THE COMPOSITION AND PROPERTIES OF BOVINE, CAPRINE AND OVINE MILK PROTEINS

by

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SUMMARY

Analytical methods of ion-exchange and gel permeation chromatography were developed to determine the composition of the caseinate complex and the whey protein fraction in bovine, caprine and ovine milk. These methods were subsequently used to study natural variation in the composition of the milk proteins, casein micellar structure and stability, and changes in the proteins during milk processing.

Initially traditional anion-exchange chromatography on DEAE-cellulose was used to examine the composition of bovine casein, but a more rapid method of anion-exchange FPLC (fast protein liquid chromatography) was introduced in which the main fractions, namely α_{s1}-, β-,α_{s2}-, κ- and γ-caseins, were separated under dissociating conditions at pH 7.0. A complementary method of cation-exchange FPLC, in which the caseins were separated under dissociating conditions at pH 5.0, was also developed to determine the compositions of bovine, caprine and ovine caseins. Both methods could also be used on a preparative scale.

In detailed studies of natural variation in the composition of bovine casein, it was found that in creamery milk in South-West Scotland there was a pronounced seasonal variation in the concentrations of the total and individual proteins but that the relative amounts of the proteins did not vary markedly. The composition of whole casein from individual cows, however, was affected by genetic polymorphism of κ-casein. The relative amount of κ-casein in whole casein varied with phenotype in the order κ-casein BB > AB > AA; on average there was about 25% more κ-casein in whole casein containing the BB phenotype than in that containing the AA phenotype. The presence of β-casein A^1, A^2 or B genetic variants had no significant effect on casein composition.

Compared with bovine casein, caprine casein contained much less α_{s1}-casein, a similar amount of α_{s2}-, and much more β- and κ-caseins. Caprine casein was also much more variable in composition, due to the occurrence of an unusual, quantitative genetic polymorphism of α_{s1}-casein. Polymorphism of caprine κ-casein was also studied and it was found that although the amount of κ-casein varied between 9 and 20% of the total casein, the variants were produced in approximately equal amounts in the heterozygotes.

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There was considerable variation in the composition of individual samples of ovine casein, mainly due to proteolysis of β-casein. Compared with bovine casein, ovine casein contained much less α_s-casein, considerably more β-casein and approximately the same amount of κ-casein. In a study of the composition of bulk milk from a commercial dairy flock, it was found that there were considerable seasonal changes in the concentrations of total and individual proteins, but the relative amounts of the proteins remained fairly constant.

The composition of the whey protein fraction of all three species was determined by gel permeation FPLC, and four main fractions were separated at pH 7.0. Compared with bovine whey protein, caprine whey protein contained less β-lactoglobulin and more α-lactalbumin, whereas ovine whey protein contained slightly more β-lactoglobulin and less α-lactalbumin.

The methods of casein analysis were combined with ultracentrifugation to examine in detail the composition and stability of casein micelles, and in particular the effect of temperature and pH on the dissociation of the caseins and calcium phosphate from the micelles. On cooling milk there was a marked increase in the level of serum casein, which was due almost entirely to β-casein dissociating from the micelles. The change in distribution of micellar and serum casein was completely reversed on re-equilibration at 20°C for 18h. In a series of studies on controlled acidification of milk in the pH range 6.7-4.9 at 30, 20 and 4°C, changes were found in the level and composition of serum casein. These were attributed to a decrease in hydrophobic interaction at lower temperatures, removal of Ca and inorganic phosphate from the micelles as the pH was lowered, and isoelectric precipitation of the caseins at low pH. Results of an investigation using controlled dialysis to selectively remove Ca or inorganic phosphate from casein micelles showed that the binding strength of individual caseins was in the order α_{s2}->α_{s1}->β->κ-casein, indicating that linkage within the micelle increases with the number of phosphate centres in the caseins. In two separate studies it was found that the relative amount of κ-casein in micellar casein increased markedly, and that of β-casein decreased, with decreasing micellar size.

A number of other important changes which take place in the caseins and the whey proteins during processing were examined. Using gel permeation FPLC, detailed quantitative information was obtained on the extent of denaturation of the individual

whey proteins of the cow, goat and sheep with increasing temperature, heating time, and pH. An increased association of the whey proteins with the caseins was observed, and there was a close correlation between the levels of denaturation of bovine β-lactoglobulin and α-lactalbumin and the amounts of these proteins retained in the curd during the pilot-scale production of Cheddar cheese. The effect of incorporation of the whey proteins into the curd on the proteolysis of the caseins during the ripening of Cheddar cheese was examined by ion-exchange FPLC.

The caseins were found to be less sensitive to heat treatment than the whey proteins, but on severe heating, such as occurs during UHT treatment, changes were detected using alkaline and SDS PAGE and anion- and cation-exchange FPLC. These changes were consistent with loss of positive and negative charges on the proteins. Comparison of laboratory and commercially prepared sodium caseinates indicated that considerable heat damage and change in functional properties had occurred in the commercial products.

In a study of acid gelation, it was found that on heating milk at functionalisation temperatures, the concentration of κ-casein in the serum increased. On controlled acidification of the heat-treated milk, Ca, inorganic phosphate and caseins dissociated from the micelles but, compared with unheated milk, maximum dissociation of the caseins at 4 and 20°C occurred at higher pH, and the overall extent of dissociation was reduced. On acidification at 30°C, the level of serum casein decreased, compared with the slight increase found for raw milk.

Results showed that a detailed knowledge of the composition and interactions of the milk proteins could result in considerable improvements and increased efficiency in milk processing.

ACKNOWLEDGEMENTS

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PUBLICATIONS

Contribution agreed with senior author, according to normal practice within Hannah Research Institute, given in order (Initiation of work:Execution of work:Writing paper)

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I declare that my contribution to the published work is as outlined above, and that this thesis has been composed solely by myself.

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GENERAL INTRODUCTION

1. INTRODUCTION

Dairy products form a substantial part of the diet in the Western world, and within the European Community the total annual production of cows' milk is about 114 million tonnes. A further 1.7 and 2.0 million tonnes are obtained from the goat and sheep, respectively (EC Facts and Figures, 1993). The proteins constitute more than 25% of the total solids in milk, and are especially important in human nutrition as a rich source of essential amino acids.

Despite their value in the diet, there is a limited amount of information about the concentrations and relative proportions of the individual proteins in the milk of the cow, goat and sheep. Also, there have been few quantitative studies on natural variation in the composition of milk proteins due to the effect of season, stage of lactation and genetic polymorphism. The main reason for the lack of this quantitative information on composition is that the milk proteins are difficult to separate and the available analytical methods have been laborious.

Precise quantitative methods are also required in order to study changes in the proteins, and to investigate problems that occur during processing. Most milk, including that for liquid consumption, undergoes some form of treatment. To meet public health requirements, milk is initially kept refrigerated, and subsequently subjected to heat treatment such as pasteurisation to reduce the growth of microorganisms. About 17% of milk is used for liquid consumption, with only slight modification such as skimming or homogenisation, but the remainder undergoes extensive processing in the manufacture of the dairy products shown in Fig. 1.

On cooling milk, or on adjusting the pH as during the manufacture of cheese or fermented milk products, there are changes in the mineral and protein equilibria which can affect processing characteristics. Similarly, heat treatment causes various problems, including the formation of deposits on heat-exchangers (Burton, 1968;
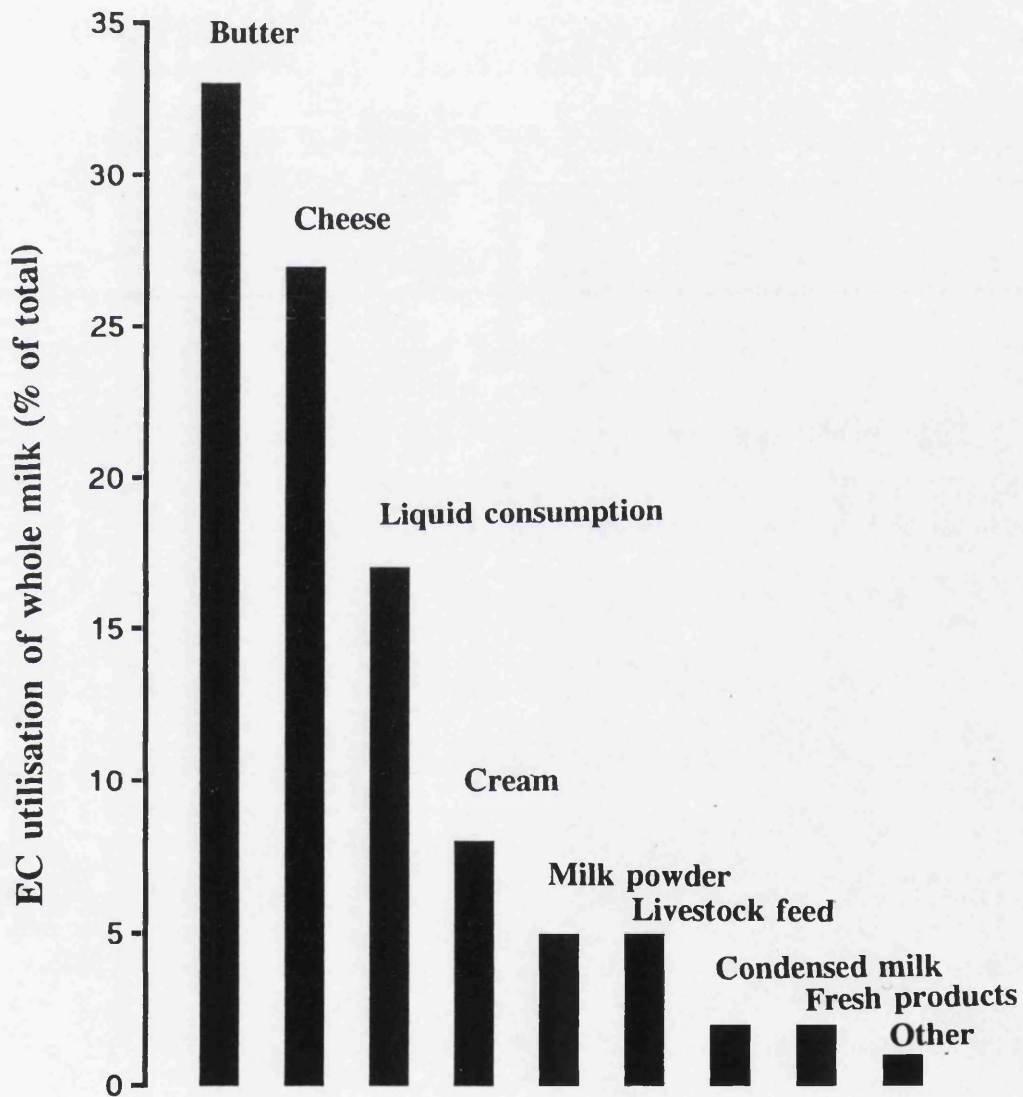
Fig.1 Utilisation of cows' milk in EC countries

Belmar-Beiny and Fryer, 1993), milk instability or gelling on heating during the production of concentrated milks (Singh and Creamer, 1992), and age-thickening of concentrated milks (Harwalkar, 1992). Difficulties are also experienced in the manufacture of specific products, such as cheese, fermented products, milk powders (Singh and Newstead, 1992), and cream liqueurs (Horne, 1992) which may be related to seasonal changes in the milk supply or to changes in the proteins during processing. The manufacture of cheese accounts for about 27% of the milk supply (Fig.1), and there is considerable potential for improving the efficiency of the manufacturing stages, increasing cheese yield, and optimising the ripening process.

This thesis describes the development of rapid, quantitative analytical methods for examining in detail the content and composition of the proteins in the milk of the cow, goat and sheep, and the use of these methods to study natural variation in the individual proteins (Chapters 2-4). The study also involved the application of these analytical methods to examine changes that occur in the proteins during milk processing, including the effect of cooling, pH adjustment (Chapter 5) and heating (Chapter 6). Work was also carried out to examine the increase in cheese yield from heat-treated milk, and to obtain more information about cheese ripening (Chapter 6). These objectives are described in detail at the end of this Chapter.

2. THE CASEINS

Biosynthesis of the main constituents in the milk of the cow, goat and sheep - protein, fat and lactose - occurs in the specialised epithelial cells of the mammary gland. Most of the milk proteins are synthesised under close genetic control from free amino acids in the secretory cells, but between 5 and 10% of the proteins, including some of the immunoglobulins, serum albumin, the transferrins and some other minor proteins, originate in the blood (Mepham *et al.*, 1992).

The milk proteins can be separated by isoelectric precipitation into two main groups - the caseins and the whey proteins. The caseins, which in the cow, goat and sheep amount to about 80% of the total protein, are defined as the group of phosphoproteins precipitated from skim-milk at pH 4.6 and 20°C (Eigel *et al.*, 1984).

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Table 1. Content and composition of protein in milk from creameries in South-West Scotland. (Values are means of 29 samples from 5 creameries)

	Conc g/l	Total protein %	Casein %	Whey protein %
Total protein	32.7			
Total casein	26.9	82.2		
Total whey protein	5.8	17.8		
CASEINS				
α_{s1}-Casein	10.3	31.3	38.1	
β-Casein	9.6	29.3	35.7	
α_{s2}-Casein	2.7	8.4	10.2	
κ-Casein	3.5	10.5	12.8	
γ-Casein	0.9	2.7	3.2	
WHEY PROTEINS				
β-Lactoglobulin	3.1	9.6		54.2
α-Lactalbumin	1.2	3.8		21.2
Serum albumin	0.5	1.4		7.8
Ig, Lactoferrin, Proteose peptones	1.0	3.0		16.8

Data from Davies and Law (1980)

Table 2. Amino acid compositions of the commonly occurring bovine caseins

Amino acid		α_{s1}-CN B-8P	β-CN A^2-5P	α_{s2}-CN A-10P	κ-CN A-1P
Asp	D	7	4	4	4
Asn	N	8	5	14	8
Thr	T	5	9	15	15
Ser	S	8	11	7	12
SerP	S	8	5	10	1
Pyroglu	E	0	0	0	1
Glu	E	25	18	25	12
Gln	Q	14	21	15	14
Pro	P	17	35	10	20
Gly	G	9	5	2	2
Ala	A	9	5	8	14
1/2 Cys	C	0	0	2	2
Val	V	11	19	14	11
Met	M	5	6	4	2
Ile	I	11	10	11	12
Leu	L	17	22	13	8
Tyr	Y	10	4	12	9
Phe	F	8	9	6	4
Lys	K	14	11	24	9
His	H	5	5	3	3
Trp	W	2	1	2	1
Arg	R	6	4	6	5
Total		199	209	207	169
M.W.		23,614	23,981	25,149	19,037

Data from sequences

```
  1  R P K H P I K H Q G   L P Q E V L N E N L   L R F F V A P F P E
 31  V F G K E K V N E L   S K D I G S E S T E   D Q A M E D I K Q M
 61  E A E S I S S S E E   I V P N S V E Q K H   I Q K E D V P S E R
 91  Y L G Y L E Q L L R   L K K Y K V P Q L E   I V P N S A E E R L
121  H S M K E G I H A Q   Q K E P M I G V N Q   E L A Y F Y P E L F
151  R Q F Y Q L D A Y P   S G A W Y Y V P L G   T Q Y T D A P S F S
181  D I P N P I G S E N   S E K T T M P L W
```

Fig.2 Primary structure and sites of phosphorylation of α_{s1}-casein B-8P

S: Phosphoserine

Mercier *et al.*(1971)

```
                                                                  ▼
  1  R E L E E L N V P G   E I V E S L S S S E   E S I T R I N K K I
 31  E K F Q S E E Q Q Q   T E D E L Q D K I H   P F A Q T Q S L V Y
 61  P F P G P I P N S L   P Q N I P P L T Q T   P V V V P P F L Q P
                                  ▼     ▼
 91  E V M G V S K V K E   A M A P K H K E M P   F P K Y P V Q P F T
121  E S Q S L T L T D V   E N L H L P P L L L   Q S W M H Q P H Q P
151  L P P T V M F P P Q   S V L S L S Q S K V   L P V P E K A V P Y
181  P Q R D M P I Q A F   L L Y Q Q P V L G P   V R G P F P I I V
```

Fig.3 Primary structure of β-casein A²

S : Phosphoserine

▼ Position of plasmin attack to give γ-caseins

Ribadeau-Dumas *et al.*(1972)

```
  1  K N T M E H V S̲ S̲ S̲   E E S I I S̲ Q E T Y   K Q E K N M A I N P
 31  S K E N L C S T F C   K E V V R N A N E E   E Y S I G S̲ S̲ S̲ E E
 61  S̲ A E V A T E E V K   I T V D D K H Y Q K   A L N E I N Q F Y Q
 91  K F P Q Y L Q Y L Y   Q G P I V L N P W D   Q V K R N A V P I T
121  P T L N R E Q L S̲ T   S̲ E E N S K K T V D   M E S̲ T E V F T K K
151  T K L T E E E K N R   L N F L K K I S Q R   Y Q K F A L P Q Y L
181  K T V Y Q H Q K A M   K P W I Q P K T K V   I P Y V R Y L
```

Fig.4 Primary structure of α_{s2}-casein A-10P

S̲: phosphoserine

Brignon *et al.* (1976)


```
  1  E E Q N Q E Q P I R   C E K D E R F F S D   K I A K Y I P I Q Y
 31  V L S R Y P S Y G L   N Y Y Q Q K P V A L   I N N Q F L P Y P Y
 61  Y A K P A A V R S P   A Q I L Q W Q V L S   N T V P A K S C Q A
 91  Q P T T M A R H P H   P H L S F M A I P P   K K N Q D K T E I P
121  T I N T I A S G E P   T S T P T T E A V E   S T V A T L E D S̲ P
151  E V I E S P P E I N   T V Q V T S T A V
```

Fig.5 Primary structure of κ-casein A

S̲: Phosphoserine

▼ Position of chymosin attack

Mercier *et al.* (1972a)

This group includes α_{s1}-, β-, α_{s2}-, κ- and the γ-caseins. The whey proteins remain in solution at pH 4.6 and include β-lactoglobulin, α-lactalbumin, serum albumin, lactoferrin and the immunoglobulins. The proteose peptones are usually also classed as whey proteins, but this fraction contains mainly peptides formed by proteolysis of the caseins. The main peptides, namely components 5, 8 slow and 8 fast, are derived from β-casein (Andrews and Alichanidis, 1983). Typical values for the concentrations and relative amounts of the individual caseins and whey proteins in creamery milks are shown in Table 1.

The caseins in the milk of the cow, goat and sheep are all derived from four main polypeptides, α_{s1}-, β-, α_{s2}- and κ- caseins, by changes in the primary structure or by post-translational modifications such as phosphorylation, glycosylation or proteolysis. The amino acid compositions of the commonly occurring bovine polypeptides are given in Table 2; the sequences are shown in Fig. 2-5. The modifications that occur in the main polypeptides are described below. They are summarised in Table 3.

2.1 Changes in Primary Structure

2.1.1 Differences between species

The sequences of the caseins of the cow, goat and sheep have been determined, and it has been shown that there is between 80 and 90% homology of the caprine and ovine caseins with the corresponding bovine caseins (Mepham et al., 1992). Most of the differences are substitutions or deletions of a few amino acids; the sequences of the three species are compared in Chapter 3 (Fig. 10-13).

2.1.2 Genetic polymorphism

Within each species it has been shown that the caseins exhibit genetic polymorphism, which is characterised by the occurrence of closely related polypeptides that differ only

Table 3. Summary of modifications to the bovine caseins

Modification	Casein			
	α_{s1}-	β-	α_{s2}-	κ-
Primary Structure[a]	A B C D E	A^1 A^2 A^3 B C D E	A B C D	A B C E
Phosphorylation[b]	8P 9P	5P C, D variants 4P	10P 11P 12P 13P	1P 2P
Glycosylation				Thr/Ser
Micelle formation				

Proteolysis[c]		γ_1- f29-209
		γ_2- f106-209
		γ_3- f108-209
		
		PP5 f1-105
		       f1-107
		
		PP8S f29-105
		       f29-107
		
		PP8F f1-28
				Para κ-
				f1-105
				
				Macro-
				peptide
				f106-169

[a] Mercier *et al.* (1972); Grosclaude *et al.* (1972); Grosclaude *et al.* (1973); Brignon *et al.* (1977)

[b] Mercier (1981)

[c] Gordon and Groves (1975); Andrews and Alichanidis (1983)

slightly in primary structure. Most instances of genetic polymorphism are due to a change in a single base pair in the DNA, leading to substitution of an amino acid in the corresponding polypeptide. For example, the change in a codon from CCU to CAU alters Pro_{67} in β-casein A^2 to His in the A^1 variant (Grosclaude *et al.*, 1973).

Larger changes involve the addition or deletion of amino acids. In bovine α_{s1}-casein A and α_{s2}-casein D (Mercier *et al.*, 1972b; Grosclaude *et al.*, 1979), caprine α_{s1}-caseins D and F (Brignon *et al.*, 1990), and ovine α_{s2}-casein (Boisnard *et al.*, 1991) the differences are caused by fairly large deletions of amino acids in the polypeptides.

The expression of each of the milk proteins is controlled by multiple-allelic genes which are transferred without dominance. The production of a particular protein is controlled by two genes, one from the male and one from the female line; the protein may contain one or both variants. The levels of expression of some genetic variants may differ, however, and there are quantitative differences in the genetic variants of β-lactoglobulin (A > B), κ-casein (B > A), α_{s1}-casein (B > A), α_{s2}-casein (A > D) and the variants of caprine α_{s1}-casein (A,B,C > E > D,F > null). The differences in the levels of expression of bovine α_{s2}-caseins A and D and caprine α_{s1}-caseins D and F have been attributed to splice-site mutations, which lead to deletions of phosphopeptides and, at the same time, reduced rate of RNA processing. On the other hand, the reduced rate of synthesis of caprine α_{s1}-casein E is not related to changes in the amino acid sequence of the protein, and must therefore, as in bovine β-lactoglobulin and κ-casein, be caused by mutations outwith the structural gene.

The genetic variants of bovine caseins are summarised in Table 4; gene frequencies of the common variants in Friesian cows are given in Table 5. Genetic variants of caprine and ovine caseins are described in Chapter 3.

2.2 Phosphorylation

The four main caseins all undergo phosphorylation at serine and, occasionally, threonine residues; it is believed that this occurs during the transfer of completed polypeptide chains from the rough endoplasmic reticulum to the Golgi (Mepham *et al.*,

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Table 4. Differences in the genetic variants of bovine caseins, and their order of occurrence

Casein	Change →	Variant	Change →	Variant
α_{s1}-Cn C	$Gly_{192} \to Glu$	B	Del 14 - 26 $Ala_{53} \to ThrP$	A D
			$Gln_{59} \to Lys$ $Glu_{192} \to Gly$	 E
β-Cn A^2	$Pro_{67} \to His$	A^1	$Ser_{122} \to Arg$	B
			$Glu_{37} \to Lys$ $SerP_{35} \to Ser$	 C
	$His_{106} \to Gln$ $SerP_{18} \to Lys$ $Glu_{36} \to Lys$	A^3 D E		
α_{s2}-Cn A	Del 50 - 58	D		
κ-Cn A	$Asp_{148} \to Ala$ $Thr_{136} \to Ile$	 B		

Grosclaude et al. (1973)
Grosclaude et al. (1979)

Table 5. Gene frequencies of the caseins from Friesian cows in the UK

Casein	Variant	Frequency
α_{s1}-	B	0.93
	C	0.07
β-	A^1	0.66
	A^2	0.24
	A^3	0.04
	B	0.06
α_{s2}-	A	mainly
κ-	A	0.83
	B	0.17

Data from Aschaffenburg (1968)

1992). The differently phosphorylated forms of the bovine caseins are shown in Table 3.

A study of more than fifty phosphorylated hydoxyamino acids in caseins from different species has shown that phosphorylation tends to occur in the tri-peptide sequences - Ser/Thr-X-A-, where X represents any amino acid residue and A is an acidic residue such as Asp, Glu, SerP or ThrP (Mercier, 1981). Closer study of the sequences has shown that the ease of phosphorylation is -Ser-X-Glu > -Thr-X-Glu > -Ser-X-Asp > -Thr-X-Asp (Mercier, 1981). The occurrence of a particular sequence, however, does not necessarily lead to complete phosphorylation, and this has been attributed to changes in the primary and secondary structures of the caseins leading to steric hindrance and less effective binding of the casein kinase. Also, at periods of maximum synthesis, there may be a deficit of casein kinase.

In bovine caseins, partial phosphorylation leads to the existence of α_{s1}-casein with either 8 or 9 phosphoseryl groups. Manson et $al.$ (1977) have shown that an extra phosphorylation can occur at Ser_{41}, where the sequence is -Ser-Lys-Asp. This is a less favourable phosphorylation site, however, and α_{s1}-casein 9P constitutes less than 15% of the total α_{s1}-casein (Manson and Annan, 1975). It has similarly been shown by Brignon et $al.$ (1976) that α_{s2}-casein exists in 10, 11, 12 and 13 P forms; there may also be microheterogeneity, with the polypeptide having a certain number of P groups differently distributed on the available serine and threonine residues.

β-Casein normally has 5 P groups but an interesting change occurs in the β-casein C variant in which the Glu_{37} is replaced by Lys, and phosphorylation is unable to take place at Ser_{35} (Grosclaude et $al.$, 1972). β-casein C, therefore, has only 4 P groups.

κ-Casein normally contains one phosphate residue but Vreeman et $al.$ (1977) have shown the existence of a second phosphorylation site at Ser_{127}. A small degree of phosphorylation is believed to occur at another unknown site (Vreeman et $al.$, 1986).

2.3 Glycosylation

κ-Casein is unique among the caseins in that it undergoes post-translational glycosylation. The degree of glycosylation varies with stage of lactation but normally

about half of the κ-casein is glycosylated (Robitaille *et al.*, 1991); on alkaline PAGE it appears as a series of bands with increasing mobility (Vreeman *et al.*, 1977). The differently glycosylated forms can be separated by anion-exchange chromatography, the more highly glycosylated κ-casein having higher net negative charge (Vreeman *et al.*, 1986). In normal milk the carbohydrate is composed of NeuNAc, Gal and GalNAc (Fournet *et al.*, 1979). Glycosylation occurs at Thr_{131}, Thr_{133}, Thr_{135} (or Thr_{136}), and Ser_{141} (or Thr_{142}) (Jollés *et al.*, 1973). The three main structures that have so far been determined (van Halbeek *et al.*, 1980) are:

(I)   NeuNAcα2 $\rightarrow$ 3Galβ1 $\rightarrow$ 3GalNAcβ1 $\rightarrow$

(II)   Galβ1 $\rightarrow$ 3GalNAcβ1 $\rightarrow$
$$6$$
$$\uparrow$$
$$\text{NeuNAc}\alpha2$$

(III)   NeuNAcα2 $\rightarrow$ 3Galβ1 $\rightarrow$ 3GalNAcβ1 $\rightarrow$
$$6$$
$$\uparrow$$
$$\text{NeuNAc}\alpha2$$
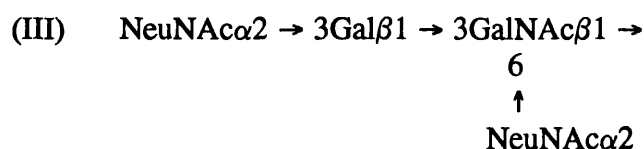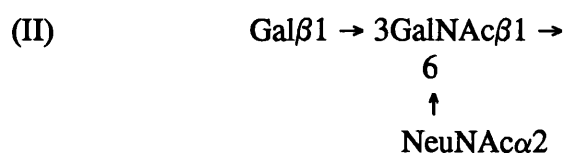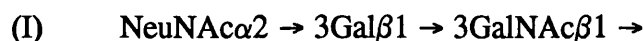
2.4 Casein Micelles

One of the characteristic properties of the caseins is that they associate in the presence of divalent ions such as calcium, and in milk at 37°C most of the caseins are in micellar form, closely linked to calcium phosphate. The micellar structure permits the presence of high concentrations of the caseins in milk without an excessively high viscosity. At the same time the micelles serve to transport high levels of calcium and phosphate which are important in nutrition. The micelles also contain small amounts of magnesium, citrate, sodium and potassium and have a high serum content. The structure and stability of casein micelles have been extensively reviewed (Waugh, 1971; Slattery, 1976; Walstra, 1990; Holt, 1992; and Rollema, 1992).

Casein micelles have a wide size distribution between 10 and 300 nm in diameter, and a volume fraction average of about 150 nm (Holt, 1992). In various studies it has been shown that the relative amount of κ-casein increases, and that of β-

casein decreases with decreasing micellar size (Davies & Law, 1983; Donnelly *et al.*, 1984; Dalgleish *et al.*, 1989).

Casein micelles are remarkably stable but on cooling more than half of the β-casein is able to dissociate out of the micelle into the serum; this is reversible on re-warming to 37°C (Rose, 1968; Downey and Murphy, 1970). Similarly, on acidification, calcium phosphate is removed from the micelles, and the caseins, including the α_{s1}- and α_{s2}-caseins which are normally more tightly bound than β- and κ-casein, are released into the serum (Dalgleish and Law, 1988). Low temperature and pH together have more than an additive effect on dissociation (Rose, 1968).

On treatment with chymosin (or rennet), as in the stomach of the calf or during the manufacture of cheese, the micelles aggregate. This occurs because of proteolysis of the κ-casein Phe_{105}-Met_{106} bond, removal of the caseinomacropeptide (f106-169), and the resulting loss of the normal stabilising effect of the κ-casein (Waugh and von Hippel, 1956).

The arrangement of the caseins, calcium phosphate and citrate within the micelle has been the subject of much research and debate, and the stucture of micelles is discussed in Chapter 5.

2.5 Proteolysis

The caseins have a fairly open conformation (Richardson *et al.*, 1992), and when they dissociate from the micelles they are susceptible to attack by proteolytic enzymes. The breakdown of β-casein by plasmin has been extensively studied. It has been shown that the γ-caseins and most of the proteose peptones, which occur in small quantities in milk, are specific fragments of the β-casein variants (Gordon and Groves, 1975), as shown in Table 3. Three different γ-caseins and five different proteose peptone peptides are derived from β-casein A^2 (Tables 6 and 7); in bulk milk from Friesian cows, corresponding fragments are also produced from β-caseins A^1 and B.

The other caseins, especially α_{s2}-casein, are also partially broken down by plasmin (Noomen, 1975; Eigel, 1977; Barry and Donnelly, 1981; Visser *et al.*, 1989).

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Table 6. Amino acid compositions of proteolytic fragments of β-casein A^2 and κ-caseins A and B

Amino acid	γ_1-A^2 29-209	γ_2-A^2 106-209	γ_3-A^2 108-209	Para-κ-A,B 1-105	CMP A 106-169	CMP B 106-169
Asp	4	2	2	2	2	1
Asn	3	1	1	5	3	3
Thr	8	4	4	3	12	11
Ser	10	7	7	7	5	5
SerP	1	0	0	0	1	1
Pyroglu	0	0	0	1	0	0
Glu	11	4	4	4	8	8
Gln	21	11	11	12	2	2
Pro	34	21	21	12	8	8
Gly	4	2	2	1	1	1
Ala	5	2	2	9	5	6
1/2 Cys	0	0	0	2	0	0
Val	17	10	10	5	6	6
Met	6	4	4	1	1	1
Ile	7	3	3	6	6	7
Leu	19	14	14	7	1	1
Tyr	4	3	3	9	0	0
Phe	9	5	5	4	0	0
Lys	10	4	3	6	3	3
His	5	4	3	3	0	0
Trp	1	1	1	1	0	0
Arg	2	2	2	5	0	0
Total	181	104	102	105	64	64
M.W.	20,521	11,823	11,557	12,268	6,787	6,755

Data from sequences

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Table 7. Amino acid compositions of proteose peptones from β-casein A^2

Amino Acid	PP8F 1-28	PP8S 29-105	PP5 1-105	PP8S 29-107	PP5 1-107
Asp	0	2	2	2	2
Asn	2	2	4	2	4
Thr	1	4	5	4	5
Ser	1	3	4	3	4
SerP	4	1	5	1	5
Pyroglu	0	0	0	0	0
Glu	7	7	14	7	14
Gln	0	10	10	10	10
Pro	1	13	14	13	14
Gly	1	2	3	2	3
Ala	0	3	3	3	3
1/2 Cys	0	0	0	0	0
Val	2	7	9	7	9
Met	0	2	2	2	2
Ile	3	4	7	4	7
Leu	3	5	8	5	8
Tyr	0	1	1	1	1
Phe	0	4	4	4	4
Lys	1	6	7	7	8
His	0	1	1	2	2
Trp	0	0	0	0	0
Arg	2	0	2	0	2
Total	28	77	105	79	107
M.W.	3,478	8,699	12,177	8,964	12,442

Data from sequences

The proteose peptone fraction may contain as many as 38 peptides, most of which are derived by the action of plasmin on the caseins (Andrews and Alichanidis, 1983).

The extent of proteolysis tends to increase towards the end of lactation and when mastitis occurs (Davies and Law, 1977b; Andrews, 1983; Andrews and Alichanidis, 1983); this is believed to be due to an increased permeability of the epithelial tight junctions and an increase in plasmin from the blood (Peaker, 1975; Aaltonen et $al.$, 1988; Politis et $al.$, 1989). The caseins may also be more available for attack, as it has been shown that the dissociation of β-casein from micelles increases towards the end of lactation (Downey and Murphy, 1970).

The specific proteolytic cleavage of κ-casein by chymosin at the Phe_{105}-Met_{106} bond causes aggregation of the casein micelles. This is important in the nutrition of young mammals in that the progress of the resulting curd through the digestive system is slowed. Chymosin action is also an important first step in the production of the curd in cheese manufacture, and has been the subject of considerable research (Dalgleish, 1992). The amino acid compositions of the single para-κ-casein and the two different caseinomacropeptides formed from κ-caseins A and B are shown in Table 6.

Proteolysis of the caseins is of particular interest in the study of flavour development in cheese where the breakdown of the caseins occurs due to a variety of endogenous and added proteolytic enzymes, including plasmin and rennin (Mulvihill and Fox, 1979; Humbert and Alais, 1979; Visser, 1981). This is discussed in more detail in Chapter 6.

2.6 Properties of the individual caseins

2.6.1 α_{s1}-Casein

α_{s1}-Casein is one of the major caseins and amounts to about 30% of the total protein in milk. It is a single polypeptide containing 199 residues; α_{s1}-casein B has a molecular weight of 23,614 (Mercier et $al.$, 1971). Most α_{s1}-casein contains eight phosphate groups, but Manson et $al.$ (1977) have shown from sequence studies that some of the α_{s1}-casein may also be phosphorylated at Ser_{41}. Seven of the phosphate groups and 12

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carboxyl groups of aspartic and glutamic residues occur between residues 43 and 80; this highly acidic segment accounts for most of the negative charge (Table 10). The clustering of the phosphoseryl residues is typical of the calcium sensitive caseins, such as β- and α_{s2}-caseins, and indicates that, together with the Asp and Glu residues, they play an important part in maintaining micelle integrity by their interaction with calcium phosphate (Dalgleish and Parker, 1979; Schmidt, 1980; Holt *et al.*, 1986).

The C-terminal half of the molecule is highly hydrophobic, and this may account for the tendency of self-association of the α_{s1}-casein monomer in aqueous solution (Farrell, 1973). The high proline content of α_{s1}-casein, and the even distribution of proline residues along the molecule indicate that the content of stable α-helical and β-sheet stuctures is low, and the protein has an open conformation. This has been confirmed directly by optical rotatory dispersion, circular dichroism and infrared analysis. These results have recently been reviewed (Holt and Sawyer, 1993; Sawyer and Holt, 1993). As the caseins have not yet been crystallised, however, it has not been possible to study three-dimensional structures using high resolution X-ray diffraction; a more recent approach has involved prediction of secondary structure from the primary structure (Creamer *et al.*, 1981; Richardson *et al.*, 1992).

At the present time four genetic variants are known in European breeds, namely A, B, C and D, and the differences are shown in Table 4 (Grosclaude, 1972). In the Holstein and Ayrshire breeds α_{s1}-casein B is the most common variant (Aschaffenburg, 1968). α_{s1}-Casein A is rare but has been extensively studied because of the effect of the deletion (14-26) on the calcium sensitivity and processing properties (Thompson and Kiddy, 1964; Sadler *et al.*, 1968; Thompson *et al.*, 1969).

2.6.2 β-Casein

β-Casein is also one of the major caseins and constitutes about 30% of the milk-proteins. It is a single polypeptide containing 209 residues, the commonly occurring variant β-casein A^2 having a molecular weight of 23,981 (Ribadeau-Dumas *et al.*, 1972). The polypeptide contains five phosphoseryl groups which are located in the N-terminal end of the molecule, four of the groups being clustered together. This end of the molecule is also rich in Asp and Glu residues and, therefore, contains most of the

negative charge. The total negative charge is, however, less than that of α_{s1}-casein (Table 10).

β-Casein is the most hydrophobic casein, and the C-terminal part of the molecule contains many hydrophobic residues, the distribution being even more pronounced than in α_{s1}-casein. Comparison of sequences of β-casein from different species shows that the polar N-terminal and the hydrophobic nature of the C-terminal domains are conserved and probably have an important biological function (Bonsing and Mackinlay, 1987). β-Casein contains even more proline residues than α_{s1}-casein; it has, therefore, an open conformation (Creamer et al., 1981).

One of the characteristic features of β-casein is that more than 50% dissociates from the micelle as the temperature is lowered and the hydrophobic interactions become weaker (Bigelow, 1967; Downey and Murphy, 1970). This may have an important effect on the processing characteristics of milk that has been refrigerated, especially since the β-casein may go back onto the surface of the micelle on re-warming (Davies and Law, 1983).

At present, the known genetic variants are A^1, A^2, A^3, B, C, D and E, and the differences are summarised in Table 4 (Grosclaude et al., 1972). β-Caseins A^1 and A^2 are common in the Friesian and Ayrshire breeds; β-casein B is present at a much lower frequency (Aschaffenburg, 1968).

β-Casein also readily undergoes proteolysis due to the action of plasmin to form the γ-caseins (Table 6). The complementary fragments - proteose peptones 5, 8 slow and 8 fast - are shown in Table 7 (Gordon and Groves, 1975; Andrews and Alichanidis, 1983). The identification of the γ-caseins as fragments of β-casein is an interesting piece of scientific detective work in which Gordon and Groves (1975) were able to establish a link between the genetic polymorphism of the γ-fragments and the parent β-caseins A^2 and B. For some time it was believed that the γ-caseins might be formed by de novo synthesis due to translation of discrete fragments of β-casein messenger RNA, but it has been established, notably by Kaminogawa and Yamauchi (1974) and Noomen (1975), that β-casein undergoes proteolysis to give γ-caseins when milk is incubated at 37°C. This is due to plasmin which is transported from the blood.

2.6.3 α_{s2}-Casein

α_{s2}-Casein was the last of the main caseins to be characterised because it occurs as differently phosphorylated forms, containing between 10 and 13 phosphate groups per molecule (Brignon *et al.*, 1976), and was difficult to separate from α_{s1}-casein. The polypeptide contains 207 residues, the 11P form having a molecular weight of 25,230 (Brignon *et al.*, 1976).

The protein can also occur as a disulphide-linked dimer due to the occurrence of two cyteine residues per polypeptide (Hoagland *et al.*, 1971). Evidence from mass spectrometry and sequence analysis has shown that all four cysteine residues are involved in disulphide linkage in the dimer; the chains can exist in parallel and antiparallel alignments (Rasmussen *et al.*, 1992). The cysteine residues are close together, and steric hindrance appears to prevent the formation of further polymers. There is, however, some evidence of disulphide linkage with κ-casein (Kudo and Mada, 1983; Rasmussen and Petersen, 1991, 1992).

The protein is the most hydrophilic of the caseins (Table 10), with three regions containing phosphoseryl and glutamic residues, and is extremely sensitive to changes in calcium concentration (Toma and Nakai, 1973). It is similar to α_{s1}-casein in having a high content of phosphoseryl residues and in being tightly bound within the micelle.

Two genetic variants are known (Table 4), but only the A variant is common in the Friesian and Ayrshire breeds (Grosclaude *et al.*, 1979). The D variant differs from the A variant due to the deletion of nine residues, including three phosphoseryl residues. This change is of particular interest because it resembles the deletion of the phosphate centre in caprine α_{s1}-caseins D and F (Chapter 3); it is also associated with a reduction in the level of synthesis of the casein.

2.6.4 κ-Casein

The κ-casein polypeptide contains 169 residues, the B variant having a molecular weight of 19,007 (Swaisgood, 1975). The molecule contains two cyseine residues (Cys_{11} and Cys_{88}), and κ-casein can exist as a series of disulphide linked polymers

containing on average five monomers (Swaisgood *et al.*, 1964; Vreeman, 1979). Three disulphide linkage patterns are possible; these are Cys_{11}-Cys_{11}, Cys_{11}-Cys_{88} and Cys_{88}-Cys_{88} (Rasmussen and Petersen, 1992). The high proline content and uniform distribution of the residues, as in the other caseins, prevent development of extensive secondary structure.

κ-Casein undergoes glycosylation at threonine and serine residues as previously described, and usually contains only one phosphoseryl residue (Vreeman *et al.*, 1986). It is, consequently, not sensitive to calcium ions, and plays an important part in micelle stabilisation (Waugh and von Hippel, 1956). On treatment with chymosin, as in the manufacture of cheese or in the calf's stomach, the Phe_{105}-Met_{106} bond is split to yield the products para-κ-casein (f1-105) and the caseinomacropeptide (106-169) (Wake, 1959; Mercier *et al.*, 1972a, b). Under the appropriate conditions of temperature and ionic strength, the micelles aggregate. Para-κ-casein contains a high proportion of hydrophobic residues, all of the aromatic residues and the two cysteine residues. The macropeptide is very hydrophilic, and also has a very high content of hydroxy amino acids, one in four of the total residues being serine or threonine. The carbohydrate, when present, is located on residues Thr_{131}, Thr_{133}, Thr_{135} (or Thr_{136}), and Ser_{141} (or Thr_{142}) (van Halbeek *et al.*, 1980; Fournet *et al.*, 1979), but does not seem to play an important part in the micelle-stabilising properties of κ-casein (Mackinlay and Wake, 1965).

Two common variants, κ-caseins A and B, occur in the milk of Friesian and Ayrshire cows, and are of particular interest because of differences in the renneting properties of the respective milks (Aschaffenburg, 1968; Jakob and Puhan, 1992).

3. THE WHEY PROTEINS

The whey proteins are defined as the proteins remaining in solution after precipitation of the caseins at pH 4.6 and 20°C (Whitney *et al.*, 1976; Eigel *et al.*, 1984). In the milk of the cow, goat and sheep, the whey proteins include β-lactoglobulin, α-lactalbumin, serum albumin, lactoferrin and the immunoglobulins. The proteose peptones are usually also classed as whey proteins, but components 5, 8 slow and 8

fast (Table 7) are derived from β-casein (Gordon and Groves, 1975; Andrews and Alichanidis, 1983). Commercial whey protein obtained from cheese manufacture may also contain small amounts of casein, the caseinomacropeptide and other proteolytic products from the caseins. The whey proteins of the goat and sheep show close homology with the bovine whey proteins (Chapter 4).

Although their structures are different, the whey proteins are similar in a number of respects. Examination of their amino acid compositions (Table 8) shows that the whey proteins are rich in cysteine residues, and have a high degree of tertiary structure which is maintained by intramolecular disulphide bonds (Fig. 6 and 7). The globular conformation makes the whey proteins less susceptible to proteolysis than the caseins.

On heating, or at extremes of pH, the whey proteins show characteristic denaturation which involves breaking of their internal disulphide bonds, and a progressive loss of globular conformation (de Wit and Swinkels, 1980; de Wit, 1981). If milk is heated above 60°C, the whey proteins become closely associated with the caseins (Smits and van Brouwershaven, 1980; Noh and Richardson, 1989; Mohammad and Fox, 1987), either through hydrophobic interaction (Bonomi and Iametti, 1991) or by formation of new intermolecular disulphide bonds (Singh and Fox, 1987; Singh, 1994). On subsequent acidification to pH 4.6, the denatured whey proteins precipitate together with the caseins (Rowland, 1937a,b; Rowland 1938). Similarly during the manufacture of cheese from heated milk, the denatured whey proteins tend to be retained in the curd, giving a substantial increase in cheese yield (Banks, 1990; Law *et al.*, 1994). The individual whey proteins undergo denaturation at different rates; in terms of loss of solubilty at pH 4.6 the ease of denaturation is immunoglobulins > serum albumin > β-lactoglobulin > α-lactalbumin (Larson and Rolleri, 1955; Dannenberg and Kessler, 1988). The individual whey proteins are discussed below.

3.1 β-lactoglobulin

β-Lactoglobulin is the main whey protein, amounting to about 10% of the total milk protein (Davies and Law, 1980). It is believed to have a physiological function in

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Table 8. Amino acid compositions of the bovine whey proteins

Amino acid	β-Lg B (Monomer)	α-La	Serum albumin	Lactoferrin[a]
Asp	10	13	39	-
Asn	5	8	12	-
(Asx)	-	-	3	62
Thr	8	7	34	37
Ser	7	7	28	44
Glu	16	7	59	-
Gln	9	6	19	-
(Glx)	-	-	1	78
Pro	8	2	28	35
Gly	4	6	16	48
Ala	15	3	46	66
1/2 Cys	5	8	35	35
Val	9	6	36	45
Met	4	1	4	9
Ile	10	8	14	4
Leu	22	13	61	66
Tyr	4	4	19	21
Phe	4	4	27	29
Lys	15	12	59	52
His	2	3	17	10
Trp	2	4	2	15
Arg	3	1	23	35
Total	162	123	582	704
M.W.	18,277	14,178	66,267	79,000

Data from sequences
[a] Buchta (1991)

```
  1  L I V T Q T M K G L   D I Q K V A G T W Y   S L A M A A S D I S

 31  L L D A Q S A P L R   V Y V E E L K P T P   E G D L E I L L Q K

 61  W E N D E C A Q K K   I I A E K T K I P A   V F K I D A L N E N

 91  K V L V L D T D Y K   K Y L L F C M E N S   A E P E Q S L V C Q

121  C L V R T P E V D D   E A L E K F D K A L   K A L P M H I R L S

151  F N P T Q L E E Q C   H I
```

Fig.6 Primary structure of β-lactoglobulin A

Frank and Braunitzer, 1967; Braunitzer *et al.*, 1972

```
  1  E Q L T K C E V F R   E L K D L K G Y G G   V S L P E W V C T T

 31  F H T S G Y D T Q A   I V Q N N D S T E Y   G L F Q I N N K I W

 61  C K D D Q N P H S S   N I C N I S C D K F   L D D D L T D D I M

 91  C V K K I L D K V G   I N Y W L A H K A L   C S E K L D Q W L C

121  E K L
```

Fig.7 Primary structure of bovine α-lactalbumin B

Brew *et al.*, 1970

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retinol binding (Papiz *et al.*, 1986), and a number of reviews on its physico-chemical properties have been published (Sawyer, 1969; McKenzie, 1971; Lyster, 1972; Hambling *et al.*, 1992). The amino acid composition is given in Table 8; the sequence is shown in Fig. 6. The polypeptide of β-lactoglobulin B has 162 residues and a molecular weight of 18,277 (Frank and Braunitzer, 1967; Braunitzer *et al.*, 1972). Between pH 5.0 and 8.0 at 20°C it exists mainly as a dimer; the two polypeptides are held together by electrostatic forces, rather than disulphide linkage (Papiz *et al.*, 1986).

β-Lactoglobulin has a high degree of secondary structure, containing 33% α-helix and 33% β-sheet (McKenzie, 1971; Sawyer and Holt, 1993), and the globular structure is maintained by intramolecular disulphide bonds. The polypeptide contains five cysteine residues and a disulphide bond is formed between residues 66 and 160. A second disulphide bond can occur between residue 106 and either residue 119 or 121 (Fig.6). This effectively leaves a free -SH equally distributed between residues 119 and 121, which can readily form intermolecular disulphide bonds on heating.

β-Lactoglobulin undergoes the characteristic denaturation shown by the whey proteins. On mild heating (20-30°C), it progressively dissociates to the monomeric form (Georges and Guinand, 1960). Above 60°C it loses secondary and tertiary structure, and becomes associated with the caseins (Singh, 1992; Noh and Richardson, 1989). The nature of the interaction between denatured β-lactoglobulin and the caseins is still a matter of speculation, and may be hydrophobic or disulphide linkage, or some combination. The evidence suggests that there is disulphide linkage of β-lactoglobulin, α-lactalbumin, and possibly some of the other whey proteins with κ-casein. This has been extensively reviewed (Sawyer, 1969; McKenzie, 1971; Jang and Swaisgood, 1990).

Six main genetic variants of β-lactoglobulin are known, and the differences are outlined in Table 9. In Friesian and Ayrshire cows, the A and B variants are the most common, having gene frequencies of 0.4 and 0.6, respectively (Aschaffenburg, 1968). These two variants have been closely studied because the A variant is associated with a higher whey protein / casein ratio, and greater heat stability (Aschaffenburg and Drewry, 1957; Dupont, 1965).

Table 9. Location of the amino acid substitutions in the genetic variants of bovine β-lactoglobulin

β-LG variant	Position of change								
	45	50	59	64	78	118	129	130	158
A				Asp		Val			
B	Glu	Pro	Gln	Gly	Ile	Ala	Asp	Asp	Glu
C			His						
D	Gln								
F		Ser[a]					Tyr[b]	Tyr[b]	Gly[a]
G					Met[a]				Gly[a]

[a] Amino acid substitutions not yet established by sequencing

[b] Tyr substitution for Asp occurs at either 129 or 130

Aschaffenburg (1968)
Ng-Kwai-Hang and Grosclaude (1992)

3.2 α-Lactalbumin

α-Lactalbumin is the second most abundant whey protein, amounting to about 4% of the total milk protein. The physico-chemical properties have been extensively reviewed (Gordon, 1971; Lyster, 1972; Brew and Grobler, 1992). The amino acid composition is given in Table 8; the sequence is shown in Fig.7. The polypeptide of the B variant contains 123 residues and has a molecular weight of 14,178 (Brew *et al*, 1970). Some minor forms contain carbohydrate which can be distinguished by polyacrylamide gel electrophoresis (Hopper and McKenzie, 1973). There are eight cysteine residues in the polypeptide, all of which are involved in intramolecular disulphide bonding which maintains the folded globular structure (Vanaman *et al.*, 1970).

α-Lactalbumin bears a striking resemblance to lysozyme in sequence and disulphide bond arrangement (Brew, 1970), but appears to have a very different biochemical role. Lysozyme acts as an anti-microbial glycosidase, whereas α-lactalbumin has a regulatory role in the synthesis of lactose, which in turn controls the volume of the aqueous phase of milk (Peaker, 1977). In lactose synthase, α-lactalbumin promotes the affinity of the enzyme galactosyltranferase for glucose in the incorporation of glucose and UDP-Gal into lactose (Gal β1-4Glucose). This reaction occurs in the Golgi, and an accumulation of lactose, which is one of the most abundant constituents of milk, causes a flow of cellular water into the Golgi to maintain osmotic pressure (Brew, 1970). α-Lactalbumin, lactose and the cellular fluid are then secreted into the milk by exocytosis. This pathway provides a means of linking the hormone-contolled synthesis of α-lactalbumin, and other proteins, with the synthesis of lactose and milk yield. α-Lactalbumin also has the property of binding divalent ions such as calcium (Kronman *et al.*, 1981), but it has not yet been established if this forms part of the regulatory mechanism.

In common with the other whey proteins, α-lactalbumin undergoes unfolding on heating, due to the disruption of intramolecular disulphide bonds (Larson and Rolleri, 1955; Chaplin and Lyster, 1986; Dannenberg and Kessler, 1988). The process reverses more readily than in the other whey proteins, however, and when measured

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in terms of loss of solubility at pH 4.6, α-lactalbumin appears more difficult to denature than the other whey proteins (Larson and Rolleri, 1955; Rüegg *et al.*, 1977).

3.3 Serum albumin

Serum albumin is a minor component of milk protein which is present in the blood, and appears in milk due to increased leakiness of the tight junctions of the epithelial cells of the mammary gland. This may be more pronounced towards the end of lactation or when mastitis occurs, and is generally accompanied by an increase in sodium and a decrease in potassium and lactose in the milk (Kitchen, 1981).

Serum albumin contains 582 residues and has a molecular weight of 66,267 (Brown, 1975). The amino acid composition is shown in Table 8. The protein has a globular structure maintained by 17 intramolecular disulphide bonds, leaving one free sulphydryl group at residue 34 (Eigel *et al.*, 1984). Serum albumin readily denatures on heating (Rüegg *et al.*, 1977) and, because of the size of the molecule and large number of cysteine residues, it may have a proportionately large effect on the behaviour of heated milks.

3.4 The immunoglobulins

The immunoglobulins are present in the milk and blood of all mammals as part of their immune defence system. Four main classes of immunoglobulins have been identified in bovine milk - IgG, IgA, IgM and IgE ; all have a similar basic structure composed of four subunits with a molecular weight that varies around 160,000 (Larson, 1992).

IgG exists in monomeric form, containing two identical light chains (MW each 25,000) and two heavy chains (MW each 55,000) giving a total MW of 160,000.

IgA and IgM are similar in basic structure to IgG, except for an additional 18 amino acids on the C-terminal end of their heavy chains. IgA occurs as a dimer with molecular weight 370,000, whereas IgM is a more complex molecule having a

molecular weight of about 1 million. IgE has a molecular weight of 190,000, and is involved with allergic reactions.

In general, mammals that transfer passive immunity before birth of their offspring (human, rabbit) have lower concentrations and different ratios of the four classes of the immunoglobulins in their milk, compared with those that transfer passive immunity in colostrum after birth, such as the cow, horse and pig (Larson, 1992). The immunoglobulins are easily denatured by heat treatment, readily losing their solubility at pH 4.6 after even mild treatment (Rüegg et al., 1977).

3.5 Lactoferrin

Lactoferrin is an iron-binding glycoprotein which constitutes about 1% of the protein in milk (Harmon et al, 1975). It is of particular interest because it has bacteriostatic properties believed to be the result of its iron-chelating ability, making iron unavailable to bacteria (Masson and Heremans, 1971; Law and Reiter, 1977; Paulsson et al., 1993). The amount in the milk of different species is variable, but usually tends to increase at the end of lactation as involution is occurring, and in the milk of cows affected by mastitis (Harmon et al., 1975). The sequence of lactoferrin is known (Goodman and Schanbacher, 1991; Schanbacher et al., 1993); the protein contains 689 residues. The amino acid composition is given in Table 8. The molecular weight, including carbohydrate, is estimated between 83,000 and 87,000 (Buchta, 1991; Hurley et al., 1993).

3.6 The proteose peptones

The proteose peptone fraction amounts to about 4% of the total protein in milk. It consists of a diverse mixture of polypeptides that remains soluble after heat treatment at 90°C for 30 min, followed by adjustment to pH 4.6 (Rowland, 1938). Andrews and Alichanidis (1983) found that this fraction, which is insoluble in 12% TCA, contained at least 38 components, most of which were derived by the action of plasmin on the

caseins. Of the major components, it has been established that proteose peptone components 5 (f1-105, f1-107), 8 slow (f29-105, f29-107) and 8 fast (1-28) are specific parts of β-casein (Gordon and Groves, 1975; Andrews, 1978a; Eigel and Keenan, 1979; Andrews, 1978b). The fragments derived from β-casein A^2 are summarised in Table 7. In milk from Friesian cows, corresponding genetic variants of the fragments are formed from β-caseins A^1 and B. Proteose peptone 3 (MW 40,800), however, is believed to be derived from the milk fat globule membrane (Ng *et al.*, 1970).

4. SCOPE OF THIS STUDY

The first part of this study involved development of rapid methods for determining the relative amounts of the individual caseins and whey proteins in the milk of the cow, goat and sheep (Chapters 2, 3 and 4). Previously, the most commonly used quantitative methods for analysis of the caseins and whey proteins involved traditional ion-exchange and gel permeation chromatography, which were laborious and time-consuming, and required large samples. Quantitative FPLC (Fast protein liquid chromatography) methods were developed in which the caseins of the three species were separated by anion- and cation-exchange chromatography, and the whey proteins by gel permeation chromatography. The methods were semi-automated and had the advantages that separations required less than 1 hour, much smaller samples could be analysed, and column packing lasted for several thousand fractionations.

In the second part of the work, the analytical methods were used to study natural variation in the composition of protein in the milk of the three species, and to examine factors which might affect processing characteristics. Previous studies had shown that there was variation in the gross composition of the casein and the whey protein. Some work had been published showing the effect on the relative amounts of the proteins, of breed and individual differences (Rolleri *et al.*, 1956), stage of lactation (Larson and Kendall, 1957; Davies and Law, 1977b; Barry and Donnelly, 1980), age and mastitis in the cow (Randolph *et al.*, 1974; Barry and Donnelly, 1981). Similary, seasonal differences had been examined in the whey proteins (de Koning *et al.*, 1974). In general, however, resolution of the earlier methods tended to be poor,

and factors affecting the concentrations and relative amounts of the milk proteins, especially of the goat and sheep, were not well documented. In this study, seasonal changes in the content and composition of casein and whey protein in the milk of the cow and sheep were examined. A further study was carried out on goats' milk to determine the effect of stage of lactation on the concentration of total casein, and the concentrations and relative amounts of the whey proteins.

Part of the work included a study of the effect of genetic polymorphism on the composition of the milk protein. As indicated earlier, even small changes in the primary structure of the caseins or whey proteins can lead to, or be associated with, quite marked changes in the processing characteristics of milk (Feagan et $al.$, 1972; McLean et $al.$, 1984; Jakob and Puhan, 1992; van den Berg et $al.$, 1992). Previous work had indicated that there was an inverse relationship between κ-casein content and micellar size (Sullivan et $al.$, 1959; McGann et $al.$, 1980). Also, there was some evidence that κ- and β-casein variants were associated with different renneting properties (van den Berg, 1992; Jakob and Puhan, 1992), but exactly how these variants affected coagulation time and gel strength was not clear. As a first step in understanding the differences, the effect of κ-casein variants A and B, and β-casein variants A^1, A^2 and B on the composition of whole casein was determined.

The effect of genetic polymorphism of caprine α_{s1}- and κ-caseins on the composition of whole casein was also studied. Earlier work demonstrated the occurrence of an unusual quantitative genetic polymorphism of α_{s1}-casein (Grosclaude et $al.$, 1987; Brignon et $al.$, 1990) in which the production of α_{s1}-casein is controlled by at least 14 possible alleles, three of which are known to give high concentrations (α_{s1}-Cn A, B and C), and four of which give lower concentrations (α_{s1}-D, E and F), or no α_{s1}-casein (α_{s1}-Cn$^\circ$). The large differences in the concentrations of total casein, and in the relative amounts of the caseins associated with the variants, indicated that the corresponding milks might have different renneting properties and give different yields of cheese (Pirisi et $al.$, 1994).

A major part of this thesis deals with the composition, structure and stability of casein micelles and, in particular, with the interaction of the caseins and calcium phosphate. Previous workers had shown that substantial amounts of the casein, especially β-casein, dissociated from the micelles at temperatures below 37°C (Rose,

1968; Downey and Murphy, 1970). In commercial practice, most milk is stored for some time at a temperature of about 5°C, and it seemed likely that this increase in serum casein might have an effect on subsequent processing. In this study, therefore, the effect of cooling milk on the dissociation of β-casein, and the reversibility on re-warming, were examined in detail. Because of the importance of acidification in the manufacture of yogurt and cottage cheese, the combined effect of reducing the temperature and pH on micellar dissociation was also studied. The relationship between micellar size and the composition of the casein and micellar calcium phosphate was also examined.

The final part of this thesis describes changes that occur in the caseins and the whey proteins during various manufacturing processes. Using gel permeation FPLC it was possible to study heat-denaturation of the whey proteins, as determined by the loss of solubility at pH 4.6, occurring during various heat-treatments. The effects of heating temperature, holding time and pH of heating on the extent of denaturation were examined; the rates of heat-denaturation of the whey proteins in the milk of the cow, goat and sheep, were compared.

It was also possible to examine heat-treatment as a means of increasing cheese yield by incorporating denatured whey proteins into the curd during the manufacture of Cheddar cheese. Anion- and cation-exchange FPLC were subsequently used to study the maturation process, and the effect of the whey proteins on cheese ripening.

Anion- and cation-exchange FPLC were also used to examine changes in the caseins, whey proteins and commercial sodium caseinate caused by severe heat treatments. This work has important implications in that the changes observed in the proteins may occur in a number of processes involving severe heat-treatment, such as the production of sterilised milk products (Singh and Creamer, 1992; Harwalkar, 1992).

In order to understand the changes taking place during lactic acid fermentation in the manufacture of yogurt and cottage cheese, a further study was carried out to examine the effect of heat-treatment and acidification on the dissociation of casein micelles.

2

THE COMPOSITION OF BOVINE CASEIN

1. INTRODUCTION

The caseins constitute about 80% of the protein in milk, and are the proteins present in most types of cheese. They are also the main constituents of milk powders and co-precipitates, and are used as sodium and calcium caseinates in the food industry in the manufacture of a wide variety of baked and confectionary products and cream liqueurs (Fox and Mulvihill, 1982; Mulvihill, 1992). As outlined in Chapter 1, however, the individual caseins have different properties, and previous studies have indicated that the concentrations and relative amounts of the individual caseins can vary.

In milk, the caseins occur in micellar form, closely associated with calcium phosphate. The structure and stability of the micelles have an important influence on the processing properties of milk, and may be affected by cold storage, pH adjustment and renneting. Changes in the micelles may lead to various problems during processing, such as heat instability and age thickening of concentrated milks. In order to determine the amount of natural variation in the composition of micellar casein, and to understand the nature of the changes occurring during processing, it is necessary to have rapid, quantitative analytical methods. Previous methods tended to be laborious, and for some purposes did not give satisfactory resolution of individual caseins.

This section describes the development of two complementary methods of anion- and cation-exchange FPLC (fast protein liquid chromatography) for the quantitative fractionation of the bovine caseins, and their application in the study of variation in the composition of casein. These methods had the added advantage that they could be used to separate the common genetic variants of κ- and β-caseins, and were applied to examine the effect of genetic polymorphism in these proteins on the composition of whole casein. Polyacrylamide gel electrophoresis (PAGE) was used to characterise the column fractions, and to identify the genetic variants of κ- and β-caseins.

2. METHODS FOR SEPARATING THE CASEINS

The two methods that have been most commonly used to separate the caseins - electrophoresis and ion-exchange chromatography - are based on charge differences in the caseins at alkaline or acid pH. A summary of the calculated net charge on the caseins and some proteolytic fragments is given in Table 10. At alkaline pH most of the negative charge is due to phosphoserine, Asp, Glu, Cys, Tyr, and the C terminal carboxyl group, whereas at acid pH the positive charge is due to Lys, Arg, His and the N terminal amino group. The net negative charge on the main caseins at alkaline pH is in the order α_{s1}- > α_{s2}- > β- > κ- > γ-caseins.

Because of the tendency for the caseins to associate, separations are usually carried out in the presence of high concentrations of a dissociating agent, such as urea (Wake and Baldwin, 1961). Also, because of the occurrence of polymers of κ-casein (Swaisgood and Brunner, 1963), a reduction or alkylation step is necessary.

The techniques of anion- and cation-exchange FPLC, and alkaline and acid urea-PAGE are discussed below; they are also compared with other methods used to separate the caseins.

2.1 Electrophoresis

2.1.1 Moving boundary electrophoresis

In early studies, moving boundary electrophoresis was used by Mellander (1939) to separate casein into three main fractions, termed α-, β- and γ-caseins, according to decreasing net negative charge. This technique was later applied to study breed differences, individual cow differences and the effect of stage of lactation (Rolleri *et al.*, 1956; Larson and Kendall, 1957). The method was difficult to use, however, and the results are of limited value because of the inability to subfractionate α-casein into its constituent α_{s1}-, α_{s2}- and κ-caseins. This method was replaced by zonal electrophoresis carried out in starch or polyacrylamide.

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Table 10. Properties of the caseins and proteolytic fragments calculated from the amino acid compositions

Casein	M.W.	A 280 coef.[a]	$H\phi$[b]	pI[c]	Charge pH 6.7[c]
α_{s1}-B 9P	23,694	10.3	1061	4.3	-23.2
8P	23,614	10.3		4.4	-21.7
α_{s2}-A 13P	25,388	10.7	997	4.6	-17.7
12P	25,308	10.7		4.7	-16.3
11P	25,228	10.8		4.8	-14.8
10P	25,149	10.8		4.9	-13.3
β-A^2	23,980	4.5	1231	4.6	-13.2
A^1	24,021	4.5		4.8	-12.9
B	24,090	4.5		4.8	-12.0
κ-A 1 Trisaccharide[d]	19,695	9.0	1094	5.0	-5.9
κ-A Non-glycosylated	19,037	9.3		5.1	-4.9
κ-B 1 Trisaccharide[d]	19,663	9.1		5.1	-4.9
κ-B Non-glycosylated	19,005	9.4		5.3	-3.9
γ_1-A^2 (f29-209)	20,521	5.3		5.7	-3.4
γ_2-A^2 (f106-209)	11,823	8.0		7.2	0.9
γ_3-A^2 (f108-209)	11,557	8.1		6.5	-0.4
PP8F (f1-28)	3,478	0		3.0	-10.0
PP5 (f1-105)	12,177	1.2		4.0	-14.3
(f1-107)	12,442	1.1		4.2	-13.0
PP8S (f29-105)	8,699	1.7		4.6	-4.4
(f29-107)	8,964	1.5		5.1	-3.1
Para-κ- (A and B)	12,268	14.5		9.0	3.6
Macropeptide A	6,787	0		3.7	-8.6
Macropeptide B	6,755	0		3.9	-7.6

[a] Molar absorbances Trp, 5,200; Tyr, 1400.   [b] Hydrophobicity, Eigel *et al.*, 1984
[c] pKa SerP, 2.1, 6.8, 12.7; C terminal 3.8; Asp, 4.0; Glu, 4.4; Cys, 9.5; Tyr, 9.6; Lys, 10.4; Arg, 12.0; His, 6.3; N terminal, 7.5; [d] Calculated NeuNAc-Gal-GalNAc-

2.1.2 Zonal electrophoresis

A significant improvement was made by Wake and Baldwin (1961) who carried out electrophoresis in a starch gel at pH 8.6, in the presence of urea (7M) as dissociating agent. These workers obtained patterns with about 20 bands, showing the complexity of whole casein. Electrophoresis was sufficiently sensitive to separate proteins differing in even a single charge, and led to the demonstration of polymorphism in the caseins and whey proteins. In early studies, Aschaffenburg (1961), using paper electrophoresis, established the occurrence of genetic polymorphism in β-casein; Thompson *et al.* (1962), using starch gel electrophoresis, demonstrated genetic polymorphism in α_{s1}-casein.

Swaisgood and Brunner (1963) showed that disulphide-linked polymers of κ-casein could be reduced by the addition of 2-mercaptoethanol. Neelin (1964), Schmidt (1964) and Woychik (1964) were later able to detect genetic polymorphism in the main non-glycosylated κ-casein bands. Peterson (1963) introduced alkaline polyacrylamide gel electrophoresis (PAGE) to examine genetic polymorphism in the caseins and whey proteins. This technique was easier to use than starch gel electrophoresis, and had the additional advantage that the concentrations of polymer (acrylamide) and cross-linking agent (bis-acrylamide) could be adjusted to give different molecular sieving effects. The technique is now widely used, and the mobilities of the most common variants that are known at present are shown in Fig. 8 and 9. Alkaline PAGE does not resolve β-casein A^1, A^2 or A^3, but these variants can be identified by acid PAGE (Peterson and Kopfler, 1966) as shown in Fig.9, and in the fourth paper in this section (Paper 4, Hollar *et al.*, 1991).

Comparison of the electrophoretic patterns of the caseins (Fig.8) with the calculated charge (Table10), shows that the major caseins migrate according to net negative charge, in the order α_{s1}- > α_{s2}- > β- > κ- > γ-caseins. Electrophoretic mobility increases with increasing levels of phosphorylation and glycosylation. The polyacrylamide gel exerts some sieving effects, however, and small, highly negatively charged peptides, such as the caseinomacropeptide and proteose peptone 8 Fast, have higher electrophoretic mobilities than α_{s1}-casein.

Differences in the caseins due to genetic polymorphism are sometimes difficult

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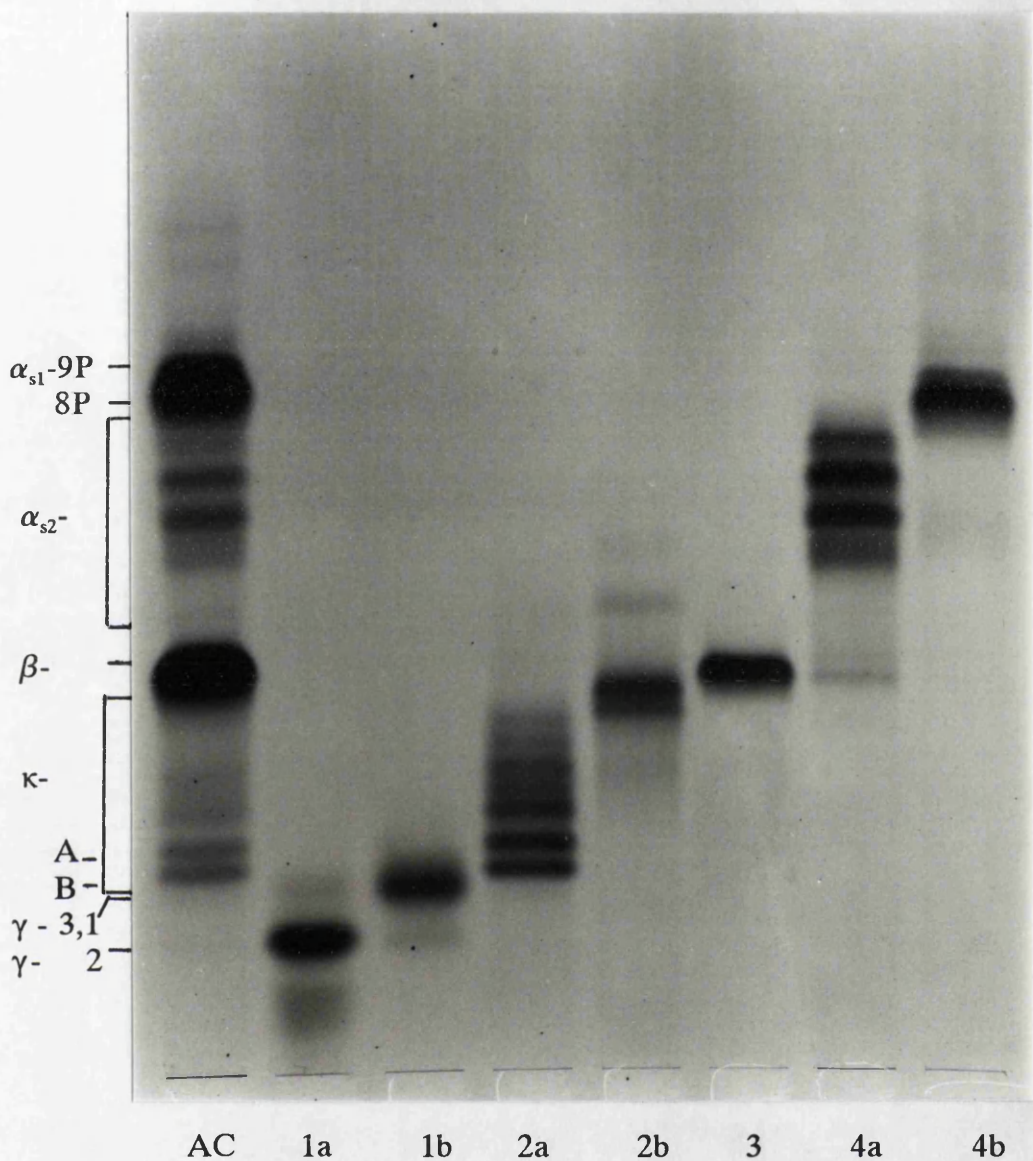
Fig. 8 Alkaline PAGE pattern obtained for whole casein (AC) and the casein fractions obtained by anion-exchange chromatography of whole casein (herd bulk) on DEAE-cellulose, according to the method of Davies and Law (1977a)

ALKALINE PAGE   ACID PAGE

A ——
D ══
αs1–   B ══
C ══
E ——

A ——   —— A   αs2–
αs2–   —— A
D ——   ══ B,C   αs1–   ↑
              —— D

A1,A2,A3 ——   —— A,B   κ–
β–   B ——   —— C
D,E ══   ══ B
C ——   ══ A1,E   β–
                     —— A2
                     —— A3

A ——
κ–   B ——

—   —— Slot
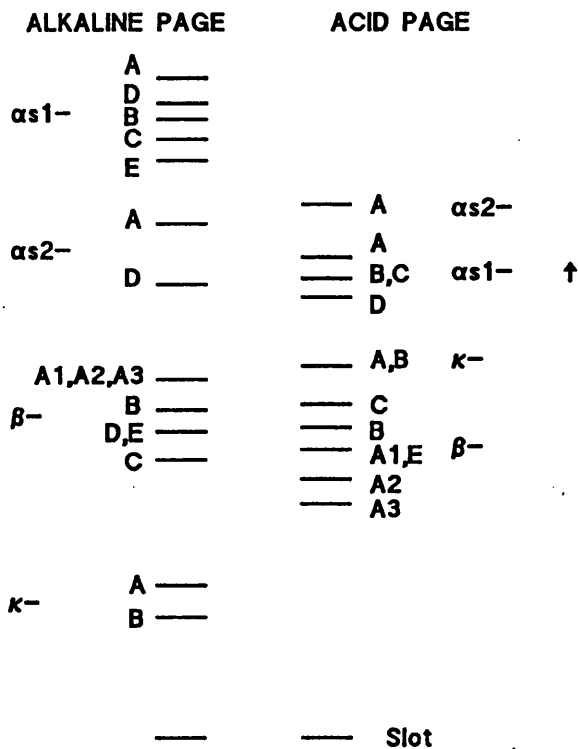
Fig.9 Relative mobilities of the common genetic variants of bovine α_{s1}-, β-, α_{s2}- and κ-caseins on alkaline and acid PAGE

Differently phosphorylated forms not shown

Grosclaude *et al.* (1972)

to detect by electrophoresis at alkaline pH, because of differences in the negative charge due to phosphorylation or glycosylation. The alkaline PAGE pattern for whole casein containing the variants α_{s1}-Cn B, β-Cn A^1 and A^2, and κ-Cn A and B are shown in Fig.8. α_{s1}-Casein can be seen as two prominent bands representing the 9P and 8P forms, in order of decreasing mobility. Similarly α_{s2}-casein is seen as four main bands representing the 13, 12, 11 and 10 P forms, again in decreasing order of mobility. κ-Casein in this sample has only one P group, but the two genetic variants, A and B, are present. The non-glycosylated forms, represented by the two most prominent bands, can easily be distinguished. The mobility of each variant increases with increasing glycosylation to give two series of bands. In this sample, β-casein contains only the A^1, and A^2 variants of β-casein, and is seen as only one band. The B variant can normally be distiguished from the A variants of β-casein at alkaline pH, and all three can be separated at acid pH (Fig.9). The electrophoretic mobilities at acid pH are β-casein B > A^1 > A^2; compared to the A^2 variant, the A^1 has an extra positively charged His, and the B variant has an extra positively charged His and Arg (Table 4).

In the work described in this Chapter, the column fractions and the genetic variants of κ- and β-caseins were identified by a combination of alkaline and acid urea-PAGE, as described above.

A number of workers have obtained quantitative results by photometric scanning of electrophoretic patterns (Kim and Bird, 1972, Dill *et al.*, 1972, McLean *et al.*, 1984). There is, however, some debate about the accuracy of the method, because of the different dye-binding properties of the individual caseins, and because of some binding of proteins to the matrix during electrophoresis. These difficulties can be partly overcome by running standards, but this makes the method more laborious.

2.1.3 SDS-PAGE

When sodium dodecyl sulphate (SDS) is added to a protein, the non-polar end of the molecule interacts with the hydrophobic groups on the protein, and the protein acquires a large negative charge. If proteins treated with SDS are subjected to polyacrylamide electrophoresis, they move towards the anode and, due to the sieving

effect of the acrylamide, are separated according to molecular size. As shown in Table 10, the main bovine caseins have a narrow molecular weight range (19,005-25,388). In theory, they should not be effectively separated by SDS-PAGE, but in practice they are resolved and migrate in decreasing order of mobility κ- > β- > α_{s1}- > α_{s2}-casein (Chapter 3). The anomalous migration of β- and α_{s1}-caseins (MW 23,980 and 23,613 respectively) has been attributed to the uneven distribution of negative charge along the polypeptide chains (Creamer and Richardson, 1984).

The technique is particularly useful for detecting smaller molecular weight proteins such as the γ-caseins and para-κ-casein (Table 10), and this is discussed in Chapter 3. Also, because of the wider molecular weight range of the whey proteins (14,178-1,000,000), SDS-PAGE is useful for identifying the individual proteins (Chapter 4). Similarly, the technique has proved invaluable in the detection of the caprine α_{s1}-casein D and F variants, which have lower molecular weights than the other variants, because of deletions of amino acids (Chapter 3).

2.1.4 Capillary electrophoresis

Recently, capillary zone electrophoresis has been used to separate the milk proteins. Good resolution of the individual caseins and whey proteins has been obtained using fused-silica capillaries (Kanning et al., 1993; de Jong et al., 1993). It has also been possible to separate the genetic variants of κ- and β-caseins and β-lactoglobulin. Negative charge on the silica capillaries can affect the resolution and quantification but this can be reduced by carrying out the separations at acid pH, or at alkaline pH using chemically coated capillaries. The technique is rapid, and separation characteristics can be modified by changing the pH or the type of coating on the capillary tubing. Separation of proteins can also be achieved on a size to charge basis, as in SDS-PAGE separations.

Quantitative values are obtained by direct UV-detection, but so far values have only been expressed on the basis of absorbance area, without correction for absorbance coefficients. A major advantage of the method is that caseins and whey proteins can be separated on a single electrophoretic run.

2.2 Ion-exchange Chromatography

2.2.1 Low pressure chromatography

Apart from electrophoresis, ion-exchange chromatography has been the most widely used technique for separating the caseins. Resolution depends on differences in the charges on the caseins (Table 10), but the separation also depends to some extent on the distribution of charge on the polypeptides, and separation characteristics are difficult to predict. On anion-exchange chromatography, the caseins elute according to their calculated net negative charge, rather than total negative charge, in the order γ-, κ-, β-, α_{s2}- and α_{s1}-casein.

The first successful fractionation of the caseins, in dissociating and reducing conditions, was carried out by Thompson (1966). He used anion-exchange chromatography on DEAE cellulose in imidazole buffer at pH 7.0, containing urea and mercaptoethanol, and separated three fractions consisting of α_s- (α_{s1}- and α_{s2}-), β- and κ-caseins. The separation was achieved because of differential binding of the negatively charged caseins to positively charged groups (diethylaminoethyl) on the anion-exchanger. On applying a gradient of NaCl, κ-casein with the lowest negative charge eluted first, β-casein was intermediate, and the α_s-caseins, having the highest negative charge eluted last (Swaisgood, 1992). Mercier *et al.* (1968) used similar conditions in a preparative method. They also determined the absorbance coefficients of the fractions, and obtained quantitative values for α_s- (α_{s1}- and α_{s2}-), β- and κ-caseins. Rose *et al.* (1969) improved on this method by using an alkylation step, instead of reduction with 2-mercaptoethanol. This avoided the problem of having a large void volume peak caused by oxidation products of 2-mercaptoethanol, and these workers were also able to determine the relative amount of γ-caseins present.

Annan and Manson (1969) used cation-exchange chromatography, under dissociating and reducing conditions, to fractionate whole casein and the α_s-casein complex. The separation was based on the differential binding of positively charged residues (mainly Lys and Arg) on the caseins to negatively charged sulphoethyl groups on the chromatographic matrix. Elution was achieved by a gradient of NaCl; the

caseins eluted in the order β-, κ-, α_{s1}- and α_{s2}-caseins.

Davies and Law (1977a) used anion-exchange chromatography on a highly substituted DEAE cellulose. They separated whole casein into five main fractions which, in order of elution were γ-, κ-, β-, α_{s2}- and α_{s1}-caseins. They used this method to examine variation in the composition of casein due to stage of lactation, individual cow differences, age of the cow, and mastitis (Law, 1977; Davies and Law, 1977b).

This method of anion-exchange chromatography was used in the initial part of the present work to examine seasonal variation in the content and composition of the casein in creamery milks in South-West Scotland (Paper 1, Davies and Law, 1980). The concentrations of total and individual caseins varied considerably over a year; the concentrations of the individual caseins, with the exception of α_{s2}-casein, were considerably higher during the summer, when the cows were at pasture. The concentration of α_{s2}-casein reached a maximum in early summer, and decreased during the summer. The relative amounts of the caseins, however, varied only slightly over the year, and the ranges were as follows: α_{s1}-casein, 37.2-39.0%; β-casein, 34.4-36.6; α_{s2}-casein, 9.2-11.5; κ-casein, 12.1-13.6; γ-casein 2.8-3.9% of the total.

2.2.2 Anion- and cation-exchange FPLC

The ion-exchange methods described above were laborious and time-consuming because columns had to be re-packed frequently, and fractionations typically took about 24 h (Yaguchi and Rose, 1971). A considerable reduction in separation times has been achieved by the introduction of new ion-exchange materials that have small, uniformly-sized beads, good flow charateritics and rapid kinetics. The beads in FPLC (Fast protein liquid chromatography) are about 8 μm in diameter, and typical back-pressures about 2 MPa, whereas in HPLC (High pressure liquid chromatography) bead sizes are 5 μm or less and back-pressures may be of the order of 10 MPa. The beads are substituted with amino or sulphonic acid groups to give anion- and cation- exchangers, respectively. Separation of the caseins is achieved by means of a salt gradient in the same way as described above for DEAE or CM substituted cellulose.

Barrefors *et al.* (1985) and Andrews *et al.* (1985) fractionated whole casein by

FPLC on Mono Q anion-exchanger, which has the positively charged trimethylaminomethyl group attached to the matrix. The casein was reduced with 2-mercaptoethanol and separations were carried out at pH 7.0, in the presence of urea, as dissociating agent. Rapid separations of the main casein fractions were obtained, and the elution profiles were similar to those obtained by traditional anion-exchange chromatography, except that resolution of the α_{s1}- and α_{s2}-caseins was poorer on Mono Q. Using similar conditions, Humphrey and Newsome (1984) fractionated casein on TSK DEAE 5PW anion-exchanger which had the charged diethylaminoethyl group attached to the matrix. They obtained an elution profile similar to that on Mono Q anion-exchanger.

In the present work, a rapid method of anion-exchange FPLC was developed which gave reliable quantitative values for five main casein fractions (Paper 2, Davies and Law, 1987). Separation of the α_{s1}- and α_{s2}-caseins, which was poor in previously published methods (Barrefors *et al.*, 1985, Andrews *et al.*, 1985), was improved by the introduction of an alkylkation step which effectively increased the positive charge on κ- and α_{s2}-caseins. The fractions were characterised by alkaline PAGE, and it was possible to determine the relative amounts of α_{s1}-, β-, α_{s2}-, κ- and γ-caseins, together with several minor fractions. Casein concentrations were calculated from peak areas using calculated specific absorbance coefficients, as shown in Table 10. In order to confirm the accuracy of the method, a comparison was carried out with anion-exchange chromatography on DEAE cellulose. Results showed that there was close agreement between the two methods; anion-exchange FPLC was also much more rapid, less laborious and required smaller samples.

Barrefors *et al.* (1985) and Dalgleish (1986) also fractionated whole casein by FPLC on Mono S cation-exchanger which is substituted with the negatively charged sulphoethyl group. Chromatography of reduced casein samples was carried out at acid pH in the presence of urea, as dissociating agent. The elution profile was similar to that obtained on sulphoethyl Sephadex by Annan and Manson (1969), but separations were obtained in 30 min. The elution pattern was also much simpler than obtained on Mono Q anion-exchanger, as the heterogeneity caused by glycosylation of κ-casein, and by phosphorylation of α_{s1}- and α_{s2}-caseins, did not affect their elution positions on the cation-exchange column.

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In the present study, a quantitative method of cation-exchange FPLC was developed which could be used to complement anion-exchange FPLC in the study of the composition of whole casein (Paper 3, Hollar *et al.*, 1991). Cation-exchange FPLC was basically similar to low pressure cation-exchange chromatography on sulphoethyl Sephadex, described above, but was much quicker. Whole casein was separated into five main fractions containing, in order of elution, γ-, β-, κ-, α_{s1}- and α_{s2}-caseins. Quantitative values were obtained from peak areas using the specific absorbance coefficients shown in Table 10. In general there was good agreement in values obtained by anion- and cation-exchange FPLC.

This method of cation-exchange chromatography had the advantage that it could be scaled up directly as a labraratory preparative method, using S-Sepharose Fast Flow (Paper 5, Leaver and Law, 1992). Results from SDS-PAGE and re-chromatography showed that the main fractions were pure. The method was especially useful for obtaining gram quantities of the different genetic variants of β-casein (A[1], A[2] and B) which eluted in the void volume fraction.

2.2.3 Reverse phase HPLC

The caseins have also been fractionated by reverse phase HPLC, which depends on the affinity of the proteins for non-polar groups (C8 and C18) attached to a chromatographic matrix. Selective elution of the caseins is achieved by a gradient of increasing hydrophobicity. Separations have been obtained at alkaline pH (Carles, 1986), and at acid pH (Visser *et al.*, 1986). The latter workers compared various types of reverse phase columns and, using trifluoroacetic acid and a gradient of acetonitrile, separated whole casein into fractions containing, in order of elution, κ-, α_{s2}-, α_{s1}- and β-caseins. There is some evidence, however, that under certain conditions reverse phase HPLC may be unsuitable for measuring the relative amounts of the individual caseins, because of incomplete recovery of α_{s2}- and κ- caseins (Visser *et al.*, 1986; 1991). These workers found that on certain types of reverse phase columns α_{s2}-casein eluted at two different concentrations of acetonitrile, indicating that there was possibly some interaction with the matrix. Also, there was a tendency for κ- and α_{s2}-caseins, which had not been completely removed from the column, to be eluted during the

following chromatographic run.

2.2.4 Hydrophobic interaction chromatography

Hydrophobic interaction FPLC is based on the same principle as reverse phase chromatography, in that the separation depends on the affinity of the caseins for hydrophobic groups on the chromatographic matrix. In hydrophobic interaction FPLC, however, the caseins are bound to the column under conditions of high ionic strength, which promotes hydrophobic bonding, and eluted using a gradient with decreasing ionic strength. Using this technique, Chaplin (1986) was able to separate whole casein into fractions containing, in order of elution, β-, α_{s2}- (plus γ-), κ- and α_{s1}-casein. Resolution of the method, however, was poorer than that obtained using reverse-phase HPLC.

3. GENETIC POLYMORPHISM OF THE CASEINS

The study of genetic polymorphism in the caseins has been important for two main reasons. Firstly, from segregation studies of alleles, it has been possible to establish that the casein genes are closely linked, and that combinations of alleles are transferred as a group. Secondly, it has become apparent that even very small differences in primary structure can lead to, or be associated with, quite marked differences in the amount and properties of the variants, and in their subsequent processing characteristics (McLean *et al.*, 1984; Jakob and Puhan, 1992; van den Berg *et al.*, 1992).

3.1 Linkage of casein genes

Direct evidence for linkage of the casein genes came from segregation studies of heterozygous bulls which showed that combinations of alleles, such as α_{s1}-Cn B - β-Cn A^2, were transferred to progeny together (Grosclaude *et al.*, 1973). Indirect evidence came from population studies showing the absence of certain combinations of alleles,

for example α_{s1}-Cn C - β-Cn B. Segregation studies also showed that there was a close linkage between the α_{s1}-Cn and κ-Cn loci, but not as close as between the α_{s1}-Cn and β-Cn loci, or the β-Cn and κ-Cn loci. Since the frequency of cross-over that occurs in chromosomes in the diploid state is proportional to the distance between loci, it could be inferred that the genes were arranged in the order α_{s1}-Cn, β-Cn, and κ-Cn (Grosclaude et al., 1973). It has since been confirmed, using a combination of physical mapping techniques, that the four casein genes are located on 200 kb of chromosome 6, in the order α_{s1}-, β-, α_{s2}-, and κ-Cn (Threadgill and Womack, 1990, Mercier and Vilotte, 1993).

From population studies it has been shown that some variants of the caseins are more widely distributed across breeds and species, and must have appeared earlier. It has, therefore, been possible to deduce the order of occurence of the various alleles. This is shown for bovine caseins in Table 4 (Grosclaude, 1988). The corresponding phylogeny of the caprine α_{s1}-casein variants is shown in Table 14. The original combination of alleles of the main caseins is believed to be α_{s1}-Cn C - β-Cn A^2 , as this occurs in European breeds of cattle, in the zebu and yak. Also Gly$_{192}$, which is present in α_{s1}-Cn C of the cow, occurs in caprine and ovine α_{s1}-casein (Ng-Kwai-Hang, 1992). The other combinations of alleles are believed to have occurred due to mutations in the original type; the common combination of alleles found in European cattle - α_{s1}-Cn B - β-Cn A^2 - has arisen by a change in Gly$_{192}$ to Glu in α_{s1}-casein. Grosclaude (1988) has found that, in the Friesian breed, the frequency of the gene combinations (or haplotypes) of the α_{s1}-, β-, and κ- alleles are: α_{s1}-Cn B, β-Cn A^2, κ-Cn A 37.1 %; α_{s1}-Cn B, β-Cn A^2, κ-Cn B 7.2 %; α_{s1}-Cn B, β-Cn A^1, κ-Cn A 31.1; and α_{s1}-Cn B, β-Cn A^1, κ-Cn B 21.4 %. A small number of rarer combinations were also found.

3.2 Genetic polymorphism and processing characteristics

The genetic variants of the caseins have been extensively used as genetic markers in the study and improvement of production characteristics, such as milk yield and fat content (McLean et al., 1984). In these instances, it is unlikely that there is a causal relationship, but there is a more direct link between genetic polymorphism of the

caseins and their processing properties, and the relative amounts and concentrations of the individual caseins.

In Friesian cows, the most common variants are κ-caseins A and B, and β-caseins A^1, A^2 and B. The gene frequencies found in Holstein-Friesian cattle in Britain are: κ-caseins A and B, 0.83 and 0.17; β-caseins A^1, A^2, A^3, and B, 0.66, 0.24, 0.04 and 0.06, respectively (Aschaffenburg, 1968). Various studies have shown that, in cheese-making, κ-casein B is associated with a decrease in clotting time, increased curd tension, better fat retention and higher cheese yield; the phenotypes are ranked BB > AB > AA (Jakob and Puhan, 1992). Similarly the B variant is associated with higher heat stability in normal and concentrated milk (McLean et al., 1987). The exact reasons for the differences in behaviour are not known. It has been shown that the B variant of κ-casein is associated with higher relative amounts of κ-casein in whole casein (Sullivan et al., 1959; McGann et al., 1980), and there is an inverse relationship between κ-casein content and micellar size (Davies and Law, 1983; Dalgleish et al., 1989). Also there are differences in the degree of glycosylation of the two κ-casein variants. For example, one of the points of attachment of the carbohydrate, Thr_{136} of the A variant, is absent in the B variant. Despite this, measurement of N-acetyl neuraminic acid content has shown that the B variant is more efficiently glycosylated than the A variant (Robitaille et al. 1991; Robitaille et al., 1993).

β-Casein variants have similarly been associated with different processing characteristics. The effect of β-casein on the renneting properties of milk are as pronounced as those of κ-casein, but the results have been less consistent, probably in part due to the difficulty in identifying the seven different alleles of β-casein. Milk containing β-Cn B, and especially β-Cn BB milk, was found to have shorter coagulation times, and gave a firmer curd than milk containing only β-Cn A (Feagan et al., 1972; Mariani et al., 1986). The β-casein C variant also gave shorter renneting times than the A variant (Jakob and Puhan, 1992).

The genetic variants discovered so far have been identified mainly by electrophoresis, and differ in charge either at alkaline or acid pH. Clearly a variant that has two residues subsituted in such a way that the overall charge, at alkaline and acid pH, remains the same, would not normally be detected by electrophoresis. Similarly any change in an uncharged amino acid that did not result in a charge difference would

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not be identified by electrophoresis. With the introduction of reverse phase chromatography, it may be possible to separate some of these silent variants. For example, Carles (1986) has identified a variant of β-casein which differs from β-Cn A^1 in the substitution of Pro by Leu.

In the study of genetic polymorphism in κ- and β-caseins described in this Chapter (Paper 6, Law, 1993), the variants of κ-casein were identified by alkaline PAGE. The relative amounts of the individual caseins were determined by anion-exchange FPLC.

Genetic polymorphism of κ-casein had a significant effect on the composition of whole casein; the relative amounts of κ-casein for phenotypes AA, AB and BB were 9.7, 10.9, and 12.1% of the total casein, respectively. This result was confirmed by cation-exchange FPLC. The relative amounts of α_{s1}- and β-caseins varied in the opposite manner (κ-casein AA > AB > BB), whereas the γ-caseins increased with κ-casein, and the relative amount of α_{s2}-casein did not vary significantly. The differences in the relative amounts of κ-casein in the three phenotypes may be particularly important because of the location of κ-casein on the surface of the micelles, and its role in micellar stability.

In a study of genetic polymorphism in β-casein, cation-exchange FPLC was used to separate the commonly occurring variants of β-casein, namely A^1, A^2 and B (Paper 4, Hollar et al., 1991). The identities of the variants were confirmed by acid PAGE, as described by Peterson and Kopfler (1966).

In a subsequent study on the effect of genetic polymorphism in β-casein on the composition of whole casein in Friesian cows (Paper 6, Law, 1993), it was found that the relative amount of β-casein was not significantly affected by the presence of A^1, A^2 or B variants; in the A^1B and A^2B heterozygotes, the levels of expression of the A and B variants were the same.

4. CONCLUSIONS

Anion-exchange chromatography on DEAE-cellulose gave good resolution of the individual caseins, and provided reliable quantitative values for the composition of whole casein. The method, however, required large samples (typically 250mg) and was laborious. Rapid methods of anion- and cation-exchange FPLC were developed, which gave similar resolution of the caseins, and comparable quantitative values.

Using anion-exchange FPLC, it was possible to determine the relative amounts of γ-, κ-, β-, α_{s2}- and α_{s1}-caseins, together with several minor fractions. Separations usually required less than 5mg of sample, and could be completed in about 36 min.

Using cation-exchange FPLC, whole casein could be fractionated into β-, κ-, α_{s1}- and α_{s2}-caseins. It was also possible to separate the A^1, A^2 and B genetic variants of β-casein. Both FPLC methods could be scaled up directly to preparative methods, and cation-exchange chromatography was particularly useful in that it was possible to obtain any of the common genetic variants of β-casein (A^1, A^2 and B) in pure form as the void volume fraction.

In a study of the protein in creamery milks in South-West Scotland, it was found that there was considerable seasonal variation in the concentrations of the total and individual caseins. The relative amounts of the individual caseins, however, showed only small variations.

A combination of anion- and cation-exchange FPLC was used to examine the effect of genetic polymorphism of κ- and β-caseins on the relative proportions of the caseins in whole casein. Genetic polymorphism of κ-casein had a significant effect on the composition of whole casein; the relative amounts of κ-casein in whole casein for phenotypes AA, AB, and BB were 8.9, 10.9 and 12.1% respectively. There were small compensatory changes in the relative amounts of the other major caseins, except α_{s2}-casein. The presence of β-casein genetic variants A^1, A^2 and B appeared to have no significant effect on the composition of whole casein, the A and B variants being produced in equal amounts in the A^1B and A^2B heterozygotes.

The content and composition of protein in creamery milks in south-west Scotland

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SUMMARY. The content and composition of protein in milk samples from creameries in south-west Scotland were determined over a period of 12 months. The composition of the whole casein was expressed in terms of α_{s1}-, β-, κ-, α_{s2}- and γ-caseins, and that of the total milk serum protein in terms of β-lactoglobulins (β-lg), α-lactalbumins, bovine serum albumin, and a mixture of immunoglobulins, proteose-peptone component 3 and lactoferrin (IPL). Concentrations of the individual caseins varied appreciably and for most, concentration was closely correlated with and showed the same seasonal pattern as total casein concentration. Concentrations of the milk serum proteins also varied but only those of β-lg and the IPL fraction were closely correlated with that of total milk serum protein and seasonal trends were not marked. Relative amounts of the individual proteins, on the other hand, showed smaller variations and so throughout the experimental period the milks contained a protein complex of comparatively constant composition. Because of this comparative constancy it would appear that seasonal variations in milk properties in south-west Scotland are unlikely to be determined to a major extent by milk protein composition, but could be more affected by protein concentration.

Statistics published by the Federation of United Kingdom Milk Marketing Boards (1978) show that approximately half of the milk sold off farms in the UK in 1977–78 was utilized for the manufacture of dairy products, with the other half going for liquid sales. Of the milk going to manufacture a considerable proportion, especially in Scotland, was used for the production of cheese, condensed milk, skim-milk powder and casein. The yield of these products depends to an appreciable extent on the protein content of the milk (Zurborg, 1978) and their quality could be influenced by the composition of the protein, i.e. the relative amounts of the various caseins and milk serum proteins present. Values for the contents of total protein, casein and whey protein in commercial milk supplies in England and Wales have been reported by Harding & Royal (1974) and for creamery milks from south-west Scotland by Holt, Muir & Sweetsur (1978), but there appears to be little information on the relative amounts of the individual caseins and milk serum proteins in such milks. Therefore we have carried out a detailed analysis of the protein in creamery milks collected over a period of one year in south-west Scotland and the results are presented here.

MATERIALS AND METHODS

Reagents. Analytical grade materials were used wherever possible and deionized water was used throughout.

Samples. Milk samples were collected over the period February 1977–February 1978 from creameries at Mauchline, Kirkmichael, Lockerbie, Sorbie and Stranraer. Those from the first 2 creameries were obtained at roughly 5-weekly intervals, with a total of 10 samples from each creamery being obtained, and those from the other 3 in February, May and November 1977 only. The samples were representative of bulk supplies ranging from 11 000–180 000 l, the milk having been kept in refrigerated tanks on farms for up to 36 h and then in some instances stored at the creamery for a further 24 h. During transport to the laboratory the temperature of samples was maintained at around 6 °C.

Skim-milk, which was used for all analyses, was obtained by collecting the material from below the fat layer after whole milk had been centrifuged at 20 °C for 30 min at 1000 g.

Preparation and analysis of casein. Samples of whole casein were prepared, alkylated and analysed as described by Davies & Law (1977a).

Preparation and analysis of milk serum protein. Samples were prepared from the filtrate after precipitation of casein at pH 4·6. The filtrate was centrifuged at 20 °C for 30 min at 70 000 g to remove the small amounts of fat, dialysed against deionized water for 48 h at 4 °C to remove lactose and salts, any precipitated protein dissolved by pH adjustment to 7·0, and the solution lyophilized.

The milk serum protein samples were fractionated at 20 °C by gel chromatography on Sephadex G-100, Superfine (Pharmacia, Fine Chemicals AB, Uppsala, Sweden) in 0·02 M-Na_2HPO_4 buffer, containing 0·1 M-NaCl and 0·02 % NaN_3 and with the pH adjusted to 6·7 ± 0·1 with 1 M-HCl. Normally about 50 mg of sample were applied in 5·0 ml buffer to a gel bed 2·5 × 90 ± 1 cm, the flow-rate was maintained at about 10 ml/h and the column eluate collected in 5·0 ml volumes. The protein content of pooled fractions was determined by a micro-biuret method (Itzhaki & Gill, 1964; Davies & Law, 1977a), using the $A_{1\,cm}^{1\,\%}$ values for the various biuret complexes which are given later.

Nitrogen analyses. Solutions for the determination of total, non-casein and non-protein nitrogen (NPN) were prepared by methods based on those of Rowland (1938), except that tungstic acid was used to prepare the filtrate containing NPN only, and their N content was determined by a micro-Kjeldahl procedure. Values for casein N and milk serum protein N were obtained by difference and were converted to values for casein and milk serum protein by multiplying by 6·51 and 6·38 respectively.

RESULTS AND DISCUSSION

Content and composition of milk protein

After an initial separation of the total milk protein into mixtures representing whole casein and total milk serum protein, by adjustment of the pH of milk to 4·6, the mixtures were further partitioned by ion-exchange chromatography and gel chromatography respectively. Ion-exchange chromatography of the whole casein yielded 5 fractions representing α_{s1}-, β-, κ-, α_{s2}- and γ-caseins respectively; the α_{s1} and α_{s2} fractions were previously referred to as $\alpha_{s1,\,0}$- and minor α_s-caseins respectively (Davies & Law, 1977b) and the nomenclature has been altered in the light of work

by Manson, Carolan & Annan (1977) and Brignon. Ribadeau Dumas & Mercier (1976). The levels of cross-contamination in fractions and the recovery of material were the same as reported previously for individual-cow and herd-bulk samples (Davies & Law. 1977a). Gel chromatography of the total milk serum protein produced 4 fractions representing respectively β-lactoglobulins (β-lg), α-lactalbumins (α-la), bovine serum albumin (BSA) (plus very small amounts of bovine serum transferrins and possibly some immunoglobulins), and a mixture of immunoglobulins, proteose-peptone component 3 and lactoferrin (IPL); the $A_{1\,cm}^{1\%}$ values at 310 nm for the biuret complexes of these fractions were 22·8, 21·7, 21·9, and 22·4 respectively. Recovery of material was similar to that reported by Davies (1974) for the albumin fraction of milk serum protein, but the resolution was slightly poorer because of the presence of additional proteo-sepeptones which led to some contamination of the β-lg fraction by material which had the same gel-electrophoretic mobility as proteose-peptone component 5. However, since proteose-peptone component 5 accounts for not more than 25 % of the total proteose-peptone (Andrews, 1978), or about 3 % of the total milk serum protein, the contamination led to only a comparatively small over-estimation of β-lg.

Analysis of variance showed that mean protein values for milks for the individual creameries did not differ significantly and therefore the results for all 29 milks from the 5 different creameries were combined to produce overall average values for south-west Scotland (Table 1). The values should be reasonably representative of much of the milk utilized for the manufacture of dairy products in Scotland since the samples were collected from a region which accounted for about 60 % of the milk produced in Scotland. The dairy cattle in the region were Ayrshire, Friesian or Ayrshire–Friesian cross-bred and the average contents of total protein, total casein and total milk serum protein were in very good agreement with the mean values of 32·29, 26·27 and 6·02 g/l respectively, for milk from breeds other than Channel Islands, which may be calculated from the results of Harding & Royal (1974) when allowance is made for fat and NPN. The average concentrations of the different caseins and the variation in values were very similar to those found for bulk milks from a herd of Ayrshire cows (Davies & Law, 1977b). Few corresponding values for the individual milk serum proteins seem to be available, but the present values were in good agreement with corresponding average values of 3·15, 1·16, 0·81, and 0·43 g/l for β-lg, α-la, IPL and BSA respectively, obtained for 3 samples of bulk milk

Table 1. *The content and composition of protein in creamery milks in south-west Scotland.*
Values are means for 29 samples from 5 creameries

	Skim-milk, g/l		Total protein, %		Total casein, %		Total milk serum protein, %	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total protein	32·71	1·80	—	—	—	—	—	—
Total casein	26·92	1·54	82·2	0·6	—	—	—	—
Total milk serum protein	5·79	0·32	17·8	0·6	—	—	—	—
α_{s1}-Casein	10·25	0·57	31·3	0·5	38·1	0·5	—	—
β-Casein	9·60	0·50	29·3	0·6	35·7	0·8	—	—
κ-Casein	3·45	0·32	10·5	0·5	12·8	0·6	—	—
α_{s2}-Casein	2·74	0·21	8·4	0·5	10·2	0·8	—	—
γ-Casein	0·88	0·15	2·7	0·4	3·2	0·4	—	—
β-Lactoglobulin	3·14	0·19	9·6	0·4	—	—	54·2	1·3
α-Lactalbumin	1·23	0·09	3·8	0·3	—	—	21·2	1·4
IPL	0·97	0·10	3·0	0·2	—	—	16·8	1·2
Bovine serum albumin	0·45	0·04	1·4	0·1	—	—	7·8	0·6

SD, Standard deviation   IPL, Immunoglobulins, proteose-peptone component 3 and lactoferrin.

collected over the same period from The Hannah Research Institute herd. Also. present values for β-lg and α-la agreed well with mean values obtained by Rolleri. Larson & Touchberry (1956), using moving-boundary electrophoresis, for a small number of milks from individual Ayrshire and Holstein cows, and were similar to values for a variety of milk samples obtained by a number of workers using immunological techniques (Larson & Hageman, 1963; Babajimopoulous & Mikolajcik. 1977; Beck & Tucker, 1977). The average value for BSA, however, was considerably higher than that of around 0·25 g/l obtained by Babajimopoulos & Mikolajcik (1977) and was also higher than the 0·3 g/l obtained by Rolleri et al. (1956) for milk from Ayrshire cows.

