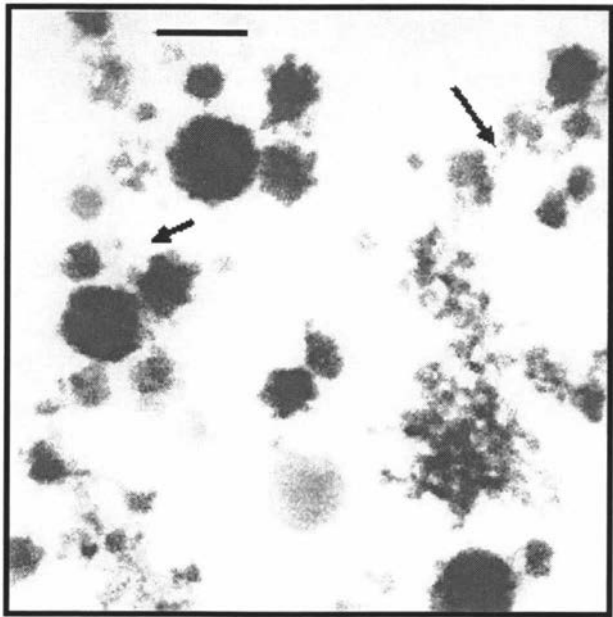


FIGURE 7.9 Evaporated skim milk microstructure by thin-section transmission electron microscopy. Bar = 0.2 μm .

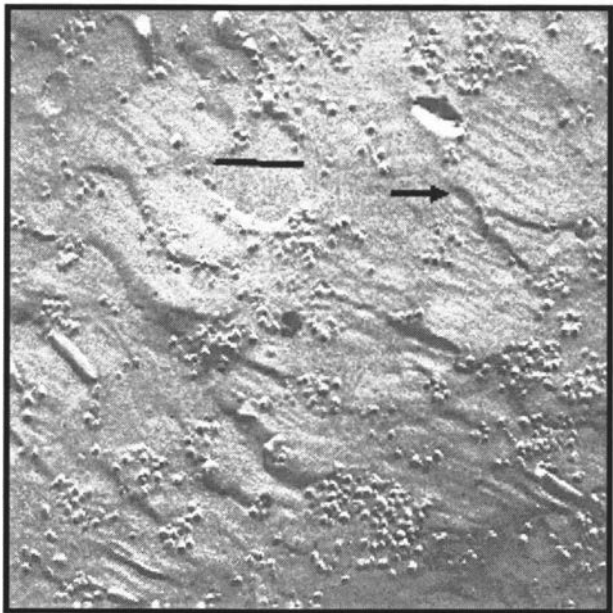


FIGURE 7.10 Evaporated skim milk microstructure by freeze-fracture transmission electron microscopy. Bar = 0.2 μm .

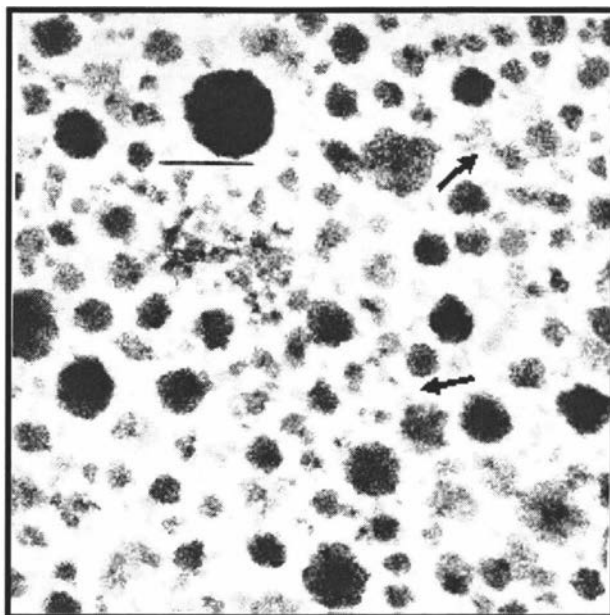


FIGURE 7.11 Reconstituted UFSMP85 (20°C for 10 min in water) microstructure by thin-section transmission electron microscopy. Bar = 0.2 μm .

7.3.2.2 Immunolabelling

The dark electron-dense dots in the micrographs are the location of the colloidal-gold-tagged antibodies to β -lactoglobulin (Figures 7.12a-7.17b) and κ -casein (Figures 7.12b-7.17c). The labelling was carried out on the embedded thin-sectioned material and thus allowed antibody labelling across the diameter of sectioned casein micelles. The control samples (not shown) indicated that non-specific binding had not been eliminated by the use of blocking agent but was at a sufficiently low level not to interfere with the results presented below.

Skim Milk

The location of the β -lactoglobulin could be seen to be mainly in the serum of the milk with some located on or near to the surface of the casein micelle (Figure 7.12a). This was in contrast to κ -casein (Figure 7.12b), which appeared to be more concentrated on and within the casein micelle than in the serum.

Ultrafiltered Milk (midway)

Retentate was collected at the end of the first loop and examined by immunolabelling. The location of β -lactoglobulin (Figure 7.13a) was similar to that observed in the skim milk, *i.e.* most of the labelling was in the serum phase.

The κ -casein-labelled milk (Figure 7.13b) showed that a change had occurred in the distribution of the κ -casein about the casein micelle. There appeared to be a slightly greater concentration of the κ -casein near the outer perimeter of the casein micelles (arrow) than was seen for the unconcentrated skim milk.

Ultrafiltered Milk (end)

There was no obvious change to the location of β -lactoglobulin in the milk from that observed earlier in the process (Figure 7.14a). The location of the κ -casein in the milk continued to alter, with an increase in labelling apparent in a ring around the outside edge of the darkly labelled casein micelles (Figure 7.14b). There appeared to be more dissociated material in the serum. This material (arrows) labelled positive for both β -lactoglobulin and κ -casein.

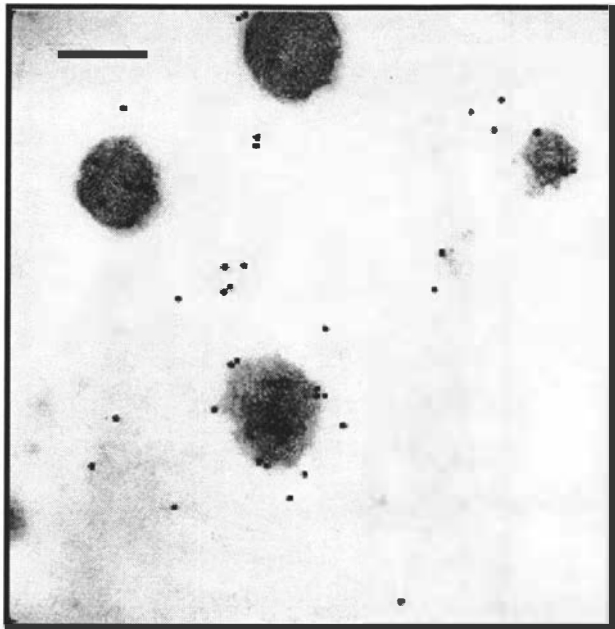


FIGURE 7.12a β -Lactoglobulin-labelled skim milk using rabbit anti-bovine β -lactoglobulin localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

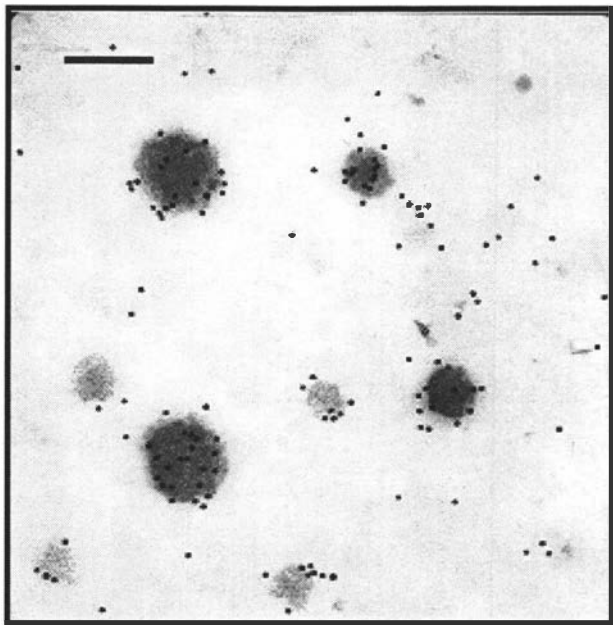


FIGURE 7.12b κ -casein-labelled skim milk using rabbit anti-bovine κ -casein localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

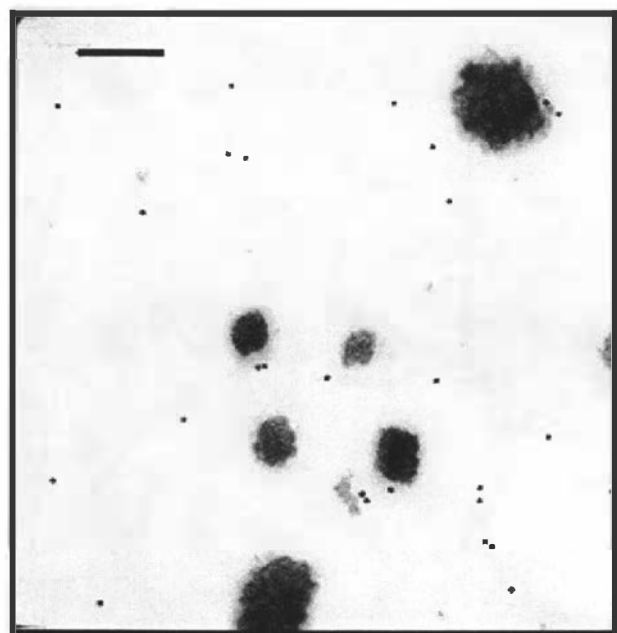


FIGURE 7.13a β -Lactoglobulin-labelled ultrafiltered (midway) skim milk using rabbit anti-bovine β -lactoglobulin localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

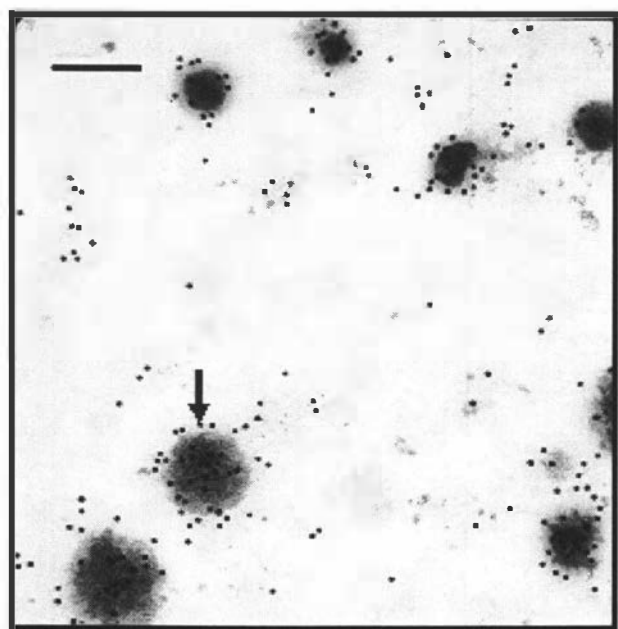


FIGURE 7.13b κ -casein-labelled ultrafiltered (midway) skim milk using rabbit anti-bovine κ -casein localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

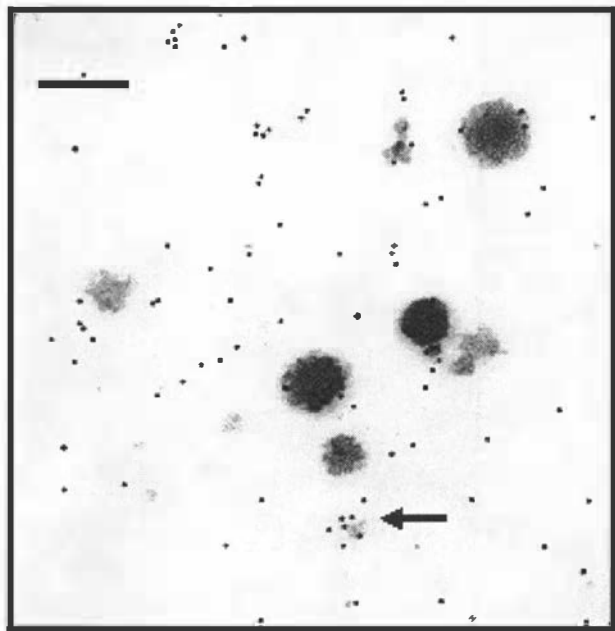


FIGURE 7.14a β -Lactoglobulin-labelled ultrafiltered (end) skim milk using rabbit anti-bovine β -lactoglobulin localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

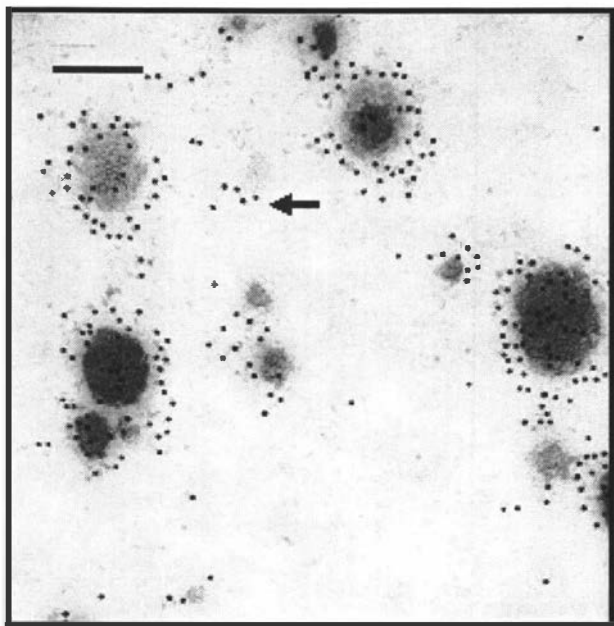


FIGURE 7.14b κ -casein-labelled ultrafiltered (end) skim milk using rabbit anti-bovine κ -casein localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

Diafiltered Milk

An apparent swelling of the micelles throughout processing, as indicated by PCS (see Section 7.3.3) and microscopy, could be observed in the milk after diafiltration. Some of the micelles appeared to swell to the extent that large gaps appeared within the micelles (Figure 7.15a). Much of the β -lactoglobulin (Figure 7.15a) and κ -casein (Figure 7.15b) appeared to be associated with regions of what appeared to be dissociated micellar material that had aggregated (arrows). Extensive dissociation of κ -casein appeared to occur from the casein micelle at this stage of processing.

Evaporated Milk

The micrographs showed that both β -lactoglobulin (Figure 7.16a) and κ -casein (Figure 7.16b) were associated with the material involved in the aggregation of the micelles after evaporation of the ultrafiltration-concentrated skim milk (arrows). There also appeared to be a greater quantity of κ -casein in the serum phase.

Reconstituted UFSMP85

The location of β -lactoglobulin appeared to be mainly in the regions between the casein micelles or attached to the surface of casein micelles within the UFSMP85 powder particle (Figure 7.17a). There was also evidence that the β -lactoglobulin was concentrated at the powder particle surface, *i.e.* a protein monolayer (rich in β -lactoglobulin) could be seen to be stripping away from the particle surface during powder reconstitution (arrow, Figure 7.17a) and at the surfaces of the bubbles formed inside the particles during drying (arrow, Figure 7.17b). The κ -casein also appeared to be located either at the surface of the micelles or in the regions between the micelles (Figure 7.17c). This was in contrast to the κ -casein in the micelles in the skim milk prior to processing, which was more concentrated within or on the surface of the micelles (Figure 7.12b).

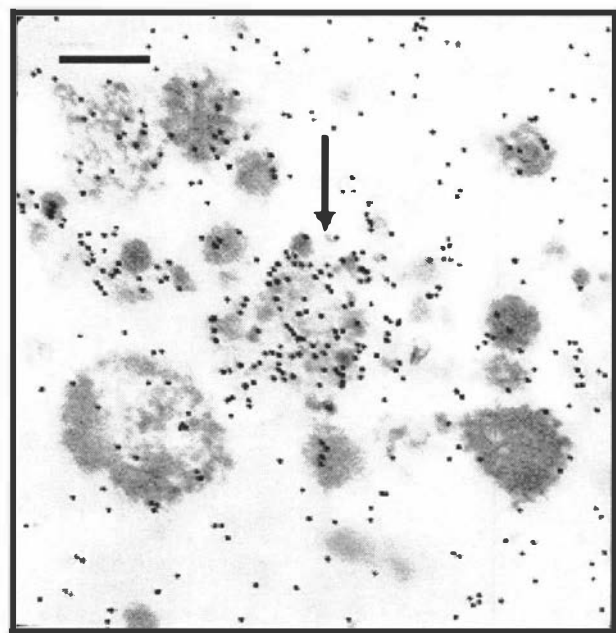


FIGURE 7.15a β -Lactoglobulin-labelled diafiltered skim milk using rabbit anti-bovine β -lactoglobulin localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

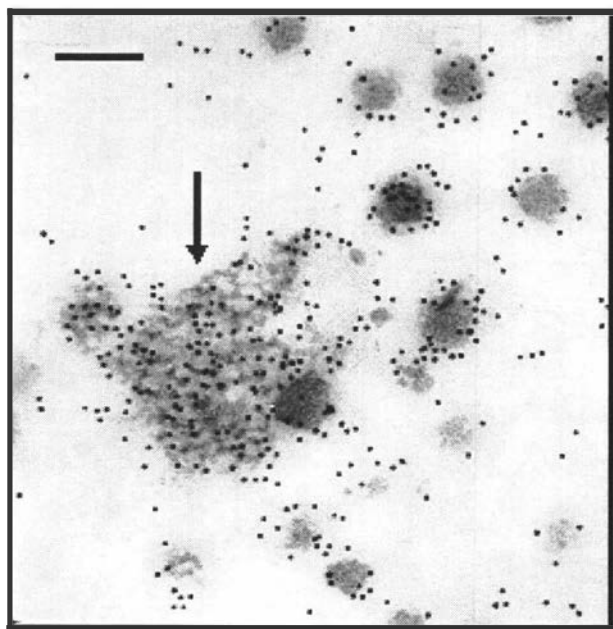


FIGURE 7.15b κ -casein-labelled diafiltered skim milk using rabbit anti-bovine κ -casein localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

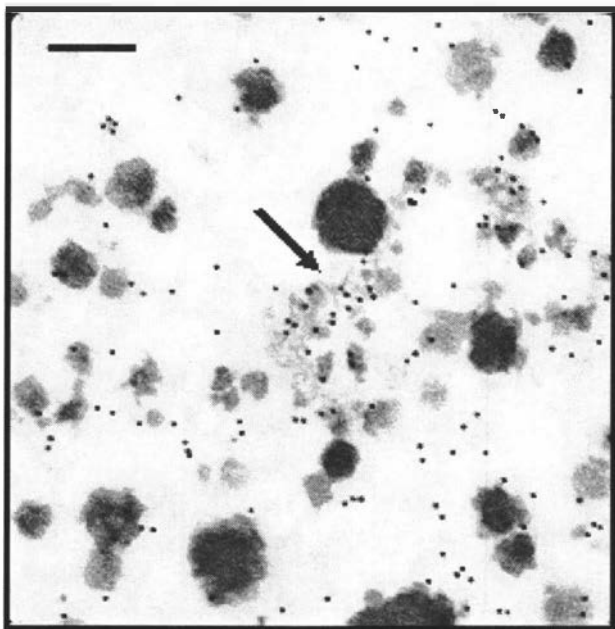


FIGURE 7.16a β -Lactoglobulin-labelled evaporated skim milk using rabbit anti-bovine β -lactoglobulin localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

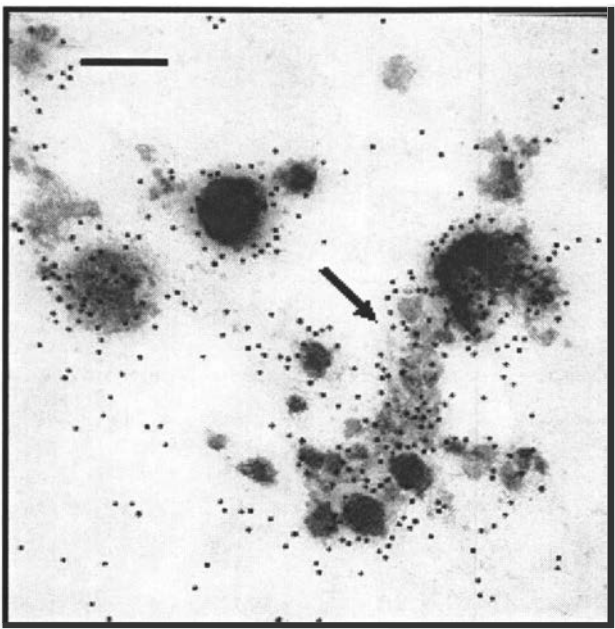


FIGURE 7.16b κ -casein-labelled evaporated skim milk using rabbit anti-bovine κ -casein localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

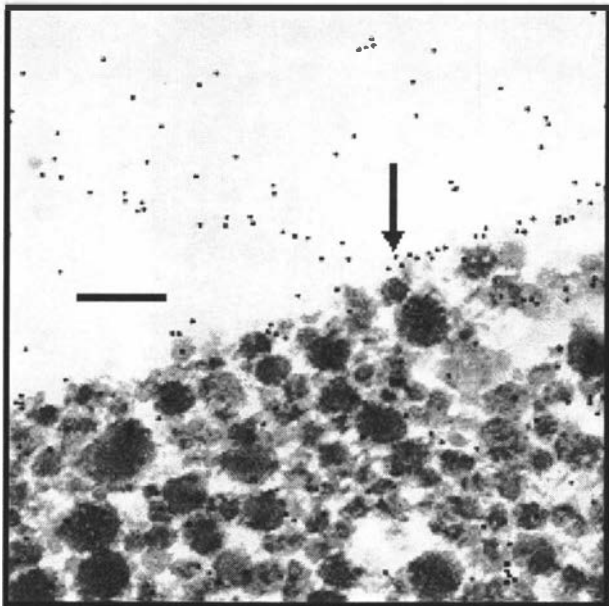


FIGURE 7.17a β -Lactoglobulin-labelled reconstituted UFSMP85 (20°C for 10 min in water) using rabbit anti-bovine β -lactoglobulin localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

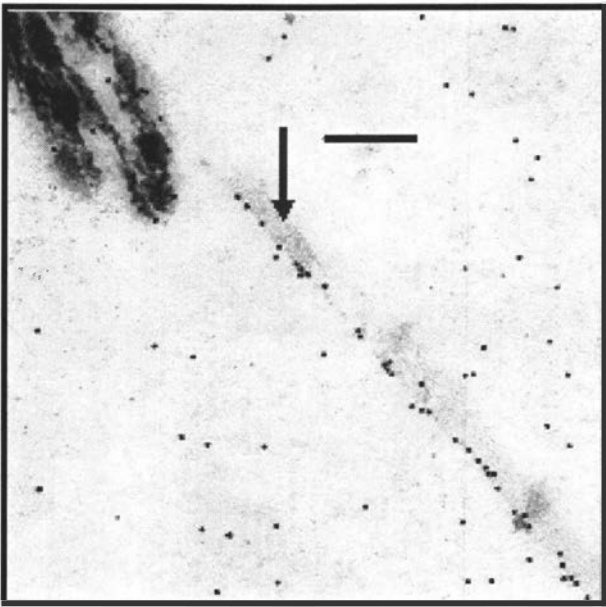


FIGURE 7.17b β -Lactoglobulin-labelled reconstituted UFSMP85 using rabbit anti-bovine β -lactoglobulin localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μ m.

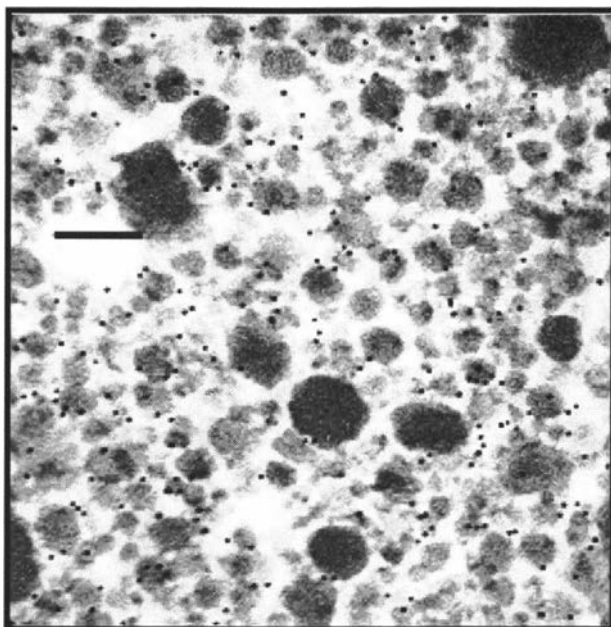


FIGURE 7.17c κ -casein-labelled UFSMP85 using rabbit anti-bovine κ -casein localised with 10 nm colloidal gold observed by thin-section transmission electron microscopy. Bar = 0.2 μm .

7.3.3 Particle Size Analysis By Photon Correlation Spectroscopy

The changes in particle size for samples taken at each stage of the process are outlined in Figure 7.18 (using both diafiltration-permeate and calcium imidazole buffer). The results in the two dispersion media were very similar and are discussed together. There was little change in particle size at the early stages of ultrafiltration; however, at the end of the ultrafiltration process and during diafiltration and evaporation and in the reconstituted product, the average particle size had increased slightly at each consecutive stage in the process. Only relatively small changes were observed in the polydispersity, which is a measure of the width of the distribution, suggesting that the size changes observed were due to the swelling of the casein micelles. This is in general agreement with the TEM results. The micelles may be expected to swell during the diafiltration process as some of the colloidal calcium phosphate is dissolved through water addition, loosening the micellar structure.

