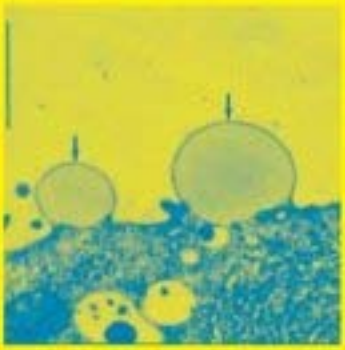
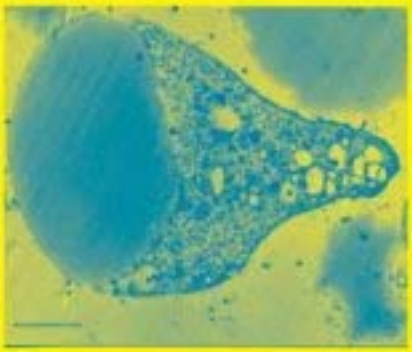
ADVANCED DAIRY CHEMISTRY

VOLUME 3

Lactose, Water, Salts and Minor Constitutents

THIRD EDITION





EDITED BY P.F. FOX AND P.L.H. McSWEENEY

Advanced Dairy Chemistry

Advanced Dairy Chemistry Volume 3: Lactose, Water, Salts and Minor Constituents

Third Edition

Edited by

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Springer

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Preface to the Third Edition

This volume completes the third edition of *Advanced Dairy Chemistry*, a series which commenced as *Developments in Dairy Chemistry* in 1982. This book provides an update of many of the topics covered in the second edition of *Advanced Dairy Chemistry* Volume 3, published in 1997, and complements Volumes 1 and 2 of the third edition (*Proteins*, 2003 and *Lipids*, 2006), making the *Advanced Dairy Chemistry* series the most comprehensive treatise on the topic.

Six (Chapters 1–4, 6, 7) of the 15 chapters in this volume are devoted to various aspects of lactose, including its chemical properties, solid and solution states, its significance in various dairy products, production and utilisation, syndromes associated with lactose malabsorption and its reaction chemistry. In recent years, galactooligosaccharides produced from lactose by the transferase activity of β-galactosidase have become important due to their prebiotic activity and Chapter 5 is devoted to this topic. The indigenous oligosaccharides in the milk of various species are discussed in Chapter 8.

The chemistry and technological aspects of milk salts and water are discussed in Chapters 9 and 11, respectively. The nutritional and health aspects of lactose, minerals and vitamins are assessed in Chapters 6, 8, 10, 12 and 13. Flavours and off-flavours in dairy products and the physico-chemical properties of milk are reviewed in Chapters 14 and 15, respectively.

Like its predecessors, this volume is intended for lecturers, senior students and research personnel working in the field of dairy chemistry and technology. Each chapter is written by an expert and is thoroughly referenced to facilitate further study of specific points.

We would like to express our sincere appreciation to the 35 authors from nine countries who contributed to this volume for sharing so willingly their knowledge of dairy chemistry, which made our task as editors a pleasure.

P. L. H. McSweeney
P. F. Fox
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Preface to the Second Edition

This book is the third volume of *Advanced Dairy Chemistry*, which should be regarded as the second edition of *Developments in Dairy Chemistry*. Volume 1 of the series, *Milk Proteins*, was published in 1992 and Volume 2, *Milk Lipids*, in 1994. Volume 3, on lactose, water, salts and vitamins, essentially updates Volume 3 of *Developments in Dairy Chemistry* but with some important changes.

Five of the eleven chapters are devoted to lactose (its physico-chemical properties, chemical modification, enzymatic modification and nutritional aspects), two chapters are devoted to milk salts (physico-chemical and nutritional aspects), one to vitamins and one to overview the flavour of dairy products. Two topics covered in the first editions (enzymes and other biologically active proteins) were transferred to Volume 1 of *Advanced Dairy Chemistry* and two new topics (water and physico-chemical properties of milk) have been introduced.

Although the constituents covered in this volume are commercially less important than proteins and lipids covered in Volumes 1 and 2, they are critically important from a nutritional viewpoint, especially vitamins and minerals, and to the quality and stability of milk and dairy products, especially flavour, milk salts and water. Lactose, the principal constituent of the solids of bovine milk, has long been regarded as essentially worthless and in many cases problematic from the nutritional and technological viewpoints; however, recent research has created several new possibilities for the utilization of lactose.

Like its predecessor, this book is intended for lecturers, senior students and research personnel; each chapter is written by an expert on the particular subject and is extensively referenced.

I wish to express my sincere thanks and appreciation to all the authors who contributed to this book and whose cooperation made my task as editor a pleasure.

P.F. Fox

Preface to the First Edition

This volume is the third in the series on the chemistry and physical chemistry of milk constituents. Volumes 1 and 2 dealt with the commercially more important constituents, proteins and lipids, respectively. Although the constituents covered in this volume are of less direct commercial importance than the former two, they are nevertheless of major significance in the chemical, physical, technological, nutritional and physiological properties of milk.

Lactose, the principal component of the milks of most species, is a rather unique sugar in many respects-it has been referred to as one of Nature's paradoxes. It is also the principal component in concentrated and dehydrated dairy products, many of the properties of which reflect those of lactose. The chemistry and principal properties of lactose have been thoroughly researched over the years and relatively little new information is available on these aspects; this new knowledge, as well as some of the older literature, is reviewed in Chapter 1.

Although lactose has many applications in the food, pharmaceutical and chemical industries, not more than 10% of the potentially available lactose is actually recovered as such. Like other sugars, lactose may be modified by a multitude of chemical reagents; some of these are reviewed in Chapter 2 and some applications of the derivatives discussed. The enzymatic hydrolysis of lactose to glucose and galactose has considerable technological as well as nutritional significance, and the recent literature on this subject is reviewed in Chapter 3. Lactose is not digestible by the majority of the world's population, and the current views on this nutritionally important problem are discussed in Chapter 4. A deficiency of either of two enzymes involved in the Leloir pathway for galactose metabolism leads to the inability to metabolize galactose produced from lactose (or other galactose-containing sugars) and causes two relatively rare congenital diseases referred to as galactosaemia, the literature on which is reviewed in Chapter 5.

Quantitatively, the salts of milk are minor constituents but they play a disproportionately important role in many of the technologically important

properties of milk, some of which have been discussed in Volume 1 of this series. Recent literature on the rather complex chemistry of the milk salts *per se* is reviewed in Chapter 6. Many of the inorganic constituents of milk, some of which are present only at trace levels, are also of very considerable nutritional significance. Since a variety of minerals are required for proper growth and development, and milk is the sole source of these requirements at a critical stage of infant growth, the significance of milk as a source of dietary minerals is discussed in Chapter 7.

The flavour/off-flavour of milk and dairy products is undoubtedly technologically important and extremely complex. This topic could easily occupy a full volume in this series but a comprehensive summary is presented in Chapter 8.

Many people may regard milk simply as a source of lipids, proteins, carbohydrates and minerals, with very little biological activity as such. This, in fact, is not the case; milk contains a great variety of biologically active species, some of which, e.g. enzymes, may cause undesirable changes in milk and dairy products during storage, while others, e.g. vitamins, immunoglobulins, are of very considerable nutritional and biological significance. Chapters 9, 10 and 11 review the recent literature on the indigenous enzymes in milk, indigenous antibacterial systems and vitamins, respectively. The importance of at least some of the indigenous enzymes and vitamins is well established but the indigenous antibacterial systems may be of much greater significance than considered heretofore, and it is hoped that Chapter 10 will stimulate further research in this area.

I wish to thank sincerely the 13 authors who have contributed to this volume; their cooperation and effort made my task as editor rather simple.

P. F. Fox

Contents

1

Lactose: Chemistry and Properties

P.F. Fox

1.1. Introduction

The principal carbohydrate in the milk of most mammalian species is the reducing disaccharide, lactose, which is composed of galactose and glucose linked by a $\beta 1 \rightarrow 4$ glycosidic bond. Its concentration varies from 0 to $\sim 10\%$, w/w, and milk is the only known significant source of lactose. Research on lactose commenced with the work of Carl Scheele about 1780; its chemistry and its important physico-chemical properties have been described thoroughly. The very extensive literature on lactose has been reviewed by Whittier (1925, 1944), Weisberg (1954), Zadow (1984, 1992), Schaafsma (2008) and Ganzle *et al.* (2008) and in all major textbooks on Dairy Chemistry or Technology, including Jenness and Patton (1959), Webb and Johnson (1965), Webb *et al.* (1974), Walstra and Jenness (1984), Fox (1985, 1997), Wong *et al.* (1988), Fox and McSweeney (1998), Walstra (2002) and Walstra *et al.* (1999, 2006).

There is little new information on the chemistry and properties of lactose *per se* but certain aspects, especially its chemical and enzymatic modification are being studied actively. The objective of this chapter is to provide a general overview of the chemistry and physico-chemical properties of lactose. Although the crystallization of lactose has been studied for a long time, it is still being researched actively and new data on the glass transition of lactose are reviewed in Chapter 2, its production and applications in the food and other industries in Chapter 3 and the problems that may be caused by the crystallization of lactose in dairy products are discussed in Chapter 4. The enzymatic and chemical modification of lactose are discussed in Chapter 5 and its ingestion and malabsorption in Chapter 6. The participation of lactose in the Maillard reaction and the consequences thereof are discussed in Chapter 7.

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The concentration of lactose in milk is inversely related to the concentration of lipids (Figure 1.1) and to the concentration of casein (Figure 1.2) (Jenness and Sloan, 1970; Jenness and Holt, 1987). The principal function of lactose and lipids in milk is as a source of energy; since lipids are ~2.2 times more energy-dense than lactose, when a highly caloric milk is required, e.g. by

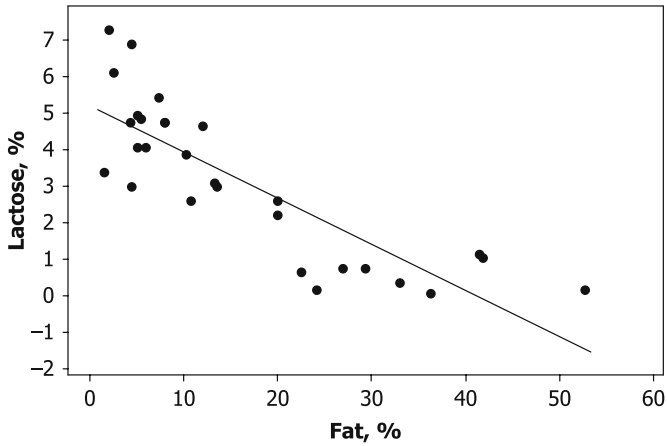

Figure 1.1. Correlation between ash and lactose in the milks of 23 species (based on data of Sloan and Jenness, 1970).

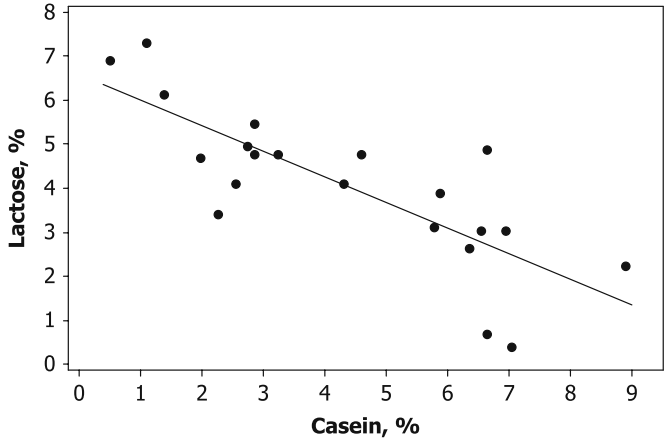

Figure 1.2. Correlation between lactose and casein in the milks of 23 species (based on data of Sloan and Jenness, 1970).

animals in a cold environment (marine mammals or polar bears), this is achieved by increasing the fat content of the milk The inverse relationship between the concentrations of lactose and casein reflects the fact that the synthesis of lactose draws water into the Golgi vesicles, thereby diluting the concentration of casein (Jenness and Holt, 1987).

Lactose is synthesized in the epithelial mammary cells from two molecules of glucose absorbed from the blood. One molecule of glucose is converted (epimerized) to galactose via the Leloir pathway which is widespread in animal tissues and bacterial cells. The galactose is phosphorylated and condensed with a second molecule of glucose through the action of a unique two-component enzyme, lactose synthase. One component is UDP-galactosyl transferase (EC 2.4.1.22) which transfers galactose from UDP-galactose to any of several acceptor molecules in the biosynthesis of glycoproteins and glycolipids. The specificity of the transferase is controlled and modified by one of the principal milk proteins, α-lactalbumin (α-La), which reduces the K_M for glucose 1000-fold and in its presence, most of the galactose is transferred to glucose, with the synthesis of lactose (see Brew, 2003). There is a positive correlation between the concentrations of lactose and α-La in milk; the milk of the California sea lion or the hooded seal which contains no lactose also lack α-La (Jenness and Holt, 1987).

The concentration of lactose in mature bovine, buffalo, ovine and caprine milk is about 4.8. 4.8, 4.6 and 4.1%, w/w, respectively; it increases slightly during the early stages of lactation but then decreases to about 70% of the maximum at the end of lactation. In contrast, the lactose content of the milk of equidae (horse, donkey and zebra) increases during lactation, reaching values in the range 6.0–7.4%, w/w, with considerable inter-individual variation (Oftedal and Jenness, 1988; Doreau and Boulot, 1989). Human milk contains ~7.5%, w/w, lactose.

Milk contains several sugars in addition to lactose, generally at low concentrations. These include glucose (~50 mg/L in bovine milk) and *N*-acetylglucosamine (Bifidus factor I), which stimulates the growth of *Bifidobacterium bifidum* and is present at quite a high level in human milk. The milk of most, probably all, species contains oligosaccharides, containing 3–10 monosaccharides which may be linear or branched. They are derivatives of lactose, which occupies the reducing end of the molecule and most contain fucose and/or *N*-acetylneuraminic acid. Human milk contains ~130 oligosaccharides at a total concentration of ~15 g/L; the milk of marsupials, bears and elephants also contains high levels of oligosaccharides which are believed to play several important functions, including brain development and bactericidal activity; they are indigestible, at least by humans, and thus affect the intestinal microflora. The oligosaccharides in milk and their significance are discussed in detail in Chapter 8.

Lactose serves two important functions in milk: it is a ready source of energy for the neonate (it provides 30% of the caloric value of bovine milk) and is responsible for about 50% of the osmotic pressure of milk, which is isotonic with blood and hence is essentially constant. The synthesis of lactose draws water osmotically into the Golgi vesicles and hence affects the volume of milk and the concentration of casein, which is packaged in the Golgi vesicles, in milk. There is an inverse correlation between the concentrations of lactose and casein in milk (Jenness and Holt, 1987).

For milk with a low level of lactose, the concentration of inorganic salts is high to maintain the osmotic pressure at the desired level. There is a strong inverse relationship (Figure 1.3) between the concentration (mM) of lactose and the osmolality (mM) of milk (Holt and Jenness, 1984; Holt, 1985) but for the milk of 29 species, the inverse relationship between the concentrations (%) of lactose and of ash is weak (Figure 1.4), probably because much of the ash arises from colloidal salts. During mastitis or in late lactation, the integrity of the mammocyte cell membranes is damaged and there is an influx of blood constituents into milk; consequently, the osmotic pressure increases, and to adjust this, the concentration of lactose is reduced. This relationship is expressed as the Koesler number (KN):

$$\text{KN} = \% \text{ chloride} \times 100/\% \text{ lactose},$$

which previously was used as a diagnostic indicator of mastitis (normally, milk has a KN <2 and a value >3 is considered abnormal) but since the

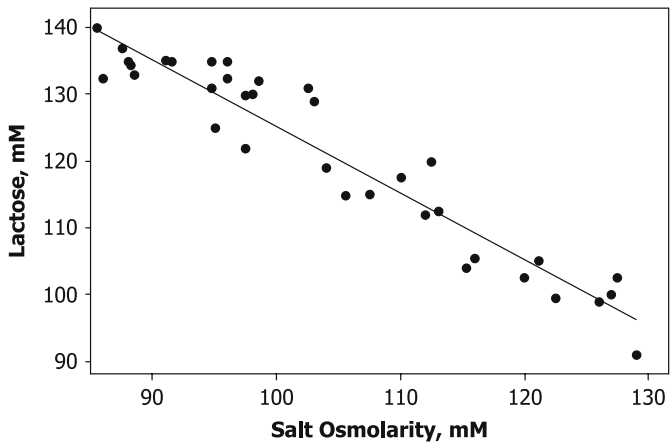

Figure 1.3. Relationship between concentration of lactose (mM) and osmolarity (mM) due to salts (redrawn from the data of Holt, 1985).

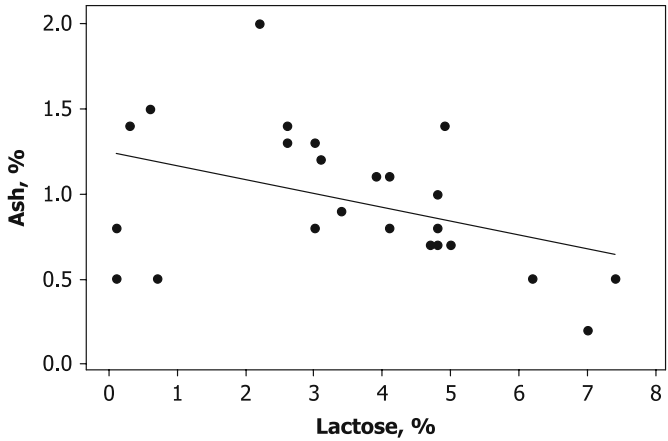

Figure 1.4. Correlation between ash and lactose in the milks of 23 species (based on data of Sloan and Jenness, 1970).

development of rapid methods for the enumeration of somatic cells, the KN is rarely used as a diagnostic test. However, the electrical conductivity of milk, which depends mainly on the milk salts and can be measured in-line during milking, is commonly used as an index of mastitis.

Why milk contains lactose rather than some other sugar(s) is not clear. The presence of a disaccharide rather than a monosaccharide can be explained on the basis that twice the mass of a disaccharide as a monosaccharide can be accommodated in milk for any particular contribution to osmotic pressure, which is fixed in milk. Maltose, which consists of two molecules of glucose, would seem to be the obvious choice of disaccharide. Since energy is expended in converting glucose to galactose, some benefits must accrue from this conversion; a possible benefit is that galactose or derivatives thereof occur in some physiologically important lipids and proteins and a galactose-containing sugar in milk provides the neonate with a ready supply of this important monosaccharide. This question was discussed by Urashima and Sato (2004).

1.2. Properties of Lactose

The properties of lactose are generally similar to those of other sugars but it differs in some technologically important respects. Some important characteristics of lactose are

- Lactose is a reducing sugar, i.e. it has a free, or potentially free, carbonyl group (an aldehyde group in the case of lactose).

- Like other reducing sugars, lactose exists partially as an open-chain form with an aldehyde group which can form a hemi-acetal and, thus, a ring structure. The formation of a hemi-acetal creates a new chiral centre (asymmetric carbon) which may exist as two isomers (enanthiomorphs), α or β. By alternatively opening and forming the ring structure, the molecule can interchange between α and β anomers, a process referred to as mutarotation.
- The α and β anomers of lactose have very different properties, the most important of which are specific rotation, $[\alpha]_D^{20}$ ($+89^\circ$ and $+35^\circ$ for α-and β-lactose, respectively) and solubility (70 and 500 g/L, for α- and β-lactose, respectively).
- Like all reducing sugars, lactose can participate in the Maillard (non-enzymatic browning) reaction, resulting in the production of (off-) flavour compounds and brown polymers. The Maillard reaction contributes positively to the flavour and colour of many foods, e.g. crust of bread, toast and deep-fried products, but the effects in dairy products are usually negative and must be avoided. (The Maillard reaction and its consequences are discussed in Chapter 7.)
- Redox titration using alkaline $CuSO_4$ (Fehling's solution) or chloramine-T is the principal standard method for the quantitative determination of lactose, although in large laboratories it is now usually determined by infrared spectrophotometry. It may also be determined by polarimetry, spectrophotometry at a visible wavelength after reaction with phenol or anthrone in strongly acid solution, enzymatically (using an enzyme assay kit) or by high-performance liquid chromatography.
- Among sugars, lactose, especially the α-enanthiomorph, has low solubility in water but when in solution, it is difficult to crystallize which may cause problems in lactose-rich dairy products, e.g. skimmed milk powder and whey powder, unless precautions are taken to induce and control crystallization (see Chapter 2).
- α- and β-lactose are soluble in water to the extent of about 70 and 500 g/L, respectively, at 20°C; at equilibrium, the ratio of α:β is about 37:63, giving a total solubility of about 180 g/L at 20°C. The solubility of α-lactose is more temperature dependent than that of the β-anomer and is the more soluble $>93.5^\circ$C (Figure 1.5). Hence, α-lactose is the form of lactose which crystallizes $<93.5^\circ$C and is the usual commercial form of lactose; β-lactose may be prepared by crystallization $>93.5^\circ$C.
- α-Lactose crystallizes as a monohydrate while β-lactose forms anhydrous crystals; thus, the yield of α-lactose is $\sim$5% higher than that of β-lactose.

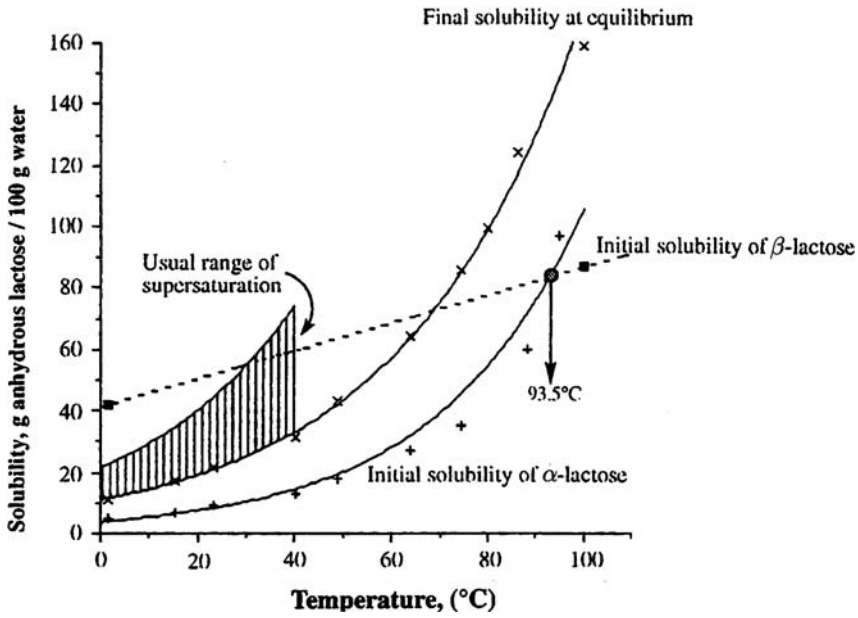

Figure 1.5. Solubility of α- and β-lactose as a function of temperature (from Fox and McSweeney, 1998).

- When milk or whey is spray-dried, any lactose which has not been pre-crystallized forms an amorphous glass which is stable if the moisture content of the powder is maintained low. However, if the moisture content increases to >6%, the lactose crystallizes as α-hydrate, the crystals of which form interlocking masses and clumps which may render the powder unusable if very extensive, i.e. inadequately crystallized powder is hygroscopic. The problem can be avoided by adequate crystallization of lactose before drying or by using effective packaging.
- Interestingly, crystalline lactose has very low hygroscopicity and is used in icing sugar blends.

Problems related to the solubility, crystallization, mutarotation and hygroscopicity of lactose are discussed in Chapters 2, 3 and 4:

- Among sugars, lactose has a low level of sweetness; it is only about 16% as sweet as sucrose at 1% in solution and hence has limited value as a sweetening agent, the principal application of sugars in foods. However, it is a useful bulking agent when excessive sweetness is undesirable.

- Lactose is important in the manufacture of fermented dairy products where it serves as a carbon source for lactic acid bacteria (LAB) which produce lactic acid.

1.2.1. Modification of the Concentration of Lactose in Milk Through Genetic Engineering

There has been considerable interest in modifying the lactose content of milk by genetic engineering (see Leaver and Law, 2003). Since the concentration of lactose is controlled by the concentration of α-La in the secretory cells, the approach to changing the concentration of lactose involves altering the level of this protein. There is interest in reducing the level of lactose for at least three reasons:

- Lactose is the least valuable constituent in milk but it costs energy on the part of the animal to synthesize it; therefore, it would be economically advantageous to reduce the lactose content of milk.
- Since lactose effectively controls the water content of milk and most dairy processes require the removal of water, it would be advantageous to reduce the amount of water in milk by reducing the level of lactose. However, if the level of lactose is reduced too much, the viscosity of the milk will be too high for easy expression of milk; the viscosity of mouse milk genetically engineered to contain no lactose was so high that the pups were unable to suckle and died (see Leaver and Law, 2003). Obviously, this problem could be overcome by reducing the level of lactose rather than eliminating it. Alternatively, it may be possible to modify the milk secretory mechanism to produce a more useful, or at least a less problematic, sugar than lactose, e.g. glucose, maltose or lactulose (which is a laxative and prebiotic), or it might be possible to increase the concentration of salts in milk.
- As discussed below, most adult humans are unable to digest lactose. If the problems arising from high viscosity were resolved, lactose-free or -reduced milk would be nutritionally desirable.

However, in some cases it would be advantageous to increase the lactose content of milk. The economic benefits of increasing the milk output of sows by increasing its lactose content were discussed by Wheeler (2003).

1.2.2. Nutritional Problems Associated with Lactose

Mammals cannot absorb disaccharides from the intestine; they are first hydrolysed in the small intestine to monosaccharides, which are absorbed. Lactose is hydrolysed by β-galactosidase (β-gal; also referred to as lactase)

which is secreted by cells in the brush border of the small intestine. The young of most mammalian species secrete an adequate level of β-gal but as the animal ages, the secretion of β-gal declines and eventually becomes inadequate to hydrolyse ingested lactose which enters the large intestine, into which it draws water, causing diarrhoea, and is metabolized by bacteria with the production of gas which causes cramps and flatulence. In humans, this may occur at 8–10 years of age. These problems cause many individuals to exclude milk from the diet. The problems may be avoided by pre-hydrolysing the lactose using exogenous β-galactosidase (see Chapters 3 and 5) or removing it by membrane technology (Chapter 3). The frequency and intensity of lactose intolerance/malabsorption varies widely among populations from $\sim$100% in south-east Asia to $\sim$5% in north-west Europe (Paige and Davis, 1985; Mustapha *et al.*, 1997; Chapter 6).

1.2.3. Production and Utilization of Lactose

Previously, whey, from cheese or casein production, was considered a waste material which was fed to farm animals, irrigated on land or disposed into sewers. Economic and environmental considerations now dictate that whey be used more efficiently. The principal product lines produced from whey are various whey powders, whey protein products produced mainly by membrane technology and lactose and its derivatives. Membrane technology is being used increasingly to concentrate and fractionate milk; the resulting permeate has a number of applications, including the production of lactose.

Lactose is prepared commercially by crystallization from concentrated whey or ultrafiltrate. The crystals are usually recovered by centrifugation; this process is essentially similar to that used for sucrose or other sugars. About 400,000 tonnes of crystalline lactose are produced annually (compared to $\sim 10^8$ tonnes of sucrose p.a.). Developments and trends in the production and utilization of lactose are discussed by de Boer and Dijksterhuis (1998) and in Chapter 4.

Owing to its relatively low sweetness and low solubility, the applications of lactose are much more limited than sucrose or glucose. Its principal application is in the production of "humanized" infant formulae based on cows' milk (human milk contains $\sim$7% lactose in comparison with $\sim$4.6% in bovine milk). The lactose used may be a crystalline product or demineralized whey (for physiological reasons, it is necessary to reduce the concentration of inorganic salts in bovine whey).

Lactose has a number of low-volume, special applications in the food industry, e.g. as a free-flowing or agglomerating agent, to accentuate/enhance the flavour of some foods, to improve the functionality of shortenings, and as a diluent for pigments, flavours or enzymes. It is widely used in the tabletting of drugs in the pharmaceutical industry where low hygroscopicity is a critical property (see Chapter 4).

1.3. Derivatives of Lactose

Like other sugars, lactose has reactive functional groups and can be converted to several more valuable food-grade derivatives (see Yang and Silva, 1995), of which the following are the most significant:

- Glucose–galactose syrups, which are $\sim 3\times$ as sweet as lactose, can be produced by hydrolysis by a strong acid but are usually prepared by β-galactosidase. The technology for the production of glucose–galactose syrups, by acid or β-galactosidase, is well developed (see Mahony, 1997; Chapter 5) but the products are not cost-competitive with other sweeteners (sucrose, glucose, high-fructose syrups or synthetic sweeteners).
- Owing to the several alcohol groups, sugars, including lactose, are reactive molecules, from which a great variety of derivatives can be produced (see Thelwall, 1997). However, the chemical derivatives of lactose are not being produced commercially, probably because similar products can be produced from other, cheaper, sugars, e.g. a trichlorinated derivative of sucrose, sucralose, commercialized under the trade name "Splenda" [E955], is a very successful artificial sweetener (up to 1000 times as sweet as sucrose or twice as sweet as saccharine and four times as sweet as aspartame). I am not aware of studies on the sweetness of chlorinated lactose.
- Probably the most commercially successful derivative of lactose is lactulose (galactose–fructose) produced by the epimerization of the glucose moiety of lactose to fructose under mildly alkaline conditions. Lactulose has many applications including use as a prebiotic and a mild laxative (see Timmermans, 1998; Aider and de Halleux, 2007; Chapter 5).
- The carbonyl group of lactose can be reduced to lactitol (the alcohol of lactose) or oxidized to lactobionic acid. The production and properties of lactitol were described by Timmermans (1998). Lactitol can be esterified with various fatty acids and used as emulsifiers analogous to sorbitans (esters of sorbitol).
- Lactobionic acid is a sweet-tasting acid, which is a very rare property and can be exploited in processed foods. It can be produced by electrochemical oxidation (Gerling, 1998), enzymatically, using glucose–fructose oxidoreductase (Satory *et al.*, 1997) or by living microorganisms (Murakami *et al.*, 2002a,b). A method for the production of lactose-free galactosaccharides was described by Splechtna *et al.* (2001); lactose was first treated with β-galactosidase to yield a mixture of galactooligosaccharides, lactose and monosaccharides. The residual lactose was oxidized by cellobiose dehydrogenase to

lactobionic acid, which was removed by ion-exchange chromatography and could be used as valuable by-product.

- Tagatose is the keto analogue of galactose. It occurs at a low level in the gum of the evergreen tree, *Sterculia setigera,* in severely heated milk or stored milk powder (Troyano *et al.*, 1992, 1994). It can be produced by treating β-galactosidase-hydrolysed lactose with a weak alkali, e.g. $Ca(OH)_2$, which converts galactose to tagatose, which can be purified by demineralization and chromatography. Tagatose is nearly as sweet as sucrose, has a good quality sweet taste and enhances the flavour of other sweeteners. It is absorbed poorly from the small intestine, serves as a prebiotic and has little effect on blood glucose. It is fermented in the lower intestine to short-chain fatty acids, which are absorbed and provide ~35% of the energy obtained from sugars catabolized in the normal way. Tagatose has GRAS status and is produced commercially by SweetGredients, a company formed by Arla Dairies and Nordzuker (Denmark).
- β-Galactosidase is normally a hydrolase but it also has transferase activity and under certain conditions this activity predominates, with the production of oligosaccharides, containing 2–10 monosaccharides. These oligosaccharides have interesting physico-chemical properties and may be useful as food ingredients but most attention today is focused on their prebiotic properties. The oligosaccharides produced by β-galactosidase (Chapter 5) should not be confused with the indigenous oligosaccharides in milk which are described in Chapter 8.
- Lactose can serve as substrate for the production of various fermentation products of which ethanol, lactic, acetic and propionic acids are the most important.

  The production of ethanol from lactose by fermentation using *Kluyveromyces lactis* or *K. fragilis* has been at a commercial level for at least 30 years. If the ethanol is used in potable products, this process is economically viable but whey-derived ethanol is not classified as potable in some countries. The increased recent interest in bioenergy sources will open new opportunities for lactose-derived ethanol but such applications may not be cost-competitive and will depend strongly on local taxation policy.

The oxidation of ethanol by *Acetobacter aceti* to acetic acid for vinegar or other applications is technically feasible but in most cases is not cost-effective.

The *in situ* fermentation of lactose by lactic acid bacteria to lactic acid is widespread in the production of fermented dairy products. The fermentation

of lactose to lactic acid for food or industrial applications (including the biodegradable plastic, polylactic acid) is technically feasible but not cost-competitive with production by the fermentation of other sugars or by chemical synthesis.

Lactic acid can be converted by *Propionibacterium* spp. to propionic acid (with acetic acid, CO_2 and H_2O as by-products), which is used as a fungicide in the food industry but like the other fermentation products described here, this conversion may not be economically feasible.

1.4. Significance of Lactose in Dairy Products

Arising from its low solubility, crystallization behaviour and hygroscoposity, lactose causes problems in concentrated, dehydrated and frozen dairy products. These problems can be avoided by the application of appropriate processing techniques, which have been developed over many years. Developments in these areas in relation to concentrated milk, milk powders, dulce de leche and ice cream will be discussed in Chapter 3.

Although commercially less important than in concentrated and dehydrated dairy products, these physico-chemical properties of lactose also cause problems in the production of frozen milk which may be economical under certain circumstances, especially if the milk is pre-concentrated. However, the casein micelles are destabilized during frozen storage and aggregate on thawing. Destabilization is caused by a decrease in pH and an increase in Ca^{2+}, both due to the formation of $Ca_3(PO_4)_2$ from $CaHPO_4$ and $Ca(HPO_4)_2$ on reducing the amount of solvent water on the formation of ice and which is reduced further by the crystallization of α-lactose monohydrate (see Fox and McSweeney, 1998). Destabilization can be avoided by pre-hydrolysing the lactose or freezing rapidly to $<-30^{\circ}C$. The aggregated casein can be redispersed by heating the thawed milk to $\sim 50^{\circ}C$; the properties of the reformed micelles have not been studied in detail. Interestingly, the cryoprecipitation of casein may be exploited for the commercial production of casein (see Moon *et al.*, 1989).

As a reducing sugar, lactose can participate in the Maillard reaction, principally with the ε-amino group of lysine, resulting in the formation of brown-coloured pigments or volatile flavoured compounds and impaired functionality and nutritional value. The Maillard reaction is the subject of Chapter 7. The reaction is particularly severe in heated products but occurs also in milk powders, especially during storage under adverse conditions of temperature and humidity (see Chapter 4). It may be a problem in cheese which is subjected to severe heating, e.g. Mozzarella, or in grated cheese during storage.

The use of *Streptococcus thermophilus*, which cannot metabolize galactose, and galactose-negative strains of lactobacilli may produce sufficient galactose to cause browning-related problems in certain types of cheese and especially in whey therefrom during drying. The crystallization characteristics of galactose are quite different from those of lactose (see Chapter 2) and may cause problems in whey powders.

The monosaccharides, glucose and galactose, are much more reactive than lactose, and hence dairy products containing hydrolysed lactose are particularly susceptible to Maillard browning. The hydrolysis of lactose by β-galactosidase markedly increases the heat stability of milk and concentrated milk, especially around the pH of minimum solubility; hydrolysis of $\geq \sim$20% has a significant effect (Tan and Fox, 1996). The mechanism of stabilization has not been elucidated fully but is probably due to the carbonyls formed in the Maillard reaction; unfortunately, such lactose-hydrolysed milk products are very susceptible to intense browning, which may render them non-viable commercially.

1.5. Conclusion

Lactose is one of the principal naturally occurring disaccharides. For reasons which are not fully clear, lactose is the principal saccharide in mammalian milks, which are the only significant sources of lactose. As for other sugars, the chemical and physico-chemical properties of lactose are well established after more than 200 years of research. However, lactose continues to be the subject of considerable research and new discoveries continue to be made and will be described in the following chapters. Moreover, lactose has become a valuable commodity, which stimulates further studies.

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2

Solid and Liquid States of Lactose

Y.H. Roos

Lactose in dairy systems can exist in various crystalline and non-crystalline forms. These forms affect lactose behaviour, particularly in processing and storage of low-water dairy foods. Crystalline α-lactose monohydrate and anhydrous β-lactose are well-known solid forms of lactose, which are relatively poorly soluble in water. Its occurrence in two anomeric forms, α- and β-lactose, makes its solubility a complex function of temperature. α-Lactose has low solubility in water at room temperature, but mutarotation to equilibrium quantities of the α- and β-forms increases the overall solubility of lactose which increases rapidly with increasing temperature, with a more rapid increase in the solubility of α-lactose. Liquid dairy systems contain dissolved lactose in a complex chemical environment and lactose is likely to exist in a composition-, temperature- and process-dependent α/β-ratio. On rapid removal of solvent water from dairy liquids on dehydration or freezing, lactose molecules retain their solution structure and, therefore, amorphous, non-crystalline solid forms of lactose are typical of dairy powders and frozen dairy desserts (Roos, 1995; Hartel, 2001).

Amorphous lactose in dairy solids may often exist in a glassy, solid state or in a syrup-like, super-cooled liquid state. The apparent glass-like solid state results from a very high viscosity exceeding 10^{12} Pa s (White and Cakebread, 1966). The state transition of amorphous solid- and liquid-like states occurs over a second-order-type state transition known as the glass transition (White and Cakebread, 1966), as described in Figure 2.1. The glass transition involves no latent heat but it can be observed from changes in heat capacity, thermal expansion coefficient, dielectric properties, various mechanical and flow properties and molecular mobility (White

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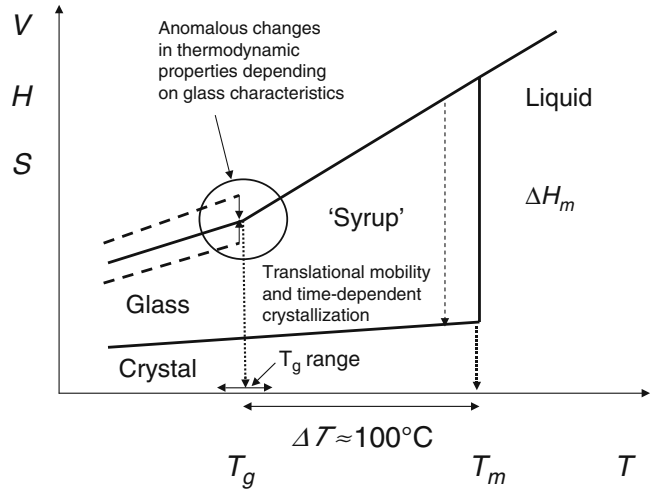

Figure 2.1. A schematic presentation of changes in enthalpy, H, entropy, S, and volume, V around glass transition temperature, T_g, and melting temperature, T_m. The glassy state is a non-equilibrium state and the glass transition occurs over a temperature range and results in a change of a solid-like material to a syrup-like liquid in sugar systems.

and Cakebread, 1966; Lai and Schmidt, 1990; Slade and Levine, 1991; Kalichevsky *et al*., 1993a; Roos, 1995). The glass transition of hydrophilic dairy solids is dominated by that of lactose in which water acts as a softener or 'plasticizer' (Jouppila and Roos, 1994a,b). Plasticization by water can be observed as a decrease in the glass transition temperature with increasing water content.

Water plasticization is an important factor contributing to dehydration characteristics and storage stability of dairy solids. A dramatic and well-documented decrease in the stability of dairy powders occurs above a critical water content and corresponding critical water activity (Supplee, 1926; Troy and Sharp, 1930; Herrington, 1934; Lea and White, 1948; King, 1965; Labuza and Saltmarch, 1981; Jouppila *et al*., 1997; Haque and Roos, 2006). These values of critical water content and water activity correspond to those at which the glass transition of lactose occurs at the storage temperature (Figure 2.2). Exceeding the glass transition conditions of lactose results in dramatic changes in the flow properties of dairy powders and the time-dependent crystallization of lactose (Roos and Karel, 1991c; 1992; Jouppila *et al*., 1997; Paterson *et al*., 2005; Haque and Roos, 2004; 2006). Many other physical and chemical changes observed in dehydrated and frozen dairy system have been shown to result from water plasticization and the glass

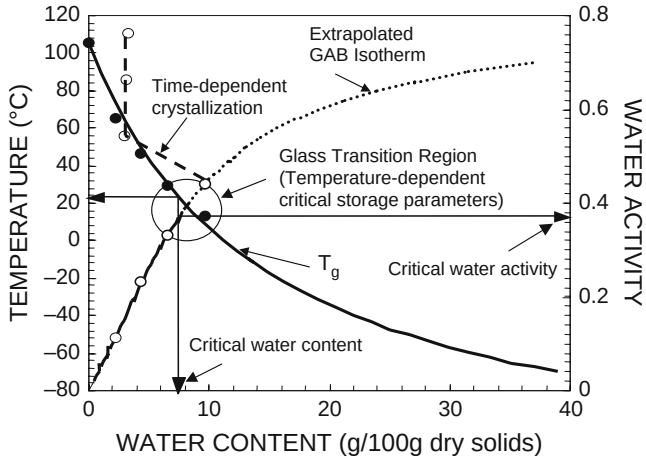

Figure 2.2. Water plasticization and glass transition temperatures of lactose at various water contents. Depression of the glass transition temperature, T_g, with water content was predicted by the Gordon–Taylor equation (1). The critical water content and water activity correspond to plasticization, depressing T_g to room temperature. Higher water levels result in stickiness, caking, increased browning rates and time-dependent lactose crystallization. Data from Haque and Roos (2004).

transition of lactose (Roos and Karel, 1991a; Slade and Levine, 1991; Jouppila *et al.*, 1997; Hartel, 2001).

The objective of this review is to highlight properties of non-crystalline lactose and its impact on dairy product characteristics at low water contents and in the frozen state. The non-crystalline state of lactose is often a non-equilibrium state showing time-dependent characteristics which may be observed, for example, from changes in flow properties and time-dependent lactose crystallization.

