A COMPARATIVE STUDY OF THE CASEIN MICELLES OF CAPRINE, OVINE AND BOVINE MILKS

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ABSTRACT

Several different hypotheses of casein micelle structure have been proposed from the data obtained in the investigations carried out on micelles over the past twenty years. The present comparative study of caprine (goat) and ovine (sheep) casein micelle systems with that of the bovine was made in an attempt to demonstrate the validity of these hypotheses.

The casein contents, mineral levels, and Ca:phosphate ratios in the skim milks varied between species, but no significant differences in the Ca:phosphate ratios in the casein micelles or the sera were found. There were major differences between the electrophoretic patterns of the casein samples from the different species. Both caprine and ovine caseins contained two prominent bands in the β-casein region, while caprine casein also contained a smaller proportion of its casein in more mobile components (α_s-caseins) than the bovine. Three major α_s-caseins were observed in ovine casein, and one major α_s-casein in the majority of the caprine casein samples.

Bovine, caprine and ovine caseins, isolated from the whole milks, were separated into their major components by ion exchange chromatography. The κ-, β-, and α_s-caseins which were isolated accounted for 15%, 35% and 50%, respectively of bovine casein, 10%, 60% and 25%, respectively of caprine casein and 10%, 45% and 35%, respectively of ovine casein. The caprine and ovine caseins were identified as α_s-, β- and κ-type caseins by their chemical and physical characteristics.

The bovine, caprine and ovine κ-caseins were readily hydrolysed by rennin and were able to stabilize the Ca sensitive α_s- or β-caseins. Caprine β_1- and β_2-caseins which were present in equimolar amounts, had nearly identical amino acid compositions, and were similar to that of bovine β-casein A[2]. The β_1 component, however, contained an additional phosphate residue.

The temperature dependent Ca sensitivities and the temperature dependent polymerization of the caprine β-caseins were similar to that of bovine β-casein. However, β_1-casein

appeared to associate more easily than β_2-casein, despite its higher net negative charge. Viscosity measurements indicated that the conformation of the two caprine β-caseins was similar to that of bovine β-casein, both at 4°C and at 25°C.

The major caprine α_s-casein was more similar to the minor bovine α_{s3}-casein than to bovine α_{s1}-casein. This was demonstrated by their behaviour on gel electrophoresis with Mg buffers, Ca sensitivities, amino acid compositions and molecular weights.

The two ovine β-caseins were similar to each other, and to the caprine and bovine β-caseins. The relationship between the ovine β-caseins was similar to that of the caprine β-caseins. Two of the three major ovine α_s-caseins were isolated. These were both similar to each other, and to bovine α_{s1}-casein.

The properties of the α_s-caseins from the three species were more variable than those of the β-caseins, which in turn were more variable than the κ-caseins.

The casein micelles from caprine, ovine and bovine milks were all highly solvated and roughly spherical. Although their size distributions varied, the micelles were in each case composed of sub-units about 12 nm in diameter. Gel chromatography of sub-micellar casein aggregates from the three species indicated that they had similar hydrodynamic sizes and appeared to be in equilibrium with their component caseins.

The finding that similar sized sub-units are formed in the casein micelles in the milks of the various species, suggests that the differing proportions of the various component caseins must compensate for the differences observed in the properties of the caseins. It appears likely that not enough emphasis has been given to the role of β-casein in micelle structure, and β- and α_s-caseins may be inter-changeable.

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CHAPTER 1: GENERAL INTRODUCTION

Milk is a complex fluid secreted by the mammary gland of mammals. Its primary function is to supply the young suckling mammal with the nutrients, such as protein, lipid, carbohydrate, minerals and water that are necessary for growth and development. However, the milk of some species has also assumed an important rôle in supplying some of the nutritional requirements of humans.

The cow (*Bos taurus* and *Bos indicus*) is the most important dairy species, and to some extent, depending on geographical location, the domestic goat (*Capra hircus*), the water buffalo (*Bubalus bubalis*) and the domestic sheep (*Ovis aries*) are also important. This thesis is concerned with a comparison of the properties of milk from the cow (bovine), with milk from the goat (caprine) and sheep (ovine).

1.1 Composition of Milk

A typical composition of normal bovine milk is presented in Table 1.1. In addition to the major constituents included in Table 1.1, normal milk contains numerous enzymes, water-soluble and lipid-soluble vitamins, as well as nitrogenous compounds, gases and trace elements (Jenness and Patton, 1959). The gross composition of bovine milk can vary widely and is dependent on factors such as inherited variation (Jenness and Patton, 1959). Other factors include the plane of nutrition, specific composition of the feed ration, seasonal variations and stage of lactation. Infections of the udder, in particular sub-clinical mastitis have one of the greatest effects on the gross composition of milk. Although few data are available, many or all of the factors which may affect the composition of bovine milk are likely to affect the composition of milk from other species.

1.2 The Caseins

Casein comprises some 80% of the total protein in bovine milk (Table 1.1). These caseins, or phospho-proteins combine with calcium, magnesium, citrate and inorganic phosphate to form large spherical colloidal aggregates known

Table 1.1

The Composition of Typical Bovine Skim Milk

	Total conc.
Water	870 g/l
Milk fat	40 g/l
Casein	25 g/l
Whey proteins	6.1 g/l
Lactose	45-50 g/l
Calcium	34 mmol/l
Magnesium	5 mmol/l
Sodium	20 mmol/l
Potassium	41 mmol/l
Citrate	9 mmol/l
Inorganic phosphate	23 mmol/l
Chloride	28 mmol/l
pH 6.68-6.72 (25°C)	

as casein micelles, which can range in diameter from 40 nm to 300 nm (Nitschmann, 1949). These casein micelles are dispersed throughout a serum phase, and "carry" more calcium phosphate, necessary for growth of the young mammal, than would otherwise be soluble in the serum. A typical composition of the serum and micelle phases of bovine milk is shown in Table 1.2.

The caseins have loosely folded open structures (Herskovits, 1966) and as such are readily accessible to proteolytic enzymes, an important feature in their nutritional role. The whey proteins, lactose and other ionic constituents in milk are largely present in the serum phase in which the micelles are suspended (Table 1.2).

Casein micelles are remarkably stable against the effects of temperature, dilution and concentration of milk, and yet are rapidly destabilized by the action of rennin, a protease isolated from the abomasum of the calf. These properties are important factors in the processing of milk to products.

Table 1.2

Approximate Composition of the Serum and Micelle Phases of Typical Bovine Skim Milk at 20°C

	Serum	Micelles
Sodium	21.4 mmol/l	9 mmol/l
Potassium	42.5 "	28 "
Magnesium	3.8 "	16 "
Calcium	12.6 "	226 "
Citrate	9.3 "	5 "
Inorganic phosphate	13.3 "	110 "
Chloride	31.0 "	?
Lactose	5.6 g/l	?
Casein	?	250 g/l
Whey Proteins	6.7 "	?

Note: Data have been calculated (Pearce, 1972) using experimental results reported by Davies and White (1960). The figures for the micelle phase were found by difference and are less reliable. The micelle phase has been assumed to occupy 10% of the skim milk volume. The composition is the same as in Table 1.1.

The casein in the micelles falls into three major classes, κ-casein, β-casein and α_s-casein, which constitute some 15%, 30% and 50% respectively of the casein in bovine milk (Jenness, 1970). These casein fractions have their own characteristic properties.

The definitions applied to the various casein fractions in milk by the American Dairy Science Association are described below since these assume special significance

in considering the nomenclature of caseins from the milks of species other than bovine (Jenness et al., 1956; Brunner et al., 1960; Thompson et al., 1965; Rose et al., 1970).

Casein: "A heterogeneous group of phospho-proteins precipitated from skim milk at pH 4.6 and 20°C."
α_s-Casein: "That fraction of α-casein precipitated by Ca at 0-40°C, and stabilized by κ-casein in the presence of Ca."
κ-Casein: "The principal casein upon which rennin acts. It is soluble in the presence of 0.40 M Ca at pH 7.0 and 0-4°C, and can prevent α_s-casein from precipitating in the presence of Ca, through the formation of soluble micelle-type complexes" (stabilization).
β-Casein: "That fraction of casein soluble in 3.3 M urea, but insoluble in 1.7 M urea at pH 4.6. β-Caseins are precipitated with calcium at 35°C, but not at 4°C, and possess ultracentrifugal association-dissociation properties at 8.5°C."

For the purpose of this thesis, this definition needs updating to: "and possess temperature dependent association-dissociation properties between 4°C and 37°C."

1.3 Aim of the Investigation

Knowledge of the fundamental structure of the casein micelle is important in understanding the stability of casein micelles during the processing of milk into milk products, and in understanding the role of the casein micelle and the caseins in nutrition.

During the last twenty years there have been numerous studies to determine the way in which caseins associate to form casein micelles. These have included an examination of the behaviour of natural micelles and also synthetic micelle systems (Waugh, 1971). Although this work has revealed a considerable number of the fundamental properties of casein micelle systems, a universally accepted model of micelle structure has yet to be proposed.

The caseins of all mammalian species that have been examined to date are thought to exist in the form of casein micelles (Jenness, 1973). A study of the micelle systems

of other species should lead to a greater understanding of the fundamental factors affecting the structure of the bovine casein micelle, since this is analogous to examining the effects of modified bovine caseins on the properties of casein micelles. As a pre-requisite to an investigation of the association of the caseins from other species to form casein micelles, it is necessary to determine their chemical and physical properties. The recent elucidation of the primary structures of the major bovine caseins has added impetus to investigations on the caseins of other species.

A knowledge of the primary structure and physical characteristics of caseins from other species will help in tracing the pattern of changes to the milk proteins, in particular the caseins, during evolution. Consequently, those caseins and regions of the casein molecules which have an important role in micelle structure and nutrition may be identified. To date, very little work has been done on the caseins of other species.

The aim of this thesis is to compare some of the fundamental properties of caprine and ovine milks with bovine milk. The composition of the milks, and some of the properties of the caprine, ovine and bovine casein micelles are compared, using electron microscopy, ultracentrifugation, viscometry and gel chromatography. Particular emphasis is given to the isolation and characterization of the major caprine and ovine caseins, and a comparison of their chemical and physical characteristics with the bovine caseins. These species were chosen for investigation since they are fairly closely related ruminant species, belonging to the family Bovidae in the order Artiodactyla (Sloan et al., 1961). Consequently, a comparison of their caseins should be of more value, than say, with those of the order Rodentia.

The thesis work is divided into three sections.

In Part I, the compositions of caprine, ovine and bovine milk are described. Some of the properties of the casein micelles from these species are also described and compared.

In Part II, the isolation of the major caprine, ovine and bovine caseins are described. Some of their chemical and physical characteristics are compared and contrasted with those of other species.

Part III briefly outlines some of the models of casein micelle structure which have been proposed. The results obtained in this thesis are discussed in the light of their application to these models.

PART I

A COMPARISON OF CAPRINE, OVINE AND BOVINE MILKS AND THEIR CASEIN MICELLES

CHAPTER 2: INTRODUCTION

In this chapter, only published studies which are directly relevant to the work presented in the following three chapters will be briefly reviewed. Other properties of casein micelles, and the various models of micelle structure are presented and discussed in Chapter 13.

2.1 Composition of Milk

In Chapter 1, typical compositions of bovine milk (Table 1.1), milk serum and casein micelles (Table 1.2) were presented. Mention was also made of some of the variables affecting the composition of bovine milk. Reference to any of the standard texts in dairy chemistry (Jenness and Patton, 1959; McKenzie, 1970; McKenzie, 1971) will provide data on the composition of bovine milk. Also, Rook and Wheelock (1967) have reviewed the literature on the water soluble constituents in bovine milk.

Data on the milks of other species is more varied in nature and scattered throughout the literature. Nejim (1963) determined the protein, fat, ash, calcium and phosphorus contents of ovine milk during a seasonal study. Djordjevic and Caric (1972) examined the distribution of calcium in ovine milk during a seasonal study.

Parkash and Jenness (1968) have comprehensively reviewed the literature on the composition and characteristics of caprine milk. Konar et al. (1971) compared the concentrations of water soluble constituents of bovine, porcine (pig), ovine and caprine milks. Major variations were observed in citric acid, chloride and potassium, with similar sodium and lactose contents. Macy et al. (1953) reviewed the literature and compiled data on the composition of human colostrum, and mature human, caprine and bovine milks.

The concentration of the individual constituents in milk are subject to considerable variation, even within each species. This can be partly attributed to whole animal effects, such as breed, state of health, plane of nutrition etc., but can also be markedly affected by variations, due to the accuracy of different analytical procedures. This is

illustrated by the range of values obtained for the citric acid content of the same milk samples, analysed using different procedures (White and Davies, 1963), and in the results of different investigations which were tabulated by Macy et al. (1953) for the constituents of caprine, human and bovine milks. However, determining the concentration of the various constituents in caprine, ovine and bovine milks using the same procedure, should provide a better basis for comparison.

2.2 Size Distribution of Casein Micelles

The size distribution of casein micelles in milk has been the subject of a number of investigations. With the exception of the recent investigations (Lin et al., 1971; Dewan and Bloomfield, 1973; Holt et al., 1973), these investigations were carried out by means of electron microscopy (Nitschmann, 1949; Knoop and Wortmann, 1960; Shimmin and Hill, 1964; Shimmin and Hill, 1965; Rose and Colvin, 1966; Calapaj, 1968; Carroll et al., 1968; Parry and Carroll, 1969; Saito, 1973; Schmidt et al., 1973).

Electron microscopy revealed that casein micelles are roughly spherical in shape and are strongly polydispersed (Nitschmann, 1949). The size distribution of casein micelles observed by Nitschmann (1949) and Rose and Colvin (1966) were two or three times lower than the values of 90-170 nm obtained by more recent measurements (Knoop and Wortmann, 1960; Calapaj, 1968; Carroll et al., 1968; Parry and Carroll, 1969; Saito, 1973).

Knoop and Wortmann (1960), using an embedding technique, obtained average micelle diameters for human and bovine milk and caprine colostrum of 42 nm, 93 nm and 133 nm, respectively. Later, Calapaj (1968) obtained an average diameter for human casein micelles of 65 nm. Schmidt et al. (1973) suggested that the micelle sizes obtained by Knoop and Wortmann (1960) may be uncertain since embedding of thin sections in methacrylate is accompanied by considerable shrinkage.

Most of the techniques used for determining the size distribution of casein micelles on electron migrographs have involved shadowing of the dried samples of milk with heavy metals such as platinum (Calapaj, 1968; Carroll et al., 1968; Parry and Carroll, 1969; Saito, 1973). Recently, Schmidt et al. (1973, 1974) obtained values for the average light-scattering diameter of unfractionated casein micelles from electron micrographs (prepared using a freeze-etching technique) and from light-scattering measurements of 236 nm and 450 nm, respectively. According to Schmidt et al. (1974) a light-scattering average diameter for casein micelles from electron micrographs of 236 nm is equivalent to a weight-average value of the diameter of 128 nm.

Lin et al. (1971) studied centrifugally fractionated micelles in solution by means of inelastic light scattering. They found 80% of the casein micelles had diameters of 100-200 nm, with a most probable diameter of 160 nm (Bloomfield and Morr, 1973). Holt et al. (1973), using the technique of clipped digital autocorrelation of scattered laser light, found casein micelle sizes similar to those found in recent electron microscopic (Carroll et al., 1968; Parry and Carroll, 1969; Saito, 1973) and inelastic light scattering studies (Lin et al., 1971), however the distributions were broader.

Electron microscopy of casein micelles revealed they are composed of roughly spherical sub-units, 10-20 nm in size (Shimmin and Hill, 1964; Shimmin and Hill, 1965; Schmidt and Buchheim, 1970; Knoop et al., 1973; Schmidt et al., 1973; Buchheim and Welsch, 1973). These sub-units are readily evident in electron micrographs prepared using the freeze-etch technique (Schmidt and Buchheim, 1970; Buchheim and Welsch, 1973; Schmidt et al., 1973) or the thin section technique (Knoop et al., 1973). According to Knoop et al., (1973) casein micelles are loosely constructed in the regions in which calcium is located, which they suggested are the interstices between the sub-units. The diameter of the sub-units was 15-20 nm and the width of the "canals" between them were about 3 nm.

2.3 Molecular Weight of Casein Micelles

From their inelastic light scattering studies on bovine casein micelles, Morr et al. (1973), Bloomfield and Morr (1973), Dewan and Bloomfield (1973) have determined the molecular weight of micelle fractions. Two fractions isolated by rate-zone ultracentrifugation, and representing small and large micelles were found to have molecular weights of 2.3×10^8 and 18×10^8, respectively. The average molecular weight of unfractionated casein micelles was found to be 5.20×10^8. From their light scattering studies Schmidt et al. (1974) obtained molecular weights of 11.0×10^8 and 1.3×10^8-3.6×10^8 for unfractionated and fractionated casein micelles, respectively. Nitschmann (1949) obtained a molecular weight of 5.56×10^8 for casein micelles, assuming a micelle diameter of 153 nm. This value was somewhat lower than that obtained for large casein micelles by Morr et al. (1973).

2.4 Voluminosity and Solvation of Casein Micelles

The degree of solvation and voluminosity (specific volume) of casein micelles are important characteristics, since micelles appear to be large porous structures which contain a considerable amount of water. Solvation is defined as the weight of water (g) bound or associated with 1 g of protein, while the voluminosity (V) is defined as the volume (ml) occupied by 1 g of dry casein micelle material.

The water can either be "tightly bound" to the casein in the micelles, or "loosely bound", occupying the holes or interstices in the interior of the micelles, and loosely associated with the exterior surface. About 0.5 g water per g dry casein micelle is "tightly bound" (Waugh, 1971), while the remainder (about 1.5 g water per g of dry casein micelle) is "loosely" associated with the casein micelle.

The degree of solvation and voluminosity of casein micelles can be determined by ultracentrifugation or viscometry. The water and protein content of micelles sedimented by ultracentrifugation was used by Thompson et al. (1969a) to determine the casein-pellet solvation.

Voluminosity and solvation of casein micelles were also measured by viscometry (Whitnah and Rutz, 1959; Dewan et al., 1973).

Casein-pellet solvation values, obtained by ultracentrifugation of bovine skim milk at 37°C, were 1.90 g water per g micelle pellet (Thompson et al., 1969a). Casein-pellet solvation values were correlated with heat stability of milk by Thompson et al. (1969a), more highly solvated casein micelles being more heat stable.

Viscosity measurements resulted in values for the voluminosity and solvation of bovine casein micelles of 4.37±0.27 ml/g micelles and 3.67 g water per g protein, respectively (Dewan et al., 1973). Micelle solvation as measured by viscometry (Dewan et al., 1973) was considerably higher than that measured by ultracentrifugation (Thompson et al., 1969a).

2.5 Temperature Dependent Dissociation of Casein Micelles

The structure of casein micelles can be investigated under conditions of controlled dissociation. Casein micelles may be dissociated by low temperature (Downey and Murphy, 1970), high pressures (Schmidt and Buchheim, 1970), high concentrations of urea (Morr, 1967a), removal of calcium phosphate by dialysis (Pyne and McGann, 1960; McGann and Pyne, 1960), gel filtration (Boulet et al., 1970; Creamer and Berry, 1975), Pyrophosphate and citrate (Morr, 1967b) or EDTA (Lin et al., 1972).

The temperature dependent dissociation of casein micelles has been investigated by ultracentrifugation (Rose, 1968; Downey and Murphy, 1970) or gel chromatography (Downey and Murphy, 1970; Creamer and Berry, 1975).

Sullivan et al. (1955) suggested that β-casein was dissociated from casein micelles on cooling. This was verified by Rose (1969) who showed β-casein constituted some 55% of the increase in serum casein, obtained by storing milk overnight at 4°C prior to centrifugation. Downey and Murphy (1970) found the total casein in supernatants prepared by

centrifugation increased from 6% to 15%, on cooling the milks from 30°C to 5°C. β-Casein accounted for 46% of this increase, while α_s- and κ-caseins constituted 30% and 23%, respectively of this casein.

Gel chromatography of samples of bovine milk on a column of Sepharose 2B in synthetic milk serum at 25°C indicated the presence of two peaks (Downey and Murphy, 1970). The first opaque peak, which was eluted at the void volume, contained the caseins, apparently in micellar form (molecular weight $>10^8$), and a second peak eluted at a volume corresponding to molecular weight $2x10^5$, contained the whey proteins. On decreasing the temperature to 5°C, about 60% of the β-casein in the casein micelles was eluted with the whey proteins. Chromatography of CPF-milk (colloidal phosphate free milk), resulted in two major peaks, one corresponding to a molecular weight of $2x10^6$, and the other resembling the peak observed with skim milk, which contained the whey proteins.

At 5°C, casein micelles in normal milk which have been chromatographed on Sepharose 2B have about 90% of their α_s- and κ-caseins, and 40% of the β-casein eluted at the void volume (Downey and Murphy, 1970). Further gel filtration of this micellar casein peak resulted in further dissociation, until chromatography for a fourth time resulted in a fraction containing 8.0:1.2:0.8 parts of α_{s1}-, β- and κ-caseins, respectively (Downey, 1973). The material in this peak was precipitated with 10 mM $CaCl_2$, whereas normal milk is unaffected by 1 M $CaCl_2$.

Creamer and Berry (1975) examined the behaviour of sub-micellar casein aggregates freed of colloidal calcium phosphate and most protein bourd Ca, by chromatography of casein micelles consecutively on columns of Sephadex G-10 and Sepharose 4B. From their results, they concluded that the individual caseins in the sub-micellar casein aggregates were in equilibrium with their constituent caseins.

CHAPTER 3: EXPERIMENTAL

3.1 Preparation of Skim Milks

Samples of bulk and individual bovine milks were obtained from cows of the Massey University No.2 twin Friesian herd and skimmed at 30°C using a separator.

Samples of individual or bulk caprine milks were obtained from British Saanen milking goats. Skim milk was prepared by centrifugation at 800 g for 10 min at 30°C, or with a commercial separator, depending on the quantity of milk available.

Ovine milk samples were obtained from Romney ewes of the Massey University flock. Some milk samples were also obtained from other breeds. The milks were bulked or kept as individual samples and the milks skimmed by centrifugation at 800 g for 10 min at 30°C. Skim milk was removed from under the cream layer using a pipette.

MILK COMPOSITION

Although there are a number of constituents which could have been compared, emphasis is placed on those which may be important to the integrity of the casein micelles.

3.2 Nitrogen Distribution

Total nitrogen (TN), non casein nitrogen (NCN), and non protein nitrogen (NPN) contents of the milks were determined by Kjeldahl digestion using the procedure of Rowland (1938), as modified by Aschaffenburg and Drewry (1959). NCN was determined in filtrates of skim milk which had been acidified to pH 4.6. NPN was measured in 12.5% trichloroacetic acid (TCA) filtrates of skim milk. Casein nitrogen (CN) was determined as:

$$CN = TN - NCN$$

Protein was 6.38 x nitrogen concentration

3.3 Inorganic Phosphate Determination

Samples were de-proteinised with TCA (final concentration, 12.5% w/v) and their phosphate content determined by the method of Chen et al. (1956), in which the phosphate is converted into a blue phosphomolybdate complex which was estimated by absorbance at 820 nm. Standards were prepared from dried AnalaR grade potassium dihydrogen phosphate.

3.4 Determination of Cation Concentrations

After the skim milk was diluted, Na and K were determined by flame emission spectroscopy. Mg was determined by atomic absorption spectroscopy using a Zeiss PMQII spectrophotometer fitted with an FA2 flame attachment and a Sargent SRL recorder. An air acetylene flame was used.

Ca plus Mg was determined in suitably diluted samples which were titrated with EDTA using calcon indicator (British Drug Houses). $CaCO_3$ dissolved in dilute HCl was used as a standard.

3.5 Cation Activity

A sample of skim milk was equilibrated with a portion of strong cation exchange resin which had been pre-equilibrated with a solution of salts having approximately the same activities as the solution under test (Pearce and Creamer, 1974). After equilibration the resin was washed, dried and later eluted with 3 M HCl. The eluent was analysed by flame photometry to give the resin composition. Hence, by use of calibrating solutions, the cation activity ratios were calculated, using K as the reference ion. From the concentration of K, the cation concentration quotient was obtained:

$$\text{e.g.} \quad \frac{a_{Ca}}{\gamma_K^2} = \frac{a_{Ca}}{a_K^2} \cdot (K)^2$$

where a_K, γ_K and (K) are the activity, activity coefficient and concentration of K respectively, and a_{Ca} is the Ca ion activity (Pearce and Creamer, 1974).

3.6 Citric Acid Content

Citric acid was measured in TCA filtrates (12.5% w/v) of milks using the method of Marier and Boulet (1958), as modified by White and Davies (1963).

3.7 Electrophoresis

Casein samples were precipitated from milks with acid and examined by gel electrophoresis using the procedures outlined in Chapter 7.6.

PROPERTIES OF CASEIN MICELLES

In this section the procedures used to examine the properties of casein micelles by electron microscopy, ultracentrifugation, viscometry and gel chromatography are described.

3.8 Freeze-etch Electron Micrographs

These were prepared by taking milk from beneath the cream layer, adding glycerol to 30% v/v, freezing it onto a specimen holder using liquid Freon 22, cutting the frozen droplet under vacuum and depositing a layer of carbon-platinum on to the lightly etched surface. After mounting the replicas on to appropriate grids they were examined with a Philips EM 200 electron microscope.

3.9 Whole Micelle Electron Micrographs

These were prepared by 100-fold dilution of the milks with 0.01 M $CaCl_2$. Further samples (30-fold dilution) were also fixed with 2% (v/v) formaldehyde solution for 30 minutes. These solutions were sprayed on to collodion coated copper grids and air dried. Some were shadowed with platinum-carbon and they were examined using a Philips EM 200 electron microscope.

Voluminosity and Solvation of Casein Micelles

The voluminosity and solvation of casein micelles, defined in Chapter 2.4, were measured by ultracentrifugation

and viscometry measurements on caprine, ovine and bovine milks. The water and protein content of pelleted micelles prepared by ultracentrifugation of milk samples were used to determine the casein-pellet solvation. Viscosity measurements on the milks and diluted milks, relative to ultracentrifuge serum were used to determine the voluminosity, and hence the solvation of casein micelles. The casein-pellet solvation (measured by ultracentrifugation) is not necessarily the same as the casein micelle solvation, measured by viscometry (Chapter 5.2).

3.10 Solvation of Casein Micelles: Ultracentrifugation

Casein-pellet solvation was determined by ultracentrifugation of skim milk using a procedure similar to that previously described by Waugh and Noble (1965).

Weighed aliquots of skim milk were centrifuged at 30±1°C in a Beckman SW 56 rotor using a Beckman L2-65 ultracentrifuge for periods of 20 minutes to 3 hours at 330 000 g. Casein-pellet solvation was also determined at 4°C and 30°C by centrifugation in a Type 65 rotor at 102 000 g for 60 minutes.

After centrifugation, the serum was carefully poured off and the tube and wet pellet weighed. After freeze drying, the tube and pellet were again weighed, the loss of weight on drying being the weight of water in the pellet.

Casein-pellet solvation (g water per g dry micelle pellet) was estimated from:

$$\text{casein-pellet solvation} = \frac{w_w - w_d}{w_d - w_t}$$

where w_w, w_d and w_t, are the weights of tube and wet pellet, tube and dry pellet, and tube, respectively.

The distribution of cations and inorganic phosphorus between the serum and micelle phases was determined by

analysing the serum and micelle pellets, after weighed aliquots of the pellets had been re-dissolved.

3.11 Solvation of Casein Micelles: Viscosity Measurements

Preparation of Samples

Samples of fresh skim milk were centrifuged at 1500 g for 15 min at 30°C. Streptopen (1 drop/500 ml) (Glaxo Laboratories, 10^5 units Streptomycin and 10^5 units penicillin per tube) was added to each milk sample to prevent bacterial growth. The samples of milk were equilibrated at 4°C and 30°C prior to viscosity measurements. Serum used to dilute the milk samples was prepared by centrifuging milk at 4°C or 30°C in a Beckman L2-65 ultracentrifuge for 1 h at 102 000 g to sediment the casein micelles. Aliquots of milk were diluted 25%, 50% and 75% by weight with 4°C or 30°C ultracentrifuge serum, and then equilibrated at 4°C or 30°C, prior to viscosity measurements.

The concentration of micellar casein in the milks was determined from the TN and NCN contents of the pelleted micelles which were obtained during the preparation of the ultracentrifuge sera. The relative densities (relative to water) of milks and ultracentrifuge sera were measured at 20°C using a 10 ml or 25 ml pycnometer. Relative densities of the diluted milk samples were obtained by interpolation of the serum and milk values.

It was considered necessary to complete all of the measurements within 12 h of the milk samples being collected. Consequently, this reduced the number of dilutions of milk which could be used to determine the intrinsic viscosity of the casein micelles.

Viscosity Measurements

The relative viscosities of milk and diluted milk samples, measured relative to ultracentrifuge serum at 4 ± 0.02°C and 30 ± 0.1°C, using Cannon Manning Semi Micro Capillary viscometers (Cannon Instrument Co., Pennsylvannia). The type 75 and type 50 viscometers were used at 4°C and 30°C, respectively, giving flow times for 1 ml samples from 200 to 400 sec.

The samples of milk, diluted milk and ultracentrifuge serum were filtered through medium porosity sintered glass filters into the viscometers and equilibrated for 10 min at 4°C or 30°C. Flow times for each sample were an average of at least three measurements, none of which was more than ±0.1 sec different from the mean. After each run the viscometers were rinsed with water, washed with chromic acid, rinsed again with deionised water and acetone, and finally dried in a stream of dry compressed air. Unless extreme care was taken in the preparation of samples for viscosity measurements, reproducible results were difficult to obtain, presumably due to the presence of dust particles and large fat globules.

Treatment of Data

The flow times, relative densities and concentrations of micellar casein were used to determine the reduced viscosity ($\eta sp/c$) of each sample from the expression:

$$\frac{\eta sp}{c} = \frac{1}{c} \cdot \frac{dt - d_o t_o}{d_o t_o}$$

where d and d_o are the relative densities, and t and t_o are the flow times of sample and ultracentrifuge serum, respectively.

The intrinsic viscosity, $[\eta]$, is related to the reduced viscosity by the Huggin's equation (Bradbury, 1970):

$$\frac{\eta sp}{c} = [\eta] + k' [\eta]^2 c$$

where k' is the Huggin's constant. The intrinsic viscosities of the casein micelles from caprine, ovine and bovine milks were determined by linear least squares analysis of a plot of reduced viscosity versus the concentration of casein in the micelles.

If it is assumed that the casein micelles are rigid, non-interacting spherical particles in solution, then it can be shown that the voluminosity, V, is related to the intrinsic viscosity by:

$$V = [\eta]/2.5$$

The derivation of this formula has been discussed in some detail by Dewan et al. (1975).

The degree of solvation of casein micelles (δ_1) can then be determined using the approximation of Tanford (1961) where:

$$V = \bar{v}_2 + \delta_1 v_1^o$$

and $\bar{v}_2$ is the partial specific volume of dry casein micelles and v_1^o is the specific volume of pure water, taken as 1.00 ml/g. The partial specific volume of dry casein was taken as 0.7 ml/g (Ford et al., 1959).

3.12 Temperature Dependent Dissociation of Casein Micelles

Casein micelles were sedimented from caprine, ovine and bovine milks by centrifugation at different temperatures and the soluble caseins present in these sera were quantitatively examined by gel electrophoresis.

Skim milk samples were equilibrated at 4°C, 20°C or 30°C for 1 h prior to centrifugation in a Beckman Type 65 rotor at 102 000 g for 1 h in a Beckman L2-65 ultracentrifuge. Four ml samples of serum were removed and acidified (Chapter 7.1), centrifuged for 15 min at 27 000 g and the supernatant removed and the precipitate (casein) redissolved in 10 ml 4.5 M urea solution containing 0.1% (v/v) 2-mercaptoethanol. Samples of milk (4 ml for bovine caprine, and 2 ml for ovine) were precipitated with acid and the caseins redissolved in 25 ml of 4.5 M urea containing 0.1% (v/v) 2-mercaptoethanol. Aliquots (0.05-0.200 ml) of these samples were examined by vertical polyacrylamide gel electrophoresis at pH 8.4 (Chapter 7.6). After staining and destaining the gels, they

were cut longitudinally and the patterns scanned using a Canalco Model G densitometer. The concentration of each soluble casein present in the sera was determined from the peak areas in the densitometer patterns, and the volume of the sample analysed. The concentrations of the caseins relative to that in whole casein were calculated from the area under each peak. No allowances were made for any differences in dye binding between the caseins.

3.13 Gel Chromatography of Casein Micelles at 6°C and 37°C

The milk and micelle fractions from the three species were examined by chromatography on a column of Sepharose 4B (Pharmacia) using the procedure described by Creamer and Berry (1975).

Three drops of streptopen and 10 mg soybean trypsin inhibitor (Sigma) per litre were added to skim milk to prevent bacterial contamination and possible enzymatic degradation of the caseins. Micelle fractions were prepared by centrifugation of skim milk in a Type 65 rotor at 57 000 g for 30 min in a Beckman L2-65 ultracentrifuge at 30°C. The upper mobile layer of small micelles was removed, and in the case of bovine and caprine milks they were recentrifuged at 102 000 g for 30 min at 30°C. The large and small micelle fractions were gently redispersed in water to give a protein concentration of approximately 5%.

The samples were chromatographed at 6°C and 37°C on a column (2.5 cm x 50 cm) consisting of an upper layer of Sephadex G-10 (Pharmacia) (2.5 cm x 16 cm) used to dissociate the casein micelles to sub-micellar casein aggregates, followed by chromatography on a lower layer (2.5 cm x 23 cm) of Sepharose 4B.

The column was equilibrated at 6°C or 37°C with several bed volumes of 0.025 M imidazole-HCl buffer pH 6.7 containing 0.1 M NaCl and 0.02% NaN_3. The pH of the buffer was adjusted at the temperature used for the chromatography. The flow rate of the column was maintained at approximately

40 ml/h at 37°C and 12 ml/h at 6°C. Fractions of approximately 3 g were collected and weighed. The absorbance of the fractions was measured at 280 nm, and used in the determination of the elution weight of the peak fractions. Some fractions were diluted with 9 M urea containing 0.1% (v/v) 2-mercaptoethanol and the proteins examined by polyacrylamide gel electrophoresis (Chapter 7.6). After destaining the gels, the protein patterns were scanned with a densitometer, and the quantity of each casein determined from the peak areas.

CHAPTER 4: COMPOSITION OF CAPRINE, OVINE AND BOVINE MILKS

The composition of caprine, ovine and bovine skim milk samples were examined on several occasions. Ovine and caprine milk samples were obtained from 6 and 12 animals, respectively, while bulk milk was obtained from a predominantly Friesian herd of cows. These milks were analysed as soon as possible after collection and the maximum time between collection and examination was 8 h for electron micrographs.

4.1 Nitrogen, Inorganic Phosphorus and Citric Acid Contents

TN, NCN and NPN contents in caprine, ovine and bovine milks were determined by Kjeldahl analysis (Table 4.1). The levels of inorganic phosphorus and citric acid found in 12.5% TCA filtrates of ovine, caprine and bovine milks are also shown in Table 4.1. TN, NCN and CN contents were fairly similar in caprine and bovine milks, although bovine milk had a slightly higher level of both TN and CN (Table 4.1). The higher level of TN in ovine milk, compared with bovine and caprine milks, is caused almost entirely by the high CN content. Protein contents were 2.3, 4.6 and 2.8 g casein/100 ml milk in caprine, ovine and bovine milks, respectively.

Macy et al. (1953) compiled the results from a number of studies which indicated an average composition for mature caprine milk of 0.558% TN, 0.166% NCN, 0.392% CN and 0.040% NPN, values which are slightly higher than those in Table 4.1. Knowles and Watkins (1938), whose results for caprine milk were not included in the review by Macy et al. (1953), were similar to those in Table 4.1 with values of 0.522% TN, 0.386% CN and 0.069% NPN.

Caprine milk from British Saanen goats is known to have a lower solids not fat content than other breeds (Parkash and Jenness, 1968), which may partly explain the lower levels of nitrogen (Table 4.1).

The nitrogen values obtained during a seasonal study of bovine milk (Table 4.1) were similar to those compiled by

Table 4.1

Composition of Milks

	Caprine		Ovine		Bovine	
	Mean	S.D.[1]	Mean	S.D.[1]	Mean	S.D.[1]
Total nitrogen g/100 ml	0.488	0.055	0.914	0.044	0.561[2]	0.048
Non casein nitrogen g/100 ml	0.130	0.029	0.189	0.008	0.121[2]	0.008
Non protein nitrogen g/100 ml	0.051	0.006	0.058	0.002	0.033[2]	0.006
Casein nitrogen g/100 ml	0.357	0.027	0.725	0.039	0.442[2]	0.044
Inorganic phosphorus mM/l	21.8	1.3	34.0	2.7	21.7	3.0
Citrate mM/l	6.69		9.77		9.6	

1. Standard deviation.
2. Results obtained during a seasonal survey of Friesian herd milk by K Pearce (1973).

Macy et al. (1953) who reported 0.550% TN, 0.110% NCN, 0.440% CN and 0.032% NPN. The NPN content of caprine and ovine milks are higher, compared with the value of 0.032% NPN obtained for bovine milk by Macy et al. (1953).

There was a similar concentration of inorganic phosphorus in caprine and bovine milks (21.8 mM and 21.7 mM, respectively), contrasted by the considerably higher level of 34.0 mM in ovine milk (Table 4.1). Bosworth and Van Slyke (1916) found 23.2-24.1 mM inorganic phosphorus in caprine milk, with one exception of 34.0 mM. These results are in good agreement with those shown in Table 4.1.

The citric acid content of caprine, ovine and bovine milks (Table 4.1) can be compared with those of Konar et al. (1971) who found 4.2, 10.7 and 11.4 mM citric acid/kg milk water in caprine, ovine and bovine milks, respectively. Macy et al. (1953) reported levels of citric acid of 7.9 and 11.7 mM in caprine and bovine milks, respectively. The citric acid levels in Table 4.1 for caprine, ovine and bovine milks are lower than those of Konar et al. (1971).

However, the citric acid content of the milks is difficult to determine with any accuracy (White and Davies, 1963), since the results obtained depend on the method used, and this may have contributed to the differences between the present results (Table 4.1) and those of Konar et al. (1971).

4.2 Cation Concentrations and Activities

The total cation concentrations in samples of ovine and caprine milk are given in Table 4.2 together with similar data for bulk factory supply bovine milk. The Na levels for the mid-lactational ovine and caprine milks were comparable at 11-14 mM. The late lactational ovine milk and the bovine milk were also comparable at 17-20 mM. Konar et al. (1971) reported average values of 21.6, 20.3 and 17.8 mmoles/kg milk water for ovine, caprine and bovine milk. These values are appreciably higher for ovine and caprine milk than the present results.

Table 4.2

Concentrations and Activities of Cations in Skimmed Milk from Animals of Several Species

Species	No. of samples pooled	Sample date	Weeks of lactation	Milk pH	Concentration, mM K	Na	Mg	Ca	Activity Quotients, mM Na	Mg	Ca
Bovine herd milk (Pearce, 1972)				6.72	39	20	5.0	34	16	0.65	1.63
Ovine	8	5/12/72	20	6.52	38.6	17.4	6.88	46.0			
	10	12/12/72	21	6.57	37.9	18.4	8.13	53.9	48.7	0.80	1.49
	1	14/12/72	21	6.47	37.0	19.4	8.10	56.3	22.6	1.02	1.74
	5	21/ 9/73	4	6.61	35.1	13.3	7.33	52.9	21.6	0.80	1.61
	5	27/ 9/73	5	6.64	36.7	11.6	6.59	49.8	13.8	0.72	1.47
	6	4/10/73	6	6.66	39.5	12.9	6.01	45.1	14.1	0.59	1.22
									Average	0.79	1.51
Caprine	1	15/12/71	-	6.90	48.1	13.6	3.76	27.0	14	0.66	1.62
	15	14/12/72	20	6.81	56.2	13.7	4.52	30.8			
	15	26/ 9/73	3	6.76	50.9	11.2	4.50	30.2	13.6	0.86	1.88
	10	1/10/73	4	6.72	50.0	11.7	4.45	29.2	14.4	0.71	1.51
									Average	0.74	1.67

The K levels in ovine and bovine milk were comparable at 36-40 mM while in the caprine milk it was slightly higher at 50 mM. However, Konar et al. (1971) reported values of 50, 71 and 61 mmoles/kg milk water for K in ovine, caprine and bovine milk respectively. As the value they reported for bovine milk was high (Rook and Campling, 1965, report ca. 40 mmoles/kg milk water) it is possible that the values reported for ovine and caprine milk were also too high.

The Ca contents of the caprine and bovine milk were similar and near 30 mM. However, ovine milk Ca content was near 50 mM. Ovine milk contains some 6% protein while bovine and caprine milk contain ca. 3.4% protein (Table 4.1). The micelle pellets from ovine milk were twice the weight of those from bovine or caprine milks (Table 4.3). As Ca is bound by casein, it is likely that the higher Ca content of ovine milk is a consequence of its higher casein content.

The Mg levels follow a similar trend to the Ca levels being highest in the ovine milk (6-8 mM) and lowest in the caprine milk (4.5 mM).

The average Ca concentration quotients $({}^{a}Ca)/(\gamma^{2}K)$ are similar for the three milks (Table 4.2). If γ_K is assumed to be 0.785 for the three milks, the average Ca ion activities are 1.01, 0.93 and 1.03 mM for bovine, ovine and caprine milk respectively.

The Mg concentration quotients are more varied, with the bovine milk giving the lowest result. The ratio of total Mg to total Ca (0.11-0.14) is lower than the ratio of the concentration quotients (0.40-0.52). This is probably caused by the stronger associations between casein, citrate, phosphate and Ca than between these anions and Mg (Pearce, 1972).

Bosworth and Van Slyke (1916) and Kondo and Mori (1932) (see review by Parkash and Jenness, 1968) obtained levels for Ca, Mg, Na and K in caprine milk of 28.6-40.7 mM, 8.5-15.3 mM, 15.3-18.2 mM and 42.4-53.3 mM respectively. These concentrations of Ca and K are similar to those of the present

study (Table 4.2), whereas the earlier results for Mg and Na were considerably higher.

Djordjevic and Caric (1972) during a seasonal study obtained an average value of 51.0 mM Ca in ovine milk, which is comparable to the results of this study (Table 4.2).

4.3 Electrophoresis of the Caseins

Casein samples prepared from the milks of the three species, gave different electrophoretic patterns (Fig.4.1). The bovine casein sample contained α_{s1}-casein B and β-casein B, with approximately equal stain intensity, and a number of minor bands. The ovine casein contained four major bands in two regions which have been designated as α_s- and β-casein (Sloan et al., 1961; O'Connor and Fox, 1973). The caprine casein components were similarly labelled, although there were marked intensity differences. A detailed analysis of the physical and chemical properties of these proteins has shown this classification (Fig.4.1) to be correct (this thesis, Part II). The caprine, ovine and bovine β-caseins all have similar electrophoretic mobilities in alkaline gels. In contrast, under these conditions the order of mobility of the α_s-caseins was bovine > ovine > caprine. It is interesting to note that some of the minor components, particularly those with lower electrophoretic mobilities than the β-caseins were similar in the three species. It is evident from Fig.4.1 that the proportion and nature of the caseins varies considerably with the different species.

A COMPARISON OF THE PROPERTIES OF THE CASEIN MICELLES

4.4 Electron Micrographs of Intact Micelles

Typical electron micrographs of the platinum shadowed micelles of bovine, ovine and caprine milk are shown in Fig.4.2. It is clear that there are more small micelles in ovine and caprine milk than in bovine milk. A characteristic of the caprine milk was the occurrence of a few large micelles, many of these were larger than any bovine micelles. Formaldehyde treated micelles showed the same characteristics

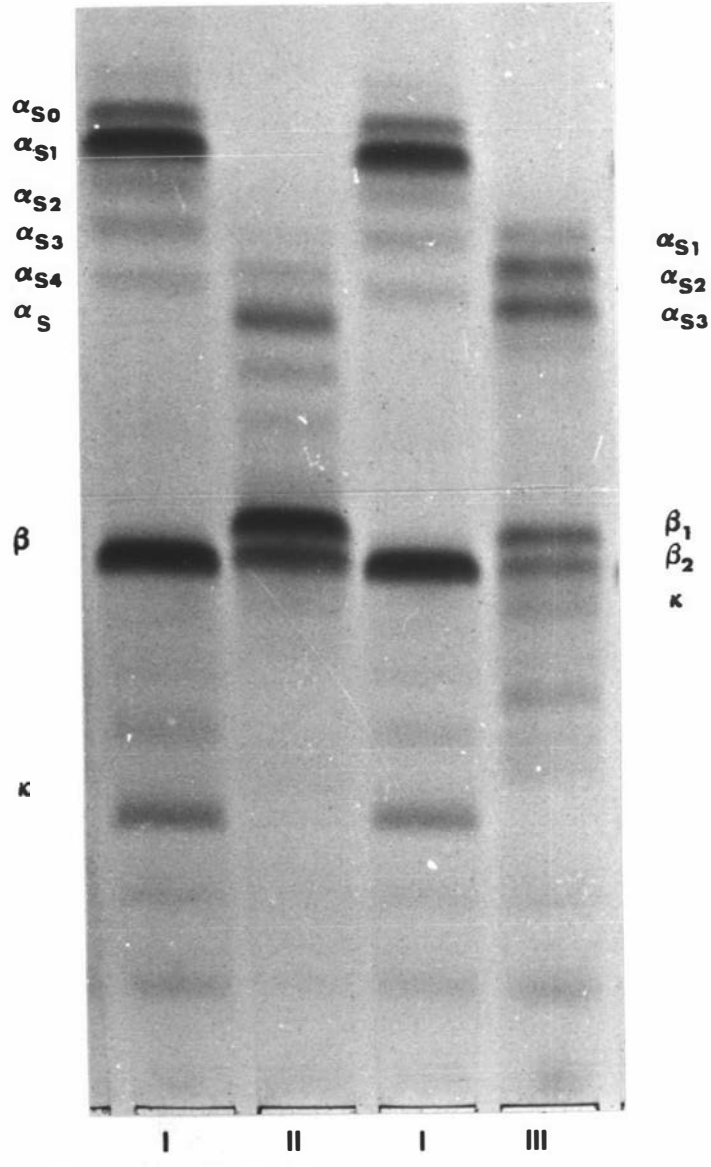


Fig.4.1: Polyacrylamide gel electrophoresis at pH 8.4 of bovine (I), caprine (II) and ovine (III) caseins. The bovine casein was obtained from a cow homozygous for α_{s_1}-Cn^B-β-Cn^B-κ-Cn^B. The bands corresponding to the major α_s- and β-caseins in caprine and ovine caseins are indicated. The β-casein with the higher electrophoretic mobility in caprine and ovine caseins was designated as β_1-casein, while the less mobile component was designated as β_2-casein. The nomenclature of these caseins is discussed in Chapter 8. The direction of electrophoresis is indicated.

but they appeared to be a little smaller. The average sizes of the caprine, ovine and bovine casein micelles were about 110 nm, despite the different size distributions.

Examination of unshadowed preparations showed that the ovine and most caprine micelles were similar to the bovine micelles, but that the large caprine micelles appeared to contain more electron dense material than large micelles of other species.

Electron microscope examination of bovine milk samples using a number of procedures showed the average micelle diameter in bovine milk was approximately 100 nm (Nitschmann, 1949; Knoop and Wortmann, 1960; Parry and Carroll, 1969; Saito, 1973), in good agreement with the present investigation. Knoop and Wortmann (1960) found that the casein micelles of caprine milk colostrum had an average size of 133 nm.

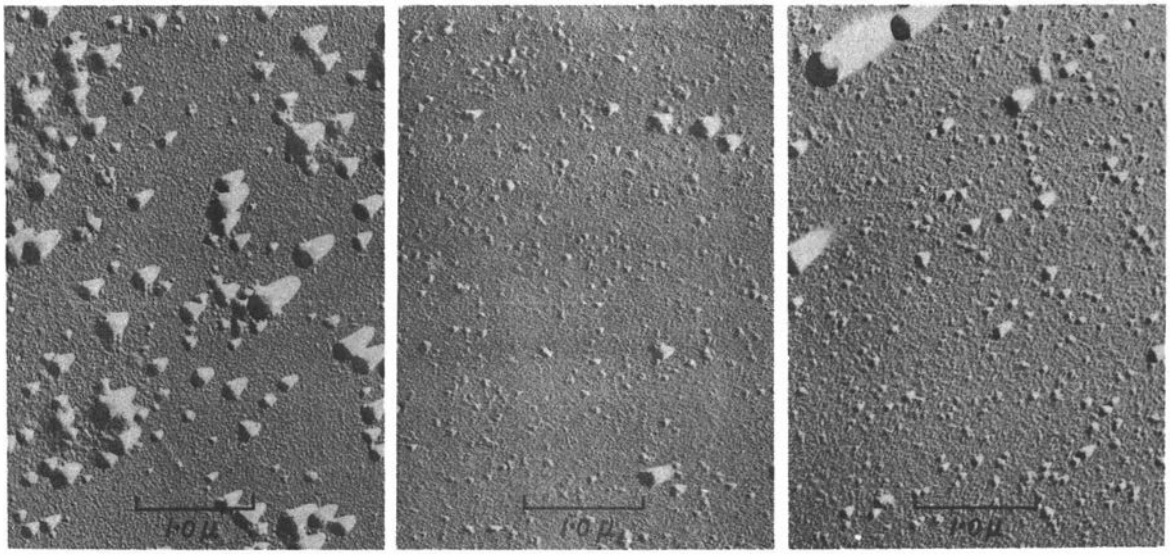
4.5 Electron Micrographs of Freeze-etch Replicas

Typical freeze-etch electron micrographs of bovine, ovine and caprine micelles are shown in Fig.4.3. It can be seen that these micelles are composed of sub-units. It can also be seen that the sizes of the sub-units of the micelles from the milk of these different species are similar at ca. 12 nm. The sizes of the sub-units in bovine milk are similar to those recently reported (Schmidt and Buchheim, 1970; Buchheim and Welsch, 1973), and slightly smaller than the size of 15-20 nm obtained by Knoop et al. (1973).

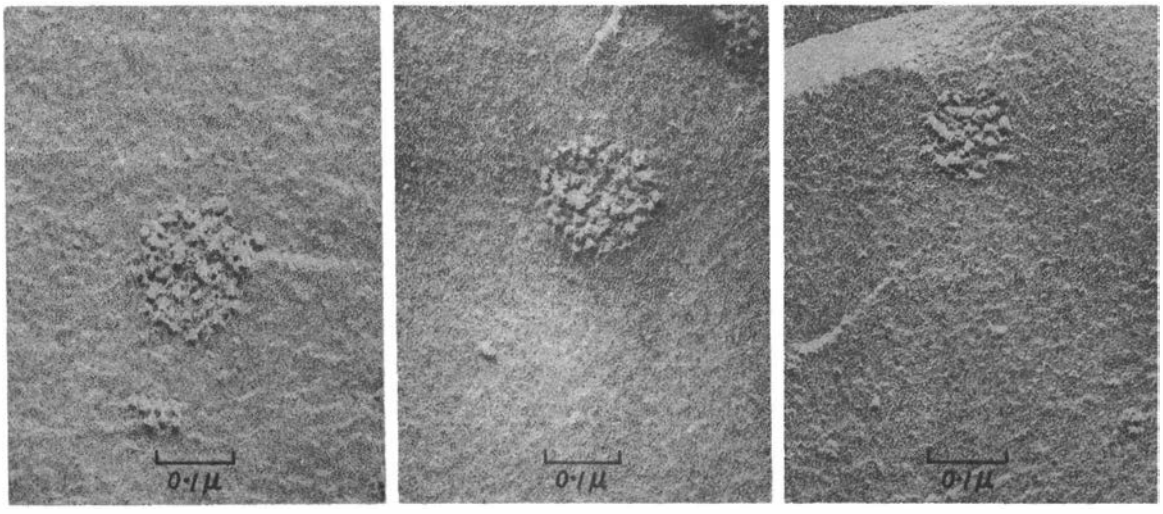
4.6 Ultracentrifugation of Casein Micelles

Under the conditions normally used for sedimenting bovine casein micelles (68 000 g for 45 min) prior to measuring their casein-pellet solvation (Thompson et al., 1969a), caprine and ovine casein micelles were not completely sedimented. Consequently, a series of centrifuge runs of different length were carried out. It was found that measured casein-pellet solvation decreased as the run length increased. Further runs at higher forces

Fig.4.2: Typical electron micrographs of shadowed bovine, ovine and caprine casein micelles on collodion grids. The milks were diluted with dilute $CaCl_2$ solution and the mixture sprayed onto the grid.



Bovine Ovine Caprine



Bovine Ovine Caprine

Fig.4.3: Freeze-etch electron micrographs of the micelles in bovine, ovine, and caprine milk. Photographs showing micelles of approximately similar size were selected.

(330 000 g for 20-180 min) were made and the results are presented in Table 4.3. It can be seen that for all milks the solvation decreased with increased time of centrifugation. The weights of the dried pellets did not alter by more than 5% from the minimum to the maximum time of centrifugation, showing that the decrease in solvation was probably caused by pellet compression and that little additional material was sedimented. These solvation values were lower than those reported by Thompson et al. (1969a) of 1.90 g water per g dry casein micelles. However, they were similar to the minimum solvations obtained by Waugh et al. (1971) for α_{s1}-casein and 1:1 ratio mixtures of α_{s1}- and β-casein in $CaCl_2$ solution. These latter results were essentially independent of field at less than 100 000 g.

Table 4.3

Variation of Casein-Pellet Solvation and Dry Pellet Weight with Species and Centrifugation Time

Species	Centrifugation time, min	Casein-pellet solvation g H_2O/g dry pellet	Dry pellet wt., g/100 g skim milk
bovine	20	2.12	3.02
	60	1.89	3.22
	180	1.51	3.20
ovine	30	2.00	6.01
	60	1.80	6.09
	120	1.62	5.97
caprine	30	1.97	2.85
	60	1.67	3.00
	120	1.54	3.02

4.7 Ca and Phosphate Distribution

The Ca and phosphate contents of milks and their derived casein pellets and sera are shown in Table 4.4. The micelle pellets from the milk of the three species were similar in their Ca and phosphate contents and their ratios

Table 4.4

Distribution of Calcium and Inorganic Phosphate Between Micellar and Serum Phases

Species	Sample date	Centrifugation time, min	Micellar content mmoles/g of dry pellets		Serum content mmoles/l serum		Ca:Phosphate ratio	
			Ca	Phosphate	Ca	Phosphate	Micellar	Serum
Bovine	(10/12/71)	0 (milk)			31.7	23.1		1.37
		20	0.718	0.345	16.2	12.4	2.08	1.30
		60	0.613	0.300	14.5	11.9	2.05	1.22
		180	0.535	0.299	15.0	12.1	2.19	1.21
Ovine	(21/ 9/73)	0 (milk)			60.2	34.8		1.73
		30	0.700	0.365	16.7	14.0	1.92	1.19
		60	0.704	0.366	16.3	13.6	1.93	1.20
		120	0.740	0.364	16.0	14.0	2.04	1.14
Caprine	(26/ 9/73)	0 (milk)			34.7	23.1		1.50
		30	0.745	0.376	12.6	10.2	1.98	1.24
		60	0.726	0.360	12.1	10.0	2.02	1.22
		120	0.758	0.365	12.0	10.2	2.08	1.18

of Ca to phosphate. The ratios of Ca to phosphate in the sera were also similar, but the absolute quantities varied, with the ovine milk containing the most Ca and the most phosphate, and the caprine milk containing the least. It is interesting to note that the milk pH's were 6.61, 6.69 and 6.76 (Table 4.2) for the ovine, bovine and caprine milks used to obtain these results, suggesting that perhaps the pH of the milk is related to the soluble Ca and phosphate in milk.

On one occasion, examination of ovine ultracentrifuge serum showed that it contained 13.6 mM Na, 35.5 mM K and 3.93 mM Mg compared with 13.3 mM Na, 35.1 mM K and 7.33 mM Mg in milk. This indicated approximately half of the Mg was associated with the ovine micelles, while Na and K were largely in the serum phase. In a sample of caprine milk, the ultracentrifuge serum contained 3.13 mM Mg compared with 4.45 mM Mg in the milk, indicating 30% of the Mg was associated with the micelles.

The greater variation in Ca:phosphate ratios among the milks than in either the sera or the micelle pellets (Table 4.4) was caused by the variation in the quantities of micellar casein in the milks (Table 4.3 gives pellet weights). As the quantity of casein in the milk increased the Ca:phosphate ratio tended towards that of the micelle phase (ca. 2.0).

Jenness and Patton (1959) reported a Ca:phosphate ratio of 2.12 in bovine casein micelles. Ca:phosphate ratios obtained by Bosworth and Van Slyke (1916) for micelles from caprine milk were more variable than those in Table 4.4. This variation was probably due to the less accurate methods of analysis available at that time.

4.8 Discussion

The results showed that the bovine, ovine and caprine casein micelles have similar sub-structures, but their size distributions were different (Figs.4.2 and 4.3).

If the sub-units are 12 nm in diameter with a volume of $9x10^{-25}$ m^3 and the average micelle is 120 nm in diameter (Schmidt et al., 1973) with a volume of $9x10^{-22}$ m^3, then a maximum of about 1 000 sub-units might fit into each micelle (assuming no space between the sub-units). If the average casein molecule is considered to have a molecular weight of 24 000, weigh about $4x10^{-20}$ g and occupy a volume of $3x10^{-26}$ m^3, then there would be about 30 molecules per sub-unit (if the sub-units contain only protein; cf. Waugh et al., 1970). If an appreciable fraction of the measured solvation is closely associated with the protein and included in the micelle sub-unit (or between the sub-units) then there would be a corresponding decrease in the number of casein molecules per sub-unit (or sub-units per micelle).

The mineral levels in the milks varied from species to species (Table 4.2) but there were no significant differences in the cation activities, or the Ca:phosphate ratios in either the casein pellets or the sera (Table 4.4). The small differences in the casein-pellet solvations (Table 4.3) may possibly be a reflection of the differences in micelle size distribution, but they were sufficiently small to be considered insignificant. The apparent differences in Ca:phosphate ratios of the skim milk were an indication of the differing quantities of casein in the milks, as the micelle pellets had Ca:phosphate ratios of ca. 2.0 and the sera had ratios of ca. 1.2.

There were major differences between the electrophoretic patterns of the casein samples. The caprine casein contained smaller quantities of the more mobile components than either the ovine or the bovine casein. However, these differences must compensate each other, so that similarly sized sub-units can be formed in the casein micelles in the milk of each species.

The sub-units however, behave differently. The caprine sub-units form either large or small micelles, ovine sub-units form small micelles and bovine sub-units form micelles with a wide size distribution. It is unlikely that variations in Ca or Mg ion activities could be of importance

because they are very similar in the three milks. It seems likely that the observed differences in micelles size distribution are related to the way in which the proteins in the various sub-units react with each other when the sub-units coalesce to form micelles.

CHAPTER 5: TEMPERATURE DEPENDENT PROPERTIES OF CASEIN MICELLES

The temperature dependent properties of casein micelles from caprine, ovine and bovine milks were compared by ultracentrifugation, viscosity measurements and gel chromatography.

5.1 Ultracentrifugation

Samples of caprine, ovine and bovine milks were centrifuged at 102 000 g for 60 min at 4°C, 20°C or 30°C, and the caseins present in the supernatant sera were precipitated with acid and examined by alkaline gel electrophoresis and densitometry (Figure 5.1). The alkaline gel electrophoresis patterns that were obtained are shown in Fig.5.1. The proportions of the "soluble" caseins, expressed relative to their concentration in milk, are presented in Table 5.1.

The results for "soluble" β-casein in caprine and ovine milks include the κ-caseins, since they are difficult to differentiate from the β-caseins and other minor components under these conditions (Chapters 9 and 10). Furthermore, the results in Table 5.1 for the γ-caseins in bovine milk may include some soluble κ-casein since no attempt was made to differentiate between these caseins.

Table 5.1

Temperature Dependent Dissociation of Casein Micelles[1]

	% Soluble Casein						
	Caprine			Bovine		Ovine	
	β-	α_s-	γ-	β-	α_s-	β-	α_s-
4°C Micelle Serum	15	-	10	16	1	21	-
20°C Micelle Serum	2	-	-	trace	-	1	-
30°C Micelle Serum	3	-	-	-	-	-	-

1. The proportion of the caseins in the ultracentrifuge sera were determined by gel electrophoresis. The gel patterns were scanned with a densitometer and the peak areas of the caseins compared with those from the gel patterns for the milks.

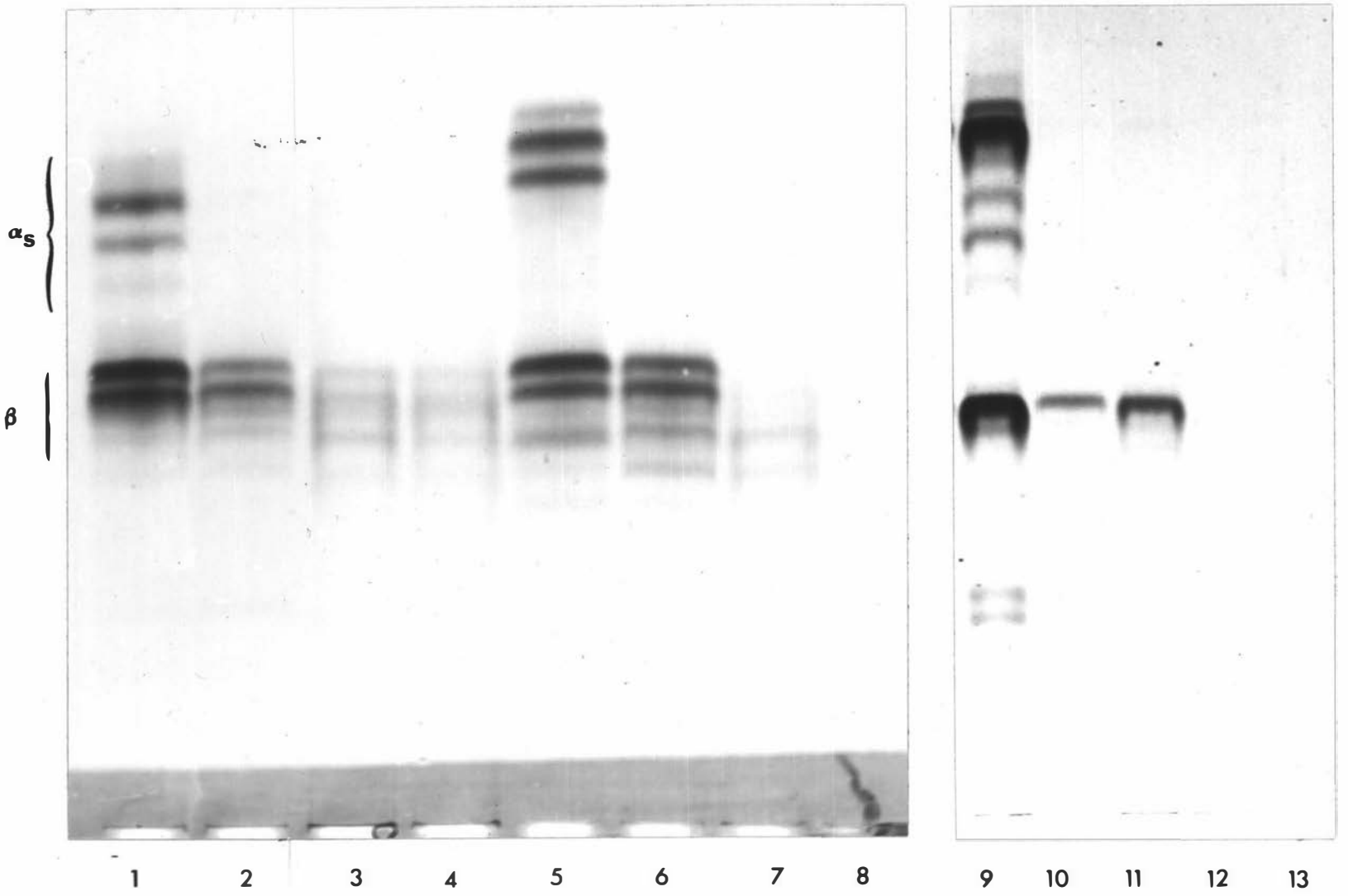


Fig.5.1: Gel electrophoresis (pH 8.4) of the caseins present in the ultracentrifuge sera, obtained by sedimenting caprine, ovine and bovine casein micelles at different temperatures. 1, whole caprine casein; 5, whole ovine casein; 9, whole bovine casein; 2, 6, 10, 11, 4°C serum [11 has twice as much sample as 10]; 3, 7, 12, 20°C serum; 4, 8, 13, 30°C serum. The direction of electrophoresis is indicated.

It is evident (Fig.5.1, Table 5.1), that a considerable amount of the β-casein in milks from the three species was soluble at 4°C. Only a trace of β-casein was soluble at 20°C in bovine milk, with none evident at 30°C. Caprine and ovine milks had 15% and 21% respectively, of their β-casein fraction soluble at 4°C, compared with 10% for bovine milk. A number of minor casein components with electrophoretic mobilities less than the β-caseins and which were soluble in caprine and ovine milks at 20°C, remained soluble in caprine milk at 30°C. The solubility of the ovine and caprine "β-caseins" at high temperature can largely be attributed to the minor caseins evident in the β-casein region.

O'Connor and Fox (1973), in a similar study, found 10% and 1%, respectively of the β- and α_s-caseins in bovine milk were soluble at 5°C, similar to the present results (Table 5.1). Their values for β-casein in caprine milk at 5°C and 20°C were higher than those in Table 5.1, being 25% and 10%, respectively. The proportion of soluble β-casein in ovine milk was similar at 5°C and 20°C to the results in Table 5.1, being 15% and 2%, respectively. Furthermore, O'Connor and Fox (1973) observed minor components in ovine and caprine milks, similar to those in Fig.5.1, that were soluble at 5°C and 20°C with electrophoretic mobilities in alkaline gels less than the β-caseins. O'Connor and Fox (1973) designated these components as "minor β-caseins", since they were not readily hydrolysed by rennin and were soluble at 5°C and 20°C. Details of a preliminary attempt made in this study to establish the identity of these caseins are outlined in Chapter 12.2.

5.2 Voluminosity and Solvation of Casein Micelles

The voluminosity and solvation of casein micelles from caprine, ovine and bovine milks were measured at 4°C and 30°C from their intrinsic viscosities.

The relative densities of the milks and sera, obtained by pycnometry, are compared with values obtained by Nejim (1963) for ovine milk and those reported by Macy et al. (1953) for caprine and bovine milks (Table 5.2). The value obtained

by Nejim (1963) for the relative density of ovine milk was considerably lower than that obtained in this study.

Table 5.2

Relative Densities of Milks and Ultracentrifuge Sera

	Ovine[1]	Caprine[1]	Bovine[2]
Milk	1.0461	1.0331	1.034
30°C serum	1.0274	1.0264	1.025
4°C serum	1.0322	1.0280	1.027
Milk	1.0366[3]	1.033[4]	1.032[4]

1. Measured at 25°C
2. Measured at 20°C
3. Value obtained by Nejim (1963) at 15°C.
4. Values reported by Macy et al. (1953).

The reduced viscosities of the milks and diluted milk fractions (relative to ultracentrifuge serum) were used to determine the intrinsic viscosities of the casein micelles at 4°C and 30°C. The intrinsic viscosities of the micelles from caprine, ovine and bovine milk were obtained by linear least squares analysis of the reduced viscosity plots, some of which are shown in Figs.5.2, 5.3 and 5.4. The values obtained for the intrinsic viscosities, voluminosities, Huggin's constant, k', and the concentration of casein in the micelles are presented in Table 5.3.

Some variation in the intrinsic viscosity, and hence voluminosity values were apparent between the different samples of milk. It is evident from Table 5.3 that with two exceptions there were no appreciable differences between the intrinsic viscosity and voluminosity values at 4°C and 30°C for the same sample of milk. The results for ovine milk (12/12/72) may be unreliable since this was an end of lactation milk sample which contained an abnormally high concentration of protein.

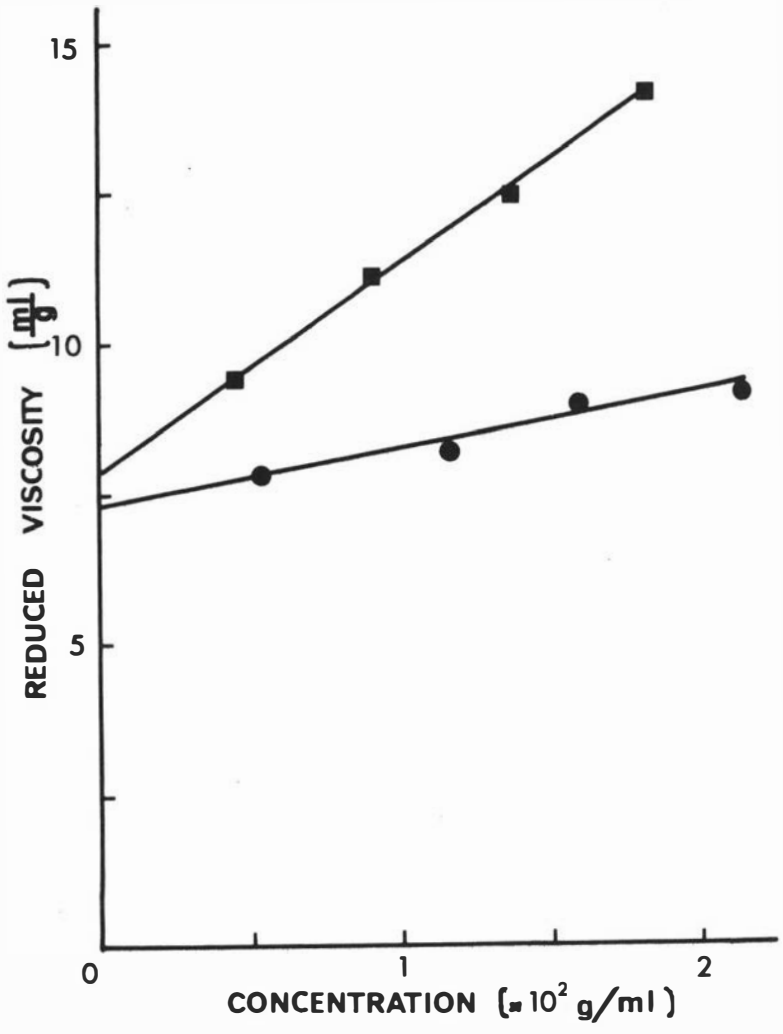


Fig.5.2: Plots of reduced viscosity for caprine casein micelles against concentration of micellar casein at 4°C (■) and 30°C (●). Ultracentrifuge sera prepared at 4°C and 30°C were used as reference solvents.

