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FORMATION AND STABILITY OF
OIL-IN-WATER CASEINATE EMULSIONS

A THESIS
PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
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Dedication

To my Parents

ABSTRACT

The main objective of this study was to gain a better understanding of the formation, stability and microstructure of oil-in-water emulsions stabilized by commercial sodium (ALANATE 180) and calcium caseinates (ALANATE 380). The study also determined the effects of heat treatment and NaCl addition on the formation and stability of these emulsions.

Emulsions were prepared using various concentrations of sodium or calcium caseinate solutions (0.5 to 5.0%) and 30% soya oil. Surface protein coverage (mg/m^2) in freshly prepared emulsions was determined from analysis of the aqueous phase after centrifugation of emulsions at 45,000 g for 40 minutes, using the Kjeldahl method. SDS-PAGE was used to identify the adsorbed protein components in the cream phase. Creaming stability was determined after storage of emulsions for 24 hours at 20°C by a low speed centrifugation method. The microstructure of these emulsions was determined using confocal laser scanning microscopy. The aggregation state of caseins in sodium and calcium caseinate solutions was determined by successive centrifugation, gel permeation chromatography and multi-angle laser light scattering techniques.

For emulsions stabilized with sodium caseinate, the surface protein concentration increased gradually with protein concentration up to 3%, but the increase was much smaller at higher concentrations. By comparison, the surface protein coverage in emulsions stabilized with calcium caseinate showed an almost linear increase with protein concentration (0.5 to 5.0%). At all protein concentrations, the surface protein coverage of emulsions stabilized with calcium caseinate was higher than that of sodium caseinate emulsions. β-Casein was adsorbed in preference to other caseins in emulsions made using ≤ 2.0% sodium caseinate, but α_s-casein (α_{s1}- + α_{s2}-) appeared to adsorb in preference to other caseins when emulsions were made using > 2.0% sodium caseinate. In calcium caseinate-stabilized emulsions, α_s-casein was found to

adsorb in preference to other caseins at all protein concentrations used.

Heat treatment (121°C for 15 min) of sodium caseinate emulsions or heat treatment of sodium caseinate solutions prior to emulsion formation, at all caseinate concentrations, resulted in an increase in surface protein coverage and altered the proportions of individual caseins at the droplet surface. The surface protein coverage of emulsions formed with calcium caseinate solutions increased markedly when the emulsions were heated (121°C for 15 min) or when calcium caseinate solutions were heated prior to emulsion formation. The preferential adsorption of α_s-casein, observed in the unheated calcium caseinate emulsions, diminished after heating, which was due to polymerization of α_s-casein during heating and/or degradation of this casein.

In sodium caseinate emulsions, the surface protein coverage and the composition of emulsion droplets were influenced by the presence of NaCl prior to emulsion formation. The surface protein coverage in emulsions made with 1 and 3% sodium caseinate increased with an increase in NaCl concentration up to 40 mM, with a large increase in the adsorption of α_s-casein at the droplet surface. Addition of NaCl beyond 40 mM had no further effects on surface coverage and composition. Similar trends were observed when NaCl was added to the emulsions after they were formed. By contrast, in calcium caseinate emulsions, the surface protein coverage decreased with increase in NaCl concentration up to 40 mM, but with further increase in NaCl concentration the surface protein coverage increased slightly. In these emulsions, the composition of the interface remained largely unaffected by NaCl addition; α_s-casein was adsorbed in preference to other caseins.

Creaming stability of calcium caseinate emulsions, after storage at 20°C for 24 hours, increased with an increase in protein concentration. However, the creaming stability of sodium caseinate emulsions decreased markedly as the protein concentration was increased above 2%. This decrease in stability was attributed to the reversible flocculation arising from a 'depletion flocculation'

mechanism. This flocculation in turn resulted in enhanced creaming at high caseinate concentrations. In sodium caseinate emulsions, the appearance of the droplets in the confocal micrographs was dependent on the concentration of protein used for making emulsions. Emulsions formed with low concentrations of sodium caseinate (0.5 and 1.0%) appeared to be homogenous with no sign of flocculation. However the emulsions made with > 2% sodium caseinate showed some irregular flocs, which appeared to be forming a network structure at higher concentrations of protein. In contrast, confocal micrographs of emulsions formed with calcium caseinate at all protein concentrations showed individual droplets. The creaming stability of these emulsions improved, when the emulsions were heated or when emulsions were made using heated sodium or calcium caseinate solutions. The presence of 200 mM NaCl prior to emulsion formation resulted in improved creaming stability and a reduced degree of flocculation.

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Chapter 1

INTRODUCTION

Commercial milk protein products represent an important source of functional ingredients for use by the food industry. For example, annual world casein and caseinate production is estimated at about 250,000 tonnes. In 1997, New Zealand exported 75,000 tonnes of casein and caseinate and about 75% of this amount was used in formulated food products (International Dairy Federation, 1997). Caseinates and caseins provide fat emulsification in coffee creamers, whipped toppings, cream liqueurs, nutritional formulations, soups and meat emulsions; foam expansion and stabilization in whipped toppings; emulsification and control of melting in cheese analogs; and contribute to viscosity in various food products. Emulsification is an important functional property of the product for about 50% of the caseins and caseinates produced in New Zealand.

It is well known that caseinates possess excellent emulsifying properties. Most of the investigations on the adsorption of proteins in emulsions have involved simple model systems using, most usually, one or may be two purified proteins (Dickinson *et al.*, 1989, 1991; Dalgleish *et al.*, 1991). Some studies have been reported on the adsorption behaviour of sodium caseinate at oil/water interfaces (Tomberg, 1978b; Robson and Dalgleish, 1987; Hunt and Dalgleish, 1994; Srinivasan *et al.*, 1996) and its ability to stabilize emulsions (Tomberg, 1978a). Caseinate is a composite of four different proteins, consisting of α_{s1}-, α_{s2}-, β- and κ-caseins, in weight proportions of approximately 4:1:4:1. During emulsion formation, all of the casein types are adsorbed at the interface and decrease the interfacial tension. β-Casein adsorbs most rapidly and causes the greatest decrease in surface tension. It is also the most hydrophobic of the caseins (Bigelow, 1967). The adsorbed layers of caseinate provide stability to the resultant emulsion with respect to coalescence and flocculation. Most of the previous studies have concentrated mainly on the adsorption of pure caseins,

mixtures of isolated proteins and on sodium caseinate made in the laboratory under relatively mild conditions (Benjamins *et al.* 1975; Dickinson *et al.* 1988a,b; Fang and Dalgleish, 1993; Hunt and Dalgleish, 1994; Agboola and Dalgleish, 1995; 1996). Studies on the adsorption behaviour of commercially produced sodium caseinate are limited. Srinivasan *et al.* (1996) studied the emulsifying properties of commercial sodium caseinate; they reported that β-casein was preferentially adsorbed in emulsions made with relatively low concentration of sodium caseinate. In the present study, their work has been extended and the adsorption behaviour of sodium caseinate has been compared with calcium caseinate in oil-in-water emulsions.

Very little information is available in the literature about the adsorption behaviour of commercial calcium caseinate at the oil-in-water interface. Mulvihill and Murphy (1991) reported that the surface protein loads of emulsions (40% soya oil and 2.5% protein) made with calcium caseinate were higher than sodium caseinate-stabilized emulsions; this was attributed to greater degree of casein aggregation in calcium caseinate solutions compared with sodium caseinate solutions. Other studies have also shown that the surface protein coverage depends on the protein aggregation state (Oortwijn and Walstra, 1979; Singh *et al.*, 1993). Thus, in this study, attempts were made to understand further the adsorption behaviour of commercial calcium caseinate in emulsions made with a range of protein concentrations.

Emulsion stability is another important aspect of emulsifying properties. Food emulsions comprise a wide variety of products where either fat or water or both are dispersed in a state of macroscopic homogeneity (Friberg, 1976). Because the contact between fat and water is energetically unfavourable, emulsions are thermodynamically unstable, and stabilizing agents are necessary to improve the shelf life (Das and Kinsella, 1989). Emulsion instability can appear visually as creaming or fat separation or a mixture of both. Aggregated caseinates of divalent cations, e.g. calcium and magnesium caseinates, have been shown to form more stable emulsions than caseinates of monovalent cations, e.g.

sodium, potassium or ammonium caseinates (Reimerdes and Lorenzen, 1983). Various studies have been reported on the creaming behaviour of sodium caseinate-stabilized emulsions (Acton and Saffle, 1971; Halling, 1981; Leman *et al.,* 1988). Relatively little information is available on the creaming stability of calcium caseinate emulsions.

Although it is known that high ionic strength may destabilize an emulsion, much less information is available on the effects of ionic strength on the adsorption behaviour of proteins in emulsions. Hunt and Dalgleish (1996) studied the effects of presence of KCl on adsorption of caseins and whey proteins only at one caseinate concentration. The effects of ionic strength on the adsorption behaviour and stability of emulsions stabilized by calcium caseinate are virtually unknown. Therefore, the secondary objective of this study was to determine the effects of ionic strength on the formation and stability of oil-in-water emulsions stabilized by commercially made sodium and calcium caseinates.

Heating of emulsions is particularly important in many food products. Heat treatment is given generally to extend the microbiological shelf life of the products. Although some information is available on the changes in sodium caseinate solutions on heating at sterilization temperature (Guo *et al.*, 1989), little is known about the changes involved in emulsifying properties during heating. Thus, the other objective was to study the heat-induced changes in oil-in-water emulsions formed with commercial sodium and calcium caseinates. The knowledge gained from the present study should provide a scientific background for the formulating emulsion products and for improving the manufacturing methods of commercial caseinates.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

Milk proteins, because of their nutritional importance and their physico-chemical properties, are key functional components in many foods and are widely consumed as a human food. Caseins and caseinates are unique amongst dairy products and they have a long history of use in the food industry, non-food industry and technical applications. Southward (1989) described comprehensively the uses of casein and caseinate in industrial and technical applications. Casein-containing products are widely used in the food industry because they have exceptional water binding capacity, fat emulsification properties, whipping ability and desirable flavour characteristics, and are viscous and soluble in neutral or alkaline conditions (Jonas *et al.,* 1976). Emulsion-type products, for example coffee whiteners, whipped toppings, cream liqueurs, and low fat spreads are an important application of caseinates in the food industry. In recent years, the use of casein and caseinate in dietary preparations, pharmaceutical industry and medical applications has increased (Southward, 1989); some of these preparations are also oil-in-water emulsions containing relatively small amounts of fat. This chapter is intended to provide a general survey on the chemistry of caseins, formation and stability of caseinate-stabilized emulsions. This review covers until the year 1996. More specific and the current (1997, 1998) literature is cited in the discussion sections of the relevant chapters.

2.2 General characteristics of milk proteins

A detailed knowledge of the inherent structural features of proteins is required in order to understand their functional properties. Bovine milk contains ~ 3.5% protein, comprising caseins and whey proteins; the caseins represent ~ 80% of the total protein. Several reviews, covering the chemistry of milk proteins, have been published during the last 18 years (Schmidt, 1980; Swaisgood, 1982, 1985, 1992; Whitney, 1988; Fox, 1989).

2.2.1 Caseins

The casein proteins, which comprise approximately 3% of cow's milk, may be distinguished from the so called "whey proteins" (0.6%) by their insolubility and tendency to precipitate and coagulate at the isoelectric point (pH 4.6) (Southward, 1985).

Bovine casein consists of four distinct proteins: α_{s1}-, α_{s2}-, β- and κ-caseins in the approximate proportions 38%, 10%, 36% and 13% (Davies and Law, 1980; Barry and Donnelly, 1980, 1981). In addition to these four caseins, which are gene products, there are several derived caseins, known as γ-caseins, resulting from the action of indigenous milk proteinases, especially plasmin, on the main caseins. There are five known variants of α_{s1}-casein (Thompson *et al.*, 1962; Grosclaude *et al.*, 1966; Eigel *et al.*, 1984), four known variants of α_{s2}-casein (Grosclaude *et al.*, 1976, 1979), seven variants of β-casein (Aschaffenburg, 1961; Peterson and Koppler, 1966; Aschaffenburg *et al.*, 1968) and two variants of κ-casein (Neelin, 1964; Schmidt, 1964). Accurate amino acid compositional data are now available for all the caseins (Swaisgood, 1992). Physico-chemical characteristics of the caseins are summarized in Table 2.1.

Table 2.1: Some physico-chemical properties of caseins. (Adapted from Walstra and Jenness, 1984).

Property	Caseins			
	α_{s1}-B	α_{s2}-A	β-A	κ-B
Molecular weight (Da)	23,614	25,230	23,983	19,023
Residues/Molecule:				
Amino acids	199	207	209	169
Proline	17	10	35	20
Cysteine	0	2	0	2
Disulphide	0	1	0	1
Phosphoserine	8	11	5	1
Isoionic point	4.96	5.19	5.19	5.43
Charge at pH 6.6	- 21.9	- 12.2	- 13.8	- 3.0
Hydrophobicity (kJ/residue)	4.9	4.7	5.6	5.1

The primary structures of the four principal caseins have been established (Swaisgood, 1992). A number of interesting and technologically important features are apparent from the primary structures.

1. The four caseins contain high levels of proline: α_{s1}-, α_{s2}-, β- and κ- caseins contain 17, 10, 35 and 20 moles proline/mole respectively. The proline residues are fairly uniformly distributed throughout the polypeptide which creates a stable poly-L-proline-type helix but which precludes long segments of α-helical or β-sheet structures (Walstra and Jenness, 1984). Thus, the caseins have a relatively open, "denatured" structure which renders them readily hydrolysable by proteinases, which may be advantageous or disadvantageous depending on the application, and also allows them to spread readily at interfaces.

2. The caseins are relatively hydrophobic proteins: α_{s1}-, α_{s2}-, β- and κ- caseins

have hydrophobicities on the Bigelow scale of 4.89, 4.64, 5.58 and 5.12 kJ/residue, respectively. However, the hydrophobic residues are not uniformly distributed along the polypeptides.

Many of the polar residues in the caseins are clustered: this is particularly marked for the phosphoseryl residues, the clustering of which is due to the sequence adjacent to the seryl residue required to catalyse phosphorylation, i.e. an acid residue (glutamic and aspartic) second next to the serine toward the carboxyl terminal (Mepham *et al.*, 1982).

1

H.Arg-Pro-Lys-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-Glu-Val-Leu-Asn-Glu-Asn-Leu-

21

Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu-

41

Ser-Lys-Asp-Ile-Gly-*SerP*-Glu-*Serp*-Thr-Glu-Asp-Gln-Ala-Met-Glu-Asp-Ile-Lys-Gln-Met

61

Glu-Ala-Glu-*SerP*-Ile-*SerP*-*SerP*-*SerP*-Glu-Glu-Ile-Val-Pro-Asn-*SerP*-val-Glu-Gln-Lys-His-

81

Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-

101

Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-*SerP*-Ala-Glu-Glu-Arg-Leu-

121

His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-

141

Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-

161

Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-

181

Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser-Glu-Lys-Thr-Thr-Met-Pro-Leu-Trp.OH

Figure 2.1: Primary structure of the variant B of α_{s1}-casein (Mercier *et al.*, 1971; Grosclaude *et al.*, 1972, 1973, 1976; Swaisgood, 1992). Sites of post-translational phosphorylations are in italics.

Figure 2.1 shows the primary structure of the variant B of α_{s1}-casein. These

sequences were obtained chemically for each of the genetic variants (Mercier *et al.*, 1971; Grosclaude *et al.*, 1972, 1973, 1976). Hydrophobic and charged residues are not uniformly distributed in the sequence. Three hydrophobic regions are discernible, roughly including residues 1 - 44, 90 - 113 and 132 - 199. However, clustering of phosphoseryl residues is perhaps the most unique feature of the primary structure of α_{s1}-casein (Swaisgood, 1992).

1

H.Lys-Asn-Thr-Met-Glu-His-Val- *SerP-SerP-SerP*-Glu-Glu-Ser-Ile-Ile-*SerP*-Gln-Glu-Thr-Tyr-

21

Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro-Ser-Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-

41

Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly- Ser-*SerP-SerP*-Glu-Glu-

61

SerP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-

81

Ala-Leu-Asn-Glu-Ile-Asn-Glu-Phe- Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-

101

Gln-Gly-Pre-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-

121

Pro-Thr-Leu-Asn-Arg-Glu-Gln-Leu-*SerP*-Thr-*SerP*-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-

141

Met-Glu-*SerP*-Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-

161

Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-

181

Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-

201   207

Ile-Pro-Tyr-Val-Arg-Tyr-Leu.OH

Figure 2.2:   Primary structure of the variant A.11P of α_{s2}-casein (Bigelow, 1967; Stewart *et al.,* 1987; Swaisgood, 1975). Sites of post-translational phosphorylation identified in the species containing 11 orthophospate groups are italics.

Figure 2.2 shows the primary structure of α_{s2}-casein. This protein is the most hydrophilic of all the caseins with three clusters of anionic phosphoseryl and glutamyl residues in its structure, residues 8-12, 56-63 and 129-133. Only two

areas of the structure are relatively hydrophobic: residues 160-207 of the C-terminal sequence, which possesses a large net positive charge, and residues 90-120 of the central structure.

1

H.Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-*SerP*-Leu-*SerP*-*SerP*-*SerP*-Glu

21

Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln-*SerP*-Glu-Glu-Gln-Gln-Gln-

41

Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-

61

Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-

81

Pro-Val--Val--Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-val-Ser-Lys-Val-Lys-Glu-

101

Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Gln-Pro-Phe-Thr-

121

Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Pro-Leu-Leu-Leu-

141

Gln-Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-

161

Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Glu-Lys-Ala-Val-Pro-Tyr-

181

Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-

201   209

Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-.OH

Figure 2.3:   Primary structure of the β-casein variant A$_2$ (Brignon *et al.*, 1970; Grosclaude *et al.*, 1972, 1973, 1974a,b, Ribadeau-Dumas *et al.*, 1972; Jimenez-Flores *et al.*, 1987; Stewart *et al.*, 1987; Swaisgood, 1992). Sites of post-translational phosphorylation are in italics.

Figure 2.3 shows the primary structure of β-casein. Of all the caseins, β-casein is the most hydrophobic and highly charged at the N-terminal domain containing the anionic phosphoserine cluster. At pH 6.6, the N-terminal 21-

residue has a net charge of - 11.5, whereas the C-terminal domain has no net charge (Swaisgood, 1992).

1

PyroGlu-Glu-Gln-Asn-Gln-Glu-Gln-Pro-Ile-Arg-Cys-Glu-Lys-Asp-Glu-Arg-Phe-Phe-Ser-Asp-

21

Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-Leu-

41

Asn-Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu-Ile-Asn-Asn-gln-Phe-Leu-Pro-Tyr-Pro-Tyr-

61

Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Ile-Leu-Gln-Trp-Gln-Val Leu-Ser-

81

Asp-Thr-Val-Pro-Ala-Lys-Ser-Cys-Gln-Ala-Gln-Pro-*Thr*-*Thr*-Met-Ala-Arg-His-Pro-His-

101

Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-*Thr*-Glu-Ile-Pro-

121

Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-*Thr*-Ser- *Thr*-Pro-Thr-[*Thr*]-Glu-Ala-Val-Glu-

141

Ser- *Thr*-Val-Ala-*Thr*-Leu-Glu-Asp-*SerP*-Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn-

161

Thr-Val-Gln-Val-*Thr* -Ser- *Thr*-Ala-Val.OH

Figure 2.4:   Primary structure of the variant B of κ-casein (Jolles *et al.*, 1970, 1972; Brignon *et al.*, 1972; Mercier *et al.*, 1972, 1973; Alexander *et al.*, 1988; Swaisgood, 1992). Sites of post-translational phosphorylation and glycosylation are in italics.

Figure 2.4 shows the primary structure of κ-casein. Comparison of κ-casein with α_{s1}- and α_{s2}-caseins indicates two unique features of this protein, viz, the absence of phosphoseryl residue clusters and glycosylation of threonyl residues. κ-Casein is very hydrophobic with an N-terminal domain encompassing roughly residues 1-105 (para-κ-casein) and a C-terminal polar domain. Distinct separation of hydrophobic and polar domains occurs as a consequence of the specific chymosin-catalysed hydrolysis of the Phe105--Met 106 peptide bond.

Thus, all four caseins have a distinctly amphipathic character with separate hydrophilic and hydrophobic domains, which renders them particularly well structured to function as emulsifiers at lipid/water or air/water interfaces. α_{s1}- and β-Caseins have no cysteine or cystine residues, whereas α_{s2}- and κ-caseins contain two cysteines which appear to form intermolecular rather than intramolecular disulphide bonds giving rise to variably sized, covalently linked polymers.

2.3 Association properties of caseins

Caseins are prone to association due to their high hydrophobicity and peculiar charge distribution. The major interactions involved in these processes are of an electrostatic and hydrophobic nature. A delicate balance between these forces, depending on experimental conditions, such as pH, temperature and ionic strength, determines the type and extent of association. The self-association behaviour of caseins has been reviewed by Swaisgood (1992) and Rollema (1992).

2.3.1 α_{s1}- and α_{s2}-caseins

Self-association of caseins, especially α_{s1}- and α_{s2}-caseins, is initiated by ionic strength. α_{s1}-Casein exhibits self-association dimers, tetramers, hexamers etc. The degree of association depends on the pH and ionic strength of the solution (Ho and Waugh, 1965; Payens and Schmidt, 1965; Schmidt and van Markwijk, 1968; Swaisgood and Timasheff, 1968; Schmidt, 1970 a,b, 1982). An increase in pH reduces the association of α_{s1}-casein, but temperature has little effect on the association (Rollema, 1992). α_{s1}-Casein is present as a monomer at low ionic strength (0.003 - 0.01M) and at neutral or alkaline pH (Swaisgood and Timasheff, 1968; Schmidt, 1970b). At higher ionic strength (0.2 M) dimers and tetramers are favoured, and as the ionic strength decreases (0.01 M) the electrostatic repulsive energy becomes predominant and at 0.01 M ionic strength monomer - dimer equilibrium occurs and at 0.003 M only monomers are present (Ho and Waugh, 1965; Schmidt, 1970b). Similarly as the pH increases, the electrostatic repulsive forces increase, decreasing the degree

of association (Swaisgood and Timasheff, 1968).

α_{s2}-Casein is more hydrophilic than $_s\alpha$ -casein and may contain inter- or intramolecular disulphide bonds (Swaisgood, 1992). The primary structure suggests that the N-terminal is a negatively charged hydrophilic domain and the C-terminal is a positively charged hydrophobic domain. With such an amphipathic, highly charged structure, the associative properties are very dependent on the ionic strength (Snoeren *et al.,* 1980). Snoeren *et al.,* (1980) showed that α_{s2}-casein associates less extensively than α_{s1}- casein as a result of stronger electrostatic repulsive forces and lower hydrophobicity. Self-association of α_{s2}-casein reached a maximum at 0.2 - 0.3M ionic strength and then decreased (Snoeren *et al.,* 1980). The decrease in association at higher ionic strengths may be due to ionic suppression of an electrostatic interaction between the N-terminal and C-terminal domains (Snoeren *et al.,* 1980).

2.3.2 β-Casein

The molecular association of β-casein is dominated by hydrophobic interactions and is less sensitive to ionic strength than that of the α_{s1}- and α_{s2} -caseins (Swaisgood, 1992). β-Casein association is extremely temperature dependent; only monomers are observed at 0 - 4°C (Payens and van Markwijk, 1963) and an increase in temperature leads to increased association (Schmidt and Payens, 1972). As the temperature increases above 4 - 5°C, β-casein undergoes a highly reversible, rapidly equilibrating, discrete self-association yielding large micelles (Schmidt and Payens, 1972; Niki *et al.,* 1977; Andrews *et al.,* 1979; Arima *et al.,* 1979; Buchheim and Schmidt, 1979; Evans *et al.,* 1979; Takase *et al.,* 1980; Schmidt, 1982; Thurn *et al.,* 1987; Kajiwara *et al.,* 1988). It appears that there is a critical concentration above which these micelles are formed, ranging from < 0.5 mg/ml to about 2 mg/ml (Schmidt and Payens, 1972; Niki *et al.,* 1977; Evans *et al.,* 1979). This critical micelle concentration depends on ionic strength, pH and temperature. The size of the micelle has been characterized by the number of monomers in the polymers (*n*). The number of monomers, *n*, in the β-casein micelle depends on

temperature, ionic strength and probably pH (Rollema, 1992). The hydrophobic interactions between the C-terminal segments and repulsive forces between the N-terminal segments limit the micellar size (Rollema, 1992).

2.3.3 κ-Casein

κ-Casein is the second most hydrophobic within the casein group and possesses the lowest net charge (- 3.9 at pH 6.6) at neutral pH. The C-terminal carries negative charge and the N-terminal carries positive charge (Rollema, 1992). This protein forms polymer micelles, like β-casein, but they are not very sensitive to temperature or ionic strength (Swaisgood *et al.*, 1964; Vreeman, 1979; Vreeman *et al.,* 1981). Vreeman *et al.,* (1981) compared the association of κ-casein with the assembly of virus coat proteins and it has been argued that polymerization of κ-casein can be described by virus assembly theories. Due to the low ester phosphate content, κ-casein is soluble in calcium containing media. This property and its unique structure are responsible for its important role in the stabilization of casein micelles (Rollema, 1992).

It has been recognized that both oxidized (S-S-containing) and reduced (SH-containing) forms of κ-casein polymerize (Swaisgood *et al.*, 1964; Talbot and Waugh, 1970; Slattery and Evard, 1973) with the reduced form showing less association than the oxidized form.

κ-Casein association is substantially different from that of β-casein association (Rollema, 1992). In the case of κ-casein, polymers of a fixed size are formed, irrespective of temperature and ionic strength. This behaviour is partly due to the low negative charge on the C-terminal. In addition, entropic repulsion between the negatively charged C-terminal and spacial requirements play an important role in the limitation of polymer size (Vreeman, 1979).

2.3.4 Interactions of caseins with metal ions

All casein components are able to bind different divalent metal ions. The

binding of calcium to the different caseins has been studied extensively because of its relevance to the structure and stability of casein micelles (Dickson and Perkins, 1971; Waugh *et al.,* 1971; Dalgleish and Parker, 1980; Ono *et al.,* 1980; Parker and Dalgleish, 1981; Payens, 1982). Spectroscopic studies have shown that calcium binds primarily to the phosphoseryl residues (Ho and Waugh, 1965; Ono *et al.,* 1976, 1980). α_{s2}-Casein is the most calcium sensitive, precipitating at a calcium concentration of less than 2 mM (Toma and Nakai, 1973; Aoki *et al.,* 1985). α_{s1}-Casein precipitates in the range of 3 - 8 mM calcium (Bingham *et al.,* 1972; Toma and Nakai, 1973; Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Aoki *et al.,* 1985; Farrell *et al.,* 1988). β-Casein precipitates in the range of 8 - 15 mM calcium at 37°C (Parker and Dalgleish, 1981; Farrell *et al.,* 1988) but remains in solution at up to 400 mM at 1°C (Farrell *et al.,* 1988). κ-Casein remains soluble under all these conditions (Swaisgood, 1992). Calcium binding to proteins is affected by pH, temperature and ionic strength. The calcium concentration that initiates precipitation increases with increasing pH and temperature but decreases with increasing ionic strength and protein concentration (Parker and Dalgleish, 1981; Bingham *et al.,* 1972; Dalgleish and Parker, 1980; Jang and Swaisgood, 1990). κ-Casein is able to protect other caseins from calcium-induced precipitation (Thompson *et al.,* 1969; Green, 1971; Toma and Nakai, 1973). This effect is responsible for the solubility of caseins in milk. In the presence of calcium, casein complexes are formed with properties differing from those of polymers of the individual caseins (Rollema, 1992). Jang and Swaisgood (1990) have reported that these complexes have higher affinities for calcium than individual casein components.

2.4 General methods of casein manufacture

A number of reviews on the principles and methods involved in the commercial production of casein have been published (Muller, 1971, 1982; Fox, 1970; King, 1970; Southward and Walker, 1980, 1982; Mulvihill, 1989; Fox and Mulvihill, 1990). A general outline of the casein manufacture process is shown in Figure 2.5. In industry, two methods are used to destabilize the casein in milk: (I)

casein may be precipitated by lowering the pH to its isoelectric point, i.e. pH 4.6, or (II) casein may be coagulated by one of a number of proteinases, eg. rennet, which hydrolyses κ-casein and render the casein micelles susceptible to precipitation by calcium ions at the natural concentration of milk.

2.4.1 Isoelectric precipitation

The pH of the skim milk may be reduced to the isoelectric point (pH 4.6) of casein by converting some of the lactose in milk to lactic acid through the action of an added culture (producing lactic casein) or by the addition of dilute mineral acids (producing mineral acid casein).

Pasteurized skim milk at pH ~ 6.7 is mixed with sulphuric acid or hydrochloric acid to a pH of approximately 4.6 at room temperature. The mixture is then heated by direct steam injection to a temperature of 50 - 55°C to cause coagulation of the casein particles (Southward and Walker, 1980; Muller, 1982).

Whole milk ⟶ Separation ⟶ Cream

Skim milk

Pasteurization

⟵ Acid or Lactic starter

Precipitation

Cooking

Casein curd + whey
(pH 4.6)

Whey ⟵ Separation

Casein curd

Washing ⟶ Water

Separation ⟶ Water/Whey

Dewatered casein curd

Drying
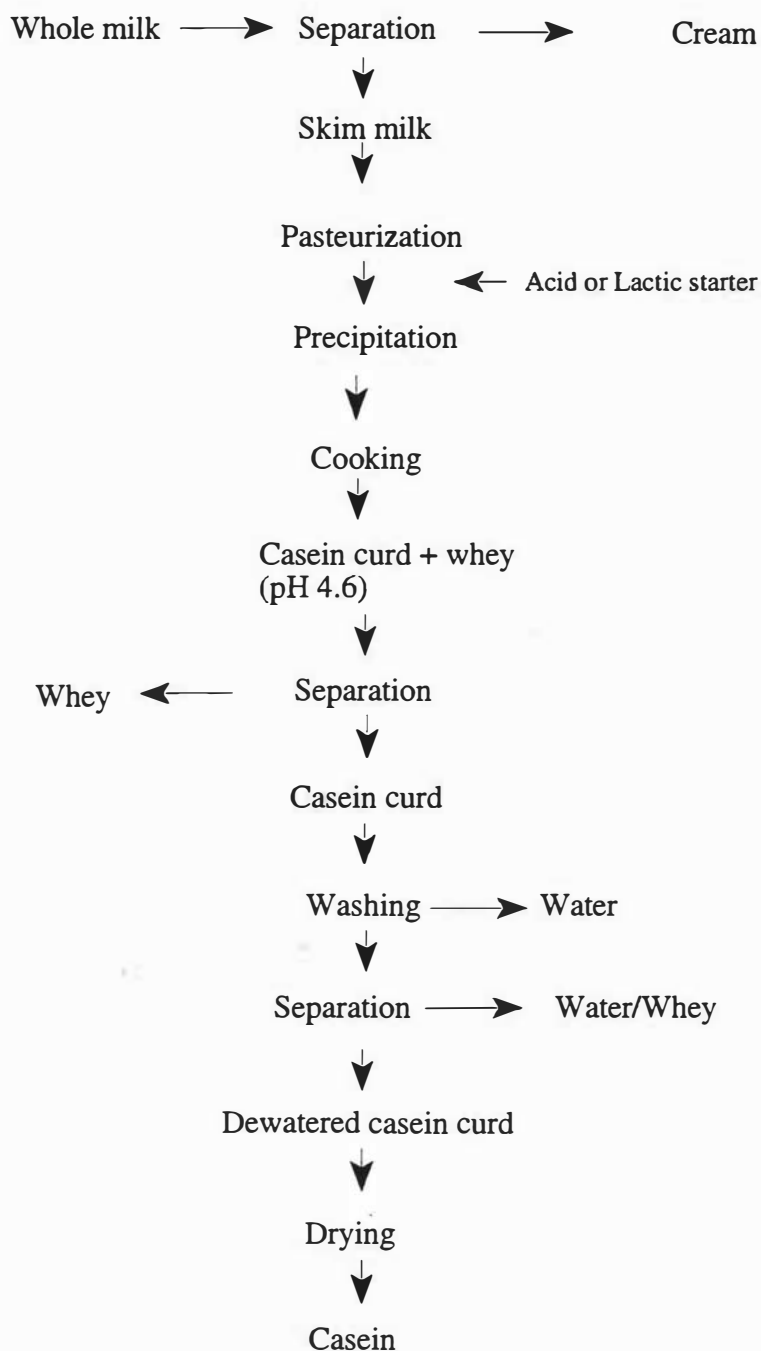
Casein

Figure 2.5: General methods of acid casein manufacture (Source Mulvihill, 1989)

In the manufacture of lactic casein, precipitation is accomplished by inoculating pasteurized skim milk with mixed or multiple defined starter cultures (Heap and Lawrence, 1984) and incubating at 22 - 26°C (Southward and Walker, 1980). During an incubation period of 14 - 16 hours, the starter ferments some of the lactose in the milk to lactic acid. As the pH of the milk slowly falls, the colloidal calcium phosphate in the casein micelles progressively dissolves (Pyne, 1962) and a casein gel network or coagulum with good water holding capacity is formed as the isoelectric pH of the casein is approached. The coagulum is then pumped to the cooking stage where direct injection heats it to about 55°C. The curd is then agitated gently to promote syneresis (Muller, 1982). When the syneresis is complete, the whey is drained off and the casein curd is washed in vats.

2.4.2 Dewheying

The production of high quality casein requires efficient separation of curd from whey (dewheying) and thorough washing to reduce the lactose content in the final product (Muller, 1971). The efficiency of the dewheying step is of the important in determining the volume of whey recovered for further processing, the efficiency of the washing operation and the quality of the final casein produced. The equipment used to achieve separation includes (a) vibratory, moving or stationary inclined screens made of nylon or fine-mesh stainless steel (Muller, 1960; King, 1970) or inclined screens made of polyester fabric laid in a cascade-like profile which subjects the curd to turning and rolling as it travels down the slope and (b) mechanical devices. Studies by Hobman and Elston (1976a) showed that the efficiency of an inclined screen for the separation of curd from whey was a function of the angle, the length of the screen and the aperture size. Continuous roller presses can also be used for removing whey from the curd, provided the temperature and pressing conditions are controlled to avoid excessive matting of the curd; the curd moisture content can be reduced to about 65%, compared with about 85% from inclined screens (Hobman and Elston, 1976b). About 20% more whey is recovered and less wash water is needed. Similar considerations have led to

the use of horizontal centrifuges for 'dewheying' (Higgs *et al.*, 1973).

2.4.3 Washing

The removal of lactose, salts and free acid from casein by thorough washing is essential in the manufacture of a high quality product. These impurities diffuse from the curd during washing with the diffusion rate depending on the size and permeability of the curd particles, and on the purity, amount, temperature and rate of movement of the wash water (Muller, 1959). The temperatures used in washing are chosen to maintain the curd in a suitable condition for agitation, pumping and separation at each stage from the wash water. Higher temperatures are needed during the washing of lactic casein to reduce to low numbers the bacteria that multiply during incubation of the milk with starter (Roeper and Elston, 1976). Temperatures of 70°C or more at the penultimate washing stage are used even with acid precipitated casein as a safeguard against bacterial contamination. The final washing stage is normally at 30 - 35°C to minimize matting of the curd during its separation from the wash water by a continuous press or horizontal centrifuge. An efficient press or centrifuge and the maintenance of optimum curd characteristics and temperatures during washing are necessary to reduce the moisture content of the curd to 55 - 60% and minimize the amount of water to be evaporated during drying (Muller, 1982).

2.4.4 Dewatering

Casein curd is mechanically dewatered following washing to produce a curd of minimum moisture content to reduce the quantity of water to be evaporated and thus minimize the energy required during drying. The properties of the casein curd following washing should allow maximum dewatering while maintaining the curd in a suitable condition for drying (Southward, 1971). Vu and Munro (1981) performed constant-pressure expression experiments on casein curd at room temperature (17 - 20°C), and found that at high applied pressures (> 104 kPa) the appearance of the drainage surface of the casein curd changed from opaque white to translucent creamy-brown. They referred to the phenomenon

as surface sealing or plasticization, which has a detrimental effect on dewatering and on drying, because the plasticized curd layer at the drainage surface acts as a relatively impermeable barrier to further moisture flow (Vu and Munro, 1981; Espie *et al.,* 1984). The physical properties of the curd were also influenced by the dewatering temperature and each dewatering machine had a characteristic temperature below which curd plasticization did not occur under the operating conditions used, i.e. ~ 30°C for the screw press and ~ 39°C for the roller press and decanter centrifuge (Munro *et al.,* 1983).

2.4.5 Drying

Several types of equipment have been developed for drying casein (Muller, 1971). To produce a stable, storable product that meets the international compositional standards for edible grade product recommended by the International Dairy Federation (1969), the casein curd is dried to less than 12% moisture. Traditionally driers were a semi-fluidized, vibrating type in which casein curd passed along vibrating perforated stainless steel conveyors while warm air was forced up through the perforations, partially fluidizing the curd which was normally minced to reduce it to evenly sized particles before entering the drier (King, 1970). Energy requirements for a number of driers of this type have been compared by Patchett (1968, 1969). The drying characteristics of a number of casein curd types have been determined in a laboratory-scale fluidized bed drier by Espie *et al.* (1984).

Dried casein is relatively hot as it emerges from the drier and the moisture content of the individual particles varies. Therefore, it is necessary to temper and blend the dried product to achieve a cooled final product of uniform moisture content (Muller, 1960; King, 1970). This is usually achieved by pneumatic circulation of the curd between a number of holding bins.

Casein is ground in roller or pin-disc mills as a final stage in manufacture to produce the small-size particles required by users of casein (Hobman, 1976).

2.5 General methods of caseinate manufacture

2.5.1 Sodium caseinate

Soluble caseinates have sodium, ammonium, potassium or magnesium as the cation. To obtain a bland flavoured caseinate, it is normal to use a fresh acid casein curd, dissolved in the appropriate alkali and spray dried (Muller, 1971). Several reviews have been published on the manufacture of spray-dried sodium caseinate (Muller, 1971, 1982; Towler, 1976; Mulvihill, 1989). Figure 2.6 shows the basic steps involved in the manufacture of spray-dried caseinates.

Addition of alkali and pH control are the most important steps in the manufacture of sodium caseinate. The alkali addition should be carefully controlled until a desired pH of 6.6 - 7.0 is obtained. Roeper (1974) found that from 4.3 to 7.6 ml of 0.1 M NaOH was required per gram of dry casein to convert an aqueous dispersion of a well-washed acid casein to a 5% sodium caseinate solution with a pH of 6.7 at 20°C.

Acid casein curd (Or Dry casein & Water)

↓

Colloid Mill ◄─── Water to 20% TS

↓

Mixer ◄─── NaOH solution to pH ~6.6
OR Ca(OH)$_2$ with Ca
~1.3%

↓

pH measurements

Dissolving Vat No 1,
Vigorous agitation

↓

Dissolving Vat No 2,
Vigorous agitation ◄─── Heat to ~75°C

↓

Heat exchanger ◄─── ~100°C

Viscosity measurements and → ↓
hot water addition if necessary

Spray drying ◄─── Inlet 300°C and
Outlet 90°C

↓
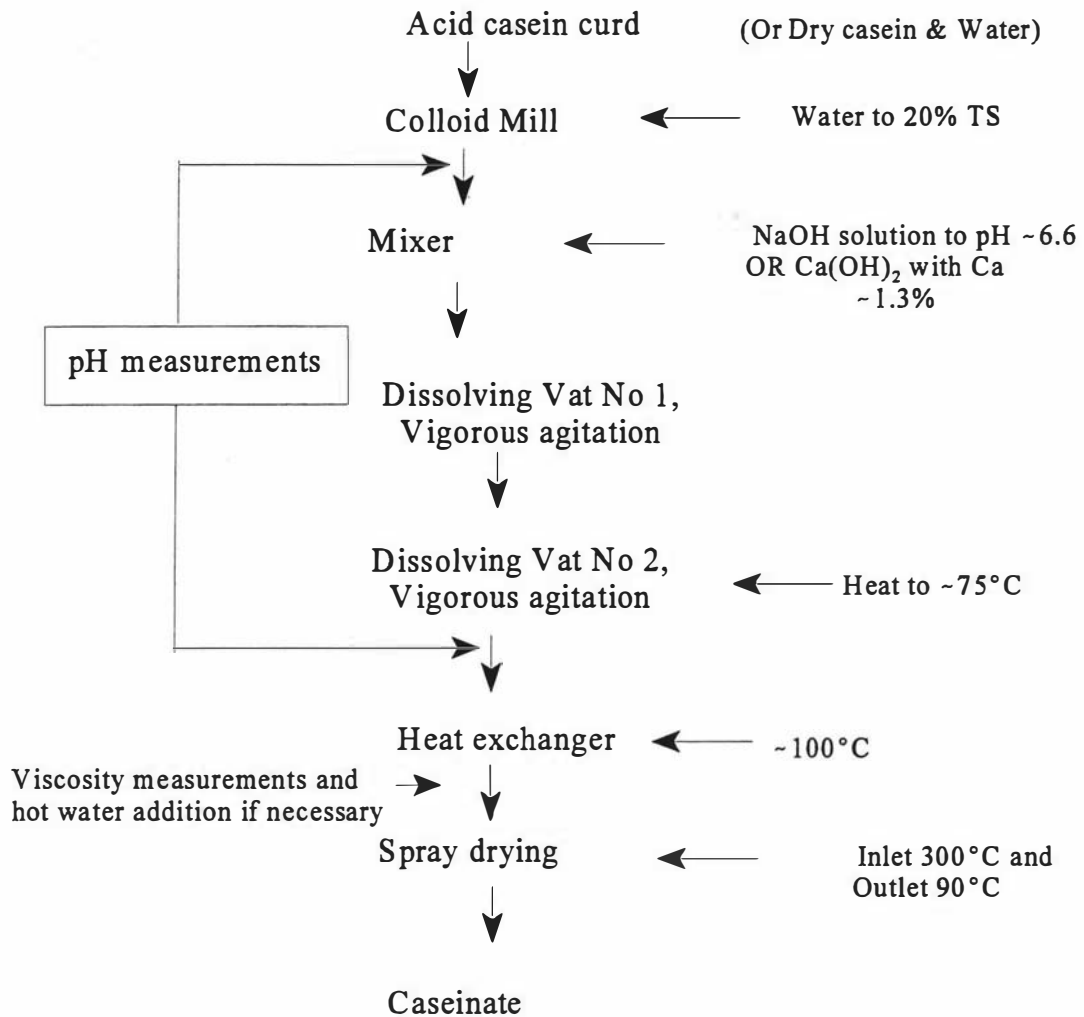
Caseinate

Figure 2.6: Caseinate manufacture (Source: Mulvihill, 1989).

The main difficulties in the manufacture of spray-dried caseinate are related to the logarithmic increase in the viscosity of caseinate solutions as their concentration increases and the tendency for the reaction of the alkali to be impeded by relatively impervious gels which form on the surface of casein particles in the presence of alkali. A satisfactory dissolving technique involves adding fresh, colloid milled wet curd and alkali progressively to water in a vat

equipped with a powerful agitator and the use of a large capacity centrifugal pump to apply shearing forces to curd particles during recirculation.

A detailed study by Towler (1976) on the factors affecting the rate of conversion of curd to sodium caseinate led to the proposal that the fresh curd and water be passed first through a colloid mill to give slurry of about 25% solids content, and then mixed with NaOH solution before passing into an agitated vat with recirculation facilities. Careful control of pH is essential. The minimum viscosity for sodium caseinate is in the pH range of 6.6 - 7.0 (Hayes and Muller, 1961). In practice it is better to maintain a pH below this value during dissolving and make any adjustment when the curd is finally in solution (Towler, 1976). It is also important to avoid exposing casein to high pH and temperature during dissolving as this can lead to loss of lysine and serine, with the formation of lysinoalanine (Creamer and Matheson, 1977).

The concentration at which the slurry can be spray dried is normally limited to only about 20% solids at 90 - 95°C due to the viscosity of the soluble caseinates. There is an optimum viscosity for spray drying, depending on the atomization system used. It is better to standardize the viscosity and temperature of the solution to be spray dried rather than to standardize on solids content as the viscosity of casein may vary throughout the year (Towler, 1976).

2.5.2 Calcium caseinate

Manufacturing details for calcium caseinate are similar to those for sodium caseinate. Calcium caseinate should preferably be prepared from freshly precipitated and washed casein curd (Roeper, 1977). This casein curd should have a moisture content of ~ 65% because low moisture curd may lead to an increase in the amount of sedimentable material in the dispersion. During the dissolving process, it has been found that the reaction between acid casein curd and calcium hydroxide proceeds at a much slower rate than that between sodium hydroxide and casein curd during sodium caseinate manufacture

(Roeper, 1977).

2.5.3 Composition of casein and caseinates

Table 2.2 : Approximate composition of commercial casein and caseinate products (on dry matter basis) (Source: Morr, 1982).

Component	Sodium caseinate	Calcium caseinate	Acid casein
Protein N x 6.38 (min)	94	93.5	95
Ash (max)	4.0	4.5	2.2
Sodium	1.3	0.05	0.1
Calcium	0.1	1.5	0.08
Phosphorus	0.8	0.8	0.9
Lactose (max)	0.2	0.2	0.2
Fat (max)	1.5	1.5	1.5
Moisture (max)	4.0	4.0	10.0
pH	6.6	6.8	--

Sodium caseinate typically contains about 1.2 - 1.4% sodium and small amounts (~ 0.1%) of calcium, whereas calcium caseinate contains 1.5% calcium and small amounts (~ 0.05%) of sodium. The caseinates are similar in broad composition to dry acid casein, but have a lower moisture content of about 4% and a higher ash content because of the alkali used in dissolution.

2.6 Effect of heat processing on caseinate solutions

As discussed earlier, all four caseins have a distinct amphiphatic character with separate hydrophobic and hydrophilic domains; this character is enhanced in α_{s1}-, α_{s2}-, and β-caseins by phosphorylation of seryl and threonyl residues. In addition, a high content and uniform distribution of proline residues along the polypeptide chains result in low levels of secondary and tertiary structures, which contributes to their stability at high temperatures. Number of reviews on heat-induced changes in caseins are available Singh and Fox (1989); Singh and Latham (1993); Zin El-Din and Aoki (1993); Singh, (1995); Guo *et al.*

(1989, 1996).

2.6.1 Aggregation

Caseinates in solution are very stable to heat treatment. Guo *et al.* (1989) measured turbidities of 1% sodium caseinate solution, at pH 7.0, after heating at 120 - 150°C for 60 min. Aggregation increased, as shown by the increase in turbidity, during heating at 130 - 140°C. Upon heating at 140°C, β-casein aggregated strongly, but aggregation seemed to be reversible on cooling to 0 - 20°C (Guo *et al.*, 1989). Kresheck *et al.* (1964) used light scattering to study the aggregation behaviour of whole and individual caseins at temperatures up to 90°C. Heat treatment had little effect on the number or weight average molecular weight or on the radius of gyration of whole casein; when individual caseins were studied separately, only κ-casein showed a marked increase in molecular weight and radius of gyration on heating at 90°C.

2.6.2 Dephosphorylation

Heating at high temperatures (110 - 140°C) causes dephosphorylation of caseinate solutions (Belec and Jenness, 1962a,b; Guo *et al.*, 1989). Sodium caseinate is completely dephosphorylated on heating at 120°C for 5 hours or 140°C for 1 hour and 50% dephosphorylation occurs within 1 hour at 120°C (Howat and Wright, 1934; Belec and Jenness, 1962a, b; Guo *et al.*, 1989; Singh, 1995). Dephosphorylation of calcium caseinate is slower than that of sodium caseinate. Howat and Wright (1934) showed that only 85% of phosphorus was released when calcium caseinate was heated at 120°C for 5 hours; α_s-casein was more susceptible to dephosphorylation than β-casein but dephosphorylation of whole casein occurred approximately at the same rate as that of α_s-casein (Belec and Jenness, 1962a).

2.6.3 Covalent changes

Heating may lead to the formation of inter- or intramolecular covalent bonds between residues of caseinates at temperatures ranging from 100 to 140°C (Van Boekel *et al.*, 1989; Singh, 1995; Cruijsen 1996). Guo *et al.* (1989)

showed some significant changes on heating sodium caseinate solution at 120°C for 1 hour using SDS-PAGE. The intensity of all casein bands decreased after heating, whereas the intensity in the region of larger protein molecules increased, probably due to aggregation.

2.6.4 Proteolysis

Formation of non-protein nitrogen (NPN) from sodium caseinate above 100°C is almost linear with time; 15-20% is solubilized after 5 hours at 120°C or 1 hour at 140°C (Howat and Wright, 1934; Guo *et al.*, 1989). Electrophoresis in urea-containing buffers and chromatography on Sephadex or Biogel showed altered patterns for heating casein (Alais *et al.*, 1966). Using gel electrophoresis, Guo *et al.* (1989) showed that sodium caseinate in solution underwent extensive degradation on heating at 120-140°C, resulting in the formation of several fast-moving, weakly staining polypeptide bands, some of which were soluble at pH 4.6 or in 12% TCA. When isolated κ-casein is heated at 120°C for 20 min, a 12% TCA-soluble peptide is released which is similar to that released by the action of chymosin on casein (Alais *et al.*, 1966).

2.7 Emulsion formation

The process of emulsification requires mechanical agitation of oil and water together with a surface active material, such as protein. Various mechanisms of emulsion formation have been described by Walstra (1993a). In general, emulsification involves three main processes.

- Oil droplets are formed, deformed and then possibly broken up into smaller droplets.
- The surface active material is adsorbed on to the newly formed droplets.
- Droplets encounter each other which, depending on the environmental conditions, may lead to the formation of a stable or unstable emulsion.

A wide variety of equipment is used in the formation of emulsions. The high pressure valve homogenizer is still the most commonly used equipment for the

formation of protein-stabilized emulsions (Tornberg, 1978a,b; Dickinson *et al.*, 1984; Leman *et al.*, 1988; Mulvihill and Murphy, 1991; Euston *et al.*, 1995; Srinivasan *et al.*, 1996). In a homogenizer, droplets are disrupted by a combination of intense laminar and turbulent flow (Walstra, 1983; Phipps, 1985; Davies, 1985). Pressure drop across the homogenizer valve, and increased turbulence on the low-pressure side of the valve, favours the formation of finer emulsions and is the main factor affecting the emulsions droplet-size distribution. The main experimental difficulty in using valve homogenizers is that small samples cannot be homogenized (Dickinson and Stainsby, 1988).

A new method of emulsification, called 'microfluidization' has been extensively used by Robin and Paquin (1991); Agboola and Dalgleish (1995); Hunt and Dalgleish (1994); Fang and Dalgleish (1993); Dalgleish *et al.* (1995). With microfluidization there are no moving parts and the energy of the moving streams is released extremely rapidly. The manufacturers claim that very fine emulsions with a narrow particle size distribution can be obtained.

It has also been shown that the particle size of protein-stabilized emulsions can vary with homogenization conditions and the type of equipment being used (Tornberg and Hermanson, 1977; Tornberg, 1978a). However, various other factors e.g. solubility of the surfactant in the continuous phase, concentration of the surfactant and the viscosity of the continuous phase, may also affect the particle size distribution.

Several studies on the influence of protein concentration on the oil droplet size of emulsions have been published recently (Tomberg *et al.*, 1990; Fang and Dalgleish, 1993; Hunt and Dalgleish, 1994; Agboola and Dalgleish, 1995; Srinivasan *et al.*, 1996). Tornberg *et al.* (1990) reported that increasing the protein concentration from 5 to 37.5 mg protein/g oil decreased the droplet size to a certain level; thereafter the droplet size remained constant. However, Fang and Dalgleish (1993) reported that the particle size in emulsions (20% soya oil)

was independent of casein concentration above 1%. Large droplets were present in emulsions made using less than 0.3% casein. Srinivasan *et al.* (1996) showed no effect on the droplet diameter in emulsions containing 30% oil with increasing concentration of caseinate from 1.0 to 7.5%.

2.8 Adsorption behaviour of proteins at the oil-in-water interface

The proteins are amphiphilic in nature which makes them suitable for adsorption at an oil/water interface. The adsorption of protein at the oil/water interface involves the following steps:

- protein migration from the aqueous phase,
- reduction in the interfacial tension,
- unfolding and spreading at the interface, and
- rearrangement of the adsorbed protein layer.

The major driving force for protein adsorption is the reduction in the contact area between non-polar groups and water, both at the oil-water interface and at the surface of protein (Dickinson and Stainsby, 1982). By adsorbing at the interface, the protein reduces the free energy of the system and hence the interfacial tension (Phillips, 1981). A protein molecule that spreads out a lot, and thus has a substantial proportion of its non-polar residues in contact with the surface is one which is also very effective in reducing the interfacial tension (Dickinson *et al.*, 1988a). A flexible protein such as casein, with higher proportions of non-polar groups is more effective in reducing the interfacial tension than globular proteins with fewer non-polar groups (Dickinson *et al.*, 1988a; Dickinson and McClements, 1995).

Once adsorbed at the interface, the protein undergoes a process of unfolding in order to attain the lowest free energy with its new environment (Dickinson and McClements, 1995). The rate and extent of unfolding of protein at the interface also depend on the flexibility of the protein molecule. Usually, the protein will reorientate itself so that substantial proportions of the non-polar amino acids are in contact with the oil phase, and the polar groups are in

contact with the aqueous phase. Depending on the relative hydrophobicity of peptide segments in a protein molecule, the adsorbed protein can assume conformations such as loops, trains and tails as shown in Figure 2.7. Dickinson (1994) reported that approximately 70% of the adsorbed β-casein monolayer is closely associated with the hydrocarbon oil/water interface in the form of trains and small loops. The rest of the segments reside in large loops and long hydrophilic tails.

The final conformation of the protein at the interface will depend on several factors such as type and concentration of protein and the state of aggregation of protein in the bulk phase. A flexible, random coil protein like β-casein unfolds more rapidly at the interface than a globular protein such as β-lactoglobulin (Figure 2.8). Formation of these loops and tails is favoured with increasing concentration of protein. At low concentration, most polypeptides have their amino acid backbone lying along the interface with few loops and tails. As the concentration increases, the polypeptides are more closely packed and hence more loops and tails are formed. Finally, the looped conformation creates enough electrostatic repulsion and steric hindrance that further protein adsorption becomes energetically unfavourable. The protein concentration at the interface is at the saturated level, and more concentrated solutions give rise to multilayer adsorption (Graham and Phillips, 1979a,b).
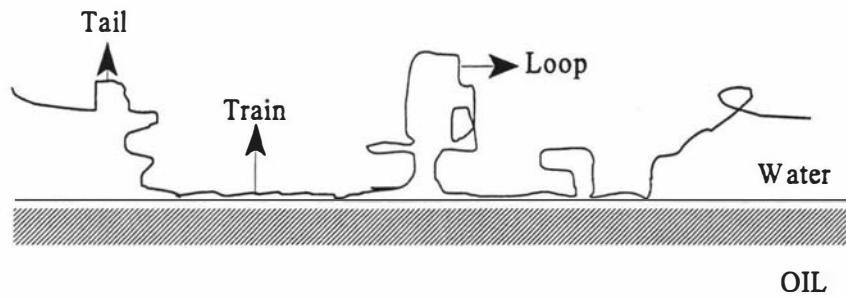
Figure 2.7: Schematic representation of linear polymer adsorbing at a plane surface according to train-loop-tail model (Dickinson, 1994)
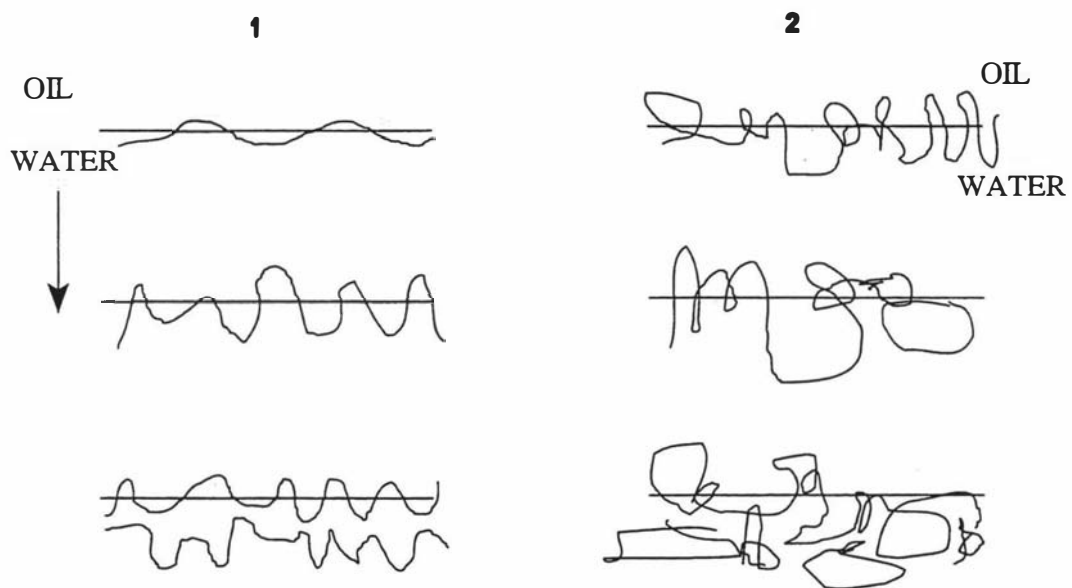

Figure 2.8: Protein structure at fluid interface (Phillips, 1977): (1) flexible, random coil proteins; (2) globular, highly structured proteins. Arrow denotes increasing protein concentration.

2.8.1 Kinetics of adsorption

Adsorption requires transport of protein material from bulk solution to the surface; this may take place by diffusion, convection or a combination of the two. In quiescent conditions, adsorption of proteins at the oil/water interface occurs via diffusion of the protein molecule from the bulk phase to the interface (Ward and Tordai, 1946; MacRitchie and Alexander, 1963; MacRitchie, 1978; Graham and Phillips, 1979 a,b,c). According to the diffusion-controlled adsorption theory, the rate of adsorption is given by (Ward and Tordai, 1946):

$$\frac{d\,\Gamma}{dt} = c_0 \left(\frac{D}{\pi t}\right)^{1/2}$$

Where Γ is the surface protein concentration, t is time, D is the diffusion coefficient, and c_0 is the initial bulk protein concentration. This equation applies only in the early stages of adsorption at a clean interface, when the protein concentration in the immediate sub-surface region is much less than c_0.

The total adsorbed protein as a function of time, therefore, can be given by:

$$\Gamma = 2\,c_0\,(Dt\,/\,\pi)^{1/2}$$

This equation provides the normal experimental test for diffusion-controlled adsorption; the plot of Γ vs. $t^{1/2}$ is linear and passes through the origin. There is reasonably convincing evidence in the literature for diffusion-controlled adsorption with a variety of proteins, though quantitative agreements with the theory have rarely been demonstrated (Dickinson and Stainsby, 1988). Under diffusion-controlled adsorption conditions, smaller protein molecules adsorb in preference to larger protein molecules (Fisher and Mitchell, 1992).

Although the theory of diffusion explains well the adsorption of molecules when the process occurs at rest, the situation is different under dynamic conditions. The emulsification process involves homogenization and, during the highly dynamic flow conditions of homogenization, much of the protein material is

transported to the oil/water interface by convection rather than by diffusion (Walstra and Oortwijn, 1982; Walstra, 1983). Walstra and Oortwijn (1982) studied the kinetics of adsorption of milk proteins during homogenization. In the initial stages, the surface protein concentration (Γ) as a function of time (t) is given by:

$$\Gamma\ (t) = K\ C_0\ d_g\ (1 + d_p\ /\ d_g)^3\ t$$

where C_0 is the bulk protein concentration, d_g and d_p are the oil droplet and protein particle sizes, respectively, and K is a constant. From this equation it can be seen that the rate of adsorption is proportional to the concentration of protein and the size of adsorbing protein molecules. In contrast to diffusion-controlled adsorption, the equation indicates that larger protein particles adsorb in preference to smaller protein particles. The equation also predicts that the preference for larger protein particles (e.g. casein micelles in milk) is greater in the case of small oil droplets, the consequence of which is that smaller oil droplets will acquire higher surface protein loads.

2.8.2 Surface protein coverage

As the adsorbed proteins form an interfacial film around the oil droplets, it is important to know how much protein is present at the interface. The amount of protein present at the interface per unit surface of dispersed phase is termed surface concentration or protein load (Γ), usually expressed as milligrams of protein per unit area of the dispersed phase. Surface concentration may determine the amount of protein required to make an emulsion with a desired oil volume and droplet size (Walstra, 1984). If the protein forms large aggregates in solution, a higher amount of protein is required to form a stable emulsion, which also suggests that the protein is a poor emulsifier. The surface concentration is also related to coalescence stability; low surface concentration enhances the coalescence process (Walstra, 1987). A technique commonly used for the determination of surface protein concentration is called the 'depletion' technique. In this technique, the emulsion is centrifuged to separate

the aqueous phase from the oil droplets, and the concentration of protein remaining in the aqueous phase is determined (Oortwijn and Walstra, 1979; de Feijter *et al.*, 1987; Dickinson and Stainsby, 1988; Srinivasan *et al.*, 1996). An alternative method, suitable for low-fat emulsions, was developed by Sharma *et al.* (1996). This method involved direct measurement of the surface protein concentration by determining the protein and fat contents of the cream phase after separation by centrifugation. Hunt and Dalgleish (1994) described similar a method for emulsions containing 20% oil, using SDS-PAGE on the separated cream layer. Whatever method is used, there are always some practical difficulties in the separation of the aqueous phase layer from the emulsion (Hunt and Dalgleish, 1994).

2.8.3 Factors affecting surface protein coverage

There are number of factors that affect the surface protein coverage in emulsions. Some of the important factors are:

- protein concentration
- oil concentration
- homogenization conditions
- type and aggregation state of protein
- ionic strength

In general, surface protein coverage at the oil/water interface increases with an increase in the bulk protein concentration. For most protein-stabilized emulsions, the surface protein coverage reaches a maximum value, and thereafter it remains constant (Walstra, 1988). Fang and Dalgleish (1993) reported that, for an emulsion containing 20% oil, a casein concentration of about 0.3% is sufficient to cover the interface (or to form a monolayer). With further increase in caseinate concentration (> 3.0%), the surface protein coverage increased to a maximum, of about 3 mg/m^2, suggesting a saturated monolayer coverage. Srinivasan *et al.* (1996) reported that in emulsions containing 30% soya oil and varying caseinate concentrations (from 1 to 7.5%), made using a valve homogenizer, the surface protein coverage increased with

an increase in caseinate concentration up to 2% but with further increase in caseinate concentration (from 2 to 4%) the surface protein coverage did not change significantly, which probably corresponded to saturated monolayer coverage of adsorbed casein molecules. When the caseinate concentration was > 4%, the surface protein coverage increased sharply, which was attributed to the formation of secondary layers or multilayers at the interface.

Varying the volume of oil before emulsification directly affects the oil droplet size and the area of the dispersed phase. A high volume of oil may result in large-sized droplets due to coalescence during emulsification, which may lead to high protein load at the interface (Oortwijn and Walstra, 1979; Tomberg, 1980; Haque *et al.*, 1988). The probability of newly created droplets not coalescing depends on the time available for the interfaces to be covered by proteins in such a way that coalescence is prevented. Phipps (1985) reported that at high oil volume concentration, the probability of encounters between droplets increases, leading to coalescence. In contrast, Srinivasan *et al.* (1996) reported that increasing the volume of oil led to an increase in the droplet diameter and reduced the surface protein load, even though the experimental conditions were slightly different.

The homogenization conditions during emulsification are important in determining the surface protein coverage. Increasing the homogenization pressure decreases the oil droplet size as long as there is enough protein to cover the increased oil surface area in such a way that coalescence is prevented. The ratio of oil surface area to protein concentration is a crucial factor in determining the surface protein coverage (Walstra, 1988; Tomberg *et al.*, 1990). Several studies have been published on the effect of homogenization pressure on surface protein coverage (Tomberg, 1978a,b; Murphy and Fox, 1991; Mulvihill and Murphy, 1991; Srinivasan *et al.*, 1996). The surface protein coverage is also affected by the type of homogenizer (Todt, 1976); when milk fat was emulsified in skim milk with a valve homogenizer, a surface protein coverage of 12 mg/m^2 was obtained compared with to a surface

protein load of ~ 5 mg/m² obtained in a blender.

The aggregation state of the protein in solution is another important factor in determining the surface protein coverage. The relationship between surface protein coverage and aggregation state have been demonstrated by Oortwijn and Walstra (1979), Mulvihill and Murphy (1991) and Singh *et al*. (1993). Mulvihill and Murphy (1991) reported that the surface protein loads were highest for highly aggregated micellar-casein-stabilized emulsions, lower for the less aggregated ethanol-precipitated casein and high calcium-caseinate-stabilized emulsions, and lowest for the least aggregated, low calcium, sodium and ammonium caseinate-stabilized emulsions. A similar relationship between protein load and state of aggregation of the milk protein used in emulsion preparation was also observed by Oortwijn and Walstra (1979), who reported protein load values of 2.5, 2.6 and 20 mg/m² in emulsions stabilized by whey protein, sodium caseinate and casein micelles, respectively. Singh *et al*. (1993) reported higher protein loads for emulsions stabilized by the whey protein/κ-casein-rich fractions (obtained by centrifugation of heated reconstituted concentrated milk at 25,000 g for 30 min) than for those stabilized by sodium caseinate or whey protein isolate. They suggested that this was due to differences in the sizes of protein aggregates in solution. When aggregated protein is adsorbed at the droplet surface, the protein layer close to the oil surface (primary protein layer) is strongly bound to the oil surface compared with the subsequent protein layer (secondary protein layer) which can be desorbed easily (Dickinson *et al*., 1988b).

In general, increasing ionic strength of caseinate solution before emulsification leads to an increase in the surface protein coverage. Tornberg (1978b) found that addition of salt, 0.2 M NaCl, enhanced the protein adsorption at the fat globule surface in sodium caseinate-stabilized emulsions. A similar effect was observed by Srinivasan *et al*. (1996) when various concentrations of $CaCl_2$ (from 0.02 to 0.20%) were added to sodium caseinate emulsions prior to emulsification. The increased surface protein adsorption at high ionic strength

has been attributed to closer packing of the protein at the surface (Tornberg, 1978b).

2.8.4 Thickness of the adsorbed layers of protein

Attempts have been made by different workers to study the thickness of the adsorbed layer of proteins (Mackie *et al.*, 1991; Dickinson *et al.*, 1993; Fang and Dalgleish 1993; Dalgleish, 1995). Caseins form much thicker adsorbed layers than whey proteins, as a result of their extended and flexible conformations (Dalgleish, 1990). This has been confirmed by a number of studies, using various techniques such as the combination of small-angle X-ray scattering with photo correlation spectroscopy (Mackie *et al.*, 1991) and dynamic laser light scattering (Dalgleish, 1993; Fang and Dalgleish, 1993).

Fang and Dalgleish (1993) found that the thickness of the adsorbed layer, determined by trypsin hydrolysis of the adsorbed protein was dependent on casein concentration. Emulsions (20% oil) with low surface coverage (about 1 mg/m^2) had a thinner layer (about 5 nm) compared with emulsions with high surface protein coverage (about 10 nm for 3 mg/m^2). They suggested that casein adopted 'extended' or 'compact' conformations depending on the amount of casein available on the interface. As expected, at low surface protein coverage, casein spreads as much as possible to cover the maximum interfacial area to form stable emulsions, whereas, at high surface protein coverage, the protein molecules were crowded together and probably do not need to spread. In an another study, Dalgleish *et al.* (1995) reported that the layer thickness was not concentration dependent. They concluded that the caseinate used in these experiments (commercial sodium caseinate) behaved differently from the freeze-dried laboratory-made caseinate used by Fang and Dalgleish (1993). Fang and Dalgleish (1993) also noted that when casein solution was added to the emulsions (6 mg/ml of casein solution was added to 3 ml of emulsion), after emulsification, containing low surface protein coverage, there was an increase in layer thickness. This observation suggests that the adsorption of casein is not completely irreversible or at least that the protein is

mobile on the interface (Dalgleish, 1995).

The thickness of the adsorbed layer in sodium-caseinate-stabilized emulsions was also affected by the presence of low molecular weight surfactant (Dalgleish *et al.*, 1995). Dalgleish *et al.* (1995) reported that in emulsions containing 20% oil and various amounts of commercial sodium caseinate in the presence of Tween, the hydrodynamic thickness of the adsorbed protein layer decreased with increase in the concentration of Tween.

2.8.5 Competitive adsorption

Food emulsions are generally prepared using mixtures of proteins, with or without other surfactants. The composition of the interfacial layer is established by competition between these surfactants during homogenization, but true equilibrium is rarely established. In such cases, there is competition between different proteins/emulsifiers in occupying the interface. For example, purified α_s- and β-caseins compete for the emulsion interface both during and after homogenization (Dickinson *et al.*, 1988b), whereas in whole caseinate, the same proteins compete less with one another (Robson and Dalgleish, 1987). The interface may therefore be occupied by more than one emulsifier in different amounts. The composition of the interface may change with time, bulk concentration of surface active materials, mode of addition etc. Studies on competitive adsorption have been carried out by Dickinson and associates using model systems containing pure proteins (Dickinson, 1986, 1989; Castle *et al.*, 1987; Dickinson and Rolfe 1989; Dickinson *et al.*, 1989, 1990, 1993; Dickinson and Tanai, 1992; Dickinson and Iveson, 1993; Courthaudon *et al.*, 1991a,b,c,d, Chen and Dickinson, 1993; 1995).

In general, studies of two different types of competitive adsorption have been carried out: competition between two different types of proteins and competition between protein and low molecular weight surfactants.

2.8.5.1 Competitive adsorption between two types of proteins

In emulsions (20% n-tetradecane) stabilized by binary mixtures of pure α_{s1}- and β-caseins (0.5% total protein), β-casein showed a preferential adsorption at the interface during or shortly after homogenization (Dickinson *et al.*, 1988b). However, as concentrations of α_{s1}-casein in the bulk phase were increased replacement of β-casein with α_{s1}-casein at the interface was observed. In emulsion systems where at least one of the proteins is globular (e.g. β-lactoglobulin), the ability of one protein to competitively displace another from the oil/water interface is slow and limited (Dickinson *et al.*, 1989).

Although sodium caseinate is mainly composed of a mixture of α_{s1}- and β-caseins, it behaves rather differently at the oil/water interface from a simple binary mixture of the two major caseins. Various studies have reported the existence of competition among proteins for adsorption in sodium caseinate emulsions (Robson and Dalgleish, 1987; Hunt and Dalgleish, 1994; Srinivasan *et al.*, 1996; Sharma and Singh, 1998). In high fat (~ 44% w/w) sodium-caseinate-stabilized emulsions containing protein concentrations in the range from ~ 27 to ~ 64 g/kg fat, Robson and Dalgleish (1987) reported no clear preference for adsorption by either α_{s1}- or β-casein immediately after homogenization. However, on aging the emulsions for several hours, some partial replacement of α_{s1}-casein by β-casein was observed. This result can be explained by the fact that the protein molecules in sodium caseinate are substantially aggregated. Both proteins go to the interface in the form of proteinaceous aggregates and the strong interaction between the two casein components at the interface then greatly reduces the kinetics of competitive adsorption (Dickinson *et al.*, 1989).

A later study by Hunt and Dalgleish (1994) found no preference for adsorption by either α_{s}-casein or β-casein at the oil/water interface either immediately after homogenization or on aging the emulsions formed with 20% soya oil and 1.0 - 3.0% protein. Hunt and Dalgleish (1994) found preferential adsorption of caseinates over whey protein. In a recent study by Srinivasan *et al.* (1996) on

emulsions containing 30% soya oil, the preferential adsorption of caseins was dependent on the protein concentration used; at protein concentrations below 2%, β-casein was adsorbed at the interface in preference to other caseins, but, at higher protein concentrations there was no clear preference for any of the caseins. An increase in the caseinate concentration in solution may affect the aggregation state of the caseins, especially that of β-casein, resulting in different interfacial behaviour and composition (Srinivasan *et al.*, 1996). In contrast, another recent study by Sharma and Singh (1998) reported that in low fat emulsions (4% milkfat) α_s-casein was adsorbed in preference to β-casein irrespective of the protein concentration in the emulsions.