2.1. State Diagram of Lactose

A state diagram may be considered as a 'map' which describes conditions at which non-crystalline systems appear as solid glasses or as super-cooled liquids at various water contents and temperatures. State diagrams describe water plasticization behaviour of hydrophilic amorphous solids and the concentration dependence of the glass transition of solutes taking into account ice formation (solvent crystallization) and its effect on solute

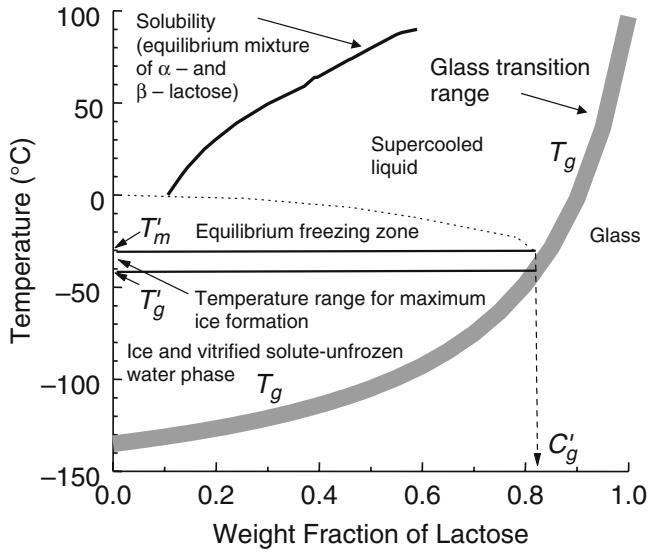

Figure 2.3. State diagram of lactose. The glass transition temperature (T_g) curve at high solids content explains the physical state dependence on temperature and water plasticization. Solvent water crystallization is controlled by equilibrium freezing as defined by solute concentration and kinetically by vitrification at solute concentration of C_g' with a glass transition of a maximally freeze-concentrated lactose at T_g' and onset temperature of ice melting at T_m'.

concentration at low temperatures. The state diagram of lactose (Figure 2.3) is useful for characterization of the physical state and physical properties of common dehydrated and frozen dairy foods.

State diagrams have been used by Levine and Slade (1988a, 1989) to characterize the effects of frozen storage temperature on food quality which is particularly important to understand the frozen state properties of ice cream and other dairy desserts. State diagrams are available for lactose, milk powders with various fat contents and with hydrolysed lactose (Jouppila and Roos, 1994b; Roos, 2002), lactose protein mixtures (Haque and Roos, 2006) and lactose–salt systems (Omar and Roos, 2006a,b). It appears that lactose governs the solid state of lactose-containing powders but the hydrolysis of lactose results in a significant change to solid properties. This change is a result of the hydrolysis of lactose to glucose and galactose which differ greatly in their sensitivity to water from that of lactose (Jouppila and Roos, 1994a,b). It is also important to note that the glass transition of dairy solids is a property of the hydrophilic, miscible components, often dominated by lactose or its mixtures with added sugar components and

products of lactose hydrolysis. Water plasticization occurs only in the solids-non-fat fraction, and state diagrams describe the solids-non-fat properties of dairy systems.

The lactose–water system is a binary solute–solvent mixture. Water, as a small molecular mass solvent, acts as a strong plasticizer and a significant depression of the glass transition temperature, T_g, occurs at low water contents (Slade and Levine, 1991). The plasticization behaviour of amorphous polymer–solvent systems is often modelled using the Gordon–Taylor relationship (Gordon and Taylor, 1952), which allows modelling of the glass transition temperature depression with increasing water content. The Gordon–Taylor relationship is shown in equation (1), where w_1 and w_2 are weight fractions of solids and water, respectively, T_{g1} and T_{g2} are the glass transition temperatures of respective components and k is a constant:

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} \tag{1}$$

The constant, k, in equation (1) can be derived from experimental data for T_g at various water contents (Roos, 1995). Water plasticization of lactose has been shown to follow this equation which allows its use for establishing the glass transition curve in the state diagram of lactose (Roos and Karel, 1991a). The Gordon–Taylor equation has also been applied to predict water plasticization of dairy powders (Jouppila and Roos, 1994b; Haque and Roos, 2006), casein (Kalichevsky *et al.*, 1993a,b) and a number of other food systems (Roos, 1995). Although numerous values have been reported for the glass transition temperature of non-crystalline water, the glass transition temperature for amorphous water is often taken as –135°C (Sugisaki *et al.*, 1968). Several equations other than the Gordon–Taylor relationship are available for predicting the effects of water plasticization and composition on the T_g of dairy solids (Roos, 1995).

Most state diagrams show equilibrium melting temperatures of ice at various water contents and kinetic limitations for ice formation. Ice formation ceases at temperatures where the equilibrium ice melting temperature approaches the glass transition of the freeze-concentrated solutes in an unfrozen solute matrix. Kinetically limited ice formation may be described as non-equilibrium ice formation, which is a typical phenomenon in rapidly cooled carbohydrate solutions and probably the most common form of ice formation in frozen dairy systems, including ice cream and frozen yoghurt. One of the first studies reporting non-equilibrium freezing was that of Troy and Sharp (1930), who found that rapid freezing of ice cream resulted in freeze-concentration and

super-saturation of lactose which, at a sufficiently low temperature, would not crystallize. Several sugars, including lactose, and sugar–protein mixtures form such super-saturated amorphous matrices in frozen systems (Bellows and King, 1973; Roos and Karel, 1991a; Slade and Levine, 1991; Goff *et al.*, 1993; Roos, 1993; Goff, 2002; Singh and Roos, 2005).

State diagrams show the T_g at various water contents. Freezing of water results in separation of ice and concentration of solutes in unfrozen water. Freezing of water ceases as the glassy state of the unfrozen water–solute phase is approached. The glass transition temperature of the maximally freeze-concentrated solute (temperature at which ice formation ceases) with corresponding solute concentration, C'_g, onset temperature for ice melting in the maximally freeze-concentrated solution, T'_m, equilibrium ice melting temperature, T_m curve, and solubility, are often included in state diagrams. The state diagram of lactose with transition temperatures and corresponding lactose concentrations is shown in Figure 2.3. The most precise C_g' values and corresponding unfrozen water contents, W_g', can be derived from state diagrams established with experimental T_g values (Roos and Karel, 1991b). The solute concentration of maximally freeze-concentrated solute matrices, including that of non-fat milk solids, has been found to be about 80% (w/w), i.e. the unfrozen water content (W_g') is 20% (w/w). These values correspond to solute and water concentrations, respectively, at which ice formation may not occur in freezing, i.e. ice formation is not possible in a system composed of 20% (w/w) water and 80% (w/w) solutes (Roos and Karel, 1991a,b; Roos, 1993; Jouppila and Roos, 1994b). Higher levels of unfrozen water may exist in maximally freeze-concentrated matrices of food polymers such as starch and proteins due to their much higher T'_g values (Roos and Karel, 1991d; Roos, 1995; Singh and Roos, 2005).

2.2. Stickiness and Caking

Stickiness and caking are phenomena which may occur when amorphous powder components are plasticized thermally as a result of heating or by exposure to high humidity, resulting in water sorption and plasticization (Peleg, 1977, 1983; Roos, 1995; Lloyd *et al.*, 1996; Paterson *et al.*, 2005; Fitzpatrick *et al.*, 2007). Stickiness and caking of dairy powders are often related to water plasticization of amorphous lactose. Water plasticization may result in glass transition and viscous flow of the non-crystalline lactose at particle surfaces which is observed as stickiness and caking. The surface viscosity of particles is an important property of amorphous powders. Downton *et al.* (1982) showed that surface viscosity governs the flow properties, stickiness and caking of amorphous powder particles. Levine and Slade (1988b) suggested

that as the viscosity decreased rapidly above the glass transition, amorphous solids could undergo numerous time-dependent structural transformations. These changes in food systems included stickiness and caking of powders, plating of particles on amorphous granules and structural collapse of dehydrated structures.

Williams *et al.* (1955) found that the viscosity of amorphous glucose above its glass transition was similar to the viscosity of other inorganic and organic glass-forming compounds. Viscosity was related to relaxation times above T_g and followed an empirical relationship known as the William–Landel–Ferry (WLF) equation (2), which was derived from the viscosity data for a number of compounds.

$$\log \frac{\eta}{\eta_s} = \frac{C_1(T - T_s)}{C_2 + (T - T_s)} \tag{2}$$

where η is viscosity at temperature, T, η_s is viscosity at a reference temperature, T_s, and C_1 and C_2 are constants.

The main cause of stickiness is water or thermal plasticization of particle surfaces, which allows a sufficient decrease in surface viscosity and enhances liquid-like behaviour and the development of surface tension for adhesion. Downton *et al.* (1982) suggested that an increase of temperature or water content caused the formation of an incipient liquid state of a lower viscosity at the particle surface, which resulted in stickiness. Downton *et al.* (1982) proposed that particles sticked together if sufficient liquid could flow to build strong enough bridges between the particles and that the driving force for the flow was surface tension, which was confirmed for dairy systems by Adhikari *et al.* (2007).

Stickiness is a time-dependent property. Since viscosity in the glassy state is extremely high, the contact time must be very long to allow adhesion. A dramatic decrease in viscosity above T_g reduces the contact time and causes stickiness which can be related to the time scale of observation. Downton *et al.* (1982) estimated that a surface viscosity lower than 10^6–10^8 Pa s at a contact time of 1–10 s was sufficient for stickiness. The sticky point was found to decrease with increasing water content. The critical viscosity for stickiness was almost independent of water content, ranging from 0.3×10^7 to 4.0×10^7 Pa s, which agreed well with the predicted viscosity range. Wallack and King (1988) reported that the critical viscosity range applied also to other amorphous powders.

Stickiness and caking may also be related to the hygroscopicity of non-crystalline sugars. Brennan *et al.* (1971) studied stickiness properties of powders in spray drying and they pointed out that two approaches may be

used to reduce the thermoplasticity and hygroscopicity and therefore to solve problems caused by wall deposition in spray drying, i.e. the use of additives as drying aids and the use of specially designed equipment. The sticky point, which describes particle adhesion and stickiness temperature, of amorphous food solids against water content follows an isoviscosity curve with about a constant temperature difference from T_g (Downton *et al.*, 1982; Roos and Karel, 1991a), and the measurement of the sticky point by the method of Lazar *et al.* (1956) can be considered as a method which, in fact, locates the glass transition of the food solids (Chuy and Labuza, 1994).

Dairy solids-non-fat are plasticized by both temperature and water. Water at a constant temperature may affect the physical properties similarly to temperature at a constant water content. Assuming that the WLF-type temperature dependence applies, the viscosity at a constant water content decreases with increasing temperature. The WLF equation with the 'universal' constants $C_1 = -17.44$ and $C_2 = 51.6$, when T_g is the reference temperature (Williams *et al.*, 1955), predicts that an isoviscosity state of 10^7 Pa s exists at about 20°C above T_g, which agrees with the experimental and predicted critical viscosity values for stickiness reported by Downton *et al.* (1982). The particular importance of the relationship between the sticky point and T_g is that the T_g of amorphous dairy powders can be used as a stability indicator. Thus, knowledge of the T_g and its dependence on water content can be used to evaluate causes of stickiness problems, especially in the production and storage of dairy and other amorphous powders, as described in Figure 2.4.

Caking of sticky powders occurs when sufficient time is allowed for surface contact. According to Peleg (1977), liquid bridging is one of the main inter-particle phenomena which result in caking of food powders. Factors that may cause liquid bridging include water sorption, melting of component compounds (e.g. lipids), chemical reactions that produce liquids (e.g. non-enzymatic browning), excessive liquid ingredients, water released due to crystallization of amorphous sugars and wetting of the powder or equipment. The most common caking mechanism in food powders is plasticization due to water sorption and subsequent inter-particle fusion (Peleg and Mannheim, 1977; Peleg, 1983). Caking of amorphous powders often results from the change of the material from the glassy to a less viscous liquid-like state, which allows liquid flow and the formation of inter-particle liquid bridges. Peleg (1983) pointed out that 'humidity caking' is the most common mechanism of caking. Humidity caking is a consequence of an increasing water content, plasticization and depression of T_g to below ambient temperature (e.g. Slade and Levine, 1991). The close relationships between stickiness and glass transition suggest that caking also occurs above the T_g with rates which are defined by the temperature difference, $T–T_g$, which for dairy powders is

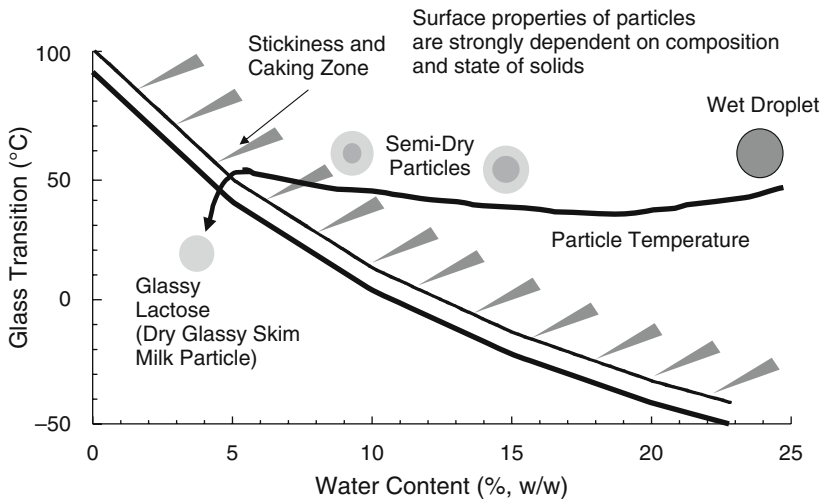

Figure 2.4. Glass transition temperature, T_g, of skim milk with a schematic representation of liquid droplets during dehydration. Dehydration to a glassy state is required for free-flowing lactose systems. The presence of lower molecular weight sugars would reduce the stickiness zone to lower temperatures and water contents while a shift to higher temperatures can be achieved by mixing lactose with higher molecular weight components. In the wet and semi-dry droplets, lactose exists as a liquid which becomes increasingly viscous as the water content decreases.

highly dependent on solids composition which is particularly important to systems with hydrolysed lactose or modified sugar composition (Jouppila and Roos, 1994a,b; Vega et al., 2005).

2.3. Crystallization and Recrystallization

The non-crystalline state of lactose is a non-equilibrium condition with a high level of super-cooling and a large driving force towards the crystalline, equilibrium state. Lactose crystallization and recrystallization in dairy powders and frozen desserts are glass transition-related, time-dependent phenomena which are governed by the mobility of lactose molecules. Crystallization in the solid, glassy state may not occur as translational mobility of lactose is not possible and crystallization is kinetically limited. Molecules in the glassy state are not able to change their spatial arrangement to the highly ordered, crystalline equilibrium state. At temperatures and water contents exceeding the critical values for the glass transition, molecular mobility increases

rapidly and results in lactose crystallization in various forms, depending on temperature and water content (Haque and Roos, 2005).

Crystallization of amorphous lactose in dairy powders and in ice cream during storage is one of the principal causes of loss of product quality (Supplee, 1926; Troy and Sharp, 1930). Supplee (1926) reported that milk powders sorbed large amounts of water at low storage relative humidities. This water often induced changes in properties of the powder, and the water content decreased at higher humidity conditions due to crystallization. Troy and Sharp (1930) reported that drying of milk and whey by spray drying and roller drying produced a glass, composed of a non-crystalline mixture of α- and β-lactose. Water sorption by whey powders caused plasticization and subsequent hardening of the material owing to lactose crystallization.

Herrington (1934) found that lactose glasses were stable at room temperature if they were protected from water. The existence of lactose in the glassy state in dairy products and lactose crystallization at high storage humidities have been confirmed in numerous studies. These studies have used polarized light microscopy, electron microscopy, differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR) and X-ray techniques to analyse the physical state of lactose in dairy powders (King, 1965; Lai and Schmidt, 1990; Roos and Karel, 1990; Jouppila *et al*., 1997; Haque and Roos, 2005). As shown in Figure 2.5, water sorption by most dehydrated dairy products, which contain lactose, shows a characteristic break in the sorption isotherm, indicating lactose crystallization (Berlin *et al*., 1968a,b; Jouppila and Roos, 1994a,b; Haque and Roos, 2006).

The crystallization behaviour of amorphous lactose in milk products is also temperature dependent. Berlin *et al*. (1970) observed that the relative humidity at which the break in sorption isotherms appeared was dependent on temperature, which was confirmed by Warburton and Pixton (1978). An increase in storage temperature shifted the break to a lower relative humidity. The temperature dependence of the water sorption properties of crystallizing amorphous sugars can be explained by changes in their physical state. DSC thermograms of milk powders show a glass transition and a crystallization exotherm for the amorphous lactose fraction (Jouppila and Roos, 1994b). Water plasticization decreases the T_g of lactose and a higher water content causes lactose crystallization at a lower temperature. Water plasticization of non-crystalline lactose and associated depression of the T_g to a lower temperature indicates that the break in the lactose sorption isotherm is both temperature and time dependent.

Amorphous lactose may crystallize in a complex manner in a number of crystalline forms and the form produced depends on the relative humidity and temperature. According to Vuataz (1988), lactose crystallizes as the anhydrous β-form at relatively low water activities or as α-lactose monohydrate

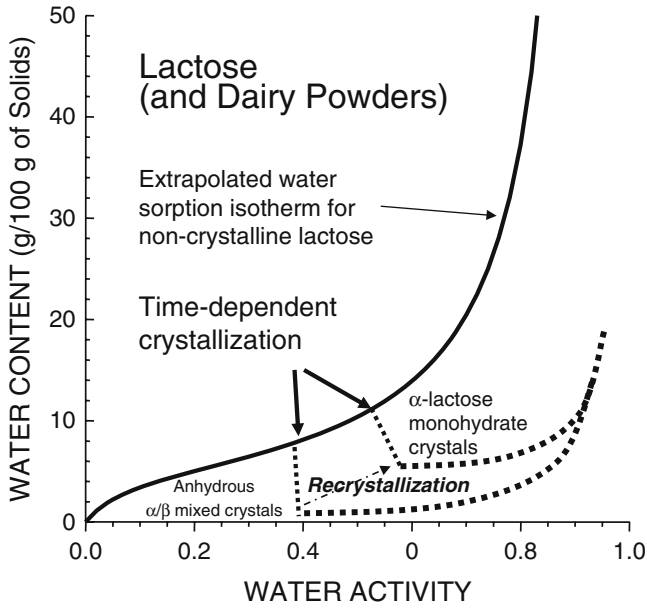

Figure 2.5. Sorption isotherm of amorphous lactose. A break in water sorption occurs as a result of lactose crystallization above the critical water content. Crystallization can be observed at varying rates at different storage relative humidities. Recrystallization of anhydrous crystals to α-lactose monohydrate crystals may be observed at higher water activities (Haque and Roos, 2005).

above a_w of 0.57 at room temperature. At higher temperatures, crystallization behaviour may change according to the stability of the crystalline form at the crystallization temperature. As shown in Figure 2.5, at intermediate water contents recrystallization of β and α/β mixed forms seems to occur and produce higher amounts of α-lactose monohydrate during storage (Haque and Roos, 2005). Various other components in milk, e.g. proteins and salts, also affect the crystallization properties and the crystalline form produced at different temperature and water conditions (Haque and Roos, 2006; Omar and Roos, 2006a,b).

The kinetics of crystallization at a constant temperature above T_g can be related to water content and water activity, which define the temperature difference, $T–T_g$. Therefore, lactose crystallization may occur above a critical water content or water activity at a constant temperature at a rate defined by the corresponding $T–T_g$ (Roos and Karel, 1992). The rate of lactose crystallization in dairy powders increases also with increasing relative humidity of

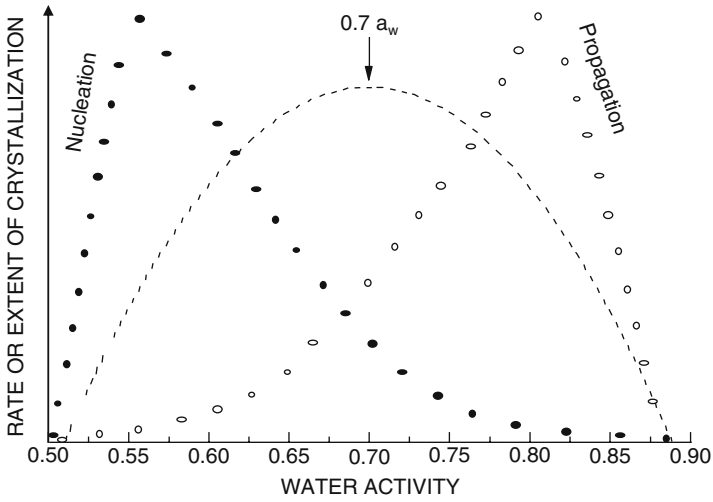

Figure 2.6. Relative nucleation (●) and crystallization (○) rates for lactose at various water activities at room temperature. The glass transition of lactose is defined by water activity, and crystallization occurs above the critical water activity. The rate of nucleation at a low water activity is high but crystal growth occurs slowly which results in a low overall rate of crystallization. The maximum rate and extent of crystallinity (---) is achieved around 0.7 a_w (Jouppila *et al.*, 1997).

storage environment (e.g. Saltmarch and Labuza, 1980; Vuataz, 1988; Jouppila *et al.*, 1997). Increasing relative humidity increases water sorption and water activity, which causes water plasticization and increases the temperature difference, T–T_g. The T–T_g of lactose defines the rate of crystallization, as shown in Figure 2.6.

Jouppila and Roos (1994b) determined glass transition temperatures for freeze-dried milk powders, which contained various amounts of fat. The T_g of non-fat solids at various water contents was almost the same as that of lactose (Figure 2.2). The water sorption properties of the non-fat solids were not affected by the fat component. Jouppila and Roos (1994b) developed state diagrams for milk powders, which defined critical values for water content and water activity for stability. Combined T_g and water sorption data suggested that a water content of 7.6 g/100 g of non-fat solids depressed T_g to 24°C. The corresponding water content for pure lactose was 6.8 g/100 g of solids. The critical a_w was 0.37. These values, being similar to those shown in Figure 2.2, are in good agreement with several studies which have found critical water contents and storage relative humidities for milk powders based on water sorption properties (e.g. Warburton and Pixton, 1978).

Milk powders with lactose hydrolysed to galactose and glucose show no break in their sorption isotherms (San Jose *et al.*, 1977; Jouppila and Roos, 1994a). It was suggested that crystallization of individual sugars in the protein–glucose–galactose mixture was delayed in comparison to lactose crystallization in skim milk and whey powders. Skim milk powders containing hydrolysed lactose showed a T_g well below that of amorphous lactose. Powders produced from skim milk containing galactose and glucose as a result of enzymatic hydrolysis of lactose had an anhydrous T_g at 49°C and a water content of 2.0 g/100 g of solids reduced the T_g to 24°C (Jouppila and Roos, 1994b). Haque and Roos (2006) have shown that the T_g of lactose-containing anhydrous skim milk powders is close to that of lactose at 105°C. However, a number of T_g values for amorphous lactose have been reported, which reflect the sensitivity of the transition to composition and water. Various criteria are also used to locate the transition temperature in DSC thermograms and it may be taken from the onset or mid-point of the transition.

Galactose and glucose show glass transitions at 30 and 31°C (Roos, 1993), respectively. Although Kalichevsky *et al.* (1993a,b) found that sugars had only a small effect on the T_g of casein, the T_g of milk powders containing hydrolysed lactose seems to be higher than is suggested by the T_g values of the component sugars. The T_g of milk powders is significantly reduced by lactose hydrolysis, which presumably is the main cause of stickiness during processing and storage, as well as of hygroscopic characteristics and higher susceptibility of the powder to non-enzymatic browning reactions. It should also be noted that although lactose is a reducing sugar, the hydrolysis of one mole of lactose produces two moles of reducing sugars, i.e. one mole of galactose and one mole of glucose.

Lactose crystallization in dairy powders results in increasing rates of non-enzymatic browning and other deteriorative changes (Labuza and Saltmarch, 1981; Saltmarch *et al.*, 1981; Miao and Roos, 2004). Saltmarch *et al.* (1981) found that the rate of browning at 45°C increased rapidly above a_w of 0.33 and showed a maximum between a_w of 0.44 and 0.53. The maximum rate of browning occurred at a lower a_w than was found for other foods. The maximum rate was coincident with extensive lactose crystallization which was observed from scanning electron micrographs. The rate of browning was significantly lower in a whey powder which contained precrystallized lactose. The loss of lysine was also found to be most rapid at water activities which allowed lactose crystallization (Saltmarch *et al.*, 1981). Crystallization of amorphous lactose in closed containers increases water activity very rapidly and accelerates the browning reaction in comparison with the rate of the reaction at the same temperature but at a constant water activity (Kim *et al.*, 1981). Compositional factors and crystallization behaviour of different sugars may also enhance lipid oxidation (Shimada *et al.*, 1991) and browning reactions (Miao and Roos, 2004; Nasirpour *et al.*, 2006).

2.4. Crystallization and Recrystallization in Frozen Systems

The viscosity of a freeze-concentrated solute phase affects time-dependent crystallization phenomena, ice formation and material properties. Levine and Slade (1988a) pointed out that the retarding effect of added maltodextrins on ice recrystallization in ice cream is due to the elevation of the glass transition of a maximally freeze-concentrated solute phase, T_g'.

At a sufficiently low temperature, the viscosity of a freeze-concentrated solute matrix becomes high enough to retard diffusion and delay ice formation (Roos and Karel, 1991b). Maximum freeze-concentration may occur at temperatures slightly below the onset temperature of ice melting, T_m', in the maximally freeze-concentrated material (Figure 2.3). Generally, the T_g' and T_m' increase with increasing molecular weight of the solute fraction (Slade and Levine, 1991; Roos and Karel, 1991d).

Lactose crystallization in frozen dairy systems may occur above the glass transition temperature of the maximally freeze-concentrated solute matrix, T_g'. Lactose is one of the least soluble sugars and the loss of quality, including a sandy mouthfeel, resulting from lactose crystallization is well known (Troy and Sharp, 1930; White and Cakebread, 1966). The solubility of lactose at 0°C is only about 12 g/100 g of water and it decreases substantially below the freezing temperature of water as a result of freeze-concentration (Nickerson, 1974). The solubility of lactose also decreases in the presence of other sugars, e.g. sucrose (Nickerson and Moore, 1972), which may significantly facilitate lactose crystallization in frozen dairy desserts and ice cream. However, crystallization of freeze-concentrated solutes can be retarded and greatly reduced by the use of sugar blends and syrups and by the addition of polysaccharides (Hartel, 2001).

Both lactose crystallization and recrystallization of ice in frozen desserts can be reduced by the addition of stabilizers which increase the viscosity of the unfrozen, freeze-concentrated solute phase. Singh and Roos (2005) also showed that in blends of polysaccharides, proteins and sugars, the T_g' was decreased but the T_m' increased as a result of retarded ice formation. The polysaccharide, protein (including) polysaccharide and protein stabilizers) and sugar composition seem to be the most important factors in formulation of frozen dairy foods with improved stability against solute crystallization and ice recrystallization.

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3

Significance of Lactose in Dairy Products

Lactose is the principal solid constituent in bovine milk, representing ~35% of the totals solids in normal milk, and is the principal constituent in many dairy products, ranging from about 40% in whole milk powder to ~70% in whey powder. Therefore, the properties of several dairy products, especially concentrated and dehydrated products, are dominated by certain properties of lactose, especially its solubility, crystallization behaviour, mutarotation properties and its propensity to Maillard browning.

In this chapter, the significance of lactose in evaporated and sweetened condensed milk, ice cream and milk powders will be considered, each in separate sections.

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I. Manufacture of Sweetened Condensed Milk and the Significance of Lactose Therein

R. Tan

I.1. Introduction

Gail Borden (1801–74) developed sweetened condensed milk (SCM) in 1856. The idea for a portable, shelf-stable canned milk product came to him during a long transatlantic trip in 1852, when cows on board the ship became too seasick to be milked and an infant died from lack of milk. He patented the process for making SCM in 1856 and founded a company known as Borden Milk Products in Burrville (CT) in 1857.

Borden did not have immediate success; his first two factories failed because the milk curdled and/or scorched. Later, inspired by the vacuum pan used by the Shaker community to condense fruit juice, he was successful in a third factory at Wassaic, NY. Historical records indicate that milk was heated first in a heating-well to about 97°C by passing a jet of live steam into it and holding for about 5 min. Sugar was then added and the mixture held in a vacuum pan at <60°C for 1–1.5 h until the batch was finished (Baldwin, 1924).

Today, there are considerable variations in the methods and equipment used to manufacture SCM. The stages of the process include heat treatment, homogenization, addition of sugar (liquid or dry, before or during concentration), concentration (or hydration if powder is used), seeding and cooling (Figure I.1). The principles involved in these stages are discussed in many publications, including Hunziker (1946) and Niewenshuije (2003). Except for seeding and cooling, the sequence of these stages varies from one processor to another and it is quite common to carry out the heat treatment, and/or

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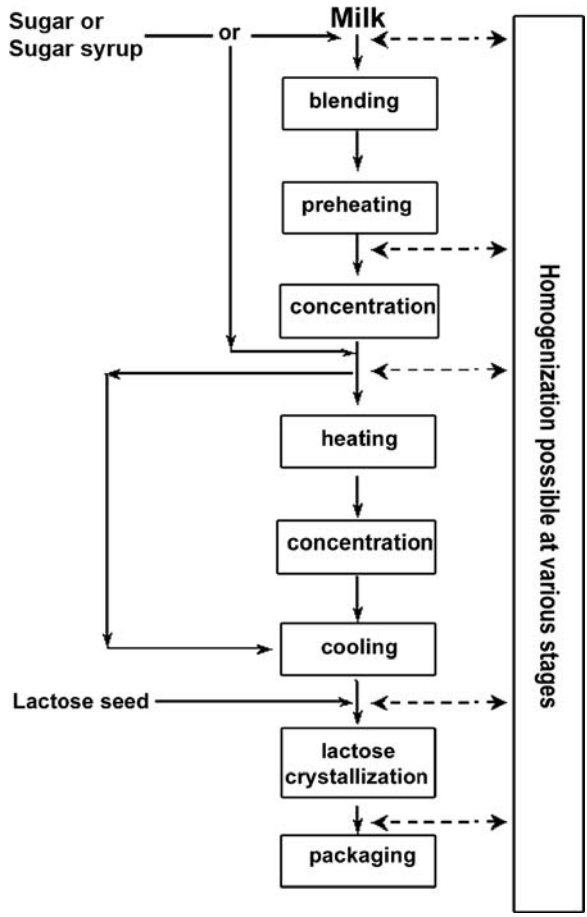

Figure I.1. Flow diagram for the manufacture of sweetened condensed milk.

concentration, in multiple stages. Different processing sequences yield products with different viscosity and age-thickening properties. Each manufacturer has its own proprietary secrets, aimed at making a product with a slight caramelized flavor, good initial viscosity and well-controlled age thickening. The initial viscosity and, especially, the rate of age thickening, depend on the stage at which sugar is added, the stage at which milk is homogenized, the extent of preheating or subsequent heating, its shear history and the storage temperature. The rate of age thickening can be logarithmic or polynomial in the order of 2–6.

I.2. Variants of Sweetened Condensed Milk

Typical SCM contains 8% milk fat, 20% milk-solids-non-fat (SNF), 45% sucrose and protein not less than 34% of the SNF (Table I.1). However, there are a few variants of SCM that contain vegetable fat or have lower milk solids. These are often called "filled SCM", "coffee whitener" or "beverage creamer". "Bonnet Bleu" (made in the Republic of Cameroon, Africa) is a SCM containing 48–49% sucrose, ~7% fat and only ~15% milk-solids-non-fat. Milk caramel or "dulce de leche" is caramelized SCM.

Filled sweetened condensed milk is SCM in which milk fat is replaced by vegetable fat to reduce its cost. This product was very unpopular initially; the "Filled Milk Act" was passed by the US Congress (21 USC Ch. 3, § 61–63) in 1923 to prohibit inter-state and foreign shipments of such products. After that, several states and countries, e.g., Australia in 1959, introduced their own versions of the Filled Milk Act, but several protests and lawsuits also followed. By 1970s, the drive to reduce the intake of cholesterol in the diet resulted in an increased demand for filled milk products. The "Filled Milk Act" was repealed in November, 1972, when a US District Court ruled that the Filled Milk Act violated the Constitution (Milnot Co. vs Richardson. Federal Supplement 350:221). The Australian Filled Milk Act was repealed in 1980 by Act No. 45.

I.3. Standards for Sweetened Condensed Milk

The USA's Code of Federal Regulation (Title 21 Section 131.120) defines SCM as a "food obtained by partial removal of water only from a mixture of milk and safe and suitable nutritive carbohydrate sweeteners. The finished food contains not less than 8% by weight of milk fat, and not less than 28% by weight of total milk solids. The quantity of nutritive carbohydrate sweetener used is sufficient to prevent spoilage. The food is pasteurized and may be homogenized." Addition of fruit juices or concentrates, coloring, natural or artificial flavors is permitted.

Filled sweetened condensed milk has been available since the 1970s, but its standard was drafted only recently (5th Session of the Codex Committee on Milk and Milk Products – April 2002 and 25th Codex Alimentarius Commission – June 30 to July 5, 2003).

For standardization or protein adjustment purposes, Codex Standard *A-4-1971 Rev 1-1999* allows the addition of milk, milk powders, cream and cream powders, milk fat products, milk retentate or permeate and lactose, in such a way as not to alter the whey protein to casein ratio of the milk. Permitted ingredients include buffering salts (stabilizers, acidity regulators)

Table I.1. Various regulations on the composition of sweetened condensed milk and related products

PRODUCT	FAT, %	Milk Solids, %	Sugar, %	Protein, %	Thickener/ buffers
CODEX STANDARD*					
Sweetened Condensed Milk	≥ 8	≥ 28	NS	≥ 34 of SNF	Allowed
Sweetened Condensed Partly Skimmed Milk	1–8	≥ 24	NS		Allowed
Sweetened Condensed Skim Milk	≤ 1	≥ 24	NS		Allowed
Unsweetened Condensed High Fat Milk	≥ 16	≥ 30	NS		Allowed
EUROPEAN STANDARD**					
Sweetened Condensed Milk	≥ 8	≥ 28	Sucrose	NS	NS
Sweetened Condensed Partly Skimmed Milk	1–8	≥ 24	Sucrose	NS	NS
Sweetened Condensed Skim Milk	≤ 1	≥ 24	Sucrose	NS	NS
Unsweetened Condensed High Fat Milk	≥ 15	≥ 26.5	NA	NS	NS
Unsweetened Condensed Milk	≥ 7.5	≥ 25	NA	NS	NS
Unsweetened Condensed Partly Skimmed Milk	1–7.5	≥ 20	NA	NS	NS
Unsweetened Condensed Skim Milk	≤ 1	> 20	NA	NS	NS
Evaporated Milk	≥ 9	≥ 31	NA	NS	NS
Evaporated Semi-skimmed Milk	4–4.5	≥ 24	NA	NS	NS
CANADIAN STANDARD***					
Sweetened Condensed Milk	≥ 8	≥ 28	Sucrose, dextrose,glucose, glucose solids, or lactose	NS	NS
Evaporated Milk	≥ 7.5	≥ 25	NA	NS	Buffer salts

(*Continued*)

Table I.1. (*Continued*)

PRODUCT	FAT, %	Milk Solids, %	Sugar, %	Protein, %	Thickener/ buffers
Evaporated Party Skim Milk	≤ 7.5, ≥ 0.3	≥ 17	NA	NS	Buffer salts
Evaporated Skim Milk	≤ 0.3	≥ 17	NA	NS	Buffer salts
CODEX ALIMENTARIUS COMMISSION 25th Session (Joint FAO/WHO), Jun 30–Jul 5, 2003 (Draft)					
Sweetened Condensed Filled Milk	7–8	SNF ≥ 20	NS	≥ 34 of SNF	NS
Evaporated Filled Milk	6–8	SNF 17.5–20	NA	≥ 34 of SNF	NS
5th Session of the Codex Committee on Milk and Milk Products, 4/8–4/12/02, US FDA, USDA (Draft)					
Sweetened Condensed Filled Milk	≥ 8	SNF ≥ 20	NS	≥ 34 of SNF	NS
Sweetened Condensed Partly Skimmed Filled Milk	$\geq$8 total fat (1–8 milk fat)	SNF 17.5–20	NS	≥ 34 of SNF	NS
Evaporated Filled Milk	≥ 7.5	SNF ≥ 20	NA	≥ 34 of SNF	NS
Partly Skimmed Evaporated Filled Milk	≥ 7.5 total fat (1–7.5 milk fat)	SNF ≥ 20	NA	≥ 34 of SNF	NS

* Codex Stan A-4-1971, Rev 1-1999
** European standards : "Condensed Milk and Dried Milk Regulation 2003", "England 2003 No. 1596"; "Northern Ireland, 2003 No. 300"; "Welsh Statutory Instrument 2003 No. 3053 W.291"; Article 1 and Annex 1 of 2001/114/EC Regulation 2,3,4 & 10 and Schedule 1 &2 SI.
*** Canadain standard : Food & Drug Regulation (C.R.C., c.870. Part B Division 8)
NS = not specified , NA = not applicable

up to 0.2% when added singly, or 0.3% when added in combinations. Carrageenan (up to 0.015%) and lecithin (emulsifier) are also allowed. In limiting the concentration of lactose in SNF, a few regulations around the world limit the added lactose seed to 0.03% (Welsh Statutory Instrument – 2003 No. 3053 W.291, and England – 2003 No. 1596).

I.4. Limitations of Lactose in Foods

Lactose has limited applications in foods because it is not very soluble (about 18 g/100 g water at room temperature). In frozen or low-moisture foods, its crystallization causes grittiness. The crystallization to α-lactose monohydrate sequesters moisture, causing instability of the casein system.

In ice cream, the so-called "MSNF factor" is used to limit the concentration of milk- solids-non-fat (MSNF), thus limiting the amount of lactose and avoiding sandiness. In the manufacture of concentrated milk products, however, seeding techniques are used to control the size of lactose crystals to prevent grittiness or sandiness. In the spray drying or fluidized bed drying of concentrated whey or milk, seeding techniques are also used to improve the properties and stability of powder, and also to increase yield.

Although SCM and caramelized SCM (dulce de leche) have been known since the 1850s and 1960s, respectively, it took years of study and trial and error to eliminate sedimentation and sandiness caused by the crystallization of lactose. The earliest studies on sandiness in SCM were probably those of Hunziker (1946) and Foster *et al*. (1957). In certain parts of the world where % protein-in-SNF is not regulated, processors who mastered the technique of seeding (controlling lactose crystallization) have been making low-cost SCM or caramel, by replacing most of the milk-solids-non-fat with lactose using whey powder (70% lactose). In caramelized SCM, phosphates and/or bicarbonates are added frequently not only to inhibit protein gelation and to catalyze browning but also to increase the solubility of lactose (see Guu and Zall, 1991). Corn syrups or maltodextrins also inhibit lactose crystallization, but they are added primarily to increase the viscosity or to replace sucrose and thus to reduce cost. Calcium nitrate, bromide or chloride has been shown to increase the solubility of lactose (Nickerson, 1974). Phosphates also increase the solubility of lactose (Guu and Zall, 1991), and surface-active substances, in general, affect its crystallization or solubility (Polyanskii, 1987; Hartel, 1993). The effect of pH on the solubility of lactose is insignificant (Smart, 1988), but the nature of the solvent and the presence of salts or other sugars influence both the rate of solubilization and mutarotation of lactose (Harper, 1992). In SCM, the presence of milk colloids at high concentration impedes the rate of lactose crystallization, its high viscosity

also reduces the rate of diffusion of lactose to the surface of lactose crystals (Hunziker, 1946).

Studies on the solubility of lactose have been published by several authors, in water (e.g., Hudson, 1908 (see Visser, 1982); Herrington, 1934; Visser, 1982), in ethanol (Machado *et al.,* 2000; Tze-Shuong, 2004) or in solution containing sucrose also (Hartel, 1993).

I.5. Crystallization of Lactose in Sweetened Condensed Milk and Related Products

Lactose in milk powder, spray-dried lactose or inhaler-grade lactose usually occurs as a combination of α-hydrate crystals, anhydrous β-crystals and amorphous lactose "glass". Amorphous lactose is formed during rapid evaporation or drying, along with the α- and β-lactose present initially in the concentrate. The glass form of lactose is also formed in ice cream during its rapid cooling and storage below its glass transition temperature ($T_g \sim -30°C$).

β-Lactose is obtained when crystallization occurs from ethanol (at various concentrations and temperatures; Olano *et al.*, 1983), or from water above 93.5°C. When dried in clusters, β-lactose looks like an uneven-sided diamond (Holsinger, 1988); when suspended in saturated solution, it looks like bundled straws. It is sweeter and much more soluble than α-lactose.

In solution, β-lactose exists in equilibrium with α-lactose. At room temperature, β- and α-lactose co-exist at a ratio of 1.6:1 (or 61% β, 39% α). Despite the greater proportion of β-lactose, the usual crystalline lactose in intermediate-moisture foods is α-lactose. This is because its solubility is much lower than that of β-lactose (below 93.5°C). On supersaturation, it crystallizes from solution as α-lactose monohydrate, leaving β-lactose in solution. On mutarotation, the concentration of α-lactose in solution is replenished, as β-lactose is converted to α-lactose. Mutarotation and crystallization continue as long as the solution is supersaturated and stop when the saturation point is reached.

α-Lactose monohydrate ($C_{12}H_{22}O_{11}.H_2O$) crystals are typically tomahawk in shape when grown in supersaturated lactose solution. In SCM or in supersaturated lactose solution containing 62% sucrose, however, the crystals are no longer tomahawk in shape, but resemble short, truncated pyramids with flat rhomboid bases and apices (Hunziker, 1946). The volume of lactose crystals in SCM is equal to that of a sphere with its diameter (D) calculated as $L/1.33$, where L is the longest axis of the crystal (Hunziker, 1946).

The crystals are sparingly soluble in water and are hard and gritty in the mouth. According to Zhizhin *et al.* (1971), SCM becomes powdery in mouthfeel when crystals of lactose exceed 16 μm, but Kruk *et al.* (1974) reported that

sandiness is perceived only when crystals are greater than 30 μm. Hunziker (1946) and Hough *et al*. (1990) related quantitatively the number and size of crystals with the degree of sandiness in caramelized SCM. According to Hunziker (1946), sandiness occurs only when there are more than 4×10^8 crystals/g of food, but according to Hough *et al.* (1990), the perception of sandiness results from a combination of the number and size of crystals. Crystals up to 105 μm are not detectable when the number of such crystals is less than 4×10^3/g, up to 45 μm when number is less than 10^5/g, or up to 15 μm when number is less than 10^7/g (Hough *et al.,* 1990). In fact, the size and number of crystals are interdependent because both depend on the solubility of lactose.