The average relative values for the various fractions, expressed as percentages of total protein (Table 1), showed as expected that total casein accounted for about 82 °₀ and total milk serum protein for about 18 % of the total protein; NPN accounted on average for 5·5 % of the total N and if this is taken into consideration then casein N and milk serum protein N accounted for 77·5 and 17·0 % respectively of the total N. Generally speaking, the average amounts of the various fractions, expressed as percentages of total protein, fell within the ranges given by Whitney et al. (1976) with the notable exception of α_s-casein which according to Whitney et al. accounts for 45–55 % of the total, whereas in this study the combined amounts of α_{s1}- and α_{s2}-caseins usually accounted for approximately 40 %. The variation in the percentage values was comparatively small with the coefficients of variation for α_{s1}-and β-caseins being around 2 %, those for κ- and α_{s2}-caseins and β-lg being about 5 %, and those for the remaining fractions, apart from γ-casein, being less than 10 %. This relative constancy in protein composition is of considerable interest since it suggests that the normal variation which can be expected in milk protein composition in south-west Scotland makes it unlikely that it is a major cause of the variation which has been observed in the properties of commercial milk supplies in the area at certain times of the year.

The amounts of the different caseins, expressed as percentages of total casein, were very similar to those found previously in herd-bulk milk (Davies & Law, 1977b), with the average over-estimation of κ-casein because of contaminants again amounting to about 2 % of the total casein. Values for γ-casein in the creamery milks were low and the reduction in β-casein values compared with those for herd bulk milks was small, thus indicating that the conditions employed during storage on the farms and at the creameries, and during milk collection, were associated with only a small increase in proteolysis over the very limited amount which occurred during considerably shorter storage periods at The Hannah Research Institute farm. Mean values for the individual milk serum protein fractions, expressed as percentages of total milk serum protein, agreed reasonably well with the corresponding values of 56·7, 21·0, 14·6 and 7·7 % for β-lg, α-la, IPL and BSA respectively which were obtained for Institute herd bulk milk collected over the same period. The value for β-lg was also the same as that found by Rolleri et al. (1956) for Holstein milk, but was considerably lower than their value of 61·3 % for milk from Ayrshires and higher than the 49·6 % found by Aschaffenburg & Drewry (1959) for Friesian milk. The present value for α-la agreed with that of Rolleri et al. while that for BSA was a little higher, and these 2 fractions together represented an amount similar to that found by Aschaffenburg & Drewry for 'residual albumins'. Aschaffenburg & Drewry found also that immuno-globulins and proteose-peptones each accounted for about 10 % of total milk serum proteins, whereas in this study the IPL fraction, which contained most of these proteins plus lactoferrin, represented slightly less than 20 % of the total. A full explanation for the differences between present values for the relative amounts of

milk serum proteins and some of those reported previously is not apparent, but the use of different analytical techniques and differences in sample type are probably major contributory factors.

Interrelationships of milk protein fractions

For the most successful utilization of milk protein in the manufacture of dairy products a knowledge of the interrelationships of the various protein fractions could be of considerable value since it is well known that some proteins interact strongly when, for example, milk is heated. To study such interrelationships, correlation coefficients were calculated and a selected portion of the matrix relating to protein concentrations is presented in Table 2. The values show that there were very highly significant positive correlations ($P < 0.001$) of total casein and total milk serum protein with total protein, and this was also true for the relationships of individual caseins (α_{s2}-casein excepted), β-lg and IPL with total protein. Since total casein accounted for about 82 % of the total protein, the same correlations with coefficients of similar magnitude were found for total casein. The correlations of the individual caseins and total casein, apart from that involving α_{s2}-casein, were generally a little closer than those found for herd bulk and individual-cow milks (Davies & Law, 1977b). This was possibly because variation associated with factors such as stage of lactation was less evident in the present creamery samples. Interrelationships involving total milk serum protein showed that the correlation coefficient with total casein was very similar to the 0.676 found by Rolleri *et al.* (1956) for milks from individual cows, and also that β-lg, the major constituent protein, was very closely related to the total, but that α-la was much less closely related. The correlation between β-lg and α-la ($r = 0.230$) was much poorer than that found by Rolleri *et al.* ($r = 0.845$). Other relationships which could be of importance were the very highly significant positive correlations ($P < 0.001$) between κ-casein and β-lg ($r = 0.595$) and between α_{s2}-casein and α-la ($r = 0.580$), the latter being noteworthy in view of the generally poor relationships between these 2 proteins and the other proteins.

Correlation coefficients for the relative amounts of the various proteins were generally considerably smaller than those for protein concentrations. However, very highly significant negative correlations ($P < 0.001$) were found between amounts of α_{s1}- and α_{s2}-caseins ($r = -0.603$) and β- and κ-caseins ($r = -0.754$), expressed as

Table 2. *Correlation coefficients for concentrations of various protein fractions with concentrations of total protein, total casein and total milk serum protein. Values calculated from results for 29 samples*

	Total protein	Total casein	Total milk serum protein
Total casein	0.991***	—	—
Total milk serum protein	0.786***	0.697***	—
α_{s1}-Casein	0.958***	0.966***	0.676***
β-Casein	0.920***	0.933***	0.628***
κ-Casein	0.926***	0.928***	0.679***
α_{s2}-Casein	0.482**	0.494**	0.281
γ-Casein	0.793***	0.784***	0.632***
β-Lactoglobulin	0.686***	0.601***	0.913***
α-Lactalbumin	0.402*	0.357	0.511**
IPL	0.737***	0.684***	0.797***
Bovine serum albumin	0.229	0.163	0.482**

Significance levels: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.
IPL, Immunoglobulins, proteose-peptone component 3 and lactoferrin.

percentages of total casein, and between β-lg and α-la ($r = -0.618$), expressed as percentages of total milk serum protein. The absence of close relationships between the relative amounts probably arose because of the comparatively small ranges in the values.

Interrelationships were also examined using molar ratios and these produced, for example, average ratios for the caseins α_{s2}: κ:β:α_{s1} of 1.0:1.7:3.7:4.0, for κ:β:α_s of 1.0:2.2:3.0, and for κ-casein:β-lg:α-la of 1.0:0.9:0.5. Whether these ratios have any biological significance is uncertain but they may, as already stated, be relevant in studies dealing with protein interactions and ion-binding.

Seasonal variation in milk protein

Seasonal variations in the properties of milk which may have an influence on the manufacture of various dairy products have been reported by a number of workers (e.g. Dellamonica *et al.* 1965; Chapman & Burnett, 1972; De Koning, Koops & Van Rooijen, 1974; Holt *et al.* 1978). Sometimes the variations have been associated with changes in levels of total protein and casein. Both the total protein and total casein contents of milk are known to vary with season (Waite, White & Robertson, 1956; Rook, 1961; Harding & Royal, 1974), but relatively little information seems to be available on seasonal variations in the concentrations of the individual proteins or their relative amounts in total milk protein. Since these factors could also affect milk properties, values were obtained for the concentrations of individual proteins at regular intervals over a period of 12 months and the results are presented in Fig. 1.

The pattern for the variation in total protein was similar to that reported by Harding & Royal (1974). Low values in late winter and early spring were followed by a considerable increase in values shortly after the cows went out to grass, with the high values being maintained throughout the summer, and then there followed a decrease in late autumn to lower values. The magnitude of the difference between summer and winter values was greater during the experimental period than appears to be normal for the region and was about twice that found in 1976 and 1978. This arose because for some unexplained reason the values at the beginning of 1977 were exceptionally low. The seasonal pattern for total casein was very similar to that for total protein, as were those for the major individual caseins, α_{s1}-and β-caseins. This was also largely true for the patterns for κ- and γ-caseins, but the pattern for α_{s2}-casein differed appreciably in that the high value reached in early summer was not sustained beyond the beginning of July. Generally speaking therefore, the results showed that milk produced when the cows were at pasture tended to be richer in all types of casein, apart from α_{s2}-casein, than milk produced at other times.

When the amounts of the various caseins were expressed as percentages of the total casein, however, little seasonal trend was found. The relative amounts showed only small variations with the overall ranges for the various caseins being as follows: α_{s1} 37.2–39.0%, β 34.4–36.6%, κ 12.1–13.6%, α_{s2} 9.2–11.5% and γ 2.8–3.9%. Thus, the composition of the whole casein remained relatively constant throughout the year even though its concentration varied by nearly 15%.

The pattern for seasonal variation in the concentration of total milk serum protein was similar to those found for total protein and total casein with the amounts in summer milk being greater than those in winter milk (Fig. 1). Trends for the individual milk serum proteins were not very marked but values for β-lg tended to be higher in summer than in winter, which is the opposite of that found by Dellamonica *et al.* (1965), while the pattern for α-la was similar to that for α_{s2}-casein with a peak value at the end of May being followed by a gradual decline to a minimum
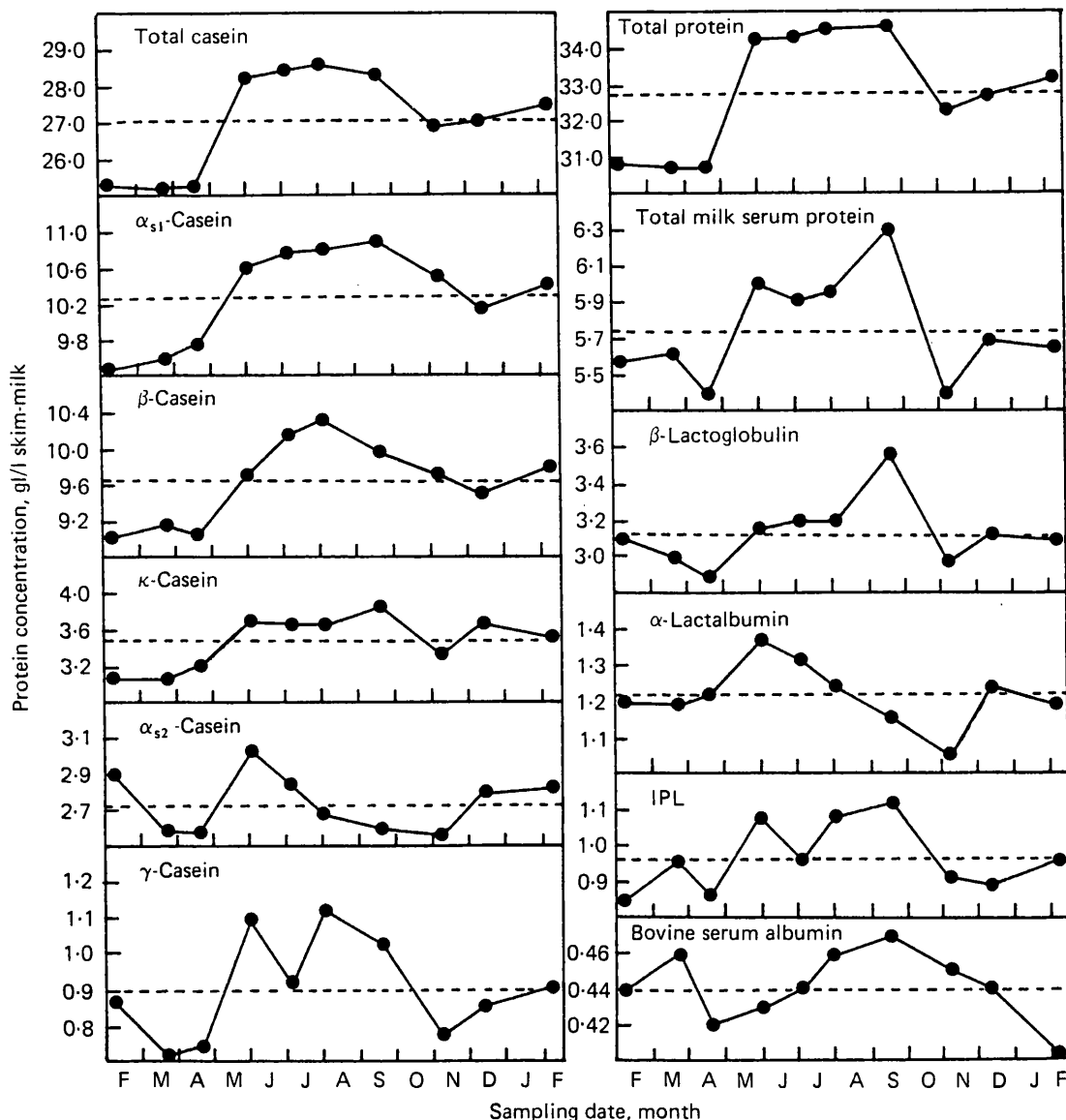
Fig. 1. Seasonal variation in concentrations of total protein, total casein, individual caseins, total milk serum protein and milk serum protein fractions. Values are means for milks from 2 creameries. (– – –, Annual mean values; IPL, immunoglobulins, proteose-peptone component 3 and lactoferrin).

in November. Variation in the IPL fraction led to most of the summer samples giving the higher values, while that for BSA was largely within the limits of experimental error.

As with the caseins, the relative amounts of the individual milk serum protein fractions showed little seasonal trend. The overall ranges for the individual fractions expressed as percentages of the total milk serum protein were as follows: β-lg 52·1–56·4 %, α-la 18·5–22·8 %, IPL 15·3–18·2 % and BSA 7·2–8·3 %. Variations from the annual means were generally within the limits of experimental error, apart from a slight tendency for the relative amount of β-lg to be low in spring and early summer and high in autumn, and for the reverse trend for α-la.

When the present results for milk protein were compared with seasonal variation in some of the properties of the milks such as their heat stability before and after concentration, little apparent interrelationship was found. High concentrations of

total protein, total casein and of the individual caseins for example were associated with both long and short coagulation times and the relative constancy in the composition of the protein moiety of the caseinate complex did not result in a uniform behaviour when it was subjected to heat. This agrees with results of Holt *et al.* (1978) which showed that variation in the composition of the milk serum, especially in urea levels, was a major factor in determining heat stability for most of the year. The results on the heat stability of the milks used in this study will be dealt with in detail elsewhere.

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Quantitative fractionation of casein mixtures by fast protein liquid chromatography

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Summary. Alkylation of whole casein samples by reaction with cysteamine and cystamine in a bis-tris-propane–urea buffer (pH 7·0) followed by fast protein liquid chromatography (FPLC) at 20 °C on a Mono Q HR5/5 column in the same buffer and using a NaCl gradient led to good resolution of the whole casein into fractions representing (i) γ_2- plus γ_3-caseins, (ii) κ-caseins, (iii) β-casein, (iv) α_{s2}-caseins and (v) α_{s1}-caseins, together with small amounts of unidentified materials. Quantitatively the FPLC values agreed well with those for α_{s1}-, β-, α_{s2}- and γ_2- plus γ_3-caseins obtained by ion-exchange chromatography on DEAE cellulose, Whatman DE52 and with those for κ-caseins obtained by gel-permeation chromatography on Sephadex G-150.

The advent of high-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) has allowed the rapid fractionation of protein mixtures and both techniques have been applied to the study of milk proteins (Pearce, 1983; Humphrey & Newsome, 1984; Andrews *et al.* 1985; Barrefors *et al.* 1985). However, the results so far obtained for the caseins suggest that the fractionation is incomplete and in some instances it appears less satisfactory than that achieved by other chromatographic methods, especially with regard to the α_s-caseins. In this study we describe a simple pretreatment of casein mixtures which leads to more effective fractionation by FPLC and the results so obtained are compared with those from chromatography on DEAE cellulose (Davies & Law, 1977) for α_{s1}-, β-, α_{s2}- and γ_2- plus γ_3-caseins, and with those from gel-permeation chromatography (Yaguchi *et al.* 1968) for κ-casein.

MATERIALS AND METHODS

Reagents

Analytical grade materials were used wherever possible and deionized water was used throughout. The 3·3 M-urea solution used in buffer preparation was filtered through a column of DEAE cellulose (Whatman DE1; 2·5 × 40 cm) and stored in glass containers at 4 °C. Buffer solutions used for FPLC were further filtered through a VCWP filter (Millipore (UK) Ltd, London, UK; pore size 0·1 µm, diam. 47 mm) and stored at 4 °C in glass containers. The buffers were warmed to 20 °C and degassed shortly before use.

Dialysis sacs

Sacs were prepared from Visking cellulose tubing which had been freed of impurities by boiling for 30 min in a solution of 0·5 M-Na_2CO_3 and 0·001 M-EDTA. Na_2 and then washed in deionized water.

Whole casein samples

Samples were prepared from skim milk, micellar casein pellets and serum casein as previously described (Davies & Law, 1977, 1983).

Ion-exchange and gel-permeation chromatography

Ion-exchange chromatography was as described by Davies & Law (1977) and gel-permeation chromatography was carried out at 4 °C essentially as described by Yaguchi *et al.* (1968).

Rennet treatment of samples

Rennet (Käselab Pulver; Chr. Hansen's Laboratorium A/S, Copenhagen, Denmark) treatment was done in phosphate buffer (pH 6·5) essentially as previously described for chymosin treatment (Davies & Law, 1977).

Fast protein liquid chromatography (FPLC)

Whole casein samples, which had been alkylated by pretreatment with cysteamine and cystamine, were subjected to FPLC on a Mono Q HR5/5 anion-exchange column (Pharmacia AB, Uppsala, Sweden).

To pretreat material for duplicate fractionations, about 12 mg lyophilized, whole casein were dissolved at 20 °C in 1·5 ml bis-tris-propane-HCl–urea (BTP) buffer (pH 7·0; 0·005 M-bis-tris-propane, 3·3 M-urea) and 1·0 mg cysteamine hydrochloride and 10·0 mg cystamine dihydrochloride added. After 5 min stirring followed by standing for about 1·5 h, the solution was transferred to a small dialysis sac (1 cm diam.) and dialysed at 4 °C against 3 × 100 ml BTP buffer over about 22 h, with the buffer changes being made after 4 h and a further 16 h; this removed excess reagents from the alkylating procedure and other impurities. The sample was then filtered through a GSWP filter (Millipore; pore size 0·22 μm, diam. 13 mm).

The FPLC was carried out at 20 °C with 500 μl sample containing ~ 2·5 mg whole casein being applied to the column which had previously been equilibrated with 10 ml BTP buffer containing 1·0 M-NaCl followed by 5 ml BTP buffer. The sample was washed onto the column with 1 ml BTP buffer and the caseins then eluted, at a flow rate of 1·0 ml/min, with a NaCl gradient formed by mixing BTP buffer containing 1 M-NaCl with the salt-free buffer so that the concentration of NaCl being fed into the column reached 0·10 M after 2 ml gradient, 0·11 M after 9 ml, 0·22 M after 14 ml, 0·28 M after 21·5 ml, 0·295 M after 29 ml and 0·43 M after 35 ml. This resulted in all the casein being eluted in a total volume of 36 ml. To ensure full control of NaCl concentration, it was necessary with our equipment to start each fractionation with the pumps at the beginning of a cycle. This then avoided the momentary drop in NaCl concentration, with the concomitant irregularity in the elution profile, which occurred if the pump supplying BTP buffer containing 1 M-NaCl had to change direction. During elution the absorbance of the eluate at 280 nm was monitored continuously. Between fractionations the column was re-equilibrated with 2 ml BTP buffer containing 1 M-NaCl followed by 5 ml salt-free buffer.

The identity of the eluted materials was established by comparing the elution
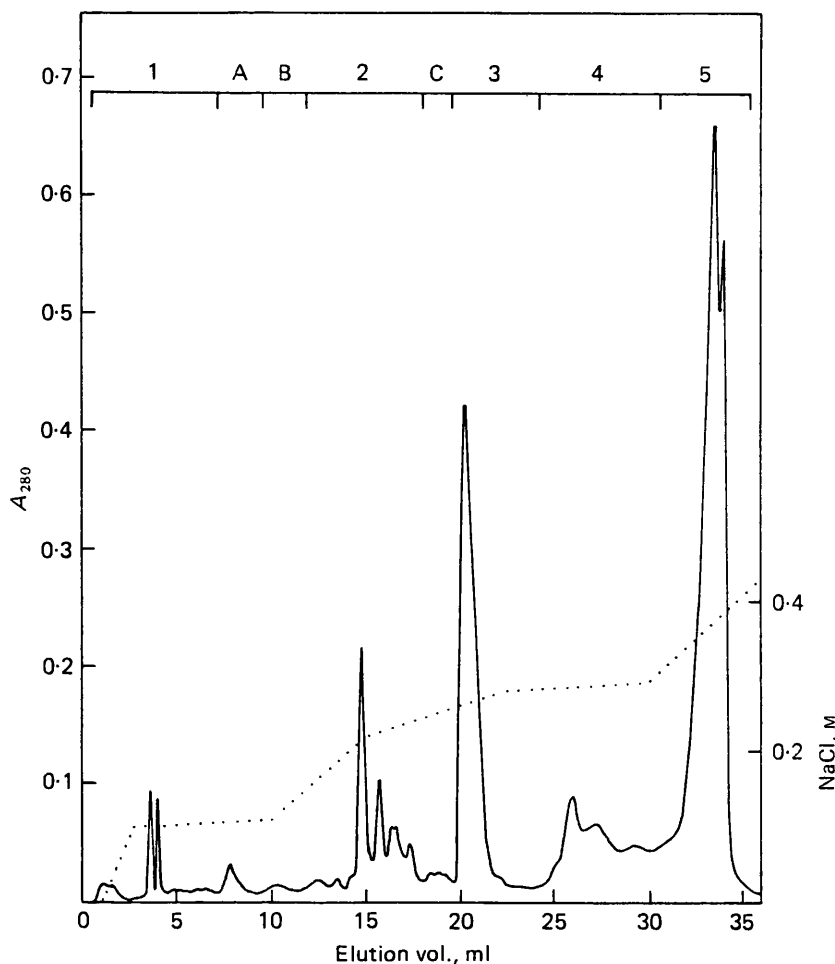
Fig. 1. Elution profile of alkylated whole casein obtained by fast protein liquid chromatography at 20 °C on a Mono Q HR5/5 column and using bis-tris-propane–urea buffer (pH 7·0; 0·005 M-bis-tris-propane, 3·3 M-urea) and a NaCl gradient. About 2·5 mg protein derived from a herd bulk milk were applied to the column and the flow rate was 1·0 ml/min. —, Absorbance; ..., NaCl gradient. Fractions were pooled (1–5 and A, B or C) as indicated.

profile of whole casein with those of pure caseins and rennet-treated caseins, and by electrophoresis in polyacrylamide gel of materials isolated from selected fractions (Davies & Law, 1977). The peak areas of the selected fractions were corrected for the baseline value, which was dependent to some extent on the back pressure of the column and normally amounted to 1–3 % of full scale deflection. The amounts of protein present were then calculated using $A_{1\,cm}^{1\%}$ values at 280 nm of 10·0 for α_{s1}-casein, 4·6 for β-casein, 9·6 for κ-casein, 10·1 for α_{s2}-casein, 8·0 for γ_2- plus γ_3-caseins (β-casein f106–209, β-casein f108–209) and 8·2 for the unidentified fractions. The value of 8·2 selected for the unidentified fractions is that determined for whole casein while the others are representative, literature values for pure caseins.

RESULTS AND DISCUSSION

Development of the FPLC procedure

Preliminary trials showed that the conditions used previously for the fractionation on DEAE cellulose of casein mixtures alkylated with iodoacetamide, namely a 0·005 M-tris buffer containing 6 M-urea, pH 8·6, and a maximum NaCl concentration
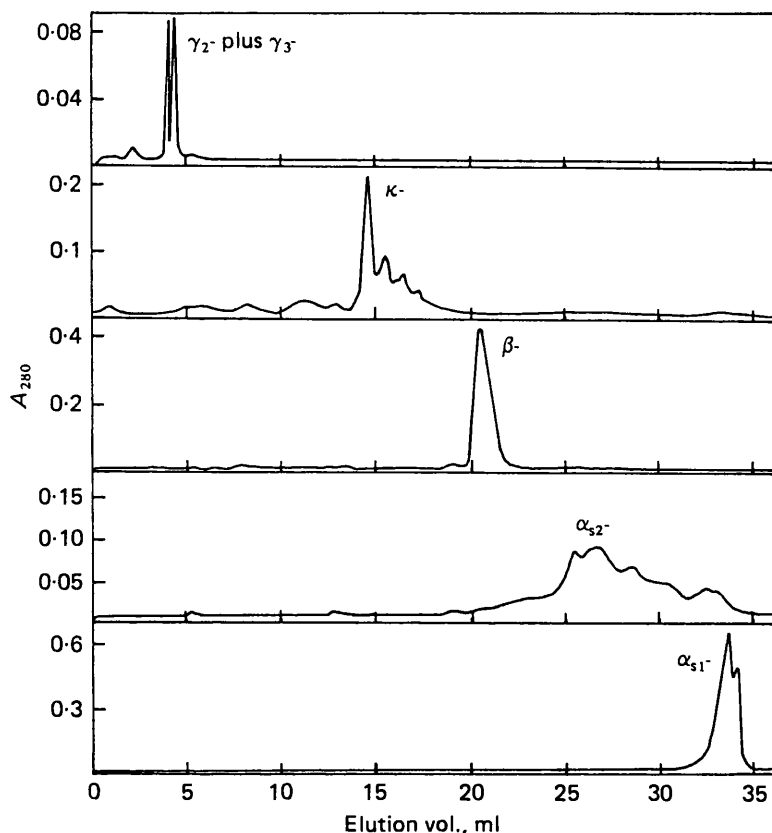
Fig. 2. Elution profiles of individual caseins obtained by fast protein liquid chromatography using the same conditions as in Fig. 1. The γ_2- plus γ_3-, β-, α_{s2}- and α_{s1}-caseins were prepared by ion-exchange chromatography on DEAE cellulose, Whatman DE52 and the κ-casein by gel-permeation chromatography on Sephadex G-150.

of 0·25 M (Davies & Law, 1977), could not be used for FPLC on the Mono Q column because much of the casein remained bound to the strong anion exchanger. While an increase in the final NaCl concentration to 0·35 M led to a release of all the bound casein, the resulting resolution was still poor, especially with regard to the α_s-caseins. Further trials were therefore carried out at pH 7·0 with a reduced urea concentration of 3·3 M as used by Mercier et al. (1968) and these produced a considerably improved resolution with elution profiles showing peaks representing the α_{s2}-caseins clearly discernible. However, quantitation of the α_{s2}-casein peaks showed that they rep- resented only about 8% of the total casein whereas the corresponding value obtained by chromatography on DEAE cellulose was about 13%, e.g. for a sample from herd bulk milk. Slight adjustments of pH, urea concentration or in the shape of the NaCl gradient brought about little or no increase in the value for α_{s2}-casein or in the resolution of the other caseins, but they did reveal that a pH of below 7·0 should be avoided because it induced increased spreading of the leading edges of peaks, so causing increased cross contamination of fractions.

It appeared therefore that a satisfactory resolution could not be achieved by simple manipulation of eluting conditions and so the effect of changing the method used to alkylate the casein was examined. Smithies (1965) describes an alkylation procedure, involving addition of cysteamine and cystamine, which results in the cleavage of protein disulphide bonds and the blocking of the resulting thiol groups with positively charged, aminoethylsulphide side chains. This, when applied to
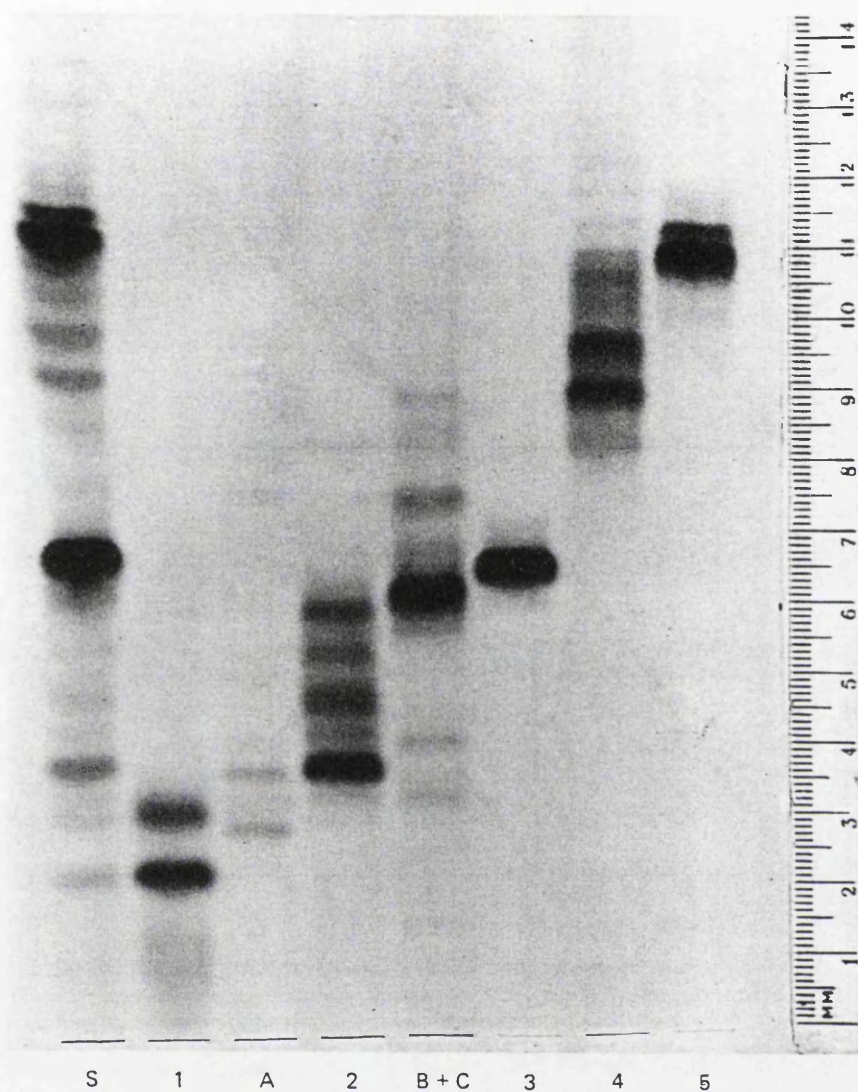
Fig. 3. Gel-electrophoretic patterns for alkylated whole casein (slot S) and for the fractions obtained by fast protein liquid chromatography, designated as in Fig. 1.

casein mixtures, should reduce the net negative charge on α_{s2}- and κ-caseins and facilitate their separation from α_{s1}- and β-caseins respectively and an example of the elution profile obtained for a sample of whole casein treated with cysteamine and cystamine is shown in Fig. 1. The casein was from a herd bulk milk and the resolution is superior to that obtained for reduced casein or casein alkylated with iodo-acetamide. Also, the profile and the value for α_{s2}-casein were similar to those from chromatography on DEAE cellulose (Davies & Law, 1977), which suggested that FPLC of cystamine-treated casein should yield reliable results.

The alkylation procedure could be varied considerably without affecting the casein partition appreciably, with comparable values for the individual caseins being obtained when, for example, the molar ratio of cystamine to cysteamine varied from 2·5 to 10 and when the reaction was carried out at pH 7·0 or 8·6 at 4 or 20 °C. The conditions finally adopted, namely a cystamine : cysteamine ratio of 5 : 1 at pH 7·0 and 20 °C, could be used with either bis-tris-propane or tris buffers. Conditions used for the FPLC of the cystamine treated casein also could be varied. Flow rates of 0·4–1·5 ml/min were satisfactory and sample solutions could be stored for at least
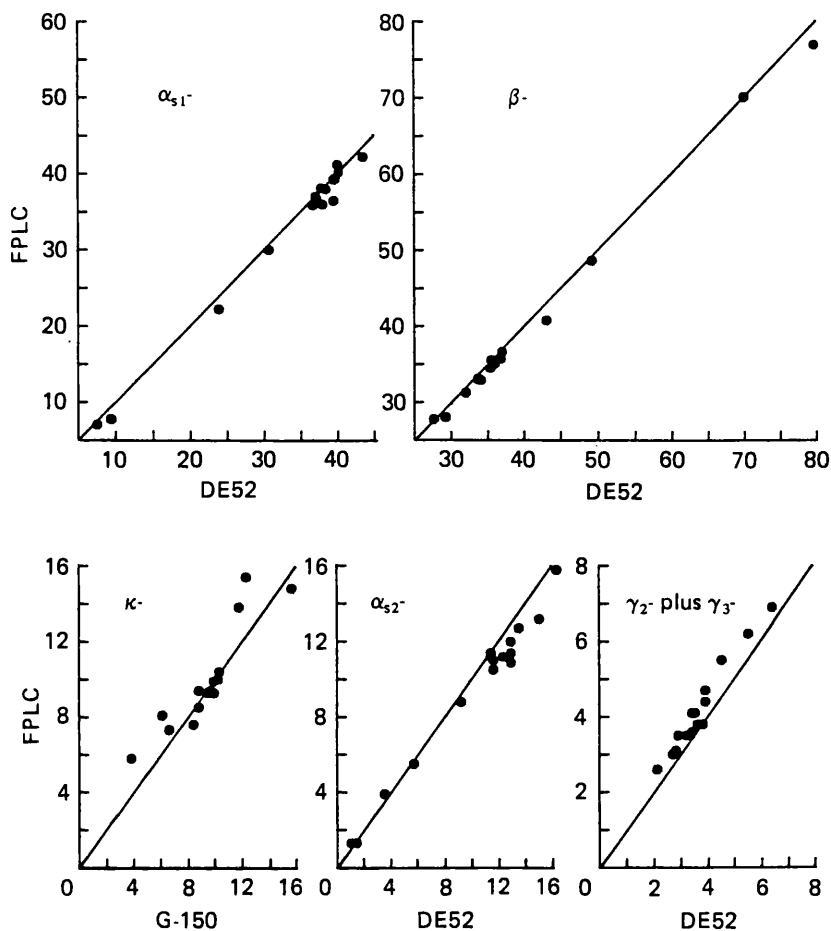
Fig. 4. Relationship between values for individual caseins obtained by fast protein liquid chromatography (FPLC) with those for α_{s1}-, β-, α_{s2}- and γ_2- plus γ_3-caseins from ion-exchange chromatography on DEAE cellulose, Whatman DE52 and those for κ-casein from gel-permeation chromatography on Sephadex G-150. Values are expressed as percentages of total casein; —, 1:1 relationship line.

24 h at 4 °C. Sample weights could range from 1·7–3·3 mg, but for maximum resolution the amount of sample was dependent to a certain extent on the exact nature of the material. For casein from cows in advanced lactation and containing the B variant of κ-casein, for example, the sample load had to be in the lower part of the range if the κ-casein was to be separated completely from other materials. Furthermore, when the composition of the sample differed markedly from that of normal whole casein precipitated at pH 4·6, it was sometimes necessary to carry out exploratory trials to establish the best conditions for FPLC.

Characterization of the materials separated by FPLC

The elution profile (Fig. 1) was obtained by FPLC of about 2·5 mg whole casein alkylated with cystamine and is representative of most samples isolated from skim milk. From results obtained by FPLC of pure caseins (Fig. 2), the profile for the whole casein was divided into a number of segments as indicated and comparison of Figs 1 and 2 shows that segment 1 represents a mixture of γ_2- and γ_3-caseins, segment 2 κ-caseins, segment 3 β-casein, segment 4 α_{s2}-caseins and segment 5 α_{s1}-caseins. The profile shows also a number of minor features, designated A, B and C. The exact nature of these was not established, but segments B and C coincide with the elution volumes of much of the chymosin resistant material in fraction 2b (Davies & Law,

1977); this was believed to represent partly dephosphorylated β-casein together with some breakdown products of other caseins.

Materials representative of the various FPLC fractions were also collected and. after dialysis and lyophilization, yielded on gel electrophoresis the patterns shown in Fig. 3. Generally speaking the patterns corroborate the results from elution profiles and in addition they show that cross contamination in the fractions was low. The patterns for 1, 2, 3 and 5 were those expected for γ_2- plus γ_3-, κ-, β- and α_{s1}-caseins respectively, but that for fraction 4, which is believed to represent α_{s2}-caseins. is considerably different from the corresponding one showing four discrete bands previously obtained (Davies & Law, 1977). The reason for the difference was not established, but it did not appear to originate in the alkylation procedure or in the temperature used for FPLC. The patterns for the unidentified fractions show that the combined fraction B+C has, as a major component, material with a mobility slightly less than β-casein which may represent the partly dephosphorylated protein, while one of the bands in the indistinct pattern for A suggests that the fraction could contain some γ_1-casein.

While the characterization of some of the FPLC fractions remained incomplete. the above results indicate that the FPLC system did provide a reasonably satisfactory method for the fractionation of casein mixtures. The inability to identify the materials in fractions A, B and C is not a serious drawback since in most samples they together represented less than 5% of the total casein although in some samples of whole casein from cows in advanced lactation and in some serum casein they accounted for up to 7%.

Quantitative analysis by FPLC and comparison of values with those obtained by other methods

FPLC of a variety of whole casein samples isolated from skim milk, and from micellar casein pellets and supernatant serum casein separated by ultracentrifugation, produced very good agreement between duplicate fractionations. Results for 15 samples showed that average differences between replicate analyses, for values expressed as percentages of total casein, were 0.43 ± 0.10, 0.32 ± 0.07, 0.07 ± 0.02, 0.36 ± 0.08 and 0.24 ± 0.07 percentage units for α_{s1}-, β-, κ-, α_{s2} and γ_2- plus γ_3-caseins respectively, and 0.21 ± 0.07 for the combined unidentified fractions. Recovery of material applied to the column varied from 95 to 110% which was reasonably satisfactory considering the very dilute solutions ($\sim 0.007\%$ protein) involved.

Comparison of FPLC values with those obtained by chromatography on DEAE cellulose (Davies & Law, 1977) for α_{s1}-, β, α_{s2}- and γ_2- plus γ_3-caseins and by gel-permeation chromatography (Yaguchi *et al.* 1968) for κ-casein gave correlation coefficients of 0.996, 0.999, 0.993, 0.977 and 0.900 respectively, which were very highly significant ($P < 0.001$). The good agreement between results is depicted in Fig. 4. Over wide ranges, values for α_{s1}- and β-caseins fell close to the line indicating a $1:1$ relationship between methods. This was true also for most κ-casein values but for some samples of serum casein, FPLC values were considerably higher because of the inclusion of some contaminant which was not sensitive to chymosin. With regard to α_{s2}-casein, the tendency for the FPLC to give slightly lower values was of little practical importance since in 90% of samples the difference in values by the two methods was < 1.5 percentage units. The slightly higher values by FPLC for γ_2- plus γ_3-caseins again were of very little practical significance since the difference amounted to < 1.0 percentage unit in all samples. The closeness of the agreement

generally obtained between the different methods does therefore indicate that the FPLC method can provide reliable, quantitative values for the different caseins and since FPLC can be carried out rapidly with small amounts of sample it offers several advantages over previous methods for the analysis of casein mixtures.

We thank Dr C. Holt for helpful discussion, especially in relation to casein alkylation.

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Separation of Major Casein Fractions Using Cation-Exchange Fast Protein Liquid Chromatography[1]

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ABSTRACT

Whole casein was separated into β-casein, κ-casein, α_{s1}-casein, and α_{s2}-casein fractions using cation-exchange fast protein liquid chromatography. The γ-caseins and several unidentified peaks were also separated. A urea-acetate buffer at pH 5 and a NaCl gradient from 0 to .26 M were used to separate the casein fractions. Several γ-caseins and unidentified fractions eluted first, followed by three β-casein peaks, several γ-casein and unidentified peaks, κ-casein, α_{s1}-casein, and α_{s2}-casein. Some γ-caseins eluted with β-casein. The four major caseins, which accounted for over 90% of the whole casein fractions, were accounted for with this method, and the calculated compositions correlated well with values obtained using anion-exchange fast protein liquid chromatography at pH 7.
(Key words: casein separation, fast protein liquid chromatography)

Abbreviation key: FPLC = fast protein liquid chromatography, UA = urea acetate.

INTRODUCTION

Many fractionation techniques have been developed for whole casein. These techniques vary in their ability to both separate and quantify the amounts of the four major casein components (α_{s1}-, α_{s2}-, β-, and κ-caseins).

Gel electrophoresis has been used to separate major and minor casein fractions (2, 12). Recent developments have shortened the time needed to produce and run PAGE gels (3, 10, 13), and densitometry has been used to quantify protein bands. Differential staining of the different proteins and nonreproducible destaining procedures make it difficult to produce repeatable results using this technique.

Reverse-phase HPLC has also been used to quantify the different casein fractions (4, 14, 15). Quantitative reproducibility is good, but it is difficult to separate the four major casein components. α_{s1}-Casein and α_{s2}-casein tend to coelute in the procedure used by Walsh (15). In the method of Carles (4), there is poor baseline separation between the major casein components, which may indicate cross-contamination. α_{s1}-Casein and α_{s2}-casein coelute in hydroxyapatite, anion-exchange chromatography, and reverse-phase HPLC procedures (14). Using anion-exchange chromatography, α_{s2}-casein elutes both before and after α_{s1}-casein, and there is tailing between some major peaks. With the Bio-Gel TSK-Phenyl RP+ column, β-casein elutes with α_{s1}-casein in a series of peaks, but the Hi-Pore reverse-phase, type RP-318 column shows good separation of κ-, α_{s2}-, α_{s1}-, and β-caseins, although γ-casein elutes with the β-casein.

Use of ion-exchange chromatography on anion-exchange materials has resulted in good separation and quantification of the major caseins (5, 7, 9, 11). Anion-exchange fast protein liquid chromatography (FPLC) using a Mono Q column at pH 7 (8) provides good separation and quantification of the major casein components with slight cross-contamination of α_{s1}- and α_{s2}-caseins, although the

intermolecular disulfide bonds in κ- and α_{s2}-caseins must be modified by treatment with cysteamine and cystamine. Small amounts of unidentified material and some γ-caseins are eluted with the series of κ-casein peaks. The procedure can be used to confirm the presence of κ-casein A and B genetic variants (6) but cannot quantify them. Cation-exchange FPLC using a Mono S column at pH 3.8 has also been used (1). This procedure rapidly separates β-, κ-, α_{s1}- and α_{s2}-caseins, although there is some loss of resolution between the major peaks in the published method due to the steep NaCl gradient required for the short (less than 20 min) separation time.

MATERIALS AND METHODS

Milk collected from the morning milking of the Hannah Research Institute herd was skimmed by centrifugation at 2000 × g for 45 min at 5°C. The skim milk was twice filtered through a double thickness of Whatman GF/A glass fiber filter paper (Whatman, Clifton, NJ). The skim milk was warmed to 30°C and acidified to pH 4.6 by adding 1N HCl with constant stirring. The precipitated casein was collected by filtration through Whatman 113V filter paper, then washed with distilled water, and refiltered twice. The precipitate was resuspended in distilled water and continuously titrated to pH 6.7 by adding 1N NaOH until the casein had dissolved. The casein was lyophilized and stored at −20°C.

The FPLC analyses of casein samples using anion-exchange chromatography on a Mono Q HR5/5 column (Pharmacia AB, Uppsala, Sweden) were performed using the method of Davies and Law (8).

Casein samples were fractionated using cation-exchange chromatography on a Mono S HR5/5 column (Pharmacia AB, Uppsala, Sweden). Urea-acetate (UA) buffer containing 6 M urea and .02 M acetate was used for cation-exchange chromatography. The buffer was made to volume and adjusted to pH 5 with 1N HCl. The UA buffer containing 1 M NaCl was prepared by dissolving NaCl in UA buffer prior to pH adjustment.

Samples for cation-exchange chromatography were prepared by dissolving about 15 mg

of lyophilized casein in 2 to 5 ml of UA buffer and treating this with 10 μl of β-mercaptoethanol at 20°C. The mercaptoethanol was required to break up disulfide-linked polymers of κ- and α_{s2}-caseins. Because the reaction is inefficient at pH conditions below 6, the pH of the samples was adjusted to 7 with 1N NaOH. After stirring for at least 1 h, samples were adjusted back to pH 5 with 1N HCl and filtered through a .2-μm Acrodisc® LC PVDF syringe filter (Gelman Sciences, Northampton, England) prior to analysis. Samples (500 μl, containing about 4 mg of whole casein) were applied to the column, which had previously been equilibrated by eluting 10 ml of UA buffer containing 1 M NaCl followed by 5 ml of UA buffer. The sample was washed onto the column with 1 ml of UA buffer and was eluted at a flow rate of 1 ml/min with the absorbance of the eluate continuously monitored at 280 nm. A gradient of NaCl was formed by mixing UA buffer containing 1 M NaCl with salt-free buffer so that the concentration of NaCl going into the column remained at 0 M for 2 ml, reached .025 M after 2.1 ml, .075 M after 16.5 ml, .15 M after 26.5 ml, and .26 M after 42.5 ml. All the applied casein was eluted in 45 ml. To maintain full control of the NaCl gradient, it was necessary to start each fractionation with both pumps at the beginning of their cycles; this prevented a momentary drop in NaCl concentration and corresponding irregularity in the elution profile when the pump containing UA buffer with 1 M NaCl changed direction. To reequilibrate the column and return both pumps to the beginning of a cycle, 8.8 ml of UA buffer containing 1 M NaCl followed by 4.6 ml of UA buffer were passed through the column.

To establish the identities of peaks found in the chromatogram, individual casein fractions were collected from chromatography of whole casein on a Mono Q column and run on a Mono S column following dialysis against UA buffer and reduction with β-mercaptoethanol. Selected fractions from chromatography on Mono S were also run on an alkaline urea-PAGE gel. Electrophoresis followed the procedure of Davies and Law (7). The 4.5% polyacrylamide gels containing 4.5 M urea and Tris-EDTA-barbitone buffer were run for 6 h

at 200 V following a prerun of 30 min at 300 V to remove impurities.

Visking cellulose dialysis tubing was prepared by boiling for 30 min in a solution containing .5 M Na_2CO_3 and .001 M Na_2-EDTA and rinsing in deionized water.

To compare quantitative estimates from Mono S and Mono Q, previously purified samples from the Hannah Research Institute laboratory were used (7). These covered a range of casein composition and included herd bulk whole, individual cow whole, and serum and micellar caseins from fresh and cooled milks.

RESULTS AND DISCUSSION

Preliminary attempts to fractionate casein by chromatography on the Mono S column were made at pH 3, 4, and 5 using a formate-based buffer. Artifact peaks diminished as the pH of the buffer system increased, so a buffer at pH 5 was selected as optimal for the analysis. Because formate does not buffer effectively at pH 5, acetate was used as the buffering agent. By flattening the NaCl gradient in the major peak regions and making it nonlinear, it was possible to get additional peak separation while minimizing the time required to separate the casein fractions. A final concentration of .26 M NaCl was needed to elute the entire casein sample. The chromatogram for a typical separation is shown in Figure 1. After the breakthrough peak (containing mainly β-mercaptoethanol), the chromatogram consisted of a series of four major peaks, separated by a number of small contributions. They were divided as shown in Figure 1. The region defined as A contained variable amounts of material. In Figure 1, there is only a small amount, but in some samples larger quantities of minor peaks were eluted in this region. Fraction 1 contained the first major peak and a minor peak immediately following it; preliminary electrophoretic studies showed that the two peaks had similar electrophoretic mobility, and they might be genetic variants of β-casein. Some minor peaks were grouped together in fraction B; they may be related molecules because they eluted at similar times.

To help identify fractions obtained from chromatography on the Mono S column, whole
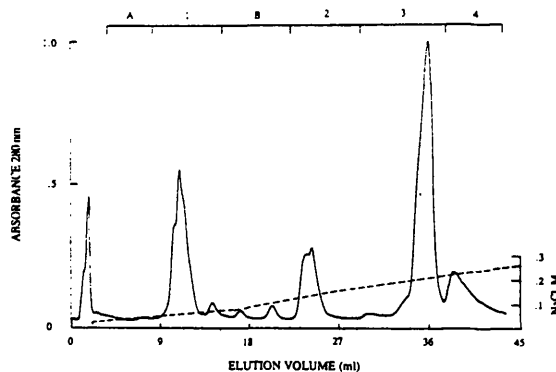
Figure 1. Elution profile of herd bulk whole casein obtained by fast protein liquid chromatography at 20°C on a Mono S HR5/5 column using urea-acetate buffer (pH 5.0; .02 M acetate; 6 M urea) and an NaCl gradient. About 2 to 5 mg of protein were applied to the column, and the flow rate was 1 ml/min. Absorbance (—); NaCl gradient (– – –). Fractions were pooled (1 to 4 and A or B) as indicated.

casein was separated, and fractions were collected using a Mono Q anion-exchange column according to the method of Davies and Law (8). After dialysis and reduction, these defined fractions were run on the Mono S cation-exchange column. Chromatograms of the fractions are shown in Figure 2. The four major casein peaks showed good separation although there was some cross-contamination between the α_{s1}- and α_{s2}-casein peaks. This can be explained because anion-exchange chromatography has difficulty separating these two caseins. Comparing Figures 1 and 2, the first major peak on the chromatogram obtained using cation-exchange chromatography on a Mono S column (fraction 1) is β-casein. κ-Casein is the next major peak (fraction 2), and the third major peak (fraction 3) is α_{s1}-casein. There are some small components at the start of fraction 3, before the α_{s1}-casein peak, possibly due to varying degrees of α_{s1}-casein phosphorylation. The last major peak (fraction 4) is α_{s2}-casein. The entire casein sample eluted in 45 ml.

The procedure of Davies and Law (8) provides a $\gamma_{2,3}$-casein fraction as well as three unidentified peaks; all are minor fractions. The $\gamma_{2,3}$-casein and the unidentified A and B fractions from Mono Q eluted on either side of the
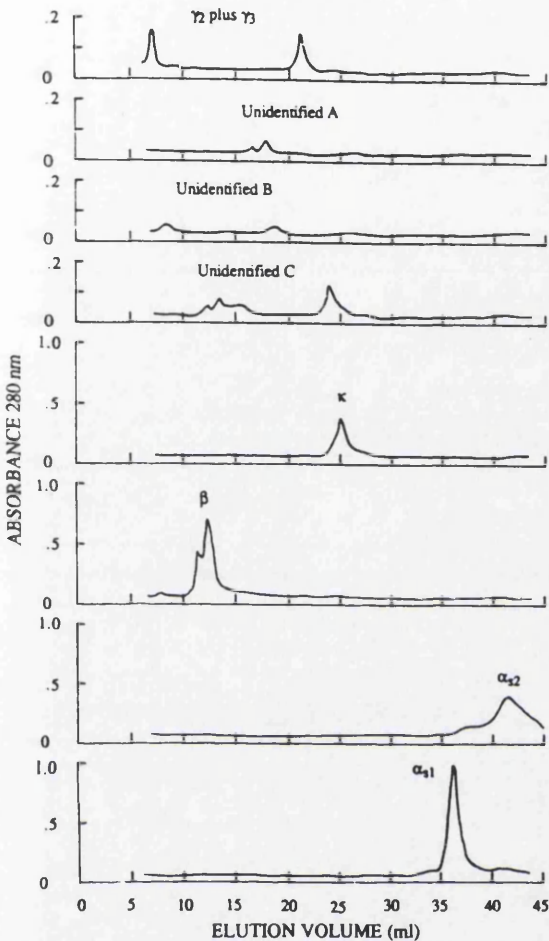
Figure 2. Elution profiles of individual caseins obtained by fast protein liquid chromatography (FPLC) on Mono S using the same conditions as in Figure 1. The identified fractions were prepared by FPLC on a Mono Q HR5/5 column using bis-Tris-propane–urea buffer (pH 7; .005 M bis-Tris-propane; 3.3 M urea) and an NaCl gradient (8).

Figure 3. Alkaline gel-electrophoretic patterns for whole casein (lane S), beginning peaks (lane BP) and fractions obtained by fast protein liquid chromatography, designated as in Figure 1.

β-casein peak in Mono S chromatography. There did not appear to be cross-contamination among these three fractions and either β- or κ-casein. The unidentified C peak from Mono Q appeared to be composed of a mixture of β- and κ-caseins. This was expected because a PAGE of this fraction showed cross-contamination, and the κ-casein series of peaks tailed into the unidentified C peak on Mono Q while the β-casein peak tailed out of it.

The fractions collected from Mono S chromatography shown in Figure 1, along with the breakthrough peak and a whole casein sample, were run on an alkaline urea-PAGE gel as shown in Figure 3. The breakthrough peak contained small amounts of unidentified material, possibly proteose-peptones or other peptide fractions. Fraction A, although it appeared as a separate peak on the Mono S chromatogram, showed one band with the same mobility on the alkaline gel as the fraction 1 (β-casein band. Fractions 1 and 3 were overloaded to see if other proteins were coeluting with the major peak. Fraction 1 (β-casein) peaks showed three very light bands that are thought to be γ-caseins. Because γ-caseins are breakdown products of β-casein, it is likely that some of them have the same retention on cation-exchange chromatography but different mobility on PAGE. Fraction B showed several light bands that may be either γ-caseins or other unidentified components. Both fractions A and B were minor components of the whole casein sample. Fraction 2 was confirmed to contain only κ-casein; the series of bands
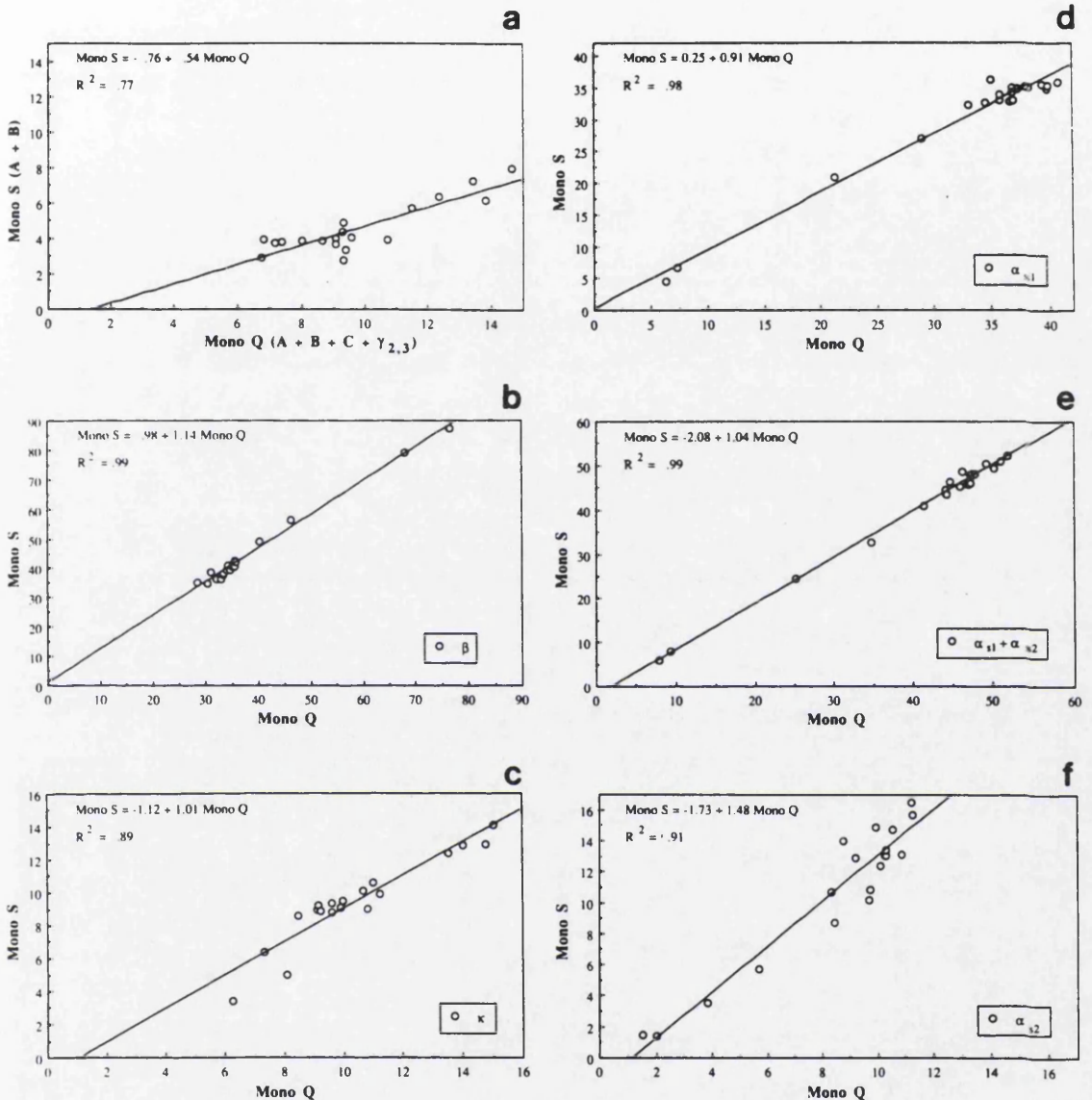
Figure 4. Relationship between values for individual caseins (β-, κ-, α_{s1}- and α_{s2}-casein), α_{s1}- and α_{s2}-casein combined, and minor fractions obtained by fast protein liquid chromatography (FPLC) on a Mono S HR5/5 column with those obtained by FPLC on a Mono Q HR5/5 column. Values are expressed as percentages of total casein; best fit lines are indicated (—).

resulted from varying degrees of glycosylation of κ-casein. α_{s1}-Casein was the major component in fraction 3 with very slight cross-contamination with α_{s2}-casein and a number of small unidentified bands. The final fraction was α_{s2}-casein. There was some tailing between the two α_s-casein peaks on the chromatogram, so some cross-contamination was

expected, but the fractions on the PAGE indicate that there was very little.

We ran samples of varying casein composition and measured peak areas with both anion-exchange chromatography on Mono Q and cation-exchange chromatography on Mono S. Absorbance extinction coefficients for these peaks were the same as those used by Davies

and Law (8). The breakthrough peak on Mono S could not be included in the calculations, although it contained small amounts of protein, because β-mercaptoethanol coeluted and had a variable absorbance. Figure 4 shows the correlations between Mono S and Mono Q chromatography for the calculated compositions of the major casein fractions. Because the unidentified and $\gamma_{2,3}$-casein fractions from Mono Q tended to elute in both the unidentified A and B areas on Mono S, the minor peaks for both columns were grouped together. Correlation between Mono S and Mono Q for the minor components was poor. The Mono S values were low because the proteins in the breakthrough fraction were not included in the calculations and small amounts of γ-casein coeluted with β-casein. The Mono Q values were higher because the unidentified C fraction contains both β- and κ-caseins. More work with the minor fractions of both procedures is required.

There was excellent correlation between Mono S and Mono Q procedures for β-casein (Figure 4). Mono S values were slightly higher because small amounts of γ-casein coeluted, and the Mono Q values were slightly low because some of the β-casein coeluted with unidentified C.

Correlation between the methods for κ-casein was fair (Figure 4). The Mono Q values were higher than those from Mono S. On Mono Q, a small amount of κ-casein eluted with unidentified C, which suggests that the κ-casein values should be low. However, amounts of γ-casein or other unidentified proteins resistant to chymosin coelute with the series of κ-casein peaks (6); this could have increased the κ-casein values found with Mono Q.

Quantitative estimates of α_{s1}-casein between the methods showed excellent correlation (Figure 4). Slight differences between the two procedures resulted when some α_{s2}-casein tailed into α_{s1}-casein on Mono Q. The α_{s2}-casein correlation was good, although the Mono Q values are lower than those for Mono S (Figure 4). A portion of α_{s2}-casein on Mono Q was not fully separated from and tailed into α_{s1}-casein. This effect was not as pronounced in the α_{s1}-casein correlation curve. When α_{s1}- and α_{s2}-caseins were combined, however, the correlation was excellent with the cross-con-

tamination of the two caseins almost balancing each other (Figure 4). Therefore, within reasonable error, Mono Q and Mono S methods of chromatography appeared to give comparable quantitative estimates.

CONCLUSIONS

The FPLC at pH 5 using a cation-exchange column provides good separation and quantitative estimates of the major casein fractions, α_{s1}-, α_{s2}-, β-, and κ-casein. It may also be possible to separate and identify some of the β-casein genetic variants. Further work is needed to separate the β-casein variants and to identify the minor casein components.

ACKNOWLEDGMENTS

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Separation of β-Casein A¹, A², and B Using Cation-Exchange Fast Protein Liquid Chromatography[1]

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ABSTRACT

β-Casein genetic variants A¹, A², and B were separated using cation-exchange fast protein liquid chromatography. β-Casein from a herd bulk casein sample eluted as a series of three peaks. Casein samples from individual cows containing known combinations of β-casein A¹, A²·, and B were used to confirm that the three peaks were β-casein genetic variants. An acid-PAGE gel confirmed the identity of the peaks that eluted from the column.

(Key words: β-casein, genetic variant, fast protein liquid chromatography)

Abbreviation key: CN = casein, FPLC = fast protein liquid chromatography, UA = urea acetate.

INTRODUCTION

In recent years, research has related β-casein (CN) genetic variants to both the composition and processing characteristics of milk (8, 12, 15). Feagan et al. (8) found that some β-CN genetic variants have significantly different "natural" curd tension. McLean et al. (12)

reported that β-CN genetic variants affected both the concentration and proportion of β-CN (A¹B, A²B > A¹A¹, A¹A², A²A², BB), α$_{s1}$-CN (A¹A¹, A²A² > BB), κ-CN (BB > A²A²), and the concentration of whey protein (A¹A² > most other β-CN variants). β-Casein A phenotypes were associated with higher milk, fat, and protein yields than other casein variants in the casein systems studied by Ng-Kwai-Hang et al. (15).

The seven β-CN genetic variants differ by one or more charged amino acids (7), which makes it possible to separate the β-CN variants. β-Casein A variants are separated under acidic conditions with PAGE, and the remaining variants can be separated under alkaline conditions (7). Both Aschaffenburg (2) and Li and Gaunt (11) studied the β-CN A¹, A², A³, B, and C gene frequencies in the five major Western dairy breeds. In both studies, β-CN A¹, A², and B accounted for over 95% of the β-CN. β-Casein A² occurs most often, followed by variants A¹, B, C, and A³. β-Casein variants D and E, which are rare, were not included in these gene frequency studies.

Starch and PAGE have improved resolution between components from what was possible with paper electrophoresis (18, 19, 20). There is a variation in the histidine content of β-CN A, found by sequencing the variants of β-CN, that makes it impossible to separate β-CN A further in alkaline media (7). Using β-CN A² as the base sequence, β-CN A¹ has a histidine replacing proline at position 67, and β-CN A³ has a glutamine replacing histidine at position 106 (7). Peterson and Kopfler developed an acid-PAGE system to separate β-CN further into A¹, A², and A³ based on changes in their histidine content (16).

Electrophoretic techniques have been improved to reduce the time required to separate the caseins and their genetic variants (1, 3, 4,

13, 20). Reduction in size of electrophoretic gels to reduce the time required to separate the caseins and their genetic variants has affected the band resolution and ease of casein genetic variant identification. Vegarud et al. (20) and Bovenhuis and Verstege (4) rapidly separated the genetic variants of caseins and whey proteins using isoelectric focusing on urea-modified gels in a Phastsystem (Pharmacia AB). This technique improved resolution of κ-CN genetic variants, but small gel size makes it difficult to identify some bands. Extra care is required when preparing samples and when modifying and running the small gels.

Addeo et al. (1) used isoelectric focusing on a thin layer gel to separate most of the common bovine casein variants. Bech and Munk (3) employed electrofocusing in agarose gels containing 7 M urea to identify quickly both casein and β-lactoglobulin variants. Both of these procedures require improvements in peak resolution. The procedure of Medrano and Sharrow (13) was a compromise between the larger acid and alkaline PAGE procedures and the smaller, faster isoelectric focusing techniques. Reducing the amount of sample loaded so that separate bands with similar mobilities remain separate would improve band resolution.

Carles (5) used reverse-phase HPLC to fractionate bovine caseins and to identify an atypical β-CN that differed by a neutral amino acid substitution. Fast protein liquid chromatography (FPLC) using anion-exchange chromatography on a Mono Q column has also been used to separate genetic variants of casein. Guillou et al. (9) separated κ-CN A and B and β-CN A[1] and C using anion-exchange chromatography. The procedure employed by Dalgleish (6) also used anion-exchange chromatography on a Mono Q column to separate κ-CN A and B. Hollar et al. (10) suggested separation of β-CN genetic variants with FPLC, using cation-exchange chromatography on a Mono S column, in which the β-CN fraction elutes in a series of three peaks. This paper describes investigations on the separation of the genetic variants of β-CN using this method.

MATERIALS AND METHODS

Whole casein samples were prepared from the milks of individual cows, which had previously been typed for their genetic variant composition using the procedure of Medrano and Sharrow (13). Whole milk from each cow was separated by centrifugation and filtration. From the skimmed milk, casein was separated from the whey proteins by isoelectric precipitation at pH 4.6. To precipitate the caseins, .3 ml each of 33.3% acetic acid and 33.3% sodium acetate at 40°C was added to 10 ml of whole milk samples (14). The pellet of casein was washed twice with acetate buffer, lyophilized, and stored at −20°C. β-Casein was typed using both alkaline and acid PAGE on a Mini-Protean II System (BioRad Corp., Richmond, CA) (13). At both pH conditions, the running gel contained 2.55% bis-acrylamide cross linker and 8% polyacrylamide, with a stacking gel of 4.6% polyacrylamide. For alkaline-PAGE, a Tris-glycine buffer at pH 8.3 was used, and PAGE was run at 50 V (constant) until the sample entered the running gel, after which it was increased to 100 V (constant). The β-CN A variants were typed using an acidic nondissociating continuous buffer system containing 8.6% glacial acetic acid and 2.5% formic acid. The gels were run at 150 V (constant).

The method of Hollar et al. (10) was used for chromatography of selected β-CN genetic variants using FPLC (Pharmacia AB, Uppsala, Sweden) with cation-exchange chromatography on a Mono S HR5/5 column. Urea-acetate (UA) buffer containing 6 M urea and .02 M acetate was made to volume and adjusted to pH 5 with 1N HCl. The UA buffer containing 1 M NaCl was prepared by dissolving NaCl in UA buffer prior to pH adjustment. Casein fractions were separated with a nonlinear NaCl gradient.

To confirm the identity of the β-CN peaks from chromatography as genetic variants of β-CN, each fraction was collected, dialyzed against deionized water, lyophilized, and run on an acid-PAGE gel. The acid-PAGE procedure of Peterson and Kopfler (16) was used to identify the peaks as β-CN genetic variants. Acid-PAGE was conducted using a vertical water-cooled cell, 10% gels, and an 8.6% glacial acetic acid and 2.5% formic acid buffer. The acid-PAGE gel was run with a combination of constant current and constant voltage for 16 h rather than 20 h to separate β-CN A[1], A[2], and B.

Purified β-CN and whole casein were treated with plasmin (EC 3.4.21.7, Sigma

Chemical Co., Poole, England) to break down the β-CN into fragments (7, 17) and to find the position of elution of the fragments using cation-exchange chromatography on the Mono S column. These fragments were previously known as γ_1-CN (A^1, A^2, A^3, B), γ_2-CN (A^2, A^3, B), and γ_3-CN (A, B) (7). Plasmin was dissolved in a .02 M Na_2HPO_4, .046 M KH_2PO_4 buffer at pH 6.5 at a concentration of 6.8 mg/ml. About 40 of mg whole casein and 15 mg of β-CN were each dissolved in 1 ml of the same phosphate buffer. After adding 100 μl of the plasmin solution to each casein solution, the casein solutions were incubated at 35°C for 10 min. To stop proteolysis, 1 ml of a .005 M bis-Tris-propane, 3.3 M urea buffer at pH 7 was added. The samples were dialyzed overnight against the UA buffer at pH 5 with three buffer changes. The whole casein sample was treated with 10 μl of β-mercaptoethanol as previously described to break up disulfide-linked polymers of κ- and α_{s2}-CN (10). The sample was filtered through a .2-μm Acrodisc® LC PVDF syringe filter (Gelman Sciences, Northampton, England) prior to chromatography.

Visking cellulose dialysis tubing was prepared by boiling for 30 min in a solution containing .5 M Na_2CO_3 and .001 M Na_2-EDTA and rinsing with deionized water.

RESULTS AND DISCUSSION

Individual cow whole casein samples of known genetic variant composition were run using cation-exchange FPLC with a Mono S column. The UA buffer at pH 5 with a non-linear NaCl gradient was used to separate the major casein components and the β-CN variants contained in the samples. Chromatograms of whole caseins containing the different genetic variants of β-CN are shown in Figure 1. Samples were both homozygous and heterozygous for β-CN A^1, A^2, and B. The flattened NaCl gradient in the β-CN region allowed for the separation of three peaks on a chromatogram of whole casein from herd bulk milk (10). Flattening the NaCl gradient further did not improve peak resolution. The casein samples from individual typed cows helped confirm the β-CN peaks observed in the β-CN region as three of the genetic variants. Figure 1 shows that, although there was good separation
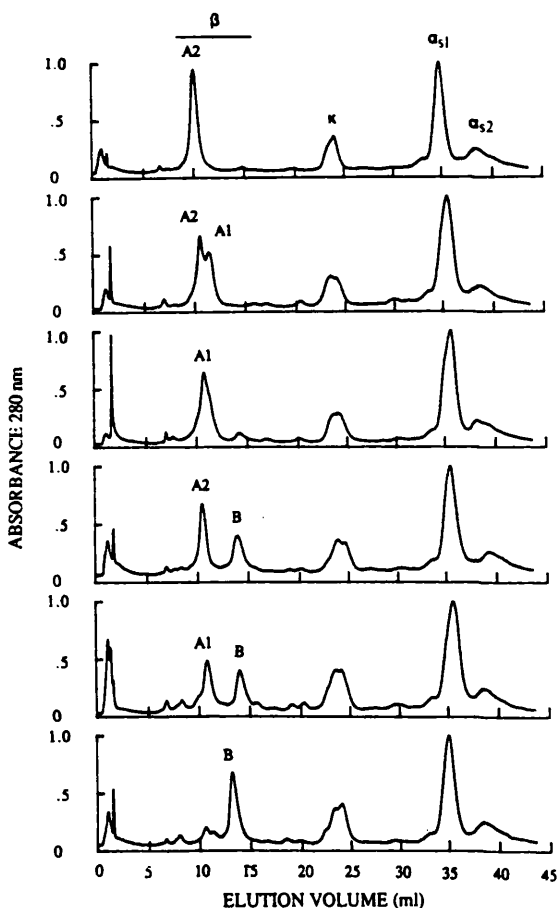
Figure 1. Elution profiles of individual cow whole casein samples of known β-casein genetic variant composition obtained by fast protein liquid chromatography at 20°C on a Mono S HR5/5 column and using urea-acetate buffer (pH 5.0; .02 M acetate, 6 M urea) and a NaCl gradient. About 2 to 5 mg of protein were applied to the column, and the flow rate was 1 ml/min. Absorbance (—).

between the β-CN A variants and β-CN B, there was some overlap between variant A^1 and A^2. The retention times of β-CN A^1 and A^2 were close to each other and varied slightly depending on whether the casein sample was homozygous or heterozygous. β-Caseins A^1 and B did not appear to overlap in heterozygous samples. To determine whether an unknown sample was homozygous A^1 or A^2, it was necessary to run previously typed β-CN or whole casein containing homozygous A^1 or A^2 and compare the elution profiles.

By running whole casein samples from individual cows, it was possible to see minor fractions that eluted with one or more of the β-

CN variants. They eluted in the β-CN region and could be proteolytic cleavage fragments of β-CN containing the amino acid substitutions of the parent β-CN genetic variants. They would be expected to have slightly different retention times than β-CN. Because of the low frequency of the remaining β-CN variants (A³, C, D, E) in western dairy breeds (2, 11), it was not possible to know if cation-exchange FPLC on a Mono S column could separate any other of the currently known β-CN genetic variants. Theoretically, such a separation is possible: for example, β-CN A³ contains one less histidine than β-CN A², which in turn contains one less histidine than β-CN A¹, and, in both cases, a proline replaces a histidine. Therefore, A³ should theoretically elute prior to β-CN A², and the separation between β-CN A³ and A² would be similar to that between A² and A¹ as shown on the chromatograms. It would be more difficult to separate and identify β-CN C, D, and E from each other and β-CN B, but this possibility cannot be ruled out.

Fractions of the three β-CN peaks were collected and run on acid-PAGE (16) as shown in Figure 2. On acid-PAGE, β-CN A² migrated most slowly, β-CN A¹ moved more rapidly, and β-CN B migrated fastest. It is necessary to run an alkaline-PAGE to identify the remaining β-CN genetic variants and to confirm the identity of β-CN B. Because the fractions were collected from milks of individual cows known to contain only combinations of variants A², A¹, and B, the B fraction shown on the acid-PAGE gel contains only β-CN B. Lane S in Figure 2 is a sample of whole casein from herd bulk milk used for much of the preliminary β-CN separation. The β-CN band intensities in this sample reflect the sizes of the peaks obtained on the FPLC chromatograms. The β-CN B band for this sample is faint, as expected, because β-CN B was a minor peak on the sample FPLC chromatogram and is present at low frequency in western dairy breeds (2, 11). An individual cow sample of unknown β-CN variant composition is shown in Lane C. This sample was run on cation-exchange FPLC, and it was tentatively typed as β-CN A²B. β-Casein A² is confirmed by the acid-PAGE, but an alkaline gel would be necessary to confirm the second allele as that of β-CN B rather than another low frequency β-CN variant (C, D, or E). The three lanes labeled A², A¹, and B were fractions collected from the individual cow samples, some of which are shown in Figure 1.

Figure 2. Acid gel-electrophoresis patterns for herd bulk whole casein (lane S), individual cow whole casein (lane C), and fractions obtained by fast protein liquid chromatography, shown in Figure 1.

Figure 3. Elution profiles of β-casein (—) and plasmin-treated β-casein (. . .) obtained by fast protein liquid chromatography using the same conditions as in Figure 1.

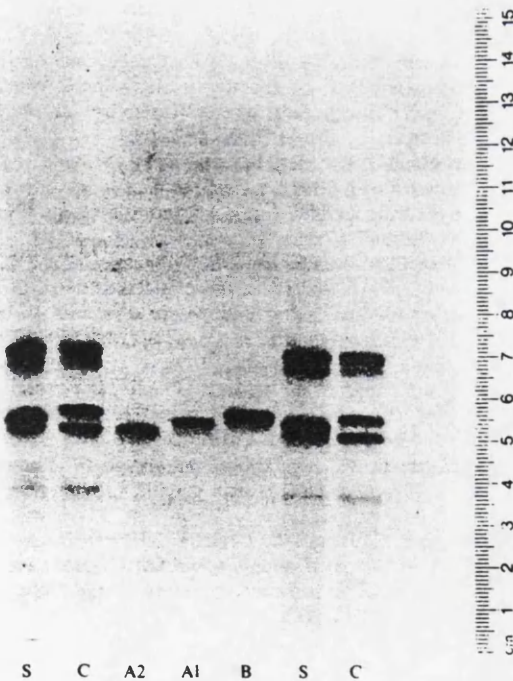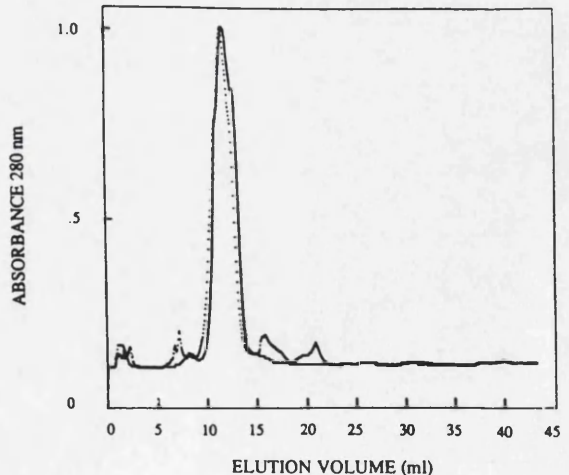There are light bands visible along with the major β-CN band in both Lanes A^2 and A^1, confirming the presence of some cross-contamination between these two peaks as expected from the chromatograms.

Proteolytic breakdown of β-CN, isolated from herd bulk milk, by plasmin is shown in Figure 3. The purified β-CN appears to contain primarily β-CN A^2 and A^1 with little β-CN B. Retention times of the various casein components are affected slightly by other components present in the sample. Purified β-CN appears to have a slightly longer retention time than β-CN in whole casein samples. In Hollar et al. (10), it was suggested that more work was required to help establish the identity of minor peaks in two unidentified fractions before β-CN A^1 and between β-CN B and κ-CN. Some minor peaks were known and others thought to be β-CN fragments of proteolytic cleavage. Because plasmin breaks β-CN into residues of different sizes (7, 17), purified β-CN and plasmin-treated purified β-CN were run using cation-exchange FPLC. The plasmin-treated sample showed formation of several minor peaks both before and after the major β-CN peak and had an increased breakthrough peak. The first minor peak in the plasmin-treated sample following the major β-CN peak elutes with β-CN B. This, along with the location of the minor peaks formed prior to the major β-CN peak, further supports the premise that the minor components that elute with β-CN are derived from β-CN.

CONCLUSIONS

The FPLC at pH 5 using cation-exchange chromatography on a Mono S column provides good separation and identification of β-CN A^1, A^2, and B. The method is capable of further development to allow quantification of the different genetic variants, but further studies on the identification of the less common A^3, C, D, and E variants are required.

ACKNOWLEDGMENTS

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Preparative-scale purification of bovine caseins on a cation-exchange resin

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A number of techniques have been developed for the purification of individual caseins from milk. These include differential precipitation (e.g. Zittle & Custer, 1963; Manson & Annan, 1971) and ion-exchange methods, e.g. fast protein liquid chromatography (FPLC; Davies & Law, 1987; St-Martin & Paquin, 1990) and conventional ion-exchange chromatography on DEAE-cellulose (Thompson, 1966; Andrews & Alichandis, 1983). The precipitation methods, whilst yielding relatively large amounts of caseins, are time consuming, require the handling of large volumes of liquid and, in our hands, yields tended to be rather varying. The FPLC techniques, whilst having the advantage of speed, produce relatively small amounts of protein in the absence of expensive preparative columns and pumps. We have therefore developed a rapid method for the preparation of multigram quantities of β-casein, based on a cation exchanger which can also be used batchwise, and also yields gram quantities of other caseins if required.

MATERIALS AND METHODS

S-Sepharose Fast Flow was purchased from Pharmacia Biosystems Ltd, Milton Keynes, as were the Mono S and Mono Q columns and Phastgels.

Preparation of samples

Acid casein was prepared from freshly skimmed bovine milk from the Hannah Institute herd. It was resuspended in one quarter of the original volume of buffer A (20 mM-acetate–6 M-urea, pH 5·0), and redissolved by adjusting the pH to 7·0 and adding 2-mercaptoethanol to a concentration of 100 μl/g casein. Solid urea was added to maintain the urea concentration at 6 M. The solution was left for 1 h to allow reduction of disulphide bonds, and the pH was adjusted to 5·0 with M-HCl.

Freeze-dried casein was also used as starting material.

Column chromatography

The casein solution was loaded at 300 ml h^{-1} via a peristaltic pump on to a column of S-Sepharose Fast Flow (bed dimensions 200 × 50 mm, bed volume 400 ml) equilibrated with buffer A. The column was washed with 600 ml buffer A at 300 ml h^{-1}, 100 ml fractions of eluent being collected. A 1600 ml linear gradient of NaCl (0·2–0·6 M) was passed through the column in order to elute the bound caseins. If only β-casein was required, the unbound β-casein was collected and bound protein was eluted in bulk by washing with buffer A containing M-NaCl. Pooled fractions

were dialysed against a large excess of deionized water to remove urea and then lyophilized.

Batch preparation of β-casein

S-Sepharose Fast Flow, equilibrated with buffer A. was added to the casein solution at pH 5·0. After stirring for 30 min at room temperature with an overhead stirrer, the resin was collected by vacuum filtration and washed with buffer A containing 0·1 m-NaCl. The resin was regenerated as for the column method.

Concentration of purified β-casein

Eluted β-casein in urea buffer could be concentrated by diluting with four volumes of distilled water. The heavy white precipitate of protein was flocculated at pH 4·6 and collected either by allowing it to settle under gravity or by centrifugation. Any remaining urea was removed by dialysis after dissolving the casein at pH 7·0.

Analysis of purified caseins

Polyacrylamide gel electrophoresis. Caseins were separated by urea-PAGE as detailed by Davies & Law (1977) after adaptation for rapid separation using a Pharmacia Phast-System. Phastgels (12·5 % acrylamide) were immersed in Tris–EDTA–barbitone (TEB) buffer, pH 7·9 containing 4·5 m-urea. After gentle stirring for 1 h, the gels were removed, drained and allowed to air dry for 15 min. Buffer strips consisted of TEB buffer containing 2 % agarose. Samples were dissolved in TEB buffer containing 8 m-urea at a protein concentration of 3 mg ml^{-1}. 1 μl 2-mercaptoethanol/mg protein being added at least 1 h before separation. Samples (0·3 μl), were applied to the gels. Electrophoresis was for 98 volt h and the proteins were stained with Coomassie blue.

Fast protein liquid chromatography. Samples were analysed at pH 5·0 using a Mono S HR5/5 cation-exchange column (Hollar *et al.* 1991). Protein reduced with 2-mercaptoethanol was eluted using a NaCl gradient in buffer A.

RESULTS AND DISCUSSION

A typical elution profile from the S-Sepharose Fast Flow column is shown in Fig. 1. The identity of the unbound material and individual pooled peak fractions was established using urea-PAGE (Fig. 2) and cation-exchange FPLC on a Mono S column (Fig. 3). Fraction 1, the unbound material, was shown to be β-casein, fraction 2 was κ-casein, 3 α_{s2}-casein and 4 α_{s1}-casein. The purity of the β-, α_{s1}-. α_{s2}- and κ-casein fractions was estimated from the FPLC profiles to be > 90 %. The order of elution of α_{s1}- and α_{s2}-caseins from the Mono S column was reversed from that from the S-Sepharose Fast Flow column, despite their similar chemistry. Overall recovery of individual caseins from a 20 g load of freeze-dried caseinate was 65 % (Table 1). Some of the losses were due to material being discarded in order to prevent cross contamination. Since the β-casein did not bind to the resin, it did not utilize any of the binding capacity and therefore more caseinate could be loaded on to the column. The capacity of the resin was determined to be sufficient to fractionate ~ 6 g caseinate/100 ml packed bed volume. Both wet, precipitated caseinate and freeze-dried caseinate were suitable as starting materials providing that the quantity of urea was adjusted in the protein solution to compensate for the water entrapped in the precipitated acid caseinate. Using the column method a complete purification took 7 h. If only the β-casein was required, the time could be reduced to 4 h,
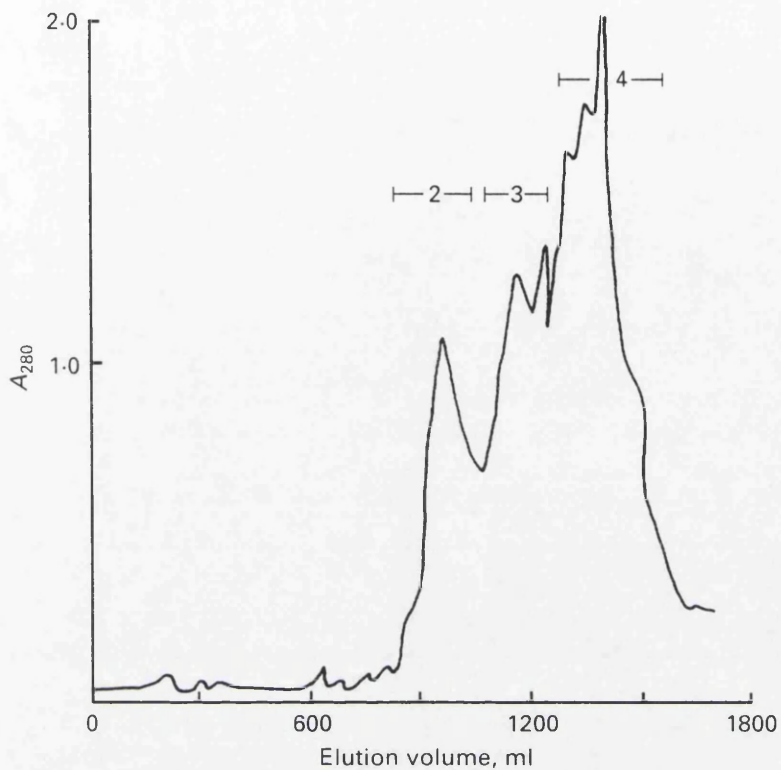
Fig. 1. Chromatography of whole casein (20 g) on a column of S-Sepharose Fast Flow carried out as described on p. 557. Fractions were pooled as shown. The unbound fraction was also collected as fraction 1.
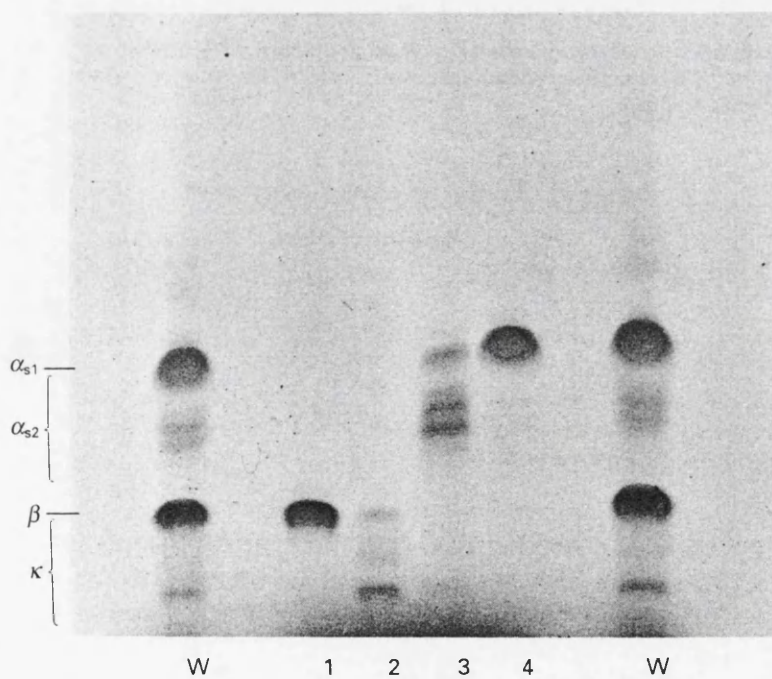
Fig. 2. Urea-PAGE of whole casein and pooled fractions from the S-Sepharose Fast Flow Column. W, whole casein; 1–4, fractions 1–4 from the separation shown in Fig. 1. Methods are described on p. 558.
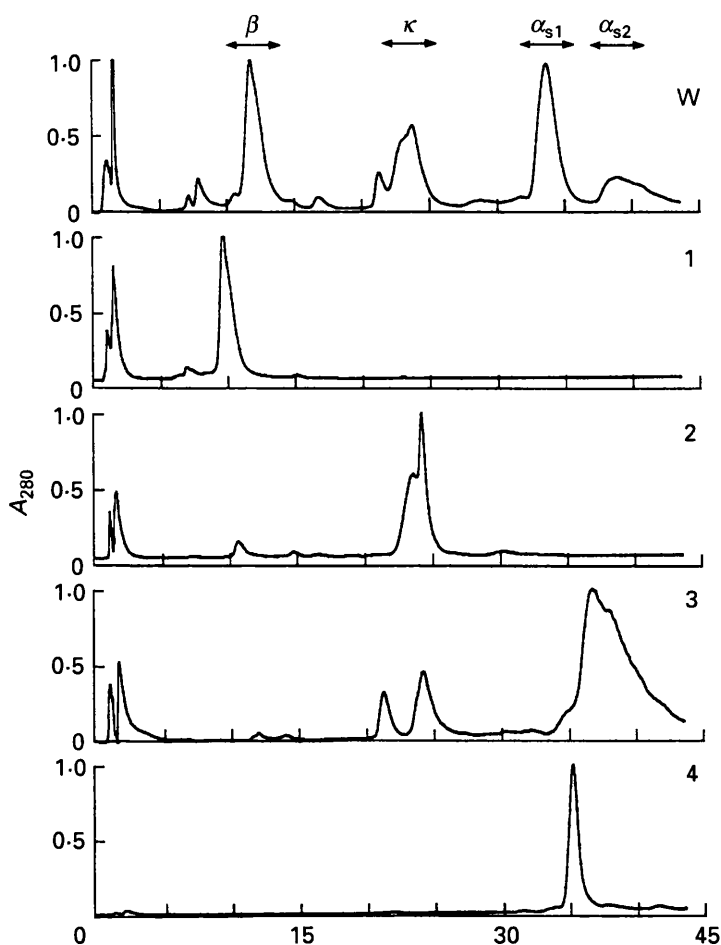
Fig. 3. Fast protein liquid chromatography on a Mono S column, as described on p. 558. W, whole casein; 1–4, fractions 1–4 from the separation shown in Fig. 1. The elution volumes of pure casein fractions are shown at the top.

Table 1. *Yields of casein fractions*†

Casein fraction	Recovered weight, g	Purity, %
β	3·8	> 95
κ	2·5	> 95
α_{s2}	2·0	90
α_{s1}	5·1	> 95

† Freeze-dried acid precipitated casein (20 g) was loaded on to a S-Sepharose Fast Flow column with a bed volume of 400 ml and eluted as described on p. 557.

permitting 8–10 g β-casein to be prepared in a working day using 300 ml resin. As a means of purifying β-casein, this method could be adapted to a batchwise mode where no column or pump was required. The purity of the β-casein was found to be the same as that obtained using the column method.

Because the β-casein was obtained in a dilute form, large volumes needed to be dialysed and freeze-dried. By diluting the eluted β-casein solutions with distilled water, most of the protein precipitated. The optimum dilution was one volume of β-casein solution to four volumes of water. Approximately 86 % of the β-casein could be recovered. Furthermore, if the pH was adjusted to 4·6, the precipitate flocculated

and quite quickly settled to the bottom of the vessel. After decanting the supernatant which contained most of the urea, the precipitated protein could be redissolved in the minimum volume of water at pH 7·0 and any remaining urea removed by dialysis.

As a method for purifying caseins. this cation-exchange technique has a number of advantages over the alternative methods. The capacity of the resin is high, as is the flow rate, permitting relatively large amounts of casein to be fractionated quickly. In addition, the individual caseins were eluted in reasonably distinct peaks thus minimizing cross contamination of individual components when fractions were pooled.

Since the separation was performed at pH 5·0, the risk of cyanate formation in the urea buffer, and with it modification of the protein (Stark *et al.* 1960; Manson, 1962) was considerably reduced. Similarly. 2-mercaptoethanol was not required in the chromatography buffers, unlike separations at pH 7·0, since at pH 5·0 the rate of disulphide exchange and oxidation is low. This method therefore minimizes the risks involved to the operator in using large quantities of this disagreeable, irritating reagent.

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Quantitative examination of genetic polymorphism in κ- and β-caseins by anion- and cation-exchange FPLC

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1. Introduction

A considerable amount of detailed information is available on genetic polymorphism of the caseins, and it is now possible to select genotypes giving different protein composition and improved processing properties of milk. κ-Casein B, for example, has been associated with a decrease in renneting time, increased curd tension and cheese yield, and improved natural heat stability (1). Similarly β-casein B gives fast clotting and a finer curd (1). It is still not known, however, how κ-casein B and β-casein B, which differ only slightly in primary structure from the corresponding A variants (2), cause these marked differences in processing properties. There is considerable evidence that κ-casein content and micellar size are negatively correlated (3, 4), and the effect of κ-casein phenotype on the composition of casein has previously been examined by gel scanning (5, 6), anion-exchange FPLC (7) and reverse phase HPLC (8).

In this study we have examined the effect of κ- and β-phenotypes on the composition of whole casein by anion- and cation-exchange FPLC, after alkylation to give optimum resolution of the κ- and α_{S2}-caseins (9). The β-casein phenotypes, and the relative amounts of the A and B variants of β-casein in A^1B and A^2B heterozygotes, were determined by cation-exchange FPLC (10).

2. Materials and methods

Milk samples. Milk samples were collected from Friesian cows mainly in the Institute herd, the remainder from farms in the North of England and from a herd in California.

Whole casein samples. Whole casein samples were precipitated from skim-milk at pH 4.6 and 20 °C by the addition of acetic acid (5 % w/v) and sodium acetate (0.2 M) according to the method of ROWLAND (11). The precipitates were washed with water at pH 4.6 and then freeze-dried.

Anion-exchange FPLC. Casein samples were alkylated with cysteamine hydrochloride and cystamine dihydrochloride, and fractionated in bis tris propane-urea buffer (pH 7.0) as described previously (9).

Cation-exchange FPLC. Casein samples were reduced with 2-mercaptoethanol and fractionated in acetate-urea buffer (pH 5.0) as described by HOLLAR *et al.* (10).

Rennet treatment of κ-casein samples. κ-Casein fractions were treated with rennet (Käselab-Pulver; Chr. Hansen's Laboratorium A/S, Copenhagen, Denmark) as described previously (12).

PAGE. κ-Casein fractions, before and after rennin treatment, were examined by alkaline PAGE following the procedure of DAVIES and LAW (12), except that the proteins were stained with Coomassie Blue dye.

3. Results

3.1 Characterisation of κ-casein variants separated by FPLC

Sections of the elution profiles obtained by anion-exchange FPLC of alkylated samples of whole casein, containing κ-caseins A, AB and B are shown in Fig. 1 a, b and c, respectively.
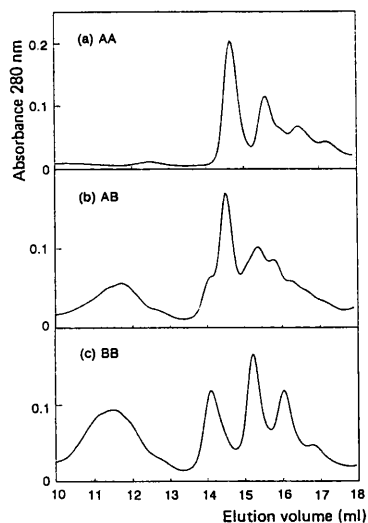
Fig. 1: κ-Casein sections of the elution profiles obtained by FPLC of alkylated whole casein at 20 °C on a Mono Q HR 5/5 column and with a bis tris propane-urea buffer (pH 7.0; 0.005 M bis tris propane; 3.3 M urea) and a NaCl gradient. (a) κ-Cn A (b) κ-Cn AB (c) κ-Cn B.

The elution profiles were basically similar to those obtained for the respective κ-casein types

by traditional anion-exchange chromatography (12, 13). κ-Casein A gave a large peak of mainly unglycosylated protein at about 14.5 ml followed by three other main peaks of κ-casein glycosylated to different degrees (Fig. 1a). κ-Casein B, which has a lower net negative charge, eluted earlier, giving a large peak of mainly unglycosylated material with a maximum at about 11.5 ml, together with four other main peaks of differently glycosylated κ-casein (Fig. 1c). κ-Casein AB appeared as a composite of the profiles of the A and B variants. The three different profiles were sufficiently distinctive to allow phenotyping of the κ-casein by anion-exchange FPLC. Alkylation of the casein samples with cysteamine hydrochloride and cystamine dihydrochloride (9) effectively reduced the net negative charge on κ-casein, which subsequently eluted earlier from the anion-exchange column. The alkylation procedure also gave a more complete separation of α_{s1}- and α_{s2}-caseins. Flattening the NaCl gradient in the κ-casein region gave a more extensive separation of the numerous κ-casein components, comparable to that shown by VREEMAN et al. (13), but did not appreciably alter quantitative values for κ-casein.

The alkaline PAGE patterns for κ-caseins A, AB and B, separated as in Fig. 1 and treated with rennin, are shown in Fig. 2 (lanes 4, 5 and 6, respectively). Electrophoresis showed that most of the material was rennin sensitive, except for a small amount of material in the most mobile bands. This may have occurred because of the resistance of highly glycosylated κ-casein to rennin treatment, or to the presence of some other unidentified protein in the region between the κ- and β-casein fractions as indicated previously (9, 12).

The elution profiles obtained by cation-exchange of whole casein samples showed that κ-caseins A, AB and B eluted as single peaks at the same position, and were as shown previously for casein from herd bulk milk containing mainly κ-casein A (10). Para-κ-casein from each of the variants eluted at the same position as the intact κ-casein.

3.2 Effect of κ-casein phenotype on the composition of whole casein

The relative amounts of the individual caseins in whole casein, determined by anion-exchange FPLC for each of the κ-casein phenotypes, and expressed as a percentage of total casein, are given in Table 1.

Fig. 2: Alkaline PAGE patterns for whole casein reduced with mercaptoethanol (AC), and the alkylated κ-casein fractions obtained by FPLC as shown in Fig. 1, before and after rennin treatment. Lane 1, κ-Cn A; lane 2, κ-Cn AB; lane 3, κ-Cn B; lanes 4, 5 and 6 κ-Cn A, κ-Cn AB and κ-Cn B after rennin treatment.

Table 1: The effect of κ-casein phenotype on the composition of whole casein

κ-Casein phenotype	Friesian			Jersey
	AA	AB	BB	BB
No of samples	100	53	44	8
Casein	% of total casein			
κ-	9.7±0.1	10.9±0.1	12.1±0.2 ***	12.3±0.5
β-	35.5±0.3	34.2±0.4	33.6±0.4 ***	33.7±0.6
α_{S1}-	36.8±0.2	36.0±0.3	33.9±0.4 ***	34.3±0.8
α_{S2}-	10.0±0.2	10.2±0.2	9.9±0.2 N.S.	10.7±0.5
Minor	8.1±0.3	8.8±0.4	10.5±0.5 ***	9.1±1.0

*** Differences between phenotypes significant P <0.001

The alkaline PAGE patterns for κ-caseins A, AB and B, separated as in Fig. 1, are shown in Fig. 2 (lanes 1, 2 and 3, respectively).

κ-Casein A shows a main band of unglycosylated κ-casein, three other main bands, and a number of interspersed minor bands. κ-Casein B shows a similar pattern except that the bands have slightly lower mobility due to the lower net negative charge on the polypeptide. κ-Casein AB gives a pattern showing the bands present in both κ-caseins A and B.

Results show that the relative amount of κ-casein in whole casein varied with phenotype in the order κ-Cn BB > AB > AA, and on average there was about 25 % more κ-casein in the BB phenotype than in the AA phenotype. The relative amounts of β- and α_{s1}-caseins varied in the opposite manner (AA > AB > BB). The relative amount of a minor fraction which contained mainly γ-caseins, including fractions 1, A, B and C described previously (9), increased with phenotype in the same way as κ-casein. The differences were significant in analysis of variance at P <0.001. The relative amount of the α_{s2}-caseins did not vary with κ-casein phenotype.

Values are also given in Table 1 for the composition of whole casein from Jersey cows of κ-Cn BB phenotype. Results show that the com-

position of casein from Friesian and Jersey cows of κ-Cn BB type was similar.

To establish the accuracy of κ-casein values, results from anion- and cation-exchange FPLC were compared (Table 2).

Cation-exchange FPLC confirmed the finding that the relative amount of κ-casein in whole casein varied with phenotype in the order κ-Cn BB > AB > AA. Also for each of the κ-casein phenotypes, values for the relative amounts of κ-casein determined by the two FPLC methods were in good agreement.

Table 2: Comparison of values from anion- and cation-exchange FPLC for the relative amounts of κ-casein variants in whole casein

κ-Casein phenotype	AA	AB	BB	All samples
No of samples	20	18	15	53
		κ-Casein, % of total casein		
Anion-exchange (Mono Q)	9.3±0.1	10.7±0.2	12.1±0.2	10.6±0.2
Cation-exchange (Mono S)	9.5±0.2	10.7±0.2	12.7±0.4	10.8±0.2
Average difference between two types of analysis	0.6±0.1	0.6±0.1	1.1±0.3	0.7±0.1

These results from FPLC are in reasonable agreement with those of McLEAN *et al.* (6) who used gel scanning and found that the relative amounts of κ-casein for phenotypes AA, AB and BB were 10.5, 11.8 and 12.9 %, respectively. Similarly AALTONEN *et al.* (7) using anion-exchange FPLC, and VAN DEN BERG (8), using reverse phase HPLC found the same trend in values for κ-casein. Each of these groups of workers found an accompanying decrease in the relative amounts of α_{s1}- or α_s-casein (AA > AB > BB) but no significant differences in the other fractions. In this study we found a small, but significant (P <0.001) decrease in β-casein (AA > AB > BB) and an accompanying increase in γ-caseins (11).

The overall distribution of κ-casein values for each of the phenotypes is shown in Fig. 3.

There was considerable variation within each phenotype, and the distributions were slightly skewed upwards. Closer inspection showed that within each phenotype higher κ-casein values tended to be associated with samples taken in late lactation, or with higher levels of γ-caseins. These results are consistent with the finding of DAVIES and LAW (14) that there is an increase in the relative amounts of κ- and γ-caseins in late lactation, but that the increase in κ-casein is not an artefact due to increased proteolysis. In the
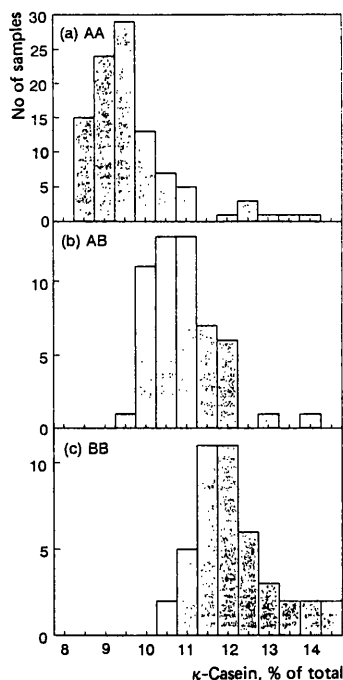
Fig. 3: Distribution of the relative amounts of κ-casein in whole casein for each of the phenotypes κ-Cn AA, AB and BB, respectively.

present study, on grouping samples according to the relative amounts of the γ-casein, it was found that the relative amount of κ-casein varied in all groups in the order BB >AB >AA.

The overall variation found in this study in the relative amount of κ-casein, within and between phenotypes, seems sufficiently large to cause a considerable difference in the micellar size distribution (4) and processing properties. The differences in the relative amounts of κ-casein, especially in view of the location of κ-casein on the surface of the micelle, may be large enough to exert a direct effect on renneting kinetics. Similarly, the increased level of κ-casein in the AB and BB phenotypes, and the increased level of the hydrophobic para-κ-casein after renneting, may account for the increase in curd firmness which is observed with κ-Cn B (1).

3.3 Effect of β-casein phenotype on the composition of whole casein

Whole casein samples, reduced with 2-mercaptoethanol, were fractionated by cation-exchange FPLC according to the method of HOLLAR *et al.* (15) and gave elution profiles which allowed identification of β-caseins A^2, A^1 and B. The relative amounts of the individual caseins in whole casein, determined by anion-exchange FPLC for each of the β-casein phenotypes, are given in Table 3.