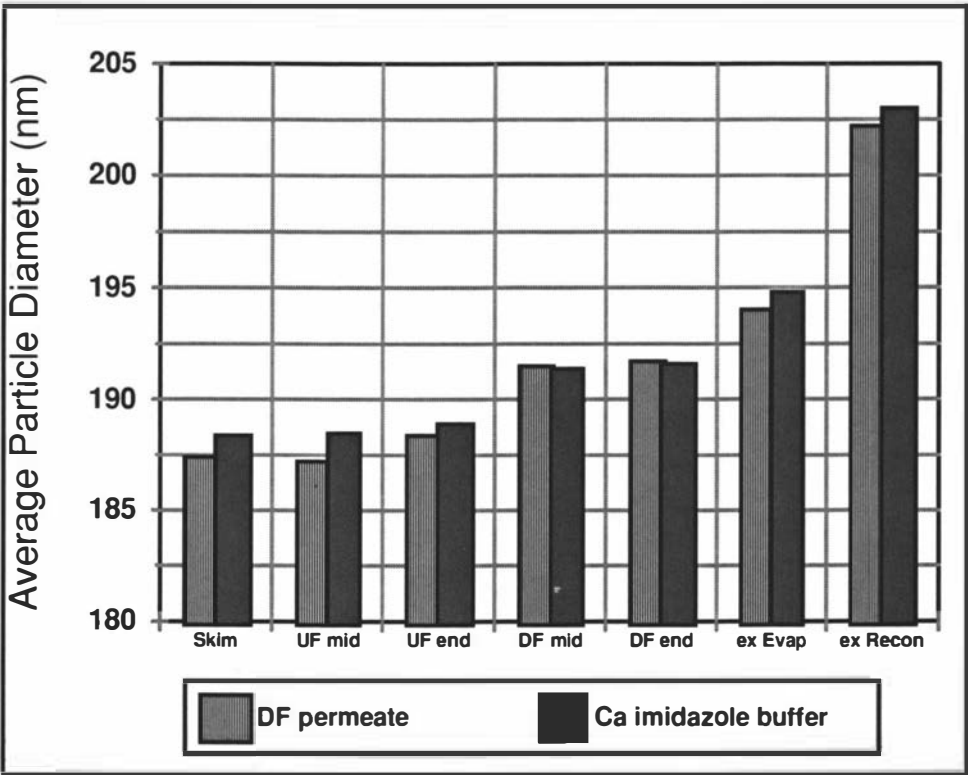


FIGURE 7.18 Average particle diameter of milk samples obtained during the manufacture and reconstitution of UFSMP85. Samples were dispersed in diafiltration (DF) permeate and calcium imidazole buffer and were evaluated using photon correlation spectroscopy.

7.3.4 Gel Electrophoresis

For the samples at each stage in the process, the UFSMP/milk sample and its ultracentrifugal supernatant were analysed for casein protein content by SDS-PAGE.

The amount of casein in the supernatant was expressed as a percentage of that found in the original sample. Each supernatant was analysed in duplicate and the results are shown in Figure 7.19. Only low levels of soluble protein were observed in all the samples analysed, which may be a consequence of the rather severe centrifugation conditions used. There was a small, but reproducible, increase in the soluble casein level at each consecutive stage in the process, but this level decreased again when the reconstituted UFSMP powder was analysed. The small increase in soluble casein indicates that the casein micelle structure was altered by the UFSMP process, possibly through the dissolution of a small amount of the colloidal calcium and phosphate which is integral in maintaining micellar structure. Only small increases in soluble casein levels and in particular κ -casein were observed. Therefore no attempt was made to completely analyse the individual casein proteins. The results from transmission electron microscopy, gel electrophoresis and particle size analysis suggest that there was a loosening or swelling of the casein micelle, resulting in the κ -casein moving away from the casein micelle surface but still being attached or connected (by protein-protein or protein-mineral bonds) to the micelles within the UFSMP85 structure.

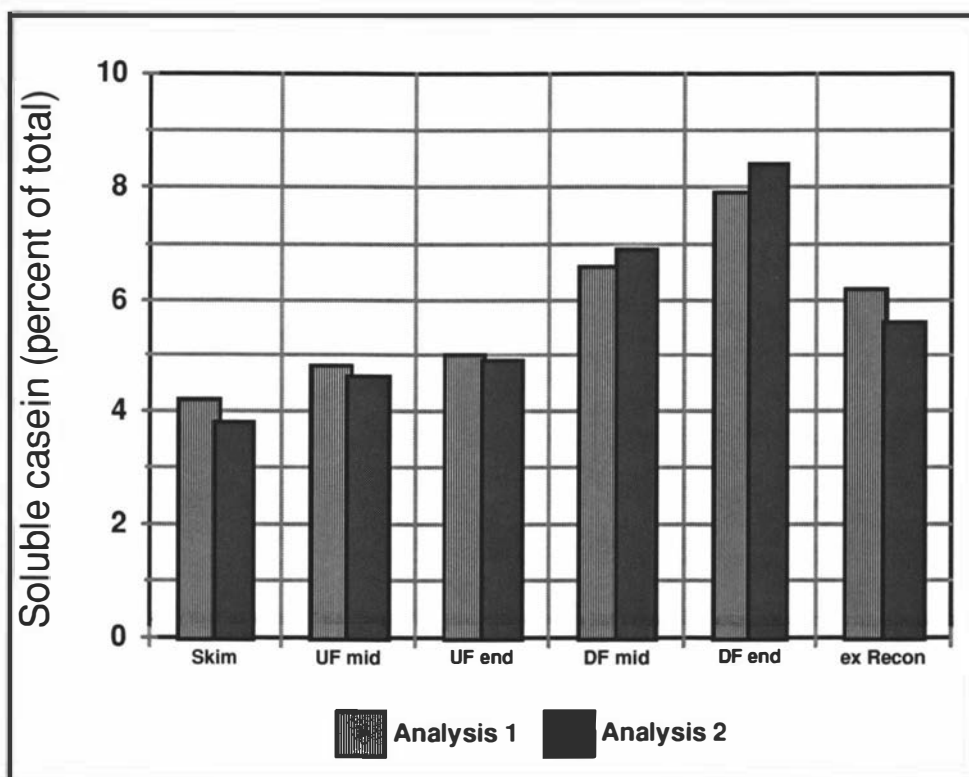


FIGURE 7.19 Percentage of casein in the serum after different stages of UFSMP85 manufacture as measured by gel electrophoresis of the ultracentrifugal supernatants (duplicate analyses of each sample).

7.4 DISCUSSION

During the course of concentration of milk by UF, and particularly during diafiltration when water is added, the micelle loses some of its colloidal calcium phosphate. This occurs during diafiltration as the casein micelles equilibrate to a mineral-depleted aqueous environment (Brule *et al.*, 1974), *i.e.* serum calcium and phosphorus are lost with the wash water disturbing the mineral equilibrium (vital for casein micelle stability). The subsequent dissolution of colloidal calcium phosphate leads to the presence of more serum calcium and phosphate. This continual loss of colloidal calcium phosphate during diafiltration results in the loosening of the micellar structure and is thus responsible for the swelling of the casein micelle (Figures 7.5, 7.6, 7.15a and 7.18). The pH of the product throughout the UFSMP process typically increases from 6.7 (skim milk) to 7.0 (dried UFSMP85) that may also influence micelle structure.

Examination of casein micelles in ultrafiltered concentrates by electron microscopy showed that there were significant differences compared with the micelles in unconcentrated milk (Srilaorkul *et al.*, 1991). The size of the highest proportion of casein micelles (at 80-100 nm in skim milk) was reduced (to 60-80 nm) in skim milk at a volume concentration factor of 5. The volume distribution and the average diameter of the casein particles were decreased. Changes in the mineral levels were claimed to effect changes in the size of the micelles (Srilaorkul *et al.*, 1991). The results of this study (volume concentration factor of 4.38) showed the opposite trend, *i.e.* the casein micelle size distribution increased with concentration by ultrafiltration and diafiltration (Figure 7.18). The difference in volume concentration factor of these two studies may explain the difference in micellar size observed. The greater the degree of concentration, the greater are the depletion of colloidal calcium phosphate and the progressive breakdown of the micellar structure from an intact micelle to a swollen diffuse micelle and finally to a smaller fragmented micellar structure.

Anema (1998) found that there was an increase in soluble κ -casein as the total solids concentration was increased in reconstituted skim milk. Green *et al.* (1984) found that, as the milk became more concentrated by ultrafiltration, the proportion of casein in the micellar form decreased slightly. The electron micrographs of the milks of Green *et al.* (1984) showed that the micelles became less spherical and more fuzzy as the milk was concentrated, indicating partial dissociation of the micelles. This micelle dissociation during ultrafiltration was also clear in this study as observed by microscopy (Section 7.3.2) and gel electrophoresis (Section 7.3.4). The results showed the movement of κ -casein from the micelle during concentration by ultrafiltration and diafiltration (Figures 7.13b and 7.14b); immunolabelling clearly showed a ring of dissociated κ -casein at the perimeter of the micelle. The dissociation of κ -casein from the micelle would result in a loss of micellar charge and an increase in the ability of the micelles to aggregate, particularly in the presence of calcium. The mechanism of interactions between the proteins dissociated from the micelles and the micelles themselves is not obvious. Further studies are needed to elucidate the nature of these interactions.

The poor cold water solubility of freshly made UFSMP is most probably related to the strength of the bonds between the dissociated micellar material and the casein micelles. The role of whey protein in the formation of the insoluble material is not known. There may be interactions between κ -casein that has dissociated from the micelle during concentration and whey proteins;

these interactions may be by way of sulphhydryl-disulphide interactions (Figures 7.15a and 7.15b). Milk protein concentrates may be more susceptible to the denaturation of whey protein. McMahon *et al.* (1993) found that heat-induced whey protein denaturation was greater in ultrafiltration-concentrated milk than in the unconcentrated milk, whereas Waungana *et al.*, (1996) found no noticeable difference in β -lactoglobulin denaturation.

Immunolabelling shows that β -lactoglobulin was concentrated on the surface skin of the UFSMP particle (Figures 7.17a and 7.17b). This may have occurred during spray drying where the whey proteins, being more surface active than casein micelles, adsorbed to the air/liquid interface. The layer of skin containing β -lactoglobulin strips off from the particle during reconstitution (Figure 7.17a). It is possible that this layer of protein may continue to thicken throughout storage and that even micelles may be involved in the formation of the water-impermeable layer as described by Schuck *et al.* (1994). During concentration of the protein and removal of lactose, the distances between the micelles are reduced. At or near the surface of a newly dried particle, the micelles are packed closely together and may even touch (micelles within the particle are not in such close proximity). This micellar contact is further promoted as the micelles become depleted in κ -casein during concentration, thus reducing steric repulsion. With such close contact and the absence of steric repulsion, other forces such as hydrophobic bonds and perhaps sulphhydryl/disulphide interchange reactions with the surface β -lactoglobulin may cause fusion of this outer micelle layer during storage. The formation of this skin during storage is the focus of Chapter 9.

7.5 CONCLUSIONS

The use of transmission electron microscopy and immunogold labelling techniques combined with photon correlation spectroscopy provided useful information about the changes in the location of proteins and the size of the casein micelles during the concentration and drying of skim milk for UFSMP85 manufacture.

κ -Casein appeared to move out of the casein micelle during ultrafiltration and diafiltration and was trapped in the UFSMP particle structure after spray drying. β -Lactoglobulin appeared to be well dispersed in the serum throughout concentration and appeared to be located at the surface (skin) of the UFSMP85 particles after spray drying. Both proteins appeared to be concentrated in large regions of aggregated non-micellar material observed in the milks after diafiltration and evaporation.

It appeared from this work that both β -lactoglobulin and κ -casein were involved in the formation of aggregates (that may or may not include mineral material) that could be involved in gluing the UFSMP85 structure together after spray drying.

This work has shown that complex changes take place in milk during concentration by ultrafiltration and evaporation and then during spray drying. The removal of lactose and the change in the protein distribution during concentration appear to predispose the product to significant protein-protein interactions during spray drying and storage.

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CHAPTER 8

MICROSTRUCTURAL CHANGES DURING THE STORAGE OF SPRAY-DRIED ULTRAFILTERED SKIM MILKS AND THEIR AFFECT ON DISSOLUTION PROPERTIES IN WATER

8.1 INTRODUCTION

Spray-dried ultrafiltered skim milk powder with 85% protein (UFSMP85) has been developed for a wide range of applications including milk fortification and cheese milk extension. The major functional problem associated with UFSMP85 is its solubility in water at and below approximately 20°C (Schuck *et al.*, 1994).

A number of workers (Jiminez-Flores & Kosikowski, 1986; Mistry & Hassan, 1992; Mistry & Pulgar, 1996) have shown that the solubility of UFSMP85 decreases over storage time particularly at elevated temperatures. The interactions of milk proteins with minerals, which are important in determining the three-dimensional structure of the protein molecules in water, may also affect some functional properties of UFSMP85 in foods, such as cheese milk extension and yoghurt. Insoluble protein aggregates in UFSMP85, intended for cheese milk extension and yoghurt, are undesirable.

In this study, the structures of skim milk powder (SMP) and UFSMP of different protein contents (56, 70, 85 and 92%) were examined after reconstitution to further elucidate the mechanism of poor solubility of the higher protein UFSMP products. In addition, the influence of shear on the solubility of UFSMP85 reconstituted in water at 20°C was examined to determine the physical strength of the insoluble aggregates. In the final part of this study, the influence of storage temperature and time on UFSMP85 microstructure was evaluated to determine how these structural changes might influence the reconstitution properties.

8.2 MATERIALS AND METHODS

8.2.1 Experimental Details

The three experiments that make up the work reported in this chapter are summarised below.

Structure and Solubility of UFSMP Containing Different Protein Concentrations

The microstructures of powders made from ultrafiltered skim milk concentrates of increasing protein content were examined after reconstitution. These powders were obtained from the New Zealand Dairy Board (Wellington) and were made in a number of different commercial manufacturing plants.

The samples examined were as follows.

SMP (6 months of storage at 20°C)
UFSMP56 (6 months of storage at 20°C)
UFSMP70 (6 months of storage at 20°C)
UFSMP85 (8 months of storage at 20°C)
UFSMP85 (2 months of storage at 20°C)
UFSMP92 (6 months of storage at 20°C)

The Influence of Shear on the Structure and Solubility of Reconstituted UFSMP85

UFSMP85 (8 months of storage at 20°C) solutions (5% w/w in water at 20°C) were sheared at 20 000 rev/min for 60 s in an Ultraturrax T25 or homogenised (Rannie laboratory homogeniser, 100 L/h) at 20°C using either 3.62, 7.25, 14.50 or 21.75 MPa. Solubility, particle size and microstructure were evaluated 60 min after the application of shear.

The Influence of Storage Time and Temperature on the Structure and Solubility of UFSMP85

The microstructure and the solubility of UFSMP85 were evaluated immediately after manufacture and then following storage (sealed foil sachets) for 1, 3, 5, 7, 9 and 12 months at 5, 20 and 30°C. After 14 months of storage at these temperatures, the UFSMP85 samples were reconstituted (5% w/w) in water at 40°C and the change in particle morphology over 7 h was evaluated by particle size analysis and confocal microscopy.

8.2.2 Transmission Electron Microscopy

The powders were dispersed in water at 45°C for 30 min before the addition of glutaraldehyde and the subsequent preparation for transmission electron microscopy (see Chapter 4 for methodology).

8.2.3 Confocal Laser Scanning Microscopy

The UFSMP85 powders were reconstituted at 40°C in water containing 0.01% w/w Fast Green FCF. The solutions were examined using a Leica confocal laser scanning microscope (model TCS 4D, Leica, Heidelberg, Germany) with a rhodamine filter block for excitation of the protein phase.

8.2.4 Particle Size Measurement

The particle size distributions of the reconstituted UFSMP85 solutions were measured using a Malvern MasterSizer E (Malvern Instruments, Malvern, UK). The solutions were added dropwise to the sample unit to obtain an obscuration in the range 20–22%. The presentation code used for the MasterSizer was 2OHD assuming the following optical parameters: a differential refractive index between the dispersed and continuous phases of 1.15, a particle absorbance of 0.1, a dispersed refractive index of 1.53 and a continuous phase refractive index of 1.33.

8.2.5 Solubility

Solutions (50 mL) of the (i) reconstituted powders of different protein concentrations, (ii) reconstituted UFSMP85 before and after homogenisation and (iii) reconstituted UFSMP85 during 12 months storage were transferred to graduated centrifuge tubes and the tubes were centrifuged at 700 g for 10 min. After centrifugation, the total solids of the original solution and the supernatant were determined and the solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Total solids of supernatant}}{\text{Total solids of original}} \times 100$$

8.3 RESULTS

8.3.1 Structure and Solubility of UFSMP Containing Different Protein Concentrations

The structures of the reconstituted milk protein powders are shown in Figures 8.1-8.6.

Skim Milk Powder (6 months of storage at 20°C)

The SMP had completely dispersed into discrete micelles after reconstitution in water for 30 min at 45°C (Figure 8.1). The casein micelles were well dispersed with very little evidence of micelle fusion or micelle aggregation.

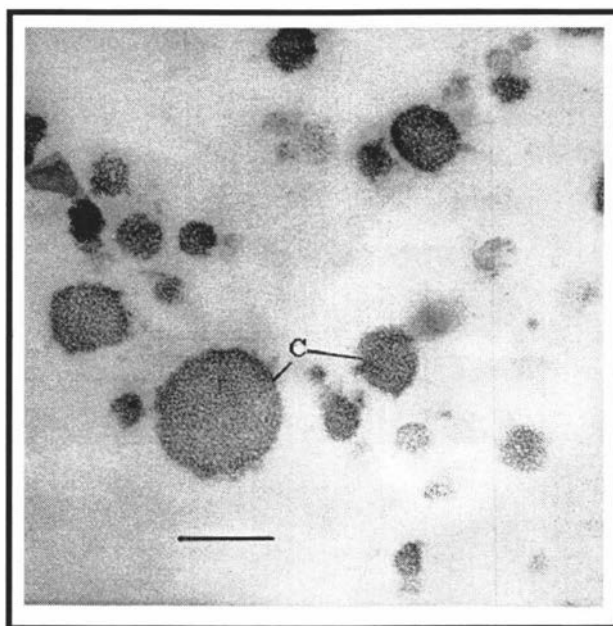


FIGURE 8.1 Transmission electron micrograph of reconstituted SMP; C = casein micelle, bar = 0.2 µm.

UFSMP56 (6 months of storage at 20°C)

The reconstituted UFSMP56 had also dissolved completely after reconstitution in water for 30 min at 45°C (Figure 8.2). The casein micelles appeared as discrete units with only a small number of aggregated micelles evident (arrow).

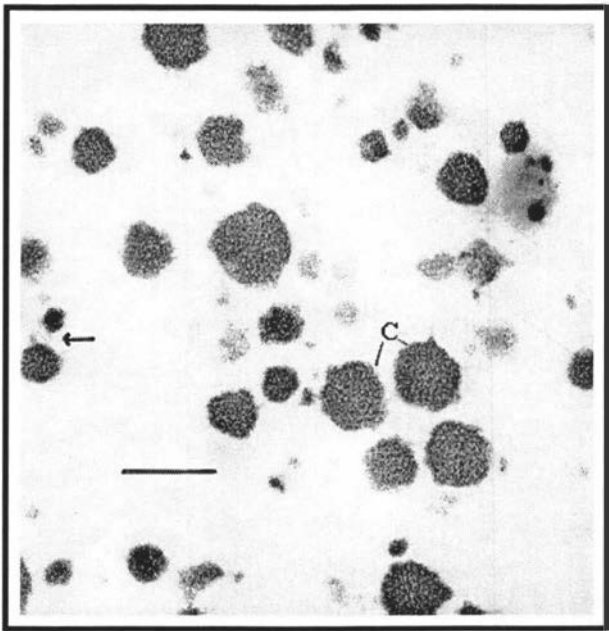


FIGURE 8.2 Transmission electron micrograph of reconstituted UFSMP56; C = casein micelle, bar = 0.2 μ m.

UFSMP70 (6 months of storage at 20°C)

The UFSMP70 particles broke down and dispersed after reconstitution (Figure 8.3). However, the micrographs showed that there were a number of casein micelles that appeared to be strongly aggregated and even fused. It is unknown where in the dried particle the aggregated micelles were located prior to reconstitution.

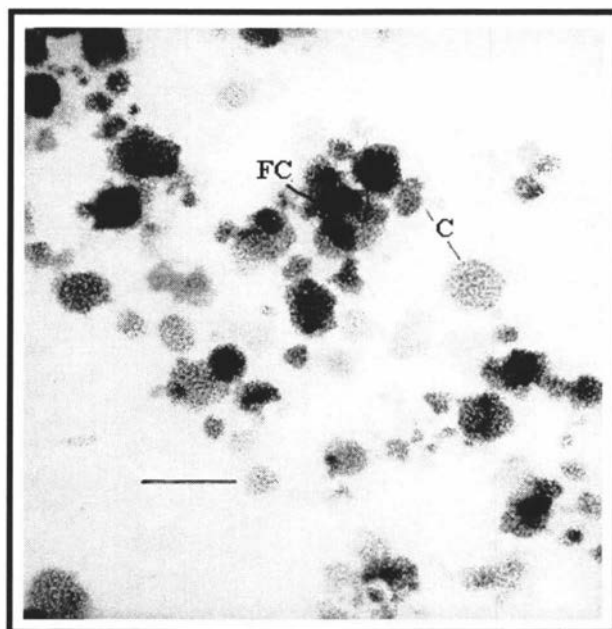


FIGURE 8.3 Transmission electron micrograph of reconstituted UFSMP70; C = casein micelle, FC = fused casein micelles, bar = 0.2 μm .

UFSMP85 (8 months of storage at 20°C)

The thin-sectioned samples showed that the structure of the powder particles was still intact and that little dispersion of the particles had occurred 30 min after reconstitution in water (Figures 8.4a and 8.4b). The structure of the UFSMP particle could be seen as undispersed casein micelles (C) with an obvious layer of aggregated/fused casein micelles (FC) on the outside of the particle (Figure 8.4b).

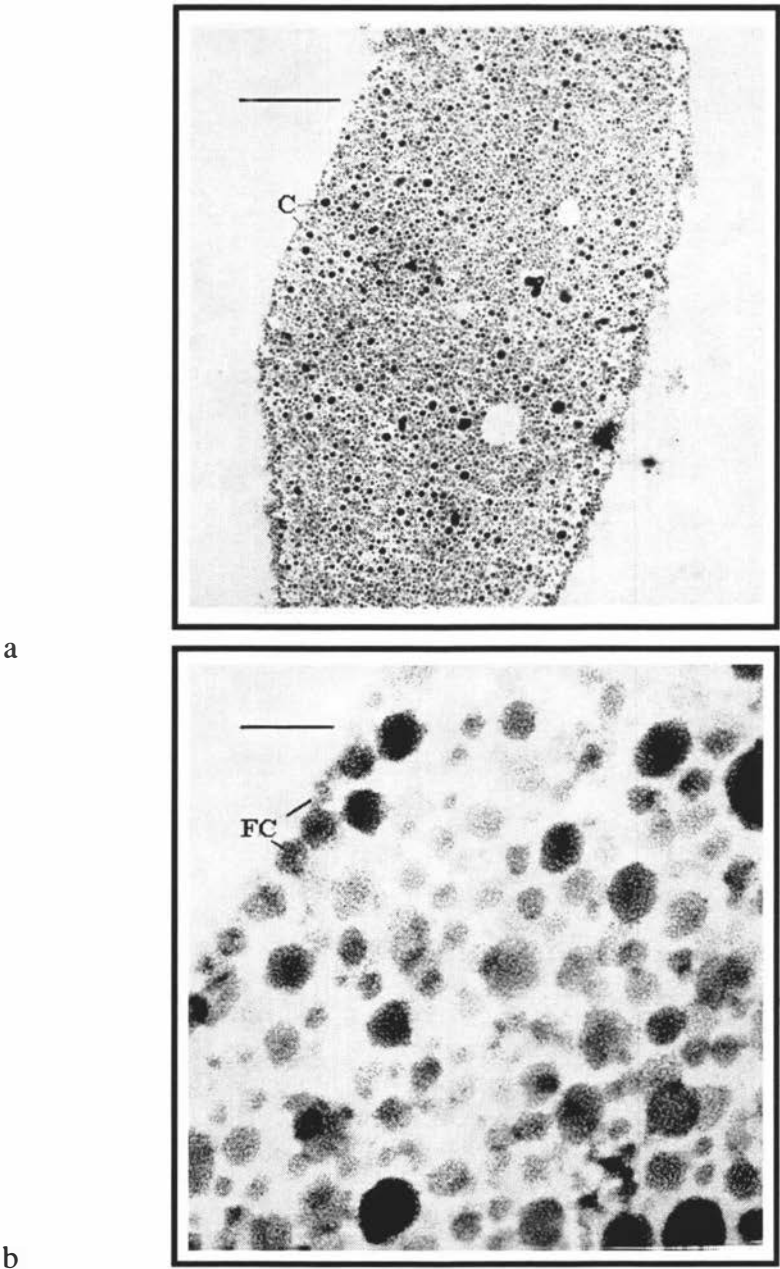


FIGURE 8.4 Transmission electron micrographs of reconstituted UFSMP85 (8 months of storage at 20°C); C = casein micelle, FC = fused casein micelles (a, bar = 2 μm; b, bar = 0.2 μm).

UFSMP85 (2 months of storage at 20°C)

Reconstitution of this powder, which was only 2 months old, resulted in better dissolution than achieved for the 8-month-old UFSMP85 (Figure 8.4). There was less evidence of an intact fused casein micelle skin on the surface of the particle and the micelles appeared to have dispersed slightly upon dissolution (Figures 8.5a and 8.5b).