2.8.5.2 Competitive adsorption between caseinate and surfactant

Competitive adsorption between surfactants (low-molecular weight emulsifiers) and proteins in oil-in-water emulsions have been reported widely in recent years. Studies on the interactions between milk protein and various surfactants have demonstrated that many surfactant molecules can displace protein from the oil/water interfaces (Courthaudon *et al.,* 1991a,b,c,d; Dickinson and Tanai, 1992; Fang and Dalgleish,1996; Euston *et al.*,1995, 1996; Hunt and Dalgleish, 1996). The degree of displacement depends not only on the specific surfactant used, but also on when it is added (i.e. before or after emulsion formation).

Competitive adsorption in model emulsion systems stabilized by pure milk proteins has shown that the addition of a water-soluble non-ionic surfactant after emulsification leads to complete removal of protein from the droplet surface at high surfactant/protein ratios (Courthaudon *et al.*, 1991a,b,c). It has also been demonstrated that the presence of an oil-soluble non-ionic or zwitterionic surfactant during emulsification leads to a reduction in protein loads of the resulting emulsion (Courthaudon *et al.,* 1991c,d).

Euston *et al.* (1995) found that addition of water soluble surfactant (Tween 60) caused a decrease in the proportion of β-casein and an increase in α_{s1}-casein at the droplet surface, especially at protein concentrations of 0.5% and 1.0%.

At higher caseinate concentration (2.0%), the casein composition at the surface remained approximately the same regardless of added Tween 60 (Euston *et al.,* 1995). An oil-soluble surfactant (Span 60) showed similar behaviour to the water-soluble surfactant (Tween 60) on partial displacement of caseinate from the oil/water interface. However, of the two surfactants, Tween 60 was the more effective in reducing surface protein concentration (Euston *et al.,* 1995).

2.9 Emulsion stability

The capacity of proteins to stabilize emulsions is the most important criterion in most food applications. A stable emulsion is one that undergoes changes rather slowly. Among other functions, including reduction of interfacial tension, an emulsifier reduces the tendency of dispersed droplets to coalesce. This is enhanced if the emulsion possesses a net repulsive force. The forces involved in stabilizing and destabilizing emulsions include van der Waals' attractive forces, electrostatic interactions and steric factors related to surface active agents, osmotic effects, hydration forces and viscosity of the continuous medium (Parker, 1987). Several reviews, dealing with the stability of protein-stabilized emulsions have been published (Leman and Kinsella, 1989; Kinsella, 1984; Halling, 1981; Walstra, 1987; Dickinson and Stainsby, 1988; Dickinson, 1994, 1997; Dalgleish, 1997; Dickinson and McClements, 1995).

As different emulsions exhibit different types of instability, the choice of method for measuring instability depends on the type of stability to be measured. There are, however, a number of methods in the literature that measure emulsion stability without specifying the kind of instability involved in the determination procedure. These include the emulsion stability index (ESI) i.e. method of Pearce and Kinsella (1978), the stability rating of Acton and Saffle (1970, 1971), and the emulsifying activity test. These methods, in both original and modified form, have been extensively used throughout the literature (Halling, 1981). These tests are empirical 'functional tests', and interpretation and valid comparison of the results of these tests are not possible because of the lack

of information on the mechanisms involved in the methods.

Emulsion stability may be categorized in a number of ways that reflect the different mechanisms involved i.e. creaming, flocculation, coalescence and phase inversion. These major forms of instability are schematically shown in Figure 2.9.

2.9.1 Creaming

Creaming refers to the gravitational separation of emulsified droplets to form a densely packed phase without changes in droplet size. Creaming is usually a reversible process because gentle shaking can redisperse the creamed layer. Extensive creaming may be controlled by reducing the droplet size, which can be achieved by high homogenization pressure provided sufficient emulsifier is present (Walstra, 1983; Leman and Kinsella, 1989).

Tornberg and Hermansson (1977) observed that the creaming stability of protein-stabilized emulsions was improved by increasing emulsification intensity and time. Leman *et al.* (1988) compared the stability of emulsions stabilized by whole milk protein, β-lactoglobulin, whey protein and casein micelles under various conditions. They found that the creaming stability improved with increasing energy input and protein concentration. Upon heating the emulsions
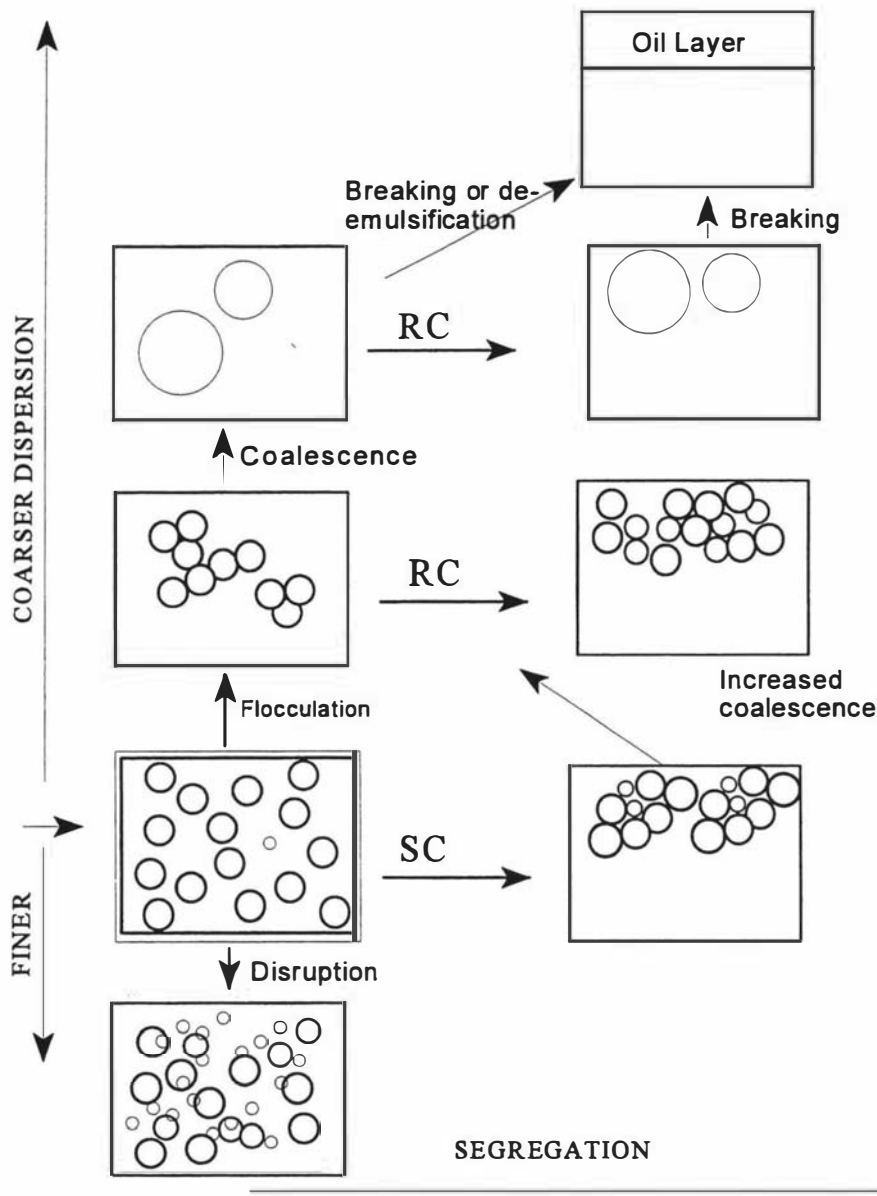

Figure 2.9: Schematic representation of several types of instability in an oil-in-water emulsion (from Walstra, 1987). SC, slow creaming; RC, rapid creaming.

(70 - 80°C) the creaming stability also improved. The creaming stability was also influenced by pH. As the pH was raised from 6 to 9, the rate of creaming decreased. Creaming is reversible by various means depending on the extent of destabilization (Dickinson and Stainsby, 1988).

An emulsion droplet surrounded by a viscoelastic film behaves hydrodynamically like a rigid sphere. So its creaming rate in highly dilute emulsions can be explained by Stokes' equation.

$$v_s = \frac{d^2 (\rho_0 - \rho) g}{18\eta}$$

where v_s is the rate of creaming, d is the particle diameter, ρ_0 is the density of the particle, ρ is the density of the continuous phase, g is the acceleration due to gravity and η is the viscosity of the continuous phase.

Stokes' equation explains that there are three ways of increasing stability with respect to creaming: reducing the droplet size, matching the densities and increasing the viscosity of the continuous phase. Darling (1987) discussed the factors affecting the validity of Stokes' equation and showed that it is not applicable in a system in which the volume of the dispersed phase is high leading to hindered separation (Darling, 1987; Srinivasan, 1995) or in a highly flocculated system (Dickinson and Stainsby, 1988).

The rate of creaming can be measured by keeping the emulsions undisturbed in long glass cylinders and measuring the changes in the fat content of the lower emulsion phase. Several methods can be used to estimate the fat content, such as gravimetric estimation or measurement of viscosity, density, turbidity (Leman *et al.,* 1988, Haque and Kinsella, 1989), particle size, conductivity or dielectric properties (Leman and Kinsella, 1989). Smith and Dairiki (1975) measured gravity-induced separation of emulsions after keeping them undisturbed for a period of 24 hours. The stability index was expressed

as the percentage of separated cream phase. However, the rate of creaming in emulsions is slow, unless the droplets are large (greater than 5 µm); therefore accelerated tests are usually employed. The most common accelerated technique is centrifugation and the instruments used range from a low-speed table centrifuge (Tornberg and Hermansson, 1977; Leman *et al.,* 1988) to the high-speed ultracentrifuge (Vold and Mital, 1972). Usually, a low gravitational speed is employed to avoid breaking the interfacial film. Tornberg and Hermansson (1977) used 180 *g* for 15 min to determine the creaming stability. After centrifugation, aliquots (5 ml) of the lower phase were removed and the fat content was determined by solvent extraction (Leman *et al.,* 1988; Haque and Kinsella, 1989). Emulsion stability was calculated as the percentage of fat remaining in the emulsion after centrifugation. Some methods employing an ultracentrifuge have actually measured the amount of separated oil (Vold and Mital, 1972) where the processes involved are a combination of creaming and coalescence. If the centrifugal field applied is not sufficient to coalesce all droplets in the creamed layer, the measured rate may not be a true reflection of the creaming rate.

Emulsions are often heated to accelerate the creaming process (Wang and Kinsella, 1976; Wu and Sexson, 1976). However, heating may cause additional changes, such as changes in protein conformation, desorption and coagulation, and may affect film stability, and the measured stability may not be attributed to creaming alone (Leman and Kinsella, 1989).

Gunning *et al.* (1988) followed the rate of rise of the creamed layer meniscus to measure the rate of creaming. One problem with the method was that it did not provide any information for the initial period before the meniscus became apparent. Walstra and Oortwijn (1975) removed samples of emulsions at different heights and determined the fat content to determine the creaming rate.

Reddy and Fogler (1981) employed a freeze-fracture electron microscopic method to determine the droplet concentration at different heights of the frozen

emulsion.

An ultrasonic interferometric method of determining creaming stability was described (Howe *et al.*, 1986). The ultrasound velocities through the oil and the aqueous phase are different. The method measures the ultrasound velocity through an emulsion sample. If the droplet size is smaller than the ultrasound wavelength, the ultrasound velocity depends only on the volume fraction of dispersed oil. Therefore, changes in the ultrasound velocity during creaming can be correlated to the change in the oil concentration profile. In recent years, this technique has been used extensively to determine the rate of creaming in emulsions (McClements, 1992, 1994; Hibberd *et al.*, 1995).

2.9.2 Flocculation

Flocculation denotes the aggregation of oil droplets to come together to form clusters. This generally modifies the physical properties of the emulsion, but the particle size distribution remains unchanged. Whether the floccule can be redispersed depends on the strength of inter-droplet interactions. Flocculation of an emulsion depends on the nature of the protein stabilizing the emulsion and on the solution conditions, such as pH and ionic strength.

There are very few studies that measure flocculation in food emulsions. Srivastava and Haydon (1964) and Eley *et al.* (1976) used a microscopic method to count the change in the number of doublets or triplets with time to find the flocculation rate constant (K_f) using the following equation:

$$\frac{1}{N_t} - \frac{1}{N_o} = K_f \, t$$

where N_o and N_t refer to the number density of droplets at time $t = 0$ and $t = t$, respectively.

Flocculation kinetics can also be determined using a Coulter counter. However,

care must always be exercised to avoid coalescence of droplets which may result in a decrease in the number of droplets. Tomberg and Ediriweera (1988) used ionic detergents to disperse the flocculi and, based on the amount of detergent required to disperse the emulsion into single droplets, they arranged the emulsions on an arbitrary scale according to their degree of flocculation. Darling (1982) evaluated the degree of clustering of emulsion droplets from the measurement of the change of viscosity at constant shear with time. A number of light scattering techniques have also been used to measure flocculation kinetics (Lips and Wills, 1973; Lips *et al.*, 1993; Dickinson *et al.*, 1994; Agboola and Dalgleish, 1995, 1996).

The droplets in a stable emulsion are prevented from flocculating either by electrostatic stabilization or by steric (polymeric) stabilization (or by a combination of the two). In electrostatic stabilization, the droplets are kept apart by repulsion between their charged surfaces; in steric stabilization, the droplets are kept apart by osmotic repulsion between their macromolecular adsorbed layers (Dickinson and Stainsby 1982). Surface active polyelectrolytes, such as proteins, can provide both electrostatic and steric stabilization (Dickinson and Stainsby, 1988). Electrostatic stabilization is favoured by low ionic strength and a pH well away from the isoelectric point. A substantial reduction in pH would reduce the charge density of protein-coated emulsion droplets, leading to loss of electrostatic stabilization (Dickinson, 1992, 1997; Dickinson and Stainsby, 1982). Whereas electrostatic stabilization is feasible at low surface concentrations of emulsifier, steric stabilization requires complete coverage of the droplet surface by a polymeric layer (Dickinson and Stainsby, 1988). However, under conditions of poor solvent quality, the entropic stabilizing steric repulsion of the adsorbed protein becomes an enthalpic destabilizing steric attraction. This results in flocculation of oil droplets, e.g when ethanol is added to emulsions (Dickinson, 1992, 1997; Dickinson and Stainsby, 1982; Agboola and Dalgleish, 1996).

When the protein concentration is insufficient to give complete coverage during

homogenization, then flocculation by macromolecular bridging may occur. In this type of flocculation, macromolecular material on one droplet becomes shared, through partial adsorption, with the initially uncoated surface of another (Dickinson and Stainsby, 1988). This type of irreversible bridging flocculation occurs during homogenization of cream (Mulder and Walstra, 1974) and during the emulsification of model emulsions prepared with binary mixtures of macromolecules of very different surface activity (Dickinson *et al.*, 1995; Dickinson and Galazka, 1991a,b).

Macromolecular bridging can also occur after homogenization. Attractive electrostatic interaction between adsorbed protein on the droplets and the polysaccharide or protein added after emulsion formation may induce flocculation by a polymer bridging mechanism (Chen and Dickinson, 1995; Dickinson, 1993). The formation of covalent disulphide bonds between adsorbed protein molecules on adjacent droplets may lead to irreversible bridging flocculation during aging of emulsions stabilized by whey proteins (McClements *et al.*, 1993).

In addition to the effect of adsorbed macromolecules on stability with respect to flocculation, it is important to realise that unadsorbed macromolecules may be involved in what is called 'depletion flocculation'. This type of flocculation arises whenever oil droplets are so close together that polymer molecules are excluded or depleted from the region of continuous phase between them. The resulting osmotic pressure gradient causes the droplets to flocculate spontaneously, because the mixing of solvent from between the particles with the bulk solution is associated with a decrease in free energy (Dickinson and Stainsby, 1988). Mixing solvent and solution can also take place by separating the droplets, which implies that the interparticle potential goes through a maximum at a separation corresponding to maximum macromolecular exclusion. Bondy (1939) first demonstrated that depletion flocculation is reversible. Figure 2.10 shows that when two particles come close together, the volume of solvent depleted is decreased, thereby decreasing the osmotic

pressure and decreasing the free energy; hence there is a driving mechanism for flocculating particles (Walstra, 1993b).
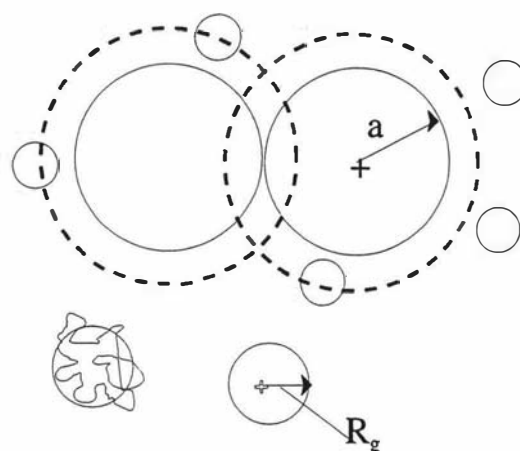

Figure 2.10: Schematic explanation of the depletion of macromolecules with radius of gyration, Rg, from the solution near the spherical particles of radius, a, and the resulting depletion flocculation (Walstra 1993b).

Asakura and Oosawa (1954) recognized this depletion flocculation in two parallel plates immersed in a solution of rigid molecules. Theories developed by Vincent *et al.*, (1988) was able to predict the complete phase diagram, including solid-liquid as well as liquid-liquid transitions. Aronson (1989) and Mclements (1994) showed that depletion flocculation can be induced in surfactant-stabilized emulsion by the presence of excess surfactant micelles in the aqueous continuous phase. Micelles are excluded from the gap between emulsion droplets at a separation distance less than the micellar diameter, with the resulting osmotic pressure gradient causing a net attraction between the droplets leading to reversible flocculation (Sperry, 1982). The factors favouring depletion flocculation are similar to those favouring thermodynamic phase separation into distinct polymer-rich and particle-rich phases. Both phenomena are reversible and both have their origin in the fact that polymer molecues are forced to compete with colloidal particles for the available space (Dickinson and Eriksson, 1991).

2.9.3 Coalescence

Coalescence is an increase in the droplet size by joining two droplets which may eventually result in the separation of the oil and aqueous phases. This process is irreversible unless the coalesced particles are disrupted by an energy input, e.g. homogenization. When two droplets approach each other, a thin film of the continuous water phase is trapped between the droplets. The behaviour of the thin film determines the degree of stability or instability of the emulsion, and the rate of thinning of the film determines the time required for the two drops to make contact. When the film has thinned to a critical thickness, it may rupture and the two droplets may unite and coalesce into a large droplet (Menon and Wasan, 1985). Although no theories have been able to successfully predict this phenomenon, several factors affect the incidence and rate of coalescence in protein-stabilized emulsions (Walstra, 1987). These include the viscoelasticity of the interfacial film, the interfacial tension, thermal motion and external vibrations.

The rate of coalescence mostly follows first-order kinetics, whereas flocculation is typically a second-order reaction. Semi-quantitative measurements of the kinetics of coalescence were done with oil-in-water emulsions made with caseinates (van Boekel and Walstra, 1981; Darling, 1982; Boode and Walstra, 1993). Various factors, such as solubility of the emulsifier, pH, salts, emulsifier concentration, phase-volume ratio, temperature and properties of the film, affect the coalescence stability of emulsions.

The coalescence of emulsion droplets causes several changes in an emulsion with time; these include an increase in average droplet size and volume of the separated phase and a decrease in the number of droplets, turbidity, emulsion viscosity etc. Therefore, the coalescence stability of emulsions can be determined experimentally by following the changes over time in any one of the above properties. Changes in droplet size and number can be followed by microscopy (Das and Chattoraj, 1980), Coulter counter (Walstra, 1968) or turbidity measurements (Walstra, 1968; Pearce and Kinsella, 1978; Das and

Kinsella, 1989). The small angle laser light scattering technique is particularly suited to detailed studies of the physics of the aggregation process although it is necessary to dilute the emulsions to allow light scattering to be measured (Lips *et al.*, 1993; Dickinson and Williams, 1994; Agboola and Dalgeish, 1996). Measurement of the volume of the separated phase to determine the coalescence stability of emulsions was employed by Tomberg and Ediriweera (1987). Viscosity measurements were used by Shioya *et al.* (1981) to determine coalescence.

Partial coalescence is a phenomenon that occurs in oil-in-water emulsions where part of the oil is crystallized. The droplets aggregate irreversibly via a network of flocculated crystals and are kept together by necks of liquid oil. This type of instability may be triggered by crystals projecting into the aqueous phase which pierce the thin film between closely approaching droplets. The extent of crystal projection, the nature of the emulsifier and the presence or absence of agitation are important factors in the rate and extent of partial coalescence (van Boekel and Walstra, 1981; Boode and Walstra, 1993). The susceptibility of emulsion droplets to coalesce or aggregate in a shear flow is known as orthokinetic stability. Boode and Walstra (1993) reported that one of the main factors affecting the orthokinetic stability of dairy emulsions containing semisolid fat droplets is the size and distribution of the fat crystals. Oil droplets containing fat crystals are very susceptible to clumping or partial coalescence when stirred, and in such dairy emulsions the nature of the fat crystal is more important than other physico-chemical factors (Dickinson, 1997).

An emulsion that has excellent long-term stability with respect to coalescence under quiescent conditions may become destabilized when subjected to vigorous stirring (Dickinson and Williams, 1994). Chen *et al.* (1993) reported the changes in the droplet size with time for β-lactoglobulin-stabilized oil-in-water emulsions under turbulent flow conditions. The destabilization time (maximum rate of increase in the volume-surface diameter) was dependent on a number of factors, such as the oil volume fraction, the initial droplet size and

the presence of low molecular weight surfactant dissolved in the aqueous phase (Dickinson, 1993; Chen *et al.*, 1993). Chen *et al.* (1993) demonstrated that in sodium-caseinate-stabilized oil-in-water emulsions the average droplet size against time was very sensitive to the concentration of added calcium ions. Dickinson *et al.* (1992) showed that the presence of significant attractive interaction between the casein-coated droplets in the presence of calcium chloride leads not only to flocculation but also to an order of magnitude increase in the effective orthokinetic coalescence rate as compared with the calcium-free emulsion.

2.9.4 Phase inversion

Phase inversion involves a change from an oil-in-water to a water-in-oil emulsion or vice versa. This may occur when the phase volume of the oil is sufficiently high. This phenomenon has been described by Mulder and Walstra (1974).

2.10 Factors affecting emulsion stability

The stability of emulsions is affected by a number of factors, including the composition of the continuous phase, droplet size and net charge, ionic strength, viscosity of the continuous phase, density of the droplet, temperature, nature of the emulsifying agent, and mechanical and physical properties of the adsorbed film (Graham and Phillips, 1976). The main physical factors affecting food emulsion stability are summarized in Table 2.3.

Table 2.3: Physical factors affecting stability of food emulsions (Dickinson, 1988).

Attributes	Creaming	Flocculation	Coalescence
Droplet size	***	**	*
Droplet size distribution	***	**	--
Volume fraction of dispersed phases	***	***	***
Density difference between phases	***	--	--
Viscosity (rheology) of continuous phase	***	***	**
Viscosity (rheology) of the adsorbed layer	--	--	***
Thickness of the adsorbed layer	--	**	***
Electrostatic interaction between droplets	*	**	**
Macromolecular interaction between droplets	--	***	**
Fat crystallization	--	--	***
Liquid crystalline phases	*	*	**

*** - Generally important, ** - often important, * - sometimes important,-- Not important

2.10.1 Droplet size

Larger sizes usually result in more rapid creaming and coalescence. The size of the droplets formed depends on the type of equipment used and on the energy intensity applied during emulsion formation.

Tomberg and Hermansson (1977) and Tomberg and Lundh (1978) studied the mechanical and equipment effects on the droplet sizes of emulsions made with several proteins. The equipment was characterized in terms of its power input

to the fluid, and of the energy density (power per unit volume) in the emulsification chamber; theory predicts that this is the crucial variable in determining the limiting droplet size. Smaller droplets were obtained when the same total energy was supplied at high power as compared with multiple passes at lower power, supporting a key role for energy density (Halling, 1981). Different proteins have been shown to give emulsions of different droplet sizes (Pearce and Kinsella, 1978; Tornberg, 1978a,b; Oortwijn and Walstra, 1979; Agboola and Dalgleish, 1995; Srinivasan *et al.,* 1996).

2.10.2 Nature of the interfacial film

The stability of an emulsion depends on the nature of the interfacial film. Surface viscosity is a measure of the susceptibility to deformation of the interface. A possible relationship between surface elasticity, surface viscosity and emulsion stability was emphasized in the past (Dickinson, 1992). The higher the surface viscosity and elasticity, the lower is the rate of coalescence. These observations have been supported by experimental data obtained with protein and non-protein emulsifiers (Rivas and Sherman, 1984). Surface viscosity plays a major role in controlling the rate of flow of film away from the point where droplets make contact, whereas surface elasticity helps to prevent coalescence on impact by accommodating deformation. According to Boyd *et al.* (1972), both surface viscosity and surface elasticity are involved in controlling the coalescence. Coalescence is caused by the force pressing adjacent globules together, giving rise to a compressive stress. With time, the stress causes breakdown of the film which results in coalescence (Boyd *et al.,* 1972).

The coalescence stability however, cannot be explained on the basis of surface viscosity alone. Casein forms highly stable emulsions and gives very small droplets, although the surface viscosity of a casein film is very low (Graham and Phillips, 1980a,b ; Tornberg, 1978a). Graham and Phillips (1980a,b) did not find any positive correlation between surface viscosity and coalescence stability. If surface viscosity is the only determining factor in coalescence

stability, emulsions with high surface load and greater film thickness of the adsorbed layer should be more stable than emulsions containing droplets with thinner adsorbed layers. Studies of β-lactoglobulin-stabilized emulsions (Das and Kinsella, 1989) indicated that around the isoelectric point emulsions with a thicker adsorbed layer were in fact least stable against coalescence. The most stable emulsions were obtained at pH 9.7. Tornberg and Ediriweera (1988) made similar observations on emulsions made with casein and whey protein.

2.10.3 Temperature

Increasing temperature, both directly and via a reduction in viscosity and surface rigidity, tends to increase all the breakdown processes in emulsions. Thermal denaturation of proteins, however, may cause an increase in viscosity of the continuous aqueous phase above certain temperatures and may even result in gelation. This may decrease the rate of emulsion breakdown because the droplet mobility is reduced (Halling, 1981).

During heat treatment the caseinate in solution or caseinate-coated emulsion droplets were destabilized and aggregated to form a coagulum. Both charge-charge repulsion and steric repulsion of caseinate residues were thought to be important in the stability of these emulsions (Dalgleish, 1995). The effect of heating at 90°C on emulsions made with whey protein isolate has been reported (Jost *et al.*, 1986; Masson and Jost, 1989; Yost and Kinsella, 1992). Heat stability of oil-in-water emulsions containing milk proteins were also studied by Hunt and Dalgleish (1995).

2.10.4 Presence of low molecular weight surfactants

Low molecular weight surfactants added to caseinate or other protein-containing food emulsions can enhance or, more likely, decrease stability against coalescence. They are surface active, but cause repulsion of the droplets only at small distance (Bergenstahl, 1988). The addition of lecithin was found to enhance coalescence stability of emulsions at very low caseinate

concentrations. Probably a stable mixed casein/lecithin surface layer was formed (Bergenstahl, 1988; Fang and Dalgleish, 1993).

Small amphiphilic molecules can displace protein films, cause disruption of the film, and result in destabilization of emulsions. These molecules have been shown to displace the caseinate from the droplet interface, thereby lowering interfacial tension and diminishing repulsion. Addition of SDS or Tween 20 to an emulsion containing caseinate, whey protein or total milk protein caused desorption of the previously adsorbed protein and the oil droplets coalesced (Oortwijn and Walstra, 1979).

2.10.5 Viscosity

The mobility of emulsified droplets affects emulsion stability by influencing the rate and extent of collisions. This is determined by the viscosity of the continuous phase. In packed food emulsions, the protein covered oil droplets are separated by an aqueous layer. The viscosity of the continuous phase determines the rate at which adjacent droplets approach. Generally, an increase in stability is observed with increasing viscosity of the continuous fluid (Walstra, 1984; Menon and Wasan, 1985).

2.10.6 Effect of pH

The behaviour of proteins is dependent on pH. A number of studies have shown that the stability of emulsions formed from proteins is lowest at or close to their isoelectric point (Masson and Jost, 1986; Dickinson and Williams, 1994; Hunt and Dalgleish, 1994). Furthermore, this is consistent with the theories of colloidal stabilization because both the electrostatic and steric repulsive potentials decrease as the isoelectric point is approached. However, studies by Lemen and Kinsella (1989) indicated that the stability of emulsions formed with proteins was improved by adjusting the pH close to the isoelectric point. The pH affects the net charge of the adsorbed layer and hence the conformation of protein molecules, which affects the protein load and electrostatic interactions at the oil/water interface, and determines the film cohesiveness and interfacial

rheology (Halling, 1981).

2.10.7 Effect of calcium

The physico-chemical aspects of interactions between calcium and proteins have been studied (Swaisgood, 1992). Dickinson *et al.* (1987) studied the association of calcium with hydrocarbon oil-in-water emulsions made with individual caseins as well as with sodium caseinate. They found from deflocculation experiments that there were stronger calcium-induced associations between the adsorbed protein layers when all the caseins were present than when only individual casein molecules are present at the interface. Dickinson *et al.* (1992) also showed that addition of calcium to the emulsion before or after homogenization influences the extent of destabilization of emulsions formed with β-casein or phosvitin. Hunt *et al.* (1993) also showed that association with calcium in sufficient quantities to cause aggregation prevents interfacial displacement with small molecule surfactants in β-casein- or phosvitin-stabilized oil-in-water emulsions. Agboola and Dalgleish (1995) studied the stability of aggregation of 20% soya oil-in-water emulsions stabilized by 0.3 to 2% sodium caseinate or β-lactoglobulin in the presence of calcium chloride solutions using light scattering and electron microscopy. They concluded that the stability of sodium caseinate emulsions depends on the amount of calcium as well as on the concentration of protein. The extent of aggregation could be associated with the extent of binding of calcium by protein in solution. Agboola and Dalgleish (1996) showed that emulsions prepared with β-lactoglobulin are as sensitive to calcium as caseinate emulsions.

Chapter 3

MATERIALS AND METHODS

3.1 Materials

Sodium caseinate (ALANATE 180) and calcium caseinate (ALANATE 380) were obtained from the New Zealand Dairy Board, Wellington, New Zealand. Soya oil (food grade) was purchased from the local supermarket.

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

3.2 Preparation of emulsions

Emulsions were usually prepared from various concentrations of sodium or calcium caseinate and with 30% (w/w) soya oil. In some cases, emulsions were made with mixtures of sodium and calcium caseinate at 1.0 and 2.5% protein. The caseinate solutions and soya oil were mixed, heated to 55°C, and then passed through a two-stage valve homogenizer without applying any pressure (Rannie, model LAB, type 12.50 H, capacity 100 l/hour, Rannie a/s, Roholmsvej 8 DK - 2620, Albertslund, Denmark). This produced a temporary oil-in-water emulsion. The mixture was then homogenized at the desired pressure, usually 3,000 psi for the first stage and 500 psi for the second stage. The resulting homogenized emulsion was stored at 20°C. At least two separate emulsions were prepared for each treatment.

3.3 Emulsion characterization

3.3.1 Droplet size distribution

The droplet size distribution of emulsions was determined using the MasterSizer (Model MS 20, Malvern Instruments Ltd., Malvern, Worcestershire, UK) at the New Zealand Dairy Research Institute, Palmerston North.

In this method, a low power laser beam is diffracted by a diluted dispersion of the emulsion and is collected over a range of scattering angles by a series of semicircular photo-electric diodes. The volume size distribution is calculated from the intensity of light diffracted at each angle using Lorenz-Mie theory (Carter and Robin, 1991; Weiner, 1984). To calculate successfully the size data, the MasterSizer requires the refractive index and absorbance of the particle being measured as well as the refractive index of the medium in which the particles are dispersed.

The parameters that were used in analysing the particle size distribution were

Presentation : (2NAD) 1.330, 1.456 + i0.00000 polydispere model

Relative refractive index = 1.33

Absorbance = 0.001

$$\frac{\text{Refractive Index of the particle}}{\text{Refractive Index of water}} = 1.095$$

The MasterSizer divides the size distribution into 32 classes across the sub-micron range (0.1 - 80 microns). The parameters obtained from the MasterSizer are shown below

d_{32}: The volume-surface mean diameter (also known as the Sauter mean diameter or D_{vs})

$$d_{32} = \frac{\Sigma d_i^3 \, N_i}{\Sigma d_i^2 \, N_i}$$

The d_{32} is the surface weighted mean diameter (i.e., the diameter of the particle having the sample mean surface area) where N is the number of particles with diameter D. D_1 is the minimum diameter in the distribution and D_2 is the maximum distribution diameter.

d_{43}: d_{43} is the weight-average diameter which is given by

$$d_{43} = \frac{\Sigma d_i \, W_i}{\Sigma \, W_i} = \frac{\Sigma d_i^4 \, N_i}{\Sigma d_i^3 \, N_i}$$

where W_i the weight of particles in the ith class size, is replaced by $d_i^3 N_i$ assuming that the density is constant for all sizes.

3.3.2 Specific surface area

The specific surface area was also obtained from the MasterSizer results, and was used for the surface load calculations. The d_{32} and specific surface area values were accurate within 3%.

3.4 Determination of surface protein concentration and composition

The amount of protein adsorbed onto the oil surface was determined by measuring the protein content of the subnatant (usually called the depletion method) after separation of the dispersed and aqueous phase by centrifugation. The details of the separation of the cream and subnatant are described below.

3.4.1 Separation of aqueous phase and cream

Emulsion (30 g) was centrifuged at 45,000 g for 40 min at 20°C in a temperature-controlled centrifuge (Sorvall RC5C, DuPont Company, USA) and the subnatant was carefully removed using a syringe. The cream layer was dispersed in deionized water (purified by reverse osmosis followed by treatment with a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) and recentrifuged at 45,000 g for 40 min. Again, the subnatant and the cream layer were collected carefully.

A similar method was adopted for calcium caseinate, but sediments are formed in calcium caseinate emulsions and these sediments were carefully removed from the centrifuge tube and analysed separately for total protein, using the Kjeldahl method (see Section 3.7).

The subnatants were then filtered through 0.45 µm and 0.22 µm filters (Millipore Corporation, Bedford, MA 01730, USA) using a Nalgene filtering unit (Nalge Company, Rochester, NY 14602-0365, USA). The filtrates were analyzed separately for total protein using the Kjeldahl method.

The surface protein concentration was calculated as follows:

Adsorbed Protein = Total protein (g) taken for making an emulsion
  − Protein (g) present in the subnatant

$$\text{Surface protein coverage (mg/m}^2) = \frac{\text{Total mg protein adsorbed}}{\text{Total oil surface area}}$$

In the case of calcium caseinate

Adsorbed protein = Total Protein (g) taken for making an emulsion
  − Protein (g) present in the subnatant
  − Protein (g) present in the sediment

The surface composition was determined by analysing the cream phase for the individual proteins using sodium dodecyl sulphate polyacrylamide gel electophoresis (SDS-PAGE), as described in section 3.11. The cream was spread on a filter paper and dried as much as possible (method adapted from Hunt and Dalgleish, 1994). After drying the cream on the filter paper, a small known amount of cream was taken and dispersed in SDS sample buffer. The mixture was heated in a water bath at 90°C for 5 minutes and then cooled to room temperature. In some cases, the subnatants obtained from emulsion samples were also dispersed in the SDS sample buffer. The dilution of the sample (subnatant) with the sample buffer was dependent on the amount of protein present in the subnatant. The cream and subnatants dispersed in sample buffer (10 µl) were applied to the slots of the SDS gel.   The reproducibility of these methods was determined by analyzing 9 separate

emulsions made with 2.5% sodium caseinate and 30% soya oil. Variations were ~ 4% for surface protein concentration, ~ 7% for α_s-casein (α_{s1}- + α_{s2}-), ~ 6% for β-casein, and ~ 10% for κ-casein.

3.5 Turbidity measurements

The turbidity of calcium caseinate dispersions with or without NaCl was determined at 900 nm using a UV 160A spectrophotometer (UV-visible recording spectrophotometer, Shimadzu Corporation, Japan) and a 2 mm quartz sample cell.

3.6 Sedimentation of calcium caseinate dispersions

The calcium caseinate dispersions (30 g) with added NaCl at pH 7.0 were centrifuged at 10,000 g for 40 min in a temperature-controlled Sorvall centrifuge. The total protein in the supernatant was determined using the Kjeldahl method.

3.7 Chemical Analysis

Total protein was measured by determining the total nitrogen by the macro-Kjeldahl method (AOAC, 1974) and multiplying by the factor 6.38. The samples were digested using a Kjeltec digester (Kjeltec 1007 Digester, Tecator, Sweden) and distilled using a Kjeltec system (Kjeltec 1026 Distilling Unit, Tecator, Sweden). The mineral analysis was carried out using plasma emission spectrometry (Ag Research, Grasslands Research Centre, Palmerston North, New Zealand).

3.8 Determination of viscosity

In order to determine the viscosity of emulsions and protein solutions, the Bohlin VOR rheometer system (Bohlin Rheologi AB, Lund, Sweden) was used in its viscometry mode. The C25 concentric cylinder measuring system, consisting of a 25 mm diameter fixed bob and a 27.5 mm diameter rotating cup, was used in all experiments. The cup and bob were first installed in the instrument. The system temperature was then adjusted to the required value,

usually 20°C. The bob was then lowered slowly into the cup until the solution just reached the top surface of the bob. The viscosities were measured at different shear rates from 20 to 750 s^{-1}.

In some cases, the emulsions made with sodium caseinate at 0.5% and 5.0% were centrifuged at 45,000 g for 40 min and the cream layer was removed carefully. This cream layer was then mixed with different sodium caseinate solutions of various concentrations (from 0.5 to 5.0%), deionized water or its own subnatant. The mixture solutions were then maintained at 20°C for about 24 hours and the viscosity was then measured using the Bohlin Rheometer as described above.

3.9 Determination of microstructure

A Leica (Heidelburg, Germany) confocal scanning laser microscope with a 100 mm oil immersion objective and an Ar/Kr laser with an excitation line of 488 nm (in such a way that only the fluorescent wavelength band can reach the detector system) was used to determine the microstructure of emulsions. Emulsions were made as described above and about 3 ml of sample was taken in a test tube and Nile Blue (approximately 0.1%) was added to it. The sample with Nile Blue (fluorescent dye) was mixed throughly and then placed on a microscope slide. The slide was then covered with a coverslip and then observed under the microscope.

3.10 Size exclusion chromatography (SEC)

Separation of milk proteins from sodium and calcium caseinate solutions and subnatants of sodium and calcium caseinate emulsions was carried out by size exclusion chromatography (SEC) on a Superose 6HR 10/30 column (Pharmacia, Uppsala, Sweden) attached to a GBC HPLC system (GBC Scientific Equipment Ltd, Victoria, Australia). A solution containing 20 mM imidazole and 50 mM NaCl at pH 7.0 was used as an elutant buffer. All the samples were filtered through 0.22 μm membrane filters. Calcium caseinate solutions were centrifuged at 5,000 g before filtration. A 50 μl portion of each

sample was injected in to the column and the absorbance (UV) was monitored at 280 nm at a flow rate of 0.4 ml/min.

The individual peaks were collected from the column, frozen immediately using dry CO_2 and then freeze-dried. The fractions were analysed by SDS-PAGE using a Mini Protean II system (Bio-Rad Laboratories, Richmond, CA, USA) as described below.

Four different proteins (thiroglobulin MW 669,000, ferritin, MW 443,000, chicken alubumin, MW 67,000 and α_s-casein, MW 23,652 Da) were used as standards to estimate the molecular weights of the peaks obtained above. SEC analysis of these standards was carried out under the same conditions as for the samples. A number of samples of the same concentration were injected into the column to check reproducibility and very similar profiles were obtained.

3.11 Electrophoresis

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

3.11.1 Preparation of stock solutions
Acrylamide/Bis (30% T, 2.67%C)

Acrylamide (30 g) and N, N-bis acrylamide (0.8 g) were dissolved in deionized water to give a final volume of 100 ml, and stored at 4°C in a dark bottle.

1.5 M Tris-HCl buffer, pH 8.8

Tris (tris hydroxymethyl aminoethane, 18.15 g), obtained from USB (United States Biochemicals Corp. Cleveland, OH, USA), was dissolved in approximately 60 ml of deionized water, the pH was adjusted to 8.8 with 1 M HCl and the volume was made up to 100 ml with deionized water. The buffer was stored at 4°C.

0.5 M Tris-HCl Buffer, pH 6.8

Tris (6 g) was dissolved in about 60 ml of deionized water. The pH of the solution was adjusted to 6.8 with 1 M HCl, and then the volume was made up to 100 ml with deionized water. The buffer was stored at 4°C.

10% SDS

SDS (10 g) was dissolved in deionized water with gentle stirring and the volume was made up to 100 ml.

SDS-reducing buffer (sample buffer), 100 ml

To 50 ml of deionized water, 0.5 M Tris-HCl buffer (12.5 ml), glycerol (10 ml), 10% (w/v) SDS (20 ml), β-mercaptoethanol (5 ml) and 0.05% (w/v) bromophenol blue (2.5 ml) were added. The pH of the sample buffer was adjusted to 6.8.

5X electrode buffer

Electrode buffer (5X) was prepared by dissolving Tris (15 g), glycine (43.2 g) and SDS (3.0 g) in deionized water. After adjusting the pH to 8.3, the volume of the buffer was made up to 1 l and the buffer was stored at 4°C. For each electrophoresis run 60 ml of this electrode buffer was diluted with 240 ml of deionized water.

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

To prepare 10 ml of resolving gel buffer, the following solutions were mixed: deionized water (2.02 ml), 1.5 M Tris-HCl buffer (2.50 ml), 10% SDS (100 μl) solution and acrylamide/bis mixture (5.3 ml). The mixture was then degassed for 15 min. Immediately, 5 μl of TEMED (tetramethylethylenediamine) and freshly prepared 10% (w/v) ammonium persulphate (50 μl) were added. After gentle mixing, 3.3 ml of the contents was poured between the electrophoresis casting plates (Bio-Rad Mini Protean, Bio-Rad, Richmond, CA, USA). A small quantity of deionized water was added to form an upper layer and the acrylamide solutions were allowed to polymerize at 20°C for about 45 min. The

water was then drained and dried with filter paper before pouring the stacking gel.

Preparation of stacking gel

To prepare 10 ml of stacking gel, the following solutions were added to 6.1 ml of deionized water: 0.5 M Tris-HCl buffer (2.5 ml), 10% SDS (100 µl) solution and 30% acrylamide/bis mixture (1.3 ml). The mixture was degassed for 15 min. Immediately TEMED (10 µl) and freshly prepared ammonium persulphate (50 µl) were added and 2.2 ml of this mixture was then poured on top of the resolving gel. The slot former (plastic comb) was immediately placed at the top of the stacking gel (between the plates) to form appropriate slots for the samples. Polymerization was carried out at 5°C overnight. Next day the plastic comb was removed and the slots were rinsed with deionised water. The excess water was then removed with filter paper. The gel plates were then placed in the electrode chamber and samples were applied to the gel slots.

3.11.2 Running of gels

The gels were run on a Mini Protean system (Bio-Rad, Richmond, CA, USA) at 200 V using a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Richmond, CA, USA) until the tracking dye moved out of the gel. The approximate running time was about 60 min after which the gels were removed from the plates and transferred gently to staining solution.

Staining

The gels were put into plastic boxes and about 50 ml of Coomassie Blue R solution (1 g brilliant blue R was dissolved in 500 ml of isopropyl alcohol and 200 ml of acetic acid and the contents were made to 2 l with distilled water) was added. The gel boxes were put on a rocking table (made at the Food Technology Department workshop) so that the gels were uniformly stained with the staining solution.

Destaining

The staining solution was drained carefully after 1 hour and replaced with destaining solution which contained a mixture of 100 ml of isopropyl alcohol and 100 ml acetic acid diluted to 1 l. The destaining solution was again changed after 1 hour with fresh destaining solution. Then the plastic box was left on the rocking table for 19 hours. After that the destaining solution is replaced with distilled water and the gel was scanned using the densitometer.

3.11.3 Densitometry

Quantitative determination of the components of the mixture separated by SDS-PAGE was performed by densitometry using a laser densitometer (LKB Ultroscan XL, LKB Produkter, AB, Sweden). In the densitometer, the protein bands on the stained gel were scanned with a narrow beam of laser light and the absorbance at 522 nm was plotted as a function of track distance. The output from the densitometer was quantified by measuring the areas under individual peaks. The scanning procedure involved cleaning the densitometer gel plate, placing the gel on the densitometer gel plate, defining the tracks and scanning each individual band. The printout was in the form of a graph of individual peaks and a table of individual peak areas. Standard protein solutions were run in conjunction with samples to aid identification of unknown protein bands in the sample.

3.12 Multi angle laser light scattering (MALLS)

Theory:

Wyatt (1993) reviewed the theory of light scattering and the use of MALLS for the absolute characterization of the molecular mass of macromolecules. Some basic light scattering principles are: (1) the amount of light scattered is proportional to the product of the molar mass and solute concentration when there is no absorbance; (2) the angular variation of scattered light is directly related to the size of the molecule.

The MALLS photometer measures the intensity of the scattered light with the

use of photodiodes placed at specific angles (θ) relative to the laser beam. This light intensity is converted to the Rayleigh ratio as follows:

$$R_{(\theta)} = \frac{I_\theta \, r^2}{I_0 \, V} \tag{1}$$

I_θ is the intensity of scattered light measured from the sample; I_0 incident beam intensity; V is volume of scattering medium; r is the distance between the scattering volume and detector. With excess scattering this R_θ will become

$$R_\theta = \frac{(I_\theta - I_\theta \text{ solvent}) \, r^2}{I_0 \, V} \tag{2}$$

The relationship between the Rayleigh excess scattering and the physical characteristics of the scattering molecule was given by Rayleigh as:

$$\frac{K^* c}{R_{(\theta)}} = \frac{1}{M_w \, P_{(\theta)}} + 2 \, A_2 c \tag{3}$$

where $P_{(\theta)}$ is

$$P_{(\theta)} \approx 1 + [\frac{16\pi^2 \, n_0^2}{3\lambda^2}] \, r_g^2 \, \text{Sin}^2(\theta/2) \tag{4}$$

and $P_{(\theta)}$ is the form factor or scattering function and substituting $P_{(\theta)}$ in equation 3.

$$\frac{K^* c}{R_{(\theta)}} = \frac{1}{M_w} [1 + \frac{16\pi^2 \, n_0^2}{3\lambda^2} \, r_g^2 \, \sin^2(\theta/2) + \ldots\ldots\ldots] + 2 A_2 c \tag{5}$$

where $R_{(\theta)}$ is the intensity of excess light scattered (Rayleigh's ratio) at an angle

θ (i.e. the intensity due to the solute), c is the concentration of solute in mg/ml, λ_0 is the wavelength of light in vacuum, M is the weight-average molecular weight, r_g^2 is the mean square radius of gyration, A is the second virial coefficient, n_0 is the refractive index, dn/dc is the refractive index increment of the solute and N_A is Avogadro's number. K* is an optical parameter which is equal to

$$K^* = 4\pi^2 n_0^2 (dn/dc)^2 \lambda_0^{-4} N_a^{-1} \qquad (6)$$

At low concentrations (e.g. commonly used in SEC) the virial coefficient term is negligible.

At the low concentration limit (c → 0), slope gives r_g

$$\frac{K_C}{R_{(0°)}} = \frac{1}{M_w} [1 + \frac{16\pi^2 n_0^2}{3\lambda^2} r_g^2 \, Sin^2(\theta/2) \ldots] \qquad (7)$$

If we measure the light scattering at small angles, the term $[16\pi^2 n_0^2 (r_g)^2 \sin^2(\theta/2)]/3\lambda^2$ will be negligible.

At the low angle limit (θ → 0) slope gives A_2

$$\frac{K^*c}{R_{(0°)}} = \frac{1}{M_w} + 2 A_2c \qquad (8)$$

And furthermore, at low angle and at low concentrations, the intercept gives molar mass,

At low concentrations and low angles (c → 0; θ → 0) intercept gives M_w

$$\frac{K^*c}{R_{(0°)}} = \frac{1}{M_w} \qquad (9)$$

Description:

Pump

↓

Injector

↓

Column

↓

UV detector   →

↓

Dawn DSP   →   Computer   (Astra Software package)

↓

RI Detector   →

↓

Waste
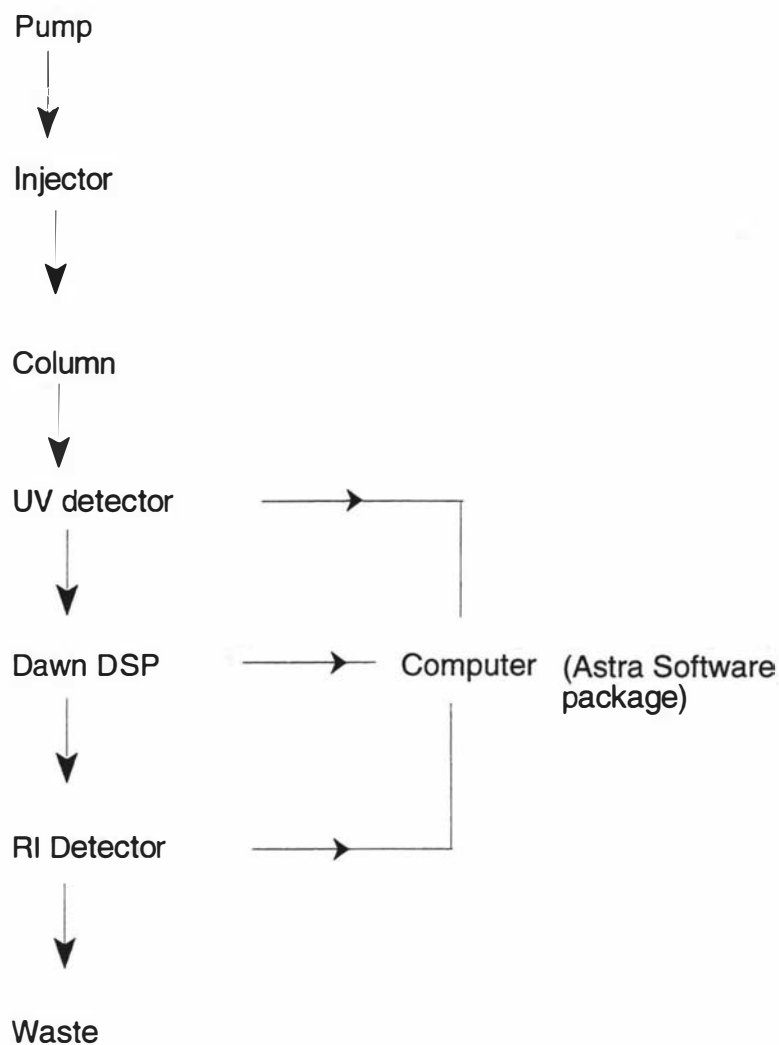
A typical on-line SEC/MALLS/RI system uses a column and three detectors in series: a SEC column, UV absorbance detector; a multiple angle laser light scattering detector; and a refractive index detector.

For on-line laser light scattering detection, a DAWN-DSP MALLS photometer (Wyatt Technology, Santa Barbara, CA) was used, equipped with a K5 flow cell and a linearly polarized He-Ne laser-light source (5 mW) with a wavelength λ

= 632.8 nm. The DAWN contained 16 detectors. Data from each detector was processed using Astra (Version 4.5) software. The RI response factor was measured by injecting a series of known NaCl concentrations directly into the detector cell with a syringe pump (Razel; Model number A-99; Razel Scientific Instruments Inc., Stamford, CA). The factor to correct the Rayleigh's ratio to 90° for instrument geometry was obtained by measuring the light scattering intensity of pure HPLC-quality toluene at 90°. The response of all the photodiodes arrayed around the scattering of all angles were normalized to the diode at 90° with monomeric bovine serium albumin (50 μl of a solution with a concentration of 10 mg/ml was injected). Prior to the measurements the DAWN-DSP MALLS photometer was calibrated with filtered (0.025 μm) HPLC-quality toluene and normalized using a bovine serum albumin (monomeric) solution. Usually the equipment is normalized with bovine serum albumin (BSA) monomeric (M = 66, 270, Sigma; lot number: 75H9305) as normalization. For normalization the sample should be monodisperse, and should have small size (~ 3 nm) so that it can scatter light isotropically (equally in all directors or angles). One of the main purposes of normalization is to normalize responses of other angles with the 90° signal. A specific refractive index increment, *dn/dc* (change in refractive index with sample concentration) was taken as 0.19 cm³/g (Payens and Heremans, 1969; Huglin, 1972).

The molecular mass M_i and the mean radius (r_i^2) of material eluting in each slice were calculated with a first-order Debye fit, i.e. plotting $K^*c/R_{(\theta)}$ against $Sin^2(\theta/2)$ where the intercept is M_i and the slope is R_g.

Sample preparation

Sodium caseinate solutions of various concentrations were prepared in 0.025 μm filtered water. Sodium caseinate solutions (20 g) were ultracentrifuged at 90,000 *g* for 60 minutes at 20°C in a temperature controlled centrifuge and about 5 ml of the subnatant was collected carefully. The subnatant was then filtered through 0.22 μm filters prior to injection in the column. A solution containing 20mM imidazol, 50 mM NaCl and 0.02% (w/w) at pH 7.0 was used as an elutant buffer, which was filtered through 0.025μm membrane filter. A number of samples of the same concentration were injected into the MALLS and very similar profiles were obtained.

3.13 Creaming stability

To determine the stability rating, the method described by Tornberg and Hermansson (1977) was adopted, where stability rating was determined on the basis of the percentage change of fat in the aqueous phase after creaming. After the emulsions were made, 30 g of emulsion was poured into a centrifuge tube and maintained at 20°C for 24 hours. After 24 hours, the samples were centrifuged at low speed (180 *g*) at 20°C for 15 min using Sorvall RC5C centrifuge, (DuPont Company, USA). After centrifugation of the emulsion, 5 ml of the lower phase was carefully removed with a syringe for fat determination by the Mojonnier method (IDF 16C : 1987). The following equation was used to determine the stability rating:

$$\text{Stability Rating} = \frac{\% \text{ Fat in lower phase}}{\% \text{ Fat in original emulsion}} \times 100$$

The reproducibility of these methods was determined by analyzing 9 separate emulsions made with 2.5% sodium caseinate and 30% soya oil. Variations were ~ 7% for the stability rating.

Chapter 4

CHARACTERIZATION OF PROTEINS IN COMMERCIAL SODIUM AND CALCIUM CASEINATES

4.1 Introduction

Caseinates are important structural components in many foods and are also used as ingredients because of their nutritional value and physico-chemical properties. Despite the extensive study of various aspects of sodium caseinates, little work has been reported on calcium caseinates; moreover little work has been reported on the mechanism of aggregation and the quantitative characterization of aggregates and particles.

Sodium and calcium caseinates consist of the casein fraction of milk which has been precipitated by acid at pH 4.6, collected, and redissolved to neutral pH by the addition of alkali. It is then spray dried. These processes can alter the native state of caseins, resulting in changes in surface charge and hydrophobicity. Consequently, the caseins can interact to form protein complexes or aggregates. The behaviour of these aggregates and complexes is critical in defining the functionality of caseinates in food applications. Although the structure and the aggregation of individual caseins has been widely studied (Schmidt, 1982; Swaisgood, 1992), there is much less understanding of how the caseins are aggregated in commercial caseinate solutions.

Consequently, the aim of this study was to characterize by size exclusion chromatography (SEC) and light scattering the aggregation state of protein in sodium and calcium caseinate solutions.

4.2 Results

4.2.1 Composition of commercial sodium and calcium caseinates

Table 4.1 presents the composition of commercial sodium and calcium caseinates. The fat, lactose, moisture and protein contents of sodium and calcium caseinates were similar to each other. The amount of sodium in calcium caseinate was small compared with that in sodium caseinate and the concentration of calcium in sodium caseinate was very low.

The proportions of individual caseins in the caseinate samples were determined using quantitative SDS-PAGE. In this electrophoresis system, α_{s1}-casein was not resolved from α_{s2}-casein and therefore the two caseins were represented together as α_s-casein ($\alpha_{s1} + \alpha_{s2}$). The proportions of α_s - and β-caseins in sodium caseinate were somewhat similar to those in calcium caseinate, whereas the proportion of κ-casein was slightly higher (Table 4.2).

Table 4.1: Chemical composition of commercial sodium and calcium caseinates.

Component (%)	Sodium caseinate	Calcium caseinate
Protein N x 6.38	94	93.5
Sodium	1.384	0.076
Calcium	0.062	1.586
Phosphorus	0.895	0.853

Table 4.2: Proportions of individual caseins in commercial sodium and calcium caseinate solutions.

Proportions of individual caseins (%)	Sodium caseinate	Calcium caseinate
α_s-casein	39.6	41.8
β-casein	41.4	43.3
κ-casein	19.0	15.0

4.2.2 Characterization of proteins in commercial sodium and calcium caseinates

4.2.2.1 Successive centrifugation

When caseinate solutions (2.5 wt%) were subjected to successive centrifugation between 100 to 90,000 g, negligible amounts of protein sedimented from the sodium caseinate solution. By contrast, considerable quantities of protein were sedimented from the calcium caseinate solution, reflecting the high degree of protein aggregation in this caseinate. A maximum of ~ 25% of the total protein could be sedimented from the calcium caseinate solution (Figure 4.1).

Quantitative SDS-PAGE showed that, with increasing centrifugation speed, the amounts of calcium sensitive α_s-casein in the supernatant decreased markedly whereas the amounts of β- and κ-caseins showed smaller decreases (Figure 4.1). This suggests that the large, sedimentable protein aggregates were rich in α_s-casein.
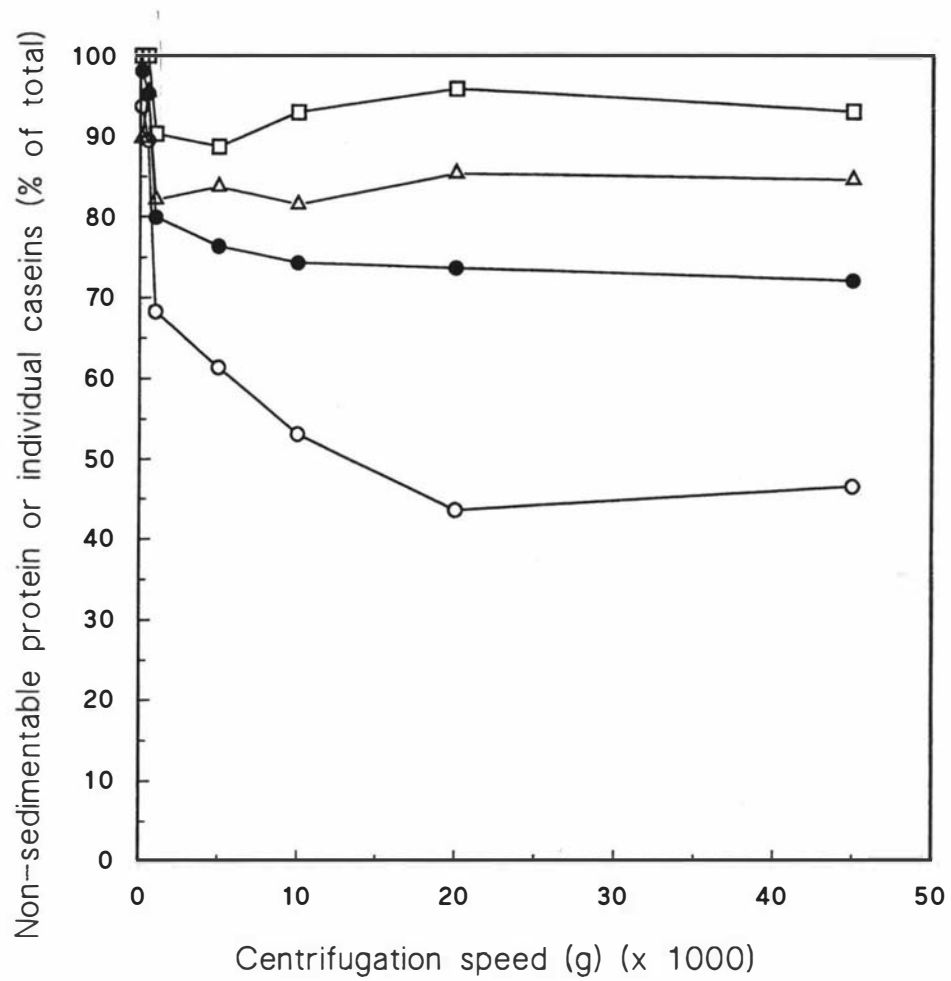
Figure 4.1: Concentrations of total protein (•), α_s-casein (○), β-casein (□) and
κ-casein (△) in the supernatant obtained from successive
centrifugation of calcium caseinate solution at different speeds.

4.2.2.2 Size exclusion chromatography (SEC)

Sodium and calcium caseinate solutions (2.5%) were chromatographed on Superose 6HR 10/30, using pH 7.0 buffer containing 50 mM NaCl. Calcium caseinate solutions were centrifuged at 5,000 g for 20 min and the supernatants were filtered through 0.22 µm filter paper prior to analysis. Sodium caseinate solutions were also filtered through 0.22 µm filter paper. The profiles obtained for sodium caseinate showed a small peak near the void volume of the column (peak I) and two partly resolved peaks (II and III) (Figure 4.2). The trailing edges of various peaks were more diffuse than the leading edges. The elution profile of calcium caseinate dispersion was qualitatively similar to that of sodium caseinate, except that the peak near the void volume was more pronounced. SDS-PAGE analysis showed that peak II contained relatively more κ-casein than α_s- and β-caseins (α_s-, β-, κ- ratio, 0.2:0.2:0.6), whereas peak III contained predominantly α_s- and β-caseins, and was relatively deficient in κ-casein (α_s-, β-, κ- ratio 0.44:0.44:0.17). It was not possible to recover enough casein material to obtain a clear pattern in peak 1.
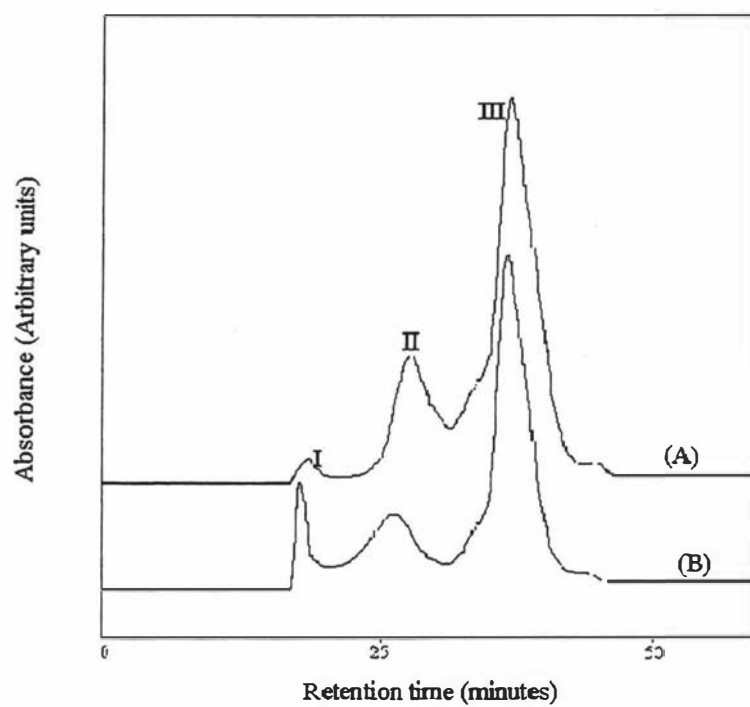
Figure 4.2:   Typical elution profiles of a 2.5% solution of sodium caseinate (A) or calcium caseinate (B) on a Superose 6HR 10/30 column. The flow rate was 0.4 ml/min and imidazole/NaCl buffer (pH 7.0) was used as the eluent.

4.2.3 Effect of protein concentration on the aggregation state of protein in sodium or calcium caseinate solutions

4.2.3.1 Size exclusion chromatography

Various concentrations of sodium and calcium caseinates (0.5 - 5.0%) were subjected to SEC on Superose 6HR 10/30 as described above. A constant volume of 50 µl of each sample was applied. As the amount of protein applied to the column increased, the size of all three peaks increased (Figure 4.3). SEC elution profiles clearly showed that the position of the major elution peaks was concentration dependent, as indicated by the changes in their retention times (Figure 4.3). The retention time of peak III (monomeric caseins) decreased with increasing concentration whereas, for peaks I and II, there was no considerable change in the retention time with increasing concentration. These observations are typical characteristics of an equilibrium mixture of associating macromolecules and suggest an increasing degree of association of monomeric casein components with increasing concentration. Similar behaviour was observed in calcium caseinate solutions (Figure 4.3B). In all cases, the peaks were fairly broad, indicating either that the sample contained a range of molecular species or that there was rapid equilibrium between polymers and monomers.

Pepper and Farrell (1982) studied interactions of soluble whole casein using gel permeation chromatography on Sephrose CL-6B in phosphate buffer and found that with increasing casein concentration the casein components associated to form sub-micelles of diameter 15 - 20 nm. A decrease in the casein concentration resulted in dissociation of κ-casein from other caseins, forming associated structures independently.

4.2.3.2 Particle size distribution

The protein particle size distribution in calcium caseinate solutions was determined by light scattering, using the Mastersizer. Protein particles in sodium caseinate solutions were too small to be measured by this technique.
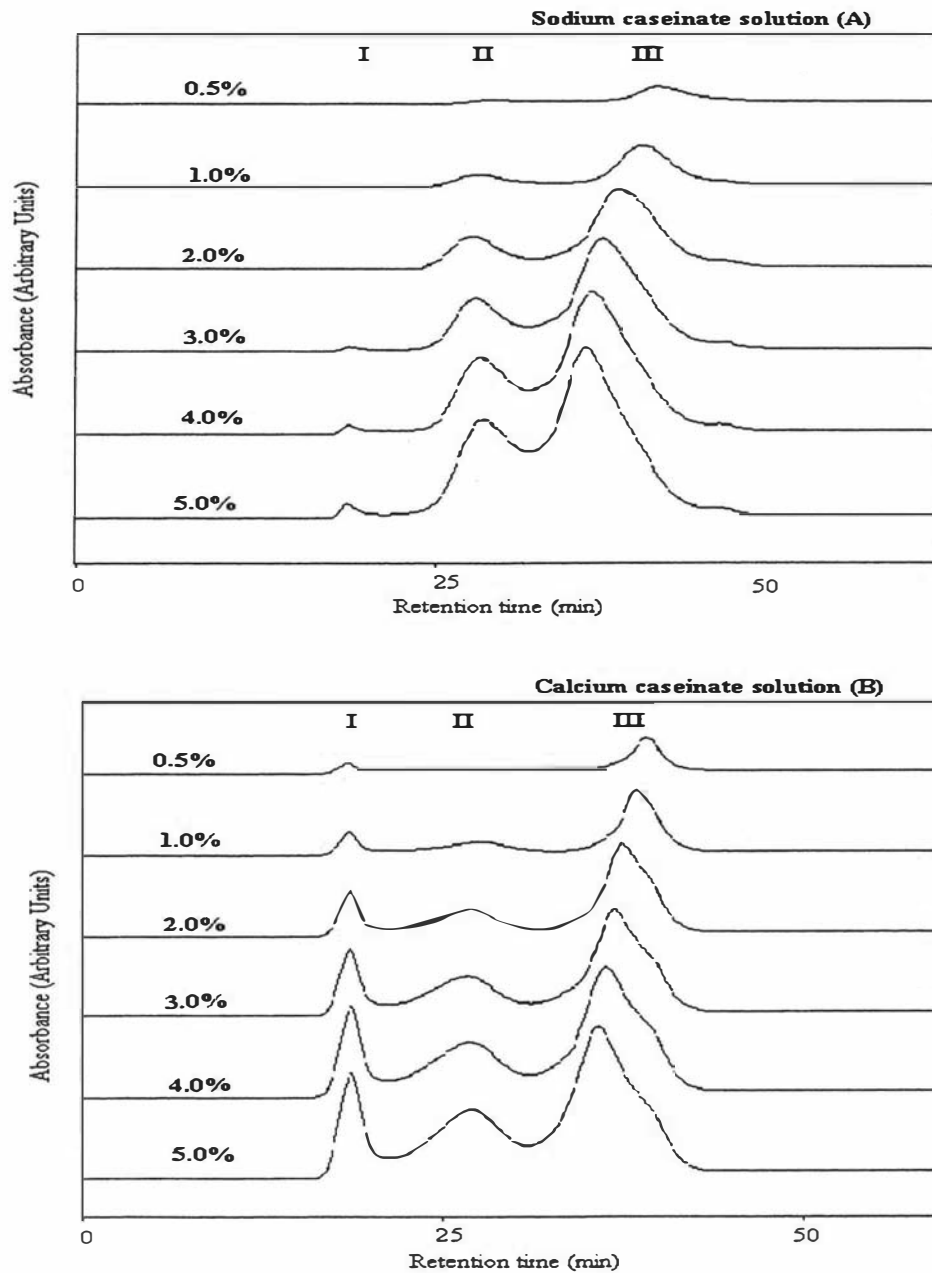
Figure 4.3: Influence of protein concentration on the elution profiles of sodium caseinate (A) or calcium caseinate (B) on a Superose 6HR 10/30 column. The flow rate was 0.4 ml/min and imidazole/NaCl buffer was used as the eluent.

Note: Concentrations of calcium caseinate shown above represent protein concentrations prior to centrifugation and filtration.

Figure 4.4 shows the change in the particle size distribution of the calcium caseinate solution as a function of protein concentration. At all concentrations of protein, a bimodal size distribution was obtained. An increase in the caseinate concentration resulted in an increase in the proportion of large particles (2 - 30 µm range), with a corresponding decrease in the proportions of particles in the range 0.1 - 2.0 µm. This indicates that aggregation of caseins in calcium caseinate solutions was enhanced at high protein concentrations.

4.2.3.3 Multi angle laser light scattering combined with SEC

Multi angle laser light scattering (MALLS) instrument was used to determine the molecular mass and the radius of gyration of caseinate solutions as a function of protein concentration.

Typical SEC-MALLS elution profiles obtained for 2% sodium caseinate solution on a Superose 6HR 10/30 column in combination with the MALLS photometer are shown in Figure 4.5. The UV and RI detector responses were very similar, but the MALLS response at 90° was very different. The MALLS system clearly detected large aggregated material (peak I) that eluted between 16 - 20 min (Figure 4.5). This material was almost invisible to the RI and UV detectors. However, the MALLS system was much less sensitive to smaller molecules, as can be seen from the sizes of peaks II and III.

