Dairy Chemistry and Biochemistry

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Preface

Milk has been the subject of scientific study for about 150 years and, consequently, is probably the best characterized, in chemical terms, of our major foods. It is probably also the most complicated and serves as the raw material for a very large and diverse family of food products. Dairy science has existed as a university discipline for more than 100 years; it is the oldest sector of food science (and technology), with the exception of brewery science. Since dairy chemistry is a major facet of dairy science, it might be expected to have been the subject of numerous books. This is, in fact, not so. During the past 40 years, as far as we are aware, only six books or series on dairy chemistry have been published in English, i.e. *Principles of Dairy Chemistry* (Jenness and Paton, 1959), *Dairy Chemistry and Physics* (Walstra and Jenness, 1984), *Fundamentals of Dairy Chemistry* (Webb and Johnson, 1964; Webb, Johnson and Alford, 1974; Wong *et al.*, 1988), *Developments in Dairy Chemistry* (Fox, four volumes, 1982, 1983, 1985, 1989), *Advanced Dairy Chemistry* (Fox, three volumes, 1992, 1995, 1997) and *Handbook of Milk Composition* (Jensen, 1995). Of these, *Principles of Dairy Chemistry* and *Dairy Chemistry and Physics* were written essentially for senior undergraduate students. The other four books/series were focused principally on lecturers, researchers, senior postgraduate students and senior production management. Thus, at present there is a lack of books written at senior undergraduate/junior postgraduate level specializing in dairy chemistry/science. This book is intended to fill that gap and should be as useful to graduates working in the dairy industry as it is to those still studying.

The book assumes a knowledge of chemistry and biochemistry but not of dairy chemistry. As the title suggests, the book has a stronger biochemical orientation than either *Principles of Dairy Chemistry* or *Dairy Chemistry and Physics*. In addition to a fairly in-depth treatment of the chemistry of the principal constituents of milk, i.e. water, lactose, lipids, proteins (including enzymes), salts and vitamins, various more applied aspects are also covered, e.g. heat-induced changes, cheese, protein-rich products and the applications of enzymes in dairy technology. The principal physical properties are also described.

To facilitate the reader, the structure of various molecules mentioned frequently in the text are given in appendices but we emphasize that a good general knowledge of chemistry and biochemistry is assumed. The chemical composition of the principal dairy products is also included.

The book does not cover the technology of the various dairy products, although brief manufacturing protocols for some products are included to facilitate discussion; however, a number of textbooks on various aspects of dairy technology are referenced. Neither are the chemical analyses, microbiology and nutritional aspects of dairy products covered, except in a very incidental manner. The effects of dairy husbandry on the composition and properties of milk are discussed briefly, as is the biosynthesis of milk constituents; in both cases, some major textbooks are referenced.

We hope that the book will answer some of your questions on the chemistry and biochemistry of milk and milk products and encourage you to undertake more extensive study of these topics.

The highly skilled and enthusiastic assistance of Ms Anne Cahalane and Ms Brid Considine in the preparation of the manuscript and of Professor D.M. Mulvihill and Dr N. O'Brien for critically and constructively reviewing the manuscript are gratefully acknowledged and very much appreciated.

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Contents

1 Production and utilization of milk

1.1 Introduction

Milk is a fluid secreted by the female of all mamalian species, of which there are more than 4000, for the primary function of meeting the complete nutritional requirements of the neonate of the species. In addition, milk serves several physiological functions for the neonate. Most of the non-nutritional functions of milk are served by proteins and peptides which include immunoglobulins, enzymes and enzyme inhibitors, binding or carrier proteins, growth factors and antibacterial agents. Because the nutritional and physiological requirements of each species are more or less unique, the composition of milk shows very marked inter-species differences. Of the more than 4000 species of mammal, the milks of only about 180 have been analysed and, of these, the data for only about 50 species are considered to be reliable (sufficient number of samples, representative sampling, adequate coverage of the lactation period). Not surprisingly, the milks of the principal dairying species, i.e. cow, goat, sheep and buffalo, and the human are among those that are well characterized. The gross composition of milks from selected species is summarized in Table 1.1; very extensive data on the composition of bovine and human milk are contained in Jensen (1995).

1.2 Composition and variability of milk

In addition to the principal constituents listed in Table 1.1, milk contains several hundred minor constituents, many of which, e.g. vitamins, metal ions and flavour compounds, have a major impact on the nutritional, technological and sensoric properties of milk and dairy products. Many of these effects will be discussed in subsequent chapters.

Milk is a very variable biological fluid. In addition to interspecies differences (Table 1.1), the milk of any particular species varies with the individuality of the animal, the breed (in the case of commercial dairying species), health (mastitis and other diseases), nutritional status, stage of lactation, age, interval between milkings, etc. In a bulked factory milk supply, variability due to many of these factors is evened out, but some variability will persist and will be quite large in situations where milk

Table 1.1 Composition (%) of milks of some species

Species	Total solids	Fat	Protein	Lactose	Ash
Human	12.2	3.8	1.0	7.0	0.2
Cow	12.7	3.7	3.4	4.8	0.7
Goat	12.3	4.5	2.9	4.1	0.8
Sheep	19.3	7.4	4.5	4.8	1.0
Pig	18.8	6.8	4.8	5.5	–
Horse	11.2	1.9	2.5	6.2	0.5
Donkey	11.7	1.4	2.0	7.4	0.5
Reindeer	33.1	16.9	11.5	2.8	–
Domestic rabbit	32.8	18.3	11.9	2.1	1.8
Bison	14.6	3.5	4.5	5.1	0.8
Indian elephant	31.9	11.6	4.9	4.7	0.7
Polar bear	47.6	33.1	10.9	0.3	1.4
Grey seal	67.7	53.1	11.2	0.7	–

production is seasonal. Not only do the concentrations of the principal and minor constituents vary with the above factors, the actual chemistry of some of the constituents also varies, e.g. the fatty acid profile is strongly influenced by diet. Some of the variability in the composition and constituents of milk can be adjusted or counteracted by processing technology but some differences may still persist. The variability of milk and the consequent problems will become apparent in subsequent chapters.

From a physicochemical viewpoint, milk is a very complex fluid. The constituents of milk occur in three phases. Quantitatively, most of the mass of milk is a true solution of lactose, organic and inorganic salts, vitamins and other small molecules in water. In this aqueous solution are dispersed proteins, some at the molecular level (whey proteins), others as large colloidal aggregates, ranging in diameter from 50 to 600 nm (the caseins), and lipids which exist in an emulsified state, as globules ranging in diameter from 0.1 to 20 μm. Thus, colloidal chemistry is very important in the study of milk, e.g. surface chemistry, light scattering and rheological properties.

Milk is a dynamic system owing to: the instability of many of its structures, e.g., the milk fat globule membrane; changes in the solubility of many constituents with temperature and pH, especially of the inorganic salts but also of proteins; the presence of various enzymes which can modify constituents through lipolysis, proteolysis or oxidation/reduction; the growth of micro-organisms, which can cause major changes either directly through their growth, e.g. changes in pH or redox potential (E_h) or through enzymes they excrete; and the interchange of gases with the atmosphere, e.g. carbon dioxide. Milk was intended to be consumed directly from the mammary gland and to be expressed from the gland at frequent intervals. However, in dairying operations, milk is stored for various periods, ranging from a few hours to several days, during which it is cooled (and perhaps

heated) and agitated to various degrees. These treatments will cause at least some physical changes and permit some enzymatic and microbiological changes which may alter the processing properties of milk. Again, it may be possible to counteract some of these changes.

1.3 Classification of mammals

The essential characteristic distinguishing mammals from other animal species is the ability of the female of the species to produce milk in specialized organs (mammary glands) for the nutrition of its newborn.

The class Mammalia is divided into three subclasses:

1. Prototheria. This subclass contains only one order, Monotremes, the species of which are egg-laying mammals, e.g. duck-billed platypus and echidna, and are indigenous only to Australasia. They possess many (perhaps 200) mammary glands grouped in two areas of the abdomen; the glands do not terminate in a teat and the secretion (milk) is licked by the young from the surface of the gland.
2. Marsupials. The young of marsupials are born live (viviparous) after a short gestation and are 'premature' at birth to a greater or lesser degree, depending on the species. After birth, the young are transferred to a pouch where they reach maturity, e.g. kangaroo and wallaby. In marsupials, the mammary glands, which vary in number, are located within the pouch and terminate in a teat. The mother may nurse two offspring, differing widely in age, simultaneously from different mammary glands that secrete milk of very different composition, designed to meet the different specific requirements of each offspring.
3. Eutherians. About 95% of all mammals belong to this subclass. The developing embryo *in utero* receives nourishment via the placental blood supply (they are referred to as placental mammals) and is born at a high, but variable, species-related state of maturity. All eutherians secrete milk, which, depending on the species, is more or less essential for the development of the young; the young of some species are born sufficiently mature to survive and develop without milk.

The number and location of mammary glands varies with species from two, e.g. human, goat and sheep, to 14–16 for the pig. Each gland is anatomically and physiologically separate and is emptied via a teat.

The wide interspecies variation in the composition (Table 1.1) and the chemistry of the constituents of milk, as discussed elsewhere, renders milk species-specific, i.e., designed to meet the requirements of the young of that species. There is also a surprisingly good relationship between milk yield and maternal body weight (Figure 1.1); species bred for commercial milk production, e.g. dairy cow and goat, fall above the line.

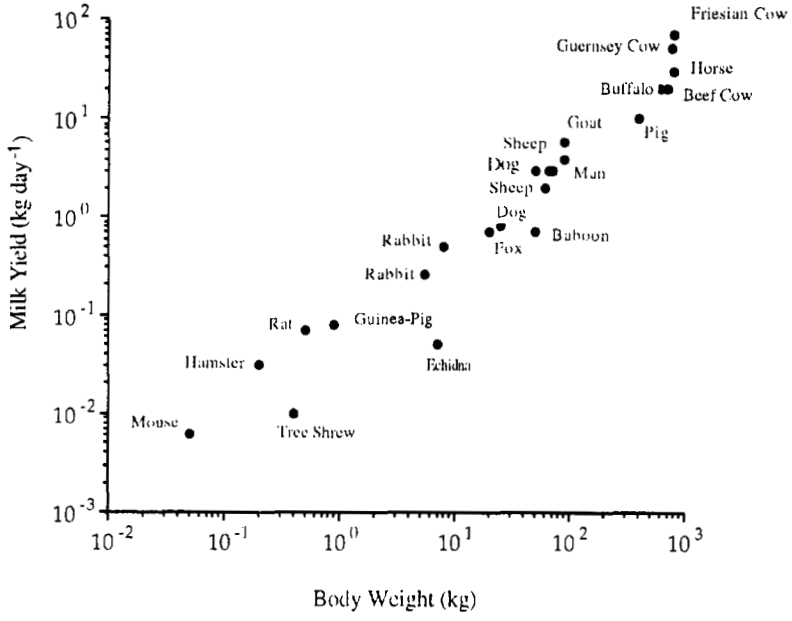

Figure 1.1 Relation between daily milk yield and maternal body weight for some species (modified from Linzell, 1972).

1.4 Structure and development of mammary tissue

The mammary glands of all species have the same basic structure and all are located external to the body cavity (which greatly facilitates research on milk biosynthesis). Milk constituents are synthesized in specialized epithelial cells (secretory cells or mammocytes, Figure 1.2d) from molecules absorbed from the blood. The secretory cells are grouped as a single layer around a central space, the lumen, to form more or less spherical or pear-shaped bodies, known as alveoli (Figure 1.2c). The milk is secreted from these calls into the lumen of the alveoli. When the lumen is full, the myoepithelial cells surrounding each alveolus contract under the influence of oxytocin and the milk is drained via a system of arborizing ducts towards sinuses or cisterns (Figure 1.2a) which are the main collecting points between suckling or milking. The cisterns lead to the outside via the teat canal. Groups of alveoli, which are drained by a common duct, constitute a lobule; neighbouring lobules are separated by connective tissue (Figure 1.2b). The secretory elements are termed the 'lobule–alveolar system' to distinguish them from the duct system. The whole gland is shown in Figure 1.2a.

Milk constituents are synthesized from components obtained from the blood; consequently, the mammary gland has a plentiful blood supply and also an elaborate nervous system to regulate excretion.

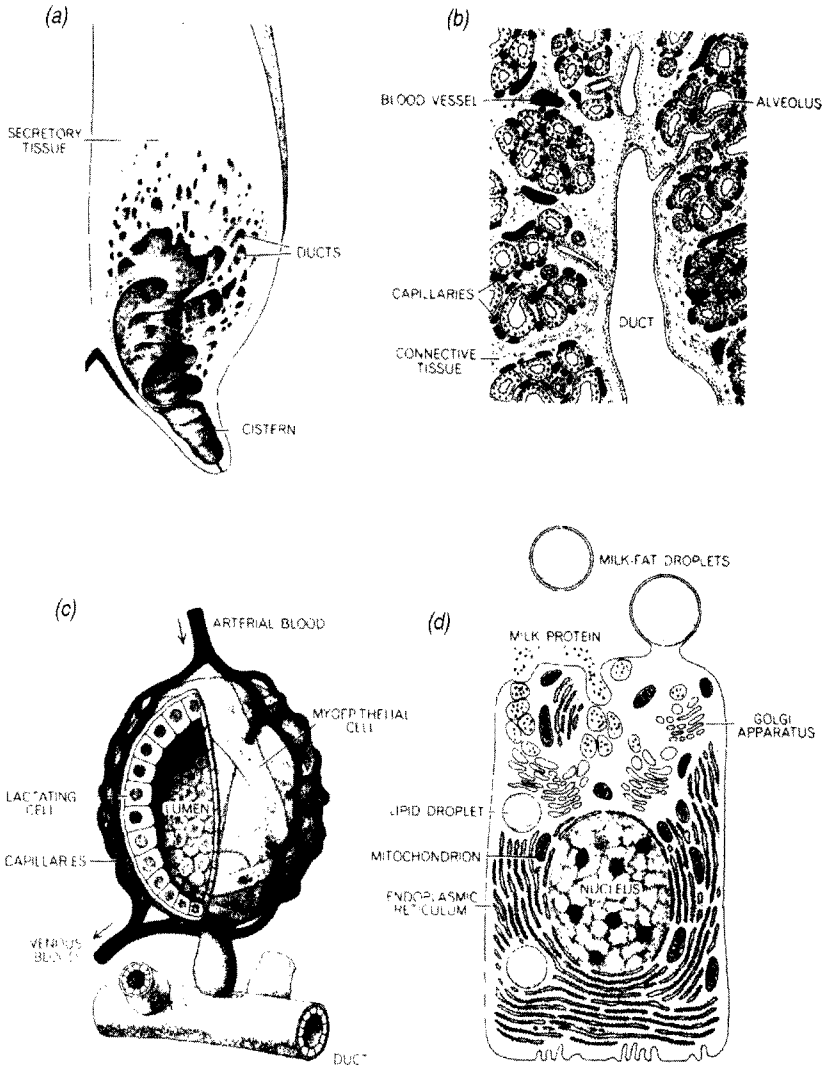

Figure 1.2 Milk-producing tissue of a cow, shown at progressively larger scale. (a) A longitudinal section of one of the four quarters of a mammary gland; (b) arrangement of the alveoli and the duct system that drains them; (c) single alveolus consisting of an elliptical arrangement of lactating cells surrounding the lumen, which is linked to the duct system of the mammary gland; (d) a lactating cell; part of the cell membrane becomes the membrane covering fat droplets; dark circular bodies in the vacuoles of Golgi apparatus are protein particles, which are discharged into the lumen. (From Patton, 1969.)

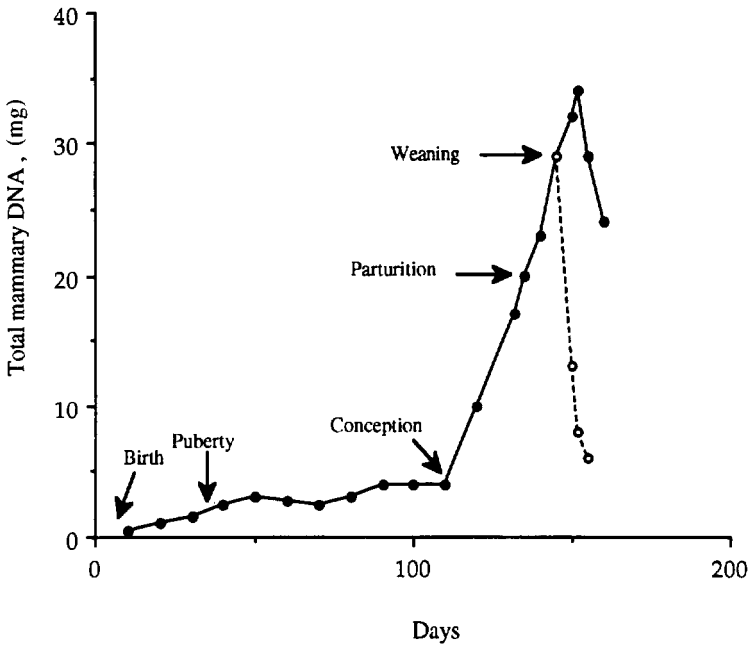

Figure 1.3 Time-course of mammary development in rats (from Tucker, 1969).

The substrates for milk synthesis enter the secretory cell across the basal membrane (outside), are utilized, converted and interchanged as they pass inwards through the cell and the finished milk constituents are excreted into the lumen across the lumenal or apical membrane. Myoepithelial cells (spindle shaped) form a 'basket' around each alveolus and are capable of contracting on receiving an electrical, hormonally mediated, stimulus, thereby causing ejection of milk from the lumen into the ducts.

Development of mammary tissue commences before birth, but at birth the gland is still rudimentary. It remains rudimentary until puberty when very significant growth occurs in some species; much less growth occurs in other species, but in all species the mammary gland is fully developed at puberty. In most species, the most rapid phase of mammary gland development occurs at pregnancy and continues through pregnancy and parturition, to reach peak milk production at weaning. The data in Figure 1.3 show the development pattern of the mammary gland in the rat, the species that has been thoroughly studied in this regard.

Mammary development is under the regulation of a complex set of hormones. Studies involving endocrinectomy (removal of different endocrine organs) show that the principal hormones are oestrogen, progesterone, growth hormone, prolactin and corticosteroids (Figure 1.4).

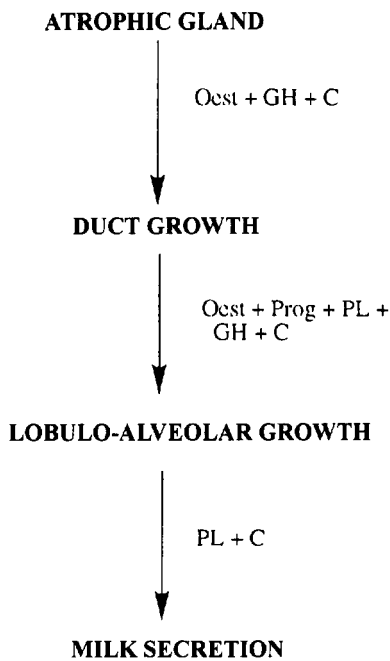

Figure 1.4 The hormonal control of mammary development in rats. Oest, Oestrogen; Prog, progesterone; GH, growth hormone; PL, prolactin; C, corticosteroids.

1.5 Ultrastructure of the secretory cell

The structure of the secretory cell is essentially similar to that of other eukaryotic cells. In their normal state, the cells are roughly cubical, *c.* 10 μm in cross-section. It is estimated that there are *c.* 5×10^{12} cells in the udder of the lactating cow. A diagrammatic representation of the cell is shown in Figure 1.2d. It contains a large nucleus towards the base of the cell and is surrounded by a cell membrane, the plasmalemma. The cytoplasm contains the usual range of organelles:

- mitochondria: principally involved in energy metabolism (tricarboxylic acid (Krebs) cycle);
- endoplasmic reticulum: located towards the base of the cell and to which are attached ribosomes, giving it a rough appearance (hence the term, rough endoplasmic reticulum, RER). Many of the biosynthetic reactions of the cell occur in the RER;
- Golgi apparatus: a smooth membrane system located toward the apical region of the cell, where much of the assembly and 'packaging' of synthesized material for excretion occur;

- lysosomes: capsules of enzymes (usually hydrolytic) distributed fairly uniformly throughout the cytoplasm.

Fat droplets and vesicles of material for excretion are usually apparent toward the apical region of the cell. The apical membrane possesses microvilli which serve to greatly increase its surface area.

1.6 Techniques used to study milk synthesis

1.6.1 Arteriovenous concentration differences

The arterial and veinous systems supplying the mammary gland (Figure 1.5) are readily accessible and may be easily cannulated to obtain blood samples for analysis. Differences in composition between arterial and venous blood give a measure of the constituents used in milk synthesis. The total amount of constituent used may be determined if the blood flow rate is known, which may be easily done by infusing a known volume of cold saline

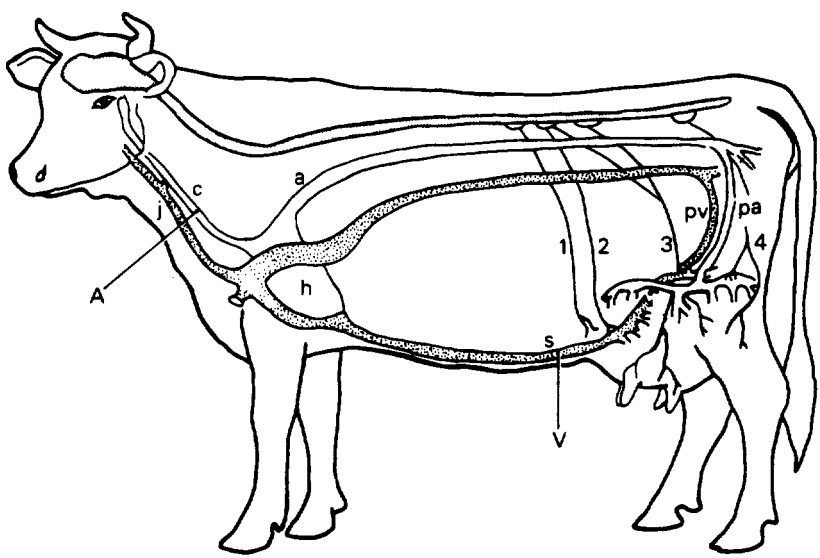

Figure 1.5 The blood vessel and nerve supply in the mammary glands of a cow. Circulatory system (arteries, white; veins, stippled): h, heart; a, abdominal aorta; pa, external pudic artery; pv, external pudic vein; s, subcutaneous abdominal vein; c, carotid artery; j, jugular vein. Nerves: 1, first lumbar nerve; 2, second lumbar nerve; 3, external spermatic nerve; 4, perineal nerve. A and V show blood sampling points for arteriovenous (AV) difference determinations (Mepham, 1987).

solution into a vein and measuring the temperature of blood a little further downstream. The extent to which the blood temperature is reduced is inversely proportional to blood flow rate.

1.6.2 Isotope studies

Injection of radioactively labelled substrates, e.g. glucose, into the blood-stream permits assessment of the milk constituents into which that substrate is incorporated. It may also be possible to study the intermediates through which biosynthesis proceeds.

1.6.3 Perfusion of isolated gland

In many species, the entire gland is located such that it may be readily excised intact and undamaged. An artificial blood supply may be connected to cannulated veins and arteries (Figure 1.6); if desired, the blood supply may be passed through an artificial kidney. The entire mammary gland may

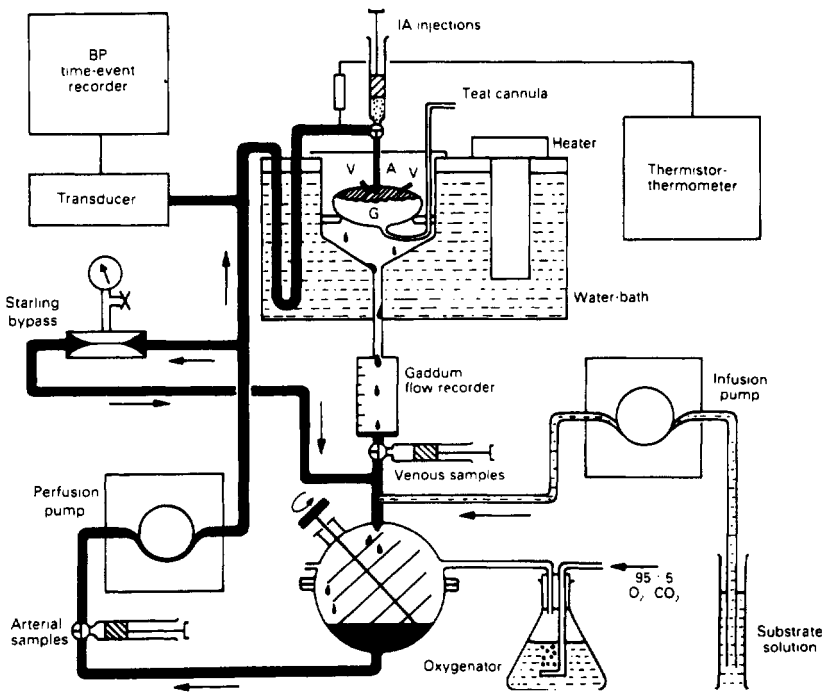

Figure 1.6 Diagram of circuit for perfusion of an isolated mammary gland of a guinea-pig., G, mammary gland; A, artery; V, veins (from Mepham, 1987).

be maintained active and secreting milk for several hours; substrates may readily be added to the blood supply for study.

1.6.4 Tissue slices

The use of tissue slices is a standard technique in all aspects of metabolic biochemistry. The tissue is cut into slices, sufficiently thin to allow adequate rates of diffusion in and out of the tissue. The slices are submerged in physiological saline to which substrates or other compounds may be added.

Changes in the composition of the slices and/or incubation medium give some indication of metabolic activity, but extensive damage may be caused to the cells on slicing; the system is so artificial that data obtained by the tissue slice technique may not pertain to the physiological situation. However, the technique is widely used at least for introductory, exploratory experiments.

1.6.5 Cell homogenates

Cell homogenates are an extension of the tissue slice technique, in which the tissue is homogenized. As the tissue is completely disorganized, only individual biosynthetic reactions may be studied in such systems; useful preliminary work may be done with homogenates.

1.6.6 Tissue culture

Tissue cultures are useful for preliminary or specific work but are incomplete.

In general, the specific constituents of milk are synthesized from small molecules absorbed from the blood. These precursors are absorbed across the basal membrane but very little is known about the mechanism by which they are transported across the membrane. Since the membrane is rich in lipids, and precursors are mostly polar with poor solubility in lipid, it is unlikely that the precursors enter the cell by simple diffusion. It is likely, in common with other tissues, that there are specialized carrier systems to transport small molecules across the membrane; such carriers are probably proteins.

The mammary gland of the mature lactating female of many species is by far the most metabolically active organ of the body. For many small mammals, the energy input required for the milk secreted in a single day may exceed that required to develop a whole litter *in utero*. A cow at peak lactation yielding 45 kg milk day^{-1} secretes approximately 2 kg lactose and 1.5 kg each of fat and protein per day. This compares with the daily weight gain for a beef animal of 1–1.5 kg day^{-1}, 60–70% of which is water. In large

measure, a high-yielding mammal is subservient to the needs of its mammary gland to which it must supply not only the precursors for the synthesis of milk constituents but also an adequate level of high-energy-yielding substrates (ATP, UTP, etc.) required to drive the necessary synthetic reactions. In addition, minor constituents (vitamins and minerals) must be supplied.

1.7 Biosynthesis of milk constituents

The constituents of milk can be grouped into four general classes according to their source:

- organ-(mammary gland) and species-specific (e.g. most proteins and lipids);
- organ- but not species-specific (lactose);
- species- but not organ-specific (some proteins);
- neither organ- nor species-specific (water, salts, vitamins).

The principal constituents (lactose, lipids and most proteins) of milk are synthesized in the mammary gland from constituents absorbed from blood. However, considerable modification of constituents occurs in the mammary gland; the constituents are absorbed from blood through the basal membrane, modified (if necessary) and synthesized into the finished molecule (lactose, triglycerides, proteins) within the mammocyte (mainly in the endoplasmic reticulum) and excreted from the mammocyte through the apical membrane into the lumen of the alveolus.

We believe that it is best and most convenient to describe the synthesis of the principal constituents in the appropriate chapter.

1.8 Production and utilization of milk

Sheep and goats were domesticated early during the Agricultural Revolution, 8000–10 000 years ago. Cattle were domesticated later but have become the principal dairying species in the most intense dairying areas, although sheep and goats are very important in arid regions, especially around the Mediterranean. Buffalo are important in some regions, especially in India and Egypt. Mare's milk is used extensively in central Asia and is receiving attention in Europe for special dietary purposes since its composition is closer to that of human milk than is bovine milk.

Some milk and dairy products are consumed in probably all regions of the world but they are major dietary items in Europe, North and South America, Australia, New Zealand and some Middle Eastern countries. Total milk production in 1996 was estimated to be 527×10^6 tonnes, of which 130,

Table 1.2 Consumption (kg caput^{-1} annum^{-1}) of liquid milk, 1993 (IDF, 1995)

Country	Total	Country	Total
Russia[a]	252	Luxembourg[a]	86
Ireland[a]	182	Netherlands	84
Iceland	180	Hungary	81
Finland	170	Estonia[a]	81
Norway	147	Canada	77
Sweden	126	France	77
Denmark	115	Italy	75
United Kingdom	115	Germany	70
Spain	115	Greece[a]	67
Switzerland	101	Belgium	65
New Zealand	101	India	51
Australia	99	Lithuania[a]	46
Czech and Slovak Reps[a]	97	Japan	42
USA	93	South Africa	38
Austria	92	Chile[a]	18

[a]Data for 1991, from IDF (1993).

Table 1.3 Consumption (kg caput^{-1} annum^{-1}) of cheese, 1993 (IDF, 1995)

Country	Fresh	Ripened	Total
France	7.5	15.5	22.8
Greece[a]	0.2	21.8	22.0
Italy	6.7	13.4	20.1
Belgium	4.7	15.1	19.8
Germany	8.0	10.5	18.5
Lithuania[a]	11.6	6.8	18.4
Iceland	5.2	11.9	17.1
Switzerland	2.8	13.6	16.4
Sweden	0.9	15.5	16.4
Luxembourg[a]	5.0	11.3	16.3
Netherlands	1.7	14.1	15.8
Denmark	0.9	14.5	15.4
Finland	2.3	12.0	14.3
Norway	0.2	14.0	14.2
Canada	0.9	12.4	13.3
USA	1.3	11.9	13.2
Austria	3.9	7.5	11.4
Czech and Slovak Reps[a]	4.0	6.6	10.6
Estonia	5.6	4.4	10.0
Australia	–	–	8.8
United Kingdom	–	–	8.3
New Zealand	–	–	8.1
Hungary	3.3	4.6	7.9
Russia[a]	2.8	4.9	7.7
Spain	–	–	7.0
Ireland[a]	–	–	5.6
Chile[a]	2.0	2.0	4.0
South Africa	0.1	1.5	1.6
Japan	0.2	1.2	1.4
India	0.2	–	0.2

[a]Data for 1991, from IDF (1993).

Table 1.4 Consumption (kg caput^{-1} annum^{-1}) of butter, 1993 (IDF, 1995)

Country	Butter
Lithuania[a]	18.8
New Zealand	9.3
Belgium	7.0
France	6.8
Germany	6.8
Russia[a]	6.5
Estonia	5.9
Luxembourg[a]	5.8
Finland	5.3
Switzerland	5.3
Czech and Slovak Reps[a]	5.0
Austria	4.3
Denmark	4.1
United Kingdom	3.5
Ireland[a]	3.4
Netherlands	3.3
Australia	3.3
Canada	3.0
Norway	2.3
Sweden	2.3
Iceland	2.2
USA	2.1
Italy	1.8
Greece[a]	1.1
India	0.1
Hungary	0.9
Japan	0.7
Chile[a]	0.6
South Africa	0.5
Spain	0.2

[a]Data for 1991, from IDF (1993).

103, 78, 26 $\times$ 10^6 tonnes were produced in western Europe, eastern Europe, North America and the Pacific region, respectively (IDF, 1996). The European Union and some other countries operate milk production quotas which are restricting growth in those areas. Data on the consumption of milk and dairy products in countries that are members of the International Dairy Federation (IDF) are summarized in Tables 1.2–1.6. Milk and dairy products are quite important in several countries that are not included in Tables 1.2–1.6 since they are not members of the IDF.

Because milk is perishable and its production was, traditionally, seasonal, milk surplus to immediate requirements was converted to more stable products, traditional examples being butter or ghee, fermented milk and cheese; smaller amounts of dried milk products were produced traditionally by sun-drying. These traditional products are still very important and many new variants thereof have been introduced. In addition, several new products have been developed during the past 130 years, e.g.

Table 1.5 Consumption (kg caput^{-1} annum^{-1}) of cream (butterfat equivalent), 1993 (IDF, 1995)

Country	Total
Sweden	3.0
Denmark	2.9
Lithuania[a]	2.9
Luxembourg[a]	2.6
Iceland	2.4
Norway	2.4
Switzerland	2.3
Russia[a]	2.1
Finland	2.0
Germany	1.8
Estonia	1.7
Hungary	1.6
Belgium	1.5
Austria	1.3
New Zealand	1.3
United Kingdom[a]	1.1
Greece[a]	1.0
France	1.0
Czech and Slovak Reps[a]	0.9
Ireland[a]	0.9
Netherlands	0.7
Canada	0.6
USA	0.6
Spain	0.4
Italy	0.3
South Africa	0.3
Japan	0.2
Chile[a]	0.2

[a]Data for 1991, from IDF (1993).

sweetened condensed milk, sterilized concentrated milk, a range of milk powders, UHT sterilized milk, ice-creams, infant foods and milk protein products.

One of the important developments in dairy technology in recent years has been the fractionation of milk into its principal constituents, e.g. lactose, milk fat fractions and milk protein products (caseins, caseinates, whey protein concentrates, whey protein isolates, mainly for use as functional proteins but more recently as 'nutraceuticals', i.e. proteins for specific physiological and/or nutritional functions, e.g. lactotransferrin, immunoglobulins).

As a raw material, milk has many attractive features:

1. Milk was designed for animal nutrition and hence contains the necessary nutrients in easily digestible forms (although the balance is designed for

Table 1.6 Consumption (kg caput^{-1} annum^{-1}) of fermented milks, 1993 (IDF, 1995)

Country	Total
Finland	37.0
Sweden	28.6
Iceland	25.9
Netherlands	20.7
France	17.3
Switzerland	17.0
India	16.1
Denmark	15.1
Lithuania[a]	14.6
Germany	12.2
Austria	11.1
Spain	9.8
Belgium	9.6
Estonia	8.8
Czech and Slovak Reps[a]	8.8
Japan	8.5
Luxembourg[a]	7.0
Greece[a]	6.8
Norway	6.3
Italy	5.0
Australia	4.8
United Kingdom[a]	4.8
Chile[a]	4.1
Hungary	3.6
South Africa	3.6
Ireland[a]	3.3
Canada	3.0
USA	2.1

[a]Data for 1991, from IDF (1993).

the young of a particular species) and free of toxins. No other single food, except the whole carcass of an animal, including the bones, contains the complete range of nutrients at adequate concentrations.

2. The principal constituents of milk, i.e. lipids, proteins and carbohydrates, can be readily fractionated and purified by relatively simple methods, for use as food ingredients.
3. Milk itself is readily converted into products with highly desirable organoleptic and physical characteristics and its constituents have many very desirable and some unique physicochemical (functional) properties.
4. The modern dairy cow is a very efficient convertor of plant material; average national yields, e.g. in the USA and Israel, are about 8000 kg annum^{-1}, with individual cows producing up to 20 000 kg annum^{-1}. In terms of kilograms of protein that can be produced per hectare, milk

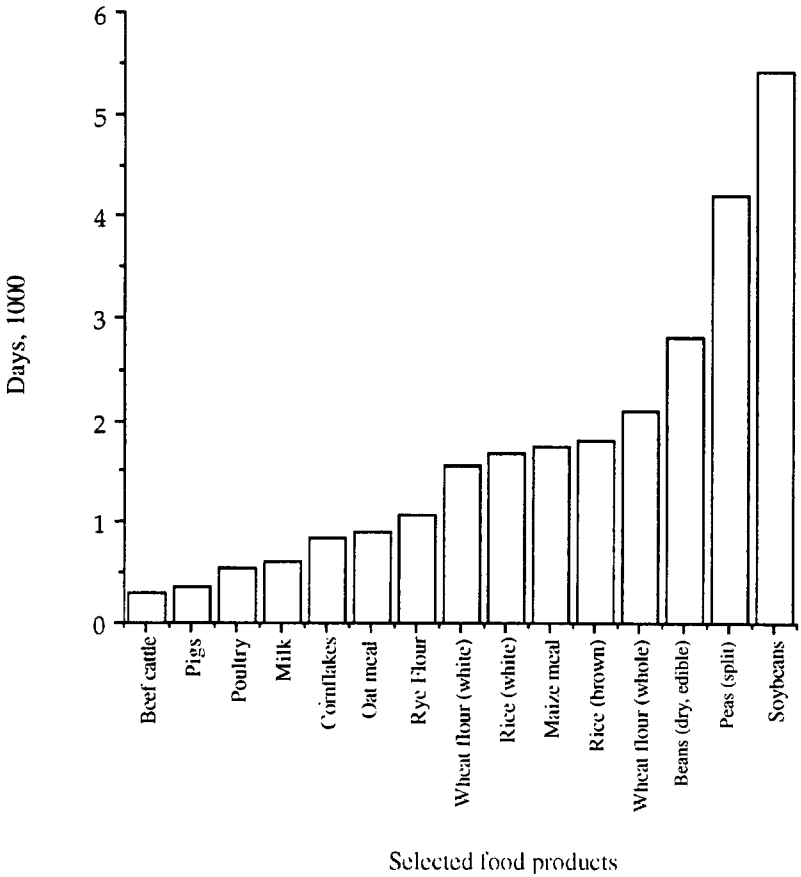

Figure 1.7 Number of days of protein supply for a moderately active man produced per hectare yielding selected food products.

production, especially by modern cows, is much more efficient than meat production (Figure 1.7) but less efficient than some plants (e.g. cereals and soybeans). However, the functional and nutritional properties of milk proteins are superior to those of soy protein, and since cattle, and especially sheep and goats, can thrive under farming conditions not suitable for growing cereals or soybeans, dairy animals need not be competitors with humans for use of land, although high-yielding dairy cows are fed products that could be used for human foods. In any case, dairy products improve the 'quality of life', which is a desirable objective *per se*.

Table 1.7 Diversity of dairy products

Process	Primary product	Further products
Centrifugal separation	Cream	Butter, butter oil, ghee Creams: various fat content (HTST pasteurized or UHT sterilized), coffee creams, wipping creams, dessert creams Cream cheeses
	Skim milk	Powders, casein, cheese, protein concentrates
Concentration thermal evaporation *or* ultrafiltration		In-container or UHT-sterilized concentrated milks; sweetened condensed milk
Concentration *and* drying		Whole milk powders; infant formulae; dietary products
Enzymatic coagulation	Cheese	1000 varieties; further products, e.g. processed cheese, cheese sauces, cheese dips
	Rennet casein	Cheese analogues
	Whey	Whey powders, demineralized whey powders, whey protein concentrates, whey protein isolates, individual whey proteins, whey protein hydrolysates, neutraceuticals Lactose and lactose derivatives
Acid coagulation	Cheese	Fresh cheeses and cheese-based products
	Acid casein	Functional applications, e.g. coffee creamers, meat extenders; nutritional applications
	Whey	Whey powders, demineralized whey powders, whey protein concentrates, whey protein isolates, individual whey proteins, whey protein hydrolysates, neutraceuticals
Fermentation		Various fermented milk products, e.g. yoghurt, buttermilk, acidophilus milk, bioyoghurt
Freezing		Ice-cream (numerous types and formulations)
Miscellaneous		Chocolate products

5. One of the limitations of milk as a raw material is its perishability – it is an excellent source of nutrients for micro-organisms as well as for humans. However, this perishability is readily overcome by a well-organized, efficient dairy industry.

Milk is probably the most adaptable and flexible of all food materials, as will be apparent from Table 1.7, which shows the principal families of milk-based foods – some of these families contain several hundred different products.

Many of the processes to which milk is subjected cause major changes in the composition (Table 1.8), physical state, stability, nutritional and sensoric

Table 1.8 Approximate composition (%) of some dairy products

Product	Moisture	Protein	Fat	Sugars[a]	Ash
Light whipping cream	63.5	2.2	30.9	3.0	0.5
Butter	15.9	0.85	81.1	0.06	2.1
Anhydrous butter oil	0.2	0.3	99.5	0.0	0.0
Ice-cream[b]	60.8	3.6	10.8	23.8	1.0
Evaporated whole milk	74.0	6.8	7.6	10.0	1.5
Sweetened condensed milk	27.1	7.9	8.7	54.4	1.8
Whole milk powder	2.5	26.3	26.7	38.4	6.1
Skim milk powder	3.2	36.2	0.8	52.0	7.9
Whey powder[c]	3.2	12.9	1.1	74.5	8.3
Casein powder	7.0	88.5	0.2	0.0	3.8
Cottage cheese, creamed	79.0	12.5	4.5	2.7	1.4
Quarg	72.0	18.0	8.0	3.0	–
Camembert cheese	51.8	19.8	24.3	0.5	3.7
Blue cheese	42.4	21.4	28.7	2.3	5.1
Cheddar cheese	36.7	24.9	33.1	1.3	3.9
Emmental cheese	36.0	28.9	30.0	–	–
Parmesan cheese	29.2	35.7	24.8	3.2	6.0
Mozzarella cheese	54.1	19.4	31.2	2.2	2.6
Processed cheese[d]	39.2	22.1	31.2	1.6	5.8
Acid whey	93.9	0.6	0.2	4.2	–

[a]Total carbohydrate.
[b]Hardened vanilla, 19% fat.
[c]Cheddar (sweet) whey.
[d]American pasteurized processed cheese.

attributes of the product; some of these changes will be discussed in later chapters.

1.9 Trade in milk products

Milk and dairy products have been traded for thousands of years and are now major items of trade. According to Verheijen, Brockman and Zwanenberg (1994), world dairy exports were US$23 $\times$ 10^9 in 1992; the major flow of milk equivalent is shown in Figure 1.8. Import and export data, as well as much other interesting statistical data on the world dairy industry, are provided by Verheijen, Brockman and Zwanenberg (1994), including a list of the principal dairy companies in the world in 1992, the largest of which was Nestlé, which had a turnover from dairy products of US$10.6 $\times$ 10^9 (*c.* 39% of total company turnover).

Traditionally, dairy products (cheese, fermented milks, butter) were produced on an artisanal level, as is still the case in underdeveloped regions and to some extent in highly developed dairying countries. Industrialization commenced during the nineteenth century and dairy manufacturing is now a well-organized industry. One of the features of the past few decades has

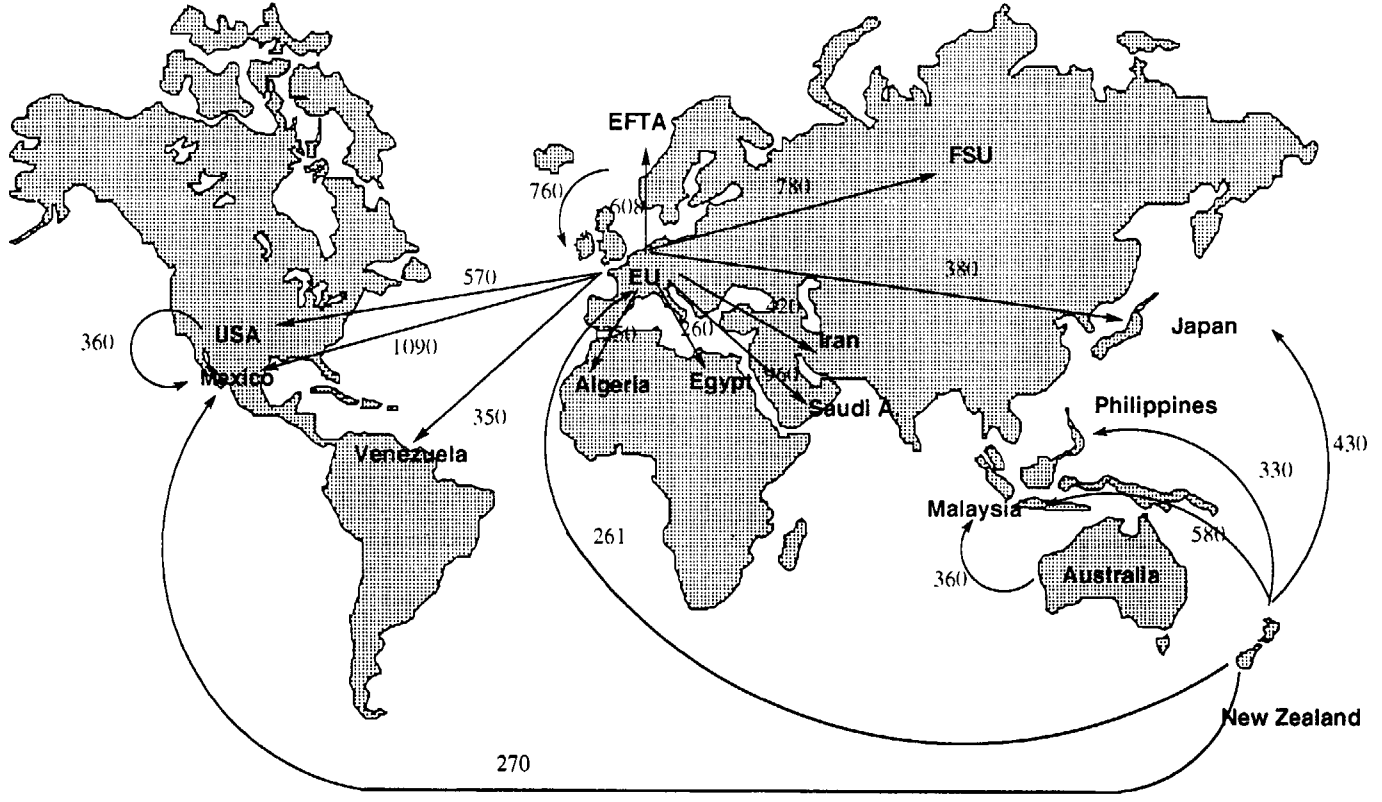

Figure 1.8 Trade flows greater than 250 000 tonnes in milk equivalents, 1992 (in 1000 tonnes) (from Verheigen, Brockman and Zwaneberg, 1994).

been the amalgamation of smaller dairy companies both within countries, and, recently, internationally. Such developments have obvious advantages in terms of efficiency and standardization of product quality but pose the risk of over-standardization with the loss of variety. Greatest diversity occurs with cheeses and, fortunately in this case, diversity is being preserved and even extended.

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2 Lactose

2.1 Introduction

Lactose is the principal carbohydrate in the milks of all mammals; non-mammalian sources are very rare. Milk contains only trace amounts of other sugars, including glucose ($50\,mg\,l^{-1}$), fructose, glucosamine, galactosamine, neuraminic acid and neutral and acidic oligosaccharides.

The concentration of lactose in milk varies widely between species (Table 2.1). The lactose content of cows' milk varies with the breed of cow, individuality factors, udder infection and especially stage of lactation. The concentration of lactose decreases progressively and significantly during lactation (Figure 2.1); this behaviour contrasts with the lactational trends for lipids and proteins, which, after decreasing during early lactation, increase strongly during the second half of lactation. Mastitis causes an increased level of NaCl in milk and depresses the secretion of lactose. Lactose, along with sodium, potassium and chloride ions, plays a major role in maintaining the osmotic pressure in the mammary system. Thus, any increase or decrease in lactose content (a secreted constituent, i.e. formed within the mammary gland) is compensated for by an increase or decrease in the soluble salt (excreted) constituents. This osmotic relationship partly explains why certain milks with a high lactose content have a low ash content and vice versa (Table 2.2).

Similarly, there is an inverse relationship between the concentration of lactose and chloride, which is the basis of Koestler's chloride–lactose test

Table 2.1 Concentration (%) of lactose in the milks of selected species

Species	Lactose	Species	Lactose	Species	Lactose
California sea lion	0.0	Mouse (house)	3.0	Cat (domestic)	4.8
Hooded seal	0.0	Guinea-pig	3.0	Pig	5.5
Black bear	0.4	Dog (domestic)	3.1	Horse	6.2
Dolphin	0.6	Sika deer	3.4	Chimpanzee	7.0
Echidna	0.9	Goat	4.1	Rhesus monkey	7.0
Blue whale	1.3	Elephant (Indian)	4.7	Human	7.0
Rabbit	2.1	Cow	4.8	Donkey	7.4
Red deer	2.6	Sheep	4.8	Zebra	7.4
Grey seal	2.6	Water buffalo	4.8	Green monkey	10.2
Rat (Norwegian)	2.6				

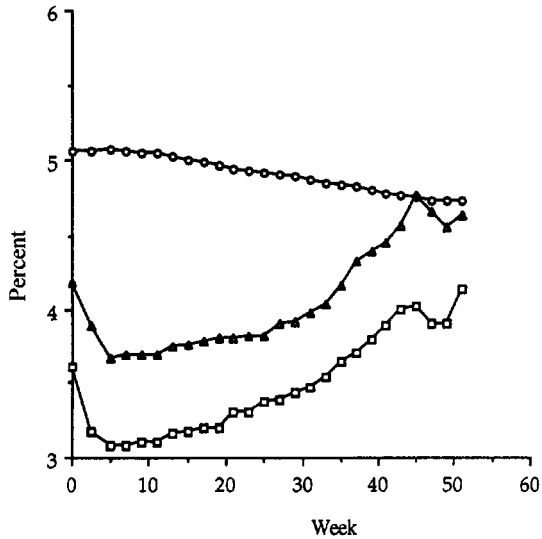


Figure 2.1 Changes in the concentrations of fat (△), protein (□) and lactose (○) in milk during lactation.

Table 2.2 Average concentration (%) of lactose and ash in the milks of some mammals

Species	Water	Lactose	Ash
Human	87.4	6.9	0.21
Cow	87.2	4.9	0.70
Goat	87.0	4.2	0.86
Camel	87.6	3.26	0.70
Mare	89.0	6.14	0.51
Reindeer	63.3	2.5	1.40

for abnormal milk:

$$\text{Koestler number} = \frac{\%\ \text{Chloride} \times 100}{\%\ \text{Lactose}}.$$

A Koestler number less than 2 indicates normal milk while a value greater than 3 is considered abnormal.

Lactose plays an important role in milk and milk products:

- it is an essential constituent in the production of fermented dairy products;

- it contributes to the nutritive value of milk and its products; however, many non-Europeans have limited or zero ability to digest lactose in adulthood, leading to a syndrome known as **lactose intolerance**;
- it affects the texture of certain concentrated and frozen products;
- it is involved in heat-induced changes in the colour and flavour of highly heated milk products.

2.2 Chemical and physical properties of lactose

2.2.1 Structure of lactose

Lactose is a disaccharide consisting of galactose and glucose, linked by a β1-4 glycosidic bond (Figure 2.2). Its systematic name is β-*O*-D-galactopyranosyl-(1-4)-α-D-glucopyranose (α-lactose) or β-*O*-D-galactopyranosyl-(1-4)-β-D-glucopyranose (β-lactose). The hemiacetal group of the glucose moiety is potentially free (i.e. lactose is a **reducing** sugar) and may exist as an α- or β-anomer. In the structural formula of the α-form, the hydroxyl group on the C_1 of glucose is *cis* to the hydroxyl group at C_2 (oriented downward).

2.2.2 Biosynthesis of lactose

Lactose is essentially unique to mammary secretions. It is synthesized from glucose absorbed from blood. One molecule of glucose is isomerized to UDP-galactose via the four-enzyme Leloir pathway (Figure 2.3). UDP-Gal is then linked to another molecule of glucose in a reaction catalysed by the enzyme, lactose synthetase, a two-component enzyme. Component A is a non-specific galactosyl transferase which transfers the galactose from UDP-Gal to a number of acceptors. In the presence of the B component, which is the whey protein, α-lactalbumin, the transferase becomes highly specific for glucose (its K_M decreases 1000-fold), leading to the synthesis of lactose. Thus, α-lactalbumin is an enzyme modifier and its concentration in the milk of several species is directly related to the concentration of lactose in those milks; the milks of some marine mammals contain neither α-lactalbumin nor lactose.

The presumed significance of this control mechanism is to enable mammals to terminate the synthesis of lactose when necessary, i.e. to regulate and control osmotic pressure when there is an influx of NaCl, e.g. during mastitis or in late lactation (lactose and NaCl are major determinants of the osmotic pressure of milk, which is isotonic with blood, the osmotic pressure of which is essentially constant). The ability to control osmotic pressure is sufficiently important to justify an elaborate control mechanism and the 'wastage' of the enzyme modifier.

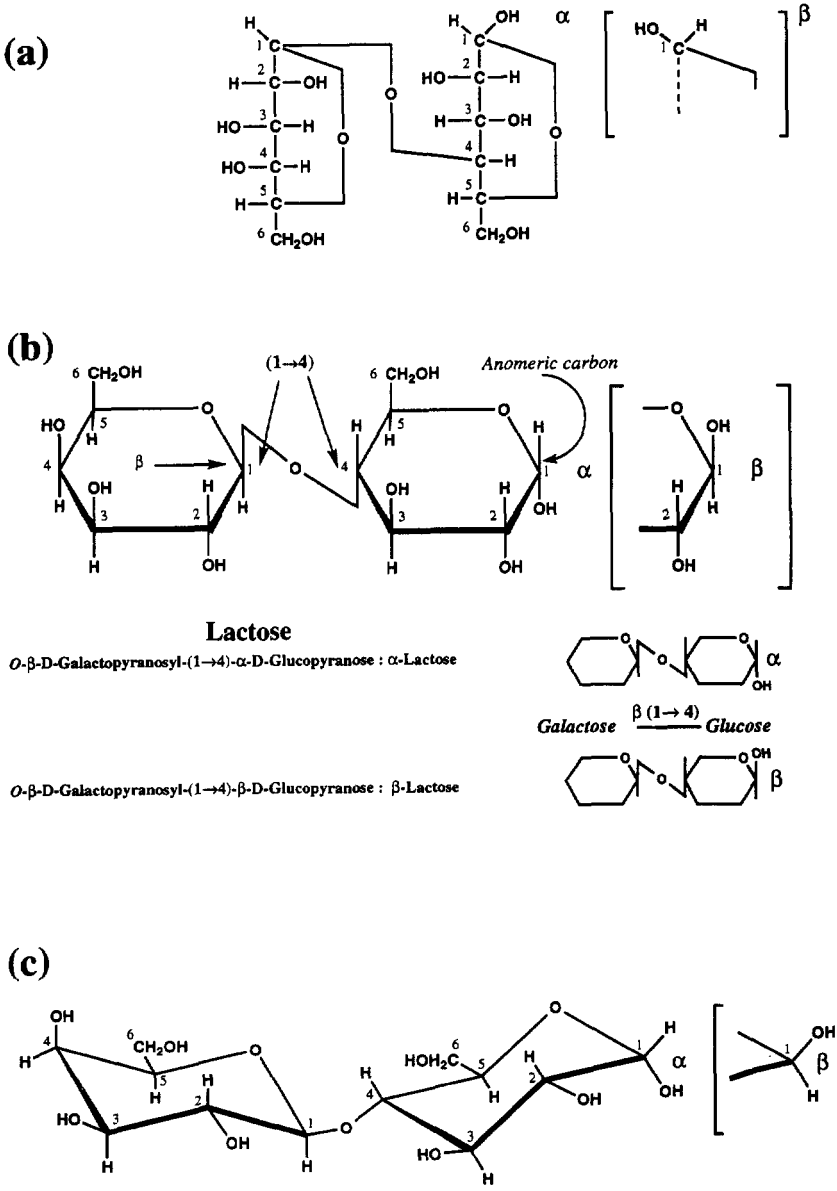


Figure 2.2 Structural formulae of α- and β-lactose. (a) Fischer projection, (b) Haworth projection and (c) conformational formula.

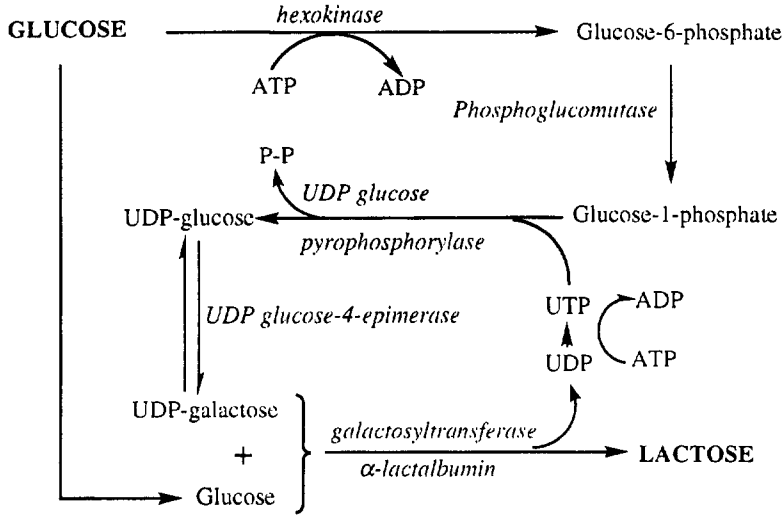


Figure 2.3 Pathway for lactose synthesis.

2.2.3 Lactose equilibrium in solution

The configuration around the C_1 of glucose (i.e. the anomeric C) is not stable and can readily change (**mutarotate**) from the α- to the β-form and vice versa when the sugar is in solution as a consequence of the fact that the hemiacetal form is in equilibrium with the open chain aldehyde form which can be converted into either of the two isomeric forms (Figure 2.2).

When either isomer is dissolved in water, there is a gradual change from one form to the other until equilibrium is established, i.e. mutarotation. These changes may be followed by measuring the change in optical rotation with time until, at equilibrium, the specific rotation is +55.4°.

The composition of the mixture at equilibrium may be calculated as follows:

	Specific rotation $[\alpha]_D^{20}$
α-form	+89.4°
β-form	+35.0°
Equilibrium mixture	+55.4°

Let equilibrium mixture = 100

Let x% of the lactose be in the α-form

Then $(100 - x)$% is the β-form

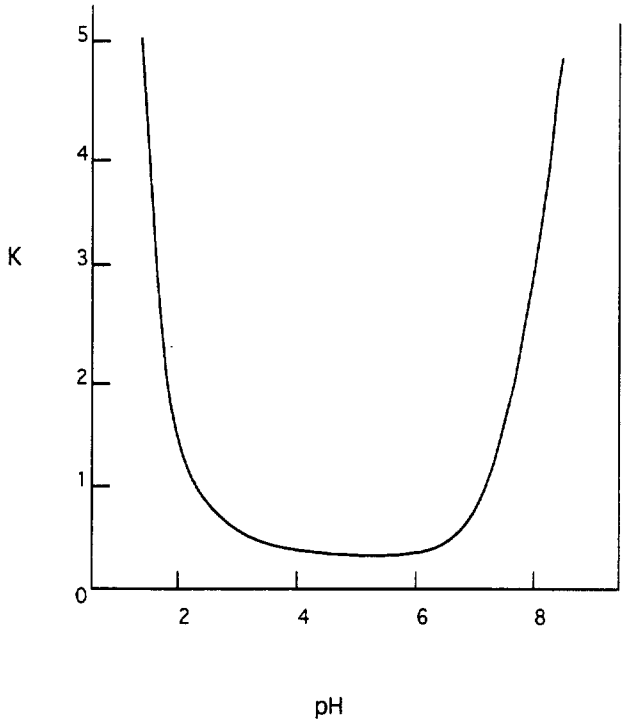


Figure 2.4 Effect of pH on the rate of mutarotation of lactose.

At equilibrium:

$$89.4x + 35(100 - x) = 55.4 \times 100$$

$$x = 37.3$$

$$100 - x = 62.7$$

Thus, the equilibrium mixture at 20°C is composed of 62.7% β- and 37.3% α-lactose. The equilibrium constant, β/α, is 1.68 at 20°C. The proportion of lactose in the α-form increases as the temperature is increased and the equilibrium constant consequently decreases. The equilibrium constant is not influenced by pH, but the rate of mutarotation is dependent on both temperature and pH. The change from α- to β-lactose is 51.1, 17.5 and 3.4% complete at 25, 15 and 0°C, respectively, in 1 h and is almost instantaneous at about 75°C.

The rate of mutarotation is slowest at pH 5.0, increasing rapidly at more acid or alkaline values; equilibrium is established in a few minutes at pH 9.0 (Figure 2.4).

2.2.4 *Significance of mutarotation*

The α- and β-forms of lactose differ with respect to:

- solubility;
- crystal shape and size;
- hydration of crystal form – hygroscopicity;
- specific rotation;
- sweetness.

Many of these characteristics are discussed in the following sections.

2.2.5 *Solubility of lactose*

The solubility characteristics of the α- and β-isomers are distinctly different. When α-lactose is added in excess to water at 20°C, about 7 g per 100 g water dissolve immediately. Some α-lactose mutarotates to the β anomer to establish the equilibrium ratio 62.7β : 37.3α; therefore, the solution becomes unsaturated with respect to α and more α-lactose dissolves. These two processes (mutarotation and solubilization of α-lactose) continue until two criteria are met: ~7 g α-lactose in solution and a β/α ratio of 1.6 : 1.0. Since the β/α ratio at equilibrium is about 1.6 at 20°C, the final solubility is 7 g + (1.6 × 7) g = 18.2 g per 100 g water.

When β-lactose is dissolved in water, the initial solubility is ~50 g per 100 g water at 20°C. Some β-lactose mutarotates to α to establish a ratio of 1.6 : 1. At equilibrium, the solution would contain 30.8 g β and 19.2 g α/100 ml; therefore, the solution is supersaturated with α-lactose, some of which crystallizes, upsetting the equilibrium and leading to further mutarotation of $\beta \rightarrow \alpha$. These two events, i.e. crystallization of α-lactose and mutarotation of β, continue until the same two criteria are met, i.e. ~7 g α-lactose in solution and a β/α ratio of 1.6 : 1. Again, the final solubility is ~18.2 g lactose per 100 g water. Since β-lactose is much more soluble than α and mutarotation is slow, it is possible to form more highly concentrated solutions by dissolving β- rather than α-lactose. In either case, the final solubility is the same.

The solubility of lactose as a function of temperature is summarized in Figure 2.5. The solubility of α-lactose is more temperature dependent than that of β-lactose and the solubility curves intersect at 93.5°C. A solution at 60°C contains approximately 59 g lactose per 100 g water. Suppose that a 50% solution of lactose (~30 g β- and 20 g α-) at 60°C is cooled to 15°C. At this temperature, the solution can contain only 7 g α-lactose or a total of 18.2 g per 100 g water at equilibrium. Therefore, lactose will crystallize very slowly out of solution as irregularly sized crystals which may give rise to a sandy, gritty texture.

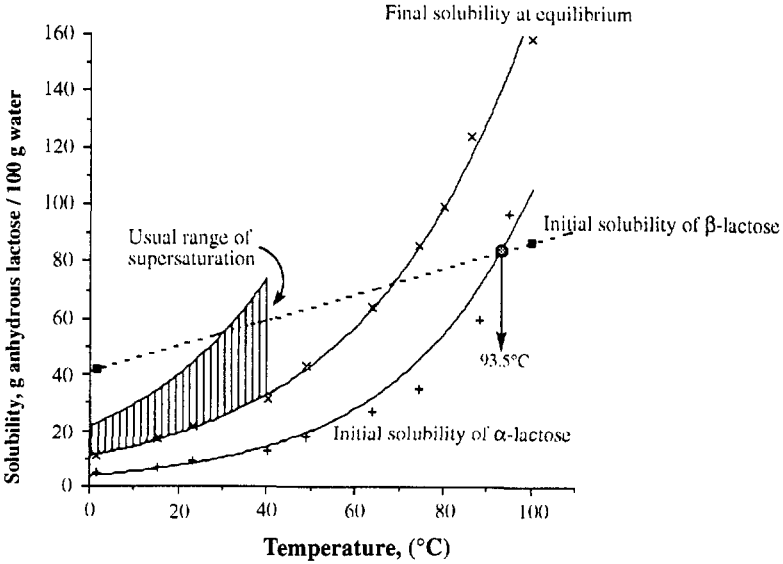


Figure 2.5 Solubility of lactose in water (modified from Jenness and Patton, 1959).

2.2.6 Crystallization of lactose

As discussed in section 2.2.5, the solubility of lactose is temperature dependent and solutions are capable of being highly supersaturated before spontaneous crystallization occurs and even then, crystallization may be slow. In general, supersolubility at any temperature equals the saturation (solubility) value at a temperature 30°C higher. The insolubility of lactose, coupled with its capacity to form supersaturated solutions, is of considerable practical importance in the manufacture of concentrated milk products.

In the absence of nuclei and agitation, solutions of lactose are capable of being highly supersaturated before spontaneous crystallization occurs. Even in such solutions, crystallization occurs with difficulty. Solubility curves for lactose are shown in Figure 2.6 and are divided into unsaturated, metastable and labile zones. Cooling a saturated solution or continued concentration beyond the saturation point, leads to supersaturation and produces a metastable area where crystallization does not occur readily. At higher levels of supersaturation, a labile area is observed where crystallization occurs readily. The pertinent points regarding supersaturation and crystallization are:

- Neither nucleation nor crystal growth occurs in the unsaturated region.
- Growth of crystals can occur in both the metastable and labile areas.
- Nucleation occurs in the metastable area only if seeds (centres for crystal growth) are added.

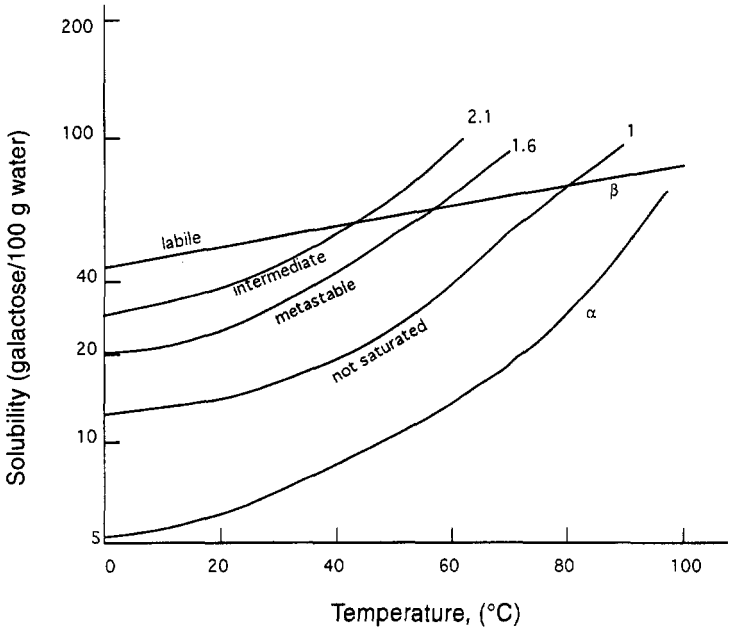


Figure 2.6 Initial solubility of α-lactose and β-lactose, final solubility at equilibrium (line 1), and supersaturation by a factor 1.6 and 2.1 (α-lactose excluding water of crystallization). (Modified from Walstra and Jenness, 1984.)

- Spontaneous crystallization can occur in the labile area without the addition of seeding material.

The rate of nucleation is slow at low levels of supersaturation and in highly supersaturated solutions owing to the high viscosity of the solution. The stability of a lactose 'glass' is due to the low probability of nuclei forming at very high concentrations.

Once a sufficient number of nuclei have formed, crystal growth occurs at a rate influenced by:

- degree of supersaturation;
- surface area available for deposition;
- viscosity;
- agitation;
- temperature;
- mutarotation, which is slow at low temperatures.

α-Hydrate. α-Lactose crystallizes as a monohydrate containing 5% water of crystallization and can be prepared by concentrating aqueous lactose solutions to supersaturation and allowing crystallization to occur below

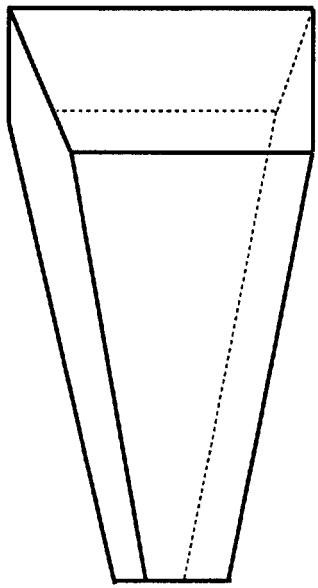


Figure 2.7 The most common crystal form of α-lactose hydrate.

93.5°C. The α-hydrate is the stable solid form at ambient temperatures and in the presence of small amounts of water below 93.5°C, all other forms change to it. The α-monohydrate has a specific rotation in water at 20°C of +89.4°. It is soluble only to the extent of 7 g per 100 g water at 20°C. It forms a number of crystal shapes, depending on the conditions of crystallization; the most common type when fully developed is tomahawk-shaped (Figure 2.7). Crystals are hard and dissolve slowly. In the mouth, crystals less than 10 μm are undetectable, but above 16 μm they feel gritty or 'sandy' and at 30 μm, a definite gritty texture is perceptible. The term 'sandy' or sandiness is used to describe the defect in condensed milk, ice-cream or processed cheese spreads where, due to poor manufacturing techniques, large lactose crystals are formed.

α-Anhydrous. Anhydrous α-lactose may be prepared by dehydrating α-hydrate *in vacuo* at temperatures between 65 and 93.5°C; it is stable only in the absence of moisture.

β-Anhydride. Since β-lactose is less soluble than the α-isomer above 93.5°C, the crystals formed from aqueous solutions at temperatures above 93.5°C are β-lactose; these are anhydrous and have a specific rotation of 35°. β-Lactose is sweeter than α-lactose, but is not appreciably sweeter than the equilibrium mixture of α- and β-lactose normally found in solution.

Table 2.3 Some physical properties of the two common forms of lactose (modified from Jenness and Patton, 1959)

Property	α-Hydrate	β-Anhydride
Melting point[a] (°C)	202	252
Specific rotaltion[b] $[\alpha]_D^{20}$	+89.4°	+35°
Solubility in water (g 100 ml^{-1}) at 20°C	7	50
Specific gravity (20°C)	1.54	1.59
Specific heat	0.299	0.285
Heat of combustion (kJ mol^{-1})	5687	5946

[a]Decomposes; values vary with rate of heating, α-hydrate loses water at 120°C.
[b]Values on anhydrous basis, both forms mutarotate to +55.4°.

Some properties of α- and β-lactose are summarized in Table 2.3. Mixed α/β crystals, e.g. $\alpha_5\beta_3$, can be formed under certain conditions. The relationship between the different crystalline forms of lactose is shown in Figure 2.8.

Lactose glass. When a lactose solution is dried rapidly, viscosity increases so quickly that crystallization is impossible. A noncrystalline form is produced containing α- and β-forms in the ratio at which they exist in solution. Lactose in spray-dried milk exists as a concentrated syrup or amorphous glass which is stable if protected from air, but is very hygroscopic and absorbs water rapidly from the atmosphere, becoming sticky.

2.2.7 Problems related to lactose crystallization

The tendency of lactose to form supersaturated solutions that do not crystallize readily causes problems in many dairy products unless adequate controls are exercised. The problems are due primarily to the formation of large crystals, which cause sandiness, or to the formation of a lactose glass, which leads to hygroscopicity and caking (Figure 2.9).

Dried milk and whey. Lactose is the major component of dried milk products: whole-milk powder, skim-milk powder and whey powder contain *c.* 30, 50 and 70% lactose, respectively. Protein, fat and air are dispersed in a continuous phase of amorphous solid lactose. Consequently, the behaviour of lactose has a major impact on the properties of dried milk products.

In freshly made powder, lactose is in an amorphous state with an α/β ratio of 1 : 1.6. This amorphous lactose glass is a highly concentrated syrup since there is not sufficient time during drying for crystallization to proceed normally. The glass has a low vapour pressure and is hygroscopic, taking up moisture very rapidly when exposed to the atmosphere. On the uptake of moisture, dilution of the lactose occurs and the molecules acquire sufficient mobility and space to arrange themselves into crystals of α-lactose

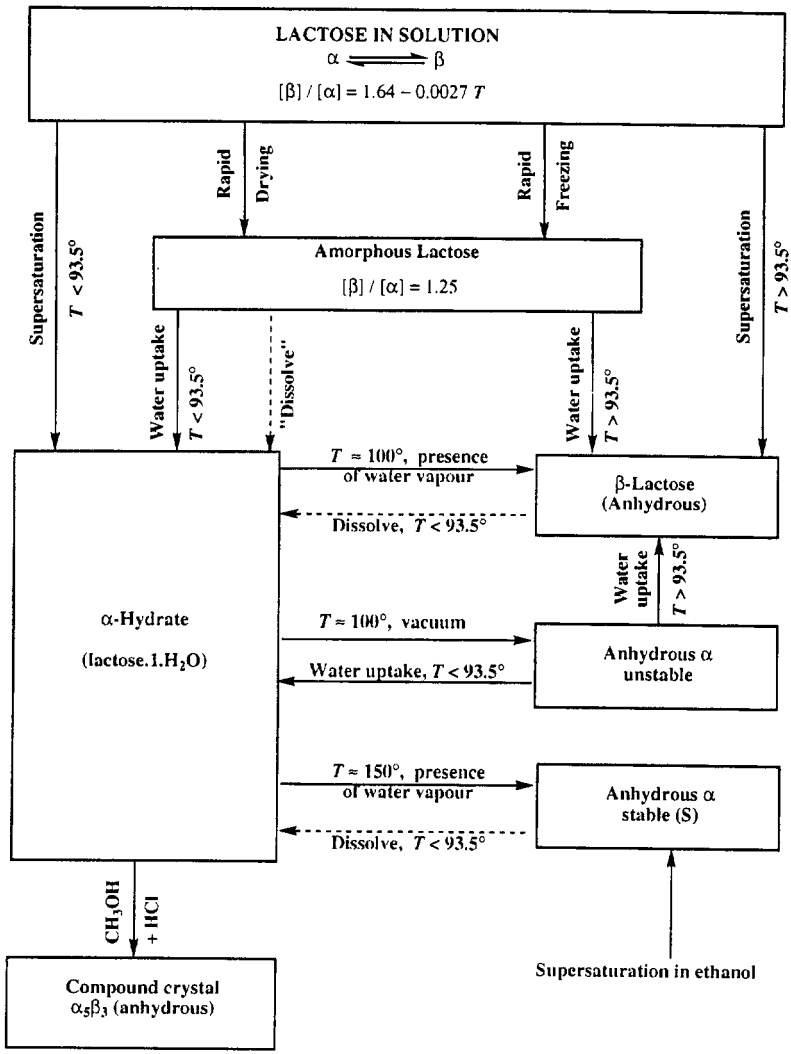


Figure 2.8 Modifications of lactose (T, temperature in °C) (from Walstra and Jenness, 1984).

monohydrate. These crystals are small, usually with dimensions of less than 1 μm. Crevices and cracks exist along the edges of the crystals, into which other components are expelled. In these spaces, favourable conditions exist for the coagulation of casein because of the close packing of the micelles and the destabilizing action of concentrated salt systems. The fat globule membrane may be damaged by mechanical action, and Maillard browning, involving lactose and amino groups of protein, proceeds rapidly when crystallization has occurred.

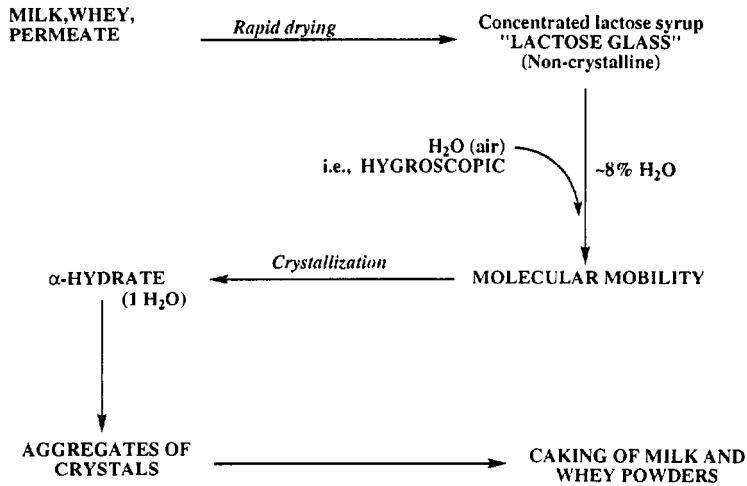


Figure 2.9 Formation and crystallization of lactose glass.

Crystallization of lactose in dried milk particles causes 'caking' of the powder into a hard mass. If a considerable portion of lactose in the freshly dried product is in the crystalline state, caking of the powder on contact with water is prevented, thereby improving the dispersibility of the powder. Lactose crystallization is achieved by rehydrating freshly dried powder to *c.* 10% water and redrying it, or by removing partly dried powder from the drier and completing drying in a fluidized bed dryer. This process is used commercially for the production of 'instantized' milk powders. Clustering of the particles into loose, spongy aggregates occurs; these agglomerates are readily wettable and dispersible. They exhibit good capillary action and water readily penetrates the particles, allowing them to sink and disperse, whereas the particles in non-instantized powder float due to their low density which contributes to their inability to overcome surface tension. Also, because of the small size of the particles in conventional spray-dried powders, close packing results in the formation of inadequate space for capillary action between the particles, thereby preventing uniform wetting. As a result, large masses of material are wetted on the outside, forming a barrier of highly concentrated product which prevents internal wetting and results in large undispersed lumps. This problem is overcome by agglomeration and, in this respect, lactose crystallization is important since it facilitates the formation of large, sponge-like aggregates.

The state of lactose has a major effect on the properties of spray-dried whey powder manufactured by conventional methods, i.e. preheating, condensing to about 50% total solids and drying to less than 4% water. The powder is dusty and very hygroscopic, and when exposed to ambient air it

has a pronounced tendency to cake owing to its very high lactose content (~70%).

Problems arising from the crystallization of lactose in milk and whey powders may also be avoided or controlled by pre-crystallizing the lactose. Essentially, this involves adding finely divided lactose powder which acts as nuclei on which the supersaturated lactose crystallizes. Addition of 0.5 kg of finely ground lactose to the amount of concentrated product (whole milk, skim milk or whey) containing 1 tonne of lactose will induce the formation of c. 10^6 crystals ml^{-1}, about 95% of which will have dimensions less than 10 μm and 100% less than 15 μm, i.e. too small to cause textural defects.

Diagrams of spray dryers with instantizers are shown in Figures 2.10 and 2.11.

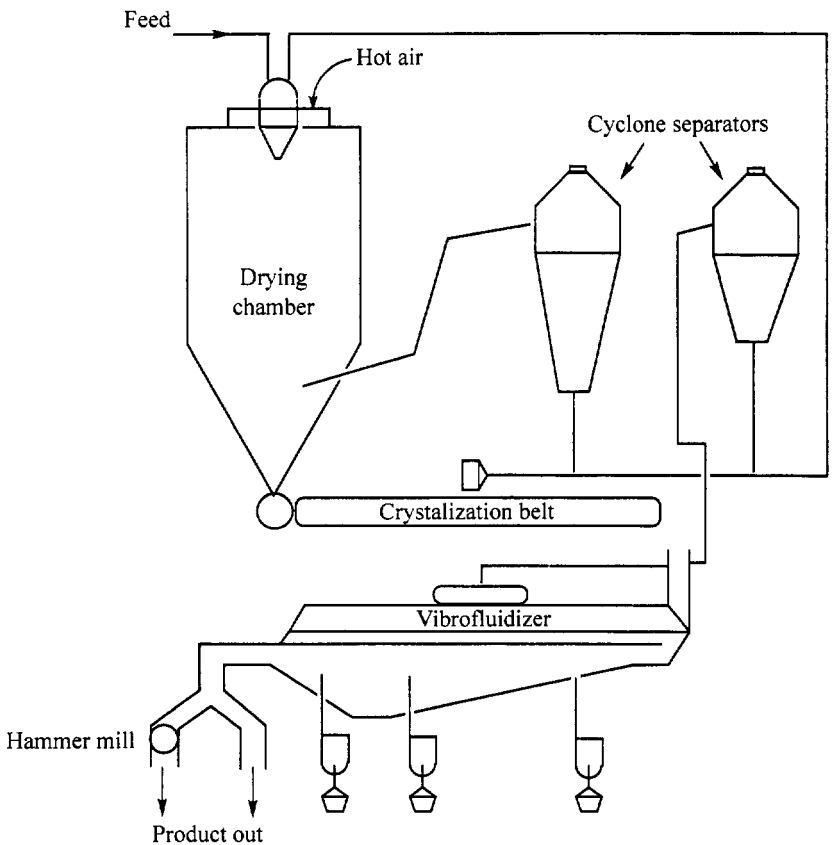


Figure 2.10 Schematic representation of a low temperature drying plant for whey (modified from Hynd, 1980).

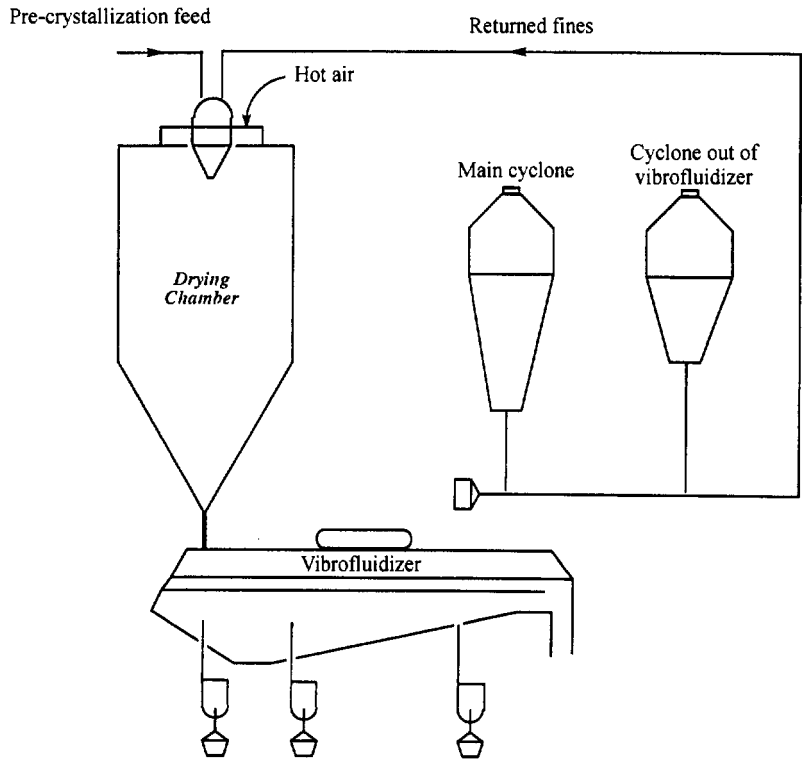


Figure 2.11 Schematic representation of a straight through drying plant for whey (modified from Hynd, 1980).

Thermoplasticity of lactose. Unless certain precautions are taken during the drying of whey or other solutions containing high concentrations of lactose, the hot, semi-dry powder may adhere to the metal surfaces of the dryer, forming deposits. This phenomenon is referred to as thermoplasticity. The principal factors influencing the temperature at which thermoplasticity occurs ('sticking temperature') are the concentrations of lactic acid, amorphous lactose and moisture in the whey powder.

Increasing the concentration of lactic acid from 0 to 16% causes a linear decrease in sticking temperature (Figure 2.12). The degree of pre-crystallization of lactose affects sticking temperature: a product containing 45% pre-crystallized lactose has a sticking temperature of 60°C while the same product with 80% pre-crystallization sticks at 78°C (Figure 2.12). Pre-crystallization of the concentrate feed to the dryer thus permits considerably higher feed concentrations and drying temperatures.

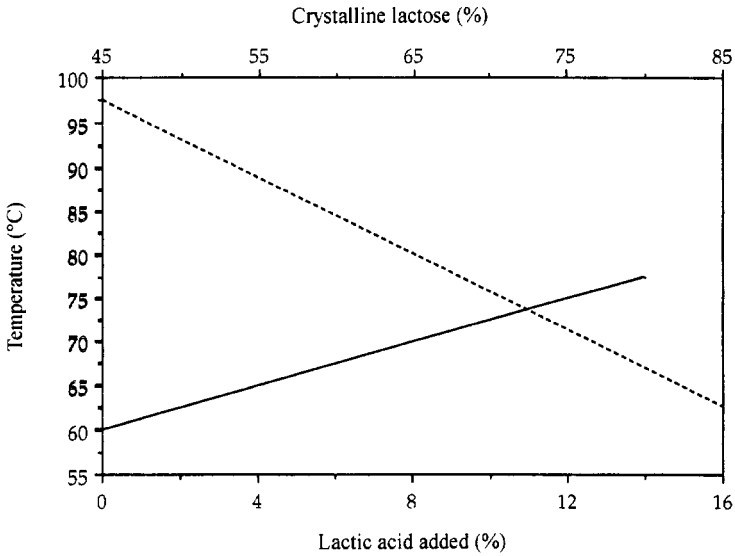


Figure 2.12 Effect of added lactic acid (----) and degree of lactose crystallization (_) on the sticking temperature of whey powder (1.5–3.5% moisture).

In practice, the most easily controlled factor is the moisture content of the whey powder, which is determined by the outlet temperature of the dryer (t_o, Figure 2.13). However, as a result of evaporative cooling, the temperature of the particles in the dryer is lower than the outlet temperature (t_p, Figure 2.13) and the difference between t_o and t_p increases with increasing moisture content. The sticking temperature for a given whey powder decreases with increasing moisture content (t_s, Figure 2.13) and where the two curves (t_s and t_p) intersect (point TPC, Figure 2.13) is the maximum product moisture content at which the dryer can be operated without product sticking during drying. The corresponding point on the outlet temperature curve (TOC) represents the maximum dryer outlet temperature which may be used without causing sticking.

Sweetened condensed milk. Crystallization of lactose occurs in sweetened condensed milk (SCM) and crystal size must be controlled if a product with a desirable texture is to be produced. As it comes from the evaporators, SCM is almost saturated with lactose. When cooled to 15–20°C, 40–60% of the lactose eventually crystallizes as α-lactose hydrate. There are 40–47 parts of lactose per 100 parts of water in SCM, consisting of about 40% α- and 60% β-lactose (ex-evaporator). To obtain a smooth texture, crystals with dimensions of less than 10 μm are desirable. The optimum temperature

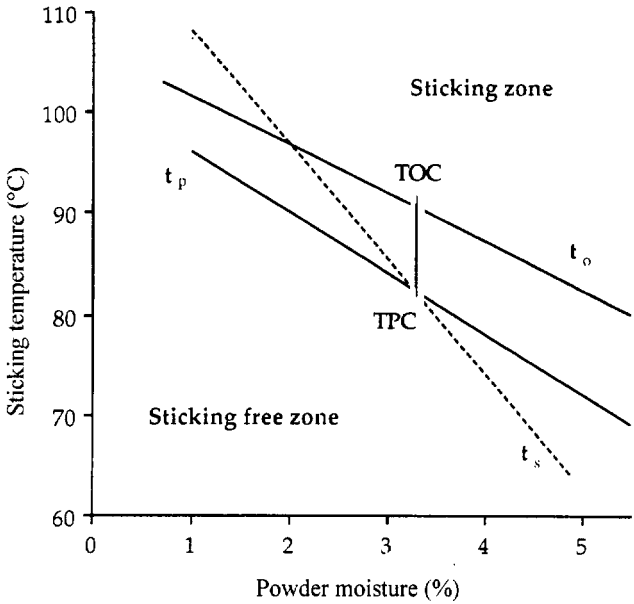


Figure 2.13 Influence of moisture content on the temperature of powder in a spray dryer (t_p), dryer outlet temperature (t_o) and sticking temperature (t_s). The minimum product temperatured required to avoid problems with sticking is at TPC with the corresponding dryer outlet temperature TOC. (Modified from Hynd, 1980.)

for crystallization is 26–36°C. Pulverized α-lactose, or preferably lactose 'glass', is used as seed. Continuous vacuum cooling, combined with seeding, gives the best product.

Ice-cream. Crystallization of lactose in ice-cream causes a sandy texture. In freshly hardened ice-cream, the equilibrium mixture of α- and β-lactose is in the 'glass' state and is stable as long as the temperature remains low and constant. During the freezing of ice-cream, the lactose solution passes through the labile zone so rapidly and at such a low temperature that limited lactose crystallization occurs.

If ice-cream is warmed or the temperature fluctuates, some ice will melt, and an infinite variety of lactose concentrations will emerge, some of which will be in the labile zone where spontaneous crystallization occurs while others will be in the metastable zone where crystallization can occur if suitable nuclei, e.g. lactose crystals, are present. At the low temperature, crystallization pressure is low and extensive crystallization usually does not occur. However, the nuclei formed act as seed for further crystallization

when the opportunity arises and they tend to grow slowly with time, eventually causing a sandy texture. The defect is controlled by limiting the milk solids content or by using β-galactosidase to hydrolyse lactose.

Other frozen dairy products. Although milk may become frozen inadvertently, freezing is not a common commercial practice. However, concentrated or unconcentrated milks are sometimes frozen commercially, e.g. to supply remote locations (as an alternative to dried or UHT milk), to store sheep's or goats' milk, production of which is seasonal, or human milk for infant feeding in emergencies (milk banks).

As will be discussed in Chapter 3, freezing damages the milk fat globule membrane, resulting in the release of 'free fat'. The casein system is also destabilized due to a decrease in pH and an increase in Ca^{2+} concentration, both caused by the precipitation of soluble CaH_2PO_4 and/or Ca_2HPO_4 as $Ca_3(PO_4)_2$, with the release of H^+ (Chapter 5); precipitation of $Ca_3(PO_4)_2$ occurs on freezing because pure water crystallizes, causing an increase in soluble calcium phosphate with which milk is already saturated. Crystallization of lactose as α-hydrate during frozen storage aggravates the problem by reducing the amount of solvent water available.

In frozen milk products, lactose crystallization causes instability of the casein system. On freezing, supersaturated solutions of lactose are formed: e.g. in concentrated milk at $-8°C$, 25% of the water is unfrozen and contains 80 g lactose per 100 g, whereas the solubility of lactose at $-8°C$ is only about 7%. During storage at low temperatures, lactose crystallizes slowly as a monohydrate and consequently the amount of free water in the product is reduced.

The formation of supersaturated lactose solutions inhibits freezing, and consequently stabilizes the concentration of solutes in solution. However, when lactose crystallizes, water freezes and the concentration of other solutes increases markedly (Table 2.4).

Table 2.4 Comparison of ultrafiltrate from liquid and frozen skim milk

Constituent	Ultrafiltrate of skim milk	Ultrafiltrate of liquid portion of frozen concentrated milk
pH	6.7	5.8
Chloride (mM)	34.9	459
Citrate (mM)	8.0	89
Phosphate (mM)	10.5	84
Sodium (mM)	19.7	218
Potassium (mM)	38.5	393
Calcium (mM)	9.1	59

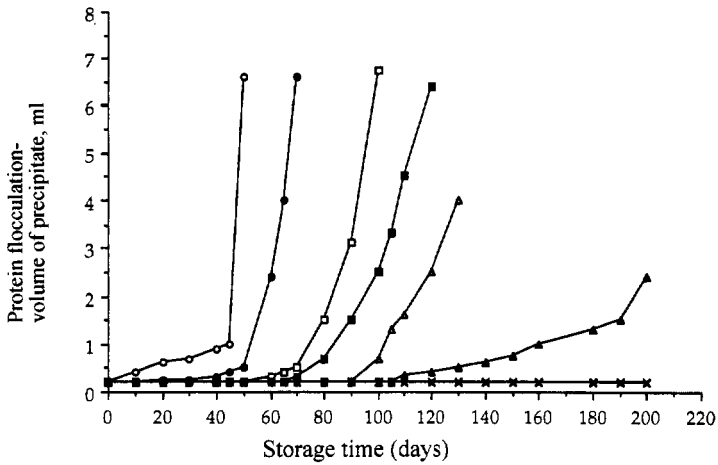


Figure 2.14 Effect of lactose hydrolysis (0, ○; 5, ●; 10, □; 15, ■; 20, △; 30, ▲; 50–85, ×, %) on the stability of milk to freezing (modified from Tumerman, Fram and Cornely, 1954).

The increase in calcium and phosphate leads to precipitation of calcium phosphate and a decrease in pH:

$$3Ca^{2+} + 2H_2PO_4^- \rightleftharpoons Ca_3(PO_4)_2 + 4H^+$$

These changes in the concentration of Ca^{2+} and pH lead to destabilization of the casein micelles.

Any factor that accelerates the crystallization of lactose shortens the storage life of the product. At very low temperatures (below −23°C), neither lactose crystallization nor casein flocculation occurs, even after long periods. Enzymatic hydrolysis of lactose by β-galactosidase before freezing retards or prevents lactose crystallization and casein precipitation in proportion to the extent of the hydrolysis (Figure 2.14).

2.3 Production of lactose

In comparison with sucrose (the annual production of which is 93×10^6 tonnes) and glucose or glucose–fructose syrups, only relatively small quantities of lactose are produced. However, it attracts commercial interest because it has some interesting properties and is readily available from whey, a by-product in the production of cheese or casein. World production of cheese is *c.* 1.4×10^7 tonnes, the whey from which contains *c.* 6×10^6 tonnes of lactose; *c.* 0.3×10^6 tonnes of lactose are contained in the whey produced during casein manufacture. According to Horton (1993),

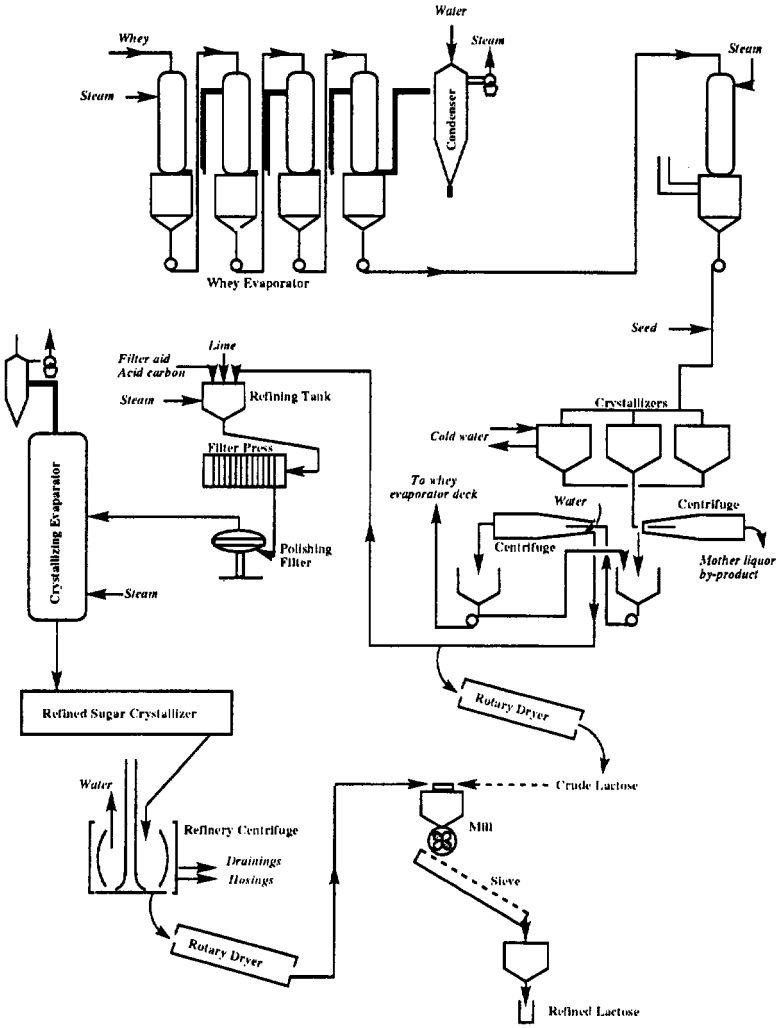


Figure 2.15 Schematic representation of plant for the manufacture of crude and refined lactose, from sweet whey.

only about 420 000 tonnes of lactose are produced annually, i.e. only about 7% of that potentially available.

Production of lactose essentially involves concentrating whey or ultrafiltration permeate by vacuum concentration, crystallization of lactose from the concentrate, recovery of the crystals by centrifugation and drying of the crystals (Figure 2.15). The first-crop crystals are usually contaminated with riboflavin and are therefore yellowish; a higher grade, and hence more

Table 2.5 Some typical physical and chemical data for various grades of lactose[a] (from Nickerson, 1974)

Analysis	Fermentation	Crude	Edible	USP[a]
Lactose (%)	98.0	98.4	99.0	99.85
Moisture, non-hydrate (%)	0.35	0.3	0.5	0.1
Protein (%)	1.0	0.8	0.1	0.01
Ash (%)	0.45	0.40	0.2	0.03
Lipid (%)	0.2	0.1	0.1	0.001
Acidity, as lactic acid (%)	0.4	0.4	0.06	0.04
Specific rotation $[\alpha]_D^{25}$	[b]	[b]	52.4°	52.4°

[a]USP, US Pharmacopoeia grade.
[b]Not normally determined.

Table 2.6 Food applications of lactose

Humanized baby foods
Determineralized whey powder or lactose
Instantizing/free-flowing agent in foods
Agglomeration due to lactose crystallization
Confectionery products
Improves functionality of shortenings
Anticaking agent at high relative humidity
Certain types of icing
Maillard browning, if desired
Accentuates other flavours (chocolate)
Flavour adsorbant
Flavour volatiles
Flavour enhancement
Sauces, pickles, salad dressings, pie fillings

Table 2.7 Relative sweetness of sugars (approx. concentration, %, required to give equivalent sweetness) (from Nickerson, 1974)

Sucrose	Glucose	Fructose	Lactose
0.5	0.9	0.4	1.9
1.0	1.8	0.8	3.5
2.0	3.6	1.7	6.5
2.0	3.8	–	6.5
2.0	3.2	–	6.0
5.0	8.3	4.2	15.7
5.0	8.3	4.6	14.9
5.0	7.2	4.5	13.1
10.0	13.9	8.6	25.9
10.0	12.7	8.7	20.7
15.0	17.2	12.8	27.8
15.0	20.0	13.0	34.6
20.0	21.8	16.7	33.3

Table 2.8 Relative humectancy of sucrose, glucose and lactose (% moisture absorbed at 20°C)

	Relative humidity		
	60%		100%
	1 h	9 days	25 days
Sugar	Humectancy		
Lactose	0.54	1.23	1.38
Glucose	0.29	9.00	47.14
Sucrose	0.04	0.03	18.35

valuable, lactose is produced by redissolving and recrystallizing the crude lactose (Table 2.5). Lactose may also be recovered by precipitation with $Ca(OH)_2$, especially in the presence of ethanol, methanol or acetone.

Lactose has several applications in food products (Table 2.6), the most important of which is probably in the manufacture of humanized infant formulae. It is used also as a diluent for the tableting of drugs in the pharmaceutical industry (which requires high-quality, expensive lactose) and as the base for plastics.

Among sugars, lactose has a low level of sweetness (Table 2.7), which is generally a disadvantage but is advantageous in certain applications. When properly crystallized, lactose has low hygroscopicity (Table 2.8), which makes it an attractive sugar for use in icings for confectionary products.

2.4 Derivatives of lactose

Although the demand for lactose has been high in recent years, it is unlikely that a profitable market exists for all the lactose potentially available. Since the disposal of whey or UF permeate by dumping into waterways is no longer permitted, profitable, or at least inexpensive, ways of utilizing lactose have been sought for several years. For many years, the most promising of these was considered to be hydrolysis to glucose and galactose, but other modifications are attracting increasing attention.

2.4.1 Enzymatic modification of lactose

Lactose may be hydrolysed to glucose and galactose by enzymes (β-galactosidases, commonly called lactase) or by acids. Commercial sources of β-galactosidase are moulds (especially *Aspergillus* spp.), the enzymes from which have acid pH optima, and yeasts (*Kluyveromyces* spp.) which produce enzymes with neutral pH optima. β-Galactosidases were considered to have

considerable commercial potential as a solution to the 'whey problem' and for the treatment of lactose intolerance (section 2.6.1). The very extensive literature on various aspects of β-galactosidases and on their application in free or immobilized form has been reviewed by Mahoney (1997). Technological problems in the production of glucose–galactose syrups have been overcome but the process is not commercially successful. Glucose–galactose syrups are not economically competitive with glucose or glucose–fructose syrups produced by hydrolysis of maize starch, unless the latter are heavily taxed. As discussed in section 2.6.1, an estimated 70% of the adult human population have inadequate intestinal β-galactosidase activity and are therefore lactose intolerant; the problem is particularly acute among Asians and Africans. Pre-hydrolysis of lactose was considered to offer the potential to develop new markets for dairy products in those countries. Various protocols are available: addition of β-galactosidase to milk in the home, pre-treatment at the factory with free or immobilized enzyme or aseptic addition of sterilized free β-galactosidase to UHT milk, which appears to be particularly successful. However, the method is not used widely and it is now considered that the treatment of milk with β-galactosidase will be commercially successful only in niche markets.

Glucose–galactose syrups are about three times sweeter than lactose (70% as sweet as sucrose) and hence lactose-hydrolysed milk could be used in the production of ice-cream, yoghurt or other sweetened dairy products, permitting the use of less sucrose and reducing caloric content. However, such applications have not been commercially successful.

The glucose moiety can be isomerized to fructose by the well-established glucose isomerization process to yield a galactose–glucose–fructose syrup with increased sweetness. Another possible variation would involve the isomerization of lactose to lactulose (galactose–fructose) which can be hydrolysed to galactose and fructose by some β-galactosidases.

β-Galactosidase has transferase as well as hydrolase activity and produces oligosaccharides (galacto-oligosaccharides, Figure 2.16) which are later hydrolysed (Figure 2.17). This property may be a disadvantage since the oligosaccharides are not digestible by humans and reach the large intestine where they are fermented by bacteria, leading to the same problem caused by lactose. However, they stimulate the growth of *Bifidobacterium* spp. in the lower intestine; a product (oligonate, 6′-galactosyl lactose) is produced commercially by the Yokult Company in Japan for addition to infant formulae. Some galacto-oligosaccharides have interesting functional properties and may find commercial applications.

2.4.2 Chemical modifications

Lactulose. Lactulose is an epimer of lactose in which the glucose moiety is isomerized to fructose (Figure 2.18). The sugar does not occur naturally and

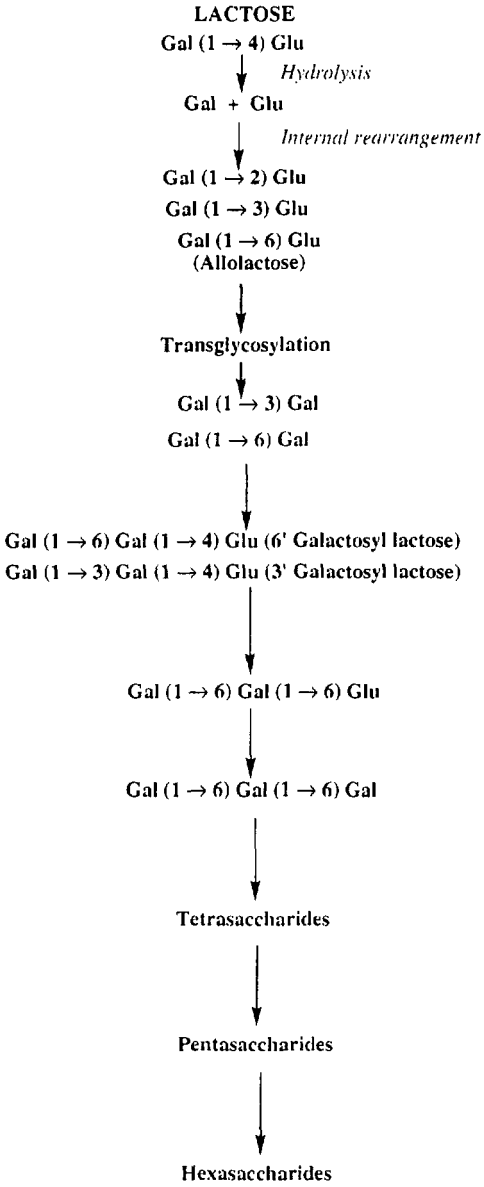


Figure 2.16 Possible reaction products from the action of β-galactosidase on lactose (from Smart, 1993).

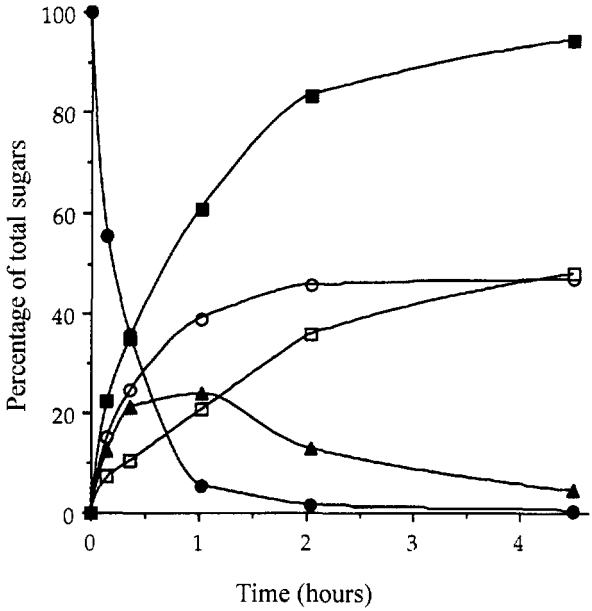


Figure 2.17 Production of oligosaccharides during the hydrolysis of lactose by β-galactosidase; ●, lactose; ■, monosaccharides; ○, glucose; ▲, oligosaccharides; □, galactose (modified from Mahoney, 1997).

was first synthesized by Montgomery and Hudson in 1930. It can be produced under mild alkaline conditions via the Lobry de Bruyn–Alberda van Ekenstein reaction and at a low yield as a by-product of β-galactosidase action on lactose. It is produced on heating milk to sterilizing conditions and is a commonly used index of the severity of the heat treatment to which milk has been subjected, e.g. to differentiate in-container sterilized milk from UHT (ultra-high temperature) milk (Figure 2.19); it is not present in raw or HTST (high temperature short time) pasteurized milk.

Lactulose is sweeter than lactose and 48–62% as sweet as sucrose. It is not metabolized by oral bacteria and hence is not cariogenic. It is not hydrolysed by intestinal β-galactosidase and hence reaches the large intestine where it can be metabolized by lactic acid bacteria, including *Bifidobacterium* spp. and serves as a bifidus factor. For this reason, lactulose has attracted considerable attention as a means of modifying the intestinal microflora, reducing intestinal pH and preventing the growth of undesirable putrefactive bacteria (Figures 2.20–2.22). It is now commonly added to infant formulae to simulate the bifidogenic properties of human milk – apparently, 20 000 tonnes annum^{-1} are now used for this and similar applications. Lactulose is also reported to suppress the growth of certain tumour cells (Figure 2.23).

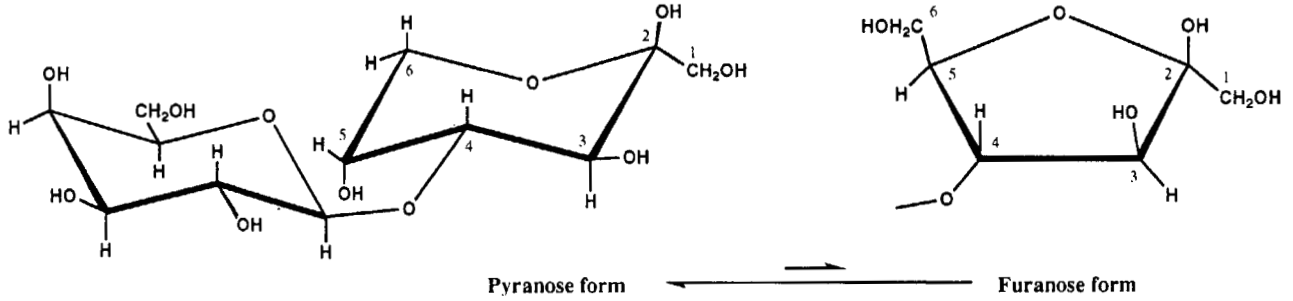


Figure 2.18 Chemical structure of lactulose.