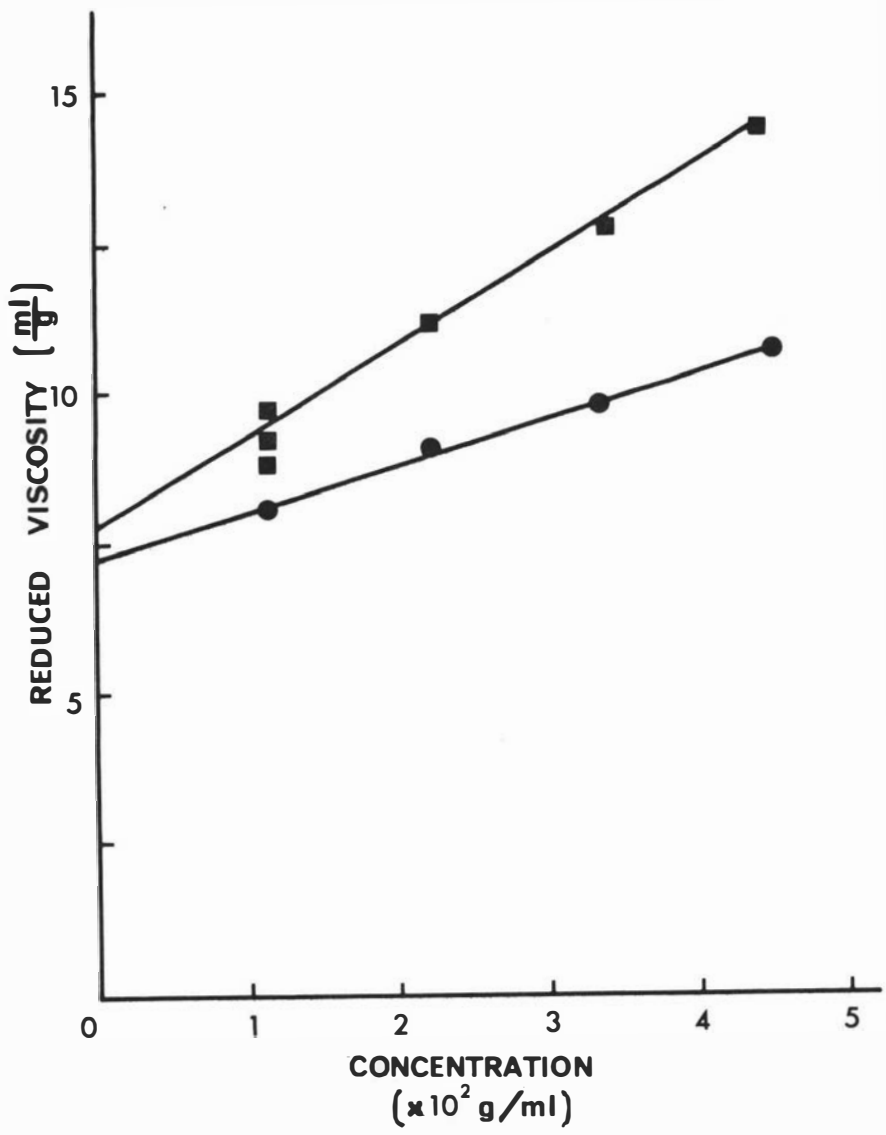


Fig.5.3: Plots of reduced viscosity for ovine casein micelles against concentration of micellar casein at 4°C (■) and 30°C (●). Ultracentrifuge sera prepared at 4°C and 30°C were used as reference solvents.

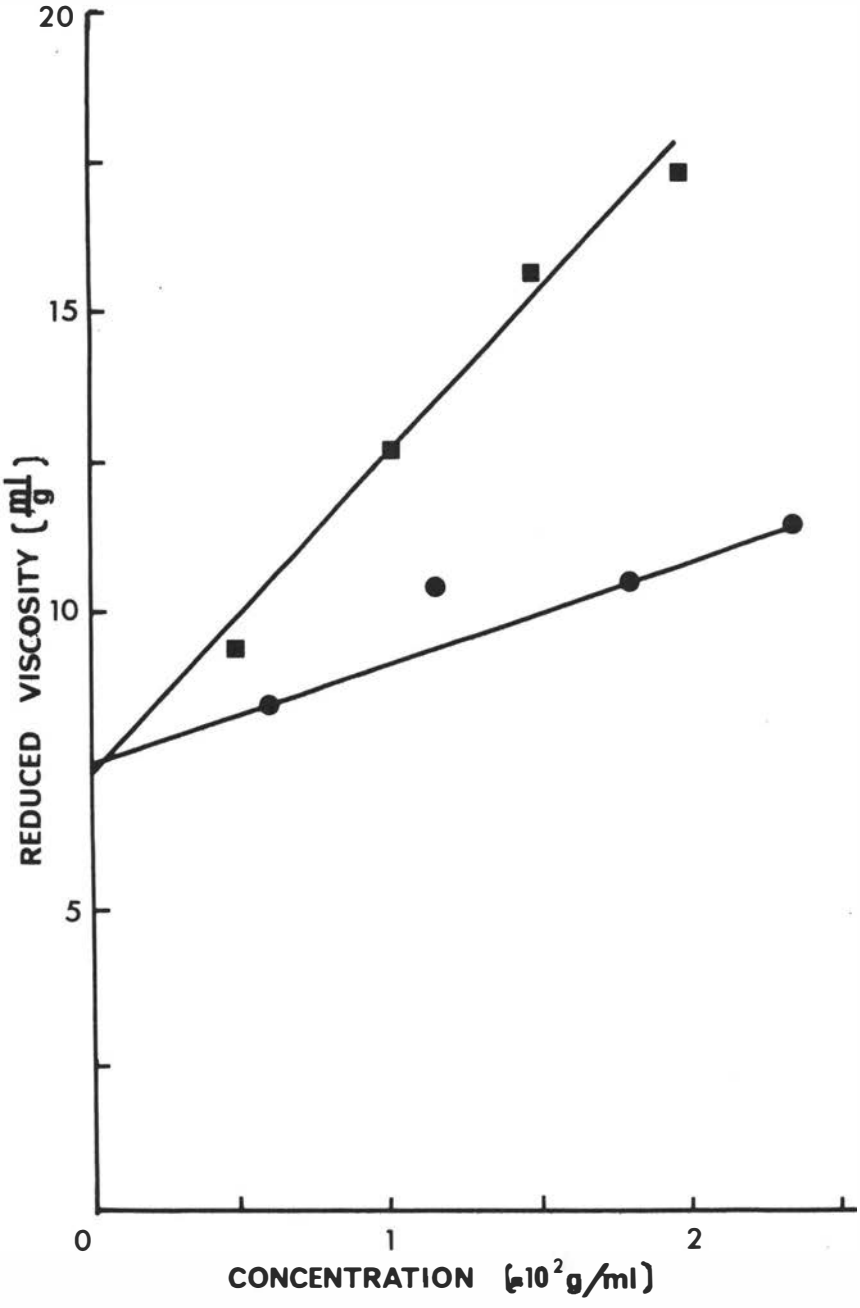


Fig.5.4: Plots of reduced viscosity for bovine casein micelles against concentration of micellar casein at 4°C (■) and 30°C (●). Ultracentrifuge sera prepared at 4°C and 30°C were used as reference solvents.

Table 5.3

Intrinsic Viscosities and Voluminosities of Caprine, Ovine and Bovine Casein Micelles

	Date	Intrinsic viscosity, [η] ml/g		Voluminosity (V) ml/g		Huggin's Constant (k')		Casein concentration g/ml	
		30°C	4°C	30°C	4°C	30°C	4°C	30°C	4°C
Ovine milk	1/12/72	6.20	-	2.48	-	0.9	-	0.0474	-
	12/12/72	5.63	6.55	2.25	2.62	0.5	1.0	0.0594	0.0587
	19/ 9/73	7.25	7.53	2.90	3.01	0.6	1.1	0.0453	0.0442
	27/ 9/73	6.43	6.30	2.57	2.52	0.9	1.9	0.0450	0.0400
	4/10/73	7.73	7.12	3.09	2.87	0.7	2.4	0.0456	0.0385
Mean				2.66	2.80				
Caprine milk	25/ 9/73	7.35	7.88	2.94	3.15	0.7	2.2	0.0214	0.0183
	1/10/73	6.53	8.90	2.61	3.56	0.6	1.2	0.0255	0.0212
	18/10/73	6.01	-	2.40	-	4.4	-	0.0192	-
Mean				2.65	3.35				
Bovine milk	9/10/73	-	8.40	-	3.36	-	1.6	-	0.0216
	16/10/73	7.95	7.25	2.96	2.90	1.2	4.1	0.0235	0.0193
Mean				2.96	3.13				

It is apparent from the voluminosities of the casein micelles from caprine, ovine and bovine milks (Table 5.3), that the micelles did not appreciably change their hydrodynamic size as the temperature was decreased from 30°C to 4°C. Furthermore, there was less casein in the micelles at 4°C than at 30°C (Table 5.3), consistent with dissociation of caseins from the micelles at low temperature.

The value of Huggin's constant, k', calculated from the gradient of the graph of reduced viscosity against concentration and the intrinsic viscosity, is generally considered to be related to the interaction between solute molecules (Bradbury, 1970) (Chapter 3.11). At 30°C, values of k' range from 0.6 to 1.2, with one exception (Table 5.3). At 4°C, values of k' were considerably higher, and with one exception, range from 1.1 to 2.4. Values of k' at 4°C were always greater than those at 30°C. This was due to the slope of the reduced viscosity plots (Figs.5.2, 5.3 and 5.4), since the intrinsic viscosity values were similar at 4°C and 30°C. The increased value of k' at 4°C, compared with 30°C may be due to increased interaction between the dissociated caseins and the casein micelles, as well as increased interaction between the micelles.

From the average voluminosities of the casein micelles (Table 5.3), values were obtained for the solvation of casein micelles from caprine, ovine and bovine milks at 4°C and 30°C (Table 5.4). These casein micelle solvation values, obtained by viscosity measurements, are compared in Table 5.4 with casein-pellet solvation values, obtained by ultracentrifugation of milk samples at 30°C (Table 4.3) and at 4°C. Casein-pellet solvation values were obtained at 4°C by centrifuging milk samples for 1 h at 102 000 g. The centrifugal force used to sediment the micelles was less than that used at 30°C (326 000 g for 20 min to 180 min).

The micelle pellets obtained at low temperatures were very "soft", compared to the "hard rubber-like" texture of the pellets at 30°C. Consequently, difficulty was experienced in separating the micelle and serum phases of pellets sedimented at low temperatures.

Table 5.4

Solvation of Casein Micelles (g water per g protein)

	Ovine	Caprine	Bovine
Viscosity Measurements[1]			
30°C	1.96	1.95	2.26
4°C	2.10	2.65	2.43
Ultracentrifugation[2]			
30°C	2.0-1.6	1.97-1.54	2.12-1.57
4°C	2.29	2.83	3.16
Dewan et al. (1973)[3]			3.67
Thompson et al. (1969a)[4]		1.60	1.90

1. Solvation values for casein micelles were calculated from the average voluminosities in Table 5.3 using the expression:

$$V = \bar{v}_2 + \delta_1 v_1^o \qquad \text{(Chapter 3.11)}$$

2. Casein-pellet solvation values (g water per g dry micelle pellet) at 30°C were obtained from Table 4.3. Values at 4°C were obtained by centrifuging milk samples for 1 h at 102 000 g.

3. Obtained at 25°C from viscosity measurements.

4. Casein-pellet solvation values obtained at 37°C by ultracentrifugation of milk samples at 68 000 g for 35 min.

Casein micelle solvation values measured at 4°C from viscosity measurements (Table 5.3) were slightly higher than those obtained at 30°C (Table 5.4). Casein-pellet solvation values (obtained by ultracentrifugation) at 30°C were similar to those obtained from viscosity measurements at 30°C. Likewise, with the possible exception of bovine milk, the casein-pellet solvation values at 4°C were similar to those obtained from viscosity measurements.

The value of casein micelle solvation obtained for bovine milk by Dewan et al. (1973) from viscosity measurements at 25°C, was considerably higher than those obtained in this study by ultracentrifugation or viscosity measurements (Table 5.4), and the casein-pellet solvation obtained by Thompson et al. (1969a), at 37°C. Dewan et al. (1973) were unable to provide an explanation for the discrepancy between their results and those of Thompson et al. (1969a). Although the number of samples of bovine milk that were examined in this study (Table 5.3) were limited, voluminosities that were obtained for the bovine casein micelles were in accordance with those of the other species.

From their intrinsic viscosity measurements, Whitnah and Rutz (1959) obtained a value for the voluminosity of bovine casein micelles at 25°C of 2.7 ml/g. From this a casein micelle solvation value of 2.0 ml/g may be calculated, which is close to the value obtained in Table 5.4 for bovine casein micelles from viscosity or ultracentrifugation measurements.

It is important to note that micelle solvation, as determined by viscosity measurements, includes the water that is occluded with the micellar domain. Consequently, one would expect the micelle solvation measured after a short time of centrifugation may correspond to the viscosity results. In fact, this appears to be the case, with the increased time of centrifugation (Table 4.3) probably resulting in more water or serum being squeezed out from between the micelles, or the micelle sub-units.

5.3 Gel Chromatography of Bovine, Caprine and Ovine Casein Micelles

Although bovine casein micelles are disrupted by the removal of Ca, the individual casein micelles remain aggregated in sub-micellar casein aggregates(Creamer and Berry, 1975). When fractions of large or small casein micelles from bovine milk were chromatographed, consecutively on Sephadex G-10 and Sepharose 4B at 37°C, ionic strength 0.1, using pH 6.7 buffer, it was found that the protein was

eluted as a single peak constituting sub-micellar casein aggregates (Creamer and Berry, 1975).

Alkaline gel electrophoresis patterns revealed the ratios of κ- to β-casein in fractions of caprine and ovine small casein micelles were higher than those in milk or fractions of large micelles. Similar results were obtained by Creamer and Berry (1975) for fractions of small bovine casein micelles. Fractions of large or small casein micelles, isolated from caprine or ovine milks by ultra-centrifugation, were eluted as a single major peak from the Sephadex G-10 and Sepharose 4B column, in the same position as the bovine casein micelle fractions. Duplicate analyses revealed that fractions eluted from the column which contained the most protein had elution weights of 83.8 g, 83.3 g and 85.6 g, respectively for caprine, ovine and bovine micelle fractions. These results suggested that the casein aggregates from the three species had similar hydrodynamic sizes.

Figure 5.5 shows the elution pattern obtained when a fraction containing large micelles from ovine milk was chromatographed consecutively on the Sephadex G-10 and Sepharose 4B column in neutral buffer at 37°C. Alkaline gel electrophoresis and densitometry of some of the fractions that were collected (Figs.5.5 and 5.6) revealed κ-casein was eluted at the leading edge, whereas β- and α_s-caseins were distributed at a constant ratio throughout the sub-micellar casein aggregate peak. Similar results were obtained to those in Figs.5.5 and 5.6 when fractions of caprine casein micelles were chromatographed on the column. κ-Casein in caprine micelle fractions could only be identified by electrophoresis of the column fractions at acid pH (Chapter 9.1), since at alkaline pH κ-casein was obscured by the β-caseins.

Creamer and Berry (1975) showed that when bovine casein micelle fractions were chromatographed on the Sephadex G-10 and Sepharose 4B column, κ-casein was eluted at the leading edge of the sub-micellar casein aggregate peak. Furthermore, densitometer measurements revealed the ratio of κ-casein to the other caseins to be 1 to 2.5 and 1 to 7

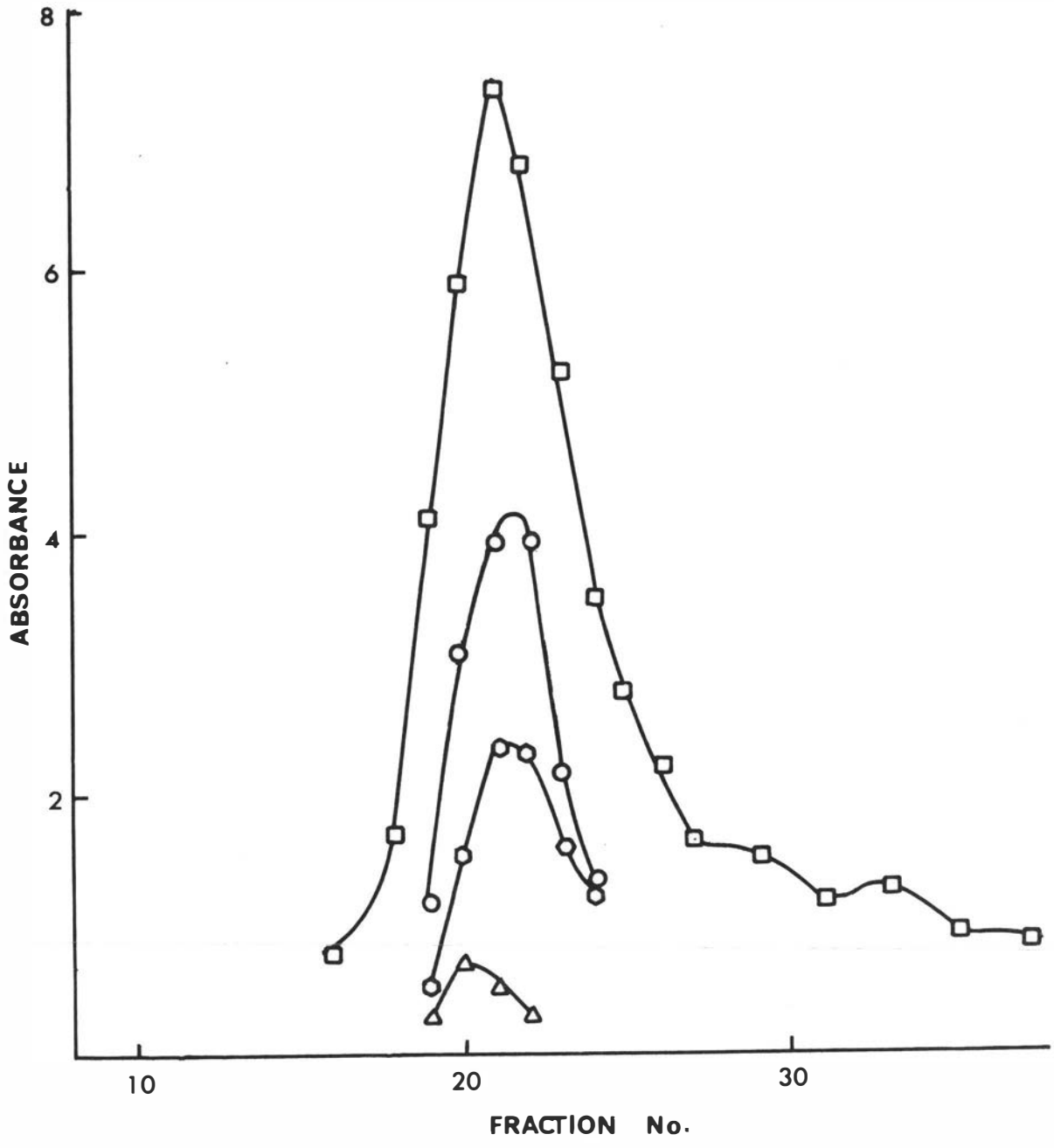


Fig.5.5: Elution of ovine sub-micellar casein aggregates from a Sephadex G-10-Sepharose 4B column at 37°C. A sample containing ovine large casein micelles was applied to the column and eluted at a flow rate of 40 ml/h with 0.025 M imidazole-HCl buffer, pH 6.7, containing 0.1 M NaCl and 0.02% (w/v) NaN_3, fractions of 3 ml being collected. Total protein was estimated as 280 nm absorbance (□). Some of the fractions containing protein were examined by gel electrophoresis (Fig.5.6) and densitometry. κ-casein (△), β-caseins (○) and α_s-caseins (●) were estimated by 615 nm absorbance of the amido black stained bands of the gel electrophoresis patterns (Fig.5.6).

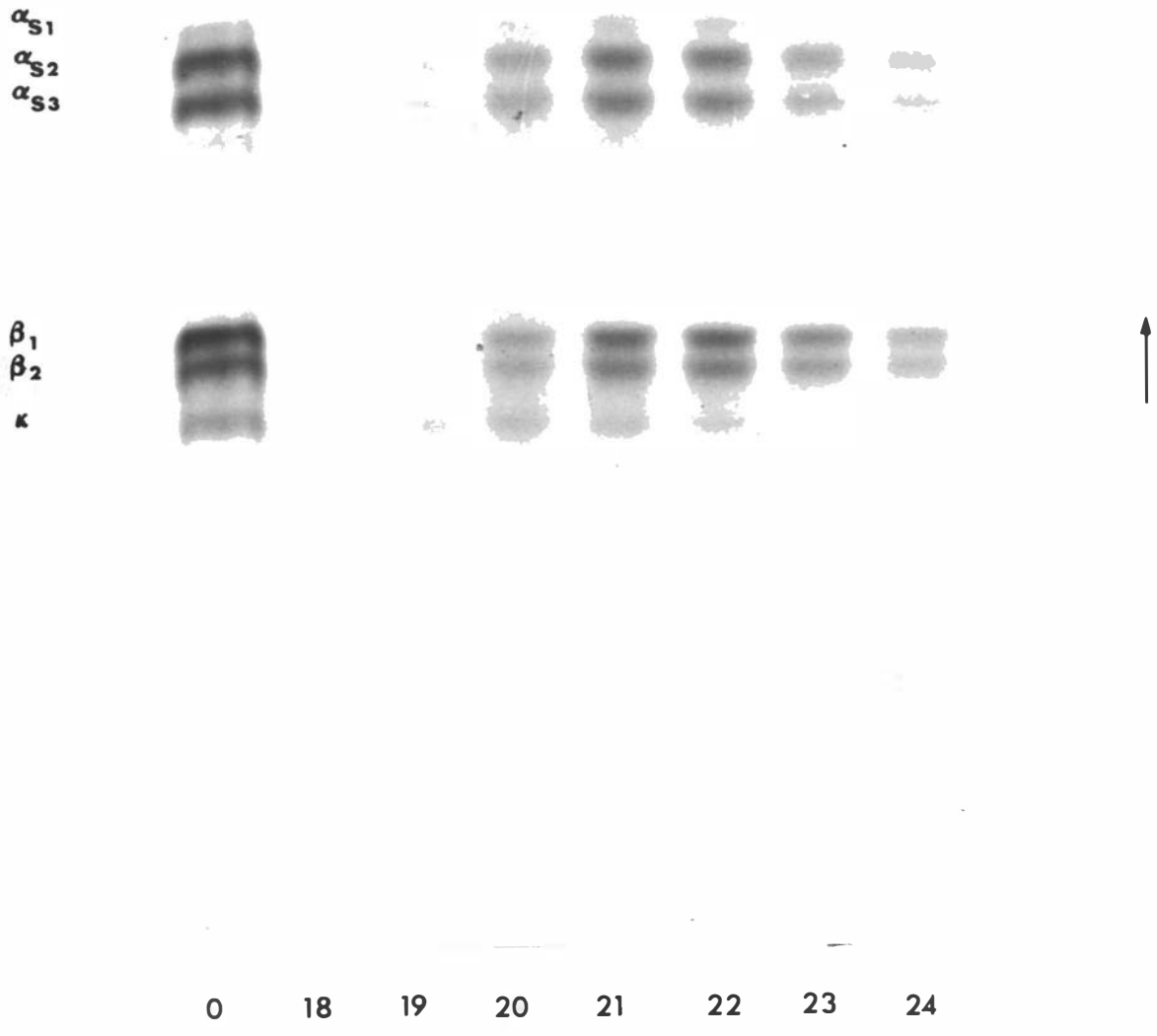


Fig.5.6: Polyacrylamide gel electrophoresis (pH 8.4) of fractions obtained by gel chromatography of ovine large casein micelles at 37°C (Fig.5.5).
0. Ovine large casein micelles prior to chromatography. The fractions from Fig.5.5 which were examined are indicated. The arrow indicates the direction of electrophoresis.

for the leading and trailing edge of the casein aggregate peak, respectively of bovine milk micelles (based on 280 nm absorbance).

At 6°C, micelle fractions from caprine, ovine and bovine milks all have similar elution patterns when chromatographed consecutively on a column of Sephadex G-10 and Sepharose 4B. However, under these conditions, considerable dissociation of the casein aggregates became apparent with the appearance of a second protein peak which eluted later than the casein aggregate peak. Figures 5.7 and 5.8 show the elution pattern obtained for small caprine micelles at 6°C, and the alkaline gel electrophoresis patterns of some of these fractions, respectively. A considerable amount of the caprine β-caseins and some α_s-caseins were dissociated from the casein aggregates at low temperature and eluted close to the whey proteins (Fig.5.8).

The sub-micellar casein aggregates from caprine, ovine and bovine micelles, at 6°C, had elution weights of 72.9 g, 75.2 g and 70.5 g, respectively, thus essentially confirming that the sub-micellar casein aggregates have similar hydrodynamic sizes. It should be noted that a column with a slightly different height of packed gel was used, compared to that used at 37°C and consequently no comparison was made between the elution volumes at 37°C and 6°C. The peak which eluted at the beginning of the chromatographic run (Fig.5.7) was distinctly turbid. Gel electrophoresis of aliquots from these fractions (Fig.5.8) revealed no protein was present. The absorbance 280 nm values (Fig.5.7) were not corrected for any turbidity in the fractions. Similar peaks, which eluted at the void volume of the column were observed by Creamer and Berry (1975).

Chromatography of caprine, ovine and bovine casein micelles consecutively on the column of Sephadex G-10 and Sepharose 4B caused dissociation to sub-micellar casein aggregates which had similar hydrodynamic sizes at both 37°C and 6°C. It should be noted that Creamer and Berry (1975) found most of the Ca was dissociated from the sub-micellar

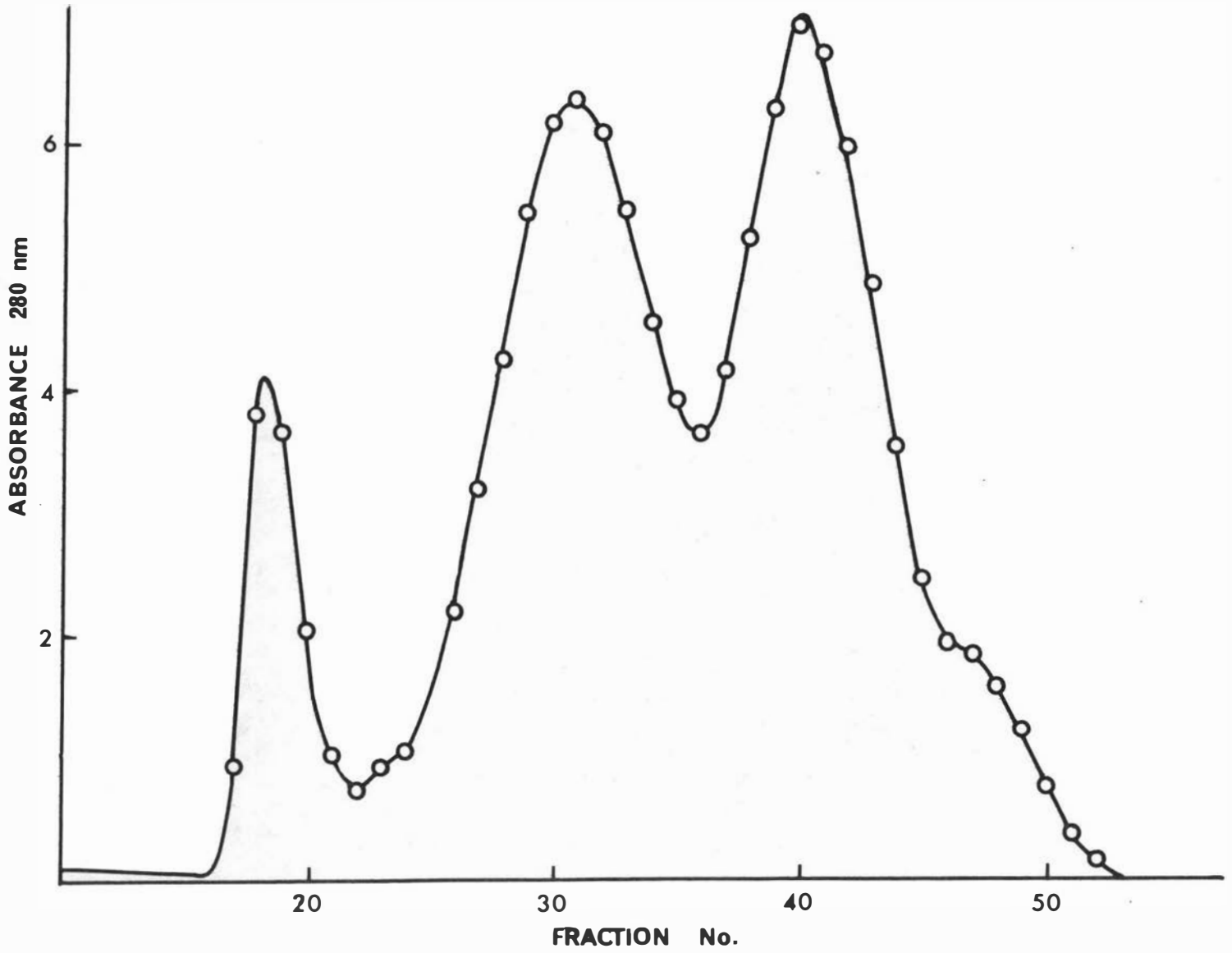


Fig.5.7: Elution of caprine sub-micellar casein aggregates obtained from a small casein micelle fraction from a Sephadex G-10-Sepharose 4B column at 6°C. Other experimental details are the same as in Fig.5.5.

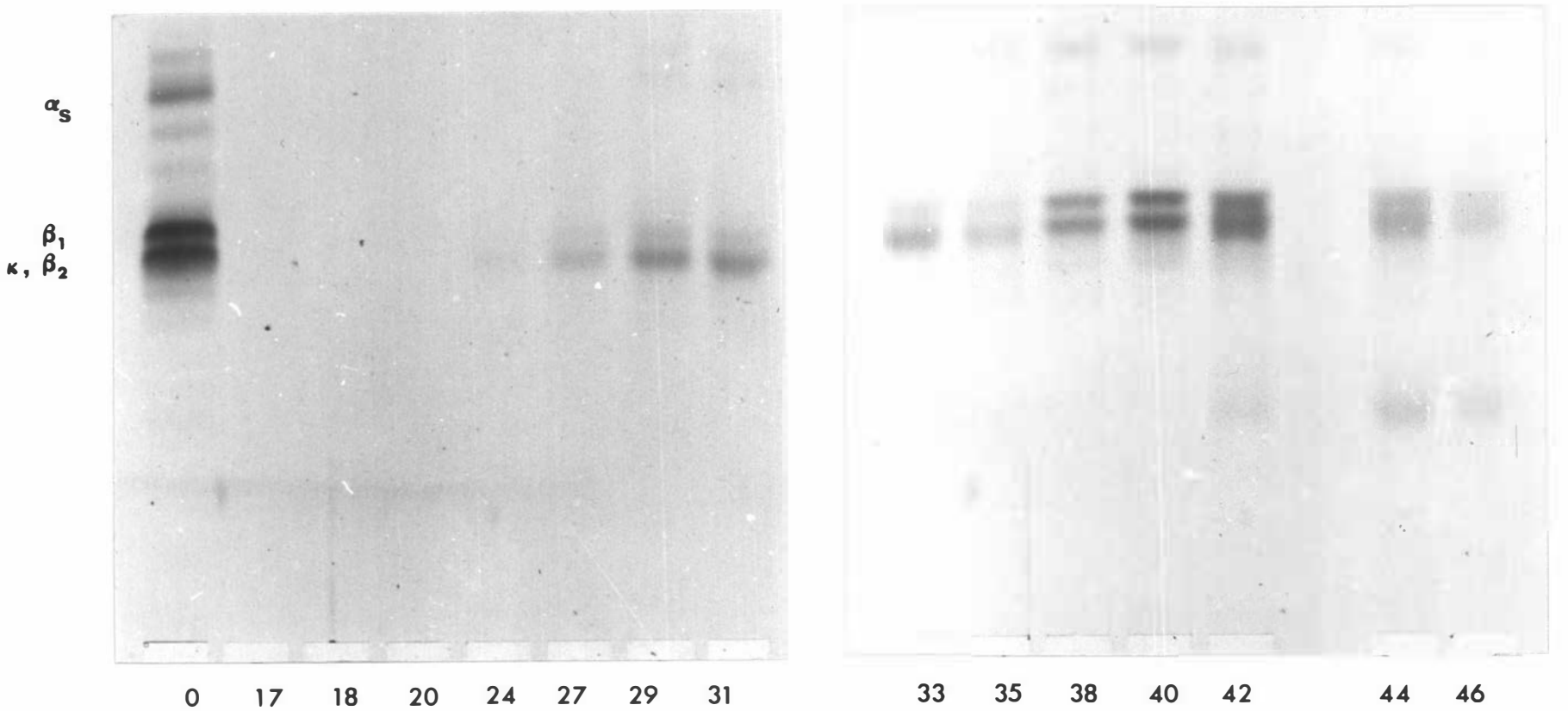


Fig.5.8: Polyacrylamide gel electrophoresis at pH 8.4 of fractions obtained by gel chromatography of caprine small casein micelles at 6°C (Fig.5.7). O, caprine small casein micelles. The fractions in Fig.5.7 which were examined are indicated. The arrow indicates the direction of electrophoresis.

casein aggregates, consistent with the removal of colloidal calcium phosphate and the loss of most of the protein bound Ca.

5.4 Discussion

Both ultracentrifugation measurements (Chapter 5.1) and gel chromatography (Chapter 5.3) of casein micelles showed that the casein micelles are porous structures which allow caseins, in particular β-caseins to dissociate at low temperatures.

The similar values obtained for micelle solvation (viscosity measurements) or casein-pellet solvation (ultracentrifugation) at 4°C and 30°C (Chapter 5.2) indicated that the bovine, caprine and ovine casein micelles are highly solvated, and have similar specific volumes despite the differences in the nature of the caseins. The reason for the difference between the voluminosity of bovine casein micelles, as obtained in this study (Table 5.3) and by Dewan et al., (1973) are not readily apparent. It is interesting to note, however, that Whitnah and Rutz (1959) obtained a similar value for the voluminosity of bovine casein micelles to that obtained in this study.

The results which have been presented in Part I of this thesis are discussed further in Chapter 13.

PART II

ISOLATION AND CHARACTERIZATION OF THE MAJOR CAPRINE AND OVINE CASEINS, AND A COMPARISON WITH THE BOVINE CASEINS.

CHAPTER 6: INTRODUCTION

The caseins, particularly those from bovine milk, have been the subject of intensive investigation during the last two decades. This has recently culminated in the elucidation, largely by the French group at Jouy-en-Josas, of the primary structures of the major bovine α_{s1}-, β- and κ-caseins and their genetic variants. This was a sterling achievement which has added impetus to further investigations on the physical and chemical properties of the bovine caseins.

Recently, there have been a number of excellent publications which have reviewed the literature on the caseins, in particular the bovine caseins (Jollès, 1966; McKenzie, 1967; McKenzie, 1970; Rose et al., 1970; McKenzie, 1971; Lyster, 1972; Mercier et al., 1972a; Swaisgood, 1973). Consequently, only a brief survey of the relevant literature is presented.

The chapter is divided into two sections, the first reviewing some of the chemical and physical characteristics of bovine caseins while the second section briefly reviews the characterization of caseins from other species.

BOVINE CASEINS

6.1 Historical

Casein was first isolated by Braconner in 1830. Hammarsten isolated and characterized acid casein some 50 years later in 1883. Nearly a century after it was first isolated, Linderstrøm-Lang and Kodama (1925) and Linderstrøm-Lang (1929) discovered casein was a heterogeneous phosphoprotein.

Use of Tiselius free-boundary electrophoresis by Mellander (1939) resulted in the separation of bovine casein into three fractions which he called α-, β- and γ-, in order of their decreasing mobilities at pH 7.6. Following this, Warner (1944) described a method to isolate α- and β-caseins by chemical fractionation and later Hipp et al. (1952) isolated the α-, β- and γ- fractions, utilizing their

different solubilities in ethanol and urea solutions. The latter method is still widely used today for isolating casein fractions.

One of the more significant advances in the chemistry of caseins was an extension of Linderstrøm-Lang's observations on solubility differences in the presence of Ca by Waugh and von Hippel (1956). They used sedimented casein micelles to isolate "first cycle casein", (casein micelles, essentially freed of Ca) and they then separated it into two fractions: Fraction S which was soluble in the presence of Ca, and Fraction P which was insoluble in the presence of Ca. Subsequently, Waugh et al. (1962) used Fraction P to isolate α_s-casein, while McKenzie and Wake (1961) used Fraction S to isolate κ-casein.

6.2 Identification of the Major Casein Fractions

There are three major bovine caseins, α_{s1}-, β- and κ-casein. The nomenclature and characteristics of these caseins have already been described briefly (Chapter 1.2) and for a more detailed description the reader is referred to the reviews by the "Committee on milk protein nomenclature, American Dairy Science Association" (Jenness et al., 1956; Brunner et al., 1960; Thompson et al., 1965; Rose et al., 1970).

The major α_s-casein fraction, which accounts for some 50% of bovine casein, contains a major component, α_{s1}-casein, and several minor components, α_{s0}-, α_{s2}-, α_{s3}-, α_{s4}- and α_{s5}-caseins (Annan and Manson, 1969; Rose et al., 1970) (Fig.4.1).

β-casein, which constitutes 30% of the total casein, has a mobility in starch and polyacrylamide gel electrophoresis less than that of α_{s1}-casein. (Fig.4.1) (Rose et al., 1970).

The κ-casein fraction contains some 15% of the total casein (Rose et al., 1970) and has an electrophoretic mobility in alkaline polyacrylamide and starch gels which is less than β-casein (Fig.4.1). The minor fraction, "whole

γ-casein", is composed of γ-, TS-, R- and S-caseins, which account for 3-7% of the total casein (Rose et al., 1970). These γ-caseins are now known to arise through the degradation of β-casein by a naturally occurring milk protease (Gordon et al., 1972; Yamauchi and Kaminogawa, 1972).

6.3 Genetic Variants of Bovine Caseins

Aschaffenburg (1961) was the first to demonstrate genetic polymorphism in the β-caseins by paper electrophoresis in the presence of urea. He found three variants which he named A, B and C, in order of their decreasing mobilities at pH 7.15. These were the result of autosomal co-dominant alleles. Later, Peterson and Kopfler (1966) using acid polyacrylamide gel electrophoresis in gels containing urea demonstrated that β-casein A was actually composed of three variants, A^1, A^2, and A^3, in order of their decreasing mobilities (Kiddy et al., 1966).

Recently, other genetic variants of β-casein have been observed in the casein from Zebu cattle (Bos indicus) which Aschaffenburg et al. (1968a) designated as the D and B_z variants. The B_z variant had an identical mobility in alkaline and acid gel electrophoresis to the B variant in Western breeds. Examination of chymotryptic digests however, revealed differences between these variants (Thompson et al., 1969b). A new variant, β-casein E, has been observed in Italian Piedmont cattle (Voglino, 1972). The isolation and partial characterization of a new β-casein variant has been described (Seitov et al., 1971) although no details of its electrophoretic mobility are available. The relative mobilities of the genetic variants of β-casein in alkaline and acid gel electrophoresis are shown in Fig.6.1.

Following the report of β-casein polymorphism, Thompson et al. (1962) using alkaline gel electrophoresis, observed three variants of α_{s1}-casein in Western cattle, which they designated as A, B and C, in order of their decreasing mobilities. Since then, Grosclaude et al. (1966) have identified a fourth variant, α_{s1}-casein D, with a mobility on alkaline gel electrophoresis intermediate

between the A and B variants (Fig.6.1). Originally this variant was thought to exist only in the milk of the French Flammande cattle, but has now been found in other breeds (Grosclaude et al., 1973).

Polymorphism has not yet been observed in the minor α_s-caseins of Western breeds (Hoagland et al., 1971), although casein of some Red Danish cattle give no α_{s3}- and α_{s4}-casein bands after starch gel electrophoresis (Michalak, 1967).

The existence of two genetic variants of κ-casein was established independently by three laboratories (Neelin, 1964; Schmidt, 1964; Woychik, 1964). These were designated as κ-casein A and B, following the nomenclature for α_{s1}- and β-caseins. κ-Casein was resolved into definite zones by electrophoresis only after the disulphide linkages were first reduced to allow complete dissociation in urea. The relative mobilities of the κ-casein genetic variants are shown in Fig.6.1.

The genetic variation in γ-casein and in the other minor fractions was shown to follow the variations in β-casein (Groves and Kiddy, 1970). In light of the recent findings on the γ-caseins (Groves et al., 1972), their relationship with β-casein is not surprising.

All of the genetic polymorphs presently characterized are products of allelic autosomal genes inherited via simple Mendelian processes with no dominance (Aschaffenburg, 1968; Li and Gaunt, 1972; Thompson, 1971).

Of the α_{s1}-caseins, the B variant is predominant in Western breeds (Bos taurus), Ayrshires being homozygous for this allele, whereas the C variant is predominant in Zebu cattle (Bos indicus) (Aschaffenburg et al., 1968a). α_{s1}-Casein A is a rare genetic variant which has only been observed in some Holstein-Friesian , New Zealand Friesian and Red Danish cattle (Farrell et al., 1971).

The A^1, A^2 and A^3 variants of β-casein A have been found in most Western breeds, although the B variant is a

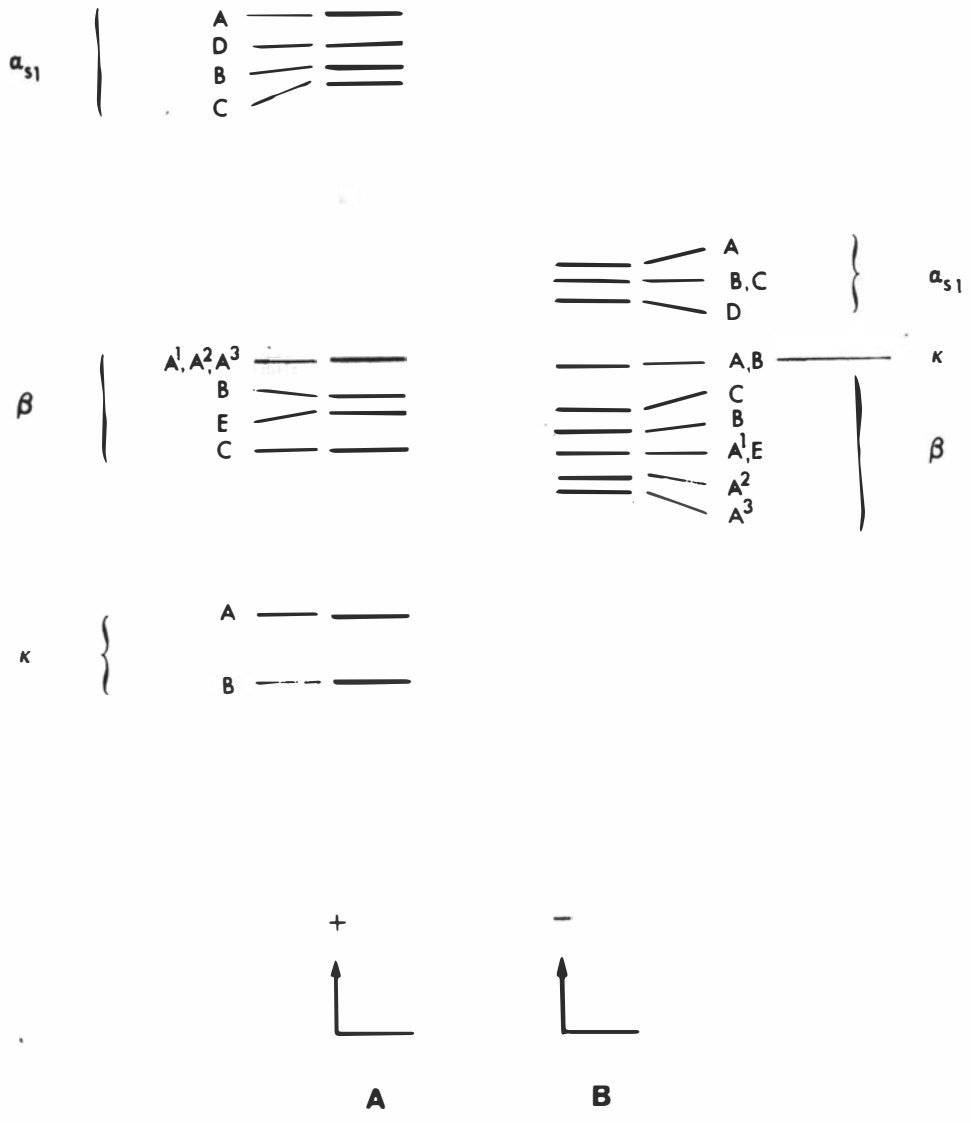


Fig.6.1: Diagrammatic representation of the relative mobilities of the genetic variants of the bovine α_{s1}-, β- and κ-caseins in starch gel electrophoresis with urea and 2-mercaptoethanol. A, pH 8.6; B, pH 3.0. Only the main band of κ-casein is indicated at pH 8.6. Reproduced from Grosclaude et al. (1973).

significant phenotype in the Jersey breed (Aschaffenburg, 1968). Both κ-casein variants occur with high frequency in all breeds, A predominantly in Holsteins and B in Jerseys (Aschaffenburg, 1968).

To date, with the exception of the β-casein B_z variant in Zebu cattle, all the genetic variants have been the result of substitution of charged amino acid residues, or in the case of α_{s1}-casein A, a deletion of 13 residues. Changes involving charged amino acids are most readily detected by gel electrophoresis. Substitutions could occur involving uncharged residues, such as in the B_z variant of β-casein (Aschaffenburg et al., 1968a; Thompson et al., 1969b) which are not as readily detected and have yet to be observed. Grosclaude et al. (1973) estimated that one in three genetic variants in bovine casein which are the result of point mutations might have substitutions involving charged amino acids. It is obvious therefore, that a large number of genetic variants involving neutral substitutions may yet be identified.

κ-CASEIN

6.4 Isolation of κ-Casein

Linderstrøm-Lang (1929) postulated the existence of κ-casein, after isolating and partially characterizing a crude casein fraction. It was later isolated from "first cycle casein" as an impure fraction soluble in Ca by Waugh and von Hippel (1956). McKenzie and Wake (1961) isolated κ-casein by a procedure involving alcohol fractionation of Fraction S, a fraction from acid casein which was soluble in Ca. Waugh (1961) showed κ-casein was the only major casein possessing disulphide bridges. These were shown to occur inter-molecularly, resulting in the formation of a series of covalent polymers ranging in size from 60 000 (trimer) to well above 150 000 with $s_{20,w}$ values of 13.25 in neutral phosphate buffer (Swaisgood and Brunner, 1963; Swaisgood et al., 1964; Mackinlay and Wake, 1964; Talbot and Waugh, 1970).

Alkaline gel electrophoresis in the presence of urea of reduced κ-casein containing a single genetic variant revealed one intensely staining band and approximately five minor bands with greater mobility (Mackinlay and Wake, 1964; Schmidt, 1964; Woychik, 1964). These rapidly migrating bands, which only account for a proportion of κ-casein, differ from the slower, more intense band in their carbohydrate content (Woychik et al., 1966; Mackinlay and Wake, 1965; Schmidt et al., 1966). The major component contains no carbohydrate, while the minor bands possess increasing amounts of hexose and sialic acid. Treatment of these caseins with neuraminadase reduces the intensity of these minor components, consistent with the removal of sialic acid from the leading bands (Schmidt et al., 1966). These bands also disappear on treatment with rennet (Rose et al., 1970; Schmidt et al., 1966). The lack of carbohydrate does not affect the micelle stabilizing ability of κ-casein (Mackinlay and Wake, 1965).

Ribadeau Dumas et al. (1964) purified κ-casein by chromatography of whole casein on DEAE-cellulose in buffer containing urea. Later, κ-casein was isolated by chromatography on DEAE-cellulose after the disulphide groups were reduced (Schmidt et al., 1966; Mackinlay and Wake, 1965; Rujolle et al., 1966; Woychik et al., 1966).

Recently, Yaguchi and Tarassuk (1967) and Yaguchi et al. (1968) described a procedure for the isolation of κ-casein from milk or casein by chromatography on Sephadex G-150 in the presence of 6 M urea. Since κ-casein is intermolecularly linked through its disulphide bonds, in the absence of 2-mercaptoethanol it is eluted as a peak at the void volume of the column. These procedures for isolating κ-caseins, utilizing chromatography on DEAE-cellulose and Sephadex G-150 and the procedure described by McKenzie and Wake (1961), do not expose the proteins to the rigorous conditions experienced using the urea-sulphuric acid fractionation method of Zittle and Custer (1963). Other methods used to isolate κ-casein are described in the review by Mackinlay and Wake (1971).

6.5 Physical Properties of κ-Casein

κ-casein is soluble in solutions containing moderate levels of Ca with 2 g atoms being bound to the protein (Dickson and Perkins, 1971). However, since only 1 phosphate group is present in κ-casein (Mercier et al., 1973), an additional binding site for Ca may exist.

Waugh and his coworkers have largely been responsible for the work on the stabilization in solution of α_{s1}-casein by κ-casein. α_{s1}-Casein and κ-casein can form soluble micelle-type complexes in the presence of Ca (stabilization), under conditions where α_{s1}-casein would normally precipitate. Although they originally believed complexes between κ-casein and α_{s1}-casein formed spontaneously, with a preferred weight ratio α_{s1}-casein:κ-casein of 4 in the absence of Ca (Waugh, 1958), recent results suggested the complex at 37°C was composed of a unit weight ratio of the two proteins (Waugh and Noble, 1965; Noble and Waugh, 1965). This work led to a study of micelle formation with α_{s1}- and κ-casein in the presence and absence of Ca. For a description of this work the reader is referred to the recent review by Waugh (1971).

6.6 Action of Rennin on κ-Casein

Addition of rennet to normal milk at room temperature results in rapid clotting. This is the result of an extremely labile phenylalanine-methionine peptide bond being cleaved (Fig.6.2), causing a loss of the stabilizing ability of κ-casein, and the generation of para-κ-casein and macropeptide (Jollès et al., 1963; Delfour et al., 1965; Jollès, 1966; Jollès et al., 1968). The macropeptide has an N-terminal methionine (Delfour et al., 1965) and a common C-terminal sequence to κ-casein (Jollès, 1966). The substitution sites responsible for the A and B genetic variants of κ-casein exist in the macropeptide (de Koning et al., 1966; Woychik et al., 1966), as well as the single phosphorylated residue (Fig.6.2). The macropeptide is also the site of attachment for the carbohydrate residues (Mackinlay and Wake, 1965; Mackinlay et al., 1966; Fiat et al., 1972). The

macropeptide fraction containing carbohydrate (glyco-macropeptide) is soluble in 12% TCA, whereas the fraction with no carbohydrate is insoluble under these conditions.

Para-κ-casein, the N-terminal region of κ-casein, contains the cysteine residues and on gel electrophoresis in urea shows a major and a minor component moving towards the cathode. Kim et al. (1969) established by gel electrophoresis and amino acid analysis that this minor band was probably due to carbamylation of the lysine residues after their exposure to concentrated solutions of urea.

6.7 Nature of the Carbohydrate Residues in κ-Casein

The carbohydrate residues attached to κ-casein have been the subject of intensive investigations (see the review by Mackinlay and Wake, 1971). Recent evidence (Tran and Baker, 1970; Fiat et al., 1972) indicates the carbohydrate moeity is a trisaccharide unit, α-N-acetyl-neuraminyl-(2→6)-β-galactosyl-(1→3, or 1→6)-N-acetyl-galactosamine, that is attached to the peptide chain through the hydroxyl groups of serine or threonine (Fiat et al., 1968). The number of trisaccharide units has been suggested to vary from 0 to 5 per mole of protein (Rose et al., 1970).

6.8 Amino Acid Composition and Primary Structure of κ-Casein

After Jollès et al. (1970) described sequences accounting for half of the κ-casein molecule, Mercier et al. (1972b) and Brignon et al. (1972) elucidated the amino acid sequences of the macropeptide and para-κ-casein, thus completing the primary structure of κ-casein B (Fig.6.2).

κ-Casein B is a single polypeptide chain containing 169 amino acid residues with a molecular weight deduced from the amino acid sequence of 19 023 (Mercier et al., 1973). Woychik et al. (1966) obtained a similar value for the monomer molecular weight of κ-casein using sedimentation equilibrium measurements in 5 M guanidine.HCl. The amino acid composition of κ-casein B deduced from its amino acid

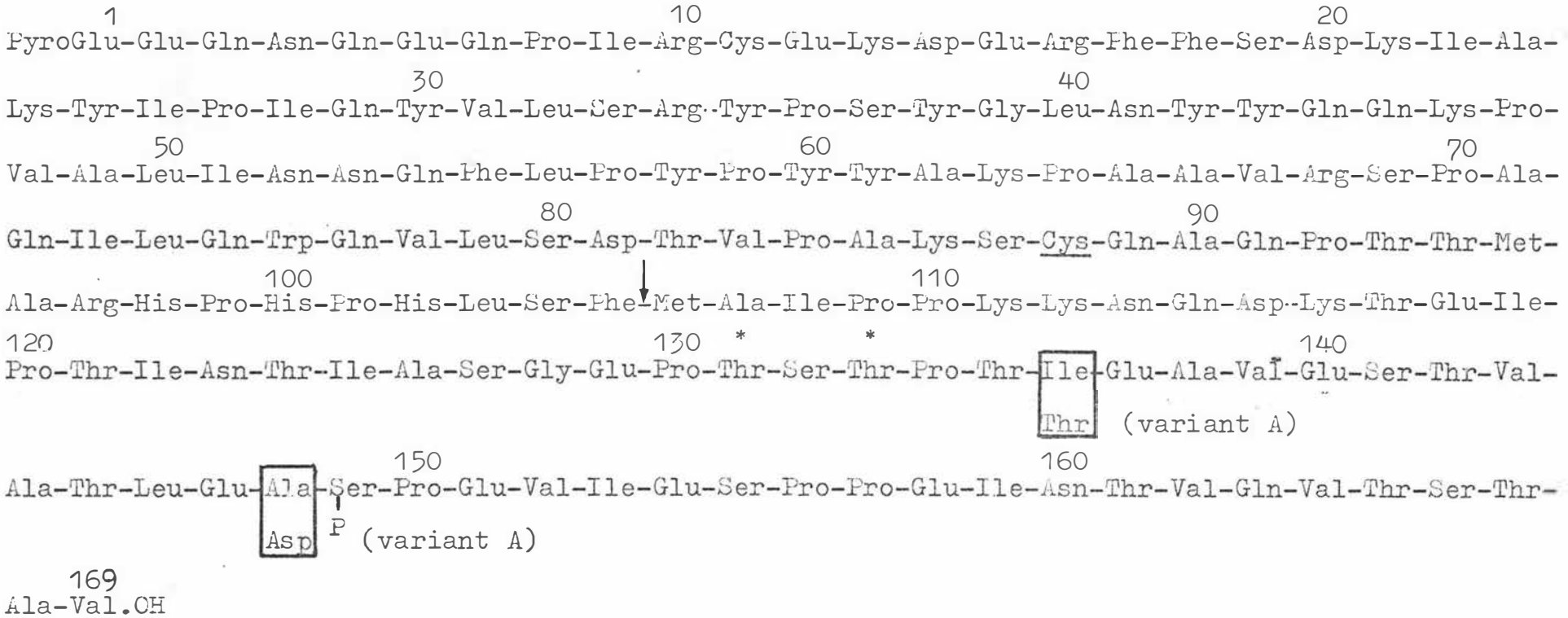


Fig.6.2: Primary structure of bovine κ-casein B. The enclosed amino acid residues are those corresponding to the double substitution differentiating the A and B genetic variants (Mercier et al., 1973). ↓ peptide bond readily cleaved by rennin.
* possible sites of attachment to the peptide chain for the trisaccharide unit in the glycopeptide characterized by Fiat et al. (1972). See text for further details.

sequence (Mercier et al., 1973) is:

Asp_4, Asn_7, Thr_{14}, Ser_{12}, $SerP_1$, Glu_{12}, Gln_{14}, $PyroGlu_1$, Pro_{20}, Gly_2, Ala_{15}, Val_{11}, Cys_2, Met_2, Ile_{13}, Leu_8, Tyr_9, Phe_4, Lys_9, His_3, Trp_1, Arg_5

which is in good agreement with those reported previously for the carbohydrate-free major component of κ-casein B (Schmidt et al., 1966; Woychik et al., 1966).

Para-κ-casein, the N-terminal region of κ-casein, has 105 residues and a molecular weight of 12 269, deduced from the amino acid sequence (Mercier et al., 1973). Its amino acid composition:

Asp_3, Asn_4, Thr_3, Ser_7, $PyroGlu_1$, Glu_4, Gln_{12}, Pro_{12}, Gly_1, Ala_9, Cys_2, Val_5, Met_1, Ile_6, Leu_7, Tyr_9, Phe_4, Lys_6, His_3, Trp_1, Arg_5

(Brignon et al., 1972) agrees fairly well with previous amino acid compositions (Kalan and Woychik, 1965; de Koning et al., 1966: Kim et al., 1969), recalculated where necessary by Swaisgood (1973) to the correct molecular weight. The presence of the N-terminal pyroglutamyl residue was deduced after mass spectrometry of the N-terminal peptide (Brignon et al., 1972). The labile peptide bond cleaved by rennin is between Phe_{105}-Met_{106}.

The B variant of the macropeptide, residues 105-169, contains 64 amino acid residues and has a molecular weight of 6754 (Mercier et al., 1972b). The amino acid compositions previously determined for this fraction (de Koning et al., 1966; Hill et al., 1970) are in good agreement with that deduced from the amino acid sequence:

Asp_1, Asn_3, Thr_{11}, Ser_5, $SerP_1$, Glu_8, Gln_2, Pro_8, Gly_1, Ala_6, Val_6, Met_1, Ile_7, Leu_1, Lys_3
(Mercier et al., 1973).

Genetic substitutions involving the A and B polymorphs previously noted by de Koning et al. (1966) and verified by Hill et al. (1970) are located at Thr/Ile_{136} and Asp/Ala_{148}

(Fig.6.2). The site of attachment of a trisaccharide characterized by Fiat et al. (1972) and Jollès et al. (1973) has tentatively been assigned as Thr_{131} or Thr_{133}, the former being the favoured position in κ-casein B.

The overall hydrophobicity of κ-casein B is between that for α_{s1}- and β-caseins at 5.37 kJ per residue and is characteristic of a hydrophobic protein (Mercier et al., 1973). The net negative charge at the pH of native milk (about pH 6.8) is very close to 3.5 for the monomer devoid of sialic acid. The high content (11.8%) and rather uniform distribution of proline residues prevent much α-helical organization of the molecule, in agreement with the previous optical rotatory dispersion measurements of Herskovits (1966). Both hydrophobic and charged amino acid residues are distributed non-uniformly along the peptide chain (Mercier et al., 1973) (Fig.6.2), whereas most of the basic residues are distributed uniformly in the section of the chain corresponding to para-κ-casein. The hydrophobic para-κ-casein contains all of the aromatic residues of κ-casein, and also the two cysteine residues which are located in two hydrophilic regions, one of these being a cluster of charged residues (Fig.6.2). Mercier et al. (1973) suggested that even if both regions are neighbouring through folding of the molecule, the charged nature of the groups surrounding the cysteine residues is unlikely to lead to favourable conditions for forming intra-molecular bonds. Instead, they are likely to be exposed and able to form inter-molecular disulphide linkages.

The macropeptide section of the κ-casein polypeptide chain has one in four of its residues as threonine or serine (Mercier et al., 1972b). According to Hill and Wake (1969) this region may be responsible for the micelle stabilizing properties of κ-casein. Mercier et al. (1973) suggest a possible biological role of the sugar residues may be to prevent phosphorylation of these threonine and serine residues, since the κ-caseins which contain carbohydrate appear to have no influence on micelle stability. Phosphorylation of these residues would undoubtedly affect the properties of κ-casein (i.e. solubility in Ca, and the

stabilization of α_{s1}- and β-caseins). They suggest that this implies glycosylation of κ-casein occurs to protect these threonine and serine residues before phosphorylation of the protein by phosphoryl kinases. Subsequent deglycosylation results in the observed mixture of proteins.

β-CASEIN

6.9 Isolation of β-Casein

β-Casein was first isolated by chemical fractionation of casein (Warner, 1944; Hipp et al., 1952). Garnier et al. (1964) purified β-casein variants by chromatography of whole casein on DEAE-cellulose in buffers containing urea. Thompson and Pepper (1964) also isolated pure β-casein A, B and C genetic variants by chromatography on DEAE-cellulose in urea of fractions obtained by the method of Hipp et al. (1952). Inclusion of 2-mercaptoethanol in the protein sample and in the buffers used for chromatography was found to remove the contaminating κ-caseins, otherwise normally associated with these purified β-caseins (Thompson, 1966). The procedures which may be used for isolating β-casein have been described in detail (McKenzie, 1967; Thompson, 1971).

6.10 Physical Properties of β-Caseins

The temperature dependent polymerization of bovine β-casein was first observed by von Hippel and Waugh (1955) and Sullivan et al. (1955).

At low temperatures, $\leq 4^{\circ}C$, β-casein exists in solution as a monomer with a molecular weight of 24 000 and a sedimentation coefficient, $s^{\circ}_{20,w}$, of approximately 1.50 S (Svedburg unit, 10^{-13} sec), which is virtually independent of protein concentration (Sullivan et al. 1955; Payens and van Markwijk, 1963; Noelken and Reibstein, 1968; Evans et al., 1971a). Payens and van Markwijk (1963) measured the intrinsic viscosity at $4^{\circ}C$ of bovine β-casein in sodium barbiturate buffer, pH 7.5, adjusted to an ionic strength of 0.2 with KCl, and obtained a value of 23 ml/mg. A similar value of 23.1 ml/g was obtained for the intrinsic viscosity of bovine β-casein B by Noelken and Reibstein (1968).

Furthermore, Noelken and Reibstein (1968) found virtually no change in the intrinsic viscosity of bovine β-casein B in going from 0.02 M EDTA-0.14 M NaCl, pH 7, at 2.5°C to 0.1 M phosphate buffer, pH 7, containing 6 M guanidine.HCl at 25°C. Tanford et al. (1967) showed using viscometry, that in 6 M guanidine.HCl a number of proteins were random coils, possessing little, if any structure. Consequently, the results of Noelken and Reibstein (1968) led them to suggest the β-casein monomer at low temperature has a conformation similar to a random coil. Optical rotatory dispersion parameters and NMR spectra suggest β-casein possesses little if any structure at low temperature (Herskovits, 1966; Garnier, 1966; Evans et al., 1971b).

Waugh et al. (1970) have considered that the high intrinsic viscosity of β-casein at low temperature could result from the highly solvated and flexible N-terminal region of the β-casein molecule which is very acidic and contains all of the organic phosphorus groups (Fig.6.3). Random coil formation, in the presence of 6 M guanidine.HCl, would decrease the contribution of the acidic peptide to viscosity, but increase the contribution of the remaining portion, so the net effect could be small.

Increasing the temperature of β-casein solutions above 4°C results in the formation of large polymers, concurrent with a decrease in the optical rotatory dispersion parameter, a_o (Garnier, 1966; Herskovits, 1966; Evans et al., 1971a, b), and a red shift in the absorption maximums of the aromatic residues (Garnier, 1966). This is indicative of a change from a more polar to a less polar environment for these residues.

At 8.5°C, and at concentrations exceeding about 0.15 g/100 ml of protein, polymers of β-casein are present. A rapidly sedimenting peak becomes apparent which has a sedimentation coefficient very dependent on protein concentration (Payens and van Markwijk, 1963). Similar results were obtained at 13.5°C. The degree of polymerization of β-casein was about 22 at 8.5°C, and even higher at 13.5°C, with a slow rate of equilibrium existing between monomers

and polymers. Payens and van Markwijk (1963) proposed that the β-casein polymer consisted of a number of inter-linked coils. Furthermore, unlike bovine α_{s1}-casein, no polymers of intermediate size were observed, the transition being of the type:

$$nM \rightleftarrows M^n$$

where n is the number of monomers (Payens et al., 1969).

Garnier (1966) as a result of optical rotatory dispersion and difference spectroscopy measurements, proposed β-casein undergoes a rapid and reversible endothermic transformation from state I at 5°C, to state II at 40°C, with a temperature of half transition of 23 to 24°C. He also suggested that β-casein at both low and high temperatures is partially folded in a left handed type poly-L-proline II helix. Evans et al. (1971a) suggested the presence of a similar structure to the poly-L-proline II helix in β-casein, as a result of their studies using 8 M LiBr. The amino acid sequence of β-casein (Fig.6.3) has 5 pairs of proline residues adjacent to one another, as well as several proline residues in alternating sequence, which are mostly located in hydrophobic regions. Swaisgood (1973) concluded from this that large regions of left handed poly-L-proline II helix were unlikely, although the existence of a similar structure could not be overruled. NMR studies (Evans et al., 1971b) suggested that the β-casein polymers, even at 25°C, still possessed a high degree of inter- and intra-chain flexibility. Spectrophotometric titration of β-casein B at 25°C indicated the 4 tyrosine residues in the molecule are still exposed to the solvent (Creamer, 1972).

Payens et al. (1969) using light scattering, showed β-casein at 21°C in neutral sodium phosphate buffer, I=0.2, had a polymer molecular weight of 725 000, and consisted of about 50 monomers. They suggested the association of β-casein closely resembles micelle formation in soap solutions (Payens, 1966; Payens et al., 1969). This is likely, considering β-casein has a hydrophilic N-terminal region of approximately 47 residues and a largely hydrophobic

tail (Fig.6.3). Sullivan et al. (1955) obtained an intrinsic viscosity for β-casein at 25°C, in neutral buffer, of 12.5 ml/g. Common globular proteins have intrinsic viscosities of 3-4 ml/g (Tanford, 1961). Consequently these results suggest the β-casein polymer may be highly solvated and highly asymmetric.

The β-casein polymer is sensitive to Ca, and is precipitated in its presence at low levels (Garnier, 1966; Thompson et al., 1969c; Waugh et al., 1970). Waugh et al. (1970) examined the behaviour of β-casein in the presence of Ca and found sedimentation rates were a function of both temperature and Ca concentration. In 0.048 M NaCl at 37°C and pH 6.6, the value of $s_{20,w}$ increased with Ca concentration until precipitation occurred at 7 to 7.5 mM. Likewise, increasing the temperature of the solution containing 0.048 M NaCl and 0.015 M Ca produced an increase in $s_{20,w}$ until precipitation occurred near 26 to 27°C. A plateau in the $s_{20,w}$ values between 21°C and 25°C occurred, with the attainment of limiting values of reduced viscosity. They also suggested the polymers had attained limiting average values of size, shape and solvation.

In further studies of these "core polymers" (Waugh et al., 1971), precipitation occurred at a site bound Ca level of 5.4 g atoms/mole and a net charge of -6, independent of NaCl concentration at pH 6.6 and 37°C. Dickson and Perkins (1971) in binding studies using ^{47}Ca, obtained a similar value (4.8 g atoms/mole). There are 5 phosphorylated serine residues in bovine β-caseins (Fig.6.3) (except the C variant), and hydrogen ion equilibria studies (Creamer, 1972) in the presence of Ca are consistent with the binding of Ca to these phosphoserine groups. Acylation of β-casein with increasing alkyl chain lengths, increases its ability to polymerize (Hoagland, 1968; Evans et al., 1971b) while increasing the negative charge on the protein by carboxyacylation decreases this ability (Evans et al., 1971a). Removal of the three C-terminal hydrophobic residues, Ile-Ile-Val-OH, from β-casein C by carboxypeptidase A altered the temperature dependent polymerization of this variant

(Thompson et al., 1967). β-Casein C tends to associate more than the A and B variants (Thompson, 1971), although their general behaviour is similar. These results all serve to indicate the importance of hydrophobic interactions in the aggregation of β-casein.

6.11 Amino Acid Composition and Sequence of β-Casein

The complete amino acid sequence of bovine β-casein A^2 has recently been elucidated by Ribadeau Dumas et al. (1972). It is a single polypeptide chain containing 209 amino acid residues with an amino acid composition deduced from the sequence of:

Asp_4, Asn_5, Thr_9, Ser_{11}, $SerP_5$, Glu_{18}, Gln_{21}, Pro_{35}, Gly_5, Ala_5, Val_{19}, Met_C, Ile_{10}, Leu_{22}, Tyr_4, Phe_9, Trp_1, Lys_{11}, His_5, Arg_4

The amino acid sequence of β-casein A^2 and the sites of genetic substitution are shown in Fig.6.3 . The molecular weights previously determined for the β-casein monomer using physical techniques (Sullivan et al., 1955; Payens and van Markwijk, 1963; Noelken and Reibstein, 1968) are in excellent agreement with that calculated from the sequence of the A^2 variant (23 983) (Ribadeau Dumas et al., 1972).