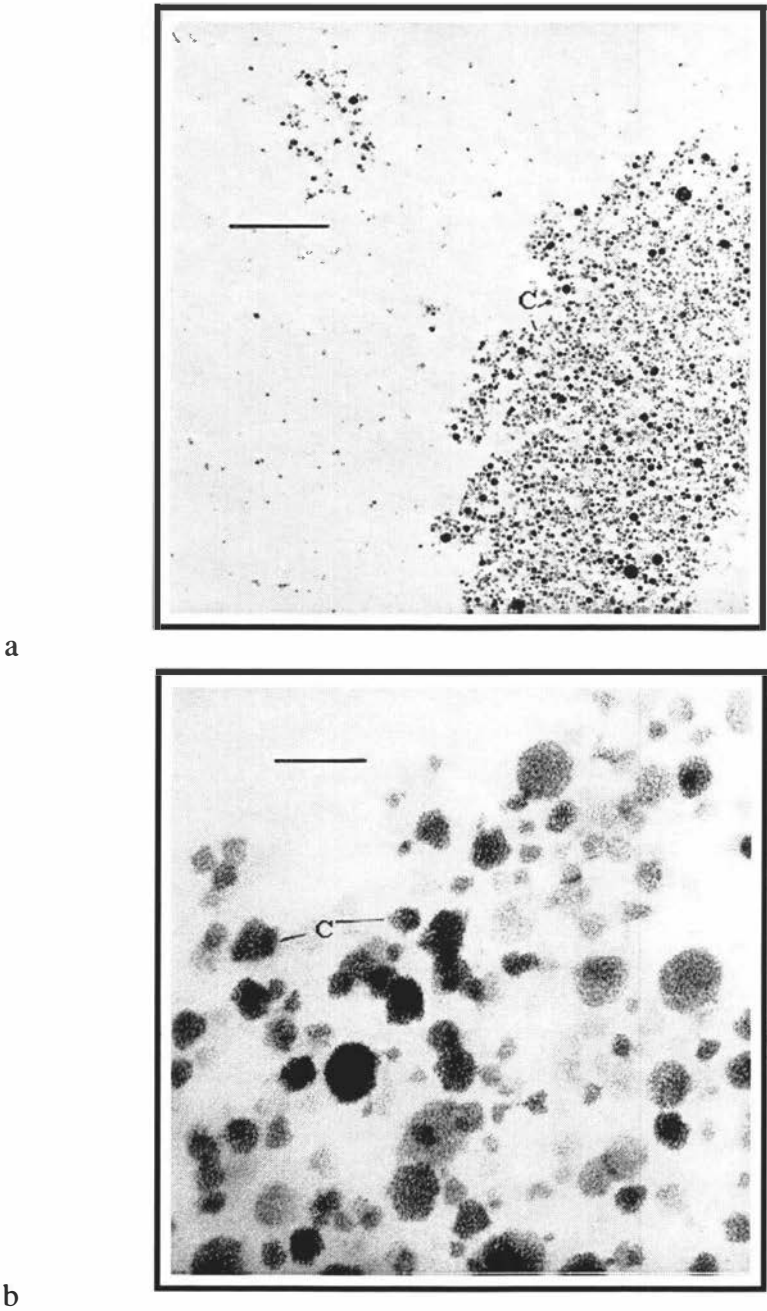


FIGURE 8.5 Transmission electron micrographs of reconstituted UFSMP85 (2 months of storage at 20°C); C = casein micelle (a, bar = 2 μm ; b, bar = 0.2 μm).

UFSMP92 (6 months of storage at 20°C)

The dispersion of this product in water at 45°C was poorer than that of the 8-month-old UFSMP85 (Figure 8.4). This powder (Figures 8.6a and 8.6b) showed a more obvious skin of fused casein micelles over the surface of the powder particle than for any of the other powders evaluated. Also this product did not reconstitute to form a dispersion of casein micelles but retained its particle structure, indicating a resistance to dispersing in water.

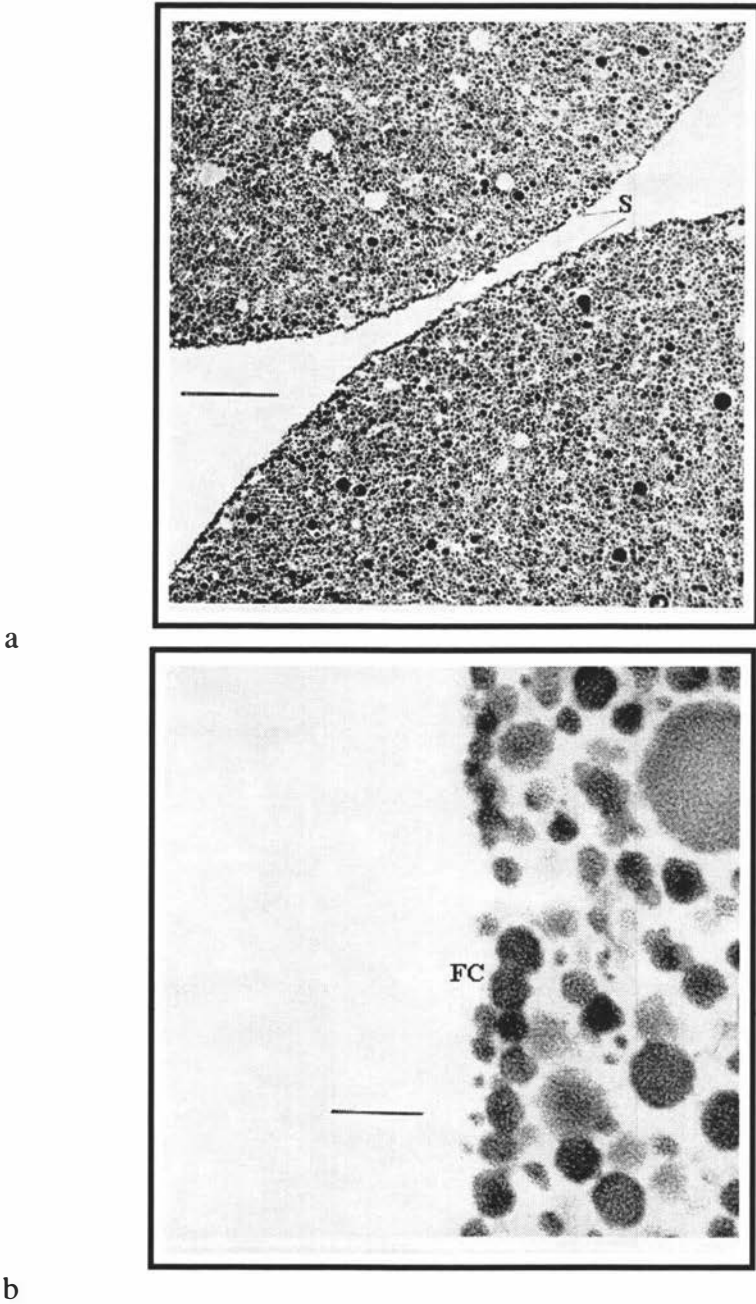


FIGURE 8.6 Transmission electron micrographs of reconstituted UFSMP92; S = skin on the surface of the particle, FC = fused casein micelles (a, bar = 2 μm ; b, bar = 0.2 μm).

The solubility of the milk protein powders decreased as the protein concentration increased (Figure 8.7). The SMP, which had the lowest amount of protein (approximately 35%), showed the highest solubility of 98%. The solubility dropped to 95% for UFSMP56, to 70% for UFSMP70 and to 35% for UFSMP85 (8-month-old powder). UFSMP92 showed the lowest solubility of only 27% in water at 20°C.

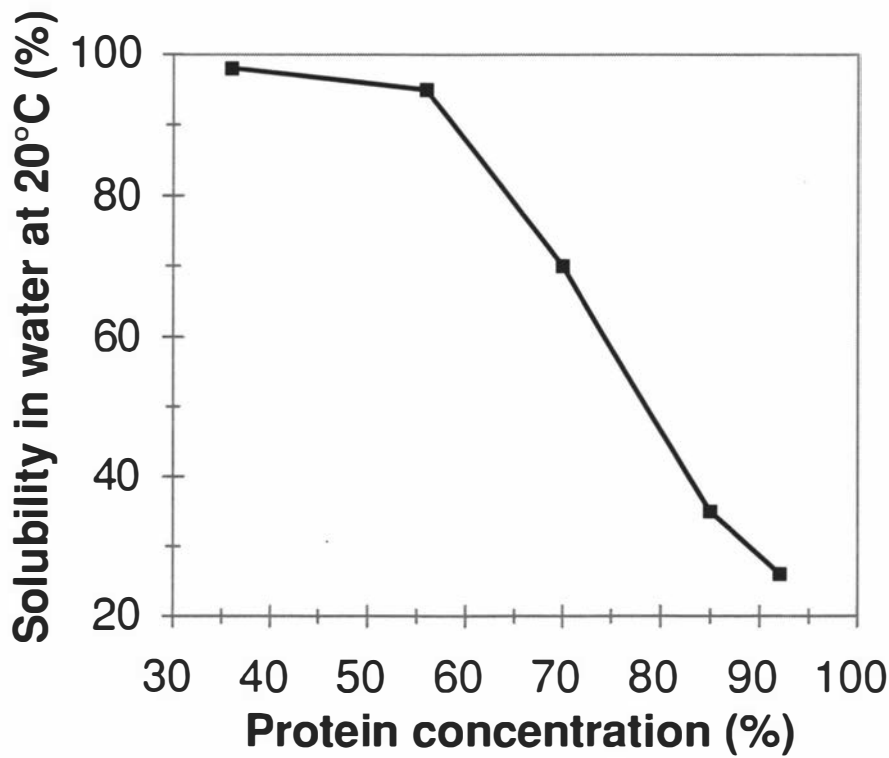


FIGURE 8.7 Solubility of milk protein products reconstituted in water at 20°C.

8.3.2 The Influence of Shear on the Structure and Solubility of Reconstituted UFSMP85

Reconstituted UFSMP85 was sheared (Ultraturrax) or homogenised (high pressure homogeniser) to investigate the strength of the insoluble protein aggregates in UFSMP85. The results for solubility are shown in Figure 8.8. It is clear that the shear applied by the Ultraturrax improved the solubility slightly but that homogenisation at pressures of 14.50 MPa or above was required to reach complete solubility. The reduction in the size of the insoluble aggregates by homogenisation can be seen in the transmission electron micrographs in Figures 8.9 -8.13. The UFSMP85 particles remained completely intact after reconstitution (Figure 8.9). There was no evidence of a skin formed by casein micelle fusion in this sample, as the outside of the particle showed signs of dissolution. The application of shear by the Ultraturrax resulted in the UFSMP85 particles being broken into large fragments, generally a half to a third the size of the intact particle (Figure 8.10). Homogenisation at 3.62 MPa resulted in a significant size reduction. The particle remnants were cross-sectional portions of the UFSMP particle, not just parts of the particle surface (Figure 8.11). Homogenisation at 7.25 MPa (Figure 8.12) and 14.50 MPa (Figure 8.13) resulted in further size reduction of the remnants seen in Figure 8.11. There was an interesting structure in the sample homogenised at 14.50 MPa, *i.e.* the presence of large numbers of bubble ghosts (arrow, Figure 8.13). Such structures have been observed before (Brooker, 1985) and usually occur when protein has been adsorbed to an interface of an air bubble prior to the collapse of this bubble. These bubbles may have been the result of some form of cavitation during homogenisation. The structures may also have been a result of surface skin from internal vacuoles of the UFSMP particle that had not been disrupted by homogenisation.

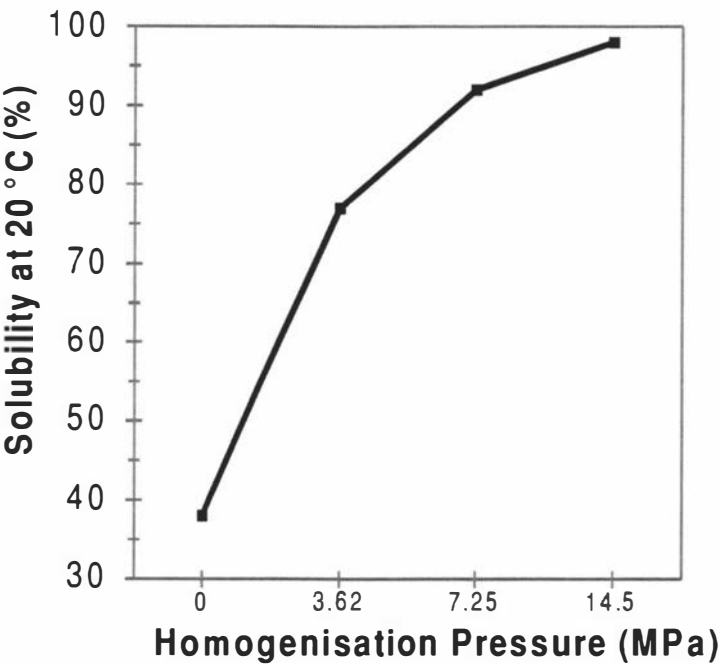


FIGURE 8.8 Solubility of reconstituted (5% TS) UFSMP85 as a function of homogenisation pressure.

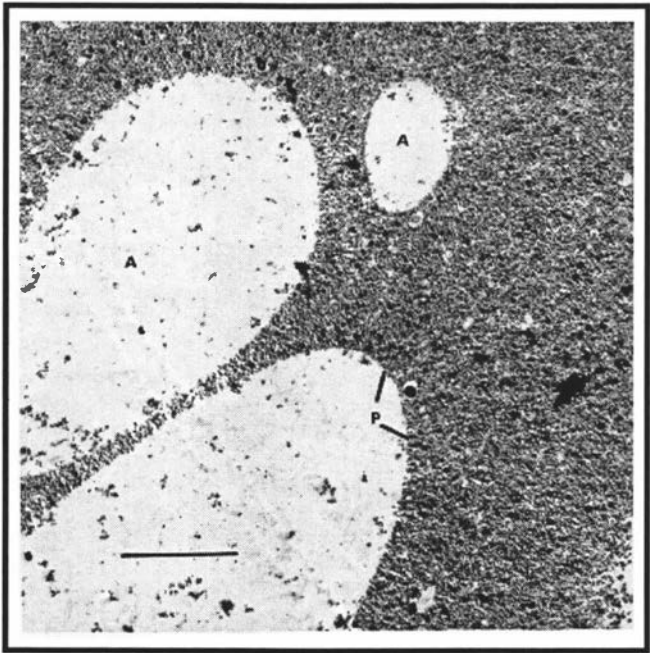


FIGURE 8.9 Transmission electron micrograph of reconstituted UFSMP85 with no shear; A = vacuole, P = particle (intact), bar = 5 μm .

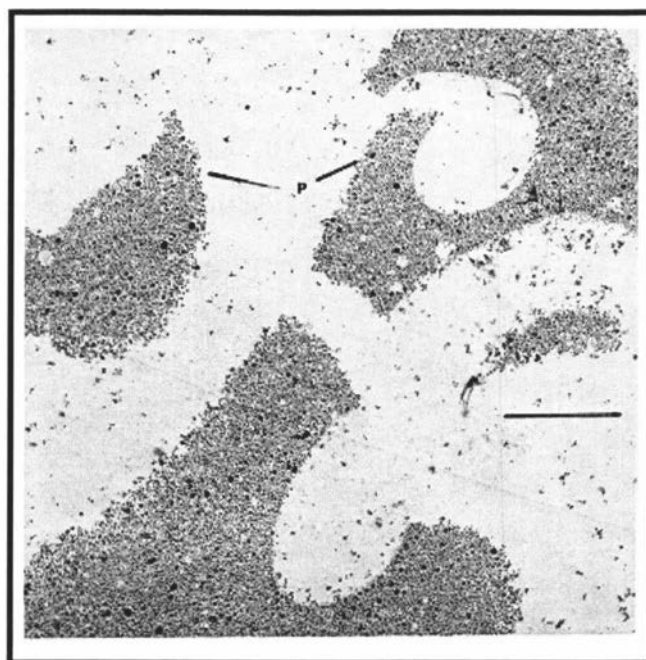


FIGURE 8.10 Transmission electron micrograph of reconstituted UFSMP85 sheared using an Ultraturrax; P = particle (fragments), bar = 5 μ m.

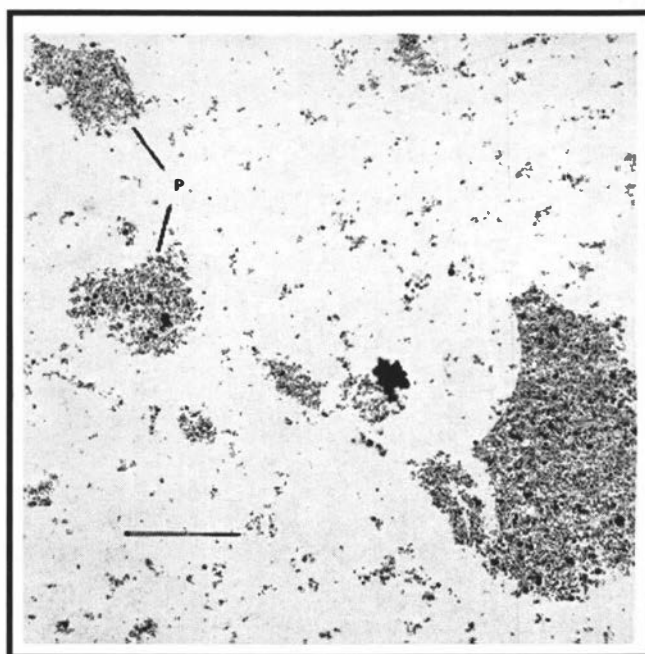


FIGURE 8.11 Transmission electron micrograph of reconstituted UFSMP85 sheared by homogenisation at 3.62 MPa; P = particle (fragments), bar = 5 μ m.

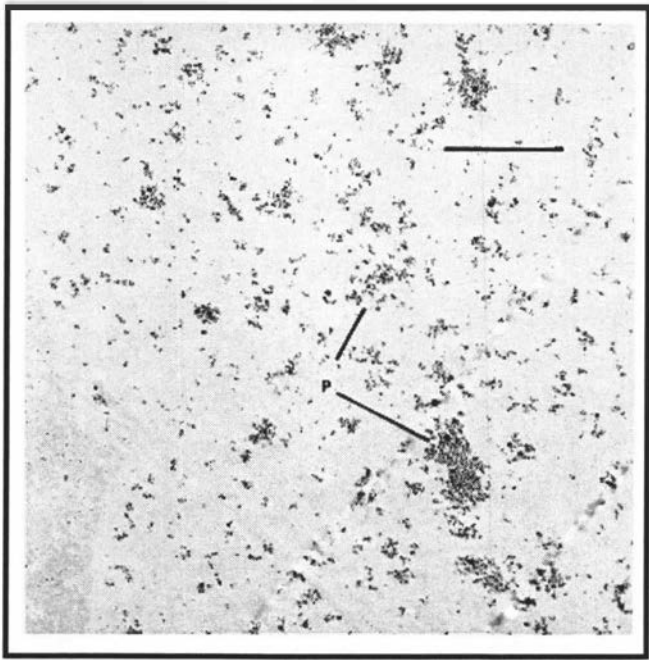


FIGURE 8.12 Transmission electron micrograph of reconstituted UFSMP85 sheared by homogenisation at 7.25 MPa; P = particle (fragments), bar = 5 μm .

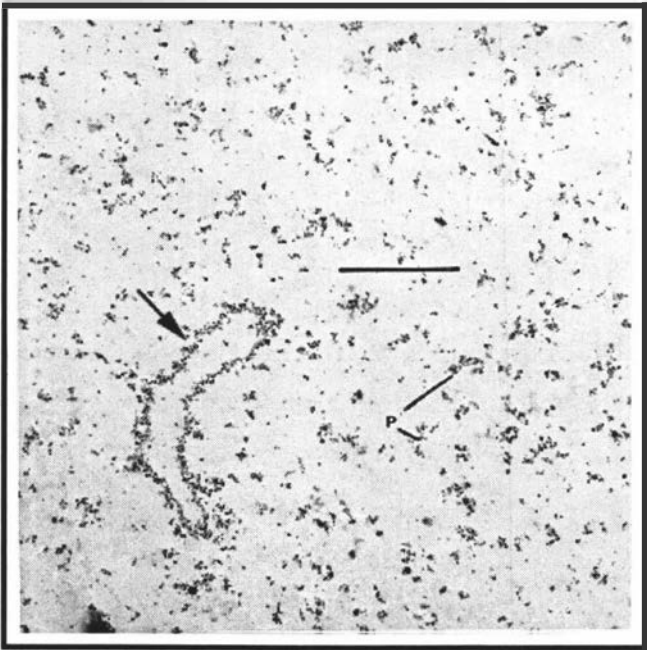


FIGURE 8.13 Transmission electron micrograph of reconstituted UFSMP85 sheared by homogenisation at 14.50 MPa; P = particle (fragments), bar = 5 μm .

8.3.3 The Influence of Storage Time and Temperature on the Structure and Solubility of UFSMP85

Prior to storage, the UFSMP85 powder showed some dissolution upon reconstitution (Figure 8.14). The surface of the particle (large arrow) showed no evidence of a skin. There was some sign of dissolution at the surface as seen by some casein micelles and groups of micelles breaking away from the particle surface. There were also signs of very small structures (small arrow), possibly non-micellar protein aggregates, leaching out of the body of the particle into a vacuole (bubble created during spray drying).

After 3 months of storage of this powder, there were subtle changes to its structure. There was a change to the structure at the interface between the protein phase and the air voids within the UFSMP85 (Figures 8.15 and 8.16) particularly in the sample stored at 30°C. A very fine layer of material had formed at this interface (arrow, Figure 8.16). There was apparently no movement of soluble protein material into the void during reconstitution, indicating that this interfacial layer was probably behaving as a barrier. It is possible that the material that formed this layer at the interface was the same material that had solubilised and was visible in the voids in the unstored UFSMP85 (Figure 8.14).

After 12 months of storage, the UFSMP85 powder particles had visibly altered with the extent of change dependent on the storage temperature. After 12 months of storage at 5°C, there was little change to the outside surface of the particle (Figure 8.17). There did appear to be a band of aggregated non-micellar material at the particle surface similar to that seen within the vacuoles of the unstored UFSMP85. The formation of a skin was obvious in the powders stored for 12 months at 20°C (arrow, Figure 8.18) and at 30°C (arrow, Figure 8.19). The thickness of this skin was greater in the sample stored at 30°C. It is the formation of this skin of aggregated casein micelles (with non-micellar protein perhaps involved) that probably influenced the solubility of the UFSMP85 even when reconstituted in water at 60°C (Figure 8.20). The influence of storage time and temperature on the solubility of UFSMP85 in 20 and 60°C water reflects the observations made by microscopy (Figures 8.14-8.19). The formation of a skin during storage resulted in a reduction in the solubility of UFSMP85. Storage at 5°C resulted in no skin formation after 12 months of storage and as a result there was no further decrease in solubility when the powder was reconstituted in water at 20 or 60°C. However, storage at 30°C for 12 months resulted in the development of a skin and as a result reduced the solubility from 40 to 26% when reconstituted in water at 20°C (Figure 8.20a) and reduced the solubility from 100 to 41% when reconstituted in water at 60°C (Figure 8.20b).

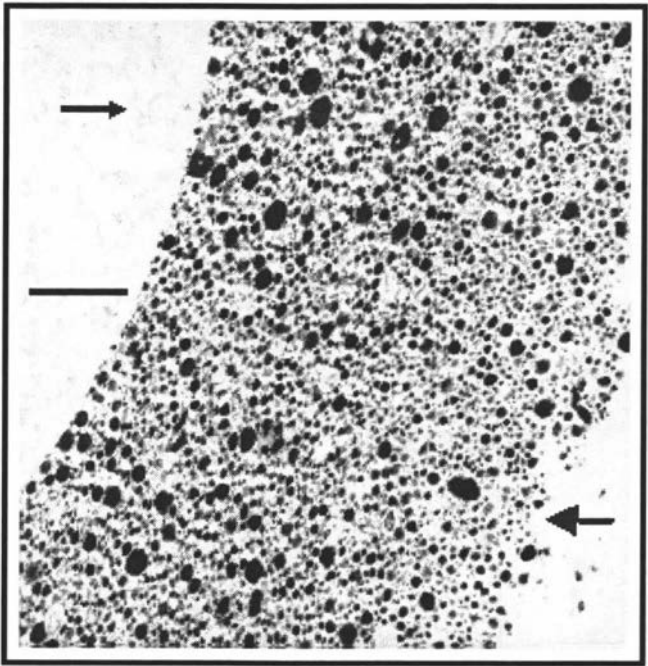


FIGURE 8.14 Transmission electron micrograph of UFSMP85 at the beginning of the storage trial; small arrow shows soluble protein material, large arrow shows the particle surface, bar = 1 μ m.

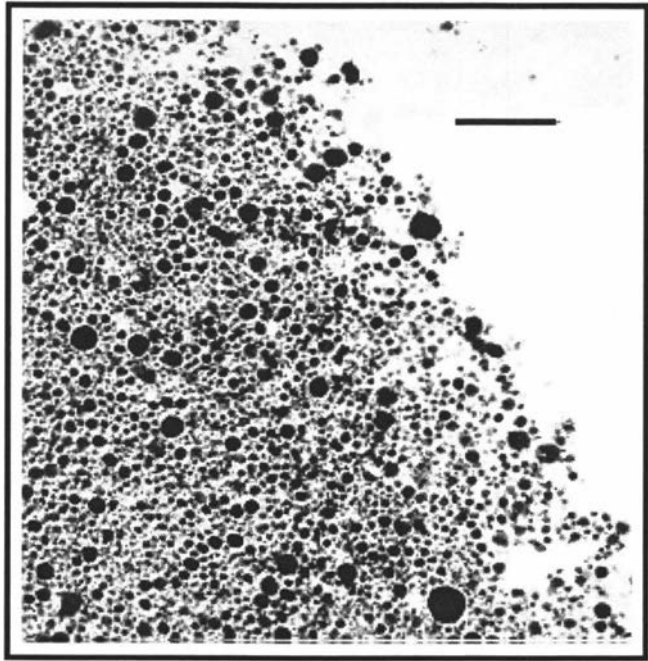


FIGURE 8.15 Transmission electron micrograph of UFSMP85 after 3 months of storage at 20°C; bar = 1 μ m.