In an attempt to remove this large aggregated material from sodium caseinate solutions, samples were ultracentrifuged at 90,000 g for 1 hour. Under these conditions, no sedimentation of protein was found to occur, but a cloudy layer was observed at the top of the centrifuge tube. This indicates that the large aggregated material consisted of some very small fat globules or fat-protein complexes that may have been generated during caseinate manufacture. When a clear subnatant layer was applied to the column, the size of the aggregate peak, detected by the MALLS instrument, decreased and the other two peaks were also visible (Figure 4.6).
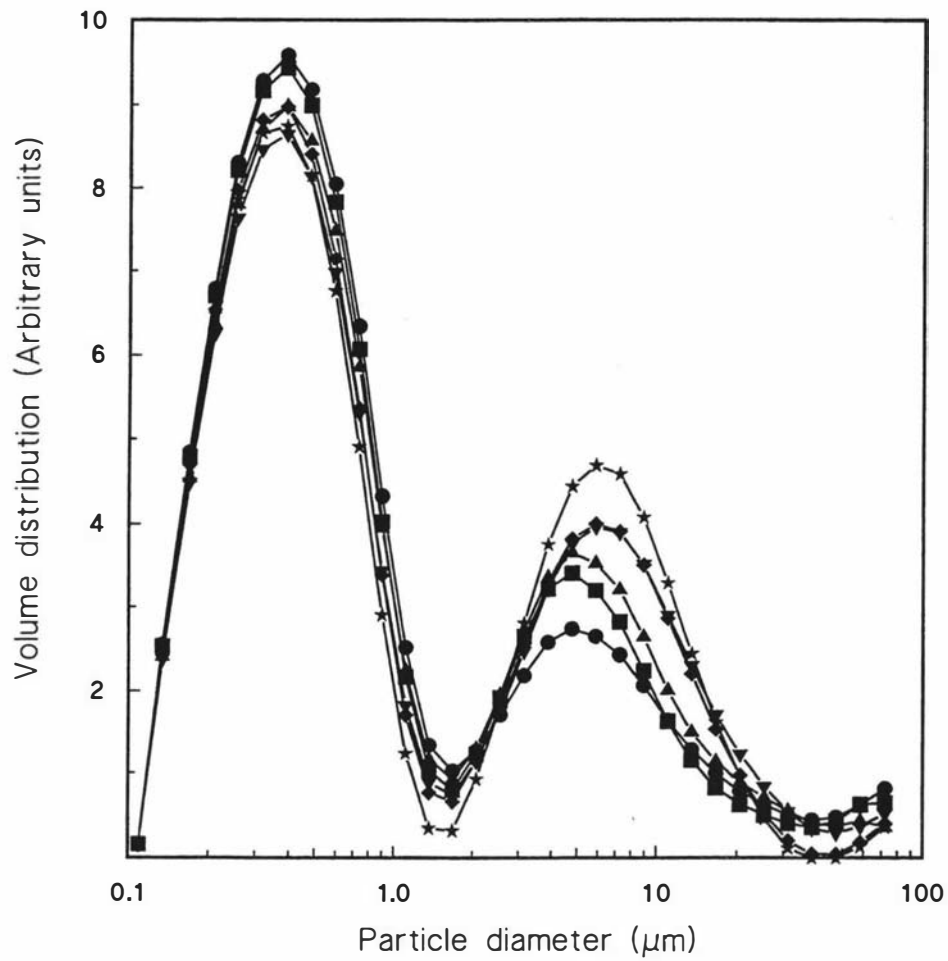
Figure 4.4: Particle size distribution of 0.5% (•); 1.0% (■); 2.0% (▲); 3.0% (▼); 4.0% (♦); 5.0% (★) calcium caseinate solutions, measured by light scattering using the Malvern Mastersizer. (Presentation used was 2NAD as per the emulsions).
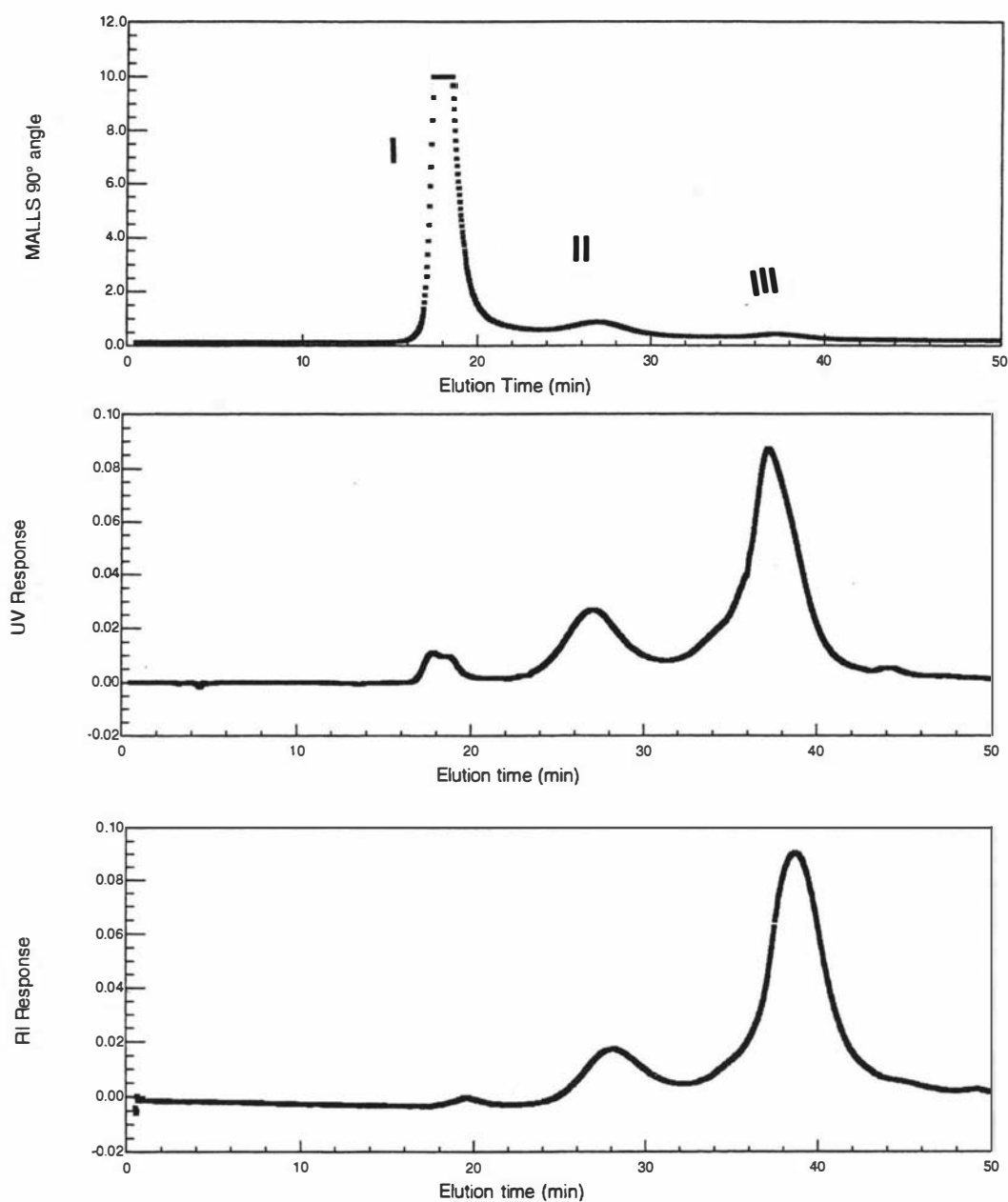
Figure 4.5: Typical elution profiles of SEC-MALLS, UV and RI detectors when 2% sodium caseinate was injected into a Superose 6HR 10/30 SEC column. Sodium caseinate solutions were filtered through 0.22 μm membrane filter.
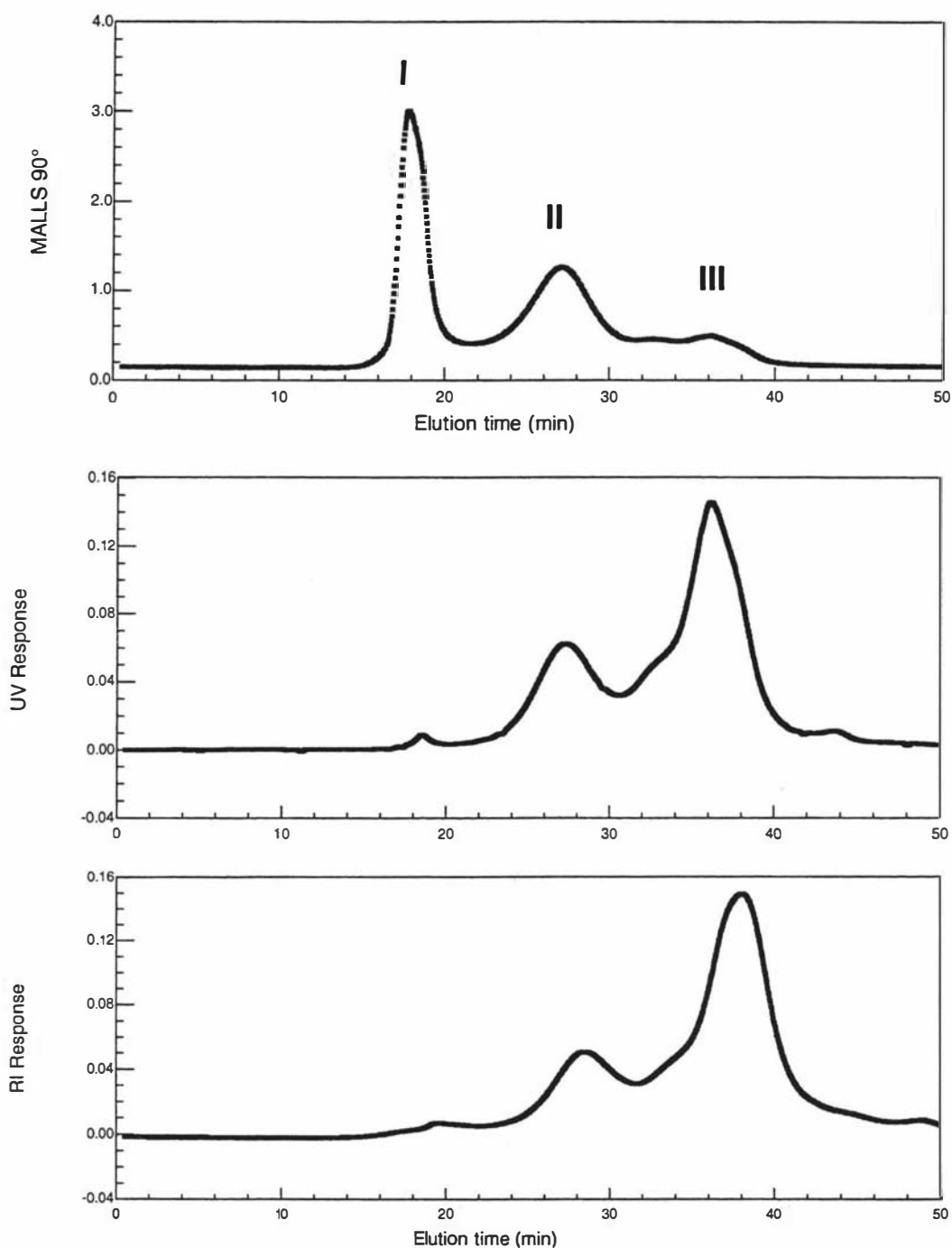
Figure 4.6: Typical elution profiles of SEC-MALLS, UV and RI detectors when 2% sodium caseinate was injected into a Superose 6HR 10/30 column. Sodium caseinate solutions were ultracentrifuged at 90,000 g for 1hour and the subnatant was filtered through 0.22 μm membrane filter, and then applied to the column.

Analysis of peak II and peak III by the Astra software of the MALLS instrument showed that peak II had a weight-average molecular mass (M_w) of ~ 4.7 ± 0.174 x 10^5 D, indicating mainly casein aggregates, and ~ 5.3 ± 0.051 x 10^4 D for peak III indicating mainly dimers and trimers.   Figure 4.7 shows the relationship between elution time and log molecular mass of a 2% caseinate solution. In general, this relationship was linear for elution times between 22 - 34 min (this corresponds to elution volume between 9 - 14 ml). At the low molecular mass end of the distribution, there was a deviation from this linear relationship, which is apparently caused by the combination of the low sensitivity of the MALLS detectors and the small amounts of material present.

The effect of caseinate concentration (0.5 - 5.0%) on molecular mass was determined (Figure 4.8). As the concentration of sodium caseinate was varied from 1.0 to 2.0%, the weight-average molecular mass for peak III increased slightly, but further increase in the protein concentration caused the weight-average molecular mass to increase considerably. In contrast, the average molecular mass for peak II showed a slight decrease with increase in protein concentration (Figure 4.9). These results suggest that, as the concentration of protein eluting in peak III increased, the weight-average molecular mass also increased, essentially confirming the SEC results (Figure 4.3).

When reporting the molecular size distribution of proteins as determined by SEC, it is necessary to keep in mind that elution is not necessarily governed by differences in molecular mass only. The gross shape of the molecule and its physico-chemical characteristics, especially net charge and hydrophobicity, may also play a role in separation (Billingham, 1977; Hoffmann et al., 1997). Light scattering provides an absolute measurement of molecular masses whereas conventional calibration relies on a relation between molecular mass and retention time, which is valid only for molecules that are similar with respect to chemical structure and molecular conformation (Hoffmann et al., 1997). Calibration curves used in SEC usually show an upward curvature close to the
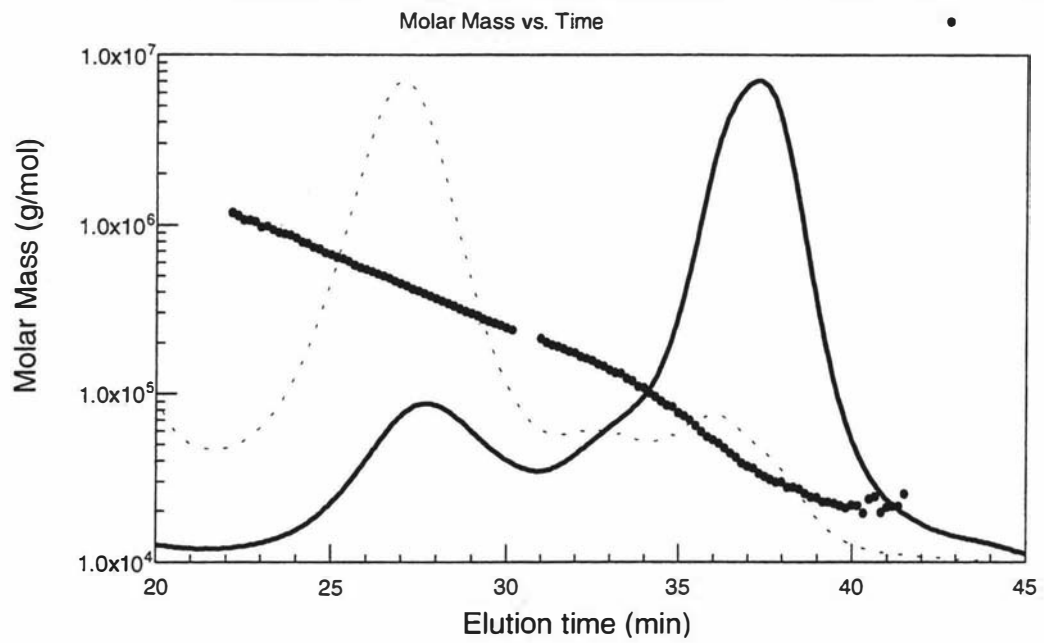
Figure 4.7: Relationship between absolute molecular mass (•) and retention time from SEC for a 2% centrifuged sodium caseinate solution. Light scattering 90° angle (------------), UV (————).
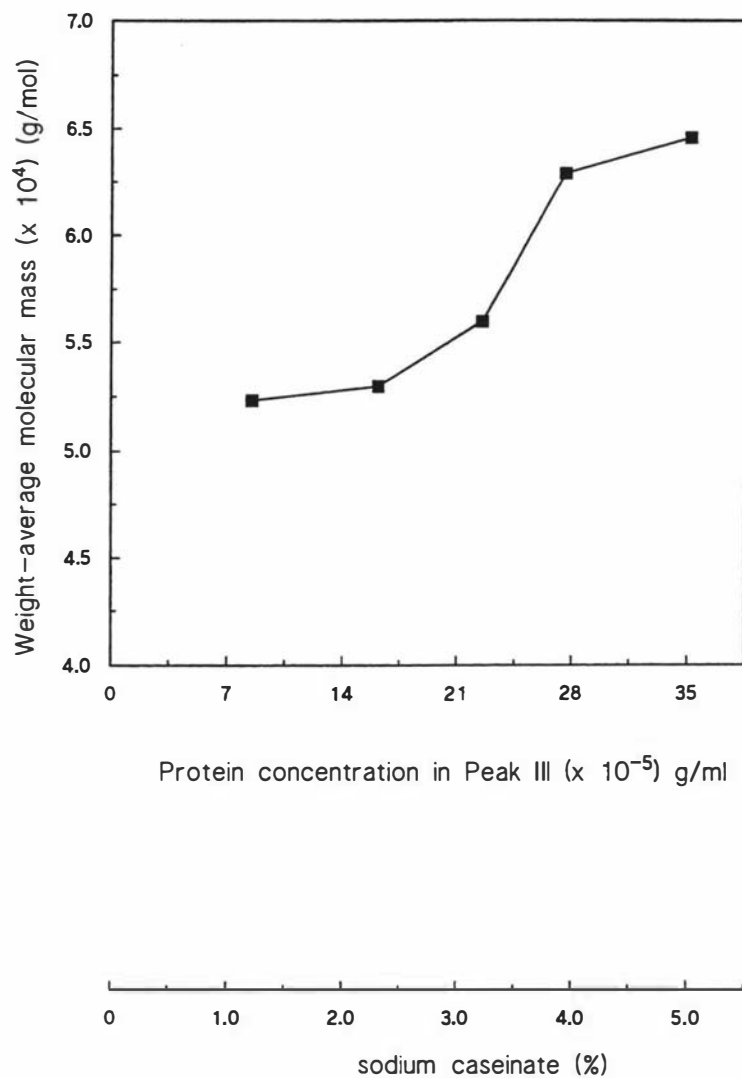
Figure 4.8: Changes in the molecular mass of protein in peak III as a function of caseinate concentration. The upper x-axis scale shows the protein concentration in peak III as calculated by the ASTRA software package of the MALLS system.
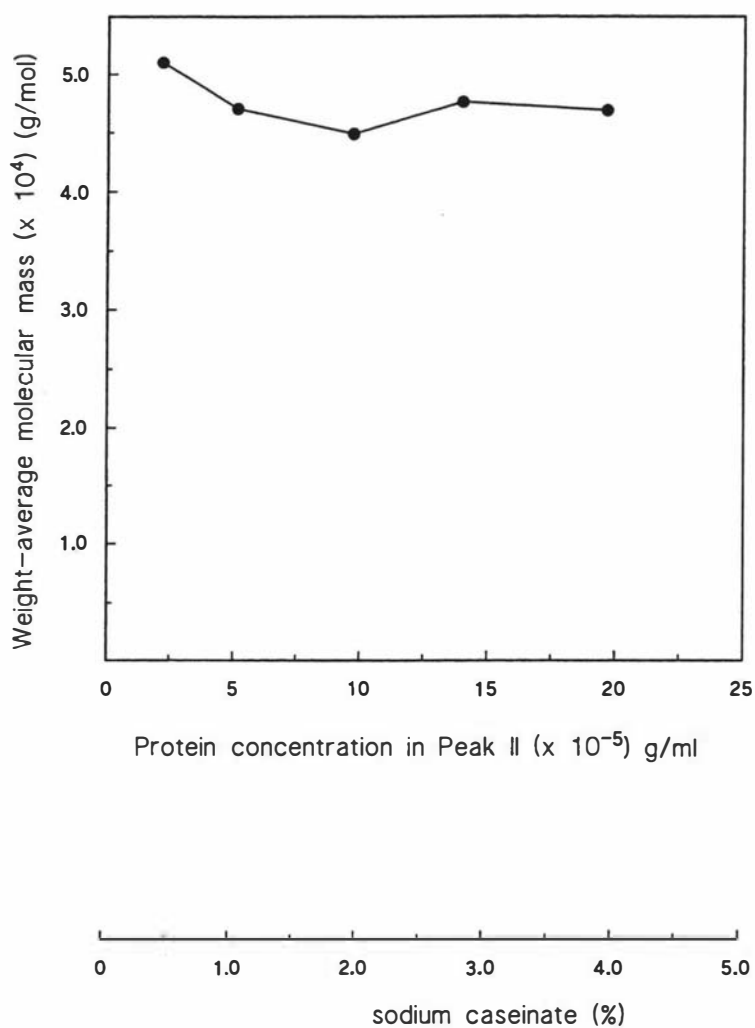
Figure 4.9: Changes in the molecular mass of protein in peak II as a function of caseinate concentration. The upper *x*-axis scale shows the protein concentration in peak II as calculated by the ASTRA software package of the MALLS system.

exclusion limit (Billingham, 1977), and in this case the use of linear extrapolation results in an underestimation of the molecular mass of the material eluting close to the void volume. So, in general, conventional SEC is expected to give an underestimation of the molecular mass of material eluting in or close to the void volume.

In the SEC-MALLS system the important parameter needed to obtain accurate molecular mass information is refractive index increment (*dn/dc*). The *dn/dc* is necessary for determining the absolute quantities of material eluting at each volume increment. This information is needed for determining the weight-fractions and for extrapolation of the light-scattering data to zero concentration in the Debye plot. The *dn/dc* value of 0.190 cm^3/g was used (Payens and Heremans, 1969; Huglinm, 1972).

The radius of gyration obtained by MALLS showed no significant variation with concentration of the sodium caseinate solution. The radius of gyration varied from ~ 11 to ~ 17 nm with no significant trends.

Light scattering experiments could not performed with calcium caseinate solutions because of some very large particles present in the calcium caseinate solutions.

4.3 Discussion

Manufacturing processes for the caseinate protein powders are well known, as described in Chapter 2. Generally, sodium and calcium caseinates are manufactured commercially from wet acid casein curd. The most important variables in sodium caseinate production are the solids level, the degree of aggregation of the initial acid casein curd, and pH/temperature/time of the conversion reaction. Conversion of the acid curd to caseinate is a gradual process and requires disaggregation and titration of the acid casein curd from a pH of ~ 4.6 to a pH of 6.8 using NaOH. The strength of the aggregates in the curd may directly affect the level of free H$^+$ ions and hence the time the casein

may be exposed to a high pH. At pH 6.8, all the caseins are solubilized, and the soluble caseins are then spray dried. Due to the dispersion properties of sodium caseinate and the resulting viscosity created, the solids levels going to the drier must be maintained under 25% even at elevated temperatures.

In the manufacture of calcium caseinate, conversion of acid casein curd to caseinate is carried out by calcium hydroxide addition. As the pH increases, caseins disaggregate and solubilize, as a result of increased electrostatic repulsions between casein molecules. Simultaneously, the binding of calcium ions to caseins increases with an increase in pH due to changes in the ionization state of phosphoserine residues, which have been identified as the primary binding sites for calcium (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981). The solubility of α_{s1}-, α_{s2}- and β-caseins is strongly affected by the binding of calcium ions, although κ-casein is not affected by the binding of calcium (Ho and Waugh, 1965; Ono *et al.*, 1976, 1980; Aoki *et al.*, 1985; Farrell *et al.*, 1988; Dalgleish and Parker, 1980; Parker and Dalgleish, 1981). Therefore, it is possible that the addition of calcium hydroxide used in the conversion process could cause extensive aggregation or even precipitation of α_{s1}- and α_{s2}-caseins. The precipitation of these caseins would be prevented, to some extent, by κ-casein (Ho and Waugh, 1965; Ono *et al.*, 1976, 1980; Aoki *et al.*, 1985; Farrell *et al.*, 1988; Dalgleish and Parker, 1980; Parker and Dalgleish, 1981) by forming complexes. The end result is probably the formation of artificial casein micelles or even larger particles in which the caseins, especially α_{s1}- and α_{s2}-caseins, are linked by calcium ions and are in equilibrium with soluble caseins. Some of the casein material that sedimented at low centrifugal speed (Figure 4.1) was probably α_{s1}- and α_{s2}-caseins that had become "insoluble" due to calcium binding during the conversion process. The SDS-PAGE results strongly support the above suggestions. Thus, it can be concluded that calcium caseinate solutions (at pH 6.8) consist of a diverse range of casein aggregates and particles. There are some very large aggregates, with high proportions of α_{s1}- and α_{s2}-caseins, some monomeric molecules, casein complexes and calcium-induced casein aggregates of

different sizes. It therefore appears that the colloidal structure of calcium caseinate particles in solution is very different from the native casein micelle structure in milk.

When commercial sodium or calcium caseinate solutions are ultracentrifuged (90,000 *g* for 1 hour) a cloudy layer floats on the top of the centrifuge tube which suggests that fat, which may have been present in the skim milk used for making caseinate, could be involved in the formation of this floating material. This material was easily detectable with the MALLS system. It is well known that very small fat globules are not separated in the cream separator. When skim milk is pasteurized the serum proteins (β-lactoglobulin and α-lactalbumin) and caseins become adsorbed to these fat globules (Sharma and Dalgleish, 1993), which consequently are precipitated along with the caseins at pH 4.6, and end up in the final caseinate product. Further work will be required to determine the nature of this material, and identify the mechanisms of its formation during the processing of caseinates. The influence of this material on caseinate functionality would be worth investigating.

Overall, the data presented here show that caseins in sodium caseinate solution exist as a dynamic system of casein monomers, casein complexes and aggregates. SEC-MALLS clearly showed that most of the protein was eluted in two peaks. A casein aggregate peak (peak II), of molecular mass in the range from ~ 2.5×10^5 to ~ 2×10^6 D, consisted predominantly of κ-casein polymers and some α_{s1}- and β-casein complexes. The second peak (peak III) had a substantial leading edge, and contained casein complexes of various molecular masses (from ~ 2×10^5 to ~ 45,000 D), and some monomers (~ 30,000 D) at the trailing end of the distribution. Concentration of casein had a major effect on the molecular mass and composition of casein aggregates and complexes. The weight-average molecular mass of caseins in peak III increased with an increase in concentration, which is consistent with the results of Pepper and Farrell (1982) who reported, using gel permeation chromatography, that, for soluble casein in solution, changes in protein

concentration resulted in a variation of association behaviour. It is interesting to note that a considerable proportion of the caseins in calcium caseinate solutions had similar aggregates to those in sodium caseinate solutions. It is estimated that ~ 50% of the total protein in calcium caseinate solution consisted of casein complexes and aggregates that were of similar molecular mass to those in sodium caseinate solution. The remaining caseins were highly aggregated, forming some particles larger than 2 μm and with a molecular mass of several hundred million.

Chapter 5

ADSORPTION BEHAVIOUR OF SODIUM AND CALCIUM CASEINATE IN OIL-IN-WATER EMULSIONS

5.1 Introduction

Milk proteins, especially caseinates, are good emulsifiers and hence are used as ingredients in a wide range of formulated food emulsions. Several studies have been reported on the adsorption behaviour of pure caseins and casein mixtures, such as sodium caseinate, in model emulsions (Tornberg, 1978b; Dickinson *et al.,* 1988b; Robson and Dalgleish, 1987, Hunt and Dalgleish, 1994). There is less information available on the adsorption behaviour of commercial calcium caseinates in which the caseins are substantially more aggregated than in sodium caseinate (Mulvihill and Murphy, 1991).

The existence of competitive adsorption between the different casein components has been clearly demonstrated in emulsions stabilized by a mixture of α_{s1}- and β-caseins (Dickinson *et al.,* 1988b); the more hydrophobic β-casein appears to adsorb in preference to α_{s1}-casein. Exchange experiments indicate that β-casein can also rapidly displace adsorbed α_{s1}-casein (Dickinson *et al.,* 1988b). However, when the emulsion is formed with sodium caseinate, there appears to be little evidence of competition between the different caseins for the oil droplet surface (Robson and Dalgleish, 1987; Hunt and Dalgleish, 1994). Recent studies (Srinivasan *et al.,* 1996) have shown that protein concentration is an important factor in determining whether or not competitive adsorption occurs in emulsions made with sodium caseinate. There is not much work reported on calcium caseinate-stabilized emulsions. In this chapter the findings of Srinivasan *et al.* (1996) have been extended and the adsorption behaviour of sodium caseinate has been compared with calcium caseinate-stabilized oil-in-water emulsions at different protein concentrations.

5.2 Results and discussion

5.2.1 Emulsion formation

Emulsions were formed by mixing 385 g of soya oil and 900 g of caseinate solution, containing 0.5, 1.0, 2.0, 3.0, 4.0 or 5.0% protein. This resulted in emulsions containing 30% oil and 0.375, 0.75, 1.5, 2.25, 3.0 or 3.75% protein in emulsions. Most of the data presented refer to the concentration of protein in the aqueous phase used for making emulsions.

5.2.2 Particle size distribution

Emulsions, at each protein concentration, were prepared on three separate occassions. Figure 5.1 shows the typical particle size distributions of emulsions formed with sodium caseinate (A) and calcium caseinate (B) at various protein concentrations. There was a gradual shift in the size distribution range towards higher particle sizes as the protein concentration was decreased from 5.0 to 0.5%, particularly in the emulsions formed with calcium caseinate. Whereas the size distribution was monomodal in all emulsions formed with sodium caseinate, the size distribution in emulsions formed with calcium caseinate was monomodal only at protein concentrations $\geq$ 2%. The range of particle sizes within these monomodal distributions was narrower (0.1 - ~ 5 µm) in sodium caseinate-stabilized emulsions compared with calcium caseinate-stabilized emulsions (0.1 - 10 µm). Calcium caseinate-stabilized emulsions at protein concentrations of 0.5 and 1.0% had a bimodal size distribution which was more pronounced in the emulsion formed with 0.5% protein. The bimodal size distribution of particles in emulsions formed with calcium caseinate at low protein concentration, i.e. at 0.5 and 1.0%, may have been due to an insufficient amount of protein being available to fully cover the oil/water interface. This may have led to the possibility of bridging flocculation between oil droplets, i.e. sharing of one protein molecule/aggregate by two or more droplets (Vincent, 1973).

In order to determine the incidence of bridging flocculation in calcium caseinate-stabilized emulsions, particle size distributions were determined after
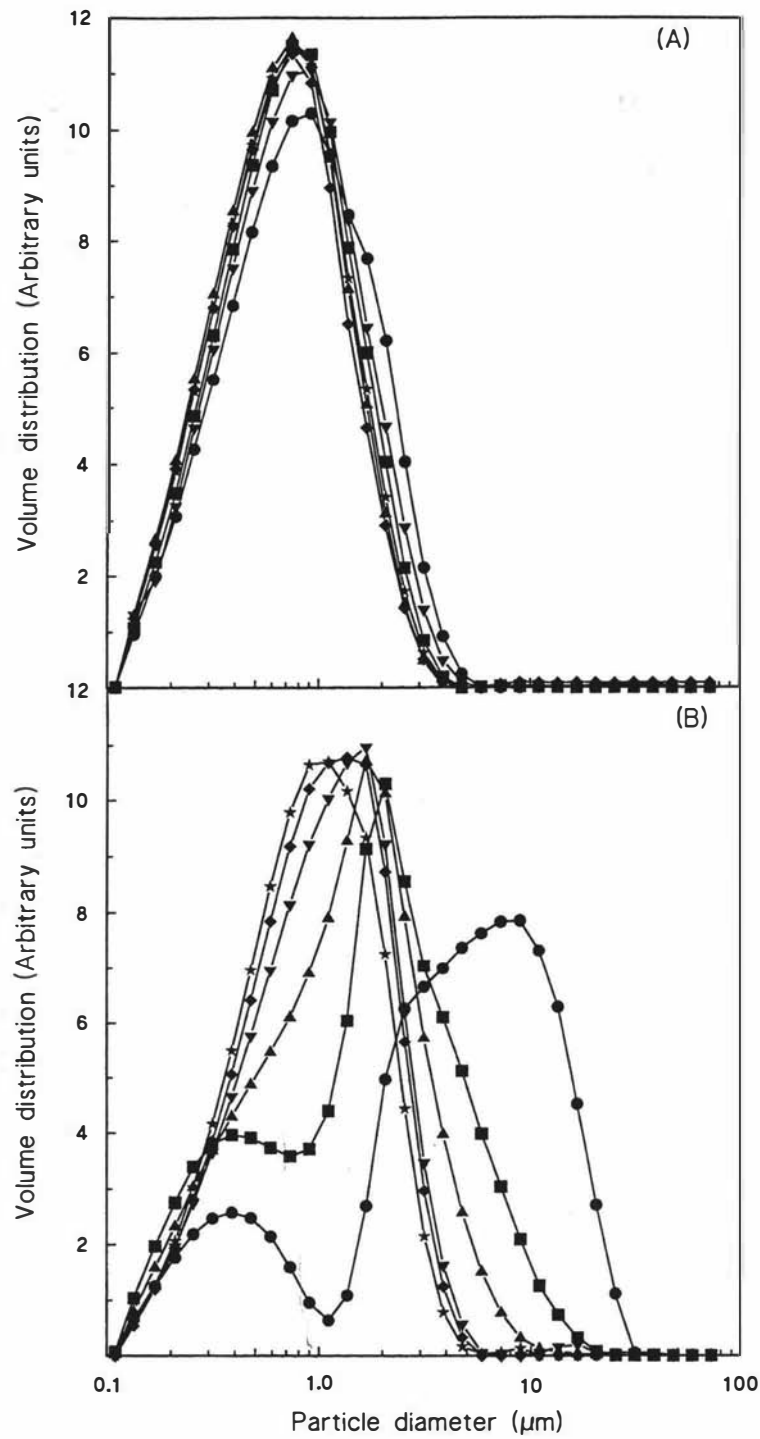
Figure 5.1: Particle size distribution of emulsions (30% oil w/w) formed with 0.5% (•); 1.0% (■); 2.0% (▲); 3.0% (▼); 4.0% (♦); 5.0% (★) sodium caseinate (A) or calcium caseinate (B).

dispersion of the emulsions in a dissociating buffer (Figure 5.2). The dissociating buffer was a mixture of 0.02% EDTA, which chelates calcium and thus causes the disintegration of calcium caseinate (Singh and Latham, 1993), and 2% SDS, which breaks the hydrophobic interactions and displaces the protein from the interface (Tomas *et al.,* 1994). Emulsions were mixed with the dissociating buffer in the ratio of 1:3 before measuring the particle size distribution. It was noted that at all protein concentrations, the particle size distributions became narrower (0.1 - ~ 5 µm) after dispersion of the emulsions in the dissociating buffer. The range of the particle size distribution in Figure 5.2 was fairly close to that of the sodium caseinate-stabilized emulsions (Figure 5.1 A). In emulsions formed with ≤ 1.0% protein, there was a very marked shift in the size distribution towards lower sizes, although emulsions formed with 0.5% protein still showed a bimodal character. As the dilution of an emulsion with EDTA and SDS displaces the protein from the surface and dissociates any large protein aggregates, these results indicate that bridging flocculation may occur between emulsion droplets, particularly at low concentrations of calcium caseinate.

Dispersion of sodium caseinate-stabilized emulsions in the dissociating buffer had no effect on their particle size distributions (results not shown), indicating no bridging flocculation in these systems.

The changes in volume-surface average droplet diameter (d_{32}) with various caseinate concentrations in emulsions are shown in Figure 5.3. An increase in the protein concentration decreased the average droplet diameter considerably in calcium caseinate-stabilized emulsions whereas in sodium caseinate-stabilized emulsions the average droplet diameter decreased only slightly with protein concentration.
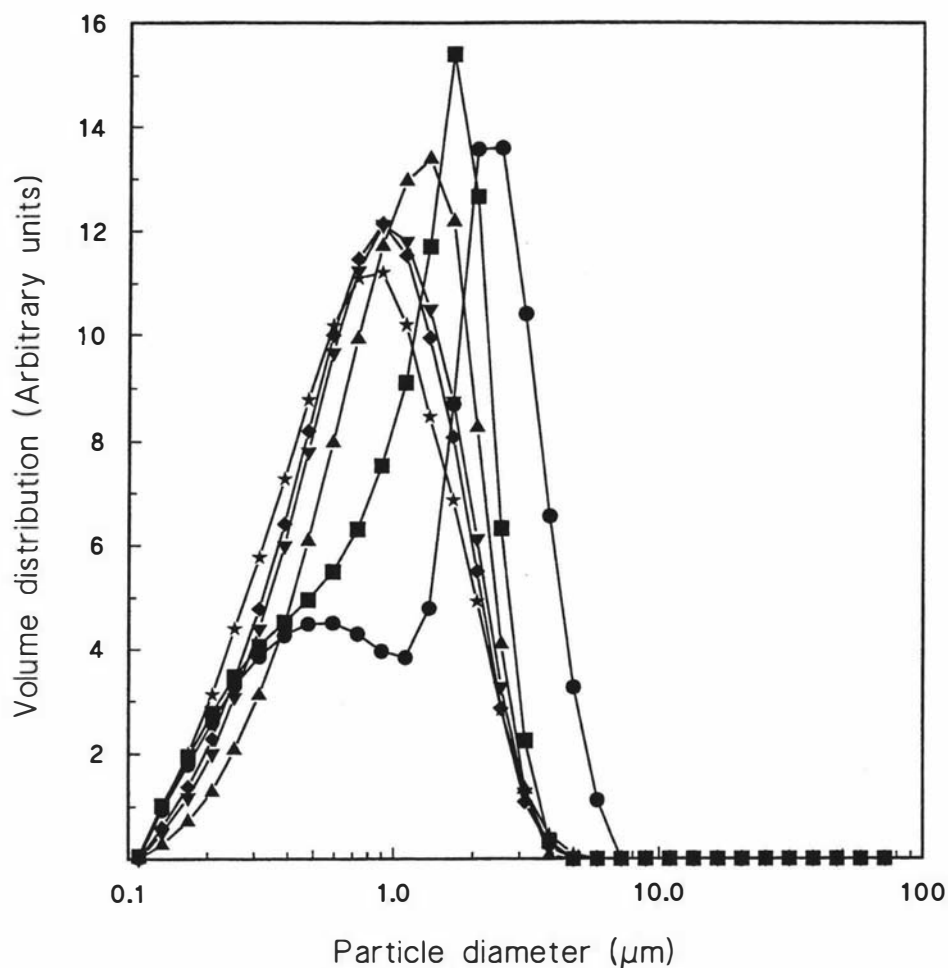
Figure 5.2: Effect of dispersion of calcium-caseinate-stabilized emulsions in a dissociating buffer (0.02% EDTA, 2% SDS) on the particle size distribution. Emulsions formed with 0.5% (•); 1.0% (■); 2.0% (▲); 3.0% (▼); 4.0% (♦); 5.0% (★) calcium caseinate.

At all protein concentrations, the average droplet diameter in calcium caseinate-stabilized emulsions was greater than that in sodium caseinate-stabilized emulsions (Figure 5.3). However, the difference in droplet diameter between sodium and calcium caseinate-stabilized emulsions decreased with increase in protein concentration. For example, the droplet diameters of sodium and calcium caseinate-stabilized emulsions were ~ 0.6 and 1.2 µm, respectively, at 0.5% protein ~ 0.54 and 0.72 µm, respectively, at 5.0% protein.

The results obtained for sodium caseinate-stabilized emulsions are essentially in agreement with those reported by Fang and Dalgleish (1993) and Srinivasan *et al.* (1996). No previous work has been reported on the effect of protein concentration on droplet sizes in calcium caseinate-stabilized emulsions. We know that the process of emulsification begins with mixing oil, as a dispersed phase, and water containing dissolved protein, as a continuous phase. Application of mechanical energy, such as homogenization, leads to the deformation of the interface between the two phases so that large droplets are formed, which are subsequently broken down into smaller ones. The forces that lead to the disruption of droplets are opposed by the Laplace pressure generated by a curved interface. Any deformation of the spherical droplet leads to changes in the radii of curvature, generating pressure gradients that tend to restore the droplet to its original size. The external pressure required to disrupt a droplet with interfacial tension γ, i.e. Laplace pressure, is given by $2\gamma/r$ for a spherical droplet of radius r. This explains why a reduction in the interfacial tension favours the emulsification process and why large droplets are easier to break than smaller droplets. It has been shown that an increase in protein concentration results in a decrease in the interfacial tension (Mulvihill and Fox, 1989; Tornberg *et al*.,1990) which consequently reduces the pressure required to disrupt the emulsion droplet.
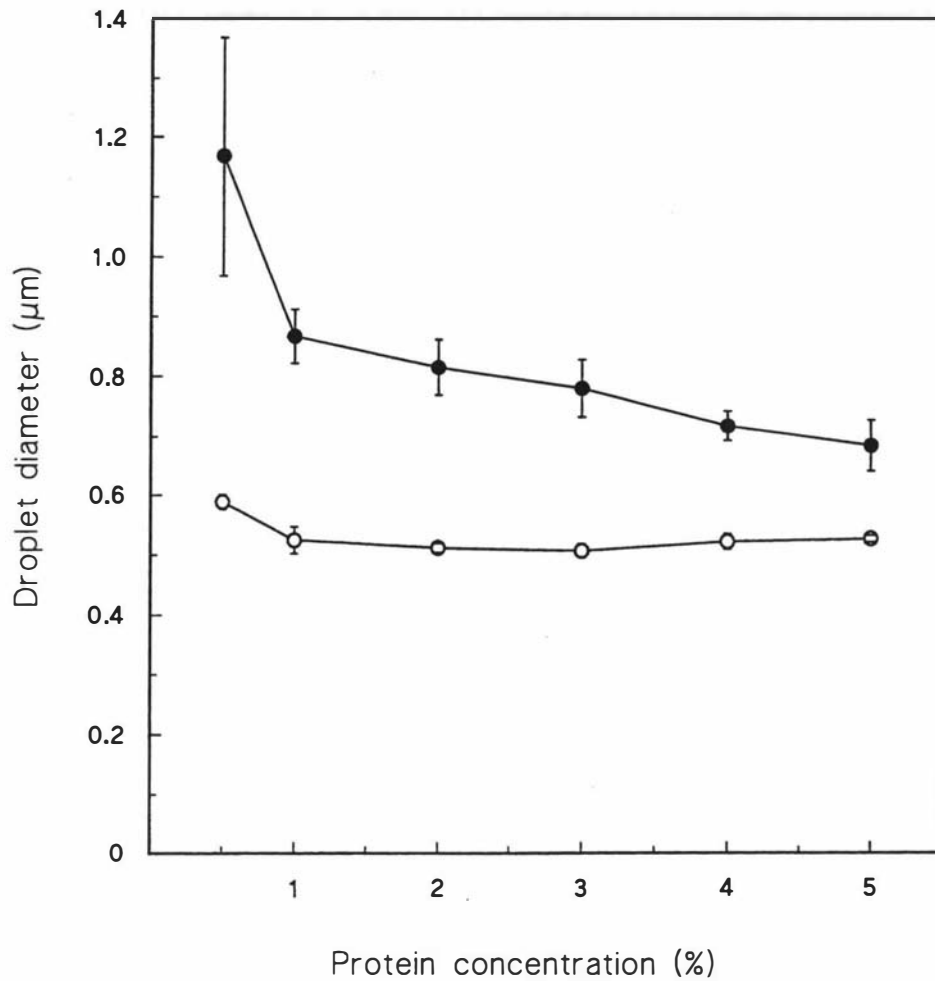
Figure 5.3: Changes in the average droplet diameter (d_{32}) of emulsions as a function of concentration (30% soya oil) of calcium caseinate (•) or sodium caseinate (○). Each data point represents average of three separate experiments. Error bars represent ± 1 SD.

5.2.3 Surface protein coverage

For emulsions formed with sodium caseinate, the surface protein concentration increased gradually with protein concentration up to 3.0%, but the increase was much smaller at higher concentrations (Figure 5.4). A maximum coverage of ~ 2.0 mg/m², which probably corresponds to monolayer adsorption of caseins, was observed under these conditions. This trend was generally similar to that reported earlier by Fang and Dalgleish (1993), Hunt and Dalgleish (1994) and Srinivasan *et al.* (1996).

The surface protein coverage in emulsions stabilized by calcium caseinate showed an almost linear increase with protein concentration (Figure 5.4). At all caseinate concentrations, the surface protein coverage of emulsions stabilized by calcium caseinate was higher than in those stabilized by sodium caseinate. The differences in surface protein concentration between the two emulsion systems increased above 2% protein. The higher levels of protein coverage in calcium caseinate emulsions suggest that casein aggregates were involved in the adsorption. Relationships between surface coverage and the aggregation state of protein have been previously demonstrated by Oortwijn & Walstra (1979), Mulvihill & Murphy (1991) and Singh *et al.* (1993).
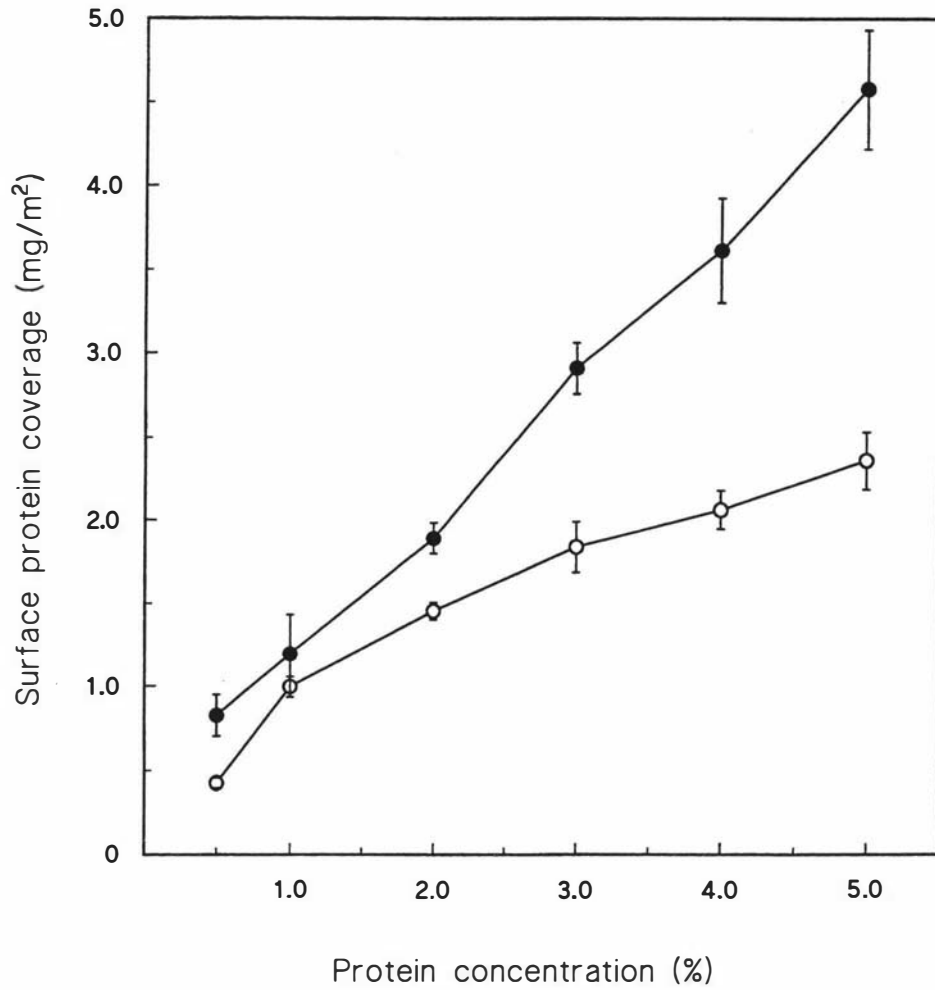
Figure 5.4: Changes in surface protein coverage (mg/m^2) of emulsion droplets as a function of concentration of calcium caseinate (•) and sodium caseinate (○).Each data point represents average of three separate experiments. Error bars represent ± 1 SD.

5.2.4 Composition of caseins at the oil/water interface (cream phase)

Electrophoretic patterns of the cream phase that had been dispersed in SDS buffer with mercaptoethanol, obtained from sodium and calcium caseinate-stabilized emulsions are shown in Figure 5.5. In the SDS-PAGE system used in this study, α_{s1}-, β-, and κ-caseins were well separated from each other but α_{s2}-casein was not resolved from α_{s1}casein. The intensities of κ-casein and α_{s} casein band appeared to increase with an increase in concentration from 0.5 to 5.0% (Figure 5.5 A), whereas the intensity of the β-casein band appeared to increase slightly initially but then decreased. Figure 5.5 B shows the electrophoretic patterns of the of caseins in calcium caseinate-stabilized emulsions using SDS-PAGE. The intensities of the α_{s}-, β- and κ-casein bands showed an increase with increase in calcium caseinate concentration from 0.5 to 5.0%. At all concentrations, the intensity of the α_{s}-casein in the cream was higher than the intensities of β- or κ-casein.

Quantitation of individual caseins by laser densitometer showed that at low caseinate concentrations (0.5 - 2.0%) the proportion of α_{s}-casein at the interface in sodium caseinate-stabilized emulsions was lower than that of β-casein (Figure 5.6) and that the ratio of α_{s}- to β-casein was considerably lower than the ratio in the original sodium caseinate (0.4:0.4). This suggests that β-casein was adsorbed in preference to α_{s}-casein under these conditions. In contrast, emulsions formed with 3 - 5% sodium caseinate showed considerably higher proportions of α_{s}-casein at the interface compared with β-casein. The proportions of κ-casein showed an increase with increase in protein concentration.

(A)

(B)
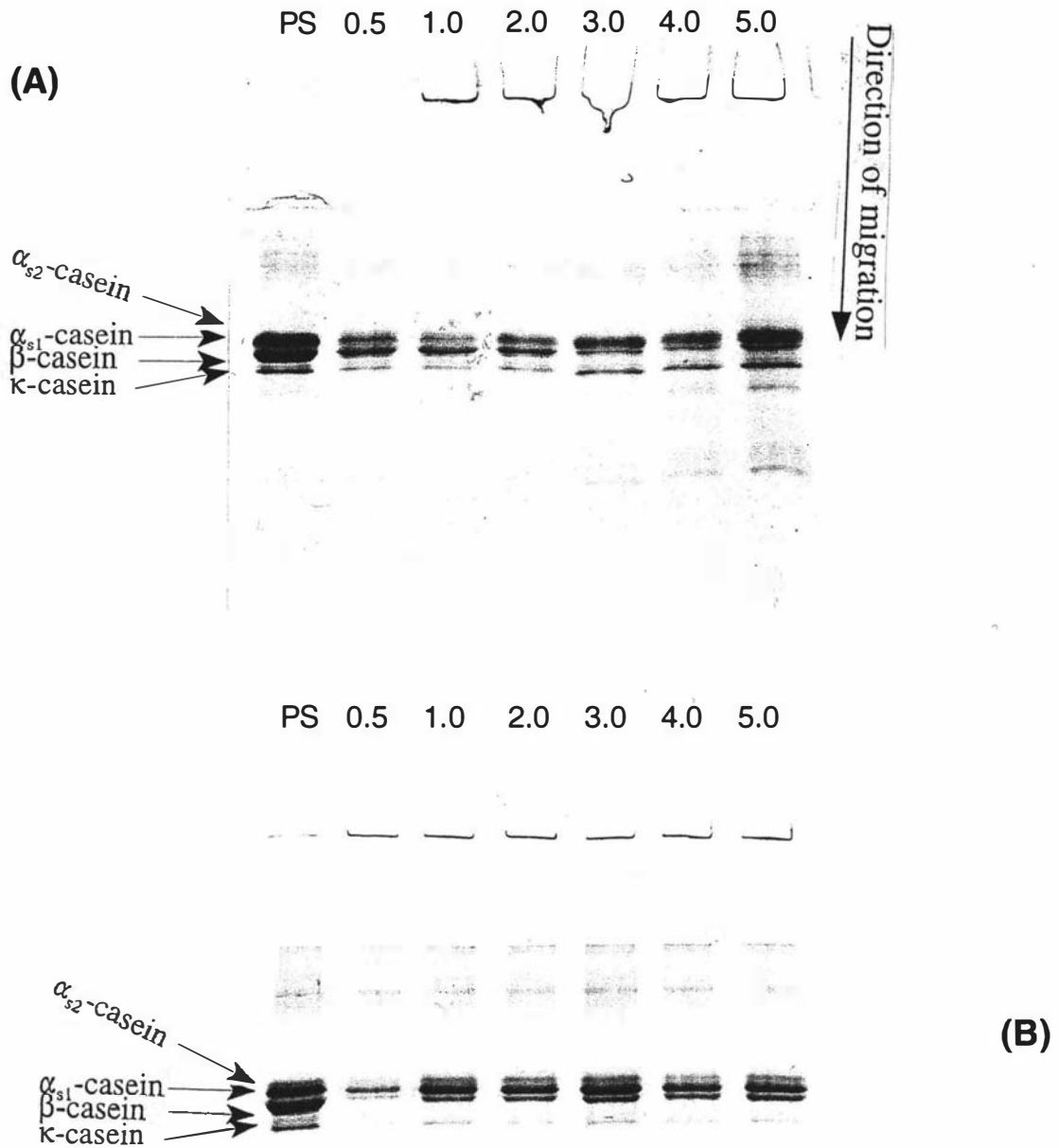
Figure 5.5: SDS-PAGE on the cream phase obtained from emulsions containing 30% soya oil and various concentrations of sodium caseinate (A) and calcium caseinate (B).

PS refers to control i.e.

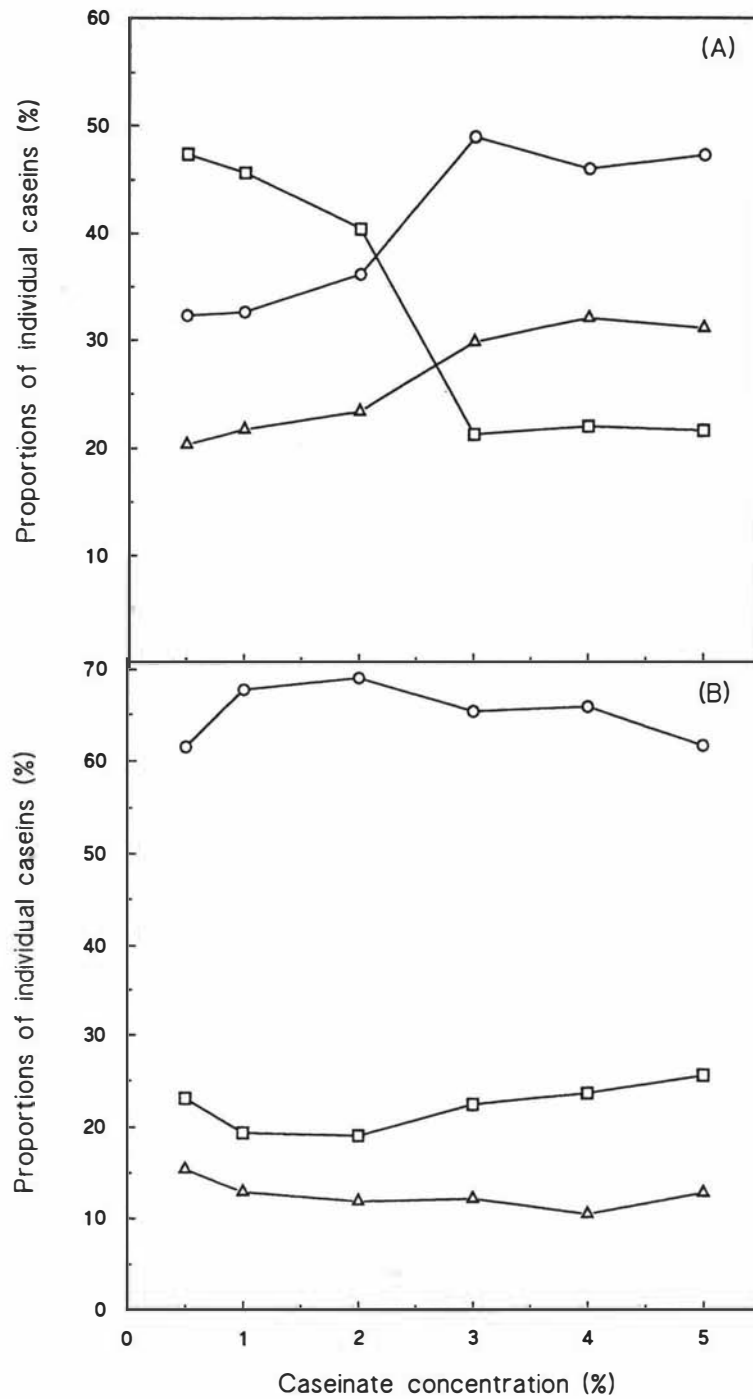(A) nomal sodium caseinate solution; (B) normal calcium caseinate solution.

Figure 5.6: Effect of caseinate concentration on relative proportions of α_s-(α_{s1}- + α_{s2} -) casein (○), β-casein (□) or κ-casein (△) at the oil/water interface in emulsions stabilized by sodium caseinate (A) or calcium caseinate (B).

The composition of the caseins at the droplet surface of emulsions formed with calcium caseinate was very different from that of sodium caseinate emulsions (Figure 5.6 B vs 5.6 A). At all concentrations used, α_s-casein was found to adsorb in preference to other caseins, as indicated by the higher ratio of α_s- to β-casein (~ 0.65:0.25) at the droplet surface compared with the original calcium caseinate (0.42:0.43). Increasing the calcium caseinate concentration did not significantly alter the proportions of individual caseins adsorbed at the droplet surface.

The surface concentrations of individual caseins, estimated from the proportions and surface coverage of sodium and calcium caseinate-stabilized emulsions, are presented in Figure 5.7. The surface concentrations of α_s- and κ-caseins increased gradually as the sodium caseinate concentration was increased from 0.5 to 5.0%. In contrast, the surface concentration of β-casein increased with an increase in caseinate concentration from 0.5 to 2.0% but decreased at 3.0% caseinate concentration. Further increase in the caseinate concentration had no effect on the surface concentration of β-casein (Figure 5.7 A). In emulsions made with $\leq$ 2.0% caseinate, the concentrations of surface caseins were in the order: β- > α_s- > κ-casein whereas in emulsions made with > 2.0% caseinate, the order was: α_s- > β- > κ-casein.

These results clearly show that the interfacial composition of emulsions formed with sodium caseinate is dependent on the amount of protein available during emulsification. β-Casein was adsorbed in preference to other caseins in emulsions made using $\leq$ 2.0% sodium caseinate. However, α_s-casein appeared to adsorb in preference to other caseins when emulsions were made using >2.0% protein.
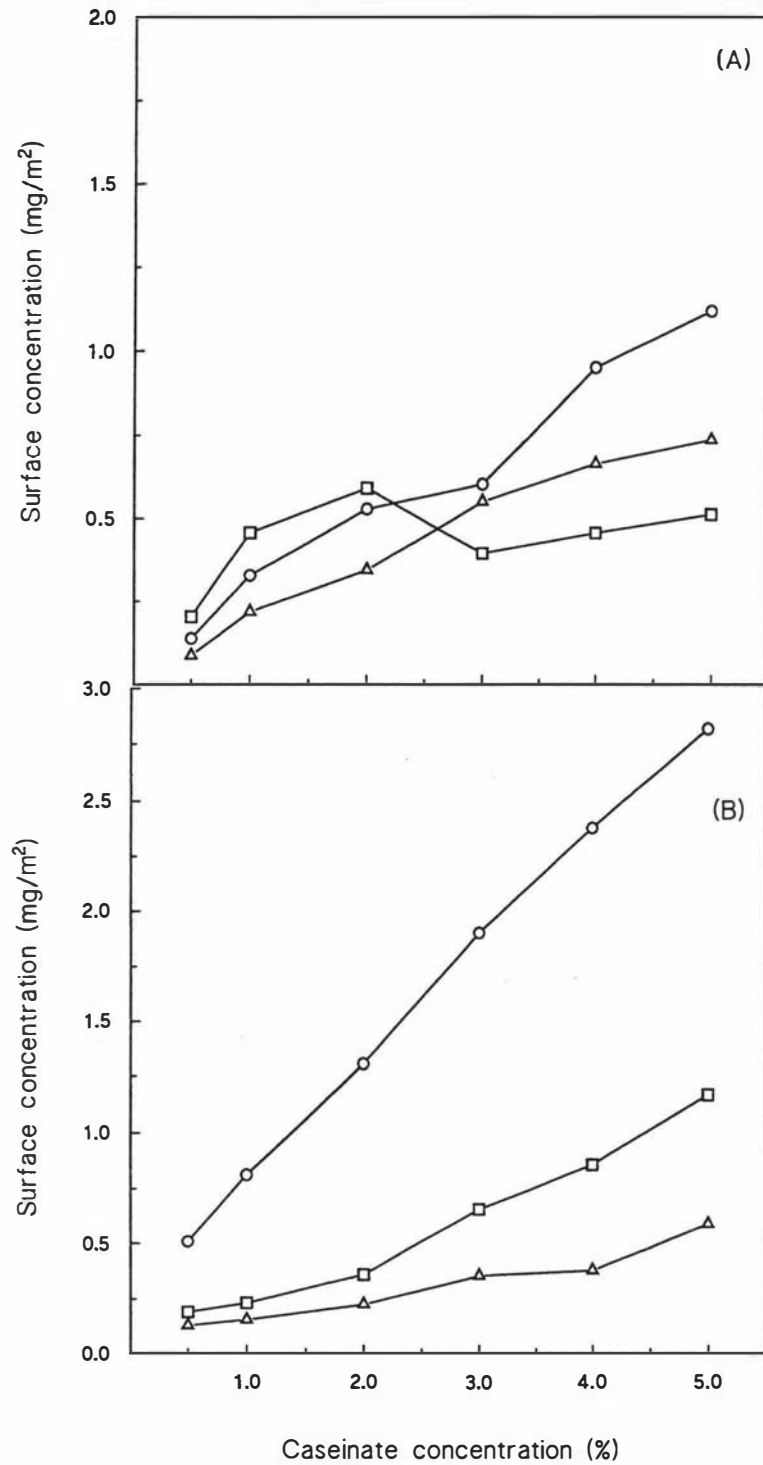
Figure 5.7: Estimated surface concentrations of α_s-casein ($\circ$), β-casein ($\square$) or κ-casein ($\triangle$) in sodium caseinate (A) or calcium caseinate (B) emulsions as a function of caseinate concentration.

On the other hand the surface concentrations of individual caseins in calcium caseinate-stabilized emulsions were very different from sodium caseinate-stabilized emulsions (Figure 5.7 B). The surface concentration of α_s-casein increased almost linearly with an increase in caseinate concentration. The surface concentrations of β- and κ-caseins also increased gradually as the calcium caseinate concentration was increased from 0.5 to 5.0%. The extent of increase followed the order: α_s- > β- > κ-casein.

The results obtained here for emulsions formed with commercial sodium caseinates confirm earlier work by Euston *et al.* (1995) and Srinivasan *et al.* (1996). However, Hunt and Dalgleish (1994) reported no preference for any of the caseins either immediately after homogenization or after ageing of emulsions formed with 20% soya oil and 1.0 - 3.0% sodium caseinate. It is likely that a commercially produced spray dried material behaved differently from the freeze-dried laboratory prepared sodium caseinate used by Hunt and Dalgleish (1994). In addition, Hunt and Dalgleish (1994) made their emulsions in a microfluidizer and obtained smaller droplet diameters.

The change in adsorption behaviour of individual caseins observed in emulsions formed with sodium caseinate may be related to the change in the aggregation state of caseins with increasing caseinate concentration (Chapter 4). The self-association behaviour of individual caseins in aqueous solutions has been well studied (Rollema, 1992). α_{s1}-Casein has been shown to associate in a series of consecutive association steps (Schmidt and Payens, 1972) whereas the association of β-casein shows detergent-like micellization, with the critical micellar concentration in the range 0.5 - 2 mg/ml, depending upon temperature and ionic strength. These associations are driven by hydrophobic interactions. If such an association involving β-casein occurs in sodium caseinate solution, it is likely that there would be fewer hydrophobic residues available for adsorption during emulsion formation, resulting in smaller proportions of β-casein at the droplet surface. However, the situation is probably more complex because the individual caseins in a caseinate

solution interact with one another to form associated structures with variable casein compositions.

The formation of aggregated structures, e.g. sub-micelles, through hydrophobic interactions between non-polar residues of casein molecules in concentrated caseinate solutions is likely to affect their ability to adsorb during homogenization. The behaviour of these aggregated structures under the high shear, turbulent conditions in the homogenizer is likely to be important as it is possible that these aggregated structures could dissociate into monomers before adsorption occurs. Another factor that needs to be considered is the exchange of casein components that may occur after homogenization. The presence of non-adsorbed aggregated structures may influence the exchange of caseins between serum and interface. For example, if serum β-casein is aggregated into micelle-like structures at higher caseinate concentrations, it may not be able to displace other caseins from the interface.

In the case of calcium caseinate, a proportion of the casein (~ 25%) is substantially aggregated (refer to Chapter 4). These large aggregates are probably linked by calcium bridges and consist of very high proportions of calcium-sensitive α_s-caseins (Chapter 4). The preferential adsorption of α_s - casein observed in calcium caseinate emulsions may by explained by assuming that aggregated material, rich in α_s-casein, was adsorbed in preference to the soluble caseins. Walstra & Oortwijn (1982) proposed that the adsorption of proteins in the homogenizer valve involves mass transfer in the turbulent flow conditions and the theory of locally isotropic turbulence predicts that large protein particles would be adsorbed in preference to monomeric proteins, which is in accordance with the results of this study.

To further investigate the preferential adsorption of α_s-casein in calcium caseinate-stabilized emulsions, calcium caseinate (2.5%) solution was treated as follows prior to making the emulsion.

(I) Homogenization at 10.3/3.45 MPa

(II) Centrifugation at 5, 000 g for 40 min.

(III) Addition of EDTA.

(I) Prehomogenization

Emulsions containing 30% oil were prepared, as described in Chapter 3.2, with 2.5% prehomogenized calcium caseinate solution. The application of mechanical energy to the protein solution prior to making emulsions may disrupt large aggregates in the calcium caseinate solution which may subsequently influence the surface composition. The proportions of individual caseins at the interface when the emulsions were made with normal or prehomogenized calcium caseinate solution are shown in Table 5.1. It can be seen that the proportions of individual caseins at the interface in both cases were almost identical. α_s-Casein was found to be adsorbed in preference to other caseins in both cases.

Table 5.1: Proportions of individual caseins at the interface (cream) obtained from emulsions made from normal calcium caseinate or calcium caseinate that had been homogenized at 10.3/3.45 MPa.

Sample type	Proportions of individual caseins (%)		
	α_s-Casein	β-Casein	κ-Casein
Protein solution	41.8	43.3	14.9
Cream 1	64.3	26.0	9.7
Cream 2	66.7	24.3	9.0

Cream 1: Emulsions made from normal calcium caseinate.

Cream 2: Emulsions made from homogenized calcium caseinate solution.

(II) Centrifugation

It was expected that, during centrifugation of calcium caseinate solution, large aggregates would be removed in the form of a sediment which is likely to affect the interfacial composition. The proportion of α_s-casein in the protein solution

after centrifugation was lower than in the normal calcium caseinate solution (Table 5.2). The proportion of α_s-casein at the interface (cream) was slightly lower than in emulsions made with normal calcium caseinate solution (Table 5.2). It was clear that α_s-casein was still adsorbed in preference to any other caseins.

Table 5.2: Proportions of individual caseins at the interface of emulsions stabilized by calcium caseinate solutions that had been centrifuged at 5,000 g for 40 min.

Sample type	Proportions of individual caseins (%)		
	α_s-Casein	β-Casein	κ-Casein
Normal protein solution	41.8	43.3	14.9
Centrifuged protein solution	35.8	44.7	19.5
Centrifuged cream	60.0	30.0	10.1

(III) EDTA addition

EDTA (10 mM) was added to normal calcium caseinate (2.5%) or centrifuged calcium caseinate solution prior to making emulsions. Emulsions were prepared with these protein solutions using 30% oil, as described before 3.2.

Table 5.3 shows the proportions of individual caseins at the interface of these emulsions. The proportion of α_s-casein in the centrifuged calcium caseinate solution with 10 mM EDTA (Table 5.3) was considerably lower than the normal calcium caseinate solution (Table 5.1). This may have been because most of the large aggregates were removed in the form of sediment during centrifugation prior to making emulsions. The interfacial compositions obtained with these emulsions showed no preferential adsorption of any caseins. Addition of EDTA to normal calcium caseinate solution resulted in a marked decrease in the proportion of α_s-casein at the interface. The proportions of β- and κ-caseins at the interface increased. Similar results were obtained when EDTA was added to centrifuged calcium caseinate solution. Addition of EDTA

to calcium caseinate solution probably caused disintegration of large α_{s1}-casein-rich aggregates by chelation of calcium. As a result, α_s-casein was adsorbed in the form of small aggregates or monomers. These results confirm that preferential adsorption of α_s-casein in calcium caseinate-stabilized emulsions was due mainly to adsorption of α_s-casein-rich aggregates which were formed through Ca^{++} mediated interactions. α_{s2}-Casein and α_{s1}-casein have been shown to be very calcium sensitive and precipitate in the ranges 2 mM and 3-8 mM calcium respectively (Toma and Nakai, 1973, Aoki *et al.*, 1985; Bingham *et al.*, 1972; Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Farrell *et al.*, 1988).

Table 5.3: Proportions of individual caseins at the interface of emulsions made from EDTA- treated calcium caseinate solutions.

Component	α_s-Casein	β-Casein	κ-Casein
Normal calcium caseinate + 10 mM EDTA cream	42.6	37.0	20.4
Centrifuged calcium caseinate + 10 mM EDTA cream	33.9	45.8	20.3

5.2.5 Competitive adsorption: analysed by size exclusion chromatography (SEC)

Once the emulsions are formed and the proteins have been adsorbed, it is generally accepted that the process is largely irreversible. Competitive adsorption between species of different sizes is a common phenomenon in emulsions. For example, the competitive adsorption of an aggregate compared with a monomer and vice-versa in a caseinate-stabilized emulsion at the oil/water interface is an important factor in governing the droplet size distribution and the surface concentration. Walstra and Oortwijn (1982) have demonstrated that large particles are adsorbed in preference to small particles. Dalgleish (1995) stated that during homogenization the controlling factor is not the different affinities of the proteins for the interface, but simply their overall

concentration. Pelan and Dickinson (1995) stated that the rate of adsorption depends only on the number of particles present in the bulk and is relatively independent of the binding affinity of the adsorbed species at the interface.

In this study attempts were made to check the competitive adsorption of an aggregate compared with a monomer using SEC in a caseinate-stabilized oil-in-water emulsion. The nature of the casein material in caseinates was determined by SEC on a Superose 6HR 10/30 column attached to a GBC HPLC system. A solution containing 20 mM imidazole and 50 mM NaCl at pH 7.0 was used as an elution buffer.