The amino acid compositions previously determined for the different variants of β-casein (Pion et al., 1965; Peterson et al., 1966; Thompson et al., 1969b) are also in good agreement with those determined from their amino acid sequences (Grosclaude et al., 1972). The variation in the number of serine residues in these amino acid compositions has been attributed to the extreme acid lability of the phosphoserine residues (Swaisgood, 1973).

Substitutions resulting in the different genetic variants were located after comparing their difference peptides with those from β-casein A^2 (Grosclaude et al., 1972). Compared to the A^2 variant, the following substitutions were found to result in the different genetic variants (Fig.6.3): A^2/A^3, His/Gln_{106}; A^2/A^1, Pro/His_{67}; A^2/B, Pro/His_{67}, and Ser/Arg_{122}; A^2/C, $SerP/Ser_{35}$, Glu/Lys_{37}, and Pro/His_{67}.

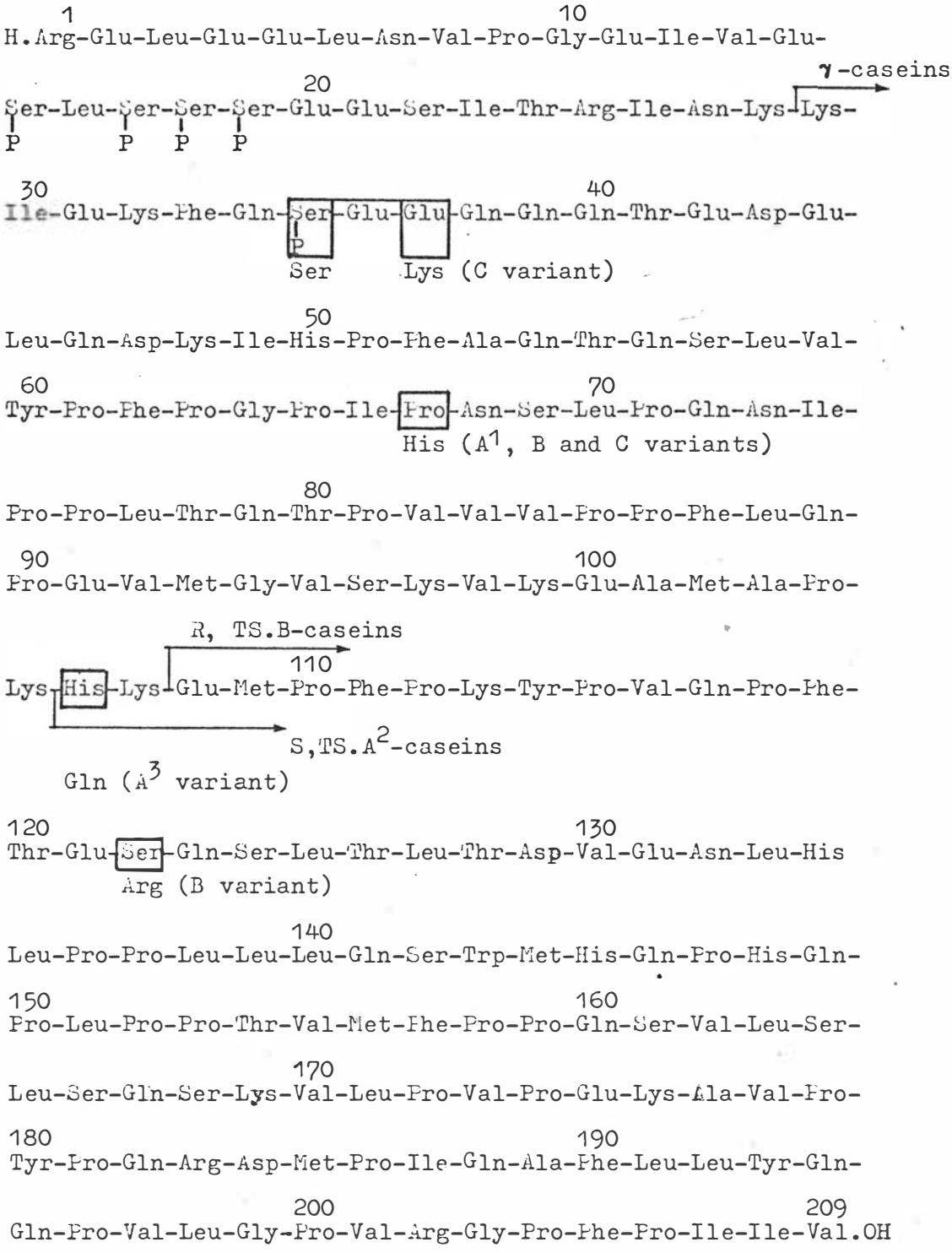


Fig.6.3: Primary structure of bovine β-casein A^2 (Ribadeau Dumas et al., 1973). The regions of the sequence which correspond to the γ-, R-, S- and TS-caseins are also indicated. Enclosed amino acid residues are those corresponding to substitutions which differentiate the A^1, A^3, B and C genetic variants from the A^2 variant.

The amino acid sequence of the β-casein B_z variant observed in Zebu cattle (Bos indicus) (Thompson et al., 1969b) has not yet been determined.

The five phosphorus residues in β-casein A^2 are covalently linked to serine residues located in the N-terminal region of the molecule (Fig.6.3), four of these phosphorylated residues being in a cluster. Peterson et al. (1958) isolated a N-terminal tryptic peptide containing four of these phosphorylated serine residues, which was later sequenced by Hanson and Annan (1971). These phosphate groups were shown to be O-esterified to the hydroxyl groups of serine residues.

The N-terminal region of β-casein A^2, with its four phosphoserine residues and glutamyl residues (Fig.6.3) constitutes a highly negatively charged zone. The net negative charge of this N-terminal region at pH 6.6 is approximately 12 (Swaisgood, 1973), while the remainder of the molecule has a net charge of essentially zero. With the exception of the N-terminal end, β-casein A^2 contains a high proportion of hydrophobic residues. Ribadeau Dumas et al. (1973) suggested the molecule has a rather loose structure at room temperature, while the high content of hydrophobic residues and the presence of clusters of such groups suggested a more or less stable structure due to hydrophobic interactions.

6.12 γ-Caseins

From the results of amino acid analyses, peptide mapping, molecular weight determinations and from studies of terminal residues, Gordon et al. (1972) recently suggested that γ-, R-, S-, and TS-caseins might be fragments of β-casein, a suggestion verified by Ribadeau Dumas et al. (1973). Individual milks containing β-casein A^1, A^2, A^3, or B always contained the corresponding γ-casein variants, which are designated in a similar manner (Groves and Kiddy, 1970). The γ-caseins, R, S and TS-caseins are derived from the C-terminal half of β-casein (Fig.6.3) (Groves et al., 1973). Previously TS-A^2 and R-casein had been observed with β-casein

A^2 variants, whereas the TS-B and S-caseins were present with β-casein B (Groves, 1969). γ-Casein corresponded to residues 29-209 of the β-casein sequence (Fig.6.3), while S- and TS-A^2 caseins corresponded to residues 106-209, and R- and TS-B to residues 108-209 (Ribadeau Dumas et al., 1972; Groves et al., 1973). The amino acid substitutions which differentiated the known variants of β-casein (Grosclaude et al., 1972) also explained the observed relationship between the polymorphism of β-, γ-, R-, S- and TS-caseins, but not the absence of the γ-C variants in milks containing the β-casein C variant (Rose et al., 1970). The similarity between some products of β-casein hydrolysis by milk protease and the minor caseins was demonstrated recently by Yamauchi and Kaminogawa (1972).

α_s-CASEINS

6.13 Isolation of α_{s1}-Caseins

α_s-Casein was first isolated by chemical fractionation (Warner, 1944; Hipp et al., 1952) and later from "first cycle" soluble casein, obtained by high speed centrifugation of skim milk at 37°C (Waugh et al., 1962). Thompson and Kiddy (1964) described a method to purify the A, B and C variants by chromatography on DEAE-cellulose of a crude α_{s1}-casein fraction prepared by the urea fractionation method of Hipp et al.(1952). α_{s1}-caseins were also purified from whole casein by chromatography on DEAE-cellulose (Thompson, 1966). McKenzie (1970, 1971) has outlined the various procedures used to isolate α_{s_1}-casein in some detail.

6.14 Physical Properties of α_s-Caseins

Bovine α_{s1}-casein in solution readily undergoes association to form polymers. Unlike β-casein, polymer formation is highly dependent on ionic strength and pH, and is virtually independent of temperature.

Sedimentation studies on α_{s1}-casein B in phosphate buffer, pH 7, (I=0.1) and light scattering measurements have indicated the dependence of $s_{20,w}$ values and apparent molecular

weights on protein concentration (Irons et al., 1973; Schmidt and Payens, 1972; Payens and Schmidt, 1966; Schmidt and van Markwijk, 1968; Schmidt, 1970a, b; Swaisgood and Timasheff, 1968). The behaviour of α_{s1}-casein is typical of rapidly equilibrating associating protein systems.

The association of α_{s1}-casein B is dependent on pH (Schmidt et al., 1967) and ionic strength (Schmidt and van Markwijk, 1968; Schmidt, 1970a), and is virtually independent of temperature (Schmidt and Payens, 1972; Irons et al., 1973), indicating the importance of both hydrophobic and electrostatic interactions. Schmidt and van Markwijk (1968) and Schmidt (1970a) employed light scattering to characterize the association of α_{s1}-casein B in pH 6.6 imidazole buffers of different ionic strength. The association of α_{s1}-casein can be described in terms of consecutive association steps, and at an ionic strength of 0.05 it was found that aggregates up to tetramer were present. Increasing the ionic strength increased the degree of polymerization. At low protein concentrations, further dissociation of α_{s1}-casein aggregates occurred and at infinite dilution, monomers of molecular weight 23 000-24 000 were obtained (Schmidt and van Markwijk, 1968). Ho and Chen (1967), by diffusion measurements in 0.01 M KCl, pH 7.02, have also shown α_{s1}-casein is dissociated to the monomer at low ionic strengths.

Optical rotatory dispersion parameters of α_{s1}-casein (Herskovits, 1966; Irons et al., 1973; Ho and Chen, 1967) indicated α_{s1}-casein in solution has only a limited amount of α-helical structure. NMR studies at 25°C (Irons et al., 1973) indicated a high degree of flexibility in the α_{s1}-casein molecule. Viscosity measurements on α_{s1}-casein B indicated little change in the intrinsic viscosity of α_{s1}-casein B between 4.9°C and 37°C in 0.01 M KCl, pH 7.02, with values ranging from 11.8 ml/g to 10.2 ml/g as the temperature was increased (Ho and Chen, 1967). In 0.1 M KCl at pH 7 and 20°C, when polymers of α_{s1}-casein are present the intrinsic viscosity was 7.7 ml/g, whereas in 6 M guanidine.HCl the

intrinsic viscosity is 19.2 ml/g. It is interesting to note that at pH 12, α_{s1}-casein C has an intrinsic viscosity of 19.5 ml/g (Swaisgood and Timasheff, 1968). The dissociation of α_{s1}-casein aggregates above about pH 9 has been attributed to the increase in the net negative charge on the protein.

Although α_{s1}-casein is not typical of a native globular protein, being asymmetric and probably highly solvated (Ho and Chen, 1967; Swaisgood and Timasheff, 1968), it nonetheless has a more compact structure than a random coil, typified by proteins in 6 M guanidine.HCl and in particular has a more compact structure than β-casein.

α_{s1}-Casein C has similar properties to the B variant, except it tends to associate to a greater extent (Swaisgood and Timasheff, 1968; Schmidt, 1970b). This increased association has been attributed to the slightly lower net charge on this variant, as a result of the amino acid substitution from glutamic acid in the B variant to glycine in the C variant. Succinylation of α_{s1}-casein B, which doubles the net negative charge at pH 6.6, decreases the extent of association, while alkylation of the protein increased the tendency to associate, despite the increase in charge (Irons et al., 1973).

Ho and Waugh (1965) using infrared spectroscopy, concluded that the phosphate groups in α_{s1}-casein were the strongest Ca binding sites. Later, Waugh et al. (1970) observed that irrespective of NaCl concentration at pH 6.6, protein precipitation was initiated at the same level of site-bound Ca and net charge, namely 8.0 and -8 [recalculated to a molecular weight of 23 600 by Swaisgood (1973)]. Dickson and Perkins (1971) using ^{47}Ca, estimated that native α_{s1}-casein bound 8.5 g atom/mole while dephosphorylated α_{s1}-casein bound 1.2 g atom/mole. From solubility studies Bingham et al. (1972) concluded that 7 g atom/mole are bound prior to precipitation, and an additional 3 moles during precipitation of the native protein, in qualitative agreement with the results of Noble and Waugh (1965). No evidence of significant binding of Ca to

dephosphorylated α_{s1}-casein was obtained, although 2 g atom/mole were bound during precipitation. Slightly higher concentrations of $CaCl_2$ are needed to precipitate dephosphorylated protein using identical protein concentrations. It appears that additional site binding of Ca to α_{s1}-casein does occur, though with a lowered binding constant, since there are 8 phosphoserine residues in the molecule.

Limited physical data is available on the behaviour of α_{s1}-casein in solution with Ca, since precipitation is initiated at low levels. α_{s1}-Casein is readily precipitated by 4-5 mM $CaCl_2$ at 37°C (Thompson et al., 1969c; Bingham et al., 1972; Noble and Waugh, 1965). The A variant which has a deletion of 13 residues (Fig.6.4) behaves in a different manner from the other variants (Thompson et al., 1969c), with pronounced solubility differences for the A and B variants between 1°C and 30°C. Additional binding of Ca to α_{s1}-casein occurs at high $CaCl_2$ levels (>80 mM), since free boundary electrophoresis showed the protein attains a net positive charge under these conditions (Bingham et al., 1972).

6.15 Amino Acid Composition and Primary Structure of α_{s1}-Caseins

The primary structure of α_{s1}-casein B was recently elucidated by Mercier et al. (1971) (Fig.6.4). The peptide chain has 199 residues and is characterized by the presence of 8 phosphoseryl residues, shown by the use of alkaline phosphatase to be O-phosphorylated (Mercier et al., 1971). Like β-casein, four of these phosphorylated residues are located close together in the highly charged region between residues 43 and 70. Three regions of the molecule are highly hydrophobic, segments 1-44, 90-113 and 132-199, with most of the aromatic residues being located in this last region (Ribadeau Dumas et al., 1973).

The amino acid composition of α_{s1}-casein B, determined from its sequence is: Asp_7, Asn_8, Thr_5, Ser_8, $SerP_8$, Glu_{24}, Gln_{15}, Pro_{17}, Gly_9, Ala_9, Val_{11}, Met_5, Leu_{17}, Tyr_{10}, Phe_8, Lys_{14}, Ile_{11}, His_5, Trp_2, Arg_6.

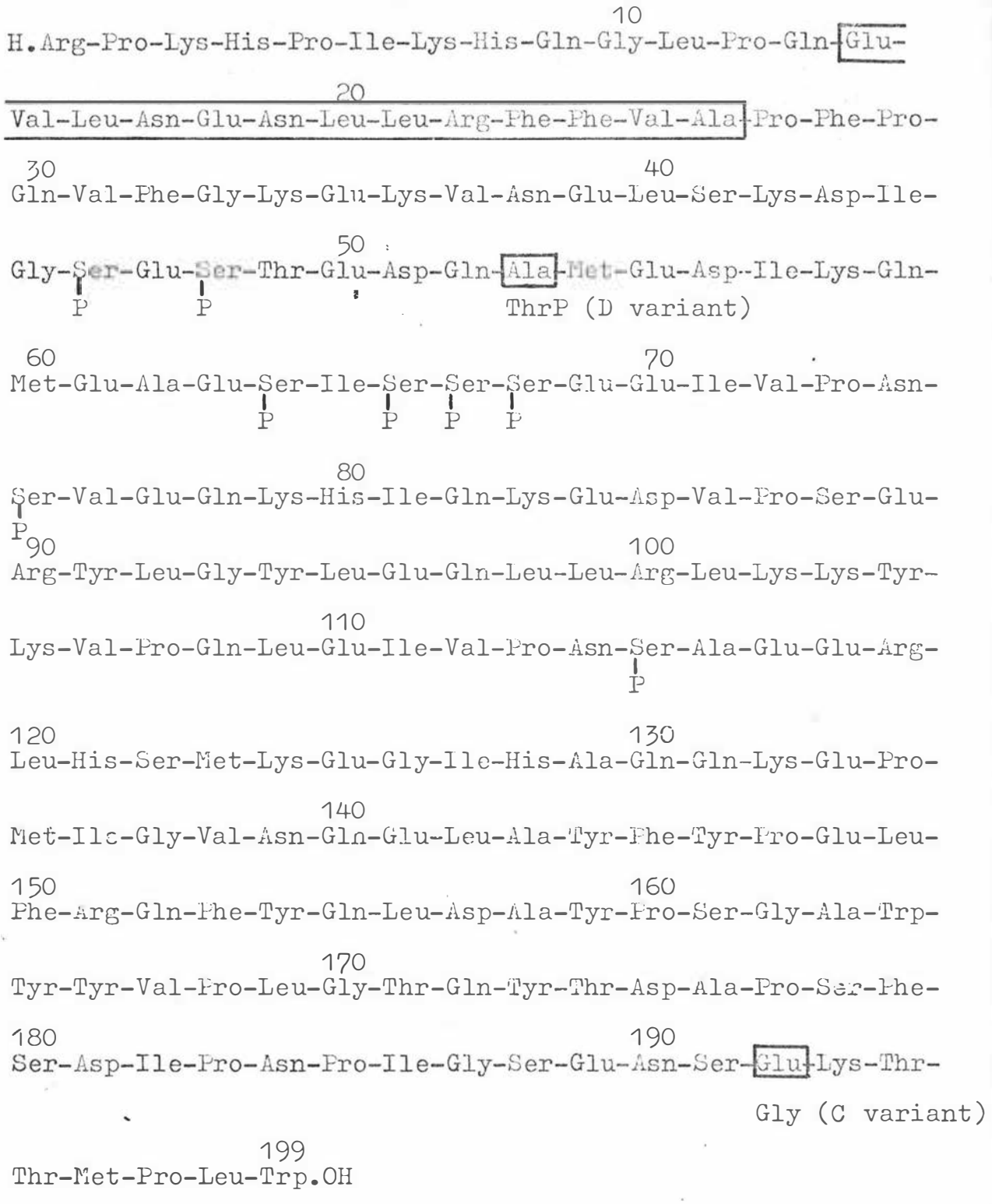


Fig.6.4: Primary structure of bovine α_{s1}-caseins (Ribadeau Dumas et al. 1973). Enclosed amino acid residues are those corresponding to mutations which differentiate A, C and D genetic variants from the B variant. The enclosed residues, 14-26, represent the sequence deleted in the A variant.

According to the sequence, the molecular weight of the monomer is 23 615 and its net negative charge at the pH of native milk (about 6.8) is very close to 22. The molecular weight values of α_{s1}-casein, determined using physical methods (Neilsen and Lillevik, 1957; Melnychyn and Wolcott, 1965; Driezen et al., 1962; Schmidt and Payens, 1963; Schmidt et al., 1967; Swaisgood and Timasheff, 1968) were in the range, 16 500 to 29 600, although the more recent results of Noelken (1967), 24 600, and Garnier (1967), 24 300, are closer to the value deduced from the sequence. The amino acid compositions of the α_{s1}-caseins previously determined by Gordon et al., (1965), de Koning and van Rooijen (1965, 1967) were similar to that calculated from the amino acid sequence (Mercier et al., 1971), providing they were recalculated to the correct molecular weight (Swaisgood, 1973).

The genetic variants, B/C, result from the substitution, Glu/Gly_{192}, whereas the B/D variants are the result of the substitution, $Ala/ThrP_{53}$ (Grosclaude et al., 1972) (Fig.6.4). α_{s1}-Casein A, when compared with the B variant has residues 14 to 26 deleted from the molecule (Grosclaude et al., 1970), possibly a result of unequal crossing-over within the α_{s1}-casein locus (Grosclaude et al., 1973). Several chemical and physical properties of this variant are also altered (Thompson, 1971). There are two polar segments in α_{s1}-casein, 45-89 and 114-131, the former being significant since it contains more than half the acidic residues, and in particular, seven of the eight phosphoseryl residues (Mercier et al., 1971).

6.16 Minor α_s-Caseins

Annan and Manson (1969) partially fractionated the group of α_s-caseins. They isolated pure α_{so}-, α_{s1}- and α_{s5}-caseins, while α_{s3}- and α_{s4}-caseins were present, together with a small amount of α_{s2}-casein, in another fraction. The C-terminal sequence of α_{so}-casein is Leu-Trp.OH, with a molecular weight determined from these C-terminal studies of 28 000 (Ribadeau Dumas et al., 1973). Annan and Manson

(1969) postulated the presence of two chains in α_{s5}-casein, α_{s3}- and α_{s4}-caseins having leucine and tyrosine respectively as C-terminal amino acids.

Ribadeau Dumas et al. (1973) isolated and partially characterized α_{s3}- and α_{s4}-caseins, and suggested a molecular weight of 26 000 for these proteins, with a C-terminal sequence of Leu-Tyr.OH. Hoagland et al. (1971) determined the amino acid composition of α_{s5}-, α_{s3}- and α_{s4}-caseins, and suggested that α_{s5}-casein was a dimer consisting of α_{s3}- and α_{s4}-caseins, the individual caseins having a molecular weight of 33 700. Gel electrophoresis of α_{s5}-casein in the absence of 2-mercaptoethanol at alkaline pH indicated a band with a mobility slightly greater than β-casein. In the presence of 2-mercaptoethanol this band was reduced with the formation of two new protein bands, α_{s3}- and α_{s4}-casein which had mobilities near to that of α_{s1}-casein (Fig.4.1). These caseins are different from the others, particularly in their cystine content and high lysine content (Hoagland et al., 1971). The fraction, α_{s5}-casein is more sensitive to Ca than bovine α_{s1}-caseins, being readily precipitated in 2 mM $CaCl_2$ (Toma and Nakai, 1973).

6.17 Homologies Within the Caseins

Mercier et al. (1971) observed homology between residues 13-21 of bovine β-casein and residues 62-70 of bovine α_{s1}-casein (Fig.6.5). Lyster (1973) suggests the two proteins may share a common evolutionary origin, since it is extremely unlikely these homologies have occurred by chance. He also found considerable homology between regions 25-39, 8-16 and 47-56 from α_{s1}-casein with regions 84-100, 148-156 and 34-43 respectively of β-caseins (Fig.6.5). There are two repeating sequences in the α_{s1}-casein molecule, 70-84 and 110-125, and 18-23 and 96-101 (Mercier et al., 1971; Lyster, 1973). No repeating sequences which could not have occurred by chance were observed in β-casein (Lyster, 1973).

6.18 A Possible Mechanism of Casein Phosphorylation

Phosphorylation of proteins is thought to occur post-ribosomally, and experiments dealing with the effect of

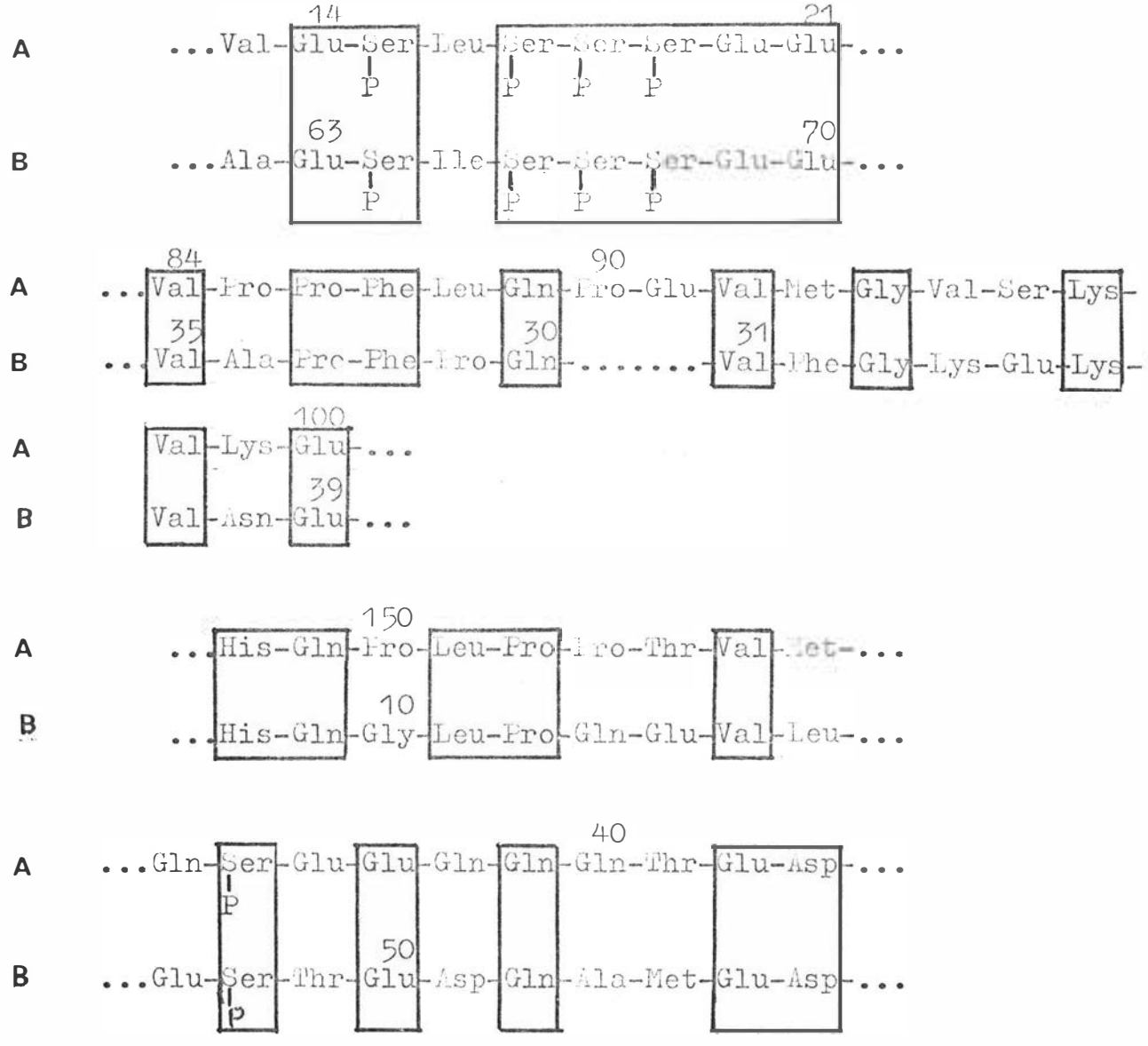


Fig.6.5: Homologies in the sequences of bovine α_{s1}- and β-caseins observed by Ribadeau Dumas et al. (1973) and Lyster (1973). A and B denote regions from the sequences of α_{s1}- and β-caseins, respectively.

puromycin on casein biosynthesis in rat and mouse mammary gland explants have supported the concept that phosphate is incorporated after completion of the protein molecule (Turkington and Topper, 1966; Singh et al., 1967).

Comparison of the sequences of β-casein and α_{s1}-casein led Mercier et al. (1972a) to suggest the sequence of amino acids around the phosphorylated and phosphate free hydroxy amino acids provided evidence of enzymatic phosphorylation. All phosphorylated residues occur in position n with relation to a glutamyl or a phosphoseryl residue in position n+2 (Ribadeau Dumas et al., 1973). Mercier et al. (1972a) suggested a phosphoryl kinase may recognise an anionic phosphorylation site corresponding to the tripeptide sequence Ser/Thr-X-Glu/SerP, where X is any amino acid.

This hypothesis was verified by examination of the α_{s1}-casein D genetic variant where an additional phosphate group is located on a threonyl residue ($ThrP_{53}$), resulting from the substitution Ala/Thr_{53}, in the tripeptide sequence $-Ala_{53}$-Met-Glu_{55}- (Grosclaude et al., 1972). Grosclaude et al. (1972) suggest this extra phosphorylated residue may be a result of the phosphoryl kinase recognising a new phosphorylation site. Similarly, the lack of one phosphate group on Ser_{35} in the β-casein C variant may be explained by the disappearance of the phosphorylation site $-Ser_{35}$-Glu-Glu_{37}- due to the amino acid substitution Glu/Lys_{37} (Mercier et al., 1972a). The macropeptide from κ-casein contains several hydroxyamino acids, which correspond to residues 127, 135, 145 (and subsequently 133 and 131) in κ-casein that are located in sites which should be phosphorylated. Mercier et al. (1972a) suggested the lack of phosphate in these potential phosphorylation sites may be due to their inaccessibility to phosphoryl kinase. Such steric hindrance may be due to tertiary configuration or to the presence of carbohydrate moieties on these hydroxyamino acid residues.

6.19 Phylogenetic Relationships of the Bovine Caseins

The elucidation of the amino acid sequences of the bovine caseins (Mercier et al., 1973; Ribadeau Dumas et al.,

1973) enabled Grosclaude et al. (1973) to draw further conclusions on their phylogenetic relationships.

The linkage between the α_{s1}-Cn and β-Cn loci was discovered by Grosclaude et al. (1964) and by King et al. (1965). The linkage of the κ-casein locus to the α_{s1}- and β-casein loci was demonstrated by Grosclaude et al. (1965) and Larsen and Thymann (1966). As well as considering the alleles of the individual loci, they also examined the "gene combinations" or "gene complexes" formed by the closely linked alleles of the "clustered loci", since these gene complexes appear to be transmitted from one generation to another. Grosclaude et al. (1972) consider the α_{s1}-Cn^{B}-β-Cn^{A2} "gene complex" as the original type from which, by successive mutations either at the α_{s1}-Cn or β-Cn locus, the other gene complexes have been derived. The phylogeny of the common α_{s1}-Cn-β-Cn gene complexes found in bovine populations is shown in Fig.6.6.

As a result of their population surveys on gene complexes, Grosclaude et al. (1972) suggested that the mutation sites corresponding to the more common α_{s1}- and β-casein variants were very close together on the chromosomal DNA, closer than to the κ-casein mutation site. They further suggested the α_{s1}- and β-casein loci were adjacent, and their mutation sites were located in their adjacent terminal parts (Grosclaude et al., 1969). Elucidation of the amino acid sequences of the bovine caseins has led Grosclaude et al. (1972) to propose that the initiator end of the β-Cn locus (N-terminal) is adjacent to the α_{s1}-Cn terminal end (C-terminal). The proposed model of the organization of the α_{s1}-Cn-β-Cn-κ-Cn "cluster of genes" is shown in Fig.6.7 (Grosclaude et al., 1973). According to Grosclaude et al. (1973) this model explains the rare occurrence of recombinant types between α_{s1}-Cn^{C} and β-Cn^{A1}, β-Cn^{B}, β-Cn^{C}, since the distances between the corresponding mutation sites on the chromosomal DNA would be only 73, 128 and 143 codons, respectively (Grosclaude et al., 1973). The κ-Cn locus is thought to be further away from the α_{s1}-Cn and β-Cn loci, since existing gene complexes, if not too rare, appear to be

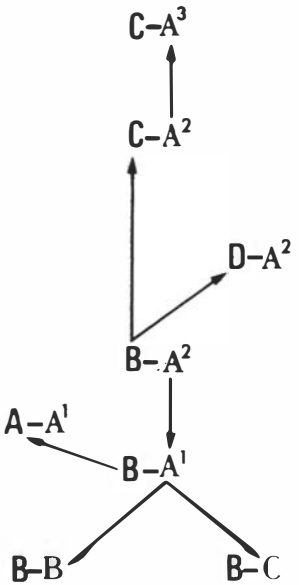


Fig.6.6: Phylogeny of the "gene complexes" formed by the alleles of the bovine α_{s1}-Cn and β-Cn loci. The α_{s1}-Cn^{B}-β-Cn^{A^2} complex ($B-A^2$ on the figure) is considered by Grosclaude et al. (1973) as the original type from which, by successive mutations either at the α_{s1}-Cn or at the β-Cn locus, the other "gene complexes" have been derived. The bold letters denote the α_{s1}-Cn loci.

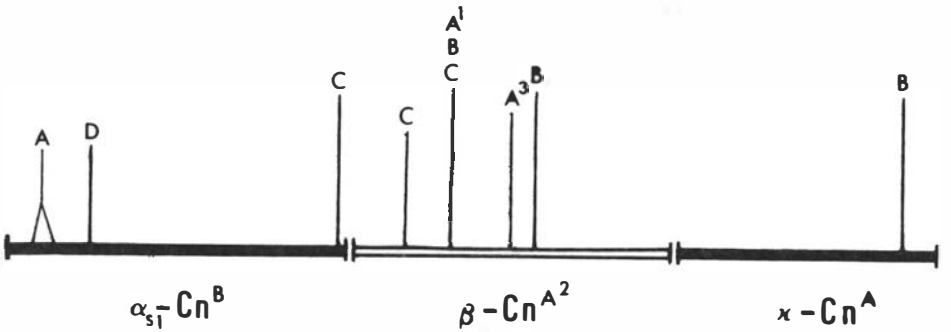


Fig.6.7: The model for the organization of the α_{s1}-Cn-β-Cn-κ-Cn "cluster of genes", proposed by Grosclaude et al. (1973). The mutation sites for the genetic variants are indicated. The longest arrows correspond to the more common variants.

associated in a nonspecific manner with κ-Cn^{A} or κ-Cn^{B}. That the κ-Cn locus is after the β-Cn locus is suggested by the association of α_{s1}-casein D with A or B variants of κ-casein (Grosclaude et al., 1973).

CASEINS FROM OTHER SPECIES

Our knowledge of the caseins of other species, at least up until the last three years was fairly limited, especially for some of the non-domestic species, and was largely restricted to electrophoretic analyses. Recently however, with the elucidation of the primary structures of the bovine caseins, attention has begun to focus on the caseins of other species. This has resulted in a considerable number of publications, in particular, on the ovine and human caseins. As a pre-requisite to an investigation of the association of the caseins from other species to form casein micelles, it is necessary to determine their chemical and physical properties.

This section is divided into subsections on the caseins of the major species (i.e. caprine, ovine, buffalo, human) and also includes some of the preliminary work on less common caseins.

6.20 Caprine Caseins

The first electrophoretic analyses of the proteins from caprine milk used free boundary electrophoresis (Dovey and Campbell, 1952) and paper electrophoresis (Schulte and Muller, 1955; Hofman, 1958a; Sloan et al., 1961). Sloan et al. (1961) examined the electrophoresis patterns produced by mammalian species, representing some eight orders of Therian mammals.

Hofman (1958a) identified two casein fractions, which he designated as α- and β-, in order of their decreasing electrophoretic mobilities. Using the method developed by Warner (1944) for bovine casein, Hofman (1958b) was able to isolate the α- and β-fractions from caprine casein. The α- fraction contained 1.02% phosphorus while the β- fraction contained 0.61% phosphorus, similar to their bovine counterparts.

Alkaline polyacrylamide gel electrophoresis in 4.5 M urea resolves caprine casein into a series of bands, some with a lower mobility than bovine α_s-casein, and two

prominent bands with a similar mobility to bovine β-casein (Fig.4.1) (Assenat, 1967; Parkash and Jenness, 1968; Zittle and Custer, 1966). Macha (1970) observed polymorphism in goat's milk by starch gel electrophoresis, one protein band being di-allelic and another tetra-allelic. These bands have yet to be positively identified and the results substantiated. Bogdanov et al. (1972) have observed polymorphism in the caprine α_s-caseins although few details are available.

Zittle and Custer (1966) employing the sulphuric acid-urea extraction method developed for bovine κ-casein, isolated κ-casein as a single major component from caprine casein. They concluded that in alkaline gel electrophoresis of whole casein, the κ-casein was obscured by the two dominant bands in the β-casein region. Caprine κ-casein was readily hydrolysed by rennin to form para-κ-casein, and was soluble in $CaCl_2$, and able to stabilize caprine α_s-casein and bovine α_s-casein against precipitation by $CaCl_2$ (Zittle, 1966; Zittle and Custer, 1966). Zittle (1967) described a method for separating α_s- and κ-casein by fractional precipitation with sodium polyphosphate from acid solutions of bovine or caprine whole casein in 2 M urea.

The sialic acid content of caprine κ-casein (0.3%) is considerably lower than that of bovine κ-casein (>2.0%) (Zittle and Custer, 1966). Jollès et al. (1964) isolated and partially characterized glycomacropeptides hydrolysed from caprine casein with rennin, and found 2.8% sialic acid, compared with 10.2% sialic acid in the glycomacropeptides isolated from bovine casein. Levels of galactosamine and galactose were also lower in caprine glycomacropeptides compared with those of their bovine counterparts (Alais and Jollès, 1961; Jollès et al., 1964). Sialic acid in the caprine glycomacropeptides was found to be a mixture of N-acetyl and N-glycollyl-neuraminic acid, unlike the bovine glycomacropeptides which only contain N-acetyl-neuraminic acid (Alais and Jollès, 1961; Jollès et al., 1964; Cabezas et al., 1968). Glutamic acid and valine were released from the C-terminal of the caprine macropeptides by the action of

carboxypeptidase A, whereas the C-terminal of bovine κ-casein, and the macropeptides has recently been shown to be Ala-Val. OH (Mercier et al., 1973).

6.21 Ovine Caseins

Paper electrophoresis of ovine casein revealed two major fractions which were analogous to the bovine α- and β-caseins (Sloan et al., 1961). Genetic polymorphism was observed in the ovine α_s - and β-caseins of several breeds by starch gel electrophoresis (King, 1966; Tanev, 1967; Tanev, 1969; Bogdanov et al., 1972; El-Negoumy and Burfening, 1972; Arave et al., 1973) and polyacrylamide gel electrophoresis (Assenat, 1967). Only two variants were observed for each casein and the frequency with which they occurred in 592 samples of milk from 6 breeds was very low, being 0.036 for $\alpha\text{-Cn}^{AB}$ and 0.17 for $\beta\text{-Cn}^{AB}$ (Arave et al., 1973). Similar results were found by King (1966), indicating there may be less variation in the ovine caseins than the widespread polymorphism evident for bovine caseins.

Alais and Jollès (1967) isolated two major κ-caseins which they suggested were genetic variants and designated them as κ-casein A and B. The presence of multiple forms of ovine κ-casein has since been reported by Ribadeau Dumas et al. (1975).

Ovine casein, when treated with rennin releases glycomacropeptides corresponding to those released from bovine and caprine whole caseins (Alais and Jollès, 1961; Jollès et al., 1961). Like their caprine counterpart, glutamic acid and valine were released from the C-terminal by carboxypeptidase A. The sialic acid content (1.0%) of the ovine glycomacropeptides was lower than in either the caprine or bovine glycomacropeptides (Jollès et al., 1961; Jollès et al., 1964), but like caprine glycomacropeptides was composed of N-acetyl- and N-glycollyl-neuraminic acid.

Mercier et al. (1968) resolved ovine casein into four major peaks by chromatography on DEAE-cellulose in buffers containing urea. Ovine κ-casein A and B were isolated from

a crude fraction by chromatography on DEAE-cellulose and their amino acid compositions were determined (Alais and Jollès, 1967). The amino acid compositions of ovine κ-casein A and B were similar. The major difference between ovine and the bovine κ-casein was the greater content of aspartic acid (Alais and Jollès, 1967; Ribadeau Dumas et al., 1975). The κ-caseins were readily hydrolysed by rennin and consisted of both major and minor components on starch gel electrophoresis similar to bovine κ-casein. The recovered weights of protein after chromatography on DEAE-cellulose indicate κ-casein comprises approximately 15% of the total casein, whereas β-casein and α_s-casein constitute 28% and 48% respectively.

Resmini et al. (1967) isolated and partially characterized the ovine κ-, β- and α_s-caseins. Shalichev and Tanev (1973) recently isolated ovine α_s-casein by chromatography of a crude α_s-casein fraction on Sephadex G-150 in 5 M urea. They obtained an extinction coefficient ($E^{1\%}_{1\ cm}$ at 280 nm) of 8.45, and a molecular weight of 47 500 for the protein. They also determined the amino acid composition of ovine α_s-casein.

Recently, Fiat et al. (1970) determined the sequence of a tryptic peptide of thirteen residues which contains the rennin sensitive phenylalanine-methione bond that was readily cleaved in bovine κ-casein by rennin and found the sequence to be identical to the corresponding region in bovine κ-casein. The N-terminal sequence of 40 residues from the ovine κ-casein macropeptide which was determined using a sequencer, has indicated a 78% homology when compared with bovine κ-casein (Fig.6.8) (Jollès et al., 1973). There is probably an insertion of two amino acids at residues 27 and 28 of the ovine glycomacropeptide, as well as seven amino acid substitutions.

Furthermore, the sequence of ovine para-κ_A-casein, determined by Jollès et al. (1974), had thirteen amino acid substitutions when compared with that of bovine para-κ-casein (Mercier et al., 1973) (Fig.6.9).

Fig.6.8: N-terminal sequences of bovine and ovine κ-casein A glycomacropeptides (Jollès et al., 1973). To optimize homologous relationships, two deletions were suggested to occur in the bovine peptide. O; deletion. The enclosed residues indicate the differences in the sequences of the two peptides.

Fig.6.9: Amino acid sequence of ovine para-κ-casein (Jolles et al., 1974), compared with that of bovine para-κ-casein (Mercier et al., 1973). The enclosed residues indicate the amino acid substitutions.

↓ indicates the site of rennin action.

6.22 Buffalo Caseins

After the earlier reports of heterogeneity in buffalo casein (Aschaffenburg and Sen, 1963; Ganguli and Bhalerao, 1964), two genetic variants of κ-casein, designated as A and B, were found by starch gel electrophoresis (Ganguli and Majumder, 1968). The B variant was found to be the most common with none of the samples examined being homozygous for the A variant (Ganguli, 1973). Aschaffenburg et al. (1968b) using starch gel electrophoresis observed two genetic variants which they designated as A and B in buffalo β-casein. The A variant was only observed in the caseins of 3 closely related buffalo from a total of 105 animals. Genetic polymorphism in the buffalo caseins appears to be a rare occurrence.

Abd El-Salam and Manson (1967) isolated buffalo κ-casein using the procedure developed by Zittle and Custer (1963) for bovine κ-casein. They found buffalo κ-casein had a phosphorus content of 0.36%, higher than bovine κ-casein, and an extinction coefficient ($E^{1\,cm}_{1\%}$ at 278 nm) of 11.0. The C-terminal sequence -Ala-Ser-Thr-Val.OH obtained by digestion of buffalo κ-casein with carboxypeptidase A is slightly different from that of bovine κ-casein (Fig.6.2).

Recently Nagasawa et al. (1973) separated buffalo casein by chromatography on DEAE-cellulose in urea into 3 major fractions, similar to their bovine counterparts. Buffalo β-casein and bovine β-casein have similar electrophoretic mobilities in alkaline polyacrylamide gels, with buffalo α_s-casein having a lower electrophoretic mobility than bovine α_{s1}-casein and being more heterogeneous. Quantitative polyacrylamide gel electrophoresis indicated buffalo casein contains 11.0% κ-, 43.4% β- and 45.6% α_s-casein. There appears to be less sialic acid in buffalo κ-casein than in bovine κ-casein. The amino acid compositions of the buffalo caseins were fairly similar to their bovine counterparts.

6.23 Human Caseins

Some of the earlier reports on the extent of rennin action on human casein were variable since glycopeptides

containing sialic acid were found in some but not all caseins (Malpress, 1962; Alais and Jollès, 1962). Alais and Jollès (1970) found difficulty in isolating casein from human milk, obtaining 0.5 g/100 ml, in contrast to bovine milk (2.5 g/100 ml). Malpress and Hytten (1964) found differences in the caseins of human milk samples, and in the sialic acid content of rennin hydrolysed caseins.

Malpress and Seid-Akhavan (1966) isolated and characterized two fractions, α_s- and κ-casein from human casein. The α_s-casein fraction isolated by Malpress and Seid-Akhavan (1966) is now thought to have been human β-casein (Nagasawa et al., 1970). The κ-fraction was insensitive to $CaCl_2$, being heterogeneous in gel electrophoresis, with the formation of soluble glycomacropeptides after rennin degradation (Alais and Jollès, 1969; Malpress and Seid-Akhavan, 1966; Nagasawa et al., 1970). Some carbohydrate is attached to the peptide through an O-glycosidic linkage to threonine and serine, and like bovine κ-casein the stabilizing power of human κ-casein is lost after rennin action (Malpress and Seid-Akhavan, 1966). Alais and Jollès (1969) also isolated two components from human caseins which they designated as κ-caseins, since they were readily hydrolysed with rennin. Groves and Gordon (1970) isolated human κ-casein and determined its amino acid composition. Toyoda and Yamauchi (1973) have also isolated a κ-casein-like fraction from human casein.

Nagasawa et al. (1967) separated human casein into 9 major fractions by chromatography on DEAE-cellulose at 10°C, in the absence of urea. The main component observed in polyacrylamide gel electrophoresis had six protein bands which migrated in the region of bovine β-casein. The patterns were very heterogeneous, with 14-26 protein bands being observed depending on the sample. No Ca sensitive fraction corresponding to cow α_s-casein was found.

Human casein was separated into three fractions by chromatography on Sephadex G-150 at 10°C in the absence of urea (Nagasawa et al., 1970). In the major fraction, six proteins were observed in alkaline polyacrylamide gels whose bands varied in their intensity, and which were

designated as β-casein A, B, C, D, E and F, in order of their decreasing electrophoretic mobilities. Human β-casein B, the second most mobile component, which was isolated by chromatography of the major fraction on DEAE-cellulose, resembled bovine β-casein in its Ca sensitivity, phosphorus content and amino acid composition (Nagasawa et al., 1970). Their results led Nagasawa et al. (1970) to propose that human casein was largely composed of a κ-casein and a β-casein fraction. The human β-casein fraction was shown by amino acid analyses and phosphorus estimation to contain one protein phosphorylated at different levels with a similar molecular weight to bovine β-casein, and containing 0-5 phosphate groups per mole of protein (Groves and Gordon, 1970; Groves and Townend, 1970). These results were verified by Nagasawa et al. (1971) in their studies on dephosphorylating human β-casein with phosphoprotein phosphatase from beef spleen. The different levels of phosphorus in the human β-caseins explains the marked sensitivity to $CaCl_2$ of the proteins containing more phosphorus (i.e. the more mobile components on gel electrophoresis) compared with the less mobile β-caseins (Nagasawa et al., 1970). Recently, Nagasawa et al. (1972a) examined the variation in gel electrophoresis patterns of human milk with the duration and temperature of storage of the milk. Human milk was found to be very susceptible to proteolysis with a marked increase in heterogeneity of the protein pattern, even after storage of the milk at 37°C for less than three hours. These observations of Nagasawa et al. (1972a) may in part explain the heterogeneity and differences observed by earlier workers (Malpress, 1962; Alais and Jolles, 1962; Malpress and Hytten, 1964; Nagasawa et al., 1967).

Voglino and Ponzone (1972) showed that the relative intensities of the A and D human β-casein bands varied and suggested that the β-caseins were under the control of two allelic genes.

Some of the physical characteristics of human β-casein have been examined by Toyoda and Yamauchi (1972) who showed human β-caseins undergo temperature dependent polymerization

similar to that of bovine β-casein, except the transition starts at a higher temperature than with bovine β-casein.

6.24 Other Caseins

Alais and Jollès (1970) examined human and rabbit caseins by starch gel electrophoresis and showed they did not have the characteristic electrophoresis pattern of caseins from the ruminants. Later, four pure components were isolated by chromatography of rabbit casein on DEAE-cellulose (Testud and Ribadeau Dumas, 1973). Some of these caseins resembled individual bovine caseins, and in particular, one fraction had an amino acid composition resembling bovine β-casein. However, it is more similar to human β-casein. It was not possible to isolate a κ-casein-like fraction in rabbit milk, although a para-κ-casein like fraction appears on electrophoresis of rennin treated rabbit whole casein.

Similarly, although canine casein was separated by DEAE-cellulose chromatography into β- and α_S-casein fractions, no fraction resembling κ-casein was found (Nagasawa et al., 1972b). Canine β-casein resembles human β-casein, at least in its amino acid composition.

Acrylamide gel electrophoresis (Feldman and Ceriani, 1970; Feldman and Hohmann, 1971) and paper electrophoresis (Sloan et al., 1961) of rat and mouse caseins have also revealed the presence of casein components, which have as yet, not been fully characterized. Their immunoelectrophoretic and electrophoretic relationships show that they are similar.

Preliminary investigations on porcine caseins have shown polymorphism in the caseins (Gerrits et al., 1969; Woychik and Wondolowski, 1969; Glasnák, 1966; Markovich, 1971). Sloan et al. (1961) showed that porcine (sow) casein is heterogeneous. Porcine α_S- and κ-caseins could be interchanged with the bovine caseins in Ca stability tests and had similar amino acid compositions to their bovine counterparts (Woychik and Wondolowski, 1969).

CHAPTER 7: EXPERIMENTAL

The methods used to isolate and characterize the major caprine and ovine caseins, and some bovine caseins are outlined in this chapter. The caseins were characterized from their electrophoretic mobilities in polyacrylamide gels, molecular weights, amino acid compositions, phosphorus contents, and extinction coefficients. The physical characteristics of the caseins were compared by measuring their solubility in the presence of $CaCl_2$, stabilization of the α_s-caseins by κ-caseins, and the temperature dependent polymerization of the β-caseins. Wherever possible all analyses were carried out using concentrated stock solutions of known protein concentration.

7.1 Preparation of Acid Caseins

Bovine Casein

Raw milk, obtained from individual Friesian cows belonging to the Massey University monozygous twin herd, was warmed to 30°C and skimmed using a separator. Acid casein was precipitated from the skim milk by slowly adjusting the pH to 4.6 with 1 M HCl. The precipitate was collected, washed several times with water, and redissolved at pH 7 by the addition of 1 M NaOH. Care was taken to maintain the pH of the solution below 8. The casein was reprecipitated at pH 4.6, washed, freeze dried and then stored at -20°C.

Caprine Casein

Samples of milk were obtained from British Saanen goats and skimmed using the same procedure for bovine milk. Acid casein was precipitated at 30°C by adjusting the pH to 4.2 with 1 M HCl. The casein was washed, redissolved, reprecipitated, freeze dried and then stored at -20°C.

Ovine Casein

Ovine milk samples were obtained by hand milking, mainly from Border Leicester-Romney crossbreed ewes belonging to the Massey University flock. These milks were either

bulked, or kept as individual samples. The milk was warmed to 30°C and centrifuged at 800 g for 15 minutes. Skim milk was removed from underneath the cream layer and diluted with an equal volume of water prior to precipitating the casein at pH 4.6 with 1 M HCl. The remainder of the procedure was the same as that used for the bovine and caprine caseins.

7.2 Polymorphism in Caprine and Ovine Caseins

A number of casein samples (25 caprine and 60 ovine) were examined by polyacrylamide gel electrophoresis for evidence of polymorphism in the caseins. Casein precipitated from 2 ml samples of caprine milk and 1 ml samples of ovine milk was dissolved in 10 ml of 4.5 M urea containing 2-mercaptoethanol (0.1%, v/v) and 10% (w/v) sucrose. Aliquots (0.02 ml) of these samples were examined by polyacrylamide gel electrophoresis at pH 8.4 and in some cases by alkaline gel electrophoresis in the presence of Mg (Chapter 7.6 and 7.7).

7.3 Chromatography of Casein on DEAE-Cellulose

Whole caprine, ovine and bovine caseins were separated into their major fractions by chromatography on DEAE-cellulose in buffer containing urea using a procedure similar to that described by Thompson (1966).

DEAE-cellulose DE-52 (Whatman) was precycled according to the procedure outlined in the Whatman handbook. The cellulose was washed in 0.5 M HCl for 30 min and washed with deionized water on a sintered glass filter until the pH of the effluent was 4. The procedure was repeated using 0.5 M NaCH and the cellulose washed with water until the pH of the effluent was 8. The DEAE-cellulose was washed with several bed volumes of 0.01 M imidazole-HCl buffer, pH 7.0, containing 4.5 M urea and 0.1% (v/v) 2-mercaptoethanol, packed into a column (2 cm x 50 cm) and equilibrated with buffer. Concentrated solutions of urea (9 M), used in the preparation of buffers, were filtered through a column of DEAE-cellulose and then stored at 4°C.

Samples of whole casein (1-2 g) were dissolved in buffer and chromatographed on the DEAE-cellulose column, the

caseins being eluted at a flow rate of 50 ml/h with a linear gradient of NaCl. Since the conditions used for chromatography varied with the nature of the sample, specific details are outlined in the results section. The effluent was continuously monitored at 280 nm with a Zeiss PMQII spectrophotometer coupled to a Sargent SRL recorder. Fractions were analysed by polyacrylamide disc gel electrophoresis (pH 8.9), pooled, dialysed and stored at -20°C. Most of the casein fractions were purified further by rechromatography on DEAE-cellulose or by chromatography on CM-cellulose. Some crude bovine α_{s1}- and β-casein fractions that had been prepared by urea fractionation of whole casein (Hipp et al., 1952) were purified by chromatography on DEAE-cellulose.

7.4 Chromatography of Casein on CM-Cellulose

Impure casein fractions were often rechromatographed on a column of Whatman CM-32 cation exchange cellulose in 0.01 M sodium formate buffer (pH 4.0) containing urea (4.5 M) and 2-mercaptoethanol (0.1%, v/v). The cellulose was precycled and packed into a column, using the same procedure as that previously described for DEAE-cellulose, except the CM-cellulose was washed first with 0.5 M NaOH and then with 0.5 M HCl. The conditions used for column chromatography on CM-32 were similar to those previously described for DEAE-cellulose.

7.5 Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gels were routinely used to identify the caseins present in the column fractions and to assess their purity.

Polyacrylamide disc gel electrophoresis at pH 8.9 in gels containing 5.5 M urea was based on the method outlined by Davis (1964). The gels consisted of an upper 1 cm layer of large pore gel used to concentrate the protein sample, and 6 cm of separation gel. These were set in 5 mm wide glass tubes. Stock solutions were prepared (Table 7.1) and from these, three working solutions were mixed (Table 7.2).

Table 7.1

Stock Solutions

Solution A, pH 8.9			Solution B, pH 6.7		
Tris	36.6	g	Tris	5.98	g
1 M HCl	48	ml	1 M HCl	48	ml
TEMED	0.23	ml	TEMED	0.46	ml
Water to	100	ml	Water to	100	ml

Solution C			Solution D		
Acrylamide	28.0	g	Acrylamide	10	g
Bis	0.735	g	Bis	2.5	g
Water to	100	ml	Water to	100	ml

Solution E

Riboflavin	4 mg
Water to	100 ml

Table 7.2

Working Solutions

Small pore solution 1

1 part A
2 parts C
1 part 9 M urea

Small pore solution 2

Ammonium persulphate solution
(0.14 g in 100 ml of 9 M urea)

Large pore solution

1 part B
2 parts D
1 part E
6 parts 9 M urea

Electrode buffer

Tris 3 g
Glycine 14.4 g
0.25 ml bromophenol blue (0.1%, v/v)
Water to 1 l

The separation gel was made by mixing equal parts of small pore solutions 1 and 2. The gel was allowed to polymerize and the spacer or large pore gel was then polymerized on top of this. Samples in urea solution were applied to the gel and electrophoresed for 1.5 h at 120 V, normally with a current of 50 mA. When the bromophenol blue dye marker reached the bottom of the gels, they were removed from the tubes and stained with 0.1% (w/v) amido black 10B in 7% (v/v) acetic acid for 1-4 h. Unbound dye was removed by electrophoresis.

7.6 Polyacrylamide Slab Gel Electrophoresis

Where it was necessary to compare the electrophoretic mobility of a number of caseins, these were examined by slab polyacrylamide gel electrophoresis. Alkaline or acid gel electrophoresis of caseins was carried out in 5% (w/v) polyacrylamide gel slabs containing 6 M urea using an E-C vertical gel electrophoresis apparatus (E-C Apparatus Corporation, Philadelphia). The method used for alkaline gel electrophoresis (Table 7.3) was similar to that used by Thompson et al. (1964) except the pH of the electrode buffer was 8.4. After the addition of ammonium persulphate, the gel was left to polymerize for 1 h. The samples, in urea, were placed in the gel slots and electrophoresis then carried out for 4 h or longer at 200 V. The gels were stained with 0.1% (w/v) amido black 10B and these were then washed in 3% (v/v) acetic acid to remove excess dye.

Samples were also examined by acid gel electrophoresis in 5% (w/v) polyacrylamide gel layers (Table 7.4). The samples were electrophoresed at 300 V for 6 h and stained and destained using the normal procedure.

7.7 Gel Electrophoresis in Buffers Containing Mg

The procedure used for gel electrophoresis in the presence of Mg has previously been described by Waugh et al. (1970). Electrophoresis in polyacrylamide gels containing Mg and 6 M urea was carried out using the E-C vertical gel electrophoresis apparatus. The composition of the gel and

Table 7.3

Stock Gel Solution

Cyanogum 41	50	g
Tris	10.80	g
$Na_2EDTA.2H_2O$	0.925	g
Boric acid	5.5	g
TEMED	1	ml
8 M urea	750	ml
Water to	1	l

Catalyst

Ammonium persulphate	0.2 g used to polymerize 200 ml of gel solution

Electrode Buffer Solution pH 8.4 (Peacock et al., 1965)

Tris	107.9	g
$Na_2EDTA.2H_2O$	9.25	g
Boric acid (crystalline)	55.0	g
Water to	10	l

Table 7.4

Acid Gel Solution (pH 4)

Cyanogum	12 g
Glacial acetic acid	12 g
9 M urea	133 ml
TEMED	1 ml
Deionized water to	200 ml

Catalyst

Ammonium persulphate	0.3 g used to polymerize 200 ml gel solution

Table 7.5

Polyacrylamide Gels Containing Mg

Stock Gel Solution

Tris	4 g
Boric acid	2 g
Magnesium acetate.$4H_2O$	0.2 g
Cyanogum	10 g
8 M urea	150 ml
TEMED	0.2 ml
Water to	200 ml

Catalyst

Ammonium persulphate	0.2 g in 200 ml gel solution

Anode Buffer (lower reservoir)

Magnesium acetate.$4H_2O$	4 g/l

Cathode Buffer (upper reservoir)

Sodium tetraborate adjusted to pH 8.6 with 3% (v/v) acetic acid	4 g/l

the electrode buffers is given in Table 7.5. Samples were applied to the gels and electrophoresis carried out for 6 h at 300 V. The gels were stained and destained as described in the previous section.

7.8 Proteolytic Degradation of Caseins with Rennet

Some column chromatography fractions were dialysed against deionized water and diluted with an equal volume of 0.2 M citrate buffer, pH 6.5. These were incubated at 37°C and aliquots of rennet (0.10 ml) (NZ Cooperative Rennet Company Ltd, Eltham) that had been diluted ten fold with buffer were added to the casein solution and incubated for 15 min. The reaction was stopped by diluting the samples with an equal volume of 9 M urea containing 2-mercaptoethanol (0.1%, v/v). Aliquots of the samples, obtained before and after rennet treatment, were examined by gel electrophoresis to identify any casein fractions which may have been hydrolysed.

Estimation of Molecular Weights

Analytical ultracentrifugation using the techniques of sedimentation velocity or sedimentation equilibrium are the commonly used procedures for determining the molecular weights of proteins. The molecular weights of the bovine caseins are difficult to determine since they readily aggregate to form polymers, whose size may be dependent on temperature, protein concentration and ionic strength (see introduction for further details). These problems can be overcome however, by ultracentrifugation of the caseins in concentrated guanidine. HCl solution (Noelken, 1967; Noelken and Reibstein, 1968).

In recent years a number of other techniques involving gel chromatography have been developed to determine the molecular weights of proteins. Gel chromatography of native globular proteins on columns calibrated with globular proteins of known molecular weight has become a widespread technique of estimating molecular weights (Andrews, 1970). Other techniques include gel chromatography on columns calibrated with proteins of known molecular weight in the presence of sodium dodecyl sulphate (Fish, 1971)

concentrated solutions of urea (Thompson and O'Donnell, 1965) or guanidine.HCl (Fish, 1971; Bryce and Crichton, 1971).

In the presence of dissociating agents, and in particular 6 M guanidine.HCl, proteins which have their disulphide linkages reduced are denatured to random coils (Tanford, 1968). There is however, some evidence of the caseins (Evans et al., 1971a) and some other proteins (Tanford, 1968) still possessing some degree of structure under these conditions. However, gel chromatography of bovine caseins in the presence of 7 M urea and in 6 M guanidine.HCl yields molecular weight values close to those estimated from their amino acid sequences. Consequently, the molecular weights of the caprine, bovine and ovine caseins were determined by gel chromatography in 7 M urea and in 6 M guanidine.HCl.

7.9 Molecular Weight of Caprine κ-Casein

The molecular weight of caprine κ-casein was estimated by gel chromatography on a column of Sephadex G-100 (2.2 cm x 90 cm) in 0.1 M Tris-HCl buffer, pH 8.0, containing 7 M urea. Pepsin (molec.wt 35 000) (Sigma), bovine β-casein B (molec.wt 24 100), bovine κ-casein B (molec.wt 19 023), haemoglobin (molec.wt 15 500) and bovine ribonuclease A (molec.wt 13 700) (Calbiochem) were used as molecular weight standards. The molecular weight of β-casein was estimated from the amino acid sequence (Ribadeau Dumas et al., 1972; Grosclaude et al., 1972).

The molecular weight of bovine κ-casein B was taken from Mercier et al. (1973). Other molecular weights were taken from Weber and Osborn (1969). Prior to chromatography all proteins except β-casein B were S-carboxymethylated (Hirs, 1967a). The flow rate of the column was maintained at 10 ml/h, 2.2 g fractions being collected and their extinctions measured at 230 nm. In each chromatographic run Blue Dextran 2000 (Pharmacia) and ε-dinitrophenyl(DNP)-lysine (BDH) were included to enable the distribution coefficient (Kd) for each protein to be calculated as described

by Fish (1971) using the formula:

$$Kd = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution weight of protein
V_o = elution weight of Blue Dextran 2000 (void volume)
V_t = elution weight of α-DNP-lysine ($V_o + V_i$, where V_i is the interstitial volume).

The logarithm of the molecular weight for each standard protein was plotted against the distribution coefficient and the molecular weight of the unknown was estimated from this curve.

7.10 Molecular Weight Analysis in 6 M Guanidine.HCl

The molecular weights of the bovine, ovine and caprine caseins, except caprine κ-casein were determined by chromatography in 6 M guanidine.HCl buffer on a column of Sepharose 6B. The column had previously been calibrated with S-carboxymethylated proteins of known molecular weight using the procedure outlined by Fish et al. (1969).

A column (2.26 cm x 84 cm) of Sepharose 6B (Pharmacia) was equilibrated with several bed volumes of 0.02 M Tris, 0.01 M Na_2EDTA buffer, pH 8.2, containing 6 M guanidine.HCl. The flow rate of the column was maintained at 5.0-5.2 ml/h using a Accuflo pump (Beckman Instruments, Spinco Division, Palo Alto). Fractions were collected at 30 min intervals using a LKB RadiRac fraction collector.

Chromatography of Proteins

The proteins, with the exception of the β-caseins were all S-carboxymethylated prior to chromatography, using a similar procedure to Hirs (1967a). The standard proteins used to calibrate the molecular weight column are presented in Table 7.6 . The protein samples (3-5 mg), α-DNP-alanine (0.02 mg) and Blue Dextran 2000 (1 mg) were dissolved in guanidine.HCl buffer containing 10% (w/v) sucrose. The

Table 7.6

Standard Proteins[1]

	Source	Molecular Weight
γ-Globulin H-chain	Sigma	50 000[2]
Ovalbumin	Sigma	43 000[2]
Chymotrypsinogen A	Sigma	25 700[2]
γ-Globulin L-chain	Sigma	23 500[2]
Myoglobin (equine)	Sigma	17 200[3]
Haemoglobin	Sigma	15 500[3]
Ribonuclease A	Miles Seravac	13 700[3]
Cytochrome C (equine)	Sigma	11 700[3]

1. Unless otherwise indicated, bovine standard proteins were used.
2. Molecular weights from Weber and Osborn (1969).
3. Molecular weights determined from the amino acid sequences listed by Dayhoff (1972).

elution weights of each protein were measured, since these result in more accurate values for the distribution coefficients than those obtained from elution volumes (Fish et al., 1969). The absorption at 640 nm, 280 nm and 365 nm of the fractions was used to estimate the elution position of Blue Dextran 2000, protein and α-DNP-alanine, respectively. The distribution coefficient (Kd) of each protein was calculated using the expression:

$$Kd = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution weight of protein
V_t = elution weight of α-DNP-alanine
V_o = void volume characterized by Blue Dextran 2000

A standard curve was prepared by plotting $Kd^{\frac{1}{3}}$ versus $(\text{molec.wt})^{\frac{1}{2}}$ for the S-carboxymethylated proteins of known molecular weight (Bryce and Crichton, 1971).

7.11 Amino Acid Compositions

The amino acid compositions of the purified caprine caseins and some bovine caseins were determined after hydrolysing the proteins with 6 M HCl. Duplicate samples of each purified protein (3-4 mg) were hydrolysed *in vacuo* in 6 M HCl at 110±1°C for 24, 48 and 72 h. Samples were placed in thick walled glass tubes and frozen in liquid nitrogen. The samples were evacuated with a vacuum oil pump and degassed as they thawed. This process was repeated several times. The sample tubes were sealed at a pressure of less than 50 microns (Moore and Stein, 1963). The ovine caseins were hydrolysed with 6 M HCl after the tubes were evacuated with a water pump and repeatedly flushed with oxygen-free nitrogen, using a similar procedure to Spitz (1973).

The hydrolysates were evaporated to dryness on a rotary evaporatory, washed with water and redried. The process was repeated twice, and the samples stored at -20°C prior to amino acid analysis.

Amino Acid Analysis

The amino acid compositions of the protein hydrolysates were determined by ion exchange chromatography using the procedure of Moore and Stein (1963).

Casein hydrolysates were analysed using the single column methodology on a Locarte Mk IV Amino Acid Analyser. Norleucine (25 nanomoles) was included as an internal standard in each chromatographic run. The analyser was calibrated with Beckman standard amino acid calibration mixtures, containing 25 nM of each amino acid. Usually, 0.100 ml aliquots of hydrolysates containing 5-100 nanomoles of each amino acid were analysed on a column of Locarte LA/49 cation exchange resin, using a three buffer program. The acidic and neutral amino acids were eluted first with buffers 1 and 2, followed by the basic amino acids which were eluted with buffer 3 (Table 7.7). After elution from the ion exchange column the concentration of each amino acid was determined photometrically after reaction with ninhydrin.

Table 7.7

Locarte Amino Acid Analyser Program

	Composition	Period of Elution (min)
Buffer 1[1]	0.2 N Na citrate pH 3.25	50
Buffer 2	0.2 N Na citrate pH 4.20	95
Buffer 3	1.0 N Na citrate pH 6.65	135
NaOH	0.2 N NaOH	30
Equilibration buffer	0.2 N Na citrate pH 3.25	90

1. After eluting the ion exchange column for 40 min the temperature was increased from 50°C to 60°C.

The buffer and ninhydrin flow rates were each maintained at 30.0 ml/h. This program resulted in a total analysis time of 6 hours 40 minutes for each sample.

Hydrolysates of caprine κ-casein were analysed using the two column methodology (Beckman 120C Instruction Manual) on a Beckman 120C Amino Acid Analyser. Norleucine (Pierce) and α-amino-β-guanidine propionic acid (Pierce) were used as internal standards in these analyses.

Cysteine

Any cysteine and cystine present in the caseins were oxidized with performic acid, prior to acid hydrolysis (Hirs, 1967b).

Tryptophan

Tryptophan in caprine κ-casein was determined by amino acid analysis after acid hydrolysis of the protein samples *in vacuo* in the presence of 4% (v/v) thioglycollic acid for 48 hours at 110°C (Matsubara and Sasaki, 1969). Tryptophan in the other caprine caseins was determined colorimetrically with p-dimethylaminobenzaldehyde after enzymatic hydrolysis with Pronase (Koch Light) as described in Procedure 'W' by Spies (1967). L-Tryptophan (Sigma) was used to prepare a standard curve.

The ovine caseins were hydrolysed in 3 M ρ-toluene sulphonic acid containing 3-(2-aminoethyl)indole (0.2% v/v) for 24 h at 110°C (Liu, 1972) and tryptophan in these digests was determined by amino acid analysis. L-Tryptophan (Sigma) was used as a standard. The method of Liu (1972) was used to determine the tryptophan content of ovine caseins, since this method was technically simpler than the method of Spies (1967). Furthermore, only 2 mg protein samples were required, compared with 10-20 mg using the method of Spies (1967).

The molar ratio of tyrosine to tryptophan in some caseins was determined from measurements made on the protein in 0.1 M NaOH at 280 nm and 294.4 nm (Beaven and Holiday, 1952).

7.12 Phosphorus

Phosphorus was determined in casein samples which had been digested using one of two methods. It was determined directly in the hydrolysates used for amino acid analysis, or in caseins (10-20 mg) which had been digested with nitric and sulphuric acids and oxidized with hydrogen peroxide using the wet digestion procedure described by McKenzie and Murphy (1970). Phosphorus was determined colorimetrically using the procedure described in Chapter 3.3.