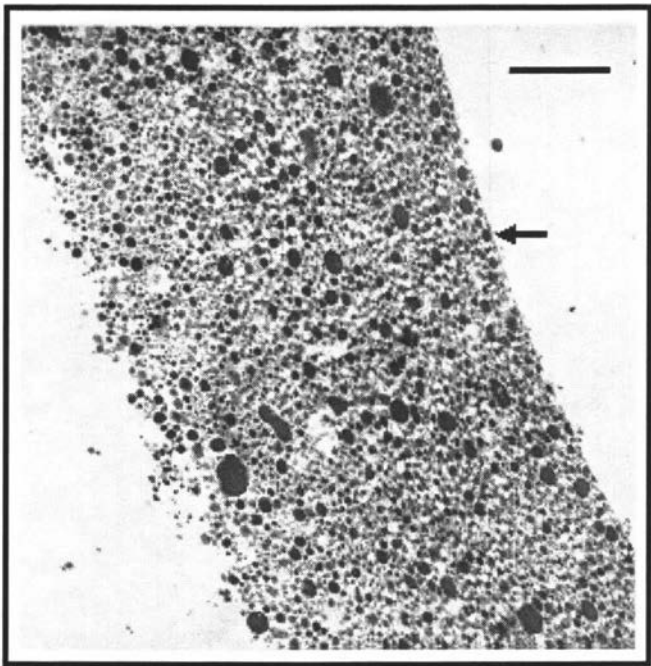


FIGURE 8.16 Transmission electron micrograph of UFSMP85 after 3 months of storage at 30°C; arrow shows a protein monolayer within a void inside the UFSMP particle, bar = 1 μ m.

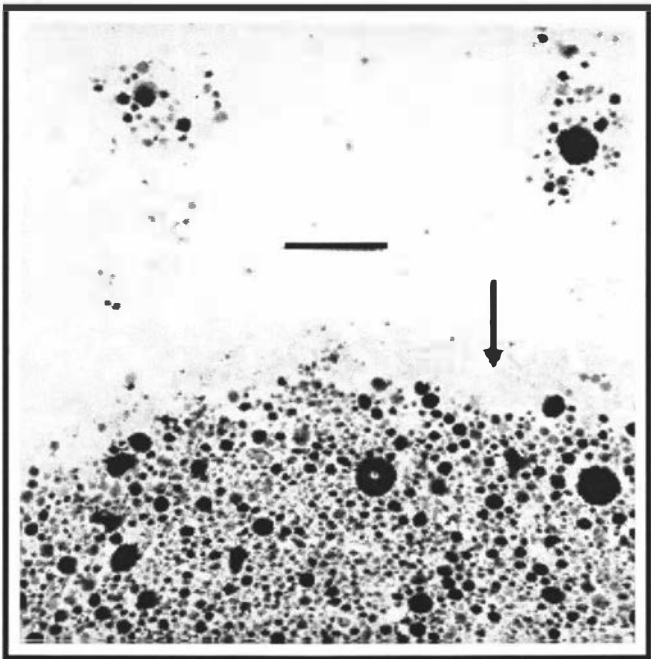


FIGURE 8.17 Transmission electron micrograph of UFSMP85 after 12 months of storage at 5°C; bar = 1 μ m.

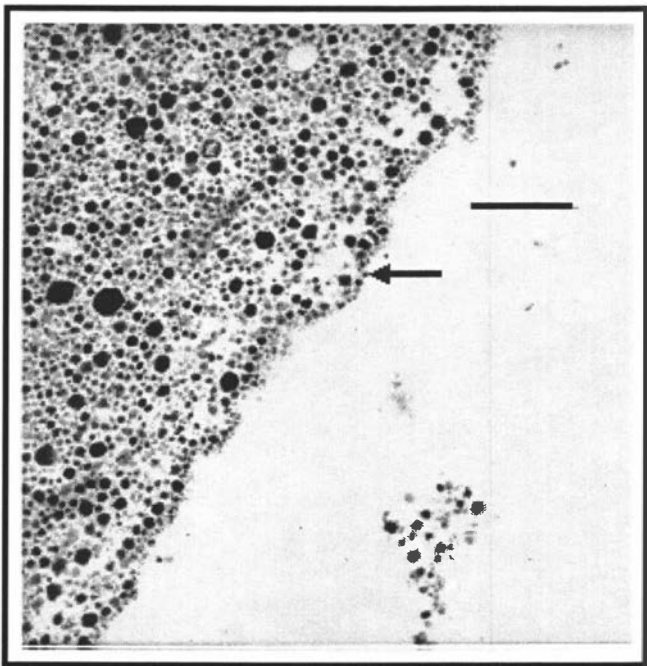


FIGURE 8.18 Transmission electron micrograph of UFSMP85 after 12 months of storage at 20°C; arrow shows the location of the skin layer on the outside of the particle, bar = 1 μm .

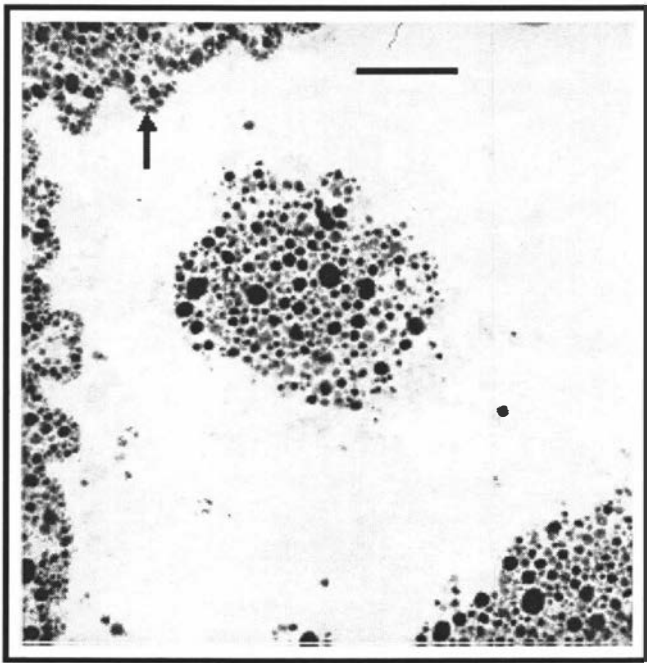
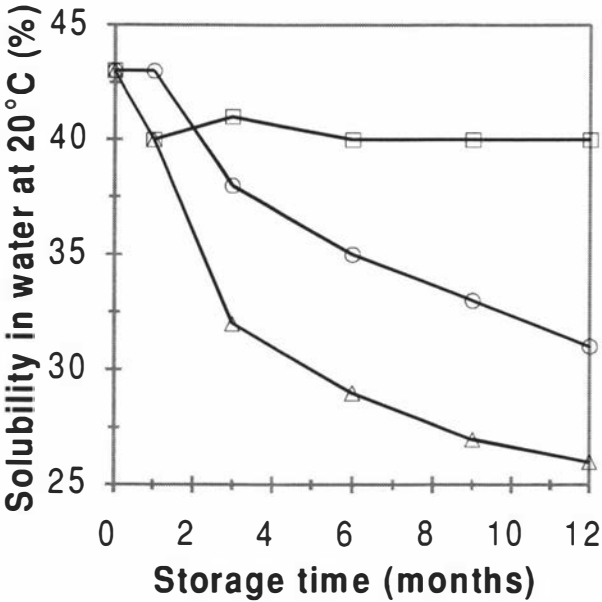


FIGURE 8.19 Transmission electron micrograph of UFSMP85 after 12 months of storage at 30°C; arrow shows the location of the skin layer on the outside of the particle, bar = 1 μm .

a



b

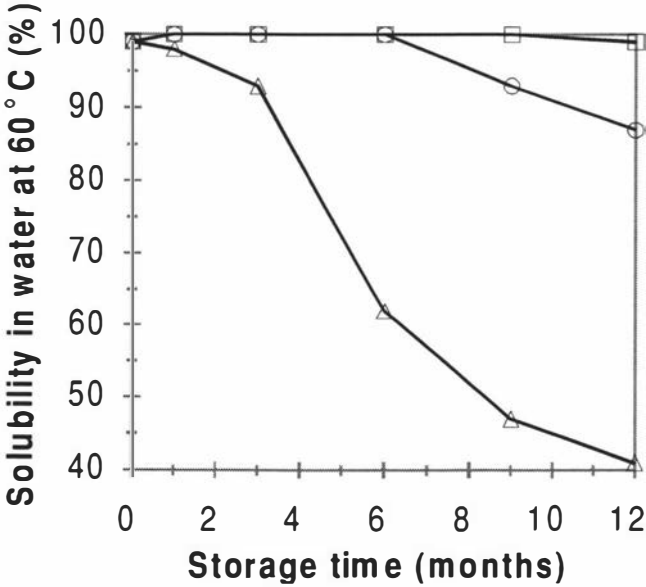


FIGURE 8.20 Change in solubility of UFSMP85 during 12 months of storage at 5 (□), 20 (○) and 30°C (Δ) (a = reconstituted in water at 20°C; b = reconstituted in water at 60°C).

After 14 months of storage, these UFSMP85 powders were reconstituted in water at 40°C (this temperature was used rather than 60°C to better distinguish between the dissolution properties of the powders over the 420 min evaluation time). The breakdown of the powder particles was observed over 300 min by confocal microscopy and after 420 min by particle size analysis.

The micrographs show that the UFSMP85 stored at 5°C for 14 months broke down slowly over the 300 min dissolution time (Figure 8.21). The particles appeared to be partially swollen after 60 min in water with some breakdown of the particle surface at this stage (Figure 8.21 b). There was no sign of any intact particle fragments after 300 min, indicating complete breakdown of the particles to micellar units (Figure 8.21c). The particle size data for this sample showed a gradual breakdown of the particles to the point where the $D(v,0.5)$ was approximately 2 μm after 420 min hydration (Figure 8.23).

The UFSMP85 stored at 30°C for 14 months and then reconstituted in water at 40°C did not break down into micellar units within 420 min (Figure 8.23). This powder swelled to a greater size but its outer skin resisted particle breakdown as shown by the confocal micrographs (Figure 8.22). The powder stored at 20°C had dissolution properties similar to those of the powder stored at 30°C, indicating that it too had formed a skin that resisted particle breakdown.

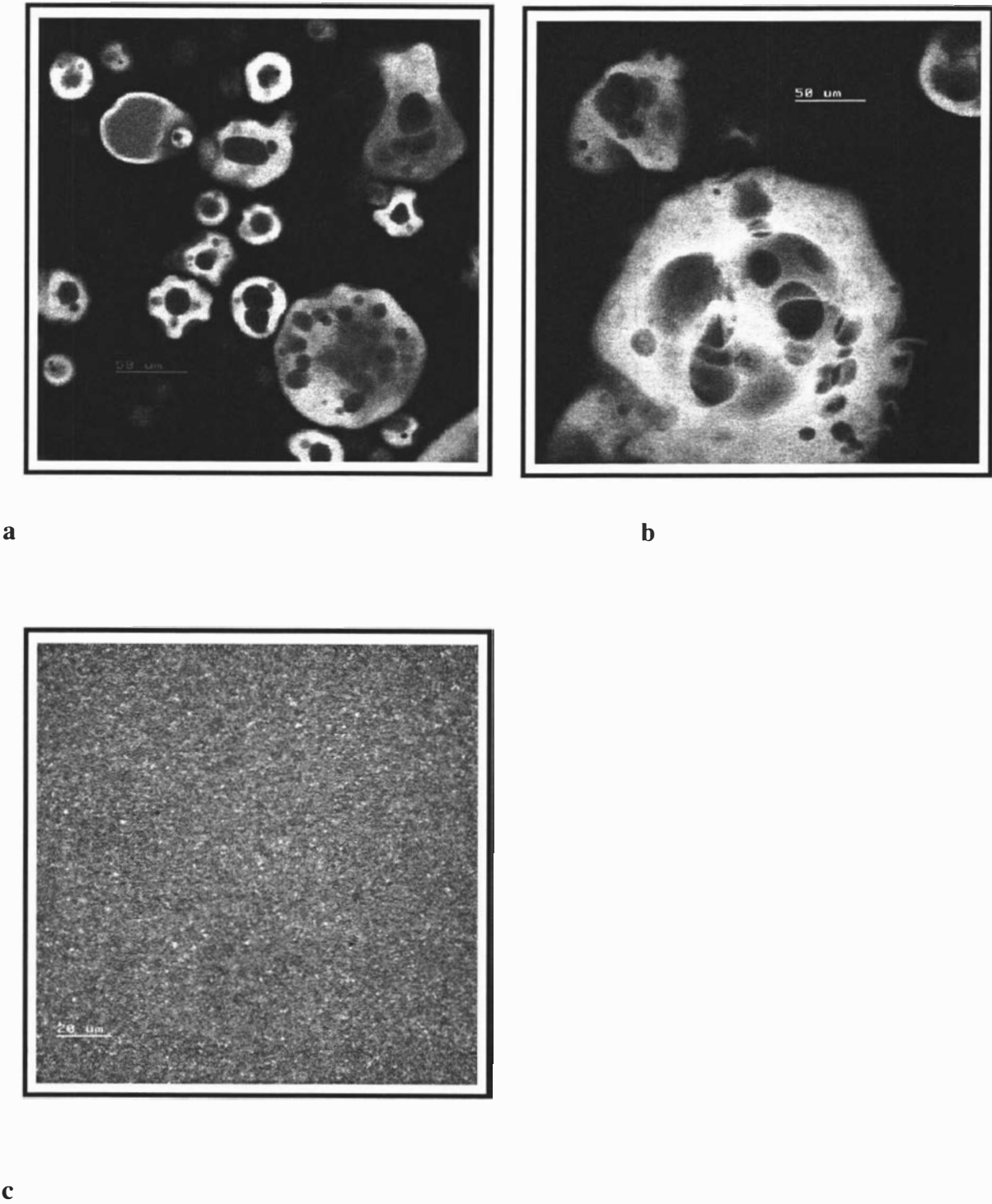
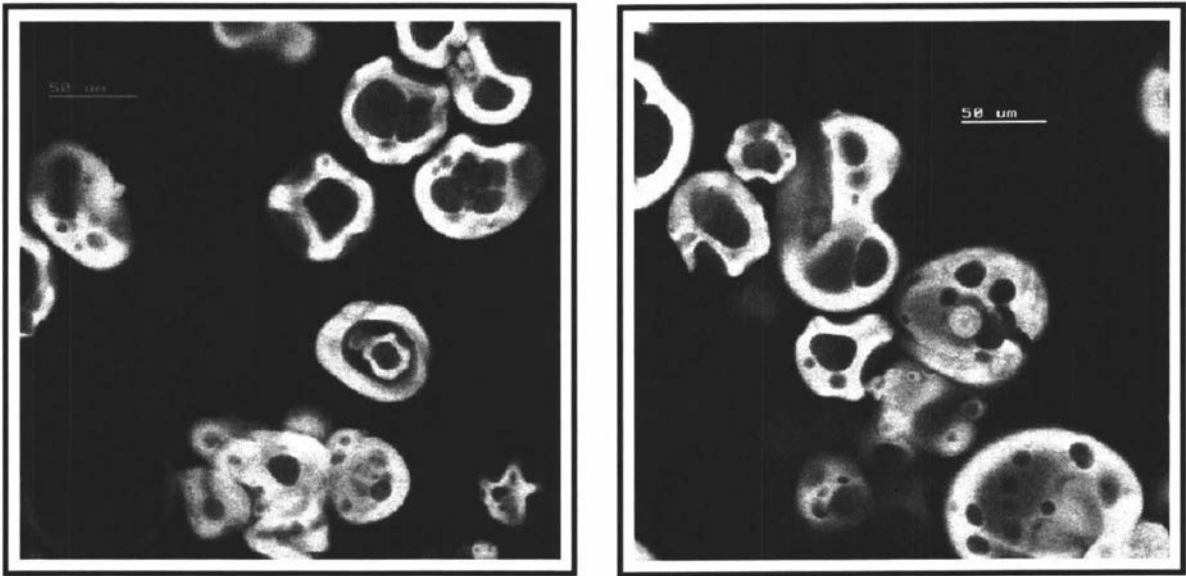
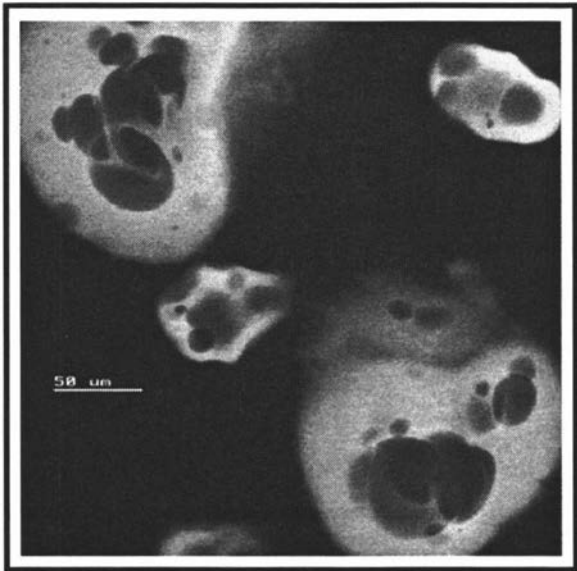


FIGURE 8.21 Confocal micrographs of UFSMP85 after 14 months of storage at 5°C (a = immediately after reconstitution; b = 60 min after reconstitution; c = 300 min after reconstitution).



a

b



c

FIGURE 8.22 Confocal micrographs of UFSMP85 after 14 months of storage at 30°C (a = immediately after reconstitution; b = 60 min after reconstitution; c = 300 min after reconstitution).

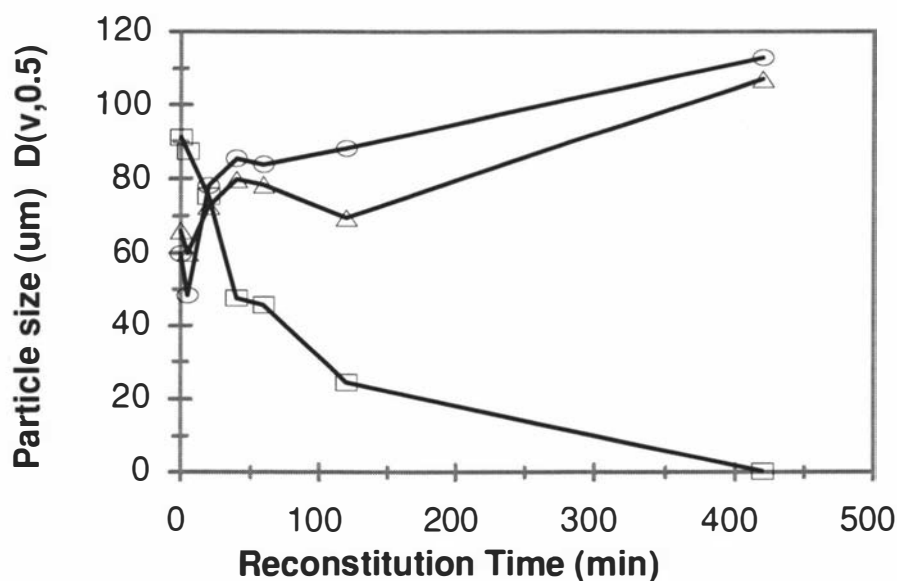


FIGURE 8.23 Changes in average particle size ($D(v,0.5)$) over 7 h of reconstitution (40°C) of UFSMP85 after 14 months of storage at 5°C ($\square$), 20°C ($\circ$) and 30°C (Δ).

8.4 DISCUSSION

Ultrafiltration followed by diafiltration is used to achieve the high protein contents in UFSMPs with protein concentrations above 80%. These processes have been shown to cause changes in the composition of skim milk (Chapter 7). It is likely that the interactions that occur during the manufacture and storage of UFSMPs with high protein contents are caused by a combination of factors.

The microstructural examination of the milk protein powders showed that there was an increase in the interaction between casein micelles with an increase in the protein concentration of the powder. The SMP dispersed well with no apparent interaction between the casein micelles (Figure 8.1). SMPs generally disperse very well and have few solubility problems.

There was some interaction between the casein micelles in UFSMP56 (Figure 8.2). This interaction appeared to involve hairy attachments, similar to those seen by Creamer & Matheson (1980) and which are considered to be denatured whey protein complexed (disulphide linked) to κ -casein on the micelle surface (Singh & Fox, 1985, 1986; Singh & Creamer, 1991). It is perhaps surprising to see these attachments in UFSMP56 as the level of denatured whey protein was low given the mild heat treatment applied during manufacture. However, it is conceivable that changes during the ultrafiltration process may have rendered the whey proteins more susceptible to this kind of interaction. The reconstituted UFSMP70 showed a considerable

increase in casein micelle interaction (Figure 8.3), as seen by the presence of protein aggregates. The interaction between these micelles was of a different kind from that seen for UFSMP56. The casein micelles were actually touching and had fused by means of some type of protein-protein or protein-mineral interaction. The poor solubility of UFSMP85 was immediately apparent from inspection of Figures 8.4 and 8.5. Large parts of the particles remained completely intact even after 30 min in water at 45°C. There may have been some resistance to dispersion of these powders, given that they were reconstituted at the same total solids concentration as for the other powders and were therefore at a higher protein concentration in solution. However, this probably did not influence the dissolution of the UFSMP85 that contained an almost complete layer of fused casein micelles around the outside of the particle (Figure 8.4b). The layer of fused micelles extending over the outside of the UFSMP and micellar casein (Figure 8.6a) powder particles probably behaved like a tough skin inhibiting the movement of water into the particle.

The UFSMP85 storage trial showed the development of the skin on the surface of the particle with storage time and increasing storage temperature. The presence of the protein skin developed during storage at 20°C and above was responsible for the reduction in UFSMP85 powder solubility. The confocal micrographs (Figure 8.22) showed that the uptake of water into the particle was severely hindered in the powders stored at 20 and 30°C because of the skin that had developed. Water was able to penetrate into the UFSMP85 particles that had been stored at 5°C (Figure 8.21) because no noticeable skin had developed at this temperature. The thickness of the skin surrounding the particle probably increased with the age of the powder particularly at 30°C. During ultrafiltration, the concentration of the protein and the removal of lactose result in micelles becoming more closely packed together. At or near the surface of a newly dried particle, the micelles are forced together by their adsorption to the particle-air interface and may even touch (micelles away from the particle surface are not in such close proximity). With such close contact and a reduction in steric repulsion (because of the depletion of κ -casein during concentration) further protein-protein interactions may increase protein aggregation or cross-linking of this outer micelle layer during storage. It is possible that protein and lactose interactions, at the particle surface, may cause protein polymerisation of the closely packed casein micelles. The phenomenon of fused casein micelles at an interface has also been shown to occur on the fat globule membrane in homogenised concentrated whole milk (Chapter 2). Holding the concentrate at 85°C resulted in an increase in protein-protein interactions between the closely packed casein micelles and probably whey protein adsorbed at the fat globule interface. Given that there is a similar adsorption of casein micelles to the concentrate-air interface during the spray drying of UFSMP, it is likely that heat and time will also result in a similar thickening of this protein-protein skin during drying and storage. Therefore, any reduction in the time the powder particles spend at temperatures above a reasonable storage temperature, say 20°C, would probably reduce the rate at which protein-protein interactions occur.

The cold solubility of UFSMP85 was seen to improve with the application of shear (Figures 8.9-8.13). However, it was interesting to note that this improved solubility was only the result of the particles being mechanically reduced to a size that stayed suspended after centrifugation. The micrographs of these sheared solutions showed that the particles were fractured during the shearing process (Figures 8.10 and 8.11) but the newly exposed surfaces did not result in any further dispersion of the internal structure. This clearly showed that interactions not related to

the surface skin were having a major influence on the poor solubility of UFSMP85 in water at 20°C.

The initial poor cold solubility of UFSMP85 is probably related to the strong interactions between the non-micellar protein components (possibly aggregates of β -lactoglobulin and κ -casein) and the casein micelles (see Chapter 7). Evidence of these interactions can be seen in Figures 8.4b and 8.5b. The dissociation of κ -casein from the micelle would result not only in a loss of micellar charge and an increase in the ability of the micelles to fuse (particularly at interfaces where they are packed closely together) but also probably in an increase in protein-protein interactions between the proteins dissociated from the micelles and the micelles themselves (probably mediated by the presence of calcium). It has been reported that lactose has a heat-stabilising influence on proteins (Plock & Kessler, 1992). As lactose is significantly reduced during ultrafiltration and diafiltration, from 48% on a solids basis in skim milk to just 4% on a solids basis after evaporation (Chapter 7), it is perhaps not surprising that there is an increase in protein-protein interactions even at relatively low temperatures during manufacture.

8.5 CONCLUSIONS

The major factor influencing the dissolution properties of UFSMP85 was probably protein-protein and protein-mineral interactions during manufacture and storage of the powder. The poor cold water solubility of freshly made UFSMP85 was probably related to the strength of the bonds between non-micellar protein (β -lactoglobulin and κ -casein aggregates) and the casein micelles. The aggregation of the material within the particle may be strongly influenced by the state of the calcium in and around the micelles. The cold insoluble UFSMP particles could be solubilised by shear only after homogenisation at a pressure of at least 14.50 MPa. During storage of the powder, the micelles at the particle surface fused as a result of protein-protein interactions to form a skin that covered the entire particle surface. This skin appeared to be very resistant to dissolution in water even at 45°C. Storage of the powder at above 20°C for 6 months or more resulted in the formation of an almost complete layer of fused casein micelles around the outside of the particle. The formation of this layer may have been promoted by the presence of β -lactoglobulin and other non-micellar protein units.

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CHAPTER 9

THE INFLUENCE OF SKIM MILK pH ON MICELLE STRUCTURE AND PROTEIN DISTRIBUTION BEFORE AND AFTER ULTRAFILTRATION

9.1 INTRODUCTION

The interactions that influence functionality of UFSMP depend on the state of the protein within the milk being processed. This can be influenced by a number of processing and compositional factors including pH, ionic strength, calcium concentration and calcium equilibrium.