I.6. Seeding Sweetened Condensed Milk with Lactose

In order to control crystal size and to avoid grittiness in SCM, it is necessary to control the number of crystals by adding an appropriate number or amount of small lactose crystals (called "seed") that act as nuclei to initiate subsequent growth. In order to ensure proper growth of the nuclei to the desired size, seeding must be done at the optimum temperature for crystal growth, and sufficient time and agitation must be provided for sufficient heat transfer and distribution of the seed. Depending on the speed of agitation and on the size of the tank, agitation may require as long as 24 h.

A few seeding procedures have been recommended but the rationale for their recommendation is unclear. Hunziker (1946) recommended seeding with the previous day's product (no quantity given) or seeding with 0.063% (w/w) lactose powder at 30°C with vigorous agitation for more than 1 h, followed by cooling to 18°C. Foster *et al*. (1957) recommended seeding with 0.05% lactose powder at 30°C with vigorous agitation for 1 h, followed by cooling to 15°C for SCM packed in tins or 21°C for bulk product. Niewenshuije (2003) recommended adding 0.02–0.05% (w/w) lactose powder or 10^5 to 10^9 1–1.5 μm crystals per ml SCM.

In making caramelized SCM, Martines *et al.* (1990) recommended seeding at 0.03% and agitating for 15 min, while Hosken (1969) recommended adding previous day's product as seed. Martines *et al.* (1990) further recommended seeding at 50°C because such product is very viscous at lower temperatures.

As a rule of thumb, lactose crystals smaller than 10 μm are desired (Niewenshuije, 2003) to avoid not only the perception of sandiness but also the sedimentation of lactose. In order to limit the crystal size to less than 10 μm, lactose crystals used for seeding should be 1–1.5 μm (Niewenshuije, 2003). However, lactose crystals available commercially are much larger

than 1 μm and are not uniform in size. In the case of fluidized bed drying of milk powder, the ideal size of crystals for optimum drying is, in fact, much larger (200–250 μm). For this reason, it becomes important for processors to know what is the correct amount of seed to use to control crystal size, or to know what crystal size to expect in the product, given a certain size of the seed used. For instance, if crystals in the seed used are 3 μm, 0.20% (w/w) lactose should be added in order to limit crystal growth to <10 μm (see below).

When the concentration of lactose in the product, its solubility and the specific gravity of its crystals are known, the number and size of lactose crystals in the final product can be estimated using simple mathematical calculations. Empirical equations derived from these calculations are useful for quick determination of the number and final size of lactose crystals in SCM. Similar equations can be derived for any other food product when the concentration of lactose (in excess of its solubility) is known.

Lactose crystal size (μm) *in SCM* (*after growth*)

$$= 0.4106 \times \text{size of seed}\,(\mu\text{m}) \times \%\,\text{seeding}^{-0.33333}$$

Maximum number of crystals/mm^3 *in SCM*

$$= (5.84 \times 10^9 \times \%\ \text{seed})/\text{specific gravity of lactose crystals} \times (\text{size of seed in}\ \mu\text{m}^3)\ \text{or}$$

$$= 3.78 \times 10^9 \times \%\ \text{seed}/(\text{size of seed in}\ \mu\text{m}^3),$$

since the specific gravity of lactose crystals is 1.545

Example: Using the equations given above, 280, 051 crystals with dimensions of 9.8 μm per mm^3 are expected when the seed used is 3 μm and 0.20% is added.

$$\text{Final lactose size} = 9.8\ \mu\text{m} = 0.410613 \times 3 \times (0.20\%)^{-0.33333}$$

$$\text{Maximum number of crystals} = 3.78 \times 10^9 \times 0.20\%/3^3$$

$$= 280,051/\text{mm}^3$$

These empirical equations are based on three parameters:

1) The solubility of lactose in SCM (15 g/100 g water; Hunziker, 1946)
2) Specific gravity of α-lactose (1.545)
3) Volume of α-lactose crystals found in SCM $\left({}^1/_6\,\pi\,d^3\right)$

where diameter d is the longest axis of the crystal divided by 1.33 (Hunziker,1946)

I.6.1. Derivation of Equations

The solubility of lactose in water is 18 g per 100 g at 20°C. According to Nickerson (1974), the solubility of lactose is reduced by 40–80% of its usual value when the concentration of sucrose also present is 40–70%. According to Hunziker (1946), its solubility in SCM, as well as in whole milk containing 62% sucrose, is reduced to 15 g per 100 g water at 18°C. SCM (at 20% milk-solids-non-fat, 27% moisture) contains ~10.8% lactose, so the amount of lactose in 100 g water is ~40 g; which means the remaining 25 g are expected to crystallize, i.e., ~6.8% of the SCM by weight.

Since the density of SCM is 1.3 mg per mm^3, 0.09 mg of lactose per mm^3 SCM (6.8% of 1.3 mg) is expected to crystallize.

If the size of lactose seed is 3 μm (0.003 mm), its volume would be 6.009×10^{-9} mm^3, weighing 9.284×10^{-9} mg per crystal (i.e., 6.009×10^{-9} $mm^3 \times$ specific gravity of the lactose, see column B in Table I.2).

If the amount of seed added to SCM is 0.20%, the amount would be 2.6×10^{-3} mg per mm^3 of SCM (i.e., 0.2% of 1.3 mg); the number of seed crystals would be about 280,000 per mm^3 of SCM ($2.6 \times 10^{-3}/9.284 \times 10^{-9}$, see column D in Table I.2). These are the seeds for the 0.09 mg of lactose mentioned above to grow upon; each seed would grow to 3.21×10^{-7} mg (i.e., 0.09/280,000, see column E in Table I.2); size of which is 9.8 μm.

Volume of each grown crystal (v)
$= 3.214 \times 10^{-7}$ mg/(1.545 mg/ mm^3) $= 2.08 \times 10^{-7}$ mm^3 (see column F in Table I.2), where 1.545 is the specific gravity of lactose crystal.

Crystal size (L, after growth)
1.33 $L = d =$ cube root of (volume $\times 6/\pi$)
$L = 0.0098$ mm $= 9.8$ μm (see column G in Table I.2)
Where (L) = diameter of a sphere/1.33 (Hunziker, 1946), and volume of sphere $= {}^1/_6\ \pi\ d^3$

Using the same method of calculation shown above, a spreadsheet like that in Table I.2 can be generated for different sets of data, i.e., different starting seed size, different levels of seed added (number of nuclei) and different expected final growth size. These data can then be used to derive graphically empirical equations like the example shown above, which are for SCM.

These calculations help processors to understand that when the size of seed is, say 1 μm, addition of as little as 0.025% is needed. If the available seed is larger, the level of addition can be adjusted to control or limit the final size of lactose crystals in the product. If the perception of sandiness is indeed a combination of both the number and size of crystals (Hough

Table I.2. Various seed sizes, level of seed added, and final crystal size in sweetened condensed milk

Size of seed crystal (μm)*	Weight of each seed crystal (mg)	Pecentage of seed added	Number of nuclei per mm^3 product	Weight (mg) of each crystal (after growth)	Volume (mm^3) of each crystal (after growth)	Size of crystals in finished product (μm)*
A	**B**	**C**	**D**	**E**	**F**	**G**
1	3.43853×10^{-10}	0.025	945171	9.522×10^{-8}	6.163×10^{-8}	6.52
1		0.05	1890341	4.761×10^{-8}	3.082×10^{-8}	5.17
1		0.10	3780682	2.381×10^{-8}	1.541×10^{-8}	4.11
1		0.15	5671023	1.587×10^{-8}	1.027×10^{-8}	3.59
1		0.20	7561364	1.190×10^{-8}	7.704×10^{-9}	3.26
3	9.28404×10^{-9}	0.025	35006	2.571×10^{-6}	1.664×10^{-6}	19.6
3		0.05	70013	1.285×10^{-6}	8.320×10^{-7}	15.5
3		0.10	140025	6.427×10^{-6}	4.160×10^{-7}	12.3
3		0.15	210038	4.285×10^{-6}	2.773×10^{-7}	10.8
3		0.20	280051	3.214×10^{-6}	2.080×10^{-7}	9.8
5	4.29817×10^{-8}	0.025	7561	1.190×10^{-5}	7.704×10^{-6}	32.6
5		0.05	15123	5.951×10^{-6}	3.852×10^{-6}	25.9
5		0.10	30245	2.976×10^{-6}	1.926×10^{-6}	20.5
5		0.15	45368	1.984×10^{-6}	1.284×10^{-6}	17.9
5		0.20	60491	1.488×10^{-6}	9.630×10^{-7}	16.3
10	3.43853×10^{-7}	0.025	945	9.522×10^{-5}	6.163×10^{-5}	65.2
10		0.05	1890	4.761×10^{-5}	3.082×10^{-5}	51.7
10		0.10	3781	2.381×10^{-5}	1.541×10^{-5}	41.1
10		0.15	5671	1.587×10^{-5}	1.027×10^{-5}	35.9
10		0.20	7561	1.190×10^{-5}	7.704×10^{-6}	32.6
20	2.75083×10^{-6}	0.025	118	7.618×10^{-4}	4.931×10^{-4}	130.4
20		0.05	236	3.809×10^{-4}	2.465×10^{-4}	103.5
20		0.10	473	1.904×10^{-4}	1.233×10^{-4}	82.1
20		0.15	709	1.270×10^{-4}	8.218×10^{-5}	71.7
20		0.20	945	9.522×10^{-5}	6.163×10^{-5}	65.2

* longest axis of α-lactose monohydrate crystal (μm).

et al., 1990), then it should be acceptable to have a product with a crystal size above the norm of 10 μm, as long as the number of crystals does not exceed its corresponding limits. For instance, if sandiness is not detectable when the number of 15 μm crystals is less than 10^7/g SCM, then it should be acceptable to use 3 μm seed, as long as up to 0.05% is added (see Table I.2).

I.7. Effect of Crystal Size on the Viscosity of Sweetened Condensed Milk

Properties of lactose related to its solubility also affect the density, viscosity, water activity, freezing and melting points and the susceptibility of the food product to browning. Apart from the concern about sandiness, there are other aspects related to the crystal size that need further consideration, i.e., sedimentability of the crystals and the effect of the number of crystals on the viscosity of the finished product. Generally, sedimentation of lactose crystals in SCM occurs within 1 month when the viscosity of product is less than 5000 mPa s (5000 cps) and crystal size is greater than 40 μm.

According to Stokes' law, the rate of sedimentation of lactose (v) is affected more significantly by its crystal size than by the viscosity of the medium (η). v is proportional to r^2, where r is the radius of the crystal. For instance, the rate of sedimentation is increased four times when crystal size is doubled, but only twofold when viscosity is halved. The two combined (doubling crystal size and reducing viscosity by 50%) increases the rate of sedimentation by eightfold.

Stokes' law

$$\nu = \frac{2r^2\, g(\rho_p - \rho_f)}{9\eta}$$

where

ν is the rate of sedimentation, r the radius of the particle, g the gravitational acceleration, ρ_p–ρ_f the difference in density between particle and medium and η the viscosity of the medium.

Stokes' equation indicates that the sedimentation of lactose reduces the viscosity of the system, and the reduction in the viscosity increases the rate of sedimentation; both are affected by crystal size.

The effect of crystal size on viscosity can be observed during the crystallization of lactose in SCM or milk concentrates. Viscosity increases to a maximum during the first half of the crystallization process because lactose crystals are still very small (large specific surface) and the mother liquor still has a relatively high solids content, which means that the friction between the crystals and the mother liquor is high. As lactose crystals grow and the solids content of the mother liquor decreases, viscosity decreases.

I.7.1. Heat Treatment Before Evaporation

Even though the size and number of lactose crystals affect the viscosity of SCM, heating during processing has a greater effect. Factors that affect the viscosity of SCM during processing include preheating of milk before

concentration, temperature of evaporation and condensation, time and temperature of heating after concentration, the stage at which sugar is added (prior to preheating or after concentration), homogenization pressure and the stage at which milk or its concentrate is homogenized, its pH, its level of fat, minerals and solids content (see Bienvenue *et al.*, 2003), its lactose crystal size, its storage temperature, shear history, shear rate applied during the measurement of viscosity and its age. It appears that preheating is one of the main factors that affect the final viscosity of SCM, probably due to its greater effect on age thickening.

Several studies have shown the effects of preheating at various temperatures, but preheating in the range of 80–85°C has, in general, been shown to have desirable effects on viscosity. The viscosity of milk, and of the concentrate made from it, increases to a maximum on preheating at 80–85°C for 15–25 min (which corresponds to the complete denaturation of whey proteins). Beyond this, and up to several hours (before the milk starts to thicken and gel), the viscosity drops to a plateau value, which is below the maximum but above the level of non-preheated milk. Preheating at temperatures higher than 80–85°C also results in lower viscosity when preheating time goes well beyond complete denaturation of whey proteins.

Preheating below 80–85°C or for <15–25 min results in a "low-heat" product with lower initial viscosity, more pronounced pseudoplasticity and thixotropicity, more sensitivity to storage temperature, greater propensity to age thickening on storage at warmer temperatures (above 18°C) and, according to Hunziker (1946), thinning results on storage at lower temperatures.

The optimum preheating conditions appear to be 80–85°C for >15–25 min, for the desired initial viscosity with slow age thickening. Perhaps due to such a long holding time, some manufacturers of SCM preheat milk at 105–110°C for 1–5 min. According to Hunziker (1946), preheating at a temperature higher than 110–120°C causes age thinning (holding time not given).

In addition to preheating, most manufacturers further control the viscosity of their SCM by either homogenization and/or by another heat treatment, after the product has been concentrated or partially concentrated. In addition to the increase in viscosity due to the emulsification of fat, homogenization increases the sensitivity of milk proteins to heat (decreases its heat stability); therefore, homogenization before heating, as opposed to homogenization after heat treatment, and homogenization of milk, as opposed to homogenization of concentrated or partially concentrated milk, have different effects on the viscosity of SCM. Heating increases the viscosity through protein gel formation (see "cooling after complete crystallization of lactose"; I.8.2). Heating is done either by tempering the finished product slowly in a temperature-controlled warehouse for several days or rapidly by heating in a hotwell tank. The latter

offers the advantage of pre-forming protein gels while the product is still in a tank. The effect of subsequent shearing (which happens during the canning process) is discussed below.

I.8. Controlling Lactose Crystal Size by Cooling

Lactose can be supersaturated by increasing the concentration of lactose in relation to water content by evaporation or by reducing its solubility through cooling.

When a solution is cooled to produce a supersaturated solution and to cause crystallization, the heat that must be removed is the sum of the sensible heat necessary to cool the solution and the heat of crystallization. Theoretically, the heat of crystallization is equal to the heat of solubilization plus the heat of dilution, but the heat of dilution is small and can be ignored. The heat of solubilization for lactose monohydrate is –15,500 kJ mol^{-1} or –43.1 kJ kg^{-1}. In order to achieve maximum heat transfer, agitation must be effective.

The most common crystallizer used in SCM manufacture is a simple open tank or vat in which the solution loses heat to its surroundings. Since cooling in this fashion is slow and not very effective, large crystals are generally produced. To reduce crystal size or increase the rate of cooling, cooling coils or jackets can be added and these crystallizers can be made continuous.

Continuous crystallizers used in the food industry are usually cylindrical, scraped-surface heat exchangers, similar in design to those for plasticizing margarine and cooking fats, and for crystallizing ice cream. It is essentially a double-pipe heat exchanger fitted with an internal scraper. The material is pumped through the central pipe and agitated by the scraper, with the cooling medium flowing through the annulus between the outer pipes.

I.8.1. Seeding/Cooling Temperature

The removal of α-lactose from the solution due to crystallization means that the ratio between α- and β-lactose changes, so that the solution contains more β-lactose than α-lactose during crystallization. Due to mutarotation, the solution of α-lactose becomes supersaturated again, and crystallization continues. This process continues for as long as the solution is supersaturated.

The rate of mutarotation is influenced directly by the temperature of the solution; it proceeds faster at high temperatures and very slowly at temperatures near the freezing point. Thus, temperature has the opposite effects on mutarotation and crystallization. This means that by cooling the concentrate too fast or to a very low temperature, crystallization proceeds quickly but the

completion of crystallization takes longer; slow mutarotation results in a small amount of β-lactose being transformed into α-lactose for further crystallization.

In practice, concentrate (lactose solution, SCM or whey) is cooled rapidly to 30°C in a flash cooler connected to the evaporator after which the concentrate is seeded with fine lactose and allowed to cool rather slowly (1–3°C/h) to about 20°C in the tank. This allows mutarotation and crystallization to proceed at reasonable rates. Throughout the crystallization process, it is very important to agitate the concentrate in the crystallization tank vigorously and continuously, in order to transport supersaturated solution to the surface of the crystals. Agitation also prevents the viscosity of the thixotropic suspension from becoming too high, which would reduce the rate of diffusion and mutarotation, and the distribution of seeds. In a study on SCM, Kruk *et al.* (1974) showed that the percentage of lactose crystals greater than 30 μm increases with an increase in viscosity, resulting in sandiness.

The optimum seeding temperature for SCM is 25–30°C because the concentration of lactose in solution (prior to crystallization) is in the "intermediate region" (Figure I.2). The percentage of total lactose in this product

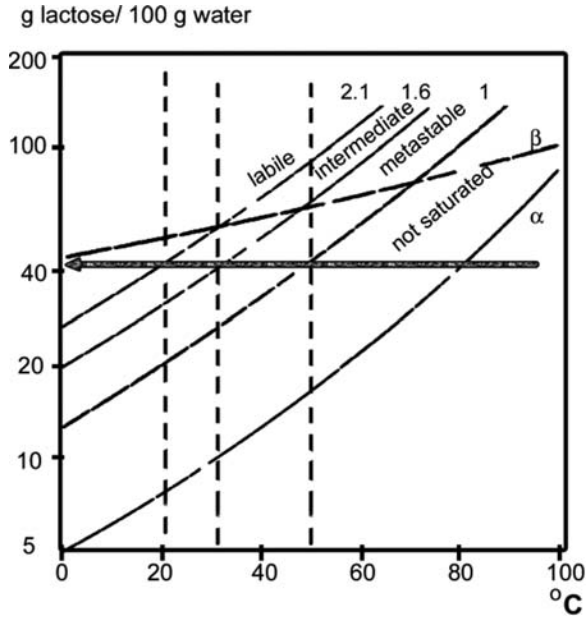

Figure I.2. The solubility of α- and β-lactose, the final solubility of lactose (line 1), and supersaturation by a factor of 1.6 and 2.1, respectively (modified from Walstra & Jenness, 1984).

(containing 27% water) is about 10.8%; its concentration in 100 g water is about 40 g. Below 20°C (labile region), lactose crystallizes instantaneously without the presence of seed. As the temperature is increased from 30 to 50°C (the meta-stable region), less lactose can crystallize and the nuclei (seed) grow slowly. At above 50°C, all lactose is in solution, and all added lactose dissolves.

In some lactose solubility diagrams (Figure I.3), no distinction is made between the intermediate and meta-state region of solubility. Another way of expressing lactose concentration is "% lactose in water", which is about 28% w/w in SCM (10.8/(10.8+27) × 100). At this concentration, the meta-stable region is also at 20–30°C. Below 20°C (labile region), lactose crystallizes instantaneously, while above 50°C, lactose is completely in solution.

Despite seeding at the optimum temperature (25–30°C), crystallization in the storage tank (with subsequent cooling to 20°C) requires about 24 h for completion in SCM. This is evidenced by a rapid change in water activity within 12 h of seeding, and a slow change afterwards. Its water activity (73% total solids, 45% sucrose, 10.8% lactose) starts at ~0.800 and reaches <0.840 upon completion of crystallization. This crystallization process can also be monitored by measuring changes in the refractive index of the concentrate

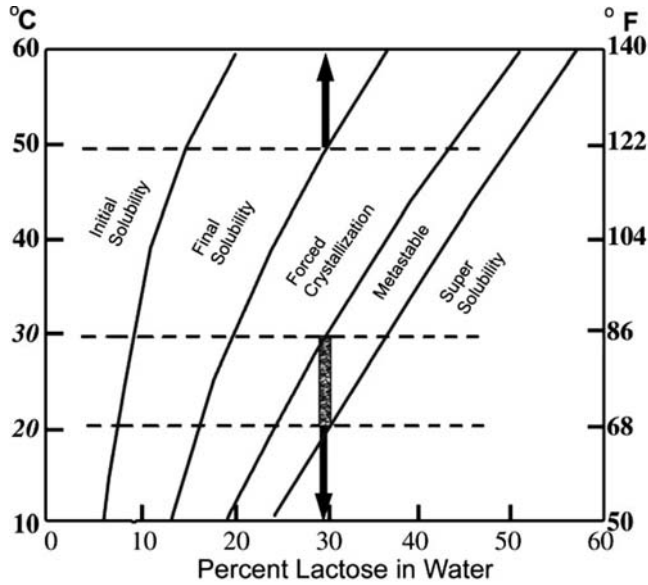

Figure I.3. Solubility of lactose (modified from Hunziker, 1946).

(Mimouni *et al.,* 2005) or by centrifugation and measuring the concentration of lactose in the supernatant (Zhizhin *et al.,* 1971).

Since SCM contains ~10.8 g lactose per 100 g, and 8.37 g are expected to crystallize, a maximum of 77.5% of the lactose is expected to crystallize.

Since the microbial stability of SCM depends solely on its water activity, the knowledge that final water activity of SCM is reached only upon complete crystallization of lactose becomes very important.

I.8.2. Cooling After Complete Crystallization of Lactose

The viscosity of SCM increases rapidly during the first 4–12 h after seeding, followed by a less rapid increase during the next 24 h, and a slower increase during subsequent storage. In the absence of seed, there is also an increase in viscosity during the first few hours after manufacture, but the magnitude is much smaller. Hence, in addition to the increase caused by lactose crystallization, there are other changes that contribute to such increases in its viscosity. In a study on concentrated milk, where the effect of lactose crystallization was not considered, Bienvenue *et al*. (2003) showed that the viscosity increased steeply after 4 h of storage and was largely reversible under high shear, but it became progressively less reversible upon longer storage. The appearance of yield stress suggests the presence of reversible flocculation arising from weak attraction between casein micelles, but as concentrated milk ages, there is a progressive transition from reversible to irreversible aggregation; particle size analysis confirmed irreversible aggregation and fusion of casein micelles during storage of concentrated milk (see Bienvenue *et al*., 2003). Hence, as SCM ages, it loses its ability to recover from shear thinning.

Since gel network formation and its transition to irreversible aggregation increases with temperature, the ability of SCM to recover from shear thinning is reduced by storage at higher temperatures. Hunziker (1946) reported that storage at or below 15°C retarded age thickening, and on storage at or below 7°C, it retained its initial viscosity permanently.

Since pumping of product from the tank to the canning line causes significant shear thinning, it is prudent to chill the product soon after the crystallization of lactose has reached completion. In addition, chilling the product may be desirable because it increases its viscosity, thereby reducing its tendency to foam; but the increase in viscosity must be such that it does not become too viscous to pump.

The longer the SCM remains in the tank, or the later the product is packed into cans, the greater will be the shear thinning (irreversible recovery of gel structure). The viscosity of SCM is always 20–50% lower throughout storage when it was packed 48 h after manufacture than when packed after 12–24 h.

I.8.3. Effect of Lactose Crystallization on the Water Activity of Sweetened Condensed Milk

The typical composition of SCM is sucrose (44–46%), lactose (10–12%), milk salts (0.25–0.35%), fat (8%), proteins (8–9%) and moisture (26–28%). The corresponding a_w is less than 0.840 upon equilibration at 25°C after seeding, or less than 0.800 before crystallization of lactose. The increase in a_w from 0.800 to 0.840 is due mainly to the crystallization of lactose (rather than possible changes to the milk salts equilibrium) because the a_w remains low (e.g., at 0.800) even after 24 h, if SCM is left undisturbed and not seeded. Similar behavior is observed in whole milk powder (Thomsen *et al.,* 2005); its a_w increased from 0.23 to 0.46 (after 147 days of storage), upon gradual transition of lactose glass to α-monohydrate.

The sucrose in SCM is undoubtedly the primary solute responsible for lowering its a_w. The desired S/(S+W) ratio (S = % sugar, W = % water) is 0.625:0.645. At a ratio of 0.625, the corresponding a_w is less than 0.85. Below a ratio of 0.625, a_w will be higher than 0.850 (microbiologically unstable) and above 0.645, sucrose will crystallize out of solution, especially when the product is stored below room temperature.

According to the British Columbia Centre of Disease Control (1997), the a_w of a sucrose solution can be estimated using an empirical equation: $a_w = 1.00/(1+ 0.27n)$, where n is the number of moles of sucrose in 100 g water (1 mole sucrose is 342 g). Based on this, the contribution of sucrose alone, to the a_w of SCM, is 0.884 (45% sucrose in 27% water or 167 g sucrose per 100 g water). It follows that the further reduction to 0.800 (prior to lactose crystallization) or to 0.840 (after crystallization) is contributed by lactose and other solutes (milk salts and proteins).

I.8.4. Effect of Lactose Crystallization on the Measurement of Moisture/Total Solids in Sweetened Condensed Milk

As explained above, lactose crystallizes as α-monohydrate on concentration or drying. As a result, the total solids (TS) in SCM are increased by the water of hydration in the monohydrate crystals. Depending on the conditions of drying, the measured value can become very high, when compared to values obtained by the Karl Fisher (wet) titration method (non-drying).

Conceivably, rapid drying encourages lactose to dry in a glassy form, so there would be little or no lactose monohydrate to increase the TS. However, due to the high concentration of sucrose in SCM, and depending on the sample size relative to the drying surface area, sucrose glass, when formed on the surface of the drying sample, can significantly inhibit complete drying.

The official AOAC 920.115 method requires drying a diluted sample of SCM in the presence of sand. The CEM (microwave) method requires drying a diluted sample on a glass fiber pad. The use of sand or a fiber pad provides a large surface area for sucrose glass formation, thus reducing the "blanketing" effect of sucrose glass on the complete drying of the sample. The use of the Mojonnier TS method, on the other hand, is very tricky (rapid drying in an open pan with no sand or pad); depending on the dilution and the sample size, the value obtained by this method can overestimate TS by as much as 1.7%.

Reproducibility of test results using the AOAC method depends on the diligence of the analyst in making sure that the surface of the sample (prior to complete drying) is mixed periodically with the sand. Since this method requires 5 h for complete drying, it is not suitable for industrial application. The Karl Fisher method, on the other hand, is very reproducible and accurate, provided that the sample has been pre-conditioned with 2–3 parts dry formamide at 60°C for 2–3 min to facilitate the release of water held tightly by the solutes, prior to the titration in dry methanol media (see Bruttel and Schlink, 2003).

I.8.5. Effect of Water Activity on the Microbial Stability of Sweetened Condensed Milk

Many microorganisms have been found in SCM produced around the world, but the a_w or composition of these products are not known. Today, more and more emphasis is given to water activity (a_w) in foods; a_w is now the primary criterion for defining the so-called "non-potentially hazardous food" (2005 Food Code of US FDA). An a_w above 0.88 is considered "potentially hazardous" for non-acid foods.

Sweetened condensed milk is a low-acid, pasteurized product (as opposed to high-acid or sterilized products). Its microbial stability at room temperature is due to the preservative action of the sugar it contains. With the right level of sugar (a_w below 0.85), only molds and osmophilic yeasts and bacteria can spoil the product. Since molds and osmophilic yeasts are typically very heat labile (readily destroyed by proper heat treatment), their presence in the product is due mainly to post-process contamination. With a sufficient level of sugar (a_w below 0.85), organisms other than osmophilic yeasts and molds should never grow in SCM to such numbers that cause spoilage.

The number of organisms required to cause spoilage varies with the food item and the type(s) of microorganisms growing in it. As a rule of thumb, 10^7 bacteria/g, 10^5 yeast/g or visible mold signals the end of microbiological shelf life. In SCM, however, various organisms die within weeks or months of storage (Rao and Ranganathan, 1970; Koroleva *et al.*, 1975; Farrag *et al.,*

1990; Chernyaeva, 1994) or grow only very slowly to a limited extent (Yankov, 1971; Jarchovska *et al.,* 1970).

Staphylococci are widespread in food plants. Some species (e.g., *Staphylococcus succinus, S. xylosus, S. pasteuri*) are more osmoresistant than others. *S. aureus* is the only known pathogen that can survive at a_w close to that of SCM but when present in SCM, it cannot produce toxins (Lukasova, 1972). It cannot grow or produce toxins at a_w below 0.864 and it needs to be present at 10^7 cells/g to cause food poisoning (Lotter and Leistner, 1978). It is highly sensitive to heat and most sanitizing agents; thus, the presence of this bacterium or its enterotoxins in processed foods or on food processing equipment is generally an indication of poor sanitation.

Zygosaccharomyces (yeasts) are osmophilic, suggesting that they live only in high solute or sugar environments (source of contamination). It has been identified in fruit concentrates and juices, jams, jellies and preserves, as well as in ketchup, salad dressings, relishes and pickles (Thomas and Davenport, 1985). Of those recognized species of *Zygosaccharomyces, Z. bailii, Z. bisporous, Z. rouxii* and *Z. florentinus* have been isolated from grape must and wine. *Z. rouxii* has been reported to grow at a_w of 0.62 in fructose, 0.65 in sucrose/glycerol solution or 0.86 in NaCl (Corry, 1978). However, *Zygosaccharomyces* has not been reported in SCM.

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II. Lactose in Dulce de Leche

E. Hynes and C. Zalazar

II.1. Introduction

Dulce de leche (DL) is a typical product of the Río de la Plata region, in South America. The main producer countries are Argentina and Uruguay, where DL is consumed as a dessert or used as an ingredient in confectionery and other food products. The consumption in Argentina is about 2.70 kg *per caput per annum*, with a stable production of 109,000 tonnes/year (Schaller, 2007). Uruguay has a minor share of the market, with the production of 9,000 tonnes/year (Zalazar, 2003); the product is also consumed in other Latin American countries but at a lower level. Recently, the export of DL to the USA and Europe has started successfully; in developed countries, DL is used almost exclusively as an ingredient for ice cream or desserts produced on an industrial scale. A small amount of DL is currently produced in Italy for this purpose.

Dulce de leche is a creamy and viscous dairy product prepared by concentrating a mixture of milk and sucrose to a solids content of 70% (Código Alimentario Argentino, 2007). The rheological behaviour of DL is intermediate between a concentrated solution and a gel, depending on the type and the solids content (Pauletti *et al*., 1990; Navarro *et al*., 1999). As a consequence of the composition of the blend and the conditions of manufacture, extensive non-enzymatic browning occurs (Maillard reactions, caramelization of sugars). These reactions are responsible for the typical attributes of the product: a light, reddish-brown colour, creamy texture and pleasant caramel-like aroma. The water activity of DL is below 0.85, as a consequence

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of the high content of sugars and low moisture (Ferramondo *et al.*, 1984); therefore, the shelf life of this product is several months. Several types of DL are available, the most popular of which, called "Familiar", is consumed mainly at homes. Other types are intended for confectionery, ice cream and other industrial uses; DL varieties with added cream, chocolate, fruits, etc. are permitted (Código Alimentario Argentino, 2007).

II.2. Technology

In the manufacture of DL, the initial mixture is composed of milk and sucrose. The fat content is adjusted by the addition of cream, and the solids content is often increased by adding whole or skimmed milk powder, in order to reduce the concentration time. A few additives are allowed: neutralizers to maintain the pH of milk during concentration, glucose in "Familiar"-type DL for partial substitution of sucrose (up to 40%), vanillin or ethylvanillin as flavours and potassium sorbate (maximum 600 mg kg^{-1}) as a fungicide (Código Alimentario Argentino, 2007).

The quantity of nutritive sweeteners added to the milk is calculated to give 68–70% total solids in the final product, of which milk solids account for at least 24%. Sucrose is most commonly used in Argentina, but glucose may also be used, in particular for "Familiar"-type DL, because it results in a glossier product and is less expensive than sucrose. The technological advantages and drawbacks of glucose addition will be discussed later.

The mixture of milk and sugars is first neutralized with $Ca(OH)_2$ or $NaHCO_3$ to obtain a pH of 7.0 or a titratable acidity equivalent to 2–10 mg lactic acid 100 mL^{-1} according to the type of DL (Arobba *et al.*, 2002). Neutralization avoids the destabilization of casein micelles as a consequence of the decrease in pH during evaporation, which in turn is due to the concentration of calcium phosphate, the formation of organic acids from lactose degradation and hydrolysis of phosphoric esters of caseins (Zalazar, 2003). Failures in DL manufacture with the separation of phases (solid proteins and liquid "whey") are due mainly to inadequate neutralization. The amount of alkali needed for neutralization is calculated taking into account the pH of the milk, and is added as a solid powder or, preferably, as an aqueous solution to improve homogeneity. The neutralized blend is transferred to the evaporation kettle where water is evaporated and the reactions leading to colour and aroma development occur.

DL can be produced by the traditional process in open kettles or by semi-continuous or continuous processes. The first is used most frequently and consists of a classic batch process, which renders the best quality DL (Figure II.1). The blend is prepared in a mixing tank and then gradually

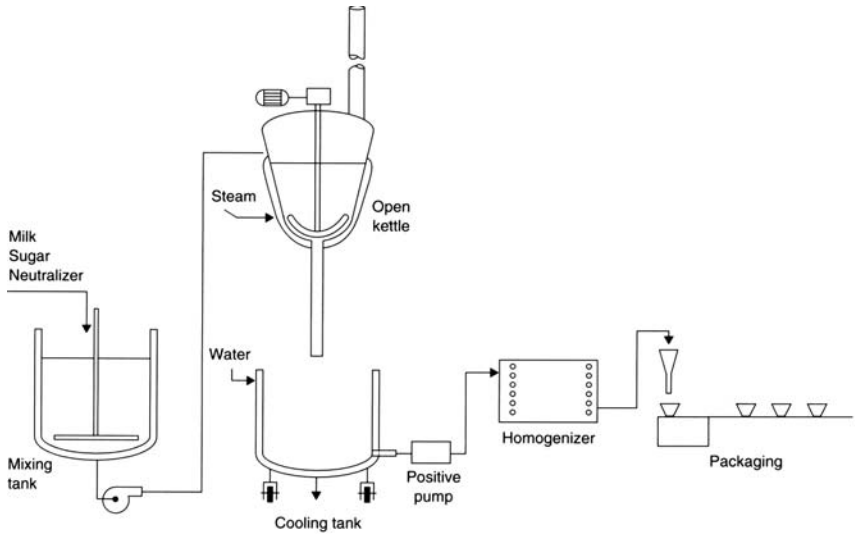

Figure II.1. Batch production of dulce de leche in open kettles. Adapted from Zalazar (2003).

transferred to the evaporating kettle. The volume in the kettle is low initially (20% of its capacity); when boiling has caused some concentration, the rest of the blend is fed slowly into the kettle while boiling continues. A stirrer fitted with heat-resistant plastic scrapers is used to prevent the product sticking to the wall of the kettle and to improve the release of vapour from the hot mass of DL. As the solids content increases, Maillard and other non-enzymatic browning reactions occur and the product attains its typical colour and flavour. Cooking is stopped when the solids content is 68%, which is assessed by refractometry. Legislation requires 70% total solids but this target value is normally attained during the following steps of the process, discharge of hot DL and cooling. After that, the product is pumped to a homogenizer, where it is homogenized to avoid clumps and improve texture. Finally, the product is packaged while still hot (60°C), to avoid microbiological contamination, in plastic containers (0.25, 0.5 and 1 kg). Glass containers are also used but they are not very common and are usually reserved for premium DL. Paste-board packages of 10 or 20 kg are used for bakery and confectionery purposes.

In the semi-continuous process, the mixture of sugar and milk is concentrated in a multiple-effect evaporator to improve the efficiency of the evaporation step; afterwards, the final solids content is obtained by boiling in the open kettle, where the colour and flavour develop. In the continuous process there is an inversion of the steps, as colour development is first

attempted in a heat exchanger by heating and rapid cooling while the product is still liquid; the control of several variables such as temperature and pH is critical in order to obtain the desired colour. The coloured blend is then fed into a multiple-effect evaporator and finished in a scraped-surface evaporator before cooling to 60°C in a tubular heat exchanger. The production of DL by the continuous process is relatively small and has not increased in recent years as the traditional batch process provides a finished product with better sensory characteristics (Zamboni and Zalazar, 1994; Zalazar, 2003).

II.3. Significance of Lactose in Dulce de Leche

II.3.1. Lactose Crystallization

II.3.1.1. Texture

DL is a concentrated dairy product and the crystallization of lactose can occur as the moisture in DL is supersaturated with lactose. If the crystals of α-lactose monohydrate grow to exceed 10 μm, they will be perceived in the mouth, giving the product a defective texture called "sandiness" or "grittiness".

Taking into consideration the average composition of the raw milk (fat: 3.3%, total proteins: 3.0%, lactose: 4.7%), and the usual concentrations of the most commonly added sugars (172.7 kg sucrose + 41.4 kg of glucose syrup, 70%, w/v, per 1000 L of milk), DL will have the composition shown in Table II.1.

These values are in agreement with those reported by Ferramondo *et al.* (1984), and imply that the concentration of sugars in the moisture of DL is 121%, 29% and 33%, w/w, for sucrose, glucose and lactose, respectively. Among these three sugars, lactose has the lowest solubility in water, ~18%, w/w, in pure water at 20°C. Even without considering the presence of glucose

Table II.1. Typical composition of commercial dulce de leche

Component	Percentage (w/w)
Sucrose	36.3
Glucose	8.7
Lactose	9.9
Fat	6.9
Total proteins	6.3
Moisture	30.0
Ash	2.0

Adapted from Zalazar (2003).

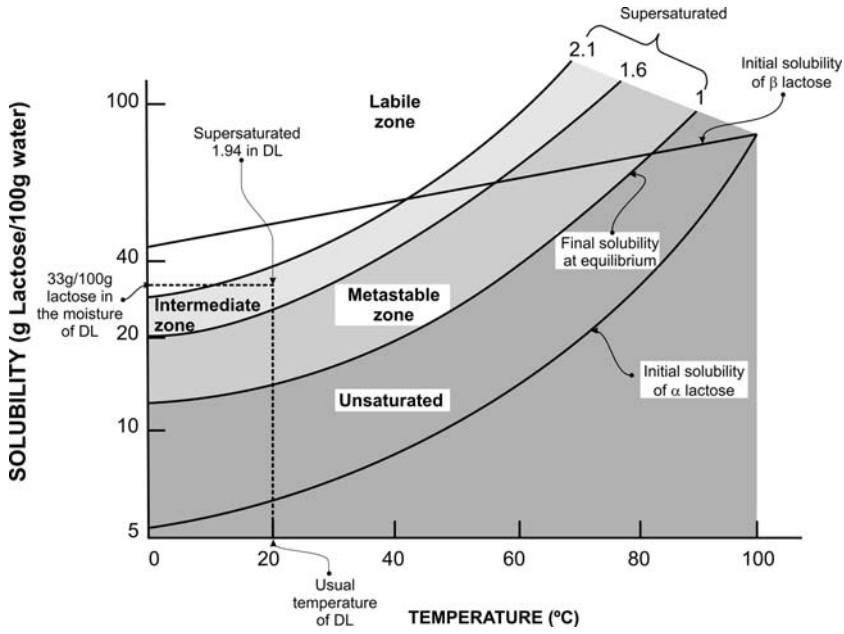

Figure II.2. Lactose solubility curves. DL: dulce de leche. Adapted from Fox and McSweeney (1998).

and sucrose in the system, the aqueous phase of DL would be supersaturated with lactose, with a concentration ca. 1.94 times higher than saturation solubility. This concentration of lactose places the aqueous phase of DL in the intermediate equilibrium zone, defined by the solubility curves of lactose (Figure II.2). In this case, lactose crystallization may be inducted by seeding with lactose crystals; however, spontaneous crystallization hardly ever occurs or it is very slow (Holsinger, 1997).

In DL, the anomeric forms of lactose mutarotate to an equilibrium:

$$\alpha \rightleftarrows \beta$$

The equilibrium ratio of α:β-lactose is 1.68. If the total (typical) concentration of lactose ($\alpha + \beta$) in DL is 9.9%, w/w, the concentration of α-lactose in DL would be 3.7%, w/w, while that of β-lactose would be 6.2%, w/w.

On the other hand, taking into account the final solubility of lactose (i.e., 18 g/L), 15 g of lactose (i.e., 33–18 g) would be available for crystallization from 100 g of the aqueous phase of DL; this is equivalent to 4.5 g of lactose per 100 g DL. With these considerations, 5.4 g of lactose would eventually

remain soluble in 100 g DL after the crystallization of the excess lactose. From the value of the equilibrium ratio, it can be calculated that of the 5.4 g of lactose in solution, 2.02 g would be α-lactose and 3.38 g β-lactose. Summarizing: 3.70–2.02 = 1.68 g of α-lactose would crystallize and 6.2–3.38 = 2.82 g of β-lactose would first mutarotate to α-lactose and then crystallize. In this context, the rate of crystallization of lactose in DL depends not only on the rate of crystal formation itself but also on the mutarotation rate, and the slowest reaction will limit the crystallization kinetics.

The presence of salts and sugars in the system impacts on the kinetics of mutarotation in opposite directions. Citrate and phosphates, at the concentrations found in milk, accelerate mutarotation to a rate twice as high as in pure water, whereas in contrast, concentrated sucrose slows down the mutarotation reaction. This latter effect is only slight up to 40% (w/v) sucrose, but above this level, the mutarotation rate decreases and the catalytic impact of milk salts is counteracted (Holsinger, 1988). Temperature and pH also influence the mutarotation rate. This effect of pH is minimum in a range of 2.5–7.5 but increases dramatically both at lower and higher pH values. The effect of temperature is shown in Table II.2.

Taking into account the environmental conditions in DL and the data above, rate of lactose mutarotation in DL should be low, as sucrose is highly concentrated, the pH is between 5.57 and 5.97 and the storage temperature is always below 20°C (Ferramondo *et al.*, 1984; Código Alimentario Argentino, 2007).