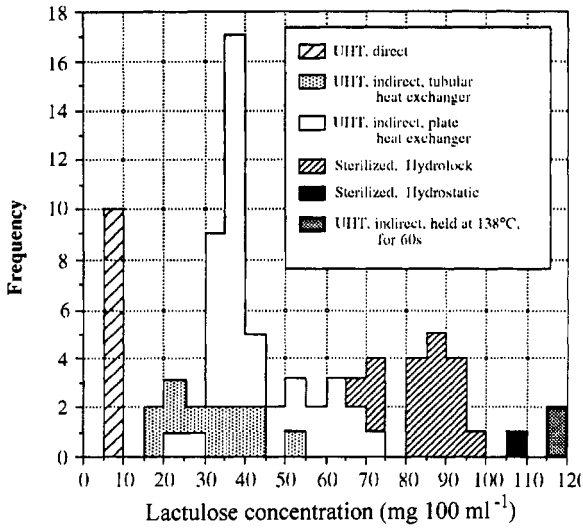


Figure 2.19 Concentration of lactulose in heated milk products (modified from Andrews, 1989).

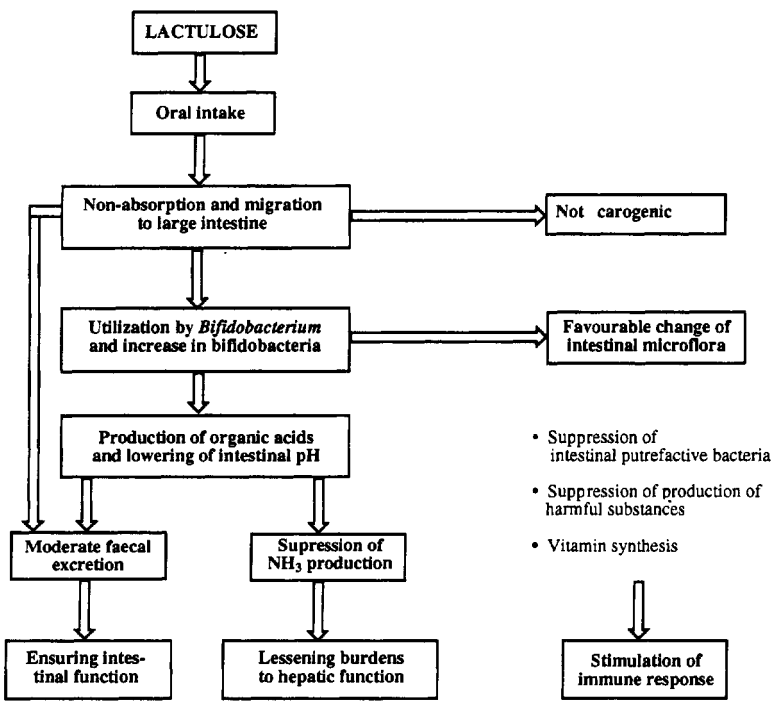


Figure 2.20 Significance of lactulose in health (modified from Tamura *et al.*, 1993).

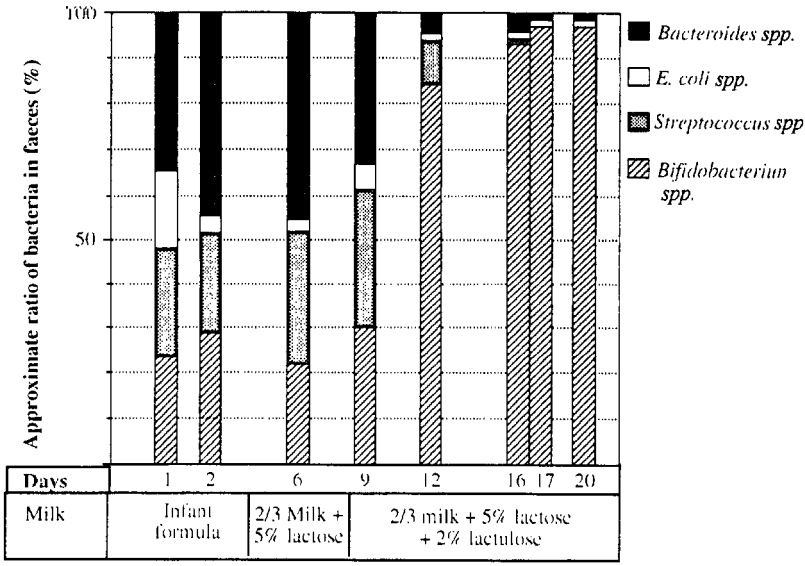


Figure 2.21 Effect of lactulose on the intestinal microflora of 2-month-old infants (modified from Tamura *et al.*, 1993).

Lactulose is usually used as a 50% syrup but a crystalline trihydrate, which has very low hygroscopicity, is now available.

Lactitol. Lactitol (4-O-β-D-galactopyranosyl-D-sorbitol), is a synthetic sugar alcohol produced on reduction of lactose, usually using Raney nickel. It can be crystallized as a mono- or di-hydrate. Lactitol is not metabolized by higher animals; it is relatively sweet and hence has potential as a non-nutritive sweetener. It is claimed that lactitol reduces the absorption of sucrose, blood and liver cholesterol levels and to be anticariogenic. It has applications in low-calorie foods (jams, marmalade, chocolate, baked goods); it is non-hygroscopic and can be used to coat moisture-sensitive foods, e.g. sweets.

It can be esterified with one or more fatty acids (Figure 2.24) to yield a family of food emulsifiers, analogous to the sorbitans produced from sorbitol.

Lactobionic acid. This derivative is produced by oxidation of the free carbonyl group of lactose (Figure 2.25), chemically (Pt, Pd or Bi), electrolytically, enzymatically or by fermentation. Its lactone crystallizes readily. Lactobionic acid has found only limited application; its lactone could be used as an acidogen but it is probably not cost-competitive with gluconic acid-δ-lactone. It is used in preservation solutions for organs prior to transplants.

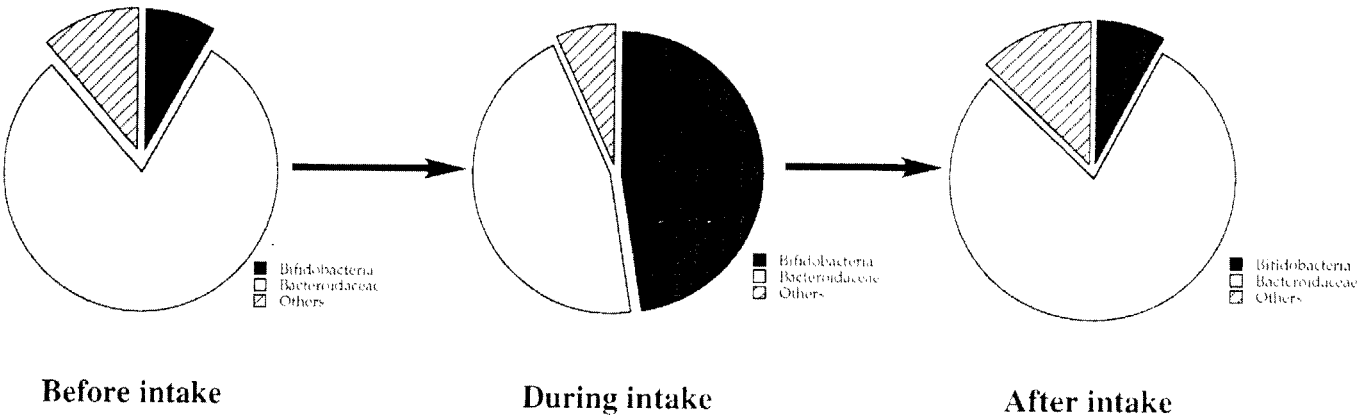


Figure 2.22 Increase in *Bifidobacterium* spp. by administration of lactulose to healthy adults (modified from Tamura *et al.*, 1993).

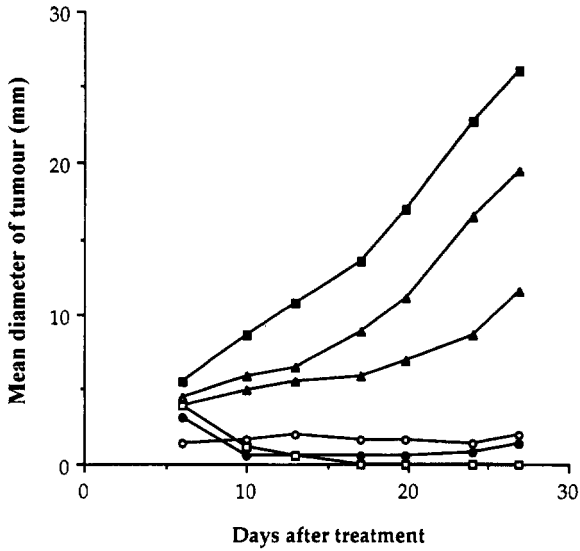
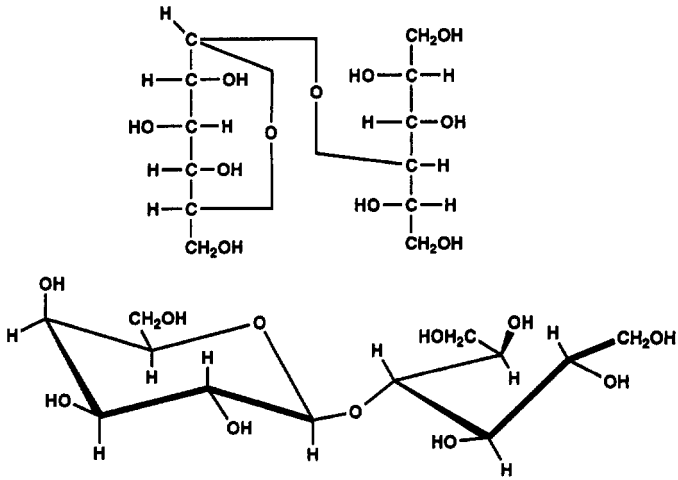


Figure 2.23 Effect of different doses of whole peptidoglycan (WPG) from *Bifidobacterium infantis* on the growth rate of Meth A tumour. Mice were inoculated subcutaneously with a mixture of 10^5 Meth A cells and 0 (■), 10 (△), 20 (▲), 25 (○), 50 (●) or 100 (□) μg of WPG. (Modified from Tamura *et al.*, 1993.)

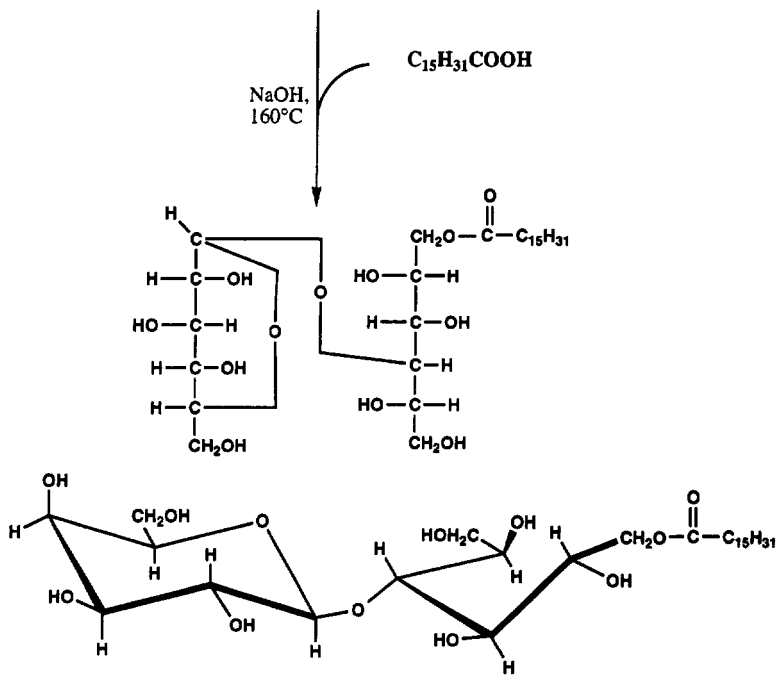
Lactosyl urea. Urea can serve as a cheap source of nitrogen for cattle but its use is limited because NH_3 is released too quickly, leading to toxic levels of NH_3 in the blood. Reaction of urea with lactose yields lactosyl urea (Figure 2.26), from which NH_3 is released more slowly.

2.4.3 Fermentation products

Lactose is readily fermented by lactic acid bacteria, especially *Lactococcus* spp. and *Lactobacillus* spp., to lactic acid, and by some species of yeast, e.g. *Kluyveromyces* spp., to ethanol (Figure 2.27). Lactic acid may be used as a food acidulant, as a component in the manufacture of plastics, or converted to ammonium lactate as a source of nitrogen for animal nutrition. It can be converted to propionic acid, which has many food applications, by *Propionibacterium* spp. Potable ethanol is being produced commercially from lactose in whey or UF permeate. The ethanol may also be used for industrial purposes or as a fuel but is probably not cost-competitive with ethanol produced by fermentation of sucrose or chemically. The ethanol may also be oxidized to acetic acid. The mother liquor remaining from the production of lactic acid or ethanol may be subjected to anaerobic digestion with the production of methane (CH_4) for use as a fuel; several such plants are in commercial use.



Lactitol, 4-O-β-D-galactopyranosyl-D-sorbitol



Lactitol monoester

Figure 2.24 Structure of lactitol and its conversion to lactyl palmitate.

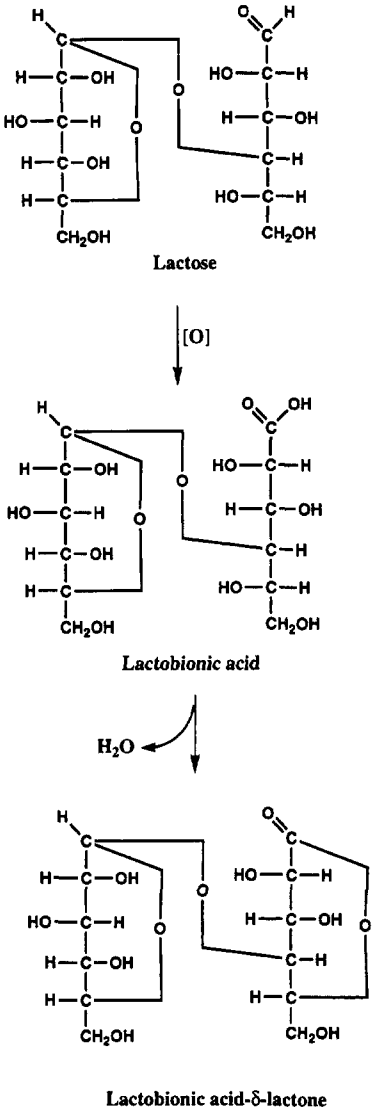


Figure 2.25 Structure of lactobionic acid and its δ-lactone.

Lactose can also be used as a substrate for *Xanthomonas campestris* in the production of xanthan gum (Figure 2.28) which has several food and industrial applications.

All the fermentation-based modifications of lactose are probably not really economical because lactose is not cost-competitive with alternative

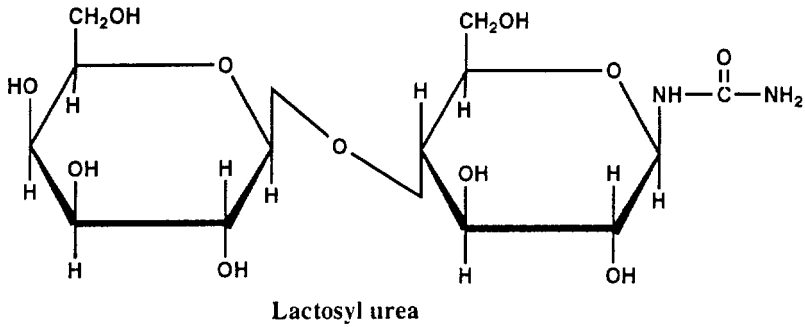


Figure 2.26 Structure of lactosyl urea.

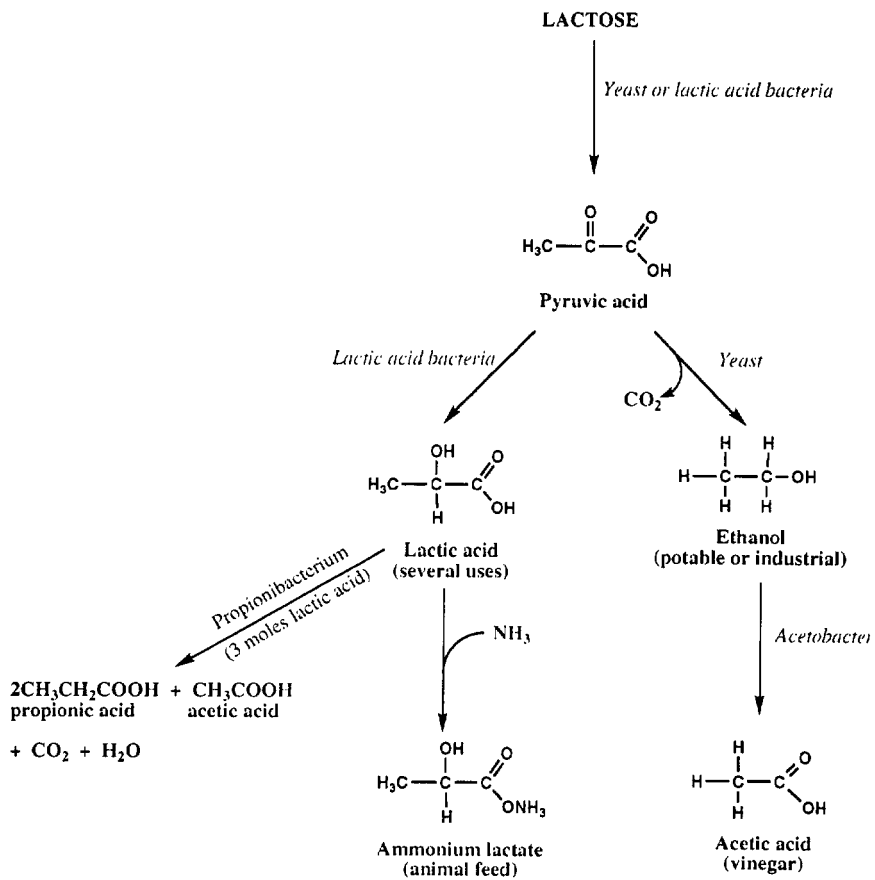


Figure 2.27 Fermentation products from lactose.

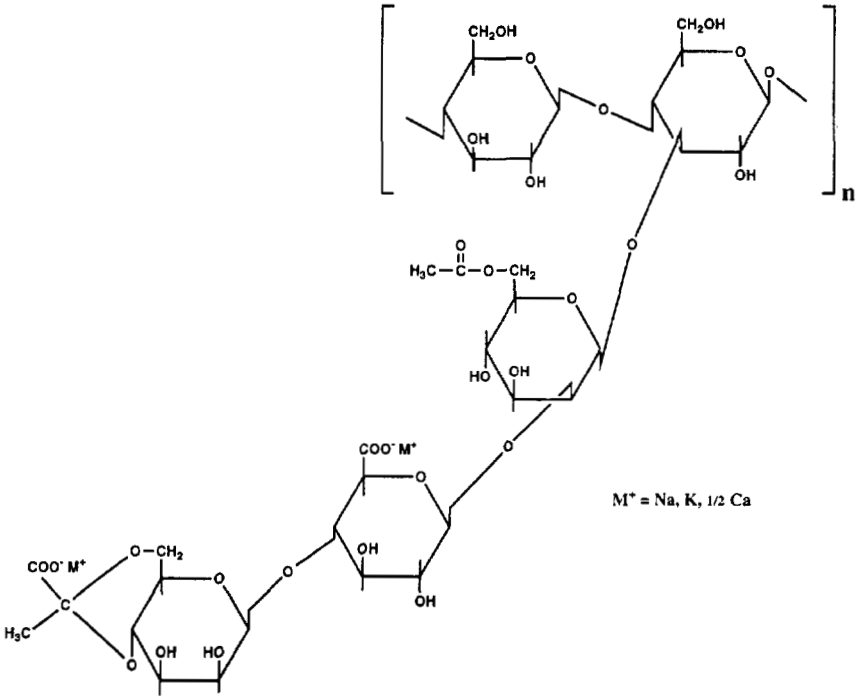


Figure 2.28 Repeating unit of xanthan gum.

fermentation substrates, especially sucrose in molasses or glucose produced from starch. Except in special circumstances, the processes can be regarded as the cheapest method of whey disposal.

2.5 Lactose and the Maillard reaction

As a reducing sugar, lactose can participate in the Maillard reaction, leading to non-enzymatic browning. The Maillard reaction involves interaction between a carbonyl (in this case, lactose) and an amino group (in foods, principally the ε-NH_2 group of lysine in proteins) to form a glycosamine (lactosamine) (Figure 2.29). The glycosamine may undergo an Amadori rearrangement to form a 1-amino-2-keto sugar (Amadori compound) (Figure 2.30). The reaction is base-catalysed and is probably first order. While the Maillard reaction has desirable consequences in many foods, e.g. coffee, bread crust, toast, french fried potato products, its consequences in milk products are negative, e.g. brown colour, off-flavours, slight loss of nutritive value (lysine), loss of solubility in milk powders (although it appears to

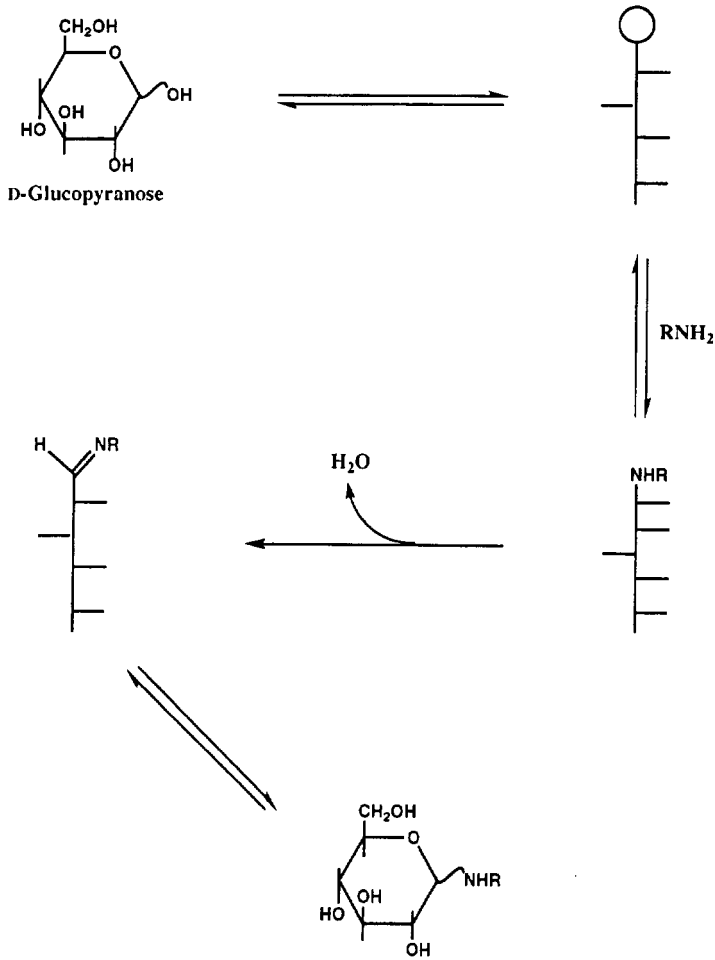


Figure 2.29 Formation of glycosylamine, the initial step in Maillard browning.

prevent or retard age-gelation in UHT milk products). Maillard reaction products (MRPs) have antioxidant properties; the production of MRPs may be a small-volume outlet for lactose.

The Amadori compound may be degraded via either of two pathways, depending on pH, to a variety of active alcohol, carbonyl and dicarbonyl compounds and ultimately to brown-coloured polymers called melanoidins (Figure 2.31). Many of the intermediates are (off-) flavoured. The dicarbonyls can react with amino acids via the Strecker degradation pathway (Figure 2.32) to yield another family of highly flavoured compounds.

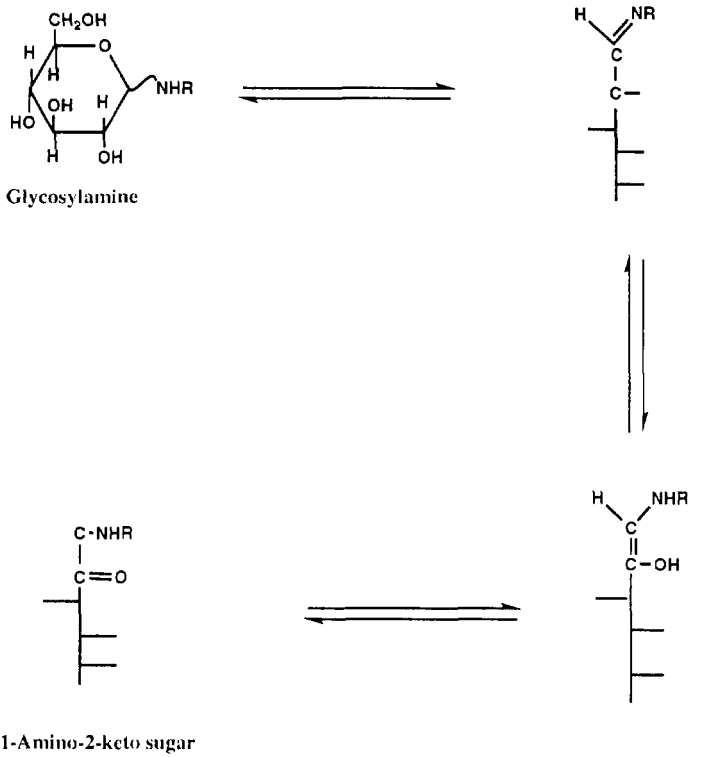


Figure 2.30 Amadori rearrangement of a glycosylamine.

2.6 Nutritional aspects of lactose

Since the milks of most mammals contain lactose, it is reasonable to assume that it or its constituent monosaccharides have some nutritional significance. The secretion of a disaccharide rather than a monosaccharide in milk is advantageous since twice as much energy can be provided for a given osmotic pressure. Galactose may be important because it or its derivatives, e.g. galactosamine, are constituents of several glycoproteins and glycolipids, which are important constituents of cell membranes; young mammals have a limited capacity to synthesize galactose.

Lactose appears to promote the absorption of calcium but this is probably due to a nonspecific increase in intestinal osmotic pressure, an effect common to many sugars and other carbohydrates, rather than a specific effect of lactose.

However, lactose has two major nutritionally undesirable consequences – lactose intolerance and galactosaemia. Lactose intolerance is caused by an insufficiency of intestinal β-galactosidase – lactose is not completely

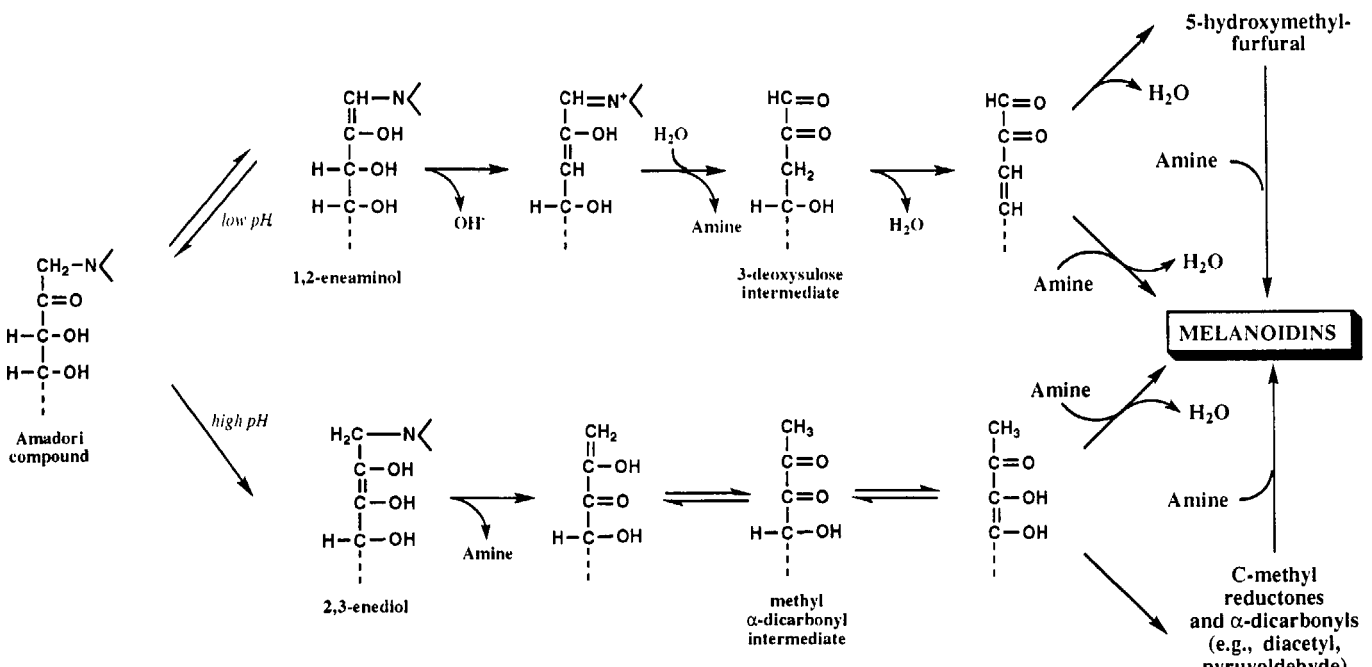


Figure 2.31 Pathways for the Maillard browning reaction.

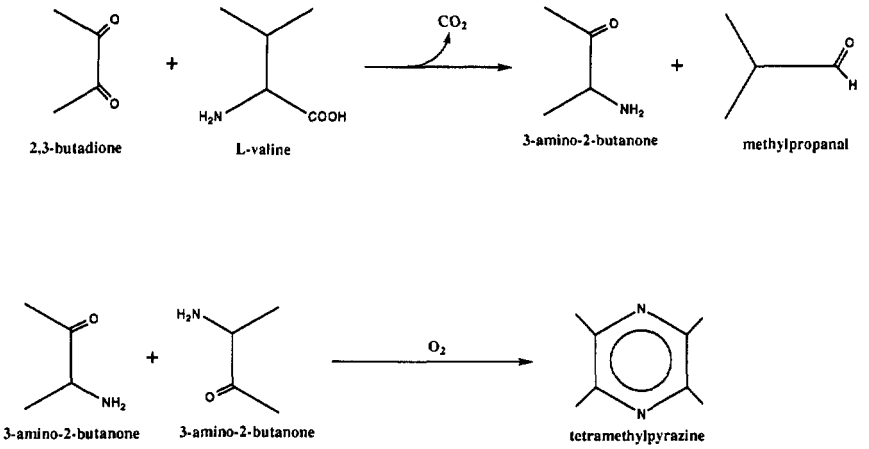


Figure 2.32 Strecker degradation of L-valine by reaction with 2,3-butadione.

hydrolysed, or not hydrolysed at all, in the small intestine and, since disaccharides are not absorbed, it passes into the large intestine where it causes an influx of water, causing diarrhoea, and is fermented by intestinal micro-organisms, causing cramping and flatulence.

2.6.1 Lactose intolerance

A small proportion of babies are born with a deficiency of β-galactosidase (inborn error of metabolism) and are unable to digest lactose from birth. In normal infants (and other neonatal mammals), the specific activity of intestinal β-galactosidase increases to a maximum at parturition (Figure 2.33), although total activity continues to increase for some time postpartum due to increasing intestinal area. However, in late childhood, total activity decreases and, in an estimated 70% of the world's population, decreases to a level which causes lactose intolerance among adults. Only northern Europeans and a few African tribes, e.g. Fulami, can consume milk with impunity; the inability to consume lactose appears to be the normal pattern in humans and other species, and the ability of northern Europeans to do so presumably reflects positive selective pressure for the ability to consume milk as a source of calcium (better bone development).

Lactose intolerance can be diagnosed by (1) jujunal biopsy, with assay for β-galactosidase, or (2) administration of an oral dose of lactose followed by monitoring blood glucose levels or pulmonary hydrogen levels. A test dose of 50 g lactose in water (equivalent to 1 litre of milk) is normally administered to a fasting patient; the dose is rather excessive and gastric

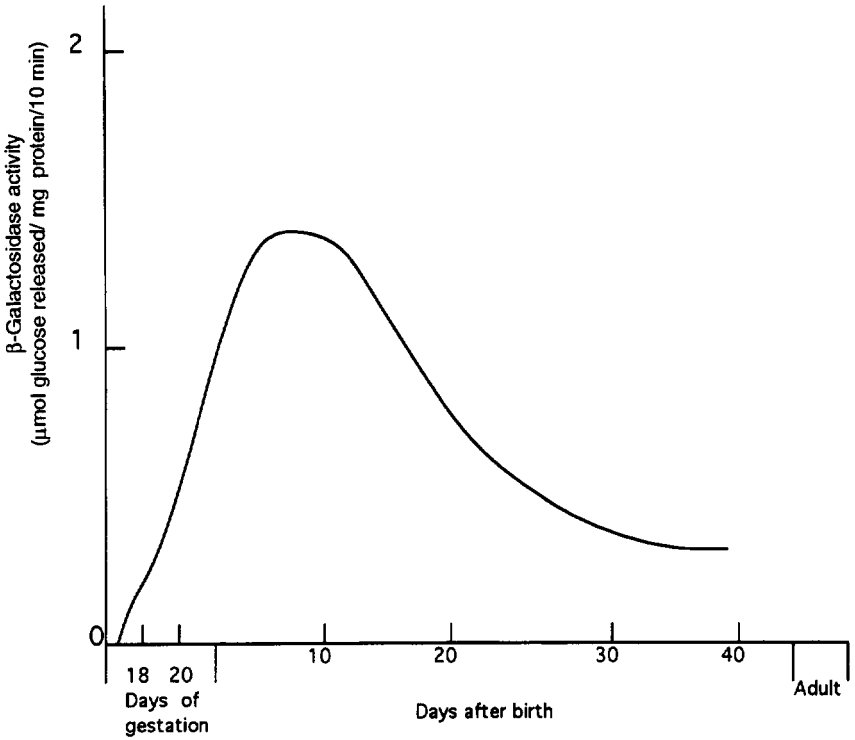


Figure 2.33 β-Galactosidase activity in homogenates from the intestine of the developing rat.

emptying is faster for a fasted than a fed subject – the presence of other constituents in the meal will delay gastric emptying. Blood glucose level will increase in a lactose-tolerant subject shortly after consuming lactose or a lactose-containing product but not if the subject has a deficiency of β-galactosidase (Figure 2.34). Pulmonary H_2 increases in lactose-intolerant subjects because lactose is metabolized by bacteria in the large intestine, with the production of H_2, which is absorbed and exhaled through the lungs.

Milk can be suitably modified for lactose-intolerant subjects by:

- ultrafiltration, which also removes valuable minerals and vitamins, and is therefore not recommended;
- fermentation to yoghurt or other fermented products in which *c.* 25% of the lactose is metabolized, and which contains bacterial β-galactosidase and is also discharged more slowly from the stomach due to its texture;
- conversion to cheese, which is essentially free of lactose;

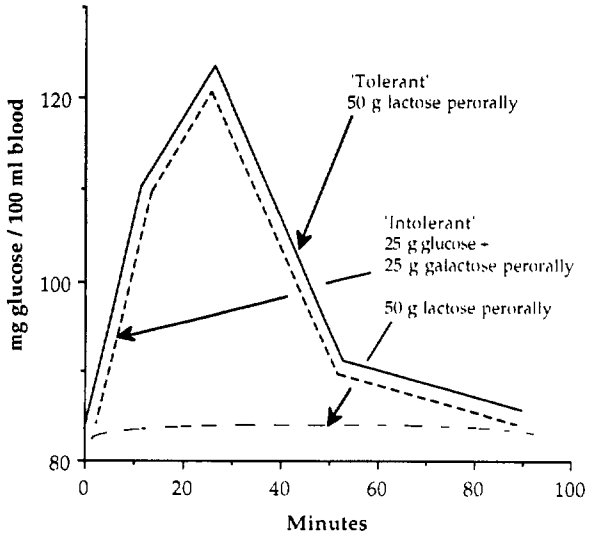


Figure 2.34 Examples of the 'lactose intolerance' test.

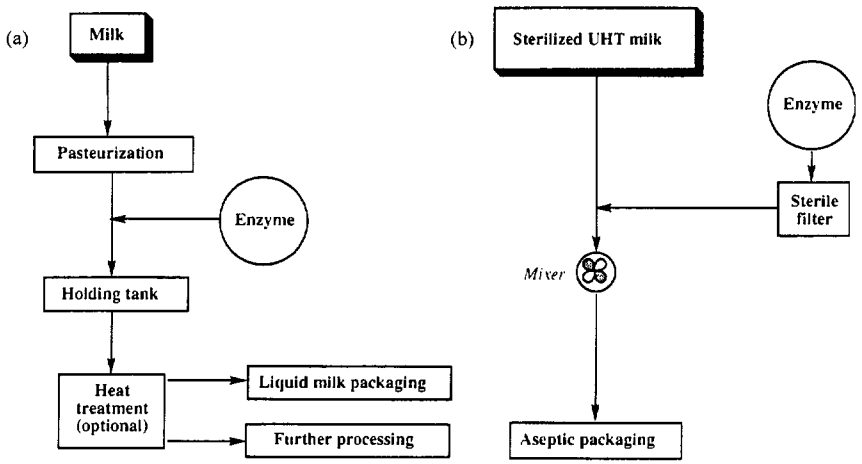


Figure 2.35 (a) Scheme for manufacture of low-lactose milk using a 'high' level of soluble β-galactosidase. (b) Scheme for the manufacture of low-lactose milk by addition of a low level of soluble β-galactosidase to UHT-sterilized milk. (Redrawn from Mahoney, 1997.)

- treatment with exogenous β-galactosidase, either domestically by the consumer or the dairy factory, using free or immobilized enzyme; several protocols for treatment have been developed (Figure 2.35).

Lactose-hydrolysed milks are technologically successful and commercially available but have not led to large increases in the consumption of

milk in countries where lactose intolerance is widespread, presumably due to cultural and economic factors. However, there are niche markets for such products.

2.6.2 *Galactosaemia*

This is caused by the inability to metabolize galactose due to a hereditary deficiency of galactokinase or galactose-1-phosphate (Gal-1-P) : uridyl transferase (Figure 2.36). Lack of the former enzyme leads to the accumulation of galactose which is metabolized via other pathways, leading, among other products, to galactitol which accumulates in the lens of the eye, causing cataract in 10–20 years (in humans) if consumption of galactose-containing foods (milk, legumes) is continued. The incidence is about 1 : 40 000. The

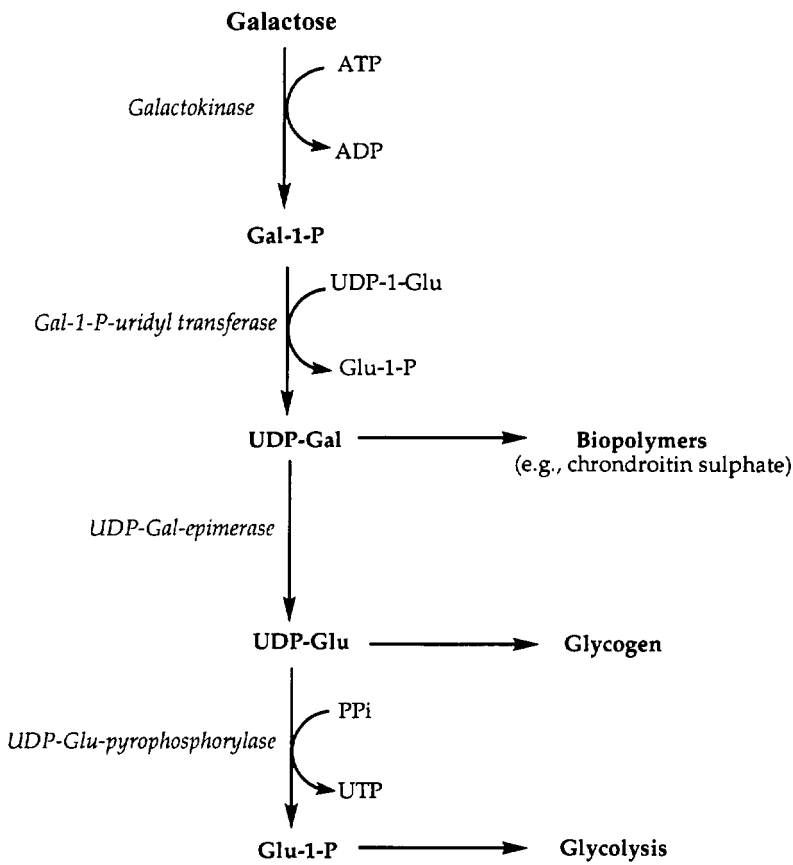


Figure 2.36 Pathways for the metabolism of galactose.

lack of Gal-1-P : uridyl transferase leads to the accumulation of Gal and Gal-1-P. The latter interferes with the synthesis of glycoproteins and glycolipids (important for membranes, e.g. in the brain) and results in irreversible mental retardation within 2–3 months if the consumption of galactose-containing foods is continued. The incidence of this disease, often called 'classical galactosaemia', is about 1 in 60 000.

The ability to metabolize galactose decreases on ageing (after 70 years), leading to cataract; perhaps this, together with the fact that mammals normally encounter lactose only while suckling, explains why many people lose the ability to utilize lactose at the end of childhood.

2.7 Determination of lactose concentration

Lactose may be quantified by methods based on one of five principles:

1. polarimetry;
2. oxidation–reduction titration;
3. colorimetry;
4. chromatography;
5. enzymatically.

2.7.1 Polarimetry

The specific rotation, $[\alpha]_D^{20}$, of lactose in solution at equilibrium is $+55.4°$ expressed on an anhydrous basis ($+52.6°$ on a monohydrate basis). The specific rotation is defined as the optical rotation of a solution containing 1 g ml^{-1} in a 1 dm polarimeter tube; it is affected by temperature (20°C is usually used; indicated by superscript) and wavelength (usually the sodium D line (589.3 nm) is used; indicated by subscript).

$$[\alpha]_D^{20} = a/lc$$

where a is the measured optical rotation; l, the light path in dm; and c, the concentration as g ml^{-1}. It is usually expressed as:

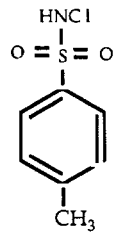
$$[\alpha]_D^{20} = 100\,a/lc$$

where c is in g per100 ml.

The milk sample must first be defatted and deproteinated, usually by treatment with mercuric nitrate ($Hg(NO_3)_2$). In calculating the concentration of lactose, a correction should be used for the concentration of fat and protein in the precipitate.

2.7.2 *Oxidation and reduction titration*

Lactose is a reducing sugar, i.e. it is capable of reducing appropriate oxidizing agents, two of which are usually used, i.e. alkaline copper sulphate ($CuSO_4$ in sodium potassium tartrate; Fehling's solution) or chloroamine-T (**2.1**).



Chloroamine-T

(2.1)

For analysis by titration with Fehling's solution, the sample is treated with lead acetate to precipitate protein and fat, filtered, and the filtrate titrated with alkaline $CuSO_4$, while heating. The reactions involved are summarized in Figure 2.37.

Cu_2O precipitates and may be recovered by filtration and weighed; the concentration of lactose can then be calculated since the oxidation of one mole of lactose (360 g) yields one mole of Cu_2O (143 g). However, it is more convenient to add an excess of a standard solution of $CuSO_4$ to the lactose-containing solution. The solution is cooled and the excess $CuSO_4$ determined by reaction with KI and titrating the liberated I_2 with standard sodium thiosulphate ($Na_2S_2O_3$) using starch as an indicator.

$$2CuSO_4 + 4KI \rightarrow CuI_2 + 2K_2SO_4 + I_2$$

$$I_2 + 2Na_2S_2O_3 \rightarrow 2NaI + Na_2S_2O_6$$

The end point in the Fehling's is not sharp and the redox determination of lactose is now usually performed using chloramine-T rather than $CuSO_4$ as oxidizing agent.

The reactions involved are as follows:

$$CH_3C_6H_4SO_2NClH + H_2O + KI \text{ (excess)}$$

$$\rightleftarrows CH_3H_6H_4SO_2NH_2 + HCl + KIO \text{ (K hypoiodate)}$$

$$KIO + \text{lactose } (-CHO) \rightarrow KI + \text{lactobionic acid } (-COOH)$$

$$KI + KIO \rightarrow 2KOH + I_2$$

The I_2 is titrated with standard $Na_2S_4O_6$ (sodium thiosulphate):

$$I_2 + 2Na_2S_2O_3 \rightarrow 2NaI + Na_2S_4O_6$$

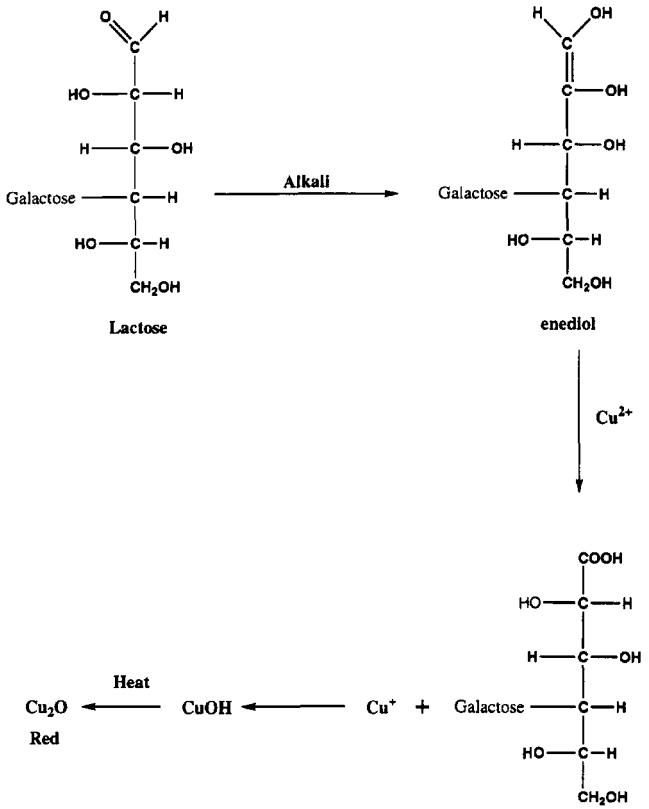


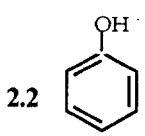
Figure 2.37 Oxidation of lactose by alkaline copper sulphate (Fehling's reagent).

One millilitre of 0.04 N thiosulphate is equivalent to 0.0072 g lactose monohydrate or 0.0064 g anhydrous lactose.

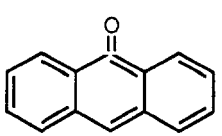
The sample is deproteinized and defatted using phosphotungstic acid.

2.7.3 Colorimetric methods

Reducing sugars, including lactose, react on boiling with phenol (**2.2**) or anthrone (**2.3**) in strongly acidic solution (70%, v/v, H_2SO_4) to give a coloured solution.



2.2 Phenol



2.3 Anthrone

The complex with anthrone absorbs maximally at 625 nm. The concentration of lactose is determined from a standard curve prepared using a range of lactose concentrations.

The method is very sensitive but must be performed under precisely controlled conditions.

2.7.4 Chromatographic methods

While lactose may be determined by gas liquid chromatography, high performance liquid chromatography (HPLC), using a refractive index detector, is now usually used.

2.7.5 Enzymatic methods

Enzymatic methods are very sensitive but are rather expensive, especially for a small number of samples.

Lactose is first hydrolysed by β-galactosidase to glucose and galactose. The glucose may be quantified using:

1. glucose oxidase using a platinum electrode, or the H_2O_2 generated may be quantified by using a peroxidase and a suitable dye acceptor; or
2. glucose-6-phosphate dehydrogenase (G-6-P-DH)

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-P} + \text{ADP}$$

$$\xrightarrow{\text{G-6-P-DH, NADP}^+} \text{Gluconate-6-P} + \text{NADPH} + \text{H}^+$$

The concentration of NADPH produced may be quantified by measuring the increase in absorbance at 334, 340 or 365 nm.

Alternatively, the galactose produced may be quantified using galactose dehydrogenase (Gal-DH):

$$\text{D-galactose} + \text{NAD}^+ \xrightarrow{\text{Gal-DH}} \text{Galactonic acid} + \text{NADH} + \text{H}^+$$

The NADH produced may be quantified by measuring the increase in absorbance at 334, 340 or 365 nm.

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3 Milk lipids

3.1 Introduction

The milks of all mammals contain lipids but the concentration varies widely between species from *c.* 2% to greater than 50% (Table 3.1). The principal function of dietary lipids is to serve as a source of energy for the neonate and the fat content in milk largely reflects the energy requirements of the species, e.g. land animals indigenous to cold environments and marine mammals secrete high levels of lipids in their milks.

Milk lipids are also important:

1. as a source of essential fatty acids (i.e. fatty acids which cannot be synthesized by higher animals, especially linoleic acid, $C_{18:2}$) and fat-soluble vitamins (A, D, E, K); and
2. for the flavour and rheological properties of dairy products and foods in which they are used.

Because of its wide range of fatty acids, the flavour of milk fat is superior to that of other fats. In certain products and after certain processes, fatty acids serve as precursors of very flavourful compounds such as methyl ketones and lactones. Unfortunately, lipids also serve as precursors of compounds

Table 3.1 The fat content of milks from various species ($g\ l^{-1}$)

Species	Fat content	Species	Fat content
Cow	33–47	Marmoset	77
Buffalo	47	Rabbit	183
Sheep	40–99	Guinea-pig	39
Goat	41–45	Snowshoe hare	71
Musk-ox	109	Muskrat	110
Dall-sheep	32–206	Mink	134
Moose	39–105	Chinchilla	117
Antelope	93	Rat	103
Elephant	85–190	Red kangaroo	9–119
Human	38	Dolphin	62–330
Horse	19	Manatee	55–215
Monkeys	10–51	Pygmy sperm whale	153
Lemurs	8–33	Harp seal	502–532
Pig	68	Bear (four species)	108–331

From Christie (1995).

that cause off-flavour defects (hydrolytic and oxidative rancidity) and as solvents for compounds in the environment which may cause off-flavours.

For many years, the economic value of milk was based mainly or totally on its fat content, which is still true in some cases. This practice was satisfactory when milk was used mainly or solely for butter production. Possibly, the origin of paying for milk on the basis of its fat content, apart from its value for butter production, lies in the fact that relatively simple quantitative analytical methods were developed for fat earlier than for protein or lactose. Because of its economic value, there has long been commercial pressure to increase the yield of milk fat per cow by nutritional or genetic means.

To facilitate the reader, the nomenclature, structure and properties of the principal fatty acids and of the principal lipid classes are summarized in Appendices 3A, 3B and 3C. The structure and properties of the fat-soluble vitamins, A, D, E and K, are discussed in Chapter 6.

3.2 Factors that affect the fat content of bovine milk

Bovine milk typically contains *c.* 3.5% fat but the level varies widely, depending on several factors, including: breed, individuality of the animal, stage of lactation, season, nutritional status, type of feed, health and age of the animal, interval between milkings and the point during milking when the sample is taken.

Of the common European breeds, milk from Jersey cows contains the highest level of fat and that from Holstein/Friesians the lowest (Figure 3.1). The data in Figure 3.1 also show the very wide range of fat content in individual-cow samples.

The fat content of milk decreases during the first 4–6 weeks after parturition and then increases steadily throughout the remainder of lactation, especially toward the end (Figure 3.2). For any particular population, fat content is highest in winter and lowest in summer, due partly to the effect of environmental temperature. Production of creamery (manufacturing) milk in Ireland, New Zealand and parts of Australia is very seasonal; lactational, seasonal and possibly nutritional effects coincide, leading to large seasonal changes in the fat content of milk (Figure 3.3), and also in the levels of protein and lactose.

For any individual animal, fat content decreases slightly during successive lactations, by *c.* 0.2% over a typical productive lifetime (about five lactations). In practice, this factor usually has no overall effect on the fat content of a bulk milk supply because herds normally include cows of various ages. The concentration of fat (and of all other milk-specific constituents) decreases markedly on mastitic infection due to impaired

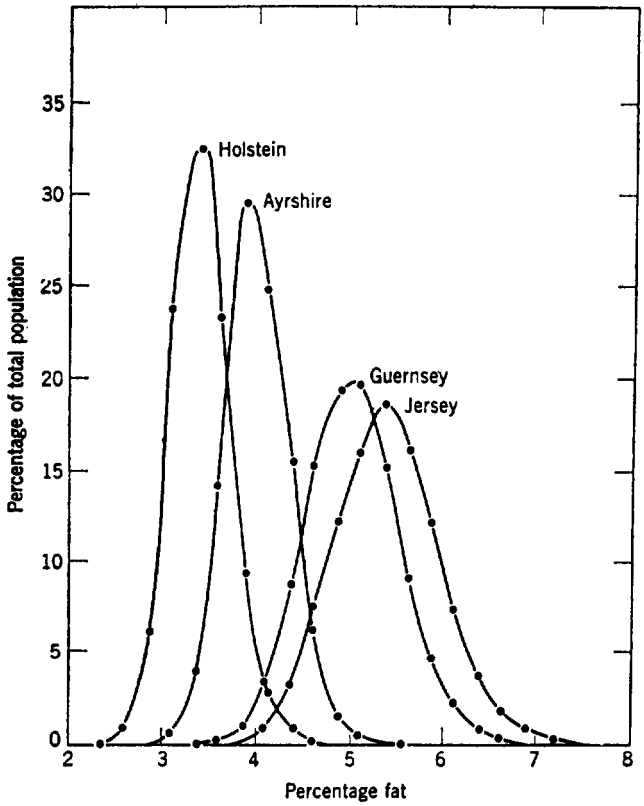


Figure 3.1 Range of fat content in the milk of individual cows of four breeds (from Jenness and Patton, 1959).

synthesizing ability of the mammary tissue; the effect is clear-cut in the case of clinical mastitis but is less so for subclinical infection.

Milk yield is reduced by underfeeding but the concentration of fat usually increases, with little effect on the amount of fat produced. Diets low in roughage have a marked depressing effect on the fat content of milk, with little effect on milk yield. Ruminants synthesize milk fat mainly from carbohydrate-derived precursors; addition of fat to the diet usually causes slight increases in the yield of both milk and fat, with little effect on fat content of milk. Feeding of some fish oils (e.g. cod liver oil, in an effort to increase the concentrations of vitamins A and D in milk) has a very marked (*c.* 25%) depressing effect on the fat content of milk, apparently due to the high level of polyunsaturated fatty acids (the effect is eliminated by hydrogenation), although oils from some fish species do not cause this effect.

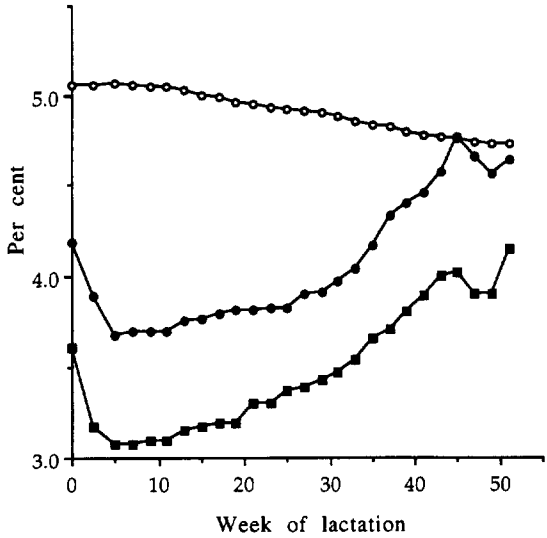


Figure 3.2 Typical changes in the concentrations of fat (●), protein (■) and lactose (○) in bovine milk during lactation.

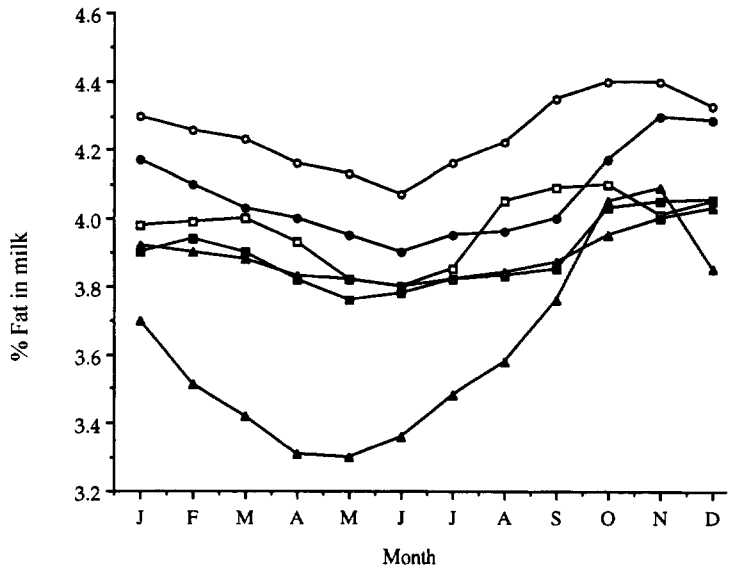


Figure 3.3 Seasonal changes in the fat content of bovine milk in some European countries: (Denmark (○), Netherlands (●), United Kingdom (□), France (■), Germany (△), Ireland (▲) (From An Foras Taluntais, 1981.)

The quarters of a cow's udder are anatomically separate and secrete milk of markedly different composition. The fat content of milk increases continuously throughout the milking process while the concentrations of the various non-fat constituents show no change; fat globules appear to be partially trapped in the alveoli and their passage is hindered. If a cow is incompletely milked, the fat content of the milk obtained at that milking will be reduced; the 'trapped' fat will be expressed at the subsequent milking, giving an artificially high value for fat content.

If the intervals between milkings are unequal (as they usually are in commercial farming), the yield of milk is higher and its fat content lower after the longer interval; the content of non-fat solids is not influenced by milking interval.

3.3 Classes of lipids in milk

Triacylglycerols (triglycerides) represent 97–98% of the total lipids in the milks of most species (Table 3.2). The diglycerides probably represent incompletely synthesized lipids in most cases, although the value for the rat probably also includes partially hydrolysed triglycerides, as indicated by the high concentration of free fatty acids, suggesting damage to the milk fat globule membrane (MFGM) during milking and storage. The very high level of phospholipids in mink milk probably indicates the presence of mammary cell membranes.

Although phospholipids represent less than 1% of total lipid, they play a particularly important role, being present mainly in the MFGM and other membraneous material in milk. The principal phospholipids are phosphatidylcholine, phosphatidylethanolamine and sphingomyelin (Table 3.3). Trace amounts of other polar lipids, including ceramides, cerobrosides and gangliosides, are also present. Phospholipids represent a considerable proportion of the total lipid of buttermilk and skim milk (Table 3.4), reflecting

Table 3.2 Composition of individual simple lipids and total phospholipids in milks of some species (weight % of the total lipids)

Lipid class	Cow	Buffalo	Human	Pig	Rat	Mink
Triacylglycerols	97.5	98.6	98.2	96.8	87.5	81.3
Diacylglycerols	0.36		0.7	0.7	2.9	1.7
Monoacylglycerols	0.027		T	0.1	0.4	T
Cholesteryl esters	T	0.1	T	0.06	–	T
Cholesterol	0.31	0.3	0.25	0.6	1.6	T
Free fatty acids	0.027	0.5	0.4	0.2	3.1	1.3
Phospholipids	0.6	0.5	0.26	1.6	0.7	15.3

From Christie (1995). T, Trace.

Table 3.3 Composition of the phospholipids in milk from various species (expressed as mol % of total lipid phosphorus)

Species	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-serine	Phosphatidyl-inositol	Sphingomyelin	Lysophospho-lipids[a]
Cow	34.5	31.8	3.1	4.7	25.2	0.8
Sheep	29.2	36.0	3.1	3.4	28.3	
Buffalo	27.8	29.6	3.9	4.2	32.1	2.4
Goat	25.7	33.2	6.9	5.6[b]	27.9	0.5
Camel	24.0	35.9	4.9	5.9	28.3	1.0
Ass	26.3	32.1	3.7	3.8	34.1	
Pig	21.6	36.8	3.4	3.3	34.9	
Human	27.9	25.9	5.8	4.2	31.1	5.1
Cat	25.8	22.0	2.7	7.8[b]	37.9	3.4
Rat	38.0	31.6	3.2	4.9	19.2	3.1
Guinea-pig	35.7	38.0	3.2	7.1[b]	11.0	2.0
Rabbit	32.6	30.0	5.2	5.8[b]	24.9	0.4
Mouse[c]	32.8	39.8	10.8	3.6	12.5	
Mink	52.8	10.0	3.6	6.6	15.3	8.3

[a]Mainly lysophosphatidylcholine but also lysophosphatidylethanolamine.
[b]Also contains lysophosphatidylethanolamine.
[c]Analysis of milk fat globule membrane phospholipids.
From Christie (1995).

Table 3.4 Total fat and phospholipid content of some milk products

Product	Total lipid (%, w/v)	Phospholipids (%, w/v)	Phospholipid as %, w/w, of total lipids
Whole milk	3–5	0.02–0.04	0.6–1.0
Cream	10–50	0.07–0.18	0.3–0.4
Butter	81–82	0.14–0.25	0.16–0.29
Butter oil	~100	0.02–0.08	0.02–0.08
Skim milk	0.03–0.1	0.01–0.06	17–30
Buttermilk	2	0.03–0.18	10

the presence of proportionately larger amounts of membrane material in these products.

Cholesterol (Appendix 3C) is the principal sterol in milk (>95% of total sterols); the level (~0.3%, w/w, of total lipids) is low compared with many other foods. Most of the cholesterol is in the free form, with less than 10% as cholesteryl esters. Several other sterols, including steroid hormones, occur at trace levels.

Several hydrocarbons occur in milk in trace amounts. Of these, carotenoids are the most significant. In quantitative terms, carotenes occur at only trace levels in milk (typically ~200 $\mu g\,l^{-1}$) but they contribute 10–50% of the vitamin A activity in milk (Table 3.5) and are responsible for the yellow colour of milk fat. The carotenoid content of milk varies with breed (milk from Channel Island breeds contains 2–3 times as much β-carotene as milk from other breeds) and very markedly with season (Figure 3.4). The latter reflects differences in the carotenoid content of the diet (since they are totally derived from the diet); fresh pasture, especially if it is rich in clover and alfalfa, is much richer in carotenoids than hay or silage (due to oxidation on conservation) or cereal-based concentrates. The higher the carotenoid content of the diet, the more yellow will be the colour of milk and milk fat, e.g. butter from cows on pasture is yellower than that

Table 3.5 Vitamin A activity and β-carotene in milk of different breeds of cows

	Channel Island breeds		Non-Channel Island breeds	
	Summer	Winter	Summer	Winter
Retinol ($\mu l\,l^{-1}$)	649	265	619	412
β-Carotene ($\mu l\,l^{-1}$)	1143	266	315	105
Retinol/β-carotene ratio	0.6	11.0	2.0	4.0
Contribution (%) of β-carotene to vitamin A activity	46.8	33.4	20.3	11.4

Modified from Cremin and Power (1985).

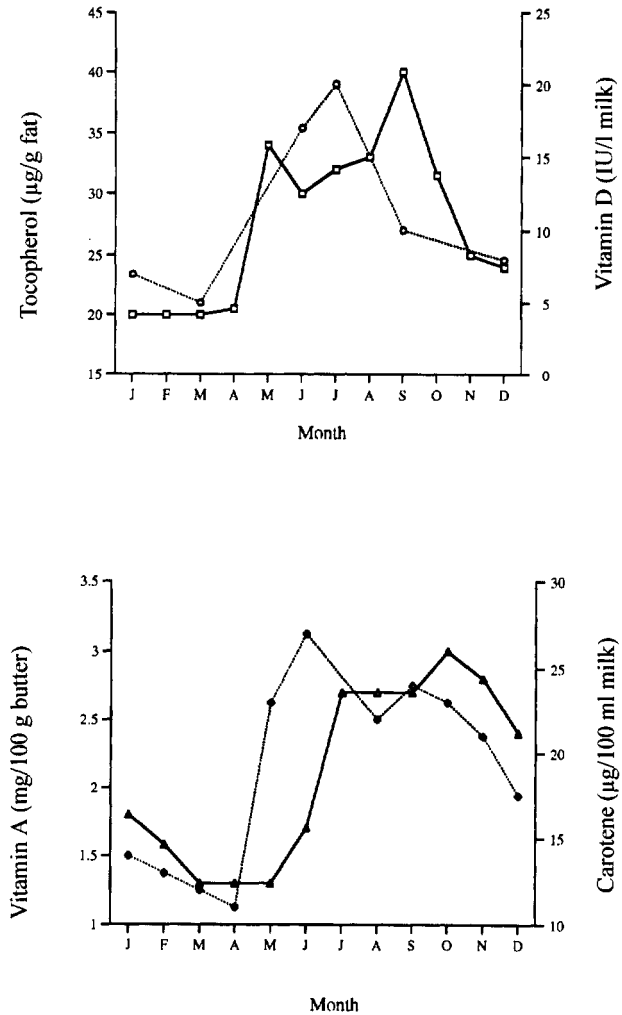


Figure 3.4 Seasonal variations in the concentration of β-carotene (◇) and of vitamins A (△), D (○) and E (□) in milk and milk products (from Cremin and Power, 1985).

from cows on winter feed, especially if the pasture is rich in clover (New Zealand butter is more yellow than Irish butter which in turn is more yellow than mainland European or US butter). Sheep and goats do not transfer carotenoids to their milks which are, consequently, much whiter than bovine milk. This may reduce the acceptability of dairy products (e.g. cheeses, butter, cream, ice-cream) made from bovine milk in regions where goats' or sheep's milk is traditional (the carotenoids may be bleached by using

peroxides, e.g. H_2O_2 or benzoyl peroxide, or masked, e.g. with chlorophyll or titanium oxide).

Milk contains significant concentrations of fat-soluble vitamins (Table 3.5, Figure 3.4) and milk and dairy products make a significant contribution to the dietary requirements for these vitamins in Western countries. The actual form of the fat-soluble vitamins in milk appears to be uncertain and their concentration varies widely with breed of animal, feed and stage of lactation, e.g. the vitamin A activity of colostrum is *c.* 30 times higher than that of mature milk.

Several prostaglandins occur in milk but it is not known whether they play a physiological role; they may not survive storage and processing in a biologically active form. Human milk contains prostaglandins E and F at concentrations 100-fold higher than human plasma and these may have a physiological function, e.g. gut motility.

3.4 Fatty acid profile of milk lipids

Milk fats, especially ruminant fats, contain a very wide range of fatty acids: more than 400 and 184 distinct acids have been detected in bovine and human milk fats, respectively (Christie, 1995). However, the vast majority of these occur at only trace concentrations. The concentrations of the principal fatty acids in milk fats from a range of species are shown in Table 3.6.

Notable features of the fatty acid profiles of milk lipids include:

1. Ruminant milk fats contain a high level of butanoic acid ($C_{4:0}$) and other short-chain fatty acids. The method of expressing the results in Table 3.6 (%, w/w) under-represents the proportion of short-chain acids – if expressed as mol %, butanoic acid represents *c.* 10% of all fatty acids (up to 15% in some samples), i.e. there could be a butyrate residue in *c.* 30% of all triglyceride molecules. The high concentration of butyric (butanoic) acid in ruminant milk fats arises from the direct incorporation of β-hydroxybutyrate (which is produced by micro-organisms in the rumen from carbohydrate and transported via the blood to the mammary gland where it is reduced to butanoic acid). Non-ruminant milk fats contain no butanoic or other short-chain acids; the low concentrations of butyrate in milk fats of some monkeys and the brown bear require confirmation.

   The concentration of butanoic acid in milk fat is the principle of the widely used criterion for the detection and quantitation of adulteration of butter with other fats, i.e. Reichert Meissl and Polenski numbers, which are measures of the volatile water-soluble and volatile water-insoluble fatty acids, respectively.

   Short-chain fatty acids have strong, characteristic flavours and aromas. When these acids are released by the action of lipases in milk or

Table 3.6 Principal fatty acids (wt % of total) in milk triacylglycerols or total lipids from various species

Species	4:0	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	C_{20}–C_{22}
Cow	3.3	1.6	1.3	3.0	3.1	9.5	26.3	2.3	14.6	29.8	2.4	0.8	T
Buffalo	3.6	1.6	1.1	1.9	2.0	8.7	30.4	3.4	10.1	28.7	2.5	2.5	T
Sheep	4.0	2.8	2.7	9.0	5.4	11.8	25.4	3.4	9.0	20.0	2.1	1.4	–
Goat	2.6	2.9	2.7	8.4	3.3	10.3	24.6	2.2	12.5	28.5	2.2	–	–
Musk-ox	T	0.9	1.9	4.7	2.3	6.2	19.5	1.7	23.0	27.2	2.7	3.0	0.4
Dall-sheep	0.6	0.3	0.2	4.9	1.8	10.6	23.0	2.4	15.5	23.1	4.0	4.1	2.6
Moose	0.4	T	8.4	5.5	0.6	2.0	28.4	4.3	4.5	21.2	20.2	3.7	–
Blackbuck antelope	6.7	6.0	2.7	6.5	3.5	11.5	39.3	5.7	5.5	19.2	3.3	–	–
Elephant	7.4	–	0.3	29.4	18.3	5.3	12.6	3.0	0.5	17.3	3.0	0.7	–
Human	–	T	T	1.3	3.1	5.1	20.2	5.7	5.9	46.4	13.0	1.4	T
Monkey (mean of six species	0.4	0.6	5.9	11.0	4.4	2.8	21.4	6.7	4.9	26.0	14.5	1.3	–
Baboon	–	0.4	5.1	7.9	2.3	1.3	16.5	1.2	4.2	22.7	37.6	0.6	–
Lemur macaco	–	–	0.2	1.9	10.5	15.0	27.1	9.6	1.0	25.7	6.6	0.5	–
Horse	–	T	1.8	5.1	6.2	5.7	23.8	7.8	2.3	20.9	14.9	12.6	–
Pig	–	–	–	0.7	0.5	4.0	32.9	11.3	3.5	35.2	11.9	0.7	–
Rat	–	–	1.1	7.0	7.5	8.2	22.6	1.9	6.5	26.7	16.3	0.8	1.1
Guinea-pig	–	T	–	–	–	2.6	31.3	2.4	2.9	33.6	18.4	5.7	T
Marmoset	–	–	–	8.0	8.5	7.7	18.1	5.5	3.4	29.6	10.9	0.9	7.0
Rabbit	–	T	22.4	20.1	2.9	1.7	14.2	2.0	3.8	13.6	14.0	4.4	T
Cottontail rabbit	–	–	9.6	14.3	3.8	2.0	18.7	1.0	3.0	12.7	24.7	9.8	0.4
European hare	–	T	10.9	17.7	5.5	5.3	24.8	5.0	2.9	14.4	10.6	1.7	T
Mink	–	–	–	–	0.5	3.3	26.1	5.2	10.9	36.1	14.9	1.5	–
Chinchilla	–	–	–	–	T	3.0	30.0	–	–	35.2	26.8	2.9	–
Red kangaroo	–	–	–	–	0.1	2.7	31.2	6.8	6.3	37.2	10.4	2.1	0.1
Platypus	–	–	–	–	–	1.6	19.8	13.9	3.9	22.7	5.4	7.6	12.2
Numbat	–	–	–	–	0.1	0.9	14.1	3.4	7.0	57.7	7.9	0.1	0.2
Bottle-nosed dolphin	–	–	–	–	0.3	3.2	21.1	13.3	3.3	23.1	1.2	0.2	17.3
Manatee	–	–	0.6	3.5	4.0	6.3	20.2	11.6	0.5	47.0	1.8	2.2	0.4
Pygmy sperm whale	–	–	–	–	–	3.6	27.6	9.1	7.4	46.6	0.6	0.6	4.5
Harp seal	–	–	–	–	–	5.3	13.6	17.4	4.9	21.5	1.2	0.9	31.2
Northern elephant seal	–	–	–	–	–	2.6	14.2	5.7	3.6	41.6	1.9	–	29.3
Polar bear	–	T	–	T	0.5	3.9	18.5	16.8	13.9	30.1	1.2	0.4	11.3
Grizzly bear	–	T	–	–	0.1	2.7	16.4	3.2	20.4	30.2	5.6	2.3	9.5

From Christie (1995).

dairy products, they impart strong flavours which are undesirable in milk or butter (they cause hydrolytic rancidity) but they contribute to the desirable flavour of some cheeses, e.g. Blue, Romano, Parmesan.

2. Ruminant milk fats contain low levels of polyunsaturated fatty acids (PUFAs) in comparison with monogastric milk fats. This is because a high proportion of the fatty acids in monogastric milk fats are derived from dietary lipids (following digestion and absorption) via blood. Unsaturated fatty acids in the diet of ruminants (grass contains considerable levels of PUFAs) are hydrogenated by rumen micro-organisms unless protected by encapsulation (section 3.16.1). The low level of PUFAs in bovine milk fat is considered to be nutritionally undesirable.
3. The milk fats from marine mammals contain high levels of long-chain, highly unsaturated fatty acids, presumably reflecting the requirement that the lipids of these species remain liquid at the low temperatures of their environments.
4. Ruminant milk fats are also rich in medium-chain fatty acids. These are synthesized in the mammary gland via the usual malonyl CoA pathway (section 3.5) and are released from the synthesizing enzyme complex by thioacylases; presumably, the higher levels of medium chain acids in ruminant milk fats compared with those of monogastric animals reflect higher thioacylase activity in the mammary tissue of the former.
5. The fatty acid profile of bovine milk fat shows a marked seasonal pattern, especially when cows are fed on pasture in summer. Data for Irish milk fat are shown in Figure 3.5; the changes are particularly marked for $C_{4:0}$, $C_{16:0}$ and $C_{18:1}$. These changes affect the Reichert Meissl, Polenski and iodine (a measure of unsaturation) (Figure 3.6) numbers and the melting point and hardness (spreadability) of butter made from these milks: winter butter, with low levels of $C_{4:0}$ and $C_{18:1}$ and a high level of $C_{16:0}$ is much harder than summer butter (Figure 3.7).
6. Unsaturated fatty acids may occur as *cis* or *trans* isomers; *trans* isomers, which have higher melting points than the corresponding *cis* isomers, are considered to be nutritionally undesirable. Bovine milk fat contains a low level (5%) of *trans* fatty acids in comparison with chemically hydrogenated (hardened) vegetable oils, in which the value may be 50% due to non-stereospecific hydrogenation.

Bovine milk fat contains low concentrations of keto and hydroxy acids (each at *c.* 0.3% of total fatty acids). The keto acids may have the carbonyl group (C=O) at various positions. The 3-keto acids give rise to methyl ketones ($R-\overset{\overset{O}{\|}}{C}-CH_3$) on heating (high concentrations of methyl ketones are produced in blue cheeses through the oxidative activity of *Penicillium roqueforti*). The position of the hydroxy group on the hydroxy acids also

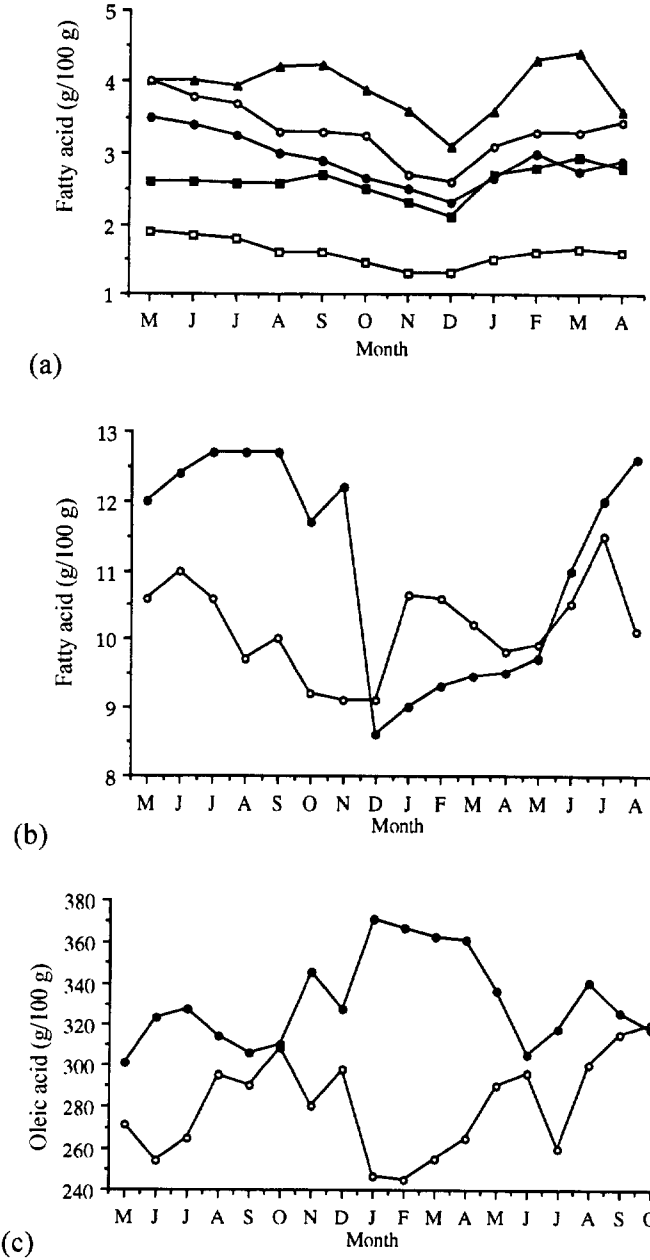


Figure 3.5 Seasonal changes in the concentration of individual fatty acids in Irish bovine milk fat. (a) $C_{4:0}$ (▲), $C_{6:0}$ (■), $C_{8:0}$ (□), $C_{10:0}$ (●), $C_{12:0}$ (○); (b) $C_{14:0}$ (○), $C_{18:0}$ (●); (c) $C_{16:0}$ (●), $C_{18:1}$ (○). From Cullinane *et al.*, 1984a.)

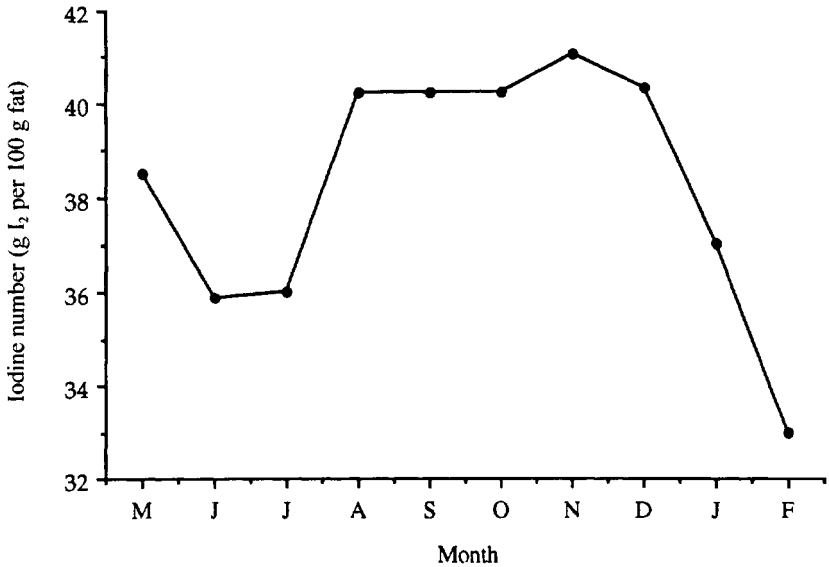


Figure 3.6 Seasonal changes in the iodine number of Irish bovine milk fat (from Cullinane *et al.*, 1984a).

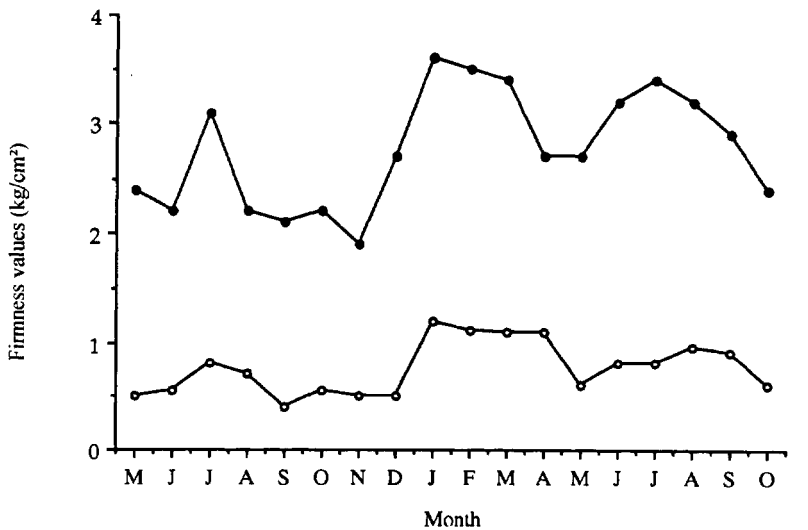


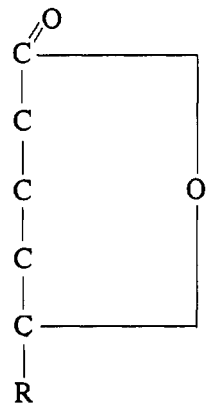
Figure 3.7 Seasonal variations in the mean firmness of Irish butter at 4°C (●) or 15°C (○) (from Cullinane *et al.*, 1984b).

Table 3.7 The fatty acid composition of cholesteryl esters, phosphatidylcholine and phosphatidylethanolamine in the milks of some species

	Fatty acid composition (wt% of the total)												
	Cow			Human			Pig		Mink			Mouse	
Fatty acid	CE	PC	PE	CE	PC	PE	PC	PE	CE	PC	PE	PC	PE
12:0	0.2	0.3	0.1	3.2	–	–			0.3	–	–	–	–
14:0	2.3	7.1	1.0	4.8	4.5	1.1	1.8	0.4	1.1	1.3	0.8	–	4.5
16:0	23.1	32.2	11.4	23.8	33.7	8.5	39.9	12.4	25.4	26.4	20.6	20.3	8.9
16:1	8.8	3.4	2.7	1.5	1.7	2.4	6.3	7.3	4.4	1.1	1.2	–	2.7
18:0	10.6	7.5	10.3	8.0	23.1	29.1	10.3	12.3	14.7	20.8	29.3	30.0	18.0
18:1	17.1	30.1	47.0	45.7	14.0	15.8	21.8	36.2	35.7	31.7	27.8	13.9	19.8
18:2	27.1	8.9	13.5	12.4	15.6	17.7	15.9	17.8	13.5	17.4	19.1	22.8	17.2
18:3	4.2	1.4	2.3	T	1.3	4.1	1.5	1.9	2.6	2.2	0.5	–	–
20:3	0.7	1.0	1.7	–	2.1	3.4	0.3	0.7	–	–	–	–	–
20:4	1.4	1.2	2.7	T	3.3	12.5	1.3	6.6	–	–	–	8.9	20.0
22:6	–	–	0.1	–	0.4	2.6	0.2	1.6	–	–	–	1.8	6.3

Abbreviations: CE, cholesteryl esters; PC, phosphatidylcholine; PE, phosphatidylethanolamine; T, trace amount. From Christie (1995).

varies; some can form lactones, e.g. the 4- and 5-hydroxy acids can form γ- and δ-lactones, respectively.



A δ-lactone

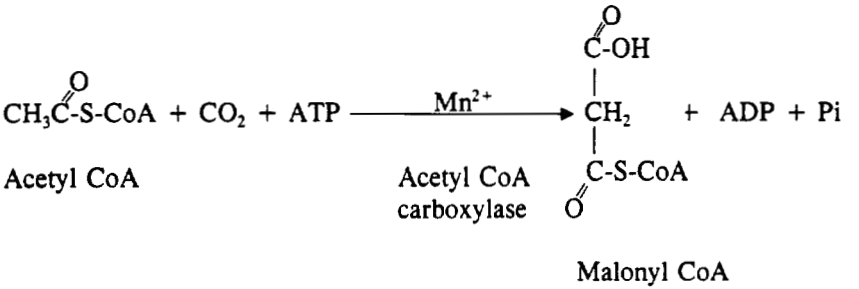
Lactones have strong flavours; traces of δ-lactones are found in fresh milk and contribute to the flavour of milk fat, but higher concentrations may occur in dried milk or butter oil as a result of heating or prolonged storage and may cause atypical flavours.

The fatty acids in the various polar lipids and cholesteryl esters are long-chain, saturated or unsaturated acids, with little or no acids of less than $C_{12:0}$ (Table 3.7; for further details see Christie, 1995).

3.5 Synthesis of fatty acids in milk fat

In non-ruminants, blood glucose is the principal precursor of fatty acids in milk fat; the glucose is converted to acetyl CoA in the mammary gland. In ruminants, acetate and β-hydroxybutyrate, produced by micro-organisms in the rumen and transported to the blood, are the principal precursors; in fact, ruminant mammary tissue has little 'ATP citrate lyase' activity which is required for fat synthesis from glucose. Blood glucose is low in ruminants and is conserved for lactose synthesis. The differences in fatty acid precursors are reflected in marked interspecies differences in milk fatty acid profiles. Restriction of roughage in the diet of ruminants leads to suppression of milk fat synthesis, possibly through a reduction in the available concentration of acetate and β-hydroxybutyrate.

In all species, the principal precursor for fatty acid synthesis is acetyl CoA, derived in non-ruminants from glucose and in ruminants from acetate or oxidation of β-hydroxybutyrate. Acetyl CoA is first converted, in the cytoplasm, to malonyl CoA:



Reduced bicarbonate supply (source of CO_2) depresses fatty acid synthesis.

Some β-hydroxybutyrate is reduced to butyrate and incorporated directly into milk fat; hence, the high level of this acid in ruminant milk fat.

In non-ruminants, the malonyl CoA is combined with an 'acyl carrier protein' (ACP) which is part of a six-enzyme complex (molecular weight *c.* 500 kDa) located in the cytoplasm. All subsequent steps in fatty acid synthesis occur attached to this complex; through a series of steps and repeated cycles, the fatty acid is elongated by two carbon units per cycle (Figure 3.8, see also Lehninger, Nelson and Cox, 1993).

The net equation for the synthesis of a fatty acid is:

$$n\ \text{Acetyl CoA} + 2(n-1)\text{NADPH} + 2(n-1)\text{H}^+ + (n-1)\text{ATP}$$
$$+ (n-1)\text{CO}_2 \rightarrow \text{CH}_3\text{CH}_2(\text{CH}_2\text{CH}_2)_{n-1}\text{CH}_2\text{C}(=\text{O})\text{—CoA} + (n-1)\text{CoA}$$
$$+ (n-1)\text{ADP} + (n-1)\text{P}_i + 2(n-1)\text{NADP} + (n-1)\text{CO}_2$$

The large supply of NADPH required for the above reactions is obtained through the metabolism of glucose-6-phosphate via the pentose pathway.

In ruminants, β-hydroxybutyrate is the preferred chain initiator (labelled β-hydroxybutyrate appears as the terminal four carbons of short- to medium-chain acids), i.e. the first cycle in fatty acid synthesis commences at β-hydroxybutyryl-S-ACP.

Synthesis of fatty acids via the malonyl CoA pathway does not proceed beyond palmitic acid ($C_{16:0}$) and mammary tissue contains an enzyme, thioacylase, capable of releasing the acyl fatty acid from the carrier protein at any stage between C_4 and C_{16}. Probable interspecies differences in the activity of thioacylase may account for some of the interspecies differences in milk fatty acid profiles.

The malonyl CoA pathway appears to account for 100% of the C_{10}, C_{12} and C_{14}, and *c.* 50% of the $C_{16:0}$ acids in ruminant milk fat, as indicated by labelling experiments (Figure 3.9). However, C_4, C_6 and C_8 are synthesized

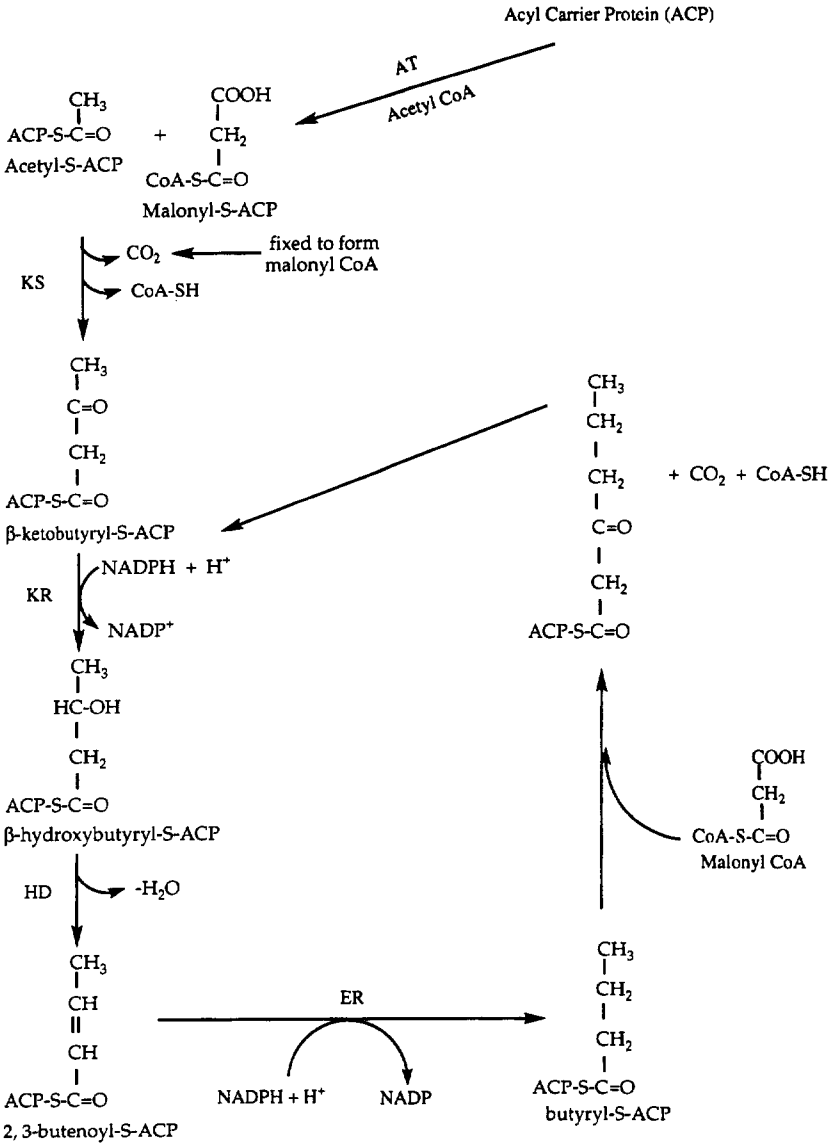


Figure 3.8 One complete cycle and the first step in the next cycle of the events during the synthesis of fatty acids. ACP = acyl carrier protein, a complex of six enzymes: i.e. acetyl CoA-ACP transacetylase (AT); malonyl CoA-ACP transferase (MT); β-keto-ACP synthase (KS); β-ketoacyl-ACP reductase (KR); β- hydroxyacyl-ACP-dehydrase (HD); enoyl-ACP reductase (ER).

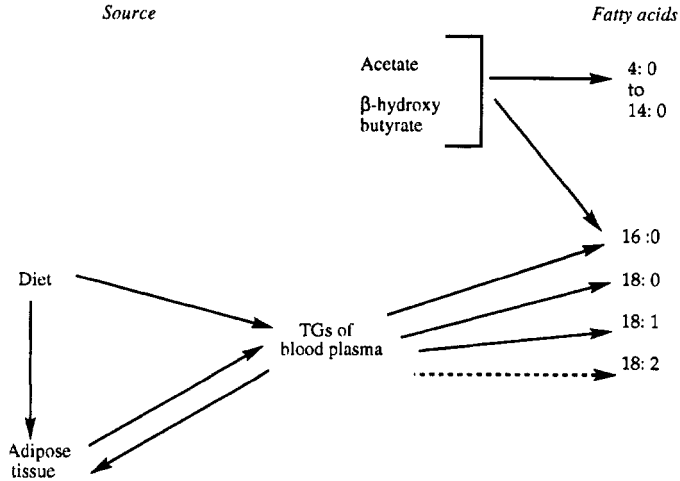


Figure 3.9 Sources of the fatty acids in bovine milk fat; TG, triglyceride (from Hawke and Taylor, 1995).

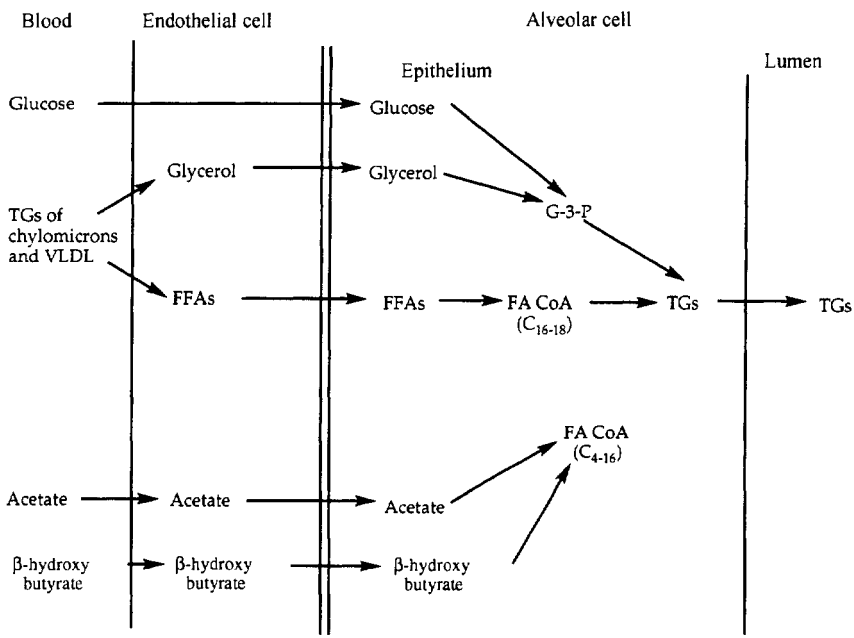


Figure 3.10 Uptake of blood constituents by the mammary gland; CoA, coenzyme A; G-3-P, glycerol-3-phosphate; FFA, free fatty acid; FA, fatty acid; TG, triglyceride, VLDL, very low density lipoprotein (from Hawke and Taylor, 1995).

from β-hydroxybutyrate and acetate mainly via two other pathways not involving malonyl CoA.

In the mammary gland, essentially 100% of $C_{18:0}$, $C_{18:2}$ and *c.* 50% of C_{16} are derived from blood lipids (chylomicrons, free triglycerides, free fatty acids, cholesteryl esters). The blood lipids are hydrolysed by lipoprotein lipase which is present in the alveolar blood capillaries, the activity of which increases eightfold on initiation of lactation. The resulting monoglycerides, free fatty acids and some glycerol are transported across the basal cell membrane and re-incorporated into triglycerides inside the mammary cell (Figure 3.10).

In blood, lipids exist as lipoprotein particles, the main function of which is to transport lipids to and from various tissues and organs of the body. There is considerable interest in blood lipoproteins from the viewpoint of human health, especially obesity and cardiovascular diseases. Lipoproteins are classified into four groups on the basis of density, which is essentially a function of their triglyceride content, i.e. chylomicrons, very low density lipoprotein particles (VLDL), low density lipoprotein (LDL) particles and high density lipoprotein (HDL) particles, containing *c.* 98, 90, 77 and 45% total lipid, respectively (Figure 3.11).

Lipoproteins, especially chylomicrons, are at an elevated level in the blood after eating, especially after high-fat meals, and give blood serum a milky appearance. They are also elevated during or after tension (so-called

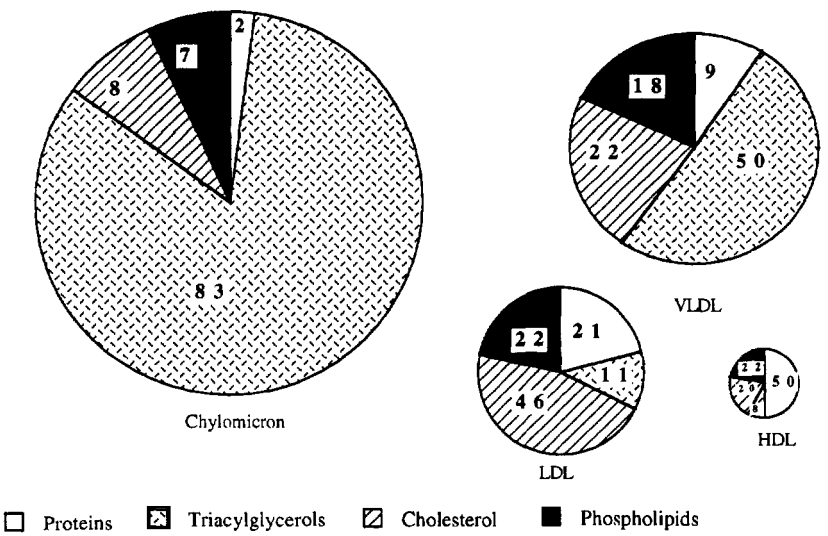
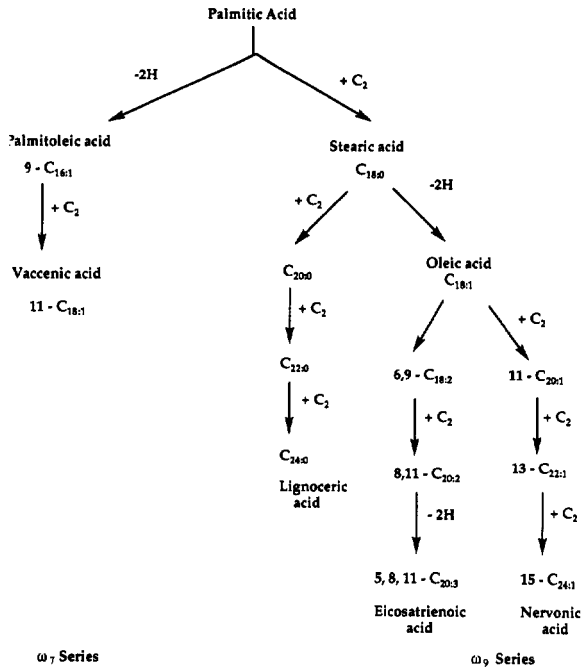


Figure 3.11 Composition (%) of human serum lipoproteins; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.



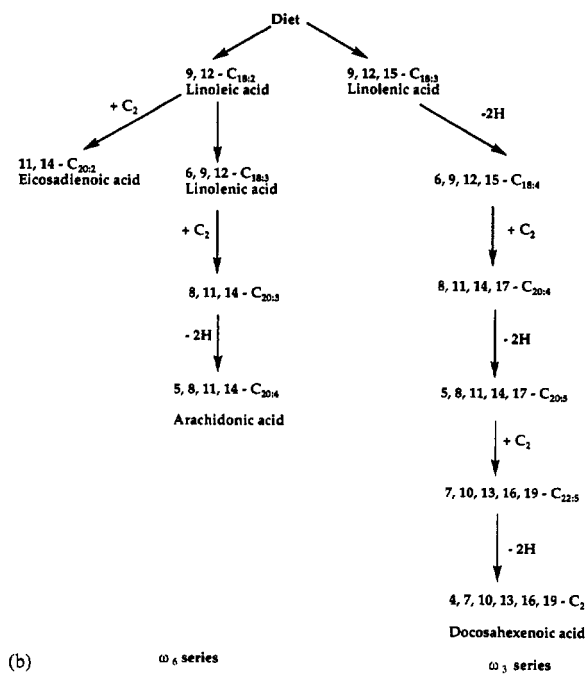


Figure 3.12 Elongation and/or desaturation of fatty acids in the mammary gland.

racing driver syndrome). Chylomicrons, which are formed in the intestinal mucosa, are secreted into the lymph and enter the blood via the thoracic duct. VLDL lipoproteins are synthesized in intestinal mucosa and liver. LDL lipoproteins are formed at various sites, including mammary gland, by removing of triglycerides from VLDL.

Since about 50% of $C_{16:0}$ and 100% of $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ are derived from blood lipids, about 50% of the total fatty acids in ruminant milk fat originate from the blood via diet or other organs.

In liver mitochondria, palmitic acid, as its CoA ester, is lengthened by successive additions of acetyl CoA. There is also a liver microsomal enzyme capable of elongating saturated and unsaturated fatty acids by addition of acetyl CoA or malonyl CoA.

The principal monoenoic acids, oleic ($C_{18:1}$) and palmitoleic ($C_{16:1}$), are derived from blood lipids but about 30% of these acids are produced by microsomal enzymes (in the endoplasmic reticulum) in the secretory cells by desaturation of stearic and palmitic acids, respectively:

$$\text{Stearyl CoA} + \text{NADPH} + O_2 \xrightarrow[\text{desaturase}]{} \text{oleoyl CoA} + \text{NADP}^+ + 2H_2O$$

Shorter chain unsaturated acids ($C_{10:1}$ to $C_{14:1}$) are probably also produced by the same enzyme.

Linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids cannot be synthesized by mammals and must be supplied in the diet, i.e. they are essential fatty acids (linoleic is the only true essential acid). These two polyenoic acids may then be elongated and/or further desaturated by mechanisms similar to stearic → oleic, to provide a full range of polyenoic acids. A summary of these reactions is given in Figure 3.12a, b.

δ-Hydroxy acids are produced by δ-oxidation of fatty acids and β-keto acids may arise from incomplete syntheses or via β-oxidation.

3.6 Structure of milk lipids

Glycerol for milk lipid synthesis is obtained in part from hydrolysed blood lipids (free glycerol and monoglycerides), partly from glucose and a little from free blood glycerol. Synthesis of triglycerides within the cell is catalysed by enzymes located on the endoplasmic reticulum, as shown in Figure 3.13.

Esterification of fatty acids is not random: C_{12}–C_{16} are esterified principally at the *sn*-2 position while C_4 and C_6 are esterified principally at the *sn*-3 position (Table 3.8). The concentrations of C_4 and C_{18} appear to be rate-limiting because of the need to keep the lipid liquid at body temperature. Some features of the structures are notable:

- Butanoic and hexanoic acids are esterified almost entirely, and octanoic and decanoic acids predominantly, at the *sn*-3 position.

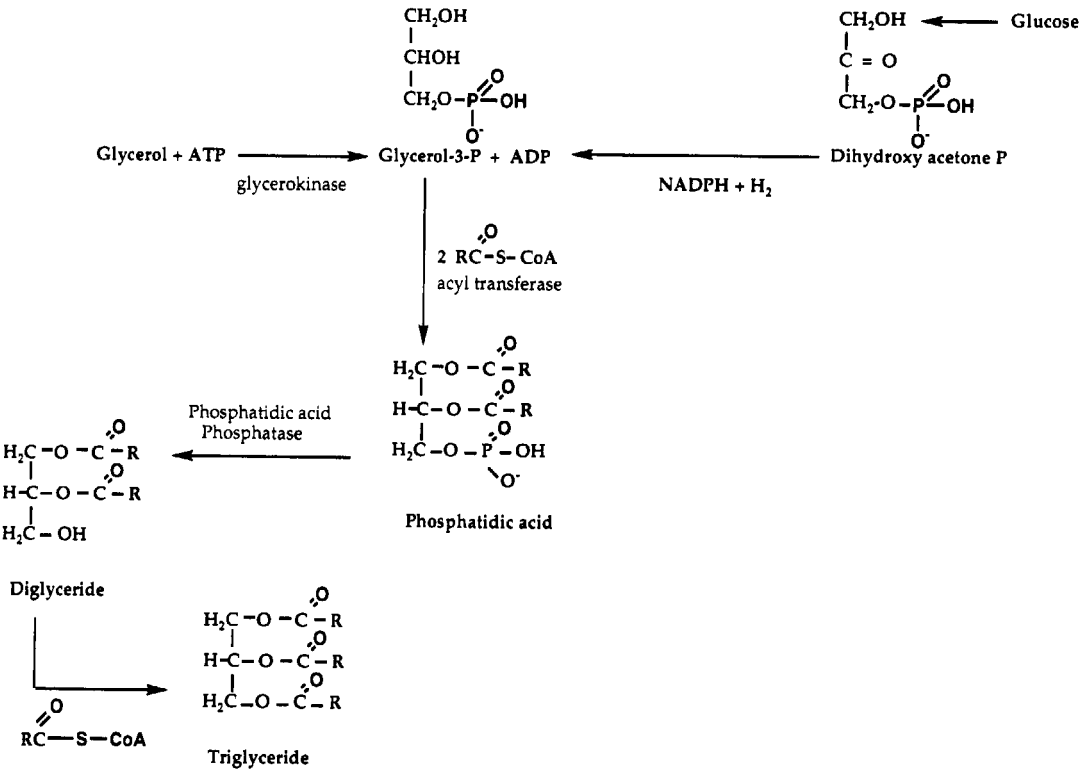


Figure 3.13 Biosynthesis of triglycerides in the mammary gland.

Table 3.8 Composition of fatty acids (mol% of the total) esterified to each position of the triacyl-*sn*-glycerols in the milks of various species

Fatty acid	Cow			Human			Rat			Pig			Rabbit			Seal			Echidna		
	sn-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3
4:0	–	–	35.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6:0	–	0.9	12.9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8:0	1.4	0.7	3.6	–	–	–	3.7	5.7	10.0	–	–	–	–	19.2	33.7	38.9	–	–	–	–	–
10:0	1.9	3.0	6.2	0.2	0.2	1.1	10.1	20.0	26.0	–	–	–	–	22.5	22.5	26.1	–	–	–	–	–
12:0	4.9	6.2	0.6	1.3	2.1	5.6	10.4	15.9	15.1	–	–	–	–	3.5	2.8	1.8	0.3	0.2	–	–	–
14:0	9.7	17.5	6.4	3.2	7.3	6.9	9.6	17.8	8.9	2.4	6.8	3.7	2.7	2.1	2.6	0.7	23.6	3.8	1.7	0.9	0.4
16:0	34.0	32.3	5.4	16.1	58.2	5.5	20.2	28.7	12.6	21.8	57.6	15.4	24.1	12.7	23.8	6.1	31.0	1.0	31.5	9.0	27.9
16:1	2.8	3.6	1.4	3.6	4.7	7.6	1.8	2.1	1.8	6.6	11.2	10.4	4.1	1.3	1.5	1.1	16.8	14.1	–	–	–
18:0	10.3	9.5	1.2	15.0	3.3	1.8	4.9	0.8	1.5	6.9	1.1	5.5	6.9	3.5	0.9	1.9	0.7	1.0	16.8	2.1	14.3
18:1	30.0	18.9	23.1	46.1	12.7	50.4	24.2	3.3	11.8	49.6	13.9	51.7	40.8	16.6	3.8	11.4	19.4	45.4	33.1	57.6	39.8
18:2	1.7	3.5	2.3	11.0	7.3	15.0	14.1	5.2	11.6	11.3	8.4	11.5	15.6	15.1	6.4	9.7	2.3	2.8	4.1	18.3	4.9
18:3	–	–	–	0.4	0.6	1.7	1.2	0.5	0.7	1.4	1.0	1.8	3.4	3.5	2.0	2.3	0.5	0.7	1.0	2.9	2.0
C_{20}–C_{22}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.8	28.7	–	–	–

From Christie (1995).