Casein micelles within milk are composed of casein proteins that are held together in an amorphous structure by minerals, mainly calcium and phosphate, referred to as colloidal calcium phosphate (CCP). It is well known that changes in the pH of milk will affect the mineral equilibria of the casein micelle and the milk serum and then will influence the protein dissociation from the micelle (van Hooydonk *et al.*, 1986; Nieuwenhuijse *et al.*, 1991). Therefore it is likely that at different pH values, the morphology of the casein micelles will alter, thus changing the ratio of soluble and micellar proteins. Concentrating milks of different pH values by ultrafiltration (UF) may result in changes to the micellar structure and composition of the retentate. It was found by St-Gelias *et al.* (1992) that diafiltration at pH 5.3 resulted in protein concentrates with the highest protein content and the lowest ash content and that under these conditions 78.3% of the calcium, 99% of the phosphorus, 75% of the magnesium, and 95% of the potassium were removed from the skim milk. Adjusting the pH of ultrafiltered retentate prior to spray drying resulted in an increase in the ash content and the solution viscosity but had little effect on the powder physical properties (El-Samragy *et al.*, 1993). The improvement in the dispersibility and solubility properties of micellar-casein-rich milk powders cannot result from anything other than a modification of the biochemical composition of the products introduced into the drying tower (Schuck *et al.*, 1994).

Preliminary work was undertaken to determine the influence of altering the pH of skim milk between 5 and 8 on the micellar structure and composition of the milk retentate after UF. No attempt was made to manufacture and examine retentate powders from this work.

9.2 EXPERIMENTAL

Skim milk was obtained from Kiwi Co-operative Dairies (Hawera, New Zealand), divided into 12 x 350 mL aliquots and subjected to the following pH adjustments: by the addition of 10% w/v sulphuric acid to pH 5.0, 5.25, 5.5, 5.75, 6.0, 6.5; by the addition of 10% w/v NaOH to pH 7.0, 7.25, 7.5, 7.75, 8.0; a control milk with no pH adjustment (pH 6.75).

UF was carried out using Amicon filtration units with PM10 (10 000 D) filters at a temperature of 10°C over approximately 24 h (Table 9.1) to achieve a concentration of 2.33 times from 350 mL to 150 mL.

The pH values of the skim milks after adjustment and UF are shown in Table 9.1.

TABLE 9.1 pH values of skim milks and ultrafiltrates

Target pH	Quantity of Acid/Alkali (mL)	Actual pH	UF Time (h)	Final pH of Retentate after UF
Control skim milk	None	6.78	29	6.74
pH 5.00	7.75 mL 10% H ₂ SO ₄	4.96	27	5.02
pH 5.25	6.20 mL 10% H ₂ SO ₄	5.25	25	5.47
pH 5.50	5.05 mL 10% H ₂ SO ₄	5.5	22	5.85
pH 5.75	3.05 mL 10% H ₂ SO ₄	5.75	23	5.98
pH 6.00	1.80 mL 10% H ₂ SO ₄	6	25	6.24
pH 6.50	0.45 mL 10% H ₂ SO ₄	6.48	20	6.6
pH 7.00	0.55 mL 10% NaOH	7	21	7.03
pH 7.25	1.05 mL 10% NaOH	7.29	26	7.18
pH 7.50	1.45 mL 10% NaOH	7.5	28	7.51
pH 7.75	1.70 mL 10% NaOH	7.77	47	7.62
pH 8.00	2.00 mL 10% NaOH	8.02	27	7.9

There is no obvious explanation for the increased UF time for the pH 7.75 sample. It is possible that for some reason the membrane used for this sample may have been defective in some way, with the pores being blocked.

A 4.5 mL sample was collected for microstructure analysis prior to and after UF. Sodium azide was added to the initial control skim milk and the skim milk retentates prior to their evaluation by the following analyses.

9.2.1 **Proximate Analyses**

The total nitrogen, fat, ash and lactose contents were measured as described in Chapter 6. Total solids, calcium and non-casein nitrogen were measured as described in Chapter 7.

Total phosphorus (mmol/kg)

The samples were acid digested and the resulting phosphate was determined colorimetrically at 820 nm using molybdenum blue.

Sodium (mmol/kg)

The sodium concentration was measured by flame emission photometry at 589 nm.

Non-protein nitrogen (% w/w)

The sample was dissolved in 12% trichloroacetic acid and the nitrogen content of the resulting filtrate was analysed using a Kjel-Tec.

9.2.2 Soluble Caseins and Whey Proteins

The content of soluble (non-micellar) individual proteins (α -lactalbumin, β -lactoglobulin, α_s -, β - and κ -caseins) in the skim milk retentates was measured by analysing the supernatant and pellet using SDS PAGE after ultracentrifugation (Beckman L2-65 ultracentrifuge with Ti80 rotor, Beckman Instruments Inc., Palo Alto, CA, USA) at 33 000 rev/min (63 000 *g* average) for 60 min at 20°C. The ultracentrifugation conditions applied here were not as strong as those applied in Chapter 7 so that more of the non micellar protein aggregates could be measured in the supernatant. Electrophoresis was performed on a mini-gel slab electrophoresis unit (Biorad Laboratories, Richmond, CA, USA), as described by Andrews (1983).

SDS-PAGE. The resolving gel contained 0.1% SDS and a 2.65:1.25:1 ratio of 30% (w/v) acrylamide (containing 2.6% Bis), resolving buffer (18.15% (w/v) Tris buffered to pH 8.8 with 6 M HCl) and milli-Q water. The stacking gel was composed of 0.1% SDS and a 1.3:2.5:6.1 ratio of 30% (w/v) acrylamide (containing 2.6% Bis), the stacking buffer (6% Tris buffered to pH 6.8 with 6 M HCl) and milli-Q water. Samples were dispersed (supernatant 1:40, pellet 1:250) in SDS sample buffer (2.0% SDS (w/v), 13% stacking buffer (v/v), 0.05% β -mercaptoethanol (reduced SDS gels only) with 0.01% bromophenol blue) and were heated (reduced SDS gels only) to 100°C for 4 min.

The gels were run at 210 V and 70 mA for approximately 1 h and then stained with Coomassie Brilliant Blue (R-250) in 25% v/v isopropanol and 10% v/v acetic acid for 1 h. This was followed by two destaining steps with 10% v/v acetic acid and 10% v/v isopropanol for a total of 20 h. Immediately after destaining, the gels were scanned on a computing laser densitometer (Molecular Dynamics model P.D., Sunnyvale, CA, USA) and the integrated intensities of the protein bands were determined using the Molecular Dynamics ImageQuant software. The results were corrected for the small differences caused by dilution. Each gel contained the control sample so that the results could be normalised over multiple gels and expressed as absorbance volumes for each protein within each gel type.

9.2.3 Transmission Electron Microscopy

Skim milk and ultrafiltered retentate samples were prepared for transmission electron microscopy (TEM) by the methodology described in Chapter 6.

9.2.4 Immunolabelling

Immunolabelling for the localisation of β -lactoglobulin and κ -casein was carried out on the retentates made from the control skim milk and the skim milks adjusted to pH 5, 6, 7, 7.5 and 8.0 as described in Chapter 6.

9.2.5 Particle Size Analysis by Photon Correlation Spectroscopy

Size measurements of the particles in the skim milk and retentate samples were made by photon correlation spectroscopy as described in Chapter 7. The skim milk and retentate samples were dispersed in their respective permeates obtained from the UF stage and allowed to stand for at least 10 min before the particle size was determined. The results presented are the cumulant z-average and the percentage volume size distribution each based on 4 sets of data.

9.3 RESULTS

There was an increase in the pH of the acidified skim milks during UF of between 0.12 and 0.35 pH units (Table 9.1). The only acidified sample that did not show a pH increase during UF was that acidified to pH 5.

The results of the chemical analyses carried out on the skim milk control and the retentates produced from the pH-adjusted skim milks are presented in Table 9.2.

TABLE 9.2 Composition of skim milk and retentates

Sample	Fat (% w/w)	Protein (% w/w)	Phosphorus (mmol/kg)	NCP* (% w/w)	Ca (mmol/kg)	Sodium (mmol/kg)	Ash (% w/w)	TS* (% w/w)	Lactose (% w/w)
Control skim milk	0.17	3.61	30.95	0.835	32.95	20.5	0.79	9.49	4.99
Control permeate	0	0.01	10.3	0.197	7.5	15.8	0.45	4.66	4.34
Control retentate	0.35	8	60.8	1.716	63.4	21.1	1.15	14.91	5.48
pH=5.00	0.35	7.99	37.7	1.104	39.2	19.1	0.9	15.25	6.05
pH=5.25	0.39	8.08	41.4	1.518	42.2	20.1	0.92	15.05	5.78
pH=5.50	0.4	8.45	46.6	1.518	50.6	21.1	0.92	15.61	5.76
pH=5.75	0.39	8.28	49.8	1.474	56.6	21.1	1.01	15.56	5.78
pH=6.00	0.32	7.41	53.8	1.34	56.5	22.3	1.06	14.26	5.48
pH=6.50	0.37	7.85	48.7	1.563	62.1	21.88	1.15	14.65	5.63
pH=7.00	0.36	7.6	53.05	1.512	62.8	25.95	1.16	14.18	5.38
pH=7.25	0.34	7.36	52.7	1.448	60.9	30.3	1.18	14.03	5.48
pH=7.50	0.33	7.25	53.7	1.448	59.2	33.7	1.2	13.87	5.41
pH=7.75	0.37	7.66	55.85	1.703	62.8	36.3	1.38	14.37	5.45
pH=8.00	0.36	7.6	56.8	1.627	62.6	41.6	1.29	14.21	5.42

* NCP - Non-casein protein.
*TS - Total solids.

9.3.1 Changes in the Protein and Mineral Content of Retentate from Ultrafiltered pH-adjusted Skim Milk

The increase in sodium between pH 7 and pH 8 (Figure 9.1) was mainly a result of sodium hydroxide addition to effect the pH adjustment in the skim milk samples. To achieve pH 8, 2.0 mL of 10% w/v NaOH was added to 350 mL of skim milk. This is equivalent to 14 mmol of additional sodium per kilogram of skim milk. The results in Table 9.2 show that sodium is lost during UF, *i.e.* the skim milk control contained 20.5 mmol/kg of sodium whereas the 2.33 times concentrated retentate contained only 21.1 mmol/kg of sodium rather than 47.8 mmol/kg (a 56% loss). If a similar sodium loss is applied to the pH 8 skim milk (containing 14 mmol/kg of additional sodium) then 36 mmol/kg of sodium would be expected to be found in the retentate. The actual concentration of sodium in the pH 8 retentate was 41 mmol/kg. This result shows that the increase in sodium concentration in the retentate with increasing pH (Figure 9.1) was related to the sodium hydroxide used to effect the pH adjustment in the skim milk.

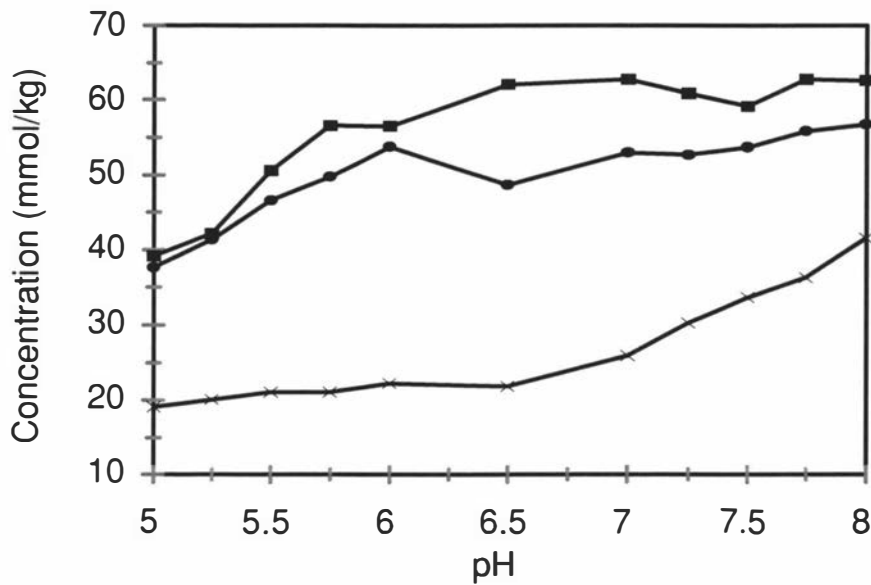


FIGURE 9.1 Influence of skim milk pH on the concentrations of ●- phosphorus, ■- calcium, x- sodium in the UF retentate.

There was a reduction in calcium and phosphorus from 63 and 57 mmol/kg respectively in the pH 8 retentate to 39 and 38 mmol/kg in the retentate from pH 5 skim milk. There were no major changes in the concentration of calcium or phosphorus in retentates made from skim milks adjusted to between pH 6.50 and pH 8.

9.3.2 Concentration of Soluble Proteins in the Retentate from Ultrafiltered pH-adjusted Skim Milk

The gels produced by SDS-non-reduced and SDS-reduced PAGE, showing the location of the major protein bands, are given in Figure 9.2. The relative concentrations of α_s -, β - and κ -caseins in the supernatant of ultracentrifuged retentates identified by SDS-non-reduced and SDS-reduced PAGE are shown in Figure 9.3a and Figure 9.3b respectively. There were no major changes in the concentrations of α -lactalbumin and β -lactoglobulin in the supernatants (results not shown).

The concentrations of protein observed in the retentate supernatant after ultra-centrifugation were seen to change considerably in the samples acidified to between pH 5.75 and pH 5.25. The micellar dissociation and release of these proteins into the serum phase was highest at approximately pH 5.25 (Figures 9.3a and 9.3b). It was interesting to note that, in the pH 5.0 ultrafiltered skim milk, there was a drop off in

these soluble caseins back to the same levels as in the control milk. There was no change in the level of soluble proteins after the addition of alkali and UF except in the pH 7.75 milk. This milk showed an unexpected increase in soluble proteins up to nearly half the increase that was observed in the pH 5.25 retentate. It is possible that this increase in soluble protein may have been due to an enzymic breakdown of the micelles rather than a pH-related structural change to the micelle. This milk was observed to take about twice the time to undergo UF than the rest of the samples (Table 9.1) which may have been sufficient time for such enzymic action to take place.

The addition of β -mercaptoethanol to the SDS buffer resulted in the reduction of disulphide bonds in the supernatant and the release of approximately 3 times the concentration of κ -casein (Figure 9.3b) than that identified by non-reduced SDS-PAGE (Figure 9.3a).

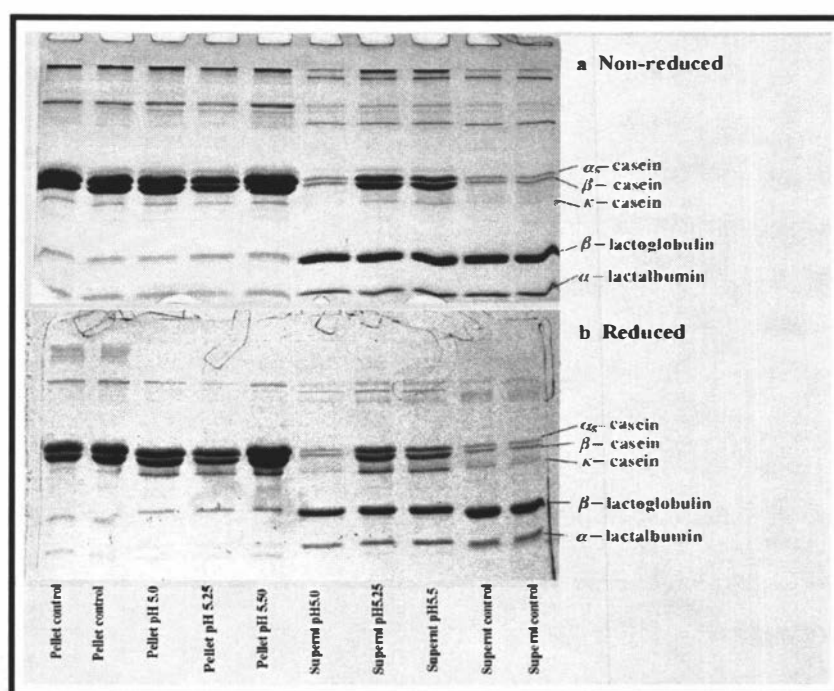


FIGURE 9.2 a, SDS-non-reduced and b, SDS-reduced PAGE of supernatants and pellets obtained from ultracentrifugation of UF retentates at 66,000 g for 1h.

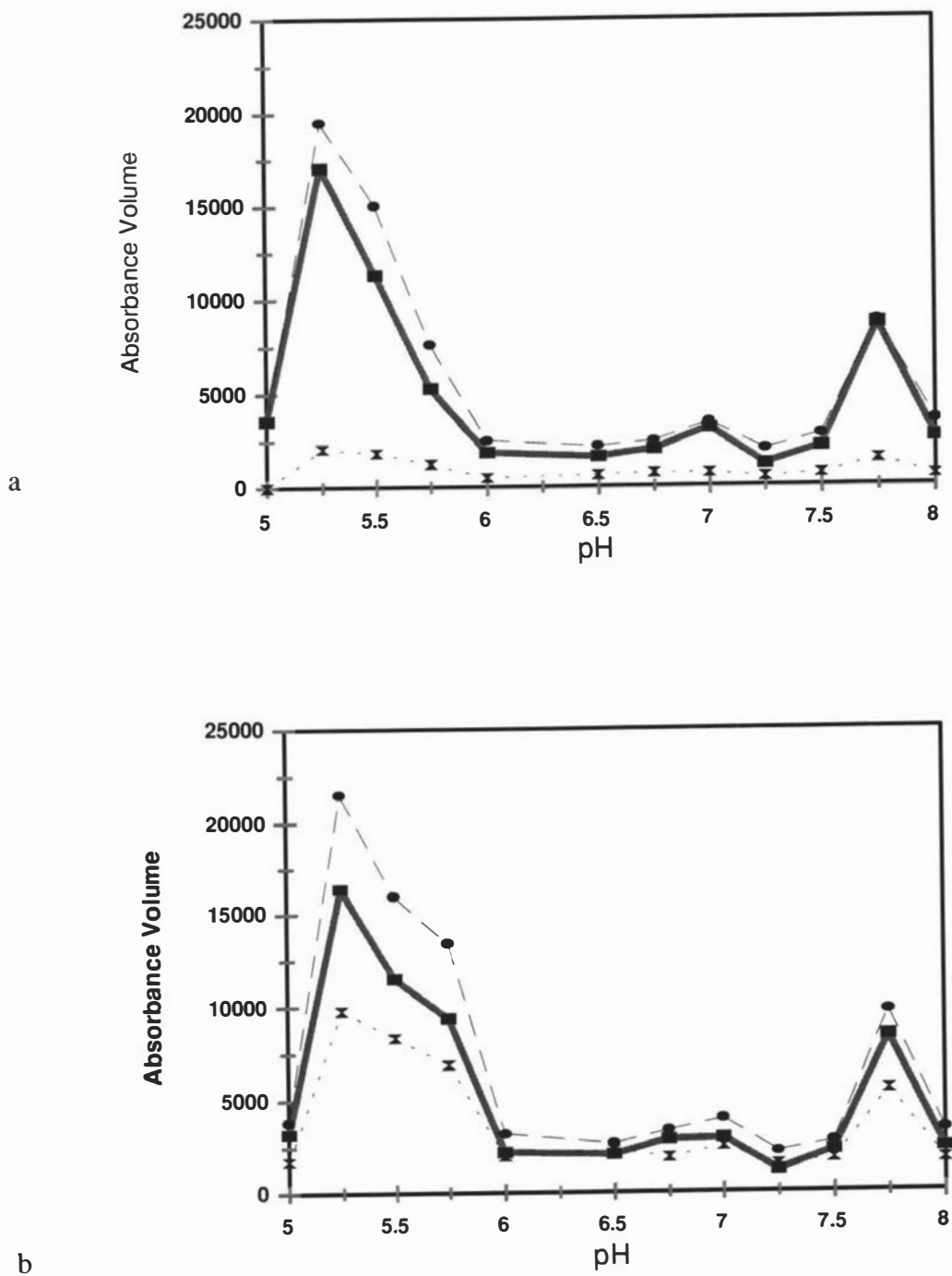


FIGURE 9.3 The influence of skim milk ^{pH} on the quantities (represented as absorbance volume) of α_s -casein (■), β -casein (●) and κ -casein (▼) in the supernatant obtained from UF retentate. The pH of skim milk was adjusted prior to UF (a, SDS-non-reduced PAGE; b, SDS-reduced PAGE).

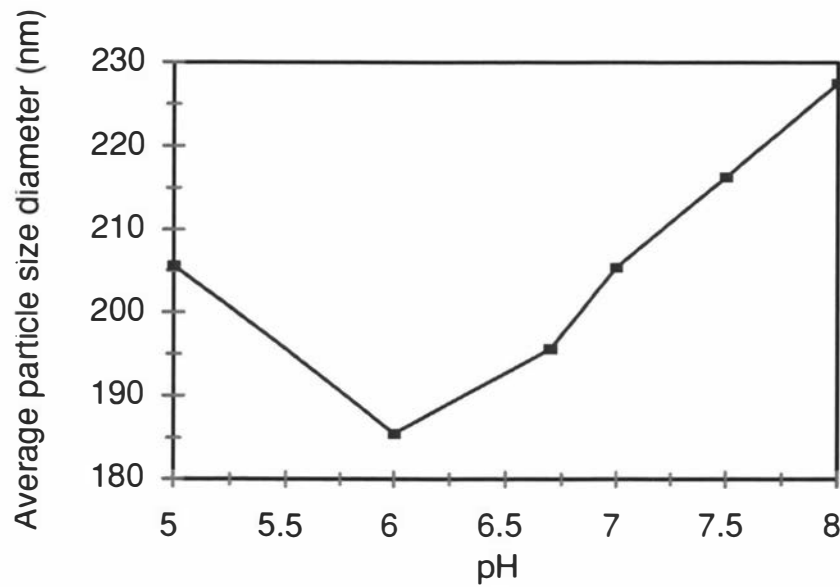


FIGURE 9.4 Influence of pH on average particle size diameter (cumulant z-average) of normal skim milk .

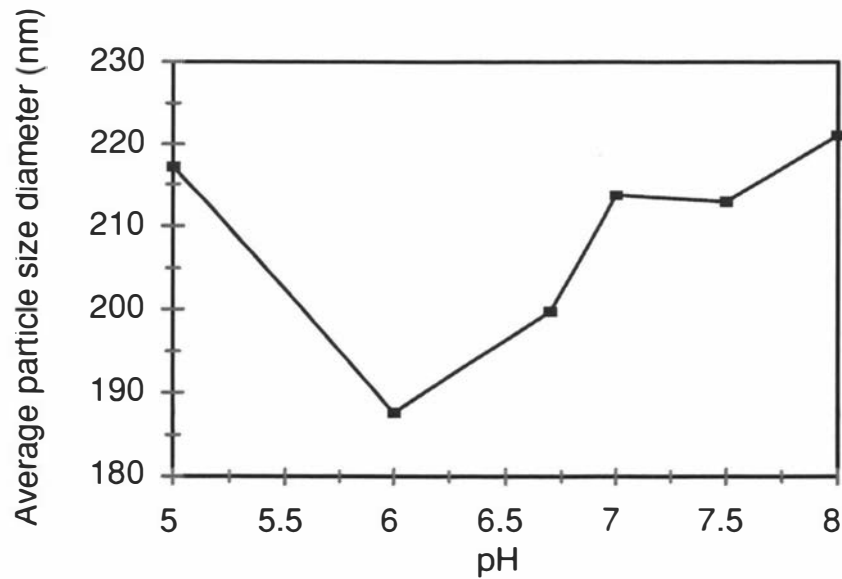


FIGURE 9.5 Influence of pH of skim milk, prior to UF, on average particle size diameter (cumulant z-average) of resulting skim milk retentate.

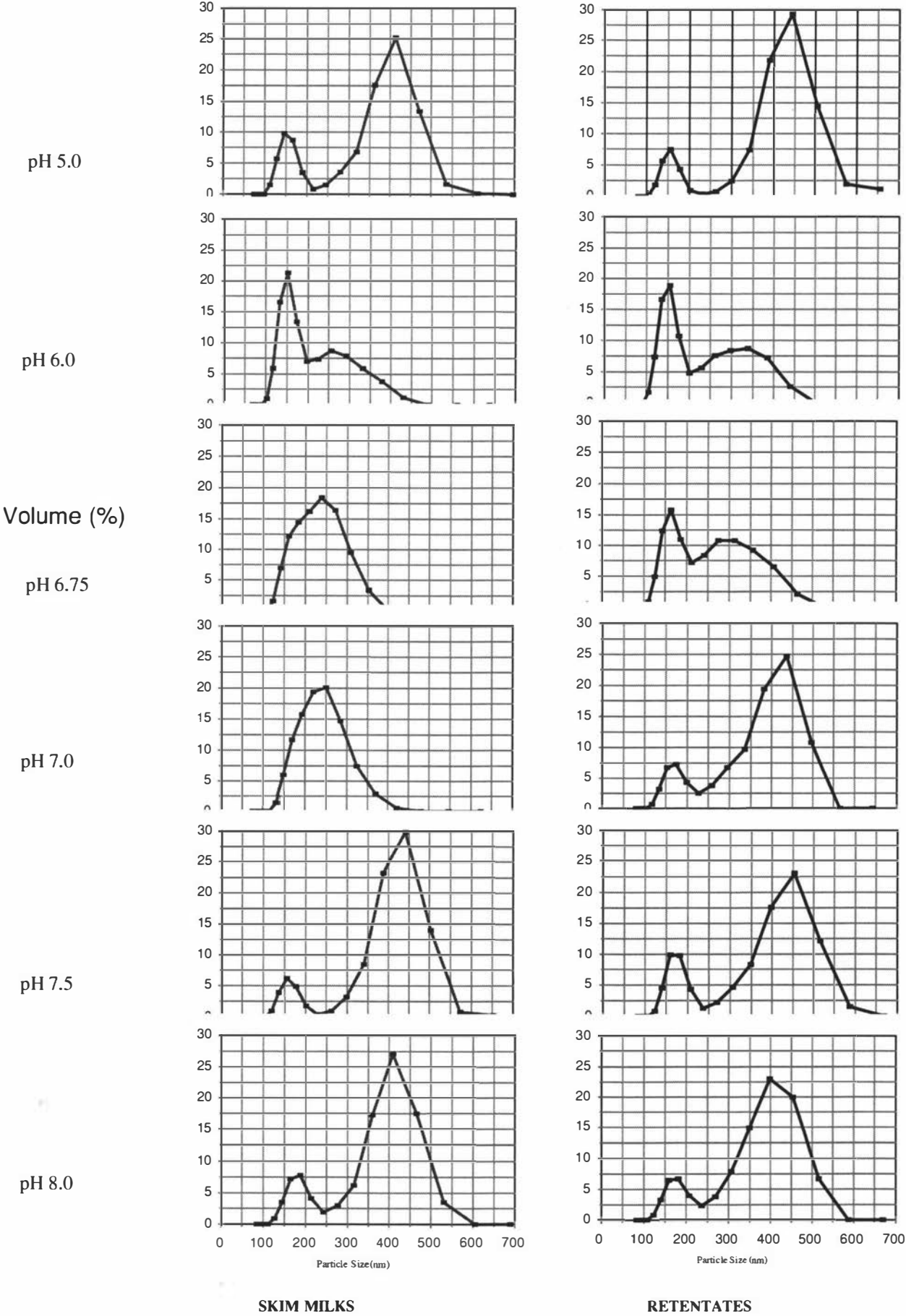


FIGURE 9.6 Particle size distribution (percentage volume basis) of pH-adjusted skim milks and resulting UF retentates.