On the other hand, the formation of lactose crystals in the intermediate zone (Figure II.2) may be very slow. In this zone, seeding with lactose crystals can induce crystallization but otherwise supersaturated solutions will be stable (Holsinger, 1997).

Either way, the slow or rate-limiting step may be mutarotation or crystallization, depending on the characteristics of the system containing the lactose. Haase and Nickerson (1966a,b) found that mutarotation is very fast and consequently crystal formation is the rate-limiting step under the environmental conditions that exist in most dairy products. Under other conditions, for example, when an extensive nucleation area is available for lactose crystallization, it has been observed that neither of the two steps can be clearly identified as the rate-controlling step (Tweig and Nickerson, 1968).

Table II.2. Effect of temperature on the rate of lactose mutarotation

Temperature, °C	75	25	15	0
Reaction completion (% in 1 h)	100 (instantaneous)	51.7	17.5	3.4

Adapted from Holsinger (1988).

The discussion presented above is only an approach to the problem of lactose crystallization in DL; in the real food matrix, the situation is even more complex than described. Mutarotation, for example, as well as crystal formation, may be impaired in DL because of the high viscosity of the medium. In contrast, the high concentration of sucrose and glucose may reduce the solubility of lactose significantly, increasing its rate of crystallization. In fact, a sucrose concentration of 70% has been reported to reduce lactose solubility to 42% of its solubility in water (Nickerson and Moore, 1974) but the combined impact of sucrose and glucose on lactose solubility has not been studied. In addition, the influence of the products of the Maillard reaction on the rates of lactose mutarotation and crystallization has not been reported to date. The available knowledge on lactose crystallization in DL is mainly empirical, based on decades of observation and experience, but systematic studies on the subject are lacking.

In good quality DL, manufactured according to the standard process, the formation of lactose crystals larger than 10 μm and detectable in the mouth does not occur before 120 days of storage at room temperature (always below 25°C). Consequently, industry has adopted this period as the usual shelf life for the product. However, due to a high demand, the food is generally consumed earlier.

On the local market, DL is most often retailed in plastic packages. When packaged in this way, it has been observed that the occurrence of plastic flavour in the DL is the first observed sensory defect and determines the shelf life of the product, before the appearance of gritty texture or "sandiness". Garitta *et al.* (2004) reported that the sensory shelf life was limited to 146 days after storage at 25°C as a consequence of a plastic flavour note developing in the DL; sandiness was not reported in the product during this period. Premium DL, on the contrary, is often packaged in glass containers and the shelf life is generally required to be longer (180 days). In this type of product, sandiness probably determines its sensory shelf life, and its development should be delayed as much as possible.

One of the most commonly used approaches to avoid lactose crystallization is the enzymatic hydrolysis of lactose to glucose and galactose, which is performed before manufacture by adding the enzyme, β-galactosidase, to the milk and incubating for several hours. The hydrolysis of 30% of the lactose is sufficient to avoid crystallization during a 180-day period. Glucose and galactose are sweeter than lactose and contribute as nutritive sweeteners to DL, reducing somewhat the costs; however, β-galactosidase is expensive and its use is justified only when a premium product with a relatively high price is manufactured.

It has been reported that lactose crystallization can be retarded by increasing the proportion of glucose in DL (Ferramondo *et al.*, 1984;

Navarro *et al.*, 1999; Arobba *et al.*, 2002). Glucose increases viscosity, thus reducing the rate of growth of the lactose crystals. However, there is little evidence to support this strategy and the use of glucose may be disadvantageous for the colour of the product due to excessive browning.

Another technological approach to avoid sandiness in DL is seeding with a certain quantity of finely divided lactose crystals as nuclei for crystallization, in order to accelerate the process and produce a large number of very small, undetectable crystals. The seeding with lactose crystals is usual in sweetened condensed milk where it is added at a level of 0.5% after evaporation (Early, 1998), but it is not a common practice in DL (Zamboni and Zalazar, 1994).

Finally, another approach has been proposed: by maintaining DL at $T>93.5^{\circ}C$ to favour the crystallization of β-lactose which proceeds in the form of uneven-sided diamond-shaped crystals too small to be perceived in the mouth (Holsinger, 1997). However, it is not known how long it would take β-lactose to crystallize above 93.5°C, and maintaining a batch of DL for a long period at such a high temperature is highly inefficient.

II.3.2. Non-enzymatic Browning Reactions

Unlike most food products, milk contains both proteinaceous material and a reducing sugar, which leads to non-enzymatic browning and changes in the nutritional value of the food when it is heated even at mild levels (Holsinger, 1997). In DL, in addition, sucrose and glucose are usually present; while sucrose is a non-reducing sugar, glucose readily reacts with proteins according to the Maillard reaction. It has been suggested that a small proportion of sucrose is hydrolyzed to its reducing monomers, glucose and fructose, during the cooking of DL in the kettle (Rozycki, 2003); however, such transformation is not significant during manufacture as environmental conditions are not favourable (Malec *et al.*, 2005). Neither fructose nor galactose, from the hypothetical hydrolysis of the disaccharides sucrose and lactose, has been detected in DL (Ferramondo *et al.*, 1984).

Lactose is usually the main reducing sugar in DL, depending on the proportion of sucrose that is replaced by glucose syrup in the initial blend, and together with milk proteins, is the main reactant for Maillard reactions. Non-enzymatic browning in DL also includes caramelization reactions, in this case, sucrose has been reported to be the main sugar involved, although lactose may contribute also (Rozicky, 2003). Caramelization and Maillard reactions continue during the storage of the product after manufacture (Garitta *et al.*, 2004).

II.3.2.1. Colour and Flavour

A typical reddish-brown colour and caramel-like flavour are major sensory attributes of DL that impact on consumer acceptability of the product. Colour, as well as typical texture, correlates positively with the overall acceptance of DL (Garitta *et al.*, 2004; Ares *et al.*, 2006). However, an excessively dark brown colour can lead to product rejection (Garitta *et al.*, 2004).

The negative changes in the level of a desirable attribute, like colour, may be due to the composition of DL or time of storage. The techniques most frequently adopted in industry to avoid texture defects (sandiness) involve higher amounts of reducing sugars in the system. Hydrolysis of lactose with β-galactosidase leads to the liberation of two molecules of reducing sugars (glucose and galactose) from each molecule of lactose.

On the other hand, if part of the sucrose is replaced by glucose, the Maillard reaction is favoured and the colour of the product may become too dark. In addition, some studies on colour formation in casein–sugar systems have shown that when glucose is present, the Maillard reaction is faster (Morales and van Boekel, 1998) although variable results have been reported using different indices of non-enzymatic browning, such as fluorescence accumulation (Morales and van Boekel, 1997; Rozycki *et al.*, 2007). An alternative method to avoid the excessive darkening of DL is to reduce the proportion of glucose or add the glucose syrup later during manufacture, after the solid content of DL reaches 55–60% (Zalazar, 2003).

A high pH of DL after neutralization (pH $>$ 7.50) may also cause too dark a colour in the product due to the catalytic effect of alkaline conditions on the Maillard reaction and brown product formation (O' Brien, 1997; Rozycki and Pauletti, 2002; see Chapter 7).

Finally, DL can darken during storage due to continuing of non-enzymatic browning in the finished product at room temperature. However, acceptability tests have shown that the colour changes during storage for the usual period (up to 180 days) are not determinants for product rejection by the consumers (Garitta *et al.*, 2004).

II.3.2.2. Nutritional Value

The impact of lactose on the nutritional properties of DL may be evaluated from two different points of view, the principal of which is the loss in nutritive value by damaging essential amino acids by Maillard reactions. The second aspect is the nutritional concern for lactose-intolerant people, as DL is rich in lactose.

In traditional DL, prepared only with sucrose as nutritive sweetener, 70% of the lysine remains available at the end of the manufacture, whereas the replacement of 10% of sucrose by reducing sugars significantly reduces the level of available lysine. This is due not only to the increase of the molar ratio of reducing sugar:lysine, but also to the fact that the monosaccharides, especially glucose, are more reactive than lactose (Malec *et al*., 2005).

As for lactose-intolerant people, no reports on the effects of DL are available. However, the product is usually consumed in low amounts (average serving is about 20 g), and along with other foods; in addition, the consistency and rich composition of the food probably favour the tolerance of lactose (Mustapha *et al*., 1997). Finally, the hydrolysis of lactose for technological purposes helps to reduce the concern about the nutritional disadvantages of the disaccharide.

II.4. Conclusions

The role of lactose in DL is related to texture quality, colour and nutritional value. The technological approaches intended to reduce costs and to improve texture and the visual aspect of DL by replacing sucrose by glucose syrup, or to retard the appearance of sandiness by hydrolysis of lactose, impact on important properties of the product such as its colour and nutritional value. Consequently, a balance should be found between the proportion of non-reducing sugars, lactose and monosaccharides in DL. Besides, the shelf life of the food should be realistically fixed as lactose crystallization will eventually occur.

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III. Significance of Lactose in Ice Cream

H. Douglas Goff

III.1. Overview of Ice Cream Ingredients and Manufacture

This section will present a brief review of the sources and functionality of lactose in ice cream. The major issues surrounding lactose in ice cream include freezing point depression, lactose crystallization and lactose digestibility. Readers are referred to previous chapters on ice cream in the *Advanced Dairy Chemistry* series for specific information related to proteins (Goff, 2003) and lipids (Goff, 2006) or to more general references on ice cream technology (Berger, 1997; Marshall *et al.*, 2003; Goff and Tharp, 2004) for further information.

The term "ice cream" in its generic sense is used here to include all whipped dairy products that are manufactured by freezing and are consumed in the frozen state, including ice cream that contains either dairy or non-dairy fats, premium, higher fat versions, light , lower fat versions, ice milk, sherbet and frozen yogurt. Ice cream mix formulations specify the content of fat, milk solids-non-fat (MSNF), sweeteners, stabilizers, emulsifiers and water that are desired (Figure III.1). Dairy and other ingredients used to supply these components are chosen on the basis of availability, cost, legislation and desired quality. Common ingredients include cream, butter or vegetable fats, as the main sources of fat; condensed skim or whole milk, skim milk powder and/or whey powder or whey protein products, as the sources of concentrated MSNF; sucrose and/or corn starch hydrolysates as the sweeteners; polysaccharides, such as locust bean gum, guar gum, carboxymethyl cellulose and/or carrageenan, as the stabilizers; mono- and di-glycerides and

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polysorbate 80, as the emulsifiers; and milk or water as the main sources of water in the formulation to balance the total solids of the components (Marshall *et al.*, 2003). Usually, one mix is used for the production of a variety of flavours.

The manufacturing process for most of these products is similar and involves the following steps (Figure III.1): preparation of a liquid mix by blending of ingredients, pasteurization (65°C for 30 min or 80°C for 25 s), homogenization, cooling to 4°C and ageing of the cold, liquid mix for 4–24 h; concomitantly whipping and freezing this mix dynamically under high shear to a soft, semi-frozen slurry with an air phase volume of 45–52% (overrun of 80–110%) at a temperature of about –5°C; incorporation of flavouring ingredients to this partially frozen mix; packaging the product; and further quiescent freezing (hardening) of the product in blast air to –30°C (Marshall *et al.*, 2003). Homogenization is responsible for the formation of the fat emulsion by forcing the hot mix through a small orifice under a pressure of 14–18 MPa, perhaps with a second stage of 3–4 MPa. Ageing allows for hydration of milk proteins and stabilizers (some increase in viscosity occurs during the ageing period), crystallization of the fat globules and a membrane rearrangement due to competitive displacement of adsorbed proteins by small-molecule

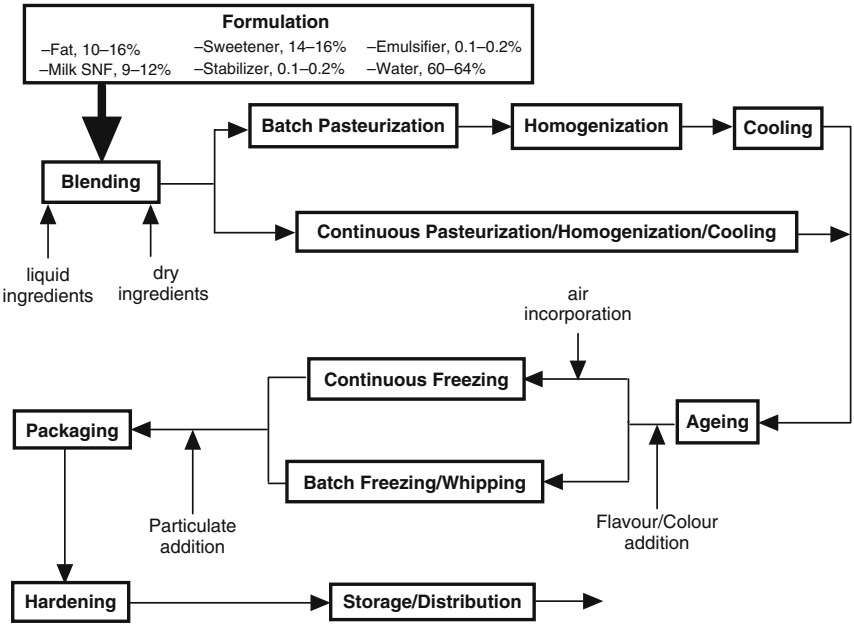


Figure III.1. Flow diagram for the production of ice cream (SNF=solids-non-fat).

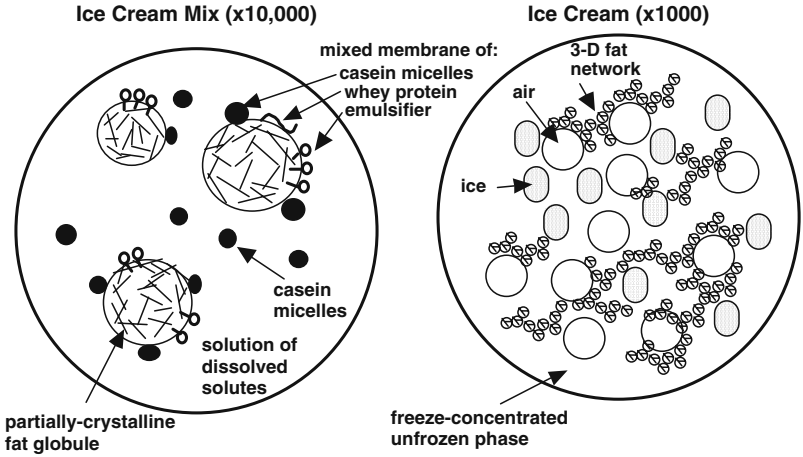


Figure III.2. Highly schematic illustration of the structure of ice cream mix and ice cream. Ice cream mix contains the partially crystalline fat globules and casein micelles as discrete particles in a solution of sugars (including lactose), salts, dispersed whey protein and stabilizers, etc. The surface of the fat globule demonstrates the competitive adsorption of casein micelles, globular, partially denatured whey proteins, β-casein and added emulsifiers. Ice cream contains ice crystals, air bubbles and partially coalesced fat globules as discrete phases within an unfrozen serum containing the dissolved material (including lactose). The partially coalesced fat agglomerates adsorb to the surface of the air bubbles, which are also surrounded by protein and emulsifier, and link the bubbles through the lamellae between them.

surfactants. The concomitant aeration and freezing process involves numerous physical changes, including the action of proteins and surfactants in forming and stabilizing the foam phase; partial coalescence of the fat emulsion, causing both adsorption of fat at the air interface and the formation of fat globule clusters that stabilize the lamellae between air bubbles; and freeze-concentration of the premix by the removal of water from solution in the form of ice. The structure of ice cream is illustrated in the diagram in Figure III.2 (Goff, 2002). Lactose is dissolved in the unfrozen phase (see further discussion regarding freezing point depression and freeze-concentration below).

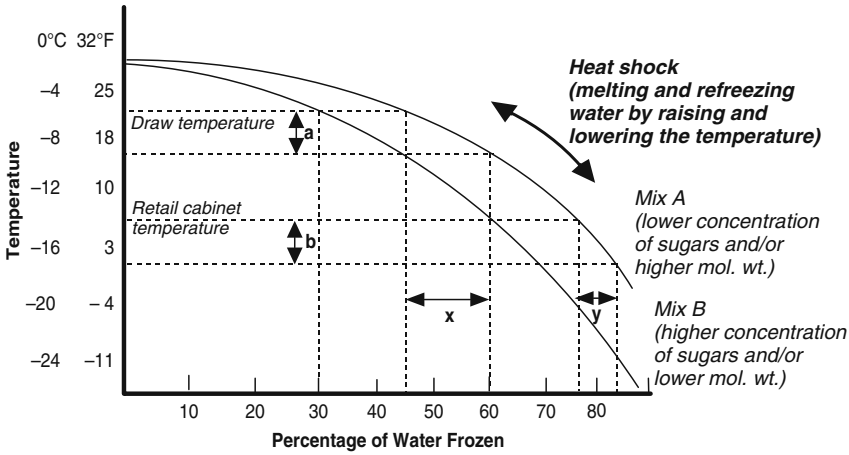
III.2. Sources of Lactose in Ice Cream

Lactose enters into mix formulations with the MSNF ingredients. Traditionally, the best sources of MSNF for high-quality products have been fresh concentrated skimmed milk or spray-dried low-heat skim milk powder. Others include those containing whole milk protein (e.g., condensed or sweetened condensed whole milk, dry or condensed buttermilk), those

containing casein (e.g., sodium caseinate) or those containing whey proteins (e.g., dried or condensed whey, whey protein concentrate, whey protein isolate) (Marshall *et al.*, 2003). Dried whey has been investigated as an ingredient for ice cream for many years, principally due to reduced cost (Leighton, 1944; Neilsen, 1963; Parsons *et al.*, 1985). However, it is high in lactose (~75–80%), which is a major limitation. It has now become quite common to supplement the traditional sources of MSNF (condensed skim milk or skim milk powder) with blended MSNF products that contribute excellent functionality from the protein (emulsification, foaming and water holding), while at the same time reducing total protein content in the dry blended ingredient from 36%, as found in skim milk powder, to 20–25%, to maintain mix costs lower than they would be if skim milk powder was used. Ingredients in the blends include milk or whey protein concentrates or isolates, perhaps modified chemically or via fermentation, perhaps also some caseinates, and whey powder or lactose, for standardization. Much experience has been gained at blending these ingredients (Goff, 2003; Marshall *et al.*, 2003) and the quality of ice cream resulting from their use can be very good. However, since most legal jurisdictions require a minimum total solids level in ice cream mix formulations, care must be taken to ensure that the lactose content in the formulation is not too high when formulating with these high-lactose ingredients, due to issues of freezing point depression and lactose crystallization.

III.3. Contribution of Lactose to Freezing Point Depression

Freezing point depression is a colligative property, governed by Raoult's law and influenced by the collective number of moles of solute in solution. Thus, freezing point depression is a function of both the concentration of all the solutes and their molecular weight. Consequently, in an ice cream mix, the major contributors to freezing point depression are the sugars and milk salts (Leighton, 1927; Smith and Bradley, 1983; Smith *et al.*, 1984; Baer and Keating, 1987; Jaskulka *et al.*, 1993, 1995). Lactose is a disaccharide, molecular weight 342 Da, and is present at a significant concentration (~6%), thus it contributes approximately 30% (although this varies with formulation) of the total freezing point depression of a mix (Marshall *et al.*, 2003). As the mix is frozen, solvent is removed with the conversion of water to ice, so that the effective concentration of solutes in the unfrozen phase continues to rise with decreasing temperature, leading to the process of freeze-concentration and establishing the equilibrium ratio of ice to water as a function of temperature (see Figure III.2). This is plotted as a freezing curve (Figure III.3), which is unique to each formulation (Bradley and Smith, 1983; Bradley, 1984; De



- ***The lower the freezing curve, the less water frozen at drawing from the barrel freezer (30% for mix B compared to 45% for mix A), hence more water to freeze out during hardening, which is the slower process yielding larger ice crystals.***
- ***The lower the freezing curve, the softer the ice cream in the retail cabinet (60% water frozen in mix B compared to 75% for mix A), hence more susceptible to heat shock.***
- ***In looking at freezing curves, on the flatter part of the curve (warmer temperature range), a given temperature change (e.g., 4°C) involves more water melting and refreezing (hence more recrystallization), while on the steeper part of the curve (lower temperature range), the same temperature change involves less water melting and refreezing (less recrystallization); for mix A, a = b but x > y.***

Figure III.3. Typical freezing curve for ice cream mixes of varying composition showing the percentage of water frozen at various temperatures.

Cindio *et al.*, 1995; Livney *et al.*, 2003; Whelan *et al.*, 2008). The significance of freezing point depression and freeze-concentration is that it dictates the harness of the ice cream as a function of temperature. In scooping or retailing operations, it is extremely important to have all ice creams close to the same degree of hardness. Hence, formulations must be adjusted to account for variable levels of sugars and/or types of sugars to ensure constancy in hardness.

Lactose is not very sweet and is contributed by the MSNF ingredients, as discussed above, rather than being considered as a sweetener. Nevertheless, an excess of lactose, for example with a high concentration of whey powder, can lead to ice cream that is too soft for typical storage/distribution temperatures and retail operations. As the amount of unfrozen water increases, the ice cream becomes more prone to problems like ice recrystallization and the development of coarse or icy textures, more prone to lactose crystallization

(see below) and more prone to shrinkage or loss of air, all which limit its shelf life (Marshall *et al.*, 2003). These are all a result of enhanced mobility of constituents within the ice cream structure. This is one of the major limitations of the use of a high level of lactose in ice cream/frozen dairy dessert formulations.

III.4. Potential for Lactose Crystallization

The crystallization of lactose in general has been well studied and is reviewed in Chapters 1, 2 and 3.I. The crystallization of lactose in ice cream has also been well studied over many decades because, in this specific application, crystallization leads to the serious texture defect known as sandiness (Zoller and Williams, 1921; Nickerson, 1954, 1956, 1962; Livney *et al.*, 1995). The solubility of α-lactose is 7 g/100 g water at 20°C, while the solubility of β-lactose is 50 g/100 g water. The mutarotation equilibrium is 1.6 β:1.0 α, so the final total solubility of lactose is 18.2 g/100 g water at 20°C at this ratio (Fox and McSweeney, 1998). Solubility and mutarotation are both a function of temperature, so at 0°C the solubility of α-lactose is approximately 2–3 g/100 g water to provide a total lactose solubility closer to 11 g/100 g water (Nickerson, 1956) and solubility continues to decline into the sub-zero region. The initial concentration of lactose in ice cream may be expected to be approximately 9–10 g/100 g water, depending on formulation (Marshall *et al.*, 2003). However, the process of freeze-concentration due to the formation of ice is critical to an understanding of lactose crystallization in ice cream, as it contributes much more strongly to supersaturation than does decreasing temperature in the absence of freezing. Solutes become freeze-concentrated in an ever-decreasing volume of solvent as the temperature is lowered and more ice is formed. The water in this unfrozen phase forms an equilibrium ratio with ice at any given temperature. The removal of solvent (water) by freezing results in a doubling of the lactose concentration at the temperature of extrusion from the ice cream freezer (–5°C) and concentrations of 3–5× at –10 to –20°C as freeze-concentration continues.

It should be obvious from the above discussion that lactose has greatly exceeded its solubility (saturation) level in frozen ice cream and, from a thermodynamic point of view, could easily crystallize. Increasing supersaturation favours crystallization (Hartel and Shastry, 1991; Hartel, 2001). However, the first step of crystallization is nucleation of the lactose and this process is constrained kinetically by both high viscosity and low temperature in the unfrozen phase, thus maintaining lactose in the supersaturated, non-crystalline state. This increased viscosity and decreased temperature, which decreases the driving force for crystallization, overwhelms the effect of

increased supersaturation, which would increase the driving force (Hartel and Shastry, 1991; Hartel, 2001). If α-lactose does nucleate, then there exists a threshold size of detection (crystals of 16–30 μm; Nickerson, 1954; Hartel, 2001; Marshall *et al.*, 2003) beyond which the textural defect of sandiness becomes increasingly evident. The typical trapezoidal wedge (tomahawk) shape of the α-lactose crystals is readily detected as a very sharp, rough particle (Figure III.4), which are easily differentiated from ice crystals as the lactose crystals do not readily melt in the mouth or between fingers. Once nucleation has occurred, crystallization can proceed quite quickly and once this level of lactose crystallization has been exceeded in packaged and flavored ice cream, then disposal is the only recourse. Therefore, it is imperative that formulation, processing and storage conditions are all optimized to inhibit completely the nucleation of lactose.

The mix formulation is the first consideration for the minimization of lactose crystallization. The more the lactose present, the greater the degree of supersaturation and the more prone the ice cream to crystallization. Recommendations regarding maximum levels of total MSNF to avoid lactose

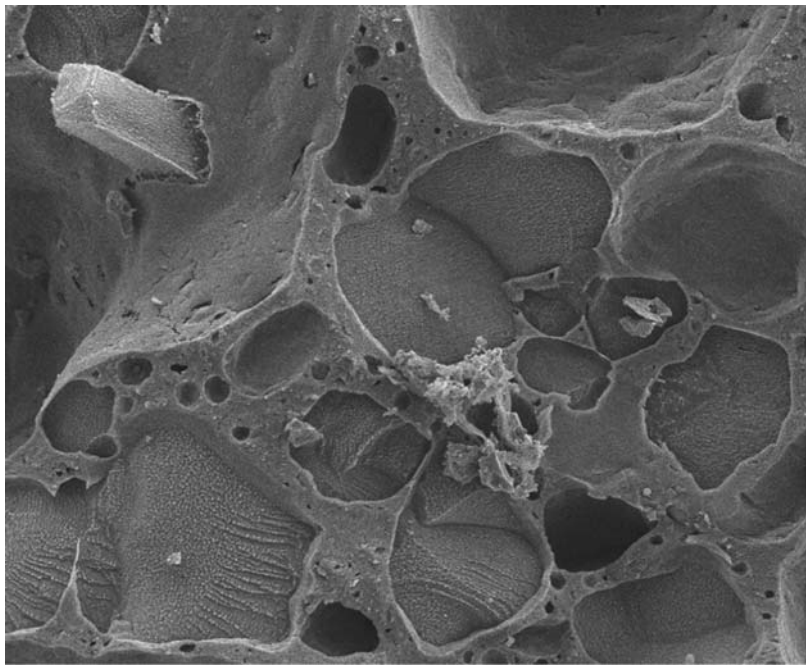


Figure III.4. Lactose crystal protruding through an air bubble in "sandy" ice cream as seen by cryoscanning electron microscopy (width of photograph=150 μm).

crystallization have been suggested to ice cream manufacturers for many years (Sommer, 1944; Nickerson, 1962); however, the modern use of polysaccharide stabilizers, as discussed below, renders obsolete some of the older recommendations for MSNF in formulations. Lactose content is certainly a consideration in the use of MSNF ingredients, such as whey powder, permeate or other MSNF sources that contain an elevated level of lactose. Nickerson and Moore (1972) showed that increasing sucrose concentration reduces lactose saturation, exacerbating the propensity for crystallization. On the other hand, sucrose may inhibit the lactose nucleation process, thereby promoting supersaturation (Livney *et al.*, 1995). Ingredients like dextrins from low DE corn syrup solids, soluble milk proteins and polysaccharide stabilizers, all of which promote solution viscosity, would be expected to have an inhibitory effect on lactose crystallization. This is one of the important contributions of the polysaccharide stabilizers to ice cream quality (Marshall *et al.*, 2003). Some flavours, especially those with nuts, seem to be associated with a higher incidence of lactose crystallization (Nickerson, 1954, 1962; Marshall *et al.*, 2003). Explanations may be the inclusion of fine particles that act as nucleation sites for lactose or localized differences in water concentration as nuts absorb water during storage. The most important storage parameter is temperature. Livney *et al.* (1995) showed that the induction time for lactose crystallization was reduced to a minimum (highest propensity for sandiness) as the temperature was lowered from –5° to –12°C but the induction time increased again at temperatures lower than –12°C. Increasing supersaturation increased the driving force as temperature was reduced but at –12°C this was offset by increasing viscosity. Crystal growth rate also followed a similar trend. Nickerson (1962) and Livney *et al.* (1995) also showed the increasing effect of temperature fluctuation on lactose crystallization which is completely inhibited as the unfrozen phase approaches or enters into the amorphous solid (glassy) state, at approximately –25 to –30°C.

III.5. Development of Lactose-Reduced Products

The consumption of lactose can be problematic for many people, due to lactose malabsorption or intolerance (see Chapter 6). Consequently, there is a significant market for reduced-lactose ice creams and frozen dairy desserts. The two approaches are enzymatic hydrolysis of lactose, either in the mix itself or from the use of lactose-hydrolyzed ingredients, and the selection and blending of milk fat and MSNF ingredients to reduce the level of lactose.

Hydrolysis of lactose either in the mix or in MSNF ingredients for use in ice cream has been studied by several researchers (Guy, 1980; Young *et al.*,

1982; Huse *et al.*, 1984; El-Neshawy *et al.*, 1988; Lindamood *et al.*, 1989; Morr and Barrantes, 1998; Matak *et al.*, 2003). Lactose hydrolysis makes the products digestible for those who are lactose-intolerant and also is a strategy to reduce the potential for the development of lactose crystallization (sandiness); however, hydrolyzed lactose causes twice the freezing point depression of an equal concentration of lactose, so that softness at storage or retailing temperatures and greater rates of ice recrystallization become the issues to overcome if hydrolysis is to be considered. Levels of lactose hydrolysis can be controlled easily from 25 to 100% based on source and activity of the β-galactosidase preparation, concentration, time and temperature of treatment (Lindamood *et al.*, 1989; Matak *et al.*, 2003). The sweetness of lactose-hydrolyzed ice cream is increased compared to its unhydrolyzed control (El-Neshawy *et al.*, 1988; Lindamood *et al.*, 1989), and this fact allows for some reduction in sucrose or blending of alternative sweeteners, such as sugar alcohols, to obtain the optimal formulation for sweetness and freezing point depression.

It is possible to formulate a lactose-free (or reduced) or a sugar-free product by selection of anhydrous milk fat or butter as the dairy fat source, or vegetable fats as the non-dairy fat source, together with high-concentration milk protein concentrates, either whole milk proteins or caseinates, as the MSNF source (Parsons *et al.*, 1985; Lee and White, 1991; Geilman and Schmidt, 1992; Rossi *et al.*, 1999; Alvarez *et al.*, 2005; Whelan *et al.*, 2008). With such a formulation, the presence of lactose in the product can be avoided. For lactose-free (or reduced) formulae, cream should not be used to supply fat and milk should not be used to supply water for formulation balancing, as these also contain lactose. The milk protein concentrate or caseinate blend must supply all of the desired functionalities of the proteins, including emulsification of the fat, aeration of the foam and water-holding capacity in the unfrozen phase.

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IV. Significance of Lactose in Milk Powders

P.M. Kelly

The expression "milk powders" is generally understood to embrace common commodity-traded dairy products such as skim milk powder, whole milk powder and whey powder, as well as an ever-increasing range of dried ingredients derived from milk, such as demineralized whey powders, delactosed whey powders, whey protein concentrate (WPC), milk protein concentrates (MPC) and permeate powders. In addition, fat-filled variations of many of these ingredients are produced. Lactose in isolated form is harvested by crystallization from either whey or permeate and dried after washing into forms suitable for food use (edible-grade lactose) or further purified for pharmaceutical applications as a drug-carrying excipient during tablet or capsule production (see Chapter 4).

Lactose occurs in an amorphous state in skim and whole milk powders – the rapid rate of concentration and spray drying does not allow sufficient time for lactose to crystallize. Hence, it is "trapped" in a relatively unstable glassy state which for the most part does not affect the free-flowing nature of spray-dried milk products provided that appropriate environmental and packing conditions are adhered to. Otherwise, because of the hygroscopic nature of lactose in the amorphous state, such powders will absorb moisture and set in train a series of physical changes that will ultimately impair product quality. Occasionally, problems may arise at an earlier stage during drying when "stickiness" is evident as powder particles adhere to the side walls of the drying chamber. Generally, the manufacture of whole and skim milk powders is not a problem in terms of significant powder deposit formation for lactose concentrations in the range 35–48%, w/w. However, the extensive range of spray-dried dairy ingredients now being manufactured from milk frequently

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includes higher levels of lactose and, possibly, other carbohydrates which may present greater challenges during processing.

IV.1. Milk Protein Standardization

The adoption in 1999 of a new Codex Standard for Milk Powders and Cream Powder, *CODEX STAN 207-1999,* provides, if desired, for the protein content of milk intended for powder production to be standardized to a minimum prescribed limit. In practical terms, this allows lactose to be added to the manufacturing milk as a means of adjusting the protein in non-fat solids to not less than 34%. The ingredients permitted for this purpose are restricted to milk permeate (i.e., permeate produced by ultrafiltration of milk), lactose or mixtures of both.

The figure of 34% was reached after the International Dairy Federation (IDF) conducted surveys of protein and solids-non-fat (SNF) levels of milks in individual countries (Higgins *et al.*, 1995). With some countries experiencing variations in protein as a percentage of SNF in the range 34–42%, *CODEX STAN 207-1999* avoids instances where milk powders with a low level of protein would not be recognized in international trade. On the other hand, considerable quantities of lactose are required by those countries which have higher milk protein values and wish to pursue protein standardization. Thus, in virtually all instances, downward adjustment of the protein content is required for standardization according to *CODEX STAN 207-1999* using permitted ingredients (i.e., milk permeate and/or lactose), in order to produce skim milk powder with 34% protein.

The consequences of milk protein standardization on milk quality were judged to be minimal by IDF experts at the time of submission to Codex. It was felt that the practice of standardizing the fat content of milk had already been in place in the early 1900s without any negative consequence. However, it is to be expected that over time owing to the diversity of functional applications encountered in the market for milk powders, it is certain to encounter some subtle change in functionality as a result of protein standardization.

IV.2. Behaviour of Lactose During Spray Drying

Early success in the mid-1900s with the introduction of spray drying as a mainstream unit process in dairy plants for skim and whole milk powder production saw this technology being extended later to drying whey. However, greater stickiness encountered with whey drying was attributed to the

impact of higher lactose levels. Pallansch (1972) established a direct relationship between the temperature of sticking and the degree of crystallization of lactose, so that by achieving up to 80% crystallization, the temperature in the spray drying chamber can now operate at normal levels without causing problems. In a further development, the acid content of whey was also found to have a negative effect on sticking temperature, which presents additional challenges when drying whey obtained from cheeses drained at a low pH (Cottage, Quarg, etc.) and acid casein production.

Today, whey powder is usually offered on the market in either standard or non-hygroscopic specifications. Extensive lactose pre-crystallization steps are implemented during the final stages of whey concentration and after subsequent discharge from the evaporator when non-hygroscopic powder is intended. The heat of lactose crystallization during whey powder manufacture is considerable, 10.63 kcal/kg, and needs to be factored into the cooling calculations during the design of crystallization tanks (Písecký, 1997). Additional post-drying crystallization may also be factored into the process, especially when handling acid whey. In this case, powder is removed from the drying chamber onto a drying belt in order to allow time for further crystallization to occur (Knipschildt, 1986).

IV.2.1. Hygroscopicity

Since the rapidity of the spray drying process limits the opportunity for lactose to crystallize as its concentration is increased, the resulting amorphous lactose in powders is hygroscopic and will attract water. A sharp increase in moisture content is observed in amorphous milk powders during storage at a relative humidity (RH) >50% (Thomas *et al*., 2004). This is, by and large, manageable during the manufacture and subsequent storage of skim and whole milk powders. In the case of whey powders, in which lactose can be as much as 70% of the dry matter, hygroscopicity manifests itself immediately during spray drying by excessive levels of stickiness of powder adhering to the chamber walls. Successful remedial measures that may be incorporated into the manufacturing process include pre-crystallization of lactose in whey concentrates before drying and post-crystallization of the spray-dried powder as it emerges from the drying chamber (Roetman, 1979). Powder hygroscopicity and caking are, thus, brought under control by lowering the level of amorphous lactose. Up to 50–75% crystallinity (i.e., extent of lactose crystallization) may be achieved in whey powder by pre-crystallization, and this may be increased close to 95% by combining pre- and post-crystallization treatments. This technological approach has enabled "non-hygroscopic" whey powders to be marketed commercially. There are

suggestions that powders may be labelled as "non-caking" at >75% crystallinity; however, there is no guarantee that lactose and milk powders that are free from amorphous lactose will not cake.

IV.2.2. Lactose Crystallization in Milk Powders

The behaviour of lactose in milk powders has important consequences for other physical and functional properties. First, powder composition and storage conditions influence crystallization changes. Secondly, it is now widely accepted that the surface composition and morphology of powder particles, more than their internal microstructure, are major factors which dictate inter-particle interaction and behaviour. Thirdly, manipulation of surface properties during microencapsulation and spray drying is desirable when attempting to protect sensitive ingredients during subsequent storage and delivery.

Because of their hydration behaviour, the presence of milk proteins in powders tends to delay lactose crystallization due to competition for available water. Crystallization in pure lactose and milk powders starts at 40% and 50% RH, respectively (Thomas *et al.*, 2004). In the case of whole milk powder, lactose crystallization does not occur until ≥66.2% RH, due to the role of milk fat which is believed to act as a hydrophobic barrier and limits the diffusion of hydrophilic molecules and the growth of lactose crystals.

The migration of internal fat to the surface of particles during the storage of milk powders is believed to be facilitated when lactose crystallization creates an internal network of capillary interstices. Morphological changes, such as surface deformation, also occur due to the build-up of lactose crystals (Thomas *et al.*, 2004). The fat content of powder has a positive influence on the surface fat coverage of powder particles, with the most dramatic effect being evident for powders in the 0–5% fat range (skim milk powder category), giving rise to a surface fat of 0–35 % (Nijdam and Langrish, 2006). Nijdam and Langrish (2006) also considered that protein along with fat preferentially migrate to the surface of particles during drying on a laboratory scale dryer at an air inlet temperature of 120°C. The migration of lactose increased when the experimental drying was conducted at an industrially realistic air inlet temperature of 200°C. However, the release of water during crystallization is likely to increase viscous flow on particle surfaces, induce lactose migration and further crystallization to the point that particle bridging and agglomeration occurs. Deformation of the surface of particles is related to uneven shrinkage of atomized droplets during the early stages of spray drying. The surface properties of high-protein powders prepared from skim milk are known to be dependent on

lactose content – the higher the lactose content, the more wrinkled the surface (Mistry *et al.*, 1992). Rosenberg and Young (1993) found that the structure of whey protein isolate (WPI)-based spray-dried microcapsules differed from that of other milk-derived powders. Deformation was evident in WPC-based particles, but not when WPI was used, which suggests that lactose (present only in the former ingredient) was also a contributory factor. The physical effects appear to be due to the solidification of particle wall solids before completion of expansion during droplet/particle dehydration. Higher drying temperatures eliminated the tendency towards the formation of shallow deformations. Rosenberg and Young (1993) believe that there is a critical viscosity determining the tendency for dents to occur, below which surface tension-driven effects are sufficient to smooth out morphological irregularities. Hence, high concentrations of whey proteins and fat in product formulations play roles in limiting surface folds.

Lactose glass is recognized as the main encapsulant of milk fat in whole milk powder (WMP) and spray-dried dairy-like emulsions made with WPC and WPI (Buma 1971; Young *et al.*, 1993a,b). Lactose is proving to be useful in microencapsulation studies aimed at optimization of ingredient formulation and process technology (homogenization; spray drying) to yield spray-dried powder particles with defined characteristics in terms of active ingredient (e.g. sensitive oil/fat) protection (Kelly, 2007). The selection of "wall" materials and the manner in which they solidify during powder particle dehydration is key to determining the amount of free fat formed, especially in the case of high-fat powders. The porosity of such "wall" materials may be critical when protecting sensitive materials such as fish oils (Keogh *et al.*, 2001). While protein is the principal emulsifying agent at the concentrate preparation stage, the presence of carbohydrate is additionally important during the subsequent spray drying step of whey protein-based emulsions. Lactose in its amorphous state acts as a hydrophilic filler or sealant that significantly limits diffusion of solvent through the wall towards the microencapsulated anhydrous milk fat droplets (Young *et al.*, 1993a). Crystalline lactose, on the other hand, reduces microencapsulation efficiency by facilitating better diffusion of solvent.

IV.3. Microstructure of Milk Powders

The surface of skim and whole milk powder particles is typically characterized by an irregular shape, shrunken appearance and shrivelled-looking surface. Puffed-looking powder particles may also occur if the powder has been exposed to unfavourable temperatures during drying. Such occurrences cause entrapped air to expand when heated and lead to rupture of the surface. When

the lactose content of milk was depleted, e.g., use of ultrafiltration to produce MPC, the surface of the resulting powder particles after spray drying was smoother than skim milk powder particles (Mistry, 2002). Hence, lactose would seem to be a major determinant of the surface morphology of spray-dried particles in milk powders, and as a result has consequences for shelf-life properties at or above ambient temperature. However, a very low air inlet temperature (120–125°C) to the spray dryer was used during that particular study.

IV.4. Phase Transitions During Milk Drying

After years of technological innovation, food material science is now being applied increasingly to improve our understanding of what happens during spray drying. The material in this instance is dominated by the behaviour of the amorphous glass structure of rapidly solidified lactose (β:α ratio of 1.2–1.4) during the conversion of milk concentrate to powder. This amorphous lactose coats the protein and fat globules in spray-dried milk powder particles. Thus, by combining material phase transitions with physicochemical changes, a more effective approach to optimizing concentration and spray drying is envisaged. The phase diagram of milk may now be augmented by tracking changes in glass transition (T_g). A graphical representation by Roos (2002) of hypothetical powder particle temperature during spray drying on a plot of T_g vs. water content provides guidance on the identification of appropriate conditions for optimal operation, e.g., use of integrated static beds within the primary drying chamber and promotion of agglomeration. Vuatez (2002) studied sorption isotherms in conjunction with glass transition as a function of concentration for both whole milk and skim milk, and proposed a universal relationship between glass transition temperature and water activity. The same author established differences in phase transitions during lactose crystallization in whole milk and skim milk. With whole milk, phase transitions were evident during nucleation of (i) α-hydrate lactose crystals in a supersaturated milk concentrate and (ii) β-anhydrous crystals in a rubbery glassy powder, while in the case of skim milk nucleation of a mixture of anhydrous β- and α-lactose occurred.