- As the chain length increases up to $C_{16:0}$, an increasing proportion is esterified at the *sn*-2 position; this is more marked for human than for bovine milk fat, especially in the case of palmitic acid ($C_{16:0}$).
- Stearic acid ($C_{18:0}$) is esterified mainly at *sn*-1.
- Unsaturated fatty acids are esterified mainly at the *sn*-1 and *sn*-3 positions, in roughly equal proportions.

Fatty acid distribution is significant from two viewpoints:

- It affects the melting point and hardness of the fat, which can be reduced by randomizing the fatty acid distribution. Transesterification can be performed by treatment with $SnCl_2$ or enzymatically under certain conditions; increasing attention is being focused on the latter as an acceptable means of modifying the hardness of butter.
- Pancreatic lipase is specific for the fatty acids at the *sn*-1 and *sn*-3 positions. Therefore, $C_{4:0}$ to $C_{8:0}$ are released rapidly from milk fat; these are water-soluble and are readily absorbed from the intestine. Medium- and long-chain acids are absorbed more effectively as 2-monoglycerides than as free acids; this appears to be quite important for the digestion of lipids by human infants who have limited ability to digest lipids due to the absence of bile salts. Infants metabolize human milk fat more efficiently than bovine milk fat, apparently owing to the very high proportion of $C_{16:0}$ esterified at *sn*-2 in the former. The effect of transesterification on the digestibility of milk fat by infants merits investigation.

3.7 Milk fat as an emulsion

In 1674, Van Leeuwenhoek reported that the fat in milk exists as microscopic globules. Milk is an oil-in-water emulsion, the properties of which have a marked influence on many properties of milk, e.g. colour, mouthfeel, viscosity. The globules range in diameter from approximately 0.1 to 20 μm, with a mean of about 3.5 μm (the range and mean vary with breed and health of the cow, stage of lactation, etc.). The size and size distribution of fat globules in milk may be determined by light microscopy, light scattering (e.g. using the Malvern Mastersizer) or electronic counting devices (such as the Coulter counter). The frequency distribution of globule number and volume as a function of diameter for bovine milk are summarized in Figure 3.14. Although small globules are very numerous (*c.* 75% of all globules have diameters < 1 μm), they represent only a small proportion of total fat volume or mass. The number average diameter of the globules in milk is only *c.* 0.8 μm. The mean fat globule size in milk from Channel Island breeds (Jersey and Guernsey) is larger than that in milk from other breeds (the fat content of the former milks is also higher) and the mean globule diameter decreases throughout lactation (Figure 3.15).

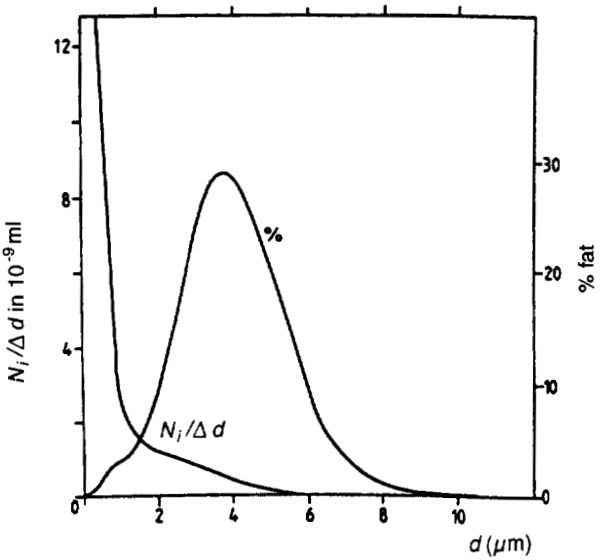


Figure 3.14 Number ($N_i/\Delta d$) and volume (% of fat) frequency of the fat globules in bovine milk (from Walstra and Jenness, 1984).

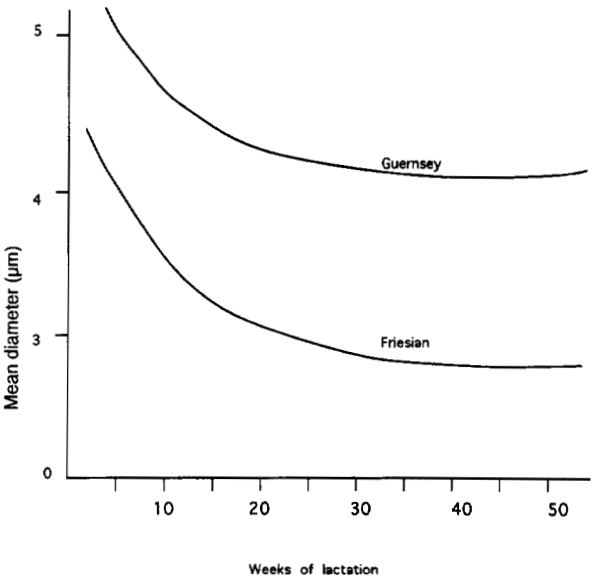


Figure 3.15 Average diameter of the fat globules in milk of Guernsey or Friesian cows throughout lactation (from Walstra and Jenness, 1984).

Milk contains $\sim 15 \times 10^9$ globules ml^{-1}, with a total interfacial area of 1.2–2.5 m^2 per g fat.

Example. Assume a fat content of 4.0%, w/v, with a mean globule diameter of 3 μm.

$$\text{Volume of typical globule} = \frac{4}{3}\pi r^3$$

$$= \frac{4}{3} \times \frac{22}{7} \times \frac{(3)^3}{2}\ \mu m^3$$

$$\sim 14\ \mu m^3.$$

$$\text{1 ml milk contains: } 0.04\text{ g fat}$$

$$= 4.4 \times 10^{10}\ \mu m^3.$$

$$\text{1 ml milk contains: } \frac{4.4 \times 10^{10}}{14} \sim 3.14 \times 10^9 \text{ globules.}$$

$$\text{Surface area of a typical globule} = 4\pi r^2$$

$$= 4 \times \frac{22}{7} \times \frac{9}{4}\ \mu m^2$$

$$= 28.3\ \mu m^2.$$

$$\text{Interfacial area per ml milk} = 28.3 \times (3.14 \times 10^9)\ \mu m^2$$

$$= 88.9 \times 10^9\ \mu m^2$$

$$= 889\ cm^2 \approx 0.09\ m^2.$$

$$\text{Interfacial area per g fat} = 88.9 \times 10^{-3} \times \frac{1}{0.04}\ m^2$$

$$= 2.22\ m^2.$$

3.8 Milk fat globule membrane

Lipids are insoluble in water and an interfacial tension therefore exists between the phases when lipids are dispersed (emulsified) in water (or vice versa). This tension *in toto* is very large, considering the very large interfacial area in a typical emulsion (section 3.7). Owing to the interfacial tension, the oil and water phases would quickly coalesce and separate. However, coalescence (but not creaming) is prevented by the use of emulsifiers (surface active agents) which form a film around each fat globule (or each water

droplet in the case of water-in-oil emulsions) and reduce interfacial tension. In the case of unprocessed milk, the emulsifying film is much more complex than that in 'artificial' emulsions, and is referred to as the milk fat globule membrane (MFGM).

In 1840, Ascherson observed an emulsion-stabilizing membrane surrounding the fat globules in milk and suggested that the membrane was 'condensed' albumin (from the skim-milk phase) aggregated at the fat/plasma interface. Babcock, in the 1880s, also felt that the milk fat emulsifier was adsorbed serum protein. Histological staining and light microscopy were employed around the turn of the century to identify the nature of the membrane material but it was early recognized that contamination of fat globules by skim-milk components presented a major problem. By analysing washed globules, it was shown that the MFGM contained phospholipids and protein which differed from the skim-milk proteins (see Brunner (1974) for historical review).

3.8.1 Isolation of the fat globule membrane

The definition of what precisely constitutes the membrane leads to considerable difficulty and uncertainty. The outer boundary is assumed to constitute everything that travels with the fat globule when it moves slowly through milk; however, the outer regions of the membrane are loosely attached and some or all may be lost, depending on the extent of mechanical damage the globule suffers. The inner boundary is ill-defined and depends on the method of preparation; there is considerable discussion as to whether a layer of high melting point triglyceride, immediately inside the membrane, is part of the membrane or not. Some hydrophobic constituents of the membrane probably diffuse into the core of the globules while components of the plasma may adsorb at the outer surface. Since the membrane contains numerous enzymes, enzymatic changes may occur.

Several methods are available for isolating all or part of the membrane. The usual initial step involves separating a cream from milk by mechanical centrifugation (which may cause some damage) or by gravity. The cream is washed repeatedly (3–6 times) with water or dilute buffer by dilution and gravity separation; soluble salts and other small molecules are probably lost into the serum. Mechanical damage may remove the loosely bound outer layers and may even cause some homogenization and adsorption of serum constituents; small globules are lost during each washing cycle.

The washed cream is destabilized by churning or freezing; then the fat (mainly triglycerides) is melted and separated from the membrane material by centrifugation. Cross-contamination of membrane with core material may be considerable, and methods must be carefully standardized. An elaborate scheme for the isolation and fractionation of the MFGM was developed by Brunner and co-workers (Brunner, 1974).

Treatment of washed cream with surfactants, usually sodium deoxycholate, releases part of the membrane, assumed to represent only the outer layer. Unless the treatment is carefully controlled, some inner material will be released also.

3.8.2 Gross chemical composition of MFGM

Yields of 0.5-1.5 g MFGM per 100 g fat have been reported; the range reflects variations in temperature history, washing technique, age, agitation, etc. The gross chemical composition of the membrane is reasonably well established and the relatively small differences reported are normally attributed to different methods used to isolate and fractionate the membrane material. The data in Table 3.9, from Mulder and Walstra (1974) and based on the investigations of many workers, give a reasonable estimate of the gross composition of the MFGM. A more detailed compositional analysis is provided by Keenan *et al.* (1983) (Table 3.10). Brunner (1965, 1974), Mulder and Walstra (1974), Patton and Keenan (1975), Keenan *et al.* (1983) and Keenan and Dylewski (1995) should be consulted for more detailed compositional data.

3.8.3 The protein fraction

Depending on the preparative method used, the membrane may or may not contain skim-milk proteins (i.e. caseins and whey proteins); if the membrane has been damaged prior to isolation, it may contain considerable amounts of these proteins. The membrane contains unique proteins which do not occur in the skim-milk phase. Many of the proteins are glycoproteins and contain a considerable amount of carbohydrate (hexose, 2.8–4.15%; hexosamine, 2.5–4.2%; and sialic acid, 1.3–1.8%).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), with silver staining of the gels, resolves MFGM proteins into as many as 60 discrete bands, ranging in molecular mass from 11 to 250 kDa (Keenan and Dylewski, 1995). Most of these proteins are present at very low concentrations (many are detectable only when gels are stained with silver but not with Coomassie blue). Some of these proteins may be genetic variants and, since the MFGM contains a plasmin-like proteinase, some of the smaller polypeptides may be fragments of larger proteins. The three principal proteins, with molecular masses (by SDS–PAGE) of 155, 67 and 48 kDa, are xanthine oxidase, butyrophilin and glycoprotein B, respectively; five or six glycoproteins have been detected by staining with Schiff's reagent.

Xanthine oxidase, which requires Fe, Mo and flavin adenine dinucleotide (FAD) as co-factors, is capable of oxidizing lipids via the production of superoxide radicals. It represents about 20% of the MFGM protein and part is readily lost from the membrane, e.g. on cooling; isoelectric focusing

indicates at least four variants with isoelectric points (p*I*) in the range 7.0–7.5.

Butyrophilin, the principal MFGM protein and so named because of its high affinity for milk lipids, is a very hydrophobic, difficult to solubilize (insoluble or only sparingly soluble in most protein solvents, including detergents) glycoprotein. Isoelectric focusing indicates at least four variants (p*I*s 5.2–5.3). The amino acid sequence of butyrophilin has been determined and its gene has been cloned, which indicates that butyrophilin is synthesized with a leader sequence; it consists of 526 amino acids and has a molecular mass, without carbohydrate, of 56 460 Da. It binds phospholipids tenaciously and perhaps even contains covalently bound fatty acids. It is located only at the apical cell surface of the mammary epithelial cells, suggesting a role in membrane envelopment of fat globules.

Several of the minor proteins of the MFGM have been isolated and partially characterized (Keenan and Dylewski, 1995). A systematic nomenclature has not been developed for the MFGM proteins and most are referred to by their relative electrophoretic mobility on SDS–PAGE and whether or not they are glycoproteins. The proteins of the MFGM represent approximately 1% of the total proteins in milk.

3.8.4 The lipid fraction

The membrane contains 0.5–1.0% of the total lipid in milk and is composed principally of phospholipids and neutral lipids in the approximate ratio 2:1, with lesser amounts of other lipids (Tables 3.9 and 3.10); contamination with core lipid is a major problem. The phospholipids are principally phosphatidylcholine, phosphatidylethanolamine and sphingomyelin in the approximate ratio 2:2:1. The principal fatty acids and their approximate percentages in the phospholipids are $C_{14:0}$ (5%), $C_{16:0}$ (25%), $C_{18:0}$ (14%), $C_{18:1}$ (25%), $C_{18:2}$ (9%), $C_{22:0}$ (3%) and $C_{24:0}$ (3%). Thus, the membrane contains a significantly higher level of polyunsaturated fatty acids than milk

Table 3.9 Gross composition of the milk fat globule membrane

Component	mg 100 g^{-1} fat globule	mg m^{-2} fat globule surface	% (w/w) of total membrane
Protein	900	4.5	41
Phospholipid	600	3.0	27
Cerebrosides	80	0.4	3
Cholesterol	40	0.2	2
Neutral glycerides	300	1.5	14
Water	280	1.4	13
Total	2200	11.0	100

From Mulder and Walstra (1974).

Table 3.10 Composition of bovine milk fat globule membranes

Constituent class	Amount
Protein	25–60% of dry weight
Total lipid	0.5–1.2 mg per mg protein
Phospholipid	0.13–0.34 mg per mg protein
Phosphatidyl choline	34% of total lipid phosphorus
Phosphatidylethanolamine	28% of total lipid phosphorus
Sphingomyelin	22% of total lipid phosphorus
Phosphatidylinositol	10% of total lipid phosphorus
Phosphatidylserine	6% of total lipid phosphorus
Neutral lipid	56–80% of total lipid
Hydrocarbons	1.2% of total lipid
Sterols	0.2–5.2% of total lipid
Sterol esters	0.1–0.8% of total lipid
Glycerides	53–74% of total lipid
Free fatty acids	0.6–6.3% of total lipid
Cerebrosides	3.5 nmoles per mg protein
Gangliosides	6–7.4 nmoles sialic acid per mg protein
Total sialic acids	63 nmoles per mg protein
Hexoses	0.6 μmoles per mg protein
Hexosamines	0.3 μmoles per mg protein
Cytochrome b_5 + P420	30 pmoles per mg protein
Uronic acids	99 ng per mg protein
RNA	20 μg per mg protein

From Keenan *et al.* (1983).

Table 3.11 Structures of glycosphingolipids of bovine milk fat globule membrane

Glycosphingolipid	Structure
Glucosyl ceramide	β-Glucosyl-(1 → 1)-ceramide
Lactosyl ceramide	β-Glucosyl-(1 → 4)-β-glucosyl-(1 → 1)-ceramide
GM_3 (hematoside)	Neuraminosyl-(2 → 3)-galactosyl-glucosyl-ceramide
GM_2	*N*-Acetylgalactosaminyl-(neuraminosyl)-galactosyl-glucosyl-ceramide
GM_1	Galactosyl-*N*-acetylgalactosaminyl-(neuraminosyl)-galactosyl-glucosyl-ceramide
GD_3 (disialohematoside)	Neuraminosyl-(2 → 8)-neuraminosyl-(2 → 3)-galactosyl-glucosyl-ceramide
GD_2	*N*-Acetylgalactosaminyl-(neuraminosyl-neuraminosyl)-galactosyl-glucosyl-ceramide
GD_{1b}	Galactosyl-*N*-acetylgalactosaminyl-(neuraminosyl-neuraminosyl)-galactosyl-glucosyl-ceramide

From Keenan *et al.* (1983).

fat generally and is, therefore, more susceptible to oxidation. The cerebrosides are rich in very long chain fatty acids which possibly contribute to membrane stability. The membrane contains several glycolipids (Table 3.11).

The amount and nature of the neutral lipid present in the MFGM is uncertain because of the difficulty in defining precisely the inner limits of the membrane. It is generally considered to consist of 83–88% triglyceride, 5–14% diglyceride and 1–5% free fatty acids. The level of diglyceride is considerably higher than in milk fat as a whole; diglycerides are relatively polar and are, therefore, surface-active. The fatty acids of the neutral lipid fraction are longer-chained than in milk fat as a whole and in order of proportion present are palmitic, stearic, myristic, oleic and lauric.

Most of the sterols and sterol esters, vitamin A, carotenoids and squalene in milk are dissolved in the core of the fat globules but some are probably present in the membrane.

3.8.5 Other membrane components

Trace metals. The membrane contains 5–25% of the indigenous Cu and 30–60% of the indigenous Fe of milk as well as several other elements, e.g. Co, Ca, Na, K, Mg, Mn, Mo, Zn, at trace levels; Mo is a constituent of xanthine oxidase.

Enzymes. The MFGM contains many enzymes (Table 3.12). These enzymes originate from the cytoplasm and membranes of the secretory cell and are present in the MFGM due to the mechanism of globule excretion from the cells.

3.8.6 Membrane structure

Several early attempts to describe the structure of the MFGM included King (1955), Hayashi and Smith (1965), Peereboom (1969), Prentice (1969) and Wooding (1971). Although the structures proposed by these workers were inaccurate, they stimulated thinking on the subject. Keenan and Dylewski (1995) and Keenan and Patton (1995) should be consulted for recent reviews.

Understanding of the structure of the MFGM requires understanding three processes: the formation of lipid droplets from triglycerides synthesized in or on the endoplasmic reticulum at the base of the cell, movement of the droplets (globules) through the cell and excretion of the globules from the cell into the lumen of the alveolus.

The MFGM originates from regions of apical plasma membrane, and also from endoplasmic reticulum (ER) and perhaps other intracellular compartments. That portion of the MFGM derived from apical plasma

Table 3.12 Enzymatic activities detected in bovine milk fat globule membrane preparations

Enzyme	EC number
Lipoamide dehydrogenase	1.6.4.3
Xanthine oxidase	1.2.3.2
Thiol oxidase	1.8.3.2
NADH oxidase	1.6.99.3
NADPH oxidase	1.6.99.1
Catalase	1.11.1.6
γ-Glutamyl transpeptidase	2.3.2.1
Galactosyl transferase	2.4.1-
Alkaline phosphatase	3.1.3.1
Acid phosphatase	3.1.3.2
N^1-Nucleotidase	3.1.3.5
Phosphodiesterase I	3.1.4.1
Inorganic pyrophosphatase	3.6.1.1
Nucleotide pyrophosphatase	3.6.1.9
Phosphatidic acid phosphatase	3.1.3.4
Adenosine triphosphatase	3.6.1.15
Cholinesterase	3.1.1.8
UDP-glycosyl hydrolase	3.2.1-
Glucose-6-phosphatase	3.1.3.9
Plasmin	3.4.21.7
β-Glucosidase	3.2.1.21
β-Galactosidase	3.2.1.23
Ribonuclease I	3.1.4.22
Aldolase	4.1.2.13
Acetyl-CoA carboxylase	6.4.1.2

From Keenan and Dylewski (1995).

membrane, termed the primary membrane, has a typical bilayer membrane appearance, with electron-dense material on the inner membrane face. The components derived from ER appear to be a monolayer of proteins and polar lipids which covers the triacylglycerol-rich core lipids of the globule before its secretion. This monolayer or coat material compartmentalizes the core lipid within the cell and participates in intracellular fusions through which droplets grow in volume. Constituents of this coat also may be involved in interaction of droplets with the plasma membrane.

Milk lipid globules originate as small lipid droplets in the ER. Lipids, presumed to be primarily triacylglycerols, appear to accumulate at focal points on or in the ER membrane. This accumulation of lipids may be due to localized synthesis at these focal points, or to accretion from dispersed or uniformly distributed biosynthetic sites. It has been suggested that triacylglycerols accumulate between the halves of the bilayer membrane and are released from the ER into the cytoplasm as droplets coated with the outer or cytoplasmic half of the ER membrane. A cell-free system has been developed in which ER isolated from lactating mammary gland can be induced to release lipid droplets which resemble closely droplets formed *in situ* in both morphology and composition. In this cell-free system, lipid

droplets were formed only when a fraction of cytosol with a molecular mass greater than 10 kDa was included in the incubation mixture, suggesting that cytosolic factors are involved in droplet formation or release from ER.

By whatever mechanism they are formed, on or in, and released from the ER, milk lipid globule precursors first appear in the cytoplasm as droplets with diameters of less than 0.5 μm, with a triglyceride-rich core surrounded by a granular coat material that lacks bilayer membrane structure, but which appears to be thickened, with tripartite-like structure, in some regions. These small droplets, named microlipid droplets, appear to grow in volume by fusing with each other. Fusions give rise to larger droplets, called cytoplasmic lipid droplets, with diameters of greater than 1 μm.

Droplets of different density and lipid : protein ratios ranging from about 1.5 : 1 to 40 : 1 have been isolated from bovine mammary gland. Triglycerides are the major lipid class in droplets of all sizes and represent increasingly greater proportions of total droplet mass in increasingly less dense droplet preparations. Surface coat material of droplets contains cholesterol and the major phospholipid classes found in milk, i.e. sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine.

SDS–PAGE shows that micro- and cytoplasmic lipid droplets have complex and similar polypeptide patterns. Many polypeptides with electrophoretic mobilities in common with those of intracellular lipid droplets are present also in milk lipid globules. Some polypeptides of MFGM and intracellular lipid droplets share antigenic reactivity. Taken together, current information suggests that lipid droplet precursors of milk lipid globules originate in the ER and retain at least part of the surface material of droplets during their secretion as milk fat globules. The protein and polar lipid coat on the surface of lipid droplets stabilizes the triglyceride-rich droplet core, preventing coalescence in the cytoplasm. Beyond a stabilization role, constituents of the coat material may participate also in droplet fusions and in droplet–plasma membrane interactions. If elements of the cytoskeleton function in guiding lipid droplets from their sites of origin to their sites of secretion from the cell, coat constituents may participate in interaction with filamentous or tubular cytoskeletal elements.

Within mammary epithelial cells, one mechanism by which lipid droplets can grow is by fusion of microlipid droplets. Microlipid droplets can also fuse with cytoplasmic lipid droplets, providing triacylglycerols for continued growth of larger droplets. The size range of lipid globules in milk can be accounted for, at least in part, by a droplet fusion-based growth process. Small milk fat globules probably arise from secretion of microlipid droplets which have undergone no or a few fusions while larger droplets can be formed by continued fusions with microlipid droplets.

While accumulated evidence favours the view that lipid droplets grow by fusion, there is no evidence as to how this process is regulated to control the ultimate size distribution of milk lipid globules. The possibility that fusion

is purely a random event, regulated only by probability of droplet–droplet contact before secretion, cannot be ruled out. Insufficient evidence is available to conclude that fusion of droplets is the sole or major mechanism by which droplets grow. Other possible mechanisms for growth, e.g. lipid transfer proteins which convey triglycerides from their site of synthesis to growing lipid droplets, cannot be excluded.

Available evidence indicates that lipid droplets migrate from their sites of origin, primarily in basal regions of the cell, through the cytoplasm to apical cell regions. This process appears to be unique to the mammary gland and in distinct contrast to lipid transit in other cell types, where triacylglycerols are sequestered within ER and the Golgi apparatus and are secreted as lipoproteins or chylomicrons that are conveyed to the cell surface via secretory vesicles.

Mechanisms which guide unidirectional transport of lipid droplets are not yet understood. Evidence for possible involvement of microtubules and microfilaments, elements of the cytoskeletal system, in guiding this transit has been obtained, but this evidence is weak and is contradictory in some cases. Cytoplasmic microtubules are numerous in milk-secreting cells and the tubulin content of mammary gland increases substantially prior to milk secretion. A general role for microtubules in the cytoplasm, and the association of proteins with force-producing properties with microtubules, provide a plausible basis for assuming the microtubules may be involved in lipid droplet translocation. Microfilaments, which are abundant in milk-secreting cells, appear to be concentrated in apical regions.

3.8.7 Secretion of milk lipid globules

The mechanism by which lipid droplets are secreted from the mammocyte was first described in 1959 by Bargmann and Knoop and has been confirmed by several investigators since (Keenan and Dylewski, 1995). The lipid droplets are pushed through and become enveloped progressively by

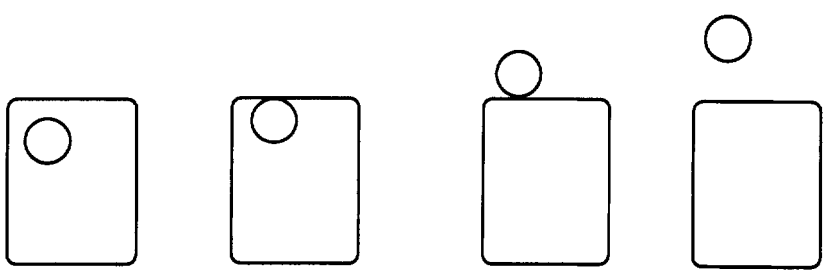


Figure 3.16 Schematic representation of the excretion of a fat globule through the apical membrane of the mammary cell.

the apical membrane up to the point where they are dissociated from the cell, surrounded entirely by apical membrane (Figure 3.16). Current concepts of the pathway by which lipid droplets originate, grow and are secreted are summarized diagrammatically in Figure 3.17.

Lipid droplets associate with regions of the plasma membrane that are characterized by the appearance of electron-dense material on the cytoplasmic face of the membrane. Droplet surfaces do not contact the plasma

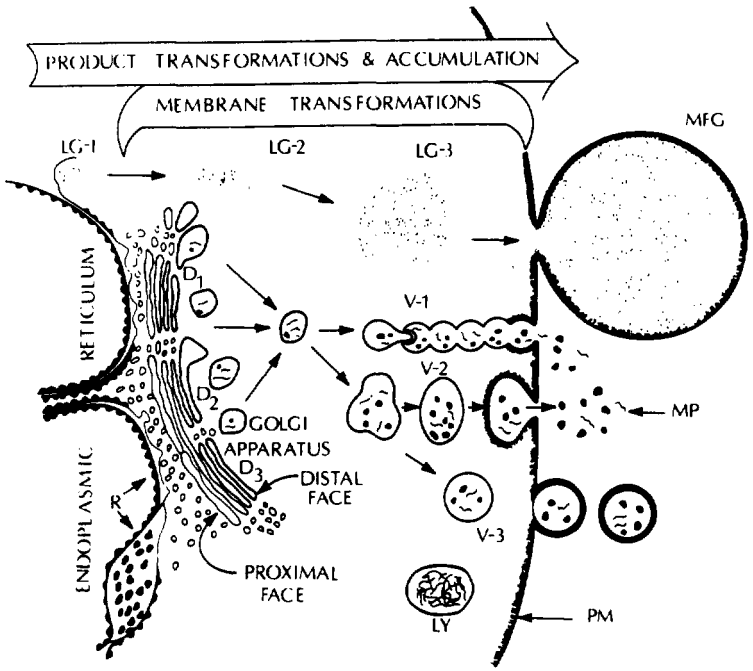


Figure 3.17 The roles of components of the endo-membrane system of mammary epithelial cells in the synthesis and secretion of the constituents of milk. Intracellular lipid globules (LG-1, LG-2, LG-3) are discharged from the cell by progressive envelopment in regions of apical plasma membrane. MFG denotes a lipid globule being enveloped in plasma membrane. Milk proteins (MP) are synthesized on polysomes of endoplasmic reticulum and are transported, perhaps in small vesicles which bleb from endoplasmic reticulum, to dictyosomes (D_1, D_2, D_3) of the Golgi apparatus. These small vesicles may fuse to form the proximal cisterna of Golgi apparatus dictyosomes. Milk proteins are incorporated into secretory vesicles formed from cisternal membranes on the distal face of dictyosomes. Lactose is synthesized within cisternal luminae of the Golgi apparatus and is incorporated into secretory vesicles. Certain ions of milk are also present in secretory vesicles. Three different mechanisms for exocytotic interaction of secretory vesicle with apical plasma membrane have been described: (1) through the formation of a chain of fused vesicles (V-1); (2) by fusion of individual vesicles with apical plasma membrane (V-2), with integration of vesicle membrane into plasma membrane; (3) by direct envelopment of secretory vesicles in apical plasma membrane (V-3). Lysosomes (LY) may function in the degradation of excess secretory vesicle membrane (from Keenan, Mather and Dylewski, 1988).

membrane directly but rather the electron-dense cytoplasmic face material; which constituents of the latter recognize and interact with constituents on the droplet surface are not known. Immunological and biochemical studies have shown that butyrophilin and xanthine oxidase, two of the principal proteins in the MFGM, are major constituents of the electron-dense material on the cytoplasmic face of apical plasma membrane. Butyrophilin, a hydrophobic, transmembrane glycoprotein that is characteristic of milk-secreting cells, is concentrated highly at the apical surface of these cells; it binds phospholipids tightly, and is believed to be involved in mediating interaction between lipid droplets and apical plasma membrane. Xanthine oxidase is distributed throughout the cytoplasm, but appears to be enriched at the apical cell surface.

In the secretion process, milk fat globules usually are enveloped compactly by apical plasma membrane, but closure of the membrane behind the projecting fat droplet occasionally entrains some cytoplasm as a so-called crescent or signet between the membrane and the droplet surface. These crescents can vary from thin slivers of cellular material to situations in which the crescent represents a greater volume than does the globule core lipid. Except for nuclei, cytoplasmic crescents contain nearly all membranes and organelles of the milk-secreting cell. Globule populations with a high proportion of crescents exhibit a more complex pattern of proteins by SDS–PAGE than low-crescent populations. Presumably, the many additional minor bands arise from cytoplasmic components in crescents. Crescents have been identified in association with the milk fat globules of all species examined to date, but the proportion of globules with crescents varies between and within species; about 1% of globules in bovine milk contain crescents.

Thus, the fat globules are surrounded, at least initially, by a membrane typical of eukaryotic cells. Membranes are a conspicuous feature of all cells and may represent 80% of the dry weight of some cells. They serve as barriers separating aqueous compartments with different solute composition and as the structural base on which many enzymes and transport systems are located. Although there is considerable variation, the typical composition of membranes is about 40% lipid and 60% protein. The lipids are mostly polar (nearly all the polar lipids in cells are located in the membranes), principally phospholipids and cholesterol in varying proportions. Membranes contain several proteins, perhaps up to 100 in complex membranes. Some of the proteins, referred to as extrinsic or peripheral, are loosely attached to the membrane surface and are easily removed by mild extraction procedures. The intrinsic or integral proteins, about 70% of the total protein, are tightly bound to the lipid portion and are removed only by severe treatment, e.g. by SDS or urea.

Electron microscopy shows that membranes are 79 nm thick, with a trilaminar structure (a light, electron-sparse layer, sandwiched between two

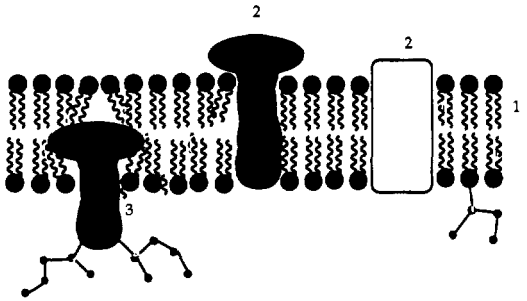


Figure 3.18 Schematic representation of a trilaminar cell membrane which is derived from the apical membrane of the mammary cell and forms the outer layer of the milk fat globule membrane following expression from the mammary cell, but which is more or less extensively lost on ageing. 1, phospholipid/glycolipid; 2, protein; 3, glycoprotein.

dark, electron-dense layers). The phospholipid molecules are arranged in a bilayer structure (Figure 3.18); the non-polar hydrocarbon chains are orientated inward where they 'wriggle' freely and form a continuous hydrocarbon base; the hydrophilic regions are orientated outward and are relatively rigid. In this bilayer, individual lipid molecules can move laterally, endowing the bilayer with fluidity, flexibility, high electrical resistance and low permeability to polar molecules. Some of the globular membrane proteins are partially embedded in the membrane, penetrating into the lipid phase from either side, others are completely buried within it, while others transverse the membrane. The extent to which a protein penetrates into the lipid phase is determined by its amino acid composition, sequence, secondary and tertiary structure. Thus, membrane proteins form a mosaic-like structure in an otherwise fluid phospholipid bilayer, i.e. the **fluid-mosaic** model (Figure 3.18).

Thus, the milk fat globules are surrounded and stabilized by a structure which includes the trilaminar apical membrane (which is replaced by Golgi membranes on secretion of proteins and lactose). The inner face of the membrane has a dense proteinaceous layer, 10–50 nm thick, probably acquired within the secretory cell during movement of the globule from the rough endoplasmic reticulum at the base of the cell, where the triglycerides are synthesized, to the apex of the cell. A layer of high melting triglycerides may be present inside this proteinaceous layer. Much of the trilaminar membrane is lost on ageing of the milk, especially if it is agitated; the membrane thus shed is present in the skim milk as vesicles (or microsomes), which explains the high proportion of phospholipids in skim milk.

McPherson and Kitchen (1983) proposed a detailed structural model of the MFGM, which appears rather speculative. Keenan *et al.* (1983), Keenan and Dylewski (1995) and Keenan and Patton (1995) describe the current

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4 Milk proteins

4.1 Introduction

Normal bovine milk contains about 3.5% protein. The concentration changes significantly during lactation, especially during the first few days post-partum (Figure 4.1); the greatest change occurs in the whey protein fraction (Figure 4.2). The natural function of milk proteins is to supply young mammals with the essential amino acids required for the development of muscular and other protein-containing tissues, and with a number of biologically active proteins, e.g. immunoglobulins, vitamin-binding, metal-binding proteins and various protein hormones. The young of different species are born at very different states of maturity, and, consequently, have different nutritional and physiological requirements. These differences are reflected in the protein content of the milk of the species, which ranges

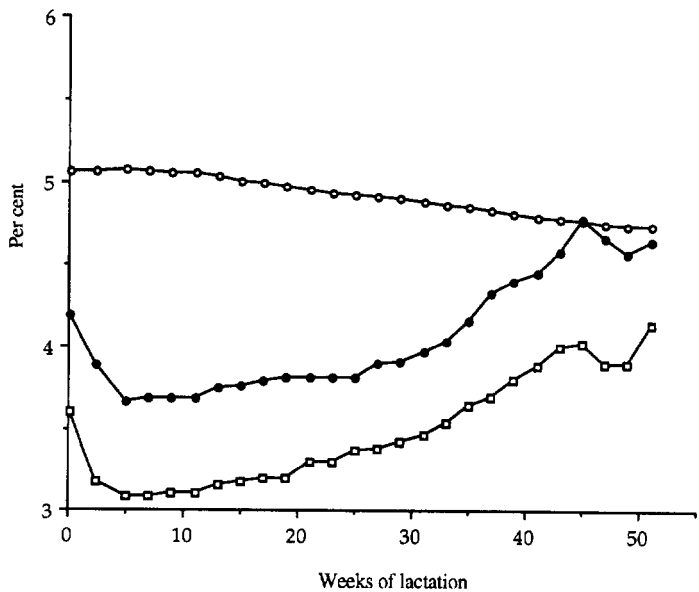


Figure 4.1 Changes in the concentrations of lactose (○), fat (●) and protein (□) in bovine milk during lactation.

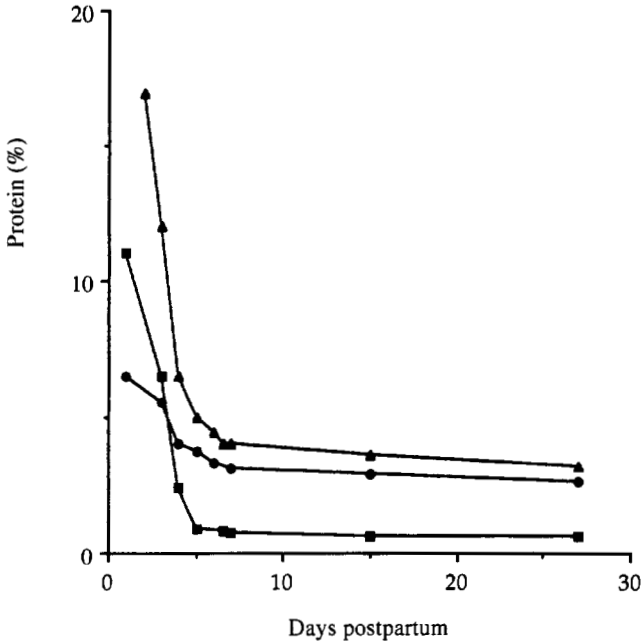


Figure 4.2 Changes in the concentration of total protein (▲) and of casein (●) and whey proteins (■) in bovine milk during the early stage of lactation.

from *c.* 1 to *c.* 24% (Table 4.1). The protein content of milk is directly related to the growth rate of the young of that species (Figure 4.3), reflecting the requirements of protein for growth.

The properties of many dairy products, in fact their very existence, depend on the properties of milk proteins, although the fat, lactose and especially the salts, exert very significant modifying influences. Casein products are almost exclusively milk protein while the production of most cheese varieties is initiated through the specific modification of proteins by proteolytic enzymes or isoelectric precipitation. The high heat treatments to which many milk products are subjected are possible only because of the exceptionally high heat stability of the principal milk proteins, the caseins.

Traditionally, milk was paid for mainly on the basis of its fat content but milk payments are now usually based on the content of fat plus protein. Specifications for many dairy products include a value for protein content. Changes in protein characteristics, e.g. insolubility as a result of heat denaturation in milk powders or the increasing solubility of cheese proteins during ripening, are industrially important features of these products.

It is assumed that the reader is familiar with the structure of proteins; for convenience, the structures of the amino acids found in milk are given in

Table 4.1 Protein content (%) in the milks of some species

Species	Casein	Whey protein	Total
Bison	3.7	0.8	4.5
Black bear	8.8	5.7	14.5
Camel (bactrian)	2.9	1.0	3.9
Cat	–	–	11.1
Cow	2.8	0.6	3.4
Domestic rabbit	9.3	4.6	13.9
Donkey	1.0	1.0	2.0
Echidna	7.3	5.2	12.5
Goat	2.5	0.4	2.9
Grey seal	–	–	11.2
Guinea-pig	6.6	1.5	8.1
Hare	–	–	19.5
Horse	1.3	1.2	2.5
House mouse	7.0	2.0	9.0
Human	0.4	0.6	1.0
Indian elephant	1.9	3.0	4.9
Pig	2.8	2.0	4.8
Polar bear	7.1	3.8	10.9
Red kangaroo	2.3	2.3	4.6
Reindeer	8.6	1.5	10.1
Rhesus monkey	1.1	0.5	1.6
Sheep	4.6	0.9	5.5
White-tailed jack rabbit	19.7	4.0	23.7

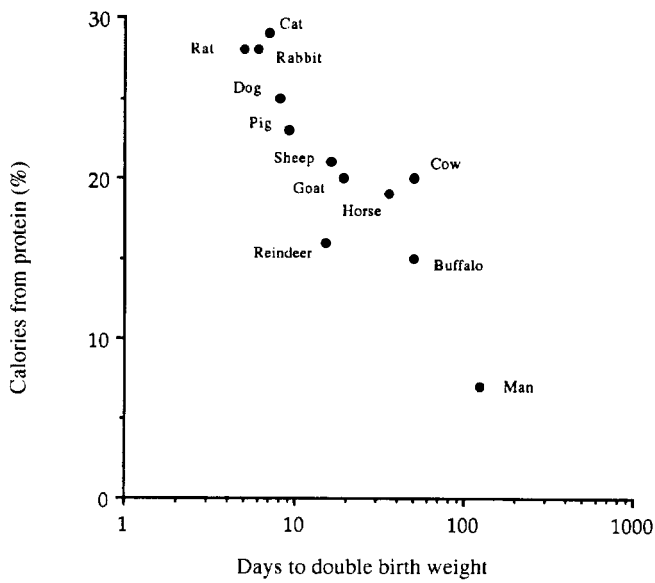


Figure 4.3 Relationship between the growth rate (days to double birth weight) of the young of some species of mammal and the protein content (expressed as % of total calories derived from protein) of the milk of that species (from Bernhart, 1961).

Appendix 4A. We have retained the term cystine to indicate two disulphide-linked cysteines.

4.2 Heterogeneity of milk proteins

Initially, it was believed that milk contained only one type of protein but about 100 years ago it was shown that the proteins in milk could be fractionated into two well-defined groups. On acidification to pH 4.6 (the isoelectric pH) at around 30°C, about 80% of the total protein in bovine milk precipitates out of solution; this fraction is now called **casein**. The protein which remains soluble under these conditions is referred to as **whey** or **serum protein** or **non-casein nitrogen**. The pioneering work in this area was done by the German scientist, Hammarsten, and consequently isoelectric (acid) casein is sometimes referred to as **casein nach Hammarsten**.

The ratio of casein : whey proteins shows large interspecies differences; in human milk, the ratio is c. 40 : 60, in equine (mare's) milk it is 50 : 50 while in the milks of the cow, goat, sheep and buffalo it is *c.* 80 : 20. Presumably, these differences reflect the nutritional and physiological requirements of the young of these species.

There are several major differences between the caseins and whey proteins, of which the following are probably the most significant, especially from an industrial or technological viewpoint:

1. In contrast to the caseins, the whey proteins do not precipitate from solution when the pH of milk is adjusted to 4.6. This characteristic is used as the usual **operational definition** of casein. This difference in the properties of the two milk protein groups is exploited in the preparation of industrial casein and certain varieties of cheese (e.g. cottage, quarg and cream cheese). Only the casein fraction of milk protein is normally incorporated into these products, the whey proteins being lost in the whey.
2. Chymosin and some other proteinases (known as rennets) produce a very slight, specific change in casein, resulting in its coagulation in the presence of Ca^{2+}. Whey proteins undergo no such alteration. The coagulability of casein through the action of rennets is exploited in the manufacture of most cheese varieties and rennet casein; the whey proteins are lost in the whey. The rennet coagulation of milk is discussed in Chapter 10.
3. Casein is very stable to high temperatures; milk may be heated at its natural pH (*c.* 6.7) at 100°C for 24 h without coagulation and it withstands heating at 140°C for up to 20 min. Such severe heat treatments cause many changes in milk, e.g. production of acids from lactose resulting in a decrease in pH and changes in the salt balance, which eventually cause the precipitation of casein. The whey proteins, on the

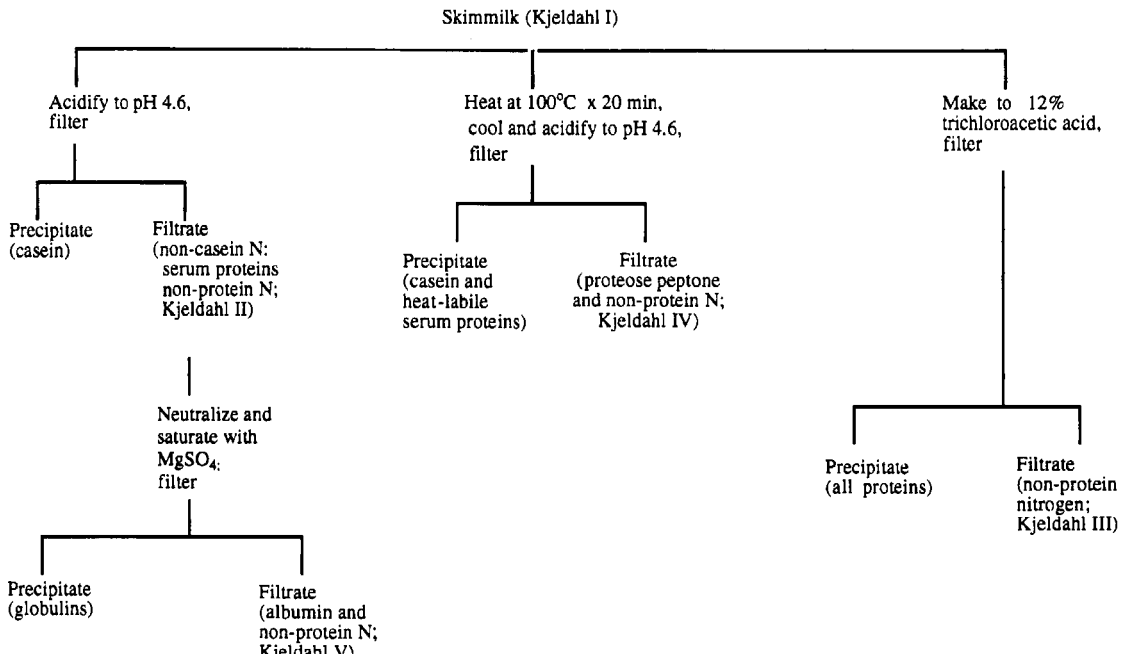
other hand, are relatively heat labile, being completely denatured by heating at 90°C for 10 min. Heat-induced changes in milk are discussed in Chapter 9.

4. Caseins are phosphoproteins, containing, on average, 0.85% phosphorus, while the whey proteins contain no phosphorus. The phosphate groups are responsible for many of the important characteristics of casein, especially its ability to bind relatively large amounts of calcium, making it a very nutritionally valuable protein, especially for young animals. The phosphate, which is esterified to the protein via the hydroxyl group of serine, is generally referred to as **organic phosphate**. Part of the inorganic phosphorus in milk is also associated with the casein in the form of **colloidal calcium phosphate** (*c.* 57% of the inorganic phosphorus) (Chapter 5).

   The phosphate of casein is an important contributor to its remarkably high heat stability and to the calcium-induced coagulation of rennet-altered casein (although many other factors are involved in both cases).
5. Casein is low in sulphur (0.8%) while the whey proteins are relatively rich (1.7%). Differences in sulphur content become more apparent if one considers the levels of individual sulphur-containing amino acids. The sulphur of casein is present mainly in methionine, with low concentrations of cysteine and cystine; in fact the principal caseins contain *only* methionine. The whey proteins contain significant amounts of both cysteine and cystine in addition to methionine and these amino acids are responsible, in part, for many of the changes which occur in milk on heating, e.g. cooked flavour, increased rennet coagulation time (due to interaction between β-lactoglobulin and κ-casein) and improved heat stability of milk pre-heated prior to sterilization.
6. Casein is synthesized in the mammary gland and is found nowhere else in nature. Some of the whey proteins (β-lactoglobulin and α-lactalbumin) are also synthesized in the mammary gland, while others (e.g. bovine serum albumin and the immunoglobulins) are derived from the blood.
7. The whey proteins are molecularly dispersed in solution or have simple quaternary structures, whereas the caseins have a complicated quaternary structure and exist in milk as large colloidal aggregates, referred to as micelles, with particle masses of 10^6-10^9 Da.
8. Both the casein and whey protein groups are heterogeneous, each containing several different proteins.

4.2.1 Other protein fractions

In addition to the caseins and whey proteins, milk contains two other groups of proteins or protein-like material, i.e. the proteose–peptone fraction and the non-protein nitrogen (NPN) fraction. These fractions were recognized as early as 1938 by Rowland but until recently very little was



Total nitrogen = Kjeldahl I
Casein = Kjeldahl I – Kjeldahl II
Non-protein nitrogen = Kjeldahl III

Proteose peptone N = Kjeldahl IV – Kjeldahl III
Serum protein = Kjeldahl II – Kjeldahl IV

Figure 4.4 Scheme for quantifying the principal protein fractions in milk.

known about them. Rowland observed that when milk was heated to 95°C for 10 min, 80% of the nitrogenous compounds in whey were denatured and co-precipitated with the casein when the pH of the heated milk was adjusted subsequently to 4.6. He considered that the heat-denaturable whey proteins represented the lactoglobulin and lactalbumin fractions and designated the remaining 20% 'proteose–peptone'. The proteose–peptone fraction was precipitated by 12% trichloracetic acid (TCA) but some nitrogenous compounds remained soluble in 12% TCA and were designated as nonprotein nitrogen.

A scheme for the fractionation of the principal groups of milk proteins, based on that of Rowland, is shown in Figure 4.4.

4.3 Preparation of casein and whey proteins

Skim milk prepared by mechanical separation (see Chapter 3) is used as the starting material for the preparation of casein and whey proteins.

4.3.1 Acid (isoelectric) precipitation

Acidification of milk to about pH 4.6 induces coagulation of the casein. Aggregation occurs at all temperatures, but below about 6°C the aggregates are very fine and remain in suspension, although they can be sedimented by low-speed centrifugation. At higher temperatures (30–40°C), the aggregates are quite coarse and precipitate readily from solution. At temperatures above about 50°C, the precipitate tends to be stringy and difficult to handle.

For laboratory-scale production of casein, HCl is usually used for acidification; acetic or lactic acids are used less frequently. Industrially, HCl is also usually used; H_2SO_4 is used occasionally but the resulting whey is not suitable for animal feeding ($MgSO_4$ is a laxative). Lactic acid produced *in situ* by a culture of lactic acid bacteria is also widely used, especially in New Zealand, the principal producer of casein.

The inorganic colloidal calcium phosphate associated with casein in normal milk dissolves on acidification of milk to pH 4.6 so that if sufficient time is allowed for solution, isoelectric casein is essentially free of calcium phosphate. In the laboratory, best results are obtained by acidifying skim milk to pH 4.6 at 2°C, holding for about 30 min and then warming to 30–35°C. The fine precipitate formed at 2°C allows time for the colloidal calcium phosphate to dissolve (Chapter 5). A moderately dilute acid (1 M) is preferred, since concentrated acid may cause localized coagulation. Acid production by a bacterial culture occurs slowly and allows time for colloidal calcium phosphate to dissolve. The casein is recovered by filtration or centrifugation and washed repeatedly with water to free the casein of lactose and salts. Thorough removal of lactose is essential since even traces of

lactose will interact with casein on heating via the Maillard browning reaction, with undesirable consequences.

The procedure used for the industrial production of acid (isoelectric) casein is essentially the same as that used on a laboratory scale, except for many technological differences (section 4.15.1).The whey proteins may be recovered from the whey by salting out, dialysis or ultrafiltration.

4.3.2 Centrifugation

Because they occur as large aggregates, micelles, most (90–95%) of the casein in milk is sedimented by centrifugation at 100 000 g for 1 h. Sedimentation is more complete at higher (30–37°C) than at low (2°C) temperature, at which some of the casein components dissociate from the micelles and are non-sedimentable. Casein prepared by centrifugation contains its original level of colloidal calcium phosphate and can be redispersed as micelles with properties essentially similar to the original micelles.

4.3.3 Centrifugation of calcium-supplemented milk

Addition of $CaCl_2$ to about 0.2 M causes aggregation of the casein such that it can be readily removed by low-speed centrifugation. If calcium is added at 90°C, the casein forms coarse aggregates which precipitate readily. This principle is used in the commercial production of some 'casein co-precipitates' in which the whey proteins, denatured on heating milk at 90°C for 10 min, co-precipitate with the casein. Such products have a very high ash content.

4.3.4 Salting-out methods

Casein can be precipitated from solution by any of several salts. Addition of $(NH_4)_2SO_4$ to milk to a concentration of 260 g l^{-1} causes complete precipitation of the casein together with some whey proteins (immunoglobulins, Ig). $MgSO_4$ may also be used. Saturation of milk with NaCl at 37°C precipitates the casein and Igs while the major whey proteins remain soluble, provided they are undenatured. This characteristic is the basis of a commercial test used for the heat classification of milk powders which contain variable levels of denatured whey proteins.

4.3.5 Ultrafiltration

The casein micelles are retained by fine-pore filters. Filtration through large-pore ceramic membranes is used to purify and concentrate casein on a laboratory scale. Ultrafiltration (UF) membranes retain both the caseins

and whey proteins while lactose and soluble salts are permeable; total milk protein may be produced by this method. The casein micelles permeate the membranes used in microfiltration (pore size $\sim 0.05-10\,\mu m$) but bacteria are retained by membranes with pores of less than $0.5\,\mu m$, thus providing a method for removing more than 99.9% of the bacteria in milk without heat treatment; microfiltration is being used increasingly in several sectors of the dairy industry.

Industrially, whey proteins are prepared by ultrafiltration or diafiltration of whey (to remove lactose and salts), followed by spray drying; these products, referred to as whey protein concentrates, contain 30–80% protein.

4.3.6 Gel filtration (gel permeation chromatography)

Filtration through cross-linked dextrans (e.g. Sephadex, Pharmacia, Uppsala, Sweden) makes it possible to fractionate molecules, including proteins, on a commercial scale. It is possible to separate the casein and whey proteins by gel filtration but the process is uneconomical on an industrial scale.

4.3.7 Precipitation with ethanol

The caseins may be precipitated from milk by *c.* 40% ethanol while the whey proteins remain soluble; lower concentrations of ethanol may be used at lower pH values.

4.3.8 Cryoprecipitation

Casein, in a mainly micellar form, is destabilized and precipitated by freezing milk or, preferably, concentrated milk, at about $-10°C$; casein prepared by this method has some interesting properties but is not produced commercially at present.

4.3.9 Rennet coagulation

Casein may be coagulated and recovered as rennet casein by treatment of milk with selected proteinases (rennets). However, one of the caseins, κ-casein, is hydrolysed during renneting and therefore the properties of rennet casein differ fundamentally from those of acid casein. Rennet casein, which contains the colloidal calcium phosphate of milk, is insoluble in water at pH 7 but can be dissolved by adding calcium sequestering agents, usually citrates or polyphosphates. It has desirable functional properties for certain food applications, e.g. in the production of cheese analogues.

4.3.10 Other methods for the preparation of whey proteins

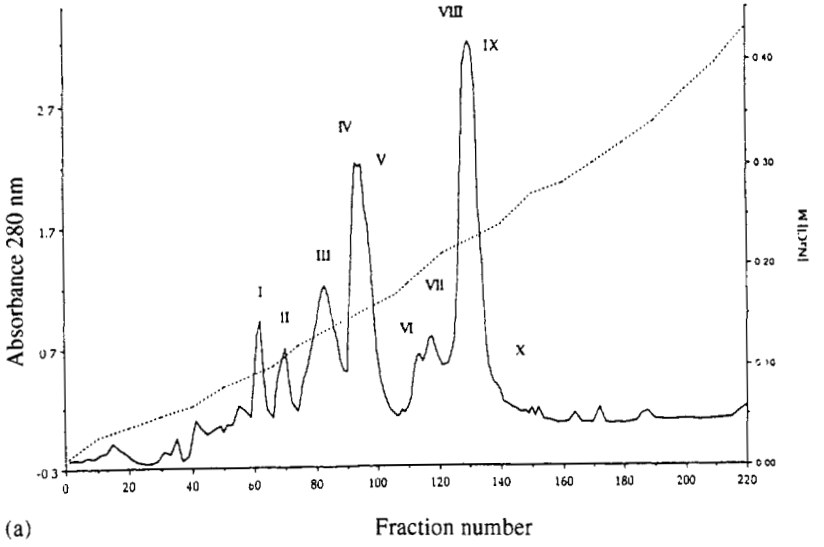
Highly purified whey protein preparations, referred to as whey protein isolates (containing 90–95% protein), are prepared industrially from whey by ion exchange chromatography. Denatured (insoluble) whey proteins, referred to as lactalbumin, may be prepared by heating whey to 95°C for 10–20 min at about pH 6.0; the coagulated whey proteins are recovered by centrifugation. The whey proteins may also be precipitated using $FeCl_3$ or polyphosphates (section 4.15.6).

4.4 Heterogeneity and fractionation of casein

Initially, casein was considered to be a homogeneous protein. Heterogeneity was first demonstrated in the 1920s by Linderstrøm-Lang and co-workers, using fractionation with ethanol-HCl, and confirmed in 1936 by Pedersen, using analytical ultracentrifugation, and in 1939 by Mellander, using free boundary electrophoresis. Three components were demonstrated and named α-, β- and γ-casein in order of decreasing electrophoretic mobility and represented 75, 22 and 3%, respectively, of whole casein. These caseins were successfully fractionated in 1952 by Hipp and collaborators based on differential solubilities in urea at *c.* pH 4.6 or in ethanol/water mixtures; the former is widely used although the possibility of forming artefacts through interaction of casein with cyanate produced from urea is of concern.

In 1956, Waugh and von Hippel showed that the α-casein fraction of Hipp *et al.* contained two proteins, one of which was precipitated by low concentrations of Ca^{2+} and was called α_s-casein (s = sensitive) while the other, which was insensitive to Ca^{2+}, was called κ-casein. α_s-Casein was later shown to contain two proteins which are now called α_{s1}- and α_{s2}-caseins. Thus, bovine casein contains four distinct gene products, designated α_{s1}-, α_{s2}-, β- and κ-caseins which represent approximately 37, 10, 35 and 12% of whole casein, respectively.

Various chemical methods were developed to fractionate the caseins but none gives homogeneous preparations. Fractionation is now usually achieved by ion-exchange chromatography on, for example, DEAE-cellulose, using urea-containing buffers; quite large (e.g. 10 g) amounts of caseinate can be fractionated by this method, with excellent results (Figure 4.5a, b). Good results are also obtained by ion-exchange chromatography using urea-free buffers at 2–4°C. High performance ion-exchange chromatography (e.g. Pharmacia FPLC™ on Mono Q or Mono S) gives excellent results for small amounts of sample (Figure 4.5c, d). Reversed-phase HPLC or hydrophobic interaction chromatography may also be used but are less effective than ion-exchange chromatography.



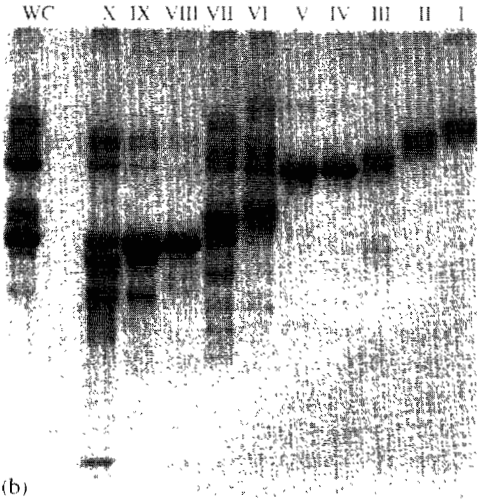
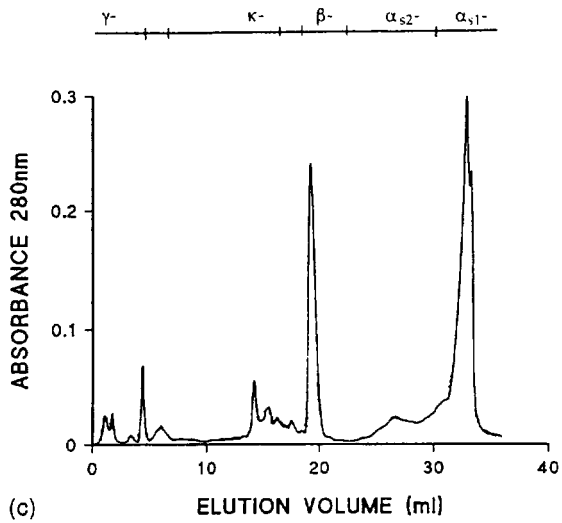
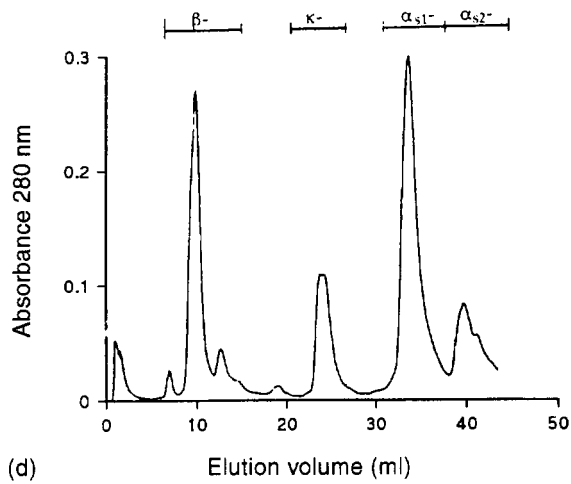


Figure 4.5 (a) Chromatogram of sodium caseinate on an open column of DEAE cellulose anion exchanger. Buffer: 5 M urea in imidazole–HCl buffer, pH 7.0; gradient: 0–0.5 M NaCl. (b) Urea polyacrylamide gel electrophoretograms of the fractions from (a). (c) Chromatogram of sodium caseinate on a Pharmacia Mono Q HR5/5 anion exchange column. Buffer: 6 M urea in 5 mM bis-tris-propane/7 mM HCl, pH 7; gradient: 0–0.5 M NaCl. (d) Chromatogram of sodium caseinate on a Pharmacia Mono S HR5/5 cation exchange column. Buffer: 8 M urea in 20 mM acetate buffer, pH 5; gradient: 0–1.0 M NaCl.



(c)



(d)

Figure 4.5 (*Continued*)

The caseins may be quantified by densitometrically scanning polyacrylamide gel electrophoretograms (section 4.4.1) but more quantitative results are obtained by ion-exchange chromatography using urea-containing buffers. However, it should be realized that the specific absorbance of the individual caseins differs greatly (Table 4.2).

Table 4.2 Properties of some milk proteins (modified from Walstra and Jenness, 1984)

	Caseins				Whey proteins		
Property	α_{s1}-B 8P	α_{s2}-A 11P	β-A^2 5P	κ-B 1P	α-la-B	β-lg-B	Serum albumin
Molecular weight	23 614	25 230	23 983	19 023[a]	14 176	18 363	66 267
Residues/molecule							
Amino acids	199	207	209	169	123	162	582
Proline	17	10	35	20	2	8	34
Cysteine	0	2	0	2	8	5	35
Intramolecular disulphide bonds	0	0	0	0	4	2	17
Phosphate	8	11	5	1	0	0	0
Carbohydrate	0	0	0	[b]	[c]	[d]	0
Hydrophobicity (kJ/residue)	4.9	4.7	5.6	5.1	4.7	5.1	4.3
Charge							
mol % residues	34	36	23	21	28	30	34
Net charge/residue	−0.10	−0.07	−0.06	−0.02[e]	−0.02	−0.04	−0.02
Distribution	Uneven	Uneven	Very uneven	Very uneven	Even	Even	
A_{280}	10.1	14.0[f]	4.5	10.5	20.9	9.5	6.6

[a]Exclusive of carbohydrate residues.
[b]Variable, see text.
[c]A small fraction of the molecules.
[d]0, except for a rare variant (Dr).
[e]Average.
[f]A_{290}.

4.4.1 Resolution of caseins by electrophoresis

Zonal electrophoresis in starch gels containing 7 M urea was used by Wake and Baldwin in 1961 to resolve casein into about 20 bands (zones); the two principal bands were α_{s1}- and β-caseins. Incorporation of urea was necessary to dissociate extensive intermolecular hydrophobic bonding. Electrophoresis in polyacrylamide gels (PAGE), containing urea or sodium dodecyl sulphate (SDS), was introduced in 1963; resolution was similar to starch gels (SGE) but since it is easier to use, PAGE has become the standard technique for analysis of caseins; a schematic representation of a urea–PAGE electrophoretogram of whole casein is shown in Figure 4.6. Owing to the presence of intermolecular disulphide bonds, κ-casein resolves poorly on

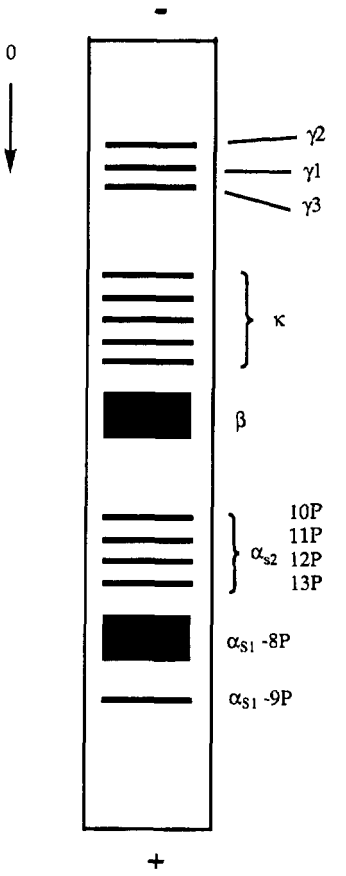


Figure 4.6 Schematic diagram of an electrophoretogram of sodium caseinate in a polyacrylamide gel containing 5 M urea in tris-hydroxymethylamine buffer, pH 8.9. 0 indicates origin.

SGE or PAGE unless it is reduced, usually by 2-mercaptoethanol ($HSCH_2CH_2OH$), or alkylated. Electrophoretic techniques for the analysis of casein were reviewed by Swaisgood (1975).

4.4.2 Microheterogeneity of the caseins

Each of the four caseins, α_{s1}, α_{s2}, β and κ, exhibits variability, which we will refer to as **microheterogeneity**, arising from five causes:

Variability in the degree of phosphorylation. Each of the four caseins is phosphorylated to a characteristic but variable level:

Casein	Number of phosphate residues
α_{s1}	8, occasionally 9
α_{s2}	10, 11, 12 or 13
β	5, occasionally 4
κ	1, occasionally 2 or perhaps 3

The number of phosphate groups in the molecule is indicated as α_{s1}-CN 8P or α_{s1}-CN 9P, etc. (CN = casein).

Disulphide bonding. The two principal caseins, α_{s1} and β, contain no cysteine or cystine but the two minor caseins, α_{s2} and κ, each contains two cysteines per mole which normally exist as intermolecular disulphide bonds. Under non-reducing conditions, α_{s2}-casein exists as a disulphide-linked dimer (previously known as α_{s5} casein) while κ-casein exists as a series of disulphide-linked molecules ranging from dimers to decamers.

Hydrolysis of primary caseins by plasmin. In 1969, Groves and coworkers showed that the γ-casein fraction, as isolated by Hipp *et al.*, is very heterogeneous, containing at least four distinct proteins: γ-casein, temperature-sensitive casein (TS, which is soluble in the cold but precipitates above 20°C), R-casein and S-casein. These four proteins were shown to be C-terminal fragments of β-casein. In 1976, the nomenclature of the γ-casein group was revised, as shown in Figure 4.7 and Table 4.3.

γ-Caseins are produced from β-casein by proteolysis by plasmin, an indigenous proteinase in milk (Chapter 8). The corresponding N-terminal fragments are the principal components of the proteose–peptone (PP) fraction, i.e. PP5 (β-CN f1–105/107), PP8 slow (β-CN f29–105/107) and PP8 fast (β-CN f1–28). Normally, the γ-caseins represent only about 3% of whole casein but levels may be very much higher (up to 10%) in late lactation and mastitic milks. Because of its high isoelectric point (6), some γ-casein may be lost on isoelectric precipitation. γ-Caseins can be readily

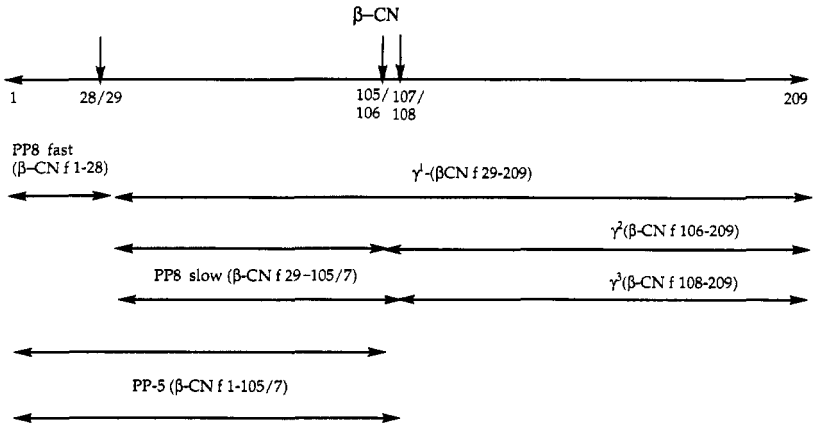


Figure 4.7 Principal products produced from β-casein by plasmin.

Table 4.3 Old and revised nomenclature for γ-caseins

Old	Trivial	Recommended nomenclature (β-casein sequence)
γ	γ-A^1, γ_1-A^2, γ_1-A^3, γ_1-B	β-CN A^1, A^2, A^3, B (f29–209)
TS-A^2	γ^2-A^2	β-CN A^2, (f106–209)
S	γ_2-B	β-CN B, (f106–209)
R	γ_3-A	β-CN A^2, (f108–209)
TS-B	γ_3-B	β-CN B (f108–209)

A and B indicate genetic variants, see p. 162.

prepared by chromatography on DEAE-cellulose since they do not adsorb even at low ionic strength (0.02 M) at pH 6.5; γ^1-casein adsorbs at pH 8.5 but γ^2- and γ^3-caseins do not.

Isolated α_{s2}-casein in solution is also very susceptible to plasmin; eight peptide bonds are hydrolysed with the production of 14 peptides. Plasmin also hydrolyses α_{s2}-casein in milk but the peptides formed have not been identified, although at least some are included in the proteose–peptone fraction.

Although less susceptible than β- and α_{s2}-caseins, isolated α_{s1}-casein in solution is also readily hydrolysed by plasmin. It has been suggested that a minor ill-defined fraction of casein, called λ-casein, consists of plasmin-produced fragments of α_{s1}-casein, but the situation is unclear.

Variations in the degree of glycosylation. κ-Casein is the only one of the principal milk proteins which is normally glycosylated but, as discussed on

p. 173, the level of glycosylation varies, resulting in 10 molecular forms of κ-casein.

Genetic polymorphism. In 1956, Aschaffenburg and Drewry discovered that the whey protein, β-lactoglobulin (β-lg), exists in two forms, A and B, which differ from each other by only a few amino acids. The milk of any individual animal may contain β-lg A or B or both, and the milk is indicated as AA, BB or AB with respect to β-lg. This phenomenon was referred to as **genetic polymorphism** and has since been shown to occur in all milk proteins; a total of about 30 variants have been demonstrated by PAGE. Since PAGE differentiates on the basis of charge, only polymorphs which differ in charge, i.e. in which a charged residue is replaced by an uncharged one or vice versa, will be detected; therefore, it is very likely that many more than 30 polymorphs exist.

The genetic variant present is indicated by a Latin letter, e.g. α_{s1}-CN A-8P, α_{s1}-CN B-8P, α_{s1}-CN B-9P, etc.

The frequency with which certain genetic variants occurs is breed-specific, and hence genetic polymorphism has been useful in the phylogenetic classification of cattle and other species. Various technologically important properties of the milk proteins, e.g. cheesemaking properties and the concentration of protein in milk, are correlated (linked) with specific polymorphs and significant research is ongoing on this subject. The genetic polymorphism of milk proteins has been comprehensively reviewed by Ng-Kwai-Hang and Grosclaude (1992) and Jakob and Puhan (1992).

4.4.3 Nomenclature of the caseins

During studies on casein fractionation, especially during the 1960s, various names were assigned to isolated fractions. To rationalize the nomenclature of milk proteins, the American Dairy Science Association established a Nomenclature Committee which published its first report in 1956 (Jenness *et al.*, 1956); the report has been revised regularly (Brunner *et al.*, 1960; Thompson *et al.*, 1965; Rose *et al.*, 1970; Whitney *et al.*, 1976; Eigel et al., 1984). An example of the recommended nomenclature is α_{s1}-CN A-8P, where α_{s1}-CN is the gene product, A is the genetic variant and 8P is the number of phosphate residues. The Committee recommends that in situations where confusion may arise through the use of a Greek letter alone, the relative electrophoretic mobility be given in brackets, thus α_{s2}-CN A-12P (1.00). The heterogeneity and nomenclature of the caseins in bovine milk is summarized in Figure 4.8.

In addition to simplifying and standardizing the nomenclature of the milk proteins, the characteristics of the various caseins and whey proteins are summarized in the above articles, which are very valuable references.

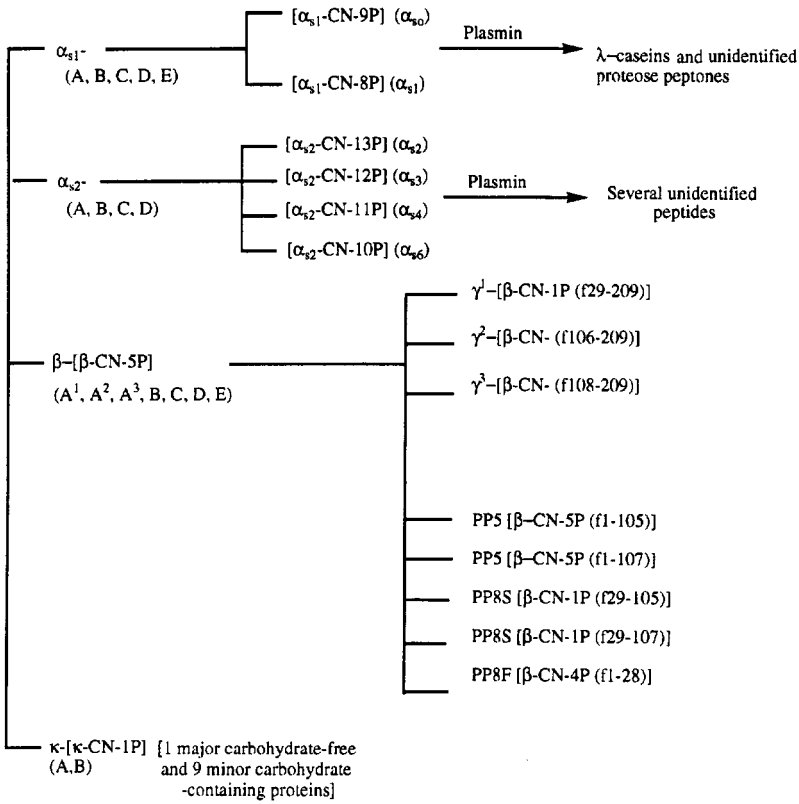


Figure 4.8 Heterogeneity of bovine casein.

4.5 Some important properties of the caseins

4.5.1 Chemical composition

The principal chemical and physicochemical properties of the principal milk proteins are summarized in Table 4.2. Some of the properties of the caseins are discussed in more detail below (see Swaisgood, 1992, for a review).

Amino acid composition. The approximate amino acid composition of the main caseins is shown in Table 4.4. Amino acid substitutions in the principal genetic variants can be deduced from the primary structures (Figures 4.9–4.12). Four features of the amino acid profile are noteworthy:

1. All the caseins have a high content (35–45%) of apolar amino acids (Val, Leu, Ile, Phe, Tyr, Pro) and would be expected to be poorly soluble in aqueous systems, but the high content of phosphate groups, low level of

Table 4.4 Amino acid composition of the major proteins occurring in the milk of western cattle (Swaisgood, 1982)

Acid	α_{s1}-Casein B	α_{s2}-Casein A	κ-Casein B	β-Casein A[2]	γ_1-Casein A[2]	γ_2-Casein A[2]	γ_3-Casein A	β-Lacto-globulin A	α-Lact-albumin B
Asp	7	4	4	4	4	2	2	11	9
Asn	8	14	7	5	3	1	1	5	12
Thr	5	15	14	9	8	4	4	8	7
Ser	8	6	12	11	10	7	7	7	7
SerP	8	11	1	5	1	0	0	0	0
Glu	24	25	12	18	11	4	4	16	8
Gln	15	15	14	21	21	11	11	9	5
Pro	17	10	20	35	34	21	21	8	2
Gly	9	2	2	5	4	2	2	3	6
Ala	9	8	15	5	5	2	2	14	3
$\frac{1}{2}$ Cys	0	2	2	0	0	0	0	5	8
Val	11	14	11	19	17	10	10	10	6
Met	5	4	2	6	6	4	4	4	1
Ile	11	11	13	10	7	3	3	10	8
Leu	17	13	8	22	19	14	14	22	13
Tyr	10	12	9	4	4	3	3	4	4
Phe	8	6	4	9	9	5	5	4	4
Trp	2	2	1	1	1	1	1	2	4
Lys	14	24	9	11	10	4	3	15	12
His	5	3	3	5	5	4	3	2	3
Arg	6	6	5	4	2	2	2	3	1
PyroGlu	0	0	1	0	0	0	0	0	0
Total residues	199	207	169	209	181	104	102	162	123
Molecular weight	23 612	25 228	19 005	23 980	20 520	11 822	11 557	18 362	14 174
$H\Phi_{ave}$ (kJ/residue)	4.89	4.64	5.12	5.58	5.85	6.23	6.29	5.03	4.68

1 Glu-Val-Leu-Asn-Glu-Asn-Leu-
H. Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-.....
21
Leu-Arg-Phe-Phe-Val-Ala-(Variants B, C, D, E)
-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu
.....(Variant A)

41 Ala (Variants A, B, C, E) Gln (Variants A, B,
Ser-Lys-Asp-Ile-Gly-*SerP*-Glu-*SerP*-Thr-Glu-Asp-Gln- -Met-Glu-Asp-Ile-Lys- -Met
ThrP (Variant D) Glu (Variant E)
61
Glu-Ala-Glu-*SerP*-Ile-*SerP*-*SerP*-*SerP*-Glu-Glu-Ile-Val-Pro-Asn-*SerP*-Val-Glu-Gln-Lys-His-

81
Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-

101
Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-*SerP*-Ala-Glu-Glu-Arg-Leu-

121
His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-

141
Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-

161
Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-

181 Glu (Variant A, B, D) 199
Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser- -Lys-Thr-Thr-Met-Pro-Leu-Trp. OH
Gly (Variant C, E)

Figure 4.9 Amino acid sequence of bovine α_{s1}-casein, showing the amino acid substitutions or deletions in the principal genetic variants (from Swaisgood, 1992).

sulphur-containing amino acids and high carbohydrate content in the case of κ-casein offset the influence of apolar amino acids. The caseins are, in fact, quite soluble: solutions containing up to 20% protein can be prepared in water at 80–90°C. High temperatures are necessary to offset high viscosity, which is the limiting factor in preparing casein solutions. The high viscosity is a reflection of the high water binding capacity (WBC) of casein, i.e. about 2.5 g $H_2O\,g^{-1}$ protein. Such high WBC gives casein very desirable functional properties for incorporation into various foods, e.g. sausage and other comminuted meat products, instant desserts, synthetic whipping creams, etc., and large quantities of casein are used commercially for these purposes.

2. All the caseins have a very high proline content: 17, 10, 35 and 20 Pro residues per mole of α_{s1}-, α_{s2}-, β- and κ-caseins, respectively (out of a total of 199, 207, 209 and 169 residues, respectively). Such high levels of proline

1
H. Lys-Asn-Thr-Met-Glu-His-Val-*SerP*-*SerP*-*SerP*-Glu-Glu-Ser-Ile-Ile-*SerP*-Gln-Glu-Thr-Tyr-

21
Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro-Ser-Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-

41
Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-*SerP*-*SerP*-*SerP*-Glu-Glu-

61
SerP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-

81
Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe-Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-

101
Gln-Gly-Pro-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-

121
Pro-Thr-Leu-Asn-Arg-Glu-Gln-Leu-*SerP*-Thr-*SerP*-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-

141
Met-Glu-*SerP*-Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-

161
Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-

181
Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-
(Leu)
201 207
Ile-Pro-Tyr-Val-Arg-Tyr-Leu. OH

Figure 4.10 Amino acid sequence of bovine α_{s2}-casein A, showing nine of the 10–13 phosphorylation sites (from Swaisgood, 1992).

result in a very low content of α-helix or β-sheet structures in the caseins. The caseins are, therefore, readily susceptible to proteolysis without prior denaturation by, for example, acid or heat. Perhaps this is an important characteristic in neonatal nutrition.

3. As a group, the caseins are deficient in sulphur amino acids which limits their biological value (80; egg albumen = 100). α_{s1}- and β-caseins contain no cysteine or cystine while α_{s2}- and κ-caseins have two cysteine residues per mole, which normally exist as intermolecular disulphides.

   The principal sulphydryl-containing protein in milk is the whey protein β-lactoglobulin (β-lg), which contains one sulphydryl group; normally, this sulphydryl group is buried within the molecule and is unreactive. Following denaturation, e.g. by heat above *c.* 75°C, the —SH group of β-lg becomes exposed and reactive and undergoes a sulphydryl–disulphide interchange with κ-casein (and possibly with α_{s2}-casein and α-lactalbumin also) with very significant effects on some of the technologically important physicochemical properties of milk, e.g. heat stability and rennet coagulability (Chapters 9 and 10).

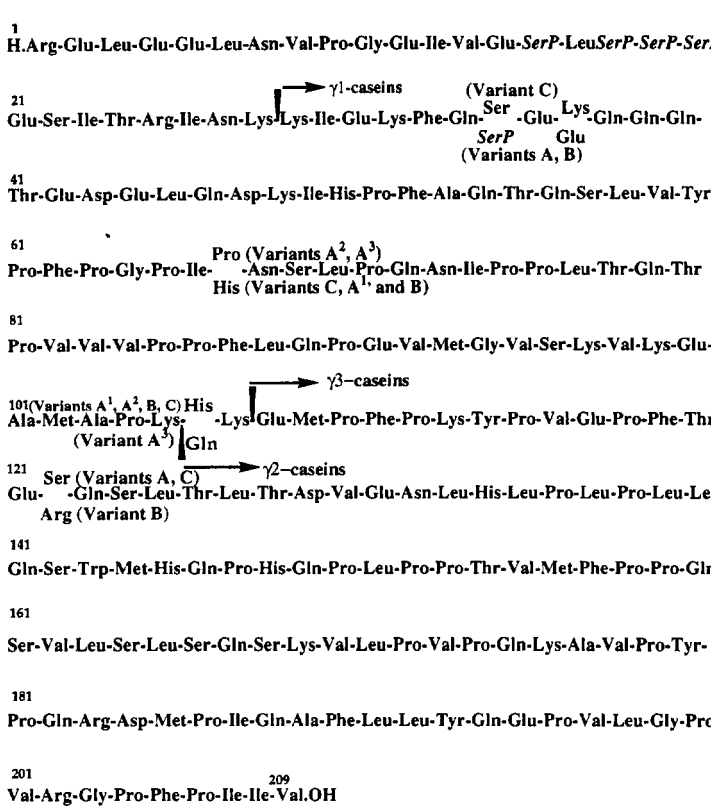


Figure 4.11 Amino acid sequence of bovine β-casein, showing the amino acid substitutions in the genetic variants and the principal plasmin cleavage sites (▼) (from Swaisgood, 1992).

4. The caseins, especially α_{s2}-casein, are rich in lysine, an essential amino acid in which many plant proteins are deficient. Consequently, casein and skim-milk powder are very good nutritional supplements for cereal proteins which are deficient in lysine. Owing to the high lysine content, casein and products containing it may undergo extensive non-enzymatic Maillard browning on heating in the presence of reducing sugars (Chapter 2).

   At pH values on the acid side of their isoelectric point, proteins carry a net positive charge and react with anionic dyes (e.g. amido black or orange G), forming an insoluble protein–dye complex. This is the principle of the rapid dye-binding methods for quantifying proteins in milk and milk products and for visualizing protein bands in gel electrophoretograms; dye-binding is normally performed at pH 2.5–3.5.

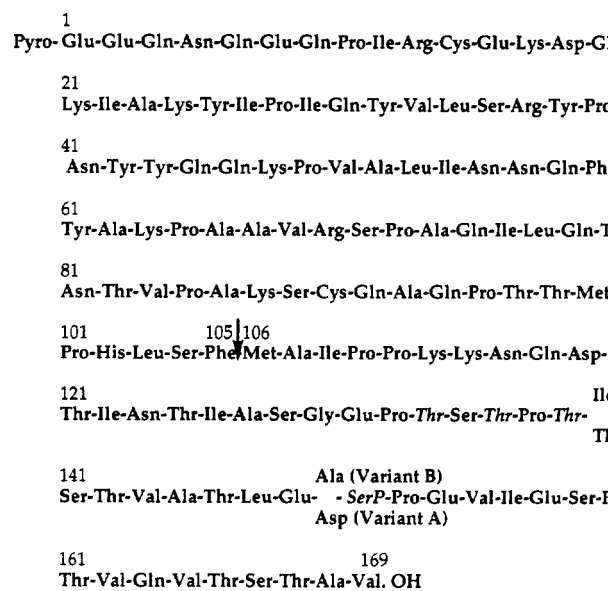


Figure 4.12 Amino acid sequence of bovine κ-casein, showing the amino acid substitutions in genetic polymorphs A and B and the chymosin cleavage site, ↓. Sites of post-translational phosphorylation or glycosylation are italicized (from Swaisgood, 1992).

Lysine is the principal cationic residue in caseins, with lesser amounts of arginine and histidine ($pK_a \sim 6$).

Since the caseins differ in lysine content (14, 24, 11 and 9 residues for α_{s1}-, α_{s2}-, β- and κ-caseins, respectively) they have different dye-binding capacities. This feature may be of some commercial significance in connection with dye-binding methods for protein analysis if the ratio of the caseins in the milks of individual animals varies (as it probably does). It should also be considered when calculating the protein concentration of zones on electrophoretograms stained with these dyes.

The absorbance of 1% solutions of α_{s1}-, α_{s2}-, β- and κ-caseins at 280 nm in a 1 cm light path is 10.1, 14.0, 4.4 and 10.5, respectively. Since the protein concentration in eluates from chromatography columns is usually monitored by absorbance at 280 nm, cognisance should be taken of the differences in specific absorbance when calculating the concentrations of individual caseins in samples.

Primary structure. The primary structures of the four caseins of bovine milk are shown in Figures 4.9–4.12. The sequences of some non-bovine caseins have been established also.

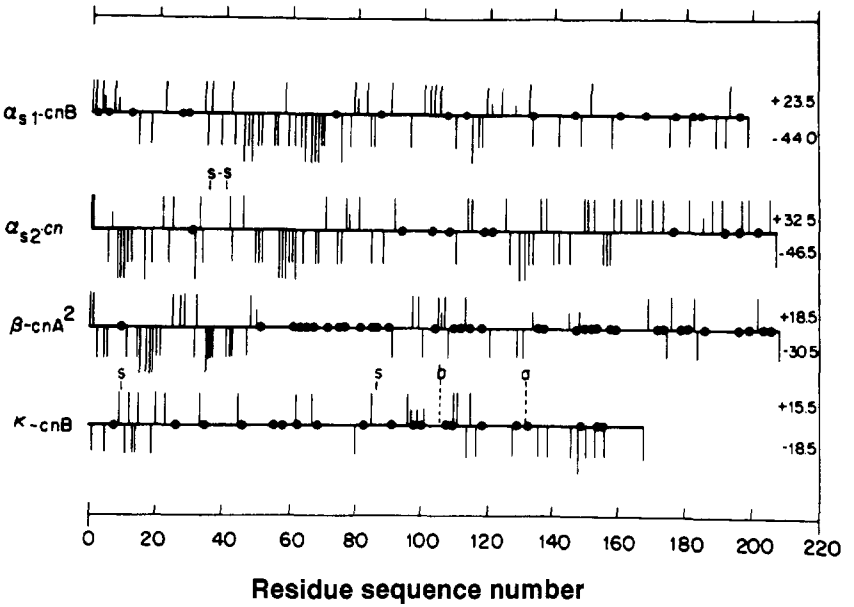


Figure 4.13 Distribution of charged residues (pH 6–7), proline (●) and cysteine (S) in α_{s1}-, α_{s2}-, β- and κ-caseins. a, Location of oligosaccharide moieties; and b, chymosin cleavage site in κ-casein (from Walstra and Jenness, 1984).

An interesting feature of the primary structures of all caseins is that polar and apolar residues are not uniformly distributed but occur in clusters, giving hydrophobic and hydrophilic regions (Figures 4.13–4.15). This feature makes the caseins good emulsifiers. The organic phosphates, which are attached to serines, occur in clusters due to the mechanism by which phosphorylation occurs (see below and section 4.14.4). The phosphate clusters bind Ca^{2+} strongly. The proline residues are fairly uniformly distributed, giving the caseins a type of poly-proline helix. β-Casein is the most hydrophobic of the caseins and α_{s2}-casein is the most hydrophilic. The C-terminal region of κ-casein is strongly hydrophilic due to a high content of sugars (in some cases), few apolar residues and no aromatic residues, while the N terminus is strongly hydrophobic; this detergent-like structure is probably important in micelle stabilization. The hydrophilic segment of κ-casein is cleaved off during rennet action, rendering the residual caseins coagulable by Ca^{2+} (Chapter 10).

The caseins are one of the most evolutionarly divergent families of mammalian proteins. Since their function is nutritional, minor amino acid substitutions or deletions are not critical. Holt and Sawyer (1993), who aligned the published sequences of α_{s1}-, β- and κ-caseins from various

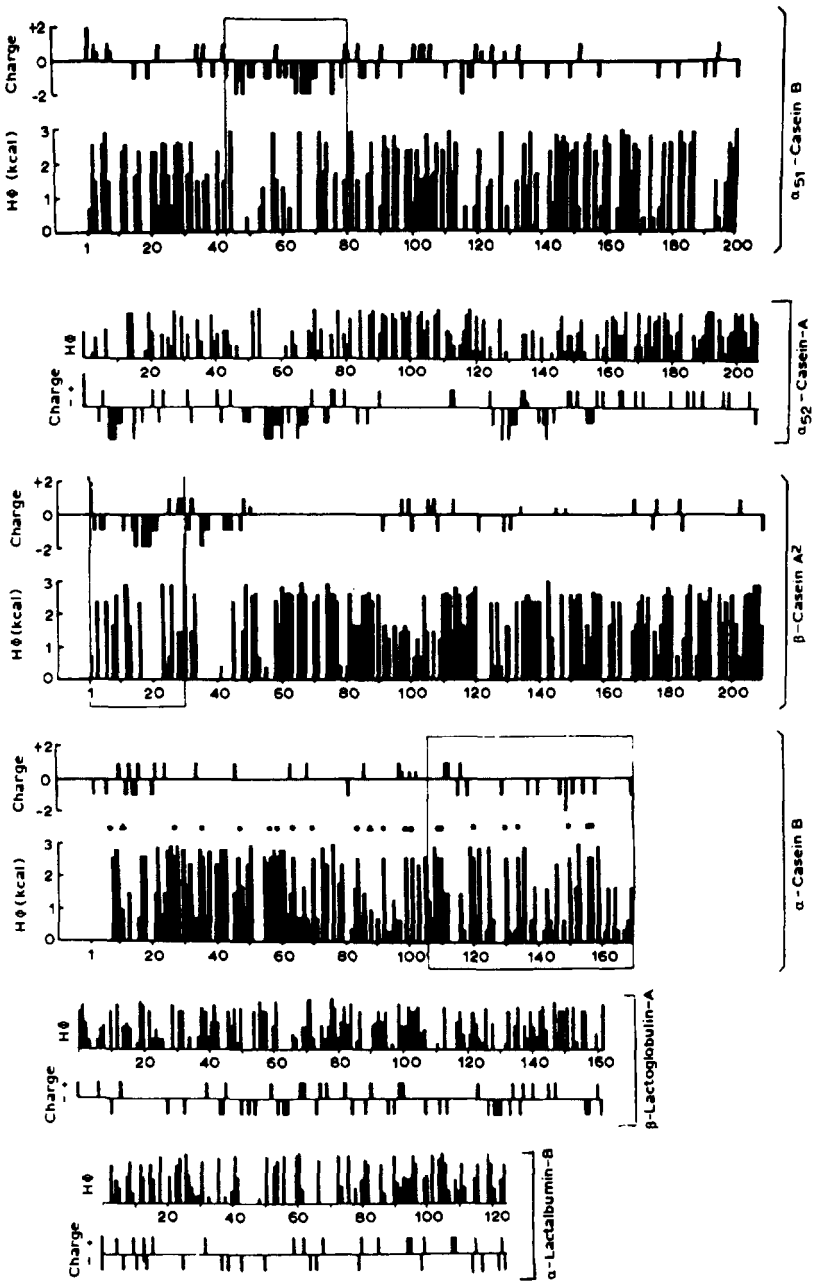


Figure 4.14 Schematic representation of the distribution of hydrophobic and charged residues in the principal milk proteins (from Swaisgood, 1992).

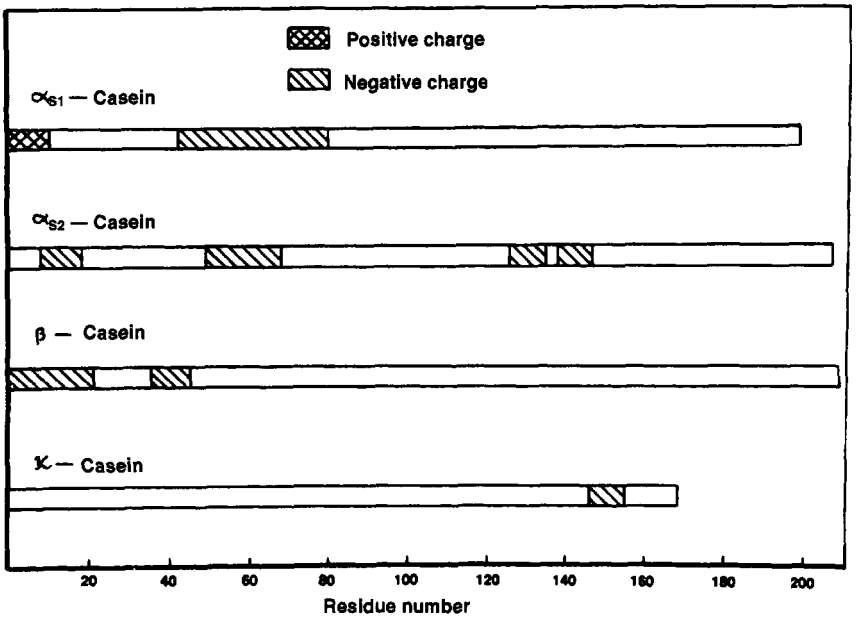


Figure 4.15 Ten residue sequences of bovine caseins with a charge density of 0.5 or greater at pH 6.6 (from Swaisgood, 1992).

species, found very little homology. Although the sequences of β-caseins from cow, sheep, mouse, rat, rabbit and human could be aligned readily, very little homology was evident between all six species (Figure 4.16): the only long homologous sequence was the signal peptide, the two N-terminal residues of the mature protein and the sequence SSEE (residues 18–21 of the mature protein, which is the principal phosphorylation site). The sequence of the signal peptides of α_{s1}- and κ-caseins also show a high degree of interspecies homology but several long insertions were required to obtain even a moderate degree of alignment of the sequences of the mature proteins.

Casein phosphorus. Milk contains about 900 mg phosphorus l^{-1}, which occurs in five types of phosphate-containing compounds, as will be discussed in Chapter 5:

- inorganic: soluble and colloidal phosphates;
- organic: phospholipids, casein and sugar phosphates, nucleotides (ATP, UTP, etc.).

Whole casein contains about 0.85% phosphorus; α_{s1}-, β- and κ-caseins contain 1.1, 0.6 and 0.16% P, respectively; on a molar basis, α_{s1}-, α_{s2}-, β- and

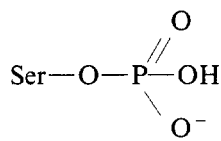
```
                10    ↓   20        30        40        50        60        70        80        90        100
COW   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRIN-KKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPG--PIP-NSLPQNIPPLTQTP
SHE   MKVLILACLVALALAREQEELNVVGETVESLSSSEESITHIN-KKIEKFQSEEQQQTEDELQDKIHPFAQAQSLVYPFTG--PIP-NSLPQNILPLTQTP
MOU   MKVFILACLVALALARE-TTFTVSSET-DSI-SSEESVEHINEQKLQKVNLMGQLQAEDVLQAKVHSSIQSQPQAFPYAQAQTISCNPVPQNIQPIAQPP
RAT   MKVFILACLVALALAREKDAFTVSSET-GSI-SSEESVEHINE-KLQKVKLMGQVQSEDVLQNKFHSGIQSEPKAIPYAQ--TISCSPIPQNIQPIAQPP
RAB   MKVLILACLVALALAREKEQLSVPTEAVGSVSSSEE-ITHINKQKLETIKHVEQLLREEKLQDKILPFIQS---LFPFAE--RIPYPTLPQNILNLAQLD
MAN   MKVLILACLVALALARE---------TIESLSSSEESITEYK-QKVEKVKHEDQQQGEDEHQDKIYPSFQPQPLIYPFVE--PIPYGFLPQNILPLAQPA
      ***+**************           *+ **** ++ ++  *+++++   • + *+ +* *+ +  *       *+    +*+    **** +++*

                110       120       130       140       150       160       170       180       190       200
COW   VVV--PPFLQPEVMGVSKVKEAMAPKHKEMPFPKYP-VEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVM-FPPQSVLSLSQSKVLPVPQKA
SHE   VVV--PPFLQPEIMGVPKVKETMVPKHKEMPFPKYP-VEPFTESQSLTLTDVEKLHLPLPLVQSWMKQPPQPLPPTVM-FPPQSVLSLSQPKVLPVPQKA
MOU   VVPSLGPVISPELESFLKAKATILPKHKQMPLLNSETVLRLINSQIPSLASLANLHLPQSLVQ-LLAQVVQAFPQTHL-VSSQTQLSLPQSKVLYFLQQV
RAT   VVPTDGPIISPELESFLKAKATVLPKHKQMPFLNSETVLRLFNSQIPSLD-LANLHLPQSPAQ-LQAQIVQAFPQTPAVVSSQPQLSHPQSKSQYLVQQL
RAB   MLL---PLLQPEIMEDPKAKETIIPKHKLMPFLKSPKTVPFVDSQILNLREMKNQHLLLPQLLPFMHQVFQPFPQTPI-PYPQALLSLPQSKFMPIVPQV
MAN   VVL---PVPQPEIMEVPKAKDTVYTKGRVMPVLKSP-TIPFFDPQIPKVTDLENLQLPLPLLQPLMQQVPQPIPQTLA-LPPQPLWSVPQPKVLPIPQQV
      ++    *+ +**++   *+* ++ +*++ **++++ + ++  +*++ +  + +++*+++  +    *+ *++*+*     +*  +* +*+*  ++ ++

                210       220       230       240
COW   VPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
SHE   VP--QRDMPIQAFLLYQEPVLGPVRGPFPILV
MOU   APFLPQDMSVQDLLQYLE-LLNPTVQ-FPATPQHSVSV
RAT   APLFQQGMPVQDLLQYLDLLLNPTLQ-FLATQQLHSTSV
RAB   VPYPQRDMPIQALQLFQELLF-PTHQGYPVVQPIAPVNV
MAN   VPYPQRAVPVQALLLNQELLLNPTHQIYPVTQPLAPVHNPISV
      +*  ++ +++*++++ ++ ++ *+ + ++
```

Figure 4.16 Homology of β-casein from a selection of species; * indicates residues identical at the same position for all species; + indicates similar residues, - an inserted space. ↓ indicates the N terminus of the mature protein (from Holt and Sawyer, 1993).

κ-caseins contain 8(9), 10–13, 5(4) and 1(2,3) moles P per mole. The phosphorus is very important:

- nutritionally, *per se*, and because it can bind large amounts of Ca^{2+}, Zn^{2+} and probably other polyvalent metals;
- it increases the solubility of caseins;
- it probably contributes to the high heat stability of casein; and
- it is significant in the coagulation of rennet-altered casein during the secondary phase of rennet action (Chapter 10).

The phosphorus is covalently bound to the protein and is removed only by very severe heat treatments, high pH or some phosphatases. The phosphate is esterified mainly to serine (possibly a little to threonine) as a monoester:



Phosphorylation occurs in the Golgi membranes of the mammary cell, catalysed by two serine-specific casein kinases. Only certain serines are phosphorylated; the principal recognition site is Ser/Thr.X.Y, where Y is a glutamyl and occasionally an aspartyl residue; once a serine residue has been phosphorylated, SerP can serve as a recognition site. X may be any amino acid but a basic or a very bulky residue may reduce the extent of phosphorylation. However, not all serine residues in a suitable sequence are phosphorylated, suggesting that there may be a further topological requirement, e.g. a surface location in the protein conformation.

Casein carbohydrate. α_{s1}-, α_{s2}- and β-caseins contain no carbohydrate but κ-casein contains about 5%, consisting of *N*-acetylneuraminic acid (sialic acid), galactose and *N*-acetylgalactosamine. The carbohydrate exists as tri- or tetrasaccharides, located toward the C-terminal of the molecule, attached through an *O*-threonyl linkage, mainly to Thr_{131} of κ-casein (Figure 4.17). The number of oligosaccharides per κ-casein molecule varies from 0 to 4. The variability of glycosylation results in at least nine, and probably 10, molecular forms of κ-casein (Table 4.5). The κ-casein in colostrum is even more highly glycosylated; more sugars are present and the structures are more complex and uncertain.

The carbohydrate is attached to the (glyco)macropeptides which are produced from κ-caseins on hydrolysis by rennets. The carbohydrate bestows on κ-casein quite high solubility and hydrophilicity. It is also

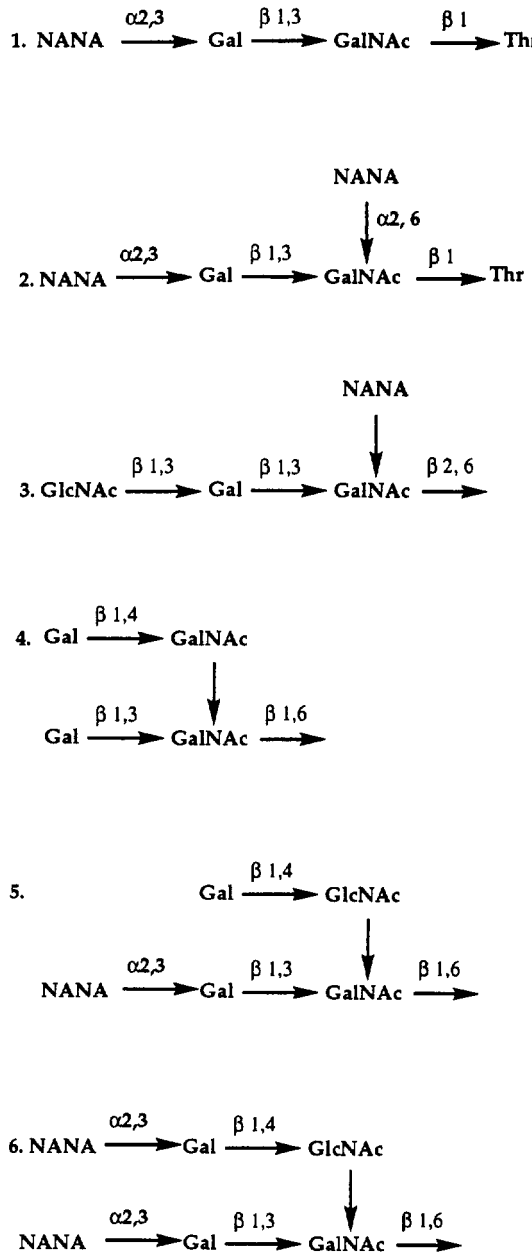


Figure 4.17 Oligosaccharides attached to casein isolated from bovine milk (1–2) or colostrum (1–6) (from Eigel *et al.*, 1984).

Table 4.5 Variability of bovine κ-casein with respect to sugars and phosphate

Fraction	Galactose	*N*-acetyl-galactosamine	*N*-acetyl-neuraminic acid	Phosphate
B-1	0	0	0	1
B-2	1	1	1	1
B-3	1	1	2	1
B-4	0	0	0	2
B-5	2	2	3	1
B-6	0	0	0(4)	3(1)
B-7	3	3	6	1
B-8	4	4	8	1
B-9	5	5	10	1

responsible for the solubility of the glycomacropeptides in 12% TCA (see Chapter 10). Although the sugars increase the hydrophilicity of casein, they are not responsible for the micelle-stabilizing properties of κ-casein, the carbohydrate-free form being as effective in this respect as the glycosylated forms.

4.5.2 Secondary and tertiary structures

Physical methods, such as optical rotary dispersion and circular dichroism, indicate that the caseins have relatively little secondary or tertiary structure, probably due to the presence of high levels of proline residues, especially in β-casein, which disrupt α-helices and β-sheets. However, theoretical calculations (Kumosinski, Brown and Farrell, 1993a, b; Kumosinski and Farrell, 1994) indicate that while α_{s1}-casein has little α-helix, it probably contains some β-sheets and β-turns. The C-terminal half of α_{s2}-casein probably has a globular conformation (i.e. a compact structure containing some α-helix and β-sheet) while the N-terminal region probably forms a randomly structured hydrophilic tail. Theoretical calculations suggest that β-casein could have 10% of its residues in α-helices, 17% in β-sheets and 70% in unordered structures. κ-Casein appears to be the most highly structured of the caseins, perhaps with 23% of its residues in α-helices, 31% in β-sheets and 24% in β-turns. Energy-minimized models of α_{s1}-, β- and κ-caseins are shown in Figure 4.18a–c. Holt and Sawyer (1993) coined the term 'rheomorphic' to describe the caseins as proteins with an open, flexible, mobile conformation in order to avoid using the 'demeaning' term, 'random coil'.

The lack of secondary and tertiary structures is probably significant for the following reasons:

1. The caseins are readily susceptible to proteolysis, in contrast to globular proteins, e.g. whey proteins, which are usually very resistant in their

native state. This has obvious advantages for the digestibility of the caseins, the natural function of which is presumably nutritional and hence easy digestibility in the 'native' state is important. The caseins are also readily hydrolysed in cheese, which is important for the development of cheese flavour and texture (Chapter 10). However, casein hydrolysates may be bitter due to a high content of hydrophobic amino acids (small hydrophobic peptides tend to be bitter). The caseins are readily hydrolysed by proteinases secreted by spoilage micro-organisms.

2. The caseins adsorb readily at air–water and oil–water interfaces due to their open structure, relatively high content of apolar amino acid residues and the uneven distribution of amino acids. This gives the caseins very good emulsifying and foaming properties, which are widely exploited in the food industry.
3. The lack of higher structures probably explains the high stability of the caseins to denaturing agents, including heat.