9.3.3 Particle Size Evaluation of Skim Milk and Ultrafiltered Skim Milk

The average particle size (nm) of the pH-adjusted skim milks (Figure 9.4) and ultrafiltered skim milks (Figure 9.5) showed similar trends, particularly the minimum seen at pH 6.0.

There was a slight decrease in average particle size (from 195.6 to 185.5 nm) as the pH of the skim milk was reduced from 6.75 to 6.0. A minimum was reached (not defined here) and then the particle size began to increase considerably (up to 205 nm) as the pH was decreased to 5.0 (Figure 9.4). There was an almost linear increase in particle size from 195.6 to 228 nm when the pH of the skim milk increased from 6.75 to 8.0 (Figure 9.4).

There was an increase in particle size from 188 to 217 nm between pH 6.0 and pH 5.0 in the ultrafiltered skim milk (Figure 9.5). The increase in particle size in the skim milks after UF was greatest in the milks between pH values of 6.75 and 7.0 (200 to 214 nm) and increased only slightly up to pH 8.0 (221 nm) (Figure 9.5).

It is evident from the particle size distributions (Figure 9.6) that there was a change in the size distribution of the proteins as the pH was altered from pH 5 through to pH 8. At pH 5, the proportion of particles in the 100-200 nm size range was low with most particles in the 300-500 nm range and a cumulant z-average of 217 nm. There was a major change at pH 6.0, with a large increase in the proportion of particles in the 100-200 nm size range, reducing the cumulant z-average value to 188 nm. This appeared to be consistent with the increase in soluble proteins at this pH as measured by PAGE (Figure 9.3). The average particle size of the ultrafiltered skim milks increased up to 214 nm at pH 7 and then dropped slightly at pH 7.5. This slight drop in cumulant average value was the result of an increase in the protein particles between 100 and 200 nm at pH 7.5 (Figure 9.6).

9.3.4 Microstructure of pH-adjusted Skim Milks and Retentates

TEM results are presented here for skim milks and retentates at all pH values whereas immunolabelling was carried out and the results presented here for only retentates from pH 5.0, 6.0, 6.75, 7.0, 7.5 and 8.0 skim milks.

Milk at pH 6.75

The casein micelles (C) in the control (pH 6.75) skim milk (Figure 9.7a) were typical of those seen in pasteurised milk products (see Chapter 3). The micelles appeared to be relatively intact and discrete with little aggregation. The micelle size range in milk is typically from 10 to 300 nm, *i.e.* from subunits to large casein micelles (Brooker, 1979). After UF (Figure 9.7b), the milk was more concentrated as seen by the greater quantity of protein matter in the micrograph. There also appeared to be a greater proportion of smaller sized protein units after UF, suggesting some degree of micellar dissociation (see protein size distribution in Figure 9.6).

The locations of β -lactoglobulin and κ -casein within the ultrafiltered skim milk retentate are shown in Figures 9.7c and 9.7d respectively. The β -lactoglobulin was concentrated in the serum with approximately 80% of

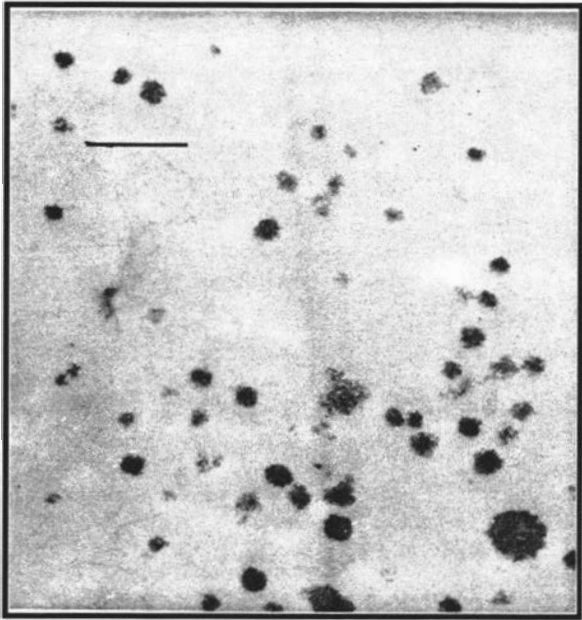
labelling in this phase (Figure 9.7c). Approximately 70% of the κ -casein labelling was closely associated with the casein micelles (Figure 9.7d).

Milk at pH 5.0

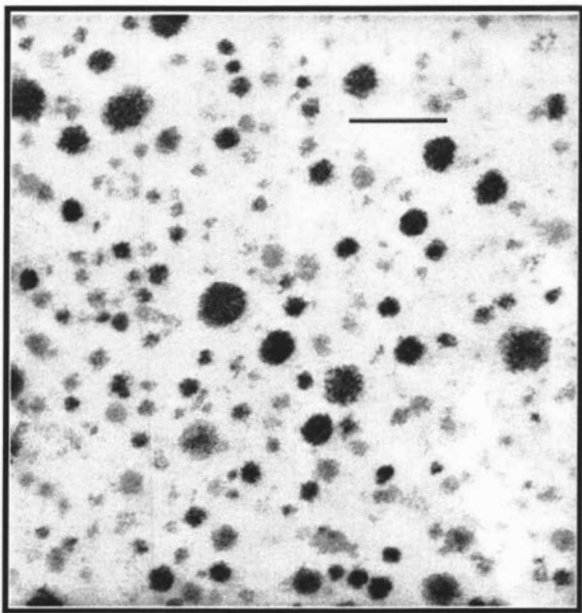
The skim milk at pH 5.0 (Figure 9.8a) showed signs of partial aggregation of the protein and some dissociation of the casein micelles. The micelles that remained at this pH had a considerably smaller size range than those observed in the control milk. The dissociation of the micelles became more pronounced after UF of the skim milk (Figure 9.8b) as shown by the presence of very small sub micellar structures and the absence of complete casein micelles. These small particles appeared to aggregate at this pH with the development of a network of fine protein structures apparent. There was some association of the β -lactoglobulin with the aggregated protein structures observed in the retentate of the pH 5 skim milk with some also located in the serum phase (Figure 9.8c). A greater proportion of κ -casein was located within these protein aggregates with over 90% of the κ -casein labelling linked to these structures (Figure 9.8d).

Milk at pH 5.25

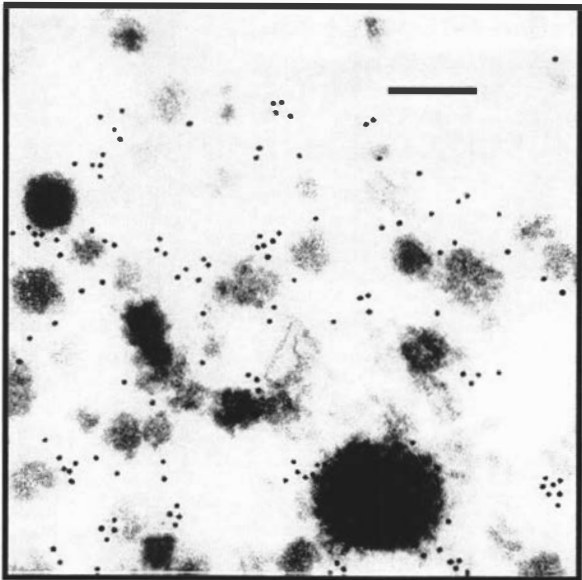
The size range of the micelles in the skim milk at pH 5.25 was greater than that for the skim milk at pH 5.0 (Figure 9.9a). There was considerable dissociation of the casein micelles in this skim milk as seen by the large quantity of sub micellar material in the micrograph. UF of this milk resulted in a further breakdown of micellar structure as seen by the increased quantity (proportionately) of dissociated micellar material (Figure 9.9b). There did not appear to be the same degree of aggregation of these dissociated micellar structures as seen in the pH 5.0 skim milk retentate (Figure 9.8b).



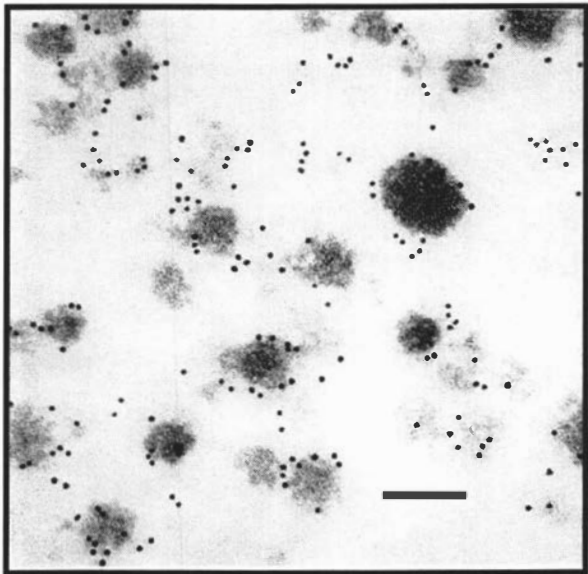
a



b

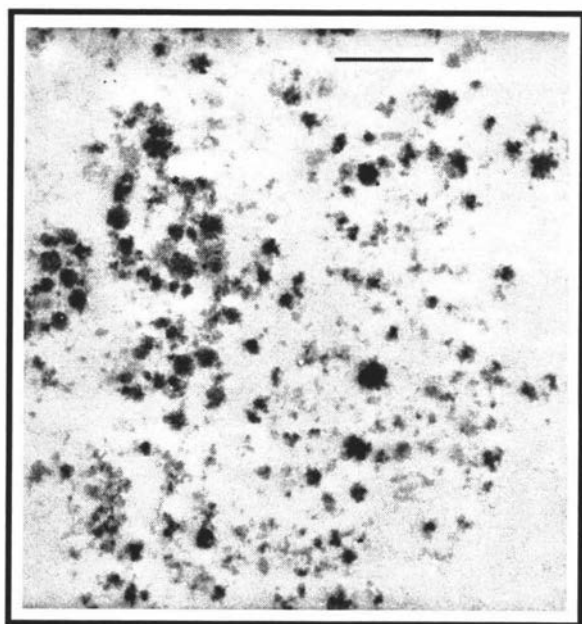


c

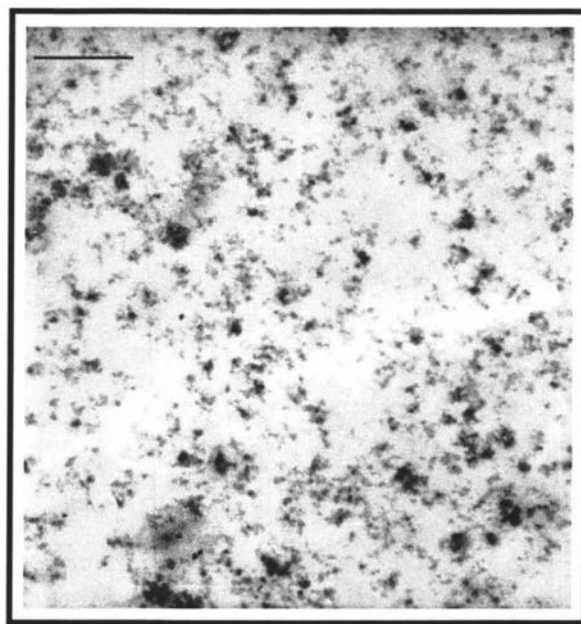


d

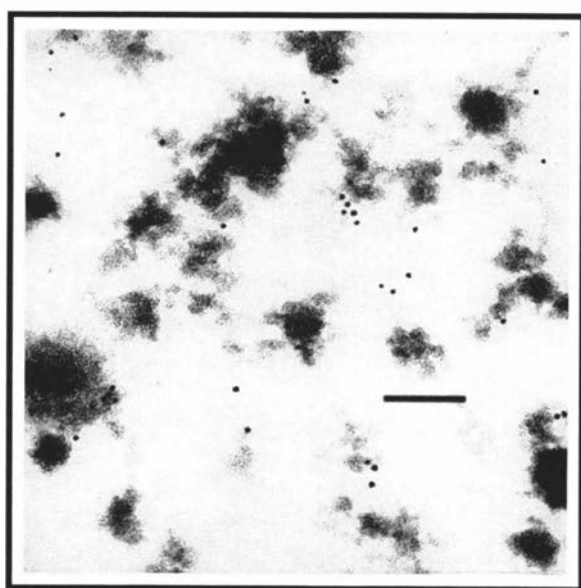
FIGURE 9.7 Micrographs of pH 6.75 (control) milk: a, control skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm); c, β -lactoglobulin-labelled retentate (bar = 0.2 μm); d, κ -casein-labelled retentate (bar = 0.2 μm).



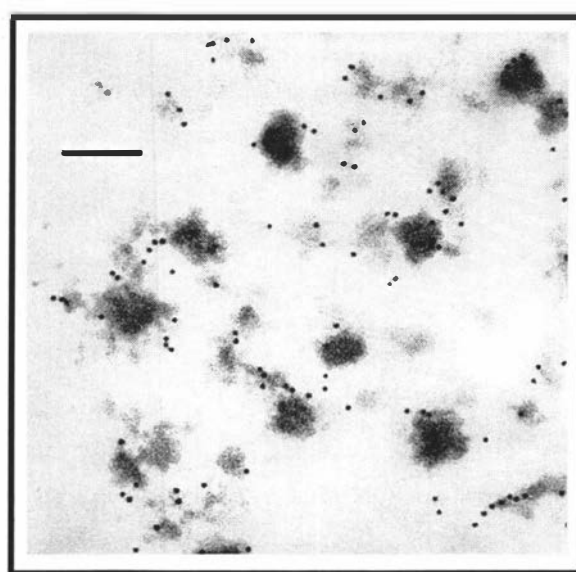
a



b



c



d

FIGURE 9.8

Micrographs of pH 5.0 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm); c, β -lactoglobulin-labelled retentate (bar = 0.2 μm); d, κ -casein-labelled retentate (bar = 0.2 μm).

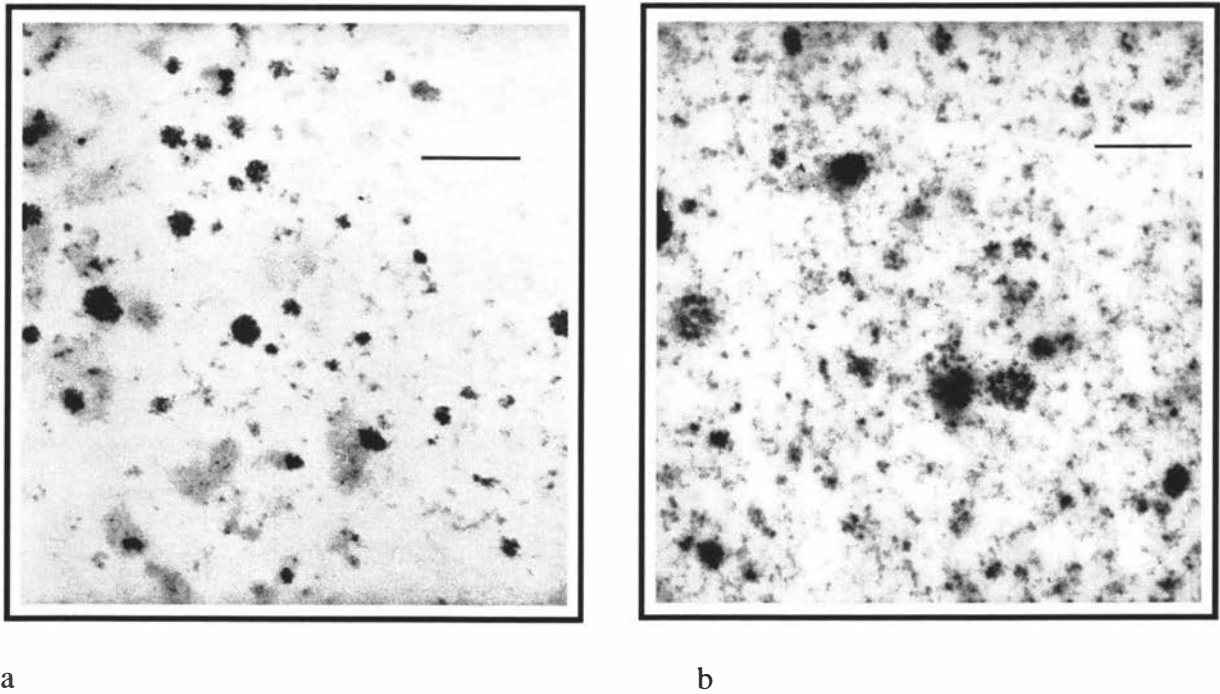


FIGURE 9.9 Micrographs of pH 5.25 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm).

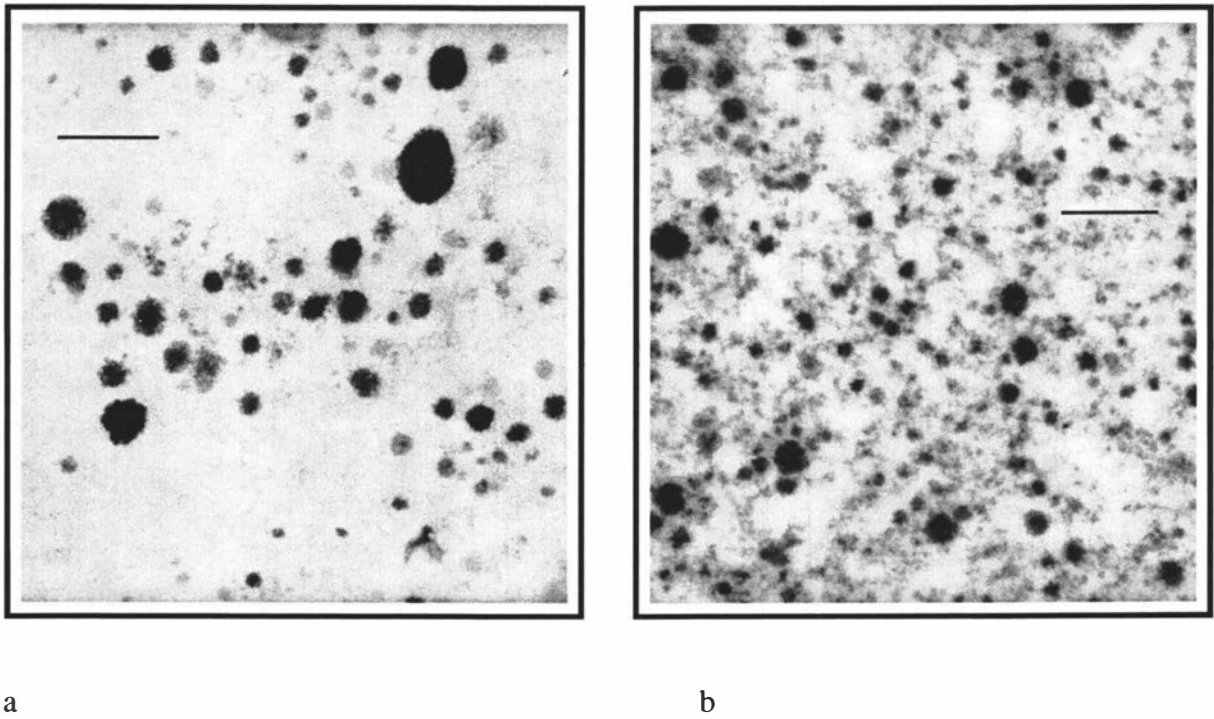


FIGURE 9.10 Micrographs of pH 5.5 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm).

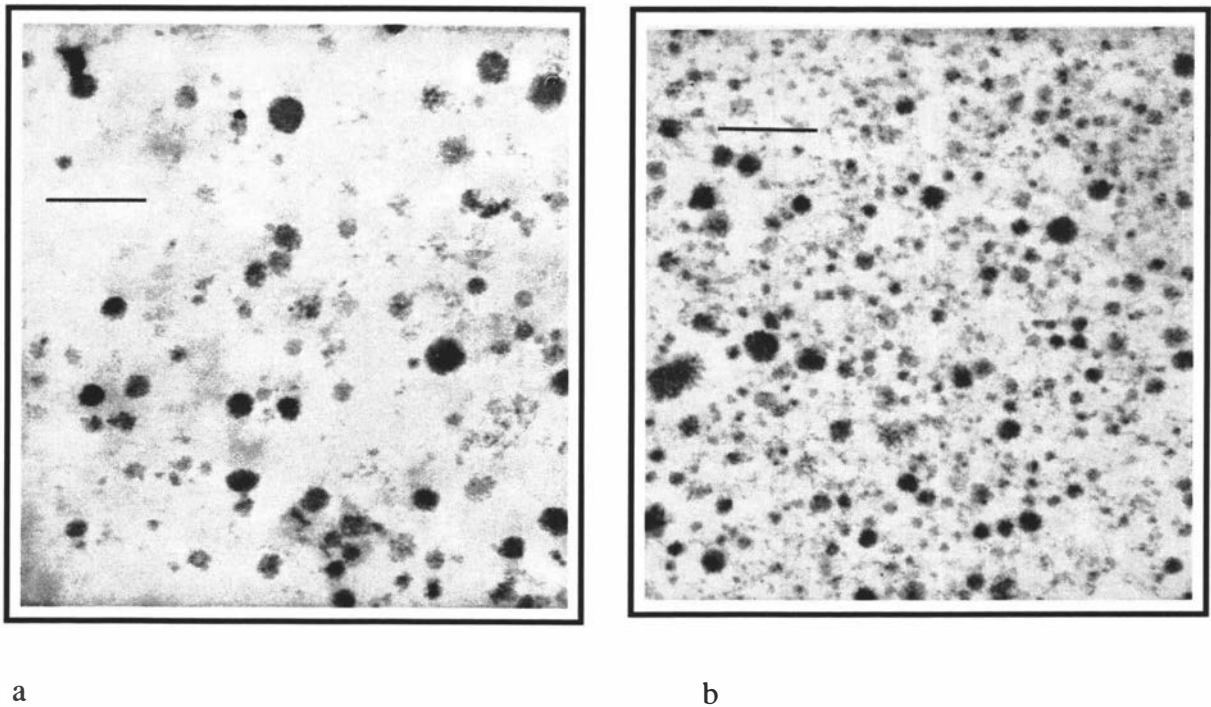


FIGURE 9.11 Micrographs of pH 5.75 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm).

Milk at pH 5.50

The size distribution of the casein micelles in the skim milk at pH 5.5 (Figure 9.10a) resembled that of the control skim milk albeit slightly swollen in appearance. UF at this pH increased the quantity of dissociated micellar material in the retentate and resulted in a reduction in the size of the micelles (Figure 9.10b).

Milk at pH 5.75

The skim milk at pH 5.75 (Figure 9.11a) and the retentate from this milk (Figure 9.11b) did not appear to be markedly different from those at pH 5.50. However, there did appear to be a larger number of small discrete micelles present in this retentate, indicating that there was not as much micelle dissociation during UF at the higher pH. The pH values after UF in these two samples were relatively close (Table 9.1) perhaps explaining the similarity in microstructure.

Milk at pH 6.00

There was little noticeable difference in the structure of the skim milk of this sample (Figure 9.12a) and the control. The retentate (Figure 9.12b) after UF of the skim milk at pH 6.0 had smaller sized micelles and more dissociated protein material than the control sample. The β -lactoglobulin again appeared to have little association with the micelles in this sample (Figure 9.12c) whereas the majority (approximately 80%) of the κ -casein was associated with the micelles (Figure 9.12d).