Table 3: The effect of β-casein phenotype on the composition of whole casein

β-Casein type	A¹A¹	A¹A²	A²A²	A¹B	A²B	BB
No of samples	8	15	11	9	7	2
Casein			% of total			
β-	33.6	34.4	35.4	33.3	31.1	29.9
α_{S1}-	36.7	37.2	37.9	36.2	36.0	37.1
κ-	10.3	10.1	9.9	10.9	11.4	12.0
α_{S2}-	9.6	9.5	8.7	10.1	9.9	8.9
Minor	9.7	8.8	8.3	9.6	11.6	12.2
β + Minor	43.3	43.2	43.7	42.9	42.7	42.1

There was some indication that the relative amount of β-casein was slightly lower, and the amount of γ-casein greater, in those samples containing β-casein B, but the level of significance in analysis of variance was low. This is contrary to the finding of McLEAN *et al.* who found that A¹B and A²B heterozygotes contained significantly more β-casein than A¹A¹, A²A² or BB types. In the present study, differences in the relative amounts of β-casein could be attributed to the level of proteolysis, and there were no significant differences between values for the sum of the β- and γ-casein fractions for each of the phenotypes (Table 3).

Also, using cation-exchange FPLC, it was possible to separate β-caseins A² and B, and A¹ and B from the heterozygotes A²B and A¹B, respectively. Results showed that the relative amounts of the A and B variants in the A¹B and A²B heterozygotes were very close to a 1:1 ratio (Table 4).

Table 4: The relative amounts of β-caseins A¹, A² and B in A¹B and A²B heterozygotes

β-Casein type	A¹B	A²B
No of samples	6	8
	% of total β-casein	
β-Casein A¹ or A²	50.8 ± 0.2	50.1 ± 0.4
β-Casein B	49.2 ± 0.2	49.9 ± 0.4

It is difficult to account for the faster clotting and finer curd formation associated with β-casein B (1) in terms of the relative amounts of the β-casein variants found here. However, there was some indication in this study that, as found previously (16), there was a closer than expected linkage between κ-casein B and β-casein B variants, and whole casein samples with β-casein B contained more κ-casein (Table 3). There was also considerable variation in the relative amounts of κ-casein within the different κ-casein phenotypes (Fig. 3). It is possible, therefore, that some of the change in renneting properties attributed to β-casein B was due to variation in the amount of κ-casein. Anion- and cation-exchange FPLC have been successfully used in this study to

identify and measure the relative amounts of κ- and β-casein variants, together with the other major caseins, and could be used in the quantitative study on the effect of the variants on the technological properties of milk.

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5. Summary

LAW, A.J.R.: **Quantitative examination of genetic polymorphism in κ- and β-caseins by anion- and cation-exchange FPLC.** Milchwissenschaft **48** (5) 243-247 (1993).

24 Casein (genetic polymorphism)

The composition of casein samples from Friesian cows was determined by anion-exchange FPLC, and the κ-casein phenotypes were identified from the elution profiles. The relative amounts of κ-casein in whole casein for phenotypes AA, AB and BB were 9.7, 10.9 and 12.1 %, respectively (P <0.001), and this result was confirmed by cation-exchange FPLC. The relative amounts of the other caseins, except α_{S2}-casein, varied significantly (P <0.001) with phenotype, casein from κ-casein BB milks containing slightly less β- and α_{S1}-caseins, and more γ-caseins. The compo-

sition of casein from Friesian and Jersey cows of κ-casein BB type was similar.

β-Casein A¹, A² and B genetic variants were identified from the elution profiles obtained by cation-exchange FPLC, and some small differences were found in the relative amounts of β-casein in whole casein for the 6 phenotypes. The variation, however, appeared to be mainly due to the amount of proteolysis, rather than to differences in the level of expression of the variants, and it was found that the A and B variants were present in the A¹B and A²B heterozygotes in equal amounts.

LAW, A.J.R.: **Quantitative Untersuchung von genetischem Polymorphismus bei κ- und β-Caseinen durch Anionen- und Kationen-Austausch-FPLC.** Milchwissenschaft **48** (5) 243–247 (1993).

24 Casein (genetischer Polymorphismus)

Es wurden die Zusammensetzung der Caseinproben von Holstein-Friesian-Kühen durch Anionen-Austausch-FPLC bestimmt und die κ-Casein-Typen in Gesamtcasein nach den Elutionsprofilen identifiziert. Die relativen κ-Caseinmengen in Gesamtcasein für die Phänotypen AA, AB und BB waren 9,7, 10,9 bzw. 12,1 % (P <0,001), und dieses Ergebnis wurde durch Kationenaustausch-FPLC bestätigt. Die relativen Mengen an anderen Caseinen, mit Ausnahme von α_{S2}-Casein, variierten signifikant (P <0,001) je nach Phänotyp, wobei Casein aus κ-Casein BB-Milch etwas weniger β- und α_{S1}-Casein und mehr γ-Casein enthielt. Die Caseinzusammensetzung bei Holstein-Friesian- und Jersey-Kühen des κ-Casein BB-Typs war ähnlich. Genetische Variationen von β-Casein A¹, A² und B wurden nach den Elutionsprofilen identifiziert und einige kleine Unterschiede der β-Caseinmengen in Gesamtcasein für die 6 Phänotypen festgestellt. Die Variation beruhte im wesentlichen eher auf dem Proteolysegrad als auf dem Unterschied des Expressionslevels der Varianten, und es wurde festgestellt, daß die Varianten A und B bei den A¹B- und A²B-Heterozygoten in gleicher Menge vorhanden waren.

3

THE COMPOSITION OF
CAPRINE AND OVINE CASEINS

1. INTRODUCTION

The proteins in the milk of the goat and the sheep have not been as extensively studied as those in cows' milk. There is, however, an increasing market for the various types of cheese and yogurt made from the milk of the goat and sheep. In order to make processing more efficient, further information is required about the content and composition of the casein fraction, and about natural variation which is caused by genetic polymorphism of the caseins, or seasonal and lactational effects. Also, further research is required into the structure and stability of the casein micelles, and the effect of heating and renneting during the manufacture of yogurt and cheese, respectively.

2. THE COMPOSITION OF MILK FROM THE COW, GOAT AND SHEEP

Some of the main properties of the milks from the three species have been summarised, and these are shown in Table 11. Compared with cows' milk, goats' milk has a similar content of the main constituents - fat, lactose and protein. There are, however, large individual variations in the concentrations (Storry *et al.*, 1983). Values for processing characteristics, such as rennet coagulation time, syneresis time and heat coagulation time are also similar to those of cows' milk. Goats' milk, however, gives a much lower coagulum strength and, because of considerable variation in cheese yield from different milks, is less satisfactory for cheese manufacture. Closer examination shows that the total concentrations of Ca and Pi in goats' milk are similar to those of cows' milk, and the ratio of Ca/Pi in casein micelles in both milks is close to 2.1. Casein micelles in

Table 11. Comparison of the properties of milk from the cow, goat and sheep

	Cow	Goat	Sheep
1. Composition of milk			
Fat %	1.38-5.10	2.75-6.43	5.79-6.45
Lactose %	3.71-4.6	3.91-4.81	4.47-4.76
Total casein %	2.28-3.27	2.14-3.18	3.78-5.20
Total whey protein %	0.16-0.76	0.37-0.70	0.88-1.04
2. Physical properties			
Rennet coag. time (min)	6.7-13.7	4.2-10.4	3.7-7.0
Coagulum strength (g)	18.2-45.8	10.9-26.6	48.5-141.5
Syneresis time (min)	27-60	24-53	53-70
Heat coag. time (min)	19.0, 26.0	31.0	Immediate
3. Micellar properties			
Micelle solvation (g H_2O/g dry pellet)	1.51	1.54	1.62
Micellar diameter (nm)	120	200 or < 80	< 80
Total Ca in milk (mM)	31.7	34.7	60.2
Total Pi in milk (mM)	23.1	23.1	34.8
Micellar ratio Ca/Pi	2.19	2.08	2.04
4. Casein composition	% of Total		
α_{s1}-Casein	38	0-20	35-47.2
α_{s2}-Casein	12	10-30	-
β-Casein	36	43-68	28-45
κ-Casein	14	15-29	10-12.1

Data compiled from:
1. Storry *et al.*, 1983
2. Storry *et al.*, 1983; Sood *et al.*, 1979
3. Richardson *et al.*, 1974
4. Remeuf & Lenoir, 1986; Anifantakis, 1986; Richardson, 1977

goats' milk show a different size distribution, tending either to be much larger or much smaller than those in cows' milk. Also, the relative amounts of the caseins in caprine casein are much more variable; caprine casein contains, on average, much less α_{s1}-casein and more β- and κ-caseins.

Compared with cows' milk, that from the sheep tends to be more concentrated, and has improved properties for cheese-making, with shorter rennet coagulation time and greater coagulum strength (Table 11). The heat stability of milk from the sheep, however, is poor. The concentrations of Ca and Pi are almost double those in cows' milk, but the ratio of Ca/Pi in the micelle is similar to that for the cow and the goat. Micelles in ewes' milk, however, are on average much smaller than those in cows' milk. Ovine casein also contains much more β- and less α_s-casein than bovine casein.

3. HOMOLOGY OF BOVINE, CAPRINE AND OVINE CASEINS

The amino acid compositions and sequences of the four main caseins of the cow, goat and sheep, namely α_{s1}-, β-, α_{s2}- and κ-, have been determined, and these are shown in Tables 12 and 13, and Fig. 10-13. The sequences of ovine α_{s1}-, β- and α_{s2}-caseins have been determined by cDNA analysis, and it has not yet been established which Ser residues are phosphorylated. Potential phosphorylation sites are indicated in Tables 12 and 13. Comparison of the sequences (Fig.10-13) shows that there is between 80 and 90% homology of caprine and ovine caseins with the corresponding bovine caseins (Mepham *et al.*, 1992; Jenness, 1980). A number of important differences occur in the individual caseins, however, which affect their processing properties, and make determination of the relative amounts of the caprine and ovine caseins more difficult than for the bovine caseins. Also, some variants of caprine α_{s1}-casein and ovine α_{s2}-casein occur because of substantial internal amino acid deletions which have marked effects on their properties, and in some cases, on their level of expression.

The alkaline PAGE patterns for whole casein from the cow, goat and sheep (Fig.14) show that caprine and ovine α_{s1}-caseins have lower electrophoretic mobilities than bovine α_{s1}-casein, and tend to migrate close to the corresponding α_{s2}-caseins. Also, caprine and ovine β-caseins each migrate as two bands (β_1- and β_2-) due to

Table 12. Amino acid compositions of bovine, caprine and ovine α_{s1}- and β-caseins

Amino acid	α_{s1}-Casein			β-Casein		
	Bovine α_{s1}-CnB	Caprine α_{s1}-CnB	Ovine	Bovine β-CnA[2]	Caprine β_1, β_2	Ovine
Asp	7	7	7	4	4	4
Asn	8	11	10	5	4	4
Thr	5	5	3	9	11	11
Ser	8	9	13	11	10, 9	10
SerP	8	9	5[a]	5	5, 6	4[a]
Glu	25	20	19	18	19	19
Gln	14	14	14	21	21	21
Pro	17	19	17	35	33	34
Gly	9	9	9	5	5	5
Ala	9	12	11	5	4	4
Cys	0	0	0	0	0	0
Val	11	9	9	19	21	21
Met	5	5	5	6	6	6
Ile	11	9	11	10	9	9
Leu	17	17	17	22	22	22
Tyr	10	11	9	4	3	3
Phe	8	7	6	9	9	9
Lys	14	13	14	11	12	13
His	5	4	4	5	5	4
Trp	2	2	2	1	1	1
Arg	6	7	6	4	3	3
Total	199	199	191	209	207	207

[a] Potential phosphorylation sites

Data from sequences

Table 13. Amino acid compositions of bovine, caprine and ovine α_{s2}- and κ-caseins

Amino acid	α_{s2}-Casein			κ-Casein		
	Bovine	Caprine	Ovine	Bovine	Caprine	Ovine
Asp	4	5	6	4	7	7
Asn	14	13	12	8	9	9
Thr	15	14	14	15	15	12
Ser	7	5	9	12	11	10
SerP	10	9	6[a]	1	2	2
Pyroglu				1		
Glu	24	25	25	12	11	11
Gln	16	16	16	14	15	15
Pro	10	13	12	20	19	19
Gly	2	2	2	2	1	1
Ala	8	9	9	14	16	19
Cys	2	2	2	2	3	3
Val	14	11	12	11	12	12
Met	4	4	4	2	1	2
Ile	11	12	11	12	10	10
Leu	13	10	11	8	8	7
Tyr	12	12	12	9	9	9
Phe	6	7	6	4	4	4
Lys	24	24	24	9	8	8
His	3	5	5	3	4	4
Trp	2	3	3	1	1	1
Arg	6	7	7	5	5	6
Total	207	208	208	169	171	171

[a] Potential phosphorylation sites

Data from sequences

55

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Cow     1   RPKHPIKHQG LPQEVLNENL LRFFVAPFPE VFGKEKVNEL SKDIGSESTE
Goat        RPKHPINHRG LSPEVPNENL LRFVVAPFPE VFRKENINEL SKDIGSESTE
Sheep       RPKHPIKHQG LSPEVLNENL LRFVVAPFPE VFRKENINEL SKDIGSESIE

Cow     51  DQAMEDIKQM EAESISSSEE IVPNSVEQKH IQKEDVPSER YLGYLEQLLR
Goat        DQAMEDAKQM KAGSSSSSEE IVPNSAEQKY IQKEDVPSER YLGYLEQLLR
Sheep       DQAMEDAKQM KAGSSSSSEE IVPNSAEQKY IQKEDVPSER YLGYLEQLLR

Cow     101 LKKYKVPQLE IVPNSAEERL HSMKEGIHAQ QKEPMIGVNQ ELAYFYPELF
Goat        LKKYNVPQLE IVPKSAEEQL HSMKEGNPAH QKQPMIAVNQ ELAYFYPQLF
Sheep       LKKYNVPQLE IVPKSAEEQL HSMKEGNPAH QKQPMIAVNQ --------LF

Cow     151 RQFYQLDAYP SGAWYYVPLG TQYTDAPSFS DIPNPIGSEN SEKTTMPLW
Goat        RQFYQLDAYP SGAWYYLPLG TQYTDAPSFS DIPNPIGSEN SGKTTMPLW
Sheep       RQFYQLDAYP SGAWYYLPLG TQYTDAPSFS DIPNPIGSEN SGKITMPLW
```

Fig.10 Primary structure and sites of phosphorylation of bovine α_{s1}-casein B-8P, caprine α_{s1}-casein B and ovine α_{s1}-casein.

S: Phosphoserine (In ovine α_{s1}-, β-, and α_{s2}-caseins indicates potential phosphorylation sites).
Differences from bovine sequences shown in bold letters.

Goat: Leroux et al., 1992; Brignon et al., 1990; Brignon et al., 1989
Sheep: Mercier et al., 1985

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Cow     1   RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH
Goat        REQEELNVVG ETVESLSSSE ESITHINKKI EKFQSEEQQQ TEDELQDKIH
Sheep       REQEELNVVG ETVESLSSSE ESITHINKKI EKFQSEEQQQ TEDELQDKIH

Cow     51  PFAQTQSLVY PFPGPIPNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE
Goat        PFAQAQSLVY PFTGPIPNSL PQNILPLTQT PVVVPPFLQP EIMGVPKVKE
Sheep       PFAQAQSLVY PFTGPIPNSL PQNILPLTQT PVVVPPFLQP EIMGVPKVKE

Cow     101 AMAPKHKEMP FPKYPVQPFT ESQSLTLTDV ENLHLPPLLL QSWMHQPHQP
Goat        TMVPKHKEMP FPKYPVEPFT ESQSLTLTDV EKLHLPLPLV QSWMHQPPQP
Sheep       TMVPKHKEMP FPKYPVEPFT ESQSLTLTDV EKLHLPLPLV QSWMKQPPQP

Cow     151 LPPTVMFPPQ SVLSLSQSKV LPVPEKAVPY PQRDMPIQAF LLYQQPVLGP
Goat        LSPTVMFPPQ SVLSLSQPKV LPVPQKAVP- -QRDMPIQAF LLYQEPVLGP
Sheep       LPPTVMFPPQ SVLSLSQPKV LPVPQKAVP- -QRDMPIQAF LLYQEPVLGP

Cow     201 VRGPFPIIV
Goat        VRGPFPILV
Sheep       VRGPFPILV
```

Fig.11 Primary structure and sites of phosphorylation of bovine β-casein A^2, caprine β-casein and ovine β-casein

Goat: Roberts et al., 1992
Sheep: Provot et al., 1989; Richardson and Mercier, 1979

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Cow      1   KNTMEHVSSS  EESI-ISQETY  KQEKNMAINP  SKENLCSTFC  KEVVRNANEE
Goat         KHKMEHVSSS  EEPINIFQEIY  KQEKNMAIHP  RKEKLCTTSC  EEVVRNANEE
Sheep        KHKMEHVSSS  EEPINISQEIY  KQEKNMAIHP  RKEKLCTTSC  EEVVRNADEE

Cow     51   EYSIGSSSEE  SAEVATEEVK  ITVDDKHYQK  ALNEINQFYQ  KFPQYLQYLY
Goat         EYSIRSSSEE  SAEVAPEEIK  ITVDDKHYQK  ALNEINQFYQ  KFPQYLQYPY
Sheep        EYSIRSSSEE  SAEVAPEEVK  ITVDDKHYQK  ALNEINQFYQ  KFPQYLQYLY

Cow    101   QGPIVLNPWD  QVKRNAVPIT  PTLNREQLST  SEENSKKTVD  MESTEVFTKK
Goat         QGPIVLNPWD  QVKRNAGPFT  PTVNREQLST  SEENSKKTID  MESTEVFTKK
Sheep        QGPIVLNPWD  QVKRNAGPFT  PTVNREQLST  SEENSKKTID  MESTEVFTKK

Cow    151   TKLTEEEKNR  LNFLKKISQR  YQKFALPQYL  KTVYQHQKAM  KPWIQPKTKV
Goat         TKLTEEEKNR  LNFLKKISQY  YQKFAWPQYL  KTVDQHQKAM  KPWTQPKTNA
Sheep        TKLTEEEKNR  LNFLKKISQY  YQKFAWPQYL  KTVDQHQKAM  KPWTQPKTNA

Cow    201   IPYVRYL
Goat         IPYVRYL
Sheep        IPYVRYL
```

Fig.12 Primary structure and sites of phosphorylation of bovine α_{s2}-casein A, caprine α_{s2}-casein and ovine α_{s2}-casein

Goat: Bouniol, 1992
Sheep: Boisnard and Petrissant, 1985

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Cow      1   EEQNQEQPIR  CEKDERFFSD  KIAKYIPIQY  VLSRYPSYGL  NYYQQKPVAL
Goat         QEQNQEQPIC  CEKDERFFDD  KIAKYIPIQY  VLSRYPSYGL  NYYQQRPVAL
Sheep        QEQNQEQRIC  CEKDERFFDD  KIAKYIPIQY  VLSRYPSYGL  NYYQQRPVAL

Cow     51   INNQFLPYPY  YAKPAAVRSP  AQILQWQVLS  NTVPAKSCQA  QPTTMARHPH
Goat         INNQFLPYPY  YAKPVAVRSP  AQTLQWQVLP  NTVPAKSCQD  QPTTLARHPH
Sheep        INNQFLPYPY  YAKPVAVRSP  AQTLQWQVLP  NAVPAKSCQD  QPTAMARHPH

Cow    101   PHLSFMAIPP  KKNQDKTEIP  TINTIASGEP  T--STPTTEA  VESTVATLED
Goat         PHLSFMAIPP  KKDQDKTEVP  AINTIASAEP  TVHSTPTTEA  IVNTVDNPEA
Sheep        PHLSFMAIPP  KKDQDKTEIP  AINTIASAEP  TVHSTPTTEA  VVNAVDNPEA

Cow    151   SPEVIESPPE  INTVQVTSTA  V
Goat         SSESIASASE  TNTAQVTSTE  V
Sheep        SSESIASAPE  TNTAQVTSTE  V
```

Fig.13 Primary structure and sites of phosphorylation of bovine κ-casein A, caprine and ovine κ-caseins

Goat: Coll *et al.*, 1991; Mercier *et al.*, 1976; Mercier *et al.*, 1976
Sheep: Furet *et al.*, 1990; Jollès *et al.*, 1974(a); Jollès *et al.*, 1974(b)
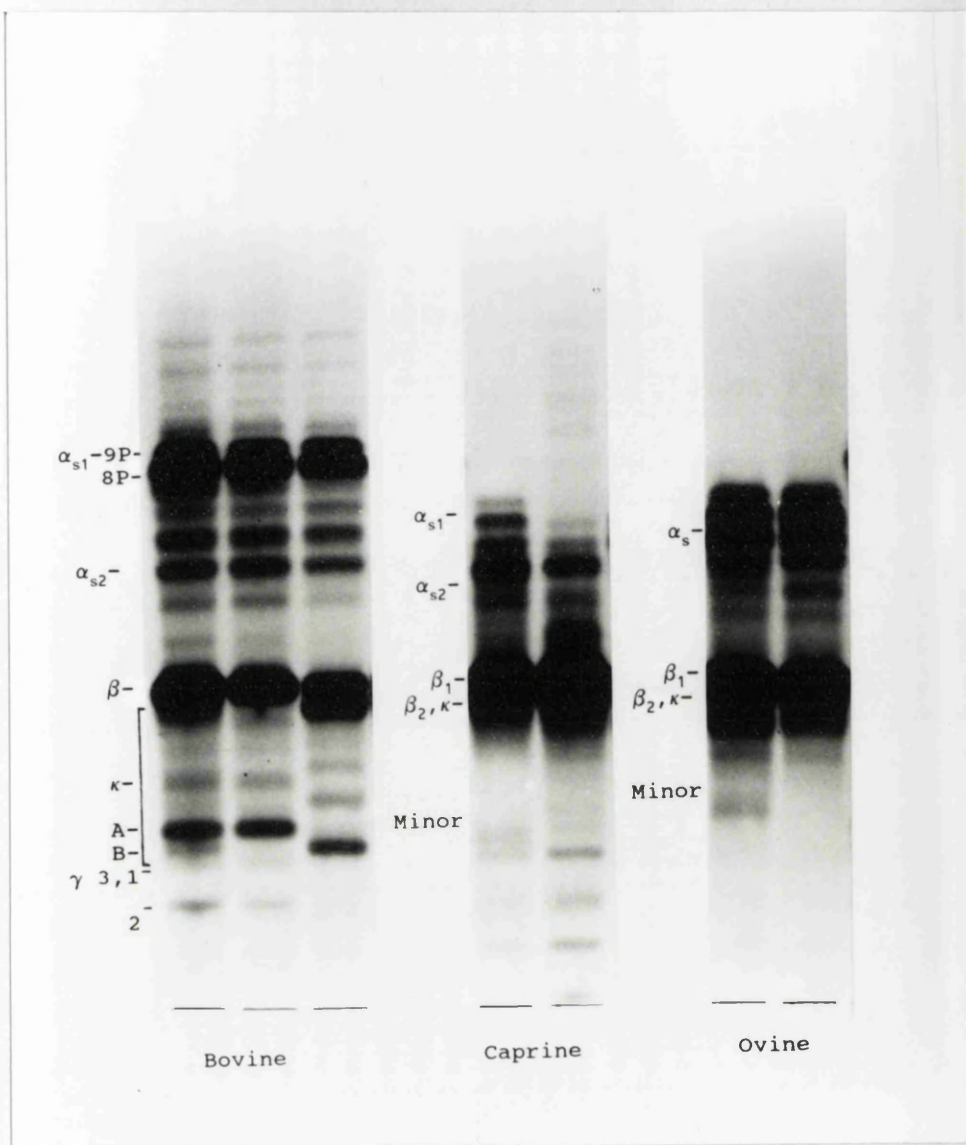
Fig. 14 Alkaline PAGE pattern obtained for whole casein from the cow goat and sheep.

Slots (left to right)
1 Bovine casein from herd bulk milk
2 Bovine casein from κ-AA cow
3 Bovine casein from κ-BB, β-AB cow

4 Caprine casein from goat producing high level of α_{s1}-casein
5 Caprine casein from goat producing low level of α_{s1}-casein

6 Ovine casein
7 Ovine casein

different levels of phosphorylation. Furthermore, caprine and ovine κ-caseins have a higher net negative charge than bovine κ-casein and overlap with the corresponding β-caseins.

Similar difficulties are experienced with anion-exchange chromatography and, although it is possible to fractionate the less tightly bound γ- and κ-caseins (Paper 8, Law and Tziboula, 1993; Papoff et al., 1993), it has not been possible to obtain a satisfactory quantitative fractionation of caprine and ovine β- and α_s- caseins. In the fractionation of caprine whole casein, this problem is compounded by the occurrence of genetic variants of α_{s1}-casein which differ in net negative charge, and elute at different positions from an anion-exchange column. As discussed below, genetic polymorphism of caprine α_{s1}-casein has pronounced effects on the concentration of α_{s1}-casein in milk, and on the processing characteristics of goats' milk. There is also evidence of the existence of genetic polymorphism in ovine α_{s1}-casein (King, 1966; Davoli et al., 1990; Papoff et al., 1993) and of quantitative genetic polymorphism in ovine α_{s2}-casein (Boisnard et al., 1991), both of which may have an effect on the properties of ovine casein.

This Chapter, therefore, describes the development of alternative methods of cation-exchange FPLC for the quantitative fractionations of caprine and ovine caseins. These methods had the advantages that results could be compared directly with those obtained by cation-exchange FPLC for bovine casein (Chapter 2), and could also be used to separate the caprine α_{s1}- and κ-casein genetic variants.

Anion- and cation-exchange FPLC were used to study the effect of genetic polymorphism in α_{s1}- and κ-caseins on the composition of caprine casein, and to examine seasonal variation in the composition of ovine casein.

4. SEPARATION AND PROPERTIES OF CAPRINE CASEINS

The techniques and conditions that have previously been used to separate the caprine caseins are similar to those described above for bovine caseins. Richardson et al. (1973) applied a combination of anion- and cation-exchange chromatography on DEAE and CM cellulose, respectively, in the presence of urea and 2-mercaptoethanol, to

obtain pure caprine κ-casein. They used alkaline PAGE to characterise the fractions and established the similarity in amino acid composition with bovine κ-casein.

Using these techniques, Addeo *et al.* (1978) showed that caprine κ-casein resembles bovine κ-casein in that it also undergoes cleavage at the Phe_{105}-Met_{106} bond, to give para-κ-casein (f1-105) and the caseinomacropeptide. They also found that, in common with bovine κ-casein, caprine κ-casein shows heterogeneity due to different degrees of glycosylation. In contrast to bovine κ-casein, however, it was found that caprine casein contains only one Met residue, and an extra Cys residue at position 10, in addition to the existing Cys_{11} and Cys_{88} (Fig. 13, Table 13). This third -SH group may lead to a different form of polymerisation from that in bovine κ-casein which usually occurs as a series of linear polymers containing, on average, five polypeptides (Swaisgood *et al.*, 1964). Also, caprine κ-casein has an extra phosphate group which is attached at Ser_{168}. The extra phosphorylation occurs because caprine κ-casein contains Glu instead of Ala at position 170, and satisfies the phosphorylation sequence Ser_{168}-X-Acidic residue (Mercier, 1981).

This extra negative charge may have an important effect on the behaviour of the casein micelles during the proteolytic and coagulation stages of renneting. Also, it has been shown by PAGE that caprine para-κ-casein has a net negative charge at alkaline pH whereas bovine para-κ-casein has a net positive charge (Addeo *et al.*, 1978). The presence of more negatively charged groups on the surface of the micelles would tend to inhibit coagulation, and may in part explain the poor coagulum strength of caprine casein.

Using the same chromatographic techniques as mentioned above, Richardson and Creamer (1974) found that the major caprine casein, β-casein, exists in two differently phosphorylated forms. β_1-Casein differs from β_2-casein, and from bovine β-casein, in having six phosphoseryl residues instead of five. This occurs because of the amino acid replacement Ile_{12} (bovine) to Thr giving the sequence Thr_{12}-X-Glu (Fig.11). This is a less favourable phosphorylation sequence, and only partial phosphorylation takes place, giving a mixture of five and six phosphate groups (Mercier, 1981).

Using anion-exchange chromatography on DEAE cellulose, Richardson and Creamer (1975) established the presence, and determined the amino acid composition

60

of α_{s2}-caseins in goats' milk. These workers also found that the relative amount of α_{s1}-casein was much lower than that in bovine casein. It has since been established that the concentration and relative amount of caprine α_{s1}-casein is variable; this is due to a quantitative genetic polymorphism in caprine α_{s1}-casein (Boulanger *et al.*, 1984; Grosclaude *et al.*, 1987).

Using a combination of electrophoresis, amino acid analysis and sequencing, it has been established that the synthesis of α_{s1}-casein is under the control of at least 14 possible alleles (Martin, 1993), giving six known polypeptides and four levels of expression. Alleles A, B and C are associated with high concentrations of α_{s1}-casein in milk (about 3.6 g/l), whereas alleles D and F are associated with low contents of α_{s1}-casein (about 0.6 g/l). Allele E (previously B') is associated with an intermediate amount (1.6 g/l), and allele α_{s1}-Cn° is associated with the absence of α_{s1}-casein (Grosclaude *et al.*, 1987; Mahé and Grosclaude, 1989; Brignon *et al.*,1990).

Some of the amino acid differences in the variants have been established, and these are summarised in Table 14. The changes in the amino acids lead to substantial differences in the properties of the variants. For example, variant B differs from A in having Glu at position 77, and this in turn allows phosphorylation of Ser_{75}, giving the B variant a higher negative charge at alkaline pH. Also, variant D differs from variant A by the deletion of 11 amino acids, including five phosphoserines. Similarly, variant F differs from variant B by the deletion of 37 amino acids, including 6 phosphoserines. Both of these deletions, therefore, lead to very large reductions in the net negative charges of the variants. The deletion of the phosphate centre leading to variant D, and the accompanying decrease in the rate of synthesis, resembles the changes associated with the bovine α_{s2}-Cn D variant (Table 4), and it has been proposed that, in both proteins, the deleted peptide corresponds to an exon. The deletion in variant F corresponds to two exons, and the reduced rate of synthesis is attributed to splice mutations giving an altered rate of processing of RNA transcripts into mature RNA (Brignon *et al.*, 1990). The reason for the reduced rate of synthesis of variant E has not been established. It is not believed to be due to changes in the amino acid sequence of the polypeptide, however, as the C variant, which has the same amino acid sequence, is synthesised at a high level (Brignon *et al.*, 1990).

The different charges on the α_{s1}-casein variants, and the lower α_{s1}-casein

Table 14. Differences in the genetic variants of caprine α_{s1}-casein, and their order of occurrence

Var.	Change →	Var.	Change→	Var.	Change →	Var.
(X)	$Glu_{77} \to Gln$	A				
	$Leu_{16} \to Pro$	B	$Arg_{100} \to Lys$ $Thr_{195} \to Ala$	(Y)	$His_8 \to Ile$	C
						E
			Del 59 - 69	D		
			Del 59 - 95	F		

Brignon *et al.* (1990)

contents associated with the D, E, F and null alleles, have a pronounced effect on the coagulation properties during the manufacture of cheese. Ambrosoli *et al.* (1988) and Remeuf (1993) found that milk with a low α_{s1}-casein content had a shorter coagulation time, whereas milk with high levels produced a firmer curd. Manfredi *et al.* (1993) and Pirisi *et al.* (1994) found that the milk from goats having a high rate of α_{s1}-casein synthesis was richer in dry matter, fat and casein and had a smaller mean micellar size. The milk also had better coagulation properties and gave high cheese yield, and these workers believed that this milk was more satisfactory for cheese manufacture.

Low pressure anion- and cation-exchange chromatography, as described above, gave satisfactory separations of some individual fractions, but were time consuming and did not give good resolution of the α_{s1}- and α_{s2}-caseins (Richardson and Creamer, 1975). Several workers have used reverse phase HPLC in acetonitrile and trifluoroacetic acid (Mikkelsen *et al.*, 1987; Mora-Gutierrez *et al.*, 1991; Jaubert and Martin, 1992); this technique has the advantage that the buffers are volatile and the proteins can be recovered easily from column fractions. There are reports, however, that recovery of some proteins, particularly bovine κ- and α_{s2}-caseins, may be incomplete (Visser *et al.*, 1991).

In the present work, preliminary trials showed that anion-exchange FPLC on a Mono Q column, in the presence of urea, gave good resolution of the γ- and κ-caseins (Paper 8, Law and Tziboula, 1993). On the other hand, the α_{s1}- and α_{s2}-caseins were not completely resolved, especially when α_{s1}-variants with lower net negative charge than the B variant were present. Also there was some ovelap of the α_{s2}- and β_1-caseins. The caseins could be separated, however, by cation-exchange FPLC as described in the previous section for bovine caseins.

The first paper in this section (Paper 7, Law and Tziboula, 1992) describes the fractionation of caprine caseins by cation-exchange FPLC on Mono S in the presence of urea. The fractions were characterised by alkaline and SDS-PAGE. The composition of whole casein varied considerably, and the overall range of values for whole casein was: α_{s1}-casein, 4.4-26.0; α_{s2}-casein, 5.0-19.2; β-casein, 42.2-64.0; κ-casein, 9.8-23.5; and minor caseins 6.9-15.0% of the total. Some of the variation was due to proteolysis of the caseins, giving substantial amounts of γ-caseins, but a considerable amount of the variation was attributed to genetic polymorphism of α_{s1}-casein.

Using cation-exchange FPLC it was possible to separate the A, B, and F variants of α_{s1}-casein, and it was found that the relative amount of α_{s1}-casein varied with the combination of alleles present. The level of expression of α_{s1}-casein followed the order A, B > E > F; mean values for the relative amounts of α_{s1}-casein associated with the corresponding phenotypes ranged from 22.7 down to 4.4% of the total.

Results showed that cation-exchange FPLC was suitable for quantitative studies of genetic polymorphism of caprine α_{s1}-casein, and could be used in future studies to examine the effect of α_{s1}-casein polymorphism on the coagulation and ripening stages during the manufacture of cheese. The method has since been used to examine the effect of stage of lactation on the concentrations and relative amounts of the individual caseins in milk from goats with high and low amounts of α_{s1}-casein (Brown *et al.*, 1995).

During the course of the work on genetic polymorphism of caprine α_{s1}-casein, a variant of κ-casein was identified which showed different behaviour on cation-exchange FPLC and alkaline PAGE. The second paper in this section (Paper 8, Law and Tziboula, 1993) describes characterisation of the κ-casein variant, and the corresponding para-κ-casein, by alkaline and SDS PAGE, chymosin action and anion-exchange FPLC. The κ-casein variant is fairly widespread (occurring in goats in Greece and Scotland), and may have a beneficial effect on the coagulation step during renneting, in that the para-κ-casein has no charge at alkaline pH, whereas the more common variant has a net negative charge. As opposed to the α_{s1}-casein variants, κ-caseins A and B were produced in equal amounts in heterozygotes.

5. SEPARATION AND PROPERTIES OF OVINE CASEINS

The ovine caseins have previously been separated by low pressure chromatography as described above for bovine and caprine caseins. Alais and Jollès (1967) used selective precipitation and anion-exchange chromatography on DEAE cellulose, in the presence of urea and 2-mercaptoethanol, to separate a pure fraction of ovine κ-casein. These workers established that ovine κ-casein was similar to bovine κ-casein in amino acid composition and in susceptiblity to rennin attack. It contained less carbohydrate and

different sugars, however, and on starch-urea gels had lower electrophoretic mobility. Mercier *et al.* (1968) used a preparative method of anion-exchange chromatography on DEAE cellulose, in the presence of urea and 2-mercaptoethanol, to isolate differently glycosylated fractions of κ-casein; they examined their rennin sensitivity and electrophoretic mobilities.

Soulier *et al.* (1975) prepared κ-casein by gel permeation chromatography in a buffer containing urea. κ-Casein appeared in the void volume, indicating the occurrence of polymers which were shown to be disulphide linked, as found for bovine κ-casein. These workers determined the amino acid composition of ovine κ-casein, and showed that it differed from bovine κ-casein in a number of respects; in particular, it contained an extra Cys and P group (Table 13). As in caprine casein, the third -SH may have an important effect on the degree of polymerisation of κ-casein, or on the reaction with β-lactoglobulin on heating. The complete sequence of ovine κ-casein has now been established by cDNA analysis, and this is compared with bovine and caprine κ-caseins in Fig. 13. As in caprine κ-casein, the extra phosphorylation of ovine κ-casein occurs at Ser_{168} because of substitution of Glu for Ala at position 170, giving the phosphorylation sequence Ser_{168}-X-Acidic residue (Mercier, 1981). It has been shown by PAGE that ovine para-κ-casein has a slight positive charge at alkaline pH (unpublished results), intermediate between the positive charge on bovine para-κ-casein and the net negative charge on caprine para-κ-casein (Addeo *et al.*, 1978).

Using anion-exchange chromatography on DEAE cellulose, Richardson and Creamer (1976) separated ovine casein into six main fractions, and established the presence of two major components, β_1- and β_2-, that were similar in amino acid composition to bovine β-casein. From the complete sequence of ovine β-casein (Fig. 11) it can be seen that two different forms occur because of the substitution of Ile_{12} (bovine) to Thr (ovine) which results in an extra phosphorylation site. As in caprine β-casein, this is a less favourable phosphorylation sequence, and partial phosphorylation takes place giving a mixture of five and six phosphate groups (Fig.11).

Richardson and Creamer (1976) also obtained a fraction from anion-exchange on DEAE cellulose which was similar to bovine α_{s1}-casein. The complete sequence of ovine α_{s1}-casein has now been established (Mercier *et al.*, 1985) and this is compared with the corresponding sequences for the cow and the goat (Fig. 10).

The method of anion-exchange chromatography on DEAE cellulose used by Richardson and Creamer (1976) was time-consuming, and did not give complete resolution of the β_1- and α_s-caseins. This problem is compounded by the occurrence of an α_{s1}-casein variant, the "Welsh" variant (King, 1966; Davoli *et al.*, 1990), which has lower electrophoretic mobility at alkaline pH; on anion-exchange chromatography, it tends to elute together with α_{s2}-casein.

Similarly, genetic polymorphism in ovine α_{s2}-casein makes quantitative fractionation difficult. Boisnard *et al.* (1991) have shown that normal genetic variation occurs due to amino acid substitution. However, for each genetic variant, they also found two other different forms of α_{s2}-casein, which differed by an internal deletion of nine amino acids, including a phosphoserine, Cys_{34} and Cys_{42}. A further deletion was found in other non-allelic forms of mRNA, and these workers suggested that the deletions arise because of incorrect processing of a unique form of pre-RNA, resulting in subsequent exon skipping and slightly smaller RNA transcripts. The changes in ovine α_{s2}-casein are similar to those already described for caprine α_{s1}-caseins D and F, and bovine α_{s2}-casein D (Section 4), and indicate that the mechanism of exon skipping is the same. The large deletion in some of the ovine α_{s2}-casein, and the removal of a serine phosphate, may cause considerable differences in its properties.

Haasnoot *et al.* (1986) used anion-exchange FPLC on a Mono Q column to separate ovine casein but did not identify the fractions. Papoff *et al.* (1993), using a similar method, were able to separate the γ-, κ- and β_2- caseins, and for most samples also obtained a reasonable separation of the β_1- and α_s-caseins. The separation of the major fractions was poorer when the α_{s1}-casein "Welsh" variant was present.

Dall'Olio *et al.* (1990) separated the κ- and α_{s2}-caseins from whole casein by binding them to a thiol-Sepharose affinity column. They were then able to identify which bands in the α_s-casein region of alkaline and acid gels represented α_{s2}-casein. However, this method could not be used quantitatively.

In the present work (Paper 9, Law *et al.*, 1992), ovine casein has been fractionated by cation-exchange FPLC chromatography on a Mono S column as described above for bovine and caprine caseins. Using alkaline and SDS PAGE, four main fractions were identified; on average ovine casein consisted of: β-casein, 50.4; α_s-casein, 34.2; κ-casein, 10.8; and γ-caseins, 4.6% of the total. There was

considerable variation in the composition of whole casein from individual sheep, and part of this was due to proteolysis of β- and α_s-caseins. This method of cation-exchange FPLC allowed direct comparison of the composition of bovine, caprine and ovine caseins, and this is shown in the first paper in this section (Paper 7, Law and Tziboula, 1992). Compared with bovine casein, ovine casein contained much more β-casein, less α_s-casein and a similar amount of κ- and γ-caseins.

The final paper in this Chapter (Paper 10, Muir *et al.*, 1993) describes the application of cation-exchange FPLC to examine seasonal changes in the composition of casein in a commercial flock over a lactation period, and the relation with compositional changes in other milk constituents. Results showed that there were marked increases in the concentrations of total and individual caseins between Spring and Autumn. As found in the study of the effect of season on the composition of bovine casein (Paper 1, Davies and Law, 1980), the relative amounts of the caseins showed little variation.

6. CONCLUSIONS

In the fractionation of caprine and ovine whole casein, anion-exchange FPLC gave satisfactory resolution of γ- and κ-caseins, but did not give complete separation of β_1-, α_{s2}- and α_{s1}-caseins, especially when less negatively charged variants of the α_{s1}-caseins were present. An alternative method of cation-exchange FPLC was developed which gave quantitative fractionations of caprine and ovine caseins.

In a study of genetic polymorphism in caprine casein, cation-exchange FPLC was used to obtain quantitative values for the relative amounts of the A, B, E and F variants of α_{s1}-casein. The A and B variants were associated with high levels of α_{s1}-casein, the E variant with an intermediate amount, and the F variant with a low level of α_{s1}-casein. The relative amount of α_{s1}-casein varied from 4.4 to 26.0% of the total casein, and the average composition of casein from British Saanen goats producing a low level of α_{s1}-casein was: β-casein (β_1 and β_2), 50.8; κ-casein, 20.5; α_{s2}-casein, 12.4; α_{s1}-casein, 6.7 ; and minor caseins, 9.7% of the total casein. Compared with bovine casein, therefore, caprine casein contained much more β- and κ-caseins, less α_{s1}-casein,

and a similar amount of α_{s2}-casein.

Genetic polymorphism of caprine κ-casein was also detected by cation-exchange FPLC, and the variants were further characterised by chymosin treatment and different types of PAGE. The polymorphism of caprine κ-casein, unlike that of the α_{s1}-casein or the bovine κ-casein (Chapter 2), did not affect the composition of whole casein, however, and the two variants were produced in equal amounts in the heterozygotes.

Results from cation-exchange FPLC showed that, on average, ovine whole casein consisted of β-casein (β_1 and β_2), 50.4; α_s-caseins, 34.2; κ-casein, 10.8; minor caseins, 4.6% of the total casein. Compared with bovine casein, therefore, ovine casein contained much more β-casein, less α_s-caseins, and similar amounts of κ- and γ-caseins. The composition of ovine casein varied considerably, however, and values for γ-caseins showed that, in some samples, there was a considerable amount of proteolysis of β- and α_s-caseins.

In a study of seasonal variation in the composition of ovine casein in a commercial Scottish flock, it was found that there were considerable changes in the concentrations of total and individual caseins, with a general increase in the concentrations from Spring to Autumn. The relative proportions of the caseins, however, stayed constant. The effect of season on bulk milk was, therefore, similar to that found in the caseins in cows' milks (Chapter 2), in that concentrations increased during the Summer, but the relative proportions of the caseins remained constant.

Quantitative fractionation of caprine casein by cation-exchange FPLC

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1. Introduction

The amino acid compositions of the major caprine caseins, β-, κ-, α_{s2}- and α_{s1}-, have been determined and their similarity to bovine caseins established (1, 2, 3, 4).

The caseins have been examined by electrophoresis and, so far, genetic polymorphism has been established in α_{s2}- and α_{s1}-caseins (5) and κ-casein (6). The polymorphism of α_{s1}-casein, which is under the control of 7 known alleles, is complicated by the occurrence of variants which differ in the concentration of α_{s1}-casein produced (5, 7, 8, 9). GROSCLAUDE et al. (7), using rocket immunoelectrophoresis, found that alleles α_{s1}-Cn^A, α_{s1}-Cn^B and α_{s1}-Cn^C are associated with a high concentration of α_{s1}-Cn in milk, whereas alleles D and F are associated with low α_{s1}- contents, and allele E is associated with an intermediate content. Allele α_{s1}-Cn^0 appears to be a true null allele, and is associated with the absence of α_{s1}-caseins in milk.

From the results of electrophoresis (5), anion-exchange chromatography (10), and from our own preliminary studies using anion-exchange FPLC, it appeared to be difficult to obtain consistent separation of the α_{s1}- and α_{s2}-caseins at alkaline pH, because of differences in the net negative charges of the variants.

In this paper, therefore, we have adapted a rapid method of cation-exchange FPLC, previously used for fractionation of bovine (11) and ovine (12) caseins, and have been able to separate caprine casein into 5 main fractions. We have then applied the method to examine the natural variation in the relative amounts of the individual caseins in two different herds of goats.

2. Materials and methods

2.1 Milk samples

Milk samples were collected from 30 British Saanen goats at the Institute, and from 29 goats in a commercial herd in Greece. Bulk milk samples were also collected from each herd. The goats were between 1 and 4 months in lactation.

2.2 Whole casein samples

Whole casein was prepared by precipitation with the addition of acetic acid (10 % v/v) and sodium acetate (1 M) (13). The precipitate was washed with water at pH 4.6 and freeze dried.

2.3 PAGE

The whole casein samples and selected fractions from chromatography were examined by alkaline and acid PAGE as described previously (14, 15), and, by sodium dodecyl sulphate (SDS) PAGE on "Phast-System" electrophoresis apparatus (Pharmacia Ltd.,

Milton Keynes, UK) with 12.5 or 20 % homogeneous gels in accordance with manufacturers instructions.

2.4 Rennet treatment

Fractions containing κ-casein were treated with rennet (Käselab Pulver; Chr. Hansen's Laboratorium A/S, Copenhagen, Denmark) in phosphate buffer (pH 6.5) as previously described (14).

2.5 Cation-exchange FPLC

The casein samples were fractionated by cation-exchange fast protein liquid chromatography (FPLC) at 20 °C on a Mono S HR 5/5 column in an acetate-urea buffer at pH 5.0, and with a NaCl gradient as described previously (11). The fractions were identified by PAGE. The peak areas were corrected for the baseline value, and the amounts of protein present were then calculated using the following $A_{1cm}^{1\%}$ values at 280 nm: minor caseins, 8.2; β-casein, 4.4; κ-casein, 8.2; α_{s1}-caseins, 9.9; α_{s2}-caseins 9.9.

3. Results and discussion

3.1 Qualitative analysis of fractions obtained by cation-exchange FPLC

The elution profile obtained by cation-exchange FPLC on Mono S is shown in Fig. 1 for whole casein from the bulk milk of 30 British Saanen goats.
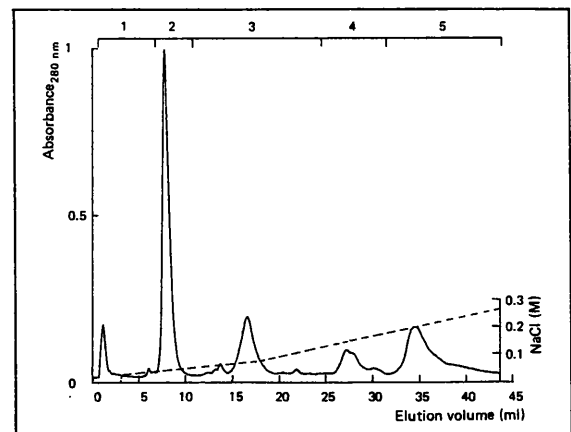
Fig. 1: Elution profile of reduced whole casein obtained by FPLC at 20 °C on a Mono S HR 5/5 column and with acetate-urea buffer (pH 5.0; 20 mM acetate, 6.0 M urea) and a NaCl gradient. About 4.0 mg casein were applied to the column, and the flow rate was 1.0 ml/min (—— absorbance, ---- NaCl gradient).

Materials from the various fractions were collected and, after dialysis and lyophilisation, gave alkaline PAGE patterns shown in Fig. 2, and SDS PAGE patterns in Figs. 3 and 4.

Fig. 4: SDS PAGE pattern on 12.5 % gel for whole casein (lane 4) and selected fractions obtained by FPLC of whole casein as shown in Fig. 1. Lane 1, fraction 1; lane 2, fraction 2; lane 3, fraction 4.
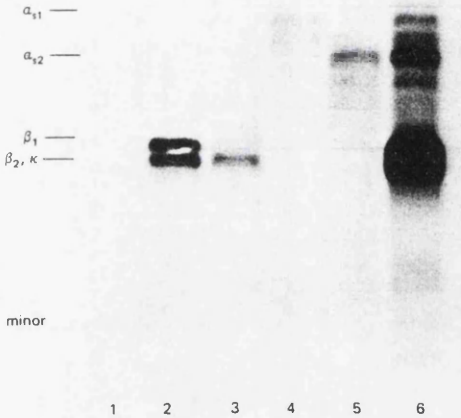
Fig. 2: Alkaline PAGE patterns for whole casein (lane 6) and the fractions obtained by FPLC of whole casein as shown in Fig. 1. Lane 1, fraction 1; lane 2, fraction 2; lane 3, fraction 3; lane 4, fraction 4; lane 5, fraction 5.
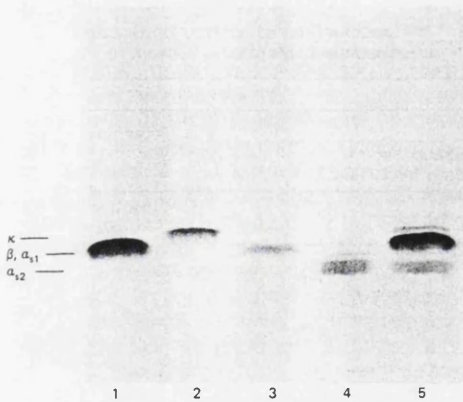
Fig. 3: SDS PAGE pattern on 20.0 % gel for whole casein (lane 5) and the fractions obtained by FPLC of whole casein as shown in Fig. 1. Lane 1, fraction 2; lane 2, fraction 3; lane 3, fraction 4, lane 4, fraction 5.

Fraction 1 contained protein of low molecular weight which was possibly a break-down product of β-casein. On alkaline PAGE (Fig. 2, lane 1) it appeared as a faint band of low mobility, similar to that of bovine γ-caseins. On SDS PAGE (Fig. 4, lane 1) it migrated well ahead of the major caseins and para-κ-casein (not shown), indicating a molecular weight of less than 12 k.

Fraction 2 contained β_1- and β_2-caseins. Alkaline PAGE (Fig. 2, lane 2) gave the characteristic pattern with two distinct bands due to the different levels of phosphorylation (1). SDS PAGE with a 20 % gel (Fig. 3, lane 1) or a 12.5 % gel (Fig. 4, lane 2) gave a single band, since the two phosphorylated levels are only slightly different in molecular weight.

Fraction 3 contained κ-casein. On alkaline PAGE (Fig. 2, lane 3) the main band had the same mobility as β_2-casein, but on SDS PAGE (Fig. 3, lane 2) the material migrated ahead of the β-casein in keeping with the smaller molecular weight of κ-casein. Also, on treatment with rennin, most of the material migrated as a single band of low molecular weight, indicative of conversion to para-κ-casein.

Fraction 4 contained α_{s1}-casein. On alkaline PAGE (Fig. 2, lane 4) of material from bulk milk, the main bands had the highest electrophoretic mobility. Several faint bands of lower mobility were also present. On SDS PAGE on a 20 % gel (Fig. 3, lane 3) the protein had the same mobility as β-casein, but on a 12.5 % gel (Fig. 4, lane 3) the protein migrated behind the β-casein (7, 8) indicating its separate identity. Acid PAGE showed that the casein from the herd of British Saanen goats contained mainly α_{s1}-casein B, together with some α_{s1}-casein A. To confirm that other genetic variants of α_{s1}-casein were successfully separated by chromatography on Mono S, fractions were collected from 11 samples of whole casein containing different genetic variants of α_{s1}-casein, and having a wide range of relative amounts of α_{s1}- and α_{s2}-caseins. Results of SDS PAGE on the α_{s1}- and α_{s2}-casein fractions showed that α_{s1}-caseins A, B and F eluted from Mono S at the same salt concentration, and that there was no detectable cross-contamination of the α_{s1}- and α_{s2}-caseins. On SDS PAGE α_{s1}-casein F was clearly detected as a broad band running ahead of κ-casein, as expected from the smaller molecular weight which results from the deletion of 37 amino acids (7, 9). In the present study no α_{s1}-casein C or D variants were detected.

Fraction 5 contained α_{s2}-casein. Alkaline PAGE (Fig. 2, lane 5) showed a number of bands of electrophoretic mobility less than the main α_{s1}-bands. On SDS PAGE (Fig. 3, lane 4) the protein migrated behind α_{s1}-casein, in keeping with its slightly higher molecular weight (7, 8). A very faint band of material, with slightly lower molecular weight than the main α_{s2}-band, could also be seen, and this has previously been identified as a minor α_{s2}-fraction (7).

3.2 Quantitative analysis by cation-exchange FPLC

Quantitative analysis of 59 whole casein samples from two different herds showed considerable variation in the relative amounts of the individual caseins (Table 1).

Table 1: The overall range of values for the relative amounts of the individual caseins in whole casein

Number of samples	59
Casein	% of total casein
α_{s1}-	4.4–26.0
α_{s2}-	5.0–19.2
β-	42.2–64.0
κ-	9.8–23.5
Minor	6.9–15.0

The distribution of the relative amounts of α_{s1}-casein in the samples is given in Fig. 5, and shows that the samples could be divided into three main groups according to their content of α_{s1}-casein. Also, it was possible to subdivide these groups according to the type of α_{s1}-casein present, as shown by acid PAGE.
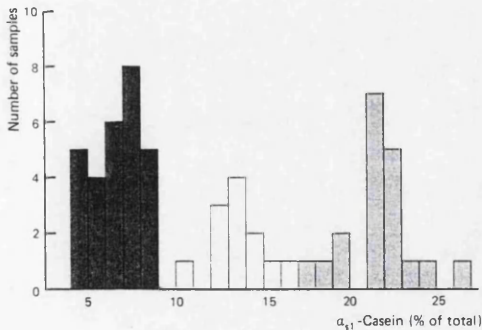
Fig. 5: Distribution of the relative amount of α_{s1}-casein in whole casein from two herds of goats.

On acid PAGE the samples which contained most α_{s1}-casein (Table 2) showed very prominent bands of either α_{s1}-casein A, α_{s1}-casein A and B, or α_{s1}-casein B only. This was in agreement with the results of BOULANGER et al. (5) who found that alleles α_{s1}-Cn^A and α_{s1}-Cn^B were associated with a high concentration of α_{s1}-casein in milk. From end group amino acid analysis BOULANGER et al. (5) found that α_{s1}-casein A amounted to 25 % of the total casein. From values in

Table 2: The composition of caprine whole casein with a high content of α_{s1}-casein

α_{s1}-Casein	A	A and B		B	
		No of samples			
British Saanen herd	0	1		0	
Greek commercial herd	1	12		5	
		% of total casein			
Casein		Mean	S.D.	Mean	S.D.
α_{s1}-	22.7	21.5	2.0	21.4	2.6
α_{s2}-	10.4	8.9	1.9	8.7	0.9
β-	46.1	46.5	2.4	46.9	1.6
κ-	12.6	12.6	1.7	12.4	2.6
Minor	8.3	10.6	2.3	10.5	1.3

Table 2 it can be seen that the composition of casein from the homozygotes and the heterozygotes was similar, indicating that the contribution of the α_{s1}-Cn^A and α_{s1}-Cn^B alleles was the same.

On acid PAGE the samples which contained an intermediate amount of α_{s1}-casein (Table 3) showed either a prominent band of α_{s1}-casein A together with a faint band of α_{s1}-casein B, or a band of medium intensity of α_{s1}-casein B only. This was consistent with the presence of either allele α_{s1}-Cn^A or α_{s1}-Cn^B, together with the allele α_{s1}-Cn^E which was associated with a diminished concentration of α_{s1}-casein B (7). The composition of casein in the two subdivisions was similar. GROSCLAUDE et al. (7) found that milk from

Table 3: The composition of caprine whole casein with an intermediate content of α_{s1}-casein

α_{s1}-Casein	A and B		B	
	No of samples			
British Saanen herd	2		0	
Greek commercial herd	5		5	
	% of total casein			
Casein	Mean	S.D.	Mean	S.D.
α_{s1}-	13.8	1.9	13.3	1.0
α_{s2}-	10.8	3.7	9.0	3.4
β-	50.9	3.9	50.2	2.9
κ-	15.4	3.2	16.2	4.0
Minor	9.2	1.3	11.3	2.5

goats of genotype α_{s1}-Cn^A/α_{s1}-Cn^E contained an intermediate concentration of α_{s1}-casein. Further calculation from those results showed that the relative amount of α_{s1}-casein was 13.9 % of the total casein, which was similar to the value found in this study.

On acid PAGE the samples containing the least amount of α_{s1}-casein (Table 4), with one exception, gave a single faint band of α_{s1}-casein B, and this was consistent with the effect of two α_{s1}-Cn^E alleles giving a very much reduced amount of α_{s1}-casein B (7).

One sample which contained only 4.4 % α_{s1}-casein (Table 4) gave, on SDS PAGE, a single band of lower molecular weight α_{s1}-casein F running ahead of κ-ca-

Table 4: The composition of caprine whole casein with a low content of α_{s1}-casein

α_{s1}-Casein	B	F
	No of samples	
British Saanen herd	27	0
Greek commercial herd	0	1

Casein	Mean	% of total casein S.D.	
α_{s1}-	6.7	1.3	4.4
α_{s2}-	12.4	2.4	6.5
β-	50.8	2.6	64.0
κ-	20.5	1.6	11.5
Minor	9.7	1.3	13.6

sein. The reduction in the amount of α_{s1}-casein appeared to be due to the effect of two α_{s1}-Cn^F alleles. GROSCLAUDE *et al.* (7) found that the double α_{s1}-Cn^F allele was associated with a low concentration of α_{s1}-casein in milk, and subsequent calculation showed that the relative amount of α_{s1}-casein F was 5.3 % of the total casein. This was in agreement with the value in this study.

Comparison of the composition of whole casein in each of the groups in Tables 2, 3 and 4 showed that, in addition to the variation in the relative amount of α_{s1}-casein, there was considerable variation in the relative amounts of the other caseins. Generally as the relative amount of α_{s1}-casein decreased, the relative amounts of β-, α_{s2}- and κ-caseins tended to increase, the increase in κ-casein being especially marked. The sample containing α_{s1}-casein F, however, had less κ-casein and considerably more β-casein than the samples with the same α_{s1}-casein content.

Genetic variation in α_{s1}-casein may, in part, explain the wide range of literature values for the composition of caprine casein. In the present study there was a considerable difference in the distribution of alleles in the two herds examined, with more of the α_{s1}-Cn^E allele in the herd of British Saanen goats and more of the α_{s1}-Cn^A and α_{s1}-Cn^B alleles in the Greek commercial herd. The whole casein from the bulk milk of the British Saanen herd consequently contained considerably less α_{s1}- and more α_{s2}-, β- and κ-caseins than that from the Greek commercial herd.

In the literature, values for the relative amount of α_{s1}-casein are between 5 and 25 % of the total casein (3, 5, 7), which are similar to the overall range found in this study. Also, where values are given in the literature for specific phenotypes (5, 7), the relative amounts of α_{s1}-casein, as discussed above, agree with corresponding values given here. Values in the literature for the relative amount of α_{s2}-casein vary between 17.3 and 25 % (3, 7), and are generally higher than found here by cation-exchange chromatography. This may partly reflect the difficulty in separating α_{s1}-casein variants from α_{s2}-casein by anion-exchange chromatography or electrophoresis. Values quoted for the relative amounts of β-casein vary between 45.2 and 60 % of the total casein (3, 7, 16) which is close to the overall range shown in the present study. Similarly, published values for the relative amount of κ-casein range from 10 to 26.6 % (3, 7, 16) which is close to the variation found in this study.

The method of cation-exchange FPLC described in this paper is particularly useful in that it permits direct comparison of the composition of caprine casein with results obtained by the same method (Table 5) for

Table 5: The composition, determined by cation-exchange FPLC on Mono S, of bovine and ovine whole casein

No. of samples	Bovine[1] Bulk sample from 100 cows	Ovine[2] 47
Casein	% of total casein	
α_{s1}-	36.2	
α_{s2}-	10.2	} 34.2
β-	40.8	50.4
κ-	10.1	10.8
Minor	2.8	4.6

[1] HOLLAR *et al.* (11)
[2] LAW *et al.* (12)

bovine (11) and ovine (12) casein. Compared with bovine casein, caprine casein contained much less α_{s1}-casein, a similar amount of α_{s2}-casein, and more β- and κ-caseins. Compared with ovine casein, caprine casein contained less α_s-casein (α_{s1}- and α_{s2}-), a similar amount of β-casein, and more κ-casein. To some extent the differences in composition of casein from the three species are due to the different content of α_{s1}-casein in caprine and bovine casein, or the different α_s-casein content of ovine casein. There was still, however, a higher proportion of κ-casein in caprine casein than in bovine or ovine casein, and this appeared to vary considerably with the α_{s1}-casein genetic variant, and, to some extent, with stage of lactation.

Cation-exchange FPLC is a rapid, automated method which is applicable to caprine, bovine and ovine caseins, and could be used in a quantitative examination of the relative amounts of the caseins in relation to their effect on the processing properties of milk.

Acknowledgement

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5. Summary

LAW, A.J.R., TZIBOULA, A.: Quantitative fractionation of caprine casein by cation-exchange FPLC. Milchwissenschaft 47 (9) 558-562 (1992).

24   Casein (fast protein liquid chromatography), goat milk (casein fractionation)

Caprine casein samples were fractionated by cation-exchange fast protein liquid chromatography (FPLC) at 20 °C on a Mono S HR 5/5 column in an acetate-urea buffer at pH 5.0 and with a NaCl gradient. Whole casein was resolved into 5 main fractions, which were characterized by PAGE, and one group of samples contained on average: (i) α_{s1}-, 21.6 %, (ii) α_{s2}-, 8.9 %, (iii) β-, 46.6 %, (iv) κ-, 12.5 % and (v) minor caseins, 10.4 %. In two other groups of samples genetic variation gave a reduced content of α_{s1}-casein. The group with an intermediate amount of α_{s1}-casein contained, on average: (i) α_{s1}-, 13.6 %, (ii) α_{s2}-, 10.0 %, (iii) β-, 50.6 %, (iv) κ-, 15.8 % and (v) minor caseins, 10.1 %. The third group with a low amount of α_{s1}-casein contained, on average, (i) α_{s1}-, 6.7 %, (ii) α_{s2}-, 12.4 %, (iii) β-, 50.8 %, (iv) κ-, 20.5 % and (v) minor caseins, 9.7 %. The compositions of caprine, bovine and ovine whole casein determined by this FPLC method are compared.

LAW, A.J.R., TZIBOULA, A.: Quantitative Fraktionierung von Ziegencasein durch Kationenaustausch-FPLC. Milchwissenschaft 47 (9) 558-562 (1992).

24   Casein (FPLC), Ziegenmilch (Caseinfraktionierung)

Ziegencaseinproben wurden durch Kationenaustausch-FPLC bei 20 °C durch eine Mono S HR 5/5-Säule in einem Acetatharnstoff-Puffer bei einem pH-Wert von 5,0 und mit einem NaCl-Gradienten fraktioniert. Das Gesamtcasein wurde in 5 wesentliche Fraktionen aufgelöst, die durch PAGE charakterisiert wurden; eine Probengruppe enthielt durchschnittlich an Caseinen: 21,6 % α_{s1}-Casein, 8,9 % α_{s2}-Casein, 46,6 % β-Casein, 12,5 % κ-Casein und an Minor-Caseinen 10,4 %. In zwei anderen Probengruppen ergab eine genetische Variation einen geringeren α_{s1}-Caseingehalt. Die Gruppe mit einem mittleren α_{s1}-Caseingehalt enthielt: 13,6 % α_{s1}-Casein, 10,0 % α_{s2}-Casein, 50,6 % β-Casein, 15,8 % κ-Casein und 10,1 % Minor-Caseine. Die dritte Gruppe mit einem niedrigen α_{s1}-Caseingehalt enthielt durchschnittlich 6,7 % α_{s1}-Casein, 12,4 % α_{s2}-Casein, 50,8 % β-Casein, 20,5 % κ-Casein und 9,7 % Minor-Caseine. Die Zusammensetzung der mit der FPLC-Methode bestimmten Ziegen-, Rinder- und Schaf-Gesamtcaseine werden verglichen.

Fractionation of caprine κ-casein and examination of polymorphism by FPLC

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1. Introduction

The primary structure of caprine κ-casein has been determined (1, 2) and the properties of the glyco-macropeptide and para-κ-casein have been described (3, 4). The occurrence of polymorphism in κ-casein has been demonstrated by electrophoresis at alkaline pH (5, 6, 7), and there is some evidence from polyacrylamide-agarose electrophoresis, isoelectric focusing (8) and reverse phase HPLC (9) that the variation is due to substitution in the para-κ-casein region.

In this paper we have separated two variants of κ-casein by cation-exchange FPLC and examined their electrophoretic properties. Also, using anion and cation-exchange FPLC, we have measured the relative amounts of κ-casein in whole casein, and have compared the relative amounts of the two variants in each of the heterozygotes.

2. Materials and methods

2.1 Milk samples

The milk samples, collected from 30 British Saanen goats at the Institute, and from 29 goats in a commercial herd in Greece, were the same as described by LAW and TZIBOULA (10). Whole casein samples were prepared by acid precipitation as described previously (10).

2.2 Cation exchange

The casein samples were fractionated by cation-exchange FPLC on a Mono S column as described in the previous publication (10).

2.3 Anion-exchange FPLC

The casein samples, reduced with mercaptoethanol, were also fractionated by anion-exchange FPLC at 20 °C on a Mono Q HR5/5 column in a bis Tris propane-urea buffer (pH 7.0; 5 mM bis Tris propane, 3.3 M urea) and with a NaCl gradient adjusted so that the concentration was 0.0 M after 1.0 ml, 0.17 M after 5.0 ml, 0.23 M after 17.5 ml, and 0.30 M after 43.5 ml.

2.4 Rennin treatment

Selected whole casein samples and κ-casein fractions were treated with rennin as described by DAVIES and LAW (11).

2.5 PAGE

Representative κ-casein fractions, before and after treatment with rennin, were examined by PAGE at alkaline pH, with normal and reversed polarity as described previously (11).

3. Results and discussion

3.1 Qualitative analysis of κ-casein fractions obtained by cation-exchange FPLC

On fractionation of the whole casein samples by cation-exchange FPLC, three different elution patterns were obtained in the κ-casein region (Fig. 1). Of 59 samples, 47 gave a single peak previously identified as κ-casein (10), eluting at about 15 ml (Fig. 1a), 2 gave a single peak at about 21 ml (Fig. 1c), and the remainig 10 gave two smaller peaks of about the same size eluting at the same positions as the two larger peaks (Fig. 1b). The difference in elution positions suggested the occurrence of two variants of κ-casein, with variant A eluting earlier from the cation-exchange column and having a smaller net positive charge at pH 5.0.
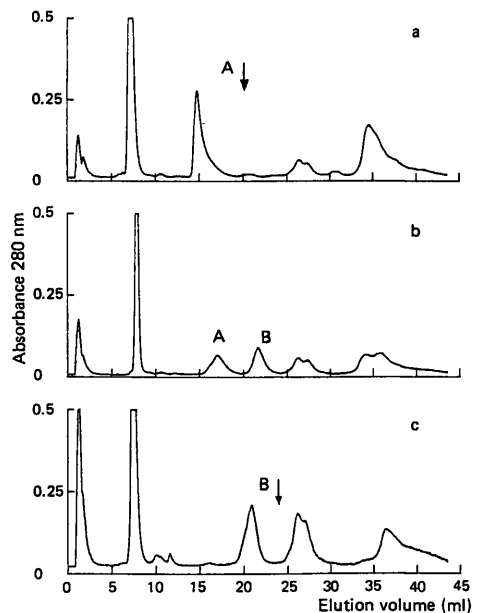
Fig. 1: Elution profiles of reduced whole casein obtained by FPLC at 20 °C on a Mono S HR5/5 column and with acetate-urea buffer (pH 5.0; 20 mM acetate, 6.0 M urea) and a NaCl gradient. About 4.0 mg casein were applied to the column, and the flow rate was 1.0 ml/min.
(a) κ-casein AA, (b) κ-casein AB, (c) κ-casein BB. The arrows show the elution positions of para-κ-caseins A and B, respectively.

Materials representative of peaks A and B were collected and, after dialysis and lyophilisation, gave PAGE patterns as shown in Fig. 2 (lanes 1 and 2

had the higher electrophoretic mobility at alkaline pH, whereas the less common variant had a higher electrophoretic mobility, and greater positive charge, at acid pH. Similarly those variants, on treatment with rennin, also gave para-κ-caseins which differed in charge in the same way as the variants described here. The variation cannot be attributed to differences in the carbohydrate since the para-κ-caseins also have different charges. Similarly a difference in the level of phosphorylation as the source of variation described here seems unlikely, since the κ-casein variants could be separated by cation-exchange FPLC, whereas the differently phosphorylated $β_1$- and $β_2$-caseins were not separated under the same conditions (10).

3.2 Qualitative analysis of κ-casein fractions obtained by anion-exchange FPLC

To help establish the accuracy of values for the relative amounts of κ-casein in whole casein samples, analysis was carried out by anion-exchange FPLC, and the results compared with those from cation-exchange FPLC. The elution profile obtained by anion-exchange FPLC of whole casein from the bulk milk of British Saanen goats, containing predominantly the κ-casein A variant, is shown in Fig. 3. The elution pattern in the κ-casein region (fraction 2) was identical for the B variant. The change in the elution pattern after treatment with rennin is indicated in Fig. 3 (dotted line),

Fig. 2: Alkaline PAGE patterns for whole casein (lane 5) and the κ-casein fractions obtained by cation-exchange FPLC of whole casein containing both variants as in Fig. 1 (b). Lane 1, κ-casein A; lane 2, κ-casein B; lane 3, κ-casein A after rennin treatment; lane 4, κ-casein B after rennin treatment.

respectively). Each of the peaks gave a prominent band with a diffuse area in front, and a fainter band about 8 mm behind the main band. The material from peak A had higher electrophoretic mobility indicating a higher net negative charge at alkaline pH.

On treatment with rennin the materials showed considerably reduced electrophoretic mobilities consistent with complete conversion of κ-casein variants to para-κ-casein (3). This was confirmed by SDS PAGE (results not shown) which showed that peaks A and B had similar molecular weight and, on treatment with rennin each gave a single band of the same molecular weight as para-κ-casein. On PAGE at alkaline pH, para-κ-casein A had a higher net negative charge and entered the gel, whereas para-κ-casein B remained in the slot. As further evidence of the difference in charge, the two forms of para-κ-casein were subjected to reverse polarity PAGE (results not shown) and the converse of the above was found, with para-κ-casein B entering the gel but para-κ-casein A remaining in the slot.

Also, when whole casein samples were treated with rennin and fractionated by cation-exchange FPLC the original κ-casein A and B peaks were absent, and corresponding peaks of para-κ-casein eluted as shown by the arrow in Fig. 1a and c respectively. The elution positions confirm that para-κ-casein A has a smaller net positive charge than para-κ-casein B at pH 5.0.

The combined results from FPLC and PAGE are consistent with the occurrence of two genetic polymorphs of κ-casein which are expressed in Mendelian fashion, giving phenotypes AA, AB and BB (47:10:2 respectively), the two variants probably arising by substitution of one or more amino acids in the para-κ-casein moiety. The variants occur in about the same frequency as those described by DI LUCCIA et al. (8) and have a number of similar properties, in that the most common variant described by those authors also

Fig. 3: Elution profile of reduced whole casein obtained by FPLC at 20 °C on a Mono Q HR5/5 column and with bis Tris propane-urea buffer (pH 7.0; 5 mM bis Tris propane, 3.3 M urea) and a NaCl gradient. About 4.0 mg casein, containing predominantly the κ-casein A variant, were applied to the column, and the flow rate was 1.0 ml/min (—— absorbance, - - - - NaCl gradient, ···· profile obtained after rennin treatment of whole casein). Fractions 1–7 were examined by PAGE as shown in Fig. 4.

and shows the removal of rennin-sensitive κ-casein and the appearance of para-κ-casein in the void volume. The identification of this fraction as κ-casein was confirmed by SDS PAGE (results not shown) and PAGE at alkaline pH. The electrophoretic pattern for κ-casein from herd bulk milk, which contained predominantly variant A, is shown in Fig. 4, lane 2. The pattern is similar to that obtained for κ-casein from
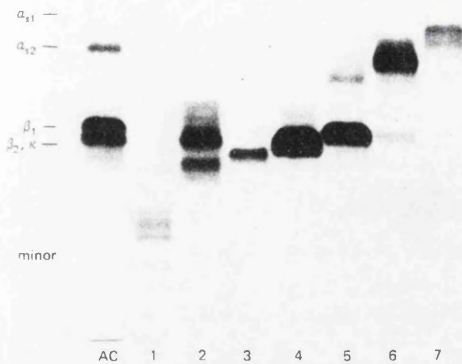
Fig. 4: Alkaline PAGE patterns for whole casein (AC) and the fractions obtained by anion-exchange FPLC of whole casein as in Fig. 3. Lanes 1-7, fractions 1-7.

Fig. 5: Correlation of the relative amounts in whole casein of κ-caseins A and B determined by cation-exchange FPLC.

cation-exchange FPLC (Fig. 2, lane 1) except that with higher loading on the gel the previously diffuse regions are seen as discrete bands. The identities of the other fractions were estab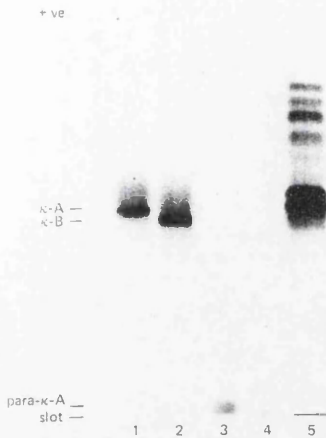lished from SDS and alkaline PAGE as follows: fraction 1, minor caseins of low molecular weight; fraction 3, unidentified material resistant to rennin attack; fraction 4, β_2-casein; fraction 5, β_1-casein together with some α_{s2}-casein; fraction 6, mainly α_{s2}-casein; fraction 7, mainly α_{s1}-casein B. Under the conditions described, anion-exchange FPLC gave good resolution of the early-eluting proteins, including κ-casein, but poorer separation of β_1-, α_{s2}- and α_{s1}-caseins, especially when variants of α_{s2}- and α_{s1}-caseins, with lower net negative charges (12, 13, 14, 15, 16) were present.

3.3 Quantitative analysis of κ-casein fractions obtained by anion- and cation-exchange FPLC

Comparison of results from anion- and cation-exchange FPLC for the relative amounts of κ-casein in whole casein showed that there was reasonable agreement between the two methods. The regression equation was: Mono S value $= 1.13 \times$ Mono Q value $- 1.15$, and the correlation coefficient was 0.897. There was considerable variation in the relative amounts of κ-casein as discussed previously (10), and it was found that in the casein from 10 different heterozygotes, the relative amounts of κ-casein (over a range 9-20 %) determined by anion-exchange FPLC were similar to the sum of the relative amounts of the A and B variants determined by cation-exchange FPLC.

Also, from the results of cation-exchange FPLC for whole casein from the heterozygotes, there was a good correlation between the relative amounts of the κ-casein A and B variants ($r = 0.979$), the two variants, within experimental error, being produced in similar amounts (Fig. 5).

The occurrence of the κ-casein variants in a herd of British Saanen goats and in a Greek commercial herd
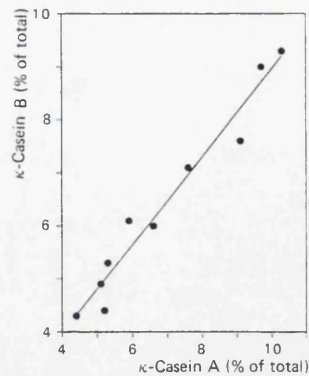
suggests that the polymorphism is widespread. Cation-exchange FPLC could be used to isolate the variants, and establish the difference in primary structures and the relation to variants already described in the literature.

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5. Summary

LAW, A.J.R., TZIBOULA, A.: **Fractionation of caprine *κ*-casein and examination of polymorphism by FPLC.** Milchwissenschaft **48** (2) 68–71 (1993).

24   Casein (fractionation), **goat milk (casein)**

Caprine casein samples from a commercial herd in Greece and from a herd of British Saanen goats were fractionated by cation- and anion-exchange FPLC, and the κ-casein fractions examined by PAGE. Cation-exchange FPLC showed the presence of two variants of κ-casein, which were expressed in a Mendelian way (47AA:10AB:2BB), and which differed in net charge in the para-κ-casein region. Values for the relative amounts of κ-casein in whole casein, determined by both methods of FPLC were in fairly close agreement and, although the amount of κ-casein varied between 9 and 20 % of the total, the variants were produced in approximately equal amounts in the heterozygotes.

LAW, A.J.R., TZIBOULA, A.: **Fraktionierung von Ziegen-*κ*-Casein und Prüfung des Polymorphismus durch FPLC.** Milchwissenschaft **48** (2) 68–71 (1993).

24   Casein (Fraktionierung), **Ziegenmilch (Casein)**

Caseinproben von Milch einer kommerziellen Ziegenherde in Griechenland und einer Herde britischer Saanen-Ziegen wurden durch Kationen- und Anionen-Austausch-FPLC fraktioniert und die κ-Caseinfraktionen mit PAGE untersucht. Die Kationen-Austausch-FPLC zeigte das Vorhandensein von zwei κ-Casein-Varianten, ausgedrückt nach Mendel (47AA:10AB:2BB), die sich in der Nettoladung im para-κ-Caseinbereich unterschieden. Die Werte für die relativen κ-Caseinmengen im Gesamtcasein, bestimmt nach beiden FPLC-Verfahren, stimmten recht gut überein. Während die κ-Caseinmenge zwischen 9 und 20 % der Gesamtmenge variierte, wurden die Varianten in etwa gleicher Menge bei den Heterozygoten gebildet.

Quantitative fractionation of ovine casein by cation-exchange FPLC

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1. Introduction

The amino acid sequences of the major ovine caseins, κ-, β-, α_{s2}- and α_{s1}-, have been determined, and their similarity to bovine caseins has been established (1, 2, 3, 4).

The caseins have been separated by starch and polyacrylamide gel electrophoresis (5, 6, 7) and quantitative values have been obtained by scanning gels (8, 9, 10).

The major caseins have also been separated and isolated by chromatography on DEAE cellulose (5) and the properties of κ-, β-, and α_s-caseins have been examined (11, 12). The method, however, is fairly lengthy and not suitable for rapid determination of the relative amounts of the individual caseins. Ovine casein has been fractionated by anion-exchange FPLC (13) but few quantitative results are available. In the present study, a rapid method of analysis by cation-exchange FPLC has been developed and used to examine natural variation in the composition of casein from Sardinian sheep.

2. Materials and methods

2.1 Milk samples

Milk samples were collected from 47 Sardinian ewes in three different flocks in North-West Sardinia. Mercuric chloride (0.01 %) was added as preservative and the samples kept refrigerated until precipitation of the casein.

2.2 Whole casein samples

Whole casein was prepared by precipitation from skim milk with the addition of acetic acid (10 % v/v) and sodium acetate (1 M) (14). The precipitate was washed twice with the above buffer, and then with water to remove non-casein material.

2.3 PAGE

The whole casein samples and selected fractions from chromatography were examined by alkaline PAGE as described previously (15), and by sodium dodecyl sulphate (SDS)-PAGE on "Phast-System" electrophoresis apparatus (Pharmacia Ltd., Milton Keynes, U.K.) with 12.5 or 20 % homogeneous gels in accordance with manufacturers instructions.

2.4 Rennet treatment of sample

Fractions containing κ-casein were treated with rennet (Käselab Pulver; Chr. Hansen's Laboratorium

A/S, Copenhagen, Denmark) in phosphate buffer (pH 6.5) as previously described (15).

2.5 Cation-exchange FPLC

Whole casein (about 8 mg) was dissolved at 20 °C in 1.0 ml acetate-urea buffer (20 mM acetate, 6.0 M urea, pH 5.0) and the pH adjusted to 7.0. Mercaptoethanol (5 μl) was added, and the solution stirred for 1 h. The pH was then adjusted back to 5.0, and the sample passed through a 0.22 μm Acrodisc LC PVDF syringe filter (Gelman Sciences, Northampton, U.K) prior to analysis.

The FPLC was carried out at 20 °C with 500 μl sample being applied to a Mono S HR 5/5 cation-exchange column (Pharmacia Ltd., Milton Keynes, U.K.), which had previously been equilibrated with 3 ml acetate-urea buffer containing 1.0 M NaCl, followed by 5 ml acetate-urea buffer. The sample was washed onto the column with 1 ml acetate-urea buffer and the caseins then eluted, at a flow rate of 1.0 ml/min, with a NaCl gradient formed by mixing acetate-urea buffer containing 1 M NaCl with the salt-free buffer so that the concentration of NaCl being fed into the column was 0.0 M after a total of 3.0 ml, 0.025 M after 3.1 ml, 0.075 M after 17.5 ml, 0.15 M after 27.5 ml and 0.26 M after 43.5 ml. Between fractionations the column was re-equilibrated as described above.

During elution the absorbance at 280 nm of the eluate was monitored continuously and data stored by a BBC microcomputer.

The identity of the eluted materials was established by PAGE of materials from selected fractions. The peak areas were corrected for the baseline value and the absorbance of mercaptoethanol in the void volume peak, and the amounts of protein present were then calculated using the following $A_{1cm}^{1\%}$ values at 280 nm: minor caseins, 6.1 (2); β-casein, 3.8 (12); κ-casein, 10.9 (11); α_s-caseins, 11.1 (12).

3. Results and discussion

3.1 Qualitative analysis of fractions obtained by cation-exchange FPLC

Ovine casein, reduced with mercaptoethanol, was fractionated in acetate-urea buffer, and the NaCl gradient adjusted to give optimum resolution. The elution profile is shown in Fig. 1.

Materials representative of the various FPLC fractions were collected and, after dialysis and lyophilisation, gave alkaline PAGE patterns shown in Fig. 2, and SDS PAGE patterns in Figs. 3 and 4.

Fraction 1 contained proteins of low molecular weights which were probably breakdown products of β- and α_s-caseins. The material was difficult to identify
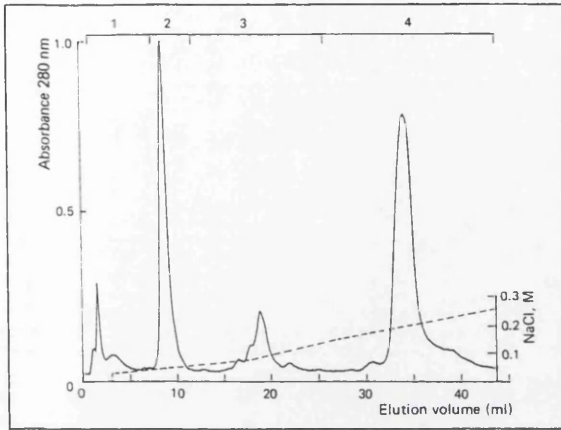
Fig. 1: Elution profile of reduced ovine whole casein obtained by fast protein liquid chromatography at 20 °C on a Mono S HR 5/5 column and with acetate-urea buffer (pH 5.0; 20 mM acetate, 6.0 mM urea) and a NaCl gradient. About 4.0 mg casein were applied to the column, and the flow rate was 1.0 ml/min. (―――― absorbance, ----- NaCl gradient)
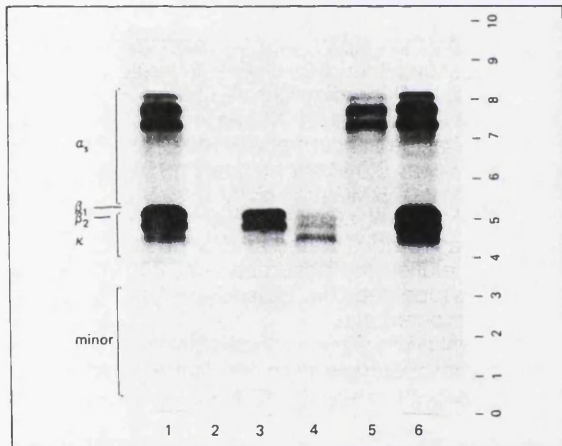
Fig. 2: Alkaline polyacrylamide gel electrophoretic patterns for ovine whole casein (lanes 1 and 6) and the fractions obtained by cation-exchange fast protein liquid chromatography of whole casein as shown in Fig. 1. Lane 2, fraction 1; lane 3, fraction 2; lane 4, fraction 3; lane 5, fraction 4.

by alkaline PAGE because of the small amount present in most samples. Although not visible in the photograph of the alkaline polyacrylamide gel, in some samples a number of faint bands could be seen about 1 and 2 cm from the slots. On SDS PAGE (Fig. 3, lane 1) fraction 1, from the whole casein sample shown in Fig. 1, appeared as a faint band of small molecular weight. Material from fraction 1 from another sample and which amounted to about 12 % of the total casein, appeared as a series of fairly prominent bands of molecular weight between about 8 and 19 K Daltons (Fig. 4, lane 3).
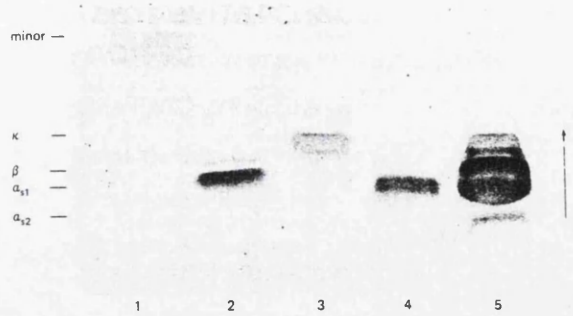
Fig. 3: SDS-polyacrylamide gel electrophoretic pattern on 12.5 % gel for ovine whole casein (lane 5) and the fractions obtained by cation-exchange fast protein liquid chromatography of whole casein as shown in Fig. 1. Lane 1, fraction 1; lane 2, fraction 2; lane 3, fraction 3; lane 4, fraction 4.
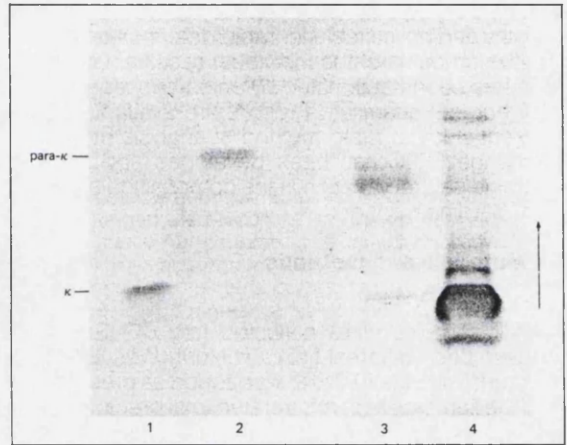
Fig. 4: SDS-polyacrylamide gel electrophoretic pattern on 20 % gel for selected fractions obtained by cation-exchange fast protein liquid chromatography. Lane 1, fraction 3 as shown in Fig. 1; lane 2, fraction 3 treated with rennin; lane 3, fraction 1 from a sample of whole casein showing extensive proteolysis; lane 4, whole casein sample showing extensive proteolysis.

From alkaline PAGE (Fig. 2, lane 3) and SDS PAGE (Fig. 3, lane 2), fraction 2 contained mainly β_1- and β_2-caseins. There was evidence, however, from both types of PAGE, that some minor proteins were present. The identity of these minor proteins is not known but, in view of their resistance to rennet attack and the occurrence on SDS PAGE of a faint band of molecular weight less than β-casein, it is possible that they are differently phosphorylated forms of β-casein.

Fraction 3 contained mainly κ-casein. The material could not be identified from alkaline PAGE alone because the migration distances of the bands were similar to those of the β-caseins. On SDS PAGE (Fig. 3, lane 3) the protein migrated as a band of molecular weight less than β-casein. After treatment of the fraction with rennet, most of the material migrated on SDS PAGE (Fig. 4, lane 2) as a band of low molecular weight, consistent with conversion to para-κ-casein.

Table 1: The composition of whole casein and the effect of proteolysis on the relative amounts of the individual caseins

No. of samples	Low 29		Degree of proteolysis Medium 14		High 4		All samples 47		
					% Total casein				
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Range
minor	2.8	1.0	5.6	0.8	14.2	5.5	4.6	3.6	0.6–20.5
β-	51.7	2.4	49.6	2.6	43.1	3.5	50.4	3.5	39.3–56.5
κ-	10.3	0.9	10.8	1.4	14.9	2.7	10.8	1.8	8.7–17.4
$α_s$-	35.1	2.6	34.0	2.5	27.8	6.5	34.2	3.6	18.6–40.4

Fraction 4, from alkaline PAGE (Fig. 2, lane 5), and SDS PAGE (Fig. 3, lane 4), contained $α_s$-caseins only.

3.2 Quantitative analysis by cation-exchange FPLC

The average composition of whole casein is given in Table 1. The values for the relative amount of β-casein were fairly close to those obtained by gel-scanning, 44.7, 40.6 and 53.8 % of the total casein (8, 9, 10). The relative amount of $α_s$-casein, however, was lower than corresponding values from gel-scanning, 42.3, 42.9 and 39.8 % obtained by the above workers.

The variation in composition of whole casein is shown in Table 1. About half of the samples contained small amounts of the minor caseins and showed little variation in composition. In the remainder of the samples, however, there was considerable variation in the relative amounts of the individual caseins, and this is shown in the overall range of the values in Table 1. Samples which contained large amounts of the low molecular weight minor caseins, contained less β- and $α_s$-caseins. These differences are consistent with proteolysis of the β- and $α_s$-caseins to form γ-caseins and other breakdown products as in bovine milk (16, 17, 18). In a few samples showing extensive proteolysis there was an appreciable increase in the value for the relative amount of κ-casein (Table 1). This was probably due to the inclusion of breakdown products which elute immediately before and after the κ-casein peak.

Comparison of the composition of ovine casein determined by the present method with the composition of bovine casein by anion-exchange FPLC (19), shows that ovine casein contains a similar amount of κ-casein, but much less $α_s$- and much more β-casein. In particular the ratio of β- : $α_s$-caseins in ovine casein is twice that in bovine casein. In view of the characteristic properties of β-casein and its tendency to dissociate from the micelle at lower temperatures, the greater proportion of β-casein may account, in part, for differences in stability or processing characteristics of ovine casein micelles. STORRY et al. (20), for example, in a study of coagulating properties of renneted milk from different species, found that the rennet coagulation time was negatively correlated with the ratio of β- : $α_s$-caseins.

The method of cation-exchange FPLC described in this paper could be used to examine such processing properties in more detail. Also, since the method offers the advantage of being a rapid, quantitative method which requires small amounts of sample, it could be used to study seasonal and lactational changes which occur in the composition of ovine casein.

4. References

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5. Summary

LAW, A.J.R., PAPOFF, C.M., DALGLEISH, D.G., CAMPUS, R.L.: Quantitative fractionation of ovine casein by cation-exchange FPLC. Milchwissenschaft 47 (5) 279–282 (1992).

24   Ovine casein (FPLC fractionation)

Casein samples from Sardinian sheep were fractionated by cation-exchange fast protein liquid chromatography (FPLC) at 20 °C on a Mono S HR 5/5 column in an acetate-urea buffer at pH 5.0 and with a NaCl gradient. Whole casein was resolved into four main fractions and contained, on average: (i) minor caseins, 4.6 %; (ii) β-casein, 50.4 %; (iii) κ-casein, 10.8 % and (iv) $α_s$-caseins, 34.2 %. In about half of the samples the degree of proteolysis was fairly slight and the composition of the casein varied little, but in the rest there were marked differences in the relative amounts of the caseins due to proteolytic breakdown of β- and $α_s$-caseins.

LAW, A.J.R., PAPOFF, C.M., DALGLEISH, D.G., CAMPUS, R.L.: Quantitative Fraktionierung von Schafmilchcasein durch Kationenaustausch FPLC. Milchwissenschaft 47 (5) 279–282 (1992).

24   Schafmilchcasein (FPLC-Fraktionierung)

Caseinproben von sardischen Schafen wurden durch Kationenaustausch-Schnell-Protein-Flüssig-Chromatographie (FPLC) bei 20 °C in einer Mono S HR 5/5-Säule in einem Acetat-Harnstoff-Puffer bei pH 5,0 und mit einem NaCl-Gradienten fraktioniert. Gesamtcasein wurde in vier Hauptfraktionen aufgespalten und enthielt durchschnittlich: 1) Minorcaseine 4,6 %; 2) β-Casein 50,4 %; 3) κ-Casein 10,8 % und 4) $α_s$-Caseine 34,2 %. In etwa der Hälfte der Proben war der Proteolysegrad sehr gering, und die Zusammensetzung des Caseins schwankte geringfügig, während in den restlichen Proben die Unterschiede im relativen Caseingehalt durch den proteolytischen Abbau der β- und $α_s$-Caseine beträchtlich waren.

Ovine Milk. 1. Seasonal changes in composition of milk from a commercial Scottish flock

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1. Introduction

Within the European Community bovine milk is in surplus and worldwide economic factors have decreed that agricultural subsidies be reduced. However, dairy farming has provided an important source of income for farmers with limited resource and has contributed positively to the social stability and welfare of regions less favored by climate or geography. In such areas, alternate sources of income are hard to find and efforts are now being focused on profitable methods of diversification.

Dairy sheep and goats exemplify this type of activity because such enterprises are ideally suited to small-scale operations and to implementation on marginal land. The retail market for sheep milk products in the United Kingdom is very small at present but, judging from consumer interest, the potential market may be substantial. Currently, at least 15,000 dairy sheep are in production in the UK and almost all ovine milk is manufactured into cheese at high added value. For the market to develop further, a greater diversity of manufactured products is required, and this presupposes a detailed knowledge of the composition of ovine milk produced under local conditions.

Although much is known about sheep milk from the traditional production areas of Southern Europe, the Middle East and North Africa (1), composition varies within wide limits as a function of breed, husbandry, stage of lactation and season. Therefore, it is not possible to extrapolate to conditions in Scotland. For this reason, information has been gathered on the composition of sheep milk from a commercial flock of dairy ewes over a full season. Associated studies have considered the effect of such changes in composition on the suitability of the milk for manufacture of yogurt (2) and on indices of stability (3).

2. Materials and methods

2.1 Husbandry of flock

As shown in Table 1, the flock comprised dairy sheep predominantly of the Friesland type which were lambed in late winter. In the early part of the year the herd was housed inside, and the main source of forage was grass silage but, from April onwards, the flock had access to fresh grass pasture. Throughout the survey period the forage was supplemented by concentrate rations. Milk yield peaked in May and reduced by late September to a level which was no longer commercially viable. Soon thereafter, the animals were dried off. Milking frequency was twice per day from March to August but once daily, when yields fell in

September. Immediately after milking, the milk was cooled to < 6 °C at which temperature it was maintained until transported to the Institute for analysis and evaluation.

Table 1: Husbandry of commercial flock from which samples were collected

	Mar	Apr	May	Jun	Jul	Aug	Sep
Number of ewes in milk	53	80	79	79	70	69	60
Milking frequency/day	2	2	2	2	2	2	1
Herd yield (l/day)	65	88	104	61	56	50	29
Diet[1]	s	s+g	g	g	g	g	g

[1] Concentrate was fed throughout lactation; s = grass silage; g = grass, grazed at pasture

2.2 Analysis of gross composition

The total solids, fat, crude protein, lactose and ash content of the bulk milk were measured by methods described previously (4).

Non-protein nitrogen and casein nitrogen were also estimated after selective precipitation (4).

Mineral analyses were carried out as described in HOLT et al. (5). In addition to measurement of the total content of calcium and inorganic phosphate, an estimate of diffusible salts was also obtained by analysis of ultrafiltrate (5).

2.3 Characterisation of milk fat

The particle size distribution of the milk fat globules was estimated by forward lobe laser light scattering (Malvern Instruments Mastersizer; Malvern, England) (6). Fat globules were dispersed in a disaggregating medium (Isoton II; Coulter Electronics, Luton, England). Particle size distributions were calculated using a log-normal distribution and a presentation factor of 0403.

Fat was isolated from the whole milk by the method of BLIGH and DYER (7), the methyl esters of the fatty acids prepared and the proportions of fatty acids in the triglyceride estimated (8).

The triglyceride distribution was also characterised by gas liquid chromatography (10).

Melting profiles of the extracted fat were measured by differential scanning calorimetry (9, 10).

2.4 Analysis of casein

The casein fractions were isolated and quantitatively fractionated as described in LAW et al. (11).

3. Results and discussion

3.1 Gross composition of milk

As shown in Table 2, the fat content was steady for 5 of the 7 months. Its drop in May parallels the phenomenon regularly found with bovine milk in South-west Scotland (4) and is associated with the transition from indoor feeding to grazing fresh, high quality grass pasture. In contrast, the crude protein content increased progressively throughout lactation, and this was mirrored by a fall in lactose content.

The casein number was remarkably constant (78.5–80) suggesting that the level of udder disease remained low throughout the season and that the lactational decline in yield observed in September had not been accompanied by substantial disruption of the physiological state of the mammary gland.

The non-protein-nitrogen content of the ovine milk was substantially higher than that associated with bovine milk and doubled in concentration over the season.

As might be expected for milk rich in protein, the calcium and inorganic phosphate content of the milk was high and tended to increase in line with crude protein content.

3.2 Composition of casein

Using cation-exchange FPLC (11), the casein was clearly discriminated into four classes: α_s-, β-, κ- and minor caseins. Seasonal variation based on this classification is shown in Table 2. The fairly constant casein composition was very similar to bovine casein (12), although the relative proportions of the various fractions were significantly different. Ovine casein contained more β-casein and less α_s-casein than bovine casein.

Small variations occurred in the proportion of minor caseins, but the overall levels (6.5 %) suggested that post-translational proteolysis was

limited in extent (12). This was consistent with the fact that the casein number also remained constant over the test period.

3.3 Fat globule size

The fat globule size distributions were compared in Table 3 with samples of bulk bovine milk collected from the Hannah Research Institute herd on the same day. On average, the fat globules were larger than the corresponding globules in bovine milk (the difference was statistically highly significant). However, whilst seasonal variation in the size of fat globules in bovine milk was modest, large changes were observed in the corresponding sheep milks. In March and April, globule size – measured by 3 separate indices – was significantly higher than in the other 5 months. There was a precipitate drop in globule size in May when the flock went out to pasture. Although, this drop was associated with other changes in composition detailed earlier there was no clear relation between particle size and composition – for example, fat content.

Table 3: Seasonal changes in indices of particle size (micron) of fat globules from sheep milk.

Particle size index[1]	Month of sample collection							Bovine milk[2]
	Mar	Apr	May	Jun	Jul	Aug	Sep	
D [0.5]	5.26	5.12	3.62	4.03	4.06	3.94	3.61	3.67
D [0.9]	9.14	8.93	7.50	7.66	8.35	8.04	7.73	7.48
D [4.3]	5.78	5.63	4.26	4.58	4.77	4.61	4.31	4.29
Span	1.17	1.17	1.59	1.38	1.56	1.54	1.68	1.55

[1] Presentation factor 0403; D [0.5] = median diameter; D [0.9] = diameter at uppermost decile; D [4.3] = volume weighted diameter; Span = a measure of the breadth of the particle size distribution.
[2] Mean values for herd bulk milk sampled at same time as sheep milk and characterised by the same technique.

Table 2: Seasonal changes in composition of sheep milk

		Month of sample collection						
		Mar	Apr	May	Jun	Jul	Aug	Sep
Total solids	%	17.52	17.53	16.49	17.97	18.37	19.08	22.23
Fat	%	7.4	7.00	5.58	7.10	7.09	7.60	9.49
Crude protein	%	4.96	4.99	5.31	5.61	5.87	6.28	7.75
Lactose	%	4.74	4.66	4.71	4.42	4.43	4.22	3.68
Casein number	%	79.5	79.1	80.0	79.3	79.1	78.5	79.2
Non-protein-nitrogen	mg %	67.2	63.9	80.3	104.4	117.3	138.2	149.5
Minor casein (% total casein)		7.7	4.9	6.9	6.1	6.5	5.9	7.2
β-Casein (% total casein)		45.9	46.4	43.3	46.3	45.0	45.5	45.9
κ-Casein (% total casein)		8.3	8.3	10.5	8.9	9.8	9.7	9.7
α_s-Casein (% total casein)		38.1	40.4	39.3	38.7	38.7	38.9	37.2
Chloride	mM/l	22.7	25.7	23.4	27.7	24.6	27.3	27.3
Citrate	mM/l	8.85	8.02	8.54	8.19	7.57	7.32	7.46
Calcium, total	mM/l	39.9	37.6	45.0	41.5	43.9	40.2	53.1
Calcium, diffusible	mM/l	6.91	7.43	6.68	6.41	5.69	5.42	6.18
Phosphate, total	mM/l	27.9	28.8	33.0	30.2	33.6	27.3	35.7
Phosphate, diffusible	mM/l	10.85	13.85	12.57	11.87	14.46	12.28	13.01

The average increase in globule size of sheep milk was consistent with its behaviour on processing. Over an extensive series of further experiments, cream separated from sheep milk had a significantly higher fat content than that separated from bulk bovine milk treated under identical conditions.

3.4 Fatty acid composition

The seasonal changes are described in Table 4. Simplification of the differences between samples was achieved by Principal Component Analysis (PCA). Analysis of the covariance matrix yielded a solution in which the first two PCs encompassed the overwhelming majority (> 97 %) of the variance. Examination of the vector loadings on these PCs indicated that the differences between samples could be best explained by differences in the proportions of decanoic (C10:0), palmitic (C16:0) and oleic (C18:1) fatty acids. Ovine milk fat, like its bovine counterpart, contained a substantial proportion of mono-unsaturated fatty acids although the proportion of poly-unsaturated acids was low.

The changes in the relative proportions of fatty acids appeared to be associated with dietary factors. In May, when the sheep were mainly eating fresh young grass, the oleic acid content of the milk fat was at its lowest, coinciding with the time at which the short chain fatty acids (SCFA) derived from *de novo* synthesis (Table 4) were at their highest level.

3.5 Triglyceride distribution

As might be expected from the changes in fatty acid composition, there were substantial seasonal changes in triglyceride distribution (Table 5). Once more, simplification was achieved by PCA. A model based on 3 PCs explained nearly 97 % of variance – 65.8, 26.6 and 4.3 % being associated with the individual components. Examination of the vector loadings on the PCs suggested that the main differences between samples lay in the proportions of triglycerides with carbon numbers 42, 52, 38 and 40. With the exception of the sample collected in May, the distribution was biomodal.