7.13 Extinction Coefficients

The extinction coefficients of the caseins were determined at 280 nm in 0.1 M phsophate buffer (pH 7.0). Aqueous stock solutions of the caseins whose protein concentrations were previously determined by measuring their dry weight (McKenzie and Murphy, 1970), were diluted with phosphate buffer and their extinctions measured at 280 nm and 320 nm against 0.1 M phosphate buffer. The extinction coefficient at 280 nm of a 1% (w/v) protein solution in a cell with a 1 cm path length was determined after correction for Rayleigh light scattering by the expression:

$$E^{1\%}_{1\ cm}\ 280\ nm = \frac{E_{280} - 1.7E_{320}}{c}$$

where c is the protein concentration in g/100 ml.

7.14 Viscosity Measurements on Caprine and Bovine β-Caseins

The intrinsic viscosity of a protein is a measure of its conformation in solution. The intrinsic viscosities of bovine and caprine β-caseins were determined in neutral buffer at several temperatures, and at 4°C in the presence of 6 M guanidine.HCl.

Stock protein solutions were prepared by precipitating caprine β_1- and β_2-caseins and bovine β-casein A^1 at their isoelectric points and redissolving them in deionized water at pH 7.0 with the addition of small quantities of 0.1 M NaOH. Their protein concentrations, determined by measuring the weight of dry material in aliquots taken from these solutions, were 1.96%, 1.51% and 1.80% for caprine β_1- and β_2-caseins and bovine β-casein A^1, respectively. These solutions were diluted with concentrated stock buffer so that they contained approximately 1% (w/v) protein in 0.02 M EDTA buffer, pH 7.0, and 0.14 M NaCl. Alternatively, they were diluted with concentrated stock buffer so the solutions contained approximately 1% (w/v) protein in 0.1 M phosphate buffer, pH 7.0, and 6 M guanidine.HCl. Reference solutions were prepared from the concentrated stock buffer solutions by diluting them to the same extent, by weight, as the protein solutions.

Guanidine.HCl (Grade 1) (Sigma), recrystallized from methanol (Nozaki and Tanford, 1967), was used in the preparation of buffers. The absorption spectrum of the guanidine.HCl that was purified by this procedure showed it to be essentially free of any contaminants (Nozaki and Tanford, 1967).

The relative density (relative to water) of the 0.02 M EDTA buffer, pH 7.0, containing 0.14 M NaCl, and 0.1 M phosphate buffer, pH 7.0, containing 6 M guanidine.HCl, were determined by pycnometry at 25°C. These measurements were repeated with the buffer solutions containing known amounts of protein. It was assumed that the change in relative density of solutions was minimal between 4 °C and 37°C.

Viscosity measurements were carried out at 4°C, 10°C, 25°C and 37°C in EDTA-NaCl buffer, pH 7.0, and at 4°C in buffer containing 6 M guanidine.HCl, using the procedure outlined in Chapter 3.11. It was necessary to filter the solutions through a medium porosity glass sintered disc before measuring their relative viscosities, otherwise reproducible viscometer flow times were difficult to obtain. At 4°C and 10°C, Cannon Manning Semi Micro Viscometers, type 75, were used as these provided a minimum flow time for water of approximately 180 sec at 10°C. At the higher temperatures the type 50 viscometers were used which provided a minimum flow time for water of approximately 380 sec at 25°C. Under these conditions the kinetic energy correction was negligible (Bradbury, 1970).

The viscometer flow times of the reference buffers and concentrated protein solutions were determined. The concentrated protein solutions were successively diluted by weight with buffer to provide a series of samples of different protein concentrations. The flow times of all of these solutions were measured at least three times to obtain results with a maximum variation of ±0.1 sec.

The specific viscosity values at different protein concentrations were determined from the expression:

$$\eta_{sp} = \frac{dt}{d_o t_o} - 1$$

where η_{sp} is the specific viscosity, and d and d_o and t and t_c, the relative densities and viscometer flow times, respectively of the protein solution and the reference solvent. The data was fitted by least squares analysis to the Huggin's equation (Bradbury, 1970):

$$\frac{\eta sp}{c} = [\eta] + k' [\eta]^2 c$$

where $[\eta]$ is the intrinsic viscosity of the protein (ml/g), k' is Huggin's constant and c is the concentration of protein in g/ml.

7.15 Sedimentation Coefficients

Sedimentation coefficients were measured in a Beckman Model E analytical ultracentrifuge using schlieren optics. The caprine β-caseins were studied in EDTA-NaCl buffer, pH 7.0 used for viscosity measurements.

Sedimentation velocity experiments were carried out at 10°C, 14°C and 25°C in a 12 mm double sector, synthetic boundary cell at 56 100 rev/min after equilibration of the protein solutions for at least 1 h prior to the run.

Sedimentation coefficients were calculated from the movement of the maximum ordinate of the schlieren curve and were corrected to standard conditions ($s_{20,w}$).

7.16 Calcium Sensitivity of Caprine, Ovine and Bovine Caseins

The solubility of caprine, ovine and bovine β- and α_s-caseins in the presence of $CaCl_2$ was measured using two procedures. The first method, used for caprine and bovine caseins, measured the concentration of soluble protein remaining in the supernatant at various $CaCl_2$ concentrations, of solutions initially containing 5 mg/ml protein in sodium cacodylate buffer, pH 6.8, at 1°C or 37°C. Due to a limited supply of some ovine caseins, it was necessary to measure their Ca sensitivity using a procedure which required less protein. The Ca sensitivity of solutions containing 0.5 mg/ml of protein, at 37°C was determined from their turbidity in the presence of various levels of $CaCl_2$. These turbidity measurements can only be used to determine the Ca concentration at which the caseins first precipitate and form colloidal aggregates, since turbidity is not necessarily related to protein concentration.

Method 1: Solutions (1 ml), which contained 6.25 mg/ml of casein in buffer, were equilibrated in 8 centrifuge tubes at 1°C or 37°C. Aliquots of $CaCl_2$ solutions were then added to each tube and rapidly mixed. Each tube then contained 5 mg/ml protein in 0.05 M sodium cacodylate buffer, pH 6.8, and 0.05 M KCl (I = 0.088). The final concentration of

$CaCl_2$ used ranged from 0 to 0.02 M $CaCl_2$ at 37°C, and 0 to 0.4 M $CaCl_2$ at 1°C. Each solution was incubated for 30 min, centrifuged at 12 000 g for 15 min and a 0.5 ml sample of the supernatant removed using pipettes equilibrated at 1°C or 37°C, and diluted with 2.0 ml H_2O. The absorbance at 280 nm and 320 nm of each fraction was measured and the results used to construct curves of casein solubility versus $CaCl_2$ concentration.

Method 2: Solutions (2.0 ml), which contained 0.75 mg/ml of protein in buffer, were equilibrated in Spectronic 20 glass cuvettes at 37°C. Aliquots of $CaCl_2$ solutions were added so the solutions (3.0 ml) contained 0.5 mg/ml protein in 0.05 M sodium cacodylate buffer, pH 6.8, and 0.05 M NaCl (I = 0.088). The $CaCl_2$ concentration in the solutions ranged from 0 to 0.02 M. These solutions were incubated for 10 min at 37°C, and their absorbance measured at 440 nm, the results being used to construct curves of turbidity versus $CaCl_2$ concentration for the individual caseins.

The ability of caprine, ovine and bovine κ-caseins to stabilize the α_s- and β-caseins from the three species in solution in the presence of $CaCl_2$ was also compared.

Solutions (0.4 ml), containing 5 mg of α_s- and β-caseins and 1 mg κ-casein in 0.01 M sodium cacodylate buffer, pH 6.8, and 0.05 M NaCl were equilibrated at 37°C. Concentrated $CaCl_2$ solution was added to each tube and rapidly mixed, giving a final concentration of 20 mM. These solutions were incubated for 15 min at 37°C and immediately centrifuged at 500 g for 5 min. An aliquot (0.2 ml) was removed from each sample and diluted with 1.8 ml of 0.05 M sodium citrate. The absorbance at 280 nm of these solutions was compared with the absorbance of the original α_s- or β-casein solutions that did not contain $CaCl_2$ or κ-casein to determine the amount of stabilization.

7.17 Temperature Dependent Polymerization of β-Casein

The increase in turbidity of solutions containing the β-caseins and $CaCl_2$, as the temperature was increased from

10°C to 40°C, showed the ability of the bovine, caprine and ovine β-caseins to undergo temperature dependent polymerization since bovine β-casein polymers are readily precipitated in $CaCl_2$ solutions.

Solutions (3 ml), in Spectronic 20 cuvettes contained 1 mg/ml of protein in 0.05 M sodium cacodylate buffer, pH 6.8, 0.05 M NaCl (I=0.10) and 20 mM $CaCl_2$ at about 10°C. The temperature of these solutions was increased slowly in approximately 2°C steps, equilibrated at that temperature for 10 min and the turbidity measured at 550 nm. From the data, curves of turbidity versus temperature were constructed. Some measurements were also made at other ionic strengths and pH's.

CHAPTER 8: CASEIN COMPOSITION AND ISOLATION OF THE COMPONENTS

The separation of whole bovine, caprine and ovine caseins into their major fractions by chromatography on columns of DEAE-cellulose is described in this chapter. The composition of the whole caseins and the polymorphism evident in a number of individual casein samples are also discussed.

8.1 Chromatography of Whole Bovine Casein on DEAE-Cellulose

Bovine casein, isolated by acid precipitation from milk of a cow homozygous for α_{s1}-Cn^{B}-β-Cn^{B}-κ-Cn^{B} was separated into three major fractions by chromatography on a column of DEAE-cellulose at pH 7.0 in buffers containing 4.5 M urea and 2-mercaptoethanol (0.1%, v/v) (Fig.8.1). The fractions indicated were pooled and their purity examined by gel electrophoresis at pH 8.4 (Fig.8.2).

Fraction 1 contained the heterogeneous group of γ-caseins which were not examined further.

Fraction 2 and 2a contained κ-casein B and the minor carbohydrate containing κ-casein fractions, respectively, which were readily hydrolysed by rennin (Fig.9.2).

Fraction 3 contained pure β-casein B.

Fraction 4 contained the minor α_{s3}- and α_{s4}-caseins as well as smaller quantities of the other caseins.

Fraction 5 contained α_{s1}-casein B and the α_{so}-casein fraction.

8.2 Composition of Bovine Casein

The composition of whole bovine casein was determined by chromatography of 2.00 g samples of 'wet' casein, containing about 0.5 g protein on DEAE-cellulose at pH 7.0. The peaks which contained protein were pooled into several fractions, similar to those in Fig.8.1. These fractions were

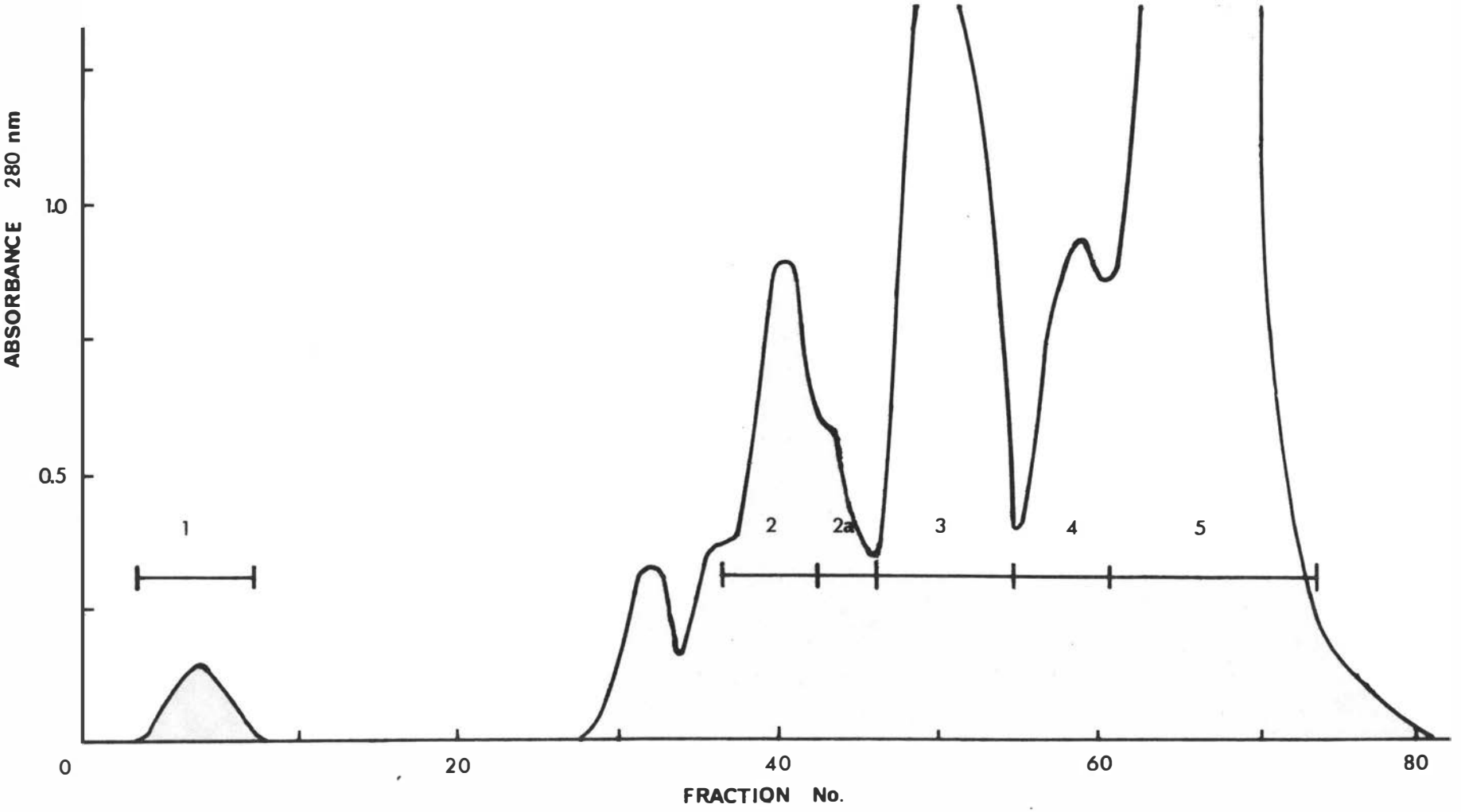


Fig.8.1: Chromatography of 1 g of whole bovine casein on DEAE-cellulose in 0.01 M imidazole-HCl buffer, pH 7.0, containing 4.5 M urea and 2-mercaptoethanol (0.1%, v/v). The flow rate was maintained at 50 ml/h, and 8 ml fractions were collected. The caseins were eluted with a 1 l linear gradient of 0.05-0.3 M NaCl. The pooled fractions, indicated by the bar, were examined by polyacrylamide slab gel electrophoresis at pH 8.4 (Fig.8.2).

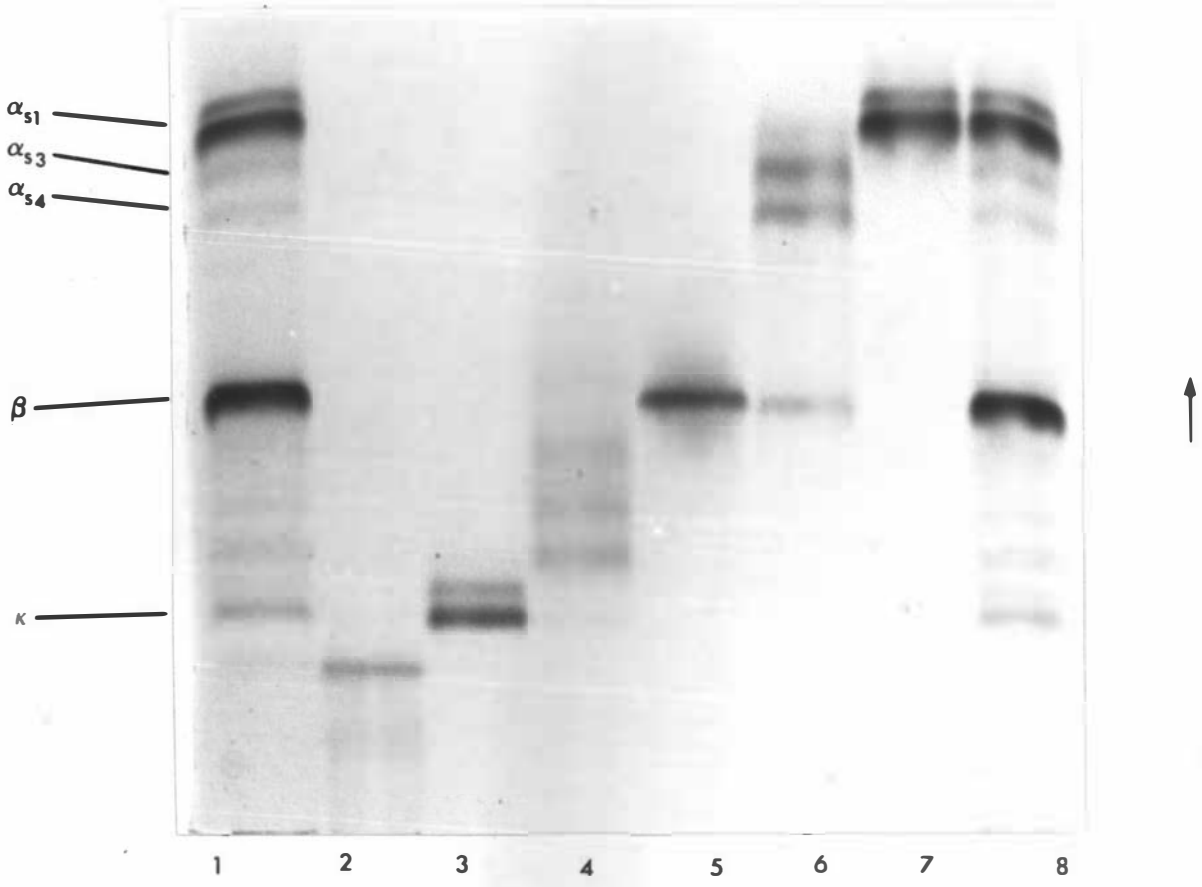


Fig.8.2: Polyacrylamide gel electrophoresis (pH 8.4) of whole bovine casein, and the fractions isolated by chromatography on DEAE-cellulose (Fig.8.1). Samples: 1, 8, whole bovine casein; 2, Fraction 1; 3, Fraction 2; 4, Fraction 2a; 5, Fraction 3; 6, Fraction 4; 7, Fraction 5. The direction of electrophoresis is indicated.

dialysed for two days against three changes of 5 l of 0.02 M NaCl and their volumes were measured before being freeze dried. Two 50 ml aliquots of 0.02 M NaCl in which the caseins were dialysed, and 10 g of 'wet' casein were also freeze dried. The protein content of each fraction was estimated from its dry weight after correcting for the salt content. After gel electrophoresis and densitometry, the proportion of the other caseins in each casein fraction was calculated by assuming that each casein had the same dye binding constant for amido black 10B. Since, in most cases there was only a small amount of contamination, this assumption should not lead to appreciable errors in the recovered weights of each protein.

It was necessary to make two further assumptions:

1. That any caseins permanently bound to the DEAE-cellulose was in the same proportion for all the caseins.
2. That none of the caseins were preferentially lost through the dialysis sac.

The percentage composition of a sample of bovine casein isolated from the milk of a single cow and based on the total recovered weight of protein is shown in Table 8.1, and compared with the compositions obtained by Ribadeau Dumas (1968) and Rose et al. (1969).

The results presented in Table 8.1 are similar to those obtained by Rose et al. (1969) who used a similar technique, except they used the corrected absorbance at 280 nm of each fraction to determine the casein composition. The values obtained for the α_s-casein fraction by Rose et al. (1969) and Ribadeau Dumas (1968) included all the α_s-caseins.

Ribadeau Dumas (1968) determined the composition of bovine casein by measuring the concentration of each amino acid released from the C-terminal of the caseins with carboxypeptidase A. His values for β-casein, listed in Table 8.1 would include the γ-caseins, since these caseins have the same C-terminal sequence (Gordon et al., 1972). The composition obtained by Ribadeau Dumas (1968) has lower values for κ- and α_s-caseins than results obtained in this study and by

Table 8.1

Composition of Bovine Casein[1]

Major Casein	Fraction	Composition (Percent of total casein) I'	II'	Rose et al.[2] (1969)	Ribadeau Dumas[2] (1968)
γ-casein	1	4.0	1.6	2.3-2.8	-
κ-casein	2+2a	14.5	17.0	14.6-14.9	10.6-12.3
β-casein	3	36.1	36.3	31.8-33.4	30.2-35.6
α_{s3}-+α_{s4}-casein	4	8.0	9.4		
α_{s1}-+α_{so}-casein	5	39.6	40.2	49.2-50.8	43.5-46.8
Recovery factor		92.0	89.0		
Expected total protein		430 mg	430 mg		

1. The composition of bovine casein was based on the total recovered weight of protein after chromatography on DEAE-cellulose.
2. Range of values obtained from three replicates. Values for α_{s1}-casein also include the minor α_s-caseins. See text for further details.

Rose et al. (1969). Ribadeau Dumas (1968) attributed the presence of phenylalanine in the carboxypeptidase A digests of whole casein to the presence of para-κ-casein. Including para-κ-casein in his results increases the κ-casein content up to a total of about 16%, in much better agreement with the other results.

8.3 Chromatography of Whole Caprine Casein on DEAE-Cellulose

Caprine casein was precipitated from the milk of a single British Saanen goat at pH 4.2. A survey of casein isolated between pH 4 and pH 5 showed that pH 4.2 gave the best yield of casein in agreement with the results of Zittle and Custer (1966).

Four major fractions were isolated by chromatography of caprine casein on DEAE-cellulose at pH 7.0 (Fig.8.3). The fractions indicated were pooled and examined by gel electrophoresis at pH 8.4 (Fig.8.4). The major peaks in the DEAE-cellulose chromatogram of caprine casein overlap to a greater degree than with bovine casein, causing each fraction to be more heterogenecus.

The caseins in fraction 1 have a similar electrophoretic mobility to the γ-caseins found in bovine casein (Figs.8.1 and 8.2). This fraction was not examined further.

Fractions 2 and 2a were shown to contain κ-casein from their sensitivity to rennin (Fig.9.2). κ-Casein has a similar electrophoretic mobility at pH 8.4 to fractions 3 and 4 (Fig.8.4) and is obscured in patterns of whole casein by these components.

Fractions 3 and 4 (Figs.8.3 and 8.4) contained β_2- and β_1-caseins, respectively. They were tentatively designated as β-caseins on the basis of their electrophoretic mobilities, which are similar to that of bovine β-casein (Fig.4.1). This designation as β-caseins was confirmed from their amino acid compositions (Chapter 9) and their temperature dependent behaviour (Chapter 11). Fraction 4 was invariably contaminated with some of the α_s-caseins which

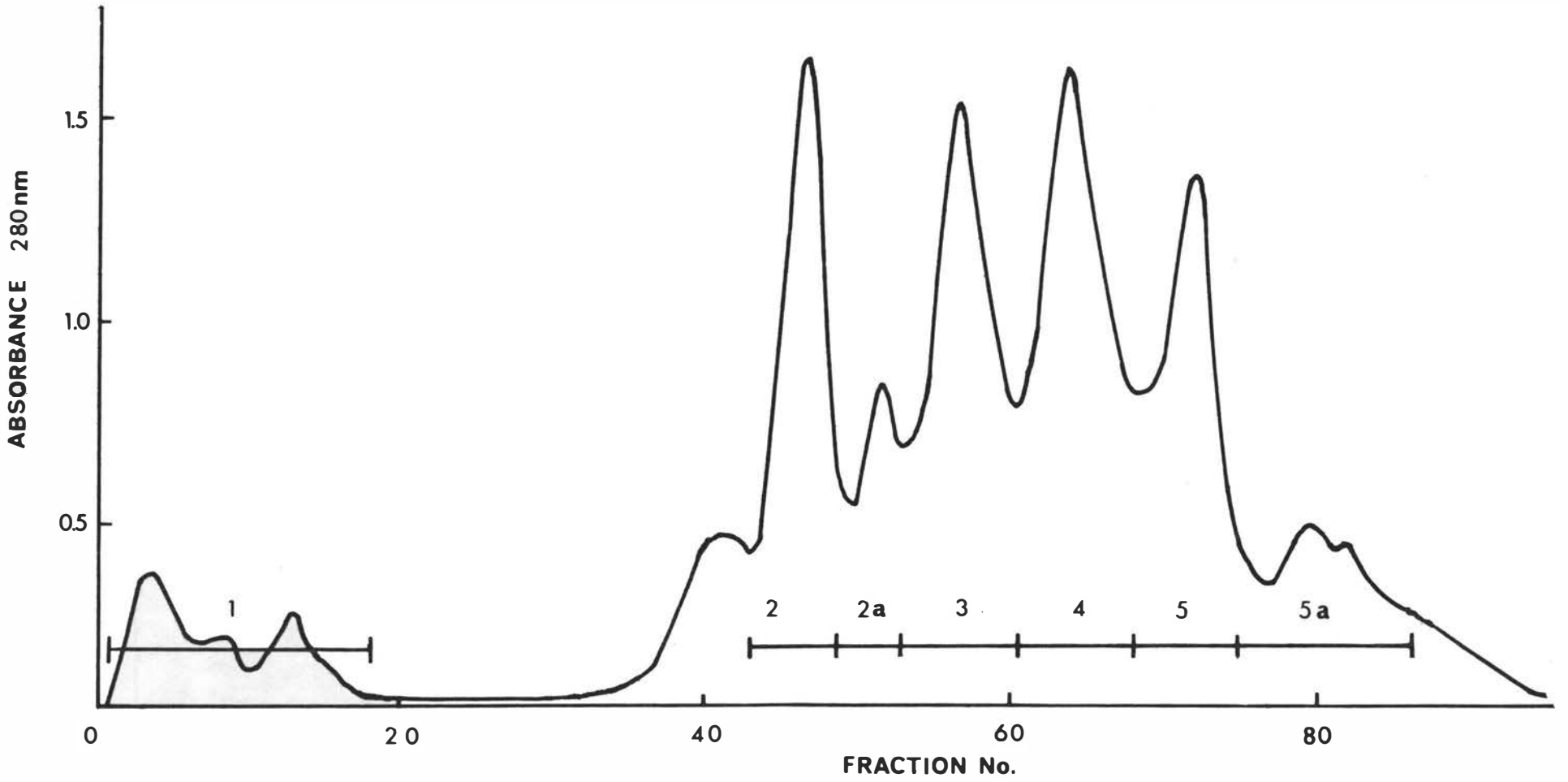


Fig.8.3: Chromatography of 1 g of whole caprine casein on DEAE cellulose at pH 7.0. Experimental details were the same as those in Fig.8.1. The fractions indicated were pooled and examined by gel electrophoresis at pH 8.4 (Fig.8.4).

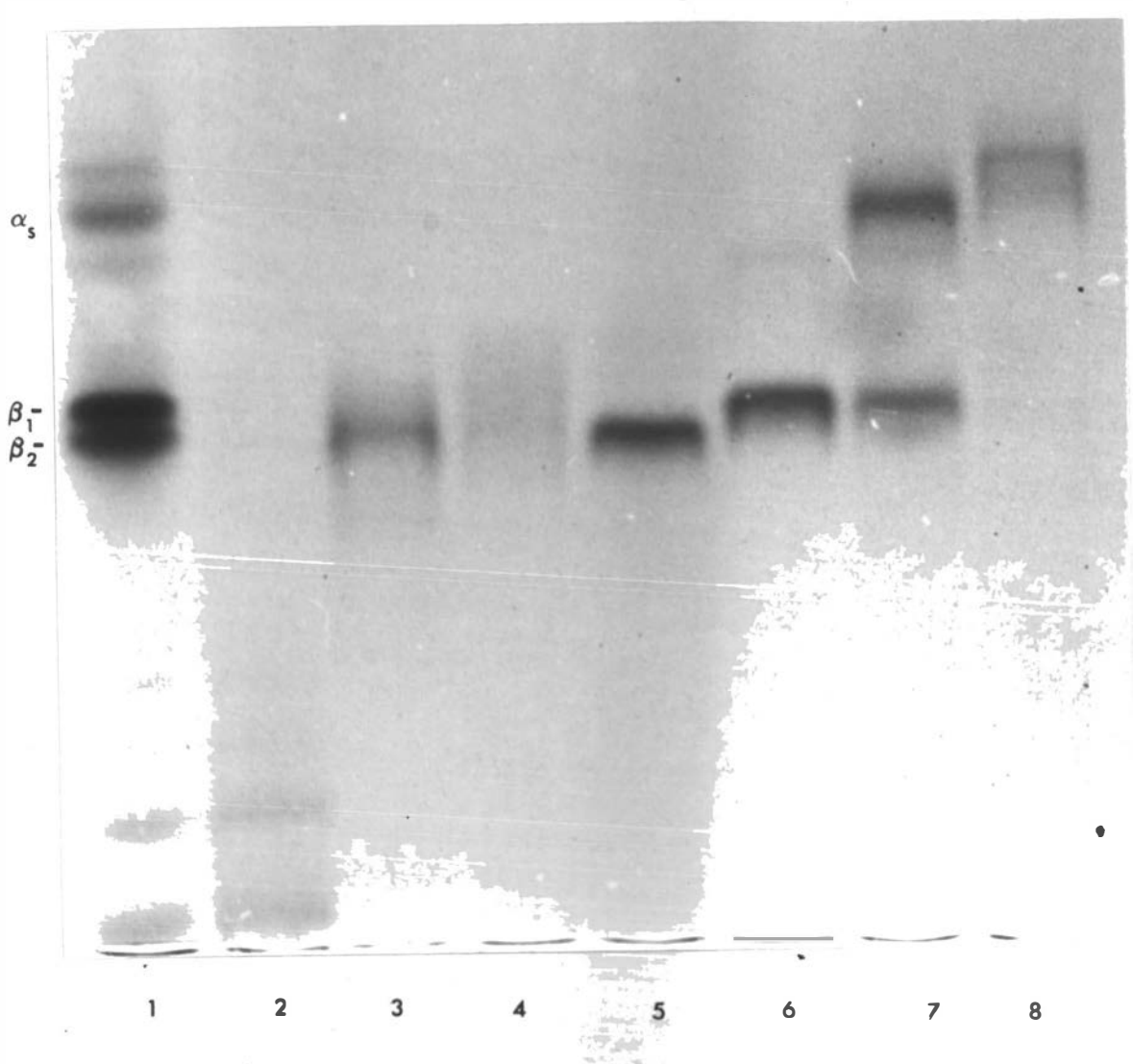


Fig.8.4: Polyacrylamide gel electrophoresis at pH 8.4 of whole caprine casein and the fractions isolated by chromatography on DEAE-cellulose (Fig.8.3). Samples: 1, whole caprine casein; 2, Fraction 1; 3, Fraction 2; 4, Fraction 2a; 5, Fraction 3; 6, Fraction 4; 7, Fraction 5; 8, Fraction 5a. The direction of electrophoresis is indicated.

have a lower mobility than the major α_s-casein. The degree of contamination of β_1-casein with these α_s-caseins was usually greater than that indicated in Fig.8.4.

Fraction 5 (Figs.8.3 and 8.4) contained the major caprine α_s-casein and some minor components, tentatively designated as α_s-caseins from their electrophoretic mobilities compared with bovine α_{s1}-casein (Fig.4.1). There was also a considerable amount of β-casein present. Only the major α_s-casein was characterized further (see Chapters 9 and 11). Fraction 5a contained the minor α_s-caseins.

These fractions were dialysed to remove urea and buffer salts, freeze dried and then stored at -20°C.

8.4 Composition of Caprine Casein

The composition of caprine casein, determined from the recovered weights of the protein fractions after chromatography of whole casein on DEAE-cellulose is shown in Table 8.2. The casein was isolated from a sample of milk obtained from a single British Saanen goat. Greater variation is evident in the duplicate chromatographic runs (Table 8.2) than was the case with bovine casein. This was probably due to overlapping of the protein peaks eluted from the DEAE-cellulose column and the increased difficulty in correcting the recovered protein weights. Samples of casein which had been stored for long periods (about 1 year) at -20°C, appeared to have been degraded, as shown by DEAE-cellulose chromatography.

It is evident from Table 8.2 that the κ-casein content of caprine casein was probably less than that of bovine casein (Table 8.1). The most notable feature of caprine casein was the high content of the β-caseins which appeared to be present in roughly equimolar amounts, constituting some 60% of the total casein compared with 36% in bovine casein. There was considerable heterogeneity in the α_s-casein region of caprine casein as shown by gel electrophoresis, with the total protein content of this region being approximately 22% of the total casein, considerably less than that present in

Table 8.2

Composition of Caprine Casein

Major casein	Fraction	Composition	
	Percent of total casein	I	II
γ-casein	1	7.2	4.7
κ-casein	2	7.7	10.7
β_2-casein	3	30.8	30.0
β_1-casein	4	28.1	35.9
α_s-casein	5	26.0	18.8
minor α_s-casein	5a		
Recovered protein		85.1%	96.7%
Expected total protein		1.000 g	1.000 g

bovine casein (Table 8.1). The major caprine α_s-casein accounts for about 15% of the total casein, compared with 40% for bovine α_{s1}-casein.

8.5 Chromatography of Whole Ovine Casein on DEAE-Cellulose

Ovine casein was precipitated from diluted skim milk at pH 4.6. The milk used was a composite sample from six Romney cross ewes, which appeared to have no differences in their caseins as shown by their gel electrophoresis patterns. Although it would have been more desirable to use casein isolated from an individual milk, problems were experienced with the removal of fat from the casein samples, and unless that fat was completely removed, this resulted in poor resolution on DEAE-cellulose. Coupled with this, the shorter period of lactation of the ewes meant only a limited number of samples of milk could be obtained.

The major components of whole ovine casein were separated by column chromatography on DEAE-cellulose (Fig. 8.5). The fractions indicated were pooled and their purity examined by gel electrophoresis at pH 8.4 (Fig.8.6).

Fractions 1 and 2 contained minor caseins which were not examined further. These caseins had similar electrophoretic mobilities to those in fraction 1 from bovine and caprine caseins.

The major protein in fraction 3 was shown to be κ-casein since it was readily hydrolysed by rennin (Fig.10.1). A minor band with a greater mobility than κ-casein, which was hydrolysed by rennin, may correspond to the carbohydrate containing κ-casein. Ovine κ-casein, in contrast to caprine κ-casein, can be clearly differentiated from the β-caseins by gel electrophoresis at pH 8.4 (Fig.8.6), and consequently there can be no confusion between them.

Ovine casein contains two β-casein components, (Fractions 4 and 5, Figs.8.5 and 8.6), which were designated as β_2- and β_1-casein respectively, because their electrophoretic mobilities were similar to that of bovine β-casein (Fig.4.1).

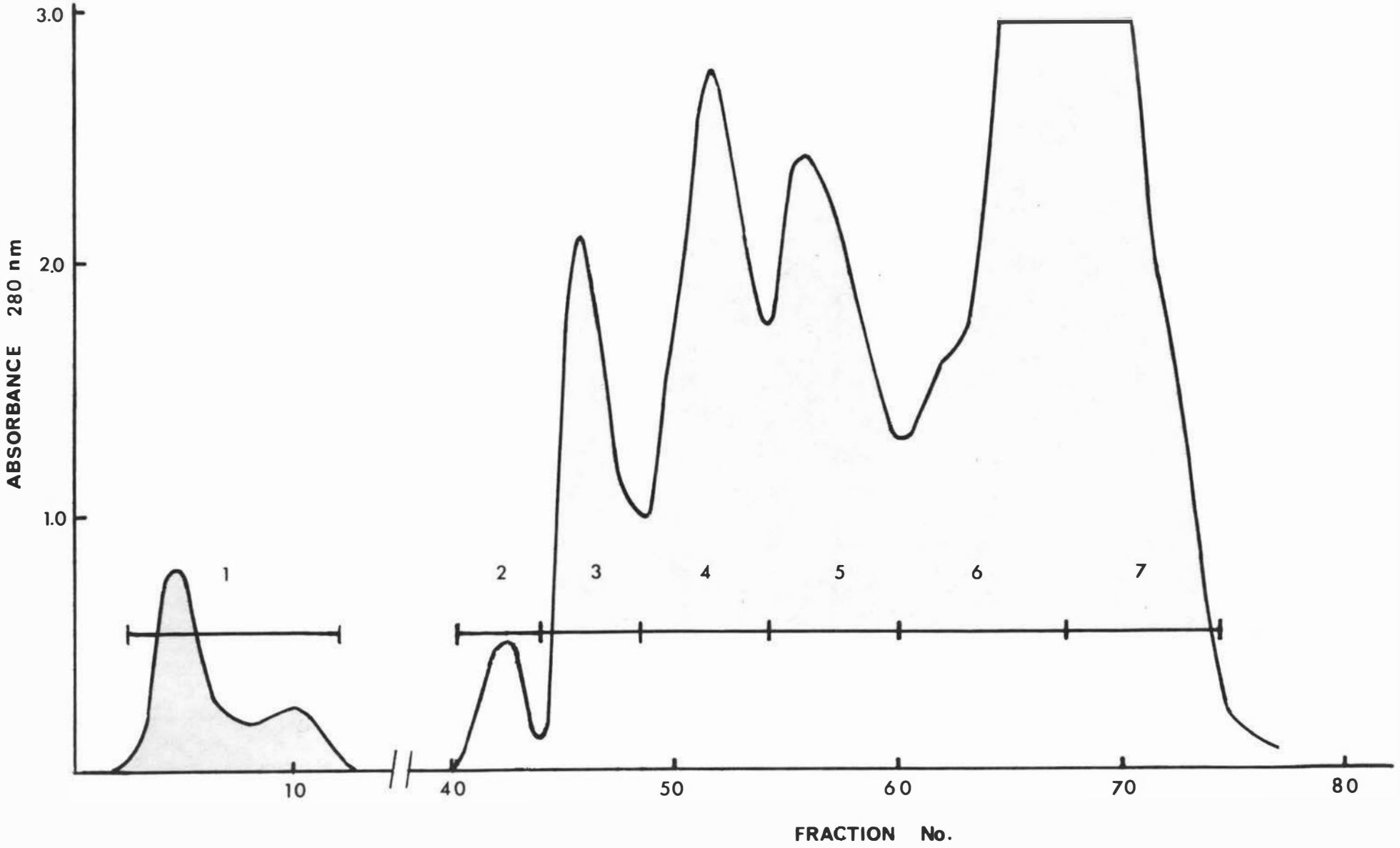


Fig.8.5: Chromatography of 1 g of ovine casein on DEAE-cellulose at pH 7. Experimental details were the same as those in Fig.8.1. The fractions indicated were pooled and examined by polyacrylamide gel electrophoresis at pH 8.4 (Fig.8.6).

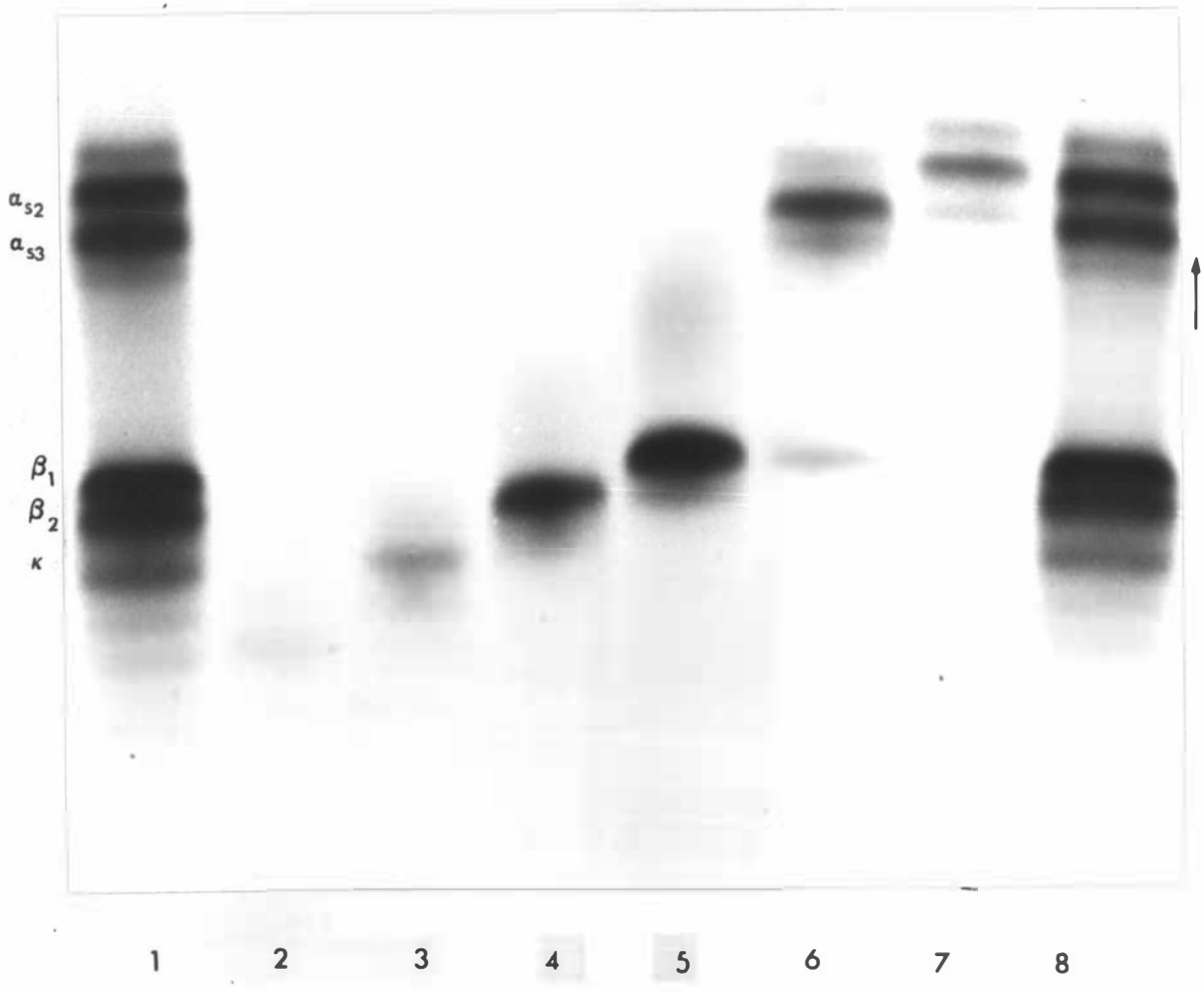


Fig.8.6: Gel electrophoresis at pH 8.4 of whole ovine casein and the fractions isolated by chromatography on DEAE-cellulose (Fig.8.5). The positions of the major caseins are indicated. Samples: 1, 8, whole ovine casein; 2, Fraction 2; 3, Fraction 3; 4, Fraction 4; 5, Fraction 5; 6, Fraction 6; 7, Fraction 7. The direction of electrophoresis is indicated.

Fractions 6 and 7 (Figs.8.5 and 8.6) contained two major α_s-casein fractions which were tentatively designated as α_{s3}- and α_{s2}-caseins, because their electrophoretic mobilities were similar to that of bovine α_{s1}-casein (Fig. 4.1). A number of other minor components with electrophoretic mobilities at pH 8.4 greater or less than the two major α_s-caseins were also observed in these fractions. The major α_s-caseins were difficult to separate from each other and were eluted on DEAE-cellulose in an incompletely resolved peak. The individual ovine caseins were more difficult to isolate in pure form than the caprine and bovine caseins because of considerable overlapping of the protein peaks in the DEAE-cellulose chromatogram.

The characteristics of these caseins and the justification for their nomenclature as κ-, β- or α_s-caseins are outlined in Chapters 10 and 11.

8.6 Composition of Ovine Casein

The composition of ovine casein in Table 8.3 was based on the results of a single experiment using a composite sample of casein. Since the ovine α_s-caseins were difficult to separate the value for α_s-casein in Table 8.3 includes a number of minor proteins in this region.

Fraction 2 which was not identified, contains some 4% of the total casein. The κ-casein content of ovine casein is similar to that of caprine casein (Table 8.2). The β-caseins exist in approximately equimolar amounts with a total content of 44.7% of the total casein, intermediate between the values for bovine and caprine caseins. Gel electrophoresis (Fig.8.6) indicated that the major α_s-caseins also appeared to exist in approximately equimolar amounts, with a total content of about 35%, again intermediate between the bovine and caprine caseins.

This composition (Table 8.3) is different from that reported by Alais and Jollès (1967) for ovine casein. They obtained a composition, based on the recovered weight of protein after chromatography on DEAE-cellulose of: minor

Table 8.3

Composition of Ovine Casein

Casein	Fraction	Composition
	Percent of total casein	
		I
γ-casein	1	6.9
X-casein	2	4.0
κ-casein	3	9.8
β_2-casein	4	20.8
β_1-casein	5	23.9
α_s-caseins	6, 7	34.6
Recovery factor		87.1
Expected protein weight		1.000 g

fractions not retained on the column, 4%; minor fractions eluted before β-casein, 4%; κ-fraction eluted in the gradient, 12%; κ-fraction eluted in 0.25 M NaOH, 4%; β-fraction, 28%; α_s-fraction, 48%.

In this study the DEAE-cellulose columns were not washed with 0.25 M NaOH after each chromatographic run. Consequently, if some κ-casein was eluted with 0.25 M NaOH, as Alais and Jollès (1967) found, this may increase the lower value of 9.8% κ-casein in Table 8.3 to a value more in keeping with their results. However, the content of α_s- and β-caseins are still considerably different to the values in Table 8.3.

8.7 Polymorphism in the Caprine Caseins

A number of individual casein samples (25), isolated from the milk of British Saanen goats and a single sample of Angora goat casein were examined by gel electrophoresis at pH 8.4 for evidence of polymorphism. Some of the representative gel electrophoretograms which were obtained are shown in Fig.8.7 and the mobilities of the caseins relative to caprine β_2-casein are presented in Table 8.4.

The most notable feature of these caprine casein gel patterns was the presence of two dominant β-casein bands, and only minor amounts of α_s-casein, unlike that of bovine casein. In the limited number of casein samples examined, both β-caseins were present in roughly equimolar amounts and did not appear to be subject to any genetic variation involving charged amino acid substitutions. A number of minor bands with a lower electrophoretic mobility than the β-caseins were also evident, particularly in Angora goat casein.

The β-casein with the greater electrophoretic mobility was designated as β_1-casein and the slower component as β_2-casein. This nomenclature appears reasonable since Zittle and Custer (1966), Ribadeau Dumas et al. (1975) and Macha (1970) have also identified two β-caseins in caprine casein.

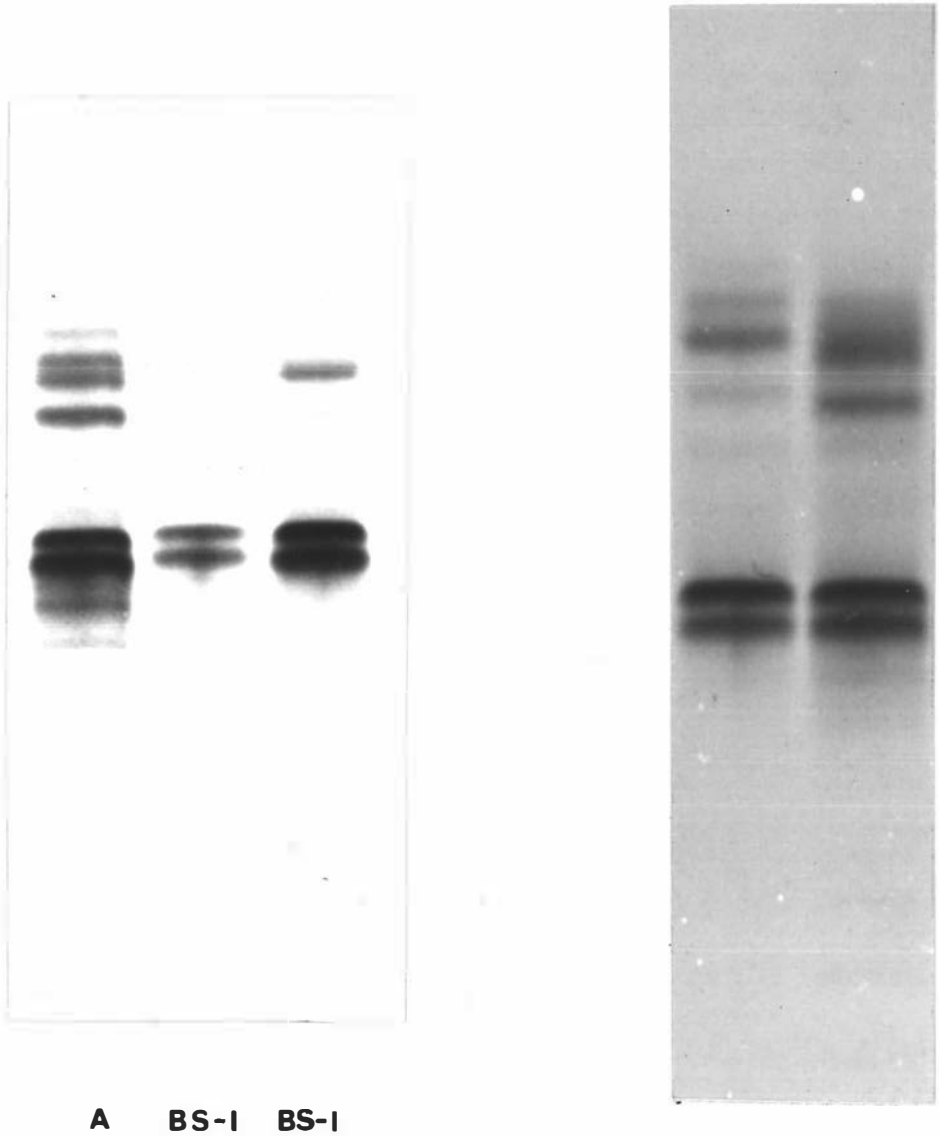


Fig.8.7: Polyacrylamide gel electrophoresis (pH 8.4) of samples of caprine casein. A, Angora goat casein; BS-I, BS-II, refer to the two types of gel electrophoresis patterns that were observed for casein from British Saanen goats.

The α_s-caseins were considerably more heterogeneous with two or three types of electrophoresis pattern being evident (Fig.8.7). One of these contains a major α_s-casein component with several minor components, while the other type, identified in three samples, has two slower major bands of approximately equal intensity which have mobilities different from the minor bands in the other α_s-casein type. Furthermore, the content of α_s-casein in these caseins with several major α_s-caseins appears to be greater than that in the samples with the single major α_s-casein. It should be noted that most of the milks were obtained from related goats and that 8 samples were from mother-daughter pairs. The sample of Angora casein had a similar pattern in the α_s-casein region to that shown by the more heterogeneous of the British Saanen caseins (Fig.8.7).

Gel electrophoresis of 10 caprine casein samples at pH 4 did not reveal any significant differences in the gel patterns. Under these conditions κ-casein is clearly evident, with a mobility intermediate between the β-caseins and α_s-caseins (Chapter 9.1). No genetic variation was observed for κ-casein.

While polymorphism was evident in the caprine caseins, particularly for the α_s-caseins, it remains to be seen whether these differences were the result of genetic variation. This can only be determined by examination of a large number of caseins from different herds and breeds, and from breeding studies. It is likely however, that genetic variants of the α_s-caseins, and perhaps the β-caseins do exist, although no conclusions can be drawn from this study.

Bogdanov et al. (1972) observed genetic variation in the α_s-caseins from goats' milk, but since their results are not readily available, they cannot be usefully compared with those of this study. Macha (1970) examined 194 samples of goats' milk by alkaline starch gel electrophoresis. A region which he called K_2 was diallelic with the occurrence of AB, BB phenotypes. This region corresponds in electrophoretic mobility to the major α_s-casein (Table 8.4). Although two bands were observed by Macha (1970) in the β-casein region, these were not subject to variation.

Table 8.4

Relative electrophoretic mobilities[1,2] of Some Bovine, Caprine and Ovine Caseins in Alkaline Gels.

Casein	Bovine	Ovine	Caprine	
			British Saanen	Angora
κ-	0.53	0.92		
β-	1.0	β_2-1.0	β_2-1.0	1.0
		β_1-1.05	β_1-1.06	1.06
		β_0-1.12		
α_{s4}-	1.49		1.24	1.24
α_{s3}-	1.59	1.51	1.33	1.34
α_{s2}-	1.66	1.58	1.42	1.40
α_{s1}-	1.73	1.64		1.45
α_{s0}-	1.79		1.54	1.54

1. The mobilities are expressed relative to β-casein in bovine casein and β_2-casein in ovine and caprine caseins, since they have similar electrophoretic mobilities and this allows for an easier comparison between the species.
2. The electrophoretic mobilities representing the major α_s-caseins are enclosed, [ ] .

8.8 Polymorphism in the Ovine Caseins

Sixty individual ovine casein samples representing Romney, Border Leicester, Perendale, Southdown and Drysdale breeds were examined by polyacrylamide gel electrophoresis for evidence of polymorphism.

Some of the typical alkaline gel electrophoresis patterns that were obtained are shown in Fig.8.8 and the electrophoretic mobilities of the caseins relative to ovine β_2-casein are compared in Table 8.4 with those of the bovine and caprine caseins. Some heterogeneity was observed between the ovine casein samples, but the major differences in the gel electrophoresis patterns were between the different breeds.

Only one major κ-casein component was observed in the casein samples from the various breeds (Fig.8.8). Alkaline gel electrophoresis indicated the presence of two β-caseins in all but two of the Romney casein samples (Fig.8.8), which contained an extra band. This was designated as β_0-casein, since it had a greater electrophoretic mobility than β_1-casein. The β-casein bands in the gel electrophoretograms varied in their intensity between different breeds of sheep (Fig.8.8). Most of the breeds of sheep that were examined are in fact Romney crossbreeds, with the exception of the Southdown, which is a "pure-breed". The Drysdales are a Romney sub-breed and the Border Leicester are Border Leicester-Romney half breeds. The Perendale originated from a Cheviot-Romney cross. In all the samples examined, except those from the Southdown and some Romney and Border Leicester ewes, where the intensity of the β-casein bands were similar, β_1-casein was the most intense, and therefore probably the major component. A decrease in the intensity of β_1-casein was observed in the two Romney samples which contained β_0-casein. Gel electrophoresis at alkaline pH in the presence of Mg caused the mobilities of the β-caseins to decrease, and to become similar so that the two ovine β-caseins were not separated (Fig.8.9).

Three major α_s-casein components were observed in all the ovine caseins examined by alkaline gel electrophoresis.

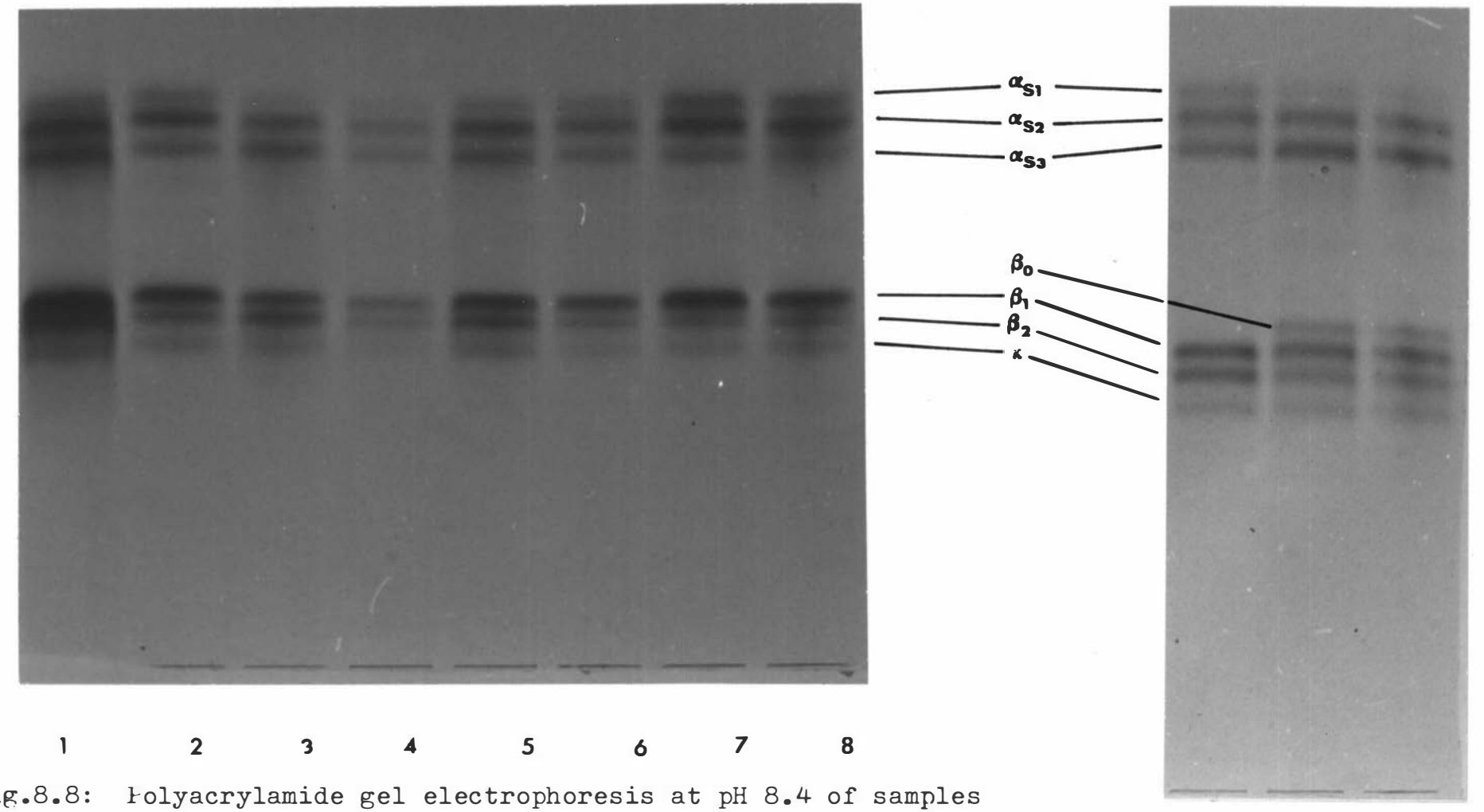


Fig.8.8: Polyacrylamide gel electrophoresis at pH 8.4 of samples of ovine casein from different breeds. 1, Romney cross-breed; 2, 3, 9, 10, 11, Romney; 4, Southdown; 5, Border Leicester; 6, Drysdale; 7, 8 Perendale. The direction of electrophoresis is indicated.

Few minor components were evident in the α_s-casein region. The major differences between casein samples, and in particular between caseins from different breeds of sheep, appeared to be in the intensity of the bands representing the three α_s-caseins, which were designated as α_{s1}-, α_{s2}- and α_{s3}-casein in order of their decreasing electrophoretic mobilities at alkaline pH (Figs.8.8 and 8.9). In the Drysdale caseins, α_{s2}-casein was the most intense band. In the Border Leicester samples α_{s2}- and α_{s3}-caseins were present in about the same concentration (Fig.8.8). However, in one Border Leicester casein sample, α_{s2}-casein was dominant, as in the Drysdale samples. In the Perendale, the major components were the α_{s1}- and α_{s2}-caseins, while in the Southdown breed the α_{s2}- and α_{s3}-caseins have the most intense bands (Fig.8.8). The Romney caseins were the most variable with either the α_{s2}- and α_{s3}-caseins or the α_{s1}- and α_{s2}-caseins being the major components (Fig.8.8).

Gel electrophoresis of the caseins at alkaline pH in the presence of Mg (Fig.8.9) did not change the intensities or relative electrophoretic mobilities of the α_s-caseins.

No attempt was made to determine the frequency of polymorphism in the ovine caseins because of the limited number of samples examined and the uncertain relationship existing between these caseins. The variation in the intensity of the casein bands was difficult to observe if the gels were overloaded with sample. The β_1- and β_2-caseins are closely related and appear to differ largely in their phosphorus contents, β_1-casein being phosphorylated to a greater extent than β_2-casein (Chapter 10). Hence, β_0-casein may contain more phosphorus than the β_1-casein since electrophoresis at alkaline pH in the presence of Mg revealed only one component in the β-casein region (Chapter 10).

Arave et al. (1973) examined 592 ovine milk samples from six breeds or crosses between these breeds by starch gel electrophoresis and revealed two variant casein types, one α_{s1}-casein and one β-casein. The overall frequency of these variants was very low, being 0.036 and 0.017 for α_{s1}-

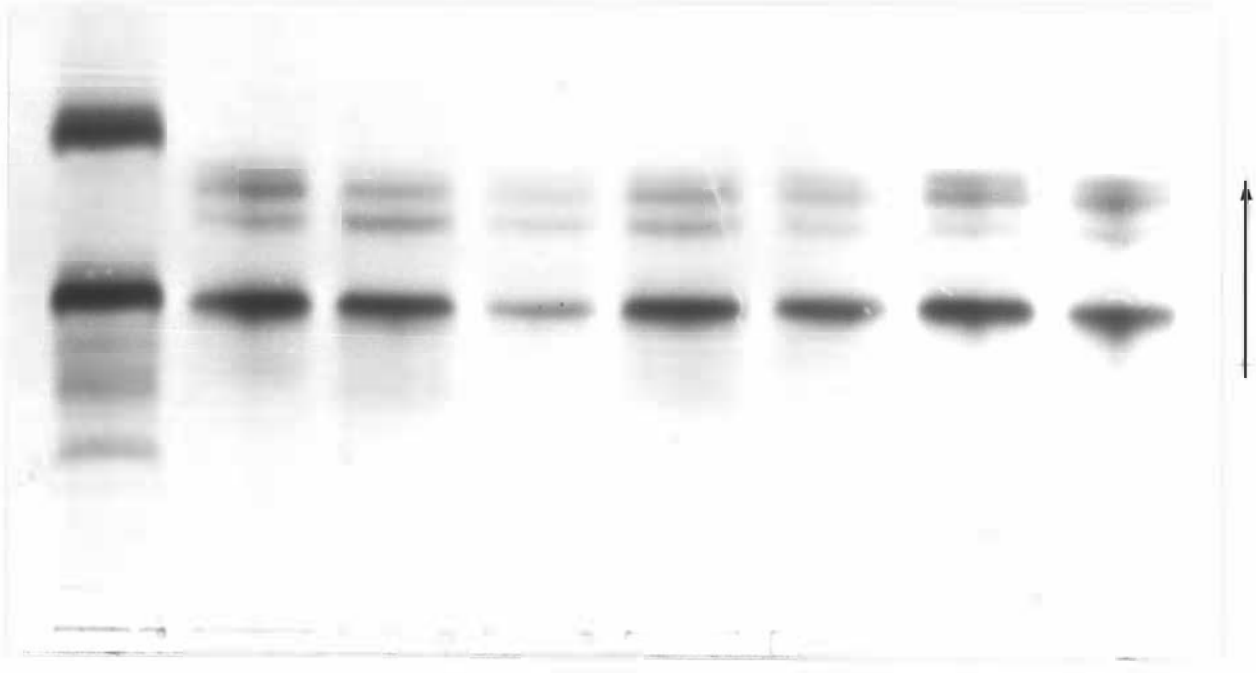


Fig.8.9: Polyacrylamide gel electrophoresis at pH 8.4 in the presence of Mg of ovine caseins isolated from different breeds of sheep.
1, bovine casein; 2, 3, Romney; 4, Southdown; 5, Border Leicester; 6, Drysdale; 7, 8, Perendale. Except for the bovine casein, the samples are the same as those in Fig.8.8. The direction of electrophoresis is indicated.

casein and β-casein, respectively. Arave et al. (1973) based their frequencies on the assumption that the variants were heterozygous in a co-dominant system. The α_s-Cn^{AB} variant observed by Arave et al. (1973) has two extra bands migrating between the three normal α_s-casein bands, designated as the A variant, and β-casein, giving a total of 5 bands instead of three. The genetic polymorphism observed by Arave et al. (1973) for β-casein was essentially similar to that observed in this study. They identified a component analogous to β_0-casein which was designated as the A variant, while β_2-casein, present in all the caseins examined, was designated as the B variant. In a sample, designated as β-Cn^{AB}, they found the intensity of the component of intermediate mobility (β_1-casein) was diminished or absent.

King (1966) in his study of 1022 individual ovine casein samples observed two α-casein variants and one β-casein variant. An α-casein variant, designated as the "Welsh" variant, was the same as the B variant described by Arave et al. (1973), and had a gene frequency of 0.048. The normal starch gel electrophoresis pattern indicated the presence of three α-casein bands, similar to the patterns in Fig.8.8 . In addition, King (1966) observed a fourth band in some casein samples that had a greater mobility than the usual three α-casein bands. Furthermore, he observed some variation in the intensity of individual α-casein bands, similar to that evident in Fig.8.8. King (1966) observed variation in the patterns of the β-caseins, and observed a band similar to that which was designated as β_0-casein in this study.

Both El-Negaimy and Burfening (1972) and Bogdanov et al. (1972) observed genetic polymorphism in the ovine caseins. However, the lack of data available for comparison with the results of this study means the type and nature of the polymorphism on which they based their results is unknown.

The lack of available information, and the arbitrary designation of various casein components as the A, B or C variants based on their electrophoretic mobilities, helps to complicate the already rather confusing nature of polymorphism in the ovine caseins.

King (1966) suggested the variation in ovine β-caseins (i.e. β_0-casein in Fig.8.8) was due to a simple alternative allele. While the nature of the variation in the β-caseins will require further investigation, alkaline gel electrophoresis in the presence of Mg does not eliminate the possibility that β_0- and β_1-caseins may differ in their phosphorus content, similar to β_1- and β_2-caseins (Chapter 10).

The variation in the ovine α_s-caseins was more obscure, although King (1966) described the "Welsh" variant, only observed in Clun Forest, Welsh Mountain and Suffolk breeds with gene frequencies of 0.04, 0.10 and 0.03, respectively, as the heterozygous form of a mutant gene. The variation in the intensity of the α_s-caseins, cannot however be explained at this time.

Aschaffenburg and Thompson (1967) observed polymorphism in the caprine α_s-caseins, although the mode of inheritance is not as simple as the bovine, and its nature is still obscure.

The dominant feature of the ovine and caprine caseins is the limited amount of polymorphism which is evident, as shown by gel electrophoresis. Clearly, further work on the inheritance and on the isolated caseins and their peptides is needed to resolve this problem.

CHAPTER 9: CHARACTERIZATION OF CAPRINE CASEINS

The isolation and chemical characterization of the major caprine caseins are described in this chapter. These caseins, which include the major α_s-, β_1-, β_2- and κ-caseins, were characterized from their electrophoretic mobilities in polyacrylamide gels, molecular weights as determined by gel chromatography, amino acid compositions, phosphorus contents and extinction coefficients at 280 nm. These characteristics were also compared with those of their bovine counterparts.

CAPRINE κ-CASEIN

9.1 Purification of Caprine κ-Casein

Five major fractions were isolated by chromatography of whole caprine casein on DEAE-cellulose (Figs.8.3 and 8.4). The major component in Fraction 2 was designated as κ-casein, since gel electrophoresis showed it was readily hydrolysed with rennet, resulting in an almost complete disappearance of the major protein bands. Fraction 2 was rechromatographed on a column of CM-32 cation exchange cellulose at pH 4.0 in 0.01 M sodium formate buffer containing 4.5 M urea and 2-mercaptoethanol (0.1%, v/v) to remove minor contaminants (Fig.9.1). The purity of caprine κ-casein isolated by this procedure and the effect of treatment with rennet are indicated by electrophoresis at pH 8.4 and pH 4 (Figs.9.2 and 9.3). Caprine para-κ-casein moved towards the anode at pH 8.4, whereas under the same conditions bovine para-κ-casein migrated toward the cathode (Fig.9.2).

Caprine κ-casein has a greater electrophoretic mobility at pH 8.4 than bovine κ-casein B (Fig.9.2). Caprine κ-casein was obscured in electrophoretograms at pH 8.4 of whole caprine casein by the more intense bands of β_1- and β_2-caseins. On electrophoresis at pH 4 (Fig.9.3), caprine κ-casein had a mobility intermediate between that of the β-caseins and α_s-casein. The failure to identify caprine κ-casein by alkaline gel electrophoresis of a sample of whole casein, because it was obscured by two major casein components, has also been reported by Zittle and Custer (1966).