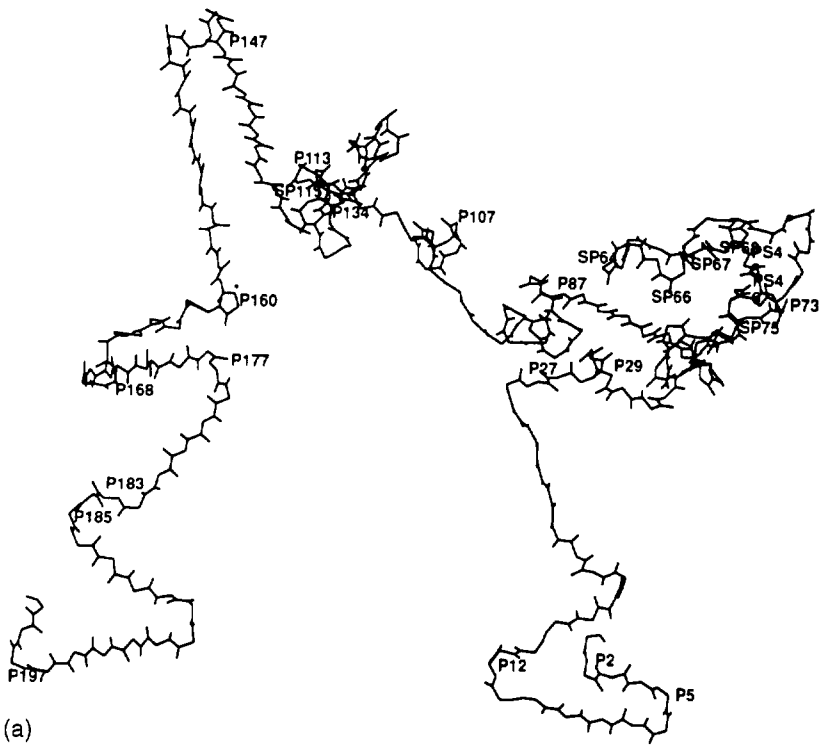


Figure 4.18 Energy-minimized models of the tertiary structures of bovine α_{s1}- (a), β- (b) and κ- (c) caseins (from Kumosinski, Brown and Farrell, 1993a, b; Kumosinski and Farrell, 1994)

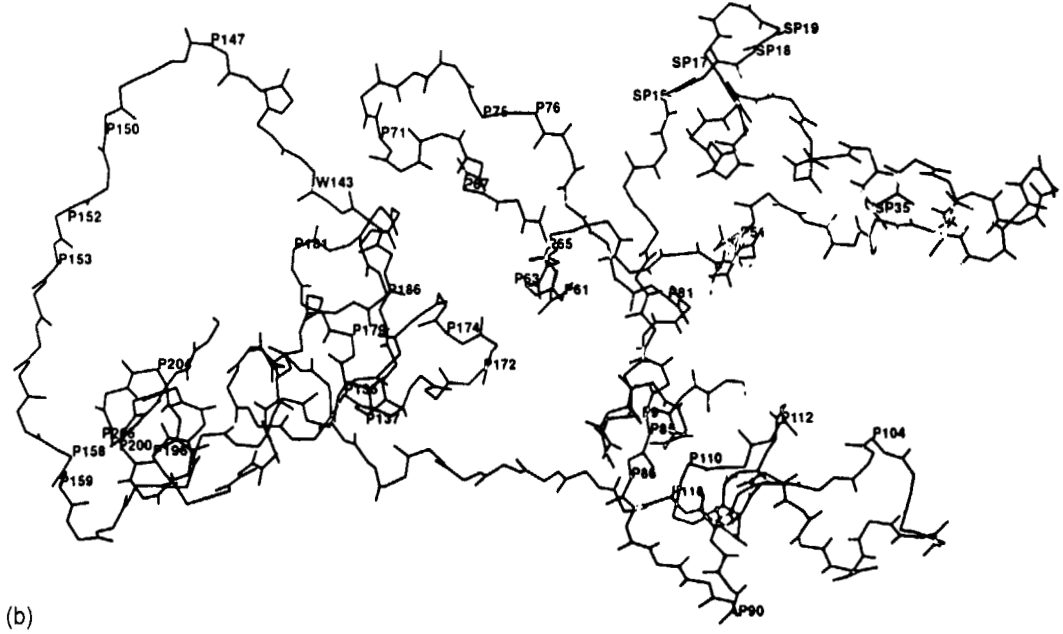


Figure 4.18 (*Continued*).

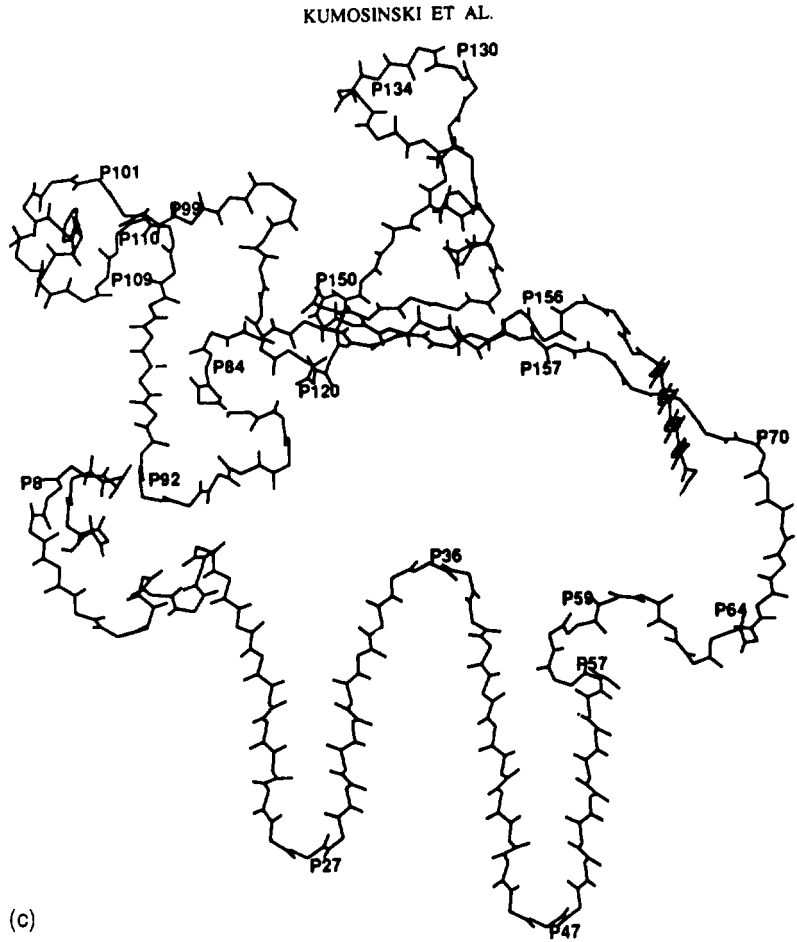


Figure 4.18 (*Continued*).

4.5.3 Molecular size

All the caseins are relatively small molecules, ranging in molecular weight from about 20 to 25 kDa (Table 4.2).

4.5.4 Hydrophobicity

The caseins are often considered to be rather hydrophobic molecules. However, consideration of the amino acid composition indicates that they are not particularly so; in fact, some are more hydrophilic than the whey protein, β-lactoglobulin (Table 4.2). However, the caseins do have high

surface hydrophobicity, in contrast to the globular whey proteins. In globular proteins, the hydrophobic residues are buried, as far as possible, within the molecule, with most of the hydrophilic residues exposed on the surface. Owing to the relative lack of secondary and tertiary structures in the caseins, such an arrangement is not possible, and hence the hydrophobic residues are rather exposed.

Thus, the caseins are relatively small, relatively hydrophobic, amphipathic, randomly or flexibly structured molecules, with relatively low levels of secondary and tertiary structures.

4.5.5 Influence of Ca^{2+} on caseins

At all temperatures, α_{s1}-CN B and C are insoluble in calcium-containing solutions and form a coarse precipitate at Ca^{2+} concentrations greater than about 4 mM. α_{s1}-CN A, from which the very hydrophobic sequence, residues 13–26, is deleted, is soluble at $[Ca^{2+}]$ up to 0.4 M in the temperature range 1–33°C. Above 33°C, it precipitates but redissolves on cooling to 28°C. The presence of α_{s1}-CN A modifies the behaviour of α_{s1}-CN B so that an equimolar mixture of the two is soluble in 0.4 M Ca^{2+} at 1°C; α_{s1}-CN B precipitates from the mixture at 18°C and both α_{s1}-CN A and B precipitate at 33°C. α_{s1}-CN A does not form normal micelles with κ-casein. Since α_{s1}-CN A occurs at very low frequency, these abnormalities are of little consequence in dairy processing but may become important if the frequency of α_{s1}-CN A increases as a result of breeding practices.

The α_{s2}-caseins are also insoluble in Ca^{2+} (above about 4 mM) at all temperatures, but their behaviour has not been studied in detail.

β-Casein is soluble at high concentrations of Ca^{2+} (0.4 M) at temperatures below 18°C, but above 18°C β-casein is very insoluble, even in the presence of low concentrations of Ca^{2+} (4 mM). Ca-precipitated β-casein redissolves readily on cooling to below 18°C. About 20°C is also the critical temperature for the temperature-dependent polymerization of β-casein and the two phenomena may be related.

κ-Casein is soluble in Ca^{2+} at all concentrations up to those at which general salting-out occurs. Solubility is independent of temperature and pH (outside the pH range at which isoelectric precipitation occurs). Not only is κ-casein soluble in the presence of Ca^{2+} but it is capable of stabilizing α_{s1}-, α_{s2}- and β-caseins against precipitation by Ca^{2+} (section 4.5.8).

4.5.6 Action of rennets on casein

This subject is dealt with in Chapter 10. Suffice it to say here that κ-casein is the only major casein hydrolysed by rennets during the primary phase of milk coagulation, which is the first step in the manufacture of most cheese varieties.

4.5.7 Casein association

All the major caseins associate with themselves and with each other. In unreduced form, κ-casein is present largely as disulphide-linked polymers. κ-Casein also forms hydrogen and hydrophobic bonds with itself and other caseins but these secondary associations have not been studied in detail.

At 4°C, β-casein exists in solution as monomers of molecular mass 25 kDa. As the temperature is increased, the monomers polymerize to form long thread-like chains of about 20 units at 8.5°C and to still larger aggregates at higher temperatures. The degree of association is dependent on protein concentration. The ability to form thread-like polymers may be important in micelle structure. β-Casein also undergoes a temperature-dependent conformational change in which the content of poly-L-proline helix decreases with increasing temperature. The transition temperature is about 20°C, i.e. very close to the temperature at which β-casein becomes insoluble in Ca^{2+}.

α_{s1}-Casein polymerizes to form tetramers of molecular mass 113 kDa; the degree of polymerization increases with increasing protein concentration and increasing temperature.

The major caseins interact with each other and, in the presence of Ca^{2+}, these associations lead to the formation of casein micelles.

4.5.8 Casein micelle structure

Composition and general features. About 95% of the casein exists in milk as large colloidal particles, known as micelles. On a dry matter basis, casein micelles contain *c.* 94% protein and 6% low molecular weight species referred to as colloidal calcium phosphate, consisting of calcium, magnesium, phosphate and citrate. The micelles are highly hydrated, binding about 2.0 g $H_2O\,g^{-1}$ protein. Some of the principal properties of casein micelles are summarized in Table 4.6.

Electron microscopy shows that casein micelles are generally spherical in shape, with diameters ranging from 50 to 500 nm (average *c.* 120 nm) and masses ranging from 10^6 to 10^9 Da (average about 10^8 Da). There are very many small micelles but these represent only a small proportion of the volume or mass (Figure 4.19). There are 10^{14}–10^{16} micelles ml^{-1} milk; they are roughly two micelle diameters (240 nm) apart, i.e. they are quite tightly packed. The surface (interfacial) area of the micelles is very large, $5 \times 10^4\,cm^2\,ml^{-1}$; hence, the surface properties of the micelles are critical to their behaviour.

Since the micelles are of colloidal dimensions, they are capable of scattering light and the white colour of milk is due largely to light scattering by the casein micelles; the white colour is lost if the micelles are disrupted, e.g. by removing colloidal calcium phosphate (by citrate, ethylene

Table 4.6 Average characteristics of casein micelles (modified from McMahon and Brown, 1984)

Characteristic	Value
Diameter	120 nm (range: 50–500 nm)
Surface area	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g cm^{-3}
Mass	2.2×10^{-15} g
Water content	63%
Hydration	3.7 g H_2O g^{-1} protein
Voluminosity	4.4 cm^3 g^{-1}
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5×10^8 Da
Number of peptide chains	10^4
Number of paticles per ml milk	10^{14}–10^{16}
Surface of micelles per ml milk	5×10^4 cm^3
Mean free distance	240 nm

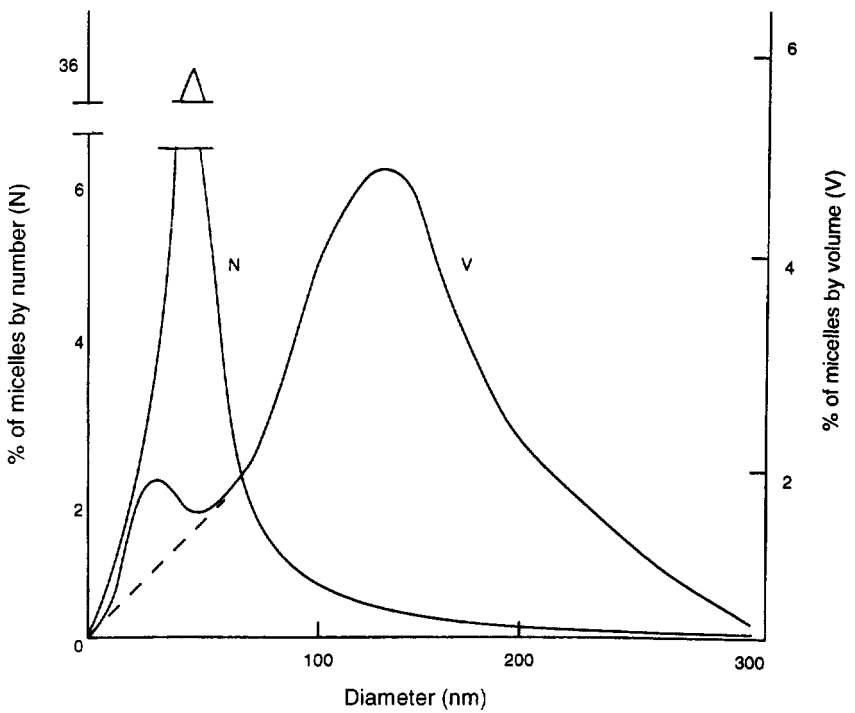


Figure 4.19 Number and volume frequency distribution of casein micelles in bovine milk (from Walstra and Jenness, 1984).

diaminetetraacetic acid (EDTA) or oxalate), by increasing pH (to greater than 9), or by the addition of urea or SDS.

Stability

1. The micelles are stable to the principal processes to which milk is normally subjected (except those in which it is intended to destabilize the micelles, e.g. rennet- and acid-induced coagulation). They are very stable at high temperatures, coagulating only after heating at 140°C for 15–20 min at the normal pH of milk. Such coagulation is not due to denaturation in the narrow sense of the word but to major changes which occur in milk exposed to such high heat treatments, including a decrease in pH due to the pyrolysis of lactose to various acids, dephosphorylation of the casein, cleavage of κ-casein, denaturation of the whey proteins and their attachment to the casein micelles, precipitation of soluble calcium phosphate on the micelles and a decrease in hydration (Chapter 9).
2. They are stable to compaction, e.g. they can be sedimented by ultracentrifugation and redispersed readily by mild agitation.
3. They are stable to commercial homogenization but are changed slightly at very high pressures (500 MPa).
4. They are stable to high $[Ca^{2+}]$, up to at least 200 mM at temperatures up to 50°C.
5. They aggregate and precipitate from solution when the pH is adjusted to the isoelectric point of caseins (*c.* pH4.6). Precipitation at this pH, which is temperature-dependent (i.e. does not occur at temperatures below 5-8°C and occurs over a wide pH range, perhaps 3.0–5.5, at higher temperatures, e.g. 70°C), occurs owing to the loss of net positive or negative charge as the pH approaches 4.6.
6. As the pH of milk is reduced, the colloidal calcium phosphate (CCP) dissolves and is completely soluble at pH 4.9 (Chapter 5). pH adjustment, followed by dialysis against bulk milk, is a convenient and widely used technique for varying the CCP content of milk. As the concentration of CCP is reduced, the properties of the micelles are altered but they retain some of their structure even after removing 70% of the CCP. Removal of more than 70% of the CCP results in disintegration of the micelles into smaller particles (aggregates).
7. Many proteinases catalyse the hydrolysis of a specific bond in κ-casein, as a consequence of which the micelles aggregate or gel in the presence of Ca^{2+} or other divalent ions. This is the key step in the manufacture of most cheese varieties (Chapter 10).
8. The micelles are destabilized by *c.* 40% ethanol at pH 6.7 and by lower concentrations if the pH is reduced.
9. They are destabilized by freezing (cryodestabilization) due to a decrease in pH and an increase in the $[Ca^{2+}]$ in the unfrozen phase of milk (Chapters 2 and 5).

Principal micelle characteristics. The structure of the casein micelles has attracted the attention of scientists for a considerable time. Knowledge of micelle structure is important because the stability and behaviour of the micelles are central to many dairy processing operations, e.g. cheese manufacture, stability of sterilized, sweetened-condensed and reconstituted milks and frozen products. Without knowledge of the structure and properties of the casein micelle, attempts to solve many technological problems faced by the dairy industry will be empirical and not generally applicable. From the academic viewpoint, the casein micelle presents an interesting and complex problem in protein quaternary structure.

Since the pioneering work of Waugh in 1958, a considerable amount of research effort has been devoted to elucidating the structure of the casein micelle, and several models have been proposed. This work has been reviewed in the references cited in the next section. The principal properties of the casein micelles are listed below and the models which best meet these requirements discussed briefly in the next section.

1. κ-Casein, which represents about 15% of total casein, is a critical feature of micelle structure and stability and must be located so as to be able to stabilize the calcium-sensitive α_{s1}-, α_{s2}- and β-caseins, which represent about 85% of total casein.
2. The κ-casein content of casein micelles is inversely proportional to their size, while the content of colloidal calcium phosphate is directly related to size.
3. Ultracentrifugally sedimented micelles have a hydration of 1.6–2.7 g $H_2O\,g^{-1}$ protein but voluminosities of 3–7 ml g^{-1} have been found by viscosity measurements and calculation of specific hydrodynamic volumes. These values suggest that the micelle has a porous structure in which the protein occupies about 25% of the total volume.
4. Chymosin and similar proteinases, which are relatively large molecules (*c.* 36 kDa), very rapidly and specifically hydrolyse most of the micellar κ-casein.
5. When heated in the presence of whey proteins, as in normal milk, κ-casein and β-lactoglobulin interact to form a disulphide-linked complex which modifies many properties of the micelles, including rennet coagulability and heat stability.
6. Removal of colloidal calcium phosphate (CCP) results in disintegration of the micelles into particles of mass $\sim 3 \times 10^6$ Da. The properties of the CCP-free system are very different from those of the normal milk system, e.g. it is sensitive to and precipitated by relatively low concentrations of Ca^{2+}, it is more stable to high temperatures, e.g. 140°C, and is not coagulable by rennets. Many of these properties can be restored, at least partially, by increased concentrations of calcium.
7. The micelles can be dispersed (dissociated) by urea or SDS, suggesting the involvement of hydrogen and hydrophobic bonds in micelle integrity.

8. The micelles can be destabilized by alcohols, acetone and similar solvents, suggesting an important role for electrostatic interactions in micelle structure.
9. As the temperature is lowered, caseins, especially β-casein, dissociate from the micelles; depending on the method of measurement, 10–50% of β-casein is non-micellar at 4°C.
10. Electron microscopy shows that the interior of the micelles are not uniformly electron dense.
11. The micelles have a surface (zeta) potential of about −20 mV at pH 6.7.

Micelle structure. Various models of casein micelle structure have been proposed and refined over the past 40 years. Progress has been reviewed regularly, including Schmidt (1982), McMahon and Brown (1984), Farrell (1988), Holt (1992, 1994), Rollema (1992) and Visser (1992).

The proposed models fall into three general categories, although there is some overlap:

1. core-coat;
2. internal structure;
3. subunit (submicelles); in many of the models in this category, it is proposed that the submicelles have a core-coat structure.

For many years there has been strong support for the view that the micelles are composed of submicelles of mass $\sim 10^6$ Da and diameter 10-15 nm. This model was introduced in 1967 by Morr who proposed that the submicelles are linked together by CCP, giving the micelle an open porous structure. On removal of CCP, e.g. by acidification/dialysis, EDTA, citrate or oxalate, the micelles disintegrate. Disintegration may also be achieved by treatment with urea, SDS or at pH greater than 9; presumably,

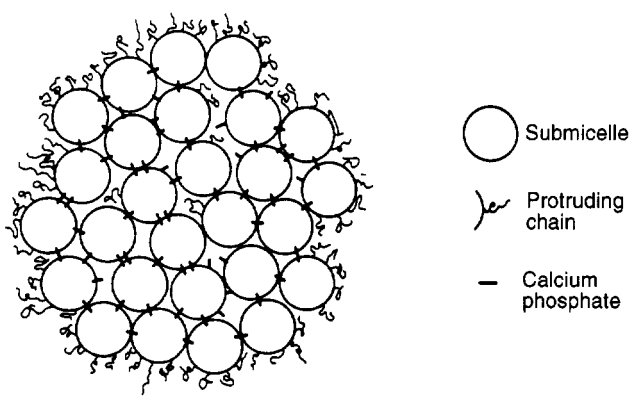


Figure 4.20 Submicelle model of the casein micelle (from Walstra and Jenness, 1984).

these treatments do not solubilize CCP, suggesting that other forces, e.g. hydrophobic and hydrogen bonds, contribute to micelle structure.

The submicellar model has undergone several refinements (see Schmidt, 1982; Walstra and Jenness, 1984; Ono and Obata, 1989). The current view is that the κ-casein content of the submicelles varies and that the κ-casein-deficient submicelles are located in the interior of the micelles with the κ-casein-rich submicelles concentrated at the surface, giving the micelles a κ-casein-rich layer but with some α_{s1}-, α_{s2}- and β-caseins also exposed on the surface. It is proposed that the hydrophilic C-terminal region of κ-casein protrudes from the surface, forming a layer 5–10 nm thick and giving the micelles a hairy appearance (Figure 4.20). This hairy layer is responsible for micelle stability through a major contribution to zeta potential (−20 mV) and steric stabilization. If the hairy layer is removed, e.g. specific hydrolysis of κ-casein, or collapsed, e.g. by ethanol, the colloidal stability of the micelles is destroyed and they coagulate or precipitate.

Although the submicellar model of the casein micelle readily explains many of the principal features and physicochemical reactions undergone by the micelles and has been widely supported, it has never enjoyed unanimous support and two alternative models have been proposed recently. Visser (1992) proposed that the micelles are spherical conglomerates of individual casein molecules randomly aggregated and held together partly by salt bridges in the form of amorphous calcium phosphate and partly by other forces, e.g. hydrophobic bonds, with a surface layer of κ-casein. Holt (1992, 1994) depicted the casein micelle as a tangled web of flexible casein

Figure 4.21 Model of the casein micelle (modified from Holt, 1994).

molecules forming a gel-like structure in which microgranules of colloidal calcium phosphate are an integral feature and from the surface of which the C-terminal region of κ-casein extends, forming a hairy layer (Figure 4.21). These models retain two of the central features of the submicellar model, i.e. the cementing role of CCP and the predominantly surface location of κ-casein.

Holt (1992, 1994) also proposed that, in addition to supplying amino acids, caseins should be considered to have a biological function, i.e. to enable a high concentration of calcium to be carried in stable form in milk; without the stabilizing effect of casein, calcium phosphate would precipitate in the mammary cells, resulting in ectopic mineralization, which might lead to the death of the mammary gland or of the whole animal. A similar situation occurs with kidney stones, gallstones and calcified synovial and salivary fluid.

Since the micelles are closely packed, intermicellar collisions are frequent; however, the micelles do not normally remain together after collisions. The micelles are stabilized by two principal factors: (1) a surface (zeta) potential of *c.* −20 mV at pH 6.7, which, alone, is probably too small for colloidal stability, and (2) steric stabilization due to the protruding κ-casein hairs.

4.6 Whey proteins

About 20% of the total protein of bovine milk belongs to a group of proteins generally referred to as **whey** or **serum proteins** or **non-casein nitrogen**. Acid and rennet wheys also contain casein-derived peptides; both contain proteose–peptones, produced by plasmin, mainly from β-casein, and the latter also contains (glyco)macropeptides produced by rennets from κ-casein. These peptides are excluded from the present discussion.

4.6.1 Preparation

The whey proteins, as a group, are readily prepared from milk by any of the methods described in section 4.3, i.e.

1. the proteins remaining soluble at pH 4.6;
2. soluble in saturated NaCl;
3. soluble after rennet coagulation of the caseins;
4. by gel permeation chromatography;
5. by ultracentrifugation, with or without added Ca^{2+}.

The whey prepared by any of the above methods, except 4, contains lactose and soluble salts. Total whey proteins may be prepared from the

wheys by dialysis and drying the retentate. The products prepared by these various methods differ: acid whey contains some γ-casein and proteose–peptones; immunoglobulins are co-precipitated with the caseins by saturated NaCl; rennet whey contains the κ-CN macropeptides produced by rennet action, plus, perhaps, very small amounts of other caseins; small casein micelles remain in the ultracentrifugal supernatant, especially if Ca is not added. The salt composition of the serum differs very considerably in wheys produced by various methods.

On a commercial scale, whey protein-rich products are prepared by:

1. Ultrafiltration/diafiltration of acid or rennet whey to remove varying amounts of lactose, and spray-drying to produce **whey protein concentrates** (30–80% protein).
2. Ion-exchange chromatography: proteins are adsorbed on an ion exchanger, washed free of lactose and salts and then eluted by pH adjustment. The eluate is freed of salts by ultrafiltration and spray-dried to yield **whey protein isolate**, containing about 95% protein.
3. Demineralization by electrodialysis and/or ion exchange, thermal evaporation of water and crystallization of lactose.
4. Thermal denaturation, recovery of precipitated protein by filtration/centrifugation and spray-drying, to yield **lactalbumin** which has very low solubility and limited functionality.

Several other methods are available for the removal of whey proteins from whey but are not used commercially. Several methods for the purification of the major and minor whey proteins on a commercial scale have also been developed and will be discussed briefly in sections 4.15.6 and 4.16.

4.6.2 Heterogeneity of whey proteins

It was recognized 60 years ago that whey prepared by any of the above methods contained two well-defined groups of proteins which could be fractionated by saturated $MgSO_4$ or half saturated $(NH_4)_2SO_4$; the precipitate (roughly 20% of total N) was referred to as **lactoglobulin** and the soluble protein as **lactalbumin**.

The lactoglobulin fraction consists mainly of immunoglobulins (Ig), especially IgG_1, with lesser amounts of IgG_2, IgA and IgM (section 4.10). The lactalbumin fraction of bovine milk contains three main proteins, β-lactoglobulin (β-lg), α-lactalbumin (α-la) and blood serum albumin (BSA), which represent approximately 50, 20 and 10% of total whey protein, respectively, and trace amounts of several other proteins, notably lactotransferrin, serotransferrin and several enzymes. The whey proteins of sheep, goat

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5 Salts of milk

5.1 Introduction

The salts of milk are mainly the phosphates, citrates, chlorides, sulphates, carbonates and bicarbonates of sodium, potassium, calcium and magnesium. Approximately 20 other elements are found in milk in trace amounts, including copper, iron, silicon, zinc and iodine. Strictly speaking, the proteins of milk should be included as part of the salt system since they carry positively and negatively charged groups and can form salts with counter-ions; however, they are not normally treated as such. There is no lactate in freshly drawn milk but it may be present in stored milk and in milk products. The major elements are of importance in nutrition, in the preparation, processing and storage of milk products due to their marked influence on the conformation and stability of milk proteins, especially caseins, and to a lesser extent the stability of lipids and the activity of some indigenous enzymes.

5.2 Method of analysis

The mineral content of foods is usually determined from the ash prepared by heating a sample at 500–600°C in a muffle furnace for about 4 h to oxidize organic matter. The ash does not represent the salts as present in the food because:

1. the ash is a mixture, not of the original salts, but of the carbonates and oxides of the elements present in the food;
2. phosphorus and sulphur from proteins and lipids are present in the ash, while organic ions, such as citrate, are lost during incineration; and
3. the temperature usually employed in ashing may vaporize certain volatile elements, e.g. sodium and potassium.

Therefore, it is difficult or impossible to relate the ash obtained from a food with its salts system, and low values are obtained for certain mineral elements by analysis of the ash compared to direct analysis of the intact food. Titrimetric, colorimetric, polarographic, flame photometric and atomic absorption spectrophotometric techniques are frequently used to analyse for the various mineral constituents; however, the quantitative estimation of

each ion in a mixture is frequently complicated by interfering ions. The major elements/ions in foods, including milk, may be determined by the following specific methods:

- Inorganic phosphate reacts with molybdate to form phosphomolybdate which may be reduced to a blue compound that can be quantified spectrophotometrically at 640 nm.
- Calcium and magnesium may be determined by titration with EDTA or by atomic absorption spectroscopy on TCA filtrates or on wet- or dry-ashed samples.
- Citrate forms a yellow complex with pyridine (which is carcinogenic) in the presence of acetic anhydride; the complex may be quantified spectrophotometrically. Alternatively, citrate can be determined by an enzymatic assay.
- Ionized calcium may be determined spectrophotometrically after reaction with murexide or using a Ca^{2+}-specific electrode.
- Sodium and potassium may be quantified by flame photometry, atomic absorption spectroscopy or ion specific electrodes.
- Chloride can be titrated with $AgNO_3$ using potentiometric or indicator end-point detection.
- Sulphate is precipitated by $BaCl_2$ and quantified gravimetrically.
- Lactate may be quantified spectrophotometrically after reaction with $FeCl_2$, or by an enzymatic assay (using lactate dehydrogenase which can quantify both D- and L-isomers) or by HPLC.

References to these and other methods can be found in Jenness (1988). Detailed analytical procedures are published in the *Official Methods of Analysis of the Association of Official Analytical Chemists* (Arlington, VA, USA) or in *Standard Methods of the International Dairy Federation* (Brussels, Belgium).

5.3 Composition of milk salts

The ash content of milk remains relatively constant at 0.7–0.8%, but the relative concentrations of the various ions can vary considerably. Table 5.1 shows the average concentration of the principal ions in milk, the usual range and the extreme values encountered. The latter undoubtedly include abnormal milks, e.g. colostrum, very late lactation milk or milk from cows with mastitic infection.

The ash content of human milk is only about 0.2%; the concentration of all principal and several minor ions is higher in bovine than in human milk (Table 5.2). Consumption of unmodified bovine milk by human babies causes increased renal load and hence demineralized bovine milk or whey should be used for infant formulae.

Table 5.1 Concentration of milk salt constituents (mg litre^{-1} milk (from various sources)

Constituent	Average content	Usual range	Extremes reported
Sodium	500	350–600	110–1150
Potassium	1450	1350–1550	1150–2000
Calcium	1200	1000–1400	650–2650
Magnesium	130	100–150	20–230
Phosphorus (total)[a]	950	750–1100	470–1440
Phosphorus (inorganic)[b]	750		
Chloride	1000	800–1400	540–2420
Sulphate	100		
Carbonate (as CO_2)	200		
Citrate (as citric acid)	1750		

[a]Total phosphorus includes colloidal inorganic phosphate, casein (organic) phosphate, soluble inorganic phosphate, ester phosphate and phospholipids.
[b]Phosphorus (inorganic) includes colloidal inorganic phosphate and soluble inorganic phosphate.

Table 5.2 Mineral composition (mg or $\mu g\,l^{-1}$) of mature human or bovine milks (from Flynn and Power, 1985)

	Mature human milk		Cows' milk	
Constituent	Mean	Range	Mean	Range
Sodium (mg)	150	110–200	500	350–900
Potassium (mg)	600	570–620	1500	1100–1700
Chloride (mg)	430	350–550	950	900–1100
Calcium (mg)	350	320–360	1200	1100–1300
Magnesium (mg)	28	26–30	120	90–140
Phosphorus (mg)	145	140–150	950	900–1000
Iron (μg)	760	620–930	500	300–600
Zinc (μg)	2950	2600–3300	3500	2000–6000
Copper (μg)	390	370–430	200	100–600
Manganese (μg)	12	7–15	30	20–50
Iodine (μg)	70	20–120	260	–
Fluoride (μg)	77	21–155	–	30–220
Selenium (μg)	14	8–19	–	5–67
Cobalt (μg)	12	1–27	1	0.5–1.3
Chromium (μg)	40	6–100	10	8–13
Molybdenum (μg)	8	4–16	73	18–120
Nickel (μg)	25	8–85	25	0–50
Silicon (μg)	700	150–1200	2600	750–7000
Vanadium (μg)	7	Tr–15	–	Tr–310
Tin (μg)	–	–	170	40–500
Arsenic (μg)	50	–	45	20–60

Tr, Trace.

5.4 Secretion of milk salts

The secretion of milk salts, which is not well understood, has been reviewed and summarized by Holt (1985). Despite the importance of milk salts in determining the processing characteristics of milk, relatively little interest has been shown in the nutritional manipulation of milk salts composition.

Three factors must be considered when discussing the milk salts system:

1. the need to maintain electrical neutrality;
2. the need to maintain milk isotonic with blood; as a result of this, a set of correlations exist between the concentrations of lactose, Na^+, K^+ and Cl^-;
3. the need to form casein micelles which puts constraints on the pH and $[Ca^{2+}]$ and requires the complexation of calcium phosphate with casein.

Skim milk can be considered as a two-phase system consisting of casein–colloidal calcium phosphate micelles in quasi-equilibrium with an aqueous solution of salts and proteins; the phase boundary is ill-defined because of the intimate association between the calcium phosphate and the caseins (phosphoproteins).

A fat-free primary secretion is formed within vesicles formed by blebbing-off of the Golgi dicytosomes; the vesicles pass through the cytoplasm to the apical membrane where exocytosis occurs. The vesicles contain casein (synthesized in the rough endoplasmic reticulum toward the base of the mammocyte); fully-formed casein micelles have been demonstrated within the Golgi vesicles. The vesicles also contain lactose synthetase (UDP:galactosyl transferase and α-lactalbumin) and there is good evidence showing that lactose synthesis occurs within the vesicles from glucose and UDP-galactose transported from the cytosol.

The intracellular concentrations of sodium and potassium are established by a Na^+/K^+-activated ATPase and Na^+ and K^+ can permeate across the vesicle membranes. Calcium is probably necessary to activate the UDP:galactosyl transferase and is transported by a Ca^{2+}/Mg^{2+}-ATPase which concentrates Ca^{2+} against an electrical potential gradient from μM concentrations in the cytosol to mM concentrations in the vesicles. Inorganic P (P_i) can be formed intravesicularly from UDP formed during the synthesis of lactose from UDP-galactose and glucose. UDP, which cannot cross the membrane, is hydrolysed to UMP and P_i, both of which can re-enter the cytosol (to avoid product inhibition); however, some of the P_i is complexed by Ca^{2+}. Ca^{2+} are also chelated by citrate to form largely soluble, undissociated complexes and by casein to form large colloidal casein micelles.

Water movement across the vesicle membranes is controlled by osmotic pressure considerations. Since lactose is a major contributor to the osmotic pressure of milk, the concentrations of both soluble and colloidal salts in

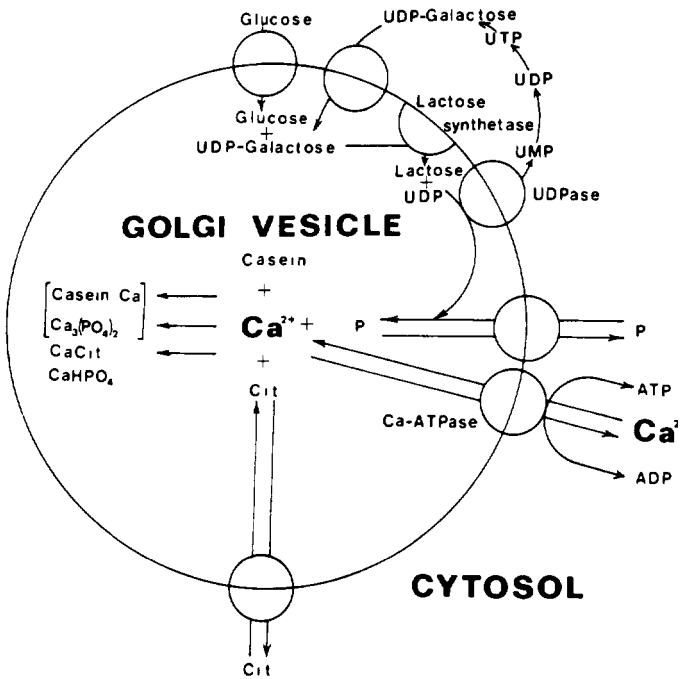


Figure 5.1 Summary of some transport mechanisms for calcium, phosphate and citrate from the cytosol of the secretory cell to the inside of Golgi vesicles (from Holt, 1981).

milk are strongly influenced by lactose concentration and the mechanism by which it is synthesized.

Inter-relationships in the biosynthesis of the principal milk salts are summarized in Figure 5.1. Transport of several ionic species via the junctions between cells (paracellular) occurs during early and late lactation and during mastitic infection when the junctions between cells are more open.

5.5 Factors influencing variation in salt composition

The composition of milk salts is influenced by a number of factors, including breed, individuality of the cow, stage of lactation, feed, mastitic infection and season of the year. The more important factors are discussed below.

5.5.1 Breed of cow

Milk from Jersey cows usually contains more calcium and phosphorus than milk from other breeds, including Holstein, but the concentrations of sodium and chloride are usually lower.

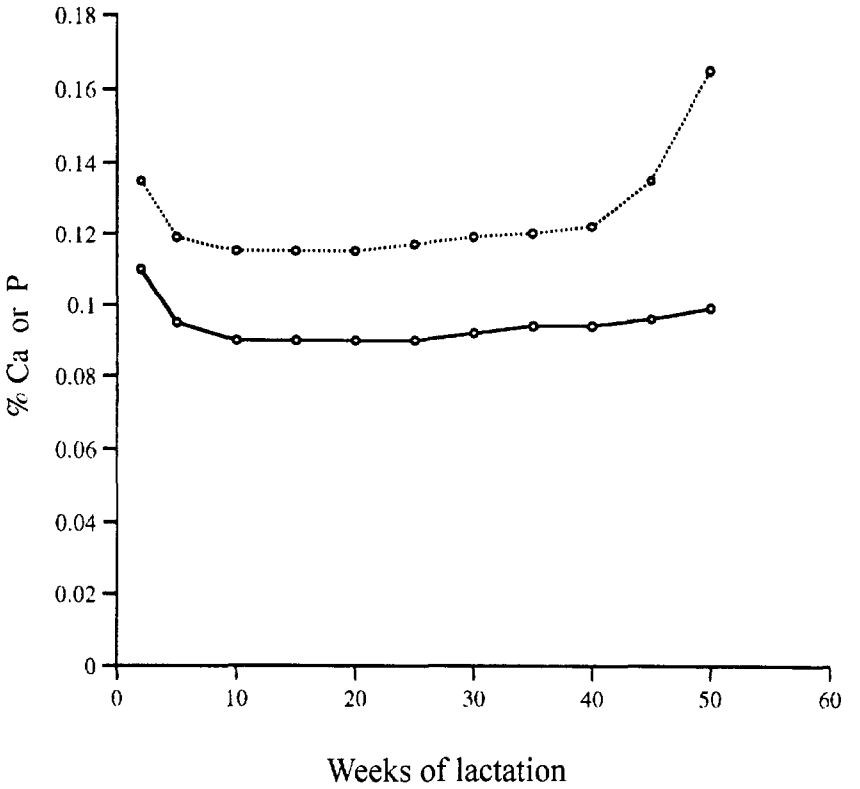


Figure 5.2 Changes in the concentrations of calcium (----) and phosphorus (—) in bovine milk during lactation.

5.5.2 Stage of lactation

The concentration of total calcium is generally high both in early and late lactation but in the intervening period no relation with stage of lactation is evident (Figure 5.2). Phosphorus shows a general tendency to increase as lactation advances (Figure 5.2). The concentrations of colloidal calcium and inorganic phosphorus are at a minimum in early and at a maximum in late lactation milk. The concentrations of sodium and chloride (Figure 5.3) are high at the beginning of lactation, followed by a rapid decrease, then increase gradually until near the end of lactation when rapid increases occur. The concentration of potassium decreases gradually throughout lactation. The concentration of citrate, which has a marked influence on the distribution of calcium, shows a strong seasonal variation (Figure 5.4), influenced more by feed than the stage of lactation. The pH of milk shows a strong

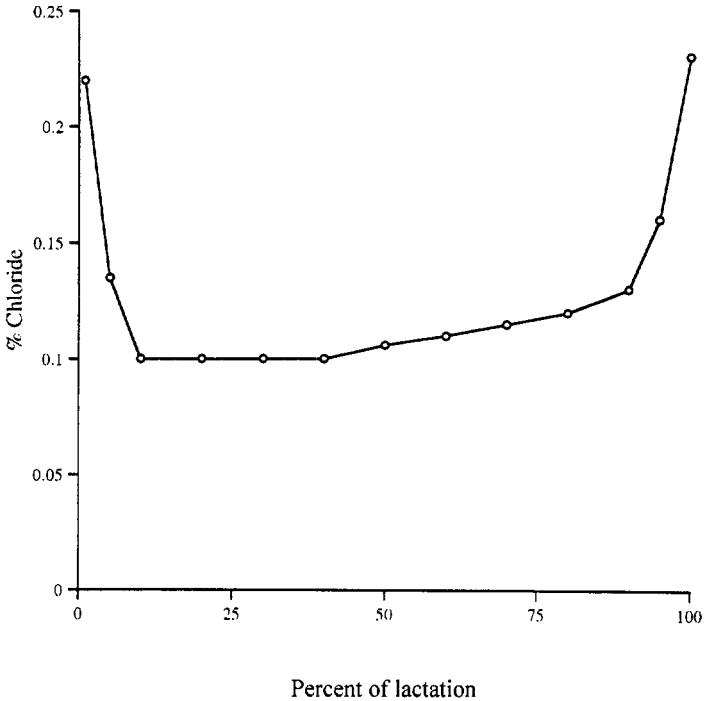


Figure 5.3 Changes in the concentration of chloride in bovine milk during lactation.

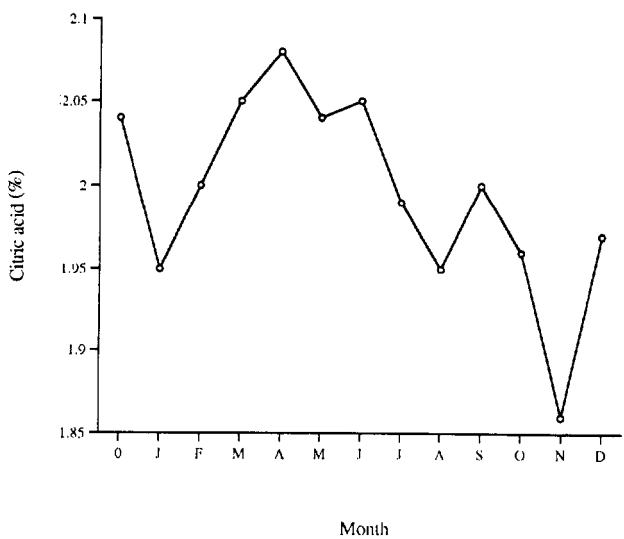


Figure 5.4 Seasonality of the concentration of citric acid in bovine milk.

seasonal trend; the pH of colostrum is about 6 but increases to the normal value of about 6.6–6.7 shortly after parturition and changes little until late lactation, when the pH raises to as high as 7.2, i.e. approaches that of blood (pH 7.4) due to degeneration of the mammary cell membrane. The pH of milk also increases during mastitic infection (e.g. 6.8–6.9), due to the influx of constituents from blood.

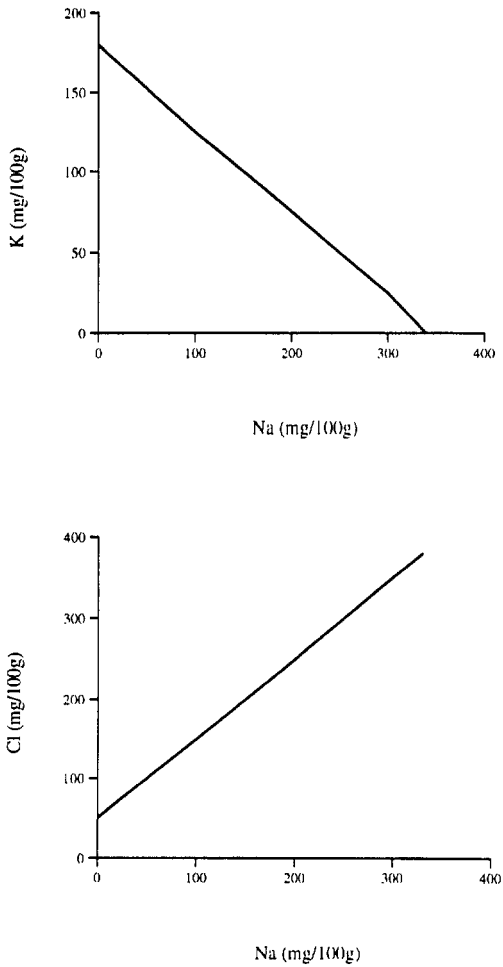


Figure 5.5 Correlations between the concentration of sodium and potassium (a) and sodium and chloride (b) in bovine milk.

5.5.3 Infection of the udder

Milk from cows with mastitic infections contains a low level of total solids, especially lactose, and high levels of sodium and chloride, the concentration of which are directly related (Figure 5.5). The sodium and chloride ions come from the blood to compensate osmotically for the depressed lactose synthesis or vice versa.

These are related by the Koestler number:

$$\text{Koestler number} = \frac{100 \times \%\,\text{Cl}}{\%\,\text{lactose}}$$

which is normally 1.5–3.0 but increases on mastitic infection and has been used as an index of such (better methods are now available, e.g. somatic cell count, activity of certain enzymes, especially catalase and *N*-acetyl-glucosamidase). The pH of milk increases to approach that of blood during mastitic infection.

5.5.4 Feed

Feed has relatively little effect on the concentration of most elements in milk because the skeleton acts as a reservoir of minerals. The level of citrate in milk decreases on diets very deficient in roughage and results in the 'Utrecht phenomenon', i.e. milk of very low heat stability. Relatively small changes in the concentrations of milk salts, especially of Ca, P_i and citrate, can have very significant effects on the processing characteristics of milk and hence these can be altered by the level and type of feed, but definitive studies on this are lacking.

5.6 Interrelations of milk salt constituents

Various milk salts are interrelated and the interrelationships are affected by pH (Table 5.3). Those constituents, the concentrations of which are related to pH in the same way, are also directly related to each other (e.g. the concentrations of total soluble calcium and ionized calcium), while those related to pH in opposite ways are inversely related (e.g. the concentrations of potassium and sodium).

Relationships between some of the more important ions/molecules are shown in Figure 5.6. Three correlations are noteworthy:

1. The concentration of lactose is inversely related to the concentration of soluble salts expressed as osmolarity. This results from the requirement that milk be isotonic with blood.

Table 5.3 Relationships between the pH of milk and the concentrations of certain milk salt constituents

Inversely related to pH	Directly related to pH
Titratable acidity	Colloidal inorganic calcium
Total soluble calcium	Caseinate calcium
Soluble unionized calcium	Colloidal inorganic phosphorus
Ionized calcium	Colloidal calcium phosphate
Soluble magnesium	Sodium
Soluble citrate	Chloride
Soluble inorganic phosphorus	Total phosphorus
Ester phosphorus	
Potassium	

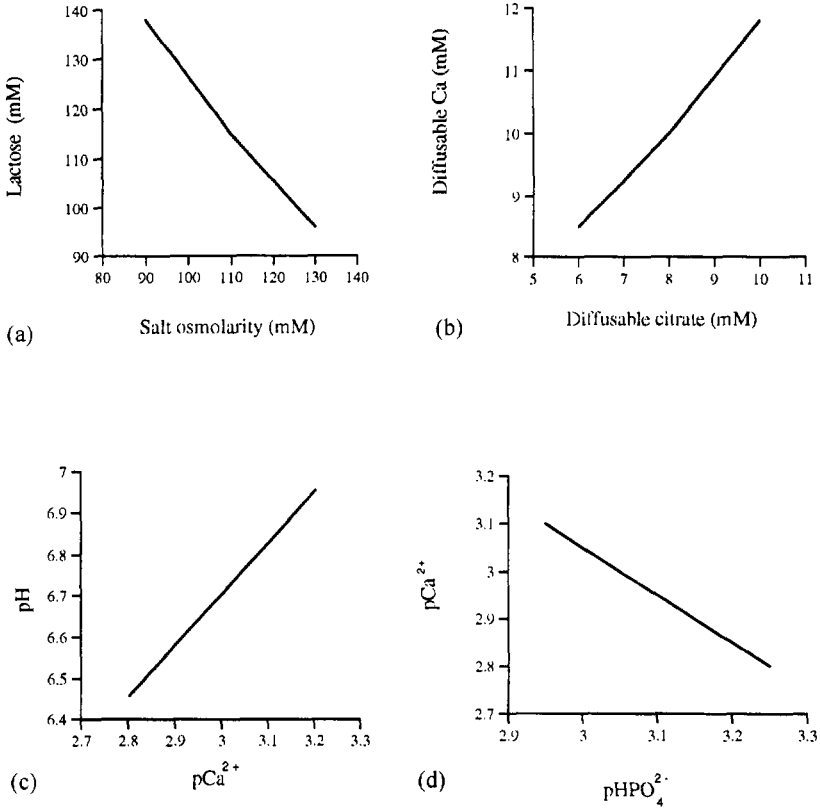


Figure 5.6 Interrelationships between lactose and soluble salts (osmolarity) and between some soluble salts in bovine milk.

2. There is a direct correlation between the concentration of diffusible Ca (and diffusible Mg) and the concentration of diffusible citrate (Figure 5.6b); this correlation, which is very good at constant pH, exists because citrate chelates Ca^{2+} more strongly than phosphate to form soluble unionized salts.
3. The ratio $HPO_4^{2-}/H_2PO_4^-$ is strongly pH dependent, as is the solubility of $Ca_3(PO_4)_2$ (section 5.8.1). As the pH is reduced, colloidal $Ca_3(PO_4)_2$ dissolves but $HPO_4^{2-} \rightarrow H_2PO_4^-$ as the pH is reduced and hence both $[Ca^{2+}]$ and soluble P_i are directly related to pH (Figure 5.6c). The $[HPO_4^{2-}]$ is inversely related to $[Ca^{2+}]$ (Figure 5.6d).

5.7 Partition of milk salts between colloidal and soluble phases

Certain of the milk salts (e.g. chlorides, and the salts of sodium and potassium) are sufficiently soluble to be present almost entirely in the dissolved phase. The concentration of others, in particular calcium phosphate, is higher than can be maintained in solution at the normal pH of milk. Consequently, these exist partly in soluble form and partly in an insoluble or colloidal form associated with casein. The state and distribution of these salts has been extensively reviewed by Pyne (1962) and Holt (1985).

The dividing line between soluble and colloidal is somewhat arbitrary, its exact position depending very much on the method used to achieve separation. However, a fairly sharp separation between the two phases is not difficult since the insoluble salts occur mainly associated with the colloidal casein micelles.

5.7.1 Methods used to separate the colloidal and soluble phases

The methods used include dialysis, ultrafiltration, high-speed centrifugation and rennet coagulation. The method used must not cause changes in equilibrium between the two phases. The two most important precautions are to avoid changes in pH (lowering the pH dissolves colloidal calcium phosphate, see Figure 5.11) and temperature (reducing the temperature dissolves colloidal calcium phosphate and vice versa). Since milk comes from the cow at about 40°C, working at 20°C and especially at 4°C will cause significant shifts in calcium phosphate equilibrium.

Ultrafiltrates obtained using cellophane or polysulphone membranes at 20°C and a transmembrane pressure of *c.* 100 kPa are satisfactory, but the concentrations of citrate and calcium are slightly low due to sieving effects which are accentuated by high pressures. Dialysis of a small volume of water against at least 50 times its volume of milk (to which a little chloroform or azide has been added as preservative) at 20°C for 48 h is the most satisfactory separation procedure and agrees closely with results obtained

Table 5.4 Effect of temperature on the composition of diffusate obtained by dialysis (modified from Davies and White, 1960)

Constituent	$mg\,l^{-1}$ milk	
	20°C	3°C
Total calcium	379	412
Ionized calcium	122	129
Magnesium	78	79
Inorganic phosphorus	318	326
Citrate (as citric acid)	1730	1750
Sodium	580	600
Potassium	1330	1330

Table 5.5 Distribution of salts ($mg\,l^{-1}$ milk) between the soluble and colloidal phases of milk (from Davies and White, 1960)

Constituent	Total in milk	Diffusate	Colloidal
Total calcium	1142	381 (33.5%)	761 (66.5%)
Ionized calcium		117	–
Magnesium	110	74 (67%)	36 (33%)
Sodium	500	460 (92%)	40 (8%)
Potassium	1480	1370 (92%)	110 (8%)
Total phosphorus	848	377 (43%)	471 (57%)
Citrate (as citric acid)	1660	1560 (94%)	100 (6%)
Chloride	1063	1065 (100%)	0 (0%)

by ultrafiltration and renneting techniques, although the latter tends to be slightly high in calcium. As mentioned above, the temperature at which dialysis is performed is important, e.g. diffusate prepared from milk at 3°C contains more total calcium, ionized calcium and phosphate than a diffusate prepared at 20°C (Table 5.4).

The partition of salts between the soluble and colloidal phases is summarized in Table 5.5. In general, most or all of the sodium, potassium, chloride and citrate, one-third of the calcium and two-thirds of the magnesium and about 40% of the inorganic phosphate are in the soluble phase.

The phosphorus of milk occurs in five classes of compounds: phospholipids, lipid, casein, small soluble organic esters, soluble and colloidal inorganic salts (Figure 5.7).

5.7.2 Soluble salts

The soluble salts are present in various ionic forms and unionized complexes. Sodium and potassium are present totally as cations, while chloride

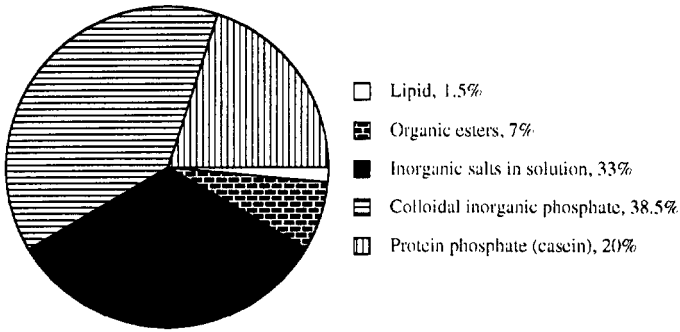


Figure 5.7 Distribution of phosphorus among various classes of compounds in bovine milk.

and sulphate, anions of strong acids, are present as anions at the pH of milk. The salts of weak acids (phosphates, citrates and carbonates) are distributed between various ionic forms, the concentration of which can be calculated approximately from the analytical composition of milk serum and the dissociation constants of phosphoric, citric and carbonic acid, after allowance has been made for binding of calcium and magnesium to citrate as anionic complexes and to phosphate as undissociated salts. The distribution of the various ionic forms can be calculated according to the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\text{salt}]}{[\text{acid}]}$$

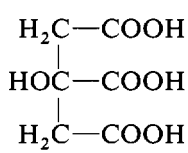
Phosphoric acid (H_3PO_4) dissociates as follows:

$$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-}$$
$$pK_a^1 = 1.96 \qquad pK_a^2 = 6.83 \qquad pK_a^3 = 12.32$$

$H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-} are referred to as primary, secondary and tertiary phosphate, respectively.

The titration curve for H_3PO_4 using NaOH is shown in Figure 5.8.

Citric acid is also triprotic while carbonic acid (H_2CO_3) is diprotic.



Citric acid

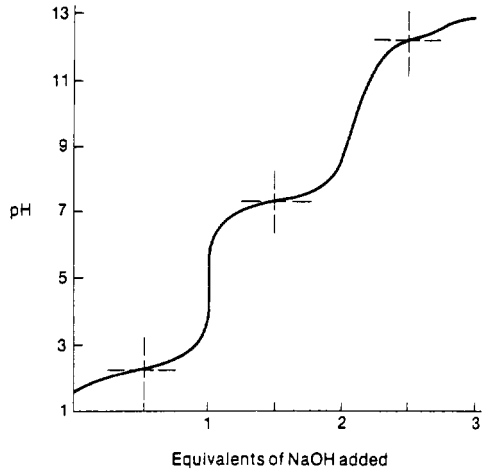


Figure 5.8 Titration curve for phosphoric acid (H_3PO_4); + indicates pK_a^1 (1.96), pK_a^2 (6.8) and pK_a^3 (12.3).

The exact value of the dissociation constants which should be used depends on the total ionic concentration and consequently, the constants used for milk are approximate. The following values are generally used:

Acid	pK_a^1	pK_a^2	pK_a^3
Citric	3.08	4.74	5.4
Phosphoric	1.96	6.83	12.32
Carbonic	6.37	10.25	

In milk, the critical dissociation constants are pK_a^3 for citric acid, pK_a^2 for phosphoric acid and pK_a^1 for carbonic acid. Bearing in mind the limitations and assumptions of the above data, the following calculations can be made for the distribution of the various ions in milk at pH 6.6.

Phosphoric acid. For the first dissociation, $H_3PO_4 \rightleftharpoons H^+ + H_3PO_4^-$; $pK_a^1 = 1.96$

$$pH = pK_a^1 + \log \frac{[salt]}{[acid]}$$

$$6.6 = 1.96 + \log \frac{[salt]}{[acid]}$$

$$\frac{[salt]}{[acid]}, \text{ i.e. } \frac{H_2PO_4^-}{H_3PO_4} = \frac{43\,700}{1}.$$

Therefore, there is essentially no H_3PO_4 in milk.

For the second dissociation, i.e. $H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+; pK_a^2 = 6.83$

$$6.6 = 6.83 + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$\log \frac{[\text{salt}]}{[\text{acid}]} = -0.23$$

$$\frac{[\text{salt}]}{[\text{acid}]}, \text{ i.e. } \frac{HPO_4^{2-}}{H_2PO_4^-} = \frac{0.59}{1}.$$

For the third dissociation, i.e. $HPO_4^{2-} \rightleftharpoons PO_4^{3-} + H^+; pK_a^3 = 12.32$

$$6.6 = 12.32 + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$\log \frac{[\text{salt}]}{[\text{acid}]} = -5.72$$

$$\frac{[\text{salt}]}{[\text{acid}]}, \text{ i.e. } \frac{PO_4^{3-}}{HPO_4^{2-}} = \frac{1.9 \times 10^{-6}}{1}.$$

Dihydrogenphosphate (primary) and monohydrogenphosphate (secondary) are the predominant forms, in the ratio of 1.0:0.59, i.e. 63% $H_2PO_4^-$ and 37% HPO_4^{2-}.

Citric acid. Using pK_as of 3.08, 4.74 and 5.4:

$$\frac{H_2\text{Citrate}^-}{H_3\text{Citric acid}} = \frac{3300}{1}$$

$$\frac{H\text{Citrate}^{2-}}{H_2\text{Citrate}^-} = \frac{72}{1}$$

$$\frac{\text{Citrate}^{3-}}{H\text{Citrate}^{2-}} = \frac{16}{1}$$

Therefore, tertiary (Citrate^{3-}) and secondary ($H\text{Citrate}^{2-}$) citrate, in the ratio 16:1, are the predominant forms.

Carbonic acid. The small amount of carbonic acid present occurs mainly as the bicarbonate anion, HCO_3^-.

Calcium and magnesium. Some calcium and magnesium in milk exist as complex undissociated ions with citrate, phosphate and bicarboante, e.g. Ca Citr^-, $CaPO_4^-$, Ca HCO_3^+. Calculations by Smeets (1955) suggest the following distribution for the various ionic forms in the *soluble* phase:

- Calcium + magnesium: 35% as ions, 55% bound to citrate and 10% bound to phosphate.

Table 5.6 Distribution of milk salts

Species	Concentration ($mg\,l^{-1}$)	Soluble		Colloidal (%)
		%	form	
Sodium	500	92	Completely ionized	8
Potassium	1450	92	Completely ionized	8
Chloride	1200	100	Completely ionized	–
Sulphate	100	100	Completely ionized	–
Phosphate	750	43	10% bound to Ca and Mg 51% H_2PO^- 39% HPO_4^{2-}	57
Citrate	1750	94	85% bound to Ca and Mg 14% $Citrate^{3-}$ 1% $HCitrate^{2-}$	
Calcium	1200	34	35% Ca^{2+} 55% bound to citrate 10% bound to phosphate	66
Magnesium	130	67	Probably similar to calcium	33

- Citrates: 14% tertiary ($Citrate^{3-}$), 1% secondary ($HCitrate^{2-}$) and 85% bound to calcium and magnesium.
- Phosphates: 51% primary ($H_2PO_4^-$), 39% secondary (HPO_4^{2-}) and 10% bound to calcium and magnesium.

Combining this information with the distribution of the various salts between the colloidal and soluble phases (Table 5.5), gives the quantitative distribution of the salts in milk shown in Table 5.6.

It should be possible to determine experimentally the concentrations of anions such as HPO_4^{2-} and $Citrate^{3-}$ in milk using ion-exchange resins or by nuclear magnetic resonance spectroscopy, but no such experimental work has been reported and available data are by calculation only.

Making certain assumptions and approximations as to the state of various ionic species in milk, Lyster (1981) and Holt, Dalgleish and Jenness (1981) developed computer programs that permit calculation of the concentrations of various ions and soluble complexes in typical milk diffusate. The outcome of both sets of calculations are in fairly good agreement and are also in good agreement with the experimentally determined values for those species for which data are available. The values calculated by Holt, Dalgleish and Jenness (1981) are shown in Table 5.7.

The ionic strength of milk is around 0.08 M.

5.7.3 Measurement of calcium and magnesium ions

Ca^{2+} and Mg^{2+}, along with H^+, play especially important roles in the stability of the caseinate system and its behaviour during milk processing, especially in the coagulation of milk by rennet, heat and ethanol. The

Table 5.7 Calculated concentrations (mM) of ions and complexes in a typical milk diffusate (from Holt, Dalgleish and Jenness, 1981)

Anion	Free ion	Cation complex			
		Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}
H_2Cit^-	+	+	+	+	+
$HCit^{2-}$	0.04	0.01	+	+	+
Cit^{3-}	0.26	6.96	2.02	0.03	0.04
$H_2PO_4^-$	7.50	0.07	0.04	0.10	0.18
HPO_4^{2-}	2.65	0.59	0.34	0.39	0.52
PO_4^{3-}	+	0.01	+	+	+
GLC-1-HPO_4^-	0.50	+	+	0.01	0.01
GLC-1-PO_4^{2-}	1.59	0.17	0.07	0.10	0.14
H_2CO_3	0.11	–	–	–	–
HCO_3^-	0.32	0.01	+	+	+
CO_3^{2-}	+	+	+	+	+
Cl^-	30.90	0.26	0.07	0.39	0.68
HSO_4^-	+	+	+	+	+
SO_4^{2-}	0.96	0.07	0.03	0.04	0.10
RCOOH	0.02	–	–	–	–
$RCOO^-$	2.98	0.03	0.02	0.02	0.04
Free ion	–	2.00	0.81	20.92	36.29

+, <0.005 μM; –, not estimated; GLC, glucose.

concentrations of these ions are also related to the solubility of the colloidal calcium phosphate. Consequently, there is considerable interest in determining their concentrations; three methods are available:

Cation-exchange resins. Using ion-exchange resins, Ca^{2+} and Mg^{2+} are adsorbed on to a cation-exchange resin added to milk; the resin is removed and the Ca^{2+} and Mg^{2+} desorbed. It is assumed that the treatment does not alter the ionic equilibrium in milk.

Interaction with murexide. The murexide method depends on the formation of a complex between Ca^{2+} and ammonium purpurate (murexide, M):

$$Ca^{2+} + M \rightleftharpoons Ca\,M$$

The free dye (M) has an absorption maximum at 520 nm while Ca M absorbs maximally at 480 nm. The concentration of Ca^{2+} can be calculated from a standard curve in which A_{480} is plotted as a function of $[Ca^{2+}]$ or preferably from a standard curve of $(A_{520} - A_{480})$ as a function of $[Ca^{2+}]$ which is less curved and more sensitive (Figure 5.9). Using this method, the $[Ca^{2+}]$ in milk was found to be 2.53–3.4 mM and appears to be 0.8 mM higher than that determined by the other methods.

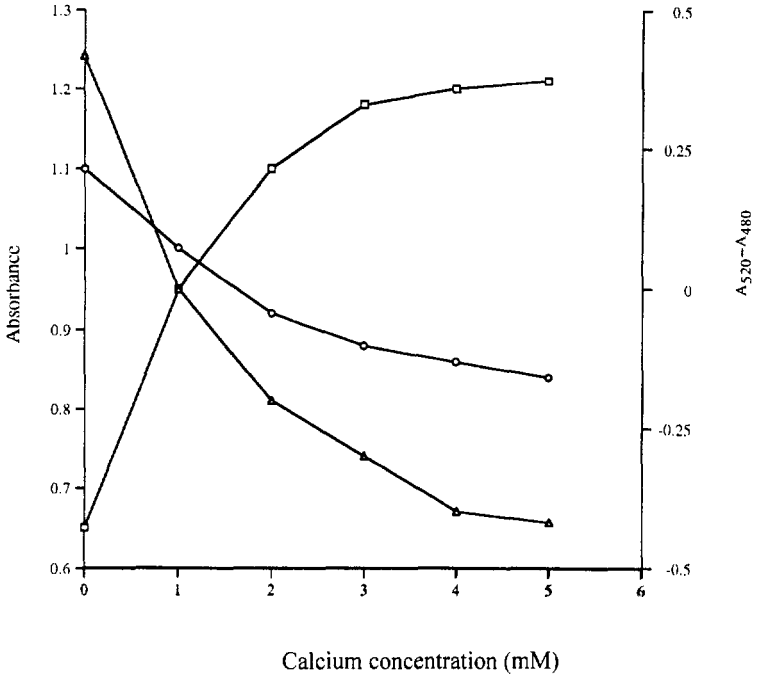


Figure 5.9 Standard curve for the absorbance of murexide at 520 nm (○) and of Ca-murexide at 480 nm (□) and $A_{520} - A_{480}$ (△).

The murexide method measures Ca^{2+} only; Mg^{2+}, at the concentration in milk, does not affect the indicator appreciably. Calculation of Mg^{2+} concentration is possible when the total calcium and magnesium (obtained by EDTA titration) is known. This is based on the assumption that the same proportion of each cation is present in the ionic form, which is justifiable since the dissociation constants of their citrate and phosphate salts are virtually identical.

Ca-ion electrode. Ca^{2+} activity (rather than concentration) can be determined rapidly and accurately using a Ca^{2+} ion-specific electrode. Care must be exercised to ensure that the potentiometer is properly standardized using solutions that simulate the composition of milk serum. The Ca^{2+} activity is lower than the Ca^{2+} concentration – values of about 2 mM have been reported.

5.7.4 Colloidal milk salts

As shown in Table 5.5, all the major ionic species in milk, with the exception of Cl^-, are distributed between the soluble and colloidal phases, but the

principal colloidal salt is calcium phosphate; about 67% and 57%, respectively, of the total calcium and phosphate are in the colloidal phase. The colloidal inorganic salts are, therefore, frequently referred to as **colloidal calcium phosphate** (CCP), although some sodium, potassium, magnesium, zinc and citrate are also present in the colloidal phase. CCP is closely associated with the casein micelles and there are two principal questions as to its nature:

- its composition and structure;
- the nature of its association with casein.

Composition and structure. All the colloidal sodium ($40\,mg\,l^{-1}$), potassium ($110\,mg\,l^{-1}$) and most of the magnesium ($30\,mg\,l^{-1}$) are probably associated with the casein as counter-ions to the negatively charged organic phosphate and carboxylic acid groups of the protein. It has been calculated that approximately 30% of the colloidal calcium (*c.* $250\,mg\,l^{-1}$) is also directly attached to these groups. According to most authors (Pyne, 1962), casein is capable of binding 25–30 moles calcium per 10^5 g casein (i.e. about 1160 g calcium per 10^5 g casein). Assuming that milk contains 25 g casein l^{-1}, the calcium-binding potential of the casein is about $300\,mg\,l^{-1}$ of milk. Since the neutralizing potential of Na^+ and K^+ is half that of Ca^{2+} and Mg^{2+}, the binding capacity of $300\,mg\,l^{-1}$ is reasonably close to the sum of the values given above.

These calculations leave about 500 mg of calcium and about 350 mg of phosphate present in the colloidal phase per litre of milk to be accounted for. The available evidence suggests that the excess CCP is present largely as tricalcium phosphate, $Ca_3(PO_4)_2$, or some similar salt.

The so-called Ling oxalate titration indicates that CCP consists of 80% $Ca_3(PO_4)_2$ and 20% $CaHPO_4$, with an overall Ca:P ratio of 1.4:1 (Pyne, 1962). However, the oxalate titration procedure has been criticized because many of the assumptions made are not reliable. Pyne and McGann (1960) developed a new technique to study the composition of CCP. Milk was acidified to about pH 4.9 at 2°C, followed by exhaustive dialysis of the acidified milk against a large excess of bulk milk; this procedure restored the acidified milk to normality in all respects except that CCP was not reformed. Analysis of milk and CCP-free milk (assumed to differ from milk only in respect of CCP) showed that the ratio of Ca:P in CCP was 1.7:1. The difference between this value and that obtained by the oxalate titration (i.e. 1.4:1) was attributed to the presence of citrate in the CCP complex, which is not measured by the oxalate method. Pyne and McGann (1960) suggested that CCP has an apatite structure with the formula:

$$3Ca_3(PO_4)_2, CaHCitr^- \quad \text{or} \quad 2.5Ca_3(PO_4)_2, CaHPO_4, 0.5\,Ca_3Citr_2^-.$$

Based on the assumption that the amount of Ca bound directly to casein is equivalent to the number of ester phosphate groups present, Schmidt

(1982) argues that CCP is most likely to be amorphous tricalcium phosphate [$Ca_3(PO_4)_2$]. The argument is as follows: It is likely that the phosphoserine residues of the caseins are potential sites for interaction with CCP. The importance of these residues in calcium binding has been demonstrated also for dentine and salivary phosphoproteins. In a casein micelle of particle weight 10^8 Da, consisting of 93.3% casein, with an ester phosphorus content of 0.83%, there are 25 000 ester phosphate groups. Such a micelle contains about 70 500 calcium atoms and about 30 000 inorganic phosphate residues, from which 5000 $Ca_9(PO_4)_6$ clusters might be formed, leaving 25 500 calcium atoms. This means that there is aproximately one calcium atom for each ester phosphate group and that about 40% of these ester phosphate groups can be linked in pairs via $Ca_9(PO_4)_6$ clusters, as shown in Figure 5.10. It is suggested that $Ca_9(PO_4)_6$ clusters adsorb two calcium atoms, which easily fit into the crystal grid, and thus acquire a positive charge and can interact electrostatically with the negatively charged ester phosphate groups of casein. The proposed structure and association with the casein micelles is shown in Figure 5.10.

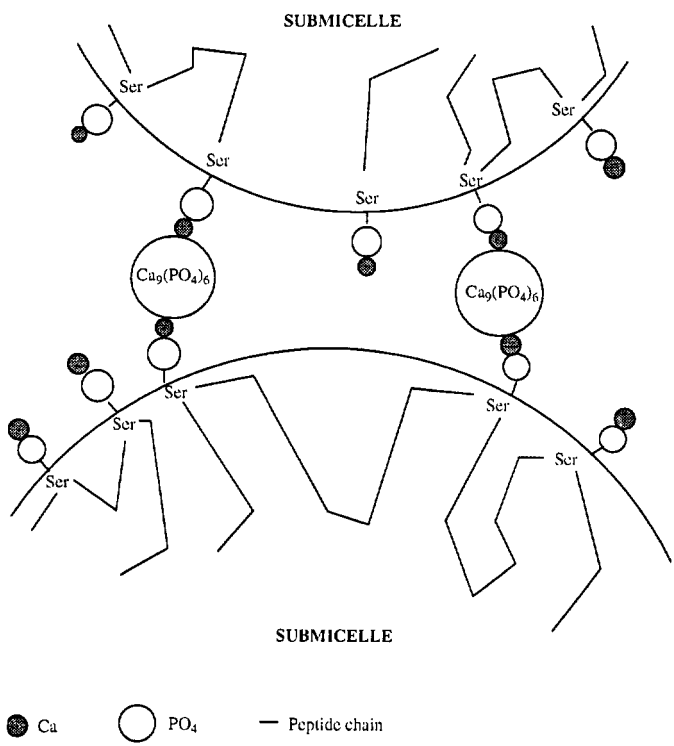


Figure 5.10 Association of colloidal calcium phosphate ($Ca_3(PO_4)_2$) with the serine phosphate groups of casein (from Schmidt, 1982).

The best physical studies, using various forms of X-ray spectroscopy, on the structure of CCP have been undertaken by Holt and colleagues (Holt, 1985). It was concluded that the most likely form of CCP is brushite ($CaHPO_4.2H_2O$), which has also been identified in bone and other calcified tissues. He explains the difference between the Ca/P ratio found by analysis, i.e. 1.51–1.6 and the Ca/P ratio of $CaHPO_4$, i.e. 1.0, as being due to the ability of the phosphate moiety of phosphoserine to substitute in surface sites of a brushite-type lattice.

Association with casein. The colloidal calcium phosphate is closely associated with the casein; it does not precipitate out of solution and is considered to be protected against precipitation by the casein. Two possible forms of protection are suggested:

- physical protection;
- chemical association between CCP and casein.

Experimental evidence strongly favours the idea of chemical association:

- CCP remains attached to the casein following treatment with protein dissociating agents (e.g. urea) or following proteolysis.
- Comparison of the potentiometric titration curves of milk and CCP-free milk shows more reactive organic phosphate groups in the latter, suggesting that CCP is attached to the organic casein phosphate groups, thereby rendering them less active.
- The formol titration is not influenced by removal of CCP, suggesting that εNH_2-groups of lysine are not involved.

The views of Schmidt and Holt on the association between CCP and casein, i.e. via a shared Ca^{2+} (Schmidt) or a shared phosphoserine, i.e. phosphoserine as part of the CCP crystal lattice (Holt), support the hypothesis of chemical association.

Although CCP represents only about 6% of the dry weight of the casein micelle, it plays an essential role in its structure and properties and hence has major effects on the properties of milk; it is the integrating factor in the casein micelle; without it, milk is not coagulable by rennet and its heat and calcium stability properties are significantly altered. In fact, milk would be a totally different fluid without colloidal calcium phosphate.

As discussed in Chapter 4 (p. 186), Holt (1994) has proposed that casein has evolved with the ability to bind high concentrations of calcium and phosphate so that milk can contain high levels of these ions, which are essential for neonatal growth, without precipitation in the ducts of the mammary glands.

5.8 Changes in milk salts equilibria induced by various treatments

The equilibria between the soluble and colloidal salts of milk are influenced by many factors, the more important of which are discussed below, and which consequently modify the processing properties of milk.

Milk serum is supersaturated with calcium phosphate, the excess being present in the colloidal phase, as described above. The balance between the colloidal and soluble phases may be upset by various factors, including changes in temperature, dilution or concentration, addition of acid, alkali or salts. The solubility product for secondary calcium phosphate, $[Ca^{2+}][HPO_4^{2-}]$ is about 1.5×10^{-5} or $pK_s = 4.85$.

5.8.1 Addition of acid or alkali

Acidification of milk is accompanied by a progressive solubilization of colloidal calcium phosphate and other colloidal salts from casein. Solubilization is complete below about pH 4.9 (Figure 5.11).

Addition of alkali has the opposite effect, and at about pH 11 almost all the soluble calcium phosphate occurs in the colloidal phase. These changes are not reversible on subsequent dialysis against untreated milk.

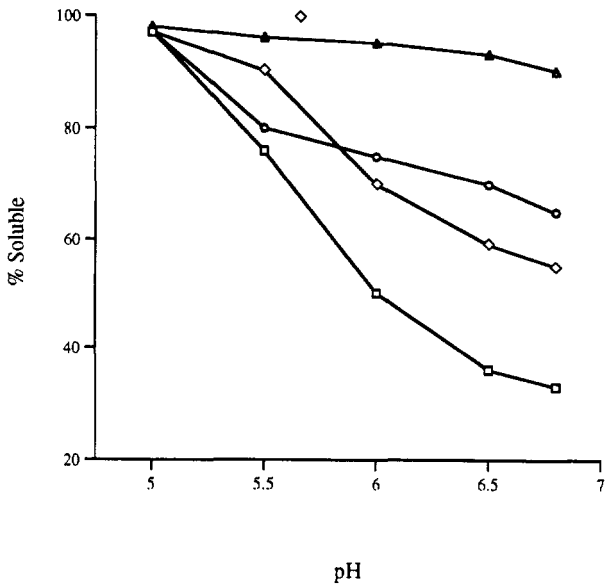


Figure 5.11 Effect of pH on the distribution of calcium (□), inorganic phosphorus (◇), magnesium (○) and citrate (△) between the colloidal and soluble phases in bovine milk.

5.8.2 *Addition of various salts*

Divalent cations. Addition of calcium to milk causes precipitation of soluble phosphate as colloidal calcium phosphate, an increase in ionized calcium, a decrease in the concentration of soluble phosphate and a decrease in pH.

Phosphate. Addition of secondary Na or K phosphate (i.e. Na_2HPO_4 or K_2HPO_4) causes the precipitation of colloidal calcium phosphate, with concomitant decreases in the concentration of soluble calcium and calcium ion. Polyphosphates, e.g. Na-hexametaphosphate, chelate Ca^{2+} strongly and dissolve CCP.

Citrate. Addition of citrate reduces the concentrations of calcium ions and colloidal calcium phosphate and increases the soluble calcium, soluble phosphate and pH.

5.8.3 *Effect of changes in temperature*

The solubility of calcium phosphate is markedly temperature-dependent. Unlike most compounds, the solubility of calcium phosphate decreases with increasing temperature; therefore, heating causes precipitation of calcium phosphate while cooling increases the concentrations of soluble calcium and

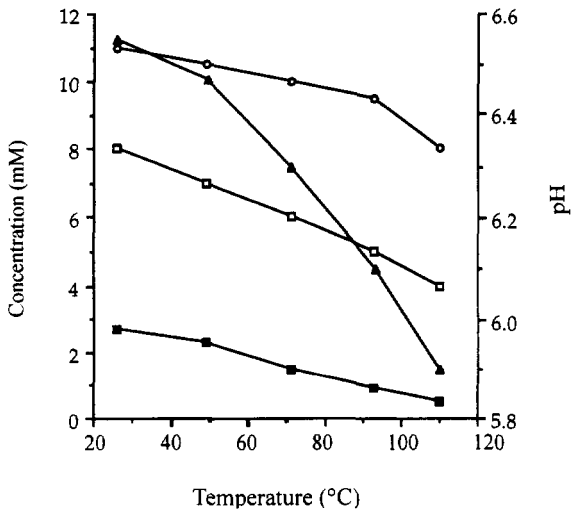


Figure 5.12 Concentration of total calcium (□), calcium ions (■), phosphate (○) and pH (▲) of ultrafiltrates prepared from milk at various temperatures (from Rose and Tessier, 1959).

phosphate at the expense of CCP. At low temperatures, shifts in the ionic balance are readily reversible, but after heating at high temperatures, reversibility becomes more sluggish and incomplete. Comparatively slight changes (20 to 3°C) cause substantial changes in equilibrium (Table 5.4) which are completely reversible. The effects of high temperature treatments were studied by Rose and Tessier (1959) using ultrafiltration of milk at various temperatures. Calcium and phosphate precipitate rapidly on heating (essentially complete within 5 min), to an extent dependent on temperature (Figure 5.12), but the distribution of Na, K, Mg or citrate are not affected. On cooling, these changes are partly reversible.

5.8.4. *Changes in pH induced by temperature*

The pH of milk is changed following heating due to changes in two salt systems. Fresh milk contains 200 mg $CO_2\,l^{-1}$; about 50% of this is lost on standing, with additional losses on heating. This results in a decrease in titratable acidity and an increase in pH. The formation of colloidal calcium phosphate during heating more than compensates for the loss of CO_2. The effect of temperature on pH is shown in Table 5.8 and Figure 5.12.

The change in pH can be described as follows:

$$3Ca^{2+} + 2HPO_4^{2-} \underset{\text{cooling}}{\overset{\text{heating}}{\rightleftharpoons}} Ca_3(PO_4)_2 + 2H^+$$

The reaction is reversible on cooling after heating to moderate temperatures but becomes only partially reversible following more severe heating. The shifts in calcium phosphate equilibrium and pH increase when milk is concentrated.

5.8.5 *Effect of dilution and concentration*

Since milk is saturated with respect to calcium and phosphate, dilution reduces the concentration of Ca^{2+} and HPO_4^{2-} and causes solution of some colloidal calcium phosphate, making the milk more alkaline. Concentration

Table 5.8 Effect of temperature on the pH of milk

Temperature (°C)	pH
20	6.64
30	6.55
40	6.45
50	6.34
60	6.23

of milk causes precipitation of colloidal phosphate and shifts the reaction of milk to the acid side, e.g. concentration by a factor of 2:1 reduces the pH to 6.2.

$$\text{Dilution:} \quad Ca_3(PO_4)_2 \xrightarrow{H_2O} 3Ca^{2+} + 2HPO_4^{2-} + 2OH^-$$

$$\text{Concentration:} \quad 3Ca^{2+} + 2HPO_4^{2-} \rightarrow Ca_3(PO_4)_2 + 2H^+$$

5.8.6 Effect of freezing

Freezing milk causes crystallization of pure water and the unfrozen liquid becomes more saturated with respect to various salts. Some soluble calcium phosphate precipitates as $Ca_3(PO_4)_2$, with the release of H^+ and a decrease in pH (e.g. to 5.8 at $-20°C$).

As discussed in Chapter 2 (p. 38), crystallization of lactose as α-monohydrate exacerbates the situation. The combination of increased concentrations of Ca^{2+} and reduced pH causes destabilization of the casein micelles.

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6 Vitamins in milk and dairy products

6.1 Introduction

Vitamins are organic chemicals required by the body in trace amounts but which cannot be synthesized by the body. The vitamins required for growth and maintenance of health differ between species; compounds regarded as vitamins for one species may be synthesized at adequate rates by other species. For example, only primates and the guinea-pig require ascorbic acid (vitamin C; section 6.4) from their diet; other species possess the enzyme gluconolactone oxidase which is necessary for the synthesis of vitamin C from D-glucose or D-galactose. The chemical structures of the vitamins have no relationship with each other. The principal classification of vitamins is based on their solubility in water. Water-soluble vitamins are the B group (thiamin, riboflavin, niacin, biotin, panthothenate, folate, pyridoxine (and related substances, vitamin B_6) and cobalamin (and its derivatives, vitamin B_{12})) and ascorbic acid (vitamin C) while the fat-soluble vitamins are retinol (vitamin A), calciferols (vitamin D), tocopherols (and related compounds, vitamin E) and phylloquinone (and related compounds, vitamin K). The water-soluble vitamins and vitamin K function as co-enzymes while vitamin A is important in the vision process, vitamin D functions like a hormone and vitamin E is primarily an antioxidant.

Milk is the only source of nutrients for the neonatal mammal during the early stage of life until weaning. Thus, in addition to providing macronutrients (protein, carbohydrate and lipid) and water, milk must also supply sufficient vitamins and minerals to support the growth of the neonate. Human beings continue to consume milk into adulthood and thus milk and dairy products continue to be important sources of nutrients in the diet of many peoples worldwide. The concentrations of macronutrients and minerals in milk have been discussed in Chapters 1 and 5; vitamin levels in milk and dairy products will be considered here. Milk is normally processed to a lesser or greater extent before consumption. Thus it is important to consider the influence of processing on the vitamin status of milk and dairy products.

Recommended dietary allowances (RDA) for vitamins are recommended intake of various vitamin to ensure the good health of a high proportion of the human population. The RDA values quoted below refer to the United States population (Whitney and Rolfes, 1996). Reference nutrient intake

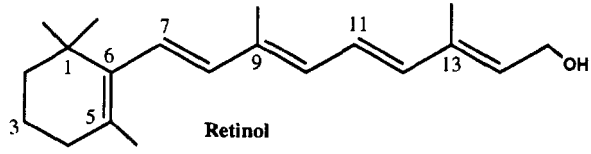
(RNI) is the quantity of a nutrient sufficient to meet the needs of 97% of the population. Nutrient intakes equal to the RNI thus pose only a very small risk of deficiency. United Kingdom RNI values (Department of Health, 1991) are also quoted below.

6.2 Fat-soluble vitamins

6.2.1 Retinol (vitamin A)

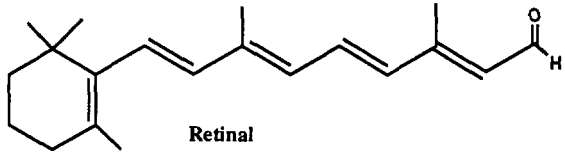
Vitamin A (retinol, **6.1**) is the parent of a range of compounds known as retinoids, which possess the biological activity of vitamin A. In general, animal foods provide preformed vitamin A as retinyl esters (e.g. **6.5**, which are easily hydrolysed in the gastrointestinal tract) while plant foods provide precursors of vitamin A, i.e. carotenoids. Only carotenoids with a β-ionone ring (e.g. β-carotene) can serve as vitamin A precursors. β-Carotene (**6.6**)

6.1



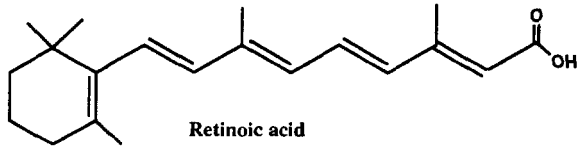
Retinol

6.2



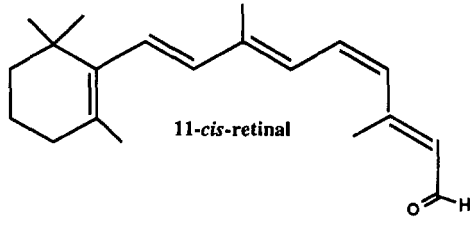
Retinal

6.3



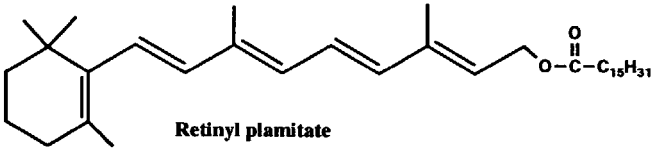
Retinoic acid

6.4



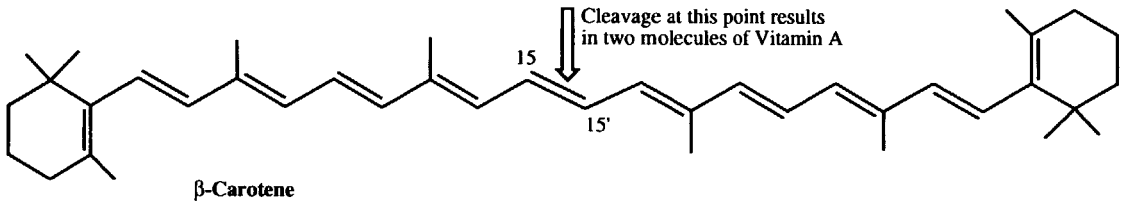
11-*cis*-retinal

6.5



Retinyl plamitate

6.6



β-Carotene

may be cleavaged at its centre by the enzyme, β-carotene-15,15′-oxygenase (present in the intestinal mucosa), to yield 2 mol retinol per mol. However, cleavage of other bonds results in the formation of only 1 molecule of retinol per molecule of β-carotene. In practice, 6 μg β-carotene will yield only 1 μg of retinol. Likewise, 12 μg other carotenes which are vitamin A precursors (i.e. which contain one β-ionone ring) are required to yield 1 μg of retinol. Thus, 1 retinol equivalent (RE) is defined as 1 μg retinol, 6 μg β-carotene or 12 μg of other precursor carotenes.

Retinol can be oxidized to retinal (**6.2**) and further to retinoic acid (**6.3**). *Cis–trans* isomerization can also occur, e.g. the conversion of all *trans*-retinal to 11-*cis*-retinal (**6.4**), which is important for vision.

Vitamin A has a number of roles in the body: it is involved in the vision process, in cell differentiation, in growth and bone remodelling and in the immune system. US RDAs for vitamin A are 1000 μg RE day^{-1} for men and 800 μg RE day^{-1} for women. UK RNI values for vitamin A are 700 and 600 μg RE day^{-1} for adult men and women, respectively. The body will tolerate a wide range of vitamin A intakes (500–15 000 μg RE day^{-1}) but insufficient or excessive intakes result in illness. Vitamin A deficiency (<500 μg RE day^{-1}) results in night blindness, xerophthalmia (progressive blindness caused by drying of the cornea of the eye), keratinization (accumulation of keratin in digestive, respiratory and urogenital tract tissues) and finally exhaustion and death. At excessive intake levels ($>15\,000$ μg RE day^{-1}), vitamin A is toxic. Symptoms of hypervitaminosis A include skin rashes, hair loss, haemorrhages, bone abnormalities and fractures, and in extreme cases, liver failure and death.

The major dietary sources of retinol are dairy products, eggs and liver, while important sources of β-carotene are spinach and other dark-green leafy vegetables, deep orange fruits (apricots, cantaloupe) and vegetables (squash, carrots, sweet potatoes, pumpkin). The richest natural sources of vitamin A are fish liver oils, particularly halibut and shark.

Vitamin A activity is present in milk as retinol, retinyl esters and as carotenes. Whole cows' milk contains an average of 52 μg retinol and 21 μg carotene per 100 g. The concentration of retinol in raw sheep's and pasteurized goats' milks is 83 and 44 μg per 100 g, respectively, although milks of these species are reported (Holland *et al.*, 1991) to contain only trace amounts of carotenes. Human milk and colostrum contain an average of 58 and 155 μg retinol per 100 g, respectively. In addition to their role as provitamin A, the carotenoids in milk are reponsible for the colour of milk fat (Chapter 11).

The concentration of vitamin A and carotenoids in milk is strongly influenced by the carotenoid content of the feed. Milk from animals fed on pasture contains higher levels of carotenes than that from animals fed on concentrate feeds. There is also a large seasonal variation in vitamin A concentration; summer milk contains an average of 62 μg retinol and 31 μg carotene per 100 g while the values for winter milk are 41 and 11 μg per

100 g, respectively. The breed of cow also has an influence on the concentration of vitamin A in milk: milk from Channel Islands breeds typically contains 65 μg and 27 μg retinol per 100 g in summer and winter, respectively, and 115 and 27 μg carotene per 100 g in summer and winter, respectively.

Other dairy products are also important sources of vitamin A (Appendix 6A). Whipping cream (39% fat) contains about 565 μg retinol and 265 μg carotene per 100 g. The level of vitamin A in cheese varies with the fat content (Appendix 6A). Camembert (23.7% fat) contains 230 μg retinol and 315 μg carotene per 100 g, while Cheddar (34.4% fat) contains 325 μg retinol and 225 μg carotene per 100 g. Whole-milk yogurt (3% fat; unflavoured) contains roughly 28 μg retinol and 21 μg carotene per 100 g while the corresponding values for ice-cream (9.8% fat) are 115 and 195 μg per 100 g, respectively.

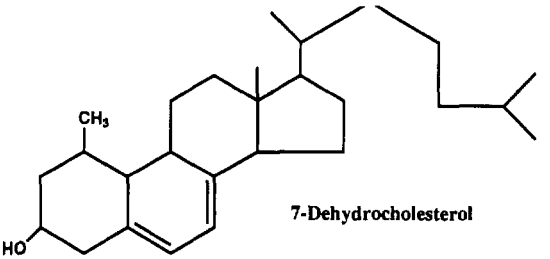
Vitamin A is relatively stable to most dairy processing operations. In general, vitamin A activity is reduced by oxidation and exposure to light. Heating below 100°C (e.g. pasteurization) has little effect on the vitamin A content of milk, although some loss may occur at temperatures above 100°C (e.g. when frying using butter). Losses of vitamin A can occur in UHT milk during its long shelf-life at ambient temperatures. Vitamin A is stable in pasteurized milk at refrigeration temperatures provided the milk is protected from light, but substantial losses can occur in milk packaged in translucent bottles. Low-fat milks are often fortified with vitamin A for nutritional reasons. Added vitamin A is less stable to light than the indigenous vitamin. The composition of the lipid used as a carrier for the exogenous vitamin influences its stability. Protective compounds (e.g. ascorbyl palmitate or β-carotene) will reduce the rate at which exogenous vitamin A is lost during exposure to light. Yogurts containing fruit often contain higher concentrations of vitamin A precursor carotenoids than natural yogurts. The manufacture of dairy products which involves concentration of the milk fat (e.g. cheese, butter) results in a *pro rata* increase in the concentration of vitamin A. The increased surface area of dried milk products accelerates the loss of vitamin A; supplementation of milk powders with vitamin A and storage at low temperatures minimizes these losses.

6.2.2 Calciferols (vitamin D)

Unlike other vitamins, cholecalciferol (vitamin D_3) can be formed from a steroid precursor, 7-dehydrocholesterol (**6.7**), by the skin when exposed to sunlight; with sufficient exposure to the sun, no preformed vitamin D is required from the diet.

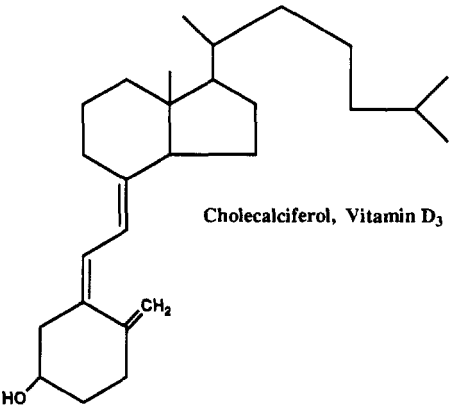
UV light (280–320 nm) causes the photoconversion of 7-dehydrocholesterol to pre-vitamin D_3. This pre-vitamin can undergo further photoconversion to tachysterol and lumisterol or can undergo a temperature-dependent isomerization to cholecalciferol (vitamin D_3, **6.8**). At body temperature, this

6.7



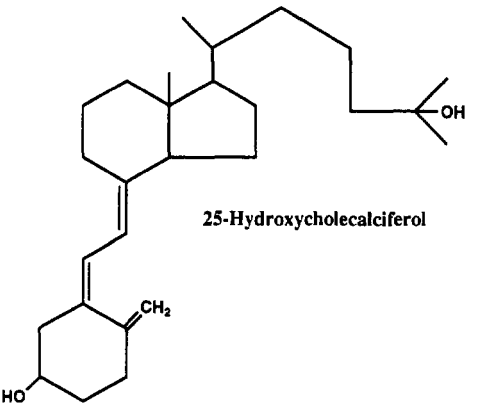
7-Dehydrocholesterol

6.8



Cholecalciferol, Vitamin D_3

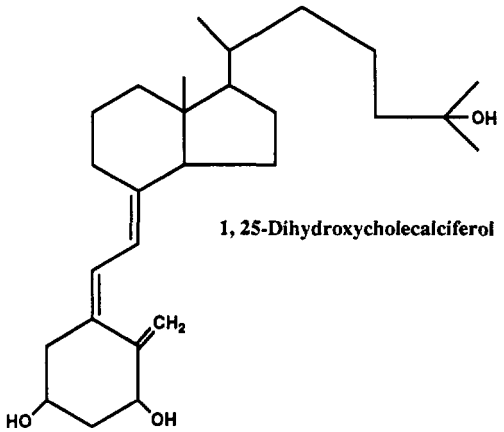
6.9



25-Hydroxycholecalciferol

conversion requires about 28 h to convert 50% of previtamin D_3 to vitamin D_3. Thus, production of vitamin D_3 in the skin can take a number of days. Preformed vitamin D_3 is obtained from the diet. Vitamin D_3 is stored in various fat deposits around the body. Regardless of the source of vitamin D_3, it must undergo two hydroxylations to become fully active. Vitamin D_3 is transported by a specific binding protein through the circulatory system to the liver where the enzyme, 25-hydroxylase, converts it to 25-hydroxy-

6.10



1, 25-Dihydroxycholecalciferol

cholecalciferol ($25(OH)D_3$; **6.9**) which is converted to 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$; **6.10**) by the enzyme, 1-hydroxylase, in the kidney. Alternatively, $25(OH)D_3$ can be hydroxylated at position 24 to form 24,25-dihydroxycholecalciferol ($24,25(OH)_2D_3$). At least 37 metabolites of vitamin D_3 have been identified, but only $3,25(OH)_2D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ have significant biological activity; $1,25(OH)_2D_3$ is the most biologically active metabolite of vitamin D_3.

Vitamin D_2 (ergocalciferol) is formed by the photoconversion of ergosterol, a sterol present in certin fungi and yeasts, and differs from cholecalciferol in having an extra methyl group at carbon 24 and an extra double bond between C_{22} and C_{23}. Ergocalciferol was widely used for many years as a therapeutic agent.

The principal physiological role of vitamin D in the body is to maintain plasma calcium by stimulating its absorption from the gastrointestinal tract, its retention by the kidney and by promoting its transfer from bone to the blood. Vitamin D acts in association with other vitamins, hormones and nutrients in the bone mineralization process. In addition, vitamin D has a wider physiological role in other tissues in the body, including the brain and nervous system, muscles and cartilage, pancreas, skin, reproductive organs and immune cells.

The RDA for vitamin D is 10 and 5 $\mu g\,day^{-1}$ for persons aged 19–24 years or over 25 years, respectively. RNI values for vitamin D are 10 $\mu g\,day^{-1}$ for persons over 65 years and for pregnant or lactating women. With the exception of these and other at-risk groups, the RNI value for dietary vitamin D is 0 $\mu g\,day^{-1}$. The classical syndrome of vitamin D deficency is rickets, in which bone is inadequately mineralized, resulting in growth retardation and skeletal abnormalities. Adult rickets or osteomalacia occurs most commonly in women who have low calcium intakes and little exposure to sunlight and have had repeated pregnancies or periods

of lactation. Hypervitaminosis D (excess intake of vitamin D) is characterized by enhanced absorption of calcium and transfer of calcium from bone to the blood. These cause excessively high concentrations of serum calcium which can precipitate at various locations in the body, causing kidney stones or calcification of the arteries. Vitamin D can exert these toxic effects if consumed continuously at only relatively small amounts in excess of the RDA.

Relatively few foods contain significant amounts of vitamin D. In addition to conversion *in situ* by the body, the principal sources of vitamin D are foods derived from animal sources, including egg yolk, fatty fish and liver. Unfortified cows' milk is not an important source of vitamin D.

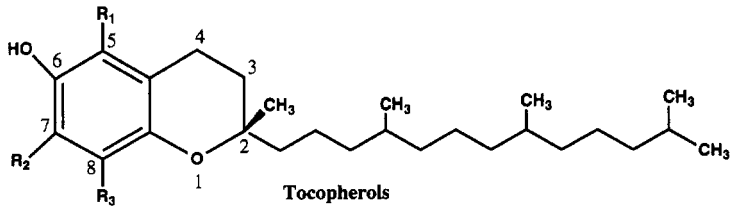
The major form of vitamin D in both cows' and human milk is $25(OH)D_3$. This compound is reported to be responsible for most of the vitamin D in the blood serum of exclusively breast-fed infants. Whole cows' milk contains only about 0.03 μg vitamin D per 100 g and 1 litre of milk per day will supply only 10–20% of the RDA. Therefore, milk is often fortified (at the level of *c.* 1–10 $\mu g\, l^{-1}$) with vitamin D. Fortified milk, dairy products or margarine are important dietary sources of vitamin D. The concentration of vitamin D in unfortified dairy products is usually quite low. Vitamin D levels in milk vary with exposure to sunlight.

As with other fat-soluble vitamins, the concentration of vitamin D in dairy products is increased *pro rata* by concentration of the fat (e.g. in the production of butter or cheese). Vitamin D is relatively stable during storage and to most dairy processing operations. Studies on the degradation of vitamin D in fortified milk have shown that the vitamin may be degraded by exposure to light. However, the conditions necessary to cause significant losses are unlikely to be encountered in practice. Extended exposure to light and oxygen are needed to cause significant losses of vitamin D.

6.2.3 Tocopherols and related compounds (vitamin E)

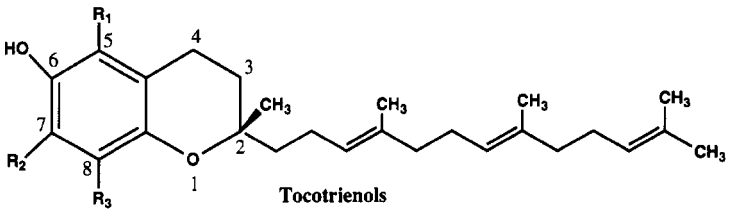
Eight compounds have vitamin E activity, four of which are derivatives of tocopherol (**6.11**) and four of tocotrienol (**6.12**); all are derivatives of 6-chromanol. Tocotrienols differ from tocopherols in having three carbon–carbon double bonds in their hydrocarbon side chain. α-, β-, γ- or δ-tocopherols and tocotrienols differ with respect to number and position of methyl groups on the chromanol ring. The biological activity of the different forms of the tocopherols and tocotrienols varies with their structure. D- and L-enantiomers of vitamin E also occur; the biological activity of the D-form is higher than that of the L-isomer. Vitamin E activity can be expressed as tocopherol equivalents (TE), where 1 TE is equivalent to the vitamin E activity of 1 mg α-D-tocopherol. The biological activity of β- and γ-tocopherols and α-tocotrienol is 50, 10 and 33% of the activity of α-D-tocopherol, respectively.

6.11



Tocopherols

6.12



Tocotrienols

α– $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = CH_3$
β– $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$
γ– $R_1 = H$, $R_2 = CH_3$, $R_3 = CH_3$
δ– $R_1 = H$, $R_2 = H$, $R_3 = CH_3$

Vitamin E is a very effective antioxidant. It can easily donate a hydrogen from the phenolic -OH group on the chromanol ring to free radicals. The resulting vitamin E radical is quite unreactive as it is stabilized by delocalization of its unpaired electron into the aromatic ring. Vitamin E thus protects the lipids (particularly polyunsaturated fatty acids) and membranes in the body against damage caused by free radicals. The role of vitamin E is of particular importance in the lungs where exposure of cells to oxygen is greatest. Vitamin E also exerts a protective effect on red and white blood cells. It has been suggested that the body has a system to regenerate active vitamin E (perhaps involving vitamin C) once it has acted as an antioxidant.

Vitamin E deficiency is normally associated with diseases of fat malabsorption and is rare in humans. Deficiency is characterized by erythrocyte haemolysis and prolonged deficiency can cause neuromuscular dysfunction. Hypervitaminosis E is not common, despite an increased intake of vitamin E supplements. Extremely high doses of the vitamin may interfere with the blood clotting process.

The RDAs for vitamin E are 10 mg and 8 mg α-TE day^{-1} for men and women, respectively. UK RNI values have not been established for vitamin E since its requirement is largely dependent on the content of polyunsaturated lipids in the diet. However, the Department of Health (1991) suggested that 4 and 3 mg α-TE day^{-1} are adequate for men and women, respectively. The major food sources of vitamin E are polyunsaturated vegetable oils and products derived therefrom (e.g. maragrine, salad dressings), green and leafy

vegetables, wheat germ, whole-grain cereal products, liver, egg yolk, nuts and seeds.

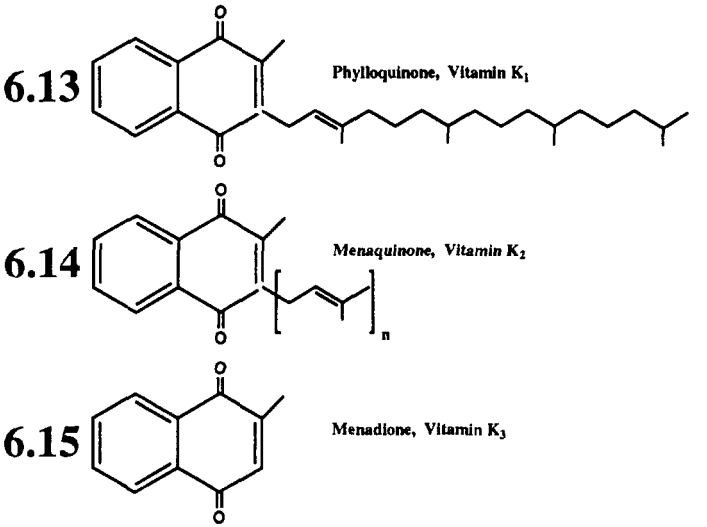
The concentration of vitamin E in cows' milk is quite low (0.09 mg per 100 g) and is higher in summer than in winter milks. Human milk and colostrum contain somewhat higher concentrations ($\sim$0.3 and $\sim$1.3 mg per 100 g, respectively). Most dairy products contain low levels of vitamin E (Appendix 6A) and thus are not important sources of this nutrient. However, levels are higher in dairy products supplemented with vegetable fat (e.g. some ice-creams, imitation creams, fat-filled dried skim milk). Like other fat-soluble vitamins, the concentration of vitamin E in dairy products is increased *pro rata* with fat content. Vitamin E is relatively stable below 100°C but is destroyed at higher temperatures (e.g. deep-fat frying). The vitamin may also be lost through oxidation during processing. Oxidative losses are increased by exposure to light, heat or alkaline pH, and are promoted by the presence of pro-oxidants, lipoxygenase or catalytic trace elements (e.g. Fe^{3+}, Cu^{2+}). Pro-oxidants increase the production of free radicals and thus accelerate the oxidation of vitamin E. Exogenous vitamin E in milk powders supplemented with this nutrient appears to be stable for long storage periods if the powders are held at or below room temperaure. The potential of feed supplemented with vitamin E to increase the oxidative stability of milk has been investigated, as has the potential use of exogenous tocopherols added directly to the milk fat.

6.2.4 Phylloquinone and related compounds (vitamin K)

The structure of vitamin K is characterized by methylnaphthoquinone rings with a side chain at position 3. It exists naturally in two forms: phylloquinone (vitamin K_1; **6.13**) occurs only in plants, while menaquinones (vitamin K_2; **6.14**) are a family of compounds with a side chain consisting of between 1 and 14 isoprene units. Menaquinones are synthesized only by bacteria (which inhabit the human gastrointestinal tract and thus provide some of the vitamin K required by the body). Menadione (vitamin K_3; **6.15**) is a synthetic compound with vitamin K activity. Unlike K_1 and K_2, menadione is water soluble and is not active until it is alkylated *in vivo*.

The physiological role of vitamin K is in blood clotting and is essential for the synthesis of at least four of the proteins (including prothrombin) involved in this process. Vitamin K also plays a role in the synthesis of a protein (osteocalcin) in bone. Vitamin K deficiency is rare but can result from impaired absorption of fat. Vitamin K levels in the body are also reduced if the intestinal flora is killed (e.g. by antibiotics). Vitamin K toxicity is rare but can be caused by excessive intake of vitamin K supplements. Symptoms include erythrocyte haemolysis, jaundice, brain damage and reduced effectiveness of anticoagulants.

The RDAs for vitamin K for people aged 19–24 years are 70 μg and 60 $\mu g\,day^{-1}$ for men and women, respectively. Corresponding values for



adults aged 25 years and over are 80 and 65 $\mu g\,day^{-1}$. The Department of Health (1991) suggested that a vitamin K intake of 1 μg per kg body weight per day is safe and adequate. The principal food sources of vitamin K are liver, green leafy vegetables and milk.

Whole cows' milk contains 0.4–1.8 μg vitamin K per 100 g while human milk contains about 0.2 μg per 100 g. Human colostrum contains higher concentrations of vitamin K, which are necessary since bacteria capable of synthesizing vitamin K take time to become established in the intestine of the neonate. Irradiation under anerobic and apolar conditions can result in *cis–trans* isomerization, resulting in loss of activity since only the *trans* isomer has vitamin K activity. However, unit operations in dairy processing are unlikely to have an effect on the stability of this nutrient.

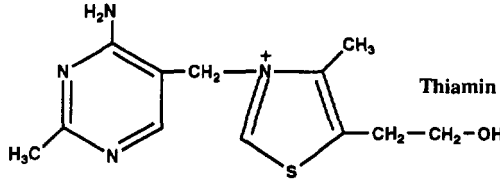
6.3 B-group vitamins

The B-group is a heterogeneous collection of water-soluble vitamins, most of which function as co-enzymes or are precursors of co-enzymes. The B-group vitamins are thiamin, riboflavin, niacin, biotin, pantothenic acid, pyridoxine (and related substances, vitamin B_6), folate and cobalamin (and its derivatives, vitamin B_{12}).

