BOVINE MILK PROTEINS: THEIR DETERMINATION, AND ASSOCIATIONS BETWEEN MILK PROTEIN GENOTYPES AND MILK YIELD AND COMPOSITION

by

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SUMMARY

BOVINE MILK PROTEINS: THEIR DETERMINATION, AND ASSOCIATIONS BETWEEN MILK PROTEIN GENOTYPES AND MILK YIELD AND COMPOSITION

A published method of estimating the casein concentration of milk using dye binding was modified: the method of casein precipitation was simplified, the number of readings on the Pro-Milk Mk. II instrument was reduced from three to two, and the use of skim milk and introduction of a centrifugation step enabled separation of casein for subsequent analysis. By using a regression equation relating the difference between dye binding and Kjeldahl values with the total protein concentration of the milk, the accuracy of estimates of casein in milk from individual cows was improved.

A method for the estimation of casein composition of milk, which is suitable for large numbers of samples from individual cows, was developed. Casein samples were treated with urea and 2-mercaptoethanol and submitted to electrophoresis in a horizontal polyacrylamide gel with a continuous tris-glycine buffer system. Separate estimates of γ- and κ-casein were obtained by also submitting a sample treated with chymosin to electrophoresis. Protein bands were stained with Coomassie blue G250 in trichloracetic acid and scanned with a densitometer.

Casein resolution appeared to be superior to other published methods and genetic variants of α_{s1}-, β- and κ-casein could be readily determined. Casein was subdivided into five fractions. The coefficients of variation of α_{s1}-casein, α_{s2}-casein (4 peaks) and β-casein were5% (expressed as % of total casein) and the coefficients

of variation of γ-casein and κ-casein were 14%.

Differences in dye binding capacities were found between genetic variants of α_{s1}-casein and β-casein. Relative dye binding capacities, given α_{s1}-casein B = 1.00, were α_{s1}-casein C 0.94, α_{s2}-casein 0.98, β-caseins A^1 0.69, A^2 0.66 and B 0.71 and para-κ-casein 0.76.

Eight large commercial herds were selected and cows lactating between 10 and 270 days were used. Single milk samples from 289 Jerseys and 249 Friesians, free of subclinical mastitis, were analysed for total solids, protein, casein, casein composition, β-lactoglobulin, α-lactalbumin and milk protein genotype. Lactation milk yield, lactation fat yield and mean fat concentration were obtained from herd recording records. Data were analysed by least-squares analysis of variance.

The α_{s1}-casein and β-lactoglobulin phenotypes in the Jersey breed showed significant deviation from expectation of independent assortment.

Significant differences in milk yield and composition were found with age and stage of lactation of the cow, and between herds within each breed. Jersey and Friesian milk had significantly different composition.

The mean percentage compositions of casein from Jersey and Friesian cows were respectively, α_{s1}-casein 31.9 and 33.6, α_{s2}-casein 12.8 and 11.7, β-casein 34.2 and 35.4, γ-casein 8.6 and 8.5 and κ-casein 12.4 and 10.8. Only the κ-casein percents were significantly different. Compared to the best estimates in the literature, the proportion of α_{s1}-casein was low due to the dye binding adjustment used, and proportion of γ-casein was high partly due to sample storage.

Significant differences in casein composition were found between

α_{s1}-casein, β-casein and κ-casein genotypes: the concentration and proportion of each casein was highly significantly associated with its respective genotype, as has been well documented in the literature with β-lactoglobulin. Significant differences in fat concentration were also detected between β-casein genotypes.

Significant differences in total solids, fat, casein, α_{s2}-casein, whey protein and β-lactoglobulin concentrations were found between β-lactoglobulin genotypes.

The associations found are discussed in relation to published literature. The genetic variants preferred for cheese manufacture, β-lactoglobulin B, β-casein B and κ-casein B, are considered to have potential as additional criteria in selection of bulls for use in artificial breeding.

DECLARATION

The work described in this thesis was performed entirely by myself, except where specifically stated to the contrary. This thesis is not substantially the same as any other thesis which has already been submitted to any University.

D.M. McLean

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CHAPTER I

GENERAL INTRODUCTION

A. Protein Composition of Bovine Milk

1. Synthesis of Milk Proteins

The principal bovine lactation-specific proteins consist of six primary translation products, α_{s1}-, α_{s2}-, β- and κ-casein, β-lactoglobulin and α-lactalbumin. These proteins comprise more than 90% of the proteins of milk taken from healthy cows in mid-lactation.

The induction and maintenance of lactation is under hormonal control (for a recent review, see Falconer 1980). The mechanism of milk protein synthesis is similar to that in all cells of other species, with each milk protein being coded for in the deoxyribonucleic acid (DNA) of each cell. Mutations of the DNA chain cause genetic variants of the milk proteins (see later).

Milk proteins are synthesised in the alveolar epithelium cells of the mammary gland, from amino acids obtained from the blood. Synthesis of milk protein precursors or prelactoproteins, having amino terminal extensions of between 15 and 21 amino acid residues (Gaye and Mercier 1979), takes place on the bound polyribosomes of the rough endoplasmic reticulum (Gaye *et al.* 1973). The hydrophobic terminal extension provides the signal for passage through the endoplasmic reticulum membrane, and is proteolytically released during the process. The proteins then pass to the Golgi apparatus where the addition of phosphate groups and carbohydrate residues probably occurs in those proteins containing these moieties. The aggregation of the casein monomers to form micelles probably also commences here. The proteins,

together with other synthesized non-lipid constituents of milk are transported to the apical portion of the cell in secretory vesicles and the contents discharged into the lumen.

Although the origin of many of the minor protein components of milk is uncertain, a number appear to be synthesized in other tissues as well as in the mammary gland (e.g. lactoferrin, secretory immunoglobulin A and most of the enzymes) and others pass unchanged from the blood stream (serum albumin, transferrin and many other blood proteins in low concentration).

2. Caseins

(i) Whole casein and its components

The primary structure of all the major milk proteins and many of the minor proteins are now known. The caseins, which account for approximately 80% of the protein in domestic cow milk, are phosphoproteins containing a high level of proline residues. The phosphate groups are linked mainly to serine and to some extent to threonine residues. A major proportion of the caseins occur in milk as micelles which are large, stable complexes containing calcium phosphate and citrate (see section vi.).

However, an operational definition of casein is required, and the caseins have been defined as "those phosphoproteins that are precipitated from raw skim milk by acidification to pH 4.6 at 20°C" (Whitney *et al.* 1976). However, as discussed by McKenzie (1971a), there are difficulties with this definition. The precipitation of phosphoprotein is probably not complete at 20°C, and small amounts of proteins that are not phosphoproteins may also be precipitated with the casein.

Other methods of isolation of casein may be employed, but these

also involve difficulties when proposing their use as a definition of casein. The method of von Hippel and Waugh (1955) involving sedimentation in the presence of calcium may not isolate all the casein, whereas with precipitation by 2M ammonium sulphate, some immunoglobulin, "proteose - peptones" and glycoproteins containing phosphorus may be included with the casein. It was proposed by McKenzie (1967) that the "proteose - peptones" are caseins and this is supported by recent work which has shown that components 5 and 8-fast arise from hydrolytic cleavage of β-casein (Andrews 1978 a,b).

According to Woodward (1976), bovine whole casein was separated into two fractions by Schlossberger as early as 1846, but it was not until the work of Linderstrøm-Lang and Kodama (1925) and Linderstrøm-Lang (1929) that the heterogeneity of casein was generally recognised. Mellander (1939) used moving boundary electrophoresis to demonstrate that whole casein gave rise to three peaks designated α-, β- and γ-caseins. In 1956, Waugh and von Hippel showed that α-casein contained two fractions which were called α_s-casein and κ-casein. They found that the κ-casein fraction was soluble in the presence of calcium and that it prevented the precipitation of α_s-casein by calcium.

Recent studies have shown that only four primary translation products make up the caseins of bovine milk, and these have been designated α_{s1}-, α_{s2}-, β- and κ-casein. Genetic variation, phosphorylation, glycosylation and proteolysis all contribute further heterogeneity. This new nomenclature is becoming accepted, but a problem now exists in designating the components of the α_{s1}- and α_{s2}-casein groups.

(ii) α_{s1}-Caseins

The major casein group in bovine milk is the α_{s1}-caseins which contribute approximately 38% of casein in mature milk (Davies and Law 1977a, b). Bovine α_{s1}-casein, the major component, is a single

phosphoprotein containing 199 amino acid residues, the B genetic variant having a molecular weight of 23 616 daltons and containing 8 phosphoseryl residues (Mercier *et al.* 1972).

α_{s0}-Casein, which moves slightly faster than α_{s1}-casein during electrophoresis at alkaline pH, differs from α_{s1}-casein only in that it contains an additional phosphate residue at position 41 (Manson *et al.* 1977). It contributes approximately 15% of the α_{s1}-casein group (Manson *et al.* 1976).

(iii) α_{s2}-Caseins

Migrating between α_{s1}-casein and β-casein in alkaline gel electrophoresis are a group of caseins which were called α_{s2}-, α_{s3}-, α_{s4}-, α_{s5}- and α_{s6}-casein or the 'minor α_s-caseins' (Annan and Manson 1969). They contain two cysteyl residues (Brignon *et al.* 1977) and it has been suggested that α_{s5}-casein consists of α_{s3}- and α_{s4}-casein molecules linked together by a disulphide bond (Annan and Manson 1969, Hoagland *et al.* 1971 and Toma and Nakai 1973).

This group of caseins, which contribute approximately 10% of whole casein (Davies and Law 1977a, b), have recently been shown to have identical peptide chains which differ only in their phosphate content (Brignon *et al.* 1976), and so have been collectively called α_{s2}-caseins. The complete amino acid sequence has been determined: they contain 207 amino acid residues, the various derivatives have 10, 11, 12 and 13 phosphorylated residues, and assuming none of the group contains carbohydrate, the total molecular weight varies from 25 150 to 25 390 daltons (Brignon *et al.* 1977).

(iv) β-Casein and related polypeptides

(a) β-Casein

The second most abundant milk protein in β-casein, contributing

approximately 36% of whole casein (Davies and Law 1977a, b). This protein consists of a single polypeptide chain of 209 amino acid residues, the β-casein A^2 variant having a molecular weight of 23 982 daltons (Ribadeau Dumas *et al.* 1972). β-Casein genetic variants, with the exception of β-casein C, contain five phosphoseryl residues, four of which are located in a group close to the N-terminal end. The fifth phosphoseryl residue, which is located in position 35, is not phophorylated in β-casein C as a lysyl residue is substituted for the glutamyl residue at position 37 (Mercier *et al.* 1972).

(b) γ-Caseins

Work by Groves and co-workers at the Eastern Region Research Centre, Philadelphia, together with early partial sequences of γ-casein determined by the French group at Jouy-en-Josas led to the conclusion that the γ-casein group of proteins are identical with large C-terminal portions of the β-casein molecule (Groves *et al.* 1972, Groves *et al.* 1973). The latest report from the Committee on the Nomenclature, Classification and Methodology of Milk Proteins has recommended that the components of 'whole-γ-casein' be renamed γ_1-, γ_2- and γ_3-casein on the basis of polypeptide chain length (Whitney *et al.* 1976). The γ_1-caseins contain one phosphoserine residue and the γ_2- and γ_3-caseins do not contain phosphorus.

(c) Proteose-peptone group

The proteose-peptones consist of a mixture of heat stable, acid soluble (pH 4.6) phosphoproteins insoluble in 12% trichloracetic acid (see Whitney *et al.* 1976). Their classification as caseins or whey proteins has been uncertain (see McKenzie 1971a). Four components (3, 5, 8-fast and 8-slow) have been described and each of these are possibly heterogeneous (Whitney *et al.* 1976).

Recently, Andrews (1978 a,b) has shown that two of the proteose-peptone components, 5 and 8-fast, are N-terminal portions of the β-

casein molecule giving strong support for the proteolytic mechanism of formation of both these proteose-peptones and the γ-caseins.

(v) κ-Casein

Bovine κ-casein was first discovered by Waugh and von Hippel (1956), and was shown to play a major role in stabilizing the casein micelle. A wide range of κ-casein concentrations have been measured in bovine casein, depending partly on the method of determination (see Table IV. 1 and 2): the most reliable methods found approximately 13% κ-casein in whole casein.

Work on the primary structure of κ-casein has been carried out in a number of laboratories, resulting in the full primary structure being published by Mercier *et al.* (1973). This has minor differences from sequences published by Jolles *et al.* (1972). It consists of a single polypeptide chain containing 169 amino acids: the molecular weight of the carbohydrate-free monomer of κ-casein B is 19 023 daltons. Chymosin (rennin) attacks the κ-casein molecule, hydrolysing a labile bond between the phenylalanyl residue at position 105 and the methionyl residue at position 106, producing para-κ-casein and macropeptides (Jolles *et al.* 1968).

The para-κ-casein portion of the molecule contains two half-cystine residues (Jolles *et al.* 1962) which according to Beeby (1974) are in the form of masked sulphydryls, one of which is more accessible than the other. Therefore, in native micelles κ-casein may be in the form of reduced monomers, whereas on isolation from milk it occurs as mixtures of polymers held together by intermolecular bonds (Swaisgood and Brunner 1963).

Most of the heterogeneity of κ-casein is present in the macropeptide portion of the molecule, and is due to genetic variation and

differences in carbohydrate content (cf. Wheelock and Sinkinson 1973). Two genetic variants of κ-casein (A and B) are known (see Section B). When homozygous κ-casein is subjected to electrophoresis in an alkaline buffer system containing urea and mercaptoethanol, one major carbohydrate-free band is found together with a number of faster migrating minor components of varying carbohydrate content (Mackinlay and Wake 1964, Neelin 1964, Schmidt 1964, Woychik 1964).

(vi) The casein micelle

In milk, the caseins are present as large, spherical aggregates known as micelles. These micelles are stable to flocculation, the calcium sensitive caseins being stablized by κ-casein against precipitation by calcium. They have a porous structure (Ribadeau Dumas and Garnier 1970) and are highly hydrated (Dewan *et al.* 1973).

Casein micelles are composed of α_{s1}-, α_{s2}-, β- and κ-caseins together with ionic calcium, colloidal calcium phosphate and citrate. Although α_{s2}-casein, an important casein component comprising approximately 10% of casein, is present in the micelle, it has been ignored in micelle models.

Bovine casein micelles exhibit a wide size distribution ranging from approximately 20-680 nm diameter, with the diameter of median volume being 130 nm (Schmidt *et al.* 1973, McGann *et al.* 1980). Particles with a diameter of less than 20 nm (submicelles) account for nearly 80% of the number of casein particles present, but comprise less than 3% of total micellar volume (Schmidt *et al.* 1973). As micelle size increases, the proportion of κ-casein in the micelle decreases (Waugh and Noble 1965).

A number of models of micelle structure have been proposed. It is now accepted that micelles are composed of spherical subunits of approximately 20 nm diameter each containing 25-30 casein monomers.

The subunit structure was first suggested by Shimmin and Hill (1964) and a model incorporating subunits was proposed by Morr (1967). The model which best fits the available data is that of Slattery and Evard (1973): in this model submicelles are composed of variable amounts of α_{s1}-, β- and κ-casein, and they are formed with hydrophilic and hydrophobic areas on their surface, due to asymmetric distribution of κ-casein.

The main driving force in association of casein monomers to form submicelles are hydrophobic interactions (Schmidt and Payens 1972, Slattery and Evard 1973, Dosako *et al.* 1980). When milk is cooled, β-casein and to a lesser extent κ-casein, dissociate from the casein micelle (Rose 1968, Downey and Murphy 1970). This has been interpreted as due to weakening of hydrophobic interactions with decreasing temperature. Electrostatic interactions are also important, particularly with α_{s1}-casein (Slattery and Evard 1973) and calcium phosphate linkages have been implicated (Morr 1967, Carroll *et al.* 1971).

Submicelles aggregate by hydrophobic interactions to form a porous structure, and growth is limited by the eventual concentration, at the micelle surface, of submicelles rich in κ-casein (Slattery and Evard 1973). Salt linkages of calcium phosphate and citrate help stabilize the micelle (Morr 1967, Slattery and Evard 1973).

3. Whey Proteins

The whey proteins consist of a complex group of proteins which are soluble at pH 4.6, the two major components being β-lactoglobulin and α-lactalbumin.

(i) β-Lactoglobulin

Bovine milk is usually considered to contain 2-3 gL^{-1} of β-lactoglobulin (see Lyster 1972), although much higher concentrations have been reported (McLean *et al.* 1974). It comprises 7-12% of skim milk

protein (Whitney *et al.* 1976). In 1955, Aschaffenburg and Drewry detected two genetically controlled, electrophoretically distinct components of β-lactoglobulin now known as A and B. Other genetic variants have since been detected (see Section B).

The primary structure of β-lactoglobulin has been established: the A variant consists of 162 amino acid residues with a molecular weight of 18 362 daltons (Braunitzer *et al.* 1972). There is one cysteine residue and two cystine residues per monomer: McKenzie and Shaw (1972) proposed that the sulphydryl group can occur in alternate positions.

Near the iso-ionic point (ca. pH 5.2) β-lactoglobulin exists as a dimer of two chains linked by non-covalent forces, and as the pH is raised or lowered from this point, dissociation to the monomer occurs (see McKenzie 1971b). However, the level of association of β-lactoglobulin in cow milk is not known.

(ii) α-Lactalbumin

The second most abundant bovine whey protein is α-lactalbumin. Blanc (1964) reported concentrations of 0.7-1.5 gL^{-1} in milk and Gordon and Kalan (1974), who compiled data from many sources, found α-lactalbumin contributed 2 to 5% of skim milk protein.

Two genetic variants, A and B, occur in Zebu cattle, but only the B type is present in Western breeds of cattle (see Section B). The primary structure of α-lactalbumin has been determined (Brew *et al.* 1970), the B variant consisting of 123 amino acid residues, with a molecular weight of 14 174 daltons.

Minor forms of α-lactalbumin have been reported (Preaux *et al.* 1965), which are due to differences in glycosylation (Gordon *et al.* 1968, Barman 1970, Hopper and McKenzie 1973), or number of amide residues (Hopper and McKenzie 1973). Normally, α-lactalbumin contains

four disulphide bridges, but Barman (1973) has reported a form with three disulphide bonds, which accounts for about 5% of the total α-lactalbumin.

Bovine α-lactalbumin and hen egg lysozyme are structurally very similar and it has been proposed that both proteins arose from a common ancestral protein (Brew *et al.* 1967). α-Lactalbumin has a unique role in the biosynthesis of lactose; this role has been reviewed by Ebner and Schanbacher (1974).

(iii) Minor whey proteins

Bovine milk contains serum albumin, which is identical to blood serum albumin, and is transported unchanged from blood to milk. The concentration, as with other blood proteins present in milk, depends on the integrity of the permeability barrier of the mammary epithelium which is affected by diseases such as mastitis.

Immunoglobulins are a group of high molecular weight glycoproteins synthesised by cells of the reticuloendothelial system and which have antibody activity. There are three major classes of bovine immunoglobulin: IgG, with subclasses IgG1 and IgG2, IgM and IgA. Immunoglobulins of mammary secretions have been reviewed by Butler (1974).

Other proteins in whey, present in lower concentrations, include transferrin and lactoferrin (see Groves 1971), ceruloplasmin (Hanson *et al.* 1967) lactollin (see Groves 1971), a large number of enzymes (for reviews, see Groves 1971, Shahani *et al.* 1973, Jenness 1974) and many serum proteins including proteins as large as α_2-macroglobulin (Gugler *et al.* 1957, McLean unpublished data). Recently, Pearce (1980) has detected a large number of heat stable, nitrogenous whey compounds which have been previously unrecognised.

B. Genetic Variants of Bovine Milk Proteins

1. Origin and Detection of Genetic Variants

Genetically controlled variation of a milk protein was first discovered in β-lactoglobulin by Aschaffenburg and Drewry (1955). Since that time, genetic variation has been detected in all of the other major milk proteins. These genetic variants are transmitted by simple Mendelian inheritance without dominance.

This genetic variation or protein polymorphism is due to a mutation in the DNA nucleotide chain which causes an amino acid substitution or deletion in the milk protein polypeptide chain. The most common type of genetic variation is a single amino acid substitution, although two substitutions occur in variants of several milk proteins (κ-casein B→A and β-lactoglobulin B→A and E→F, see Table I. 1). Amino acid substitution may change the number of phosphate residues (e.g. β-casein C) or carbohydrate content (e.g. β-lactoglobulin Droughtmaster). A deletion of a section of the polypeptide chain occurs with two milk protein variants, α_{s1}-casein A and α_{s2}-casein D.

With few exceptions, all milk protein genetic variants have been detected by electrophoresis, as the amino acid substitution or deletion has resulted in a change in charge of the molecule. When subjected to zone electrophoresis the protein genetic variants move at different rates and so two bands are formed with a heterozygous sample.

Paper electrophoresis was originally used to discover many of the milk protein genetic variants e.g. β-lactoglobulin (Aschaffenburg and Drewry 1955 and 1957), α-lactalbumin (Blumberg and Tombs 1958) and β-casein variation (Aschaffenburg 1961). However, starch and acrylamide gel electrophoresis, with their superior resolving power, are now the most commonly used electrophoresis support media. The majority of milk protein variants are detected by electrophoresis in an alkaline medium

TABLE I. 1

Alterations in amino acid sequences specific for the genetic variants of the major milk proteins*

Protein	Compared variants	Alterations of the protein
α_{s1}-casein	B → A	Deletion of residues 14 to 26
	B → C	192 Glu → Gly
	B → D	53 Ala → ThrP
	→ E(Bali) (=E(yak)?)	
α_{s2}-casein	A → B	
	A → C	
	A → D	Deletion of residues 50-58 or 51 to 59, or 52 to 60
β-casein	A^2 → A^1	67 Pro → His
	A^1 → B	122 Ser → Arg
	A^1 → C	(37 Glu → Lys (35 SerP→ Ser
	A^2 → A^3	106 His → Gln
κ-casein	B → A	(148 Ala → Asp (136 Ile → Thr
β-lactoglobulin	B → A	(64 Gly → Asp
		(118 Ala → Val
	B → C	59 Gln → His
	B → D	45 Glu → Gln
	B → E(=D yak)	158 Glu → Gly
	E → F	(129 or 130 Asp → Tyr (50 Pro → Ser
	E → G	78 Ile → Met
	A → Dr	28 Asp → Asn + CHO
α-lactalbumin	B → A	10 Arg → Gln
	B → C	Asp → Asn or Glu → Gln

* Compiled from Mercier *et al.* (1972), Lyster (1972), Grosclaude *et al.* (1979), Bell, Hopper and McKenzie (1981) and Bell, McKenzie and Shaw (1981)

but Kiddy *et al.* (1966) were able to resolve β-casein A into its A^1, A^2 and A^3 components by electrophoresis in an acid medium. Many methods have been published for genetically typing milk proteins: these have been reviewed by Thompson (1970), McKenzie (1971a) and Swaisgood *et al.* (1975).

It has been estimated that the charge of the protein is changed in only about a third of single amino acid substitutions (Harris 1971b and Lewontin 1973). More elaborate methods, such as accurate amino acid analysis and sequencing, are needed to detect electrophoretically neutral or silent variants. Boyer (1972) found a high incidence of electrophoretically neutral variants among primate haemoglobins. However, Townend *et al.* (1965) performed amino acid analysis on β-lactoglobulin samples from 33 individual Holstein and Ayrshire-Holstein crossbred cows but were unable to find any differences other than the previously known A-B amino acid difference. Aschaffenburg *et al.* (1968) detected the β-casein B_z variant, which replaces β-casein B in Zebu cattle, by non-electrophoretic means (mapping chymotryptic peptides) and recently Bell, McKenzie and Shaw (1981) detected a neutral substitution in β-lactoglobulin in milk from Bali cattle.

2. Milk Protein Genetic Variants

The specific differences between the genetic variants of α_{s1}-, β- and κ-casein and the relative mobilities of these variants in alkaline and acid gel electrophoresis have been summarised by Mercier *et al.* (1972) (see Table I. 1. and Figure I. 1.).

(i) α_{s1}-Caseins

Five genetic variants of α_{s1}-casein are known: A, D, B, C and E in order of decreasing electrophoretic mobility in an alkaline buffer system. α_{s1}-Casein A, B, and C were discovered by Thompson *et al.*

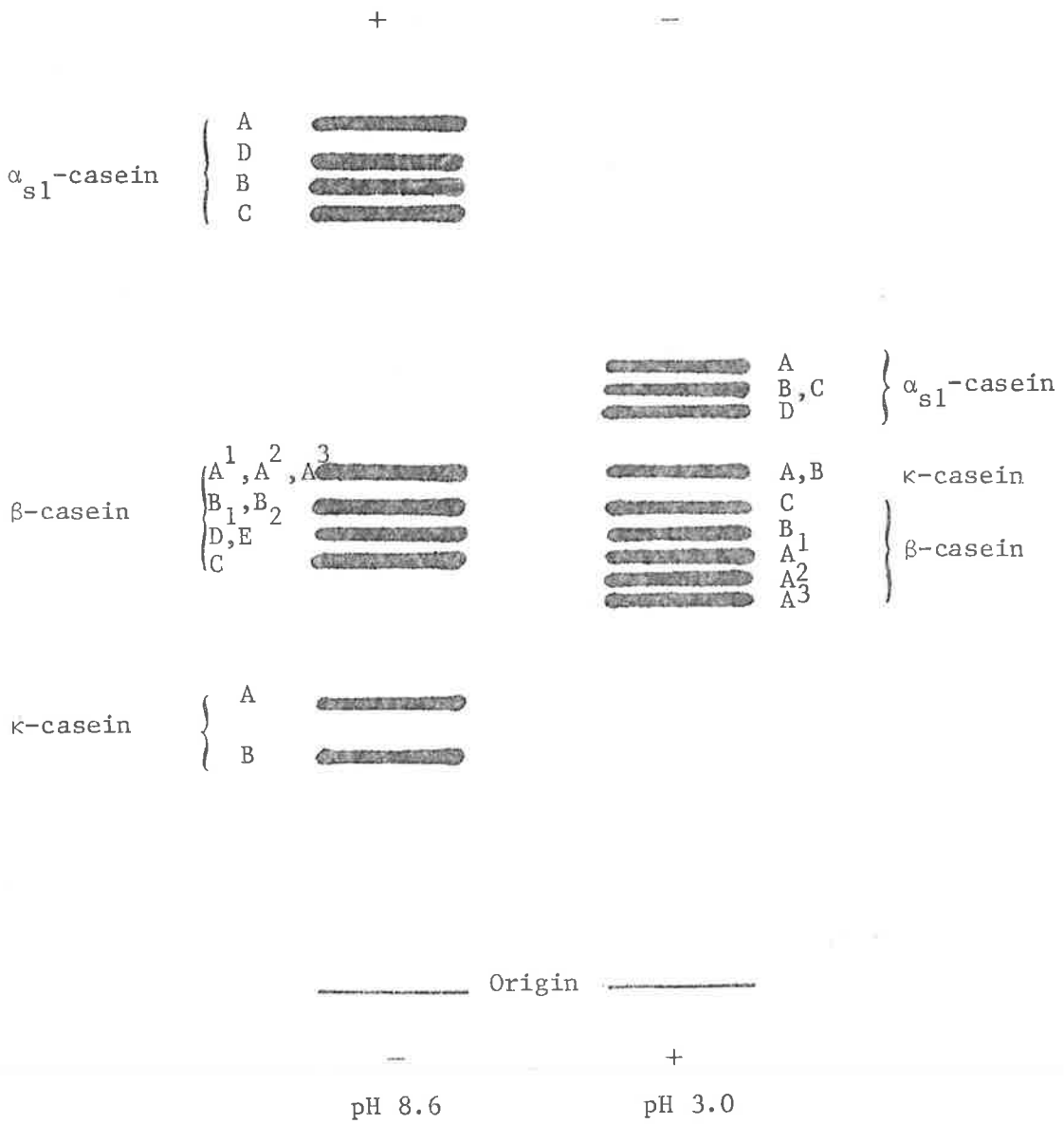


FIGURE I.1

Diagrammatic representation of the relative mobility of some genetic variants of α_{s1}, β- and κ-casein during electrophoresis in starch gel containing urea and mercaptoethanol. Only the main κ-casein bands are indicated (from Mercier *et al.* 1972 and Whitney *et al.* 1976).

(1962), α_{s1}-casein D by Grosclaude *et al.* (1966), α_{s1}-casein E (yak) by Grosclaude *et al.* (1976a) and α_{s1}-casein E(Bali) by Bell, Hopper and McKenzie (1981).

Variants B and C occur in all breeds, with B being predominant in Western breeds and C predominant in Zebu breeds (Aschaffenburg 1968). α_{s1}-Casein A is rare, having been detected in the Holstein-Friesian in U.S.A. (Thompson *et al.* 1962), Australia (Munro, personal communication) and New Zealand (Creamer, reported by Thompson and Farrell 1974) and in the Red Danish breed (Thymann and Larsen 1965). α_{s1}-Casein D is also rare, but has been detected in several European breeds of cattle (Grosclaude *et al.* 1973).

The α_{s1}-casein A variant is due to a deletion of 13 codons in the DNA chain coding for α_{s1}-casein (Kalan *et al.* 1966, Thompson *et al.* 1969). It would be expected that α_{s0}-casein type derivations of the genetic variants of α_{s1}-casein would also occur (Manson *et al.* 1977).

(ii) α_{s2}-Caseins

A genetic variant which simultaneously affects the four α_{s2}-casein fractions has recently been discovered (Grosclaude *et al.* 1976b). Milk of bovines, zebus and yaks in a high Nepalese valley were investigated, and in addition to the classical variant α_{s2}-casein A, two other variants were found: α_{s2}-casein B in bovines and zebus and α_{s2}-casein C in yaks.

An additional variant, α_{s2}-casein D, has been found in two bovine breeds of eastern France (Vosgienne and Montbeliarde) by Grosclaude *et al.* (1979). This differs from α_{s2}-casein A by the deletion of a very acidic nonapeptide.

(iii) β- and γ- Caseins

Genetic variation of β-casein (A, B, and C) was first detected

by Aschaffenburg (1961) using paper electrophoresis in an alkaline buffer system. The A band was resolved into three distinct variants (A^1, A^2 and A^3) by acid gel electrophoresis after it was found that tryptic peptides contained heterogeneity with respect to histidine residues (Peterson and Kopfler 1966, Kiddy *et al.* 1966). A fourth A variant, β-casein A^4, has recently been found in Bali cattle (Bell, Hopper and McKenzie 1981).

Aschaffenburg *et al.* (1968) discovered a further genetic variant, β-casein D, in the Indian Deshe and KenyanBoran and also demonstrated that in Zebu cattle, the β-casein B_Z variant replaces the normal β-casein B variant of Western breeds of cattle. Another variant of β-casein called β-casein E, has been detected in Italian Piemont cattle (Voglino 1972) and Creamer and Richardson (1975) reported a further variant which they called β-casein B^2.

β-Casein A is common in all breeds of dairy cattle. The A^2 variant generally has a higher frequency than A^1, with A^3 being rare. The B gene is fairly common in Jerseys and Brown Swiss, has a lower frequency in Holstein-Friesians, Guernseys and Zebus and has not been detected in Ayrshire cattle. β-Casein C, although originally thought to be restricted to certain breeds, has now been detected in low frequency in most Western breeds of cattle (see Grosclaude *et al.* 1973). β-Casein D has a low frequency in Indian and African Zebus, but has not been observed in Western breeds of cattle (Aschaffenburg 1968). The frequency of the newly detected β-casein B^2 and E variants is not known.

The existence of γ-casein polymorphism was suggested by Aschaffenburg (1961) using paper electrophoresis and confirmed by Groves and Kiddy (1967, 1968) using disc electrophoresis. The genetic variants of the γ-casein fragments are determined by the β-casein locus

and the variation present depends on the position of cleavage so separate typing is not necessary.

(iv) κ-Casein

By using gel electrophoresis of casein in the presence of urea and mercaptoethanol, variations of κ-casein in individual milks was demonstrated independently by three workers (Neelin 1964, Schmidt 1964, Woychik 1964). This variation was shown to be genetically controlled by Thymann and Larsen (1965), and Grosclaude *et al.* (1965). The polypeptide chains of the two genetic variants, A and B, differ by two amino acid residues (Woychik *et al.* 1966, de Koning *et al.* 1966). Recently the discovery of a third κ-casein variant which was called C, was claimed by Di Stasio and Merlin (1979). However, verification by amino acid analysis or genetic studies is necessary, as the C band could have been a carbohydrate containing κ-casein band or a γ-casein band.

The A allele tends to predominate in the majority of breeds, although there are several exceptions, including the Jersey (Aschaffenburg 1968).

(v) β-Lactoglobulin

Aschaffenburg and Drewry (1955, 1957) demonstrated that bovine milk contains two genetic variants of β-lactoglobulin, which they designated as A and B. A third variant, β-lactoglobulin C, was detected in Australian Jerseys by Bell (1962 , 1967) and Grosclaude *et al.* (1966) detected a fourth, designated β-lactoglobulin D.

The breed distribution of β-lactoglobulin A, B, C and D has been compiled by Aschaffenburg (1968): the B allele is predominant in the majority of breeds tested, with the A allele usually present in lower frequencies. β-Lactoglobulin C is present in low frequencies

only in the Jersey, although an unconfirmed single heterozygote was reported in the South African Nguni (Osterhoff and Pretorius 1966). β-Lactoglobulin D has been found in several European breeds but its frequency is low.

Bell *et al.* (1966) reported a rare genetic variant in Australian Droughtmaster beef cattle which was called β-lactoglobulin Droughtmaster and which is due to a substitution of Asn for Asp at position 28, with the carbohydrate moiety attached through the Asn (H.A. McKenzie, W.H. Murphy and D.C. Shaw, quoted by Bell and McKenzie 1976). Three new β-lactoglobulin variants E, F and G, have been found in Bali cattle in Australia (Bell, McKenzie and Shaw 1981). The amino acid substitutions involved in the genetic variants of β-lactoglobulin are given in Table I. 1.

(vi) α-Lactalbumin

Blumberg and Tombs (1958) found two forms of α-lactalbumin, when milks from the Nigerian Zebu were examined by paper electrophoresis. One form corresponded to the mobility of the single band found in Western breeds of cattle (α-lactalbumin B) and the other, α-lactalbumin A, moved more rapidly. Recently, Bell, Hopper and McKenzie (1981) have reported a third α-lactalbumin variant, C, which is present in Bali cattle.

(vii) Summary of milk protein genotypes and common types in Western cattle breeds

Five genetic variants of α_{s1}-casein are known (A, B, C, D, E), B being predominant and C occurring in lower frequencies in Western breeds of cattle. Four α_{s2}-casein variants have been detected (A, B, C, D), but only α_{s2}-casein A is common in Western breeds. Ten variants of β-casein (A^1, A^2, A^3, A^4, B, B_Z, B^2, C, D, E) have been reported,

A^1, A^2 and B being most common in Western breeds. Two variants of κ-casein are known with A and B both common. β-Lactoglobulin has eight known genetic variants (A, B, C, D, Dr, E, F, G), A and B being the most common in Western breeds. Only one α-lactalbumin type (B) is known in Western breeds.

3. Linkage between Casein Genes

Linkage between the α_{s1}-casein and β-casein was discovered independently by Grosclaude *et al.* (1964) who used segregation data collected from suitable families and by King *et al.* (1965) who showed in contingency tables the non-association between α_{s1}-casein C and β-casein B variants. The linkage of the κ-casein locus to the α_{s1}- and β-casein loci was demonstrated independently by Grosclaude *et al.* (1965) and Larsen and Thymann (1966) both using segregation data.

It has been proposed that the genes responsible for the synthesis of the casein fractions are located together on the same chromosone in the order α_{s1}-, β-, κ-casein (Grosclaude *et al.* 1973). This cluster of genes or "gene complex" appear to be transmitted from one generation to another with a very low probability of recombination.

Recent genetic studies (Grosclaude *et al.* 1976b, Grosclaude *et al.* 1979) have demonstrated that the four α_{s2}-casein fractions are synthesised by the same structural gene and that this gene is closely linked to the genes coding for α_{s1}-, β- and κ-caseins. As the γ-caseins have been shown to result from proteolysis of the β-casein molecule, all of the major fractions of casein are now known to be synthesised by this gene complex.

CHAPTER II

THE AIMS OF THE PRESENT INVESTIGATION

The major proteins of bovine milk and their genetic variation were reviewed in Chapter I. The main aim of this investigation was to determine whether significant associations exist between these milk protein genetic variants and the milk yield of the cow and the composition of the milk, particularly the protein composition.

Although dairy farmers in Australia are still paid on the basis of the amount of fat produced, the value of the protein component of milk is being increasingly recognised. Milk protein has an economic value to the manufacturer similar to that of milk fat, it is seen as a major nutritional ingredient of dairy products and it makes an important contribution to the manufacturing properties of the milk. In the manufacture of cheese and casein, the casein concentration determines the yield for a given volume of milk, and means of increasing casein concentration of milk by using possible associations with milk protein genotype were sought.

Little information was available on the relationship between milk protein genotype and concentrations of individual milk proteins, other than the well documented relationship between genotype and concentration of β-lactoglobulin. Therefore, investigation of associations between genotypes of the caseins and their concentrations was an important aim of this study.

Numerous studies of relationships between milk protein genotypes and milk yield, gross composition and manufacturing properties have been reported in the literature. Statistical significance was often not demonstrated, and some significant differences were conflicting.

Many studies used small numbers of samples and often environmental variation and genetic variation other than that due to the specific genotype was not well controlled. The investigation described in Chapter V aimed to select milk samples from a large number of cows in commercial dairy herds in South Australia and to eliminate or control variation other than due to milk protein genotypes. A limitation of this work was that milk composition, other than fat concentration, could only be determined on a single milk sample from each cow.

A rapid and reliable method for estimating the casein concentration in large numbers of milk samples from individual cows was required. A published method, based on the dye binding principle, was modified to suit these requirements, and this work is described in Chapter III.

A suitable method for determining the casein composition of these samples was also required. Quantitative gel electrophoresis was selected but specific problems were encountered. There are few estimates of the dye binding capacities of individual caseins in the literature and no information on possible differences in dye binding between casein genetic variants. As the associations between casein genotypes and concentrations of individual caseins was to be investigated, the results were dependent on determining any differences in dye binding between variants and correction of casein concentrations if differences occurred. The development of the quantitative gel electrophoresis method used is described in Chapter IV.