Emulsions (30% soya oil) were prepared with various concentrations of sodium or calcium caseinate. The emulsions were centrifuged at 45,000 g for 40 min and the subnatant was carefully collected. The subnatant was filtered through 0.22 µm membrane filters before injecting into the column. Typical elution profiles of sodium and calcium caseinate subnatants are shown in Figure 5.8. The profiles for both sodium and calcium caseinate subnatants showed a peak near the void volume of the column (peak I), containing very large particles, and two other peaks (peak II - intermediate molecular weight aggregates; peak III - non-aggregated, monomeric casein). The results obtained for peak I were variable, and showed no consistent trend with increasing protein concentrations. This was apparently because some small oil droplets apparently passed through the 0.22 µm filter and eluted in the void volume. The sizes of peak II and peak III increased with an increase in protein concentration in both subnatants. The elution time for peak III showed a considerable decrease with increase in protein concentration, essentially similar to that observed for caseinate solutions (refer to Chapter 4). SDS-PAGE on peak II showed that it was mainly composed of κ- and α_s-caseins. SDS-PAGE on peak III showed more β- than α_s-casein. Similar results were observed for peak II and peak III for the subnatants obtained from calcium caseinate emulsions when analysed by SDS-PAGE.
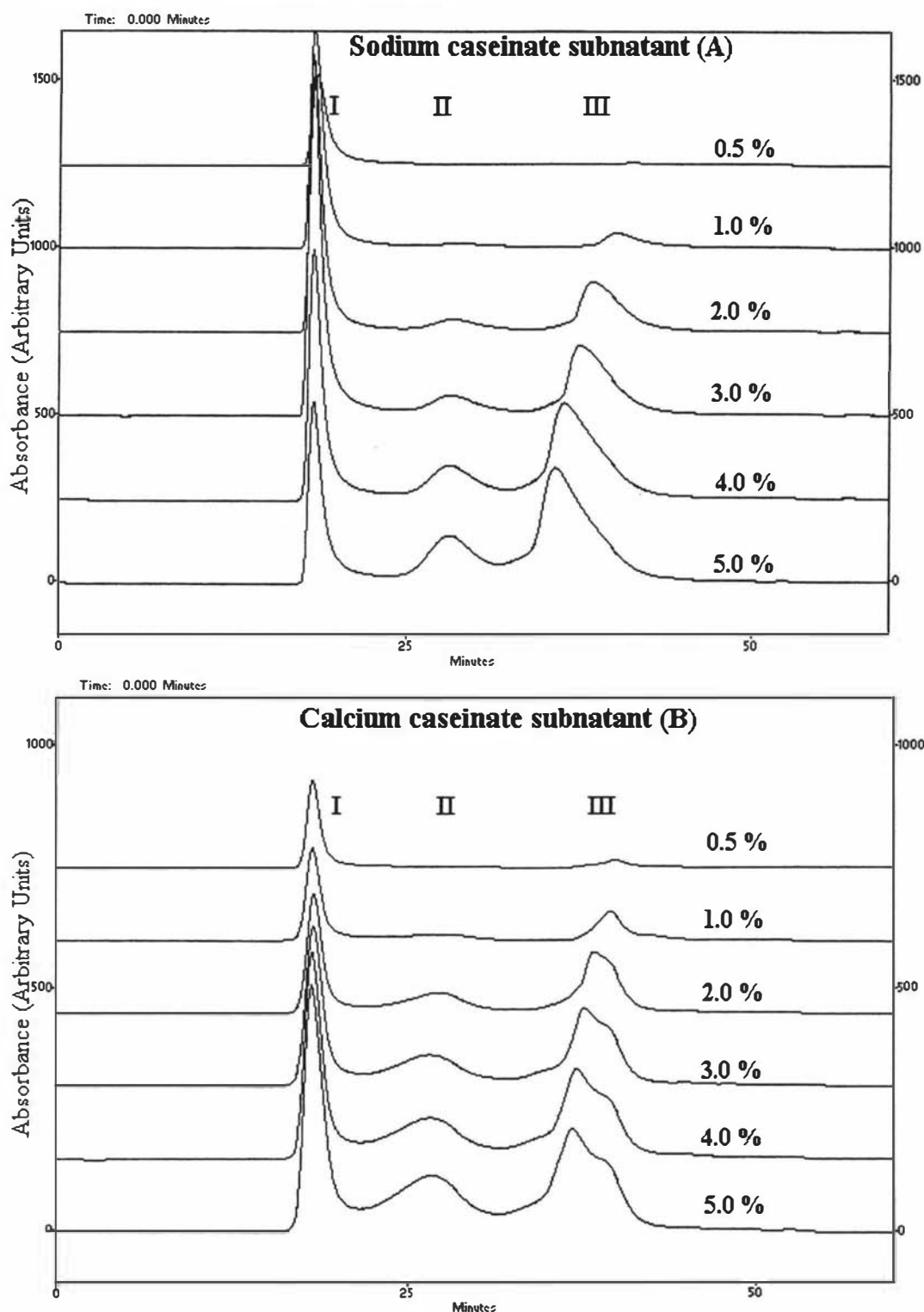
Figure 5.8: Elution profiles of subnatants (obtained by centrifugation of emulsions at 45,000 g for 40 min) on a Superose 6HR 10/30 obtained from the emulsions formed with sodium caseinate (A) and calcium caseinate (B).
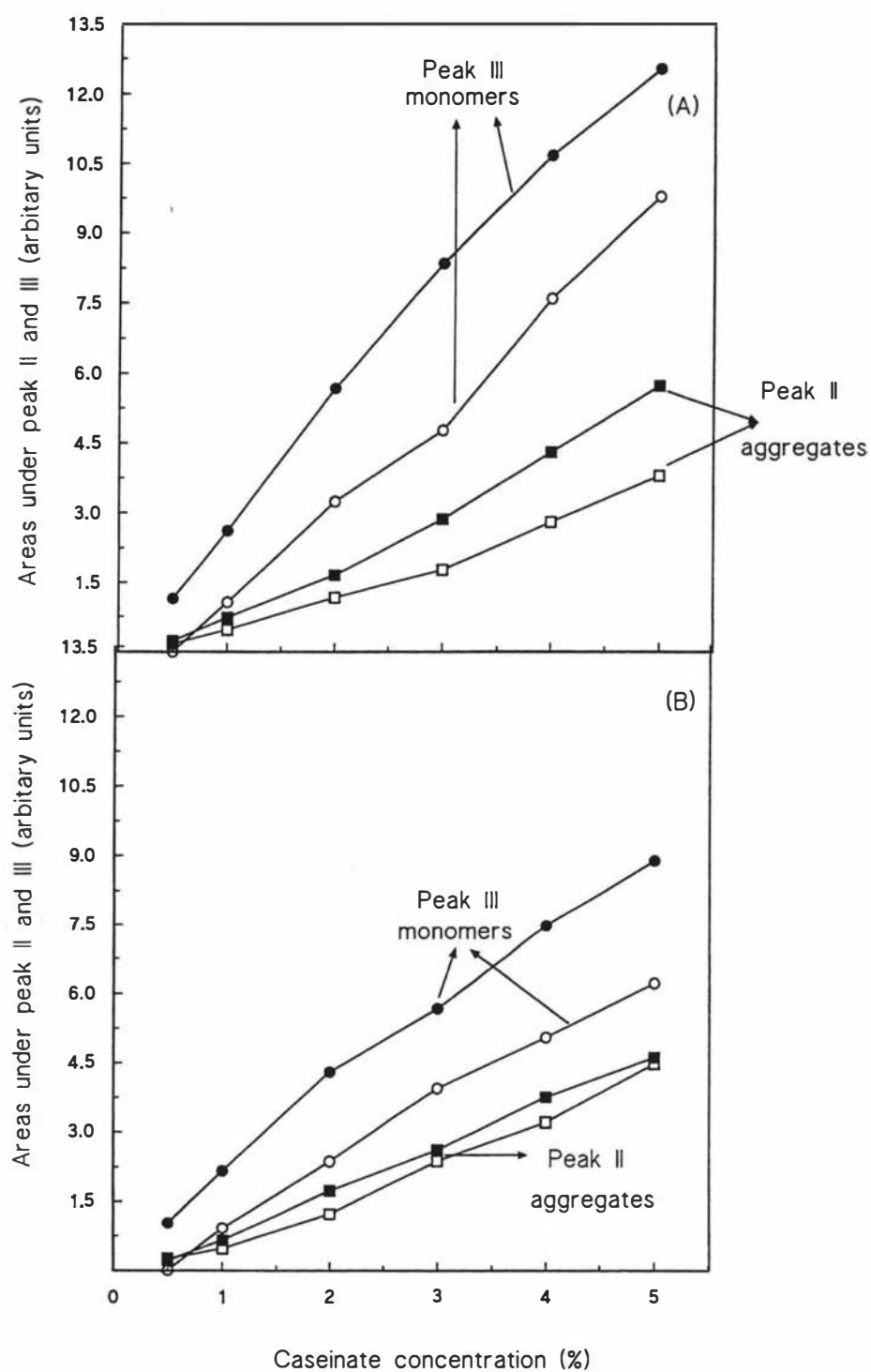
Figure 5.9: Areas of peak II (□, ■) and peak III (○, •) as a function of sodium caseinate (A) or calcium caseinate (B) concentration both in solution (•, ■) and in the subnatant (○,□).

Changes in the areas of peak II and peak III for the original caseinate solutions and the emulsion subnatants as a function of protein concentration are shown in Figure 5.9. It was clear that, both in the protein solution and in the subnatant, protein material increased with concentration. As expected the concentrations in the subnatant were lower than in the protein solution at each concentration for both types of system.

In sodium caseinate solution, the ratio of peak II to peak III increased with an increase in caseinate concentration (Figure 5.10 A). However, the ratio of peak II to peak III in the subnatant obtained from sodium caseinate-stabilized emulsions showed no significant change with an increase in protein concentration > 2.0%. This ratio decreased markedly with an increase in protein concentration from 1 to 2%. At 3% or below, the ratio of peak II to peak III in the subnatant was greater than that in the original sodium caseinate solutions. This suggests that under these conditions casein complexes and monomers (peak III) were adsorbed in preference to aggregates (peak II). In contrast, at 4 and 5% protein, the peak II to peak III ratio in the subnatant was lower than that in the original sodium caseinate solution indicating that the aggregates (peak II) were adsorbed in preference to monomers and casein monomers and complexes (peak III). These results are consistent with the surface composition of the individual caseins shown in Figure 5.6. From the surface composition data (Figure 5.6) it was observed that, in emulsions made with 0.5 - 2.0% sodium caseinate, β-casein was preferentially adsorbed (peak III in the SEC contained more β-casein than α_s-casein). In contrast, in the emulsions formed with > 2.0% caseinate, a high proportion of α_s-casein was found at the droplet surface compared with β-casein (peak II in the SEC contained more α_s-casein than β-casein). In case of calcium caseinate solutions, the ratio of areas under peak II to peak III was lower than in the subnatants (Figure 5.10 B) suggesting that peak II was adsorbed in preference to peak III at all caseinate concentrations. These results are also in agreement with the surface composition of the individual caseins shown in Figure 5.6.
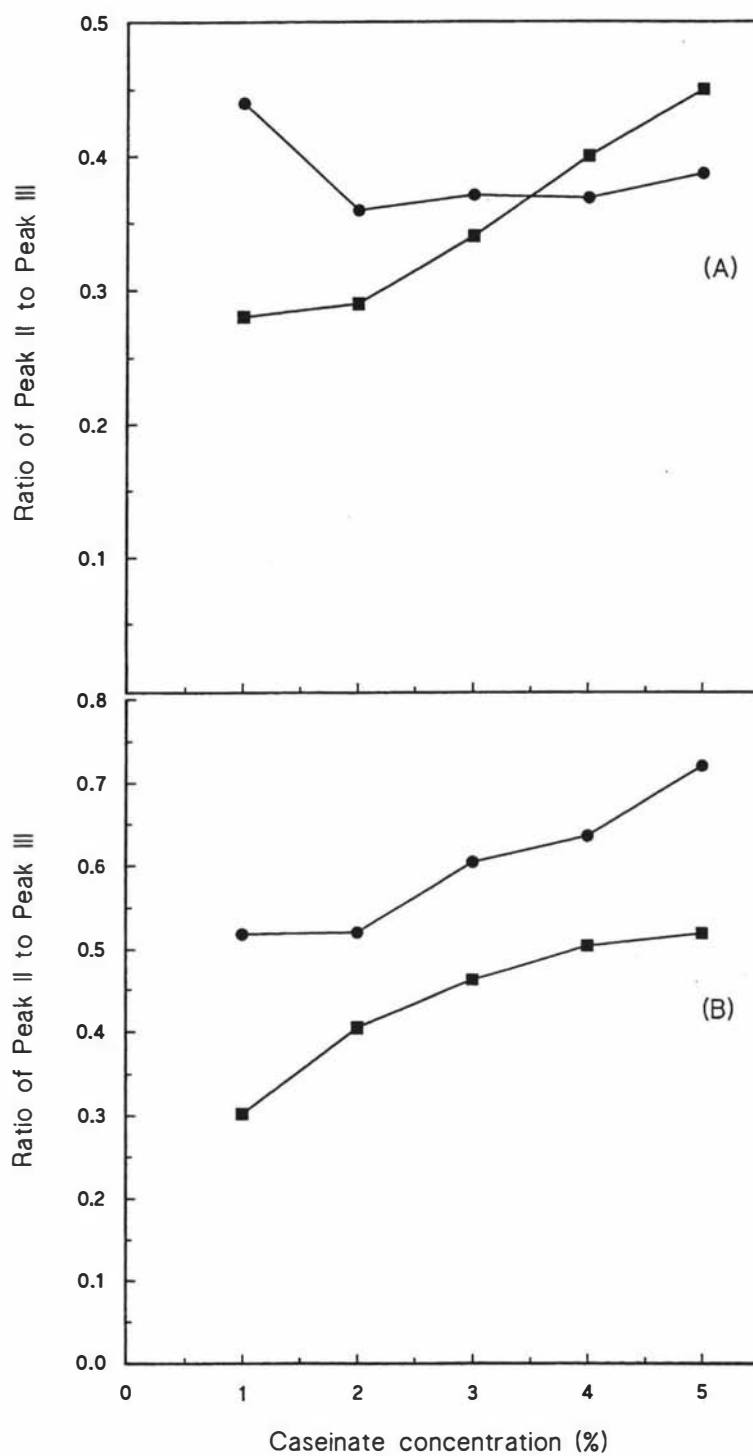
Figure 5.10: Ratio of peak II to peak III as a function of caseinate concentration in protein solution (■) or emulsion subnatant (●). Emulsions stabilized by sodium caseinate (A) or calcium caseinate (B).

5.2.6 Adsorption behaviour of mixtures of calcium and sodium caseinates in emulsions

Sodium and calcium caseinate solutions were mixed in different ratios at a total protein concentration of 1.0 or 2.5% and emulsions were made as described in Section 3.2.3.

5.2.6.1 Particle size distribution and droplet diameter

Figure 5.11 shows the particle size distribution of emulsions formed with mixtures (1 and 2.5% total protein) of sodium and calcium caseinate. In the 1% mixture, as the sodium caseinate concentration was increased, there was a shift towards smaller droplet sizes. When the concentration of sodium caseinate in the mixture was $\geq$ 0.4% the particle size distribution was monomodal, whereas below this concentration a bimodal size distribution of particles was observed.

In the case of emulsions made with a mixture concentration of 2.5% (total protein), the particle size distribution was monomodal at all ratios of sodium to calcium caseinate (Figure 5.11 B). As the sodium caseinate concentration was increased in the mixture, the particle size distribution showed a shift towards smaller droplet sizes. The range of particle sizes in calcium caseinate-stabilized emulsions were 0.1 - ~ 8.0 µm. The range of particle sizes became narrower (0.1 - ~ 5.0 µm) with the addition of sodium caseinate ($\geq$ 0.2%).

The average droplet diameter decreased with an increase in sodium caseinate concentration in the mixture at both 1.0 (Figure 5.12) and 2.5% (Figure 5.13) total protein, confirming the earlier results (Figure 5.3) that calcium caseinate-stabilized emulsions form larger droplets than sodium caseinate-stabilized emulsions. In the 1% mixture, addition of 0.2% sodium caseinate caused a decrease in the droplet diameter (from~ 0.83 µm to ~ 0.74 µm). There was no significant variation in the droplet diameter when the concentration of sodium caseinate in the mixture was increased from 0.2 to 0.6%. With further increase
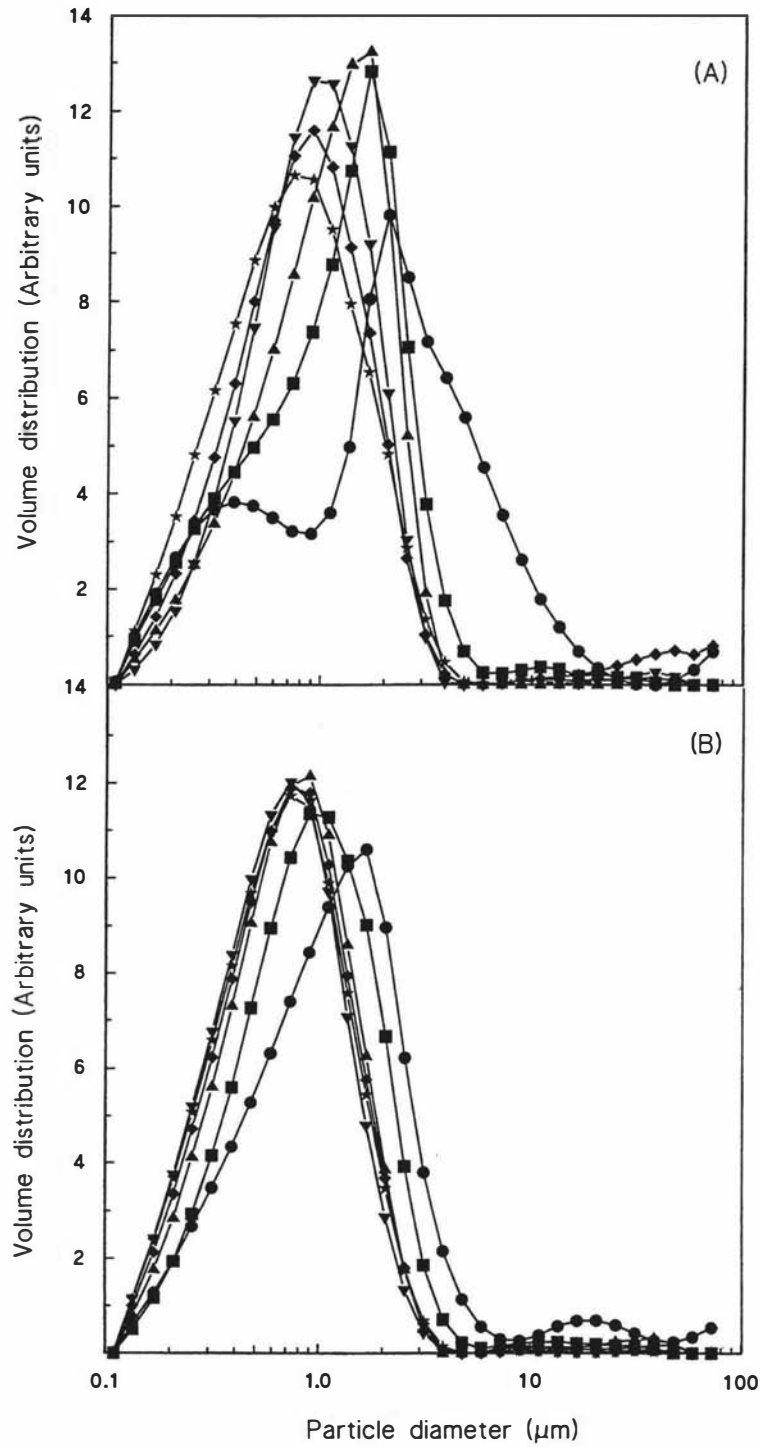
Figure 5.11: Particle size distribution of emulsions (30% oil w/v) formed with sodium and calcium caseinate solutions mixed in different ratios at a total protein concentration of 1.0% (A) or 2.5% (B). Amounts of sodium caseinate in the mixture: 0% (•), 20% (■), 40% (▲), 60% (▼), 80% (♦) and 100% (★).
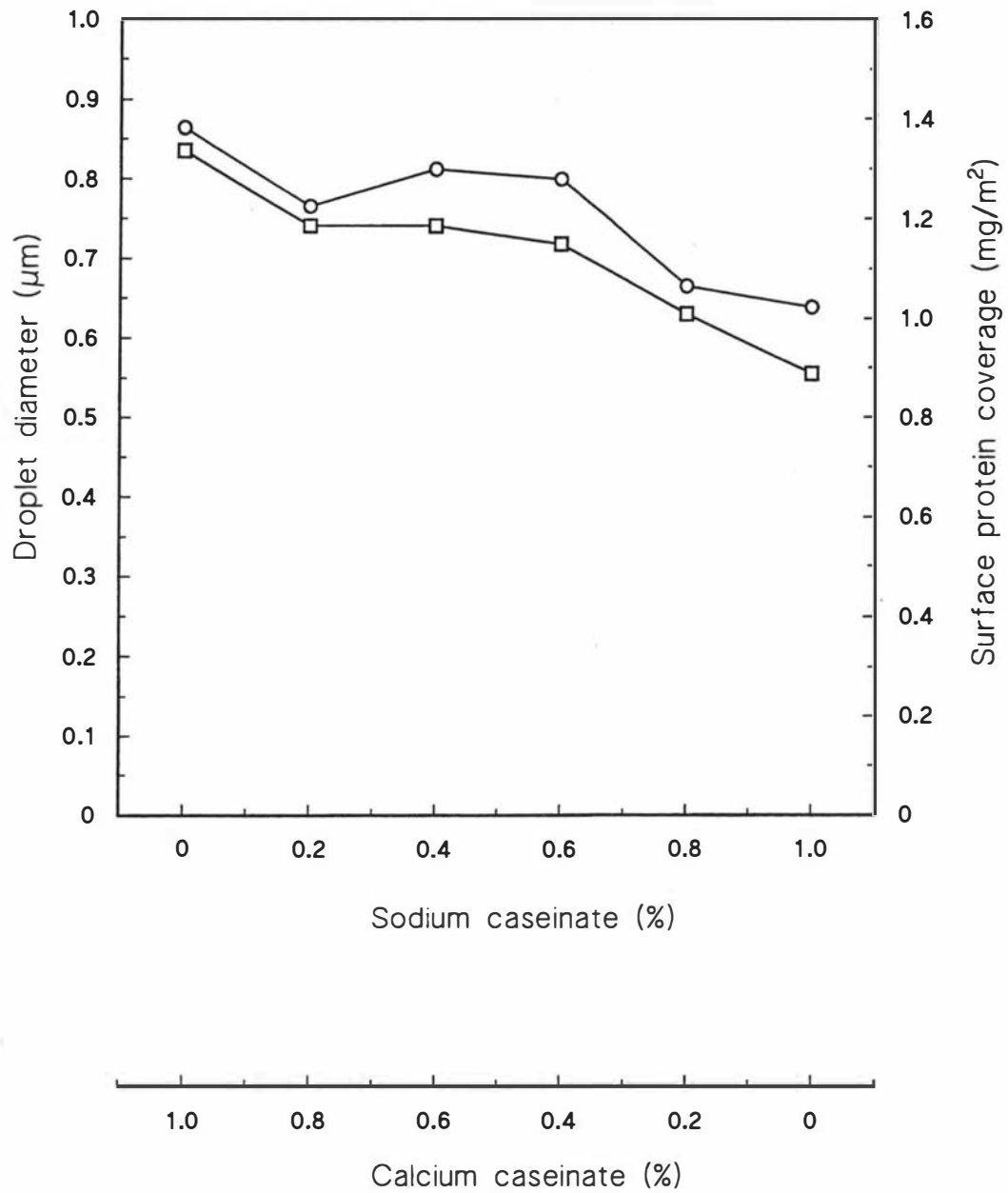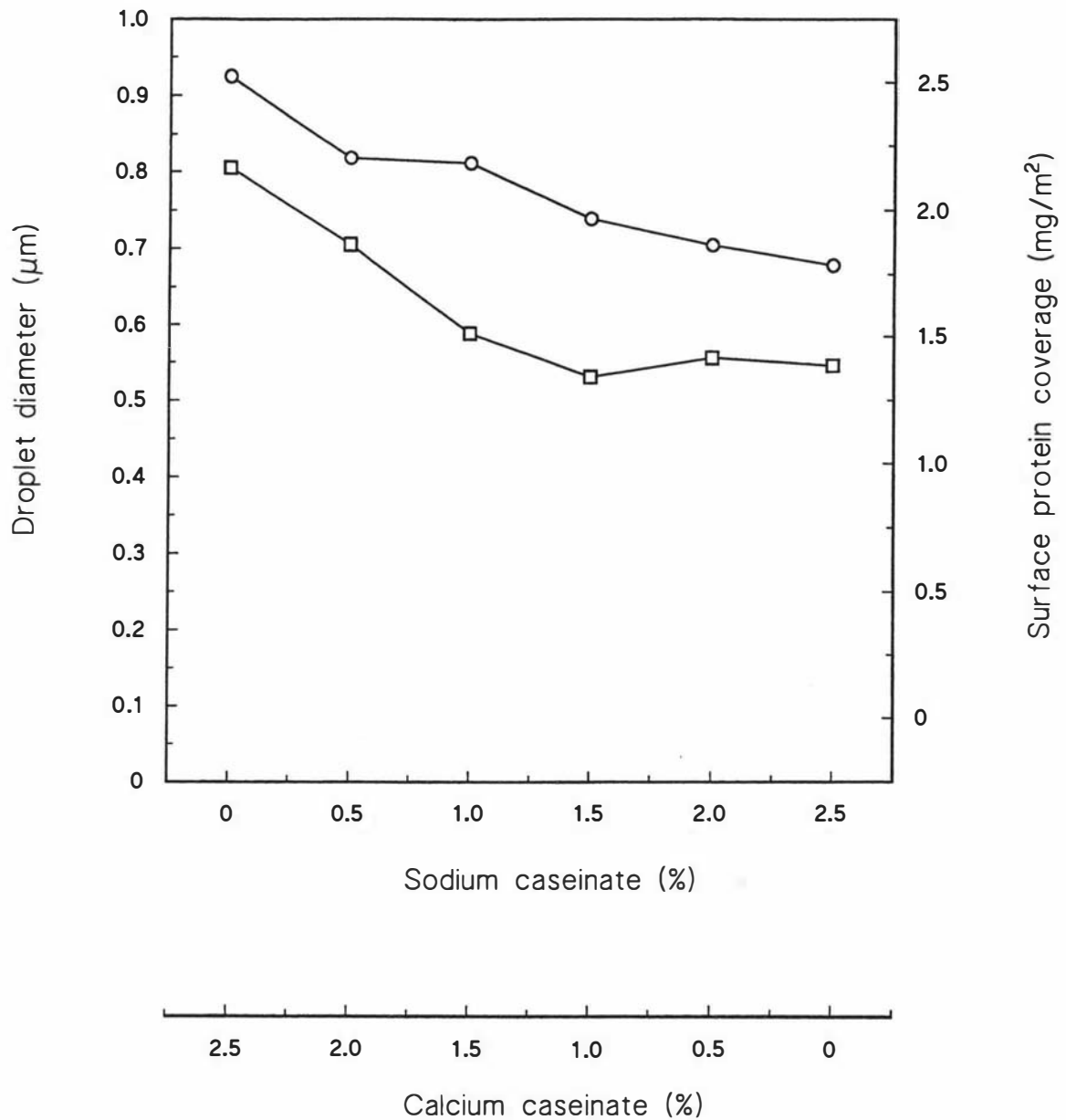
Figure 5.12: Droplet diameter (□) and surface protein coverage (○) in emulsions formed with mixtures of sodium and calcium caseinates at a total protein concentration of 1.0%.

Figure 5.13: Droplet diameter (□) and surface protein coverage (○) in emulsions formed with mixtures of sodium and calcium caseinates at a total protein concentration of 2.5%.

in sodium caseinate, the droplet diameter decreased from ~ 0.63 to ~ 0.55 μm. In the case of emulsions made with a mixture concentration of 2.5%, the droplet diameter decreased almost linearly with an increase in the concentration of sodium caseinate in the mixture from 0 to 0.6%, but there was no additional change in the droplet diameter with further increase in the sodium caseinate concentration in the mixture.

5.2.6.2 Surface protein coverage

In the case of the 1.0% mixture (Figure 5.12), the surface protein showed a slight decrease as the concentration of sodium caseinate in the mixture was increased. On the other hand, in the 2.5% mixture (Figure 5.13) the protein coverage decreased gradually with an increase in sodium caseinate concentration in the mixture. It was also observed that the surface protein coverage in the mixtures at a particular concentration was not a simple average surface protein concentration of these two caseinates. As these surface protein concentrations appeared to be higher than expected, it appears that calcium caseinate was adsorbing from the mixtures in greater quantities than sodium caseinate, presumably because it contains a higher proportion of large aggregates. The results are in accordance with the observations of Oortwijn and Walstra (1979) whereby larger particles adsorb in preference to smaller particles.

5.2.6.3 Proportions of individual caseins at the interface

Table 5.4 represents the interfacial composition (cream phase) of droplet emulsions formed from mixtures at a total protein concentration of 1.0%. As the sodium caseinate concentration in the mixture was increased from 0 to 0.4%, the proportion of α_s-, β- and κ-caseins at the interface showed no significant changes. However, when the concentration was increased to 0.6% or above, the proportion of α_s-casein decreased considerably and the proportion of β-casein increased and κ-casein showed an increase. From these results we can conclude that when the concentration of sodium caseinate in the mixture was ≤ 0.4%, the ratio of α_s- to β-casein (0.7:0.2) on the interface was higher than

in the original caseinate solutions (0.4:0.4) indicating that α_s-casein was preferentially adsorbed. When the concentration of sodium caseinate in the mixture was $\geq 0.8\%$, β-casein was adsorbed in preference to other caseins. At 0.6% sodium caseinate in the mixture, the proportions of all caseins were almost equal to those in the original caseinate solutions suggesting no preferential adsorption.

In 2.5% mixtures, addition of sodium caseinate caused no significant changes in the proportions of individual caseins at the droplet surface (Table 5.5). At all concentrations α_s-casein was found to adsorb in preference to other caseins, as indicated by the higher ratio of α_s- to β-casein (~ 0.6:0.2) at the droplet surface compared with the original caseinate solution (0.4:0.4).

The surface concentrations of individual caseins estimated from the proportions of individual caseins and the surface coverage of emulsions stabilized by a 1% or 2.5% (total protein in the mixture) mixture of sodium and calcium caseinates are presented in Figure 5.14 and 5.15, respectively. The surface concentration of β-, κ- and α_s-caseins showed no change when the concentration of sodium caseinate in the mixture was increased to 0.4%. With further increase in the sodium caseinate concentration, the surface concentration of β-casein increased, whereas the surface concentration of α_s-casein decreased markedly. κ-Casein showed a gradual increase when the sodium caseinate concentration in the mixture was > 0.2%. When the sodium caseinate concentration was $\leq 0.6\%$, the concentrations of caseins at the interface were in the order α_s- > β- > κ-, whereas, when the sodium caseinate concentration was $\geq 0.8\%$, the surface concentrations were in the order β- > α_s- $\approx$ κ-casein.

Table 5.4:   Proportions of individual caseins in the cream phase of emulsions made from a mixture of sodium and calcium caseinates (total protein, 1.0%).

% Sodium caseinate in the mixture (1.0%)	Proportions of individual caseins (%)		
	α_s-Casein	β-Casein	κ-Casein
0	67.7	19.4	12.9
0.2	71.1	22.2	6.7
0.4	68.0	22.2	9.8
0.6	47.5	35.5	17.0
0.8	26.9	50.0	23.1
1.0	20.0	56.7	23.3

Table 5.5:   Proportions of individual caseins in the cream phase of emulsions made from a mixture of sodium and calcium caseinates (total protein, 2.5%).

% Sodium caseinate in the mixture (2.5%)	Proportions of individual caseins (%)		
	α_s-Casein	β-Casein	κ-Casein
0	65.9	23.9	10.2
0.5	63.4	22.5	14.1
1.0	64.5	20.8	14.6
1.5	61.9	22.5	15.7
2.0	60.5	24.0	15.5
2.5	54.3	33.8	18.8
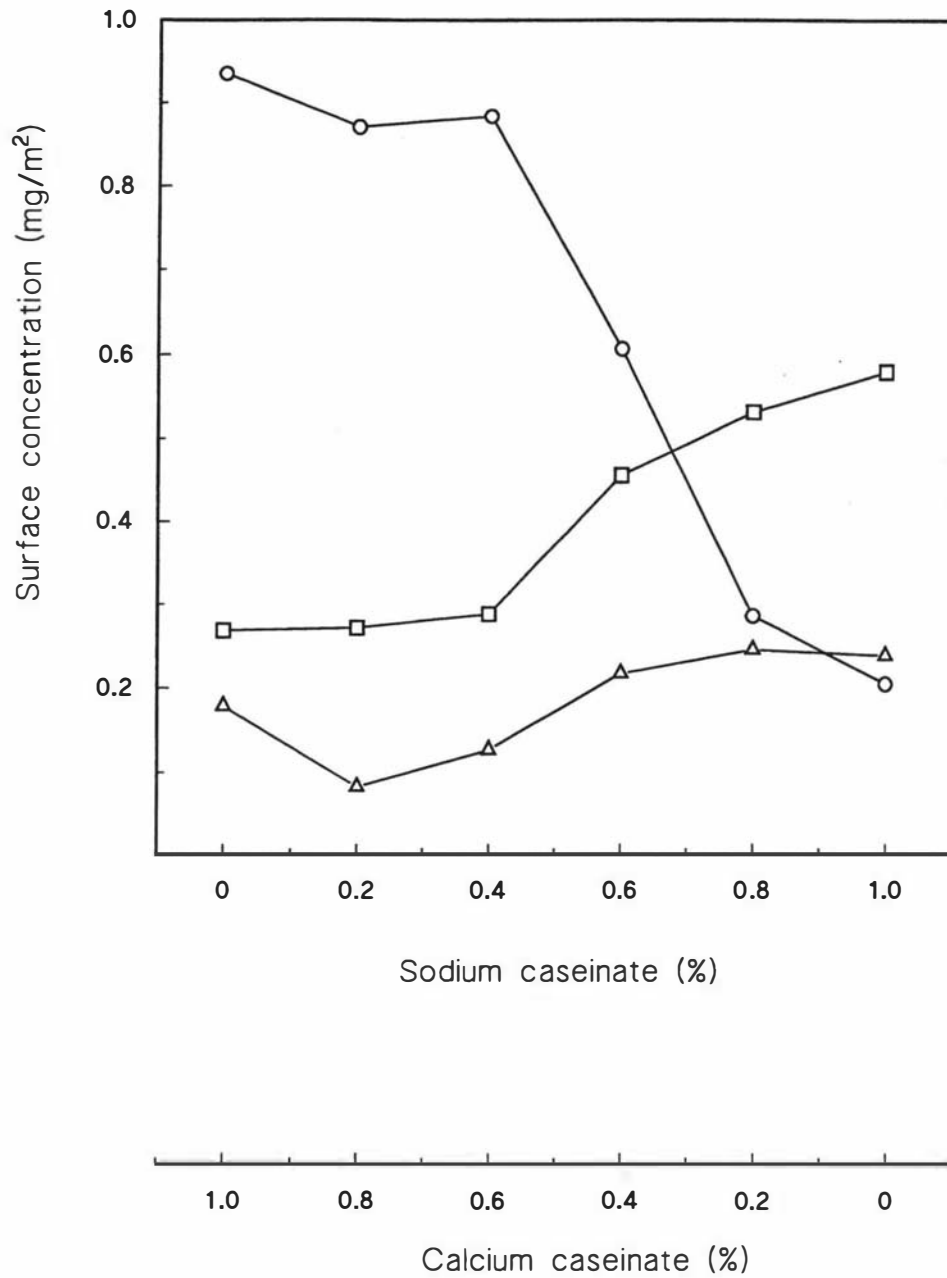
Figure 5.14: Estimated surface concentrations of α_s-casein ($\circ$), β-casein ($\square$) or
κ-casein ($\triangle$) on the interface in emulsions formed with a sodium
and calcium caseinate mixture at a total protein concentration of
1.0%.
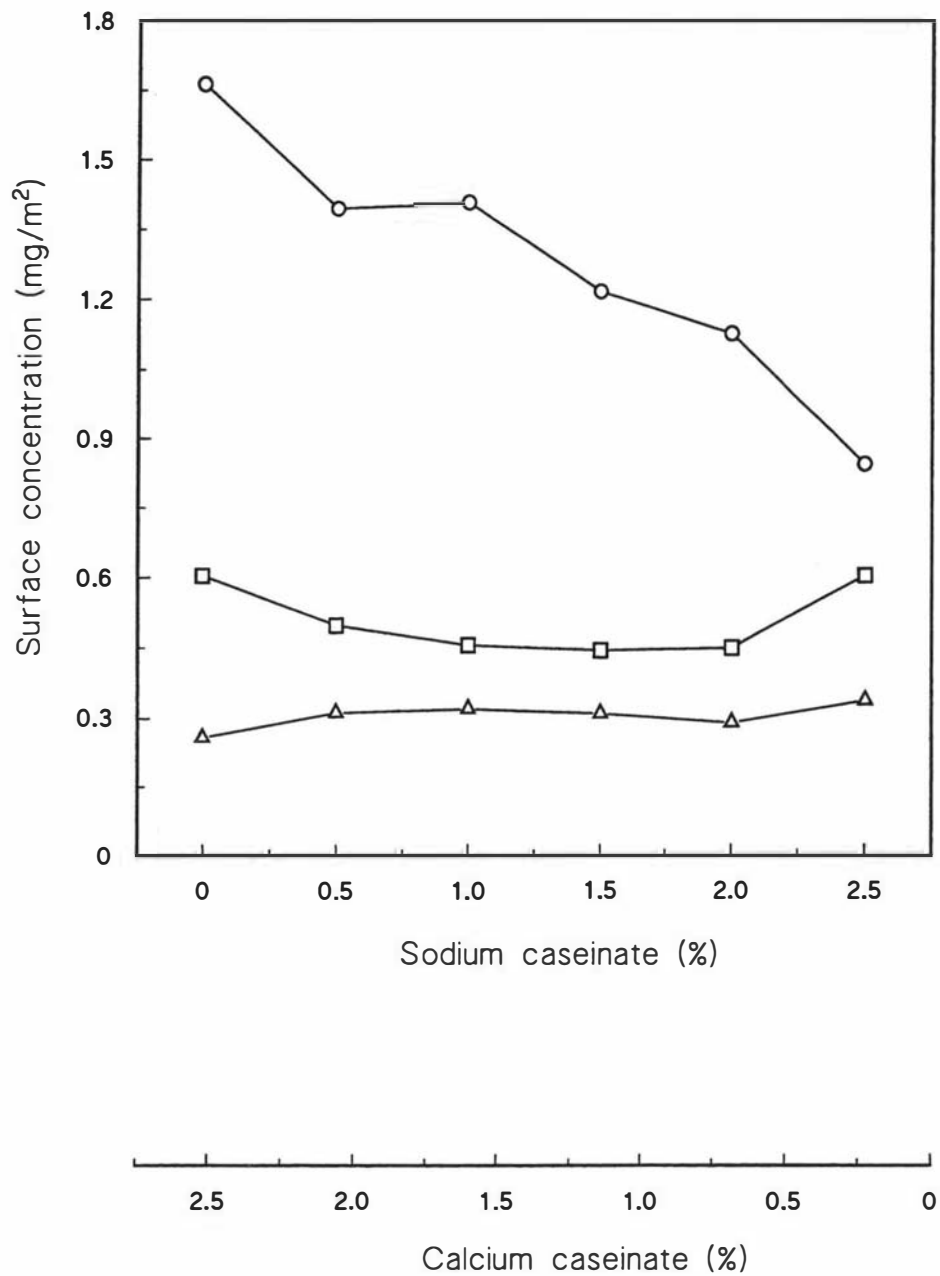
Figure 5.15: Estimated surface concentrations of α_s-casein (○), β-casein (□) or κ-casein (△) on the interface in emulsions formed with sodium and calcium caseinate mixture at a total protein concentration of 2.5%.

The surface concentrations of individual caseins in emulsions stabilized by a 2.5% mixture of sodium and calcium caseinates are presented in Figure 5.15. As the sodium caseinate concentration in the mixture was increased from 0 to 2.5% the surface concentration of α_s-casein decreased almost linearly from ~ 1.7 to 0.9 mg/m^2. The surface concentrations of β- and κ-caseins showed slight variations with an increase in sodium caseinate in the mixture. The results presented in Figure 5.13 show that the surface coverage of these emulsions decreased with an increase in sodium caseinate concentration in the mixture, and it appears that this decrease in surface coverage was mainly due to the decrease in α_s-casein on the interface rather than any other caseins (Figure 5.15).

Chapter 6

STABILITY OF OIL-IN-WATER EMULSIONS FORMED WITH SODIUM AND CALCIUM CASEINATES

6.1 Introduction

Food emulsions comprise a wide variety of products where either fat or water or both are dispersed in a state of macroscopic homogeneity (Friberg, 1976). Because the contact between fat and water is energetically unfavourable, emulsions are thermodynamically unstable, and stabilizing agents are necessary to improve the shelf life (Das and Kinsella, 1989). Emulsion instability can appear visually as a creaming or fat separation or mixture of both. Various studies have been reported on the creaming behaviour of caseinate-stabilized emulsions (Leman *et al.*, 1988; Halling, 1981; Acton and Saffle, 1971; Walstra, 1975; Walstra and Oortwijn, 1975; Dickinson and Golding, 1997; Dickinson *et al.,* 1997). Very few studies have been reported on the creaming behaviour of commercially made sodium caseinate and calcium caseinate-stabilized oil-in-water emulsions.

In this chapter the creaming stability of sodium and calcium caseinate emulsions was studied as a function of protein concentration. The creaming behaviour of emulsions formed with a mixture of sodium and calcium caseinate has also been described.

6.2 Results and discussion

Table 6.1 shows the values of the protein concentration in the bulk aqueous phase following centrifugation (C_b), as a function of the bulk protein concentration (C_{tot}) used to make the emulsion (oil + aqueous phase) and the total protein content (C_{aq}) with respect to the aqueous phase. Also tabulated is the estimated surface protein coverage (Γ) calculated from the oil fraction and the specific surface area (refer to Chapter 5).

Table 6.1: The concentration of unadsorbed protein (C_b) and calculated surface protein coverage (Γ) (mg/m^2) as a function of total protein content (C_{tot}) in the emulsion (30% soya oil, pH 7.0) and total protein content (C_{aq}) orginally present in the aqueous phase.

Total protein content (C_{aq}) in protein solution (%)	Total protein content in emulsion (C_{tot}) (%)	Sodium caseinate		Calcium caseinate	
		Unadsorbed protein (C_b) (%)	Protein load (Γ) mg/m^2	unadsorbed protein (C_b) (%)	Protein load (Γ) mg/m^2
0.5	0.375	0.2333	0.4279	0.2238	0.8273
1.0	0.750	0.4978	0.9978	0.5599	1.1976
2.0	1.500	1.2697	1.4558	1.2242	1.8917
3.0	2.250	2.0413	1.8385	1.8296	2.9094
4.0	3.000	2.9135	2.0609	2.4359	3.6113
5.0	3.750	3.9030	2.3560	3.0403	4.5739

The creaming stability of sodium and calcium-caseinate-stabilized emulsions after storage at 20°C for 24 hours was determined as described in Chapter 3.13. Stability or instability of these emulsions was expressed as a stability rating (refer to Chapter 3). These experiments were carried out three times.

Figure 6.1 shows the changes in stability rating as a function of caseinate concentration. The stability rating of emulsions formed with calcium caseinate increased rapidly with an increase in protein concentration from 0.5 to 2.0%. Further increase in the caseinate concentration had no further significant effect on the stability rating. By contrast, the stability rating of emulsions formed with sodium caseinate showed a very peculiar behaviour (Figure 6.1); the stability rating decreased markedly with an increase in protein concentration from 0.5 to 3.0%, but further increase in the sodium caseinate concentration from 3.0 to
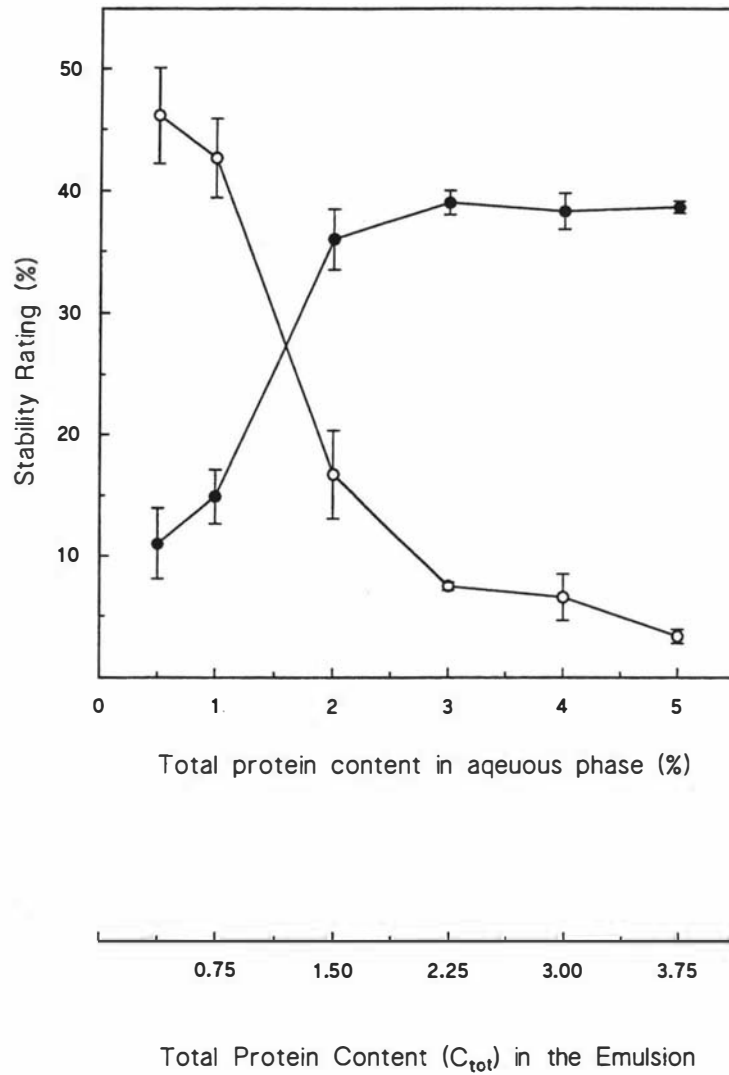
Figure 6.1: Changes in stability rating (%) as a function of caseinate concentration in emulsions containing 30% soya oil and varying amounts of calcium caseinate (•) or sodium caseinate (○). The emulsions were stored at 20°C for 24 hours.

5.0% caused only a slight decrease in stability rating.

Generally, the creaming behaviour of emulsion droplets can be described by Stokes' equation (refer Chapter 2.9.1; Page 42). The increase in creaming stability of calcium caseinate emulsions with protein concentration could be attributed to a combination of factors, including a decrease in d and $\Delta\rho$ (due to an increase in surface protein coverage) and an increase in the viscosity of the continuous phase (Figure 6.3). Although similar changes occurred in sodium caseinate-stabilized emulsions (Figure 6.2), the creaming stability, surprisingly, decreased with an increase in the protein concentration.

In order to further understand the unusual creaming behaviour of sodium caseinate-stabilized emulsions, emulsions were examined by confocal laser microscopy and viscometry.

6.2.1 Confocal microscopy

Figures 6.4 and 6.5 show the microstructure of emulsions formed using calcium or sodium caseinate. Emulsions formed with calcium caseinate showed more individual oil droplets at all caseinate concentrations used to prepare the emulsions (Figure 6.4 A to F). The droplet size of emulsions at caseinate concentrations < 2.0% was considerably larger than for emulsions made at higher caseinate concentrations (also refer to Chapter 5). There appeared to be some aggregation of particles above 3% caseinate concentration (Figure 6.4 D to F) but this did not seem to affect the stability rating of these emulsions.

The emulsions formed with low concentrations of sodium caseinate (0.5 and 1.0%) appeared to be homogeneous with no signs of flocculation and no visible creaming (Figure 6.5 A and B). As the caseinate concentration was increased, it could be seen that there was an increase in the extent of flocculation. In emulsions made with 3.0% sodium caseinate, large irregular flocs with diameter $\geq$ 10 μm were observed (Figure 6.5 D). With further increase in the sodium caseinate concentration, these flocs appeared to be forming a network
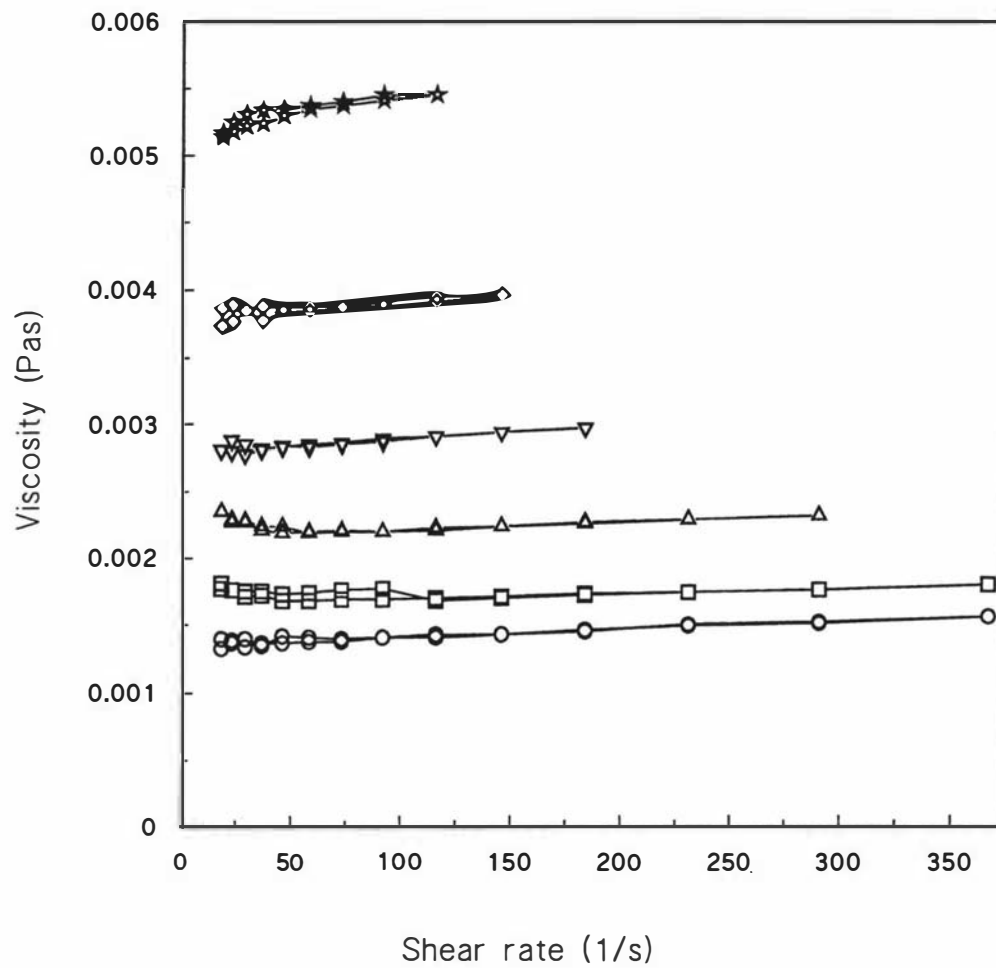
Figure 6.2:   Apparent viscosity of 0.5% (○); 1.0% (□); 2.0% (▵) ;3.0% (▽); 4.0% (◇) and 5.0%(☆) solutions of sodium caseinate.
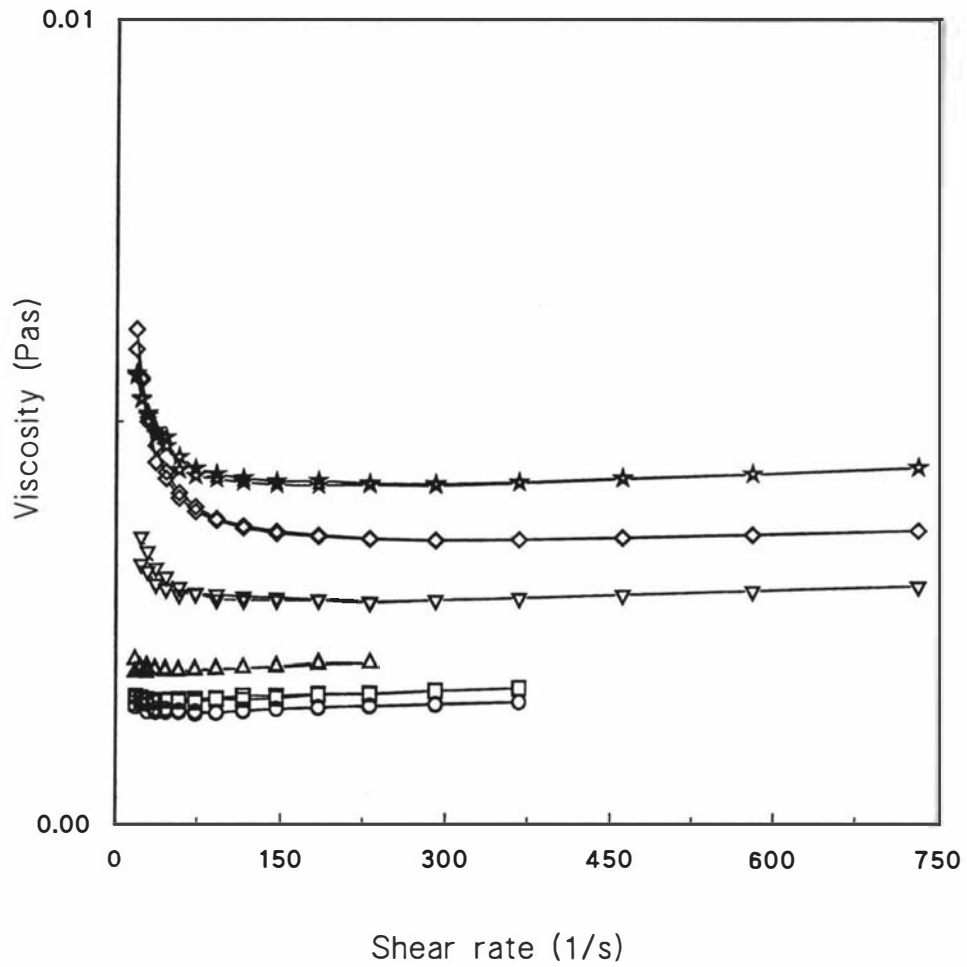
Figure 6.3: Apparent viscosity of 0.5% (○); 1.0% (□); 2.0% (△) ;3.0% (▽); 4.0% (◇) and 5.0%(☆) solutions of calcium caseinate.
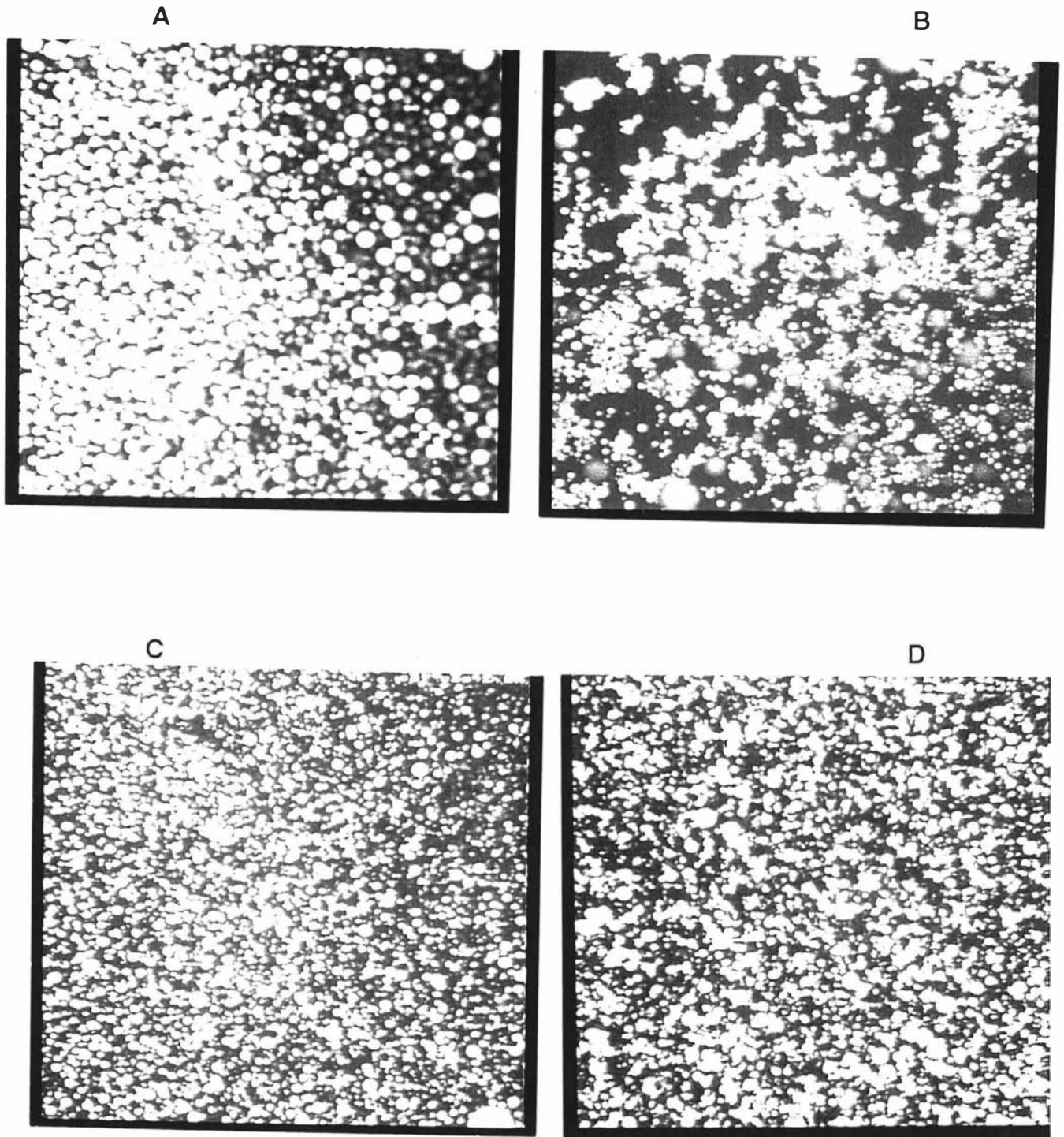
Figure 6.4: Confocal micrographs of calcium caseinate emulsions (30%) formed with 0.5% (A); 1.0% (B); 2.0% (C); 3.0% (D); 4.0% (E); or 5.0% (F) protein.   Scale bar represents 10 μm
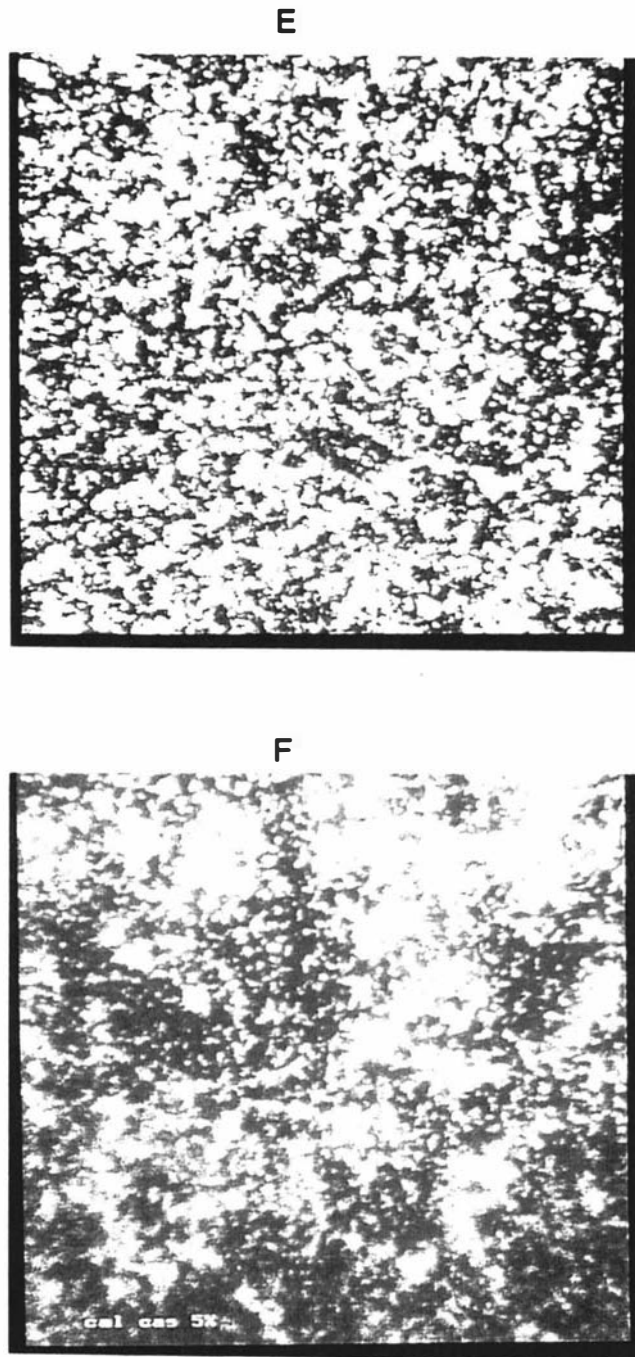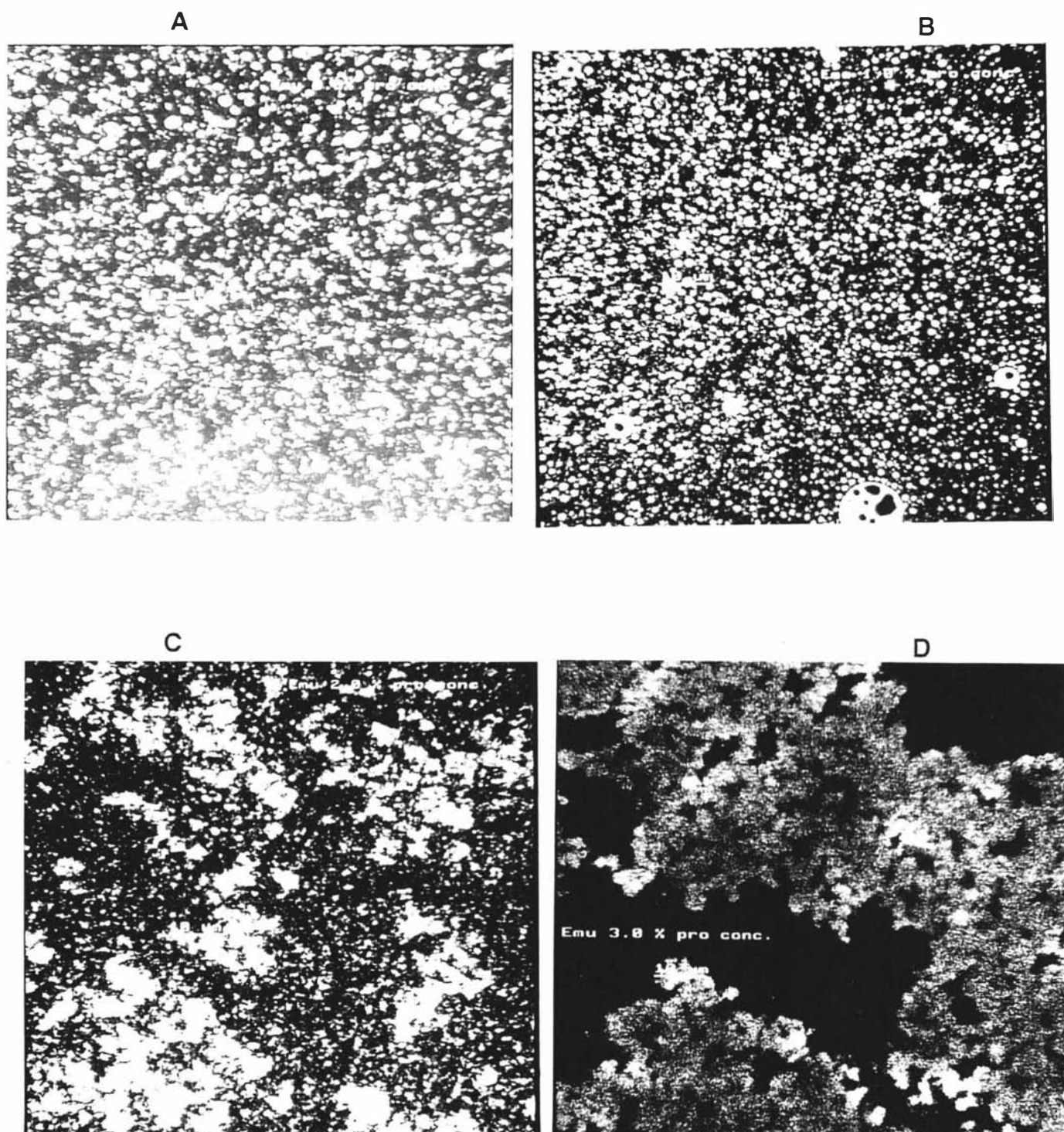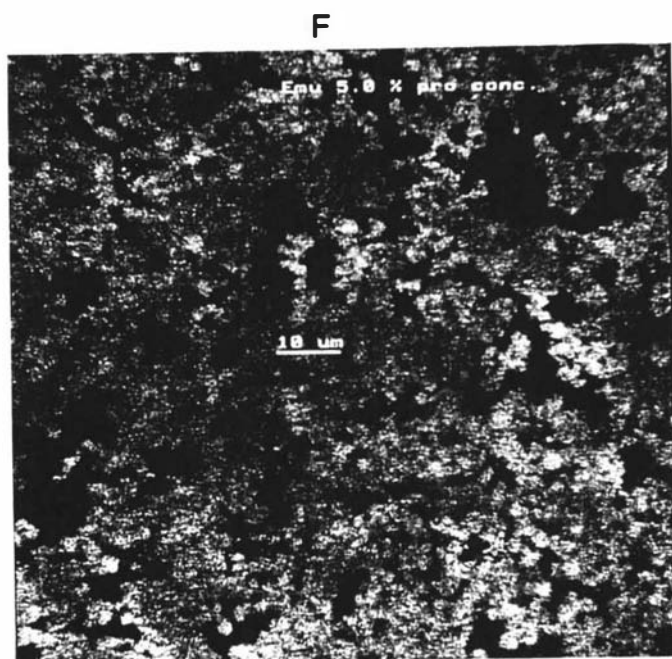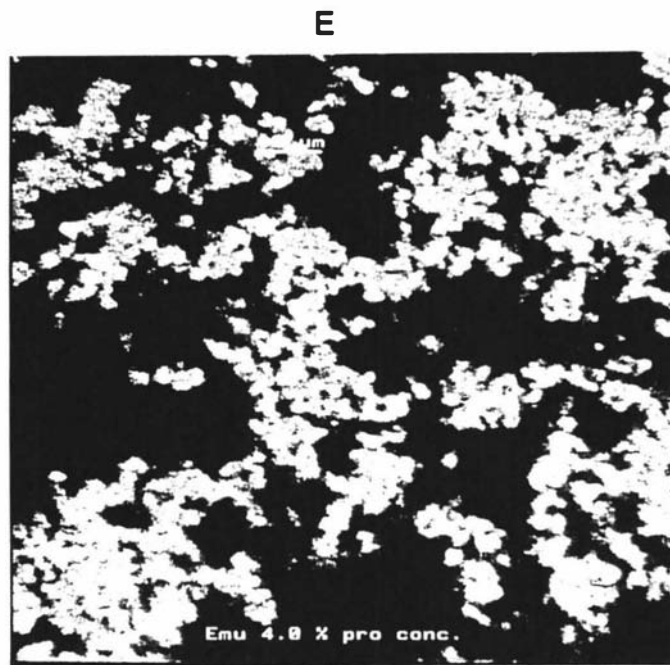
E

F

Figure 6.4:   Continued...

Figure 6.5: Confocal micrographs of sodium caseinate emulsions (30%) formed with 0.5% (A); 1.0% (B); 2.0% (C); 3.0% (D); 4.0% (E);or 5.0% (F) protein.

E

F

Figure 6.5: Continued...

structure (Figure 6.5 E and F). In this case, visible creaming occurred within a few hours of emulsion preparation.

6.2.2 Viscometry

Figures 6.6 A and B show the changes in apparent viscosity of the emulsions formed with sodium and calcium caseinate as a function of applied shear (emulsions were subjected to an upward shear sweep (29.2 - 730 s^{-1}) immediately followed by a downwards shear sweep). At a given shear rate, the viscosity of the emulsions increased with an increase in protein concentration for both sodium and calcium caseinate emulsions. The emulsions formed with low concentrations of sodium caseinate (0.5 - 2.0%) showed an almost Newtonian behaviour, as expected for non-flocculated emulsions. As the protein concentration was increased above 2%, there was a rapid increase in viscosity at low shear rates. Emulsions formed with 4 and 5% sodium caseinate showed a clear pseudoplastic behaviour, i.e. the emulsions exhibited shear thinning over a wide range of shear rates.

At a given concentration, the viscosities of calcium caseinate-stabilized emulsions were much lower than those of sodium caseinate-stabilized emulsions.

Figure 6.6 B shows the changes in apparent viscosity of the emulsions formed with calcium caseinate as a function of shear. With increasing concentration of protein, the viscosity increased. At any concentration of protein, Newtonian behaviour was observed in calcium caseinate emulsions. The flow curves of the emulsions were linear on a log-log plot of shear stress versus shear rate (Figure 6.7), indicating that they might be adequately described by the power law model:
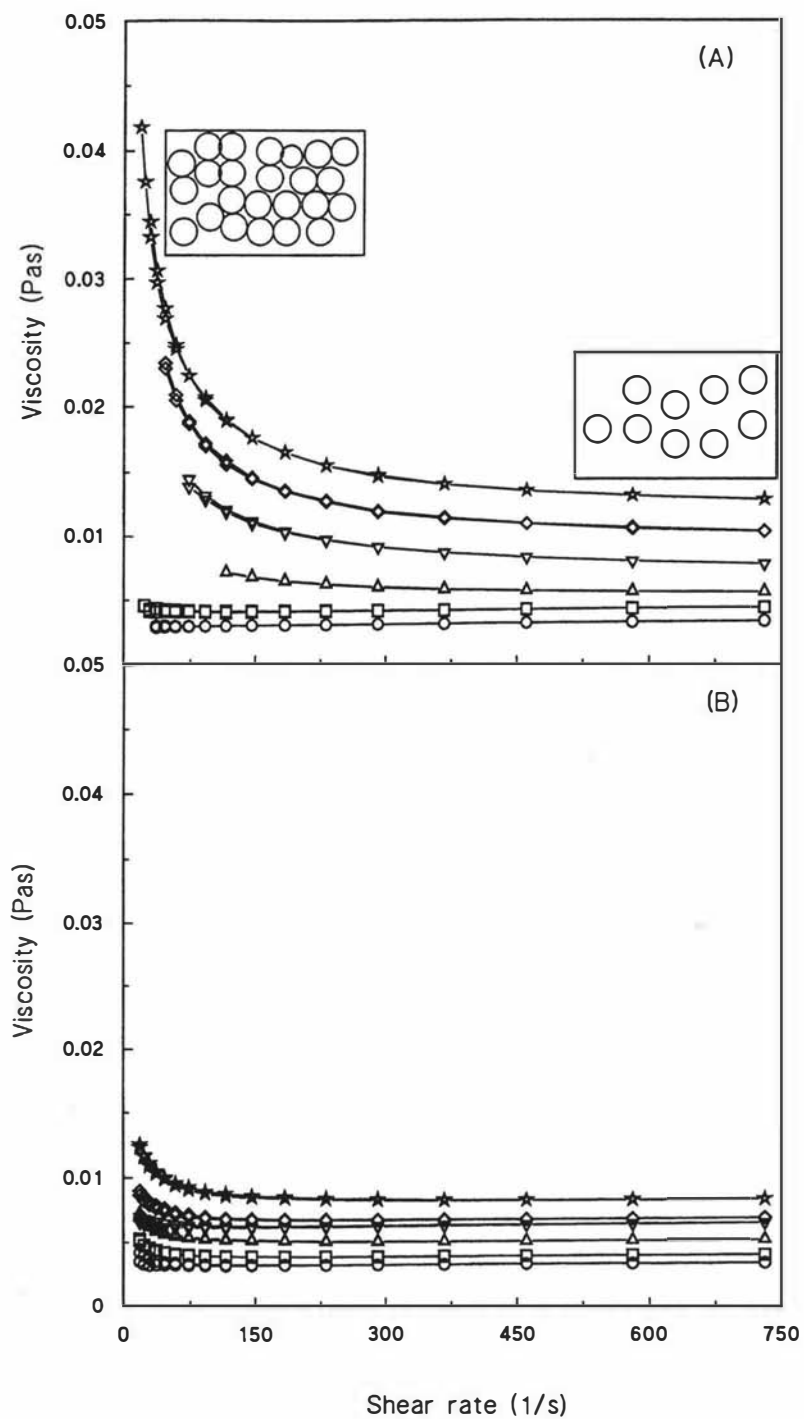
Figure 6.6: The change in apparent viscosity as a function of shear rate (1/s) for emulsions (30% oil) formed with (○) 0.5%; (▫) 1.0%; (△) 2.0%; (▽) 3.0%; (◇) 4.0% and (☆) 5.0% sodium caseinate (A) or calcium caseinate (B).

$$\tau \;=\; k\,\gamma^n$$

where τ is the shear stress (Pa), γ is the shear rate (1/s), k is the fluid consistency index and n is the flow behaviour index. For Newtonian fluids, $n=1$, and any other values indicate a deviation from Newtonian flow. For example, $n < 1$ indicates pseudoplastic behaviour (shear thinning) and $n > 1$ indicates dilatant behaviour (shear thickening).