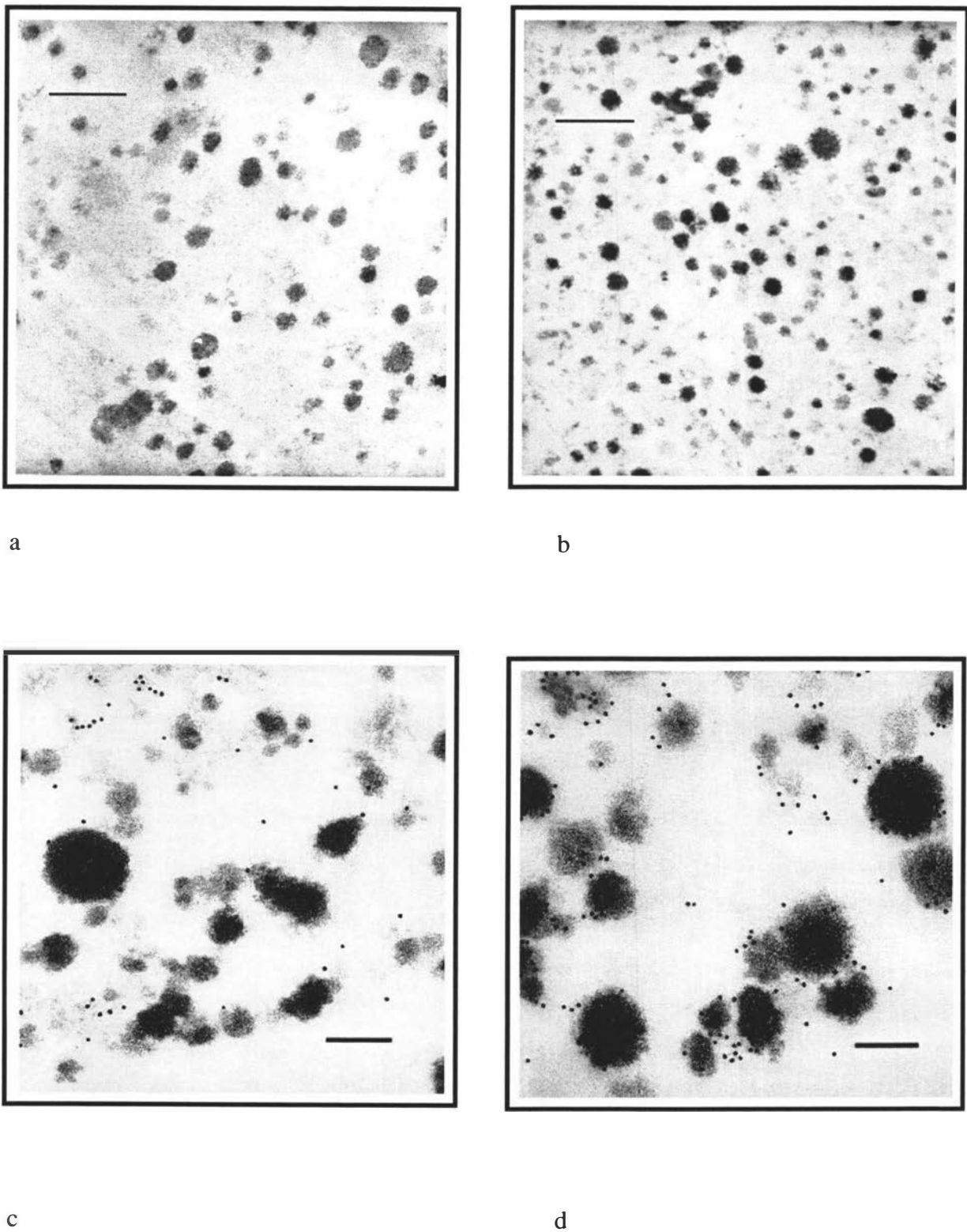
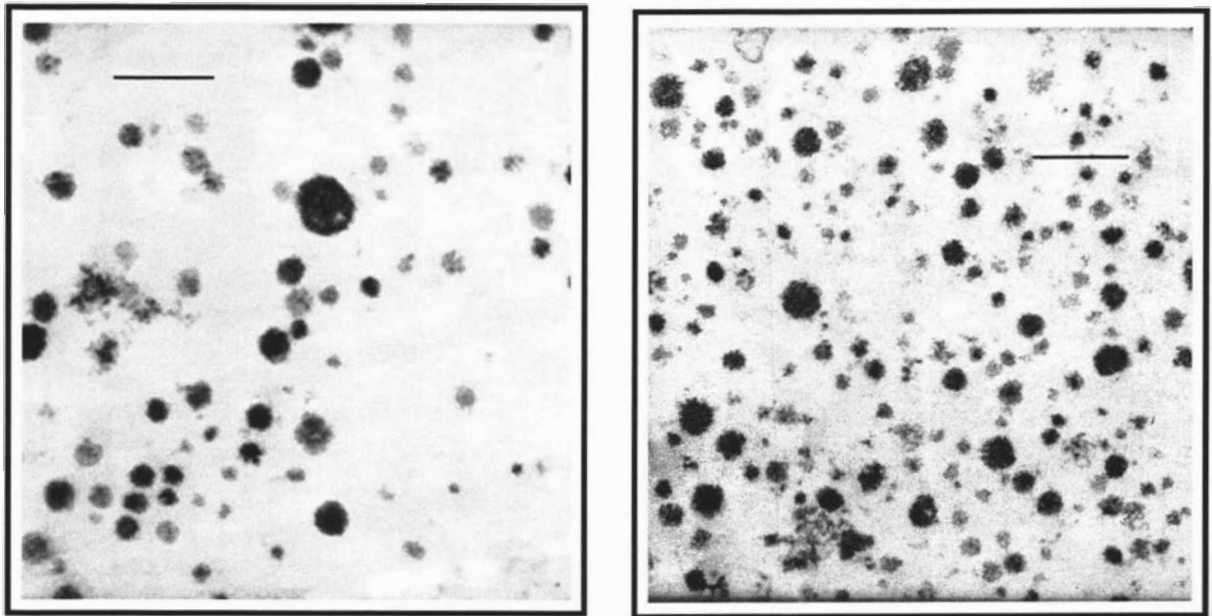


FIGURE 9.12 Micrographs of pH 6.0 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm); c, β-lactoglobulin-labelled retentate (bar = 0.2 μm); d, κ-casein-labelled retentate (bar = 0.2 μm).



a

b

FIGURE 9.13 Micrographs of pH 6.5 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm).

Milk at pH 6.50

The only noticeable structural difference observed between this sample (pH 6.5) and the control was evidence of slight micelle dissociation after UF (Figure 9.13b).

Milks at pH 7.00 and pH 7.25

There was little structural difference between the control sample and the skim milks adjusted to pH 7.00 and pH 7.25 (Figures 9.14a and 9.15a) and their retentates (Figures 9.14b and 9.15b). The β -lactoglobulin in the retentate from the pH 7.0 skim milk was again located mainly (approximately 90%) in the serum phase (Figure 9.14c). The κ -casein was located mainly (approximately 70%) on the casein micelles (Figure 9.14d).

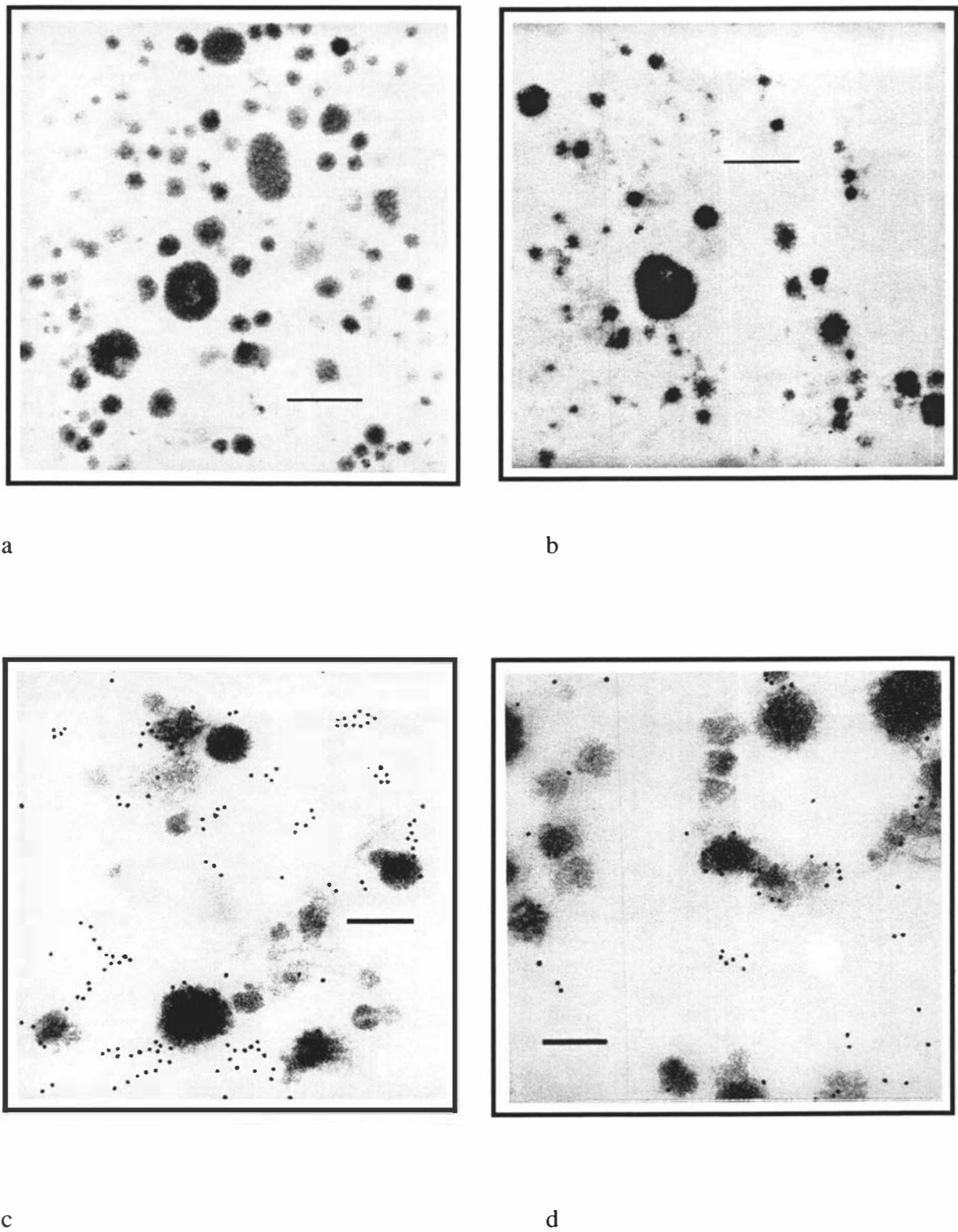
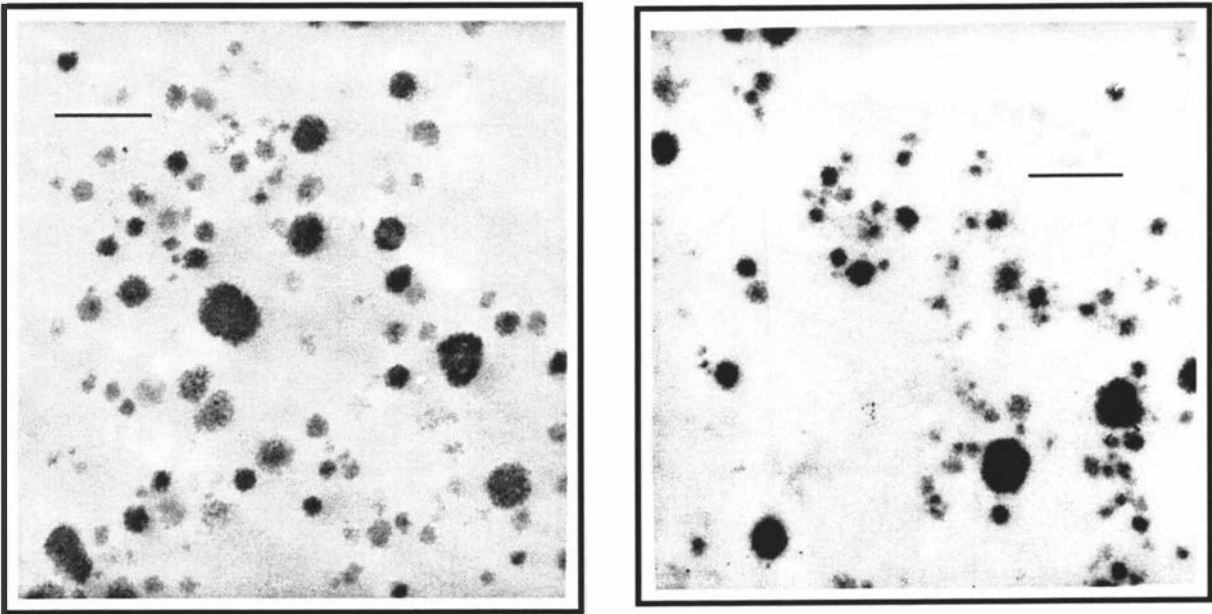


FIGURE 9.14 Micrographs of pH 7.0 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm); c, β-lactoglobulin-labelled retentate (bar = 0.2 μm); d, κ-casein-labelled retentate (bar = 0.2 μm).



a

b

FIGURE 9.15 Micrographs of pH 7.25 milk: a, skim milk (bar = 0.5 μ m); b, retentate (bar = 0.5 μ m).

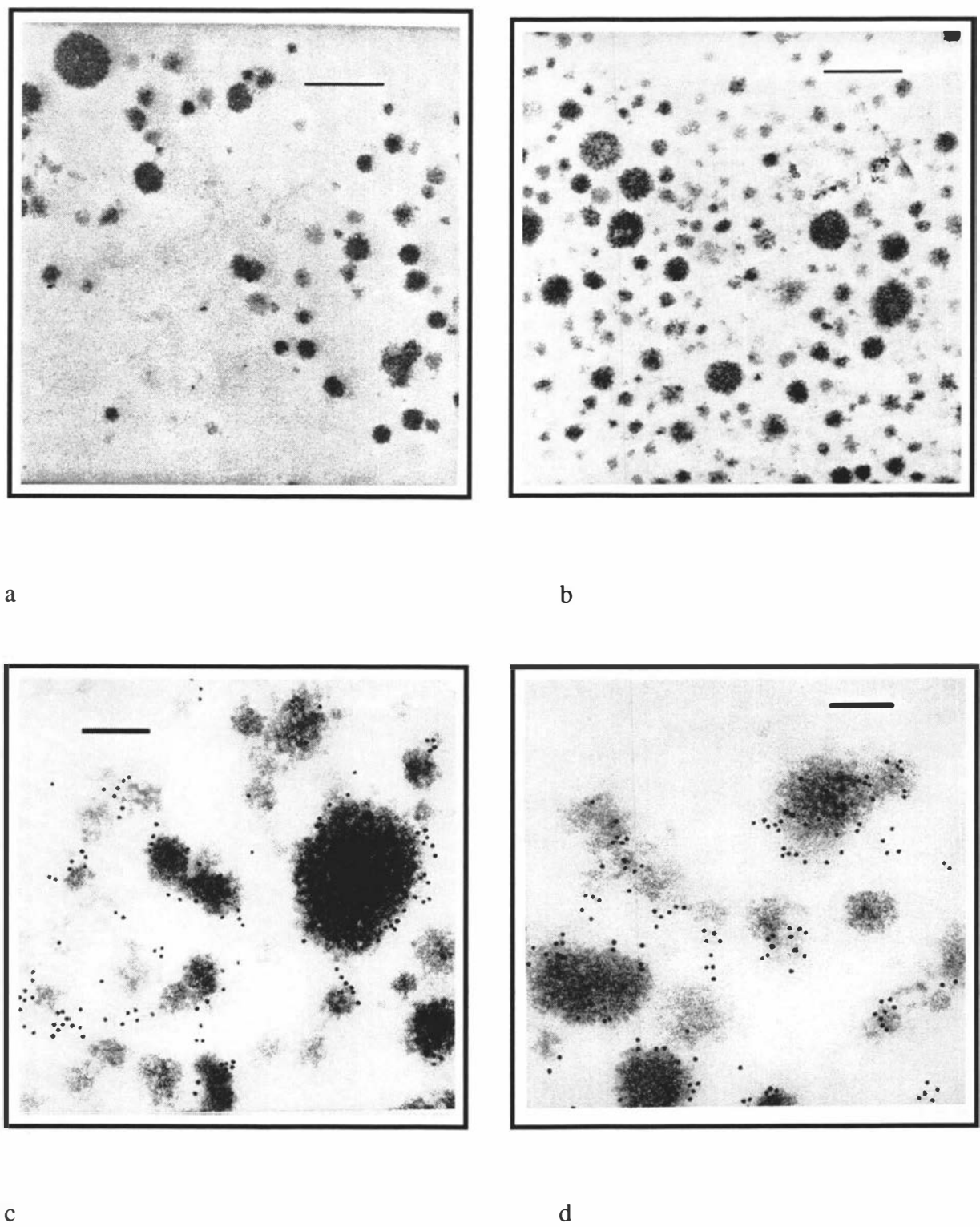
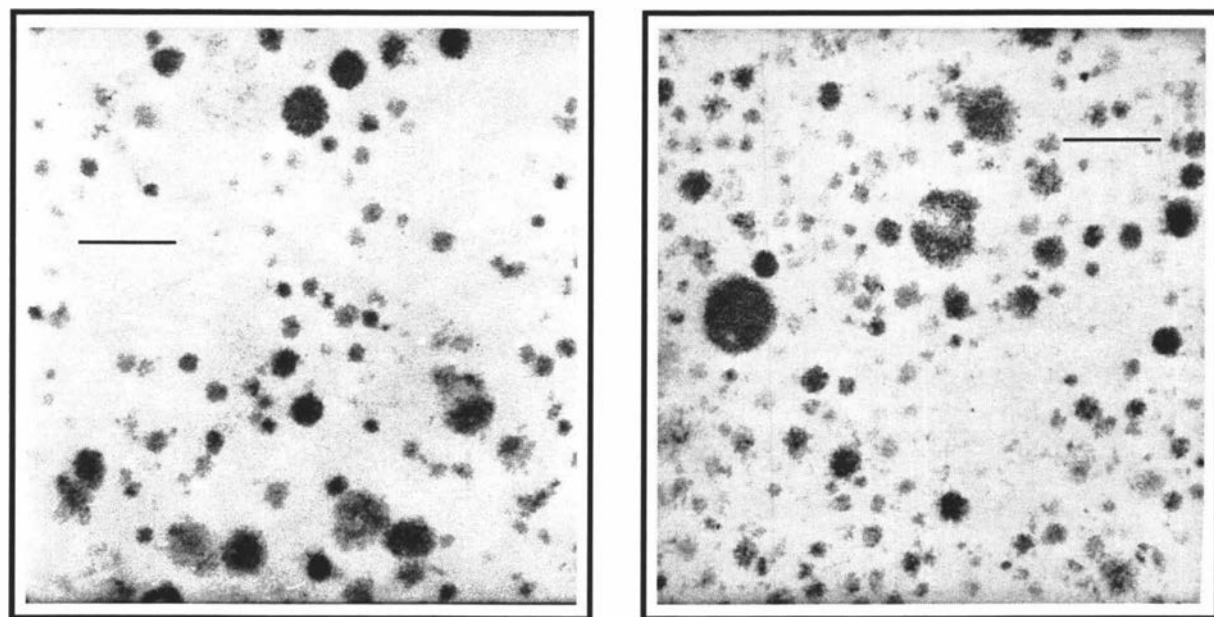


FIGURE 9.16 Micrographs of pH 7.5 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm); c, β-lactoglobulin-labelled retentate (bar = 0.2 μm); d, κ-casein-labelled retentate (bar = 0.2 μm).



a

b

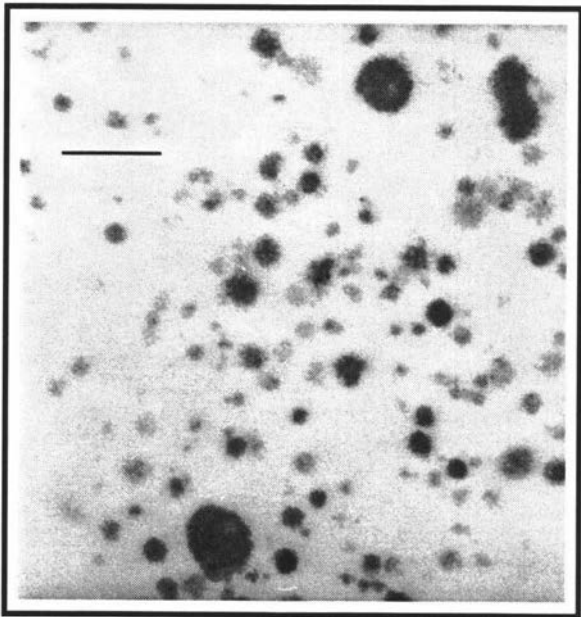
FIGURE 9.17 Micrographs of (a) pH 7.75 skim milk and (b) resulting UF retentate (bar = 0.5 μm).

Milk at pH 7.50

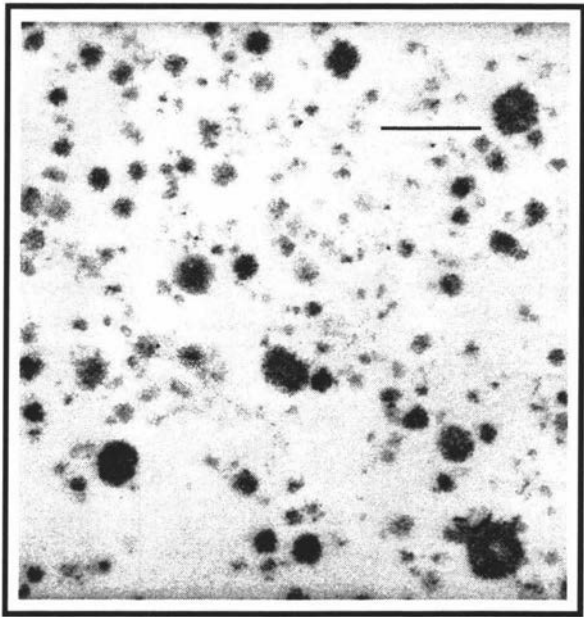
There appeared to be an increase in the amount of dissociated material in the retentate after UF (Figure 9.16b). The micelles in the retentate also appeared to have become more diffuse and less dense. The β -lactoglobulin in this sample appeared to be more closely associated with the micelle than in previous lower pH samples. Approximately 70% of this protein was linked to the casein micelle (Figure 9.16c). More of the κ -casein (approximately 90%) appeared to be associated with the micelles at this pH than in the pH 7.0 sample (Figure 9.16d).

Milks at pH 7.75 and pH 8.00

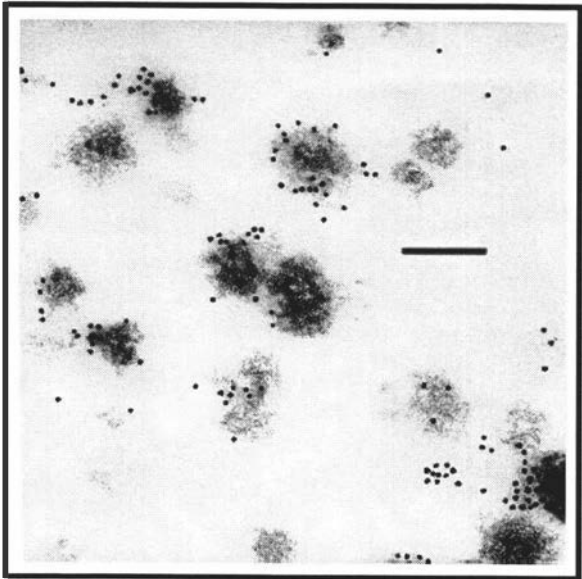
In the milks adjusted to pH 7.75 and pH 8.0 there appeared to be further swelling of the casein micelles and the development of more dissociated material (Figures 9.17a and 9.18a). Following UF of these samples, more small structures were apparent in the milk (dissociated micellar material) and there was a further increase in aggregation between the micelles and the dissociated material particularly in the pH 8.0 sample (Figure 9.18b). There did appear to be a change in the aggregation and distribution of the β -lactoglobulin and κ -casein within the retentate of the pH 8.0 milk. In lower pH samples, there was little aggregation of these proteins. In this sample, the β -lactoglobulin appeared to be mainly (approximately 80%) associated with the casein micelles in clumps rather than as individual units (Figure 9.18c). The κ -casein appeared to be aggregated in a similar way to the β -lactoglobulin on the casein micelles (Figure 9.18d). This aggregation effect was more obvious at a lower magnification (Figure 9.18e).



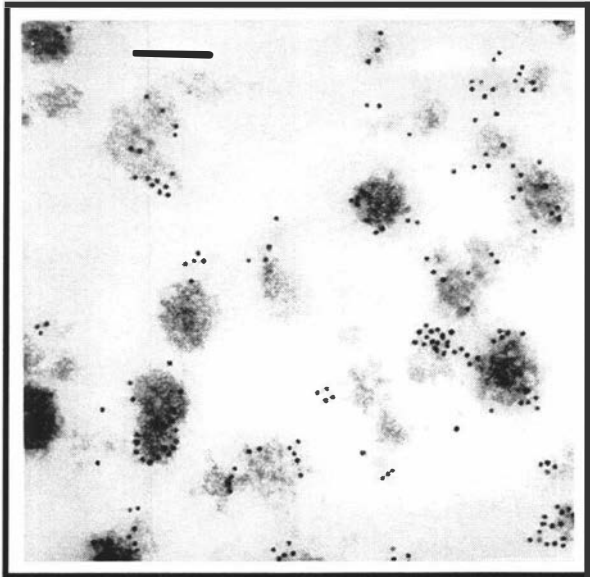
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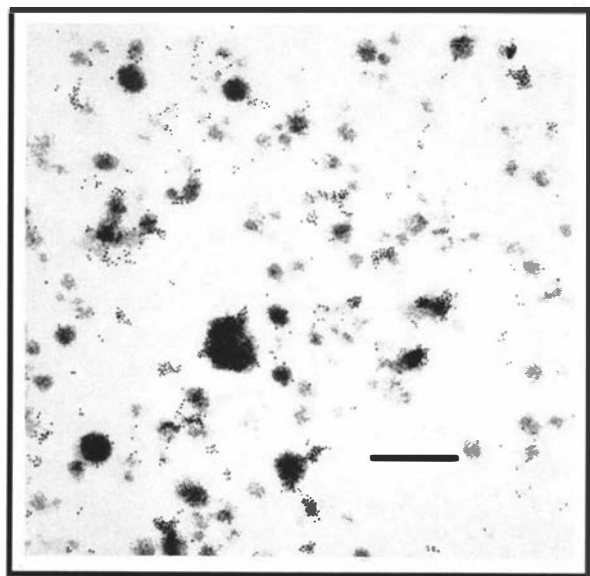
b



c



d



e

FIGURE 9.18 Micrographs of pH 8.0 milk: a, skim milk (bar = 0.5 μm); b, retentate (bar = 0.5 μm); c, β -lactoglobulin-labelled retentate (bar = 0.2 μm); d, κ -casein-labelled retentate (bar = 0.2 μm); e, κ -casein-labelled retentate (bar = 0.5 μm).