CHAPTER III

DETERMINATION OF THE CONCENTRATION OF TOTAL PROTEIN, CASEIN AND WHEY PROTEIN IN MILK

A. Determination of Total Protein

1. Total Nitrogen

The most accurate method of determining the total nitrogen (TN) concentration in milk is by Kjeldahl analysis and this is usually used as the reference method. There has been considerable variation in the method of determining nitrogen concentration in milk by Kjeldahl analysis: a relatively rapid and precise method has been outlined by McKenzie and Wallace (1954).

Early estimates of the average value for nitrogen content of purified acid-precipitated casein was 15.68%(m/m), and although the nitrogen content of the principal whey proteins were lower, a multiplying factor of 6.38 (100/15.68) was considered acceptable in calculating crude protein concentration (see White 1980). Renner (1980) has suggested 'total nitrogenous substances' for this fraction, but as the term 'crude protein' is so widely used, it will be retained in the present study. The conversion factor depends on the composition of the milk, and may vary between 6.37 and 6.40 (Kroger 1973), so the protein concentration determined in this way is not absolute. Early estimates of nitrogen content of milk proteins may be in error due to uncertainty about the salt and water contents of the test samples. Based on recent values for the nitrogen content of the individual proteins compiled by Jenness (1970) and a recent estimate of protein composition of milk (Davies and Law 1980), White (1980) calculated that the average

nitrogen content of milk protein was 15.89% and he suggested that crude protein concentration be obtained by multiplying total nitrogen content by the factor 6.29 instead of by 6.38. However, the factor of 6.38 is universally accepted at present.

Approximately 6% of the nitrogen content of milk consists of non-protein constituents, and so the true protein concentration is obtained by the formula (total nitrogen - non protein nitrogen) x 6.38. Non-protein nitrogen (NPN) can be determined by Kjeldahl analysis of the filtrate after precipitation of protein using 120 gL^{-1} trichloracetic acid (Rowland 1938b).

Many workers ignore the NPN when determining the protein concentration of milk (e.g. Kiddy 1963, Sherbon 1978, Standards Association of Australia 1974). McDowell (1972) suggested that values for protein concentration of milk should refer only to 'true protein', and that in the absence of an analytical result for NPN, the subtraction of an average value from TN x 6.38, would be justified. White (1980) agreed that a constant factor could be used to obtain true protein of bulk milk samples, i.e. 6.29 (total N x 0.94), but with individual cow samples, such a scheme was not practicable due to variation of NPN content. Renner (1980) also considered that use of a constant factor would give incorrect true protein results due to variation of NPN content.

The Kjeldahl method is the official method for determination of the protein concentration of milk (e.g. Standards Association of Australia 1974). However, it is time consuming, costly and impractical for routine determination of large numbers of samples, and simpler methods of determining the protein concentration of milk have been developed. These can be grouped into the following categories: biuret reaction, Folin reaction, refractometry, spectrophotometry including the use of direct infra-red energy absorption due to peptide linkages, fluoremetry, acidometric methods, formol titration, alkaline steam distillation

(Kofranyi) and dye binding (for reviews see Booy *et al.* 1962 and Kroger 1973). Of these methods only two, dye binding and infra-red absorption, are sufficiently accurate and precise and are suitable for routine use on a large scale. Commercial instruments based on both principles have been developed for milk protein estimation.

2. Dye Binding

The basic principles of dye binding were first described by Fraenkel-Conrat and Cooper (1944) and the use of dye binding for the determination of milk protein was introduced by Schober and Hetzel (1956) using the dye amido black 10B and by Udy (1956) using orange G. Measured volumes of milk and buffered dye solution are mixed and insoluble complexes are formed due to reaction of the basic amino acid residues of the protein, particularly lysine residues, with acid groups on the dye and also due to hydrophobic interactions. These insoluble complexes are removed by filtering or centrifugation and the excess dye is measured photometrically.

The individual proteins of milk bind different amounts of dye, with whole casein binding less dye than the whey proteins (Ashworth and Chaudry 1962) and so the dye binding method depends on the milk protein composition remaining relatively constant. As protein composition changes with such factors as stage of lactation and level of mastitis, it may be expected that dye binding methods could be inaccurate when used to estimate protein content of individual cow samples. However, Waite and Smith (1972) found the accuracy of the dye binding method was little affected even in cases of severe subclinical mastitis, but that the accuracy was reduced in milk produced in the first and last month of lactation.

Dye binding methods are normally calibrated against the Kjeldahl method. As non-protein nitrogen does not bind any significant amount

of dye (Ashworth and Seals 1958), it is logical to calibrate the dye binding method to 'true protein'. Calibration may consist of determining a regression between the amount of dye bound and the protein concentration of the calibrating samples or by adjustment of the volume of dye solution delivered so that the protein read-out is equal to that obtained by Kjeldahl determination. Because the relationship between Kjeldahl (N x 6.38) and dyebinding method is influenced by changes in protein composition and proportion of NPN, and because purity of dye lots vary, the method should be regularly calibrated using the same type of milk as will be routinely tested. During a one year study to evaluate the Pro-Milk Mark II, an instrument measuring milk protein by the dye binding method, Szijarto *et al.* (1973) found that six calibration adjustments were required throughout the year, three when changing dye lots and three with the same dye lot, due to seasonal changes in mean dye binding capacity of milk proteins.

Clark (1979) and Grappin *et al.* (1980) have proposed using standardised reconstituted milk samples as a secondary reference standard to eliminate the necessity of carrying out Kjeldahl determinations for calibration purposes in every laboratory using dye binding methods and to reduce variation between laboratories. While achieving the aim of reducing variation between laboratories, the use of a reference protein sample does not allow re-calibration for seasonal changes in the milk protein dye binding capacity.

The accuracy of dye binding methods of measuring milk protein is good, as standard deviations (s.d.) of the difference between determinations by the Kjeldahl method and dye binding methods of between $\pm$ 0.4 to $\pm$ 1.1 gL^{-1} have been reported (Renner and Omeroglu 1972, Szijarto *et al.* 1973, Sherbon 1974, McGann 1978). Precision (repeatability) of dye binding methods is exceptionally good with the standard deviation

generally better than $\pm$ 0.1 gL^{-1} (McGann 1978).

3. Infra-red Spectroscopy

The protein concentration of milk and also the concentration of the other two major constituents of milk, fat and lactose, can be measured by infra-red spectroscopy as each has an absorption peak in the infra-red regions where the other components have little absorption (Goulden 1964). The wavelengths selected by Goulden (1964) were 5.73 µm for the carbonyl groups of fat (frequency 1 745 cm^{-1}), 6.46 µm for the amide groups of protein (1 548 cm^{-1}) and 9.60 µm for the hydroxyl groups of lactose (1 042 cm^{-1}). Problems involving strong water absorption bands and light scattering by fat globules and casein micelles have been overcome and several instruments using infra-red absorption are available commercially.

As with dye binding methods of measuring milk protein concentration, the infra-red instruments must be checked routinely with results obtained by acceptable reference methods. The accuracy, expressed as standard deviation of the difference between values obtained by infra-red instruments and reference method, is acceptable: Grappin and Jeunet (1976) found $\pm$ 0.3 gL^{-1} and $\pm$ 0.5 gL^{-1} for herd and individual cow milks respectively, and McGann (1978) reported $\pm$ 0.4 - 0.6 gL^{-1} for individual cow milks. The precision of the infra-red method was also satisfactory, with a standard deviation of duplicate measurements of $\pm$ 0.15 gL^{-1} (Grappin and Jeunet 1976) and $\pm$ 0.2 gL^{-1} (McGann 1978).

4. Other Methods

A nephelometric procedure for determination of milk protein (and fat) has recently been developed (Beitz *et al.* 1977) but the precision of this method (s.d. $\pm$ 0.83 gL^{-1}) was not as good as dye binding and infra-red spectroscopy instruments.

Renner and Omeroglu (1972) studied the accuracy of a number of methods for the determination of the protein concentration of milk based on a photometric principle. They found that the biuret method was not sufficiently accurate (s.d. $\pm$ 1.0 gL^{-1}), the manual amido black (s.d. $\pm$ 0.6 gL^{-1}), orange G (s.d. $\pm$ 0.6 gL^{-1}) and formol titration (s.d. $\pm$ 0.7 gL^{-1}) had satisfactory accuracies and the Infra Red Milk Analyser (s.d. $\pm$ 0.5 gL^{-1}), Kofranyi method (s.d. $\pm$ 0.4 gL^{-1}) and amido black instruments (s.d. $\pm$ 0.4 gL^{-1}) had very high accuracies.

For the rapid determination of the protein concentration of large numbers of milk samples, instruments based on dye binding and infra-red spectroscopy are suitable, and are sufficiently accurate and precise. For the present study, a Pro-Milk Mark II dye binding instrument was available, and as this was also suitable for estimating the casein concentration of milk as discussed in the following section, this was the method selected.

B. Determination of Casein and Whey Protein

1. Precipitation of Casein

The first step in determining the concentration of casein and whey protein in milk is the separation of these two fractions, and this has most commonly been carried out by acidification to the isoelectric point of casein (pH 4.6 - 4.7). Rowland (1938a) found that maximum precipitation of casein from milk samples of varying casein concentrations resulted from the addition to 10 mL of milk of about 80 mL of water at 40°C and 1.0 mL 100 gL^{-1} acetic acid solution, followed after 10 minutes by 1.0 mL of M sodium acetate solution. The reaction of acid with caseinate was not instantaneous, and acetic acid was added first to increase the rate of precipitation by giving a pH value slightly on the acid side of the isoelectric point, then the sodium

acetate was added to bring the pH to the isoelectric point. However, the actual proteins precipitated was not tested by Rowland (1938a). It is possible that some whey proteins are precipitated by the addition of acetic acid which do not redissolve in the period before filtering (McKenzie, personal communication).

Rowland's (1938a) method of adding acetic acid and sodium acetate separately to diluted milk is widely used and is incorporated in various standard methods (e.g. International Dairy Federation 1964 and Standards Association of Australia 1974).

Other methods of removing the casein have been used in various rapid methods of measuring casein concentration. Ashworth (1956) precipitated casein by adding an acetic acid-sodium acetate buffer containing 240 gL^{-1} ethanol to give approximately 1:10 dilution of skim milk. McGann *et al.* (1972) precipitated the casein with minimum dilution of whey, by separately adding equal volumes of acetic acid and sodium acetate which together amounted to only 6% of the volume of the milk and which approximately counter-balances the contraction in volume of milk due to the removal of the casein and fat co-precipitate.

Methods other than acid precipitation may also be used to separate the casein and non-casein fractions, but the protein composition of the casein fractions will be different, as discussed earlier. Ammonium sulphate precipitation was used by Wagner *et al.* (1974) and coagulation by chymosin action was used by Mickelson and Shukri (1975).

However, Sherbon (1978) still considered that there was a critical need for a faster, easier method to remove casein.

2. Methods of Measuring the Protein Concentrations of Casein or Whey

(i) Total nitrogen

The classical (Rowland 1938b, Aschaffenburg and Drewry 1959) and standard (e.g. International Dairy Federation 1964, Standards Association

of Australia 1974) methods use Kjeldahl analysis to determine the nitrogen content of the filtrate after casein precipitation. By subtracting the non-casein nitrogen (whey proteins + NPN) from total nitrogen, casein nitrogen is obtained. A volume correction factor is normally applied to allow for the volume of precipitate. Rowland (1938b) used 0.995 for whole milk and the International Dairy Federation (1964) and Standards Association of Australia (1974) use 0.994 for whole milk and 0.998 for skim milk.

(ii) Rapid methods utilizing dye binding

To avoid slow and tedious Kjeldahl analysis, a number of rapid methods have been published which utilize dye binding. The precipitated casein was re-dispersed and measured by Ashworth (1965), Renner and Omeroglu (1973) and Wagner *et al.* (1974). Haenlein *et al.* (1968) and Michelson and Shukri (1975) were able to measure whey protein directly by using a suitable whey-dye ratio. In the Pro-Milk difference (PMD) method of McGann *et al.* (1972) the milk sample and the filtrate were mixed with dye solution to transpose the readings to a more sensitive part of the scale.

Amido black (McGann *et al.* 1972, Renner and Omeroglu 1973 and Wagner *et al.* 1974) and acid orange G (Ashworth 1965, Haenlein *et al.* 1968 and Mickelson and Shukri 1975) dyes have both been used in rapid methods of measuring casein concentration.

The casein values obtained by rapid, dye binding methods must be adjusted using a correction factor or regression equation relating them to values obtained with a suitable reference method. McGann *et al.* (1972) found that when 0.4 gL^{-1} was added to the Pro-Milk difference (PMD) values they agreed with the non-casein protein values determined by the standard Kjeldahl procedure (International Dairy Federation 1964). However, Renner and Ando (1974) used the Pro-Milk difference

method of McGann *et al.* (1972) and found a different regression equation for measuring whey protein concentration (whey protein in gL^{-1} = 7.641 PMD + 0.44).

The accuracy and precision of the various dye binding methods of measuring the casein concentration of milk are very good. McGann *et al.* (1972) obtained standard deviations of the difference between the dye binding and Kjeldahl analysis methods of $\pm$ 0.46 gL^{-1} and $\pm$ 0.22 gL^{-1} for whey protein and casein estimations of bulk milks respectively and a s.d. of $\pm$ 0.46 gL^{-1} for casein estimation of individual milks. Mickelsen and Shukri (1975) found that their dye binding method was more precise than methods utilizing Kjeldahl analysis and formol titration.

(iii) The method selected and modifications required for the present study

In the present study a rapid, convenient and precise method of measuring the casein and whey protein concentrations in small samples of milk from a large number of individual cows was required. The most suitable method available was the Pro-Milk difference method of McGann *et al.* (1972), but various modifications to the method were necessary.

In the method of McGann *et al.* (1972) the casein and fat are co-precipitated by the addition of acetic acid and sodium acetate solutions to 100 mL of whole milk at 40°C and the whey obtained by filtration. Three readings per milk sample are carried out on the Pro-Milk Mk. II instrument -

Reading I - 1 mL of milk plus 20 mL of dye solution

Reading II - 1 mL of milk plus 1 mL of distilled water plus 20 mL of dye solution

Reading III - 1 ml of milk plus 1 mL of whey plus 20 mL of dye solution.

Reading I gives the total protein concentration of the milk.

Reading II is used to determine the dilution of excess dye resulting when both milk and whey are mixed with the dye solution, and this is subtracted from Reading III to give the Pro-Milk difference (PMD) an uncorrected measure of the whey protein concentration.

In the present study, the method of McGann *et al.* (1972) was modified by (1) reducing the volume of milk required, (2) precipitating the casein from skim milk rather than whole milk, (3) using a single addition of acetic acid - sodium acetate buffer and (4) adding a centrifugation step. Modifications (2) and (4) were necessary to obtain casein for quantitative analysis as well as whey for protein determination. The method of converting the Pro-Milk readings to whey protein concentration and the necessity of the three Pro-Milk readings were reassessed.

Data on the casein concentration in milk from only 8 individual cows was presented by McGann *et al.* (1972) whereas in the present study all determinations were on milk from individual cows, so information on the accuracy of the dye binding method for estimating casein and whey protein concentrations of milk from individual cows has been extended.

C. The Present Investigation: Estimation of Total Casein and Whey Protein Concentration

1. Experimental

(i) Milk sampling and analysis

Individual cow samples were collected from the Northfield Research Centre herd on three occasions. The presence of subclinical mastitis was tested using the Wisconsin Mastitis Test (Thompson and Postle 1964) and samples with a score of more than 15 mm were discarded. The casein and non-casein fractions were separated by a different method

with each of the three collections, as outlined below. Duplicate Kjeldahl determinations were carried out on the whey, using the method described by McKenzie and Wallace (1954), except that the amount of K_2SO_4 was increased to 2.0g (this was found necessary with the burners used, to increase the digestion temperature) and 0.5 mL of 30% H_2O_2 free of N-containing preservatives was added to the digest mixture to reduce foaming (Dr. H.A. McKenzie, personal communication).

Just before each collection, the volume of dye delivered by the Pro-Milk Mk. II (Foss-Electric, Hillerød, Denmark) was adjusted so that the indicated protein content of a herd milk sample was identical to the crude protein value (TN x 6.38) estimated by duplicate Kjeldahl determinations.

Method A - Whey separation by the method of McGann *et al.* (1972)

Thirty-five individual cow samples were used to prepare whey as described by McGann *et al.* (1972), single Pro-Milk readings RI, RII and RIII were taken and the PMD calculated. No volume correction was applied to the Kjeldahl values of the whey as the 6% dilution of the whole milk by acetic acid and sodium acetate approximately counter-balances the contraction in volume of milk due to the removal of the casein and fat co-precipitate (McGann *et al.* 1972).

Method B - Modified method of whey separation

The method of whey separation of McGann *et al.* (1972) was modified by (1) using skim milk instead of whole milk, (2) reducing the milk volume from 100 mL to 10 mL and (3) introducing a centrifugation step to obtain a casein pellet for further quantitative analysis. This method was used to prepare whey from 51 individual cow samples.

The modified method was as follows -

Whole milk was centrifuged at 3 000 rpm (1 000 *g*) and 2°C for 5 minutes and the skim milk decanted. 10 mL of skim milk was pipetted into a 15 mL centrifuge tube and warmed to 40°C. 0.30 mL of 333 gL^{-1}

w/v solution of acetic acid was added while mixing on a vibrating stirrer, then the stoppered tube was inverted several times for complete mixing. After 10 minutes, 0.30 mL 3.33 M solution of sodium acetate was added, mixing as previously. After about 3 minutes, the tubes were centrifuged at 10 000 rpm (12 000 *g*) and 20°C for 15 minutes and the whey decanted through a 9 cm Whatman No. 41 filter paper.

As with whey samples prepared by method A, single Pro-Milk readings RI, RII and RIII were taken and the PMD calculated.

A correction factor of 0.966 was applied to the whey volume used for Kjeldahl analysis. This factor was experimentally determined: the volume obtained when 6 mL of acid buffer was added to 100 mL skim milk was determined, and then the volume of casein ,which was determined by drying the precipitated casein in a vacuum oven at 102°C for 2 hours and by using displacement, was subtracted to give the actual whey volume.

Method C - Further modifications to method B, using a single addition of acetic acid - sodium acetate buffer

Whey separation method B was further modified by combining the acetic acid and sodium acetate before adding to the milk i.e. 0.6 mL of a 1:1 mixture of 333 gL^{-1} w/v solution of acetic acid and 3.33 M solution of sodium acetate was added to 10 mL of skim milk at 40°C. After waiting at least 10 minutes, the tubes were centrifuged and filtered as described for method B.

Whey from 33 individual cow samples was prepared, single Pro-Milk readings RI and RIII were taken, RII calculated using a regression equation (see later) and the PMD calculated.

Kjeldahl determinations were carried out on the skim milk, whey and a 120 gL^{-1} trichloracetic acid filtrate (NPN). The whey volume used for Kjeldahl analysis was multiplied by a correction factor of 0.966, determined as described previously.

Prior to using this modified method of whey separation, the effect of adding a single volume of acetic acid - sodium acetate buffer was assessed.

When the volumes and concentrations of acetic acid outlined by International Dairy Federation (1964) and McGann *et al.* (1972) were added to 100 mL of whole milk at 40°C, the pH of the mixtures measured after 10 minutes were approximately 4.4 and 4.3 respectively. After addition of the respective volumes and concentrations of sodium acetate, the pH increased to approximately 4.8 and 4.7. A final pH of approximately 4.7 was measured when 6.0 mL of a 1:1 mixture of 333 gL^{-1} w/v acetic acid and 3.33 M sodium acetate was added to 100 mL of skim milk at 40°C.

By adding the acetic acid and sodium acetate together, the PMD of the whey was found to be 2 gL^{-1} higher than when the casein was precipitated by adding the same acetic acid and sodium acetate solutions separately. When 1N hydrochloric acid was added to skim milk at 20°C to a final pH of 4.60, the PMD was 5 gL^{-1} higher than the PMD measured using whey obtained by adding acetic acid and sodium acetate solution separately.

(ii) Simplification of Pro-Milk readings

With the aim of reducing the work required to determine the casein concentration, the need to carry out reading RII on the Pro-Milk was investigated. Using the data from casein determinations on the milk from 147 individual cow samples collected over several years from the Northfield Research Centre herd, the relationship between RII and the total protein concentration (RI) was determined.

2. Results

(i) Modification of the correction factor

The mean and range of crude protein concentrations determined using the Pro-Milk Mk. II, and whey protein concentrations determined

by Kjeldahl analysis on the whey filtrate (KWP) of the milk from the three samplings are shown in Table III.1. The mean difference between the Kjeldahl protein values of the whey filtrate and the PMD, for the three methods of whey preparation, are also shown in Table III.1.

The differences, KWP-PMD, were significantly ($P < 0.001$) related to the total protein concentration of the milk, with each method of whey separation (see Table III.1.).

This relationship was used to correct the Pro-Milk whey protein determinations instead of using a simple mean difference as used by McGann *et al.* (1972). The rearranged equations used to calculate whey protein concentration are shown as Equation 1 in Table III.1. The accuracy (mean difference between Kjeldahl and Pro-Milk estimations $\pm$ standard deviation of the difference) of Pro-Milk estimations of whey protein and casein concentrations, corrected using the simple mean differences and using regression equations 1, are shown in Table III.2.

With samples collected for whey separation methods A and B the only Kjeldahl analysis was on the whey filtrate and so the accuracy of the Pro-Milk casein estimation could not be determined. However, the samples collected for whey separation by method C were also used for Kjeldahl analyses of the skim milk to give crude protein and of the 120 gL^{-1} T.C.A. filtrate to enable calculation of true protein.

The mean difference between the crude protein concentration determined by Kjeldahl analysis and using the Pro-Milk was + 0.16 $\pm$ 0.453 gL^{-1} corresponding to a coefficient of variation (c.v.) of 1.2%, referred to the mean crude protein concentration. The mean difference between Kjeldahl true protein and Pro-Milk protein estimations was + 1.69 $\pm$ 0.371 gL^{-1} (c.v. = 1.0%).

The casein concentration of the skim milk was calculated for both Kjeldahl and Pro-Milk methods as the difference between their respective

TABLE III. 1

The protein content of whey samples[a] prepared by 3 methods and regression equations to correct the Pro-Milk readings

	Method of whey separation		
	A	B	C
Number of milk samples	35	51	33
Mean total protein[b]	35.7 ± 2.58[d]	35.1 ± 3.01	37.4 ± 3.60
Range of total protein	31.4 – 43.8	26.9 – 43.7	29.3 – 43.0
Mean whey protein[c]	8.0 ± 0.78	8.0 ± 0.79	8.3 ± 1.12
Range of whey proteins	6.6 – 10.1	6.6 – 10.3	6.6 – 10.9
Mean difference: Kjeldahl whey protein (KWP) – Pro-Milk difference (PMD)	0.84 ± 0.39	1.19 ± 0.45	1.39 ± 0.55
Relationship between KWP-PMD (y) and crude protein (RI): y =	0.11 RI-2.89	0.10 RI-2.32	0.12 RI-3.09
Regression coefficient (r)	0.70	0.67	0.78
Equation 1: Whey protein (using R II) =	PMD+0.11RI-2.89	PMD+0.10RI-2.32	PMD+0.12RI-3.09
Equation 2: Whey protein (without R II) =	RIII - 0.857RI - 4.90	RIII - 0.867RI - 4.33	RIII - 0.847RI -5.10

[a] All protein values are expressed as gL^{-1}

[b] Crude protein values determined by Pro-Milk Mk II

[c] Whey protein values determined by Kjeldahl analysis of the whey filtrate (N x 6.38)

[d] Standard deviation

TABLE III. 2

The accuracy of Pro-Milk estimates of casein and whey protein content using various methods of correction

Method of correction of Pro-Milk values	Whey protein (gL^{-1})			Casein (gL^{-1})
	A	B	C	C
Mean Kjeldahl whey protein (KWP)[a]	8.01	8.27	8.32	28.93[b]
Using mean difference from KWP				
Mean Pro-Milk (PM1)[c]	7.97	8.28	8.33	29.08[f]
Mean difference (KWP-PM1)	+0.04	-0.01	-0.01	-0.15
S.D. of difference	±0.39	±0.45	±0.55	±0.48
c.v.[g]	4.9%	5.4%	6.6%	1.7%
Using regression equations 1				
Mean Pro-Milk (PM2)[d]	8.03	8.28	8.33	29.08
Mean difference (KWP-PM2)	-0.02	-0.01	-0.01	-0.15
S.D. of difference	±0.28	±0.34	±0.34	±0.50
c.v.	3.5%	4.1%	4.1%	1.7%
Using regression equations 2				
Mean Pro-Milk (PM3)[e]	8.22	8.31	8.32	29.09
Mean difference (KWP-PM3)	-0.21	-0.04	0.00	-0.16
S.D. of difference	±0.27	±0.34	±0.35	±0.50
c.v.	3.3%	4.1%	4.2%	1.7%

[a] Kjeldahl analysis of whey filtrates prepared with minimal dilution (N x 6.38)

[b] Casein was calculated as the difference between Kjeldahl protein (TN x 6.38) and Kjeldahl whey protein content

[c] Whey protein contents determined by adding the respective mean differences (KWP-PMD) to the PMD (for mean differences, see Table III. 1)

[d] Whey protein contents determined from PMD and RI using the respective regression equations 1 in Table III. 1

[e] Whey protein contents determined without taking reading RII and calculated using the respective regression equations 2 in Table III. 1

[f] Pro-milk estimates of casein content were calculated as the difference between Pro-Milk total protein and whey protein contents

[g] Coefficient of variation = standard deviation of the difference divided by the mean Pro-Milk whey protein content

total crude protein and whey protein concentrations and so gives true casein concentration. The mean difference and standard deviation of the difference between Kjeldahl and Pro-Milk methods of determining casein concentration, after whey protein concentration had been corrected using either the mean difference or the regression equation relating KWP-PMD with crude protein (Equation 1) are shown in Table III.2.

(ii) Simplification of Pro-Milk readings

With milk samples from individual cows which ranged in protein concentration from 25.5 to 48.0 gL^{-1} the mean and standard deviation of the difference between Pro-Milk readings RII and RI was 0.87 ± 0.17 gL^{-1}. The difference between RII and RI (total protein)was significantly ($P < 0.001$) related to the total protein concentration of the milk -

$$y = 2.01 - 0.033\ x \qquad (r = -0.76)$$

where

y = RII - RI

and x = RI (total protein)

i.e.

$$RII = 0.967\ RI + 2.01$$

This regression equation was combined with equations 1 in Table III.1 to give equations 2, also shown in Table III.1., which can be used to calculate the whey protein concentration without taking reading RII.

Table III.2. shows the effect of eliminating reading RII on the accuracy of the Pro-Milk estimates of whey protein and casein concentrations of skim milk.

(iii) Investigation of atypical milks

The largest difference between the Kjeldahl whey protein concentration and uncorrected PMD for all samples in the three collections (a total of 119 milk samples) was 2.6 gL^{-1} (the largest difference after correction of the PMD using the appropriate regression equation was

0.9 gL^{-1}). However, prior to the three sample collections described in this experiment, uncorrected differences of up to 4.5 gL^{-1} had been found (corrected difference of 2.4 gL^{-1}). Milk samples which gave these large differences between estimates of whey protein by Kjeldahl and Pro-Milk determinations were from cows in late lactation with very high total protein concentrations. In the above case, where the uncorrected difference was 4.5 gL^{-1}, the milk was from a Jersey cow in its tenth month of lactation producing very little milk which contained 47.7 gL^{-1} total protein.

It was thought that these high protein, late lactation milks may contain high levels of non-protein nitrogen (NPN) which does not bind dye, or high levels of immunoglobulins which have a lower dye binding capacity than the major whey proteins, β-lactoglobulin and α-lactalbumin. Milk samples from 6 cows selected to give a range of (KWP-PMD) values were analysed for NPN and 3 samples were analysed for whey protein distribution (Aschaffenberg and Drewry 1959). No obvious relationship was found between (KWP-PMD) and whey composition. Further work is required to determine why whey prepared from high protein, late lactation milk has abnormal dye binding properties.

3. Discussion

In the present study the Pro-Milk was calibrated to give the same reading as the crude protein concentration determined by Kjeldahl (N x 6.38). Although White (1980) has suggested multiplying by the factor of 6.29, the factor of 6.38 has been retained in the present study because of its universal acceptance. The mean difference between crude protein estimates by Kjeldahl analysis and Pro-Milk of + 0.16 $\pm$ 0.45 gL^{-1}, obtained with milk from individual cows, indicates that the Pro-Milk estimations in the present study are nearly as accurate as

those of McGann *et al.* (1972) obtained using bulk milk samples (-0.05 $\pm$ 0.38 gL^{-1}) and superior to the results of Sherbon (1974) who analysed milks from individual cows (0.27 $\pm$ 1.09 gL^{-1}). However, it should be noted that in the present study, samples were collected a short time after the Pro-Milk had been calibrated, whereas in the studies of McGann *et al.* (1972) and Sherbon (1974) milk samples were collected over a period of one and two years respectively, and so a number of calibrations would have been carried out during these periods.

As would be expected, a larger difference was obtained between the Pro-Milk estimate and true protein measured by Kjeldahl analysis, due to the method of Pro-Milk calibration. The difference, + 1.69 $\pm$ 0.371 gL^{-1}, reflects the non-protein nitrogen content of the milk (mean of 1.70 $\pm$ 0.167 gL^{-1} expressed as 'protein' i.e. NPN x 6.38). This mean NPN value is lower than the mean NPN, expressed as 'protein', of 2.23 gL^{-1} found by McDowell (1972) for Friesian milk in New Zealand.

Many workers have ignored NPN and use Kjeldahl total nitrogen x 6.38 to calculate the protein concentration of samples for Pro-Milk standardization (see Kiddy 1963, Sherbon 1978). Heinrich (1978) has recommended using crude protein in preference to true protein as the reference method for calibrating rapid routine methods. Determination of total protein in milk by Kjeldahl analysis (Standards Association of Australia 1974) ignores the NPN content, and a recent draft Australian Standard for determination of protein in milk by dye binding methods describes both alternatives without making a definite recommendation.

However, a number of authors have recently recommended that true protein concentration should be determined for standardization (McDowell 1972, Clarke 1979). Certainly it is more logical to standardize to true protein as dye binding methods are not affected by NPN. In fact, since the present study was carried out, the Pro-Milk in the authors laboratory

has been standardized by adjustment to the true protein concentration determined by Kjeldahl analysis.

Kjeldahl analysis of whey filtrates gives an estimate of (whey protein nitrogen + NPN) x 6.38 and as the Pro-Milk values are corrected to this Kjeldahl estimate using a regression equation, the Pro-Milk 'whey protein' concentrations also include NPN. Subtraction of whey protein concentration from crude protein will, therefore, give an estimate of true casein concentration. When the Pro-Milk is standardized to give true protein concentration, the correction of the Pro-Milk whey protein concentrations to give true whey protein is necessary, so that true casein can be obtained by difference.

The isoelectric point of casein is usually considered to be pH 4.6 - 4.7 (Rowland 1938a). At the isoelectric point, solubility of protein is at a minimum and this is utilized in the acid precipitation of casein from milk. However, casein is not homogeneous, but consists of four proteins, each of which may be present in different forms, and a single pH giving complete precipitation of all caseins, without precipitation of whey proteins, cannot be specified. The problems of defining casein were discussed in Chapter I.

Addition of acetic acid to whole milk at 40°C as in the method of Rowland (1938b), International Dairy Federation (1964), Standards Association of Australia (1974) and McGann *et al.* (1972), resulted in a pH below the isoelectric point and the pH after subsequent addition of sodium acetate solution was above the isoelectric point. The pH of 4.7 measured after the addition of acetic acid-sodium acetate buffer was the same as the final pH when acetic acid and sodium acetate were added separately in the method of McGann *et al.* (1972).

Addition of acetic acid - sodium acetate buffer resulted in a higher PMD of the whey than when they were added separately, and

adding hydrochloric acid to skim milk at 20°C as in the definition of casein (Whitney *et al.* 1976) resulted in an even higher PMD of the whey. This indicates that either casein precipitation was incomplete when using these two methods or that some whey proteins were being precipitated with casein when acetic acid was added or to a lesser extent by the acetic acid-sodium acetate buffer and which did not re-dissolve on the addition of sodium acetate. Rowland (1938a) studied conditions of maximum protein precipitation, without investigating the specific proteins precipitated and concluded that addition of acetic acid and sodium acetate at the same time resulted in incomplete casein precipitation. However, by adding the two solutions together, the amount of protein precipitated is closer to that precipitated by conditions defined by Whitney *et al.* (1976).

In method A, where whey was prepared as described by McGann *et al.* (1972), the PMD was on average 0.84 $\pm$ 0.39 gL^{-1} lower than the Kjeldahl protein value. McGann *et al.* (1972) found that the PMD values were 0.7 gL^{-1} lower on average than the Kjeldahl protein values of whey prepared without significant dilution and 0.4 gL^{-1} lower than the Kjeldahl protein values on diluted wheys prepared using the International Dairy Federation (1964) standard method. These authors used 0.4 gL^{-1} as a correction factor to obtain the Pro-Milk whey protein concentration.

When modifications were made to the method of whey separation of McGann *et al.* (1972), the difference between the PMD and the respective Kjeldahl whey protein value increased, and so the adjustment necessary to correct to the Kjeldahl whey protein value was larger (see Table III.1.). This is possibly reflected in the increased standard deviation of the difference between Kjeldahl and Pro-Milk estimations of whey protein concentration, with all methods of correction of the Pro-Milk data, as shown in Table III.2.

The accuracy, expressed as mean ± standard deviation of the difference between Kjeldahl and Pro-Milk estimations, are not strictly comparable to the results of McGann *et al.* (1972) as they used the International Dairy Federation (1964) Kjeldahl method as a standard, whereas in the present study Kjeldahl determinations were made on the whey prepared with minimal dilution. Using the method of casein separation of McGann *et al.* (1972) and correcting the PMD by adding the mean difference from the Kjeldahl protein value, the mean ± s.d. of the difference from the Kjeldahl protein value was + 0.04 ± 0.39. McGann *et al.* (1972) did not publish any whey protein values for individual cows, but the mean and s.d. of the differences in whey protein concentration estimated by Kjeldahl analysis and by using the Pro-Milk, for 24 bulk milk samples, was - 0.04 ± 0.46 gL^{-1}. For casein concentration in milk from 8 individual cows, the mean difference and s.d. was + 0.16 ± 0.46 gL^{-1}. McGann *et al.* (1972) found that Pro-Milk estimates of whey protein were not as accurate as estimates of casein and that estimates of casein in individual cow milk was not as accurate as those of bulk milks. Therefore the accuracy of the method of McGann *et al.* (1972), determined in the present study, compares very favourably with that determined in the original study.

An improvement in the accuracy of the method of determining whey protein concentration results if a regression equation relating the difference (KWP-PMD) with Pro-Milk total protein concentration is used to correct the PMD values instead of using the simple mean difference (see Table III.2.). This improvement in accuracy occurred with all methods of casein separation. The difference, KWP-PMD, is also significantly ($P < 0.01$) related to stage of lactation ($r = 0.47$). Total protein concentration increases with lactation length, and protein composition changes, with increasing proportions of serum albumin and

immunoglobulins towards the end of lactation. As these proteins have a lower dye binding capacity this results in an increase in the difference, KWP-PMD.

By utilizing the relationship between the (Reading II - Reading I) and the total protein concentration (Reading I), whey protein concentration can be obtained by making only two Pro-Milk readings instead of three used by McGann *et al.* (1972). The accuracy of the method was not reduced; in fact, the standard deviation of the difference between mean Kjeldahl and corrected Pro-Milk whey protein concentrations are the same or slightly less than when three Pro-Milk readings were taken. However, with one method of casein separation (Method A - that of McGann *et al.* 1972) the mean difference was larger.

When the casein concentration was calculated for milk samples used for method C, it was found that accuracy was not improved by using a regression equation instead of the simple mean difference to correct the whey protein concentrations. The accuracy of the Pro-Milk determination of casein using the modified method C in the present study ($- 0.16 \pm 0.50$) was not as good as found by McGann *et al.* (1972) for bulk milks ($- 0.04 \pm 0.46$), but is similar to that found for milk from milk from individual cows ($+ 0.16 \pm 0.46$).

In the present study a simple method of measuring casein and whey protein concentration in large numbers of milk samples from individual cows, and which the separated casein could be used for further analysis, was required. Method C, where casein was precipitated by addition of acetic acid-sodium acetate buffer to skim milk at $40^{\circ}C$ and the casein was separated by centrifugation, was the method selected for use in the study described in Chapter V. If the casein fraction was not required, the method could be simplified by adding the acetic acid-sodium acetate buffer to whole milk at $40^{\circ}C$ and omitting the centrifugation step. In

this case it would be necessary to re-determine the relationship between the PMD and Kjeldahl analysis of the whey filtrates. However the difference in whey protein concentration calculated with different regression equations is not large: using extreme total protein and PMD values obtained in the experiment described in Chapter V, and calculating whey protein concentration using regression equations for Methods A and C, the same whey protein values were found at low total protein concentrations (25 gL^{-1}) and a 2.6% difference resulted at high total protein concentrations (50 gL^{-1}).

CHAPTER IV

DETERMINATION OF INDIVIDUAL PROTEINS OF MILK

A. Caseins

1. Introduction

In milk, the individual caseins are bonded together to form the casein micelle, and in most methods of estimating casein composition the micelle must be dissociated into the casein monomers. Wake and Baldwin (1961) disaggregated casein using concentrated solutions of urea, revealing many new components on starch gel electrophoresis. However, κ-casein was still present as a smear: use of treatment to break disulphide bonds by Mackinley and Wake (1964), Neelin (1964), Schmidt (1964) and Woychik (1964) resulted in many distinct bands.

Urea, the disaggregating agent most commonly used during fractionation of casein by electrophoresis or column chromatography, ruptures hydrogen, hydrophobic and salt linkages. To prevent κ-casein from reaggregating into disulphide bonded polymers, 2-mercaptoethanol or dithiothreitol is used. Urea and E.D.T.A. were used by Manning *et al.* (1971) when estimating κ-casein concentration by sulphydryl analysis.

In the methods which rely on amino acid release by carboxypeptidase and macropeptide release by chymosin, the casein micelle was not dissociated as carboxypeptidase and chymosin are able to move freely within the porous structure of the casein micelle (Ribadeau Dumas and Garnier 1970).

Several recent reviews have mentioned the lack of suitable accurate and precise methods for the estimation of the concentration

of the individual milk proteins, particularly the caseins (Jenness 1970, Wheelock *et al.* 1972, Alais and Blanc 1975).