Using the power law model, the flow behaviour index (n) and the fluid consistency index (k) were calculated and plotted (Figure 6.8). From Figure 6.8 A, it is clear that, in sodium caseinate emulsions, with an increase in concentration of protein in the emulsions the flow behaviour index (n) decreased indicating that emulsions made with low protein concentrations ($\leq$ 1.0%) were Newtonian and emulsions made with higher concentrations of protein ($\geq$ 2.0%) were pseudoplastic. The value of n decreased rapidly as the protein concentration was increased from 1 to 3%; with further increase in the concentration ($\geq$ 3.0%), the value of n showed no significant change.

It is interesting to note that the stability rating of the emulsions (Figure 6.1) showed similar trends to the flow behaviour index (n). It may be concluded that the stability rating (Figure 6.1) of sodium caseinate emulsions was proportional to the pseudoplasticity (Figure 6.9) and inversely proportional to the fluid consistency index. By contrast, the flow behaviour index (n) and fluid consistency index (k) showed no significant change with an increase in the concentration of protein in calcium caseinate emulsions (Figure 6.9B). In these emulsions, it appears that the stability rating was independent of n and k.

No hysteresis was evident in the flow curves (Figure 6.7) indicating that the emulsions did not exhibit thixotropic (time dependent) behaviour.
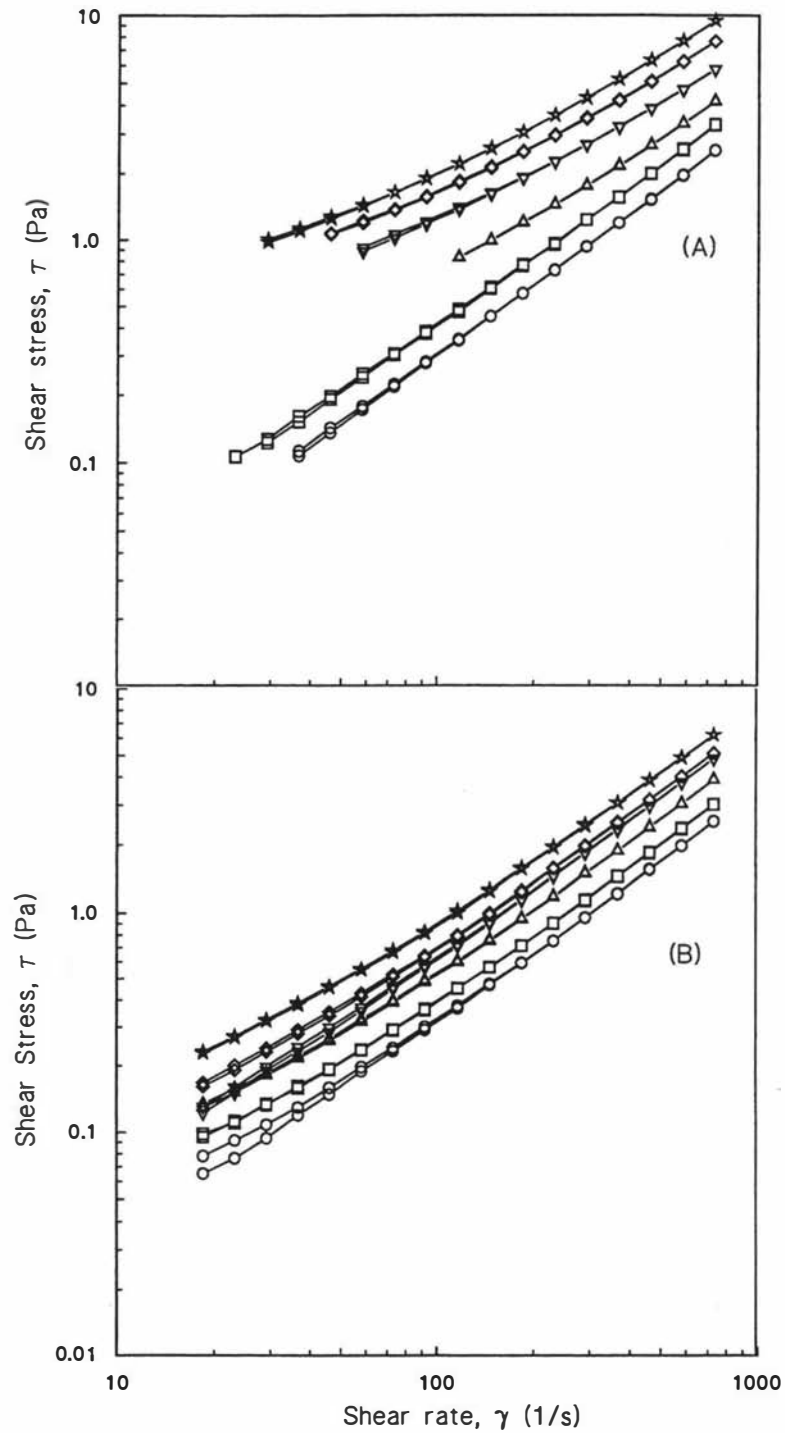
Figure 6.7: Flow curves of emulsions (30% oil) made with 0.5% (○); 1.0% (□);
2.0% (△); 3.0% (▽) ; 4.0% (◇) and 5.0% (☆) sodium caseinate (A)
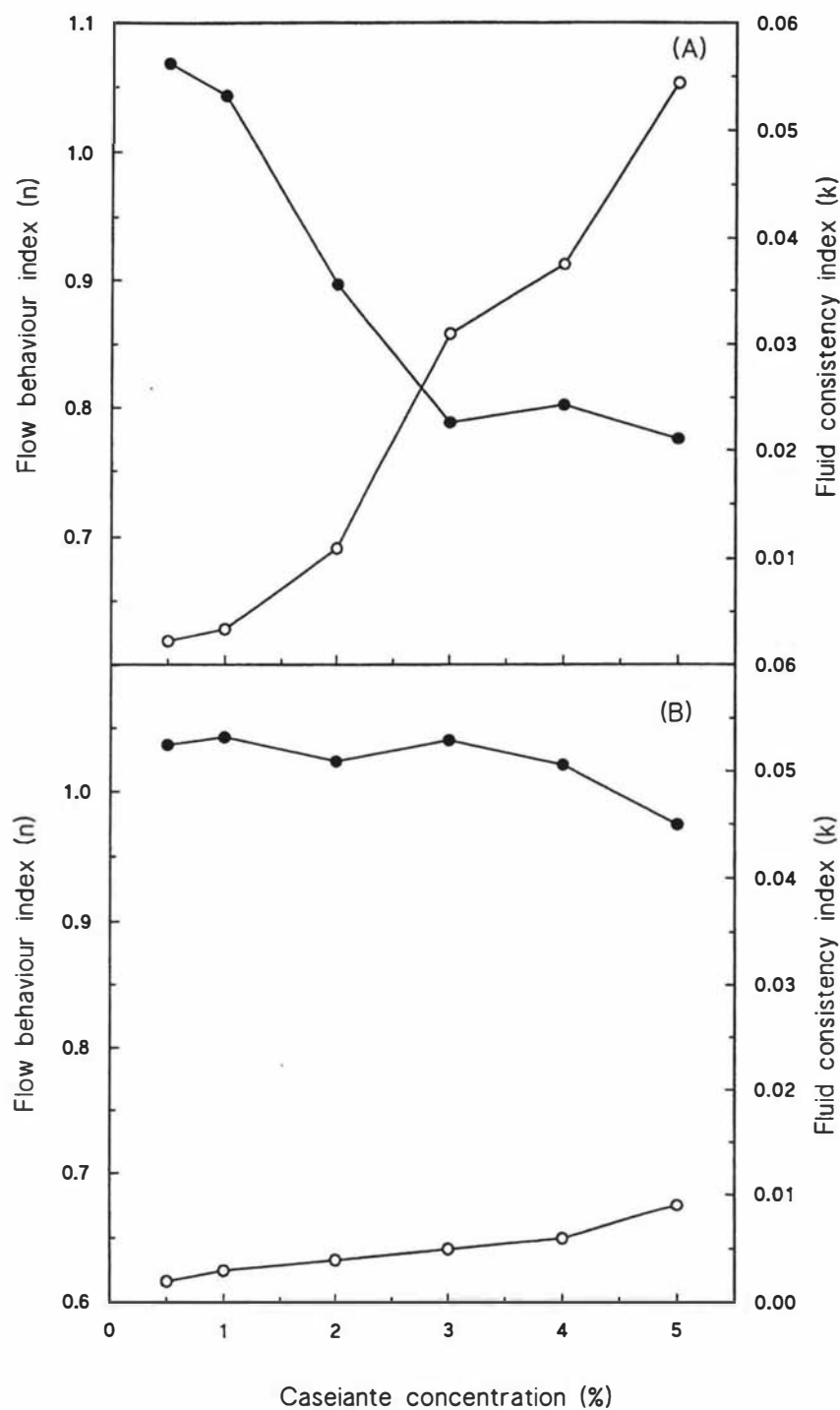or calcium caseinate (B).

Figure 6.8: Change in flow behaviour index (n) (•) and fluid consistency index (k) (○) with change in concentration of protein for sodium caseinate (A) and calcium caseinate (B) in the aqueous phase
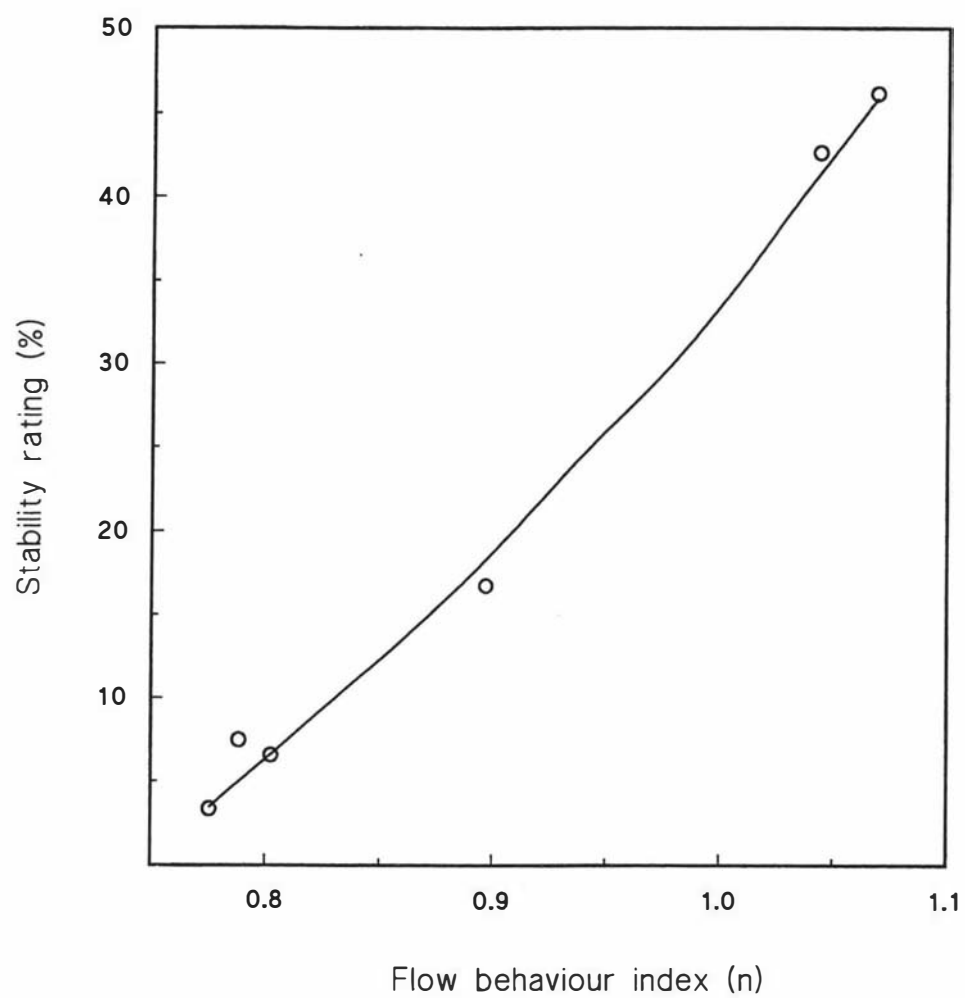
Figure 6.9: Stability rating as a function of flow behaviour index (n) for sodium caseinate emulsions.

Emulsions made with calcium caseinate showed Newtonian behaviour at all protein concentrations used (Figures 6.7 and 6.8 B) (illustrated by a value of *n* approximately equal to one).

It is clear that the viscosity of sodium caseinate emulsions formed with relatively high protein concentrations decreased with an increase in shear rate and seemed to increase again with a decrease in shear rate (Figure 6.6). This shear thinning behaviour, in general, can be explained by:

(A) progressive orientation of droplets in the direction of flow, so that frictional resistance is reduced;

(B) deformation of the protein hydration sphere in the direction of flow (if the protein is highly hydrated and dispersed);

(C) rupture of hydrogen and other weak bonds resulting in dissociation of protein aggregates or networks.

From the confocal micrographs, the emulsion droplets are seen to be spherical, indicating that shear thinning is unlikely to be accounted for by orientation of the particles. Therefore the shear thinning in this study is probably the result of phenomena (B) and (C). This may indicate reversible aggregation or flocculation of emulsion droplets. With increasing shear rate, these flocs break up and some continuous phase liquid is released (Campanella *et al.,* 1995), resulting in a decrease in the effective dispersed phase concentration and a reduction in viscosity. Shear does not affect the viscosity when the flocs are completely broken (Figure 6.6 A and B). The absence of thixotropy in these emulsions indicates that the deflocculation phenomenon is reversible; that is, flocs will form again when the shear rate is reduced.

From the confocal microscopy and viscometry results, it is clear that sodium caseinate-stabilizied emulsions made with relatively high concentrations of protein ($\geq$ 3%) undergo reversible flocculation of emulsion droplets.

Further experiments were carried out to obtain an understanding of the relative

importance of adsorbed and unadsorbed protein in the emulsions made with sodium caseinate.

Emulsions were made with 0.5% or 5.0% sodium caseinate and 30% oil and samples (30 g) of these emulsions were centrifuged at 45,000 g for 40 min and the cream layer was carefully separated. The subnatant was weighed, and replaced with equal amounts of sodium caseinate solution of various concentrations (0.5 to 5.0%). In some cases, the cream layer was mixed with water and its own subnatant. This mixture was then mixed gently at a constant low stirring speed and maintained at 20°C for 24 hours. After 24 hours, the emulsions were examined by confocal microscopy and viscometry.

Figure 6.10 shows the apparent viscosity as a function of shear rate for the 5.0% cream layer mixed with different caseinate solutions. The apparent viscosities of 5% cream mixed with water, 0.5% and 1% caseinate solution were almost Newtonian (Figure 6.10), similar to the emulsions made with 0.5% and 1% caseinate solution (Figure 6.6 A). The cream obtained from 5% sodium caseinate emulsions and mixed with 5%, 4%, 3% and 2% caseinate solutions showed marked shear thinning behaviour. At a given shear rate, the viscosity of the emulsions increased with an increase in the concentration of sodium caseinate in the aqueous phase, particularly at low shear rates. The viscosity of 5% cream mixed with 5% caseinate solution was slightly higher than the viscosity of 5% cream mixed with its own subnatant which may have been due to the slight increase in the concentration of protein.

Figure 6.11 shows the change in the apparent viscosity with shear rate for the cream obtained from 0.5% sodium caseinate emulsion and mixed with caseinate solutions of various concentrations. The results were similar to those for emulsions consisting of cream from 5% sodium caseinate and mixed with caseinate solutions (Figure 6.10), i.e. emulsions containing $\geq$ 2% sodium caseinate in the aqueous phase showed considerable shear thinning. However, the viscosities of these emulsion systems, at a given shear rate, were

considerably lower than those shown in Figure 6.10. These two emulsion systems had similar average particle size (refer to Chapter 5) but had different surface protein loads. The higher surface load of the 5% sodium caseinate emulsion systems may have contributed to their higher emulsion viscosities. The viscosity of 0.5% cream mixed with 0.5% caseinate solution was similar to the viscosity of 0.5% cream mixed with its own subnatant.

Confocal microscopy studies were also conducted on these emulsions. It can be seen that, when cream obtained from 5% sodium caseinate emulsions was mixed with 0.5% sodium caseinate solution, the resulting emulsion system appeared to be homogeneous with no sign of flocculation and visible creaming (Figure 6.12 A), similar to the emulsion made with 0.5% sodium caseinate (Figure 6.5 A). When this cream was mixed with 1.0% sodium caseinate solution, irregular large flocs were observed (Figure 6.12 B), similar to the emulsion made with 1.0% sodium caseinate (Figure 6.5 B). With further increase in concentration of sodium caseinate in the aqueous phase, these flocs appeared to be forming a network structure (Figure 6.12 C to G). In contrast, when this cream was mixed with water, these flocs seemed to disappear and the emulsion appeared to be homogeneous with no sign of flocculation (Figure 6.12 H).

Figure 6.13 (A to H) represents the confocal micrographs of cream obtained from 0.5% sodium caseinate emulsions and mixed with different sodium caseinate solutions. When this cream was mixed with 0.5% sodium caseinate solution, the resulting emulsion system appeared to be homogeneous with no flocculation and visible creaming (Figure 6.13 A); however when it was mixed with 1.0% sodium caseinate solution the flocs were beginning to form (Figure 6.12 B). As the concentration of sodium caseinate was increased from 2.0 to 5.0%, these flocs appeared to be forming a network structure (Figure 6.13 C to F). These confocal micrographs were similar to those obtained for the standard emulsions made with similar concentrations of sodium caseinate (Figure 6.5 C to F).
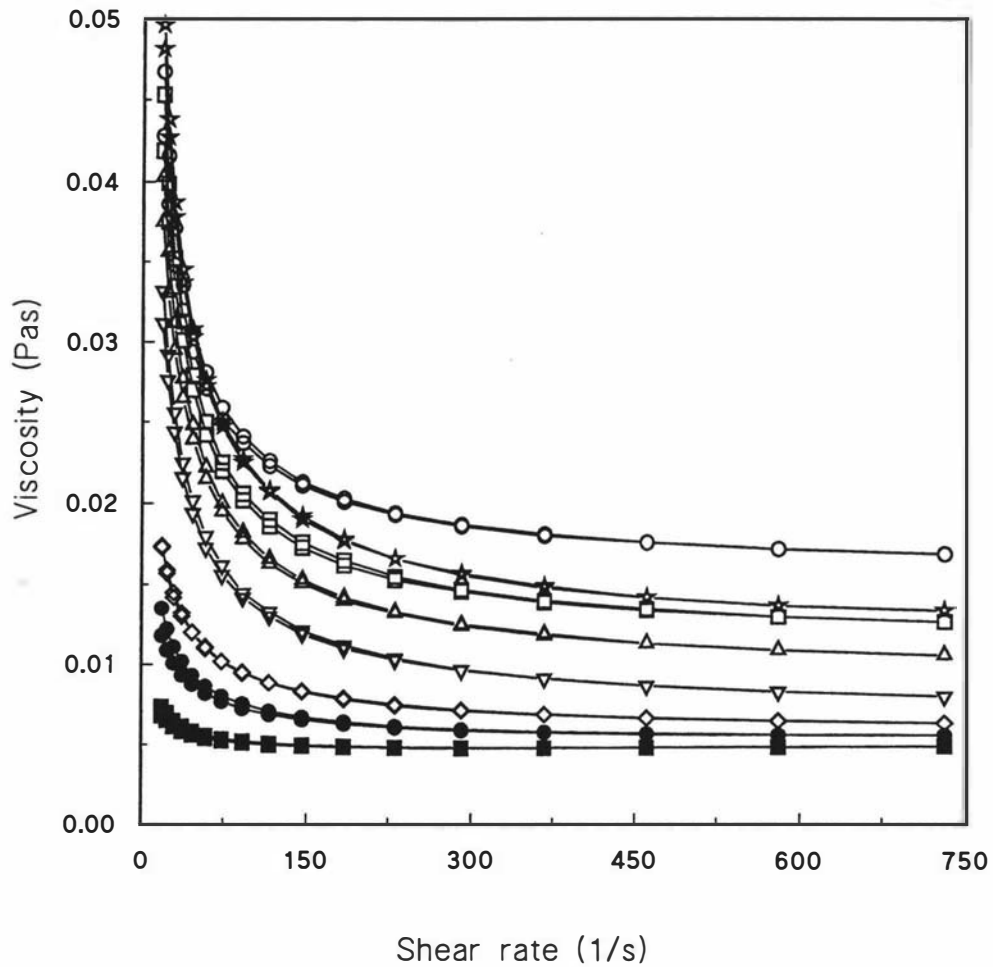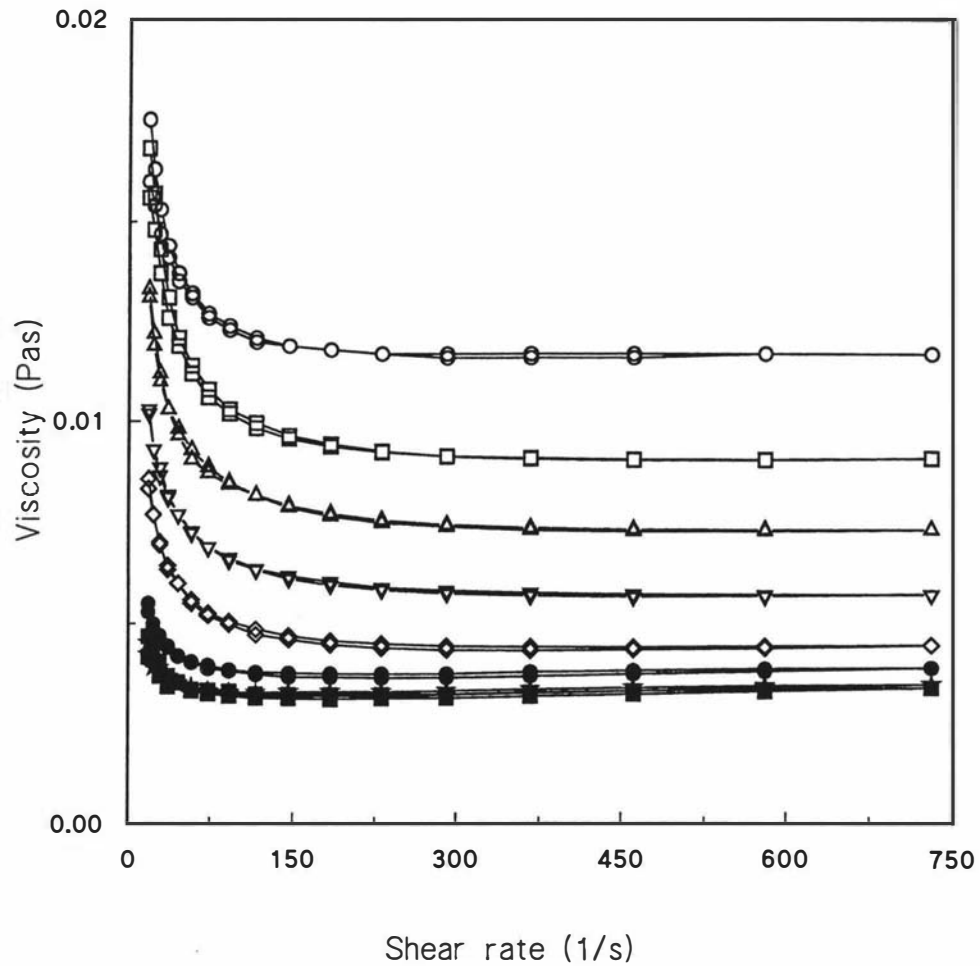
Figure 6.10: Apparent viscosity as a function of shear rate (1/s) of cream obtained from 5% sodium caseinate emulsion and mixed with varying concentrations of caseinate solutions. 5.0% (o); 4.0% (□); 3.0% (∆); 2.0% (▽); 1.0% (◇); 0.5% (•) and own subnatant (☆); water (■).

Figure 6.11: Apparent viscosity as a function of shear rate (1/s) of cream obtained from 0.5% sodium caseinate emulsion and mixed with varying concentrations of caseinate solutions. 5.0% (○); 4.0% (□); 3.0% (△); 2.0% (▽); 1.0% (◇); 0.5% (•) and own subnatant (☆); water (■).

Overall, these results show that the concentration of unadsorbed caseinate in the system is important in inducing flocculation of oil droplets. The surface coverage does not appear to be important, as both emulsion systems, with low and high surface loads, were flocculated above 2% sodium caseinate in the aqueous phase.
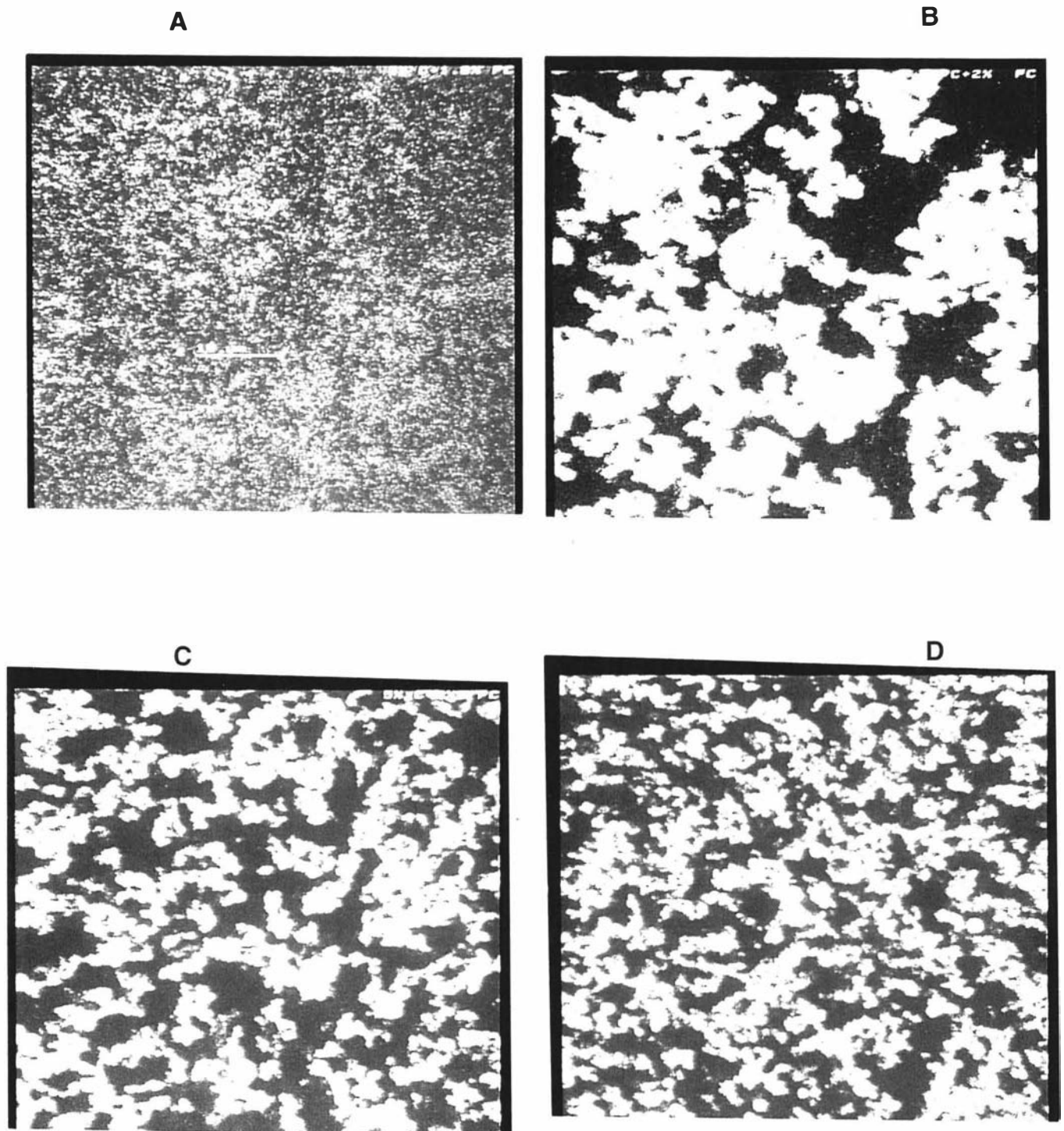
Figure 6.12: Confocal micrographs of 5.0% sodium caseinate cream mixed with various concentrations of caseinate solutions. 0.5% (A); 1.0% (B); 2.0% (C); 3.0% (D); 4.0% (E); 5.0% (F); its own subnatant (G) and water (H).
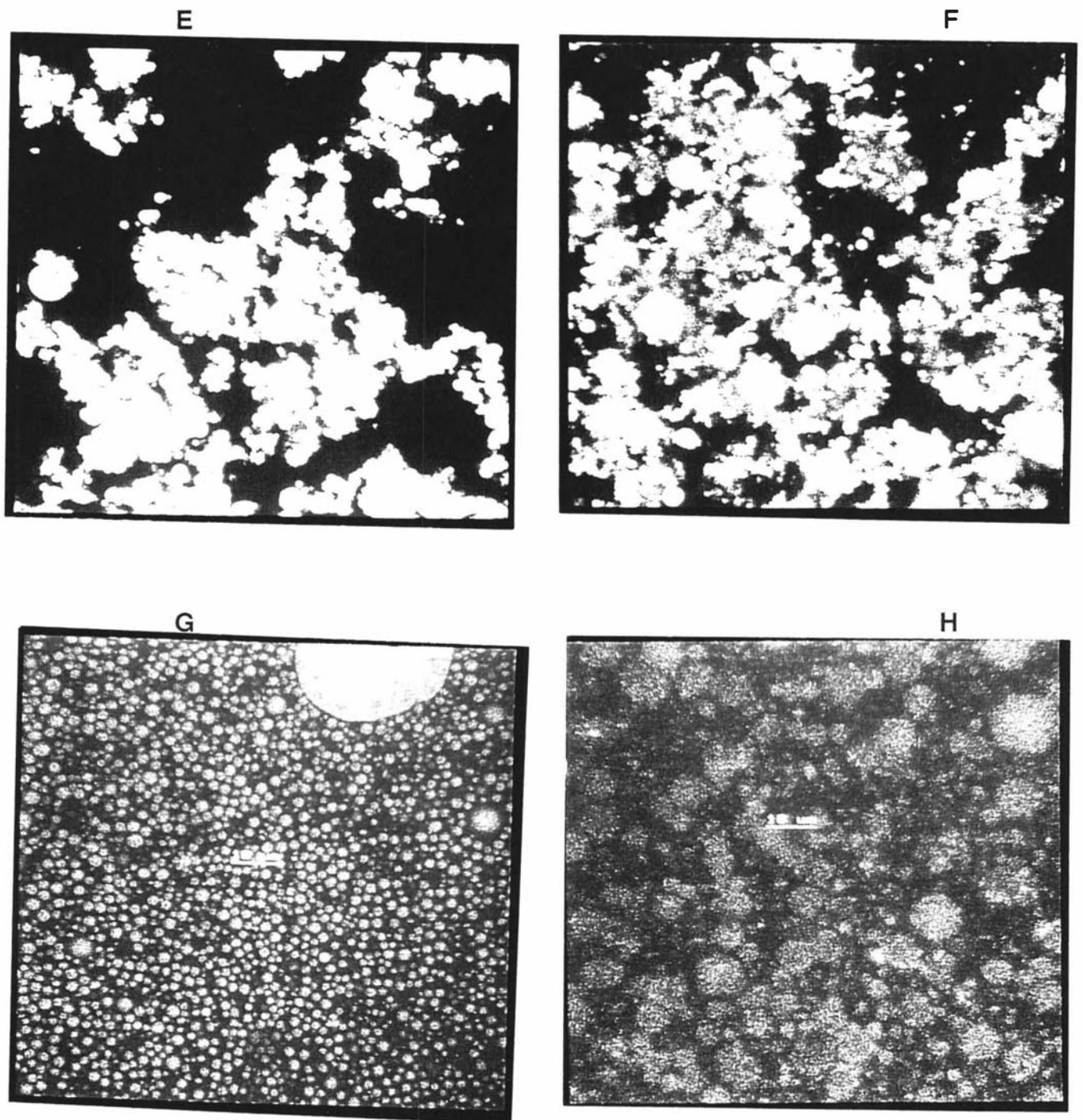
E

F

G

H
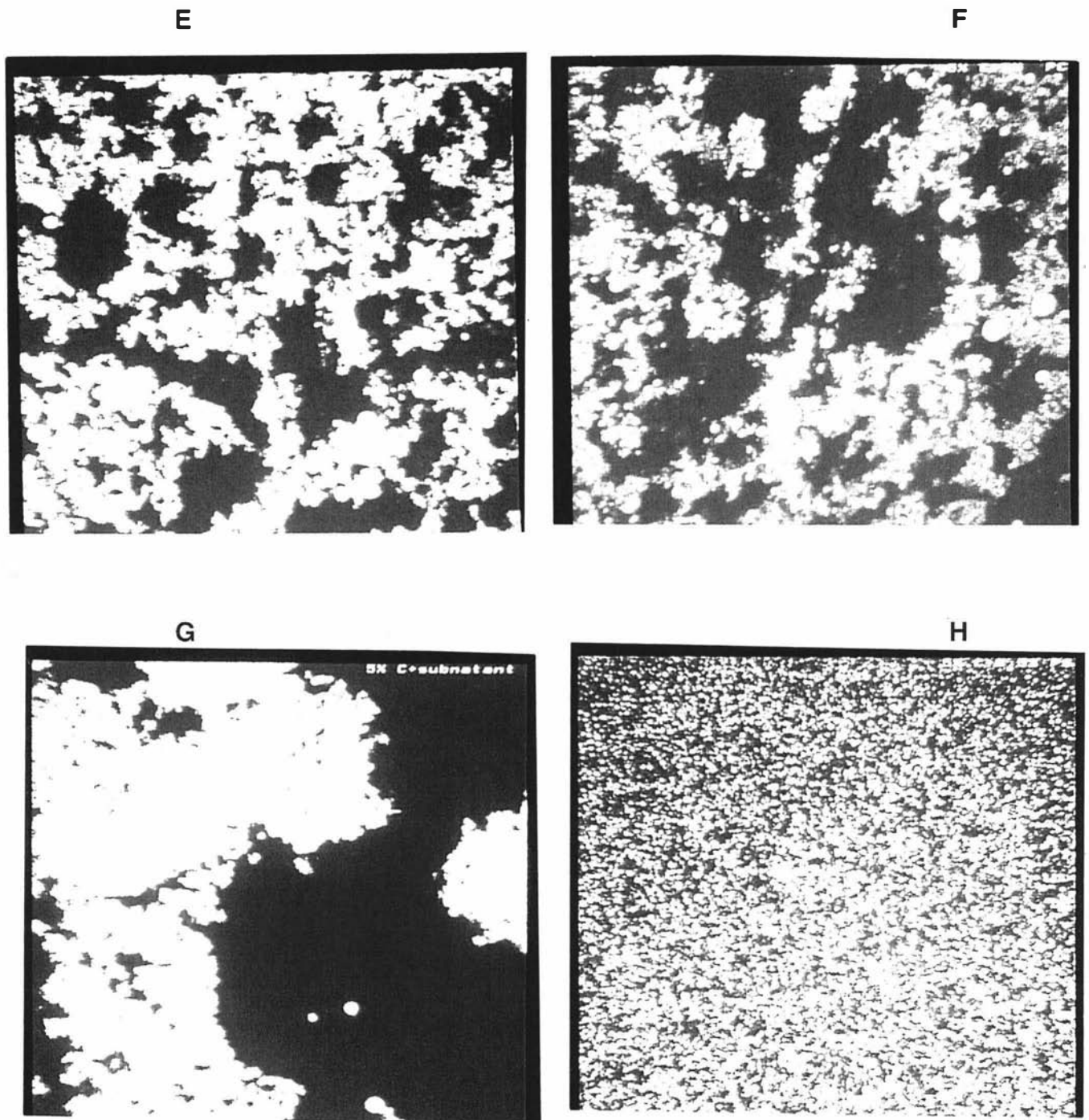
5X C+subnatant

Figure 6.12: continued..
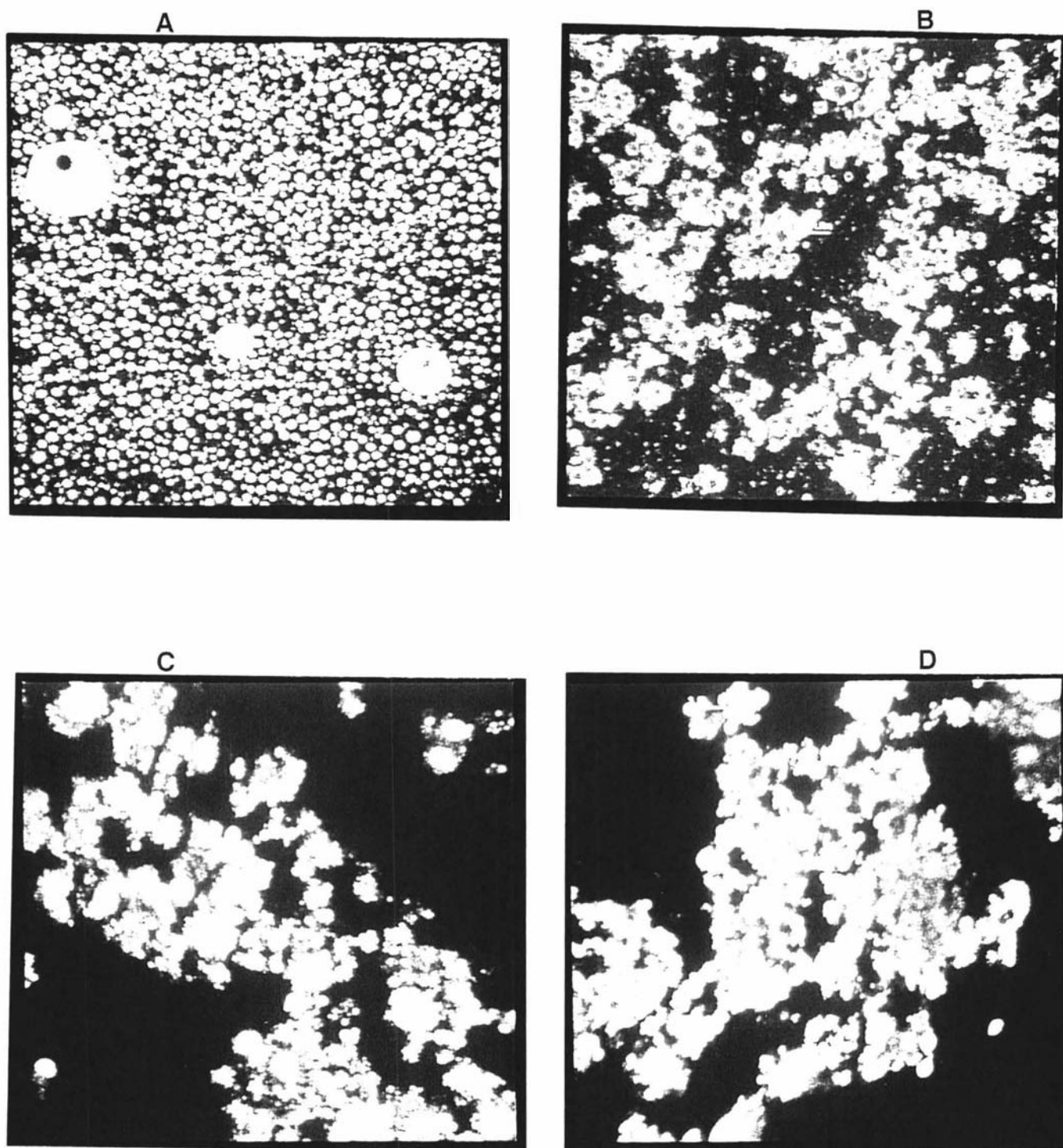
Figure 6.13: Confocal micrographs of 0.5% sodium caseinate cream mixed with various concentrations of caseinate solutions. 0.5% (A); 1.0% (B); 2.0% (C); 3.0% (D); 4.0% (E); 5.0% (F); its own subnatant (G) and water (H).

Figure 6.13: continued..

6.2.3 Creaming stability of emulsions made with mixtures of sodium and calcium caseinate

Sodium and calcium caseinate solutions were mixed in different ratios, while keeping the total protein concentration at 1.0 or 2.5% and emulsions were made as described in Chapter 3. Creaming stability was determined as described in Chapter 3.

In emulsions containing 1% protein, the stability rating increased gradually with an increase in the sodium caseinate concentration in the mixture (Figure 6.14). However, in emulsions containing 2.5% protein, the stability rating decreased rapidly from ~ 37% to ~ 15% as the sodium caseinate concentration in the mixture was increased to 0.5% (Figure 6.15). Further increase in the sodium caseinate concentration in the mixture caused a smaller but gradual decrease in the stability rating.

Confocal micrographs of emulsions made with 2.5% calcium caseinate solution or a mixture of 0.5% sodium caseinate and 2.0% calcium caseinate are shown in Figure 6.16 A and B, respectively. Emulsions made with 2.5% calcium caseinate were homogeneous with no or little sign of flocculation and no visible creaming. In contrast, emulsions made with mixture containing 0.5% sodium caseinate and 2.0% calcium caseinate showed large irregular flocs which resulted in a marked decrease in the stability rating (Figure 6.16 B).
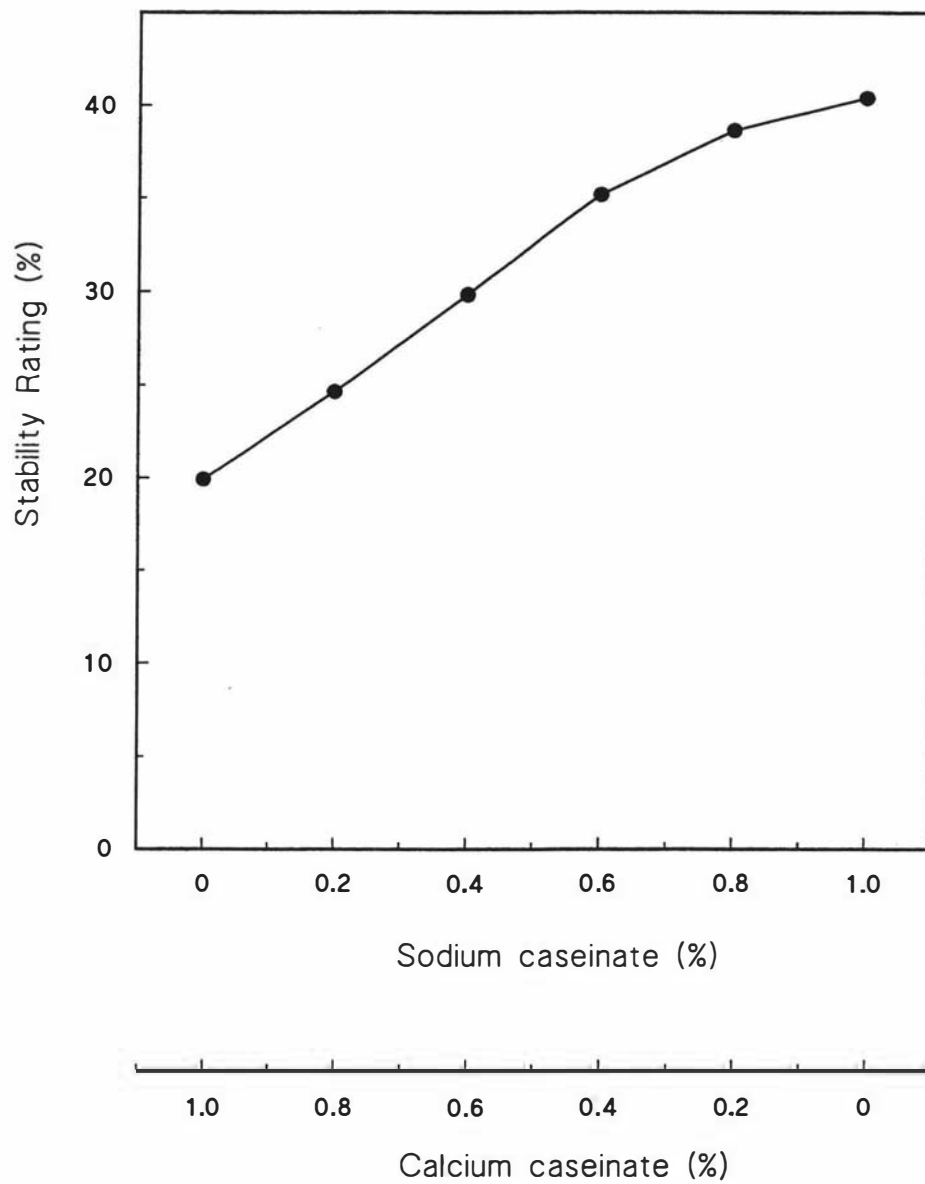
Figure 6.14: Creaming stability (after 24 hours of storage at 20°C) of emulsions containing 30% soya oil and mixtures of sodium and calcium caseinate, at a total protein concentration of 1.0%.
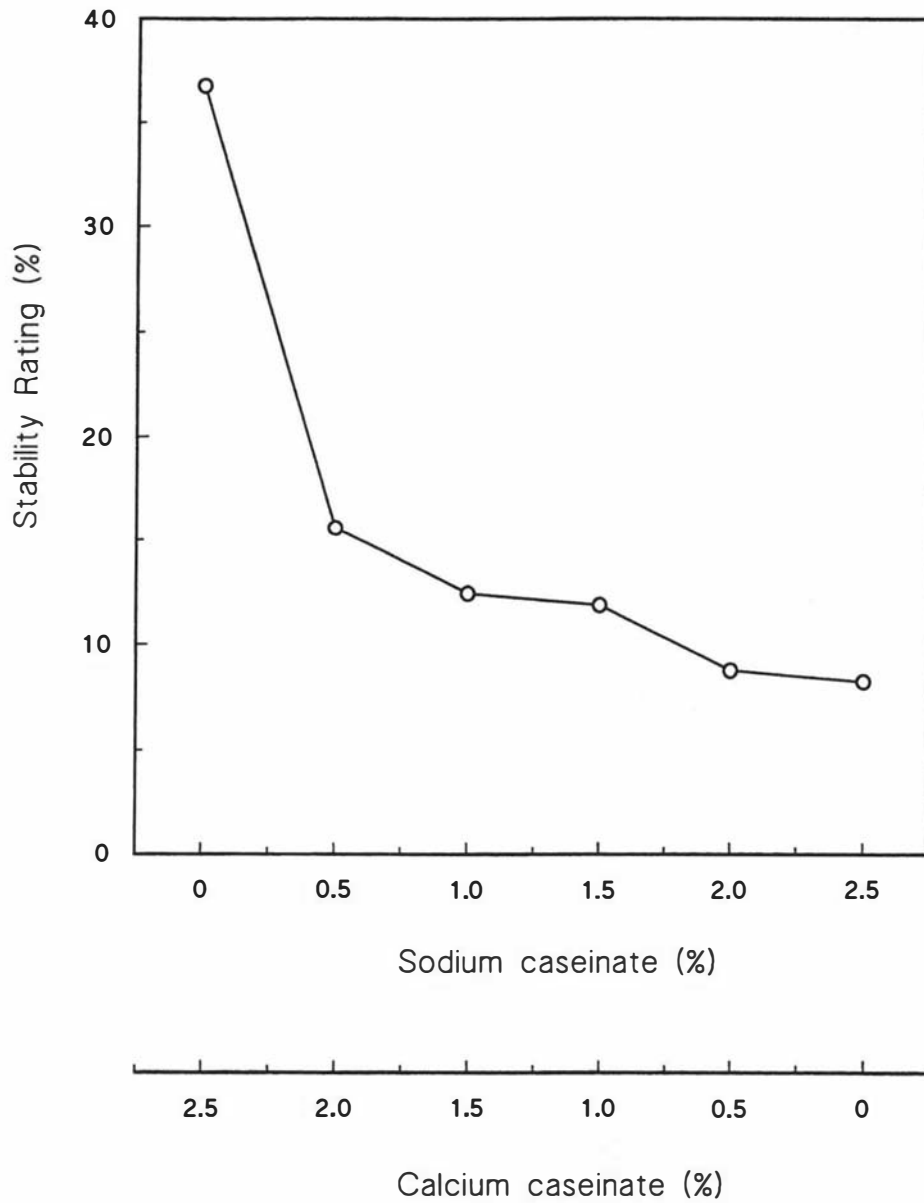
Figure 6.15: Creaming stability (after 24 hours of storage at 20°C) of emulsions containing 30% soya oil and mixtures of sodium and calcium caseinate, at a total protein concentration of 2.5%.
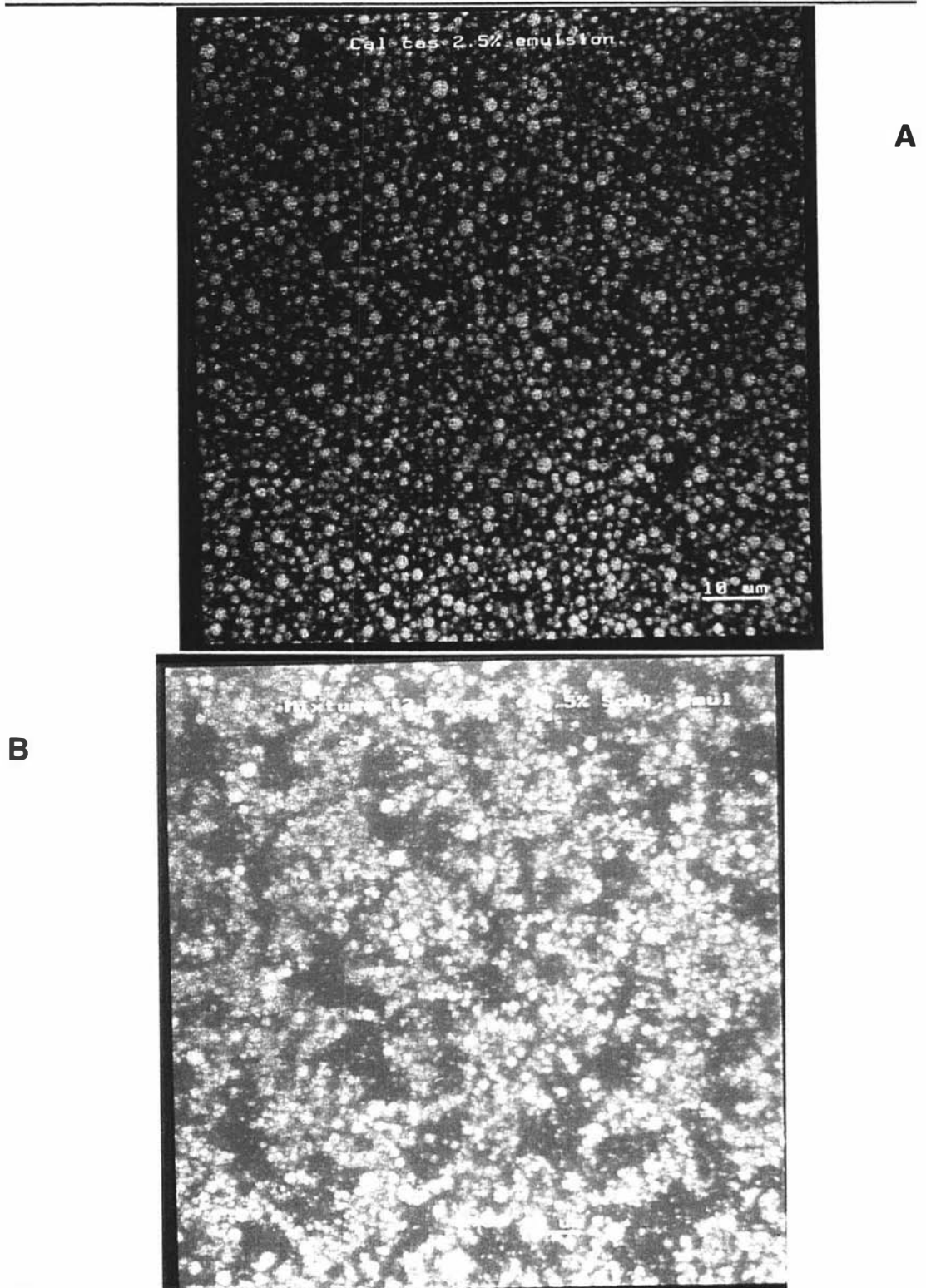
Figure 6.16: Confocal micrographs of emulsions containing 30% oil and mixtures of sodium and calcium caseinate, at a total protein concentration of 2.5%. Calcium caseinate (2.5%) (A); calcium caseinate (2%) + sodium caseinate (0.5%) (B).

6.3 General discussion

During the course of this research, Dickinson and Golding (1997) published a paper explaining the abnormal creaming behaviour of sodium caseinate-stabilized emulsions. They attributed the reversible flocculation in these systems and the resulting enhanced creaming to a 'depletion flocculation' mechanism, which exists between pairs of large spherical particles dispersed in a dilute solution of non-adsorbing polymer molecules or small solid particles. That depletion flocculation is reversible to dilution was first demonstrated by Bondy (1939) for the creaming of natural rubber in the presence of water-soluble polymer.

It is well established that depletion flocculation can be induced in surfactant-stabilized emulsion systems by the presence of excess surfactant micelles in the aqueous phase (Aronson, 1989; McClements, 1994; Calderon *et al.*, 1993). The surfactant micelles are excluded from the space between the droplet surfaces, with the resulting osmotic pressure gradient causing reversible flocculation. The extent of flocculation is dependent on the average droplet size and the concentration and aggregation number of the micelles (Dickinson and Golding, 1997). Casein coated emulsion droplets have been shown to become flocculated in the presence of high molecular weight polysaccharides (dextrans, xanthan etc.) by a depletion mechanism. The depletion flocculation results in enhanced emulsion creaming under quiescent storage conditions as the flocs tend to separate faster than the individual droplets (Dickinson and Golding, 1997).

Depletion flocculation may indicate that initial floc formation occurs above a certain polymer concentration which Dickinson *et al.* (1997) termed the 'critical flocculation concentration' c^*. From Figure 6.17 it is clear that the concentration of unadsorbed protein increases almost linearly with an increase in the total protein content in the aqueous phase from 0.5 to 5.0%. In contrast, the surface protein coverage increased rapidly initially, but the increase was slower above 2% protein (Figure 6.17). Dickinson and Golding (1997) suggested that the

initial discernible 'onset' of flocculation occurred at an aqueous caseinate concentration somewhere between 2 and 3% w/w. But from Figures 6.1, 6.5 C and 6.12 B and C, it is evident that flocculation begins even when the total protein content in the aqueous phase is 2%, i.e. at an unadsorbed protein content of ~ 1.2%. So under these conditions the critical flocculation concentration is well below 2%. With increasing total caseinate concentration in the emulsion above this critical flocculation concentration c^*, there is a steady increase in the concentration of unbound protein, resulting in a corresponding enhancement in the extent of flocculation of the droplets, from the initial formation of discrete individual flocs at lower caseinate concentrations (Figures 6.5 C and D) through to the development of a more expansive droplet network structure at higher concentrations (Figures 6.5 E and F).

It is likely that depletion flocculation in the sodium caseinate emulsions was caused by the presence of caseinate sub-micelles formed from self-assembly of sodium caseinate in the aqueous phase of the emulsion at concentrations above 2 wt%. At the simplest level of approximation, the maximum contact value of the depletion attraction for large spheres of radius R due to small spheres of diameter d is given by

$$U_{dep} = -3kTR\phi_s / d$$

where k is the Boltzmann constant, T is the temperature, and ϕ_s is the volume fraction of small spheres. Assuming sub-micelles of diameter 10 nm and a droplet diameter of 0.6 μm Dickinson *et al.* (1997) obtained an interparticle depletion interaction of $U_{dep} \approx$ - 3 kT, which they suggested was strong enough to induce incipient phase separation and hence greatly enhance creaming in an emulsion stored under gravity.
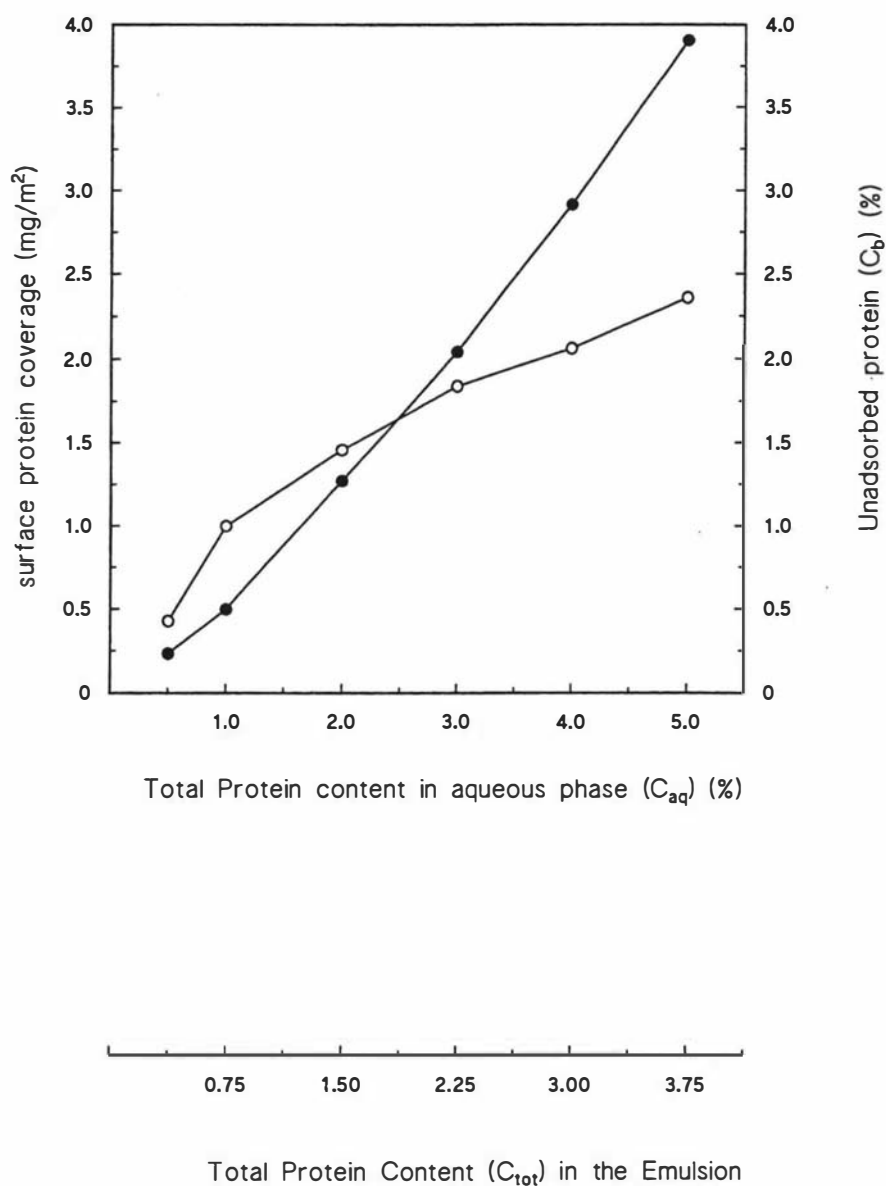
Figure 6.17: Emulsifier distribution between bulk aqueous phase and interface in sodium-caseinate-stabilized emulsions (30% oil, pH 7.0). The concentration of unadsorbed protein (C_b) (•) and the surface protein coverage (mg/m²) (○) are plotted as a function of total protein content in the emulsion (C_{tot}) or total protein content (C_{aq}) in the aqueous phase.

The absence of depletion flocculation in calcium caseinate emulsions at the same total protein concentrations may be due to the different state of casein aggregation in calcium caseinate solutions. Calcium caseinate consists of protein particles ranging in diameter from 0.02µm to > 100µm. The presence of large particles probably prevents depletion flocculation from occurring. Assuming an average diameter of protein particles in calcium caseinate solutions of 100 nm and droplet diamteter (~ 1 µm), the value of U_{dep} will be very low.

It is interesting to note that emulsions formed with a mixture of 2.0% calcium caseinate and 0.5% sodium caseinate showed extensive flocculation, although emulsions formed individually with either 0.5% sodium caseinate or 2.0% calcium caseinate were very stable and showed no signs of flocculation. It is possible that the addition of sodium caseinate caused dissociation of large protein aggregates into smaller calcium caseinate aggregates, which was sufficient to induce depletion flocculation. The decreased stability rating of emulsions made with mixtures of sodium and calcium caseinate (total protein concentration 2.5%) could be attributed to competition by the sodium ions for the specific binding sites on the phosphoserine groups of caseins, thereby reducing the destabilizing effects of Ca^{2+} (Agboola and Dalgleish, 1995). This is in accordance with the known decrease in calcium binding to both α_{s1}-casein (Dalgleish and Parker, 1980) and β-casein (Parker and Dalgleish, 1981) with increase in ionic strength as dictated by the molarity of NaCl. Further studies are required to clarify this phenomenon.

6.4 General conclusions

It can be concluded that caseins in sodium caseinate solution exist as a dynamic system of casein monomers, casein complexes and aggregates. The caseins in calcium caseinate solutions consist of diverse range of casein aggregates and particles (Chapter 4). The results in Chapter 5 show that in sodium caseinate-stabilized emulsions made with low protein concentrations (≤ 2.0%), casein monomers and smaller complexes are adsorbed in preference

to aggregates, but at higher concentrations, larger aggregates are adsorbed in preference to monomers. In these emulsions, β-casein is adsorbed in preference to α_s-casein, when the emulsions are made with ≤ 2.0% protein and α_s-casein is adsorbed preferentially when the caseinate concentration in the continuous phase is ≥ 3.0%. However, in calcium caseinate-stabilized emulsions, at all protein concentrations α_s-casein is adsorbed in preference to any other caseins at all concentrations (Chapter 5).

Sodium caseinate-stabilized emulsions made with higher concentrations (> 2.0%) show extensive destabilization (reversible) which is apparently due to 'depletion flocculation'. In contrast, the stability rating of calcium caseinate emulsions increases with increase in protein concentration.

Overall, key findings in the area of sodium caseinate-stabilized emulsions include

(1)   the casein composition of the interface is dependent on the concentration of protein used to make emulsions.

(2)   depletion flocculation occurs when the concentration of protein in the aqueous phase exceeds 2.0%.

It appears that the concentration of casein has a major effect on its molecular mass and composition of casein aggregates and complexes, which in turn influences the adsorption behaviour during emulsion formation and hence the surface casein concentration and composition. As depletion flocculation depends on the concentration and size of the unadsorbed protein, it is likely that variations of the composition of casein aggregates and complexes would also effect depletion flocculation. The state of protein can be changed by changing the environmental conditions, such as pH, temperature and ionic strength. Therefore, the next two chapters of this thesis were focussed on exploring the effects of ionic strength (by NaCl addition) and heat treatment of caseinate solutions on these properties.

Calcium caseinate in solution exists as casein aggregates and particle, and the aggregation state of calcium caseinate solutions influences the protein coverage and surface composition. It is likely that the state of aggregation can be affected by different treatments of the calcium caseinate solutions. The remaining sections of this thesis would explore the effects of heating and addition of NaCl on calcium caseinate systems, as these treatments are likely to influence protein aggregation state. Addition of NaCl is likely to cause dissociation of large calcium linked casein aggregates while heat treatment is likely to promote aggregation of casein particles.

Chapter 7

EFFECT OF HEAT TREATMENT (RETORT CONDITIONS) ON THE FORMATION AND STABILITY OF CASEINATE EMULSIONS

7.1 Introduction

Many food products, based on emulsions, are subjected to heat treatment prior to distribution. The heat treatment is applied generally to extend the microbiological shelf life of the product. Heat-induced changes in proteins of skim milk systems have been studied extensively and models have been advanced describing the coagulation of proteins in skim milk systems (Fox, 1982; Singh, 1988; Singh and Creamer, 1992). Heat-induced changes in sodium caseinate solutions were also studied by Guo *et al.* (1989). However, limited information is available on the heat-induced changes in sodium and calcium caseinate-stabilized emulsions.

Studies on the effects of heating on protein functionality have been limited mainly to whey proteins (Mangino *et al.,* 1987). The heat stability of oil-in-water emulsions containing milk proteins has also been studied by Hunt and Dalgleish (1995). Very little work has been published on the effects of heating on the emulsifying properties of sodium caseinates, especially at ultra-high temperatures. In this chapter, the effects of heating on the adsorption behaviour and stability of caseinate emulsions were investigated. The effect of heating caseinate solutions prior to emulsion formation were also explored.

7.2 Emulsion formation

Emulsions were prepared with 30% soya oil with various concentrations of sodium and calcium caseinate. The emulsions were sealed in glass bottles and then heated at 121°C for 15 min in an autoclave. This heat treatment was similar to many retort treatments used in the food industry. In some cases, the caseinate solutions were heated at 121°C for 15 min in an autoclave, and then

mixed with soya oil followed by emulsification. The emulsions were analysed for particle diameter, droplet surface concentration and composition, and stability as described in Chapter 3.

7.3 Results and discussion

7.3.1 Particle size distribution and droplet diameter

7.3.1.1 Sodium caseinate

The particle size distributions of unheated sodium caseinate emulsions formed with various concentrations of protein have been described in Chapter 5 (Figure 7.1 A). Heat treatment of emulsions made using $\geq$ 1.0% sodium caseinate had no considerable effect on the size distribution of the emulsion droplets (Figure 7.1 B). However, when emulsions made with 0.5% sodium caseinate were subjected to heat treatment, slight broadening of the particle size distribution was observed. Similar results were observed when emulsions were formed with sodium caseinate solutions heated at 121°C for 15 min (Figure 7.1 C).

7.3.1.2 Calcium caseinate

In contrast to sodium caseinate emulsions, heat treatment of calcium caseinate emulsions resulted in marked changes in the size distribution of the emulsion droplets (Figure 7.2). Heat treatment of emulsions made using $\leq$ 2.0% calcium caseinate caused a major shift in the particle size distributions towards higher particle size ranges. For example, the emulsions formed with 0.5 and 1.0% protein and heated at 121°C for 15 min had particles > ~ 80 µm (Figure 7.2 B); some of these particles could not be measured accurately with the Mastersizer using a 45 mm lens. Emulsions formed with higher concentrations of calcium caseinate showed considerable broadening of the particle size distribution after heat treatment. Generally, similar trends were observed when emulsions were formed with calcium caseinate solutions heated at 121°C for 15 min (Figure 7.2 C), although the size distributions were slightly different.
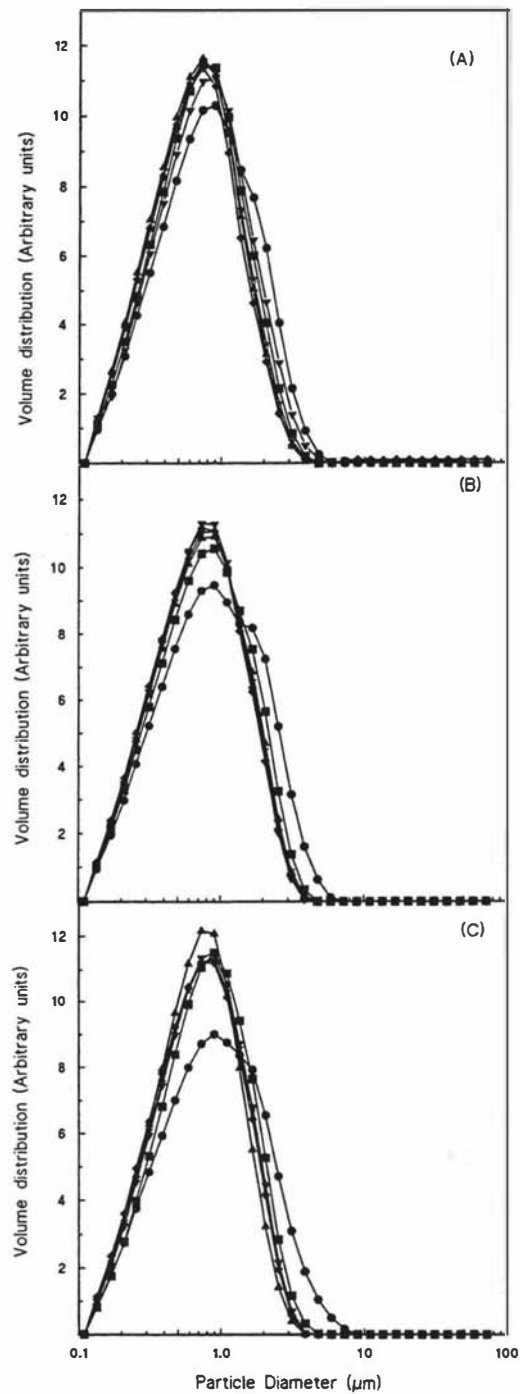
Figure 7.1: Particle size distributions of oil droplets in unheated emulsions (A), emulsions heated at 121°C for 15 min (B) or emulsions formed from sodium caseinate solutions that had been heated at 121°C for 15 min. The emulsions contained 30% oil and 0.5% (•); 1.0% (■); 2.0% (▲); 3.0% (▼); 4.0% (♦); 5.0% (★) sodium caseinate.
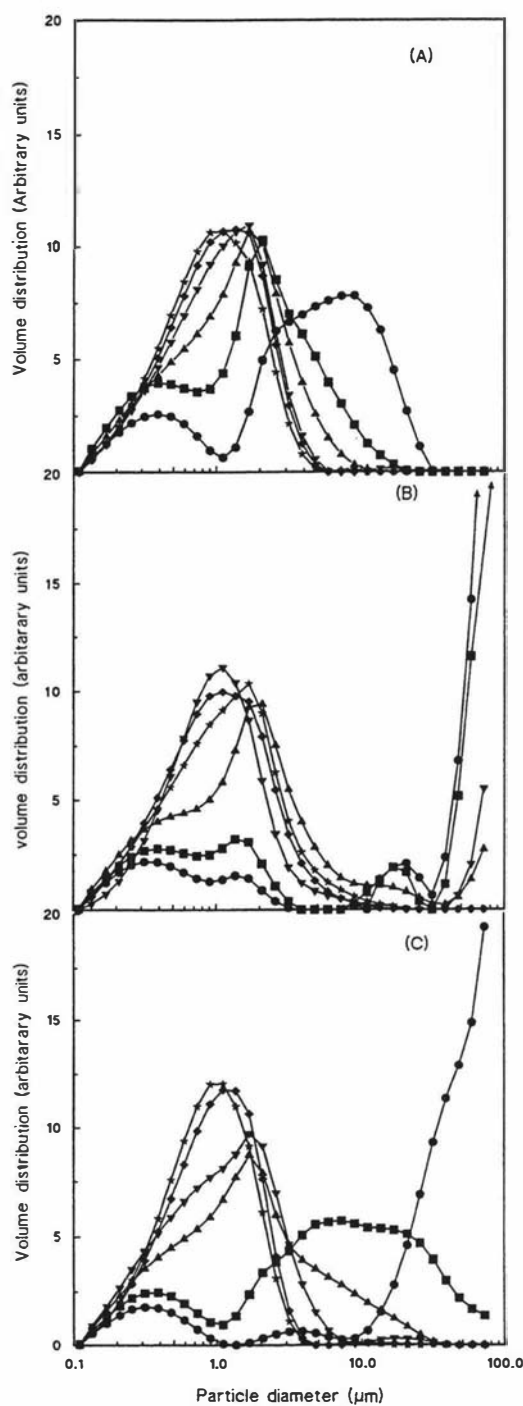
Figure 7.2: Particle size distributions of oil droplets in unheated emulsions (A), emulsions heated at 121°C for 15 min (B) or emulsions formed from calcium caseinate solutions that had been heated at 121°C for 15 min. The emulsions contained 30% oil and 0.5% (•); 1.0% (■); 2.0% (▲); 3.0% (▼); 4.0% (♦); 5.0% (★) calcium caseinate.