6.3.1 Thiamin (vitamin B_1)

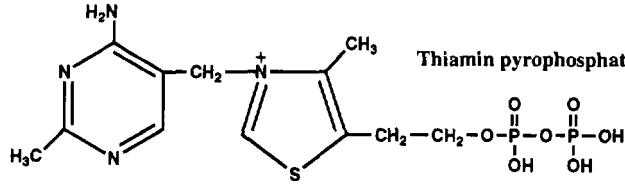
Thiamin (vitamin B_1; **6.16**) consists of two heterocyclic rings (substitued pyrimidine and substituted thiazole), linked by a methylene bridge. Thiamin acts as a co-enzyme in the form of thiamin pyrophosphate (TPP; **6.17**)

6.16



Thiamin (Vitamin B_1)

6.17



Thiamin pyrophosphate

which is an essential co-factor for many enzyme-catalysed reactions in carbohydrate metabolism. TPP-dependent pyruvate dehydrogenase catalyses the conversion of pyruvate ($CH_3COCOOH$) to acetyl CoA (CH_3CO-CoA) in mitochondria. The acetyl CoA produced in this reaction enters the Krebs cycle and also serves as a substrate for the synthesis of lipids and acetylcholine (and thus is important for the normal functioning of the nervous system). TPP is necessary in Krebs cycle for the oxidative decarboxylation of α-ketoglutarate ($HOOCCH_2CH_2COCOOH$) to succinyl CoA ($HOOCCH_2CH_2CO$-CoA) by the α-ketoglutarate dehydrogenase complex. TPP also functions in reactions involving the decarboxylation of ketoacids derived from branched-chain amino acids and in transketolase reactions in the hexose monophosphate pathway for glucose metabolism.

The characteristic disease caused by prolonged thiamin deficiency is beriberi, the symptoms of which include oedema, enlarged heart, abnormal heart rhythms, heart failure, wasting, weakness, muscular problems, mental confusion and paralysis.

Thiamin is widespread in many nutritious foods but pig meat, liver, whole-grain cereals, legumes and nuts are particularly rich sources. Because of its importance in energy metabolism, the RDA for thiamin is quoted in terms of energy intake (0.12 mg kJ^{-1} day^{-1}; 1 mg day^{-1} minimum). This is approximately equivalent to 1.5 mg and 1.1 mg per day for men and women, respectively. The RNI value for thiamin is 0.4 mg per 1000 kcal (4186 kJ) per day for adults.

Milk contains, on average, 0.03 mg thiamin per 100 g. Most (50–70%) of the thiamin in bovine milk is in the free form; lesser amounts are phosphorylated (18–45%) or protein-bound (7–17%). The concentration in mature human milk is somewhat lower (*c.* 0.02 mg per 100 g). Human colostrum contains only trace amounts of thiamin which increase during lactation. Pasteurized milk from goats and Channel Island breeds of cow contain about 0.04 mg per 100 g, while values for raw sheep's milk are somewhat higher, with an average of 0.08 mg per 100 g. Most of the thiamin

in bovine milk is produced by micro-organisms in the rumen and, therefore, feed, breed of the cow or season have relatively little effect on its concentration in milk.

Thiamin levels in milk products (Appendix 6A) are generally 0.02–0.05 mg per 100 g. As a result of the growth of the *Penicillium* mould, the rind of Brie and Camembert cheese is relatively rich in thiamin (0.5 and 0.4 mg per 100 g, respectively).

Thiamin is relatively unstable and is easily cleaved by a nucleophilic displacement reaction at its methylene carbon. The hydroxide ion (OH^-) is a common nucelophile which can cause this reaction in foods. Thiamin is thus more stable under slightly acid conditions. Thiamin is reported to be relatively stable to pasteurization and UHT heat treatment ($\leqslant 10\%$ losses) and during the storage of pasteurized milk, but losses of 20–40% have been reported for UHT milks stored for long periods of time (1–2 years). The light sensitivity of thiamin is less than that of other light-sensitive vitamins.

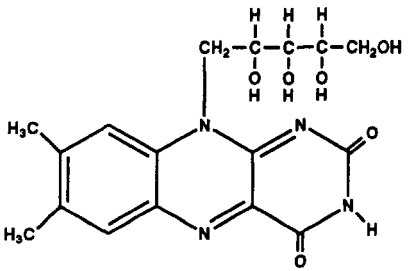
6.3.2 Riboflavin (vitamin B_2)

Riboflavin (vitamin B_2; **6.18**) consists of an isoalloxazine ring linked to an alcohol derived from ribose. The ribose side chain of riboflavin can be modified by the formation of a phosphoester (forming flavin mononucleotide, FMN, **6.19**). FMN can be joined to adenine monophosphate to form flavin adenine dinucleotide (FAD, **6.20**). FMN and FAD act as co-enzymes by accepting or donating two hydrogen atoms and thus are involved in redox reactions. Flavoprotein enzymes are involved in many metabolic pathways. Riboflavin is a yellow-green fluorescent compound and, in addition to its role as a vitamin, it is responsible for the colour of milk serum (Chapter 11).

Symptoms of riboflavin deficiency include cheilosis (cracks and redness at the corners of the mouth), glossitis (painful, smooth tongue), inflamed eyelids, sensitivity of the eyes to light, reddening of the cornea and skin rash. The US RDA for riboflavin is expessed in terms of energy intake (*c.* 0.14 mg kJ^{-1} day^{-1}, equivalent to about 1.7 and 1.3 mg day^{-1} for men and women, respectively). Corresponding UK RNI values are 1.3 and 1.1 mg day^{-1} for adult men and women, respectively. Important dietary sources of riboflavin include milk and dairy products, meat and leafy green vegetables. Cereals are poor sources of riboflavin, unless fortified. There is no evidence for riboflavin toxicity.

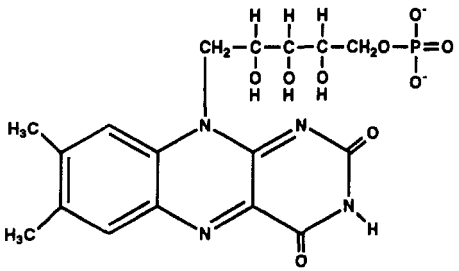
Milk is a good source of riboflavin; whole milk contains about 0.17 mg per 100 g. Most (65–95%) of the riboflavin in milk is present in the free form; the remainder is present as FMN or FAD. Milk also contains small amounts (about 11% of total flavins) of a related compound, 10-(2′-hydroxyethyl) flavin, which acts as an antivitamin. The concentration of this compound must be considered when evaluating the riboflavin activity in milk. The concentration of riboflavin in milk is influenced by the breed of

6.18



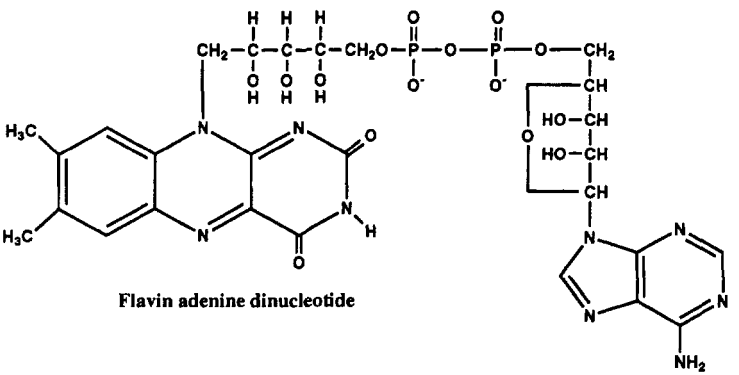
Riboflavin

6.19



Flavin mononucleotide

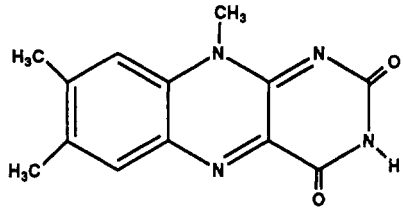
6.20



Flavin adenine dinucleotide

cow (milk from Jersey and Guernsey cows contains more riboflavin than Holstein milk). Summer milk generally contains slighly higher levels of riboflavin than winter milk. Interspecies variations in concentration are also apparent. Raw sheep's milk contains about 0.32 mg per 100 g while the mean value for pasteurized goats' milk (0.13 mg per 100 g) is lower; human milk contains 0.03 mg per 100 g. Dairy products also contain significant amounts

6.21



Lumiflavin

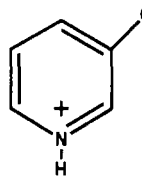
of riboflavin (Appendix 6A). Cheese contains 0.3–0.5 mg per 100 g and yogurt about 0.3 mg per 100 g. The whey protein fraction of milk contains a riboflavin-binding protein (RfBP) which probably originates from blood plasma, although its function in milk is unclear.

Riboflavin is stable in the presence of oxygen, heat and at acid pH. However, it is labile to thermal decomposition under alkaline conditions. The concentration of riboflavin in milk is unaffected by pasteurization and little loss is reported for UHT-treated milks. The most important parameter affecting the stability of riboflavin in dairy products is exposure to light (particularly wavelengths in the range 415–455 nm). At alkaline pH, irradiation cleaves the ribitol portion of the molecule, leaving a strong oxidizing agent, lumiflavin (**6.21**). Irradiation under acidic conditions results in the formation of lumiflavin and a blue fluorescent compound, lumichrome. Lumiflavin is capable of oxidizing other vitamins, particularly ascorbate (section 6.4 and Chapter 11). Loss of riboflavin in milk packaged in materials that do not protect against light can be caused by either sunlight or by lights in retail outlets. Packaging in paperboard containers is the most efficient method for minimizing this loss, although glass containing a suitable pigment has also been used. Riboflavin is more stable in high-fat than in low-fat or skim milk, presumably as a result of the presence of antioxidants (e.g. vitamin E) in the milk fat which protect riboflavin against photo-oxidation.

6.3.3 Niacin

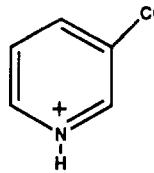
Niacin is a generic term which refers to two related chemical compounds, nicotinic acid (**6.22**) and its amide, nicotinamide (**6.23**); both are derivatives of pyridine. Nicotinic acid is synthesized chemically and can be easily converted to the amide in which form it is found in the body. Niacin is obtained from food or can be synthesized from tryptophan (60 mg of dietary tryptophan has the same metabolic effect as 1 mg niacin). Niacin forms part of two important co-enzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which are co-factors for many enzymes that participate in various metabolic pathways and function in electron transport.

6.22



Nicotinic acid

6.23



Nicotinamide

The classical niacin deficiency disease is pellagra, which is characterized by symptoms including diarrhoea, dermatitis, dementia and eventually death. High-protein diets are rarely deficient in niacin since, in addition to the preformed vitamin, such diets supply sufficient tryptophan to meet dietary requirements. Large doses of niacin can cause the dilation of capillaries, resulting in a painful tingling sensation.

The RDA for niacin is expressed in terms of energy intake; 6.6 mg niacin equivalent (NE, 1 mg niacin or 60 mg tryptophan) per 1000 kcal (4186 kJ) per day is recommended (13 NE day^{-1} minimum). This is approximately equivalent to 19 and 15 mg NE day^{-1} for men and women, respectively. The UK RNI value for niacin is 6.6 mg NE per 1000 kcal (4186 kJ) per day for adults. The richest dietary sources of niacin are meat, poultry, fish and whole-grain cereals.

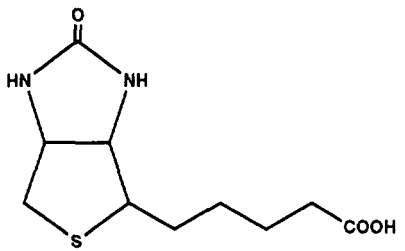
Milk contains about 0.1 mg niacin per 100 g and thus is not a rich source of the preformed vitamin. Tryptophan contributes roughly 0.7 mg NE per 100 g milk. In milk, niacin exists primarily as nicotinamide and its concentration does not appear to be affected greatly by breed of cow, feed, season or stage of lactation. Pasteurized goats' (0.3 mg niacin and 0.7 mg NE from tryptophan per 100 g) and raw sheep's (0.4 mg niacin and 1.3 mg NE from tryptophan per 100 g) milk are somewhat richer than cows' milk. Niacin levels in human milk are 0.2 mg niacin and 0.5 mg NE from tryptophan per 100 g. The concentration of niacin in most dairy products is low (Appendix 6A) but is compensated somewhat by tryptophan released on hydrolysis of the proteins.

Niacin is relatively stable to most food-processing operations. It is stable to exposure to air and resistant to autoclaving (and is therefore stable to pasteurization and UHT treatments). The amide linkage of nicotinamide can be hydrolysed to the free carboxylic acid (nicotinic acid) by treament with acid but the vitamin activity is unaffected. Like other water-soluble vitamins, niacin can be lost by leaching.

6.3.4 *Biotin*

Biotin (**6.24**) consists of an imidazole ring fused to a tetrahydrothiophene ring with a valeric acid side chain. Biotin acts as a co-enzyme for carboxylases involved in the synthesis and catabolism of fatty acids and for branched-chain amino acids and gluconeogenesis.

6.24



Biotin

Biotin deficiency is rare but under laboratory conditions it can be induced by feeding subjects with large amounts of raw egg white which contains the protein, avidin, which has a binding site for the imidazole moiety of biotin, thus making it unavailable. Avidin is denatured by heat and, therefore, biotin binding occurs only in raw egg albumen. Symptoms of biotin deficiency include scaly dermatitis, hair loss, loss of appetite, nausea, hallucinations and depression.

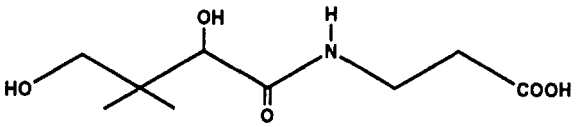
Biotin is widespread in foods, although its availability is affected somewhat by the presence of binding proteins. Biotin is required in only small amounts. Although US RDA values have not been established, the estimated safe and adequate intake of biotin is 30–100 $\mu g\,day^{-1}$ for adults. The Department of Health (1991) suggested that biotin intakes between 10 and 200 $\mu g\,day^{-1}$ are safe and adequate. Biotin is reported to be non-toxic in amounts up to at least 10 $mg\,day^{-1}$.

Milk contains about 1.9 μg biotin per 100 g, apparently in the free form. Pasteurized caprine, raw ovine and human milks contain 3.0, 2.5 and 0.7 μg per 100 g, respectively. The concentration of biotin in cheese ranges from 1.4 (Gouda) to 7.6 (Camembert) μg per 100 g (Appendix 6A). Skim-milk powder contains high levels of biotin (*c.* 20 μg per 100 g) owing to the concentration of the aqueous phase of milk during its manufacture. Biotin is stable during food processing and storage and is unaffected by pasteurization.

6.3.5 *Panthothenic acid*

Pantothenic acid (**6.25**) is a dimethyl derivative of butyric acid linked to β-alanine. Pantothenate is part of the structure of co-enzyme A (CoA), and

6.25



Pantothenic acid

as such is vital as a co-factor for numerous enzyme-catalysed reactions in lipid and carbohydrate metabolism.

Pantothenate deficiency is rare, occurring only in cases of severe malnutrition; characteristic symptoms include vomiting, intestinal distress, insomnia, fatigue and occasional diarrhoea. Pantothenate is widespread in foods; meat, fish, poulty, whole-grain cereals and legumes are particularly good sources. Although no RDA or RNI value has been established for panthothenate, safe and adequate intake of this vitamin for adults is estimated to be 3–7 mg day^{-1}. Pantothenate is non-toxic at doses up to 10 g day^{-1}.

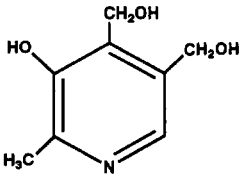
Milk contains, on average, 0.35 mg panthothenate per 100 g. Pantothenate exists partly free and partly bound in milk and its concentration is influenced by breed, feed and season. Raw ovine and pasteurized caprine milks contain slightly higher concentrations of this nutrient (averaging 0.45 and 0.41 mg per 100 g, respectively). The values for pantothenate in human milk vary widely; values ranging from 0.2 to 0.7 mg per 100 g have been reported. Mean concentrations of pantothenate in cheese vary from about 0.3 (cream cheese, Gouda) to 0.7 (Stilton) mg per 100 g (Appendix 6A). Pantothenate is stable at neutral pH but is easily hydrolysed by acid or alkali at high temperatures. Pantothenate is reported to be stable to pasteurization.

6.3.6 Pyridoxine and related compounds (vitamin B_6)

Vitamin B_6 occurs naturally in three related forms: pyridoxine (**6.26**; the alcohol form), pyridoxal (**6.27**; aldehyde) and pyridoxamine (**6.28**; amine). All are structurally related to pyridine. The active co-enzyme form of this vitamin is pyridoxal phosphate (PLP; **6.29**), which is a co-factor for transaminases which catalyse the transfer of amino groups (**6.29**). PLP is also important for amino acid decarboxylases and functions in the metabolism of glycogen and the synthesis of sphingolipids in the nervous system. In addition, PLP is involved in the formation of niacin from tryptophan (section 6.3.3) and in the initial synthesis of haem.

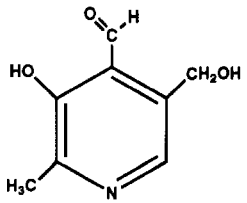
Deficiency of vitamin B_6 is characterized by weakness, irritability and insomnia and later by convulsions and impairment of growth, motor

6.26



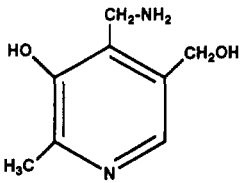
Pyridoxine

6.27



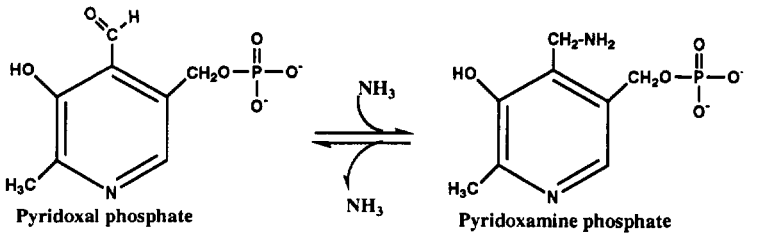
Pyridoxal

6.28



Pyridoxamine

6.29



Pyridoxal phosphate

Pyridoxamine phosphate

functions and immune response. High doses of vitamin B_6, often associated with excessive intake of supplements, are toxic and can cause bloating, depression, fatigue, irritability, headaches and nerve damage.

Since vitamin B_6 is essential for amino acid (and hence protein) metabolism, its RDA is quoted in terms of protein intake (0.016 mg per g protein per day, equivalent to about 2.0 and 1.6 mg day^{-1} for men and women,

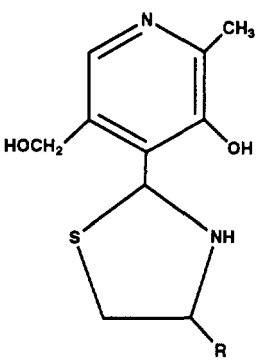
respectively). The corresponding UK RNI value for B_6 is 15 $\mu g\ g^{-1}$ protein for adults. Important sources of B_6 include green, leafy vegetables, meat, fish and poultry, shellfish, legumes, fruits and whole grains.

Whole milk contains, on average, 0.06 mg B_6 per 100 g, mainly in the form of pyridoxal (80%); the balance is mainly pyridoxamine (20%), with trace amounts of pyridoxamine phosphate. Concentrations in raw ovine and pasteurized caprine milks are similar to those in cows' milk (0.08 and 0.06 mg per 100 g, respectively). The concentration of B_6 varies during lactation; colostum contains lower levels than mature milk. Seasonal variation in the concentration of vitamin B_6 has been reported in Finnish milk; levels were higher (14%) when cattle were fed outdoors than when they were fed indoors. Mature human milk contains about 0.01 mg B_6 per 100 g.

In general, dairy products are not major sources of B_6 in the diet. Concentrations in cheeses and related products vary from about 0.04 (fromage frais, cream cheese) to 0.22 (Camembert) mg per 100 g (Appendix 6A). Whole-milk yogurt contains roughly 0.1 mg per 100 g and the concentration in skim-milk powder is *c.* 0.6 mg per 100 g.

All forms of B_6 are sensitive to UV light and may be decomposed to biologically inactive compounds. Vitamin B_6 may also be decomposed by heat. Losses of 45% and 20–30% can occur on cooking meat and vegetables, respectively. The aldehyde group of pyridoxal and the amine group of pyridoxamine show some reactivity under conditions that may be encountered during milk processing. An outbreak of B_6 deficiency in 1952 was attributed to the consumption of heated milk products. Pyridoxal and/or its phosphate can react directly with the sulphydryl group of cysteine residues in proteins, forming an inactive thiazolidine derivative (**6.30**). Losses during pasteurization and UHT treatments are relatively small, although losses of up to 50% can occur in UHT milk during its shelf-life.

6.30

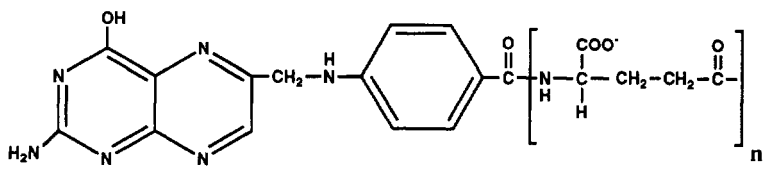


Thiazolidine derivative of pyridoxal

6.3.7 *Folate*

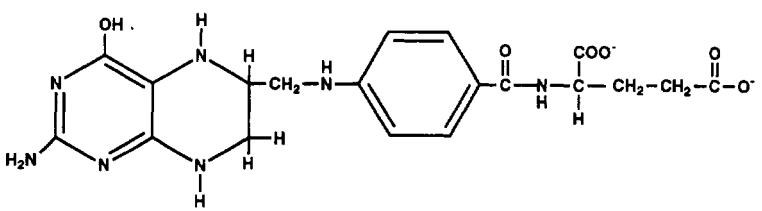
Folate consists of a substituted pteridine ring linked through a methylene bridge to *p*-aminobenzoic acid and glutamic acid (**6.31**). Up to seven glutamic acid residues can be attached by γ-carboxyl linkages, producing polyglutamyl folate (**6.31**) which is the major dietary and intracellular form of the vitamin. Reductions and substitutions on the pteridine ring result in tetrahydrofolate (H_4 folate; **6.32**) and 5-methyl tetrahydrofolate (5-methyl-H_4 folate; **6.33**). Folate is a co-factor in the enzyme-catalysed transfer of single carbon atoms in many metabolic pathways, including the biosynthesis of purines and pyramidines (essential for DNA and RNA) and interconversions of amino acids. Folate interacts with vitamin B_{12} (section 6.3.8) in the enzyme-catalysed synthesis of methionine and in the activation of 5-methyl-H_4 folate to H_4 folate. H_4 Folate is involved in a complex and inter-linked series of metabolic reactions (Garrow and James, 1993).

6.31



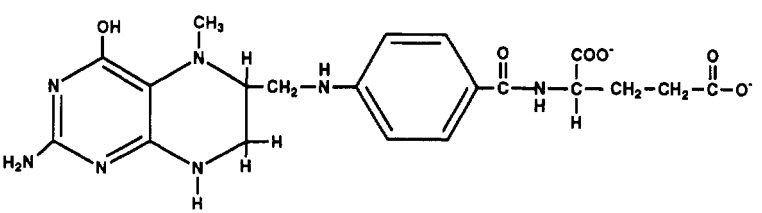
Folate

6.32



Tetrahydrofolate

6.33



5-methyl tetrahydrofolate

Folate deficiency impairs cell division and protein synthesis; symptoms include megaloblastic anaemia, digestive system problems (heartburn, diarrhoea, constipation), suppression of the immune system, glossitis and problems with the nervous system (depression, fainting, fatigue, mental confusion). The RDA for folate is 3 μg per kg body weight per day (equivalent to *c.* 200 and 180 $\mu g\,day^{-1}$ for men and women, respectively). The RNI value for adults is 200 $\mu g\,day^{-1}$. Higher intakes of folate have been suggested for women of child-bearing age to prevent the development of neural tube defects in the developing foetus.

Rich dietary sources of folate include leafy green vegetables, legumes, seeds and liver. Milk contains about 6 μg folate per 100 g. The dominant form of folate in milk is 5-methyl-H_4 folate. Folate in milk is mainly bound to folate-binding proteins and about 40% occurs as conjugated polyglutamate forms. The folate binding proteins of milks of various species have been characterized (Fox and Flynn, 1992). It has been suggested that protein binding increases the bioavailability of folate. Winter milk is reported to contain higher concentrations of folate than summer milk (7 and 4 μg per 100 g, respectively). Raw sheep's milk contains, on average, 5 μg per 100 g while the value for pasteurized goats' milk is 1 μg per 100 g. Folate levels in human milk increase from 2 to 5 μg per 100 g as colostrum changes to mature milk. Folate levels in some dairy products are shown in Appendix 6A. Whipping cream contains about 7 μg per 100 g while the value for cheese varies widely from 30–40 μg per 100 g (Edam, Cheddar) to greater than 100 μg per 100 g (Camembert); the high concentration found in mould-ripened varieties presumably reflects biosynthesis of folate by the mould. The concentration of folate in yogurt is about 18 μg per 100 g, principally in the form of formyl folate. The higher level of folate in yogurt is due to biosynthesis, particularly by *Streptococcus salivarius* subsp. *thermophilus*, and perhaps to some added ingredients.

Folate is a relatively unstable nutrient; processing and storage conditions that promote oxidation are of particular concern since some of the forms of folate found in foods are easily oxidized. The reduced forms of folate (dihydro- and tetrahydrofolate) are oxidized to *p*-aminobenzoylglutamic acid and pterin-6-carboxylic acid, with a concomitant loss in vitamin activity. 5-Methyl-H_4 folate can also be oxidized. Antioxidants (particularly ascorbic acid in the context of milk) can protect folate against destruction. The rate of the oxidative degradation of folate in foods depends on the derivative present and the food itself, particularly its pH, buffering capacity and concentration of catalytic trace elements and antioxidants.

Folate is sensitive to light and may be subject to photodecomposition. Heat treatment influences folate levels in milk. Pasteurization and the storage of pasteurized milks have relatively little effect on the stability of folate but UHT treatments can cause substantial losses. The concentration of oxygen in UHT milk (from the headspace above the milk or by diffusion

through the packaging material) has an important influence on the stability of folate during the storage of UHT milk, as have the concentrations of ascorbate in the milk and of O_2 in the milk prior to heat treatment. Folate and ascorbic acid (section 6.4) are the least stable vitamins in powdered milks.

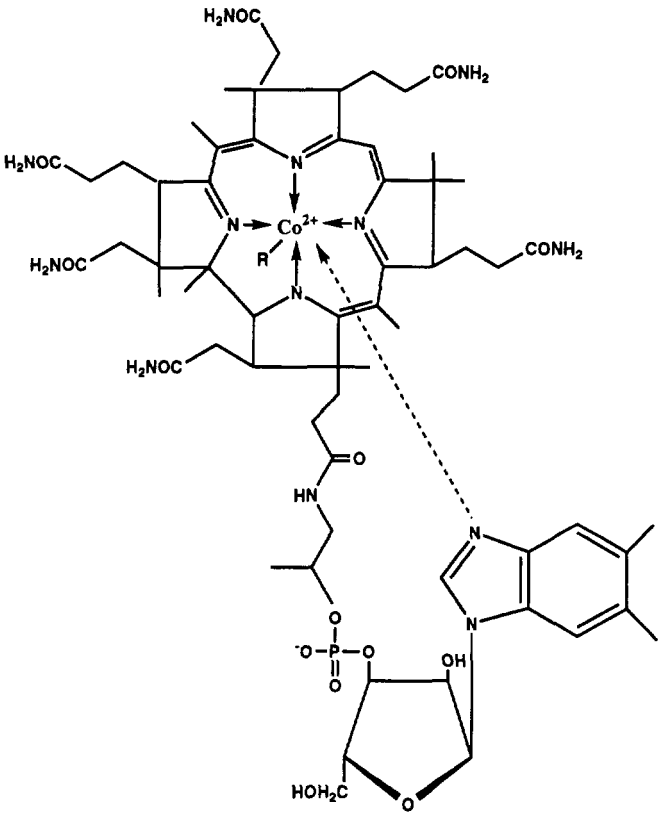
The heat stability of folate-binding proteins in milk should also be considered in the context of folate in dairy foods. Breast-fed babies require less dietary folate (55 μg folate day^{-1} to maintain their folate status) than bottle-fed infants (78 μg day^{-1}). The difference has been attributed to the presence of active folate-binding proteins in breast milk; folate-binding proteins originally present in milk formulae are heat-denatured during processing. However, a study involving feeding radiolabelled folate to rats together with dried milks prepared using different heat treatments showed no differences in folate bioavailability (Öste, Jägerstad and Andersson, 1997).

6.3.8 Cobalamin and its derivatives (vitamin B_{12})

Vitamin B_{12} consists of a porphyrin-like ring structure, with an atom of Co chelated at its centre, linked to a nucleotide base, ribose and phosphoric acid (**6.34**). A number of different groups can be attached to the free ligand site on the cobalt. Cyanocobalamin has -CN at this position and is the commercial and therapeutic form of the vitamin, although the principal dietary forms of B_{12} are 5′-deoxyadenosylcobalamin (with 5′-deoxyadenosine at the R position), methylcobalamin (-CH_3) and hydroxocobalamin (-OH). Vitamin B_{12} acts as a co-factor for methionine synthetase and methylmalonyl CoA mutase. The former enzyme catalyses the transfer of the methyl group of 5-methyl-H_4 folate to cobalamin and thence to homocysteine, forming methionine. Methylmalonyl CoA mutase catalyses the conversion of methylmalonyl CoA to succinyl CoA in the mitochondrion.

Vitamin B_{12} deficiency normally results from indequate absorption rather than inadequate dietary intake. Pernicious anaemia is caused by vitamin B_{12} deficiency; symptoms include anaemia, glossitis, fatigue and degeneration of the peripheral nervous system and hypersensitivity of the skin. The adult RDA and RNI for B_{12} are 2 and 1.5 μg day^{-1}, respectively. Unlike other vitamins, B_{12} is obtained exclusively from animal food sources, such as meat, fish, poultry, eggs, shellfish, milk, cheese and eggs. Vitamin B_{12} in these foods is protein-bound and released by the action of HCl and pepsin in the stomach.

Bovine milk contains, on average, 0.4 μg B_{12} per 100 g. The predominant form is hydroxycobalamin and more than 95% of this nutrient is protein bound. The concentration of B_{12} in milk is influenced by the Co intake of the cow. The predominant source of B_{12} for the cow, and hence the ultimate origin of B_{12} in milk, is biosynthesis in the rumen. Therefore, its concentra-



6.34

Vitamin B_{12}

tion in milk is not influenced greatly by feed, breed or season. Higher concentrations are found in colostrum than in mature milk.

The B_{12}-binding proteins of human milk have been studied in detail. The principal binding protein (R-type B_{12}-binding protein) has a molecular mass of *c.* 63 kDa and contains about 35% carbohydrate. Most or all of the B_{12} in human milk is bound to this protein. A second protein, transcobalamin II, is present at low concentrations.

Raw ovine and pasteurized caprine milks contain 0.6 and 0.1 μg B_{12} per 100 g, respectively. Human colostrum contains 0.1 μg per 100 g but the mature milk contains only traces of B_{12}. Concentrations of B_{12} in dairy products (Appendix 6A) include about 0.3 μg per 100 g for cream and 1 μg per 100 g for many cheese varieties. Yogurt contains roughly 0.2 μg per 100 g of this nutrient.

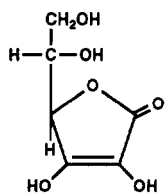
Vitamin B_{12} is stable to pasteurization and storage of pasteurized milks (< 10% loss). UHT heat treatment, and in particular storage of UHT milk, causes greater losses. Storage temperature has a major influence on the

stability of B_{12} in UHT milk. Losses during storage at 7°C are minimal for up to 6 months but at room temperature (the normal storage conditions for UHT milk), losses can be significant after only a few weeks. Oxygen levels in UHT milk do not appear to influence the stability of B_{12}.

6.4 Ascorbic acid (vitamin C)

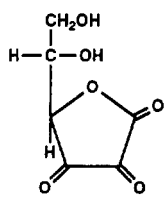
Ascorbic acid (**6.35**) is a carbohydrate which can be synthesized from D-glucose or D-galactose by most species with the exception of primates, guinea-pigs, an Indian fruit bat and certain birds. Ascorbate can be oxidized reversibly to dehydroascorbate (**6.36**) in the presence of transition metal ions, heat, light or mildly alkaline conditions without loss of vitamin activity. Dehydroascorbate can be oxidized irreversibly to 2,3-diketogulonic acid (**6.37**) with loss of activity. 2,3-Diketogulonic acid can be broken down to oxalic and L-threonic acids and ultimately to brown pigments.

6.35



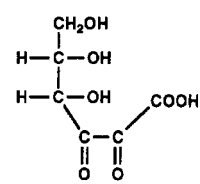
Ascorbic acid

6.36



Dehydroascorbic acid

6.37



2, 3-Diketogulonic acid

Ascorbic acid is a strong reducing agent and therefore is an important antioxidant in many biological systems. It is also necessary for the activity of the hydroxylase that catalyses the post-translational conversion of proline to hydroxyproline and lysine to hydroxylysine. This post-translational hydroxylation is vital for the formation of collagen, the principal protein in connective tissue. Ascorbate functions to maintain iron in its correct oxidation state and aids in its absorption. Vitamin C also functions in amino acid metabolism, in the absorption of iron and increases resistance to infection. The classical vitamin C deficiency syndrome is scurvy, the symptoms of which include microcytic anaemia, bleeding gums, loose teeth, frequent infections, failure of wounds to heal, muscle degeneration, rough skin, hysteria and depression. The popular scientific literature has suggested major health benefits associated with ascorbate intakes far in excess of the RDA. While many of these claims are spurious, they have led to the widespread use of vitamin C supplements. Toxic effects of vitamin C have been reported and include nausea, abdominal cramps, diarrhoea, urinary tract problems and kidney stones. The RDA and RNI for vitamin C are 60 and 40 mg day^{-1}, respectively. However, ascorbate requirements vary with sex, physical stress and perhaps with age. The richest sources of ascorbic acid are fruits and vegetables; milk is a poor source. Milk contains about 1 mg ascorbate per 100 g, although reported values range from about 0.85 to 2.75 mg per 100 g. These differences reflect the fact that ascorbate levels can be reduced markedly during the handling and storage of milk. A ratio of ascorbate to dehydroascorbate in milk of 4 : 1 has been reported, although this ratio is greatly influenced by oxidation. Some authors have reported seasonal differences in the concentration of vitamin C in milk (highest in winter milk) but the influence of this factor is unclear.

Human milk and colostrum contain about 4 and 7 mg ascorbate per 100 g, respectively. Raw sheep's milk contains more ascorbate (*c.* 5 mg per 100 g) than bovine milk, although reported values for pasteurized caprine milk are similar to those for cow's milk. Ascorbate is readily oxidized at the pH of milk. The rate of oxidation is influenced by factors including temperature, light, the concentration of oxygen and the presence of catalytic trace elements. Ascorbic acid is of great importance in establishing and maintaining redox equilibria in milk (as discussed in detail in Chapter 11), the protection of folate (section 6.3.7) and in the prevention of oxidized flavour development in milk. The photochemical degradation of riboflavin (section 6.3.2) catalyses the oxidation of ascorbate.

At least 75% of the vitamin C in milk survives pasteurization, and losses during storage of pasteurized milk are usually minimal. However, considerable losses of vitamin C have been reported in milk packaged in transparent containers. The extent of losses during UHT treatment depends on the amount of oxygen present during heat treatment and subsequent storage, and on storage temperature. The concentration of ascorbate in creams and

yogurts is similar to, or a little lower than, that in milk (Appendix 6A); cheese contains only trace amounts.

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Appendices

Appendix 6A Vitamin and vitamin precursor concentrations (per 100 g) in dairy products (modified from Holland et al., *1991)*

Product	Retinol (μg)	Carotone (μg)	Vitamin D (μg)	Vitamin E (mg)	Thiamin (mg)	Ribo-flavin (mg)	Niacin (mg)	Trp÷60 (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (μg)	Folate (μg)	Panto-thenate (mg)	Biotin (μg)	Vitamin C (mg)
Skimmed milk														
pasteurized	1	Tr	Tr	Tr	0.04	0.18	0.1	0.8	0.06	0.4	6	0.32	2.0	1
UHT, fortified	61	18	0.1	0.02	0.04	0.18	0.1	0.8	0.05	Tr	4	0.33	1.5	35[a]
Whole milk														
pasteurized	52	21	0.03	0.09	0.04	0.17	0.1	0.7	0.06	0.4	6	0.35	1.9	1
summer	62	31	0.03	0.10	0.04	0.17	0.1	0.7	0.06	0.4	4	0.35	1.9	1
winter	41	11	0.03	0.07	0.04	0.17	0.1	0.7	0.06	0.4	7	0.35	1.9	1
sterilized, in container	52	21	0.03	0.09	0.03	0.14	0.1	0.8	0.04	0.1	Tr	0.28	1.8	Tr
Channel Island milk														
whole, pasteurized	46	71	0.03	0.11	0.04	0.19	0.1	0.9	0.06	0.4	6	0.36	1.9	1
summer	65	115	0.04	0.13	0.04	0.19	0.1	0.9	0.06	0.4	5	0.36	1.9	1
winter	27	27	0.03	0.09	0.04	0.19	0.1	0.9	0.06	0.4	7	0.36	1.9	1
semi-skimmed, UHT	14	22	0.01	0.04	0.04	0.19	0.1	0.9	0.05	0.2	1	0.34	1.5	Tr
Dried skimmed milk[b] (fortified)	350	5	2.10	0.27	0.38	1.63	1.0	8.5	0.60	2.6	51	3.28	20.1	13
with vegetable fat (fortified)	395	15	10.50	1.32	0.23	1.20	0.6	5.5	0.35	2.3	36	2.15	15.0	11
Evaporated milk, whole	105	100	3.95[c]	0.19	0.07	0.42	0.2	2.0	0.07	0.1	11	0.75	4.0	1
Goat's milk, pasteurized	44	Tr	0.11	0.03	0.04	0.13	0.3	0.7	0.06	0.1	1	0.41	3.0	1
Human milk,														
colostrum	155	(135)	N	1.3	Tr	0.03	0.1	0.7	Tr	0.1	2	0.12	Tr	7
transitional	85	(37)	N	0.48	0.01	0.03	0.1	0.5	Tr	Tr	3	0.20	0.2	6
mature	58	(24)	0.04	0.34	0.02	0.03	0.2	0.5	0.01	Tr	5	0.25	0.7	4
Sheep's milk, raw	83	Tr	0.18	0.11	0.08	0.32	0.4	1.3	0.08	0.6	5	0.45	2.5	5
Fresh whipping cream, pasteurized (39.3% fat)	565	265	0.22	0.86	0.02	0.17	Tr	0.5	0.04	0.2	7	0.22	1.4	1
Cheeses														
Brie	285	210	0.20	0.84	0.04[d]	0.43	0.4	4.5	0.15	1.2	58	0.35	5.6	Tr
Camembert	230	315	(0.18)	0.65	0.05[e]	0.52	1.0	4.9	0.22	1.1	102	0.36	7.6	Tr
Cheddar, average	325	225	0.26	0.53	0.03	0.40	0.1	6.0	0.10	1.1	33	0.36	3.0	Tr

Cheddar-type (15% fat)	165	100	0.11	0.39	0.03	0.53	0.1	7.4	0.13	1.3	56	0.51	3.8	Tr
Cheese spread, plain	275	105	0.17	0.24	0.05	0.36	0.1	3.2	0.08	0.6	19	0.51	3.6	Tr
Cottage cheese														
plain	44	10	0.03	0.08	0.03	0.26	0.1	3.2	0.08	0.7	27	0.40	3.0	Tr
reduced fat (1.4% fat)	16	4	0.01	0.03	(0.03)	(0.26)	(0.1)	3.1	(0.08)	(0.7)	(27)	(0.40)	(3.0)	Tr
Cream cheese	385	220	0.27	1.00	0.03	0.13	0.1	0.7	0.04	0.3	11	0.27	1.6	Tr
Danish blue	280	250	(0.23)	0.76	0.03	0.41	0.5	4.7	0.12	1.0	50	0.53	2.7	Tr
Edam	175	150	(0.19)	0.48	0.03	0.35	0.1	6.1	0.09	2.1	40	0.38	1.8	Tr
Feta	220	33	0.50	0.37	0.04	0.21	0.2	3.5	0.07	1.1	23	0.36	2.4	Tr
Fromage frais														
fruit	82	N	0.04	(0.01)	0.02	0.35	0.1	1.6	0.04	1.4	15	N	N	Tr
plain	100	Tr	0.05	0.02	0.04	0.40	0.1	1.6	0.10	1.4	15	N	N	Tr
very low fat (0.2% fat)	3	N	Tr	Tr	(0.03)	(0.37)	(0.1)	1.8	(0.07)	(1.4)	(15)	N	N	Tr
Gouda	245	145	(0.24)	0.53	0.03	0.30	0.1	5.6	0.08	1.7	43	0.32	1.4	Tr
Parmesan	345	210	(0.25)	0.70	0.03	0.44	0.1	9.3	0.13	1.9	12	0.43	3.3	Tr
Processed cheese, plain	270	95	0.21	0.55	0.03	0.28	0.1	4.9	0.08	0.9	18	0.31	2.3	Tr
Stilton, blue	355	185	0.27	0.61	0.03	0.43	0.5	5.3	0.16	1.0	77	0.71	3.6	Tr
Drinking yogurt, UHT	Tr	Tr	Tr	Tr	0.03	0.16	0.1	0.7	0.05	0.2	12	0.19	0.9	0
Low-fat yogurt, plain	8	5	0.01	0.01	0.05	0.25	0.1	1.2	0.09	0.2	17	0.45	2.9	1
Whole-milk yogurt														
plain	28	21	0.04	0.05	0.06	0.27	0.2	1.3	0.10	0.2	18	0.50	2.6	1
fruit	39	16	(0.04)	(0.05)	0.06	0.30	0.1	1.3	0.07	0.1	10	0.30	2.0	1
Ice-cream														
dairy, vanilla	115	195	0.12	0.21	0.04	0.25	0.1	0.8	0.08	0.4	7	0.44	2.5	1
non-dairy, vanilla	1	6	Tr	0.84	0.04	0.24	0.1	0.7	0.07	0.5	8	0.43	3.0	1

Tr, Trace; N, nutrient present in significant quantities but there is no reliable information on amount; ( ), estimated value.

[a]Unfortified milk would contain only traces of vitamin C.

[b]Unfortified skimmed milk powder contains approximately 8 μg retinol, 3 μg carotene, Tr vitamin D and 0.01 mg vitamin E per 100 g. Some brands contain as much as 755 μg retinol 10 μg carotene and 4.6 μg vitamin D per 100 g.

[c]This is for fortified product. Unfortified evaported milk contains approximately 0.09 μg vitamin D per 100 g.

[d]The rind alone contains 0.5 mg thiamin per 100 g.

[e]The rind alone contains 0.4 mg thiamin per 100 g.

7 Water in milk and dairy products

7.1 Introduction

The water content of dairy products ranges from around 2.5 to 94% (w/w) (Table 7.1) and is the principal component by weight in most dairy products, including milk, cream, ice-cream, yogurt and most cheeses. The moisture content of foods (or more correctly their water activity, section 7.3), together with temperature and pH, are of great importance to food technology. As described in section 7.8, water plays an extremely important role even in relatively low-moisture products such as butter (*c.* 16% moisture) or dehydrated milk powders (*c.* 2.5–4% moisture). Water is the most important diluent in foodstuffs and has an important influence on the physical, chemical and microbiological changes which occur in dairy products. Water is an important plasticizer of non-fat milk solids.

7.2 General properties of water

Some physical properties of water are shown in Table 7.2. Water has higher melting and boiling temperatures, surface tension, dielectric constant, heat capacity, thermal conductivity and heats of phase transition than similar molecules (Table 7.3). Water has a lower density than would be expected from comparison with the above molecules and has the unusual property of expansion on solidification. The thermal conductivity of ice is approximately four times greater than that of water at the same temperature and is high compared with other non-metallic solids. Likewise, the thermal diffusivity of ice is about nine times greater than that of water.

The water molecule (HOH) is formed by covalent (σ) bonds between two of the four sp^3 bonding orbitals of oxygen (formed by the hybridization of the 2s, $2p_x$, $2p_y$ and $2p_z$ orbitals) and two hydrogen atoms (Figure 7.1a). The remaining two sp^3 orbitals of oxygen contain non-bonding electrons. The overall arrangement of the orbitals around the central oxygen atom is tetrahedral and this shape is almost perfectly retained in the water molecule. Due to electronegativity differences between oxygen and hydrogen, the O-H bond in water is polar (a vapour state dipole moment of 1.84 D). This results in a partial negative charge on the oxygen and a partial positive charge on each hydrogen (Figure 7.1b). Hydrogen bonding can occur between the two lone electron pairs in the oxygen atom and the hydrogen atoms of other

Table 7.1 Approximate water content of some dairy products (modified from Holland *et al.*, 1991)

Product	Water (g/100 g)
Skimmed milk, average	91
pasteurized	91
fortified plus SMP	89
UHT, fortified	91
Whole milk, average	88
pasteurized[a]	88
summer	88
winter	88
sterilized	88
Channel Island milk, whole, pasteurized	86
summer	86
winter	86
semi-skimmed, UHT	89
Dried skimmed milk	3.0
with vegetable fat	2.0
Evaporated milk, whole	69
Flavoured milk	85
Goats' milk, pasteurized	89
Human milk, colostrum	88
mature	87
Sheep's milk, raw	83
Fresh cream, whipping	55
Cheeses	
Brie	49
Camembert	51
Cheddar, average	36
vegetarian	34
Cheddar-type, reduced fat	47
Cheese spread, plain	53
Cottage cheese, plain	79
with additions	77
reduced fat	80
Cream cheese	46
Danish blue	45
Edam	44
Feta	57
Fromage frais, fruit	72
plain	78
very low fat	84
Full-fat soft cheese	58
Gouda	40
Hard cheese, average	37
Lymeswold	41
Medium-fat soft cheese	70
Parmesan	18
Processed cheese, plain	46
Stilton, blue	39
White cheese, average	41
Whey	94
Drinking yogurt	84
Low-fat plain yogurt	85
Whole-milk yogurt, plain	82
fruit	73
Ice-cream, dairy, vanilla	62
non-dairy, vanilla	65

[a]The value for pasteurized milk is similar to that for unpasteurized milk.

Table 7.2 Physical constants of water and ice (from Fennema, 1985)

Molecular weight	18.01534
Phase transition properties	
Melting point at 101.3 kPa (1 atm)	0.000°C
Boiling point at 101.3 kPa (1 atm)	100.00°C
Critical temperature	374.15°C
Critical pressure	22.14 MPa (218.6 atm)
Triple point	0.0099°C and 610.4 kPa (4.579 mmHg)
Heat of fusion at 0°C	6.012 kJ (1.436 kcal) mol^{-1}
Heat of vaporization at 100°C	40.63 kJ (9.705 kcal) mol^{-1}
Heat of sublimation at 0°C	50.91 kJ (12.16 kcal) mol^{-1}

Other properties at	20°C	0°C	0°C (ice)	−20°C (ice)
Density ($kg\,l^{-1}$)	0.9998203	0.999841	0.9168	0.9193
Viscosity (Pa s)	1.002×10^{-3}	1.787×10^{-3}	–	–
Surface tension against air ($N\,m^{-1}$)	72.75×10^{-3}	75.6×10^{-3}	–	–
Vapor pressure (Pa)	2.337×10^{3}	6.104×10^{2}	6.104×10^{2}	1.034×10^{2}
Specific heat ($J\,kg^{-1}\,K^{-1}$)	4.1819	4.2177	2.1009	1.9544
Thermal conductivity ($J\,m^{-1}\,s^{-1}\,K^{-1}$)	5.983×10^{2}	5.644×10^{2}	22.40×10^{2}	24.33×10^{2}
Thermal diffusivity ($m^2\,s^{-1}$)	1.4×10^{-5}	1.3×10^{-5}	$\sim 1.1 \times 10^{-4}$	$\sim 1.1 \times 10^{-4}$
Dielectric constant,				
static[a]	80.36	80.00	91[b]	98[b]
at 3×10^{9} Hz	76.7	80.5	–	3.2
	(25°C)	(1.5°C)	(−12°C)	–

[a]Limiting value at low frequencies.
[b]Parallel to *c*-axis of ice; values about 15% larger if perpendicular to *c*-axis.

Table 7.3 Properties of water and other compounds (from Roos, 1997)

Property	Ammonia (NH_3)	Hydrofluoric acid (HF)	Hydrogen sulphide (H_2S)	Methane (CH_4)	Water (H_2O)
Molecular weight	17.03	20.02	34.08	16.04	18.015
Melting point (°C)	−77.7	−83.1	−85.5	−182.6	0.00
Boiling point (°C)	−33.35	19.54	−60.7	−161.4	100.00
Critical T (°C)	132.5	188.0	100.4	−82.1	374.15
Critical P (bar)	114.0	64.8	90.1	46.4	221.5

molecules which, due to the above-mentioned differences in electronegativity, have some of the characteristics of bare protons. Thus, each water molecule can form four hydrogen bonds arranged in a tetrahedral fashion around the oxygen (Figure 7.1d). The structure of water has been described as a continuous three-dimensional network of hydrogen-bonded molecules, with a local preference for tetrahedral geometry but with a large number of strained or broken hydrogen bonds. This tetrahedral geometry is usually

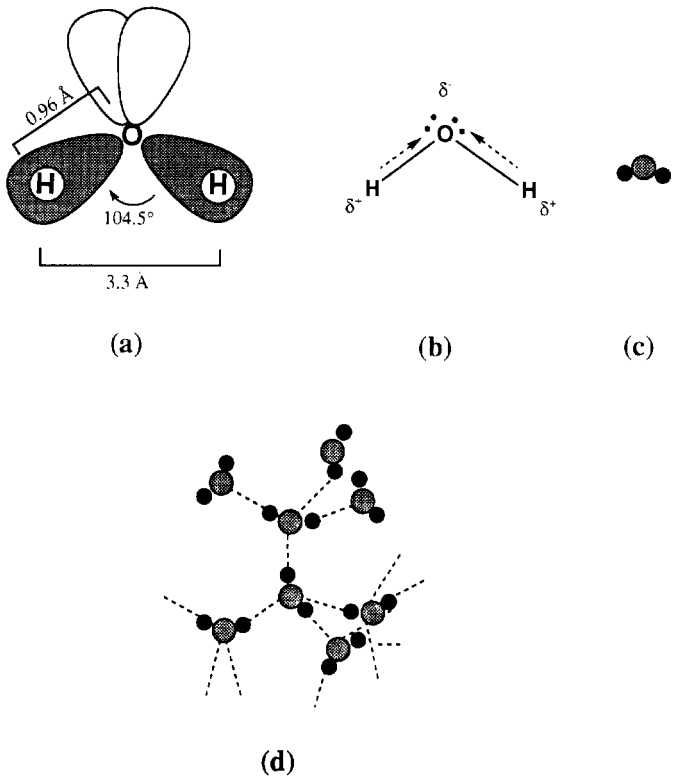

Figure 7.1 Schematic representations (a–c) of a water molecule and hydrogen bonding between water molecules (d).

maintained only over short distances. The structure is dynamic; molecules can rapidly exchange one hydrogen bonding partner for another and there may be some unbonded water molecules.

Water crystallizes to form ice. Each water molecule associates with four others in a tetrahedral fashion as is apparent from the unit cell of an ice crystal (Figure 7.2). The combination of a number of unit cells, when viewed from the top, results in a hexagonal symmetry (Figure 7.3). Because of the tetrahedral arrangement around each molecule, the three-dimensional structure of ice (Figure 7.4) consists of two parallel planes of molecules lying close to each other ('basal planes'). Basal planes of ice move as a unit under pressure. The extended structure of ice is formed by stacking of several basal planes. This is the only crystalline form of ice that is stable at a pressure of 1 atm at 0°C, although ice can exist in a number of other crystalline forms, as well as in an amorphous state. The above description of ice is somewhat simplified; in practice the system is not perfect due to the presence of ionized

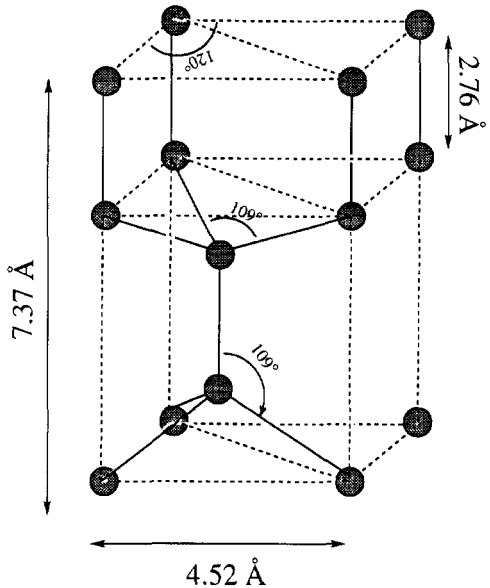

Figure 7.2 Unit cell of an ice crystal at 0°C. Circles represent the oxygen atoms of water molecules, — indicates hydrogen bonding. (Modified from Fennema, 1985.)

water (H_3O^+, OH^-), isotopic variants, solutes and vibrations within the water molecules.

With the exceptions of water vapour and ice, water in dairy products contains numerous solutes. Thus, the interactions of water with solutes is of great importance. Hydrophilic compounds interact strongly with water by ion–dipole or dipole–dipole interactions while hydrophobic substances interact poorly with water and prefer to interact with each other ('hydrophobic interaction').

Water in food products can be described as being free or bound. The definition of what consitiutes 'bound' water is far from clear (see Fennema, 1985) but it can be considered as that part of the water in a food which does not freeze at −40°C and exists in the vicinity of solutes and other non-aqueous constituents, has reduced molecular mobility and other significantly altered properties compared with the 'bulk water' of the same system (Fennema, 1985). The actual amount of bound water varies in different products and the amount measured is often a function of the assay technique. Bound water is not permanently immobilized since interchange of bound water molecules occurs frequently.

There are a number of types of bound water. Constitutional water is the most strongly bound and is an integral part of another molecule (e.g. within the structure of a globular protein). Constitutional water represents only a

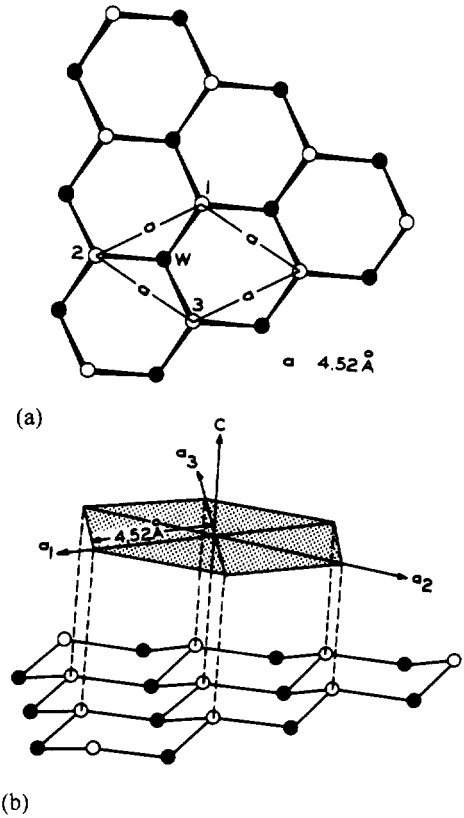

Figure 7.3 The 'basal plane' of ice (combinations of two planes of slightly different elevations) viewed from above. The closed circles represent oxygen atoms of water molecules in the lower plane and the open circles oxygen atoms in the upper plane, (a) seen from above and (b) from the side (from Fennema, 1985).

small fraction of the water in high-moisture foods. 'Vicinal' or monolayer water is bound to the first layer sites of the most hydrophilic groups. Multilayer water occupies the remaining hydrophilic sites and forms a number of layers beyond the monolayer water. There is often no clear distinction between constitutional, monolayer and multilayer water since they differ only in the length of time a water molecule remains associated with the food.

The addition of dissociable solutes to water disrupts its normal tetrahedral structure. Many simple inorganic solutes do not possess hydrogen bond donors or acceptors and therefore can interact with water only by dipole interactions (e.g. Figure 7.5 for NaCl). Multilayer water exists in a structurally disrupted state while bulk-phase water has properties similar to

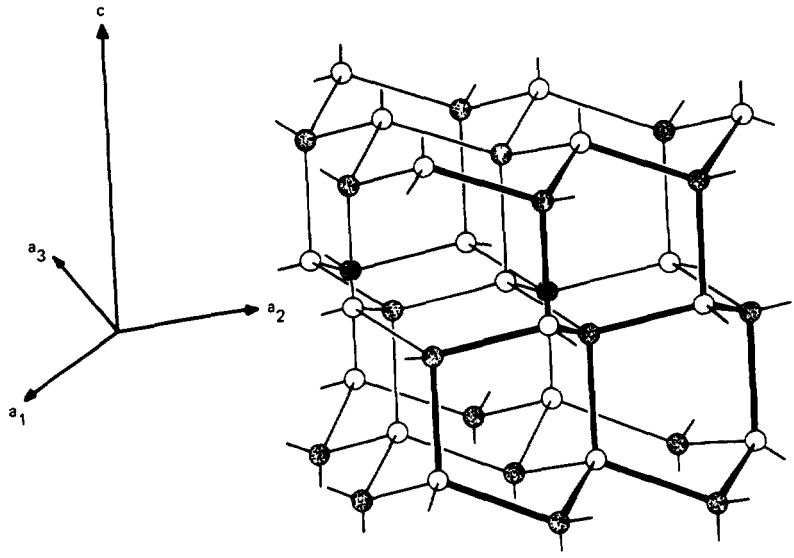

Figure 7.4 The extended structure of ice. Open and shaded circles represent oxygen atoms of water molecules in the upper and lower layers, respectively, of a basal plane (from Fennema, 1985).

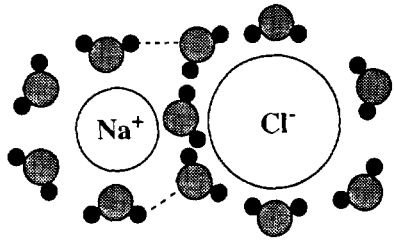

Figure 7.5 Arrangement of water molecules in the vicinity of sodium and chloride ions (modified from Fennema, 1985).

those of water in a dilute aqueous salt solution. Ions in solution impose structure on the water but disrupt its normal tetrahedral structure. Concentrated solutions probably do not contain much bulk-phase water and structures caused by the ions predominate. The ability of an ion to influence the structure of water is influenced by its electric field. Some ions (principally small and/or multivalent) have strong electric fields and loss of the inherent structure of the water is more than compensated for by the new structure resulting from the presence of the ions. However, large, monovalent ions have weak electric fields and thus have a net disruptive effect on the structure of water.

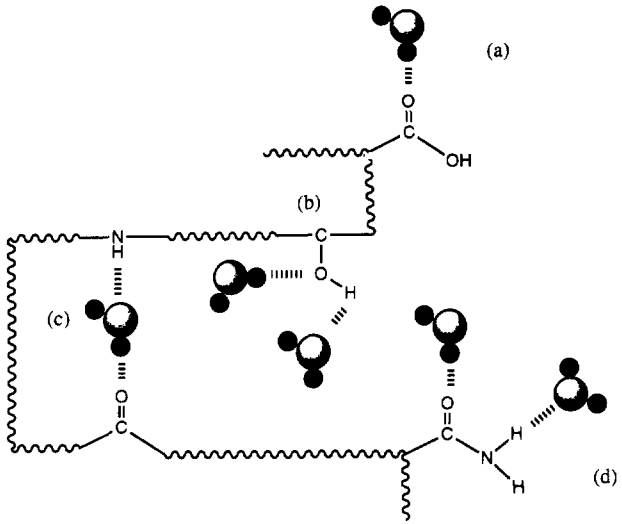

Figure 7.6 Schematic representation of the interaction of water molecules with carboxylic acid (a), alcohol (b), -NH and carbonyl groups (c) and amide groups (d).

In addition to hydrogen bonding with itself, water may also form such bonds with suitable donor or acceptor groups on other molecules. Water–solute hydrogen bonds are normally weaker than water–water interactions. By interacting through hydrogen bonds with polar groups of solutes, the mobility of water is reduced and, therefore, is classified as either constitutional or monolayer. Some solutes which are capable of hydrogen bonding with water do so in a manner that is incompatible with the normal structure of water and therefore have a disruptive effect on this structure. For this reason, solutes depress the freezing point of water (Chapter 11). Water can potentially hydrogen bond with lactose or a number of groups on proteins (e.g. hydroxyl, amino, carboxylic acid, amide or imino; Figure 7.6) in dairy products.

Milk contains a considerable amount of hydrophobic material, especially lipids and hydrophobic amino acid side chains. The interaction of water with such groups is thermodynamically unfavourable due to a decrease in entropy caused by increased water–water hydrogen bonding (and thus an increase in structure) adjacent to the non-polar groups.

7.3 Water activity

Water activity (a_w) is defined as the ratio between the water vapour pressure exerted by the water in a food system (p) and that of pure water (p_o) at the

same temperature:

$$a_w = \frac{p}{p_o}. \tag{7.1}$$

Due to the presence of various solutes, the vapour pressure exerted by water in a food system is always less than that of pure water (unity). Water activity is a temperature-dependent property of water which may be used to characterize the equilibrium or steady state of water in a food system (Roos, 1997).

For a food system in equilibrium with a gaseous atmosphere (i.e. no net gain or loss of moisture to or from the system caused by differences in the vapour pressure of water), the equilibrium relative humidity (ERH) is related to a_w by:

$$\text{ERH}(\%) = a_w \times 100. \tag{7.2}$$

Thus, under ideal conditions, ERH is the % relative humidity of an atmosphere in which a foodstuff may be stored without a net loss or gain of moisture. Water activity, together with temperature and pH, is one of the most important parameters which determine the rates of chemical, biochemical and microbiological changes which occur in foods. However, since a_w presupposes equilibrium conditions, its usefulness is limited to foods in which these conditions exist.

Water activity is influenced by temperature and therefore the assay temperature must be specified. The temperature dependence of a_w is described by the Clausius–Clapeyron equation in modified form:

$$\frac{\mathrm{d}\ln(a_w)}{\mathrm{d}(1/T)} = \frac{-\Delta H}{R} \tag{7.3}$$

where T is temperature (K), R is the universal gas constant and ΔH is the change in enthalpy. Thus, at a constant water content, there is a linear relationship between $\log a_w$ and $1/T$ (Figure 7.7). This linear relationship is not obeyed at extremes of temperature or at the onset of ice formation.

The concept of a_w can be extended to cover sub-freezing temperatures. In these cases, a_w is defined (Fennema, 1985) relative to the vapour pressure of supercooled water ($p_{o(SCW)}$) rather than to that of ice:

$$a_w = \frac{p_{ff}}{p_{o(SCW)}} = \frac{p_{ice}}{p_{o(SCW)}} \tag{7.4}$$

where p_{ff} is the vapour pressure of water in the partially frozen food and p_{ice} that of pure ice. There is a linear relationship between $\log a_w$ and $1/T$ at sub-freezing temperatures (Figure 7.8). The influence of temperature on a_w is greater below the freezing point of the sample and there is normally a pronounced break at the freezing point. Unlike the situation above freezing

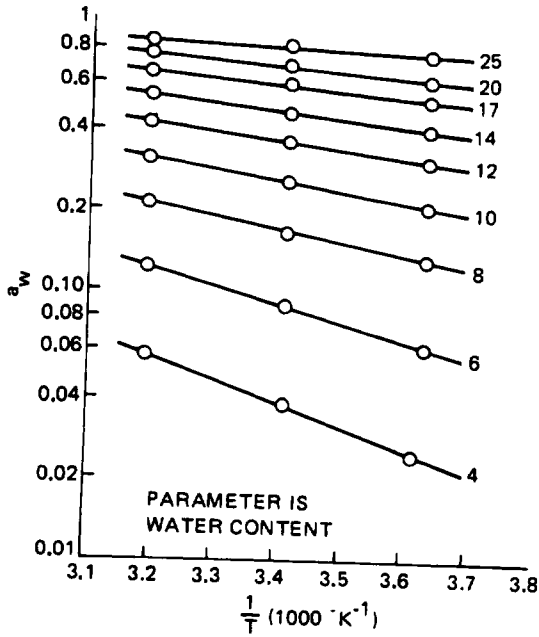

Figure 7.7 Clausius–Clapeyron relationship between water activity and temperature for native potato starch. Numbers on curves indicate water content, in g per g dry starch (from Fennema, 1985).

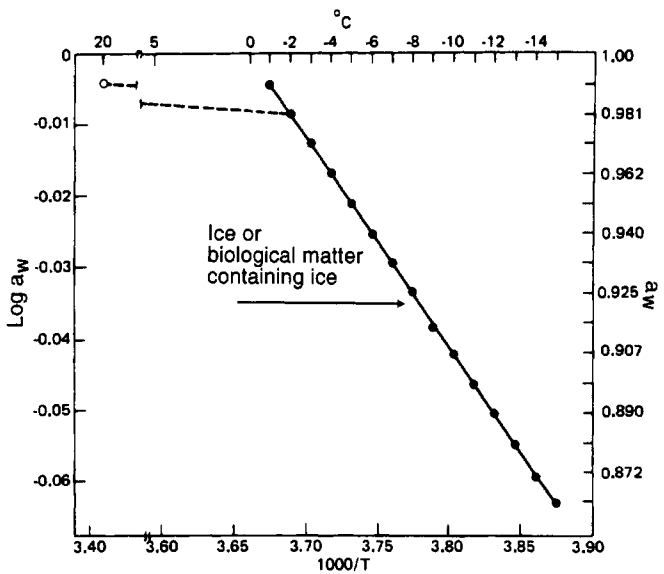

Figure 7.8 Relationship between water activity and temperature for samples above and below freezing (from Fennema, 1985).

(where a_w is a function of composition and temperature), a_w below freezing is independent of sample composition and is influenced only by temperature. Thus, a_w values of foods at sub-freezing temperatures cannot be used to predict the a_w of foods above freezing. Sub-freezing a_w values are far less useful indicators of potential changes in foods than a_w values determined above the freezing point.

Water activity may be measured by a number of techniques (Marcos, 1993). Comparison of manometric readings taken simultaneously on a food system and on pure water is the most direct technique. a_w can also be measured in dilute solutions and liquid foods with low solute concentrations by cryoscopy, since under certain conditions a_w can be considered as a colligative property. In these cases, the Clausius–Clapeyron equation is valid:

$$a_w = \gamma[n_2/(n_1 + n_2)] \tag{7.5}$$

where n_1 and n_2 are the number of moles of solute and water, respectively, and γ is the activity coefficient (approximately one for dilute solutions); n_2 can be determined by measuring the freezing point from the relation:

$$n_2 = \frac{G\Delta T_f}{1000K_f} \tag{7.6}$$

where G is the grams of solvent in the sample, ΔT_f is the freezing point depression (°C) and K_f is the molal freezing point depression constant for water, i.e. 1.86.

Water activity may also be measured by determining the ERH for a food sample, using equation 7.2.

ERH may be estimated by measuring the relative humidity of the headspace over a food in a small, sealed container hygrometrically, psychrometrically or directly by measuring the moisture content of the air by gas chromatography. ERH can be estimated by moisture-related colour changes in paper impregnated with cobalt thiocyanate ($Co(SCN)_2$) and compared to standards of known a_w.

Differences in the hygroscopicity of various salts may also be used to estimate a_w. Samples of the food are exposed to a range of crystals of known a_w; if the a_w of the sample is greater than that of a given crystal, the crystal will absorb water from the food.

Alternatively, a_w may be measured by isopiestic equilibration. In this method, a dehydrated sorbent (e.g. microcrystalline cellulose) with a known moisture sorption isotherm (section 7.4) is exposed to the atmosphere in contact with the sample in an enclosed vessel. After the sample and sorbent have reached equilibrium, the moisture content of the sorbent can be measured gravimetrically and related to the a_w of the sample.

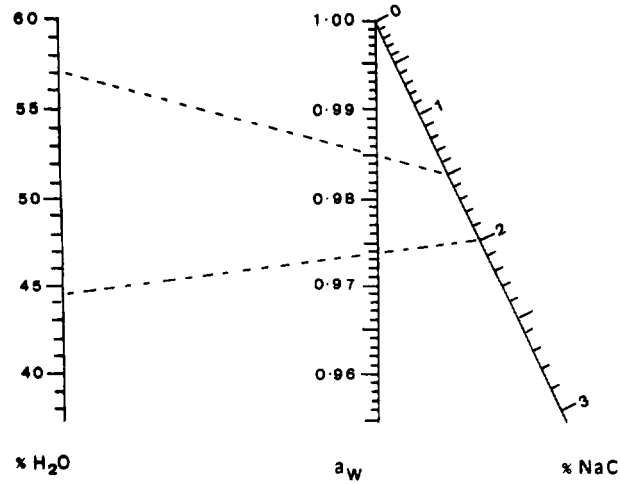

Figure 7.9 Nomograph for direct estimation of water activity (a_w) of unripe cheeses from % H_2O and % NaCl. Examples: If % H_2O = 57.0, and % NaCl = 1.5, then a_w = 0.985; if % H_2O = 44, % NaCl = 2.0, then a_w = 0.974 (from Marcos, 1993).

The a_w of a sample can also be estimated by exposing it to atmospheres with a range of known and constant relative humidities (RH). Moisture gains or losses to or from the sample may then be determined gravimetrically after equilibration. If the weight of the sample remains constant, the RH of the environment is equal to the ERH of the sample. The a_w of the food may be estimated by interpolation of data for RH values greater and less than the ERH of the sample.

For certain foodstuffs, a_w may be estimated from chemical compostion. A nomograph relating the a_w of freshly made cheese to its content of moisture and NaCl is shown in Figure 7.9. Likewise, various equations relating the a_w of cheese to [NaCl], [ash], [12% trichloroacetic acid-soluble N] and pH have been developed (see Marcos, 1993).

7.4 Water sorption

Sorption of water vapour to or from a food depends on the vapour pressure exerted by the water in the food. If this vapour pressure is lower than that of the atmosphere, **absorption** occurs until vapour pressure equilibrium is reached. Conversely, **desorption** of water vapour results if the vapour pressure exerted by water in the food is greater than that of the atmosphere. **Adsorption** is regarded as sorption of water at a physical interface between a solid and its environment. Absorption is regarded as a process in

which adsorption occurs in the interior of the substance (Kinsella and Fox, 1986).

The water sorption characteristics of dairy products (like those of most other foodstuffs) are governed by their non-fat constituents (principally lactose and proteins). However, in many milk and whey products, the situation is complicated by structural transformations and/or solute crystallization.

The relationship between the water content of a food (g H_2O per g dry matter) and a_w at a constant temperature is known as a **sorption isotherm**. Sorption isotherms are prepared by exposing a set of previously dried samples to atmospheres of high RH; desorption isotherms can also be determined by a similar technique. Isotherms provide important information regarding the difficulty of removing water from a food during dehydration and on its stability, since both ease of dehydration and stability are related to a_w. A typical sorption isotherm is shown in Figure 7.10. Most sorption isotherms are sigmoidal in shape, although foods which contain large amounts of low molecular weight solutes and relatively little polymeric material generally exhibit J-shaped isotherms. The rate of water sorption is temperature dependent and for a given vapour pressure, the amount of water lost by desorption or gained by resorption may not be equal and therefore sorption hysteresis may occur (Figure 7.11).

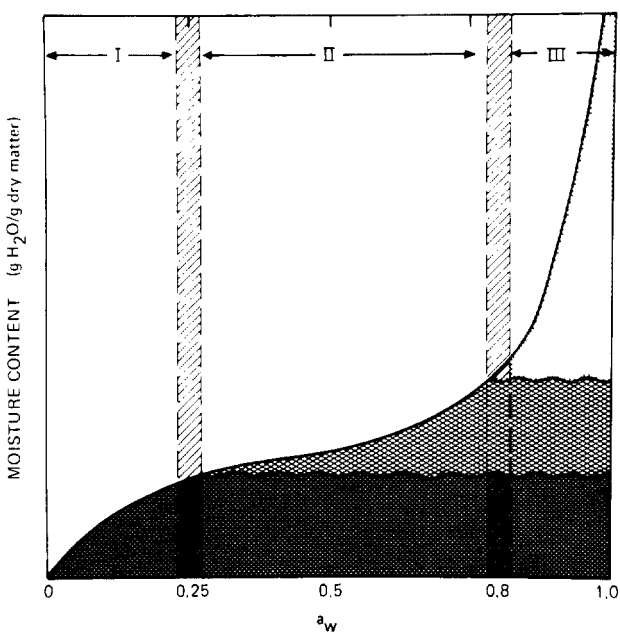

Figure 7.10 Generalized moisture sorption isotherm for a food (from Fennema, 1985).

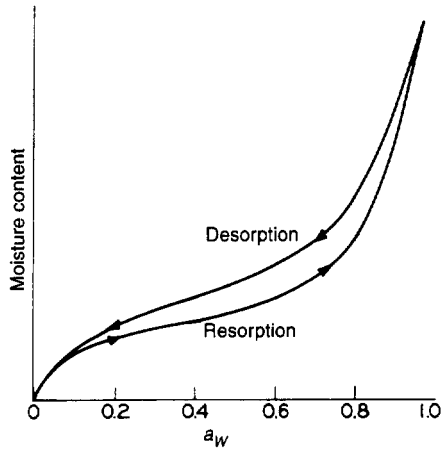

Figure 7.11 Hysteresis of a moisture sorption isotherm (from Fennema, 1985).

The moisture present in zone I (Figure 7.10) is the most tightly bound and represents the monolayer water bound to accessible, highly polar groups of the dry food. The boundary between zones I and II represents the monolayer moisture content of the food. The moisture in zone II consists of multilayer water in addition to the monolayer water, while the extra water added in zone III consists of the bulk-phase water.

Water sorption isotherms may be determined experimentally by gravimetric determination of the moisture content of a food product after it has reached equilibrium in sealed, evacuated desiccators containing saturated solutions of different salts. Data obtained in this manner may be compared with a number of theoretical models (including the Braunauer–Emmett–Teller model, the Kühn model and the Gruggenheim–Andersson–De Boer model; see Roos, 1997) to predict the sorption behaviour of foods. Examples of sorption isotherms predicted for skim milk by three such models are shown in Figure 7.12.

The sorption behaviour of a number of dairy products is known (Kinsella and Fox, 1986). Generally, whey powders exhibit sigmoidal sorption isotherms, although the characteristics of the isotherm are influenced by the composition and history of the sample. Examples of sorption isotherms for whey protein concentrate (WPC), dialysed WPC and its dialysate (principally lactose) are shown in Figure 7.13. At low a_w values, sorption is due mainly to the proteins present. A sharp decrease is observed in the sorption isotherm of lactose at a_w values between 0.35 and 0.50 (e.g. Figure 7.13). This sudden decrease in water sorption can be explained by the crystallization of amorphous lactose in the α-form, which contains one mole of water of crystallization per mole. Above a_w values of about 0.6, water sorption is principally influenced by small molecular weight components (Figure 7.13).

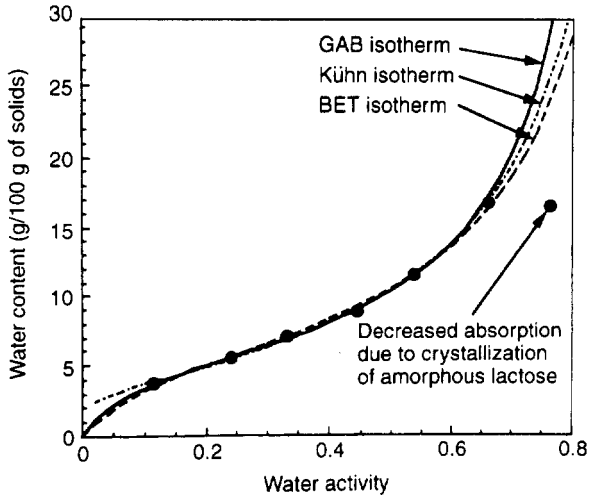

Figure 7.12 Adsorption of water by skim milk and sorption isotherms predicted by the Braunauer–Emmett–Teller (BET), Kühn and Guggenheim–Andersson–De Boer (GAB) sorption models (from Roos, 1997).

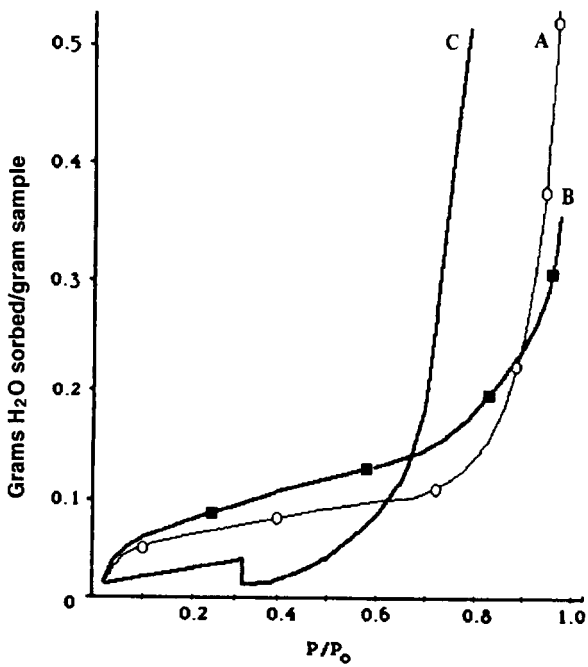

Figure 7.13 Water vapour sorption by whey protein concentrate (A), dialysed whey protein concentrate (B) and dialysate (lactose) from whey protein concentrate (C) (from Kinsella and Fox, 1986).

Despite some conflicting evidence (Kinsella and Fox, 1986), it appears that denaturation has little influence on the amount of water bound by whey proteins. However, other factors which may accompany denaturation (e.g. Maillard browning, association or aggregation of proteins) may alter protein sorption behaviour. Drying technique affects the water sorption characteristics of WPC. Freeze-dried and spray-dried WPC preparations bind more water at the monolayer level than do roller-, air- or vacuum-dried samples, apparently due to larger surface areas in the former. As discussed above, temperature also influences water sorption by whey protein preparations. The sorption isotherm for β-lactoglobulin is typical of many globular proteins.

In milk powders, the caseins are the principal water sorbants at low and intermediate values of a_w. The water sorption characteristics of the caseins are influenced by their micellar state, their tendency towards self-association, their degree of phosphorylation and their ability to swell. Sorption isotherms for casein micelles and sodium caseinate (Figure 7.14) are generally sigmoidal. However, isotherms of sodium caseinate show a marked increase at a_w between 0.75 and 0.95. This has been attributed to the

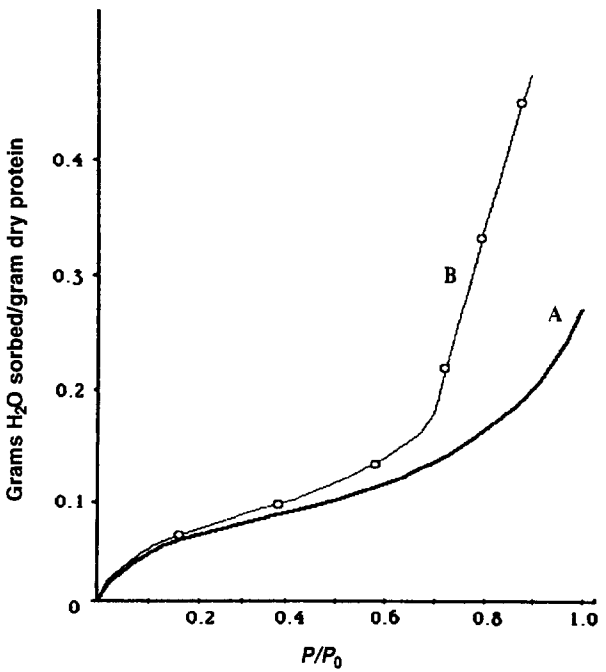

Figure 7.14 Sorption isotherm for casein micelles (A) and sodium caseinate (B) at 24°C, pH 7 (from Kinsella and Fox, 1986).

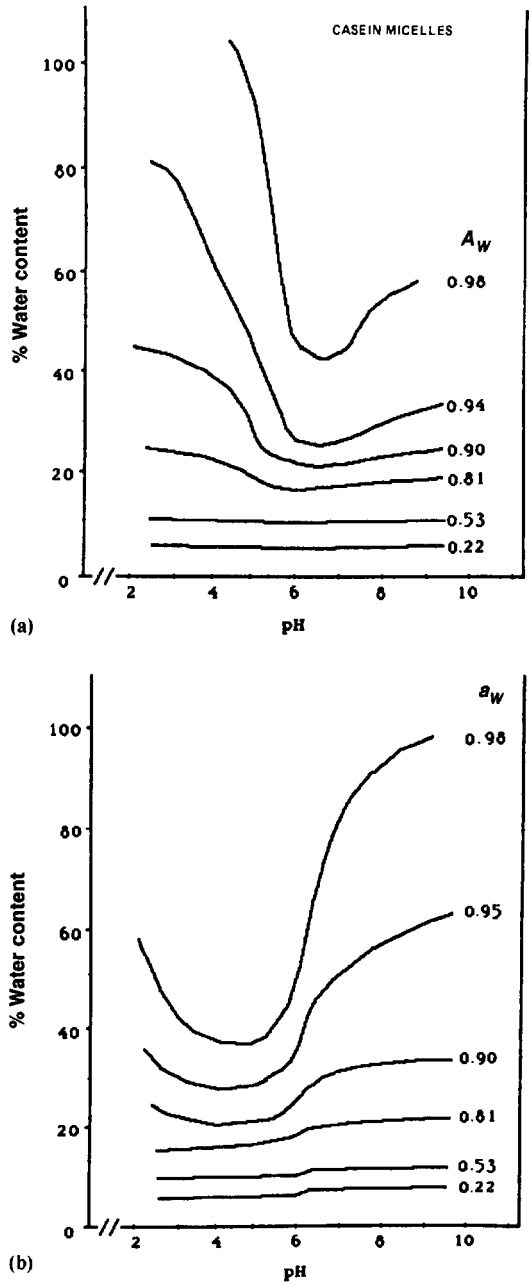

Figure 7.15 Equilibrium water content of (a) casein micelles and (b) sodium caseinate and casein hydrochloride as a function of pH and changing water activities (isopsychric curves) (from Kinsella and Fox, 1986).

presence of certain ionic groups, bound Na^+ or the increased ability of sodium caseinate to swell.

Heating of casein influences its water sorption characteristics, as does pH. With some exceptions at low pH, the hydration of sodium caseinate increases with pH (Figure 7.15b). Minimum water sorption occurs around the isoelectric pH (4.6). At low and intermediate values of a_w, increasing pH, and thus $[Na^+]$, has little influence on water sorption. At low a_w values, water is bound strongly to binding sites on the protein while at higher a_w both protein and NaCl sorb available water in multilayer form. Water sorption by casein micelles (Figure 7.15a) has a minimum at about pH 6–7 at high a_w. This difference in sorption minima between caseinate and casein micelles is because hydration of caseinate is due mainly to ion effects (Na^+ being more effective in this respect than Cl^-). Hydration behaviour of casein micelles, on the other hand, reflects effects of pH on micelle integrity. Hydrolysis of κ-casein by rennet appears to have only a small influence on its ability to bind water, although the chemical modification of amino groups has a greater effect. Genetic variation in the amino acid sequences of the caseins caused by genetic polymorphism also influences water sorption. The addition of NaCl to isoelectric casein greatly increases water sorption.

The greatest consequences of water sorption are in the context of dehydrated dairy products. In addition to being influenced by relative humidity, temperature and the relative amounts and intrinsic sorption properties of its constituents, the amount of water sorbed by milk powders is influenced by the method of preparation, the state of lactose, induced changes in protein conformation and swelling and dissolution of solutes such as salts. As discussed in Chapter 2, amorphous lactose is hygroscopic and may absorb large amounts of water at low relative humidities, while water sorption by crystalline lactose is significant only at higher relative humidities and thus water sorption by milk products containing crystallized lactose is due mainly to their protein fraction.

7.5 Glass transition and the role of water in plasticization

The non-fat solids in low-moisture dairy products (e.g. milk powders) or frozen milk products (since dehydration occurs on freezing) are amorphous in most dairy products (except those containing pre-crystallized lactose). The non-fat solids exist in a metastable, non-equilibrium state as a solid glass or a supercooled liquid. Phase changes can occur between these states with a phase transition temperature range called the glass transition (T_g; Roos, 1997). Changes in heat capacity, dielectric properties, volume, molecular mobility and various mechanical properties occur on glass transition. The temperature of onset of the glass transition of amorphous water (i.e. the transformation of a solid, amorphous glass into a supercooled liquid and

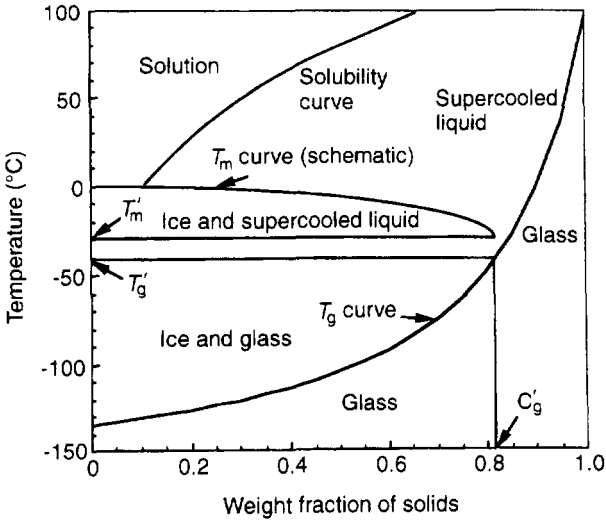

Figure 7.16 State diagram of lactose (from Roos, 1997).

vice versa) is about −135°C. T_g increases with increasing weight fraction of solids (Figure 7.16). The addition of water causes a sharp decrease in T_g.

The stability of dairy products decreases sharply above a critical water activity (section 7.8). This decrease in stability is related to the influence of water on the glass transition and the role of water as a plasticizer of amorphous milk constituents (Roos, 1997).

7.6 Non-equilibrium ice formation

Cooling solutions to below their freezing point results in the formation of ice. If solutions of sugars are cooled rapidly, non-equilibrium ice formation occurs. This is the most common form of ice in frozen dairy products (e.g. ice-cream). Rapid freezing of ice-cream mixes results in the freeze concentration of lactose and other sugars, resulting in supersaturated solutions if the temperature is too low to permit crystallization. The rapid cooling of lactose results in the formation of a supersaturated, freeze-concentrated amorphous matrix.

Various thermal transitions can occur in rapidly cooled solutions, including glass transition, devitrification (ice formation on warming a rapidly-frozen solution) and melting of ice. The relationship between temperature, weight fraction of solids, solubility and glass transition of lactose is shown in Figure 7.16.

7.7 Role of water in stickiness and caking of powders and crystallization of lactose

As discussed in section 2.2.7, drying of whey or other solutions containing a high concentration of lactose is difficult since the semi-dry powder may stick to the metal surfaces of the dryer. The influence of dryer temperature and other process parameters on stickiness during the drying of whey are discussed in Chapter 2. The role of agglomeration on the wetting and reconsitiution of dairy powders was also discussed in Chapter 2.

The principal cause of sticking and caking is the plasticization of amorphous powders by heating or by exposure to high relative humidities. As discussed by Roos (1997), heating or the addition of water reduces surface viscosity (thus permitting adhesion) by creating an incipient liquid state of lower viscosity at the surface of the particle. If sufficient liquid is present and flowing by capillary action, it may form bridges between particles strong enough to cause adhesion. Factors that affect liquid bridging include water sorption, melting of components (e.g. lipids), the production of H_2O by chemical reactions (e.g. Maillard browning), the release of water of crystallization and the direct addition of water.

The viscosity of lactose in the glassy state is extremely high and thus a long contact time is necessary to cause sticking. However, above T_g, viscosity decreases markedly and thus the contact time for sticking is reduced. Since T_g is related to sticking point, it may be used as an indicator of stability. Caking of powders at high RH results when the addition of water plasticizes the components of the powder and reduces T_g to below the ambient temperature.

The crystallization of amorphous lactose was discussed in Chapter 2.

7.8 Water and the stability of dairy products

The most important practical aspect of water in dairy products is its effect on their chemical, physical and microbiological stability. Chemical changes which are influenced by a_w include Maillard browning (including loss of lysine), lipid oxidation, loss of certain vitamins, pigment stability and the denaturation of proteins. Physical changes involve crystallization of lactose. Control of the growth of micro-organisms by reduction in a_w is of great significance for the stability of a number of dairy products. The relationship between the stability of foods and a_w is summarized in Figure 7.17.

Milk is the only naturally occurring protein-rich food which contains a large amount of a reducing sugar. Maillard browning is undesirable in the context of nearly all dairy foods. Since lactose is a reducing sugar, it can participate in these browning reactions and essentially all dairy products (with the exceptions of butter oil, butter and dairy spreads) have sufficient

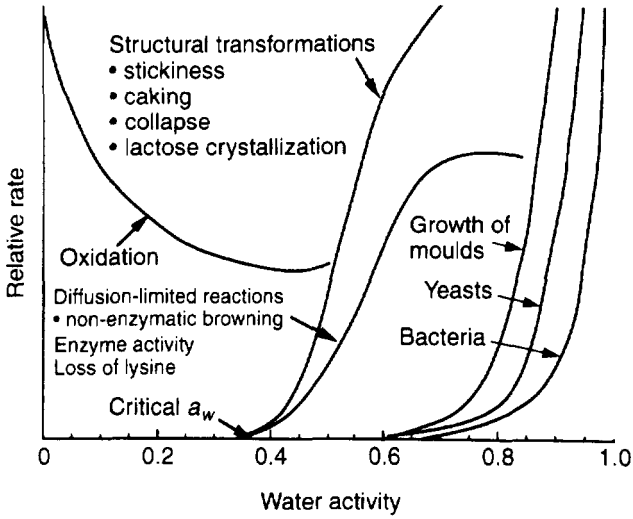

Figure 7.17 Stability map for non-fat milk solids showing schematic rates of various deteriorative changes and growth of micro-organisms as a function of water activity (from Roos, 1997).

protein to supply the necessary amino groups. Many of the stages of Maillard browning (Chapter 2) have high activation energies and thus the process is accelerated at high temperatures. The combination of the presence of lactose and high temperatures occurs during the production of many milk and whey powders, processed cheese and when dairy products are heated during cooking (e.g. the browning of Mozzarella cheese during baking of pizzas). The loss of lysine accompanies the early stages of the Maillard reaction in which its ε-amino group participates. Loss of lysine is significant from a nutritional standpoint since it is an essential amino acid. Loss of lysine may occur without visible browning.

For a given product composition and temperature, the rate of browning is affected by a_w. The influence of water on the rate of Maillard browning depends on the relative importance of a number of factors. Water imparts mobility to reacting species (thus increasing the rate of browning) but may also dilute reactants (thus reducing the rate of browning). At low values of a_w, the increase in molecular mobility is most significant, while at higher values of a_w, the dilution effect predominates. At lower a_w values, water can also dissolve new reacting species. The presence of water can retard certain steps in browning in which water is released as a product (product inhibition, e.g. the initial glycosylamine reaction) or enhance other reactions (e.g. deamination). For many foods, the rate of Maillard browning usually

reaches a maximum at intermediate moisture levels ($a_w \approx$ 0.40–0.80). However, the maximum rate is greatly influenced by the presence of other constituents in the food, such as glycerol or other liquid humectants which can shift the maximum to lower a_w values. The rate of browning of milk powders is also accelerated by the crystallization of lactose.

Lipid oxidation can cause defects in high-fat dairy products. The mechanism of lipid oxidation is discussed in Chapter 3. At low a_w, the rate of oxidation decreases with increasing a_w and reaches a minimum around the monolayer value and then increases at higher a_w. The antioxidant effect of water at low values of a_w has been attributed to bonding of hydroperoxide intermediates and the hydration of metal ions, which act as catalysts. The increased rate of oxidation at higher a_w is a consequence of increased mobility of reactants. In general, water may influence the rate of lipid oxidation by affecting the concentration of initiating radicals, the degree of contact, the mobility of reacting species and the relative importance of radical transfer versus recombination events. Side reactions associated with lipid oxidation (e.g. cross-linking of proteins, enzyme inactivation by peroxidation products, degradation of amino acids) are also influenced by a_w.

The stability of some vitamins is influenced by a_w. In general, the stability of retinol (vitamin A), thiamin (vitamin B_1) and riboflavin (vitamin B_2) decreases with increasing a_w. At low a_w (below 0.40), metal ions do not have a catalytic effect on the destruction of ascorbic acid. The rate of loss of ascorbic acid increases exponentially as a_w increases. The photodegradation of riboflavin (Chapter 6) is also accelerated by increasing a_w.

Water activity influences the rate of thermal denaturation of proteins, including enzymes. Generally, the denaturation temperature increases with decreasing a_w. The rate of nearly all enzyme-catalyzed reactions increases with increasing a_w, as a consequence of increased molecular mobility.

The emulsification state of water in butter (i.e. the water droplet size) is very important for the quality of the product. Bacteria in butter can grow only in the aqueous emulsified phase. A finely divided aqueous phase restricts bacterial growth since the nutrients available in small droplets will quickly become limiting. Also, unless bacterial contamination is high, it is likely that most small water droplets in butter are sterile.

Together with pH and temperature, a_w has a major influence on the rate of growth of micro-organisms. Indeed, reduction of a_w by drying or the addition of salt or sugars is one of the principal traditional techniques used to preserve food. The minimum a_w required for microbial growth is about 0.62, which permits the growth of xerophilic yeasts. As a_w increases, moulds and other yeasts can grow and, finally, bacteria (above about 0.80). a_w also controls the growth of pathogenic micro-organisms; *Staphylococcus aureus* will not grow below $a_w \sim 0.86$ while the growth of *Listeria monocytogenes* does not occur below $a_w \sim 0.92$.

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8 Enzymology of milk and milk products

8.1 Introduction

Like all other foods of plant or animal origin, milk contains several indigenous enzymes which are constituents of the milk as secreted. The principal constituents of milk (lactose, lipids and proteins) can be modified by exogenous enzymes, added to induce specific changes. Exogenous enzymes may also be used to analyse for certain constituents in milk. In addition, milk and most dairy products contain viable micro-organisms which secrete extracellular enzymes or release intracellular enzymes after the cells have died and lysed. Some of these enzymes may cause undesirable changes, e.g. hydrolytic rancidity of milk and dairy products, bitterness and/or age gelation of UHT milks, bittiness in cream, malty flavours or bitterness in fluid milk, or they may cause desirable flavours, e.g. in ripened cheese.

This chapter is devoted mainly to the significance of indigenous enzymes in milk. The principal applications of exogenous enzymes have been dealt with in other chapters, e.g. rennets and lipases in cheese production (Chapter 10), β-galactosidase to modify lactose (Chapter 2). Some minor or potential applications of exogenous enzymes are presented here. Enzymes derived from contaminating bacteria, which may be significant in milk and some dairy products, will not be discussed. The interested reader is referred to McKellar (1989) for a comprehensive review of enzymes produced by psychrotrophs which are the principal spoilage microorganisms in refrigerated milk and milk products. The significance of enzymes from microbial cultures in cheese ripening is discussed in Chapter 10.

8.2 Indigenous enzymes of bovine milk

8.2.1 Introduction

As many as 60 indigenous enzymes have been reported in normal bovine milk. With the exception of α-lactalbumin, which is an enzyme modifier in lactose synthesis (Chapter 2) most, if not all, of the indigenous enzymes in milk have no obvious physiological role. They arise from three principal sources:

- the blood via defective mammary cell membranes;

- secretory cell cytoplasm, some of which is occasionally entrapped within fat globules by the encircling fat globule membrane (MFGM) (Chapter 3);
- the MFGM itself, the outer layers of which are derived from the apical membrane of the secretory cell, which, in turn, originates from the Golgi membranes (Chapter 3); this is probably the principal source of indigenous enzymes.

Thus, most enzymes enter milk due to peculiarities of the mechanism by which milk constituents, especially the fat globules, are excreted from the secretory cells. Milk does not contain substrates for many of the enzymes present, while others are inactive in milk owing to unsuitable environmental conditions, e.g. pH.

Many indigenous milk enzymes are technologically significant from five viewpoints:

1. Deterioration (lipase (commercially, probably the most significant enzyme in milk), proteinase, acid phosphatase and xanthine oxidase) or preservation (sulphydryl oxidase, superoxide dismutase) of milk quality.
2. As indices of the thermal history of milk: alkaline phosphatase, γ-glutamyl transpeptidase, lactoperoxidase.
3. As indices of mastitic infection: catalase, *N*-acetyl-β-D-glucosaminidase, acid phosphatase; the concentration of several other enzymes increases on mastitic infection.
4. Antimicrobial activity: lysozyme, lactoperoxidase (which is exploited as a component of the lactoperoxidase – H_2O_2 – thiocyanate system for the cold pasteurization of milk).
5. As commercial source of enzymes: ribonuclease, lactoperoxidase.

With a few exceptions (e.g. lysozyme and lactoperoxidase), the indigenous milk enzymes do not have a beneficial effect on the nutritional or organoleptic attributes of milk, and hence their destruction by heat is one of the objectives of many dairy processes.

The distribution of the principal indigenous enzymes in milk and their catalytic activity are listed in Table 8.1. In this chapter, the occurrence, distribution, isolation and characterization of the principal indigenous enzymes will be discussed, with an emphasis on their commercial significance in milk.

8.2.2 Proteinases (EC 3.4.–.–)

The presence of an indigenous proteinase in milk was suggested by Babcock and Russel in 1897 but because it occurs at a low concentration or has low activity in milk, it was felt until the 1960s that the proteinase in milk may be of microbial origin. Recent changes in the dairy industry, e.g. improved hygiene in milk production, extended storage of milk at a low temperature

Table 8.1 Indigenous enzymes of significance to milk

Enzyme	Reaction	Importance
Lipase	Triglycerides + H_2O → fatty acids + partial glycerides + glycerol	Off flavours in milk; flavour development in Blue cheese
Proteinase (plasmin)	Hydrolysis of peptide bonds, particularly in β-casein	Reduced storage stability of UHT products; cheese ripening
Catalase	$2H_2O_2 \rightarrow O_2 + 2H_2O$	Index of mastitis; pro-oxidant
Lysozyme	Hydrolysis of mucopolysaccharides	Bacteriocidal agent
Xanthine oxidase	Aldehyde + $H_2O + O_2 \rightarrow$ Acid + H_2O_2	Pro-oxidant; cheese ripening
Sulphydryl oxidase	$2RSH + O_2 \rightarrow RSSR + H_2O_2$	Amelioration of cooked flavour
Superoxide dismutase	$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	Antioxidant
Lactoperoxidase	$H_2O_2 + AH_2 \rightarrow 2H_2O + A$	Index of pasteurization; bacteriocidal agent; index of mastitis; pro-oxidant
Alkaline phosphomonoesterase	Hydrolysis of phosphoric acid esters	Index of pasteurization
Acid phosphomonoesterase	Hydrolysis of phosphoric acid esters	Reduce heat stability of milk; cheese ripening

at the farm and/or factory and altered product profile, e.g. UHT processing of milk, have increased the significance of indigenous milk proteinase which has, consequently, been the focus of considerable research.

Milk contains at least two proteinases, plasmin (alkaline milk proteinase) and cathepsin D (acid milk proteinase) and possibly several others, i.e. two thiol proteinases, thrombin and an aminopeptidase. In terms of activity and technological significance, plasmin is the most important of the indigenous proteinases and has been the subject of most attention. The relevant literature has been reviewed by Grufferty and Fox (1988) and Bastian and Brown (1996).

Plasmin (EC 3.4.21.7)

The physiological function of plasmin (fibrinolysin) is to dissolve blood clots. It is part of a complex system consisting of plasmin, its zymogen (plasminogen), plasminogen activators, plasmin inhibitors and inhibitors of plasminogen activators (Figure 8.1). In milk, there is about four times as much plasminogen as plasmin and both, as well as plasminogen activators, are associated with the casein micelles, from which they dissociate when the pH is reduced to 4.6. The inhibitors of plasmin and of plasminogen activators are in the milk serum. The concentration of plasmin and plasminogen in milk increase with advancing lactation, mastitic infection and number of lactations.

Plasmin is usually extracted from casein at pH 3.5 and purified by precipitation with $(NH_4)_2SO_4$ and various forms of chromatography, including affinity chromatography. Plasmin is optimally active at about pH 7.5 and 35°C; it exhibits *c.* 20% of maximum activity at 5°C and is stable over the pH range 4 to 9. Plasmin is quite heat stable: it is partially inactivated by heating at 72°C × 15 s but its activity in milk increases following HTST pasteurization, probably through inactivation of the indigenous inhibitors of plasmin or, more likely, inhibitors of plasminogen activators. It partly survives UHT sterilization and is inactivated by heating at 80°C × 10 min at pH 6.8; its stability decreases with increasing pH in the range 3.5–9.2.

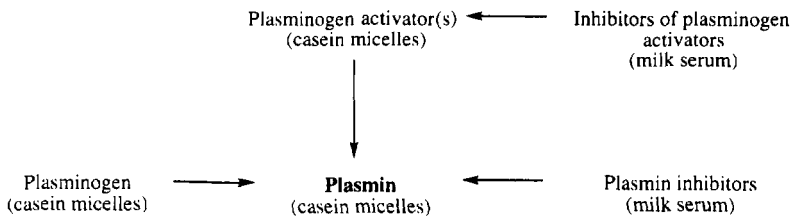


Figure 8.1 Schematic representation of the plasmin system in milk.

Plasmin is a serine proteinase (inhibited by diisopropylfluorophosphate, phenylmethyl sulphonyl fluoride and trypsin inhibitor) with a high specificity for peptide bonds to which lysine or arginine supplies the carboxyl group. Its molecular weight is about 81 Da and its structure contains five intramolecular disulphide-linked loops (kringles) which are essential for its activity.

Activity of plasmin on milk proteins. β-Casein is the most susceptible milk protein to plasmin action; it is hydrolysed rapidly at Lys_{28}–Lys_{29}, Lys_{105}–His_{106} and Lys_{107}–Glu_{108}, to yield γ^1 (β-CN f29–209), γ^2 (β-CN f106–209) and γ^3 (β-CN f108–209) caseins and proteose-peptone (PP)5 (β-CN f1–105/7), PP8 slow (β-CN f29–105/7) and PP8 fast (β-CN f1–29) (Chapter 4). In solution, β-casein is also hydrolysed at Lys_{113}–Tyr_{114} and Lys_{183}–Asp_{184}, but it is not known if these bonds are hydrolysed in milk. γ-Caseins normally represent about 3% of total N in milk but can be as high as 10% in late lactation milk; the concentration of proteose peptones is about half that of the γ-caseins.

α_{s2}-Casein in solution is also hydrolysed very rapidly by plasmin at bonds Lys_{21}–Gln_{22}, Lys_{24}–Asn_{25}, Arg_{114}–Asn_{115}, Lys_{149}–Lys_{150}, Lys_{150}–Thr_{151}, Lys_{181}–Thr_{182}, Lys_{187}–Thr_{188} and Lys_{188}–Ala_{189} (see Bastian and Brown, 1996) but it is not known if it is hydrolysed in milk. Although less susceptible than α_{s2}- or β-caseins, α_{s1}-casein in solution is also readily hydrolysed by plasmin (see Bastian and Brown, 1996) but it does not appear to be hydrolysed to a significant extent in milk although it has been suggested that λ-casein is produced from α_{s1}-casein by plasmin. Although κ-casein contains several Lys and Arg residues, it appears to be quite resistant to plasmin, presumably due to a relatively high level of secondary and tertiary structure. β-Lactoglobulin, especially when denatured, inhibits plasmin, presumably via sulphydryl–disulphide interactions which rupture the structurally important kringles.

Significance of plasmin activity in milk. Plasmin and plasminogen accompany the casein micelles on the rennet coagulation of milk and are concentrated in cheese in which plasmin contributes to primary proteolysis of the caseins, especially in cheeses with a high-cook temperature, e.g. Swiss and some Italian varieties, in which the coagulant is totally or largely inactivated (Chapter 10). Plasmin activity may contribute to age gelation in UHT milk produced from high-quality raw milk (which contains a low level of *Pseudomonas* proteinase). It has been suggested that plasmin activity contributes to the poor cheesemaking properties of late-lactation milk but proof is lacking. The acid precipitability of casein from late lactation milk is also poor but evidence for the involvement of plasmin is lacking. Reduced yields of cheese and casein can be expected to result from plasmin action since the proteose peptones are, by definition, soluble at pH 4.6.

Cathepsin D (EC 3.4.23.5). It has been known for more than 20 years that milk also contains an acid proteinase, (optimum pH $\approx$ 4.0) which is now known to be cathepsin D, a lysozomal enzyme. It is relatively heat labile (inactivated by 70°C $\times$ 10 min). Its activity in milk has not been studied extensively and its significance is unknown. At least some of the indigenous acid proteinase is incorporated into cheese curd; its specificity on α_{s1}- and β-caseins is quite similar to that of chymosin but it has very poor milk-clotting activity (McSweeney, Fox and Olson, 1995). It may contribute to proteolysis in cheese but its activity is probably normally overshadowed by chymosin, which is present at a much higher level.

Other proteinases. The presence of low levels of other proteolytic enzymes in milk has been reported (see Fox and McSweeney, 1996). Most of these originate from somatic cells, and their level increases during mastitic infection. The presence of cathepsin D, a lysozomal enzyme, in milk suggests that all the lysozomal proteinases are present in milk although they may not be active. These minor proteinases are considered to be much less significant than plasmin, but more work on the subject is necessary.

8.2.3 Lipases and esterases (EC 3.1.1.–)

Lipases catalyse the development of hydrolytic rancidity in milk, and, consequently, lipases and lipolysis in milk have been studied extensively.

Milk contains three types of esterase:

1. A-type carboxylic ester hydrolases (arylesterases; EC 3.1.1.2), which hydrolyse aromatic esters, e.g. phenylacetate; they show little activity on tributyrin, and are not inhibited by organophosphates.
2. B-type esterases (glycerol tricarboxyl esterases, aliphatic esterases, lipases; EC 3.1.1.3): they are most active on aliphatic esters although they show some activity on aromatic esters; they are inhibited by organophosphates.
3. C-type esterases (cholinesterase; EC 3.1.1.7; EC 3.1.1.8): they are most active on choline esters but hydrolyse some aromatic and aliphatic esters slowly; they are inhibited by organophosphates.

In normal milk, the ratio of A : B : C esterase activity is about 3 : 10 : 1 but the level of A-esterase activity increases considerably on mastitic infection. A and C esterases are considered to be of little technological significance in milk.

Classically, lipases hydrolyse ester bonds in emulsified esters, i.e. at a water/oil interface, although some may have limited activity on soluble esters; they are usually activated by blood serum albumin and Ca^{2+} which bind free fatty acids, which are inhibitory. Little lipolysis normally occurs in

milk because more than 90% of the lipase is associated with the casein micelles while the triglyceride substrates are in fat globules surrounded, and protected, by the fat globule membrane (MFGM). When the MFGM is damaged, lipolysis occurs rapidly, giving rise to hydrolytic rancidity.

Lipase was first isolated from skim milk and characterized by Fox and Tarassuk in 1967. The enzyme was optimally active at pH 9.2 and 37°C and found to be a serine enzyme (inactivated by organophosphates). A lipoprotein lipase (LPL; activated by lipoprotein co-factors) was demonstrated in milk by Korn in 1962 and was isolated by Egelrud and Olivecrona in 1972. LPL is, in fact, the principal indigenous lipase in milk and most recent work has been focused accordingly. The molecule has been characterized at the molecular, genetic, enzymatic and physiological levels (see Olivecrona *et al.*, 1992).

In addition to LPL, human milk contains a bile salts-activated lipase, which probably contributes to the metabolism of lipids by breast-fed babies who have limited pancreatic lipase activity. Bovine milk and milks from other dairy animals do not contain this enzyme.