2. Methods Used to Determine Casein Composition

(i) Quantitative electrophoresis

Various methods of electrophoresis, including moving boundary and zone electrophoresis in different media have been the most commonly used methods of estimating casein composition of milk.

(a) Moving boundary electrophoresis

Moving boundary electrophoresis has been used to determine individual caseins quantitatively, giving values which range from 65-75% α-casein, 22-32% β-casein and 3-9% γ-casein (Mellander 1939, Hipp *et al.* 1952, Rolleri *et al.* 1956, Larson and Kendall 1957). The method is tedious, requires expensive equipment and does not estimate α_{s1}-, α_{s2}- and κ-casein. Waugh and von Hippel (1956) separated casein fractions by calcium precipitation at 37°C and using moving boundary electrophoresis estimated that casein contains very approximately 55% α-casein, 30% β-casein and 15% κ-casein.

(b) Zone electrophoresis

Zone electrophoresis is a more convenient and versatile method than moving boundary electrophoresis. Paper was the first medium used for zone electrophoresis but resolution is poor compared to more recently developed methods utilizing starch and polyacrylamide gel. Both starch and polyacrylamide gels have been used for quantitative electrophoresis of casein and when considering resolution and band sharpness neither appears to be superior. The major advantage of polyacrylamide gel is that it is transparent, so can be scanned by transmission densitometry without further treatment. However, protein bands separated by starch gel electrophoresis,

where the background gel is opaque, may be estimated using reflectance densitometry (Murphy and Downey 1969), by scanning photographic transparencies of the rinsed gels (Kim and Bird 1972) or by rendering the background gel transparent (Michalak 1973a).

Protein bands separated by electrophoresis are most commonly stained and scanned with a densitometer, although unstained bands can be estimated using nephelometry (Lontie *et al.* 1963), ultraviolet absorbance (Watkin and Miller 1970) or fluorescence emission of aromatic amino acids (Easton *et al.* 1971). The major source of error in quantitative densitometry of stained protein bands is variation in the amount of dye bound by the components of the protein mixture: many studies of casein composition have failed to correct for these differences.

The casein composition of milk estimated using quantitative zone electrophoresis is summarised in Table IV.1. It is difficult to compare the results of these studies because different samples and methods of preparation were used and the degree of protein separation and peak splitting were different. However, the wide variation in composition found should be noted. Only MacRae and Baker (1958) and Michalak (1973a) applied a correction for dye-binding differences: many estimations of κ-casein by electrophoretic means are low compared with other methods of estimation, due to failure to correct for differences in dye binding capacities of the caseins.

(ii) Column chromatography

(a) Gel filtration

Yaguchi *et al.* (1968) used Sephadex G-150 gel filtration in tris-citrate-urea buffer, to prepare κ-casein and found that based on peak areas, uncorrected for differences in absorbance, acid casein contained 13% κ-casein. This gel filtration method was used by

TABLE IV. 1

Casein composition of bovine milk as determined by quantitative zone electrophoresis

Medium	Casein composition (as % of total casein)[a]								Reference
	α	α_s	α_{s1}	α_{s2}	β	γ	κ	$\gamma+\kappa$[b]	
Paper	60				32	8			MacRae and Baker(1958)
	55				39	6			Ganguli and Bhalerao (1963)
		46			42			12	Pavel *et al.* (1966)
Starch		54			41			5	Morr *et al.*(1971)
		46			35	8	11		Mariani(1972)
		48			37	1	15		Kim and Bird(1972)[c]
			33	14	38			16	Michalak(1973a)[d]
Polyacrylamide		56			32			12	Maki *et al.* (1971)
		59			37			4	Dill *et al.*(1972)[e]
Pre-α_1[f]			48	7	38			6	Haenlein *et al.* (1973)[g]
			50	6	35			8	Ramachandran *et al.* (1973)[h]
		51			44			3	Randolph *et al.* (1974)[i]
		48-52			37-41			9-13	Rothenbuhler(1974)[j]

Footnotes:

[a] All figures rounded to the nearest percent

[b] In urea containing gels, where proportions of both κ- and γ-casein have not been given, this fraction has been called γ- + κ-casein

[c] Casein prepared by method B or C

[d] Skim milk samples used; relative casein concentrations recalculated as % of total casein

[e] Figures quoted are for total machine milk samples

[f] Fast moving protein bands ahead of α_{s1}-casein

[g] Figures quoted are Holsteins with a leucocyte content <250 000 per mL

[h] Ammonium sulphate casein

[i] WMT negative milk

[j] Skim milk samples used, but whey proteins ignored - so semi-quantitative

Creamer and Berry (1975) to estimate κ-casein in column effluents and by Davies and Law (1977b) to confirm κ-casein concentrations obtained by ion-exchange chromatography. In Table IV.2 the proportion of κ-casein found by gel filtration is compared with the casein composition found by methods other than electrophoresis.

(b) Anion-exchange chromatography

Ribadeau Dumas *et al.* (1964), Mercier *et al.* (1968), Rose *et al.* (1969) and El-Negoumy (1976) have published methods of estimating casein composition by DEAE - cellulose column chromatography in urea. With these methods resolution was incomplete. DEAE-Sephadex A-25 has also been used (Hladik and Kas 1973), but separation was poor.

Davies and Law (1977a) modified the method of Rose *et al.* (1969) by using an improved ion exchanger, Whatman DEAE-cellulose DE52 and changing the buffer and method of protein determination. Excellent resolution of α_{s1}-, α_{s2}-, β-, γ- and κ-caseins was obtained but electrophoresis patterns indicated that the κ-casein fraction contained an unknown impurity and so κ-casein was overestimated by 10-30%. Davies and Law (1977b and 1980) applied this method to study casein composition of factory, herd and individual cow samples.

(c) Cation-exchange chromatography

Annan and Mason (1969) treated whole casein with mercaptoethanol and obtained good separation of fractions by chromatography on a column of sulphoethyl Sephadex C-50 in the presence of 8 M urea. No quantitative results were presented.

Snoeren *et al.* (1977) obtained excellent separation of α_{s2}-caseins, κ-casein and other caseins using Amberlite CG 50 and step-wise elution with EDTA-NaCl-urea buffers. Estimates of α_{s2}- caseins and κ-casein contents were 8.4% and 10.8% respectively.

TABLE IV. 2

Casein composition of bovine milk as determined by methods other than electrophoresis

Method	Casein composition (as % of total casein)						Reference
	α_s	α_{s1}	α_{s2}	β	γ	κ	
Column chromatography -gel filtration						13	Yaguchi *et al.* (1968)
-anion exchange		38	6	30		11	Ribadeau Dumas *et al.* (1964)
	40			30	5	15	Mercier *et al.* (1968)
	50			33		15[a]	Rose *et al.* (1969)
	37-55			28-41	3-9	12-18	Hladik and Kas (1973)
	49			34	3	13	El-Negoumy (1976)
		38	10	36	3	13	Davies and Law (1977a,b)
	52			33		15	Ekstrand and Larsson-Raznikiewicz (1978)
-cation exchange			8			11	Snoeren *et al.* (1977)
-hydroxyapatite	52			35		14	McGann *et al.* (1979)
	49			34	5	10[b]	Barry and Donnelly (1980)
Sialic acid estimation						16	Sullivan *et al.* (1959)
						11-26	Marier *et al.* (1963)
Sialic acid and turbidimetric estimation	49-60			19-36		10-23	Tessier *et al.* (1963)
Amino acids released by carboxypeptidase		44-47	7	30-36		11-12[c]	Ribadeau Dumas (1968) Ribadeau Dumas *et al.* (1975)
Sulphydryl group analysis						23[d]	Manning *et al.* (1971)
Laurell's immunoelectrophoresis						27-30[e]	Thymann (1972)
Macropeptide release by chymosin						19	Alais (1963)
						18	Kirchmeier (1967)
						4-9[f]	Creamer *et al.* (1973)
						11-13	Hossain and Gravert (1976)

[a] Includes γ-casein

[b] Also 3% unidentified components

[c] 3-4% para-κ-casein was also measured

[d] Also includes α_{s2}-casein

[e] Recalcuated as a % of casein

[f] Determined in micelle size fractions - small micelles contained 8-9% κ-casein and large micelles 4-6% κcasein

(d) Hydroxyapatite chromatography

Using column chromatography on hydroxyapatite with buffer containing phosphate, urea and dithiothreitol, Donnelly (1977) obtained good separation of α_s-, β- and κ-caseins. This method was used by McGann *et al.* (1979), and Barry and Donnelly (1980) to determine casein composition. A major disadvantage of this method is its failure to separate α_{s1}- and α_{s2}- caseins, two distinctly different protein groups.

In general, column chromatography methods tend to be slow and not suitable for analysing large numbers of samples. Possibly the ion-exchange chromatography method of Davies and Law (1977a), and for α_{s2}- and κ-casein, the method of Snoeren *et al.* (1977), could be used as reference methods for other less accurate but more rapid methods.

(iii) Sialic acid and turbidimetric determinations

Sullivan *et al.* (1959) and Marier *et al.* (1963) used sialic acid (N-acetylneuraminic acid) as an index of κ-casein concentration in bovine skim milk. However, these estimates are unreliable due to variation in the proportion of carbohydrate rich components.

Tessier *et al.* (1963) estimated the amount of β- + κ-casein in whole casein by turbidimetric measurements. By subtracting the κ-casein content determined by sialic acid analysis (Marier *et al.* 1963) the β-casein concentration was obtained. However, turbidity measurements are subject to errors caused by presence of other caseins and by unidentified changes in casein components (Rose *et al.* 1969).

(iv) Analysis of amino acids released by carboxypeptidase

Ribadeau Dumas (1968) measured the C-terminal amino acids released by the action of carboxypeptidase A to determine the α_{s1}-, β-, κ- and para-κ-casein concentrations of whole casein. In addition,

Ribadeau Dumas *et al.* (1975) also measured the concentration of the α_{s2}- caseins. This method could be subject to many errors, as it depends on the quantitative cleavage of the C-terminal amino acids, and also any non-specific proteolysis or contaminating peptides could give rise to false results. It is relatively time consuming and requires specialized and expensive equipment.

(v) Sulphydryl group analysis

Manning *et al.* (1971) outlined a procedure for estimating κ-casein concentration by sulphydryl group analysis, and found a mean κ-casein content of 22.5% for 25 bulk skim milk samples. However, the α_{s2}- caseins also contain sulphydryl groups and so this estimate agrees fairly well with the 19.2% obtained by Snoeren *et al.* (1977) for α_{s2}- + κ-casein.

(vi) Immunological methods

The immuno electrophoretic method of Laurell (1966) was used by Thymann (1972) to estimate κ-casein content of skim milk. The κ-casein content was found to be much higher than previous estimates (26.6 - 29.9% of total casein assuming 80% casein content of milk protein). Possibly differences in the state of aggregation between the κ-casein standard and the casein micelles caused different diffusion rates in the agarose gel as no reducing agents were used. Another disadvantage with this approach is the problem of preparing specific antisera due to the difficulty in preparing immunologically pure individual caseins and to their poor antigencity.

(vii) Macropeptide release by chymosin

Several workers have estimated κ-casein concentration in milk by measuring macropeptides released by chymosin action (Alais 1963, Kirchmeier 1967, Creamer *et al.* 1973, Hossain 1976 and Beeby 1980).

Variation in details of the method have resulted in wide variation in κ-casein contents obtained (see Table IV.2.). The earlier methods relied on Kjeldahl analysis to measure the increase in nitrogen soluble in 2-3% T.C.A., but Beeby (1980) reacted the T.C.A. filtrate with fluorescamine at pH 6.0 and measured the fluorescence, so eliminating the Kjeldahl analysis. Under the conditions employed by Beeby (1980), all available κ-casein was cleaved and other casein components were not measurably affected by chymosin. The method gave reproducible results and was capable of detecting variations of the order of 5% in the level of κ-casein. Unfortunately, only absolute κ-casein contents were presented by Beeby (1980), so comparison with other methods could not be made.

3. Summary of Methods for Determining Casein Composition and Selection of the Method for the Present Study

Earlier electrophoretic methods (moving boundary, paper, agar) are inferior in resolution to starch and polyacrylamide gel electrophoresis. For quantitative work, polyacrylamide has the advantage of being transparent, although methods are available for use with starch gels. Improved resolution of the caseins, use of standards and allowance for differences in dye binding capacities between proteins have resulted in quantitative gel electrophoresis becoming increasingly accurate and precise. Also it is suitable for routine use with large numbers of samples. However, many workers do not allow for differences in dye binding capacities: there is a need for further work on the relative dye binding capacities of caseins and their genetic variants.

Of the other methods of determining casein composition, several can be discarded because of inherent problems (sialic acid and turbidimetric analysis, immunological methods, sulphydryl analysis).

Ion exchange chromatography (e.g. the method of Davies and Law 1977a) appears to give a realistic estimate of casein composition but is time consuming.

Apart from gel electrophoresis and ion exchange chromatography, the only other method which provides complete casein composition is the analysis of amino acids released by carboxypeptidase, and this is also time consuming and requires expensive, specialised equipment.

Gel filtration and chromatography using Amberlite CG 50, although time consuming, could be used to estimate κ-casein concentration. Beeby's (1980) method of estimating κ-casein by release of macropeptide by chymosin is rapid and sensitive.

Although Davies and Law (1980) found little variation in the relative amounts of individual caseins in factory milk samples, considerable variation was found in composition of individual cow milk samples (Davies and Law 1977a). However, a reasonable estimate of bovine casein composition, based on the results of Michalak (1973a), Yaguchi *et al.* (1968), Davies and Law (1977a,b, 1980) Snoeren *et al.* (1977) and Hossain and Gravert (1976) is approximately 39% α_{s1}- caseins, 10% α_{s2}- caseins, 35% β-casein, 3% γ-casein and 13% κ-casein.

For the purposes of the present study, a method for the determination of the casein composition of large numbers of milk samples from individual cows was required, and quantitative gel electrophoresis was selected. Because polyacrylamide gel is transparent, it was selected in preference to starch. The method should be simple and convenient to use and horizontal gels with a continuous buffer system appeared most suitable. Also, resolution of the caseins and their genetic variants should be such that casein is separated into its major components for quantitation and genetic variants (except β-casein A variants) are identifiable. However, gel

electrophoresis does have problems when used for quantitative analysis, particularly in obtaining a linear relationship between dye and protein concentration and because of different dye binding capacities of caseins and their genetic variants, and these aspects were investigated in some detail.

B. Whey Proteins

1. Introduction

As with the estimation of casein composition numerous methods have been used to estimate the concentrations of the whey proteins, including chemical fractionation, various types of electrophoresis, gel filtration, ion-exchange chromatography and immunoassays. Alais and Blanc (1975) and Whitney *et al.* (1976) have compiled tables showing whey protein composition.

2. Methods of Determining Whey Protein Composition

(i) Chemical Separations

Chemical fractionation followed by nitrogen determination was used by Rowland (1938a,b) to determine total albumins, globulins and proteose-peptone concentrations. Values obtained for the globulins and proteose-peptones fractions were overestimated by this method and Ashaffenburg and Drewry (1959) modified the method to give four fractions, the values obtained being comparable to values obtained by moving boundary electrophoresis.

The method of Aschaffenburg and Drewry (1959) has been widely used for measuring non-casein proteins in milk, but is subject to error (Armstrong *et al.* 1967 and Farah 1979). Also, Pearce (1980) has shown that Aschaffenburg and Drewry's (1959) 'total albumin fraction' contains as much as 20% of previously unrecognised heat stable proteins classified tentively as proteose-peptones, and so

revealed a major inaccuracy in Aschaffenburg and Drewry's method of whey protein fractionation.

(ii) Electrophoresis

Moving boundary electrophoresis was used by Larson and Rolleri (1955), Rolleri *et al.* (1956) and Larson and Kendall (1957) to measure concentrations of whey proteins. Electrophoresis on paper (Lecce and Legates 1959, Pavel *et al.* 1966), cellulose acetate (Maki *et al.* 1971, Luck *et al.* 1976, Bell and Stone 1979) in starch (Michalak 1973a) and polyacrylamide gel (Hartman and Swanson 1955, Dill *et al.* 1972, Haenlein *et al.* 1973, Randolph *et al.* 1974, Darling and Butcher 1976, Hillier 1976, Anderson and Andrews 1977) have been used for quantitation of whey proteins. Of these studies, Michalak (1973a), Darling and Butcher (1976), Hillier (1976) and Anderson and Andrews (1977) have allowed for differences in dye binding capacities of the whey proteins.

The use of zone electrophoresis for determining casein composition was discussed in the previous section: the same advantages and problems apply to the use of electrophoresis for measuring whey proteins.

(iii) Column chromatography

Gel filtration and ion-exchange chromatography have been used for the separation of whey proteins and several quantitative methods have been published. Armstrong *et al.* (1970) separated the whey proteins into five fractions by gel filtration on Sephadex G-75 or G-100. The quantitative gel filtration method of Davies (1974) used Sephadex G-100, but separated only the albumin fraction of the whey proteins. Davies and Law (1980) used a similar method to fractionate total whey proteins into 4 fractions with only slightly poorer resolution. A quantitative method using DEAE - Sephadex was

used by Hladik and Kas (1973) but separation was not complete.

(iv) Immunological methods

A number of workers have used immunological methods for the estimation of whey proteins. The Oudin immunodiffusion method was used to measure concentrations of β-lactoglobulin (Larson and Twarog 1961) and α-lactalbumin (Larson and Hageman 1963). The improved radial immunodiffusion method of Mancini *et al.* (1963) has been used to measure the concentration of β-lactoglobulin (McLean *et al.* 1974, Hodate and Johke 1976, Komatsu *et al.* 1977), α-lactalbumin (Hodate and Johke 1976, Komatsu *et al.* 1977, Bartsch *et al.* 1979) and many other minor proteins.

Babajimopoulos and Mikolajcik (1977) compared Oudin's immunodiffusion, Mancini's radial immunodiffusion and Laurell's (1966) immunoelectrophoresis method to measure the concentrations of β-lactoglobulin and serum albumin in milk. Laurell's immunoelectrophoresis method was the most rapid and sensitive method and required the least amount of reagents. Guidry and Pearson (1979) described several improvements to the radial immunodiffusion technique that have reduced the coefficients of variation to below 5% for the milk and serum proteins tested.

Crossed immunoelectrophoresis (Clarke and Freeman 1968) enables many proteins to be estimated simultaneously and does not require a specific antisera. It has been used for the analysis of whey proteins (Lowenstein *et al.* 1975) but no quantitative data was presented.

3. Summary of Methods Used to Determine Concentrations of Whey Proteins

Quantitative gel filtration and ion-exchange chromatography methods are relatively time consuming and have generally not given good separation of whey proteins. Recently published methods of

quantitative polyacrylamide gel electrophoresis (e.g. Hillier 1976, Darling and Butcher 1976) have improved precision and overcome problems of different dye binding capacities. Immuno assays provide a rapid and convenient method for estimating individual proteins in large numbers of samples, the main disadvantage being the necessity of a specific antisera. A method for measuring the concentration of the two major whey proteins, β-lactoglobulin and α-lactalbumin, in large numbers of samples was required, and as suitable antisera were available, radial immunodiffusion was selected.

C. The Present Investigation:

Determination of Individual Proteins of Milk

1. Experimental and Results

(i) Casein composition

(a) Materials and apparatus

Reagents used in this study were generally analytical grade. Sources of less common reagents are listed:

Chymosin - Sigma rennin (E.C. No. 3.4.4.3) crystallized and lyophilized powder from calf stomach.

Urea - The cyanate level of the urea was tested by measuring the absorbance of a 4 M solution over a range of wavelengths and the sample with the lowest absorbance between 220 and 250 nm was used.

Acrylamide - Cyanogum-41, which consists of a mixture of 95% w/w acrylamide monomer and 5% w/w N:N′ methylene-bis-acrylamide from B.D.H. Chemicals Ltd., Poole, England. Canalco ultra pure acrylamide and bis-acrylamide from Miles Laboratories Inc., Elkhart, U.S.A. were also used.

Paper wicks - for application of samples into the gel slab were cut from Whatman 3 MM chromatography paper, Whatman Biochemicals Ltd., Maidstone, England.

Stains - The purity of the amido black (Merck Amido schwarz 10B) and Coomassie blue G250 (Gurr Xylene Brill Cyanin G, Searle Diagnostic), was tested by measuring absorptivity, a, as carried out by Wilson (1979). The a_{620} of amido black (0.01 gL^{-1} dissolved in 10 gL^{-1} acetic acid), was 79.3 and according to Wilson (1973) pure amido black has an absorptivity value of 90.0, so the Merck amido black sample was 80% pure. The wavelength of maximum absorbance of the 0.01 gL^{-1} solution of amido black in 10 gL^{-1} acetic acid was 620 nm, as reported by Wilson (1979).

The a_{605} of Coomassie blue G250 (0.02 gL^{-1} dissolved in methanol) was 55.0 which is slightly higher than the most pure of 3 samples tested by Wilson (1979). The wavelength of maximum absorbance of this Coomassie blue G250 solution was 609 nm, and the wavelength of maximum absorbance of an α_{s1}-casein band stained with Coomassie blue G250 by the method of Diezal *et al.* (1927) was 606 nm.

Milk samples - Herd milk samples and individual cow milk samples of known genetic type were collected from the Northfield Research Centre's grade Friesian herd and used for studies on the gel electrophoresis method for determining casein composition. Milk homozygous for casein genetic variants was obtained from the Northfield herd and a commercial Jersey herd and used for protein preparation.

Electrophoresis apparatus - Three electrophoresis units were used,

(1) a Multiphor unit from LKB Produckter AB, Bromma, Sweden,

(2) a unit constructed from Perspex utilizing LKB glass plates and

(3) a unit having a brass cooling plate coated with Melanex (Paton Industries Pty. Ltd., Stepney, South Australia) as described by Bailey (1968a) and also utilizing LKB glass plates.

Circulating water was maintained at constant temperature using a Lauda refrigerated thermostat (Messgerate-Werk Lauda, West Germany). Initially a Vokam power supply (Shandon Scientific Co. Ltd., London, England) was used, but when electrophoresis of three gels was conducted simultaneously an Ames Model 300 C power supply (Ames Company, Division Miles Laboratories Inc., Elkhart, U.S.A.), which has three power outlets, was used.

Densitometers - Two transmission densitometers were used: a Pye Unicam SP 1800 ultraviolet spectrophotometer fitted with a Pye Unicam SP 1809 scanning densitometer accessory (Pye Unicam Ltd., Cambridge, England) and connected to a Linear Instruments Model 252 A electronic integrating chart recorder (Linear Instruments Corp., Irvine, U.S.A.) and a Helena Quick Scan Junior TLC densitometer (Helena Laboratories Corp., Beaumont, U.S.A.).

(b) Sample preparation

Casein was precipitated from skim milk by method C as described in Chapter III. The volume and concentrations of the imidazole-HCl buffer used to redissolve the casein were chosen to give a casein solution of approximately the same volume as the original skim milk (i.e. 10 mL) and to overwhelm the acetic acid-sodium acetate buffer sufficiently to give a pH value of 6.6 ± 0.03. Under these conditions the casein dissolved readily and gave a final pH value sufficiently low so subsequent cleavage of κ-casein by chymosin was complete.

When separated by alkaline gel electrophoresis the κ- and γ-casein bands migrate between β-casein and the application slot. To obtain an estimate of each component, two accurately measured samples of each casein preparation were examined by electrophoresis, one being treated with chymosin prior to reduction and used to estimate γ- and para-κ-casein and the other diluted with a volume of water equal to

the chymosin volume and used to estimate other components.

Optimum chymosin concentration was determined. Dilutions of a 2 gL^{-1} stock solution of chymosin were prepared and 10 µL of these dilutions was added to 50 µL of 25 gL^{-1} acid casein solution to give the concentrations shown in Figure IV.1. After 5 minutes at 37°C a solution of urea and mercaptoethanol was added. The effects of chymosin on the various components is shown in Figure IV.1. A chymosin concentration of 0.0083 gL^{-1} caused complete cleavage of κ-casein, no proteolysis of α_{s1}-casein and a 3.6% loss of β-casein, and was therefore used in subsequent experiments. The α_{s2}-caseins fraction included the degraded β-casein peak in this particular subdivision, so this fraction increased with increasing chymosin concentration above 0.0083 gL^{-1}.

Urea and 2-mercaptoethanol were used to disaggregate the casein micelles before electrophoresis. Urea and dithiothreitol and urea, dithiothreitol and iodoacetamide (alkylation) also gave good resolution of caseins under the right conditions.

Sample buffer concentration did not appear to affect resolution of the caseins, although in one gel, samples in more dilute buffer produced sharper bands (see Hjerten *et al.* 1965). In the method adopted the sample buffer concentration was one-tenth that in the gel. Details of the gel electrophoresis method finally adopted are given in Section (o).

(c) Gel and buffer system

A large number of variables affect the resolution of the caseins using acrylamide gel electrophoresis. Some of these variables, such as type and concentration of acrylamide and buffer, concentrations of urea and 2-mercaptoethanol and electrophoresis temperature were investigated in the present study. It was difficult to test

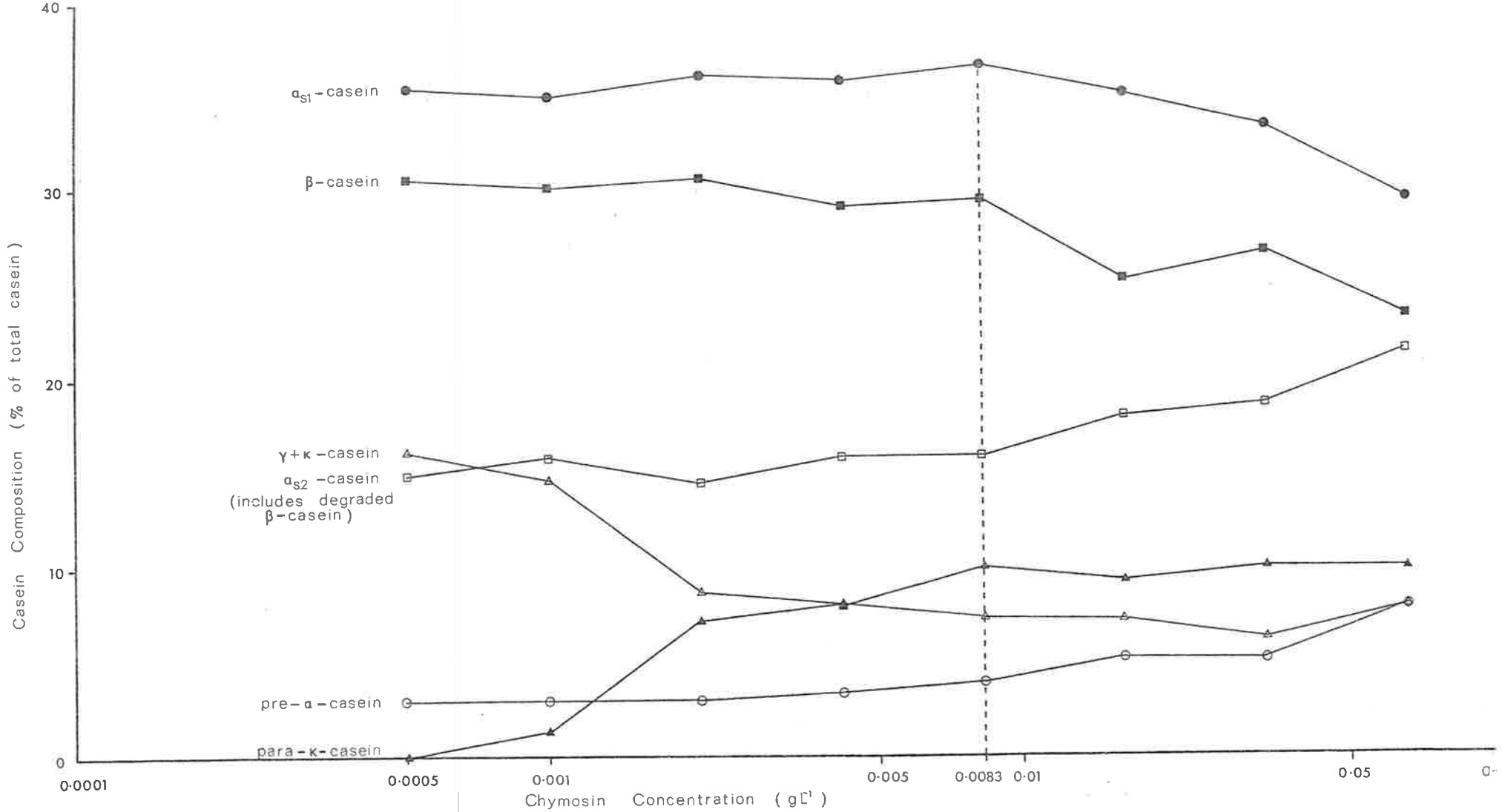


FIGURE IV. 1: The effect of concentration of chymosin, added to whole casein at 37°C, on the proteolysis of casein components. Casein composition was determined by gel electrophoresis. The concentration of chymosin selected for subsequent experiments was 0.0083 gL^{-1}.

systematically all variables as often after a variable had been shown to give optimum resolution, it was necessary to retest it after another variable was changed.

Several different types of buffer systems were examined (e.g. those of Thompson *et al.* 1964, Melachouris 1969) but a continuous tris-glycine system was selected for more intensive study. Several combinations of tris and glycine concentration (0.20 M tris and 0.385 M glycine, 0.05 M tris and 0.385 M glycine and 0.10 M tris and 0.19 M glycine) were used for a period with good results but the 0.10 M tris- 0.19 M glycine system of McKenzie (1973, personal communication) was finally adopted.

Both Cyanogum-41 and Canalco ultra pure acrylamide and bis-acrylamide were used with equally good results. Lower acrylamide concentrations (50 gL^{-1}) appeared to give better resolution with the 0.2 M tris, 0.385 M glycine buffer, whereas an acrylamide concentration of 70 or 80 gL^{-1} gave better resolution with the 0.1 M tris, 0.19 M glycine buffer system. With the 50 gL^{-1} acrylamide gels, the κ-casein bands overlapped one of the major γ-casein bands making genetic typing difficult, whereas with acrylamide concentrations above 70 gL^{-1}, the κ-casein A and B bands migrated between the two major γ-casein bands.

Gel urea concentrations between 3.5 and 8 M were tried and it was found that gels should contain at least 7 M urea for optimum resolution.

(d) Electrophoresis conditions

The three electrophoresis units appeared to give identical patterns. Initially, electrophoresis was carried out with the temperature maintained at $10^{o}C$. However, it was often found that poor band resolution was due to the proteins moving faster at the

top gel surface than the surface next to the cooling plate. Increasing the temperature to 25°C improved resolution but the edges of the gel dried out, and although this could be overcome by covering the gel surface with a thin film of plastic, a temperature of 20°C was adopted.

For ease of peak subdivision (see later) it was desirable that a given protein band migrate the same distance in each gel. However, with the same initial potential gradient (10 V cm^{-1}), the rate of increase of the gradient varied during electrophoresis, according to the number of times the electrode buffer had been used, resulting in slightly different migration distances being obtained with different gels. A dye marker, bromophenol blue, was tried, but as its migration rate was much faster than α_{s1}- casein, it was not used.

(e) Sample application

Application of samples into the slab of polyacrylamide gel for horizontal electrophoresis is a critical step in obtaining sharp, straight protein bands. Cutting a slot with the sharpened end of a spatula and adding the sample (usually 1.5 µL) in a small wick cut from chromatography paper gave the best resolution. For quantitative analysis, a wick length of 4 mm enabled the whole width of the stained protein bands to be scanned (Fazekas *et al.* 1963) when using a 5 mm slit in the densitometer and allowing for slight divergence of the bands during electrophoresis.

(f) Staining and rinsing

In some experiments, protein bands were stained with amido black, but for the majority, Coomassie blue G250 staining according to the method of Diezal *et al.* (1972) was used. The protein was fixed by immersing the gel in 125 gL^{-1} trichloroacetic acid (TCA) for 5

minutes, then a volume of 2.5 gL^{-1} aqueous solution of Coomassie blue G250 equal to one-twentieth of the TCA volume was added. Fifteen minutes after the addition of dye, the gel was placed in 50 gL^{-1} acetic acid.

The binding between protein and Coomassie blue G250 was examined using gels containing replicates of a casein sample which were stained overnight by the method of Diezal *et al.* (1972) then rinsed in 50 gL^{-1} acetic acid for 0, 2, 5, 7 or 8 days. Equal areas of gel were immersed in equal volumes of 50 gL^{-1} aqueous ammonia for a day and the absorbance at 610 nm of dye removed by ammonia solution was determined.

The results (see Fig. IV.2) show that rinsing in 50 gL^{-1} acetic acid progressively removed dye, presumably unbound dye from the gel background, and an equilibrium was reached by approximately 5 days. When the gel was placed in the ammonia solution for one day, further dye was removed but a proportion of dye remained bound to the protein.

Rinsing of the stained gels in 50 gL^{-1} acetic acid was the usual method of destaining. Use of alcohol in the destaining solution increases the efficiency of the destaining process, but may result in loss of dye (Bertolini *et al.* 1976). Electrophoretic destaining was not used as Fishbein (1972) found it to be variable and uneven. Some gels, mainly gels stained with amido black, were destained by circulating the rinsing solution through a short column of activated charcoal granules to remove the dye. With Coomassie blue G250 this was found to substantially reduce band intensity and was not further used.

Linearity of peak area versus protein content was examined using

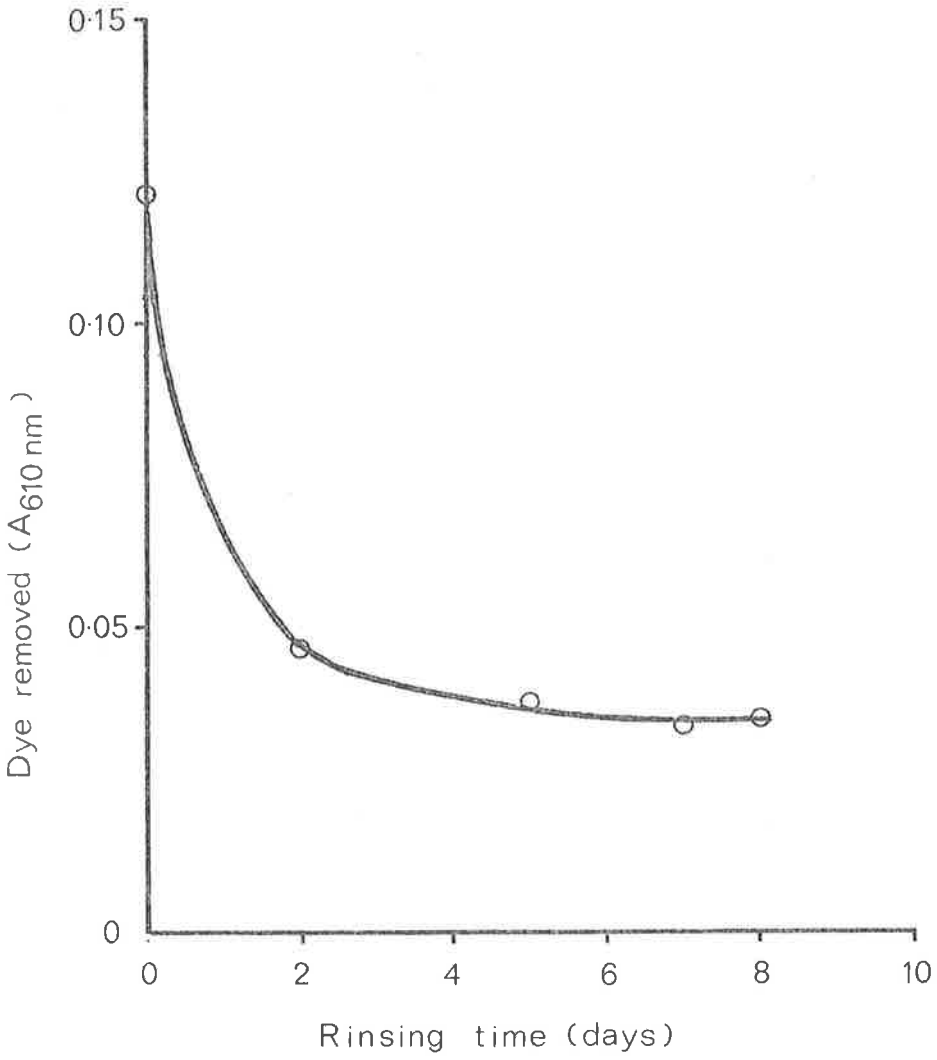


FIGURE IV.2

The release of dye by 50 gL^{-1} ammonia solution from protein bands in polyacrylamide gels stained with Coomassie blue G250 after rinsing the gel for various times in 50 gL^{-1} acetic-acid.

dilutions of whole casein or individual caseins. Initially, gels were stained for 15 minutes using Coomassie blue G250 as described by Diezal *et al.* (1972). It was found that when gels had been left in the 50 gL^{-1} acetic acid rinsing solution for long periods of time (e.g. 38 days) peak area versus protein weight was linear, whereas a non-linear relationship resulted when gels were only rinsed for 1-3 days. Therefore the effect of rinsing time was examined in more detail.

The relationship of peak area with protein weight for gels rinsed 2 hours, 1, 2, 5 and 9 days after staining for 15 minutes is shown in Figure IV.3. Although curves were obtained for all rinsing times, peak area increased for 2 days then there appeared to be a slight redistribution of stain, with bands containing more than approximately 5 µg β-casein staining more intensely and peak areas of bands with less than 5 µg decreasing slightly.

To examine the effect of migration distance on the relationship between area and protein weight, using the 15 minute staining method of Diezal *et al.* (1972), dilutions of α_{s1}- casein were submitted to electrophoresis for 1, 2, 3 and 4 hours giving migration distances of 1.5, 3.0, 4.2 and 5.5 cm. The stained gel was rinsed for 7 days in 50 gL^{-1} acetic acid before scanning and the peak area versus protein weight for each migration distance are shown in Figure IV.4.