Table 5: Seasonal changes in distribution of carbon number (mole %) of triglyceride extracted from sheep milk

Carbon number	Month of sample collection						
	Mar	Apr	May	Jun	Jul	Aug	Sep
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28	0.4	0.5	0.8	0.1	0.4	0.2	0.3
30	1.7	0.9	3.2	0.7	1.8	1.4	1.8
32	3.2	1.6	4.2	1.5	2.2	2.0	2.7
34	5.7	4.5	6.8	3.0	5.1	3.8	5.2
36	10.3	9.2	10.9	8.0	8.3	8.5	8.0
38	19.7	19.2	16.3	16.5	13.2	12.8	12.5
40	14.5	14.3	15.0	13.0	15.7	16.2	12.3
42	7.4	6.0	11.8	6.1	9.9	9.7	11.8
44	5.7	5.6	8.6	5.3	8.2	9.1	9.7
46	5.8	4.4	6.6	5.2	7.4	8.2	8.9
48	6.4	6.2	5.1	7.0	6.8	7.6	7.5
50	8.6	10.8	4.6	10.8	9.0	7.7	7.6
52	8.4	13.4	4.9	17.6	8.7	9.6	9.0
54	2.1	3.8	1.2	5.4	3.6	3.3	2.8

3.6 Melting properties of fat

Table 6: Seasonal changes in melting behaviour of milk fat isolated from ovine milk

Month	Fraction (%) melting within range (°C)				
	<5	5–10	10–15	15–20	>20
March	45.1	14.2	14.4	5.0	20.9
April	44.7	5.0	8.2	9.2	32.9
May	35.3	10.3	13.4	12.9	28.0
June	36.0	9.7	13.5	6.4	34.4
July	29.4	13.6	16.7	6.1	34.2
August	37.3	11.0	12.1	8.3	31.3
September	36.8	9.6	10.7	12.8	30.1

Table 4: Seasonal changes in fatty acid composition (mole %) of triglyceride extracted from sheep milk

Fatty acid[1]	Month of sample collection						
	Mar	Apr	May	Jun	Jul	Aug	Sep
4:0	11.0	10.4	9.0	8.9	7.8	7.0	6.7
6:0	4.0	3.3	4.9	3.0	4.0	3.8	3.7
8:0	2.5	2.2	4.1	2.0	3.2	2.9	3.0
10:0	5.2	4.2	10.4	4.5	7.5	7.3	8.2
12:0	3.6	3.0	5.6	2.9	4.1	4.1	5.0
14:0	7.8	7.1	9.8	6.7	8.9	9.3	10.2
14:1	1.1	1.3	1.8	1.4	1.6	1.7	1.9
16:0	24.8	26.1	20.3	23.2	20.1	20.5	18.3
16:1	1.9	2.6	2.0	3.7	3.7	4.0	4.2
18:0	12.0	11.8	7.5	10.3	8.9	9.1	7.6
18:1	22.7	23.8	19.2	26.8	23.4	23.1	22.3
18:2	2.4	3.1	2.6	4.0	3.0	3.4	3.6
18:3	0.9	1.2	2.8	2.6	3.7	3.9	5.2
Ratio 18:0/18:1	0.53	0.50	0.39	0.38	0.38	0.39	0.34
SCFA[2]	35.2	31.5	45.6	29.4	37.1	36.1	38.7

[1] First digit defines carbon number; second describes degree of unsaturation
[2] SCFA = Short chain fatty acids C4:0–C14:1

The ratio of stearic to oleic acid was higher in the early spring when the sheep were housed indoors, but fell abruptly in May and remained constant thereafter at a value similar to that found in bovine milk.

As shown in Table 6, there were substantial differences in softness as evidenced by the proportion of the fat which melted below 5 °C. By analogy with bovine milk fat, it was expected that the proportions of SCFA and oleic acid might influence melting properties. The fraction melting below 5 °C was related to both fractions and a statistically significant relation was found which explained 84 % of variance:

Fraction melting <5 °C
$$= 282 - 2.84 [SCFA] - 6.14 [C18:1]$$

The fitted *versus* predicted values together with the standard deviations of the fitted values are shown in Fig. 1. Clearly, the relation is sufficiently tight to promise some predictive value. Nevertheless, this finding does not imply causality. Whilst the relation found between melting properties and SCFA parallels that for bovine milk, the result for oleic acid is opposite to that found for bovine milk This aspect deserves further study.
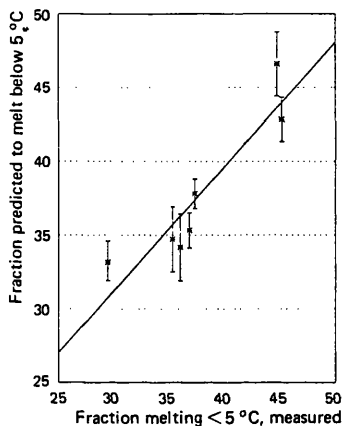
Fig. 1: Prediction of melting behaviour of ovine milk fat from fatty acid distribution. Values predicted from the relation Fraction melting <5 °C = 282 − 2.84* [Short chain fatty acids] − 6.14*[oleic acid]; regression accounted for 84.2 % variance. Values shown are of predicted values with standard deviation of fitted value shown as error bars.

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5. Summary

MUIR, D.D., HORNE, D.S., LAW, A.J.R., STEELE, W.: **Ovine milk. 1. Seasonal changes in composition of milk from a commercial Scottish flock.** Milchwissenschaft **48** (7) 363–366 (1993).

24 Sheep milk (composition)

The seasonal changes in the composition of milk from a commercial flock of ewes have been studied. The milk composition exhibited seasonal changes in fat and protein content similar to those found in bovine milk produced under an equivalent feeding regime, albeit at substantially higher concentrations. There were significant differences in casein composition between species. Ovine casein had a higher proportion of β-casein and a lower proportion of α_s-casein than bovine casein. The average size of milk fat globules in ovine milk was greater than the bovine equivalent. There were significant seasonal variations in fat composition, the most notable being associated with the proportion of short chain fatty acids (SCFA) and in the relative amounts of oleic and palmitic acid. Corresponding changes in triglyceride distribution and melting behaviour were also noted.

MUIR, D.D., HORNE, D.S., LAW, A.J.R., STEELE, W.: **Schafmilch. 1. Jahreszeitliche Veränderungen in der Zusammensetzung der Milch einer kommerziellen schottischen Herde.** Milchwissenschaft **48** (7) 363–366 (1993).

24 Schafmilch (Zusammensetzung)

Die jahreszeitlichen Veränderungen in der Zusammensetzung der Milch einer gewerblichen Schafherde wurden untersucht. Die Milchzusammensetzung zeigte deutliche saisonale Veränderungen beim Fett- und Proteingehalt. Diese ähnelten denen der Kuhmilch, die unter vergleichbaren Fütterungsbedingungen erzeugt wird, wenn auch bei deutlich höheren Konzentrationen. Es bestanden signifikante Unterschiede in der Caseinzusammensetzung zwischen den Species. Schafcasein hat einen höheren Anteil von β-Casein und einen niedrigeren Anteil von α_s-Casein als Kuhcasein. Die durchschnittliche Größe der Milchfettkügelchen in Schafmilch ist größer als in Kuhmilch. Es bestand eine signifikante saisonale Variation in der Fettzusammensetzung. Besonders beachtenswert sind der Anteil kurzkettiger Fettsäuren (SCFA) und die Unterschiede der relativen Mengen an Öl- und Palmitinsäure. Entsprechende Veränderungen bestehen auch hinsichtlich der Triglyceridverteilung und des Schmelzverhaltens.

THE COMPOSITION OF BOVINE, CAPRINE AND OVINE WHEY PROTEINS

1. INTRODUCTION

The whey proteins constitute about 20% of the total protein in milk, and are usually obtained as by-products in the manufacture of cheese, or rennet and acid casein. Whey proteins remaining after acid precipitation of the caseins from milk include β-lactoglobulin, α-lactalbumin, serum albumin, lactoferrin and immunoglobulins. The proteose peptones, which are mainly proteolytic fragments of β-casein, are also present. Whey proteins obtained during the manufacture of cheese or rennet casein also contain the caseinomacropeptide, small amounts of caseins and some other proteolytic fragments of the caseins.

Initially, recovery of whey products, including the whey proteins, was carried out to avoid the problem of disposing of material which, in cheese-making, amounted to about 90% by weight of the original milk. It is now recognised, however, that the whey proteins have high nutritional value, and are a rich source of essential amino acids. They also have useful functional properties as emulsifiers, stabilisers and gelling agents, and are added to a wide variety of processed foods (Mulvihill, 1992).

Various methods have been used to recover the proteins from whey, including precipitation, ion-exchange and gel permeation chromatography and ultrafiltration. Another way of retaining the whey proteins during processing is to heat milk, and cause their denaturation and close association with the caseins. On subsequent renneting, the whey proteins become incorporated into the curd, giving an increase in cheese yield. Similarly on acid precipitation from heated milk, the denatured whey proteins precipitate together with the caseins (Chapter 6).

In the present study, a rapid analytical method of gel permeation FPLC was developed which could be used to examine the composition of whey proteins in the milk of the cow, goat and sheep. This allowed direct comparison of the composition

of the whey proteins from the three species. Gel permeation FPLC was also used to examine natural variation in the composition of the whey proteins and, in particular, lactational changes in the caprine whey proteins. This technique was also suitable for studying heat denaturation of the whey proteins of the three species, and their subsequent retention in cheese (Chapter 6).

2. SEPARATION OF THE BOVINE WHEY PROTEINS

Rowland (1938) used selective precipitation to partition the proteins in milk, and three of these fractions - albumin, globulin and proteose-peptones - contained mixtures of the individual whey proteins. The albumin fraction, which remains soluble in 20% ammonium sulphate, and contains the main whey proteins, was sub-fractionated by Kiddy *et al.* (1965) using ion-exchange chromatography. Three fractions, namely β-lactoglobulin, α-lactalbumin and serum albumin, were obtained by cation-exchange chromatography on CM cellulose. Re-chromatography of the β-lactoglobulin fraction on DEAE cellulose separated the A and B genetic variants, which could also be identified by alkaline PAGE (Fig. 15). These workers showed that the A and B variants were produced in approximately equal amounts.

Armstrong *et al.* (1970) separated the albumin fraction using gel permeation chromatography on Sephadex G-75 and G-100; they obtained four main fractions which contained, in decreasing molecular weight, lactoferrin (83,000-87,000), serum albumin (66,267), β-lactoglobulin (dimer 36,726) and α-lactalbumin (14,178). Whole whey protein also could be fractionated by gel permeation chromatography, and the immunoglobulins (MW 158,000-1,000,000), which were present in the globulin fraction described by Rowland (1938), eluted near the void volume.

The proteose-peptone fraction described by Rowland (1938) is a complex mixture containing at least 38 peptides, most of which are derived by the action of plasmin on the caseins, particularly β-casein (Andrews and Alichanidis, 1983; Chapter 1). The peptides remain soluble after heating milk at 95°C for 30 min and adjusting to pH 4.6, but precipitate in TCA at a concentration of 12.5%. The proteose peptones, which amount to about 20% of the total whey proteins, are usually present as
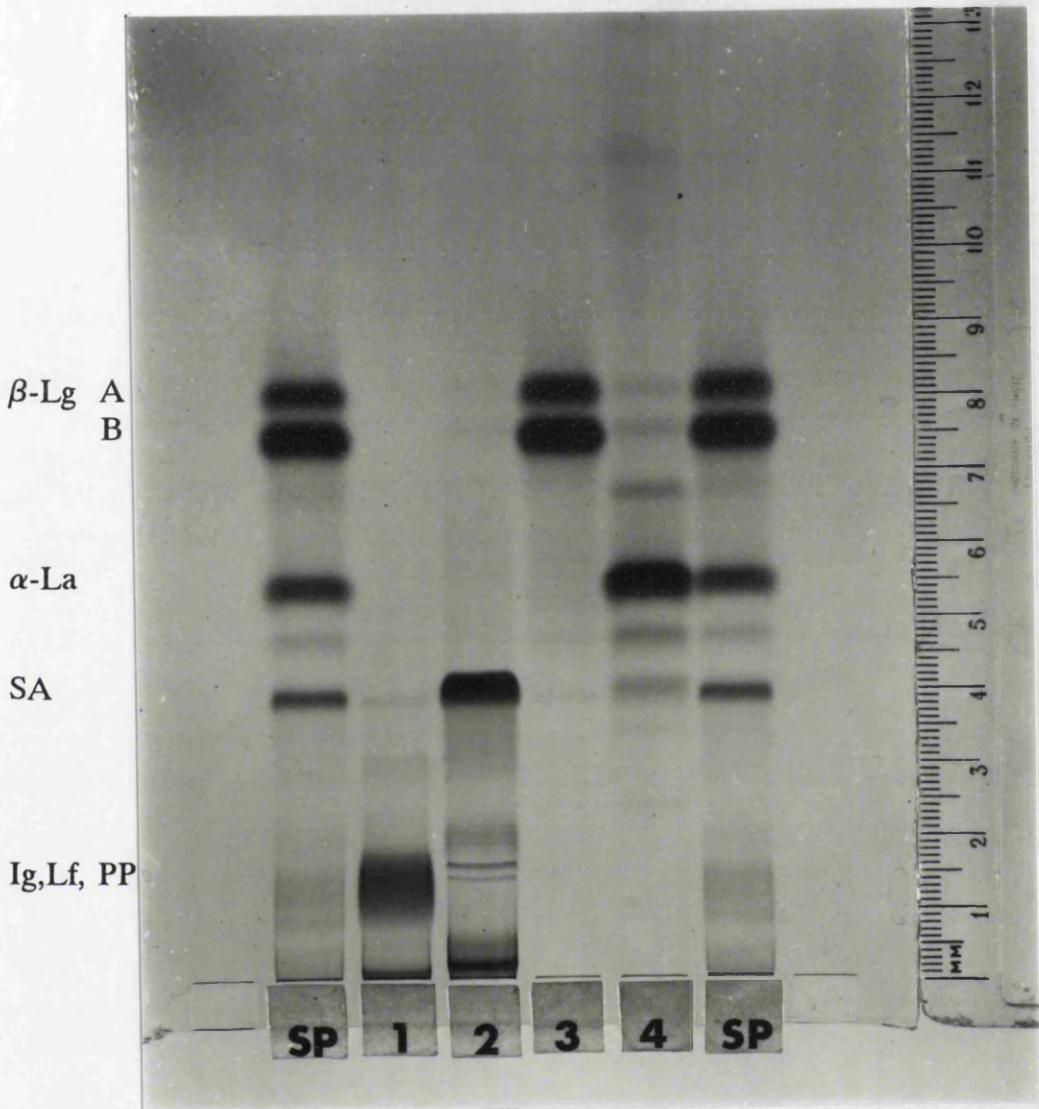
Fig.15 Alkaline PAGE pattern for whole whey protein and the fractions obtained by gel permeation chromatography on G-100 Sephadex (Davies, 1974)

β-Lg, β-lactoglobulin; α-La, α-lactalbumin; SA, serum album; Ig, immunoglobulins; SA, serum albumin; PP, Proteose peptones

Slots
SP Whole whey protein
1-4 Fractions in order of decreasing molecular weight

contaminants of ion-exchange and gel permeation fractions, but their absorbance at 280nm is low (Table 10), and their presence does not affect quantitative values at this wavelength.

Davies (1974) separated the whey proteins by gel permeation chromatography on Sephadex G-100; the alkaline PAGE pattern for the whey protein fractions is shown in Fig.15. β-Lactoglobulin A contains an extra Asp residue at position 64 and has, therefore, higher electrophoretic mobility than the B variant. The α-lactalbumin fraction contains one main band, and several other minor bands which represent differently glycosylated forms (Hopper and McKenzie, 1973). The other fractions of higher molecular weight tend to be heterogeneous. Fraction one contains a mixture of immunoglobulins and lactoferrin, whereas fraction two contains mainly serum albumin, together with serum transferrins.

In the present study (Paper 1, Davies and Law, 1980), the above method of gel permeation on Sephadex G-100 was used to examine seasonal variation in the content and composition of the whey proteins in creamery milks in South-West Scotland. Results showed that concentrations of total and individual whey proteins increased considerably during the Summer, but the relative amounts of the individual whey proteins varied little. The overall ranges for the relative amounts of the individual fractions were: immunoglobulins (plus proteose peptones and lactoferrin), 15.3-18.2; serum albumin, 7.2-8.3; β-lactoglobulin, 52.1-56.4; and α-lactalbumin, 18.5-22.8 % of the total.

Several workers have obtained quantitative values for the relative amounts of the individual whey protein fractions by scanning alkaline gels (Hillier, 1976; Storry et al., 1983), but this requires careful comparison with standards to overcome the difficulties due to different dye-binding of the whey proteins. Because of the wide range in their molecular weights, the whey proteins can be separated by SDS-PAGE (Hurley et al., 1993). This method has been used in the present work to characterise the bovine, caprine and ovine whey proteins; the results are described in the two papers in this section.

Cifuentes et al. (1993) and Otte et al. (1994) have used capillary electrophoresis to separate the whey proteins and obtained quantitative values for β-lactoglobulin, α-lactalbumin and serum albumin, by direct UV determination. This technique offers

considerable promise in that the separation characteristics can be modified by applying different coatings to the capillaries, changing the pH, or using polymers in the separation buffer to achieve a fractionation according to molecular size.

Traditional methods of chromatography gave good resolution of the main whey proteins, but were time-consuming. With the introduction of column materials having smaller bead size and rapid kinetics, it was possible to obtain rapid fractionation of the whey proteins by ion-exchange and gel permeation HPLC and FPLC. Various workers have used anion-exchange on Mono Q to fractionate the whey proteins (Andrews *et al.*, 1985; Manji *et al.*, 1985; Giradet *et al.*, 1989). This method gave a good separation of the main fractions and the A and B variants of β-lactoglobulin, but did not usually resolve the minor components.

Several groups have used reverse-phase chromatography to fractionate whey proteins (Pearce, 1983; Bican and Spahni, 1991; Parris and Baginski,1991); they have been able to separate the main whey proteins and the A and B variants of β-lactoglobulin. In the more rapid methods there is usually loss of resolution of the minor whey proteins. There is also evidence that, under some conditions, recovery of protein may be incomplete (de Frutos *et al.*, 1992); some care may be required, therefore, in using reverse-phase chromatography for quantitative fractionation of the whey proteins.

The whey proteins have also been separated by gel permeation HPLC (Diosady *et al.*, 1980; Gupta, 1983; van den Bedem and Leenheer, 1988). Andrews *et al.* (1985) were able to fractionate the proteins in acid whey by gel permeation FPLC on a column of Superose 12, but did not obtain complete separation of the two major whey proteins, β-lactoglobulin and α-lactalbumin.

In the present study the whey proteins were fractionated by gel permeation FPLC on a column of Superdex 75 HR 10/30, which has a more suitable fractionation range than Superose 12, and gave a satisfactory separation of the whey proteins, including β-lactoglobulin and α-lactalbumin. The first paper in this section (Paper 11, Law *et al.*, 1993) describes characterisation of the whey protein fractions, and the application of the method to determine the relative amounts of the proteins in acid whey. On average, whey protein from bulk milk consisted of: immunoglobulins, 9.8; serum albumin/lactoferrin, 10.7; β-lactoglobulin, 61.4; and α-lactalbumin, 18.0% of the total.

On heating milk above 60°C, the whey proteins undergo denaturation which involves breaking of internal disulphide bonds and a loss of globular conformation (van Boekel, 1992). The denatured whey proteins then become associated with the casein micelles, either by hydrophobic interaction or through disulphide linkage (Noh and Richardson, 1989). On acidification, the denatured whey proteins precipitate together with the caseins. Also, on renneting, the denatured whey proteins are incorporated into the curd. The extent to which the individual whey proteins lose their solubility at pH 4.6 can, therefore, be used as a measure of their irreversible denaturation. The first paper in this section (Paper 11, Law *et al.*, 1993) shows that gel permeation FPLC can also be used to measure the amounts of the individual whey proteins in acid filtrate before and after heat treatment, and is suitable for studying the relative rates of denaturation of the whey proteins. Denaturation of the whey proteins, and their incorporation into the curd during the manufacture of cheese, is discussed in detail in Chapter 6.

3. COMPARISON OF BOVINE, CAPRINE AND OVINE WHEY PROTEINS

The whey proteins, namely β-lactoglobulin, α-lactalbumin, serum albumin, lactoferrin, immunoglobulins and proteose peptones, are all present in the milk of the cow, goat and sheep, but the concentrations vary considerably (Fig.18). Representative values for the main whey proteins are shown in Table 15. Compared with cows' milk, that from the goat contains similar concentrations of total and individual whey proteins, whereas milk from the sheep contains considerably more total whey protein and more β-lactoglobulin and α-lactalbumin. As may be seen from Table 15, the variation in concentrations within each species is considerable. Few values are available for the relative amounts of the whey proteins in the three species, but results from the present work are shown in Table 15, and are discussed below.

The amino acid compositions and sequences of β-lactoglobulin and α-lactalbumin of the three species have been determined (Table 16, Fig.16 and 17). Comparison of the sequences shows that there is more than 90% homology of the

Table 15. Comparison of the content and composition of bovine, caprine and ovine whey proteins

Whey Protein	Cow	Goat	Sheep
	Concentration (g/l)[a]		
Total	1.6-7.6	3.7-7.0	8.8-10.4
β-Lactoglobulin	2.3-4.9	1.8-2.8	2.7-5.0
α- Lactalbumin	0.8-1.2	0.6-1.1	1.2-2.6
Serum albumin	0.2-0.4	0.1-1.1	0.3-0.5
	% of Total whey protein[b]		
β-Lactoglobulin	59.4-63.9	39.2-72.1	61.1
α-Lactalbumin	17.1-18.7	17.8-33.3	10.8
Serum albumin /lactoferrin	8.7-12.2	5.1-21.5	8.1
Immunoglobulins	8.2-11.1	4.6-21.4	20.0

Data compiled:
[a] Storry *et al.*, 1983
[b] Law *et al.*, 1993a; Law and Brown, 1994; Law, 1995

Table 16. Amino acid compositions of bovine, caprine and ovine β-lactoglobulin and α-lactalbumin

Amino acid	β-Lactoglobulin			α-Lactalbumin		
	Bovine β-Lg A	Caprine	Ovine β-Lg A (B)	Bovine	Caprine	Ovine
Asp	11	8	8	13	14	14
Asn	5	6	7	8	8	8
Thr	8	8	8	7	6	6
Ser	7	6	6	7	6	6
Glu	16	15	15	7	6	6
Gln	9	9	9	6	7	7
Pro	8	8	8	2	2	2
Gly	3	5	5	6	5	5
Ala	14	16	16	3	5	6
Cys	5	5	5	8	8	8
Val	10	10	10	6	6	5
Met	4	4	4	1	0	0
Ile	10	10	10	8	8	8
Leu	22	21	21	13	13	13
Tyr	4	4	4 (3)	4	4	4
Phe	4	4	4	4	4	4
Lys	15	16	15	12	13	13
His	2	2	2 (3)	3	3	3
Trp	2	2	2	4	4	4
Arg	3	3	3	1	1	1
Total	162	162	162	123	123	123

Data from sequences

```
Cow     1 LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP
Goat    1 IIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP
Sheep   1 IIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR VYVEELKPTP

Cow    51 EGDLEILLQK WENDECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK
Goat   51 EGNLEILLQK WENGECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK
Sheep  51 EGNLEILLQK WENGECAQKK IIAEKTKIPA VFKIDALNEN KVLVLDTDYK

Cow   101 KYLLFCMENS AEPEQSLVCQ CLVRTPEVDD EALEKFDKAL KALPMHIRLS
Goat  101 KYLLFCMENS AEPEQSLACQ CLVRTPEVDK EALEKFDKAL KALPMHIRLA
Sheep 101 KYLLFCMENS AEPEQSLACQ CLVRTPEVDN EALEKFDKAL KALPMHIRLA

Cow   151 FNPTQLEEQC HI
Goat  151 FNPTQLEGQC HV
Sheep 151 FNPTQLEGQC HV
```

Fig.16 Primary structure of bovine β-lactoglobulin A, caprine β-lactoglobulin and ovine β-lactoglobulin A

Differences from bovine sequences shown in bold letters

Goat: Godovac-Zimmermann and Braunitzer, 1987
Sheep: Kolde and Braunitzer, 1983

```
Cow     1 EQLTKCEVFR ELKDLKGYGG VSLPEWVCTT FHTSGYDTQA IVQNNDSTEY
Goat    1 EQLTKCEVFQ KLKDLKDYGG VSLPEWVCTA FHTSGYDTQA IVQNNDSTEY
Sheep   1 EQLTKCEAFQ KLKDLKDYGG VSLPEWVCTA FHTSGYDTQA IVQNNDSTEY

Cow    51 GLFQINNKIW CKDDQNPHSS NICNISCDKF LDDDLTDDIM CVKKILDKVG
Goat   51 GLFQINNKIW CKDDQNPHSR NICNISCDKF LDDDLTDDIV CAKKILDKVG
Sheep  51 GLFQINNKIW CKDDQNPHSR NICNISCDKF LDDDLTDDIV CAKKILDKVG

Cow   101 INYWLAHKAL CSEKLDQWLC EKL
Goat  101 INYWLAHKAL CSEKLDQWLC EKL
Sheep 101 INYWLAHKAL CSEKLDQWLC EKL
```

Fig.17 Primary structure of bovine, caprine and ovine α-lactalbumin

Goat: Kumagai *et al.*, 1987; Vilotte *et al.*, 1991; McGillivray *et al.*, 1979, Shewale *et al.*, 1984
Sheep: Gaye *et al.*, 1987; Mercier *et al.*, 1978

caprine and ovine β-lactoglobulins and α-lactalbumins with the corresponding bovine whey proteins. Caprine and ovine β-lactoglobulins contain fewer Asp and Glu residues, however, and at alkaline pH have a lower net charge than bovine β-lactoglobulin. Also, caprine β-lactoglobulin contains an extra Lys residue. These charge differences are reflected in the results from alkaline PAGE in which the order of mobility of β-lactoglobulin is bovine $>>$ ovine $>$ caprine (Amigo et al., 1989). Also, on anion-exchange chromatography, the less negatively charged ovine and caprine β-lactoglobulins elute earlier than bovine β-lactoglobulin (Laezza et al., 1991). Differences also occur in the charged amino acids of the α-lactalbumins, but these are less pronounced, and their electrophoretic mobilities at alkaline pH are only slightly different (Amigo et al.,1991).

Information about homology of the other whey proteins is fragmentary. The complete sequence of bovine lactoferrin (Schanbacher et al., 1993) and a partial sequence for ovine lactoferrin (Buchta, 1991) have been established; these show more than 80% homology. The amino acid compositions of the two lactoferrins are similar (Buchta, 1991), and CD spectral analysis showed that they have similar secondary and tertiary structures (Shimazaki et al., 1991).

The complete sequences of bovine and ovine serum albumin have been determined (Brown, 1975; Brown et al., 1989) and these show close homology, but little information is available for caprine serum albumin. Similarly, because of the diversity of the fractions, it has been difficult to compare the amino acid compositions of the respective immunoglobulins and proteose peptones. Ramos et al. (1988) found that, compared with bovine proteose peptones, the fraction from ovine milk contained much more carbohydrate, mainly galactose, mannose and glucosamine. The sialic acid content followed the order ovine $>$ caprine $>$ bovine. Mati et al. (1991) examined the proteose peptones and a hydrophobic fraction, and found that these were similar in amino acid composition in the cow, goat and sheep.

4. SEPARATION OF THE CAPRINE WHEY PROTEINS

Early methods of purification of the caprine whey proteins involved ion-exchange and gel permeation on CM and DEAE cellulose and Sephadex. These methods have been

reviewed by Yaguchi and Rose (1971). Most of the quantitative values for the concentrations and relative amounts of the individual caprine whey proteins have been obtained by Storry *et al.* (1983), who scanned polyacrylamide gels using the method of Hillier (1976). Values were obtained for β-lactoglobulin, α-lactalbumin and serum albumin; these are shown in comparison with bovine whey proteins in Table 15.

Reverse-phase HPLC has also been used to fractionate caprine whey proteins (de Frutos *et al.*, 1992), but the minor fractions were not separated, and quantitative values were not given. Hill and Kakuda (1990) fractionated caprine whey proteins by gel permeation FPLC, and obtained six protein-containing fractions, but resolution of the two main fractions, β-lactoglobulin and α-lactalbumin, was poor.

In the present work (Paper 12, Law and Brown, 1994), the method of gel permeation FPLC that was used to fractionate bovine whey proteins was applied to the caprine whey proteins. Using SDS PAGE, four main fractions similar to those in cows' milk were identified. The method was used to examine natural variation in the composition of caprine whey protein, and the effect of stage of lactation on the concentrations and relative amounts of the individual whey proteins. On average, whey protein from the milk of British Saanen goats consisted of immunoglobulins, 11.3; serum albumin/lactoferrin, 8.6; β-lactoglobulin, 54.8; and α-lactalbumin, 25.2 % of the total. Compared with bovine whey protein, therefore, that from the goat contained less β-lactoglobulin, more α-lactalbumin and similar amounts of the higher molecular weight fractions.

The content and composition of whey protein from different goats varied considerably, however, partly due to the effect of stage of lactation. In a study of lactational effects in five goats (Paper 12, Law and Brown, 1994), it was found that the concentrations of total and individual whey proteins tended to be high at the beginning and end of lactation, but lower in mid-lactation. The total yields of β-lactoglobulin and α-lactalbumin decreased with advancing lactation, whereas yields of immunoglobulins and serum albumin/lactoferrin did not change markedly. The composition of the whey proteins varied within narrow limits; the relative amounts of β-lactoglobulin and α-lactalbumin decreased slightly with advancing lactation stage, whereas the relative amounts of the higher molecular weight fractions, which originate in the blood, increased slightly.
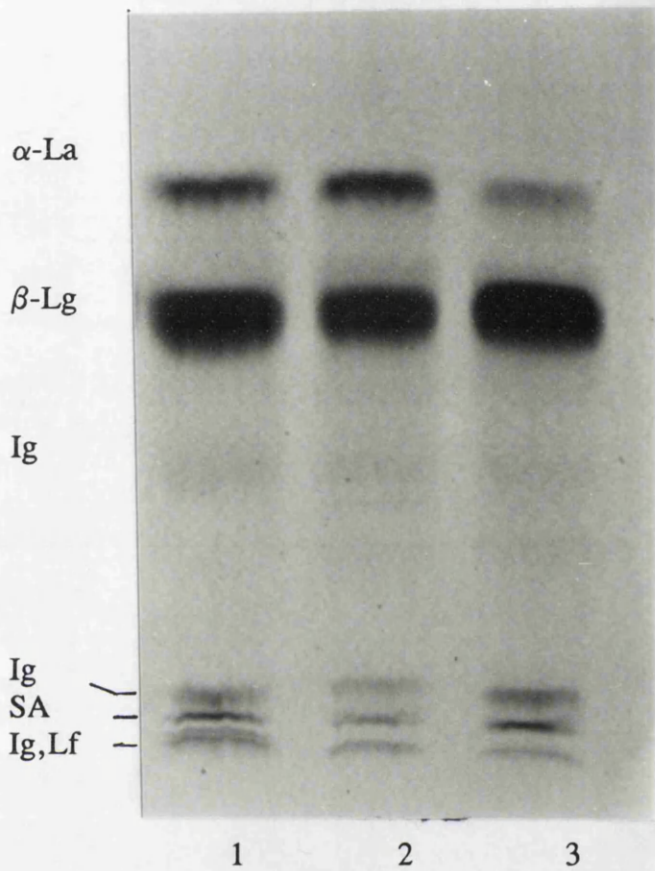
Fig.18 SDS-PAGE of bovine, caprine and ovine whey proteins

α-La, α-lactalbumin; β-Lg, β-lactoglobulin; Ig, immunoglobulins; SA, serum albumin; Lf, lactoferrin

Slot 1, Whey proteins from bulk milk of Friesian cows
Slot 2, Whey proteins from bulk milk of British Saanen goats
Slot 3, Whey proteins from bulk milk of Friesland sheep

5. SEPARATION OF THE OVINE WHEY PROTEINS

In two early studies, lactational variation in the composition of ovine whey protein fractions was examined by the precipitation methods of Rowland (1938), followed by nitrogen determination (Bouchard and Brisson, 1969; Poulton and Ashton, 1970). This method was laborious, however, and did not give values for the minor fractions.

As for the caprine whey proteins, most of the quantitative values for the composition of ovine whey proteins have been obtained by Storry et al. (1983), using gel scanning; some of these results are presented in Table 15. This electrophoretic method had the advantage that the the A and B variants of ovine β-lactoglobulin could be separated. The differences in charge and electrophoretic mobility of the variants are caused by substitution of His_{20} in the B variant for Tyr (Gaye et al., 1986). Lopez-Galvez et al. (1994) have shown that both variants are common.

Most of the early chromatographic methods used to fractionate or purify the ovine whey proteins are similar to those described for the caprine whey proteins; these have been reviewed by Yaguchi and Rose (1971). De Frutos et al. (1992) obtained a rapid separation of ovine whey proteins using reverse-phase HPLC but, as found for the caprine whey proteins, this did not give complete resolution of the minor whey proteins.

Because the molecular weights of the corresponding whey proteins of the three species are similar, the method of gel permeation FPLC described for bovine and caprine whey proteins was also suitable for the study of the ovine whey proteins. The second paper in Chapter 6 (Paper 19, Law, 1995) describes characterisation of the ovine whey proteins, and comparison of the composition of bovine, caprine and ovine whey proteins in bulk milks. As for bovine and caprine whey protein, four main fractions from gel permeation FPLC were identified by SDS PAGE. On average, ovine whey protein consisted of: immunoglobulins, 20.0; serum albumin/lactoferrin, 8.1; β-lactoglobulin, 61.1; and α-lactalbumin, 10.8% of the total. Compared with bovine whey protein, therefore, that from the sheep contained slightly more β-lactoglobulin and much less α-lactalbumin. Ewes' milk was more concentrated, however, and the concentrations of total whey protein, β-lactoglobulin, immunoglobulins and the serum

albumin/lactoferrin fraction were higher than in cows' milk. On the other hand, the concentrations of α-lactalbumin were similar in both types of milk. The effect of composition on the relative rates of denaturation of the individual whey proteins of the cow, goat and sheep is discussed in Chapter 6.

5. CONCLUSIONS

The whey proteins were initially fractionated by gel-permeation chromatography on Sephadex G-100. In a study of seasonal variation in the composition of whey proteins in creamery milks in South-West Scotland, it was found that the concentrations of total and individual whey proteins increased markedly in the summer, but the relative amounts of the individual whey proteins tended to remain within narrow limits.

This method of chromatography was time-consuming, however, and a rapid method of gel-permeation FPLC was developed which was suitable for the fractionation of bovine, caprine and ovine whey proteins. Four main whey protein fractions were identified for each species, and quantitative values were similar to those obtained by traditional gel-permeation chromatography. On average, whey protein from the milk of Friesian cows consisted of: immunoglobulins, 9.8%; serum albumin and lactoferrin, 10.7%; β-lactoglobulin, 61.4%; and α-lactalbumin, 18.0% of the total. Compared with bovine whey protein, caprine whey protein contained less β-lactoglobulin and more α-lactalbumin, whereas ovine whey protein contained slightly more β-lactoglobulin and considerably less α-lactalbumin. The concentrations of the whey proteins, with the exception of α-lactalbumin, were much higher in ewes' milk.

In a lactational study of caprine whey proteins, results showed that the concentrations of total and individual whey proteins varied considerably; they tended to be high at the begining and end of lactation, but lower in mid lactation. The relative amounts of the whey proteins did not vary widely but, as lactation progressed, there was a slight decrease in the relative amounts of β-lactoglobulin and α-lactalbumin, and a corresponding increase in the relative amounts of immunoglobulins, serum albumin and lactoferrin.

Quantitative fractionation of whey proteins by gel permeation FPLC

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1. Introduction

The whey proteins have previously been separated by various methods including anion-exchange FPLC (1, 2), reverse phase HPLC (3) and gel scanning (4). Gel permeation chromatography has also been used, and has the advantage of being relatively unaffected by high ionic strength or by changes in charge or hydrophobicity which can occur in whey proteins during heat treatment (5, 6). Traditional methods of gel permeation (7, 8) gave good resolution of the main whey proteins but were fairly slow, whereas HPLC methods (9, 10, 11, 12) typically require less than 1 h. FPLC gel permeation, which is carried out on materials with larger bead diameter (about 13 μm) and at much lower pressures than corresponding HPLC methods, also gives rapid separation of the whey proteins (2, 13). Resolution of the two main proteins, α-lactalbumin and β-lactoglobulin, however, has tended to be poor.

In this paper we describe a method of FPLC gel permeation which separates whey proteins into 4 main fractions and gives good resolution of α-lactalbumin and β-lactoglobulin.

We have applied this method to measure the relative amounts of the individual whey proteins in acid filtrate and whey from raw and heated milks, and have shown that the method can be used to examine denaturation of the whey proteins and their recovery in cheese-making with heated milk.

2. Materials and methods

Milk samples. Milk samples were collected from the bulk milk of Friesian cows in the Institute herd.

Heat treatment. Some of the milk samples were heated on pilot-scale heating equipment (APV Junior) which included a plate heat-exchanger to give rapid heating to the holding temperature and subsequent rapid cooling.

Acid filtrate. Milk samples were skimmed by centrifugation at 1000g for 30 min and adjusted to pH 4.6 at 20 °C by the addition of 1M HCl. The supernatant was passed through Whatman No. 42 filter paper and then a 0.22 μm nylon filter.

Freeze-dried whey protein. Whole whey protein was obtained by dialysing acid filtrate against deionised water, and freeze-drying.

Whey. Whey was obtained about 3 h after addition of rennet in pilot-scale production of Cheddar cheese, and filtered through a 0.22 μm nylon filter.

Whey protein standards. Standards of IgM, IgG, lactoferrin, serum albumin, β-lactoglobulin and α-lactalbumin were obtained from Sigma Chemical Company (Poole, Dorset, UK).

Proteose-peptones. A sample of proteose-peptones was prepared by heating skim-milk at 95 °C for 30 min, precipitating the casein and denatured whey proteins at pH 4.6, and then precipitating the proteose peptones by addition of trichloroacetic acid to a concentration of 12.5 %. The precipitate was washed with water and freeze dried.

Gel permeation FPLC. FPLC was carried out on 50 μl aliquots of solutions of freeze-dried whey proteins, acid filtrate or whey containing about 0.3 mg of protein. The samples were fractionated by gel permeation FPLC at 20 °C on a Superdex 75 HR 10/30 column (Pharmacia Biotech, Milton Keynes, UK) in tris-HCl buffer (pH 7.0, 100 mM tris, 0.5 M NaCl) at a flow rate of 0.5 ml/min. The absorbance of the eluate was monitored at 280 or 214 nm, and a total volume of 26 ml was passed through the column to ensure complete elution of absorbing material. Peak areas were corrected for the baseline value, and concentrations of the individual whey proteins calculated using $A_{1cm}^{1\%}$ 280 values: immunoglobulins, 12.1; serum albumin, lactoferrin, 6.9; β-lactoglobulin, 9.5; α-lactalbumin, 20.1 (15).

SDS-PAGE. Whey proteins and proteose-peptones were examined by sodium dodecyl sulphate (SDS)-PAGE on Phastsystem electrophoresis equipment (Pharmacia Biotech, Milton Keynes, UK) using 20 % homogeneous gels, in accordance with the manufacturers instructions.

3. Results and discussion

3.1 Characterisation of fractions obtained by gel permeation FPLC

The elution profile of a typical sample of freeze-dried whey proteins from herd bulk milks is shown in Fig. 1. To help in a preliminary identification of the peaks, standards of the whey proteins were run under the same conditions and at the concentrations they occur in milk (Fig. 2). The standards eluted in order of decreasing molecular weight (15) and mainly as single peaks. From these results it was possible to identify the main fractions shown in Fig. 1 as: (1) IgM (MW 1,000,000) and IgG (158,000), (2) Lactoferrin (83,000–87,000) and serum albumin (66,267), (3) β-lactoglobulin (36,726) and (4) α-lactalbumin (14,147). A graph of log MW
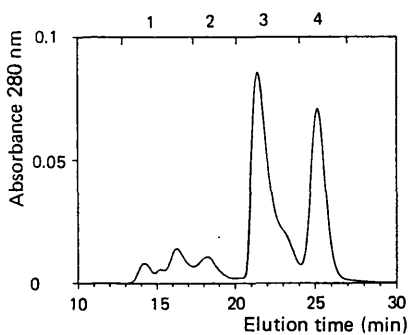
Fig. 1: Elution profile of freeze-dried whole whey proteins obtained by gel permeation FPLC in tris-HCl buffer (pH 7.0; 100 mM tris, 0.5 M NaCl) on a column of Superdex 75 HR 10/30. A 50 µl solution containing about 0.3 mg whey proteins was applied to the column and eluted at a flow rate of 0.5 ml/min.
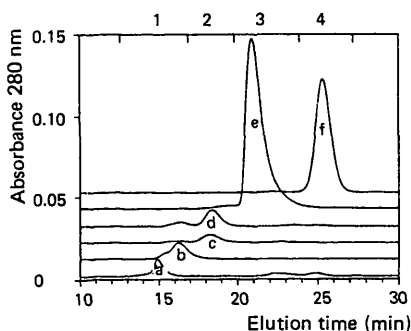
Fig. 2: Elution profiles of standard whey proteins at approximately the concentrations they occur in milk. (a) IgM, (b) IgG, (c) lactoferrin, (d) serum albumin, (e) β-lactoglobulin, (f) α-lactalbumin. Conditions of chromatography as in Fig. 1.

of the whey proteins against elution time showed almost ideal behaviour of the whey proteins on the gel permeation column (Fig. 3).
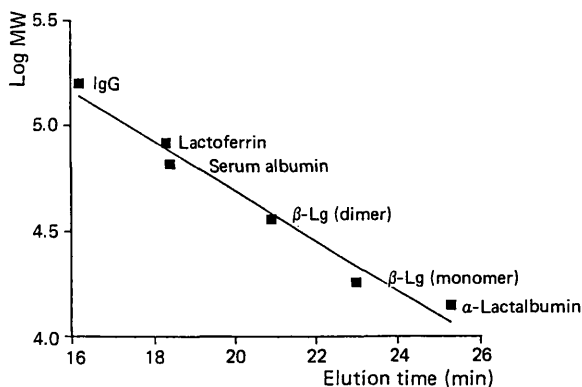
Fig. 3: Relation between log MW of the individual whey proteins and their elution time (min) as shown in Figs. 1 and 2.

As a check on their purity, the fractions obtained from freeze-dried whey protein were collected and examined by SDS-PAGE (Fig. 4). The electrophoretic patterns for the fractions were also compared with those of the whey protein standards
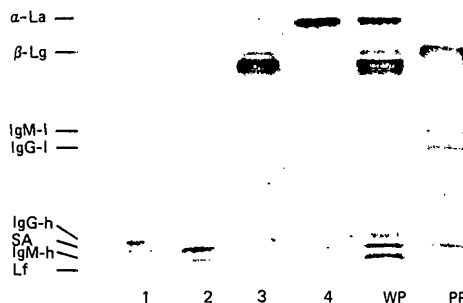
Fig. 4: SDS-PAGE pattern on a 20 % homogeneous gel for whole whey proteins (WP), the proteose-peptone fraction (PP), and the fractions obtained by gel permeation FPLC as shown in Fig. 1. Lanes 1–4, fractions 1–4 respectively. The proteins are: α-La, α-lactalbumin; β-Lg, β-lactoglobulin; IgM-l, light chain IgM; IgG-l, light chain IgG; IgG-h, heavy chain IgG; SA, serum albumin; IgM-h, heavy chain IgM; Lf, lactoferrin.

(results not shown) and generally confirmed the results from gel permeation FPLC. Fraction 1 contained mainly IgG together with some IgM, but there was some indication of cross-contamination of fraction 1 with serum albumin. Fraction 2 contained mainly serum albumin together with lactoferrin. Fractions 3 and 4 contained β-lactoglobulin and α-lactalbumin respectively. Electrophoresis also indicated (results not shown) that the shoulder on the peak of β-lactoglobulin at elution time 23.5 min (Fig. 1) was in fact β-lactoglobulin, and is believed to be the monomeric form, which exists in equilibrium with the dimer at pH 7.0 (16). Further evidence comes from the observed decrease in the dimer peak and a corresponding increase in the size of the smaller molecular weight peak on dilution (16), and the close fit of the elution position of the peak to the log of the monomeric molecular weight (Fig. 3).

There was also some indication from SDS-PAGE that a number of the fractions, particularly the β-lactoglobulin fraction and possibly also the immunoglobulin fraction, contained material which was present in the heterogeneous proteose-peptone fraction (14). This contamination did not significantly affect quantitative values for the fractions because, as explained below, the absorbance at 280 nm of the proteose peptone fraction was very low.

3.2 Quantitative analysis of whey proteins by gel permeation FPLC

The gel permeation method was used to examine changes in the relative amounts of the whey

proteins in acid filtrate and whey from raw and heated milk. The elution profile of undialysed acid filtrate is shown in Fig. 5. The small peak eluting at 35 min represents material of molecular weight less than about 6,000, and the larger peaks at elution times of 40 and 50 min have been identified as orotic acid and other low molecular weight materials respectively (2). All of the absorbing material eluting after 30 min could be removed by dialysis, and the relative amounts of the individual whey proteins in acid filtrate (Fig. 5) were identical to those in dialysed, freeze-dried whey protein (Fig. 1).
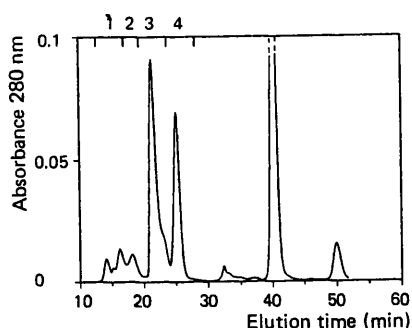
Fig. 5: Elution profile obtained by gel permeation FPLC of 50 µl acid filtrate, according to the conditions in Fig. 1.

The overall separation of the whey proteins by this method was similar to that obtained by traditional gel chromatography (7,8). Separation of the main whey proteins, α-lactalbumin and β-lactoglobulin, however, was more complete than in previously published gel permeation FPLC methods (2, 13).

The average composition of whey protein obtained from 8 samples of herd bulk milk over a period of 3 months is given in Table 1. Compared to traditional gel permeation (8), and gel permeation HPLC (10), the present method gave slightly lower values for immunoglobulins, slightly higher values for β-lactoglobulin and similar values for the relative amounts of the serum albumin and α-lactalbumin fractions. Some of the differences from literature values could be due to natural variation, and within the bulk milk over a period of 3 months there were substantial seasonal changes in most fractions (Table 1).

Gel permeation FPLC was also used to fractionate fresh whey and gave an elution profile similar to that shown in Fig. 5 for acid filtrate. The relative amounts of the whey proteins in the acid filtrate, and the corresponding whey were similar apart from a slight increase in the amount of the void volume peak of the whey.

On heating milk above 60 °C the whey proteins undergo denaturation involving unfolding of the proteins (12), an increase in hydrophobicity (5, 6) and disulphide linkage with other proteins (17). which leads to aggregation and a decrease in solubility at pH 4.6 (9, 18, 19). Although denaturation and aggregation are separate processes, the precipitation of the denatured whey proteins with casein at pH 4.6 provides a useful means of examining the effect of heat treatment. Various workers have measured changes in the total whey protein N distribution (18, 19), and a number of groups have used chromatography to examine changes in the concentrations of the individual proteins in acid filtrate from milk before and after heating (6. 10, 12).

Typical elution profiles obtained by gel permeation FPLC of acid filtrate from milk heated to different extents, are shown in Fig. 6. On heating at 90 °C for 30 sec, most of the immunoglobulins. serum albumin and lactoferrin, and about 50 °= of the β-lactoglobulin precipitated at pH 4.6 whereas the solubility of the α-lactalbumin was hardly affected. SDS-PAGE confirmed that the identities of the main peaks remained unchanged on heating, and there was no evidence of increased cross-contamination of the fractions due, for example, to polymerisation of α-lactalbumin or β-lactoglobulin. On more severe heat treatment at 95 C for 30 min, all of the major whey proteins precipitated at pH 4.6, and only the proteose-peptone fraction, representing less than 5 °₀ of the total area at 280 nm, remained. The proteose-peptone fraction. which amounts to about 20 °₀ of the whey proteins measured by nitrogen analysis (19) and com-

Table 1:	The average composition of whey protein from herd bulk milk		
Fraction	% of Total		
	Mean	S.D.	Range
Immunoglobulins	9.8	1.0	8.2 – 11 1
Serum albumin/lactoferrin	10.7	1.3	8.7 – 12.2
β-Lactoglobulin	61.4	1.8	59.4 – 63.9
α-Lactalbumin	18.0	0.7	17.1 – 18.7
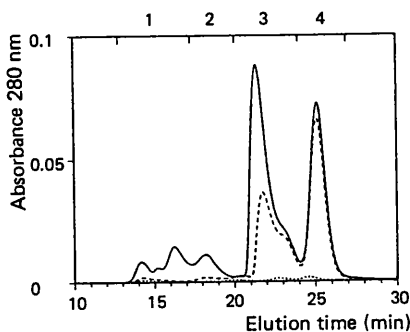
Fig. 6: Elution profiles obtained by gel permeation FPLC showing the decrease in solubility of the whey proteins at pH 4.6 with increasing severity of heat treatment. Conditions as in Fig. 1. — Acid filtrate from raw milk. ---- acid filtrate from milk heated at 90 C for 30 sec. · proteose-peptones in acid filtrate from milk heated at 95 °C for 30 min.

prises at least 38 components (14), has a low absorbance coefficient at 280 nm, and therefore does not appreciably affect determination of the other whey proteins. The proteose-peptones can, however, be detected by measuring the absorbance of the column eluate at 214 mn, and the profile obtained for the proteose-peptone fraction prepared by heating milk at 95 °C for 30 min (data not shown) confirms the finding from SDS-PAGE (Fig. 4) that proteins in the proteose-peptone fraction could also be detected in the β-lactoglobulin and immunoglobulin fractions.

Our results show that the method of gel permeation FPLC described here can be used to measure the relative amounts of the whey proteins in freeze-dried whole whey protein, acid filtrate and whey from raw and heated milk. The method can, therefore, also be used to study the extent of denaturation of the whey proteins and, in particular, the effect of heating on the extent of incorporation of whey proteins into the curd during cheese-making. This will be discussed in more detail in a future publication.

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5. Summary

LAW, A.J.R., LEAVER, J., BANKS, J.M., HORNE, D.S.: **Quantitative fractionation of whey proteins by gel permeation FPLC.** Milchwissenschaft **48** (12) 663–666 (1993).

24 Whey proteins (gel permeation FPLC)

Whey proteins were fractionated by gel permeation FPLC in tris-HCl buffer at pH 7.0 and 20 °C on a column of Superdex 75 HR 10/30. Four main fractions were identified by comparison of their elution positions with known standards, and by SDS-PAGE. On average, whey protein from the bulk milk of Friesian cows consisted of: (1) immunoglobulins, 9.8 %; (2) serum albumin and lactoferrin, 10.7 %; (3) β-lactoglobulin, 61.4 %; (4) α-lactalbumin, 18.0 % of the total. The proteose-peptone fraction had very low absorbance at 280 nm and was not determined along with the other column fractions, but could be detected at 214 nm after heat-denaturation and precipitation of the other whey proteins. The FPLC method was used to measure the relative amounts of the individual whey proteins in acid filtrate and whey from raw and heated milk, and was suitable for measuring denaturation of whey proteins and the extent of their incorporation into the curd during cheese-making with heat-treated milk.

LAW, A.J.R., LEAVER, J., BANKS, J.M., HORNE, D.S.: **Quantitative Fraktionierung von Molkenproteinen durch Gelpermeations-FPLC.** Milchwissenschaft **48** (12) 663–666 (1993).

24 Molkenproteine (Gelpermeations-FPLC)

Molkenproteine wurden durch Gelpermeations-FPLC in tris-HCl-Puffer bei pH 7,0 und 20 °C auf einer Superdex 75 HR 10/30-Säule fraktioniert. Durch Vergleich der jeweiligen Elutionspositionen mit bekannten Standards und durch SDS-PAGE wurden 4 Hauptfraktionen identifiziert. Die Molkenproteine der Sammelmilch von Friesiankühen bestanden im Mittel aus folgenden Komponenten: 1. Immunglobuline, 9,8 %; 2. Serumalbumin und Laktoferrin, 10,7 %; 3. β-Laktoglobulin, 61,4 %; 4. α-Laktalbumin, 18,0 % (jeweils bezogen auf die Gesamtmolkenproteine). Die Proteose-Pepton-Fraktion zeigte eine sehr geringe Absorption bei 280 nm und wurde nicht zusammen mit den anderen Säulenfraktionen gemessen. Ein Nachweis war jedoch bei 214 nm nach Hitzedenaturierung und Ausfällung der anderen Molkenproteine möglich. Die FPLC-Methode wurde herangezogen, um die relativen Mengen der einzelnen Molkenproteine im sauren Filtrat und in Molke roher und erhitzter Milch zu messen. Die Technik ist geeignet, um die Denaturierung von Molkenproteinen und das Ausmaß ihrer Einbeziehung in den Käsebruch während der Käseherstellung aus wärmebehandelter Milch zu ermitteln.

Compositional changes in caprine whey proteins

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1. Introduction

Cheese yield can be increased considerably by the incorporation of whey proteins into the curd using controlled heat-denaturation (1) or ultrafiltration (2). Also, in the manufacture of yogurt, the texture can be altered by careful heat-denaturation of the whey proteins, which leads to association of the denatured whey proteins with casein micelles (3,4). Few studies, however, have been carried out to examine variation in the concentrations of the individual whey proteins in goats' milk, or differences in their relative rates of denaturation.

Seasonal variation in the total whey protein fraction has been examined by Kjeldahl nitrogen analysis (5), and natural variation in the concentrations of β-lactoglobulin, α-lactalbumin and serum albumin has been studied by gel-scanning (6). The major whey proteins, namely β-lactoglobulin and α-lactalbumin, have been separated by reverse phase HPLC (7,8), but in the more rapid methods the minor fractions are not separated completely from the major whey proteins. The whey proteins have been fractionated by gel permeation FPLC (Fast protein liquid chromatography) but β-lactoglobulin and α-lactalbumin are not completely resolved (9).

In this study we have fractionated the whey proteins by a method of gel permeation FPLC that was previously used for bovine whey proteins (10), and have identified four main fractions. We have applied this method to examine natural variation in the composition of whey protein from individual goats. We have also studied the effect of stage of lactation on the concentrations, overall yields, and relative amounts of the individual whey proteins. In addition, we have shown that gel permeation FPLC can be used to study the relative rates of heat-denaturation of the individual whey proteins.

2. Materials and methods

2.1 Milk samples

Milk samples were collected from 41 British Saanen goats in a herd in South-West Scotland. A further 75 milk samples were collected from 5 British Saanen goats in the Institute herd at regular intervals throughout lactation.

2.2 Acid filtrate

Milk samples were skimmed by centrifugation at 1000g for 30 min. The skim-milk was diluted 1:1 with water and adjusted to pH 4.6 at 20°C by the addition of 1M HCl. The supernatant was passed through a 0.22 μm filter.

2.3 Heat treatment

Skim-milk samples (2.5 ml) were placed in thin-walled glass test tubes having an internal diameter of 7 mm. The tubes were then stoppered and placed in a waterbath at 90°C, allowed 1 min to warm to temperature, and maintained at this temperature for intervals between 15 s and 30 min. At the end of the heating period the tubes were rapidly cooled in ice. Acid filtrate was prepared as above.

2.4 Gel permeation FPLC

Chromatography was carried out as described previously for bovine whey proteins (10), on a Superdex 75 HR 10/30 column (Pharmacia Biotech, St Albans, UK).

2.5 SDS-PAGE

Fractions from the gel permeation column were examined by SDS-PAGE on Phastsystem electrophoresis equipment (Pharmacia Biotech, St Albans, UK) and 20% homogeneous gels, in accordance with the manufacturers instructions.

2.6 Acid PAGE

A combination of SDS-PAGE and acid PAGE was used to determine the α_{s1}-casein genotype of the goats used in the lactational study. Acid-PAGE was carried out on Phastsystem electrophoresis equipment and 20% gels, essentially using the conditions previously described (11).

2.7 Determination of total casein

Total casein nitrogen was determined directly by a micro-Kjeldahl method, with the ammonia content being determined colorimetrically (12).

3. Results and discussion

3.1 Identities of the fractions obtained by gel permeation FPLC

The elution profile of a sample of acid filtrate from herd bulk milk is shown in Fig. 1. The elution pattern of the proteins was similar to that obtained previously for bovine whey proteins (10), and identities could be tentatively assigned to the fractions on the basis of their elution positions and corresponding molecular weights. The identities of the fractions were confirmed by SDS-PAGE (Fig. 2), and it was found that fraction 1 contained mainly IgM and IgG, which, under the dissociating conditions of electrophoresis, were separated into heavy and light chains. There also appeared to be some cross-contamination of fraction 1 with serum albumin. Fraction 2 contained serum albumin and lactoferrin, and fractions 3 and 4 contained β-lactoglobulin and α-lactalbumin, respectively. The fractionation was similar to that previously obtained by gel permeation FPLC of caprine whey proteins on Superose 12 (9), but resolution of the two main proteins – β-lactoglobulin and α-lactal-
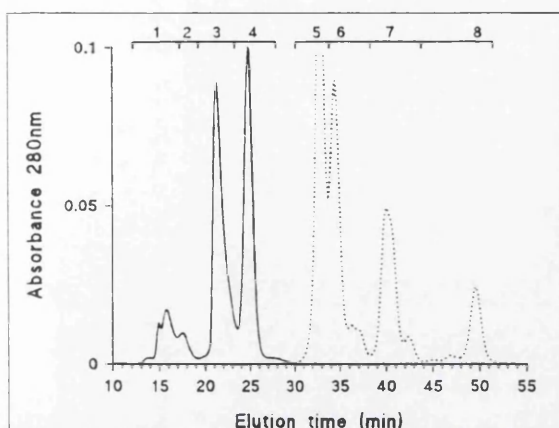
Fig. 1: Elution profile obtained by gel permeation of acid filtrate in tris-HCl buffer (pH 7.0, 100 mM tris, 0.5 M NaCl) on a column of Superdex 75 HR 10/30. A solution containing 0.3 mg whey protein in 50 µl was applied to the column and eluted at a flow rate of 0.5 ml/min. —— Protein fractions, ······· Non-protein material.
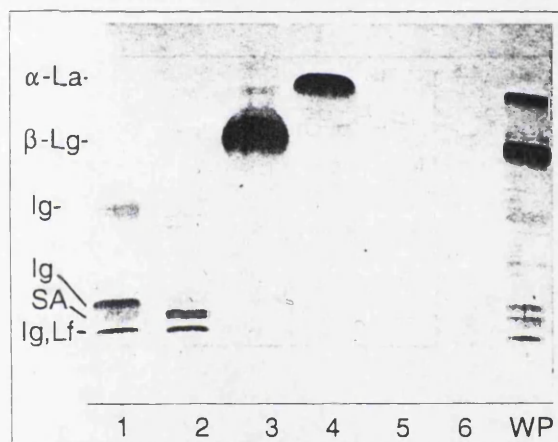
Fig. 2: SDS-PAGE pattern on a 20% homogeneous gel for whole whey proteins (WP), and the fractions obtained by gel permeation FPLC as shown in Fig.1. Lanes 1-6, fractions 1-6, respectively. The proteins are α-La, α-lactalbumin; β-Lg, β-lactoglobulin; Ig, immunoglobulins; SA, serum albumin; Lf, lactoferrin.

bumin – was improved in the method described here. Fractions 5 and 6, which were also present in ovine acid filtrate but absent from bovine acid filtrate (10), could not be detected by alkaline PAGE, and were mainly non-protein material with a molecular weight of about 3,000. The material had an absorption maximum at 260 nm and a minimum at 230 nm, instead of the characteristic absorption spectrum of protein with a maximum at 280 nm. The materials in peaks 7 and 8 had low molecular weights, and have previously been identified as mainly orotic and uric acids respectively (9).

Proteose peptones were present as minor contaminants of most fractions, but it was established that their absorbance at 280 nm was very low (Fig.7), and their presence did not significantly

affect quantitative values for the other whey proteins. Concentrations of the individual whey proteins were calculated using the following absorbance coefficients at 280 nm: immunoglobulins, 12.1; serum albumin, lactoferrin, 6.9; β-lactoglobulin, 9.5; α-lactalbumin, 20.1.

3.2 Natural variation in the composition of whey protein

Values are given in Table 1 showing the average composition of whey protein in a herd of British Saanen goats. Compared with values obtained by gel permeation FPLC for bovine whey protein (10), caprine whey protein contained less β-lactoglobulin, more α-lactalbumin and similar amounts of the minor fractions. Using gel-scanning, STORRY et al. (6) found that caprine whey protein contained, on average, less β-lactoglobulin but a similar amount of α-lactalbumin to bovine whey protein. In the present study, there was considerable overall variation in the composition of caprine whey protein, and it was found that those samples of whey protein that contained more of the immunoglobulin and serum albumin/lactoferrin fractions, contained less β-lactoglobulin.

Table 1: The average composition of whey protein from a herd of British Saanen goats

Fraction	% of total		
	Mean	S.D.	Range
Immunoglobulins	11.3	4.2	4.6–21.4
Serum albumin / lactoferrin	8.6	2.8	5.1–21.5
β-Lactoglobulin	54.8	6.5	39.2–72.1
α-Lactalbumin	25.2	3.7	17.8-33.3

3.3 The effect of stage of lactation

There were marked lactational changes in the concentrations of total and individual whey proteins, and mean values for 5 goats are shown in Fig.3. The concentration of total whey protein was high at the beginning of lactation, decreased in mid-lactation and increased towards the end of lactation. The concentrations of immunoglobulins, serum albumin/lactoferrin and β-lactoglobulin showed the same changes and were closely correlated with the total concentration (r=0.819, 0.746, 0.907, respectively, P<0.001). The concentration of α-lactalbumin, however, showed no marked trend. There was a small increase in the concentrations of all the whey proteins about 14 weeks from the beginning of lactation, and this was believed to be due to a change in nutrition.

Of the 5 goats used in this lactational study, four were of α_{s1}-Cn EE genotype, and produced milk with a low concentration of α_{s1}-casein. The other goat was of Cn AB genotype, and produced a high concentration α_{s1}-casein. The concentrations of total casein, and the corresponding concentrations of total whey proteins, for each of the α_{s1}-casein genotypes are shown in Fig. 4. Milk from the goat producing a high concentration of α_{s1}-casein had a higher concentration of total casein throughout lactation. The high concentration
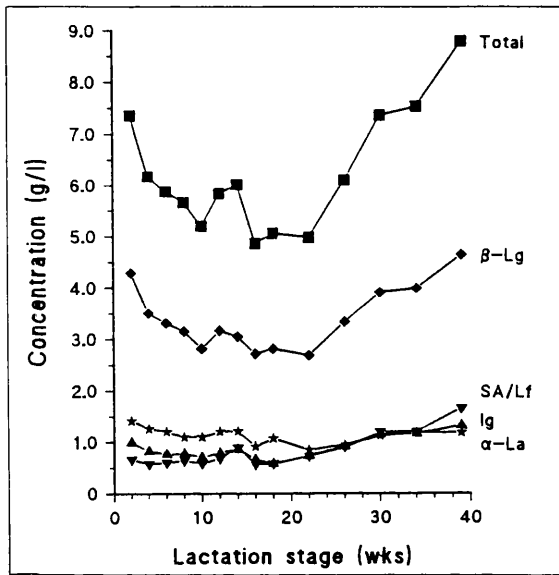
Fig. 3: Changes in the concentrations of total and individual whey protein fractions with stage of lactation.

and 5b, respectively. The milk yield increased rapidly within the first 4 weeks, remained fairly constant for about 10 weeks and then decreased rapidly. The yield of total protein, β-lactoglobulin and α-lactalbumin generally decreased throughout lactation, but there were temporary increases at 14 and 32 weeks of lactation. The yield of the immunoglobulin and serum albumin/lactoferrin fractions did not show any marked changes. There was a fairly close correlation between the milk yield and the total output of α-lactalbumin ($r=0.791$, $P<0.001$). This is consistent with the role of α-lactalbumin in the lactose synthase system, and the rate of lactose synthesis regulating the secretion of milk serum (13).

The relative amounts of the individual whey proteins also varied with stage of lactation (Fig. 6), but the changes were small compared with those in the concentrations and yields. The relative amount of β-lactoglobulin tended to decrease slightly with stage of lactation, whereas the relative amounts of the immunoglobulins and serum albumin/lactoferrin tended to increase. The relative amount of α-lactalbumin was initially fairly

Fig. 4: Lactational changes in the total casein and total whey protein in the milk of goats having either a high or low content of α_{s1}-casein. Total casein: ◆, high level of α_{s1}-casein; ▲, low level of α_{s1}-casein. Total whey protein: ▼, high level of α_{s1}-casein; ■, low level of α_{s1}-casein.

of α_{s1}-casein did not appear to be produced at the expense of the other caseins or whey proteins, however, and the concentration of whey protein was also slightly higher for most of lactation in the milk of the goat producing a high level of α_{s1}-casein.

Mean values for the change in milk yield, and the yield of the individual whey proteins from a single gland of each goat are shown in Fig. 5a

Fig. 5: Lactational changes in (a) milk yield (l) and (b) yield of total and individual whey proteins from a single gland/day. ■ Total whey protein, ◆ β-lactoglobulin, ★ α-lactalbumin, ▲ immunoglobulins, ▼ serum albumin/lactoferrin.
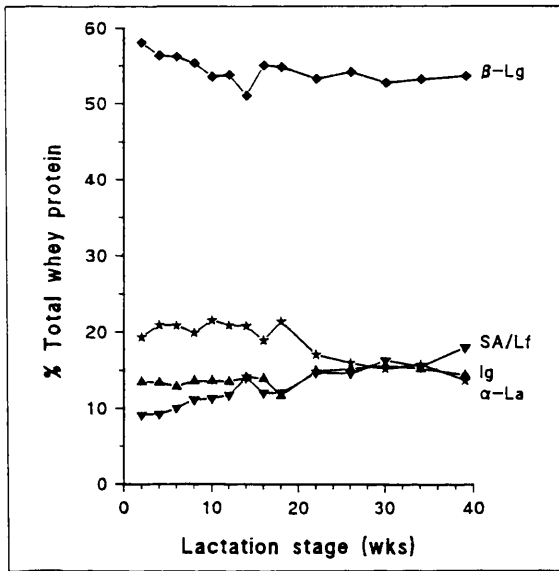
Fig. 6: Changes in the relative amounts of the individual whey proteins with stage of lactation.

constant but decreased from the middle to the end of lactation.

3.4 Denaturation of the whey proteins

Elution profiles are shown in Fig. 7 for the protein-containing fractions in acid filtrate from raw milk and milk heated at 90°C for 15 s, 3 min and 20 min. As found previously for bovine whey proteins (10,14), the caprine whey proteins were denatured on heating, and precipitated together with the caseins at pH 4.6. On the basis of this loss of solubility at pH 4.6, the fractions containing the larger molecular weight proteins, such as the immunoglobulins, serum albumin and lactoferrin, were most easily denatured, β-lactoglobulin was intermediate, and α-lactalbumin was most
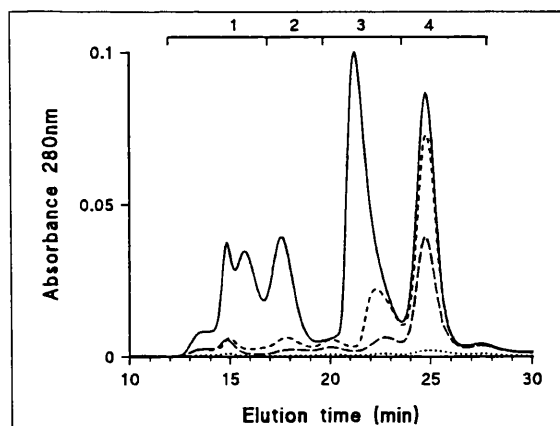
Fig. 7: Elution profiles obtained by gel permeation FPLC (conditions as in Fig. 1) showing the decrease in solubility of the whey proteins at pH 4.6 on heating milk at 90°C. Heating times were: —— Raw milk, --- 15 s, – – 3 min, ······ 20 min.

difficult to denature. On heating at 90°C for 15 s, for example, most of the immunoglobulins, serum albumin and lactoferrin, and a large proportion of the β-lactoglobulin were irreversibly denatured, whereas there was only a slight change in α-lactalbumin. On heating at 90°C for 3 min, almost all the β-lactoglobulin was denatured and more than half of the α-lactalbumin. On increasing the heating time to 20 min, only the proteose peptones remained in solution at pH 4.6. This fraction had a low absorbance at 280 nm and, therefore, did not substantially affect quantitative values for the other whey protein fractions.

This study shows that there is considerable variation in the content and composition of whey proteins in goats' milk, and that part of this is due to a lactational effect. Also there are large differences in the rates of denaturation of the individual whey proteins. In view of these results, it seems worthwhile to study in more detail the effect of heat-treatment on the incorporation of caprine whey proteins into the curd during cheese-making. The effect of stage of lactation on the concentrations, total yields and relative amounts of the corresponding casein fractions will also be reported in a separate publication.

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5. Summary

LAW, A.J.R., BROWN., J.R.: **Compositional changes in caprine whey proteins.** Milchwissenschaft **49** (12) 674–678 (1994).

38 Goat milk (whey proteins)

The composition of caprine whey protein was examined by gel permeation FPLC, in tris-HCl buffer at pH 7.0, on a column of Superdex 75 HR 10/30. The whey proteins were separated into 4 main fractions, and these were identified from their elution positions, and by SDS-PAGE. The average composition of whey protein from a herd of British Saanen goats was (1) immunoglobulins, 11.3%; (2) serum albumin and lactoferrin, 8.6%; (3) β-lactoglobulin, 54.8%; (4) α-lactalbumin, 25.2% of the total. There was considerable variation in the composition of whey protein, however, and part of this variation was due to the effect of stage of lactation. A lactational study of 5 goats showed that there were marked changes in the concentrations of all fractions, except α-lactalbumin. The total yields of β-lactoglobulin and α-lactalbumin decreased with advancing lactation, whereas the yields of immunoglobulins and serum albumin/lactoferrin did not change markedly. Changes in the relative amounts of the individual whey proteins were generally small compared to changes in the concentrations and total yields. The relative amounts of β-lactoglobulin and α-lactalbumin tended to decrease with advancing lactation, whereas the relative amounts of the immunoglobulin and serum albumin/lactoferrin fractions increased slightly.

A preliminary study of acid filtrates from heated milks showed that gel permeation FPLC could also be used to study the rates of denaturation of the caprine whey proteins.

LAW, A.J.R., BROWN, J.R.: **Veränderungen in der Zusammensetzung von Ziegen-Molkenproteinen.** Milchwissenschaft **49** (12) 674–678 (1994).

38 Ziegenmilch (Molkenproteine)

Die Zusammensetzung von Molkenprotein in Ziegenmilch wurde durch Gelpermeations-FPLC untersucht, und zwar in tris-HCl-Puffer bei pH 7,0 auf einer Superdex 75 HR 10/30-Säule. Die Molkenproteine wurden in 4 Hauptfraktionen unterteilt; diese wurden durch ihre Elutionspositionen und durch SDS-PAGE identifiziert. Die durchschnittliche Molkenproteinzusammensetzung einer Herde von British Saanen-Ziegen lag wie folgt (von der Gesamtmasse): 1. Immunglobuline 11,3%; 2. Serumalbumin und Laktoferrin 8,6%; 3. β-Laktoglobulin 54,8%; 4. α-Laktalbumin 25,2%. Jedoch gab es beträchtliche Variationen in der Molkenproteinzusammensetzung, die teilweise durch das Laktationsstadium verursacht war. Eine Laktationsstudie bei 5 Ziegen zeigte, daß es deutliche Unterschiede in der Konzentration aller Fraktionen, außer bei α-Laktalbumin, gab. Die Gesamtausbeute an β-Laktoglobulin und α-Laktalbumin nahm mit fortschreitender Laktation ab, während sich die Gehalte an Immunglobulinen und Serumalbumin/Laktoferrin nicht wesentlich veränderten. Veränderungen in den relativen Mengen der einzelnen Molkenproteine waren im allgemeinen gering im Vergleich zu den Veränderungen der Konzentrationen und der Gesamtmengen. Die relativen Mengen an β-Laktoglobulin und α-Laktalbumin tendierten dazu, mit fortschreitender Laktation zu sinken, während die relativen Mengen der Immunglobulin- und Serumalbumin/Laktoferrin-Fraktionen leicht anstiegen.

Eine vorläufige Untersuchung der Säurefiltrate aus erhitzter Milch zeigte, daß die Gelpermeations-FPLC auch zur Untersuchung des Denaturierungsgrades von Ziegen-Molkenproteinen eingesetzt werden kann.

5

MICELLAR STRUCTURE AND STABILITY

1. INTRODUCTION

Most of the casein in milk is in micellar form, closely associated with calcium phosphate and, to a large extent, the processing characteristics of milk are determined by the structure and stability of the casein micelles. On cooling milk, as during on-farm storage, β-casein dissociates from the micelles, and some colloidal calcium phosphate dissolves into the serum. This process is at least partly reversed when milk is re-warmed, as during pasteurisation, but it is not known if the micelles return exactly to their original structure. On reducing the pH, as would occur in the manufacture of cheese, fermented products or acid casein, calcium phosphate is fairly rapidly removed from the micelles, and they tend to disintegrate, with the release of all types of the caseins into the serum. Also, on heating milk above 60°C, the denatured whey proteins interact with micellar caseins; this subsequently affects renneting and acid precipitation (Chapter 6).

This Chapter deals in detail with the changes that take place in the mineral and protein composition of the micelles on adjusting the temperature between 30 and 4°C, and reducing the pH. A series of studies was also carried out to examine the association of calcium phosphate and the caseins within the micelle, and their distribution between the colloidal and serum phases. The relation between micellar size and casein composition was also investigated.

2. MICELLAR STRUCTURE

Studies involving ultracentrifugation have shown that in cows' milk stored above 30°C, more than 90% of the casein is in micellar form (Rose, 1968; Downey and Murphy, 1970). The composition of micellar casein varies considerably with micellar size

(Rose, 1965; Donnelly *et al.*, 1984), and between species. Compared with bovine casein, caprine and ovine caseins contain much more β-casein and less α_s-caseins. In caprine casein, the relative amount of α_{s1}-casein can vary from 0 to above 20% of the total (Tables 11 and 17). Also, as described in more detail below, on storing cows' milk at 4°C, more than half of the β-casein can dissociate from the micelle.

About 66% of the calcium and 50% of the P_i (inorganic phosphate) in milk are in colloidal form (White and Davies, 1960); this colloidal calcium phosphate is usually also considered to include the Ca^{2+} bound directly to the caseins (about 5mM), and smaller amounts of Mg^{2+} and citrate. Typical values for the concentrations of the main salts in cows' milk, milk serum and micelles are given in Table 18. Micelles in the milk of the cow, goat and sheep have similar ratios of Ca/P_i, and a similar, high water content (micelle solvation), and these are shown in Table 11. Comparatively little research has been carried out on the properties of caprine and ovine micelles, and the discussion below refers mainly to bovine micelles.

2.1 Micellar Models

As information about the relative amounts and properties of the caseins has been acquired, several micellar models have been proposed to account for the behaviour of micelles during various processes, such as cooling, heating, acidification and renneting. These models fall into three main groups. The earlier coat-core models were based on the solubility properties of the main caseins in the presence of calcium, whereas later models included internal structures based on the known composition and properties of the individual caseins. Recent models are based on a subunit structure with the presence of an outer hydrophilic "hairy" layer containing the caseinomacropeptide fragment of κ-casein. The various micellar models have been exhaustively reviewed (Farrell, 1973; Slattery, 1976; Walstra, 1990; Holt, 1992; Rollema, 1992). Some of the micellar models that have aspects relevant to the present view of the micelle are discussed below.

The coat-core model of Waugh and Noble (1965) and Noble and Waugh (1965) was based on work which showed essentially that α_s- (α_{s1}- and α_{s2}-) and β-caseins

Table 17. Average composition of bovine, caprine and ovine whole casein

	Bovine[a]	Caprine[b]			Ovine[c]
	% of total casein				
α_{s1}-	36.2	21.5	13.8	6.7	34.2
α_{s2}-	10.2	8.9	10.8	12.4	
β-	40.8	46.5	50.9	50.8	50.4
κ-	10.1	12.6	15.4	20.5	10.8
Minor	2.8	10.6	9.2	9.7	4.6

[a] Hollar *et al.*, 1991
[b] Law and Tziboula, 1992
[c] Law *et al.*, 1992

Table 18. Concentrations of the salts in milk, milk serum and micelles

	Skim-milk	Milk serum	Micellar
	Concentration mM at 20°C		
Ca	29.4	9.1	20.3
P_i	20.3	10.8	9.5
Mg	5.0	3.2	1.8
Citrate	9.2	8.2	1.0
Na	22.8	21.8	1.0
K	39.7	37.5	2.2

From data of White and Davies, 1960

aggregated in the presence of calcium ions, whereas κ-casein was not sensitive to calcium, and could prevent the precipitation of these caseins. These workers suggested that, in the absence of calcium, a complex of α_{s1}- and κ-caseins was formed. In the presence of calcium ions, α_{s1}- and β-caseins aggregated to form a micellar core; precipitation of this complex was prevented by formation of a monolayer of the α_{s1}-/κ-casein complex. They considered that the κ-casein was located on the surface of the micelle, and micellar size varied inversely with κ-casein content. In a study of the association of the caseins, Payens (1966) found that the degree of polymerisation of β-casein decreased with temperature, and at $4\,°C$ it existed in the monomeric state. He therefore proposed a similar coat-core model, but with a micellar core containing a network of open coils of β-casein and folded α_{s}-casein molecules attached by hydrophobic bonds. κ-Casein was located on the surface of the micelle, and this was consistent with the loss of stability on proteolysis of κ-casein by chymosin.

Rose (1969) proposed that β-casein monomers self-associated into chain-like polymers, and that α_{s1}- and κ-caseins became attached. β-Casein was oriented inwards, whereas most κ-casein was directed outwards. In a study of the relation between micellar and serum casein, however, Rose found that the level of serum casein increased as the temperature was reduced, and as colloidal calcium phosphate was removed by lowering the pH. He therefore suggested that colloidal calcium phosphate was incorporated into the network of micellar caseins as a stabilising agent. Using anion-exchange chromatography, Rose found that the ratio of caseins in the micelle was $3\alpha_{s}$-casein (α_{s1}- and α_{s2}-) : 2 β-casein : 1 κ-casein.

Garnier and Ribadeau-Dumas (1970) and Garnier (1973) suggested an alternative model in which the micelle had an open sponge-like structure and a high water content. This was based on evidence that fluorescent dye and large molecules such as carboxypeptidase A (MW 34,600) could enter the micelles. They suggested that the micelle was formed by an irregular network of cross-linked polymers, with κ-casein located at the nodes of the lattice, and α_{s1}- and β-caseins forming the arrays. The size distribution of the micelles was, therefore, controlled by the relative rates of synthesis of the κ-, α_{s1}- and β-caseins.

The present view of micellar structure includes some of the main features of these models, but is based on a subunit structure, typified by the model (Fig. 19)

proposed by Walstra (1990). Evidence for a subunit structure comes from dissociation studies, electron microscopy, neutron diffraction and X-ray diffraction.

The subunit structure of micelles was proposed by Morr (1967) after carrying out studies on the effect of urea and oxalate on the dissociation of micelles. He suggested that micelles contained loosely packed subunits composed of an inner core of α_s- and β-caseins surrounded by an outer layer rich in α_s- and κ-caseins. The subunits were joined through calcium, and colloidal calcium phosphate and calcium citrate linkages, between phosphoserine and carboxyl groups on the caseins. Using gel chromatography and gel electrophoresis, Pepper (1972) showed that micellar casein depleted of calcium by treatment with EDTA (first cycle casein) dissociated at low protein concentration to a unit complex with a diameter of about 10nm and molecular weight of 270,000. The unit complex contained each of the caseins.

Using electron microscopy, Shimmin and Hill (1964) showed that micelles appeared to be built up of units about 10nm in diameter and having a molecular weight of about 300,000. In later studies, Bucheim and Welsch (1973) examined lactating rat mammary tissue. They showed that a large number of small particles, about 10nm in diameter, occurred in Golgi vesicles and that these particles eventually condensed to form the individual casein micelles that are extruded into the glandular lumen. Vacuoles near the dictyosomes and endoplasmic reticulum were believed to contain micelles in the process of formation, whereas Golgi vacuoles nearer the apical end of the cell contained more completely formed micelles. On the basis of these observations, it has been proposed that after synthesis on the ribosomes, the caseins interact to form subunits composed of several monomers; the composition of the subunits depends on the relative rates of synthesis of the caseins. On reaching the Golgi, these subunits become phosphorylated to form the electron dense particles observed in the early stages of micellar formation. Addition of calcium causes polymerisation of these particles, and deposition of calcium phosphate gives the complete micelle.

Neutron diffraction (Stothart and Cebula, 1982) and X-ray diffraction (Pessen et al., 1989) confirm the existence of discrete subunits in the micelle. Using the latter technique, Pessen et al. (1989) found that subunits formed in the absence of calcium had a molecular weight of 285,000. These workers also found that the subunits consisted of two regions of different electron density. The inner core was more electron
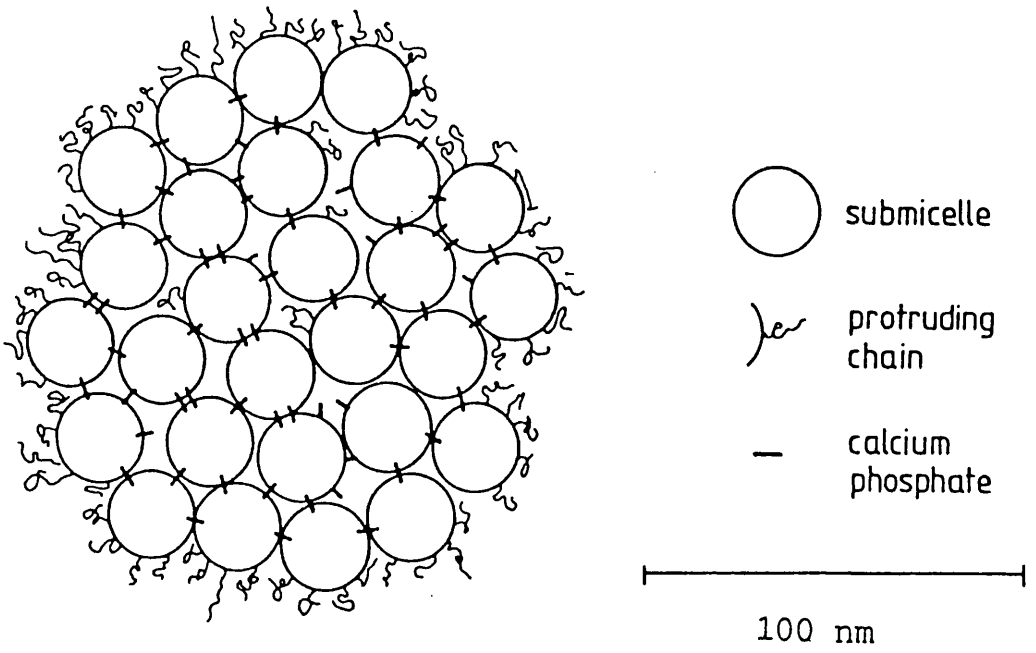
Fig.19 Subunit micellar model (Walstra, 1990)

dense, consistent with higher hydrophobicity, whereas the outer region was less electron dense and hydrophilic.

In the micellar model of Walstra (1990), the submicelles contain between 15 and 25 molecules, and are between 10 and 15nm in diameter (Fig.19). The submicelles are held together by hydrophobic interaction and salt bridges; the submicelles are bound into micelles by colloidal calcium phosphate. κ-Casein tends to be located at the outside of the micelle, and the hydrophilic caseinomacropeptide sticks out from the micelle into the serum as flexible hairs. The hairs prevent aggregation of the micelles and form a layer about 7nm thick. Results from light scattering (Walstra *et al.*, 1981; Horne and Davidson, 1993) show that, on the addition of chymosin, the hairs are removed, destabilising the micelle and decreasing its hydrodynamic radius by about 5-7nm. Similarly, in studies of ethanol stability, addition of ethanol causes the hairs to collapse, reducing the micellar hydrodynamic radius by 5 to 10nm.

The papers included in this Chapter relate to various aspects of micellar structure and stability, and these are discussed separately below.

3. MICELLAR DISSOCIATION AT LOW TEMPERATURE

Using ultracentifugation, Rose (1968) found that when milk was cooled to 4°C and stored for several hours, the level of serum casein increased considerably and was, on average, about 25% of the total casein. There was, however, considerable variation between cows in the level of serum casein (14-44% of the total casein), although serum calcium and phosphate levels were within the normal range. On analysing serum casein using alkaline PAGE, Rose found that it contained α_{s1}-, β- and κ-caseins, with β-casein accounting for 55% of the total increase in serum casein. On diluting sedimented micellar pellets in ultrafiltrate the new level of serum casein remained considerably below that in the original milk. Rose concluded that micellar and serum casein do not form an equilibrium controlled by the solubilities of the caseins. Instead, he attributed the increase in serum casein to weaker hydrophobic interaction, and especially to less extensive polymerisation of β-casein, at the lower temperature.

Downey and Murphy (1970) similarly found an increase in the level of serum casein on cooling milk, but on average only about 15% of the casein was present in the supernatants. β-Casein accounted for about half of the increase in soluble casein, with α_s- and κ-caseins accounting for almost equal proportions of the remainder. There was considerable variation in the level of serum casein in the milks of different cows, however, the concentration being especially high in the milks of cows in late lactation or affected by mastitis. Several other workers have found high levels of serum casein associated with mastitis (Sharma and Randolph, 1974: Ali et al., 1980c).

Creamer et al. (1977) carried out a detailed study on the diffusion of β-casein, within the micelle and from the surface, by following the transfer of ^{14}C-labelled β-casein, and the A and B genetic variants of β-casein. Results showed that when milk was cooled to 0°C, the level of serum casein increased rapidly and reached a maximum in about two hours. Their results were consistent with dissociation of β-casein from the surface of the micelle on cooling, followed by movement of β-casein from the interior to the surface. On re-warming to 37°C this process was reversed; β-casein re-deposited on the surface of the micelle, and then re-distributed within the micelle.

On studying changes in the mineral equilibrium when milk was cooled, Davies and White (1960) found a small but significant increase in the diffusible calcium, ionised calcium, and P_i in milk that had been been stored at 3°C for 24h. Pierre and Brule (1981) found a larger increase in the level of ultrafiltrable Ca and P_i when milk was cooled. They concluded that calcium phosphate was normally present as a saturated solution in the soluble phase of milk. When milk was cooled the solubility of calcium phosphate in the serum increased and calcium phosphate was able to dissolve off the micelles. These workers also believed that part of the β-casein was held by hydrophobic interaction and dissociated as the temperature was reduced, but that demineralisation, caused by cooling or addition of EDTA, led to release of more β-casein.

Several workers have indicated that the increase in serum casein, on storing milk at low temperatures, may affect the processing properties. Fox (1970), and Fox and Guiney (1973) showed that caseins in micellar form were not susceptible to proteolysis but, on removal of colloidal phosphate, the micelles disintegrated and the caseins were then more open to enzymatic breakdown. Their results suggested that

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about 50% of the β-casein was able to diffuse from the micelle at low temperatures, whereas α_s-casein (α_{s1}- and α_{s2}-) was firmly fixed within the micelle, and only became susceptible to proteolysis when the micellar structure was totally disrupted by removal of colloidal calcium phosphate. Leaver and Thompson (1993) studied proteolysis of the caseins with soluble and immobilised trypsin. On cooling milk to 4°C, 25% of the β-casein, 3% of the κ-casein and 2% of the α_s-caseins diffused into the serum. On re-warming to 37°C, the rates of trypsinolysis of the individual caseins were similar to those in uncooled milk, suggesting that the caseins which diffused from the micelles on cooling, returned to a similar position on re-warming.

Ali *et al.* (1980a, b) studied the influence of storage time and temperature on the distribution of individual milk proteins between micellar and soluble phases, and its effect on cheese-making properties. They found that when milk was stored at 4 or 7°C for 48h there was a marked increase in dissociation of micellar caseins, particularly β-casein, and of calcium and P_i. On making cheese with these milks, the curd structure was weaker, the curds were more moist, and cheese yield was slightly lower. The effects of cold storage could be partly reversed by heating at 72°C for between 30 and 60s, but determination of the level of soluble casein showed that reversal was incomplete.

The first paper in this Chapter (Paper 13, Davies and Law, 1983) describes variation in the level and composition of bovine casein micellar and serum casein with temperature. Micellar fractions were sedimented by step-wise ultracentrifugation, and the concentrations and relative amounts of the caseins in the micellar fractions and in serum casein were determined by Kjeldahl N analysis and anion-exchange chromatography on DEAE cellulose. As in previous studies discussed above (Rose, 1968; Downey and Murphy, 1970; Creamer *et al*, 1977), it was found that when milk was cooled there was a large increase in the serum concentrations of β-casein. Results are presented showing the composition of the serum and micellar casein fractions in milk at 20° and 4°C. In contrast to other studies, however, there was no significant increase in the serum concentration of any of the other caseins, apart from a slight increase in the γ-caseins, which were derived from β-casein. In this study, the centrifugation time was increased to make allowance for the increase in viscosity that occurs as milk is cooled to 4°C, and at each temperature the supernatant containing the

serum casein was clear. It appears, therefore, that the earlier reported increases in serum α_{s1}- and κ-caseins may have resulted from incomplete sedimentation of micelles, and the inclusion of some micellar casein with the serum casein.

This paper also describes in detail the reversibility of the changes caused by cooling milk to 4°C, and shows that, after re-warming to 20°C for 18h, the amount and casein composition of the different micellar size classes were similar to those in uncooled milk.

4. COLLOIDAL CALCIUM PHOSPHATE

In the micellar model of Walstra (1990), the submicelles are bound by colloidal calcium phosphate, which includes all of the calcium, inorganic phosphate (P_i) and small amounts of Mg^{2+} and citrate that are present in the micelle. Pyne and McGann (1960) determined the composition of colloidal calcium phosphate by a method in which they prepared colloidal phosphate-free milk by controlled acidification of chilled milk to pH 4.8-5.0, followed by prolonged dialysis against the original milk to restore the level of Ca^{2+}. By comparing the colloidal phosphate-free milk with skim-milk, which was identical in all respects except for the colloidal calcium phosphate, these workers obtained a ratio for colloidal Ca/P_i of 1.68.

The composition of colloidal calcium phosphate has also been determined experimentally in dialysis equilibrium experiments as the difference between total and diffusible calcium and P_i (White and Davies, 1960). In these experiments, however, a small amount of Ca^{2+}, remained bound directly to the caseins, and it was difficult to measure the exact ratio of Ca to P_i in colloidal calcium phosphate.

Various other dissociation experiments have been carried out in which colloidal calcium phosphate has been removed by EDTA (Holt, 1982; Griffin et al., 1988) and by acidification (Chaplin, 1984; van Hooydonk et al.,1986). Usually a linear relationship has been found between micellar calcium and P_i; the slope of the line has been between 1.61 and 1.98. In each case, the intercept of the line on the axis representing calcium concentration is usually taken as a measure of the amount of Ca^{2+} remaining tightly bound to negatively charged groups on the casein. These groups are

believed to be mainly phosphoserine residues. The calcium binding capacity of the caseins decreases in the order α_{s2}- > α_{s1}- > β- > κ-casein, according to decreasing phosphoserine content (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Baumy *et al.*, 1989). There is some infrared spectroscopic evidence, however, that the negatively charged Asp and Glu residues may also bind Ca^{2+} (Byler and Farrell, 1989).

The term micellar calcium phosphate is used to describe the complex formed between the colloidal calcium phosphate and the casein in the micelles. The evidence suggests that the interaction with calcium phosphate occurs through the phosphoserine and phosphothreonine residues in the caseins (Aoki *et al.*, 1987; Schmidt and Poll, 1989) but other groups, such as the positive charges on Lys and Arg, may also be involved (Visser *et al.*, 1979).

Two different structures have been proposed for micellar calcium phosphate. If the phosphoserines and phosphothreonines of the caseins, together with their bound calcium, are not part of the micellar calcium phosphate, the molar ratio of Ca/P_i is about 1.5, and the calcium phosphate may be of the tricalcium phosphate type. If, however, the ester phosphates are considered as an integral part of the calcium phosphate, the ratio of Ca/P is close to 1.0, and the calcium phosphate resembles brushite (Holt *et al.*, 1989). Results from X-ray absorption spectroscopy (Holt *et al.*, 1982), and measurement of the solubility product (Chaplin, 1984) also indicate that the micellar calcium phosphate is similar to brushite ($CaHPO_4.2H_2O$), but further confirmation is required.

The second paper in this Chapter (Paper 14, Holt *et al.*, 1986) describes the investigation of the composition of colloidal calcium phosphate, and the way in which calcium phosphate is associated with the caseins. Controlled dissociation of the micelles at constant temperature, pH, ionic strength and osmolarity, was carried out by dialysing milk against buffer containing calcium phosphate and different levels of Ca^{2+}, or phosphate-free buffer with 3 or 6mM Ca^{2+}. The dialysed milks were centrifuged at high speed, and the extent of dissociation of the individual casein fractions from the micelle was determined by anion-exchange chromatography (Davies and Law, 1977a). Using the first buffer, there was no change in colloidal P_i, but colloidal Ca decreased when the free Ca^{2+} fell to about 1mM, which is slightly below the normal level of 2mM in milk. At this concentration, there was marked dissociation of the caseins from

the micelles, and the extent to which the caseins were retained in the micelles was in the order α_{s2}- > α_{s1}- > β- > κ-casein. Using the second buffer, both colloidal Ca and P_i were reduced, and when more than 30% was removed from the micelles the caseins dissociated from the micelles, again the order of retention being α_{s2}- > α_{s1}- > β- , κ-caseins. These results showed the importance of calcium phosphate in maintaining micellar structure. Also, the degree of binding of the caseins was in the same order as the number of phosphoserine groups in the respective caseins. This indicated that phosphate centres in the caseins were important in the linkage with calcium phosphate. This was confirmed by enzymic hydrolysis of micellar calcium phosphate, followed by amino acid analysis, which showed a high content (72%) of serine, phosphoserine, glutamic acid and glutamine. Glutamic acid is often present in the tripeptide sequence that favours phosphorylation of the caseins - Ser/Thr-X-A-, where A is an acidic residue such as Glu, Asp, SerP or ThrP (Mercier, 1981).

5. MICELLAR DISSOCIATION AT LOW pH

Fermentation of milk by lactic acid bacteria is an essential first step in the manufacture of various dairy products, including cheese, cottage cheese and yogurt. Growth of the bacteria causes a decrease in the pH, and accompanying changes in the properties of the micelles. The most important effects of the reduction in pH are that the colloidal calcium phosphate and small amounts of magnesium and citrate are solubilised, and caseins are liberated into the serum.

Pyne and McGann (1960) acidified milk at 0°C with concentrated HCl, and then dialysed against the original milk. They were able to remove all of the colloidal calcium phosphate, except the caseinate-bound Ca^{2+}, from the micelles without precipitation of the caseins. Re-introduction of the calcium phosphate, however, did not usually restore the properties of the original milk (McGann and Pyne, 1960).

Using dialysis and ultrafiltration, Davies and White (1960) measured the concentrations in diffusate of calcium, total phosphorus, magnesium and citrate. As the pH was reduced to 5.6, there was a marked increase in all of these constituents in the serum, and at pH 4.6 most of the calcium, magnesium and citrate had been removed

from the micelles. More than 73% of the total phosphorus was removed from the micelles, the remainder being the organic phosphorus on the caseins.

In addition to changes in the colloidal calcium phosphate, Rose (1968) found a large increase in the amount of soluble casein when milk was acidified by the addition of HCl and then equilibrated overnight at 4°C. The level of serum casein increased as the pH was reduced to 5.3, and then decreased as the isoelectric point was approached. The effect of pH was much less when the milk was re-equilibrated to 35°C for 2h. Rose concluded that the combined effect of low temperature and low pH was more than additive in causing dissociation of caseins from the micelle.

Van Hooydonk *et al.* (1986) carried out a quantitative study on the acidification of milk. They obtained a linear relationship between micellar calcium and inorganic phosphate in the pH range pH 6.7 - 5.6. The calculated micellar ratio of Ca/P_i was 1.66, and the intercept was 4.64, indicating the presence of about 4.6mM of caseinate bound Ca^{2+}, but almost complete removal of the P_i, about pH 5.3. Using SDS-PAGE, these workers showed that at 30°C maximum dissociation of the caseins occurred at pH 5.6, and all of the major caseins were present in the serum. They also found that the voluminosity of the micelles was at a maximum at pH 5.3.

Ali *et al.* (1980b) examined the effect of acidification of milk and storage at 4°C for up to 72h. On keeping the milks at 4°C for 48h without pH adjustment, there were increases in the serum concentrations of α_{s1}-, β- and κ-caseins, as determined by gel-scanning, and there were slight increases in serum Ca and P_i. On adjusting the pH to 6.2, and then storing at 4°C, there were large increases in the serum concentrations of α_{s1}- and β-caseins, Ca and P_i. The concentration of κ-casein in the serum was less in the treatment involving acidification. The results for α_{s1}- and β-caseins confirm those of Rose (1968) that low temperature and acidification together cause more dissociation from the micelle than separately.

The third and fourth papers in this section (Paper 15, Dalgleish and Law, 1988; Paper 16, Dalgleish and Law, 1989) describe in detail the combined effect of pH and temperature on dissociation of the caseins and colloidal calcium phosphate from bovine casein micelles. After acidification and storage at 30, 20 or 4°C the milks were centrifuged at high speed; the level and composition of the serum and micellar caseins were examined by Kjeldahl N analysis and FPLC anion-exchange FPLC as described

in Chapter 2 (Davies and Law, 1987). The concentrations of Ca, P_i and Mg in the serum were also measured. There were only small increases in the serum concentrations of Ca and P_i as the temperature was lowered. On reducing the pH, however, serum concentrations of Ca and P_i increased considerably, and below pH 5.3 most of the Ca and all of the P_i was removed from the micelle. The ratio of micellar Ca/P_i was between 1.75-1.85, close to values found by other workers, as discussed above. At the natural pH of milk, the level of serum casein increased at lower temperatures, and this was mainly due to weakening of hydrophobic interaction, and dissociation of β-casein from the micelle. On lowering the pH, at 20° and especially at 4°C, the levels of all caseins in the serum tended to increase, confirming the results of Rose (1968) that the combined effects of pH and low temperature were more than additive in causing dissociation from the micelle. The results were consistent with calcium phosphate playing an important part in binding the caseins in the micelle. At 30°C, however, all of the calcium phosphate could be removed from the micelle without any appreciable increase in the level of serum casein. This shows that, in addition to calcium phosphate, hydrophobic interaction is also important in maintaining the integrity of the micelle.

6. MICELLAR SIZE AND COMPOSITION

6.1 Determination of Micellar Size

The average size and size distribution of casein micelles have been determined by electron microscopy (Schmidt *et al.*, 1973; Schmidt *et al.*, 1974; Donnelly *et al.*, 1984; Vreeman *et al.*, 1991). There has been some criticism that, as micelles have a high water content, artefacts may be introduced during the preparation of sections for electron microscopy. The above workers used the technique of freeze-etching, which is less likely to alter the structure during the preparation of sections. This involves rapid freezing in the presence of glycerol to prevent the formation of ice crystals. The freeze-fractured material is then shadowed with very fine platinum and carbon. To obtain values for micellar size from electron micrographs, it is necessary to make a

relative number fraction (%)
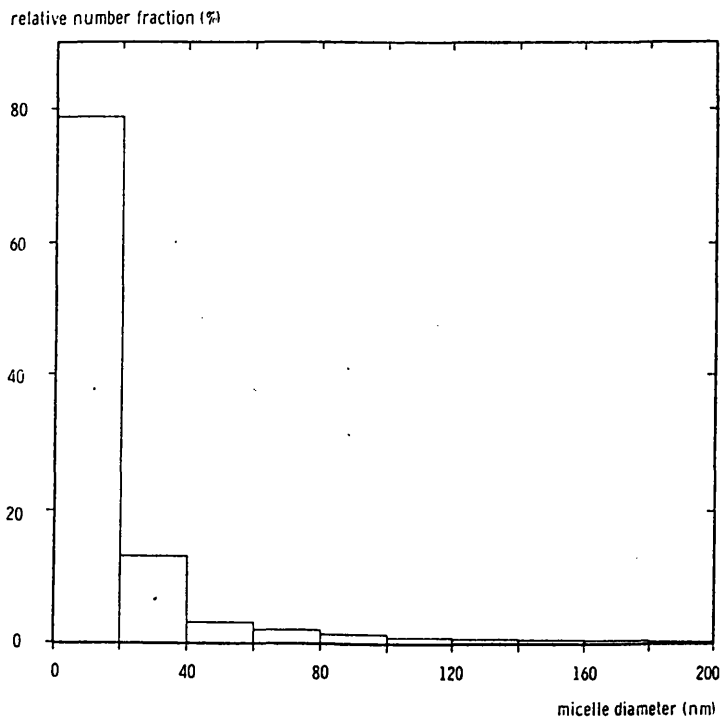
Fig.20 Number frequency distribution of casein micelles in bulk milk

relative volume fraction (%)
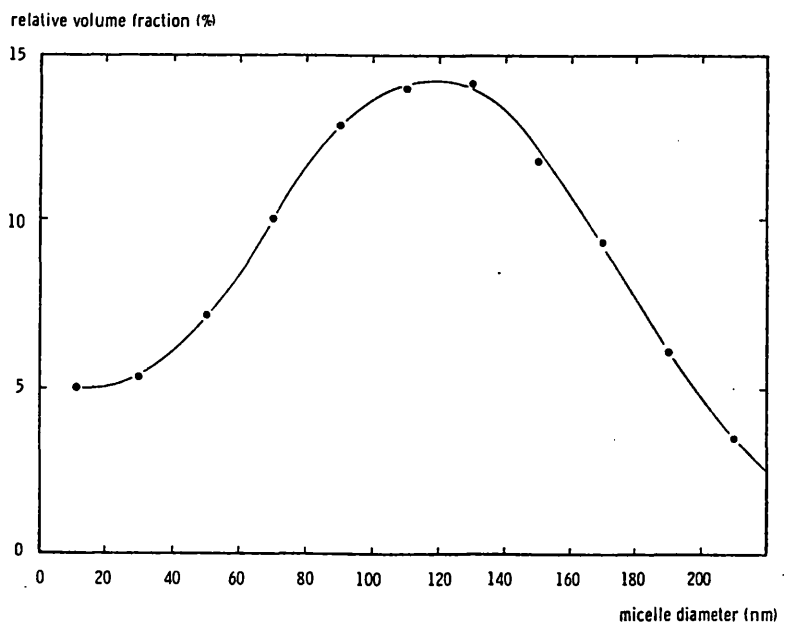
Fig.21 Volume frequency distribution of casein micelles

Walstra (1990)

statistical correction to take into account that the observed section is not usually through the diameter of the micelle, and that the probability of the micelle being sectioned at all depends on micellar size. Using this method, Schmidt *et al.* (1973) found that about 80% of the micelles had diameters less than 20nm; the number of micelles per size class decreased very rapidly with increasing diameter, up to about 180nm (Fig.20). Because micellar volume (and weight) varies with the radius[3], a small number of very large micelles have a big effect on the volume (and weight) average distribution. On making this correction, the volume average micellar diameter was about 120nm (Fig.21).

Various workers have measured micellar size by light scattering techniques. Dewan *et al.* (1974) obtained values for the weight-average micellar diameter of 160nm, and for the mean molecular weight of 2.5×10^8. There was, however, a broad distribution of molecular weights. Average micellar size has been measured by quasi-elastic laser light-scattering (Holt *et al.*, 1973) and by elastic light-scattering (Holt, 1975; Holt *et al.*, 1975), which is based on the wavelength dependence of turbidity. These workers found that the volume-average micellar diameter in the milk of different cows was between 180 and 260nm, and that the molecular weight varied from 2.6×10^8 to 15×10^8.

Electron microscopy and light-scattering gave the same type of curves for the number average micellar size distribution, in which there was a very large number of small particles, decreasing in number with increasing diameter. On comparing values for the volume average micellar diameter, Schmidt *et al.* (1974) and Holt *et al.* (1978a) found that most values obtained by light-scattering were about twice the values obtained by electron microscopy. Schmidt *et al.* (1974) suggested that the number of large micelles was being underestimated by electron microscopy, and that a water-rich protein layer surrounding the micelles was not being detected. Holt *et al.* (1978a) believed that high values from light-scattering might in part be due to the presence of small fat globules.

Using light-scattering, Holt and Baird (1978) showed that there was considerable variation in the average micellar size in the milks of different cows. Also, in a study of creamery bulk milks Holt and Muir (1978) found that there was a

pronounced seasonal trend, with smaller average micellar sizes in summer than in winter.

6.2 Size and Composition

Casein micelles have been fractionated on a size basis by two different methods - ultracentrifugation and gel permeation chromatography. In some early work, micelles were sedimented by differential centrifugation, and κ-casein was determined by measuring the sialic acid concentration (Sullivan et al, 1959; Decelles, 1967). These workers found that the smaller, more slowly sedimenting micelles contained more κ-casein. There is some doubt about the accuracy of the quantitative values, however, as it has been shown that the sialic acid content of κ-casein is variable (Rose et al., 1969).