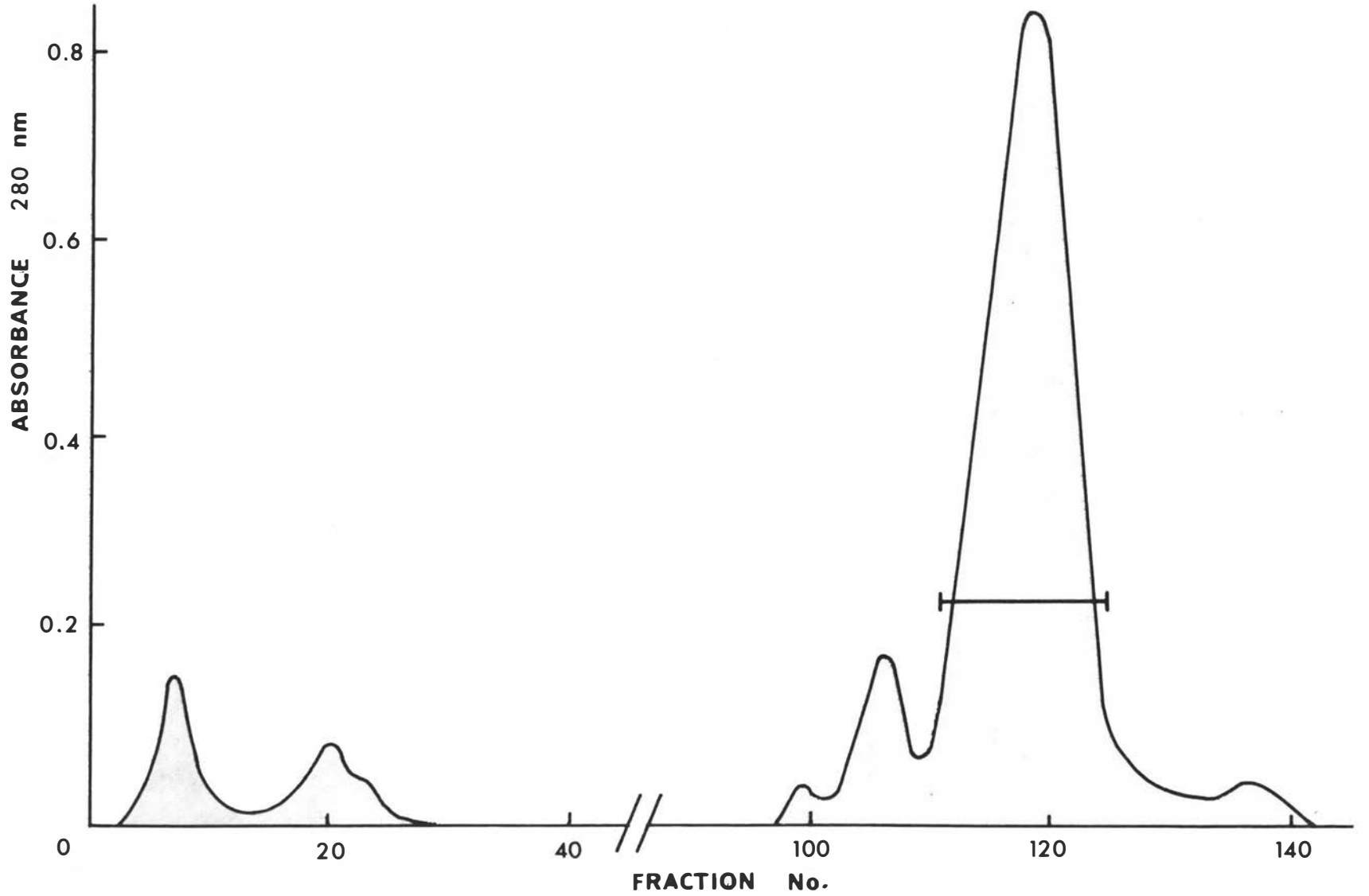
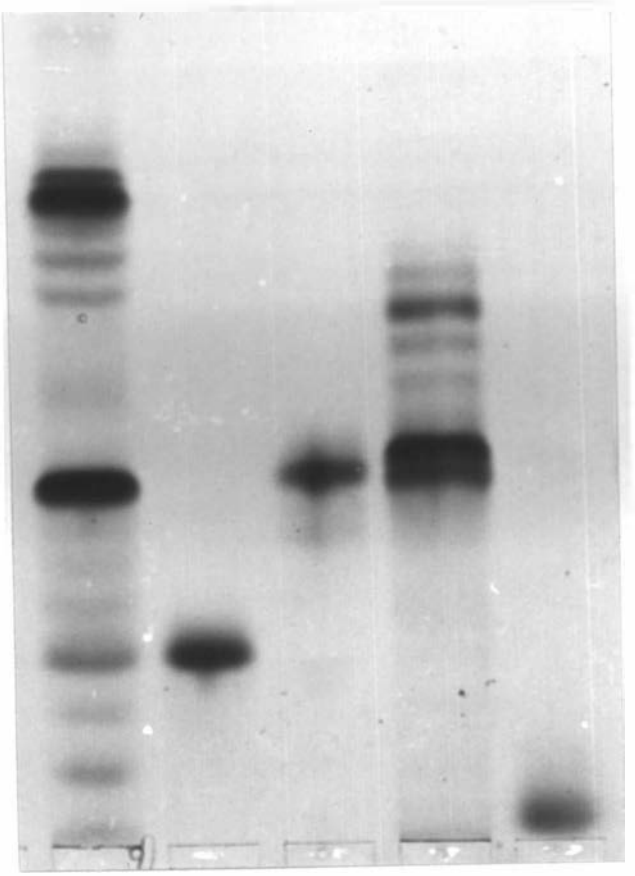
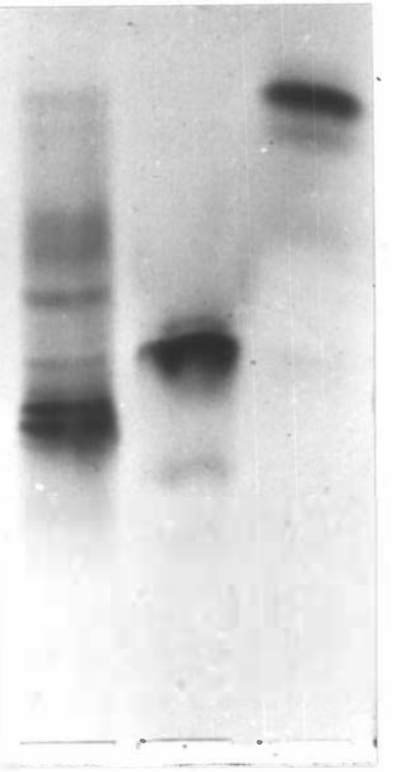


Fig.9.1: Chromatography of caprine κ-casein on a CM-cellulose column (2 cm x 50 cm) in 0.01 M sodium formate buffer, pH 4.0, containing 4.5 M urea and 2-mercaptoethanol (0.1% v/v). The column was eluted with a 1 l linear gradient from 0-0.12 M NaCl. The fraction containing κ-casein, indicated by the bar, was pooled and its purity examined by alkaline and acid gel electrophoresis (Figs.9.2 and 9.3).



1 2 3 4 5

Fig.9.2: Alkaline gel electrophoresis patterns (pH 8.4) of whole caprine casein (4), purified κ-casein (3) and the effect of rennet on this fraction (5), compared with whole bovine casein (1) and purified κ-casein (2). The direction of electrophoresis is indicated.



1 2 3

Fig.9.3: Acid gel electrophoresis (pH 4) of whole caprine casein (1), purified caprine κ-casein (2) and the effect of rennet on this fraction (3).

Alkaline gel electrophoresis indicated that the κ-casein preparation obtained by a combination of DEAE- and CM-cellulose chromatography was fairly pure (Fig.9.2). However, acid gel electrophoresis revealed that κ-casein contained traces of two minor impurities (Fig.9.3). The level of the impurities in some other preparations were less than those indicated in Figs.9.2 and 9.3. The minor band with a greater mobility than κ-casein, observed by alkaline gel electrophoresis, may be the carbohydrate containing fraction of κ-casein. Alais and Jollès (1961) and Jollès et al. (1964) found the level of carbohydrate in the glycomacropeptide fraction of caprine casein to be considerably less than in the bovine glycomacropeptide fraction. They obtained values for the bovine and caprine glycomacropeptides of 14.3% and 3.0% sialic acid, respectively. Consequently, caprine κ-casein should not be as heterogeneous as bovine κ-casein on gel electrophoresis.

9.2 Molecular Weight of Caprine κ-Casein

The molecular weight of caprine κ-casein was estimated by chromatography in buffer containing 7 M urea on a column of Sephadex G-100 which had previously been calibrated with carboxymethylated proteins of known molecular weight (Fig.9.4). Caprine κ-casein was eluted from the column in a similar position to bovine κ-casein. The distribution coefficient obtained for each protein was the average of at least three determinations. The distribution coefficient obtained for caprine κ-casein indicated a molecular weight of 18 400±500 which is similar to the molecular weight of 18 500 obtained for bovine κ-casein by sedimentation equilibrium in 5 M guanidine.HCl (Woychik et al., 1966). The molecular weight obtained for caprine κ-casein is close to the molecular weight (19 023) estimated from the amino acid sequence of bovine κ-casein B (Mercier et al., 1973).

The molecular weights of the other caseins were not determined using this method because of the high slope in Fig.9.4, indicating the low sensitivity of elution weight to molecular weight, at molecular weights above 20 000. It would appear that under the conditions used for chromatography

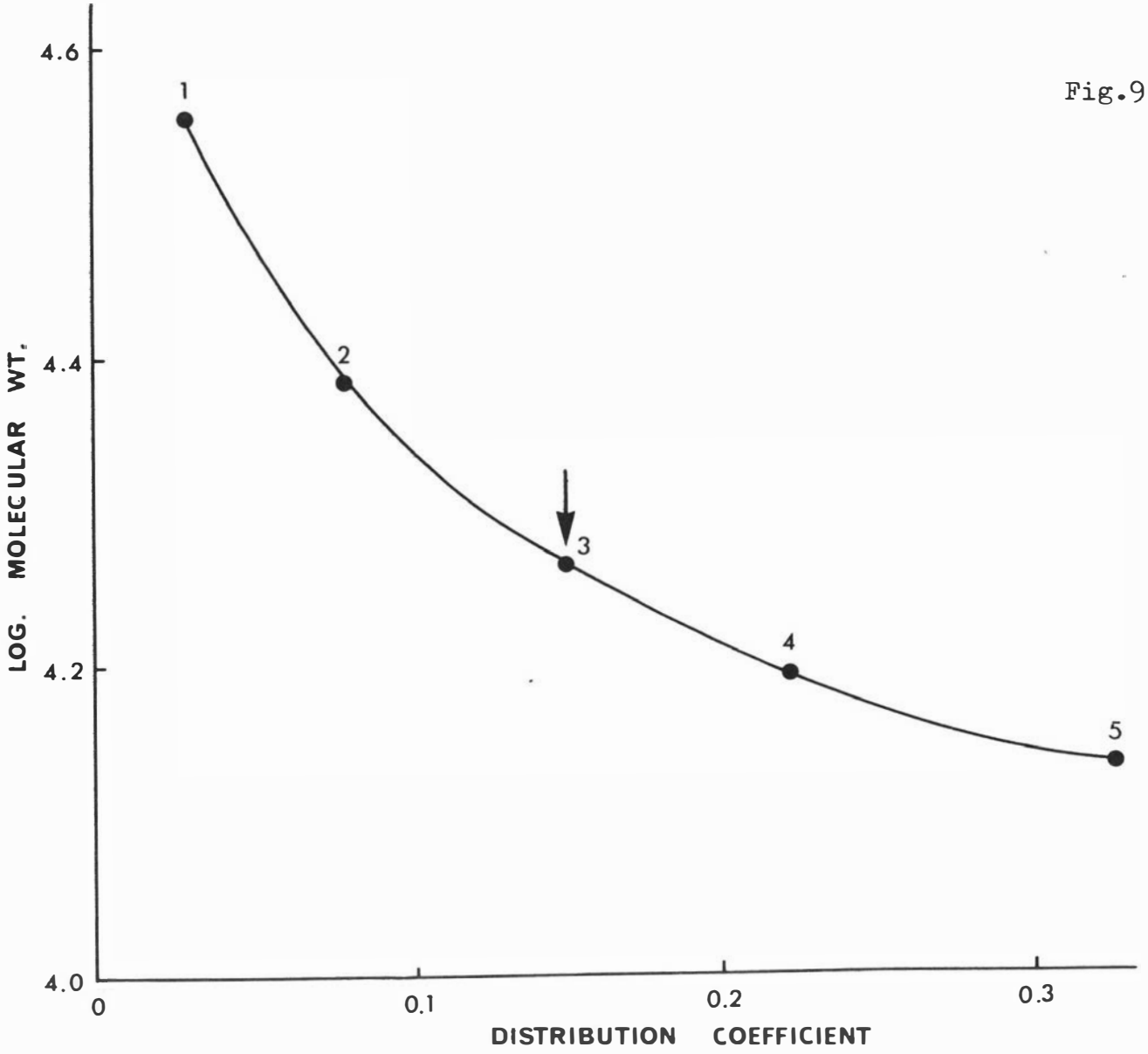


Fig.9.4: Plot of the logarithm of the molecular weight versus the distribution coefficient for carboxymethylated proteins chromatographed in 0.1 M Tris-HCl buffer, pH 8.0, containing 7 M urea on Sephadex G-100. The standard proteins used to calibrate the column were: 1, pepsin; 2, bovine β-casein B; 3, bovine κ-casein; 4, haemoglobin; 5, ribonuclease A. The position at which caprine κ-casein is eluted is indicated. Further details are outlined in the text.

on Sephadex G-100 (i.e. buffer containing 7 M urea), proteins with molecular weights above 20 000 are only partly included in the gel.

9.3 Amino Acid Composition of Caprine κ-Casein

The amino acid composition of caprine κ-casein was calculated from twelve analyses. This was determined by duplicate analysis of duplicate samples hydrolysed for 24, 48 and 72 h. The number of residues per mole of protein were initially calculated on the basis that the protein had a molecular weight of 18 400 and contained 8 residues of lysine. It was refined by assuming the protein had 8 lysine residues, 15 aspartic acid residues, 14 alanine residues, 7 tyrosine residues and 4 phenylalanine residues. The results are shown in Table 9.1, together with the reported compositions of bovine (Mercier et al., 1973) and ovine κ-caseins (Alais and Jollès, 1967; Ribadeau Dumas et al., 1975). The composition of bovine κ-casein B was based on the amino acid sequence determined by Mercier et al. (1973). With the exception of serine, threonine, valine and isoleucine the number of residues was taken as the average of twelve replicates including three hydrolysis times. The molecular weights calculated from the amino acid compositions and shown in Table 9.1, do not take into account the amides present or the sugar residues bound to the protein, but include the phosphate residues.

The amino acid composition of caprine κ-casein was very similar to that of bovine κ-casein B (Mercier et al., 1973) (Table 9.1) based on its amino acid sequence. The number of residues of eight amino acids in the compositions of bovine κ-casein and caprine κ-casein were the same, and seven amino acids differed by only one residue. The number of residues for isoleucine and aspartic acid differed by three and four respectively. A close similarity was also observed between the compositions of caprine κ-casein and ovine κ-casein (Alais and Jollès, 1967; Ribadeau Dumas et al., 1975). The extinction coefficient (absorbance at 280 nm of a 1% (w/v) solution, 1 cm in depth) of caprine κ-casein was 8.80, a value in agreement with an analysis of 1 tryptophan,

Table 9.1

Amino Acid Compositions of Caprine, Ovine and Bovine κ-Caseins
(Residues per Mole of Protein)

Residue	Caprine κ-Casein			Bovine κ-Casein B	Ovine κ-Casein B	Ovine κ-Casein
	Mean No. of Residues	Standard Deviation	Probable No. of Residues	Mercier et al. (1973)	Alais and Jolles (1967)	Ribadeau Dumas et al. (1975)
Asp	15.13	0.26	15	11	17	16
Thr[1]	13.4		13	14	10	12
Ser[1]	12.1		12	13	10-12	12
Glu	26.60	1.08	27	27	25-27	26-27
Pro	19.70	1.23	20	20	21	19-20
Gly	2.20	0.53	2	2	2	2
Ala	14.06	0.33	14	15	17	18-19
Cys[2]	2.11		2	2	1- 2	3
Val[1]	10.97	0.29	11	11	10-11	12
Met	0.98	0.18	1	2	2- 3	2
Ile[1]	9.57	0.16	10	13	10	9
Leu	8.09	0.52	8	8	8- 9	7
Tyr	7.16	0.27	7	9	8- 9	8
Phe	3.99	0.25	4	4	4	4
His	3.81	0.16	4	3	3- 4	4
Lys	7.89	0.38	8	9	9	8
NH_3				21		
Arg	4.13	0.28	4	5	5- 6	6
Trp[3]	0.96		1	1	1- 2	1
Phosphorus residues	1.4		1	1	2	2
Molecular weight (amino acid composition)			18 291	19 023	19 180	19 050
No. of residues			163	169	163-174	168-171

1. Values for serine and threonine were obtained by extrapolation to zero time. Valine and isoleucine were taken as the 72 h results.
2. Measured as cysteic acid from duplicate analyses.
3. Tryptophan was estimated from a single analysis where thioglycollic acid was present during hydrolysis (Matsubara and Sasaki, 1969).

7 tyrosine and 4 phenylalanine residues (Table 9.1). Bovine κ-casein B which contains two more tyrosine residues than caprine κ-casein (Table 9.1) has been reported to have an extinction coefficient of 9.6 (Talbot and Waugh, 1970).

9.4 Discussion

About 10% of the protein in caprine casein is κ-casein, which can be readily isolated by a combination of chromatography on DEAE- and CM-cellulose. In contrast, the proportion of κ-casein in bovine casein is about 15%.

The glycopeptides isolated from ovine and caprine caseins do not appear to be as heterogeneous as those from bovine casein, presumably due to their lowered content of sialic acid (Alais and Jollès, 1961; Jollès et al., 1964). The most notable feature of caprine and ovine κ-caseins is their high aspartic acid level, compared with bovine κ-casein. The phosphorus content of 0.23% for caprine κ-casein was similar to that for bovine κ-casein (Alais and Jollès, 1961), there being 1.4 residues of phosphorus per mole of caprine κ-casein. Alais and Jollès (1967) reported a phosphorus content of 0.35% and 0.40% (2.1 and 2.5 residues per molecule) for two ovine κ-casein fractions which they designated as the A and B genetic variants. Ribadeau Dumas et al. (1975) found 2 residues of phosphorus in ovine κ-casein.

The molecular weight of 18 400 for caprine κ-casein, obtained by chromatography in 7 M urea on a Sephadex G-100 column, was in good agreement with the value of 18 290 determined from the amino acid composition. This value is close to the molecular weight of 19 023 obtained for bovine κ-casein B (Mercier et al., 1973) from the amino acid sequence.

The greater mobility of caprine κ-casein on gel electrophoresis at pH 8.4 is undoubtedly linked to its amino acid composition with one less lysine and arginine and four more aspartic acid residues than bovine κ-casein (Table 9.1), since the phosphorus content of the two caseins is similar (Richardson et al., 1973). It is likely that much of this

charge difference resides in the para-κ-casein region of the κ-caseins, because of the relative electrophoretic mobility of caprine and bovine para-κ-caseins in alkaline gel electrophoresis (Assenat, 1967). Under these conditions, caprine para-κ-casein had the greatest electrophoretic mobility, followed by ovine para-κ-casein, while bovine para-κ-casein had a negative mobility (moved towards the cathode).

CAPRINE β-CASEINS

9.5 Isolation of Caprine β_1- and β_2-Caseins

Two caprine β-caseins, designated as β_1-casein (Fraction 4, Figs.8.3 and 8.4) and β_2-casein (Fraction 3, Figs.8.3 and 8.4) were isolated by chromatography of whole caprine casein on DEAE-cellulose. Their nomenclature was discussed in Chapter 8.7. β_2-Casein, the slower component on electrophoresis at pH 8.4 was eluted more readily from the DEAE-cellulose column than β_1-casein (Fig.8.4). Each β-casein fraction was normally rechromatographed on DEAE-cellulose before being purified further by chromatography on a column of CM-cellulose in 0.01 M sodium formate buffer, pH 4.0, containing 4.5 M urea. A typical chromatogram obtained for β_2-casein is shown in Fig.9.5. Rechromatography on DEAE-cellulose was more effective in removing the other β-casein component, while chromatography on CM-cellulose removed the minor α_s-casein fractions normally found in impure β-casein preparations (Fig.8.4). The purity of the caprine β-caseins isolated using this procedure is shown in Fig.9.6. A fraction containing crude bovine β-casein B, isolated by urea fractionation of whole casein, was purified by chromatography on DEAE-cellulose. The fractions containing pure protein were pooled after being examined by gel electrophoresis.

Gel electrophoresis at pH 4 reversed the order of mobility of the caprine β-caseins so that β_2-casein became more mobile than β_1-casein. In the presence of Mg in the electrophoresis buffer the mobilities of β_1- and β_2-caseins decreased and the two components merged (Fig.9.6). This suggested that β_1- and β_2-caseins differed in the number of

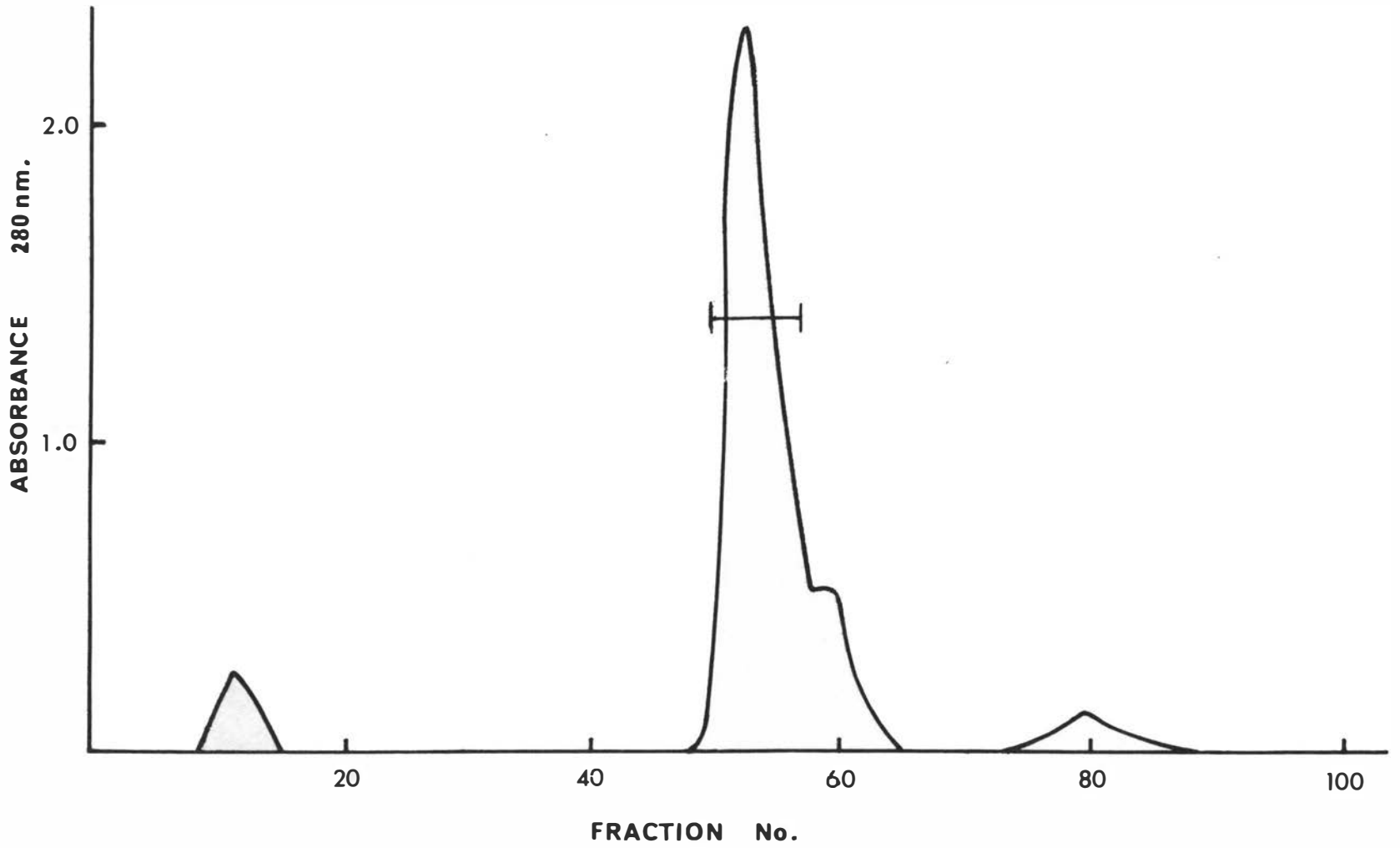


Fig.9.5: Chromatography of crude caprine β_2-casein on CM-cellulose. A 1 l linear gradient of 0-0.15 M NaCl was used to elute the caseins. The other experimental details are the same as those in Fig.9.1. The fraction indicated was pooled and its purity examined by gel electrophoresis (Fig.9.6).

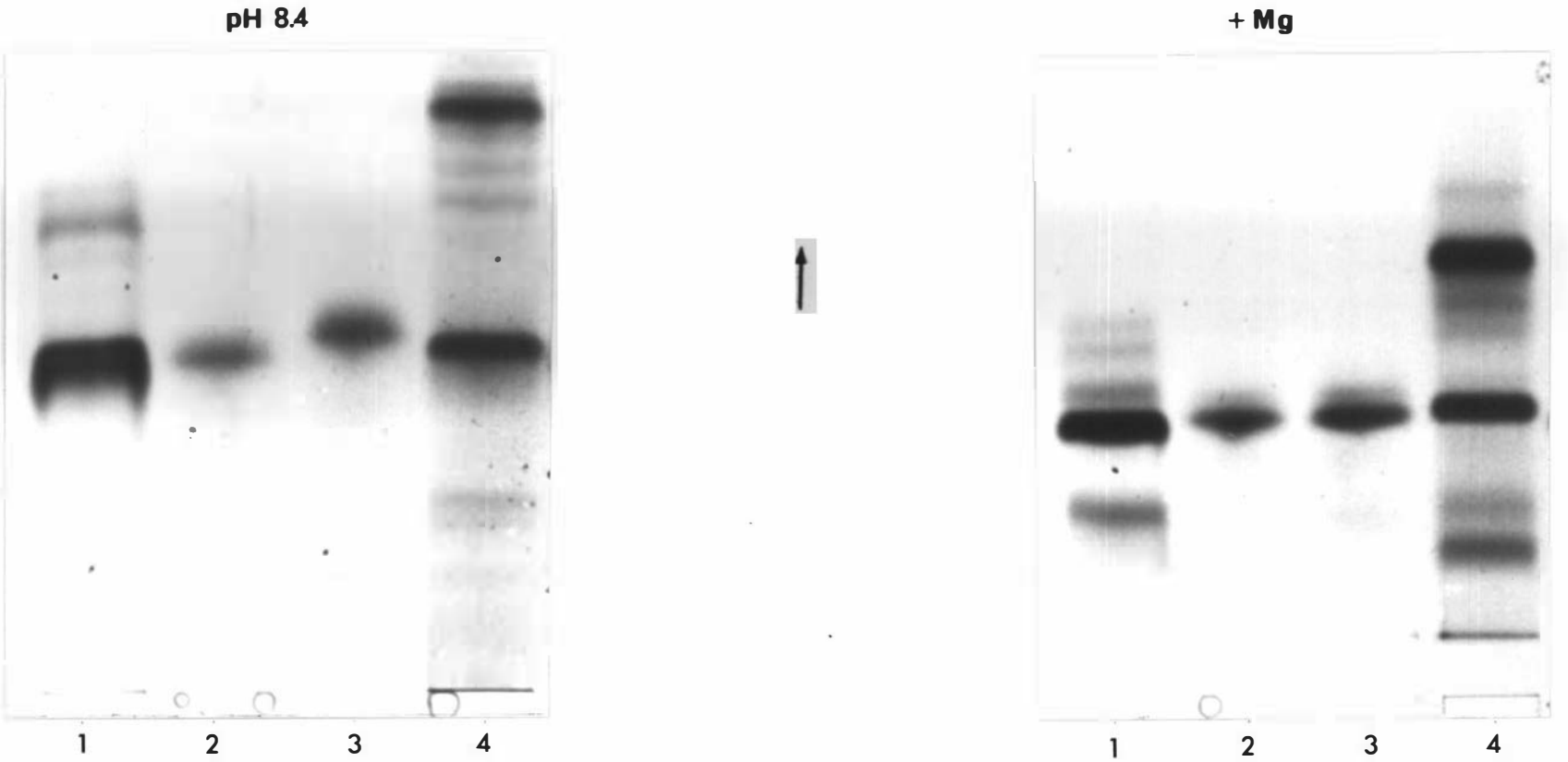


Fig.9.6: Gel electrophoresis at alkaline pH of the caprine β-caseins and at alkaline pH in the presence of Mg. Samples: 1, caprine casein; 2, caprine β_2-casein; 3, caprine β_1-casein; 4, bovine casein. The direction of electrophoresis is indicated.

phosphate groups that each contained, since Mg associates strongly with the phosphate groups of the caseins and largely neutralizes their charge. This conclusion is supported by the results from acid gel electrophoresis which showed that β_1-casein carries more negative charge than β_2-casein at both pH 4 and pH 8.6. At these pH values the phosphate groups would carry single and double negative charges, respectively.

The mobility of caprine β_2-casein on alkaline gels was similar to that of bovine β-casein B (Fig.9.6).

9.6 Molecular Weights of Caprine β-Caseins

The molecular weights of the major caprine, bovine and ovine caseins, except caprine κ-casein, were estimated by chromatography on a column of Sepharose 6B in buffer containing 6 M guanidine.HCl. A typical elution pattern obtained from a calibration run is shown in Fig.9.7. The distribution coefficients (Kd) for the standard carboxymethylated proteins and the caprine and bovine caseins were the average of at least three determinations.

The results were analysed using the procedure as described by Bryce and Crichton (1971). Using this procedure, values of $Kd^{\frac{1}{3}}$ were plotted against $(\text{molec.wt})^{\frac{1}{2}}$ for the standard proteins (Fig.9.8) and this calibration curve was then used to estimate the molecular weights of the caseins. Linear least squares analysis of the data obtained for the standard proteins resulted in the expression:

$$Kd^{\frac{1}{3}} = 1.01546 - 0.00188\ (\text{molec.wt})^{\frac{1}{2}}$$

The molecular weights obtained for the caprine and bovine caseins are shown in Table 9.2, although these results will be discussed in their respective sections.

The value obtained for bovine α_{s1}-casein B (Table 9.2) was nearly identical to the molecular weight of 23 615 estimated from the amino acid sequence (Mercier et al., 1971).

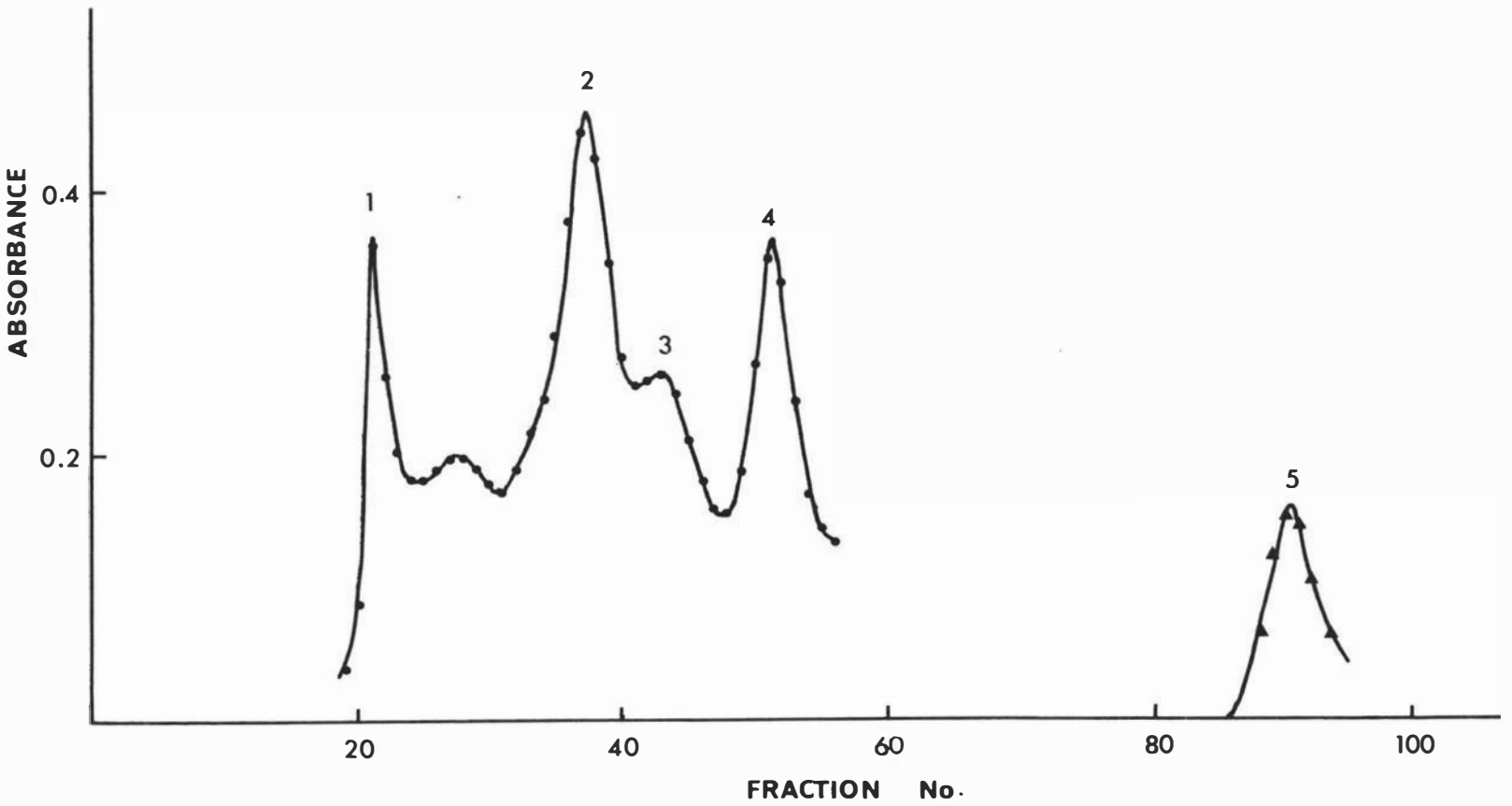


Fig.9.7: A typical chromatographic run used to calibrate the molecular weight column. The sample, containing 1 mg of blue dextran 2000 (1) and 0.02 mg α-DNP-alanine (5), and the carboxymethylated proteins, γ-globulin (5.25 mg), consisting of the H-chain (2) and the L-chain (3) and cytochrome C (2.6) mg (4) were chromatographed on a column (2 cm x 90 cm) of Sepharose 6B in 0.02 M Tris, 0.01 M EDTA, pH 8.2, buffer containing 6 M guanidine. HCl. The fractions were approximately 2.5 g, and the flow rate was maintained at 5.2 g/h.

● absorbance at 280 nm. ▲ absorbance at 365 nm.

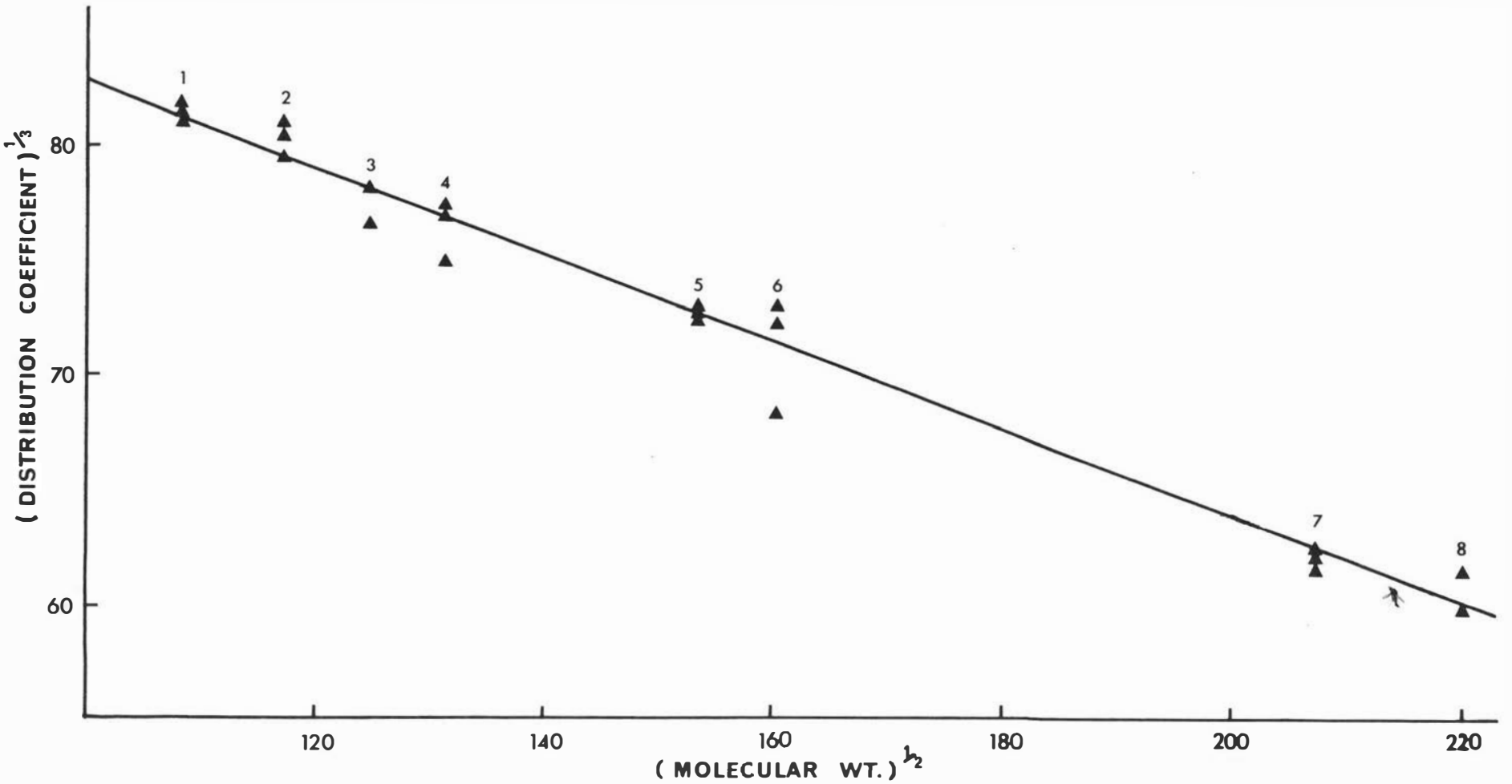


Fig.9.8: The molecular weight calibration curve obtained by chromatography of S-carboxymethylated proteins of known molecular weight on a column of Sepharose 6B in buffer containing 6 M guanidine HCl (Fig.9.7). The proteins used to calibrate the column were: 1, cytochrome C; 2, ribonuclease A; 3, haemoglobin; 4, myoglobin; 5, γ-globulin L-chain; 6, chymotrypsinogen A; 7, ovalbumin; 8, γ-globulin H-chain. The molecular weights of these proteins are listed in Table 7.6.

Likewise, the molecular weight obtained for bovine β-casein B was close to the molecular weight of 24 089 estimated from the amino acid sequence (Swaisgood, 1973). These results agree closely with those obtained using other physical techniques that were previously outlined in Chapter 6.11 and 6.15.

Table 9.2

Molecular Weights of Caprine and Bovine Caseins

	Molecular weight	Standard[1] deviation
Caprine α_s-casein	25 700	±1500
Caprine β_2-casein	23 500	± 900
Caprine β_1-casein	25 500	±1200
Bovine α_{s1}-casein B	24 100	±1600
Bovine β-casein B	25 100	± 600
Bovine α_{s3}-casein	25 400	±1200

[1] Given by $\sqrt{\sum_i (X_i - \bar{X})^2 / n-1}$ where X_i is an individual determination and $\bar{X}$ is the mean of n determinations.

The precision of $Kd^{\frac{1}{3}}$ values for caprine β_2-casein was about 1%, resulting in an accuracy for the molecular weight of 8%. In general, for the other caseins, the precision in estimating $Kd^{\frac{1}{3}}$ was approximately 1%, with an accuracy in estimating the molecular weight of about 10%.

Molecular weights of 23 500 and 25 500 were obtained for caprine β_2- and β_1-caseins, respectively (Table 9.2). In addition, a sample containing both β_1- and β_2-caseins was chromatographed on the molecular weight column and the fractions collected as before. After weighing, each fraction was dialysed to remove salts, and after gel electrophoresis and densitometry the ratio of β_1- and β_2-casein was found to be constant across the protein peak. This showed that the molecular weights of these proteins were not distinguishable within the limits of this technique

(±500). Consequently, the caprine β-caseins were assumed to have the same molecular weight, namely 24 500. Using the technique (described above) of gel filtration on the molecular weight column and examination of the fractions by electrophoresis, enabled the peaks of bovine α_{s1}-I (the rennin degradation product of α_{s1}-casein B), bovine α_{s1}-casein A and α_{s1}-casein B to be clearly resolved (Creamer and Richardson, 1974). These caseins have molecular weights of 20 800, 22 098 and 23 615 for α_{s1}-I, α_{s1}-casein A and B, respectively.

9.7 Amino Acid Compositions of the Caprine β-Caseins and Bovine β-Casein B

The amino acid compositions of caprine β_1- and β_2-caseins and bovine β-casein B were determined by analysis in triplicate of duplicate samples hydrolysed *in vacuo* in 6 M HCl for 24, 48 and 72 h. Threonine and serine results obtained from 24 h hydrolysates were increased 5% and 10% respectively, to allow for decomposition of these residues (Moore and Stein, 1963). Values for isoleucine and valine were 72 h results, as both of these amino acids are capable of forming acid resistant peptide bonds which result in the slower release of these amino acids (Moore and Stein, 1963). Incomplete release of isoleucine and valine after hydrolysis for 24 and 48 h was observed in this study.

A typical amino acid chromatogram obtained for caprine β_2-casein using the Locarte Mk IV amino acid and peptide analyser is presented in Fig.9.9.

Molar ratios of amino acids in the caprine β-caseins were determined assuming the proteins each had a molecular weight of 24 500 and contained 9 aspartic acid residues, 20 leucine residues, 9 phenylalanine residues and 12 lysine residues in each molecule. The amino acid composition of bovine β-casein B was based on 9 aspartic acid residues, 5 glycine residues, 4 tyrosine residues, 9 phenylalanine residues and 11 lysine residues in each molecule of protein. These amino acid compositions are compared with that of bovine β-casein A[2] (estimated from its amino acid sequence; Mercier et al., 1972a) and those of the caprine β-caseins (reported by Ribadeau Dumas et al., 1975) (Table 9.3).

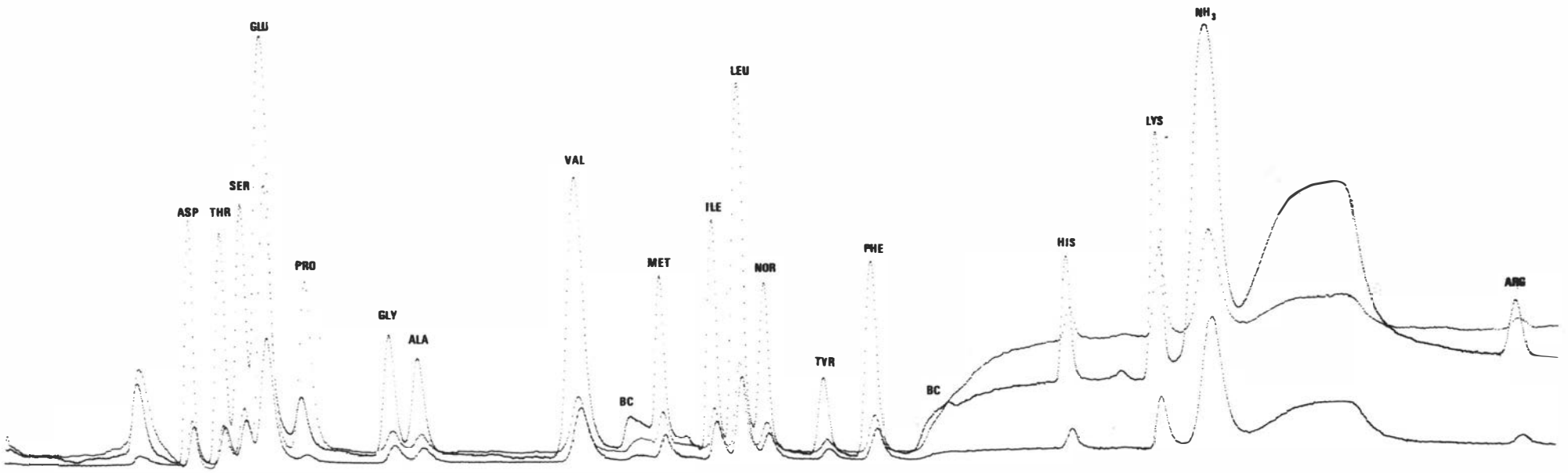


FIGURE 9.9: Amino acid analysis chromatogram obtained for caprine β_2–casein using a Locarte Mk.IV analyser. Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glumatic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Nor, norleucine; Tyr, tyrosine; Phe, phenylalahine; His, histidine; Lys, lysine; NH_3, ammonia; Arg, arginine; BC, buffer change.

Table 9.3

Amino Acid Composition of β-Casein (Residues Amino Acid/Mole Protein)

Residue	Caprine β_1-Casein Mean No. Residues	S.D.[2]	Caprine β_2-Casein Mean No. Residues	S.D.[2]	Bovine β-Casein B Mean No. Residues	Caprine β_1-Casein Probable No. Residues	Caprine β_2-Casein Probable No. Residues	Bovine β-Casein A[2] Ribadeau Dumas et al. (1973)	Caprine β-Caseins[1] Ribadeau Dumas et al. (1975)
Asp	9.0	0.22	9.1	0.12	9.3	9	9	9	9-12
Thr[1]	11.5		11.6		8.9	12	12	9	12
Ser[1]	14.8		14.7		15.2	15	15	16	15
Glu	43.2	1.31	43.0	0.89	39.8	43	43	39	41
Pro	32.8	1.36	32.6	1.13	35.0	33	33	35	33
Gly	5.6	0.14	5.6	0.20	5.1	6	6	5	5
Ala	4.7	0.18	4.7	0.16	5.7	5	5	5	5
Val[1]	21.0	0.26	21.3	0.52	18.4	21	21	19	19-20
Met	5.8	0.14	6.3	0.24	5.6	6	6	6	6
Ile[1]	8.6	0.17	9.0	0.31	9.0	9	9	10	9
Leu	20.2	0.49	20.5	0.47	21.8	20	20	22	21
Tyr[1]	3.6	0.07	3.4	0.06	4.0	4	4	4	4
Phe	8.8	0.15	9.0	0.14	8.8	9	9	9	9
His	5.1	0.08	5.2	0.14	6.1	5	5	5	5
Lys	12.1	0.40	11.7	0.20	11.0	12	12	11	12
Arg	3.3	0.07	3.1	0.15	4.4	3	3	4	3
Trp[1]	0.7		0.8		-	1	1	1	1
NH_3[1]						29	29	26	-
Phosphorus residues	6.2		5.3		-	6	5	5	4/5
Molecular weight (from Sepharose 6B column)	25 500		23 500		25 100				
Molecular weight (from amino acid composition)						24 439	24 360	23 982	23 950
No. of residues						213	213	209	210

1. See text.
2. Standard deviation.

The amino acid compositions of caprine β_1- and β_2-caseins were nearly identical, differing mainly in their phosphorus content (Table 9.3). The amino acid composition of bovine β-casein B was very similar to that estimated from the amino acid sequence (Swaisgood, 1973). It is interesting to note the slightly lower content of isoleucine, and the correspondingly higher level of alanine in the composition of β-casein B (Table 9.3), compared with values obtained by other workers (Swaisgood, 1973). If these are real differences they may be the result of a neutral substitution, Ile/Ala, which could not be differentiated by gel electrophoresis. In fact, only one case of a neutral substitution has been reported, being that for the B_z variant of β-casein from Zebu cattle (Thompson et al., 1969b).

The amino acid compositions of the caprine β-caseins are similar to that of bovine β-casein A^2 (Table 9.3). Caprine β_2-casein compared with bovine β-casein A^2 has seven amino acid residues the same, with five differing by one residue, three differing by two residues and one differing by three and four residues. The amino acid composition of caprine β_2-casein determined by Ribadeau Dumas et al. (1975) differs from β_2-casein (this study) in glutamic acid, glycine, valine and possibly leucine (Table 9.3).

Caprine β_1- and β_2-caseins contained 0.62% and 0.67% tryptophan, respectively. Determinations on acid hydrolysed samples showed caprine β_1- and β_2-caseins contained 0.78% and 0.67% phosphorus, respectively. Assuming a molecular weight of 24 500, β_1- and β_2-casein therefore contain 6.2 and 5.3 residues of inorganic phosphorus per molecule of protein, respectively.

The absorbance of 1 cm solutions of β_1- and β_2-casein (1%, w/v) at 260 nm (corrected for Rayleigh scattering) were 4.2 and 4.5, respectively. These were similar to reported values for bovine β-casein (4.5-4.7) (Thompson, 1971), suggesting that the caprine β-caseins and bovine β-casein contain the same number of tyrosine and tryptophan residues. The absorbance in 0.1 M NaOH solution at 280 nm and 294 nm showed the ratio of tyrosine to tryptophan to be 3.8 and 3.7

for β_1- and β_2-caseins, respectively. Consequently, tryptophan was taken as one residue per molecule and tyrosine as four residues per molecule, despite the lower values obtained from the amino acid composition (Table 9.3).

9.8 Discussion

The mobility in polyacrylamide gels at pH 8.4 of bovine β-casein B is similar to that of caprine β_2-casein, the molecular weight of the proteins is similar and hence the net charge is probably similar. The number of histidine residues was 5 and 6 respectively for caprine β_2-casein and bovine β-casein B, whereas the number of lysine plus arginine residues was one more for the B variant of bovine β-casein (Mercier et al., 1972a). Consequently, β_2-casein (or β_1-casein) should have one less carboxyl residue than bovine β-casein B, i.e. 21 aspartic acid and glutamic acid residues and 31 asparagine plus glutamine residues. The bovine casein contains 26 amide groups (Grosclaude et al., 1973) and 22 carboxyl groups . As the only difference in charged residues between β_1- and β_2-caseins was the number of phosphate groups, the relative number of carboxyl and amide groups would be the same for the two caprine caseins.

The amino acid compositions of the caprine β-caseins were very close to that published for bovine β-casein A^2 (Table 9.3). The sum of the lysine and arginine residues is constant at 15, and the sum of leucine, isoleucine and valine residues is similar at 50 and 51, respectively for caprine β_2- and bovine β-casein A^2. The only major difference between the two caprine β-caseins was their different phosphate levels with β_1- and β_2-caseins containing six and five phosphate residues, respectively per molecule.

Ribadeau Dumas et al. (1975) have recently isolated two caprine β-caseins with similar amino acid compositions (Table 9.3). They also differ principally in their phosphorus content, with β_2-casein and β_1-casein having 4 and 5 phosphate groups, respectively. However, reinvestigation of their data revealed 5 and 6 phosphate groups, respectively for β_2- and β_1-caseins (Ribadeau Dumas, 1974, personal communication).

The different levels of phosphorylation found in bovine β-casein are known to be the result of genetic variation in the sequence (Mercier et al., 1972a). In contrast, human β-casein, similar in composition and properties to its bovine counterpart (Nagasawa et al., 1970), contains six β-caseins varying only in their level of phosphorylation (containing 0-5 phosphate groups)(Groves and Gordon, 1970). Since such proteins have not yet been isolated in caprine casein, the same mechanism of multiple phosphorylation may not occur in the synthesis of the caprine β-caseins. An alternative possibility, however, is that a gene duplication and a subsequent mutation occurred for one of these proteins such that one casein contains five phosphate residues whilst the other contains six.

CAPRINE α_s-CASEIN

This section describes the isolation and chemical characterization of the major caprine α_s-casein. Caprine α_s-casein was initially compared with bovine α_{s1}-casein. However, when the amino acid composition of caprine α_s-casein was determined, a close analogy was observed with the minor bovine α_{s3}- and α_{s4}-caseins, particularly in regard to their cysteine content. Consequently, the properties of both bovine α_{s1}- and α_{s3}-caseins were compared with caprine α_s-casein.

9.9 Isolation of the α_s-Caseins

The major caprine α_s-casein (Fraction 5, Figs.8.3 and 8.4) was isolated by chromatography on DEAE-cellulose and purified free from minor contaminants by rechromatography on CM-cellulose. The minor α_s-casein fractions were not examined further. This procedure removed most of the contaminants having a similar electrophoretic mobility to caprine α_s-casein (Fig.9.10). Caprine α_s-casein has a lower electrophoretic mobility at pH 8.4 than bovine α_{s1}-casein and bovine α_{s3}-casein, with a mobility closer to that of bovine α_{s4}-casein.

Crude bovine α_{s1}-casein B, isolated by urea fractionation of whole casein (Hipp et al., 1952), was further purified

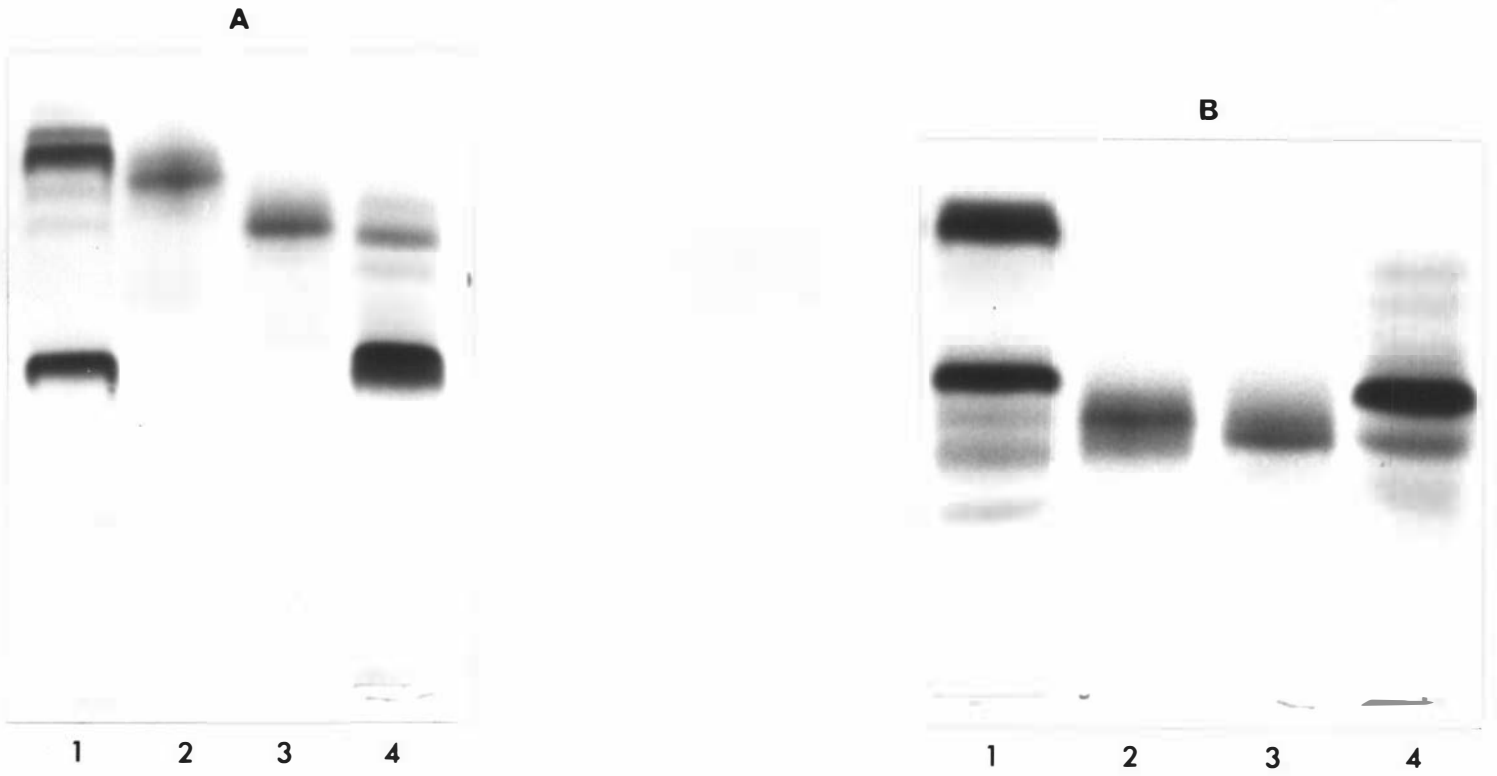


Fig.9.10: Alkaline gel electrophoresis of purified caprine α_s-casein and purified bovine α_{s3}-casein in the presence (B) and absence of Mg (A). 1, whole bovine casein; 2, purified bovine α_{s3}-casein; 3, purified caprine α_s-casein; 4, whole caprine casein.

by chromatography on DEAE-cellulose and the purity examined by gel electrophoresis.

The bovine α_{s3}- and α_{s4}-caseins were isolated by chromatography on DEAE-cellulose using a similar procedure to that previously described by Hoagland et al. (1971). Whole bovine casein was initially fractionated by chromatography on DEAE-cellulose and the fractions that contained bovine α_{s3}- and α_{s4}-caseins were pooled and dialysed. These caseins were separated into two fractions by rechromatography on DEAE-cellulose and the fractions isolated were examined by gel electrophoresis (Fig.9.10). The peak fractions containing pure protein were pooled, dialysed and then stored at $-20^{o}C$.

Although bovine α_{s3}- and α_{s4}-caseins were resolved into two distinct peaks by chromatography, the α_{s4}-casein fraction which was pooled appeared to be contaminated with α_{s3}-casein. The level of α_{s3}- and α_{s4}-caseins in whole bovine casein (approximately 10% of the total casein) and their similarity made them difficult to isolate in large quantities.

The amino acid compositions of bovine α_{s3}- and α_{s4}-caseins have been shown to be nearly identical (Hoagland et al., 1971) and consequently the more easily isolated α_{s3}-casein was used for comparison with caprine α_{s}-casein.

Electrophoresis of these caseins in the presence of Mg (Fig.9.10), which binds to the phosphorus residues of the caseins and largely neutralizes their charge, showed caprine α_{s}-casein and bovine α_{s3}-casein maintain their relative mobilities, although their electrophoretic mobilities decrease markedly relative to bovine α_{s1}-casein B. The relative mobilities of the caseins, in particular caprine α_{s}-casein, bovine α_{s1} and α_{s3}-caseins are shown in Table 9.4 . Although gel electrophoresis at pH 8.4 showed caprine α_{s}-casein and bovine α_{s1}-casein were fairly homogeneous, when Mg was included in the gel some heterogeneity became evident (Fig.9.10).

Table 9.4

Relative Mobilities of Casein Components in the Presence and Absence of Mg

Component	Distance travelled, mm		Ratio of distances travelled
	Alkaline gel	Mg gel	
Bovine			
α_{s1}-casein	106	58	0.55
α_{s3}-casein	98	31	0.31
β-casein	65	41	0.63
Caprine			
α_s-casein	89	29	0.32
β_2-casein	62	37	0.60

9.10 Molecular Weights of the α_s-Caseins

The molecular weights of the S-carboxymethylated α_s-caseins were determined on a column of Sepharose 6B in 6 M guanidine.HCl (Chapter 9.6). Molecular weights of 25 700, 25 400 and 24 100, respectively, were obtained for caprine α_s-casein, bovine α_{s3}-casein and bovine α_{s1}-casein B (Table 9.2). The molecular weight obtained for bovine α_{s1}-casein B was in close agreement with the value calculated from the amino acid sequence (Mercier et al., 1972a). The molecular weight obtained for bovine α_{s3}-casein was considerably lower than the value of 33 800 reported by Hoagland et al. (1971), for α_{s3}-casein, however, it was much closer to the value of 26 000 recently reported by Ribadeau Dumas et al. (1973).

A sample containing both caprine α_s-casein and bovine α_{s3}-casein was chromatographed on the molecular weight column and the fractions collected as before. After weighing, each fraction was dialysed to remove the buffer salts, and after gel electrophoresis and densitometry the ratio of caprine α_s-casein to bovine α_{s3}-casein was found to be constant across the protein peak, indicating the molecular weights of the proteins were similar. This confirmed the results obtained when they were chromatographed individually. A similar experiment using caprine α_s-casein and bovine α_{s1}-casein showed that caprine α_s-casein had an appreciably greater molecular weight, thus confirming the results obtained earlier. Figure 9.11 shows the chromatographic pattern and gel electrophoresis patterns obtained for these two proteins. It can be seen that the leading edge of the peak contained predominantly caprine α_s-casein, whereas the trailing edge largely contained bovine α_{s1}-casein. This technique is especially useful for directly comparing the molecular weights of two proteins, particularly when one can be used as a sensitive molecular weight marker.

9.11 Amino Acid Compositions of the α_s-Caseins

The amino acid compositions of caprine α_s-casein and bovine α_{s3}-casein were determined using samples hydrolysed in

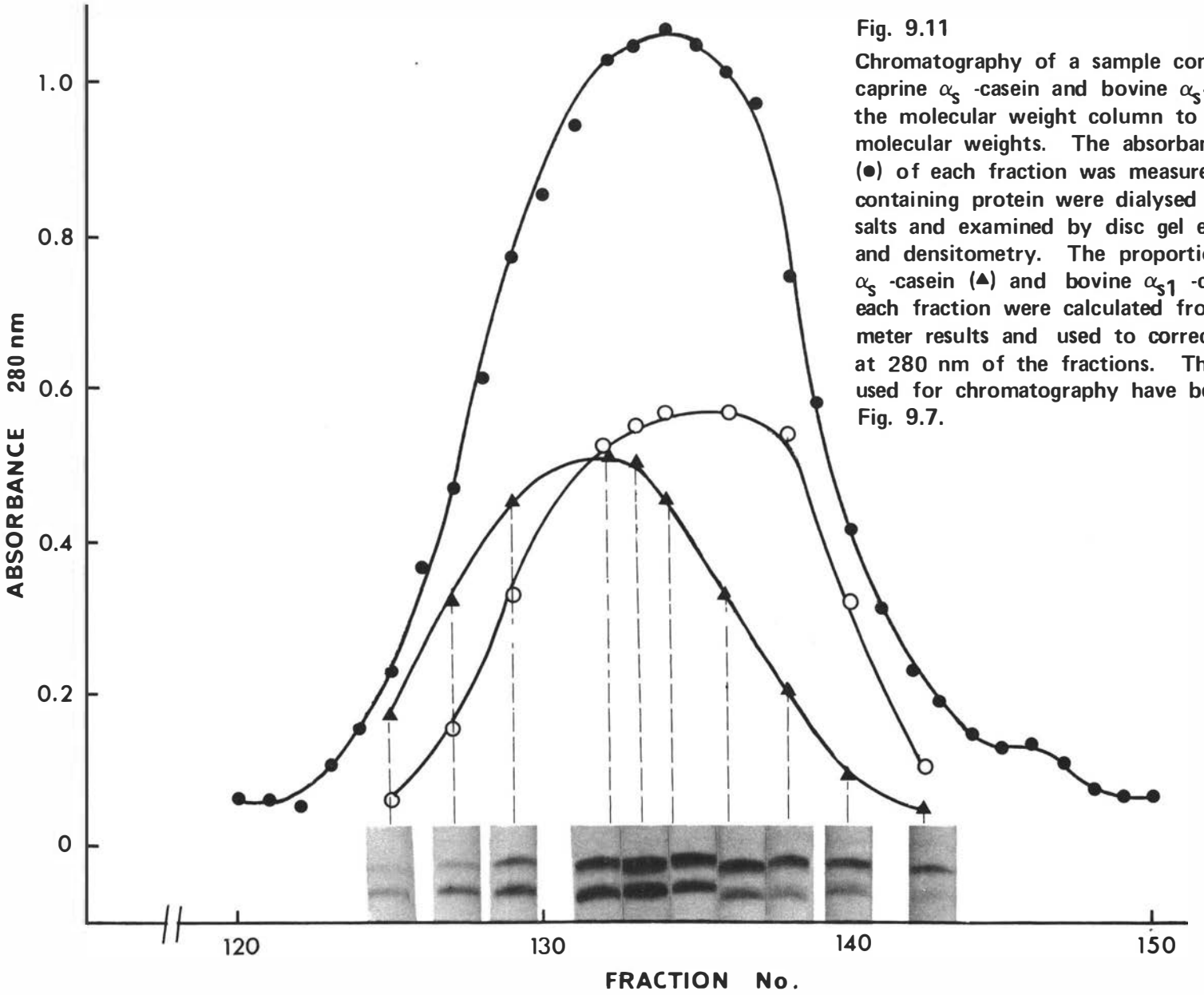


Fig. 9.11

Chromatography of a sample containing the major caprine α_s -casein and bovine α_{s1} -casein B on the molecular weight column to compare their molecular weights. The absorbance at 280 nm (●) of each fraction was measured and those containing protein were dialysed free of buffer salts and examined by disc gel electrophoresis and densitometry. The proportion of caprine α_s -casein (▲) and bovine α_{s1} -casein B (○) in each fraction were calculated from the densitometer results and used to correct the absorbance at 280 nm of the fractions. The conditions used for chromatography have been outlined in Fig. 9.7.

6 M HCl for 24, 48 and 72 h. Molar ratios of amino acids in caprine α_s-casein were initially calculated on the basis that the protein had a molecular weight of approximately 25 700 and contained 22 residues of lysine. It was refined by assuming that the protein had 17 aspartic acid residues, 4 glycine residues, 10 alanine residues and 11 tyrosine residues (Table 9.5).

Molar ratios of amino acids in bovine α_{s3}-casein were calculated assuming the protein had a molecular weight of approximately 25 400 and contained 23 lysine residues. The composition was further refined assuming each mole of protein contained 23 lysine residues, 19 aspartic residues, 4 glycine residues, 11 tyrosine residues and 6 arginine residues.

The compositions are compared in Table 9.5 with those for bovine α_{s1}-casein B (Mercier et al., 1972a) and bovine α_{s3}-casein (Ribadeau Dumas et al., 1975; Hoagland et al., 1971), after recalculation of Hoagland's data to give a more appropriate molecular weight.

It is clear that with the exception of proline, and possibly valine, the composition of caprine α_s-casein is very close to that of bovine α_{s3}-casein (this study), and those of Ribadeau Dumas et al. (1975) and Hoagland et al. (1971). However, there are large differences between caprine α_s-casein and bovine α_{s1}-casein, notably in the case of threonine, glycine, leucine, lysine and cysteine residues (Table 9.5). All three proteins, however, have 7 or 8 phosphate residues and similar numbers of aromatic residues.

The tryptophan content of caprine α_s-casein was 1.82% (Table 9.5). Phosphorus contents, estimated in duplicate samples of hydrolysates used for amino acid analyses, were 0.92% and 0.88% for caprine α_s-casein and bovine α_{s3}-casein, respectively, corresponding to 7 phosphorylated residues per molecule of protein (Table 9.5). Previous results had shown that a crude fraction of caprine α_s-casein contained 1.02% phosphorus (Hofman, 1958b). Annan and Manson (1969) found 1.03% phosphorus in bovine α_{s3}-casein. Recently, however, Ribadeau Dumas et al. (1975) also reported bovine

Table 9.5

Amino Acid Composition of Caprine and Bovine α_s-Caseins (Residues Amino Acid/Mole Protein)

	Caprine α_s-casein			Bovine α_{s3}-casein					Bovine α_{s1}-casein
				Present results					
	Mean No. Residues	S.D.	Probable No. Residues	Mean No. Residues	S.D.	Probable No. Residues	Hoagland et al.(1971) Recalculated Mean	Ribadeau Dumas et al. (1975) No. Residues	Mercier et al. (1972a) No. Residues
Asp	17.20	0.46	17	18.60	0.44	19	19.7	19.2	15
Thr	14.02		14	14.40		14	15.5	15.1	5
Ser	13.78		14	16.03		16	17.3	14.1	16
Glu	44.75	1.20	45	44.81	0.77	45	43.2	44.2	39
Pro	17.86	0.48	18	12.93	0.25	13	11.9	10.6	17
Gly	4.12	0.11	4	3.96	0.09	4	3.1	2.4	9
Ala	9.63	0.26	10	9.01	0.26	9	8.5	8.8	9
Cys	1.8		2	2.3		2	1.1	-	0
Val	12.42	0.33	12	14.36	0.29	14	14.4	14.5	11
Met	4.31	0.12	4	4.04	0.06	4	4.2	3.9	5
Ile	10.80	0.29	11	10.51	0.21	11	11.6	10.7	11
Leu	12.40	0.33	12	13.79	0.26	14	13.3	13.4	17
Tyr	10.90	0.29	11	11.05	0.22	11	10.7	11.8	10
Phe	7.53	0.20	8	6.25	0.13	6	6.2	6.2	8
His	5.30	0.14	5	3.71	0.09	4	4.1	3.0	5
Lys	21.91	0.59	22	23.07	0.40	23	22.8	26.4	14
Arg	6.44	0.17	6	6.06	0.16	6	5.7	5.9	6
Trp	2.32		2	-		2	1.5	1.8	2
Phosphorus	7.3		7	7.0		7	-	7.1	8
Total no. of residues			217			217			199
Molecular weight									
(a) Amino acid analysis			26 009[1]			25 938[1]			23 615
(b) Sepharose 6B column	25 700			25 400					24 100

1. Molecular weight excludes amide groups.

α_{s3}-casein as having 7 phosphorylated residues, in agreement with the results in Table 9.5, and considerably lower than the results of Annan and Manson (1969).

The extinction coefficients (absorbance of a 1% (w/v) solution 1 cm in depth) measured in 0.1 M phosphate buffer, pH 7.0, were 10.1 and 10.3, respectively for caprine α_s-casein and bovine α_{s3}-casein. Bovine α_{s5}-casein which is thought to be an equimolar mixture of α_{s3}- and α_{s4}-caseins (Hoagland et al., 1971), was reported to have an extinction coefficient of 10.1 (Toma and Nakai, 1973).

9.12 Discussion

The present study has shown that the major α_s-casein of caprine milk has an amino acid composition and molecular weight close to that of the minor bovine protein, α_{s3}-casein, (Table 9.5). In contrast, bovine α_{s1}-casein has a molecular weight lower than, and a composition substantially different from the major caprine α_s-casein. It should be noted that bovine α_{s4}-casein has a similar composition to α_{s3}-casein and these two proteins occur in milk in equilibrium with α_{s5}-casein, a disulphide bonded dimer which may be composed of one molecule each of α_{s3}- and α_{s4}-caseins (Hoagland et al., 1971; Toma and Nakai, 1973), but could be a mixture of 3 possible disulphide dimers.

The marked decrease in the electrophoretic mobilities of caprine α_s-casein and bovine α_{s3}-casein in the presence of Mg (Fig.9.10, Table 9.4) may be attributed to these caseins binding more Mg than bovine α_{s1}-casein. Bovine α_{s1}-casein, which has one more phosphate group than caprine α_s-casein and bovine α_{s3}-casein was not affected to the same extent, suggesting Mg may be bound to binding sites other than the phosphate groups.