The physical state of skim powders is effectively determined by lactose; Jouppila and Roos (1991) showed that the T_g of such powders was almost equal to that of pure lactose (see Chapter 2). Stickiness is also recognized as a surface phenomenon that is governed by the T_g behaviour of lactose. Hence, current research aims to produce "sticky curves" in conjunction with T–T_g plots in order to identify sticky and non-sticky conditions.

IV.4.1. Glass Transition

The glass transition represents a change between the solid- and liquid-like states of an amorphous phase, and hence is useful to describe the physical changes taking place in lactose and lactose-containing powders, especially as the non-equilibrium nature of their amorphous lactose is time dependent. The glass transition temperature (T_g) of lactose is identified by a characteristic change in heat capacity (ΔCp) at the interface between solid- and liquid-like states as measured by differential scanning calorimetry (DSC) when such powders are exposed to a range of storage temperatures under defined humidity conditions. For example, at room temperature, the T_g of amorphous lactose occurs at a water content of 6.8%, expressed on a lactose basis, which corresponds to an equilibrium relative humidity of 37% and a_w of 0.37 (Roos, 2002).

Depending on the lactose concentration, the T_g of milk powders has been found to be very similar to that of pure lactose (Jouppila and Roos, 1994). During spray drying, the glass transition of spray-atomized powder increases as the water content is reduced towards the end of the drying operation, and in reality, particle temperature rises in the advanced stage of dehydration and will approach that of the dryer outlet air if the residence time permits. Hence, two- and three-stage spray drying interrupt this process by separating the slower stage of dehydration from that which occurs in the primary drying zone (spray dryer chamber) and facilitates the secondary drying of particles at a slower pace and lower temperature in either a static integrated bed or external fluidized bed. High temperatures or residual water contents at the later stages of drying may cause stickiness, caking, browning and adhesion of powder particles to the drying chamber (Roos, 2002). Stickiness and caking tend to occur at approximately 10°C above T_g as measured by DSC (Roos, 2002). The effect of water content on T_g may be predicted using the Gordon–Taylor equation:

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2},$$

where T_g is the glass transition temperature of a mixture of solids with a weight fraction of w_1, anhydrous T_{g1}, and water with a weight fraction of w_2 and glass transition temperature of T_{g2} for pure water (frequently assumed to be –135°C) and k is a constant.

When T_g data are combined with that of water sorption properties, insights into the extent of water plasticization of milk powders are generated for various storage conditions (Roos, 2002). Such information may be used to

differentiate between the critical water content of isolated lactose and skim milk solids of 6.8 and 7.6 g/100 g solids, respectively.

The effect of non-lactose solids on the T_g of powder may be predicted using the extended Couchman–Karasz equation, i.e., more than three constituents (water, amorphous lactose, glucose and galactose, casein and whey proteins) are considered (Schuck, 2005). The Couchman–Karasz equation for tertiary mixtures (casein, carbohydrates and water) takes the form

$$T_g = \frac{W_1 \Delta Cp_1 T_{g1} + W_2 \Delta Cp_2 T_{g2} + W_3 \Delta Cp_3 T_{g3}}{W_1 \Delta Cp_1 + W_2 \Delta Cp_2 + W_3 \Delta Cp_3},$$

where T_{gi} is the glass transition temperature (K) of component i, ΔCp_i is the change in heat capacity (J/kg/°C) of this component at T_{gi} and W_i is its weight fraction.

IV.4.2. Caking

Caking is a problem that arises when a low-moisture, free-flowing powder first becomes lumpy, then agglomerates into a solid and finally transforms into a sticky mass. These physical and microstructural changes take place during caking of skim milk powder while equilibrated at 43–94% RH (Aguilera *et al.*, 1995). Initial changes are evident when powder reaches >54% RH while at ≥74% RH, lactose crystallization as well as bridging between particles occurs. The caking of milk powder is preceded by viscous flow on the surface of particles as amorphous lactose becomes sticky on exposure to humid air. Viscous flow was measured as an increase in density of lactose plugs within a cylindrical compaction apparatus after incubation under defined temperature/time conditions (Lloyd *et al.*, 1996). The onset temperature of viscous flow decreased with increasing a_w and corresponded to the onset temperature of T_g (Lloyd *et al.*, 1996). Viscous flow at $T > T_g$ increases the potential for caking to occur. It is speculated that other forms of lactose (α-lactose monohydrate, anhydrous α-lactose and the compound crystals of β/α-lactose) in addition to electrostatic forces between particles, the presence of mineral salts and proteins, surface wetting followed by water equilibration or cooling and pressure also play significant roles. In the case of fat-containing powders, fat-induced caking becomes a problem when total or surface fat exceeds 41 and 12.6%, respectively (Foster *et al.*, 2005). The caking in this instance is attributed to fat crystallization in the liquid bridges between particles, crystallization being triggered by cooling of the powder.

Methods for quantifying the degree of caking (flowability test, shear and tensile strength measurement, visual observation of lumps, microscopic and image analyses, penetration force test and crushing test) are largely empirical, and not always reproducible. A caking index, developed by Aguilera *et al.* (1995), is defined as the state of the system at any time relative to that of the initial state. Two morphological indicators of the state of the system are the ratio of the porosity of the instant system to that of the initial system $p_{(t)}/p_o$; and the ratio of inter-particle bridge diameter to particle diameter, $D_{bridge}/D_{particle}$. These authors proposed that bridging occurs at the onset of caking as a result of surface deformation and sticking at contact points between particles, without a measurable decrease in system porosity. During this early phase, small inter-particle bridges may disintegrate under even mild shaking. Agglomeration follows and involves an irreversible consolidation of bridges but the high porosity of the particulate system is maintained. The compaction that occurs at an advanced stage of caking is associated with a pronounced loss of system integrity as a result of thickening of inter-particle bridges owing to flow, reduction of inter-particle spaces and deformation of particle clumps under pressure.

Many methods have been proposed for eliminating, minimizing or controlling caking, e.g. (i) controlling storage conditions below a_w 0.57 if no amorphous lactose is present, and below a_w 0.25 if it is present (Bronlund, 1997); (ii) avoiding mixing of particles with different initial humidity and temperature (Fito *et al.*, 1994); (iii) cooling the product immediately to an appropriate temperature, well below T_g before packaging (Bronlund, 1997; Bhandari and Howes, 1999); (iv) minimizing temperature variation in different parts of powder silos or bags during storage (Fito *et al.*, 1994); (v) conditioning of powders by ventilation in silos during storage (Bagster, 1970; Lenniger and Beverloo, 1975); (vi) adding a glidant or flow conditioner to improve powder flow (Lai *et al.*, 1986), high molecular weight carbohydrates (e.g., maltodextrin) in infant formula (Aguilera *et al.*, 1995) to raise the T_g of the samples, and dessicant, e.g., silicon dioxide and sodium silicoaluminate (Lai *et al.*, 1986) to improve dehydration characteristics and to reduce stickiness (Chuy and Labuza, 1994).

Technological options (i)–(iv) above target the stabilization of amorphous lactose during storage. These are common techniques to delay caking but they do not prevent it. The availability of accurate data on free moisture, water of crystallization and amorphous lactose in lactose powders may assist in selecting the suitable parameters for processing and storage conditions for controlling caking. Method (v) is intended to eliminate any amorphous material and to allow sufficient time for the removal of bound moisture in bulk powder. The final attempt (vi) is desirable to reduce caking and to improve the physical properties of milk and lactose powders.

IV.4.3. Stickiness and Caking of Milk Powders

Adhesion of powder particles to dryer chamber walls during processing is generally referred to as the stickiness behaviour of a product. Excess stickiness gives rise to product loss, fouling of process surfaces, risk of compromising product quality because of increased residence of deposits which may be dislodged and re-entrained with product flow and a risk of spontaneous combustion within the drying chamber (Ozmen and Langrish, 2002).

Stickiness of powders arises from plasticization of particle surfaces. Adsorbed water in contact with the surface of powder particles has a plasticizing effect by lowering T_g and also reducing viscosity. Direct methods for measuring stickiness are based on the use of physical indices, such as resistance to shear, viscosity and optical properties (Schuck *et al.*, 2006). T_g can be correlated indirectly with stickiness. In fact, T_g measured by DSC is virtually the same as the sticky point temperature measured using a thermo-mechanical test (Ozmen and Langrish, 2002). Using a blow tester-based method, Patterson *et al.* (2005) used the parameter (T–T_g) to characterize the rate of stickiness development for a range of conditions (37–67°C and 0.15–0.35 a_w), and found that at a given T–T_g value, the level of stickiness increased linearly with time. Patterson *et al.* (2007) also showed that stickiness curves for powders run above and parallel those for the T_g of amorphous lactose, and succeeded in establishing a critical point $(T–T_g)_{crit}$ to characterize the initiation of powder stickiness.

Caking follows when inter-particle liquid bridges provide an environment for dissolution of milk components and the resulting lactose crystallization transforms these interfaces into solid bridges (Thomas *et al.*, 2004). Sticking is typically a problem encountered during drying, while caking is more prevalent during the subsequent storage of powder (Schuck *et al.*, 2005). Stickiness and caking of whole and skim milk powders are different due to the significant difference in their surface compositions (Özkan *et al.*, 2002). The high-fat surface coverage and its melting behaviour was associated with stickiness occurring at a lower temperature in whole milk powders, while the higher temperature at which cakiness occurred in skim milk powder was influenced by $T>T_g$ so that the rubber phase transition to the crystalline phase facilitated the formation of strong junctions between skim milk powder particles (Özkan *et al.*, 2002).

A close relationship appears to exist between the measured sticky point temperature of skim milk powder and that of lactose (Boonyai *et al.*, 2004). However, the approaches used to correlate the sticky point and T_g are not very precise. First, the sticky point temperature may be taken at 20°C above T_g for general and simple estimations, but this may not be sufficient when spray drying some products at a very high temperature where precise

temperature control is generally required. A shift in temperature of a few degrees may cause stickiness.

A device developed by Hennigs *et al.* (2001) was used to measure the sticky point temperature of SMP. This technique measured electric resistance of a stirrer in contact with powder which was then translated into stickiness behaviour. At the sticky point temperature, the voltage increased sharply and the stirrer consequently stopped. It was found that the sticky point temperature of SMP could be predicted precisely from the T_g of lactose, using suitable curvature parameters. A limitation of the method is the risk of void formation during movement of the powder by the stirrer.

The caking temperature of powders (T_c) may be linked to variations in T_g. Both models share the same curvature index for lactose, emphasizing that lactose is mainly responsible for the stickiness of milk powders. The caking temperature model is distinguished from the glass transition model by inclusion of a coefficient of temperature difference (d) (Hennigs *et al.*, 2001):

$$T_g = \frac{W_L.T_g(l) + k.W_w.T_g(w)}{W_L + k.W_w},$$

$$T_c = \frac{T_g(mp) + k.X.T_g(w)}{1 + k.X} + d,$$

where T_g is the glass transition temperature of lactose; W_L and W_w are the weight fractions of lactose and water; k is the curvature index (=7.4 for pure lactose); $T_g(l)$ is the glass transition temperature of anhydrous lactose; $T_g(w)$ is the glass transition temperature of water; T_c is the caking temperature; T_g(mp) is the glass transition temperature of anhydrous skim milk powder; X is the ratio of water to dry powder; and d is a coefficient (=23.3 K for skim milk powder).

Schuck *et al.* (2005) described a sticking and caking sensitivity index (SCSI), ranging in value from 0 to 10, which they claimed may be used to anticipate powder behaviour during drying and storage – the more favourable situation being an SCSI = 4 (i.e., no sticking and/or no caking), while SCSI $\geq$ 6 suggests that there is a high to very high risk of sticking or caking. Computation of the SCSI takes into consideration powder characteristics such as temperature in the form of T–T_g and changes in heat capacity during glass transition (ΔCp). A points system is allocated to these physical characteristics according to Table IV.1.

Stickiness behaviour is also proving to be an important parameter in the latest efforts to model particle dehydration during spray drying. Simulation of spray drying processes is being used increasingly in order to facilitate more efficient dryer operation and implementation of advanced control systems.

Table IV.1 Calculation of Stickiness and Caking Index (SCSI)* modified from Schuck et al (2005)

$T–T_g$ (°C)	Number of points	ΔCp (J/g/°C)	Number of points
≤ 5	0	< 0.1	0
$> 5; \leq 10$	1	$\geq 0.1; < 0.2$	1
$> 10; \leq 15$	2	$\geq 0.2; < 0.3$	2
$> 15; \leq 20$	3	$\geq 0.3; < 0.4$	3
$> 20; \leq 30$	4	$\geq 0.4; < 0.5$	4
> 30	5	≥ 0.5	5

*SCSI = Sum of the number of points for $[T–T_g]$ + number of points for [ΔCp]. For example, a powder with a $[T–T_g] = 16$°C and ΔCp = 0.25 (J/g/°C) has a SCSI of 5.

A particular challenge occurs when considering the drying profile of a range of particles. One of the difficulties is to be able to predict surface moisture content and temperature, which are important variables for determining whether the T_g of surface lactose has been exceeded and what are the prospects of the particles becoming sticky (Nijdam and Langrish, 2006).

IV.4.4. Instantization/Agglomeration

Regular spray-dried milk powders do not disperse readily or completely when poured onto water.

In order to improve reconstitution properties, agglomeration was developed as a process whereby larger powder particles (more likely as particle clusters) are created, largely by the adhesion of smaller to the larger particles – the smaller particles limit the instant dispersion of powders in water. The resulting more open powder structure allows greater penetration of water in the course of subsequent wetting and dispersing.

The key to agglomeration in practice is to create an environment for adequate mixing of "wet", sticky partially dried powder particles with recycled dried fines (i.e., fine powder particles collected by cyclonic separation), so that the smaller particles adhere to the larger ones and form clusters around them. The surface properties of partially dried powder in the wet zone of the spray drying chamber influence the nature of particle bridges that may be formed. If the starting material is a dried powder, then partially rewetting and heating in excess of T_g will provide a viscous surface for adjacent particles to adhere to and form strong bridges (Bhandari and Howes, 1999). There is a dependence on the presence of fat and amorphous lactose to provide the necessary stickiness to facilitate agglomeration. Nijdam and Langrish (2006) expressed a concern that inadequate amounts of lactose may be present because of competitive displacement by protein. However, since most

agglomeration now takes place in the primary drying chamber (*straight-through drying/agglomeration process),* it is likely that most agglomerating particles in this zone of the dryer are wet and have a surface composition more typical of the matrix before migration occurs during the final stages of particle dehydration.

Later, surface properties will also prevail during reconstitution along with other factors, such as the interplay between liquid (water), gaseous (air between particles) and solid phases (powder particles) during powder wetting. Efficient wettability and dispersibility are essential in order to prevent a viscous layer forming at the interface around grouped particles which delays dissolution of the powder in water.

Powder agglomeration may be regarded as intentional caking as a result of forced compaction under controlled conditions – particles being forced to interact and form granules (Listiohadi *et al*., 2005). Some insights into the effects of force during the recycling of fines when spray drying are gleaned from the descriptions of Písecký (1997). When the fines return is positioned close to the spray atomizer, considerable penetration of wet primary particles occurs, which in turn, become covered by concentrate from the incoming spray. These newly forming agglomerates possess high moisture, plasticity and stickiness. On the other hand, if the fines return is positioned at a distance from the atomizer, less compact agglomerates, displaying "raspberry- like" and "grape-like" microstructures, are formed. The objective is to strive as far as possible to achieve a "compact grape" structure as an optimal process condition where the powder has simultaneously good instant properties and sufficient mechanical strength to withstand the rigours of subsequent handling and packaging (Table IV.2). "Onion-structured" agglomerates have also been described. These have high mechanical strength, bulk density and appear as slowly dispersible particles on reconstitution.

Two distinct processes for agglomerating milk powders are used in practice (Wulff, 1980), the straight-through method (accomplished during spray drying) and the rewet method (using powder that has already been prepared). Both processes exploit similar principles of instantization – (i) wetting of particle surfaces (by steam, water or a mixture in the case of the rewet method), (ii) agglomerating, (iii) drying or redrying (rewet), (iv) cooling and (v) classifying according to particle size in order to remove particles that are too large or too small.

IV.4.5. Maillard Reactions

Non-enzymatic browning of milk powders during prolonged storage has been associated with reactions taking place between proteins and lactose functioning as a reducing sugar (Thomas *et al*., 2004; see Chapter 7). Early

Table IV.2. Influence of spray atomization/fines return-mediated agglomerate structures on the physical properties of agglomerated particles (adapted from Písecký, 1997)

	Agglomerate structure			
Physical property	"Onion-"	"Raspberry-"	"Compact grape-*"	"Loose grape-*"
Particle moisture at collision	High		Low	
Mechanical stability	High		Low	
Bulk density (no attrition)	High		Low	
Bulk density (after attrition)	High	Low	High	
Slowly dispersible particles	Many		Few	
Dispersibility (after attrition)	Poor	Good	Poor	

*Optimum agglomerate structure

Maillard reactions (EMR) develop more readily in WPCs with a high (WPC-35 to WPC-50) than with a low lactose content (Morgan 2005). A comparison of skim milk powder and WPC with similar protein and lactose contents (35% and 51%, respectively) showed that the development of EMR was similar for both, except at prolonged heating times where a break in the progress of the amino-sugar reaction was observed for SMP (Morgan, 2005). These studies were undertaken under accelerated storage conditions at 60°C and it is believed that differences in molecular mobility between WPC and SMP may occur at this temperature.

IV.5. Some Ingredient Applications Where the Role of Lactose Is Emphasized

IV.5.1. Infant Milk Formula

As the lactose content of human milk is significantly higher than that of bovine milk, infant milk formula manufacturers use edible-grade lactose and whey powders as ingredients during processing in order to "humanize" their product formulations. Lactose loading of formulations in this instance is driven by the necessity of nutritional demand, and in processing terms is facilitated by sourcing demineralized and partially demineralized whey powders (containing typically 70%, w/w, lactose). Such dairy ingredients provide,

in addition to lactose, minerals and other desired key minor constituents. However, a greater challenge is faced when drying powders of such high-lactose content.

IV.5.2. Chocolate

Lactose may be generally regarded as having a secondary role in milk chocolate, its presence being largely opportunistic by virtue of being a constituent of the milk powder used. However, attempts at explaining variations in chocolate properties, especially viscosity, have led to greater scrutiny of the functional quality of such dairy ingredients. When Aguilar and Ziegler (1995) partially substituted sucrose in milk chocolate by lactose provided by whole milk powder, the physical state of the lactose had important effects on chocolate properties – higher concentrations of amorphous lactose reduced chocolate viscosity, increased particle size of chocolate mass post-refining and reduced the requirement for surface-active agents to achieve the desired Casson yield value, while increasing the content of crystalline lactose had the opposite effect.

Spray-dried whole milk powder (WMP), with its relatively low level of free fat, is sub-optimal as a dairy ingredient for use in chocolate production compared to roller-dried WMP. However, adapting our knowledge of lactose behaviour may now be the basis of a technological approach to circumventing this problem. Baechler *et al.* (2005) showed that the phase diagram of whole milk powder may be exploited to achieve greater functionality of WMP for use in chocolate. They demonstrated that careful control of the water activity of WMP before heating at 90°C for 70 min could bring about a desired release of free fat (>70% of total fat), while maintaining WMP in powder form and avoiding the induction of browning. This release of free fat onto the surface of WMP was correlated with increased lactose crystallization in the β-form.

IV.6. Role of Lactose in Microencapsulation

Microencapsulation involves the optimization of ingredient formulation and process technology (homogenization, spray drying) to produce stable emulsions that may be spray dried to yield powder particles with defined characteristics. The protection of an active ingredient (e.g., oils/fats that are particularly susceptible to oxidation) either during processing, delivery or subsequent storage is usually the objective. The process differs from conventional whole milk powder production in that emulsion composition and formation are finely tuned to take advantage of structural changes that

occur in powder particles during spray drying. The selection of "wall" materials and the manner in which they solidify during powder particle dehydration is key to determining the amount of free fat formed, especially in the case of high-fat powders. The porosity of such "wall" materials may be critical when protecting sensitive materials, such as fish oils (Keogh *et al*., 2001).

While protein is the principal emulsifying agent at the concentrate preparation stage, the presence of carbohydrate is additionally important during the subsequent spray drying of whey protein-based emulsions. Lactose in its amorphous state acts as a hydrophilic filler or sealant that significantly limits the diffusion of solvent through the wall towards the microencapsulated AMF droplets (Young *et al*., 1993a). Crystalline lactose, on the other hand, reduces microencapsulation efficiency by facilitating greater solvent diffusion. Thus, microencapsulation efficiency is increased first by emulsifying anhydrous milk fat in a solution prepared from WPI and carbohydrate, and homogenizing the resulting emulsions at 50 MPa in four successive passes before spray drying (Young *et al*., 1993b). Increasing the number of homogenization passes also helps counterbalance the negative effect of salts when WPC-35 is used instead of WPI as the principal emulsifying agent (Keogh and O Kennedy, 1999).

The presence of lactose is also important in obtaining complete encapsulation of fat after spray drying sodium caseinate-stabilized soybean oil emulsions (Fäldt and Bergenståhl, 1995). The normally hydrated protein-based interfacial surface film in an emulsion is believed to shrink because of the loss of water during drying (Fäldt & Bergenståhl, 1995). However, the presence of lactose may replace water to some extent and keep the protein solubilized after drying to reduce shrinkage. Thus, the stability of a sodium caseinate film on powder surfaces is increased, and less fat leaks out onto the powder surface during the drying process (Fäldt and Bergenståhl, 1995).

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V. Reduced Lactose and Lactose-Free Dairy Products

Shakeel-Ur-Rehman

V.1. Introduction

The malabsorption of lactose by lactose-intolerant individuals, which has been recognized since the 1950s (Holsinger and Kligerman, 1991), resulted in the development of lactose-reduced or lactose-free milks, dairy products and dairy beverages. The availability of lactose-reduced or lactose-free milks offers an opportunity for the dairy industry to gain new consumers who would otherwise avoid milk. Two approaches are commercially used to manufacture lactose-free or lactose-reduced milks and dairy products, i.e., treatment with β-D-galactosidase and membrane processing. In dairy products like yoghurt and cheese, some of the lactose is converted to lactic acid by bacterial action and in cheese most of the lactose ($\sim$98%) is lost in the whey. Yoghurt and cheese, therefore, are naturally lactose-modified or reduced-lactose dairy products. The β-D-galactosidase produced by some lactic acid bacteria during the fermentation of lactose helps in lactose absorption in the human intestine (Flatz, 1987).

V.2. Methods for the Manufacture of Lactose-Free or Lactose-Reduced Milk Products

Three methods are used to reduce/modify or eliminate lactose in milk and milk products.

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V.2.1. Treatment of Milk with β-Galactosidase

Lactose-reduced milks and milk products are made by treatment of cows' milk with lactase (β-D-galactosidase), a process which began with the commercial availability of this enzyme from microbial sources in early 1970s. Treatment of milk and milk products with β-D-galactosidase to reduce their lactose content is an appropriate method for increasing their potential uses and to deal with the problems of lactose insolubility and lack of sweetness (Mahoney, 1997). Moreover, treatment of milk with β-D-galactosidase makes it a suitable food for a large number of adults and children who are intolerant to lactose. The hydrolysis of lactose by β-D-galactosidase into its constituent monosaccharides, D-glucose and D-galactose, allows most consumers who are lactose-intolerant to digest comfortably products that contain lactose. The constituent monosaccharides of lactose are more soluble than lactose and hence lactose hydrolysis improves the mouthfeel of fluid milks. This is especially important in fluid milks where most of the lactose is removed by filtration technology and the residual lactose is converted by β-D-galactosidase to glucose and galactose. Lactose solubility is a problem in concentrated fresh milk products manufactured by reverse osmosis (RO), especially when the lactose content is more than 12%. The use of β-D-galactosidase pills started with individual customers adding the enzyme to a glass of milk; however, with improvements in processing techniques, hydrolyzing the lactose before packaging certain dairy products has become more common. Three techniques are used commercially to perform lactose hydrolysis, single-use batch system, recovery systems (enzyme re-use) and immobilized enzymes (Mahoney, 1997). The factors that govern the hydrolysis of lactose by β-D-galactosidase are the amount of lactose in the product, the temperature at which the product is held and the time of contact.

V.2.1.1. Reduced-Lactose or Lactose-Free Fluid Milks

A range of reduced-lactose and lactose-free milks is available on US market; the three most popular lactose-reduced or lactose-free products on the US market are Lactaid, Dairy Ease and Mootopia. Lactose-free milk brands available in Europe include those of Hyla (Valio, Finland, Sweden, Belgium), Emmi (Switzerland), Kaiku plus sin lactosa (Spain) and Lactofree (Arla, United Kingdom). The reduced-lactose or lactose-free milks manufactured by treatment with β-D-galactosidase have a lower freezing point and are far sweeter than normal milk. The treatment of fluid milks with β-D-galactosidase offers certain specific challenges to processors. The average lactose content in cows' milk is 4.8% and its conversion to glucose

and galactose by β-D-galactosidase results in milks that are too sweet and often disliked by consumers. The excessive sweetness of β-D-galactosidase-treated milks has caused some lactose-intolerant subjects to change to soy or oat "milks". The treatment of full lactose milk with β-D-galactosidase increases the chances of Maillard browning, especially in UHT milks (O' Brien, 1997). The monosaccharides formed from lactose react faster than lactose with amino acids, resulting in extensive browning; the extent of the change will depend on the percent hydrolysis. The β-D-galactosidase treatment of fluid milks increases the cryoscopic value of milk from 0.454 to 0.650°C making it difficult to assess the adulteration of milk with water by cryoscopic methods. Some of the commercially available preparations of β-D-galactosidase contain some proteinase activity which is very heat stable and may not be inactivated by pasteurization or even ultra-high temperature sterilization of milk, resulting in poor shelf life of the lactose-free milk. The β-D-galactosidase treatment increases the cost of fluid milk by ~ $0.06–$0.08/L.

V.2.1.2. Lactose-Free Ice Creams

Lactose makes up approximately 20% of the carbohydrates in ice cream (Marshall and Arbuckle, 1996). The percentage of lactose in ice cream depends on the formulation, including the amount of non-fat milk solids and fat in the mixture. Lactose-hydrolyzed ice cream requires less added sugar to get desirable sweetness as the relative sweetness of lactose is only one-fifth that of sucrose (Fennema, 1996); hydrolysis of 70% of the lactose in milk increases its sweetness by an amount comparable to the addition of approximately 2% sucrose (Zadow, 1986). The monosaccharides produced from lactose depress the freezing point of ice cream mix, increase relative sweetness and promote the ease of dippability of ice cream (Iversen, 1983). The freezing point of ice cream mix is directly proportional to the number of particles in solution (Mitchell, 1989). Lindamood *et al.* (1989) reported that the freezing point of ice cream decreases proportionately to the extent of lactose hydrolysis in the ice cream mix. The freezing point of ice cream mix not treated with β-D-galactosidase (10% milk fat, 12% MSNF, 12% sucrose, 5% corn syrup solids and 0.25% stabilizer–emulsifier blend) was –1.45°C while the freezing point of mixes that had undergone 25, 50 or 100% lactose hydrolysis were –1.62, –1.67 and –1.92°C, respectively (Lindamood *et al.*, 1989). The low freezing point is often responsible for the accelerated melting of ice cream (Marshall and Arbuckle, 1996). The hydrolysis of lactose in ice cream results in a smoother product.

V.2.1.3. Condensed Milks

The defect of sandiness and poor texture in concentrated milks can be overcome by hydrolyzing its lactose. Sabioni *et al.* (1984) reported less sandiness in lactose-hydrolyzed sweetened condensed milk compared to the condensed milk in which lactose was not hydrolyzed.

V.2.2. Reduction of Lactose by Membrane Technology Techniques

Membrane techniques such as microfiltration and ultrafiltration (UF) are used commercially to modify the proportion of lactose in milk and milk products. These processes have resulted in lactose-modified ice creams, milk powders, yoghurts and a series of fluid milks and dairy beverages. The addition of UF retentate of milk changes the physical and chemical properties of all dairy products to which it is added. UF membranes retain all the fat and practically all the protein in milk. The retention coefficients of the non-protein nitrogen compounds are generally 20–40%, and higher for the high-concentration factors (Grandison and Glover, 1997). Urea and amino acids are mainly lost through the membrane. Retention of lactose during ultrafiltration may be up to 10%. The minerals and other ions retained during the membrane processing of milk by ultrafiltration are those that are attached to the proteins, like calcium, magnesium, phosphate and citrate, while others pass into the permeate. Likewise, the fat-soluble and protein-bound vitamins are retained completely. A process in which lactose can be removed completely from the milk is a modification of ultrafiltration, referred as diafiltration. During diafiltration, water is added to the milk or to the ultrafiltration concentrate of milk in order to wash out components able to pass through the membranes. Diafiltration helps to remove more permeate and more small molecules and therefore is a purification process. The combination of ultrafiltration and diafiltration is a technique for manufacturing milk enriched in protein and fat, and very low in lactose and salts. During 3X (the most commonly used concentration factor in industry) ultrafiltration of milk, 66–67% of lactose passes into the permeate and 33–34% is retained in the UF milk retentate. From the initial lactose content of 4.8 g/100 g cows' milk, 3.2 g pass into the permeate during 3X UF and 1.6 g are retained in the UF concentrate. When the UF concentrate is diluted to the level of proteins and fat in the original milk by addition of water, the lactose content in the reconstituted milk is now only 1.6%. The residual lactose is then converted to glucose and galactose by treatment with β-D-galactosidase. The resulting product is 100% lactose free and has a sweetness similar to that of ordinary whole milk. Select Milk Producers in USA

have patented a combination of membrane technology for manufacturing lactose-free milks (Dunker *et al.*, 2007; Shakeel-Ur-Rehman *et al.*, 2007). The product is 100% lactose free, has 75% more protein and 65% less sugar than cows' milk and contains 4 g of hydrolyzed lactose per serving (240 mL). Recently, patented processes (Tossavainen and Sahlsten, 2003) have been developed to reduce the lactose in milk to less than 3% and the residual lactose is hydrolyzed by β-D-galactosidase to glucose and galactose in order to get a sweetness similar to normal milk. These milks are becoming popular in Europe and America. The reduction of lactose by membrane technologies or chromatographic processes has resulted in labeling issues with regulatory authorities. Milk in which lactose is reduced by filtration technology to less than 2.0% results in a lower cryoscopic value than that of ordinary whole milk and, even after lactose hydrolysis, the cryoscopy value is lower than regular cows' milk. The health authorities consider the reduction in cryoscopic values as adulteration in milk. The popularity of foods with a low glycemic value has also necessitated the manufacture of milk products with reduced milk sugar. In Europe, Valio has developed a range of lactose-free and lactose-reduced dairy beverages using membrane technologies. The products, in which the level of lactose in milk is reduced by membrane technologies, may be suitable for diabetic people.

The use of UF concentrates in the manufacture of sweetened condensed milk has been found to avoid sandiness (Sepra Alverez *et al.*, 1979) as the condensed milk is low in lactose.

Delactosed high-protein milk powders have been manufactured by use of ultrafiltration and diafiltration processes (Mistry and Hasan, 1991). The delactosed milk protein powder has potential in the manufacture of new products like low-fat yoghurts.

Milk protein concentrate (MPC) is a relatively new dairy ingredient produced by ultrafiltration of skim milk. The proportion of protein in the MPC depends on the removal of lactose by ultrafiltration/microfiltration and diafiltration. The proportion of caseins and whey proteins in the MPC is similar to that in the original milk but MPC contains little lactose. Due to the high casein content and low lactose content in MPC, it is an attractive ingredient for enriching the casein content of cheese milk. MPC80 contains 6.5% lactose compared to 51% in non-fat dry milk (NFDM); the use of MPC instead of NFDM in cheese milk results in a low residual lactose level in cheese and reduces the chances of undesirable fermentation during ripening (Shakeel-Ur-Rehman *et al.*, 2003). The ratio of protein to lactose is higher in MPC than in condensed milk or NFDM. The use of MPC to standardize milk for Pizza cheese manufacture results in an increased yield and less browning.

V.2.3. Reduction of Lactose in Milk by Chromatographic Methods

Chromatography processes use charged resins to separate proteins and other charged ions in milk from lactose. The proteins and charged ions bind to oppositely charged resin while lactose does not bind and passes directly through the system. Milk from which lactose is to be separated is passed through a column containing cation exchange resin (Harju, 1989). The cation exchange resin is balanced in such a way that an ionic balance is obtained with milk. After balancing the resin, skim milk or concentrated milk is passed through the bed. The lactose fraction is eluted at the bottom of the bed, while the protein and mineral fraction is eluted with the help of water in another stream. The main disadvantages of chromatographic processes are that they are time-consuming and involve expensive equipment.

V.3. Conclusion

Awareness about lactose intolerance has considerably increased in past decade, so has the market for lactose-free milks. Conversion of lactose in ordinary milk by treatment of β-galctosidase results in high levels of sweetness which may be unacceptable to many consumers. Reducing lactose in fluid milks by chromatographic or membrane technology followed by conversion of residual lactose by β-galctosidase to its constituent monosaccharides has helped manufacturers to provide a fluid milk with a genuine taste of milk; however, the products are expensive and there are some concerns about labeling the products as "milk". The chromatographic method of lactose removal is too expensive and has not found widespread use in industry. Although removal of lactose from fluid milks by membrane technology increases the price by about 20–30%, it is still a method of choice for most of the manufacturers to provide a lactose-free milk with a genuine taste of milk. This has helped many consumers to again drink milk who had otherwise stopped drinking milk.

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4

Production and Uses of Lactose

A.H.J. Paterson

4.1. Theoretical Approach to Production

Lactose has been produced industrially for over 100 years (Dryden, 1992). The objectives of the lactose manufacturer were summed up by Herrington (1934): "In the manufacture of lactose, it is desirable to secure a maximum yield of crystals in a minimum time, and to secure crystals which may be readily washed with a minimum of loss." These objectives are still valid for the modern lactose manufacturer and this chapter will examine how these objectives might be met.

The production of lactose from solution generally follows a standard crystallisation process involving concentration, nucleation, growth and harvesting/washing. The overall yield is determined by the conditions used in all four phases of production. Figure 4.1 shows the lactose solubility data in water as gleaned from the literature. The equation for the fitted line in the figure is

$$C_{LS} = 10.9109 \exp^{0.02804T}$$

C_{LS} = concentration of anhydrous lactose (g lactose/100 g water)
T = temperature (°C).

This equation, the same as that given by Butler (1998), can be used to demonstrate how various processing conditions impact on the overall yield of a process.

Whey arriving at a factory needs to be concentrated, usually by reverse osmosis and evaporation, until the lactose concentration is typically around 110 g lactose per 100 g water (58% TS for whey permeates). It is then cooled

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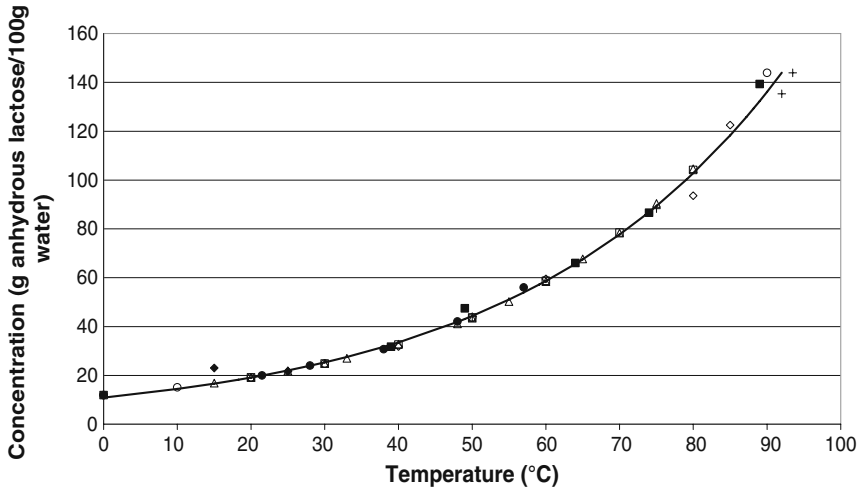

Figure 4.1. Solubility data for lactose from the literature (McLeod, 2007) (♦ Hudson, 1904, ■ 1908; • Saillard, 1919; + Gillis, 1920; ○ Herrington, 1934; □ Rozanov, 1962; ◊ Foremost Foods, 1970; Δ Visser, 1982).

over a period up to 48 h (but times as short as 12 h have been used) to allow first nucleation and then growth of lactose crystals. In many industrial plants, the final temperature will be between 15 and 25°C. If it is assumed that the concentrate is cooled to 20°C, that sufficient time is allowed for the solution to come to equilibrium with the crystals and that all crystals are of sufficient size, that there is 100% recovery through the recovery and washing zone, and ignoring the losses incurred by recycled lactose from the washing stages, then the theoretical yield can be calculated as follows: in concentrate, there are 110 g of lactose per 100 g of water; at 20°C, 100 g of water can contain 19.1 g of lactose in solution, at equilibrium, therefore, 90.9 g lactose will crystallise, giving a theoretical yield of 90.9 g/110 g or 82.6% w/w.

Normal yields will be less than this because of losses due to (i) dissolved lactose and fine crystals that are not recovered during the recovery/washing operations and are lost in the mother liquor and (ii) recycled lactose from the washing operation increasing the water load through the plant.

To increase this yield, operating conditions must be changed; several things can be done to improve the yield.

1. Increase the concentration exiting the evaporators. The limit to this, without crystallisation occurring in the evaporators, is the solubility limit at the final temperature of the evaporators. If the concentration

can be increased to 130 g lactose/100 g water (62% TS for whey permeates), the potential yield would be increased to 85.3%.

2. Cool the crystallisation batch to a lower temperature. If it could be cooled to 5°C, the concentration in the mother liquor at equilibrium would be 12.6 g lactose/100 g water, resulting in a theoretical maximum yield of 88.6% with an evaporator concentration of 110 g lactose/100 g water and yield of 90.3% if the evaporator concentration is 130 g lactose/100 g water.
3. By allowing crystallisation to occur in the evaporator, even higher concentrations could conceivably be achieved and this enables even higher yields to be obtained. If the concentration can reach 200 g lactose/100 g water (71% TS for whey permeates) and that the batch is cooled to 5°C, the yield would be 93.7%.

These examples have not taken into account other practical limitations that might occur, such as calcium deposits in the evaporators or crystalline concentrates too viscous to pump. The actual limits that can be achieved will vary between factories depending on whey purity, plant cleanliness and the specifics of the plant design.

The precipitation of calcium phosphate on the evaporator tubes limits the run time of the evaporators. Steps can be taken to minimise this problem, such as the addition of agents to sequester calcium ions or the use of ion exchange or ion chromatography to remove the calcium phosphate from the whey before it reaches the evaporator.

Actual plant data are commercially sensitive and hence the figures given here are indicative to show how plant yield can be influenced by changing plant conditions.

Actual plant yields are usually considerably below the theoretically possible yields due to inefficiencies in the harvesting and washing stages in the plants. According to APV (2007), a 65% yield is typical. Plants have reported yields as low as 50%, which is completely unacceptable. It should be possible to improve the yield to the mid-60% range by altering the plant operating conditions. Many of the problems can often be traced to the generation of fines within the system which end up passing out in the mother liquor or causing an increased load on the evaporators through recycling which leads to more water going through the process and hence to larger losses. Control of nucleation within the plant is the only way to tackle this problem as it is nucleation that determines the number of crystals in the concentrate and hence the final particle size distribution.

Incorrect cooling procedures can also lead to local nucleation events occurring at the walls within the crystallisers or even to induce a second major

nucleation event, creating a batch with bimodal particle size distribution. These events lead to batches with too much fines, leading to significant losses.

Recycling of lactose through the plant leads to further losses, as every kilogram of lactose that is recycled from the washing stage to the evaporator leads to loss of 6.3–17.4% of the recycled lactose (depending on the conditions under which the plant is run). Hence, any change in the process that leads to increased recycling of lactose within the plant leads to increased losses.

Microbial fermentation of dilute streams also represents an area where losses can occur directly, and indirectly as a result of effects of the by-products on downstream processing. Dryden (1992) reported the production of small flat lactose crystals in several New Zealand plants, resulting in considerable loss of yield due to the removal of fines in the mother liquor. The problem was traced back to whey which had been held too long and which had fermented.

Another area where unwanted fermentation of lactose within the plant can cause problems is that the production of endotoxins and/or the growth of bacteria such as enterobacteria. The carry-over of these into the product due to adsorption is greater when small lactose crystals are produced, as small crystals have a much larger surface area than larger crystals and hence the level of these contaminants is greater. These problems can carry over into the production of pharmaceutical-grade lactose.

4.2. Edible-Grade Lactose

The CODEX definition of lactose is "Lactose: A natural constituent of milk normally obtained from whey with an anhydrous lactose content of not less than 99.0%, w/w, on a dry basis. It may be anhydrous or contain one molecule of water of crystallisation or be a mixture of both forms."

http://www.codexalimentarius.net/download/standards/338/CXS_212e.pdf accessed 29 August 2007.

Some producers give the specifications of their products on their websites, a list of which is given for the reader who wants more detail.

http://www.merricks.com/lactose.htm
http://www.biolac.com/appl/FI/FI129/FI129D02.NSF/O/77ADDC8F940151B7C12571950039D76A
http://www.bmi-eg.com/en_lactose.php
http://lactoseindia.com/spec3.html
http://www.foodnavigator-usa.com/news-by-product/indexbyCpyEntry.asp? id=3347

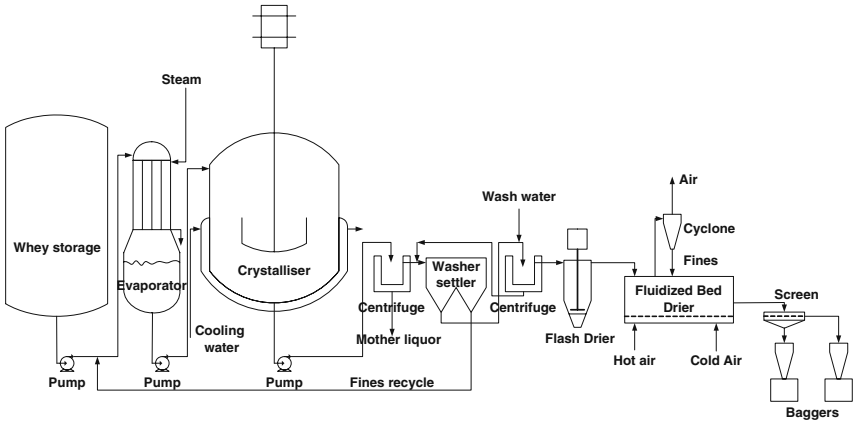

Figure 4.2. Process flow diagram of a plant for the manufacture of edible-grade α-lactose monohydrate.