The effect of staining time was studied in an attempt to extend the range of linearity of area versus protein weight. Dilutions of whole casein were subjected to electrophoresis, stained with Coomassie blue G250 for 15 minutes, 1 hour and overnight by the method of Diezal *et al.* (1972) and scanned after 1½ and 6 days. With the major caseins, α_{s1}- and β-casein, 15 minute and one hour staining times with 1.5 and 6 days rinsing resulted in a non-linear relationship between densitometer area and protein weight. Overnight

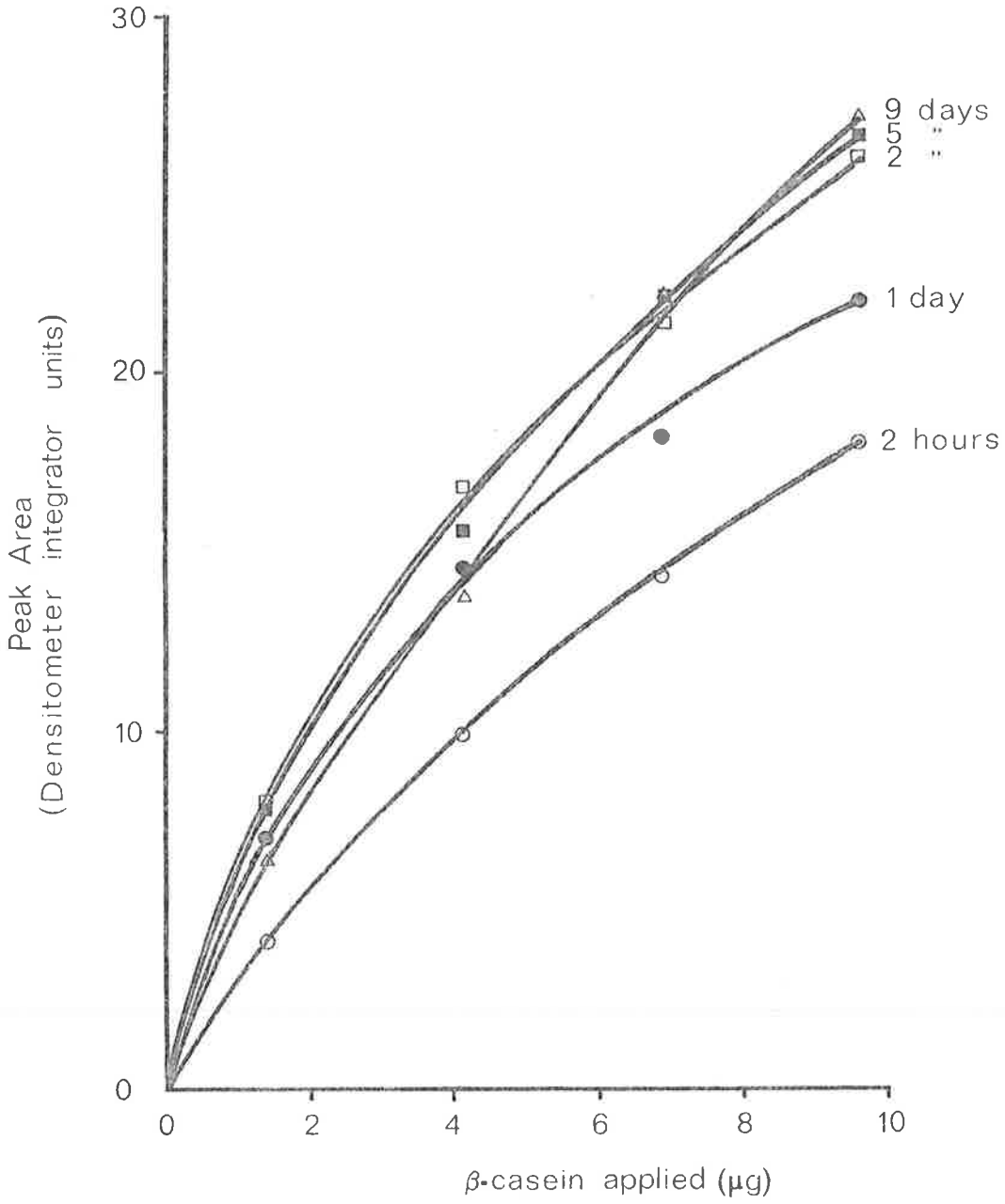


FIGURE IV.3

The effect of rinsing time on the relationship between peak area and protein weight. β-Casein was submitted to electrophoresis stained for 15 minutes with Coomassie blue G250 and rinsed for various times in 50 gL^{-1} acetic acid before scanning.

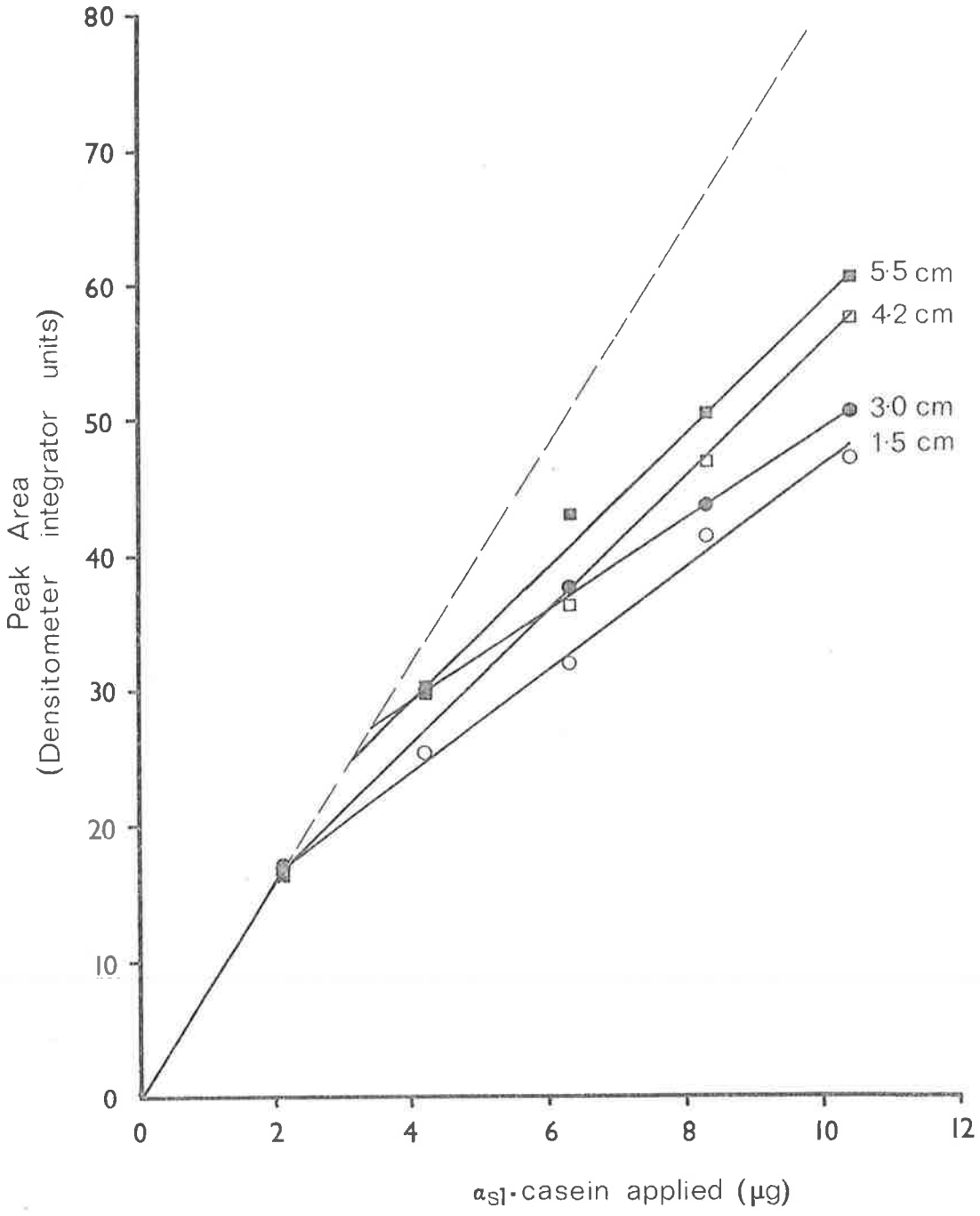


FIGURE IV.4

The effect of migration distance on the relationship between peak area and protein weight.

α_{s1}-Casein was submitted to electrophroesis for various times, stained for 15 minutes with Coomassie blue G250, rinsed for 7 days and scanned.

staining resulted in a linear relationship after rinsing for 1½ days or 6 days (see Figure IV.5).

Some later gels showed that 3 days rinsing was insufficiently long to attain a linear area versus protein weight relationship whereas after 6 days rinsing a straight line resulted.

Based on these results, it was decided to use overnight staining and 7 days rinsing for all quantitative gels including those in the experiment described in Chapter V. However, after the quantitative electrophoresis of samples collected in Chapter V was finished, electrophoresis of purified casein genetic variants to determine dye binding capacities showed that a linear response was not always obtained using overnight staining and rinsing for 7 days (see Section 1).

The area versus protein weight relationship obtained with the staining method modified from Diezal *et al.* (1972) was compared to that obtained using the Coomassie blue G250 staining method of Anderson and Andrews (1977) and with 5 gL^{-1} amido black. The two Coomassie blue G250 staining methods gave similar results, with curves for the two major caseins, α_{s1}- and β-casein and straight lines with the minor fractions, α_{s2}- and para-κ-casein. A linear peak area to protein weight relationship was found with amido black staining with these gels although a linear relationship has not been consistently found with amido black in this work.

(g) Densitometry

With the Pye Unicam densitometer, the scanning procedure, in which gel strips were placed in a silica cuvette containing rinsing solution, was extremely tedious. Resolution was checked using a test slide supplied by Pye Unicam and linearity of response examined with either dilutions of $K_2Cr_2O_7$ solution or a Kodak optical density

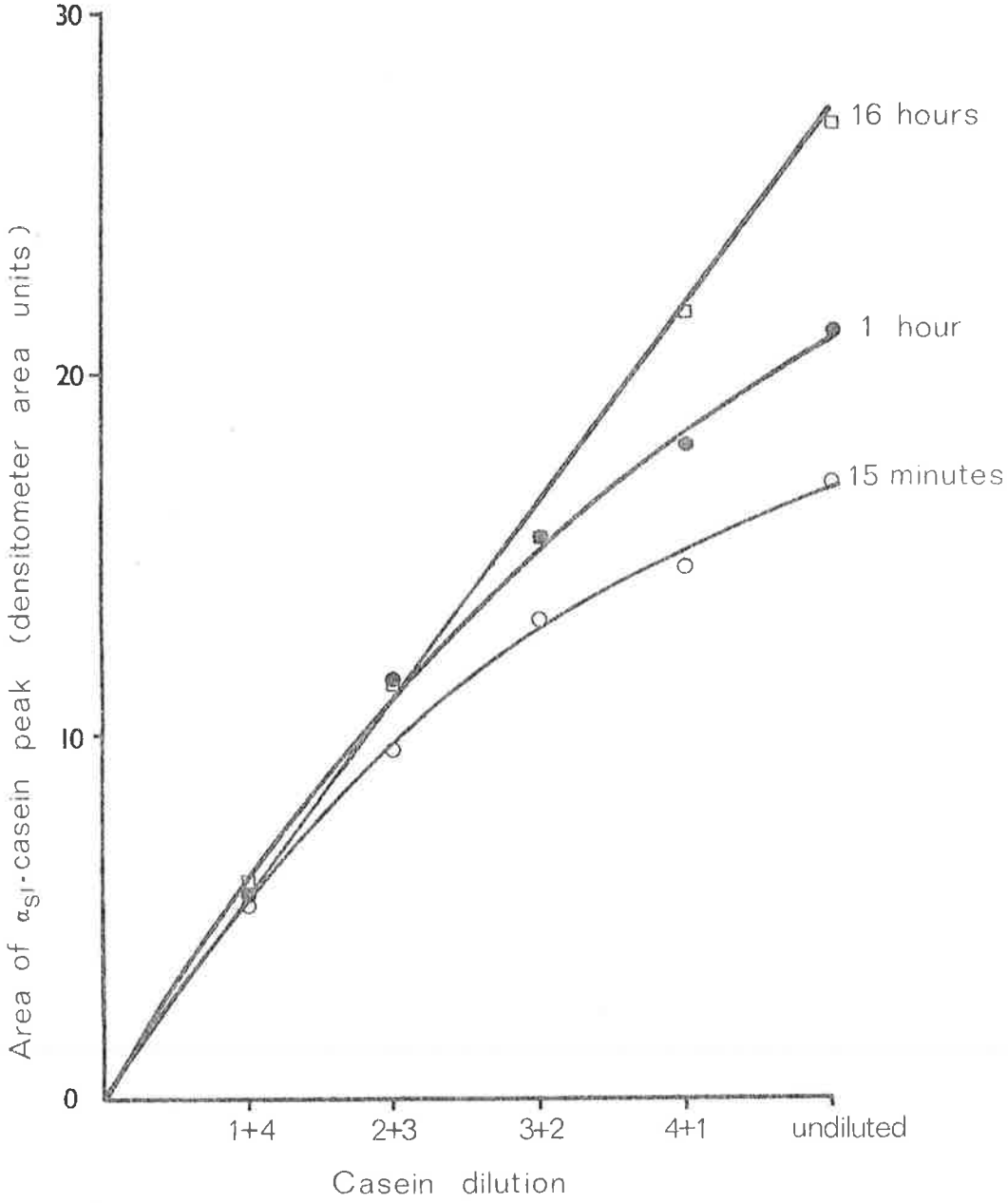


FIGURE IV.5

The effect of staining time on the relationship between peak area and casein dilution. Caseins were separated by electrophoresis, stained for either 15 minutes, 1 hour or overnight (16 hours) with Coomassie blue G250 and rinsed for 6 days. Areas of the α_{s1}-casein peaks are shown. Assuming a casein content of 29 gL^{-1}, 7µg of α_{s1}-casein would be applied to the gel with the undiluted sample.

step tablet. A straight line was obtained to 1.2 absorbance units with both methods of checking linearity and sample concentration was adjusted so that 1.2 absorbance units was not exceeded.

For the majority of the densitometry reported in this study, including the gel electrophoresis described in Chapter V, the Quick Scan densitometer was used. Scanning gel slabs with this instrument was much simpler and quicker than with the Pye Unicam scanning accessory. Gels were scanned at 610 nm using a 0.5 x 5 mm slit and the high chart speed. The base line and integrator settings are extremely critical, particularly for minor components, and ideally they should be constant while scanning the whole gel. However adjustments were sometimes necessary during the time required to scan a gel due to instrument variation.

When testing linearity of response of the Helena Quick Scan densitometer using the optical density step tablet, at the gain setting usually used for scanning casein samples (6), height and area (integrator deflections for a fixed chart distance) were both linearly related to nominal optical density up to the maximum obtainable (0.8), whereas with a gain setting of 1 the instrument was linear to O.D. = 1.7.

In scanning gels, the densitometer was adjusted to zero with the gel positioned to measure absorbance in front of all casein bands. With most gels the instrument also recorded zero on the positive side of the sample slot, and with chymosin treated samples, on both sides of the para-κ-casein peak. However, with a few gels there was a slight increase in background staining towards the negative end of the gel and it was necessary to apply a baseline correction. To determine integrator deflections per unit area, the main pen was adjusted over a range of heights using both the

densitometer zero and an optical density step tablet. Integrator deflections per unit area was constant only when pen height was at least 2 cm (0.028 deflections per sq. mm). With the majority of gels where baseline corrections was necessary, the increase in baseline above zero was less than 2 mm (with larger deviations the whole gel was repeated) and so the integrator deflections per square millimetre at 2 mm (0.035) was used for baseline correction.

(h) Casein resolution

An acrylamide gel containing resolved casein components after electrophoresis is in Figure IV.6, and in Figure IV.7, several electrophoresis patterns and their corresponding densitometer traces are shown.

With casein samples untreated with chymosin the densitometer peaks were separated into five groups -

pre-α-fraction. These protein bands migrate faster than α_{s0}-casein and appear to be formed by degradation of α_{s1}-caseins. All casein samples showed at least some of these bands, and the number and intensity increased with mastitis, age of cow, storage and chymosin action. Two peaks resulting from proteolysis of heterozygous α_{s1}-casein could be distinguished in the pre-α-fraction with some samples (see Figure IV.7).

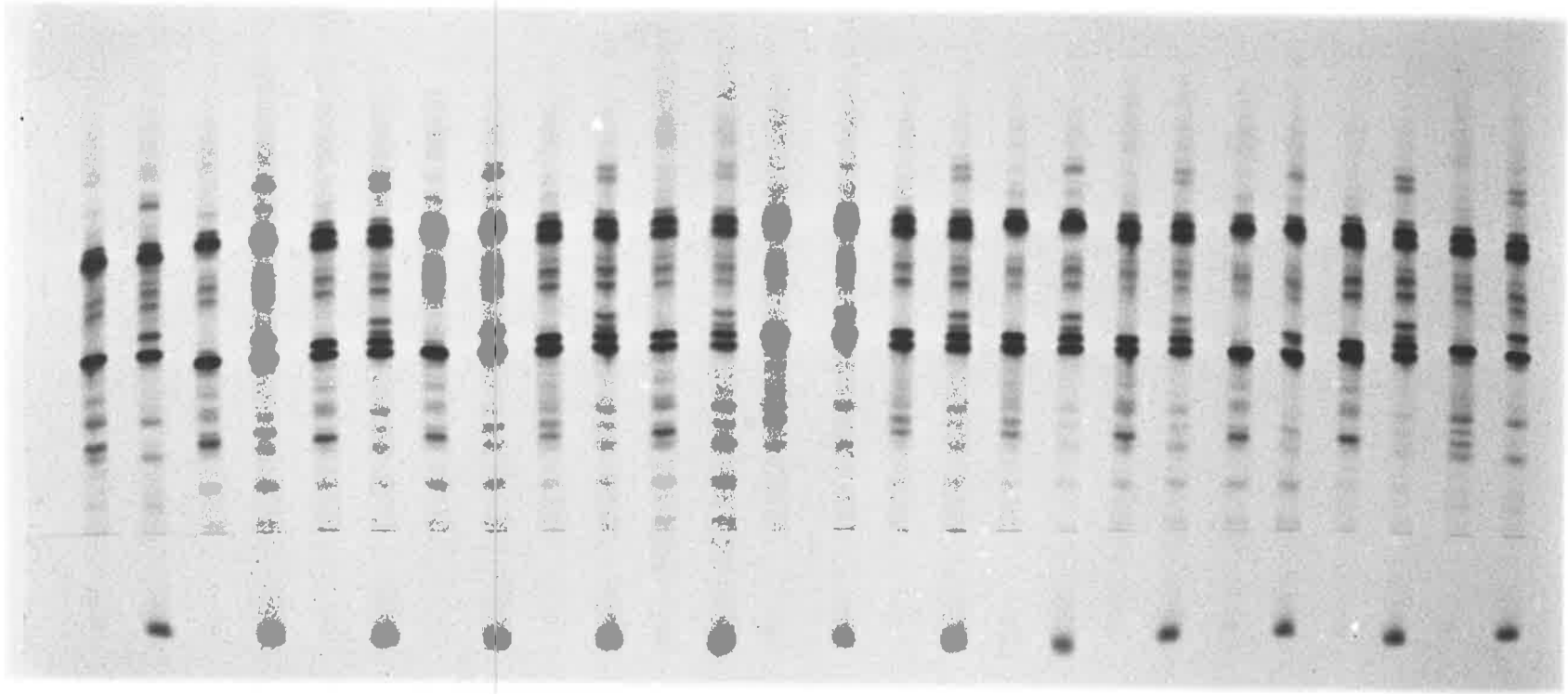
α_{s1}-caseins. In the homozygote, 2 bands are produced due to the major α_{s1}-casein component and a weaker slightly faster migrating α_{s0}-casein component. The appearance of α_{s0}-casein in densitometer scans varied, being either barely visible, a shoulder or sometimes with a trough between separate peaks (see Figure IV.7). Samples containing α_{s1}-casein BC produced 3 bands, the α_{s1}- B band presumably overlapping the α_{s0}- C band.

α_{s2}-caseins. Four bands (and densitometer peaks) could be

FIGURE IV.6

An acrylamide gel used for genetic typing of α_{s1}-, β- and κ-casein and quantitative determination of individual caseins. The gel contains 12 individual cow casein samples (from Jersey herd 3 in Chapter V) and the casein standard; each sample was run with and without prior chymosin treatment. Casein genotypes are as follows:

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Genotype:													
α_{s1}-casein	C	B	BC	B	BC	BC	Casein	BC	B	BC	B	BC	BC
β-casein	A	B	AB	B	AB	AB	std.	AB	AB	AB	B	AB	A
κ-casein	B	B	B	B	AB	B		AB	AB	B	B	B	AB


1
2
3
4
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12
13

FIGURE IV.7

Electrophoresis patterns of individual cow casein samples and the corresponding densitometer traces

a. cow 602 (α_{s1}-casein B, β-casein AC, κ-casein AB)

b. cow 680 (α_{s1}-casein BC, β-casein A, κ-casein AB)

c. cow 743 (α_{s1}-casein B, β-casein AB, κ-casein AB)

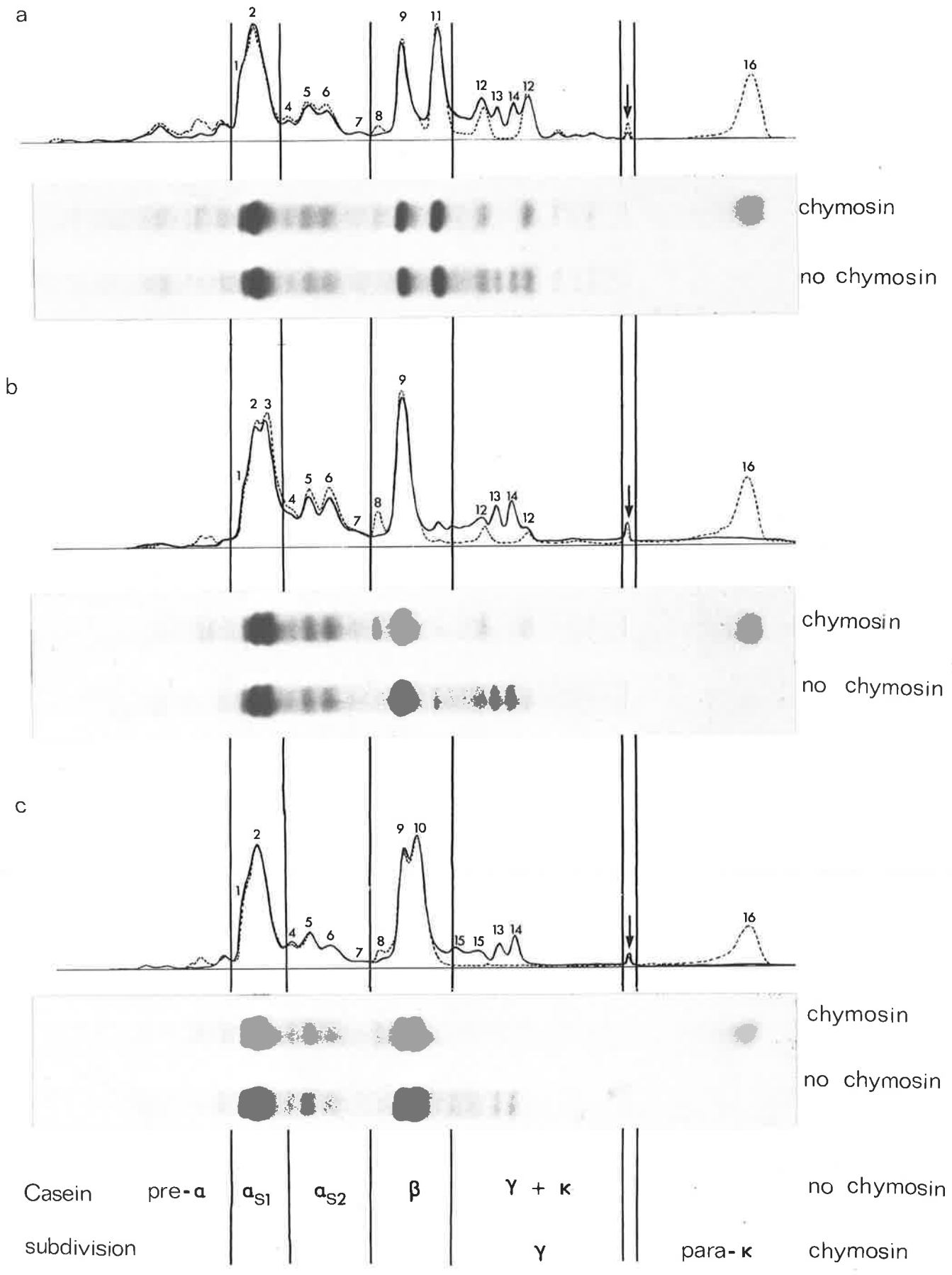
The lower electrophoresis pattern and the solid densitometer trace shows the casein sample without chymosin treatment and the upper electrophoresis pattern and the dotted densitometer trace shows the effect of chymosin

Protein peaks are numbered as follows:

1	α_{s0}-casein B	9	β-casein A
2	α_{s1}-casein B	10	β-casein B
3	α_{s1}-casein C	11	β-casein C
4	α_{s2}-casein	12	γ-casein
5	α_{s3}-casein	13	κ-casein A
6	α_{s4}-casein	14	κ-casein B
7	α_{s6}-casein	15	glycosylated κ-casein
8	degraded β-casein	16	para-κ-casein

The position of the application slot is indicated by an arrow

Note the increasing content of pre-α and γ-casein with age of cow: cow 602 was 5½ years old, cow 680 was 3½ years old and cow 743 was 2 years old


a
b
c
chymosin
no chymosin
Casein subdivision
pre-α
α_{S1}
α_{S2}
β
γ + κ
γ
para-κ

distinguished, corresponding to $\alpha_{s2,\ 3,\ 4}$ and $_{6}$-casein (α_{s5}-casein is split by 2-mercaptoethanol to form α_{s3}- and α_{s4}-casein, so is not present in these gels). With samples containing α_{s1}-casein C, the α_{s2}-casein peak appeared as a shoulder on the α_{s1}-casein peak, making fraction subdivision difficult (see later). Although the α_{s2}-caseins were not normally quantitated separately, an approximate estimate of their proportion of the total area of α_{s2}-caseins, calculated as the mean of 15 individual cow samples, was 16% α_{s2}-, 37% α_{s3}-, 38% α_{s4}- and 9% α_{s6}- casein, or 2.0%, 4.6%, 4.8% and 1.1% as a proportion of total casein, assuming 12.5% α_{s2}-casein in total casein.

β-casein. The 3 β-casein variants detected using the alkaline buffer system (A, B and C) are shown in Figure IV.7. With β-casein AC, virtually complete separation was achieved. These 3 casein samples were prepared on the day following milk collection and had been stored at -15°C for 16 days. Degraded β-casein (peak 8) can be detected in all 3 samples, but as this fraction was grouped with β-casein little error should result.

γ- + κ-casein. The γ- and κ-casein bands migrate in the area between β-casein and the application slot. With samples heterozygous for κ-casein, the two major unglycosylated protein bands migrated between two major γ-casein bands with little overlap, so κ-casein could be readily typed for genetic variants. Differences in the number and intensity of γ-casein bands between individual cow samples was obvious.

Chymosin splits κ-casein and as para-κ-casein is the only fraction migrating towards the negative electrode, it can be readily scanned. With the splitting of κ-casein the only protein bands migrating between β-casein and the application slot are numerous

γ-casein components, so an independent estimate of these can be made.

(i) Subdivision of casein fractions

If the areas of the casein were determined by drawing a vertical line from the minimum point between fractions to the integrator trace, then the two peaks resolved in heterozygotes would require a longer scan length than would homozygotes. The possibility exists that minor proteins of similar mobility might be included in the area scanned for the heterozygote caseins and not for that of the homozygote. In that case the heterozygote would be overestimated. As an extreme example, β-casein AC has two widely separated peaks with β-casein C overlapping γ- or κ-casein bands (see Figure IV.7a).

If electrophoresis could be conducted so that the migration distances of each protein was constant in each gel run, then the components could be subdivided by using a constant densitometer chart length regardless of whether the protein was homozygous or heterozygous. However, in practice the same initial V cm^{-1} gradient and electrophoresis time resulted in varying migration distances.

The method of fraction subdivision adopted was as follows: with all samples in each gel a constant peak width, the mean peak width of heterozygotes within that gel, was used for both α_{s1}- and β-casein. For gels containing few or no heterozygotes, the peak width was calculated by multiplying the standard casein (see Section j) peak width of that gel by a factor of 1.1. This factor was calculated for both α_{s1}- and β-casein by dividing the mean heterozygote peak width for all gels in the experiment described in Chapter V by the peak width of the standard casein averaged over all gels (this factor was relatively constant with varying migration distance). In the

experiment described in Chapter V, no β-casein C variants were detected, simplifying peak splitting for β-casein. The length of the scan for γ-casein in the chymosin treated sample was the same as that between the β-casein/γ- + κ-casein subdivision and the application slot of the untreated sample.

(j) Whole casein standard

A sample of casein was submitted to electrophoresis with the unknown samples in each gel to correct for between gel variability and to determine concentrations of individual caseins (see Darling and Butcher 1976).

The method of preparation of this casein gel standard was similar to that used for all casein samples prepared for electrophoresis. A bulk milk sample from the Northfield Research Centre herd, which contained mixed genetic variants, was skimmed and casein precipitated by the addition of acetic acid-sodium acetate mixture as described in Chapter III. The centrifuged casein was dissolved in imidozole-HCl buffer and stored at -11^{o}C.

A sample of the casein standard was submitted to electrophoresis on each of the 55 gels used to determine casein composition in the study described in Chapter V. The mean concentration of casein components, expressed as a percent of total casein and uncorrected for differences in dye binding, were pre-α-casein 6.5 ± 1.98%, α_{s1}-caseins 34.8 ± 2.29%, α_{s2}-caseins 11.5 ± 1.63%, β-casein 29.6 ± 2.21%, γ-casein 8.3 ± 2.37 and para-κ-casein 9.4 ± 1.75%. It was intended to correct these gels for between gel differences using the standard casein mean composition but this correction caused increased variability of minor components (see Section m), so was not used.

It was also intended to determine the composition of the standard casein sample by submitting the standard and purified casein

fractions of known concentration to electrophoresis simultaneously. However by the time suitable purified casein fractions were prepared (see Section j) the standard casein solution had degraded appreciably. Therefore the casein composition of unknown samples was determined by correcting the peak area for differences in dye binding capacity (area/weight of protein - see section 1) and calculating protein weight as a percent of total protein weight.

(k) Purification of casein components

α_{s0}-Casein B and C, α_{s1}-casein B and C, $\alpha_{s3,4}$-casein, β-casein A^1, A^2 and B and κ-casein were purified to enable the determination of relative dye binding capacities.

Casein was precipitated by acid from skim milks selected for particular genetic variants and for absence of mastitis, washed twice in distilled water, redissolved in imidazole - HCl buffer and stored at $-16^{o}C$. The casein fractions were separated by ion-exchange chromatography on Whatmans DEAE-cellulose (Davies and Law 1977a). DEAE-Sephacel was also used but resolution was slightly inferior.

Protein fractions from column separations were grouped according to absorbance at 278 nm and concentrated by dialysis against 100 gL^{-1} polyethylene glycol or by ultra-filtration, and filtered through an 0.45 μm Millipore membrane. κ-Casein fractions were also dialysed against distilled water to lower the pH for optimum chymosin reaction. Problems were experienced in obtaining pure γ- and κ-casein fractions using ion exchange chromatography: their concentration was low and during concentration a cloudy precipitate formed. No estimate of the dye binding capacity of γ-casein could be obtained, and κ-casein prepared by Dr. E.R.B. Graham using calcium precipitation at $37^{o}C$ to remove other caseins, column chromatography on Sepharose 4B and

and column electrophoresis, was used to estimate the dye binding capacity of para-κ-casein.

All fractions selected for dye binding studies were electrophoretically pure except for κ-casein where the estimated concentration was corrected for absorbance of impurities. The α_{s2}-caseins fraction consisted mainly of α_{s3}- and α_{s4}-casein, the two major components.

The concentration of purified casein components was estimated by subtracting the absorbance at 350 nm, as a correction for scattered light, from that at 278 nm and using the absorptivities shown in Table IV.3.

Thompson and Kiddy (1964) found a small difference in absorptivity between genetic variants of α_{s1}-casein and their values were used in the present study (see Table IV.3).

Estimates of 1.04 and 1.07 for the absorptivity of α_{s0}-casein by Annan and Manson (1969) and Manson *et al.* (1976) respectively were similar to Annan and Manson's (1969) estimation of 1.07 for α_{s1}-casein so the same value was used for both α_{s0}- and α_{s1}-casein.

The absorptivity of α_{s2}-casein determined by Annan and Manson (1969) was 1.01. As this value was lower than their estimate for α_{s1}-casein, it was decided to reduce the value used in the present study to 1.00.

For β-casein, the absorptivity values determined for the genetic variants by Thompson and Pepper (1964) were used. κ-Casein concentration was estimated using an absorptivity value of 1.05 (Garnier 1963) in preference to 1.22 measured by Zittle and Custer (1963) as this value gave a more realistic gel electrophoresis estimate of κ-casein.

TABLE IV. 3

Estimates of absorptivity, a^*, of casein components measured at 278 nm and the values used in the present study

Casein component	a_{278}	Reference
pre-α-fraction	1.00	No reported values, so same value as used for α_{s1}-casein
α_{s0}-casein	1.04	Annan and Manson (1969)
α_{s0}-casein	1.07	Manson *et al.* (1976)
α_{s0}-casein B & C	1.00	Used in the present study
α_{s1}-casein B	1.005	Thompson and Kiddy (1964) and used in the present study
α_{s1}-casein C	1.003	Thompson and Kiddy (1964) and used in the present study
α_{s1}-caseins	1.007	Annan and Manson (1969)
α_{s2}-caseins	1.01	Annan and Manson (1969)
α_{s2}-caseins	1.00	Used in the present study
β-casein A	0.46	Thompson and Pepper (1964), Garnier *et al.* (1964) and used in the present study for β-casein A^1 and A^2
β-casein B	0.47	Thompson and Pepper (1964) and used in the present study
κ-casein	1.22	Zittle and Custer (1963)
κ-casein	1.05	Garnier (1963) and used in the present study

* $a = \frac{A}{bc}$ where A = absorbance, b = sample path length in centimetres and c = concentration in gL^{-1}

(1) Dye binding capacities of the caseins

The relative dye binding capacities of α_{s1}-casein B and C, α_{s2}-caseins, β-casein A^1, A^2 and B and para-κ-casein when stained with Coomassie blue G250 were determined after electrophoresis in 6 gels, although the results obtained were supported by additional work. Each protein was diluted to give a range of 5 concentrations and each gel contained dilutions of 5 different proteins or variants. One of the six gels (gel 6 in Table IV.3) is in Figure IV.8 and the relationship between densitometer area and weight of protein is in Figure IV.9. A curved relationship resulted with α_{s1}- and β-casein. The dye binding capacity (area/μg protein) of proteins with a curved relationship was measured at approximately their protein concentration in milk. As the absolute dye binding capacity varied between gels and with densitometer settings and because all caseins and variants could not be compared within the same gel, dye binding capacities relative to that of α_{s1}-casein B were calculated (see Table IV.4).