The lipolytic system in most milks becomes active only when the milk MFGM is damaged by agitation, homogenization or temperature fluctuations. However, some individual cows produce milk which becomes rancid spontaneously, i.e. without apparent activation. Spontaneous rancidity was considered to be due to a second lipase, termed **membrane lipase**, which was believed to be associated with the MFGM, but recent evidence suggests that LPL is responsible for spontaneous rancidity following activation by a lipoprotein (co-lipase) from blood serum; normal milk will become spontaneously rancid if blood serum is added, suggesting that 'spontaneous milks' contain a higher than normal level of blood serum. Dilution of 'spontaneous milk' with normal milk prevents spontaneous rancidity, which consequently is not normally a problem with bulk herd milks; presumably, dilution with normal milk reduces the lipoprotein content of the mixture to below the threshold necessary for lipase adsorption.

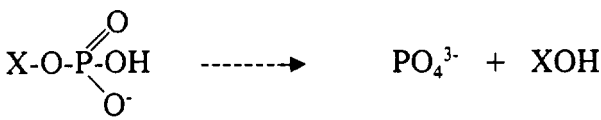
Natural variations in the levels of free fatty acids in normal milk and the susceptibility of normal milks to lipolysis may be due to variations in the level of blood serum in milk.

Significance of lipase. Technologically, lipase is arguably the most significant indigenous enzyme in milk. Although indigenous milk lipase may play a positive role in cheese ripening, undoubtedly the most industrially important aspect of milk lipase is its role in hydrolytic rancidity which renders liquid milk and dairy products unpalatable and eventually unsaleable. Lipolysis in milk has been reviewed extensively (Deeth and Fitz-Gerald, 1995). As discussed in Chapter 3, all milks contain an adequate level of lipase for rapid lipolysis, but become rancid only after the fat globule membrane has been damaged.

8.2.4 Phosphatases

Milk contains several phosphatases, the principal ones being alkaline and acid phosphomonoesterases, which are of technological significance, and ribonuclease, which has no known function or significance in milk. The alkaline and acid phosphomonoesterases have been studied extensively (see Andrews (1993) for references).

Alkaline phosphomonoesterase (EC 3.1.3.1). The existence of a phosphatase in milk was first recognized in 1925. Subsequently characterized as an alkaline phosphatase, it became significant when it was shown that the time–temperature combinations required for the thermal inactivation of alkaline phosphatase were slightly more severe than those required to destroy *Mycobacterium tuberculosis*, then the target micro-organism for pasteurization. The enzyme is readily assayed, and a test procedure based on alkaline phosphatase inactivation was developed for routine quality control of milk pasteurization. Several major modifications of the test have been developed. The usual substrates are phenyl phosphate, *p*-nitrophenyl-phosphate or phenolphthalein phosphate which are hydrolysed to inorganic phosphate and phenol, *p*-nitrophenol or phenolphthalein, respectively:



where XOH = phenol, *p*-nitrophenol or phenolphthalein.

The release of inorganic phosphate may be assayed but the other product is usually determined. Phenol is colourless but forms a coloured complex on reaction with one of several reagents, e.g. 2,6-dichloroquinonechloroimide, with which it forms a blue complex. *p*-Nitrophenol is yellow while phenolphthalein is red at the alkaline pH of the assay (10) and hence the concentration of either of these may be determined easily.

Isolation and characterization. Alkaline phosphatase is concentrated in the fat globule membrane and hence in cream. It is released into the buttermilk on phase inversion; consequently, buttermilk is the starting material for most published methods for the purification of alkaline phosphatase. Later methods have used chromatography on various media to give a homogeneous preparation with 7440-fold purification and 28% yield. The characteristics of milk alkaline phosphatase are summarized in Table 8.2. The enzyme appears to be similar to the alkaline phosphatase of mammary tissue.

Table 8.2 Characteristics of milk alkaline phosphatase

Characteristic	Conditions
pH optimum	Casein: 6.8 *p*-nitrophenylphosphate: 9.65 *p*-nitrophenylphosphate: 10.5
Temperature optimum	37°C
K_m	0.69 mM on *p*-nitrophenylphosphate
Activators	Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+}
Molecular weight	170–190 kDa
Association/dissociation	2 subunits of molecular weight 85 kDa formed on heating (100°C for 2 min or acidification to pH 2.1)
Polymorphic forms	4

Reactivation of phosphatase. Much work has been focused on a phenomenon known as 'phosphatase reactivation', first recognized by Wright and Tramer in 1953, who observed that UHT-treated milk was phosphatase-negative immediately after processing but became positive on standing; microbial phosphatase was shown not to be responsible. Bulk HTST milk never showed reactivation, although occasional individual-cow samples did; HTST pasteurization after UHT treatment usually prevented reactivation and reactivation was never observed in very severely heated milk. Reactivation can occur following heating at temperatures as low as 84°C for milk and 74°C for cream; the optimum storage temperature for reactivation is 30°C, at which reactivation is detectable after 6 h and may continue for up to 7 days. The greater reactivation in cream than in milk may be due to protection by fat but this has not been substantiated. Mg^{2+} and Zn^{2+} strongly promote reactivation; Sn^{2+}, Cu^{2+}, Co^{2+} and EDTA are inhibitory, while Fe^{2+} has no effect.

Sulphydryl -(SH) groups appear to be essential for reactivation; perhaps this is why phosphatase becomes reactivated in UHT milk but not in HTST milk. The role of -SH groups, supplied by denatured whey proteins, is considered to be chelation of heavy metals, which would otherwise bind to -SH groups of the enzyme (also activated on denaturation), thus preventing renaturation. The role of Mg^{2+} or Zn^{2+} is seen as causing a conformational change in the denatured enzyme, necessary for renaturation.

Reactivation of alkaline phosphatase is of considerable practical significance since regulatory tests for pasteurization assume the absence of phosphatase activity. An official AOAC method used to distinguish between renatured and residual native alkaline phosphatase is based on the increase in phosphatase activity resulting from addition of Mg^{2+}: the activity of renatured alkaline phosphatase is increased about 14-fold but that of the native enzyme is increased only two-fold.

Although it can dephosphorylate casein under suitable conditions, as far as is known, alkaline phosphatase has no direct technological significance

in milk and milk products; perhaps its pH optimum is too far removed from that of milk; it is also inhibited by inorganic phosphate.

Acid phosphomonoesterase (EC 3.1.3.2). Milk contains an acid phosphatase which has a pH optimum at 4.0 and is very heat stable (LTLT pasteurization causes only 10–20% inactivation and 30 min at 88°C is required for full inactivation). Denaturation of acid phosphatase under UHT conditions follows first-order kinetics. When heated in milk at pH 6.7, the enzyme retains significant activity following HTST pasteurization but does not survive in-bottle sterilization or UHT treatment. The enzyme is not activated by Mg^{2+} (as is alkaline phosphatase), but it is slightly activated by Mn^{2+} and is very effectively inhibited by fluoride. The level of acid phosphatase activity in milk is only about 2% that of alkaline phosphatase; activity reaches a sharp maximum 5–6 days post-partum, then decreases and remains at a low level to the end of lactation.

Milk acid phosphatase has been purified to homogeneity by various forms of chromaotgraphy, including affinity chromatography; purification up to 40 000-fold has been claimed. The enzyme shows broad specificity on phosphate esters, including the phosphoseryl residues of casein. It has a molecular mass of about 42 kDa and an isoelectric point of 7.9. Many forms of inorganic phosphate are competitive inhibitors, while fluoride is a powerful non-competitive inhibitor. The enzyme is a glycoprotein and its amino acid composition is known. Milk acid phosphatase shows some similarity to the phosphoprotein phosphatase of spleen but differs from it in a number of characteristics.

Although casein is a substrate for milk acid phosphatase, the major caseins, in the order $\alpha_s(\alpha_{s1} + \alpha_{s2}) > \beta > \kappa$, also act as competitive inhibitors of the enzyme when assayed on *p*-nitrophenylphosphate, probably due to binding of the enzyme to the casein phosphate groups (the effectiveness of the caseins as inhibitors is related to their phosphate content).

Significance. Although acid phosphatase is present in milk at a much lower level than alkaline phosphatase, its greater heat stability and lower pH optimum may make it technologically significant. Dephosporylation of casein reduces its ability to bind Ca^{2+}, to react with κ-casein, to form micelles and its heat stability. Several small partially dephosphorylated peptides have been isolated from Cheddar and Parmesan cheese. However, it is not known whether indigenous or bacterial acid phosphatases are mainly responsible for dephosphorylation in cheese. Dephosphorylation may be rate-limiting for proteolysis in cheese ripening since most proteinases and peptidases are inactive on phosphoproteins or peptides. It has been suggested that phosphatase activity should be included in the criteria for starter selection.

The acid phosphatase activity in milk increases by a factor of 4–10 during mastitic infection; three isoenzymes are then present, only one of which is indigenous milk acid phosphatase, the other two being of leucocyte origin; these latter isoenzymes are more thermolabile and are inactivated by HTST pasteurization.

8.2.5 Lysozyme (EC 3.2.1.17)

Lysozyme (muramidase, mucopeptide *N*-acetylmuramylhydrolase) is a widely distributed enzyme which lyses certain bacteria by hydrolysing the β(1-4)-linkage between muramic acid and *N*-acetylglucosamine of mucopolysaccharides of the bacterial cell wall.

Lysozyme was isolated from human milk in 1961 by Jolles and Jolles, who believed that bovine milk was devoid of lysozyme. Milks of many species have since been shown to contain lysozyme and several have been isolated and characterized. Human and equine milks are an exceptionally rich source, containing 130 mg l^{-1} (3000 times the level of bovine milk) and about 800 mg l^{-1}, respectively (see Farkye, 1992).

The pH optima of human milk lysozyme (HML), bovine milk lysozyme (BML) and egg-white lysozyme (EWL) are 7.9, 6.35 and 6.2, respectively. BML has a molecular weight of 18 kDa compared with 15 kDa for HML and EWL. The amino acid composition of BML is reported to be considerably different from that of HML or EWL. All lysozymes are relatively stable to heat at acid pH values (3–4) but are relatively labile at pH greater than 7. Low concentrations of reducing agents increase the activity of BML and HML by about 330%.

Significance. Presumably, the physiological role of lysozyme is to act as a bactericidal agent; in the case of milk it may simply be a 'spill-over' enzyme or it may have a definite protective role. If the latter is true, then the exceptionally high level of lysozyme in human and equine milk may be nutritionally significant. Breast-fed babies generally suffer less enteric problems than bottle-fed babies. While there are many major compositional and physicochemical differences between bovine and human milks which may be responsible for the observed nutritional characteristics (Chapter 4), it has been suggested that the disparity in lysozyme content may be significant. A number of investigators have recommended fortification of bovine milk-based infant formulae with EWL, especially for premature babies. Feeding studies are equivocal on the benefits of this practice and recent trials failed to demonstrate any beneficial effect due to inactivation of EWL in the human stomach.

No beneficial effects from lysozyme on the shelf-life of milk have been reported. Addition of lysozyme to milk reduces its heat stability but the level

of indigenous lysozyme is probably too low to contribute to the natural variations in the heat stability of milk.

8.2.6 N-Acetyl-β-D-glucosaminidase (EC 3.2.1.30)

N-Acetyl-β-D-glucosaminidase (NAGase) hydrolyses terminal, non-reducing *N*-acetyl-β-D-glucosamine residues from glycoproteins. It is a lysosomal enzyme and originates mainly from somatic cells and mammary gland epithelial cells. Consequently, NAGase activity increases markedly and correlates highly with the intensity of mastitis. A field test for mastitis based on NAGase activity has been developed, using chromogenic *N*-acetyl-β-D-glucosamine-*p*-nitrophenol as substrate; hydrolysis yields yellow *p*-nitrophenol. NAGase is optimally active at 50°C and pH 4.2 and is inactivated by HTST pasteurization (70–71°C × 15–18 s) (see Farkye, 1992).

8.2.7 γ-Glutamyl transpeptidase (transferase) (EC 2.3.2.2)

γ-Glutamyl transpeptidase (GGT) catalyses the transfer of γ-glutamyl residues from γ-glutamyl-containing peptides:

$$\gamma\text{-glutamyl-peptide} + X \rightleftharpoons \text{peptide} + \gamma\text{-glutamyl-}X,$$

where X is an amino acid.

GGT, which has been isolated from the fat globule membrane, has a molecular mass of about 80 kDa and consists of two subunits of 57 and 26 kDa. It is optimally active at pH 8–9, has a pi of 3.85 and is inhibited by iodoacetate, diisopropylfluorophosphate and metal ions, e.g. Cu^{2+} and Fe^{3+}

It plays a role in amino acid transport in the mammary gland. γ-Glutamyl peptides have been isolated from cheese but since γ-glutamyl bonds do not occur in milk proteins, their synthesis may be catalysed by GGT. The enzyme is relatively heat stable and has been proposed as a marker enzyme for milks pasteurized in the range 72–80°C × 15 s. GGT is absorbed from the gastrointestinal tract, resulting in high levels of GGT activity in the blood serum of newborn animals fed colostrum or early breast milk. Since GGT is inactivated by the heat treatment to which infant formulae are subjected, the level of GGTase activity in infants can be used to distinguish breast-fed from formula-fed infants (see Farkye, 1992).

8.2.8 Xanthine oxidase (EC 1.2.3.2)

It has been recognized for about 80 years that milk contains an enzyme capable of oxidizing aldehydes and purines. The enzyme is now generally referred to as xanthine oxidase (XO); milk is a very good source of XO, at

least part of which is transported to the mammary gland via the bloodstream. A similar enzyme is found in various animal tissues and several bacterial species (Farkye, 1993).

Isolation. Numerous methods have been developed for the purification of XO from milk; since the enzyme is concentrated in the MFGM, in which it is one of the principal proteins, all methods employ cream as starting material, use a dissociating agent to liberate XO from membrane lipoproteins and some form of chromatography for further purification.

Milk XO has a molecular weight of *c.* 300 kDa and consists of two subunits. The pH optimum is about 8.5 and the enzyme requires flavin adenine dinucleotide (FAD), Fe, Mo and an acid-labile compound as co-factors; cows deficient in Mo have low XO activity. The amino acid composition of XO has been determined by a number of workers; at least five genetic polymorphic forms have been reported.

Activity in milk. Various processing treatments affect the XO activity of milk. Activity is increased by about 100% on storage at 4°C for 24 h, by 50–100% on heating at 70°C for 5 min and by 60–90% on homogenization. These treatments cause the transfer to XO from the fat phase to the aqueous phase, rendering the enzyme more active. The heat stability of XO is very dependent on whether it is a component of the fat globules or is dissolved in the aqueous phase; ageing and homogenization increase susceptibility and explain the inconsistency of early work in which the history of the sample was unknown or unrecorded. XO is most heat stable in cream and least in skim milk. Homogenization of concentrated milk prepared from heated milk (90.5°C for 15 s) partially reactivates XO, which persists on drying the concentrate, but no reactivation occurs following more severe heating (93°C for 15 s); apparently, homogenization releases potentially active, undenatured XO from the MFGM. All the major milk proteins can act as either activators or inhibitors of XO, depending on their concentration, and may have some significance in the activation, inactivation and reactivation of the enzyme.

Significance of xanthine oxidase

Lipid oxidation

XO, which can excite stable triplet oxygen ($^3O_2$), is a pro-oxidant. Milk which undergoes spontaneous rancidity contains about 10 times the normal level of XO, and spontaneous oxidation can be induced in normal milk by the addition of XO to about four times normal levels. Heat-denatured or flavin-free enzyme is ineffective and the susceptibility of unsaturated fatty acids to oxidation increases with the degree of unsaturation.

Atherosclerosis

It has been suggested that XO from homogenized milk enters the vascular system and may be involved in atherosclerosis via oxidation of plasmalogens (Appendix 3B) in cell membranes. However, the experimental evidence in support of this view is very weak and the hypothesis has been disclaimed (see Farkye, 1992).

8.2.9 Sulphydryl oxidase (EC 1.8.3.–)

Milk contains an enzyme, sulphydryl oxidase (SO), capable of oxidizing sulphydryl groups of cysteine, glutathione and proteins to the corresponding disulphide (reviewed by Farkye, 1992). The enzyme is an aerobic oxidase which catalyses the following reaction:

$$2RSH + O_2 \rightleftharpoons RSSR + H_2O_2$$

It undergoes marked self-association and can be purified readily by chromatography on porous glass. The enzyme has a molecular weight of about 89 kDa, a pH optimum of 6.8–7.0, and a temperature optimum of 35°C. Its amino acid composition, its requirement for iron but not for molybdenum and FAD, and the catalytic properties of the enzyme, indicate that sulphydryl oxidase is a distinct enzyme from xanthine oxidase and thiol oxidase (EC 1.8.3.2).

SO is capable of oxidizing reduced ribonuclease and restoring enzymic activity, suggesting that its physiological role may be the non-random formation of protein disulphide bonds, e.g. during protein biosynthesis.

SO immobilized on glass beads has the potential to ameliorate the cooked flavour arising from sulphydryl groups exposed upon protein denaturation, but the commercial viability of this system is not known.

The production of sulphur compounds is believed to be very important in the development of Cheddar cheese flavour. Residual sulphydryl oxidase activity may play a role in initially reoxidizing sulphydryl groups exposed upon heating cheesemilk; the sulphydryl groups thus protected may be reformed during the ripening process.

8.2.10 Superoxide dismutase (EC 1.15.1.1)

Superoxide dismutase (SOD) scavenges superoxide radicals, $O_2^{\cdot-}$ according to the reaction:

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

The H_2O_2 formed may be reduced by catalase, peroxidase or suitable reducing agents. SOD has been identified in many animal and bacterial cells; its biological function is to protect tissue against oxygen free radicals in anaerobic systems (reviewed by Farkye, 1992).

SOD, isolated from bovine erythrocytes, is a blue-green protein due to the presence of copper, removal of which by treatment with EDTA results in loss of activity, which is restored by adding Cu^{2+}; it also contains Zn^{2+}, which does not appear to be at the active site. The enzyme, which is very stable in 9 M urea at neutral pH, consists of two identical subunits of molecular weight 16 kDa held together by one or more disulphide bonds. The amino acid sequence has been established.

Milk contains trace amounts of SOD which has been isolated and characterized; it appears to be identical to the bovine erythrocyte enzyme. SOD inhibits lipid oxidation in model systems. The level of SOD in milk parallels that of XO (but at a lower level), suggesting that SOD may be excreted in milk in an attempt to offset the pro-oxidant effect of XO. However, the level of SOD in milk is probably insufficient to explain observed differences in the oxidative stability of milk. The possibility of using exogenous SOD to retard or inhibit lipid oxidation in dairy products has been considered.

SOD is more heat stable in milk than in purified preparations; in milk it is stable at 71°C for 30 min but loses activity rapidly at even slightly higher temperatures. Slight variations in pasteurization temperature are therefore critical to the survival of SOD in heated milk products and may contribute to variations in the stability of milk to oxidative rancidity.

8.2.11 Catalase (EC 1.11.1.6)

Indigenous milk catalase was first recognized in 1907. Although about 70% of the catalase activity of whole milk is in the skim-milk phase, cream has a higher specific activity; the pellet obtained from buttermilk on centrifugation at 10 000 g is a particularly rich source, from which catalase has been highly purified (reviewed by Farkye, 1992).

Milk catalase is a haem protein with a molecular weight of 200 kDa, and an isoelectric pH of 5.5; it is stable between pH 5 and 10 but rapidly loses activity outside this range. Heating at 70°C for 1 h causes complete inactivation. Like other catalases, it is strongly inhibited by Hg^{2+}, Fe^{2+}, Cu^{2+}, Sn^{2+}, CN^- and NO_3^-.

Catalase activity in milk varies with feed, stage of lactation and especially with mastitic infection, of which it may be used as an index. It may act as a lipid pro-oxidant via its haem iron.

8.2.12 Lactoperoxidase (EC 1.11.1.7)

The occurrence of a peroxidase, lactoperoxidase (LPO), in milk was recognized as early as 1881. It is one of the most heat-stable enzymes in milk; its destruction was used as an index of flash pasteurization (now very rarely used) and is now used as an index of super-HTST pasteurization.

LPO was first isolated in 1943; several isolation procedures have since been published (reviewed by Björck, 1993).

LPO is a haem protein containing about 0.07% Fe, with an absorbance peak (Soret band) at 412 nm ($A_{412}/A_{280} \sim 0.9$); the pH optimum is around 8.0; its molecular weight is 77.5 kDa and it consists of two identical subunits. Two principal forms (A and B) occur, each of which exhibits micro-heterogeneity with regard to amide groups (glutamine and/or asparagine) and carbohydrate content, giving a total of 10 variants.

Significance. Apart from its exploitation as an index of flash or super-HTST pasteurization, LPO is also technologically significant for a number of other reasons:

1. It is a possible index of mastitic infection; although the level of LPO in milk increases on mastitic infection, it is not well correlated with somatic cell count.
2. LPO causes non-enzymic oxidation of unsaturated lipids, probably acting through its haem group; the heat-denatured enzyme is more active than the native enzyme.
3. Milk contains bacteriostatic or bactericidal substances referred to as lactenins. One of these is LPO, which requires H_2O_2 and thiocyanate (SCN^-) to cause inhibition. The nature, mode of action and specificity of the LPO–H_2O_2–SCN^- system has been widely studied. LPO and thiocyanate, which is produced in the rumen by enzymic hydrolysis of thioglycosides from *Brassica* plants, occur naturally in milk, but H_2O_2 does not. However, H_2O_2 can be generated metabolically by catalase-negative bacteria, or produced *in situ* through the action of exogenous glucose oxidase on glucose, or it may be added directly.

   The peroxidase system has been found to have good bactericidal efficiency for the cold pasteurization of fluids or sanitization of immobilized enzyme columns. The generation of H_2O_2 *in situ* through the action of immobilized glucose oxidase on glucose is effective against Gram-negative bacteria in thiocyanate and glucose-enriched milk and whey. A self-contained LPO–H_2O_2–SCN^- system using coupled β-galactosidase and glucose oxidase, immobilized on porous glass beads, to generate H_2O_2 *in situ* from lactose in milk containing 0.25 mM thiocyanate has been developed. Indigenous xanthine oxidase, acting on added hypoxanthine, may also be exploited to produce H_2O_2 for the LPO–H_2O_2–SCN^- system. The bactericidal effects of the LPO–H_2O_2–SCN^- system may be used to cold pasteurize milk in situations where refrigeration and/or thermal pasteurization is lacking. LPO is cationic at the pH of milk and may be readily isolated on cation-exchange resins. Addition of isolated LPO to milk replacers for calves or piglets reduces the incidence of enteritis.

Table 8.3 Other enzymes that have been isolated from milk and partially characterized but which are of no known significance (Farkye, 1992)

Enzyme		Reaction catalysed	Comment
Glutathione peroxidase	EC 1.11.1.9 2	$GSH + H_2O \rightleftharpoons GSSH$	Contains Se
Ribonuclease	EC 3.1.27.5	Hydrolysis of RNA	Milk is a very rich source; similar to pancreatic RNase
α-Amylase	EC 3.2.1.1	Starch	
β-Amylase	EC 3.2.1.2	Starch	
α-Mannosidase	EC 3.2.1.24		Contains Zn^{2+}
β-Glucuronidase	EC 3.2.1.31		
5′-Nucleotidase	EC 3.1.3.5 5′	Nucleotides + $H_2O \rightleftharpoons$ ribonucleosides + P_i	Diagnostic test for mastitis
Adenosine triphosphatase	EC 3.6.1.3	$ATP + H_2O \rightleftharpoons ADP + P_i$	
Aldolase	EC 4.1.2.13	Fructose 1,6 diP $\rightleftharpoons$ glyceraldehyde-3-P + dihydroxyacetone-P	

4. Acid production in milk by some starters is reported to be retarded by severe heat treatment of milk (77–80°C for 10 min) but can be restored by addition of LPO; the mechanism involved is unknown.

8.2.13 Other enzymes

In addition to the enzymes described above, a number of other indigenous enzymes (Table 8.3) have been isolated and partially characterized (see Farkye, 1992). Although fairly high levels of some of these enzymes occur in milk, they have no apparent function in milk and will not be discussed further.

Nearly 40 other enzymic activities have been detected in milk but have not been isolated and limited information on their molecular and biochemical properties in milk are available; some of these are listed in Table 8.4.

8.3 Exogenous enzymes in dairy technology

8.3.1 Introduction

Crude enzyme preparations have been used in food processing since prehistoric times; classical examples are rennets in cheesemaking and papaya leaves to tenderize meat. Added (exogenous) enzymes are attractive in food processing because they can induce specific changes, in contrast to chemical or physical methods which may cause non-specific undesirable changes. For some applications, there is no viable alternative to enzymes, e.g. rennet-coagulated cheeses, whereas in some cases, enzymes are preferred

Table 8.4 Partial list of minor enzymes in milk (modified from Farkye, 1992)

Enzyme	Reaction catalysed	Source	Distribution in milk
EC 1.1.1.1 Alcohol dehydrogenase	Ethanol + NAD^+ ⇌ acetaldehyde + NADH + H^+		
EC 1.1.1.14 L-Iditol dehydrogenase	L-Iditol + NAD^+ ⇌ L-sorbose + NADH	–	SM
EC 1.1.1.27 Lactate dehydrogenase	Lactic acid + NAD^+ ⇌ pyruvic acid + NADH + H^+		
EC 1.1.1.37 Malate dehydrogenase	Malate + NAD^+ ⇌ oxaloacetate + NADH	Mammary gland	SM
EC 1.1.1.40 Malic enzyme	Malate + $NADP^+$ ⇌ pyruvate + CO_2 + NADH	Mammary gland	SM
EC 1.1.1.42 Isocitrate dehydrogenase	Isocitrate + $NADP^+$ ⇌ 2-oxoglutarate + CO_2 + NADH	Mammary gland	SM
EC 1.1.1.44 Phosphoglucuronate dehydrogenase (decarboxylating)	6-Phospho-D-gluconate + $NADP^+$ ⇌ D-ribose-5-phosphate + CO_2 + NADPH	Mammary gland	SM
EC 1.1.1.49 Glucose-6-phosphate dehydrogenase	D-Glucose-6-phosphate + $NADP^+$ ⇌ D-glucono-1,5-lactone-6-phosphate + NADPH	Mammary gland	SM
EC 1.4.3.6 Amine oxidase (Cu-containing)	$RCH_2NH_2 + H_2O + O_2 \rightleftharpoons RCHO + NH_3 + H_2O_2$	–	SM
– Polyamine oxidase	Spermine → spermidine → putrescine	–	SM
– Fucosyltransferase	Catalyses the transfer of fucose from GDP L-fucose to specific oligosaccharides and glycoproteins	–	SM
EC 1.6.99.3 NADH dehydrogenase	NADH + acceptor ⇌ NAD^+ + reduced acceptor	–	FGM
EC 1.8.1.4 Dihydrolipomide dehydrogenase (diaphorase)	Dihydrolipomide + NAD^+ ⇌ lipoamide + NADH	–	SM/FGM
EC 2.4.1.22 Lactose synthetase A protein: UDP-galactose: D-glucose, 1-galactosyltransferase; B protein: α-lactalbumin	UDP-galactose + D-glucose ⇌ UDP + lactose	Golgi apparatus	SM
EC 2.4.1.38 Glycoprotein 4-β-galactosyltransferase	UDP-galactose + *N*-acetyl-D-glucosaminyl-glycopeptide ⇌ UDP + 4,β-D-galactosyl-*N*-acetyl-D-glucosaminyl-glycopeptide	–	FGM
EC 2.4.1.90 *N*-Acetyllactosamine synthase	UDP-galactose + *N*-acetyl-D-glucosamine ⇄ UDP *N*-acetyllactosamine	Golgi apparatus	–
EC 2.4.99.6 CMP-*N*-acetyl-*N*-acetyl-lactosaminide α-2,3-sialyltransferase	CMP-*N*-acetylneuraminate + β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminyl-glycoprotein ⇌ CMP + α-*N*-acetylneuraminyl-1,2,3-β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminyl-glycoprotein	–	SM

EC 2.5.1.3 Thiamin-phosphate pyrophosphorylase	2-Methyl-4-amino-5-hydroxymethylpyrimide diphosphate + 4-methyl-5-(2-phosphono-oxyethyl)-thiazole $\rightleftharpoons$ pyrophosphate + thiamine monophosphate	–	FGM
EC 2.6.1.1 Aspartate aminotransferase	L-Aspartate + 2-oxoglutarate $\rightleftharpoons$ oxaloacetate + L-glutamate	Blood	SM
EC 2.6.1.2 Alanine aminotransferase	L-Alanine + 2-oxoglutarate $\rightleftharpoons$ pyruvate + L-glutamate	Blood	SM
EC 2.7.5.1 Phosphoglucomutase	–	–	–
EC 2.7.7.49 RNA-directed DNA polymerase	*n* Deoxynucleoside triphosphate $\rightleftharpoons$ *n* pyrophosphate + DNA_n	–	SM
EC 2.8.1.1 Thiosulphate sulphur transferase	Thiosulphate + cyanide $\rightleftharpoons$ sulphite + thiocyanate	–	SM
EC 3.1.1.8 Cholinesterase	An acylcholine + H_2O $\rightleftharpoons$ choline + a carboxylic acid anion	Blood	FGM
EC 3.1.3.9 Glucose-6-phosphatase	D-Glucose-6-phosphate + H_2O $\rightleftharpoons$ D-glucose + inorganic phosphate	–	FGM
EC 3.1.4.1 Phosphodiesterase	–	–	–
EC 3.1.6.1 Arylsulphatase	Phenol sulphate + H_2O $\rightleftharpoons$ phenol + sulphate	–	–
EC 3.2.1.21 β-Glucosidase	Hydrolysis of terminal non-reducing β-D-glucose residues	Lysosomes	FGM
EC 3.2.1.23 β-Galactosidase	Hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides	Lysosomes	FGM
EC 3.2.1.51 α-Fucosidase	An α-L-fucoside + H_2O $\rightleftharpoons$ an alcohol + L-fucose	Lysosomes	–
EC 3.4.11.1 Cytosol aminopeptidase (leucine aminopeptidase)	Aminoacyl-peptide + H_2O $\rightleftharpoons$ amino acid + peptide	–	SM
EC 3.4.11.3 Cystyl-aminopeptidase (oxytocinase)	Cystyl-peptides + H_2O $\rightleftharpoons$ amino acid + peptide	–	SM
EC 3.4.21.4 Trypsin	Hydrolyses peptide bonds, preferentially Lys-X, Arg-X	–	SM
EC 3.6.1.1 Inorganic pyrophosphatase	Pyrophosphate + H_2O $\rightleftharpoons$ 2-orthophosphate	–	SM/FGM
EC 3.6.1.1 Pyrophosphate phosphorylase	–	–	–
EC 3.6.1.9 Nucleotide pyrophosphorylase	A dinucleotide + H_2O $\rightleftharpoons$ 2 mononucleotides	–	SM/FGM
EC 4.2.1.1 Carbonate dehydratase	H_2CO_3 $\rightleftharpoons$ $CO_2 + H_2O$	–	SM
EC 5.3.1.9 Glucose-6-phosphate isomerase	D-Glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate	–	SM
EC 6.4.1.2 Acetyl-CoA carboxylase	ATP + acetyl CoA + HCO_3 $\rightleftharpoons$ ADP + orthophosphate + malonyl CoA	–	FGM

SM, Skim milk; FGM, fat globule membrane.

over chemical methods because they cause fewer side-reactions and consequently give superior products, e.g. hydrolysis of starch.

Although relatively few enzymes are used in the dairy industry on a significant scale, the use of rennets in cheesemaking is one of the principal of all industrial applications of enzymes.

The applications of exogenous enzymes in dairy technology can be divided into two groups:

1. Technological, in which an enzyme is used to modify a milk constituent or to improve its microbiological, chemical or physical stability.
2. Enzymes as analytical reagents. Although the technological applications are quantitatively the more important, many of the analytical applications of enzymes are unique and are becoming increasingly important.

Since the principal constituents of milk are proteins, lipids and lactose, proteinases, lipases and β-galactosidase (lactase) are the principal exogenous enzymes used in dairy technology. Apart from these, there are, at present, only minor applications for glucose oxidase, catalase, superoxide dismutase and lysozyme. Lactoperoxidase, xanthine oxidase and sulphydryl oxidase might also be included, although at present the indigenous form of these enzymes is exploited.

The application of enzymes in food technology has been widely reviewed (Fox, 1991; Nagodawithana and Reed, 1993). Reviews on applications of exogenous enzymes in dairy technology include Fox and Grufferty (1991), Fox (1993) and Brown (1993).

8.3.2 Proteinases

There is one major (rennet) and several minor applications of proteinases in dairy technology.

Rennets. The use of rennets in cheesemaking is the principal application of proteinases in food processing and is second only to amylases among industrial applications of enzymes. The sources of rennets and their role in milk coagulation and cheese ripening are discussed in Chapter 10 and will not be considered here.

Accelerated cheese ripening. Cheese ripening is a slow, expensive and partially uncontrolled process; consequently, there is increasing interest, at both the research and industrial levels, in accelerating ripening. Various approaches have been investigated to accelerate ripening, including a higher ripening temperature (especially for Cheddar-type cheese which is usually ripened at 6–8°C), exogenous proteinases and peptidases, modified starters (e.g. heat-shocked or lactose-negative) and genetically engineered starters or

starter adjuncts (Fox *et al.*, 1996). The possible use of exogenous proteinases and peptidases attracted considerable attention for a period but uniform distribution of the enzymes in the cheese curd is a problem. Microencapsulation of enzymes offers a possible solution but is not commercially viable at present. Exogenous proteinases/peptidases are not used commercially in natural cheeses but are being used to produce 'enzyme modified cheese' for use in processed cheese, cheese dips and sauces. Selected genetically modified and adjunct cultures appear to be more promising.

Protein hydrolysates. Protein hydrolysates are used as flavourings in soups and gravies and in dietetic foods. They are generally prepared from soy, gluten, milk, meat or fish proteins by acid hydrolysis. Neutralization results in a high salt content which is acceptable for certain applications but may be unsuitable for dietetic foods and food supplements. Furthermore, acid hydrolysis causes total or partial destruction of some amino acids. Partial enzymatic hydrolysis is a viable alternative for some applications but bitterness due to hydrophobic peptides is frequently encountered. Bitterness may be eliminated or at least reduced to an acceptable level by treatment with activated carbon, carboxypeptidase, aminopeptidase, ultrafiltration, hydrophobic chromatography or by the plastein reaction. Caseins yield very bitter hydrolysates but the problem may be minimized by the judicious selection of the proteinase(s) (so as to avoid the production of very bitter peptides) and by using exopeptidases (especially aminopeptidases) together with the proteinase.

A novel, potentially very significant, application of proteinases in milk protein technology is the production of biologically active peptides (Chapter 4). Carefully selected proteinases of known specificity are required for such applications, but the resulting products have high added value.

Modification of protein functionality. The functional properties of milk proteins may be improved by limited proteolysis. Acid-soluble casein, free of off-flavour and suitable for incorporation into beverages and other acid foods (in which casein is insoluble) has been produced by limited proteolysis. The antigenicity of casein is destroyed by proteolysis and the hydrolysate is suitable for use in milk protein-based foods for infants allergic to cows' milk formulations. Controlled proteolysis improves the meltability of directly acidified cheese but excessive proteolysis causes bitterness. Partial proteolysis of lactalbumin (heat-coagulated whey proteins), which is insoluble and has very poor functional properties, yields a product that is almost completely soluble above pH 6; although the product is slightly bitter, it appears promising as a food ingredient. Limited proteolysis of whey protein concentrate reduces its emulsifying capacity, increases its specific foam volume but reduces foam stability and increases heat stability.

8.3.3 β-Galactosidase

β-Galactosidases (commonly referred to as lactase), which hydrolyse lactose to glucose and galactose, are probably the second most significant enzyme in dairy technology. Twenty years ago, β-galactosidase was considered to have very considerable potential but this has not materialized although there are a number of significant technological or nutritional applications. The various aspects of lactose and applications of β-galactosidase are considered in Chapter 2.

8.3.4 Lipases

The principal application of lipases in dairy technology is in cheese manufacture, particularly hard Italian varieties. The characteristic 'piccante' flavour of these cheeses is due primarily to short-chain fatty acids resulting from the action of lipase(s) in the rennet paste traditionally used in their manufacture. Rennet paste is prepared from the stomachs of calves, kids or lambs slaughtered after suckling; the stomachs and contents are held for about 60 days and then macerated. The product, which has proteolytic (rennet) and lipolytic activities, is considered to be unhygienic and its use is not permitted in some countries. The lipase in rennet paste, generally referred to as pregastric esterase (PGE), is secreted by a gland at the base of the tongue, which is stimulated by suckling; the secreted lipase is washed into the stomach with the ingested milk. The physiological significance of PGE, which is secreted by several species, is to assist in lipid digestion in the neonate which has limited pancreatic function. The considerable literature has been reviewed by Nelson, Jensen and Pitas (1977) and Fox and Stepaniak (1993). PGE shows a high specificity for short-chain fatty acids, especially butanoic acid, esterified on the *sn*-3 position of glycerol, although some interspecies differences in specificity have been reported.

Semi-purified preparations of PGE from calf, kid and lamb are commercially available and give satisfactory results; slight differences in specificity renders one or other more suitable for particular applications. Connoisseurs of Italian cheese claim that rennet paste gives superior results to semipurified PGE, and it is cheaper.

Rhizomucor miehei secretes a lipase that is reported to give satisfactory results in Italian cheese manufacture. This enzyme has been characterized and is commercially available as 'Piccantase'. Lipases secreted by selected strains of *Penicillium roqueforti* and *P. candidum* are considered to be potentially useful for the manufacture of Italian and other cheese varieties.

Extensive lipolysis also occurs in Blue cheese varieties in which the principal lipase is secreted by *P. roqueforti* (Chapter 10). It is claimed that treatment of Blue cheese curd with PGE improves and intensifies its flavour but this practice is not widespread. Several techniques have been developed

for the production of fast-ripened Blue cheese-type products suitable for use in salad dressings, cheese dips, etc. Lipases, usually of fungal origin, are used in the manufacture of these products or to pre-hydrolyse fats/oils used as ingredients in their production.

Although Cheddar cheese undergoes relatively little lipolysis during ripening, it is claimed that addition of PGE, gastric lipase or selected microbial lipases improves the flavour of Cheddar, especially that made from pasteurized milk, and accelerates ripening. It is also claimed that the flavour and texture of Feta and Egyptian Ras cheese can be improved by adding kid or lamb PGE or low levels of selected microbial lipases to the cheese milk, especially if milk concentrated by ultrafiltration is used.

Lipases are used to hydrolyse milk fat for a variety of uses in the confectionary, sweet, chocolate, sauce and snack food industries and there is interest in using immobilized lipases to modify fat flavours for such applications (Kilara, 1985). Enzymatic interesterification of milk lipids to modify rheological properties is also feasible.

8.3.5 Lysozyme

As discussed in section 8.2.5, lysozyme has been isolated from the milk of a number of species; human and equine milks are especially rich sources. In view of its antibacterial activity, the large difference in the lysozyme content of human and bovine milks may have significance in infant nutrition. It is claimed that supplementation of baby food formulae based on cows' milk with egg-white lysozyme gives beneficial results, especially with premature babies, but views on this are not unanimous.

Nitrate is added to many cheese varieties to prevent the growth of *Clostridium tyrobutyricum* which causes off-flavours and late gas blowing. However, the use of nitrate in foods is considered to be undesirable because of its involvement in nitrosamine formation, and many countries have reduced permitted levels or prohibited its use. Lysozyme, which inhibits the growth of vegetative cells of *Cl. tyrobutyricum* and hinders the germination of its spores, is an alternative to nitrate for the control of late gas blowing in cheese but is not widely used at present. Lysozyme also kills *Listeria* spp. Lysozyme addition permits the use of lower temperatures in food sterilization. Co-immobilized lysozyme has been proposed for self-sanitizing immobilized enzyme columns; although the technique may be uneconomical for large-scale operations, it was considered feasible for pilot-scale studies, especially on expensive enzymes.

8.3.6 Catalase

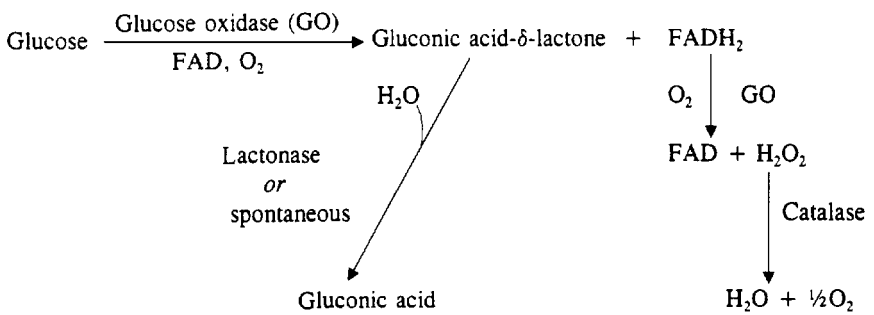
Hydrogen peroxide is a very effective chemical sterilant and although it causes some damage to the physicochemical properties and nutritional value

of milk protein, principally by oxidizing methionine, it is used as a milk preservative, especially in warm countries lacking refrigeration, and is permitted in the US for treatment of cheesemilk. Excess H_2O_2 may be reduced following treatment by soluble exogenous catalase (from beef liver, *Aspergillus niger* or *Micrococcus lysodeiktieus*). Immobilized catalase has been investigated for this purpose but the immobilized enzyme is rather unstable.

As discussed in section 8.3.7, catalase is frequently used together with glucose oxidase in many of the food applications of the latter; however, the principal potential application of glucose oxidase in dairy technology is for the *in situ* production of H_2O_2, for which the presence of catalase is obviously undesirable.

8.3.7 Glucose oxidase

Glucose oxidase (GO) catalyses the oxidation of glucose to gluconic acid (via gluconic acid-δ-lactone) according to the following reaction:



The H_2O_2 formed is normally reduced by catalase present as a contaminant in commercial GO preparations (from *P. notatum*, *P. glaucum* or *A. niger*) or added separately. Glucose oxidase, which has a pH optimum of about 5.5, is highly specific for D-glucose and may be used to assay specifically for D-glucose in the presence of other sugars.

In the food industry, glucose oxidase has four principal applications:

1. Removal of residual trace levels of glucose. This application, which is particularly useful for the treatment of egg white prior to dehydration (although alternative procedures using yeast fermentation are used more commonly), is of little, if any, significance in dairy technology.
2. Removal of trace levels of oxygen. Traces of oxygen in wines and fruit juices cause discolouration and/or oxidation of ascorbic acid. Chemical

reducing agents may be used to scavenge oxygen but enzymatic treatment with GO may be preferred. Glucose oxidase has been proposed as an antioxidant system for high-fat products such as mayonnaise, butter and whole-milk powder, but it does not appear to be widely used for this purpose, probably because of cost *vis-à-vis* chemical antioxidants (if permitted) and the relative effectiveness of inert gas flushing in preventing lipid oxidation in canned milk powder.

3. Generation of H_2O_2 *in situ*. The H_2O_2 generated by glucose oxidase has a direct bactericidal effect (which appears to be a useful side-effect of GO applied to egg products) but its bactericidal properties can be much more effectively exploited as a component of the lactoperoxidase – H_2O_2 – SCN^- system. Glucose required for GO activity may be added or produced by the action of β-galactosidase on lactose (both β-galactosidase and glucose oxidase have been immobilized on porous glass beads). H_2O_2 may also be generated *in situ* by the action of xanthine oxidase on added hypoxanthine. It is likely that exogenous H_2O_2 will be used in such applications rather than H_2O_2 generated by glucose oxidase or xanthine oxidase.
4. Production of acid *in situ*. Direct acidification of dairy products, particularly cottage and Mozzarella cheeses, is fairly common. Acidification is normally performed by addition of acid or acidogen (usually gluconic acid-δ-lactone) or by a combination of acid and acidogen. *In situ* production of gluconic acid from added glucose or from glucose produced *in situ* from lactose by β-galactosidase or from added sucrose by invertase has been proposed; immobilized glucose oxidase has been investigated. However, it is doubtful whether immobilized glucose oxidase could be applied to the acidification of milk because of the high probability of fouling by precipitated protein, even at low temperatures, which would lead to less casein precipitation. We are not aware if glucose oxidase in any form is used commercially for direct acidification of milk. Production of lactobionic acid from lactose by lactose dehydrogenase has also been proposed for the direct acidification of dairy and other foods.

8.3.8 Superoxide dismutase

Superoxide dismutase (SOD), an indigenous enzyme in milk, was discussed in section 8.2.10. A low level of exogenous SOD, coupled with catalase, was shown to be a very effective inhibitor of lipid oxidation in dairy products. It has been suggested that SOD may be particularly useful in preserving the flavour of long-life UHT milk which is prone to lipid oxidation. Obviously, the commercial feasibility of using SOD as an antioxidant depends on cost, particularly *vis-à-vis* chemical methods, if permitted.

8.3.9 Exogenous enzymes in food analysis

Exogenous enzymes have several applications in food analysis (Whitaker, 1991). One of the principal attractions of enzymes as analytical reagents is their specificity, which eliminates the need for extensive clean-up of the sample and makes it possible to quantify separately closely related molecules, e.g. D- and L-glucose, D-and L-lactic acid, which are difficult to quantify by chemical or physical methods. Enzymatic assays can be very sensitive; some can detect concentrations at the picomole level. Enzymes can be immobilized as enzyme electrodes and as such can be used continuously to monitor changes in the concentration of a substrate in a product stream. Disadvantages of enzymes as analytical reagents are their relatively high cost, especially when few samples are to be analysed, relatively poor stability (due to denaturation or inhibition) and the need to use highly purified enzymes.

Enzymes are rarely used by industrial food laboratories but find regular application in more specialized analytical or research laboratories. Important applications are summarized in Table 8.5 (see Boehringer Mannheim (1986) for methods). There are alternative chemical and/or physical methods, especially some form of chromatography, for all these applications, but extensive clean-up and perhaps concentration may be required.

The use of luciferase to quantify ATP (Blum and Coulet, 1994) in milk is the principle of modern rapid methods for assessing the bacteriological quality of milk based on the production of ATP by bacteria. Such methods have been automated and mechanized.

Table 8.5 Some examples of compounds in milk that can be analysed by enzymatic assays

Substrate	Enzyme
D-Glucose	Glucose oxidase; glucokinase; hexokinase
Galactose	Galactose dehydrogenase
Fructose	Fructose dehydrogenase
Lactose	β-Galactosidase, then analyse for glucose or galactose
Lactulose	β-Galactosidase, then analyse for fructose or galactose
D- and L-Lactic acid	D- and L-Lactate dehydrogenase
Citric acid	Citrate dehydrogenase
Acetic acid	Acetate kinase + pyruvate kinase + lactate dehydrogenase
Ethanol	Alcohol dehydrogenase
Glycerol	Glycerol kinase
Fatty acids	Acyl-CoA synthetase + Acyl-CoA oxidase
Amino acids	Decarboxylases; deaminases
Metal ions (inhibitors or activators)	Choline esterase; luciferase; invertase
ATP	Luciferase
Pesticides (inhibitors)	Hexokinase; choline esterase
Inorganic phosphate	Phosphorylase a
Nitrate	Nitrate reductase

Enzyme-linked immunosorbent assays. An indirect application of enzymes in analysis is as a marker or label in enzyme-linked immunosorbent assays (ELISA). In ELISA, the enzyme does not react with the analyte; instead, an antibody is raised against the analyte (antigen or hapten) and labelled with easily assayed enzyme, usually a phosphatase or a peroxidase. The enzyme activity is proportional to the amount of antibody in the system, which in turn is proportional, directly or indirectly depending on the arrangement used, to the amount of antigen present (Morris and Clifford, 1984).

Either of two approaches may be used: competitive and non-competitive, each of which may be used in either of two modes.

Competitive ELISA

On the basis of enzyme-labelled antigen

The antibody (Ab) is adsorbed to a fixed phase, e.g. the wells of a microtitre plate. An unknown amount of antigen (Ag, analyte) in the sample to be assayed, together with a constant amount of enzyme-labelled antigen (Ag–E), are then added to the well (Figure 8.2b). The Ag and Ag–E compete for the fixed amount of Ab and amount of Ag–E bound is inversely proportional to the amount of Ag present in sample. After washing away the excess of unbound antigen (and other materials), a chromogenic substrate is added and the intensity of the colour determined after incubation for a fixed period. The intensity of the colour is inversely proportional to the concentration of antigen in the sample (Figure 8.2b).

On the basis of enzyme-labelled antibody

In this mode, a fixed amount of unlabelled antigen (Ag) is bound to microtitre plates. A food sample containing antigen is added, followed by a fixed amount of enzyme-labelled antibody (Ab–E) (Figure 8.2a). There is competition between the fixed and free antigen for the limited amount of Ab–E. After an appropriate reaction time, unbound Ag (and other materials) are washed from the plate and the amount of bound enzyme activity assayed. As above, the amount of enzyme activity is inversely proportional to the concentration of antigen in the food sample.

Noncompetitive ELISA. The usual principle here is the sandwich technique, which requires the antigen to have at least two antibody binding sites (epitopes). Unlabelled antibody is first fixed to microtitre plates; a food sample containing antigen (analyte) is then added and allowed to react with the fixed unlabelled antibody (Figure 8.3). Unadsorbed material is washed out and enzyme-labelled antibody then added which reacts with a second site on the bound antigen. Unadsorbed Ab–E is washed off and enzyme activity assayed; activity is directly related to the concentration of antigen.

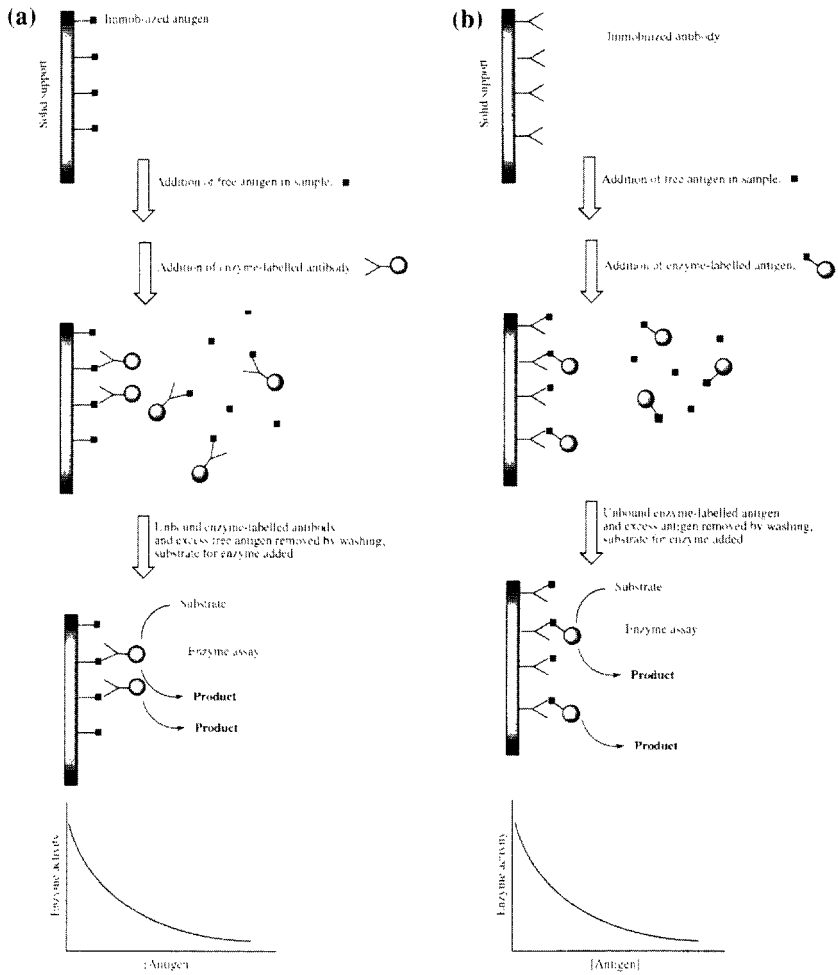


Figure 8.2. Schematic representation of a competitive enzyme-linked immunosorbent assay using (a) immobilized antigen or (b) immobilized antibody.

Examples of the use of ELISA in dairy analyses include:

- quantifying denaturation of β-lactoglobulin in milk products (native and denatured β-lg react differently with antibodies);
- detection and quantitation of adulteration of milk from one species with that from other species, e.g. sheep's milk by bovine milk;
- authentication of cheese, e.g. sheep's milk cheese;
- detection and quantitation of bacterial enzymes in milk, e.g. from psychrotrophs;

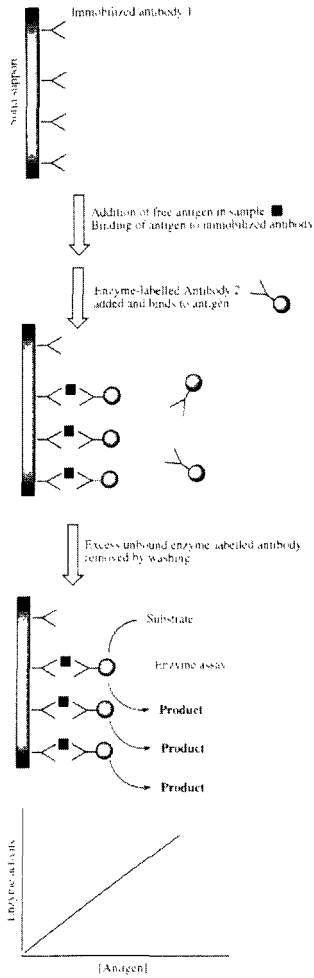


Figure 8.3. Schematic representation of a non-competitive enzyme-linked immunosorbent assay using the 'sandwich' technique.

- quantitation of antibiotics;
- potential application of ELISA includes monitoring proteolysis in the production of protein hydrolysates or in cheese.

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9 Heat-induced changes in milk

9.1 Introduction

In modern dairy technology, milk is almost always subjected to a heat treatment; typical examples are:

Thermization	e.g. 65°C × 15 s
Pasteurization	
LTLT (low temperature, long time)	63°C × 30 min
HTST (high temperature, short time)	72°C × 15 s
Forewarming (for sterilization)	e.g. 90°C × 2–10 min, 120°C × 2 min
Sterilization	
UHT (ultra-high temperature)	130–140°C × 3–5 s
In-container	110–115°C × 10–20 min

The objective of the heat treatment varies with the product being produced. Thermization is generally used to kill temperature-sensitive micro-organisms, e.g. psychrotrophs, and thereby reduce the microflora of milk for low-temperature storage. The primary objective of pasteurization is to kill pathogens but it also reduces the number of non-pathogenic micro-organisms which may cause spoilage, thereby standardizing the milk as a raw material for various products. Many indigenous enzymes, e.g. lipase, are also inactivated, thus contributing to milk stability. Forewarming (preheating) increases the heat stability of milk for subsequent sterilization (as discussed in section 9.7.1). Sterilization renders milk shelf-stable for very long periods, although gelation and flavour changes occur during storage, especially of UHT-sterilized milks.

Although milk is a very complex biological fluid containing complex protein, lipid, carbohydrate, salt, vitamins and enzyme systems in soluble, colloidal or emulsified states, it is a very heat-stable system, which allows it to be subjected to severe heat treatments with relatively minor changes in comparison to other foods if subjected to similar treatments. However, numerous biological, chemical and physico-chemical changes occur in milk during thermal processing which affect its nutritional, organoleptic and/or technological properties. The temperature dependence of these changes

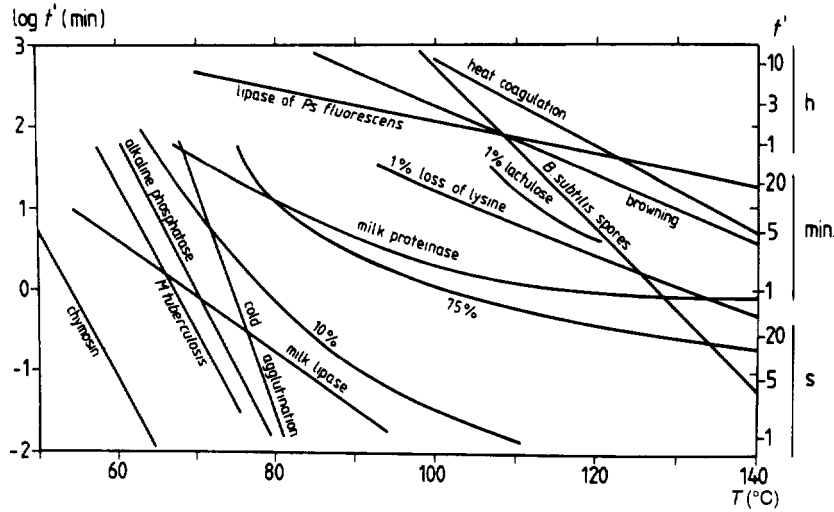


Figure 9.1 The time needed (t') at various temperatures (T) to inactivate some enzymes and cryoglobulins; to kill some bacteria and spores; to cause a certain degree of browning; to convert 1% of lactose to lactulose; to cause heat coagulation; to reduce available lysine by 1%; and to make 10% and 75% of the whey proteins insoluble at pH 4.6 (from Walstra and Jenness, 1984).

Table 9.1 Approximate values for the temperature dependence of some reactions in heated milk (modified from Walstra and Jennes, 1984)

Reaction	Activation energy (kJ mol^{-1})	Q_{10} at 100°C
Many chemical reactions	80–130	2.0–3.0
Many enzyme-catalysed reactions	40–60	1.4–1.7
Autoxidation of lipids	40–100	1.4–2.5
Maillard reactions (browning)	100–180	2.4–5.0
Dephosphorylation of caseinate	110–120	2.6–2.8
Heat coagulation of milk	150	3.7
Degradation of ascorbic acid	60–120	1.7–2.8
Heat denaturation of protein	200–600	6.0–175.0
Typical enzyme inactivation	450	50.0
Inactivation of milk proteinase (plasmin)	75	1.9
Killing vegetative bacteria	200–600	6.0–175.0
Killing of spores	250–330	9.0–17.0

varies widely, as depicted in general terms in Figure 9.1 and Table 9.1. The most significant of these changes, with the exception of the killing of bacteria, will be discussed below. In general, the effect(s) of heat on the principal constituents of milk will be considered individually, although there are interactions between constituents in many cases.

9.2 Lipids

Of the principal constituents, the lipids are probably the least affected by heat. However, significant changes do occur in milk lipids, especially in their physical properties, during heating.

9.2.1 Physicochemical changes

Creaming. The chemical and physicochemical aspects of the lipids in milk were discussed in Chapter 3. The principal effect of heat treatments on milk lipids is on creaming of the fat globules. As discussed in Chapter 3, the fat in milk exists as globules, 0.1–20 μm in diameter (mean, 3–4 μm). The globules are stabilized by a complex membrane acquired within the secretory cell and during excretion from the cell. Owing to differences in density between the fat and aqueous phases, the globules float to the surface to form a cream layer. In cows' milk, the rate of creaming is far in excess of that predicted by Stokes' law, owing to aggregation of the globules which is promoted by cryoglobulins (a group of immunoglobulins). Buffalo, ovine or caprine milks do not undergo cryoglobulin-dependent agglutination of fat globules and cream very slowly with the formation of a compact cream layer.

When milk is heated to a moderate temperature (e.g. 70°C × 15 min), the cryoglobulins are irreversibly denatured and hence the creaming of milk is impaired or prevented; HTST pasteurization (72°C × 15 s) has little or no effect on creaming potential but slightly more severe conditions have an adverse effect (Figure 9.2).

Homogenization, which reduces mean globule diameter to below 1 μm, retards creaming due to the reduction in globule size but, more importantly, to the denaturation of cryoglobulins which prevents agglutination. In fact, there are probably two classes of cryoglobulin, one of which is denatured by heating, the other by homogenization.

Changes in the fat globule membrane. The milk fat globule membrane (MFGM) itself is altered during thermal processing. Milk is usually agitated during heating, perhaps with foam formation. Agitation, especially of warm milk in which the fat is liquid, may cause changes in globule size due to disruption or coalescence; significant disruption occurs during direct UHT processing. Foaming probably causes desorption of some membrane material and its replacement by adsorption of skim-milk proteins. In these cases, it may not be possible to differentiate the effect of heating from the total effect of the process.

Heating *per se* to above 70°C denatures membrane proteins, with the exposure and activation of various amino acid residues, especially cysteine.

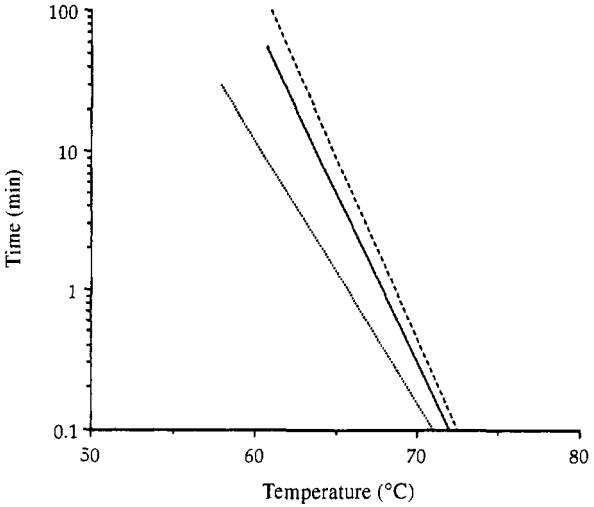


Figure 9.2 Time-temperature curves for the destruction of *M. tuberculosis* (...), inactivation of alkaline phosphatase (_) and creaming ability of milk (– – –) (from Webb and Johnson, 1965).

This may cause the release of H_2S (which can result in the development of an off-flavour) and disulphide interchange reactions with whey proteins, leading to the formation of a layer of denatured whey proteins on the fat globules at high temperatures (>100°C). The membrane and/or whey proteins may participate in Maillard browning with lactose and the cysteine may undergo β-elimination to dehydroalanine, which may then react with lysine to form lysinoalanine or with cysteine residues to form lanthionine, leading to covalent cross-linking of protein molecules (section 9.6.3). Membrane constituents, both proteins and phospholipids, are lost from the membrane to the aqueous phase at high temperatures. Much of the indigenous copper in milk is associated with the MFGM and some of it is transferred to the serum on heat processing. Thus, severe heat treatment of cream improves the oxidative stability of butter made from it as a result of the reduced concentration of pro-oxidant Cu in the fat phase and the antioxidant effect of exposed sulphydryl groups.

The consequences of these changes in the MFGM have been the subject of little study, possibly because severely heated milk products are usually homogenized and an artificial membrane, consisting mainly of casein and some whey proteins, is formed; consequently, changes in the natural membrane are not important. Damage to the membrane of unhomogenized products leads to the formation of free (non-globular) fat and consequently to 'oiling-off' and the formation of a 'cream plug' (Chapter 3).

Severe heat treatment, as is encountered during roller drying and to a lesser extent spray drying, results in at least some demulsification of milk fat, with the formation of free fat, which causes (Chapter 3):

- the appearance of fat droplets when such products are used in tea or coffee;
- increased susceptibility of the fat to oxidation, since it is not protected by a membrane;
- reduced wettability/dispersibility of the powder;
- a tendency of powders to clump.

9.2.2 *Chemical changes*

Severe heat treatments, e.g. frying, may convert hydroxyacids to lactones, which have strong, desirable flavours and contribute to the desirable attributes of milk fat in cooking.

Release of fatty acids and some interesterification may also occur, but such changes are unlikely during the normal processing of milk.

Naturally occurring polyunsaturated fatty acids are methylene-interrupted but may be converted to conjugated isomers at high temperatures. Four

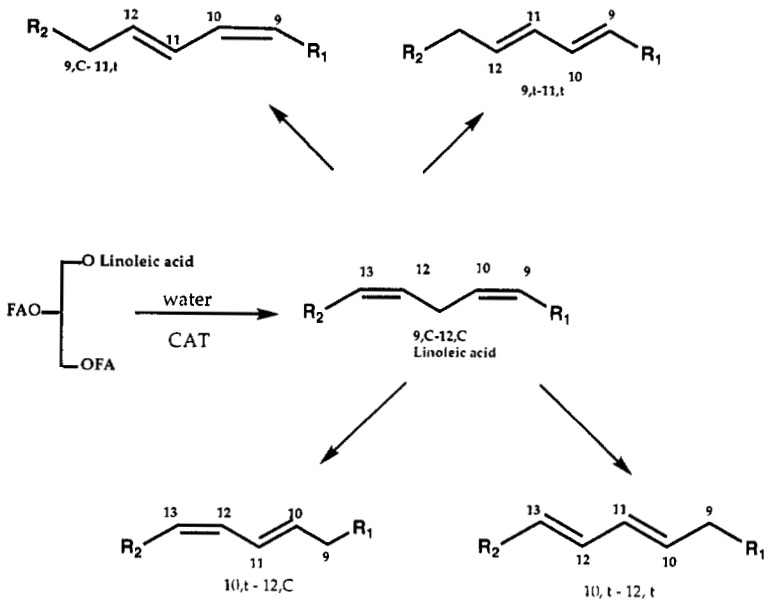


Figure 9.3 Isomers of conjugated linoleic acid.

Table 9.2 Concentration of conjugated linoleic acid (CLA) isomers in selected foods (modified from Ha, Grimm and Pariza, 1989)

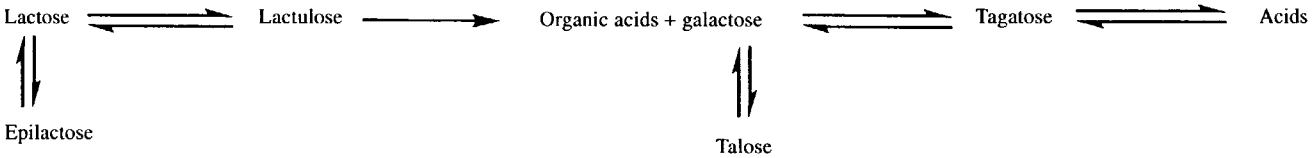
Sample	mg CLA/kg food	Fat content (%)	CLA in fat (mg kg^{-1})
Parmesan cheese	622.3 ± 15.0	32.3 ± 0.9	1926.7
Cheddar cheese	440.6 ± 14.5	32.5 ± 1.7	1355.7
Romano cheese	356.9 ± 6.3	32.1 ± 0.8	1111.9
Blue cheese	169.3 ± 8.9	30.8 ± 1.5	549.8
Processed cheese	574.1 ± 24.8	31.8 ± 1.1	1805.3
Cream cheese	334.5 ± 13.3	35.5 ± 1.0	942.3
Blue spread	202.6 ± 6.1	20.2 ± 0.8	1003.0
Cheese whiz	1815.0 ± 90.3	20.6 ± 1.1	8810.7
Milk			
pasteurized whole	28.3 ± 1.9	4.0 ± 0.3	707.5
non-pasteurized whole	34.0 ± 1.0	4.1 ± 0.1	829.3
Ground beef			
grilled	994.0 ± 30.9	10.7 ± 0.3	9289.7
uncooked	561.7 ± 22.0	27.4 ± 0.2	2050.0

isomers of conjugated linoleic acid (CLA) are shown in Figure 9.3. It is claimed that CLA has anticarcinogenic properties. The mechanism of CLA formation in foods in general is not clear but heat treatment, free radical-type oxidation and microbial enzymatic reactions involving linoleic and linolenic acids in the rumen are thought to be major contributors. Rather high concentrations of CLA have been found in heated dairy products, especially processed cheese (Table 9.2). It has been suggested that whey proteins catalyse isomerization.

9.3 Lactose

The chemistry and physicochemical properties of lactose, a reducing disaccharide containing galactose and glucose linked by a β(1-4)-bond, were described in Chapter 2.

When severely heated in the solid or molten state, lactose, like other sugars, undergoes numerous changes, including mutarotation, various isomerizations and the formation of numerous volatile compounds, including acids, furfural, hydroxymethylfurfural, CO_2 and CO. In solution under strongly acidic conditions, lactose is degraded on heating to monosaccharides and other products, including acids. These changes do not normally occur during the thermal processing of milk. However, lactose is relatively unstable under mild alkaline conditions at moderate temperatures where it undergoes the Lobry de Bruyn–Alberda van Ekenstein rearrangement of aldoses to ketoses (Figure 9.4).



[Epilactose = 4-O-β-D-galactopyranosyl-D-mannopyranose
Lactulose = 4-O-β-D-galactopysanosyl-D-fructofuranose]

Figure 9.4 Heat-induced changes in lactose under mild alkaline conditions.

Lactose undergoes at least three heat-induced changes during the processing and storage of milk and milk products.

9.3.1 Formation of lactulose

On heating at low temperatures under slightly alkaline conditions, the glucose moiety of lactose is epimerized to fructose with the formation of lactulose, which does not occur in nature. The significance of lactulose has been discussed in Chapter 2. Lactulose is not formed during HTST processing but is formed during UHT sterilization (more during indirect than direct heating) and especially during in-container sterilization; therefore, the concentration of lactulose in milk is a useful index of the severity of the heat treatment to which the milk has been subjected (see Figure 2.19). The concentration of lactulose is probably the best index available at present for differentiating between UHT and in-container sterilized milks and a number of assay procedures have been developed, using HPLC or enzymatic/spectrophotometric principles.

9.3.2 Formation of acids

Milk as secreted by the cow contains about 200 mg CO_2 l^{-1}. Owing to its low concentration in air, CO_2 is rapidly and, in effect, irreversibly lost from milk on standing after milking; its loss is accelerated by heating, agitation

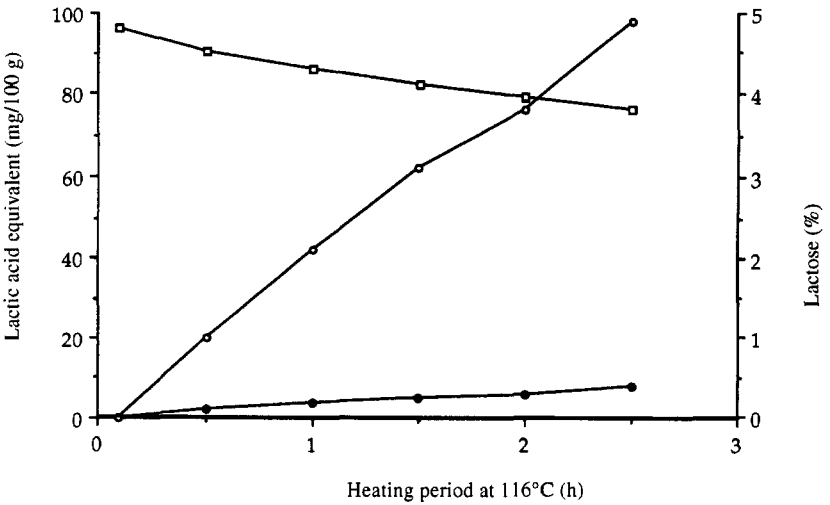


Figure 9.5 Changes in titratable acidity (○), lactic acid (●) and lactose (□) on heating homogenized milk in sealed cans at 116°C. Titratable acidity expressed as mg lactic acid/100 g milk (from Gould, 1945.)

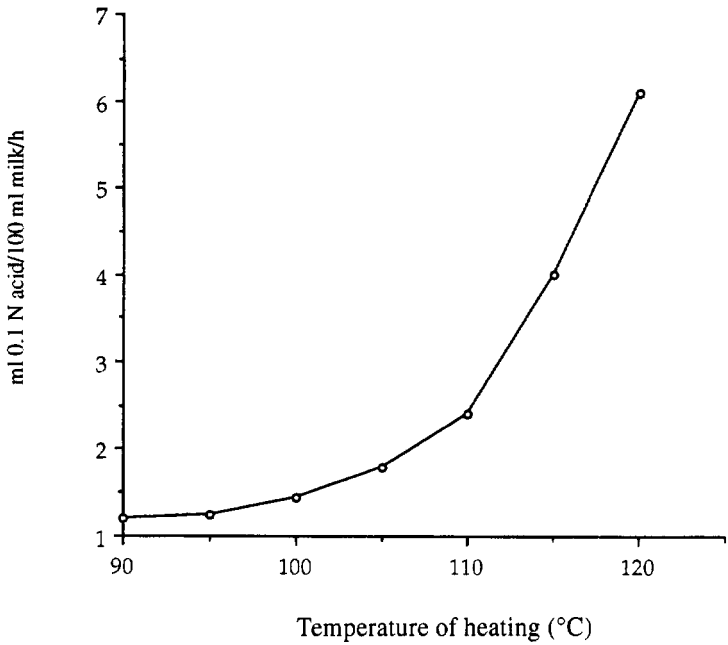


Figure 9.6 Effect of temperature on the rate of heat-induced production of acid in milk (from Jenness and Patton, 1959).

and vacuum treatment. This loss of CO_2 causes an increase in pH of about 0.1 unit and a decrease in the titratable acidity of nearly 0.02%, expressed as lactic acid. Under relatively mild heating conditions, this change in pH is more or less offset by the release of H^+ on precipitation of $Ca_3(PO_4)_2$, as discussed in section 9.4.

On heating at temperatures above 100°C, lactose is degraded to acids with a concomitant increase in titratable acidity (Figures 9.5, 9.6). Formic acid is the principal acid formed; lactic acid represents only about 5% of the acids formed. Acid production is significant in the heat stability of milk, e.g. when assayed at 130°C, the pH falls to about 5.8 at the point of coagulation (after about 20 min) (Figure 9.7). About half of this decrease is due to the formation of organic acids from lactose; the remainder is due to the precipitation of calcium phosphate and dephosphorylation of casein, as discussed in section 9.4.

In-container sterilization of milk at 115°C causes the pH to decrease to about 6 but much of this is due to the precipitation of calcium phosphate; the contribution of acids derived from lactose has not been quantified accurately. Other commercial heat treatments, including UHT sterilization, cause insignificant degradation of lactose to acids.

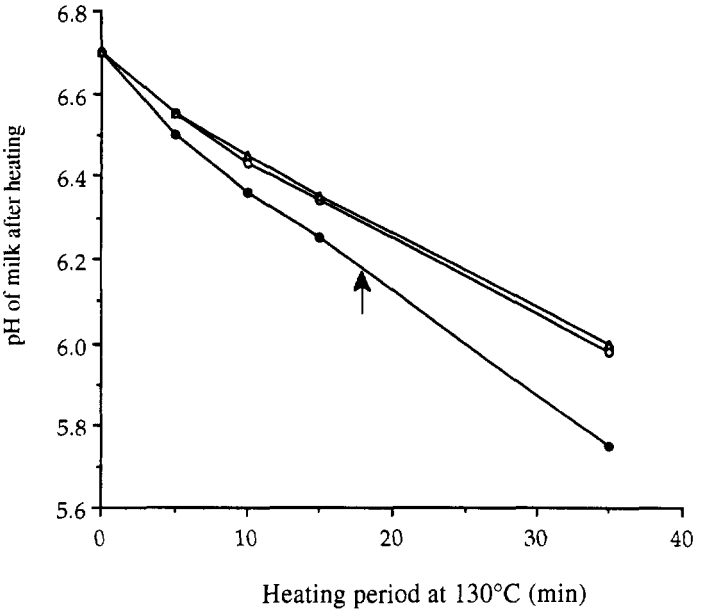


Figure 9.7 The pH of samples of milk after heating for various periods at 130°C with air (○), O_2 (●) or N_2 (△) in the headspace above the milk; ↑, coagulation time (from Sweetsur and White, 1975).

9.3.3 Maillard browning

The mechanism and consequences of the Maillard reaction were discussed in Chapter 2. The reaction is most significant in severely heat-treated products, especially in-container sterilized milks. However, it may also occur to a significant extent in milk powders stored under conditions of high humidity and high temperature, resulting in a decrease in the solubility of the powder. If cheese contains a high level of residual lactose or galactose (due to the use of a starter unable to utilize galactose; Chapter 10), it is susceptible to Maillard browning, especially during cooking on pizza, e.g. Mozzarella (Pizza) cheese. Browning may also occur in grated cheese during storage if the cheese contains residual sugars; in this case, the water activity of the cheese ($a_w \sim 0.6$) is favourable for the Maillard reaction. Poorly washed casein and especially whey protein concentrates (which contain 30–60% lactose) may undergo Maillard browning when used as ingredients in heat-treated foods.

Maillard browning in milk products is undesirable because:

1. The final polymerization products (melanoidins) are brown and hence dairy products which have undergone Maillard browning are discoloured and aesthetically unacceptable.

2. Some of the by-products of Maillard browning have strong flavours (e.g. furfural, hydroxymethylfurfural) which alter the typical flavour of milk.
3. The initial Schiff base is digestible but after the Amadori rearrangement, the products are not metabolically available. Since lysine is the amino acid most likely to be involved and is an essential amino acid, Maillard browning reduces the biological value of proteins. Interaction of lysine with lactose renders the adjacent peptide bond resistant to hydrolysis by trypsin, thereby reducing the digestibility of the protein.
4. The polymerized products of Maillard browning can bind metals, especially Fe.
5. It has been suggested that some products of the Maillard reaction are toxic and/or mutagenic but such effects are, at most, weak and possibly due to other consequences of browning, e.g. metal binding.
6. The attachment of sugars to the protein increases its hydrophilicity; however, solubility may be reduced, probably due to cross-linking of protein molecules.
7. The heat stability of milk is increased by the Maillard reaction, probably via the production of carbonyls (section 9.7).

The formation of brown pigments via the Maillard reaction, especially in model systems (e.g. glucose–glycine), usually follows zero-order kinetics, but the loss of reactants has been found to follow first- or second-order kinetics in foods and model systems. Activation energies of 109, 116 and 139 kJ mol^{-1} have been reported for the degradation of lysine, the formation of brown pigments and the production of hydroxymethylfurfural (HMF), respectively.

Browning can be monitored by measuring the intensity of brown colour, the formation of hydroxymethylfurfural (which may be measured spectrophotometrically, after reaction with thiobarbituric acid, or by HPLC, but which is not regarded as a very good indicator of Maillard browning), loss of available lysine (e.g. by reaction with 2,4-dinitrofluorobenzene) or by the formation of furosine. Furosine is formed on the acid hydrolysis of lactulosyl lysine (the principal Maillard product formed during the heating of milk). During acid hydrolysis, lactulosyl lysine is degraded to fructosylysine which is then converted to pyridosine, furosine and carboxymethyl lysine (Figure 9.8). Furosine may be determined by ion-exchange chromatography, GC or HPLC, and is considered to be a very good indicator of Maillard browning and the severity of heat treatment of milk (Erbersdobler and Dehn-Müller, 1989). The effects of time and temperature on the formation of furosine are shown in Figure 9.9. The concentration of furosine is highly correlated with the concentrations of HMF and carboxymethyl lysine. The concentration of furosine in commercial UHT milks is shown in Figure 9.10.

Dicarbonyls, which are among the products of the Maillard reaction, can react with amines in the Strecker reaction, producing a variety of flavourful

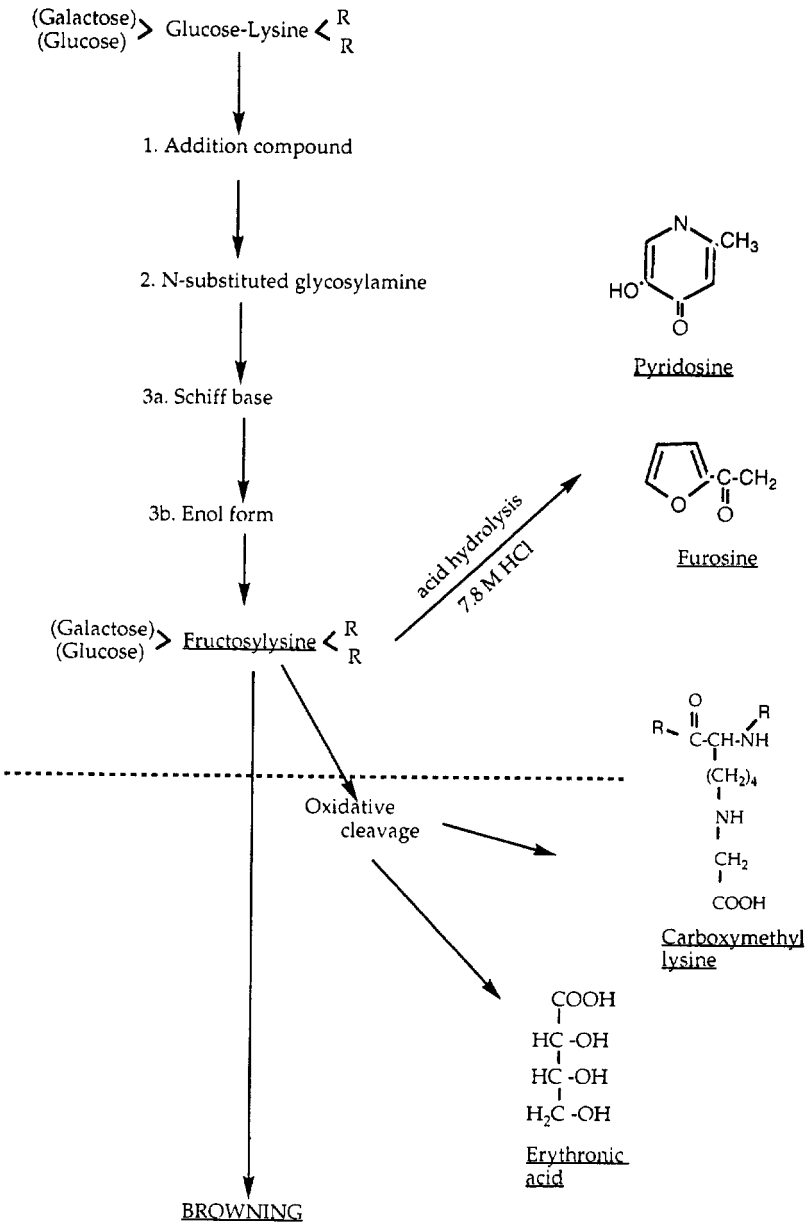


Figure 9.8 Initial steps of the Maillard reaction with the formation of furosine (after hydrolysis with 7.8 M HCl) as well as of *N*-ε-carboxymethyl lysine and erythronic acid (from Erbersdobler and Dehn-Müller, 1989).

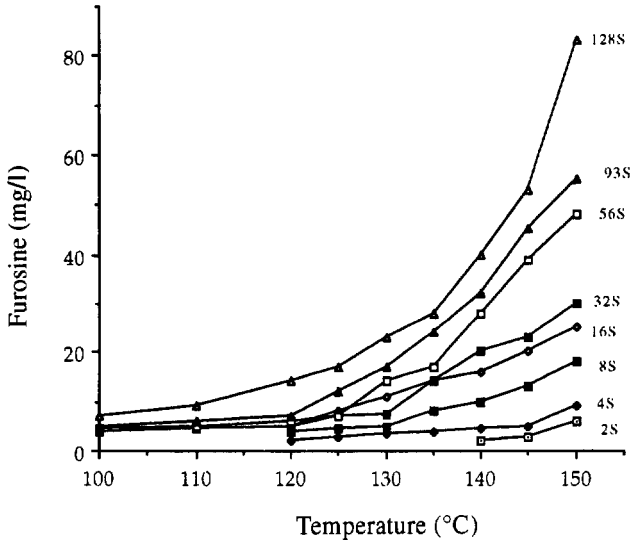


Figure 9.9 Effect of heating temperature and time on the concentration of furosine in directly heated UHT milks (from Erbersdobler and Dehn-Müller, 1989).

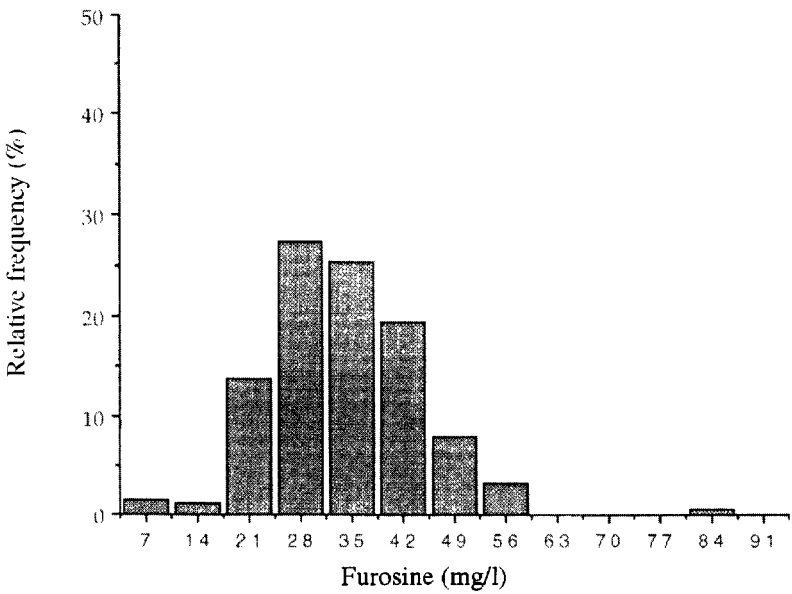


Figure 9.10 Relative distribution of the furosine concentrations in 190 commercial UHT milks in increments of 7 mg furosine (from Erbersdobler and Dehn-Müller, 1989).

compounds (Figure 2.32). The Maillard and especially the Strecker reactions can occur in cheese and may be significant contributors to flavour; in this case, the dicarbonyls are probably produced via biological, rather than thermal, reactions.

9.4 Milk salts

Although the organic and inorganic salts of milk are relatively minor constituents in quantitative terms, they have major effects on many aspects of milk, as discussed in Chapter 5. Heating has little effect on milk salts with two exceptions, carbonates and calcium phosphates. Most of the potential carbonate occurs as CO_2 which is lost on heating, with a consequent increase in pH. Among the salts of milk, calcium phosphate is unique in that its solubility decreases with increasing temperature. On heating, soluble calcium phosphate precipitates on to the casein micelles, with a concomitant decrease in the concentration of calcium ions and pH (Chapter 5). These changes are reversible on cooling if the heat treatment was not severe. Following severe heat treatment, the heat-precipitated calcium phosphate is probably insoluble but some indigenous colloidal calcium phosphate dissolves on cooling to partly restore the pH. The situation becomes rather complex in severely heated milk due to the decrease in pH caused by thermal degradation of lactose and dephosphorylation of casein.

The cooling and freezing of milk also cause shifts in the salts equilibria in milk, including changes in pH, as discussed in Chapters 2, 5 and 11.

9.5 Vitamins

Many of the vitamins in milk are relatively heat labile, as discussed in Chapter 6.

9.6 Proteins

The proteins of milk are probably the constituents most affected by heating. Some of the changes involve interaction with salts or sugars and, although not always fully independent of changes in other constituents, the principal heat-induced changes in proteins are discussed in this section.

9.6.1 Enzymes

As discussed in Chapter 8, milk contains about 60 indigenous enzymes derived from the secretory cells or from blood. Stored milk may also contain enzymes produced by micro-organisms. Both indigenous and bacterial

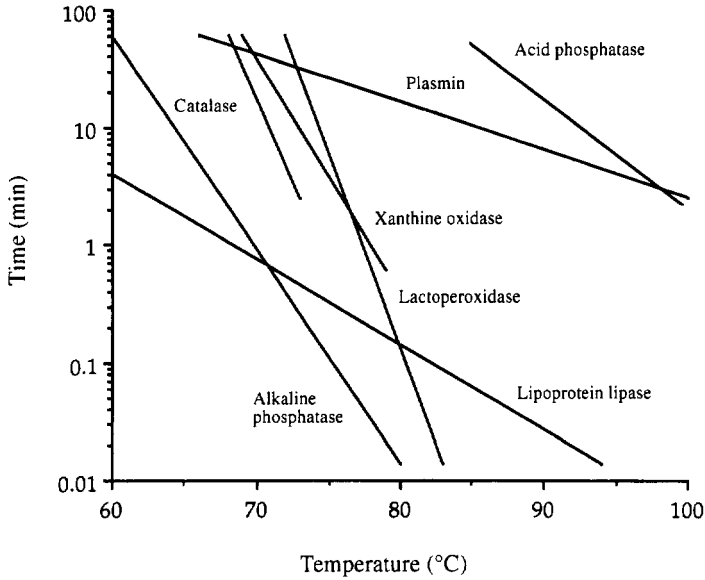


Figure 9.11 Time-temperature combinations required for which milk must be heated to a certain temperature to inactivate some indigenous milk enzymes (from Walstra and Jenness, 1984).

enzymes can have undesirable effects in milk and dairy products. Although not the primary objective of thermal processing, some of the indigenous enzymes in milk are inactivated by the commercially used heat processes, although many are relatively heat stable (Figure 9.11).

The thermal denaturation of indigenous milk enzymes is important from two major viewpoints:

1. To increase the stability of milk products. Lipoprotein lipase is probably the most important in this regard as its activity leads to hydrolytic rancidity. It is extensively inactivated by HTST pasteurization but heating at 78°C × 10 s is required to prevent lipolysis. Plasmin activity is actually increased by HTST pasteurization due to inactivation of inhibitors of plasmin and/or of plasminogen activators.
2. The activity of selected enzymes is used as indices of thermal treatments, e.g. alkaline phosphatase (HTST pasteurization), γ-glutamyl transpeptidase (index of heating in the range 72–80°C) or lactoperoxidase (80–90°C).

Microbial enzymes. The widespread use of refrigerated storage of milk at farm and factory for extended periods has led to psychrotrophs, especially

Pseudomonas fluorescens, becoming the dominant micro-organisms in raw milk supplies. Psychrotrophs are quite heat labile and are readily killed by HTST pasteurization and even by thermization. However, they secrete extracellular proteinases, lipases and phospholipases that are extremely heat stable – some are not completely inactivated by heating at 140°C for 1 min and thus partially survive UHT processing. If the raw milk supply contains high numbers of psychrotrophs ($>10^6$ per ml), the amounts of proteinase and lipase that survive UHT processing may be sufficient to cause off-flavours, such as bitterness, unclean and rancid flavours, and perhaps gelation.

One of the very curious characteristics of the proteinases and lipases secreted by many psychrotrophs is that they have relatively low stability in the temperature range 50–65°C, Figure 9.12 (the precise value depends on the enzyme). Thus, it is possible to reduce the activity of these enzymes in milk by a low temperature inactivation (LTI) treatment (e.g. 60°C × 5–10 min) before or after UHT processing. Inactivation of the proteinase by LTI appears to be due mainly to proteolysis; in the native state, the enzyme is tightly folded and resistant to proteolysis by other proteinase molecules in its neighbourhood but at about 60°C, some molecules undergo conformational changes, rendering them susceptible to proteolysis by proteinase molecules which are still active. On increasing the temperature further, all proteinase molecules are denatured and inactive but they can renature on

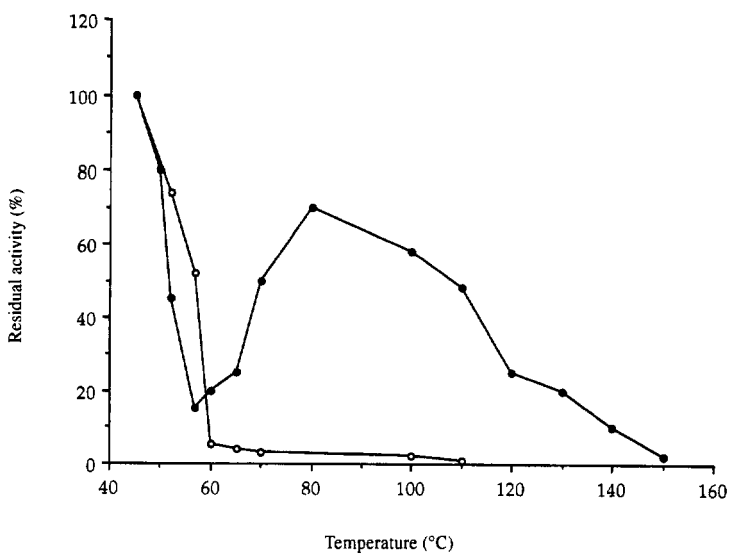


Figure 9.12 Thermal inactivation of *Ps. fluorescens* AFT 36 proteinase on heating for 1 min in 0.1 M phosphate buffer, pH 6.6 (○) or in a synthetic milk salts buffer, pH 6 (●) (from Stepaniak, Fox and Daly, 1982).

cooling. Since this mechanism does not apply to purified lipase, the mechanism of LTI of lipase is not clear (for reviews on enzymes from psychrotrophs see Driessen (1989) and McKellar (1989)).

9.6.2 Denaturation of other biologically active proteins

Milk contains a range of biologically active proteins, e.g. vitamin-binding proteins, immunoglobulins, metal-binding proteins, antibacterial proteins (lactotransferrin, lysozyme, lactoperoxidase), various growth factors and hormones (Chapters 4 and 8). These proteins play important nutritional and physiological functions in the neonate. All these proteins are relatively heat labile – some are inactivated by HTST pasteurization and probably all are inactivated by UHT and more severe heat treatments. Inactivation of these biologically active proteins may not be particularly important when milk is used in the diet of adults but may be highly significant in infant formulae; consequently, supplementation of infant formulae with some of these proteins is advocated.

9.6.3 Denaturation of whey proteins

The whey proteins, which represent about 20% of the proteins of bovine milk, are typical globular proteins with high levels of secondary and tertiary structures, and are, therefore, susceptible to denaturation by various agents, including heat. The denaturation kinetics of whey proteins, as measured by loss of solubility in saturated NaCl at pH 4.6, are summarized in Figure

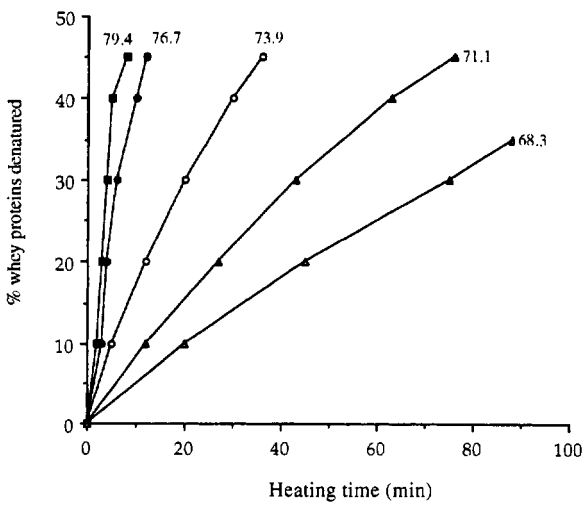


Figure 9.13 Heat denaturation of whey proteins on heating skim milk at various temperatures (°C) as measured by precipitability with saturated NaCl (from Jenness and Patton, 1959).

9.13. Thermal denaturation is a traditional method for the recovery of proteins from whey as 'lactalbumin'; coagulation is optimal at pH 6 and about 90°C for 10 min (Chapter 4).

The order of heat stability of the whey proteins, measured by loss of solubility, is: α-lactalbumin (α-la) > β-lactoglobulin (β-lg) > blood serum albumin (BSA) > immunoglobulins (Ig) (Figure 9.14). However, when measured by differential scanning calorimetry, quite a different order is observed: Ig > β-lg > α-la > BSA. In the case of α-la, the discrepancy appears to be explained by the fact that it is a metallo (Ca)-protein which renatures quite readily following thermal denaturation. However, the Ca-free apoprotein is quite heat labile, a fact which is exploited in the isolation of α-la. The Ca^{2+} is bound in a pocket to the carboxylic acid groups of three Asp residues and the carbonyls of an Asp and a Lys residue (Chapter 4). The carboxylic acid groups become protonated below about pH 5 and lose their ability to bind Ca; the apoprotein can be aggregated by heating to about 55°C, leaving mainly β-lg in solution. Apo-lactoferrin is also considerably less stable than the intact protein.

The denaturation of α-la and β-lg in milk follows first- and second-order kinetics, respectively (Figure 9.15). Both proteins show a change in the temperature-dependence of denaturation at about 90°C (Figure 9.15).

The mechanism of the thermal denaturation of β-lg has been studied extensively; the sequence of events is shown schematically in Figure 9.16. At about 20°C in the pH range 5.5–7.0, β-lg exists as an equilibrium between

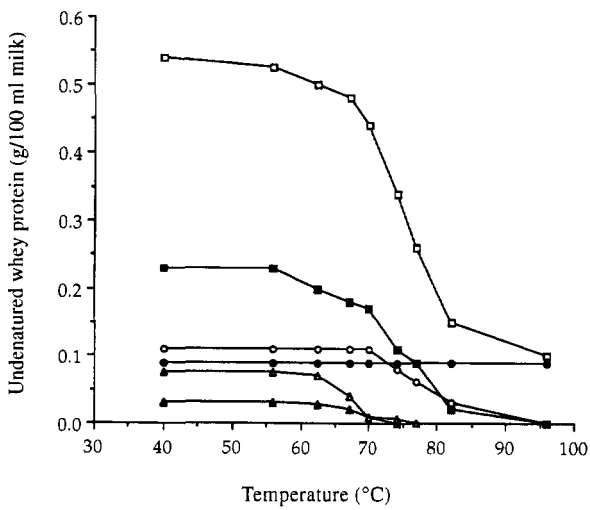


Figure 9.14 The denaturation of the total (□) and individual whey proteins in milk, heated at various temperatures for 30 min; β-lactoglobulin (■), α-lactalbumin (○), proteose peptone (●), immunoglobulins (△), and serum albumin (▲) (from Webb and Johnson, 1965).

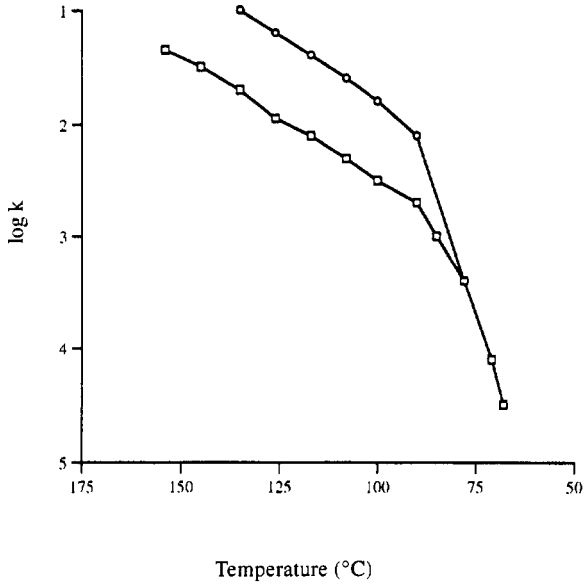


Figure 9.15 Arrhenius plot of the rate constant for the heat treatment of α-lactalbumin (□) and β-lactoglobulin (○) (from Lyster, 1970).

its dimeric (N_2) and monomeric (2N) forms. Between pH 7 and 9, it undergoes a reversible conformational change, referred to as the $N \rightleftarrows R$ transition. Both equilibria are pushed to the right as the temperature is increased, i.e. $N_2 \rightarrow 2N \rightarrow 2R$. Above about 65°C, β-lg undergoes reversible denaturation ($R \rightleftarrows D$) but at about 70°C, denaturation becomes irreversible via a series of aggregation steps. The initial type I aggregation involves the formation of intermolecular disulphide bonds while the later type II aggregation involves non-specific interactions, including hydrophobic and electrostatic bonding. Type III aggregation involves non-specific interactions and occurs when the sulphydryl groups are blocked.

Some of the most important consequences of the heat denaturation of whey proteins are due to the fact that these proteins contain sulphydryl and/or disulphide residues which are exposed on heating (Figure 9.17). They are important for at least the following reasons:

1. The proteins can participate in sulphydryl–disulphide interchange reactions at temperatures above about 75°C at the pH of milk, but more rapidly at or above pH 7.5. Such interactions lead to the formation of disulphide-linked complexes of β-lg with κ-casein, and probably α_{s2}-casein and α-la, with profound effects on the functionality of the milk protein system, such as rennet coagulation and heat stability.

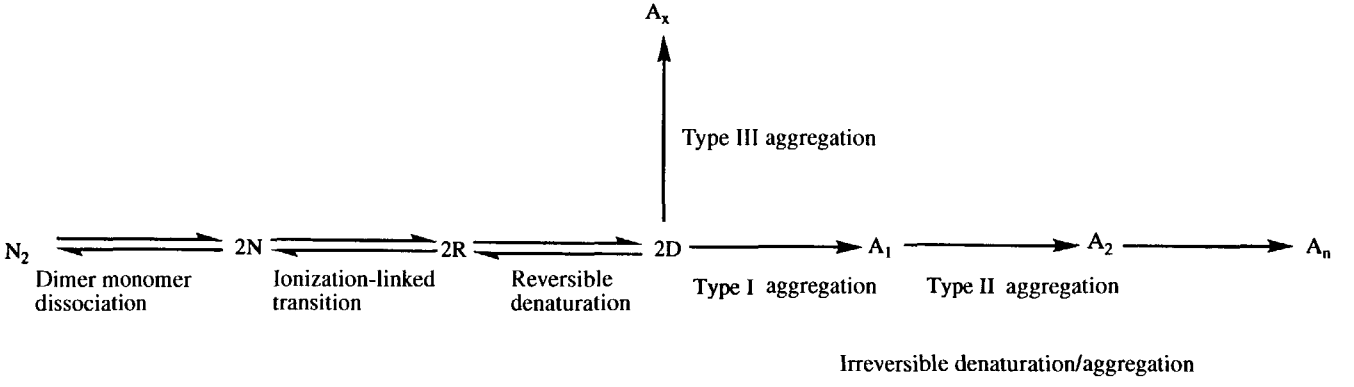


Figure 9.16 Stages in the thermal denaturation of β-lactoglobulin (from Mulvihill and Donovan, 1987).

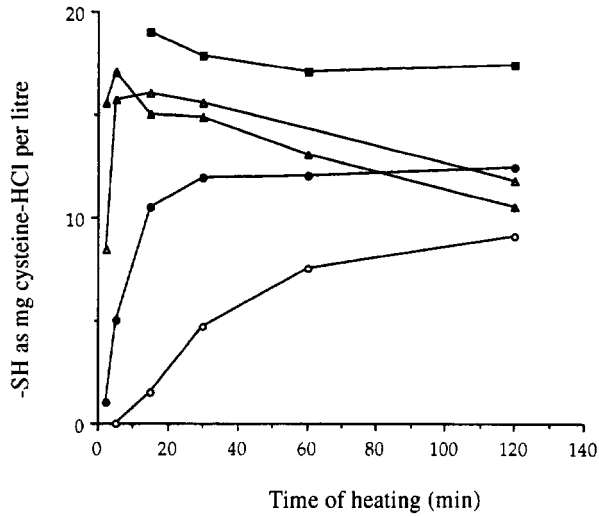


Figure 9.17 Exposure of sulphydryl groups by heating milk at 75 (○), 80 (●), 85 (△) or 95 (▲) °C; de-aerated milk heated at 85°C (■) (from Jenness and Patton, 1959).

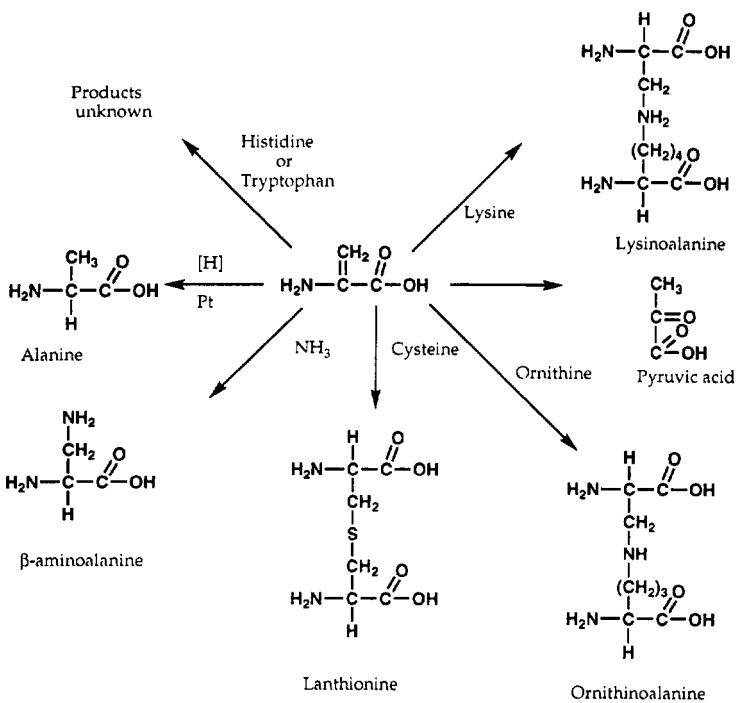


Figure 9.18 Interaction of dehydroalanine with amino acids.

2. The activated sulphydryls may decompose with the formation of H_2S and $H_3C\text{-}S\text{-}CH_3$, which are responsible for the cooked flavour of severely heated milk, including UHT milk. These compounds are volatile and unstable and disappear within about 1 week after processing so that the flavour of UHT milk improves during the first few weeks after processing.
3. Serine, serine phosphate, glycosylated serine, cysteine and cystine residues can undergo β-elimination with the formation of dehydroalanine. Dehydroalanine is very reactive and can react with various amino acid residues, especially lysine, leading to the formation of lysinoalanine, and to a lesser extent with cysteine with the formation of lanthionine (Figure 9.18). These reactions lead to intra- or intermolecular cross-linking which reduce protein solubility, digestibility and nutritive value (because the bonds formed are not hydrolysed in the intestinal tract and lysine is an essential amino acid). Although there are reports to the contrary, lysinoalanine is not normally found in UHT milk or cream.

9.6.4 *Effect of heat on caseins*

As discussed in Chapter 4, the caseins are rather unique proteins. They are rather small (20–25 kDa), relatively hydrophobic molecules, with little higher structure, few disulphide bonds (present only in the two minor caseins, α_{s2} and κ) and no sulphydryl groups. All the caseins are phosphorylated (8–9, 10–13, 4–5 and 1 mole P per mole protein for α_{s1}-, α_{s2}-, β- and κ-casein, respectively); due to their high levels of phosphorylation, α_{s1}-, α_{s2}- and β-caseins bind calcium strongly, causing them to aggregate and precipitate, and affecting their general stability, including heat stability.

Within the strict sense of the term, the caseins are not susceptible to thermal denaturation, e.g. sodium caseinate (pH 6.5–7.0) may be heated at 140°C for more than 1 h without any visible physicochemical changes. However, severe heat treatments do cause substantial changes, e.g. dephosphorylation (about 100% in 1 h at 140°C), aggregation (as indicated by changes in urea–PAGE or gel permeation chromatography), possibly due to the formation of intermolecular disulphide and intermolecular isopeptide bonds, cleavage of peptide bonds (formation of peptides soluble at pH 4.6 or in 12% TCA). β-Elimination of serine, serine phosphate and cysteine residues may also occur, especially at pH values above 7. Such heat-induced changes are evident in commercial sodium caseinate.

The remarkably high heat stability of the caseins allows heat-sterilized dairy products to be produced without major changes in physical properties (reviewed by Fox, 1982; Singh and Creamer, 1992). The heat stability of unconcentrated milk is almost always adequate to withstand the temperature treatments to which it is normally subjected; only rarely is a defect known as the 'Utrecht phenomenon' encountered, when milk coagulates on HTST heating. This defect is due to a very high Ca^{2+} concentration owing

to a low concentration of citrate, arising from poor feed. However, the heat stability of milk decreases sharply on concentration and is usually inadequate to withstand in-container or UHT processing unless certain adjustments and/or treatments are made. Although the heat stability of concentrated milk is poorly correlated with that of the original milk, most of the research on the heat stability of milk has been done on unconcentrated milk.

9.7 Heat stability of milk

Studies on the heat stability of milk date from the pioneering work of Sommer and Hart, which commenced in 1919. Much of the early work concentrated on attempts to relate heat stability to variations in milk composition, especially the concentrations of milk salts. Although the heat coagulation time (HCT) of milk is inversely related to the concentrations of divalent cations (Ca^{2+} and Mg^{2+}) and positively with the concentrations of polyvalent anions (i.e. phosphate and citrate), the correlations are poor and unable to explain the natural variations in HCT. This failure was largely explained in 1961 by Rose who showed that the HCT of most milks is extremely sensitive to small changes in pH in the neighbourhood of 6.7. In effect, the influence of all other factors on the HCT of milk must be considered against the background of the effect of pH.