The process for making edible-grade lactose is relatively straightforward in that the basic procedure is to concentrate whey, and then allow it to cool, so that the lactose crystallises out of the mother liquor. The crystals are then separated from the mother liquor, washed, dried and packed. A description of this process was given by Weisberg (1954). Figure 4.2 is a process flow diagram showing a typical lactose production plant. Most manufactures follow this method, with some variations, depending on the site. Some producers like to include various steps either before or after concentration and different concentration techniques can be used, including reverse osmosis before a final concentration by evaporation. All the processes known to the author include a final evaporation step to achieve the desired solids level.

The critical steps in the process are as follows:

1. Removing as much water as possible in the final stages of evaporation.
2. Transfer of the high-solids concentrate from the evaporator to the crystalliser without uncontrolled nucleation. Where a process uses higher concentrations than it was originally designed for, it may require an appropriate increase in temperature before transfer to the crystalliser to limit supersaturation. The key here is to maintain the same absolute level of α-lactose supersaturation as it enters the crystalliser, thus maintaining similar conditions to those that occurred in the original process at the time of nucleation. This is because the rate of nucleation is a strong function of the absolute level of α-lactose supersaturation as well as the fluid dynamics of the crystalliser.

3. Cooling to as low a temperature as is economically feasible; a lower temperature means lower lactose solubility, allowing a greater mass of lactose to crystallise. Again, there are limits, as the rate of crystal growth is quite slow at these low levels of supersaturation and requires long crystallisation times; this in turn means more capital for larger crystallisers.
4. Cooling at the appropriate rate is critical. Aggressive early cooling may result in too many crystals being formed during nucleation, leading to crystals that are too small for convenient downstream washing and processing. If early cooling is too slow not enough nuclei form, rendering it highly likely that a secondary nucleation event will occur, giving a bimodal distribution with a large tail of fines in the particle distribution. Traditionally, these problems have led to the mystique that lactose crystallisation is an art rather than a science. Research has started to unravel some of this mystique (Kendrew and Moelwyn-Hughes, 1940; Haase and Nickerson, 1966b; van Kreveld 1969; Butler, 1998; Kauter, 2003; McLeod, 2007).
5. Separating the crystals from the mother liquor: This is usually completed with a decanter centrifuge and subsequent washing stages. The washing plant can consist of a series of hydro-cyclones or a series of counter-current mixing and settling separators but other designs have also been used. There is very little information in the literature on the performance of the alternative washing regimes and is an area identified as having potential for further research.
6. Drying of the crystals: The final operation involves separating the washed crystals in a centrifuge from which the lactose cake at 5 and 12% (w/w) free moisture is discharged into a dryer. Today, most lactose plants use a flash dryer with an inlet air temperature of 120–180°C. This process flashes off the water and produces a small layer of amorphous lactose on the surface of the crystals. This amorphous lactose is likely to crystallise in the fluid bed dryer following the flash dryer. Planned research, which is just starting in the author's laboratory, on the reaction kinetics of amorphous lactose crystallisation at higher temperatures over a range of moisture content should show whether this is the case or not.
7. The temperature of packing: The fluid bed dryer is usually run in two compartments, with the first compartment running with hot air (110°C) being used to drive off the final moisture and/or to crystallise the amorphous lactose layer from the flash dryer. The second compartment uses cold air to cool the product before it goes to the sieves and then the packaging lines via pneumatic conveying lines. It is important that the product is cooled to below 40°C before it is

packed, because temperature gradients cause moisture movement within the bags during storage (Bronlund and Paterson, 2008; Paterson and Bronlund, 2009). This is the major cause of caking of bulk lactose. It has been demonstrated that for caking to occur, the relative humidity in the air spaces within the product must rise above 80% so that significant amounts of capillary condensation can occur.

A temperature gradient within the product will cause moisture to move from the hot region to the cold region (Paterson and Bronlund, 2009). It is this moisture movement, caused by day–night temperature fluctuations as the product is transported about the world, that can cause free-flowing product to arrive at its destination as solid 900 kg blocks (Bronlund and Paterson, 2008; Paterson and Bronlund, 2009). In order to prevent caking during transport, it is vital that the moisture level be reduced to below a critical moisture content. This water content can be determined most easily by measuring the water activity and relating this to the moisture content via the isotherm for the lactose crystals which is shown in Figure 4.3 (Bronlund and Paterson, 2004). It is obvious from this figure that the water activity is a much more sensitive index than the moisture content and it is also quicker and easier to measure. Hence, it is recommended that the water activity should be the preferred quality control method of determining whether a dried product is suitable for shipping or long-term storage.

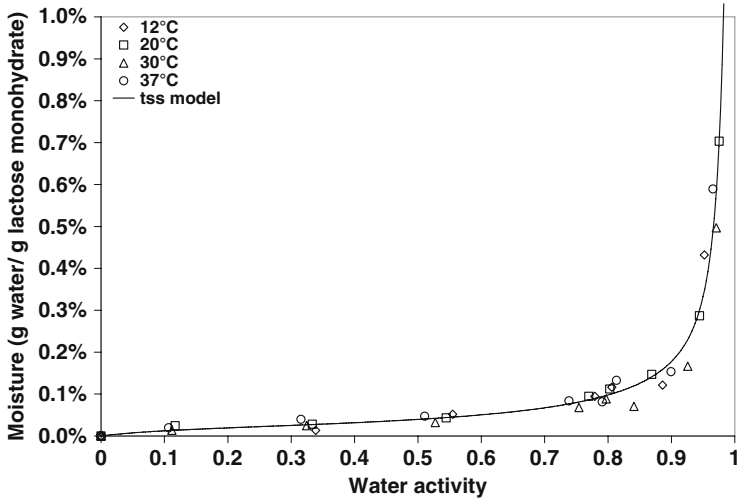

Figure 4.3. Effect of temperature on the adsorption isotherm of α-lactose monohydrate, with the *t*hird *s*tage *s*orption (tss) model fitted to the data. (Reproduced from Bronlund and Paterson, 2004, with permission.).

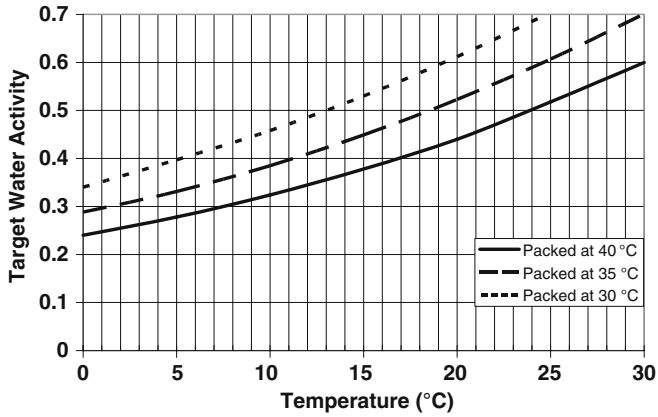

Figure 4.4. Graph showing the hot packed temperature, the cold storage temperature and water activity combinations that lead to relative humidity conditions in the bed exceeding 80%, which precedes caking (from Paterson and Bronlund, 2009, with permission.).

Figure 4.4 shows a graph that has been produced based on the model developed by Paterson and Bronlund (2009) and it shows that if a product is packaged at 40°C and then placed in a warehouse at 10°C for storage, in order to avoid caking, the water activity of the powder must be below 0.32. The figure can also be used as a guideline for the target water activity to be achieved in the dryer in order to prevent caking during transport. To use Figure 4.4 in this way, one assumes that the entire bag is heated to the maximum temperature and then has the outside of the bag subjected to a cold environment. It is obvious that provided the final product has a water activity below 0.3, no caking should occur under most conditions that are likely to be encountered during transport from cold to hot climates, such as when product is shipped across the equator.

The KELLER^{TM} edible lactose process is the most common turnkey lactose plant. It is marketed by Relco of the USA (http://www.relco.net). It follows the traditional path, with a concentration leaving the evaporator of about 58% TS. The cooling curve with time is considered confidential and is a key part of its success. By controlling the cooling curve in the standard Keller design crystallisers, it is possible to control nucleation to produce the right number of crystals, allowing growth of optimally sized crystals for the washing and recovery section of the plant.

To summarise, the main objective of plants producing edible-grade lactose is to achieve the maximum yield. The main parameters are to have the solids level exiting the evaporator as high as possible and to cool the batch to as low a temperature as can be achieved. But it is critical to control α-lactose

supersaturation at all points and at all times during the crystallisation process, so that the final size distribution is optimal. Maximising the plant yield is a matter of finding the optimum balance.

4.3. Pharmaceutical-Grade Lactose

Pharmaceutical-grade lactose must meet the standards for contaminants described by Anonymous (1993) and USP-25 (2001). These two standards are almost identical and the test procedures that must be followed are prescribed. As a general guideline, heavy metals must be below 5 μg per g, the microbial count must be less than 100 per g, with no *Escherichia coli* present and with a combined mould and yeast count below 50 per g. There are also ash, clarity and light-absorbing tests which must be passed.

In general, the process for the manufacture of edible-grade lactose described above produces a product that does not meet the ash, protein and light absorption standards for pharmaceutical-grade lactose. The impurities usually consist of riboflavin, a variety of proteins, lactose phosphate and lactic acid. The process of producing USP-grade lactose from edible-grade lactose is to re-dissolve the lactose in clean water and then to remove the impurities by a combination of adsorption and filtration processes, followed by re-crystallisation (Kellam, 2007). This process is shown in Figure 4.5.

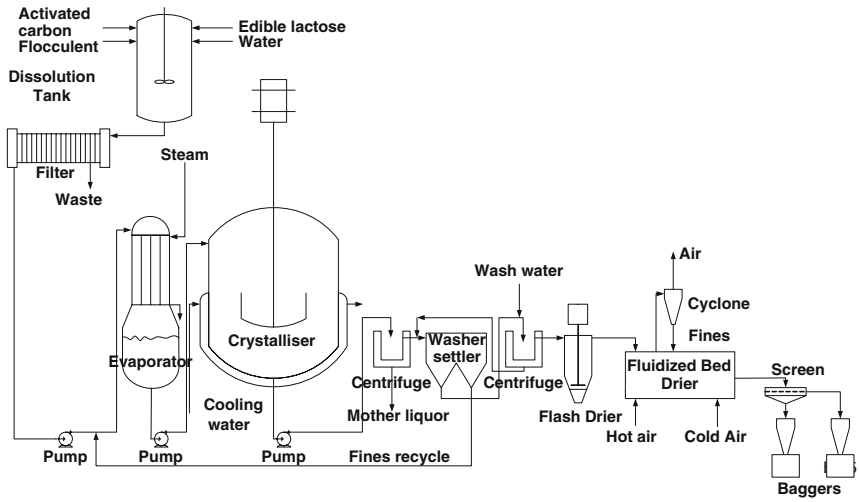

Figure 4.5. Process flow diagram for a pharmaceutical-grade α-lactose monohydrate plant.

Durham *et al.* (2007) claim to produce USP lactose directly from whey permeates by using ion exchange, nanofiltration, chromatography, evaporation and crystallisation, without the need for a second crystallisation step, with a yield approaching 95%.

Pharmaceutical-grade lactose is generally sold by mesh size, the different products being milled to different degrees, possibly in conjunction with air or sieve classification. As well as the traditional α-lactose monohydrate, two other forms are sold to the pharmaceutical industry, i.e. anhydrous lactose (β-lactose) and spray-dried lactose.

4.3.1. Anhydrous Lactose

This has the same heavy metal, microbial and colour specifications as USP-grade α-lactose monohydrate. Anhydrous lactose is usually made by roller drying, a solution of USP-grade lactose at a temperature greater than 93°C which produces a flaked-type product consisting of very fine crystals of β-lactose caked together. The flaked cake is then milled to the required size distribution (Whittier, 1944).

4.3.2. Spray-Dried Lactose

Fine pharmaceutical-grade α-lactose monohydrate crystals are partially dissolved in clean water and the slurry is then spray dried. This produces a product that has crystals of monohydrate lactose joined together by amorphous lactose into roughly spherical agglomerates. Because most of the amorphous lactose is in the centre of the agglomerates, the resultant powder is free flowing without becoming too sticky (Darcy and Buckton, 1998). The amount of amorphous lactose present can be controlled by adjusting the temperature of the water or lactose solution in which the α-lactose monohydrate crystals are suspended.

4.4. Uses of Lactose

Lactose has many uses in the food and pharmaceutical industries. Affertsholt-Allen (2007) gave a breakdown of the uses of lactose in Europe and in the USA as shown in Figures 4.6 and 4.7.

In the food industry, its uses are based on its relative sweetness and as a source of energy. It is less sweet than sucrose, with up to 3.3 times the concentration of lactose being required to give the same level of sweetness as sucrose (Parrish *et al.*, 1981). This means that more lactose can be used without making the product too sweet. Lactose maintains the crystallised

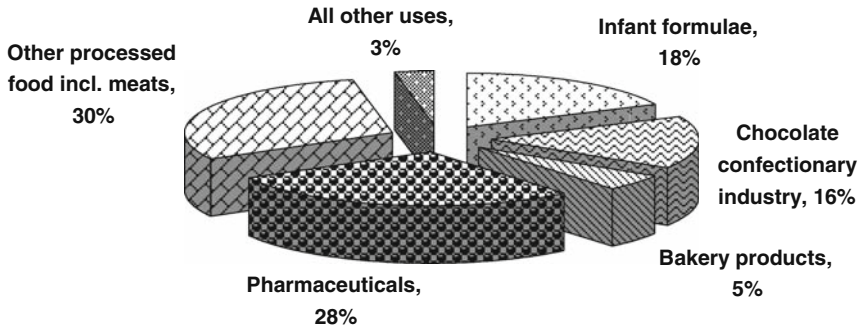

Figure 4.6. Usage of 325,000 tonnes of lactose in the EU in 2005 (from Affertsholt-Allen, 2008, reproduced with permission.).

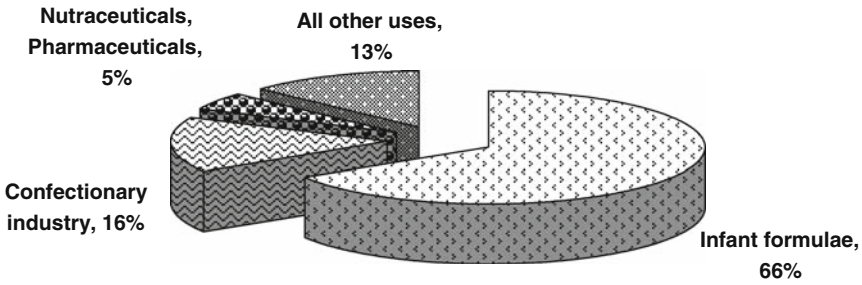

Figure 4.7. Usage of 130,000 tonnes of lactose in the USA in 2005 (from Affertsholt-Allen, 2007, reproduced with permission.).

sugar texture without causing the food to become too sweet (Burrington, 2007).

Lactose is used in the confectionary industry to produce caramel flavours through the Maillard reaction, usually with milk proteins, often added with the lactose in the form of sweetened condensed milk (Weisberg, 1954; Anonymous, 2007b). The Maillard reaction is also important for its use in the baking industry where it is used to promote crust browning as the yeast used during the rising process cannot utilise lactose, leaving it as a reducing sugar available to undergo the browning reaction. Lactose can also adsorb food dyes and flavours and it finds uses in confectionery where this property is exploited.

Lactose is a major energy source in milk, including that of humans. The level of lactose in human milk is about 7% compared to 4.7% in cows' milk. This was recognised by Henri Nestlé who enriched milk powder made

from cows' milk with lactose to produce the first infant formulae in 1867 (Anonymous, 2007a). This has been one of the major uses of lactose since.

The period from 2004 to 2007 has seen a dramatic increase in the price of lactose from around US$440 to a high of US$2000/tonne and decreasing later to US$1100/tonne (Affertsholt-Allen, 2008). This price increase is likely to see a swing in the uses of lactose as it moves from being a low-cost energy source to a relatively expensive source. For comparison, the cost of sucrose in 2007 fluctuated around US$175/tonne. The reason for the increase in the price of lactose can be traced back to the market forces of supply and demand. In this case, the demand has been stimulated by the standardisation of milk powders. In countries, such as New Zealand, where the lactation of the national herd is largely synchronised to match seasonal grass availability, the protein content of milk powder fluctuates markedly throughout the year. The addition of lactose to standardise protein level is now permitted, provided that the adjustment does not alter the whey protein to casein ratio of the milk being adjusted. Standardisation of milk for protein as well as fat levels is being introduced within Europe also. The result has been a great demand for lactose causing a worldwide shortage. This effectively means that lactose has an inherent value approaching that of skim milk powder, rather than being a substance that has to be disposed of so that it does not cause problems in the environment due to its high biological oxygen demand (BOD). The price of lactose is likely to fall again when the supply matches the increased demand, as has happened by March 2008. At any point in history, there are usually multiple lactose production projects waiting to be considered by cheese-producing companies.

Lactose is limited as a food ingredient in that many people around the world are lactose intolerant (hypolactasia), meaning that their body does not produce lactase, the enzyme which hydrolyses lactose in the gut into glucose and galactose, which can be absorbed. Consequently, the lactose passes into the lower intestine where it provides a ready source of energy for anaerobic bacteria. These bacteria produce gas as a by-product and this can cause cramping, flatulence and perhaps diarrhoea.

In the pharmaceutical industry, lactose is used as the main carrier (about 70% of tablets contain lactose) for drugs because it is not sweet, it is safe, it is available in highly refined form and it makes good quality tablets. It has found uses in the industry in a number of different product forms. The main one is α-lactose monohydrate which can be used as a tablet excipient, but it can also be finely milled to produce inhaler-grade lactose. Here, the lactose acts as a carrier for micronised drug materials to reach the lungs. Both anhydrous lactose (β-lactose) and spray-dried lactose are also used to make tablets. The form of the lactose is critical for consistent tabletting formulations, and much emphasis is placed on the reproducibility between batches of

the particle properties that are required to produce consistent tablets, with an even spread of the active drug dispersed within the lactose powder being used as an excipient.

For some of the uses of lactose, it is important for the customer to dissolve lactose into water. It is important, therefore, to understand how lactose dissolves. Since α-lactose monohydrate is the cheapest form of lactose available on the market, it is the form that is generally used. Hodges *et al.* (1993) have shown that the mechanism that determines the rate of dissolution depends mainly on the desired concentration in solution. At levels above the solubility level of α-lactose, the rate of dissolution is governed largely by the rate of mutarotation of α-lactose to β-lactose (Haase and Nickerson, 1966a). Below the α-lactose solubility limit, the rate has been shown to be modelled by the mass transfer rate of moving the α-lactose into solution (Lowe and Paterson, 1998). These mechanisms have been combined in a mathematical model and then summarised into a series of graphs (Figures 4.8 and 4.9) for different particle sizes (Lowe, 1993) which have been rearranged to emphasise the effect of particle size. These graphs show the dissolution times expected for producing a desired concentration of lactose (expressed as kg anhydrous lactose per m^3 of solution) from mono-sized α-lactose monohydrate crystals of 50, 150 or 400 μm size.

Figure 4.8 covers the concentration range of 40–200 kg/m^3 and temperatures of 10–40°C. The graphs can be used as follows: if it is desired to produce a solution with a concentration of 90 kg/m^3, at 10°C, the dissolution

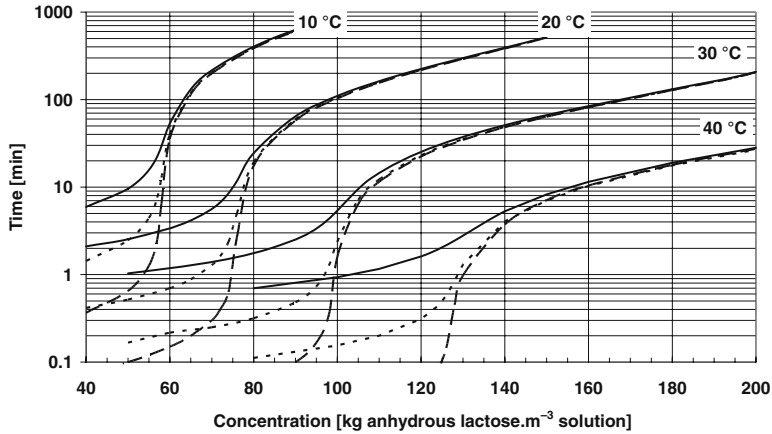

Figure 4.8. Dissolution times for producing solutions with lactose concentrations of 40–200 kg/m^3 [—— —— 50 μm, - - - 150 μm, —— 400 μm] (data from Hodges *et al.*, 1993; Lowe and Paterson, 1998).

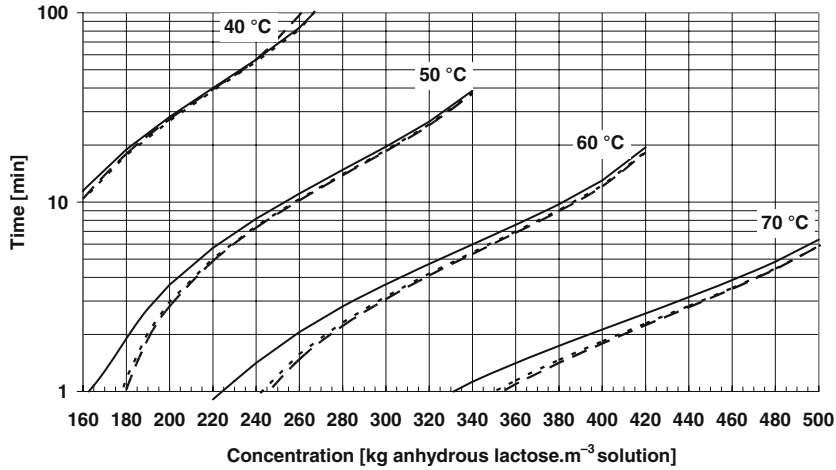

Figure 4.9. Dissolution times for producing solutions with lactose concentrations of 160–500 kg/m^3 [— — 50 μm, - - - 150 μm, —— 400 μm] (data from Hodges *et al.*, 1993; Lowe and Paterson, 1998).

process takes 600 min and is governed totally by the mutarotation kinetics and particle size has very little effect. This is still largely true at 20°C, where the increased temperature reduces the dissolution time to 60 min; but at 30°C, the mutarotation reaction is so fast that the mechanism governing the dissolution is dependent on mass transfer and the particle size has a large effect with the time required being 2.5 min for 400 μm crystals, 0.5 min for 150 μm and 0.1 min for 50 μm crystals. Figure 4.9 covers the range of concentrations from 160 to 500 kg anhydrous lactose per m^3 of solution.

If the lactose powder contains a range of particle sizes, then the dissolution time for the largest particle size is the best one to use, but it will underestimate the time required, especially if near-saturated solutions are being produced. The curves have been stopped short of the saturated limits. However, within these limitations, the graphs can be used to estimate the approximate dissolution times required to achieve a given concentration when dissolving α-lactose monohydrate in water at a given temperature.

4.5. The Future for Lactose

Lactose has changed over the last 30 years from being a problem component in dairy wastewater with a high BOD to being a valuable by-product of the dairy industry. Lactose will continue to have a market as a required

supplement to increase the energy value of bovine milk to that of human milk in infant food formulations. The increased use of standardisation of milk powders for both fat and protein levels will continue to keep the price of lactose higher than it has been over the last 20 years, although some corrections in the market place will occur as more cheese manufacturers start to realise what a valuable asset they have in their lactose. Much current research is being carried out in the area of lactose derivatives, with the aim of producing high-value neutraceuticals. This is an area in which more commercial activity is likely to be seen over the next 10–15 years and if it works, then it will be another driver in increasing the price of lactose.

On the pharmaceutical side, there is research on alternatives to lactose as an excipient, but it is expected that it will be some years before these are serious replacement threats to the position pharmaceutical-grade lactose currently holds.

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5

Galacto-oligosaccharides and Other Products Derived from Lactose

M.J. Playne and R.G. Crittenden

5.1. Introduction

Lactose is the precursor for a number of compounds derived by chemical, physical or enzymatic conversion that have an established and expanding place in the health and food industries. These include lactulose and galacto-oligosaccharides both of which are manufactured in large tonnages worldwide. Lactitol and lactosucrose are produced commercially, but in much smaller amounts, while lactobionic acid is produced for limited industrial and medical applications and for research use. The only other lactose derivative of commercial interest is an isomer of galactose called tagatose in which there is an emerging interest as a sweetener.

None of these compounds occur naturally in cow's milk except in trace amounts. Yet, lactose-derived oligosaccharides occur naturally in human milk at relatively high concentrations (5–12 g/L) and play a major role in directing the development of the intestinal microbiota of infants. One of the major and emerging uses of lactose derivatives has been to emulate the physiological effects of these natural oligosaccharides, and β-galacto-oligosaccharides (GOSs) and lactulose in particular have found applications as prebiotics in functional foods and beverages. βGOSs are also classified nowadays as dietary fibre. Prebiotic α-galacto-oligosaccharides are also available commercially, but since they are produced typically from soybean, they are not discussed here. Lactulose is an important pharmaceutical used in the treatment of constipation

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and hepatic encephalopathy, and lactitol is increasingly used in similar applications. Galactose, lactic acid and lactates are also produced from lactose, but are not reviewed here (refer to Chapters 1 and 4).

Extensive reviews on GOSs have been published in recent years (Matsumoto *et al.*, 1993; Playne and Crittenden, 1996; Crittenden and Playne, 1996; Tanaka and Matsumoto, 1998; Sako *et al.,* 1999; Schoterman, 2001; Playne, 2002a; Meyer and Tungland, 2001; Nakakuki, 2002). Readers may also like to compare the commercially produced GOSs to the complex oligosaccharides found in human milk, which have been reviewed thoroughly by Kunz *et al.* (2000) and Boehm and Stahl (2003, 2007). New methods for manufacturing lactose derivatives, as well as new applications, are emerging constantly. Patents on methods of the manufacture of oligosaccharides, lactulose, lactosucrose, lactitol, lactobionic acid and tagatose are extensive. The full patents provide a wealth of information on manufacturing procedures, the enzymes and microorganisms involved, analytical methods, separation and purification procedures and applications and uses of the products. Readers are advised to examine these resources and internet access is available free, for at least all US patents. In this chapter, we have drawn on this extensive body of scientific literature to provide an overview of the current state of the art in production, applications and physiological effects of lactose derivates.

5.1.1. Definitions

Oligosaccharides

– are usually defined as glycosides composed of 2–10 covalently linked monomer sugar units. However, disaccharides, such as lactose and lactulose, are often not regarded as oligosaccharides and some saccharides of longer chain length than ten monomer units are called oligosaccharides, provided they are of defined length, composition and structure.

Non-digestible Oligosaccharides (NDOs)

– not all oligosaccharides are NDOs. NDOs can be distinguished from other carbohydrates on the basis of being not digested in the stomach and small intestine, and therefore not digested under acid conditions and by pancreatic hydrolytic enzymes. NDOs include some larger saccharide molecules, such as inulin.

Polysaccharides

– are high molecular weight polymers of one or more monosaccharides. They are of greater molecular size than oligosaccharides and usually of undefined length. Examples are starch and cellulose.

Galacto-oligosaccharides

- are oligosaccharides composed primarily of galactose monomers linked together in a number of different structural configurations. They usually consist of a number of $\beta(1\rightarrow6)$ linked galactopyranosyl units linked to a terminal glucopyranosyl residue *via* an $\alpha(1\rightarrow4)$ glycosidic bond. They are sometimes referred to as *trans*-galacto-oligosaccharides (TOSs).
- abbreviations used are GOS and TOS.

Lactulose (4-*O*-β-D-Galactopyranosyl-D-fructofuranose; $C_{12}H_{22}O_{11}$; FW 342.30 Da)

- is an isomer of lactose, wherein the glucose moiety of lactose is converted by alkaline isomerization to fructose. The disaccharide, lactulose, is therefore composed of β-D-galactose linked to β-D-fructose in a (1→ 4) configuration.

Lactosucrose (β-D-Fructofuranosyl-4-*O*-β-D-galactopyranosyl-α-D-glucopyranoside; $C_{18}H_{34}O_{17}$; FW 522 Da)

- is a trisaccharide formed from lactose and sucrose by an enzymatic transglycosylation.

Lactitol (4-*O*-β-D-Galactopyranosyl-D-glucitol; $C_{12}H_{24}O_{11}$; FW 344.32 Da)

- is a sugar alcohol derived from lactose by catalytic hydrogenation.
- synonyms are lactit, lactositol, lactobiosit

Lactobionic acid (4-*O*-β-D-Galactopyranosyl-D-gluconic acid; $C_{12}H_{22}O_{12}$; FW 358.30 Da)

- is an oxidation product of lactose.

Tagatose (D-(–)-Tagatose; $C_6H_{12}O_6$; FW 180.16 Da)

- is a D-lyxo-hexulose with a molecular weight of 180.16. It can occur naturally and is derived from galactose by alkaline isomerization.

Prebiotic

- a prebiotic is a selectively fermented ingredient that allows specific changes, in both the composition and/or the activity in the gastrointestinal microflora, that confer benefits upon host well-being and health (Gibson *et al.*, 2004; Roberfroid, 2007). These ingredients are normally restricted to certain carbohydrates (particularly oligosaccharides), but could include certain proteins, peptides and lipids. The concept of a prebiotic ingredient arose initially from the idea of

compounds called "bifidus factors" which could enhance the growth of bifidobacteria within the intestinal microbiota.

Synbiotic

– are foods containing both probiotic bacteria and prebiotic ingredients to provide a diet in which the intestinal growth and/or metabolic activity of the probiotic bacteria is selectively enhanced by the presence of the prebiotic, thus promoting the chance of the probiotic bacteria becoming established in the gut.

5.2. History of Prebiotics

5.2.1. The Genus *Bifidobacterium*

The concept of prebiotics arose from the realization that some compounds could enhance the growth of bifidobacteria in the intestinal tract of humans. These compounds were called the "bifidus factors", and followed Tissier's early work in France around 1900 on bifidobacteria and their presence in the gastrointestinal tract of babies. He realized that bifidobacteria could possibly control diarrhoea in infants. At that time, bifidobacteria were called *Bacillus bifidus.* Although the name *Bifidobacterium* was proposed as early as 1920, it did not gain official recognition as a separate genus until 1974. There are now 36 recognized species within the genus *Bifidobacterium* (German Culture Collection, 2007).

5.2.2. Bifidus Factors

In 1953, Gyorgy discovered a strain of *Bifidobacterium bifidum* which would grow only in the presence of human milk, or more specifically in the presence of derivatives of *N*-acetylglucosamine. Further specific requirements for the growth of different strains were recorded, e.g., human casein hydrolysates (Gyorgy *et al.*, 1954a,b). In 1953, lactulose as a growth factor for bifidobacteria in infant milk was being studied, and infant milk containing lactulose was being sold by Morinaga in Japan as early as 1960. Lactulose was recognized as a "bifidus" factor by Petuely (1957). Bifidus factors have been summarized by Modler *et al.* (1990) and are shown in Table 5.1.The value of a range of oligosaccharides and polysaccharides as bifidus factors was recognized by Yazawa *et al.* (1978) and Yazawa and Tamura (1982). Ballongue (1998) and Tamura (1983), respectively, have described the historical developments of the genus *Bifidobacterium* and the recognition of bifidus factors summarized above.

Table 5.1. Compounds and products claimed to be "bifidus growth factors"

BIFIDUS GROWTH FACTOR 1 (AMINO SUGARS)
Glycoproteins with:
N-acetyl glucosamines
–Lacto-*N*-tetraose
–Lacto-*N*-neotetraose
–Lacto-*N*-fucopentaose
–Lacto-*N*-difucopentaose
N-Acetylgalactosamine
N-Acetylmannosamine
BIFIDUS GROWTH FACTOR 2
Casein peptides (non-glycosylated) (after hydrolysis using a proteinase)
BIFIDUS GROWTH FACTOR 3
Pantethine
OLIGOSACCHARIDES
Stachyose
Fructo-oligosaccharides
Galacto-oligosaccharides
Iso-malto oligosaccharides
Xylo oligosaccharides
DI- AND TRI-SACCHARIDES
Raffinose
Lactulose
Lactosucrose
CELL-FREE EXTRACTS
Propionibacterium freudenreichii
LACTOFERRIN
Fe, Cu and Zn complexes

5.2.3. Oligosaccharides as Prebiotics

The earliest scientific publications on oligosaccharides as prebiotics occurred in Japan (Yazawa *et al*., 1978) and were followed soon after by a number of papers by Japanese researchers examining, particularly, various galacto- and fructo-oligosaccharides (for example, see Minami *et al*., 1983). The earliest patents on methods for the production of oligosaccharides occurred in 1982, also in Japan. Most of the Japanese commercial development of various food-grade oligosaccharides occurred during that decade. However, the widespread use of prebiotics in products in Japan did not really start until 1990. By the end of 1999, prebiotics dominated the ingredients in FOSHU (Foods for Specified Health Use) – approved products in Japan (see Section 5.10.1).

The actual use of the word "prebiotic" is credited to Gibson and Roberfroid (1995), and this word is now widely used to include oligosaccharides, polysaccharides, inulin, lactulose, lactitol, glucans, resistant starches and many dietary fibres. The word "prebiotic" relates to "probiotics", which are live microbial food ingredients, such as lactobacilli and bifidobacteria, that are consumed with the aim of supplementing the intestinal microbiota and improving health. The development of prebiotics in Europe did not occur until the early 1990s, with the start of inulin and fructo-oligosaccharide production in Belgium and later the development of galacto-oligosaccharide production in Holland and the research on gluco-oligosaccharides in Toulouse. Meiji (Japan) also formed alliances with Beghin Say in France and with Coors in the USA to produce fructo-oligosaccharides. However, European researchers have dominated the scientific literature on prebiotics since then, and the European food industry has very much claimed the "prebiotic concept" in recent years. Other than the development of fructo-oligosaccharide production in Korea and in Taiwan, there has been little development outside Europe and Japan. However, companies in a number of countries, including Australia, produce galacto-oligosaccharides in-house primarily for infant milk formula markets. The commercial development of the prebiotic carbohydrate market is shown in Table 5.2.

Table 5.2. The Commercial Evolution of Different Classes of Prebiotic Carbohydrates

Prebiotic Compound	Year*
Lactulose	1953
Fructo-oligosaccharides	1983
Soy-oligosaccharides	1983
Galacto-oligosaccharides	1985
Lactitol	1987
Inulin	1990
Lacto-sucrose	1993
Xylo-oligosaccharides	1994
Resistant starches	1996
β-glucans	1998
Cereal oligosaccharides	1998
Polysaccharides	1998
Dietary fibres	1999

*estimated first year of commercial production with a prebiotic claim.

5.2.4. Use of β-Galactosidases for the Synthesis of Oligosaccharides

The ability of glycosidase enzymes to carry out synthetic reactions by reversing the equilibrium conditions has long been known (Croft-Hill, 1898). The enzymatic synthesis of galacto-oligosaccharides from lactose was first studied in detail by Pazur (1953, 1954) and Pazur *et al.* (1958). A number of papers published in the 1970s and 1980s examined the production of oligosaccharides from lactose using enzymes derived from a number of sources (for example, Toba *et al.*, 1985). The mechanisms of the action of β-galactosidases on lactose were first described by Wallenfels and Malhotra in 1960 (see Prenosil *et al.*, 1987a). Prenosil *et al.* (1987a,b) described the nature of the oligosaccharide products formed from lactose by β-galactosidases from a number of different microbial sources. However, Wallenfels (1951) first described enzymatic synthesis of oligosaccharides from disaccharides.

5.3. Chemistry – Structures and Reactivity

Glycans, with nucleic acids and proteins, are widely distributed in living organisms. All of these polymers are covalently linked moieties, but glycans have characteristics not found in the other two. Notably, the nature of the linkage between monomeric units in glycans is much more variable than those found in the other polymers, and this leads to a much greater variety in the sequence of the biopolymer. Thus, this leads to a huge structural diversity of oligosaccharides in glycosylated compounds (glycoproteins, glycolipids).

The number of combinations of structural linkages between monomers is high. For example, a galactose unit can be linked to a mannose unit at four positions (C2, C3, C4 and C6), and thus form four isomeric structures. Additionally, a galactose moiety can take two anomeric configurations, meaning that the number of combinations rises to eight. Furthermore, a galactose moiety can occur in both furanose and pyranose forms; thus, there are 16 possible isomeric structures of this Gal-Man disaccharide. As the number of linkages expands, so does the seemingly endless possible combinations. In contrast to proteins and nucleic acids, glycans are not limited in their molecular structure, as they can branch three-dimensionally. Neither are glycans constrained by genetic templates as is the case with nucleic acids and proteins. This structural diversity (and flexibility) has led to the emerging science of glycobiology and its application in medicine in the development of targetted drugs.

The nomenclature of carbohydrates has been described fully in a series of publications authorized by the International Union of Pure and Applied Chemistry (IUPAC). Detailed information on currently accepted nomenclature and its historical development can be accessed on-line (Queen Mary

University of London, 2008). This web site details the 1996 Recommendations of the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN) of the IUPAC and the International Union of Biochemistry and Molecular Biology (IUBMB). The full 1996 recommendations have been published (IUPAC, 1996) and are available as a pdf file at www.iupac.org/publications/pac/1996/pdf/6810x1919.pdf.

5.4. Synthesis and Manufacturing Methods

The synthesis of oligosaccharides from simple sugars has been studied extensively using classical methods of carbohydrate chemistry. The use of enzymes in the synthesis of oligosaccharides has now overtaken direct chemical pathways because of the ability of enzymes to be specific in the formation of particular linkages between monomers. The scope of the earlier work on oligosaccharide synthesis has been ably summarized by Bailey (1965) and Pazur (1970). Chemical synthetic methods based on lactose have been discussed by Thelwall (1997).

The interest of the food industry in these ingredients since 1980 has resulted in the development of manufacturing methods, most of which are based on enzymatic conversions, and only limited purification steps to reduce cost. Highly purified product is not warranted for most food applications. However, products of greater purity and specific structure are necessary in some applications, particularly those for pharmaceutical use.

Prebiotic oligosaccharides are prepared by several methods. Some are extracted from plant materials and used directly, e.g., inulin, some resistant starches, soybean and many dietary fibres. Others are modified enzymatically after extraction of the crude parent feedstock from plants (e.g., xylo-oligosaccharides and some fructo-oligosaccharides).

β-Galacto-oligosaccharides are generally synthesized from lactose by transgalactosylation of lactose by the enzyme β-galactosidase. In contrast, lactosucrose is prepared by a different enzymatic route. Here, lactose, in the presence of sucrose, acts as the acceptor of fructose in a transfructosylation reaction catalysed by the enzyme β-fructofuranosidase. Fructo-oligosaccharides are also synthesized from sucrose by similar enzymatic routes.

Lactulose and lactitol are prepared by two different chemical syntheses from lactose. Lactulose is prepared by alkaline isomerization of lactose, while lactitol is synthesized by catalytic hydrogenation of lactose. Lactulose can also be prepared by an enzymatic route, though this is not used commercially. Lactobionic acid is prepared by dehydrogenation of lactose at high pH using a metal catalyst. However, it can also be prepared in high yield by microbial bioconversion and by enzymic oxidation of lactose. Tagatose was prepared

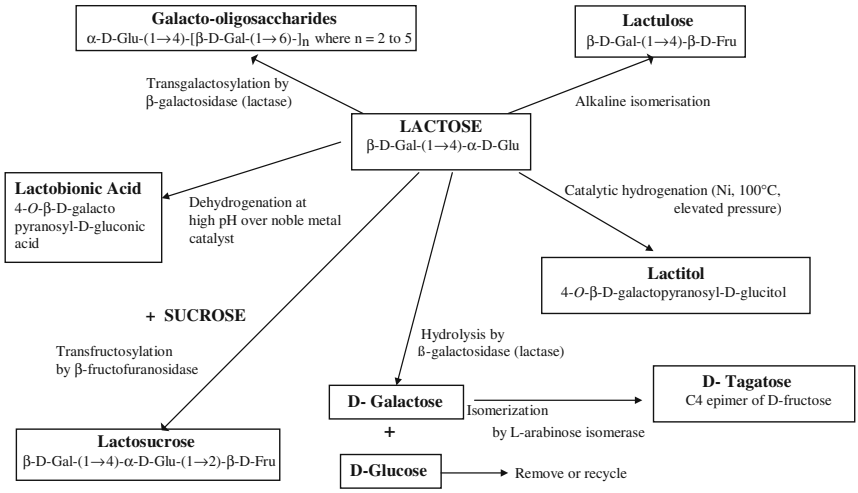

Figure 5.1. The preparation of galacto-oligosaccharides, lacto-sucrose, lactulose, lactitol, lactobionic acid and tagatose from lactose.

initially from galactose by alkaline isomerization in a manner parallel to the preparation of the disaccharide, lactulose from lactose, but it is now manufactured by an enzymatic route from lactose.

The basis of the preparation of galacto-oligosaccharides, lactulose, lactitol and lactosucrose from lactose is shown in Figure 5.1.