Good agreement for the dye binding capacities of β-casein variants measured in 2 additional gels was obtained: the mean relative to β-casein A^2 = 0.66 was β-casein A^1 = 0.70 and β-casein B = 0.72. However, the mean relative dye binding capacity of α_{s1}-casein C, measured in 3 gels, was only 0.84 compared to α_{s1}-casein B = 1.00. α_{s0}-Casein B had a higher dye binding capacity than α_{s0}-casein C. The dye binding capacity of γ-casein was not determined as a pure fraction of suitable concentration could not be prepared. As these fractions are degradation products of β-casein and the proportion of basic groups (lysine, arginine, histadine and terminal amino group) in the γ-caseins is similar to that in β-casein, the dye binding capacities used were the same as the appropriate genetic

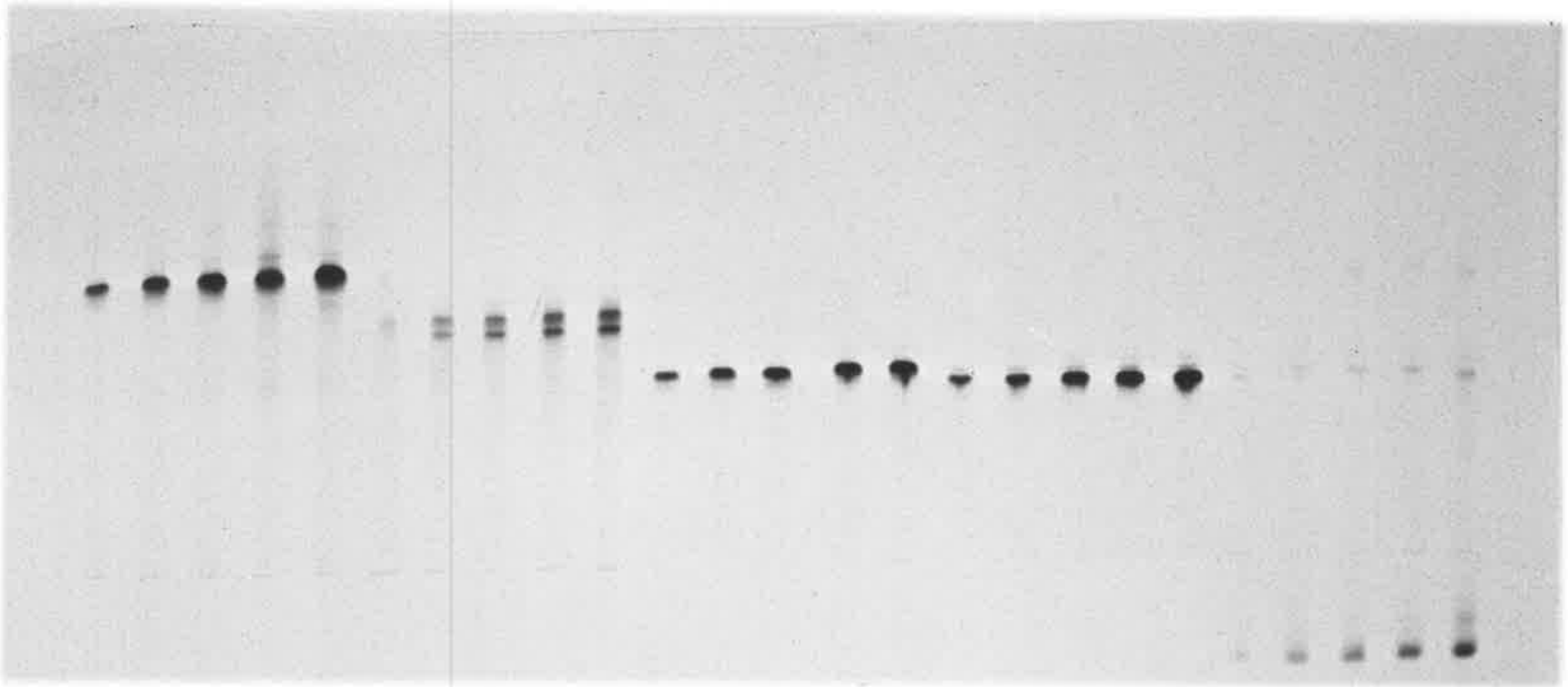
FIGURE IV. 8

Polyacrylamide gel electrophoresis patterns of purified caseins used for the determination of relative dyebinding capacities (gel 6 in Table IV.4):

(a) α_{s1}-casein B, (b) α_{s2}-caseins

(c) β-casein A^2 (d) β-casein B

(e) para-κ-casein



a b c d e

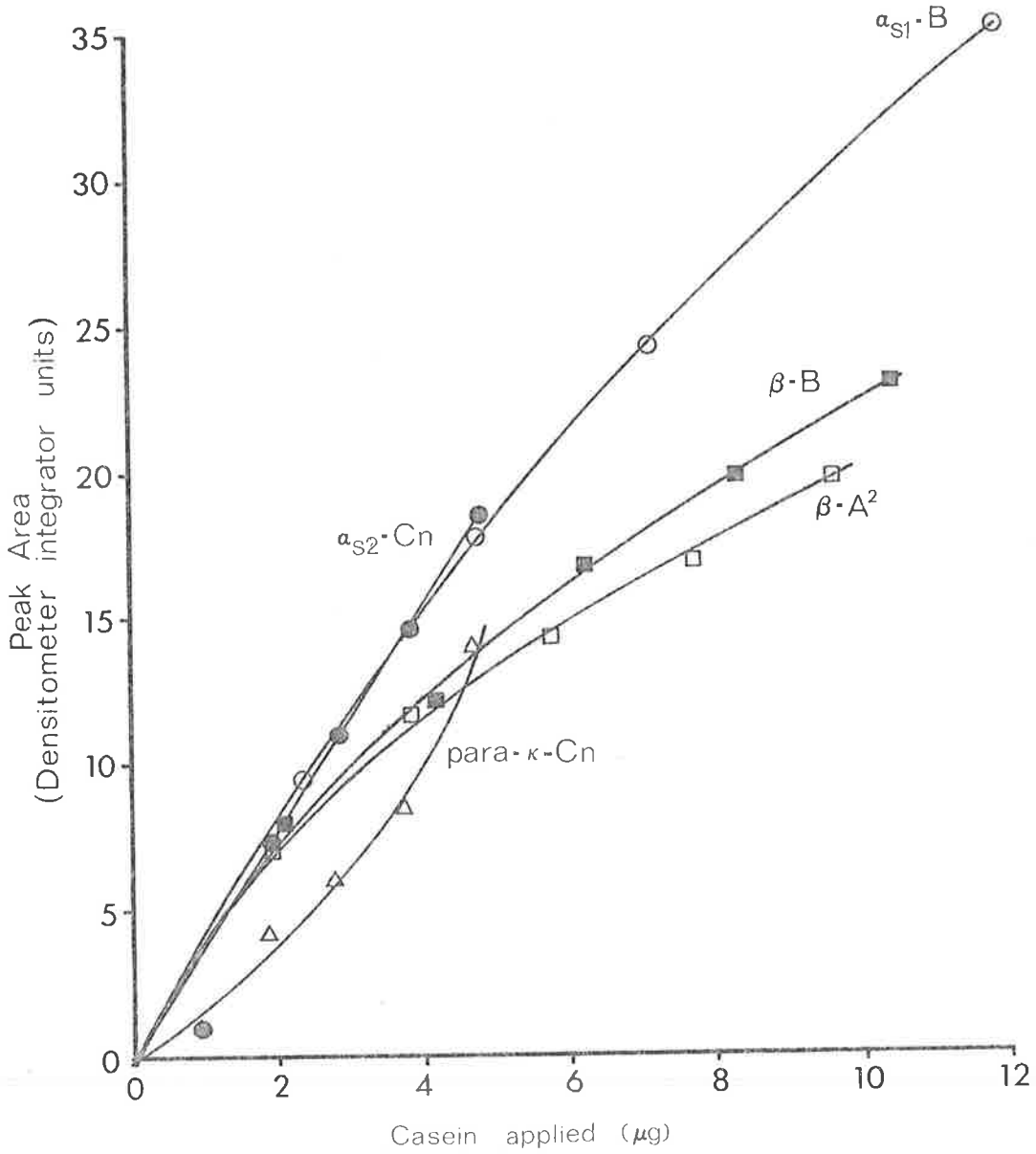


FIGURE IV. 9

The estimation of relative dye binding capacities of casein components subjected to electrophoresis in polyacrylamide gel. The relationship between densitometer area and protein weight for α_{s1}-casein B, α_{s2}- caseins, β-casein B and A^2 and para-κ-casein dilutions on a single gel (Gel 6 in Table IV. 4) is shown. Protein bands were stained with Coomassie blue G250 for 16 hours and rinsed for 7 days before scanning.

TABLE IV. 4

The relative dye binding capacities of casein components subjected to gel electrophoresis and stained with Coomassie blue G250

Casein	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Mean
α_{s1}-casein B	1.00	1.00		1.00	1.00	1.00	1.00
α_{s1}-casein C	0.91	0.93		1.06	0.84		0.94
α_{s2}-caseins	0.99	0.88		1.00		1.05	0.98
β-casein A^1			0.71		0.66		0.69
β-casein A^2		0.65	0.66	0.74	0.60	0.66	0.66
β-casein B		0.72	0.70		0.67	0.74	0.71
para-κ-casein	0.87		0.82	0.73		0.61	0.76

variant of β-casein.

(m) Precision

The precision of the determination of individual caseins was determined with gels scanned with both densitometers.

The gels scanned with the Pye Unicam system contained 70 gL^{-1} Cyanogum and 8 M urea with 0.05 M tris - 0.385 M glycine buffer system. Five gels each contained 8 replicates, with and without chymosin treatment, of an acid precipitated casein sample prepared from herd milk. Protein bands were stained with Coomassie blue G250 (Diezal *et al.* 1972) and scanned after 6 weeks in 50 gL^{-1} acetic acid.

The mean coefficient of variation of integrator area units of the 5 gels, (c.v. - standard deviation divided by the mean expressed as a percent) for α_{s1}- (including pre-α-), α_{s2}-, β-, γ- and κ-caseins (uncorrected for baseline deviations, between gel differences and dye binding differences) was 5.8, 6.8, 5.7, 42.4 and 12.8% respectively. The corresponding coefficients for each casein component, expressed as a % of total casein, was 2.1, 3.5, 2.1, 59.5, 12.1% respectively.

Protein bands scanned with the Quick Scan densitometer were separated using the gel electrophoresis method outlined in section (o). Four gels, each containing 4 individual cow casein samples and whole standard casein, in duplicate, with and without chymosin treatment were scanned. The coefficient of variation of each casein component for both the integrator area and the component as a percent of total casein are shown in Table IV.5. The integrator areas of the casein components of the individual cow samples were corrected for between-gel differences by dividing the component area by the mean area of the whole casein standard within the gel and multiplying by the

TABLE IV. 5

Precision of estimation of casein components using the Quick Scan densitometer. Coefficients of variation for integrator areas and casein components expressed as a percent of total casein, are presented before and after correction for between gel differences*

Casein component	Integrator area		Casein component as % of total casein	
	Without correction	Between gel correction	Without correction	Between gel correction
pre-α	24.4	17.5	20.8	14.7
α_{s1}-caseins	5.0	5.7	4.9	4.3
α_{s2}-caseins	8.3	7.4	5.1	5.7
β-casein	4.6	4.8	4.8	4.5
γ-casein	15.3	17.8	13.7	15.9
κ-casein	18.1	17.7	14.6	16.2

* Between gel correction: $\text{component area} \times \frac{\text{overall mean area of standard}}{\text{mean area of standard within the gel}}$

The overall mean area of standard, for each casein component, was the mean of 4 gels.

overall mean of the standard. Coefficients of variation of corrected areas were compared with the coefficients of variation without correction.

(n) Values obtained for casein composition

The overall mean casein composition, expressed as a percent of total casein, of 287 milk samples from Jersey cows and 236 milk samples from Friesian cows which were sampled in the experiment described in Chapter V, was α_{s1}-casein 31.9 and 33.6, α_{s2}-casein 12.8 and 11.7, β-casein 34.2 and 35.4, γ-casein 8.6 and 8.5 and κ-casein 12.4 and 10.8, respectively. The only significant difference in casein composition between breeds was in the proportion of κ-casein (see later).

(o) Summary of gel electrophoresis method adopted

The alkaline urea-acrylamide gel electrophoresis method which was adopted to genetically type α_{s1}-, β- (except A variants) and κ-casein and to estimate casein composition, and which was used in the study described in Chapter V, is outlined below.

Preparation of samples

Solutions

Imidazole - HCl solution: 0.05 M imidazole, 0.008 M HCl

Chymosin stock solution: 2 gL^{-1} Sigma crystalline chymosin. Store at $2^{o}C$.

Chymosin working solution: 0.05 gL^{-1}. Dilute stock solution 1 in 40.

Urea - 2ME - buffer mixture: 10 M Urea, 0.02 M Tris, 0.38 M Glycine, 2% (v/v) 2-mercaptoethanol (prepare daily).

Method

The precipitated casein pellet was drained for at least 15 minutes and dissolved in 9.2 ml of imidazole-HCl solution using stirring. If it was not immediately required it was stored at - 16°C. Ten µL of either water or chymosin working solution was added to 50 µL of each acid casein sample in 2 mL Autoanalyzer vials (chymosin concentration 0.0083 gL^{-1}). The vials were capped, gently shaken and placed in a 37°C water bath for 5 minutes. Then 60 µL of the urea-2ME-buffer mixture was added and the samples gently mixed and stored overnight at 2°C.

Electrophoresis

Solutions

Tris-glycine stock solution: 0.5 M Tris, 0.95 M Glycine

Electrode buffer: 0.1 M Tris, 0.19 M Glycine pH 8.8. Dilute tris-glycine stock solution 1 in 5 with water. Store at 2°C and replace each week.

Ammonium persulphate stock solution: 200 gL^{-1}

Method

Gels contained 80 gL^{-1} acrylamide, 0.10 M tris, 0.19 M glycine, 7 M urea and 0.25% (v/v) 2-mercaptoethanol. Each gel of approximately 60 mL was prepared by dissolving 25.2 g urea in 25 mL water and 12 mL tris-glycine stock solution, adding 4.8 g Cyanogum, 60 µL 3-dimethylaminopropionitrile (DMAPN), 150 µL 2-mercaptoethanol and finally 300 µL 200 gL^{-1} ammonium persulphate solution. After mixing, the solution was poured into a cell prepared as described by the LKB Multiphor instructions, and left overnight.

Pre-electrophoresis was carried out at 10 V cm^{-1} and 20°C for 30 minutes. Using a 0-5 µL micro-syringe, 1.5 µL of sample was added to a 4 x 1.5 mm wick cut from Whatman 3 MM chromatography paper and

the wick inserted in a 5 mm long slot cut in the gel using the sharpened end of a spatula. The position of the 26 slots cut in each 248 x 115 x 2 mm gel was located 32 mm from the cathodic edge using a template. After adjusting the power supply to initially give a potential gradient of 10 V cm^{-1} across the gel, electrophoresis was carried out at 20°C for 4.0 to 4.5 hours, depending on rate of increase of the potential gradient.

Staining and rinsing

Solutions

125 gL^{-1} trichloracetic acid

2.5 gL^{-1} aqueous solution of Coomassie blue G250

50 gL^{-1} acetic acid

Method

The protein was fixed by immersing each gel in 100 mL of 125 gL^{-1} trichloracetic acid for 5 minutes and stained by adding 5 mL of dye solution and leaving overnight. The dye solution was then discarded, the gel was rinsed in 50 gL^{-1} acetic acid, and left for 7 days in 50 gL^{-1} acetic acid in the dark.

Densitometry

Stained gels were scanned using a Helena Quick Scan densitometer, with a 610 nm filter and using a 0.5 x 5 mm slit and high chart speed. The baseline and integrator settings were adjusted to zero in a gel area free of stained bands. The peak widths of the major components, α_{s1}- and β-casein, was constant within each gel and was the mean peak width of heterozygotes within that gel, or for gels containing few or no heterozygotes the peak width was the standard casein peak width multiplied by 1.1 (see Section h). The peak width of the γ-casein fraction on chymosin treated samples was the distance between the β-casein/γ- + κ-casein subdivision

and the slot in the untreated sample. Peak areas of casein fractions where the baseline deviated from zero were corrected by adding or subtracting the area of deviation (sq. mm multiplied by 0.035 to give area in integrator units - see section f).

Protein fraction areas were divided by the relevant relative dye binding capacity to give weight of protein and expressed as a percent of total casein which was determined by the dye binding method described in Chapter III.

(ii) Whey protein composition

The concentration of β-lactoglobulin and α-lactalbumin in skim milk was determined by radial immunodiffusion (Mancini *et al.* 1963).

β-Lactoglobulin and α-lactalbumin were prepared from mastitis free skim milk by the ammonium sulphate fractionation methods, Ia and IIb respectively, of Armstrong *et al.* (1967). They were further purified by column chromatography on Sephadex G-100 and DEAE-Sephadex A50 as described by Armstrong *et al.* (1970). The absence of apparent heterogeneity of protein fractions were tested by starch gel electrophoresis and by immunoelectrophoresis using rabbit anti-bovine skim milk, anti-bovine whey and anti-bovine serum.

Rabbits were immunized using a method similar to that used by Avrameas (1969). The specificity of the antisera was checked by immunoelectrophoresis against skim milk and bovine serum and strongly reacting specific antisera were combined.

The general procedures used were similar to those described by Weeke (1973) for immunoelectrophoresis. The optimum dilution of antisera in agar and dilution of protein standard and skim milk were determined as described by Fahey and McKelvey (1965). Each gel was loaded with four dilutions of the purified protein standard in duplicate, and the diluted skim milk samples were loaded in duplicate.

After 2 days diffusion the gel was rinsed, dried and the precipition rings stained with Coomassie blue G250. The diameter of the stained rings was measured after projection using a photographic enlarger. A standard graph was drawn by plotting concentration of the standards against the square of the precipitation ring diameter (Mancini *et al.* 1963, Berne 1972).

To test whether each of the genetic variants of β-lactoglobulin react equally with rabbit antisera prepared against a single genetic variant, purified β-lactoglobulin A, B and C were each diluted to give a range of concentrations normally used for standards and radial immunodiffusion carried out using an antiserum produced against a single variant. Each of genetic variants reacted equally with this antisera and it was concluded that quantitation of β-lactoglobulin by radial immunodiffusion would not be affected by the genetic variant present.

During preliminary determinations of β-lactoglobulin concentration in skim milk, some repeatable values well outside the range expected from concentrations in the literature were obtained. To test the validity of these apparently high values, the method of standard additions was used. The concentrations of β-lactoglobulin obtained were as expected and it was concluded that the high β-lactoglobulin values obtained using radial immunodiffusion were valid.

2. Discussion

The method of casein precipitation was as used for the estimation of total casein concentration, and is discussed in Chapter III. El-Negoumy (1966) also precipitated casein by adding an equal mixture of sodium acetate and acetic acid for the rapid preparation of samples for gel electrophoresis. Kim and Bird (1972) showed that the method of preparation of isoelectic casein can affect the relative

percent of casein components. To minimise loss of minor components such as γ-casein, the precipitated casein was dissolved without any washing, and this may be the reason that the concentration of pre-α- and γ-casein fractions found in the present study was higher than in some other studies. No contamination of the casein samples with whey protein was apparent in the gel electrophoresis patterns.

Some studies (Murphy and Downey 1969, Michalak 1973a, Rothenbuhler 1974 and Anderson and Andrews 1977) submitted skim milk samples to electrophoresis for quantitation of all milk protein components in a single analysis. However, although the time consuming preparation of casein is eliminated, densitometry is complicated by the problem of overlapping peaks.

Good resolution of casein groups was obtained using the horizontal polyacrylamide gel electrophoresis system with continuous tris-glycine buffer. In fact, resolution appears to be superior to other published quantitative electrophoresis methods of estimating casein composition. Genetic variants of α_{s1}-casein, β-casein (except for A variants) and κ-casein could be readily determined with the same gel. The reports of many studies using quantitative electrophoresis of casein give no indication of resolution as the densitometer traces obtained are not presented. Exceptions are Murphy and Downey (1969) and Michalak (1973a) who both obtained similar traces with good resolution, although protein peaks overlapped due to the use of skim milk instead of casein. Haenlein *et al.* (1973) obtained good resolution with casein samples but the method used by Kim and Bird (1972) gave poor resolution.

The densitometer peaks were divided into 6 groups of casein components: pre-α-, α_{s1}-, α_{s2}- β-, γ- and κ-casein. Most studies of normal milk

have ignored the protein bands migrating more rapidly than α_{s0}-casein, although they have been reported in several studies of mastitic milk (Kiddy *et al.* 1968, Haenlein *et al.* 1973, Randolph *et al.* 1974). It appears that the pre-α fraction, or at least some of its components, are formed by proteolysis of the α_{s1}-caseins in a similar manner to that shown for the γ-caseins. This is supported by Aimutus and Eigel (1980), who also showed that this fraction has many similarities to λ-casein (see Long *et al.* 1958 and El-Negoumy 1973). The concentration of pre-α-casein found with mastitis-free milk was higher than the proportion of 1.5% found by Haenlein *et al.* (1973). Both pre-α and γ-caseins vary considerably in concentration in individual cow milk samples and this depends on the age and mastitis status of the cow and storage and chymosin treatment of the sample.

The 4 separate peaks obtained for $\alpha_{s2,3,4}$ and $_6$- casein were similar to those obtained by Haenlein *et al.* (1973) although these authors did not identify the components. Michalak (1973a) obtained peaks for two of the α_{s2}-caseins, with the other two components presumably being masked by β-lactoglobulin and α-lactalbumin peaks, and other studies have not obtained sufficient resolution to estimate the α_{s2}-caseins. The proportion of α_{s3}- and α_{s4}-casein in total casein was slightly higher than 4% obtained by Richardson and Creamer (1976), although this difference may be partly because Richardson and Creamer's values are as a percent of total micellar material.

In alkaline gel electrophoresis of casein the γ- and κ-casein bands are normally interspersed in the region between β-casein and the origin. Consequently reported figures of κ-casein concentration often include γ-casein, although Kim and Bird (1972) and Mariani (1972) have reported separate estimations. Proteolysis of κ-casein with

chymosin allowed separate estimates of γ- and κ-casein in the present study. Recently, methods of determining para-κ-casein by gel electrophoresis after reaction of milk with chymosin have been published by Dalgleish (1979) and Chaplin and Green (1980).

The major problem with the present quantitative electrophoresis method was the failure to consistantly produce a linear relationship between densitometer peak area and protein weight. When tested using an optical density step tablet both densitometers showed a linear response between pen height or area and optical density within the range of optical densities found using the casein sample preparation described in section (b). These results indicate that the densitometers were not responsible for the non-linear response often obtained. Therefore the effect of the staining method on the relationship between peak area and protein weight was investigated.

The Coomassie blue G250 staining method of Diezal *et al.* (1972) was selected for investigation because of its sensitivity and lack of background staining, and because amido black, which is commonly used for quantitative electrophoresis, was not considered satisfactory by Fazekas *et al.* (1963).

Datyner and Finnimore (1973) listed the requirements for a quantitative staining method as (i) the dye should form a stoichiometric complex with the protein, to obtain a linear dye/protein relationship, (ii) the staining method should be reproducible (to achieve this, sufficient time should be allowed for dye to penetrate all parts of the protein zone so as to attain an equilibrium situation) and (iii) all excess dye which is not bound to the protein should be removed.

To obtain a stoichiometric dye-protein complex it is desirable that the dye-protein bond be covalent. Rinsing stained gels in

50 gL^{-1} acetic acid for more than 5 days removed all unbound dye from the gel background. Further dye, which was electrostatically bound to the protein was released by 50 gL^{-1} ammonia solution, as this deprotonates the sites for anion adsorption (Daytner and Finnimore 1973). A proportion of dye remained bound to the protein, and this was considered by Daytner and Finnimore (1973) to be covalently bound, although Fishbein (1972) considered that Coomassie blue does not form covalent bonds. Hydrophobic interactions between non-polar regions are also involved (Fullington *et al.* 1980). Therefore the dye-protein binding with Coomassie blue G250 appears to be partly electrostatic and partly hydrophobic, and this may explain the failure to consistently obtain a linear response and the poor reproducibility of the present results. However, linear relationships between densitometer area and protein weight have been obtained using amido black (e.g. Kruski and Narayan 1974, Darling and Butcher 1976) although Racusen (1973) showed that binding was electrostatic in nature.

Fazekas *et al.* (1963) introduced Procion brilliant blue RS for quantitative work on cellulose acetate, because it can form covalent bonds with protein, and found it superior in accuracy and precision to Coomassie blue R250 and amido black. However, it could not be used to stain protein bands in polyacrylamide gel because of difficulty in removing unbound background dye (Datyner and Finnimore 1973). There is a need for a dye capable of forming covalent bonds with protein, giving the advantages of stoichiometry and precision and which, if unbound, can readily be removed from polyacrylamide gel.

The intensity of Coomassie blue G250 bound to protein bands is dependent on both staining time and rinsing time, and the dye

concentration became stable only after 16 hours staining and 7 days rinsing. Little work appears to have been done on staining and rinsing times for quantitative electrophoresis. Fish *et al.* (1969) presented densitometer tracings of gels stained with Coomassie blue R250 which showed that longer staining times (3 hours) intensified all bands. Minor bands were best seen after 16 hours staining. When introducing the G250 form of Coomassie blue, Diezel *et al.* (1972) showed that band intensity increased with storage of the gel in 50 gL^{-1} acetic acid, and Reisner *et al.* (1975) using this method found that attainment of maximum colouration was significantly slower than with other stains. Using a Coomassie blue G250 perchloric acid solution for staining, Reisner *et al.* (1975) found that overnight staining was necessary for the dye to completely penetrate the gel.

The migration distance of protein bands by electrophoresis affected the relationship between peak area and protein weight. As migration distance decreases, the area versus protein weight relationship deviates from linearity, as found by Fishbein (1972). However, even at the longest migration distance of 5.5 cm, linearity was not achieved. This experiment was carried out prior to the experiments studying staining time and the 15 minute staining method of Diezal *et al.* (1972) was used: a more linear relationship may have been obtained at the longest migration distance with overnight staining.

At the longest migration distance (10-11 cm), Fishbein (1972) found that area was proportional to protein weight up to 55 µg, but at 3-4 cm migration distance (the migration distance of β-casein in the present study) the relationship was linear to approximately 15 µg. Fishbein (1972) suggested that progressive compaction of the protein bands at shorter migration distances prevent stoichiometric uptake

of dye molecules once a critical protein concentration has been exceeded.

Using the casein preparation and sample application of the present method, the weight of α_{s1}- or β-casein applied with a milk sample of average casein content, would be approximately 7 µg. Fenner *et al.* (1975), using Coomassie blue R250, obtained a linear relationship between dye uptake and amount of protein in the range 1-10 µg protein, and Hillier (1976), using Coomassie blue G250, found a linear relationship over the range 0-4 µg protein.

Although consistent results were not obtained, some gels showed linear relationships between peak area and protein weight with the minor components, α_{s2}- and para-κ-casein (up to approximately 5 µg protein), but with the major components, α_{s1} and β-casein, a curve was obtained. It is fortunate that components of higher concentration in milk generally migrate further than those of lower concentration. Possibly the protein weights applied to the gel were too high, but further dilution would increase the difficulty in obtaining estimates of the minor components, γ- and κ-casein, which already have lower precision.

The range over which linearity is obtained can be extended by eluting the dye (Kruski and Narayan 1974, Fenner *et al.* 1975), but this would be difficult with a complex mixture of proteins such as casein,

The variation in dye binding capacities in a protein mixture such as the caseins and between-gel differences in degree of staining can be overcome by simultaneously separating a standard protein mixture prepared either by grouping known amounts of purified proteins (Hillier 1976) or by using a protein mixture which have been standardized previously using purified proteins (Darling

and Butcher 1976). The second alternative is preferably for economy of purified proteins which are time consuming and difficult to prepare, and it was intended to use this technique in the present study. However, degradation of the casein standard used in the study described in Chapter V before the purification of casein components was completed prevented the use of this technique. Casein to be used as a standard should have been freeze dried and stored at $-16^{o}C$.

Few estimates of the precision of quantitative gel electrophoresis methods are available. Using the transmission mode to scan 6 different proteins submitted separately to cellulose acetate electrophoresis, Fazekas *et al.* (1963) found that precision, expressed as coefficient of variation, was 2.0 - 13.5% with Procion blue and 10.0 - 32.0% with Coomassie blue R250. Michalak (1973a), using starch gel electrophoresis and amido black staining, reported that the precision of estimates of major milk protein components, α_{s1}- and β-casein and β-lactoglobulin was good (c.v. = 9.3, 7.3 and 13.5% respectively), whereas the precision of estimates of proteins with lower concentration, κ-casein, zones 1.00 and 1.04 (α_{s2}-caseins) and α-lactalbumin, was lower (c.v. = 19.8, 16.8 and 16.1% respectively). The use of an internal standard reduced the coefficient of variation from 7.6 - 8.5% to 2.6 - 3.5% when estimating four whey proteins stained with Coomassie blue G250 after polyacrylamide gel electrophoresis (Hillier 1976).

The accuracy of the present method was not estimated but it would have been higher if a linear relationship had existed between area and protein weight.

By expressing each casein component as a percent of total casein, the variability was reduced. Although scanning protein bands using

the Pye Unicam scanning accessory was slow and tedious, precision of estimates of all fractions except γ-casein was slightly better than with the Quick Scan densitometer.

The correction for differences between gels using the standard casein solution which was applied to gels scanned using the Quick Scan densitometer resulted in reduced variability of some fractions (pre -α, α_{s1}-, β-casein) and an increase in variability of the other fractions and so was not used in the casein composition estimates in Chapter V.

The coefficient of variation of the α_{s1}-, α_{s2}- and β-casein fractions was less than 5% with both instruments and with the Quick Scan instrument, a c.v. of approximately 14% was obtained for γ- and κ-casein. The lower precision of estimates of minor fractions γ- and κ-caseins results because very slight changes in baseline adjustment made relatively large differences to peak area (see also Law *et al.* 1979).

There have been few estimates of the absorptivity at 278 nm of casein components, particularly genetic variants of caseins, and the value of absorptivity used will affect the relative dye binding capacities and so the final casein composition determined by quantitative gel electrophoresis. Differences in absorptivity between genetic variants of both α_{s1}- and β-casein have been found.

The determination of the relative dye binding capacities used in the present study (Table IV.4) suffer from two problems: the relationship between densitometer area and protein weight was not always linear particularly with major caseins and the precision of estimates of relative dye binding capacities was poor.

Although gel electrophoresis has been frequently used to estimate casein composition, there have been few estimates of the

dye binding capacity of casein components and correction of densitometer areas for differences in dye binding.

By mixing known amounts of protein and amido black, Ashworth and Chaudry (1962) estimated that the relative dye binding capacities of α-casein, β-casein and κ-casein was 1.00: 0.90: 1.03 and Tarassuk *et al.* (1967) estimated the relative dye binding capacities of α-casein, β-casein, γ-casein and κ-casein was 1.00: 0.85: 0.93: 0.90. MacRea and Baker (1958) scanned casein bands separated by paper electrophoresis and stained with azocarmine B and found relative dye binding capacities of α:β:γ = 1.00: 0.63: 0.26. Michalak (1973a) stained protein bands separated by starch gel electrophoresis with amido black and corrected scanned areas using the dye binding capacities estimated by Zwierzchowski and Michalak (1971). These relative dye binding capacities were α_{s1}: β : κ = 1.00: 0.91: 0.67.

The only estimate of dye binding capacity of caseins using Coomassie blue G250 was by Anderson and Andrews (1977) and they found that the densitometer peak area/μg protein of β-casein was 1.18 times that of α_{s1}-casein. This difference in dye binding capacities is the reverse to that found with amido black and in the present study using Coomassie blue G250 and this explains the very high ratio of α_{s1}-casein to β-casein reported by Anderson and Andrews (1977). The dye binding capacity of κ-casein and other minor caseins was not reported by Anderson and Andrews (1977) as it was considered that κ-casein could not be readily or accurately quantified by the polyacrylamide gel electrophoretic procedure used.

It can be seen that large differences have been found for the relative dye binding capacities of casein fractions, due presumably to differences in the purity of casein fractions used, the type of dye used and method of determining the dye binding capacity.

The most reliable estimates of casein composition of bovine milk are those of Davies and Law (1977a,b, 1980) who found that on average casein consisted of 38% α_{s1}-casein, 10% α_{s2}-casein, 36% β-casein, 3% γ-casein and 13% κ-casein. The average casein composition of the milk samples collected in the experiment described in Chapter V differs from this, the main differences being the low proportion of α_{s1}-casein and high proportion of γ-casein. The casein composition obtained by gel electrophoresis depends largely on the dye binding capacities used for adjustment of integrator areas.

The relative dye binding capacity of β-casein estimated in the present study (0.66 - 0.71 for genetic variants) is lower than that found by Ashworth and Chaudry (1962), Tarassuk *et al.* (1967) and Zwierzchowski and Michalak (1971) and when used to correct densitometer areas results in a lower α_{s1}-casein concentration relative β-casein than expected when compared to the estimate of casein composition by Davies and Law (1980). Therefore it is considered that the dye binding capacities of β-caseins used in the present study are too low. Michalak (1973a) also reported a higher proportion of β-casein than α_{s1}-casein, and he considered his estimate of the dye binding capacity of α_{s1}-casein was slightly high.

The proportion of α_{s2}-casein obtained was slightly higher than that reported by Davies and Law (1980), but no estimate of the dye binding capacity of this fraction could be found in the literature.

In the present study, κ-casein concentration was estimated by correcting the area of the para-κ-casein peak for differences in dye binding from other caseins. No other estimates of the relative dye binding capacity of para-κ-casein was found in the literature.

κ-Casein has a low dye binding capacity (Zwierzchowski and Michalak 1971) and in studies where no correction for differences in dye binding capacity were made, low κ-casein concentrations were reported (e.g. Morr *et al.* 1971, Dill *et al.* 1972, Haenlein *et al.* 1973, Randolph *et al.* 1974). Using the dye binding adjustment for para-κ-casein in the present study, the proportion of κ-casein obtained was slightly lower than that of Davies and Law (1980), who considered their estimate of κ-casein to be too high due to inclusion of chymosin-insensitive protein in the κ-casein fraction (Davies and Law 1977a).

Very high values of γ-casein were obtained in the present study, compared to those of Davies and Law (1977a,b, 1980), although estimates in the literature have ranged up to 9%. Published estimates of γ-caseins may be low due to losses occurring during precipitation and washing steps of casein preparation, although Davies and Law (1977a) claimed only about 1% loss of casein during washing. Estimates of γ-casein using electrophoresis may be low due to inclusion of γ-casein with κ-casein (see Kim and Bird 1972). In the present study, the same relative dye binding capacity was used for γ-casein as for β-casein. However, Tarassuk *et al.* (1967) found that γ-casein binds more dye than β-casein, so the use of a higher dye binding capacity would have resulted in lower γ-casein concentrations. However, much of the increase in γ-casein in the present study may have resulted from prolonged storage of the casein samples.

In the study described in Chapter V, the effect of casein genotypes on the concentrations of individual caseins in milk was studied. If the genetic variants have different capacities for binding dye, concentrations of casein components estimated without

correction for this difference would be in error and false associations could be found. To the present authors knowledge there has not been any studies of the dye binding capacities of the genetic variants of caseins. Reimerdes and Mehrens (1978) reported that β-lactoglobulin B bound nearly twice as much Coomassie blue G250 as the A variant, although Hillier (1976) also using Coomassie blue G250, found little difference in their dye binding capacities.

In the present study, differences in dye binding capacity were found between the genetic variants of α_{s1}- and between those of β-casein. Therefore the casein concentrations of milk from individual cows of known casein genotype were corrected for these differences. The relative dye binding capacity used for heterozygotes was midway between the homozygotes. As κ-casein concentration was estimated by scanning para-κ-casein, this estimation was not influenced by dye binding differences between variants.

The differences in dye binding capacities between caseins and their genetic variants are generally as expected when the proteins content of basic amino acids is considered. The number of basic groups in the caseins, expressed as a percent of total amino acid residues, is α_{s1}-casein B and C 13.1, α_{s2}-caseins 16.4, β-casein B 11.0, A^1 10.5 and A^2 10.0, κ-casein A and B 10.1 and para-κ-casein 13.3. The α_{s2}-caseins, with the highest content of basic groups, would be expected to have the highest dye binding capacity, but a similar dye binding capacity to α_{s1}-casein was found. Although the number of basic groups of the α_{s1}-caseins B and C are similar, a consistent difference in dye binding capacity was found. The dye binding of β-casein genetic variants was as expected considering their content of basic groups. Para-κ-casein has a similar content of basic groups to α_{s1}-casein, but its dye binding capacity was

much lower. Based on content of basic groups, the dye binding capacity of κ-casein would be lower than that of para-κ-casein, although the carbohydrate content of κ-casein would also reduce dye binding.

The quantitative gel electrophoresis method described in this Chapter was used to estimate casein composition in the study described in Chapter V. Although the proportions of casein components were not the same as the most reliable estimates available, this is not a limitation in a comparison of composition between groups of milk samples, such as in Chapter V.

CHAPTER V

ASSOCIATION BETWEEN MILK PROTEIN GENOTYPE AND MILK YIELD AND COMPOSITION

A. Review

1. Introduction

Economically important characteristics of dairy cattle, such as milk yield and fat and protein concentration of the milk, show continuous variation. Genetic and non-genetic (environmental) factors contribute to this variation. In the past, the major genetic improvements in milk production have been made using the methods of quantitative genetics (see e.g. Falconer 1960); in particular, by the estimation of a bull's breeding value using progeny testing, the selection of superior bulls, and their use by artificial insemination.

The heritability of a quantitative trait is the proportion of the total phenotypic variance generated by the summed additive genetic effects of all quantitative loci affecting the trait. The number of loci affecting a quantitative trait was until recently generally considered to be large, with each gene having a small and essentially unidentifiable effect. However, there is considerable evidence suggesting that genetic variation in a continuous quantitative trait may often be controlled by relatively few genes. (see Robertson 1966, Stewart 1969 and Shire 1976).

In this chapter, literature on the association between milk protein loci and various quantitative traits is reviewed, and an investigation of the association between these milk protein genotypes

and milk yield and composition is described.

2. Genetic Variation and its Causes

The genetic variation of proteins discovered by the widespread use of electrophoresis is an example of the enormous variation of genes coding for proteins found in most species studied. In Chapter I.B. the genetic variants of bovine milk proteins were reviewed: all major milk proteins are now known to be polymorphic. Harris (1971) estimated that in man any individual may be heterozygous at about 16% of all gene loci coding for the structures of enzymes. Lewontin (1973) compiled data from studies on a number of species and when it was assumed that electrophoresis detects only one-third of all allelic variation, the average proportion of loci which were polymorphic was 66% and average heterozygosity per individual was 29%. It seems likely that at most gene loci coding for protein structure different alleles occur among members of natural populations.

The classic "Darwinian" view is that polymorphisms are the consequence of differential selection and represent stable balanced equilibria due to heterozygote advantage, frequency dependant selection or other processes, or they may represent intermediary stages of gene evolution (see Harris 1971 and Clarke 1975).

Natural selection of polymorphisms can be assessed only through investigation of survival rates and fecundity. Some examples of the maintenance of balanced polymorphism by selection differential exist, the best known one being sickle cell haemoglobulin polymorphism. A number of studies have provided direct evidence of natural selection by showing a correlation between environmental conditions and allelic frequency of enzymes (see review by Lewontin 1973). Clarke (1975) described experiments with genetic variants of alcohol dehydrogenase of *Drosophila melanogaster* which demonstrated the operation of frequency

dependant selection: a polymorphic population was able to exploit its environment more effectively and grew larger than a genetically uniform population.

Functional differences in the products of common alleles have been demonstrated with many proteins. Harris (1971) classified the twenty-three human enzyme polymorphisms detected at that time, and found that there was evidence for quantitative differences in enzyme activity in sixteen cases, and in most of the other seven cases the effect had not been closely examined.

To explain the wealth of genetic variability and the fact that in many cases protein polymorphisms appear to have no visible phenotypic effects and no obvious correlation with environmental conditions, Kimura and his colleagues proposed the neutral theory of molecular evolution (see Kimura 1979). This theory proposes that at the molecular level most evolutionary change and most of the variability within a species are caused not by selection but by random drift of mutant genes that are selectively equivalent.

The controversy of the relative importance of natural selection and random drift of neutral genes on the observed genetic variation has not been settled at the present time.

3. Genetic Variation and Milk Production

Numerous studies have investigated the association between genetic variants of blood antigens, enzymes and serum and milk proteins and various performance traits in dairy cattle. These have been recently reviewed by Kiddy (1979). Associations between blood groups (A, B, FV, J, L, M, S and Z systems) and economically important traits have been demonstrated (see Mather 1977), but the effects are usually small and often not consistent. Genetic polymorphism of the serum protein transferrin has received considerable attention and although some results

are conflicting, there is evidence that 2% of milk production variation is due to the transferrin locus (Ashton and Hewetson 1969). There are few studies of genetic variation of enzymes and milk production of dairy cattle but functional differences between enzyme genetic variants have been demonstrated in other species. The present study is concerned with the association between milk protein genetic variants and milk yield and composition: many significant effects have been found in other studies and these will be reviewed in a later section.

Brum *et al.* (1968) suggested that possible explanations of association between polymorphism type and production difference include pleiotropic effects of the genes at the polymorphic loci or chromosomal linkage of these genes to other genes which exert major influences upon the production traits. Neimann-Sørenson and Robertson (1961) and Brum *et al.* (1968) could find no indication of linkage between blood group genes and genes with measurable effects on the production characters studied.

Soller (1978) obtained some quantitative estimates of the possible contribution to dairy cattle improvement programmes of several selection procedures utilizing individual loci. He concluded that only selection for identifiable quantitative loci having direct physiological or molecular effects that are inherited in a simple Mendelian manner could make a significant contribution to dairy production.