Previous estimates of the molecular weight of the proteins, α_{s3}-, α_{s4}- and α_{s5}-caseins have been by sedimentation in a preparative ultracentrifuge fitted with a schlieren optical system (Toma and Nakai, 1973), by C-terminal analysis (Annan and Manson, 1969; Ribadeau Dumas et al., 1973) and

from the amino acid composition (Hoagland et al., 1971; Ribadeau Dumas et al., 1975). These studies gave molecular weights of 31 800 and 65 750 for α_{s3}- and α_{s5}-caseins (Toma and Nakai, 1973), 14 000 or 28 000 for α_{s3}- and α_{s4}-caseins (Annan and Manson, 1969) and 33 700 for α_{s3}- and α_{s4}-caseins (Hoagland et al., 1971). The present results show the molecular weight of bovine α_{s3}-casein to be 25 400 ± 1500 by chromatography on Sepharose 6B in 6 M guanidine.HCl, a result which is significantly lower than the values obtained by other workers but similar to the molecular weight of 26 000 obtained by Ribadeau Dumas et al. (1973). However, the molecular weights of α_{s3}- and α_{s5}-caseins determined by ultracentrifugation were measured at high protein concentrations. If the proteins had any tendency to associate this would have led to the values reported. Annan and Manson (1969) concluded that the molecular weight was 14 000, because the release of amino acids, leucine and tyrosine by carboxypeptidase A was virtually simultaneous, each was the end group of α_{s3}- and α_{s4}-caseins. Ribadeau Dumas et al. (1973) found the C-terminal sequence of both bovine α_{s3}- and α_{s4}-caseins to be Leu-Tyr.OH, thus doubling the estimate of molecular weight of Annan and Manson (1969) from 14 000 to 28 000.

The bovine α_{s3}-, α_{s4}- and α_{s5}-caseins all together only amount to a small proportion of the total α_s-caseins (Annan and Manson, 1969) and possibly 10% (by weight) of the total bovine casein (Chapter 8.4). The major caprine α_s-casein accounts for about 15% of the total caprine casein. However, there may not be a protein in the group of caprine caseins corresponding to bovine α_{s1}-casein.

The possibility that caprine α_s-casein exists as a polymer in caprine milk, through the formation of inter-or intra-molecular disulphide linkages should be considered. Alkaline gel electrophoresis of caprine casein before and after treatment with 2-mercaptoethanol revealed no differences in the protein patterns. It is interesting to note that bovine α_{s5}-casein, the dimer of α_{s3}- and α_{s4}-caseins, has a mobility in alkaline gel electrophoresis slightly greater than that of β-casein, whereas α_{s3}- and α_{s4}-caseins have mobilities nearly equivalent to α_{s1}-casein (Fig.9.10).

Consequently, if caprine α_s-casein existed as a dimer in the absence of 2-mercaptoethanol, it would seem likely that some change in its electrophoretic mobility would have been observed.

CHAPTER 10: CHARACTERIZATION OF THE MAJOR OVINE CASEINS

Five major ovine caseins, κ-, β_2-, β_1, α_{s2}- and α_{s3}-caseins were isolated by chromatography of whole casein on DEAE-cellulose. This chapter describes their characterization and a comparison with their respective bovine and caprine caseins.

10.1 Ovine κ-Casein

Ovine κ-casein was isolated by chromatography of whole casein on a column of DEAE-cellulose (Fraction 3, Figs.8.5 and 8.6) and purified further by chromatography on CM-cellulose at pH 4.0. It was identified as κ-casein from its electrophoretic mobility in polyacrylamide gels at pH 8.4 and from its sensitivity to rennin (Fig.10.1).

Gel electrophoresis at pH 8.4 showed ovine κ-casein has a lower mobility than the ovine β-caseins and, unlike its caprine counterpart, can be readily identified in patterns of whole casein. Minor bands with a greater electrophoretic mobility than ovine κ-casein were observed in some preparations. These proteins were readily hydrolysed by rennin and were probably κ-caseins glycosylated at different levels.

Alais and Jollès (1961) found the level of carbohydrate in the glycomacropeptide from ovine casein to be less than that from bovine casein. They reported levels of 1.1% sialic acid in the ovine glycomacropeptide, and 14.3% and 3.0% in the bovine and caprine glycomacropeptides, respectively.

A molecular weight of 20 500 was obtained for ovine κ-casein by chromatography of the S-carboxymethylated protein in 6 M guanidine.HCl buffer on a column of Sepharose 6B. This molecular weight was similar to values previously reported of 19 050 (Ribadeau Dumas et al.,1975) and 20 000 (Alais and Jollès, 1967).

No further characteristics of ovine κ-casein were determined since the amino acid sequences of ovine para-κ-casein (Jollès et al., 1974) and the partial sequence of the

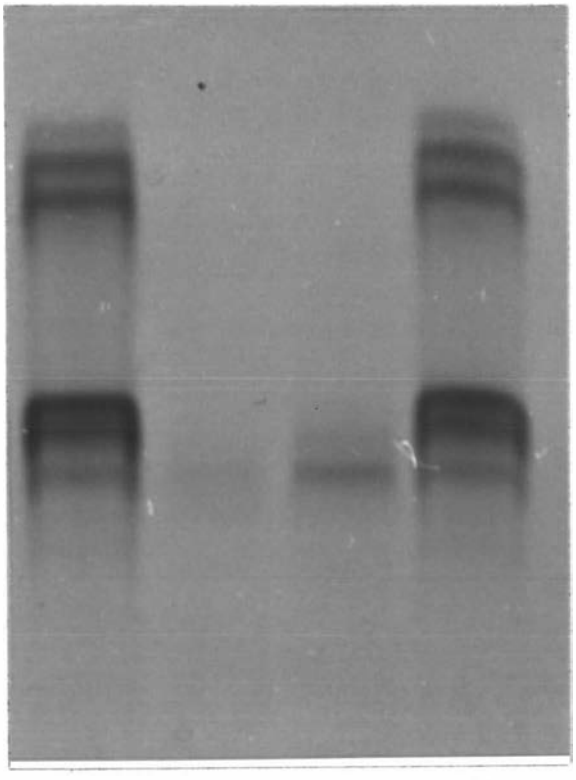


Fig.10.1: Alkaline gel electrophoresis of whole ovine casein (1,4), purified ovine κ-casein (3), and the effect of rennet on this fraction (2). The direction of electrophoresis is indicated.

ovine macropeptide (Jollès et al., 1973) have recently been determined.

OVINE β-CASEINS

10.2 Isolation of Ovine β-Caseins

Two β-caseins (Fractions 4 and 5, Figs.8.5 and 8.6) were separated from whole casein by chromatography on DEAE-cellulose at pH 7.0. These caseins, present in equimolar amounts were tentatively designated as β_2-casein (Fraction 4) and β_1-casein (Fraction 5), since β_2-casein had a similar electrophoretic mobility at pH 8.4 to bovine β-casein B and caprine β_2-casein. Since there was a poor separation of peaks from the DEAE-cellulose column after chromatography of whole ovine casein (Fig.8.5), fractions were invariably contaminated with one another and required further purification. They were repurified, initially by rechromatography on columns of DEAE-cellulose, followed by chromatography on CM-cellulose at pH 4.0. Alkaline gel electrophoresis showed that this procedure yielded highly purified β-caseins (Fig.10.2), although some heterogeneity became evident in the presence of Mg. The ovine β-caseins were much more difficult to purify than their caprine counterparts, which in turn were more difficult to purify than bovine β-casein.

Ovine β_1-casein has a greater electrophoretic mobility at pH 8.4 than β_2-casein (Fig.10.2) and was less readily eluted from DEAE-cellulose (Fig.8.5). The electrophoretic mobility of bovine β-casein B at pH 8.4 was similar to that of ovine β_2-casein, while the mobilities of ovine β_1- and β_2-caseins were similar to those of caprine β_1- and β_2-caseins, respectively (Fig.4.1). At pH 4.0, the mobilities of ovine β_1- and β_2-caseins were reversed, so ovine β_2-casein had the greater electrophoretic mobility. When Mg was included in the electrophoresis buffer, the mobilities of ovine β_1- and β_2-caseins decreased and the two components merged (Fig.10.2). This suggested that β_1- and β_2-caseins differed in the number of phosphate groups that each contained, since Mg associates strongly with the phosphate groups of the caseins, and largely neutralizes their charge. This conclusion was supported by the results

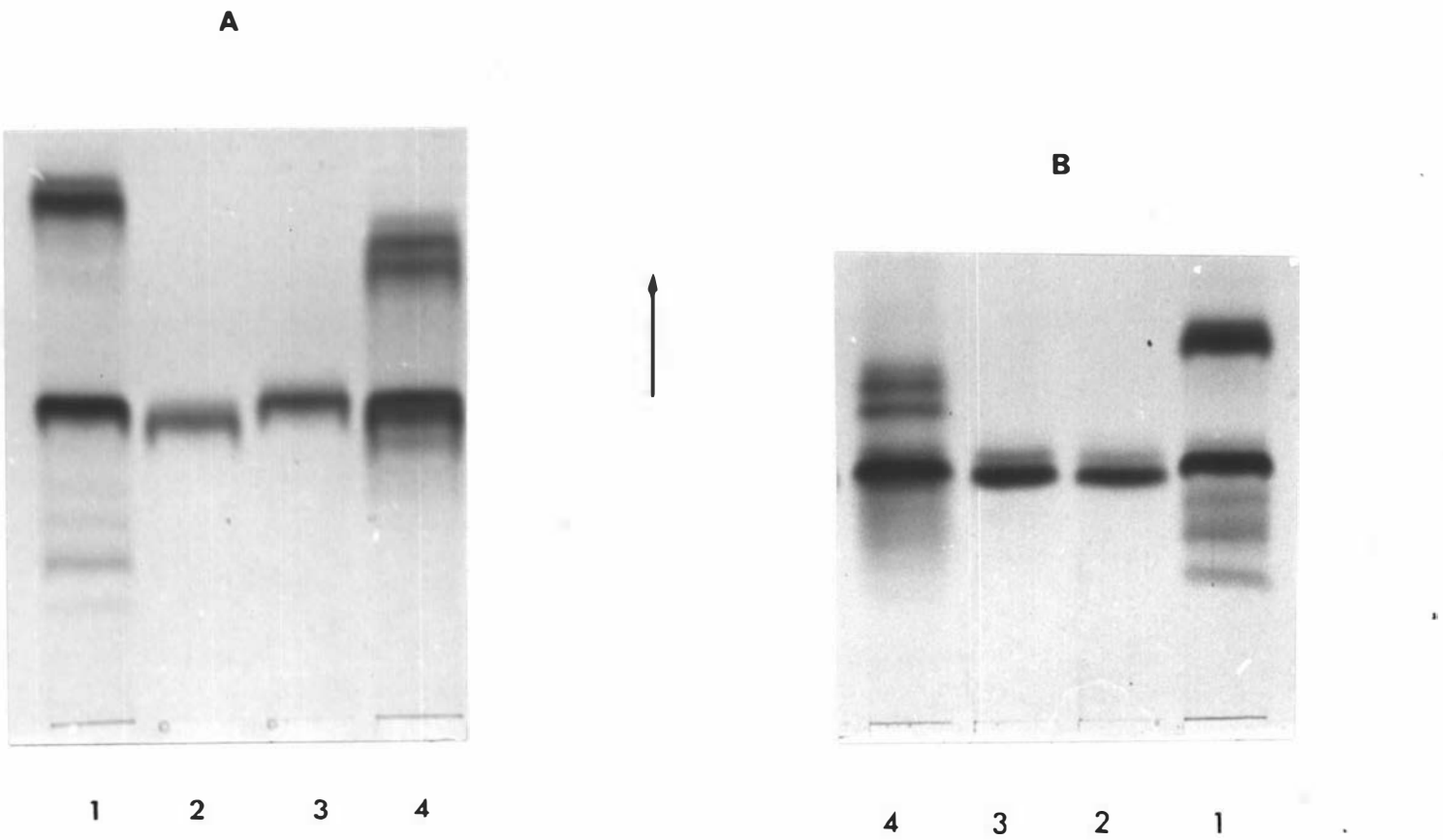


Fig.10.2: Alkaline gel electrophoresis of the purified ovine β-caseins in the absence (A) and presence of Mg (B). 1, whole bovine casein; 2, ovine β_2-casein; 3, ovine β_1-casein; 4, whole ovine casein. The direction of electrophoresis is indicated.

from acid gel electrophoresis where the mobilities suggested that β_1-casein carried more negative charge than β_2-casein at pH 4.0 and pH 8.4. The behaviour of these ovine β-caseins is similar to that observed for the caprine β-caseins (Chapter 9.5).

10.3 Molecular Weights of Ovine β-Caseins

Molecular weights of 25 000 and 24 900 were obtained for ovine β_1- and β_2-caseins, respectively, by chromatography in 6 M guanidine.HCl on a calibrated column of Sepharose 6B (Chapter 9.6). These molecular weights were the average of duplicate analyses.

To determine whether the molecular weights of the ovine β-caseins were actually higher than that of bovine β-casein a sample containing both ovine β_1-casein and bovine β-casein B was chromatographed on the molecular weight column. After dialysing each fraction, gel electrophoresis and densitometry showed the ratio of ovine β_1-casein to bovine β-casein B was constant across the peak, suggesting that the molecular weights were similar at 24 00-25 000. This verified the previous results when the proteins were chromatographed individually on the molecular weight column.

10.4 Amino Acid Compositions of the Ovine β-Caseins

The amino acid compositions of the ovine β_1- and β_2-caseins were determined from nine analyses. Samples of protein which had been hydrolysed with 6 M HCl for 24, 48 and 72 h were analysed in triplicate. The molar ratios of amino acids were initially calculated on the basis that the β-caseins contained 8 aspartic acid residues and had molecular weights of 24 000-25 000. They were refined by assuming ovine β_1- and β_2-caseins contained 8 aspartic acid, 5 glycine, 9 phenylalanine, 12 lysine and 3 arginine residues per mole of protein. The amino acid compositions of the ovine β_1- and β_2-caseins are presented in Table 10.1, and compared with those of caprine β_2-casein and bovine β-casein A^2, and with the composition of ovine β-casein obtained by Resmini et al. (1967), after it was recalculated assuming a molecular weight for the protein of 24 000.

Table 10.1

Amino Acid Compositions of the Ovine β-Caseins (Residues of Amino Acid per Mole of Protein)

	Ovine							Caprine	Bovine
	β_2-Casein Mean No. of Residues	S.D.[3]	β_1-Casein Mean No. of Residues	S.D.[3]	β-Casein[2] Resmini et al. (1967)	β_2-Casein Most Probable No. of Residues	β_1-Casein Most Probable No. of Residues	β_2-Casein Table 9.3	β-Casein A[2] Ribadeau Duma et al. (1973)
Asp	8.08	0.20	8.13	0.19	9.8	8	8	9	9
Thr[1]	10.62		10.58		10.9	11	11	12	9
Ser[1]	13.78		13.22		13.3	14	13	15	16
Glu	39.02	1.42	39.92	1.01	44.3	39	40	43	39
Pro	37.27	2.86	37.41	1.14	31.8	37	37	33	35
Gly	5.10	0.10	5.11	0.13	5.2	5	5	6	5
Ala	4.70	0.29	4.39	0.12	6.3	5	4	5	5
Val[1]	21.88	0.52	21.69	0.57	18.6	22	22	21	19
Met	5.30	0.26	5.11	0.12	5.2	5	5	6	6
Ile[1]	8.53	0.27	8.77	0.21	9.0	9	9	9	10
Leu	21.27	0.48	21.31	0.53	21.0	21	21	20	22
Tyr	2.97	0.07	3.00	0.08	3.9	3	3	4	4
Phe	9.02	0.19	8.90	0.21	8.2	9	9	9	9
His	4.67	0.28	4.65	0.13	4.4	5	5	5	5
Lys	11.86	0.25	11.57	0.30	11.8	12	12	12	11
Arg	2.99	0.11	2.89	0.08	3.3	3	3	3	4
Trp	1.1		1.1		0.8	1	1	1	1
Phosphorus	4.1		4.6		-	4	5	5	5
Molecular weight									
(a) Amino Acid analysis						23 773	23 823	24 360	23 982
(b) Sepharose 6B column	24 900		25 000						
No. of residues						209	208	213	209

1. Threonine and serine values obtained from 24 h hydrolysates were increased by 5% and 10% respectively. Isoleucine and valine were 72 h results.

2. Results of Resmini et al. (1967) were recalculated assuming a molecular weight of 23 500, excluding the phosphate groups.

3. Standard deviation.

The compositions of the ovine β_1- and β_2-caseins were very similar, with only minor differences between them (Table 10.1). The differences in the number of glutamic acid residues were probably not significant, considering their standard deviations. The proline content of ovine β_2-casein was rather uncertain, due to the large standard deviation, however it appears unlikely there are any significant differences between these two proteins (Table 10.1).

Ovine β_2-casein and bovine β-casein A^2 have the same number of glutamic acid, glycine, alanine, phenylalanine, histidine and tryptophan residues. Caprine β_2-casein and ovine β_2-casein also have similar compositions (Table 10.1), with the levels of seven amino acid residues the same, eight amino acids which differ by one residue and two amino acids which differ by two residues. Tryptophan in samples of the ovine β-caseins was determined directly by amino acid analysis after the proteins were hydrolysed for 24 h with 3 M p-toluene sulphonic acid. The molar ratios of tryptophan to arginine were determined by amino acid analysis. On the basis of three arginine residues per mole, the tryptophan content was 0.84% for both β_1- and β_2-caseins.

Inorganic phosphorus, determined in acid hydrolysed samples of the β-caseins, was 0.59% and 0.53% for ovine β_1- and β_2-caseins, respectively. The absorbance of 1 cm solutions of the β_1- and β_2-caseins (1% w/v), pH 7.0, at 280 nm (corrected for Rayleigh scattering) were 3.9 and 3.7, respectively. Bovine and caprine β-caseins, which contain one tryptophan and four tyrosine residues, have extinction coefficients of 4.2-4.7 (Chapter 9.7). The lower values for the extinction coefficients of the ovine β-caseins are consistent with their content of one tryptophan and three tyrosine residues.

10.5 Discussion

The two ovine β-caseins are similar to the two caprine β-caseins with almost identical electrophoretic mobilities in alkaline polyacrylamide gels, molecular weights, amino acid compositions and with a difference in phosphorus content between

the β_1- and β_2-caseins (Table 10.1). The composition obtained by Resmini et al. (1967) for ovine β-casein is close to those of ovine β_1- and β_2-caseins which were obtained in this study (Table 10.1), although some differences are apparent in the aspartic acid, glutamic acid and proline contents.

The electrophoretic mobilities at pH 8.4 of the ovine and caprine β_2-caseins and bovine β-casein B are similar, and since they all have the same molecular weights, the net charges on the proteins are probably similar. The sum of the lysine, histidine and arginine residues is the same for the ovine and caprine β-caseins and bovine β-casein A^2, and consequently the sum of the negatively charged residues is probably similar. Furthermore, the sum of isoleucine, valine and leucine residues is similar at 49, 51 and 52, respectively, for caprine β_2-casein, ovine β_2-casein and bovine β-casein A^2 (Table 10.1). The ovine β_2- and β_1-caseins contain 4 and 5 residues of phosphorus, respectively and are similar to the corresponding caprine β-caseins which contain 5 and 6 phosphorus residues. The ovine β-caseins may have a similar mechanism of phosphorylation to that of the caprine β-caseins. It is interesting to note that the variation observed in the intensity of the β-casein bands in some ovine casein samples (Chapter 8.8) has not yet been observed in the caprine caseins.

OVINE α_s-CASEINS

10.6 Isolation of Ovine α_{s2}-Casein and α_{s3}-Casein

Alkaline gel electrophoresis of a number of individual ovine casein (Chapter 8.8) revealed the presence of three major components having electrophoretic mobilities which were lower than that of bovine α_{s1}-casein (Fig.4.1). Two of these α_s-caseins were isolated by DEAE-cellulose chromatography of whole ovine casein and designated as α_{s2}- and α_{s3}-caseins, since they corresponded in electrophoretic mobility to the slower components of the three α_s-caseins. These components (Fractions 6 and 7, Figs.8.5 and 8.6) were only partially separated by chromatography on DEAE-cellulose and it was

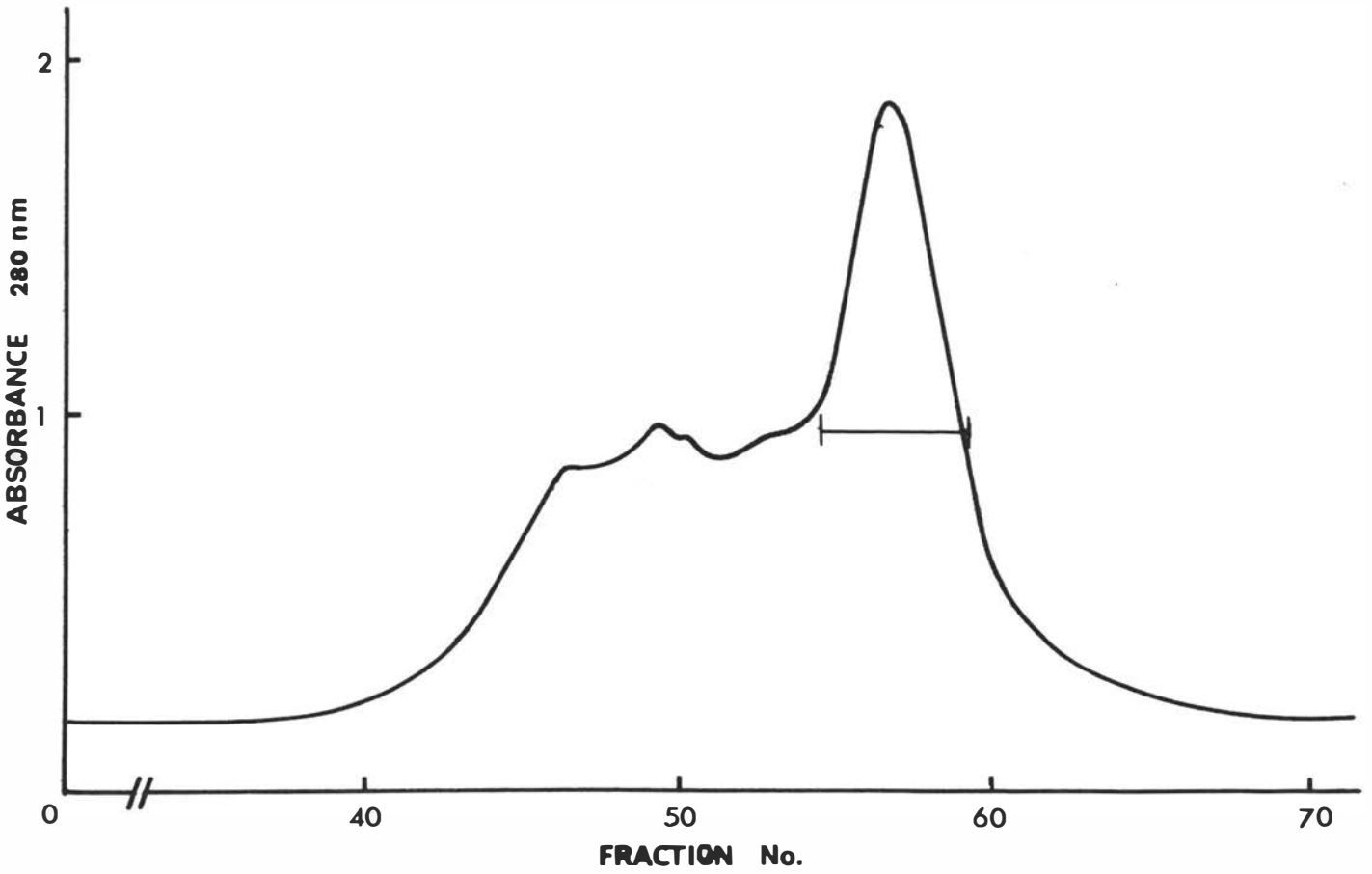


Fig.10.3: Chromatography of ovine α_{s3}-casein on DEAE-cellulose in 0.01 M imidazole-HCl buffer, pH 7, containing urea (4.5 M) and 2-mercaptoethanol (0.1%,v/v), and using a 1l gradient from 0-0.15 M $MgCl_2$ to elute the caseins. Other experimental details are similar to those outlined in Fig.8.1. The fraction indicated was pooled and its purity examined by alkaline gel electrophoresis (Fig.10.4).

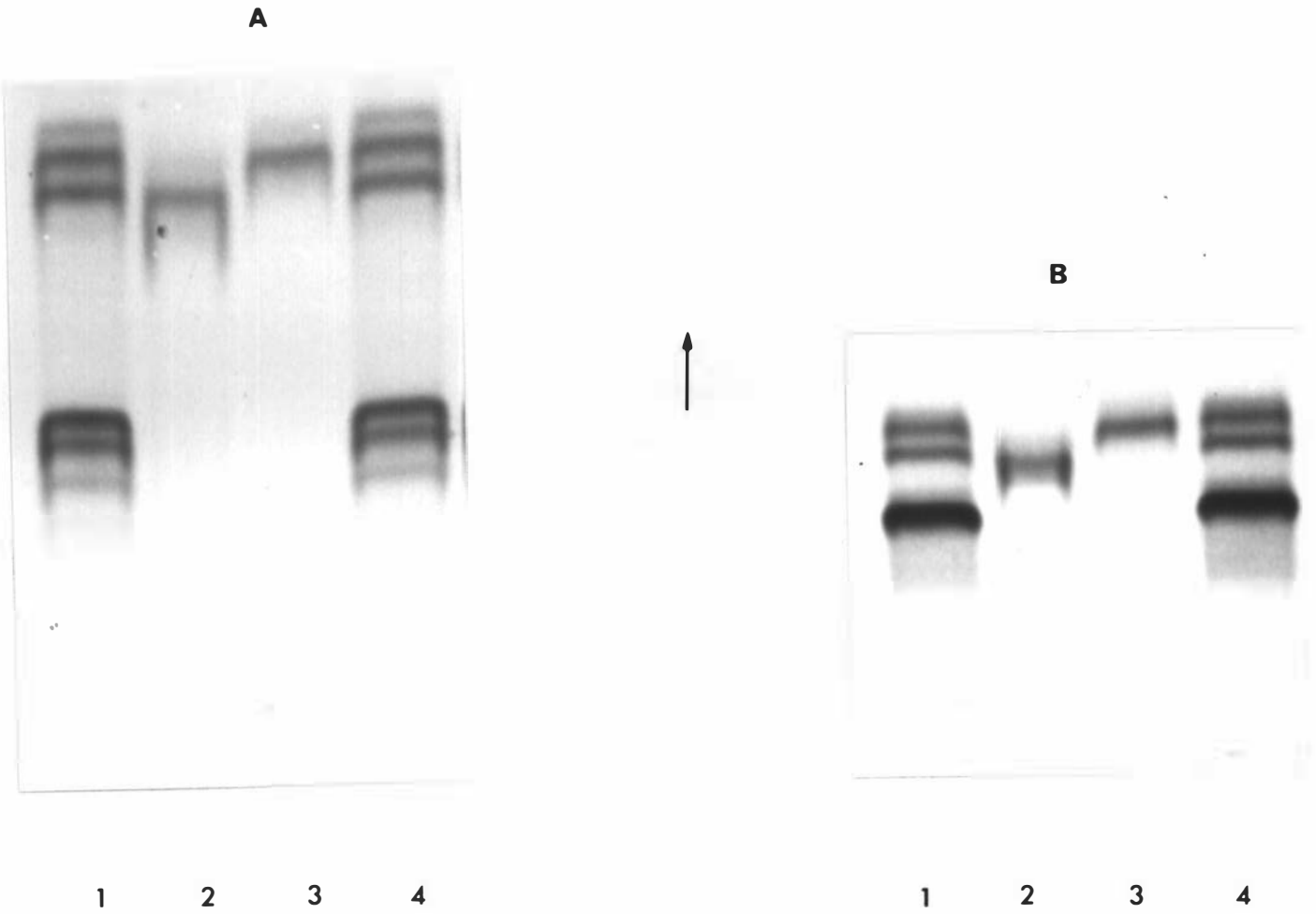


Fig.10.4: Polyacrylamide gel electrophoresis of the ovine α_s-caseins at pH 8.4 (A) and at pH 8.4 in the presence of Mg (B). 1, 4, whole ovine casein; 2, ovine αs_3-casein; 3, ovine αs_2-casein.

necessary to purify them further by rechromatography on columns of DEAE-cellulose and CM-cellulose. Although alkaline gel electrophoresis indicated the ovine α_s-caseins were pure, electrophoresis at alkaline pH in the presence of Mg revealed 30% contamination of α_{s3}-casein with another casein component. This contaminant behaved in a similar manner to bovine α_{s3}-casein and caprine α_s-casein, since in the presence of Mg, its electrophoretic mobility was reduced and became less than that of the ovine β-caseins. Consequently, ovine α_{s3}-casein was repurified by chromatography on DEAE-cellulose at pH 7 in buffers containing Mg (Fig.10.3). The purity of the ovine α_{s2}- and α_{s3}-caseins, as shown by alkaline gel electrophoretic mobilities, are shown in Fig.10.4. In the presence of Mg the mobilities of α_{s2}- and α_{s3}-caseins were reduced, but they did not merge together, and seemed to behave in a similar manner to bovine α_{s1}-casein. This would suggest the difference in net charge on the two ovine α_s-caseins is not due to differences in their phosphorus content.

10.7 Molecular Weights of Ovine α_s-Caseins

The molecular weights of the ovine α_s-caseins were determined by chromatography on a calibrated column of Sepharose 6B in 6 M guanidine.HCl. A sample of pure ovine α_s-casein, which contained α_{s2}- and α_{s3}-caseins yielded an average molecular weight determined from duplicate analyses of 24 100. In addition, a sample which contained the ovine α_s-caseins and bovine α_{s1}-casein B, was chromatographed on the molecular weight column and the fractions examined by gel electrophoresis. The ratio of ovine α_{s2}- and α_{s3}-caseins to bovine α_{s1}-casein B was found to be constant across the peak, suggesting the molecular weights were the same within the error of the method and verifying the results obtained for the individual proteins.

10.8 Amino Acid Compositions of the Ovine α_s-Caseins

The amino acid compositions of the ovine α_s-caseins were determined in a similar manner to that used for the

β-caseins. They were based on single samples of protein hydrolysed for 24, 48 and 72 h, and analysed in triplicate.

The amino acid compositions of ovine α_{s2}-casein and α_{s3}-casein were initially based on the supposition that the proteins had a molecular weight of approximately 24 000 and contained 17 residues of aspartic acid. The composition of α_{s2}-casein was further refined by assuming it contained 17 aspartic acid, 9 glycine, 12 alanine, 14 lysine and 6 arginine residues per molecule of protein. The composition of α_{s3}-casein was refined by assuming it contained 17 aspartic acid, 11 glycine, 12 alanine, 13 lysine and 6 arginine residues per molecule of protein.

The amino acid compositions of the ovine α_{s2}- and α_{s3}-caseins are compared with those of caprine α_s-casein and bovine α_{s1}-casein B in Table 10.2. The amino acid compositions of ovine α_s-casein determined by Resmini et al. (1967) and Shalichev and Tanev (1973) are also shown after recalculation to a more appropriate molecular weight.

The amino acid compositions of the ovine α_{s2}- and α_{s3}-caseins were similar, with minor differences in their threonine, serine, glutamic acid, glycine, valine, methionine, isoleucine, phenylalanine, lysine, and perhaps arginine contents (Table 10.2). The composition of ovine α_{s2}-casein, in particular, was close to that obtained by Resmini et al. (1967). Considerably more differences were observed between the composition of α_{s2}-casein and that of Shalichev and Tanev (1973).

The compositions of the ovine α_s-caseins are much more similar to bovine α_{s1}-casein B than to caprine α_s-casein (Table 10.2). The ovine α_s-caseins and bovine α_{s1}-casein B, in particular, differ from caprine α_s-casein in their content of threonine, glycine, cysteine, lysine and leucine. The tryptophan content of the ovine α_s-caseins was 1.7%, based on the molar ratio of tryptophan to arginine determined by amino acid analysis, assuming the proteins contained six arginine residues per mole.

Table 10.2

Amino Acid Compositions of the Ovine α_s-Caseins (Residues of Amino Acid per Mole of Protein)

	Ovine								Bovine	Caprine
	α_{s3}-Casein Mean No. of Residues	S.D.[4]	α_{s2}-Casein Mean No. of Residues	S.D.[4]	α_s-Casein[3] Resmini et al. (1967)	α_s-Casein Shalichev and Tanev (1973)	α_{s3}-Casein Probable No. of Residues	α_{s2}-Casein Probable No. of Residues	α_{s1}-Casein B Ribadeau Dumas et al. (1973)	α_s-Casein Table 9.4
Asp	17.20	0.26	17.20	0.31	18.0	18.3	17	17	15	17
Thr[1]	4.55	0.45	3.64	0.04	4.6	5.1	5	4	5	14
Ser[1]	18.47	0.41	16.61	0.16	16.4	14.8	18	17	16	14
Glu	42.29	0.48	38.89	0.72	39.5	39.6	42	39	39	45
Pro	22.21	0.76	22.35	0.47	19.2	20.4	22	22	17	18
Gly	10.74	0.20	9.39	0.16	10.0	10.3	11	9	9	4
Ala	12.23	0.66	12.23	0.29	13.7	14.6	12	12	9	10
Cys										2
Val[1]	10.53	0.38	9.72	0.20	8.6	10.3	11	10	11	12
Met	3.76	0.30	4.64	0.11	4.5	3.2	4	5	5	4
Ile[1]	9.96	0.22	10.66	0.13	10.0	9.7	10	11	11	11
Leu	17.66	0.42	17.80	0.42	18.0	18.1	18	18	17	12
Tyr[2]	8.92	0.18	10.48	0.23	11.0	6.5	10	10	10	11
Phe	6.30	0.30	6.81	0.13	6.6	5.6	6	7	8	7
His	4.24	0.35	4.16	0.09	3.5	6.3	4	4	5	5
Lys	13.23	0.40	14.25	0.16	14.0	19.8	13	14	14	22
NH_3										
Arg	5.55	0.66	6.44	0.33	5.4	6.5	6	6	6	6
Trp	2.16		2.16		2.8	-	2	2	2	2
Phosphorus	9.3		8.7		-	-	9	9	8	7
Molec. weight amino acid composition							24 623	24 353	23 615	26 010
Sepharose 6 B Column	24 100		24 100							
No. of Residues							211	207	199	216

1. Threonine and serine values obtained from the 24 h analyses were increased by 5% and 10% respectively. Isoleucine and valine were 72 h results.

2. See text.

3. The compositions obtained by Resmini et al. (1967) and Shalichev and Tanev (1973) were recalculated to a molecular weight of 23 500, excluding the phosphate groups.

4. Standard deviation.

Inorganic phosphorus measured in the acid hydrolysates used for amino acid analysis were 1.06% and 1.20% for ovine α_{s2}- and α_{s3}-caseins, respectively. The absorbances of 1% solutions (w/v) at 280 nm, pH 7.0 (after correcting for Rayleigh scattering) were 11.20 and 10.9. These are higher than the extinction coefficient for bovine α_{s1}-casein B of 10.1 (Thompson, 1971). Amino acid analysis revealed 8.92 residues of tyrosine in ovine α_{s3}-casein (Table 10.2). The molar ratio of tyrosine:tryptophan measured in 0.1 M NaOH (Beaven and Holiday, 1952) was 5.04. This is equivalent to 10 tyrosine residues per mole in α_{s3}-casein. Consequently, both ovine α_s-caseins were assumed to have 10 tyrosine residues in each mole of protein. The higher values for the extinction coefficients of the ovine α_s-caseins compared with that for bovine α_{s1}-casein which contains 2 tryptophan and 10 tyrosine residues, suggests there may be 11 tyrosine residues in the ovine α_s-caseins rather than 10 residues.

10.9 Discussion

Two major α_s-caseins, designated as α_{s2}- and α_{s3}-casein, were isolated by chromatography on columns of DEAE-cellulose and CM-cellulose. Although this procedure was satisfactory for the purification of α_{s2}-casein, it was necessary to purify α_{s3}-casein by chromatography on DEAE-cellulose in the presence of Mg in order to remove an impurity which behaved in a similar manner to bovine α_{s3}-casein and the major caprine α_s-casein during gel electrophoresis in the presence and absence of Mg.

The amino acid compositions and molecular weights of the ovine α_{s2}- and α_{s3}-caseins were similar to each other and more closely related to bovine α_{s1}-casein B than to bovine α_{s3}-casein or caprine α_s-casein (Table 10.2). Amino acid analysis of the impure ovine α_{s3}-casein fraction revealed the contaminant was similar to bovine α_{s3}-casein.

The similar phosphorus contents, molecular weights and behaviour on gel electrophoresis in the presence of Mg of the ovine α_s-caseins suggested they differ in the number of charged amino acid residues which they contain and not in

the number of phosphorylated residues. Since α_{s2}-casein has a greater electrophoretic mobility at pH 8.4 than α_{s3}-casein, despite its extra lysine residue, it must also have fewer glutamine and or asparagine residues. Although there are a number of differences in the compositions of the ovine α_{s2}- and α_{s3}-caseins, these caseins must be considered as being more closely related to each other than bovine α_{s1}-casein is to β-casein, or bovine α_{s1}-casein is to bovine α_{s3}-casein.

Shalichev and Tanev (1973) isolated and purified ovine α_s-casein by urea fractionation of ovine casein and chromatography of a crude α_s-casein fraction on Sephadex G-150 in Tris-citrate buffer containing urea. They obtained a molecular weight of 47 500 for α_s-casein by chromatography on Sephadex G-150 in Tris-citrate buffer, pH 7.5, in the absence of urea. The molecular weight column was previously calibrated with native globular proteins. Under these conditions ovine α_s-casein, since it has an amino acid composition close to that of bovine α_{s1}-casein, is unlikely to be similar in molecular conformation to the proteins used to calibrate the column. Under similar conditions, depending on the protein concentration, bovine α_{s1}-casein would be present in a particular polymerization form, and as a mixture of polymers (Schmidt, 1970b). If one assumes the ovine α_s-caseins are associating proteins this high molecular weight value is not surprising.

Ovine α_{s2}- and α_{s3}-caseins appear to be fairly closely related to each other, and they are each more closely related to bovine α_{s1}-casein, with their similar amino acid compositions, molecular weights, behaviour in alkaline gel electrophoresis in the presence and absence of Mg, than to caprine α_s-casein.

CHAPTER 11: PHYSICAL CHARACTERIZATION OF THE CAPRINE, OVINE AND BOVINE CASEINS

This chapter outlines some of the physical characteristics of the caprine, ovine and bovine κ-, β- and α_s-caseins.

The temperature dependent polymerization of caprine and bovine β-caseins was investigated by ultracentrifugation and viscometry. The effect of temperature on the solubility of the proteins in the presence of $CaCl_2$ was determined for caprine, ovine and bovine β-caseins. The solubilities of the α_s- and β-caseins from the milks of the three species were compared in the presence of $CaCl_2$, at 37°C, and the effectiveness of the bovine, caprine and ovine κ-caseins in stabilizing these caseins in 20 mM $CaCl_2$ was assessed.

11.1 Ultracentrifugation

The caprine β-caseins were examined in the analytical ultracentrifuge at various temperatures for evidence of temperature dependent polymerization. All measurements were made in 0.02 M EDTA buffer, pH 7.0, containing 0.14 M NaCl, and at protein concentrations of 0.80% (w/v) and 0.88% (w/v) for caprine β_1- and β_2-casein, respectively.

At 10°C, only single slow moving peaks with sedimentation coefficients ($s_{20,w}$) of 1.17S and 1.15S were observed for caprine β_1- and β_2-caseins, respectively (Table 11.1). At 14°C, a single slowly sedimenting peak was observed for caprine β_2-casein (Table 11.1).

These sedimentation coefficients were consistent with caprine β_1- and β_2-caseins being monomers at 10°C, and also at 14°C for β_2-casein, even at protein concentrations of 0.80-0.88% (w/v) (Table 11.1). Evans et al. (1971b) obtained a sedimentation coefficient, $s^o_{20,w}$ ($s_{20,w}$ extrapolated to zero protein concentration) of 1.15S for the monomer forms of bovine β-casein A at 3°C.

At 25°C, ultracentrifugation revealed caprine β_1- and β_2-caseins contained rapidly sedimenting and slowly sedimenting peaks (Table 11.1), indicating the presence of an associating

Table 11.1

Sedimentation Coefficients[1] of Caprine β-Caseins

	β_2-Casein[2]		β_1-Casein[2]	
	$s_{20,w}$ monomer	$s_{20,w}$ polymer	$s_{20,w}$ monomer	$s_{20,w}$ polymer
10°C	1.15S	-	1.17S	-
14°C	1.24S	-		
25°C	1.37S	16.18S	2.20S	10.18S
	1.51S	15.23S		

1. Sedimentation coefficients are in Svedburg units (S).
2. These measurements were made in 0.02 M EDTA buffer, pH 7.0, containing 0.14 M NaCl, at fixed protein concentrations of 0.08% and 0.88% for β_1- and β_2-caseins, respectively. The viscosity of the buffer at 25°C (relative to water) was used to convert apparent sedimentation coefficients to $s_{20,w}$ values. No account was taken of the effect of protein concentration on the viscosity of the buffer.

The partial specific volume of caprine β_1-casein, estimated from its amino acid composition, was 0.744 (Schachman, 1957).

protein system. The sedimentation coefficient for the rapidly sedimenting peak in β_2-casein was less than that for β_1-casein and may reflect differences in the size of the polymers (Table 11.1). At 20°C, Evans et al. (1971b) observed both slowly and rapidly sedimenting peaks in the ultracentrifuge for bovine β-casein A. The rapidly sedimenting peak had a $s_{20,w}$ value of 12.68.

Consequently, the caprine β-caseins, like their bovine counterpart, appear to undergo temperature dependent polymerization from monomer to polymer, as the temperature is increased from 10°C to 25°C.

11.2 Viscosity Measurements on the Caprine and Bovine β-Caseins

The intrinsic viscosities of caprine β_1- and β_2-caseins were compared with bovine β-casein A^1 at temperatures from 4°C to 37°C in neutral buffer, and in buffer containing 6 M guanidine.HCl at 4°C.

The intrinsic viscosity, $[\eta]$, of a protein, previously defined in Chapter 7.14, is a measure of the specific volume (in units of ml/g) of the domain of a macromolecule in solution, and its measurement can give information on that macromolecule in solution (Tanford, 1961).

The reduced viscosity plots obtained for caprine β_1- and β_2-caseins at 4°C, 10°C, 25°C and 37°C in neutral buffer, and at 4°C in the presence of 6 M guanidine.HCl are presented in Figs.11.1 and 11.2. Similar results obtained for bovine β-casein A^1 at 4°C and 25°C are presented in Fig.11.3. The intrinsic viscosities and the values of Huggin's constant, k', obtained for caprine β_1- and β_2-caseins and bovine β-casein A^1 are presented in Table 11.2.

The curvature of some of the reduced viscosity plots observed at low protein concentrations has been previously noted for bovine β-casein (Sullivan et al., 1955; von Hippel and Waugh, 1955; Payens and van Markwijk, 1963). Toyoda and Yamauchi (1972) also observed curvature in reduced viscosity plots for human β-casein. The reason for

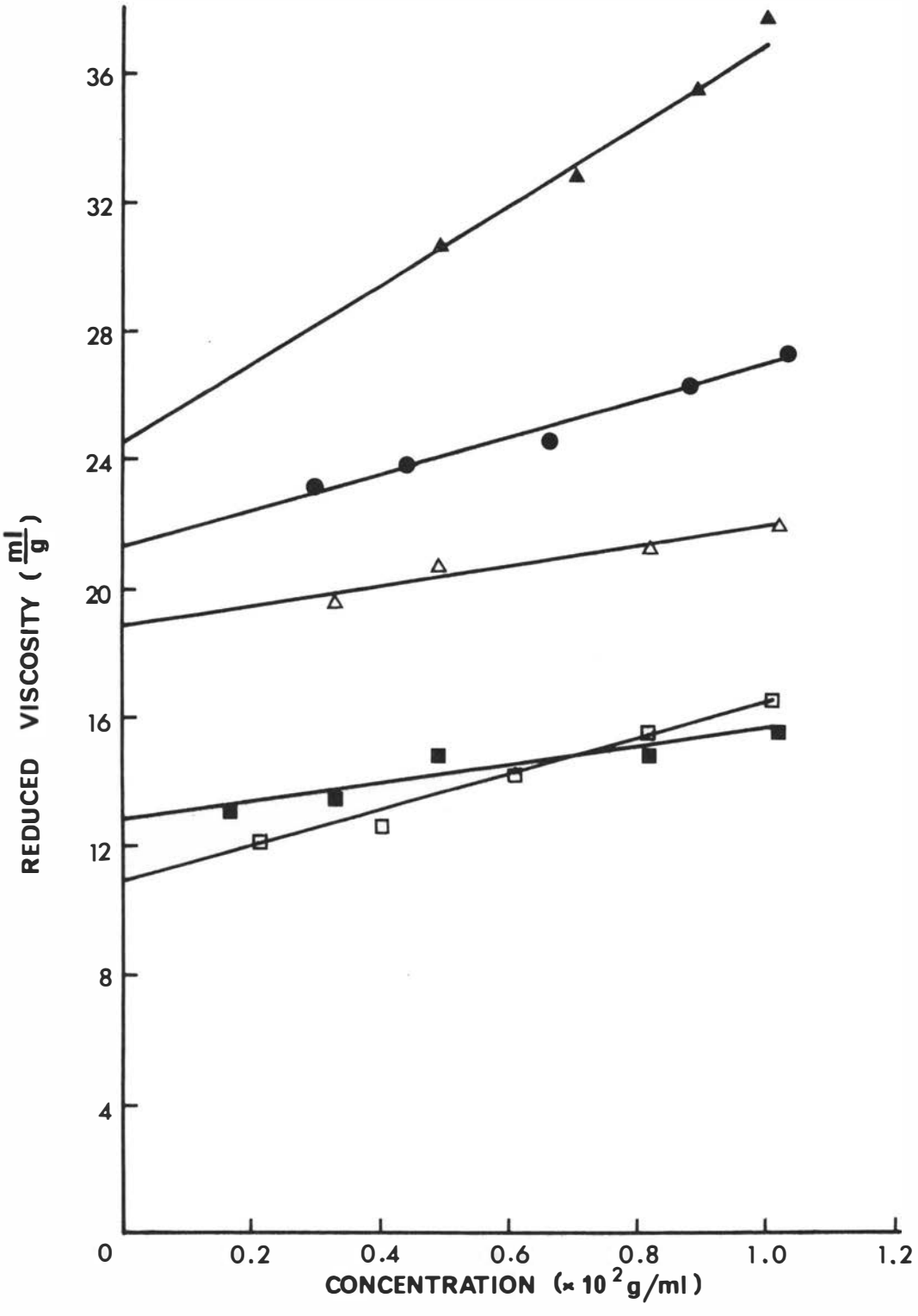


Fig.11.1: Reduced viscosity plots for caprine β_1-casein at different temperatures and in different solvents. 0.1 M phosphate buffer, pH 7, containing 6 M guanidine.HCl, 4°C (▲): 0.02 M EDTA buffer, pH 7, containing 0.14 M NaCl at 4°C (●), 10°C (△), 25°C (■), 37°C (□).

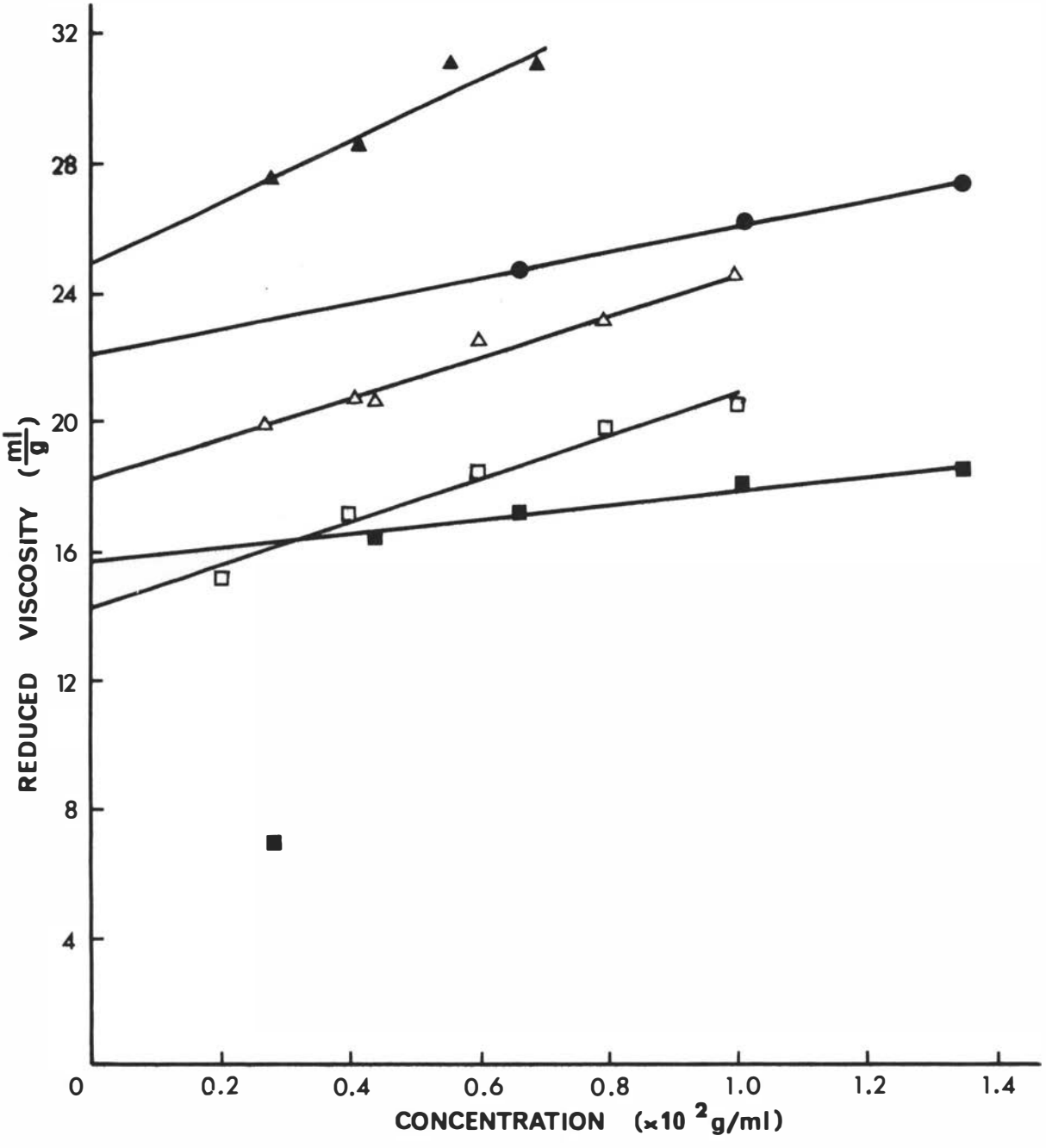


Fig.11.2: Reduced viscosity plots for caprine β_2-casein at 4°C in 6 M guanidine.HCl (▲), and in EDTA-NaCl buffer at 4°C (●), 10°C (△), 25°C (■), 37°C (□).

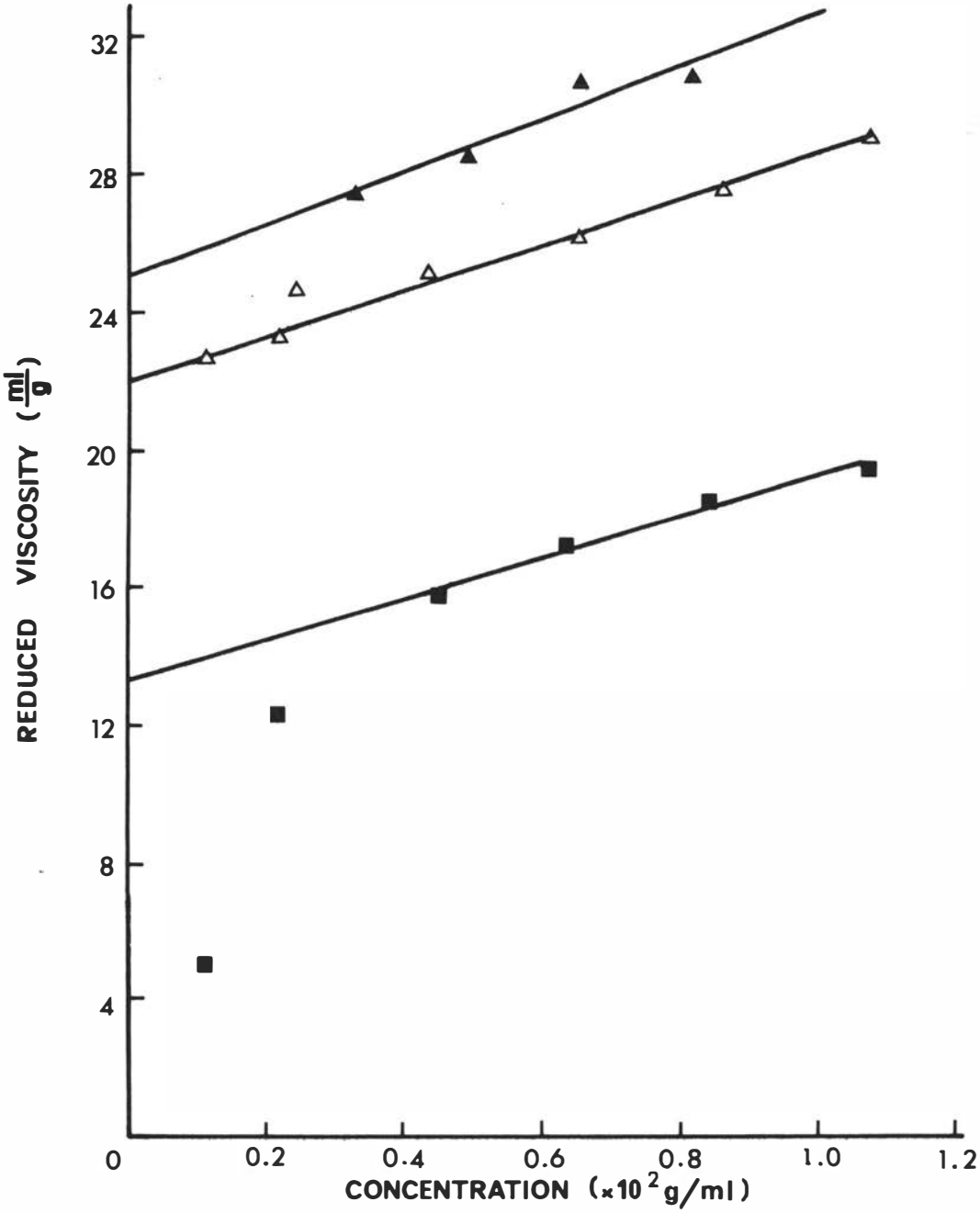


Fig.11.3: Reduced viscosity plots for bovine β-casein A[1] at 4°C in 6 M guanidine.HCl (▲), and in EDTA-NaCl buffer at 4°C (△) and 25°C (■).

the curvature of these graphs is obscure. In the present study, protein was not lost from solution, as measured by 280 nm absorbance, and consequently it was unlikely to have been caused by adsorption of protein to the wall of the viscometer.

As the temperature was increased from 4°C to 37°C, the intrinsic viscosities of bovine and caprine β-caseins in EDTA-NaCl buffer decreased (Table 11.2). This was either a result of a change in the conformation of the protein as the temperature was increased, or was due to a decrease in solvation of the protein (Tanford, 1961). At 4°C, the intrinsic viscosities of the β-caseins were all similar and only increased slightly in value in going from neutral buffer to one containing 6 M guanidine.HCl (Table 11.2).

These results suggested that the caprine and bovine β-caseins have a conformation in neutral buffer at low temperatures which approximates that of a random coil, since proteins in 6 M guanidine.HCl have been shown to have conformations closely resembling that of a random coil (Tanford, 1963). Noelken and Reibstein (1968) obtained similar results for bovine β-casein B (Chapter 6.10).

The intrinsic viscosity of bovine β-casein A^1 in neutral buffer at 4°C was similar to the value of 23.1 ml/g obtained for the B variant at 2.5°C by Noelken and Reibstein (1968). The intrinsic viscosity of β-casein B at 25°C in 6 M guanidine.HCl was 22.2 ml/g, lower than the value of 25.0 ml/g obtained for the A^1 variant at 4°C (Table 11.2). It is interesting to note that Toyoda and Yamauchi (1972) observed temperature dependence for the intrinsic viscosity of human β-casein in 6 M guanidine.HCl. At 4°C and 10°C, the intrinsic viscosities of human β-casein in 6 M guanidine.HCl were 25.2 ml/g and 24.8 ml/g respectively, and 21.0 ml/g at 25°C.

At 10°C, the intrinsic viscosity of caprine β_1-casein had not changed appreciably from that at 4°C, however, the value for caprine β_2-casein had decreased to 18.3 ml/g. At 25°C, the intrinsic viscosities of bovine β-casein A^1 and

Table 11.2

Intrinsic Viscosities of Caprine and Bovine β-Caseins

	Bovine β-casein A			Caprine β_2-casein			Caprine β_1-casein		
	$[\eta]$ ml/g	S.E.[1]	k'[2]	$[\eta]$ ml/g	S.E.[2]	k'[2]	$[\eta]$ ml/g	S.E.[1]	k'[2]
6 M guanidine.HCl									
4°C	25.0	1.0	1.4	24.9	1.2	1.6	24.7	1.0	2.0
0.02 M EDTA -0.14 M NaCl, pH 7.0									
4°C	22.1	0.1	1.4	22.2	0.3	0.8	21.6	0.4	0.3
10°C	-			18.3	0.1	1.9	21.5	0.3	1.2
25°C	11.5	0.9	6.2	15.7	0.3	0.9	12.9	0.5	1.8
37°C	-			13.8	0.2	4.1	10.8	0.5	5.0

1. S.E., Standard error of the intrinsic viscosities.
2. Huggin's constant, k'.

caprine β_1- and β_2-caseins have decreased to values of 11.5 ml/g, 12.9 ml/g and 15.7 ml/g, respectively (Table 11.2). The intrinsic viscosity of bovine β-casein A^1 at 25°C was in good agreement with the value of 12.5 ml/g obtained at 25°C by Sullivan et al. (1955). The difference in intrinsic viscosity of caprine β_2-casein at 25°C, and also at 37°C, when compared with bovine β-casein A^1 and caprine β_1-casein may be attributed to differences in conformation and solvation at these temperatures.

The values of k', Huggin's constant, listed in Table 11.2 are similar to values obtained for bovine β-casein at low temperature by Noelken and Reibstein (1968) and Payens and van Markwijk (1963). In 6 M guanidine.HCl, Noelken and Reibstein (1968) obtained a value for k', from bovine β-casein B, of 1.0, while in aqueous neutral buffer at low temperature the value was also 1.0. Payens and van Markwijk (1963) obtained a value for k' in neutral buffer of 0.93 for bovine β-casein. In Table 11.2 the values of k' for bovine β-casein at 4°C were slightly higher than the values obtained by Noelken and Reibstein (1968) and higher than the highest values (0.29-0.95) found by Tanford et al. (1967) for proteins in 6 M guanidine.HCl. The limited data available, and the lack of adequate theory for k', other than for rigid, non-solvated spheres (Bradbury, 1970) would suggest little interpretation may be placed on these results.

11.3 The Effect of Temperature on the Solubility of the Caprine, Ovine and Bovine β-Caseins in the Presence of $CaCl_2$

Bovine β-casein is known to undergo temperature dependent polymerization to form large polymers which readily precipitate in the presence of low levels of $CaCl_2$ (Garnier, 1966; Thompson et al., 1969c). Polymerization appears to be dependent not only on temperature, but also on protein concentration. Consequently, use was made of the sensitivity to Ca of these polymers in making a preliminary comparison of the temperature dependent association of the β-caseins from bovine, caprine and ovine caseins.

The turbidity at 550 nm of solutions containing 1 mg/ml protein in sodium cacodylate buffer, pH 6.8, and 20 mM $CaCl_2$, was measured as the temperature was increased from about 14°C to 40°C. The behaviour of bovine β-casein B and caprine β_1- and β_2-caseins were compared at ionic strengths (excluding Ca) of 0.05, 0.10 and 0.15 (Figs.11.4, 11.5 and 11.6). As the ionic strength was increased from 0.05 to 0.15, the temperature at which precipitation first became apparent in the solutions increased quite markedly. The turbidity of the β-casein solutions reached a maximum value at about 38-40°C. Caprine β_2-casein solutions were possibly exceptional, since a maximum in turbidity was not attained within the experimental temperature range. Values of the temperature of half transition (the midpoint temperature between the minimum and maximum value of the turbidity of a β-casein solution) obtained for the β-caseins are presented in Table 11.3.

Table 11.3

Temperature of Half Transition Values for the β-Caseins

Ionic strength	Bovine β-casein B T°C	Caprine β_1-casein T°C	Caprine β_2-casein T°C
0.05	23	28	~33
0.10	29	34	~40
0.15	36	37	-

At any ionic strength, turbidity became apparent in solutions of bovine β-casein at a lower temperature than for caprine β_1-casein, which in turn was turbid at a lower temperature than β_2-casein (Figs.11.4, 11.5 and 11.6). Increasing the pH to 7.1, or decreasing it to 6.5 did not significantly alter the shape or position of the temperature turbidity curves. Furthermore, the colloidal aggregates redissolved again as the temperature decreased, though the turbidity curves followed a different path.

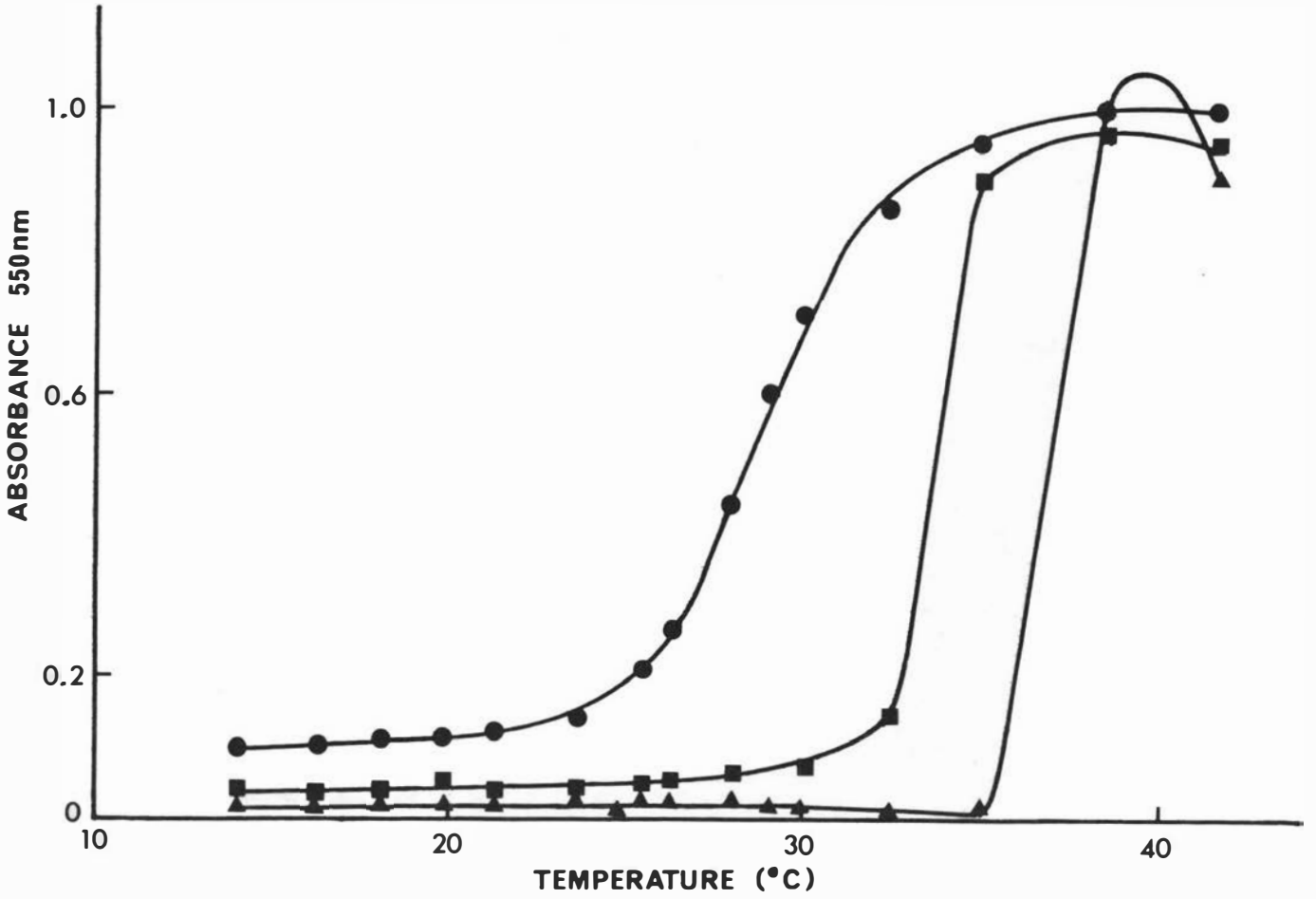


Fig.11.4: The effect of temperature and ionic strength on the turbidity of caprine β_1-casein solutions containing 1 mg/ml protein in 20 mM $CaCl_2$ and 0.05 M sodium cacodylate buffer, pH 6.8, with NaCl added to ionic strengths, excluding $CaCl_2$, of 0.05 (●), 0.10 (■) and 0.15 (▲).

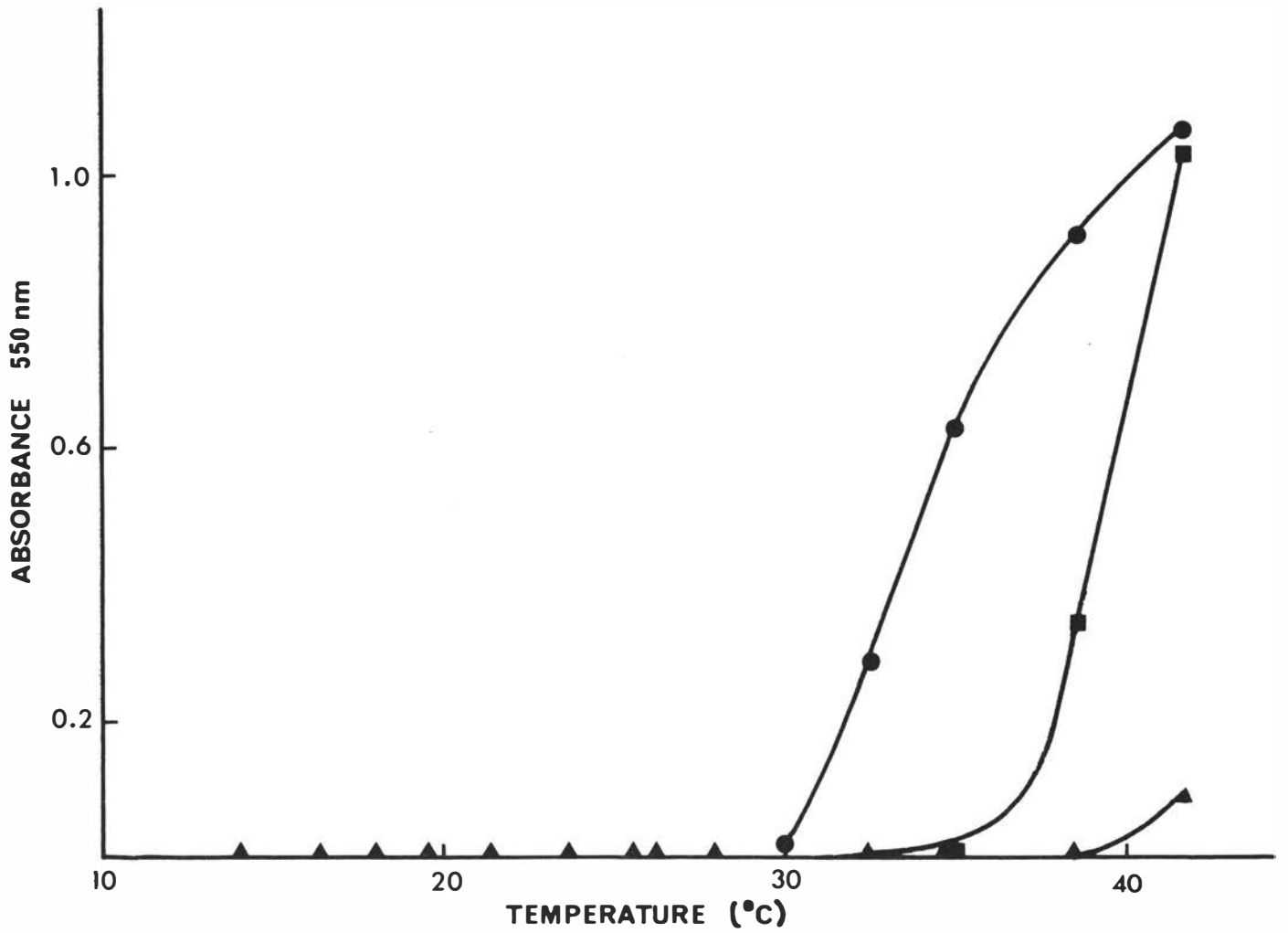


Fig.11.5: The effect of temperature and ionic strength on the turbidity of caprine β_2-casein solutions containing 1 mg/ml protein in 20 mM $CaCl_2$ and 0.05 M sodium cacodylate buffer, pH 6.8, with NaCl added to ionic strengths of 0.05 (●), 0.10 (■) and 0.15 (▲).