5.4.1. β-Galacto-oligosaccharides

A typical flow chart for the manufacture of galacto-oligosaccharides is shown in Figure 5.2. Similar methods are used by the main galacto-oligosaccharide manufacturers (Yakult, Friesland Foods Domo and Nissin Sugar). The process depends on reversing the normal degradative hydrolytic action of β-galactosidase. Instead of producing glucose and galactose from the feedstock lactose, the enzyme is "pushed" into synthesis of a mixture of tri, tetra and penta galacto-oligosaccharides. This is achieved by having a high concentration of lactose in solution (e.g., 400 g/L or higher). This is achievable only if the temperature is elevated to between 50 and 80°C and the enzyme chosen to catalyse the reaction must be active at these temperatures. The enzyme chosen must also have minimal hydrolytic activity to avoid the simultaneous formation of excessive amounts of glucose and galactose. The β-galactosidases used may produce either $\beta 1 \rightarrow 4$ or $\beta 1 \rightarrow 6$ linkages in the oligosaccharide chain. The ratio of products

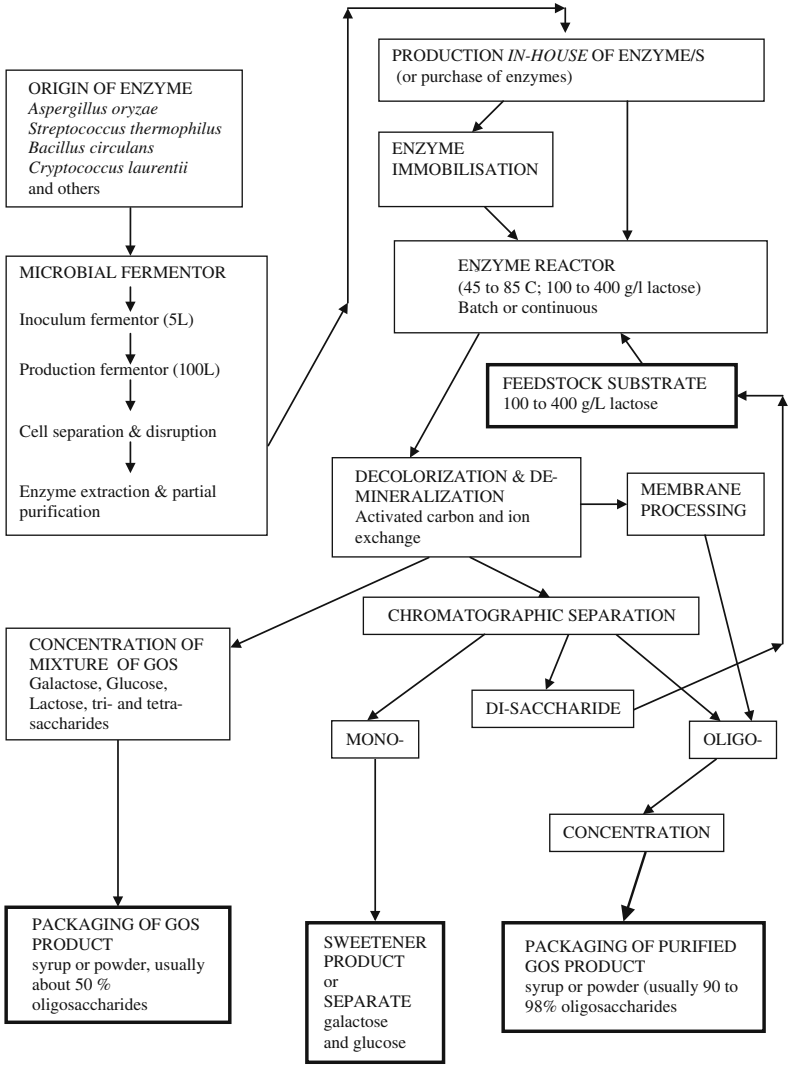

Figure 5.2. Manufacturing process for the production of galacto-oligosaccharide (GOS) syrups and powders from lactose.

containing either linkage can be controlled by using dual enzyme systems. Generally, the production of trisaccharides predominates, and thus trisaccharides are dominant in most commercial GOS products. Enzymes derived from *Bacillus circulans* or *Cryptococcus laurentii* will produce

4′-galactosyllactose, while enzymes from *Aspergillus oryzae* or *Streptococcus thermophilus* will produce 6′-galactosyllactose. The former enzymes are used by the Nissin Company to produce their Cup-Oligo product which is a $\beta 1 \rightarrow 4$ galactosyl lactose, whereas Yakult Pharmaceuticals have used the latter enzymes for their Oligomate products which have predominantly $\beta 1 \rightarrow 6$ linkages, although Yakult now also uses *B. circulans* to produce $\beta 1 \rightarrow 4$ linked product. Thus, the choice of enzyme is crucial to the outcome of the commercial process. Similar considerations apply to the production of fructo-oligosaccharides from sucrose. Figure 5.2 shows the steps required for the simple production of a mixture of oligosaccharides (tri- to hepta-saccharides), lactose, glucose and galactose, when no purification steps are included. Additional separation of oligosaccharides, from the substrate lactose, and the hydrolytic products, glucose and galactose, can be achieved, but is an expensive process. Separation is usually by a chromatographic process. Following the batch reaction with enzymes, the product mixture is decolourized and demineralized, then filtered and concentrated to produce either a syrup or a powder. Attempts to reduce enzyme costs have been made by immobilization of the enzymes, but it is believed that this is not used commercially. A wide range of microorganisms continue to be evaluated as sources of suitable β-galactosidases. For example, the enzyme from *Sterigmatomyces elviae* has given high oligosaccharide yields from lactose at 200 g/L at 85°C. Active patenting activity occurs in this area in particular. Mahoney (1997) listed the wide range of microbial species which have β-galactosidases able to utilize lactose. Since that publication, considerable research has been performed on new sources of thermostable enzymes, the development of recombinant enzymes and enzymes from *Bifidobacterium* species. However, the choice of a suitable commercial enzyme depends on its ability to perform the reaction at a high temperature, and this will continue to determine the selection of the enzyme.

5.4.1.1. Type of Enzyme and Mechanisms of Reaction

Two types of enzyme have been used in the preparation of oligosaccharides, the glycosyltransferases (EC 2.4 series) and the glycosidases (EC 3.2 series). The commercial processes used in the food industry which are described in the previous section all use glycosidases.

Glycosyltransferases: Glycosyltransferases catalyse the stereo- and regio-specific transfer of a monosaccharide from a donor substrate (such as a glycosyl nucleotide) to an acceptor substrate. The specificity of such reactions is high and good yields can be obtained. The presence of multiple functional groups and stereo isomers in complex oligosaccharides makes

them difficult and time-consuming to synthesize by organic chemistry. Chemical methods for the preparation of peptides and oligonucleotides have been developed which are robust and automated. This has not been possible for the chemical preparation of complex oligosaccharides and glyco-conjugates. Thus, researchers have turned to enzymatic methods.

Enzymatic synthesis of oligosaccharides using glycosyltransferases of the Leloir pathway can overcome the problems encountered in the chemical synthesis of specific oligosaccharides. However, these enzymatic syntheses require sugar nucleotides and glycosyltransferases, both of which have not been readily available or, at least, not cost-effectively. Such synthetic methods will not be covered in detail in this chapter, but are well reviewed elsewhere (Prenosil *et al*., 1987a; Nilsson, 1988; Rastall and Bucke, 1992; Koizumi *et al*., 1998). Koizumi *et al*. (1998) discuss the large-scale production of uridine 5′-diphosphate (UDP)-galactose and globotriose. Globotriose is the trisaccharide portion of a verotoxin receptor and has the structure α-D-Gal (1→4)-β-D-Gal (1→4)-D-Glc.

The instability of glycosyltransferases as synthetic reagents has led some workers to develop immobilized glycosyltransferases for oligosaccharide synthesis (Rastall and Bucke, 1992). For specific synthesis of oligosaccharides of medical interest, a large number of different glycosyltransferases are required, and their availability is a limiting factor. As more glycosyltransferases are isolated from nature or formed by recombinant techniques, it has become increasingly possible to form the specific physico-chemical structures required.

The usual substrates for galactosyltransferases are UDP-galactose and either a free *N*-acetylglucosamine or an *N*-acetylglucosamine bound to a protein molecule. The complexity and cost of methods involving glycosyltransferases have precluded their use in food applications to date. However, several companies are interested in the production of longer chain, specifically tailored oligosaccharides for use in the functional food sector. The main application of synthesis systems using glycosyltransferases remains in the biomedical and pharmaceutical areas.

In 2007, the Sigma-Aldrich Company (USA) listed four glycosyltransferases (Table 5.3) in their catalogue, which were also available in different formulations and as kits with accompanying substrates and cofactors (Sigma-Aldrich, 2007). Other major biochemical supply houses carry some glycosyltransferase products. There are also a number of specialist glycoscience commercial groups worldwide, some of which are developing specific oligosaccharide pharmaceuticals, others providing specialist analytical services in the area and others offering custom manufacture and the supply of specific oligosaccharides (see Playne, 2002b for details).

Table 5.3. Glycosyltransferases listed by Sigma-Aldrich (2007)

Name	Description
(α1→3)galactosyltransferase, mouse, recombinant, expressed in *Escherichia coli*	catalyses the transfer of galactose from UDP-galactose to *N*-acetyllactosamine
(β1→4)galactosyltransferase 1, human, recombinant, expressed in *Saccharomyces cereviseae*	transfers galactose from UDP-galactose to D-glucose in the presence of α-lactalbumin
(β1→4)galactosyltransferase from bovine milk	transfers galactose from UDP-galactose to D-glucose in the presence of α-lactalbumin
(α1→3)fucosyltransferase VI, human, recombinant, expressed in *Pichia pastoris*	transfers L-fucose from GDP-L-fucose to *N*-acetyl-D-lactosamine

Glycosidases: Glycosidases can transfer the glycosyl moiety of a substrate to hydroxyl acceptors. Hydrolysis represents merely a special case where water serves as the hydroxyl acceptor. Most glycosidases are able to catalyse either hydrolysis or transglycosidation, with the reaction outcome dependent on the relative abundance of the hydroxyl acceptors. Glycosidases are broadly classified as *exo*- and *endo*-glycosidases. The *exo*-glycosidases act on the linkage at the non-reducing end of the saccharide chain, whereas the *endo*-glycosidases act on the glycosidic linkages within the saccharide molecule. Glycosidases are less specific in their ability to catalyse certain cleavages than the glycosyltransferases and are available from a wide range of sources, e.g., viruses, microorganisms, plant and animal cells. They do not require expensive cofactors for synthesis reactions and generally are regarded as suitable for the synthesis of short-chain oligosaccharides. The use of glycosidases as synthetic enzymes dates back many years, but only since 1980 has their use become of major commercial importance.

Glycosidase reversal can be achieved by a kinetic approach or by an equilibrium approach. The kinetic approach recognizes that the hydrolysis of glycosidic bonds is a two-stage process involving a covalently linked sugar–enzyme intermediate. For oligosaccharide synthesis, the covalent bond is cleaved by a nucleophilic displacement reaction by an acceptor molecule. In this case, the normal acceptor molecule, water, is replaced by an alcohol or by a carbohydrate.

The equilibrium approach is based on the fact that, in principle, all enzymatically catalysed reactions are reversible. To synthesize oligosaccharides by this method requires, therefore, the use of very high concentrations of the sugar substrate to reduce the water activity. High temperatures are normally used (a) to speed up the otherwise rather slow reaction and (b) to increase the solubility of the sugar substrate so that a high concentration in solution can be achieved. The enzyme is protected from denaturation by the

high temperatures, the stabilizing effects of the high sugar and low water concentrations (Johansson *et al.*, 1989). The temperature tolerance of the enzyme also depends on its source. The manufacturing processes for food-grade oligosaccharide mixtures described in Section 5.4.1 use the equilibrium principle and the glycosidase, β-galactosidase.

The microbial β-galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23) have been well studied, but it is often not appreciated that their catalytic action is best described as a transgalactosylation rather than a hydrolysis. The enzyme transfers the galactose moiety of a β-galactoside to an acceptor containing a hydroxyl group. If the acceptor is water, then galactose is formed, but if lactose is used as the acceptor molecule, then new oligosaccharides will be synthesized. The different microbial origins of β-galactosidases not only determine their temperature tolerance but also the characteristic mixture of di- to hexa-saccharides formed and whether a $\beta1\rightarrow4$ or a $\beta1\rightarrow6$ linkage is formed. The formation of oligosaccharides reaches a time-course maximum during a batch reaction. Continuation of the reaction will lead to the hydrolysis of the formed oligosaccharides to monosaccharides (Smart, 1993).

Allolactose (β-D-Gal-(1$\rightarrow$6)-D-Glu) is always formed initially in the reaction to produce oligosaccharides from lactose (Prenosil *et al.*, 1987a). Galactobiose (β-D-Gal-(1$\rightarrow$6)-D-Gal) is also formed, but allolactose is the dominant initial product. A range of higher oligosaccharides is then formed, including galactotriose, [4-β-galactobiosylglucose, 6-β-galactobiosylglucose and 6-β-galactotriosylglucose (Asp *et al.,* 1980; Rastall and Bucke (1993)]. The products of synthesis from lactose have been thoroughly reviewed by Prenosil *et al.* (1987a). Table 5.4 shows the numerous possible reaction products from lactose resulting from hydrolysis, internal rearrangement and transgalactosylation.

Methods to enhance the effectiveness and reduce the cost of processing lactose using β-galactosidase have been examined intensively in recent years. They include immobilization of the enzyme by encapsulation, by entrapment in fibre matrices, in gels or on semi-permeable membranes. A variety of adsorption and covalent attachment techniques have also been described.

5.4.1.2. Microorganisms Used to Produce β-Galactosidases (Lactase)

Historically, β-galactosidase has been an important industrial enzyme due to its many applications in the dairy industry. These include the hydrolysis of lactose to prevent symptoms of lactose intolerance, lactose crystallization problems in processed foods, in cheese manufacture, cheese whey waste reduction, improvement of whey protein concentrates and in ethanol production. Since about 1985, the use of lactases for the production of oligosaccharides has added a new dimension, which has required a

Table 5.4. Possible reaction products formed from lactose by hydrolysis, and by internal rearrangement and transgalactosylation

Common name	Component linkage	Process
Lactose	Gal (1→4) Glu	
Galactose	Gal	hydrolysis
Glucose	Glu	hydrolysis
DISACCHARIDES		
	Gal (1•2) Glu	internal rearrangement
	Gal (1•3) Glu	internal rearrangement
Allolactose	Gal (1•6) Glu	internal rearrangement
	Gal (1•3) Gal	transgalactosylation
Galactobiose	Gal (1•6) Gal	transgalactosylation
TRISACCHARIDES		
6′-galactosyl lactose	Gal (1•6) Gal (1•4) Glu	transgalactosylation
3′-galactosyl lactose	Gal (1•3) Gal (1•4) Glu	transgalactosylation
	Gal (1•6) Gal (1•6) Glu	internal rearrangement
Galactotriose	Gal (1•6) Gal (1•6) Gal	transgalactosylation
Tetrasaccharides Pentasaccharides Hexasaccharides Septasaccharides Octasaccharides	similar rearrangements and further transgalactosylations, including some side chain formation	

reassessment of the properties of the range of lactases available in different microorganisms. A summary review of lactose hydrolysis processes using lactases has been compiled by Sienkiewicz and Riedel (1990), including the use of permeabilization agents, immobilization of enzymes and membrane systems.

β-Galactosidases are generally found only in microorganisms and in the mammalian intestinal tract. However, there are some exceptions, such as the lactase derived from the marine mollusc, *Charonia lampas*. The choice of enzyme to use for the production of lactose hydrolysis products and of galacto-oligosaccharides is critical to a commercial process for the reasons outlined earlier. The use of these enzymes commercially for many years in lactose hydrolysis has resulted in the ready commercial availability of a number of well-described sources. Dominant among these is the yeast lactase from *Kluyveromyces* spp. and the fungal lactase from *Aspergillus* spp. For the large-scale commercial production of galacto-oligosaccharides, other microbial sources are used also (e.g., *B. circulans, S. thermophilus, C. laurentii* and *S. elviae*). A list of microorganisms used to produce lactase is shown in Table 5.5. The enzymes differ in their temperature optimum and their optimum pH, as well as their preference in cleavage of $\beta(1\rightarrow3)$ or $\beta(1\rightarrow4)$ linkages. This information, where it is known, is included in Table 5.5.

Table 5.5. Microbial sources of β-galactosidases used for the production of galacto-oligosaccharides

Microbial species	Strain name or trade name/s (if available)	Reference (see below)	Optimum pH	Optimum temp., °C	Reactive linkage and initial product
Kluyveromyces marxianus ssp. lactis	Maxilact GODO-YNL2	1	6.0–7.0	37–45	6′-galactosyl lactose
Kluyveromyces fragilis		2	6.9–7.3	40–44	6′-galactosyl lactose
Aspergillus oryzae	ACIDOLACT	3	3.5–6.5	50–55	6′-galactosyl lactose, and 3′-galactosyl lactose
Aspergillus niger		4	3.0–4.0	55–65	
Bacillus circulans	Biolacta N5	5	6.0	55–60	4′-galactosyl lactose
Bacillus stearothermophilus		6	6.0–6.4	50–65	4′-galactosyl lactose
Streptococcus thermophilus	TS2	7	6.5–7.5	55–57	3′-galactosyl lactose
Lactobacillus spp.		8	5.0–7.0	40	6′-galactosyl lactose
Bifidobacterium spp.	DSM 20083 NCIMB 41171	9	7.5 6.8–7.0	39 40	6′-galactosyl lactose
Cryptococcus laurentii	OKN-4	10	4.3	55–60	4′-galactosyl lactose
*Sporobolomyces singularis (*syn. *Bullera singularis*	mutants of ATCC 24193	11			4′-galactosyl lactose
Sterigmatomyces elviae	CBS 8119	12	4.5–5.0	80–85	4′-galactosyl lactose
Rhodotorula minuta	IFO 879	13			?
Geotrichum amycelium	ATCC 56046	14			?
Sirobasidium magnum	CBS 6803	15	4.5–5.5	65	4′-galactosyl lactose
Sulfolobus solfataricus	P-2	16	2.5–3.5	75–80	?
Thermotoga maritima	recombinant	17	6.0	80	?
Thermus sp.	Z-1 recombinant	18	6.0 5.0–6.0	70–80 70–90	?
Thermus caldophilus	GK-24 recombinant				

(*Continued*)

Table 5.5. (*Continued*)

Microbial species	Strain name or trade name/s (if available)	Reference (see below)	Optimum pH	Optimum temp., °C	Reactive linkage and initial product
Saccharopolyspora rectivirgula	SAM 1400	19	6.0–7.5	50–55	?
Escherichia coli		20	7.0	40–45	

1. Burvall *et al*., 1979; Asp *et al*., 1980; Prenosil *et al*., 1987b; **2**. Toba and Adachi, 1978; Mahoney *et al*., 1975; Mahoney and Whitaker, 1978; Prenosil *et al*., 1987b; **3**. Toba *et al*., 1985; Prenosil *et al*., 1987b; **4.** Toba and Adachi, 1978; Prenosil *et al*., 1987b; **5.** Griffiths and Muir, 1978; Mozaffar *et al*., 1984; Nakanishi *et al*., 1983; Usui *et al*., 1993; Yanahira *et al*., 1995; **6.** Griffiths and Muir, 1978; Sienkiewicz and Riedel, 1990; **7.** Greenberg and Mahoney, 1982, 1983; Smart, 1991; Linko *et al*., 1992; **8.** Toba *et al*., 1981; Kobayashi *et al*., 1990; **9**. Rabiu *et al*., 2001; Tzortzis *et al*., 2005a; **10.** Ohtsuka *et al*., 1990; Ozawa *et al*., 1991; **11**. Gorin *et al*., 1964; Shin *et al*., 1995; Shin *et al*., 1998; Cho *et al*., 2003; Ishikawa *et al*., 2005; **12.** Onishi and Tanaka, 1995; Onishi *et al*., 1995; **13**. Onishi and Tanaka, 1996; **14.** Onishi *et al*., 1995; **15.** Onishi *et al* 1996; Onishi and Tanaka, 1997; **16.** Akiyama *et al.,* 2001; Grogan, 1991; Pisani *et al*., 1990; She *et al*., 2001; **17**. Ji *et al*., 2005; **18**. Choi *et al*., 2004a; **19**. Nakayama *et al*., 1992, 1993; Nakao *et al*., 1994; **20**. Sienkiewicz and Riedel, 1990, p256; Saito *et al*., 1992.

5.4.1.3. Some Suppliers of β-Galactosidases (EC 3.2.1.23)

Examples of some suppliers of the enzyme are listed in Table 5.6. Generally, the fungal lactases function optimally at low pH and a relatively high temperature, whereas the yeast lactases perform best at neutral pH and a lower temperature. The yeast lactases respond to nutrient additions of magnesium and potassium ions.

5.4.1.4. Investigations of the Major Transgalactosylation Enzyme Sources

Kluyveromyces marxianus syn. *K. fragilis* and syn. *Candida pseudotropicalis* is an anamorph of *Candida kefyr*.
and
Kluyveromyces lactis syn. *K. marxiansus var. lactis.* is an anamorph of *Candida sphaerica* (NCBI, 2007)

These two yeasts were described in earlier years as belonging to the genus *Saccharomyces* and also have a number of synonymous names. *Saccharomyces* is still used sometimes as the generic name for these species. The β-galactosidase in these species is intracellular, and production is inducible. Thus, enzyme activity increases rapidly in the presence of carbohydrate sources, such as lactose, galactose or lactobionic acid. The activity is growth-associated and displays optimal activity at neutral pH and a temperature of about 40°C. The regulation of enzyme activity in these yeasts is

Table 5.6. Some suppliers of β-galactosidase enzymes

Catalogue No.	Source	Optimum pH	Recommended Reaction temperature (°C)
Seikagaku Corporation, Tokyo, Japan			
100570	jack bean, lactase	3.5	37
100572	*Charonia lampas (a marine mollusc), lactase*	3.6	45
100573	*Streptococcus* 6646 k, lactase	5.5	40
Sigma-Aldrich Corporation, USA			
G1560	*Aspergillus oryzae, lactase F*	4.0–4.5	30
G1875	bovine liver	7.3	37
G4142	bovine testes	4.4	25
G2513 etc	*Escherichia coli*, *lactase*	7.3	37
G3665	*Kluyveromyces lactis,* lactase	–	–
G3782	*Saccharomyces fragilis*, lactase	7.2	37
Megazyme International Ireland Ltd, Wicklow, Ireland			
E-LACTS	*Kluyveromyces fragilis,* lactase	6.5–7.0	45
E-BGLAN	*Aspergillus niger*	4.5	60
Boehringer Mannheim GmbH, Mannheim, Germany			
903 345	bovine testes	4.3	37
1 088 718	*Diplococcus pneumoniae*	6.0-6.5	37
105 031 and 634 395	*E. coli* overproducer, lactase	7.0	25
Novozymes A/S, Bagsvaerd, Denmark			
Lactozym		6.5	37
Enzyme Solutions, Melbourne, Australia			
GODO YNL	*Kluyveromyces fragilis,* lactase	6.5–7.0	37–45
ACIDOLACT	*Aspergillus oryzae,* lactase	4.5–5.0	50–55

complex and responds to ionic concentrations of a range of elements, namely, K, Mn and Mg. The enzymes are inhibited by Na^+ and Ca^{2+}. Finkelman (1989) has described the process of strain selection of yeasts for maximal enzyme activity. Although these yeast lactases suffer from low acid and temperature tolerance, and operate at neutral pH, this industrial disadvantage is outweighed by the high yields, the ease of cultivation and the established safety record of the yeasts. Yeast lactases from *Kluyveromyces* remain the most popular source of the enzyme. There is considerable strain-to-strain variation in the activity of the β-galactosidase present in the strain, and much research has been conducted to select the best strains.

One of the first descriptions of the use of these enzymes for the production of galacto-oligosaccharides is that of Roberts and Pettinati (1957).

Recently, Rodriguez *et al.* (2006) expressed the intracellular β-galactosidase of *k. lactis* as an extracellular enzyme in *Aspergillus niger*.

Aspergillus oryzae and *Aspergillus niger*

β-Galactosidases from both of these fungal species have been used for the production of galacto-oligosaccharides, but the enzyme from *A. oryzae* has been the one mostly used in industry to produce galacto-oligosaccharides. Consequently, it has been studied extensively (Toba and Adachi, 1978; Toba *et al*., 1985; Prenosil *et al*., 1987b; Sienkiewicz and Riedel, 1990).

Enzymes from *A. oryzae* include glycosidases which may act as α-galactosidases or as β-galactosidases. They can also express α-mannosidase activity and can perform fructosidation as well as galactosidation reactions. Their β-galactosidase is extracellular, in contrast to the intracellular enzyme in yeasts.

The mould lactases of *Aspergillus* are more thermostable and acid stable than the *Kluyveromyces* lactases and are less exacting in their requirements for activators and stabilizers. They are produced by solid-substrate fermentation and, hence, the enzyme yields and activities are lower than those obtained with *Kluyveromyces* fermentations. In immobilized enzyme systems, these *Aspergillus* lactases show good stability. From an industrial viewpoint, their maximum operating temperature under long-term exposure was still considered too low for optimal reaction kinetics.

Bacillus circulans

Bacillus circulans is an aerobic, Gram-positive, spore-forming organism that has been investigated as a source of active enzymes for a number of biotechnological products. Nakanishi *et al.* (1983) and Mozaffar *et al.* (1984) first reported that β-galactosidase from *B. circulans* synthesizes oligosaccharides from lactose, but did not describe their structures. Subsequently, these have been described further by Sakai *et al*. (1992) and Usui *et al*. (1993) who reported that this β-galactosidase preferentially formed a β(1→4) linked galactosyl-disaccharide. Yanahira *et al*. (1995) described 11 oligosaccharides formed in the reaction, including 5 newly described oligosaccharides ranging in size up to an octasaccharide. They noted that although 95% of the trisaccharide initially formed was a 4′-galactosyl lactose, after time, trisaccharides with 3, 2 and 6 linkages occurred. This finding emphasizes the importance of the duration of the reaction on the final structural composition of the oligosaccharides formed. Griffiths and Muir (1978) examined the thermostability of the β-galactosidase of a thermophilic *Bacillus* and compared the properties of the enzyme in whole cells and in cells entrapped in polyacrylamide gel. They noted optimal activity of the enzyme at 65–75°C and at pH 6.2–6.6.

Streptococcus thermophilus

S. thermophilus is used widely in fermented dairy foods and is a GRAS organism. The use of non-GRAS status bacteria limits their commercial use in many countries. For example, the use of *Escherichia coli* strains is not permitted in the food industry in most countries. The β-galactosidase of *S. thermophilus* is a useful enzyme for commercial hydrolysis of lactose in milk and cheese whey (Greenberg and Mahoney, 1982). These authors pointed out that the thermal stability of the enzyme is greater in milk and whey than in buffer. The enzyme from this bacterium is more heat stable than that of *K. marxianus* (Linko *et al*., 1992) and it is produced intracellularly. The inhibitors and stimulants of the enzyme have been studied (Greenberg and Mahoney, 1982). The (1→3) glycosyl linkage is cleaved first by this enzyme, but later it also cleaves the (1→4) and (1→6) linkages.

The ability of the β-galactosidase of *S. thermophilus* to undergo transgalactosylation reactions was recognized by Toba *et al*. (1981) and by Greenberg and Mahoney (1983) and further studied by Smart and co-workers in more detail (Smart, 1989, 1991, 1993; Garman *et al*., 1996). Playne *et al*. (1993) have also described successful laboratory production of oligosaccharide mixtures from lactose using a crude enzyme extract from a strain of *S. thermophilus*. Oligosaccharide production was higher than that by commercial enzymes from *Aspergillus* and *Kluyveromyces*. The company, Yakult Honsha Co., Ltd., uses a β-galactosidase from *S. thermophilus* as well as an enzyme from *A. oryzae* in a two-stage process to produce their oligosaccharide product (Matsumoto *et al.*, 1993).

Because of its long-standing importance in the manufacture of yoghurt and in other fermented dairy products, a considerable body of knowledge has been established for *S. thermophilus*. The complete genomic sequences for two strains have been determined (Bolotin *et al*., 2004). This genomic analysis allows a new understanding of its biochemistry and physiology.

Lactobacillus Species

Most species of *Lactobacillus* possess high β-galactosidase activity. Thus, there have been a number of investigations of enzymes from this source and their possible application for the production of β-galacto-oligosaccharides (Toba *et al*., 1981). Garman *et al*. (1996), in a study of a number of species of *Lactobacillus* and *S. thermophilus,* found that a strain of *Lb. delbrueckii* subsp. *bulgaricus* possessed a β-galactosidase with transgalactosylation activity similar to the enzyme from *S. thermophilus*. As an example of the use of

lactobacilli, Kobayashi *et al.* (1990) patented a method for producing a processed milk containing galacto-oligosaccharide. In their patent, milk was treated with a β-galactosidase derived from *S. thermophilus* or *Lb. delbrueckii* subsp. *bulgaricus* so as to change at least 15% of the lactose in the milk into galacto-oligosaccharide. The *Lactobacillus* enzyme was found to be useful as it performed transgalactosylation reactions even when the lactose concentration was quite low. Other enzyme sources, such as that from the well-established *A. oryzae*, act largely hydrolytically when the lactose is in low concentrations as found in milk.

Bifidobacterium Species

Tzortzis *et al.* (2005a) have used whole cells of *B. bifidum* NCIMB 41171 to produce galacto-oligosaccharides from lactose. Optimum enzyme activity occurred at pH 6.8–7.0 and 40°C. A 50% (w/w) solution of lactose gave a 20% mixture of oligosaccharides. The mixture comprised 25% disaccharides (other than lactose), 35% trisaccharides, 25% tetrasaccharides and 15% pentasaccharides. These proportions seemed to be produced consistently by this organism. Current interest in bifidobacteria as a source of β-galactosidase may be generated by the ability of at least some strains of *Bifidobacterium* to produce higher proportions of longer chain oligosaccharides than most other lactases studied to date.

Van Laere *et al.* (2000) studied the ability of several strains of *Bifidobacterium* to metabolize a GOS mixture, which had been purified so that it contained 6% tri-, 17% tetra-, 37% penta-, 27% hexa- and 8.5% hepta-oligosaccharide. These proportions were different from most commercial GOS products which contain predominantly trisaccharide and only low concentrations of the higher oligosaccharides. This study found that *Bifidobacterium adolescentis* DSM 20083 could utilize the higher oligosaccharides better than other species. Thus, it may be that longer chain oligosaccharides are of value for the manufacture of particular symbiotic mixtures of prebiotic and probiotic, aimed to stimulate the growth of particular strains of bifidobacteria.

Rabiu *et al.* (2001) have shown, in a study of five strains of *Bifidobacterium*, that their β-galactosidases predominantly produced (1→6) GalP linkages as opposed to the (1→4) linkages generated by the β-galactosidases of many microbial species (see Table 5.4). These authors also noted the production of unusual higher chain oligosaccharides by these bifidobacterial enzymes. Tzortzis *et al.* (2005b) also recorded mostly Gal β(1→6) Gal linkages in their oligosaccharide mixture produced by *B. bifidum* NCIMB 41171.

5.4.1.4.1 Other Yeasts

Cryptococcus laurentii

The production of galactose transfer products by *C. laurentii* IFO 609 has been examined by Onishi and Yokozeki (1992). The enzyme of this species produces a 4′-galactosyl lactose from lactose. A yield of 47 g/L of 4′-galactosyl lactose was produced from 100 g/L of lactose in studies using *C. laurentii* OKN-4 conducted by the Nissin Sugar Manuf. Co. Ltd., Japan (Ozawa *et al*., 1991; Ohtsuka *et al.,* 1992).

This yeast can be an opportunistic pathogen in immuno-compromised patients. It can cause superficial infections and is described as causing fungemia. It has been implicated in meningitis and is regarded as a human pathogen. Thus, its direct use as a food-processing aid is restricted.

Sporobolomyces singularis (syn. *Bullera singularis*)

It has long been recognized that this basidiomycetous yeast possesses a β-hexosidase able to behave with galactosidase-like activity (Gorin *et al*., 1964). The nature of this action has been investigated in detail by Ishikawa *et al*. (2005). It seems that the basidiomycetous yeasts commonly possess a β-glucosidase which performs like a β-galactosidase when presented with lactose as a substrate and is a strong producer of galacto-oligosaccharides. Dombou *et al*. (1994) described a method for the production of galacto-oligosaccharides from lactose using basidiomycetous yeasts. Preferred yeasts belonged to the genera of *Rhodotorula, Pichia, Sporobolomyces, Kluyveromyces, Debaryomyces, Candida, Torulopsis, Cryptococcus, Trichosporon, Lipomyces* and *Brettanomyces*. The inventors preferred an isolate of *Lipomyces starkeyi* for the description of the invention.

The purification and biochemical properties of a galacto-oligosaccharide-producing β-galactosidase from *B. singularis* has been described by Cho *et al*. (2003). Shin *et al*. (1995, 1998) examined optimal culture conditions for *B. singularis* and continuous production of galacto-oligosaccharides in a chitosan-immobilized system for the enzyme.

Sterigmatomyces elviae

The basidiomycete species *Sterigmatomyces* has been investigated for its potential as a source of a β-galactosidase for the production of galacto-oligosaccharides (Onishi and Tanaka, 1995; Onishi *et al*., 1995). The main transgalactosylation product was a 4′-galactosyl lactose. In comparison with a number of other bacteria and yeasts, the authors considered the enzyme from *S. elviae* CBS 8119 to be the best galacto-oligosaccharide producer. The

other high-yielding enzymes were from the yeast species *Rhodotorula minuta* and *Sirobasidium magnum*. With the enzyme from the *Sterigmatomyces* sp. strain, the optimum reaction temperature was 80°C, but the yield was only 37%, which occurred when cells were permeabilized with toluene and resting cells used to produce the oligosaccharide. An improved fermentation system where cell growth consumed excess glucose resulted in an increased yield of 64%. Onishi and Tanaka (1995) purified the β-galactosidase from the *Sterigmatomyces* strain and examined its properties in more detail. They noted that the yeast would not grow above 40°C despite having an optimum temperature for the enzyme in the toluene-permeabilized resting cells of 80°C. They found that optimal pH for activity was between 4.5 and 5.0. The thermostable nature of the enzyme from a mesophile was found to be unusual as most thermostable enzymes were derived from thermophiles. The enzyme was similar to that found in *C. laurentii* except that it was far more thermo-tolerant, and for this reason was regarded as a superior enzyme.

Rhodotorula minuta

Onishi and Tanaka (1996) reported the properties of a glycosidase that can produce galacto-oligosaccharides from a strain of this yeast species. This basidomycete is found in the environment and in dairy products. It may colonize plants, humans and other mammals. While being considered as a common contaminant, *Rhodotorula* may infect individuals with predisposing risk factors. For this reason, it may not be approved by regulatory authorities for the production of a food-processing aid.

Geotrichum amycelium (syn. *Trichosporon ovoides*)

This is another basidiomycetous yeast claimed to be able to produce substantial yields of galacto-oligosaccharides from lactose. However, there is only limited published information on this enzyme activity of the species (Onishi *et al.*, 1995).

Sirobasidium magnum

Research conducted at the laboratories of the Ajinomoto Co. Inc. in Japan has demonstrated the potential of this basidiomycetous yeast as a source of a β-galactosidase to produce galacto-oligosaccharide (Onishi *et al.*, 1996) A 4′-galactosyl lactose was produced with a yield of over 200 g/L at up to 50°C using toluene-treated cells to improve cell wall permeability and using glucose oxidase to remove glucose formed as a hydrolysis product. In the following year, the group purified the β-galactosidase and found that the optimum

conditions for enzyme activity were 65°C and pH 4.5–5.5 (Onishi and Tanaka, 1997).

A patent for the production of galacto-oligosaccharides was taken out by Onishi and Yokozeki (1992); it included *Sirobasidium* with a number of other species.

5.4.1.4.2 The Extremophiles

Microorganisms isolated from environmentally extreme conditions are of potential use for the industrial production of galacto-oligosaccharides. A number of these have been studied with this objective. It would be a major advantage if enzymatic conversions to oligosaccharides by the equilibrium route could be performed at a very high temperature under acidic conditions. A number of thermophiles have been examined for suitable β-galactosidases, including strains of *Thermus, Thermoaerobacter, Sulfolobus* and *Thermotoga* spp. Examples are given below.

Sulfolobus solfataricus

Sulfolobales are hyperthermophilic archaea from terrestrial volcanic sites that grow in sulfur-rich hot acid springs, with optimum growth at 75–80°C and pH 2–3. *S. solfataricus* grows optimally at temperatures ranging from 70 to 90°C and at pH 2–4. It can grow either lithoautotrophically by oxidizing sulphur or chemoheterotrophically on reduced carbon compounds. Pisani *et al*. (1990) studied the properties of a β-galactosidase in the species and found it to be thermostable. Grogan (1991) examined in more detail the properties of the β-glycosidase present and found that the same enzyme exhibited both β-galactosidase and β-glucosidase activity. He also noted the optimal reaction temperature to be 77–87°C and the optimal pH for β-galactosidase activity to be pH 4.9. Other thermophilic microorganisms such as *Thermotoga maritima* can also produce galacto-oligosaccharides by a thermostable recombinant β-galactosidase (She *et al*., 2001).

Thermotoga maritima

Thermotoga maritima, a rod-shaped bacterium belonging to the order Thermotogales, was originally isolated from a geothermal marine sediment. The organism has an optimum growth temperature of 80°C. The species metabolizes many simple and complex carbohydrates.

Ji *et al*. (2005) prepared a recombinant β-galactosidase from a strain of *T. maritima* in *E. coli*. They determined the stability and productivity of this enzyme at a range of pH and up to 95°C. Optimal conditions were pH 6 at

80°C in the presence of manganese ions. Lactose at 500 g/L yielded 91 g/L galacto-oligosaccharides in 300 min with 1.5 units enzyme/ml.

Thermus Species

The production of galacto-oligosaccharides has also been explored in strains of *Thermus* species. Recombinant production of a thermostable β-glucosidase, expressed in *E. coli* K 12, has been used to investigate the production of galacto-oligosaccharides from lactose. The yield of galacto-oligosaccharides from 300 g/L lactose at 70°C was 40% and trisaccharides comprised two-thirds of the products formed (Akiyama *et al*., 2001). Choi *et al*. (2004a) have also developed a similar method with the production of a recombinant enzyme from a β-glycosidase in *Thermus caldophilus.*

5.4.1.4.3 Thermoactinomycetes

Saccharopolyspora rectivirgula

Some thermoactinomycetes have been shown to produce β-galactosidases which have high β-D-galactosyltransferase activity, high heat stability and which can act in the neutral pH range. Some strains from the genera *Saccharopolyspora, Thermomonospora* and *Thermoactinomyces* have been found to possess effective enzymes. In particular, a strain of *S. rectivirgula* has been studied (Nakayama *et al*., 1992, 1993; Nakao *et al*., 1994). Nakayama *et al*. (1993) pointed out that other β-galactosidases derived from species such as *Paecilomyces variori* also possess high heat stability and are capable of repeated use at high temperatures, but that the *Paecilomyces* enzyme is not suitable for many applications because it is effective only at low pH (3.5). The most advantageous property of the *Saccharopolyspora* enzyme is its heat stability over long periods. Nakao *et al.* (1994) reported a 41% yield of galacto-oligosaccharides from 1.75 M lactose.

Saccharopolyspora rectivirgula was previously named *Faenia rectivirgula.* Species of *Saccharopolyspora* are implicated as causal agents of infection for a condition known as farmer's lung disease. In nature, the species is found in mouldy hay. It can also cause allergic reactions in humans.

5.4.1.5. Development of Modified Galacto-oligosaccharide Structures

There is considerable scope for the development of novel galacto-oligosaccharides with specific functionalities for human health. Oligosaccharides have been developed to act as alternative receptors to absorb lectin-like toxins from toxigenic bacteria (VTEC, ETEC, *Clostridium difficile*)

in the gut. Zopf and Roth (1996) have discussed the use of oligosaccharides as anti-infective agents, using a decoy oligosaccharide in the mucous layer to bind the pathogen's carbohydrate-binding proteins. They claim that such oligosaccharides (as found in human milk) seem to prevent pathogens attaching to intestinal mucosa. Kunz and Radloff (1996) state that "Human oligosaccharides are considered to be soluble receptor analogues of epithelial cell surfaces, participating in the non-immunological defence system of human milk-fed infants". Their view is that these specialized oligosaccharides are potent inhibitors of bacterial adhesion to epithelial cells. Thus, there is scope for specifically designed galacto-oligosaccharides to be able to act as anti-infective reagents, particularly in the small intestine. However, care needs to be taken to ensure that the oligosaccharide did not also interfere with colonization in the large intestine by the normal intestinal microbiota, including any added probiotics.

Thus, there are opportunities for the synthesis of galacto-oligosaccharides which more closely mimic the structures found in human milk. Human milk contains 5–12 g/L oligosaccharides, and colostrum has even higher concentrations, but cow's milk contains very little (0.03–0.06 g/L) and most of that is as sialyl lactose. The monomers of human milk oligosaccharides are D-glucose, D-galactose, *N*-acetylglucosamine, L-fucose and sialic acid (*N*-acetyl neuraminic acid). The core oligosaccharide molecule normally carries lactose at its reducing end and generally has the following structure:

$$[\mathrm{Gal}(\beta 1 \rightarrow 3/4)\mathrm{GlcNAc}(\beta 1 \rightarrow]_{n=0-25}3/6)\mathrm{Gal}(\beta 1 \rightarrow 4)\mathrm{Glc}$$

Both fucose and sialic acid can attach in a number of different ways to this core. Predominantly, fucose attaches with an $\alpha 1 \rightarrow 2$ linkage to galactose, but with $\alpha 1 \rightarrow 3/4$ linkages to *N*-acetylglucosamine. Readers are referred to Kunz and Rudloff (2002) and Boehm and Stahl (2003) for more details on possible oligosaccharide structures. The major oligosaccharides in human milk are lacto-*N*-tetraose and lacto-*N*-fucopentaose (Kunz and Radloff, 1996) (see also Chapter 8).

There is scope for manufacturers to develop cost-effective procedures to produce a range of fuco-galacto-oligosaccharides. At present, procedures are multi-step processes and are expensive (Crout and Vic, 1998). Of relevance to this chapter is the possibility of economic manufacture of fucose-containing and *N*-acetylglucosamine-containing galacto-oligosaccharides.

The use of recombinant DNA technology to modify the metabolic pathways of microorganisms for oligosaccharide synthesis is complex, and hence expensive. It also involves the use of sugar nucleotides, such as UDP, as cofactors in the enzyme reactions. Use of whole microbial cells reduces costs, and concentrations of the synthesized oligosaccharide obtained are

increasing (Ruffing and Chen, 2006). For example, Koizumi *et al.* (1998) achieved a yield of 188 g/L of the galacto-oligosaccharide, globotriose (Galα1→4Galβ1→4Glc) and no oligosaccharide by-products were observed. The synthesis simultaneously produced high yields of UDP-galactose from galactose and orotic acid.