When the emulsions formed with various concentrations of calcium caseinate and heated at 121°C for 15 min were mixed with the dissociating buffer (ratio 1:3) before measuring the particle size distribution, the particle size distributions became narrower (0.1 - ~ 5 µm). Heated emulsions made with 0.5 and 1.0% calcium caseinate showed a bimodal distribution and a slightly higher range of particle sizes compared with the emulsions made with higher caseinate concentrations (Figure 7.3).

The changes in the volume-surface average diameter (d_{32}) in emulsions, as a function of sodium caseinate concentration, are shown in Figure 7.4 A . At a given protein concentration, the average droplet diameters of sodium caseinate heated emulsions were slightly larger than for the unheated control emulsions. Heating sodium caseinate solutions before emulsion formation had a similar effect; the droplet diameter varied between ~ 0.65 to ~ 0.55 µm.

The average diameter of droplets in unheated and heated calcium caseinate emulsions decreased with an increase in calcium caseinate concentration (Figure 7.4 B). Heat treatment of emulsions, made using > 2.0% calcium caseinate, had no significant effect on the droplet diameter, but the droplet diameter of emulsions made with 0.5 and 1.0% calcium caseinate increased markedly after heat treatment. The average droplet diameters in emulsions formed from heat-treated calcium caseinate solutions were similar to those in heated emulsions.

7.3.2 Surface protein coverage

When emulsions formed with various quantities of sodium caseinate were heated at 121°C for 15 min the surface protein concentration was found to increase at all sodium caseinate concentrations (Figure 7.5 A). However, the difference in the surface protein coverage between heated and unheated emulsions increased with an increase in the concentration of caseinate. The surface protein coverage of emulsions formed with sodium caseinate solutions, heated at 121°C for 15 min, also showed similar trends, although the surface
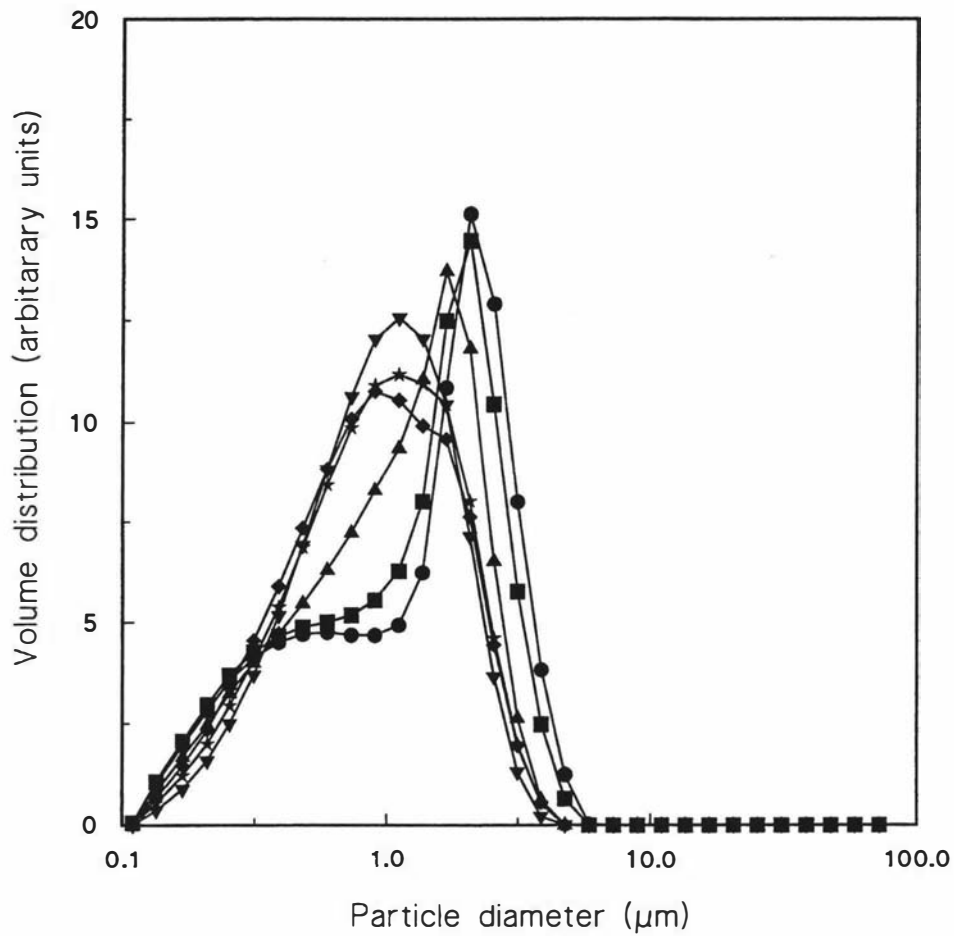
Figure 7.3: Effect of dispersion of calcium caseinate-stabilized emulsions in a dissociating buffer (0.02% EDTA, 2% SDS) on the particle size distributions. Emulsions formed with 0.5% (•); 1.0% (■); 2.0% (▲); 3.0% (▼); 4.0% (◆); 5.0% (★) calcium caseinate
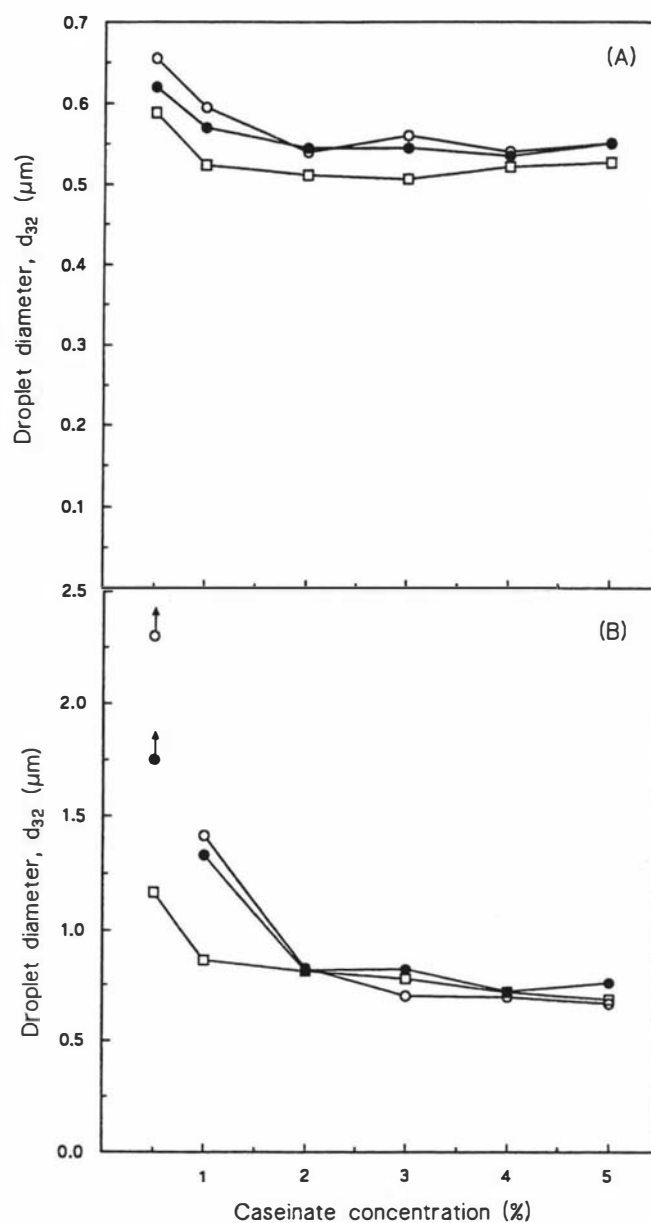
Figure 7.4: Changes in the average droplet diameter (d_{32}) as a function of caseinate concentration in emulsions containing 30% soya oil and varying amounts of sodium caseinate (A) or calcium caseinate (B). Unheated emulsions (□), emulsions heated at 121°C for 15 minutes (●) or emulsions formed with caseinate solutions heated at 121°C for 15 min (○). Data represents average of two determinations. Arrow indicates that the real d_{32} may be higher, as a number of droplets in the emulsions were found to be outside the Mastersizer range (Figure 7.2).
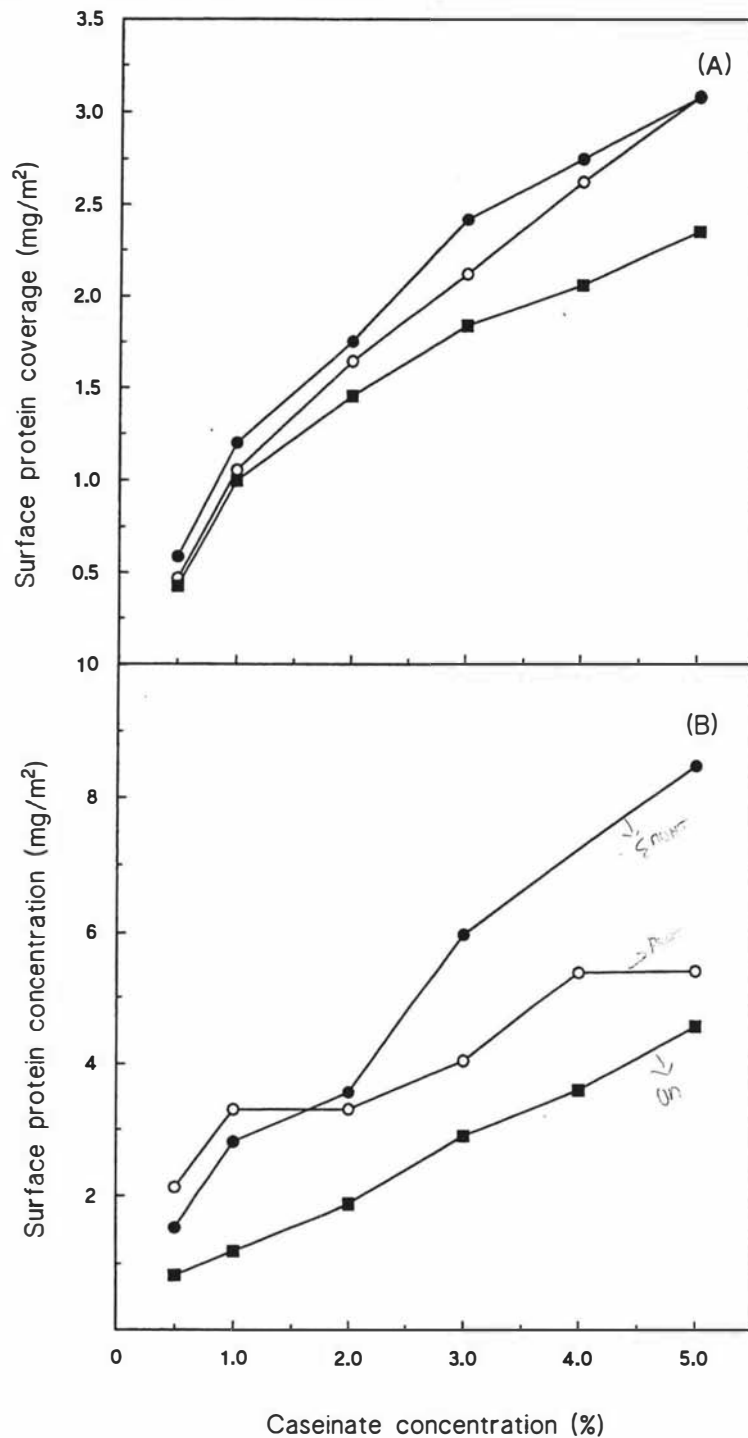
Figure 7.5: Changes in surface protein coverage (mg/m²) as a function of protein concentration in emulsions stabilized by sodium caseinate (A) or calcium caseinate (B). Unheated emulsions (■); emulsions heated at 121°C for 15 minutes (•); emulsions formed with caseinate solution heated at 121°C for 15 min (○). Data represents average of two determinations.

protein coverage for these emulsions was slightly lower than when the emulsions were heated.

Heat treatment of calcium caseinate emulsions at 121°C for 15 minutes also resulted in an increase in the surface protein coverage at all caseinate concentrations (Figure 7.5 B); this increase was much larger than that observed in sodium caseinate emulsions after heating. At 0.5 and 1.0% calcium caseinate, the surface protein coverage in emulsions formed with calcium caseinate solutions heated at 121°C for 15 min was slightly higher than that in heated emulsions but it was considerably lower in emulsions made with higher calcium caseinate concentrations.

7.3.3 SDS-PAGE

SDS-PAGE patterns of the various protein solutions and emulsions in Figure 7.6.

7.3.3.1 Sodium caseinate

7.3.3.1.1 SDS-PAGE of heated sodium caseinate solutions

SDS-PAGE showed noticeable changes in sodium caseinate solutions on heating at 121°C for 15 min (Figure 7.6 A). Comparing the electrophoretic patterns of heated and unheated 2% sodium caseinate (Figure 7.6 A), it appears that the intensity of all casein bands, particularly α_{s1}-casein, decreased after heating. α_{s2}-Casein appeared to be very sensitive to heat treatment. The decrease in intensity of the casein bands was probably caused by both aggregation, as indicated by the appearance of slower-moving diffuse, protein material (labelled as X) and the formation of degradation products (labelled as Y). These faster moving peptides and the formation of polymeric material were more visible in heated 5% caseinate solutions. At least three bands, labelled P, Q and R, arising from the heat-induced degradation of caseins could be clearly seen (Figure 7.6 A).

These results are essentially in agreement with Guo *et al.* (1989) who showed

that, when sodium caseinate was heated at 120 and 130°C and analysed by SDS-PAGE, the intensities of the major casein bands were reduced and several new peptides were formed due to protein degradation on heating. The degradation of sodium caseinate was also observed by gel filtration on a Sephadex G-150 column. Fox (1982) and Singh and Fox (1985) also showed that α_{s2}-casein was the most susceptible casein to thermal treatment in skim milk systems.

7.3.3.1.2 SDS-PAGE of cream phase of emulsions formed with heated sodium caseinate solutions

The SDS-PAGE patterns of the cream phase obtained from emulsions formed with autoclaved (121°C for 15 min) protein solutions are shown in Figure 7.6 B. Emulsions made with 0.5% heated sodium caseinate showed a very low intensity of α_s-casein at the droplet surface but β- and κ-caseins were clearly visible. Emulsions made with $\geq$ 1.0% heated sodium caseinate solutions · showed no major difference between the relative intensities of the major casein bands. In emulsions made with unheated sodium caseinate (Chapter 5), it was found that when the sodium caseinate concentration was $\leq$ 2.0% β-casein was preferentially adsorbed and when the concentration was $\geq$ 3.0% α_s-casein was adsorbed in preference to other caseins. No such preferential adsorption was noted when the emulsions were made using autoclaved sodium caseinate solutions except at 0.5% where β-casein appeared to be adsorbed in preference. One notable feature was the accumulation of significant amounts of faster moving peptides at the droplet surface. Three bands, labelled as P, Q and R, were clearly visible at the droplet surface and their intensity was greater than that in the original heated caseinate solution. This indicates that some of these degradation products were more readily adsorbed than the parent protein. Some polymerized casein material, which remained in the slots, was visible in all samples.

7.3.3.1.3 SDS-PAGE of cream phase of heated sodium caseinate-stabilized emulsions

The SDS-PAGE patterns of the cream phase of emulsions heated at 121°C for 15 min are shown in Figure 7.6 C. The pattern appears to be similar to that of the cream phase obtained from emulsions formed with heated sodium caseinate solutions (Figure 7.6 B). Emulsions made with 0.5% protein and heated showed a very low intensity of α_s-casein at the droplet surface but β- and κ-caseins were clearly visible. When emulsions containing higher caseinate concentrations ($\geq$ 1.0%) were heated (121°C for 15 min) some of the protein material could not be resolved and remained on the top of the stacking gel. The intensity of polymerized protein in heated emulsions appeared to be greater than in emulsions made with heated sodium caseinate solutions (Figure 7.6 C). Bands P, Q and R, were clearly visible at the droplet surface and their intensity was greater than in the original caseinate solution.

Previous studies have shown that heat treatment of caseinate solutions at high temperatures ($\geq$ 120°C) causes a number of physical and chemical changes (Guo *et al.*, 1989). Dephosphorylation of serine-phosphate groups occurs during heating which may reduce the negative charge on the caseinate, possibly promoting casein-casein interaction. The level of pH 4.6 and 12% TCA-soluble nitrogen increases considerably on heating sodium caseinate solutions, indicating degradation of peptide bonds (Guo *et al.*, 1989). The caseiantes may also undergo polymerization, involving covalent bonds other than disulfide bonds. Cystine or phosphoserine residues may undergo β-elimination, yielding dehydroalanine residues which may then react with lysine to form lysinoalanine cross-links, or with the thiol groups of cysteine residues to form lanthionine cross-links when protein solutions are heated at moderately high pH (Bohak, 1964; de Groot and Slump, 1969). Guo *et al.* (1989) found that levels of available lysine were reduced by 10-15% on heating an aqueous solution of sodium caseinate at 140°C for 30 min.

When emulsions formed with sodium caseinate are heated, it appears that

similar changes would occur in the caseinate adsorbed at the droplet surface. The SDS-PAGE results on the cream phase obtained from heated emulsions indicated that a substantial proportion of adsorbed caseinate was polymerized to form high molecular weight aggregates (Figure 7.6 C). This could arise from covalent bond formation between adsorbed casein molecules or between caseinate molecules in solution and those at the emulsion droplets or both. The increase in surface protein concentration (Figure 7.5 A) on heating indicates that interactions between unadsorbed caseinate molecules and caseinate at the droplet surface may be occurring during heating. Relatively higher proportions of casein degradation products at the droplet surface (Figure 7.6 C) indicate that the adsorbed caseinate molecules were more susceptible to fragmentation during heating than those in solution. This may be due to different structures and conformations of caseins at the droplet surface.

In emulsions formed with sodium caseinate solution that had been heat treated prior to emulsification, the surface concentration and composition are determined by the state of the caseinate molecules in solution. It appears that both polymerized caseinate material and degradation products are adsorbed efficiently during emulsification (Figure 7.6 B). In fact, some of the degradation products appear to be more surface active and therefore accumulate readily at the droplet surface.

SDS-PAGE in the presence of mercaptoethanol showed that the proteins gradually polymerized to form higher molecular weight protein which failed to enter the gel. As all the forces involved in protein-protein interactions including disulphide bonds are disrupted in SDS and mercaptoethanol, it appears that this high molecular weight material was linked through covalent bonds other than disulphide bonds; α_{s1}- and α_{s2}-caseins were particularly sensitive. These covalent bonds may be formed between caseinate molecules at the surface of the same droplet or between two molecules at the surface of two different droplets or between molecules on the surface and those in the bulk aqueous phase. The latter would lead to higher surface protein concentrations.
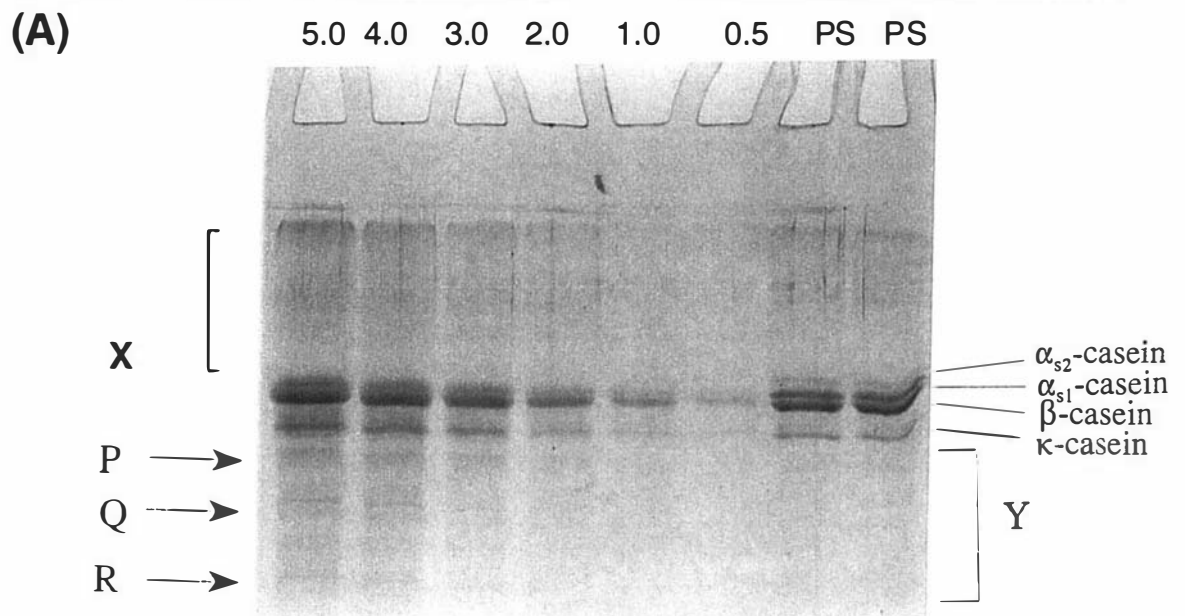
(A)

Figure 7.6: SDS-PAGE electrophoretic patterns of emulsions formed with sodium caseinate either before or after heating at 121°C for 15 min.

(A) Heated sodium caseinate solutions

(B) Cream phase of emulsions formed with heated sodium caseinate solutions.

(C) Cream phase of heated sodium caseinate-stabilized emulsions

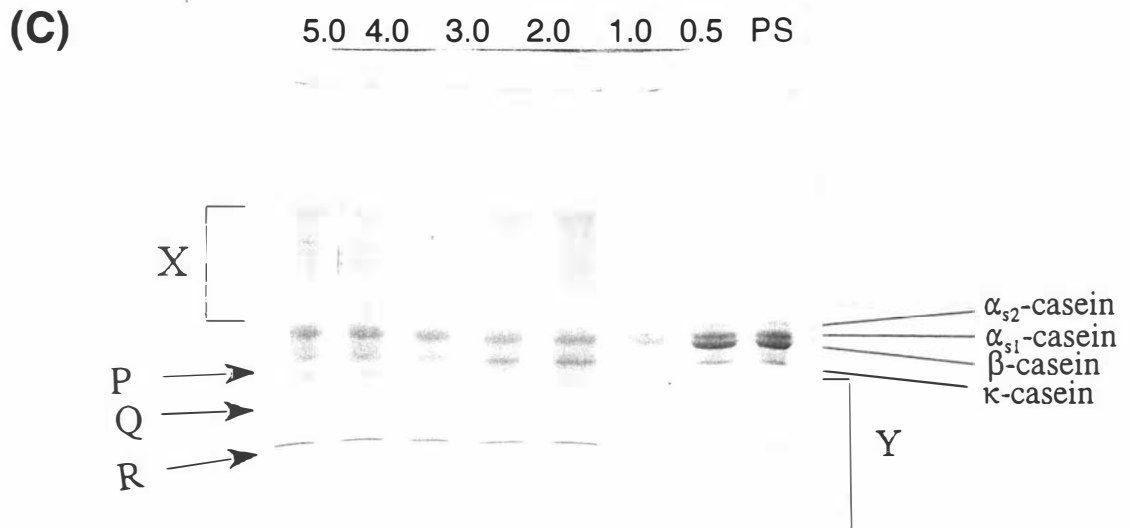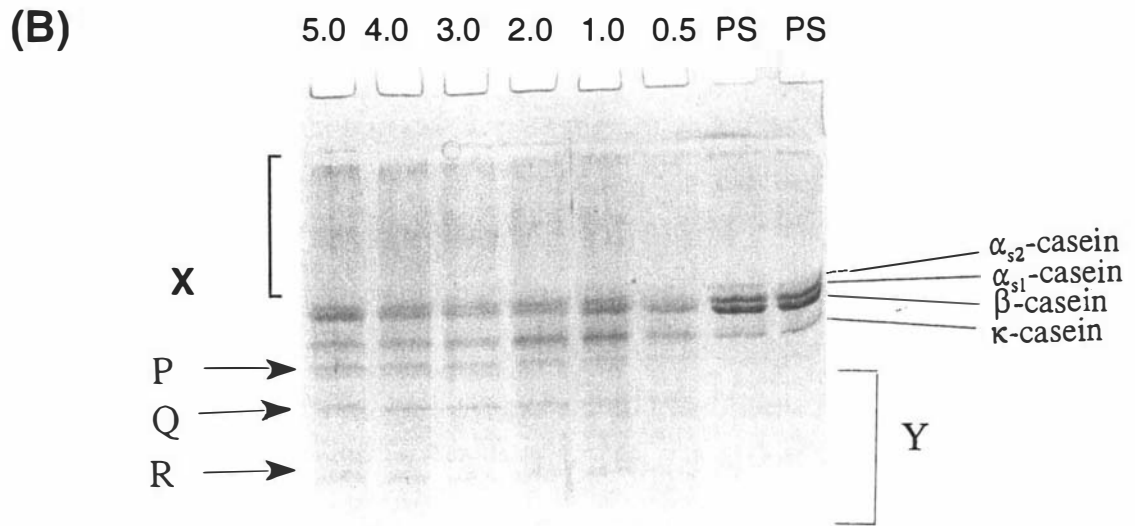PS refers to control i.e. nomal unheated sodium caseinate solution.

(B)

(C)

Figure 7.6 continued..

7.3.3.2 Calcium caseinate

7.3.3.2.1 SDS-PAGE of heated calcium caseinate solutions

The SDS-PAGE patterns of calcium caseinate solutions heated 121 °C for 15 min are shown in Figure 7.7 A. Comparing the electrophoretic patterns of heated and unheated calcium caseinate (2%) (Figure 7.7 A), it appears that the intensity of all casein bands decreased after heating at all concentrations. The α_{s2}-casein band had disappeared completely and the intensity of the α_{s1}casein band was very low. There was an increase in the intensity of high molecular weight protein material (labelled as X in Figure 7.7 A) that remained on the top of the resolving gel after heating. Simultaneously, there was an increase in the formation of degradation products (labelled as Y in Figure 7.7 A). Bands P, Q and R, that were observed in heated sodium caseinate solution (Figure 7.6 A) were also seen in heated calcium caseinate solution.

Comparison of the SDS-PAGE patterns in Figure 7.7 A with those in Figure 7.6 A, shows that heat treatment of calcium caseinate solution caused more extensive polymerization of caseins than heat treatment of sodium caseinate solutions. It is interesting to note that α_{s1}- and α_{s2}-caseins were more sensitive to heating in calcium caseinate solutions than in sodium caseinate solutions.

7.3.3.2.2   SDS-PAGE of various phases from heated calcium-caseinate-stabilized emulsions

The SDS-PAGE patterns of the cream phase obtained from heated calcium caseinate emulsions are shown in Figure 7.7 B. These electrophoretic patterns were different from those obtained for heated sodium caseinate emulsions (Figure 7.6 D). In the case of unheated calcium caseinate-stabilized emulsions, the relative proportions of α_s-casein, adsorbed at the droplet surface, were higher than those of any other caseins at all caseinate concentrations used (see Chapter 5). When the emulsions were heated (121°C for 15 min), the intensity of the α_{s1}- and α_{s2}-casein bands at the droplet surface was reduced markedly. Some of the protein material could not be resolved and remained at the top of the stacking and resolving gels. As in the case of heated sodium

caseinate emulsions, accumulation of considerable amounts of faster moving peptides (labelled as Y) at the droplet surface was also noted. The three bands which were seen in heated sodium caseinate emulsions (labelled P, Q, R in Figure 7.6 D) were also seen in the heated calcium caseinate emulsions (labelled P, Q and R in Figure 7.7 B). As in the case of sodium caseinate, in calcium-caseinate-stabilized emulsions all the three major casein bands (i.e. α_s-, β - and κ-casein) were visible at all concentrations. However, the intensity of the α_s-casein band was relatively low compared with unheated emulsions.

The SDS-PAGE patterns of the subnatants, obtained after centrifugation of heated calcium caseinate emulsions (45,000 g for 40 min), are shown in Figure 7.7 C. It is interesting to note that the subnatant (unadsorbed protein) contained mainly β- and κ-caseins and the α_s-casein band was not visible at all, which may suggest that α_s-casein was either on the interface or had polymerized to form large aggregates that sedimented during centrifugation.

The SDS-PAGE patterns of the sediments, obtained after centrifugation of heated calcium caseinate emulsions (45,000 g for 40 min), are shown in Figure 7.7 D. The sediment contained relatively high proportions of protein material that could not be resolved and remained at the top of the stacking and resolving gels. Surprisingly, the sediment also contained considerable proportions of faster moving peptides. The three bands labelled as P, Q and R were clearly visible in the sediment.

7.3.3.2.3 SDS-PAGE of various phases from emulsions formed with heated calcium caseinate solutions

The SDS-PAGE patterns of the cream phase obtained from emulsions formed with heated (121°C for 15 min) calcium caseinate solutions were somewhat similar to those obtained for heated emulsions (Figure 7.7 E). However, at all caseinate concentrations, when emulsions were formed with heated calcium caseinate solutions some of the protein material was polymerized and was not resolved and remained at the top of the resolving and stacking gels. The

intensity of this unresolved material was much greater than in heated calcium caseinate emulsions. As in the case of heated calcium caseinate emulsions, the intensities of slower moving peptides (labelled as X) increased with an increase in concentration and the intensity of α_{s1}- and α_{s2}-casein bands at the droplet surface reduced markedly. The three bands labelled as P, Q and R, were clearly visible at the droplet surface and their intensity was greater than that of the heated calcium caseinate solutions or in heated emulsions. The electrophoretic patterns of the subnatant (unadsorbed protein) and the sediments (Figure 7.7 F and G) were similar to those for the heated emulsions (Figure 7.7 D).

From these results it appears that there may be at least three types of reactions that could be involved during heating of emulsions or when emulsions are formed with heated calcium caseinate solutions.

(I)   Polymerization and degradation of caseinate during heating.

(II)   Interaction between the serum phase proteins and the proteins on the interface.

(III)   Aggregation of caseinate coated emulsion droplets induced by calcium ions in calcium caseinate emulsions.

(IV)   Interactions between adsorbed protein molecules.

In emulsions formed with calcium caseinate solution that had been heated prior to emulsification, the surface concentration and composition are influenced by the presence of polymerized caseinate molecules as well by the degradation products in heated solution. Some of the degradation products appear to adsorb rapidly during emulsion formation.

Calcium caseinate, in solution, contains large calcium-induced aggregates. During emulsification, these large casein aggregates are adsorbed on the interface giving higher protein loads (refer to Chapter 5). On heating calcium caseinate emulsions, some of the caseins were polymerized to form even larger aggregates; adsorption of these larger aggregates at the droplet surface would give rise to higher protein loads.
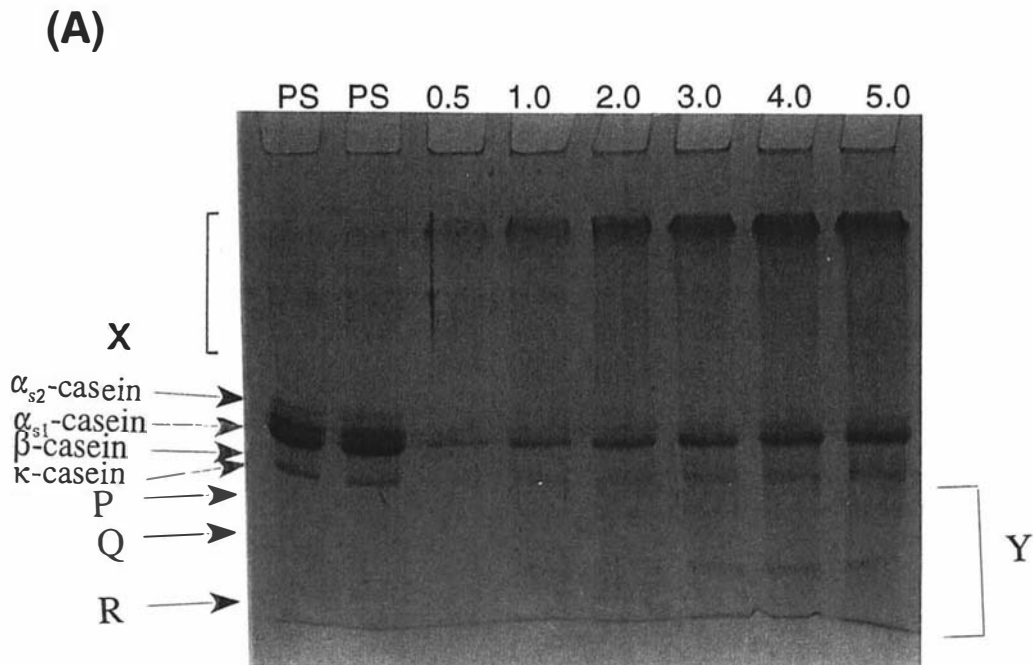
(A)

Figure 7.7: SDS-PAGE patterns of emulsions formed with calcium caseinate either before or after heating at 121°C for 15 min.

(A) Heated calcium caseinate solutions.

(B) Cream phase of heated calcium-caseinate-stabilized emulsions.

(C) Subnatant obtained from heated calcium-caseinate-stabilized emulsions.

(D) Sediment obtained from heated calcium-caseinate-stabilized emulsions.

(E) Cream phase of emulsions formed with heated calcium caseinate solutions.

(F) Subnatant obtained from emulsions formed with heated calcium caseinate solutions.

(G) Sediment obtained from emulsions formed with heated calcium caseinate solutions.

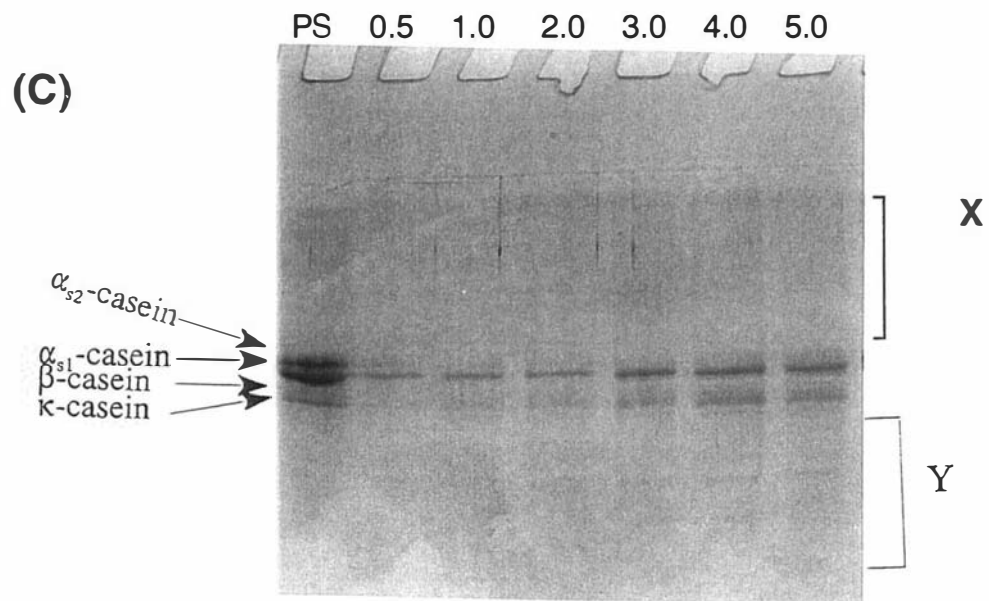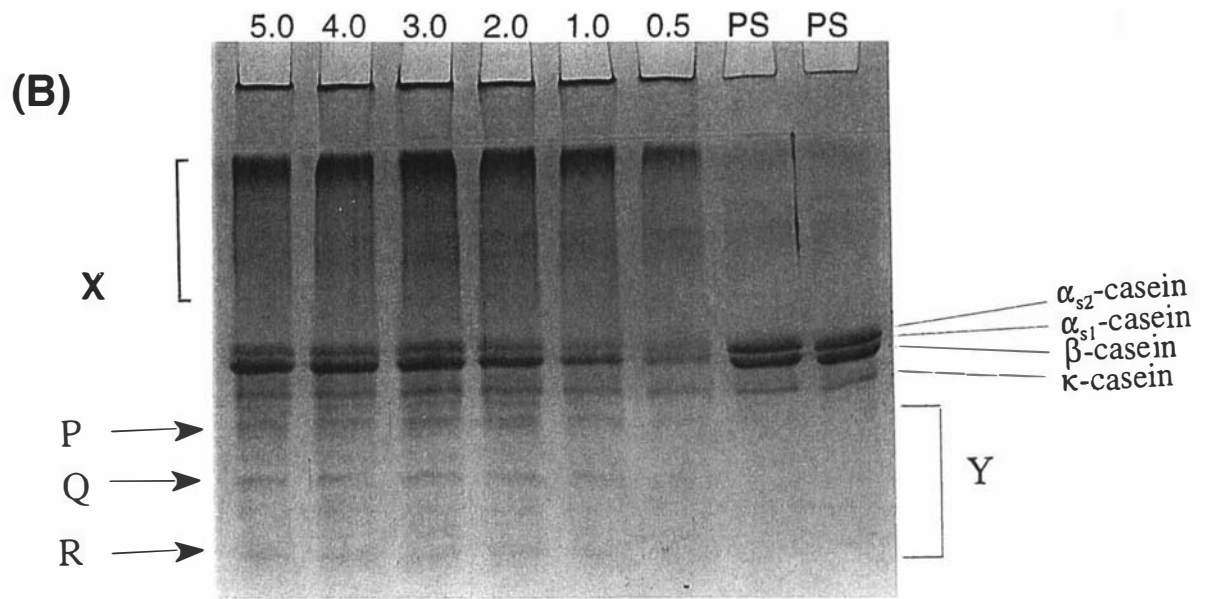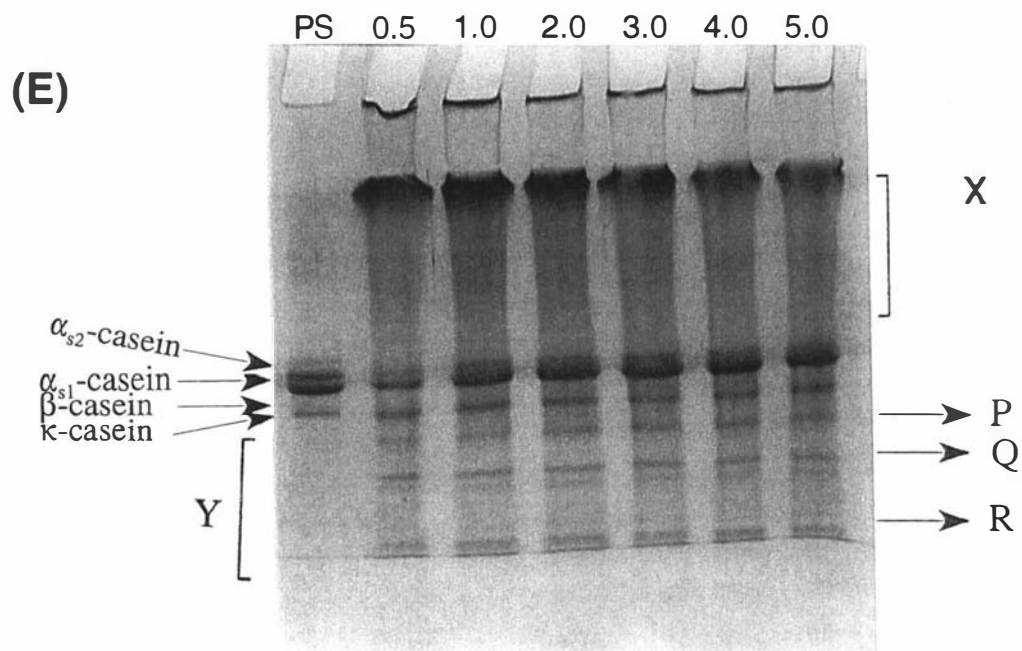PS refers to control i.e. nomal unheated calcium caseinate solution.

(B)

(C)

Figure 7.7: Continued..

5.0   4.0   3.0   2.0   1.0   PS

(D)

X

α_{s2}-casein
α_s-casein
β-casein
κ-casein

P
Q
R

Y

PS   0.5   1.0   2.0   3.0   4.0   5.0

(E)

X

α_{s2}-casein
α_{s1}-casein
β-casein
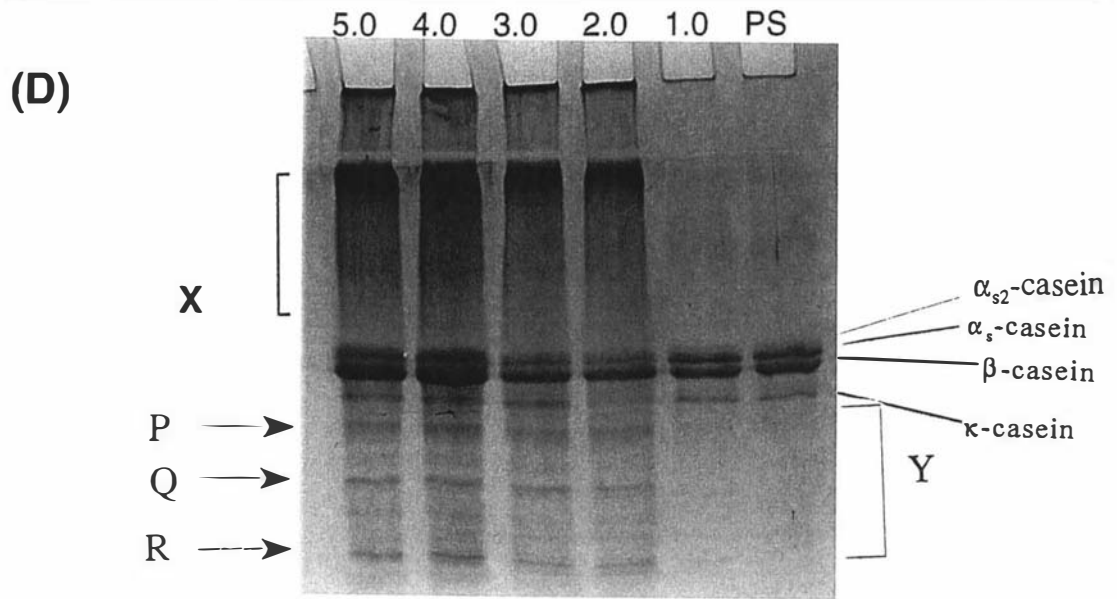κ-casein
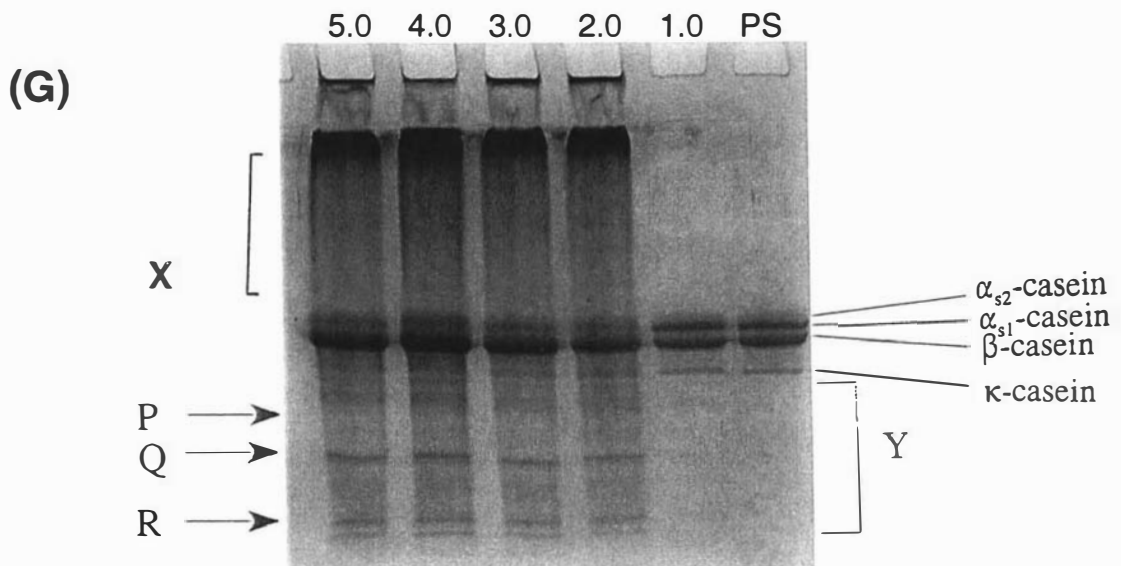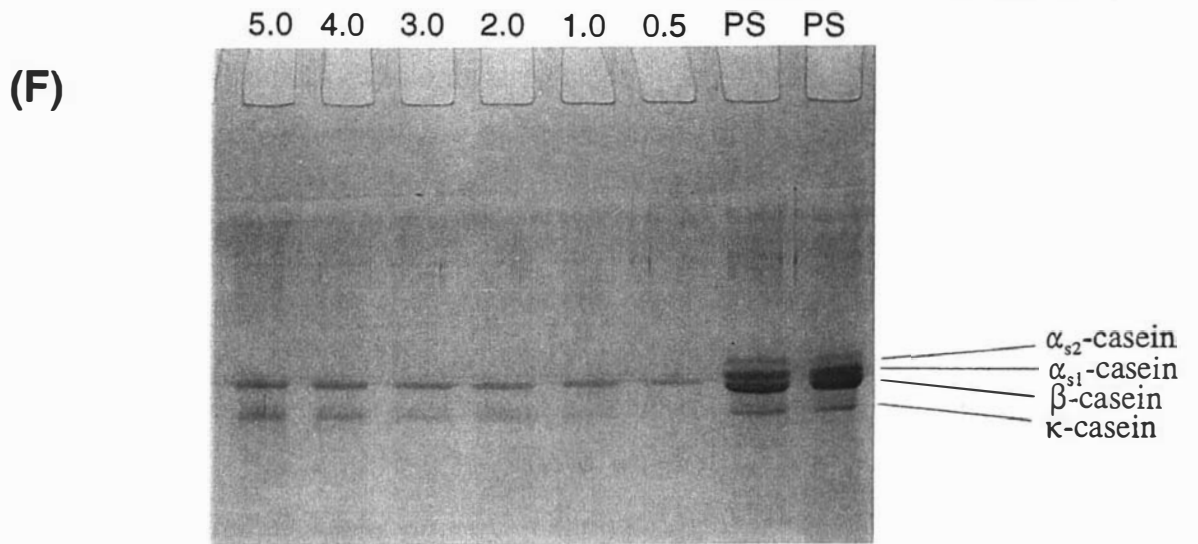
Y

P
Q
R

Figure 7.7: Continued..

Figure 7.7: Continued..

The extent of polymerization in calcium caseinate emulsions was much higher compared with sodium caseinate emulsions. It is possible that the extent of binding of calcium with negatively charged groups on the caseinate increases during heating, resulting in greater polymerization in calcium caseinate solutions. Binding of calcium to caseinate effectively reduces the negative charge, which diminishes the electrostatic repulsion between the caseinate chains (Cruijsen, 1996). The extent of polymerization/aggregation is also affected by dephosphorylation. Cleavage of phosphate groups during heating will affect the charge on the caseinate molecules and the sensitivity of caseinate against precipitation by calcium ions (Hekken and Strange, 1993). Therefore, it is likely that on heating at high temperatures, the negative charge on the caseinate gradually diminishes, as a result of dephosphorylation, causing aggregation of caseinate.

When the emulsions formed with calcium caseinate were heated, it appears that similar polymerization and degradation reactions occurs at the emulsion droplet surface. In addition, serum (unadsorbed) caseinate may interact with adsorbed casein to form polymerized products.

7.3.4 Stability of emulsions

7.3.4.1 Sodium caseinate

The changes in creaming stability of heated emulsions or emulsions formed with heated solutions of sodium or calcium caseinate after storage at 20°C for 24 hours are shown in Figure 7.8.

The stability rating of unheated sodium caseinate emulsions decreased rapidly with an increase in protein concentration from 0.5 to 3.0%, but further increase in sodium caseinate concentration from 3.0 to 5.0% caused only a slight decrease in stability rating (refer to Chapter 6). The creaming profile of heated (121°C for 15 min) sodium caseinate emulsions was similar to that of the unheated emulsion, but the stability rating values of heated emulsions were higher at all caseinate concentrations. Emulsions formed with heated (121°C for 15 min) sodium caseinate solutions also showed similar creaming profiles to those of unheated emulsions (Figure 7.8 A). As in the case of heated emulsions, the stability rating values obtained for the emulsions formed with heated sodium caseinate solutions at all caseinate concentrations were considerably higher than those of unheated emulsions. However, the stability rating values obtained for emulsions formed with heated sodium caseinate solutions were also higher than those of heated emulsions, except at 2 and 5.0% where the values were almost the same.

Heating of emulsions or sodium caseinate solutions prior to emulsion formation improved the creaming stability. This change in the stability rating due to heating caseinate solutions may be attributed to the higher viscosity, due to more aggregated forms in sodium caseinate solutions during heating, and also to the increase in the surface protein coverage.
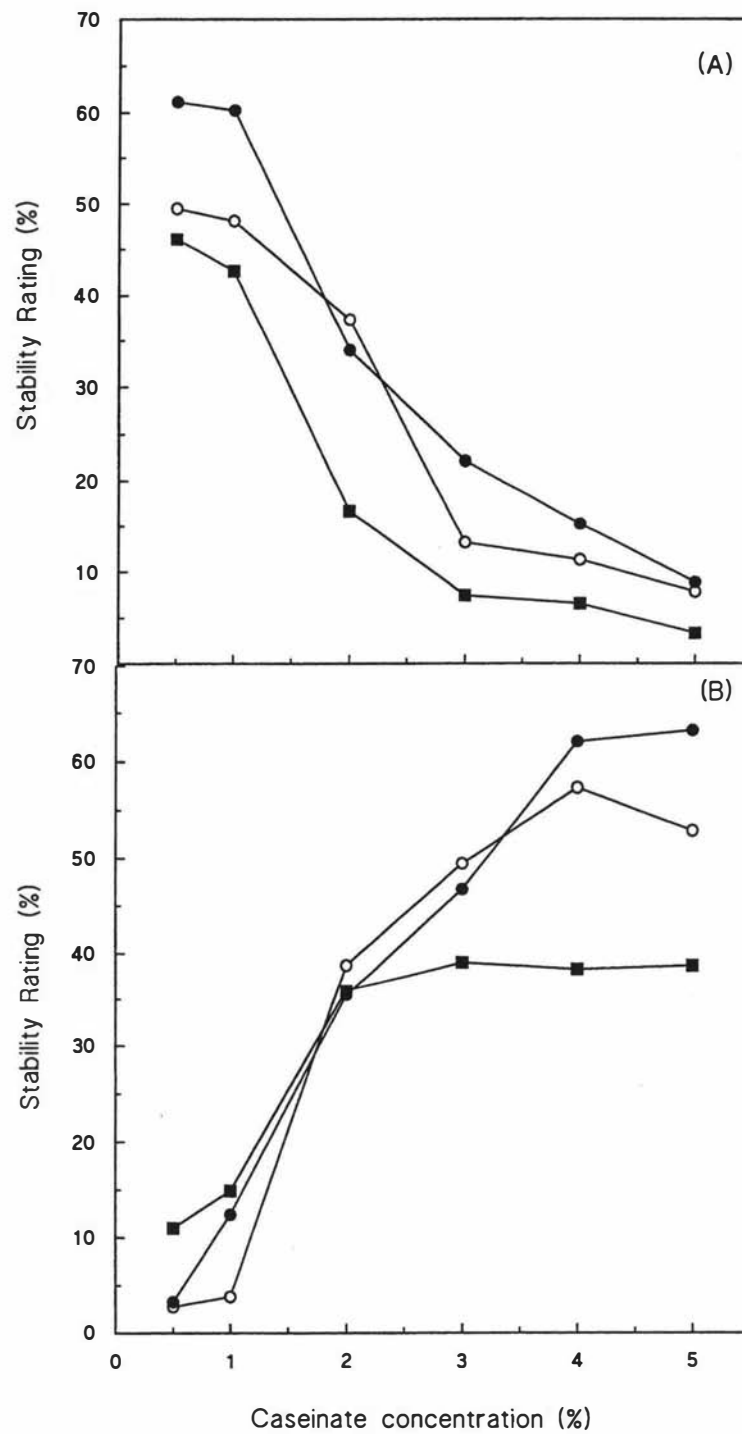
Figure 7.8: Effect of caseinate concentration on the stability rating of emulsions (30% oil) stabilized by sodium caseinate (A) or calcium caseinate (B). Unheated emulsions (■); emulsions heated at 121°C for 15 min (○); emulsions formed with heated (121°C for 15 min) caseinate solution (●).

7.3.4.2 Confocal microscopy

The emulsions made with 0.5 and 1.0% unheated sodium caseinate emulsions showed no flocculation and the droplets appeared to be separated and evenly distributed (refer to Chapter 6). At caseinate concentrations > 2.0%, there appeared to be the formation of irregular flocs. These flocs appeared to join together to form a network structure as the caseinate concentration was increased from 2.0 to 5.0%. Heated sodium caseinate emulsions with a caseinate concentration of 0.5 or 1.0% (Figures 7.9 A and B) showed no flocculation and the droplets appeared to be individual and evenly distributed. At 2% (Figure 7.9 C) there appeared to be the beginning of the formation of some flocs, but there was no network formation of these flocs. At caseinate concentrations > 3.0% (Figures 7.9 D to F), the flocs appeared to join together to form a network structure.

The confocal micrographs of emulsions formed with heated sodium caseinate solutions (Figure 7.10 A to F), showed that the emulsions formed with 0.5 and 1.0% caseinate concentrations (Figure 7.10 A and B) had no flocculation and the droplets appeared to be separated and evenly distributed, as was observed in unheated emulsions. At caseinate concentrations > 2% (Figures 7.10 C to F), there appeared to be the formation of irregular flocs. These flocs appeared to join together to form a network structure as the caseinate concentration was increased from 2.0 to 5.0%. It is surprising to note that, to some extent, the confocal micrographs of emulsions formed with heated caseinate solutions were similar to those of unheated sodium caseinate-stabilized emulsions.

Dickinson and Golding (1997) reported that the extent of flocculation is mainly dependent on the concentration of unbound or unadsorbed protein, with the initial discernible onset of flocculation occurring at an aqueous caseinate concentration somewhere between 2 and 3% (w/w). As discussed in Chapter 6, depletion flocculation may indicate that the initial floc formation occurs above a certain polymer concentration, which Dickinson *et al.* (1997) termed as 'critical flocculation concentration' c^*. In the present study, it was found that, in

unheated sodium caseinate emulsions, this c^{*} is ~ 1.2% (refer to Chapter 6). However, in the case of heated emulsions or emulsions formed with heated sodium caseinate solutions, c^{*} is slightly higher at ~ 2% (Table 7.1). As the extent of flocculation is also dependent on the size of the unadsorbed species (Dickinson and Golding, 1997; Dickinson *et al.*, 1997), it is also possible that the heating of emulsions or protein solutions increased the particle size of the unadsorbed protein, hence decreasing the depletion attraction.

Table 7.1: Measured protein concentration in the bulk phase (C_b) and calculated surface protein coverage (Γ) (mg/m^2) as a function of total protein content (C_{tot}) in the emulsion (30% soya oil, pH 7.0) and total protein content (C_{aq}) originally present in the aqueous phase.

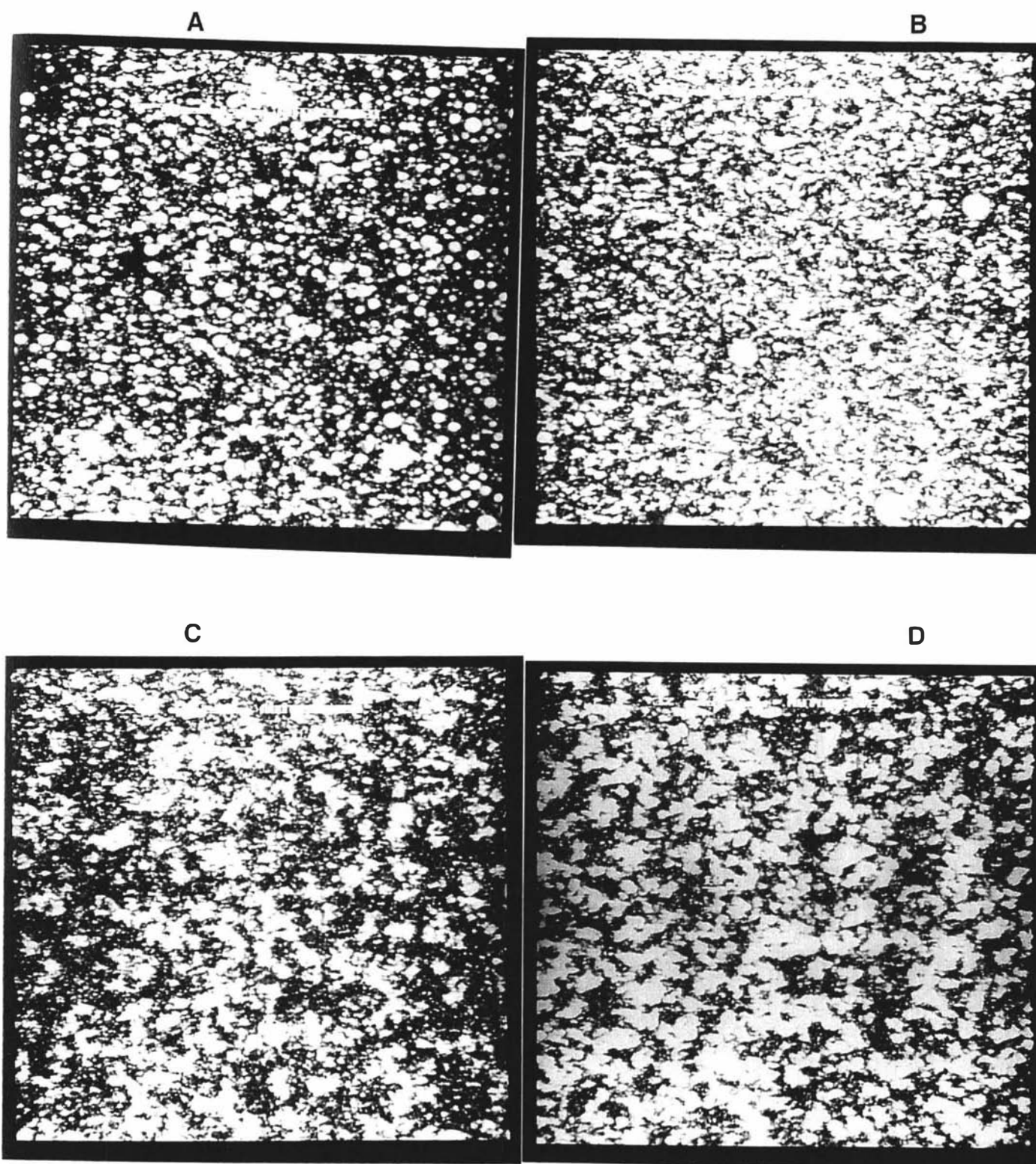| Total protein content (C_{aq}) in aqueous phase (%)	Total protein content in emulsion (C_{tot}) (%)	Sodium caseinate emulsions heated		Emulsions formed with heated sodium caseinate solutions	
		Unadsorbed protein (C_b) (%)	Protein load (Γ) (mg/m^2)	Unadsorbed protein (C_b) (%)	Protein load (Γ) (mg/m^2)
0.5	0.375	0.1884	0.5889	0.2288	0.4693
1.0	0.75	0.4249	1.2019	0.4718	1.0552
2.0	1.5	1.1904	1.7535	1.2168	1.6458
3.0	2.25	1.9523	2.4195	2.1357	2.1221
4.0	3.0	2.8695	2.7499	2.9004	2.6262
5.0	3.75	3.7583	3.0822	3.8298	3.0646

Figure 7.9: Confocal micrographs of heated sodium caseinate emulsions made with: 0.5% (A); 1.0% (B); 2.0% (C); 3.0% (D); 4.0% (E) or 5.0% (F) protein.
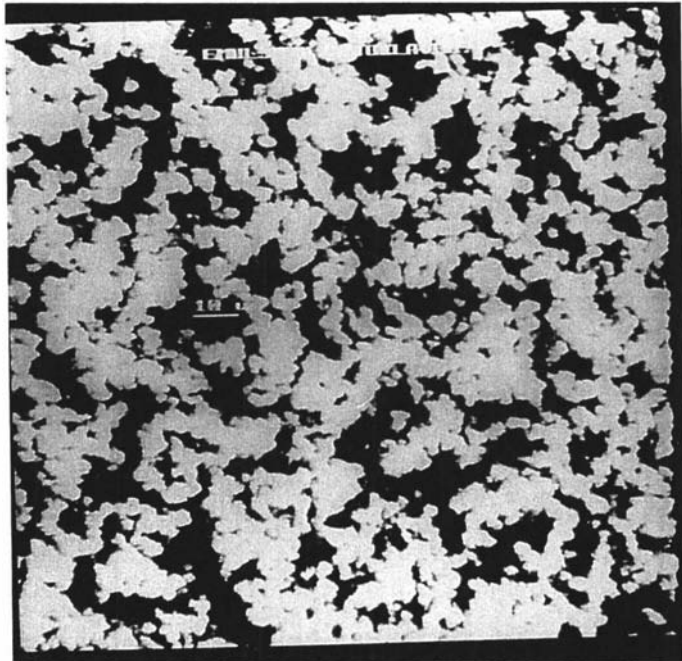
E
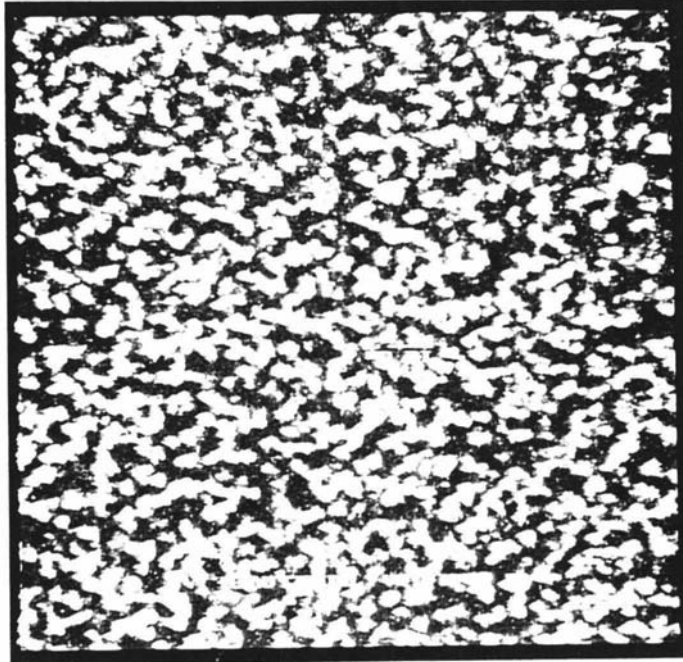
F

Figure 7.9: Continued..
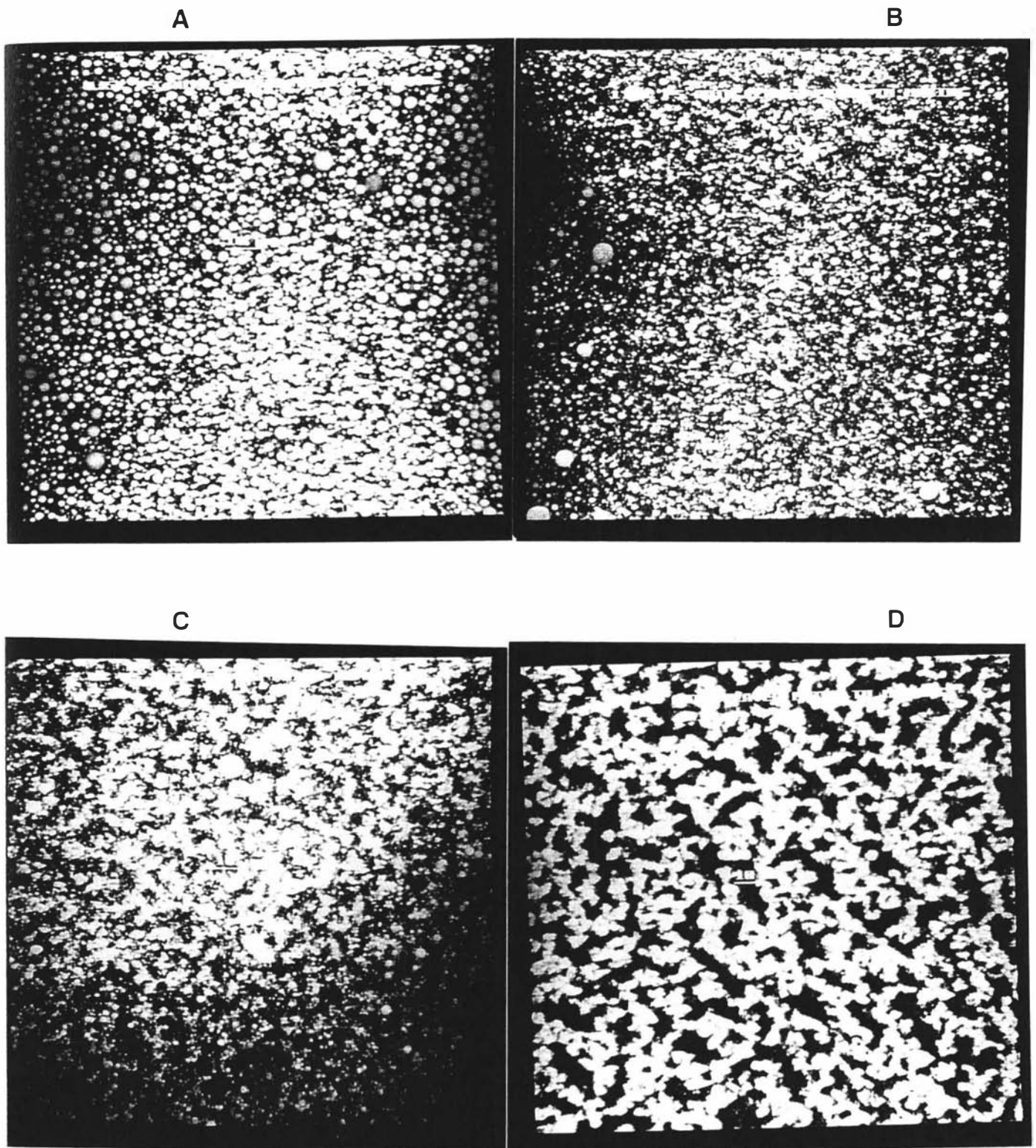
A

B

C

D

Figure 7.10: Confocal micrographs of emulsions formed with heated sodium caseinate: 0.5% (A); 1.0% (B); 2.0 (C); 3.0% (D); 4.0% (E) or 5.0% (F).
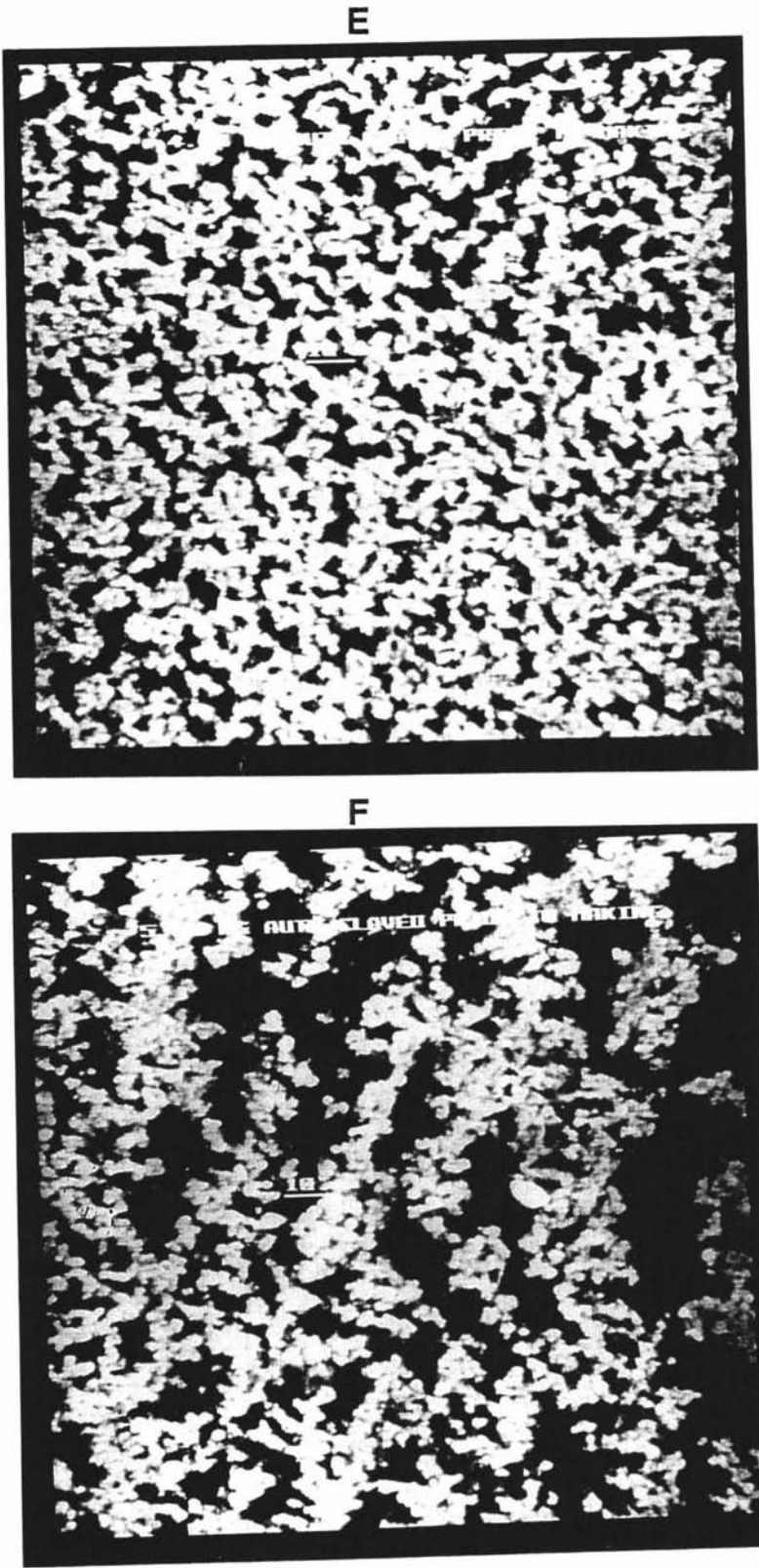
E

F

Figure 7.10: Continued..

7.3.4.3 Calcium caseinate

The changes in the creaming stability of unheated and heated emulsions or emulsions formed with heated calcium caseinate solutions after storage at 20°C for 24 hours are shown in Figure 7.10 B. The stability rating of unheated calcium caseinate emulsions increased rapidly with an increase in protein concentration from 0.5 to 2.0%. An increase in caseinate concentration beyond 2% had no further significant effect on stability rating (refer to Chapter 6).