Using ultracentifugation, Rose et al. (1969) prepared four micellar fractions, and analysed the caseins by anion-exchange chromatography. With decreasing micellar size, the relative amount of α_s-casein increased slightly, β-casein decreased and κ-casein increased markedly.

Several groups have used gel permeation chromatography to fractionate micelles. Ekstrand and Larsson-Raznikiewicz (1978) fractionated micelles on controlled-pore glass granules (CPG-10/3000), in synthetic milk ultrafiltrate at 4°C, and obtained five micellar fractions. The larger micelles eluted from the column first, whereas the much smaller whey proteins eluted last. These workers were unable to detect monomeric caseins that might correspond to a serum casein fraction. With decreasing micellar size, the relative amount of α_{s1}-casein determined by anion-exchange chromatography decreased slightly, whereas β- and κ-caseins increased.

Using a similar method of gel permeation chromatography, at 20°C, McGann et al. (1979) obtained three micellar fractions, and a whey protein fraction containing serum casein. With decreasing micellar size, the relative amount of α_s-casein determined by chromatography on hydroxyapatite decreased, whereas β- and κ-caseins increased. These workers obtained essentially the same results with glutaraldehyde-fixed micelles, showing that no appreciable amount of dissociation was taking place during chromatography.

Donnelly *et al.* (1984) fractionated micelles on porous glass (CPG-10, 50nm followed by CPG-10, 300nm) at 30°C and obtained eight micellar fractions. The micellar sizes were determined by electron microscopy, and their composition was examined by chromatography on hydroxyapatite. With decreasing micellar size, the relative amounts of α_s- and β-caseins decreased, but the relative amount of κ-casein increased. On drawing a plot of κ-casein fractional content versus micelle surface to volume ratio, these workers obtained a straight line with a correlation coefficient of 0.98.

In addition to casein composition, various other properties have been related to micellar size. Sood *et al.* (1976) prepared three micellar fractions by ultracentrifugation and determined the voluminosity, defined as the volume (ml) occupied by one gram of dry material, by viscosity measurements. They found that the smaller micelles contained more bound water.

Conflicting results have been obtained regarding the carbohydrate content of differently sized micelles. Creamer *et al.* (1973) studied the solubilities in trichloroacetic acid of the glycomacropeptides released from large and small micelles. These workers found that although the larger micelles contained less κ-casein, a greater proportion of the κ-casein was glycosylated than in small micelles. Slattery (1978) examined the relationship between the glycosylation pattern and micellar size in more depth. He found that κ-casein in the largest micelles was the most highly glycosylated. The proportion of glycosylated κ-casein in five classes of smaller micelles, however, appeared to increase with decreasing micellar size. On this basis, Slattery suggested that glycosylation occurs after micellar assembly, and small micelles, having the larger surface area/volume ratio, contain proportionately more carbohydrate. On the other hand, Dalgleish (1985), using anion-exchange FPLC, found that the ratio of unglycosylated to glycosylated κ-casein did not vary with micellar size.

Results on the coagulation properties of differently sized micelles are also conflicting. Ekstrand *et al.* (1981) found that the largest and smallest micelles, separated by gel permeation chromatography, had longer rennet coagulation times than medium size micelles. Dalgleish *et al.* (1981), however, separated micelles into different size classes by centrifugation, and found that the rates of aggregation of the

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re-suspended micelles were the same. Ford and Grandison (1986), on the other hand, found that the smaller micelles had slightly shorter coagulation times.

The first and last papers in this section (Paper 13, Davies and Law, 1983; Paper 17, Dalgleish *et al.*, 1989) describe the examination of the relation between micellar size and composition. In both studies different size classes of micelles were obtained by ultracentrifugation. Determination of the sizes of the micelles in different pellets by light scattering confirmed that the more slowly sedimenting micelles had, in fact, smaller diameters. In the first study (Davies and Law, 1983), the composition of the micellar casein was determined by anion-exchange chromatography on DEAE cellulose whereas, in the second study, the relative amounts of the caseins were determined by anion-exchange FPLC, as described in Chapter 2. In both studies, the smaller more slowly sedimenting micelles contained more κ-casein, as found by other workers. The relative amount of κ-casein in the larger micelles (diameter 298nm) was 5.7% of the total casein, whereas the relative amount of κ-casein in the smaller micelles (diameter 106nm) was 12.9% of the total. The increase in κ-casein with decreasing micellar size was balanced almost exactly by a decrease in the relative amount of β-casein. In both studies it was found that the relative amounts of α_{s1}- and α_{s2}-caseins did not vary with micellar size. These results differ from those of Donnelly *et al.* (1984) who separated micelles by gel permeation and found that the relative amount of α_{s1}-casein also decreased with decreasing micellar size. In the present study, there was a slight decrease in micellar P_i with decreasing micellar size, but the level of micellar Ca stayed almost constant. The ratio of Ca/P_i, therefore, increased with decreasing micellar size, and this may reflect different calcium phosphate binding in the smaller micelles.

7. CONCLUSIONS

When milk was stored at 4°C for several hours, there was a large increase in the concentration of serum casein, which was due almost entirely to dissociation of micellar β-casein together with a small amount of γ-caseins. There were also small increases in the serum concentrations of Ca and P_i. On prolonged cooling, more than

50% of the β-casein dissociated into the serum. There was no increase, however, in the serum concentrations of α_{s1}-, α_{s2}- or κ-caseins on cooling. The increases in the serum concentrations of these caseins on cooling reported by others may have resulted from incomplete sedimentation of micelles, and the inclusion of micellar casein with the serum casein. The changes in composition and micellar distribution were reversed when milk was warmed to 20°C for 18h.

In a study of micellar stability, using controlled dialysis of milk to remove colloidal calcium, or colloidal calcium and phosphate, there was marked dissociation of the caseins from the micelles, showing the importance of calcium phosphate in maintaining micellar structure. The caseins were retained in the micelles in the order α_{s2}- > α_{s1}- > β-/κ-casein, and this indicated that the phosphoserine groups on the caseins were important in linkage with the calcium phosphate.

In a study of the combined effect of pH and temperature on micellar dissociation, it was found that, on lowering the pH of milk, serum concentrations of Ca and P_i increased rapidly, and below pH 5.3 most of the Ca and all of the P_i was removed from the micelle. On lowering the pH at 4°C, there was a marked increase in the level of serum casein, which was due to the release of all types of the caseins. The increase in serum casein, on reducing both pH and temperature, was much more than obtained by adjusting each separately. On lowering the pH at 20°C, there was a moderate increase in the level of serum casein. At 30°C, however, all of the calcium phosphate could be removed from the micelles, without any appreciable increase in serum casein. This indicated that in addition to calcium phosphate, hydrophobic interactions are also important in maintaining micellar structure.

On studying micellar composition and size, it was found that the relative amount of κ-casein in micellar casein was inversely related to micellar size. The increase in κ-casein with decreasing micellar diameter was balanced almost exactly by a decrease in the relative amount of β-casein. The relative amounts of α_{s1}- and α_{s2}-caseins, however, did not vary with micellar size. There was also a slight decrease in micellar P_i with decreasing micellar size, but the level of micellar Ca stayed almost constant.

Variation in the protein composition of bovine casein micelles and serum casein in relation to micellar size and milk temperature

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SUMMARY. The caseinate complex in bovine milk was partitioned by differential centrifugation at both 20 and 4 °C into 4 micellar fractions and a fraction representing serum casein, and the protein composition of the fractions determined. At both temperatures the relative amount of κ-casein in the micellar caseins increased markedly and that of β-casein decreased appreciably with decreasing micelle size. The relative amount of α_{s2}-casein also tended to decrease with decreasing micelle size, but the relative amounts of α_{s1}- and γ-caseins, and an unidentified casein fraction, showed little systematic variation. The serum casein differed appreciably in composition from the micellar caseins, being very rich in β-casein and comparatively poor in α_{s1}- and α_{s2}-caseins, and the amount present at 4 °C was considerably greater than at 20 °C, with the increase being due almost entirely to β-casein, but with γ-casein also making a significant contribution. The changes in the composition and distribution of micellar and serum caseins induced by cooling milk at 4 °C were completely reversible when the milk was re-equilibrated at 20 °C for 18 h.

The caseinate complex in bovine milk exists largely as a polydisperse system of colloidal particles, the caseinate micelles, together with some less aggregated material which is often referred to as serum or soluble casein; in this paper the term serum casein is used. By differential centrifugation, or chromatography in controlled-pore glass or other large-pore media, the complex can be divided somewhat arbitrarily into a number of micelle size classes and a fraction taken to represent serum casein. Analyses of these materials have indicated that the composition of the micelles varies according to their size and that the distribution of the casein between micellar and serum phases is influenced by temperature. For example, a number of studies have shown that the relative amount of κ-casein in the micelles increases with decreasing micellar size (Sullivan *et al.* 1959; Rose *et al.* 1969; McGann *et al.* 1979, 1980), and that the amount of serum casein increases when the temperature of milk is reduced within the range 37–4 °C (Rose, 1968; Downey & Murphy, 1970; Sharma & Randolph, 1974; Ali *et al.* 1980). Other aspects of micellar composition, such as the relationships between micelle size and the relative amounts of caseins other than κ-casein, however, are less clearly defined, and there appears to be comparatively little information on the reversibility of temperature-induced changes in micellar composition and in the distribution of the caseins. In this study the amounts of the different individual caseins in micelles of different size classes and in serum casein, prepared by differential centrifugation, have been determined, and the reversibility on rewarming, of changes resulting from the cooling of milk to about 4 °C, has been examined.

Reagents

Analytical grade materials were used wherever possible and deionized water was used throughout.

Samples

Milk samples were collected from the Institute herd of Ayrshire cows and included herd-bulk milks and milks from healthy individual cows. During collection care was taken that the temperature of the milks did not fall below 20 °C. In the laboratory, the temperature of samples was reduced to 20 °C and skim-milk, which was used for all analyses apart from somatic cell count determinations, was obtained by collecting the material from below the fat layer after whole milk had been centrifuged at 20 °C for 30 min at 1000 g. The skim-milks, with 0·01 g NaN_3/100 ml added as preservative, were stored at either 20 °C for about 15 h, 4 °C for 40 h, or 4 °C for 48 h followed by re-equilibration at 20 °C for 18 h, before being analysed.

Somatic cell counts were determined in whole milks by a direct microscopic method.

Isolation of micellar casein pellets and serum casein

Normally 4 micellar casein pellets (designated pellets 1, 2, 3 and 4 in order of decreasing micelle size) and a sample of serum casein were isolated from each skim-milk at a given temperature by differential centrifugation using an MSE Superspeed 65 ultracentrifuge and a 6 × 38 ml swing-out rotor (MSE Scientific Instruments, Crawley, Sussex, UK). The conditions used at 20 °C to obtain the pellets, and supernatant 4, which was the source of serum casein, are given in Table 1, while at 4 °C centrifuging times were twice the corresponding times at 20 °C. At both temperatures, acceleration and deceleration of the rotor each took about 10 min when 23000 g was used and about 20 min when 70000 g was used. Supernatants 1, 2 and 3 were collected by decanting whereas supernatant 4 was collected with a syringe so as to exclude the poorly consolidated 'fluffy layer' lying immediately above pellet 4. The 'fluffy layer', which is made up largely of membrane materials (Plantz *et al.* 1973; Anderson *et al.* 1974), was included with pellet 4.

The centrifuging technique gave reproducible results at both 20 and 4 °C with differences in the amount of casein sedimented in different tubes from the same centrifugation, and from different centrifugations carried out under the same conditions, normally amounting to less than 2 % of the total casein.

Preparation of whole casein samples

Samples of whole casein were prepared from skim-milks, solutions of casein pellets and supernatant 4 (serum casein) essentially as described by Davies & Law (1977*a*). For skim-milk casein a double acid-precipitation was used and residual fat was removed before the second precipitation by centrifuging the casein solution at 20 °C for 30 min at 70000 g. A double acid-precipitation was used also for serum casein but no fat removal was required. Before precipitation of casein from pellets, they were dissolved by adding 2 ml EDTA solution (2·5 % $EDTA.Na_2$, 2·5 % $EDTA.Na_4$; pH 6·8) per pellet and the solutions were then diluted with water to give a protein concentration similar to that in skim-milk diluted with an equal volume of water. Only a single acid-precipitation was carried out because most of the contaminating

Table 1. *Conditions used for the isolation of micellar casein pellets and serum casein*
at 20 °C

Step	Starting material	Centrifuging conditions*	Products
1	Skim-milk	6 min. 23000 g	Pellet 1 and supernatant 1
2	Supernatant 1	9 min, 70000 g	Pellet 2 and supernatant 2
3	Supernatant 2	30 min. 70000 g	Pellet 3 and supernatant 3
4	Supernatant 3	120 min, 70000 g	Pellet 4 and supernatant 4†

* g Values for $r_{av.}$ 12·3 cm.
† Serum casein prepared from supernatant 4.

milk serum proteins were removed during the centrifuging steps. The final casein solutions were lyophilized and stored at −20 °C.

Analysis of casein samples

Casein samples were alkylated and fractionated by ion-exchange chromatography as described by Davies & Law (1977a), and fractionated by gel chromatography at 4 °C essentially as described by Yaguchi et al. (1968).

The protein contents of chromatographic fractions were determined by a micro-biuret method (Davies & Law, 1977a).

N analyses

The total and non-casein N contents of skim-milks and supernatants were determined by a micro-Kjeldahl method based on that of Rowland (1938), but with the ammonia content of diluted digests being determined colorimetrically, after reaction with an alkaline mixture of salicylate and dichloroisocyanurate in the presence of nitroprusside (Reardon et al. 1966). Values for casein N were obtained by difference and were converted to casein values by multiplying by 6·51.

RESULTS AND DISCUSSION

Choice of conditions for isolating micellar casein pellets and serum casein

The polydisperse system of caseinate polymers which exists in milk can be separated by differential centrifugation into a number of fractions, with the separation being influenced by both the molecular weight and the hydrodynamic radius of the particles. The fractions so obtained are not monodisperse, but separation into a series of pellets, apparently comprising casein micelles with decreasing weight-average molecular weights (Dalgleish et al. 1981), and a residual fraction representing serum casein, can be achieved. In the present study the average sizes of micelles in the different casein pellets prepared were not determined directly because of the uncertainty that the pelleted material, on redispersion in milk serum, would yield particles with the same size distribution as before sedimentation. However, the root-mean-square Z-average radius of micelles in skim-milks, and in supernatants 1, 2 and 3 obtained by centrifugation (Table 1), were determined from the wavelength dependence of the turbidity (Holt et al. 1975) and gave values for herd-bulk samples of around 200, 150, 120 and < 90 nm respectively, indicating that a fractionation dependent at least partly on particle radius had been achieved. The average radius of particles in supernatant 4, the source of serum casein, was too small to be determined by this method.

A number of different combinations of time and centrifugal force have been used for partitioning the caseinate system in milk, but with little comment as to why a particular set of conditions was selected. In this study the aim was to produce a partition into the following: (1) a relatively small fraction representing the largest micelles, (2) 3 further micellar fractions representing micelles of progressively decreasing size and each accounting for about the same amount of the remaining micellar casein and (3), a clear supernatant representing serum casein. While this partition was not achieved for all milks, the conditions chosen for separation at 20 °C (Table 1) did produce, for 10 milks, fractionations in which pellet 1 accounted for 10–20 % of the total casein, pellets 2 and 3 each accounted for about 25–35 %, pellet 4 accounted for 15–25 %, and supernatant 4, which was invariably a clear liquid, free of turbidity, accounted for 3–12 % of the total casein. Moreover, a doubling of the time used for the final centrifuging step resulted normally in only a little more of the total casein being sedimented at that stage, thus indicating that all of the serum casein represented material which was relatively small.

At 4 °C, centrifuging times were twice those used at 20 °C, to compensate for the approximate doubling in the viscosity which occurs when skim-milk is cooled from 20 °C (Whitaker et al. 1927). From values for the root-mean-square Z-average radii of the micelles in the various supernatants, the fractionations achieved on the basis of particle radius appeared to be similar to those obtained at 20 °C. The amounts of casein in the various fractions, however, were somewhat different with pellet 1 accounting for 5–15 % of the total, pellet 2 15–25 %, pellet 3 15–30 %, pellet 4 15–35 % and serum casein 15–25 %, indicating that there had been a considerable increase in serum casein and some decrease in amounts in pellets 1 and 2. These changes probably arose from a combination of factors including some dissociation of micellar casein, with a concomitant loss of micelle mass, and some increase in the number of the smallest micelles, at 4 °C. The increase in serum casein with the reduction in temperature is in agreement with that already reported (Rose, 1968; Downey & Murphy, 1970) and in this study, values obtained after storage for 72 h were usually slightly higher than those after 40 h, and there was little evidence of the reduction in the amount of serum casein found by Ali et al. (1980), after the longer storage period.

Protein composition of micellar and serum caseins isolated at 20 °C

Micellar and serum caseins were isolated from 3 herd-bulk and 2 individual-cow milks after storage at 20 °C for about 15 h. The results for one of the herd-bulk samples, which generally speaking were representative of all 5 samples, are given in Table 2. The amounts of the 4 micellar caseins (pellets 1–4) and of the serum casein are expressed as percentages of the total casein in the skim-milk and their percentage protein compositions, together with that of the whole casein in the skim-milk, are given in terms of the individual caseins. The values for α_{s1}-, β-, α_{s2}- and γ-caseins were obtained after fractionation by ion-exchange chromatography, those for κ-casein after fractionation by gel chromatography and those for the unidentified fraction, which may represent partly dephosphorylated β-casein, as the difference between κ-casein values obtained by ion-exchange chromatography and gel chromatography. κ-Casein values obtained by gel chromatography are more accurate than those from ion-exchange chromatography (Davies & Law, 1977 a, b), especially for serum casein samples, and agree well with values calculated from amounts of para-κ-casein obtained from ion-exchange chromatography of chymosin-treated samples.

The values for the percentage composition of the micellar caseins show that the major changes associated with changing micelle size were a marked increase in the

Table 2. *Distribution and percentage protein composition of micellar and serum caseins isolated from a sample of herd-bulk milk at 20 °C after storage of the milk at 20 °C for 15 h, together with the composition of the whole casein in the stored skim-milk, and the composition of the total micellar casein calculated from the compositions of the 4 micellar pellets*

Fraction	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Serum casein	Skim-milk casein	Total micellar casein
Total casein in skim-milk. %	11·6	28·0	26·8	23·8	9·8	—	—
				Composition. %			
α_{s1}-Casein	39·5	39·3	39·7	38·5	24·2	38·3	39·2
β-Casein	35·5	34·3	31·8	29·6	42·8	33·6	32·5
κ-Casein	6·6	8·8	10·5	16·3	17·7	11·2	11·0
α_{s2}-Casein	11·4	11·0	10·3	9·3	2·5	10·2	10·4
γ-Casein	4·5	4·8	5·0	5·2	6·0	4·6	4·9
Unidentified	2·5	1·8	2·7	1·1	6·8	2·1	2·0

relative amount of κ-casein and a decrease in the relative amount of β-casein with decreasing size; these changes were also found in the other 4 samples analysed. Table 2 shows also that the relative amount of α_{s2}-casein tended to decrease with decreasing micelle size, and while this trend did not occur in all samples, generally speaking the smallest micelles were poorer in α_{s2}-casein than the largest. The relative amounts of α_{s1}-, γ- and unidentified caseins showed little or no systematic variation with changing micelle size. A marked increase in κ-casein values with decreasing micelle size has already been widely reported (Sullivan *et al.* 1959; Rose *et al.* 1969; McGann *et al.* 1980; Dalgleish *et al.* 1981) and in this study the κ-caseins in the various pellets yielded, qualitatively, very similar gel electrophoretic patterns, which is somewhat different from Slattery's (1978) findings. The decrease in the relative amount of β-casein with decreasing micelle size is in agreement with results by Rose *et al.* (1969) and Saito (1973) after fractionation by differential centrifuging but is the opposite of what was found by Morr *et al.* (1971) for fractions from rate-zone ultracentrifuging in a sucrose gradient and by McGann *et al.* (1979) for fractions from chromatography in controlled-pore glass. The reason for this difference is not fully apparent. In the centrifuging procedure used in this study all of the casein pellets apart from pellet 1 were subjected to a maximum pressure of approximately 75 MPa, which has been found to be sufficient to cause a reversible dissociation of β-casein polymers (Payens & Heremans, 1969; Schmidt & Payens, 1972) and to disrupt large casein micelles (Walstra, 1980), and similar pressure-induced changes may therefore be responsible for the present pattern in the relative amounts of β-casein. On the other hand, the pattern of β-casein values found in fractions after chromatography in certain very porous media could arise because of some dissociation of β-casein as reported by Downey (1973) and Creamer *et al.* (1977), but no appreciable dissociation is considered to occur during rapid fractionations carried out in controlled-pore glass at 20 °C in the presence of synthetic milk serum (McGann *et al.* 1979). This discrepancy in the β-casein values from the 2 systems of analysis is being further investigated. The relative constancy in the proportion of α_{s1}-casein may be significant in the light of the suggestion of Lin *et al.* (1972) that casein micelles are composed of a size-determining framework which is predominantly α_s-caseins and colloidal phosphate.

In this study the percentage composition of total micellar casein was not determined directly, but when calculated from the compositions of the individual pellets it was found to be very similar to that of the total casein in skim-milk

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Table 3. *Distribution and percentage protein composition of micellar and serum caseins isolated from a sample of herd-bulk milk at 4 °C after storage of the milk at 4 °C for 40 h. together with the composition of the whole casein in the stored skim-milk and the composition of the total micellar casein calculated from the compositions of the 4 micellar pellets*

(The milk sample is the same as for Table 2.)

Fraction	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Serum casein	Skim-milk casein	Total micellar casein
Total casein in skim-milk, %	7·0	16·8	18·5	34·5	23·2	—	—
				Composition, %			
α_{s1}-Casein	42·6	43·1	43·8	42·0	10·8	38·3	42·7
β-Casein	32·4	29·8	28·0	26·6	68·9	35·8	28·1
κ-Casein	8·4	9·1	11·1	15·8	7·4	10·6	12·6
α_{s2}-Casein	13·3	13·3	12·4	11·8	2·0	10·2	12·4
γ-Casein	3·3	3·2	3·4	3·4	6·8	3·6	3·4
Unidentified	0·0	1·5	1·3	0·4	4·1	1·5	0·8

(Table 2). This calculation should yield an accurate value since the composition of the skim-milk casein, when calculated from the compositions of the micellar and serum caseins, was in very good agreement with that obtained by direct analysis. The composition of the serum casein, on the other hand, was very different from that of the skim-milk casein (Table 2), being much poorer in α_{s1}- and α_{s2}-caseins and richer in the other caseins, and it was also different from the compositions of the 4 micellar caseins. These differences were found also in the other 4 milks.

Protein composition of micellar and serum caseins isolated at 4 °C

Since milk producers in Scotland are expected to cool milk destined for sale to about 4 °C, the distribution and compositions of micellar and serum caseins isolated at 4 °C were determined. Table 3 shows values obtained for a sample of herd-bulk milk after storage at 4 °C for 40 h; similar results were obtained for a second herd-bulk milk and for a sample from an individual cow. The results in Tables 2 and 3 are for the same milk and they show in relation to the distribution of the fractions some of the changes referred to earlier, with reductions at 4 °C in the percentages of the total casein appearing in pellets 1, 2 and 3 and increases in the amounts in pellet 4 and serum casein. Despite these differences the changes in micellar composition with decreasing micelle size were the same as at 20 °C, with the marked increase in the relative amount of κ-casein and the decrease in β-casein again clearly discernible (Table 3). A comparison of the compositions of the individual pellets obtained at 4 °C with those of corresponding ones isolated at 20 °C showed, however, that the former were richer in α_{s1}- and α_{s2}-caseins and, in some instances κ-casein, and poorer in β-, γ- and unidentified caseins. The reduced amounts of β-casein were a consequence of its appreciable dissociation from the micelle on the reduction in temperature (Rose, 1968; Downey & Murphy, 1970), probably as a result of the weakening of hydrophobic bonds (Scheraga *et al.* 1962; Némethy & Scheraga, 1962), and the same may be true for the γ- and unidentified caseins, but the reduced levels of these caseins may also be due partly to less degradation of the β-casein during storage at 4° than at 20 °C. The increase in the relative amounts of α_s- and κ-caseins were probably merely a reflection, arithmetically, of the reduced β-casein values.

The composition of total micellar casein, calculated from results for individual

Table 4. *Amounts of whole and individual caseins in the serum phase of a herd-bulk milk at 20 and 4 °C, together with the increase at 4 °C*

(Values are expressed in g/l skim-milk and as percentages of the corresponding total amount of the casein in the milk, and for the increases, as percentages of the total increase.)

	20 °C		4 °C		Increase at 4 °C	
	g/l	Total, %	g/l	Total, %	g/l	Increase, %
α_{s1}-Casein	0·72	6·2	0·78	6·5	0·06	1·4
β-Casein	1·28	12·5	4·97	44·7	3·69	87·4
κ-Casein	0·53	15·5	0·53	16·1	0·00	0·0
α_{s2}-Casein	0·08	2·6	0·14	4·4	0·06	1·4
γ-Casein	0·18	12·9	0·49	43·8	0·31	7·4
Unidentified	0·20	31·2	0·30	63·8	0·10	2·4
Whole casein	2·99	9·8	7·21	23·2	4·22	—

pellets, differed considerably from that for skim-milk casein, showing a lower β-casein value and higher values for α_{s1}-, κ- and α_{s2}-caseins (Table 3). These differences were largely absent from the corresponding comparison at 20 °C.

The serum casein isolated at 4 °C was much richer in β-, γ- and unidentified caseins and much poorer in α_{s1}- and α_{s2}-caseins, and poorer in κ-casein, than the corresponding skim-milk and 4 micellar caseins (Table 3). The differences, apart from that for κ-casein, were of the same kind as found at 20 °C but generally speaking were more marked. Further comparison of the serum caseins obtained at 4 and 20 °C is made in Table 4 where values for the individual caseins in the serum phase are expressed in g/l skim-milk and as percentages of the corresponding total in the milk. Table 4 presents also the increases in amounts at 4 °C in g/l skim-milk and as percentages of the total increase. The values show that the concentration and the percentage of the total of β- and γ-caseins were much greater, and those of the unidentified casein considerably greater at 4 °C, whereas the results for α_{s1}-, κ- and α_{s2}-caseins showed little variation with temperature. The increase in total serum casein at 4 °C was therefore due very largely to β-casein, but with γ-casein also making a significant contribution. Similar results were obtained for serum caseins prepared from a further sample of herd-bulk milk and from the milk of an individual cow, when the micellar casein was removed by single-step centrifugations at 70000 *g* for 4 h and 2 h at 4 and 20 °C respectively. In these 2 samples, β-casein accounted for 87·6 and 93·0 % respectively of the increase at 4 °C. The large contribution made by β-casein to the increase in serum casein at low temperatures has also been found by others (Rose, 1968; Downey & Murphy, 1970) but the present results differ from those obtained previously with respect to the α_s- and κ-caseins since in this study these caseins made virtually no contribution to the increase at low temperature whereas in the earlier work each accounted for 20–30 % of the increase. The reason for the difference probably lies in the different conditions used to isolate the serum casein, with the absence in the earlier studies of any compensation for changes in the viscosity of milk with temperature possibly being the significant factor.

Reversibility of changes induced by storing milk at 4 °C

Because storage at 4 °C led to considerable changes in the caseinate complex (Tables 2 and 3) and since cold-storage of milk followed by some form of heat treatment is common practice in the dairy industry, a study was carried out to determine whether the changes induced at 4 °C were reversible. This involved storing milk at 4 °C for 48 h and then transferring it to an incubator, which was at 20 °C,

Table 5. *Distribution and percentage protein composition of micellar and serum caseins isolated from a sample of herd-bulk milk at 20 °C after storage of the milk at 4 °C for 48 h and then at 20 °C for 18 h, together with the composition of the whole casein in the stored skim-milk, and the composition of the total micellar casein calculated from the composition of the 4 micellar pellets*

(The milk sample is the same as in Tables 2 and 3.)

Fraction	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Serum casein	Skim-milk casein	Total micellar casein
Total casein in skim-milk, %	11·1	24·9	28·2	25·6	10·2	—	—
					Composition, %		
α_{s1}-Casein	39·3	39·4	40·1	38·0	24·7	38·4	39·2
β-Casein	34·8	33·7	31·9	29·2	40·6	33·1	32·0
κ-Casein	6·5	8·5	10·5	15·7	17·9	11·2	11·0
α_{s2}-Casein	11·4	11·1	10·3	9·2	3·4	10·3	10·3
γ-Casein	5·1	5·1	5·0	5·6	6·7	4·7	5·2
Unidentified	2·9	2·2	2·2	2·3	6·7	2·3	2·3

where the milk was allowed to stand for 18 h, before the micellar and serum caseins were isolated at 20 °C. The very mild re-equilibration conditions avoided the use of relatively high temperatures which could lead to the formation of artifacts, and a long time was used to accommodate the relatively slow rates of some of the reactions involved in re-establishing the equilibria which determine micellar composition (Pyne, 1945). The results from such a re-equilibration experiment for the herd-bulk milk which also provided the results in Tables 2 and 3 are shown in Table 5. The composition of the casein isolated from the skim-milk after the re-equilibration (Table 5) was the same as that from the control milk after storage at 20 °C for 15 h (Table 2), thus indicating that no further change, through proteolysis for example, had occurred despite the considerable storage time involved. The results show also that the changes induced in the distribution of casein between micellar fractions and serum casein by storage at 4 °C (Table 3) had been reversed to yield a pattern very similar to that in the control milk. A similar re-establishing of serum casein levels was found by Downey & Murphy (1970) on warming at 30 °C milk which had been stored at 5 °C, but they did not report on how the casein was redistributed in the micelles. Finally, the results in Tables 2 and 5 show, most significantly, that the protein compositions of the various fractions obtained from the re-equilibrated milk were, within the limits of experimental error, the same as for the corresponding fractions from the control milk after storage at 20 °C for 15 h. This reversibility of the temperature-induced changes in the protein composition is analogous to that found by Davies & White (1960) for the other important micellar constituents, Ca and phosphate. The reversibility is in general agreement with results obtained by Creamer et al. (1977) for the exchange of β-casein between serum and micellar phases, and by Ali et al. (1980) for levels of α_{s1}-, β- and κ-caseins in serum casein. However, the completeness of the reversibility achieved in this study was not found in their work, possibly because considerably shorter re-equilibration times, more akin to those employed in commercial practices, were used. Because of the difference, a further study of the effects of procedures based on those used in commercial practices would be worthwhile.

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Effects of colloidal calcium phosphate content and free calcium ion concentration in the milk serum on the dissociation of bovine casein micelles

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SUMMARY. The strength of binding of the individual caseins and the nature of the bonding within bovine casein micelles were examined through dissociation of the micelles by dialysis of skim milk either against phosphate-free buffers containing 3 or 6 mM-CaCl$_2$, or against buffers that were nearly saturated with respect to micellar calcium phosphate, but which had a free Ca^{2+} concentration in the range 0·4–5·9 mM. Dissociation was followed by ultracentrifuging the dialysed milks and determining the partition of the total and the individual caseins between the pellet and serum. During dialysis against the phosphate-free buffers both colloidal Ca and P$_i$ in the milks decreased and about 30 % of the P$_i$ could be removed without significant casein dissociation. With further loss of P$_i$, however, increasing dissociation occurred and the proportions of the individual caseins retained in the casein pellet were in the order α_{s2}- > α_{s1}- > β- $\approx$ κ-casein. Dialysis against the calcium phosphate buffers resulted in no loss of colloidal P$_i$ but colloidal Ca increased with the free Ca^{2+} concentration of the buffer. Little change in the casein partition occurred in the presence of more than 1 mM free Ca^{2+}, but serum casein increased markedly at lower levels, and the strength of binding of the individual caseins in the pelleted casein was in the order α_{s2}- > α_{s1}- > β- > κ-casein. In both types of buffer, dissociation is considered to occur through the breaking of linkages between the caseins and inorganic constituents. Analysis of the amino acids in a calcium phosphate-rich material obtained after exhaustive proteolytic digestion of casein micelles suggests that these linkages involve the phosphate centres of the caseins.

Approximately 80 % of the protein in bovine milk consists of a mixture of four phosphoproteins, the α_{s1}-, α_{s2}-, β- and κ-caseins, mostly in the form of a polydisperse distribution of colloidal particles, the casein micelles. The caseins account for ~ 92 % of the dry weight of micelles, with Ca and P$_i$ forming a further 7 %, and the remainder being made up from small amounts of Mg and citrate and traces of some other constituents (Schmidt, 1980; McGann *et al.* 1983). It is believed that the micellar Ca exists partly as a calcium phosphate salt and partly as Ca^{2+} bound only to the protein, the so-called caseinate Ca. However, in this paper, the terms micellar calcium phosphate (MCP) and bound Ca^{2+} are used to describe the fractions of micellar Ca that are independent of, and dependent on, the free Ca^{2+} concentration in the serum respectively, but without specifying the nature of the ion binding sites.

Micellar Ca is known to be essential for maintaining micelle structure and stability, as demonstrated by studies of micelle dissociation in acidified milk at low

temperature (McGann & Pyne, 1960), or when Ca is complexed by addition of a chelating agent (Noble & Waugh, 1965; Rose, 1968; Lin *et al.* 1972). Such work, however, involved an initial drastic alteration of the ionic environment of the micelles and, although demonstrating the essential requirement for Ca, did not distinguish between MCP and bound Ca^{2+} as factors in maintaining micelle structure. Furthermore, little is known about the behaviour of the individual caseins when the micelles dissociate, even though this behaviour could provide additional information about micelle structure.

In this study, an attempt was made to alter the Ca content of micelles in two different ways while the pH, ionic strength and osmolarity of the serum were maintained constant. In one procedure, micellar Ca but not P_i was changed by dialysis of milk against buffers that were nearly saturated with respect to calcium phosphate, but with free Ca^{2+} concentrations in the range 0·4–5·9 mM. In the other procedure, micellar Ca and P_i were reduced by dialysis of milk against phosphate-free buffers containing either 3 or 6 mM-$CaCl_2$, to maintain free Ca^{2+} concentration at or above the level in the starting milk. After dialysis, the casein was partitioned into a pellet and a serum fraction by ultracentrifugation, and the extent of casein dissociation and the strength of binding of the individual caseins in the pellet, and hence in the native casein micelles, related to changes in the partition of Ca and P_i. Also, evidence of the nature of a possible linkage between colloidal calcium phosphate (CCP) and the caseins was sought by analysis of a calcium phosphate-rich material that remained after exhaustive proteolytic digestion of the casein micelles.

MATERIALS AND METHODS

Milk samples

Samples were obtained from the pooled milk of the Institute herd of cows, care being taken that the milk temperature did not fall below 20 °C during collection. In the laboratory, milk temperature was reduced to 20 °C and the fat separated by centrifugation at 1000 *g* for 30 min. Skimmed milk, which was used in all experiments, was collected by suction, and 0·1 g NaN_3 and 0·1 g trypsin inhibitor (Type 1-S, Sigma London Chemical Co., Poole, Dorset, UK) added per litre to control bacterial growth and the activity of native milk proteinases.

Buffer solutions

Two types of buffer were used in dialysis, namely calcium phosphate buffers and phosphate-free buffers, the compositions of which are given in Tables 1 and 2 respectively. All buffers had a pH of 6·70, an osmolarity of ∼ 300 mM and an ionic strength of ∼ 80 mM, which are characteristic of milk of average composition.

The composition of each of the calcium phosphate buffers was calculated with the aid of a computer program used to model the ion equilibria in milk ultrafiltrate (Holt *et al.* 1981). The buffers had free Ca^{2+} concentrations in the range 0·4–5·9 mM and a free Mg^{2+} concentration of 0·9 mM. To avoid dissolving MCP during dialysis, buffers contained sufficient Ca and P_i to bring the ion activity product for MCP to over 70 % of its average value in milk ultrafiltrate (Holt, 1982). Special care was required in the preparation of the buffers to achieve the desired pH and to avoid precipitation of calcium phosphate. A phosphopeptide derived from β-casein (Manson & Annan, 1971) was found to inhibit the formation of calcium phosphate precipitates during preparation of the buffers. Occasionally, buffers were slightly opalescent immediately after preparation and, in the absence of the phosphopeptide, the opalescence

Table 1. *Composition of calcium phosphate buffers, pH 6·70**

Buffer	A	B	C	D	E	F	G	H
					mM			
CaCl$_2$	1·1	3·0	7·0	8·0	11·5	4·5	14·0	16·0
Mg acetate	3·0	4·5	5·0	4·5	3·0	1·4	2·5	2·2
NaCl	0	0	11·0	20·0	29·0	57·0	32·0	32·6
Na$_3$ citrate	0	4·0	9·0	9·0	9·0	0	9·0	9·0
NaH$_2$PO$_4$	37·7	26·7	12·5	10·3	4·0	3·3	1·7	1·1
K$_2$HPO$_4$	31·3	23·3	12·5	9·7	4·5	4·7	3·8	3·4
Calculated values								
[Ca^{2+}]	0·4	0·6	1·0	1·4	3·1	3·1	4·6	5·9
[Mg^{2+}]				0·9				
Ionic strength	90	83	80	83	78	79	84	88
% Saturation†	71	75	74	82	84	81	81	83

* The buffers also contained lactose (155 mM), NaN$_3$ (1·5 mM), and a barium phosphopeptide (0·02 %).

† Calculated ion activity product for micellar calcium phosphate as a percentage of the average value for milk ultrafiltrate.

Table 2. *Composition of phosphate-free buffers, pH 6·70*

Buffer	PF3	PF6
	mM	
CaCl$_2$	3·0	6·0
NaCl	60·0	55·0
Imidazole	20·0	20·0
Lactose	155	155
NaN$_3$	1·5	1·5

increased to form a precipitate within 24 h, but in its presence the opalescence remained constant, suggesting that the peptide stabilized the calcium phosphate nuclei.

Preparation of buffers

The exact volumes of 1 M solutions of CaCl$_2$, Mg acetate, Na$_3$ citrate, NaCl, NaH$_2$PO$_4$ and K$_2$HPO$_4$ required to prepare a given volume of buffer (Table 1) were calculated. From the sum of these volumes, the volume of an aqueous solution containing the required weights of lactose, NaN$_3$ and phosphopeptide was calculated. To this latter solution the volumes of CaCl$_2$, Mg acetate, Na$_3$ citrate and NaCl were added, followed by slow additions of portions of the two phosphate solutions, under rapid stirring and with the pH being continuously monitored, so that the final pH was attained with the final addition of the more basic K$_2$HPO$_4$ solution.

For the two phosphate-free buffers (Table 2), adjustment to pH 6·7 was with 5 M-HCl.

Dialysis procedures

For dialysis against calcium phosphate buffers, milk samples were placed in sacs (washed Visking tubing, 14 mm diam.), the sacs weighed and placed in separate flasks containing 3 times the volume of a calcium phosphate buffer. The flasks were then continuously but gently agitated in a water bath at 20 °C for 2–2·5 d. Buffers were changed after ~ 7 or 16 h, and then twice more at later stages, so that by the end of the dialysis procedure the diffusible ions in the dialysed milk samples were close to those in the dialysis buffer. For the buffers listed in Table 1, average differences

in concentrations of Ca and P_i between buffers and the corresponding ultrafiltrates prepared from the dialysed milks were 0·13 and 0·52 mM respectively, confirming that replacement of the original diffusible ions had occurred. At the end of dialysis. sacs were reweighed and. generally, a change of only 2–3 % on starting weights was found. indicating that the osmotic flow of water had been kept small.

For dialysis against the phosphate-free buffers (Table 2), milk samples were placed in a series of sacs (washed Visking tubing, 25 mm diam.), the sacs weighed and placed in about 10 l buffer maintained at 20 °C. The buffer contained 1–2 g/l of a weak anion exchange resin (BioRad P12 or Amberlite IRA 167) to complex phosphate ions and the ratio of buffer to milk was never < 8:1 and was usually > 10:1. The buffer was stirred continuously and buffer changes made after 16 and 40 h. and again after longer dialysis times. Sacs were removed at intervals during the course of dialysis, which in one experiment extended over 160 h, and reweighed. Generally there was a gradual increase in weight with increasing dialysis time, amounting to nearly 10 % of the initial weight at the longest dialysis time, but usually it was no more than 4–6 %. This slow increase in weight was probably due to an osmotic flow of water arising from small concentration gradients of salts caused by the gradual solution of CCP. Concentrations of non-diffusable components were corrected for dilution during dialysis, as appropriate.

The partition and determination of Ca and P_i

Total Ca and P_i in the original and dialysed milks were determined on filtrates prepared after precipitating milk protein with 15 % trichloroacetic acid. Diffusable Ca and P_i were determined on ultrafiltrates prepared using a membrane of 10000 mol. wt cut-off under a pressure of 80 kPa. All ultrafiltrates were prepared immediately after removing milks from the dialysis sacs. The P_i was measured by a molybdenum blue method (Allen, 1940) and Ca by atomic absorption spectroscopy, after quantitative precipitation as the oxalate (Davies & White, 1962) to avoid matrix effects (Holt *et al.* 1981).

Colloidal Ca and P_i concentrations were calculated as the difference between total and diffusable values and are expressed as mol/mol casein, taking the mol. wt of casein as 23300.

Nitrogen analysis

Total and non-casein N contents of starting and dialysed milks, and of ultracentrifuge serum were determined by a micro-Kjeldahl method based on that of Rowland (1938), but with the ammonia content of diluted digests being determined colorimetrically (Reardon *et al.* 1966). Casein N values were obtained by difference and converted to casein concentrations with the factor 6·37.

Isolation of pellet and serum caseins and preparation and analysis of whole casein samples

The casein in starting and dialysed milks was partitioned into a pellet and a serum fraction by centrifuging at 70000 *g* for 2 h at 20 °C in the 6 × 38 ml rotor of a Superspeed 65 ultracentrifuge (MSE Scientific Instruments, Crawley, W. Sussex, UK). This method produced firm pellets and clear or slightly opalescent sera. Results for five milks dialysed against phosphate-free buffer and four milks dialysed against calcium phosphate buffers showed that the concentration of P_i in the serum obtained by centrifuging exceeded that in the corresponding ultrafiltrate by, on average, only

0·3 mM, thus indicating that there was little CCP, and hence intact, native casein micelles, in the supernatant.

Whole casein samples were prepared from starting milks, sedimented pellets and centrifuged sera by acid precipitation at pH 4·6 (Davies & Law, 1983), but the initial solution of pellets was achieved by adding 5 ml per pellet of the EDTA solution. The relative amounts of α_{s1}-, α_{s2}-, β- and γ-caseins in samples were determined after alkylation and fractionation by ion-exchange chromatography (Davies & Law, 1977), and that of κ-casein by gel-permeation chromatography at 4 °C essentially as described by Yaguchi *et al.* (1968). The protein contents of the chromatographic fractions were determined by a micro-biuret method (Davies & Law, 1977).

The partition of whole casein and of the individual caseins is expressed as the percentage of the whole or individual casein that was pelleted by the standard centrifugation procedure. These values provide a measure of the relative strength of binding of the individual caseins in the pellet, since allowance is thereby made for the different amounts originally present in milk. Results presented here were obtained by analysis of pelleted casein, but in principle the partition can be obtained also by analysis of serum casein. A number of milk samples were analysed by both procedures and it was confirmed that very similar values were obtained.

Preparation of calcium phosphate-rich material from casein micelles

The procedure used was adapted from that of Rose & Colvin (1966). Casein micelles from ∼ 450 ml milk were sedimented by centrifuging at 70000 *g* for 1·5 h at 20 °C and the pellet resuspended in about 60 ml milk ultrafiltrate. The suspension was placed in a dialysis sac (washed Visking tubing, 25 mm diam.) and 2 ml milk ultrafiltrate, containing 60 mg each of porcine fibrinolysin, protease type XIV from *Streptomyces griseus* and potato acid phosphatase, and 200 μl papain type IV (all from Sigma London Chemical Co.), were added. The sac was sealed, its contents mixed and the sac placed immediately in about 5 l milk containing 0·01 % NaN$_3$ and dialysis carried out, with stirring, for 24 h at room temperature. The sac was then removed, the addition of enzymes repeated and dialysis resumed against a further 5 l milk for 24 h. At the end of dialysis, the solution in the sac was opalescent and contained a few pieces of clotted protein. The clots were removed by low-speed centrifuging and the MCP sedimented by centrifuging at 85000 *g* for about 16 h at 20 °C. The sedimented material was frozen and lyophilized; the yield was typically ∼ 740 mg.

RESULTS

Dialysis of milk against calcium phosphate buffers

Equilibrium dialysis against the calcium phosphate buffers should allow the free Ca^{2+} concentration in milk to be changed without altering the content of colloidal P_i to any significant extent. Results from such dialysis with eight buffers and three different starting milks (Fig. 1) showed that colloidal P_i remained essentially constant, and independent of free Ca^{2+} concentration in the buffer. The mean value for all samples of 8·7 mol P_i/mol casein is in very good agreement with the 8·6 mol P_i/mol casein which may be calculated from previous results for pooled milk from the Institute herd (White & Davies, 1958) and indicates that very little, if any, of the colloidal P_i dissolved during dialysis. The colloidal Ca, on the other hand, increased with free Ca^{2+} concentration in the manner of a binding isotherm (Fig. 1).
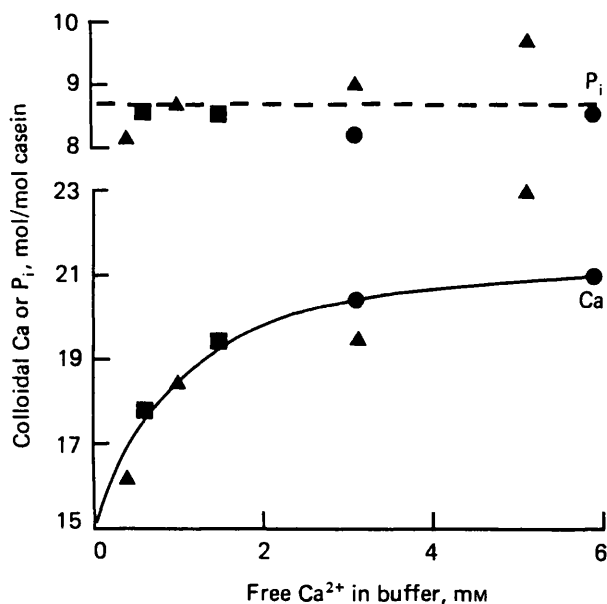
Fig. 1. Partition of Ca and P_i after dialysis of skim milk against calcium phosphate buffers having different free Ca^{2+} concentrations. ●, ■, ▲, Three different milks used in three separate experiments; - - - -, mean value for colloidal P_i in all the dialysed milks.
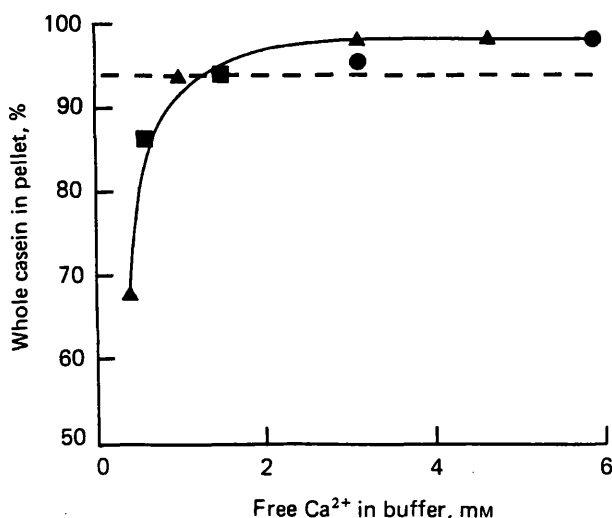
Fig. 2. Partition of whole casein after dialysis of skim milk against calcium phosphate buffers having different free Ca^{2+} concentrations. - - -, Mean partition of whole casein in the undialysed starting milks; ●, ■, ▲, as in Fig. 1.

Extrapolation of the points to zero free Ca^{2+} concentration by means of a Scatchard plot (Tanford, 1961; results not shown) gave an intercept of about 15 mol Ca/mol casein. At a free Ca^{2+} concentration of 2 mM, which is typical of milk of average composition (Holt *et al.* 1981), the colloidal Ca is about 20 mol/mol casein, indicating that there are normally about 5 mol bound Ca^{2+}/mol casein. Bound Ca^{2+} is defined here as the fraction of the micellar Ca which depends on the free Ca^{2+} concentration in the milk serum.

The effect of dialysis against the calcium phosphate buffers on the partition of whole casein is shown in Fig. 2 and the effects on the individual caseins are presented
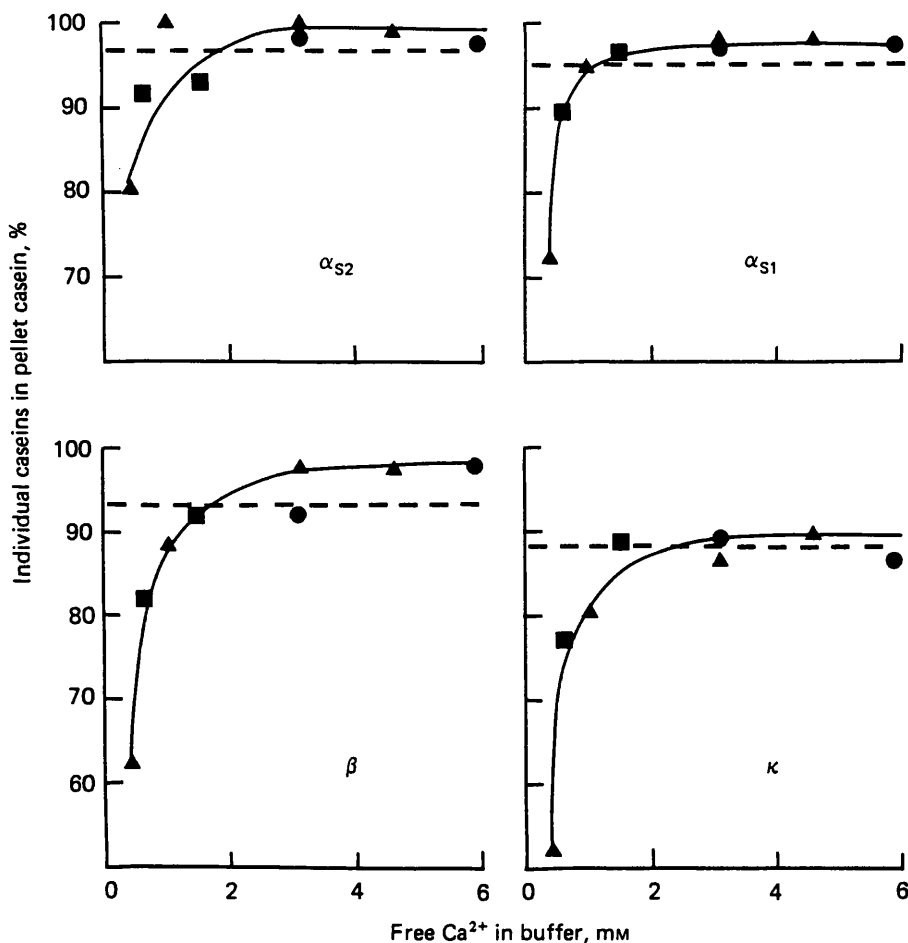
Fig. 3. Partition of individual caseins after dialysis of skim milk against calcium phosphate buffers having different free Ca^{2+} concentrations. — — —, Mean partition of the individual caseins in the undialysed starting milks; ●, ■, ▲, as in Fig. 1.

in Fig. 3. Significant casein dissociation occurred only when free Ca^{2+} concentration was < 1 mM. In the presence of 1·0–1·5 mM free Ca^{2+}, casein partition was little different from that of the undialysed starting milks, whereas at concentrations of 3 mM and above some of the serum casein in the starting milk was converted to a more aggregated form, possibly by incorporation in the micelles. The patterns for the individual caseins (Fig. 3) show that when significant dissociation occurred the percentages pelleted were in the order α_{s2}- > α_{s1}- > β- > κ-casein. After dialysis against buffers containing more than 2 mM free Ca^{2+} some increase occurred in the percentages pelleted of all except κ-casein. The latter appeared to be nearly independent of free Ca^{2+} concentration above 1 mM, whereas at free Ca^{2+} levels above 3 mM the other caseins were nearly completely sedimented.

Dialysis of milk against phosphate-free buffer containing 3 mM-$CaCl_2$

Results for three different milks, involving 25 samples and dialysis for up to 110 h, showed that the concentrations of Ca and P_i in the milks decreased with increasing dialysis time. The decrease was most rapid in the first 20 h, especially for P_i, largely because of the loss of diffusible components. Diffusible Ca fell from about 10 to 5 mM in 20 h; after 40 h the value was about 4 mM and this decreased slowly to a little above the 3 mM of the buffer. Diffusible P_i decreased from about 12 to 2 mM
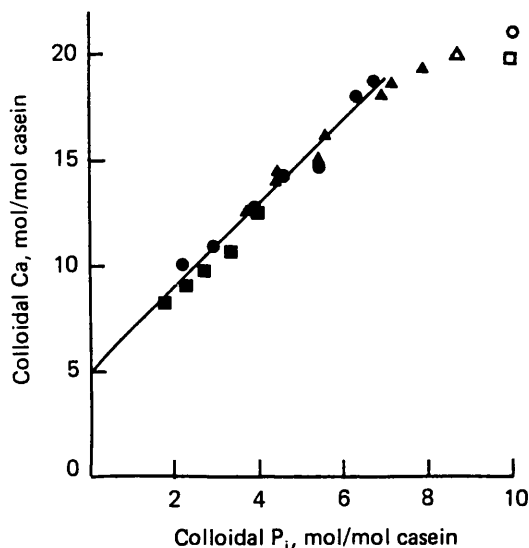
Fig. 4. Relationship between colloidal Ca and P_i in skim milks dialysed against phosphate-free buffer containing 3 mM-$CaCl_2$. ●, ■, ▲, Three different milks used in three separate experiments; ○, □, △ represent the same milks undialysed.

in 20 h, followed by a very gradual decrease to ~ 0·5 mM. Changes in colloidal Ca were closely correlated with those in colloidal P_i, and after dialysis for more than ~ 24 h the relationship was linear (Fig. 4), with a slope of 1·98±0·01. This slope is somewhat larger than estimates of the Ca/P_i ratio in CCP (McGann et al. 1983; Holt, 1982; Chaplin, 1984), possibly because bound Ca^{2+} were released simultaneously at this stage of dialysis.

During the first 24 h of dialysis the decrease in pelleted casein was small, and in one milk a small increase was observed, possibly because the increase in free Ca^{2+} concentration caused a small amount of serum casein in the starting milk to bind to the micelles. After the first 24 h, pelleted casein decreased gradually and then more rapidly until, for two of the milks, the level fell to ~ 50 % of that in the starting milk at the end of the dialysis period and for the third milk to ~ 80 %. Although there were these differences between milks in the reduction in the amount of pelleted casein, for all samples the reduction was very closely related to the reduction in colloidal P_i (Fig. 5). A similar relationship was obtained between colloidal Ca and pelleted casein, but the scatter of points was greater, possibly because of differences in the capacity of different milks to bind Ca^{2+}. The close relationship in Fig. 5 for all three milks provides good evidence that the loss of colloidal P_i allows the casein to dissociate even in the presence of a free Ca^{2+} concentration comparable to or greater than that in milk (Holt et al. 1981; Geerts et al. 1983). Initially, a decrease in colloidal P_i had little effect on the casein partition. Further decrease, however, led to increasing dissociation, so that only about 45 % of the casein was pelleted when the colloidal P_i had been reduced to a little less than 20 % of its original value.

Likewise, there was a clear relationship between the partition of the individual caseins and the amount of colloidal P_i (Fig. 6). This was especially true for α_{s1}-, β- and κ-caseins, but for α_{s2}-casein the degree of dissociation was more limited and the scatter of points greater. Fig. 6 shows also that there were differences in the partition of the different caseins. A reduction in colloidal P_i from 10 to 7·5 mol P_i/mol casein had little effect on any of the caseins, with the partitions of α_{s2}-, α_{s1}-, β- and κ-caseins
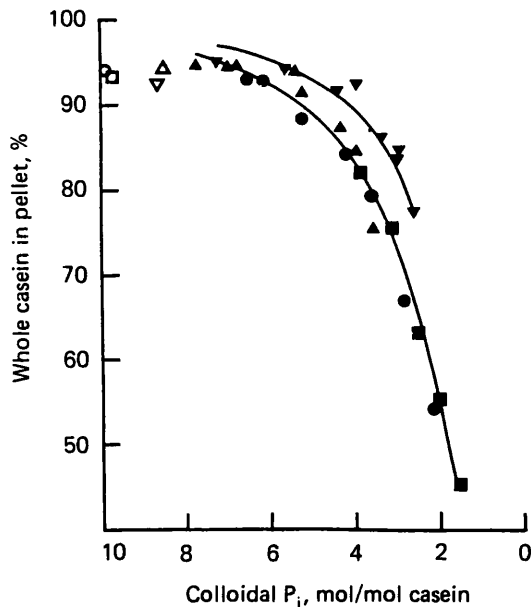
Fig. 5. Partition of whole casein after dialysis of skim milk against phosphate-free buffer containing 3 mM-($\bullet$, $\blacksquare$, $\blacktriangle$) or 6 mM-($\blacktriangledown$) $CaCl_2$; $\bigcirc$, $\square$, $\triangle$, $\triangledown$ represent the same milks undialysed. Dialysis time increased from left to right.

remaining at about 94, 97, 94 and 88 % respectively. However, further reduction to 5 mol P_i/mol casein, for example, reduced the corresponding casein partitions to 91, 96, 85 and 83 %, and a reduction to 2·5 mol P_i/mol casein gave values of 80, 72, 51 and 52 % respectively. From these values and the trends depicted in Fig. 6 it appears that the percentages of the individual caseins pelleted were in the order α_{s2}- > α_{s1}- > β- and κ-caseins, with the less readily sedimented β- and κ-caseins behaving somewhat similarly to each other.

Dialysis of milk against phosphate-free buffer containing 6 mM-$CaCl_2$

To provide additional evidence of the effects of free Ca^{2+} concentration and MCP on the dissociation of casein micelles, another milk sample was dialysed against a phosphate-free buffer containing 6 mM- rather than 3 mM-$CaCl_2$. The main objective of this experiment was to determine whether the casein still dissociated in the presence of the higher free Ca^{2+} concentration, even though it has been shown that the individual caseins, apart from κ-casein, aggregate in solutions containing more than 5 mM free Ca^{2+} at neutral pH (Thompson *et al.* 1969; Toma & Nakai, 1973; Parker & Dalgleish, 1981). Also, the higher free Ca^{2+} concentration might accentuate the difference in the calcium sensitivities of β- and κ-caseins and thus induce a greater difference in their dissociation.

Results for the salt partition showed that the rates of solution of colloidal Ca and P_i was significantly slower than in buffer containing 3 mM-$CaCl_2$. However, Fig. 5 shows that dissociation of casein still occurred even though the free Ca^{2+} concentration was about 3 times that in normal milk, although for a given degree of dissociation there was less colloidal P_i at the higher free Ca^{2+} concentration. Patterns for the individual caseins (Fig. 7) indicate that, apart from κ-casein, there were initially slight increases in pelleted values, probably caused by some aggregation of the serum casein fractions, possibly with the micelles, to increase the fraction sedimented. Thereafter, as more colloidal P_i dissolved, some dissociation was induced. This was
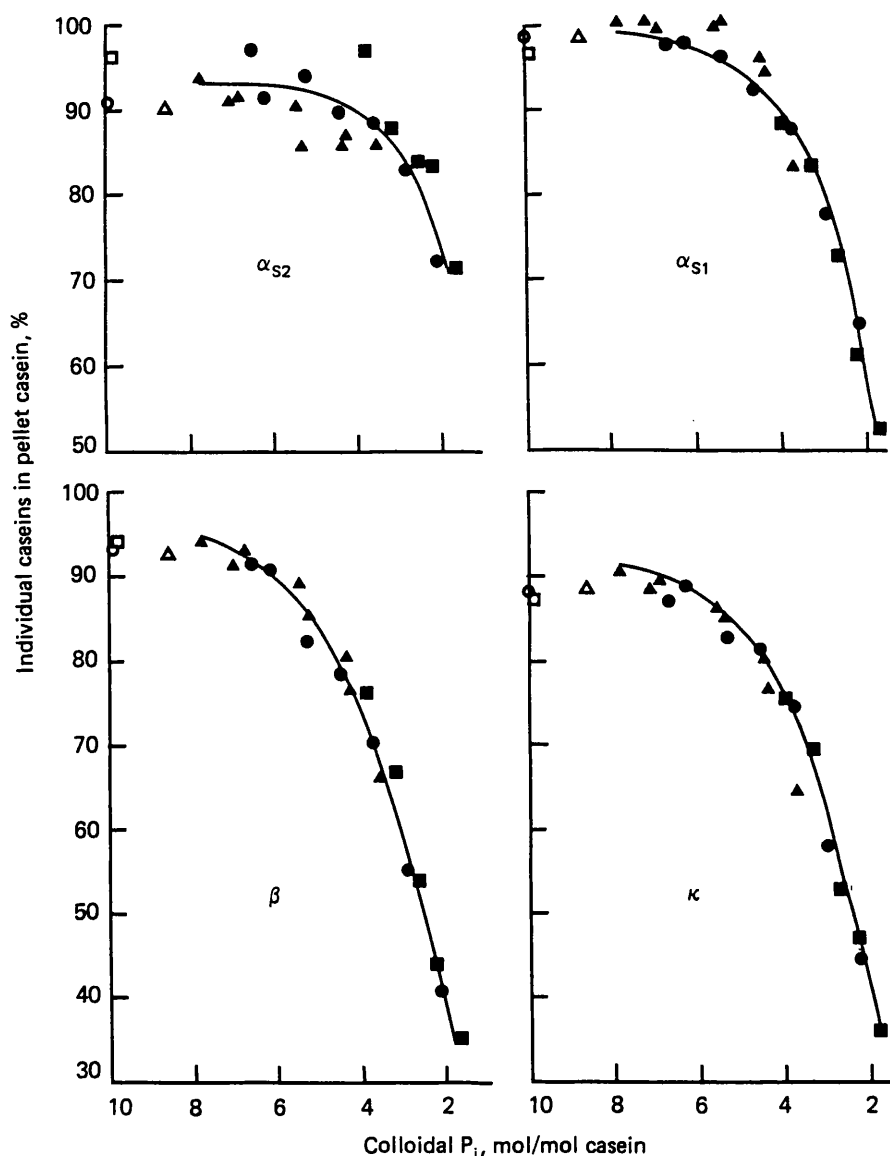
Fig. 6. Partition of individual caseins after dialysis of skim milk against phosphate-free buffer containing 3 mM-CaCl₂; ●, ■, ▲ and ○, □, △ as in Fig. 5. Dialysis time increased from left to right.

limited for α_{s1}- and α_{s2}-caseins and greater for β- and κ-caseins, but again the latter two caseins behaved similarly and there was no indication of the more Ca-sensitive β-casein being pelleted to a greater extent.

Composition of the calcium phosphate-rich material prepared by enzymic digestion of casein micelles

The material prepared by enzymic digestion of micelles is not pure calcium phosphate, but contains significant amounts of N and phosphate esters (Holt *et al.* 1982), together with small amounts of Zn, citrate and traces of ATP (McGann *et al.* 1983). The phosphate esters and N are of particular interest in the present context because they may indicate that some material from the protein–calcium phosphate interface has survived the digestion, thus providing information on how the calcium phosphate acts to maintain micelle structure and integrity.
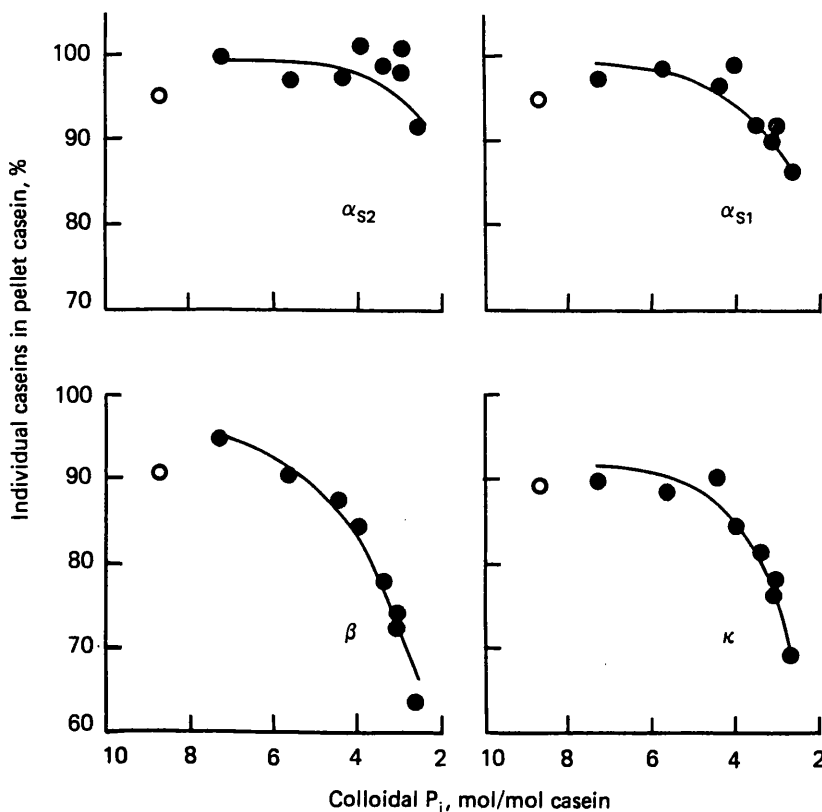
Fig. 7. Partition of individual caseins after dialysis of skim milk against phosphate-free buffer containing 6 mM-CaCl$_2$. ○, Undialysed milk. Dialysis time increased from left to right.

Table 3. *Chemical composition of calcium phosphate-rich material prepared from a dialysed, enzymic digest of casein micelles*

	mg/g	mmol/g
Ca	140·9	3·52
Total P	96·6	3·12
P$_i$	64·9	2·09
Mg	5·3	0·22
Na	4·4	0·19
K	3·8	0·10
Citrate	48·4	0·25
N	33·8	2·41

The chemical composition of a preparation of micellar calcium phosphate is given in Table 3. The sample contained only a trace of lactose and only small amounts of Na and K, indicating that it was not significantly contaminated with residues from milk serum. The predominant cation was Ca and, in agreement with previous results (Holt *et al.* 1982; McGann *et al.* 1983), the molar ratio of Ca to P$_i$ was close to 1·6 (1·68). Also, in further agreement with previous results (Holt *et al.* 1982), there was an appreciable content of phosphate esters so that the ratio of Ca to total P was much closer to unity (1·13).

The amino acid content of the sample, determined after hydrolysis with 6 M-HCl for 24 h, accounted for 88 % of the N present. The amino acid composition (Table 4, column 2) is distinctly different from that of whole casein, with substantial proportions of certain amino acids having been removed by the enzymic treatment:

Table 4. *Amino acid composition of calcium phosphate-rich material prepared from a dialysed, enzymic digest of casein micelles, and the composition of the phosphate centres of α_{s1}-, β- and α_{s2}-caseins, calculated as described in the text*

	Calcium phosphate sample		Phosphate centres calculated,
Component	μmol/g	mol %	mol %
Ser + SerP*	706	37·3	36·5
Glx	662	35·0	38·0
Ile	143	7·6	6·6
Val	82	4·3	3·6
Leu	77	4·1	3·6
Ala	62	3·3	2·9
Asx	52	2·7	2·9
Gly	39	2·1	0·7
Thr	23	1·2	3·6
Lys	19	1·0	Absent
His	15	0·8	0·7
Arg	12	0·6	Absent
Tyr	Trace	—	0·7
Phe	Trace	—	Absent
Pro	Trace	—	Absent
Met	Absent	—	Absent
Trp	ND	—	Absent

* Corrected assuming 10 % loss during acid digestion.
ND Not determined.

only a trace of proline and only small amounts of leucine and lysine were found, whereas these are among the most abundant amino acids in whole casein. Together, glutamic acid + glutamine and serine + phosphoserine accounted for 72 % of the amino acids found, while only small amounts of hydrophobic and basic amino acids were present. The measured serine content of the acid digest can account for about 70 % of the phosphate ester content of the material, depending on the proportion of serine which was phosphoserine and the extent of degradation of these two amino acids during digestion. Table 4 shows also the calculated amino acid composition of residues 46–51 and 61–70 of α_{s1}-casein, 11–21 of β-casein and 5–12, 49–61 and 126–133 of α_{s2}-casein (Eigel *et al.* 1984), which constitute the so-called phosphate centres of the caseins, with due allowance being made for the α_{s1}-, β- and α_{s2}-caseins being present in whole casein in molar ratios of $\sim$ 4:4:1 (Davies & Law, 1980). Apart from threonine, agreement between the experimental and calculated compositions is reasonably good, providing evidence that the peptides comprising the phosphate centres are the major sources of amino acids in the calcium phosphate sample. Apparently the acid phosphatase, added as one of the mixture of enzymes, was unable to bring about hydrolysis of the phosphoseryl residues, possibly because it was itself hydrolysed by one of the proteinases. Since the phosphate centres contain only one residue of serine and one of glutamine, the results suggest that the interface between casein and the calcium phosphate could be rich in phosphoserine and glutamic acid residues. It has been suggested (ter Horst, 1963; Visser *et al.* 1979) that MCP interacts with casein through the lysyl residues. Such interactions, if present at all in the native micelle, did not survive the enzymic digestion and sedimentation procedures.

DISCUSSION

Casein micelles appeared to exhibit considerable stability under the conditions used in the present dialysis experiments, since appreciable change in the colloidal Ca and P_i levels or in the free Ca^{2+} concentration in the serum were required before appreciable dissociation occurred. For example, on dialysis against the phosphate-free buffer containing 3 mM-$CaCl_2$, a reduction of $\sim 30\%$ in the amount of colloidal P_i could occur without any significant reduction in the amount of pelleted casein (Fig. 5), while dialysis against the calcium phosphate buffers demonstrated that the free Ca^{2+} concentration could be reduced to about 1 mM, compared to values of around 2 mM normally found in milk, without affecting the casein partition (Fig. 2). However, the results show also that further solution of colloidal P_i was accompanied by casein dissociation even with the phosphate-free buffer in which the free Ca^{2+} concentration was about 3 times greater than that in normal milk. This suggests that the colloidal P_i is of considerable importance in maintaining micelle integrity, and that this integrity cannot be preserved simply by the presence of relatively high free Ca^{2+} concentrations in the serum. Nevertheless, an increase in free Ca^{2+} concentration can retard dissociation (Fig. 5).

For the individual caseins, the partition patterns appeared similar in the two types of dialysis buffer (Figs 3, 6 and 7). The patterns show that at a given level of colloidal P_i or free Ca^{2+} concentration, dissociation was greater for β- and κ-caseins than for α_{s1}- and α_{s2}-caseins, which is in agreement with some results from the addition of EDTA to milk (Lin *et al.* 1972). Moreover, the mechanisms by which the caseins dissociated appear to be the same in both phosphate-free and calcium phosphate buffers, since when the percentages of β-, κ- and α_{s2}-caseins pelleted are plotted against the corresponding α_{s1}-casein partition (Fig. 8), experimental points fall more or less on the same lines except at low levels of dissociation of α_{s2}-casein, where appreciable scatter is evident. Generally the dissociations indicate that the percentage retention of the individual caseins in the pelleted casein was in the order α_{s2}-casein $> \alpha_{s1}$-casein $> \beta$-casein $\geqslant \kappa$-casein. If this order is compared with the amino acid composition of the caseins (Eigel *et al.* 1984), it is found to be directly related to the total number of basic residues (predominantly lysyl) and also to the total number of acidic residues (mainly phosphoseryl and glutamyl) in the proteins. Residues of either group could bind to the CCP, providing linkages to maintain micelle structure and stability. That lysyl residues might bind to the calcium phosphate has been suggested previously (ter Horst, 1963; Visser *et al.* 1979), but in the present work very little lysine or other basic amino acids were found in the calcium phosphate-rich material isolated from micelles after proteolytic digestion (Table 4). Most of the N fraction in the isolated material was accounted for by the amino acids phosphoserine and glutamic acid, suggesting that the phosphate centres of the caseins provide strong sites of interaction with the CCP. This is in agreement with earlier proposals (McGann & Pyne, 1960; Rose, 1965; Schmidt, 1982) and with studies on individual caseins and their partly or fully dephosphorylated derivatives, which have demonstrated the importance of phosphoseryl residues in the binding of Ca^{2+} and the aggregation of the caseins (Ho & Waugh, 1965; Whikehart & Rafter, 1970; Dickson & Perkins, 1971). The strength of binding of the individual caseins within native casein micelles is therefore probably largely dependent on the number of phosphoseryl residues they contain. However, other factors must also be involved, since the dissociation patterns of β- and κ-caseins were similar, yet they contain five and one or two phosphoseryl residues per monomer respectively. Here the temperature-
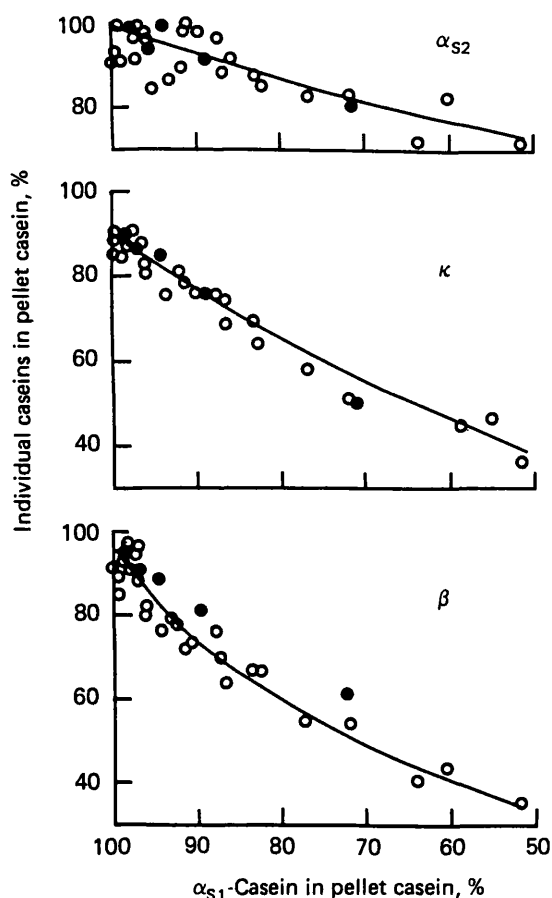
Fig. 8. Partition of α_{s2}-, κ- and β-caseins as a function of the partition of α_{s1}-casein. $\bigcirc$, Samples dialysed against phosphate-free buffers (either 3 mM- or 6 mM-$CaCl_2$); $\bullet$, samples dialysed against calcium phosphate buffers.

dependent dissociation of a considerable fraction of micellar β-casein (Rose, 1968; Downey & Murphy, 1970; Davies & Law, 1983) and the probable existence of micellar κ-casein as a disulphide-linked polymer (Mackinlay & Wake, 1964; Talbot & Waugh, 1970; Swaisgood, 1982) could be complicating factors.

As to the nature of the bonding material within the micelle, two forms of micellar Ca have been distinguished previously, namely the Ca in the calcium phosphate and the caseinate (or carboxyl-linked) Ca (e.g. White & Davies, 1958; McGann & Pyne, 1960; Holt, 1982; Schmidt, 1982). If this model is accepted, then from Fig. 1 the colloidal Ca at zero free Ca^{2+} concentration (~ 15 mol/mol casein) would be the calcium phosphate Ca, and additional bound Ca^{2+} the caseinate Ca. Clearly, both forms could act to maintain micelle structure and integrity, but a distinction between them may be artificial, since bound Ca^{2+} may be associated with the surface of the calcium phosphate or with the calcium phosphate–casein interface. Thus the slope of the line relating the level of colloidal Ca to that of colloidal P_i in milks dialysed against one of the phosphate-free buffers (Fig. 4) could reflect a change in both the supposed forms of colloidal Ca, even though free Ca^{2+} concentration was relatively constant, so it is difficult to attribute unequivocally the changes in the casein partition on dialysis against the phosphate-free buffers to change in any particular fraction of micellar Ca. Nevertheless, it is likely that the level of bound Ca^{2+} in these experiments was always considerably greater than those at which appreciable

dissociation occurred on dialysis against the calcium phosphate buffers, and therefore the dissociation of the casein probably resulted from the dissolving of CCP and not from changes in the amount of bound Ca^{2+}. Thus it would appear that the CCP is an important bonding material in casein micelles.

In the dialysis experiments with the calcium phosphate buffers there was little, if any, change in milk colloidal P_i (Fig. 1), so changes in casein partition could have been produced by the differences in free Ca^{2+} concentration in the buffers, through their effect on bound Ca^{2+} levels. At free Ca^{2+} levels of 3 mM and above, pelleted casein increased above the average for the starting milks (Fig. 2), in agreement with earlier studies involving small additions of Ca (Rose, 1968; Lin *et al.* 1972). Similarly, the pelleted proportions of the Ca-sensitive α_{s1}-, α_{s2} and β-caseins, but not the Ca-insensitive κ-casein, also increased. On the other hand, micelle dissociation was appreciable at free Ca^{2+} concentrations below 1 mM, but this may not be due solely to a reduction in bound Ca^{2+} since another possibility must be considered. The buffers with the lowest free Ca^{2+} concentrations contained the largest concentrations of P_i, this being essential to preserve their saturation with respect to MCP, and this high P_i could displace phosphoseryl residues involved in linkages with the calcium phosphate and thereby induce dissociation. It has not been possible therefore to obtain a true measure of the importance of bound Ca^{2+}.

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pH-Induced dissociation of bovine casein micelles. I. Analysis of liberated caseins

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SUMMARY. The dissociation of caseins of different types from casein micelles in milk, acidified to different pH values in the range 4·9–6·7, and at temperatures of 4, 20 and 30 °C, has been studied. In contrast to a number of previous findings, it was shown that caseins of all types were dissociated from the micelles, although in all cases β-casein was in highest concentration. The amounts and proportions of all of the caseins were found to be pH- and temperature-dependent, especially the former. Studies of the proportions of the different caseins liberated suggested that, at a defined temperature, the proportions of κ- and α_{s2}-caseins were independent of pH, while the proportions of β- and α_{s1}-caseins were variable, changes in one being compensated by changes in the other. The manner in which the proportions of the α_{s1}-casein and β-caseins changes with pH was found to be temperature-dependent.

It is known that casein micelles are affected by pH and temperature in a number of ways. As the pH is decreased from that of normal milk ($\sim 6·7$), the micellar Ca phosphate begins to dissociate (Van Hooydonk *et al.* 1986; Chaplin, 1984). Since this material is largely responsible for maintaining the integrity of the micelle (Pyne & McGann, 1960; McGann & Pyne, 1960), its dissociation in turn can cause dissociation of individual casein molecules (Holt *et al.* 1986). This reaction is strongly temperature-dependent: in milk stored at 4 °C the micelles partly dissociate even if the pH is not changed (Rose, 1968; Reimerdes & Klostermeyer, 1976; Davies & Law, 1983): in this case, the predominant protein liberated from the micelles is β-casein. Conversely, at higher temperatures, the dissociation of the caseins from the micelles in milk is inhibited even if the pH is low (Rose, 1968).

Some results (Rose, 1968; Snoeren *et al.* 1984; Van Hooydonk *et al.* 1986) suggest that β-casein is the protein which is predominantly dissociated from the casein micelles as the pH is decreased. There are however no quantitative studies of this. A preliminary study (Roefs *et al.* 1985) showed that, at 4 °C, all types of caseins were dissociated from the micelle, and not simply β-casein: this was especially the case at substantially lowered pH values.

Decrease in the pH also affects some of the other properties of the micelle, notably the voluminosity (Tarodo de la Fuente & Alais, 1975; Snoeren *et al.* 1984; Van Hooydonk *et al.* 1986), and arguably the ζ-potential (Schmidt & Poll, 1986), although the validity of calculation of the ζ-potential (from measurements of the electrophoretic mobility) in particles such as partly dissociated casein micelles is open to question. The changes in the micelles occur because some form of micellar framework is apparently maintained even when a large proportion of the original

micellar material has been removed (Roefs et al. 1985). During processing, the pH is adjusted in the manufacture of cheese, Cottage cheese and yogurt, and the structures of the curds formed in these products depend on the structures of the casein particles. However, as the pH is decreased, there will come a point at which the casein micelles no longer possess any of their former structure. Experiments on the manufacture of cheese from heated milk have shown that the effects of heat may be partly reversed by changes in pH (Banks et al. 1987).

We have studied the effects of pH, specifically on the dissociation of the caseins from the casein micelles. Three temperatures were chosen for the study. The first, 4 °C, is that at which milk is stored, and the second, 30 °C, is the temperature at which many manufacturing processes occur. A third, 20 °C, was chosen so that the results could be compared with those of Holt et al. (1986) on the dissociation of micelles induced by other means.

MATERIALS AND METHODS

Milk samples

In all experiments, fresh bulk milk from the Institute's herd was used. Before use, the milk was skimmed by centrifugation at either 4 or 20 °C (depending on the final required temperature) for 30 min, at a centrifugal force of 1000 g.

Acidification of milks

Two approaches were used to alter the pH of the milk. In the first, defined amounts of glucono-δ-lactone (GDL) were added, with stirring, and the milk was left for 23–25 h to attain pH. The amounts of GDL were determined in advance by experimentally measuring a calibration curve. The second method was to use the technique employed by Holt (1982) to acidify, using HCl. Measured amounts of HCl (2·0 M) were put into dialysis sacs, and these were then dropped into milk samples, with stirring. The stirring was continued for 23–25 h. In all cases, sets of 18 samples of milk were treated so as to give a range of pH values between 4·9 and 6·7, measured at the temperature of the experiment. In experiments at 20 and 30 °C the milks were treated with 0·01 % Na azide to prevent bacterial growth and 0·01 % soyabean trypsin inhibitor to minimize the activity of plasmin.

Of the three temperatures used, (4, 20 and 30 °C) experiments involving acidification with GDL and with HCl were performed at 4 and 30 °C only. At 20 °C, only GDL was used since, as will be seen, the method of acidification did not appear to be important.

Centrifugation and casein separation

Soluble caseins were defined as those caseins which did not sediment from milk during centrifugation at 70000 g at the times specified below. A Superspeed 65 centrifuge (Fisons Scientific Instruments, Crawley, Sussex, UK) was used with a 6 × 38 ml swing-out rotor, at a speed of 22500 rev./min. Because the sedimentation had to be carried out at the temperature at which the milks had been stored, the time of centrifugation was altered to allow for the higher viscosity of the milk at lower temperatures (Davies & Law, 1983). Thus, centrifugation times were, at 4 °C, 4 h; at 20 °C, 2 h; at 30 °C, 105 min. In principle, further corrections could be made to the centrifugation time to account for changes in the voluminosity and density of the micelle as the pH is altered (Van Hooydonk et al. 1986). However, it was not possible to estimate in detail the magnitudes of the necessary changes in conditions. The

effect, in the pH range 5·0–6·6, is likely to be small. After centrifugation, the supernatant liquid was carefully removed by syringe and allowed to attain room temperature. The pH was then adjusted to 4·6 and all of the casein precipitating from the serum was collected, and washed with water at pH 4·6 to remove residual serum proteins. The micellar pellets remaining after centrifugation were resuspended in 5 ml of 2·5% Na_2-EDTA and 2·5% Na_4-EDTA, pH 6·8, and stirred for 15 min. Water (25 ml) was then added and stirring was continued for 1 h at 20 °C and a further 16 h at 4 °C (Davies & Law, 1983). The solutions were then acid-precipitated as for the serum casein. All of the casein samples were then lyophilized.

Nitrogen analyses

Total and non-casein N contents of the milks before pH adjustment were determined by a micro-Kjeldahl method (Davies & Law, 1983). This allowed the determination of the concentrations of whey proteins, which were assumed not to change as the pH was varied. Each of the serum samples after pH adjustment was analysed in the same way, for total N only. The original value for non-casein N was subtracted to give the concentration of casein in the serum, and hence the partition between micellar and serum casein.

Chromatographic analysis of the caseins

Analysis of the casein samples was by fast protein liquid chromatography (FPLC, Pharmacia (UK) Ltd, Milton Keynes, Bucks, UK), as described by Davies & Law (1987). This technique relies upon modification of the casein with cystamine/cysteamine, and sample preparation was exactly as described in the publication. Preliminary quantification of the individual casein components relied on the integration performed by the FPLC controller, and more detailed analysis of the elution profiles was achieved using a BBC Master microcomputer linked to the FPLC equipment.

<div align="center">RESULTS</div>

Liberation of total casein

Fig. 1 shows the amounts of caseins dissociated from micelles into the serum as the pH of the milks was decreased, at the three different temperatures used. It was clear from the result that there was no difference between GDL and HCl in respect of the liberation of casein, probably because the pH-adjusted milks had been left to equilibrate for a sufficiently long period. Some problems have been reported over the use of GDL (Trop & Kushelevsky, 1985), but we found that it did not affect the partition of the proteins in our experiments, nor did it apparently induce any modifications to the casein which could be detected. The breakdown of micelles at 4 °C was similar to that observed by Rose (1968) and Roefs *et al.* (1985) in overall shape, but appeared to have a peak at a lower pH (5·1 compared with Rose's value of 5·3). All results concurred in showing that about half of the total casein was rendered soluble at the pH of maximum micellar breakdown. This also compares well with the results of Rose (1968).

At 20 °C considerably less casein was solubilized, although at maximum dissociation the non-micellar casein formed approximately one-third of the total casein. The pH at which maximum dissociation occurred at 20 °C was higher than at 4 °C (pH 5·4 compared with 5·1). This trend appeared to be continued at higher temperature, because the results at 30 °C showed that, although only small amounts
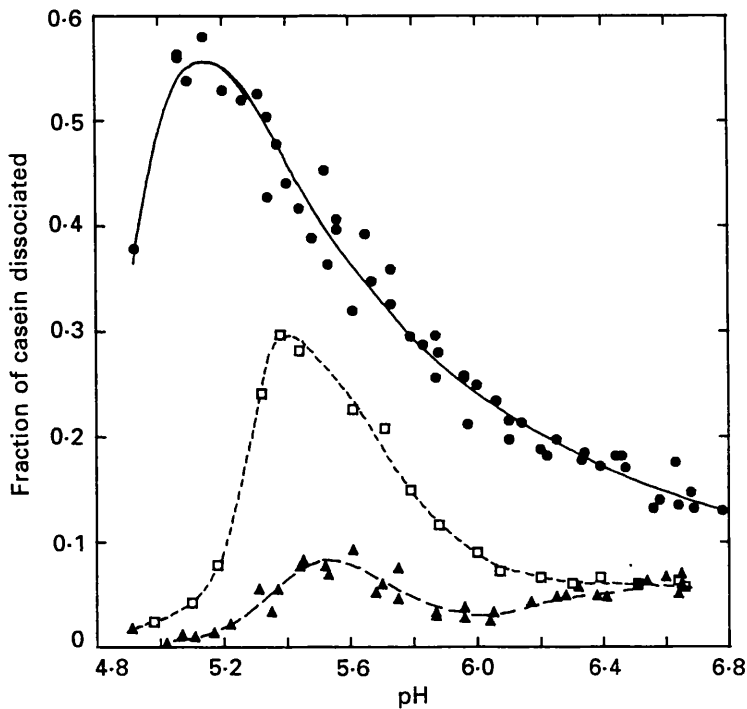
Fig. 1. Total amounts of casein dissociated as functions of pH at temperatures of: 4 °C, ● ; 20 °C, □ ; 30 °C, ▲. Points are expressed as fractions of the total casein (soluble and micellar).
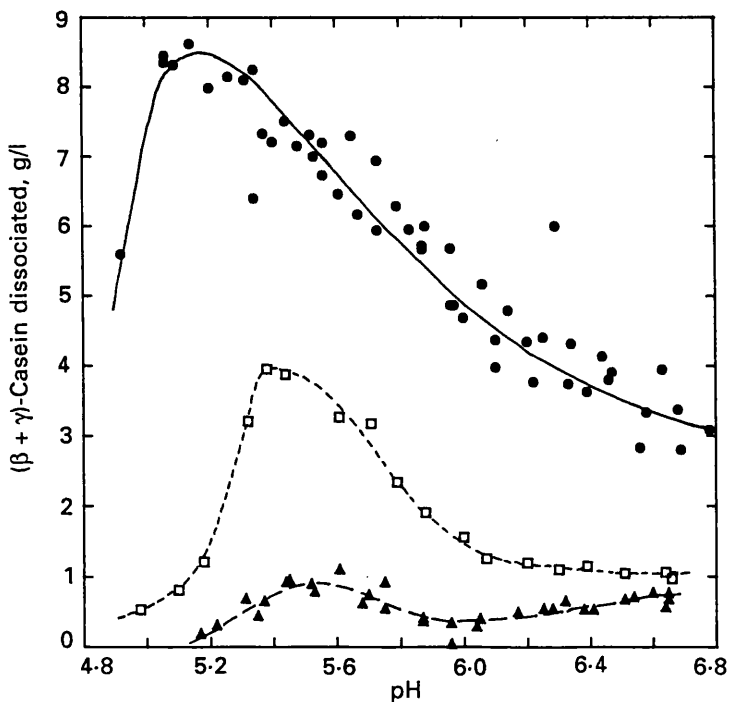
Fig. 2. The sum of β- and γ-caseins dissociated from the micelles as functions of pH at temperatures 4, 20 and 30 °C. Symbols as for Fig. 1. Amounts are expressed as concentrations in the serum.

of casein (less than one-tenth of the total) were dissociated, the maximum dissociation occurred at pH 5·5. The amount of non-micellar casein at 30 °C decreased between pH 6·7 and 6·0: this was the only temperature at which such a phenomenon was observed. We originally attributed this to an increase in the content of soluble γ-casein, formed by proteolysis of β-casein by plasmin (Gordon & Groves, 1975),
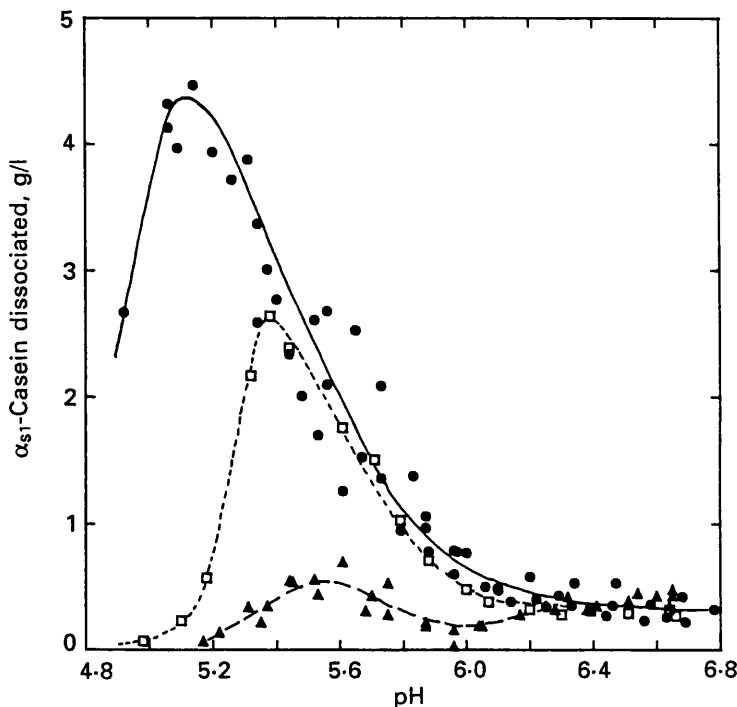
Fig. 3. Concentrations in the serum of α_{s1}-casein dissociated from the micelles as functions of pH and temperature. Symbols as for Fig. 1.

despite the presence of trypsin inhibitors. However (see below), the behaviour was observed for α_{s1}-casein and κ-casein as well as for β-casein. Apart from the decrease between pH 6·7 and 6·0, our results at 30° are similar to those of Rose (1968) at 35 °C, although Rose's result did not show such a pronounced maximum at low pH.

Dissociation of individual caseins

Individual caseins can be considered either in terms of their total concentration in the serum or their proportion of the total casein complex. The concentration of $(\beta+\gamma)$-casein liberated into the serum increased almost linearly as the pH was lowered at 4 °C (Fig. 2). Conversely, a different shape of curve was found for the liberation of the $(\beta+\gamma)$-caseins at 20 and 30 °C, these curves being similar to those for the release of total casein at these temperatures shown in Fig. 1. In all of these cases, the fraction $(\beta+\gamma)$-casein was plotted, to avoid problems of breakdown. It is known that γ-casein derives from β-casein (Gordon & Groves, 1975), but it was not certain at what stage in our experiments the γ-casein had formed from the β-casein, whether before or after the latter had dissociated from the micelles. However, it was evident that in all cases the concentrations of the γ-caseins were highest at the natural pH of milk and lowest at pH $\sim 5\cdot5$.

Unlike β-casein, the dissociation of α_{s1}-casein at 4 °C was virtually independent of pH at pH values above 6·0: indeed, milks at all three temperatures gave similar, low, concentrations of α_{s1}-caseins in the serum above pH 6·2 (Fig. 3). The behaviour and concentrations of dissociated α_{s1}-casein were very similar at 4 and 20 °C down to pH 5·4, below which the concentration of α_{s1}-casein in the serum at 20 °C dropped sharply, in parallel with the decrease in the total concentration of serum casein. At 30 °C, the concentration of α_{s1}-casein in the serum was low compared with the amounts found at other temperatures, because of the generally low concentration of dissociated casein at this temperature.
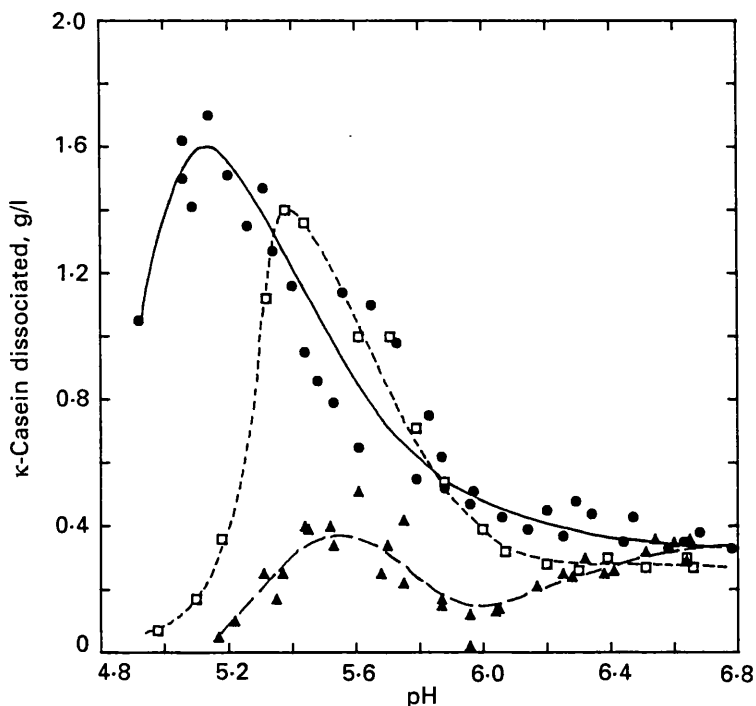
Fig. 4. Concentrations in the serum of κ-casein dissociated from the micelles, as functions of pH
and temperature. Symbols as for Fig. 1.
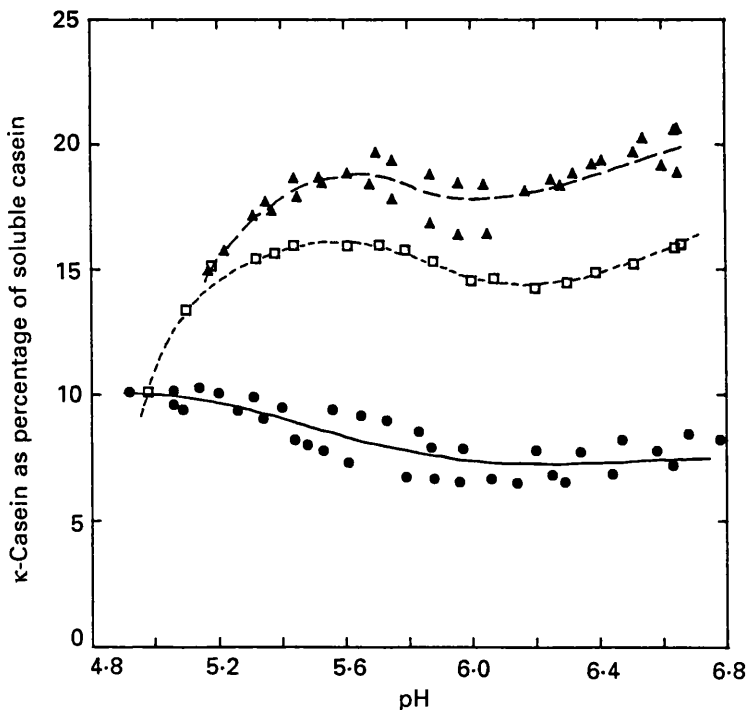
Fig. 5. Proportions of κ-casein in the dissociated casein as functions of pH and temperature, expressed
as % of the total soluble casein. Symbols as for Fig. 1.

It was clear that the amounts of dissociated α_{s1}-casein showed a lesser dependence
on temperature compared to β-casein, especially at pH values above 6·0. This may
be attributable to the greater hydrophobic nature of β-casein (Swaisgood, 1982), or
to its ability to form larger aggregates than α_{s1}-caseins as the temperature is raised
(Schmidt, 1982).
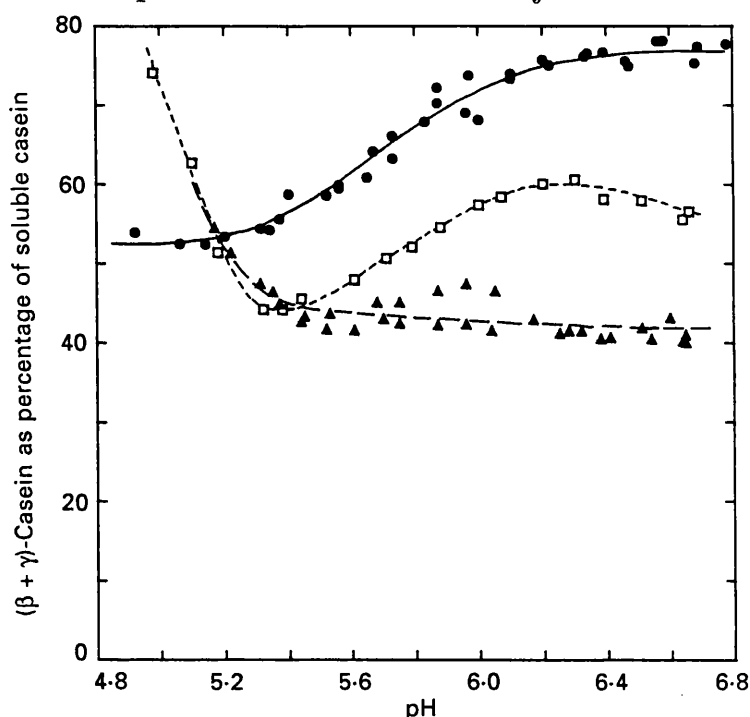
Fig. 6. Proportions of β-+γ-casein in the dissociated casein as functions of pH and temperature, expressed as % of the total soluble casein. Symbols as for Fig. 1.

The concentrations of dissociated κ-casein (Fig. 4) were similar to those of α_{s1}-casein in behaviour, although smaller in magnitude. Less temperature dependence was shown than for the β-casein release. Analysis of the κ-casein fraction in the dissociated casein (Fig. 5) showed that the proportion of κ-casein in the dissociated casein was to a first approximation virtually independent of pH above 5·4, although it was strongly dependent upon the temperature: at higher temperature, κ-casein formed a larger fraction of the dissociated casein ($\sim$20% compared with $\sim$8% for the dissociation at 4 °C). Although the temperature dependence was no doubt an overall reflection of the greater dissociation of β-casein at low temperatures, the constancy of the proportion of κ-casein over a range of pH was remarkable in view of the considerable variations in the proportions of α_{s1}-casein and β-casein described below.

α_{s2}-Caseins were measured but will not be considered in detail here: as expected, they formed only a small amount (in none of the experiments greater than 6%) of the total soluble casein. In the original milks, the amount of α_{s2}-casein was $(10·5\pm0·21)$% of the total casein. The caseins which dissociated during the change in pH were therefore all relatively depleted in α_{s2}-casein compared with the starting milk. The casein remaining in the micelles was correspondingly enriched in this particular component, so that at maximum dissociation of the micelles, the proportion of α_{s2}-casein in the micelles was as high as 15%. The proportions of the undefined minor peaks A, B and C in the chromatographs of the caseins (Davies & Law, 1987) remained approximately constant, within experimental error.

Considerable changes were observed in the proportions of α_{s1}- and β-caseins which dissociated from the micelles at different pH and temperature values. The behaviour is shown in Figs 6 and 7. Approximately, the proportions of κ-casein and α_{s2}-casein in the dissociated casein remained constant as the pH was altered, but the amounts of α_{s1}- and β-casein changed to compensate for one another as the pH was lowered. As
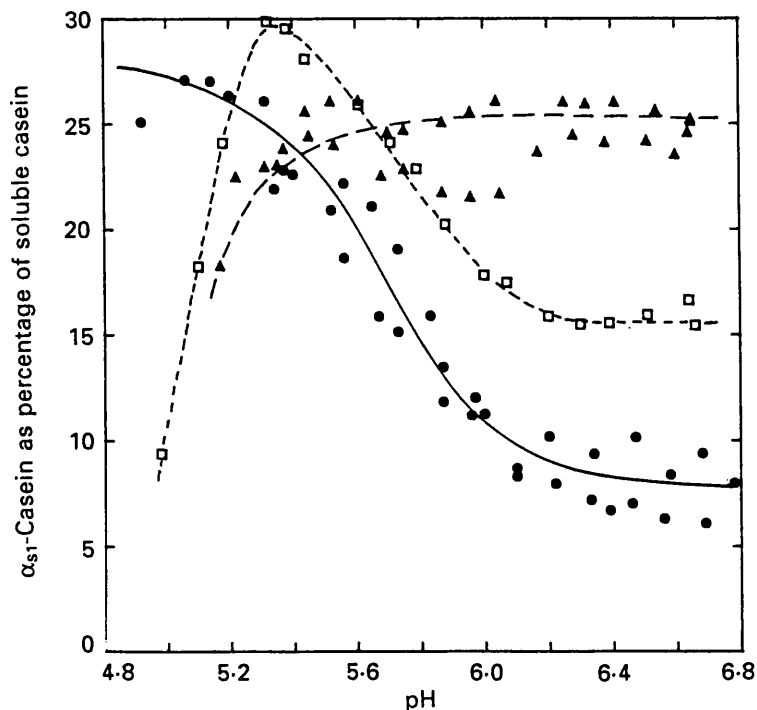
Fig. 7. Proportions of α_{s1}-casein in the dissociated casein as functions of pH and temperature,
expressed as % of the total soluble casein. Symbols as for Fig. 1.

was shown above, the concentrations of the dissociated caseins changed at the same
time as their compositions, and it was the dissociation of the individual caseins at
different rates which produced the effects shown in Figs 6 and 7. There appeared to
be two combined effects, if the shapes of the curves were examined, the limiting types
of behaviour being given at 4 and 30 °C.

At 4 °C, the proportion of β-casein in the dissociated casein decreased sigmoidally
between pH 6·4 and 5·2 and α_{s1}-casein increased to compensate: in these results, a
single pH-dependent effect appeared to take place. Conversely, at 30 °C, the
proportions of α_{s1}- and β-caseins remained almost constant above pH 5·4, but below
this pH value the proportion of β-casein increased rapidly as the pH was reduced
further (although Fig. 1 shows that the total concentration of soluble casein under
these conditions was decreasing rapidly with decreasing pH). The behaviour at 20 °C
was intermediate between the effects at 4 and 30 °C. The proportion of β-casein
decreased with pH down to pH 5·4 (as at 4 °C), but then rose very sharply (as at
30 °C). Although the situation was complicated by the different concentrations of
dissociated caseins at the three temperatures, it may nevertheless be the case that
there are two patterns of dissociation of caseins, depending on the temperature.