9.4 DISCUSSION

The structural changes observed here by thin section TEM of the acidified ($\text{pH} < 6.75$) skim milks were similar to those reported by Visser *et al.* (1986) who carried out TEM by freeze fracturing. Acidification of milk results in a gradual dissociation of CCP (Chaplin, 1984; van Hooydonk *et al.*, 1986; Dalglish & Law, 1988, 1989; Singh *et al.*, 1996) and therefore a release of casein components from the micelles. It was shown by Roefs *et al.* (1985) that most of the colloidal phosphate and a considerable part of the casein dissociated from the micelles in skim milk at pH 5.4. All casein fractions showed a distinct increase in solubility around pH 5.4 consistent with dissociation of most of the calcium phosphate from the micelles. It was suggested that a micellar skeleton (of α_{s1} -casein) remained even when all the CCP had been completely released at pH 5.1 (Visser *et al.*, 1986). The solubilisation of phosphorus was almost complete at about pH 5.2, whereas calcium solubilisation was complete at about pH 4.8 (Singh *et al.*, 1996). This would indicate that some micellar-like structure would remain in the milks acidified to pH 5.0. This was what was observed in the skim milks acidified to pH 5.0 in this work (Figure 9.8a). However, it appeared that these micellar-like structures in the pH 5.0 skim milk dispersed further upon UF (Figure 9.8b) perhaps due to the removal of calcium into the permeate and further solubilisation of CCP. Some workers reported that the released casein consisted predominantly of β -casein (Snoeren *et al.*, 1984; van Hooydonk *et al.*, 1986) whereas others demonstrated that all casein components dissociated from the casein micelles upon acidification (Roefs *et al.*, 1985; Dalglish & Law, 1988, 1989). Singh *et al.* (1996) showed that serum protein (β -casein) increased linearly (between pH 6.6 and pH 5.6) in non-heated skim milk that was acidified at 5°C . When acidifying at 22°C , the amount of β -casein increased in the serum only at $\text{pH} < 6.1$. In this study, the samples were ultrafiltered at 10°C , immediately

after pH adjustment, for approximately 24 h. The dissociation of casein protein from the micelles in the ultrafiltered concentrated skim milk did not occur until below pH 6.0 (Figure 9.3). These soluble caseins were dominated by β - and α_s -caseins with κ -casein contributing the least. This was in contrast to the findings of Roefs *et al.* (1985), Dalgleish & Law (1988) and Singh *et al.* (1996) all of whom found that the soluble caseins in pH-adjusted skim milks were predominantly β - and κ -caseins (equal amounts) with α_s -casein being less in quantity. It is possible that the lower contribution of κ -casein to the soluble casein in the ultrafiltered skim milk in this work may have been the result of the increased protein levels and the proportional increase in protein interactions particularly involving κ -casein.

The extent of micelle dissociation upon acidification appears to change at around pH 5.2 where isoelectric precipitation of the dissociated protein occurs and aggregated particles appear (Visser *et al.*, 1986). These workers related this aggregation of protein at between pH 5 and pH 5.2 (see Figure 9.8) specifically to the loss of charge on β -casein. There was some evidence of this protein interaction in the skim milk adjusted to pH 5.0 in the current study where dissociated material and the residual micelles appeared to have aggregated (Figure 9.8a). The aggregation of dissociated micellar material appeared to be more extensive in this milk after UF when a network of aggregated material appeared to have formed (Figure 9.8b). This network formation is likely to have been more extensive in the concentrated milks as there was more chance of the proteins colliding and forming aggregates because of the higher concentration of components. The SDS-PAGE results showed that there was a dramatic decrease in soluble proteins in the pH 5.0 retentate as this aggregated structure formed.

Increasing the pH of milk and heating result in an increase in κ -casein dissociation and an increase in the supersaturation of calcium phosphate. At a pH above 6.7 electrostatic repulsion increases but steric repulsion decreases (due to κ -casein dissociation) (Nieuwenhuijse *et al.*, 1991). When the pH of the skim milk was increased, the average particle size diameter increased but the SDS-PAGE results did not show any increase in dissociation of protein material from the micellar to the soluble phase after UF. However, electron microscopy of the pH 7.75 and pH 8.0 ultrafiltered skim milks showed that some swollen micelles had dissociated to produce a considerable quantity of smaller structures in the milk and that these had aggregated with the micelles. This aggregation may explain the increase in particle size with increasing pH. It is possible that the aggregation of protein caused the dissociated material to be caught up in this matrix and prevented it from being separated out in the supernatant during ultracentrifugation. It is also possible that the dissociated material was still large enough to be sedimented by ultracentrifugation. An increase in viscosity was observed in the higher pH samples (not measured) which confirmed an increase in protein interactions and the reduction in protein material being released into the supernatant as measured by SDS-PAGE.

The aggregation of β -lactoglobulin and κ -casein on to the micelles in the pH 8.0 retentate suggested that at this pH the dissociated protein probably first aggregated in the serum and then aggregated on to the micellar structures.

Holt (1995) has shown that above pH 7 the calcium within the milk begins to change from the dicalcium phosphate form to the β -tricalcium phosphate form. This change of form may be partly responsible for the structural change in the high pH skim milks and retentates shown here.

Modification of the milk pH prior to UF may have a number of processing ramifications. St-Gelias *et al.* (1992) found that the UF of acidified milk at temperatures below 10°C resulted in the dissociation of the casein micelles, an increase in solubility of the final product and an

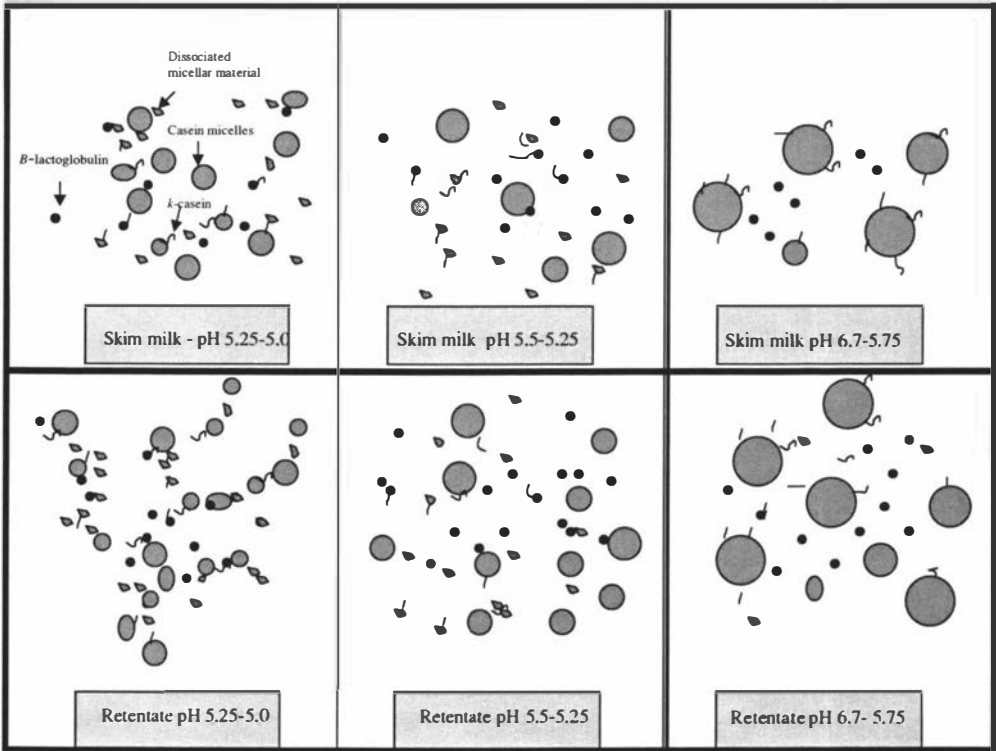
increase in viscosity of the retentate. This led to an increased fouling of the UF membranes and reduced the permeation or flux rate. An increase in pH will also have the effect of increasing viscosity, reducing flux and probably increasing fouling rates. The cost of these UF processing changes would need to be balanced against the benefits achieved in final product performance before any commitment to changing a commercial process could be made.

9.5 CONCLUSIONS

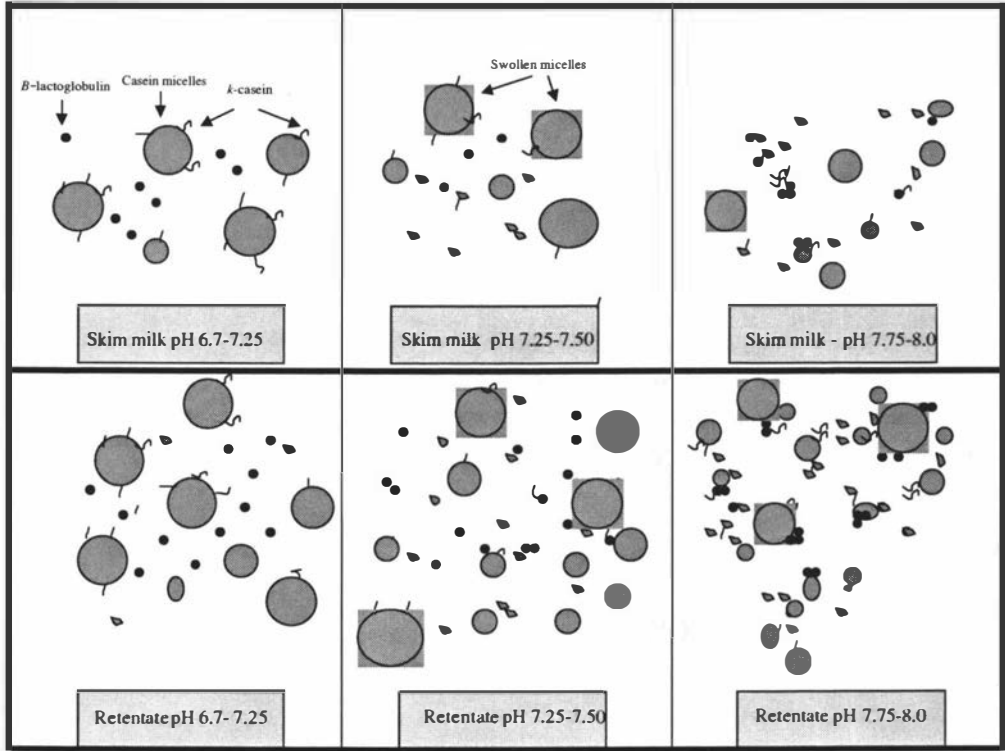
A summary of the changes that occurred during the pH adjustment of skim milk followed by UF is shown in the schematic diagram Figure 9.19.

When the pH of the skim milk was reduced to pH 5.0, a number of structural changes occurred prior to and after UF (as depicted in Figure 9.19a). The dissociation of CCP resulted in an increasing amount of material that dissociated from the micelle as the pH was reduced. The skim milk at pH 5.0 contained much smaller micellar structures than the control milk (pH 6.75). The ultrafiltered retentates of the pH-adjusted skim milks showed that there was a further increase in micellar dissociation following the UF process. The SDS-PAGE results showed that there was an increase in dissociated α_s -, β - and κ -caseins in the ultrafiltered retentates only in samples from skim milk at pH < 6.0. At pH 5.0, an aggregated network of protein material was formed by charge neutralisation of protein and isoelectric precipitation. The network was more evident after concentration by UF.

The structural changes that occurred in the skim milk and ultrafiltered retentate as the skim milk pH was increased to pH 8.0 are shown schematically in Figure 9.19b. The average particle diameter of the skim milk and ultrafiltered retentate increased as the pH of the skim milk was increased. Electron microscopy showed that the micelles increased in size and became more diffuse and less dense up to pH 7.75. The structure of the pH 8.0 skim milk showed that the micelles had started to disperse and that following UF this dispersed or dissociated micellar material aggregated with the remaining micellar structures. It appeared that at this pH the dissociated protein material had aggregated in the serum and then aggregated on to the micellar structures.



a



b

FIGURE 9.19 Schematic diagram of the structural changes that occur after pH adjustment of skim milk followed by UF (a) adjustment of the control down to pH 5.0; (b) adjustment of the control up to pH 8.0.

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CHAPTER 10

CONCLUDING DISCUSSION

The goal of this thesis was to develop further understanding about the influence of processing factors on component interactions during the manufacture of whole milk powder (WMP) and high protein powders, using microscopy as a major investigative tool.

The following sections provide a summary of the work carried out in the thesis and how the results have contributed towards this goal.

10.1 MICROSTRUCTURAL CHANGES DURING THE MANUFACTURE AND RECONSTITUTION OF WMP

The application of confocal laser scanning microscopy to WMP overcomes many difficulties associated with the microscopic examination of milk powder such as the inability to focus on a thin focal plane (light microscopy), the inability to locate many of the components (scanning electron microscopy) and the long preparatory techniques (transmission electron microscopy). Confocal laser scanning microscopy of WMP required only simple sample preparation and, using fluorescent stains or the reflectance mode of the microscope, resulted in images highlighting the location of various components within the powder particles. The size and distribution of fat globules within the powder sample could be evaluated. Lactose crystals could also be evaluated without dissolving away the other components of the milk powder. A phospholipid stain was used to follow the movement of lecithin (wetting agent) on the surface of the powder particles during storage.

The changes in structure during the different stages of WMP manufacture from standard milk and whey-protein-depleted milks were evaluated using thin-section transmission electron microscopy. The fat-protein and protein-protein interactions during the manufacture of WMP contribute to the reconstitution properties of the powder. Whey protein denaturation during preheating and the activation of sulphhydryl groups predispose the whey protein to further interactions with casein micelles and fat globule membrane protein during further processing. Homogenisation changes the composition of the fat globule membrane, with casein micelles becoming adsorbed in large numbers. The resulting fat globules can act like large casein micelles, effectively increasing the volume fraction of this reactive material. Powders containing highly homogenised fat globules, with a resulting high protein load on the fat globule membrane, are less stable when reconstituted in water and coffee. Powder manufactured from milk with a low pH, perhaps as a result of poor microbiological quality, had a higher degree of protein interactions during processing perhaps leading to poor reconstitution. Removal of up to 32% of the whey protein from the milk led to a marked reduction in the aggregation of adjacent casein micelles. Removal of 40% of the whey protein appeared to cause an increase in direct micelle-micelle contact. The functional properties of the powders produced indicated that reducing the whey protein concentration in the milk may improve functional properties by reducing coffee sediment and slowly dissolving particles.

A number of different instant WMP (IWMP) samples obtained from overseas markets were evaluated to determine the influence of structural and chemical properties on the reconstitution

properties of the powders. The IWMP with the worst functional performance (solubility in coffee and hot and cold water and dispersibility) had a low whey protein nitrogen index (WPNI), a high fat globule protein load (excessive shear during processing) and a high degree of protein-protein interactions. The IWMP sample with the most favourable functional properties (good solubility in hot and cold water and coffee, excellent dispersibility and good agglomerate structure) had a high WPNI (low preheating) and relatively few protein-protein interactions. The manufacturers of this IWMP appeared to have optimised the processing conditions (*i.e.* low temperature processing and no excessive shear) to produce a powder that when reconstituted had similar properties to fresh milk.

WMP that is manufactured from high quality milk, with minimum heat treatment, mild homogenisation conditions and careful drying conditions, may have sufficiently good reconstitution properties to produce milk of similar quality to fresh milk.

A summary of the interactions occurring during the processing of WMP, observed during this study, is represented schematically in Figure 10.1.

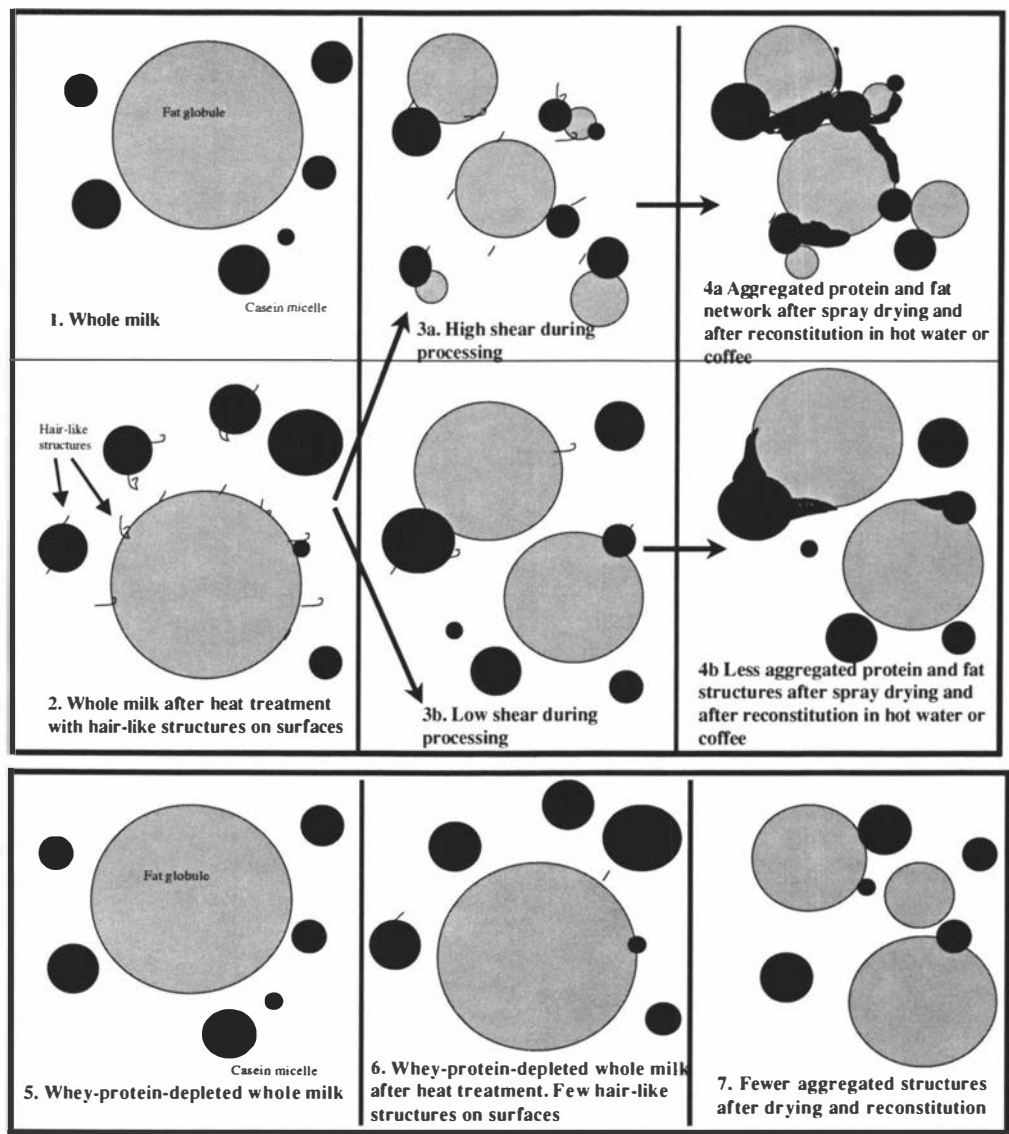


FIGURE 10.1 Schematic diagram of component interactions that occur during the manufacture and reconstitution of WMP.

10.2 MICROSTRUCTURAL CHANGES DURING THE MANUFACTURE AND STORAGE OF 85% PROTEIN ULTRAFILTERED SKIM MILK POWDER (UFSMP85)

The cause of the poor solubility of UFSMP85 upon reconstitution in water at and below ambient temperatures (generally $\leq 25^{\circ}\text{C}$) has not been fully described by other workers. A combination of microstructure techniques, chemical techniques and particle size analysis provided valuable insights into the physical and chemical changes that occur during membrane concentration, evaporation, spray drying and storage of UFSMP85.

This work showed that there are changes that occur during membrane concentration and evaporation that predispose the concentrated milk to protein-protein and protein-mineral

interactions upon drying. The extent of these protein interactions increases with an increase in protein content of the UFSMP. These interactions result in a solubility of approximately 40% when UFSMP85 is reconstituted in water at 20°C. The solubility is approximately 98% when UFSMP85 is reconstituted in water at 60°C. Upon storage at temperatures of 20°C and above, a skin that reduces water permeation develops on the particle surface. This skin eventually (after 6 months of storage) reduces the powder solubility even in water at 60°C. The major changes during the processing of UFSMP85 are summarised in Table 10.1.

TABLE 10.1 Summary of the changes that occur during the manufacture and storage of UFSMP85

Stage of Process	Observations of Changes During Processing	Influence on UFSMP85 Solubility
Skim milk	Intact micelles with little interaction.	
Ultrafiltration	Movement of proteins (κ -casein followed) from micelle to serum phase. Swelling of micelles as calcium moves to serum phase. Loss of lactose.	
Diafiltration	Movement of proteins from micelle to serum phase. Appearance of aggregates of dissociated micellar material. Some aggregation of micelles due to further loss of lactose and reduced steric repulsion.	Increased aggregation may predispose the powder to poor solubility. Reducing lactose concentration reduces the solvent properties and makes the protein less stable and more likely to cause protein interactions.
Evaporation	Increased aggregation of micelles with other micelles and dissociated micellar material.	
Spray drying	Formation of a particle-air interface. Presence of β -lactoglobulin at the interface. Close packing of casein micelles at this interface. Possible 'setting' of the internal structure as water is removed and the micelles, minerals and non-micellar protein are forced together. High temperatures within the drier may denature proteins at the particle surface.	Poor solubility in ambient (20°C) water 24 h after manufacture. Completely soluble when reconstituted in water at 60°C.
Storage	Little change when stored at 7°C and below. Storage at temperatures of 20°C and above for 6 months resulted in the formation of two distinct layers at the particle surface. The first layer consisted of an aggregated, thickened skin layer of β -lactoglobulin (and possibly other proteins). The second skin layer was made up of a layer of aggregated casein micelles that appeared to act as a water-impermeable skin.	The solubility of UFSMP85 after storage above 20°C was influenced by the formation of a skin of whey protein and aggregated casein micelles that held the particle together upon reconstitution. These micelles may have aggregated due to covalent linkages or through Maillard reactions. The formation of the skin layers during storage reduced the solubility of the powder even when reconstituted at 60°C. Homogenisation at increasing pressures resulted in a decreasing particle size and increased solubility.

A schematic representation of the changes that occur during the manufacture and storage of UFSMP85 is shown in Figure 10.2.

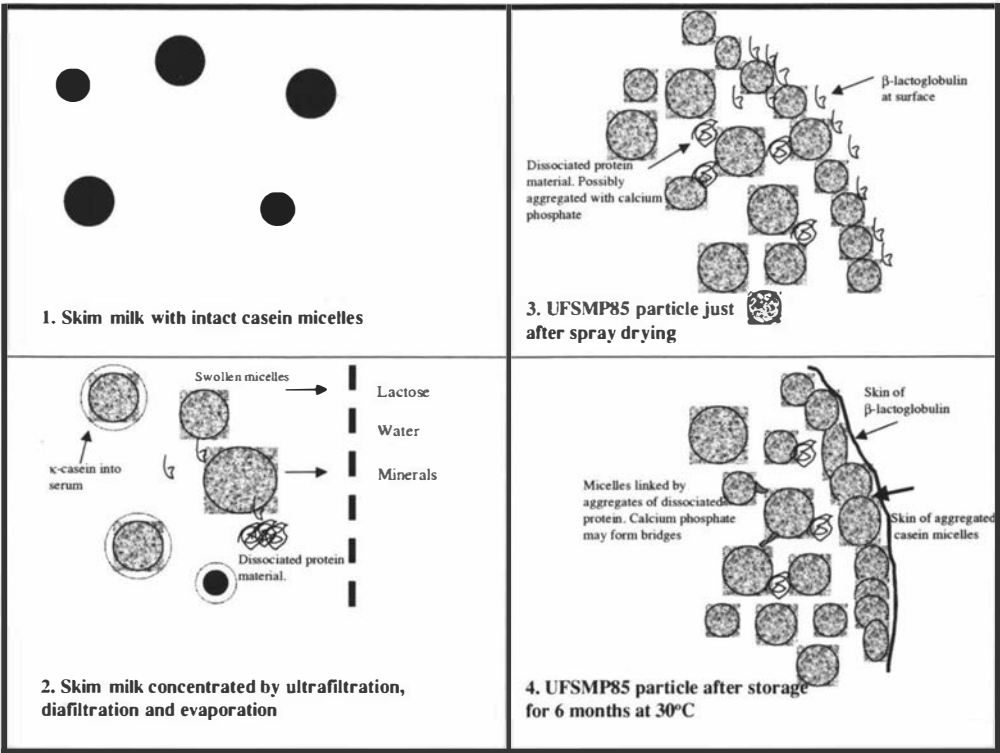


FIGURE 10.2 Schematic diagram of the protein-protein interactions that occur during the manufacture and storage of UFSMP85.

There may be improvements that can be made to the cold water solubility of UFSMP products with protein contents above 80%. Some alteration in the micellar structure of the skim milk, *e.g.* by pH adjustments, may result in reduced micellar interaction and therefore a reduced set network upon drying, possibly producing a more soluble powder.

Small improvements in solubility may be achieved by reducing the heating applied during manufacture, particularly the temperature and time within the drying chamber where the protein-air interfaces form. Reducing temperatures during transportation and storage will reduce the rate at which the skin forms and thus will keep the powder soluble in water at 60°C but this will not improve cold water solubility.

10.3 COMMON THEMES

The work presented in this thesis shows that there are a number of common themes that influence the performance of WMP and UFSMP85 upon reconstitution in water. The protein-protein interactions that occur during manufacture of these powders have a major influence on solubility.

During WMP manufacture, these protein interactions occur when the casein micelles come into close contact at the surface of fat globules. During UFSMP85 manufacture, the protein interactions occur when lactose is removed during ultrafiltration and diafiltration forcing the casein micelles close together. It appears in both cases that whey proteins are also involved in protein interactions.

The work indicates that there are compositional and processing options that may be used to reduce protein aggregation during the processing of WMP. The depletion of whey protein in the milk prior to processing, reduction in shear (homogenisation pressure) and minimising heat treatment throughout processing all appeared to reduce protein interactions.

It is not clear from this work how to minimise protein interactions during UFSMP85 manufacture. It is possible that a number of the options applied during WMP manufacture to reduce protein interactions may also help during UFSMP85 manufacture. These options and other approaches could be evaluated in future studies to improve the solubility of UFSMP85.