Loci having direct effect on milk production could do this in a number of ways. Change in the nucleotide sequence coding for the precurser of a particular milk protein may directly affect the rate of synthesis of that protein. Also genes coding for enzymes which release the hydrophobic terminal extension or add phosphate groups and carbohydrate residues may affect the rate of synthesis or function

of a milk protein. Substitutions could also occur in nucleotide sequences of genes coding for enzymes and peptide hormones involved in the synthesis of milk components and secretion of milk. Even substitutions in enzymes and hormones involved in general body metabolism could have an effect on milk production.

Possible explanations for lack of uniformity of results or lack of significant relationships in studies of associations between polymorphisms and production traits are:

1) Although the effect of a polymorphism on a given trait may be unimportant biologically, standard tests may erroneously indicate significance due to chance alone 5% of the time (Robertson 1966).

2) The number of loci affecting a quantitative trait to an important extent may be small whereas the number of polymorphisms is large, giving a low probability of selecting the right one to study (Robertson 1966). Alternatively, a relatively large number of loci may affect a quantitative trait with a small probability that any one locus would have a major effect (Arave *et al.* 1971). As discussed earlier, the number of loci affecting quantitative traits is not known. It would be expected that the number of loci affecting the concentration of a single milk protein such as β-lactoglobulin would be less than the number affecting a more complex trait such as milk yield.

3) The effects of a polymorphic loci on a trait may depend on the genetic constitution of the population in which they are found (Robertson 1966). For example, differences in significance between breeds was found by Neimann-Sørenson and Robertson (1961), Hoogendoorn *et al.* (1969), and Munro (1978). Differences in production traits due to the cows sire can be removed by least-squares analysis (Brum *et al.* 1968, Arave *et al.* 1971 and Mather 1977).

The effect of a genetic variant may be affected by variation at other loci i.e. interactions between loci (Shire 1976). For example, El-Negoumy (1972) found that the effect of a genetic variant on the stability of casein micelles was dependant on the genetic variants of the other milk proteins present.

There is a possibility, particularly in studies involving small numbers of animals, that the group of animals containing the variant being investigated may have a higher incidence of another protein variant and so affect the relationship found. Mather (1977) suggested that analysis of relationships between genetic markers and production should be on models containing several markers and interactions, so that estimates of each marker is not really attributable to others. Another approach was used by an Italian group (Mariani *et al.* 1976, Mariani *et al.* 1979) where milk was produced by pairs of cows, one being homozygous for one allele and the other homozygous for the other allele, with other milk protein genetic types being identical. However, it is probable that there were differences in many other genetic loci, some of which could have had an effect on the production traits measured. Many studies have relied on an equal distribution of other genotypes.

4) Results of studies on the effect of polymorphisms on production may depend on how effectively environmental effects have been eliminated by selection of animals included in the study or by correction of the data. Environmental effects are taken to include herd effects (e.g. nutrition and management), season, age, stage of lactation and disease (in particular mastitis). Eidrigevich *et al.* (1972) found that the effect of genetic variants on milk yield varied substantially between farms and Janicki (1974) found differences between high and low yielding herds. Semenenko (1971b) and Marinchuk (1978) found differences in superiority in different lactation numbers.

To minimise non-genetic causes of variation, Neimann-Sørenson and Robertson (1961) used cows at the Danish Progeny Testing Stations where heifers of approximately the same age and date of calving are kept under uniform conditions during their first lactation. Michalak (1973b) used selected cows which were fed identically during their first lactation and Mariani *et al.* (1976) and Mariani *et al.* (1979) used selected pairs of cows in the same herd with comparable number and stage of lactation.

Another approach to minimise environmental variation is the use of correction factors to adjust production data for the effects of age, season and lactation length and by the use of least-squares procedures (Brum *et al.* 1968, Hoogendoorn *et al.* 1969, Arave *et al.* 1971 and Mather 1977).

5) Arave *et al.* (1971) considered that a further possible reason for the lack of uniformity of results or lack of significant relationships between polymorphisms and production traits could be linkage between polymorphic loci and genes affecting quantitative traits. Presumably, this could be because genes may be correlated in one population and uncorrelated in another, although selection is the same in both cases (see Lewontin 1973).

4. Association between Milk Protein Genotype and Milk Yield and Composition

(i) β-Lactoglobulin genotype

In searching for the possible physiological significance of the recently discovered β-lactoglobulin genetic variants A and B, Aschaffenburg and Drewry (1957) measured the casein and β-lactoglobulin concentration in milk from 24 individual cows. The results suggested some form of quantitative relationship between β-lactoglobulin and casein synthesis. When β-lactoglobulin concentration was plotted

against casein concentration, linear relationships were found with each genotype, with slope constants: $K_A = 0.150$, $K_{AB} = 0.122$ and $K_B = 0.087$. Taking the data from Aschaffenburg and Drewry's figure 1 to show the effect of β-lactoglobulin genotype on β-lactoglobulin and casein concentration separately, the mean β-lactoglobulin concentrations in gL^{-1} were A 3.6, AB 3.3 and B 2.5 and mean casein concentrations were A 24.0, AB 27.0 and B 28.3.

The association between β-lactoglobulin genotype and concentration of β-lactoglobulin in milk is the most consistently found association of a milk protein genotype with any milk protein component or production trait (Aschaffenburg and Drewry 1957, Moustgaard *et al.* 1960, Rose 1962, Murphy and Downey 1969, Feagan *et al.* 1972, Michalak 1973b, McLean *et al.* 1974, Cerbulis and Farrell 1975, Komatsu *et al.* 1977, Mariani *et al.* 1979). Mean β-lactoglobulin concentration decreases in order of β-lactoglobulin genotype A, AB, B. Cerbulis and Farrell (1975) and Mariani *et al.* (1979) also found significant differences in whey protein content between β-lactoglobulin genotypes (A > AB > B).

The increase in casein concentration with β-lactoglobulin genotype in order A, AB, and B, as found by Aschaffenburg and Drewry (1957), has been confirmed by Moustgaard *et al.* (1960) ($P < 0.0005$) and Mariani *et al.* (1979) ($P < 0.05$) and was also indicated in some data collected by the present author from cows in a controlled feeding situation. Moustgaard *et al.* (1960) and Mariani *et al.* (1979) also demonstrated that β-lactoglobulin B milk had a significantly ($P < 0.0005$ and $P < 0.001$, respectively) higher proportion of casein in the total protein, and Michalak (1973b) and Cerbulis and Farrell (1975) reported the same trend.

The results of Michalak (1973b) showed a trend of higher α_{s1}- and κ-casein contents and lower β-casein content, expressed as a percent of total protein, with β-lactoglobulin B cows when compared to β-lactoglobulin A cows (significance not given). Although Mariani *et al.* (1979) found no significant differences in casein components, expressed as a percent of total casein, the α_{s1}- and κ-casein concentrations were significantly ($P < 0.05$) higher in β-lactoglobulin B milk than in type A milk.

The results of studies on the association between β-lactoglobulin genotype and total protein concentration in milk are conflicting. Janicki (1974) and Golikova (1975) claimed β-lactoglobulin A was significantly superior for protein production, Semenenko (1971b) and Mityut'ko (1974) claimed that β-lactoglobulin B was significantly superior, Pyanovs'ka and Meshcheryakova (1974) claimed that the heterozygote, β-lactoglobulin AB, was significantly superior and Dvorak and Macha (1970), Kiddy and Kral (1973), Cerbulis and Farrell (1975), Janicki (1978) and Mariani *et al.* (1979) found no significant difference.

The results of studies on the association between β-lactoglobulin genotype and milk yield are inconsistent although most studies found that β-lactoglobulin A was superior. When comparing the A and B genotypes, Comberg *et al.* (1964), Thatcher (1965), Macha and Mullerova (1969), Arave *et al.* (1971), Kamenskaya (1973), Mityut'ko (1974), Kuzmenko *et al.* (1977) and Munro (1978) found β-lactoglobulin A significantly superior and Eidrigevich *et al.* (1972), Osipenko and Mityut'ko (1973) and Janicki (1978) found β-lactoglobulin B significantly superior.

The only studies reporting a significant association between β-lactoglobulin genotype and fat concentration were Golikova (1975)

(β-lactoglobulin A superior) and Sherbon *et al.* (undated) and Hoogendoorn *et al.* (1969) (β-lactoglobulin B superior).

(ii) α_{s1}-Casein genotype

Hoogendoorn *et al.* (1969) found a significant association between α_{s1}-casein genotype and milk protein content with Jersey cows but the superior genotype was not given. Dvorak and Macha (1970) and Munro (1977, 1978) found that α_{s1}-casein BC cows produce milk with significantly higher protein content than α_{s1}-casein B cows, whereas Mityut'ko (1974) found that the reverse was true.

Golikova and Panin (1972) found that α-casein content (expressed as a percent of total casein) was higher and β-casein content was lower for α_{s1}-casein C cows when compared to α_{s1}-casein B cows (significance not given).

Mityut'ko (1974) reported that α_{s1}-casein B cows had significantly higher milk yield than BC cows although other studies where significance was not demonstrated have reported the reverse trend.

Munro (1978) found that α_{s1}-casein BC Friesians had a higher milk fat content than type B. A significantly higher fat yield was obtained from α_{s1}-casein BC cows compared to B type cows although milk yields were not significantly different (Semenenko 1971a).

(iii) β-Casein genotype

Hoogendoorn *et al.* (1969) found that in Milking Shorthorns, the β-casein genotype significantly affected the milk protein content and Munro (1977, 1978) showed that milk containing β-casein B genetic types had significantly higher protein contents, with both Friesian and Jersey cows.

Heterozygous β-casein AB cows synthesized significantly more

total protein, casein, β-casein and whey "globulin" than the homozygotes (Mityukov 1974). However, Golikova and Panin (1972) found that milk from β-casein A cows had a higher percent α-casein and lower percent β-casein when compared to β-casein B cows.

With regard to the association between β-casein genotype and milk yield, Kamenskaya (1973) found type A and AB were higher than type B, Mityut'ko (1974) found that type A was higher than AB, Timoshenko (1973) found type AB was higher than type A and Eidrigevich *et al.* (1972) found the heterozygote AB gave the highest milk yields.

Hoogendoorn *et al.* (1969) found a significant association between β-casein genotype and fat content in Shorthorns, Mityut'ko (1974) found β-casein AB had a significantly higher fat content than type A and Munro (1978) reported a significant relationship in Jerseys.

(iv) κ-Casein genotype

Two studies have shown that milk from κ-casein B cows is significantly superior to other types for milk protein content (Hoogendoorn *et al.* 1969 with Jerseys and Munro 1977, 1978 with Jerseys and Friesians) and this trend was also found in several other studies where significance was not given (Michalak 1973b, Mariani *et al.* 1976 and Pavlyuchenko *et al.* 1977).

Mityukov (1974) found that animals heterozygous for the κ-casein genotype were capable of more intensive synthesis of milk proteins, but only the α_{s1}-casein fraction was significantly different. Casein concentrations calculated from the data of Michalak (1973b) show κ-casein AB is associated with higher casein content than κ-casein A, and Mariani *et al.* (1976) found that κ-casein B milk had a higher content of casein than κ-casein A milk, but the significance of the results of these two studies was not given. Michalak (1973b) and Mariani *et al.* (1976) found similar effects of κ-casein genotype on

individual protein concentrations: κ-casein B milk contained a lower α_{s1}-casein content and higher β- and κ-casein contents, with similar whey protein contents.

Munro (1978) reported that κ-casein genotype had a significant association with milk yield, but no significant association with fat content has been reported.

(v) Casein genotype

The genes responsible for the synthesis of α_{s1}, β- and κ-caseins are closely linked (see section I.B.3), and so the effect of the casein genotypes on milk production and composition should be considered as a 'casein gene complex'.

Hoogendoorn *et al.* (1969) found that when the four milk protein loci (α_{s1}, β- and κ-casein and β-lactoglobulin) were combined, ignoring breeds, significant differences ($P < 0.005$) in percent protein was observed. Larsen (1972) showed that casein polymorphism significantly affected the protein content of milk. In Jerseys, 7.5% of the variation in percent protein was accounted for by the casein system, with lesser amounts in two other breeds.

Mityut'ko and Pavlyuchenko (1973) found that cows with the casein genotype β B - κ A gave the highest milk yield while β AB - κ A gave the lowest. Zhebrovskii *et al.* (1975) reported that α_{s1} B - β A - κ A - β1g A was the most promising genotype when considering milk, fat and protein yield.

5. Association between Milk Protein Genotype and Other Parameters

Earlier sections have reviewed associations between milk protein genotypes and milk yield, fat and protein content and protein composition, those being the parameters directly involved in the present investigation. Before a decision could be made with regard

to selection for a particular genetic variant, other parameters of economic importance would also have to be considered, such as fertility, feed efficiency, mastitis resistance and suitability of the milk for manufacture of products.

Few studies of the association between milk protein genotype and fertility have been reported. Janicki (1977) found that β-lactoglobulin AB cows were significantly younger at first calving than type A or B. Hargrove *et al.* (1980) found that the probability of conception was significantly associated with α_{s1}-casein (BC > B), and β-casein (A^1A^3 > others) genotype.

The feed efficiency of β-lactoglobulin A cows was significantly greater than that of β-lactoglobulin B cows (Arave *et al.* 1971). Resistance to mastitis was greater with β-lactoglobulin heterozygotes (AB) than with the homozygotes (Kriventsov and P'yankova 1971, Osterhoff *et al.* 1973).

Casein variants with a lower net negative charge (i.e. the slower variants during electrophoresis in an alkaline medium) have shorter chymosin clotting times resulting in firmer curds at a given cutting time. For cheesemaking, α_{s1}-casein C gives a firmer curd than B (Sadler *et al.* 1968), and with β-casein and κ-casein, type B gives a firmer curd than A (Feagan *et al.* 1972). Mariani *et al.* (1976) found that milk containing κ-casein B was more suited for manufacture of Parmesan cheese, and Morini *et al.* (1979) showed that the cheese produced had better physical, chemical and organoleptic characteristics than cheese from κ-casein A milk.

6. Conclusions

A number of studies have shown associations between milk protein genotype and milk yield, milk composition and other parameters, but in very few cases are they significant and consistent. Also, little work

has been published on genotypes present in low frequencies, such as α_{s1}-casein C, β-casein C and β-lactoglobulin C.

β-Lactoglobulin genotype affects the concentration of β-lactoglobulin in the milk and appears to affect the ratio of casein to whey protein, without any consistent effect on the total protein content of the milk. For cheesemaking, β-lactoglobulin B milk would be preferable to β-lactoglobulin A milk. However, a number of studies have indicated that β-lactoglobulin A is associated with higher milk yields, and one study has shown that the feed efficiency of β-lactoglobulin A cows is significantly higher than β-lactoglobulin B cows. As milk yield is negatively correlated with concentrations of milk components, considerations of commercial breeding may have to accomodate conflicting effects of a genotype.

There has generally been insufficient studies to draw definite conclusions about the association between casein genotypes and milk yield and composition. α_{s1}-Casein B cows appear to produce more milk containing lower concentrations of fat and protein, when compared to α_{s1}-casein BC cows. With β-casein genotype, type A cows appear to produce more milk with lower protein content, when compared to type B cows. κ-Casein B cows appear to produce more milk containing a higher content of protein and casein, compared to κ-casein A cows.

For cheesemaking, the α_{s1}-casein C, β-casein B and, κ-casein B genes are superior.

In the present investigation, the association between milk protein genotype and milk yield and composition was studied in two breeds of dairy cattle in South Australia. Apart from the known association between β-lactoglobulin genotype and β-lactoglobulin concentration, there have been few studies on the direct effect of milk protein genotype on the concentration of that protein, and in the present

investigation the association between casein genotypes and individual casein concentrations was studied. The association between β-lactoglobulin genotype and total casein concentration in milk, demonstrated in several studies, could be of practical significance for manufacture of cheese and other products and was also investigated.

B. The Present Investigation

1. Experimental

(i) Herd selection

Herd recording in South Australia is carried out by the Herd Improvement Services Co-operative of South Australia Limited (H.I.S.C.O.L.). Only herds participating in the H.I.S.C.O.L. monthly herd recording service were included in the present investigation as this facilitated collection of milk samples, provided milk yield and fat content on the day of milk collection and also provided records of the breed, sire, date of birth, calving date and lactation data for each cow. To facilitate milk collection, the study was restricted to herds in the Central and Southern Hills areas of South Australia. Within these two areas, H.I.S.C.O.L. has two testing centres at Hahndorf and Yankalilla respectively. Both 'herd recorder sampled' and 'owner sampled' herds were used, and both stud and grade herds were used.

Eight large herds were selected to give the maximum number of suitable samples within each herd. When selecting these herds, uniformity of breed, seasonality of calving and herd cell count were also used as criteria. Cows in the same herd were assumed to be under uniform management and nutritional conditions.

This study was restricted to the Jersey and Friesian breeds, which are the two major breeds of dairy cattle in South Australia,

and four herds of each breed were selected. Where herds contained mixed breeds, herd size selection was based on the number of purebred animals indicated by the herd manager. With one herd (herd 6) H.I.S.C.O.L. records later indicated that only 29 of the 84 milk samples selected were from pure Friesian cows. The herds selected, breed and sampling date are in Table V.1.

To reduce seasonal effects, the herds were sampled within the shortest time possible - a four month period between May and September, 1978. This time was selected to give the maximum number of milk samples from cows in mid-lactation. The order of sampling of the selected herds depended on the seasonal calving pattern of the herd and the H.I.S.C.O.L. test date.

(ii) Milk collection, milk yield and fat concentration

A subsample of milk from each cow was taken during the evening and morning milking using Tru-test milk meters (Tru-test Plastics Ltd., Auckland, New Zealand). These separate p.m. and a.m. samples were transported to the H.I.S.C.O.L. testing centre at either Hahndorf or Yankalilla, warmed to 40^{o}C, mixed, and weighed to obtain the daily milk yield in litres. A subsample was taken for fat determination and the remainder was immediately transported to the Northfield Research Centre. The fat concentration of the composite p.m.-a.m. milk sample was determined by H.I.S.C.O.L. using the Babcock method (Standards Association of Australia 1976).

(iii) Sample selection

Lactation length was restricted to between 10 and 270 days so that variation in protein composition due to early and late lactation milk was reduced. At the time of sampling, calving dates were not known, and samples from cows with monthly test numbers of 9 or greater

TABLE V. 1

The breed and sampling date of selected herds and various cow and milk sample numbers

Herd No.	Major breed	Date sampled	Total cows in milk	No. of pure bred cows of the major breed	No. of samples initially selected[a]
1	Jersey	10.5.78	118	118	80
2	Jersey	7.6.78	153	106	68
3	Jersey	22.8.78	126	88	70
4	Jersey	11.9.78	121	121	90
5	Friesian	22.6.78	124	124	80
6	Friesian	11.7.78	160	29	84[b]
7	Friesian	19.7.78	140	139	104
8	Friesian	16.8.78	105	77	47

[a] From cows indicated by the farm manager to be pure bred, with monthly test number of 8 or less and W.M.T. reading of 15 or less

[b] Only 29 samples were later found to be from pure bred animals: other samples, although from cows more than 3/4 Friesian, were not included in the gene frequencies and genotype/production analysis.

were discarded. When the calving date became available, and the lactation length at sampling could be calculated, 5 cows were found to have been lactating for less than 10 days and 17 cows for more than 270 days, and so the results of these samples were not used.

To eliminate the effect of subclinical mastitis on protein composition the milk samples were tested using the Wisconsin Mastitis Test (Thompson and Postle 1964) and samples with a reading of more than 15 mm were discarded.

(vi) Fat separation

Whole milk was centrifuged at 3000 r.p.m. (1000 *g*) and 2°C for 5 minutes using a M.S.E. High Speed 18 centrifuge (Measuring and Scientific Equipment Ltd., London, U.K.) or a Damon/IEC B-60 ultra-centrifuge (Damon/IEC Division, Needham Heights, Massachusetts, U.S.A.). The hard fat layer was broken with a spatula and the skim milk decanted.

(v) Skim milk composition

(a) Total solids concentration

The total solids concentration of skim milk samples was determined by drying on a hot plate at 108°C for 25 minutes. An aluminium milk bottle top containing two filters (Argus Rex, Nykoping, Sweden) was heated on a hot plate for 30 minutes and quickly weighed while hot. One mL of skim milk was added to the filters using a glass barrelled, stainless steel syringe and the bottle tops again placed on the hot plate. After drying for 25 minutes, they were again quickly weighed while hot. The total solids concentration obtained was corrected for an average fat content of 0.9 gL^{-1} in the skim milk. This correction factor was determined by selecting 5 milk samples containing a range of fat concentrations, centrifuging at 3000 r.p.m. and 2°C for 5 minutes

as used to prepare the skim milk, and determining the residual fat concentration using a Milko-tester Mark II (Foss-Electric, Hillerød, Denmark). There was no trend in residual fat concentration with initial fat content and the mean (0.9 ± 0.32 gL^{-1}) was used to correct the total solids concentration of skim milk.

(b) Protein concentration

The protein concentration of the skim milk samples was determined using a Pro-Milk Mark II (Foss-Electric, Hillerød, Denmark). Prior to the sampling of each herd, the volume of dye delivered by the Pro-Milk was adjusted so that the protein reading obtained for a bulk milk sample was the same as the crude protein value obtained by duplicate Kjeldahl determinations. The syringe used for milk sampling was adjusted to deliver 1 g of distilled water.

(c) Protein composition

The casein concentration of the skim milk samples was determined using method C as described in Chapter III. With a small proportion of samples, the protein concentration of skim milk and whey exceeded 55 gL^{-1}, the maximum reading on the Pro-Milk scale. When this occurred, a skim milk sample of lower protein concentration was used as the base to obtain reading III.

The casein precipitated from the skim milk samples during estimation of whey protein and casein concentration was redissolved in imidozole-HCl buffer as described in Chapter IV and stored at $-16^{o}C$. The casein composition was determined by quantitative polyacrylamide gel electrophoresis as described in Chapter IV. This was not completed until approximately one year after sample collection due to difficulties in obtaining a linear relationship between the amount of purified protein added and densitometer peak area.

The concentrations of β-lactoglobulin and α-lactalbumin were determined by radial immunodiffusion as described in Chapter IV. The diluted skim milk samples were loaded on the agar plates on the day after sample collection.

(vi) Lactation data

Lactation data (milk yield, fat yield, mean fat content and lactation length) was collected for the 1978-79 recording year from H.I.S.C.O.L. records. This lactation data was missing from the H.I.S.C.O.L. records for 37 of the cows. This could be due to two reasons:- (1) the cow failed to lactate for a minimum 90 day period required for compiling a lactation summary or (2) the lactation summary contains lactation data for all cows completing their 90-300 day lactation before 31st March. Some cows, particularly in herds sampled later, may not have finished lactating within the 1978-79 recording year and the 1979-80 recording year lactation summaries were not available.

(vii) Genetic variants

(a) α_{s1}-, β- and κ-Casein variants in alkaline gel

The genetic variants of α_{s1}-, β- and κ-casein, with the exception of β-casein A variants, were obtained from the acrylamide gels used for determining casein composition (see Chapter IV).

(b) β-Casein A variants in acid gel

Skim milk samples for genetic typing of β-casein A and β-lactoglobulin were stored at -16°C. β-Casein A genetic variants were determined by thin layer starch gel electrophoresis using the buffer system of T.K. Bell (personal communication).

The gel contained 20 g hydrolysed starch, 29 g urea and 100 mL of lithium formate buffer of pH 3.0 made up of 0.017 M lithium hydroxide

and 0.083 M formic acid. The electrode buffer of pH 3.7 was made up of 0.079 M lithium hydroxide and 0.116 M formic acid.

The starch slurry was poured into a mould consisting of a Perspex frame and glass plates, to produce a starch layer 246 x 113 x 1.5 mm on a 1 mm thick glass plate.

The skim milk samples were diluted with an equal volume of 7 M urea solution and loaded into the gel as described for the quantitative acrylamide gels (Chapter IV). Electrophoresis was carried out for 5 hours using an LKB Multiphor apparatus (LKB Produckter AB, Bromma, Sweden) with the power source adjusted initially to 260 volts, and with water at 15°C circulating through the cooling plate.

The gels were stained for 1 hour with 0.0069 gL^{-1} nigrosine in methanol: water: glacial acetic acid (15:15:2, v:v:v) and rinsed in methanol: water: glacial acetic acid (5:5:1, v:v:v).

Four samples had deteriorated and could not be typed for β-casein as 3 bands of varying intensity were present. The acid casein preparations of these four samples were also tested, with the same result.

(c) β-Lactoglobulin variants

The β-lactoglobulin genetic variants were determined by starch gel electrophoresis using the buffer system of Gahne (1963). The gel contained 13.5 g hydrolysed starch and 100 mL of pH 8.5 buffer made up as 0.060 M Tris, 0.005 M citric acid, 0.008 M lithium hydroxide and 0.031 M boric acid. The pH 8.5 electrode buffer was made up as 0.050 M lithium hydroxide and 0.191 M boric acid.

The method of gel preparation was similar to that used for acid β-casein starch gels. Skim milk samples were loaded into the gel without dilution. Electrophoresis was carried out at 16°C for 2.5 hours with initial settings of 250-260 V and 65-80 mA giving 13-14 Vcm^{-1}.

The gel was stained for 1 minute in 0.064 gL^{-1} amido black in methanol: water: glacial acetic acid (5.6:5:1, v:v:v) and rinsed in methanol: water: glacial acetic acid (5:5:1, v:v:v).

(viii) Statistical analysis

Comparison of genotype frequencies between breeds and between herds within breeds was carried out using j x k contingency tables (Mather 1964). Non-independent segregation between pairs of milk protein genes within each breed was also tested using j x k contingency tables.

The association between milk protein genotype and milk and fat yield and milk composition variables was analysed by least-squares analysis of variance (Harvey 1975). The model fitted included breed, herds within breed, age of cow and genetic type as main effects, and the two way interaction terms breed by age of cow, breed by genetic type and age of cow by genetic type. Stage of lactation was included as a linear covariate. Breed, age of cow and genetic type were treated as fixed effects, whereas herds within breed was treated as a random effect. The four milk proteins studied which exhibit genetic variation were included in the model one at a time. The effect of sires within herds was ignored, as the number of sires used in each herd was large and so the number of daughters of each sire was often small with large variation in numbers between sires. The sires of 80 cows in 5 herds was unknown or not available from H.I.S.C.O.L. results. Fifty seven known sires were used in the 4 Jersey herds and 45 of these had 3 or less daughters and 39 sires were used in the Friesian herds and 31 had 3 or less daughters.

2. Results

(i) Milk yield and composition

The mean lactation milk and fat yield and fat concentration, and the concentrations of components in the test day sample, for each herd and the two breeds, are in Table V. 2. The proportion of casein in total protein, proportion of casein components in total casein and proportion of β-lactoglobulin and α-lactalbumin in total whey protein are in Table V. 3.

The significant differences obtained due to the effect of breed, herd within breed, age of cow and stage of lactation on lactation milk and fat yield, mean fat concentration and the composition of the test day milk sample are in Tables V. 4. and 5. The least-squares means and standard errors of these variables for breed and age of cow are in Tables V. 6. and 7.

The concentrations of total solids, fat, protein, casein, α_{s1}-, β- and κ-casein and whey protein were significantly higher in skim milk from Jerseys compared to concentrations in Friesian milk. Lactation milk and fat yields were not significantly different. The only significant difference in casein composition between breeds, was a higher proportion of κ-casein in milk from Jerseys ($P < 0.05$).

Significant differences between herds within breed was obtained for all yields and concentrations measured.

Lactation milk and fat yield increased significantly ($P < 0.001$) with increasing age. Significant differences between age groups were found with total solids, fat, whey protein and β-lactoglobulin concentrations: the concentration of each reached a maximum in the second or third lactation and then declined with increasing age. Similar, non-significant, trends were indicated with protein and casein concentrations. The concentration and proportion of α_{s2}-, β- and κ-casein declined and that of γ-casein increased with increasing age.

TABLE V. 2

The mean, standard deviation and range of lactation milk and fat yield, fat concentration and the concentrations of components in the test day milk sample, for each herd and breed

Variable	Jersey herds				Jersey mean (range)	Friesian herds				Friesian mean (range)	Overall mean (range)
	1	2	3	4		5	6	7	8		
Lactation data:											
Number of observations	73	63	59	68	263	77	27	97	37	238	501
Milk yield (L)	2850 ± 619.5[#] (312 - 4110)	2029 ± 570.3 (410 - 3316)	3230 ± 548.1 (2134 - 4875)	4614 ± 782.6 (2685 - 6439)	3195 (312 - 6439)	2887 ± 818.1 (737 - 4889)	3192 ± 706.8 (1023 - 4264)	3775 ± 831.3 (1111 - 5484)	4191 ± 985.6 (2120 - 6453)	3486 (737 - 6453)	3333 (312 - 6453)
Fat yield (kg)	162 ± 33.8 (13 - 224)	104 ± 26.8 (24 - 164)	172 ± 30.9 (112 - 259)	247 ± 38.7 (158 - 335)	172 (13 - 335)	123 ± 33.3 (22 - 189)	134 ± 33.2 (64 - 186)	152 ± 34.8 (51 - 276)	171 ± 40.0 (79 - 251)	144 (22 -276)	159 (13 - 335)
Fat concentration (gL^{-1})	57 ± 4.7 (47 - 70)	52 ± 5.1 (42 - 62)	53 ± 3.8 (46 - 65)	54 ± 5.0 (42 - 65)	54 (42 - 70	43 ± 4.3 (30 - 55)	42 ± 6.2 (35 - 63)	40 ± 4.4 (29 - 53)	41 ± 3.7 (35 - 49)	41 (29 - 63)	48 (29 - 70)
Test day data:											
Number of observations	73	63	69	84	289	77	29	101	42	249	538
Milk yield (L)	8.9 ± 2.68 (3.0 - 15.3)	7.5 ± 2.73 (2.6 - 13.4)	11.9 ± 3.58 (4.1 - 20.1)	17.7 ± 5.19 (1.9 - 29.5)	11.9 (1.9-29.5)	12.3 ± 4.54 (4.9 - 22.9)	15.0 ± 3.60 (6.6 - 22.2)	13.0 ± 3.98 (3.9 - 23.8)	13.7 ± 3.87 (7.3 - 20.1)	13.1 (3.9 - 23.8)	12.5 (1.9 - 29.5)
Concentrations (gL^{-1})											
Total solids	169 ± 14.0 (144 - 204)	151 ± 10.9 (130 - 185)	153 + 10.5 (130 - 189)	160 + 13.0 (134 - 188)	159 (130 - 204)	134 ± 10.9 (115 - 167)	135 ± 9.7 (115 - 159)	131 ± 8.0 (106 - 155)	130 ± 9.4 (111 - 144)	132 (106 - 167)	147 (106 - 204)
Fat	63 ± 9.7 (42 - 97)	52 ± 7.6 (34 - 75)	52 ± 6.3 (33 - 66)	53 ± 7.4 (42 - 70)	55 (33 - 97)	42 ± 6.9 (26 - 60)	39 ± 6.3 (28 - 57)	41 ± 5.6 (20 - 57)	41 ± 5.9 (30 - 52)	41 (20 - 60)	49 (20 - 97)
Protein	41.8 ± 4.24 (33.3 - 52.3)	39.6 ± 3.64 (23.5 - 49.2)	37.6 ± 3.11 (31.7 - 43.9)	42.6 ± 3.61 (32.2 - 50.3)	40.6 (23.5 - 52.3)	33.5 ± 3.30 (27.3 - 42.3)	32.8 ± 2.68 (28.3 - 39.9)	30.1 ± 2.31 (25.1 - 38.9)	32.4 ± 3.10 (26.1 - 38.2)	31.9 (25.1 - 42.3)	36.6 (23.5 - 52.3)
Casein	32.4 ± 3.49) (25.4 - 40.7)	31.0 ± 3.02 (23.5 - 41.3)	29.3 ± 2.77 (22.2 - 35.1)	33.6 ± 3.34 (24.9 - 41.8)	31.7 (22.2 - 41.8)	25.5 ± 2.61 (20.9 - 31.4)	26.3 ± 2.54 (22.4 - 33.1)	23.3 ± 2.00 (18.6 - 32.2)	25.5 ± 2.39 (20.2 - 29.5)	24.7 (18.6 - 33.1)	28.5 (18.6 - 41.8)
α_{s1}-casein	10.3 + 1.44 (7.9 - 14.4)	10.3 + 1.29 (7.6 - 13.7)	9.3 + 1.06 (6.9 - 11.6)	10.5 + 1.00 (7.5 - 13.2)	10.1 (6.9 - 14.4)	8.6 + 1.03 (6.6 - 11.6)	8.3 + 0.84 (6.5 - 10.2)	8.0 + 0.82 (5.6 - 10.4)	8.5 ± 0.78 (7.2 - 10.2)	8.3 (8.0 - 11.6)	9.3 (6.9 - 14.4)
α_{s2}-casein	3.8 + 0.68 (2.3 - 5.4)	3.3 + 0.62 (1.8 - 4.4)	3.8 + 0.69 (2.1 - 5.3)	5.1 + 0.96 (2.9 - 7.1)	4.1 (1.8 - 7.1)	3.0 ± 0.69 (1.3 - 4.7)	3.1 ± 0.73 (1.3 - 4.6)	2.7 ± 0.65 (1.5 - 5.6)	3.0 ± 0.56 (1.3 - 4.4)	2.9 (1.3 - 5.6)	3.6 (1.3 - 7.1)
β-casein	11.6 ± 1.49 (8.1 - 14.8)	10.4 ± 1.47 (7.0 - 15.2)	10.5 ± 1.18 (7.7 - 13.4)	10.8 ± 1.23 (8.1 - 13.7)	10.8 (7.0 - 15.2)	9.2 ± 1.03 (6.5 - 12.0)	9.3 ± 1.17 (7.2 - 11.9)	8.2 ± 0.95 (5.9 - 11.0)	8.8 ± 1.09 (5.7 - 11.2)	8.8 (5.7 - 12.0)	9.9 (5.7 - 15.2)
γ-casein	2.6 ± 1.19 (0.6 - 6.0)	3.2 ± 1.40 (0.5 - 7.0)	2.1 ± 0.88 (0.5 - 4.8)	3.0 ± 0.87 (0.9 - 6.3)	2.7 (0.5 - 7.0)	1.9 ± 0.84 (0.4 - 5.2)	2.4 ± 0.75) (1.1 - 4.5)	1.9 ± 0.75 (0.4 - 4.4)	2.6 ± 0.96 (1.2 - 4.9)	2.1 (0.4 - 5.2)	2.4 (0.4 - 7.0)
κ-casein	4.2 ± 0.76 (2.7 - 5.9)	3.8 ± 0.81 (1.4 - 5.6)	3.6 ± 0.59 (2.1 - 5.2)	4.1 ± 0.79 (2.4 - 6.3)	3.9 (1.4 - 6.3)	2.6 ± 0.68 (1.2 - 4.3)	3.3 ± 0.53 (2.5 - 4.5)	2.5 ± 0.47 (1.4 - 3.5)	2.7 ± 0.62 (1.7 - 4.5)	2.7 (1.2 - 4.5)	3.4 (1.2 - 6.3)
Whey protein	9.3 ± 1.46 (6.1 - 12.9)	8.6 ± 2.03 (5.3 - 16.7)	8.3 ± 1.02 (5.5 - 11.0)	9.0 ± 1.01 (6.5 - 11.0)	8.8 (5.3 - 16.7)	7.6 ± 1.54 (4.9 - 13.6)	6.5 ± 0.91 (4.2 - 7.9)	6.3 ± 0.94 (4.6 - 9.9)	6.9 ± 1.27 (4.9-9.7)	7.0 (4.2 - 13.6)	8.0 (4.2 - 16.7)
β-lactoglobulin	4.1 ± 0.83 (2.4 - 5.9)	3.9 ± 0.75 (2.6 - 5.5)	3.0 ± 0.48 (2.1 - 4.1)	3.6 ± 0.65 (2.5 - 5.1)	3.6 (2.1 - 5.9)	3.2 ± 0.58 (2.0 - 4.4)	2.9 ± 0.50 (2.0 - 3.8)	3.0 ± 0.47 (1.9 - 4.1)	2.7 ± 0.47 (1.8 - 3.5)	3.0 (1.8 - 4.4)	3.3 (1.8 - 5.9)
α-lactalbumin	0.95 ± 0.126 (0.70 - 1.37)	1.18± 0.114 (0.94- 1.56)	1.12 ± 0.073 (0.98 - 1.33)	1.26 ± 0.129 (1.01 - 1.54)	1.13 (0.70 - 1.56)	0.97 ± 0.125 (0.74 - 1.38)	1.02 ± 0.102 (0.81 - 1.27)	1.01 ± 0.116 (0.69 - 1.38)	0.88 ± 0.105 (0.62- 1.14)	0.98 (0.62 - 1.38)	1.06 (0.62 - 1.56)

[#] mean ± standard deviation with the range in brackets underneath.