<div align="center">DISCUSSION</div>

It is apparent that, although β-casein is the largest single component in the
dissociated casein, it is by no means the only casein dissociated at any of the pH and
temperature combinations which we used. Our observations therefore do not support
the studies of Van Hooydonk et al. (1986) and of Snoeren et al. (1984), where β-casein
appeared to be by far the most important of the liberated proteins. Our results rather
tend to support the limited electrophoresis study of Rose (1968), which showed that
appreciable amounts of α_s-caseins were present in the serum at low pH values. Some

of the differences may arise from different heat treatments of the milks, or from the use of reconstituted milk powder in one case.

It is a possibility that, especially if dissociation occurs via 'submicelles', there will be correlations between the dissociation behaviour of the different proteins, i.e. that the dissociation of one type of protein accompanies the dissociation of another. The results presented here demonstrated that at 4 °C there was little correlation between the behaviour patterns of the α_{s1}-, β- and κ-caseins: the three proteins appeared to dissociate independently of each other, and it appeared unlikely that, at this temperature, the dissociation took place via the loss of small complexes of constant composition. The results at 30 °C and above pH 5·4 were consistent with the dissociation of the caseins in constant proportions, although this does not prove that it is small complexes of constant composition which dissociate, rather than individual casein molecules. If it is the case that complexes dissociate, then their composition is not representative of the micelles as a whole, being enriched in κ-casein and depleted in α_s-caseins.

Three processes can be identified which affect the dissociation of caseins from micelles when the pH is lowered. These are: (1) the hydrophobic effect (Tanford, 1980), where dissociation is greatest at low temperature and decreases as temperature increases but should have no obvious pH-dependence; (2) the dissolution of Ca and phosphate from the micelles, causing a loss of casein which increases as the pH decreases and more micellar Ca phosphate is dissolved; and (3), the isoelectric precipitation of the caseins as the pH falls to a sufficiently low value. The last of these effects is both pH- and temperature-dependent. The second of the effects will be considered in detail in a subsequent publication, but it should be pointed out that the dissociation of the caseins from the micelles is not simply a result of removing some or all of the micellar Ca phosphate, since the dissociation patterns of casein at 4 and 30 °C were very different, although the micellar Ca phosphate had dissociated to similar extents in both cases.

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pH-Induced dissociation of bovine casein micelles
II. Mineral solubilization and its relation to casein release

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SUMMARY. Measurements of the release of Ca, Mg and inorganic phosphate(P_i) from the casein micelles of bovine milk have been made, as functions of the pH, in the range 4·9–6·7, and at temperatures of 4, 20 and 30 °C. The results are in general agreement with earlier published studies in giving a value of 1·75–1·84 for the micellar Ca:P_i ratio. Mg appeared to behave similarly to Ca, although the amounts of micellar material were much smaller. The results on the acid-solvation of calcium phosphate are considered in relation to published quantitative studies of the pH-induced dissociation of the different types of caseins from the micelle, and of the micellar dissociation caused when micellar calcium phosphate is dissolved at neutral pH. It is evident from this that at present it is not possible to derive a universal relation between the dissociation of minerals and of caseins from the micelles at different temperatures and under different conditions.

Casein micelles in normal bovine milk are made up of the different casein proteins in combination with calcium phosphate ('micellar' or 'colloidal' calcium phosphate). The nature of the interactions between the proteins and the mineral have been studied, as well as the composition of the mineral itself (Pyne & McGann, 1960; McGann & Pyne, 1960; Holt, 1982; Holt *et al.* 1982; Chaplin, 1984; Holt *et al.* 1986; Aoki *et al.* 1988), but it has so far been impossible to define precisely the function of the mineral in maintaining the micellar structure. For example, it has been proposed on the one hand that the calcium phosphate acts as a binding agent between micellar subunits (Schmidt, 1980, 1982), and on the other that a calcium phosphate/casein complex exists in which the phosphoseryl residues of the caseins form part of the calcium phosphate matrix (Holt *et al.* 1982, 1986).

Alteration of the pH of milk, particularly in the acid direction, is fundamental to a number of manufacturing processes (e.g. manufacture of many types of cheese, formation of structure in Cottage cheese and yogurt), and it is already established that during this alteration in pH profound changes can occur in the micellar composition and hence presumably in the micellar structure. The acidification of milk solubilizes partly or wholly the micellar calcium phosphate (Chaplin, 1984; Van Hooydonk *et al.* 1986) and this can under some circumstances be accompanied by extensive dissociation of casein from the micelle (Rose, 1968; Davies & Law, 1983; Van Hooydonk *et al.* 1986). A major determinant of this pH-induced dissociation of the casein is the temperature, so that at 30 °C a decrease in pH causes virtually no release of casein, while at 4 °C about 60 % of the casein is soluble at pH 5·5 (Dalgleish & Law, 1988).

When considering the formation and structures of gels or coagula from milk at reduced pH, it is important to understand the nature of the particles involved in forming these structures. Several publications have dealt with the dissociation of minerals or of caseins from micelles during pH adjustment (Chaplin, 1984; Roefs et al. 1985; Snoeren et al. 1984; Van Hooydonk et al. 1986), but few of these have been sufficiently detailed to allow comparison of the dissociation of the minerals and of casein, nor have they been concerned with the effect of temperature upon the dissociation process. We have already described the behaviour of the casein fractions during acidification (Dalgleish & Law, 1988) and in the present paper we describe the behaviour of the mineral fraction of the micelles, in an attempt to establish quantitative relationships between the behaviour of the protein and mineral fractions during the acidification of milk.

MATERIALS AND METHODS

Milk samples

Milk samples were collected from the bulk tank of the Institute farm. Skim milk was prepared by centrifuging whole milk at either 4 or 20 °C (depending on the temperature at which the milk was to be stored) at 1000 g and collecting the material below the separated fat layer.

Acidification of milks

To alter the pH of the milk, defined amounts of glucono-δ-lactone (GDL) were added, with stirring, and the milk was left at the appropriate temperature for 23–25 h to attain pH and to allow equilibrium dissociation to be established. The necessary amounts of GDL were determined in advance by experimentally measuring the relation between the concentration of added GDL and the decrease in pH. Sets of samples of milk were treated in order to give a range of pH values between 4·9 and 6·7, measured at the temperature of the experiment. Three different temperatures 4, 20 and 30 °C were used. In experiments at 20 and 30 °C the milk was also treated with 0·01 % Na azide to prevent bacterial growth and 0·01 % soyabean trypsin inhibitor to minimize the activity of plasmin.

Centrifugation

At the end of the storage period, each milk sample was partitioned into a pellet of micellar material and a serum fraction by centrifugation at 70000 g in a Superspeed 65 ultracentrifuge (Fisons Scientific Instruments, Crawley, Sussex, UK) using a 6 × 38 ml swing-out rotor, at a speed of 22500 rev./min. The sedimentation was carried out at the temperature at which the milks had been stored and the time of centrifugation was adjusted to compensate for the higher viscosity of the milk at lower temperatures (Davies & Law, 1983). Thus, centrifugation times were, at 4 °C, 4 h; at 20 °C, 2 h; at 30 °C, 105 min. After centrifugation, the supernatant liquid was carefully removed by syringe and allowed to attain room temperature.

Ultrafiltration

As an alternative to centrifugal fractionation of the milk after pH adjustment, ultrafiltration was used to prepare serum (for experiments at 20 °C only). About 300 ml milk was circulated through a hollow-fibre ultrafiltration membrane with a nominal M_r cut-off of 500000 (AG Technology Corp., Needham, MA 02194, USA). Ultrafiltrate was obtained at a rate of 4 ml/min. The first 5 ml were discarded, and

the next 2 ml retained for analysis. Previous experiments had established that at 20 °C no caseins passed through the membrane.

Nitrogen analyses

Total and non-casein N contents of the milks before pH adjustment, and total N in the supernatants obtained by centrifugation, were determined by a micro-Kjeldahl method (Davies & Law, 1983). The concentrations of casein N in the starting milks and in the supernatants were calculated by subtracting the non-casein N values from the total N values. A factor of 6·51 was used to convert casein N values to casein concentrations and the partition of micellar and serum casein was calculated by subtracting the concentration of casein in the serum from the total casein concentration.

Ca, Mg and inorganic phosphate (P_i) analyses

Total Ca, Mg and P_i contents of the original milks, and of the centrifugal supernatants and permeates from ultrafiltration, were determined by high pressure ion chromatography (Dionex (UK) Ltd, Milton Keynes, Bucks., UK). Ca and Mg were analysed on a cation exchange column (CS3 Ion Pac column) fitted with a guard column (CG3), and P_i was analysed on an anion exchange column (AS4A) with a guard column (AG4A). Before analysis, the samples of milks, supernatants or permeates were acidified to pH 4·6 by the addition of 1 M-HCl, and stirred for 20 min at 20 °C, to ensure precipitation of any caseins. The resulting solutions were either filtered through Whatman No. 42 filter paper or passed through a micro-ultrafiltration unit (Aimer Products Ltd, London, UK) by centrifuging for 30 min at 1100 *g*. The samples were diluted 100-fold before chromatography. Heights of the peaks were compared with those given by standard solutions. The concentrations of total Ca, Mg and P_i in the original samples were calculated with a correction for the volume of HCl required to precipitate the casein, and for the volume of the casein precipitate as estimated from the casein N determination. The partition of Ca, Mg and P_i between micelles and serum was calculated by subtracting the concentrations in the serum from the total concentrations in the milks.

RESULTS AND DISCUSSION

Dissociation of Ca and P_i from micelles

Fig. 1 shows the extents of dissociation of the Ca and P_i from the micelles into the serum as functions of the pH. The results shown are for 20 °C but essentially similar results were obtained for all temperatures. At pH values above 6·0 there was somewhat greater solubilization of Ca and P_i at 4 °C compared with that at either 20 or 30 °C, although the differences were small (Davies & White, 1960). As shown by Van Hooydonk *et al.* (1986) the plots of the solubilization of mineral against pH were sigmoid in shape. We did not, however, observe as large a difference between the Ca and P_i titration curves as was apparent in the latter publication, although we confirm their observation that P_i appears to titrate at a higher pH than Ca. We did not confirm the suggestion by Evenhuis & De Vries (1959) that all micellar Ca is solubilized at pH values below 5·2 (Visser *et al.* 1986): even at pH 4·9 we estimate that 1 mM-Ca is sedimentable and non-diffusible (Fig. 1)

We found that the method of separation of the serum (i.e. centrifugation or ultrafiltration) had some effect upon the measurements of the amounts of P_i and Ca which were dissociated from the casein micelles. Ca was on average 1·09 mM higher
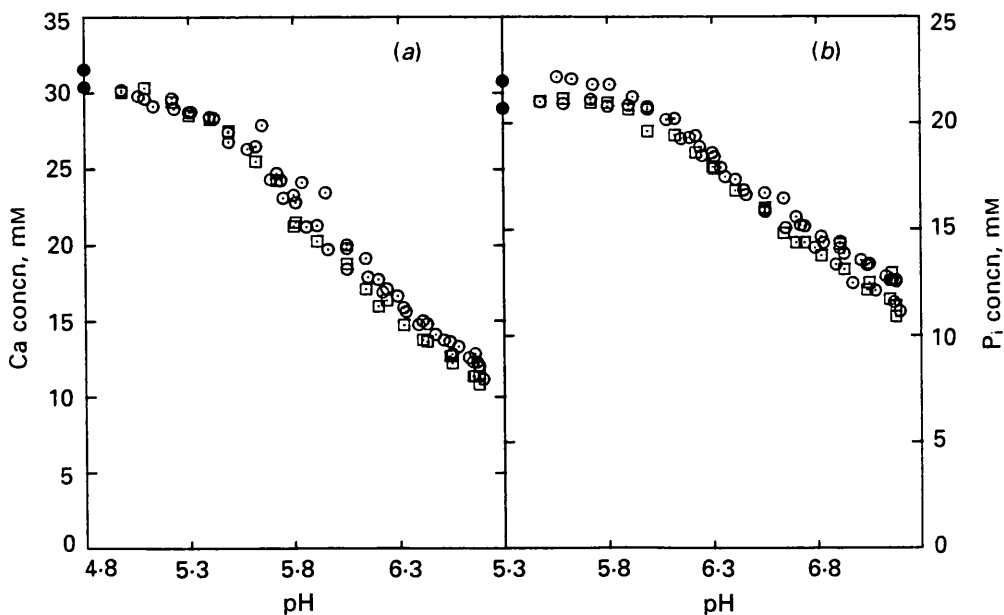
Fig. 1. Solubilization of (a) Ca and (b) inorganic phosphate (P$_i$) from milk samples as the pH is decreased at 20 °C. Concentrations of both materials in the milk sera are shown. Also demonstrated is the difference between sera produced by ultracentrifugation (O) and ultrafiltration (□) of the same milk samples. Points on the axis represent total Ca and P$_i$ measured after precipitation of the casein at pH 4·6: maximum and minimum values for the range of milks used are shown.

in the supernatant from centrifugation than in the permeate from ultrafiltration, and the corresponding value for P$_i$ was 0·87 mM (both these values for milks treated at 20 °C). The analyses for both Ca and P$_i$ were performed using a specific method which cannot be interfered with by the presence of other materials, so that it is apparent that a genuine difference exists between the centrifuged and ultrafiltered materials. While it would be possible to explain the difference in Ca by postulating that some Ca^{2+} is bound to non-diffusible materials such as caseins (Dalgleish & Parker, 1980; Parker & Dalgleish, 1981) or serum proteins (Baumy & Brulé, 1988), a similar explanation cannot be used for P$_i$. The additional Ca and P$_i$ in the centrifuged serum do not partake in the general stoichiometry (see below) of dissociated material. It is therefore possible that, at some pH values, there is some non-micellar calcium phosphate in a non-diffusible state. At pH values below 5·4 the differences between the estimates of Ca and P$_i$ in the centrifuged and ultrafiltered solutions became much less, indicating that a titration of the materials involved had taken place by that pH value.

Composition of the micellar calcium phosphate

The composition of the calcium phosphate which was dissolved from the casein micelles was similar to previously published estimates, inasmuch as the plots of colloidal Ca against colloidal P$_i$ were approximately linear at pH values above 5·5, and gradients of these plots gave ratios of 1·75–1·85 mol Ca/mol P$_i$ (Fig. 2). These values are in the range of previous values obtained by depleting the mineral fraction of the casein micelles. Chaplin (1984) has quoted a value of 1·61 and Van Hooydonk et al. (1986) a value of 1·66 for the pH-induced depletion, but other values have been measured when the calcium phosphate was dissolved without changing the pH (Holt, 1982, 1·61; Holt et al. 1986, 1·98; Griffin et al. 1988, 1·92). It has been suggested (Holt
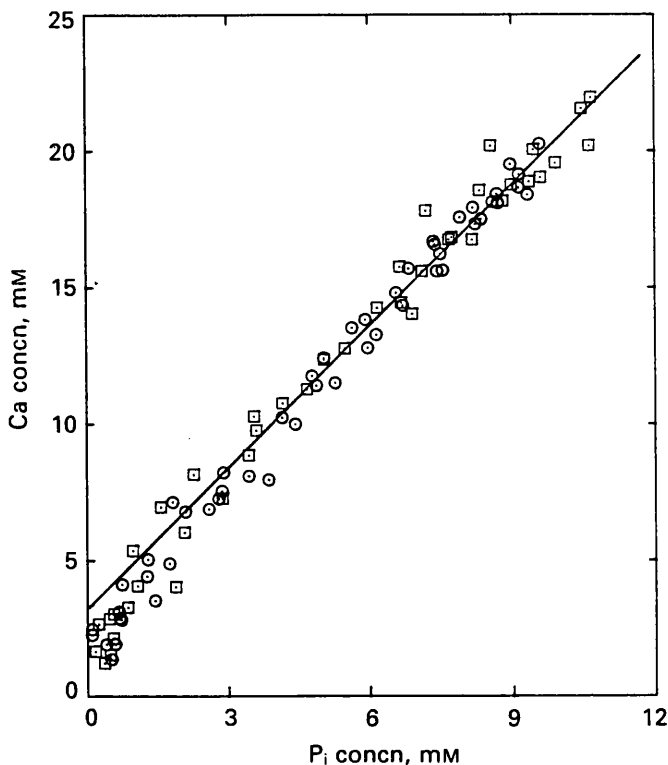
Fig. 2. Concentrations of Ca and inorganic phosphate (P$_i$) in micelles at different pH values compared at 20 °C. Results from centrifugation and ultrafiltration are shown using the same symbols as in Fig. 1.

et al. 1986) that the higher values are likely to be related to the simultaneous release of calcium phosphate and of casein-bound Ca: however this will also apply to experiments where calcium phosphate is dissolved by decreasing pH. The existence of two components in the dissociation pattern is a possibility, since the plot in Fig. 2 is clearly non-linear (see also Van Hooydonk *et al.* 1986) at low concentrations of P$_i$. This is consistent with decreasing pH causing the calcium phosphate to dissolve, but also removing Ca which is not bound to phosphate. We do not know enough about the two effects to be able to separate them, but it appears that the non-specific effect is minimal at high pH values, whereas calcium phosphate is dissolved over all of the pH range (i.e. the pK of the non-specific binding groups is less than that of phosphate).

No significant temperature dependence of the slopes of the plots of Ca against P$_i$ was observed: experiments at all three temperatures showed similar behaviour to that at 20 °C, in respect of the slopes of the plots of Ca against P$_i$ at pH values greater than 5·8 (i.e. on the 'linear' part of Fig. 2). Thus, the composition of the micellar calcium phosphate and the manner of its dissociation appeared to be largely unaffected by the temperature of the milk. However, extrapolation of the linear portion of the plot to P$_i$ = 0 gave a somewhat lower intercept at 4 °C than at the other two temperatures. Because the intercept can be interpreted as that amount of Ca which is bound to casein without being involved in calcium phosphate formation, the smaller value at 4 °C can be explained by reduced amounts of such binding at the lower temperature. All of the intercepts which we measured (between 1·8 and 3·0 mol Ca/mol casein) are lower than the value of about 4 mol Ca/mol protein found by a

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number of other studies (Holt, 1982; Holt et al. 1986; Chaplin, 1984; Van Hooydonk et al. 1986), although the differences are relatively small. The estimates of the intercept also depend on the amount of non-linearity which is in the curve of Fig. 2: the intercept and gradient of the calculated line are in fact dependent on one another.

Mg in the casein micelle

The total concentrations of Mg in the milks were measured to be between 4·3 and 4·9 mM. In the centrifugal supernatants prepared from milks with no added GDL (i.e. at their natural pH of 6·7), concentrations of Mg were between 3·2 and 3·6 mM. Therefore, the average concentration of Mg associated with the casein micelles is of the order of 1·3 mM (cf. Holt, 1982). This micellar Mg was progressively released as the pH was decreased from 6·7 to 4·9, at which point only about 0·2 mM of the Mg remained in the micelles. If the presence of P_i is the determining factor for the amount of Mg in the micelles, the dependence of micellar Mg on micellar P_i is stronger than that shown by Holt (1982) for micelles dissociated by EDTA. However, as for Ca^{2+}, Mg^{2+} may bind to the non-phosphorylated binding sites in the caseins in a pH-dependent way. It appeared from our results that the behaviour of the small amount of micellar Mg paralleled that of the Ca. At pH 4·9, the lowest pH which was used in the experiments, there was still some Ca (and Mg) remaining on the casein (Fig. 2), which was displaced between pH 4·9 and 4·6, although by pH 4·9 there was no micellar P_i remaining.

Dissociation of caseins in relation to micellar calcium phosphate

The results shown above allow us to attempt to relate the extents of dissolution of the micellar calcium phosphate to the solubilization of caseins from the micelle. It is immediately apparent from the results presented in this paper and from the quantitative description of the dissociation of casein, described in our previous publication (Dalgleish & Law, 1988), that the relation between the mineral and protein contents of micelles is complex, especially when the effect of temperature is taken into consideration. We have not been able to substantiate either the assertion of Van Hooydonk et al. (1986) that mainly β-casein was liberated by decreasing the pH at 30 °C, nor that of Griffin et al. (1988) that all caseins were liberated into the serum in their original proportions when micellar calcium phosphate was dissolved by treatment with EDTA. In our experiments at 30 °C, virtually no casein was dissociated from the micelles even at pH values where the calcium phosphate had been extensively solubilized. On the other hand, at 4 °C extensive dissociation of casein occurred even at the natural pH of milk (6·7), and the caseins dissociated even more extensively as the pH was decreased (Rose, 1968; Davies & Law, 1983; Dalgleish & Law, 1988). Thus, 'micellar' casein (i.e. that casein which sediments from the milk under the defined conditions of centrifugation) at 30 °C was independent of the integrity of the micellar calcium phosphate but this was not the case at 20 and 4 °C.

Some support for the pseudo-integrity of the casein micelle during pH decrease has been provided by a study by Rollema & Brinkhuis (1989). Studies of the proton magnetic resonance of casein micelles showed that the caseins remained immobilized as the micellar calcium phosphate was removed by decreasing pH. Conversely, when the solubilization was achieved by the use of EDTA, caseins become more mobile.

The micellar dissociation patterns caused by decreasing pH may be compared with those described by Holt et al. (1986), since these authors provide sufficient detail for an exact comparison to be made of the effects of pH-induced dissociation and of
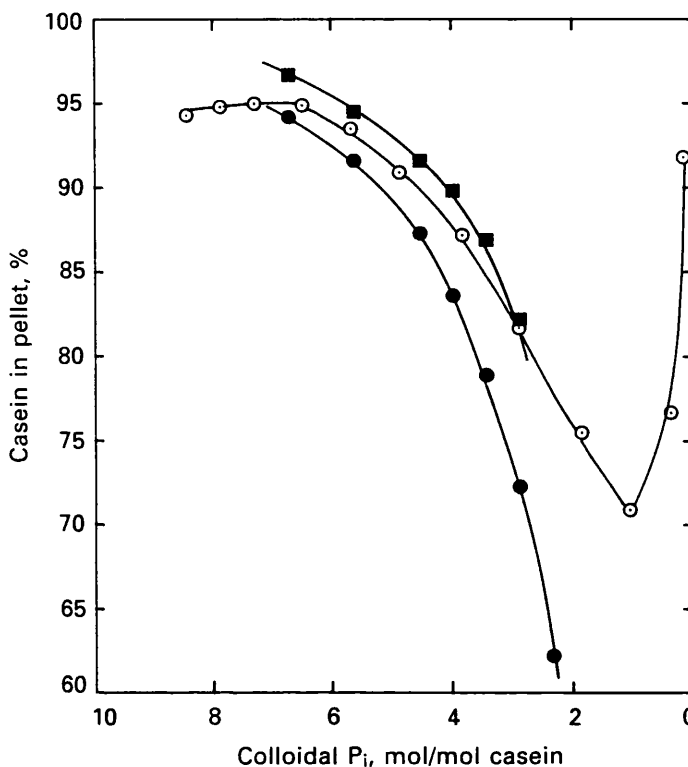
Fig. 3. Comparison of the dissociation of casein from micelles by pH and by dialysis. Micellar casein is shown as a function of the remaining micellar inorganic phosphate (P_i), for experiments involving dialysis against buffers containing 3 mM-Ca^{2+} (●) and 6 mM-Ca^{2+} (■); (Data taken from Holt *et al.* 1986). Experiments involving pH decrease (○) use data from the present work and from the earlier publication (Dalgleish & Law, 1988). All results were obtained at a temperature of 20 °C.

dissociation induced by dialysis of micelles against phosphate-free buffers (Fig. 3). For casein micelles whose calcium phosphate was intact (i.e. in untreated milk), the extent of dissociation of the caseins from the micelle (about 5%) was very similar in both experiments. However, as the amount of colloidal P_i was reduced, it became apparent that the effect of altering pH could not be compared with the effect of dialysis against 3 mM-Ca^{2+}. Less casein dissociated when calcium phosphate was removed by decreasing the pH. At sufficiently low pH, less dissociation was found even by comparison with the effects of dialysis against 6 mM-Ca^{2+} (which is more effective than 3 mM-Ca^{2+} for preventing micellar dissociation). The effects of dissociation caused by pH decrease and by dialysis against 6 mM-Ca^{2+} coincided when the pH was about 5·7 (Fig. 3) at which point (Fig. 1) the amount of micellar calcium phosphate is about one-third of its original value, and the concentration of Ca^{2+} in the serum is about 24 mM. Thus, the effect of pH is not simply to increase the concentration of Ca^{2+} to that which produces the same amount of dissociation in the absence of phosphate. Considerably more Ca^{2+} is dissolved into the serum as the pH drops, and at most only part of the behaviour observed during acidification can be explained by the increase in the serum Ca^{2+} and its binding to non-phosphorylated sites in caseins. The fact that only small differences existed between ultrafiltrate and ultracentrifugate suggested that extensive binding of Ca^{2+} to caseins was not of great significance at the pH values in question. Therefore, although binding of Ca^{2+} to casein is likely to be the cause of the increased retention of caseins in the micelle

between 3 mM and 6 mM-Ca^{2+} found by Holt *et al.* (1986) at neutral pH, it cannot explain the behaviour of the caseins during the pH decrease.

As pH decreases below about 5·5 at 20 °C, any similarity between the pH- and dialysis-induced dissociation disappears, because the isoelectric precipitation of the caseins becomes important in the former process (Fig. 3). Whereas the progressive removal of the micellar calcium phosphate caused only increasing dissociation during dialysis at pH 7·0, the caseins began to reassociate at low pH (Dalgleish & Law, 1988), independently of the amount of calcium phosphate remaining in the micelle (about 1 mol P_i/mol casein at 20 °C).

Therefore, the forces which maintain caseins in the colloidal state are only partly dependent upon the presence of intact micellar calcium phosphate. At 30 °C, it is possible to remove virtually all of the phosphate without dissociating the casein. However, although the casein remains in a sedimentable state at reduced pH, it is perhaps misleading to refer to the particles present as casein micelles, since in composition and structure they differ considerably from the 'native' casein micelles found in milk at its normal pH (Van Hooydonk *et al.* 1986; Visser *et al.* 1986). It has often been assumed that the low solubility of the caseins at 30 °C and the much higher solubility at 4 °C, when the micelles are depleted in calcium phosphate, is a consequence of hydrophobic interactions between the caseins. The fact that a structure remains even when the micelles are partly dissociated may however be evidence for more specific bond formation. It seems to be the case that at 30 °C a number of compensating reactions occur to maintain the casein in a particulate state, which are partly absent at lower temperatures. While hydrophobic interactions will undoubtedly play a part in this, it is also necessary to consider the effects of decreasing charge on the protein, as acidic groups are titrated, and the effect of specific ionic interactions between the proteins as other sources of binding are removed.

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Size-related differences in bovine casein micelles

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The casein micelles from bovine milk have been fractionated by centrifugation to give eight fractions containing particles of different sizes. For each fraction, the contents of whole casein, calcium and inorganic phosphate were determined, and detailed analyses of the casein fraction were made. The results demonstrated the expected increase in the proportion of κ-casein as the micelle size decreased, but also showed that the proportion of α_s-caseins was size-independent. The contents of calcium and inorganic phosphate were also largely independent of micellar size. These results are discussed in terms of their structural implications for the casein micelle.

Introduction

Several studies [1–3] have examined the composition of bovine casein micelles as a function of their size, in attempts to describe the different functions of the four major classes of casein proteins (α_{s1}, α_{s2}, β and κ-caseins). These functions include, for example, the stabilization of casein micelles by κ-casein [4] and the interactions of κ-casein with carrageenans [5]. The fractionation of the original polydisperse micellar fractions can be made most effectively using either differential high-speed centrifugation [3,6], centrifugation in sucrose density gradients [7,8] or permeation chromatography on controlled-pore glass (CPG) [1,2,9]. Of these methods, the last two have been subject to criticism because of the possibility of artificially altering the micellar size distribution.

During fractionation employing CPG, it is distinctly possible for an unsuitable choice of experimental conditions to cause the micelles to dissociate partially, and to release β-casein into the solution. This may create an impression that the smaller micelles, having longer retention times, are enriched in β-casein. This tendency to produce distorted results has been examined in detail by Donnelly et al. [2], and has been shown to occur at temperatures as high as 30°C. Micelles are known to dissociate to an increasing extent at lower temperatures,

and results obtained using CPG-chromatography at temperatures lower than 30°C are likely to be significantly in error, unless the micelles have been fixed with glutaraldehyde. This latter step, however, makes the subsequent analysis for individual caseins virtually impossible.

The generally accepted conclusion of most of the studies is that the proportion of κ-casein in micelles increases as the micellar size decreases [1,2,6,10]. This result has been used in support of models of the casein micelle in which all of the κ-casein is on the micellar surface [11], a conclusion which is partly reinforced by studies of the renneting and alcohol stability of the micelles [12–14]. However, other results suggest that much of the κ-casein is near, rather than on, the micellar surface [4,15], and indeed evidence has been presented for the presence of κ-casein in the interiors of micelles [16,17]. In contrast to the general agreement on the distribution of κ-casein, studies differ in their estimates of the distribution of the other caseins. Micellar fractionation using CPG-chromatography suggested that the contents of both α_s- and β-caseins decreased with decreasing micellar size [2]. This is consistent with a model of the casein micelle having a 'coat' of κ-casein and a 'core' of mixed α_s- and β-caseins [18]. On the other hand, one limited centrifugal fractionation gave four micellar fractions whose contents of α_s-casein were independent of size, but whose contents of β-casein decreased to compensate for the increase in the content of κ-casein [3]. Such results are incompatible with a coat-core model of the micelle, at least if the core is to be considered as being of constant composition.

A further investigation has therefore been undertaken to try to resolve the question of the compositions

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of α_s- and β-caseins in micelles of different sizes. The mineral contents of the different micellar groups were also investigated, to determine the effect of the changing proportions of the different caseins on the calcium phosphate of the micelles.

Materials and Methods

Milk, collected from the bulk tank of the Institute farm after morning milking, was skimmed (3000 × g, 20 min, 20°C) and the casein micelles in the skim milk were fractionated using a sequence of eight consecutive centrifugation steps [19]. The conditions of centrifugation were deliberately different in speeds and times from those used by Davies and Law [3], to obtain a greater number of fractions, and to attempt to circumvent the possibility of β-casein dissociating from the micelle as a result of the high pressure experienced in the centrifuge [20]. After separation, the micellar fractions were collected and dissolved in distilled water at pH 7.0 (to dissociate the micelles), and were then made up to 100 ml. Measurements of calcium and inorganic phosphate were made on these solutions using ion chromatography, and the contents of total and casein nitrogen were estimated by micro-Kjeldahl techniques. Samples of the micellar caseins were obtained by acid-precipitation of the solutions. Analysis of the whole casein fractions into $\gamma_{2,3}$, κ, β, α_{s2} and α_{s1} caseins was performed using fast protein liquid chromatography (FPLC, Pharmacia Ltd.), using the method previously described [21].

The sizes of the micelles in the various fractions were measured in separate experiments using photon correlation spectroscopy (PCS), with light from a He–Ne laser ($\lambda = 632.8$ nm) and a scattering angle of 90° [22]. For these measurements, the micellar pellets produced by centrifugation were resuspended in milk ultrafiltrate and measurements were made within 1 h of resuspension, to avoid any problems of redistribution of particle sizes [8]. We have observed no alteration in the diameters of micelles resuspended in this way, over a period of at least 2 h [15]. The fractions obtained by centrifugation are relatively monodisperse [22], so it is legitimate to consider that the z-average diameters which are measured using PCS may be taken also to be good representations of the number-average diameters. The diameters of the particles were calculated from the measured diffusion coefficients by the Stokes–Einstein relation, assuming that the particles were non-draining systems, although strictly speaking this is not true for casein micelles, which appear to have a partly-draining outer 'hairy' layer [15].

Results

Results from a number of measurements of the composition of the casein fractions are shown in Table I. It is apparent that the casein remaining in the serum is not greatly enriched in β-casein, and so only small quantities of β-casein, if any, are liberated from the micelles by the high pressure during centrifugation. About 5% of the original casein is left in the serum after the final centrifugation step, and of this only some 30% is β-casein. This is in agreement with the conclusion reached by Davies and Law earlier [3]. The different centrifugation conditions and the similarity of our results to the previous work appears to confirm the lack of distortion of the composition of the fractions during different centrifugation steps, and that pressure-induced dissociation is not important during the centrifugal separation.

As expected, the content of κ-casein increased as the micellar size decreased, from 5.7% in the largest micelles to 12.9% in the smallest. This is very similar to the range of values found by Donnelly et al. [2] for CPG-fractionated micelles. However, our results do not agree with those of the latter authors in respect of the α_s- and β-caseins. In our results the increase in the content of κ-casein is almost exactly balanced by a decrease in the content of β-casein, and the proportions of both of the α_s-caseins were independent of micelle size. The de-

TABLE I

Proportions of different caseins in the micellar fractions

Fraction	$\gamma_{2,3}$	A *	B *	κ	C *	β	α_{s2}	α_{s1}	Radius (nm)
1	0.041	0.015	0.010	0.057	0.011	0.392	0.113	0.362	149
2	0.039	0.014	0.010	0.067	0.015	0.374	0.120	0.361	127
3	0.037	0.014	0.011	0.075	0.013	0.370	0.123	0.360	112
4	0.037	0.013	0.011	0.080	0.013	0.362	0.118	0.366	100
5	0.040	0.014	0.011	0.087	0.015	0.353	0.116	0.364	87
6	0.044	0.017	0.012	0.094	0.014	0.347	0.115	0.356	77
7	0.045	0.020	0.011	0.107	0.015	0.335	0.113	0.361	66
8	0.041	0.019	0.011	0.129	0.020	0.314	0.106	0.360	53
Serum	0.059	0.029	0.014	0.201	0.039	0.270	0.072	0.316	–

* Terminology of Davies and Law [21] for undefined casein fractions.

TABLE II

Phosphate and calcium contents of micellar fractions

Fraction	Content (mol per mol casein)		
	Calcium	Inorganic phosphate	$\dfrac{Ca}{P_i}$
1	21.5	11.5	1.87
2	19.1	10.0	1.91
3	19.7	10.6	1.86
4	19.6	10.1	1.94
5	18.8	9.7	1.94
6	19.8	9.7	2.04
7	19.2	9.4	2.04
8	18.5	9.2	2.01

crease in the β-casein content does not arise from proteolysis by plasmin, because the proportion of $\gamma_{2,3}$-casein is effectively constant over all of the fractions. There are three small peaks (named A, B and C in Table I) of unidentified material in all of the casein chromatograms [21]: these may increase to a small but not important extent (since the sum of the three is always less than 0.05 of the total casein) as the micellar size decreases.

The changes in casein composition of the fractions were not accompanied by large changes in their contents of calcium and phosphate. The content of phosphate appeared to decrease to a small extent as the micellar size decreased, but the calcium content remained essentially constant (Table II). The result of this was to increase the ratio of calcium to inorganic phosphate from 1.90 for the largest particles to 2.00 for the smallest. This ratio is rather larger than that suggested for casein micelles [23], although some recent results [24] suggest results of this order. As the micelles decrease in size, the amounts of casein phosphate decrease because the β-casein (five phosphoserines) is replaced by κ-casein (one phosphoserine). This may suggest a lesser involvement of calcium phosphate in micelle formation, and consequently a lower content of phosphate.

Discussion

From the analyses of the casein fractions given in Table I, it is clear that the contents of κ- and β-caseins compensate for one another, and that the content of α_s-casein remains constant as the micellar size changes. The content of κ-casein has been shown to vary with the surface/volume ratio of the particles [1]. However, such surface-to-volume ratios are not dimensionless, and they cannot be used to assign molecules between the surface and interior of the micelle. A simple calculation to describe the distribution of the individual casein molecule between the surface of the micelle and its interior is given as follows.

A casein micelle of volume V and radius R is assumed to be composed of molecules of volume v. The total number of molecules is then

$$N_t = \frac{4\pi R^3}{3v} \tag{1}$$

If the micelle is considered to have a surface layer which is one molecule thick, then this thickness will be $2r$, where r is the radius of a casein molecule, relating to volume by

$$v = 4\pi r^3/3 \tag{2}$$

The volume of the outer shell is given by $4\pi(R - 2r)^2 \cdot 2r$, so that the number of molecules in the surface layer is

$$N_s = \frac{8\pi(R - 2r)^2 r}{v} \tag{3}$$

Thus

$$N_t = \left(\frac{R}{r}\right)^3 \quad \text{and} \quad N_s = 6\left(\frac{R}{r} - 2\right)^2 \tag{4}$$

Although R is measured in experiments, r is not defined. We may relate it to the degree of hydration of the micelle, as follows. If the hydration factor (grams of H_2O per gram protein) in casein micelles is h, the mass of the protein in a micelle is the total weight divided by $h + 1$. If the mass of a casein molecule is m, then the total number of molecules is

$$N_t = \frac{4\pi R^3 \rho}{3m(h + 1)} \tag{5}$$

where ρ is the average density of a micelle. From Eqns. 1, 5 and 2, with $m = 23\,000$ and Avogadro's number equal to $6.02 \cdot 10^{23}$ and taking $\rho = 1.0$ (the value will lie between those of water, 1.00, and of protein, 1.4, the precise value depending on the value of h) we find

$$r^3 = 9.12 \cdot 10^{-21}(h + 1) \tag{6}$$

For the range of h used in the calculations, values of r lie in the range 2.8–3.6 nm, which lies within the limits to be expected for a non-globular protein. Since we are considering the dimensions of protein monomers, these radii are less than the dimensions of caseins in solution [25], where some aggregation has already occurred. For a given estimate of hydration, we may define the surface/volume ratio, on a protein molecular basis,

$$\frac{N_s}{N_t} = \frac{6r}{R} \tag{7}$$

where r may be calculated from Eqn. 6.

For any of the protein types in the micelle, we assume a distribution between the surface layer and the interior. For example because of the relation between κ-casein and surface/volume, it is clear that the κ-casein is mostly to be found in the surface layers of the micelles. Conversely, because the proportion of α_s-caseins is independent of micellar size, the surface layer and the interior of the micelle must contain the same proportion of α_s-caseins. For any of the caseins, an equation of the type

$$\text{fractional content} = \frac{(aN_i + bN_s)}{N_t}$$

$$= a + (b - a)\frac{N_s}{N_t} \qquad (8)$$

can be defined, where a and b are the fractional contents of the casein in the interior and on the surface respectively, and N_i is the number of interior molecules. The values of a and b can be determined by plotting the fractional content of the individual casein against N_s/N_t (as calculated by Eqn. 7) over the range of different micellar sizes. The plots for the total α_s, the ($\beta + \gamma_{2,3}$) and the κ-caseins, calculated using $h = 3.0$ are
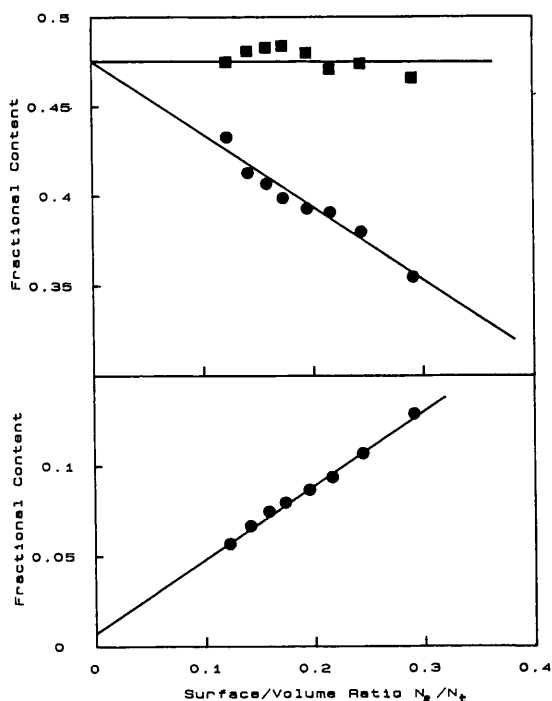
Fig. 1. Plot of the contents of the different caseins in micelles of different sizes, as in Eqn. 8. Top figure: ●, sum of $\beta + \gamma_{2,3}$-caseins; ■, sum of $\alpha_{s1} + \alpha_{s2}$-caseins. Lower figure: ●, κ-casein. A value of $h = 3.0$ g per g was used for the hydration to allow calculation of r.

Hydration (g per g)	κ-Casein		β-Casein	
	interior	surface	interior	surface
5.0	0.006	0.385	0.477	0.102
4.5	0.006	0.391	0.476	0.96
4.0	0.007	0.399	0.476	0.089
3.5	0.008	0.407	0.475	0.080
3.0	0.008	0.417	0.474	0.071
2.5	0.009	0.428	0.473	0.060
2.0	0.010	0.442	0.472	0.046
1.5	0.011	0.460	0.471	0.028
1.0	0.012	0.484	0.470	0.005

shown in Fig. 1. The plots, as predicted by Eqn. 8 are straight lines for all values of h. The plot of κ-casein content against N_s/N_t showed a definite small positive intercept (Fig. 1): this is in contrast to the results shown by Donnelly and coworkers [1,2] which have a negative intercept according to the simple surface/volume calculation (as these authors show, the results of Schmidt et al. [26] also produce a negative intercept): such a phenomenon is not physically reasonable. The plots confirm that the κ- and β-casein fractions vary with the surface/volume ratio, but that the α_s-caseins do not. The proportions of the latter caseins in the surface layer and in the interior of the micelle are constant at 0.475. Thus, approximately half of the surface layer and of the interior are composed of the two α_s-caseins.

The distribution pattern for the β- and κ-caseins between the surface and the interior depends on the value taken for micellar hydration, although not strongly so. No definite agreed value is available for micellar hydration, although a value of between 2 and 3 g per g appears to be most likely [27]. Table III shows that some general conclusions may be drawn on the positions of these two caseins. Although the amounts vary to a small extent with hydration, it is clear that virtually all of the κ-casein will be found on the surface of the micelle. The calculated content of the κ-casein in the interior of the micelle is between 0.6 and 1.2%. Conversely, about 47% of the interior casein is β-casein (+γ-casein). The interior of the micelle is therefore composed of equimolar amounts of β- and α_s-caseins, with only small amounts of κ-casein: it is perhaps this small amount which produces the results found in previous studies [16,17].

Calculations of the proportions of the different caseins on the surface depend rather more on the value chosen for hydration (Table III). κ-Casein can be calculated to form between 39% and 48% of the surface, and β-casein between 10% and 0%. Whatever values are taken, it is clear that the surface of the micelle is predominantly composed of κ- and α_s-caseins, with only small amounts of β-casein. It has been suggested

previously that all of the casein types are present on the micellar surface [28], and our results confirm this, although the earlier work did not indicate the small amount of β-casein. The presence of β-casein in the surface layer is compatible with the observation of Chaplin and Green [29] that some β-casein can be hydrolyzed by immobilized carboxypeptidase.

The presence of α_s-caseins on the micellar surface need not alter the principle that κ-casein is responsible for sterically stabilizing the micelle, and indeed only a small amount of the κ-casein may be involved in this activity [4,15]. It is perhaps this α_s-casein component which is responsible for the calcium-dependence of the aggregation of renneted casein micelles [30], since the para-κ-casein moieties on the surface of the micelle are believed to act via hydrophobic interactions.

It is not clear how much these results and calculations are relevant to the question of whether or not caseins form submicellar particles. The calculation above is not predicated on either the presence or absence of submicelles, and it is not apparent that the overall conclusions would be greatly altered by postulating a submicellar structure, apart from the fact that the composition of the surface layer as we define it would correlate with the composition of layers deeper within the micelle. Clearly, if casein micelles are formed of subunits which themselves are random associations of different caseins [11,31], then the distribution of caseins among micelles of different sizes can tell us nothing about the possibility of submicelles. More fixed submicellar structures, as postulated by Ono et al. [32,33] seem unlikely to give the distribution which we determine, but it is possible that the casein aggregates described by Britten et al. [34] may better fit the observed distribution of caseins (although not to give a micellar model as described by Paquin et al. [35]). The question of the substructure of the casein micelle must remain, as before, open.

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6

CHANGES IN THE MILK PROTEINS DURING PROCESSING

1. HEAT DENATURATION OF THE WHEY PROTEINS

1.1 Introduction

Milk is normally subjected to heat treatment to reduce the growth of microorganisms, or to modify its processing properties. Additional heating may also be required at several different stages in the manufacture of evaporated products, milk powders and caseinates. The most commonly used commercial heat treatments, in order of increasing temperature, are thermisation (50-65°C), pasteurisation (65-75°C), functionalisation (75-90°C) and sterilisation (90-140°C).

Thermisation is used to improve the keeping quality of milk at refrigeration temperatures; it is carried out by heating the milk between 50 and 65°C for 10 to 20 s. The treatment is believed to denature bacterial enzymes, and prevent the subsequent growth of psychrotrophic bacteria.

Pasteurisation consists of heating milk below boiling point, but at a temperature sufficiently high to kill all pathogenic microorganisms and about 99% of other bacteria. In the high-temperature short-time (HTST) process, milk is typically heated at 72°C for about 15s.

At functionalisation temperatures, the main purpose of heating is to denature the whey proteins, and cause a change in the properties of a particular product. For example in the manufacture of yogurt, heating at 85°C for 30 to 45 min, or 90-95°C for 5 to 10 min gives an improvement in the texture of the yogurt (Tamime and Robinson, 1988). It has also been found that the heat stability of concentrated milks is improved by preheating at 85°C for about 30 min. This treatment can be used to alter the viscosity of products such as sweetened condensed milk. Also, in the manufacture

Table 19. Changes in proteins on heating milk

1. <u>MILD HEATING (20-30°C)</u> β-Lg dimer $\rightarrow$ monomer[a]

2. <u>THERMISATION (50-65°C)</u>   Reversible denaturation[b]
     serum albumin   (62.2°C)

3. <u>PASTEURISATION (65-75°C)</u>   Reversible denaturation[b]
     α-Lactalbumin   (65.2°C)
     Lactoferrin   (69.0°C)
     β-Lactoglobulin   (72.8°C)
     Immunoglobulins (72.9°C)

	% Irreversible Denaturation[c] 72°C/5min
Immunoglobulins	56.4
Serum albumin/lactoferrin	46.2
β-Lactoglobulin	11.0
α-Lactalbumin	7.9

4. <u>FUNCTIONALISATION (75-90°C)</u>

	% Irreversible Denaturation[c]	
	80°C/5min	90°C/5min
Immunoglobulins	88.5	91.6
Serum albumin/lactoferrin	77.5	86.1
β-Lactoglobulin	43.0	87.2
α-Lactalbumin	17.8	43.2

5. <u>STERILISATION (90-140°C)</u>

	% Irreversible Denaturation[c] 5min			
	100°C	110°C	120°C	140°C
β-Lactoglobulin	93.7	94.7	96.0	96.0
α-Lactalbumin	66.8	78.9	91.5	96.5

[a] Georges and Guinand (1960); [b] Ruegg *et al.* (1977); [c] Law *et al.* (1994)

of cheese, it is possible to obtain a substantial increase in yield by a prior heat treatment which causes denaturation of the whey proteins and their incorporation into the curd. This is discussed in detail below.

Sterilisation allows storage of dairy products for long periods without refrigeration, and typically involves heating milk between 90 and 140°C. Older methods of sterilisation usually caused complete denaturation of the whey proteins, and gave marked changes in the caseins, including Maillard reaction with lactose, and cooked flavours. In the ultra-high temperature (UHT) process, milk is usually heated between 130 and 150°C for between 1 and 5s, and the levels of denaturation of the whey proteins and modification of the caseins are lower.

In this study (Paper 18, Law *et al.*, 1994) milk was heated at temperatures between 72 and 140°C, for between 15s and 5 min. The extent of denaturation of the whey proteins was determined by gel permeation FPLC as described in Chapter 4. The effect of heating on the caseins was examined by ion-exchange FPLC and PAGE, and is discussed in Section 4.

Denaturation of the whey proteins occurs when milk is heated above about 60°C, and involves unfolding of the compact globular proteins into less organised structures, followed by hydrophobic and disulphide interaction with the casein micelles. The unfolding is reversible if the heating is mild, but on severe heating it is usually followed by protein aggregation, and a loss of solubility at pH 4.6. The two processes of unfolding and aggregation are quite distinct, however, and may occur to different extents depending on the pH of heating, protein concentration and ionic strength. These two stages are described below.

1.2 Conformational Changes in the Whey Proteins on Heating

Several methods have been applied to study the unfolding process during heat-denaturation. Georges and Guinand (1960), using light scattering, showed that β-lactoglobulin exists mainly as dimers in milk at 20°C but dissociates to the monomer form as the temperature is raised above 30°C. Dupont (1965) used optical rotatory dispersion, and established that initially a reversible change occurred in the

conformation of monomeric β-lactoglobulins A and B at 66.5 and 67.5°C respectively, and that this was followed by irreversible polymerisation. Extending this work, Mills (1976) examined unfolding of β-lactoglobulin by measuring differences in the intensity of the fluorescence of tryptophan. As the temperature was raised above 50°C, one of the tryptophan residues became accessible, and this was reversed on cooling to 20°C. On heating above 70°C, the second tryptophan became exposed, but this conformational change was not reversed on cooling; the author attributed this to polymerisation through disulphide bonds.

Rüegg *et al.* (1977) used differential scanning calorimetry (DSC) to examine unfolding of the whey proteins on heating. The results gave information about the enthalpy changes accompanying the thermal transitions of the proteins, and the temperature of maximum heat absorption was taken as the temperature of denaturation. These workers found that the denaturation temperatures of the whey proteins were: serum albumin, 62.2; α-lactalbumin, 65.2; lactoferrin, 69.0, β-lactoglobulin, 72.8, and immunoglobulins, 72.9°C. The temperatures and the order of denaturation were different from those obtained by methods based on loss of solubility at pH 4.6. Rüegg *et al.* (1977) also measured renaturation of the whey proteins on cooling; they showed that for α-lactalbumin, which is normally considered to be the most heat-resistant of the whey proteins, this occurred readily. It has been suggested that α-lactalbumin renatures because initially it has no free -SH group which can form aggregates. β-Lactoglobulin, on the other hand, has a free -SH group, and readily undergoes irreversible denaturation involving disulphide linkage.

De Wit and Swinkels (1980) also used DSC, and found a similar denaturation temperature of 70.4°C for β-lactoglobulin. They also found that aggregation of denatured whey proteins was markedly increased when heating was carried out in the presence of Ca^{2+}. A further effect of heating was observed by DSC near 130°C, which they attributed to unfolding of residual protein structure.

Several workers have measured changes in the surface hydrophobicity of the whey proteins by following the binding of a fluorescent hydrophobic dye to accessible hydrophobic sites (Pagliarini *et al.*, 1990; Bonomi and Iametti, 1991; Regester *et al.*, 1992). One of the advantages of this method was that it could be used to examine the changes occurring at high temperatures, rather than after cooling. This is discussed

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more fully below in relation to hydrophic interaction of the whey proteins and the caseins.

1.3 Interaction of Denatured Whey Proteins and Caseins

Rowland (1937a,b) showed that when milk was heated above 90°C and subsequently adjusted to pH 4.6 at room temperature, the denatured whey proteins precipitated together with the caseins. The denatured whey proteins also precipitated at pH 4.6 in the absence of the caseins. It appeared, therefore, that the conformation of the whey proteins changed on heating and they became insoluble near their isoelectric points. There is a considerable amount of evidence that in heated milk, the denatured whey proteins become closely associated with the casein micelles, initially through hydrophobic interaction and then by disulphide linkage. This is important commercially in that denatured whey proteins can be recovered together with casein by acid precipitation, as co-precipitate. Also, in the manufacture of cheese, the denatured whey proteins remain associated with the micelles during rennet coagulation and are retained in the curd, giving a substantial increase in yield. The nature of the hydrophobic and disulphide interaction between denatured whey proteins and casein micelles is described in more detail below.

1.3.1 Hydrophobic interaction

In a study of the interaction of β-lactoglobulin and κ-casein in the micelles, Smits and van Brouwershaven (1980) showed that although disulphide bonding was important in the complex, the ionic strength, Ca^{2+}, concentration of the proteins, and pH also affected the degree of association. This indicated that hydrophobic interaction might also be involved in formation of the complex.

Pagliarini *et al.* (1990) investigated the effect of commercial heat treatments on denaturation of the whey proteins, and on the accessibility of hydrophobic groups on the surface of the proteins in the serum. They found that whey proteins in unheated

milk had little affinity for a hydrophobic, fluorescent dye (ANS). On heating milk above 70°C, however, the whey proteins became denatured, and had higher affinity for the fluorescent dye. On more severe heating, the affinity for the dye again decreased. These results showed that the whey proteins had low surface hydrophobicity in their native state because hydrophobic groups, which helped maintain their structure, were buried inside. On heating above 70°C, however, the proteins began to unfold, and the hydrophobicity of their surfaces increased. On more prolonged heating, aggregation occurred, and effectively the surface hydrophobicity decreased.

Regester *et al.* (1992) also studied the effect of heating milk on the increase in surface hydrophobicity of the whey proteins, as measured by the binding of hydrophobic *cis*-parinaric acid. On heating between 68 and 80°C, β-lactoglobulin showed a larger increase in surface hydrophobicity than α-lactalbumin. Also, in the manufacture of Cheddar cheese from heated milk, there was a close correlation between the increase in surface hydrophobicity of β-lactoglobulin and α-lactalbumin and their incorporation into the rennet curd.

Haque and Kinsella (1988) examined formation of the complex when κ-casein and β-lactoglobulin were heated at 70°C; they found that, in the initial stage, hydrophobic interaction maintained the complex, and covalent bonds were formed later. Aggregation of the mixture of κ-casein and β-lactoglobulin occurred within about 1.5 min, and susceptibility to chymosin decreased significantly within 3 min, but covalent bonding could not be detected until after about 1 h.

1.3.2 Disulphide linkage

Disulphide linkage of denatured whey proteins and casein micelles has been examined by a variety of techniques. Using alkaline PAGE, Purkayastha *et al.* (1966) showed that when milk or a solution of κ-casein and β-lactoglobulin was heated, a complex of the two proteins was formed. In the absence of a reducing agent such as 2-mercaptoethanol during electrophoresis, the intensity of the κ-casein and β-lactoglobulin bands decreased, and a diffuse band representing the complex was produced. Using reducing conditions during electrophoresis, only separate κ-casein and β-lactoglobulin

bands could be seen. Also, when κ-casein was alkylated to form the S-carboxyamidomethyl derivative, and then heated with β-lactoglobulin, no complex was formed. The results indicated that the complex between the two proteins involved disulphide linkage. Similar early studies have confirmed the heat-induced association of κ-casein and β-lactoglobulin; these have been reviewed by Sawyer (1969).

Results from electron microscopy (Davies *et al.*, 1978) showed that when milk was heated at 95 °C for 10 min, the micelles acquired filamentous appendages. Heating micelles in protein-free ultrafiltrate or in the presence of α-lactalbumin had no effect. Heating micelles with β-lactoglobulin caused formation of the appendages, but this could be prevented by addition of an alkylation agent (N-ethylmaleimide), indicating that the β-lactoglobulin was usually disulphide linked. Extending this work, Mottar *et al.* (1989) showed by means of electron microscopy and immunochemical methods that both β-lactoglobulin and α-lactalbumin were present on the surface of the micelle. β-Lactoglobulin was more readily denatured and formed filamentous appendages, but at higher temperatures α-lactalbumin also was denatured and tended to fill the spaces between the appendages, giving the micelles a smoother surface.

From turbidity measurements, Jeurnink (1992) showed that when milk was heated above 60°C there was an increase in micellar size. This was due to formation of complexes of β-lactoglobulin with the micelle surface; the amount of complex increased with the concentrations of β-lactoglobulin and calcium phosphate.

Smits and van Brouwershaven (1980), using ultracentrifugation and ^{3}H-labelled β-lactoglobulin, showed that β-lactoglobulin became associated with casein micelles on heating. They suggested that initially hydrophobic interactions were involved, but found that disulphide bonds were important in maintaining the complex between κ-casein and β-lactoglobulin.

Using radiolabelled whey proteins, Noh and Richardson (1989) carried out a detailed study of the incorporation of heat-denatured β-lactoglobulin and α-lactalbumin onto casein micelles, and subsequently into the curd obtained on renneting. As the extent of denaturation of β-lactoglobulin and α-lactalbumin increased, their concentrations in the serum decreased, and the amounts associated with the micellar fraction increased. This work also clearly showed the possibilty of increasing cheese yield by incorporating denatured whey proteins into the curd. In a later study, Noh *et*

al. (1989) showed that on heating skim milk at 95°C, both β-lactoglobulin and α-lactalbumin became associated with κ-casein in the micelles. Using gel permeation on Sephacryl S-300, together with radiolabelled κ-casein and whey proteins, they were able to separate a micellar fraction, which became progressively richer in β-lactoglobulin and α-lactalbumin as the time of heating at 95°C increased. Further examination of the resultant complex showed that it could be fully dissociated into monomers in the presence of 2-mercaptoethanol and guanidine HCl, but not in the absence of the reducing agent. This indicated that the complex of κ-casein, β-lactoglobulin and α-lactalbumin was disulphide linked. When β-lactoglobulin and α-lactalbumin were heated together with the other whey proteins, only monomers, dimers and a very small amount of larger aggregates were formed. This confirmed that the complex formed on heating was mainly between the whey proteins and the casein micelles.

1.4 Determination of the Extent of Denaturation

Because of the stability of the complex formed by caseins and denatured whey proteins, the whey proteins precipitate together with the caseins at pH 4.6, and it is possible to use their change in solubility as a measure of the extent of denaturation. Rowland (1937a,b) showed that when milk was heated at temperatures above 90°C for more than 5 min, there was an increase in the amount of protein which precipitated when the pH was adjusted to pH 4.6. At the same time, the concentrations in the serum of the albumin and globulin fractions, which are now known to comprise the main whey proteins, decreased. On heating at 90°C for between 30 and 60 min, denaturation of the main whey proteins was complete; the amount of soluble nitrogen at pH 4.6 reached a constant value representing about 20% of the total original soluble protein. The heat-stable proteose-peptone fraction remaining is now known to be a complex mixture of polypeptides derived mainly from caseins (Andrews and Alichanidis, 1983).

Using the above method of selective precipitation, together with moving boundary electrophoresis, Larson and Rolleri (1955) showed that when milk was heated at temperatures between 56 and 96°C for 30 min, there was a progressive decrease in

the concentrations of the main whey proteins remaining soluble at pH 4.6. Taking this loss of solubility as a measure of denaturation, they found that the immunoglobulins and serum albumin were most easily denatured, β-lactoglobulin was intermediate, and α-lactalbumin was least heat sensitive. Unfortunately, although moving boundary electrophoresis gave good resolution of the individual whey proteins, it was a difficult technique to use.

Lontie and Préaux (1967) fractionated whey proteins by gel permeation chromatography on G-100 Sephadex. They were able to compare the effects of various heat treatments, including pasteurisation and sterilisation, on denaturation of the individual whey proteins. The method was laborious, however, and separations typically required about 12h.

Several groups have used the more rapid technique of gel permeation HPLC to examine the extent of denaturation of the whey proteins in whey concentrate (Li-Chan, 1983) and in skim-milk powders (Gupta, 1983; van den Bedem and Leenheer, 1988). Gel permeation FPLC was used by Andrews et al. (1985) to examine the composition of whey proteins in raw milk and whey, and gave good resolution of the minor whey proteins, but poorer resolution of β-lactoglobulin and α-lactalbumin.

Anion-exchange FPLC has also been applied in the study of heat-processed milks (Manji and Kakuda, 1987). One advantage of the method was that the A and B genetic variants of β-lactoglobulin could be quantified, but separation of the minor fractions tended to be poor.

Reverse phase HPLC has been used to study whey protein denaturation (Parris and Baginski, 1991; Bican and Spahni, 1991; Luf et al., 1992; Kessler et al., 1992). Most of these methods also resolved the A and B genetic variants, but there was some indication that, under certain conditions, recovery of whey proteins was incomplete (de Frutos et al., 1992). Also, since the hydrophobicity of the whey proteins is known to change on heating, the elution positions of the denatured proteins could be different from the original proteins.

Quantitative PAGE has also been used to study the kinetics of the denaturation of the whey proteins (Hillier and Lyster, 1979; Chaplin and Lyster, 1986; Dannenberg and Kessler, 1988). This is discussed in more detail below.

In the present work, denaturation of the whey proteins was determined by gel

permeation FPLC, as described in the first paper in Chapter 4 (Law *et al.*, 1993). Whey protein was separated into four main fractions containing immunoglobulins, serum albumin and lactoferrin, β-lactoglobulin and α-lactalbumin. The concentrations of the individual whey proteins, before and after heating, were determined in acid filtrate. This measure of denaturation was, therefore, based on loss of solubility at pH 4.6 caused by aggregation of the denatured whey proteins, rather than on the degree of unfolding of the whey proteins, as would be measured, for example, by direct scanning calorimetry. There is some justification in using this definition of denaturation, in that the loss of solubility at pH 4.6 was found to be closely related to the extent to which denatured whey proteins were incorporated into the curd during the manufacture of cheese from heat-treated milk. This is discussed in more detail below.

The first paper in this section describes the effect of temperature, heating time and pH of heating on the degree of denaturation of the four whey protein fractions in cows' milk. Results showed that the order of denaturation of the whey proteins was immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. This was in agreement with other results based on loss of solubility, but differed from results obtained by direct scanning calorimetry, which reflect the degree of unfolding of the proteins. α-Lactalbumin, in particular is able to renature and, therefore, appears more stable on the basis of solubility than indicated by direct scanning calorimetry.

Results showed that when milk was heated at 70°C for 15s, there was a small amount of denaturation of the immunoglobulins and the serum albumin/lactoferrin fraction. Commercial heat treatments such as pasteurisation or thermisation, therefore, may lead to changes in the properties of the higher molecular weight whey proteins. β-Lactoglobulin and α-lactalbumin did not seem to be appreciably denatured by these heat treatments. At higher temperatures (functionalisation temperatures), or with longer holding times, there were substantial changes in all of the whey proteins, except the proteose peptone fraction. On heating milk at 90° for 5 min, for example, most of the immunoglobulins, serum albumin/lactoferrin, and β-lactoglobulin were denatured, but only about 40% of the α-lactalbumin. On more severe heat treatment, as would occur during sterilisation, the whey proteins were completely denatured; there were other marked changes in both the whey proteins and the caseins (Section 4).

2. INCREASE IN CHEESE YIELD BY INCORPORATION OF DENATURED WHEY PROTEINS

2.1 Introduction

In traditional cheese making, milk is converted into a gel by the addition of rennet, which splits off the caseinomacropeptide from the micelles, and causes their aggregation. The caseins and fat are retained in the curd, but the milk serum and the whey proteins, which amount to about 20% of the total milk protein, are expelled during subsequent syneresis. The whey proteins, however, have a high content of essential amino acids. Because of the nutritional value of the whey proteins, and the considerable potential for increasing cheese yield, various methods have been developed for their incorporation into the rennet curd.

2.2 Methods for the Incorporation of Whey Proteins into Cheese

Maubois and Mocquot (1975) used an ultrafiltration membrane to concentrate the casein and fat before the addition of rennet during the manufacture of soft or ripened cheeses. The whey proteins were physically entrapped in the curd giving an increase in yield, on the basis of protein, of about 16-20%. This technique was also used by Chapman *et al.* (1974) for the production of hard cheeses such as Cheddar and Cheshire. Brown and Ernstrom (1982), on the other hand, concentrated cheese whey by ultrafiltration, denatured the whey proteins by heating at 75°C for 30 min, and then added the whey protein back to milk for the production of cheese. This gave a substantial increase in yield without significant loss of cheese quality.

Banks and Muir (1985) denatured whey proteins in whey concentrate by heating at 95°C for 20 min, and then precipitated the whey protein by acidification to pH 4.5. On adding the precipitate of whey proteins to cheese milk, they were able to prepare Cheddar cheese with satisfactory texture and flavour.

In each of the above methods, the whey proteins were physically entrapped in the rennet curd. Marshall (1986), however, heated cheese milk at 97°C for 15s to

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denature the whey proteins. During this heat treatment the denatured whey proteins became associated with the casein micelles, through hydrophobic and disulphide interaction, as discussed above. On adding rennet, the denatured whey proteins were incorporated directly into the curd. This association of the whey proteins and caseins tended to inhibit gelation following the action of rennet, but on reducing the pH of renneting, and adding calcium, normal coagulation and curd-firming was restored. In the manufacture of Cheshire cheese, an increase in yield of 4.5%, on a dry mattter basis, was obtained.

Banks (1988, 1990) also increased the yield of Cheddar cheese by heating milk at 90°C for 60s to denature the whey proteins. As found by Marshall (1986) and Harper et al. (1989), the complex of whey proteins and κ-casein tended to inhibit gelation, following addition of rennet. This problem was overcome by reducing the pH of renneting. At low pH, more rennet was retained in the curd, and tended to cause bitter flavour. On reducing the amount of rennet, however, the ripened cheese had an acceptable flavour and texture. The cheese also had the unusual property that, on heating, the fat appeared to be bound to the protein, and was not released as in normal Cheddar. This was shown to be due to the presence of denatured whey proteins.

In most of the above studies, the retention of whey proteins was measured directly as the increase in yield, on a dry matter basis. Singh et al. (1988), however, determined retention of the whey proteins in the curd indirectly, by measuring the concentration of nitrogen in the whey. Using this method they found that about 50% of the whey proteins could be transferred to the curd in heated milks. There was an increase in the rennet coagulation time after heating, but this could be reduced by acidification to less than pH 6.0, or by acidification to pH 5.5, followed by re-neutralisation to pH 6.6. A similar decrease in renneting time was achieved by the addition of $CaCl_2$, and this indicated that in each of the above procedures the decrease in renneting time was achieved by an increase in serum Ca^{2+}.

In the present study, incorporation of denatured whey proteins into Cheddar cheese produced from heated milk has been examined in detail. Results presented in the first paper in this chapter (Paper 18, Law et al., 1994) showed that as the temperature of heating and holding time increased, there was a decrease in the amounts of the individual whey proteins remaining soluble after acidification to pH 4.6. There

was also a corresponding decrease in the amounts of the individual whey proteins remaining in the whey after rennet coagulation, and there was a close correlation between the loss of solubility at pH 4.6 of the whey proteins, and the extent of their retention in the curd during the manufacture of Cheddar cheese. Results showed, therefore, that FPLC gel permeation was a suitable technique for determining the degree of denaturation of the individual whey proteins during heat treatment, and the extent of their subsequent incorporation into cheese.

2.3 Effect of pH of Heating on Denaturation of Whey Proteins

A further study was carried out to examine the effect of the pH of heat-treatment on the incorporation of whey proteins into Cheddar cheese. The effect of the pH of heating on both the unfolding and aggregation steps during denaturation of the whey proteins has been examined previously. Using direct scanning calorimetry, Bernal and Jelen (1984, 1985) measured the temperatures of conformation change in the whey proteins; they found that as the pH was reduced from 6.7 to 3.5, α-lactalbumin was more easily denatured, whereas β-lactoglobulin and serum albumin were less readily denatured. Using the same technique, Rüegg et al. (1977) showed that β-lactoglobulin unfolded more readily as the pH of heating was raised from 6.46 to 7.25.

Lyster (1970) studied the effect of pH of heating on denaturation of β-lactoglobulin and α-lactalbumin; he found that within the range 6.2-6.9, the rates of denaturation, as measured by changes in solubility at pH 4.6, were independent of pH of heating. Outside this range, however, the rates of denaturation increased.

Singh et al. (1988) measured the incorporation of whey protein into rennet curd prepared from milk heated between pH 6.5 and 6.8. There was increased incorporation of the whey proteins with increasing temperature on heating for 10 min between 70 and 90°C, at all values of pH. Maximum incorporation of whey proteins occurred at pH 6.5-6.6, and decreased as the pH increased.

Results showed, therefore, that unfolding of some whey proteins occurs more readily at alkaline pH, whereas aggregation of the denatured whey proteins tends to be inhibited, possibly because of the increase in negative charge on the proteins at higher

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pH.

In the present study (Paper 18, Law *et al.*, 1994), milk was heated between pH 6.2 and 9.1, at 90°C for 30s, but was then re-adjusted to pH 6.7. On the basis of loss of solubility at pH 4.6, determined by gel permeation FPLC, the levels of denaturation of β-lactoglobulin, α-lactalbumin and total whey protein were lowest at pH 6.5, 6.4 and 6.3 respectively. The levels of denaturation were slightly higher at pH 6.2, but increased markedly at alkaline pH. The most rapid increase in the degree of denaturation of β-lactoglobulin occurred between pH 6.5 and 8.0, whereas that for α-lactalbumin was between 7.3 and 9.2; there was an overall increase in the denaturation of whey proteins amounting to about 24% of the total, indicating that greater incorporation of whey proteins into rennet curd may be achieved at alkaline pH. This study also indicated that it may be possible to reduce the severity of heating, and obtain the same increase in cheese yield by heating the milk at alkaline pH. These results differ from those of Singh *et al.* (1988) who obtained a decrease in the whey protein retained in the curd as the pH of heating was increased from 6.5 to 6.8. This difference may arise because, in the present study, the milk was re-adjusted to pH 6.7 after heating, and then stored at 4°C for 17h. This treatment would reduce the negative charge on the caseins, increase the serum calcium concentration, and promote the aggregation of denatured whey proteins with the casein micelles.

3. COMPARISON OF DENATURATION OF BOVINE, CAPRINE AND OVINE WHEY PROTEINS

3.1 Introduction

Although a considerable amount of work has been carried out on the heat denaturation of bovine whey proteins, comparatively little has been done on caprine and ovine whey proteins. Milk from the goat and sheep, however, are commonly used for the manufacture of cheese and, as found for cows' milk, the yield may be increased by incorporating denatured whey proteins into the curd. Similarly, in the manufacture of yogurt from these milks, controlled denaturation of the whey proteins leads to their interaction with the casein micelles, and modification of the texture and viscosity of the product. Detailed information about the relative rates of denaturation of the whey proteins of the two species is, therefore, required in order to increase the efficiency of these processes.

In one of the few studies that have been carried out into the relative rates of denaturation of the whey proteins of the goat and sheep, Ramos (1978) examined the effect of different heat treatments on the nitrogen fractions in the milk of the cow, goat and sheep, and found that the ovine whey proteins were considerably more sensitive. During pasteurisation at 63°C for 30 min, 15% of the water soluble proteins in ewes' milk were denatured, compared with 2.3% in cows' milk. Whey proteins in goats' milk were unaffected. On closer examination it was found that, under the same conditions, 34% of ovine, and 1.8% of bovine β-lactoglobulin was denatured, but caprine β-lactoglobulin was unaffected. After more severe heat treatments, the differences in protein denaturation among the species were less pronounced.

In this study (Paper 19, Law, 1995), the relative rates of denaturation of the individual whey proteins of the cow, goat and the sheep were measured by gel permeation FPLC as described in Chapter 4. The kinetics of denaturation of the whey proteins of the three species was examined.

3.2 Kinetics of the Denaturation of Bovine Whey Proteins

Various groups have measured the kinetics of denaturation of the major bovine whey proteins. Lyster (1970) used an immunodiffusion method to examine irreversible denaturation of α-lactalbumin and β-lactoglobulin in skim-milk. He found that denaturation of α-lactalbumin was a first order reaction between 90 and 155°C. Denaturation of β-lactoglobulin was a second order reaction, but the reaction was more complex than it appeared. Following second order kinetics, the time taken for β-lactoglobulin to reach 50% denaturation should have varied inversely with the initial concentration. Lyster found, however, that doubling the initial concentration of β-lactoglobulin had no effect on the time taken to reach 50% denaturation. He therefore concluded that with respect to concentration the denaturation followed first order kinetics, but the reaction was second order with respect to time. On constructing Arrhenius plots of the kinetics constants for α-lactalbumin and β-lactoglobulin against the temperature in °K, the lines showed a change in slope at 80 and 90°C, respectively. This indicated that denaturation of the two whey proteins involved a mechanism with at least two successive steps. Lyster considered that both proteins undergo an initial first-order reversible change in conformation, followed by a second order reaction which involves irreversible disulphide linkage.

Using quantitative PAGE, Hillier and Lyster (1979) confirmed these results for the denaturation of α-lactalbumin and β-lactoglobulin, but considered that denaturation of α-lactalbumin was probably a second order reaction displaying pseudo first order kinetics. Denaturation of serum albumin appeared more complex than α-lactalbumin or β-lactoglobulin, and conformed equally well to equations describing first or second order kinetics.

Dannenberg and Kessler (1988) used quantitative PAGE, and carried out a very detailed examination of the denaturation of α-lactalbumin and the A and B genetic variants of β-lactoglobulin. These workers showed that denaturation of α-lactalbumin followed an apparent first order reaction between 70 and 150°C. They found, however, that denaturation of β-lactoglobulin had a reaction order of 1.5 between 70 and 150°C. In common with Hillier and Lyster (1979), they found that Arrhenius plots for α-lactalbumin and β-lactoglobulin gave lines with changes in slope at 80 and 90°C,

respectively. Calculation showed that for α-lactalbumin the activation energy was smaller between 70 and 80°C than between 85 and 150°C. Also the activation energy for β-lactoglobulin was lower in the range 70-90°C than in the range 95-150°C. The authors believed that these results were due to irreversible denaturation proceeding by several reaction steps. In the first reversible step, the protein molecule would unfold; in the second irreversible step aggregation reactions would predominate.

Dalgleish (1990) studied the relation between denaturation of whey proteins and the extent of aggregation. Using chromatography to examine heated milks under non-dissociating and dissociating conditions (EDTA and urea), he established that between 75 and 90°C, the decrease in soluble whey proteins was matched by an increase in aggegated, disulphide linked material. Results showed that denaturation of β-lactoglobulin was a pseudo first order reaction at the higher temperatures, but that at lower temperatures there was a slower initial reaction which preceded more rapid denaturation.

Using reverse phase HPLC, Kessler *et al.* (1992) found that the order of reaction for denaturation of β-lactoglobulin varied with the ratio of casein/whey proteins. In whey, denaturation fitted a reaction order of 2.0, but in milk fitted a reaction order of 1.5. On adding casein to whey, there was a gradual decrease in the reaction order from 2.0 down to 1.5. They concluded that casein played an important part in the denaturation process.

Fewer kinetics studies have been carried out on the minor whey proteins, but Luf *et al.* (1992) studied denaturation of the immunoglobulins by reverse-phase HPLC, and confirmed previous results that they are the most heat sensitive of the whey proteins. They found that the reaction was first order, but an Arrhenius plot showed a change in slope at 76°C, indicating a variation in the reaction mechanism, as found for α-lactalbumin and β-lactoglobulin.

The variation found in literature values of the reaction orders and rate constants may result in part from the use of different techniques to determine levels of denaturation, but could also be due to different heating rates. The occurrence of fractional values for orders of reaction and the change in slope in the Arrhenius plots discussed above indicate that denaturation of the whey proteins is a complex process probably involving several simultaneous reactions with different temperature

dependence. The evidence discussed above suggests that denaturation involves two main steps, namely unfolding of the compact globular structure of the proteins, and irreversible association of the denatured whey proteins with the caseins. Using direct scanning calorimetry, de Wit and Swinkels (1980) examined the kinetics of the unfolding of β-lactoglobulin, as distinct from the aggregation stage. They found that between 65 and 72°C the reaction was first order; they concluded that below about 70°C β-lactoglobulin was unfolding, but above this temperature aggregation was occurring. Different heating conditions may affect the relative predominance of these reactions. In the present study, laboratory heating conditions and levels of denaturation were similar to those obtained previously using pilot-scale heating equipment with a heat-exchanger (Paper 18, Law *et al.*, 1994), and comparable results should be obtained on commercial heating equipment.

3.3 Relative Rates of Denaturation of Bovine, Caprine and Ovine Whey Proteins

In this study (Paper 19, Law, 1995) the relative rates of denaturation of the whey proteins in the milk of the cow, goat and sheep were determined by gel permeation FPLC as described previously for bovine and caprine whey proteins (Paper 11, Law *et al.*, 1993a; Paper 12, Law and Brown, 1994). In preliminary work, the individual fractions of ovine whey proteins were identified by SDS-PAGE; it was confirmed that whey proteins of all three species were separated by gel permeation into fractions containing immunoglobulins, serum albumin/lactoferrin, β-lactoglobulin and α-lactalbumin. On heating the milks at temperatures of 70, 80 and 90°C for times between 0.25 and 30 min, and subsequently adjusting to pH 4.6, it was found that the solubility of the whey proteins of all three species decreased with increasing severity of heating. Based on the loss of solubility at pH 4.6, the ease of irreversible denaturation of the whey proteins of the goat and sheep was, as found previously for bovine whey proteins, in the order immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin.

The orders of reaction and the rate constants for denaturation of the immunoglobulins, β-lactoglobulin and α-lactalbumin were determined by fitting the

data to two expressions derived from the general rate equation, as in previous studies on the kinetics of bovine whey proteins (Hillier and Lyster, 1979; Dannenberg and Kessler, 1988). Results showed that the corresponding whey proteins of the three species also had similar reaction orders for their denaturation, and these were: immunoglobulins 1.5, β-lactoglobulin 1.5, and α-lactalbumin 1.0. It seems likely, therefore, that denaturation of heat denaturation of caprine and ovine whey proteins occurs in the same way as for bovine whey proteins, with an initial unfolding of the whey proteins, an increase in their surface hydrophobicity, and hydrophobic interaction and disulphide linkage with the casein micelles.

Differences were found in the rates of denaturation of the corresponding whey proteins of the three species. On heating at 70°C, or at 80°C with short holding times, caprine whey proteins were less extensively denatured than the corresponding bovine or ovine whey proteins. On more severe heating at 80 and 90°C, the order of ease of denaturation of the whey proteins was usually ovine > caprine > ovine. On heating at 90°C, however, denaturation of the immunoglobulins and serum albumin/lactoferrin occurred rapidly and differences in these proteins were less pronounced.

Some of the disparity in the rates of denaturation of the corresponding whey proteins of the three species may be due to the small differences in the respective sequences of the whey proteins. The amino acid sequences of caprine β-lactoglobulin and ovine β-lactoglobulin A show 95.1% homology with bovine β-lactoglobulin A. Similarly, the amino acid sequences of caprine and ovine α-lactalbumin show 94.3 and 93.5% homology with bovine α-lactalbumin. Studies have shown that small differences in the sequences of bovine β-lactoglobulins are associated with changes in the rates of denaturation. For example, β-lactoglobulin B differs from the A variant by only two residues (64 and 118) but at temperatures below 95°C denatures more readily than the A variant (Hillier and Lyster, 1979; Dannenberg and Kessler, 1988). In the present study, however, on severe heating, all of the whey proteins of the goat and sheep were more easily denatured than those of the cow, indicating that factors other than differences in the amino acid sequences might be having an effect.

As discussed above, Kessler *et al.* (16) have shown that the caseins play an important part in the kinetics of denaturation of the bovine whey proteins. Comparison of the composition and content of the caseins in skim-milk of the three species shows

that the concentrations of caprine and ovine κ-caseins were considerably higher than that of bovine κ-casein. Caprine and ovine κ-caseins also each contain three -SH groups, whereas bovine κ-casein contains two -SH groups. Compared with cows' milk, therefore, caprine and ovine milks contained almost twice the concentration of -SH available for disulphide bonding to the whey proteins. Also, the concentration of caprine β-casein was slightly higher, and that of ovine β-casein considerably higher, than the concentration of bovine β-casein. As β-casein is the most hydrophobic of the caseins, the higher concentration in ewes' milk may promote the initial hydrophobic interaction of denatured whey proteins and the caseins.

Other factors that affect the availability of κ-casein for reaction with the whey proteins may play a part in determining the relative rates of denaturation of the whey proteins of the three species. As indicated above, compared with bovine whey proteins, caprine whey proteins were less readily denatured on mild heat treatment, but more easily at 90°C. This may reflect differences in the structure and heat stability of the micelles of the species, but more work is required to study these factors.

In previous work, using a heat treatment of 90°C for 30s for cows' milk, it was possible to obtain substantial increases in the yield of Cheddar cheese while retaining satisfactory flavour and texture characteristics (Paper 18, Law *et al.*, 1994). From the results of the present work showing the relation between heat-treatment and the extent of irreversible denaturation of the whey proteins, suitable heating conditions could be selected to give comparable increases in cheese yield from the milk of the goat and sheep.

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4. CHANGES IN THE CASEINS AND WHEY PROTEINS AT STERILISATION TEMPERATURES

4.1 Introduction

Milk is heated above functionalisation temperatures during in-container sterilisation (110-120°C), UHT sterilisation (130-150°C), and as a pre-treatment in the manufacture of evaporated milk and heat-stable milk powders. This heat-treatment improves the long term keeping quality by destroying microorganisms, and causes changes in the flavour and processing properties of the milk. Various problems are encountered on heating at high temperature, however, and these include formation of deposits on heat exchangers (Burton, 1968), instability of concentrated milks during heating (Singh and Creamer, 1992), subsequent age-gelation of sterilised milks (Harwalkar, 1992), and production of cooked flavours (Calvo and de la Hoz, 1992).

On heating milk at 110°C for 5 min, most of the whey proteins undergo denaturation in which they lose their globular conformation, become associated with the casein micelles, and become less soluble at pH 4.6 (Section 1). In contrast, the caseins have comparatively open and flexible conformations (Holt and Sawyer, 1993), with no intramolecular disulphide bonding. They show no marked changes at this temperature, apart from the way in which they associate within the micelles, or with the whey proteins. On heating milk above 110°C, as in sterilisation, changes occur in the amino acid residues of the caseins, including loss of phosphate from phosphoseryl residues, reaction of ϵ-amino groups of lysine with carbonyl groups of lactose in the formation of a series of Maillard products, and the formation of lysinoalanine. On prolonged heating, proteolysis may occur, and the level of proteose peptones increases. There is evidence that some of these modifications also occur during the manufacture of other milk products. Changes in functional properties due to processing have been reported for skim-milk powders (Singh and Newstead, 1992), co-precipitates (Southward and Goldman, 1975; Grufferty and Mulvihill, 1987), and sodium caseinates (Muir and Dalgleish, 1987; Schmidt and McNeill, 1993). In the present work, the changes occurring in sodium caseinates (Paper 20, Dalgleish and Law, 1988) and in the caseins in heated milk (Paper 21, Law *et al.*, 1994) were examined. The

modifications that have previously been found in the caseins on heating are discussed below.

4.2 Heat-induced Modifications of the Caseins

4.2.1 Dephosphorylation of the caseins

Howat and Wright (1934) studied the rate of release of phosphorus on heating solutions of sodium and calcium caseinates. After heating at 120°C for 30 min, about 30% of the phosphorus on the sodium caseinate, and 20% of that on the calcium caseinate, was removed. On prolonged heating for 5h, all of the phosphorus was removed from the sodium caseinate, and about 80% from the calcium caseinate. As dephosphorylation occurred, the calcium binding capacity of the caseinates decreased, and the level of Ca^{2+} in solution increased. This increase in Ca^{2+}, combined with the decrease in pH on heating, and the reduction in negative charge on the caseins, promoted the coagulation of the caseinates. These results were confirmed by Pyne (1958) and Davies and White (1958).

Dephosphorylation of the individual caseins has also been examined. Belec and Jenness (1962a) carried out a detailed study of the effect of heating, between 110 and 140°C, on the release of phosphorus from solutions of whole, α- (α_{s1}-, α_{s2}- and κ-) and β- sodium caseinates. They found that the reaction was first order, but that the removal of phosphorus from β-caseinate was slower than for whole or α-caseinate. The rate of hydrolysis of phosphorus was unaffected by changes in acidity between pH 6 and 7.

Various studies have shown that dephosphorylation causes changes in the properties of the caseins. In a study of the behaviour of dephosphorylated caseins in the micelle, Pepper and Thomson (1963) showed that dephosphorylated κ-casein retained its stabilising ability, whereas dephosphorylated α_s- (α_{s1}-and α_{s2}-) was less easily stabilised by κ-casein.

Guo et al. (1989) measured the release of phosphate on heating a solution of sodium caseinate; they found that as heating was increased from 110 to 140°C for 60 min, the amount of phosphate rendered soluble increased from 20 to 100% of the total.

These workers were able to relate the changes in the level of phosphorylation to different behaviour of the caseins on anion-exchange chromatography.

Similar changes occurred in milk. Continuing their studies on milks heated between 110 and 140°C, Belec and Jenness (1962b) found that there was extensive hydrolysis of phosphorus from the caseins. For example, after heating at 120°C for 10 min, about 8% of the phosphorus was removed from the caseins, and at 140°C about 30% was released. Removal of phosphorus was slower in milk than in caseinate solutions, and these workers concluded that the inhibition was caused by Ca^{2+} ions.

4.2.2 Maillard reaction

The Maillard reaction in milk is a complex series of chemical changes in which a condensation initially occurs between free amino groups on proteins, especially the ϵ-amino group of lysine, and the carbonyl group of lactose. The intermediates are colourless, but the final products are brown pigments (melanoidins) which, after severe heating, may impart unpleasant flavours to milk products. Browning is not usually obvious after UHT processing, but may occur during in-container sterilisation of milk (Burton, 1984). A number of intermediates are formed in the Maillard reaction; some of these such as hydroxymethylfurfural, furosine, pyridosine and lactulose have been used to determine the extent of heat-damage in milk (Resmini *et al.*, 1992; Hewedy *et al.*, 1994). The formation of these compounds has been extensively reviewed (Olano and Martinez-Castro, 1989; O'Brien and Morrissey, 1989; Erbersdobler and Dehn-Müller, 1989; Andrews, 1989).

One of the main consequences of the Maillard reaction is that the amount of lysine available for nutrition is reduced. Fox (1981) found that there was an almost linear decrease in the dye binding capacity when milk was heated at 140°C; after heating for 20 min, the available lysine was reduced by 15%. In a later study on solutions of sodium caseinate, Guo *et al.* (1989) found a decrease of 10-15% in the available lysine after heating at 140°C for 30 min.

Lysine groups, however, show a wide range in reactivity, depending on their location in the protein. In a detailed study of the reaction between β-casein A^1 and

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lactose in ultrafiltrate, Henle and Klostermeyer (1992) established that during the initial stages, five specific Lys residues reacted to form lactuloselysine, and the other six were unmodified. On more severe heating a further four residues reacted, but the remaining two were unaltered even after heating at 90°C for 1h. These workers attributed the differences in reactivity to the nature of the amino acids adjacent to the Lys residues. Charged groups had an enhancing effect, whereas hydrophobic residues tended to inhibit glycosylation of the ϵ-amino groups.

4.2.3 Formation of lysinoalanine

Lysinoalanine is present in various high protein foods and food ingredients that are prepared by heating at alkaline pH (Sternberg and Kim, 1975). It occurs in some milk products, and is of particular interest because of its possible toxicity (de Groot and Slump, 1969). Bohak (1964) showed that lysinoalanine was formed at alkaline pH as a product of the reaction between the ϵ-amino groups of proteins and dehydroalanine. Dehydroalanine, in turn, is formed in milk proteins by β-elimination from phosphoserine (Manson and Carolan, 1972) and, possibly, from cysteine residues.

Lorient (1979) examined the effect of heating solutions of pure caseins and peptides. He showed that formation of lysinoalanine was negligible on heating α_{s1}-casein at pH 6.0 and 120°C for 30 min, but increased rapidly with increasing pH. On heating at pH 7.0 and 120°C for 2h, about one Lys residue/mol casein was converted to lysinoalanine, whereas at pH 11.9 and 90°C for 1h, four residues of lysinoalanine/mol casein were formed. Similar results were obtained for β-casein. Enzymic dephosphorylation of phosphoseryl residues of α_{s1}- and β-caseins reduced the formation of lysinoalanine; this was attributed to inhibition of formation of dehydroalanine from phosphoserine.

In a further study of the reactivity of the caseins at alkaline pH, Manson and Carolan (1980) showed that in α_{s1}-casein 8P and 9P, and β-casein, lysinoalanine was formed by reaction of lysine residues with dehydroalanine at phosphoseryl residues which occupied isolated positions in the structure. The contribution to lysinoalanine production by phophoseryl clusters, however, was not significant.

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Table 20. Reactions of amino acids in caseins at high temperature (>120°C)

1. Dephosphorylation

$$R\text{-}CH_2\text{-}O\text{-}PO_3^{2-} + H_2O \rightarrow R\text{-}CH_2\text{-}OH + HPO_4^{2-}$$

Phosphoserine   Serine

$$R\text{-}CH_2\text{-}O\text{-}PO_3^{2-} \quad\quad \rightarrow R=CH_2 + HPO_4^{2-}$$

Phosphoserine   Dehydroalanine

2. Formation of lysinoalanine

$$R\text{-}CH_2\text{-}O\text{-}PO_3^{2-} \quad\quad \rightarrow R=CH_2 + HPO_4^{2-}$$

Phosphoserine   Dehydroalanine

$$R\text{-}CH_2\text{-}S^- \quad\quad\quad \rightarrow R=CH_2 + HS^-$$

Cysteine   Dehydroalanine

$$R\text{-}(CH_2)_4\text{-}NH_3^+ + H_2C=R' \rightarrow R\text{-}(CH_2)_4\text{-}NH\text{-}CH_2\text{-}R'$$

Lysine   Dehydroalanine   **Lysinoalanine**

3. Maillard Reaction

$$R\text{-}(CH_2)_4\text{-}NH_3^+ + \text{Lactose carbonyl} \rightarrow \textbf{Maillard products}$$

Lysine   Hydroxymethyfurfural

Furosine, Pyrodisine

Lysinoalanine

Lactulose

Melanoidins

(Walstra *et al.*, 1984; Burton, 1984; Singh and Fox, 1989)

4.2.4 Heat induced proteolysis

On heating solutions of sodium and calcium caseinates at 120°C for 30 min, Howat and Wright (1934) obtained an increase in acid soluble nitrogen (NPN) of about 5% of the total nitrogen in milk. After heating for 5h, the level of NPN increased to about 20% of the total nitrogen.

These results were confirmed by Alais *et al.* (1966), who found that when a solution of whole casein, κ-casein or the α_s-caseins was heated at 120°C for 20 min, there was an increase in NPN of about 0.6% of the total. Alais *et al.* (1967) showed that one of the peptides causing the increase in NPN was almost identical to the caseinomacropeptide which is normally formed from κ-casein on renneting.

On heating milk between 50 and 120°C for 30 min, Davies and White (1958) showed that there was appreciable proteolysis of the caseins which increased with temperature. At 120°C, there was an increase in the level of the breakdown fraction (proteose peptones), amounting to about 3% of the total protein.

Guo *et al.* (1989) found a similar increase in the level of NPN on heating sodium caseinate above 110°C. The level of NPN increased from 4% of the total nitrogen on heating for 60 min at 110°C, to 20% on heating at 150°C. The level of NPN, on heating between 130 and 140°C, was independent of pH in the range 6.5-7.2, but increased as the casein concentration was increased from 2 to 8% (w/v). Using SDS-PAGE and gel permeation chromatography to examine the acid soluble fraction, they found that there was an increase in the amounts of small molecular weight peptides, consistent with increased proteolysis of the caseins at higher temperatures.

4.2.5 Changes in charge-related properties of the caseins

Creamer and Matheson (1980) examined changes in the caseins in milks heated at 110 and 130°C for 20 min. The electrophoretic patterns obtained by alkaline PAGE for whole casein showed that the casein bands, especially those of α_{s2}-casein, became broader and less distinct as the temperature of heating was increased.

Guo *et al.* (1989) also used alkaline PAGE, together with anion-exchange

chromatography, to investigate changes in solutions of sodium caseinate heated between 120 and 150°C for 1 h. Control and heated sodium caseinates were fractionated on DEAE cellulose in the presence of urea. Compared with the unheated caseins, those heated at 130 and 140°C gave less distinct peaks, and tended to elute earlier from the column. When chromatographic fractions were examined by alkaline PAGE, the patterns obtained for the heated caseins tended to be smeared, and became less distinct with increasing temperature. Analysis of the sodium caseinates, heated at 130 and 140°C for 1h, showed that 65 and 100% of the organic phosphate, respectively, had been removed from the caseins. Results from chromatography and electrophoresis confirmed, therefore, that on heating at high temperatures, the caseins lost negative charge and this appeared to be due to dephosphorylation.

4.3 Sodium Caseinates

Sodium caseinates are used in a wide variety of processed foods because of their ability to bind water, increase viscosity and form gels. Also, because of the combination of hydrophobic and polar regions on the caseins, they are surface active and are used as emulsifiers in products such as cream liqueurs (Mulvihill, 1992). Cream liqueurs are essentially oil-in-water emulsions in 10-20% ethanol, with a high sugar content. The emulsion is normally stabilised by the addition of sodium caseinate which forms a layer on the fat droplets (Horne, 1992). For reasons that are not completely understood, however, the emulsions are susceptible to destabilisation or thickening on storage, and they are particularly affected by high ionic strength and the presence of Ca^{2+}. There is evidence that some of the stability problems may be related to the method of manufacture of the sodium caseinates. This is discussed in more detail below.

In a preliminary study, Muir and Dalgleish (1987) studied the behaviour in alcoholic media of commercial sodium caseinates of different origins. The gross composition of the caseinates, including calcium, total phosphate and ester phosphate contents, was determined. The relative amounts of the individual caseins were examined by anion-exchange FPLC, according to the method of Davies and Law (1987). There was no obvious relation between the gross composition and the ability

of the caseinates to stabilise cream liqueurs, but differences were found in the FPLC elution patterns of the caseinates which reflected their method of preparation. There were particularly marked differences between the elution pattern of laboratory prepared acid casein and those of commercial sodium caseinates, especially in the regions of the κ- and α_{s2}-caseins.

The third paper in this Chapter (Paper 20, Dalgleish and Law, 1988) describes a more detailed chromatographic examination of differences in commercial spray- and roller-dried sodium caseinates obtained from different manufacturers. On anion-exchange FPLC, the peaks of the major caseins, especially in the regions of κ- and α_{s2}-caseins, were more distinct in laboratory prepared acid casein than in either spray- or roller-dried commercial caseinates. Also, there was considerable variation in the chromatograms of the commercial caseinates, which indicated that differences during manufacture caused changes in the charge on the caseins. The main changes appeared to be due to loss of negative charge on the major caseins, causing them to elute earlier from the positively charged anion-exchange column. The order of the extent of the modifications to the caseinates was roller-dried > spray dried > laboratory freeze-dried. An investigation of the calcium sensitivity of the caseinates showed that the order of sensitivity was the reverse of the above. The combined results showed that severe heating during the production of commercial caseinates, especially the roller-dried product, caused loss of negative charge leading to decreased binding and sensitivity to Ca^{2+}. The heat-damage may occur during the drying process in which there is a fine balance between obtaining a totally dry product, and avoiding overheating as water is removed.

4.4 Heat-induced Changes in the Caseins in Milk

The fourth paper in this Chapter (Paper 21, Law et al., 1994) describes the changes that occurred in the caseins and the whey proteins when milk was heated between 72 and 140°C. As described previously (Paper 18, Law et al., 1994), on heating between 72 and 110°C for 5 min, there was progressive irreversible denaturation of the whey proteins, which precipitated together with the caseins on adjusting to pH 4.6. At

110°C, most of the whey proteins were denatured, but the caseins were comparatively stable, and appeared unaltered. In an examination of the changes occurring at higher temperatures, the caseins from heated milks were subjected to alkaline and SDS-PAGE and a combination of anion- and cation-exchange FPLC, according to the methods described in Chapter 2. On heating above 110°C, the bands obtained by alkaline PAGE for the individual caseins showed smearing which became more pronounced with increasing temperature, indicative of changes in the net negative charges on the caseins. Casein obtained from milk heated at 140°C for 5 min showed very marked smearing of the electrophoretic pattern. Casein obtained at this temperature also showed the brown discolouration typical of products formed in the Maillard reaction, indicating that at least some of the modification of the caseins involved reaction of the ϵ-amino groups of lysine with carbonyl groups of lactose. The amount of proteolysis of the caseins, as determined by NPN analysis and from the SDS-PAGE pattern, was fairly small even at 140°C,

Results from anion-exchange FPLC showed that when milk was heated above 110°C for 5 min, the peaks of the individual caseins became broader and less distinct, and the effect increased with temperature. To a large extent, binding of the caseins to the positively charged Mono Q anion-exchange column depends on the number of phosphoseryl residues, and the changes in the chromatographic profile of heated caseins were consistent with loss of negative charge due to dephosphorylation of the caseins. This is in agreement with previous results showing a loss of organic phosphate on heating solutions of sodium caseinate (Guo *et al.*, 1989).

When caseins from heated milk were fractionated by cation-exchange FPLC, the peaks again appeared broader, and the caseins tended to elute earlier. There was an especially large increase in the amount of material eluting in the region before α_{s1}-casein, and this has since been shown to be derived from α_{s1}-casein. Results indicated that the heated caseins were less tightly bound to the negatively charged Mono S column, possibly because of removal of positively charged amino groups from the caseins. The effect was more pronounced on heating at higher temperatures, and the characteristic browning of the casein samples indicated that ϵ-amino groups of lysine were involved in Maillard reaction with the carbonyl group of lactose.

Comparison of the results obtained by alkaline PAGE, anion- and cation-

exchange FPLC showed that the types of changes taking place in commercial sodium caseinates and in casein from heated milk were similar. This demonstrated that the changes in the caseins are due to the effect of heat, rather than to their interaction with the whey proteins. The overall changes in the sodium caseinates and caseins from heated milk are consistent with loss of both negative and positive charges from the amino acid residues in the proteins.

5. PROTEOLYSIS DURING CHEESE-RIPENING

5.1 Introduction

The manufacture of cheese accounts for more than 27% of the total milk produced in EC countries (Fig.1), and even a small increase in the efficiency of the process could result in considerable saving. Part of this could be obtained by achieving consistency in the maturation stage, or by shortening the time required for cheese ripening. Also, there exists the possibility of developing new types of cheese and cheese flavourings. In order to understand the ripening process, and ultimately achieve precise control of maturation, it is essential to have analytical methods with high resolution that can be used to measure the rate of degradation of the original proteins, and examine the formation of proteolytic products. The development of two new methods based on anion- and cation-exchange FPLC, and their application to the study of proteolysis in cheese are described below.

Breakdown of the caseins, fat and residual lactose is important in the process of cheese ripening, but the exact contribution of each to the flavour of matured cheeses has not been fully estabished. In the early stages of ripening, chymosin and plasmin cause proteolysis of the caseins to large and intermediate-sized peptides, and may produce hydrophobic peptides that have bitter flavour (Visser *et al.*, 1975; Visser *et al.*, 1983). At later stages, proteolysis of the caseins to peptides, and ultimately to amino acids, has a direct effect on the flavour and texture of most varieties of matured cheeses.

In the manufacture of Cheddar cheese, the milk is usually pasteurised, and a culture of lactic starter bacteria is added. Growth of the bacteria causes a reduction in pH, which partially dissolves the colloidal calcium phosphate, and reduces the net negative charge on the micelles. On addition of rennet, the caseinomacropeptide is removed from κ-casein on the surface of the micelles, and coagulation occurs. Some of the rennet, which contains chymosin and varying amounts of other proteolytic enzymes such as pepsin, is usually retained in the curd. The process of proteolysis continues due to the action of these enzymes, together with plasmin which is present in milk, and other proteolytic enzymes from the starter bacteria and the milk. If the

milk has been heated to pasteurisation temperature or above, some of the whey proteins are retained in the curd together with the caseins, although the extent to which they undergo proteolysis is believed to be limited. The initial mixture of proteins is therefore complex, and a considerable number of proteolytic fragments may be generated.

5.2 Main Changes Occurring During Proteolysis

The relative contributions of enzymes from rennet, starter bacteria and milk to proteolysis in cheese have been studied. The evidence shows that rennet and, to a lesser extent plasmin, acts on the caseins to produce large and intermediate sized peptides, whereas enzymes from starter bacteria cause further breakdown to small peptides and amino acids (O'Keefe *et al.*, 1976; Visser, 1977; O'Keefe *et al.*, 1978).

Proteolysis of α_{s1}-casein is caused mainly by the action of chymosin, whereas breakdown of β-casein is initiated by plasmin. In various studies on the action of chymosin on the caseins, it has been found that the enzyme tends to act on Phe-X and Leu-X peptide bonds. Peptides have been identified from α_{s1}-casein (Mulvihill and Fox, 1979; McSweeney *et al.*, 1993a), β-casein (Creamer *et al.*, 1971; Creamer, 1976; Carles and Ribadeau-Dumas, 1984) and α_{s2}-casein (McSweeney, 1993a). After the initial proteolysis of κ-casein, the caseinomacropeptide is lost in the whey; para-κ-casein, which is hydrophobic, remains in the curd. Green and Foster (1974) have suggested that para-κ-casein is resistant to further proteolysis, but other workers have found that there is extensive breakdown of this protein during ripening (Pihlanto-Leppälä *et al.*, 1993).

Plasmin is present in milk and, during storage, causes proteolysis of β-casein to form the γ-caseins and the corresponding proteose peptones 5, 8 slow and 8 fast (Gordon and Groves, 1975; Andrews and Alichanidis, 1983; Noomen, 1975). Plasmin has specificity similar to trypsin, and acts on Lys-X and Arg-X bonds to cause proteolysis of α_{s1}- and α_{s2}-caseins (Noomen, 1975; Eigel, 1977; Barry and Donnelly, 1981). The pH of cheese is below the optimum for the action of plasmin (about pH 7.5), but there is considerable evidence that the action of plasmin continues in cheese,

and has an appreciable effect on the ripening process (Visser and de Groot-Mostert, 1977; Farkye and Fox, 1991; McSweeney, 1993b).

The further breakdown of the large and intermediate peptides peptides by enzymes from starter and other bacteria has been reviewed previously (Visser, 1981; McSweeney, 1993c; McSweeney *et al.*, 1993b).

5.3 Direct Methods for Determination of Proteolysis

The extent of proteolysis during ripening of cheese has been determined by Kjeldahl analysis of nitrogen in various extracts of cheese (Lindqvist and Storgårds, 1959; O'Keefe *et al.*, 1976; Visser, 1977; Kuchroo and Fox, 1982a, b). Different extraction procedures have been compared, and standardised methods have been developed (Reville and Fox, 1978; Kuchroo and Fox, 1982a, b). Extracts obtained with water, $CaCl_2$ or at pH 4.6 have similar compositions; the medium and small peptides and free amino acids are separated from the larger, insoluble proteins. The soluble nitrogen content at pH 4.6 increases as maturation proceeds and, as the intact caseins are insoluble at pH 4.6, the ratio of soluble nitrogen to total nitrogen can be used to determine the extent of proteolysis. Other more specific extraction procedures have been used; these have been reviewed by Christensen *et al.* (1991). An alternative approach has been to subfractionate aqueous or pH 4.6 extracts using electrophoresis or chromatography. In the present study (Paper 22, Calvo *et al.*, 1992), the proteins remaining insoluble after extraction with water were examined by anion- and cation-exchange FPLC.

Because the Kjeldahl method is laborious, other methods have been developed to study proteolysis. The protein, peptide and amino acid contents of cheese fractions have been determined by direct spectrophotometric measurement at 280nm of tryptophan and tyrosine (Vakaleris and Price, 1959). The obvious disadvantage of this method is that some peptides may not contain these amino acids and will therefore not be taken into account.

A more reliable procedure involves the measurement of free amino groups which are produced as the result of proteolysis; these groups have been determined by

direct titration, or reaction with trinitrobenzene sulfonic acid (TNBS), ninhydrin, or o-pthaldialdehyde (OPA). These methods have the advantage that they can be used on crude extracts of cheese (Ardö and Meisel, 1991).

5.4 Electrophoresis in the Study of Proteolysis

The above methods have been useful for measuring gross changes in the various protein, peptide and amino acid fractions, but more specific techniques are required to establish which caseins are being degraded, and to identify the corresponding proteolytic fragments.

In early studies, moving boundary electrophoresis at alkaline pH was used to examine ripening of several types of cheese (Lindqvist and Storgårds, 1959). Different ripening patterns of the cheeses could be seen, but the α_{s1}-, α_{s2}- and para-κ-caseins were not well resolved.