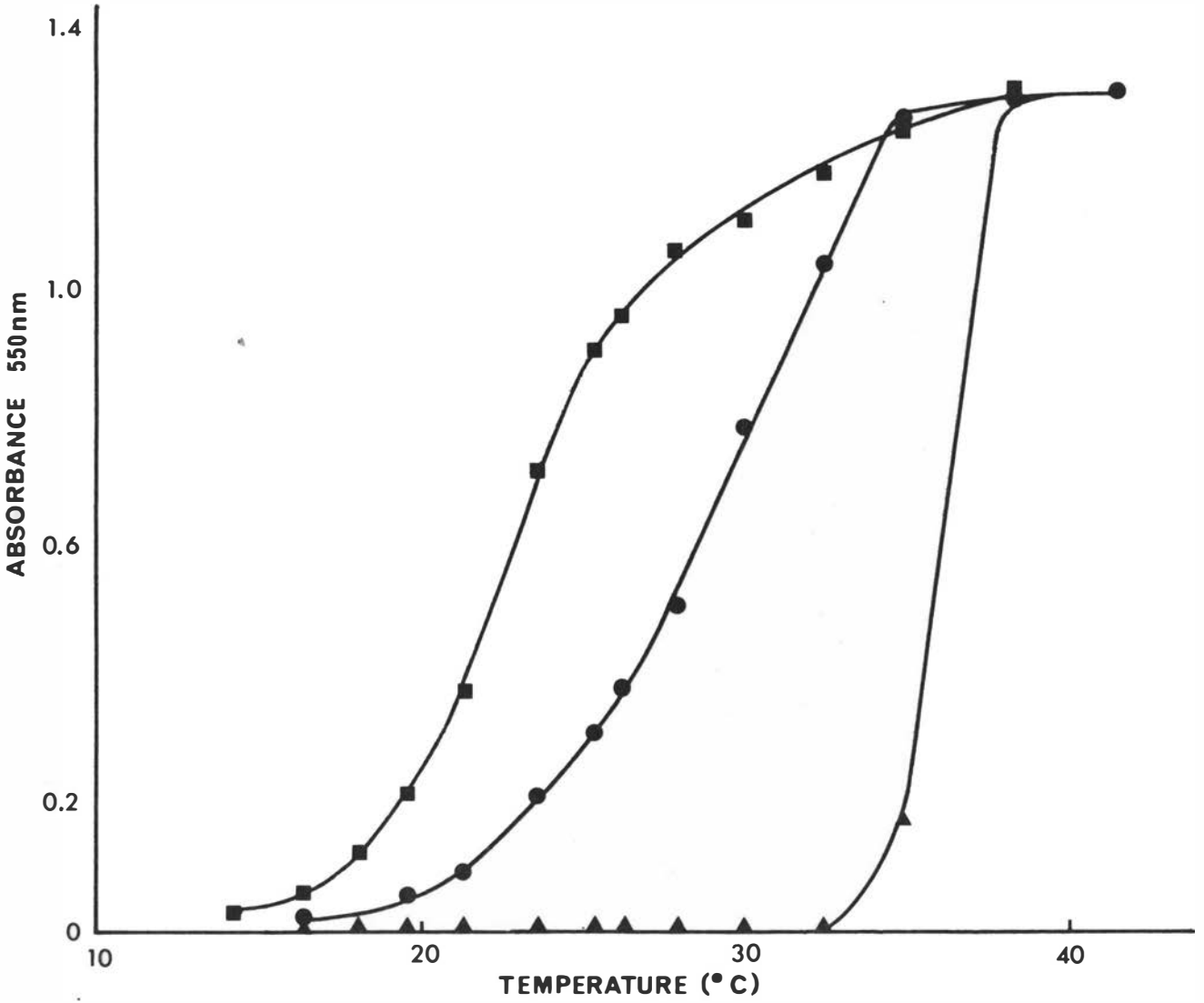


Fig.11.6: The effect of temperature and ionic strength on the turbidity of bovine β-casein B solutions containing 1 mg/ml protein in 20 mM $CaCl_2$ and 0.05 M sodium cacodylate buffer, pH 6.8, with NaCl added to ionic strengths of 0.05 (■), 0.10 (●) and 0.15 (▲).

Some preliminary measurements were made comparing the ovine β-caseins with bovine β-casein A^1 at an ionic strength of 0.06. These indicated that ovine β_2-casein solutions behaved in a similar manner to bovine β-casein solutions with colloidal aggregates being evident for both proteins at 26°C. Turbidity became apparent in ovine β_1-casein solutions at 20-21°C.

These results suggested that the ovine and caprine β-caseins undergo temperature dependent polymerization similar to that of bovine β-casein, since only the polymers of β-casein appear to be sensitive to Ca (Chapter 11.4).

11.4 Calcium Sensitivity of the Caprine, Ovine and Bovine Caseins

The sensitivity of caprine, ovine and bovine caseins to the presence of varying amounts of $CaCl_2$, was measured in sodium cacodylate buffer, pH 6.8, containing 5 mg/ml protein at both 1°C and 37°C, or in solutions containing 0.5 mg/ml protein at 37°C.

β-Caseins

At 37°C, and an initial protein concentration of 5 mg/ml, caprine β_1-casein was more sensitive to the presence of $CaCl_2$ than caprine β_2-casein (Fig.11.7). The amount of soluble caprine β_1-casein decreased rapidly between 6 and 8 mM $CaCl_2$, compared with 8 to 10 $CaCl_2$ for caprine β_2-casein. At 37°C, in 0.01 M imidazole-HCl buffer, pH 7.0, and an initial protein concentration of 10 mg/ml, Thompson et al. (1969c) found the amount of soluble bovine β-casein C decreased between 5 and 10 mM $CaCl_2$, until at 10 mM 40% of the protein remained soluble. However, valid comparisons between different studies are difficult, since the purity of the caseins and the experimental conditions have an important bearing on the results obtained.

The sensitivity of ovine β_1- and β_2-caseins to $CaCl_2$ at 37°C, at an initial protein concentration of 0.5 mg/ml, was measured from their initial increase in turbidity with increasing $CaCl_2$ concentration. These results are presented

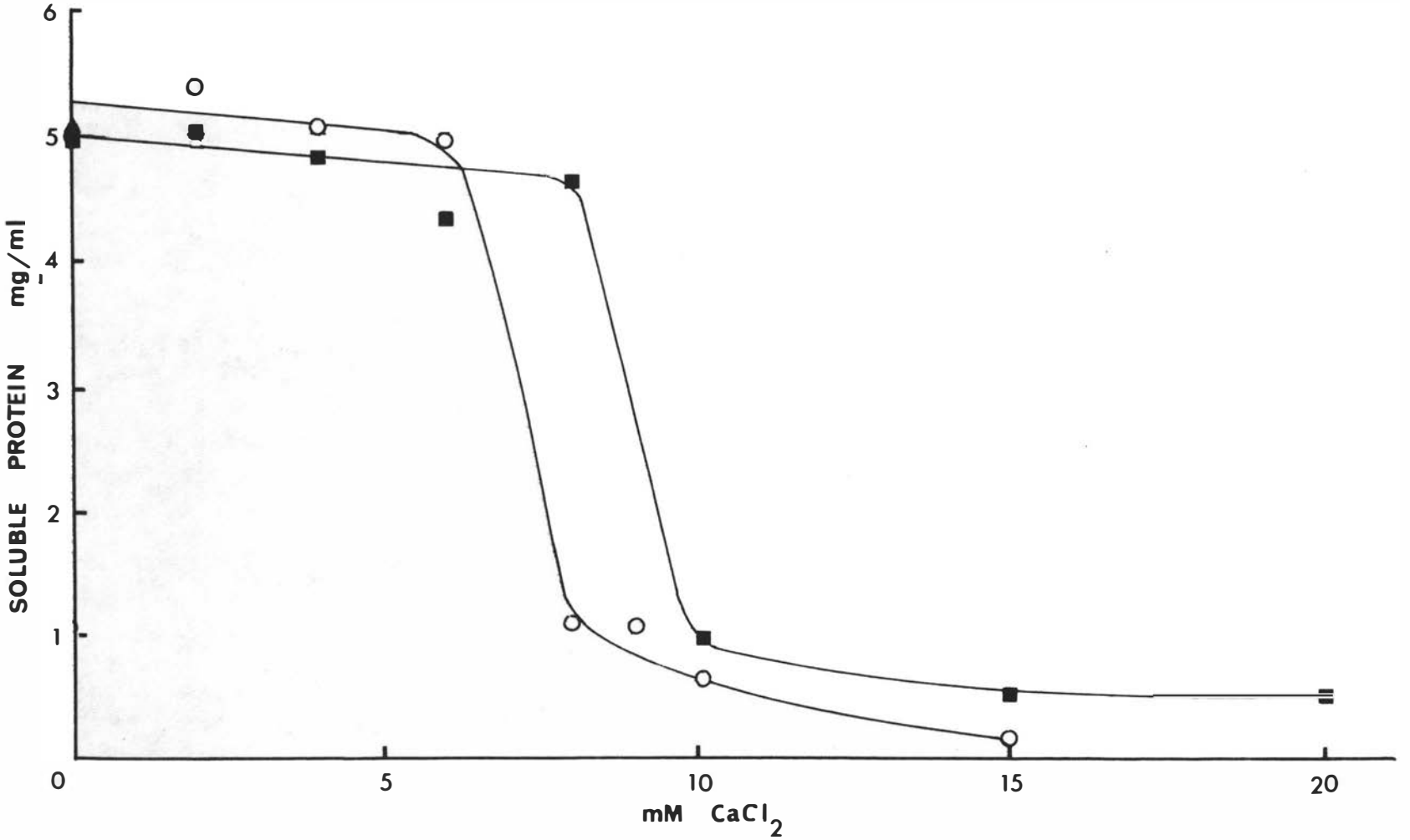


Fig.11.7: Calcium sensitivity of caprine β_1-casein (O) and β_2-casein (■) at 37°C, in 0.05 M cacodylate buffer, pH 6.8, containing 0.05 M KCl (I = 0.088). The initial protein concentration was 5 mg/ml.

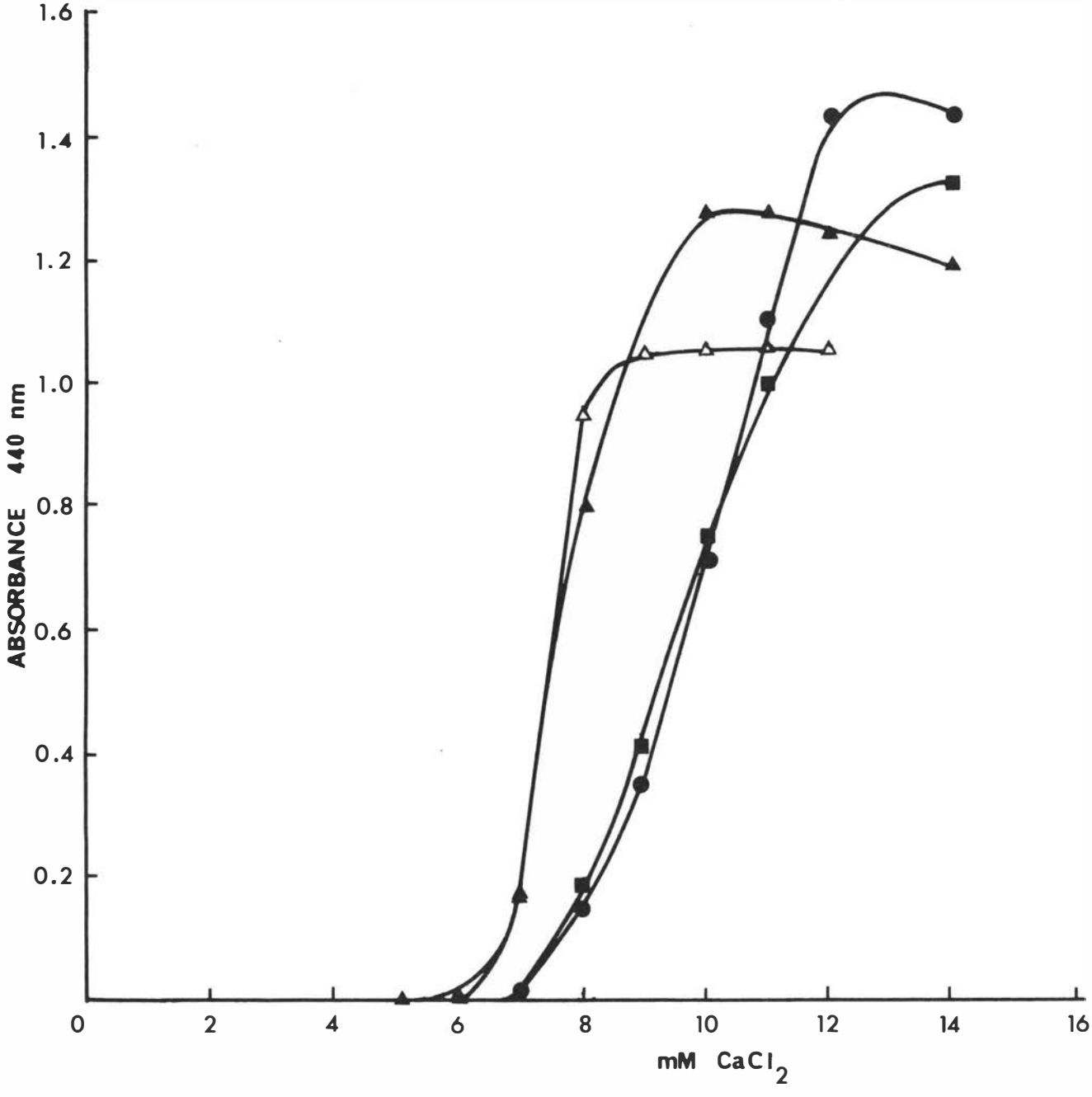


Fig.11.8: Calcium sensitivity of caprine, ovine and bovine β-caseins at 37°C in 0.05 M cacodylate buffer, pH 6.8, containing 0.05 M NaCl (I = 0.088). The initial protein concentration was 0.5 mg/ml. ▲, caprine β_1-casein; △, ovine β_1-casein; ■, ovine β_2-casein; ●, bovine β-casein A^1.

in Fig.11.8, along with similar results obtained for caprine β_1-casein and bovine β-casein. Under these conditions, ovine β_1-casein was more sensitive to $CaCl_2$ than ovine β_2-casein, and had a similar sensitivity to caprine β_1-casein (Fig.11.8). The sensitivity to $CaCl_2$ of ovine β_2-casein and bovine β-casein A^1 was also similar, and the protein started to form colloidal aggregates at 7 mM $CaCl_2$. Since turbidity measurements are not necessarily related to the amount of protein in the colloid, the only conclusions that may be made with any accuracy are those regarding the initial point of precipitation.

At 1°C, and at a protein concentration of 5 mg/ml, the caprine β-caseins remained soluble, at least up to 0.2 M $CaCl_2$. Ovine β-casein solutions containing 0.5 mg/ml of protein and 20 mM $CaCl_2$ at 37°C which were initially turbid, were clear after standing overnight at 4°C.

α_s-Caseins

The solubility of the major caprine α_s-casein in the presence of $CaCl_2$, at a protein concentration of 5 mg/ml, was compared with bovine α_{s1}-casein B and one of the minor bovine caseins, α_{s3}-casein. At 37°C, caprine α_s-casein was the most sensitive, followed by bovine α_{s3}-casein, and bovine α_{s1}-casein B (Fig.11.9). The result for α_{s3}-casein was similar to that previously reported for bovine α_{s5}-casein (Toma and Nakai, 1973), where 20% of the original protein (1 mg/ml) was shown to be soluble in solutions containing 2 mM $CaCl_2$, at pH 7.5 and 37°C. Bovine α_{s5}-casein is thought to be a disulphide dimer, composed of equimolar amounts of α_{s3}- and α_{s4}-caseins (Annan and Manson, 1969; Hoagland et al., 1971). The solubility curve for bovine α_{s1}-casein B (Fig.11.9) was similar to that previously obtained at the same initial protein concentration by Noble and Waugh (1965), who found precipitation of the protein was initiated close to 4 mM $CaCl_2$.

At 1°C, all the caseins were more soluble in the presence of $CaCl_2$, but the order of sensitivity remained the same. At a $CaCl_2$ concentration of 5 mM, caprine

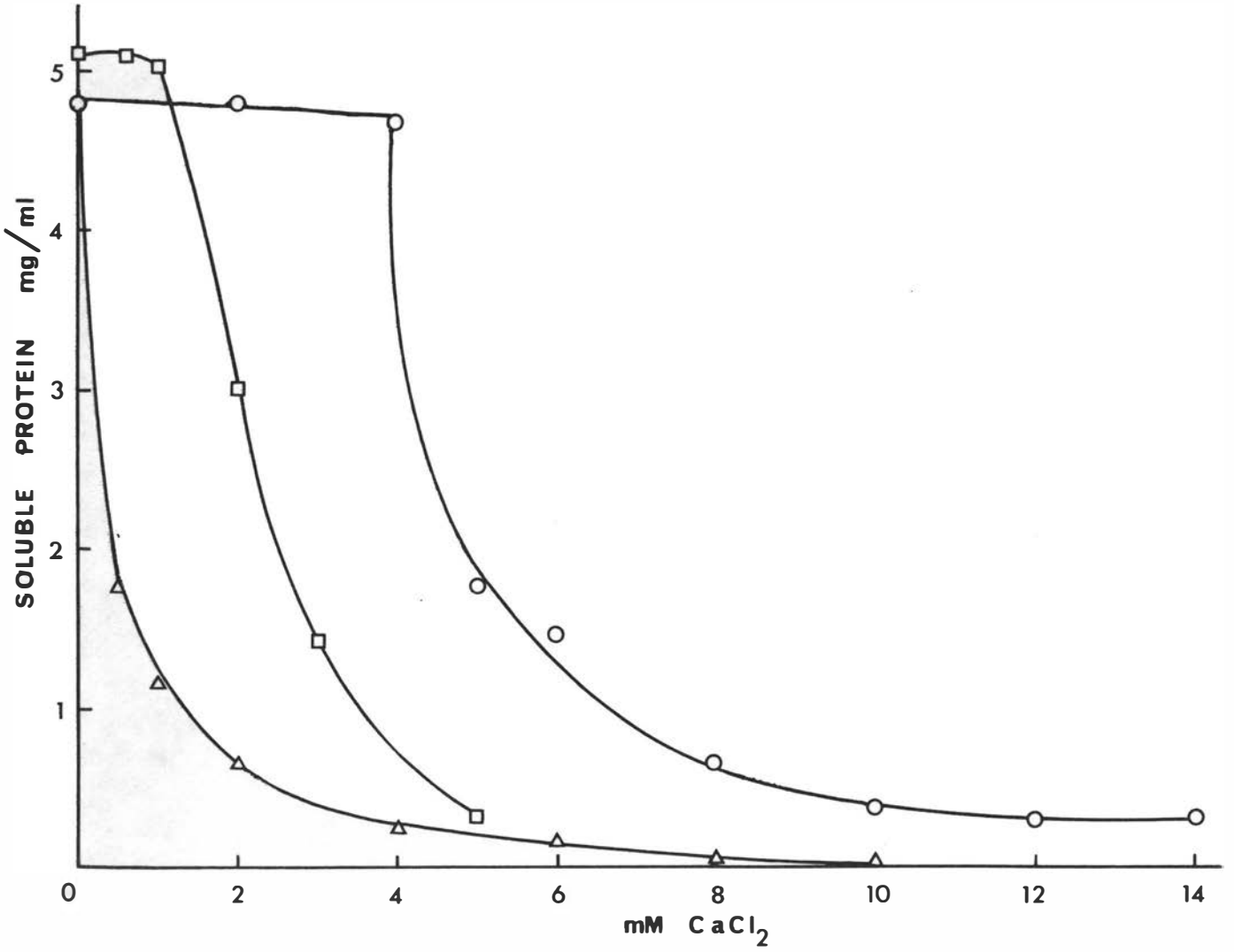


Fig.11.9: Calcium sensitivity of the α_s-caseins at 37°C. △, caprine α_s-casein; □, bovine α_{s3}-casein; ○, bovine α_{s1}-casein B. Other experimental details are the same as in Fig.11.7.

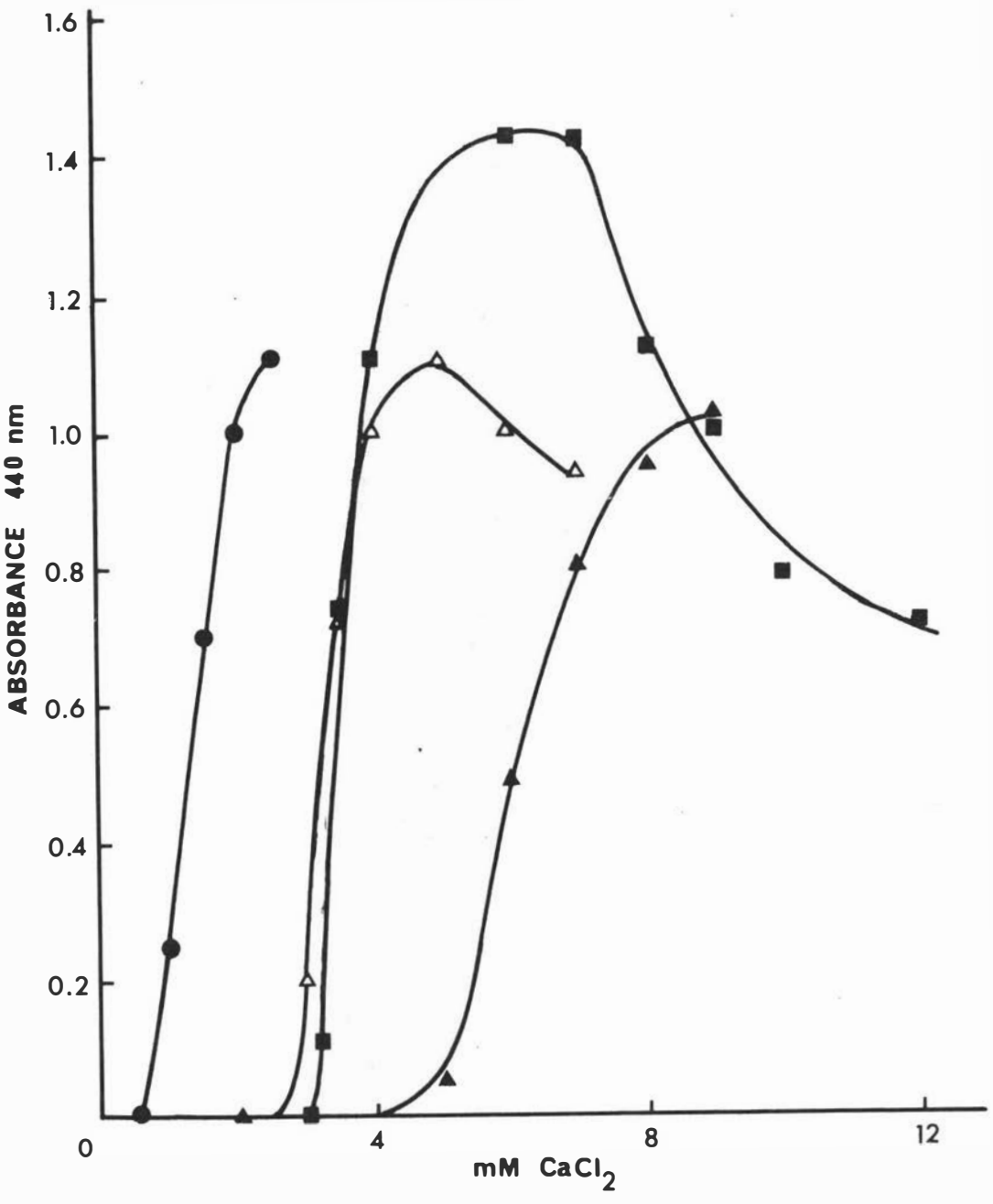


Fig.11.10: Calcium sensitivity of the caprine, ovine and bovine α_s-caseins at 37°C. ● , caprine α_s-casein; △ , ovine α_{s2}casein; ■ , ovine α_{s3}-casein; ▲ , bovine α_{s1}-casein B. Other experimental details were outlined in Fig.11.8.

α_s-casein was soluble to 0.3 mg/ml, bovine α_{s3}-casein was soluble to 1.0 mg/ml and bovine α_{s1}-casein B was soluble to 3.8 mg/ml. At a $CaCl_2$ concentration of 0.2 M, caprine α_s-casein was soluble to 2.5 mg/ml, whereas bovine α_{s1}-casein B was soluble to 0.5 mg/ml. Thompson et al. (1969c) found similar results for bovine α_{s1}-casein B at 1°C, and attributed the increased solubility at high $CaCl_2$ concentrations to the proteins becoming positively charged. Caprine α_s-casein appeared to bind more Ca under these conditions, and thus become more soluble than bovine α_{s1}-casein.

At 37°C, and an initial protein concentration of 0.5 mg/ml, the sensitivity to $CaCl_2$ of ovine α_{s2}- and α_{s3}-caseins was similar (Fig.11.10), precipitating at 3 mM $CaCl_2$. On the other hand, bovine α_{s1}-casein B and caprine α_s-casein initially precipitated at 5 mM and 0.5 mM $CaCl_2$, respectively, in agreement with the earlier results (Fig.11.9).

11.5 Stabilization of the Caprine, Ovine and Bovine α_s- and β-Caseins in the Presence of $CaCl_2$ by κ-Caseins

The ability of the caprine, ovine and bovine κ-caseins to stabilize the individual α_s- and β-caseins in solution in the presence of 20 mM $CaCl_2$ through the formation of micelle type complexes were compared. The results, presented in Table 11.4, showed that at a weight ratio of α_s- or β-casein: κ-casein of 5:1, all of the κ-caseins can stabilize the proteins in solution with varying degrees of success.

Bovine and ovine κ-caseins appeared to be slightly more effective than caprine κ-casein in stabilizing the α_s- and β-caseins, although ovine κ-casein could only stabilize 47% of bovine α_{s3}-casein in solution (Table 11.4). The somewhat reduced efficiency of caprine κ-casein may have been due to a decrease in stabilizing ability as a result of some pretreatment during the purification or storage of κ-casein. It was evident though that the caprine, ovine and bovine κ-caseins can stabilize the α_s- and β-caseins in solution in the presence of $CaCl_2$, since in the absence of κ-casein about 95% of the α_s-caseins and more than 85% of the β-caseins were precipitated (Table 11.4).

Table 11.4

Stabilization[1] of α_s- and β-Caseins by the κ-Caseins

	κ-Caseins			No added κ-casein
	Bovine	Caprine	Ovine	
	Percent soluble protein[2]			
Bovine α_{s3}-casein	88	88	47	2
Bovine α_{s1}-casein	95	85	97	3
Bovine β-casein A[1]	100	68	82	12
Caprine α_s-casein	80	68	73	3
Caprine β_2-casein	91	82	68	15
Ovine α_{s2}-casein	84	60	77	3
Ovine β_2-casein	82	63	76	7

1. Stabilization tests were performed at α_s- or β-casein:κ-casein weight ratios of 5:1 in sodium cacodylate buffer, pH 6.8, at 37°C in the presence of 20 mM $CaCl_2$. The initial concentration of α_s- or β-casein was 5 mg/ml.
2. These values were obtained from the absorbance of the soluble protein, after correction for the amount of added κ-casein, and are expressed as a percentage of the absorbance of the α_s- or β-casein prior to adding $CaCl_2$.

Noble and Waugh (1965) found that at a weight ratio of bovine α_{s1}-casein:κ-casein of 10:1, about 95% of the α_{s1}-casein was soluble in 20 mM $CaCl_2$. Similar results were obtained for α_{s1}-casein in 10 mM $CaCl_2$ by Toma and Nakai (1973) at a α_s-casein:κ-casein weight ratio of 5:1, although under these conditions only 55% of the bovine α_{s5}-casein was soluble. In the present study, bovine α_{s3}-casein was nearly as effectively stabilized as bovine α_{s1}-casein (Table 11.4). Zittle and Custer (1966) found 80% of the impure caprine α_s-casein remained soluble at a caprine α_s-casein: caprine κ-casein weight ratio of 7:1 in 20 mM $CaCl_2$.

11.6 Discussion

Viscosity Measurements

At low temperatures (≤4°C), caprine β_1- and β_2-caseins are present in solution as monomers, as shown by ultracentrifugation (Chapter 11.1). Similar studies have shown that monomers of bovine β-casein are also present under these conditions (Payens and van Markwijk, 1963; Noelken and Reibstein, 1968; Evans et al., 1971a).

At 4°C, caprine β_1- and β_2-caseins and bovine β-casein A^1 have intrinsic viscosities in EDTA-NaCl buffer, pH 7.0, ranging from 21.6 to 22.2 ml/g, which increased to about 25 ml/g in 6 M guanidine.HCl. Noelken and Reibstein (1968), who also found that the intrinsic viscosity of bovine β-casein was similar in EDTA buffer at 4°C, and in 6 M guanidine.HCl, proposed that bovine β-casein B had a random structure at low temperature. Subsequently, Waugh et al. (1970) proposed that the high intrinsic viscosity of bovine β-casein at low temperatures could result from the highly solvated and flexible N-terminal portion of the β-casein molecule, which is very acidic and contains all of the organic phosphate groups. Random coil formation in the guanidine.HCl solution would reduce the contribution of the acidic peptide to viscosity, but increase the contribution of the remaining portion. This same hypothesis may be used to explain the behaviour of the caprine β-casein at low temperature, since they also contain considerable amounts of phosphorus (Chapter 9.7).

At 25°C, the caprine and bovine β-caseins have fairly high intrinsic viscosities ranging from 11.5 to 15.7 ml/g (Table 11.2). Even at 37°C, the intrinsic viscosities of caprine β_1- and β_2-caseins were 10.8 and 13.8 ml/g, respectively. These intrinsic viscosity values indicate the caprine and bovine β-caseins, even at 37°C, are highly solvated or asymmetric molecules, since it is known that the common globular proteins are neither highly solvated nor very asymmetric and that their intrinsic viscosities vary from 3.3 to 4.0 ml/g (Tanford, 1961). Certainly, neither the caprine β-caseins nor bovine β-casein have this characteristic behaviour.

It is interesting to note that human β-casein which also has a disorganized structure at low temperature, as shown by viscosity measurements, optical rotatory dispersion and circular dichroism, differs from bovine β-casein in its temperature dependent polymerization behaviour (Toyoda and Yamauchi, 1972). Only monomers of β-casein were evident in sedimentation patterns until approximately 20°C, when rapidly sedimenting peaks became apparent. However, human β-casein is not a single species but a mixture of six proteins which have from 0 to 5 phosphate groups (Groves and Gordon, 1970). This would be sufficient to alter their individual physical properties, since each protein has a different sensitivity to $CaCl_2$ (Nagasawa et al., 1970).

Polymerization of the β-Caseins

Ultracentrifugation showed that monomers of bovine β-casein, present at low temperatures (≤4°C), aggregated to form large polymers as the temperature was increased (Payens and van Markwijk, 1963; Evans et al., 1971a, b). Thompson et al. (1967) found that removal of the C-terminal residues, -Ile-Ile-Val.OH of bovine β-casein (Fig.6.3) with carboxypeptidase A altered the temperature dependent association properties of bovine β-casein, since less polymer was formed at 8.5°C, and at 20°C, than with the untreated bovine β-casein. Creamer and Berry (1974, personal communication) have verified by gel chromatography, that removal of the two hydrophobic C-terminal residues of bovine β-casein affects

the equilibrium between monomer and polymer, compared with results obtained for the untreated bovine β-casein. Furthermore, bovine β-I, a rennet degradation product of β-casein (Creamer et al., 1971), that has 20 C-terminal residues removed (Pelissier et al. 1974) does not undergo temperature dependent polymerization, as shown by gel chromatography (Creamer and Berry, 1974, unpublished results).

Intrinsic fluorescence measurements on the caprine β-caseins showed that they undergo the same type of temperature dependent polymerization from monomer to polymer as bovine β-casein A^1 (Pearce, 1974, unpublished results). However, the temperature at which caprine β_1-casein polymerized was lower than that for β_2-casein, despite the higher net negative charge on β_1-casein, with its extra phosphorus residue compared to β_2-casein.

The polymer form is in equilibrium with the monomer and if the position of the equilibrium is determined by the opposing forces of attraction between hydrophobic regions on the separate monomers, and repulsion between monomers with a net negative charge, then, because the net charge on caprine β_1-casein is greater than β_2-casein, the attractive forces between β_1-casein monomers must be greater than between the β_2-casein monomers. This suggests that despite the apparent similarity of the caprine β_1- and β_2-caseins, there may be differences in their sequences, particularly in the C-terminal region, since this region appears to be involved in the polymerization of bovine β-casein.

One or more conservative changes in the C-terminal region may be sufficient to affect the temperature dependent polymerization of these caseins. This suggestion can only be ratified by the determination of the C-terminal sequences of these proteins, in conjunction with an investigation of their physical properties. Furthermore, possible changes in the primary structure, particularly in a region important to the associative properties of the β-caseins may be sufficient to explain the differences in associative properties of the β-caseins from the various species.

From the few results for the experiments on ovine β-caseins, it is likely that the ovine β-caseins could behave in a similar manner to that proposed for the caprine β-caseins.

An important characteristic of the caprine and bovine β-casein monomers is their solubility in the presence of $CaCl_2$. Although limited data are available on the ovine β-caseins they were soluble in the presence of $CaCl_2$ at low temperature (6°C), and insoluble at 37°C (Chapter 11.4). These results are consistent with a change from monomer to polymer for the ovine β-caseins.

At higher temperatures (25°C), caprine, ovine and bovine β-casein solutions are sensitive to $CaCl_2$ (Chapter 11.3), presumably as a result of the presence of β-casein polymers. Ultracentrifugation of the caprine β-caseins at 25°C and protein concentrations of 0.80-0.88% (w/v), revealed the presence of β-casein polymers. The sensitivities to $CaCl_2$ of the ovine and caprine β_2-caseins were similar, both being precipitated at 37°C in solutions containing 0.5 or 5 mg/ml of protein by the presence of 7 to 9 mM $CaCl_2$ (Figs.11.7 and 11.8). Similarly, ovine and caprine β_1-caseins were more sensitive than the other caseins, being precipitated at approximately 6 mM $CaCl_2$ (Figs.11.7 and 11.8).

As the temperature of caprine β-casein solutions containing 20 mM $CaCl_2$ were increased, colloidal aggregates of caprine β_1-casein were formed at a lower temperature than were colloidal aggregates of caprine β_2-casein (Chapter 11.3). These colloidal aggregates, which were only present at temperatures greater than 20-25°C, are due to two independent equilibrium reactions. There is the temperature dependent transition from monomer to polymer, followed by the transition, in the presence of $CaCl_2$, from polymer to colloidal aggregate. Ho and Waugh (1965) found that the primary Ca binding sites in the caseins were the phosphoserine residues. At a constant ionic strength and $CaCl_2$ concentration, it is likely that the transition of the caprine β-caseins from monomer to polymer would not be affected by the consequent

transition from polymer to colloidal aggregate. However, increasing the ionic strength from 0.05 to 0.15 increased the temperature of half transition values for the caprine β-caseins (Table 11.3). This may have been due to a reduction in the Ca activity in the solution, and reduced Ca binding by the protein, until higher temperatures were reached, as well as changes in the monomer to polymer equilibrium.

Solubility of the α_s-Caseins

The caprine, ovine and bovine α_s-caseins are all sensitive to $CaCl_2$ at 37°C. The reason for the increased sensitivity of caprine α_s-casein and bovine α_{s3}-casein is uncertain, since they both contain 7 phosphorylated residues, in contrast to 8 in bovine α_{s1}-casein B (Table 9.4). Dickson and Perkins (1971), Bingham et al. (1972) and Waugh et al. (1971) suggested that binding sites for Ca to the carboxyl groups of aspartic acid and glutamic acid may exist, in conjunction with the primary phosphate binding sites (Ho and Waugh, 1965). Bingham et al. (1972) found 7 moles of Ca were bound per mole of bovine α_{s1}-casein, while the addition of a further 3 moles of Ca resulted in precipitation, although there are only 8 phosphate groups in bovine α_{s1}-casein. Indeed, the marked decrease in the electrophoretic mobilities of bovine α_{s3}-casein and caprine α_s-casein relative to bovine α_{s1}-casein B in the presence of urea and Mg (approximately 5 mM), compared with their mobilities in normal alkaline gel electrophoresis (Fig.9.10) and the solubility of caprine α_s-casein at high Ca concentrations provides indirect evidence that Ca or Mg binding sites other than the phosphate groups exist in these proteins.

The ovine α_{s2}- and α_{s3}-caseins contain 9 phosphates compared to 8 in bovine α_{s1}-casein B (Table 10.2), which may account for the increased sensitivity of the ovine α_s-caseins to $CaCl_2$ (Chapter 11.4). Despite the differences in the Ca sensitivity of the α_s-caseins, they can all be effectively stabilized by caprine, ovine and bovine κ-caseins in the presence of $CaCl_2$, a property which is important to the integrity of casein micelles.

CHAPTER 12: A COMPARISON OF THE PROPERTIES OF CASEINS FROM THE MILK OF SEVERAL SPECIES

Certain chemical and physical characteristics of caprine and ovine κ-, β- and α_s-caseins have been compared with those of their bovine counterparts in Chapters 8, 9, 10 and 11. In this chapter the characteristics of these caseins will be reiterated and compared with those characteristics which are known for caseins from buffalo, rabbit, dog (canine), and human milks.

The milks of the species so far examined appear to contain casein, which precipitates between pH 4 and 5, and which is present in the form of casein micelles. One of the striking differences between the milks from different species is the variation in protein content, and in particular, the levels of casein. For instance, human milk contains 0.5% casein (Alais and Jollès, 1970). Caprine, bovine and ovine milks contain 2.3%, 2.8% and 4.6% casein, respectively (Chapter 4.1), while rabbit milk contains 8.5% casein (Alais and Jollès, 1970). Marked species differences were observed both in the nature and heterogeneity of the caseins, as shown by electrophoresis on paper (Sloan et al., 1961), starch gels (Jenness, 1973) and polyacrylamide gels (O'Connor and Fox, 1973). The relationships between the caseins of milk from any animal cannot be determined solely from electrophoretic patterns, but must await isolation of the proteins and their chemical and physical characterization. Those caseins for which only electrophoresis data is available will not be considered here.

12.1 κ-Caseins

The caseins isolated from ovine, caprine and bovine milk contain a κ-casein fraction which may be readily isolated by column chromatography techniques. These κ-caseins were characterized from their sensitivity to rennin, being hydrolysed at a sensitive peptide bond to yield para-κ-casein (Assenat, 1967), and the soluble macropeptide fraction (Alais and Jollès, 1961; Jollès et al., 1961; Jollès et al., 1964). Fractions corresponding to bovine κ-casein have

been isolated from buffalo casein (Nagasawa et al., 1973), porcine casein (sow) (Woychik and Wondolowski, 1969) and human casein (Malpress and Seid-Akhavan, 1966; Alais and Jollès, 1969; Nagasawa et al., 1970; Groves and Gordon, 1970).

Fractions corresponding to κ-casein have not been isolated from canine casein (dog) (Nagasawa et al., 1972b) and rabbit casein (Testud and Ribadeau Dumas, 1973), although a para-κ-casein-like fraction was identified in rabbit casein. It seems likely however, that all mammalian milks possess a fraction resembling κ-casein, or at least contain a protein capable of fulfilling a function similar to that of κ-casein.

In the species studied in this thesis, κ-casein accounts for 10 to 15% of the total casein. The κ-casein content of bovine casein (15%) is higher than that of caprine (about 10%) and ovine caseins (10%). Quantitative gel electrophoresis indicated buffalo casein contains 11% κ-casein (Nagasawa et al., 1973). No results were available for the other species.

Although Groves and Gordon (1970) have reported a preliminary amino acid composition for human κ-casein, the nature of these caseins is still rather uncertain. Alais and Jollès (1969) have isolated at least two fractions from human casein which were analogous to bovine κ-casein, and Nagasawa et al. (1970) isolated a component, which like bovine κ-casein, yielded a spreading zone when examined by alkaline gel electrophoresis in the absence of 2-mercaptoethanol.

Two genetic variants of κ-casein have been observed in bovine κ-casein (Grosclaude et al., 1973) and in ovine κ-casein (Alais and Jollès, 1967). Caprine casein appears to contain one major κ-casein, with no observed genetic variants (Chapter 8.7). Several different forms of ovine κ-casein appear to be present, differing in their phosphorus and carbohydrate contents (Ribadeau Dumas et al., 1975; Alais and Jollès, 1967). Polymorphism was not observed in buffalo κ-casein by Ganguli (1973), although examination of

a number of buffalo casein samples (70) indicated the presence of two components which they designated as κ-casein variants (κ-Cn^A and κ-Cn^B), the relative concentration of the B variant being considerably higher than that of the A variant. The actual relationship between those two κ-caseins has yet to be determined. It is interesting to note that Aschaffenburg et al. (1968b) observed that in addition to the major buffalo κ-casein, two less mobile and two more mobile components were present. All five components disappeared after treatment with rennin. The possibility that the A variant observed by Ganguli (1973) was a κ-casein fraction containing carbohydrate cannot be discounted.

The κ-casein from the various species have variable levels of carbohydrate associated with the peptide chain. Bovine κ-casein has the highest level of sialic acid (1.89%) (Tripathi and Gehrke, 1970), whereas ovine (Alais and Jollès, 1967), caprine (Zittle and Custer, 1966) and buffalo κ-caseins (Ganguli and Majumder, 1968; Nagasawa et al., 1973) contain 0.3%, 0.3% and 0.6% sialic acid, respectively. The sialic acid content of human casein appears to be extremely variable (Malpress and Hytten, 1964).

The reason for variation in the carbohydrate and in particular sialic acid contents of the various κ-caseins is uncertain. Mackinlay and Wake (1965) found that the carbohydrate residues in κ-casein have no functional role in the stabilization of casein micelles. Mercier et al. (1973) suggested that the carbohydrate residues may have a functional role in protecting those serine and threonine residues in κ-casein which would otherwise be phosphorylated, thus drastically altering the properties of the protein. If this proposal is correct, then the level of carbohydrate in the κ-caseins may simply be related to the level and nature of the deglycosylating enzymes present in the mammary glands of the different species.

Synthesis of milk proteins occurs in the mammary gland on the ribosomes of the endoplasmic reticulum (Farrell et al., 1971). Following synthesis, the proteins migrate

to the vicinity of the Golgi apparatus and it is in the Golgi region, or in the Golgi vacuoles that addition of carbohydrate and phosphorylation of the caseins is believed to occur.

The κ-caseins from caprine, ovine and bovine caseins appear to have molecular weights close to 19 000. For the purpose of comparison, the published amino acid compositions of buffalo (Nagasawa et al., 1973) and human κ-caseins (Groves and Gordon, 1970) were recalculated, assuming a molecular weight of about 19 000. The amino acid compositions of the κ-caseins from bovine (Mercier et al., 1973), caprine (Table 9.1), ovine (Ribadeau Dumas et al., 1975), buffalo (Nagasawa et al., 1973), and human caseins (Groves and Gordon, 1970) are compared in Table 12.1. Included are the molecular weights (calculated from the amino acid composition) and the Bigelow hydrophobicity values (Bigelow, 1967).

The content of serine, glutamic acid, proline, glycine, valine, tyrosine, phenylalanine, histidine, lysine and tryptophan residues is almost invariant with the different species (Table 12.1). However, the content of aspartic acid, cysteine, isoleucine and leucine residues varies quite considerably. In particular, human κ-casein has considerably more leucine and methionine and considerably less alanine and threonine than the κ-caseins from other species (Table 12.1). The average hydrophobicities of the κ-caseins are fairly similar, ranging from 4.72 to 5.12 kJ/residue. These values are typical of fairly hydrophobic proteins, and are intermediate between values for the α_s- and β-caseins. The amino acid composition of human κ-casein is considerably different from those of the ruminants κ-caseins, as might be expected.

Fiat et al. (1970) isolated a tryptic peptide from ovine κ-casein which contained the rennin sensitive region, and found that it had an identical sequence to that of residues 99 to 111 from bovine κ-casein B (Fig.6.2) (Mercier et al., 1973). The rennin sensitive peptide bond in bovine κ-casein is located between Phe_{105}-Met_{106}. Furthermore, Jollès et al. (1973) determined the N-terminal sequence of

Table 12.1

Amino Acid Compositions of κ-Caseins from Different Species
(Amino acid residues/mole of protein)

	Bovine κ-Casein B Mercier et al. (1973)	Caprine κ-Casein Table 9.1	Buffalo[2] κ-Casein Nagasawa et al. (1973)	Ovine κ-Casein Ribadeau Dumas et al. (1975)	Human[1,2] κ-Casein Groves and Gordon (1970)
Asp	11	15	12	16	15
Thr	14	13	16	12	8
Ser	13	12	12	12	11
Glu	27	27	28	26-27	29
Pro	20	20	20	19-20	21
Gly	2	2	2	2	3
Ala	15	14	15	18-19	10
Cys	2	2	1	3	1
Val	11	11	13	12	11
Met	2	1	2	2	5
Ile	13	10	12	9	9
Leu	8	8	9	7	14
Tyr	9	7	8	8	8
Phe	4	4	4	4	4
His	3	4	3	4	4
Lys	9	8	8	8	8
NH_3	21	-	-	-	-
Arg	5	4	4	6	8
Trp	1	1	1	1	-
No. of phosphorus residues	1	1	1	2	-
No. of residues	169	163	170	169-172	169
Molecular weight (calculated)	19023	18300	19000	19050	19300
Average hydrophobicity kJ/residue (Bigelow, 1967)	5.12	4.82	5.04	4.72	5.04

1. Composition based on a 24 h analysis, therefore values for isoleucine and valine may be low.
2. Data recalculated, assuming a molecular weight of about 19 000.

the first 40 residues in the macropeptide from ovine κ-casein (Fig.6.8). The sequence was similar to that of the macropeptide from bovine casein, with 7 amino acid substitutions and a probable insertion of two residues in the ovine macropeptide. It is interesting to note that 6 of the 7 substitutions are favourable changes, i.e. point mutations, and the unfavourable substitution from Ile_{31} in the bovine macropeptide to Pro_{33} in the ovine macropeptide has been previously observed (Dayhoff, 1972). Recently, the sequence of para-κ-casein from the A variant of ovine κ-casein was determined by Jollès et al. (1974) (Fig.6.9). It has 105 residues and 13 amino acid substitutions when compared with bovine para-κ-casein. These substitutions are all favourable point mutations (Dayhoff, 1972). The most notable feature is the substitution from Arg_{10} in bovine para-κ-casein to Cys_{10} in ovine para-κ-casein, in a position adjacent to one of the other cysteine residues (Fig.6.9). With these results and observations in mind, it is therefore hardly surprising that the κ-caseins from the various species are capable of stabilizing their Ca sensitive casein fractions, analogous to bovine κ-casein. Caprine κ-casein can stabilize caprine, ovine and bovine α_s- and β-caseins (Chapter 11.5) while human κ-casein can stabilize bovine α_{s1}-casein and the human and bovine β-casein fractions in the presence of Ca (Nagasawa et al., 1970).

It seems likely that the κ-caseins, particularly those from caprine, ovine and bovine caseins are all closely related, and probably have a common ancestral protein. This can only be determined after the κ-caseins from the species have been isolated, and their primary structures determined.

12.2 β-Caseins

A major casein fraction having an electrophoretic mobility in alkaline gel or paper electrophoresis similar to that of bovine β-casein has been observed in all of the mammalian milks so far examined (Sloan et al., 1961; O'Connor and Fox, 1973; Jenness, 1973). Some of these casein fractions, namely those from bovine (Ribadeau Dumas et al., 1973), caprine (Chapter 9), ovine (Chapter 10; Resmini et al.,

1967), buffalo (Nagasawa et al., 1973), rabbit (Testud and Ribadeau Dumas, 1973), canine (Nagasawa et al., 1972b) and human caseins (Nagasawa et al., 1970; Groves and Gordon, 1970) have been examined in more detail.

In bovine casein, the proportion of β-casein is 30-35%, of the total protein, whereas in caprine and ovine caseins, β-casein accounts for 60% (Table 8.2) and 45% respectively of the total protein (Table 8.3). Nagasawa et al. (1973) found 43% β-casein in buffalo casein, while human casein appears to consist largely of β-casein fractions (Nagasawa et al., 1970). Testud and Ribadeau Dumas (1973) have isolated and characterized aβ-casein fraction from rabbit casein.

Bovine β-casein is subject to considerable genetic variation. These closely related genetic variants are the result of amino acid substitutions, due to single nucleotide base changes in the triplet codons of the messenger RNA coding for each amino acid (Grosclaude et al., 1973). Similar detailed studies have not been made on the β-caseins from animals of the other species.

In this study, two major β-caseins have been isolated and characterized in caprine and in ovine caseins. All four β-caseins had similar molecular weights, amino acid compositions, and the different mobilities of the β_1- and β_2-caseins in alkaline gel electrophoresis appeared to be due to differences in their phosphorus content (Chapters 9.7 and 10.4). In caprine β-caseins, the more mobile component in alkaline gel electrophoresis had six phosphorylated residues compared with five in the slower component. The ovine β-caseins contained five and four phosphorylated residues respectively for β_1- and β_2-caseins. The β-caseins in caprine casein do not appear to be subject to genetic variation to any extent, since only two components have been observed by gel electrophoresis, both in this study and by other workers (Zittle and Custer, 1966; Macha, 1970; Ribadeau Dumas et al., 1975).

The situation is not as simple with the ovine β-caseins, and although alkaline gel electrophoresis indicated few

differences between caseins from the same breed of sheep, there was significant variation between the caseins from different breeds of sheep (Chapter 8.8). This was due to changes in the relative intensities of the β_1- and β_2-caseir bands in ovine caseins, and in two samples of casein, the observation of an extra band with a greater electrophoretic mobility at alkaline pH than β_1-casein. This band, designated as β_0-casein, was similar to that observed by King (1966), which he suggested may be due to a simple alternative allele.

In human β-casein there are six β-casein components, designated A, B, C, D, E and F, in order of their decreasing electrophoretic mobilities in alkaline gels (Nagasawa et al., 1970). These β-caseins appear to be the same protein phosphorylated to different levels (Groves and Gordon, 1970; Nagasawa et al., 1971), for example, β-casein A has five phosphate residues while β-casein F has none. Voglino and Ponzone (1972) examined 80 human casein samples and found that the dominant A and D components of β-casein were variable in intensity. They suggested that the synthesis of these β-caseins is under the control of a single locus with two co-dominant alleles. Aschaffenburg et al. (1968b) observed polymorphism in buffalo β-casein in 3 out of a total of 105 casein samples.

The β-caseins from buffalo (Nagasawa et al., 1973) caprine and ovine (this thesis), human (Nagasawa et al., 1970) and rabbit caseins (Testud and Ribadeau Dumas, 1973) are all soluble in the presence of $CaCl_2$ at low temperature, yet are easily precipitated at 30-37°C. This is indicative of a temperature dependent polymerization of β-casein, since only the polymers appear to be sensitive to Ca. Measurements of intrinsic viscosity, analytical ultracentrifugation or the effect of temperature on the turbidity of calcium containing β-casein solutions indicated caprine β-caseins and probably ovine β-caseins undergo temperature dependent polymerization from monomer to polymer as the temperature was increased from 4°C to 40°C (Chapter 11). The β_1-caseins of caprine and ovine caseins, and human β-casein A and B (i.e. those

containing more phosphorus) are more sensitive to $CaCl_2$ than the equivalent β-caseins with less phosphorus (caprine and ovine β_2-caseins, human β-caseins C to F). The human β-caseins also undergo temperature dependent polymerization, as determined by ultracentrifugation, optical rotatory dispersion and viscosity measurements (Toyoda and Yamauchi, 1972). The temperature at which polymer formation becomes evident in these β-caseins, compared with bovine β-casein appears to be different, and is likely to be related to the differences in the primary structures of the human and bovine β-caseins.

The amino acid compositions of the β-caseins from the various species are presented in Table 12.2. For the purpose of comparison, the amino acid compositions of buffalo and canine β-caseins were recalculated assuming a molecular weight for these proteins of 24 000, since no molecular weight data was available.

It is evident from Table 12.2 that these β-caseins have several features in common, namely, high levels of glutamic acid and proline. The sum of lysine, histidine and arginine in caprine, ovine, bovine and buffalo caseins is constant at 20, with values of 19 for human β-casein, 22 for canine β-casein and 23 for rabbit β-casein. Furthermore, the sum of the hydrophobic residues, isoleucine, leucine and valine is 50 for caprine β_2-casein, 51 for bovine β-casein, 52 for both ovine and buffalo β-caseins, and 58 for human, canine and rabbit β-caseins. The increased content of these amino acids is reflected in the increased hydrophobicity of the human, canine and rabbit β-caseins compared with those from the ruminants. These observations, along with the other differences in the amino acid compositions, particularly the aspartic acid, serine, glycine, alanine, valine, methionine, isoleucine, tyrosine and arginine residues supports the suggestion that the caseins from the closely related ruminant species, including the cow, goat, sheep and buffalo, have very similar β-caseins, while the other species have different β-caseins. However, the average hydrophobicities of these caseins are comparable and typical of very hydrophobic proteins (Bigelow, 1967).

Table 12.2

Amino Acid Compositions of β-Caseins from Different Species

(Amino acid residues/mole of protein)

	Bovine β-Casein A[2] Mercier et al. (1972a)	Caprine β_2-Casein Table 9.3	Ovine β_2-Casein Table 10.1	Buffalo β-Casein Nagasawa et al. (1973)	Human β-Casein Groves and Gordon (1970)	Canine β-Casein Nagasawa et al. (1972b)	Rabbit β-Casein Testud and Ribadeau Dumas (1973)
Asp	9	9	8	9	11	13	11
Thr	9	12	11	8	6	6	10
Ser	16	15	14	17	9	11	10
Glu	39	43	39	38	39	38	39
Pro	35	33	37	37	39	32	27-28
Gly	5	6	5	6	3	-	4
Ala	5	5	5	5	7	9	8
Cys	-	-	-	-	-	-	-
Val	19	21	22	18	19	15	14
Met	6	6	5	6	3	6	3
Ile	10	9	9	10	13	12	13
Leu	22	20	21	24	26	32	31
Tyr	4	4	3	4	7	3	3
Phe	9	9	9	9	5	8	11
His	5	5	5	5	5	8	3
Lys	11	12	12	12	11	9	14
NH_3	27	-	-	-	-	-	-
Arg	4	3	3	3	3	5	6
Trp	1	1	1	1	1	-	-
No. of phosphorus residues	5	5	4	5	0-5	4	6
No. of residues	209	213	209	212	210	207	207-208
Molecular weight	23982	24420	24360	24000	23712-24108	24000	23850
Bigelow hydrophobicity kJ/residue	5.58	5.33	5.63	5.67	6.03	5.78	5.75

The human β-caseins appear to be a group of proteins with the same amino acid compositions, which differ only in their phosphorus content (Groves and Gordon, 1970). The possibility that the ovine and caprine β_1- and β_2-caseins, which also differ in their phosphorus content (Table 12.2), are the result of multiple phosphorylation in a similar manner to that of the human caseins cannot be discounted. In caprine and ovine milks, caseins other than the β_1- and β_2-caseins were soluble at low temperature (4°C) (O'Connor and Fox, 1973) (Chapter 5.1). O'Connor and Fox (1973) designated these components as "minor β-caseins", since they were soluble from 4°C to 37°C, and were not readily hydrolysed by rennin.

Gel electrophoresis of these caseins, before and after rennet treatment, and in the presence of Mg (this study) could neither discount nor confirm the premise that these caseins are "minor β-caseins". Consequently, their relationship to the caprine and ovine β-caseins must be determined after their isolation and characterization.

12.3 α_s-Caseins

Fractions analogous in their properties to bovine α_{s1}-casein have been isolated from caprine and ovine caseins (this study), buffalo (Nagasawa et al., 1973), rabbit (Testud and Ribadeau Dumas 1973), and canine caseins (Nagasawa et al., 1972b). Although Malpress and Seid-Akhavan (1966) isolated a fraction from human casein which they designated as α_s-casein, Nagasawa et al. (1970) later showed this to be a β-casein fraction.

The proportion of α_s-casein in the caseins differ widely between the various species. Bovine, ovine and caprine α_s-caseins are 50%, 35% and 23%, respectively of the total casein. In buffalo casein, 46% of the casein was α_s-casein, as shown by quantitative gel electrophoresis (Nagasawa et al., 1973). It appears that no major α_s-casein fraction exists in human milk (Nagasawa et al., 1970), although Ponzone and Voglino (1972) have observed polymorphic protein bands in human casein, which they designated as α_{s1}-

caseins, based on their electrophoretic mobilities. The identification of these protein bands as α_s-caseins has yet to be verified. The relationship between the A, B, C and D genetic variants of bovine α_{s1}-casein has been previously discussed (Chapter 6). Polymorphism has not yet been observed in the minor α_{s0}-, α_{s2}-, α_{s3}-, α_{s4}- and α_{s5}-caseins from the caseins of Western cattle (Bos taurus) or Zebu cattle (Bos indicus) (Aschaffenburg, 1968a; Hoagland et al., 1971). No genetic polymorphism has been observed in buffalo α_s-casein (Aschaffenburg et al., 1968b). Polymorphism of the type observed in bovine α_{s1}-casein appears to be a rare occurrence in the α_s-caseins of caprine and ovine milks (Chapter 8). Certainly, this is the case for ovine casein (King, 1966; Arave et al., 1973).

The α_s-caseins from bovine, caprine and ovine caseins were all precipitated by low levels of $CaCl_2$ (Chapter 11.4), yet were all stabilized by any of the κ-caseins from the three species (Chapter 11.5). Woychik and Wondolowski (1969) have shown porcine α_s-casein can be stabilized by porcine or bovine κ-caseins. Little work has been done on the α_s-caseins from other species, apart from canine (Nagasawa et al., 1972b) and rabbit α_s-caseins (Testud and Ribadeau Dumas, 1973) where they have been shown to be insoluble in low levels of $CaCl_2$, at 37°C.

The amino acid compositions of the α_s-caseins are presented in Table 12.3. For the purposes of comparison, a molecular weight of 24 000, including the phosphate residues, was assumed for buffalo and canine α_s-caseins since no molecular weight data were available.

There appears to be few similarities between the amino acid compositions of the α_s-caseins of the various species. The amino acid compositions of bovine α_{s1}-casein, buffalo α_s-casein and ovine α_{s2}- and α_{s3}-caseins appear to be basically similar, with average hydrophobicities (Table 12.3) that are typical of hydrophobic proteins. Rabbit α_s-casein resembled bovine α_{s3}-casein and caprine α_s-casein in its threonine and glycine contents. It does not, however, contain cysteine or have a high lysine content, unlike the

Table 12.3

Amino Acid Compositions of α_s-Caseins from Different Species
(Residues amino acid/mole of protein)

	Bovine α_{s1}-Casein B	Bovine α_{s1}-Casein	Buffalo α_{s1}-Casein	Caprine α_s-Casein	Ovine α_{s3}-Casein	Ovine α_{s2}-Casein	Rabbit α_s-Casein	Canine α_s-Casein
	Mercier et al. (1972a)	Table 9.4	Nagasawa et al. (1973)	Table 9.4	Table 10.3	Table 10.3	Testud & Ribadeau Dumas (1973)	Nagasawa et al. (1972b)
Asp	15	19	17	17	17	17	15	16
Thr	5	14	7	14	5	4	14	5
Ser	16	16	14	14	18	17	13	9
Glu	39	45	39	45	42	39	63	45
Pro	17	13	18	18	22	22	18	22
Gly	9	4	11	4	11	9	3	2
Ala	9	9	10	10	12	12	11	13
Cys	-	2	-	2	-	-	-	-
Val	11	14	12	12	11	10	19	10
Met	5	4	4	4	4	5	3	2
Ile	11	11	12	11	10	11	8	10
Leu	17	14	18	12	18	18	30	25
Tyr	10	11	10	11	10	10	13	7
Phe	8	6	8	8	6	7	7	6
His	5	4	4	5	4	4	3	4
Lys	14	23	15	22	13	14	15	4
NH_3	22	-	-	-	-	-	-	-
Arg	6	6	5	6	6	6	17	17
Trp	2	2	3	2	2	2	-	2
No. of phosphorus residues	8	7	7	7	9	9	12	7
No. residues	199	217	207	217	211	207	252	199
Bigelow hydrophobicity kJ/residue	4.99	4.56	4.99	4.70	4.71	4.97	4.72	5.03
Molecular weight	23615	25940	24300	26010	24623	24353	36800	24000

α_s-caseins from the other species (Table 12.3). The similarity between caprine α_s-casein and the minor bovine α_{s3}-casein, and between bovine α_{s1}-casein, buffalo α_s-casein and the ovine α_s-caseins, suggests that caprine milk is the only milk of these four species that does not contain a "bovine α_{s1}-casein" type of protein. It would be interesting to compare the amino acid sequences of bovine α_{s3}- and α_{s1}-caseins to determine whether there is a close genetic relationship between them.

12.4 Conclusions

The biological function of milk is to supply the young suckling mammal with the nutrients it requires for rapid growth and development. The casein micelles and the whey proteins are an excellent source of protein, phosphorus and calcium. In addition, α-lactalbumin, the B protein of lactose synthetase, is involved in the biosynthesis of lactose (Brew, 1970). The caseins from the milks of the other species that have been isolated all contained a considerable amount of phosphorus. Some of them, particularly the β-caseins, appear to have "loose structures" typical of bovine caseins, thereby allowing easy access for the proteolytic enzymes of the digestive tract.

Although only a limited number of κ-caseins have been examined, they appear to be similar in amino acid composition and in their properties. They have an important role in micelle stabilization, which may have limited the number of successful mutations and therefore changes in protein structure which these caseins have undergone.

The β-caseins are another similar group of proteins, with minor differences in their properties. All of the β-caseins so far examined are sensitive to $CaCl_2$ at 37°C, and are soluble in 20 mM $CaCl_2$ at low temperatures. Genetic variation in the β-caseins of species other than bovine appears to be a rare event.

The α_s-caseins, comprising a large number of major and minor caseins, appear to be the most heterogeneous group of caseins, although genetic variation other than for bovine

α_{s1}-caseins also appears to be a fairly rare event. The results in Tables 12.1, 12.2 and 12.3 suggest that in terms of protein evolution, the ruminant caseins form a close group, with the human caseins in particular being much more distantly related. The relationships are not dissimilar from the phylogenetic tree proposed by Dayhoff (1972) from the amino acid sequences of cytochrome C and the α and β chains of haemoglobin.

The ability of the mammary gland to secrete milk has been traced some hundred thousand years to the age of the Therian and monotreme mammals (Sloan et al., 1961). It seems likely that the milks of primitive mammals contained a single protein which was soluble in the presence of Ca, and which could be coagulated by rennin or a similar proteolytic enzyme. The characteristics of such an ancestral protein, and the κ-, β- and α_s-caseins from the caseins of the present-day studies, can only be inferred from the characterization of these latter proteins and a knowledge of their primary structures.

PART III

MODELS OF BOVINE CASEIN MICELLE STRUCTURE AND THEIR RELATIONSHIP TO CAPRINE AND OVINE CASEIN MICELLES.

CHAPTER 13: DISCUSSION

Several models have been proposed for the structure of the bovine casein micelle (Waugh and Noble, 1965; Payens, 1966; Morr, 1967a; Rose, 1969; Parry and Carroll, 1969; Garnier and Ribadeau Dumas, 1970; Waugh et al., 1970; Waugh, 1971; Fox and Guiney, 1973; Slattery and Evard, 1973).

Before considering some of the various models of casein micelle structure, and their relevance to caprine and ovine casein micelles, it is as well to consider some of the characteristics of bovine casein micelles.

There are a number of recent reviews on the models of casein micelle structure that have been proposed (Rose, 1969; Waugh, 1971; Lyster, 1972; Swaisgood, 1973; Thompson and Farrell, 1973; Garnier, 1973).

13.1 Hydrophobic, Electrostatic and Hydrogen Bonding

Hydrophobic, electrostatic and hydrogen bonding are the forces largely responsible for the maintenance of protein structure. The nature of these interactions has been discussed by Mahler and Cordes (1967).

The driving force for formation of hydrophobic bonds is largely the positive entropy change, resulting from the liberation of solvent molecules organized about the apolar (hydrophobic) residues, that accompanies the transfer of these residues from an aqueous to a nonaqueous environment. Consequently, a more stable system may result if hydrophobic residues are in the interior of the molecule, and not in contact with the water. Hydrophobic interactions are highly temperature dependent, being minimal below $5^{\circ}C$ and maximal at higher temperatures. Klotz (1970) pointed out that for proteins whose crystallographic structure is known, many apolar side chains do exist, fully or partially exposed to the solvent, and therefore these proteins have regions which are available for interaction with other proteins.

The primary structures of κ-, β- and α_{s1}-caseins (Figs.6.2, 6.3 and 6.4) have a number of clusters of hydrophobic residues, and rank among the most hydrophobic of the proteins tabulated by Bigelow (1967). Dissociation of the β- and κ-caseins, and to some extent the α_{s1}-casein from the bovine casein micelle at low temperatures, can be explained in terms of decreased hydrophobic bonding, consistent with the known properties of β-casein. The suggestion by Thompson and Farrell (1973) that the casein micelle may be partially stabilized by hydrophobic interactions seems likely.

In essence, the ionic side chains in the proteins, whose crystallographic structure is known, are exposed to the solvent (Klotz, 1970). The role of inter- and intramolecular ionic bonds among α_{s1}-, β- and κ-caseins in the stabilization of micelle structure is difficult to assess, although they may be important in the stabilization of α_{s1}-casein with κ-casein (Pepper et al., 1970; Hill, 1970).

The role of phophoserine residues in calcium binding has been demonstrated by Ho and Waugh (1965), Dickson and Perkins (1971) and by Bingham et al. (1972). Although dephosphorylated α_{s1}-casein was still precipitated with calcium, there was decreased stabilization with κ-casein, and electron microscopy revealed larger, but fewer micelle-type structures were present (Bingham et al., 1972). Hence, the formation of micelle-type structures is not entirely dependent on the formation of calcium phosphate bonds between the caseins (Thompson and Farrell, 1973).

Many globular proteins are stabilized by a high degree of α-helical, or β-pleated sheet structure, through hydrogen bonds (Mahler and Cordes, 1967). The caseins appear to possess little of these structures, although the possibility of other types of secondary structure being present cannot be overlooked (Herskovits, 1966; Garnier, 1966; Evans et al., 1971a; Irons et al., 1973).

13.2 Role of Disulphide Bonds

Disulphide bonds between cysteine residues serve to stabilize the peptide chains in a preformed conformation (Blow and Steitz, 1970). Woychik et al. (1966) demonstrated that reduced and alkylated κ-casein stabilized α_{s1}-casein against calcium precipitation as well as native κ-casein. Toma and Nakai (1973) found that the disulphide linked dimer, α_{s5}-casein was not as readily stabilized by κ-casein, as was α_{s1}-casein.

Thompson and Farrell (1973) suggested that while the disulphide bridges of the casein may contribute to the stability of the micelles, they are not the central feature of micelle formation.

13.3 Colloidal Calcium Phosphate

The total calcium content of bovine milk is about 30 mM (Table 4.2), while the Ca ion activity quotient is 1.63 mM. The importance of colloidal calcium phosphate in micelle structure is well documented (McGann and Pyne, 1960; Pyne and McGann, 1960; Downey and Murphy, 1970; Morr et al., 1971). Colloidal calcium phosphate appears to resemble tricalcium phosphate with a calcium:phosphate ratio of 1:5 (White and Davies, 1958), although this value is somewhat uncertain (See Thompson and Farrell, 1973).

Pyne and McGann (1960) prepared CPF-milk (colloidal phosphate free milk) by lowering the pH of a sample of skim milk to 4.9 and dialysing it at 5°C against the original milk. CPF-milk is translucent compared with normal milk, and has a greatly increased viscosity. Whereas CPF-milks are precipitated with 25 mM $CaCl_2$, addition of $CaCl_2$ up to 1 M at 30°C, has little effect on normal milk. Jenness et al. (1966) and Lin et al. (1972) found a marked increase in serum or nonmicellar casein as the colloidal calcium phosphate contents of milks or centrifugally fractionated micelles were reduced by the addition of EDTA. Using gel chromatography Downey and Murphy (1970) found that the major peak in CPF-milk was eluted at a volume consistent with a molecular weight of 2×10^6, whereas normal milk micelles had

molecular weights in excess of 10^8. Morr et al. (1971) showed by electron microscopy that sub-micellar casein complexes in CPF-milk were heterogeneous and irregularly shaped, and ranged in size from 30-50 nm. Removal of colloidal calcium phosphate and protein bound Ca from bovine casein micelles, by chromatography on Sepharose 4B, resulted in dissociation of the casein micelles to sub-micellar casein aggregates which are thought to be in equilibrium with their component caseins (Creamer and Berry, 1975). The importance of calcium phosphate in the stabilization of micelles appears evident, but the mechanism of stabilization is unknown.

Proposed Models for Bovine Casein Micelle Structure

Models of micelle structure can be classified into three groups, the coat-core models, internal structure models, and sub-unit models.

13.4 Coat-Core Models

The model proposed by Waugh and Noble (1965) and Waugh et al. (1970) and reviewed by Waugh (1971) is primarily based on studies of the calcium solubilities of the caseins. The model describes the formation of low weight ratio complexes of α_{s1}- and β-casein in the absence of calcium. Upon addition of calcium ions, the α_{s1}- or β-caseins begin to aggregate to a limiting size (the caseinate core). In the presence of unit or low weight ratio α_{s1}:κ-complexes, precipitation of the α_{s1}-casein is prevented by the formation of a monolayer of these low weight ratio α_{s1}:κ-complexes which envelopes the core aggregates.