For the majority of individual-cow and all bulk milks, the HCT increases with increasing pH from 6.4 to about 6.7, then decreases abruptly to a minimum at around pH 6.9 but increases continuously with further increases in pH (Figure 9.19). The HCT decreases sharply below pH 6.4. Milks which show a strong dependence of heat stability on pH are referred to as type A milks. Occasionally, the HCT of individual-cow milks increases continuously with increasing pH, which is as would be expected due to increasing protein charge with increasing pH; these are referred to as type B milks.

The maximum HCT and the shape of the HCT–pH profile are influenced by several compositional factors, of which the following are the most significant:

1. Ca^{2+} reduces HCT throughout the pH range 6.4–7.4.
2. Ca-chelators, e.g. citrate, polyphosphate, increase stability.
3. β-Lg, and probably α-la, increase the stability of casein micelles at pH 6.4–6.7 but reduce it at pH 6.7–7.0; in fact, the occurrence of a maximum–minimum in the HCT–pH profile depends on the presence of β-lg.
4. Addition of κ-casein to milk increases stability in the pH range of the HCT minimum.

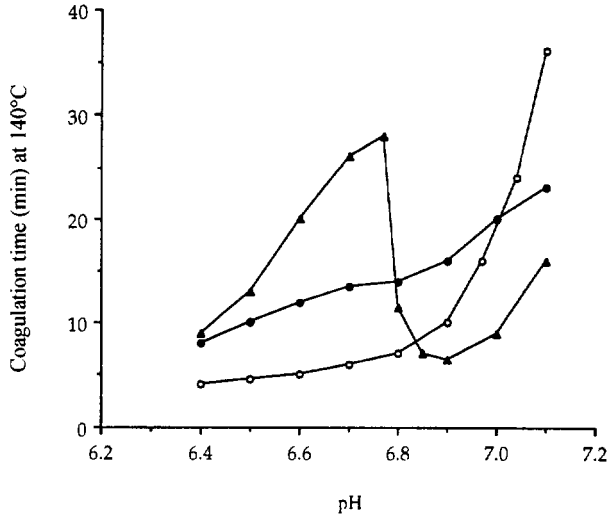


Figure 9.19 Effect of pH on the heat stability of type A milk (▲), type B milk (●) and whey protein-free casein micelle dispersions (○) (from Fox, 1982).

5. Reducing the level of colloidal calcium phosphate increases stability in the region of the HCT maximum.
6. Natural variations in HCT are due mainly to variations in the concentration of indigenous urea due to changes in the animals' feed.

The current explanation for the maximum–minimum in the HCT–pH profile is that on heating, κ-casein dissociates from the micelles; at pH values below about 6.7, β-lg reduces the dissociation of κ-casein, but at pH values above 6.7, it accentuates dissociation. In effect, coagulation in the pH range of minimum stability involves aggregation of κ-casein-depleted micelles, in a manner somewhat analogous to rennet coagulation, although the mechanism by which the altered micelles are produced is very different.

As would be expected, heating milk at 140°C for an extended period causes very significant chemical and physical changes in milk, of which the following are probably the most significant:

1. Decrease in pH. After heating at 140°C for 20 min, the pH of milk has decreased to about 5.8 due to acid production from pyrolysis of lactose, precipitation of soluble calcium phosphate as $Ca_3(PO_4)_2$, with the release of H^+, and dephosphorylation of casein with subsequent precipitation of the liberated phosphate as $Ca_3(PO_4)_2$ with the release of H^+. The heat-induced precipitation of $Ca_3(PO_4)_2$ is partially reversible on cooling so that the actual pH of milk at 140°C at the point of coagulation is much lower than the measured value and is probably below 5.0.

2. Precipitation of soluble calcium phosphate as $Ca_3(PO_4)_2$ with the release of H^+. After heating at 140°C for 5–10 min, most (>90%) of the soluble phosphate has been precipitated.
3. Dephosphorylation of casein, which follows first-order kinetics. After heating at 140°C for 60 min, >90% of the casein phosphate groups have been hydrolysed.
4. Maillard browning, which occurs rapidly at 140°C. Since Maillard browning involves blocking of the ε-amino group of proteins with a concomitant reduction in protein charge, it would be expected that Maillard browning would reduce HCT, but in fact the Maillard reaction appears to increase heat stability, possibly owing to the formation of low molecular weight carbonyls.
5. Hydrolysis of caseins. During heating at 140°C there is a considerable increase in non-protein N (12% TCA-soluble), apparently following zero-order kinetics. κ-Casein appears to be particularly sensitive to heating and about 25% of the *N*-acetylneuraminic acid (a constituent of κ-casein) is soluble in 12% TCA at the point of coagulation.
6. Cross-linking of proteins. Covalent cross-linking of caseins is evident (by gel electrophoresis) after even 2 min at 140°C and it is not possible to resolve the heat-coagulated caseins by urea– or SDS–PAGE.
7. Denaturation of whey proteins. Whey proteins are denatured very rapidly at 140°C; as discussed in section 9.6.3, the denatured proteins associate with the casein micelles, via sulphydryl–disulphide interactions with κ-casein, and probably with α_{s2}-casein, at pH values below 6.7. The whey proteins can be seen in electron photomicrographs as appendages on the casein micelles.
8. Association and shattering of micelles. Electron microscopy shows that the casein micelles aggregate initially, then disintegrate and finally aggregate into a three-dimensional network.
9. Changes in hydration. As would be expected from many of the changes discussed above, the hydration of the casein micelles decreases with the duration of heating at 140°C. The decrease appears to be due mainly to the fall in pH – if samples are adjusted to pH 6.7 after heating, there is an apparent increase in hydration on heating.
10. Surface (zeta) potential. It is not possible to measure the zeta potential of casein micelles at the assay temperature but measurements on heated micelles after cooling suggest no change in zeta potential, which is rather surprising since many of the changes discussed above would be expected to reduce surface charge.

All the heat-induced changes discussed would be expected to cause major alterations in the casein micelles, but the most significant change with respect to heat coagulation appears to be the decrease in pH – if the pH is readjusted occasionally to pH 6.7, milk can be heated for several hours at 140°C without coagulation. The stabilizing effect of urea is, at least partially,

due to the heat-induced formation of NH_3 which reduces or delays the fall in pH; however, other mechanisms for the stabilizing effect of urea have been proposed.

9.7.1 Effect of processing operations on heat stability

Concentration. Concentration by thermal evaporation markedly reduces the heat stability of milk, e.g. concentrated skim milk containing about 18% total solids coagulates in roughly 10 min at 130°C. The stability of the concentrate is strongly affected by pH, with a maximum at around pH 6.6, but stability remains low at all pH values above about 6.8 (Figure 9.20). Concentration by ultrafiltration has a much smaller effect on HCT than thermal evaporation, due to a lower concentration of soluble salts in the retentate.

Homogenization. Homogenization of skim milk has no effect on HCT but it destabilizes whole milk, the extent of destabilization increasing with fat content and the severity of homogenization (Figure 9.21). Destabilization probably occurs because the fat globules formed on homogenization are stabilized by casein and consequently they behave as 'casein micelles', in effect increasing the concentration of coagulable material.

Forewarming (preheating). Heating an unconcentrated milk, especially at 90°C × 10 min, before a heat stability assay, reduces its heat stability,

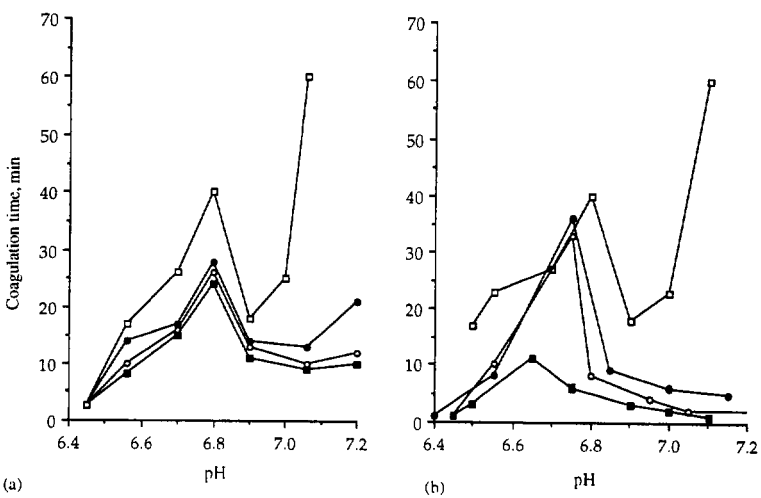


Figure 9.20 Effect of total solids (TS) content on the heat stability at 130°C of skim milk □, 9.3% TS; ●, 12.0% TS; ○, 15.0% TS; ■, 18.4% TS. (a) Concentrated by ultrafiltration, (b) concentrated by evaporation (from Sweetsur and Muir, 1980).

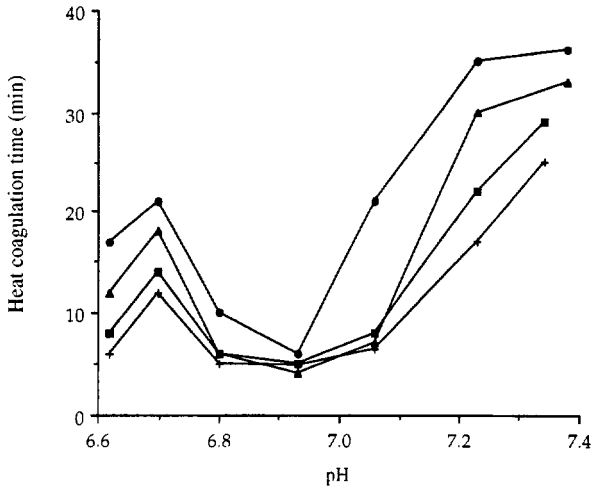


Figure 9.21 Effect of pressure (Rannie homogenizer) on the heat coagulation time (at 140°C) of milk, unhomogenized (●) or homogenized at 3.5 MPa; (▲); 10.4 MPa (■) or 20.7/3.5 MPa (+) (from Sweetsur and Muir, 1983).

mainly by shifting its natural pH; maximum heat stability is affected only slightly or not at all. However, if milk is preheated before concentration, the heat stability of the concentrate is increased. Various preheating conditions are used, e.g. 90°C × 10 min, 120°C × 2 min or 140°C × 5 s; the last is particularly effective but is not widely used commercially. The stabilizing effect is probably due to the fact that the heat-induced changes discussed previously are less detrimental if they occur prior to concentration rather than in concentrated milk which is inherently less stable.

Additives. Orthophosphates, and less frequently citrates, have long been used commercially to increase the stability of concentrated milk. The mechanism was believed to involve Ca-chelation but pH adjustments may be the principal mechanism.

Numerous compounds increase heat stability (e.g. various carbonyls, including diacetyl, and ionic detergents) but few are permitted additives. Although added urea has a major effect on the stability of unconcentrated milk, it does not stabilize concentrated milks, although it does increase the effectiveness of carbonyls.

9.8 Effect of heat treatment on rennet coagulation of milk and related properties

The primary step in the manufacture of most cheese varieties and rennet casein involves coagulation of the casein micelles to form a gel. Coagulation

involves two steps (phases), the first of which involves enzymatically hydrolysing the micelle-stabilizing protein, κ-casein, by selected proteinases, referred to as rennets. The second step of coagulation involves coagulation of rennet-altered micelles by Ca^{2+} above 20°C (Chapter 10).

The rate of rennet coagulation is affected by many compositional factors, including the concentrations of Ca^{2+}, casein and colloidal calcium phosphate and pH. Coagulation is adversely affected by heat treatment of the milk at temperatures above about 70°C due to interaction of denatured β-lg (and α-la) with κ-casein. The primary and, especially, the secondary phases of rennet coagulation are adversely affected by the interaction and, if the heat treatment is sufficiently severe (e.g. 80°C × 5–10 min), the milk does not coagulate on renneting. The effect on the primary phase is presumably due to blockage of the rennet-susceptible bond of κ-casein following interaction with β-lg. The adverse effect of heating on the second phase arises because the whey protein-coated micelles are unable to interact properly because the aggregation sites, which are unknown, are blocked.

The adverse effects of heat treatment on the rennetability of milk can be offset by acidifying or acidifying–reneutralizing the heated milk or supplementing it with Ca^{2+}. The mechanism by which acidification offsets the adverse effects of heating is not known but may involve changes in Ca^{2+} concentration.

The strength of the rennet-induced gel is also adversely affected by heat treatment of the milk, again presumably because the whey protein-coated micelles are unable to participate properly in the gel network. Gels from severely heat-treated milk have poor syneresis properties, resulting in high-moisture cheese which does not ripen properly. Syneresis is undesirable in fermented milks, e.g. yoghurt, the milk for which is severely heat-treated (e.g. 90°C × 10 min) to reduce the risk of syneresis.

9.9 Age gelation of sterilized milk

Two main problems limit the shelf-life of UHT sterilized milks: off-flavour development and gelation. Age gelation, which also occurs occasionally with in-container sterilized concentrated milks, is not related to the heat stability of the milk (provided that the product withstands the sterilization process) but the heat treatment does have a significant influence on gelation, e.g. indirectly heated UHT milk is more stable to age gelation than the directly heated product (the former is the more severe heat treatment). Plasmin may be responsible for the gelation of unconcentrated UHT milk produced from good-quality milk, while proteinases from psychrotrophs are probably responsible if the raw milk was of poor quality. It is possible that physicochemical phenomena are also involved, e.g. interaction between whey proteins and casein micelles.

Table 9.3 Substances making a strong contribution to the flavour of indirectly heated UHT milk, those contributing to differences in flavour of milk heat-treated in different ways, and those used in a synthetic UHT flavour preparation (from Manning and Nursten, 1987)

	UHT-i[a]	UHT-i-LP[b]	UHT-i-UHT-d[c]	Synthetic UHT flavour[d] (mg per kg LP)
Dimethyl sulphide	+	0	1	
3-Methylbutanal	+	1	1	
2-Methylbutanal	+	0	1	
2-Methyl-1-propanethiol	+	1	1	0.008
Pentanal	+	1	1	
3-Hexanone	+			
Hexanal	+	1	1	
2-Heptanone	+	4	2	0.40
Styrene	+			
Z-4-Heptenal[e]	+	1	0	
Heptanal	+			
2-Acetylfuran	+			
Dimethyl trisulphide	+	2	0	
Cyanobenzene	+			
1-Heptanol	+			
1-Octen-3-one[e]	+			
Octanal	+	1	1	
p-Cymene	+			
Phenol	+			
Indene	+			
2-Ethyl-1-hexanol	+			
Benzyl alcohol	+			
Unknown	+			
Acetophenone	+	1	0	
1-Octanol	+			
2-Nonanone	+	4	2	0.21
Nonanal	+			
p-Cresol	+			
m-Cresol	+			
E-2,*Z*-6-Nonadienal	+			
E-2-Nonenal	+			
3-Methylindene	+			
Methylindene	+			
Ethyldimethylbenzene	+			
Decanal	+			
Tetraethylthiourea	+			
Benzothiazole	+	1	0	0.005
γ-Octalactone	+	1	0	0.025
2,3,5-Trimethylanisole	+			
δ-Octalactone	+	1	0	
1-Decanol	+	1	1	
2-Undecanone	+	2	1	0.18
2-Methylnaphthalene	+			
Indole	+			
δ-Decalactone	+	1	0	0.650
Hydrogen sulphide		2	1	0.03
Diacetyl		2	1	0.005
Dimethyl disulphide		2	1	0.002
2-Hexanone		2	1	

Table 9.3 (*Continued*)

	UHT-i[a]	UHT-i-LP[b]	UHT-i-UHT-d[c]	Synthetic UHT flavour[d] (mg per kg LP)
γ-Dodecalactone		2	1	0.025
δ-Dodecalactone		2	1	0.1
Methanethiol		1	1	0.002
2-Pentanone		1	1	0.29
Methyl isothiocyanate		1	1	0.01
Ethyl isothiocyanate		1	1	0.01
Furfural		1	1	
Benzaldehyde		1	0	
2-Octanone		1	0	
Naphthalene		1	0	
γ-Decalactone		1	0	
2-Tridecanone		1	0	
Acetaldehyde		−1	0	
1-Cyano-4-pentene		−1	0	
2-Methyl-1-butanol		−1	1	
Ethyl butyrate		−1	0	
3-Buten-1-yl isothiocyanate		−1	0	
E-2,*E*-4-nonadienal		−1	0	
2,4-Dithiapentane			1	
Maltol				10.00

[a]Indirectly heated UHT milk; + indicates a component that makes a strong contribution to the flavour. In addition to the components listed, a further 12 unknowns made strong contributions.
[b]Components contributing to a difference in flavour between indirectly heated UHT milk and low temperature pasteurized (LP) milk. Scale for difference: 1, slight; 2, moderate; 3, strong; 4, very strong.
[c]Components contributing to a difference in flavour between indirectly and directly heated UHT milks. Scale for difference as in [b].
[d]Composition of synthetic UHT flavour.
[e]Tentative identification.

In the case of concentrated UHT milks, physicochemical effects appear to predominate, although proteolysis also occurs, e.g. the propensity of UHT concentrated milk reconstituted from high-heat milk powder to age gelation is less than those from medium- or low-heat powders, although the formation of sediment is greatest in the concentrate prepared from the high-heat powder (see Harwalkar, 1992).

9.10 Heat-induced changes in flavour of milk

Flavour is a very important attribute of all foods; heating/cooking makes a major contribution to flavour, both positively and negatively. Good-quality fresh liquid milk products are expected to have a clean, sweetish taste and essentially no aroma; any departure therefrom can be considered as an

off-flavour. Heat treatments have a major impact on the flavour/aroma of dairy foods, either positively or negatively.

On the positive side, thermization and minimum pasteurization should not cause the formation of undesirable flavours and aromas and should, in fact, result in improved flavour by reducing bacterial growth and enzymatic activity, e.g. lipolysis. If accompanied by vacuum treatment (vacreation), pasteurization removes indigenous off-flavours, i.e. those arising from the cow's metabolism or from feed, thereby improving the organoleptic qualities of milk.

Also on the positive side, severe heat treatment of cream improves the oxidative stability of butter produced therefrom due to the exposure of antioxidant sulphydryl groups. As discussed in section 9.2.2, lactones formed from hydroxyacids are major contributors to the desirable cooking quality of milk fats but contribute to off-flavours in other heated products, e.g. milk powders.

UHT processing causes substantial deterioration in the organoleptic quality of milk. Freshly processed UHT milk is described as 'cooked' and 'cabbagy', but the intensity of these flavours decreases during storage, giving maximum flavour acceptability after a few days. These off-flavours are due to the formation of sulphur compounds from the denatured whey proteins, as discussed in section 9.6.3. After this period of maximum acceptability, quality deteriorates, the milk being described as stale. At least 400 volatiles have been detected in UHT milk, about 50 of which (Table 9.3) are considered to make a significant contribution to flavour (Manning and Nursten, 1987). The shelf-life of UHT milk is usually limited by gelation and/or bitterness, both of which are due to proteolysis, as discussed in section 9.6.1.

Since sulphur compounds are important in the off-flavour of UHT milk, attempts to improve its flavour have focused on reducing the concentration of these, e.g. by adding thiosulphonates, thiosulphates or cystine (which react with mercaptans) or sulphydryl oxidase, an indigenous milk enzyme (which oxidizes sulphydryls to disulphides; Chapter 8).

The products of Maillard browning have a significant negative impact on the flavour of heated milk products, especially in-container sterilized milks and milk powders.

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10 Chemistry and biochemistry of cheese and fermented milks

10.1 Introduction

Cheese is a very varied group of dairy products, produced mainly in Europe, North and South America, Australia and New Zealand and to a lesser extent in North Africa and the Middle East, where it originated during the Agricultural Revolution, 6000–8000 years ago. Cheese production and consumption, which vary widely between countries and regions (Appendices 10A and 10B), is increasing in traditional producing countries (2–4% p.a. for several years) and is spreading to new areas. On a global scale, 30% of all milk is used for cheese; the proportion is about 40% in North America and about 50% in the European Union.

Although traditional cheeses have a rather high fat content, they are rich sources of protein and in most cases of calcium and phosphorus and have anticarigenic properties; some typical compositional data are presented in Table 10.1. Cheese is the classical example of a convenience food: it can be used as the main course in a meal, as a dessert or snack, as a sandwich filler, food ingredient or condiment.

There are at least 1000 named cheese varieties, most of which have very limited production. The principal families are Cheddar, Dutch, Swiss and Pasta filata (e.g. Mozzarella), which together account for about 80% of total cheese production. All varieties can be classified into three superfamilies based on the method used to coagulate the milk, i.e. rennet coagulation (representing about 75% of total production), isoelectric (acid) coagulation and a combination of heat and acid (which represents a very minor group).

Production of cheese curd is essentially a concentration process in which the milkfat and casein are concentrated about tenfold while the whey proteins, lactose and soluble salts are removed in the whey. The acid-coagulated and acid/heat-coagulated cheeses are normally consumed fresh but the vast majority of rennet-coagulated cheeses are ripened (matured) for a period ranging from 3 weeks to more than 2 years, during which numerous microbiological, biochemical, chemical and physical changes occur, resulting in characteristic flavour, aroma and texture. The biochemistry of cheese ripening is very complex and is not yet completely understood.

Table 10.1 Composition of selected cheeses (per 100 g)

Cheese type	Water (g)	Protein (g)	Fat (g)	Cholesterol (mg)	Energy (kJ)
Brie	48.6	19.3	26.9	100	1323
Caerphilly	41.8	23.2	31.3	90	1554
Camembert	50.7	20.9	23.1	75	1232
Cheddar	36.0	25.5	34.4	100	1708
Cheshire	40.6	24.0	31.4	90	1571
Cottage	79.1	13.8	3.9	13	413
Cream cheese	45.5	3.1	47.4	95	1807
Danish blue	45.3	20.1	29.6	75	1437
Edam	43.8	26.0	25.4	80	1382
Emmental	35.7	28.7	29.7	90	1587
Feta	56.5	15.6	20.2	70	1037
Fromage frais	77.9	6.8	7.1	25	469
Gouda	40.1	24.0	31.0	100	1555
Gruyere	35.0	27.2	33.3	100	1695
Mozzarella	49.8	25.1	21.0	65	1204
Parmesan	18.4	39.4	32.7	100	1880
Ricotta	72.1	9.4	11.0	50	599
Roquefort	41.3	19.7	32.9	90	1552
Stilton	38.6	22.7	35.5	105	1701

10.2 Rennet-coagulated cheeses

The production of rennet-coagulated cheeses can, for convenience, be divided into two phases: (1) conversion of milk to curds and (2) ripening of the curds.

10.2.1 Preparation and treatment of cheesemilk

The milk for most cheese varieties is subjected to one or more pre-treatments (Table 10.2). The concentrations of fat and casein and the ratio of these components are two very important parameters affecting cheese quality. While the concentrations of these components in cheese are determined and controlled by the manufacturing protocol, their ratio is regulated by adjusting the composition of the cheesemilk. This is usually done by adjusting the fat content by blending whole and skimmed milk in proportions needed to give the desired fat : casein ratio in the finished cheese, e.g. 1.0 : 0.7 for Cheddar or Gouda. It should be remembered that about 10% of the fat in milk is lost in the whey while only about 5% of the casein is lost (unavoidably, see section 10.2.2).

With the recent commercial availability of ultrafiltration, it has become possible to increase the concentration of casein, thus levelling out seasonal variations in milk composition and consequently in gel characteristics and

Table 10.2 Pre-treatment of cheese milk

Standardization of fat : protein ratio
Addition of skim milk
Removal of some fat
Addition of ultrafiltration retentate
Addition of $CaCl_2$
Adjustment of pH (e.g. by gluconic acid-δ-lactone)
Removal or killing of contaminating bacteria
Thermization (e.g. 65°C × 15 s)
Pasteurization (e.g. 72°C × 15 s)
Bactofugation
Microfiltration

cheese quality. The capacity of a given plant is also increased by pre-concentrating milk by ultrafiltration.

The pH and the concentration of calcium in milk also vary, with consequential effects on the properties of renneted milk gels. The addition of $CaCl_2$ to cheesemilk (0.02%) is widely practised and adjustment and standardization of milk pH by using the acidogen, gluconic acid-δ-lactone (GDL), is recommended and commercially practised on a limited scale.

Although raw milk is still widely used for cheese manufacture, e.g. Parmigiano-Reggiano (Italy), Emmental (Switzerland), Comté and Beaufort (France) and many less well known varieties, both on a factory and farmhouse scale, most Cheddar and Dutch-type cheeses are produced from pasteurized milk (HTST; *c.* 72°C × 15 s). Pasteurization is used primarily to kill pathogenic and spoilage bacteria. However, desirable indigenous bacteria are also killed by pasteurization and it is generally agreed that cheese made from pasteurized milk ripens more slowly and develops a less intense flavour than raw milk cheese, apparently because certain, as yet unidentified, indigenous bacteria are absent. At present, some countries require that all cheese milk should be pasteurized or the cheese aged for at least 60 days (during which time pathogenic bacteria die off). A global requirement for pasteurization of cheesemilk has been recommended but would create restrictions for international trade in cheese, especially for many of those with 'Appellation d'Origine Protégée' status. Research is under way to identify the important indigenous microorganisms in raw milk cheese for use as inoculants for pasteurized milk. While recognizing that pasteurization is very important in ensuring safe cheese, pH (below about 5.2) and water activity (a_w, which is controlled by addition of NaCl) are also critical safety hurdles.

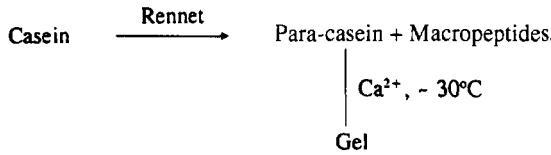
Milk may be thermized (*c.* 65°C × 15 s) on receipt at the factory to reduce bacterial load, especially psychrotrophs, which are heat labile. Since thermization does not kill pathogens, thermized milk is usually fully pasteurized before cheesemaking.

Clostridium tyrobutyricum (an anaerobic spore-former) causes late gas blowing (through the production of H_2 and CO_2) and off-flavours (butanoic acid) in many hard ripened cheeses; Cheddar-type cheeses are major exceptions. Contamination of cheese milk with clostridial spores can be avoided or kept to a very low level by good hygienic practices (soil and silage are the principal sources of clostridia) but they are usually prevented from growing through the use of sodium nitrate ($NaNO_3$) or, less frequently, lysozyme, and/or removed by bactofugation (centrifugation) or microfiltration.

10.2.2 Conversion of milk to cheese curd

Typically, five steps, or groups of steps, are involved in the conversion of milk to cheese curd: coagulation, acidification, syneresis (expulsion of whey), moulding/shaping and salting. These steps, which partly overlap, enable the cheesemaker to control the composition of cheese, which, in turn, has a major influence on cheese ripening and quality.

Enzymatic coagulation of milk. The enzymatic coagulation of milk involves modification of the casein micelles via limited proteolysis by selected proteinases, called rennets, followed by calcium-induced aggregation of the rennet-altered micelles:



If present, the fat globules are occluded in the gel but do not participate in the formation of a gel matrix.

As discussed in Chapter 4, the casein micelles are stabilized by κ-casein, which represents 12–15% of the total casein and is located mainly on the surface of the micelles such that its hydrophobic N-terminal region reacts hydrophobically with the calcium-sensitive α_{s1}-, α_{s2}- and β-caseins while its hydrophilic C-terminal region protrudes into the surrounding aqueous environment, stabilizing the micelles by a negative surface charge and steric stabilization.

Following its isolation in 1956, it was found that κ-casein is the only casein hydrolysed during the rennet coagulation of milk and that it was hydrolysed specifically at the Phe_{105}–Met_{106} bond, producing para-κ-casein (κ-CN f1–105) and macropeptides (f106–169; also called glycomacropeptides since they contain most or all of the sugar groups attached to κ-casein) (Figure 10.1). The hydrophilic macropeptides diffuse into the surrounding medium while the para-κ-casein remains attached to the

Figure 10.1 Amino acid sequence of κ-casein, showing the principal chymosin cleavage site (↓); oligosaccharides are attached at some or all of the threonine residues shown in italics.

micelle core (the macropeptides represent *c.* 30% of κ-casein, i.e. 4–5% of total casein; this unavoidable loss must be considered when calculating the yield of cheese). Removal of the macropeptides from the surface of the casein micelles reduces their zeta potential from about −20 to −10 mV and removes the steric stabilizing layer. The proteolysis of κ-casein is referred to as the **primary (first) phase** of rennet-coagulation.

When about 85% of the total κ-casein in milk has been hydrolysed, the colloidal stability of the micelles is reduced to such an extent that they coagulate at temperatures greater than about 20°C (*c.* 30°C is used in cheesemaking), an event referred to as the **secondary phase** of rennet coagulation. Calcium ions are essential for the coagulation of rennet-altered micelles (although the binding of Ca^{2+} by casein is not affected by renneting).

The Phe_{105}–Met_{106} bond of κ-casein is several orders of magnitude more sensitive to rennets than any other bond in the casein system. The reason(s) for this unique sensitivity has not been fully established but work on synthetic peptides that mimic the sequence of κ-casein around this bond has provided valuable information. The Phe and Met residues themselves are not essential, e.g. both Phe_{105} and Met_{106} can be replaced or modified without drastically changing the sensitivity of the bond – in human, porcine and rodent κ-caseins, Met_{106} is replaced by Ile or Leu, and the proteinase from *Cryphonectria parasitica* (section 10.2.2.2), hydrolyses the bond Ser_{104}–Phe_{105} rather than Phe_{105}–Met_{106}. The smallest κ-casein-like pept-

Table 10.3 Kinetic parameters for hydroloysis of κ-casein peptides by chymosin at pH 4.7 (compiled from Visser *et al.*, 1976; Visser, Slangen and van Rooijen, 1987)

Peptide	Sequence	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)
S.F.M.A.I.	104–108	0.33	8.50	0.038
S.F.M.A.I.P.	104–109	1.05	9.20	0.114
S.F.M.A.I.P.P.	104–110	1.57	6.80	0.231
S.F.M.A.I.P.P.K.	104–111	0.75	3.20	0.239
L.S.F.M.A.I.	103–108	18.3	0.85	21.6
L.S.F.M.A.I.P.	103–109	38.1	0.69	55.1
L.S.F.M.A.I.P.P.	103–110	43.3	0.41	105.1
L.S.F.M.A.I.P.P.K.	103–111	33.6	0.43	78.3
L.S.F.M.A.I.P.P.K.K.	103–112	30.2	0.46	65.3
H.L.S.F.M.A.I	102–108	16.0	0.52	30.8
P.H.L.S.F.M.A.I	101–108	33.5	0.34	100.2
H.P.H.P.H.L.S.F.M.A.I.P.P.K.	98–111	66.2	0.026	2509
	98–111[a]	46.2[a]	0.029[a]	1621[a]
κ-Casein[b]		2–20	0.001–0.005	200–2000
L.S.F.(NO_2)Nle A.L.OMe		12.0	0.95	12.7

[a]pH 6.6.
[b]pH 4.6.

ide hydrolysed by chymosin is Ser.Phe.Met.Ala.Ile (κ-CN f104–108); extending this peptide from its C and/or N terminus increases its susceptibility to chymosin (i.e. increases k_{cat}/K_m); the peptide κ-CN f98–111 is as good a substrate for chymosin as whole κ-casein (Table 10.3). Ser_{104} appears to be essential for cleavage of the Phe_{105}–Met_{106} bond by chymosin, and the hydrophobic residues, Leu_{103}, Ala_{107} and Ile_{108} are also important.

Rennets. The traditional rennets used to coagulate milk for most cheese varieties are prepared from the stomachs of young calves, lambs or kids by extraction with NaCl (*c.* 15%) brines. The principal proteinase in such rennets is chymosin; about 10% of the milk-clotting activity of calf rennet is due to pepsin. As the animal ages, the secretion of chymosin declines while that of pepsin increases; in addition to pepsin, cattle appear to secrete a chymosin-like enzyme throughout life.

Like pepsin, chymosin is an aspartyl (acid) proteinase, i.e. it has two essential aspartyl residues in its active site which is located in a cleft in the globular molecule (molecular mass ~36 kDa) (Figure 10.2). Its pH optimum for general proteolysis is about 4, in comparison with about 2 for pepsins from monogastric animals. Its general proteolytic activity is low relative to its milk-clotting activity and it has moderately high specificity for bulky hydrophobic residues at the P_1 and P'_1 positions of the scissile bond. Its physiological function appears to be to coagulate milk in the stomach of the neonate, thereby increasing the efficiency of digestion, by retarding discharge into the intestine, rather than general proteolysis.

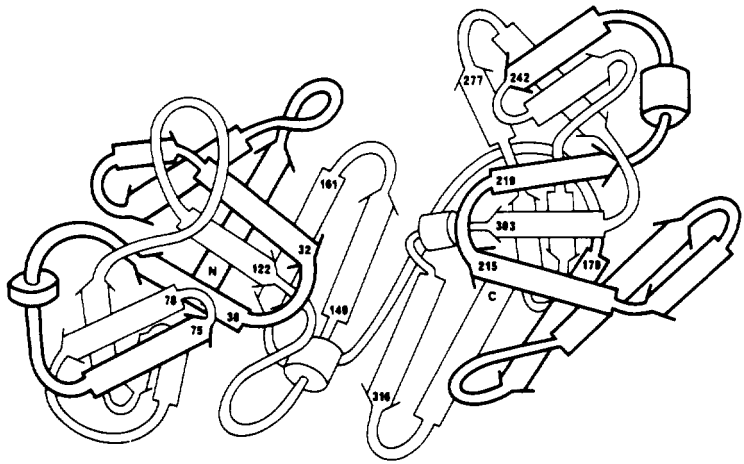


Figure 10.2 Schematic representation of the tertiary structure of an aspartyl proteinase, showing the cleft which contains the active site; arrows indicate β structures and cylinders the α-helices (from Foltmann, 1987).

Due to increasing world production of cheese and the declining supply of young calf stomachs (referred to as vells), the supply of calf rennet has been inadequate for many years. This has led to a search for suitable substitutes. Many proteinases are capable of coagulating milk but most are too proteolytic relative to their milk-clotting activity, leading to a decrease in cheese yield (due to excessive non-specific proteolysis in the cheese vat and loss of peptides in the whey) and defects in the flavour and texture of the ripened cheese, due to excessive or incorrect proteolysis. Only six proteinases are used commercially as rennet substitutes: porcine, bovine and chicken pepsins and the acid proteinases from *Rhizomucor miehei*, *R. pusillus* and *Cryphonectria parasitica*. Chicken pepsin is quite proteolytic and is used widely only in Israel (for religious reasons). Porcine pepsin enjoyed limited success about 30 years ago, usually in admixtures with calf rennet, but it is very sensitive to denaturation at pH values above 6 and may be denatured extensively during cheesemaking, leading to impaired proteolysis during ripening; it is now rarely used as a rennet substitute. Bovine pepsin is quite effective and many commercial calf rennets contain up to 50% bovine pepsin. *Rhizomucor miehei* proteinase, the most widely used microbial rennet, gives generally satisfactory results. *Cryphonectria parasitica* proteinase is, in general, the least suitable of the commercial microbial rennet substitutes and is used only in high-cooked cheeses in which extensive denaturation of the coagulant occurs, e.g. Swiss-type cheeses.

The gene for calf chymosin has been cloned in *Kluyveromyces marxianus* var. *lactis*, *Aspergillus niger* and *E. coli*. Microbial (cloned) chymosins have

given excellent results in cheesemaking trials on various varieties and are now widely used commercially, although they are not permitted in some countries. Significantly, they are accepted for use in vegetarian cheeses. The gene for *R. miehei* proteinase has been cloned in *A. oryzae*; the resultant product, Marzyme GM, is commercially available (Texel, Stockport, UK) and is reported to be a very effective coagulant.

Coagulation of rennet-altered micelles. When *c.* 85% of the total κ-casein has been hydrolysed, the micelles begin to aggregate progressively into a gel network. Gelation is indicated by a rapid increase in viscosity (η) (Figure 10.3). Coagulation commences at a lower degree of hydrolysis of κ-casein if the temperature is increased, the pH reduced or the Ca^{2+} concentration increased.

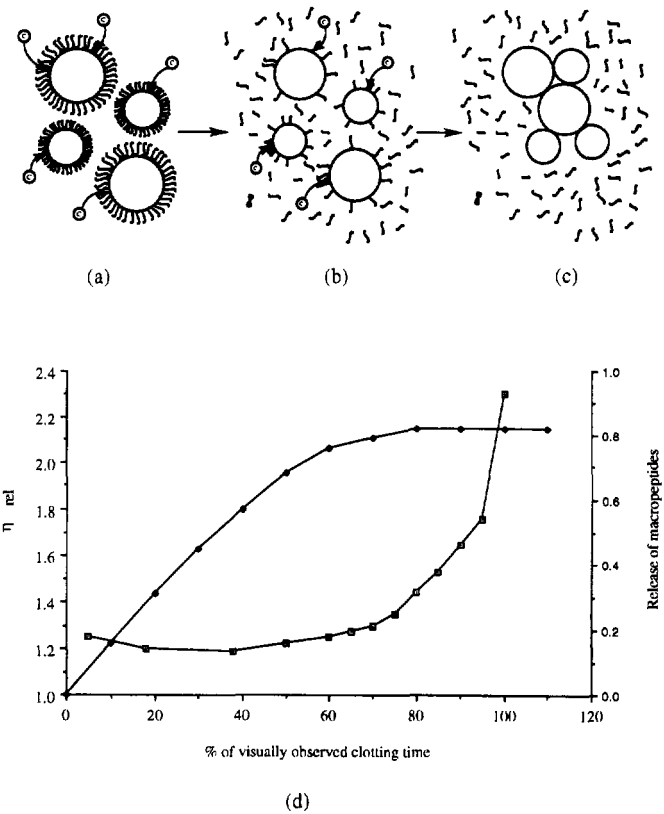


Figure 10.3 Schematic representation of the rennet coagulation of milk. (a) Casein micelles with intact κ-casein layer being attacked by chymosin (C); (b) micelles partially denuded of κ-casein; (c) extensively denuded micelles in the process of aggregation; (d) release of macropeptides (♦) and changes in relative viscosity (⊡) during the course of rennet coagulation.

The actual reactions leading to coagulation are not known. Ca^{2+} are essential but Ca-binding by caseins does not change on renneting. Colloidal calcium phosphate (CCP) is also essential: reducing the CCP concentration by more than 20% prevents coagulation. Perhaps, hydrophobic interactions, which become dominant when the surface charge and steric stabilization are reduced on hydrolysis of κ-casein, are responsible for coagulation (the coagulum is soluble in urea). The adverse influence of moderately high ionic strength on coagulation suggests that electrostatic interactions are also involved. It is claimed that pH has no effect on the secondary stage of rennet coagulation, which is perhaps surprising since micellar charge is reduced by lowering the pH and should facilitate coagulation. Coagulation is very temperature-sensitive and does not occur below about 18°C, above which the temperature coefficient, Q_{10}, is approximately 16.

Factors that affect rennet coagulation. The effect of various compositional and environmental factors on the primary and secondary phases of rennet coagulation and on the overall coagulation process are summarized in Figure 10.4.

No coagulation occurs below 20°C, due mainly to the very high temperature coefficient of the secondary phase. At higher temperatures (above 55–60°C, depending on pH and enzyme) the rennet is denatured. Rennet coagulation is prolonged or prevented by preheating milk at temperatures above about 70°C (depending on the length of exposure). The effect is due to the interaction of β-lactoglobulin with κ-casein via sulphydryl-disulphide interchange reactions; both the primary and, especially, the secondary phase of coagulation are adversely affected.

Measurement of rennet coagulation time. A number of principles are used to measure the rennet coagulability of milk or the activity of rennets; most measure actual coagulation, i.e. combined first and second stages, but some specifically monitor the hydrolysis of κ-casein. The most commonly used methods are described below.

The simplest method is to measure the time elapsed between the addition of a measured amount of diluted rennet to a sample of milk in a temperature-controlled water-bath at, e.g. 30°C. If the coagulating activity of a rennet preparation is to be determined, a 'reference' milk, e.g. low-heat milk powder reconstituted in 0.01% $CaCl_2$, and perhaps adjusted to a certain pH, e.g. 6.5, should be used. A standard method has been published (IDF, 1992) and a reference milk may be obtained from Institut National de la Recherche Agronomique, Poligny, France. If the coagulability of a particular milk is to be determined, the pH may or may not be adjusted to a standard value. The coagulation point may be determined by placing the milk sample in a bottle or tube which is rotated in a water-bath (Figure 10.5); the fluid milk forms a film on the inside of the rotating bottle/tube but

Factor	First phase	Second phase	Overall effect, see panel
Temperature	+	++	a
pH	+++	-	b
Ca	-	+++	c
Pre-heating	++	++++	d
Rennet concentration	++++	-	e
Protein concentration	+	++++	f

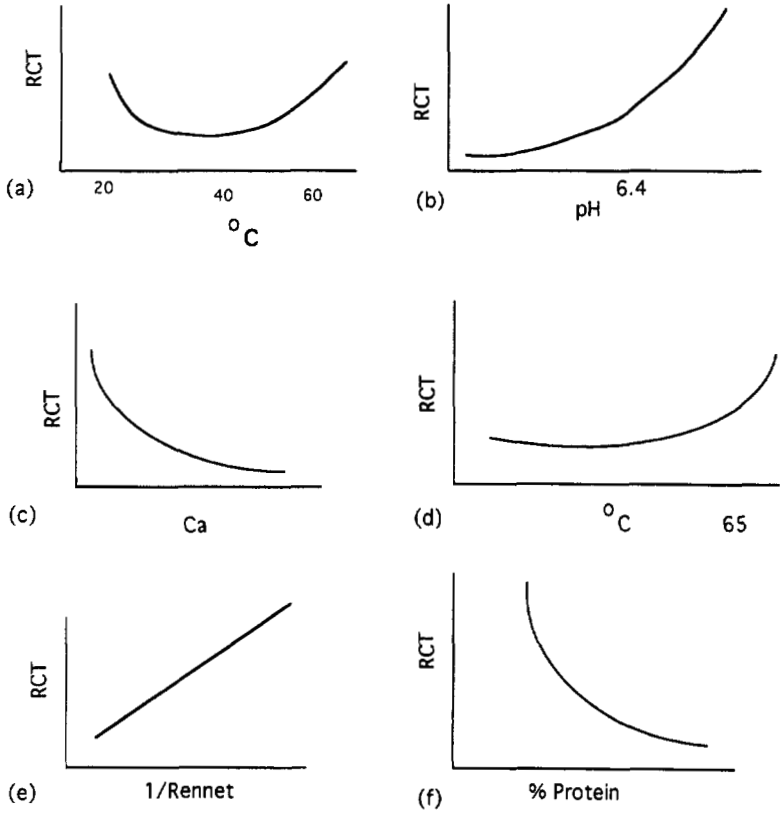


Figure 10.4 Principal factors affecting the rennet coagulation time (RCT) of milk.

flocs of protein form in the film on coagulation. Several types of apparatus using this principle have been described.

As shown in Figure 10.3, the viscosity of milk increases sharply when milk coagulates and may be used to determine the coagulation point. Any type of viscometer may, theoretically, be used but several dedicated pieces

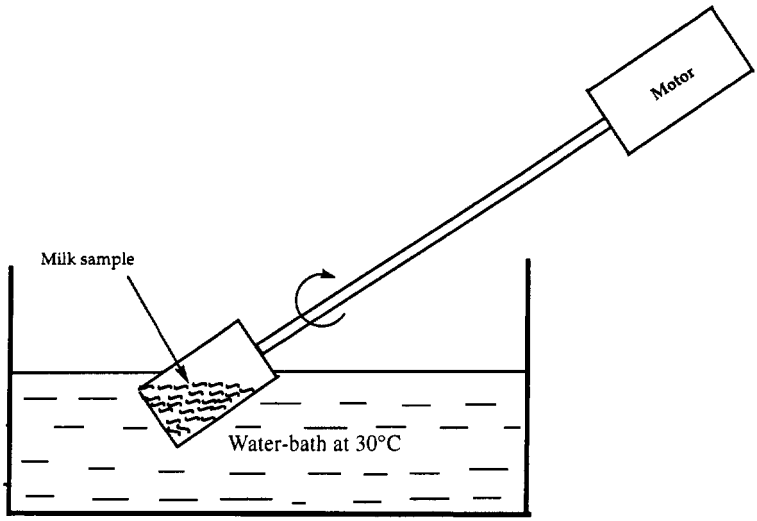


Figure 10.5 Apparatus for visual determination of the rennet coagulation time of milk.

of apparatus have been developed. The most popular of these, although with limited use, is the Formograph (Foss Electric, Denmark), a diagram of which is shown in Figure 10.6a. Samples of milk to be analysed are placed in small beakers which are placed in cavities in an electrically heated metal block. Rennet is added and the loop-shaped pendulum of the instrument placed in the milk. The metal block is moved back and forth, creating a 'drag' on the pendulum in the milk. The arm to which the pendulum is attached contains a mirror from which a flashing light is reflected on to photosensitive paper, creating a mark. While the milk is fluid, the viscosity is low and the drag on the pendulum is slight and it scarcely moves from its normal position; hence a single straight line appears on the paper. As the milk coagulates, the viscosity increases and the pendulum is dragged out of position, resulting in bifurcation of the trace. The rate and extent to which the arms of the trace move apart is an indicator of the strength (firmness) of the gel. A typical trace is shown in Figure 10.6b. A low value of r indicates a short rennet coagulation time while high values of a_{30} and k_{20} indicate a milk with good gel-forming properties.

A recently developed, and apparently industrially useful, apparatus is the hot wire sensor. A diagram of the original assay cell is shown in Figure 10.7a. A sample of milk is placed in a cylindrical vessel containing a wire of uniform dimensions. A current is passed through the wire, generating heat which is dissipated readily while the milk is liquid. As the milk coagulates, generated heat is no longer readily dissipated and the temperature of the

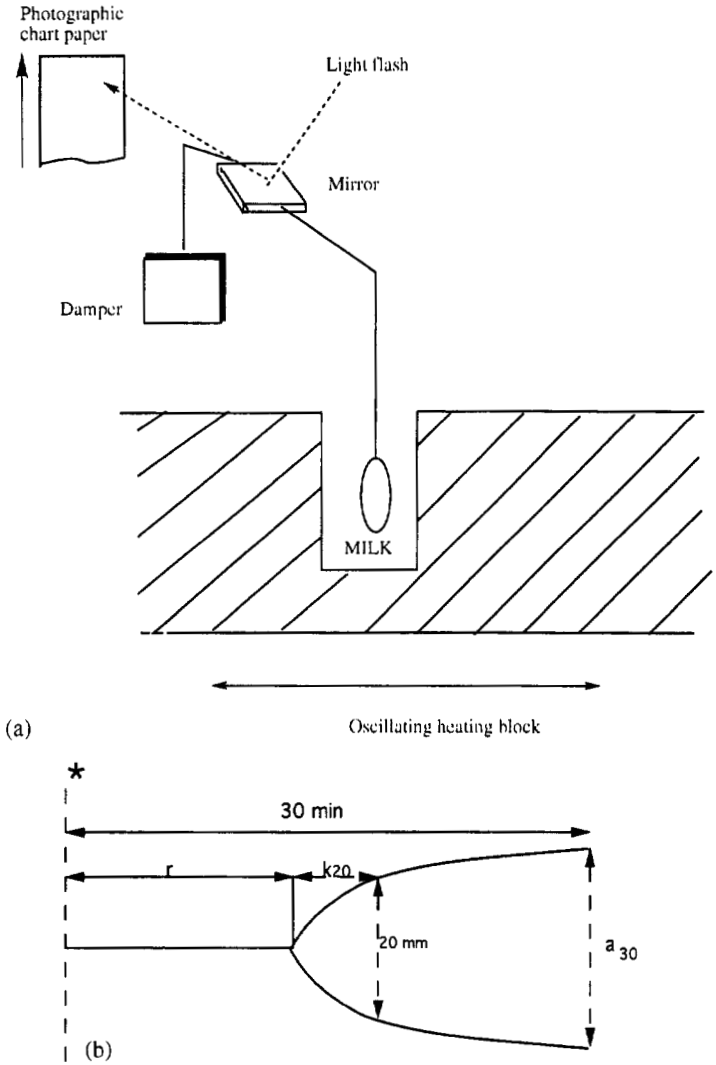


Figure 10.6 (a) Schematic representation of the Formograph apparatus for determining the rennet coagulation of milk. (b) Typical formogram. * Point of rennet addition, r is rennet coagulation time, k_{20} is the time required from coagulation for the arms of the formogram to bifurcate by 20 mm, a_{30} is the extent of bifurcation 30 min after rennet addition (the approximate time at which the coagulum is cut in cheesemaking).

wire increases, causing an increase in its conductivity; a typical trace is shown in Figure 10.7b. The principle has been commercialized by Stoelting Inc. (Kiel, Wisconsin). The wire probe, in a stainless steel shield, is inserted through the wall of the cheese vat. The output from the wire is fed to a computer which can be used to switch on the gel-cutting knife, permitting

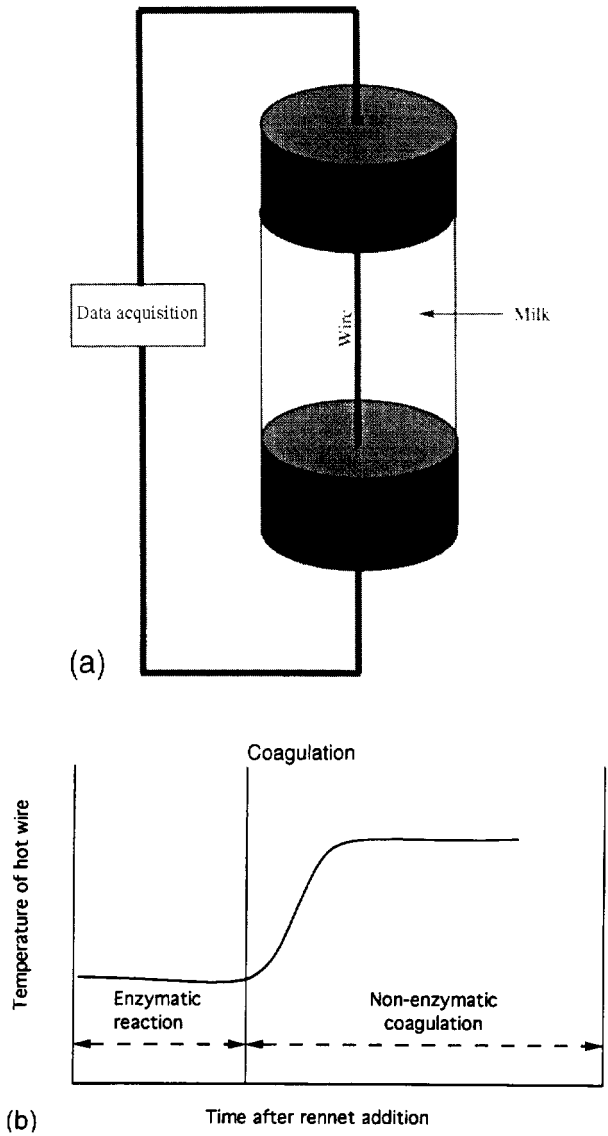


Figure 10.7 (a) Hot wire sensor for objectively measuring the rennet coagulation of milk. (b) Changes in the temperature of the hot wire during the course of the rennet coagulation of milk.

automation and cutting of the gel at a consistent strength, which is important for maximizing cheese yield.

The primary phase of rennet action may be monitored by measuring the formation of either product, i.e. para-κ-casein or the GMP. Para-κ-casein may be measured by SDS–polyacrylamide gel electrophoresis (PAGE),

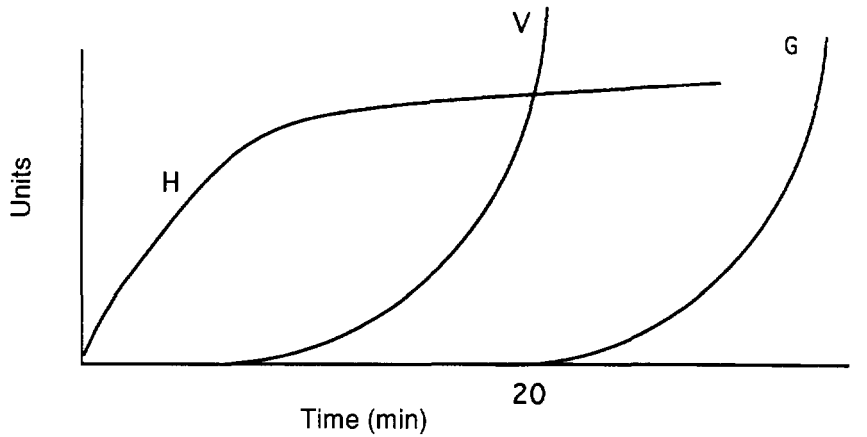


Figure 10.8 Schematic representation of hydrolysis and gel formation in renneted milk; H = hydrolysis of κ-casein; V = changes in the viscosity of renneted milk (second stage of coagulation), G = changes in the viscoelastic modulus (gel formation).

which is slow and cumbersome, or by ion-exchange high performance liquid chromatography (HPLC). The GMP is soluble in TCA (2–12% depending on its carbohydrate content) and can be quantified by the Kjeldahl method or more specifically by determining the concentration of *N*-acetylneuraminic acid or by reversed phase HPLC (RP-HPLC).

The activity of rennets can be easily determined using chromogenic peptide substrates, a number of which are available.

Gel strength (curd tension). The gel network continues to develop for a considerable period after visible coagulation (Figure 10.8). The strength of the gel formed, which is very important from the viewpoints of syneresis (and hence moisture control) and cheese yield, is affected by several factors – the principal ones are summarized in Figure 10.9.

The strength of a renneted milk gel can be measured by several types of viscometers and penetrometers. As discussed on p. 389, the Formograph gives a measure of the gel strength but the data can not be readily converted to rheological terms. Penetrometers give valuable information but are single-point determinations. Dynamic rheometers are particularly useful, allowing the buildup of the gel network to be studied.

Syneresis. Renneted milk gels are quite stable if undisturbed but synerese (contract), following first-order kinetics, when cut or broken. By controlling the extent of syneresis, the cheesemaker can control the moisture content of cheese curd and hence the rate and extent of ripening and the stability of the cheese – the higher the moisture content, the faster the cheese will ripen

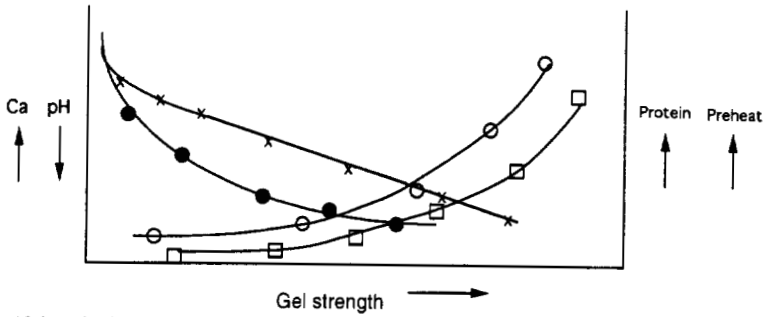


Figure 10.9 Principal factors that affect the strength of renneted milk gels (curd tension); pH (●), calcium concentration (○), protein concentration (□), preheat treatment (×).

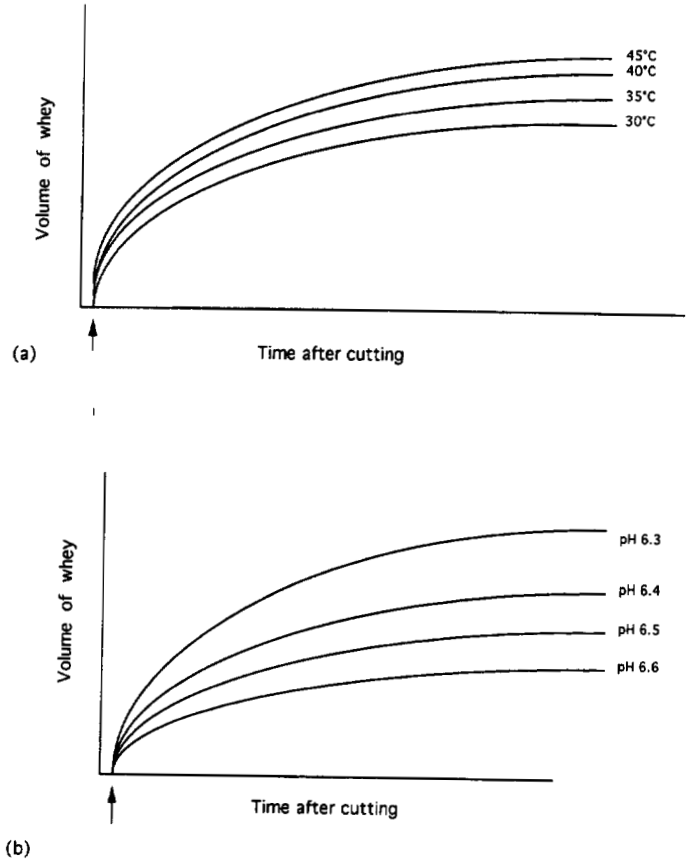


Figure 10.10 Effect of temperature (a) and pH (b) on the rate and extent of syneresis in cut/broken renneted milk gels.

but the lower its stability. Syneresis is promoted by:

- cutting the curd finely, e.g. Emmental (fine cut) versus Camembert (large cut);
- low pH (Figure 10.10b);
- calcium ions;
- increasing the cooking temperature (Camembert, *c.* 30°C; Gouda, *c.* 36°C; Cheddar, *c.* 38°C; Emmental or Parmesan, 52–55°C) (Figure 10.10a);
- stirring the curd during cooking;
- fat retards syneresis, while increasing the protein content (up to a point) improves it; at high protein concentrations, the gel is too firm and does not synerese (e.g. UF retentate).

Gels prepared from heated milk synerese poorly (assuming that the milk does coagulate). Such reduced syneresis properties are desirable for fermented milk products, e.g. yoghurt (milk for which is severly heated, e.g. 90°C × 10 min) but are undesirable for cheese.

Good analytical methods for monitoring syneresis are lacking. Principles that have been exploited include: dilution of an added marker, e.g. a dye, which must not adsorb on to or diffuse into the curd particles, measurement of the electrical conductivity or moisture content of the curd or by measuring the volume of whey released (probably the most commonly used method although only one-point values are obtained).

10.2.3 Acidification

Acid production is a key feature in the manufacture of all cheese varieties – the pH decreases to about 5 (±0.3, depending on variety) within 5–20 h, at a rate depending on the variety (Figure 10.11). Acidification is normally achieved via the bacterial fermentation of lactose to lactic acid, although an acidogen, usually gluconic acid-δ-lactone, alone or in combination with acid, may be used in some cases, e.g. Mozzarella.

Traditionally, cheesemakers relied on the indigenous microflora of milk for lactose fermentation, as is still the case for several minor artisanal varieties. However, since the indigenous microflora varies, so does the rate of acidification and hence the quality of the cheese; the indigenous microflora is largely destroyed by pasteurization. 'Slop-back' or whey cultures (starters; the use of whey from today's cheesemaking as an inoculum for tomorrow's milk) have probably been used for a very long time and are still used commercially, e.g. for such famous cheese as Parmigiano-Reggiano and Comté. However, selected 'pure' cultures have been used for Cheddar and Dutch-type cheeses for at least 80 years and have become progressively more refined over the years. Single-strain cultures were introduced in New Zealand in the 1930s as part of a bacteriophage control programme. Selected phage-unrelated strains are now widely used for Cheddar cheese;

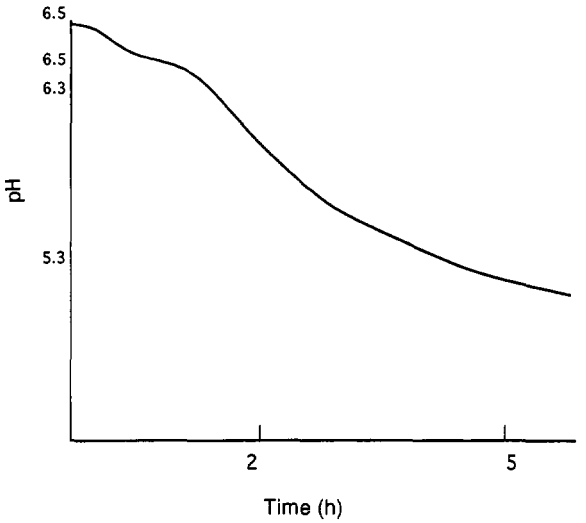


Figure 10.11 pH profile of Cheddar during cheese manufacture.

although selected by a different protocol, highly selected cultures are also used for Dutch and Swiss-type cheeses.

Members of three genera are used as cheese starters. For cheeses that are cooked to a temperature below about 39°C, species of *Lactococcus*, usually *Lc. lactis* ssp. *cremoris*, are used, i.e. for Cheddar, Dutch, Blue, surface mould and surface-smear families. For high-cooked varieties, a thermophilic *Lactobacillus* culture is used, either alone (e.g. Parmesan) or with *Streptococcus salivarius* ssp. *thermophilus* (e.g. most Swiss varieties and Mozzarella). *Leuconostoc* spp. are included in the starter for some cheese varieties, e.g. Dutch types; the function is to produce diacetyl and CO_2 from citrate rather than acid production.

The selection, propagation and use of starters will not be discussed here. The interested reader is referred to Cogan and Hill (1993).

The primary function of cheese starter cultures is to produce lactic acid at a predictable and dependable rate. The metabolism of lactose is summarized in Figure 10.12. Most cheese starters are homofermentative, i.e. produce only lactic acid, usually the L-isomer; *Leuconostoc* species are heterofermentative. The products of lactic acid bacteria are summarized in Table 10.4.

Acid production plays several major roles in cheese manufacture:

- Controls or prevents the growth of spoilage and pathogenic bacteria.
- Affects coagulant activity during coagulation and the retention of active coagulant in the curd.

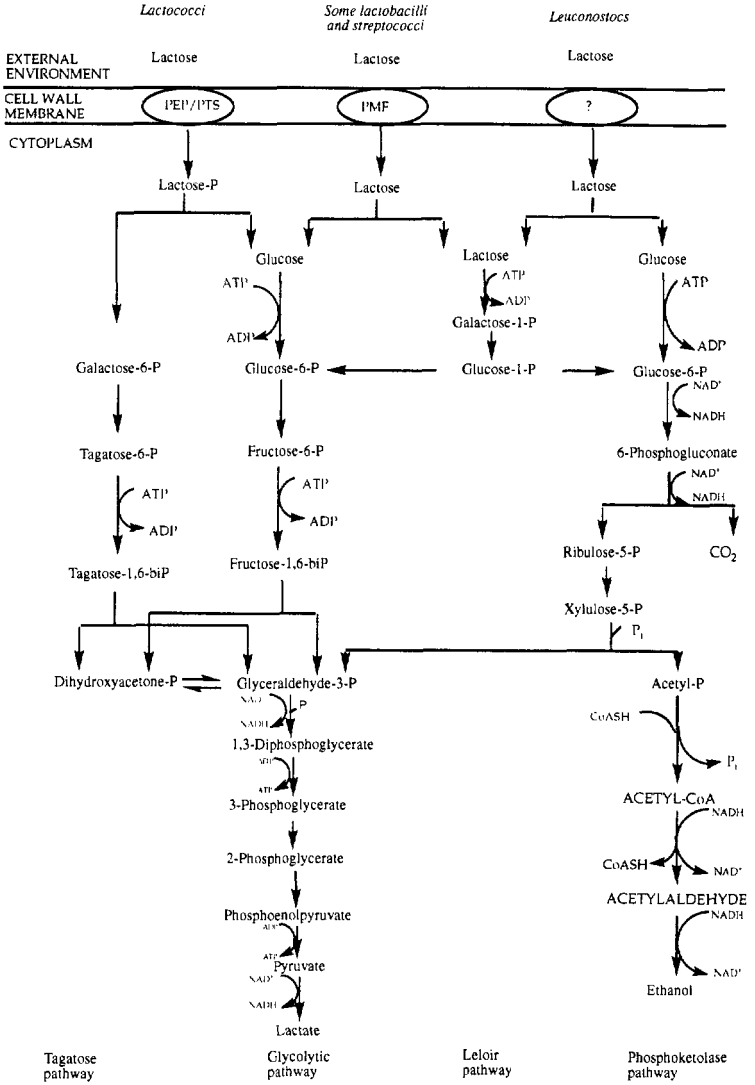


Figure 10.12 Metabolism of lactose by lactic acid bacteria; many *Lactobacillus* species/strains can not metabolize galactose (from Cogan and Hill, 1993).

- Solubilizes of colloidal calcium phosphate and thereby affects cheese texture; rapid acid production leads to a low level of calcium in the cheese and a crumbly texture (e.g. Cheshire) and vice versa (e.g. Emmental).
- Promotes syneresis and hence influences cheese composition.
- Influences the activity of enzymes during ripening, and hence affects cheese quality.

Table 10.4 Salient features of lactose metabolism in starter culture organisms (from Cogan and Hill, 1993)

Organism	Transport[a]	Cleavage[b] enzyme	Pathway[c]	Products (mol mol^{-1} lactose)
Lactococcus spp.	PTS	pβgal	GLY	4 L-Lactate
Leuconostoc spp.	?	βgal	PK	2 D-Lactate + 2 ethanol + 2CO_2
Str. salivarius subsp. *thermophilus*	PMF	βgal	GLY	2 L-Lactate[d]
Lb. delbrueckii subsp. *lactis*	PMF?	βgal	GLY	2 D-Lactate[d]
Lb. delbrueckii subsp. *bulgaricus*	PMF?	βgal	GLY	2 D-Lactate[d]
Lb. helveticus	PMF?	βgal	GLY	4 L- (mainly) + D-lactate

[a]PTS, phosphotransferase system; PMF, proton motive force.
[b]pβgal, phospho-β-galactosidase; βgal, β-galactosidase.
[c]GLY, glycolysis; PK, phosphoketolase.
[d]These species metabolize only the glucose moiety of lactose.

The primary starter performs several functions in addition to acid production, especially reduction of the redox potential (E_h, from about +250 mV in milk to −150 mV in cheese), and, most importantly, plays a major, probably essential, role in the biochemistry of cheese ripening. Many strains produce bacteriocins which control the growth of contaminating micro-organisms.

The ripening of many varieties is characterized by the action, not of the primary starter, but of other micro-organisms, which we will refer to as a secondary culture. Examples are *Propionibacterium* in Swiss-type cheeses, *Penicillium roqueforti* in Blue cheeses, *Penicillium camemberti* in surface mould-ripened cheeses, e.g. Camembert and Brie, *Brevibacterium linens* and yeasts in surface smear-ripened cheese, *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp. in Dutch-type cheeses. The specific function of these micro-organsims will be discussed in section 10.2.7 on ripening. Traditionally, a secondary culture was not used in Cheddar-type cheeses but there is much current interest in the use of cultures of selected bacteria, usually mesophilic *Lactobacillus* spp. or lactose-negative *Lactococcus* spp., for Cheddar cheese with the objective of intensifying or modifying flavour or accelerating ripening; such cultures are frequently referred to as 'adjunct cultures'.

10.2.4 Moulding and shaping

When the desired pH and moisture content have been achieved, the curds are separated from the whey and placed in moulds of traditional shape and size to drain and form a continuous mass; high-moisture curds form a

Figure 10.13 A selection of cheese varieties, showing the diversity of cheese size, shape and appearance.

continuous mass under their own weight but low-moisture varieties are pressed.

Cheeses are made up in traditional shapes (usually flat cylindrical, but also sausage, pear-shaped or rectangular) and size, ranging from around 250 g (e.g. Camembert) to 60–80 kg (e.g. Emmental; Figure 10.13). The size of cheese is not just a cosmetic feature; Emmental must be large enough to prevent excessive diffusion of CO_2, which is essential for eye development, while Camembert must be quite small so that the surface does not become over-ripe while the centre is still unripe (this cheese softens from the surface to the centre).

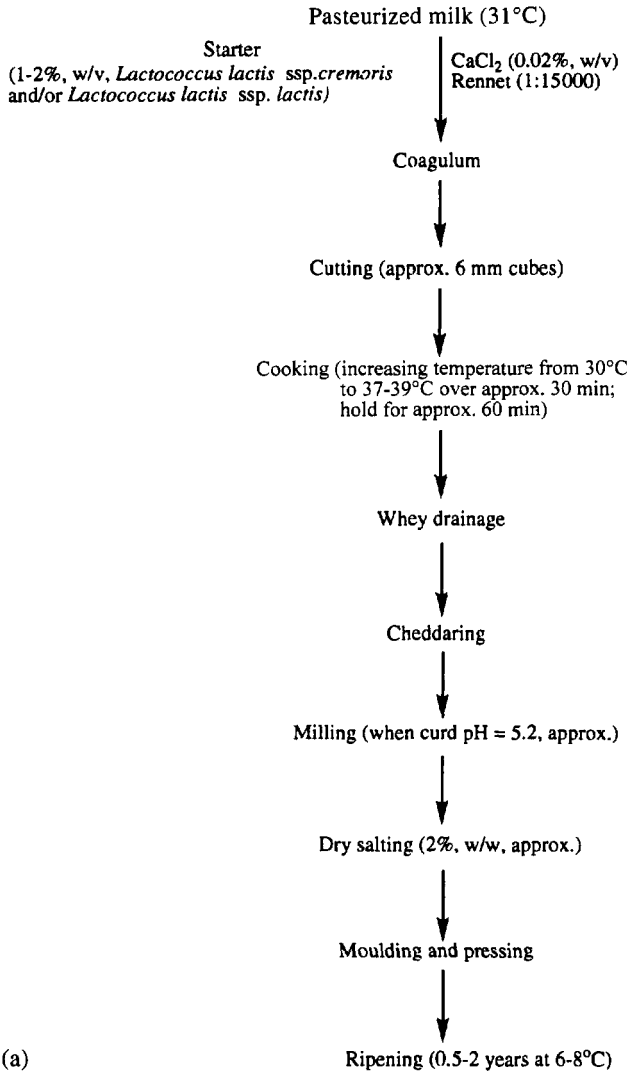
Curds for the *Pasta filata* cheeses, e.g. Mozzarella, Provolone and Halloumi, are heated in hot water (70–75°C), kneaded and stretched when the pH reaches about 5.4; this gives the cheeses a characteristic fibrous structure.

10.2.5 Salting

All cheeses are salted, either by mixing dry salt with the drained curd (confined largely to English varieties), rubbing dry salt on the surface of the pressed cheese (e.g. Romano or Blue cheeses), or by immersion of the pressed cheeses in brine (most varieties). Salt concentration varies from *c.* 0.7% (*c.* 2% salt-in-moisture) in Emmental to 7–8% (*c.* 15% salt-in-moisture) in Domiati.

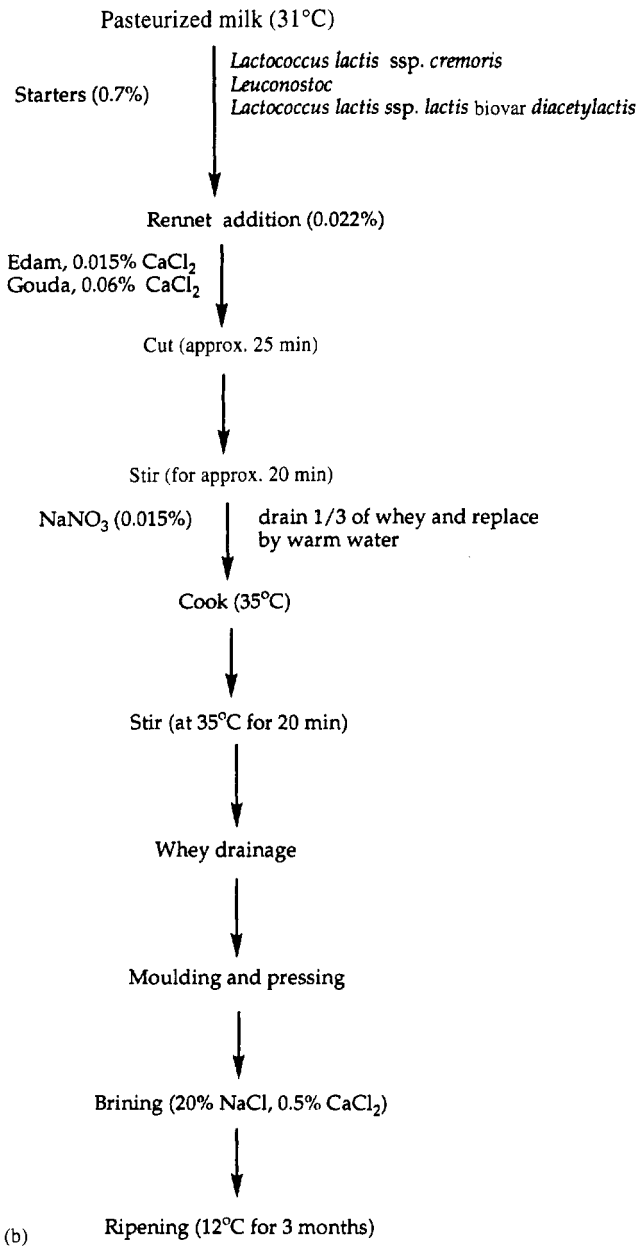
Salt plays a number of important roles in cheese:

- It is the principal factor affecting the water activity of young cheeses and has a major effect on the growth and survival of bacteria and the activity of enzymes in cheese, and hence affects and controls the biochemistry of cheese ripening.



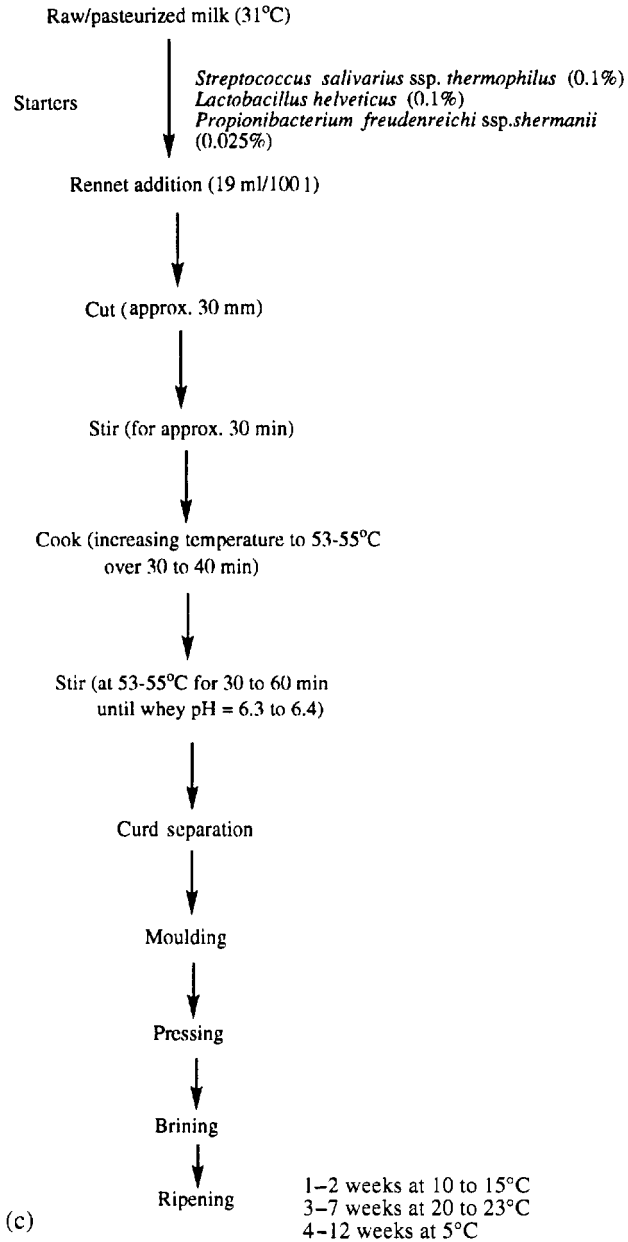
(a)

Figure 10.14 Protocols for the manufacture of (a) Cheddar, (b) Gouda, (c) Emmental and (d) Parmigiano-Reggiano.



(b)

Figure 10.14 (*Continued*).



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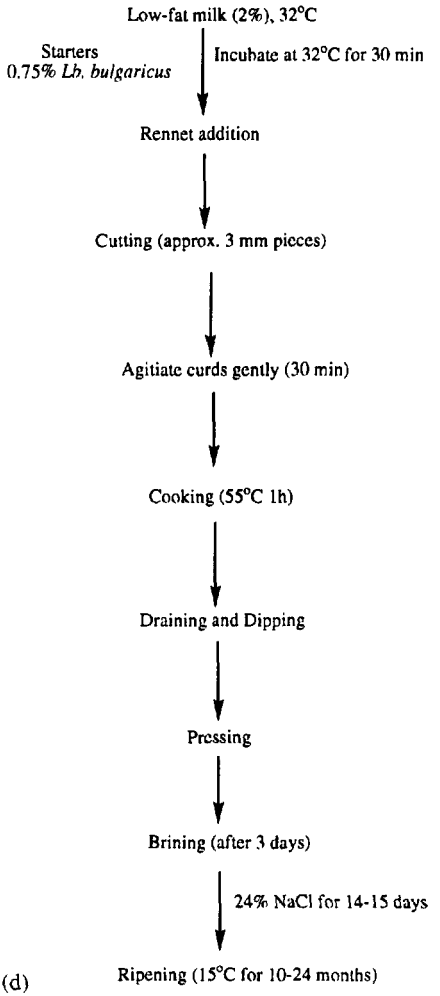


Figure 10.14 (*Continued*).

- Salting promotes syneresis and hence reduces the moisture content of cheese; about 2 kg of water are lost for each kilogram of salt absorbed.
- It has a positive effect on flavour.
- Cheese contributes to dietary sodium, high levels of which have undesirable nutritional consequences, e.g. hypertension and osteoporosis.

10.2.6 Manufacturing protocols for some cheese varieties

The manufacturing protocols for the various cheese varieties differ in detail but many elements are common to many varieties. The protocols for the principal varieties are summarized in Figures 10.14a–d.

10.2.7 Cheese ripening

While rennet-coagulated cheese curd may be consumed immediately after manufacture (and a little is), it is rather flavourless and rubbery. Consequently, rennet-coagulated cheeses are ripened (matured) for a period ranging from about 3 weeks for Mozzarella to more than 2 years for Parmesan and extra-mature Cheddar. During this period, a very complex series of biological, biochemical and chemical reactions occur through which the characteristic flavour compounds are produced and the texture altered.

Four, and in some cheeses five or perhaps six, agents are responsible for these changes:

1. The cheese milk. As discussed in Chapter 8, milk contains about 60 indigenous enzymes, many of which are associated with the fat globules or casein micelles and are therefore incorporated into the cheese curd; the soluble enzymes are largely removed in the whey. Many of the indigenous enzymes are quite heat stable and survive HTST pasteurization; at least three of these (plasmin, acid phosphatase and xanthine oxidase) are active in cheese and contribute to cheese ripening; some indigenous lipase may also survive pasteurization. The contribution of other indigenous enzymes to cheese ripening is not known.
2. Coagulant. Most of the coagulant is lost in the whey but some is retained in the curd. Approximately 6% of added chymosin is normally retained in Cheddar and similar varieties, including Dutch types; the amount of rennet retained increases as the pH at whey drainage is reduced. As much as 20% of added chymosin is retained in high-moisture, low-pH cheese, e.g. Camembert. Only about 3% of microbial rennet substitutes is retained in the curd and the level retained is independent of pH.

   Porcine pepsin is very sensitive to denaturation at pH 6.7 but becomes more stable as the pH is reduced.

   The coagulant is major contributor to proteolysis in most cheese varieties, notable exceptions being high-cooked varieties, e.g. Emmental and Parmesan, in which the coagulant is extensively or totally denatured during curd manufacture.

   A good-quality rennet extract is free of lipolytic activity but a rennet paste is used in the manufacture of some Italian varieties, e.g. Romano and Provolone. Rennet paste contains a lipase, referred to as pre-gastric esterase (PGE), which makes a major contribution to lipolysis in, and to the characteristic flavour of, these cheeses. Rennet paste is considered unhygienic and therefore semi-purified PGE may be added to rennet extract for such cheeses (Chapter 8).
3. Starter bacteria. The starter culture reaches maximum numbers at the end of the manufacturing phase. Their numbers then decline at a rate depending on the strain, typically by 2 log cycles within 1 month. At least some of the non-viable cells lyse at a rate dependent on the strain. As far as is known, the only extracellular enzyme in *Lactococcus*, *Lactobacillus*

and *Streptococcus* is a proteinase which is attached to the cell membrane and protrudes through the cell wall; all peptidases, esterases and phosphatases are intracellular and therefore cell lysis is essential before they can contribute to ripening.

4. Non-starter bacteria. Cheese made from pasteurized, high-quality milk in modern factories using enclosed automated equipment contains very few non-starter bacteria ($<50\,\text{cfu}\,\text{g}^{-1}$) at one day but these multiply to 10^7–$10^8\,\text{cfu}\,\text{g}^{-1}$ within about 2 months (at a rate depending on, especially, temperature). Since the starter population declines during this period, non-starter bacteria dominate the microflora of cheese during the later stages of ripening.

   Properly made cheese is quite a hostile environment for bacteria due to a low pH, moderate-to-high salt in the moisture phase, anaerobic conditions (except at the surface), lack of a fermentable carbohydrate and the production of bacteriocins by the starter. Consequently, cheese is a very selective environment and its internal non-starter microflora is dominated by lactic acid bacteria, especially mesophilic lactobacilli, and perhaps some *Micrococcus* and *Pediococcus*.
5. Secondary and adjunct cultures. As discussed in section 10.2.3, many cheese varieties are characterized by the growth of secondary microorganisms which have strong metabolic activity and dominate the ripening and characteristics of these cheeses.
6. Other exogenous enzymes. An exogenous lipase is added to milk for a few varieties, e.g. pre-gastric lipase (in rennet paste) for Romano or Provolone cheese. In recent years, there has been considerable academic and commercial interest in adding exogenous proteinases (in addition to the coagulant) and/or peptidases to accelerate ripening. The enzymes may be added to the milk or curd in various forms, e.g. free, microencapsulated or in attenuated cells.

The contribution of these agents, individually or in various combinations, has been assessed in model cheese systems from which one or more of the agents was excluded or eliminated, e.g. by using an acidogen rather than starter for acidification or manufacturing cheese in a sterile environment to eliminate non-starter lactic acid bacteria (NSLAB). Such model systems have given very useful information on the biochemistry of ripening.

During ripening, three primary biochemical events occur, glycolysis, lipolysis and proteolysis. The products of these primary reactions undergo numerous modifications and interactions. The primary reactions are fairly well characterized but the secondary changes in most varieties are more or less unknown. An overview of the principal biochemical changes follows.

Glycolysis. Most (about 98%) of the lactose in cheese-milk is removed in the whey as lactose or lactic acid. However, fresh cheese curd contains 1–2%

lactose which is normally metabolized to L-lactic acid by the *Lactococcus* starter within a day for most varieties or a few weeks for Cheddar. In most varieties, the L-lactate is racemized to DL-lactate by NSLAB within about 3 months and a small amount is oxidized to acetic acid at a rate dependent on the oxygen content of the cheese and hence on the permeability of the packaging material.

In cheese varieties made using *Streptococcus salvarius* ssp. *thermophilus* and *Lactobacillus* spp. as starter, e.g. Swiss types and Mozzarella, the metabolism of lactose is more complex than in cheese in which a *Lactococcus* starter is used. In these cheeses, the curd is cooked to 52–55°C, which is above the growth temperature for both components of the starter; as the curd cools, the *Streptococcus*, which is the more heat-tolerant of the two starters, begins to grow, utilizing the glucose moiety of lactose, with the production of L-lactic acid, but not galactose, which accumulates in the curd. When the curd has cooled sufficiently, the *Lactobacillus* spp. grow, and, if a galactose-positive species/strain is used, it metabolizes galactose, producing DL-lactate (Figure 10.15). If a galactose-negative strain of *Lactobacillus* is used, galactose accumulates in the curd and can participate in Maillard browning, especially during heating, which is undesirable, especially in Pizza cheese.

Swiss-type cheeses are ripened at about 22°C for a period to encourage the growth of *Propionibacterium* spp. which use lactic acid as an energy

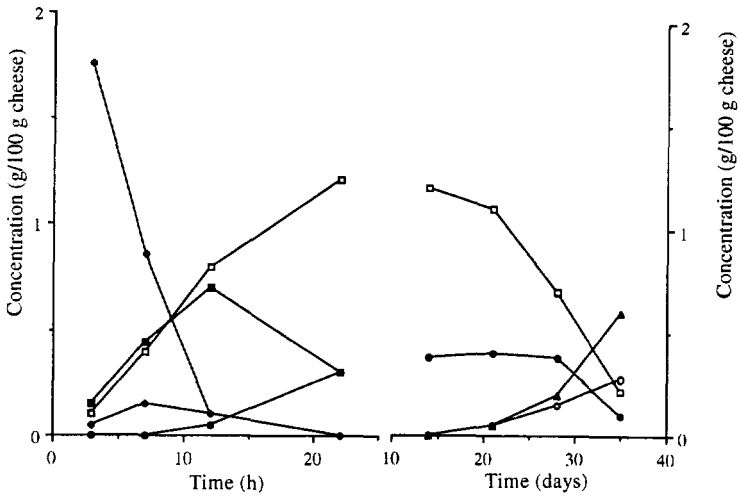


Figure 10.15 Metabolism of lactose, glucose, galactose, D- and L-lactic acid in Emmental cheese. Cheese transferred to hot room (22–24°C) at 14 days. ●, D-lactate; ○, acetate; ■, galactose; □, L-lactate; ♦, glucose; ◇, lactose; ▲, propionate.

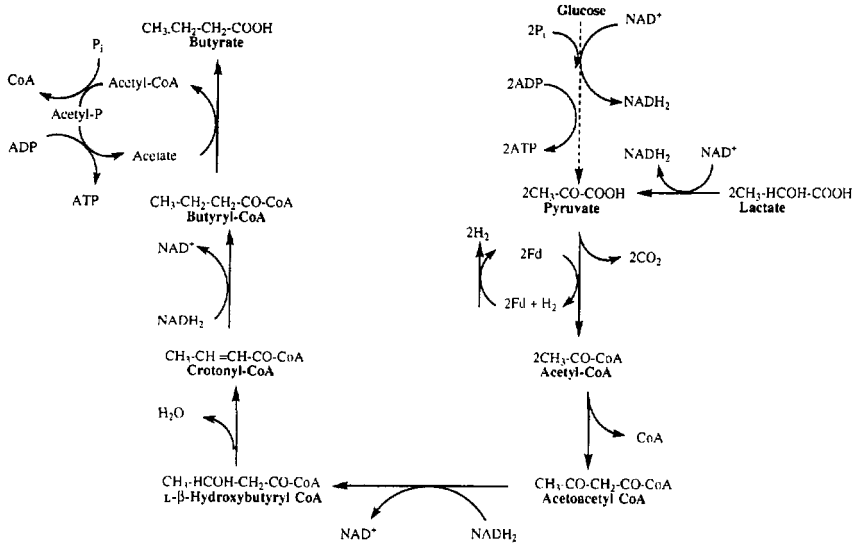


Figure 10.16 Metabolism of glucose or lactic acid by *Clostridium tyrobutyricum* with the production of butyric acid, CO_2 and hydrogen gas.

source, producing propionic acid, acetic acid and CO_2 (Figure 10.15):

$$3CH_3CHOHCOOH \rightarrow 2CH_3CH_2COOH + CH_3COOH + CO_2 + H_2O$$

Lactic acid — Propionic acid — Acetic acid

Propionic and acetic acids probably contribute to the flavour of Swiss-type cheeses, while the CO_2 is responsible for their large characteristic eyes. Lactic acid may be metabolized by *Clostridium tyrobutyricum* to butyric acid, CO_2 and hydrogen (Figure 10.16); butyric acid is responsible for off-flavours and the CO_2 and H_2 for late gas blowing. Clostridia are controlled by good hygienic practices, addition of nitrate or lysozyme, bactofugation or microfiltration. The principal sources of clostridia are soil and silage.

In surface mould-ripened cheeses, e.g. Camembert and Brie, *Penicillium camemberti*, growing on the surface, metabolizes lactic acid as an energy source, causing the pH to increase. Lactic acid diffuses from the centre to the surface, where it is catabolized. Ammonia produced by deamination of amino acids contributes to the increase in pH which reaches about 7.5 at the surface and 6.5 at the centre of the cheese. Ripening of Camembert and Brie is characterized by softening (liquefaction) of the texture from the surface towards the centre. Softening is due to the increase in pH, proteolysis and diffusion of calcium phosphate to the surface, where it precipitates due to the high pH. These events are summarized in Figure 10.17.

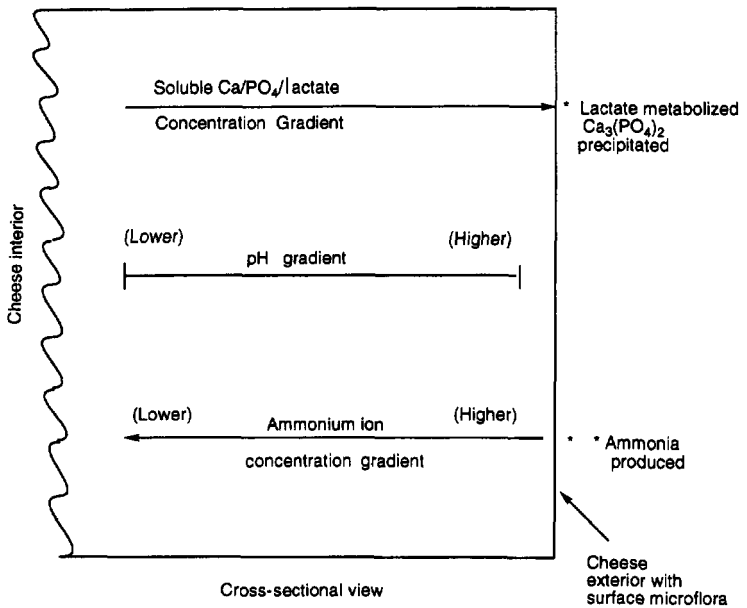


Figure 10.17 Schematic representation of the gradients of calcium, phosphate, lactic acid, pH and ammonia in ripening of Camembert cheese.

In surface smear-ripened cheeses, e.g. Munster, Limburger, Tilsit, Trapist, the surface of the cheese is colonized first by yeasts which catabolize lactic acid, causing the pH to increase, and then by *Brevibacterium linens*, the characteristic micro-organism of the surface smear but which does not grow below pH 5.8, and various other micro-organisms, including *Micrococcus*, *Arthrobacter* and coryneform bacteria.

Lipolysis. Some lipolysis occurs in all cheeses; the resulting fatty acids contribute to cheese flavour. In most varieties, lipolysis is rather limited (Table 10.5) and is caused mainly by the limited lipolytic activity of the starter and non-starter lactic acid bacteria, perhaps with a contribution from indigenous milk lipase, especially in cheese made from raw milk.

Extensive lipolysis occurs in two families of cheese in which fatty acids and/or their degradation products are major contributors to flavour, i.e. certain Italian varieties (e.g. Romano and Provolone) and the Blue cheeses. Rennet paste, which contains pre-gastric esterase (PGE) rather than rennet extract, is used in the manufacture of these Italian cheeses. PGE is highly specific for the fatty acids on the *sn*-3 position of glycerol, which, in the case of milk lipids, are predominantly highly flavoured short-chain fatty acids (butanoic to decanoic). These acids are principally responsible for the characteristic piquant flavour of these Italian cheeses.

Table 10.5 Free fatty acids in a selection of cheese varieties (Woo and Lindsay, 1984; Woo, Kollodge and Lindsay, 1984)

Variety	FFA (mg kg^{-1})	Variety	FFA (mg kg^{-1})
Sapsago	211	Gjetost	1658
Edam	356	Provolone	2118
Mozzarella	363	Brick	2150
Colby	550	Limburger	4187
Camembert	681	Goats' milk	4558
Port Salut	700	Parmesan	4993
Moneterey Jack	736	Romano	6743
Cheddar	1028	Roquefort	32453
Gruyere	1481	Blue (US)	32230

Blue cheeses undergo very extensive lipolysis during ripening; up to 25% of all fatty acids may be released. The principal lipase in Blue cheese is that produced by *Penicillium roqueforti*, with minor contributions from indigenous milk lipase and the lipases of starter and non-starter lactic acid bacteria. The free fatty acids contribute directly to the flavour of Blue cheeses but, more importantly, they undergo partial β-oxidation to alkan-2-ones (methyl ketones; ($R{-}\overset{\overset{O}{\|}}{C}{-}CH_3$) through the catabolic activity of the mould (Figure 10.18). A homologous series of alkan-2-ones from C_3 to C_{17} is formed (corresponding to the fatty acids from C_4 to C_{18}), but heptanone and nonanone predominate; typical concentrations are shown in Table 10.6. The characteristic peppery flavour of Blue cheeses is due to alkan-2-ones. Under anaerobic conditions, some of the alkan-2-ones may be reduced to the corresponding alkan-2-ols (secondary alcohols), which cause off-flavours.

Proteolysis. Proteolysis is the most complex, and perhaps the most important, of the three primary biochemical events in the ripening of most cheese varieties. In internal, bacterially ripened cheeses, e.g. Cheddar, Dutch and Swiss varieties, it is mainly responsible for the textural changes that occur during ripening, i.e. conversion of the tough rubbery texture of fresh curd to the smooth, pliable body of mature cheese. Small peptides and free amino acids contribute directly to cheese flavour and amino acids serve as substrates in several flavour-generating reactions, e.g. decarboxylation, deamination and desulphuration. Amino acids may also react chemically with carbonyls via the Maillard reaction and Strecker degradation, with the production of a great diversity of sapid compounds (Chapter 2). Excessive amounts of hydrophobic peptides may be produced under certain circumstances and may lead to bitterness which some consumers find very objectional; however, at an appropriate concentration, and when properly balanced by other compounds, bitter peptides probably contribute positively to cheese flavour.

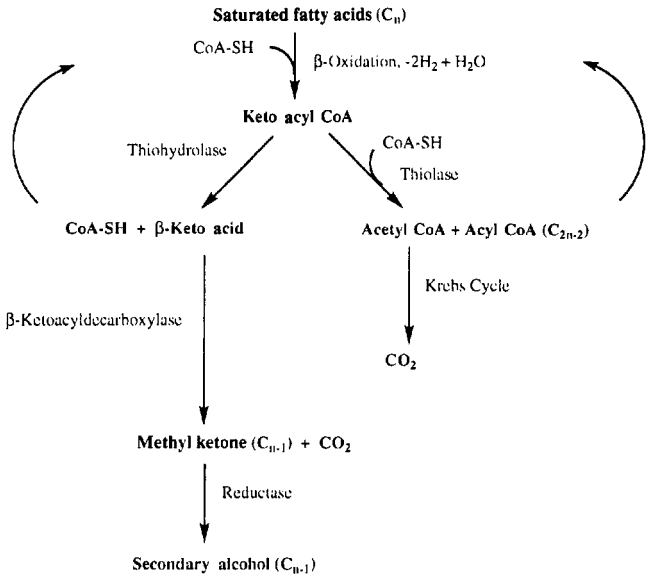


Figure 10.18 β-Oxidation of fatty acids to methyl ketones by *Penicillium roqueforti* and subsequent reduction to secondary alcohols.

Table 10.6 Typical concentrations of alkan-2-ones in Blue cheese (from Kinsella and Hwang, 1976)

	μg per 10 g dry Blue cheese							
2-Alkanone	A[a]	B[a]	C[a]	D[b]	E[b]	F[b]	G[c]	H[c]
2-Propanone	65	54	75	210	–	0	60	T[d]
2-Pentanone	360	140	410	1022	367	51	372	285
2-Heptanone	800	380	380	1827	755	243	3845	3354
2-Nonanone	560	440	1760	1816	600	176	3737	3505
2-Undecanone	128	120	590	136	135	56	1304	1383
2-Tridecanone	–	–	–	100	120	77	309	945
Total	1940	1146	4296	5111	1978	603	9627	9372

[a]Commercial samples of ripe Blue cheese.
[b]Samples D, E and F of Blue cheese ripened for 2, 3 and 4 months, respectively.
[c]Samples G and H of very small batches of experimental Blue cheese ripened for 2 and 3 months, respectively.
[d]Trace.

The level of proteolysis in cheese varies from limited (e.g. Mozzarella) through moderate (e.g. Cheddar and Gouda) to very extensive (e.g. Blue cheeses). The products of proteolysis range from very large polypeptides, only a little smaller than the parent caseins, to amino acids which may, in turn, be catabolized to a very diverse range of sapid compounds, including amines, acids and sulphur compounds.

Depending on the depth of information required, proteolysis in cheese is assessed by a wide range of techniques. Electrophoresis, usually urea–PAGE, is particularly appropriate for monitoring primary proteolysis, i.e. proteolysis of the caseins and the resulting large polypeptides. Quantifying the formation of peptides and amino acids soluble in water, at pH 4.6, in TCA, ethanol or phosphotungstic acid, or the measurement of free amino groups by reaction with ninhydrin, *o*-phthaldialdehyde, trinitrobenzene or fluorescamine, is suitable for monitoring secondary proteolysis. Reversed phase HPLC is especially useful for fingerprinting the small peptide profile in cheese and is now widely used. High-performance ion-exchange or size exclusion chromatography are also effective but are less widely used.

Proteolysis has not yet been fully characterized in any cheese variety but considerable progress has been made for Cheddar and, as far as is known, generally similar results apply to other low-cook, internal bacterially ripened cheeses (e.g. Dutch types). Proteolysis in Cheddar will be summarized as an example of these types of cheese.

Urea–PAGE shows that α_{s1}-casein is completely hydrolysed in Cheddar within 3–4 months (Figure 10.19). It is hydrolysed by chymosin, initially at Phe_{23}–Phe_{24} and later at Leu_{101}–Lys_{102}, and to a lesser extent at Phe_{32}–Gly_{33}, Leu_{98}–Lys_{99} and Leu_{109}–Glu_{110}. Although β-casein in solution is readily hydrolysed by chymosin, in cheese β-casein is very resistant to chymosin but is hydrolysed slowly (*c.* 50% at 6 months) by plasmin at Lys_{28}–Lys_{29}, Lys_{105}–His/Gln_{106} and Lys_{107}–Glu_{108}, producing γ^1, γ^2- and γ^3-caseins, respectively, and the corresponding proteose–peptones (PP5, PP8 slow and PP8 fast; Chapter 4). Chymosin and, to lesser extent, plasmin

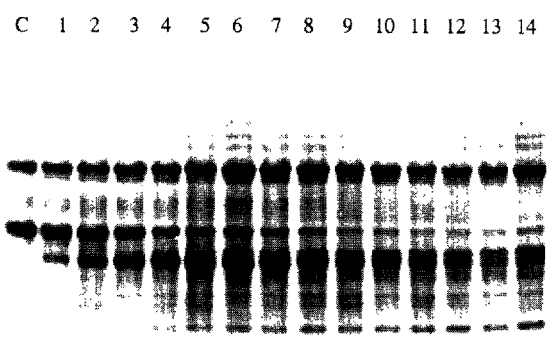


Figure 10.19 Urea-polyacrylamide gel electrophoretograms of Cheddar cheese after ripening for 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18 or 20 weeks (lanes 1–14); C, sodium caseinate. (Supplied by S. Mooney.)

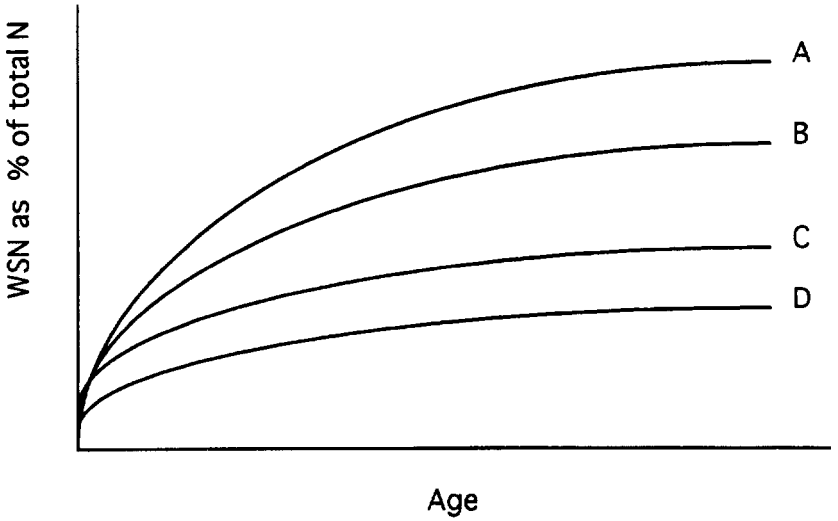


Figure 10.20 Formation of water-soluble nitrogen (WSN) in: (A) Cheddar cheese with a controlled microflora (free of non-starter bacteria); (B) controlled microflora chemically-acidified (starter-free) cheese; (C) controlled microflora, rennet-free cheese; (D) controlled microflora, rennet-free, starter-free cheese.

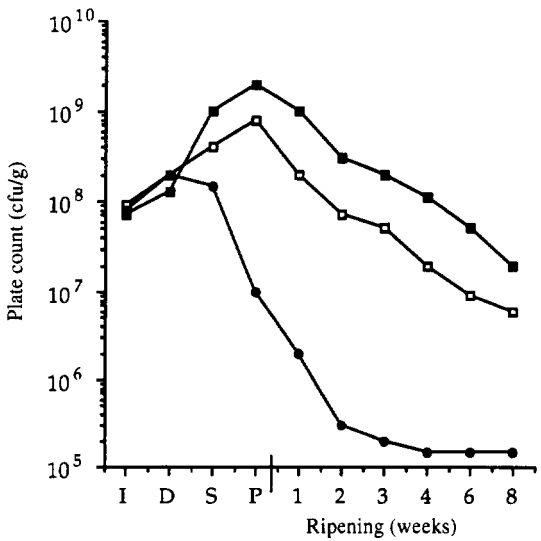


Figure 10.21 Changes in the population of starter cells in cheese made using different single strain starters. I, Inoculation; D, whey drainage; S, salting; P, after pressing.

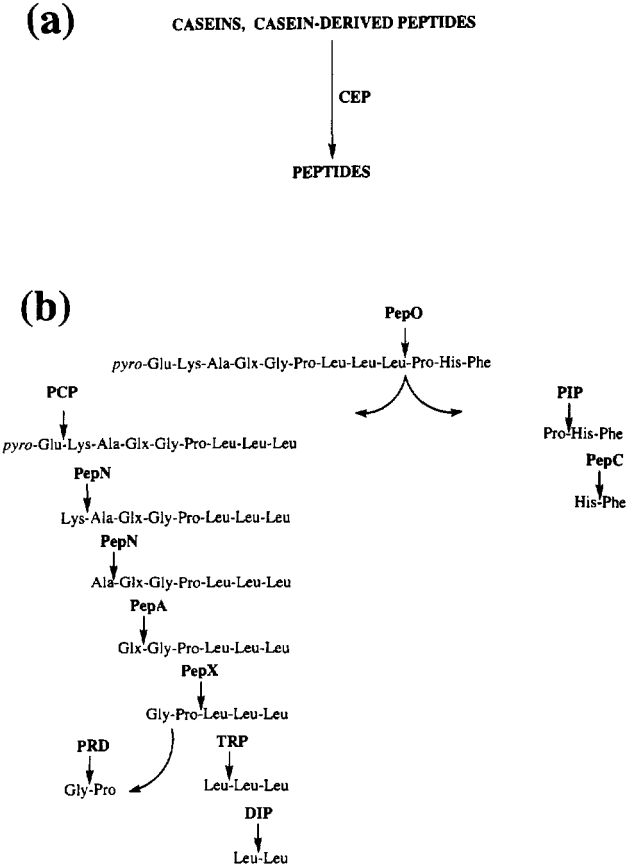


Figure 10.22 Schematic representation of the hydrolysis of casein (a) by lactococcal cell envelope proteinase (CEP), and (b) degradation of an hypothetical dodecapeptide by the combined action of lactococcal peptidases: oligopeptidase (PepO), various aminopeptidases (PCP, PepN, PepA, PepX), tripeptidase (TRP), prolidase (PRD) and dipeptidase (DIP).

are mainly responsible for primary proteolysis, i.e. the formation of water (or pH 4.6)-soluble N, as summarized in Figure 10.20.

Although *in vitro*, the cell wall-associated proteinase of the *Lactococcus* starters is quite active on β-casein (and that from some strains on α_{s1}-casein also), in cheese, they appear to act mainly on casein-derived peptides, produced by chymosin from α_{s1}-casein or by plasmin from β-casein.

The starter cells begin to die off at the end of curd manufacture (Figure 10.21); the dead cells may lyse and release their intracellular endopeptidases (Pep O, Pep F), aminopeptidases (including Pep N, Pep A, Pep C, Pep X), tripeptidases and dipeptidases (including proline-specific peptidases) which produce a range of free amino acids (Figure 10.22). About 150 peptides have

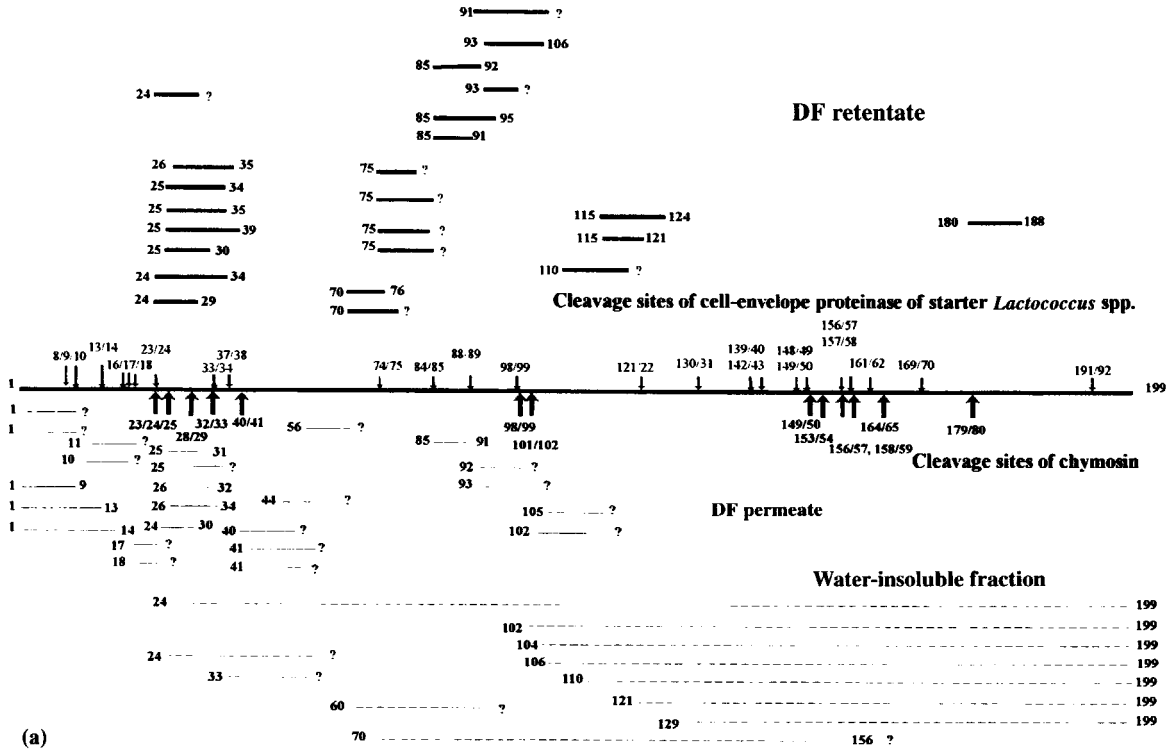


Figure 10.23 Water-insoluble and water-soluble peptides derived from α_{s1}-casein (A), α_{s2}-casein (B) or β-casein (C) isolated from Cheddar cheese; DF = diafiltration. The principal chymosin, plasmin and lactococcal cell-envelope proteinase cleavage sites are indicated by arrows (data from T.K. Singh and S. Mooney, unpublished).