Improved resolution was obtained by carrying out electrophoresis in a support medium of starch or polyacrylamide, in the presence of dissociating and reducing agents (Morr, 1971). At alkaline pH the caseins and peptides were separated according to their net negative charges. PAGE has the advantage that its separation characteristics can be altered by adjusting the amounts of polymer (acrylamide) and cross-linking agent (bisacrylamide). Using alkaline PAGE, Creamer and Mills (1971) showed that rennin cleaved β-casein in three distinct regions of the molecule to give peptides designated β-I, β-II and β-III, in order of increasing electrophoretic mobility. These peptides were subsequently isolated and identified (Creamer, 1976).

Similarly, Mulvihill and Fox (1979), using alkaline PAGE, studied the initial products formed by the action of chymosin on α_{s1}-casein, and were able to separate five main peptides, α_{s1}-I to α_{s1}-V. Alkaline PAGE has been used in various other studies of proteolysis in cheese (Green and Foster, 1974; Visser and de Groot-Mostert, 1977; Farkye and Fox, 1991; McSweeney et al., 1993a). Quantitative values have been obtained by scanning gels (Visser and de Groot-Mostert, 1977; Marcos et al, 1979). The caseins and peptides have different dye-binding properties, however, and it is difficult to obtain accurate quantitative values without frequent comparison with

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standards.

At alkaline pH, para-κ-casein has a net positive charge, and therefore does not migrate towards the anode in the alkaline PAGE methods described above. It can, however, be separated by alkaline PAGE if the polarity is reversed (McLean *et al.*, 1982; Davies and Law, 1977a).

Acid PAGE has been carried out on caseins (Peterson and Kopfler, 1966), and can be used to separate proteins and peptides that differ in net positive charge. The gels are more difficult to form at acid pH and, compared with alkaline PAGE, separations generally take longer because the net positive charge on the caseins is lower at acid pH than the corresponding negative charge at alkaline pH. Some of these difficulties have been overcome by the development of miniature gels which are polymerised at alkaline pH, and then equilibrated with buffer at low pH before electrophoresis. Separation times are generally less than one hour.

SDS-PAGE is used to separate proteins according to molecular weight, and has been applied to study the formation of peptides in the maturation of cheese (Harper *et al.*, 1989). Shalabi and Fox (1987) compared SDS-PAGE and different variations of alkaline PAGE, and were of the opinion that separations in SDS gave poorer resolution. Nevertheless, SDS-PAGE can be useful for detecting whey proteins or peptides which have molecular weights considerably different from the caseins. It is, therefore, suitable for the study of proteolysis in cheese made from heated milk which has denatured whey proteins retained in the curd; it has been used in the present study to characterise fractions obtained by anion- and cation-exchange FPLC.

5.5 Chromatography in the Study of Proteolysis

Lindqvist *et al.* (1953) used paper chromatography to examine extracts containing amino acids from different types of cheeses, and they detected differences in the distribution of the free amino acids. This technique was not suitable for peptides or caseins, however, and most of the earlier methods of chromatography involved separation of the proteins, peptides and amino acids into fractions according to molecular weight.

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5.5.1 Gel permeation chromatography

Gel permeation chromatography of cheese extracts has been carried out on various types of Sephadex (polydextrans) with different molecular weight fractionation ranges. Extracts of proteins and peptides from Cheddar cheese were fractionated on Sephadex G-100 (fractionation range 4,000-150,000) in the presence of urea as dissociating agent, and dithiothreitol to reduce disulphide bonds (Foster and Green, 1974). Using alkaline PAGE, these workers identified five different molecular weight fractions. They subsequently used gel permeation chromatography to examine the different rates of proteolysis of the caseins with different coagulants.

The buffer used in the above method contained high concentrations of urea, which had to be dialysed from column fractions before the proteins and peptides could be further characterised. This difficulty was overcome by using volatile buffers containing n-propanol and water on Sephadex LH-20 (Visser, 1977). Using this technique, Visser *et al.* (1983) were able to isolate and identify small peptides of β-casein that caused bitterness in Gouda cheese.

The smaller molecular weight peptides have been examined by gel permeation chromatography of water soluble, pH 4.6 soluble, and bitter extracts on Sepadex G-15 (Aston and Creamer, 1986), Sephadex G-25 (O'Keeffe *et al.*, 1978; Edwards and Kosikowski, 1981) and G-50 Sephadex (O'Keeffe *et al.*, 1976; Visser, 1977). The fractionations of extracts of Cheddar cheese, in water and more specific solvents, on Sephadex G-10, G-25, G-50 and G-150 have been compared by Kuchroo and Fox (1982a, 1983a,b). These workers preferred the use of distilled water as eluent as opposed to the dissociating buffers in most of the above gel permeation fractionations. They considered, however, that gel permeation did not yield homogeneous fractions of peptides, and was more useful as a preliminary fractionation procedure.

5.5.2 Reverse phase chromatography

Whole casein has been fractionated by reverse phase HPLC at acid pH (Visser *et al.*, 1986) and alkaline pH (Carles, 1986). There is some indication that, under certain

conditions, recovery of α_{s2}-caseins, and possibly κ-casein may be incomplete (Visser *et al.*, 1991). However, most of the buffers used are volatile, and this facilitates further characterisation of the fractions. Also, as the buffers generally have low UV absorbance, it is possible to detect peptides that do not contain tryptophan and tyrosine by measuring the absorbance of column eluate below 280nm. Reverse phase HPLC has been used to study the proteolysis of β- and α_{s1}-caseins (Christensen *et al.*, 1989; Singh *et al.*, 1994), and the specificity of chymosin on β-casein (Carles and Ribadeau-Dumas, 1984) and α_{s1}-casein (McSweeney *et al.*, 1993a). It has also been used to examine the action of plasmin on α_{s2}-casein (Le Bars and Gripon, 1989), and the action of chymosin, plasmin and trypsin on κ-casein (Pihlanto-Leppälä *et al.*, 1993). Reverse phase HPLC has also been found useful in the study of non-starter bacteria in the maturation of Cheddar cheese (McSweeney *et al.*, 1993b).

5.5.3 Ion-exchange chromatography

Anion-and cation-exchange chromatography have been successfully used to obtain quantitative separations of the caseins (Chapter 2), but these techniques have not been widely applied in the fractionation of peptides in cheese. Mulvihill and Fox (1979), however, obtained fractions enriched in peptides α_{s1}-I to IV by cation-exchange chromatography on cellulose phosphate in dissociating and reducing conditions. In a later study, Kuchroo and Fox (1983a) fractionated the water soluble and ethanol soluble fractions from Cheddar cheese on DEAE cellulose at pH 6.5. The separation was not carried out in the presence of a dissociating agent, and they obtained heterogeneous fractions.

Haasnoot *et al.* (1986) applied anion-exchange FPLC on Mono Q to detect bovine caseins in cheese made from the milk of the goat or sheep. The technique was more rapid than earlier ion-exchange methods, but these workers did not identify the fractions or obtain quantitative values.

In the present study (Paper 22, Calvo *et al.*, 1992), water insoluble fractions were prepared from Cheddar cheese throughout a ripening period of twelve weeks. After reduction with 2-mercaptoethanol, the materials were fractionated by anion- and

cation-exchange FPLC, essentially according to the methods in Chapter 2. The column fractions were characterised by SDS-PAGE; it was established that anion-exhange FPLC on Mono Q gave good resolution of residual β-casein, but poorer resolution of the α_{s1}- and α_{s2}-caseins, which eluted together with some peptides. Conversely, cation-exchange FPLC gave a good separation of para-κ- and α_{s1}- caseins, and approximate levels of α_{s2}-casein could be obtained. A combination of anion- and cation-exchange FPLC, therefore, gave the best quantitative values for the extent of degradation. The methods were also suitable for examining the ripening of cheese made from overheated milk, as the denatured whey proteins tended to elute from the ion-exchange columns at low salt concentrations (Calvo *et al.*, 1992a, c).

6. THE EFFECT OF HEAT TREATMENT AND ACIDIFICATION ON CASEIN MICELLES

6.1 Introduction

During most manufacturing processes, milk undergoes an initial heat treatment to control the growth of microorganisms. In the manufacture of several products such as yogurt and cottage cheese, this heat treatment is followed by lactic acid fermentation which causes a reduction in pH and leads to acid coagulation (Tamime and Robinson, 1988; Mulvihill and Grufferty, 1995). The severity of heat treatment may affect the pH at which coagulation occurs, and change the texture and stability of the acid gel. Various studies have been carried out on unheated milk to examine changes in the micellar calcium phosphate and caseins on acidification of milk at different temperatures (Chapter 5). In order to fully understand and effectively control the formation of acid gels, similar detailed information is required on the dissociation of casein micelles in heat-treated milk. This study, therefore, deals with the effect of heat-treatment at functionalisation temperatures (85°C for 10 min), and the effect of subsequent acidification and storage at 4, 20 or 30°C, on the serum-micellar distribution of Ca, P_i, individual caseins and denatured whey proteins.

6.2 Effect of Whey Proteins on Acid Gelation

In some early work it was found that heat-treated milk coagulated more readily during lactic acid fermentation, and gave a much firmer curd than unheated milk. Grigorov (1966a,b) showed that milk pasteurised at 85°C and incubated at 45°C, coagulated at pH 4.7, whereas milks heated between 85 and 95°C for 30 min coagulated between pH 5.16 and 5.08. Coagulation times were shorter for heated milks, and a satisfactory gel was obtained by heating at 85°C for 20-30 min. Heating at higher temperatures caused deterioration of the gel. Emmons *et al.* (1955) also found an increase in the pH at which heated milk coagulated, and attributed this difference to denaturation of whey proteins. On heating a synthetic skim-milk that did not contain whey proteins, they

found that the pH of acid coagulation was the same as in the unheated milk. On adding whey proteins to the synthetic milk, the pH of coagulation increased considerably (up to 0.5 of a pH unit), the increase being closely correlated with the ratio of denatured whey proteins to casein in the milk. Horne and Davidson (1992) examined acid gel formation in heat-treated milk by using diffusing wave spectroscopy to measure changes in particle mobility as the gel formed. They found that, in unheated milk, gelation at 30°C occurred at pH 4.9, but as the temperature of pre-heating was increased from 75 to 90°C (for 10 min), the pH of coagulation increased, and the coagulation time decreased markedly. On heating milk at 90°C for 10 min, acid gelation at 30°C occurred at pH 5.5.

Kalab *et al.* (1976), using scanning electron microscopy, examined the microstructure of the acid gel in yogurt. They were able to relate changes in the physical properties to the appearance of the casein micelles and whey proteins. In unheated milk incubated at 44°C, the first signs of gelation occurred at pH 5.14 and the gelation was in full process at pH 4.92. In skim-milk heated to 90°C and incubated at 44°C, however, the corresponding changes occurred at pH 5.36 and 5.17, respectively. Davies *et al.* (1978), also using electron microscopy, detected filamentous appendages on the surface of the casein micelles, and they believed that they were composed of β-lactoglobulin attached to micellar κ-casein. Evidence in support of this was that development of the appendages was inhibited by the addition of the sulphydryl blocking agent N-ethylmaleimide to the milk before heating. Electron microscopy showed that the appendages prevented micellar coalescence giving rise to a firmer curd with a lower tendency to syneresis. Parnell-Clunies *et al.* (1987) used a wider range of heat treatments; they confirmed that, as opposed to the more open structure in raw milk, there was an extensive, compact gel network of micelles and whey proteins in heat-treated milk. They believed that this structure improved the water retention properties of the gel.

Dannenberg and Kessler (1988a,b) examined the effect of different heat treatments of milk (70-130°C) on the physical properties of yogurt gels, and used electrophoresis to measure the extent of irreversible denaturation of β-lactoglobulin in the milks. They found that there was a close correlation between the level of denaturation of β-lactoglobulin and the texture of the acid gel. The susceptibility of the

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gel to syneresis decreased with increasing denaturation of β-lactoglobulin, rather than the temperature or time of heating. Prolonged heating beyond the point required to cause 99% denaturation of β-lactoglobulin, however, caused a deterioration in the consistency of the gel.

Because of the difficulty in measuring the extent of irreversible denaturation of the minor whey proteins, most of the reports on the effect of heating milk on the structure of the acid gels have related to the role of denaturation of β-lactoglobulin and its association with κ-casein. Mottar *et al.* (1989), however, used electron microscopy to study the effect of different heat treatments of milk on the microstructure of the acid gel. They showed that α-lactalbumin tends to fill spaces on the micellar surface between the appendages of β-lactoglobulin described by Davies *et al.* (1978). On heating milk under conditions sufficiently severe to cause denaturation of α-lactalbumin, the micellar surface became more hydrophobic, and the water binding capacity and viscosity of the acid gel increased.

6.3 Effect of Heat Treatment and Acidification on Ca and P_i

On heating milk there is a decrease in diffusible Ca and P_i which, depending on the severity of heating, reverses on cooling. Hilgeman and Jenness (1951) found that after heating milk at 80°C for 30 min, there was a 12-18% decrease in the soluble Ca and P_i. On subsequently holding the milk at 5°C for up to 48h, there was a slow increase to the original serum concentrations. These results were confirmed by later studies (Tessier and Rose, 1958; Kannan and Jenness,1961). Rose and Tessier (1959) prepared ultrafiltrate directly from hot milk, and showed that the change in serum Ca and P_i was much greater than had been estimated from heated milks that had been cooled for a short time before analysis. Ultrafiltrate from milk heated at 93°C contained only 50% of the Ca and 82% of the P_i found in the ultrafiltrate from skim-milk at 27°C. Pouliot *et al.* (1989) found that when milk was heated to 85°C for 40 min there was a transfer of Ca and P_i from the serum to the colloidal phase, but on holding the milk at temperatures between 4 and 60°C, the transfer was 90-95% and 93-99% reversible for Ca and P_i, respectively.

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Using more severe heating conditions (115°C for 15min or 110°C for 30 min), Evenhuis and de Vries (1956) found that re-solubilisation of the calcium phosphate was slow, even when the pH was reduced to 4.7. They concluded that, on severe heating, calcium phosphate was precipitated and re-crystallised as hydroxyapatite. Visser *et al.* (1986) similarly found that, on heating milk at 100°C for 15 min and cooling, the serum P_i concentration was only about 85% of that in the serum of unheated milk. Results from IR analysis showed that the spectrum of the crystalline calcium phosphate formed on severe heating was identical to that of hydroxyapaptite.

Few studies have been carried out on the effect of acidification on calcium phosphate in heat-treated milk. Evenhuis and de Vries (1956) found that solubilisation of colloidal calcium phosphate in severely heated milk was fairly slow at pH 4.7, but proceeded rapidly at pH 2.0. Visser *et al.* (1986) examined changes in the Ca and P_i on acidification of heated milk using hydrolysis of glucono-δ-lactone to gluconic acid to produce conditions similar to acid gelation in yogurt. On incubation at 43°C, gelation took place at pH 5.2, and at that pH almost all of the P_i had been solubilised.

6.4 Effect of heat treatment and acidification on the caseins and whey proteins

Sullivan *et al.* (1957) showed that when milk was heated between 70 and 100°C for 1 min there was an overall decrease in the concentration of total protein in the supernatant obtained by ultracentrifugation. Using [35]P-labelled whey proteins they found that more than 50% of the whey proteins were associated with the micellar pellet. There is considerable evidence, presented below, that on heating there is also a transfer of casein from the micelles into the serum. It is difficult, therefore, to obtain quantitative information about the levels of denatured whey proteins and caseins present in the serum and micellar phases. This difficulty is compounded by the fact that denatured whey proteins are associated with the caseins through hydrophobic interactions and disulphide linkage, and precipitate together with the serum and micellar caseins at pH 4.6 (Section 1). It is also difficult to reduce and dissociate this mixture of whey proteins and caseins.

On ultracentrifugation of heated milk, Morr (1973a,b,c) separated an opalescent layer immediately above the main micellar pellet. From analysis for sialic acid, thiol

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and disulphide groups he established that this fraction contained a high proportion of a disulphide-linked complex of β-lactoglobulin and κ-casein. Smits and Brouwershaven (1980) studied the formation of this complex by heating ^{3}H-labelled β-lactoglobulin and casein micelles resuspended in milk salt buffer. On ultracentrifugation, they obtained an interphase layer similar to the opalescent layer described above, which contained β-lactoglobulin and κ-casein in ratios between 1:1 and 3:1. The amount of β-lactoglobulin associated with the casein micelles was pH dependent, increasing between pH 6.8 and 5.8, and decreasing between 6.8 and 7.3.

Aoki *et al.* (1974, 1975) examined the level and composition of serum casein in concentrated whey-protein-free milk. On heating at 140°C for times from 15s to 4 min 15s, the levels of soluble casein increased markedly; the relative amount of κ-casein, determined by sialic acid analysis, increased from 14 to 39-43% of the soluble casein. These results were confirmed by data from anion-exchage chromatography which showed that, on heating at 135°C for 15s, the soluble casein consisted of α_s-casein, 21.8%; β-casein, 25.0%; κ-casein, 49.2%; and minor caseins, 4.0% of the total. In a further study involving the preparation of concentrated milks with different sizes of micelles, Aoki and Kako (1983) found that, on heating at 135-140°C, more soluble casein was formed in the milks containing smaller micelles, but the composition of the soluble casein did not vary; for each micellar class more than 50% of the soluble casein was κ-casein.

Several studies have shown that the extent to which the complex of β-lactoglobulin and κ-casein becomes associated with either the serum or micellar phases varies with pH. Creamer *et al.* (1978) heated milk at 100°C for 30 min, and examined the complex of β-lactoglobulin and κ-casein by electron microscopy, amino acid analysis and gel permeation chromatography. They found that on heating at pH 6.5 or pH 6.8, the same type of β-lactoglobulin-κ-casein complex was formed, but at the higher pH it was present in the serum, and at the lower pH was associated with the casein micelles. On heating milk at 140°C between pH 6.2 and 7.2, Kudo (1980) similarly found that below pH 6.5 substantial quantities of whey proteins sedimented with the micelles on ultracentrifugation, whereas above pH 6.8 most of the whey proteins remained in the supernatant together with caseins released from the micelles.

The pH-dependence of the dissociation of κ-casein from the micelles was

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confirmed by other workers using milder heating conditions (Singh and Fox, 1986; Singh et al., 1988). Singh and Fox (1987a) indicated that, on heating milk at 90°C for 10 min, below pH 6.9 β-lactoglobulin became associated with the micelles and tended to prevent dissociation of κ-casein. At pH 6.9 or above, however, the complex of β-lactoglobulin and κ-casein dissociated from the micelles, and high concentrations of β-lactoglobulin promoted dissociation. Reducing the colloidal calcium concentration content of milk by 40%, or increasing it by 20%, had no significant effect on the dissociation of κ-casein on heating, but decreasing P_i or increasing Ca reduced the amount of κ-casein dissociation (Singh and Fox, 1987b). As found on adjusting the pH of heating above or below pH 6.9, chemical modification of the amino and carboxyl groups, to increase or decrease the net negative charge on the caseins, similarly promoted and inhibited, respectively, dissociation of κ-casein from the micelles.

Van Hooydonk et al. (1987) studied the dissociation of κ-casein into the serum in milk heated at 85°C for 5-10 min, and stored at 4°C for 24h. On preparing supernatants by ultracentrifugation, and determining the levels of κ-casein by the release of caseinomacropeptide on renneting, they found that 30-40% of the total κ-casein dissociated into the serum on heating. Results from electrophoresis showed that the serum casein also contained α_s- and β-caseins. Similarly, on preparing UHT milk by heating at 140°C for 2, 10 and 30s, there was an increase in the level of serum κ-casein with increasing heating time. On storing the heated milks at 5°C for 24h, there was a further marked increase in the level of serum κ-casein.

It is clear from the changes in the whey proteins, Ca, P_i and caseins, discussed above, that acid gelation in heated milk is a complex process, and cannot be regarded simply as aggregation of intact micelles as the net negative charge on the caseins is reduced. Several accounts of the main changes occurring before acid gelation have been proposed (Heertje et al., 1985; Visser et al., 1986). On evidence from electron microscopy, Heertje et al. (1985) have suggested that in milk heated at 90°C for 15 min and then incubated at 30°C, the micelles remain intact as the pH is reduced between pH 6.6 and 5.9. As colloidal calcium is progressively solubilised, the caseins begin to dissociate from the micelles; between pH 5.5 and 5.2 an aggregated network of modified micelles is formed. As the pH is lowered further to about 5.1, gelation begins; at pH 5.0 most of the Ca and P_i is removed from the micelles. The exact

reasons for the changes at low pH are not fully understood. Heertje *et al.* (1985) have suggested that the net suface charge on the micelles and the hydrated outer layer of the micelles may affect aggregation. The zeta potential of casein micelles, which is a measure of the net negative surface charge, shows anomalous behaviour at low pH, reaching a minimum at pH 5.2, but increasing at pH 5.0, and then decreasing to zero near pH 4.6. The positions of the minimum and maximum, however, are altered considerably for micelles in heat-treated milk or for micelles heated in the presence of whey proteins (Schmidt and Poll, 1986). The voluminosity of the micelles does not appear to be affected by heat treatment (Creamer and Matheson, 1980) but increases with decreasing temperature, being about 4.0 at 25°C and 4.7 at 5°C (Walstra, 1979). The voluminosity also increases with decreasing pH, reaching a maximum at about pH 5.4 and then decreasing (Roefs *et al.*, 1985). Heertje *et al.* (1985) attributed the effect of the whey proteins in increasing the pH of gelation to their forming a hydrophobic layer on the micellar surface, and reducing the influence of the outer hydrated layer.

The present work (Paper 23, Law, 1995) was carried out to obtain more information about changes in the minerals and proteins on heating milk, and particularly about the changes in the serum and micellar concentrations of the whey proteins, Ca, P_i and caseins on acidification and storage at different temperatures. Milk was heated at functionalisation temperatures (85°C for 10 min), and acidified by the addition of glucono-δ-lactone to reduce the pH in a controlled manner, as during lactic acid fermentation. The milks were stored at 4, 20 or 30°C for 22h, and serum and micellar fractions were prepared by ultracentrifugation.

Results showed that after heating and storing for 22 h at 4, 20 or 30°C, the concentrations of Ca and P_i in the sera were similar to those in the corresponding sera of unheated milk. It was concluded that any transfer of Ca and P_i onto the micelles on heating, as discussed above, was completely reversed on cooling and storing for 22h. On acidifying the heat-treated milk and storing for 22h at 4, 20 or 30°C, there was an increase in the concentrations of Ca and P_i in the serum with decreasing pH, which was independent of the storage temperature. Below pH 5.5, more than 90% of the micellar Ca and P_i was removed, and the material sedimented by ultracentrifugation was, therefore, substantially different from intact micelles. At pH 5.0, almost all of the Ca and P_i was removed from the micelles. Between pH 6.7 and 5.6, there was a close

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correlation between the concentrations of Ca and P_i remaining in the micelles; the slope of the line was 1.98, which was similar to that previously found in unheated milk (van Hooydonk *et al.*, 1986; Dalgleish and Law, 1989). Results indicated, therefore, that solubilisation of the colloidal calcium phosphate was similar to that in unheated milk.

On heating at 85°C for 10 min, about 86% of the whey proteins were irreversibly denatured, and only 8% of the β-lactoglobulin and 25% of the α-lactalbumin remained undenatured. The serum and micellar caseins and the associated denatured whey proteins were analysed by SDS-PAGE and by anion- and cation-exchange FPLC according to the methods of Davies and Law (1987) and Hollar *et al* (1991). From Kjeldahl N analysis and anion-exchange FPLC it was calculated that the denatured whey proteins were about equally distributed between the serum and micellar phases. On heating milk and storing for 22h, there was a substantial increase in the concentration of κ-casein in the serum at each storage temperature, and on average 27.3% of the total κ-casein dissociated from the micelles, compared with 13.2% in raw milk. This level of dissociation was slightly lower than found by Aoki *et al.* (1974, 1975) and van Hooydonk *et al.* (1987), as discussed above. At 20 and 4°C, there was also a slight increase in the concentration of β-casein in the serum, but the concentrations of α_{s1}- and α_{s2}-caseins were not appreciably affected by heating.

On acidifying the heat-treated milk and storing at 4 or 20°C for 22h, the concentrations of κ-, β-, α_{s2}- and α_{s1}-caseins in the serum increased as the pH was reduced to 5.5 and 5.7, respectively, but decreased as the pH was reduced further. The increase in serum concentrations of the individual caseins was lower at 20°C than at 4°C. At each storage temperature, maximum dissociation occurred at a higher pH than in raw milk, and the overall increases in the individual caseins on acidifying were lower than previously found in raw milk (Rose, 1968; Dalgleish and Law, 1988). The concentrations of κ-, β-, α_{s2}- and α_{s1}-caseins in the serum of heat-treated milk stored at 30°C decreased steadily as the pH was reduced; they did not show the increase found in raw milk at pH 5.5 (Dalgleish and Law, 1988).

On acidification of heat-treated milk and storage at 4, 20 and 30°C, the denatured whey proteins tended to remain associated with the micellar fraction. As discussed above, it has previously been shown that β-lactoglobulin forms a complex with κ-casein and, depending on the pH, the complex remains attached to the micelle

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or dissociates into the serum (Creamer *et al.*, 1978; Singh and Fox, 1987a, b). In the present study there appeared to be substantial amounts of κ-casein in the serum even at pH 6.7; the level increased at 20 and 4°C as the pH was reduced to about 5.7 and 5.5, respectively. Some additional work showed that the increased dissociation of κ-casein occurred either during or soon after heating, and the level increased only slightly during the subsequent storage period.

The increase in the pH of maximum dissociation of the caseins in heated milk, and the close association of about half of the denatured whey proteins with the micelles, indicate that the denatured whey proteins promote aggregation of the micelles. The whey proteins may interact with κ-casein and increase the hydrophobicity of the outer layer of the micelles, thus promoting aggregation as the pH is reduced.

Results show that there is no simple relation between solubilisation of colloidal calcium phosphate and dissociation of the caseins. At pH 6.7, without much change in the extent of solubilisation of the colloidal calcium phosphate, there is a substantial increase in serum casein at lower temperatures, due mainly to dissociation of β-casein as hydrophobic interaction becomes weaker. On the other hand, at 30°C the colloidal calcium phosphate can be removed completely, and the level of serum casein decreases. The results confirm previous findings that at 30°C hydrophobic interactions are important in maintaining micellar structure. At 4 and 20°C, however, removal of calcium phosphate promotes dissociation of the caseins into the serum (Dalgleish and Law, 1989). Studies involving ^{1}H-NMR have shown that although casein micelles have fairly open structures, most of the caseins are immobilised and form part of a rigid structure (Rollema and Brinkhuis, 1989). These NMR studies have also shown that, on acidification between 5.8 and 7.5, there is no change in the mobility of the caseins, whereas on addition of EDTA to remove Ca they are able to move more freely. The differences appear to arise because, apart from removing micellar calcium phosphate, acidification also causes a reduction in the net negative charge on the caseins, and reduces repulsion within the micelles. At the same time, acidification increases the ionic strength of the serum, thereby increasing interaction between the micellar caseins.

7. CONCLUSIONS

A combination of gel permeation and ion-exchange FPLC was used to study changes in the whey proteins and caseins during heat-treatment, cheese manufacture and maturation, and acid gelation.

Gel permeation FPLC was used to examine the effect of different heat treatments on the level of denaturation of four whey protein fractions. As the temperature of heating (72-140°C) and the holding time (15s to 5min) were increased, each of the whey proteins became increasingly insoluble at pH 4.6; the decrease in their concentrations in acid filtrate, measured by gel permeation FPLC, gave a good indication of the extent of their irreversible denaturation.

The level of denaturation of the whey proteins increased with temperature and holding time, and the ease of denaturation was immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. On heating milk at 80°C for 5 min, for example, most of the immunoglobulins and the serum albumin/lactoferrin fraction were denatured, but only about 40% of the β-lactoglobulin and less than 20% of the α-lactalbumin. On heating at 120°C for 5 min, all of the whey proteins were denatured.

The pH at which milk was heated had a pronounced effect on the extent of denaturation of the whey proteins. On heating milk between pH 6.2 and 9.1, at 90°C for 30s, the levels of denaturation of β-lactoglobulin, α-lactalbumin and total whey protein were lowest at pH 6.5, 6.4 and 6.3 respectively. The level of denaturation of each was slightly higher at pH 6.2, but increased markedly at alkaline pH up to 9.2, to give an overall increase in denaturation of about 24% of the total whey protein.

In a study of the manufacture of Cheddar cheese, it was found that there was substantial incorporation of whey proteins into cheese when the milk was pre-heated at 90°C for 30s to partially denature the whey proteins. There was a close correlation between the extent of denaturation of the individual whey proteins, as measured by loss of solubility at pH 4.6, and their retention in the curd. Gel permeation FPLC could, therefore, have a practical application in determining the levels of denaturation in industrial heating equipment, and the recovery of whey proteins in the curd during the manufacture of cheese.

In a comparison of the rates of denaturation of the whey proteins of the cow,

goat and sheep, it was found that the ease of denaturation of the whey proteins of all three species was in the order immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. The reaction orders for denaturation of the corresponding whey proteins of the three species were the same, but there were differences in their rates of denaturation. On heating at 70°C, or at 80°C with short holding times, caprine whey proteins were less extensively denatured than the corresponding bovine or ovine whey proteins. On more severe heating at 80 and 90°C, the order of ease of denaturation of the whey proteins was usually ovine > caprine > ovine. On heating at 90°C, however, denaturation of the immunoglobulins and serum albumin/lactoferrin occurred rapidly and differences in these proteins were less pronounced. The results indicated that, as for cows' milk, considerable increases in the yield of cheese could be obtained by incorporating denatured whey proteins into the rennet curd during the manufacture of cheese from heated milk of the goat or sheep.

On heating cows' milk at 110°C for 5 min, most of the whey proteins were denatured, but there were no obvious changes in the caseins, as determined by alkaline PAGE and ion-exchange FPLC. On heating above this temperature, however, the electrophoretic patterns of the caseins became increasingly smeared and indistinct, and at 140°C the casein showed a brown discolouration typical of products formed in the Maillard reaction. Similarly, on anion- and cation-exchange FPLC, the peaks from caseins subjected to severe heat-treatment tended to be broader, and the caseins tended to elute from the columns earlier. There was a particularly marked increase in the amount of material which, on cation-exchange FPLC, eluted after κ-casein, and this was shown to be derived from α_{s1}-casein. The changes in the caseins observed on electrophoresis and ion-exchange FPLC were consistent with loss of negative charge through dephosphorylation of the casein, and loss of positive charge due to reaction of ϵ-amino groups of lysine to form Maillard products and lysinoalanine. The amount of heat-induced proteolysis of the caseins on heating at 140° for 5 min was fairly small.

In a study of commercial spray-dried and roller-dried sodium caseinates obtained from different manufacturers, and used in the manufacture of cream liqueurs, it was found that the elution profiles obtained by anion-exchange FPLC differed considerably. Compared to the peaks obtained for laboratory prepared sodium caseinate, those obtained for the commercial caseinates were less distinct.

The modifications in the commercial sodium caseinates were similar to those seen in the caseins in heated milk. The main changes appeared to be due to loss of negative charge, causing the caseins to elute earlier from the anion-exchange column. The order of the extent of modification to the caseinates was roller-dried > spray-dried > laboratory freeze-dried, showing that the severity of heating during the drying process was an important factor in causing modifications in the caseinates.

In a study of proteolysis of the caseins during the ripening of Cheddar cheese, anion- and cation-exchange FPLC were used to obtain quantitative information about the relative rates of proteolysis of the individual caseins. These methods were also used to examine cheese which had been prepared from heat-treated milk, and contained denatured whey proteins. Anion-exchange FPLC gave good resolution of residual β-casein, whereas cation-exchange FPLC could be used to separate para-κ-casein and α_{s1}-casein, and to obtain approximate values for α_{s2}-casein. A combination of anion- and cation-exchange gave the best quantitative results on the extent of proteolysis. Both techniques could be used in combination with other methods, such as PAGE and reverse phase HPLC, for further characterisation of the peptides.

A study was carried out into the effect of heat treatment and acidification on the dissociation of casein micelles. After heating at functionalisation temperatures and storing at 4, 20 or 30°C, the serum concentrations of Ca and P_i were restored to their original levels after 22h. Levels of κ-casein increased considerably on heating, however, and about 27% of the total κ-casein dissociated. At 4 and 20°C, the levels of β-casein in the serum also increased on heating. On acidification of heat-treated milk and storage at 4, 20 or 30°C, micellar Ca and P_i were completely removed at pH 5.0. As the pH decreased from 6.7, there were marked increases in the dissociation of all caseins at 20°C, and especially at 4°C, reaching maxima at pH 5.7 and 5.5, respectively; thereafter serum concentrations decreased rapidly with decreasing pH. Compared with raw milk, the pH of maximum dissociation was higher, and overall levels of dissociation were reduced in heat-treated milk. At 30°C, the amount of dissociation decreased to negligible levels with decreasing pH, despite complete removal of all the colloidal Ca and P_i at low pH. The results indicate that denaturation of the whey proteins, and their association with the caseins, promoted aggregation of the micelles as the pH was reduced.

Denaturation of the whey proteins in heated milk and their incorporation into Cheddar cheese

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1. Introduction

The whey proteins are sensitive to heat at temperatures above 60 °C and undergo denaturation which involves unfolding of the globular conformation (1), an increase in surface hydrophobicity (2, 3) and disulphide linkage with other proteins (4). If the heat treatment is sufficiently severe the changes become irreversible, and the denatured whey proteins, which become closely associated with the micelles through disulphide or hydrophobic interaction (5), precipitate together with the caseins on acidification to pH 4.6 (6, 7). Similarly, on rennet treatment of heated milk, depending on the extent of heating, the denatured whey proteins are incorporated into the curd, giving increased cheese yield (8, 9).

Denaturation of total whey protein in heated milk has previously been examined by measuring changes in the soluble whey protein nitrogen at pH 4.6 (6, 7). Also, the extent of denaturation of the individual whey proteins, on the basis of decreased solubility at pH 4.6, has previously been studied by anion-exchange FPLC (10, 11), reverse phase HPLC (12), gel scanning (13, 14) and gel permeation (1, 15).

In this study we have used a method of gel permeation FPLC (16) to examine the effect of temperature, holding time, and pH of heating on the extent of denaturation of four whey protein fractions. We have also studied the effect of denaturation of the individual whey proteins on the extent of their incorporation into the curd during the pilot-scale production of Cheddar cheese.

2. Materials and methods

2.1 Milk samples

Milk samples were collected from the bulk milk of Friesian cows in the Institute herd, and skimmed in a fat separator. Fat was added back to the milk to give a casein:fat ratio of 0.7.

2.2 Heat treatment

Whole milk was heated on a modified pilot-scale heat-exchanger (APV Junior) at fixed temperatures of 72, 80, 90, 100, 110, 120 or 140 °C for holding times of 0.25, 0.5, 1.0, 2.0 or 5.0 min. The heat-exchanger gave rapid heating to the holding temperature and subsequent rapid cooling.

In part of the study, milk samples were adjusted to pH values between 6.2 and 9.1 by the addition of HCl or NaOH before heating, and then re-adjusted to pH 6.7. All samples were cooled and kept at 4 °C for about 17 h before being used for cheese-making.

2.3 Acid filtrate

Milk samples were skimmed by centrifugation at 1000 g for 30 min and adjusted to pH 4.6 at 20 °C by the addition of 1M HCl. The supernatant was passed through Whatman No. 42 filter paper and then a 0.22 μm nylon filter.

2.4 Whey

Whey was collected during the pilot-scale production of Cheddar cheese by a modified method (8), and filtered through a 0.22 μm nylon filter.

2.5 Gel permeation FPLC

Samples of acid filtrate and whey from raw and heated milks were fractionated by gel permeation FPLC as described previously (16).

2.6 Nitrogen analysis

Solutions for the determination of total, non-casein and non-protein nitrogen were prepared by methods based on those of Rowland (7), and their N content was determined by a micro-Kjeldahl procedure.

3. Results and discussion

3.1 Denaturation of the whey proteins

The effect of temperature. Milk samples were heated at selected temperatures between 72 and 140 °C for 1 min, and the denatured whey proteins were precipitated together with the casein by adjusting the pH to 4.6. The relative amounts of four whey protein fractions remaining in the serum were examined by gel permeation FPLC, as described previously (16), and typical profiles are shown in Fig. 1. The four fractions were: (1) immunoglobulins (2) serum albumin and lactoferrin (3) β-lactoglobulin and (4) α-lactalbumin. The solubility of each of the whey proteins at pH 4.6, and the corresponding peak area, decreased with increasing temperature, and at 140 °C mainly proteose peptones, representing less than 5 % of the total area at 280 nm, remained (16). SDS-PAGE confirmed that the composition of the fractions was unchanged on heating, and that the shoulder which became increasingly prominent on the β-lactoglobulin peak, at an elution time of 23 min, was monomeric β-lactoglobulin (16).

The level of denaturation of each fraction was calculated as the decrease in area of each peak, expressed as a percentage of the area of the corresponding peak in acid filtrate from raw milk. Values for each of the whey proteins, at temperatures between 72 and 140 °C and a holding time

of 1 min, are shown in Fig. 2. The immunoglobulin and serum albumin fractions were most heat sensitive, and about 40 % was irreversibly denatured at 72 °C. Less than 10 % of β-lactoglobulin was denatured at 72 °C, but there was a rapid increase between 80 and 90 °C, and more than 80 % was denatured at 100 °C. α-Lactalbumin was the most heat resistant and was almost unaffected by heating at 80 °C for 1 min, but the level of irreversible denaturation increased linearly to above 80 % at 140 °C. The extent of denaturation of total whey protein, determined from the level of soluble whey protein nitrogen at pH 4.6, also increased up to about 120 °C but increased slowly above this temperature due to the presence of the heat-stable proteose peptones (7).
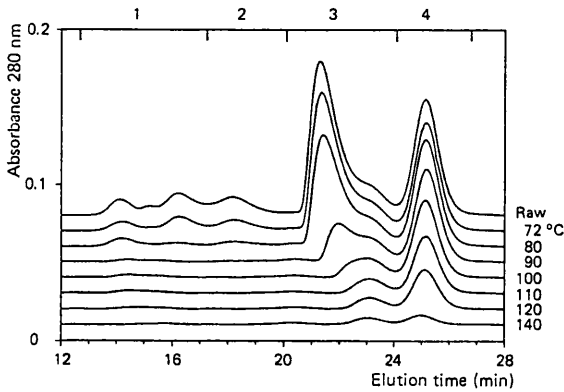

Fig. 1: Elution profiles obtained by gel permeation FPLC of acid filtrates prepared from milk heated for 1 min at temperatures between 72 and 140 °C. A 50 µl solution containing about 0.3 mg whey proteins was applied in tris-HCl buffer (pH 7.0; 100 mM tris, 0.5 M NaCl) to a column of Superdex 75 HR 10/30, and eluted at a flow rate of 0.5 ml/min.
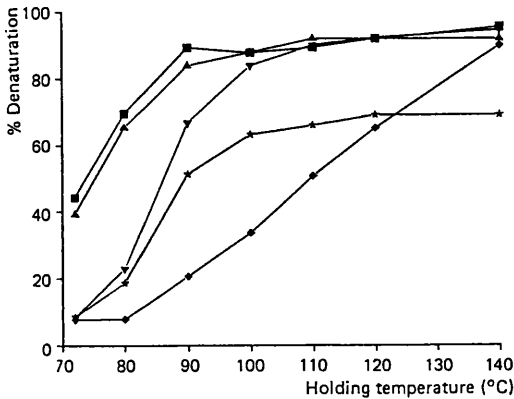

Fig. 2: The effect of temperature, at a holding time of 1 min, on the extent of denaturation of the individual and total whey protein. ■ Immunoglobulins, ▲ serum albumin/lactoferrin, ▼ β-lactoglobulin, ♦ α-lactalbumin, * total whey protein.

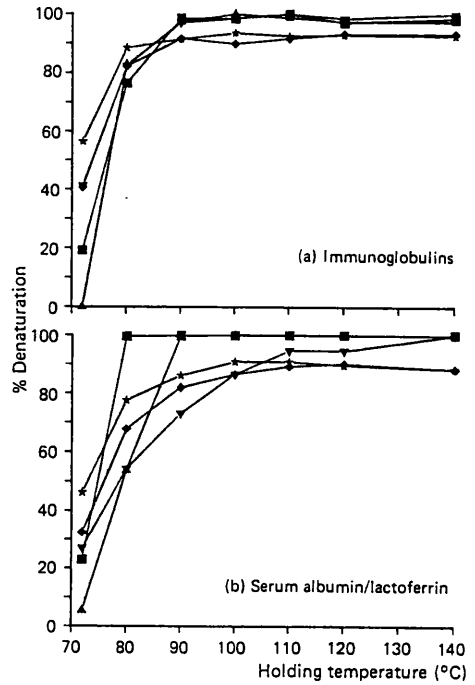Fig. 3: The effect of holding time and temperature on denaturation of (a) immunoglobulins and (b) serum albumin and lactoferrin. Holding times were: ■ 15 s, ▲ 30 s, ▼ 1 min, ♦ 2 min and * 5 min.
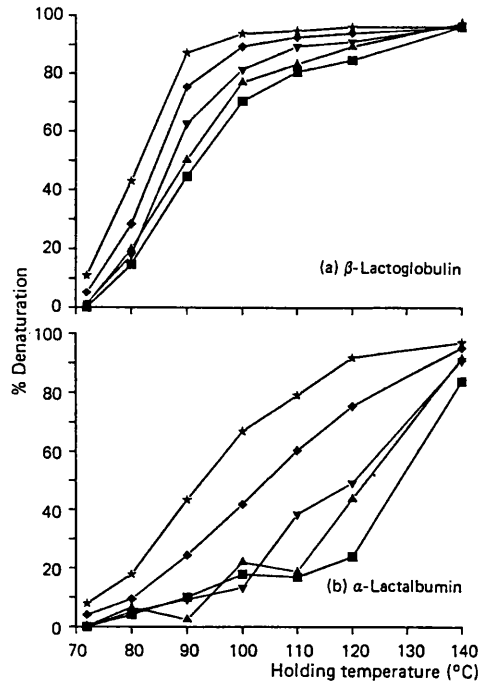

Fig. 4: The effect of holding time and temperature on the denaturation of (a) β-lactoglobulin (b) α-lactalbumin and (c) total whey protein. Holding times were: ■ 15 s, ▲ 30 s, ▼ 1 min, ♦ 2 min, and * 5 min.

The effect of holding time. The effect of holding times between 15 s and 5 min, at temperatures between 72 and 140 °C, is shown in Fig. 3 and 4. The level of denaturation of each whey protein increased with holding time but the immunoglobulin and serum albumin fractions were most affected at temperatures between 72 and 90 °C, whereas β-lactoglobulin was most affected between 72 and 120 °C, and α-lactalbumin between 72 and 140 °C. Denaturation of total whey proteins, measured by nitrogen analysis, increased with holding time over the range 72–120 °C, but there was a slight apparent decrease at holding times of 2 and 5 min at 140 °C which is believed to be caused by increased breakdown of the caseins.

Results for the effect of temperature and holding times agree with other methods based on the decrease in solubility of the denatured whey proteins at pH 4.6. LARSON and ROLLERI (17) found that the immunoglobulins and serum albumin were least heat resistant, β-lactoglobulin intermediate, and α-lactalbumin most heat resistant. The results also agree with those of DANNENBERG and KESSLER (13) who obtained quantitative values for the effects of temperature and holding time, and showed that β-lactoglobulin was more easily denatured than α-lactalbumin. The present results differ, however, from those of RÜEGG *et al.* (18) who found that the denaturation temperatures of the whey proteins, measured as the temperatures of maximum heat absorption by direct scanning calorimetry, were considerably lower. The differences between the methods are especially marked for β-lactoglobulin and α-lactalbumin and can be attributed to the initial reversibility of the heat-induced changes in these proteins, followed by irreversible denaturation at higher temperatures (19). In the present study, as discussed below, we found the loss of solubility of the whey proteins at pH 4.6 a useful measure of their denaturation because it was also closely correlated with the extent to which they became associated with the micelles and were incorporated into the curd on renneting heat-treated milk.

In the acid filtrate the ratio of β-lactoglobulin : α-lactalbumin was about 3.6, but this decreased rapidly with increasing temperature and holding time so that, after heating, at 140 °C for 5 min, giving 67 % denaturation of the total whey protein, the ratio was about 0.5. This variation in the ratio of β-lactoglobulin and α-lactalbumin, which arises because of their different rates of denaturation, could be used as a means of estimating the extent of heat treatment and denaturation in total whey protein or whey.

The effect of pH of heating. The effect of heating milk between pH 6.2 and 9.1, at 90 °C for 30 s, is shown in Fig. 5. The levels of denaturation of β-lactoglobulin and α-lactalbumin determined by gel permeation FPLC, and of total whey protein determined by nitrogen analysis were lowest at pH 6.5, 6.4 and 6.3 respectively. The level of denaturation of each was slightly higher in milk heated at pH 6.2, but increased considerably at alkaline
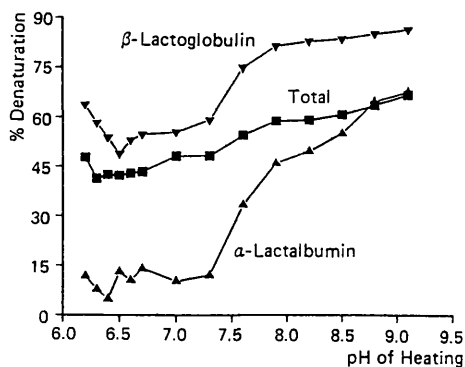

Fig. 5: The effect of pH of milk, heated at 90 °C for 30 s, on the levels of denaturation of β-lactoglobulin, α-lactalbumin and total whey protein.

pH. The most rapid increase in the level of denaturation of β-lactoglobulin occurred between pH 6.5 and 8.0, whereas that for α-lactalbumin was between 7.3 and 9.2. The overall increase in denaturation was about 24 % of the total whey protein. Several workers have suggested that the whey proteins become associated with the casein micelles on heating milk below pH 6.7, but at alkaline pH there is effectively a depletion of calcium in the serum and an increase in the negative charge of the caseins, and the whey protein/κ-casein complexes instead tend to be found in the serum (9, 20). In the present study, however, the milk was re-adjusted to pH 6.7 after heating, and then stored at 4 °C for 17 h. This treatment, reducing the negative charge and increasing the serum calcium concentration, could promote re-association of the denatured whey proteins with the casein micelles.

3.2 Incorporation of denaturated whey proteins into Cheddar cheese

Gel permeation FPLC was used to examine the effect of denaturation of the individual whey proteins on the extent of their incorporation into Cheddar cheese. The elution profiles obtained for whey were similar to those for acid filtrate (Fig. 1), except for a slight increase in the size of the void volume peak at an elution time of 14 min. The peak was established by SDS-PAGE to contain mainly non-protein material and was not included with the other fractions. The relative amounts of the individual whey proteins in acid filtrate and the corresponding whey, obtained after the preparation of Cheddar cheese from milk pasteurised at 72 °C for 16 s, were almost identical (Table 1 (a)). Also, the relative amounts of the whey proteins in acid filtrate and the corresponding whey from milk heated at 90 °C for 30 s were similar (Table 1 (b)). However, compared to the whey protein from milk pasteurised at 72 °C for 16 s, that from the heat-treated milk contained less of the immunoglobulin, serum albumin and β-lactoglobulin fractions, and substantially more α-lactalbumin.

Correlations were calculated between the fraction of β-lactoglobulin and α-lactalbumin remaining

soluble in acid filtrate, and the corresponding fractions remaining in the whey obtained during the production of Cheddar cheese. The acid filtrates and wheys were prepared from milk pasteurised at 72°C for 16 s or milks heated at 90°C for 30 s between pH 6.2 and 9.1. The results (data not shown) demonstrate that, regardless of heat-treatment, there was a close correlation between the amounts of β-lactoglobulin (r=0.973) and α-lactalbumin (r=0.898) soluble in acid filtrate and the amounts lost in the whey. This suggests that the changes which occur on denaturation and cause the loss of solubility at pH 4.6, such as increased hydrophobicity (2,3), disulphide linkage (4) and association with casein micelles (5), also promote the incorporation of the denatured whey proteins into the curd on renneting.

Table 1: The relative amounts of the individual whey proteins in acid filtrate and fresh whey from (a) milk pasteurised at 72°C for 16 s and (b) milk heated at 90°C for 30 s. Values are means for 3 different milks and wheys.

Whey proteins	(a) Pasteurised 72°C/16 s		(b) Heated 90°C/30 s	
	Acid filtrate	Whey	Acid filtrate	Whey
		(% of total)		
Immunoglobulins	6.5	8.9	1.7	3.3
Serum albumin/ lactoferrin	9.1	8.7	7.1	6.9
β-Lactoglobulin	65.5	64.1	59.7	60.0
α-Lactalbumin	18.8	18.2	31.5	29.9

Our results show that the method of gel permeation FPLC described here provides a useful quantitative means of examining factors affecting denaturation of the individual whey proteins and their incorporation into Cheddar cheese, and could be used to monitor the performance of industrial heating equipment, and the recovery of whey proteins during cheese manufacture.

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5. Summary

LAW, A.J.R., BANKS, J.M., HORNE, D.S., LEAVER, J., WEST, I.G.: **Denaturation of the whey proteins in heated milk and their incorporation into Cheddar cheese.** Milchwissenschaft **49** (2) 63–67 (1994).
54 Cheddar cheese (whey proteins)

Gel permeation FPLC (Fast protein liquid chromatography) was used to examine the effect of different heat treatments on the level of denaturation of four whey protein fractions, namely immunoglobulins, serum albumin/lactoferrin, β-lactoglobulin and α-lactalbumin. As the severity of heating increased, each of the whey proteins showed a loss of solubility at pH 4.6, and the decrease in their concentrations in acid filtrate, as determined by gel permeation FPLC, was taken as a measure of the levels of irreversible denaturation.

The extent of denaturation of the total whey protein increased with temperature between 72 and 140°C and with holding times between 15 s and 5 min. At holding times of 1 min, the immunoglobulin and serum albumin fractions were most affected at temperatures between 72 and 90°C, whereas β-lactoglobulin was most affected over the range 72 to 120°C, and α-lactalbumin at temperatures between 72 and 140°C. On adjusting the pH of milk and then heating at 90°C for 30 s, the levels of denaturation of β-lactoglobulin, α-lactalbumin and total whey protein increased considerably as the pH was raised from about 6.5 to 9.1.

There was a close correlation between the levels of denaturation of β-lactoglobulin and α-lactalbumin and the amounts of these proteins retained in the curd during the pilot-scale production of Cheddar cheese.

Results indicate that gel permeation FPLC could be used to examine levels of denaturation of the whey proteins in industrial heating equipment, and their recovery in the curd during cheese manufacture.

LAW, A.J.R., BANKS, J.M., HORNE, D.S., LEAVER, J., WEST, I.G.: **Denaturierung von Molkenproteinen in erhitzter Milch und deren Verwendung bei Cheddarkäse.** Milchwissenschaft **49** (2) 63–67 (1994).

54 Cheddarkäse (Molkenproteine)

Die Gelpermeations-FPLC (Fast Protein Liquid Chromatography) wurde eingesetzt, um die Auswirkungen unterschiedlicher Wärmebehandlungsverfahren auf den Denaturierungsgrad von 4 Molkenproteinfraktionen zu untersuchen. Hierzu gehörten Immunglobuline, Serumalbumin/Laktoferrin, β-Laktoglobulin und α-Laktalbumin. Mit Zunahme der Erhitzungsintensität zeigte jedes der Molkenproteine einen Löslichkeitsverlust bei pH 4,6. Die Abnahme der Konzentrationen im sauren Filtrat (Bestimmung durch Gelpermeations-FPLC) diente als Maß für den Grad der irreversiblen Denaturierung.

Das Denaturierungsausmaß des Gesamtmolkenproteins erhöhte sich bei Temperaturen zwischen 72 und 140 °C und bei Heißhaltezeiten zwischen 15 s und 5 min. Bei Heißhaltezeiten von 1 min wurden die Immunglobulin- und Serumalbuminfraktionen besonders bei Temperaturen zwischen 72 und 90 °C beeinflußt, während beim Laktalbumin die größte Wirkung im Temperaturbereich zwischen 72 und 120 °C und bei α-Laktalbumin im Bereich zwischen 72 und 140 °C festgestellt wurde. Bei Einstellung des pH-Wertes der Milch und anschließender Erhitzung bei 90 °C für die Dauer von 30 s erhöhten sich die Denaturierungsgrade bei β-Laktoglobulin, α-Laktalbumin und Gesamtmolkenprotein erheblich mit Zunahme des pH-Wertes von etwa 6,5 auf 9,1.

Es bestand eine enge Korrelation zwischen den Denaturierungsgraden von β-Laktoglobulin und α-Laktalbumin und den Anteilen dieser Proteine, die im Bruch bei der Herstellung von Cheddarkäse im Pilot-Maßstab zurückgehalten wurden.

Die Ergebnisse weisen darauf hin, daß die Gelpermeations-FPLC eingesetzt werden kann, um die Denaturierungsgrade von Molkenproteinen unter industriellen Erhitzungsbedingungen und deren Verbleib im Käsebruch während der Käseherstellung zu untersuchen.

Heat denaturation of bovine, caprine and ovine whey proteins

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1. Introduction

Denaturation of bovine whey proteins occurs when milk is heated above 60 °C, and involves an initial reversible step in which the intramolecular disulphide bonds are broken and the proteins lose their globular configuration (1, 2). On more severe heating, the whey proteins undergo irreversible denaturation, and become associated with the caseins through hydrophobic interaction and disulphide linkage (3, 4, 5, 6). On subsequent acidification to pH 4.6, the denatured whey proteins precipitate together with the caseins (7), and the decrease in concentration of the individual whey proteins in the acid filtrate can be used to determine the extent of their irreversible denaturation. The order of ease of irre-

versible denaturation of the bovine whey proteins has been found to be immunoglobulins >serum albumin >β-lactoglobulin >α-lactalbumin (8,9,10,11).

The close association of denatured whey proteins with the casein micelles leads to changes in the processing characteristics of heated milk. In the manufacture of yogurt, for example, controlled heat denaturation of the whey proteins gives improved texture (12). Also, during the manufacture of cheese from heated milk, the denatured whey proteins are retained in the rennet-curd, giving a substantial increase in cheese yield (13). Denaturation of bovine whey proteins has been extensively studied (14, 15, 16), but comparatively little information is available about the relative rates of denaturation of caprine and ovine whey proteins.

In previous studies, gel permeation FPLC was used to study the rates of denaturation of the individual bovine whey proteins and their incorporation into Cheddar cheese (17, 18). In this study, gel permeation FPLC has been used to examine the content and composition of the whey proteins in the milk of the cow, goat and sheep, and to determine the relative rates of denaturation of the individual whey proteins of the three species.

2. Materials and methods

2.1 Milk samples

Samples were collected from the bulk milks of Friesian cows and British Saanen goats at the Institute, and from Friesland sheep in South-West Scotland. The milks were skimmed by centrifugation at 1000 g for 30 min.

2.2 Heat treatment

Skim-milk samples (2.5 ml) were heated in stoppered, thin-walled glass tubes (1 mm wall, 7 mm internal diameter) in a waterbath at 70, 80 and 90°C. The milks were allowed to warm up for 1 min, and were held at the required temperature for additional periods of between 0.25 and 30 min. At the end of the holding times, the milks were rapidly cooled in ice.

2.3 Gel permeation FPLC

As described previously (17).

2.4 SDS-PAGE

As described previously (17).

2.5 Cation-exchange FPLC

The composition of the casein in the milk of each species was determined by cation-exchange FPLC (21).

3. Results and discussion

3.1 Characterisation of fractions obtained by gel permeation FPLC

The elution profiles obtained by gel permeation of dialysed, freeze-dried whey proteins from the cow, goat and sheep are shown in Fig. 1. Characterisa-
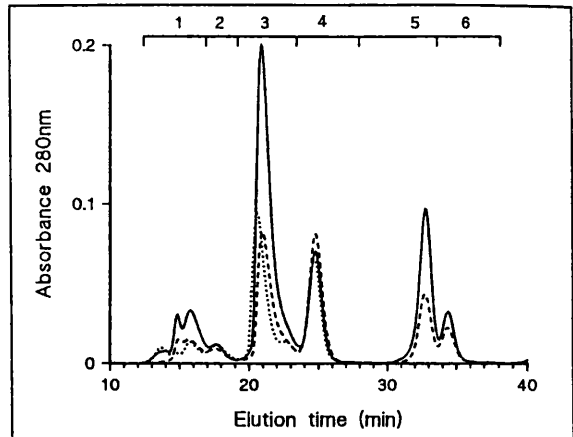

Fig. 1: Elution profiles obtained by gel permeation FPLC of freeze-dried whole whey proteins in tris-HCl buffer (pH 7.0; 100mM tris, 0.5M NaCl) on a column of Superdex 75 HR 10/30. About 0.3-0.6 mg whey proteins were applied to the column and eluted at a flow rate of 0.5 ml/min. — Bovine, ---- caprine, — ovine.

tion of the fractions obtained by gel permeation FPLC of bovine and caprine whey proteins has been described in previous publications (17, 22). The elution profile of ovine whey protein was similar to those obtained for bovine and caprine whey protein, and the identities of the fractions were determined by SDS-PAGE (Fig. 2). Fraction 1 contained IgG and IgM, which were separated by SDS-PAGE into heavy and light chains. There also appeared to be some protein with the same electrophoretic mobility as serum albumin in the fraction. Fraction 2 contained mainly serum albumin, together with a small amount of lactoferrin. Fraction 3 consisted of β-lactoglobulin, together with some proteose peptones, and fraction 4 contained α-lactalbumin. Fractions 5 and 6, which were also present in caprine acid filtrate (22), but absent from bovine acid filtrate (17), gave
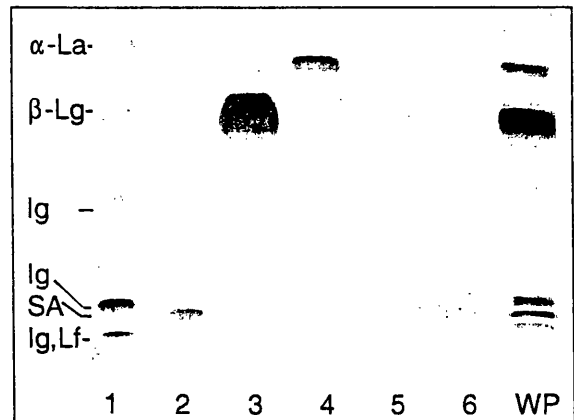

Fig. 2. SDS-PAGE pattern on a 20% homogeneous gel for ovine whole whey protein (WP), and the fractions obtained by gel permeation FPLC as shown in Fig.1. Lanes 1-6, fractions 1-6, respectively.

only very faint bands on SDS-PAGE, and contained mainly non-protein material with a molecular weight of about 3,000. The materials eluting after fraction 6 had low molecular weights and, in bovine acid filtrate, have been identified as mainly orotic and uric acids, respectively (19).

3.2 The composition of bovine, caprine and ovine whey proteins

Using anion-exchange FPLC (19, 20), it was established that β-lactoglobulin in the cows' milk used in this study consisted of 54 and 46% of the A and B genetic variants, respectively. Also, the milks from the goat and sheep each contained only one variant of β-lactoglobulin, that in ewes' milk being β-lactoglobulin A. The relative amounts and concentrations of the whey proteins in bulk skim-milks of the three species, determined by gel permeation FPLC, are shown in Table 1.

Table 1: The composition and content of the whey proteins in unheated bulk skim-milks of the cow, goat and sheep

	Cow	Goat	Sheep
		% of Total	
Immunoglobulins	15.0	11.5	20.0
Serum albumin/lactoferrin	9.5	12.8	8.1
β-Lactoglobulin	59.3	54.2	61.1
α-Lactalbumin	16.2	21.4	10.8
		Concentration (g/l)	
Immunoglobulins	0.97	0.71	2.15
Serum albumin/lactoferrin	0.61	0.79	0.87
β-Lactoglobulin	3.83	3.33	6.58
α-Lactalbumin	1.05	1.31	1.16
Total whey protein	6.46	6.14	10.76

3.3 Denaturation of the individual whey proteins

When milk from the cow, goat or sheep was heated between 70 and 90°C and subsequently adjusted to pH 4.6, there were changes in the gel-permeation elution profiles of the acid filtrates that indicated a progressive loss of solubility of the whey proteins at pH 4.6 with increasing severity of heating. These changes in the elution profiles of bovine and caprine whey proteins have been described previously (18, 22), and are consistent with irreversible denaturation of the whey proteins, involving their close association with the caseins through hydrophobic interaction or disulphide bonds, and their subsequent acid precipitation together with the caseins.

Apart from at very low and very high levels of denaturation, the ease of denaturation over the range of temperature and holding time for all three species was in the order immunoglobulins >serum albumin/lactoferrin >β-lactoglobulin >α-lactalbumin (Fig. 3 and 4). Various workers have found the same order of irreversible denaturation for the bovine whey proteins (8, 9, 10, 11, 18) but comparatively little information is available for the whey proteins of the goat and sheep.
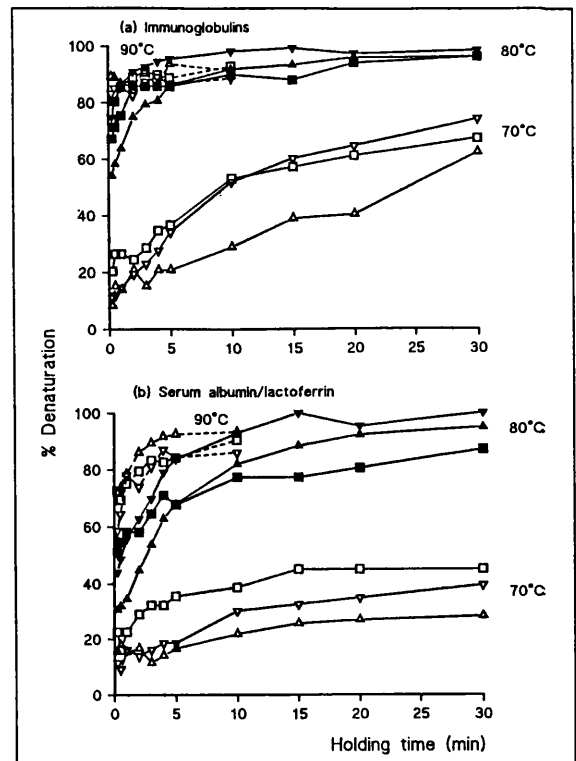
Fig. 3: The effect of holding time at 70, 80 and 90 °C on the extent of denaturation of (a) immunoglobulins and (b) serum albumin/lactoferrin of the cow, goat and sheep. ▢– cow, △– goat, ▽– sheep, 70°C; ■ Cow, ▲ goat, ▼ sheep, 80°C; ▢--- Cow, △--- goat, ▽--- sheep, 90°C. Values at 90°C for the cow and sheep are means of 2 different milks. Values at 70 and 80°C for the goat are means of 2 milks, and those at 90°C means of 3 different milks.

3.4 Species differences in denaturation of the whey proteins

Differences were found in the rates of denaturation of the corresponding individual whey proteins of the three species (Fig. 3 and 4). On heating at 70°C for times between 0.25 and 30 min, the rates of denaturation of the respective individual bovine and ovine whey proteins were fairly similar, but were greater than for the corresponding caprine whey proteins. With short heating times at 80°C, the rates of denaturation of the individual whey proteins generally followed the order ovine >bovine >caprine. On heating at 80°C for longer times, however, there were considerable differences in the rates of denaturation of the whey proteins of the 3 species, and the ease of denaturation was in the order ovine >caprine >bovine. At 90°C, denaturation of the immunoglobulins, and the serum albumin/lactoferrin fraction occurred rapidly, and differences between species were small. The rates of denaturation of the respective β-lactoglobulins and α-lactalbumins, however, were considerably different and again in the order ovine >caprine >bovine. RAMOS (23) examined changes in the levels of 6 nitrogen fractions in milk,
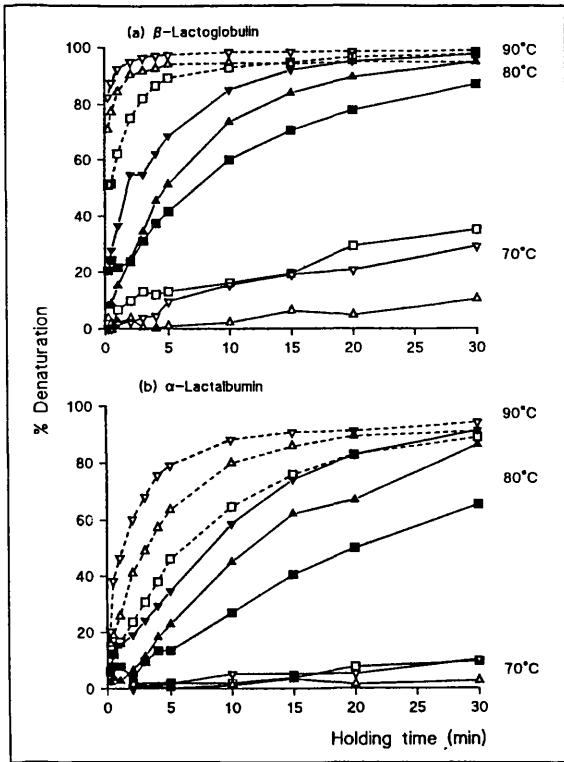
Fig. 4: The effect of holding time at 70, 80 and 90 °C on the extent of denaturation of (a) β-lactoglobulin and (b) α-lactalbumin of the cow, goat and sheep. □– Cow, △– goat, ▽– sheep, 70°C; ■ Cow, ▲ goat, ▼ sheep, 80°C; □--- Cow, △--- goat, ▽--- sheep, 90°C. Replicates as in Fig.3.

and found, as in the present study, that ewes' milk showed higher sensitivity to heat treatment than that of the cow or goat.

3.5 Kinetics of denaturation of β-lactoglobulin and α-lactalbumin

The orders of reaction and rate constants for denaturation of the individual whey proteins (Table 2) were determined by examining the closeness of fit of

the data to two expressions derived from the general rate equation, as described previously (10, 14, 16). The reaction orders of the corresponding whey proteins of the 3 species were the same, but the rate constants differed. At 70°C the rate constants for denaturation of caprine immunoglobulins, β-lactoglobulin and α-lactalbumin were lower than those for the corresponding bovine and ovine whey proteins. At 80 and 90°C, however, the order was ovine > caprine > bovine (Table 2).

Some of the differences in the rates of denaturation of the corresponding individual whey proteins of the three species may be due to the small differences in the sequences of the whey proteins. In the present study, however, on severe heating, all of the caprine and ovine whey proteins were more easily denatured than the corresponding bovine whey proteins, indicating that other factors might be involved.

Comparison of the content of the caseins in skimmilk of the three species (Table 3) shows that the concentrations of caprine and ovine κ-caseins were considerably higher than for bovine κ-casein. Caprine and ovine κ-caseins also each contain 3 -SH groups, whereas bovine κ-casein contains only 2. Compared with cows' milk, therefore, caprine and ovine milks contained almost twice the concentration of -SH on κ-casein available for disulphide bonding to the whey proteins. Also, the concentration of caprine β-casein was slightly higher, and that of ovine β-casein considerably higher than the concentration of bovine β-casein. As β-casein is the most hydrophobic of the caseins, the higher concentration in ewes' milk may promote the initial hydrophobic interaction of denatured whey proteins and the caseins.

Table 3: The composition and content of the caseins in bulk skim-milks of the cow, goat and sheep

	Cow	Goat % of Total	Sheep
Minor	6.2	2.5	4.8
κ-Casein	9.4	13.2	8.8
β-Casein	37.5	50.1	43.5
α_{s1}-Casein	33.0	18.4	
α_{s2}-Casein	13.9	15.8	} 42.9
	Concentration (g/l)		
Minor	1.51	0.53	1.72
κ-Casein	2.29	2.80	3.16
β-Casein	9.14	10.64	15.60
α_{s1}-Casein	8.04	3.91	
α_{s2}-Casein	3.39	3.36	} 15.39
Total casein	24.37	21.24	35.87

Results of casein and whey protein analyses (Tables 1 and 3) show that there is considerable potential for increasing cheese yield by the incorporation of denatured whey proteins into the curd. Assuming the loss of caseinomacropeptide from κ-casein (Table 3) into the whey, and quantitative retention of the denatured whey proteins in the curd, as found previously for cows' milk (18), the calculated possible increases in protein yield from heat

Table 2: Rate constants for denaturation of whey proteins

		Immuno-globulins $n = 1.5$	β-Lacto-globulin $n = 1.5$ k sec^{-1} x 10^3	α-Lact-albumin $n = 1$
70°C	Cow	0.74	0.25	0.05
	Goat	0.36	0.05	0.009
	Sheep	1.05	0.21	0.06
80°C	Cow	3.09	1.82	0.57
	Goat	4.43	3.69	1.08
	Sheep	18.55	5.55	1.36
90°C	Cow	—	11.6	1.41
	Goat	—	15.4	2.48
	Sheep	—	25.9	4.46

treated milk of the the cow, goat and sheep were 27.4, 30.3 and 31.0 %, respectively. The information presented in this work could be used in the selection of suitable heat treatments to increase cheese yield from the milk of the goat and sheep, as described previously for cows' milk (24).

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5. Summary

LAW, A.J.R.: **Heat denaturation of bovine, caprine and ovine whey proteins.** Milchwissenschaft **50** (7) 384–388 (1995).

66 Whey proteins (heat denaturation)

The whey proteins in the milk of the cow, goat and sheep were separated by gel permeation FPLC into 4 main fractions containing immunoglobulins, serum albumin/lactoferrin, β-lactoglobulin and α-lactalbumin. Differences were found in the relative amounts and concentrations of the individual whey proteins of the three species and, compared with cows' milk, ewes' milk contained considerably more total whey protein, immunoglobulins and β-lactoglobulin. Gel permeation FPLC was also used to examine the relative rates of irreversible denaturation of the individual whey proteins, and on heating between 70 and 90°C the order of ease of denaturation for all 3 species was immunoglobulins >serum albumin/lactoferrin >β-lactoglobulin >α-lactalbumin. There were differences in the rates of denaturation of the corresponding whey proteins of the 3 species. On heating at 70°C, or at 80°C with short holding times, caprine whey proteins were less extensively denatured than bovine or ovine whey proteins. On more severe heating at 80 and 90°C, the order of ease of denaturation of the whey proteins was usually ovine > caprine >bovine. On heating at 90°C, however, denaturation of the immunoglobulins and serum albumin/ lactoferrin occurred rapidly, and differences between species in these proteins were less pronounced. Results showed that, as found previously for cows' milk, there was considerable potential for increasing cheese yield by the incorporation of denatured caprine and ovine whey proteins into the curd during cheese manufacture, and suitable heat treatments could be determined from the denaturation levels presented.

LAW, A.J.R.: **Hitzedenaturierung von Kuh-, Ziegen- und Schaf-Molkenproteinen.** Milchwissenschaft **50** (7) 384–388 (1995).

66 Molkenproteine (Hitzedenaturierung)

Die Molkenproteine der Kuh-, Ziegen- und Schafmilch wurden durch Gelpermeations FPLC in 4 Hauptfraktionen getrennt, die Immunglobuline, Serumalbumin/Laktoferrin, β-Laktoglobulin und α-Laktalbumin enthielten. Unterschiede wurden in den relativen Mengen und Konzentrationen der einzelnen Molkenproteine der drei Species festgestellt. Verglichen mit Kuhmilch enthielt Schafmilch wesentlich mehr Gesamtmolkenprotein, Immunglobuline und β-Laktoglobulin. Die Gelpermeations-FPLC wurde auch zur Untersuchung der relativen Grade der irreversiblen Denaturierung der einzelnen Molkenproteine verwendet. Nach dem Erhitzen auf 70 bis 90°C lag die Reihenfolge der Denatuierungsneigung für alle 3 Species wie folgt: Immunglobuline >Serumalbumin/Laktoferrin >β-Laktoglobulin >α-Laktalbumin. Es gab Unterschiede im Denaturierungsgrad der entsprechenden Molkenproteine der 3 Species. Beim Erhitzen auf 70 oder 80°C mit kurzen Haltezeiten waren die Ziegenmolkenproteine weniger denaturiert als die von Kuh oder Schaf. Bei stärkerem Erhitzen auf 80 oder 90°C lag die Denaturierungsneigung der Molkenproteine in der Reihenfolge Schaf > Ziege >Kuh. Bei Erhitzen auf 90°C ging die Denaturierung der Immunglobuline und Serumalbumin/ Laktoferrin jedoch schnell vor sich, und die Unterschiede zwischen den Species waren für diese Proteine weniger ausgeprägt. Die Ergebnisse zeigten, daß, wie für Kuhmilch festgestellt, die Käseausbeute durch den Zusatz denaturierter Ziegen- und Schafmolkenproteine zum Bruch im Verlauf der Käseherstellung deutlich erhöht werden kann. Geeignete Hitzebehandlungen sind aus den hier dargestellten Denaturierungsgraden abzuleiten.

Sodium caseinates — composition and properties of different preparations

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A number of samples of commercially available spray-dried and roller-dried caseinates have been compared with freshly prepared caseinate from the laboratory. The compositions of the materials were measured using chromatography, and their calcium susceptibilities were determined using turbidity measurements. The chromatographic analysis showed that the commercial caseinates differed substantially from the fresh material, especially in the regions where x-casein and α_{s2}-caseins were eluted. The fresh caseinate was more affected by the presence of calcium ions than the others, and there were also differences between the individual spray-dried caseinates in this respect. The roller-dried caseinates were very insensitive to the presence of calcium ions.

Sodium caseinate consists 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- or roller-dried. Ideally, there should be no difference between the material prepared in the laboratory and that which is prepared on a large scale by industrial processes. However, in the course of recent work it has become apparent to us that different batches of caseinates from the same or different manufacturers possess different stability properties in the presence of ethanol (Muir & Dalgleish, 1987).

We have, therefore, undertaken detailed comparisons of different commercially available caseinates. Two particular aspects were investigated, namely the detailed composition of the caseinate (which can be analysed by chromatography (Davies & Law, 1987)) and the calcium susceptibility of the different samples. The functional properties of the casein in, for example, cream liqueurs, depend to some extent on its interaction with calcium (Banks, Muir & Wilson, 1981) and the aggregation of caseins with calcium has been widely studied (Schmidt, 1982). This aggregation can be taken to be a good indication of differences between caseinates. Individual α_s-and β-caseins are precipitated by sufficient calcium (Horne & Dalgleish, 1980), but the whole casein, containing the stabilizing protein x-casein, forms more stable aggregated particles in the presence of moderate amounts of calcium ions (Schmidt *et al*, 1974). The extent of such aggregation can be estimated by measuring the turbidity of the solutions.

MATERIALS AND METHODS

Samples of spray- and roller-dried caseinates of Danish origin and spray-dried caseinate of Scottish origin were provided respectively by Sibner Hegner (Kent) and Scottish Pride (Stranraer). Fresh sodium caseinate was prepared in the laboratory from skimmed milk from the institute's herd, using the following method: 1 litre of milk was diluted with an equal volume of water, and the pH was adjusted to 4.6 with HCl (1M). The temperature was raised to 35°C, and the mixture was left for one hour. The precipitated caseins were collected by filtration through a Whatman No. 1 filter paper, washed and resuspended in 1 litre of water. The pH of this mixture was adjusted to 7.0, and the mixture was stirred, with continual readjustment of the pH to 7.0, until all of the casein had dissolved. The caseinate was then dialysed exhaustively against distilled water and was freeze-dried.

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Before analysis, samples of the caseinates were alkylated so that (i) their sulphydryl residues were incapable of interacting and (ii) the charges of the α_{s2}-caseins were reduced to help in their chromatographic separation (Davies & Law, 1987). After dialysis to remove excess reagents, the alkylated caseinate samples were suspended in a buffer containing 3.3 M urea and 5 mM bis-tris-propane at a pH of 7.0 (Buffer A). After suspension, the solutions were filtered through membranes of pore size 0.22 μm. Some of the solutions required filtration through membranes of larger pore size prior to the final filtration. After filtration, the caseins were analysed using Fast Protein Liquid Chromatography (FPLC) (Pharmacia, UK), as described by Davies & Law (1987).

For studies of the calcium sensitivity, caseinates were resuspended to a concentration of about 20 mg ml^{-1} in a buffer of 20 mM imidazole, 50 mM NaCl, pH 7.0. The temperature was raised during this process to 80°C to facilitate solution, and then the solutions were allowed to cool. All of the solutions were cloudy, and were filtered through cellulose nitrate filters of successively smaller pore sizes down to 0.22 μm. Some of the caseinates, especially the roller-dried material, proved to be unfilterable even through the largest pore size (3.0μm). It was not clear initially whether this was because of large amounts of residual fat or because of incomplete solution of the protein, but when centrifuged at 65000 × g for 60 min the suspensions yielded pellets of insoluble protein and no appreciable fat layers. The caseinates in question therefore contained appreciable amounts of non-dispersible protein. For these caseinate solutions which had required centrifugation, the supernatant remaining after centrifugation was filtered and used for the experiments. The filtered casein solutions were then made up to a concentration of 14.65 mg/ml, as gauged by their absorbance at 280 nm, using an absorbance coefficient of 0.80 for a 1 mg ml^{-1} solution. This coefficient was calculated from the known absorbances of the individual caseins, and an estimate that whole casein contains 40% of each of α_{s1}- and β-caseins and 10% of each of the α_{s2}- and x-caseins. The concentrations of the caseinate solutions were confirmed using Kjeldahl analysis of the nitrogen content, and were found to agree with those calculated from the absorbance. The Kjeldahl measurements also showed that the caseinates contained effectively no non-casein nitrogen (ie, nitrogen which cannot be precipitated at pH 4.6).

Aliquots (5 ml) of these casein solutions were then mixed with equal volumes of solutions of calcium chloride of defined concentrations in the range 0 to 21 mM, made up in the imidazole buffer used to dissolve the caseins. These mixtures

were stirred vigorously to ensure rapid and complete mixing, and were then left for 90 minutes to equilibrate at a temperature of 20°C. After this time, the turbidity of the solutions was measured, in cuvettes of 1 mm path length, at a wavelength of 600 nm, using a Cecil 292 spectrophotometer. What is measured is the absorbance of the solution, from which turbidity can be calculated by multiplying by 2.303: in the results which follow, the measured absorbance at 600 nm is quoted.

RESULTS AND DISCUSSION

The elution profiles of the different casein samples which were obtained using FPLC are shown in Figs. 1-3. Caseinate prepared in the laboratory (HRI caseinate) showed a number of peaks in the chromatogram, most of which can be identified with the major casein fractions (Fig. 1). The two major peaks are from β- and α_s-caseins: the method which we used gave good separation of the α_{s1}- and α_{s2}-caseins, the α_{s2}-caseins appearing between the β-casein and the α_{s1}-casein fractions. The x-casein was eluted as a series of peaks ahead of the β-casein. All of the peaks in the elution profile were sharp, especially the x- and α_{s2}-casein fractions. The commercial sodium caseinate samples showed a pattern which was qualitatively similar but significantly different in detail. All of the peaks were broadened, especially in the regions were α_{s2}-casein and x-casein were normally eluted. In the former of these two regions, all of the caseinate samples, compared with that prepared in the laboratory, showed a broad area of eluted material, rather than a series of peaks as seen in the elution profile of the HRI caseinate (Fig. 2). The roller- and spray-dried caseinates also differed from one another in this region. Since the material did not elute as peaks, we have as yet been unable to identify its composition. However, it made a significant contribution to the total casein analysis (Table 1). A further point of interest was the appearance of a quantity of material which eluted after the β-casein peak: there was more of this in the roller-dried caseinate, but it was not identified.

The elution profiles of the caseinates in the x-casein region were considerably different from the HRI caseinate (Fig. 3). The roller- and spray-dried caseinates resembled each other over part of the profile, but the spray-dried caseinates all gave an extra peak between the positions of the x-casein and the β-casein. This peak was almost entirely lacking in the HRI caseinate, and was diminished in the roller-dried caseinate. The different peaks in the x-casein fraction of HRI caseinate are known to be the differently-glycosylated x-casein fractions (Vreeman et al, 1977). The breakdown of the pattern in the commercial caseinates suggested that the manufacturing process had caused some deglycosylation of the protein. x-casein contains one seryl phosphate residue (Vreeman et al, 1977): the shift in the peak pattern may also be caused by the dephosphorylation of this group.

Dephosphorylation may also be responsible for the appearance of the peak which eluted ahead of β-casein, and also for the appearance of the material in the region of the α_{s2}-casein. All of the caseins apart from x-casein contain multiple phosphate groups (Swaisgood, 1982) and the reneutralization

Fig. 1. Elution profile of HRI sodium caseinate on Pharmacia Mono-Q exchanger, column type HR5/5. The peaks are identified from previous work by Davies & Law (1987), but the fractions considered here are composites of some of the previously determined fractions. The dotted line shows the gradient of NaCl used to elute the sample.
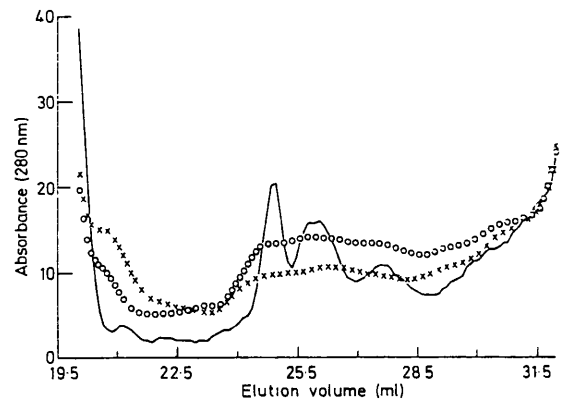
Fig. 2. Elution of the α_{s2}-casein region (cf Fig. 1) of HRI caseinate (—); average of spray-dried caseinates (○○○); and average of roller-dried caseinates (× × ×).

Fig. 3. Elution profiles of the χ-casein region (cf Fig. 1) of HRI caseinate (—); average of spray-dried caseinates (○○○) and average of roller-dried caseinates (× × ×).

TABLE 1

Compositions of the caseinates. The proportions of the different casein fractions in each caseinate, as measured by Fast Protein Liquid Chromatography, and expressed as percentages of the total. The separation between fractions is as in Fig. 1

Sample	γ + other	x	Unknown	β	α_{s2}	α_{s1}
Spray 1	6.8	9.4	2.1	31.8	14.3	35.5
Spray 2	9.1	9.7	2.9	28.2	27.1	23.1
Spray 3	8.9	10.1	3.5	27.1	24.1	26.3
Spray 4	8.7	11.3	2.0	29.5	13.9	34.6
Spray 7	8.6	10.3	3.1	28.6	17.8	31.6
Spray 8	8.4	10.0	3.4	28.3	14.4	35.5
Roller 5	9.0	11.4	1.9	29.4	13.9	34.3
Roller 6	8.7	12.3	2.2	28.1	16.0	32.6
HRI	8.1	9.2	0.9	33.1	11.7	36.9
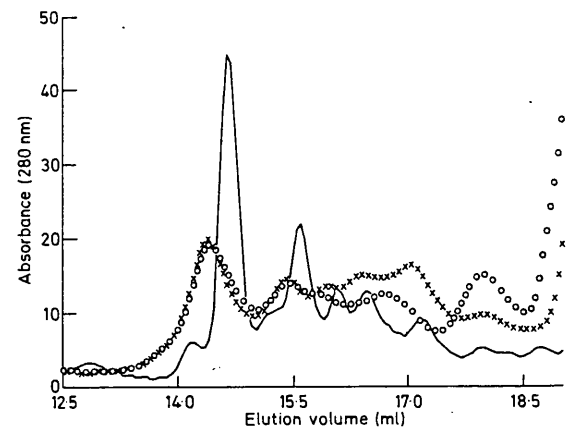
of the acid caseinate, combined with heat, may cause some hydrolysis of the phosphoseryl residues (Manson, 1973). This will reduce the overall charge on the molecules and decrease their affinity for the column packing material, so that they will elute earlier than the unmodified protein.

It is therefore apparent that compositional differences exist between different caseinates (and in fact other results, which are not shown here, demonstrate that differences exist between batches from the same manufacturer and indeed the same plant). There are several reasons why commercial caseinates may differ from those produced in the laboratory, which may explain, either singly or in combination, the sources of variation. The more rigid control of pH in the laboratory, combined with a lower temperature regime during neutralization prevent alkaline degradation of the phosphoseryl residues of the caseins. Moreover, the lower temperature in the laboratory will prevent any reactions between caseins and residual lactose. It is possible for residual serum protein to denature at high temperature and bind to the x-casein (Sawyer, 1969), but this reaction did not appear to be significant, since no traces of denatured serum protein were detected.

A major difference between the caseinates is the method of drying: the laboratory casein is freeze-dried rather than roller- or spray-dried. There are differences between caseins dried by the three different methods and it is possible that the nature of the drying process may be the most important factor in determining the composition of the product.

The effects of different processing treatments were also demonstrated by the different behaviour of the turbidities of the materials when they were subjected to treatment with calcium ions (Figs. 4,5). In all cases, the turbidity remained low (ie, aggregation of the caseinate did not occur) until the solutions contained a total of about 6 mM Ca^{2+}. At concentrations of Ca^{2+} above this value the casein began to aggregate and the turbidity increased, although the calcium concentrations at which the increases were observed were not the same for all samples. The HRI caseinate started to aggregate at the lowest concentration of Ca^{2-}, and the spray-dried preparations were the next most calcium-sensitive. However, there was a variation between the spray-dried materials, some of which required as much as 2 mM more Ca^{2+} than did others (Fig. 5). The roller-dried caseinates were much less calcium-sensitive, giving much smaller increases in turbidity than the other samples. Some turbidity increase was observed at total concentrations of Ca^{2-} of above 6 mM, but the effect was very small (Figs. 4,5).

All of the caseinates, apart from the roller-dried material, showed a plateau in the turbidity at concentrations of Ca^{2+} above 13 mM (Fig. 4). The turbidities of the solutions of spray-dried caseinates in their plateau regions were similar (Fig. 5), but the HRI caseinate gave a turbidity at the plateau of more than twice the values obtained for the spray-dried material. None of the samples precipitated within the time scale of the experiments. Turbidity is essentially dependent upon the weights of the particles which are in suspension, for suspensions having the same concentration, and therefore it was expected that the high turbidities of HRI caseinate would mean that these suspensions contained the largest particles. This was confirmed by using the 90° scattering of laser light to determine the average diameters of the particles in solution. The results for solutions containing 16 mM Ca^{2+} are given in Table 2, and confirm the difference in the particle sizes for HRI caseinate and spray- and roller-dried caseinates.

The roller-dried caseinates did not give a plateau in their turbidity plots, in contrast to the others, but gave slowly increasing turbidity as the concentration of Ca^{2+} was increased. Since the concentrations of the caseinates were the same in all of the experiments, the differences in turbidity cannot be caused by differences in the concentrations of material causing different extents of reaction. The roller-dried material was very insensitive to the presence of Ca^{2+}.

The decreased tendency to aggregation in the presence of
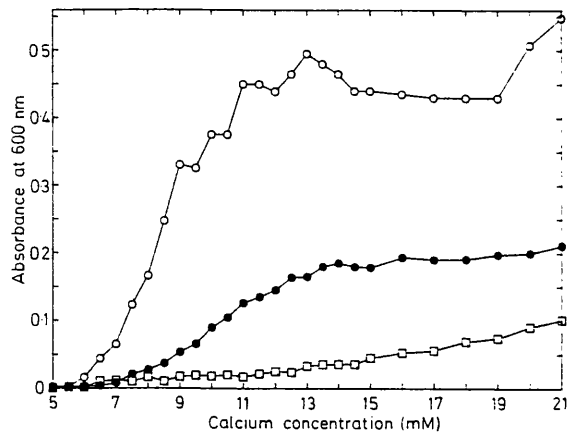
Fig. 4. Calcium sensitivity of the caseinates: $\bigcirc$ = HRI caseinate; $\bullet$ = average of all spray-dried caseinates; $\square$ = average of roller-dried caseinates.
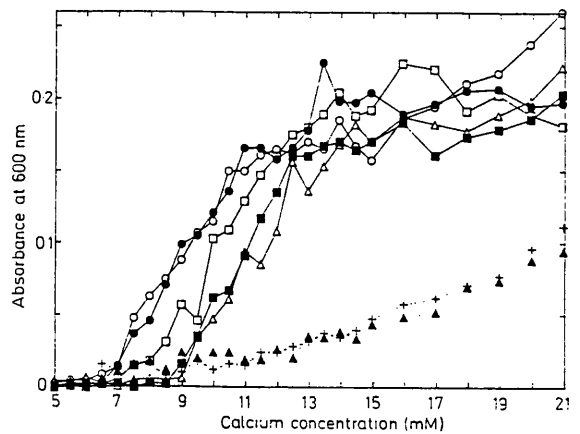
Fig. 5. Calcium sensitivity of the caseinates: $\bigcirc$ = spray-dried caseinate 1; $\bullet$ = spray-dried caseinate 2; $\square$ = spray-dried caseinate 3; $\triangle$ = spray-dried caseinate 4; $\blacksquare$ = spray-dried caseinate 8; $\blacktriangle$ = roller-dried caseinate 5; + = roller-dried caseinate 6.

TABLE 2

Diameters of particles formed from caseinates in solutions containing 15 mM Ca^{2+}

Casein sample	Diameter (nm)
Spray 1	178
Spray 2	208
Spray 3	188
Spray 7	166
Roller 5	112
Roller 6	109
HRI	645

Ca^{2+} of the commercial caseinates, compared with freshly prepared material, can arise from two causes. Either the caseinate has lost the capacity to bind calcium, or the binding of calcium to the proteins can no longer induce aggregation. For example, if extensive dephosphorylation has occurred, less Ca^{2+} will bind to the caseins (Bingham, 1976). Although our studies showed some evidence for dephosphorylation of the caseinates (Muir & Dalgleish, 1987) it did not seem to have occurred on a very large scale.

It may be, therefore, that the processing of the caseinate causes an alteration in the capacity of the caseinates to aggregate, although the source of this modification remains to be established.

CONCLUSION

The conclusion from these studies appears to be that quality control of caseinate may be difficult to achieve, since there is inter-batch variation in the composition and the properties. There is a clear distinction in properties as we measured them, and there is thus a strong suggestion that the functional properties of the different caseinates may be different. Indeed, this may prove to be beneficial, but it must first be ascertained why the caseinates differ before the effect on the functional properties can be adequately described.

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Heat-induced changes in the whey proteins and caseins

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1. Introduction

On heating milk above 60°C the whey proteins increasingly lose their globular conformation (1), become associated with the casein micelles through disulphide or hydrophobic interactions (2, 3, 4), and precipitate together with the caseins on acidification to pH 4.6 (5, 6). On the basis of loss of solubility at pH 4.6, the immunoglobulins and serum albumin are most easily denatured, β-lactoglobulin is intermediate and α-lactalbumin is most heat-stable (7, 8).

The caseins have comparatively little secondary and tertiary structures, but on heating they undergo changes in the way they associate within the micelles and with the denatured whey proteins (9). Also, on severe heating, changes occur in the amino acid residues of the caseins, including removal of phosphate from phosphoserine (10, 11), formation of lysinoalanine (12), and reaction of ε-amino groups of lysine with carbonyl groups of lactose as the first step in the formation of a series of Maillard products (13). On prolonged heating at high temperatures, some proteolysis of the caseins occurs, as indicated by an increase in the level of proteose-peptone nitrogen (5).

The heat-induced changes in the caseins have previously been studied, using dissociating and reducing conditions, by alkaline PAGE (14, 15, 16, 17), SDS-PAGE (14,15) and anion-exchange chromatography (15,18).

In this work, in addition to SDS and alkaline PAGE, we have used gel permeation and anion- and cation-exchange FPLC to examine the effect of heating milk at different temperatures and holding times on denaturation of the whey proteins, and to study the heat-induced changes in the caseins.

2. Materials and methods

2.1 Heat treatment

Whole milk samples were heated at selected temperatures of 72, 80, 90, 100, 110, 120 or 140°C for holding times of 0.25, 0.5, 1.0, 2.0 or 5.0 min as described previously (19).

2.2 Protein samples

Milk samples were skimmed by centrifugation at 1000 g for 30 min, and the denatured whey proteins and the caseins were precipitated at pH 4.6 by the addition of 1M HCl.

2.3 Nitrogen analysis

Solutions for the determination of total, non-casein and non-protein nitrogen were prepared by methods based on those of ROWLAND (6), and their N content was determined by a micro-Kjeldahl procedure.

2.4 Gel permeation FPLC

The extent of denaturation of four whey protein fractions was determined by gel permeation FPLC of the acid filtrates from raw and heated milks as described in a previous publication (20).

2.5 PAGE

The mixtures of denatured whey proteins and caseins were examined by SDS- and alkaline PAGE (20, 21). The proteins on SDS-PAGE were silver stained and on alkaline PAGE were stained with Coomassie Blue.

2.6 Anion-exchange FPLC

The denatured whey proteins and caseins were alkylated and fractionated by anion-exchange

FPLC on a Mono Q HR 5/5 column, according to the method of DAVIES and LAW (22), except that the samples were dissolved in buffer with urea at a concentration of 8.0 M.

2.7 Cation-exchange FPLC

The protein samples were reduced with 2-mer-captoethanol and fractionated by cation-exchange FPLC on a Mono S HR 5/5 column, as described by HOLLAR et al. (23), except that the concentration of urea in the sample solvent was 8.0 M.

3. Results and discussion

3.1 Gel permeation FPLC

The effect of heating milk for 5 min at temperatures between 72 and 140 °C on denaturation of the individual whey proteins is shown in Fig. 1. The extent of denaturation, as determined by loss of solubility at pH 4.6, increased with the temperature, and most of the immunoglobulins and serum albumin, β-lactoglobulin and α-lactalbumin were denatured and precipitated together with the casein at pH 4.6 when milks were heated for 5 min at 80, 90 and 110 °C, respectively. The combined effect of temperature and holding times between 15 s and 5 min on denaturation of the individual whey proteins has been described in a previous publication (19). The effect of heating milk for 5 min at temperatures between 72 and 140 °C on the extent of denaturation of total whey protein, determined by nitrogen analysis, is also shown in Fig. 1. The total amount of denatured whey protein, based on the loss of solubility at pH 4.6, increased rapidly between 72 and 100 °C, but remained almost constant between 100 and 120 °C. The proteosepeptones, amounting to about 25% of the total whey protein, were heat-stable and did not precipitate at pH 4.6. There was a small decrease in the amount of protein precipitating at pH 4.6, after heating for 5 min at 140 °C, which was believed to be caused by an increase in the extent of proteolysis of the caseins (5).
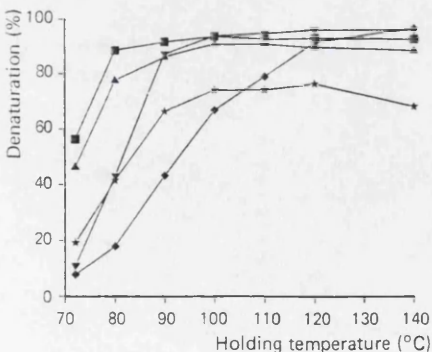
Fig. 1: The effect of temperature, at a holding time of 5 min, on the extent of denaturation of the individual and total whey proteins. ■ Immunoglobulins, ▲ serum albumin/lactoferrin, ▼ β-lactoglobulin, ◆ α-lactalbumin, ★ total whey protein.

3.2 SDS-PAGE

The patterns from SDS-PAGE are shown in Fig. 2 for the mixture of denatured whey proteins and caseins precipitated at pH 4.6 from raw milk and milk heated for 5 min at temperatures between 72 and 140 °C. The amounts of immunoglobulins and serum albumin in the precipitate increased on heating for 5 min at 72 and 80 °C, whereas the amount of β-lactoglobulin increased at temperatures between 72 and 90 °C and the amount of α-lactalbumin increased between 80 and 110 °C.

Fig. 2: SDS-PAGE pattern on a 20% homogeneous gel for the proteins precipitated at pH 4.6 from raw milk and milk heated for 5 min at different temperatures. Lanes 1–8, raw, 72, 80, 90, 100, 110, 120 and 140 °C respectively. The whey proteins are: α-La, α-lactalbumin; β-Lg, β-lactoglobulin; IgG-h, heavy chain immunoglobulin G; SA, serum albumin; IgM-h, heavy chain immunoglobulin M; Lf, lactoferrin. The caseins are γ-, κ-, β-, αs-.

The patterns for the caseins from milk heated for 5 min at temperatures between 72 and 110 °C were similar to that for whole casein from unheated milk, apart from a slight decrease in the intensity of a γ-casein band on heating. At 120 and 140 °C, there was an increase in the amount of protein migrating in the region between κ-casein and β-lactoglobulin. At 140 °C there was an increase in the material remaining in the slot, and there was a marked smearing of most bands. There was not, however, any appreciable increase in the amount of low molecular weight peptides caused by proteolysis, or in the amount of high molecular weight proteins caused by covalent linkage of the caseins.

3.3 Alkaline PAGE

The patterns from alkaline PAGE are shown in Fig. 3 for the proteins precipitated at pH 4.6 from raw milk and milk heated for 5 min at temperatures between 72 and 140 °C. The minor whey proteins could not be detected, but the amount of β-lactoglobulin increased on heating at 80 and 90 °C, and then remained constant. The α-lact-

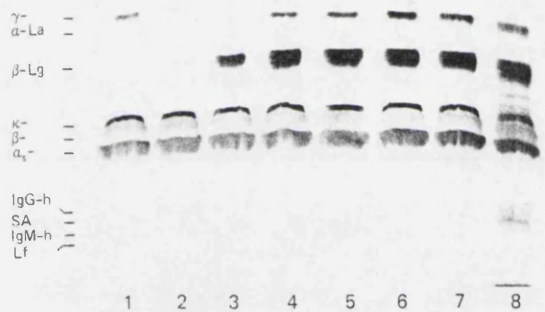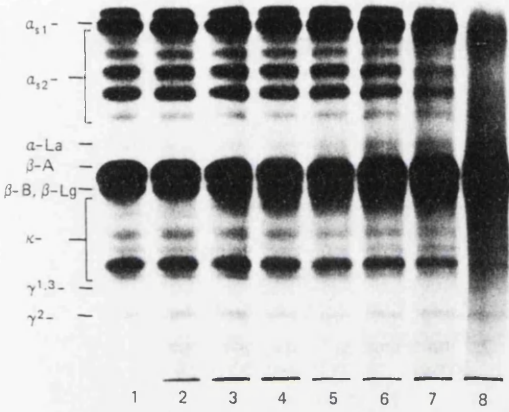Fig. 3: Alkaline PAGE pattern for the proteins precipitated at pH 4.6 from raw milk and milk heated for 5 min at different temperatures. Lanes 1–8, raw, 72, 80, 90, 100, 110, 120 and 140 °C, respectively. The whey proteins are: α-La, β-Lg. The caseins are: α_{s1}-, α_{s2}-, β-A, β-B, κ-, $\gamma_{1,3}$-, γ_2-.

Fig. 4: Elution profiles obtained by anion-exchange FPLC of the proteins precipitated at pH 4.6. ····· Raw milk, —— milk heated at 90 °C for 5 min, ----- milk heated at 140 °C for 5 min. WP, whey proteins. Caseins: minor, κ-, C, β-, α_{s2}-, α_{s1}-.

albumin band, however, increased in intensity with temperature between 80 and 110 °C.

On heating between 72 and 110 °C for 5 min, there was no appreciable change in the patterns for the caseins, but at 120 °C the bands became less distinct, and at 140 °C there was a marked smearing of all the bands. CREAMER and MATHESON (14) and GUO et al. (15), using longer heating times, also found blurring of electrophoretic patterns, and attributed this to a wider range of net negative charge on each of the main components. The typical brown discolouration of the denatured whey proteins and casein from milk heated for 5 min at 140 °C in this study suggested that at least some of the change in charge could be due to Maillard reaction of ϵ-amino groups of lysine with carbonyl groups of lactose (13).

3.4 Anion-exchange FPLC

When the mixture of denatured whey proteins and caseins was fractionated by anion-exchange FPLC, the whey proteins eluted together with the minor caseins between 2 and 10 ml, before the main casein fractions (Fig. 4). There was an increase in the amount of denatured whey proteins in the mixture as the temperature of heating increased from 72 to 110 °C for a holding time of 5 min. The amount of whey proteins in the mixture, determined on the basis of absorbance, was closely correlated (r=0.942) with the amount precipitated from milk at pH 4.6, as determined by nitrogen analysis.

There was little change in the elution patterns for the major caseins when milk was heated for 5 min at temperatures between 80 and 110 °C

(Fig. 4), but at 120 °C and, to a greater extent at 140 °C (Fig. 4), the profiles of each of the major caseins, and of the whey proteins, became less distinct. The β- and α_{s1}-casein peaks showed an increase in the amount of protein on their leading and tailing edges.

The changes in the relative amounts of the various casein fractions, determined by anion-exchange FPLC, with increasing temperature at a holding time of 5 min are shown in Fig. 5. Values for the relative amounts of the individual caseins are expressed as a percentage of the total casein, excluding the minor caseins eluting between 2 and 10 ml. The relative amounts of protein in the β-casein region and, to a lesser extent, in the α_{s1}-casein region decreased with increasing temperature whereas the amounts of protein increased in the κ- and α_{s2}-casein regions and in the unidentified fraction C.

Fig. 5: The change in the relative amounts of protein in the casein fractions, determined by anion-exchange FPLC, with increasing temperature at a holding time of 5 min. Abbreviations see Figs. 3, 4.

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Law, Whey proteins

On heating milk at 140°C the changes in the relative amounts of the fractions were almost directly proportional to the heating time between 15 s and 5 min. Similar changes in the caseins were found in different preparations of sodium caseinate by DALGLEISH and LAW (18) using the same method, and by GUO et al. (15) using chromatography on DEAE cellulose. Some of the changes in the elution profiles were consistent with a reduction in negative charge on the major caseins, possibly because of removal of phosphate from phosphoseryl residues (10, 11, 15), leading to earlier elution of the modified caseins from the positively charged anion-exchange column. The shoulders on the tailing edges of β- and α_{s1}-caseins may have been caused by loss of positive charge due, possibly, to Maillard reaction of amino groups of lysine (13).
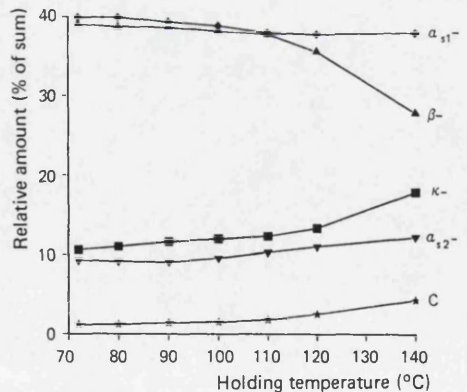
3.5 Cation-exchange FPLC

The elution profiles obtained by cation-exchange FPLC of the proteins precipitated at pH 4.6 from milks heated for 5 min between 72 and 100 °C were similar to that obtained for casein from unheated milk (23), except that the denatured whey proteins eluted between 7 and 12 ml together with β-casein (Fig. 6). On heating for 5 min at 120°C and, to a greater extent at 140°C (Fig. 6), the peaks became broader and less distinct. An additional peak (fraction P), eluting at about 28 ml, also appeared and increased with temperature and holding time (Fig. 6).
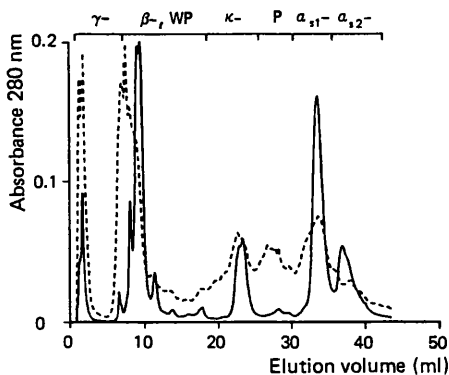
Fig. 6: Elution profiles obtained by cation-exchange FPLC of the proteins precipitated at pH 4.6 from milk heated for 5 min. —— 100°C, ----- 140°C. Abbreviations see Figs. 3, 4.