Parry and Carroll (1969) were unsuccessful in locating this outer layer of κ-casein using ferritin labelled antibodies and electron microscopy, and concluded that κ-casein may act as a point of nucleation, about which the calcium insoluble caseins might cluster and be stabilized by calcium phosphate. However, Ashoor et al. (1971) demonstrated that papain, which had been crosslinked by glutaraldehyde into a large insoluble polymer, caused proteolysis of all three major components of casein. This would rule out any preferential localization of κ-casein.

13.5 Internal Structure Models

The second class of models are based on the known properties of the isolated casein components, which in turn cause or direct the formation of the internal structure of the casein micelles.

Garnier and Ribadeau Dumas (1970) proposed a model which places considerable emphasis on κ-casein. Trimers of κ-casein were linked to three chains of α_{s1}- and β-caseins which radiated from the κ-casein node (Y-like structure). These chains of α_{s1}- and β-caseins may be connected with other κ-nodes to form a loosely packed network. This model, based on experiments with carboxypeptidase A (molec. wt 34 500) and myoglobin (molec. wt 17 000), favours an open porous structure for casein micelles (Ribadeau Dumas and Garnier, 1970).

However, this model imposes considerable restriction on κ-casein, and aggregates of κ-casein larger than trimer have been shown to exist (Talbot and Waugh, 1970). The model assigns no definite role to casein interaction and ignores colloidal calcium phosphate, known to be involved in the stabilization of casein micelles.

Rose (1969) used the known endothermic polymerization of β-casein as the basis of his micelle model. In this model, β-casein monomers self associate into chain-like polymers to which α_{s1}-casein monomers are attached, and κ-casein interacts with the α_{s1}-casein monomers. As the micelle forms, colloidal calcium phosphate is incorporated into the network as a stabilizing agent.

13.6 Sub-unit Models

Shimmin and Hill (1964) proposed a sub-unit model based on electron microscopy and predicted a diameter of 10 nm for the sub-units of the casein micelle.

Based on studies of the disruption of casein micelles with oxalate and urea, Morr (1967a) postulated that casein micelles are composed of numerous, "loosely" packed, calcium-

caseinate complex units, joined in association by a combination of hydrophobic bonding, calcium and colloidal calcium phosphate linkages, between casein phosphoserine and carboxyl groups. Morr (1967a) suggested each of these sub-units is composed of an inner core of α_s- and β-caseins, surrounded by an outer layer of predominantly α_{s1}- and κ-caseins, according to the suggestion of Waugh and Noble (1965) and Waugh et al. (1970).

Schmidt and Buchheim (1970) found dialysis or compression of bovine casein micelles caused them to dissociate into sub-units with a diameter of 10-15 nm, as shown from electron micrographs. They suggested that bovine casein micelles were composed of sub-units which were linked by Ca bridges between the phosphate and carboxyl groups to form casein micelles.

Slattery and Evard (1973) have recently proposed a sub-unit model based on their sedimentation and viscosity measurements of mixtures of κ-, α_{s1}-, and β-caseins. They proposed that sub-units, containing 25-30 monomers each, and with a diameter of about 20 nm, are formed in all systems. These polymers are micelle sub-units in which the nonpolar portion of each monomer is orientated radially inwards, while the charged acidic peptides of the Ca sensitive caseins and the hydrophilic, carbohydrate-containing portion of κ-casein are near the surface. Asymmetric distribution of κ-casein in a micelle sub-unit results in hydrophilic and hydrophobic areas on the sub-unit surface. Aggregation through hydrophobic interactions forms a porous micelle, growth of which is limited by the eventual concentration, at the micelle surface, of sub-units rich in κ-casein. The model of Slattery and Evard (1973) accounts for the destabilization of the micelle by dephosphorylation of Ca sensitive caseins, or by high concentrations of NaCl or $CaCl_2$ and for the events associated with clotting by rennin action.

Since the recent electron micrographic studies (Schmidt and Buchheim, 1970; Knoop et al., 1973; Schmidt et al., 1973; Richardson et al., 1974) have shown bovine casein micelles to

be composed of sub-units, only those models consistent with the sub-unit concept will be considered. This discounts the early Waugh model (Waugh and Noble, 1965) and the models of Payens (1966), Rose (1969) and Garnier and Ribadeau Dumas (1970).

13.7 Caprine and Ovine Casein Micelles and their Relationship to the Models of Bovine Casein Micelle Structure

Caprine, ovine and bovine milks have most of their casein present in the form of casein micelles which also contain Ca, Mg and phosphorus.

Although the mineral levels in the caprine, ovine and bovine milks varied (Table 4.2), there were no significant differences in the cation activities, or the Ca:phosphate ratios in either the casein pellets (sedimented by ultracentrifugation) or the sera (Table 4.4). The apparent differences in the Ca:phosphates ratios of the milks (Table 4.4) were an indication of the differing quantities of casein, as the micelle pellets had Ca:phosphate ratios of about 2.0 and the sera had ratios of about 1.2 (Table 4.4).

Bovine, caprine and ovine casein micelles are highly solvated, with about 2.0 g water per g protein, irrespective of whether micelle solvations were determined by viscometry or ultracentrifugation (Table 5.4). The voluminosities (specific volumes) of caprine, ovine and bovine casein micelles obtained from viscosity measurements (Chapter 5.2), were similar at about 2.8 ml/g and did not change significantly as the temperature was reduced from 30°C to 4°C, despite the 12% decrease in the level of micellar casein. Ultracentrifugation of caprine, ovine and bovine milks at low temperature showed that the caseins which dissociated from the casein micelles were predominantly β-caseins.

These results are consistent with the concept of caprine, ovine and bovine casein micelles as being highly solvated, porous structures which have similar specific volumes, and which readily allow caseins, or sub-units to dissociate at low temperatures.

A porous sponge-like structure was proposed to account for the structure of bovine casein micelles by Ribadeau Dumas and Garnier (1970) who found that carboxypeptidase A (molec. wt 35 700) and myoglobin (molec. wt 17 200) were able to penetrate to the interior of the micelle. Furthermore, Lin et al. (1972) found that lowering the Ca ion activity of fractionated bovine casein micelles resulted in the release of soluble caseins from the micelles, without altering their hydrodynamic radii, as measured by inelastic light scattering, until the Ca activity reached a critical level when the micelles abruptly dissociated. They suggested that the removal of Ca initially caused dissociation of weakly bound caseins from the micelle, while a size-determining micellar framework remained intact.

Electron micrographs of caprine, ovine and bovine casein micelles indicated they were roughly spherical in shape with diameters ranging from about 35 nm to 350 nm (Chapter 4.4). Gel electrophoresis of fractions containing small and large caprine and ovine micelles revealed that the small casein micelles contained more κ-casein than the large micelles, similar to results obtained with fraction of large and small bovine casein micelles (Rose et al., 1969; Waugh, 1971; Creamer et al., 1973).

Electron micrographs of freeze-etch replicas showed that caprine, ovine and bovine casein micelles are composed of sub-units, about 12 nm in diameter (Chapter 4.4). Sub-unit structure of casein micelles was previously shown from electron micrographs of bovine casein micelles (Shimmin and Hill, 1964; Shimmin and Hill, 1965; Calapaj, 1968; Schmidt and Buchheim, 1970; Knoop et al., 1973; Schmidt et al., 1973), electron micrographs of lactating rat mammary glands (Carroll et al., 1971; Buchheim and Welsch, 1973) and electron micrographs of human casein micelles (Calapaj, 1968). Electron micrographs have shown that the casein micelles of all the species so far examined are composed of sub-units which vary in size from 10-20 nm.

Removal of colloidal calcium phosphate from caprine and ovine casein micelles caused dissociation to sub-micellar

casein aggregates, similar to those obtained with bovine casein micelles by Creamer and Berry (1975). The casein aggregates from caprine, ovine and bovine micelles were eluted from a column of Sepharose 4B at 37°C at the same position, suggesting that the stable casein aggregates were similar in hydrodynamic size (Chapter 5.3). Dissociation of casein micelles to casein aggregates by gel chromatography further indicates the importance of calcium phosphate in maintaining micelle structure. It should be noted that under these conditions most of the protein bound Ca is also removed from the casein aggregates.

Gel chromatography and ultracentrifugation of purified bovine caseins showed that at 30-37°C stable casein aggregates with a diameter of 10-20 nm were formed (Waugh et al., 1970; Pepper, 1972; Slattery and Evard, 1973; Creamer and Berry, 1975). This lends support to the sub-unit concept of micelle structure.

Quantitative column chromatography and gel electrophoresis of whole caprine, ovine and bovine caseins showed that there were considerable differences between them with respect to their content of α_s- and β-caseins, and net charge on the caseins. Ovine milk has nearly twice the concentration of casein (4.6 g per 100 ml milk) as caprine or bovine milks (2.3 and 2.8 g per 100 ml milk, respectively). The caseins differ quite markedly in their composition, since bovine casein contains 15% κ-, 30% β- and 50% α_s-casein, compared with 10% κ-, 60% β- and 25% α_s-casein in caprine casein and 10% κ-, 45% β- and 35% α_s-casein in ovine casein. The major α_s-casein in caprine casein was shown to be similar in its properties to the minor bovine α_{s3}-casein (Chapter 9), which as yet, has not been assigned an important role in bovine casein micelle structure. Two of the ovine α_s-caseins that have been investigated, α_{s2}- and α_{s3}-caseins, were shown to be similar to each other, and to bovine α_{s1}-casein (Chapter 10).

The proportion of β-caseins in the caprine, ovine and bovine caseins varies considerably. The β-caseins have similar chemical properties (Chapters 9 and 10), although they

differ in their temperature dependent assocation-dissociation behaviour (Chapter 11). The caprine, ovine and bovine κ-caseins are also similar in their chemical properties, in particular, their ability to stabilize the α_s- and β-caseins in the presence of $CaCl_2$.

Despite the differences between the individual caseins from caprine, ovine and bovine milks, they all associate to form sub-units with a similar size, as shown by electron micrographs, and form casein aggregates which have a similar size, as shown by gel chromatography. Thus, the differences between the caprine, ovine and bovine caseins must be self compensating.

Gel chromatography of sub-micellar casein aggregates (Chapter 5.3) showed the κ-caseins of caprine, ovine and bovine milks were eluted at the leading edge of the casein aggregate peak, whereas α_s- and β-casein were evenly distributed across the peak. As a result of their studies on bovine casein aggregates, Creamer and Berry (1975) suggested that α_{s1}- and κ-casein polymers were larger than α_{s1}- and β-casein polymers, consistent with the ultracentrifugation results obtained for bovine α_{s1}-, β- and κ-casein mixtures by Slattery and Evard (1973). In light of the results obtained for caprine and ovine casein aggregates, it would appear that their κ- and α_s-casein polymers may also be larger than α_s- and β-casein polymers and that the casein aggregates are probably in rapid equilibrium with their constituent caseins; a situation similar to that proposed for bovine casein aggregates (Creamer and Berry, 1975).

The temperature dependent effects observed for the caseins in caprine, ovine and bovine milks are unlikely to be very significant in casein micelle structure since milk, in fulfilling its natural role is kept at a temperature of about 37°C. There are moderate differences between the caprine, ovine and bovine β-caseins, but they all show temperature dependent behaviour either by themselves as shown from ultracentrifugation, viscosity measurements and Ca solubility measurements (Chapter 11), or with other proteins as shown by gel chromatography of sub-micellar

aggregates (Chapter 5.3), or ultracentrifugation of casein micelles at low temperature (Chapter 5.1).

O'Connor and Fox (1973) examined the temperature dependent dissociation characteristics of casein micelles from bovine, caprine, ovine, canine, porcine, equine (mares) and ass's milk, as measured by rennin proteolysis, ultracentrifugation and gel filtration. They found that if the canine milk was omitted, the other six species fell into two groups: bovine, caprine and ovine milks in group 1 and ass, equine and porcine milks in group 2. Although the members of group 1 differed qualitatively, all exhibited considerable temperature dependent dissociation of micellar β-casein (as also shown in this study), while those of group 2 showed very low levels of soluble β-casein at any temperature. This suggests that the most studied, yet "closely related" ruminant milk β-caseins may show atypical behaviour and that "β-caseins" do not normally show a temperature dependence in their association-dissociation behaviour. Clearly this concept can only be examined by further investigation of the β-caseins in the milks from non-ruminant species.

β-Casein most probably assumes an important role in the structure of caprine casein micelles, since it constitutes about 60% of the total casein. Presumably the major caprine α_s-casein, which accounts for about 15% of the total casein and which has markedly different properties from bovine α_{s1}-casein, although it can be stabilized by the κ-caseins from the three species, probably fulfils a similar role to that of bovine α_{s3}-casein with the β-casein partly fulfilling the role of α_{s1}-casein. The ovine and bovine casein micelles seem to be similar in most respects.

As a consequence, it seems likely that in the models which have been proposed for bovine casein micelle structure, not enough emphasis has been assigned to β-casein, although Fox and Guiney (1973) and Downey (1973) suggest some of the β-casein is important in micelle structure. Since the predominant components of human casein are κ- and β-caseins, with α_{s1}-casein having yet to be positively identified (Nagasawa et al., 1970), it is likely that β-casein has an

important role in the structure of human casein micelles. The investigations by O'Connor and Fox (1973) of the micelle systems of other species led them to consider that β-casein had a role in micelle structure.

The characteristics which have been described for caprine and ovine casein micelles are most consistent with the sub-unit model of bovine casein micelle structure proposed by Morr (1967a), and in particular, that proposed by Slattery and Evard (1973), with some modifications regarding the role of the constituent caseins.

As implied by Waugh et al. (1970), an alternative explanation may be that neither the α_s- or β-caseins have a particularly specific role in micelle structure, unlike that of κ-casein, and are interchangable in their roles, depending on the nature of the caseins constituting whole casein. After all, the role of casein micelles is to provide a source of nutrients for the young mammal, which can be readily digested by proteolytic enzymes. The specific structural requirements of secondary and tertiary structure, normally necessary for an enzyme to be active, are not required for casein micelles. Consequently, the micelle may be formed from the non-specific interaction of caseins, with a definite role being assumed for κ-casein alone.

REFERENCES

Abd El-Salam, M.H. and Manson, W. (1967): The C-terminal sequence of amino acid residues of κ-casein isolated from buffalo's milk. J.Dairy Res. 34, 85-88.

Alais, C. and Jollès, P. (1961): Comparative study of the caseino-glycopeptides obtained after rennin digestion of the caseins of the milk of cow, sheep and goat. II. Study of the non-peptidic part. Biochim.Biophys.Acta 51, 315-322.

Alais, C. and Jollès, P. (1962): Human casein and its caseinoglycopeptide. Nature 196, 1098-1099.

Alais, C. and Jollès, P. (1967): Isolation, purification, and analysis of two κ-casein-like fractions from sheep casein. J.Dairy Sci. 50, 1555-1561.

Alais, C. and Jollès, P. (1969): The chromatographic purification of human kappa-casein. J.Chromatog. 44, 573-580.

Alais, C. and Jollès, P. (1970): Comparative electrophoretical studies of human and rabbit caseins. Int.J.Biochem. 1, 546-552.

Andrews, P. (1970): Estimation of molecular size and molecular weights of biological compounds by gel filtration. Metn.Biochem.Analysis 18, 1-53.

Annan, W.D. and Manson, W. (1969): A fractionation of the α_s-casein complex of bovine milk. J.Dairy Res. 36, 259-268.

Arave, C.W., Gillett, T.A., Price, D.A. and Matthews, D.H. (1973): Polymorphisms in caseins of sheep milk. J.Anim.Sci. 36, 241-244.

Aschaffenburg, R. (1961): Inherited casein variants in cow's milk. Nature 192, 431-432.

Aschaffenburg, R.(1968): Reviews of the progress of dairy science. J.Dairy Res. 35, 447-460.

Aschaffenburg, R. and Drewry, J. (1959): New procedure for the routine determination of the various non-casein proteins of milk. Proc.15th Int.Dairy Congr., London 3, 1631-1637.

Aschaffenburg, R. and Sen, A. (1963): Comparison of the caseins of buffalo's and cow's milk. Nature 197, 797.

Aschaffenburg, R. and Thompson, M.P. (1967): Unpublished results. Cited in Thompson (1971).

Aschaffenburg, R., Sen, A. and Thompson, M.P. (1968a): Genetic variants of casein in Indian and African Zebu cattle. Comp.Biochem.Physiol. 25, 177-184.

Aschaffenburg, R., Sen, A. and Thompson, M.P. (1968b): The caseins of buffalo milk. Comp.Biochem.Physiol. 27, 621-623.

Ashoor, S.H., Sair, R.A., Olson, N.F. and Richardson, T. (1971): Use of a papain superpolymer to elucidate the structure of bovine casein micelles. Biochim.Biophys.Acta 229, 423-430.

Assenat, L. (1967): Contribution a l'étude d'une méthode d'identification des laits et fromages au moyen de l'électrophorése sur gel de polyacrylamide. Le Lait 47, 393-414.

Beaven, G.H. and Holiday, E.R. (1952): Ultraviolet absorption spectra of proteins and amino acids. Adv.Prot.Chem. 7, 319-386.

Bigelow, C. (1967): On the average hydrophobicity of proteins and the relation between it and protein structure. J.Theoret.Biol. 16, 187-211.

Bingham, E.W., Farrell, H.M., Jr. and Carroll, R.J. (1972): Properties of dephosphorylated α_{s1}-casein. Precipitation by calcium ions and micelle formation. Biochemistry 11, 2450-2454.

Bloomfield, V.A. and Morr, C.W. (1973): Structure of casein micelles: physical methods. Neth.Milk Dairy J. 27, 103-120.

Blow, D.M. and Steitz, T.A. (1970): X-ray diffraction studies of enzymes. Ann.Rev.Biochem. 39, 63-100.

Bogdanov, L.V., Polyakovskii, V.I., Lazovskii, A.A., Martsinkevich, I.S., Pogonyailo, D.I., Nikonchik, L.I., Zotova, V.S., Troshchenkova, L.V. and Dzhumkov, V.A. (1972): Genetic polymorphism of some blood and milk proteins in animals kept in Belorussia. Byulleten' Nauchnykh Rabot, Vsesoyuznyi Ordena Trudovogo Krasnogo Znameni Nauchno-issledovatel'skii Institut Zhivotnovodstva No. 26, 42-48. cited in Dairy Sci.Abstr. 35, [2297], (1973).

Bosworth, A.W. and Van Slyke, L.L. (1916): J.Biol.Chem.24, 177 cited in Parkash and Jenness (1968).

Boulet, M., Yang, A. and Riel, R.R. (1970): Examination of the mineral composition of the micelle of milk by gel filtration. Can.J.Biochem. 48, 816-822.

Bradbury, J.H. (1970): Viscosity. p.100-145. In Leach, S.J. ed. Physical Principles and Techniques of Protein Chemistry. Part B. New York, Academic Press.

Brew, K. (1970): Lactose synthetase: Evolutionary origin, structure and control. p.93-118. In Campbell, P.N. and Dickens, F. eds. Essays in Biochemistry, Vol.6. London, Academic Press.

Brignon, G., Mercier, J.-C., Ribadeau Dumas, B. and Das, B.C. (1972): Structure primaire de la paracaseine κ-bovine. FEBS Letters 27, 301-305.

Brunner, J.R., Ernstrom, C.A., Hollis, R.A., Larson, B.L., Whitney, R. McL. and Zittle, C.A. (1960): Nomenclature of the proteins of bovine milk - First revision. J.Dairy Sci. 43, 901-911.

Bryce, C.F.A. and Crichton, R.R. (1971): Gel filtration of proteins and peptides in the presence of 6 M guanidine hydrochloride. J.Chromatog. 63, 267-280.

Buchheim, W. and Welsch, U. (1973): Evidence for the submicellar composition of casein micelles on the basis of electron microscopical studies. Neth.Milk Dairy J. 27, 163-180.

Cabezas, J.A., Frois, M.D. and Vázques-Torto, J. (1968): Sialic acids. IX. Nature and concentration of the acyl neuraminic acids isolated from various goat products. Revta esp. Fisiol. 24, 133-135 cited in Dairy Sci.Abstr. 31, [3116], (1969).

Calapaj, G.G. (1968): An electron microscope study of the ultrastructure of bovine and human casein micelles in fresh and acidified milk. J.Dairy Res. 35, 1-6.

Carroll, R.J., Thompson, M.P. and Nutting, G.C. (1968): Glutaraldehyde fixation of casein micelles for electron microscopy. J.Dairy Sci. 51, 1903-1908.

Carroll, R.J., Farrell, H.M., Jr. and Thompson, M.P. (1971): Electron microscopy of the casein micelle - forces contributing to its integrity. J. Dairy Sci. 54, 752.

Chen, P.S., Jr., Toribara, T.Y. and Warner, H. (1956): Microdetermination of phosphorus. Anal.Chem. 28, 1756-1758.

Creamer, L.K. (1972): Hydrogen ion equilibria of bovine β-casein B. Biochim.Biophys.Acta 271, 252-261.

Creamer, L.K. and Berry, G.P. (1975): A study of the properties of dissociated bovine casein micelles. J.Dairy Res. in press.

Creamer, L.K. and Richardson, B.C. (1974): Identification of the primary degradation product of α_{s1}-casein in cheddar cheese. N.Z. J.Dairy Sci.Tech. 9, 9-13.

Creamer, L.K. and Waugh, D.F. (1966): Calcium binding and precipitate solvation of Ca-α_s-caseinates. J.Dairy Sci. 49, 706.

Creamer, L.K., Mills, O.E. and Richards, E.L. (1971): The action of rennets on the caseins. J.Dairy Res. 38, 269-280.

Creamer, L.K., Wheelock, J.V. and Samuel, D. (1973): The distribution of glyco-κ-casein and carbohydrate-free κ-casein between large and small bovine casein micelles, and its implication in micelle structure. Biochim. Biophys.Acta 317, 202-206.

Davies, D.T. and White, J.C.D. (1960): The use of ultrafiltration and dialysis in isolating the aqueous phase of milk and in determining the partition of milk constituents between the aqueous and disperse phases. J.Dairy Res. 27, 171-190.

Davis, B.J. (1964): Disc electrophoresis - II. Method and application to human serum proteins. Ann.N.Y. Acad.Sci. 121, 404-427.

Dayhoff, M.O. (1972): In Dayhoff, M.O. ed. Atlas of Protein Sequences and Structure. Vol.5. Washington, National Biomedical Research Foundation. 418 p.

de Koning, P.J. and van Rooijen, P.J. (1965): Genetic variants of α_{s1}-casein; amino acid composition of the variants B, C, and BC. Biochem.Biophys.Res.Commun. 20, 241-245.

de Koning, P.J. and van Rooijen, P.J. (1967): Amino acid composition of α_{s1}-casein D. Nature 213, 1028-1029.

de Koning, P.J., van Rooijen, P.J. and Kok, A. (1966): Location of amino acid differences in the genetic variants of κ-casein A and B. Biochem.Biophys.Res.Commun. 24, 616-621.

Delfour, A., Jollès, J., Alais, C. and Jollès, P. (1965): Caseinoglycopeptides: characterization of a methionine residue and of the N-terminal sequence. Biochem.Biophys. Res.Commun. 19, 452-455.

Dewan, R.K. and Bloomfield, V.A. (1973): Molecular weight of bovine casein micelles from diffusion and viscosity measurements. J.Dairy Sci. 56, 66-68.

Dewan, R.K., Bloomfield, V.A., Chudgar, A. and Morr, C.V. (1973): Viscosity and voluminosity of bovine casein micelles. J.Dairy Sci. 56, 699-705.

Dickson, I.R. and Perkins, D.J. (1971): Studies on the interactions between purified bovine caseins and alkaline-earth-metal ions. Biochem.J. 124, 235-240.

Djordjevic, J. and Caric, M.M. (1972): La dynamique des particles de caséine de diamètres différents dans le lait de brebis au cours de la lactation. Le Lait 52, 558-566.

Dovey, A. and Campbell, P.N. (1952): A comparison of the electrophoretic patterns of cow, goat and rabbit casein. Nature 169, 1014-1015.

Downey, W.K. (1973): Structure of bovine casein micelles. Neth.Milk Dairy J. 27, 218-219.

Downey, W.K. and Murphy, R.F. (1970): The temperature-dependent dissociation of β-casein from bovine casein micelles and complexes. J.Dairy Res. 37, 361-372.

Driezen, P., Noble, R.W. and Waugh, D.F. (1962): Light-scattering studies of $\alpha_{s1,2}$-caseins. J.Am.Chem.Soc. 84, 4938-4943.

El-Negoumy, A.M. and Burfening, P.J. (1972): Polymorphic composition of ewe's casein and whey proteins. J.Dairy.Sci. 55, 673.

Evans, M.T.A., Irons, L. and Jones, M. (1971a): Physico-chemical properties of β-casein and some carboxyacyl derivatives. Biochim.Biophys.Acta 229, 411-422.

Evans, M.T.A., Irons, L. and Petty, J.H.P. (1971b): Physico-chemical properties of some acyl derivatives of β-casein. Biochim.Biophys.Acta 243, 259-272.

Farrell, H.M., Jr. and Thompson, M.P. (1971): Biological significance of milk protein polymorphism. J.Dairy Sci. 54, 1219-1228.

Farrell, H.M., Jr.,Thompson, M.P. and Larson, B. (1971): Verification of the occurrence of the α_{s1}-casein A allele in Red Danish cattle. J.Dairy Sci. 54, 423-425.

Feldman, M.K. and Ceriani, R.L. (1970): A comparitive immunologic and electrophoretic analysis of rat and mouse caseins. Comp.Biochem.Physiol. 37, 421-427.

Feldman, M.K. and Hohmann, P. (1971): Identification of mouse casein components following immunodiffusion and electrophoretic analysis. Int.J.Biochem. 2, 477-480.

Fiat, A.-M., Alais, C. and Jollès, P. (1968): Contribution to the study of the linkage between the peptide and sugar moeities in cow's κ-casein. Chimia 22, 137-139.

Fiat, A.-M., Alais, C. and Jollès, J. (1970): Further data on the specificity of rennin: The rennin sensitive sequence of sheep κ-casein. Chimia 24, 220.

Fiat, A.-M., Alais, C. and Jollès, P. (1972): The amino acid and carbohydrate sequences of a short glycopeptide isolated from bovine κ-casein. Eur.J.Biochem. 27, 408-412.

Fish, W.W. (1971): Gel chromatography in denaturing solvents: A method for the study of protein subunit composition. J.Agr.Food Chem. 19, 660-664.

Fish, W.W., Mann, K.G. and Tanford, C. (1969): The estimation of polypeptide chain molecular weights by gel filtration in 6 M guanidine hydrochloride. J.Biol.Chem. 244, 4989-4994.

Ford, T.F., Ramsdell, G.A. and Alexander, T.G. (1959): Apparent specific volume of the calcium caseinate-calcium phosphate complex in milk. J.Dairy Sci. 42, 397-408.

Fox, P.F. and Guiney, J. (1973): Casein micelle structure: susceptibility of various casein systems to proteolysis. J.Dairy Res. 40, 229-234.

Ganguli, N.C. (1973): State of the casein micelle in buffalo milk. Neth.Milk Dairy J. 27, 258-272.

Ganguli, N.C. and Bhalerao, V.R. (1964): A comparitive study on the caseins of buffalo and cow milks by paper disc electrophoresis. Milchwissenschaft 19, 535-538.

Ganguli, N.C. and Majumder, G.C. (1968): κ-Casein variants in buffalo milk. J.Dairy Sci. 51, 796-797.

Garnier, J. (1966): Conformation de la caseine β en solution analyses d'une transition thermique entres et 40°C. J.Molec.Biol. 19, 586-590.

Garnier, J. (1967): Extension of the continuous variation method to protein interactions: α_{s1}- and β-caseins associations with κ-casein. Biopolymers 5, 473-475.

Garnier, J. (1973): Models of casein micelle structure. Neth.Milk Dairy J. 27, 240-248.

Garnier, J. and Ribadeau Dumas, B. (1970): Structure of the casein micelle. A proposed model. J.Dairy Res. 37, 493-504.

Garnier, J., Ribadeau Dumas, B. and Mocquot, G. (1964): A new method for the preparation of an immunologically homogeneous β-casein. J.Dairy Res. 31, 131-136.

Gerrits, R.J., Kraeling, R.R. and Kincaid, C.M. (1969): Polymorphism in a casein fraction of sows' milk. Biochem.Genet. 3, 355-358.

Glasnák, V. (1966): Protein polymorphism in sows' milk. p.433-435. In Polymorphismes Biochimques Des Animaux. Paris, Institut National de la Recherche Agronomique.

Glasnák, V. (1968): Inter- and intra-specific differences in milk proteins of cattle and swine. Comp.Biochem. Physiol. 25, 355-357.

Gordon, W.G., Basch, J.J. and Thompson, M.P. (1965): Genetic polymorphism in caseins of cow's milk. VI Amino acid compositions of α_{s1}-caseins A, B and C. J.Dairy Sci. 48, 1010-1013.

Gordon, W.G., Groves, M.L., Greenberg, R., Jones, S.B., Kalan, E.B., Peterson, R.F., and Townend, R.E. (1972): The probable identification of γ-, TS-, R-, and S-caseins as fragments of β-casein. J.Dairy Sci. 55, 261-263.

Grosclaude, F., Garnier, J., Ribadeau Dumas, B. and Jeunet, R. (1964): Relationship between the loci controlling the polymorphism of α_s- and β-caseins. C.r. hebd. séanc.Acad. Sci. (Paris) 259, 1569-1571.

Grosclaude, F., Pujolle, J., Garnier, J. and Ribadeau Dumas, B. (1965): Genetic determinism of the κ-caseins of cows' milk: the close linkage between the κ-Cn locus and the α_s-Cn and β-Cn loci. C.r. hebd. séanc. Acad Sci. (Paris) 261, 5229-5232.

Grosclaude, F., Pujolle, J., Garnier, J., and Ribadeau Dumas, B. (1966): Evidence for two additional variants in proteins of cows' milk: α_{s1}-casein D and β-lactoglobulin D. Ann.Biol.Anim.Biochim.Biophys. 6, 215-222.

Grosclaude, F., Mercier, J.-C. and Ribadeau Dumas, B. (1969): On the localization, in the C-terminal peptide of bovine α_{s1}-casein of the glycine/glutamic acid substitution which differentiates the genetic band C variants. C.r. hebd. séanc. Acad. Sci. (Paris) 268, 3133-3136.

Grosclaude, F., Mahé, M.-F., Mercier, J.-C. and Ribadeau Dumas, B. (1970): Localisation, dans la partie NH_2-terminale de la caséine α_{s1}-bovine, d'une délétion de 13 acides aminés différenciant le variant a des variants B et C. FEBS Letters 11, 109-112.

Grosclaude, F., Mahé, M.-F., Mercier, J.-C. and Ribadeau Dumas, B. (1972): Caractérisation des variants génétiques des caséines α_{s1} et β bovines. Eur.J.Biochem. 26, 328-337.

Grosclaude, F., Mercier, J.-C. and Ribadeau Dumas, B. (1973): Genetic aspects of cattle casein research. Neth.Milk Dairy J. 27, 328-340.

Groves, M.L. (1969): Some minor components of casein and other phosphoproteins in milk. A review. J.Dairy Sci. 52, 1155-1165.

Groves, M.L. and Gordon, W.G. (1969): Evidence from amino acid analysis for a relationship in the biosynthesis of γ- and β-casein. Biochim.Biophys.Acta 194, 421-432.

Groves, M.L. and Gordon, W.G. (1970): The major component of human casein: A protein phosphorylated at different levels. Arch.Biochem.Biophys. 140, 47-51.

Groves, M.L. and Kiddy, C.A. (1970): γ-Caseins isolated from milk samples typed β-casein A^1 and A^3. J.Dairy Sci. 53, 931-933.

Groves, M.L. and Townend, R.E. (1970): Molecular weight of some human and cow caseins. Arch.Biochem.Biophys. 139, 406-409.

Groves, M.L., Gordon, W.G., Kalan, E.B. and Jones, S.B. (1972): Composition of bovine γ-caseins A^1 and A^3, and further evidence for a relationship in biosynthesis of γ- and β-caseins. J.Dairy Sci. 55, 1041-1049.

Groves, M.L., Gordon, W.G., Kalan, E.B., and Jones, S.B. (1973): TS-A^2, TS-B, R- and S-caseins: their isolation, composition and relationship to the γ-, β-casein polymorphs A^2 and B. J.Dairy Sci. 56, 558-568.

Gupta, S.K. and Ganguli, N.C. (1965): Sialic acid content of casein preparations from cow and buffalo milks. Milchwissenschaft 20, 10-14.

Herskovits, T.T. (1966): On the conformation of caseins. Optical rotatory properties. Biochemistry 5, 1018-1026.

Hill, R.D. (1970): The effect of the modification of arginine side chains in the casein on the coagulation of rennin-altered casein. J.Dairy Res. 37, 187-192.

Hill, R.J. and Wake, R.G. (1969): Amphiphile nature of κ-casein as the basis for its micelle stabilizing property. Nature 221, 635-639.

Hill, R.J., Naughton, M.A. and Wake, R.G. (1970): The major genetic variant macropeptides of κ-casein - A comparison of their amino acid contents and tryptic peptides. Biochim.Biophys.Acta 200, 267-274.

Hipp, N.J., Groves, M.L., Custer, J.H. and McMeekin, T.L. (1952): Separation of α-, β- and γ-caseins. J.Dairy Sci. 35, 272-281.

Hirs, C.H.W. (1967a): Reduction and S-carboxymethylation of proteins. p.199-203. In Hirs, C.H.W. ed. Methods in Enzymology. Vol.11. New York, Academic Press.

Hirs, C.H.W. (1967b): Performic acid oxidation. p.197-199. In Hirs, C.H.W. ed. Methods in Enzymology. Vol.11. New York, Academic Press.

Ho, C. and Chen, A.H. (1967): The polymerization of bovine α_{s1}-casein B. J.Biol.Chem. 242, 551-553.

Ho, C. and Waugh, D.F. (1965): Interaction of bovine caseins with divalent ions. J.Am.Chem.Soc. 87, 889-892.

Hoagland, P.D. (1968): Acylated β-caseins. Effect of alkyl group size on calcium ion sensitivity and on aggregation. Biochemistry 7, 2542-2546.

Hoagland, P.D., Thompson, M.P. and Kalan, E.B. (1971): Amino acid composition of α_{s3}-, α_{s4}- and α_{s5}-caseins. J.Dairy Sci. 54, 1103-1110.

Hofman, T. (1958a): Inhomogeneity of α-casein from goat milk. Nature 181, 633-634.

Hofman, T. (1958b): Casein and phosphatases. Biochem.J. 69, 139-145.

Holt, C., Dalgleish, D.G. and Parker, T.G. (1973): Particle size distributions in skim milk. Biochim.Biophys.Acta 328, 428-432.

Irons, L., Evans, M.T.A., Jones, M. and Miller, P.J. (1973): The effect of succinylation and acylation on the physico-chemical properties of α_{s1}-casein B. Biochim.Biophys. Acta 328, 433-447.

Jenness, R. (1970): Protein composition of milk. p.17-43. In McKenzie, H.A. ed. Milk Proteins. Vol.1. New York, Academic Press.

Jenness, R. (1973): Caseins and caseinate micelles of various species. Neth.Milk Dairy J. 27, 251-257.

Jenness, R. and Patton, S. (1959): Principles of Dairy Chemistry. New York, John Wiley and Sons, Inc. 446 p.

Jenness, R., Larson, B.L., McMeekin, T.L., Swanson, A.M., Whitnah, C.H. and Whitney, R.McL(1956): Nomenclature of the proteins of bovine milk. J.Dairy Sci. 39, 536-541.

Jenness, R., Morr, C.V. and Josephson, R.V. (1966): Some properties of colloidal phosphate-free skimmilk. J.Dairy Sci. 49, 712.

Jollès, P. (1966): Progress in the chemistry of casein. Angew.Chem.Int.Ed.Engl. 5, 558-566.

Jollès, P., Alais, C. and Jollès, J. (1961): Comparative study of the caseino-glycopeptides obtained after rennin digestion of the caseins of the milk of cow, sheep and goat. I. Study of the peptidic part. Biochim.Biophys. Acta 51, 309-314.

Jollès, P., Alais, C. and Jollès, J. (1963): Étude de la caséine κ de vache caratérisation de la liason sensible a l'action de la présure. Biochim.Biophys.Acta 69, 511-517.

Jollès, P., Alais, C., Adam, A., Delfour, A. and Jollès, J. (1964): Recherches préliminaires sur la structure de la partie glucidique des caséino-glycopeptides. Chimia 18, 357-358.

Jollès, J., Alais, C. and Jollès, P. (1968): The tryptic peptide with the rennin-sensitive linkage of cow's κ-casein. Biochim.Biophys.Acta 168, 591-593.

Jollès, J., Alais, C. and Jollès, P. (1970): Chemical structure studies of cow κ-casein: study of the soluble tryptic peptides. Helv.Chim.Acta 53, 1918-1926.

Jollès, J., Fiat, A.-M., Alais, C. and Jollès, P. (1973): Comparative study of cow and sheep κ-caseinoglycopeptides: determination of the N-terminal sequences with a sequencer and location of the sugars. FEBS Letters 30, 173-176.

Jollès, J., Schoentgen, F., Hermann, J., Alais, C. and Jollès, P. (1974): The sequence of sheep κ-casein: Primary structure of para-κ_A-casein. Eur.J.Biochem. 46, 127-132.

Kalan, E.E. and Woychik, J.H. (1965): Action of rennin on κ-casein, the amino acid compositions of the para-κ-casein, and glycomacropeptide fractions. J.Dairy Sci. 48, 1-6.

Kiddy, C.A., Peterson, R.F. and Kopfler, F.C. (1966): Genetic control of the variants of β-casein A. J.Dairy Sci. 49, 742.

Kim, Y.K., Yaguchi, M., and Rose, D. (1969): Isolation and amino acid composition of para-kappa-casein. J.Dairy Sci. 52, 316-320.

King, J.W.B., (1966): The caseins of sheep milk. p.427. In Polymorphismes Biochimiques des Animaux. Paris, Inst.Nat. Recherche Agronomique.

King, J.W.B., Aschaffenburg, R., Kiddy, C.A. and Thompson, M.P. (1965): Non-independent occurrence of α_{s1}- and β-casein variants of cow's milk. Nature 206, 324-325.

Klotz, I.M. (1970): Comparison of molecular structures of proteins: Helix content; distribution of apolar residues. Arch.Biochem.Biophys. 138, 704-706.

Knoop, V.E. and Wortmann, A. (1960): Studies on the size distribution of casein particles in cow's milk, goat's milk and human milk. Milchwissenschaft 15, 273-281.

Knoop, A.M., Knoop, E. and Wiechen, A. (1973): Electron microscopical investigations on the structure of casein micelles. Neth.Milk Dairy J. 27, 121-127.

Knowles, F. and Watkins, J.E. (1938): The milk of the goat. J.Dairy Res. 9, 153-165.

Konar, A, Thomas, P.C. and Rook, J.A.F. (1971): The concentration of some water soluble constituents in the skim milks of cows, sows, ewes and goats milk. J.Dairy Res. 38, 333-341.

Kondo, K. and Mori, S. (1932): J.Chem.Soc.Japan 53, 1163. cited in Parkash and Jenness (1968).

Larsen, B. and Thymann, M. (1966): Studies on milk protein polymorphism in Danish cattle and the interaction of the controlling genes. Acta vet.Scand. 7, 189-205.

Li, F.H.F. and Gaunt, S.N. (1972): A study of genetic polymorphisms of milk β-lactoglobulin, α_{S1}-casein, β-casein, and κ-casein in five dairy breeds. Biochem.Genet. 6, 9-20.

Lin, S.H.C., Dewan, R.K., Bloomfield, V.A. and Morr, C.V. (1971): Inelastic light-scattering study of the size distribution of bovine milk casein micelles. Biochemistry 10, 4788-4793.

Lin, S.H.C., Leong, S.L., Dewan, R.K., Bloomfield, V.A. and Morr, C.V. (1972): Effect of calcium ion on the structure of native bovine micelles. Biochemistry 11, 1818-1821.

Linderstrøm-Lang, K. (1929): Studier over kasein. C.R. Trav.Lab.Carlsberg 17, 1-116.

Linderstrøm-Lang, K. and Kodama, S. (1925): Studier over kasein. C.R. Trav.Lab.Carlsberg 16, 1-62.

Liu, T.-Y. (1972): Determination of tryptophan. p.44-55. In Hirs, C.H.W. and Timasheff, S.N. ed. Methods in Enzymology. Vol.25. New York, Academic Press.

Lyster, R.L.J. (1972): Reviews of the progress of dairy science. Section C. Chemistry of milk proteins. J.Dairy Res. 39, 279-318.

Lyster, R.L.J. (1973): Studies on the amino acid sequences of caseins. Neth.Milk Dairy J. 27, 323-327.

McGann, T.C.A. and Pyne, G.T. (1960): The colloidal phosphate of milk. J.Dairy Res. 27, 403-417.

McKenzie, H.A. (1967): Milk proteins. Adv.Prot.Chem. 22, 55-234.

McKenzie, H.A. (1970): In McKenzie, H.A. ed. Milk Proteins. Vol.1. New York, Academic Press. 519p.

McKenzie, H.A. (1971): In McKenzie, H.A. ed. Milk Proteins. Vol.2. New York, Academic Press. 552p.

McKenzie, H.A. and Murphy, W.H. (1970): General methods and elemental analysis. p.127-180. In McKenzie, H.A. ed. Milk Proteins. Vol.1. New York, Academic Press.

McKenzie, H.A. and Wake, R.G. (1961): An improved method for isolation of κ-casein. Biochim.Biophys.Acta 47, 240-242.

Macha, J. (1970): Polymorphism of proteins in the goats milk. Živočisna Vyŕoba 15, 801-806.

Mackinlay, A.G. and Wake, R.G. (1964): The heterogeneity of κ-casein. Biochim.Biophys.Acta 93, 378-386.

Mackinlay, A.G. and Wake, R.G. (1965): Fractionation of S-carboxymethyl-κ-casein and characterization of the components. Biochim.Biophys.Acta 104, 167-180.

Mackinlay, A.G. and Wake, R.G. (1971): κ-Casein and its attack by rennin (chymosin). p.175-215. In McKenzie, H.A. ed. Milk Proteins. Vol.2. Academic Press, New York.

Mackinlay, A.G., Hill, R.J. and Wake, R.G. (1966): The action of rennin on κ-casein. The heterogeneity and origin of the insoluble products. Biochim.Biophys.Acta 115, 103-112.

Macy, I.G., Kelly, H.J. and Sloan, R.E. (1953): The composition of milks. Nat.Acad.Sci.-Publ.Nat.Res.Council No. 254, Washington, D.C.

Mahler, H.R. and Cordes, E.H. (1967): Biochemical Chemistry. Tokyo, John Weatherhill Inc. 872p.

Majumder, G.C. and Ganguli, N.C. (1970): An appraisal of caseins from different species of animals by starch gel electrophoresis. Ind.J.Dairy Sci. 23, 179-187.

Malpress, F.H. (1962): Sialic acid in casein preparations from human milk. Biochem.J. 85, 33P.

Malpress, F.H. and Hytten, F.E. (1964): Studies on human casein preparations from single milk samples. Biochem.J. 91, 130-136.

Malpress, F.H. and Seid-Akhavan, M. (1966): Studies on human α_s- and κ-casein fractions and human caseinoglycomacropeptide. Biochem.J. 101, 764-773.

Manson, W. and Annan, W.D. (1971): The structure of a phosphopeptide derived from β-casein. Arch.Biochem.Biophys. 145, 16-26.

Markovich, L.G. (1971): Genetic polymorphism of β_1 and β_2-caseins in the milk of large white sows. Byulleten' Nauchnykh Rabot Vsesoyuznyĭ Ordena Trudovogo Krasnogo Znameni Nauchno-issledovatel' skiĭ Institut Zhivotnovodstva No.24, 24-26. cited Dairy Sci.Abstr. 35, [5327] (1973).

Marier, J.R. and Boulet, M. (1958): Direct determination of citric acid in milk with an improved pyridine-acetic anhydride method. J.Dairy Sci. 41, 1683-1692.

Matsubara, H. and Sasaki, R.M. (1969): High recovery of tryptophan from acid hydrolysates of proteins. Biochem. Biophys.Res.Commun. 35, 175-181.

Mellander, O. (1939): Elektrophoretische untersuchung von casein. Biochem.Z. 300, 240-245.

Melnychyn, P. and Wolcott, J.M. (1965): Simple procedure for isolation of α_s-caseins. J.Dairy Sci. 48, 780.

Mercier, J.-C., Maubois, J.L., Poznanski, S. and Ribadeau Dumas, B. (1968): Fractionnement préparatif des caséines de vache et de brebis par chromatographie sur D.E.A.E. cellulose, en milieu urée et 2-mercaptoéthanol. Bull.Soc. Chim.Biol. 50, 521-530.

Mercier, J.-C., Grosclaude, F. and Ribadeau Dumas, B. (1971): Structure primaire de la caséine α_{s1}-bovine. Eur.J. Biochem. 23, 41-51.

Mercier, J.-C., Grosclaude, F. and Ribadeau Dumas, B. (1972a): Primary structure of bovine caseins. A review. Milchwissenschaft 27, 402-408.

Mercier, J.-C., Uro, J., Ribadeau Dumas, B. and Grosclaude, F. (1972b): Structure primaire du caséinomacropeptide de la caséine κ B_1 bovine. Eur.J.Biochem. 27, 535-547.

Mercier, J.-C., Ribadeau Dumas, B. and Grosclaude, F. (1973): Amino acid composition and sequence of bovine κ-casein. Neth.Milk Dairy J. 27, 313-322.

Michalak, W. (1967): Anomalous electrophoretic pattern of milk proteins. J.Dairy Sci. 50, 1319-1320.

Moore, S. and Stein, W.H. (1963): Chromatographic determination of amino acids by the use of automatic recording equipment. P.819-831. In Colowick, S.P. and Kaplan, N.O. ed. Methods in Enzymology. Vol.6. New York, Academic Press.

Morr, C.V. (1967a): Effect of oxalate and urea upon ultracentrifugation properties of raw and heated skimmilk casein micelles. J.Dairy Sci. 50, 1744-1751.

Morr, C.V. (1967b): Some effects of pyrophosphate and citrate ions upon the colloidal caseinate-phosphate micelles and ultrafiltrate of raw and heated skimmilk. J.Dairy Sci. 50, 1038-1044.

Morr, C.V., Josephson, R.V., Jenness, R. and Manning, P.B. (1971): Composition and properties of submicellar casein complexes in colloidal phosphate-free skimmilk. J.Dairy Sci. 54, 1555-1563.

Morr, C.V., Lin, S.H.C., Dewan, R.K. and Bloomfield, V.A. (1973): Molecular weights of fractionated bovine milk casein micelles from analytical ultracentrifugation and diffusion measurements. J.Dairy Sci. 56, 415-418.

Nagasawa, T., Ryoki, T., Kiyosawa, I. and Kuwahara, K. (1967): Studies on human casein. I. Fractionation of human casein by diethylaminoethyl cellulose column chromatography. Arch.Biochem.Biophys. 121, 502-507.

Nagasawa, T., Kiyosawa, I. and Kuwahara, K. (1970): Human casein. II. Isolation of human β-casein fraction and human β-casein B. J.Dairy Sci. 53, 136-145.

Nagasawa, T., Kiyosawa, I. and Kuwahara, K. (1971): Human casein. III. DEAE-cellulose-urea chromatography of human casein and dephosphorylation of casein fractions. J.Dairy Sci. 54, 987-993.

Nagasawa, T., Kiyosawa, I., Kuwhara, K., Fukuwatari, Y. and Suzuki, T. (1972a): Changes in disc gel electrophoretic patterns of human milk proteins with duration and temperature of storage of the milk. J.Dairy Sci. 55, 289-293.

Nagasawa, T., Kiyosawa, I., Kato, R. and Kuwahara, K. (1972b): Isolation of canine α_s-casein and major whey protein component A and their amino acid composition. J.Dairy Sci. 55, 1550-1556.

Nagasawa, T., Kiyosawa, I., Kuwahara, K. and Ganguli, N.C. (1973): Fractionation of buffalo milk casein by acrylamide gel electrophoresis and DEAE cellulose column chromatography. J.Dairy Sci. 56, 61-65.

Neelin, J.M. (1964): Variants of κ-casein revealed by improved starch gel electrophoresis. J.Dairy Sci. 47, 506.

Nejim, H.T. (1963): The composition of Iraqi sheep's milk. J.Dairy Res. 30, 81-85.

Nielsen, H.C. and Lillevik, H.A. (1957): Molecular weight determination by osmotic pressure measurements of caseins in 6.6 M urea. J.Dairy Sci. 40, 598.

Nitschmann, H. (1949): Elektronenmikroskopische grössenbestimmung der calciumcaseinatteilchen in kuhmilch. Helv.Chim.Acta 32, 1258-1264.

Noble, R.W., Jr. and Waugh, D.F. (1965): Casein micelles. Formation and structure I. J.Am.Chem.Soc. 87, 2236-2245.

Noelken, M. (1967): The molecular weight of α_{s1}-casein B. Biochim.Biophys.Acta 140, 537-539.

Noelken, M. and Reibstein, M. (1968): Conformation of β-casein B. Arch.Biochem.Biophys. 123, 397-402.

Nozaki, Y. and Tanford, C. (1967): Examination of titration behaviour. p.715-734. In Hirs, C.H.W. ed. Methods in Enzymology. Vol.11. New York, Academic Press.

O'Connor, P. and Fox, P.F. (1973): Temperature-dependent dissociation of casein micelles from the milk of various species. Neth.Milk Dairy J. 27, 199-217.

Parkash, S. and Jenness, R. (1968): The composition and characteristics of goats' milk: A review. Dairy Sci.Abstr. 30, 67-87.

Parry, R.M., Jr. and Carroll, R.J. (1969): Location of κ-casein in milk micelles. Biochim.Biophys.Acta 194, 138-150.

Payens, T.A.J. (1966): Association of caseins and their possible relation to structure of the casein micelle. J.Dairy Sci. 49, 1317-1324.

Payens, T.A.J. and Schmidt, D.G. (1966): Boundary spreading of rapidly polymerizing α_{s1}-casein B and C during sedimentation. Arch.Biochem.Biophys. 115, 136-145.

Payens, T.A.J. and van Markwijk, B.W. (1963): Some features of the association of β-casein. Biochim.Biophys.Acta 71, 517-530.

Payens, T.A.J., Brinkhuis, J.A. and van Markwijk, B.W. (1969): Self association in non-ideal systems. Combined light scattering and sedimentation measurements in β-casein solutions. Biochim.Biophys.Acta 175, 434-437.

Peacock, A.C., Bunting, S.L., and Queen, K.G. (1965): Serum protein electrophoresis in acrylamide gel. Patterns from normal human subjects. Science 147, 1451-1453.

Pearce, K.N. (1972): Electrolyte systems relating to milk. Thesis, PhD., Massey University.

Pearce, K.N. (1973): New Zealand Dairy Research Institute Annual Report, p.25-26.

Pearce, K.N. and Creamer, L.K. (1974): Determination of cation activities in solutions by the ion exchange method. Analyt.Chem. 46, 457-458.

Pelissier, J.-P., Mercier, J.-C. and Ribadeau Dumas, B. (1974): Étude de la protéolyse des caséines α_{s1} et β bovines par la présure. Ann.Biol.anim.Biochem.Biophys. 14, 343-362.

Pepper, L. (1972): Casein interactions as studied by gel chromatography and ultracentrifugation. Biochim.Biophys. Acta 278, 147-154.

Pepper, L., Hipp, N.J. and Gordon, W.G. (1970): Effects of modification of ϵ-amino groups on the interaction of κ- and α_{s1}-caseins. Biochim.Biophys.Acta 207, 340-346.

Peterson, R.F. and Kopfler, F.C. (1966): Detection of new types of β-casein by polyacrylamide gel electrophoresis at acid pH. A proposed nomenclature. Biochem.Biophys.Res. Comm. 22, 388-392.

Peterson, R.F., Nauman, L.W., and McMeekin, T.L. (1958): The separation and amino acid composition of a pure phosphopeptone prepared from β-casein by the action of trypsin. J.Am.Chem.Soc. 80, 95-99.

Peterson, R.F., Nauman, L.W., and Hamilton, D.F. (1966): Amino acid composition of six distinct types of β-casein. J.Dairy Sci. 49, 601-607.

Pion, R., Garnier, J., Ribadeau Dumas, B., de Koning, P.J., and van Rooijen, P.J. (1965): Amino acid composition of β-casein genetic variants. Biochem.Biophys.Res.Commun. 20, 246-250.

Pujolle, J., Ribadeau Dumas, B., Garnier, J., and Pion, R. (1966): A study of κ-casein components. Biochem.Biophys. Res.Commun. 25, 285-290.

Pyne, G.T. and McGann, T.C.A. (1960): The colloidal phosphate of milk. J.Dairy Res. 27, 9-17.

Resmini, P., Semenza, F. and Zanini, A. (1967): Studi sui latti di diverse specie. Nota VI: Composizione ammino-acidica delle frazioni caseiniche vaccine e pecorine. La Ricerca Scientifica 37, 74-78.

Ribadeau Dumas, B. (1968): Simultaneous determination of α_{s1}-, β-, and κ-caseins in whole casein by using carboxy-peptidase A. Biochim.Biophys.Acta 168, 274-281.

Ribadeau Dumas, B. and Garnier, J. (1970): Structure of the casein micelle. The accessibility of the subunits to various reagents. J.Dairy Res. 37, 269-278.

Ribadeau Dumas, B., Maubois, J.L., Mocquot, G. and Garnier, J. (1964): Étude de la constitution de la caséine de vache par chromatographie sur colonnes de diéthylaminoéthyl-cellulose en milieu urére. Biochim.Biophys.Acta 82, 494-506.

Ribadeau Dumas, B., Brignon, G., Grosclaude, F. and Mercier, J.-C. (1972): Structure primaire de la caséine β-bovine. Eur.J.Biochem. 25, 505-514.

Ribadeau Dumas, B., Mercier, J.-C., and Grosclaude, F. (1973): Amino acid composition and sequence of bovine α_{s1}- and β-caseins. Neth.Milk Dairy J. 27, 304-312.

Ribadeau Dumas, B., Grosclaude, F. and Mercier, J.-C. (1975): Primary structure of the polymorphs of the caseins. Modern Prob.in Pediatrics 15, in press.
B. Ribadeau Dumas (1974), personal communication.

Richardson, B.C., Creamer, L.K. and Munford, R.E. (1973): Comparative micelle structure. I. The isolation and chemical characterization of caprine κ-casein. Biochim.Biophys.Acta 310, 111-117.

Richardson, B.C., Creamer, L.K., Pearce, K.N. and Munford, R.E. (1974): Comparative micelle structure: II. Structure and composition of casein micelles in ovine and caprine milk as compared with those in bovine milk. J.Dairy Res. 41, 239-247.

Rook, J.A.F. and Campling, R.C. (1965): Effect of stage and number of lactation on the yield and composition of cow's milk. J.Dairy Res. 32, 45-55.

Rook, J.A.F. and Wheelock, J.V. (1967): Reviews of the progress of dairy science. J.Dairy Res. 34, 273-287.

Rose, D. (1968): Relation between micellar and serum casein in bovine milk. J.Dairy Sci. 51, 1897-1902.

Rose, D. (1969): A proposed model of micelle structure in bovine milk. Dairy Sci.Abstr. 31, 171-175.

Rose, D. and Colvin, J.R. (1966): Appearance and size of micelles from bovine milk. J.Dairy Sci. 49, 1091-1097.

Rose, D., Davies, D.T. and Yaguchi, M. (1969): Quantitative determination of the major components of casein mixtures by column chromatography on DEAE-cellulose. J.Dairy Sci. 52, 8-11.

Rose, D., Brunner, J.R., Kalan, E.B., Larson, B.L., Melnychyn, P., Swaisgood, H.E. and Waugh, D.F. (1970): Nomenclature of the proteins of cow's milk: Third revision. J.Dairy Sci. 53, 1-17.

Rowland, S.J. (1938): The determination of the nitrogen distribution in milk. J.Dairy Res. 9, 42-46.

Saito, Z. (1973): Electron microscopic and compositional studies of casein micelles. Neth.Milk Dairy J. 27, 143-162.

Schachmann, H.K. (1957): Ultracentrifugation, diffusion and viscometry. p.32-103. In Colowick, S.P. and Kaplan, N.O. ed. Methods in Enzymology. Vol.4. New York, Academic Press.

Schmidt, D.G. (1964): Starch gel electrophoresis of κ-casein. Biochim.Biophys.Acta 90, 411-414.

Schmidt, D.G. (1970a): The association of α_{s1}-casein B at pH 6.6. Biochim.Biophys.Acta 207, 130-138.

Schmidt, D.G. (1970b): Differences between the association of the genetic variants B, C and D of α_{s1}-casein. Biochim.Biophys.Acta 221, 140-142.

Schmidt, D.G. and Buchheim, W. (1970): Elektronenmikroskopische untersuchung der feinstruktur von caseinmicellen in kuhmilch. Milchwissenschaft 25, 596-600.

Schmidt, D.G. and Payens, T.A.J. (1963): The purification and some properties of a calcium-sensitive α-casein. Biochim. Biophys.Acta 78, 492-499.

Schmidt, D.G. and Payens, T.A.J. (1972): The evaluation of positive and negative contributions to the second virial coefficient of some milk proteins. J.Colloid.Interface Sci. 39, 655-662.

Schmidt, D.G. and van Markwijk, B.W. (1968): Further studies on the associating subunit of α_{s1}-casein. Biochim.Biophys. Acta 154, 613-614.

Schmidt, D.G., Both, P. and de Koning, P.J. (1966): Fractionation and some properties of κ-casein variants. J.Dairy Sci. 49, 776-782.

Schmidt, D.G., Payens, T.A.J., van Markwijk, B.W. and Brinkhuis, J.A. (1967): On the subunit of α_{s1}-casein. Biochem.Biophys.Res.Comm. 27, 448-455.

Schmidt, D.G., Walstra, P. and Buchheim, W. (1973): The size distribution of casein micelles in cow's milk. Neth.Milk Dairy J. 27, 128-142.

Schmidt, D.G., Both, P., van Markwijk, B.W. and Buchheim, W. (1974): The determination of size and molecular weight of casein micelles by means of light-scattering and electron microscopy. Biochim.Biophys.Acta 356, 72-79.

Schulte, K.E. and Müller, F. (1955): Elektrophorestische studien an milchproteinen. Milchwissenschaft 10, 228-232.

Seitov, Z.S., Zhumashev, Zh., Ognesvskaya, A.Ya. (1971): Isolation and composition of β_1-casein of cow's milk. Biokhimiya 36, 1217-1221.

Shalichev, J. and Tanev, G. (1973): Isolation, purification, and determination of some chemical and physicochemical characteristics of sheep α_s-casein. J.Dairy Sci. 56, 171-176.

Shimmin, P.D. and Hill, R.D. (1964): An electron microscope study of the internal structure of casein micelles. J.Dairy Res. 31, 121-123.

Shimmin, P.D. and Hill, R.D. (1965): Further studies on the internal structure of the casein micelles of milk. Aust.J.Dairy Tech. 20, 119-122.

Singh, V.N., Dave, S.S. and Venkitasubramanian, T.A. (1967): Effect of puromycin in vitro on the phosphorylation of precursor protein in lactating rat mammary-gland slices. Biochem. J. 104, 48c-50c.

Slattery, C.W. and Evard, R. (1973): A model for the formation and structure of casein micelles from subunits of variable composition. Biochim.Biophys.Acta 317, 529-538.

Sloan, R.E., Jenness, R., Kenyon, A.L. and Regehr, E.A. (1961): Comparative biochemical studies of milks. I. Electrophoretic analysis of milk proteins. Comp.Biochem.Physiol. 4, 47-62.

Spies, J.R. (1967): Determination of tryptophan in proteins. Anal.Chem. 39, 1412-1416.

Spitz, H.D. (1973): A new approach to sample preparation of protein hydrolysates for amino acid analysis. Anal.Biochem. 56, 66-73.

Sullivan, R.A., Fitzpatrick, M.M., Stanton, E.K., Annino, R., Kissel, G. and Palermiti, F. (1955): The influence of temperature and electrolytes upon the apparent size and shape of α- and β-casein. Arch.Biochem.Biophys. 55, 455-468.

Swaisgood, H.E. (1973): The caseins. Critical Reviews in Food Tech. 4, 375-414.

Swaisgood, H.E. and Brunner, J.R. (1963): Characteristics of κ-casein in the presence of various dissociating agents. Biochem.Biophys.Res.Commun. 12, 148-151.

Swaisgood, H.E. and Timasheff, S.N. (1968): Association of α_{s1}-casein C in the alkaline pH range. Arch.Biochem. Biophys. 125, 344-361.

Swaisgood, H.E., Brunner, J.R., and Lillevik, H.A. (1964): Physical parameters of κ-casein from cow's milk. Biochemistry 3, 1616-1623.

Talbot, B. and Waugh, D.F. (1970): Micelle-forming characteristics of monomeric and covalent polymeric κ-caseins. Biochemistry 9, 2807-2813.

Tanev, G. (1967): Thin-layer starch-gel electrophoretic separation of the principal proteins of ewe's milk. I. Zhivotn.Nauki: Sof. 4 , 123-129

Tanev, G. (1969): Thin layer starch gel electrophoretic separation of the principal proteins of ewe's milk. II. Proportions of individual fractions. Zhivotn.Nauki: Sof. 6 , 101-104.

Tanford, C. (1961): Physical Chemistry of Macromolecules. New York, John Wiley and Sons. 710p.

Tanford, C. (1968): Protein denaturation. Adv.Protein Chem. 23, 121-282.

Tanford, C., Kawahara, K. and Lapanje, S. (1967): Proteins as random coils. I. Intrinsic viscosities and sedimentation coefficients in concentrated guanidine hydrochloride. J.Am.Chem.Soc. 89, 729-736.

Testud, M. and Ribadeau Dumas, B. (1973): Etude des caseines de lait de lapine. Biochimie 55, 1085-1093.

Thompson, E.O.P. and O'Donnell, I.J. (1965): Studies on reduced wool. V. A comparison of the two major components. Aust.J.Biol.Sci. 18, 1207-1225.

Thompson, M.P. (1966): DEAE-cellulose-urea chromatography of casein in the presence of 2-mercaptoethanol. J.Dairy Sci. 49, 792-795.

Thompson, M.P. (1971): α_s- and β-caseins. p.117-173. In McKenzie, H.A. ed. Milk Proteins. Vol.2. New York, Academic Press.

Thompson, M.P. and Farrell, H.M., Jr. (1973): The casein micelle - the forces contributing to its integrity. Neth.Milk Dairy J. 27, 220-239.

Thompson, M.P. and Kiddy, C.A. (1964): Genetic polymorphism in caseins of cows' milk. III. Isolation and properties of α_{s1}-caseins A, B and C. J.Dairy Sci. 47, 626-632.

Thompson, M.P. and Pepper, L. (1964): Genetic polymorphism in caseins of cow's milk. IV. Isolation and properties of β-caseins A, B and C. J.Dairy Sci. 47, 633-637.

Thompson, M.P., Kiddy, C.A., Pepper, L. and Zittle, C.A. (1962): Variations in the α_s-casein fraction of individual cow's milk. Nature 195, 1001-1002.

Thompson, M.P., Kiddy, C.A., Johnston, J.C. and Weinberg, R.M. (1964): Genetic polymorphism in caseins of cow's milk. II. Conformation of the genetic control of β-casein variation. J.Dairy Sci. 47, 378-381.

Thompson, M.P., Tarassuk, N.P., Jenness, R., Lillevik, H.A., Ashworth, U.S. and Rose, D. (1965): Nomenclature of the proteins of cow's milk. Second revision. J.Dairy Sci. 48, 159-169.

Thompson, M.P. Kalan, E.B. and Greenberg, R. (1967): Properties of caseins modified by treatment with carboxypeptidase A. J.Dairy Sci. 50, 767-769.

Thompson, M.P., Boswell, R.T., Martin, V., Jenness, R. and Kiddy, C.A. (1969a): Casein-pellet-solvation and heat stability of individual cow's milk. J.Dairy Sci. 52, 796-798.

Thompson, M.P., Gordon, W.G., Pepper, L. and Greenberg, R. (1969b): Amino acid composition of β-caseins from the milks of Bos indicus and Bos taurus cows: A comparative study. Comp.Biochem.Physiol. 30, 91-98.

Thompson, M.P., Gordon, W.G., Boswell, R.T. and Farrell, H.M., Jr. (1969c): Solubility, solvation and stabilization of α_{s1}- and β-caseins. J.Dairy Sci. 52, 1166-1173.

Toma, S.S. and Nakai, S. (1973): Calcium sensitivity and molecular weight of α_{s5}-casein. J.Dairy Sci. 56, 1559-1562.

Toyoda, M. and Yamauchi, K. (1972): Conformation and some properties of β-casein-like fraction of human casein. Biochim.Biophys.Acta 263, 555-563.

Toyoda, M. and Yamauchi, K. (1973): Preparation and properties of human κ-casein-like fraction. Agric.Biol. Chem. 37, 783-788.

Tran, V.D. and Baker, B.E. (1970): Casein. IX. Carbohydrate moiety of κ-casein. J.Dairy Sci. 53, 1009-1012.

Tripathi, K.K. and Gehrke, C.W. (1970): Chemical and chromatographic isolation of κ-casein. J.Chromatog. 46, 280-285.

Turkington, R.W. and Topper, Y.J. (1966): Casein biosynthesis: Evidence for phosphorylation of precursor proteins. Biochim.Biophys.Acta 127, 366-372.

Voglino, G.F. (1972): A new β-casein variant in Piedmont cattle. Anim.Blood Grps.Biochem.Genet. 3, 61-62.

Voglino, G.F. and Ponzone, A. (1972): Polymorphism in human casein. Nature. New Biology 238, 149-150.

von Hippel, P.H. and Waugh, D.F. (1955): Casein: monomers and polymers. J.Am.Chem.Soc. 66, 4311-4319.

Warner, R.C. (1944): Separation of α- and β-caseins. J.Am.Chem.Soc. 66, 1725-1731.

Waugh, D.F. (1958): The interactions of α_s-, β- and κ-caseins in micelle formation. Faraday Soc.Discuss. No.25, 186-192.

Waugh, D.F. (1961): Casein interactions and micelle formation. J.Phys.Chem. 65, 1793-1797.

Waugh, D.F. (1971): Formation and structure of casein micelles. p.3-85. In McKenzie, H.A. ed. Milk Proteins. Vol.2. New York, Academic Press.

Waugh, D.F. and Noble, R.W., Jr. (1965): Casein micelles. Formation and structure. II. J.Am.Chem.Soc. 87, 2246-2257.

Waugh, D.F. and von Hippel, P.H. (1956): κ-Casein and the stabilization of casein micelles. J.Am.Chem.Soc. 78, 4576-4582.

Waugh, D.F., Ludwig, M.L., Gillespie, J.M., Melton, B., Foley, M. and Kleiner, E.S. (1962): The α_s-caseins of bovine milk. J.Am.Chem.Soc. 84, 4929-4938.

Waugh, D.F., Creamer, L.K., Slattery, C.W. and Dresdner, G.W. (1970): Core polymers of casein micelles. Biochemistry 9, 786-795.

Waugh, D.F., Slattery, C.W. and Creamer, L.K. (1971): Binding of cations to casein. Site binding, Donnan binding, and system characteristics. Biochemistry 10, 817-823.

Weber, K. and Osborn, M. (1969): The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. J.Biol.Chem. 244, 4406-4412.

White, J.C.D. and Davies, D.T. (1958): The relation between the chemical composition of milk and the stability of the caseinate complex. J.Dairy Res. 25, 236-255.

White, J.C.D. and Davies, D.T. (1963): The determination of citric acid in milk and milk sera. J.Dairy Res. 30, 171-189.

Whitnah, C.H. and Rutz, W.D. (1959): Some physical properties of milk. VI. The voluminosity of caseinate complex in milk, and reconstituted sediments. J.Dairy Sci. 42, 227-231.

Woychik, J.H. (1964): Polymorphism in κ-casein of cow's milk. Biochem.Biophys.Res.Commun. 16, 267-271.

Woychik, J.H. and Wondolowski, M.J. (1969): Chromatographic isolation and amino acid composition of sow α_{s1}-, β-, and κ-caseins. J.Dairy Sci. 52, 901.

Woychik, J.H., Kalan, E.B., and Noelken, M.E. (1966): Chromatographic isolation and partial characterization of reduced κ-casein components. Biochemistry 5, 2276-2282.

Yaguchi, M. and Tarassuk, N.P. (1967): Gel filtration of acid casein and skimmilk on Sephadex. J.Dairy Sci. 50, 1985-1988.

Yaguchi, M., Davies, D.T. and Kim, Y.K. (1968): Preparation of κ-casein by gel filtration. J.Dairy Sci. 51, 473-477.

Yamauchi, K. and Kaminogawa, S. (1972): Decomposition of milk proteins by milk protease. Agric.Biol.Chem. 36, 249-254.

Zittle, C.A. (1966): Purification of goat κ-casein and its stabilization of bovine α_s-casein. J.Dairy Sci. 49, 706.

Zittle, C.A. (1967): Precipitation of caprine and bovine caseins from acidic solutions by sodium polyphosphate: Influence of pH and urea. Utilization for separation of α_s- and κ-caseins. J.Dairy Sci. 50, 1352-1355.

Zittle, C.A. and Custer, J.H. (1963): Purification and some of the properties of α_s-casein and κ-casein. J.Dairy Sci. 46, 1183-1188.

Zittle, C.A. and Custer, J.H. (1966): Identification of the κ-casein among the components of whole goat casein. J.Dairy Sci. 49, 788-791.