TABLE V. 3

The mean, standard deviation and range of the proportion of casein in total protein and proportion of casein components in total casein, and mean proportion of whey protein in total whey protein, for each herd and breed

Variable	Jersey herds				Jersey mean (range)	Friesian herds				Friesian mean (range)	Overall mean (range)
	1	2	3	4		5	6	7	8		
Casein as % of Total Protein	77.5± 2.67[#] (71.3-83.0)	78.3± 4.14 (63.9-84.7)	77.9± 2.53 (70.0-83.7)	78.9± 2.35 (73.3-83.5)	78.1 (63.9-84.7)	76.1± 3.54 (67.6-84.2)	80.2± 2.75 (74.8-86.0)	77.4± 2.69 (67.1-84.3)	78.7± 2.91 (71.9-83.7)	77.4 (67.1-86.0)	77.9 (63.9-86.0)
Casein components as % of total casein											
α_{s1}-casein	31.7± 2.57 (26.1-38.7)	33.2± 2.73 (25.9-39.6)	31.9± 2.27 (25.4-36.9)	31.3± 2.05 (26.4-37.6)	31.9 (25.4-39.6)	33.8± 2.65 (29.4-39.9)	31.3± 1.55 (28.6-34.8)	34.5± 2.39 (23.2-39.3)	33.3± 1.55 (30.1-36.4)	33.6 (23.2-39.9)	32.6 (23.2-39.9)
α_{s2}-casein	11.6± 1.57 (7.5-14.6)	10.6± 1.64 (6.7-14.5)	12.9± 1.76 (8.4-16.3)	15.3± 1.88 (10.9-18.4)	12.8 (6.7-18.4)	11.9± 2.08 (5.1-16.8)	11.7± 2.36 (5.5-16.1)	11.5± 2.07 (5.9-17.4)	11.6± 1.81 (6.4-15.4)	11.7 (5.1-17.4)	12.5 (5.1-18.4)
β-casein	35.8± 3.08 (26.6-41.7)	33.6± 4.20 (23.7-43.8)	35.9± 2.68 (29.6-41.9)	32.1± 2.24 (25.6-37.6)	34.2 (23.7-43.8)	36.3± 3.22 (27.9-43.5)	35.2± 2.88 (28.7-42.0)	35.3± 2.88 (27.6-42.1)	34.4± 3.13 (28.2-39.7)	35.4 (27.6-43.5)	34.7 (23.7-43.8)
γ-casein	7.9± 3.68 (2.3-19.5)	10.4± 4.36 (1.9-23.7)	7.1± 2.82 (1.9-16.8)	9.0± 2.52 (2.8-16.2)	8.6 (1.9-23.7)	7.6± 3.08 (1.7-17.9)	9.1± 2.83 (4.3-14.4)	8.1± 3.31 (1.7-20.2)	10.2± 3.61 (4.3-18.5)	8.5 (1.7-20.2)	8.5 (1.7-23.7)
κ-casein	12.9± 1.85 (9.8-18.5)	12.3± 2.26 (4.4-18.7)	12.3± 1.65 (9.3-17.0)	12.3± 1.61 (6.7-15.9)	12.4 (4.4-18.7)	10.3± 2.32 (4.7-15.9)	12.6± 1.41 (9.4-14.7)	10.6± 1.82 (6.4-14.8)	10.5± 2.04 (6.9-16.2)	10.8 (4.7-16.2)	11.8 (4.4-18.7)
Whey proteins as % of total whey protein											
β-lactoglobulin	44.1	45.4	36.1	40.0	40.9	42.1	44.6	44.1	39.1	42.9	41.3
α-lactalbumin	10.2	13.7	13.5	14.0	12.8	12.8	15.7	14.9	12.8	14.0	13.3

[#] mean (calculated from the mean concentrations in Table V. 2) + standard deviations with range in brackets underneath

TABLE V. 4.a

Least-squares analysis of variance of lactation milk and fat yield, fat concentration and the concentrations of total solids, protein, casein, whey protein, β-lactoglobulin and α-lactalbumin in the test day milk sample when β-lactoglobulin genotype was included in the model

Source of variation	d.f.	Mean squares-Complete lactation data			d.f.	Mean squares-Concentrations of components in test day sample (gL^{-1})					
		Milk yield (L)	Fat yield (kg)	Fat content (gL^{-1})		Total solids	Protein	Casein	Whey Protein	β-Lacto-globulin	α-Lact-albumin
Breed (B)	1	5,610.91	103,687.76	9,456.55***	1	41,175.77*	4,678.64**	2,971.98**	190.33*	33.49	2.103
Herds (within breeds)	6	40,727,617.99***	109,992.86***	246.13***	6	3,351.74***	304.58***	215.22***	19.82***	8.27***	0.650***
Age of cow (A)	3	11,114,818.73***	27,917.09***	59.19*	3	267.55*	16.90	9.16	7.71***	0.49*	0.005
β-lactoglobulin genotype (G)	4	190.610.63	600.64	49.93*	4	229.46*	27.24**	23.84**	7.40***	14.22***	0.029
B x A	3	1,723,958.16**	2,266.66*	46.35	3	88.05	8.85	17.90*	1.27	0.06	0.006
B x G	2	179,332.65	414.67	17.30	2	7.50	1.43	5.11	6.96**	0.68*	0.006
A x G	12	368,244.41	978.79	19.48	12	170.85*	6.08	6.21	1.73	0.10	0.008
Stage of lactation (linear)	1	158,629.87	53.11	2.99	1	13,795.77***	1,737.57***	865.29***	149.64***	26.53***	0.285***
Remainder	467	404,265.38	812.97	20.47	504	91.30	7.44	6.22	1.22	0.15	0.013

* P<0.05; ** P<0.01; *** P<0.001

TABLE V. 4.b

Least-squares analysis of variance of lactation milk and fat yield, fat concentration and the concentrations of total solids, protein, casein, whey protein, β-lactoglobulin and α-lactalbumin in the test day milk sample when α_{s1}-casein genotype was included in the model

Source of variation	d.f.	Mean squares-Complete lactation data			d.f.	Mean squares-Concentrations of components in test day sample (gL^{-1})					
		Milk yield (L)	Fat yield (kg)	Fat content (gL^{-1})		Total solids	Protein	Casein	Whey Protein	β-Lacto-globulin	α-Lact-albumin
Breed (B)	1	493,021.29	15,584.73	764.70	1	3,241.18	372.28	364.62	0.02	0.09	0.297
Herds (within breeds)	6	45,360,227.44***	121,613.55***	224.52***	6	3,029.02***	283.94***	193.39***	22.13***	9.55***	0.647***
Age of cow (A)	3	8,321,679.90***	20,146.25***	23.22	3	151.99	9.57	7.00	2.76	0.59	0.002
α_{s1}-Casein genetic type(G)	2	27,669.44	181.67	44.26	2	9.93	0.06	2.43	2.22	0.33	0.002
B x A	3	698,226.45	546.74	22.45	3	68.24	2.92	6.71	0.68	0.28	0.008
B x G	2	89,123.16	26.72	1.60	2	13.74	0.80	8.13	5.09*	0.46	0.004
A x G	6	93,140.19	611.00	26.02	6	44.93	2.65	1.78	1.10	0.21	0.006
Stage of lactation (linear)	1	65,110.95	5.21	0.87	1	13,375.57***	1,689.27***	829.62***	151.52***	28.75***	0.306***
Remainder	476	416,055.57	846.16	20.64	513	95.70	7.70	6.40	1.39	0.31	0.013

* P<0.05; ** P<0.01; *** P<0.001

TABLE V.4.c

Least-squares analysis of variance of lactation milk and fat yield, fat concentration and the concentrations of total solids, protein, casein, whey protein, β-lactoglobulin and α-lactalbumin in the test day milk sample when β-casein genotype was included in the model

Source of variation	d.f.	Mean squares-Complete lactation data			d.f.	Mean squares-Concentration of components in test day sample (gL^{-1})					
		Milk yield (L)	Fat yield (kg)	Fat content (gL^{-1})		Total solids	Protein	Casein	Whey Protein	β-Lacto-globulin	α-Lact albumin
Breed (B)	1	2,565,667.45	2,729.70	1,527.18*	1	9,251.93	956.77	516.97	67.13	13.00	0.136
Herds (within breeds)	6	45,878,901.00***	119,520.62***	206.88***	6	2,933.66***	261.99***	180.64***	21.14***	9.04***	0.682***
Age of cow (A)	3	10,452,209.58***	20,469.37***	46.51	3	139.05	10.02	5.32	5.99**	1.72***	0.001
β-Casein genotype (G)	5	70,137.13	372.72	52.89*	5	154.01	12.01	5.22	4.04*	0.37	0.025
B x A	3	1,637,581.13**	1,720.51	33.28	3	106.10	3.35	6.29	1.18	0.26	0.003
B x G	4	277,194.22	143.50	28.32	4	84.71	9.47	5.86	3.85**	0.30	0.001
A x G	15	562,127.94	861.50	14.45	15	80.50	7.89	4.56	0.83	0.51	0.009
Stage of lactation (linear)	1	253,962.09	106.86	4.10	1	13,975.82***	1,749.43***	898.30***	139.75***	85.78***	0.253***
Remainder	461	402,563.59	844.21	20.34	498	93.90	7.50	6.39	1.37	0.31	0.013

*P<0.05; ** P<0.01; *** P<0.001

TABLE V. 4.d

Least-squares analysis of variance of lactation milk and fat yield, fat concentration and the concentrations of total solids, protein, casein, whey protein, β-lactoglobulin and α-lactalbumin in the test day milk sample when κ-casein genotype was included in the model

Source of variation	d.f.	Mean squares-Complete lactation data			d.f.	Mean squares-Concentration of components in test day sample (gL^{-1})					
		Milk yield (L)	Fat yield (kg)	Fat content (gL^{-1})		Total solids	Protein	Casein	Whey Protein	β-Lacto-globulin	α-Lact-albumin
Breed (B)	1	8,456,442.34	23,636.44	8,661.39***	1	35,978.46*	3,704.50*	2,293.28*	166.85*	34.72	1.995
Herds (within breeds)	6	45,819,651.78***	122,254.76***	235.34***	6	3,118.04***	284.37***	195.61***	21.98***	9.47***	0.726***
Age of cow (A)	3	21,601,365.09***	48,323.12***	47.69	3	345.50*	17.69	14.75	10.73***	2.12***	0.001
κ-Casein genotype (G)	2	489,540.10	533.74	30.19	2	41.11	0.23	2.62	4.06	1.38*	0.067**
B x A	3	288,752.30	245.38	60.21*	3	104.37	8.19	10.01	0.13	0.21	0.0004
B x G	2	831,077.88	1,406.44	0.61	2	77.98	13.02	7.35	0.89	0.46	0.034
A x G	6	403,140.46	848.95	27.70	6	50.26	2.99	2.73	0.31	0.07	0.006
Stage of lactation (linear)	1	264,250.76	199.67	0.82	1	13,702.97***	1,751.95***	896.36***	140.59***	25.91***	0.327***
Remainder	476	409,205.44	838.27	20.54	513	95.62	7.66	6.36	1.41	0.31	0.013

* P<0.05; ** P<0.01; *** P<0.001

TABLE V.5.a

Least-squares analysis of variance of concentrations of casein components and casein components expressed as a percent of total casein when β-lactoglobulin genotype was included in the model

Source of variation	d.f.	Mean squares-Concentration of casein components (gL^{-1})					Mean squares-Casein components as a % of total casein				
		α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein	α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein
Breed (B)	1	159.50*	102.54	229.58*	26.63	107.81**	308.93	144.24	133.72	5.85	216.26*
Herds (within breeds)	6	15.00***	21.98***	23.29***	10.92***	6.06***	56.59***	119.30***	141.74***	84.25***	19.37***
Age of cow (A)	3	0.47	0.68	3.21	7.64***	0.67	6.70	7.67	29.13*	90.79***	4.04
β-lactoglobulin genotype (G)	4	1.18	1.45*	1.90	1.20	0.83	11.68	6.74	4.25	8.04	2.87
B x A	3	2.78*	0.32	2.38	1.36	0.66	1.50	7.21	1.76	9.44	1.60
B x G	2	1.93	0.33	4.12*	2.16	0.45	14.86	3.85	18.42	32.12*	5.79
A x G	12	0.78	0.19	1.27	0.79	0.52	6.36	1.90	8.25	7.84	3.64
Stage of lactation (linear)	1	103.58***	12.89***	27.22***	58.80****	7.93***	7.81	0.35	282.07***	310.90***	9.20
Remainder	489	0.93	0.48	1.32	0.75	0.43	5.38	3.47	8.46	9.21	3.65

*P<0.05; ** P<0.01; *** P<0.001

TABLE V. 5.b

Least-squares analysis of variance of concentrations of casein components and casein components expressed as a percent of total casein when α_{s1}-casein genotype was included in the model

Source of variation	d.f.	Mean squares-Concentration of casein components (gL^{-1})					Mean squares-Casein components as a % of total casein				
		α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein	α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein
Breed (B)	1	18.81	12.88	25.75	4.95	14.56	53.13	14.53	23.20	6.13	33.88
Herds (within breeds)	6	12.81***	21.81***	20.15***	11.18***	5.58***	58.85***	128.74***	115.58***	88.18***	20.33***
Age of cow (A)	3	1.36	1.08	2.60	4.31***	0.83	8.97	9.88*	18.24	53.35***	6.66
α_{s1}-Casein genotype (G)	2	10.18***	0.27	0.88	0.87	1.46*	112.73***	4.78	3.74	10.34	15.97*
B x A	3	0.88	0.05	1.85	1.44	0.71	6.78	4.28	7.98	11.71	3.08
B x G	2	1.79	0.12	0.46	0.51	0.15	0.51	1.03	0.82	4.89	0.37
A x G	6	0.78	0.36	0.45	1.47	0.31	4.51	3.21	8.41	12.66	2.70
Stage of lactation (linear)	1	93.03***	12.53***	26.70***	60.79***	7.37***	3.01	0.28	265.08***	331.14.***	9.71
Remainder	498	0.82	0.48	1.40	0.78	0.43	4.56	3.45	8.57	9.62	3.61

* P<0.05; ** P<0.01; *** P<0.001

TABLE V. 5.c

Least-squares analysis of variance of concentrations of casein components and casein components expressed as a percent of total casein when β-casein genotype was included in the model

Source of variation	d.f.	Mean squares-Concentration of casein components (gL^{-1})					Mean squares-Casein components as a % of total casein				
		α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein	α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein
Breed (B)	1	42.79	8.87	33.08	1.05	18.51	0.34	3.79	27.65	7.24	43.23
Herds (within breeds)	6	10.64 ***	19.31 ***	19.44 ***	10.27 ***	6.24 ***	43.00 ***	115.91 ***	123.56 ***	79.83 ***	25.93 ***
Age of cow (A)	3	0.42	0.70	2.56	1.59	0.32	2.83	4.02	11.46	25.44 *	2.24
β-Casein genotype (G)	5	2.80 **	0.93	8.23 ***	1.20	1.28 **	46.86 ***	8.29 *	65.85 ***	11.99	11.36 **
B x A	3	0.09	0.34	1.82	0.81	0.70	6.14	1.68	3.28	7.13	2.59
B x G	4	1.41	0.32	1.37	0.50	0.53	3.13	1.05	5.79	7.07	3.24
A x G	15	0.50	0.65	1.08	1.20	0.31	3.16	5.25	7.52	14.02	2.94
Stage of lactation (linear)	1	96.03 ***	13.19 ***	38.57 ***	55.51 ***	10.05 ***	0.34	0.68	204.19 ***	278.10 ***	4.59
Remainder	483	0.83	0.48	1.22	0.78	0.42	4.21	3.36	7.29	9.59	3.46

* P <0.05; ** P<0.01; *** P<0.001

TABLE V. 5.d

Least-squares analysis of variance of concentrations of casein components and casein components expressed as a percent of total casen when κ-casein genotype was included in the model

Source of variation	d.f.	Mean squares-Concentration of casein components (gL^{-1})					Mean squares-Casein components as a % of total casein				
		α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein	α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein
Breed (B)	1	212.60**	65.61	185.44*	18.17	45.25*	14.07	65.66	79.20	0.56	14.49
Herds (within breeds)	6	13.30***	22.65***	20.61***	10.82***	5.09***	44.73***	129.46***	144.03***	83.05***	14.33***
Age of cow (A)	3	1.11	2.08**	7.23**	8.07***	1.47**	3.16	15.21**	30.58*	121.32***	8.75*
κ-Casein genotype (G)	2	2.60	0.03	1.36	0.79	6.41***	50.72***	1.04	7.93	11.37	80.68***
B x A	3	3.75**	0.28	1.35	2.25*	0.15	9.35	4.50	5.66	20.87	2.01
B x G	2	0.75	1.11	2.83	0.37	0.33	5.15	7.86	23.37	5.36	8.20
A x G	6	0.98	0.12	1.28	1.06	0.30	5.74	0.49	7.43	15.44	2.76
Stage of lactation (linear)	1	99.39***	13.97***	31.50***	53.84***	11.81***	1.77	0.09	263.05***	264.49***	1.13
Remainder	498	0.93	0.49	1.38	0.79	0.39	5.16	3.51	8.68	9.57	3.03

* P< 0.05; ** P<0.01; *** P<0.001

TABLE V. 6.a

Least-squares means and standard errors of lactation milk and fat yield, fat concentration and the concentrations of total solids, protein, casein, whey protein, β-lactoglobulin and α-lactalbumin in the test day milk sample when β-lactoglobulin genotype was included in the model.

	No.of obsv.	Complete lactation data			No.of obsv.	Concentration of components in test day sample (gL^{-1})					
		Milk yield (L)	Fat yield (kg)	Fat content (gL^{-1})		Total solids	Protein	Casein	Whey protein	β-lacto-globulin	α-lact-albumin
Overall mean	500	3172±586.4	149±30.5	48.0±1.43	537	145.2±5.02	35.8±1.51	27.9±1.27	7.9±0.38	3.31±0.249	1.04±0.070
Breed											
Jersey	262	3168±682.6	170±35.5	54.1±1.66a[#]	288	157.6±5.71a	39.9±1.72a	31.2±1.45a	8.7±0.44a	3.67±0.284	1.13±0.080
Friesian	238	3177±798.3	129±41.5	41.8±1.95b	249	132.8±7.01b	31.6±2.11b	24.5±1.77b	7.0±0.54b	2.96±0.348	0.95±0.098
β-Lactoglobulin genotype											
AA	61	3241±594.3	147±30.8	46.0±1.59a	64	142.3±5.22a	35.9±1.57a	27.4±1.33a	8.5±0.42a	4.08±0.256a	1.01±0.072
AB	218	3237±586.5	154±30.5	48.3±1.43b	231	146.0±5.02b	36.6±1.51a	28.5±1.27b	8.1±0.38b	3.56±0.249b	1.06±0.070
BB	169	3163±586.9	152±30.5	48.4±1.44b	185	147.7±5.03b	36.4±1.52a	28.6±1.27b	7.7±0.39c	2.94±0.250c	1.06±0.070
AC	23	3172±604.0	150±31.2	47.7±1.76ab	25	144.5±5.41ab	35.8±1.62a	27.9±1.38ab	7.9±0.45bc	3.41±0.263b	1.03±0.074
BC	29	3049±606.6	145±31.3	49.4±1.81b	32	145.3±5.49ab	34.1±1.64b	26.8±1.40a	7.2±0.46c	2.59±0.265d	1.02±0.075
Age of cow (years)											
2	80	2673±593.9a	122±30.8a	46.2±1.58a	94	141.4±5.19a	34.9±1.56	27.7±1.32	7.2±0.41a	3.22±0.255a	1.03±0.072
3	109	2957±594.1a	139±30.8b	48.4±1.58b	117	147.3±5.17b	36.4±1.55	28.5±1.31	8.0±0.41b	3.45±0.254b	1.05±0.071
4	68	3370±597.4b	163±30.9c	49.5±1.64b	70	146.5±5.30b	35.7±1.59	27.3±1.35	8.4±0.43b	3.33±0.259ab	1.03±0.073
5+	243	3690±587.5c	174±30.5c	47.7±1.45ab	256	145.4±5.05b	36.0±1.52	28.1±1.28	7.9±0.39b	3.25±0.250a	1.04±0.070

[#]Means not followed by the same letter are significantly ($P < 0.05$) different

TABLE V. 6.b

Least-squares means and standard errors of lactation milk and fat yield, fat concentration and the concentrations of total solids, protein, casein, whey protein, β-lactoglobulin and α-lactalbumin in the test day milk sample when α_{s1}-casein genotype was included in the model

	No.of obsv.	Complete lactation data			No.of obsv.	Concentration of components in test day sample (gL^{-1})					
		Milk yield (L)	Fat yield (kg)	Fat content (gL^{-1})		Total solids	Protein	Casein	Whey protein	β-lacto-globulin	α-lact albumin
Overall mean	501	3176±1452.3	152± 75.2	48.5±3.20	538	145.9±11.63	36.3±3.56	27.8±2.94	8.4±0.99	3.46±0.653	1.06±0.170
Breed											
Jersey	263	3310± 619.1	176± 32.1	53.8±1.37	289	156.7±4.79	39.9±1.47	31.5±1.21	8.5±0.41	3.52±0.269	1.17±0.070
Friesian	238	3043±2911.7	128±150.8	43.2±6.41	249	135.1±23.01	32.6±7.05	24.2±5.81	8.4±1.96	3.40±1.292	0.96±0.337
α_{s1}-Casein genotype											
BB	319	3193±1448.0	150± 75.1	47.6±3.10	340	145.7±11.51	36.2±3.53	28.2±2.91	8.1±0.97	3.39±0.646	1.05±0.169
BC	150	3151±1450.7	154± 75.2	49.3±3.17	163	146.5±11.60	36.3±3.55	28.3±2.93	8.0±0.98	3.30±0.651	1.05±0.170
CC	32	3186±1488.8	152± 76.7	48.6±3.95	35	145.4±12.63	36.3±3.83	27.1±3.20	9.2±1.16	3.70±0.711	1.08±0.179
Age of cow (years)											
2	81	2687±1456.6a[#]	126± 75.4a	47.6±3.30	95	144.0±11.72	35.7±3.59	27.6±2.96	8.1±1.01	3.33±0.658	1.07±0.171
3	109	2984±1455.2a	143± 75.3b	48.7±3.27	117	148.4±11.71	36.9±3.58	28.4±2.96	8.4±1.00	3.57±0.658	1.07±0.171
4	68	3366±1456.8b	163±75.4c	49.6±3.30	70	145.8±11.76	36.2±3.60	27.5±2.97	8.8±1.01	3.55±0.661	1.06±0.171
5+	243	3669±1452.1c	175± 75.2c	48.1±3.19	256	145.2±11.62	36.2±3.56	27.8±2.94	8.4±0.99	3.39±0.653	1.06±0.170

[#] Means not followed by the same letter are significantly ($P<0.05$) different

TABLE V. 6.c

Least-squares means and standard errors of lactation milk and fat yield, fat concentration and the concentrations of total solids, protein, casein, whey protein, β-lactoglobulin and α-lactalbumin in the test day milk sample when β-casein genotype was included in the model

	No.of. obsv.	Complete lactation data			No.of obsv.	Concentration of components in test day sample (gL^{-1})					
		Milk yield (L)	Fat yield (kg)	Fat content (gL^{-1})		Total solids	Protein	Casein	Whey protein	β-lacto-globulin	α-lact albumin
Overall mean	500	3246± 904.1	154±46.2	47.8±1.90	537	147.5± 6.09	36.8±1.82	28.7±1.51	8.1±0.52	3.39±0.338	1.05±0.093
Breed											
Jersey	263	3030±1260.8	161±64.4	53.1±2.64a #	289	159.6± 9.63	40.7±2.88	31.6±2.39	9.1±0.81	3.85±0.534	1.10±0.147
Friesian	237	3462±1479.7	147±75.5	42.5±3.11b	248	135.4±10.02	32.9±3.00	25.9±2.49	7.1±0.85	2.94±0.556	1.00±0.153
β-Casein genotype											
A^1A^1	91	3405± 935.9	156±47.5	45.8±2.56ab	95	149.6± 7.13	37.9±2.10	28.7±1.79	9.2±0.68a	3.74±0.399	1.01±0.102
A^1A^2	132	3246± 905.4	148±46.2	45.9±1.93a	135	143.9± 6.17	35.8±1.84	28.1±1.53	7.7±0.53b	3.31±0.342	1.03±0.094
A^1B	19	3171± 920.2	155±46.8	49.1±2.26b	22	146.2± 6.54	36.5±1.94	28.8±1.63	7.7±0.59b	3.28±0.364	1.02±0.097
A^2A^2	117	3257± 904.3	155±46.2	48.6±1.90b	123	146.9± 6.13	36.4±1.83	28.5±1.52	8.0±0.52b	3.33±0.340	1.08±0.093
A^2B	108	3265± 931.6	156±47.3	48.4±2.48ab	125	151.3± 6.55	37.3±1.95	29.5±1.64	7.8±0.59b	3.29±0.365	1.09±0.097
BB	33	3131± 914.3	152±46.6	49.1±2.13b	37	147.2± 6.41	37.0±1.91	28.9±1.60	8.1±0.57ab	3.41±0.357	1.07±0.096
Age of cow (years)											
2	81	2760± 909.4a	130±46.4a	47.3±2.02	95	146.5± 6.24	36.4±1.86	28.8±1.55	7.6±0.54a	3.18±0.347a	1.05±0.094
3	109	3003± 907.6a	145±46.3b	48.8±1.98	117	149.5± 6.21	37.3±1.85	29.9±1.54	8.3±0.53b	3.57±0.345b	1.05±0.094
4	68	3496± 907.9b	167±46.3c	48.2±1.99	70	147.7± 6.22	36.9±1.86	28.6±1.55	8.3±0.54b	3.49±0.346bc	1.05±0.094
5+	242	3723± 905.1c	174±46.2c	46.9±1.92	255	146.3± 6.13	36.6±1.83	28.4±1.52	8.2±0.52b	3.33±0.340ac	1.05±0.093

Means not followed by the same letter are significantly ($P < 0.05$) different

TABLE V. 6.d

Least-squares means and standard errors of lactation milk and fat yield, fat concentration and the concentrations of total solids, protein, casein, whey protein, β-lactoglobulin and α-lactalbumin in the test day milk sample when κ-casein genotype was included in the model

	No.of obsv.	Complete lactation data			No.of obsv.	Concentration of components in test day sample (gL^{-1})					
		Milk yield (L)	Fat yield (kg)	Fat content (gL^{-1})		Total solids	Protein	Casein	Whey protein	β-lacto-globulin	α-lact-albumin
Overall mean	501	3205±523.3	151±27.0	48.0±1.18	538	145.8±4.07	36.3±1.23	28.3±1.02	8.0±0.34	3.35±0.224	1.05±0.062
Breed											
Jersey	263	2997±823.9	162±42.6	54.7±1.85a[#]	289	158.7±6.37a	40.4±1.92a	31.5±1.59a	8.9±0.53a	3.75±0.351	1.15±0.097
Friesian	238	3412±669.3	140±34.6	41.4±1.50b	249	133.0±5.27b	32.2±1.59b	25.0±1.32b	7.1±0.44b	2.95±0.290	0.96±0.081
κ-Casein genotype											
AA	126	3125±531.1	149±27.3	48.9±1.34	133	146.8±4.26	36.3±1.28	28.1±1.07	8.3±0.37	3.53±0.236a	1.07±0.064a
AB	192	3188±524.2	151±27.1	47.6±1.20	208	145.5±4.10	36.3±1.24	28.3±1.03	8.0±0.34	3.30±0.226b	1.07±0.062a
BB	183	3302±526.5	154±27.2	47.5±1.24	197	145.1±4.16	36.2±1.25	28.5±1.04	7.8±0.36	3.22±0.230b	1.02±0.063b
Age of cow (years)											
2	81	2687±527.6a	124±27.2a	47.1±1.27	95	144.5±4.17a	35.8±1.26	28.3±1.05	7.5±0.36a	3.15±0.230a	1.05±0.063
3	109	3039±526.3b	146±27.2b	48.8±1.24	117	148.4±4.16b	36.8±1.25	28.8±1.04	8.1±0.36b	3.49±0.229c	1.05±0.063
4	68	3409±528.2c	162±27.2c	48.4±1.28	70	145.3±4.21a	36.4±1.27	28.1±1.06	8.3±0.37b	3.45±0.233bc	1.05±0.063
5+	243	3684±524.1d	173±27.1d	47.7±1.19	256	144.9±4.10a	36.2±1.24	28.0±1.03	8.2±0.35b	3.32±0.226b	1.05±0.062

[#]Means not followed by the same letter are significantly ($P<0.05$) different

TABLE V. 7.a

Least-squares means and standard errors of concentrations of casein components and casein components expressed as a percent of total casein when β-lactoglobulin was included in the model

	No.of obsv.	Concentration of casein components (gL^{-1})					Casein components as a % of total casein				
		α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein	α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein
Overall mean	522	9.3±0.34	3.4±0.41	9.8±0.42	2.2±0.29	3.2±0.22	33.3±0.66	12.0±0.96	35.3±1.04	7.9±0.80	11.4±0.38
Breed											
Jersey	286	10.0±0.37a[#]	4.0±0.45	10.8±0.47a	2.5±0.32	3.9±0.24a	32.2±0.72	12.8±1.06	34.6±1.15	8.1±0.88	12.3±0.42a
Friesian	236	8.5±0.48b	2.8±0.59	8.9±0.60b	1.9±0.41	2.6±0.31b	34.4±0.94	11.3±1.37	36.1±1.49	7.7±1.14	10.5±0.54b
β-lactoglobulin genotype											
AA	61	9.0±0.37	3.3±0.43a	9.6±0.46	2.4±0.32	3.1±0.24	33.3±0.74	11.9±1.00	35.3±1.13	8.5±0.92	11.1±0.48
AB	226	9.3±0.34	3.6±0.41b	9.9±0.42	2.3±0.29	3.3±0.22	32.7±0.66	12.5±0.96	35.0±1.04	8.2±0.80	11.6±0.38
BB	179	9.3±0.34	3.5±0.41ab	10.0±0.43	2.4±0.29	3.4±0.22	32.8±0.66	12.1±0.96	35.1±1.05	8.3±0.81	11.7±0.39
AC	24	9.5±0.40	3.3±0.44ab	10.0±0.50	2.0±0.35	3.2±0.26	34.0±0.84	11.7±1.05	35.8±1.23	7.2±1.05	11.2±0.57
BC	32	9.1±0.41	3.2±0.44a	9.5±0.50	1.9±0.50	3.1±0.26	33.8±0.85	12.0±1.05	35.7±1.24	7.3±1.07	11.3±0.58
Age of cow (years)											
2	91	9.3±0.36	3.4±0.42	9.9±0.45	1.9±0.31a	3.2±0.23	33.8±0.73	12.1±0.99	36.0±1.12a	6.7±0.90a	11.4±0.46
3	107	9.4±0.36	3.5±0.42	10.2±0.45	2.0±0.31ac	3.4±0.23	33.1±0.73	12.3±0.99	35.9±1.11a	7.0±0.90a	11.7±0.46
4	69	9.1±0.39	3.4±0.43	9.5±0.48	2.4±0.33bc	3.0±0.25	33.4±0.79	12.2±1.02	34.9±1.18ab	8.6±0.98b	10.9±0.52
5+	255	9.2±0.34	3.3±0.41	9.7±0.43	2.6±0.29b	3.3±0.22	33.0±0.70	11.6±0.97	34.6±1.06b	9.3±0.82b	11.6±0.40

[#] Means not followed by the same letter are significantly ($P<0.05$) different

TABLE V.7.b

Least-squares means and standard errors of concentration of casein components and casein components expressed as a percent of total casein when α_{s1}-casein genotype was included in the model

	No.of obsv.	Concentration of casein components (gL^{-1})					Casein components as a % of total casein				
		α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein	α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein
Overall mean	523	9.3±0.75	3.5±0.98	9.7±0.94	2.3±0.70	3.2±0.49	33.6±1.60	12.3±2.38	34.8±2.24	8.0±1.95	11.3±0.93
Breed											
Jersey	287	10.1±0.31	4.2±0.41	10.6±0.39	2.7±0.29	3.9±0.21	32.2±0.67	13.1±1.00	33.8±0.94	8.5±0.82	12.4±0.39
Friesian	236	8.5±1.45	2.8±1.89	8.7±1.81	1.8±1.35	2.5±0.95	35.0±3.10	11.6±4.60	35.7±4.34	7.5±3.78	10.2±1.80
α_{s1}-Casein genotype											
BB	327	8.9±0.73a[#]	3.5±0.97	9.9±0.91	2.4±0.68	3.4±0.48a	31.9±1.55a	12.3±2.35	35.3±2.18	8.6±1.87	11.9±0.87a
BC	161	9.8±0.74b	3.4±0.98	9.9±0.93	2.2±0.69	3.1±0.49b	34.6±1.58b	11.8±2.37	34.9±2.22	7.8±1.92	10.9±0.91b
CC	35	9.2±0.88ab	3.5±1.04	9.2±1.11	2.1±0.83	3.1±0.59ab	34.3±1.93b	12.9±2.55	34.1±2.68	7.6±2.50	11.1±1.33ab
Age of cow (years)											
2	92	9.1±0.76	3.6±0.98	9.7±0.95	2.0±0.71a	3.3±0.50	33.1±1.63	12.9±2.39a	35.3±2.28	7.2±2.00a	11.7±0.97
3	107	9.5±0.76	3.6±0.98	9.9±0.95	2.1±0.71a	3.6±0.50	33.5±1.63	12.6±2.39ab	35.0±2.28	7.3±2.00a	11.6±0.97
4	69	9.4±0.76	3.3±0.99	9.5±0.96	2.3±0.71ab	3.0±0.51	34.3±1.64	12.1±2.40ab	34.7±2.30	8.2±2.03ab	10.7±0.99
5+	255	9.3±0.75	3.3±0.98	9.4±0.93	2.6±0.70b	3.2±0.49	33.6±1.60	11.9±2.37b	34.0±2.24	9.3±1.95b	11.3±0.93

[#] Means not followed by the same letter are significantly ($P<0.05$) different

TABLE V. 7.c

Least-squares means and standard errors of concentrations of casein components and casein components expressed as a percent of total casein when β-casein genotype was included in the model

	No.of obsv.	Concentration of casein components (gL^{-1})					Casein components as a % of total casein				
		α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein	α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein
Overall mean	522	9.2±0.38	3.6±0.51	10.1±0.51	2.4±0.37	3.5±0.29	32.1±0.76	12.4±1.26	35.3±1.29	8.3±1.03	11.9±0.59
Breed											
Jersey	287	10.1±0.56	4.0±0.76	10.9±0.76	2.6±0.55	4.1±0.43	32.0±1.13	12.6±1.86	34.6±1.91	8.0±1.53	12.8±0.87
Friesian	235	8.4±0.66	3.2±0.89	9.3±0.89	2.3±0.64	2.9±0.50	32.2±1.31	12.1±2.17	36.0±2.24	8.7±1.79	11.0±1.02
β-Casein genotype											
A^1A^1	91	9.4±0.52bc[#]	3.6±0.58	9.6±0.66a	2.5±0.50	3.7±0.38ab	32.9±1.09bc	12.4±1.44ab	33.6±1.66a	8.5±1.57	12.6±0.92ab
A^1A^2	132	9.4±0.39bc	3.5±0.52	9.6±0.52a	2.2±0.38	3.3±0.30a	33.5±0.78b	12.5±1.27b	34.5±1.32a	8.0±1.07	11.6±0.61a
A^1B	22	8.9±0.44ac	3.2±0.54	10.9±0.58b	2.4±0.43	3.4±0.33ab	30.9±0.91a	11.2±1.33a	38.0±1.45b	8.3±1.28	11.6±0.74ab
A^2A^2	117	9.6±0.38 b	3.6±0.52	9.7±0.52a	2.3±0.38	3.3±0.29a	33.8±0.77b	12.6±1.26b	34.3±1.31a	8.1±1.06	11.3±0.60a
A^2B	123	9.2±0.46ab	3.8±0.55	11.1±0.60b	2.3±0.45	3.5±0.34ab	30.9±0.96ac	12.4±1.36ab	37.5±1.50b	7.5±1.36	11.6±0.79ab
BB	37	8.8±0.42a	3.9±0.53	9.7±0.56a	2.8±0.41	3.8±0.32b	30.6±0.87a	13.2±1.31b	33.4±1.41a	9.5±1.22	12.8±0.70b
Age of cow (years)											
2	92	9.2±0.40	3.7±0.52	10.2±0.54	2.4±0.39	3.5±0.30	31.8±0.81	12.6±1.28	35.6±1.35	8.0±1.12a	12.1±0.65
3	107	9.3±0.40	3.7±0.52	10.3±0.53	2.3±0.39	3.6±0.30	32.2±0.80	12.5±1.28	35.6±1.34	7.7±1.10a	12.1±0.63
4	69	9.2±0.40	3.6±0.52	10.0±0.53	2.6±0.39	3.4±0.30	32.3±0.80	12.3±1.28	35.2±1.34	8.6±1.11ab	11.6±0.63
5+	254	9.2±0.38	3.5±0.51	9.9±0.52	2.6±0.38	3.4±0.29	32.2±0.77	12.1±1.26	34.8±1.30	9.1±1.05b	11.9±0.60

[#] Means not followed by the same letter are significantly ($P<0.05$) different

TABLE V. 7.d

Least-squares means and standard errors of concentrations of casein components and casein components expressed as a percent of total casein when κ-casein genotype was included in the model

	No.of obsv.	Concentration of casein components (gL^{-1})					Casein components as a % of total casein				
		α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein	α_{s1}-casein	α_{s2}-casein	β-casein	γ-casein	κ-casein
Overall mean	523	9.3±0.26	3.5±0.35	9.8±0.33	2.4±0.24	3.3±0.16	33.0±0.48	12.2±0.83	34.7±0.87	8.4±0.66	11.7±0.27
Breed											
Jersey	287	10.3±0.42a #	4.0±0.55	10.7±0.52a	2.7±0.38	3.8±0.26a	32.8±0.76	12.7±1.31	34.1±1.38	8.4±1.04	12.0±0.43
Friesian	236	8.3±0.34b	2.9±0.44	8.8±0.42b	2.1±0.31	2.9±0.21b	33.3±0.62	11.6±1.06	35.3±1.12	8.3±0.84	11.4±0.35
κ-Casein genotype											
AA	128	9.5±0.29	3.5±0.36	9.6±0.36	2.5±0.26	3.0±0.18a	34.0±0.57a	12.2±0.86	34.4±0.95	8.8±0.77	10.5±0.35a
AB	199	9.3±0.27	3.5±0.35	9.9±0.33	2.3±0.24	3.6±0.17b	32.9±0.50b	12.3±0.83	35.0±0.88	8.0±0.68	11.8±0.28b
BB	196	9.1±0.28	3.5±0.35	9.9±0.35	2.4±0.25	3.7±0.17c	32.1±0.52c	12.1±0.84	34.7±0.91	8.3±0.71	12.9±0.31c
Age of cow (years)											
2	92	9.3±0.28	3.6±0.35a	9.9±0.35ac	2.2±0.25a	3.4±0.17ac	32.8±0.53	12.4±0.85ac	35.2±0.92ac	7.7±0.72a	12.0±0.32a
3	107	9.5±0.28	3.6±0.35a	10.1±0.35a	2.2±0.25a	3.5±0.17ac	32.9±0.52	12.5±0.84ac	35.2±0.91ac	7.6±0.71a	11.9±0.31a
4	69	9.3±0.29	3.4±0.36ab	9.6±0.35bc	2.5±0.26b	3.3±0.18bc	33.3±0.55	12.1±0.85bc	34.3±0.93bc	8.8±0.74b	11.5±0.33ab
5+	255	9.2±0.27	3.3±0.35b	9.6±0.33b	2.7±0.24b	3.3±0.17b	33.0±0.49	11.8±0.83b	34.3±0.88b	9.5±0.67b	11.5±0.28b

Means not followed by the same letter are significantly ($P<0.05$) different

When the composition of the single test day milk sample was analysed for effect of stage of lactation, significant ($P < 0.001$) increases in the concentration of total solids, protein, casein, all casein components, whey proteins and β-lactoglobulin and a decrease in the concentration of α-lactalbumin was found. Also, the proportion of γ-casein in total casein increased and the proportion of β-casein decreased with increasing stage of lactation ($P < 0.001$).