The changes in the relative amounts of the casein fractions, determined by cation-exchange FPLC, with increasing temperature at a holding time of 5 min are shown in Fig. 7. Values for the relative amounts of the casein fractions are expressed as a percentage of the total casein, excluding the γ- and β-caseins. The relative amounts
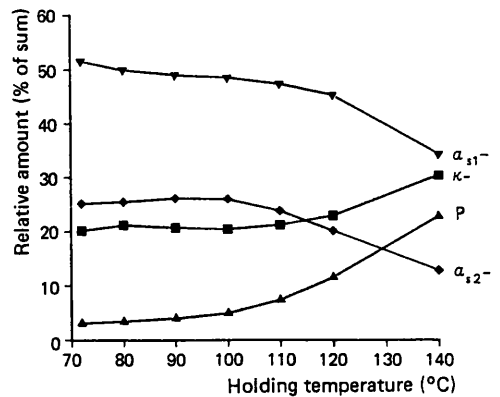
Fig. 7: The change in the relative amounts of protein in the caseins fractions, determined by cation-exchange FPLC, with increasing temperature at a holding time of 5 min. Abbreviations see Figs. 3, 4.

of protein in the α_{s1}- and α_{s2}-casein regions decreased with increasing holding time, whereas that in the κ-fraction and fraction P increased. On heating milk at 140°C the relative amounts of the fractions changed linearly with heating time between 15 s and 5 min. SDS-PAGE showed that the protein in fraction P had the same molecular weight as α_{s1}-casein. Also, the close negative correlation of the amounts of the two fractions suggested that on heating milk, α_{s1}-casein may lose positive charge, possibly through reaction of the ϵ-amino groups of lysine, and elute earlier from the negatively charged cation-exchange column. GUO et al. (15) found a decrease in available lysine on prolonged heating of sodium caseinate at 140°C, and it is well established that on heating, lysine can form lysinoalanine (12) and combine with carbonyl groups of lactose in the Maillard reaction (13).

In this study most of the whey proteins were denatured on heating for 5 min at 110°C, and above this temperature there were heat-induced changes in the caseins and the denatured whey proteins. The combined results from the techniques used here showed that, at the temperatures and holding times studied, although denaturation of the whey proteins was extensive, the amount of proteolysis of the caseins was fairly slight. The changes in behaviour of the caseins on alkaline PAGE and on anion- and cation-exchange FPLC were consistent with changes in the charges of the positively and negatively charged amino acid residues. In view of the effect of charge differences on the processing properties of the caseins, more detailed study of the heat-induced changes seems worthwhile.

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5. Summary

LAW, A.J.R., HORNE, D.S., BANKS, J.M., LEAVER, J.: **Heat-induced changes in whey proteins and caseins.** Milchwissenschaft 49 (3) 125–129 (1994).

34 Whey proteins (heated milk)

Changes in the whey proteins and caseins in milk heated at temperatures between 72 and 140°C for holding times between 15 s and 5 min were examined by PAGE and gel permeation and ion-exchange FPLC The extent of denaturation of the whey proteins, as measured by loss of solubility at pH 4.6 increased with the severity of heating, and most of the immunoglobulins and serum albumin, β-lactoglobulin and α-lactalbumin were denatured when milks were heated for 5 min at 80, 90 and 110°C respectively, and precipitated with the caseins at pH 4.6.

The caseins were less sensitive than the whey proteins to heating, and only slight changes could be detected on heating milk for 5 min at temperatures up to 110°C. On heating for 5 min at 120°C, or between 1 and 5 min at 140°C, however, the patterns of the major caseins on alkaline PAGE and the elution profiles on ion-exchange FPLC became less distinct. On anion-exchange FPLC there was a marked decrease in the amount of protein in the β-casein region and an increase in the κ- and α_{s2}- regions. On cation-exchange FPLC there was a decrease in the amount of protein in the α_{s1}- and α_{s2}- regions, and an increase in the amount of protein in the κ-region and in the amount of a fraction believed to be derived from α_{s1}-casein. Results from SDS-PAGE and nitrogen analysis showed that, at the temperatures and holding times studied, the degree of proteolysis was slight, and the differences in behaviour of the caseins on alkaline PAGE and ion-exchange chromatography were consistent with changes in the charges of the negatively and positively charged amino acid residues on heating.

LAW, A.J.R., HORNE, D.S., BANKS, J.M., LEAVER, J.: **Hitzeinduzierte Veränderungen bei Molkenproteinen und Caseinen.** Milchwissenschaft 49 (3) 125–129 (1994).

34 Molkenproteine (Milcherhitzung)

Mit Hilfe von PAGE und Gelpermeation sowie Ionenaustausch-FPLC wurden die Veränderungen der Molkenproteine und Caseine in Milch nach Erhitzung auf Temperaturen zwischen 72 und 140 °C bei Heißhaltezeiten zwischen 15 s und 5 min untersucht. Das Ausmaß der Molkenprotein-Denaturierung, gemessen durch den Löslichkeitsverlust bei pH 4,6, erhöhte sich mit der Erhitzungsintensität. Der größte Anteil der Immunglobuline und von Serumalbumin, β-Laktoglobulin und α-Laktalbumin wurden dann denaturiert, wenn die Milchproben 5 min bei 80, 90 bzw. 110 °C erhitzt wurden. Bei pH 4,6 kam es gemeinsam mit den Caseinen zu einer Ausfällung.

Die Caseine waren im Vergleich zu den Molkenproteinen gegenüber einer Erhitzung weniger empfindlich. Bei einer Erhitzung der Milch für die Dauer von 5 min auf Temperaturen bis zu 100 °C konnten lediglich geringe Änderungen festgestellt werden. Bei einer Erhitzung für die Dauer von 5 min bei 120 °C bzw. zwischen 1 und 5 min bei 140 °C wurden die Muster der wichtigsten Caseine in der alkalischen PAGE und die Elutionsprofile in der Ionenaustausch-FPLC weniger eindeutig. Bei der Anionen-Austausch-FPLC kam es zu einem deutlichen Abfall der Proteinmenge in der β-Caseinregion und zu einem Anstieg der Proteinanteile in den κ- und α_{s2}-Bereichen. Bei der Kationenaustausch-FPLC zeigte sich ein Abfall der Proteinmengen in den α_{s1}- und α_{s2}-Regionen und eine Erhöhung der Proteinmengen in der κ-Region. Gleiches erfolgte in einem Fraktionsbereich, der sich vom α_{s1}-Casein ableiten dürfte. Ergebnisse der SDS-PAGE und der Stickstoffanalyse zeigten, daß bei den untersuchten Temperaturen und Heißhaltezeiten der Proteolysegrad gering war. Die Unterschiede im Verhalten der Caseine bei der alkalischen PAGE und in der Ionenaustausch-Chromatographie stimmten überein mit Veränderungen der Ladungen der negativ und positiv geladenen Aminosäureresten bei der Erhitzung.

Analysis of caseins in cheese using ion-exchange chromatography

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1. Introduction

The taste and texture of cheese is affected by both the rate and extent of the proteolysis which occurs during the ripening period (1). Changes in the manufacturing process *e.g.* heating of the milk, nature of starter culture etc., can alter the kinetics of the proteolysis and hence the properties of the mature cheese.

Various methods have been used to quantify the changes in the caseins occurring during ripening. Electrophoresis is probably the most widely used technique (2, 3, 4) but densitometric scanning of the stained proteins is difficult to quantify. Gel permeation chromatography is similarly difficult to quantify and is relatively non-selective (5). Recently, CHRISTENSEN et al. (6, 7) used reverse-phase HPLC to quantify changes in casein levels.

Since ion-exchange fast protein liquid chromatography (FPLC) has been used successfully to separate and quantify individual casein fractions in milk (8), we have examined its usefulness in their analysis during cheese ripening, the results of which are reported here.

2. Materials and methods

Cheese manufacture

Cheddar cheese was manufactured on a 45 kg scale using procedures described elsewhere (9, 10).

Preparation of samples for FPLC

Water insoluble fractions were prepared as described by KUCHROO and FOX (11).

Grated cheese (10 g) was homogenized with 20 ml of distilled water at room temperature for 3 min. The samples were then heated at 40 °C and held at this temperature for 1 h prior to centrifugation at 3,000 g for 30 min. The pellets, which contained the caseins and large peptides were freeze dried.

Analysis by FPLC

FPLC analysis of the freeze dried proteins was carried out using anion-exchange chromatography on a Mono Q HR5/5 column and cation-exchange chromatography on a Mono S HR5/5 column (Pharmacia AB, Uppsala, Sweden). Detection was at 280 nm.

Samples for anion-exchange chromatography were prepared by dissolving 10 mg of freeze-dried protein extract in 10 ml of bis-tris-propane (urea buffer, BTP buffer; 5 mM BTP, 3.3 M urea adjusted to pH 7.0 with HCl). After stirring for 5 min the pH was adjusted to 7.0, 10 µl of 2-mercaptoethanol was added, and

stirring continued for 1 h. The samples were then filtered through a GSWP filter (Millipore, pore size 0.22 µm, diameter 13 mm).

500 µl of the sample were applied to the column and the samples were separated at room temperature at a flow rate of 1.0 ml/min using a sodium chloride gradient as detailed by DAVIES and LAW (8) (0.10 M after 2 ml, 0.22 M after 14 ml, 0.28 M after 21.5 ml, 0.295 M after 29 ml and 0.43 M after 35 ml). A modified salt gradient was also used (0.10 M after 2 ml, 0.11 M after 9 ml, 0.22 M after 14 ml and 0.35 M after 57.5 ml).

Samples for cation-exchange chromatography were prepared by dissolving 10 mg of the freeze dried protein extract in 10 ml of urea-acetate buffer (pH 5.0, 10 M urea, 0.02 M sodium acetate). 2-Mercaptoethanol is required to reduce disulphide bonds. Because the reaction is inefficient at pH values below 6, the pH of the samples was adjusted to 7 with 1 M NaOH prior to adding 10 µl of 2-mercaptoethanol. After stirring for 1 h, samples were adjusted back to pH 5 with 1 M HCl and filtered through a GSWP filter.

FPLC analysis of the samples using cation-exchange chromatography was carried out at room temperature using a urea-acetate buffer (pH 5.0, 6.0 M urea, 0.02 M acetate), and a gradient of sodium chloride (0.025 M after 2 ml, 0.075 M after 16.5 ml, 0.15 M after 26.5 ml, 0.26 M after 42.5 ml and 0.72 M after 52.5 ml). The flow rate was 1 ml/min and 500 µl samples were applied to the column.

The identity of the eluted materials in the anion and cation-exchange chromatography was established by comparing the elution profile of the samples with those of pure standards prepared in our laboratory and by polyacrylamide gel electrophoresis (PAGE) of materials isolated from selected fractions.

Gel electrophoresis

Selected fractions obtained from the FPLC analysis were dialysed exhaustively against distilled water and lyophilised. They were then analysed by PAGE using "Phast-System" electrophoresis equipment (Pharmacia U.K., Milton Keynes, Beds.). Sodium dodecyl sulphate (SDS)-PAGE was performed on 20 % homogeneous gels in accordance with the manufacturer's instructions. Gels were stained with Coomassie Blue and on occasions counterstained with silver.

3. Results

Extracts were prepared from cheeses throughout a 12 week ripening period. By extracting many of the water soluble peptides, interference with the determination of the cheeses in the pellets was reduced.

Initial FPLC analysis of the proteins from 4 week old cheese was carried out using anion-exchange chromatography and the salt gradient system devised by

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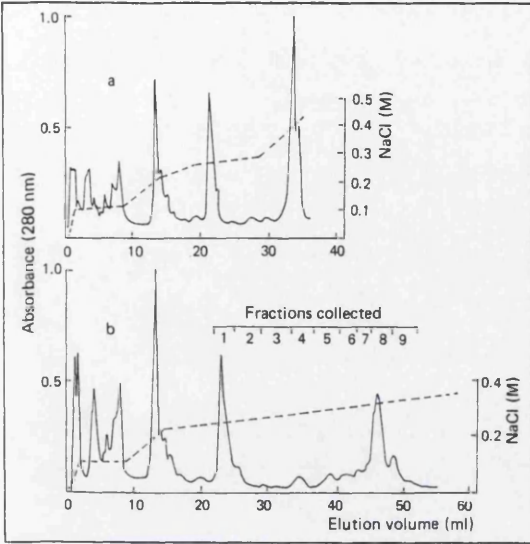
DAVIES and LAW (8) (Fig. 1a). This method gave good resolution of the β-casein. However, the $α_{s1}$- and $α_{s2}$-caseins were eluted very close together and some peptides were present in the region of the $α_s$-caseins. In an attempt to improve the resolution of the $α_{s1}$- and $α_{s2}$-caseins further separations were performed using a modified salt gradient.

Nine component fractions were isolated (Fig. 1b) and analyzed by SDS-PAGE (Fig. 2). Fraction 1 consisted of β-casein and fraction 2 which was a shoulder to the major peak in fraction 1 contained β-casein and a peptide of lower molecular weight. However, even under these conditions, the fractions containing the $α_s$-caseins still contained a number of peptides formed during ripening.

Analysis of the caseins and large peptides by anion-exchange chromatography although useful for studying the changes in the level of β-casein during ripening, cannot be used to study the changes in the $α_s$-caseins. Further analyses were performed using cation-exchange chromatography.

Fig. 3 shows the analysis of a two week old cheese by cation exchange. Nine component fractions were isolated and analyzed by SDS-PAGE (Fig. 4). Fractions 1 and 3 contained a number of peptides. Fraction 2 consisted mainly of β-casein. Some products of

degradation were eluted in the same position as the β-casein in cheeses older than 2 weeks. Fraction 4 contained only para κ-casein which elutes in the region of the γ-caseins when anion-exchange chromatography is used. Fractions 5 and 6 consisted of various peptides, fraction 7 contained the α_{s1}-casein and fraction 8 contained the α_{s2}-casein and some high molecular weight components. Fraction 9 contained a variety of high molecular weight components.

Changes in the para κ-casein and α_{s1}-casein and during ripening can therefore be studied by cation-exchange FPLC and approximate levels of α_{s2}- can also be obtained. Due to peptide contamination, β-casein cannot be quantified by this technique.

κ-Casein was not detected in any of the cheeses by any of the analytical techniques indicating that renneting was completed by the time the first samples were taken.

4. Conclusions

SDS-PAGE followed by densitometric scanning of the stained protein bands is the most widely used method for measuring proteolysis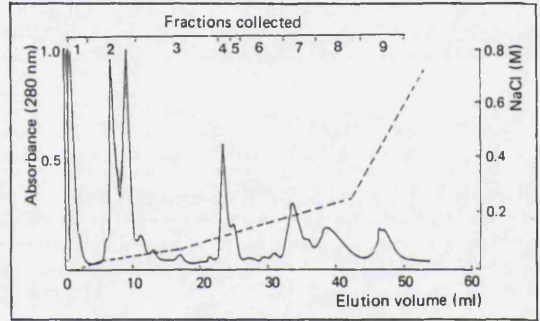 in cheese throughout the ripening period. Unfortunately, the various caseins have a very similar mobility in this system and determining the concentration of individual caseins can be very difficult since stained bands tend to merge. We have found that ion-exchange FPLC is a very useful alternative to PAGE with the advantage that quantification of caseins by integration of peak areas is much simpler. However, a combination of cation-exchange chromatography and anion-exchange chromatography is required for optimum quantification, neither method on its own being sufficient due to interference from peptides formed during the maturation period.

The maturation of Cheddar-type cheese prepared using pasteurized and overheated milks has been followed using ion-exchange chromatography and the results are shown in the accompanying paper.

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6. Summary

CALVO, M.M., LEAVER, J., LAW, A.J.R., BANKS, J.M.: Analysis of caseins in cheese using ion-exchange chromatography. Milchwissenschaft 47 (7) 417–419 (1992).

54   Cheese ripening (casein)

Ion-exchange fast protein liquid chromatography has been assessed as a method for quantifying the levels of the various major caseins present in the water-insoluble fraction of Cheddar-type cheese. Anion-exchange chromatography gave good resolution of the β-casein. Quantification of the α_{s1}- and para κ-caseins could be achieved using cation-exchange chromatography, and an estimate of α_{s2}-casein levels could be made. By combining the two forms of ion-exchange chromatography, a good measure of the levels of the caseins could be achieved.

CALVO, M.M., LEAVER, J., LAW, A.J.R., BANKS, J.M.: Analyse der Caseine in Käse mit Hilfe der Ionenaustausch-chromatographie. Milchwissenschaft 47 (7) 417–419 (1992).

54   Käsereifung (Casein)

Die Ionenaustausch-Protein-Schnell-Flüssig-Chromatographie wurde als Verfahren zur Bestimmung der Menge der verschiedenen wesentlicheren Caseine in der wasserunlöslichen Fraktion von Käse nach Cheddar-Art eingesetzt. Die Anionenaustausch-Chromatographie ergab eine gute Auflösung des β-Caseins. Die Quantifizierung der α_{s1}- und para κ-Caseine konnte mit der Kationenaustausch-Chromatographie vorgenommen werden. Durch Kombination dieser beiden Ionenaustausch-Chromatographie-Verfahren war eine gute quantitative Bestimmung der Caseine möglich.

The effect of heat treatment and acidification on the dissociation of bovine casein micelles

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SUMMARY. The effects of heat treatment and subsequent acidification of milk on the distribution of proteins, Ca and P_i (inorganic phosphate) between the serum and micellar phases were examined using ultracentrifugation. On heating milk at 85°C for 10 min, and storing for 22h at 4, 20 or 30°, κ-casein dissociated from the micelles and, on average, 27.3 % of the total κ-casein was present in the serum, compared with 13.2% in that from raw milk. At 4 and 20°C, there was also slightly more β-casein in the serum from heat-treated milk than in that from raw milk. The whey proteins were extensively denatured, and were almost equally distributed between the supernatants and micellar pellets. After storage for 22h, the distribution of serum and colloidal Ca and inorganic phosphate (P_i) in heat-treated milk was similar to that in raw milk.

On acidifying heat-treated milk by the addition of glucono-δ-lactone, and storing for 22h at 4, 20 or 30°C, there was progressive solubilisation of colloidal calcium phosphate with decreasing pH, and at pH 5.0 almost all of the Ca and P_i was present in the serum. At 20°C, and even more so at 4°C, serum concentrations of the individual caseins increased considerably with decreasing pH, reaching maximum levels of about 25 and 40% of the total casein at pH 5.7 and 5.5, respectively, and then decreasing rapidly at lower pH. Compared with raw milk, maximum dissociation in heat-treated milks at 4 and 20°C occurred at higher pH, and the overall levels of dissociation of individual caseins from the micelles were lower. At 30°C, the concentrations of individual caseins in the serum of heat-treated milk decreased steadily as the pH was reduced. The possible role of the denatured whey proteins in promoting dissociation of κ-casein on heating, and the subsequent aggregation of the micelles as the pH is reduced, is discussed.

INTRODUCTION

During processing, milk may be subjected to quite extreme changes in temperature and pH which affect the distribution of the minerals, whey proteins and caseins between the micellar and serum phases. In previous studies on the cold storage of milk, it has been shown that at 4°C a small amount of the colloidal calcium phosphate slowly dissolves into the serum. There is also a marked increase in the level of serum casein which is due mainly to the dissociation of β-casein from the micelles as hydrophobic bonding becomes weaker at low temperatures (Rose, 1968; Downey & Murphy, 1970; Creamer *et al.*, 1977). These changes are at least partly reversible on re-warming the milk to room temperature (Ali *et al.*, 1980; Davies & Law, 1983).

In several processes, such as the manufacture of yogurt and cheese, the pH of milk is reduced by lactic acid fermentation. As the pH of unheated milk decreases, colloidal calcium phosphate, and small amounts of magnesium and citrate are fairly rapidly dissolved, and the caseins are liberated into the serum (Pyne & McGann, 1959; Rose, 1968). The extent of the dissociation varies with temperature and pH. On reducing the pH at 30°C, most of the colloidal calcium phosphate can be removed, but hardly any casein dissociates into the serum (Dalgleish & Law, 1988, 1989). On reducing the pH at 20 or 4°C, colloidal Ca and P_i (inorganic phosphate) are dissolved, and the level of serum casein increases markedly, the effect of low temperature and pH being more than additive. As the pH is reduced further, depending on the temperature, isoelectric precipitation of the caseins occurs.

The above studies relate to unheated milk, but most manufacturing processes require an initial heat treatment of the milk. In the production of yogurt, for example, milk is heated at functionalisation temperatures (typically 85°C for 10 min) in order to control the growth of microorganisms and modify the texture (Davies *et al.*, 1978; Tamime & Robinson, 1988). Under these conditions, the whey proteins are denatured and become associated with the casein micelles, initially through reversible hydrophobic interaction and then through disulphide bonding (Haque & Kinsella, 1988; Noh & Richardson, 1989). The association of denatured whey proteins with micellar caseins on heating tends to prevent micellar coalescence at low pH, and gives improved yogurt texture and gel strength (Davies *et al.*, 1978).

The present study was carried out to obtain further information about the effect of heat treatment and subsequent acidification, on the micellar-serum distribution of Ca, P_i, denatured whey proteins and individual caseins. The pH of heat-treated milk was reduced in a controlled manner by the addition of glucono-δ-lactone, and changes in the levels of serum and micellar Ca, P_i, individual caseins and denatured whey proteins were examined using ultracentrifugation. Serum and micellar caseins, together with the associated denatured whey proteins, were analysed by anion- and cation-exchange FPLC. As in previous studies on the pH-induced dissociation of casein micelles in unheated milk (Dalgleish & Law, 1988; 1989), the effect of pH was investigated at three different temperatures, namely 4, 20 and 30°C. The first is the temperature at which milk is usually stored, and the last is that at which various manufacturing processes, including lactic acid fermentation, are carried out. The study at 20°C was carried out to allow comparison with previous results.

METHODS AND MATERIALS

Milk Samples

Milk samples were collected from the bulk milk of Friesian cows in the Institute herd. The milks were skimmed by centrifugation at 1000g for 30 min.

Heat treatment

Skim-milk was heated in a waterbath to 85°C, and maintained at this temperature for 10 min. The milk was then rapidly cooled in ice to 4, 20 or 30°C, depending on the subsequent storage temperature.

Acidification of milks

The pH of 50 ml aliquots of heat-treated milks was reduced by the addition of pre-determined amounts of glucono-δ-lactone (GDL). The milks were stored at 30, 20 or 4°C for about 22h, during which time the GDL hydrolysed slowly to lactic acid, and gave a range of milks between pH 6.7 and 5.0.

Separation of serum and micellar proteins

After storage, raw and heat-treated milks at their normal pH, and heat-treated milks that had been pH-adjusted, were centrifuged at 50,000g in a fixed-angle rotor in a Sorvall centrifuge at the appropriate storage temperature (4, 20 or 30°C). Centrifugation times were adjusted to allow for the increase in viscosity of milk at lower temperatures, and at 4, 20 and 30°C these were 3h, 1h 30 min and 1h 19 min, respectively. After centrifugation, the supernatant and interphase layer were poured off and adjusted to room temperature. Serum casein and the associated denatured whey proteins were precipitated at pH 4.6 by the addition of 1M-HCl. The precipitates were washed once with water at pH 4.6, and freeze dried. The corresponding micellar pellets obtained by centrifugation were re-suspended in 5ml of 2.5% Na_2EDTA and Na_4EDTA at pH 6.8, and stirred for 15 min. Water (25ml) was added and

stirring continued for about 16h, with occasional addition of 1M-NaOH to maintain the pH at 7.0, until the pellets were completely re-suspended. The micellar caseins and associated denatured whey proteins were prepared as described for the serum caseins, by a single acid precipitation and wash at pH 4.6, followed by freeze-drying.

Nitrogen analysis

Total, non-casein and non-protein N concentrations of the raw and heated skim-milks were determined by a micro-Kjeldahl method (Davies & Law, 1983). The total N contents of the supernatants obtained by centrifugation were also determined. From the values for raw milk, the initial concentrations of casein and whey proteins, and the distribution of casein between the micellar and serum phases were obtained. In heated milks, denatured whey proteins precipitated together with the caseins at pH 4.6, and the total amounts of acid precipitable protein in the serum and micellar phases were calculated. The total amount of denatured whey protein in the heat-treated milk was calculated from the difference in NCN values for raw and heated milk.

Anion-exchange FPLC

The serum and micellar caseins, and associated denatured whey proteins, were alkylated and fractionated by anion-exchange FPLC on a Mono Q HR 5/5 column, as described by Law et al. (1994). This method gave values for the relative amounts of β-, α_{s1}-, α_{s2}- and the minor caseins.

Cation-exchange FPLC

The relative amount of κ-casein in the serum and micellar caseins was determined by cation-exchange FPLC on a Mono S HR 5/5 column as described by Law et al. (1994). The relative amounts of whey proteins in the protein mixtures were calculated by difference using this value for the relative amount of κ-casein, and the values obtained by anion-exchange FPLC for the other caseins.

Gel permeation FPLC

The concentrations of four main whey protein fractions - immunoglobulins, serum albumin/lactoferrin, β-lactoglobulin and α-lactalbumin - in raw and heated milks and in the supernatants of pH-adjusted milks were obtained by gel permeation of the respective pH 4.6 acid filtrates, as described previously (Law et al., 1993). The extent of irreversible denaturation of each fraction, based on the loss of solubility at pH 4.6, was calculated as the decrease in area of each peak, expressed as a percentage of the area of the corresponding peak in acid filtrate from raw milk. The proteose peptones remained in solution at pH 4.6 even after severe heating and, as they had very low absorbance coefficients at 280nm, did not affect determination of the other whey proteins.

PAGE

Serum and micellar proteins from raw and heat-treated milks were examined by SDS-PAGE, as described previously (Law et al., 1993).

Ca and inorganic phosphate (P$_i$) analyses

The concentrations of total Ca in raw and heat-treated milks and in the supernatants obtained by ultracentrifugation were determined. Casein was precipitated from each of the samples at pH 4.6 by the addition of 1M-HCl and with continuous stirring for 20 min. The supernatants were filtered through Whatman No. 42 filter paper and diluted 100-fold. Ca in the acid filtrates was determined by a colorimetric method based on the formation of a complex with *o*-cresolphthalein complexone, in the presence of 8-hydroxyquinoline to prevent

interference by Mg (Connerty & Briggs, 1966). Colloidal Ca was calculated as the difference between the total in skim-milk and the Ca in the corresponding supernatant obtained by ultracentrifugation.

The concentrations of total inorganic phosphate (P_i) in raw and heat-treated skim-milks and in the supernatants obtained by ultracentifugation were determined. P_i of the heat-treated skim-milk and serum was determined in the pH 4.6 filtrates, as above, using the colorimetric method of Fiske & Subbarow (1925). Total P_i in raw skim-milks and the corresponding supernatants was determined in trichloroacetic acid (12%) filtrates, in order to prevent interference by whey proteins. Colloidal P_i was calculated as the difference between the total in skim-milk and the P_i in the corresponding supernatant obtained by centrifugation.

RESULTS AND DISCUSSION

Effect of heat treatment and storage on the distribution of Ca and P_i

On heating skim-milk at 85° for 10 min and storing for 22h at 4, 20 or 30°C, the concentrations of serum and colloidal Ca and P_i in heat-treated milks, at each storage temperature, were similar to the corresponding concentrations in raw milk (Table 1). Various studies have shown that, on heating milk, the concentrations of diffusible Ca and P_i decrease, and calcium phosphate precipitates onto the casein micelles (Kannan & Jenness, 1961). Rose & Tessier (1959) found that, on heating milk at 93°C for 20 min, the hot ultrafiltrate contained only 50% of the Ca and 82% of the P_i found in ultrafiltrate from raw milk at 27°C. They also found that, on cooling to 4°C, Ca and P_i slowly diffused into the serum, and after 22h the concentrations of diffusible Ca and P_i were only slightly lower than those in unheated milk. The present work shows that on less severe heating (85°C for 10 min) and similar storage conditions (22h) the changes that took place on heating were reversed, and the partition of Ca and P_i was similar to that in unheated milk. Further study showed that the reversal occurred fairly rapidly, and after heat-treatment and storage for 1.5h at 20°C, the concentrations of colloidal Ca and P_i were only 5.4 and 9.3% above the corresponding values for raw skim-milk.

Effect of pH on the solubilisation of colloidal calcium phosphate

On acidifying heat-treated milk and holding at 4, 20 or 30°C for 22h there was an increase in serum Ca and P_i with decreasing pH (Fig.1). The rate of change of solubilisation of colloidal calcium phosphate with pH did not occur in a linear fashion, however, but tended to be more rapid in the middle of the pH range. The removal of Ca and P_i from the micelles was not markedly affected by the temperature of storage and, at each storage temperature, below pH 5.0 all of the colloidal Ca and P_i was dissolved. There are few results available on heat-treated milk with which to compare the present findings. Visser *et al.* (1986) found that on acidification of milk heated at 90°C for 15 min, almost all of the micellar P_i was removed when the pH was reduced to pH 5.2. The present results show that the rate of release of Ca and P_i from micelles in heated milk was similar to that in raw milk (Dalgleish & Law, 1989).

The relation between the concentrations of Ca and P_i remaining in the micelles on acidification of heat-treated milk is shown in Fig.2. Over most of the pH range, there was a linear relation between the concentrations of colloidal Ca and P_i, but there was some deviation from linearity at low pH. The slope of the line over the linear part of the plot was 1.98, and the intercept of the line with the Y axis was 3.1. Previous workers, removing colloidal calcium phosphate by acidification of unheated skim-milk, have found slightly lower values for the slope of the line ranging 1.58-1.85 (Chaplin, 1984; van Hooydonk *et al.*, 1986; Dalgleish & Law, 1989). Other workers, using EDTA or prolonged dialysis to remove coloidal calcium

Table 1. *Concentrations of total, serum and micellar Ca and inorganic phosphate (P$_i$) in raw and heat-treated skim-milk (85°C for 10 min) stored for 22h at 4, 20 or 30°C. Serum prepared by ultracentrifugation. Values at 4, 20 and 30°C are for different bulk milks*

Storage Temperature	4°C		20°C		30°C	
	Raw	Heated	Raw	Heated	Raw	Heated
			mM			
Total Ca	35.7	34.9	33.9	31.4	33.3	33.4
Serum Ca	13.0	12.4	11.2	10.1	11.4	11.2
Colloidal Ca	22.7	22.5	22.7	21.3	21.9	22.2
Total P$_i$	21.3	22.9	22.0	21.7	19.6	20.5
Serum P$_i$	12.6	13.0	11.5	12.1	11.1	10.5
Colloidal P$_i$	8.7	9.9	10.5	9.6	8.5	10.0

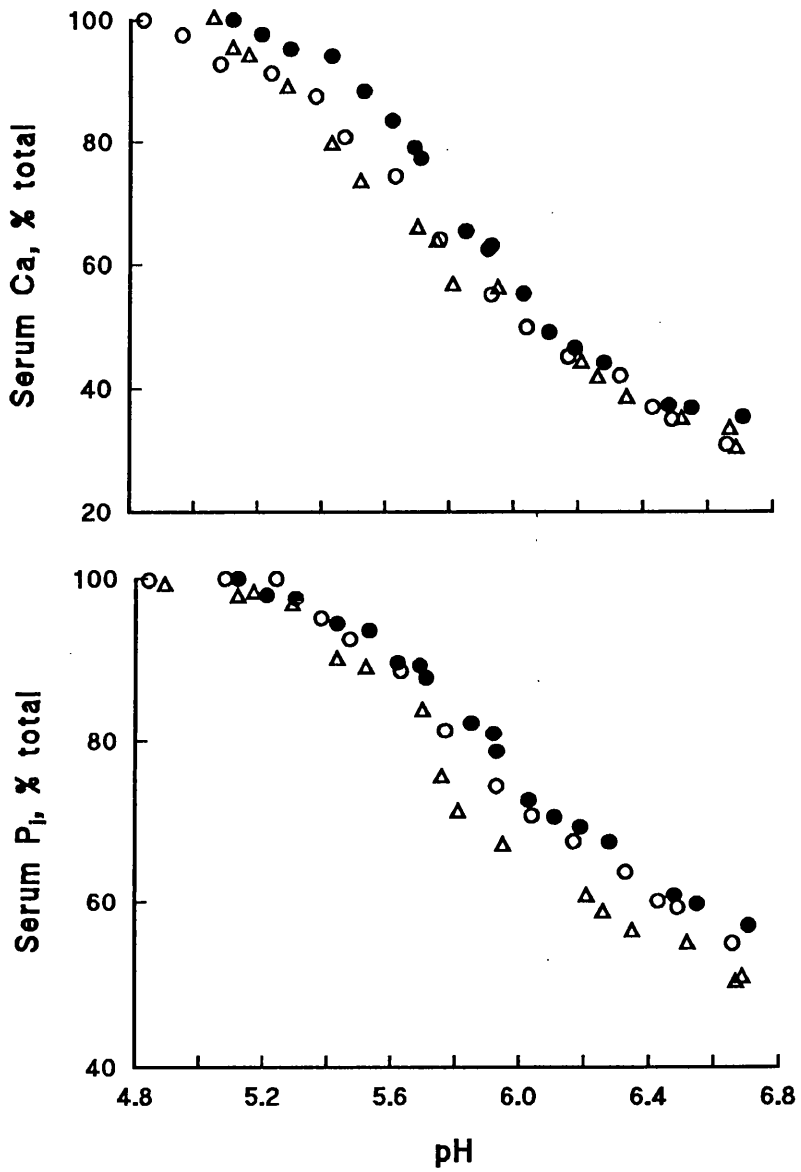Fig. 1. Increase in serum concentrations of Ca and inorganic phosphate (P$_i$) on acidification of heat-treated milk (85°C for 10 min) and storage for 22h at different temperatures: ●, 4°C; ○, 20°C; ▲, 30°C.
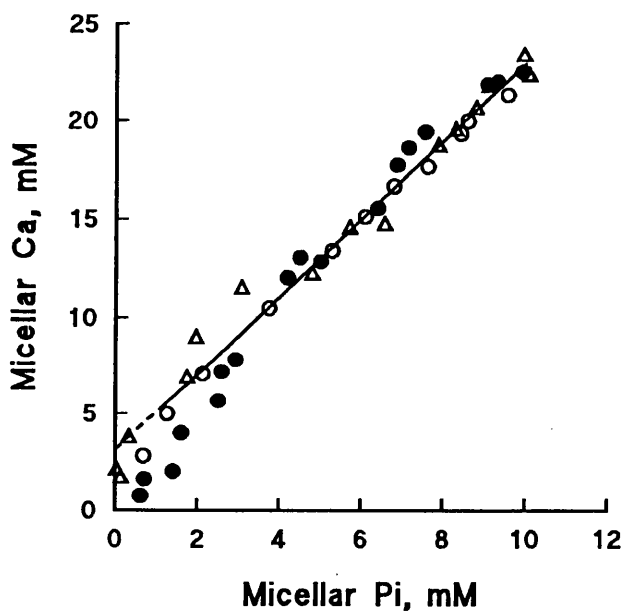
Fig.2. Relation between micellar Ca and inorganic phosphate (P_i) on acidification of heat-treated milk (85°C for 10 min) and storage at different temperatures: ●, 4°C; ○, 20°C; △, 30°C.

phosphate have found different values (1.61-2.08) for the ratio of colloidal Ca to P_i (Holt, 1982; Holt *et al.*, 1986; Griffin *et al.*, 1988). It has previously been proposed that colloidal calcium phosphate comprises micellar calcium phosphate and caseinate bound Ca, and that deviation from linearity of the plot of colloidal Ca and P_i, and the positive intercept on the axis representing Ca concentration at low pH, reflects the difficulty in removing the more tightly bound caseinate Ca (Dalgleish & Law, 1989). The difference between the value for the ratio of colloidal Ca to colloidal P_i obtained in the present work (1.98) for heat-treated milk and that from the previous study of Dalgleish & Law (1989) on raw milk (1.75-1.85) was small, and may be due to differences in the analytical methods used to determine Ca and P_i. Results suggest, therefore, that heating milk at 85°C for 10 min had little effect on either the rate or the extent of the subsequent pH-induced dissociation of colloidal calcium phosphate.

Effect of heat-treatment and storage on the caseins and whey proteins

The acid-precipitable proteins in the micellar and serum fractions obtained by ultracentrifugation of raw and heat-treated milk, stored for 22h at 20°C, were compared by SDS-PAGE (Fig.3). The electrophoretic patterns show that whole casein and the micellar and serum caseins from heat-treated milks contained considerable amounts of the whey proteins. There was also a marked increase in the relative amount of κ-casein in the serum casein fraction from the heated milk. Electrophoresis of the proteins from heated milk in the absence of reducing agent (results not presented) showed that the denatured whey proteins and κ-casein remained in the slots of the gel, and must be present as disulphide linked polymers. It was not possible to determine from this experiment, however, the extent to which the whey proteins formed homopolymers or were disulphide linked to κ-casein.

SDS-PAGE patterns for the acid-precipitable proteins in the serum fractions from milk heated and stored at 4, 20 or 4°C (Fig.4), show that each of the fractions contained considerable amounts of whey proteins. Compared with serum casein from raw milk, at each storage temperature, that from heat-treated milk contained more κ-casein, and the relative proportion increased with temperature (30>20>4°C). The relative amount of β-casein in serum casein from raw and heat-treated milk increased as the temperature of storage was reduced.

In order to quantify changes in the relative amounts and concentrations of the individual serum and micellar caseins and the extent of their association with the denatured whey proteins, the acid-precipitable proteins in the serum and micellar fractions were alkylated in dissociating conditions, and analysed by anion-exchange FPLC. The elution profiles for serum caseins from raw and heated milks (85° for 10 min) stored at 20°C for 22h are shown in Fig. 5. The quantitative analysis of mixtures of whey proteins and caseins is normally difficult to achieve. In heated milk the whey proteins and caseins become disulphide linked and are difficult to dissociate even in the presence of reducing agents. Comparison of quantitative values from anion-exchange FPLC of mixtures of whey proteins and caseins prepared by tungstic acid precipitation from raw skim-milk, and the corresponding denatured whey proteins and caseins precipitated at pH 4.6 from the same milk after heat treatment at 85°C for 10 min, showed that there was good agreement for the relative amounts of β-, α_{s1}- and α_{s2}-caseins. Closer examination of the whey proteins by anion-exchange FPLC showed that although most of the whey proteins were alkylated and eluted between 3 and 10 ml (Fig. 5), about 15% of the denaturable whey proteins eluted in the κ-casein region. In whole casein from heat-treated milk the error in the analysis was small, but in serum casein containing a high proportion of denatured whey proteins the error was appreciable. Values for the relative amounts of κ-casein were, therefore, determined by cation-exchange FPLC in which κ-casein was clearly separated from the denatured whey proteins (Law *et al.*, 1994).

Table 2 shows the distribution and composition of serum and micellar caseins obtained
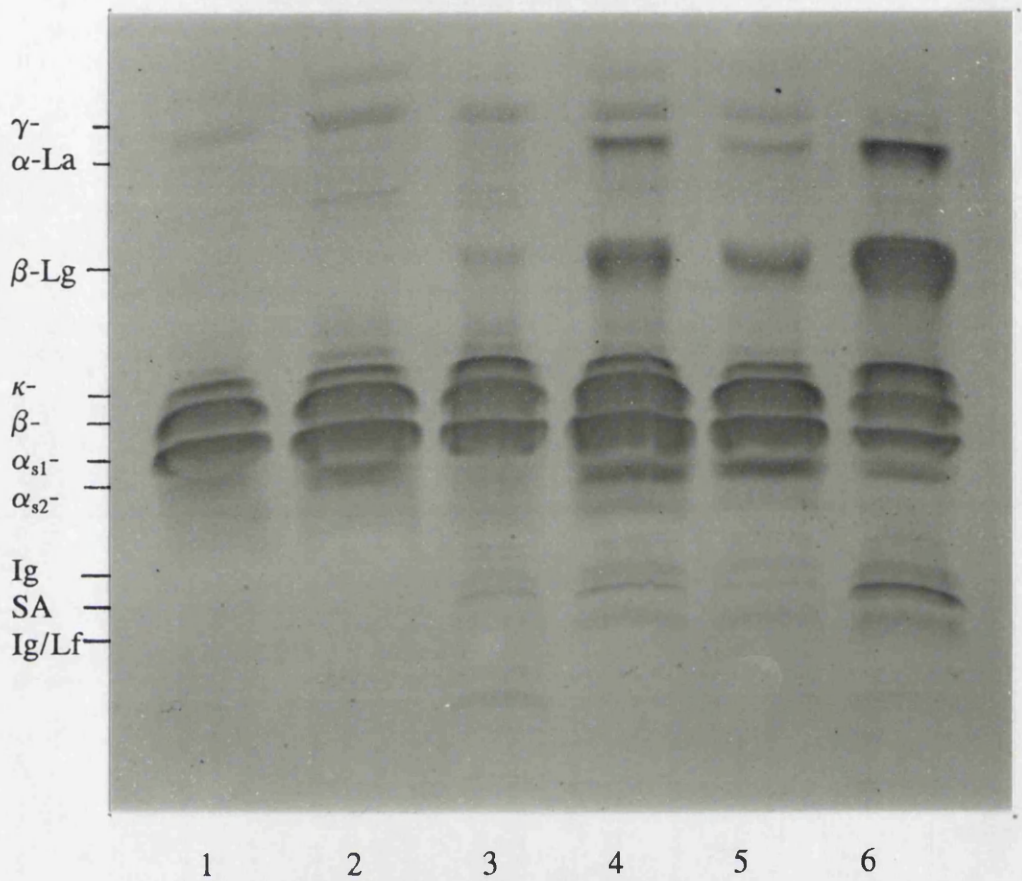
Fig.3. SDS-PAGE pattern of proteins in whole, micellar and serum caseins from raw (slots 1-3) and heat-treated milk (85°C for 10 min) stored for 22 h at 20°C.
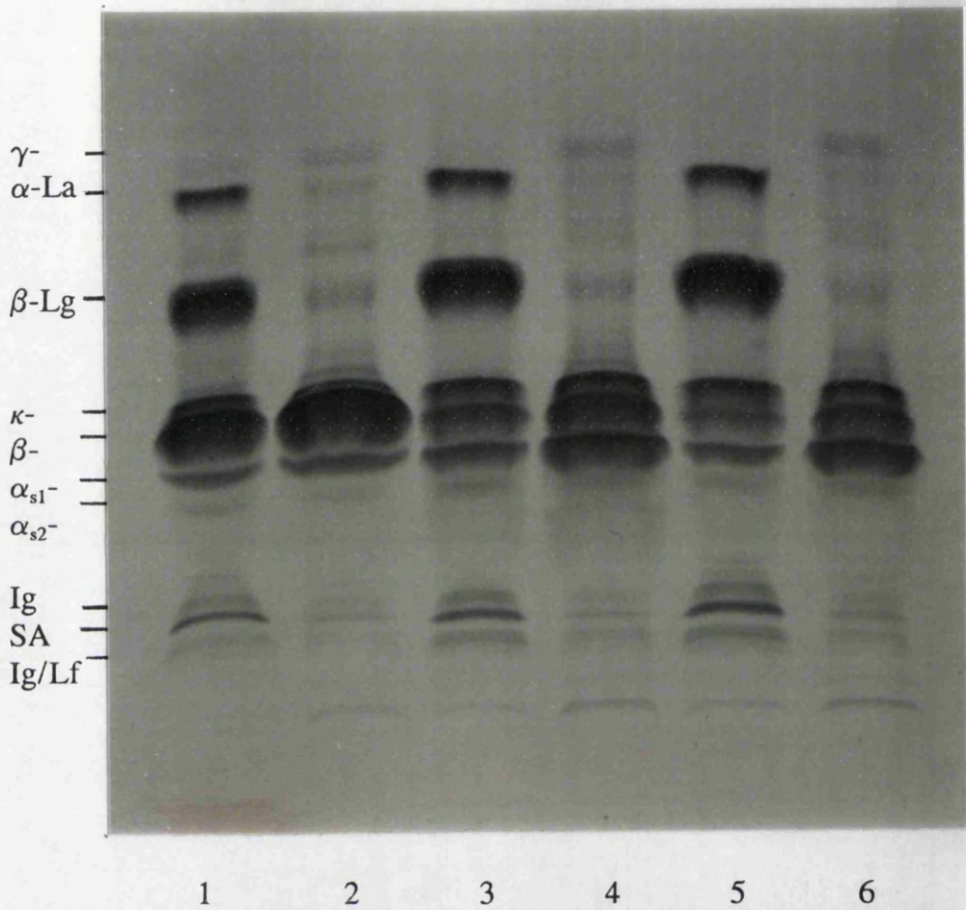
Fig.4. SDS-PAGE pattern of serum caseins from raw milk stored for 22 h at 4, 20 and 30°C (slots 2, 4 and 6), and from heat-treated milk (85°C for 10 min) stored for 22 h at 4, 20 and 30°C (slots 1, 3 and 5).
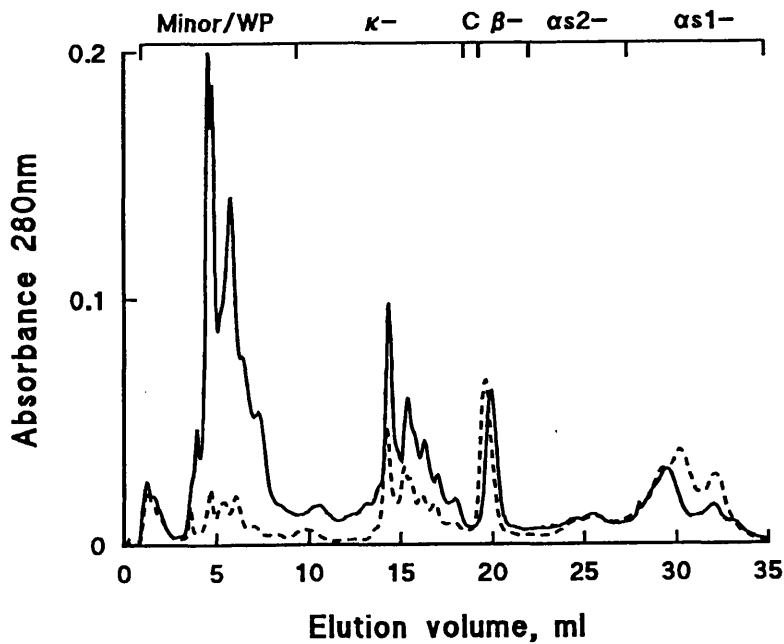
Fig.5. Elution profile of alkylated serum casein and the associated denatured whey proteins obtained by fast protein liquid chromatography (FPLC) at 20°C on a Mono Q HR 5/5 anion-exchange column, and using bis-tris-propane buffer (pH 7.0; 0.005M-bis-tris-propane-urea buffer, 3.3M-urea) and a NaCl gradient. Alkylation was carried out in a similar buffer, 8.0M with respect to urea. ----, Serum casein from raw skim-milk kept at 20°C for 22h.

———, Serum casein and the associated denatured whey proteins from milk heated at 85°C for 10 min, and stored at 20°C for 22h.

Table 2. *Distribution and percentage composition of serum and micellar caseins obtained by ultracentrifugation of raw and heat-treated skim-milk (85°C for 10 min) stored for 22h at 4, 20 and 30°C*

| | Whole casein | 4°C | | 20°C | | 30°C | |
		Raw	Heated	Raw	Heated	Raw	Heated
				Serum casein			
Total casein in skim-milk, %	-	11.0	14.8	8.0	12.6	7.3	7.0
				Composition, %			
κ-Casein	10.1	8.5	16.7	18.3	26.6	21.4	33.1
β-Casein	32.7	68.0	60.9	27.8	26.0	23.0	20.7
α_{s2}-Casein	11.9	1.9	2.0	7.4	7.9	5.8	5.4
α_{s1}-Casein	34.0	10.9	8.3	30.2	20.2	33.5	20.1
Minor caseins	11.3	10.7	12.1	16.3	19.3	16.3	20.7
				Micellar casein			
Total casein in skim-milk, %		89.0	85.2	92.0	87.4	92.7	93.0
				Composition, %			
κ-Casein		9.7	8.5	9.1	7.6	9.2	7.4
β-Casein		30.2	31.7	33.3	34.2	32.6	35.1
α_{s2}-Casein		13.2	12.9	11.9	13.8	9.8	11.5
α_{s1}-Casein		38.5	39.8	36.4	35.9	36.3	35.4
Minor caseins		8.4	7.1	9.3	8.5	12.1	10.6

by ultracentrifugation of skim-milk and heat-treated skim-milk (85°C for 10 min), stored for 22 h at 4, 20 and 30°C. Results for the composition are expressed as % of the total serum and micellar casein respectively, excluding the associated denatured whey proteins. The level of serum casein in raw and heat-treated milk increased with decreasing temperature, the increase at 4 and 20°C being slightly greater for heated milk. Serum casein from both raw and heated milk at lower temperatures contained much more β-casein and proportionately less of the other caseins (4 > 20 > 30°C). These changes were consistent with increased dissociation of β-casein at low temperatures, as reported previously (Rose, 1968; Downey & Murphy, 1970; Davies & Law, 1983). Compared with serum casein from raw milk, at each storage temperature that from heat-treated milk contained much more κ-casein. Results for the composition of the micellar caseins (Table 2) confirmed the above findings, in that micellar caseins at low temperatures contained less β-casein (4 < 20 < 30°C). Similarly, compared with micellar casein from raw milk, that from heat-treated milk contained less κ-casein.

The changes in concentrations of serum caseins on heat treatment and storage are shown in Table 3. There was a marked increase in the total concentration of serum casein in raw and heated milk as the temperature of storage was reduced (4 > 20 > 30°C), which was partly due to an increase in the serum concentration of β-casein. At 20 and 4°C, the concentrations of β-casein in the serum from heat-treated milk were higher than in that from raw milk. Compared with serum from raw milk, that from heated milk at each storage temperature also contained much more κ-casein, and on average the level of dissociation of κ-casein from the micelles increased from 13.2 to 27.3% of the total κ-casein (Table 3). The levels of dissociation of α_{s1}- and α_{s2}-caseins in raw and heat-treated milk, at all storage temperatures, were fairly low, and at 20 and 4°C the concentrations in the sera from raw and heated milk showed no major differences. On storage at 30°C, the concentration of α_{s1}-casein was lower in the serum from heat-treated milk than in that from raw milk.

Van Hooydonk *et al.* (1987) also found that there was increased dissociation of κ-casein into the serum in milk heated at 85°C for 5-10 min, and stored at 4°C for 24h. On determining the levels of κ-casein in the serum by the release of caseinomacropeptide on renneting, they found that 30-40% of the total κ-casein dissociated into the serum on heating. Results from electrophoresis also showed the presence of α_s- and β-caseins in the serum, but quantitative values were not given for the extent of their dissociation. They also found that on storing heat-treated milk at 5°C for 24h, there was a further increase in the level of κ-casein in the serum. In the present study, some additional work showed that when milk was heated at 85°C for 10 min and then centrifuged immediately at 20°C, the concentration of κ-casein in the serum was considerably higher than in that from raw milk (Table 3). The amount of κ-casein dissociated from the micelles increased to 30.5% of the total κ-casein, compared with 14.6% in the serum from raw milk. This level of dissociation was only slightly lower than that obtained after storage for 22h (33.8%), showing that the storage time at 20°C had little effect, and most of the dissociation of κ-casein occurred during heating or soon afterwards. The concentrations of β-, α_{s1}- and α_{s2}-caseins decreased slightly on heating but increased again on storage for 22h.

Results from gel permeation chromatography showed that when milk was heated at 85°C for 10 min, 86% of the whey proteins were irreversibly denatured, and only 8% of β-lactoglobulin and 25% of α-lactalbumin remained undenatured. The concentrations of denatured whey proteins which precipitated together with the serum and micellar caseins at pH 4.6 are presented in Table 4. Results show that, on high-speed centrifugation, the denatured whey proteins were almost equally distributed between the supernatants and the micellar pellets. Smits & Brouwershaven (1980) found that when casein micelles and β-lactoglobulin were heated at 90°C for 20 min, and then ultracentrifuged, 44% of the β-lactoglobulin was associated with the micellar pellet, and a further 31% was in the interphase layer immediately above the pellet. In the present study, the interphase layer was included with the superantant, and the serum would, therefore, be enriched in the complex of β-lactoglobulin and κ-casein.

Table 3. *Concentrations of serum caseins, and extent of dissociation of each of the caseins in raw and heat-treated milk (85°C for 10 min), stored at 4, 20 and 30°C*

| | 4°C | | 20°C | | | 30°C | |
	Raw	Heated	Raw	Heated	Heated	Raw	Heated
Storage time, h	22	22	22	1.5	22	22	22
			Concentrations in serum, g l^{-1}				
κ-Casein	0.26	0.68	0.41	0.85	0.94	0.42	0.62
β-Casein	2.05	2.48	0.62	0.59	0.92	0.45	0.39
α_{s2}-Casein	0.06	0.08	0.16	0.11	0.28	0.11	0.10
α_{s1}-Casein	0.33	0.34	0.67	0.50	0.72	0.66	0.38
Minor caseins	0.32	0.49	0.36	0.33	0.64	0.32	0.38
Total	3.02	4.07	2.22	2.38	3.50	1.96	1.87
			% Dissociated				
κ-Casein	9.5	24.8	14.6	30.5	33.8	15.6	23.2
β-Casein	25.6	30.9	7.0	5.8	10.5	4.7	4.0
α_{s2}-Casein	1.4	1.9	4.8	4.2	8.1	3.9	3.4
α_{s1}-Casein	4.4	4.5	7.3	4.3	7.8	6.8	3.9

Table 4. *Concentrations of proteins in serum from raw and heat-treated skim-milk (85°C for 10 min) stored for 22h at 4, 20 and 30°C, and distribution of denatured whey proteins between the supernatants and micellar pellets obtained by ultracentrifugation*

	4°C		20°C		30°C	
	Raw	Heated	Raw	Heated	Raw	Heated
			Concentrations, g l^{-1}			
Total acid-precipitable protein (pH 4.6) in serum	3.02	6.70	2.22	6.23	1.96	4.24
Serum casein	3.02	4.07	2.22	3.50	1.96	1.87
Denatured whey proteins						
Serum	-	2.63	-	2.73	-	2.37
Micellar pellet	-	2.16	-	2.84	-	2.40
Total	-	4.79	-	5.57	-	4.77

Using radiolabels, Noh and Richardson (1989) showed that α-lactalbumin also was associated with the casein micelles after heating. Their results also indicated that on heating skim-milk, some of the denatured whey proteins remained in the serum rather than being associated with the casein micelles. They found that the denatured whey proteins in the serum were complexes with molecular weights between those of whey proteins and the colloidal complexes.

It is not clear from previous studies to what extent the association of denatured whey proteins with the casein micelles may affect the dissociation of κ-casein. Singh and Fox (1987) showed that dissociation of κ-casein from the micelles was dependent on the pH of heat-treatment. On heating milk at 90°C for 10 min below pH 6.9, β-lactoglobulin became associated with the micelles, and tended to prevent dissociation of κ-casein. At pH 6.9 or above, as the negative charge on the proteins increased, the complex of β-lactoglobulin and κ-casein dissociated from the micelles. Aoki *et al*. (1974), however, found that even on heating casein micelles at 140°C for 15s in the absence of whey proteins, there was still an increase in the level of soluble casein, and that almost half of this was κ-casein.

The effect of pH on the dissociation of caseins and denatured whey proteins

Changes in the concentrations of the caseins and whey proteins in the serum on acidification of heat-treated milk and storage for 22h at 4, 20 and 30°C are shown in Fig. 6. At 4°C, there was a marked increase in the level of total serum casein especially below pH 6.0, reaching a maximum of 39.7% of the total casein at pH 5.5, and then decreasing rapidly at lower pH. At 20°C, the increase in serum casein was less than in milk stored at 4°C. Between pH 6.7 and 6.0 there was only a slight increase in serum casein, but below pH 6.0 the level increased considerably, reaching a maximum of 24.8% of the total casein at pH 5.7, and then decreasing rapidly at lower pH. On storing acidified heat treated milk at 30°C, there was a decrease in the level of serum casein, and below pH 5.7 most of the casein was sedimented. The increases in the levels of total serum casein in the pH ranges 6.7-5.5 and 6.7-5.7 for heated milks stored at 4 and 20°C were similar to those found in unheated milk over the corresponding ranges (Dalgleish & Law, 1989). In heated milks stored at 4 and 20°C, however, maximum dissociation took place at pH 5.5 and 5.7, whereas in raw milk stored at 4 and 20°C release of caseins into the serum continued down to pH 5.1 and 5.4, respectively, so that about 55 and 30% of the total casein dissociated. In heat-treated milk, therefore, the overall levels of serum casein at 4 and 20°C were substantially lower than in unheated milk (Dalgleish & Law, 1988). In heat-treated milk stored at 30°C, there was a steady decrease in serum casein with decreasing pH, whereas in raw milk there was a small increase in serum casein with a maximum at pH 5.5 (Dalgleish & Law, 1988).

On storing heat-treated milk at 4 and 20°C, the concentrations of all the individual caseins in the serum showed the same trends as the the total serum casein, and increased as the pH was reduced to 5.5 and 5.7, respectively, but decreased as the pH was reduced further (Fig.6). The levels of maximum dissociation of the caseins at 4°C, expressed as a percentage of the total of each individual casein in skim-milk, were: κ-casein, 53.7%; β-casein, 63.7%; α_{s2}-casein, 16.9% and α_{s1}-casein, 36.1%. The increase in serum concentrations of the individual caseins with decreasing pH was lower at 20°C than at 4°C. The corresponding levels of maximum dissociation at 20°C were: κ-casein, 46.2%; β-casein, 26.9%; α_{s2}-casein, 14.9% and α_{s1}-, 19.5%.

The increases in serum concentrations of κ-, β- and α_{s1}-caseins in heated milk stored at 4 and 20°C were similar to those for the corresponding raw milks within the same pH ranges (Dalgleish & Law, 1988). At each storage temperature, however, maximum dissociation of the caseins in heat-treated milk occurred at a higher pH than in raw milk, and the overall increases in the individual caseins on adjusting the pH of heat-treated milk were considerably lower than the corresponding increases in raw milk (Dalgleish & Law, 1988). As described above, on
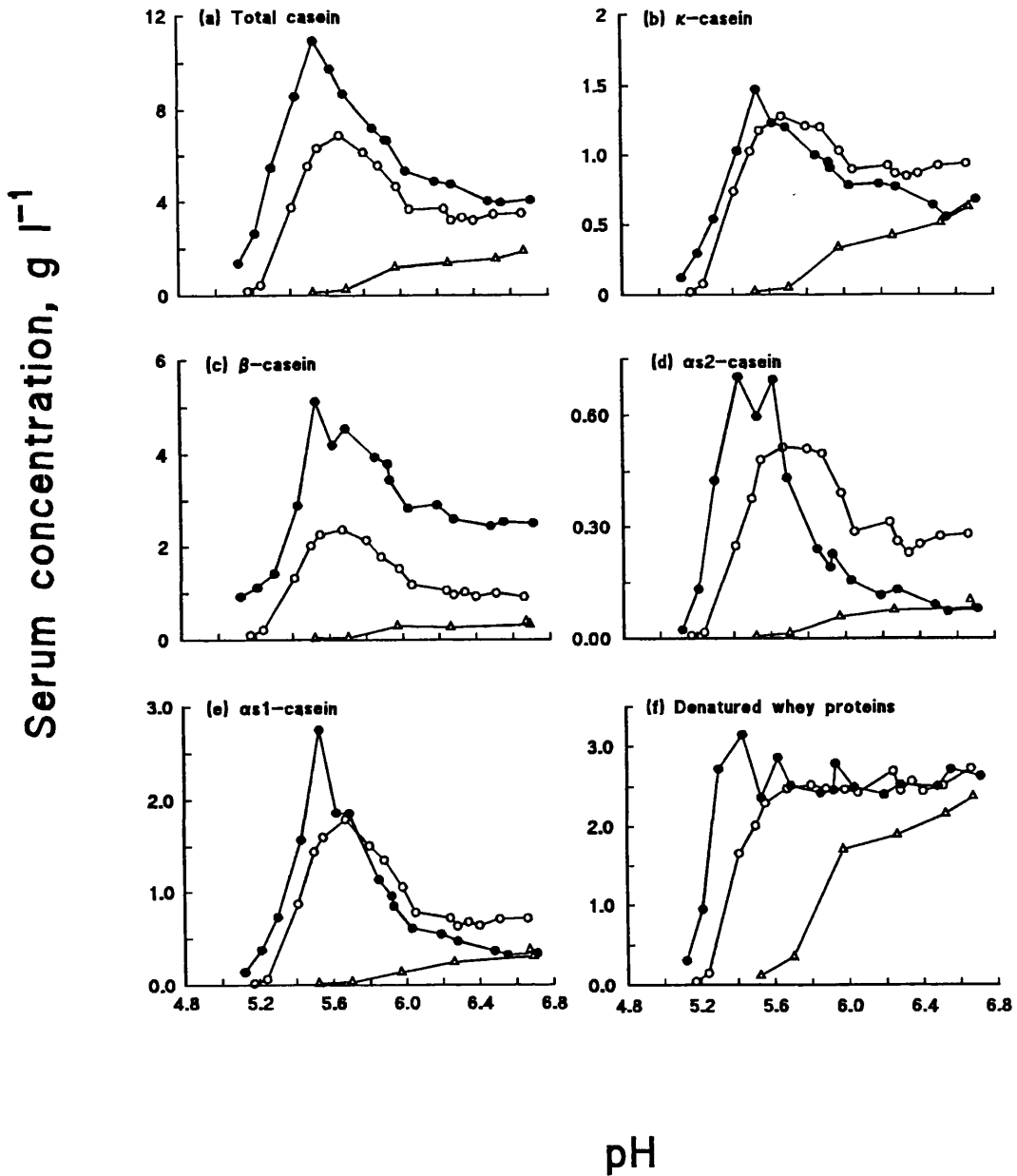
Fig.6. Changes in the serum concentrations of the caseins and denatured whey proteins on acidification of heat-treated milk (85°C for 10 min) and storage at different temperatures: ●, 4°C; ○, 20°C; △, 30°C.

heating milk there was a marked dissociation of κ-casein from the micelles, and at pH 6.7 the concentration of κ-casein in the serum was considerably higher than that in the serum from raw milk. In heat-treated milks stored at 4 and 20°C, the overall levels of serum κ-casein above pH 5.5 and 5.7, respectively, were much higher than in the corresponding raw milks. At lower pH, less dissociation of κ-casein took place in heat-treated milk than in raw milk. At 30°C, the concentrations of κ-, β-, α_{s2}- and α_{s1}-caseins in the serum of heat-treated milk decreased rapidly as the pH was lowered, and did not show the small increase found in raw milk at about pH 5.5 (Dalgleish & Law, 1988). Because of the dissociation of κ-casein on heating milk, however, the concentration of κ-casein in the serum of heat-treated milk at 30°C above pH 6.0 was higher than that in the serum from raw milk.

On acidification of heat-treated milk, and storage at 4, 20 and 30°C, the denatured whey proteins showed less change than the caseins in their distribution between the serum and micellar pellets. At 4°C, the concentrations of the denatured whey proteins in the serum increased slightly with decreasing pH, reaching a maximum at pH 5.4 and then decreasing rapidly at lower pH. At 20°C, their serum concentrations showed no marked change between and 6.7 and 5.7, but decreased rapidly at lower pH. At 30°C, the serum concentrations of denatured whey proteins decreased gradually with decreasing pH, the level being negligible below pH 5.5.

It has been shown previously that acid gelation occurs at higher pH after heat-treatment (Grigorov, 1966; Horne & Davidson, 1992). Emmons et al. (1955) showed that this was due to denaturation of the whey proteins. On heating a synthetic skim-milk that did not contain whey proteins, the pH of acid coagulation was the same as in the unheated milk, but on adding whey proteins and heating, the pH of subsequent coagulation increased by up to 0.5 of a pH unit; the increase was closely correlated with the ratio of denatured whey proteins to casein in the milk. To a large extent, therefore, the increased tendency for the residual micelles to aggregate may occur because of interaction of denatured whey proteins with κ-casein on the surface of the micelles, possibly reducing the effect of the macropeptide hairs which normally help to stabilise the micelles. The increased dissociation of κ-casein from the micelles on heating may also cause a depletion of the hydrophilic hairs, and further reduce the stability of the micelles.Using ^{1}H-NMR in studies of micellar structure, Rollema and Brinkhuis (1989) have shown that, on heating between 60 and 90°C, there is a breakdown in the rigid structure of the micelles and that, in the absence of whey proteins, this is reversible. It is possible, however, that in the presence of substantial amounts of denatured whey proteins during heating, quite considerable structural reorganisation of the caseins occurs within the micelles.

Comparison of the results obtained for the changes in the serum concentrations of Ca, P_i and caseins shows that, as found previously for raw milk, the relation between dissociation of calcium phosphate and the individual caseins from the micelles is complex. Although calcium phosphate is important in maintaining micellar structure, other forces must be involved. The rate of removal of calcium phosphate from the micelles on acidification of heat-treated milk was not markedly affected by the temperature. On reducing the pH at 30°C, however, all of the calcium phosphate was removed without any increase in dissociation of the caseins from the micelles. On acidification at 20°C, and even more so at 4°C, there was considerable dissociation of caseins from the micelles. Results also showed that the rate of solubilisation of micellar calcium phosphate in heat-treated milk was similar to that found previously for raw milk. The overall levels of dissociation of the caseins, however, were less than found for raw milk. From NMR studies, Rollema and Brinkhuis (1989) have shown that, on acidification of casein micelles down to pH 5.8, there is no change in the mobility of the caseins, whereas on addition of EDTA to remove Ca they are able to move more freely. It appears, therefore, that acidification not only causes removal of calcium phosphate, but reduces the net negative charge on the caseins that would otherwise tend to promote dissociation. At the same time, removal of micellar calcium phosphate by acidification increases the ionic strength of the serum, thereby

increasing hydrophobic interaction between the micellar caseins. This effect may be enhanced because of the hydrophobic nature of the denatured whey proteins on the surface of the micelles after heating.

The results of the present work show that the Ca, P_i, casein and whey protein content of micelles can be changed considerably from that in raw milk by a combination of heat-treatment, acidification and different storage conditions, and these changes may affect the physical properties of the micelles. Futher information about the kinetics of the changes in the micellar-serum distribution of calcium phosphate and the proteins could help to increase the efficiency of processes involving acidification of heat-treated milk.

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7

GENERAL DISCUSSION

1. INTRODUCTION

Analytical methods including anion- and cation-exchange FPLC and gel permeation FPLC were developed and used to examine natural variation in the content and composition of the caseins and whey proteins in milk of the cow, goat and sheep. These methods were applied to study changes taking place in the milk proteins during various stages of processing, such as cooling, acidification, heating, renneting and proteolysis.

2. COMPOSITION OF BOVINE CASEIN

2.1 Ion-exchange FPLC

Initially the composition of bovine casein was determined by low-pressure anion-exchange chromatography on DEAE cellulose, but more rapid methods of anion- and cation-exchange FPLC were developed. These methods were less laborious than low-pressure chromatography; where comparison was possible, they gave similar resolution and quantitative results. For example, in the analysis of bovine casein, quantitative values for the individual caseins obtained by anion-exchange FPLC on Mono Q (Paper 2, Davies and Law, 1987) were in close agreement with those obtained by anion-exchange chromatography on DEAE cellulose (Davies and Law, 1977a). Values obtained by cation-exchange FPLC on Mono S (Paper 3, Hollar *et al.*, 1991) were also closely correlated with the corresponding values obtained by anion-exchange FPLC.

The anion- and cation-exchange FPLC methods also required much less sample, but sufficient protein could be recovered from analytical columns to carry out further characterisation by other techniques such as electrophoresis or re-chromatography. Both

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methods could be scaled up directly for laboratory preparation of the major caseins by substituting similar ion-exchange materials having larger bead sizes and correspondingly lower back-pressures (Paper 5, Leaver and Law, 1993).

A major advantage of cation-exchange FPLC was that it could be used to fractionate the caseins of the cow, goat and sheep and, therefore, allowed direct comparison of the composition of casein of the three species. The method was also used to separate the common variants of bovine β-casein (A^1, A^2 and B), and was applied to study the effect of genetic polymorphism on the composition of whole casein.

One drawback of the FPLC analytical methods, in common with most other available techniques, was that the composition of the caseins and whey proteins could not be determined in a single fractionation. The whey proteins, therefore, were fractionated separately by gel permeation FPLC, after precipitation of the caseins. It was possible, however, to obtain quantitative values for the individual caseins in the presence of native and denatured whey proteins (Paper 21, Law *et al.*, 1994). This was particularly useful in the study of changes in the caseins and whey proteins at high temperatures, and in the examination of proteolysis in cheese manufactured from heated milk (Paper 22, Calvo *et al.*, 1992a). Simultaneous fractionation of the major caseins and whey proteins has been obtained by reverse phase HPLC but some doubt has been cast on the accuracy of quantitative results because of poor recovery, under certain conditions, of κ- and α_{s2}-caseins (Visser *et al.*, 1991) and whey proteins (de Frutos *et al.*, 1992). The major caseins and whey proteins have also been separated by a single run on capillary electrophoresis, but few quantitative values are available at present (de Jong *et al.*, 1993).

2.2 Seasonal Variation

Results from anion-exchange chromatography on DEAE cellulose (Paper 1, Davies and Law, 1987) showed that there was considerable seasonal variation in the concentrations of the caseins in creamery milks in South-West Scotland (Chapter 2). In the summer months, when cows were at pasture, milk generally contained more total and individual

caseins, apart from α_{s2}-casein which reached a maximum in early summer and then declined through the summer. The relative amounts of the caseins in bulk milk, however, showed comparatively little variation. Previous workers found lactational changes in the composition of casein from individual cows (Davies and Law, 1977b; Barry and Donnelly, 1980), which to a large extent were caused by increased proteolysis of β-casein, and possibly α_{s1}- and α_{s2}-caseins, especially towards the end of lactation, and in older cows. Some lactational effect on the composition of casein in creamery milks was expected, because of the increased number of cows calving in the spring, but these changes tended to be averaged out in the bulk milks.

Seasonal variation in the concentrations of the individual caseins may have a direct effect on the processing properties of milk. For example, it seems likely that cheese yields would follow the same trend as total casein, being low in the Autumn and Winter, but increasing rapidly in the Spring when cows return to pasture. Variation in the concentrations of the caseins could also have a secondary effect in that, as the total concentration and yield increase in early Summer, there may be less efficient phosphorylation of α_{s1}- and α_{s2}-caseins and less complete glycosylation of κ-casein. Lactational changes have been found in the degree of glycosylation of κ-casein (Robitaille et al., 1991) but further work is required to determine if these changes are related to casein concentrations. Results from other work on creamery bulk milks (Holt and Muir, 1978) showed that there was a pronounced seasonal trend in micellar size, and that average sizes were smaller in Summer when casein concentrations tended to be higher. Also, the average micellar size was positively correlated with the amount of colloidal P_i per unit weight of casein and negatively correlated with casein-bound Ca.

2.3 Genetic Polymorphism

Genetic polymorphism of κ-casein had a significant effect on the composition of whole casein; on average, the relative amounts of κ-casein in whole casein for phenotypes AA, AB and BB were 9.7, 10.9 and 12.1%, respectively. The relative amount of β-casein decreased with increasing κ-casein, whereas the relative amounts of α_{s1}- and α_{s2}-caseins did not change significantly. The differences in the relative amount of the κ-

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casein variants are particularly important because of the role of κ-casein in stabilising casein micelles. Various workers have found that κ-casein B is associated with a decrease in clotting time, increased curd tension, better fat retention and higher cheese-yield (BB > AB > AA). The reason for the association of κ-casein B with these properties is still not clear, especially since, on renneting, the caseinomacropeptide, which contains the substituted amino acids (Ala_{148} and Ile_{136}), is removed from the micellar surface, and the remaining para-κ-casein from both genetic variants is the same. Similarly, it has been suggested that the differences in glycosylation of the A and B variants might cause changes in the processing properties, but the carbohydrate is removed together with the caseinomacropetide on renneting.

A number of workers have shown that there is a negative correlation between κ-casein content and micellar size (McGann *et al.*, 1979; Donnelly *et al.*, 1984; Paper 17, Dalgleish *et al.*, 1989). Using dynamic light scattering, Horne *et al.* (1993a) confirmed that, as expected from the above values for the relative amounts of κ-casein in the different genotypes, κ-casein BB milks contained, on average, smaller micelles than κ-casein AA milks. There was also a positive correlation between rennet coagulation time and micellar diameter. Recent results (unpublished work) show, however, that within each phenotype, micellar size is independent of the κ-casein content.

In a study of the proteolytic and coagulation stages of renneting, Horne and Muir (1994) have shown that the rates of proteolysis of κ-caseins A and B are similar, and that the shorter coagulation time of κ-casein BB milks appears to be due to less effective stabilisation of the micelles by κ-casein B. Dalgleish (1979) has shown that rennet coagulation can occur when about 80% of the κ-casein has been hydrolysed. It has been proposed that the lower negative charge or different glycosylation on residual unhydrolysed κ-casein B, compared to the A variant, may promote coagulation.

Various studies have shown that the levels of expression of some genetic variants are equal, whereas others, such as bovine κ-caseins A and B, differ. Results from this study have shown that the level of expression of κ-casein B is about 25% greater than that of the A variant. Similarly, in a study of β-lactoglobulin, Aschaffenburg and Drewry (1957) found that, for equal yields of milk of similar casein content, cows homozygous in β-lactoglobulin A produced almost twice as much β-

lactoglobulin as the β-lactoglobulin B homozygotes; the output of the heterozygotes was intermediate. In the present study, however, it was found that the bovine β-casein variants A^1, A^2 and B are produced in equal amounts. Similarly, the A and B variants of caprine κ-casein (Paper 8, Law and Tziboula, 1993) are produced in equal amounts.

Differences have been found by other workers in the levels of expression of variants of bovine α_{s1}-casein (B>A), α_{s2}-casein (A>D), caprine α_{s1}-caseins (A > D and F) and ovine α_{s2}-casein. It has been shown that the variants produced at the lower levels arise because of deletions of segments of amino acids which, with the exception of that in bovine α_{s1}-casein A, are also phosphopeptides. It has been suggested that each of the deleted peptides corresponds to one exon (two in the case of caprine α_{s1}-casein F), and that these variants occur because of splice-site mutations leading to abnormal splicing of RNA, and subsequent exon skipping (Brignon et al., 1990; Boisnard et al., 1991; Mohr et al., 1994). The lower rate of synthesis of the variants has been attributed to a reduced rate of processing of the altered RNA. The A and B variants of κ-casein and β-lactoglobulin, however, each differ by only two amino acid substitutions, and it seems unlikely that the different levels of expression of these variants occurs by the above mechanism. It is possible, however, that the variants are genetically linked to changes outwith the structural gene which cause different levels of expression, but further research is required to establish this.

Information from DNA and amino acid sequences of the milk proteins has shown that some mutations at the DNA level may have gone undetected, and that the true extent of genetic polymorphism in milk proteins may be more than three times the present known level (Grosclaude, 1988). As the genetic code is degenerative, and more than one codon may give the same amino acid, changes may occur in the DNA sequence which are not translated into amino acid substitutions. Also, only about 33% of the amino acid substitutions involve changes in net charge that can be detected by electrophoresis, which has been the most commonly used technique for separating genetic variants. It is probable, therefore, that other genetic variants, with different processing characteristics, will be detected by techniques such as reverse-phase HPLC or sequencing that do not depend on charge differences.

The genetic variants described in this study are due in most instances to substitution of one or two amino acids or to deletions of small peptides (Tables 4, 9

and 14). Techniques are now available, however, to introduce new genetic material from other species and to delete quite large segments of existing genetic material. Most of this work has been carried out in small animals because of their short generation time. Various proteins have been expressed in the milk of the mouse, including β-casein from the rat and goat (Lee et al., 1988; Persuy, 1992), α-lactalbumin from the cow and guinea-pig (Maschio et al.,1991; Vilotte et al., 1989), and β-lactoglobulin from the sheep (Simons et al., 1987). Similarly, it has been possible to delete the murine β-casein gene (Kumar et al., 1994).

Using cation-exchange FPLC, Stevenson and Leaver (1994) fractionated the proteins of mouse milk, and studied changes in the content and composition of protein in the milks from genetically altered mice. Addition of the ovine β-lactoglobulin gene to the mouse genome resulted in the expression of β-lactoglobulin and a reduction in the levels of the endogenous proteins, so that the overall concentration of protein was unchanged (Stevenson, 1993). On deleting the mouse β-casein gene, the total concentration of milk proteins decreased by about 10%. The decrease was less than expected, as β-casein normally acccounts for about 21% of the total protein; it was found that there were compensatory increases amounting to 25% of the total κ-casein, 16% of the total casein, and 25% of the whey proteins (Stevenson, 1993). Results show, therefore, that by adding or removing genes coding for the milk proteins, it is possible to obtain large differences in the composition of the casein and whey protein, without necessarily changing the overall protein concentration. Also, there was no evidence of instability of the micelles in the milk of β-casein deficient mice, although micelles from β-casein deficient mice were smaller than those from the wild-type (Kumar et al., 1994).

At present, it is still extremely difficult to produce transgenic animals but, clearly, if these techniques were applied to add new genes or to duplicate or remove existing genes coding for the individual milk proteins in the cow, goat and sheep, substantial changes could be made to the structure of the micelles and to their processing properties. Some possible gene modifications and their effects on properties have been outlined by Jimenez-Flores and Richardson (1988). The FPLC methods described in the present work could be used to examine induced changes in the composition of the caseins or whey proteins of the cow, goat and sheep.

3. CAPRINE AND OVINE CASEIN

3.1 Caprine Casein

Anion-exchange FPLC gave satisfactory resolution of caprine γ- and κ-caseins, but did not give complete separation of β_1, α_{s2}- and α_{s1}-caseins. Cross-contamination was particularly marked when α_{s1}-casein variants A and F were present, as these had lower net negative charges and tended to elute earlier from the anion-exchange column than α_{s1}-caseins B and E. It was found, however, that the caprine caseins, including the α_{s1}-casein variants, could be satisfactorily fractionated (Paper 7, Law and Tziboula, 1992) by the same method of cation-exchange FPLC used for bovine caseins. This had the added advantage that the compositions of whole casein from both species could be compared directly.

As discussed below, the composition of caprine casein varied considerably due to quantitative genetic polymorphism of α_{s1}-casein. Compared with bovine casein, that from the goat contained, on average, much less α_{s1}-casein, a similar amount of α_{s2}-casein, and more β- and κ-caseins (Table 17). These differences were especially pronounced in goats producing low levels of α_{s1}-caseins, due to the E and F alleles.

In a study of genetic polymorphism of caprine α_{s1}-casein, it was found that the relative amount of α_{s1}-casein varied between 26.0 and 4.4% of the total casein (variant A, B > E > F); the relative amounts of the other caseins increased in proportion. In a lactational study of caprine caseins, using the same method of cation-exchange FPLC, it was also found that the concentration of α_{s1}-casein in milk was related to the type of α_{s1}-casein variant present (Brown et al., 1995). Recent studies by other workers, have shown that the synthesis of α_{s1}-casein is under the control of at least 14 alleles. These produce six different polypeptides (Boulanger et al., 1984; Grosclaude et al., 1987; Mahé and Grosclaude, 1989; Martin, 1993), and four different levels of expression of α_{s1}-casein (A,B,C > E > D, F > null allele). Brignon et al. (1990) have suggested that the reduced rates of synthesis of the D and F variants are due to splice mutations giving an altered rate of processing of RNA.

A number of studies have shown that there are commercial advantages in having goats with the high-producing A, B and C alleles as opposed to the D, E and F alleles.

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High-producing α_{s1}-casein alleles are associated with higher concentrations of total protein and fat, and higher protein yield per lacatation (Manfredi *et al.*, 1993; Pirisi *et al.*, 1994). Similarly, the physicochemical properties of the high-producing α_{s1}-casein alleles are more satisfactory for cheese-making (Ambrosoli *et al.*, 1988; Pirisi *et al.*, 1994). In particular, Remeuf (1993) found that α_{s1}-casein type was associated with micellar size (AA < EE < FF) and rennet gel strength (AA > EE > FF). Also, Heil and Dumont (1993) noted that high α_{s1}-casein content was associated with less intensity of goat flavour in cheese, and firmer and smoother texture. It is worthwhile, therefore, carrying out more work to compare the different α_{s1}-casein variants in the manufacture of cheese, and to establish the gene frequencies of the variants in different breeds. The method of cation-exchange FPLC described here can be used to obtain quantitative information on the composition of casein associated with the various possible combinations of the α_{s1}-casein variants.

In a further study of genetic polymorphism, using cation-exchange FPLC, it was found that two variants of caprine κ-casein were present in milk samples from a commercial herd in Greece and from a herd of British Saanen goats. The two variants were transferred in Medelian fashion, and differed in net charge in the para-κ-casein region. Although the total amount of κ-casein varied between 9 and 20% of the total casein, the variants were produced in approximately equal amounts. This differs from the polymorphism of bovine κ-casein, in which the levels of expression of the variants are unequal. However, because of the charge diference in the para-κ-casein regions, it is possible that, as found for bovine κ-caseins A and B, the two caprine κ-casein variants have different processing properties. Further work is required to establish this, and to identify the amino acid differences in the variants.

In a study of variation in the composition of casein from individual goats, results from cation-exchange FPLC showed that the relative amounts of the early-eluting fraction varied from 6.9 to 15.0 % of the total casein. SDS and alkaline PAGE patterns showed that these were low molecular weight peptides analagous to bovine γ-caseins. There appeared to be, therefore, a considerable amount of proteolysis of caprine β-casein, as found previously in bovine caseins (Davies and Law, 1977b; Barry and Donnelly, 1981). In a study of the lactational changes occurring in the relative amounts of the caseins, it was found that there was a marked increase in the relative

amount of the γ-caseins with advancing lactation, and as the milk yield decreased (Brown *et al.*, 1995). This is similar to increase in γ-caseins observed in bovine caseins in late lactation, when there is increased permeability of the mammary gland, raised levels of plasmin, and increased proteolysis of β-casein (Aaltonen *et al.*, 1988; Politis *et al.*, 1989).

3.2 Ovine Casein

In preliminary work, it was found that anion-exchange FPLC gave satisfactory resolution of ovine γ-, κ- and β_2-caseins but poorer resolution of the β_1- and α_s-caseins (Papoff *et al.*, 1993). As found for caprine casein, cross-contamination was particularly marked when genetic variants of α_{s1}-casein, such as the "Welsh" variant, were present. Applying the same method of cation-exchange FPLC that was used for bovine and caprine caseins, however, it was possible to separate ovine whole casein into four main fractions; the separation was not affected by the presence of the α_{s1}-casein "Welsh" variant.

Compared with bovine casein, that from the sheep contained less α_s-casein (α_{s1}- and α_{s2}-), a similar amount of κ-casein, and more β-caseins (Table 17). Ovine casein and caprine casein containing a high level of α_{s1}-casein were, therefore, similar in composition. It was not possible to sub-fractionate the ovine α_s-caseins and, at present, there is no single-step quantitative method available for the fractionation of these caseins.

Cation-exchange FPLC was used to examine natural variation in the composition of ovine casein. In a study of seasonal variation in the content and composition of casein in a commercial Scottish flock, it was found that the concentration of total and individual caseins increased from Spring to Autumn as the average stage of lactation increased. The relative proportions in bulk milks, however, showed very little variation. On the other hand, the composition of casein from individual sheep in Sardinia varied considerably. About half of the samples contained small amounts of minor caseins, and showed little variation in composition, but the remainder contained large amounts of low molecular weight proteins and less β- and α_s- caseins. A

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considerable part of the variation, therefore, could be attributed to proteolysis of β- and α_s-caseins. Further work indicated that some of the variation in composition might also be due to the occurrence of the "Welsh" genetic variant of α_{s1}-casein, but the exact nature of this variant has not been established (Papoff *et al.*, 1993).

Boisnard *et al.* (1991) have also shown the occurrence of an unusual polymorphism in ovine α_{s2}-caseins. Two allelic forms of α_{s2}-casein have been found, which differ by amino acid substitutions. For each genetic variant, two non-allelic forms of α_{s2}-casein occur in milk, and differ by an internal deletion of nine amino acids. The evidence suggests that the shorter forms of α_{s2}-casein occur because of incorrect processing of a unique form of pre-mRNA, which results in exon skipping, as described above for the α_{s1}-casein variants of the goat. The deletion of amino acids involves removal of two Cys and one phosphoseryl residue and may, therefore, have a marked effect on the properties of the smaller α_{s2}-casein.

4. THE WHEY PROTEINS

Initially in this study, gel permeation chromatography on Sephadex G-100 was used to determine the composition of whey protein (Paper 13, Davies and Law, 1983). The method was laborious, however, and a method of gel permeation FPLC was developed which gave rapid separation of bovine whey proteins (Paper 11, Law *et al.*, 1993). Comparison of the fractionations and quantitative results for whey protein from bulk milks showed that there was general agreement between the two methods. The same FPLC method was also suitable for the fractionation of caprine and ovine whey proteins; this allowed direct comparison of the composition of whey protein from the three species.

Compared with bovine whey protein, that from the goat contained less β-lactoglobulin and more α-lactalbumin. On the other hand, compared with bovine whey protein, that from the sheep, contained a similar amount of β-lactoglobulin, more immunoglobulins, and less α-lactalbumin. Also, in this study, the concentration of total whey proteins in the milk of the cow and goat were similar, but that in milk of the sheep was almost twice the level. The concentrations of α-lactalbumin, therefore, were

similar in the milk of the three species, but the milk of the sheep contained almost twice as much β-lactoglobulin as milk from the cow or goat. These differences could have substantial effects on the relative rates of denaturation of the whey proteins of the three species.

In a study of creamery milks in South-West Scotland, it was found that the concentrations of total and individual whey proteins varied considerably; total whey protein concentrations were higher in the Summer months, as found for the caseins. There were no clear trends in the individual whey proteins, however, apart from a tendency for the concentration of β-lactoglobulin to increase, and that of α-lactalbumin to decrease, from Spring to Autumn. The relative amounts of the whey proteins, on the other hand, showed little seasonal variation.

Gel permeation FPLC was used to study the effect of stage of lactation on the content and composition of caprine whey proteins. The concentrations of total and individual whey proteins, except α-lactalbumin, showed large variations, and tended to be high at the beginning and end of lactation, but lower in mid lactation. The total yields of β-lactoglobulin and α-lactalbumin decreased with advancing lactation, whereas the yields of immunoglobulins and serum albumin/lactoferrin did not change markedly. The relative amounts of the whey proteins showed less variation but, with advancing lactation, the relative amounts of β-lactoglobulin and α-lactalbumin decreased, and the relative amounts of the immunoglobulin and serum albumin/lactoferrin fractions increased slightly. This may reflect the increased permeability of the mammary gland towards the end of lactation, and the transfer of serum albumin, lactoferrin and immunoglobulins from blood plasma into milk.

In a study of the combined seasonal and lactational changes in the ovine whey proteins in milk of a commercial Scottish flock, it was found that the concentrations of total and individual whey proteins, except α-lactalbumin, tended to be higher at the beginning and end of the lactation in the Spring and Autumn, respectively. The relative amount of β-lactoglobulin increased and that of α-lactalbumin decreased as lactation progressed, whereas the relative amounts of the immunoglobulin and serum albumin/lactoferrin remained almost constant throughout the period of study.

5. MICELLAR STRUCTURE AND STABILITY

The large differences in the composition of the casein of the cow, goat and sheep (Table 17) indicate that the structure and stability of the micelles of the three species may be different. Both caprine and ovine casein contain considerably more β-casein. A higher proportion of casein may, therefore, be able to dissociate from the micelles on cooling to 4°C, compared with that found for bovine casein micelles. Also, compared with whole casein from the cow and sheep, the relative amount of κ-casein is much higher in caprine casein. If the same negative correlation between κ-casein content and micellar size in cows' milk also exists in goats' milk, then caprine micelles, on average, should be smaller. The available evidence shows that the caprine micellar size distribution is bi-modal, with average micellar diameters of 260 or <80nm (Remeuf and Lenoir, 1986), compared to about 120nm for bovine micelles. Also, in the present study, the relative amount of κ-casein was found to increase with decreasing content of α_{s1}-casein, and the micellar size would also be expected to decrease. Remeuf (1993) found that micellar diameter actually decreased with increasing α_{s1}-casein content (AA<BB<FF). Other factors, therefore, may be involved in the structure and stability of caprine and ovine micelles. Compared with bovine β-casein, which has five phosphoseryl groups, β-casein from these two species contains a mixture of five and six phosphoseryl groups residues; the more highly phosphorylated β_1-casein may increase interaction with colloidal calcium phosphate. Also, the net negative charge on caprine and ovine α_{s1}- and α_{s2}-caseins as determined by gel electrophoresis (Fig. 14) is lower than on the corresponding bovine α_{s1}-casein, and there may be less repulsion between the caseins within the caprine and ovine micelles. Further study of caprine and ovine casein micelles is clearly required to investigate the relation between micellar casein composition and size, and to determine the changes in the level and composition of serum casein on cooling.

In the present study, when cows' milk was stored at 4°C for 15 h, there was a marked increase in the concentration of serum casein, which was due mainly to dissociation of β-casein from the micelle. On prolonged cooling, more than 50% of the β-casein dissociated into the serum, and there were small increases in the serum concentrations of Ca and P_i. Also, these changes were reversed when milk was re-

equilibrated at 20°C for 18h. Cooling and re-warming commonly occur during the initial storage and processing of milk; the equilibration time after these adjustments in temperature could affect the extent of the changes in the equilibrium between micellar and serum phases.

In a study of the combined effect of pH and temperature on micellar stability, it was found that, on lowering the pH of milk, the serum concentrations of Ca and P_i increased. On lowering the pH at 4°C, the level of serum casein increased due to dissociation of each of the caseins from the micelles; the combined effect of temperature and pH was more than additive. On lowering the pH at 20°C, there was only a moderate increase in the level of serum casein. At 30°C, all of the calcium phosphate could be removed from the micelles by acidification, without any appreciable increase in the level of serum casein. This suggests that hydrophobic bonding, in addition to casein interaction with calcium phosphate, may be important in maintaining the micellar structure. This series of experiments also indicates how different combinations of temperature and pH could be used to alter the amount of colloidal calcium phosphate and structure of the micelles during the production of cheese and fermented products. Clearly, at higher temperatures, calcium phosphate can be removed without disruption of micellar structure whereas, at 4°C, both calcium phosphate and the caseins, especially β-casein, are removed from the micelles. The equilibration time after adjustment of temperature and pH is also likely to be important. In this study, although temperature and pH adjustment was rapid, the equilibration time was about 24 h. Obviously further work is necessary to provide information about the extent of the changes taking place within shorter equilibration times, as might occur during processing.

Part of this work involved the study of the relation between micellar size and composition. As found by other workers (McGann *et al.*, 1979; Donnelly *et al.*, 1984), there was an inverse relation between the relative amount of κ-casein and micellar diameter. With decreasing micellar diameter, the relative amount of κ-casein increased and β-casein decreased, but the α_{s1}- and α_{s2}-caseins did not vary. From results on the relative amounts of the different κ-casein variants in whole casein (BB > AB > AA), and studies of micellar size and composition, milk containing κ-casein B would tend on average to have smaller micelles than milk containing κ-casein A. In recent studies

(Horne *et al.*, 1993a), it has been found that this relation holds, but that within the BB or AA genotype there is no relation between the relative amount of κ-casein and micellar diameter (unpublished results). This shows that the relation between κ-casein variants and micellar size may depend on the actual properties of the variants, rather than their different relative amounts. This is currently being investigated.

6. CHANGES IN THE MILK PROTEINS DURING PROCESSING

6.1 Heat-Denaturation of Bovine, Caprine and Ovine Whey proteins

In a study of denaturation of whey proteins in cows' milk, it was found that as the temperature of heating (72-140°C) and holding time were increased (15s to 5 min), the whey proteins became increasingly insoluble at pH 4.6. The change in concentrations of the individual whey proteins was determined by gel permeation FPLC, and was taken as a measure of irreversible denaturation of the whey proteins. The level of denaturation increased with temperature and holding time; the ease of denaturation was immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. This order of denaturation differs from results obtained by direct scanning calorimetry, which essentially shows the temperature at which the whey proteins unfold. Rüegg *et al.* (1977) found that the ease with which the whey proteins unfold was in the order serum albumin > α-lactalbumin > lactoferrin > β-lactoglobulin > immunoglobulins (Chapter 6, Section 1). The disparity between the two methods in determining the extent of denaturation is believed to be due to the reversibility of the unfolding process. α-Lactalbumin, for example, is the most resistant to irreversible denaturation as measured on the basis of loss of solubilty, but actually unfolds at a low temperature (65.2°C); it readily re-natures because initially it has no free -SH group that can form aggregates with other proteins.

In this study, the measure of irreversible denaturation of whey proteins obtained by gel permeation FPLC proved to have practical value, and gave a good indication of the amount of denatured whey protein that was incorporated into the curd during cheese manufacture from heated milk. Gel permeation FPLC could also be used to

determine the extent of denaturation of the individual whey proteins in industrial heating equipment during the manufacture of other products such as yogurt and co-precipitate.

On examining denaturation of the whey proteins in the milk of the goat and sheep, it was found that after heat treatment these whey proteins also showed increasing loss of solublity at pH 4.6 with increasing severity of heating. As for bovine whey proteins, the relative ease of denaturation of the whey proteins was immunoglobulins > serum albumin/lactoferrin > β-lactoglobulin > α-lactalbumin. Results indicated, therefore, that controlled denaturation of the whey proteins could also be used to increase the yield of cheese from the milk of the goat and sheep.

A number of problems arise during the manufacture of cheese from heated milk, including an increase in the rennet coagulation time, changes in proteolysis during ripening, and loss of melting properties in the final product. These changes occur because of interaction of denatured whey proteins with the casein micelles, and possibly also because of changes in the micellar-serum distribution of calcium phosphate. The rennet coagulation time of milk increases with the severity of heat treatment and extent of denaturation of the whey proteins (Marshall, 1986; van Hooydonk et al., 1987; Singh et al., 1988). It is not clear from the literature if heat treatment causes inhibition of the proteolytic stage, but it is generally agreed that denatured whey proteins become associated with the casein micelles through hydrophobic interaction and disulphide bonding, and inhibit the aggregation phase (van Hooydonk et al., 1987). Various reports have shown, however, that increasing the concentration of serum Ca^{2+} of heated milks restores the rennet coagulation time and improves the gel strength. The level of serum Ca^{2+} can be increased by the addition of low concentrations of $CaCl_2$ (Marshall, 1986; Singh et al., 1988) or by reducing the pH of the heated milk to pH 5.5, and re-neutralising after holding for between 2 and 24h (van Hooydonk et al., 1987; Lucey et al., 1993).

6.2 Changes in the Caseins and Whey Proteins at Sterilisation Temperatures

On heating milk at temperatures up to 110°C for 5 min, the whey proteins were denatured but there were no marked changes in the electrophoretic patterns or ion-exchange elution profiles of the caseins. Above this temperature, however, there were changes in the electrophoretic and chromatographic properties of the caseins that were consistent with loss of negative charge, through dephosphorylation, and loss of positive charge through reaction of ϵ-amino groups of lysine to form Maillard products and lysinoalanine.

Similar modifications were observed in commercial sodium caseinates that had been obtained from different manufacturers. Closer examination of the sodium caseinates indicated that the severity of heating during the drying process was an important factor in causing changes in the caseinates; the degree of modification to the caseinates was roller-dried > spray-dried > laboratory freeze-dried. Because of the importance of sodium caseinates in a wide variety of food products, work is continuing to study the changes in the caseinates, especially in relation to their ability to stabilise cream liqueurs.

The modifications which occur in the caseins at high temperatures may also have a direct effect on the stability of milk on heating (Singh and Creamer, 1992), and on the age-thickening of concentrated milks (Harwalkar, 1992). Fox (1982) has indicated that the reduction in pH which occurs during heating, and which lowers the net charge on the caseins, may be the most important factor in promoting coagulation. Dephosphorylation of the caseins which occurs on heating milk could also lead to a considerable reduction in the net negative charge on the caseins and, therefore, promote aggregation of micelles. Conversely, various reagents such as aldehydes, ketones and urea, which are believed to react with ϵ-amino groups on the caseins, increase the heat stability of milk over a wide pH range (Holt *et al.*, 1978b; Muir and Sweetsur, 1976; 1977). In this study, changes were also found in the positive charge of the caseins that were believed to be due to reaction of amino groups to form Maillard products and lysinoalanine. These changes, therefore, may have a similar stabilising effect. Both anion- and cation-exchange FPLC could be used to examine the effect of alteration in the negative and positive charges of the caseins on heat stability.

6.3 Proteolysis During Cheese-Ripening

Anion- and cation- exchange FPLC were found to be useful quantitative methods for determining the extent of proteolysis of the major caseins during the ripening of Cheddar cheese. A combination of both methods gave the best results. Anion-exchange FPLC gave good resolution of residual β-casein, whereas cation-exchange FPLC gave good resolution of para-κ- and α_{s1}-caseins, and approximate levels of α_{s2}-caseins. The methods were rapid, and could be used with other techniques to resolve the original caseins and large peptides.

The ion-exchange FPLC methods were also versatile; they were used to examine proteolysis in cheese made from heated milk (Calvo *et al.*, 1992a,b,c), and from the milk of the goat and sheep. Previous studies showed that atypical flavours were produced in Cheddar cheese containing either native or denatured whey proteins, and that the whey proteins were not broken down during ripening. Using anion-exchange FPLC and PAGE in a study of the ripening of Cheddar cheese made from heated milk, Calvo *et al.* (1992b,c) confirmed that β-lactoglobulin was disulphide linked to the casein, and was not broken down during proteolysis. Differences were found in the breakdown pattern of the caseins, however, which may account for the atypical cheese flavour. In cheese made from pasteurised or overheated milk, the extent of proteolysis of α_{s1}-casein was greater than that of β-casein. In cheese made from heat-treated milk, as opposed to raw milk, there was a more marked initial decrease in α_{s1}-casein, and more extensive proteolysis of β-casein. In all cheeses the levels of both para-κ-casein and α_{s2}-casein decreased throughout the ripening period.

In Cheddar cheese, proteolysis of α_{s1}-casein is usually more extensive than that of β-casein. Compared to bovine casein, however, caprine and ovine caseins, contain more β-casein and much less α_{s1}-casein. The difference in composition is especially pronounced in caprine casein containing the D, E, F and null variants alleles of α_{s1}-casein which are associated with reduced amounts of α_{s1}-casein. Heil and Dumont (1993) found that high α_{s1}-content was associated with less intensity of goat flavour and firmer and smoother texture. The overall ripening pattern in cheese made from the milk of the goat or sheep, therefore, may be substantially different from that made from cows' milk, and requires further examination.

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Further detailed research into proteolysis during cheese ripening has considerable potential, and could ultimately lead to a reduction in the length of the maturation period, consistency in ripening, and the development of new flavours.

6.4 Effect of Heat-Treatment and Acidification on the Dissociation of Micelles

On heating milk at 85°C for 10 min and storing at 4, 20 or 30°C, there was a marked increase in the level of κ-casein in the serum; on average 27% of the κ-casein dissociated from the micelles. Several workers have reported similar levels of dissociation (Aoki et al., 1974, 1975; van Hooydonk et al., 1987), but others have indicated that the dissociation of κ-casein from micelles occurs more readily above pH 6.8-6.9 (Creamer et al., 1978; Singh and Fox, 1987a, b). In the present work and that of van Hooydonk et al. (1987), there were substantial amounts of κ-casein in the serum at pH 6.7, and some additional work showed that most of the dissociation of κ-casein occurred during heating or soon afterwards. If a similar degree of dissociation occurs on shorter heat treatments, the high levels of κ-casein in the serum could have pronounced effects on other properties such as the rennet coagulation time, or heat stability.

On acidification of heat-treated milk and storage, there was an increase in the concentrations of Ca and P_i in the serum with decreasing pH, so that at pH 5.0 all of the micellar Ca and P_i had been removed. At 30°C, however, the extent of dissociation of micellar caseins decreased with decreasing pH. The results show, therefore, that although calcium phosphate helps to maintain micellar structure, other forces must be involved. At 30°C, hydrophobic interaction would be important, but results from ^{1}H-NMR studies show a difference between the mobility of caseins in which Ca has been removed by pH adjustment, as opposed to removal by EDTA. It appears, therefore, that the simultaneous reduction in charge and increase in ionic strength, as calcium phosphate is removed by acidification, may promote specific electrostatic interactions of the micellar caseins and inhibit dissociation.

On acidification of heat-treated milk, compared with raw milk, there was an increase in the pH of maximum dissociation on storing at 20 or 4°C. This increase was

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attributed to the effect of denatured whey proteins interacting with the casein micelles. Similar interactions of denatured whey proteins during the production of cheese from heat-treated milk could affect renneting times, cheese ripening and the melting properties of the cheese. The methods described here could be used to investigate this further.

7. CONCLUSIONS

Quantitative methods of ion-exchange and gel permeation FPLC were developed to determine the content and composition of the caseins and whey proteins in the milk of the cow, goat and sheep. These methods were successfully used to study natural variation in the composition, and to examine changes in the proteins during processing.

There was considerable variation in the content and composition of the caseins and whey proteins within each species. This could be attributed to the combined effect of stage of lactation, season, and genetic polymorphism. Some of the variation in the caseins occurred because of proteolysis of β-casein to give the γ-caseins. There were also considerable differences between species in the concentrations and relative amounts of the individual caseins and whey proteins. In bulk milk, differences in the composition of whole casein from individual animals tended to be averaged out, and the composition varied within narrow limits. There were pronounced seasonal trends, however, in the concentrations of total and individual caseins and whey proteins.

The quantitative analytical methods were used to study changes that occur in micellar structure and stability during the various stages of milk processing, including cooling, acidification, heating, renneting and proteolysis in cheese ripening. The first part of this study gives detailed information about the level and composition of micellar and serum casein, the release of calcium phosphate from the micelles, and the relation between micellar size and composition.

In a study of the effect of temperature and holding time on the milk proteins, gel permeation was used to determine the extent of irreversible denaturation of the individual whey proteins. Results are presented on the kinetics of denaturation of the whey proteins in the milk of the cow, goat and sheep, and on the effect of pH on the level of denaturation of bovine whey proteins. Gel permeation was also used to show the relation between the extent of denaturation of the whey proteins and their incorporation into rennet curd during the manufacture of cheese from heated milk.

Anion- and cation-exchange FPLC were used to examine changes in the caseins when milk was heated above 110°C. These modifications were similar to those found in commercial sodium caseinates, and involved changes in negatively and positively charged residues of the caseins, consistent with dephosphorylation and reaction of

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amino groups to form Maillard products and lysinoalanine.

The extent of proteolysis of the individual caseins during the ripening of Cheddar cheese from raw and heated milk was examined by anion- and cation-exchange FPLC. A combination of the two methods gave quantitative information about the rate of proteolysis of the main bovine caseins, and could also be used to study maturation of cheese from the milk of the goat and sheep.

Finally, in a study of acid gelation of heat-treated milk, it was found that, on heating at functionalisation temperatures and then cooling, there were no marked changes in the concentrations of Ca and P_i in the serum, but there was a pronounced increase in the level of soluble κ-casein; on average 27% of the total κ-casein dissociated from the micelles. On acidification, colloidal Ca and P_i were extensively solubilised, and at 20°C, and especially at 4°C, dissociation of micellar caseins occurred. Compared with raw milk, however, maximum dissociation occurred at higher pH, and the overall levels of dissociation were reduced. These differences in heat-treated milks were attributed to interaction of denatured whey proteins with the casein micelles, promoting aggregation of the micelles as the pH was reduced. At 30°C, the level of dissociation of micellar casein decreased with decreasing pH, despite extensive removal of the colloidal Ca and P_i. This indicates that although binding of calcium phosphate to the caseins helps to maintain micellar structure, other interactions must be involved.

These studies showed that there was considerable natural variation in the composition of the milk proteins of the three species, and that changes and interactions of the proteins during processing were complex. Results indicated, however, that the detailed studies of the caseins and whey proteins led to a better understanding of the changes taking place, and could give substantial improvements in existing processing methods, and help in the development of novel products.

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