The creaming profile of heated (121°C for 15 min) calcium caseinate emulsions was different from that of unheated emulsions. The stability rating of heated emulsions increased almost linerarly with an increase in caseinate concentration from 1 to 4%, with no further increase at higher caseinate concentrations. The stability rating values of the heated emulsions were higher than those of the unheated emulsions at caseinate concentrations $\geq$ 3.0%. However, when the caseinate concentration was < 2%, the stability rating values of the unheated emulsions were slightly higher than those of the heated emulsions (Figure 7.10 B).

The creaming profiles of emulsions formed with heated (121°C for 15 min) calcium caseinate solutions were similar except that, at 4 and 5% caseinate concentration, the stability rating values of emulsions formed with heated calcium caseinate solutions were slightly higher than those of the heated emulsions.

Generally, the creaming behaviour of emulsion droplets can be described by Stokes' equation (refer to Chapter 6). The increase in creaming stability in calcium-caseinate-stabilized emulsions with increasing protein concentration can be attributed to a combination of factors, including a decrease in droplet diameter, (Figure 7.4 A) and an increase in viscosity of the continuous phase (refer to Chapter 6).

7.3.4.5 Confocal microscopy

Figures 7.11 A to F show the microstructure of calcium caseinate-stabilized emulsions heated at 121°C for 15 min. Comparing the confocal micrographs of unheated emulsions and heated calcium caseinate emulsions, it appears that the 0.5 and 1.0% unheated emulsions showed more individual oil droplets. However, in heated emulsions at these concentrations, the droplets appeared to flocculate. The extent of flocculation at 0.5% was considerably higher than at 1.0%. This could have been mainly due to bridging flocculation between oil droplets, i.e. sharing of protein molecules/aggregates by two droplets (Vincent, 1973). When the calcium caseinate concentrations in the emulsions were 2.0 and 3.0% (Figures 7.11 C and D), the droplets seemed to be homogeneous and more individual droplets were observed. There appeared to be association of droplets above 4% (Figures 7.11 E and F) caseinate concentration, but this did not seem to affect the stability rating of these emulsions (Figure 7.8 B). At protein concentrations $\geq$ 3.0%, the heated emulsions were found to be more stable than the unheated emulsions and, when the concentrations were $\leq$ 1.0%, the unheated emulsions were more stable than the heated emulsions.

Figures 7. 12 A to F show the confocal micrographs of emulsions formed with heated calcium caseinate solutions. Comparing these micrographs with those of the unheated emulsions, we can say that, when the caseinate concentration was $\geq$ 1.0%, these emulsions appeared to be similar to the unheated emulsions. Emulsions formed with heated calcium caseinate showed more individual oil droplets at concentrations $\geq$ 1.0% (Figures 7.12 B to F), similar to the unheated emulsions. At 0.5% concentration, the emulsions formed with heated calcium caseinate solutions showed some very large droplets and less individual droplets. There were more flocs observed at this concentration and hence the stability was low.

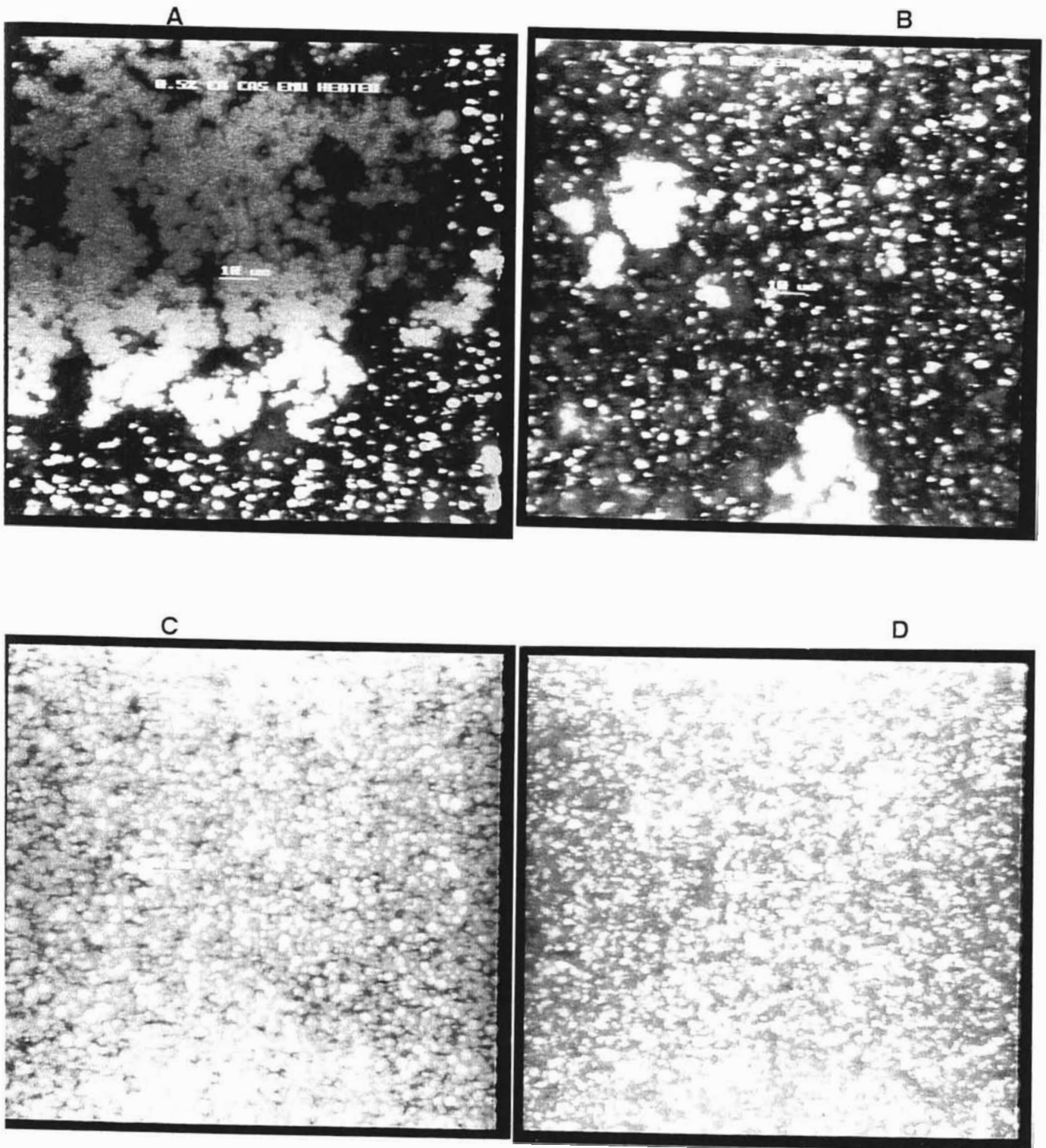**Figure 7.11**: Confocal micrographs of heated calcium caseinate emulsions formed with 0.5 % (A); 1.0% (B); 2.0% (C); 3.0% (D); 4.0% (E) or 5.0% (F) protein.

E

F
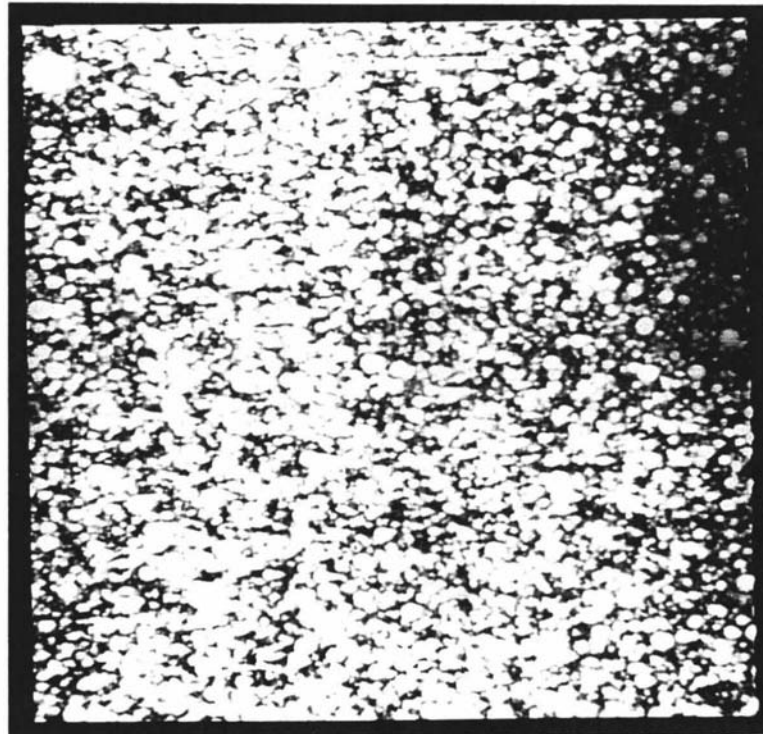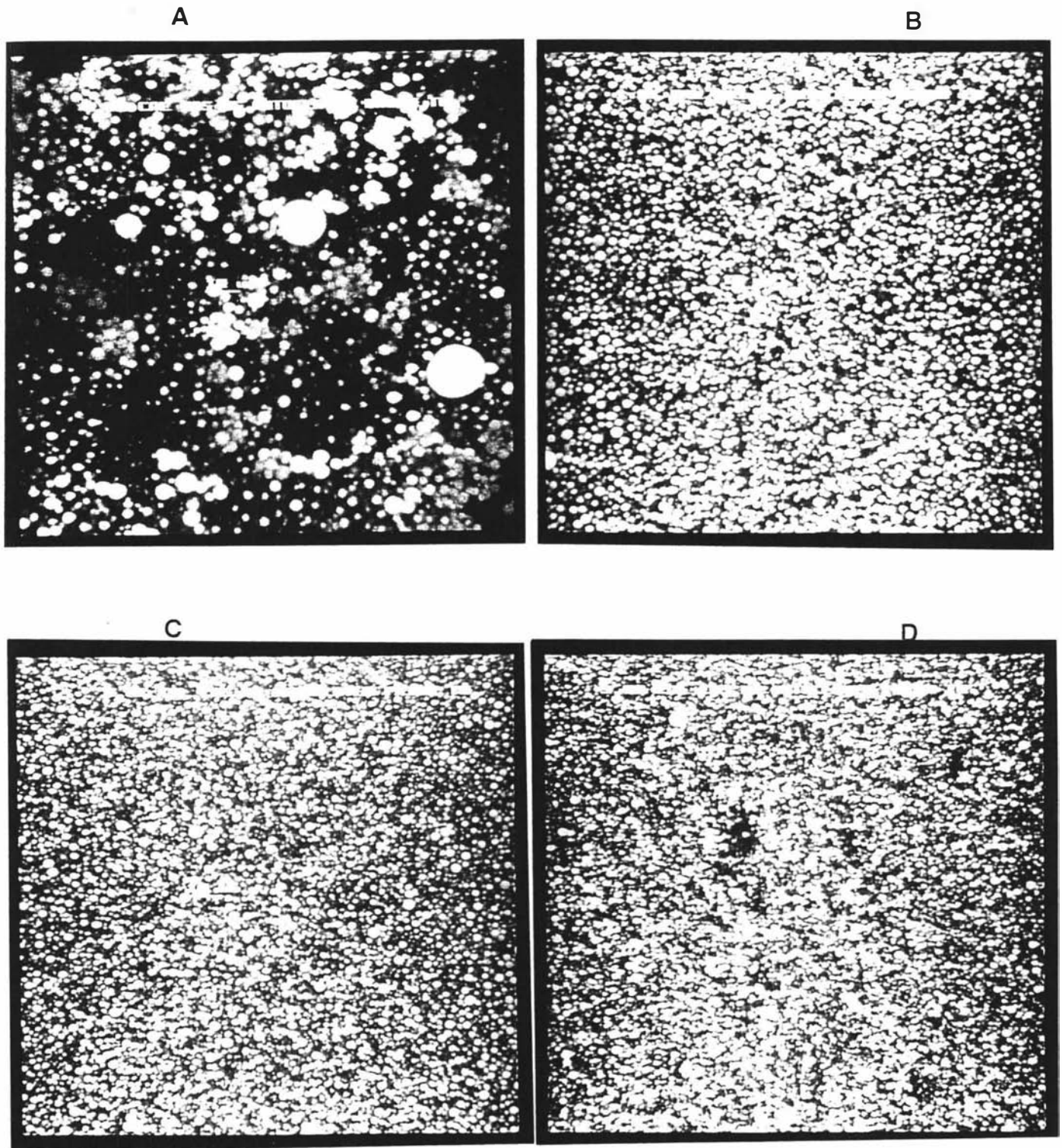

Figure 7.11: Continued..

Figure 7.12: Confocal micrographs of emulsions formed with heated calcium caseinate solutions.0.5 % (A); 1.0% (B); 2.0 (C); 3.0% (D); 4.0% (E) or 5.0% (F).
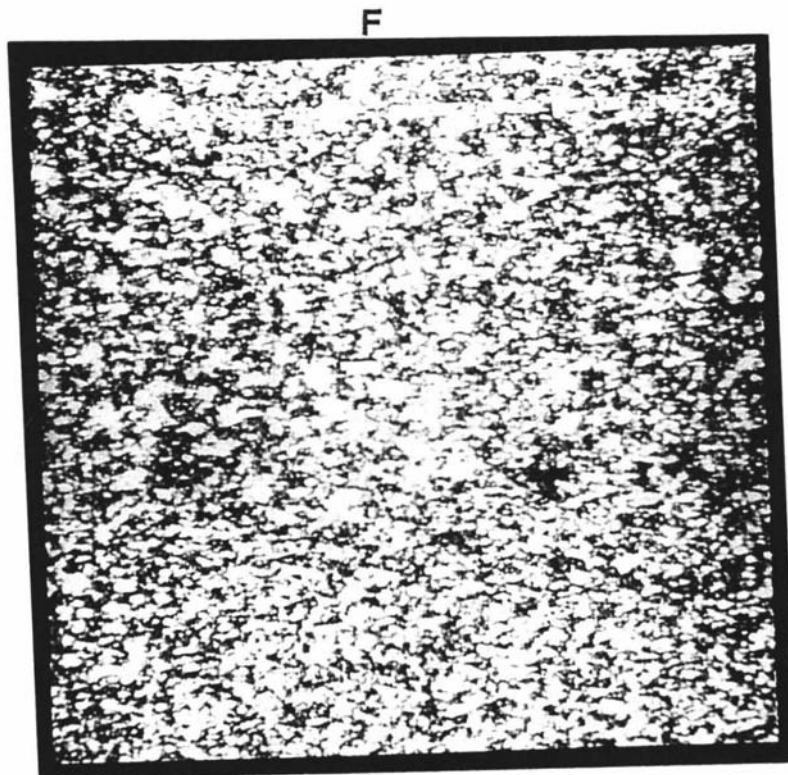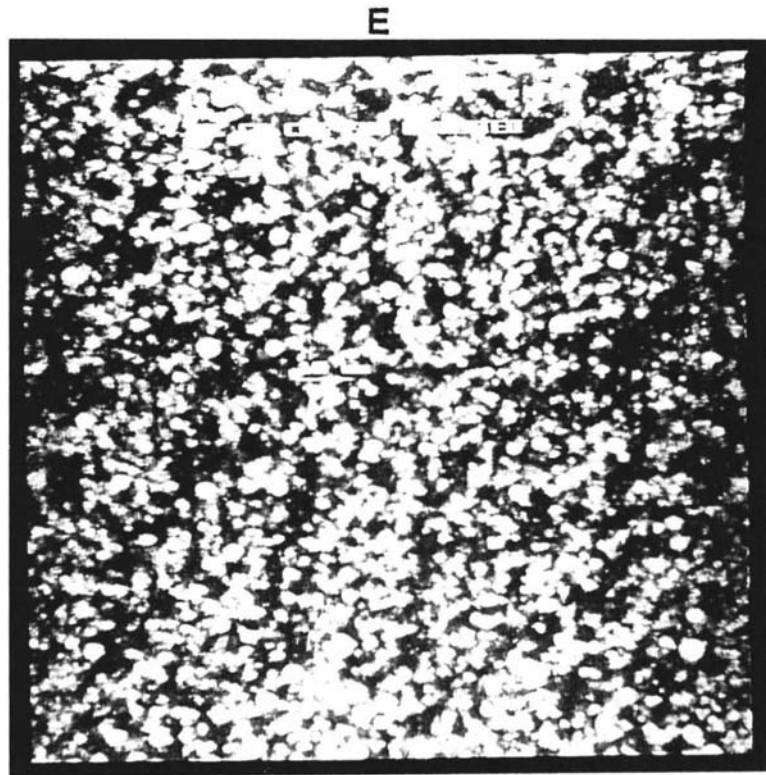
E

F

Figure 7.12: Continued..

Chapter 8

THE EFFECT OF ADDITION OF NaCl ON THE FORMATION

AND STABILITY OF CASEINATE EMULSIONS

8.1 Introduction

Milk proteins, especially caseinates, are good emulsifiers and hence are used as ingredients in a wide range of formulated food emulsions. An important factor to consider during the manufacture of food emulsions is that different concentrations of salts are added for various food products, thus it is important to understand whether the interfacial composition of emulsions is altered and whether this has any effect on the stability of the emulsions. Several studies have been reported on the adsorption behaviour of pure caseins and casein mixtures, such as sodium caseinate, in model emulsions (Tomberg, 1978a,b; Dickinson *et al.,* 1988a,b; Robson and Dalgleish, 1987; Hunt and Dalgleish, 1994; Srinivasan *et al.,* 1996).

Although it is known that high ionic strength may destabilize an emulsion, much less information is available on the effects of ionic strength on the adsorption of proteins in emulsions. Hunt and Dalgleish (1996) reported the effect of KCl on the adsorption behaviour of caseinate and whey proteins. They have reported that increasing the concentration of KCl beyond 25 mM affected the droplet diameter and surface composition. In emulsions containing 20% soya oil and 2% caseinate, α_{s1}-casein increased at the expense of β-casein when the KCl concentration was increased up to 200mM. The study by Hunt and Dalgleish (1996) was done at single caseinate concentration (2%). Srinivasan *et al.* (1996) reported that the total protein content affects the competitive adsorption: β-casein was preferentially adsorbed in emulsions made at relatively low levels of sodium caseinate, but opposite is the case in emulsions containing substantial levels of unadsorbed caseinate. It would be of interest to study the effect of ionic strength on these emulsions at different protein concentrations, particularly at 1 and 3%.

Results shown in Chapter 6 show that emulsions made with higher protein concentrations were found to be more unstable than emulsions made with low protein concentrations. The instability was explained as so called 'depletion flocculation' which occurred due to the increase in the concentration of unadsorbed protein. The effect of ionic strength on the creaming stability is also not known. It is important to know the effect of NaCl on the extent of depletion flocculation. Most of the previous reports on the interfacial properties have focused on sodium caseinate (Tomberg, 1978a,b, Robson and Dalgleish, 1987; Fang and Dalgleish, 1993; Hunt and Dalgleish, 1994, 1996).

From Chapter 4 we know that calcium caseinate in solution consists of a diverse range of casein aggregates and particles. The results in Chapter 5 showed that in emulsions formed with calcium caseinate, the surface protein coverage increased almost linearly with increase in protein concentration and α_s-casein was adsorbed in preference to other caseins at all concentrations. We also know from Chapter 6 that sodium caseinate emulsions exhibit depletion flocculation at higher protein concentrations. It is likely that addition of NaCl to calcium caseinate would decrease the binding of calcium caseinate, resulting in dissociation of calcium caseinate in to smaller aggregates or complexes. In other words, calcium caseinate may be atleast partly converted in to sodium caseinate after addition of NaCl. This would influence the adsorption behaviour of calcium caseinate during emulsion formation as well as the creaming stability of emulsions. No previous work has been reported on the effect of NaCl on adsorption behaviour and stability of calcium caseinate emulsions. Therfore, the objective was to study the effects of changing concentration of NaCl on the adsorption behaviour and creaming stability of calcium caseinate-stabilized emulsions.

This chapter is divided into two parts. **Part A** describes the influence of NaCl on the adsorption behaviour, creaming stability and microstructure of sodium caseinate-stabilized emulsions, and **Part B** describes the influence of NaCl on the formation and stability of calcium-caseinate-stabilized emulsions.

Part A: **The effect of addition of NaCl on the formation and stability of sodium caseinate emulsions.**

8.2 Results

8.2.1 Emulsion formation

Emulsions (30% soya oil) were made with 1 or 3% sodium caseinate. Various concentrations of NaCl were added into the caseinate solution prior to making the emulsions or to emulsions made in the absence of NaCl.

8.2.2 Particle size distribution and droplet diameter

The effect of the presence of NaCl during emulsion formation and addition of NaCl to the emulsion prepared in the absence of NaCl on the average diameter (d_{32}) of emulsion droplets, made with 1.0 or 3.0% sodium caseinate at pH 7.0, is shown in Figure 8.1 A and B. For both emulsions, the presence of NaCl at concentrations in the range of 0 to 1000 mM did not affect the size distribution of the emulsion droplets and the d_{32} remained constant at close to ~ 0.58 µm for emulsions made with 1% sodium caseinate and ~ 0.55 µm for emulsions made with 3% sodium caseinate. Similar results were found when NaCl was added to emulsions after they were formed.

These results are essentially in agreement with those obtained by Hunt and Dalgleish (1996) who reported that the addition of 0 - 200 mM KCl to caseinate emulsions, prior to emulsification, had no influence on the particle size distribution and the weight-average diameter.

8.2.3 Surface protein coverage

The changes in the surface coverage in emulsions made with 1% sodium caseinate in the presence of NaCl are shown in Figure 8.2 A.

As the NaCl concentration was increased from 0 to 40 mM, the surface coverage increased markedly from 1.1 to 1.7 mg/m², but increasing the NaCl concentration beyond 40 mM changed the surface coverage only slightly. When NaCl was added to the emulsions made with 1% sodium caseinate, the
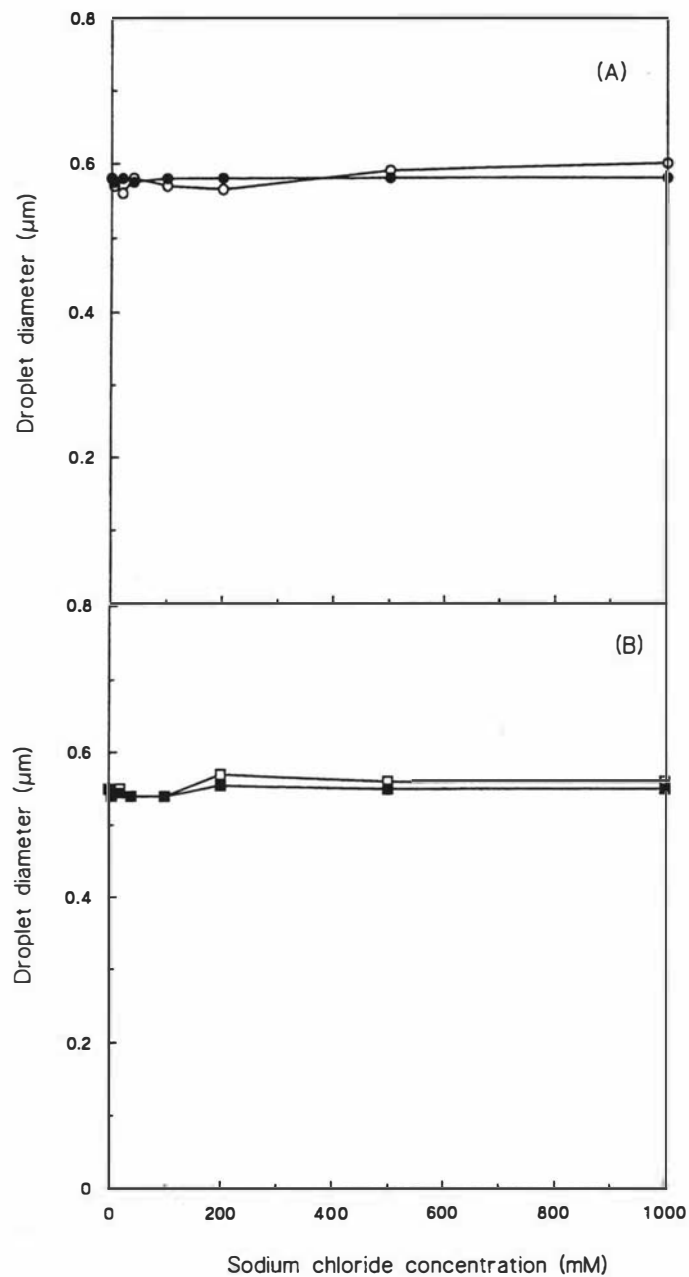
Figure 8.1: Changes in average droplet diameter, d_{32} (volume-to-surface) as a function of NaCl concentration in emulsions made using 30% soya oil and 1% sodium caseinate (A) (NaCl added before (○) or after (●) emulsion formation) or 3% sodium caseinate (B) (NaCl added before (□) or after (■) emulsion formation).
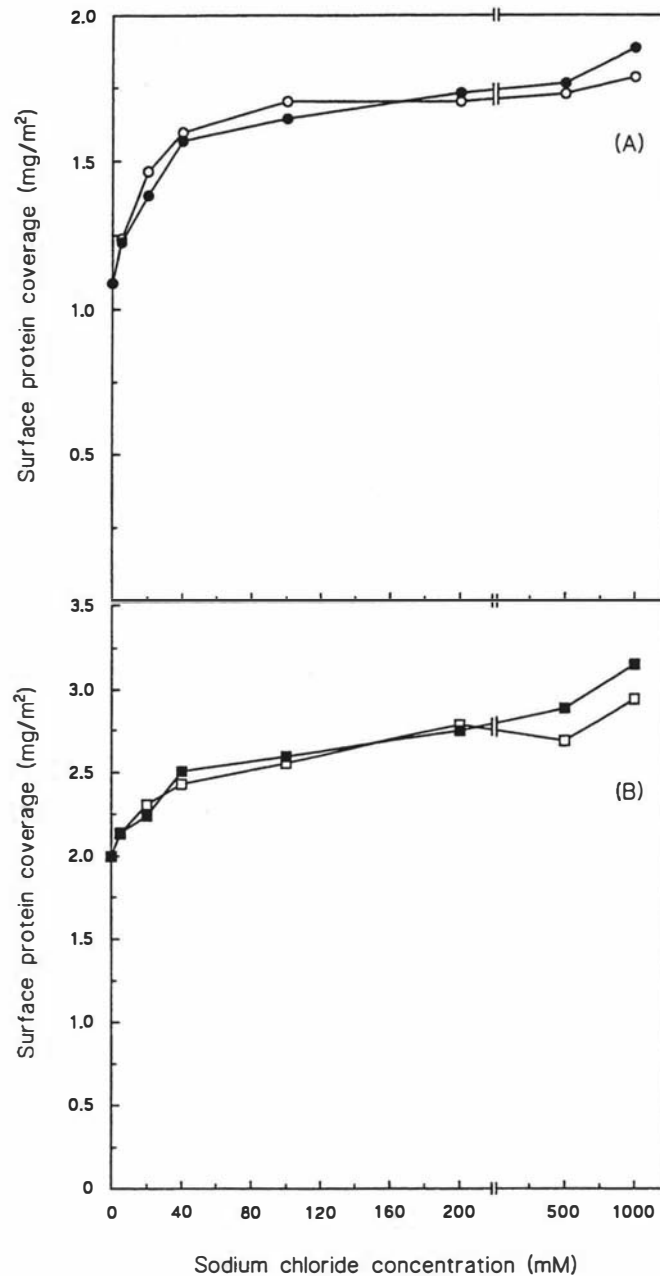
Figure 8.2: Changes in surface protein coverage (mg/m^2) as a function of
NaCl concentration of emulsions (30% oil) stabilized by 1%
sodium caseinate (A)   (NaCl added before (o) or after (•)
emulsion formation) or 3% sodium caseinate (B) (NaCl added
before (□) or after (■) emulsion formation). Each data represents
average of two separate experiments.

changes in the surface coverage were similar to those in the emulsions made in the presence of NaCl (Figure 8.2 A). However, at 1000 mM the surface coverage in emulsions made in the presence of NaCl was slightly lower compared with when the same concentration of NaCl was added to the emulsion after it was made.

Figure 8.2 B represents the changes in the surface coverage in emulsions made with 3% sodium caseinate in the presence of various concentrations of NaCl. The effect of the addition of NaCl to emulsions after they were made is also shown in Figure 8.2 B. An increase in NaCl concentration from 0 to 40 mM resulted in an increase in surface coverage, but with further increase in NaCl concentration from 200 to 1000 mM, the surface coverage increased only slightly. This increase in surface coverage was also observed in emulsions in which NaCl was added after emulsion formation. The values obtained in both cases, i.e. emulsions made in the presence NaCl and NaCl added to emulsions, were found to be almost identical. The surface coverage values found in the absence of NaCl were similar to those reported in Chapter 5 and by Srinivasan *et al.* (1996) and Euston *et al.* (1995).

8.2.4 Composition of caseins at the oil/water interface (cream phase)

8.2.4.1 Emulsions made with 1% caseinate

In emulsions made in the absence of NaCl (1% protein), the proportions of adsorbed caseins were: α_s-casein, $(\alpha_{s1}- + \alpha_{s2}-) \sim 28\%$, β-casein $\sim 49\%$ and κ-casein $\sim 22\%$ as compared with the proportions in the original caseinate solution (α_s- 42%, β- 39% and κ-casein 19%). This suggests that under these conditions β-casein was adsorbed in preference to α_s-casein (see also Chapter 5). As the NaCl concentration was increased from 0 to 40 mM prior to emulsion formation, the relative proportion of α_s-casein ($\alpha_{s1}- + \alpha_{s2}$-casein) increased from ~ 28 to $\sim 43\%$ (Figure 8.3 A and B). In the case of emulsions in which NaCl was added after they were made, the proportion of α_s-casein increased from $\sim 28\%$ to $\sim 46\%$ as the NaCl concentration was increased from 0 to 40 mM. There were corresponding decreases in the proportions of adsorbed β- and κ-

caseins, with β-casein showing a greater decrease. Addition of NaCl from 40 to 1000 mM did not significantly further affect the interfacial composition of the emulsion droplets.

8.2.4.2 Emulsions made with 3% caseinate

The interfacial compositions of emulsions made with 3.0% sodium caseinate in the absence of NaCl solutions was different (Figure 8.4 A and B) from that of emulsions made with 1% sodium caseinate in the absence of NaCl. Increasing the concentration of NaCl prior to emulsion formation affected the proportions of adsorbed caseins. The proportions of both α_s- and β- caseins adsorbed at the interface increased at the expense of κ-casein as the concentration of NaCl was increased up to 500 mM. An increase in the concentration of NaCl to 1000 mM caused only slight further changes. In the case of emulsions in which NaCl was added after they were formed, similar results were obtained. However, β-casein showed a greater increase whereas α_s-casein showed a slight decrease as the NaCl concentration was increased from 200 to 1000 mM. Comparison of the results presented in Figure 8.4 with those in Figure 8.3 indicated that the surface composition of the emulsion droplets in emulsions made with 1% sodium caseinate was affected differently by the presence of NaCl from that in emulsions made with 3.0% sodium caseinate.

Figure 8.3: Effect of concentration of NaCl on the relative proportions of α_s-casein (•), β-casein (▲) and κ-casein (▼) at the droplet surface (cream phase) of emulsions formed with 30% soya oil and 1% sodium caseinate. NaCl added before (A) or after (B) emulsion formation. Each data represents average of two separate experiments.
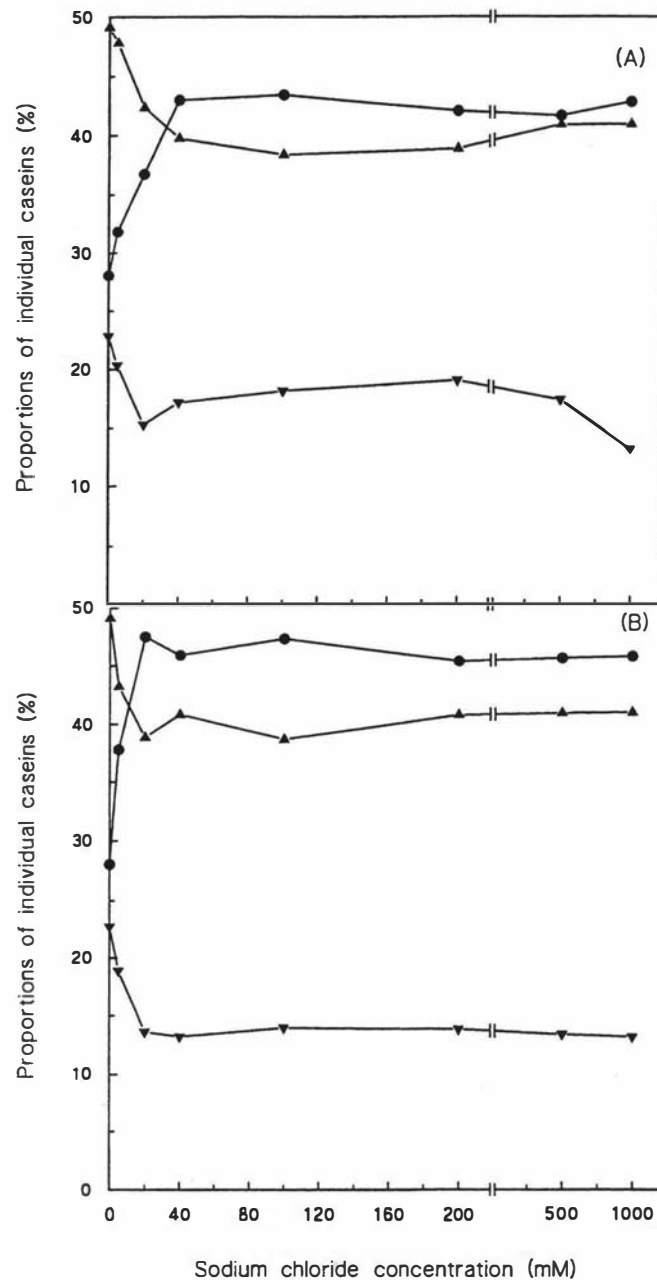
Figure 8.4: Effect of concentration of NaCl on the relative proportions of α_s-casein (•), β-casein (▲) and κ-casein (▼) at the droplet surface (cream phase) of emulsions formed with 30% soya oil and 3% sodium caseinate. NaCl added before (A) or after (B) emulsion formation. Each data represents average of two separate experiments.
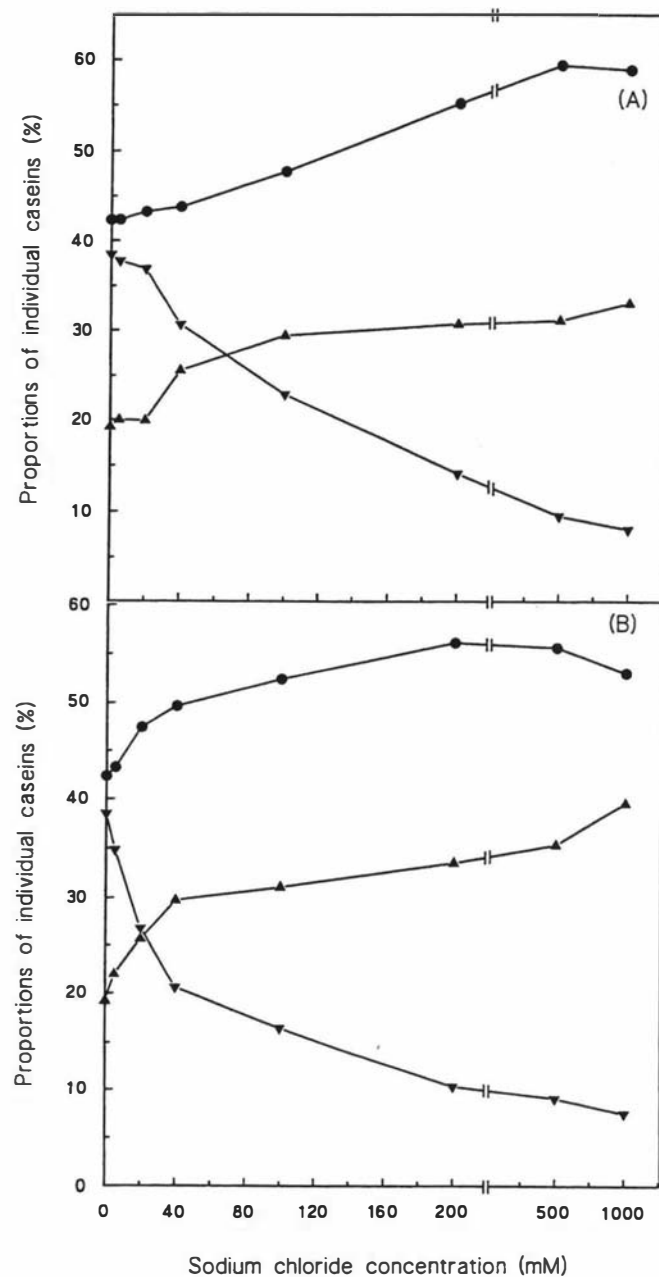
Hunt and Dalgleish (1996) reported that the presence of KCl in emulsions made with 2% (w/w) caseinate and 20% soya oil affected the surface composition of emulsion droplets. They found that adsorbed α_{s1}-casein increased at the expense of β-casein when > 25 mM KCl was present in the emulsion. The percentages of κ- and α_{s2}-caseins at the interface remained constant with further increase in KCl concentration. The presence of NaCl in the present study appeared to cause changes in the surface composition at much lower concentrations (5 mM) in the emulsions stabilized with 1% sodium caseinate.

8.2.5 Surface concentration

The surface concentrations of individual caseins in various emulsions were estimated and the results are shown in Figure 8.5 A and B. In emulsions made with 1% sodium caseinate, the surface concentration of κ-casein essentially remained constant with an increase in NaCl concentration in the emulsion mixture (Figure 8.5 A). The surface concentration of β-casein increased from ~ 0.53 to ~ 0.63 mg/m^2 as the NaCl concentration was increased from 0 to 40 mM, but further increase in NaCl had very little further effect. When the concentration of NaCl was increased from 0 to 40 mM, the surface concentration of α_s-casein (α_{s1}- + α_{s2}-casein) showed a marked increase from 0.30 to ~ 0.69 mg/m^2. However, increasing the concentration of NaCl above 40 mM did not further affect the surface concentration of α_s-casein. Similar trends were found when the same concentrations of NaCl were added to the emulsions after they were formed (Figure 8.5 B).

In emulsions made with 3% sodium caseinate in the presence of NaCl, the surface concentration of κ-casein decreased as the concentration of NaCl was increased from 0 to 1000 mM (Figure 8.6 A). In contrast, the surface concentrations of α_{s1}- and β-casein increased markedly with an increase in NaCl concentration. Similar changes in the surface concentrations of α_s-, β- and κ-caseins were observed when NaCl was added after emulsion formation (Figure 8.6 B). The increase in the surface concentrations of α_{s1}- and β-casein
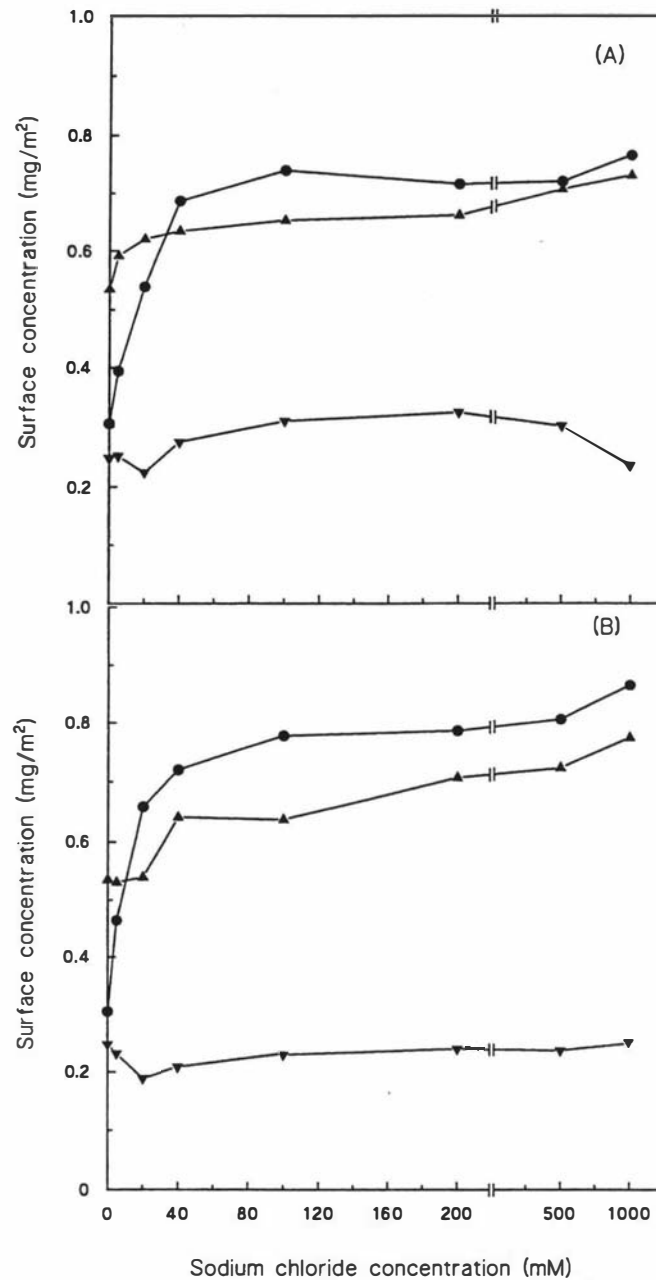
Figure 8.5: Changes in estimated surface concentrations (mg/m^2) of α_s-casein (α_{s1} +α_{s2}) (•), β-casein (▲) and κ-casein (▼) as a function of NaCl concentration in emulsions (30% oil) formed with 1% sodium caseinate. NaCl added before (A) or after (B) emulsion formation.

Figure 8.6:   Changes in estimated surface concentrations (mg/m^2) of α_s-
casein (α_{s1}- + α_{s2}-) (•), β-casein (▲) and κ-casein (▼) as a function
of NaCl concentration in emulsions (30% oil) formed with 3%
sodium caseinate. NaCl added before (A) or after (B) emulsion
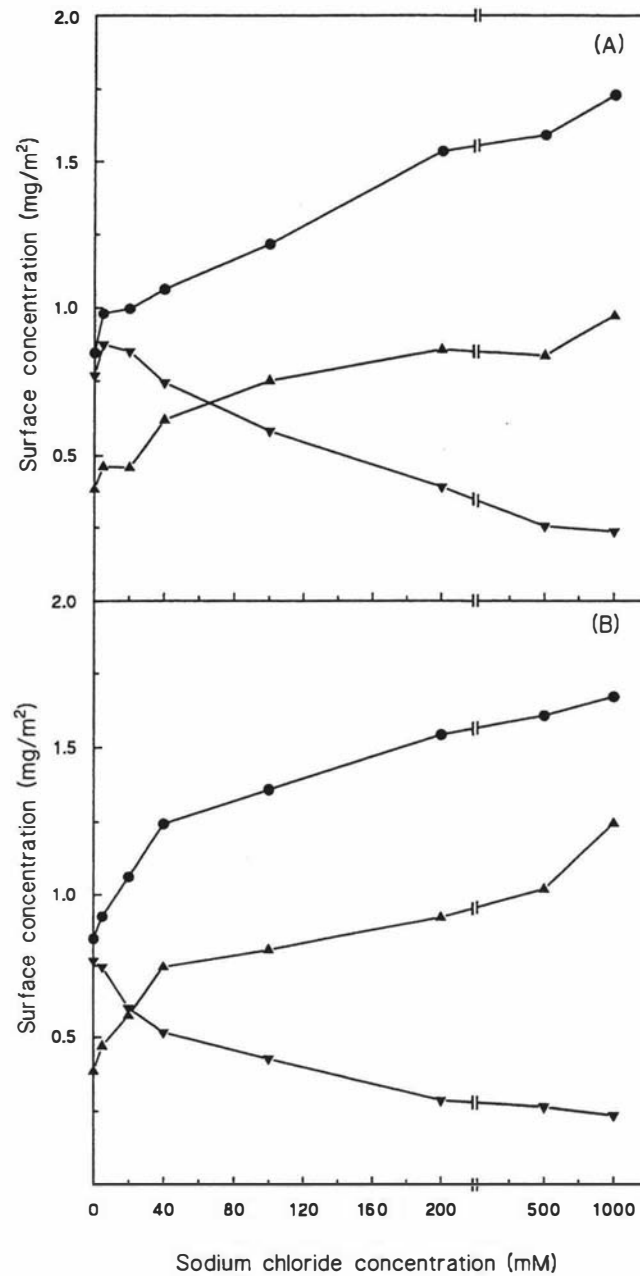formation.

followed a similar trend to that of the surface coverage (Figure 8.2).

8.2.6 Stability of emulsions

The creaming stability of emulsions made with 1.0% sodium caseinate was markedly influenced by the addition of NaCl (Figure 8.7 A). The presence of NaCl up to 40 mM caused an increase in stability rating from ~ 40% to ~ 60%. With further addition of NaCl up to 500 mM, the stability rating showed no further change. However, above 500 mM NaCl, the stability rating decreased slightly. Addition of NaCl to emulsions after they were formed resulted in similar changes in the stability rating (Figure 8.7 A).

The stability rating was dramatically affected by the addition of NaCl in the case of emulsions made with 3% sodium caseinate. As the NaCl concentration was increased from 0 to 200 mM, the stability rating increased from ~ 5% to ~ 58% in the case of emulsions made in the presence of NaCl and from ~ 5% to ~ 63% in the case emulsions in which NaCl was added after they were made. Beyond 200 mM NaCl, the stability rating showed no significant further change in both cases.

8.2.6.1 Microstructure

The microstructure of these emulsions was examined using confocal microscopy. From the confocal micrographs, it is clear that the addition of NaCl before or after emulsification to the emulsions made with 1.0% sodium caseinate had no effect on the appearance of emulsion droplets (Figure 8.8 A and B). The emulsions appeared to be homogeneous with no sign of flocculation.

The emulsions made with 3% sodium caseinate, in the absence of NaCl, showed significant flocculation of oil droplets. These flocs were almost forming a network structure. The presence of such flocculation was responsible for the enhanced creaming observed (under gravity) for these emulsions upon storage for 24 hours (Figure 8.7 B), in agreement with the results presented in
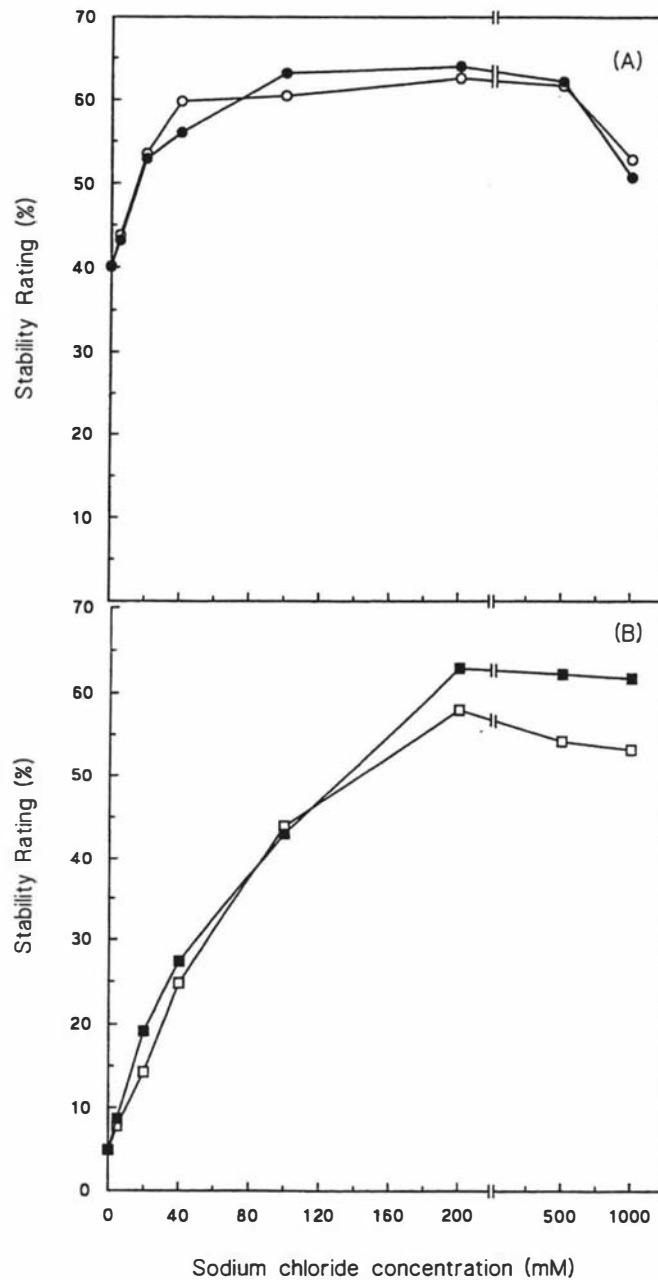
Figure 8.7: Changes in the stability rating (%) as a function of NaCl concentration in emulsions (30% oil) formed with 1% (A) or 3% (B) sodium caseinate. NaCl was added before (○, □) or after (●, ■) emulsion formation. Each data represents   average of two separate experiments.
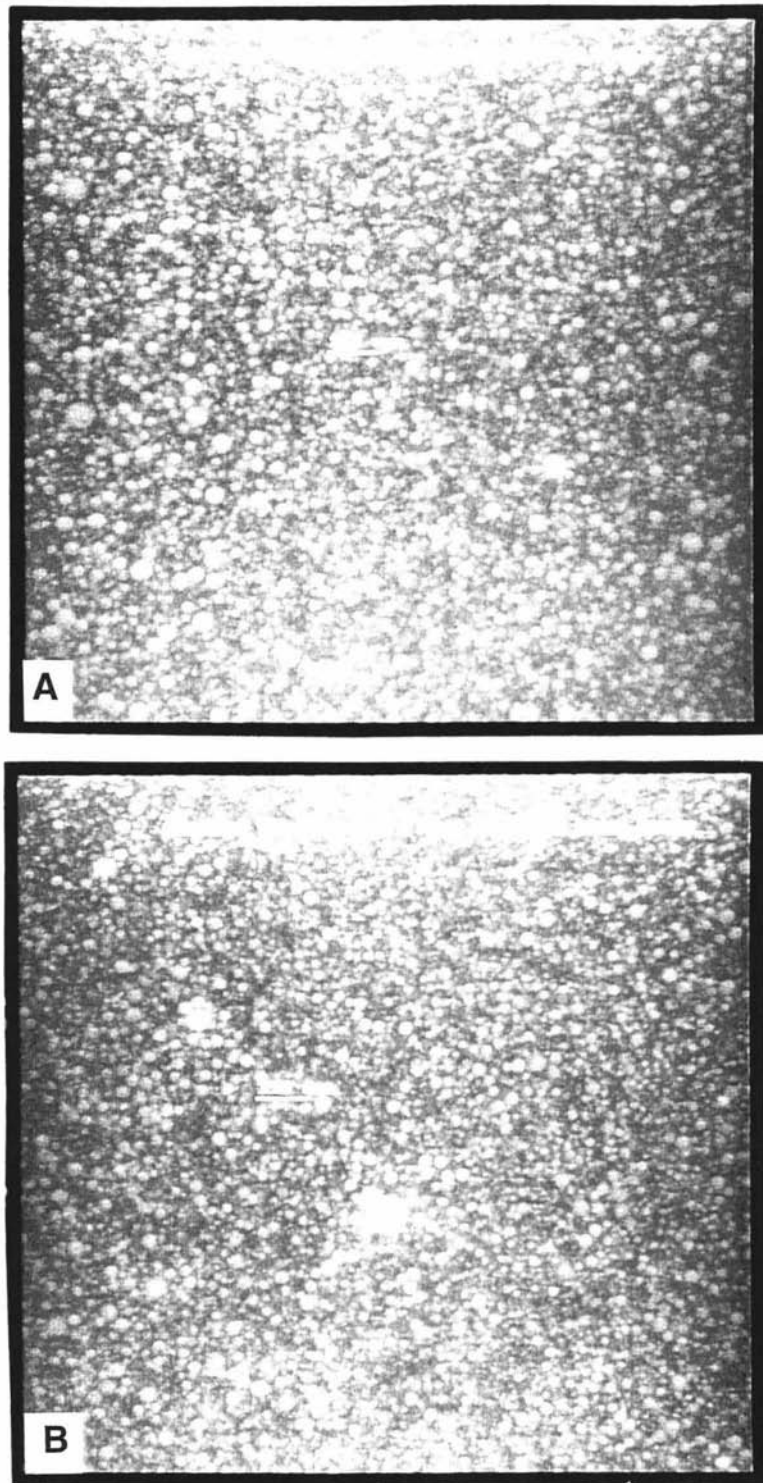
Figure 8.8:   Confocal micrographs of 1% sodium caseinate emulsions made in the presence of NaCl. Emulsions containing: no NaCl (A); 500 mM NaCl (B). Emulsions appear homogeneous with no sign of flocculation.

Chapter 6. The extent of flocculation and network formation, observed in Figure 8.9 A, appeared to diminish gradually with increasing NaCl concentration from 0 to 200 mM (Figure 8.9 B - H). At 200 mM and above, there was no network formation and the emulsions appeared to be relatively homogeneous with the droplets being well separated from each other.

Figure 8.9: Confocal micrographs of 3% sodium caseinate emulsions made in the presence of NaCl. Emulsions containing: no NaCl (A); 5 mM (B); 20 mM (C); 40 mM (D); 100 mM (E); 200 mM (F); 500 mM (G) or 1000 mM (H).

Figure 8.9:   Continued...

8.3   Effect of NaCl on the adsorption behaviour of caseins in emulsions made with various protein concentrations

From the above experiments, it is clear that the emulsifying properties of sodium caseinate are affected by the addition of NaCl before or after emulsification. M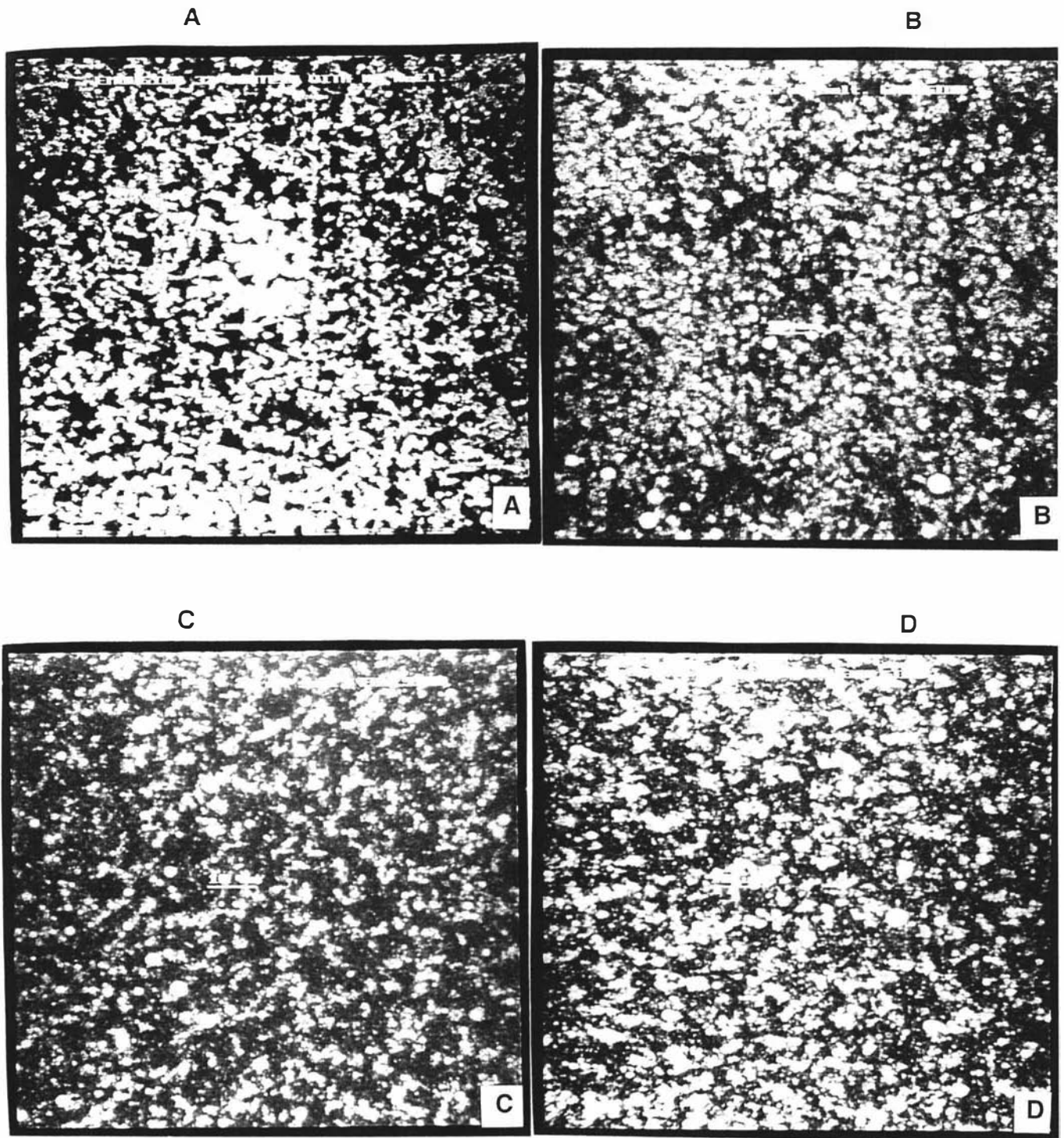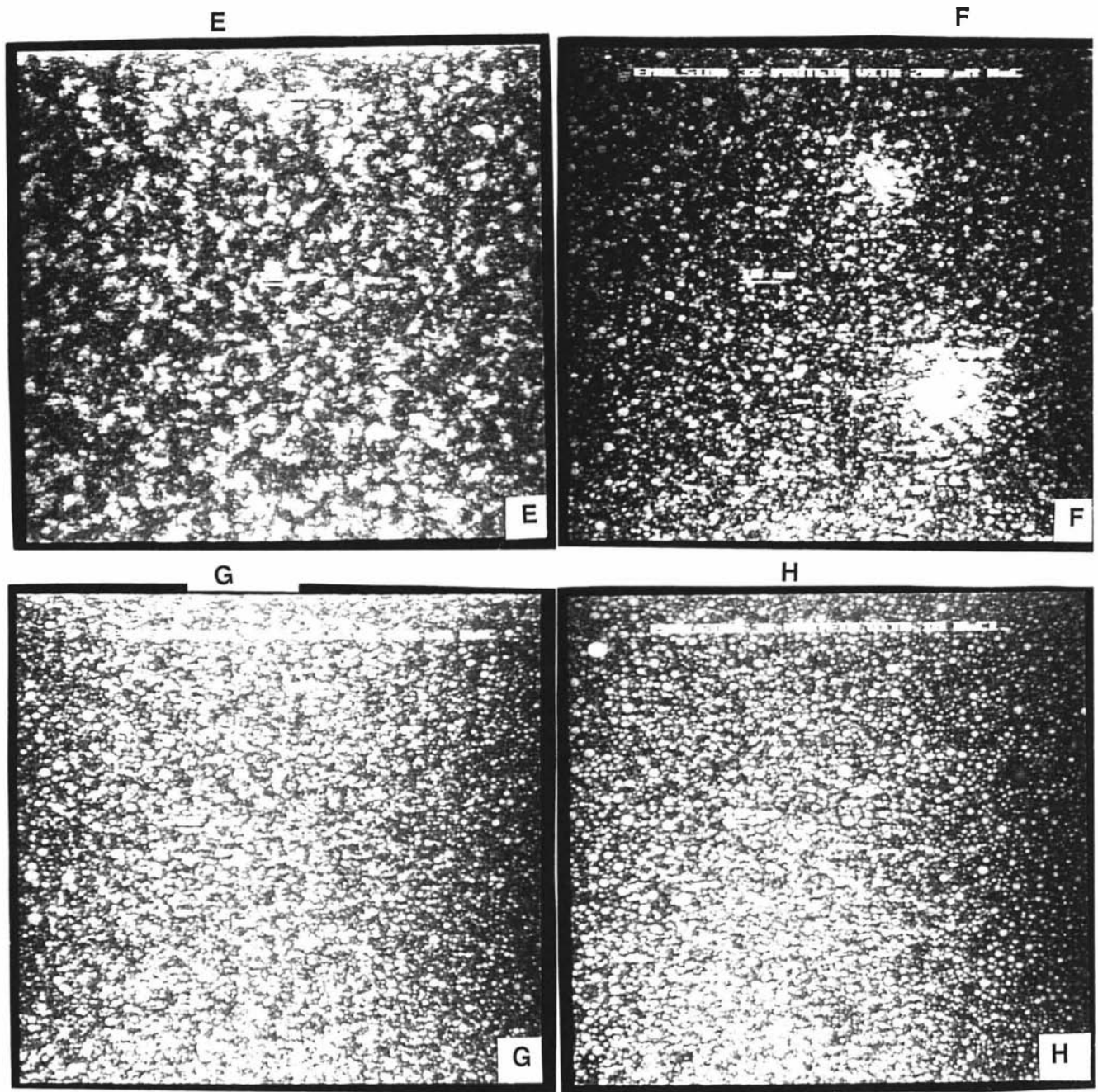ost of the changes in the adsorption behaviour of the caseins and the creaming stability of the emulsions occurred when the NaCl concentration was in the range 5 - 200 mM. Further experiments were carried out to determine how the adsorption behaviour and creaming stability were affected by various caseinate concentrations in the presence of 200 mM NaCl.

8.3.1 Droplet diameter

Figure 8.10 shows the changes in the droplet diameter as a function of caseinate concentration in emulsions made with and without 200 mM NaCl. The emulsions made in the presence of NaCl showed a slight decrease in the volume-surface diameter (d_{32}) as the concentration of protein was increased from 0.5 to 1.0%. With further increase in caseinate concentration, the droplet diameter showed no further change. A similar trend was observed when the emulsions were made in the absence of NaCl. The droplet diameters of all emulsions made in the presence of NaCl were slightly higher than for those made without NaCl.

8.3.2 Surface protein coverage

The surface coverage in emulsions made in the presence of 200 mM NaCl gradually increased from ~ 1.0 to ~ 3.2 mg/m² as the protein concentration was increased from 0.5 to 5.0% (Figure 8.11). In the case of emulsions made in the absence of NaCl, the surface coverage also showed an increase with an increase in protein concentration. At all concentrations, the surface coverage in emulsions, made in the presence of 200 mM NaCl, was higher than in the emulsions made in the absence of NaCl. At 0.5% protein, the surface coverage in emulsions made in the presence of NaCl was ~ 40% higher than in emulsions made in the absence of NaCl. At all other concentrations, there was ~ 60% increase in the surface coverage of emulsions made in the presence of
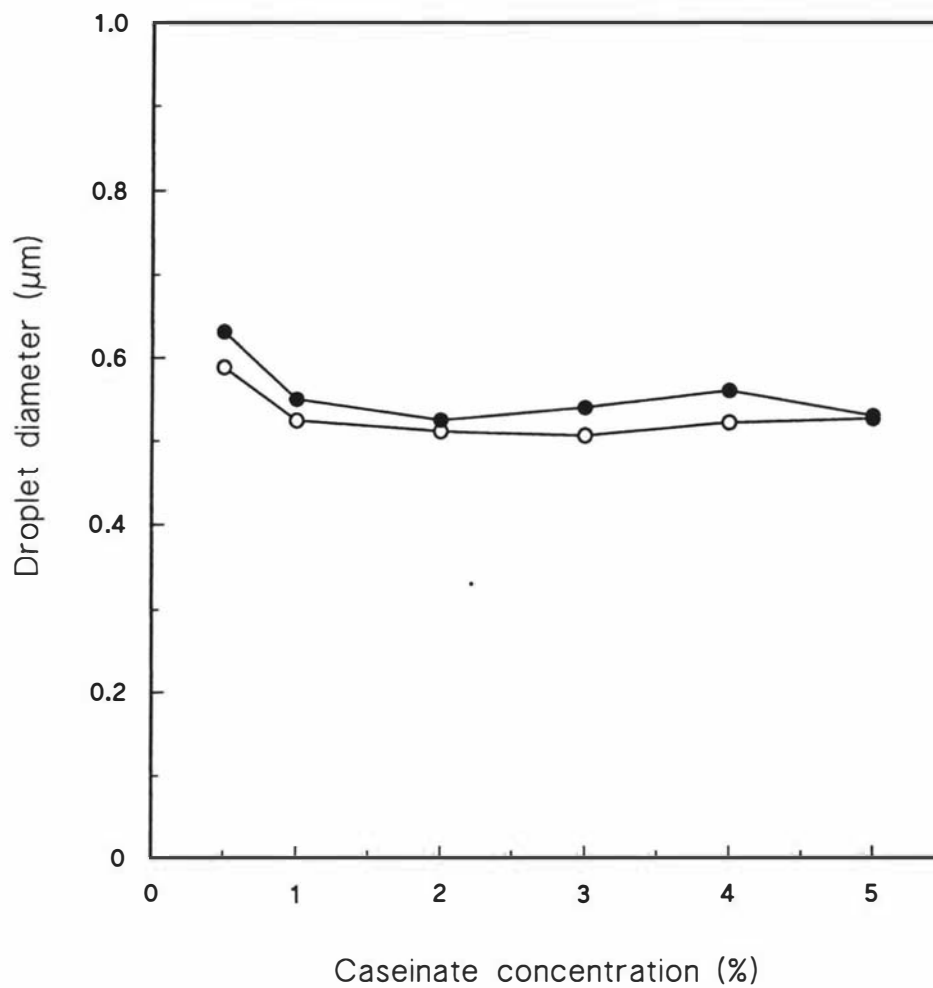
Figure 8.10: Changes in average droplet diameter, d_{32} (volume-to-surface) as a function of protein concentration in emulsions made using 30% soya oil and various amounts of sodium caseinate without added NaCl (○) or with 200 mM NaCl added prior to emulsion formation (●).

Figure 8.11: Changes in surface protein coverage (mg/m²) as a function of
protein concentration in emulsions made using 30% soya oil and
various amounts of sodium caseinate without added NaCl (○) or
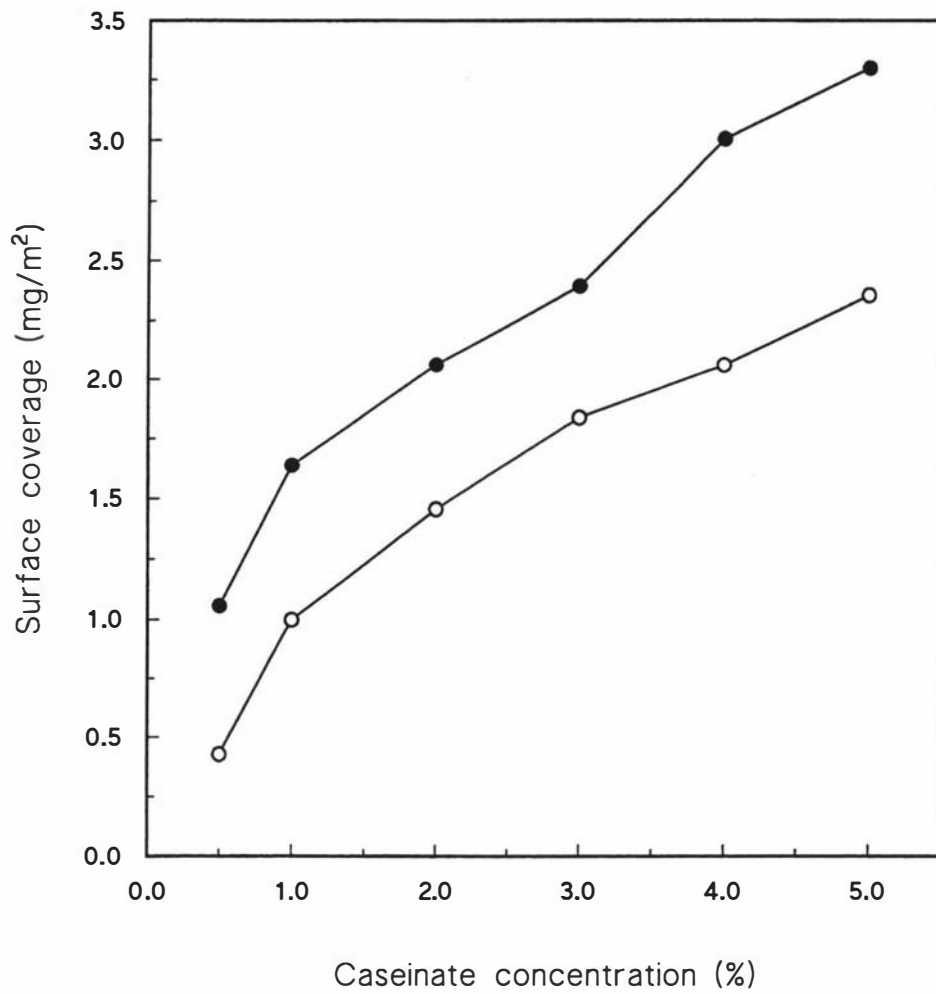with 200 mM NaCl added prior to emulsion formation (•).

200 mM NaCl compared with the emulsions made in the absence of NaCl.

8.3.3 Composition of caseins at the oil/water interface (cream phase)

The changes in the proportions of individual caseins at the oil/water interface of sodium caseinate-stabilized emulsions in the presence or absence of 200 mM NaCl are shown in Figure 8.12. The proportion of κ-casein showed no change with increasing protein concentration in the emulsions made in the presence of 200 mM NaCl. At 0.5% protein, the proportion of β-casein was slightly higher than that of α_s-casein (α_{s1}- + α_{s2}-) suggesting that β-casein was preferentially adsorbed under these conditions. In emulsions made with 1.0% sodium caseinate in the presence of 200 mM NaCl, the proportions of α_s- and β-caseins were almost similar, indicating that there was no preferential adsorption under these conditions. As the concentration of protein was increased beyond 2.0% the proportion of α_s-casein at the droplet surface increased whereas that of β-casein decreased. These results indicate that, in emulsions made with sodium caseinate above 2.0% in the presence of NaCl, α_s-casein was adsorbed in preference to β-casein.