(ii) Milk protein gene frequencies

The genotype and gene frequencies for the four milk proteins, α_{s1}-, β- and κ-casein and β-lactoglobulin, for each herd and for the two breeds, are in Table V. 8 and 9, respectively. Significant differences in genotype frequencies between herds within breeds were detected with each milk protein for both breeds, as indicated in Table V. 8. The overall genotype frequency of the two breeds was significantly different with each of the four milk proteins (Table V. 8).

The genotype distribution was tested for deviation from Hardy-Weinberg expectation (Falconer 1960), and no deviation was detected with the four milk proteins within each breed. However, when the overall genotype distribution was tested, significant ($P < 0.05$) deviation was detected with β-casein.

The significant associations found between milk protein systems using j x k contingency tests are in Table V. 10. Only combinations with expected values greater than 1.00 were included. Significant differences from expectation of independent assortment were found with α_{s1}- and β-casein phenotypes and with β- and κ-casein phenotypes in Jerseys, and with α_{s1}- and κ-casein phenotypes in Friesians. In the Jersey breed, significant differences from expectation was also found with the α_{s1}-casein and β-lactoglobulin phenotypes. When the

TABLE V. 8

Number of each milk protein genotype according to herd and breed

(a) α_{s1}-casein

Breed	Herd No.	Genotype			Total cows	Significance*
		BB	BC	CC		
Jersey	1	38	37	5	80	a
	2	20	40	8	68	b
	3	31	35	4	70	b
	4	25	47	18	90	c
Friesian	5	71	8	1	80	a c
	6	25	4	0	29	b
	7	102	2	0	104	c
	8	44	1	1	46	b c
Jersey Total		114	159	35	308	a
Friesian Total		242	15	2	259	b

(b) β-casein

Breed	Herd No.	Genotype								Total cows	Significance*
		A^1A^1	A^1A^2	A^1B	A^2A^2	A^2A^3	A^2B	A^3B	BB		
Jersey	1	1	5	2	29	0	33	0	9	79	a b
	2	1	8	5	24	0	24	0	4	66	a
	3	0	7	6	15	0	31	0	11	70	b
	4	0	5	3	29	0	37	0	16	90	a b
Friesian	5	40	34	0	6	0	0	0	0	80	a
	6	6	14	4	3	0	1	1	0	29	b
	7	33	51	2	16	0	2	0	0	104	c
	8	19	20	2	3	1	1	0	0	46	c
Jersey Total		2	25	16	97	0	125	0	40	305	a
Friesian Total		98	119	8	28	1	4	1	0	259	b

TABLE V. 8 (continued)

(c) κ-casein

Breed	Herd No.	Genotype			Total cows	Significance*
		AA	AB	BB		
Jersey	1	4	39	37	80	a
	2	6	24	38	68	b
	3	1	18	51	70	c
	4	4	29	57	90	b
Friesian	5	36	36	8	80	a
	6	4	16	9	29	b
	7	54	41	8	103	a
	8	28	14	5	47	a
Jersey Total		15	110	183	308	a
Friesian Total		122	107	30	259	b

(d) β-lactoglobulin

Breed	Herd No	Genotype						Total cows	Significance*
		AA	AB	BB	AC	BC	CC		
Jersey	1	8	29	20	13	7	2	79	a
	2	4	22	24	8	10	0	68	b
	3	4	34	27	1	4	0	70	c
	4	10	36	26	7	11	0	90	b
Friesian	5	19	39	21	0	0	0	79	a
	6	3	13	13	0	0	0	29	b
	7	15	47	42	0	0	0	104	a
	8	3	21	23	0	0	0	47	b
Jersey Total		26	121	97	29	32	2	307	a
Friesian Total		40	120	99	0	0	0	259	b

*Differences in genotype distribution between herds within breed and between breeds: distributions not followed by the same letter are significantly ($P < 0.05$) different

TABLE V. 9

Gene frequencies for α_{s1}, β- and κ-casein and β-lactoglobulin for each herd and for the Jersey and Friesian breed *

Breed	Herd No.	α_{s1}-casein		β-casein			κ-casein			β-lactoglobulin		
		B	C	A^1	A^2	A^3	B	A	B	A	B	C
Jersey	1	0.706	0.294	0.057	0.608	0	0.335	0.294	0.706	0.367	0.481	0.152
	2	0.588	0.412	0.114	0.606	0	0.280	0.265	0.735	0.279	0.588	0.132
	3	0.693	0.307	0.093	0.486	0	0.421	0.143	0.857	0.307	0.657	0.036
	4	0.539	0.461	0.044	0.556	0	0.400	0.206	0.794	0.350	0.550	0.100
Jersey, overall		0.628	0.372	0.074	0.564	0	0.362	0.227	0.773	0.329	0.565	0.106
Friesian	5	0.938	0.063	0.712	0.288	0	0	0.675	0.325	0.487	0.513	0
	6	0.931	0.069	0.517	0.362	0.017	0.103	0.414	0.586	0.328	0.672	0
	7	0.990	0.010	0.572	0.409	0	0.019	0.723	0.277	0.370	0.630	0
	8	0.967	0.033	0.652	0.304	0.011	0.033	0.745	0.255	0.287	0.713	0
Friesian, overall		0.963	0.037	0.625	0.348	0.004	0.025	0.678	0.322	0.386	0.614	0

* The number of samples in each herd and breed are given in Table V. 8

Table V. 10

Detection of non-independent assortment of genotypes among the four milk protein loci in the Jersey and Friesian breeds by j x k contingency tables

Loci	Jersey			Friesian		
	df	χ^2	P	df	χ^2	P
α_{s1}- and β-casein	8	181.92	<0.001***	2	4.28	<0.30
α_{s1}- and κ-casein	4	3.14	<0.70	2	6.34	<0.05*
β- and κ-casein	4	65.41	<0.001***	4	9.16	<0.10
α_{s1}-casein and β-lactoglobulin	8	18.22	<0.05*	2	1.75	<0.50
β-casein and β-lactoglobulin	16	19.53	<0.30	6	1.03	<0.99
κ-casein and β-lactoglobulin	8	5.05	<0.80	4	1.59	<0.90

α_{s1}-casein C and β-lactoglobulin C homozygotes were not included in the analysis (lowest expected number 9.3) the differences from expectation was still significant (χ^2 = 14.68, 4 d.f. P < 0.01).

As the α_{s1}-, β- and κ-casein loci are closely linked on the same chromosome, the frequency of casein phenotypes were determined. The most common casein phenotypes in Jerseys were α_{s1}-casein BC-β-casein A^2B - κ-casein B (21.6%), α_{s1} B - β B - κ B (13.1%), α_{s1} BC - β A^2 - κ AB (10.8%) and α_{s1} B - β A^2B - κ AB (9.8%) and in Friesians α_{s1} B - β A^1A^2 - κ A (22.1%), α_{s1} B - β A^1 - κ A (18.2%), α_{s1} B - β A^1 - κ AB (15.9%) and α_{s1} B - β A^1A^2 - κ AB (14.7%).

(iii) Associations between milk protein genotype and milk yield and composition

For each of the milk proteins, the significant differences found between the genetic variants for lactation milk and fat yield, fat concentration and the milk composition variables determined using the test day skim milk sample, are indicated in Tables V. 4 and 5. Any interactions of genotype with breed or age of cow is also indicated. The least-squares means and standard errors of these variables, for the genetic variants of each milk protein, and an indication of which variants are significantly different, is in Tables V. 6 and 7. A summary of the significant relationships between milk protein genotypes and milk yield and composition is in Table V. 11.

(a) β-Lactoglobulin genotype

The β-lactoglobulin A milk was significantly (P < 0.05) lower in total solids and fat concentrations when compared to milk of AB and B genotypes. The BC genotype had significantly lower protein concentration than other β-lactoglobulin genotypes i.e. there was no significant difference between the A, AB, AC and B genotypes.

The β-lactoglobulin A genotype was also significantly (P < 0.01)

TABLE V. 11

Summary of the significance of relationships between milk protein genotype and milk yield and composition

Variable	Milk protein genotype			
	α_{s1}-casein	β-casein	κ-casein	β-lactoglobulin
Complete lactation data				
Milk yield				
Fat yield				
Fat concentration		*		*
Concentrations in test day sample				
Total solids				*
Protein				**
Casein				**
α_{s1}-casein	***	**		
α_{s2}-casein				*
β-casein		***		
γ-casein				
κ-casein	*	**	***	
Whey protein		*		***
β-lactoglobulin			*	***
α-lactalbumin			**	
Casein components, expressed as % of total casein, in test day sample				
α_{s1}-casein	***	***	***	
α_{s2}-casein		*		
β-casein		***		
γ-casein				
κ-casein	*	**	***	

* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$

lower in casein concentration when compared to the AB and B genotypes. However, the only significant difference between β-lactoglobulin genotypes in casein composition was in α_{s2}-casein concentration ($P < 0.05$). The difference in mean casein concentration between β-lactoglobulin A and B milks was 4.3%.

Significant differences between β-lactoglobulin genotypes were found in whey protein (A > AB > B; $P < 0.001$) and β-lactoglobulin (A > AB, AC > B > BC; $P < 0.001$) concentrations. The difference in mean β-lactoglobulin concentration between β-lactoglobulin A and B milks was 32.5%. With whey protein concentration there was a significant breed by genotype interaction: differences were not significant between Friesian genotypes and with Jerseys only type A was significantly greater than type B. A significant breed by genotype interaction was also obtained with β-lactoglobulin concentration and within each breed the same significant trend was found (A, AB > B).

Breed by genotype interactions for β-casein concentration and proportion of γ-casein in total casein were found but within breed differences were not significant.

(b) α_{s1}-Casein genotype

There were significant differences between α_{s1}-casein genotypes in concentration and proportion of total casein of both α_{s1}-casein ($P < 0.001$) and κ-casein ($P < 0.05$). The C allele was associated with a higher concentration of α_{s1}-casein and a lower concentration of κ-casein in the milk when compared with the B allele.

There was a significant ($P < 0.05$) interaction between breed and genotype for whey protein content. α_{s1}-Casein genotype was not significantly associated with whey protein concentration in the combined breed analysis, and within breeds there were non-significant trends in opposite directions.

(c) β-Casein genotype

Significant differences between β-casein genotypes were found with α_{s1}-, β- and κ-casein concentrations, and with the proportions of α_{s1}-, α_{s2}-, β- and κ-casein in total casein. With three common alleles (A^1, A^2, B) at the β-casein locus, it was more difficult to determine the order of gene superiority. With both concentration and proportion of β-casein, the homozygotes A^1, A^2 and B were not significantly different. However, the B type heterozygotes, A^1B and A^2B, were significantly higher both in concentration and proportion of β-casein than A^1, A^1A^2 and A^2 and also the B homozygote ($P < 0.001$). The β-casein B allele had significantly lower α_{s1}-casein concentration ($B < A^1, A^2$; $P < 0.01$) and higher κ-casein concentration ($B > A^2$; $P < 0.01$) than the A allele.

Significant differences between β-casein genotypes were also found in fat concentration ($A^1 < A^2$, B; $P < 0.05$) and whey protein concentration ($A^1 > A^2$; $P < 0.05$). With whey protein concentration, there was a significant ($P < 0.01$) interaction between breed and genotype. The whey protein concentration of milk from Jersey β-casein A^1 cows was significantly higher than that with all other genotypes, whereas there were no significant differences between Friesian β-casein genotypes.

(d) κ-Casein genotype

Significant differences in concentration of κ-casein in skim milk were detected between κ-casein genotypes ($A < AB < B$; $P < 0.001$). When casein components were expressed as a % of total casein, κ-casein B milk had a significantly ($P < 0.001$) lower proportion of α_{s1}-casein and higher proportion of κ-casein. Significant differences between κ-casein genotypes were also found in β-lactoglobulin ($A > AB, B$; $P < 0.05$) and α-lactalbumin ($A, AB > B$; $P < 0.01$) concentrations.

3. Discussion

The milk yield and fat concentration was not considered in the selection of the four Jersey and four Friesian herds sampled. The herds were generally representative of commercial dairy herds in the Central and Southern Hills areas of South Australia, with milk production of some herds being above the State breed averages, and some being lower (the mean milk yield, fat yield and fat concentration of herd-tested Jerseys during the 1978-79 season was 2871 L, 151 kg and 53 gL^{-1} respectively, and the corresponding figures for Friesians was 4310 L, 171 kg and 40 gL^{-1}). The average milk and fat yield of the four Jersey herds was above the State average yields for Jerseys and the average of the four Friesian herds was below the State average. It should be noted that the mean milk yield of herd 4 was well above the average for Jerseys and was higher than all Friesian herds sampled.

The milk yield on the test day showed considerable variation between cows, mainly reflecting stage of lactation. The Pro-Milk used to estimate protein concentration was standardized against Kjeldahl total nitrogen x 6.38 and so total protein concentrations include non-protein nitrogen (i.e. crude protein). Casein concentrations are true protein values. Casein nitrogen, expressed as a percent of total nitrogen, has been called the casein number by Rowland (1938c). The mean casein number was 77.9 which is similar to that found in other studies (Rowlands 1938c, Waite and Smith 1972, Haenlein *et al.* 1973, Cerbulis and Farrell 1975). Rowland (1938c) found that in normal milk variability of the casein number was low, ranging from 77.3 and 80.7. Casein number was reduced by mastitis and so has been proposed as an indication of mastitis (Rowland and Zein-el-dine 1938, Ashworth 1965). However, in the present study, in which samples affected by subclinical mastitis were eliminated, casein number varied widely,

ranging from 63.9 to 86.0 (which is similar to the range reported by Cerbulis and Farrell 1975), and so was not considered a good indication of subclinical mastitis.

There was no evidence that the distribution of casein number of Jerseys was narrower than with Friesians, as found by Cerbulis and Farrell (1975).

Considerable variation in the proportion of casein components was found in milk samples from 523 individual cows. Mean coefficient of variation of α_{s1}-, α_{s2}-, β-, γ- and κ-casein was 7%, 16%, 9%, 38% and 16% respectively. The most variable fractions were α_{s2}- and γ-casein, and to a lesser extent β-casein, and the coefficients of variation for α_{s1}- and κ-casein were only slightly higher than the precision of the method of estimation (Table IV. 5). Considerable variation of casein composition in individual cow samples was also found by Davies and Law (1977a), and Davies and Law (1977b) showed that much of this variation was due to samples collected in early and late lactation. The range of values found in the present study was wider than that reported by Davies and Law (1977b) (only 42 milk samples), with lower α_{s1}-, β- and κ-casein values and higher γ-casein values being found.

The mean β-lactoglobulin concentration of 3.6 and 3.0 gL^{-1} for the Jersey and Friesian breeds was similar to the mean concentrations of 3.9 and 3.0 gL^{-1} found by Rolleri *et al.* (1956) for these breeds. The proportion of β-lactoglobulin in total whey protein (including NPN equivalent) varied between 36 and 45%, which was higher than the mean of 26% found by Cerbulis and Farrell (1975) who used the method of Aschaffenburg and Drewry (1959). Together β-lactoglobulin and α-lactalbumin made up approximately 55% of whey protein. This agrees with the whey composition found by Davies and Law (1980), when their

data is calculated as a percent of whey protein + NPN i.e. 41.0% β-lactoglobulin, 16.0% α-lactalbumin, 12.7% IPL (immunoglobulins, proteose-peptone, lactoferrin) 5.9% serum albumin and 24.4% NPN. Pearce (1980) considered that on a nitrogen basis, up to 40% of polypeptide material in whey should be classified as proteose-peptone, if the heat stable portion of the 'total albumin' fraction is added to the conventional 'proteose-peptone fraction'. However, this proportion seems too high, when other components making up the whey protein + NPN are considered.

The higher concentrations of components in the milk from Jerseys compared to Friesians was as expected (Wilcox *et al.* 1971, Rolleri *et al.* 1956) and presumably reflect the attributes selected for in the past (Munro 1976). The differences in concentration of β-lactoglobulin between breeds was not significant, although Rolleri *et al.* (1956) and McLean *et al.* (1974) had shown that the concentration in Jersey milk was higher than in milk from Friesians. That significance of the difference in milk yield between breeds was not achieved was possibly because the mean of the Friesian herds selected was below the State average and Jersey herds were above average.

The only significant breed effect on casein composition was a higher proportion of κ-casein in Jersey milk, although a non-significant trend of higher proportion of α_{s2}-casein and lower proportion of α_{s1}- and β-casein in Jersey milk was found. The higher proportion of κ-casein in Jersey milk was due to the higher κ-casein concentration in κ-casein B milk compared to κ-casein A milk and the higher frequency of B genes (0.77) in Jerseys compared to Friesians (0.32). Rolleri *et al.* (1956) found that casein of the Holstein breed contained significantly less β-casein and more γ-casein, but approximately the same amount of α_s -casein.

The increase in lactation milk and fat yield with increasing age is well known (e.g. Waite *et al.* 1956, Wilcox *et al.* 1971). Although the differences did not reach significance in all components, the concentrations of total solids, fat, protein, casein, whey protein and β-lactoglobulin increased to a maximum in either the second or third lactation and then declined with increasing age. Waite *et al.* (1956) and Rook and Campling (1965) showed that the concentrations of all major constituents in milk (fat, solids-not-fat, lactose, total N, casein N and β-lactoglobulin N) declined with increasing age. Waite *et al.* (1956) found that the fall was least for crude protein, as a slight rise in non-casein N concentration in the milk of older cows partly compensated for the fall in casein concentration. In the present study, whey protein concentration was significantly lower in the first lactation than in later lactations, indicating that serum proteins increase with age due to the effects of mastitis, as shown by Waite (1968). Although cow numbers were small (three) in the study by Rook and Campling (1965), some milk components from two of the cows increased to a maximum in the second lactation then declined, as found in the present study.

The concentration and proportion of γ-casein in the milk increased and concentration and proportion of β-casein decreased with increasing age of the cow, indicating increased proteolysis of β-casein in milk from older cows. The concentration and proportion of α_{s2}- and κ-casein also declined with age, but that of α_{s1}-casein remained relatively stable. The long storage of milk samples may have emphasised the differences in β- and γ-casein concentrations by allowing time for higher levels of proteolytic enzymes in milk from older cows to react.

In the least-squares analysis of variance (Harvey 1975), stage of lactation was included as a linear covariate. Significant regression

coefficients with stage of lactation were obtained for all concentrations of components measured: all increased during the lactation except for α-lactalbumin. The trends in gross milk composition associated with stage of lactation of the cow are well characterized (for review see Rook 1961). The concentration of fat and protein are high in early lactation, fall to a minimum, which occurs at about 10 weeks for fat and about 6 weeks for protein, then increase, most rapidly in the later stages, until the end of lactation (Azarme 1938, Waite *et al.* 1956, Rook and Campling 1965, Munro *et al.* 1974). Casein concentration follows the same trend as protein (Azarme 1938, Waite *et al.* 1956).

α-Lactalbumin was the only protein to decline in concentration during the lactation. After an initial increase, the concentration of lactose also declines (Waite *et al.* 1956). In the biosynthesis of lactose, α-lactalbumin acts as a protein modifier of an enzyme, galactosyltransferase, and the control of lactose biosynthesis appears to be directly related to the concentration and rate of flow of α-lactalbumin through the Golgi apparatus (Ebner and Schanbacher 1974). This unique system explains the relationship between α-lactalbumin and lactose concentrations during lactation.

The increase in proportion of γ-casein and decrease in β-casein during the cow's lactation was also found by Larson and Kendall (1957), before the relationship between β-casein and γ-casein had been demonstrated by Groves *et al.* (1972). Davies and Law (1977b) found that in early and late lactation, the relative amount of β-casein was lower and those of κ- and γ-caseins were higher than in mid-lactation. Stage of lactation appeared to have little effect of the relative amounts of α_{s1}- and α_{s2}-casein.

The significant differences between the Jersey and Friesian breeds in gene frequencies of all milk protein tested was as expected

(Aschaffenburg 1968).

The genotype of each cow results from the genotype of the dam and sire. However, although many sires were used in each herd sampled, a majority of the cows were sired by a few bulls, and so bull selection by the farm manager was a major cause of the significant differences in genotype frequency found between herds within each breed.

Aschaffenburg (1968) discussed this problem of between herd variation, and he stressed the need for adequate numbers of samples giving a representative cross-section of the population, when determining gene frequencies.

The gene frequencies of the four milk proteins were compared with those reported in other studies (the review by Aschaffenburg 1968, Hoogendoorn *et al.* 1969, Li and Gaunt 1972, Hines *et al.* 1977, Munro 1977, 1978). Except for β-casein A^1 and β-lactoglobulin C, the gene frequencies found in the Jersey breed were within the range reported in other studies. The frequency of β-casien A^1 was very low (0.074) and Munro (1977) reported a frequency of 0.207 in a similar population of cows.

The frequency of β-lactoglobulin C varied widely between herds (0.036 - 0.152) and the overall frequency for Jerseys (0.106) was higher than most other reported values, although it is similar to the frequency of 0.10 found by Bell (1967) who typed 448 Australian Jersey cows in 5 herds. However, Aschaffenburg (1968) considered this frequency was abnormally high due to the startling concentration of the C allele in a single herd. Estimates of the frequency of the C allele in Australian Jerseys by Bailey (1968b) (0.06 for 229 cows in 6 herds) and Munro (1977) (0.06 for 348 cows) are also higher than frequencies of 0.01 found by Aschaffenburg (1965) in 80 cows in the

U.K. and 0.04 found by Larsen and Thymann (1966) in 159 Danish Jerseys. Other studies, such as those of Kiddy *et al.* (1965), Hoogendoorn *et al.* (1969) and Li and Gaunt (1972) have failed to detect the C allele, due to the use of phenotyping methods which either do not resolve β-lactoglobulin C from the B variant or it is masked by β-casein A.

β-Casein C was not detected, although Li and Gaunt (1972) and Munro (1977) have reported a low frequency in Jerseys. The absence of β-casein A^3 in Jerseys agrees with data reviewed by Aschaffenburg (1968) and with Hoogendoorn *et al.* (1969) and Li and Gaunt (1972).

The gene frequencies in the Friesian breeds were also generally within the range reported in the literature, except that the frequency of β-casein A^3 was lower. The α_{s1}-casein A variant was not detected, although a heterozygote has been found in a similar population of cows (Munro, personal communication). The absence of β-casein C in Friesians agrees with other studies.

If non-independant assortment among α_{s1}-, β- and κ-casein loci can be attributed to close linkage (i.e. linkage disequilibrium), one would expect significant results between any pair of these loci (Li and Gaunt 1972), but this was not found in the present study or by Li and Gaunt (1972).

Two-way contingency tables do not separate the effects of linkage from interloci interaction, but can demonstrate the absence or very low frequency, of certain combinations of loci which are considered to be recombinants. This was shown, in particular, by the absence of α_{s1}-casein C - β-casein B phenotypes (BC - A^1B, BC - B, C - A^1B, C - A^2B, C - B) in the present study.

Linkage can be directly proven by collecting segregation data from suitable families: this was the method used to demonstrate linkage

between α_{s1}-casein and β-casein (Grosclaude *et al.* 1964) and, between κ-casein and the α_{s1}- and β-caseins (Grosclaude *et al.* 1965, Larsen and Thymann 1966).

The significant ($P < 0.05$) difference from expectation of independant assortment between the α_{s1}-casein and β-lactoglobulin loci in Jerseys was unexpected, although Li and Gaunt (1972) found non-independent assortment of genotype between the κ-casein and β-lactoglobulin loci in Brown Swiss cows ($P < 0.01$). Linkage between the caseins and β-lactoglobulin loci has not been demonstrated using segregation data and presumably the associations found in these studies are due to inter-loci interaction.

The relationships between milk protein genotypes and milk yield and composition were analysed using least-squares analysis of variance (Harvey 1975). The least-squares approach was able to equalize the effects of unequal numbers, and allowance was made for the effects of breed, herd and stage of lactation. In this study each protein genotype was analysed separately, without including interactions between genotypes. However, according to Mather (1977), this approach does not determine whether effects are additive between genotypes. Mather (1977) considered three different approaches: analysis of each genotype separately, analysis of combinations of genotypes and analysis of combinations and genotype interactions. He concluded that the third, more complex model, gave the truest picture. However, Soller (1978) found the usefulness of linked marker genes would not be increased if a number of marker alleles were followed simultaneously.

The relationship between β-lactoglobulin genotype and concentration of β-lactoglobulin in the milk (A > AB > B), which has been reported in numerous previous studies, was again confirmed in the present study. Because of its low frequency and its occurrence only in the Jersey breed, the effect of the C allele has been less studied.

β-Lactoglobulin AC milk had a lower β-lactoglobulin concentration than β-lactoglobulin A milk, and β-lactoglobulin BC milk had a lower β-lactoglobulin concentration than β-lactoglobulin B milk, but these differences were not significant. McLean *et al.* (1974) found that the β-lactoglobulin concentration of milk containing the C variant (AC, BC or CC) was not significantly different from β-lactoglobulin B milk, but was less than in β-lactoglobulin A milk.

The highly significant and consistently found association between β-lactoglobulin genotype and β-lactoglobulin concentration in the milk is most probably a direct effect of the β-lactoglobulin gene on the rate of synthesis of β-lactoglobulin in the mammary gland, although an unlikely alternative explanation is differences in breakdown of the molecule after synthesis.

The significant differences in whey protein concentration between β-lactoglobulin genotypes, which were also found by Cerbulis and Farrell (1975) and Mariani *et al.* (1979), are due to the contribution of β-lactoglobulin, which is the major whey protein component.

The significant association between β-lactoglobulin genotype and casein concentration in milk confirms the results of studies by Moustgaard *et al.* (1960) and Mariani *et al.* (1979) who also showed significant differences, and Aschaffenburg and Drewry (1959) whose work first indicated this relationship. Only Cerbulis and Farrell (1975) have reported the reverse trend in concentration (not significant) but when their results were expressed as a percent of total protein, β-lactoglobulin B milk had a higher percent than type A milk.

Except for α_{s2}-casein concentration (AB > A), no significant association was found between β-lactoglobulin genotype and concentration and proportion of casein components, but with each of the other casein components there was a non-significant trend of higher concentrations

with the β-lactoglobulin B genotype. Mariani *et al.* (1979) found that the concentration of α_{s1}- and κ-casein was significantly ($P < 0.05$) higher in β-lactoglobulin B milk than in type A milk.

The only significant association between β-lactoglobulin genotype and total protein concentration of the milk was the lower concentration in β-lactoglobulin BC milk samples compared to other genotypes. The results of studies of the association between β-lactoglobulin genotype and total protein concentration reported in the literature are conflicting: where significance has been demonstrated, the superior gene was not consistent, and the majority of studies have not been able to demonstrate a significant difference between genotypes. It appears that as β-lactoglobulin B is substituted for the A gene, the concentration and proportion of whey protein (β-lactoglobulin in particular) decreases, and that of casein increases with the total protein concentration remaining constant.

The superiority of the β-lactoglobulin B allele for fat concentration supports the studies of Sherbon *et al.* (undated) and Hoogendoorn *et al.* (1969), but conflict with Golikova (1975) who found that β-lactoglobulin A was superior, and many other studies have failed to find a significant association.

β-Lactoglobulin B milk also had a higher total solids concentration, presumably due to its higher fat concentration. However, Arave *et al.* (1971) found that β-lactoglobulin A milk had significantly higher total solids concentration.

The only significant associations found with the α_{s1}-casein genotype were with casein composition: the concentration and proportion of α_{s1}-casein was higher and the concentration and proportion of κ-casein was lower with α_{s1}-casein C when compared to type B. The magnitude of change in mean α_{s1}-casein and κ-casein concentration

were equal, and so no significant change in total casein concentration was detected. Golikova and Panin (1972) had noted a trend of higher proportion of α-casein and lower proportion of β-casein with α_{s1}-casein C cows compared to α_{s1}-casein B cows.

Some significant but often conflicting associations between α_{s1}-casein genotype and total protein concentration of milk have been reported in the literature (Hoogendoorn *et al.* 1969, Dvorak and Macha 1970, Mityut'ko 1974 and Munro 1978) but differences in the present study were not significant. Also, differences in milk and fat yield and fat concentration were not significant, although Munro (1978) found that milk from α_{s1}-casein BC Friesian cows had higher milk fat concentration than from type B cows.

β-Casein genotype was significantly associated with changes in the concentration and proportion of most of the casein components. Tests of significance of the relationship between β-casein genotype and both concentration and proportion of β-casein, indicated that the B heterozygotes, A^1B and A^2B, were superior to other genotypes, but it should be noted that the type B cows were all of the one breed (Jerseys), although no breed by genotype interaction was found. A higher β-casein concentration with β-casein B milk compared to type A milk and a lower α_{s1}-casein concentration of milk from β-casein B cows compared to A^1 and A^2 genotypes agrees with the trend found by Golikova and Panin (1972). The higher κ-casein concentration of β-casein B cows, compared to A^2 cows, has not been previously reported.

The changes in concentration of the casein components which were associated with the β-casein genotype balance out, and so no significant differences between genotypes were detected with total casein concentration.

The higher fat concentration of β-casein A^2 and B compared to

A^1 conflicts with the findings of Munro (1977, 1978) who found A^1 was superior to other types in Jerseys. However, a large study by Babukov (1978) reported that β-casein B milk had the highest fat concentration, but the significance of this result was not given. Most studies of the association between β-casein genotype and milk yield and composition have not considered the specific β-casein A variants.

The significantly higher concentration and proportion of κ-casein and lower proportion of α_{s1}-casein in milk of κ-casein B cows when compared to type A cows confirms the trends reported by Michalak (1973b), Mariani *et al.* (1976) and Davies and Law (1977b), although these studies did not demonstrate significance. Changes in casein components did not cause a significant difference in total casein concentration, although a trend of higher casein concentration with κ-casein B milk has been reported (Michalak 1973b and Mariani *et al.* 1976). Also, significant differences in total protein concentration which have been demonstrated for κ-casein genotype (Hoogendoorn *et al.* 1969, Munro 1978) were not found in the present study. The significant association between κ-casein genotype and the concentration of the two major whey proteins, β-lactoglobulin and α-lactalbumin, has not been previously reported.

Milk yield was not significantly associated with any of the four milk protein genotypes tested. This result was not unexpected as many studies reported in the literature have also been unable to detect a significant difference and the results of different studies which detected significant differences are often conflicting. Perhaps the most consistent association between milk protein genotype and milk yield found in the literature, is the superiority of the β-lactoglobulin A gene (Comberg *et al.* 1964, Thatcher 1965, Macha and Mullerova 1969,

Arave *et al.* 1971, Kamenskaya 1973, Mityut'ko 1974, Kuz'menko *et al.* 1977 and Munro 1978). As there is a negative genetic correlation between milk yield and the concentration of components such as fat and protein (Wilcox *et al.* 1971), and as the β-lactoglobulin B gene is associated with higher concentrations of some components (fat, total solids and casein), the finding by other studies of lower milk yield with β-lactoglobulin B would not be unexpected. However, at least one component, β-lactoglobulin has a lower concentration in β-lactoglobulin B milk.

The cows fat yield, which is the most important economic parameter to the dairy farmer supplying milk for manufacture, as he is paid on the basis of amount of fat produced, was not associated with any milk protein genotype. The higher fat concentration found with the β-casein A^2 and B genes when compared to the A^1 gene and with β-lactoglobulin B gene compared to the A gene, did not cause a significant difference in fat yield. Semenenko (1971a) found fat yield to be significantly higher with α_{s1}-casein BC cows when compared to type B cows.

The results of the study by Soller (1978) showed that loci associated with quantitative traits in dairy cattle can only make a significant contribution to improvement when they directly affect milk production. Although the milk protein loci were not associated with milk yield and fat yield, the two most economically important traits, a number of significant associations were found with fat concentration and protein composition.

As the link between the DNA nucleotide sequence causing the protein variants and the variable being investigated becomes less direct (possibly more genes having in effect on the variable) it becomes more likely that any effect will be masked and that conflicting

associations will be found by different workers. Other possible reasons for lack of uniformity of results or lack of significant relationships in studies of associations between genotypes and milk production variables, were reviewed in Section V.A.3. It appears that for a relationship to be considered "real" it should be consistently and significantly demonstrated by different workers in different cow populations. However, it is possible that "real" effects could be masked in certain studies by the environmental conditions at the time. For example, Feagan *et al.* (1972) were able to demonstrate a relationship between β-lactoglobulin genotype and the type of heat coagulation time - pH curve of milk during the dry summer/autumn period, but not during winter and spring.

The genes responsible for synthesis of α_{s1}-, β- and κ-casein are very closely linked, and so these genotypes should be considered as a 'casein gene complex'. It is intended to analyse the casein variants as a complex, and to include interactions between genotypes in the model in future work.

This study has investigated relationships between milk protein genotypes and the yield and composition of the milk, but before any decision is made on which are the most favourable genes to breed for, other properties such as the manufacturing properties of the milk must be considered. In South Australia, the majority of milk in excess of liquid milk requirement is used for cheesemaking, and so the properties of milk of different genotype for cheesemaking will be especially considered.

The B variant of β-lactoglobulin is preferable for cheesemaking as these cows produce milk with higher total solids, fat and casein concentrations, and so would be expected to give a higher yield of cheese. The firmer curd formed with β-lactoglobulin B milk after

chymosin addition (Sherbon *et al.* 1967) may the the result of higher casein concentration (Weisberg *et al.* 1933, Zviedrans, personal communication), although White and Davies (1958) found no consistent relationship between casein concentration and curd firmness. However, Feagan *et al.* (1972) did not find a significant difference between β-lactoglobulin genotypes in curd firmness. At present, the gene frequency of β-lactoglobulin B in South Australia is 0.57 in Jerseys and 0.61 in Friesians.

The association of α_{s1}-casein genotype with casein composition is not of direct economic importance. However, significant differences in curd firmness between genotypes were found by Sadler *et al.* (1968) (A < AB < B < BC) but not by Feagan *et al.* (1972).

The association of β-casein genotype B with higher fat (B, $A^2 > A^1$), and κ-casein content (present study), shorter chymosin clotting time (A > AB > AC > B, El-Negoumy 1972), and higher curd firmness (A < B, Sherbon *et al.* 1967, Feagan *et al.* 1972, Corradini 1974) suggest that for cheesemaking, the B variant is preferable. Its low frequency (0.36 in Jerseys and 0.03 in Friesians) allows potential for selection.

In the present study, κ-casein was associated only with concentrations of individual proteins, but a number of studies have consistently shown that κ-casein B milk clots faster on chymosin addition and so forms a firmer curd (Sherbon *et al.* 1967, El-Negoumy 1972, Feagan *et al.* 1972, Losi *et al.* 1973, Corradini 1974 and Mariani *et al.* 1976). κ-Casein B milk is also more suited to cheese manufacture (Mariani *et al.* 1976) and the cheese produced is superior to that produced from κ-casein A milk (Morini *et al.* 1979). The B variants of both β- and κ-casein have higher concentrations of κ-casein, which is the chymosin-sensitive protein in milk. A high κ-casein concentration may be beneficial to curd formation.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

The dye binding method of estimating casein concentration developed by McGann *et al.* (1972) and modified in the present study allowed rapid and accurate determination of large numbers of skim milk samples from individual cows. The precipitated casein was suitable for analysis of casein components. The modified method also saved dye solution and time. The use of a regression equation to adjust the Pro-Milk difference values improved the accuracy of the method compared to the original method of McGann *et al.* (1972). Because of its simplicity, this method could be used in cheese factories to obtain an accurate estimation of casein concentration so that milk can be adjusted to the correct casein to fat ratio. At present, methods which assume a constant proportion of casein in total protein are used, and this proportion is known to vary seasonally. The method should not be used to determine the casein concentration of milk from individual cows in late lactation which contain high levels of protein. Calibration of the Pro-Milk to read 'true' total protein and recalculation of the regression equation to give 'true' whey protein concentration is recommended.

The method developed for the estimation of casein composition consisted of electrophoresis of treated casein samples in a horizontal polyacrylamide gel slab with continuous buffer system. The protein bands were stained and scanned using a densitometer.

The casein components were subdivided into five major groups, whereas other published methods do not give separation of all these groups. The densitometer trace appeared to be superior to other

published methods, and casein genetic variants could also be determined. Good precision was obtained with estimates of major caseins, but it was lower with estimates of γ- and κ-casein.

The inclusion of adjustments for different dye binding capacities for the caseins and the genetic variants of α_{s1}- and β-casein made the method suitable for individual cow samples. Adjustment for dye binding differences between κ-casein genotypes was not necessary as separate estimates of γ- and κ-casein were obtained by adding chymosin to casein samples and scanning the para-κ-casein band.

However, these estimates of dye binding capacities may not be accurate, and as the precision of estimates of dye binding capacities were low, estimates of certain casein components differed from the best estimates available in the literature. Further work on dye binding of caseins is needed.

A problem with this method, in which proteins were stained with Coomassie blue G250, was the failure to consistently obtain a linear relationship between densitometer peak area and protein weight. Migration distance and staining and rinsing times were shown to have an important effect on the linearity of response. There is a need for a dye which is capable of covalently binding with proteins forming a stoichiometric complex, and which can readily be removed from polyacrylamide gel when unbound.

The direct association between a proteins genotype and its rate of synthesis, which has been well documented with β-lactoglobulin, has now been demonstrated with the three caseins, α_{s1}-, β- and κ-casein: their concentrations in milk were all highly significantly associated with their respective genotypes.

The casein genotypes were associated with the casein composition of the milk, but not with total casein or total protein concentration. In contrast, the β-lactoglobulin genotype was associated with total

casein concentration, but not with casein components except with α_{s2}-casein.

Consideration of the results of the present study and of published reports, indicate that some of the associations found could have economic importance. The greatest potential role of milk protein genotypes appears to be in improving the yield and quality of the final product, rather than in increasing milk and fat yield from the cow, as no significant associations were found with these variables. The association between β-lactoglobulin genotype and total solids, fat and casein concentrations could be exploited in dairy cattle breeding. β-Lactoglobulin B milk, which has higher concentrations of these components, will give a higher yield of cheese from a given volume of milk. Other studies have shown that the B variants of both β-casein and κ-casein are superior to the A variants, for cheese manufacture.

Genotypes with favourable attributes could be considered as additional criteria in the final selection of bullls for widespread use in artificial breeding. The milk protein genes carried by a bull can be determined by genetically typing milk from the bull's daughters. Bull selection is dependent on progeny testing now, so no further delay would result. Mather (1977) considered that selection based on genetic markers could aid in selection of animals at a much earlier age. However, it is not envisaged by the present author that the use of milk protein variants could replace progeny testing as carried out at present.

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