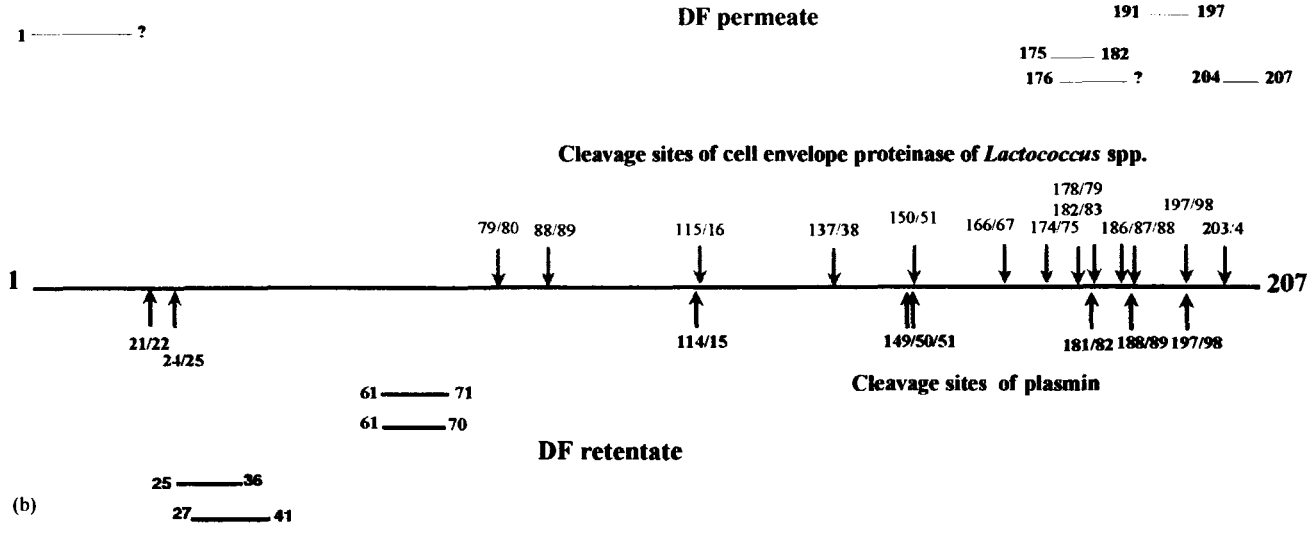
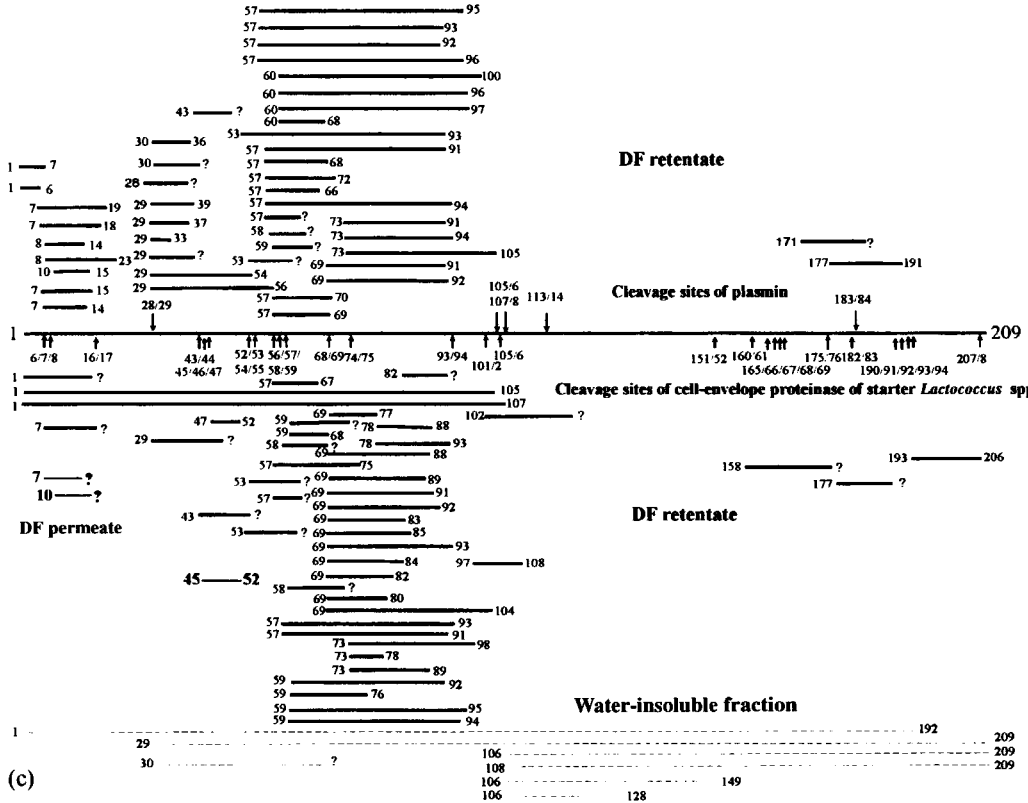


Figure 10.23 (*Continued*).



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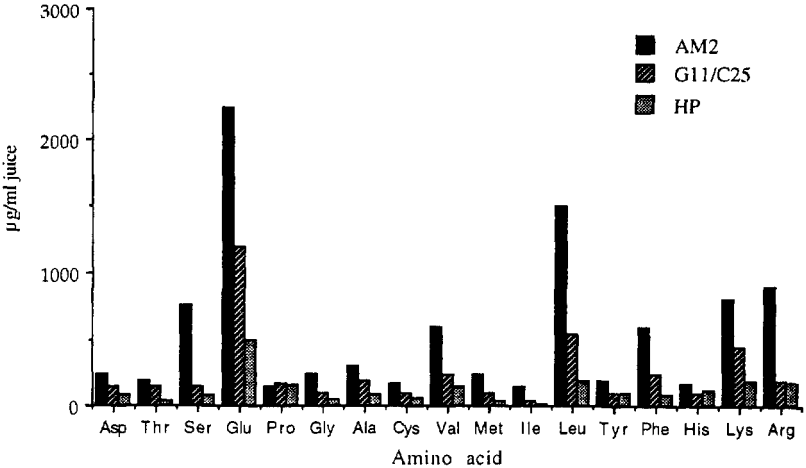

Figure 10.24 Concentration of individual amino acids in 60-day-old Cheddar cheese, made with a single-strain starter *Lactococcus lactis* ssp. *cremoris* AM_2, G11/C25 or HP (from Wilkinson, 1992).

been isolated from the water-soluble fraction of Cheddar, and characterized (Figure 10.23). These show that both lactococcal proteinase and exopeptidase contribute to proteolysis in cheese. The proteinases and peptidases of the NSLAB (mainly mesophilic lactobacilli) appear to contribute little to proteolysis in Cheddar, except in the production of amino acids.

The principal amino acids in Cheddar are shown in Figure 10.24.

10.2.8 Cheese flavour

Although interest in cheese flavour dates from the beginning of this century, very little progress was made until the development of gas liquid chromatography (GC) in the late 1950s, and especially the coupling of GC and mass spectrometry (MS). More than 200 volatile compounds have been identified in cheese by GC–MS (principal compounds are listed in Table 10.7). The volatile fraction of cheese may be obtained by taking a sample of headspace but the concentration of many compounds is too low, even for modern GC–MS techniques. The volatiles may be concentrated by solvent extraction or distillation. In the former, a large solvent peak may mask important constituents while the latter may generate artefacts, even at moderately low temperatures. Trapping of volatiles, e.g. on adsorbants or in cold traps, is probably the most satisfactory method for concentration.

The taste of cheese is concentrated in the water-soluble fraction (peptides, amino acids, organic acids, amines, NaCl) while the aroma is mainly in the volatile fraction. Initially, it was believed that cheese flavour was due to one

Table 10.7 Volatile compounds which have been identified in Cheedar cheese (modified from Urbach, 1993)

Acetaldehyde	Dimethyl disulfide	2-Methylbutanol
Acetoin	Dimethyl trisulfide	3-Methylbutanol
Acetone	δ-Dodecalactone	3-Methyl-2-butanone
Acetophenone	Ethanol	3-Methylbutyric acid
1,2-Butanediol	Ethyl butanol	2-Nonanone
n-Butanol	2-Ethyl butanol	δ-Octalactone
2-Butanol	Ethyl butyrate	*n*-Octanoic acid
Butanone	Ethyl hexanoate	2-Octanol
n-Butyl acetate	2-Heptanone	2,4-Pentanediol
2-Butyl acetate	*n*-Hexanal	*n*-Pentanoic acid
n-Butyl butyrate	*n*-Hexanoic acid	2-Pentanol
n-Butyric acid	*n*-Hexanol	Pentan-2-one
Carbon dioxide	2-Hexanone	*n*-Propanol
p-Cresol	Hexanethiol	Propanal
γ-Decalactone	2-Hexenal	Propenal
δ-Decalactone	Isobutanol	*n*-Propyl butyrate
n-Decanoic acid	Isohexanal	Tetrahydrofuran
Diacetyl	Methanethiol	Thiophen-2-aldehyde
Diethyl ether	Methional	2-Tridecanone
Dimethyl sulfide	Methyl acetate	2-Undecanone

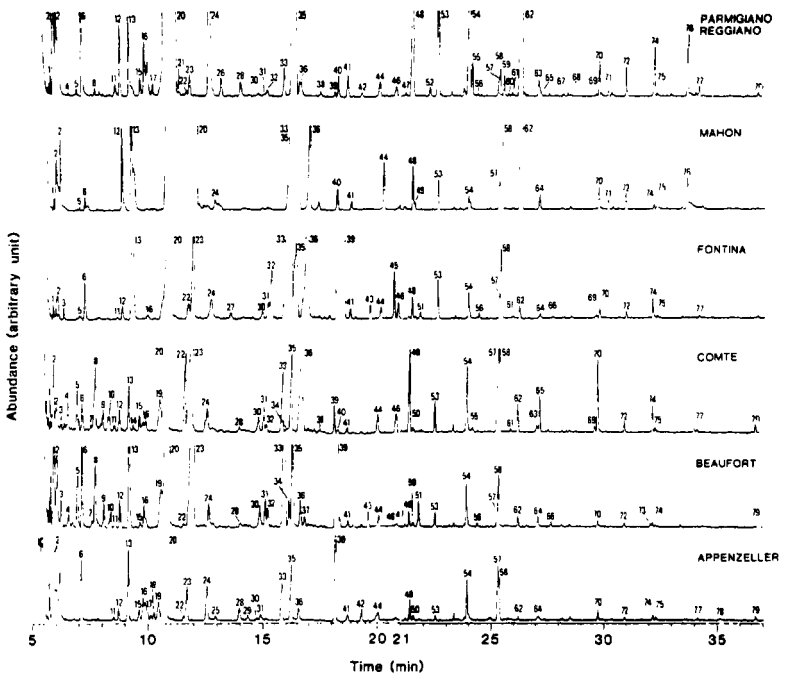

Figure 10.25 GC-MS chromatograms of the headspace volatiles of six cheese varieties (from Bosset and Gauch, 1993).

or a small number of compounds, but it was soon realized that all cheeses contained essentially the same sapid compounds. Recognition of this led to the component balance theory, i.e. cheese flavour is due to the concentration and balance of a range of compounds. Although considerable information on the flavour compounds in several cheese varieties has been accumulated, it is not possible to fully describe the flavour of any variety, with the possible exception of Blue cheeses, the flavour of which is dominated by alkan-2-ones.

Many cheeses contain the same or similar compounds but at different concentrations and proportions; chromatograms of some cheese varieties are shown in Figure 10.25. The principal classes of components present are aldehydes, ketones, acids, amines, lactones, esters, hydrocarbons and sulphur compounds; the latter, e.g. H_2S, methanethiol (CH_3SH), dimethyl sulphide ($H_3C\text{-}S\text{-}CH_3$) and dimethyl disulphide ($H_3C\text{-}S\text{-}S\text{-}CH_3$), are considered to be particularly important in Cheddar cheese. The biogenesis of flavour compounds has been reviewed by Fox *et al.* (1993, 1996a) and Fox, Singh and McSweeney (1995).

10.2.9 Accelerated ripening of cheese

Since the ripening of cheese, especially low moisture varieties, is a slow process, it is expensive in terms of controlled atmosphere storage and stocks. Ripening is also unpredictable. Hence, there are economic and technological incentives to accelerate ripening, while retaining or improving characteristic flavour and texture.

The principal approaches used to accelerate cheese ripening are:

1. Elevated ripening temperatures, especially for Cheddar which is now usually ripened at 6–8°C; most other varieties are ripened at a higher temperature, e.g. around 14°C for Dutch types or 20–22°C for Swiss types and Parmesan, and hence there is little or no scope for increasing the ripening temperature.
2. Exogenous enzymes, usually proteinases and/or peptidases. For several reasons, this approach has had limited success, except for enzyme-modified cheeses (EMC). These are usually high-moisture products which are used as ingredients for processed cheese, cheese spreads, cheese dips or cheese flavourings.
3. Attenuated lactic acid bacteria, e.g. freeze-shocked, heat-shocked or lactose-negative mutants.
4. Adjunct starters, especially mesophilic lactobacilli.
5. Use of fast-lysing starters which die and release their intracellular enzymes rapidly.
6. Genetically modified starters which super-produce certain enzymes; unfortunately, the key enzymes are not yet known.

The lack of definitive information on the key flavour-generating reactions in cheese is hampering efforts to accelerate ripening, which are, at present, empirical. Considerable in-depth information on the biochemistry of cheese ripening is now becoming available which will facilitate the genetic engineering of starter cultures with improved cheesemaking properties. Acceleration of cheese ripening has been reviewed by Fox *et al.* (1996b).

10.3 Acid-coagulated cheeses

On acidification to pH 4.6, the caseins coagulate, which is the principle used to manufacture of a family of cheeses which represent about 25% of total cheese consumption and are the principal cheeses in some countries (Appendix 10B). Acidification is traditionally and usually achieved by *in situ* fermentation of lactose by a *Lactococcus* starter but direct acidification by acid or acidogen (gluconic acid-δ-lactone) is also practised. The principal

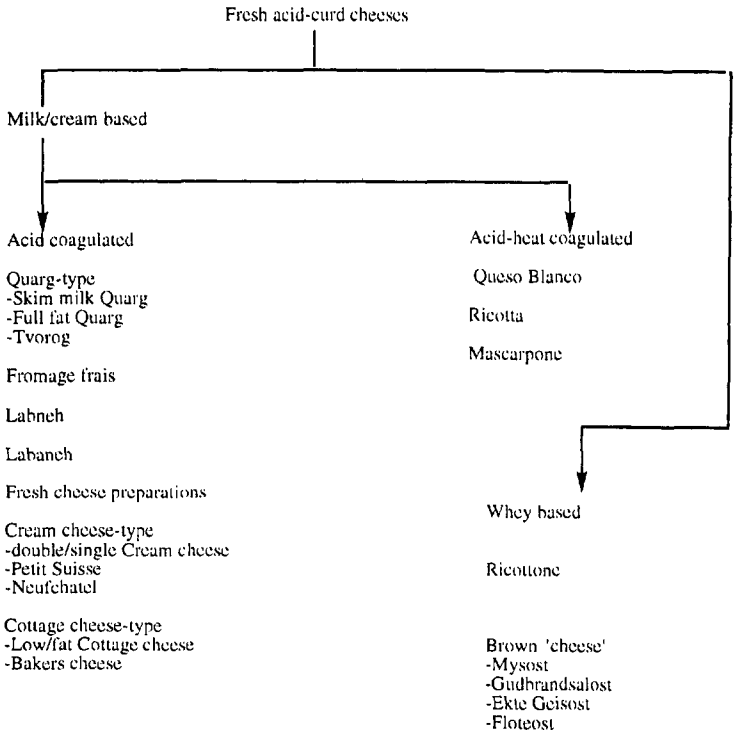

Figure 10.26 Examples of acid-coagulated or heat-acid coagulated or whey-based cheese varieties (from Fox *et al.*, 1996a).

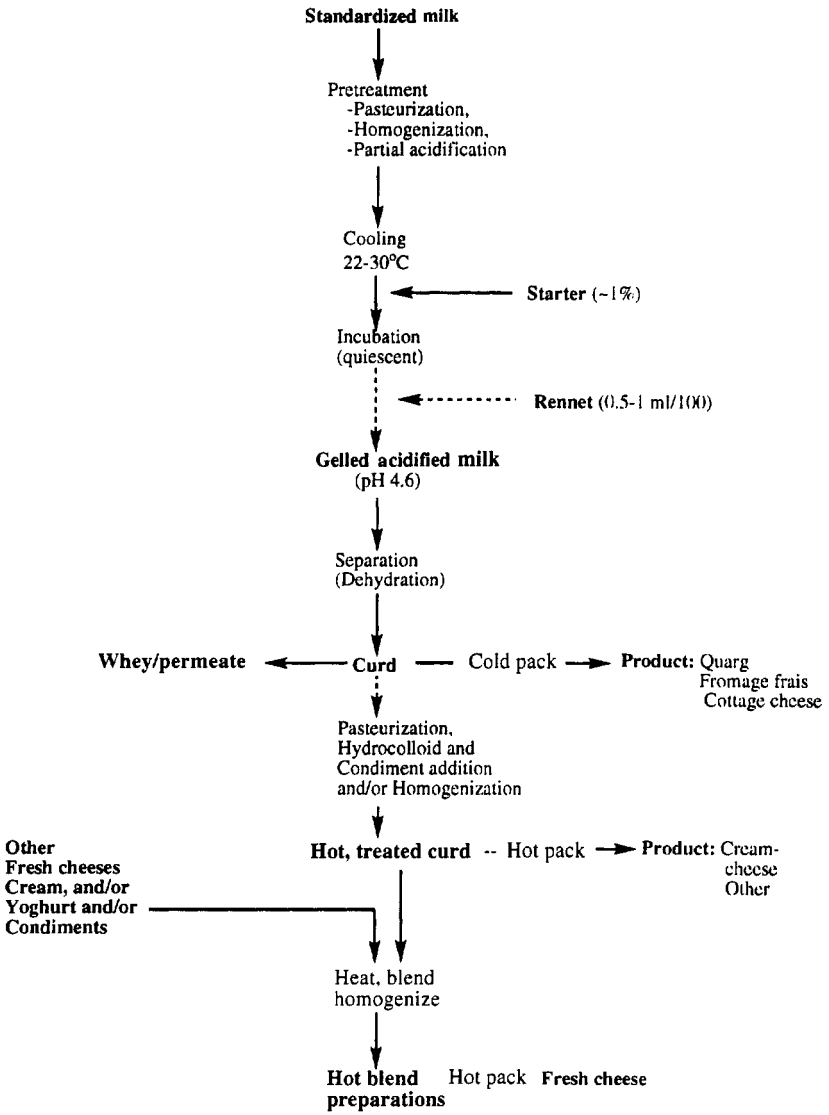

Figure 10.27 Protocol for the manufacture of fresh acid-coagulated cheese (from Fox *et al.*, 1996a).

families of acid-coagulated cheeses are illustrated in Figure 10.26 and a typical manufacturing protocol is shown in Figure 10.27.

Acid-coagulated cheeses are usually produced from skim milk and are consumed fresh. Major varieties include quarg, (American) cottage cheese, cream cheese and petit suisse. These cheeses may be consumed in salads, as

food ingredients and serve as the base for a rapidly expanding group of dairy products, i.e. fromage frais-type products.

The casein may also be coagulated at a pH above 4.6, e.g. about 5.2, by using a higher temperature, e.g. 80–90°C. This principle is used to manufacture another family of cheeses, which include Ricotta (and variants thereof), Anari, and some types of Queso Blanco. These cheeses may be made exclusively from whey but usually from a blend of milk and whey and are usually used as a food ingredient, e.g. in lasagne or ravioli.

10.4 Processed cheese products

Processed cheese is produced by blending shredded natural cheese of the same or different varieties and at different degrees of maturity with emulsifying agents and heating the blend under vacuum with constant agitation until a homogeneous mass is obtained. Other dairy and non-dairy ingredients may be included in the blend. The possibility of producing processed cheese was first assessed in 1895; emulsifying salts were not used and the product was not successful. The first sucessful product, in which emulsifying salts were used, was introduced in Europe in 1912 and in the USA in 1917 by Kraft. Since then, the market for processed cheese has increased and the range of products expanded.

Although established consumers may regard processed cheeses as inferior products compared to natural cheeses, they have numerous advantages compared to the latter:

1. A certain amount of cheese which would otherwise be difficult or impossible to commercialize may be used, e.g. cheese with deformations, cheese trimmings or cheese after removal of localized mould.
2. A blend of cheese varieties and non-cheese components may be used, making it possible to produce processed cheeses differing in consistency, flavour, shape and size.
3. They have good storage stability at moderate temperatures, thus reducing the cost of storage and transport.
4. They are more stable than natural cheeses during storage, which results in less wastage, a feature that may be especially important in remote areas and in households with a low level of cheese consumption.
5. They are amenable to imaginative packing in various conveniently sized units.
6. They are suitable for sandwiches and fast food outlets.
7. They are attractive to children who generally do not like or appreciate the stronger flavour of natural cheeses.

Today, a wide range of processed cheese products is available, varying in composition and flavour (Table 10.8).

Table 10.8 Compositional specifications and permitted ingredients in pasteurized processed cheese products[a] (modified from Fox *et al.*, 1996a)

Product	Moisture (%, w/w)	Fat (%, w/w)	Fat in dry matter (%, w/w)	Ingredients
Pasteurized blended cheese	≤43	–	≥47	Cheese; cream, anhydrous milk fat, dehydrated cream (in quantities such that the fat derived from them is less than 5% (w/w) in finished product); water; salt; food-grade colours, spices and flavours; mould inhibitors (sorbic acid, potassium/sodium sorbate, and/or sodium/calcium propionates), at levels ≤0.2% (w/w) finished product
Pasteurized processed cheese	≤43	–	≥47	As for pasteurized blended cheese, but with the following extra optional ingredients: emulsifying salts (sodium phosphates, sodium citrates; 3% (w/w) of finished product), food-grade organic acids (e.g. lactic, acetic or citric) at levels such that pH of finished product is ≥5.3
Pasteurized processed cheese foods	≤44	≥23	–	As for pasteurized blended cheese, but with the following extra optional ingredients (milk, skim milk, buttermilk, cheese whey, whey proteins – in wet or dehydrated forms)
Pasteurized processed cheese spreads	40–60	≥20	–	As for pasteurized blended cheese, but with the following extra optional ingredients: food-grade hydrocolloids (e.g. carob bean gum, guar gum, xanthan gums, gelatin, carboxymethylcellulose, and/or carageenan) at levels <0.8% (w/w) of finished products; food-grade sweetening agents (e.g. sugar, dextrose, corn syrup, glucose syrup, hydrolysed lactose)

[a]Minimum temperatures and times specified for processing are 65.5°C for 30 s.

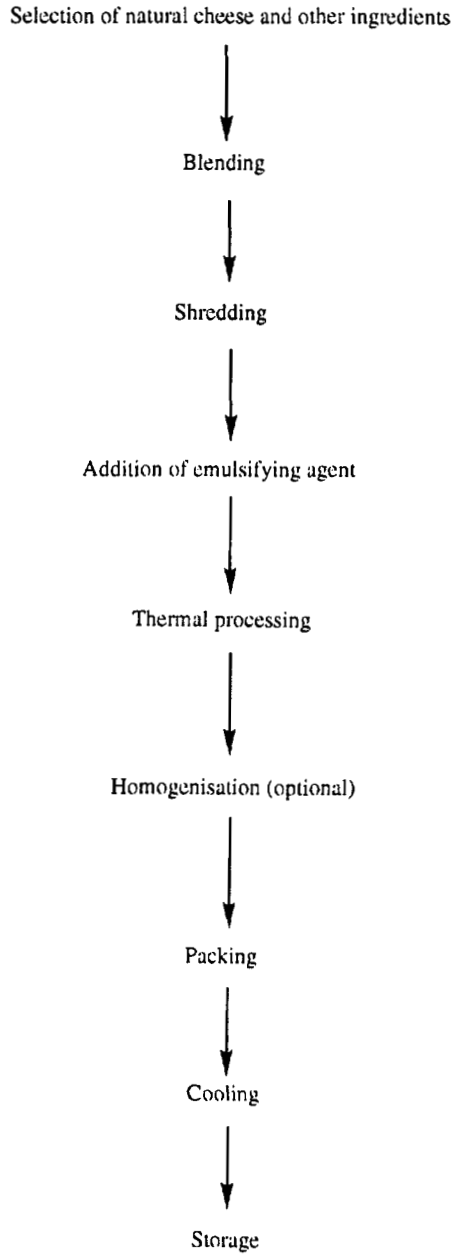

Figure 10.28 Protocol for the manufacture of processed cheese.

10.4.1 Processing protocol

The typical protocol for the manufacture of processed cheese is outlined in Figure 10.28.

The important criteria for selecting cheese are type, flavour, maturity, consistency, texture and pH. The selection is determined by the type of processed cheese to be produced and by cost factors.

A great diversity of non-cheese ingredients may be used in the manufacture of processed cheese (Figure 10.29).

Emulsifying salts are critical in the manufacture of processed cheese with desirable properties. The most commonly used salts are orthophosphates, polyphosphates and citrates but several other agents are used (Tables 10.9 and 10.10). Emulsifying salts are not emulsifiers in the strict sense, since they are not surface active. Their essential role in processed cheese is to supplement the emulsifying properties of cheese proteins. This is accomplished by sequestering calcium, solubilizing, dispersing, hydrating and swelling the proteins and adjusting and stabilizing the pH.

The actual blend of ingredients used and the processing parameters depend on the type of processed cheese to be produced; typical parameters are summarized in Table 10.11.

One of the major advantages of processed cheese is the flexibility of the finished form, which facilitates usage. The texture may vary from firm and sliceable to soft and spreadable. These cheeses may be presented as large blocks (5–10 kg), suitable for industrial catering, smaller blocks, e.g. 0.5 kg,

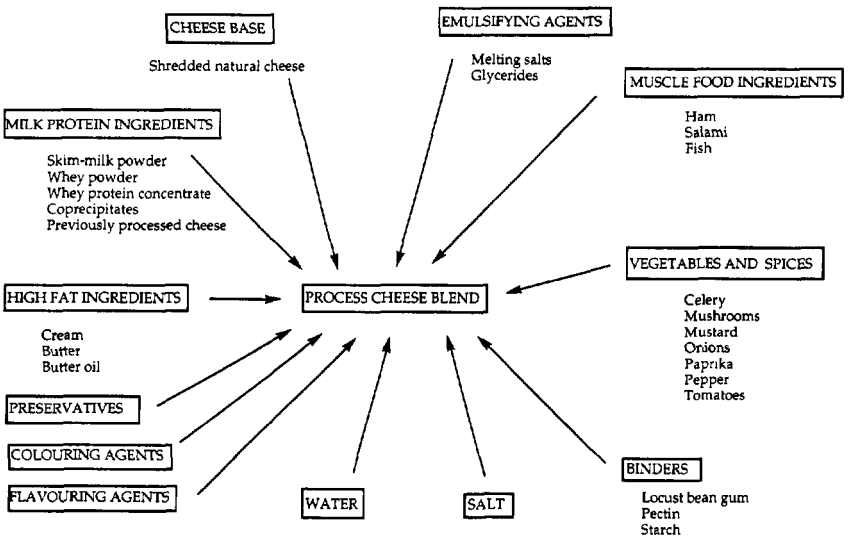

Figure 10.29 Examples of non-cheese ingredients used in processed cheese (from Caric and Kalab, 1987).

Table 10.9 Properties of emulsifying salts for processed cheese products (from Caric and Kalab, 1987)

Group	Emulsifying salt	Formula	Solubility at 20°C (%)	pH value (1% solution)
Citrates	Trisodium citrate	$2Na_3C_6H_5O_7.1H_2O$	High	6.23–6.26
Orthophosphates	Monosodium phosphate	$NaH_2PO_4.2H_2O$	40	4.0–4.2
	Disodium phosphate	$Na_2HPO_4.12H_2O$	18	8.9–9.1
Pyrophosphates	Disodium pyrophosphate	$Na_2H_2P_2O_7$	10.7	4.0–4.5
	Trisodium pyrophosphate	$Na_3HP_2O_7.9H_2O$	32.0	6.7–7.5
	Tetrasodium pyrophosphate	$Na_4P_2O_7.10H_2O$	10–12	10.2–10.4
Polyphosphates	Pentasodium tripolyphosphate	$Na_5P_3O_{10}$	14–15	9.3–9.5
	Sodium tetrapolyphosphate	$Na_6P_4O_{13}$	14–15	9.0–9.5
	Sodium hexametaphosphate (Graham's salt)	$Na_{n+2}P_nO_{3n+1}$ ($n = 10$–25)	Very high	6.0–7.5
Aluminium phosphates	Sodium aluminium phosphate	$NaH_{14}Al_3(PO_4)_8.4H_2O$	–	8.0

Table 10.10 General properties of emulsifying salts in relation to cheese processing (from Fox *et al.*, 1996a,b)

Property	Citrates	Orthophosphates	Pyrophosphates	Polyphosphates	Aluminium
Ion exchange (calcium sequesterization)	Low	Low	Moderate	High–very high	Low
Buffering action in the pH range 5.3–6.0	High	High	Moderate	Low–very low	–
para-Caseinate dispersion	Low	Low	High	Very high	–
Emulsification	Low	Low	Very high	Very high ($n = 3$–10) –low	Very low
Bacteriostatic	Nil	Low	High	High–very high	–

Table 10.11 Chemical, mechanical and thermal parameters as regulating factors in the cheese processing procedures (from Caric and Kalab, 1993)

Process conditions	Processed cheese block	Processed cheese slice	Processed cheese spread
Raw material			
a. Average of cheese	Young to medium ripe, predominantly young	Predominantly young	Combination of young, medium ripe, overipe
b. Water-insoluble N as a % of total N	75–90%	80–90%	60–75%
c. Structure	Predominantly long	Long	Short to long
Emulsifying salt	Structure-building, not creaming, e.g. high molecular weight polyphosphate, citrate	Structure-building, not creaming, e.g. phosphate/citrate mixtures	Creaming, e.g. low and medium molecular weight polyphosphate
Water addition	10–25% (all at once)	5–15% (all at once)	20–45% (in portions)
Temperature	80–85°C	78–85°C	85–98°C (150°C)
Duration of processing (min)	4–8	4–6	8–15
pH	5.4–5.7	5.6–5.9	5.6–6.0
Agitation	Slow	Slow	Rapid
Reworked cheese	0–0.2%	0	5–20%
Milk powder or whey powder 5–12%		0	0
Homogenization	None	None	Advantageous
Filling (min)	5–15	As fast as possible	10–30
Cooling	Slowly (10–12 h) at room temperature	Very rapid	Rapidly (15–30 min) in cool air

for household use, small unit packs, e.g. 25–50 g, or slices which are particularly suited for industrial catering and fast food outlets.

10.5 Cheese analogues

Cheese analogues represent a new range of cheese-like products which probably contain no cheese. The most important of these are Mozzarella (Pizza) cheese analogues which are produced from rennet casein, fat or oil (usually vegetable) and emulsifying salts. The function of emulsifying salts is essentially similar to those in processed cheese, i.e. to solubilize the proteins. The manufacturing protocol is usually similar to that used for processed cheese, bearing in mind that the protein is dried rennet casein rather than a blend of cheeses (Figure 10.30).

The main attributes required of cheese analogues used in pizzas are meltability and stretchability; flavour is provided by other ingredients of the

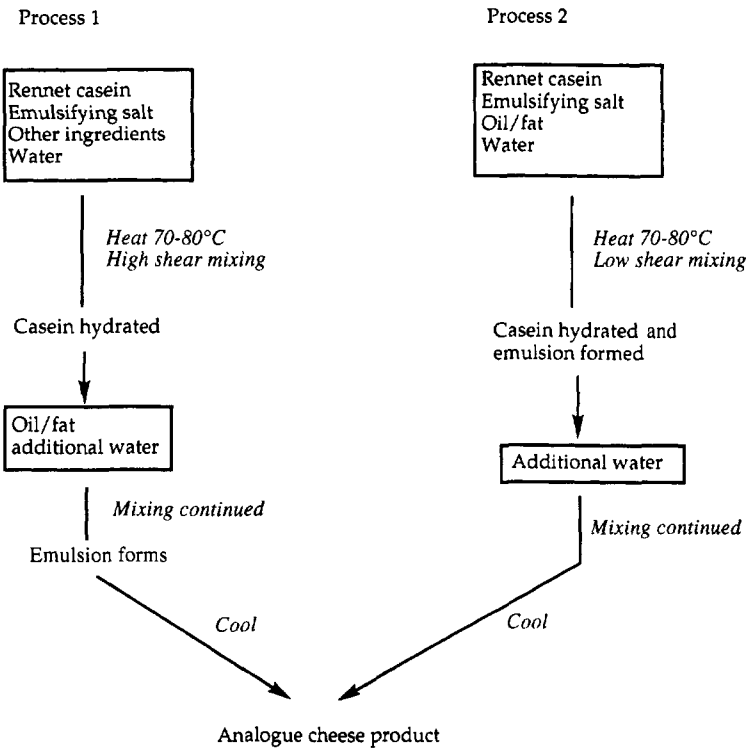

Figure 10.30 Typical protocols for the manufacture of cheese analogue from rennet casein.

pizza, e.g. tomato paste, sausage, peppers, spices, anchovies, etc. It may be possible to produce analogues of other cheeses by adding biochemically or chemically generated cheese flavours. Apart from the use of some casein (rennet or acid) in processed cheese blends, cheese analogues, other than Mozzarella, are not widely used at present. As discussed in section 10.2.8, the flavour and texture of natural cheeses are very complex and cannot be simulated readily. The usual approach is to accelerate the ripening of natural cheese (section 10.2.9), although this approach has enjoyed limited success to date.

10.6 Cultured milks

Acidified (cultured) milk products may very well be the oldest dairy products. If removed aseptically from a healthy udder, milk is essentially sterile but, in practice, milk becomes contaminated by various bacteria, including lactic acid bacteria (LAB) during milking. During storage, these contaminants grow at rates dependent on the temperature. LAB probably dominate the microflora of uncooled milk expressed by hand. Since LAB are well suited for growth in milk, they grow rapidly at ambient temperature, metabolizing lactose to lactic acid and reducing the pH of the milk to the isoelectric point of caseins (about pH 4.6), at which they form a gel under quiescent conditions, thus producing cultured milks. Such products have existed since the domestication of dairy animals and some form of cultured milk is produced throughout the world; the principal products are

Table 10.12 Some typical examples of starter cultures employed in the manufacture of fermented milks (from Robinson and Tamime, 1993)

Type of culture	Product	Micro-organisms involved
Mesophilic	Taetmojolk	*Lactococcus lactis* subsp. *lactis*
	Folkjolk	*Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* *Leuconostoc mesenteroides* subsp. *cremoris*
	Ymer	*Lc. lactis* subsp. *cremoris* *Lc. lactis* subsp. *lactis* biovar. *diacetylactis*
	Kefir	Kefir grains – thermophilic lactobacilli and *Kluyveromyces marxianus*
Typical fermentation temperature 20–22°C		
Thermophilic	Yoghurt	*Streptococcus salvarius* subsp. *thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus*
	Yakult	*Lactobacillus casei* subsp. *casei*
	Acidophilus milk	*Lactobacillus acidophilus*
	A/B milk	*Lb. acidophilus* *Bifidobacterium bifidum*
	A/B yoghurt	As above plus yoghurt culture
Typical fermentation temperatures 37–42°C		

listed in Table 10.12 (Tamime and Robinson, 1985); yoghurt in its various forms, is probably the most important type but consumption varies widely (Table 1.6).

The production of fermented milks no longer depends on acid production by the indigenous microflora. Instead, the milk is inoculated with a carefully selected culture of LAB and for some products with LAB plus lactose-fermenting yeasts (Table 10.12). The principal function of LAB is to produce acid at an appropriate rate via the pathways summarized in Figure 10.12. The yoghurt fermentation is essentially homofermentative but the characteristic flavour of cultured buttermilk is due mainly to diacetyl which is produced from citrate by *Lactococccus lactis* ssp. *lactis* biovar *diacetylactis*, which is included in the culture for this product (Figure 10.31).

Kefir and Koumiss contain about 1 and 6% ethanol, respectively, which is produced by lactose-fermenting yeasts, usually *Kluyveromyces marxianus*. The ethanol modifies the flavour of the products and the CO_2 produced in the fermentation affects both their flavour and texture. Koumiss, which is produced traditionally from mares' milk, mainly in Russia and surrounding areas of Asia, is not in fact coagulated.

The technology of fermented milks will not be discussed in detail and the interested reader is referred to Tamime and Robinson (1985), Tamime and Marshall (1997) and Marshall and Tamime (1997). A flow diagram of the manufacturing protocol of yoghurt is presented in Figure 10.32. Depending on the product, the milk used may be full-fat, partially skimmed or fully skimmed. If it contains fat, the milk is homogenized at 10–20 MPa to prevent creaming during fermentation. For yoghurt, the milk is usually supplemented with skim-milk powder to improve gel characteristics. Acid milk gels are quite stable if left undisturbed but if stirred or shaken, they synerese, expressing whey, which is undesirable. The tendency to synerese is reduced by heating the milk at, for example, 90°C × 10 min or 120°C × 2 min. Heating causes denaturation of whey proteins, especially β-lactoglobulin, and their interaction with the casein micelles via κ-casein. The whey protein-coated micelles form a finer (smaller whey pockets) gel than that formed from unheated or HTST pasteurized milk, with less tendency to synerese.

In some countries, it is common practice to add sucrose to the milk for yoghurt, to reduce the acid taste. It is also very common practice to add fruit pulp, fruit essence or other flavouring, e.g. chocolate, to yoghurt, either to the milk (set yoghurt) or to the yoghurt after fermentation (stirred yoghurt).

In the manufacture of Labneh and other Middle Eastern fermented milks, the fermented product is concentrated by removing part of the serum (whey). This was done traditionally by stirring the yoghurt and transferring it to muslin bags to partially drain. Concentration can now be achieved by ultrafiltration, before, but preferably after, fermentation.

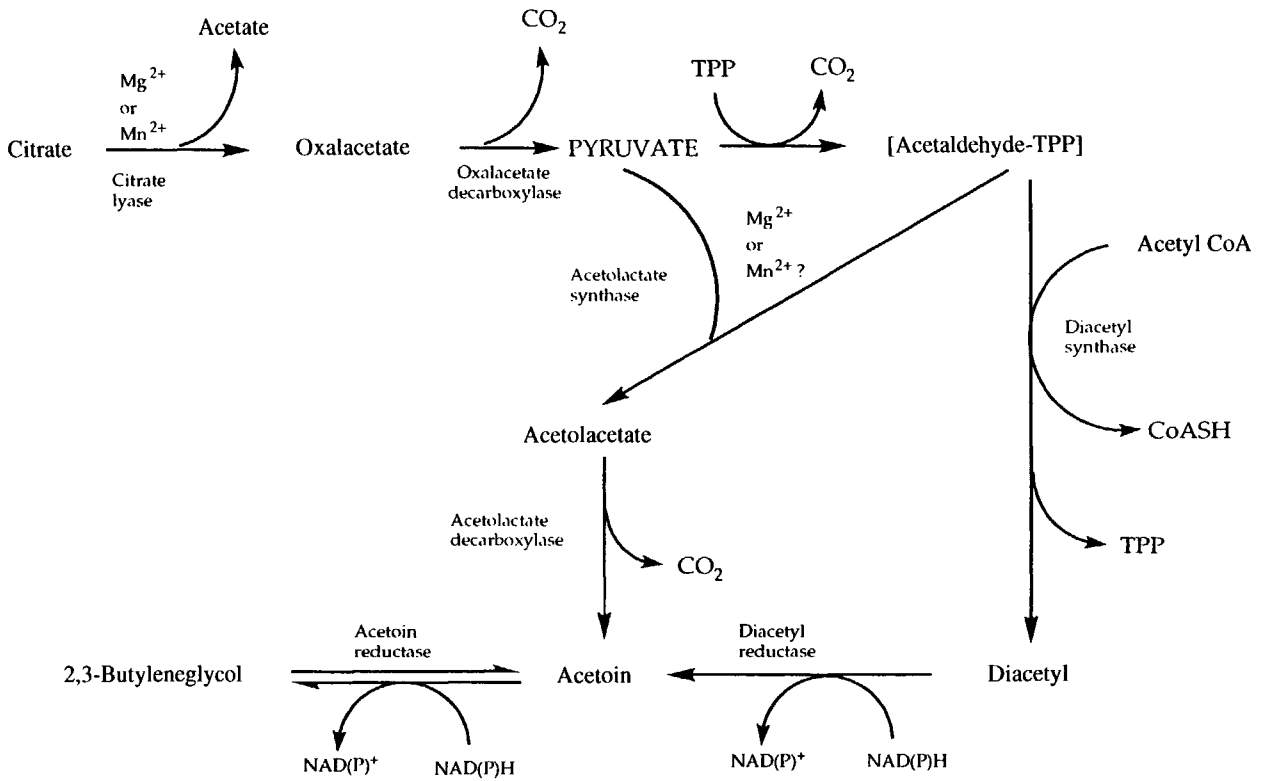

Figure 10.31 Citrate metabolism by *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* or *Leuconostoc* spp. (from Cogan and Hill, 1993).

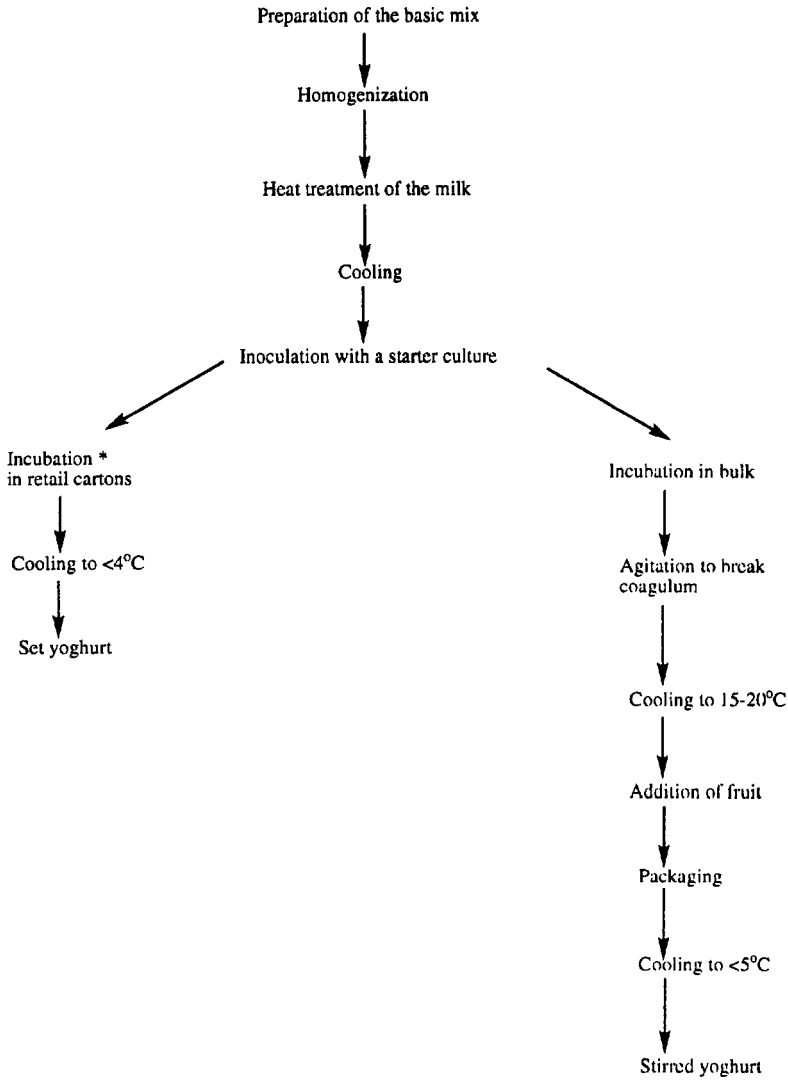

Figure 10.32 Protocol for the manufacture of yoghurt. *, Sucrose and/or fruit (fruit flavours) may be added at this point. (From Robinson and Tamime, 1993.)

Fermented milk products exhibit thixotropic rheological properties, i.e. the viscosity (resistance to flow) decreases as the rate of shear increases; a typical relationship is shown in Figure 10.33. The rheological properties are major parameters of quality and are controlled by varying the total solids content of the milk, the heat treatment and homogenization of the milk and the use of hydrocolloids, e.g. gelatin or carageenan.

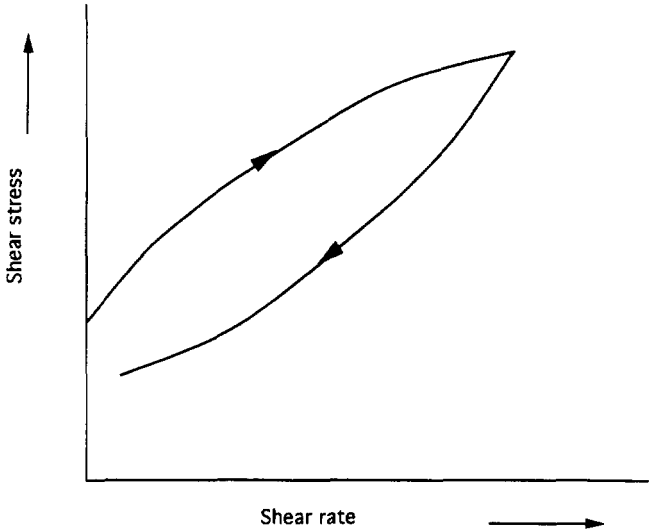

Figure 10.33 Representation of shear stress as a function of shear rate for yoghurt displaying rheological hysteresis.

Fermented milk products developed by chance but the increased storage stability and desirable organoleptic properties of such products were soon appreciated. Special therapeutic properties of yoghurt were claimed by Metchnikoff in 1910 and have been a controversial subject since. It is now generally accepted that fermented milk products have nutritional benefits above those of their gross chemical constituents. It has been documented that some *Lactobacillus* spp., and in particular *Bifidobacterium* spp., contained in yoghurt can colonize the large intestine, reduce its pH and control the growth of undesirable micro-organisms. Some of these bacteria also produce probiotics. Yoghurts containing such cultures, often referred to as bioyoghurt, are enjoying considerable commercial success. Legislation in many countries specifies a minimum number of viable micro-organisms in yoghurt.

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Appendices

Appendix 10A World cheese production, 1994 (FAO, 1994)

(See facing page)

Country	Cheese production (tonnes)	Country	Cheese production (tonnes)
World	**14 880 089**	Jordan	4612
Africa	**495 298**	Japan	98 000
Algeria	1045	Kazakhstan	93 000
Angola	1007	Kyrgyzstan	25 000
Botswana	1498	Lebanon	14 744
Egypt	333 950	Mongolia	1764
Eritrea	216	Myanmar	27 622
Ethiopia	4600	Oman	411
Kenya	210	Syria	78 638
Mauritania	1664	Tajikistan	16 000
Morocco	6947	Turkey	139 177
Namibia	70	Turkmenistan	7000
Niger	12 064	Uzbekistan	46 000
Nigeria	7022	Yemen	9155
South Africa	38 000	**Europe**	**7 075 705**
Sudan	72 479	Albania	15 400
Tanzania	1200	Austria	109 600
Tunisia	7060	Belarus	109 000
Zambia	1069	Belgium–Luxembourg	68 000
Zimbabwe	5197	Bosnia–Hercegovina	13 500
North and Central		Bulgaria	66 000
America	**3 861 927**	Croatia	16 701
Canada	305 100	Czech Republic	117 449
Costa Rica	5960	Denmark	288 100
Cuba	14 600	Estonia	23 000
Dominican Republic	2500	Finland	92 193
El Salvador	2580	France	1 562 496
Guatemala	11 700	Germany	1 371 174
Honduras	8310	Greece	210 300
Mexico	116 360	Hungary	77 496
Nicaragua	5318	Iceland	2050
Panama	4500	Ireland	91 250
USA	3 385 000	Italy	919 373
South America	**613 158**	Latvia	18 000
Argentina	330 000	Lithuania	4000
Bolivia	6738	Macedonia, FYR of	8100
Brasil	60 150	Malta	83
Chile	44 599	Moldova Republic	30 500
Colombia	51 000	Netherlands	647 640
Ecuador	6288	Norway	80 300
Peru	19 983	Poland	296 200
Uruguay	20 400	Portugal	64 400
Venezuela	74 000	Romania	51 204
Asia	**873 757**	Russian Federation	708 000
Afghanistan	15 600	Slovakia	42 202
Armenia	14 750	Slovenia	10 000
Azerbaijan	43 000	Spain	159 000
Bangladesh	1000	Sweden	138 854
Bhutan	2021	Switzerland	134 640
China	164 646	United Kingdom	362 000
Cyprus	5600	Ukraine	308 770
Georgia	54 600	Yugoslavia, FR	60 000
Iran	200 089	**Oceania**	**423 625**
Iraq	24 733	Australia	233 635
Israel	85 944	New Zealand	190 000

The following countries are included in FAO (1994) but no data for cheese production are available: Burkina Faso, Burundi, Chad, Madagascar, Guinea, Rwanda, Senegal, Somalia, Swaziland, Jamaica, Trinidad and Tobago, Suriname, India, Indonesia, Republic of Korea, Malaysia, Nepal, Pakistan, Philippines, Saudi Arabia, Sri Lanka, Thailand, United Arab Emirates and Fiji.

Appendix 10B Consumption of cheese (kg per caput, 1993) (IDF, 1995)

Country	Ripened cheese[a]	Fresh and cottage cheese	Total
France	15.5	7.3	22.8
Italy	13.4	6.7	20.1
Belgium	15.1	4.7	19.8
Germany	10.5	8.0	18.5
Iceland	11.9	5.2	17.1
Switzerland	13.6	2.8	16.4
Sweden	15.5	0.9	16.4
Netherlands	14.1	1.7	15.8
Denmark	14.5	0.9	15.4
Finland	12.0	2.3	14.3
Norway	14.0	0.2	14.2
Canada	12.4	0.9	13.3
USA	11.9	1.3	13.2
Austria	7.5	3.9	11.4
Estonia	4.4	5.6	10.0
Australia	8.7	0.8	9.5
United Kingdom	—— 8.3 ——		8.3
Spain	—— 8.1 ——		8.1
New Zealand	—— 8.1 ——		8.1
Hungary	4.6	3.3	7.9
Ireland[b]	—— 5.6 ——		5.6
South Africa	1.5	0.1	1.6
Japan	—— 1.4 ——		1.4

[a]Including processed cheese.
[b]Data for Ireland 1991.

11 Physical properties of milk

Milk is a dilute emulsion consisting of an oil/fat dispersed phase and an aqueous colloidal continuous phase. The physical properties of milk are similar to those of water but are modified by the presence of various solutes (proteins, lactose and salts) in the continuous phase and by the degree of dispersion of the emulsified and colloidal components.

Data on the physical properties of milk are important since such parameters can influence the design and operation of dairy processing equipment (e.g. thermal conductivity or viscosity) or can be used to determine the concentration of specific components in milk (e.g. use of the elevation in freezing point to estimate added water or specific gravity to estimate solids-not-fat), or to assess the extent of biochemical changes in the milk during processing (e.g. acidification by starter or the development of a rennet coagulum). Some important physical properties of milk are summarized in Table 11.1.

Table 11.1 Some physical properties of milk (Walstra and Jenness, 1984; Sherbon, 1988; Singh, McCarthy and Lucey, 1997)

Osmotic pressure	~700 kPa
a_w	~0.993
Boiling point	~100.15°C
Freezing point	−0.522°C (approx.)
Refractive index, n_D^{20}	1.3440–1.3485
Specific refractive index	~0.2075
Density (20°C)	~1030 kg m^{-3}
Specific gravity (20°C)	~1.0321
Specific conductance	~0.0050 ohm^{-1} cm^{-1}
Ionic strength	~0.08 M
Surface tension (20°C)	~52 N m^{-1}
Coefficient of viscosity	2.127 mPa s
Thermal conductivity (2.9% fat)	~0.559 W m^{-1} K^{-1}
Thermal diffusivity (15–20°C)	~1.25×10^{-7} m^2 s^{-1}
Specific heat	~3.931 kJ kg^{-1} K^{-1}
pH (at 25°C)	~6.6
Titratable acidity	1.3–2.0 meq OH^- per 100 ml (0.14–0.16% as lactic acid)
Coefficient of cubic expansion (273–333 K)	0.0008 m^3 m^{-3} K^{-1}
Redox potential (25°C, pH 6.6, in equilibrium with air)	+0.25 to +0.35 V

11.1 Ionic strength

The ionic strength, I, of a solution is defined as:

$$I = \tfrac{1}{2}\Sigma c_i z_i^2 \tag{11.1}$$

where c_i is the molar concentration of the ion of type i and z_i is its charge. The ionic strength of milk is *c.* 0.08 M.

11.2 Density

The density (ρ) of a substance is its mass per unit volume, while its specific gravity (SG) or relative density is the ratio of the density of the substance to that of water (ρ_w) at a specified temperature:

$$\rho = m/V \tag{11.2}$$

$$\text{SG} = \rho/\rho_w \tag{11.3}$$

$$\rho = \text{SG}\rho_w \tag{11.4}$$

The thermal expansion coefficient governs the influence of temperature on density and therefore it is necessary to specify temperature when discussing density or specific gravity. The density of milk is of consequence since fluid milk is normally retailed by volume rather than by mass. Measurement of the density of milk using a hydrometer (lactometer) has also been used to estimate its total solids content.

The density of bulk milk (4% fat and 8.95% solids-not-fat) at 20°C is approximately $1030\,\text{kg}\,\text{m}^{-3}$ and its specific gravity is 1.0321. Milk fat has a density of about $902\,\text{kg}\,\text{m}^{-3}$ at 40°C. The density of a given milk sample is influenced by its storage history since it is somewhat dependent on the liquid to solid fat ratio and the degree of hydration of proteins. To minimize effects of thermal history on its density, milk is usually prewarmed to 40–45°C to liquify the milk fat and then cooled to the assay temperature (often 20°C).

The density and specific gravity of milk vary somewhat with breed. Milk from Ayrshire cows has a mean specific gravity of 1.0317 while that of Jersey and Holstein milks is 1.0330. Density varies with the composition of the milk and its measurement has been used to estimate the total solids content of milk. The density of a multicomponent mixture (like milk) is related to the density of its components by:

$$1/\rho = \Sigma(m_x/\rho_x) \tag{11.5}$$

where m_x is the mass fraction of component x, and ρ_x its apparent density in the mixture. This apparent density is not normally the same as the true density of the substance since a contraction usually occurs when two components are mixed.

Equations have been developed to estimate the total solids content of milk based on % fat and specific gravity (usually estimated using a lactometer). Such equations are empirical and suffer from a number of drawbacks; for further discussion see Jenness and Patton (1959). The principal problem is the fact that the coefficient of expansion of milk fat is high and it contracts slowly on cooling and therefore the density of milk fat (Chapter 3) is not constant. Variations in the composition of milk fat and in the proportions of other milk constitiuents have less influence on these equations than the physical state of the fat.

In addition to lactometry (determination of the extent to which a hydrometer sinks), the density of milk can be measured by pycnometry (determination of the mass of a given volume of milk), by hydrostatic weighing of an immersed bulb (e.g. Westphal balance), by dialatometry (measurement of the volume of a known mass of milk) or by measuring the distance that a drop of milk falls through a density gradient column.

11.3 Redox properties of milk

Oxidation–reduction (redox) reactions involve the transfer of an electron from an electron donor (reducing agent) to an electron acceptor (oxidizing agent). The species that loses electrons is said to be oxidized while that which accepts electrons is reduced. Since there can be no net transfer of electrons to or from a system, redox reactions must be coupled and the oxidation reaction occurs simultaneously with a reduction reaction.

The tendency of a system to accept or donate electrons is measured using an inert electrode (typically platinum). Electrons can pass from the system into this electrode, which is thus a half-cell. The Pt electrode is connected via a potentiomenter to another half-cell of known potential (usually, a saturated calomel electrode). All potentials are referred to the hydrogen half-cell:

$$\tfrac{1}{2}H_2 \rightleftarrows H^+ + e^- \tag{11.6}$$

which by convention is assigned a potential of zero when an inert electrode is placed in a solution of unit activity with respect to H^+ (i.e. pH = 0) in equilibrium with H_2 gas at a pressure of 1.013×10^5 Pa (1 atm). The redox potential of a solution, E_h, is the potential of the half-cell at the inert electrode and is expressed as volts. E_h depends not only on the substances present in the half-cell but also on the concentrations of their oxidized and reduced forms. The relationship between E_h and the concentrations of the oxidized and reduced forms of the compound is described by the Nernst equation:

$$E_h = E_o - RT/nF \ln a_{red}/a_{ox} \tag{11.7}$$

where E_o is the standard redox potential (i.e. potential when reactant and

product are at unit activity), n is the number of electrons transferred per molecule, R is the universal gas constant ($8.314\,J\,K^{-1}\,mol^{-1}$), T is temperature (in Kelvin), F is the Faraday constant ($96.5\,kJ\,V^{-1}\,mol^{-1}$) and a_{red} and a_{ox} are activities of the reduced and oxidized forms, respectively. For dilute solutions, it is normal to approximate activity by molar concentration. Equation 11.7 can be simplified, assuming a temperature of 25°C, a transfer of one electron and that activity $\approx$ concentration:

$$E_h = E_o + 0.059 \log [Ox]/[Red]. \quad (11.8)$$

Thus, E_h becomes more positive as the concentration of the oxidized form of the compound increases. E_h is influenced by pH since pH affects the standard potential of a number of half-cells. The above equation becomes:

$$E_h = E_o + 0.059 \log [Ox]/[Red] - 0.059\ pH. \quad (11.9)$$

The E_h of milk is usually in the range $+0.25$ to $+0.35$ V at 25°C, at pH 6.6 to 6.7 and in equilibrium with air (Singh, McCarthy and Lucey, 1997). The influence of pH on the redox potential of a number of systems is shown in Figure 11.1.

The concentration of dissolved oxygen is the principal factor affecting the redox potential of milk. Milk is essentially free of O_2 when secreted but in equilibrium with air, its O_2 content is about 0.3 mM. The redox potential of anaerobically drawn milk or milk which has been depleted of dissolved oxygen by microbial growth or by displacement of O_2 by other gases is more negative than that of milk containing dissolved O_2.

The concentration of ascorbic acid in milk ($11.2–17.2\,mg\,l^{-1}$) is sufficient to influence its redox potential. In freshly drawn milk, all ascorbic acid is in the reduced form but can be oxidized reversibly to dehydroascorbate, which is present as a hydrated hemiketal in aqueous systems. Hydrolysis of the lactone ring of dehydroascorbate, which results in the formation of 2,3-diketogulonic acid, is irreversible (Figure 11.2).

The oxidation of ascorbate to dehydroascorbate is influenced by O_2 partial pressure, pH and temperature and is catalysed by metal ions (particularly Cu^{2+}, but also Fe^{3+}). The ascorbate/dehydroascorbate system in milk stabilizes the redox potential of oxygen-free milk at *c.* 0.0 V and that of oxygen-containing milk at $+0.20$ to $+0.30$ V (Sherbon, 1988). Riboflavin can also be oxidized reversibly but its concentration in milk (*c.* 4 μM) is thought to be too low to have a significant influence on redox potenial. The lactate–pyruvate system (which is not reversible unless enzyme-catalysed) is thought not to be significant in influencing the redox potential of milk since it, too, is present at very low concentations. At the concentrations at which they occur in milk, low molecular mass thiols (e.g. free cysteine) have an insignificant influence on the redox potential of milk. Thiol–disulphide interactions between cysteine residues of proteins influence the redox properties of heated milks in which the proteins are denatured. The free

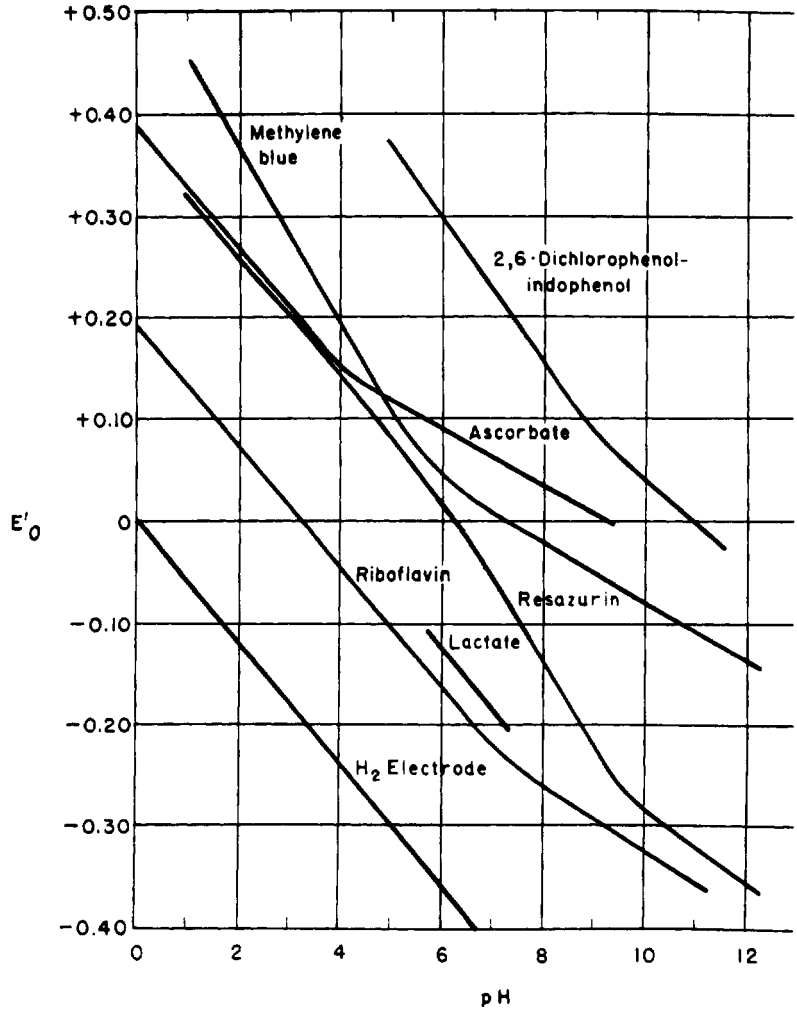


Figure 11.1 Effect of pH on the oxidation–reduction potential of various systems (from Sherbon, 1988).

aldehyde group of lactose can be oxidized to a carboxylic acid (lactobionic acid) at alkaline pH but this system contributes little to the redox properties of milk at pH 6.6.

The E_h of milk is influenced by exposure to light and by a number of processing operations, including those which cause changes in the concentration of O_2 in the milk. Addition of metal ions (particularly Cu^{2+}) also influences the redox potential. Heating of milk causes a decrease in its E_h,

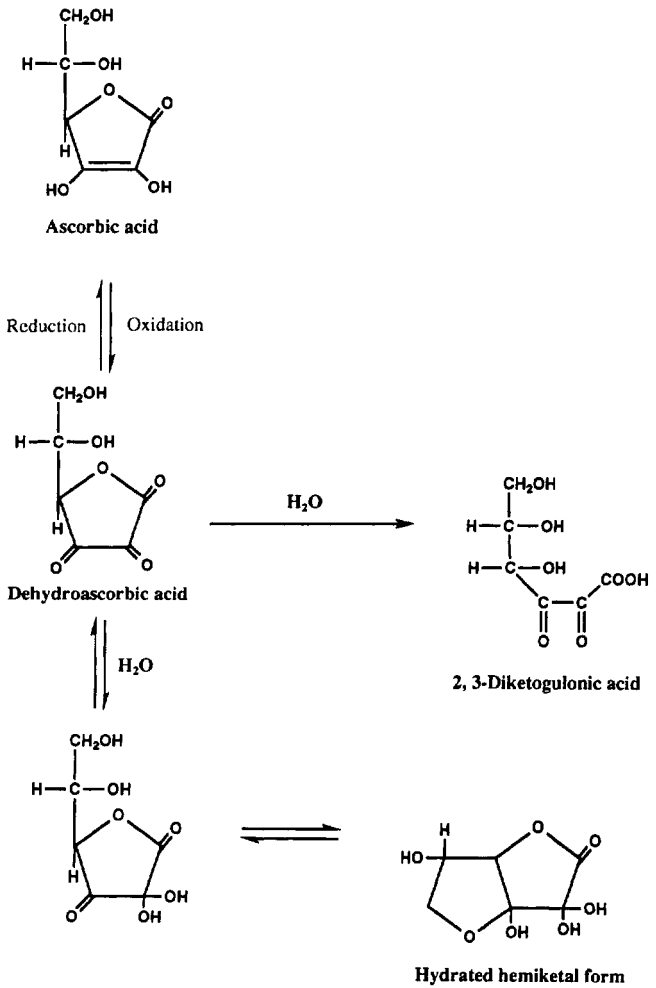


Figure 11.2 Chemical structures of ascorbic acid and its derivatives.

due mainly to the denaturation of β-lactoglobulin (and the consequent exposure of —SH groups) and loss of O_2. Compounds formed by the Maillard reaction between lactose and proteins can also influence the E_h of heated milk, particularly dried milk products.

Fermentation of lactose during the growth of micro-organisms in milk has a major effect on its redox potential. The decrease in the E_h of milk caused by the growth of lactic acid bacteria is shown in Figure 11.3. A rapid decrease in E_h occurs after the available O_2 has been consumed by the bacteria. Therefore, the redox potential of cheese and fermented milk products is negative. Reduction of redox indicators (e.g. resazurin or

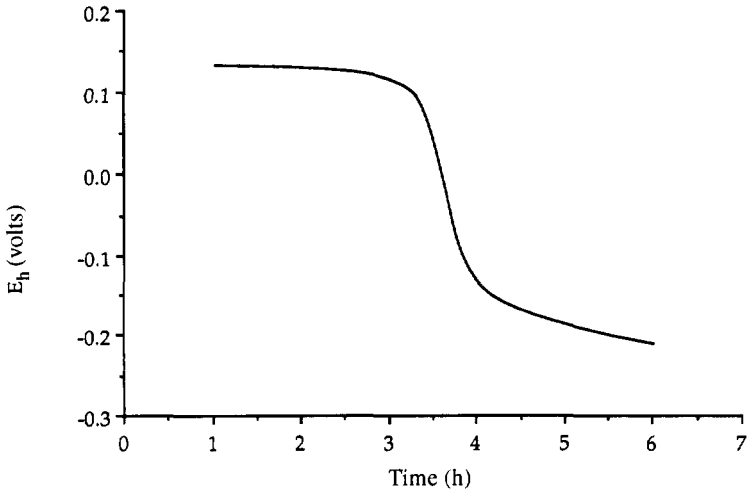
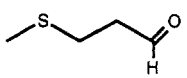


Figure 11.3 Decrease in the redox potential of milk caused by the growth of *Lactococcus lactis* subsp. *lactis at* 25°C.

methylene blue) can be used as an index of the bacterial quality of milk by measuring the 'reduction time', at a suitable temperature, of milk containing the dye.

Riboflavin absorbs light maximally at about 450 nm and in doing so can be excited to a triplet state. This excited form of riboflavin can interact with triplet O_2 to form a superoxide anion O_2^- (or H_2O_2 at low pH). Excited riboflavin can also oxidize ascorbate, a number of amino acids and proteins and orotic acid. Riboflavin-catalysed photo-oxidation results in the production of a number of compounds, most notably methional (**11.1**) which is the principal compound responsible for the off-flavour in milk exposed to light.

11.1



Methional

Photo-oxidation of milk constituents was discused in detail by Walstra and Jenness (1984).

11.4 Colligative properties of milk

Colligative properties are those physical properties which are governed by the number, rather than the kind, of particles present in solution. The important colligative properties of milk are its freezing and boiling points (*c.* −0.522 and 100.15°C, respectively) and its osmotic pressure (approxi-

mately 700 kPa at 20°C), all of which are interrelated. Since the osmotic pressure of milk remains essentially constant (because it is regulated by that of the cow's blood), the freezing point is also relatively constant.

The freezing point of an aqueous solution is governed by the concentration of solutes in the solution. The relationship between the freezing point of a simple aqueous solution and concentration of solute is described by a relation based on Raoult's law:

$$T_f = K_f m \tag{11.10}$$

where T_f is the difference between the freezing point of the solution and that of the solvent, K_f is the molal depression constant (1.86°C for water) and m is the molal concentration of solute. However, this equation is valid only for dilute solutions containing undissociated solutes. Raoult's law is thus limited to approximating the freezing point of milk.

The freezing point of bovine milk is usually in the range −0.512 to −0.550°C, with a mean value close to −0.522°C (Sherbon, 1988) or −0.540°C (Jenness and Patton, 1959). Despite variations in the concentrations of individual solutes, the freezing point depression of milk is quite constant since it is proportional to the osmotic pressure of milk (approximately 700 kPa at 20°C), which is regulated by that of the cow's blood. The freezing point of milk is more closely related to the osmotic pressure of mammary venous blood than to that of blood from the jugular vein.

Owing to their large particle or molecular mass, fat globules, casein micelles and whey proteins do not have a significant effect on the freezing point of milk, to which lactose makes the greatest contribution. The freezing point depression in milk due to lactose alone has been calculated to be 0.296°C. Assuming a mean freezing point depression of 0.522°C, all other constituents in milk depress the freezing point by only 0.226°C. Chloride is also an important contributor to the colligative properties of milk. Assuming a Cl^- concentration of 0.032 M and that Cl^- is accompanied by a monovalent cation (i.e. Na^+ or K^+), the freezing point depression caused by Cl^- and its associated cation is 0.119°C. Therefore, lactose, chloride and its accompanying cations together account for about 80% of the freezing point depression in milk. Since the total osmotic pressure of milk is regulated by that of the cow's blood, there is a strong inverse correlation between lactose and chloride concentrations (Chapter 5).

Natural variation in the osmotic pressure of milk (and hence freezing point) is limited by the physiology of the mammary gland. Variations in the freezing point of milk have been attributed to seasonality, feed, stage of lactation, water intake, breed of cow, heat stress and time of day. These factors are often interrelated but have relatively little influence on the freezing point of milk. Likewise, unit operations in dairy processing which do not influence the net number of osmotically active molecules/ions in solution do not influence the freezing point. Cooling or heating milk causes

transfer of salts to or from the colloidal state. However, evidence for an effect of cooling or moderate heating (e.g. HTST pasteurization or minimum UHT processing) on the freezing point of milk is contradictory, perhaps since such changes are slowly reversible over time. Direct UHT treatment involves the addition of water (through condensed steam). This additional water should be removed during flash cooling, which also removes gases, e.g. CO_2, from the milk, causing a small increase in freezing point. Vacuum treatment of milk, i.e. vacreation (to remove taints), has been shown to increase its freezing point, presumably by degassing. However, if vacuum treatment is severe enough to cause a significant loss of water, the freezing point will be reduced, thus compensating fully or partially for the loss of CO_2. Fermentation of milk has a large effect on its freezing point since fermentation of 1 mol lactose results in the formation of 4 mol lactic acid. Likewise, fermentation of citrate influences the freezing point of milk.

Accurate measurement of the freezing point depression in milk requires great care. The principle used is to supercool the milk sample (by 1.0 to 1.2°C), to induce crystallization of ice, after which the temperature increases rapidly to the freezing point of the sample (Figure 11.4). For water, the temperature at the freezing point will remain constant until all the latent heat of fusion has been removed (i.e. until all the water is frozen). However, for milk the temperature is stable at this maximum only momentarily and falls rapidly because ice crystallization causes concentration of solutes which leads to a further depression of freezing point. The observed freezing point of milk (maximum temperature after initiation of crystallization) is not the same as its true freezing point since some ice crystallization will have occurred before the maximum temperature is reached. Correction factors have been suggested to account for this but, in practice, it is usual to report

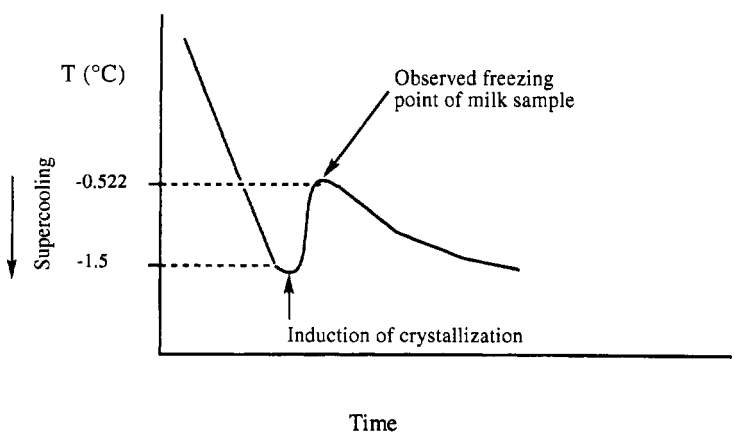


Figure 11.4 Temperature–time curve for the freezing of milk.

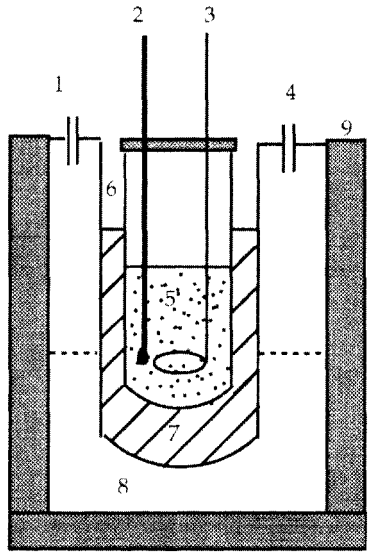


Figure 11.5 Schematic representation of a Hortvet cryoscope. 1,4, Inlet and outlet for air or vacuum supply; 2, thermometer calibrated at 0.001°C intervals; 3, agitator; 5, milk sample; 6, glass tube; 7, alcohol; 8, ether cooled by evaporation; 9, insulated jacket.

the observed freezing point when other factors (particularly the degree of supercooling) have been standardized. Therefore, the observed freezing point of milk is empirical and great care is necessary to standardize methodology.

The Hortvet technique (originally described in 1921) has been used widely to estimate the freezing point of milk. The original apparatus consisted of a tube, containing the milk sample and a thermometer calibrated at 0.001°C intervals, which was placed in ethanol in a Dewar flask which was cooled indirectly by evaporation of ether (caused by pulling or pumping air through the ether, Figure 11.5). This apparatus has been modified to include mechanical refrigeration and various stirring or tapping devices to initiate crystallization. The early Hortvet cryoscopes used thermometers calibrated in degrees Hortvet (°H; values in °H are about 3.7% lower than in °C). The difference between °H and °C originates from differences in the freezing points of sucrose solutions measured using the Hortvet cryoscope and procedure and their true freezing points. IDF (1983) suggested the following formulae to interconvert °H and °C:

$$°C = 0.96418°H + 0.00085$$

$$°H = 1.03711°C - 0.00085$$

However, it is now recommended that thermometers be calibrated in °C. More recently, thermistors have been used instead of mercury thermometers. Cryoscopes based on dew point depression have also been approved for use. These latter instruments also use thermistors and are based on changes in osmotic pressure. Thermistor cryoscopes are now used more widely than Hortvet instruments.

Measurement of the freezing point depression of milk is used to estimate the degree of adulteration of milk with added water. Assuming an average freezing point of 0.550°C, the amount of added water can be calculated from:

$$\% \text{ added water} = \frac{0.550 - \Delta T}{0.550} \times (100 - \text{TS}) \qquad (11.11)$$

where ΔT is the observed freezing point depression of the test sample and TS is the % total solids in the milk. Interpretation of freezing point values when assaying milk suspected of being adulterated with water requires care. Milk with a freezing point of −0.525°C or below is usually presumed to be unadulterated. Due to greater variation in the freezing point of milks drawn from individual animals than of bulk milk, specifications for the freezing point of bulk milk are more stringent than those for milks from individual animals. Finally, it should be noted that estimation of the adulteration of milk with water depends on the constancy of the freezing point (as discussed above). Adulteration of milk with isotonic solutions, e.g. ultrafiltration permeate (which is being considered for standardization of the protein content of milk, see Rattray and Jelen, 1996), will not be detected by this technique.

11.5 Interfacial tension

A phase can be defined as a domain bounded by a closed surface in which parameters such as composition, temperature, pressure and refractive index are constant but change abruptly at the interface. The principal phases in milk are its serum and fat and the most important interfaces are air/serum and fat/serum. If present, air bubbles, and ice, fat or lactose crystals will also constitute phases. Forces acting on molecules or particles in the bulk of a phase differ from those at an interface since the former are attracted equally in all directions while those at an interface experience a net attraction towards the bulk phase (Figure 11.6).

This inward attraction acts to minimize the interfacial area and the force which causes this decrease in area is known as the interfacial tension (γ). If one phase is air, the interfacial tension is referred to as surface tension. Interfacial tension can be expressed as force per unit length (N m^{-1}) or the energy needed to increase the interfacial area by a unit amount (J m^{-2} or N m^{-1}).

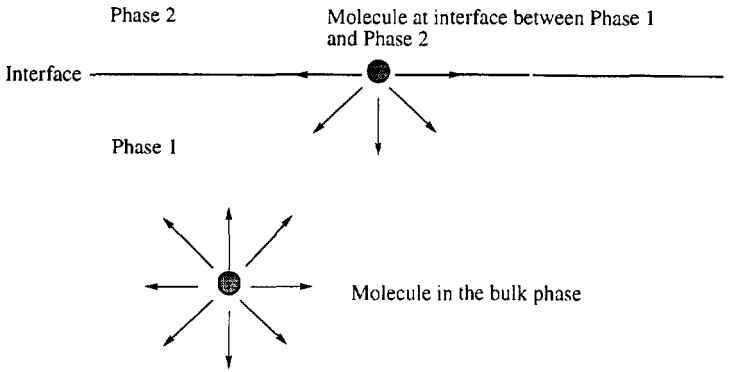


Figure 11.6 Schematic representation of the forces acting on a molecule or particle in a bulk phase or at an interface.

In addition to temperature (which decreases γ), the properties of interfaces are governed by the chemistry of the molecules present, their concentration and their orientation with respect to the interface. Solutes adsorbed at an interface which reduce interfacial tension are known as surface active agents or surfactants. Surfactants reduce interfacial tension by an amount given, under ideal conditions, by the Gibb's equation:

$$d\gamma = -RT\Gamma d\ln a \tag{11.12}$$

where Γ is the excess concentration of the solute at the interface over that in the bulk solution, a is the activity of the solute in the bulk phase and R and T are the universal gas constant and temperature (in Kelvin), respectively. Therefore, the most effective surfactants are those which accumulate most readily at an interface.

Interfacial tension may be measured by a number of techniques, including determining how far a solution rises in a capillary, by measuring the weight, volume or shape of a drop of solution formed at a capillary tip, measuring the force required to pull a flat plate or ring from the surface or the maximum pressure required to form a bubble at a nozzle immersed in the solution. Ring or plate techniques are most commonly used to determine γ of milk.

Reported values for the interfacial tension between milk and air vary from 40 to 60 $N\,m^{-1}$, with an average of about 52 $N\,m^{-1}$ at 20°C (Singh, McCarthy and Lucey, 1997). At 20–40°C, the interfacial tension between milk serum and air is about 48 $N\,m^{-1}$ while that between sweet cream, buttermilk and air is about 40 $N\,m^{-1}$ (Walstra and Jenness, 1984). Surface tension values for rennet whey, skim milk and 25% fat cream are reported to be 51–52, 52–52.5 and 42–45 $N\,m^{-1}$, respectively (Jenness and Patton, 1959).

The principal surfactants in milk are its proteins, phospholipids, mono- and diglycerides and salts of free fatty acids. The immunoglobulins are less effective surfactants than other milk proteins. Salts and lactose do not contribute significantly to the interfacial tension of milk. The difference in interfacial tension between milk serum/air and buttermilk/air can be attributed to the higher concentration of very surface active proteins and protein–phospholipid complexes of the fat globule membrane in buttermilk. The interfacial tension between milk fat globules and the milk serum is about $2\,N\,m^{-1}$, while the interfacial tension between non-globular, liquid milk fat and milk serum is about $15\,N\,m^{-1}$, indicating the effectiveness of milk fat globule membrane material in reducing interfacial tension. The surface tension of whole milk is a little lower than that of skim milk, possibly due to the presence of higher levels of material from the fat globule membrane and traces of free fat in the former. Surface tension decreases with increasing fat content up to about 4%. Lipolysis reduces the surface tension of milk due to the liberation of free fatty acids and attempts have been made to estimate hydrolytic rancidity by exploiting this fact, although such approaches have not been very successful (see Sherbon (1988) for references).

In addition to its composition, various processing parameters can influence the surface tension of milk. The surface tension of whole and skim milk decreases with increasing temperature. Surface tension also varies with the temperature history and age of the milk and with the time required for measurement. Homogenization of raw milk reduces surface tension because lipolysis by the indigenous milk lipase is stimulated and surface-active fatty acids released. Homogenization of pasteurized milk causes a slight increase in surface tension. Pasteurization of milk has little effect on its surface tension although heating milk to sterilization temperatures causes a slight increase in surface tension, resulting from denaturation and coagulation of proteins which are then less effective as surfacants.

11.6 Acid–base equilibria

The acidity of a solution is normally expressed as its pH, which may be defined as:

$$pH = -\log_{10} a_{H^+} \tag{11.13}$$

or

$$pH = -\log_{10} f_H[H^+] \tag{11.14}$$

where a_{H^+} is the activity of the hydrogen ion, $[H^+]$ its concentration and f_H its activity coefficient. For many dilute solutions, $f_H \approx 1$ and pH can thus be closely approximated by the negative logarithm of the hydrogen ion concentration.

The pH of milk at 25°C is usually in the range 6.5–6.7, with a mean value of 6.6. The pH of milk is influenced much more by temperature than is the pH of dilute buffers, principally due to the temperature dependence of the solubility of calcium phosphate (Chapter 5). pH varies with stage of lactation; colostrum can have a pH as low as 6.0. Mastitis tends to increase the pH since increased permeability of the mammary gland membranes means that more blood constituents gain access to the milk; the pH of cow's blood is 7.4. The difference in pH between blood and milk results from the active transport of various ions into the milk, precipitation of colloidal calcium phosphate (CCP; which results in the release of H^+) during the synthesis of casein micelles, higher concentrations of acidic groups in milk and the relatively low buffering capacity of milk between pH 6 and 8 (Singh, McCarthy and Lucey, 1997).

An important characteristic of milk is its buffering capacity, i.e. resistance to changes in pH on addition of acid or base. A pH buffer resists changes in the $[H^+]$ (ΔpH) in the solution and normally consists of a weak acid (HA) and its corresponding anion (A^-, usually present as a fully dissociatable salt). An equilibrium thus exists:

$$HA \rightleftharpoons H^+ + A^- \tag{11.15}$$

The addition of H^+ to this solution favours the back reaction while the addition of base favours the forward reaction. The weak acid/salt pair thus acts to minimize ΔpH. An analogous situation exists for buffers consisting of a weak base and its salt. The pH of a buffer can be calculated from the concentration of its components by the Henderson–Hasselbalch equation

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \tag{11.16}$$

where pK_a is the negative logarithm of the dissociation constant of the weak acid, HA. A weak acid/salt pair is most effective in buffering against changes in pH when the concentrations of acid and salt are equal, i.e. at pH = pK_a of HA. The effectiveness of a buffer is expressed as its buffering index

$$\frac{dB}{dpH} \tag{11.17}$$

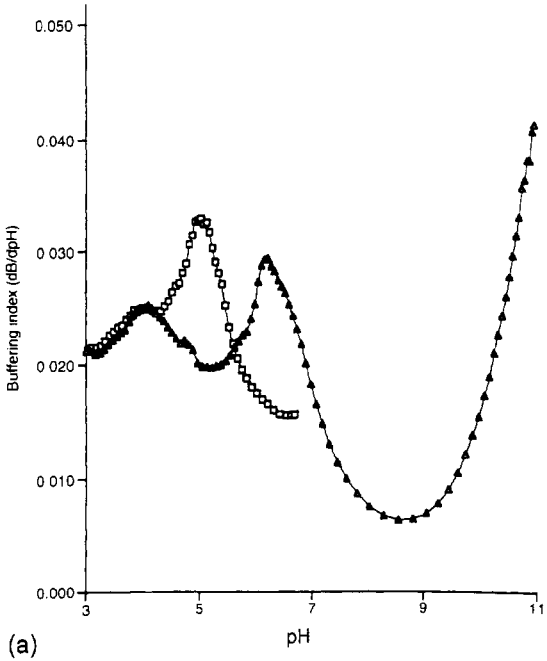
Milk contains a range of groups which are effective in buffering over a wide pH range. The principal buffering compounds in milk are its salts (particularly soluble calcium phosphate, citrate and bicarbonate) and acidic and basic amino acid side-chains on proteins (particularly the caseins). The contribution of these components to the buffering of milk was discussed in detail by Singh, McCarthy and Lucey (1997).

In theory, it should be possible to calculate the overall buffering properties of milk by combining the titration curves for all components but in

practice this is not done since K_a values for many milk constituents are uncertain. Titration curves obtained for milk are very dependent on the technique used, and forward and back titrations may show a marked hysteresis in buffering index (Figure 11.7a). The buffering curve for milk titrated from pH 6.6 to pH 11.0 (Figure 11.7b) shows decreasing buffering from pH 6.6 to about pH 9. Milk has good buffering capacity at high pH values (above pH 10), due principally to lysine residues and carbonate anions. When milk is back titrated from pH 11.0 to pH 3.0, little hysteresis is apparent (Figure 11.7b). Buffering capacity increases below pH 6.6 and reaches a maximum around pH 5.1. This increase, particularly below pH 5.6, is a consequence of the dissolution of CCP. The resulting phosphate anions buffer against a decrease in pH by combining with H^+ to form HPO_4^{2-} and $H_2PO_4^-$. If an acidified milk sample is back titrated with base (Figure 11.7a), buffering capacity is low at about pH 5.1 and the maximum in the buffering curve occurs at a higher pH value (about 6.3), due to the formation of CCP from soluble calcium phosphate with the concomitant release of H^+. Ultrafiltration (UF) causes a steady increase in the buffering capacity of UF retentates due to increased concentrations of caseins, whey proteins and colloidal salts and makes it difficult to obtain an adequate decrease in pH during the manufacture of cheese from UF retentates.

Acid–base equilibria in milk are influenced by processing operations. Pasteurization causes some change in pH due to the loss of CO_2 and precipitation of calcium phosphate. Higher heat treatments (above 100°C) result in a decrease in pH due to the degradation of lactose to various organic acids, especially formic acid (Chapter 9). Slow freezing of milk causes a decrease in pH since the formation of ice crystals during slow freezing concentrates the solutes in the aqueous phase of milk, with the precipitation of calcium phosphate and a concomitant release of H^+. Rapid freezing does not have this effect since there is insufficient time for the above changes to occur. Concentration of milk by evaporation of water causes a decrease in pH as the solubility of calcium phosphate is exceeded, resulting in the formation of more colloidal calcium phosphate. Conversely, dilution causes colloidal calcium phosphate to go into solution, with a corresponding decrease in $[H^+]$ (Chapter 5).

The buffering capacity of milk is often estimated by determining its titratable acidity, which involves titrating a sample of milk, containing a suitable indicator (usually phenolphthalein), with NaOH and thus is a measure of the buffering capacity of the milk between its natural pH and the phenolphthalein endpoint (i.e. between about pH 6.6 and 8.3). Titratable acidity is normally used to estimate the freshness of milk and to monitor the production of lactic acid during fermentation. Fresh milk typically requires 1.3–2.0 milliequivalents OH^- to titrate 100 ml from pH 6.6 to pH 8.3 (13–20 ml of 0.1 M NaOH), i.e. fresh milk has a titratable acidity of 0.14 to 0.16%, expressed as lactic acid.



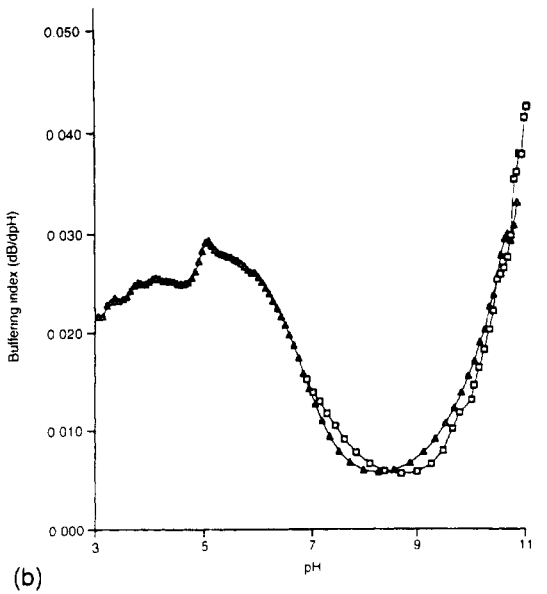


Figure 11.7 (a) Buffering curves of milk titrated from its initial pH (6.6) to pH 3.0 with 0.5 N HCl [□] and back-titrated to pH with 11.0 with 0.5 N NaOH [△].(b) Buffering curves of milk titrated from its initial pH (6.6) to pH 11.0 with 0.5 N NaOH [□] and back-titrated to pH with 3.0 with 0.5 N HCl [△]. (From Singh, McCarthy and Lucey, 1997.)

A high titratable acidity for fresh milk suggests high concentrations of proteins and/or other buffering constituents. Titratable acidity varies only slightly with the breed of cow, although the values for individual cows can vary more widely (0.08–0.25% as lactic acid). The liberation of fatty acids on lipolysis can interfere with the estimation of titratable acidity in high-fat products. Precipitation of calcium phosphate (with a concomitant decrease in pH) and 'fading of the phenolphthalein end-point' can occur during titration and thus the titratable acidity value obtained is influenced by the speed of titration.

11.7 Rheological properties

11.7.1 Newtonian behaviour

Under certain conditions (e.g. moderate shear rates, at fat contents below 40% and at temperatures above 40°C, at which the fat is liquid and no cold agglutination occurs) milk, skim milk and cream are, in effect, fluids with Newtonian rheological properties. Newtonian behaviour can be described by the equation:

$$\tau = \eta\dot{\gamma} \tag{11.18}$$

where τ is the shear stress (force per unit area, Pa), $\dot{\gamma}$ the shear rate (rate of change of velocity across the stream, s^{-1}) and η is the coefficient of viscosity (Pa s). The coefficient of viscosity for a Newtonian fluid is independent of shear rate but is influenced by temperature and pressure.

The coefficient of viscosity for whole milk at 20°C, but not affected by cold agglutination of fat globules, is about 2.127 mPa s. Values for water and milk plasma at 20°C are 1.002 and 1.68 mPa s, respectively. Casein, and to a lesser extent fat, are the principal contributors to the viscosity of milk; whey proteins and low molecular mass species have less influence.

The viscosity of milk and Newtonian milk products is influenced by composition, concentration, pH, temperature, thermal history and processing operations.

The Newtonian coefficient of viscosity at a given temperature for milk, creams and some concentrated milk products is related to the concentration of individual components by Eiler's equation:

$$\eta = \eta_0\left(1 + \frac{1.25\sum(\phi_i)}{1 - \sum(\phi_i)/\phi_{max}}\right)^2 \tag{11.19}$$

where η_0 is the coefficient of viscosity of the portion of the fluid consisting of water and low molecular mass species other than lactose and ϕ is the volume fraction of all dispersed particles that are at least an order of magnitude larger than water. The volume fraction of any component is

given by

$$\phi_i = V_i c_{v,i} \tag{11.20}$$

where V_i is the voluminosity of component i (in $m^3 kg^{-1}$ dry component) and $c_{v,i}$ is the volume concentration of the component in the product ($m^3 kg^{-1}$ product). The voluminosity of fat in fat globules is *c.* $1.11 \times 10^{-3} m^3 kg^{-1}$, that of casein micelles is *c.* $3.9 \times 10^{-3} m^3 kg^{-1}$, whey proteins *c.* $1.5 \times 10^{-3} m^3 kg^{-1}$ and lactose *c.* $1 \times 10^{-3} m^3 kg^{-1}$. For milk

$$\phi \approx \phi_f + \phi_c + \phi_w + \phi_l \tag{11.21}$$

where ϕ_f, ϕ_c, ϕ_w, ϕ_l are the volume fractions of fat, casein, whey proteins and lactose, respectively. ϕ_{max} is the assumed value of $\sum(\phi_i)$ for maximum packing of all dispersed particles (0.9 for fluid milk products).

Increasing pH increases viscosity slightly (perhaps as a consequence of micellar swelling) while a small decrease in pH reduces viscosity, although a large decrease in pH causes aggregation of casein micelles. Viscosity is inversely related to temperature. The viscosity of milk shows thermal hysteresis; it usually shows greater viscosity during heating than during subsequent cooling, probably due to the melting and crystallization behaviour of milk triglycerides.

The viscosity of milk and creams tends to increase slightly with age, due in part to changes in ionic equilibria. Heating skim milk to an extent that denatures most of the whey proteins increases its viscosity by about 10%. Homogenization of whole milk has little effect on its viscosity. The increase in the volume fraction of fat on homogenization is compensated by a decrease in the volume fractions of casein and whey proteins because some skim milk proteins are adsorbed at the fat–oil interface. Pasteurization has no significant effect on the rheology of whole milk.

11.7.2 Non-Newtonian behaviour

Raw milks and creams exhibit non-Newtonian rheological properties when they are held under conditions which favour cold agglutination of fat globules (below 40°C and low shear rates). Under such conditions, they show thixotropic (shear thinning) behaviour, i.e. their apparent viscosity (η_{app}) is inversely related to shear rate. Aggregates of fat globules and the milk serum trapped in their interstitial spaces have a large effective volume due to their irregular shapes. Increasing the shear rate causes increased shear stress to be applied to the aggregates which can disperse, yielding smaller or more rounded ones. Disaggregation reduces the interstitial space between fat globules, thereby reducing the total volume fraction of the fat phase and consequently reducing the η_{app} of the product. When the shearing force applied to the fluid increases in excess of the forces which hold the aggregates together, increases in shear rate cause increasingly smaller

changes in apparent viscosity. Thus, at high shear rates the fluid will exhibit Newtonian behaviour.

Increasing the fat content and/or reducing the temperature favours non-Newtonian behaviour. Low temperatures promote cold agglutination of fat globules and thus increase both η_{app} and deviation from Newtonian behaviour. The temperature at cream separation also influences the rheological properties of the resulting cream. Cream prepared by separation above 40°C shows less deviation from Newtonian behaviour since cryoglobulins are lost in the skim milk, resulting in less agglutination. Apparent viscosity is also influenced by the shear history of the product. The reformation of bonds between fat globules in aggregates requires time and thus the η_{app} versus shear rate ($\dot{\gamma}$) curves exhibit hysteresis. η_{app} increases after cessation of shearing (as aggregates are reformed) but usually does not return to its original value. Hysteresis is apparent in products containing aggregates caused by cold agglutination or homogenization.

Coalescence of fat globules does not change η_{app} since the volume fraction of the fat is not changed. However, partial coalescence can result in an increase in η_{app} due to entrapment of milk serum in aggregates. Indeed, high-fat creams can exhibit rheopectic (shear thickening) behaviour since shearing can cause partial coalescence of fat globules.

In addition to the general decrease in viscosity with increasing temperature, heating milk can also influence its rheology by heat-induced denaturation of cryoglobulins and/or other whey proteins. Concentration of milk, e.g. by ultrafiltration, prior to heating results in a greater increase in η_{app} than in milk heated before concentration.

The addition of hydrocolloids (e.g. carrageenans, pectins or carboxymethyl cellulose) as thickening agents will greatly increase the apparent viscosity of the product. The production of extracellular polysaccharides by certain bacteria will also increase the viscosity of milk products.

11.7.3 Rheology of milk gels

Gels are viscoelastic bodies, the rheological properties of which can be described by two parameters, the storage modulus (G', which is a measure of its elasticity) and the loss modulus (G'', which is a measure of its viscous nature). The combined viscoelastic modulus (G^*) is a measure of the overall resistance of a gel to deformation. These moduli are often highly dependent on the time-scale of deformation. Another important parameter of a food gel is its yield stress.

Although the gelation properties of whey proteins are of great importance in many foods (Mulvihill, 1992) and it is possible to form a weak gel in creams by the formation of a continuous network of fat globules, most important milk gels are those involving casein micelles which can be made to form a gel matrix either by isoelectric precipitation (acid-induced gel) or by the action of a proteolytic enzyme (rennet-induced gel). Both gel types

are relatively similar but, over long deformation times, rennet-induced gels have more liquid character than acid gels, which means that the former can flow under their own weight while acid gels are more likely to retain their shape. Rennet-induced gels also have a greater tendency to synerese and have a higher yield stress than acid-induced gels.

The firmness of acid- and rennet-induced milk gels is increased by such factors as time elapsed after aggregation of the micelles, gelation at elevated temperature, increasing casein and calcium phosphate concentrations and reduced pH (Walstra and Jenness, 1984). Heat-induced denaturation of whey proteins decreases the firmness of rennet-induced gels but increases the firmness of acid-induced gels. Fat globules weaken casein gels by interrupting the gel matrix. Casein molecules on the surface of fat globules in homogenized milk can participate in gel network formation. However, in practice this is influenced by a number of other factors, including preheating, homogenization pressure and temperature, and type of gel (Walstra and Jenness, 1984). Indeed, the yield stress of a rennet-induced milk gel may be reduced by homogenization.

11.7.4 Rheological properties of milk fat

The rheological properties of milk fat are greatly influenced by the ratio of solid to liquid fat and by the crystal form of the solid fat. At room temperature (20°C), milk fat is partially solid and has a plastic consistency, i.e. it exhibits viscoelastic properties; at small deformations (below 1%), it is almost completely elastic due to interactions between the fat crystals which form a weak network but it will begin to flow when subjected to greater deformations. As discussed by Walstra and Jenness (1984), the important parameters in determining the firmness of milk fat include the fraction of solid fat, the shape and size of fat crystals, heterogeneity throughout the fat and the extent to which fat crystals form a network throughout the mass of fat.

The structure of butter and other dairy spreads are further complicated by the presence of aqueous phase droplets and intact fat globules. Water droplets tend to weaken the structure and fat crystals inside intact fat globules cannot participate in the formation of a network thoughout the product (Chapter 3).

11.8 Electrical conductivity

The specific resistance (ρ, ohm cm) of a substance is related to its dimensions by:

$$\rho = \alpha R/l \tag{11.22}$$

where α is the cross-sectional area (cm^2), l is length (cm) and R the measured resistance (ohms). The specific conductance, K ($ohm^{-1}\,cm^{-1}$), is the reciprocal of specific resistance. The specific conductance of milk is usually in the range 0.0040–0.0055 $ohm^{-1}\,cm^{-1}$. Ions (particularly Na^+, K^+ and Cl^-) are responsible for most of the electrical conductivity of milk which is increased by the bacterial fermentation of lactose to lactic acid. Measurement of the specific conductance of milk has been used as a rapid method for detecting subclinical mastitis. The conductivity of solutions is altered by concentration and dilution. However, the usefulness of this in the context of milk (e.g. to detect adulteration with water) is reduced considerably by the influence of concentration or dilution on the precipitation or solubilization of colloidal calcium phosphate. Direct conductivity measurements are thus unsuitable for assessing the amount of water added to milk.

11.9 Thermal properties of milk

The specific heat of a substance is the amount of heat energy, in kJ, required to increase the temperature of 1 kg of the substance by 1 K. The specific heat of skim milk increases from 3.906 to 3.993 $kJ\,kg^{-1}\,K^{-1}$ from 1 to 50°C. Values of 4.052 and 3.931 $kJ\,kg^{-1}\,K^{-1}$ have been reported for skim and whole milks, respectively, at 80°C (Sherbon, 1988). The specific heat of milk is inversely related to its total solids content, although discontinuities have been observed around 70–80°C. Skim-milk powder usually has a specific heat in the range 1.172–1.340 $kJ\,kg^{-1}\,K^{-1}$ at 18–30°C.

The specific heat of milk fat (solid or liquid) is about 2.177 $kJ\,kg^{-1}\,K^{-1}$. The specific heat of milk and cream is therefore strongly influenced by their fat content. Over most commonly encountered temperature ranges, the specific heat of high-fat dairy products is complicated by the latent heat absorbed by melting fat (about 84 $J\,g^{-1}$). The observed specific heat of these products, at temperatures over which milk fat melts is thus the sum of the true specific heat and the energy absorbed to provide the latent heat of fusion of milk fat. Specific heat is thus influenced by factors such as the proportion of fat in the solid phase at the beginning of heating, and thus the composition of the fat and its thermal history. The apparent specific heat of high-fat dairy products (sum of 'true' specific heat and the energy absorbed by melting of fat) is usually maximal at 15–20°C and often has a second maximum or inflexion around 35°C.

The rate of heat transfer through a substance by conduction is given by the Fourier equation for heat conduction:

$$\frac{dQ}{dt} = -kA\frac{dT}{dx} \tag{11.23}$$

where dQ/dt is the quantity of heat energy (Q) transferred per unit time (t), A is the cross-sectional area of the path of heat flow, dT/dx is the temperature gradient and k is the thermal conductivity of the medium. The thermal conductivity of whole milk (2.9% fat), cream and skim milk is roughly 0.559, 0.384 and 0.568 $W\,m^{-1}\,K^{-1}$, respectively. The thermal conductivity of skim milk, whole milk and cream increases with increasing temperature but decreases with increasing levels of total solids or fat, particularly at higher temperatures. In addition to their composition, the thermal conductivity of dried-milk products depends on bulk density (weight per unit volume) due to differences in the amount of air entrapped in the powder.

Thermal diffusivity is a measure of the ability of a material to dissipate temperature gradients within it. Thermal diffusivity (α, $m^2\,s^{-1}$) is defined as the ratio of thermal conductivity (k) to volumetric specific heat (density times specific heat, ρc):

$$\alpha = k/\rho c \tag{11.24}$$

The thermal diffusivity of milk (at 15–20°C) is about $1.25 \times 10^{-7}\,m^2\,s^{-1}$.

11.10 Interaction of light with milk and dairy products

The refractive index (n) of a transparent substance is expressed by the relation:

$$n = \frac{\sin i}{\sin r} \tag{11.25}$$

where i and r are the angles between the incident ray and the refracted ray of light, respectively, and a perpendicular to the surface of the substance. The refractive index of milk is difficult to estimate due to light scattering by casein micelles and fat globules. However, it is possible to make accurate measurements of the refractive index of milk using refractometers in which a thin layer of sample is used, e.g. the Abbé refractometer. The refractive index of milk at 20°C using the D-line of the sodium spectrum ($\sim$589 nm), n_D^{20}, is normally in the range 1.3440–1.3485. The refractive index of milk fat is usually in the range 1.4537–1.4552 at 40°C. Although there is a linear relationship between the solids content (weight per unit volume) and refractive index, determination of percentage solids in milk by refractometry is difficult, since the contributions of various milk components differ and are additive. The relationship between the refractive index of milk and its total solids content varies with changes in the concentration and composition of the solutes in milk. However, attempts have been made to measure the total contribution of solids and casein in milk and milk products by estimating

the refractive index (Sherbon, 1988). The specific refrative index (refractive constant), K, is calculated from:

$$K = \frac{n^2 - 1}{n^2 + 2} \cdot \frac{1}{\rho} \tag{11.26}$$

where n is the refractive index and ρ is density. Milk has a specific refractive index of about 0.2075.

Milk contains not only numerous dissolved chemical components but it is also an emulsion with a colloidal continuous phase. Therefore, milk absorbs light of a wide range of wavelengths and also scatters ultraviolet (UV) and visible light due to the presence of particles. Milk absorbs light of wavelengths between 200 and about 380 nm due to the proteins present and between 400 and 520 nm due to fat-soluble pigments (carotenoids). A number of functional groups in milk constituents absorb in the infrared (IR) region of the spectrum; the OH groups of lactose absorb at *c.* 9.61 μm, the amide groups of proteins at 6.465 μm and the ester carbonyl groups of lipids at 5.723 μm (Singh, McCarthy and Lucey, 1997). Since light scattering is reduced at longer wavelenghts in the IR region, the absorbance of IR light of specific wavelengths can be used to measure the concentrations of fat, protein and lactose in milk. Instruments using this principle are now widely used in the dairy industry. However, since milk contains about 87.5% water (which absorbs IR light strongly), it is opaque to light throughout much of the IR region of the spectrum.

Milk contains about 1.62 mg kg^{-1} riboflavin which fluoresces strongly on excitation by light of wavelenghts from 400 to 500 nm, emitting light with a λ_{max} = 530 nm. Milk proteins also fluoresce due to the presence of aromatic amino acid residues; part of the light absorbed at wavelengths around 280 nm is emitted at longer wavelengths.

Scattering of light by the colloidal fat particles present in milk has been used to estimate its fat content. A commercial apparatus (Milko-Tester™) has been developed which exploits this principle. Milk is diluted (to avoid multiple scatterings) using an EDTA solution which disperses the casein micelles. The milk sample is homogenized to ensure a uniform fat globule size and the extent of scattering of white light is determined.

11.11 Colour of milk and milk products

The white colour of milk results from scattering of visible light by casein micelles and fat globules. Homogenization of milk results in a whiter product due to increased scattering of light by smaller, homogenized, fat globules. The serum phase of milk is greenish due to the presence of riboflavin which is responsible for the characteristic colour of whey.

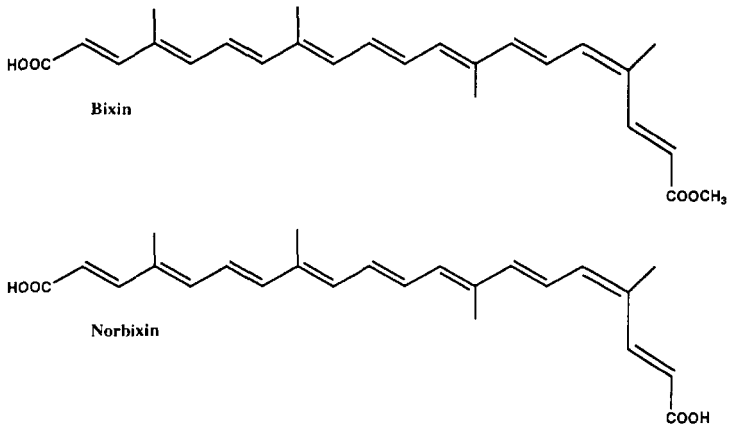


Figure 11.8 Structures of *cis*-bixin and norbixin, apocarotenoid pigments in annatto.

The colour of dairy products such as butter and cheese is due to fat-soluble pigments, especially carotenoids, which are not synthesized by the animal but are obtained from plant sources in the diet. Therefore, feed has a major effect on the colour of milk-fat. Cows fed on grass produce a more yellow-coloured fat than animals fed on hay or concentrates. The ability of cattle to metabolize carotenes to vitamin A varies between breeds and between individuals (Chapter 6).

The most widely used added colorant in dairy products is annatto (E160b) which is a yellow-orange preparation containing apocarotenoid pigments obtained form the pericarp of the seeds of the tropical shrub, *Bixa orellana*. The principal pigment in annatto is *cis*-bixin (methyl 9′-*cis*-6,6′-diapocarotene-6,6′-diooate) with smaller amounts of norbixin (*cis*-6,6′-diapocarotene-6,6′-dioic acid) (Figure 11.8). The heat treatment used in extraction normally converts *cis*-bixin to *trans*-bixin which is red and soluble in oil. Annatto is used to give a yellow colour to margarine and to colour 'red' Cheddar and other cheeses.

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