Comparison of the results obtained for emulsions made in the presence of NaCl with those obtained for emulsions made without NaCl (Figure 8.12) showed that the proportions of κ-casein were lower in NaCl containing emulsions at all protein concentrations. At 0.5% protein, the proportion of β-casein in the emulsions made in the presence of 200 mM NaCl was found to be almost identical to the proportion of β-casein in the emulsions made in the absence of NaCl (Figure 8.12). When the caseinate concentration was ≤ 2.0% the proportion of β-casein was found to be slightly lower in emulsions made in the presence of NaCl, but when the protein concentration was ≥ 3.0% the proportion of β-casein was higher than in the emulsions made in the absence of NaCl. At all concentrations, the proportion of α_s-casein was found to be higher in emulsions made in the presence of NaCl compared with emulsions made in the absence of NaCl (Figure 8.12). The details of the interfacial composition of the oil-in-water emulsions made in the absence of NaCl were
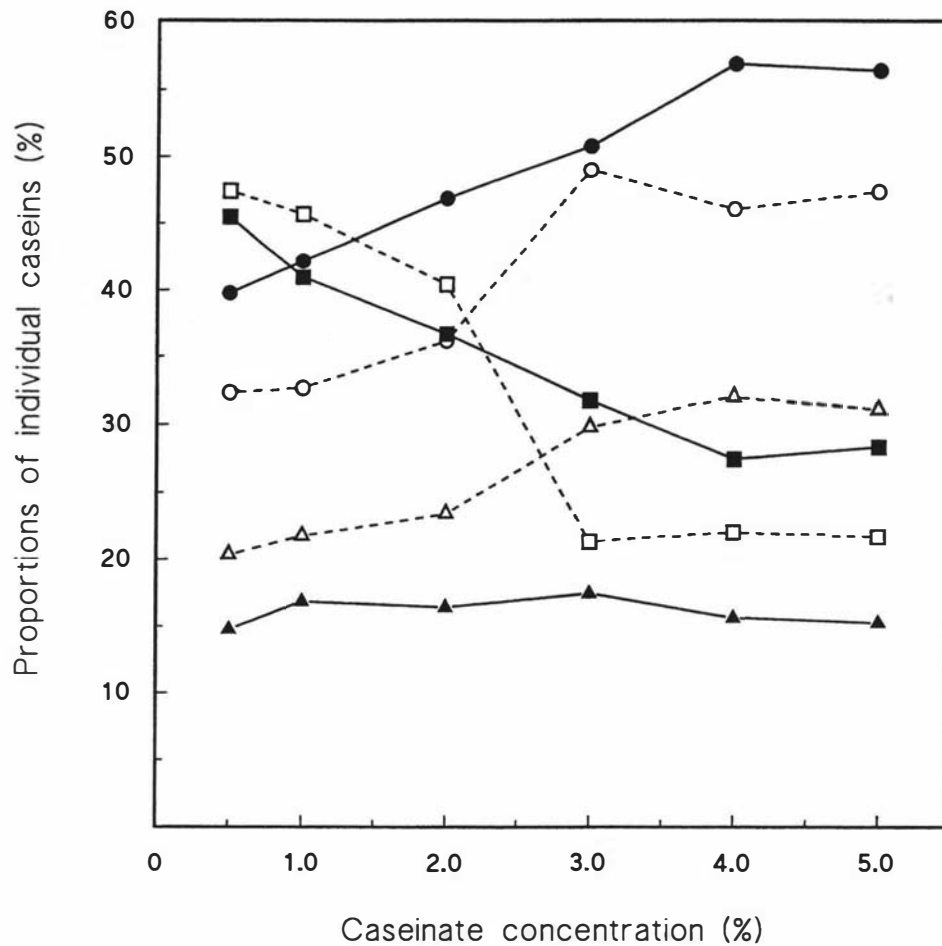
Figure 8.12: The relative proportions of α_s-casein (•, ○), β-casein (■, □) and κ-casein (▲, △) found in the cream phase of emulsions made with various concentrations of sodium caseinate without added NaCl (- - - -) or sodium caseinate with 200 mM NaCl added prior to emulsion formation (———).

discussed in Chapter 5.

8.3.4 Estimated surface concentrations

The surface concentrations of individual caseins in emulsions made with various sodium caseinate concentrations in the presence of 200 mM NaCl are shown in Figure 8.13. For comparison, the surface concentrations of emulsions made in the absence of NaCl are also shown in Figure 8.13. The surface concentration of κ-casein showed a gradual increase from ~ 0.15 to ~ 0.5 mg/m^2 with an increase in sodium caseinate concentration from 0.5 to 5.0% in emulsions made with 200 mM NaCl. The surface concentrations of α_s- and β-caseins in emulsions made with 0.5 and 1.0 % sodium caseinate in the presence of NaCl were almost identical. As the concentration of caseinate was increased from 1.0 to 5.0%, the surface concentration of β-casein increased slightly. In contrast, the surface concentration of α_s-casein showed a marked increase from ~ 0.7 to ~ 1.9 mg/m^2 with an increase in protein concentration $\geq$ 1.0%. In emulsions made with > 1.0% caseinate, the concentrations of surface caseins were in the order: α_s-casein > β-casein > κ-casein.

Comparison of results obtained for emulsions in the presence of NaCl with those in the absence of NaCl (Figure 8.13) showed that the surface concentrations of α_s- and β-caseins in emulsions made in the presence of 200 mM NaCl were considerably higher at all protein concentrations. In contrast, the surface concentration of κ-casein was found to be lower for emulsions made in the presence of NaCl, at protein concentrations > 2.0% compared with emulsions made in the absence of NaCl. Below 2% protein, the concentrations of κ-casein were almost similar in the emulsions made with or without NaCl.
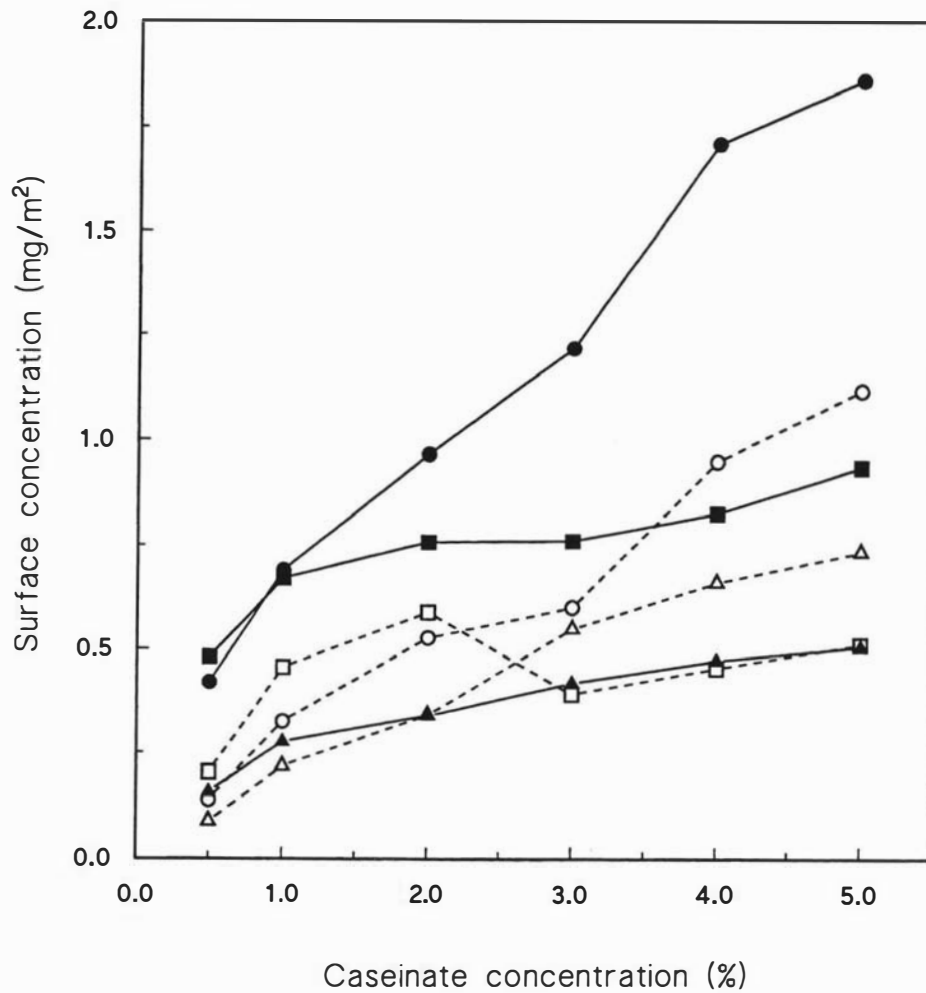
Figure 8.13: The surface concentrations of α_s-casein (•, ○), β-casein (■, □) and κ-casein (▲, △) found in the cream phase of emulsions made with various concentrations of sodium caseinate without added NaCl (- - - -) or sodium caseinate with 200 mM NaCl added prior to emulsion formation (————).

8.3.5. Stability of emulsions

The changes in the creaming stability of emulsions made with various sodium caseinate concentrations in the presence or absence of 200 mM NaCl are presented in Figure 8.14. As the concentration of caseinate was increased from 0.5 to 2.0%, the stability rating of emulsions made in the presence of NaCl showed no change, but with further increase in caseinate concentration from 2.0 to 5.0% the stability rating decreased rapidly from ~ 65% to ~ 27%.

The stability rating of emulsions made in the absence of NaCl was different from that of the emulsions made in the presence of NaCl. In the case of the emulsions, made in the absence of NaCl, the stability rating decreased rapidly as the concentration of caseinate was increased > 1.0%. At a given caseinate concentration, the stability rating of emulsions made in the presence of NaCl was considerably higher than that of emulsions made in the absence of NaCl. When the caseinate concentration was varied from 0.5 to 5.0%, the stability rating values of emulsions made in the absence of NaCl varied with protein concentration from ~ 45% to ~ 4.0%, whereas in emulsions made in the presence of NaCl the stability rating values decreased from ~ 65% to ~ 27%.

Comparison of confocal micrographs of emulsions made in the presence of NaCl (Figures 8.15) with those of emulsions made in the absence of NaCl (refer to Chapter 6, Figure 6.5) showed that the extent of flocculation in the two emulsion systems was entirely different, particularly in emulsions made with caseinate concentrations > 1.0%. The emulsions made with 0.5 and 1.0% caseinate in the absence of NaCl showed no flocculation and the droplets appeared to be separated and evenly distributed within the emulsions. At caseinate concentrations > 2.0%, there appears to be formation of irregular flocs. These flocs appeared to join together to form a network structure as the caseinate concentration was increased from 2.0 to 5.0%. However, the emulsions made with caseinate concentrations of 0.5, 1.0 or 2.0% in the presence of NaCl showed no flocculation and the droplets appeared to be separated and evenly distributed. At 3.0%, there appears the beginning of the

Total caseinate content in the emulsion
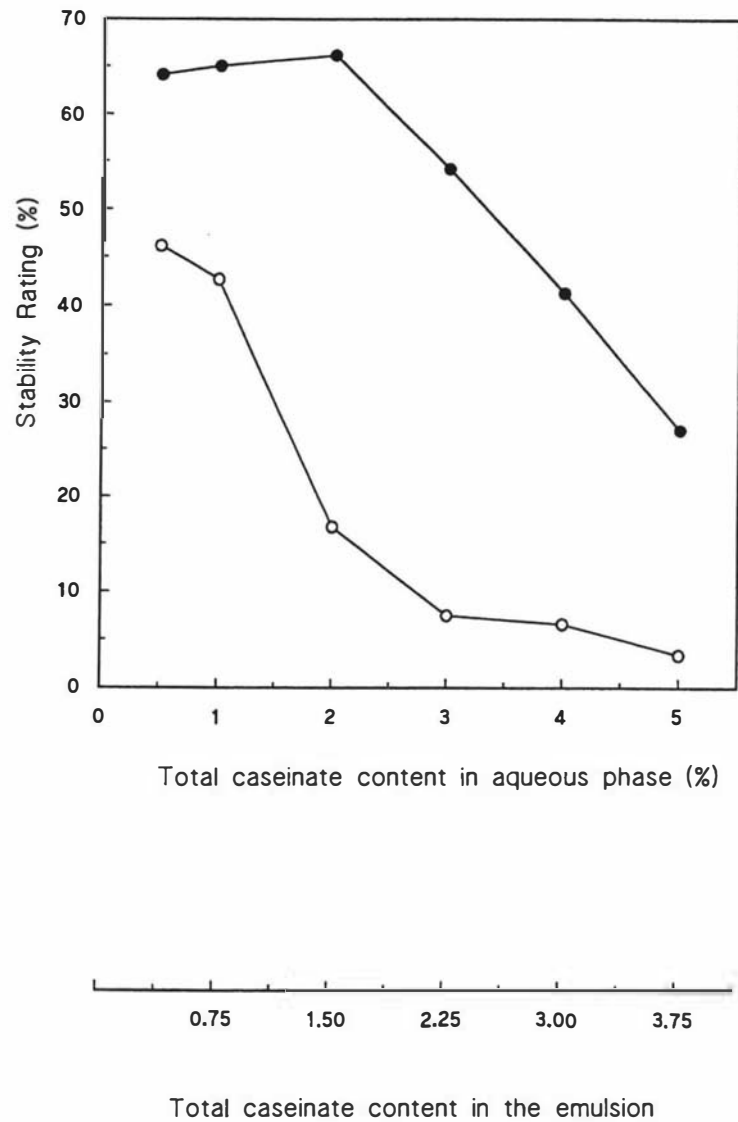
Figure 8.14: Change in stability rating in emulsions using 30% soya oil and various concentrations of sodium caseinate (○) or sodium caseinate with 200 mM NaCl added prior to emulsion formation (●).

A

B

C

D
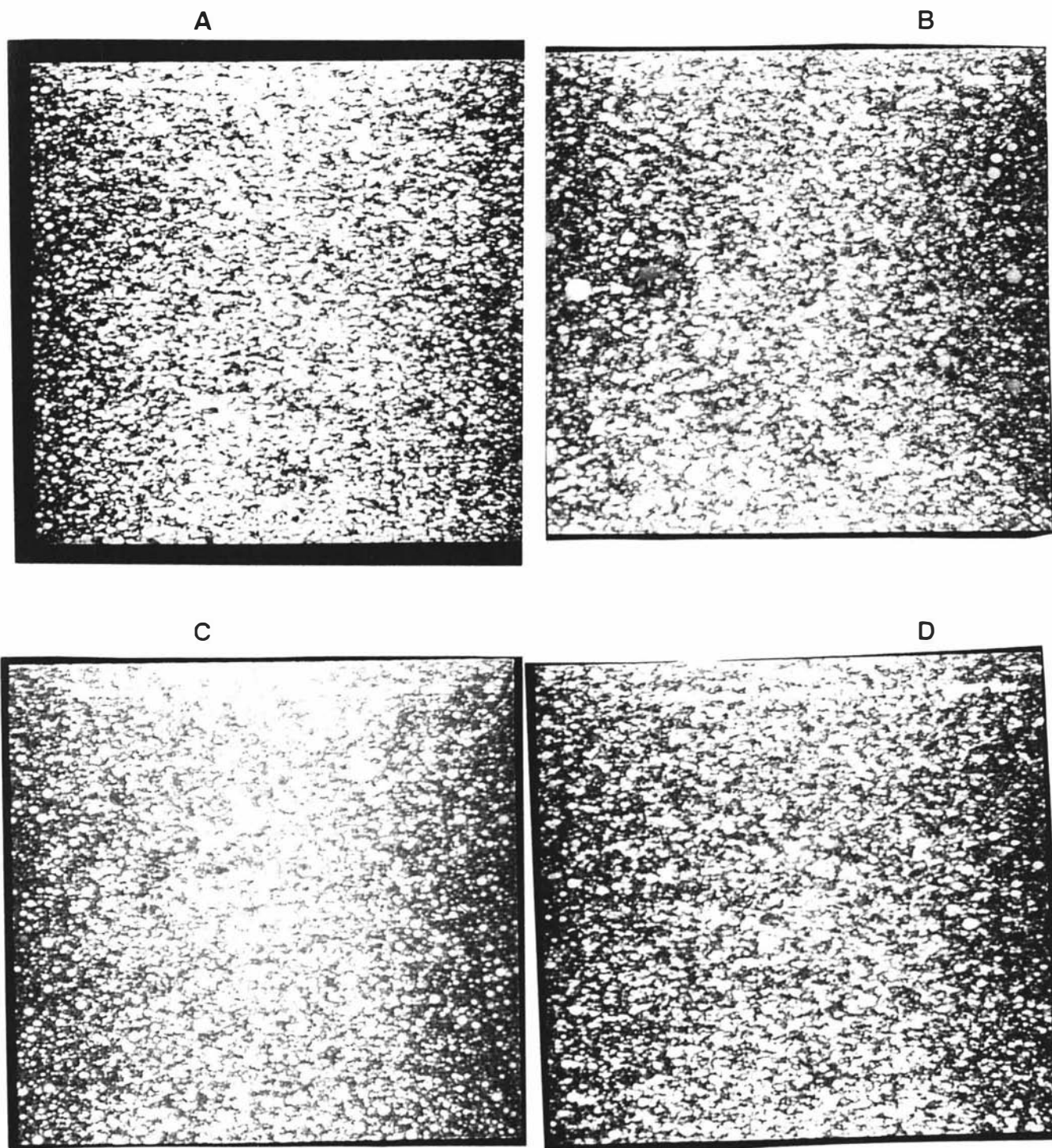
Figure 8.15: Confocal micrographs of sodium caseinate emulsions made with various concentrations of sodium caseinate in the presence of 200 mM NaCl: 0.5% (A); 1.0% (B); 2.0% (C); 3.0% (D); 4.0% (E) or 5.0% sodium caseinate (F).

E
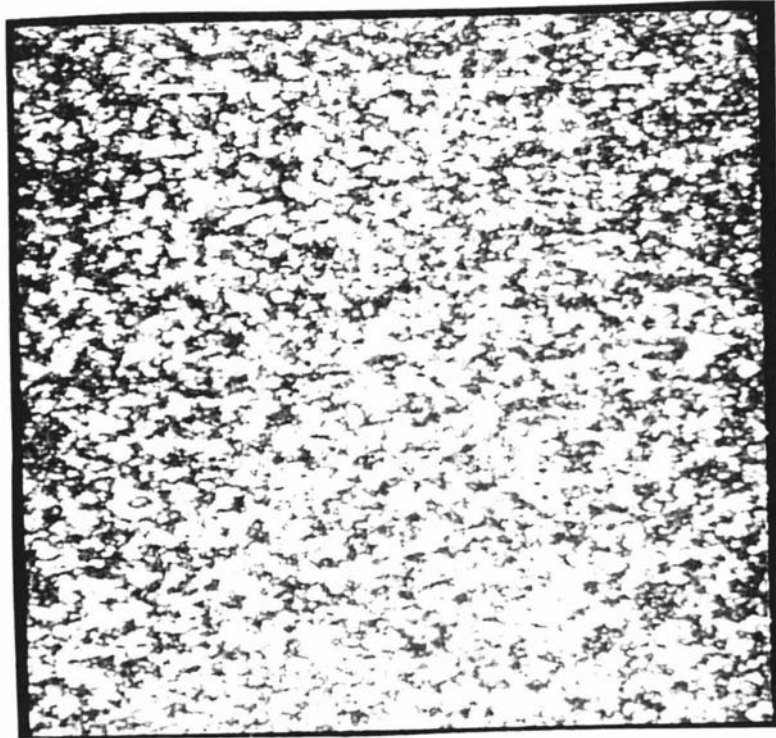
F
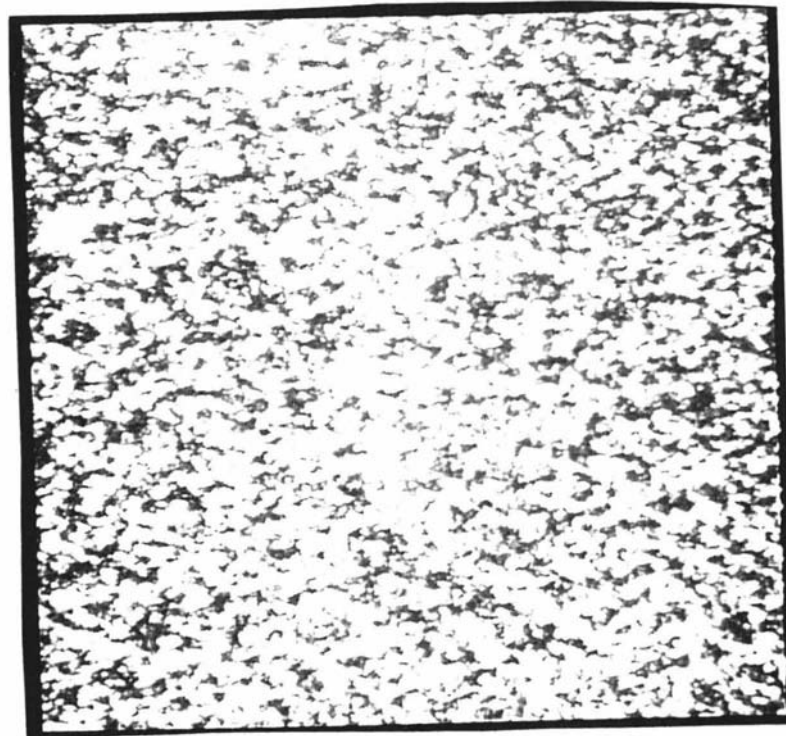
Figure 8.15: Continued...

formation of some flocs. However, there were more individual droplets than in the 3% sodium caseinate-stabilized emulsions made in the absence of NaCl. At caseinate concentrations > 3.0%, the flocs appeared to join together to form a network structure, but the extent of flocculation was less than in emulsions made in the absence of NaCl.

8.4 Discussion

The presence of NaCl increases the surface protein coverage (Figure 8.2) of emulsion droplets, not only during homogenization but also when the NaCl is added to preformed emulsions. This may be attributed to more compact packing of the adsorbed casein molecules, resulting from decreased repulsive interactions between adsorbed casein molecules with an increase in ionic strength. Alternatively, raising the ionic strength may induce aggregation of caseins in solution and adsorption of the resulting aggregates to the interface may lead to high surface coverage. By contrast, Tornberg (1978a) reported that addition of 0.2 M NaCl to whey protein concentrate solutions reduced their adsorption on a soya oil/water interface in emulsions formed using a valve homogenizer. No previous work has been reported on the changes in the surface coverage of caseins in emulsions stabilized by sodium caseinate in the presence of NaCl.

The present results clearly showed that the addition of a neutral salt to sodium caseinate-stabilized emulsions affected the competition between different caseins for adsorption to an oil/water interface and influenced the creaming and flocculation stability of the emulsion droplets. In emulsions made with 1% sodium caseinate, raising the ionic strength markedly increased the amount of α_{s1}-casein at the droplet surface. This may again be due to screening of charges on the polyelectrolyte residues, resulting in more compact packing of α_{s1}-casein molecules at the interface. Previous studies on model α_{s1}-casein solutions have shown that, at ionic strengths greater than 0.003 and pH 6.6, the α_{s1}-casein monomers exist in an equilibrium with oligomers, and the extent of association increases with an increase in ionic strength (Ho & Waugh, 1965; Schmidt & van Markwijk, 1968; Schmidt, 1970a). Addition of NaCl to caseinate solution may cause α_s-casein (α_{s1}- + α_{s2}-) to form dimers, tetramers, hexamers etc., which may subsequently become adsorbed, resulting in a high concentration of α_{s1}-casein at the interface. When NaCl was added to the preformed emulsion, it is possible that the unadsorbed α_{s1}-casein interacted with adsorbed α_{s1}-casein at the droplet surface, giving higher surface

concentrations of α_s-casein. In emulsions made with 3% protein, the presence of NaCl also increased the adsorption of β-casein and κ-casein at the interface (Figure 8.3 and 8.4). The reasons for the increased adsorption of β- and κ-casein are not clear. It is likely that NaCl addition affected the aggregation state of various casein complexes that exist in concentrated caseinate solutions.

Hunt and Dalgleish (1996) reported that the adsorption behaviour of sodium caseinate (2%) in oil-in-water emulsions (20% oil) was influenced by KCl at concentration > 25 mM. The present study showed that changes in the adsorption behaviour in the presence of NaCl occur even at 5 mM NaCl.

Hunt and Dalgleish (1996) reported that α_{s1}-casein adsorbed preferentially at the expense of β-casein when the KCl concentration in the emulsions was above 25 mM. Similar results in the proportions of α_s-casein and β-casein were observed in the present study for emulsions made in the presence of NaCl (Figure 8.12). However, when these results were considered in terms of the surface concentration of α_{s1}- and β-caseins, it appears that the concentration of β-casein at the interface remained largely unaffected by NaCl addition, whereas the concentration of α_{s1}-casein increased. Hunt and Dalgleish (1996) did not report the surface protein concentrations in their emulsions.

Dickinson *et al.* (1987) and Dickinson, (1997) found that emulsions made with α_{s1}-casein were more susceptible to flocculation by NaCl than β-casein-stabilized emulsions. Addition of NaCl caused extensive flocculation of α_{s1}-casein coated oil droplets at ionic strengths of 0.1 M or above but no flocculation of droplets occurred in emulsions stabilized by β-casein or sodium caseinate. The present results (Figure 8.7) showed that the presence of NaCl during emulsion formation or the addition of NaCl to preformed emulsions did not cause any (irreversible) flocculation of droplets, as indicated by the changes in the particle size distribution (Figure 8.1). However, the creaming stability was improved by addition of NaCl, with the effect being much larger in emulsions stabilized with relatively high concentrations of caseinate (above 2.0% w/w).

The improvement in creaming stability with NaCl addition observed in emulsions made with low caseinate concentrations (~ 1%) may be attributed to changes in surface protein coverage and/or changes in surface composition. In emulsions made with 1% caseinate, the surface coverage increased with added NaCl which was largely due to increased adsorption of α_{s1}-casein (Figures 8.11 and 8.13).

In emulsions formed with relatively high concentrations of caseinate, the creaming stability improved markedly with NaCl addition. The instability of the systems containing no added NaCl has been attributed to depletion flocculation as discussed in Chapter 6.

Dickinson and Golding (1997) reported that the extent of depletion flocculation is mainly dependent on the unbound or unadsorbed protein, with the initial discernible onset of flocculation occurring at an aqueous caseinate concentration somewhere between 2 and 3% (w/w). In the present study, it was observed that with increasing concentration of NaCl, the surface protein coverage increased and hence the concentration of unadsorbed protein decreased. The remaining unadsorbed protein probably did not reach the critical flocculation concentration; hence the depletion flocculation disappeared and the creaming stability increased. The critical flocculation concentration in the presence of NaCl with sodium caseinate-stabilized emulsions was different from that in emulsions made in the absence of NaCl. The onset of flocculation occurred at an aqueous phase caseinate concentration somewhere > 3.0% (Figure 8.16).

As the extent of flocculation is also dependent on the size of the unadsorbed species (Dickinson and Golding, 1997; Dickinson *et al.*, 1997), it is also possible that added NaCl increased the particle size of the unadsorbed protein, hence decreasing the depletion attraction.
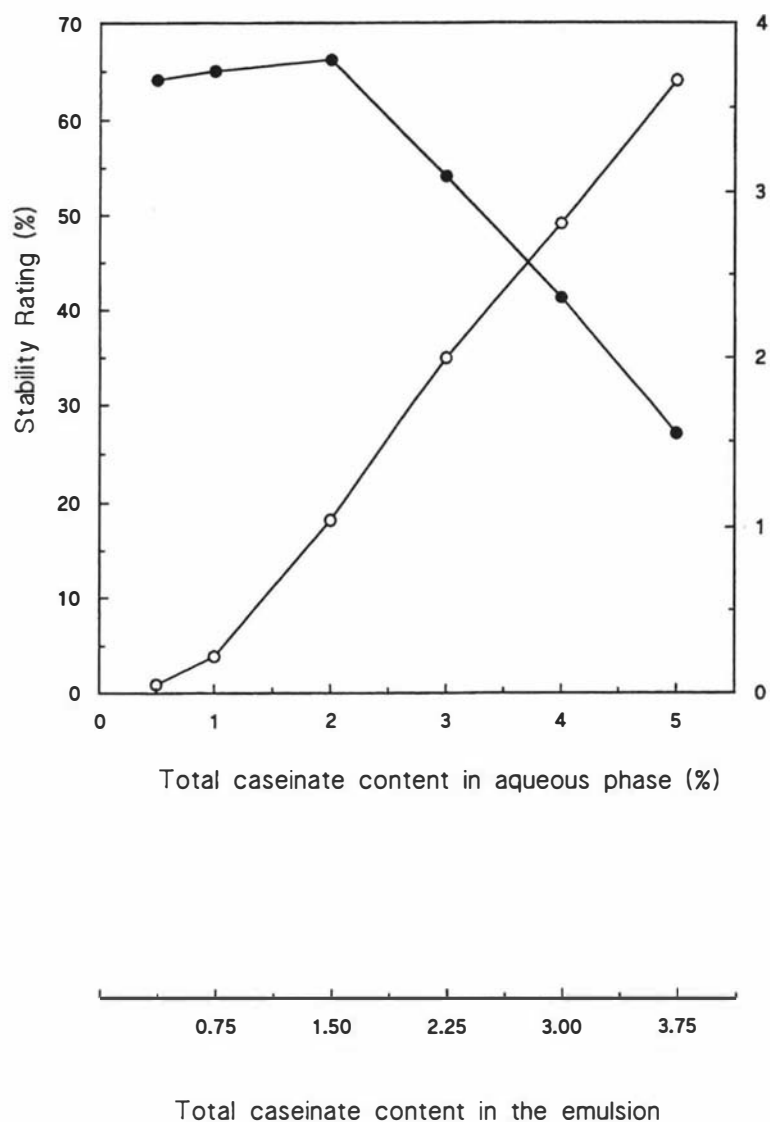
Figure 8.16: Stability rating (•) of sodium caseinate emulsions in the presence of 200 mM NaCl (30% oil, pH 7.0) and the concentration of unbound protein or protein in the bulk aqueous phase (○) as a function of total protein in the emulsion or total protein content in the aqueous phase.

Part B: The effect of addition of NaCl on the formation and stability of calcium caseinate emulsions.

8.5 Results

8.5.1 Emulsion formation

Emulsions (30% soyal oil) were made with 2.5% calcium caseinate. Various amounts of NaCl were added to the caseinate solution prior to emulsification.

8.5.2 Particle size distribution and droplet diameter

Figure 8.17 shows the particle size distributions of emulsions formed with 2.5% calcium caseinate at various concentrations of NaCl. As the concentration of NaCl was increased, the particle size distribution became slightly narrower (0.1 - ~ 0.3 µm). There was no significant variation in the particle size distribution when the concentration of NaCl was between 20 and 150 mM. At all NaCl concentrations, the particle size showed a monomodal distribution.

The effect of various concentrations of NaCl during emulsion formation on the average diameter (d_{32}) of emulsion droplets, made with 2.5% calcium caseinate and 30% soya oil at pH 7.0, is shown in the Figure 8.18. The diameter of the emulsion droplet decreased from 0.73 to 0.60 µm as the concentration of NaCl was increased from 0 to 20 mM; with further increase in NaCl, the droplet diameter remained constant (~ 0.60 µm).

8.5.3 Surface protein coverage

The surface protein coverage of emulsion made with 2.5% calcium caseinate and various concentrations of NaCl decreased gradually from 2.5 to ~ 2.2 mg/m² with an increase in NaCl concentration from 0 to 50 mM (Figure 8.18). As the concentration of NaCl was increased from 50 to 200 mM, the surface protein coverage increased slightly from ~ 2.2 to 2.4 mg/m². It is interesting to note that the surface protein coverages of emulsions made in the absence of NaCl and with 200 mM NaCl were almost similar. The surface protein coverage values found in the absence of NaCl are similar to the values reported earlier (Chapter 5).
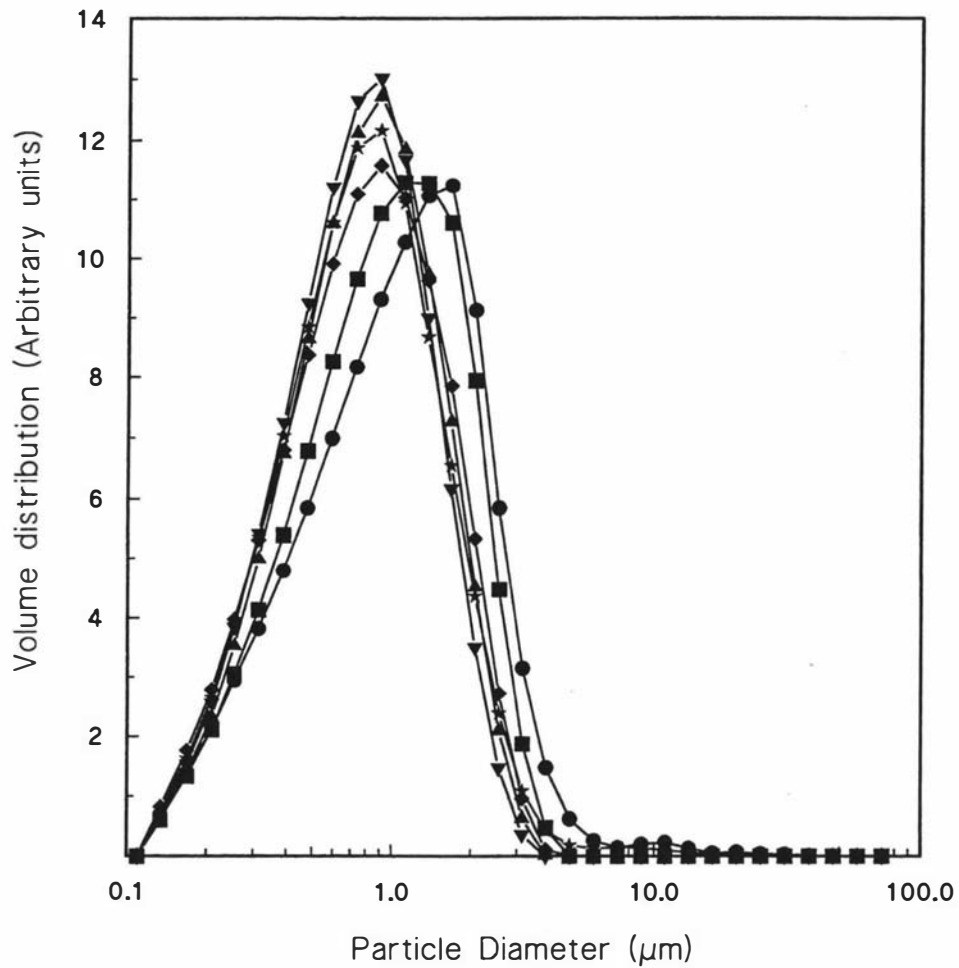
Figure 8.17: Change in the particle size distribution of 2.5% calcium caseinate emulsions as a function of concentrations of NaCl. No NaCl (•); 5 mM NaCl (■); 20 mM NaCl (▲); 40 mM NaCl (▼); 80 mM NaCl (♦) or 150 mM NaCl (★).
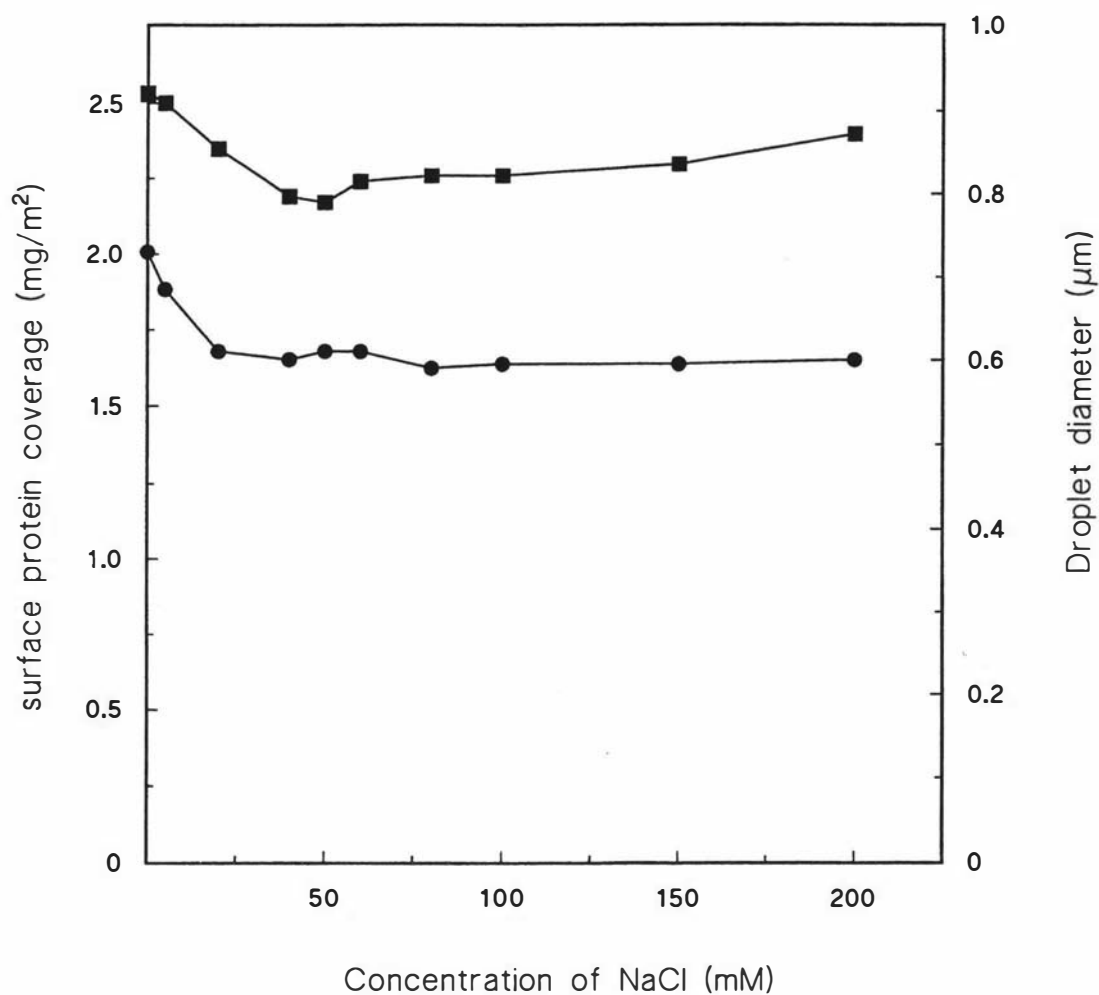
Figure 8.18: Change in surface protein coverage (mg/m^2) and droplet diameter (μm) in emulsions containing 30% oil and 2.5% calcium caseinate at pH 7.0 as a function of concentration of NaCl. Surface protein coverage (■) and average droplet diameter (d$_{32}$) (•).

8.5.4 Composition of caseins at the oil/water interface (cream phase)

From Chapter 5, it is clear that in calcium caseinate emulsions at any concentration, α_s-casein was preferentially adsorbed in the absence of NaCl. Addition of various concentrations of NaCl to 2.5% calcium caseinate-stabilized emulsions did not significantly alter the proportions of individual caseins adsorbed at the droplet surface (results not shown).

8.5.5 Stability of emulsions

The presence of NaCl up to 50 mM caused an increase in the stability rating from ~ 50% to ~ 67% (Figure 8.19). With further addition of NaCl up to 200 mM, the stability rating showed no further change.

8.6   Effect of NaCl on the emulsions made with various calcium caseinate concentrations

From the above experiments, it is clear that the emulsifying properties are affected by the addition of NaCl before emulsification. Most of the changes in the adsorption behaviour of proteins and the creaming stability of emulsions occurred when the NaCl concentration was < 150 mM. Further experiments were carried out to determine how the adsorption behaviour and creaming stability were affected at caseinate concentrations (from 0.5 to 5.0%) in the presence of 150 mM NaCl.
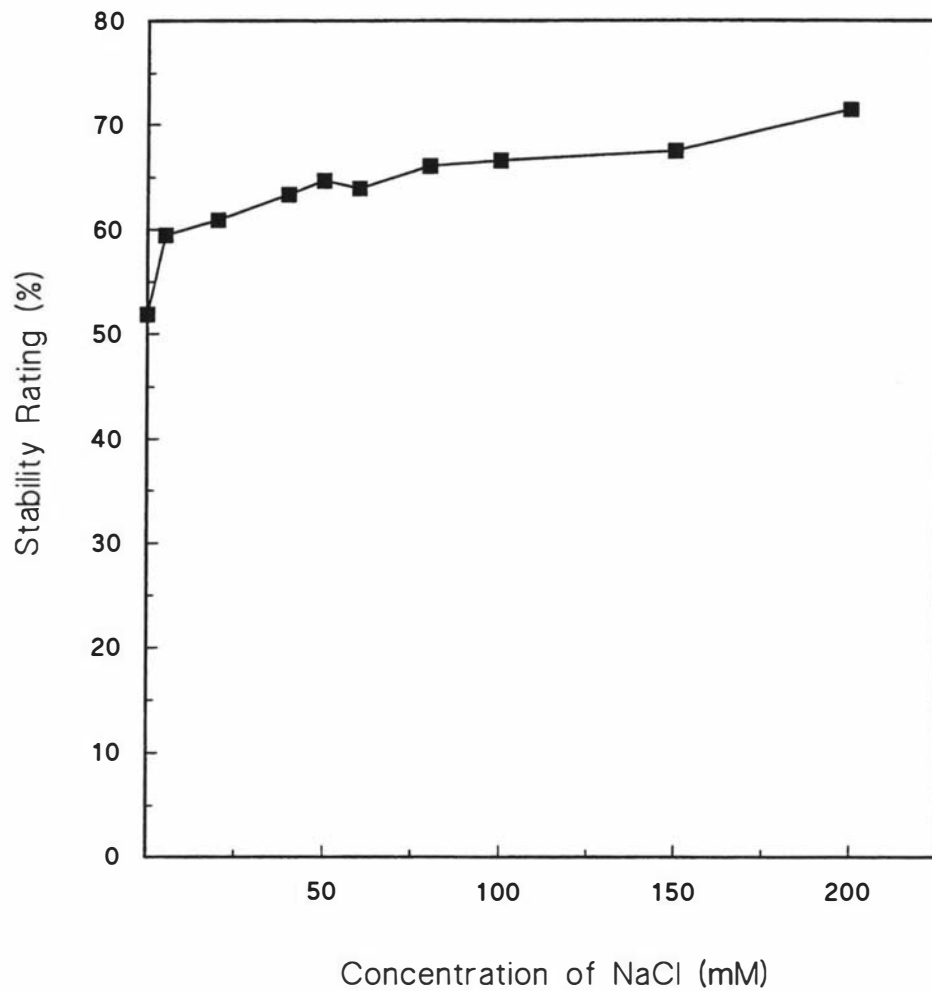
Figure 8.19: Changes in stability rating of emulsions made with 30% soya oil and 2.5% calcium caseinate at pH 7.0 as a function of NaCl addition prior to emulsification.

8.6.1 Particle size distribution and droplet diameter

As shown in Chapter 5, the particle size of calcium caseinate emulsions showed a bimodal distribution when emulsions were made with 0.5 and 1.0% caseinate concentration (Figure 8.20 A). In the presence of 150 mM NaCl, the particle size of emulsions made with 0.5 and 1.0% caseinate concentration showed a monomodal distribution (Figure 8.21 B). It was noted that the effect of NaCl on the particle size distribution mainly occurred in emulsions made with < 2.0% calcium caseinate. At all caseinate concentrations, in the presence of 150 mM NaCl, the particle size distributions became narrower (0.1 to ~ 3.0 μm). The range of the particle size distributions in Figure 8.21 B was fairly close to those for sodium-caseinate-stabilized emulsions.

The changes in volume-surface average droplet diameter (d_{32}) with caseinate concentrations in emulsions made in the presence or absence of 150 mM NaCl are shown in Figure 8.21. At any concentration, the droplet diameter of calcium caseinate emulsions made in the absence of NaCl (refer to Chapter 5) was higher than in emulsions made in the presence 150 mM NaCl. The diameter of emulsion droplets of calcium caseinate emulsions made in the absence of NaCl decreased from ~ 1.2 to ~ 0.7 μm, whereas that in emulsions made in the presence of 150 mM NaCl decreased from ~ 0.77 to 0.6 μm, as the calcium caseinate concentration was increased from 0.5 to 5.0%.
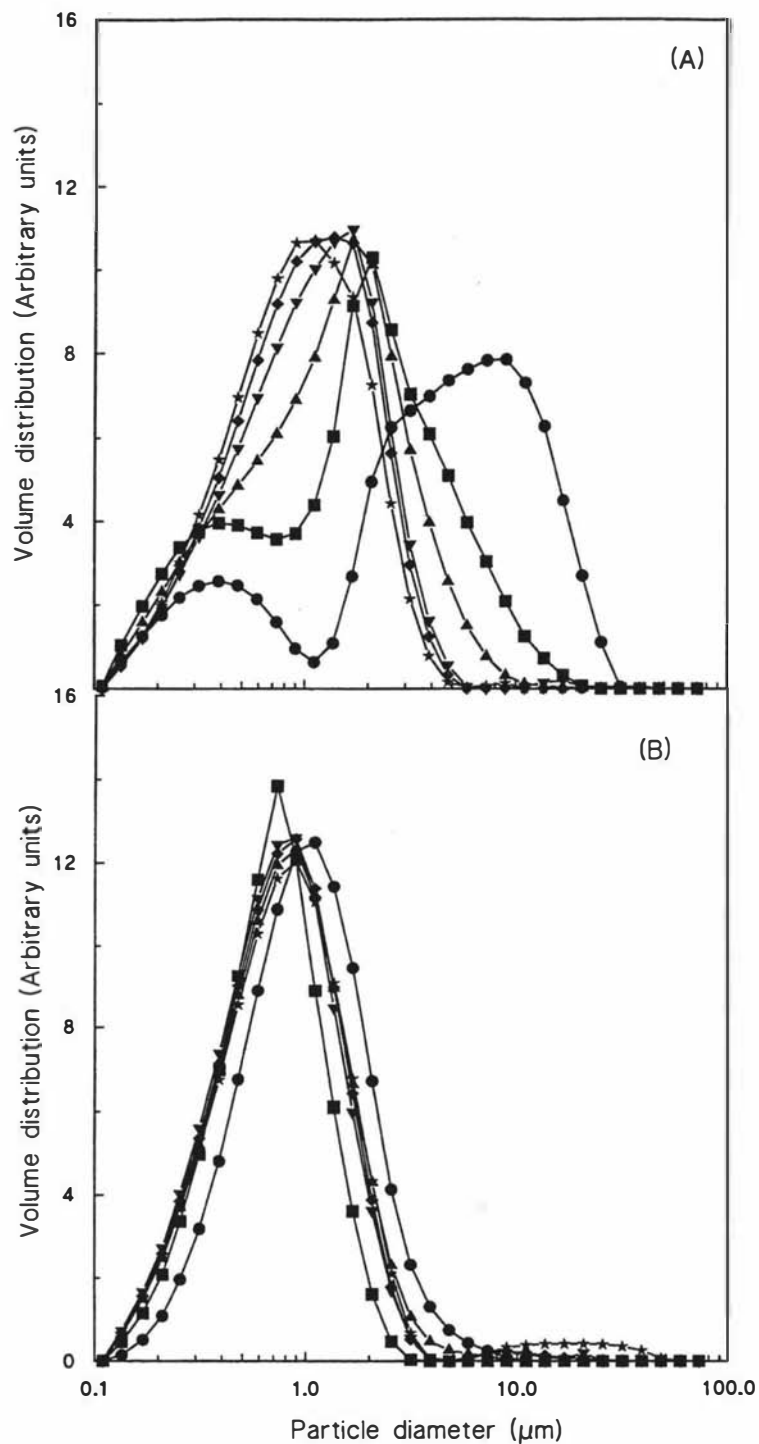
Figure 8.20: Particle size distribution of emulsions (30% oil) formed with 0.5% (•); 1.0% (■); 2.0% (▲); 3.0% (▼); 4.0% (♦); 5.0% (★) calcium caseinate and with 30% oil, containing no NaCl (A) or 150 mM NaCl (B).
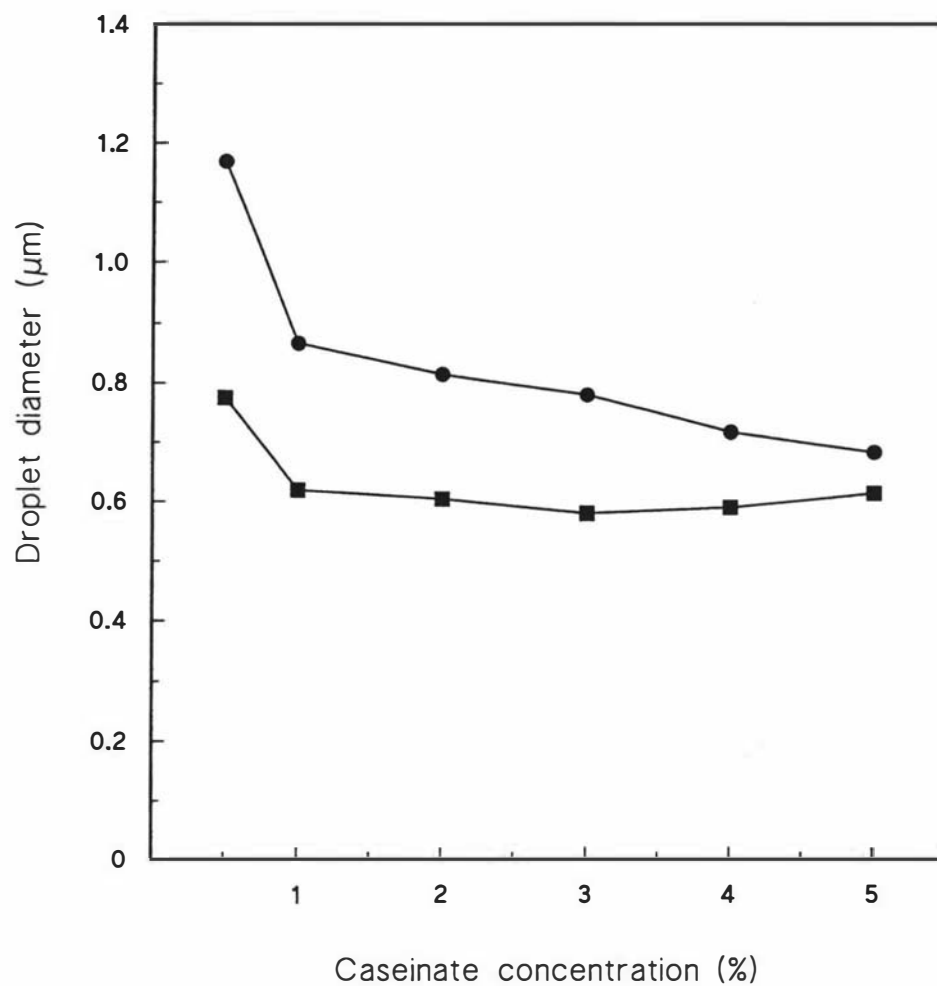
Figure 8.21: Changes in average droplet diameter (volume-to-surface, d_{32}) as a function of calcium caseinate concentrations in emulsions made with no added NaCl (•) or 150 mM NaCl (■).

8.6.2 Surface protein coverage

The surface protein coverages of emulsion droplets, in the absence or presence of 150 mM NaCl, are shown in Figure 8.22. In the case of emulsions made in the absence of NaCl, the surface protein coverage increased almost linearly with an increase in calcium caseinate concentration (refer to Chapter 5). The surface protein coverage of emulsions made with ≤ 2.0% protein and with 150 mM NaCl was higher than in the emulsions made in the absence of NaCl. However, the surface protein coverage of emulsions made with ≥ 3.0% protein in the presence of 150 mM NaCl was lower than in the emulsions made in the absence of NaCl. At 3.0% protein the surface protein coverages of emulsions made with or without NaCl were similar.

8.6.3 Stability of emulsions

In the absence of NaCl, the creaming stability increased markedly with an increase in protein concentration from 0.5 to 2.0%, but with further increase in the caseinate concentration from 3.0 to 5.0% there was no significant change in the creaming stability (Figure 8.23; also refer to Chapter 6). Addition of 150 mM NaCl enhanced the creaming stability of the emulsions at all caseinate concentrations.
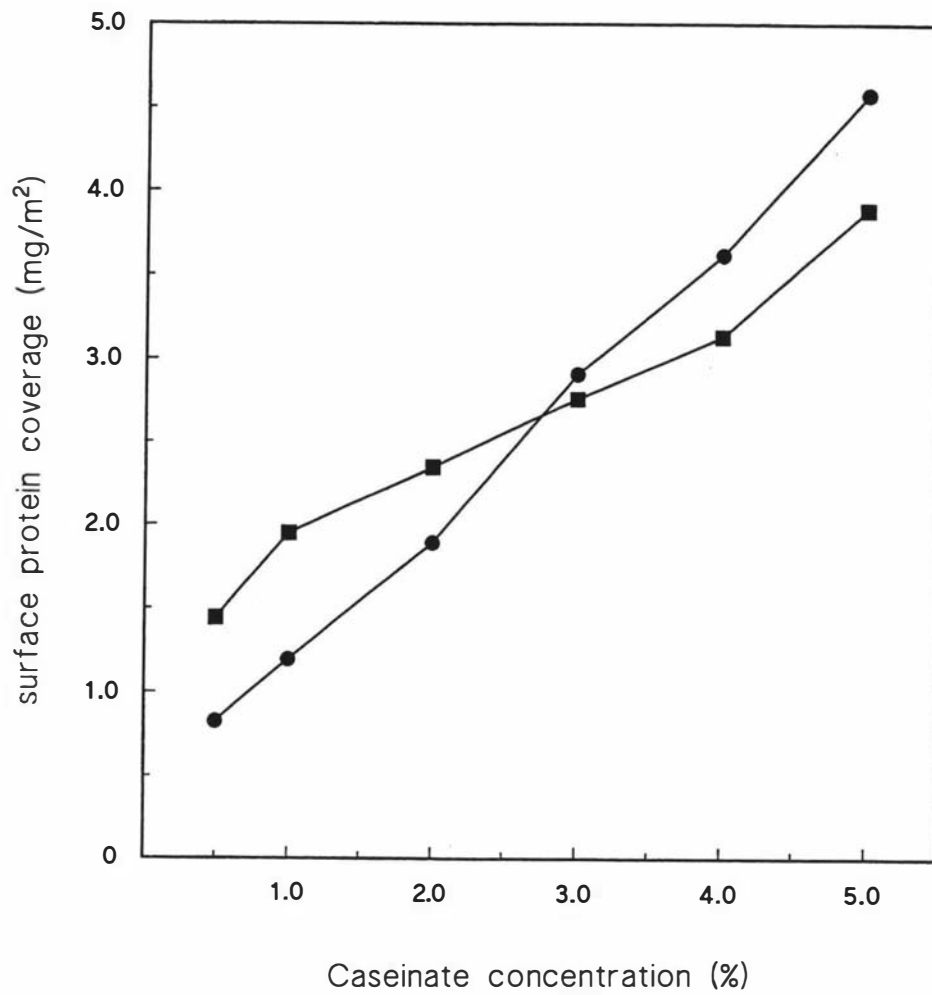
Figure 8.22: Changes in surface protein coverage (mg/m^2) of emulsions (30% oil) made with various calcium caseinate concentrations and with 0 mM NaCl (•) or 150 mM NaCl (■) at pH 7.0.
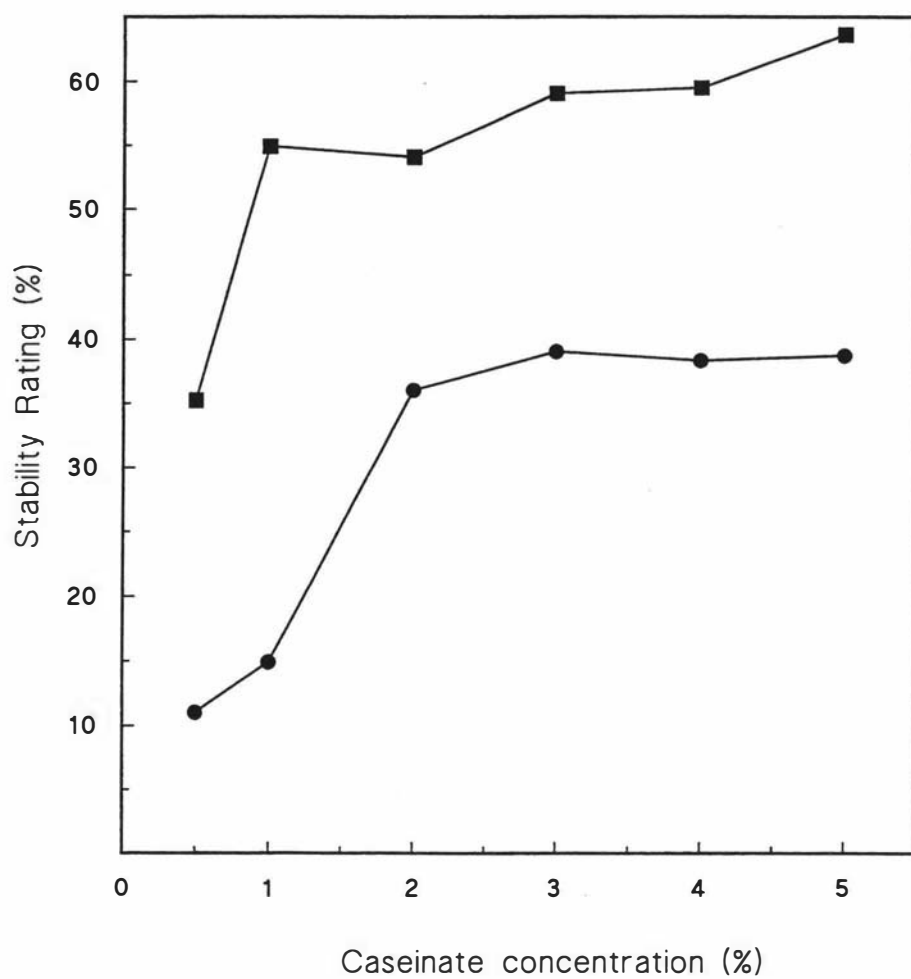
Figure 8.23: The stability rating (%) of emulsions as a function of calcium caseinate concentration. Emulsions formed with no added NaCl (•) or 150 mM NaCl (■) at pH 7.0.

8.7 Discussion

The present results clearly showed that an increase in NaCl concentration from 0 to 40 mM decreased the surface protein coverage and resulted in lower droplet sizes (Figure 8.18). This may suggest that the casein aggregates in the calcium caseinate dispersion dissociated gradually with increasing NaCl concentration. Mulvihill and Murphy (1991) reported that highly aggregated proteins had lower fat surface areas in the same power input range. Previous work (Dickinson *et al.*, 1992; Agboola and Dalgleish, 1995; Srinivasan *et al.*, 1996) showed that the addition of calcium to sodium caseinate increased the particle size of oil-in-water emulsions because of calcium-induced aggregation.

The dissociation of aggregates in calcium caseinate with NaCl addition was confirmed by the changes in turbidity and protein sedimentation of 2.5% calcium caseinate dispersions (Figure 8.24). The turbidity and protein sedimentation in calcium caseinate dispersions decreased markedly with an increase in NaCl concentration from 0 to 50 mM. No significant changes in the turbidity or sedimentable protein were observed when the NaCl concentration was > 50 mM. These results indicated that most of the aggregates in calcium caseinate had dissociated with the addition of 50 mM NaCl. It is likely that the binding of Ca^{2+} to the caseins was reduced with the addition of NaCl. The solubility of α_{s1}-, α_{s2}- and β-caseins is strongly affected by the binding of calcium ions. Binding of Ca^{2+} to the caseins at neutral pH reduces their negative charge, which diminishes the electrostatic repulsion between the molecules. In this way, calcium binding promotes hydrophobic interactions which can lead to increasing association and ultimately to precipitation (Swaisgood, 1992). The strength of binding of Ca^{2+} to α_{s1}- and β-caseins is decreased by increasing ionic strength (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Baumy *et al*, 1989).
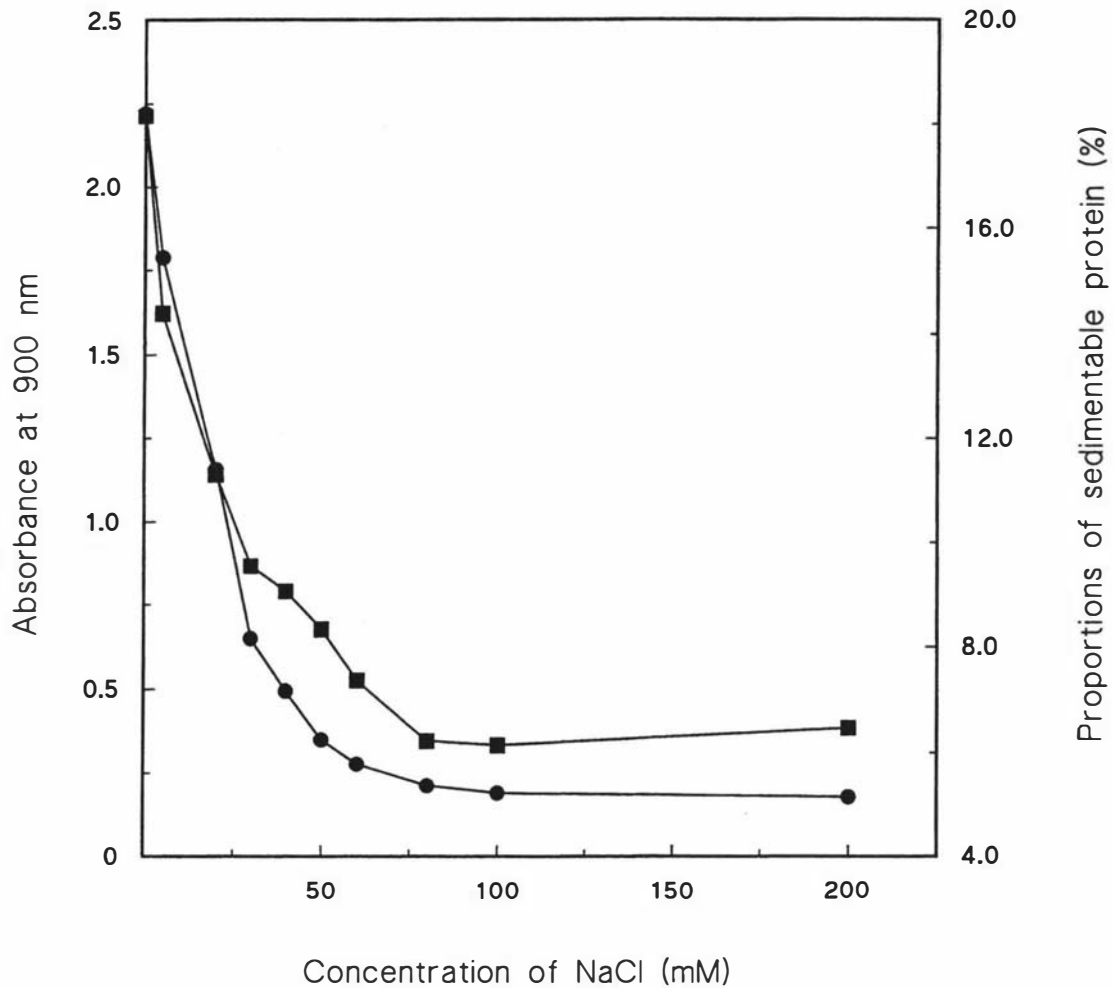
Figure 8.24: Changes in turbidity (absorbance at 900 nm) (•) and the
proportions of sedimentable protein (%) (■) (10,000 *g* for 40 min)
as a function of added NaCl in 2.5% calcium caseinate
dispersions.

It is not immediately apparent why further addition of NaCl (beyond 50 mM) caused an increase in surface protein coverage (Figure 8.19). It may be that the caseins aggregated again at higher ionic strength, but this aggregation may not be related to Ca^{2+}. We can assume that most of the aggregates, formed as a result of binding of Ca^{2+} to caseins, were dissociated at about 50 mM NaCl and then self-association of caseins started at higher concentrations of NaCl.

We know from Stokes' equation, that the rate of creaming is mainly dependent on the droplet size, the difference in density between the droplet and the aqueous phase and the viscosity of the continuous phase (refer to Chapter 6). Mulvihill and Murphy (1991) reported that the fat surface area had a major influence on the stability of emulsions stabilized by less aggregated caseinates. In the present work, the stability rating was mainly dependent on the particle size of the emulsion. A decrease in droplet size resulted in an increase in creaming stability. The trend of the change in stability rating was very similar to the trend observed for changes in emulsion droplet size (Figure 8.19 and Figure 8.21). It seems that the creaming stability was relatively independent of the surface protein coverage and the viscosity of the continuous phase. The marked change in surface protein coverage with a change in concentration (2.0 to 5.0%) of calcium caseinate did not cause any significant change in the stability rating (Figure 8.19 and Figure 8.22).

Chapter 9

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDIES

The work presented in this thesis has contributed to the understanding of the major factors (protein content, ionic strength, temperature) that influence the nature of the adsorbed layer in oil-in-water emulsions formed with commercial calcium and sodium caseinates. Useful insights have been provided into the instability processes of creaming and flocculation in these emulsions.

Since the structure and composition of the adsorbed layers and the resulting stability of emulsions are dependent on the surface activity, aggregation state and concentration of the protein species used, initial work attempted to identify the molecular aggregation state of caseinates in solutions. Multi-angle laser light scattering (MALLS) combined with SEC proved to be very useful technique in determining the molecular weights and sizes of various aggregates and complexes in caseinate solutions. It was found that caseinates consist of complex mixture of caseins in various state of aggregation, with the aggregation state of casein in calcium caseinate solutions being different from that of sodium caseinate solutions.

The study of aggregation state of caseinates by MALLS and SEC could only be carried out in relatively dilute conditions, which may lead to dissociation of some of the aggregates. The use of techniques that may allow studying the concentrated solutions of caseinates, such as osmotic pressure measurements or diffuse wave spectroscopy, could be explored for these systems. Because proteins adsorb to the interfaces mainly by hydrophobic interactions, it would be of interest to determine the hydrophobicity of various casein complexes and aggregates.

The study clearly showed that the surface protein coverage and composition

are dependent on the concentration of protein. An increase in the amount of caseinate used leads to a steady increase in adsorbed protein (from 0.4 - 2.5 mg/m^2) with possible formation of multilayers at high concentrations. It is possible to make a stable emulsions containing caseinates even when the surface concentration is as little as 0.4 mg/m^2. For an emulsion containing 30% oil, this casein concentration can be about 0.5%. This study made an attempt to determine the competitive adsorption between species of different sizes in caseinate solutions. At low protein concentrations ($\leq$ 2.0%) casein monomers and smaller complexes are adsorbed in preference to aggregates, but at higher concentrations, larger aggregates are adsorbed in preference to monomers and complexes. Because of different casein compositions of smaller complexes and larger aggregates, the emulsions made with low caseinate concentrations ($\leq$ 2.0%) show preferential adsorption of β-casein, while the emulsions stabilized with higher protein concentration (> 2.0%) show greater adsorption of α_s-casein.

In case of calcium caseinate-stabilized emulsions an increase in the amount of caseinate used leads to an almost linear increase in the surface protein coverage (0.8 - 4.5 mg/m^2). The calcium caseinate solution has a wide range of casein aggregates and particles, some very large particles with high proportions of α_{s1}- and α_{s2} -caseins, some monomeric molecules, casein complexes and calcium-induced casein aggregates of different sizes. It appears that larger aggregates, rich in α_s-casein are adsorbed in preference to monomers or casein complexes. Thus, at all protein concentrations, this results in higher surface coverage than in sodium caseinate emulsions and preferential adsorption of α_s-casein.

In sodium caseinate emulsions, the surface coverage and, in particular, surface composition is greatly influenced by the addition of NaCl before or after emulsion formation. Increase in NaCl up to 40 mM increases the surface protein coverage, which is mainly due to increased adsorption of α_s-casein. In calcium caseinate emulsions, the surface protein coverage is also affected by

the addition of NaCl. Addition of up to 40 mM NaCl prior to emulsion formation dissociates the large aggregates, resulting in a decrease in the droplet diameter and lower surface protein coverage.

In emulsions formed with mixtures of calcium and sodium caseinate solutions, (total protein concentration of 2.5%), the surface protein coverage decreases markedly with decrease in the calcium caseinate concentration in the mixture. This decrease is mainly due to the decrease in α_s-casein on the droplet surface. The surface coverage of β- and κ-casein remains unaffected.

These results suggest that it is possible to produce a range of emulsions with different surface protein concentration and compositions by manipulation of concentration of protein used, ratio of sodium and calcium caseinates and ionic strength. More effort will need to be diverted towards understanding the self-assembly and aggregation properties of concentrated solutions of mixed caseins and caseinates and how these are influenced by environmental conditions, e.g. pH, temperature, ionic strength. It would be interesting to determine the effects of processing conditions (cooking, washing, drying temperature etc.) used during the manufacture of caseinates on the molecular aggregation state of caseins in the final products.

The present study showed that heat treatment of caseinate solutions or emulsions results in a number of changes in the caseins, including the formation of some degradation products. In fact, some of these degradation products or peptides produced from caseinates during heating appear to be adsorbed at the droplet surface in preference to the parent protein. Thus the caseinates do not need to be intact to be good emulsifiers. It is also worth noting that the changes occurring during heating in adsorbed caseinates are qualitatively similar to that in the serum (unadsorbed) caseinate. Further studies are required to investigate the nature of degradation products and their adsorption behaviour during emulsion formation.

The present study clearly showed that sodium caseinate emulsions are destabilized reversibly when the concentration of unadsorbed caseinate in the emulsions increases above 2%. This is due to depletion flocculation which is usually associated with the presence of unadsorbed macromolecule that occupy sufficient volume to flocculate the emulsion droplets. This effect is related to the concentration and molecular assembly of adsorbed casein molecules. Addition of NaCl prior to emulsion formation or its addition to the preformed emulsion decreased the extent of depletion flocculation. Confocal microscopy proved to be very useful technique in determining the microstructure of these emulsions and also provided further clarifications on the flocculation mechanism particularly in sodium caseinate emulsions. Further studies on the molecular aggregation of caseins, as a function of casein and NaCl concentration, will help to better understand this phenomenon.

In contrast to sodium caseinate emulsions, the creaming stability of calcium caseinate emulsions shows no depletion flocculation, and their behaviour can be adequately described by the Stoke's equation. Addition of NaCl or heating of caseinate solutions prior to emulsification or heating of emulsions after formation improves the creaming stability.

The work presented here on caseinates formed a good basis for the future study of interactions of caseinates with other food components in real food emulsion systems. Most of the food emulsions contains not only protein but also other components, such as small molecule surfactant, polysaccharide and minerals etc. It would be interesting and commercially important to explore how caseinates interact with polysaccharide and other proteins (e.g. whey proteins, soya proteins) in model food systems.

Some key differences in the emulsifying properties of sodium and calcium caseinates.

	Sodium caseinate	Calcium caseinate
1.	In solution, sodium caseinate, exist as dynamic system of casein monomers, casein complexes and aggregates.	In solution, calcium caseinate, contains some very large aggregates with high proportions of α_{s1}- and α_{s2} -caseins, casein complexes and calcium induced aggregates of different sizes.
Emulsifying properties		
2.	Monomodal size distribution was obtained with a range of particle sizes from 0.1 - ~ 5.0 µm in emulsions formed with protein ranging from 0.5 to 5.0%.	Biomodal size distribution was obtained with the range of particle sizes from 0.1 - ~ 30.0 µm when the caseinate concentration was ≤ 1.0%. This could be due to bridging flocculation between oil droplets. Monomodal size distribution was obtained when the caseinate concentration was ≥ 2.0% with a range of particle sizes from 0.1 to ~ 5.0 µm.
3	The surface protein coverage increased from 0.4 to 2.4 mg/m^2 with increase in caseinate concentration from 0.5 to 5.0%.	The surface protein coverage increased from 0.8 to 4.5 mg/m^2 with increase in caseinate concentration from 0.5 to 5.0%.

4.	β-Casein was preferentially adsorbed at ≤ 2.0% and α_s-casein was adsorbed in preference to other caseins at ≥ 2.0% caseinate concentration.	α_s-Casein was adsorbed in preference to other caseins at all caseinate concentrations
5.	Emulsions with low protein concentrations (< 2.0%) were more stable than emulsions with higher protein concentrations.	Emulsions with high protein concentrations (> 2.0%) were more stable than emulsions with low protein concentrations.
6.	Depletion flocculation was observed when the caseinate concentration was ≥ 2.0%.	No depletion flocculation behaviour was observed.
7.	In the presence of NaCl, β-Casein was preferentially adsorbed when the caseinate concentration was 0.5%. α_s-Casein was preferentially adsorbed when the caseinate concentration was ≥ 2.0%.	NaCl had no effect on the preferential adsorption. α_s-Casein was preferentially adsorbed at all caseinate concentrations.
8.	Depletion flocculation was observed only at caseinate concentrations of 4 and 5% in the presence of NaCl.	No depletion flocculation behaviour was observed in the presence of NaCl.
9.	Heating emulsions or protein solutions prior to emulsification had no effect on the particle size distribution and the droplet diameter	Heating emulsions or protein solutions prior to emulsification shifted the particle size distribution towards larger sizes in emulsions formed ≤ 2.0%.

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