Crout and Vic (1998) favoured the use of glycosidases over glycosyltransferases because they are better suited to cheap synthesis methods. They also point out that *exo*-glycosidases currently used commercially only allow glycosyl transfer at the non-reducing terminal monomer of the substrate, and this restricts the types of oligosaccharide structures that can be produced. They advocate the use of *endo*-glycosidases which may allow branched structures to be formed. To improve the reverse hydrolysis required for this type of synthesis, organic solvents have been used in a number of studies (Crout and Vic, 1998). The use of glycosidases with novel approaches may lead to synthesis of useful new oligosaccharides.

For the synthesis of a fucose-containing galacto-oligosaccharide, it will first be necessary to produce the L-fucose required. L-fucose occurs in nature only at low concentrations and is present in plant species of the *Convolvulaceae* family. On the other hand, D-fucose is found in some seaweeds or can be produced from D-galactose. Fucose is a hexose deoxy sugar (Figure 5.3) and

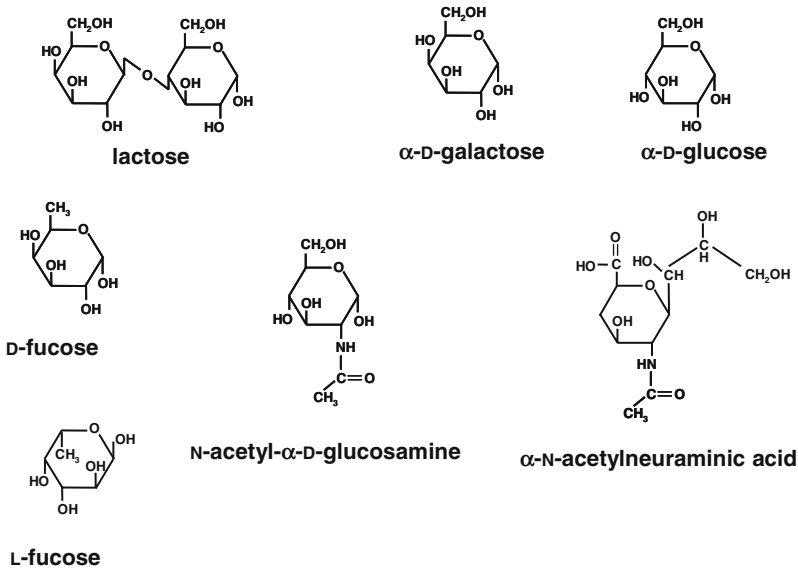

Figure 5.3. Chemical structures (Haworth) of compounds found in human milk (fucose, *N*-acetyl glucosamine and *N*-acetyl neuraminic acid) compared to those of lactose, galactose and glucose.

can be formed using an α-fucosidase. While one can envisage synthesis of D-fucose from D-galactose, it is difficult to see how L-fucose could be formed easily.

Economic sources of *N*-acetylglucosamine may be chitin in the shells of crustaceans. β-1,4-Linked *N*-acetyl-D-glucosamine can be derived readily from chitosan (Izume *et al*., 1992) and it may be feasible to link it enzymatically to galacto-oligosaccharides during their synthesis. There is much scope to investigate such methods in order to produce a new generation of modified galacto-oligosaccharide products. Chemical structures of L- and D-fucose, D-glucose, D-galactose, *N*-acetylglucosamine, *N*-acetyl neuraminic acid are shown in Figure 5.3.

5.4.2. Lactulose

The disaccharide, lactulose (Figure 5.4), is made from lactose by a semi-synthetic isomerization reaction under alkaline conditions. Lactulose can also be formed in small quantities in milk which has been heat-treated, e.g., UHT milk. Lactulose is used primarily as a pharmaceutical, but also as a

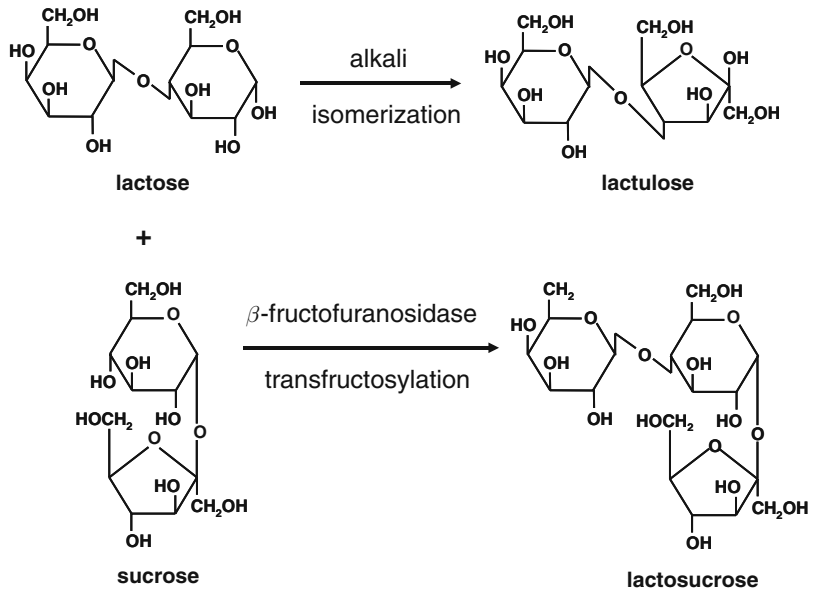

Figure 5.4. Chemical structures (Haworth) of lactulose and its conversion from lactose, and of lacto-sucrose and its formation from lactose and sucrose.

functional food ingredient. The Solvay Company started manufacturing lactulose in Europe in the 1960s and produces about 50% of world production (see Table 5.8). A considerable body of research has evolved over the last 40 years to improve yields and the purity and safety of the product, and reduce costs. The essential principle of the synthesis has remained the same – alkaline isomerization using heated solutions. These isomerization reactions to convert lactose to lactulose all produce a number of side products derived from lactose, principally galactose, epi-lactose, tagatose and fructose. Epi-lactose is another isomer of lactose (4-*O*-β-D-galactopyranosyl-D-mannose).

Lactulose was first prepared by a Lobry deBruyn–Alberda van Ekenstein alkaline isomerization of lactose in dilute $Ca(OH)_2$ solution (Montgomery and Hudson, 1930). Strong alkalis, such as NaOH, and strong organic bases have also been used in the isomerization reaction. All these methods resulted in low yields of lactulose because of side reactions which lead to the formation of a number of unwanted products. Separation and purification methods were required to remove the unwanted products and the brownish colour occurring due to these side products.

Subsequent methods for producing lactulose included complexing reagents, such as aluminate (Guth and Tumerman, 1970) and borate ions (Mendicino, 1960; Carubelli, 1966; Kozempel and Kurantz, 1994; Carobbi *et al.*, 2001), to shift the transient equilibrium established during base-catalysed isomerization in favour of the ketose. The yield of lactulose was much improved, but both aluminates and borates were difficult to remove from the reaction mixture. A very high ratio of borate to sugar (e.g., 50:1) was required for optimum yields of lactulose. Under such conditions, removal of the borate was very difficult, and the cost of borate proved prohibitive. This meant that methods to recycle the borate had to be devised.

Considerable research has been conducted to improve the alkaline isomerization process. Efforts have concentrated on reducing the costs of the complexing agent, borate (Hicks *et al.*, 1986). For example, Dorscheid and Krumbholz (1991) used a combination of electrodialysis and ion exchange to purify the lactulose and recycle the borate. Other methods have been devised to remove borate by precipitation as insoluble salts (Fu and Song, 1993). The yield of lactulose has often been quite low (<20%).

Amines have been used as catalysts for the isomerization process. However, only the tertiary amine, triethylamine, avoided the problems of the primary and secondary amines, which formed adducts with reducing sugars (Parrish, 1970)

Later, the combination of the isomerization of lactose in the presence of borate (at a molar ratio of 1:1) with a tertiary amine (Hicks and Parrish, 1980; Hicks, 1981) gave 90% yield of lactulose with a minimal use of borate and reduced the formation of side products.

Other reagents such as basic magnesium salts and sodium hydrosulphite have also been used (Carobbi *et al*., 1985). Whilst offering some advantages, these compounds also created new problems of purification and of disposal of spent materials in an environmentally friendly way.

Attempts have been made to develop continuous reaction systems, as opposed to batch reactors. One example is the dual reactor tank system coupled to a tubular reactor described by Kozempel and Kurantz (1994). The yield of lactulose from lactose was 70% in this system.

Developing commercially viable processes has proved difficult, but the use of borate appears to be the preferred approach, mainly because high yields are obtained. Effective separation of lactulose from the product mix has proved very difficult to achieve economically. Most pharmaceutical-grade lactulose syrups contain substantial concentrations of lactose, epi-lactose and galactose.

Research has been conducted to develop better methods to obtain lactulose in powder or crystalline forms. As lactulose is highly soluble in water, it is difficult to obtain in the form of a stable powder and it is generally produced as a syrup. Nevertheless, complicated processes have been developed to prepare powdered or crystallized products. Crystallization methods using alcohols, such as ethanol and methanol, have been developed to obtain lactulose crystals. Highly pure lactulose crystals can be obtained, but contamination with alcohol remains a problem. Another problem is the presence of lactose, galactose and other side products which have to be removed also, and this adds to the cost. Alternative methods that avoid the use of alcohols have been developed. These are based on seeding crystals into the concentrated syrup and then freezing the mixture. The Morinaga Company has developed a process to form crystalline lactulose trihydrate. This product, it is claimed, is purer and more stable under atmospheric storage conditions than anhydrous lactulose or the monohydrate. The process is based on a crystallization precipitation procedure (Tomito *et al*., 1994).

Lactulose can also be prepared by an enzymatic route (Lee *et al*., 2004), but this route is not used currently for commercial production.

5.4.3. Lactosucrose (β-D-Gal-(1→4)-α-D-Glu-(1→2)-β-D-Fru)

Lactosucrose is a trisaccharide produced enzymatically by transfer of the fructosyl moiety of sucrose to lactose as an acceptor molecule. The structure is shown in Figure 5.4. The enzyme used is a β-fructofuranosidase (EC 3.2.1.26). It is known to conduct the transfructosylation reaction, as well as hydrolysis, in a manner analogous to that described for β-galactosidase. Similarly, the microbial origin of the fructofuranosidase is important. Enzymes from different sources exhibit different degrees of acceptor types

and specificity. Some may prefer lactose as an acceptor, others sucrose. Thus, research has been conducted into the characteristics of the enzyme from a number of microorganisms (Takaichi *et al.*, 1995; Choi *et al.*, 2004b; Park *et al.*, 2005). The reaction conditions, such as substrate concentration and reaction time, are also important (Fujita *et al.*, 1992a). The β-fructofuranosidase is sometimes called "levansucrase". Microorganisms belonging to the genera *Arthrobacter* and *Bacillus* are mostly used both in research and industrially to produce β-fructofuranosidase (Fujita *et al.*, 1992b; Hara *et al.*, 1992; Kawase *et al.*, 2001; Pilgrim *et al.*, 2001). A variety of methods have been used in production – some in which whole microbial cells are used, others have used crude cell extracts and still others have used purified enzyme. Fujita *et al.* (1992a) describe an industrial method for producing lactosucrose using β-fructofuranosidase from *Arthrobacter* sp. strain K1, which seems to be the one used by Ensuiko Sugar Refining Co., Ltd. and by the Hayashibara Company. These two Japanese-based companies are the main producers of commercial lactosucrose. The yield of lactosucrose from the two substrates (sucrose and lactose) is low (5–30% of total sugars in the reactants). By careful selection of strains and the use of optimal conditions, batch reactions have been able to produce 181 g lactosucrose from 225 g sucrose and 225 g lactose (40% conversion), but the concentration of lactosucrose in the reactor has only been about 18% (see Park *et al.*, 2005). Those authors used a strain of *B. subtilis* at pH 6 and 55°C for a 10 h reaction. This result is typical of the published data for batch reactions. Kawase *et al.* (2001), who described the production of lactosucrose using a simulated moving bed reactor, claim that this continuous process increases the yield to 56% compared to 48% typical of a batch fermentation.

There is a major problem with the presence of other carbohydrate products and residual amounts of unused lactose and sucrose in the process. Thus, it is typical for crude product to be decolourized, demineralized and purified by column chromatography using a strongly acidic cation-exchange resin. A fermentation method to remove monosaccharides, like glucose, has also been used. The lactosucrose is spray dried (Hara *et al.*, 1992, 1994a). The aim is to obtain a purified solution of lactosucrose of 45%, w/v, prior to spray drying. The two major producers sell lactosucrose as a powder with different degrees of purity (40, 55 or about 70% lactosucrose in the powder). Thus, a considerable concentration of other carbohydrates remains in the product.

In a series of publications, Petzelbauer *et al.* (1999, 2000, 2002a,b) used thermostable purified enzymes from *S. solfataricus* and *Pyrococcus furiosus* to produce lactosucrose in a continuous stirred-tank reactor at 70°C, coupled to a cross-flow ultrafiltration module. They also used an immobilized enzyme system. Their data do not show that they were able to achieve the yields obtained in commercial systems. However, they demonstrated the stability of

these thermostable enzymes at high temperatures for a prolonged period under realistic bioprocessing conditions.

The properties of the β-fructofuranosidase extracted from *Arthrobacter* sp. K1 have been described by Fujita *et al.* (1990a). The enzyme has an isoelectric point of 4.3, an optimum pH of 6.5–6.8, but the enzyme remains stable between pH 5.5 and 10.00. It has an optimum temperature of 55°C, but is stable between 45 and 60°C. It is inhibited by several heavy metals. Fujita *et al.* (1990b) also describe the acceptor specificity for a wide range of mono- and oligo-saccharides and glycosides.

5.4.4. Lactitol

Lactitol (Figure 5.5) is well established as a replacement sweetener for low-calorie foods. In recent years, interest in it as a prebiotic carbohydrate has developed. This sugar alcohol was discovered in 1912 and was first used in foods in the 1980s. It is formed when lactose is hydrogenated in the presence of Raney-nickel catalyst. The conversion of a sugar to a sugar alcohol always involves the reduction of a carbonyl group. The preferred reducing agent is hydrogen gas under high pressure (e.g., 40 bar) at 100°C in the presence of a nickel catalyst. This synthesis has been used widely at both laboratory and industrial scale for many years. Ipatiew (1912) first produced a lactitol syrup by such a process, but lactitol was first crystallized by Senderens (1920). Lactitol is produced either as a syrup, as dihydrate or monohydrate crystals or in anhydrous form. Van Velthuijsen (1979) has described a typical modern industrial process and specifications of the product obtained in such processes. Less than 2.5% other polyols and 0.1% reducing sugars are present in the food-grade lactitol products which are 97.5% lactitol. Thus, this

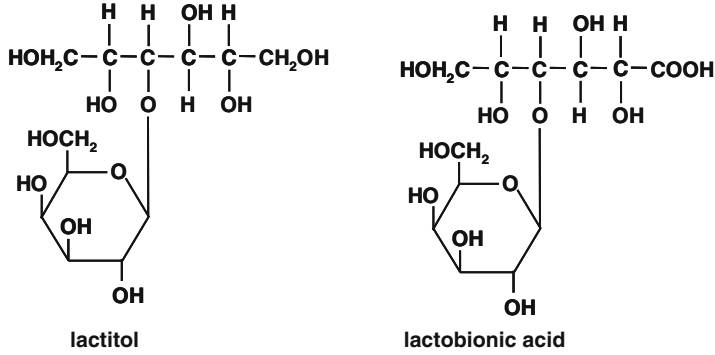

Figure 5.5. The chemical structures (Haworth) of lactitol and lactobionic acid.

compound is sold as a much purer product for food-grade use than galacto-oligosaccharides, lactosucrose and lactulose. The food grades of these other products all contain substantial amounts of other reactant and product carbohydrates. Lactitol can also be prepared by reducing lactose using $NaBH_4$ (Scholnick *et al.*, 1975; Saijonmaa *et al.*, 1978), but industrially, lactitol is prepared by the catalytic hydrogenation process (Van Velthuijsen, 1979). The reaction is carried out in an autoclave at over 40 bar and over 100°C. A lactose solution of 30–40% is used. The ratio of Raney-nickel catalyst to lactose is critical for efficient conversion. After hydrogenation is completed, the catalyst is sedimented and filtered. The lactitol solution is treated with an ion-exchange resin and activated carbon, and the purified solution is concentrated. Crystals of lactitol are removed from the mother liquor by centrifugation and the process repeated. After repeated crystallization, the mother liquor can be used as a 64% syrup of lactitol (Van Velthuijsen, 1979; Booy, 1987).

Preparation of derivatives of lactitol has also been studied. Polyalcohol esters can be used as non-ionic emulsifying agents. For example, sorbitan esters and sucrose esters of fatty acids are well known. Van Velthuijsen (1979) described the preparation of esters like lactitol palmitate and their properties and possible applications as laundry detergents.

Research has also demonstrated the formation of a range of oligosaccharides from lactitol using β-galactosidase (Yanahira *et al.*, 1992); they were able to form six different oligosaccharides – all as a trisaccharide containing a lactitol unit. The general chemistry and properties of the sugar alcohols are described by Benson (1978). Comprehensive reviews on lactitol are by Van Velthuijsen (1979), Booy (1987) and Mesters and Brokx (2000).

5.4.5. Lactobionic Acid

Lactobionic acid is formed by oxidation of lactose and is 4-*O*-D-galactopyranosyl-D-gluconic acid (Figure 5.5). Heterogeneous catalytic oxidation and microbiological/enzymatic oxidation of lactose have been researched. The facile dehydrogenation of lactose at high pH over a noble metal catalyst is used commercially (Figure 5.1).

The aldehyde group of the glucose in the lactose molecule is oxidized to the carboxyl group by either (a) chemical oxidation or (b) biochemical oxidation. Electrolytic methods of oxidation are also possible. For chemical oxidation, a mild treatment with hypobromite or hypoiodite produces an equilibrium mixture of lactobionic acid and its δ-lactone. Biochemical oxidation can be achieved with enzymes isolated from microorganisms or by using the microorganisms themselves for the bioconversion. For the latter process,

a number of species of *Pseudomonas* have been studied extensively (Sienkiewicz and Riedel, 1990). Some examples of recent research are given below.

An ultrafiltration membrane bioreactor was used in batch, fed-batch or continuous modes to oxidize aldose sugars to their corresponding aldonic acids. The enzyme used for the reaction was glucose-fructose oxidase obtained from a *Zymomonas mobilis* strain. The enzyme was selective and a high yield of lactobionic acid was obtained from lactose (Satory *et al.*, 1997).

A novel enzymatic process to convert lactose to lactobionic acid has been developed using cellobiose dehydrogenase. The electron acceptor in the reaction is regenerated in a continuous process with laccase, a copper-containing oxidase. Specific productivity to lactobionic acid was high at 25 g/L/h/kU (Ludwig *et al.*, 2004).

Miyamoto *et al.* (2000) reported the laboratory-scale production of lactobionic acid from cheese whey and from lactose in fed-batch cultures of *Pseudomonas* sp. LS13-1. The yield of lactobionic acid was reported to exceed 80%. The reaction time was 155 h, and concentrations achieved reached 290 g/L lactobionic acid.

5.4.6. Tagatose

Galactose, produced by hydrolysis of lactose and removal of the glucose co-product, is used as the substrate to produce tagatose (Figures 5.1 and 5.6). The galactose is isomerized under alkaline conditions, using, for example, $Ca(OH)_2$, to form tagatose. The mixture is purified and solid tagatose is produced by crystallization. Considerations for the efficient production of tagatose parallel those described in Section 5.4.2 for the production of lactulose from lactose. This was the original commercial method used by Spherix Inc. and there are patents that describe it in more detail (Beadle *et al.*, 1991, 1992).

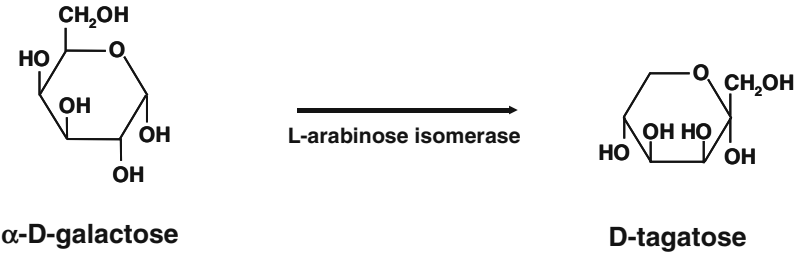

Figure 5.6. The chemical structures (Haworth) of D-tagatose and D-galactose.

More recently, enzymatic methods have been developed (Bertelsen *et al.*, 2006), including the use of thermostable isomerases (Pyun *et al.*, 2005; Hansen *et al.*, 2006). An enzymatic method is currently used by the present producer of food-grade tagatose, NUTRILAB, in Belgium and the process has been described by Bertelsen *et al.* (2006). A 40% solution of lactose is passed through an enzyme bioreactor at an elevated temperature. A thermostable β-galactosidase (lactase) is used for hydrolysis. The stream is then treated chromatographically to remove glucose and lactose, with the lactose being recycled. The eluate containing mainly galactose is passed through a second enzyme bioreactor. In this case, a thermostable L-arabinose isomerase is used to convert the galactose to D-tagatose. The eluate typically contains about 15% tagatose, 35% galactose and 50% glucose. It is again treated chromatographically to remove glucose and galactose, and the galactose stream is recycled. The eluate contains the tagatose product. Various configurations of this process have been tested, including the incorporation of both enzyme systems in one bioreactor. The thermostable enzymes are obtained from a number of thermophilic microorganisms, such as *Bacillus, Sulfolobus, Thermoanaerobacter, Thermus* and *Pyrococcus.* Kim and his colleagues in South Korea have published a suite of papers on improvements to the enzymatic process for the production of tagatose, and this research has been summarized by Kim (2004).

5.5. Commercial Producers and Products

5.5.1. Estimated Production of the Lactose Derivatives

There have been a number of reviews in which the manufacturing processes for oligosaccharides, major commercial oligosaccharide manufacturers and the tonnage of the different oligosaccharide products produced have been summarized (Matsumoto *et al.*, 1993; Playne and Crittenden, 1996; Crittenden and Playne, 1996). Estimates made in 1994/1995 of the annual global production of different oligosaccharides and allied products were galacto-oligosaccharides 12,000–14,000 t; lactulose 20,000 t; lactosucrose 1,600 t (Playne and Crittenden, 1996). Estimates from a survey conducted in 2004 were that annual global tonnages were galacto-oligosaccharides12,000–14,000 t; lactulose 40,000 t; lactosucrose 3,000 t; lactitol 10,000 t; lactobionic acid no data; and tagatose 500 t (3A Business Consulting, 2005). The same authors predicted that world production in 2009 would be galacto-oligosaccharides 21,000 t; lactulose 45,000 t; lactitol 11,000 t; and tagatose 800 t. Thus, annual growth rates around 10% or more were predicted for galacto-oligosaccharides and for tagatose, with slower growth rates for lactulose and lactitol. However, a higher estimate for lactulose production of over 50,000 t annually has also been published (LFRA, 2000).

In-house production of galacto-oligosaccharides in a number of countries makes it difficult to obtain precise data on world production. Such production is used directly for other products such as animal feed and infant milk foods. We estimate that total world production of all the above lactose-derived products in 2009 will be 83,000–90,000 t. In addition, there will be substantial production of lactose hydrolysate and galactose.

5.5.2. β -Galacto-oligosaccharides

Galacto-oligosaccharides (GOSs) have been manufactured commercially since the mid-1980s, principally by three Japanese companies – Yakult Honsha Co. Ltd., Snow Brand Milk Products and Nissin Sugar Manufacturing Co. Ltd. Commercial production in Europe commenced in 1995 in the Netherlands. In-house production has also occurred elsewhere, including in Australia and New Zealand.

The early establishment of a gut microflora in babies dominated by bifidobacteria, particularly in breast-fed babies, has been attributed to the presence of oligosaccharides in human milk. Although highly diverse, these human milk oligosaccharides have a backbone that is structurally similar to GOS. Hence, the inclusion of GOS as bifidogenic factors in infant products has been an important driver of the commercial production of GOS. The companies principally involved in the manufacture and marketing of GOS are Yakult Honsha Co Ltd., Nissin Sugar Manufacturing Co. Ltd. and Friesland Foods Domo (formerly Borculo Domo Ingredients) (Table 5.7). Yakult produces three galacto-oligosaccharide products: Oligomate 55 (syrup), Oligomate 55P (powder) and TOS-100 (a purified powder containing 99% oligosaccharides). Nissin also produces a syrup (Cup-Oligo H70) and a powder (Cup-Oligo P), while Friesland Foods Domo produces a syrup, Vivinal GOS. On the other hand, Snow Brand has now sub-contracted production and includes galacto-oligosaccharides in infant milk formula powders (e.g., P7 powder containing 1.2% oligosaccharide). With the exception of the purified powder produced by Yakult, the other commercial food-grade galacto-oligosaccharides all contain 40–70% of tri- and tetrasaccharides.

5.5.3. Lactulose

The two major manufacturers of lactulose are Solvay Pharmaceuticals and Morinaga Milk Industry. The Solvay production seems to be geared towards pharmaceutical applications, while Morinaga's production

Table 5.7. Major galacto-oligosaccharide manufacturers for the food industry

Company Name and Address	Product Name	Product Description
Yakult Honsha Co. Ltd. 1-1-19 Higashi-shimbashi, Minato-ku, Tokyo 105 Japan.	Oligomate 55	Syrup containing 75%(w/v) solids. Oligosaccharides >55% of solids.
	Oligomate 55P	Powder >55% oligosaccharides.
	TOS-100	Powder >99% oligosaccharides.
Nissin Sugar Mfg. Co. Ltd. 14-1 Nihonbashi-koamicho, Chuo-ku, Tokyo Japan.	Cup-Oligo H-70	Syrup containing 75% (w/v) solids. Oligosaccharides ≈ 70% of solids.
	Cup-Oligo P	Powder 70% oligosaccharides.
Friesland Foods Domo Head Office Hanzeplein 25, 8017 JD Zwolle The Netherlands (factory located at Borculo NL)	TOS-Syrup	Syrup containing 75% (w/v) solids.
	Vivinal-GOS	Oligosaccharides ≈ 60% of solids.
San-ei Sucrochemical Co., Ltd. 24-5 Kitahama-machi, Chita City, Aichi, Japan	garakutoorigo [origomeito] 55 N	Syrup

emphasizes uses in the food and feed markets. The other manufacturers of note are shown in Table 5.8. Commercially, lactulose is available in either dried form (powder, crystals or granulated) or as a syrup of 50–72%, w/v, lactulose.

Table 5.8. Major manufacturers of lactulose

Company name and address	Product name	Product description
Solvay Pharmaceuticals GmbH , Hans-Buckler-Alles 20, D-30173, Hannover, Germany (Head Office); (production sites at Weesp, The Netherlands and Victoriaville, Quebec, Canada)	Duphalac	Syrup –72% ,w/v, solids.
	Bifiteral	Syrup –66.7%,w/v, solids
	Chronulac, Cephulac	
	Lactulose	Powder >95% lactulose.
Morinaga Milk Industry Co. Higashihara 5-1-83, Zama-Shi, Kanagawa 228 Japan. (production sites at Fuji, Japan, and at Milei GmbH, Kemptener Str. 91 88299 Leutkirch/Germany,)	MLS-50	Syrup containing 70% solids. Lactulose ≈ 70% of solids.
	MLP-40	Powder 41% lactulose.
	MLC-A	Powder (anhydride) 98% lactulose.

(*Continued*)

Table 5.8. (*Continued*)

Company name and address	Product name	Product description
	MLC-H	Powder (hydrate) 86% lactulose.
	Milei lactulose	syrup 68% solids with <10% lactose
Chephasaar Chem.-Pharm. Fabrik GmbH., Muhlstrasse 50, 66386 St Ingbert, Germany	Lactulose MIP	syrup 65%
Inalco S.p.A., Via Calabiana 18, 20139 Milano, Italy (also in USA as Inalcopharm) manufactures in Vicchio, Italy		lactulose, crystals lactulose solution, 50% USP
Danipharm A/S is part of the Biofac Group, Denmark. A subsidiary, PharmaDan A/S produces lactulose at Osterbelle, Jutland	Danilax	lactulose purum liquidum Solution BP/Ph. Eur.
Fresenius Pharma Austria GmbH, Austria acquired Laevosan GmbH in Linz in 1997	Laevolac	syrup 66 g/L lactulose

5.5.3.1. Purity of Lactulose

Just as galacto-oligosaccharides are normally sold as impure mixtures, this is also the case with lactulose, in which substantial amounts of galactose and lactose are usually present in the commercial products. Examples are given for some Solvay products. The Solvay syrup product which contains 667 g lactulose/L contains <59 g lactose, <112 g galactose, <45 g epi-lactose, <14 g tagatose and <7 g fructose. The crystalline pharmaceutical product contains >95% lactulose, <2% lactose, <2.5% galactose, <3% tagatose and <1.5% epi-lactose [Solvay, personal communication June 1995]. In a more recent analysis, their Canadian factory reported that relative to lactulose, the Solvay 667 g/L syrup contains <3% tagatose, <1% fructose, <15% galactose, <7% epi-lactose, <9% lactose [Canlac certif. analysis, Sept. 2003].

5.5.4. Lactosucrose

The manufacture of lactosucrose appears to be restricted to the two companies listed in Table 5.9. However, a number of other Japanese companies distribute the product (e.g., Maruha Corporation) and develop new applications (e.g., Otsuka Pharmaceutical Co. Ltd., Tokyo). Lactosucrose production was developed in Japan as a cooperative venture between Ensuiko, Hayashibara and Bifermin Pharmaceutical Co. Ltd.

Table 5.9. Major manufacturers of lactosucrose

Company name and address	Product name	Product description
Ensuiko Sugar Refining Co. 13-46 Daikoku-cho, Tsurumi-ku, Yokohama 230 Japan.	Nyuka-Origo LS-40L	Syrup containing 72% (w/v) solids. Lactosucrose ≈ 42% of solids.
	Nyuka-Origo LS-55L	Syrup containing 75% (w/v) solids. Lactosucrose ≈ 55% of solids.
	Nyuka-Origo LS-55P	Powder. 55% lactosucrose.
	Pet-Oligo L55	Syrup containing 75% (w/v) solids. Lactosucrose ≈ 57% of solids.
	Pet-Oligo P55	Powder. 45% lactosucrose.
Hayashibara Shoji Inc. 2-3 Shimoishii 1-chome, Okayama 700-0907 Japan.	Newka-Oligo LS-35	Syrup containing 72% (w/v) solids. Lactosucrose ≥ 35% of solids.
	Newka-Oligo LS-55L	Syrup containing 75% (w/v) solids. Lactosucrose ≥ 55% of solids.
	Newka-Oligo LS-55P	Powder. ≥ 55% lactosucrose.
	Nyuuka-oligo 700	68-73% 0f solids as lacto-sucrose

5.5.5. Lactitol

The two major world manufacturers are PURAC Biochem and Danisco. Some 11,000 t are produced annually, and the growth rate is around 5% per annum. The price per kg is approximately US$2.00–2.50. Thus, it is a much cheaper product than food-grade GOS and lactosucrose, but similar to prices for lactulose syrup.

The Hayashibara Company of Okayama, Japan, took out early patents on lactitol production. Manufacturers are listed in Table 5.10.

5.5.6. Lactobionic Acid

Both Solvay, Germany, and Friesland Foods Domo, Netherlands, are major producers of the relatively small tonnage of lactobionic acid produced annually (see Tables 5.7 and 5.8 for the addresses of these companies).

5.5.7. Tagatose

The development of tagatose as a commercial product has followed a tortuous path, since its discovery. Licensing disagreements have dominated its development. The original inventor was Dr Gilbert Levin who formed BioSpherix Inc. in 1967. Later, the name was changed to Spherix Inc. In 1988, a patent was taken out on the use of D-tagatose as a sweetener and the product developed in-house until 1996. In 1996, a license was granted to the

Table 5.10 Major manufacturers of lactitol

Company name and address	Product name	Product description
PURAC Biochem b.v. Div of CSM Gorinchem The Netherlands	Lacty-M	food and pharmaceutical grades 96% powder, monohydrate
Danisco DK Texturants and Sweeteners Division, Danisco A/S, Langebrogade 1, DK-1001-K, Denmark.	Lactitol AC Lactitol ACM Lactitol CM Lactitol MC Finlac DC	anhydrous and monohydrate produced, and directly compressible grade (FinlacDC)
Mitsubishi Shoji Food Tech Co Ltd, previously Towa Chemical Industry Co Ltd ., 2-8-7, Yaesu, Chuo-ku, Tokyo, 104-0028 Japan	Milchen	lactitol monohydrate powder

Danish firm, MD Foods, to develop the food uses for tagatose, while Spherix retained the rights to non-food uses. MD Foods later took over the Swedish company, Arla Foods, and used its name. A period of slow development occurred as Arla gathered evidence and sought listing of D-tagatose as a GRAS ingredient. This was granted by the US Food and Drug Administration in 2001. As no product was on the market by 2002, the agreement between Spherix and Arla Foods was re-negotiated with new royalty agreements. Meanwhile in 2002, Arla Foods had commenced a joint venture with the German sugar producer, Nordzucker, to manufacture tagatose at a plant near Hannover, Germany. The joint venture company was SweetGredients GmbH & Co. KG. Commercial production of tagatose began in 2003 and the product was marketed as Gaio®tagatose. After an active period of global marketing of the product, SweetGredients decided to put tagatose production on hold in March 2006 as they had not been able to create a large enough market for the sweetener and manufacturing was closed down in Germany. Later in 2006, the Belgian Company NUTRILAB NV took over the stocks and project from Arla Foods and began to set up a new manufacturing site with a new enzymatic process for manufacture. The new product brand name is NUTRILATOSE. The process is being scaled-up to produce up to 10,000t annually. The plant is planned for completion by late 2009. The original tagatose patents assigned to Spherix Inc. expired in 2006 in the USA and 2007 in Europe.

Global annual production capacity is currently estimated to be about 500 t, but this is expected to increase markedly after 2009. The market may grow quite quickly for this product in the health foods area for low GI foods. Manufacturers are listed in Table 5.11.

Table 5.11. Current manufacturers of tagatose

Company name and address	Product name	Product description
Spherix Inc., 12051 Indian Creek Court Beltsville, MD 20705 USA	Naturlose	non-food uses
NUTRILAB N.V., Driesstraat 54 A, B-3461 Bekkevoort, Belgium	Nutrilatose	food uses

5.6. Analytical Methods

The choice of analytical methods for complex carbohydrates will depend on the required outcomes. For example, it may suffice to use a simple HPLC procedure with water as solvent and a standard ion-exchange column, such as the BioRad HPX87C (calcium form). This will lead to adequate separations of monomer sugars and of disaccharides and the 3–6 unit oligosaccharides (Crittenden and Playne, 2002). However, usually it will not differentiate isomers. In many cases, this may not be necessary. More sophisticated highly alkaline systems with pulsed amperometric detection (PAD) will give improved separations. More complex approaches are necessary if full structural analysis is necessary. Some useful references to the analysis of monomer sugars and oligosaccharides are Townsend *et al.* (1988), Hardy and Townsend (1988), Lee (1990), Reddy and Bush (1991) and Van Riel and Olieman (1991). A standard method (method 2001.02) for analysis of galacto-oligosaccharides in foods has been developed (AOAC, 2006). This method is based on high-performance anion-exchange chromatography (HPAEC) using a sodium acetate gradient under alkaline conditions. The eluate is monitored using pulsed amperometric detection (PAD) with a gold electrode. A commonly used apparatus is a Bio-LC system (Dionex Corporation, Sunnyvale, California) (for example, see Hardy and Townsend, 1988; Quemener *et al.*, 1994; van Laere *et al.*, 2000; De Slegte *et al.*, 2002).

Prior to the widespread adoption of HPLC methods, oligosaccharides were commonly analysed by gas chromatography of derivatized monomer sugars. Samples were methanolized (methanolic 0.5 M HCl, 80°C, 24 h), *N*-acetylated and trimethylsilyated. The glycosides so formed were separated by gas chromatography (e.g., Lemoine *et al.*, 1997). Other methods for preparing methylated derivatives have been described (Rabiu *et al.*, 2001). Thin layer chromatography has also been used widely for qualitative and semi-quantitative analyses of mono- and oligo-saccharides (e.g., Rabiu *et al.*, 2001). NMR spectroscopy has been used for analysis of carbohydrates where structural

elucidation of complex products is needed (see Thelwall, 1997). Yanahira *et al.* (1995) examined the composition and structure of galactosaccharides using methylation analysis, mass spectrometry and NMR spectroscopy. Lemoine *et al.* (1997) carried out extensive studies using NMR spectroscopy to examine the structures of extracellular polysaccharides of *S. thermophilus.* Boehm and Stahl (2003) have provided an excellent overview of analytical methods suitable for glyco-conjugates present in milk. They review methods for chromatography, mass spectrometry, spectroscopy, electrophoresis and separation techniques, such as crystallization and filtration.

Traditionally, dietary fibre was considered to comprise largely of plant cell walls, namely cellulose and lignin (Van Soest, 1978). Southgate *et al.* (1978) defined dietary fibre as "the sum of lignin and the polysaccharides that are not digested by the endogenous secretions of the human digestive tract". This physiologically based definition includes both water-soluble and -insoluble constituents. Thus, fructo- and galacto-oligosaccharides and possibly other prebiotic carbohydrates would be considered as "dietary fibre". Appropriate methods of analysis of the water-soluble components have long been a challenge for analysts. Thebaudin *et al.* (1997) discussed the heterogeneous composition of this fraction and methods of analysis in more detail. The 1998 Codex guidelines on food labelling defined dietary fibre as "edible plant or animal material not hydrolysed by the endogenous enzymes of the human digestive tract" (Codex Alimentarius, 1998). The historical development of the dietary fibre concept, its definition and the ensuing development of suitable analytical methods has been discussed extensively (AACC, 2001; IFST, 2007). The present accepted definition, which has been adopted widely by food regulators and manufacturers, includes generalized health benefits based mainly on epidemiological data generated primarily from studies using fruits, vegetables and whole grain cereal foods. This definition is

> Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation and/or blood cholesterol attenuation and/or blood glucose attenuation (AACC, 2001).

In 2006, FAO/WHO commissioned experts to further consider the definition of dietary fibre. They recommended that the definition be restricted to "dietary fibre consists of intrinsic plant cell wall polysaccharides", thus restricting the previous all-encompassing physiological definition. This recommendation was considered in late 2007 (IFST, 2007). What does this all mean for the analysis of galacto-oligosaccharides?

For food labelling reasons, manufacturers of fructo- and galacto-oligosaccharides have been keen to be able to label those ingredients as dietary fibre. The standard official method of analysis of dietary fibre adopted in 1985 was AOAC method 985.29. Because the method was inadequate for fructans, complementary methods were developed, such as AOAC 997.08 (based on ion-exchange chromatography) and AOAC 999.03 (an enzymatic/spectrophotometric method). Quemener *et al.* (1994) developed a method for measurement of *trans*-galacto-oligosaccharides by enzymatic treatment with β-galactosidase, followed by high-performance anion-exchange chromatography, using PAD (HPAEC-PAD). This method was tested further in collaborative studies (De Slegte *et al.*, 2002) and adopted as the official AOAC method (AOAC 2001.02). It has been published as Method 32-33 by the AACC (2002). The chromatography supply company, Dionex Corporation, has recently released an Application Note No. 155 which examines the use of different potential waveforms in the PAD and improves the current method.

An enzymatic method to determine the lactulose content of milk in the presence of lactose has been adopted by the IDF (1995). The method is suitable for determining lactulose in the presence of much higher concentrations of lactose. Nagendra and Rao (1992) published a colorimetric method to measure lactulose in the presence of lactose. However, the HPAEC-PAD method described above is suitable for the determination of lactulose, lactosucrose, lactitol and tagatose, as well as complex mixtures of oligosaccharides.

Lactulose is often determined by enzymatic methods using kits supplied by companies like Merck and Boehringer–Mannheim. The principle of the determination is to first hydrolyse the lactulose with a galactosidase to fructose and galactose, then to convert the fructose in the presence of ATP with a hexokinase to fructose-6-phosphate. The fructose-6-phosphate is then converted to glucose-6-phosphate using phosphoglucose isomerase. Finally, in a reaction of the glucose-6-phosphate with NADP using glucose-6-phosphate dehydrogenase, the amount of NADPH formed is measured spectrophotometrically.

5.7. Properties

5.7.1. Properties of Oligosaccharides

Food-grade oligosaccharides are not pure products, but are mixtures containing oligosaccharides of different degrees of polymerization (dp), the parent polysaccharide or disaccharide and monomer sugars. A typical commercial product composition for an unpurified GOS may comprise glucose and galactose 10%; disaccharides (lactose) 70%; trisaccharides 18.7%; tetrasaccharides 1.2%; and pentasaccharides 0.06%. More advanced manufacturing processes result in much higher concentrations of galacto-oligosaccharides in the

product. For example, Oligomate 50 produced by the Yakult Company contains 50–52% galacto-oligosaccharides, 10–13% lactose and 36–39% monosaccharides (Matsumoto *et al.*, 1993). Purified products are available from many manufacturers, but costs increase considerably because of the extra processing steps required. Purified products usually contain between 85 and 99% galacto-oligosaccharides. It is obvious that there are important differences in the properties and actions of food-grade oligosaccharide mixtures depending on the mixture purchased. The applications of oligosaccharides as components in foods will therefore vary considerably.

General properties of oligosaccharides are listed in Table 5.12. The data provided by producers, not all of which have been possible to reproduce in this review, usually include the following factors: sweetness (relative to sucrose), viscosity, water activity, moisture retention, osmotic pressure, freezing point depression, heat stability, storage stability at different pH values and colouring by heat.

Most producers also provide safety and toxicological data for acute, sub-acute and chronic toxicity. Data are also commonly provided to demonstrate changes in colonic microbial flora following ingestion of oligosaccharides. Usually, substantial increases in bifidobacteria are demonstrated relative to the other bacterial classes measured.

The physico-chemical properties of oligosaccharides are largely dependent on the molecular weight of the particular oligosaccharide product. These effects are shown in Table 5.13.

Table 5.12. General properties of galacto-oligosaccharides

Appearance: translucent / colourless
Sweetness: typically 0.3–0.6 times that of sucrose.
Calorific value: lower than sucrose
Water activity: similar to that of sucrose
Viscosity: similar to that of high-fructose glucose syrup
Heat stability: stable to 160°C for 10 min at pH7 and to 100°C for 10 min at pH 2. More heat stable than fructo-oligosaccharides at low pH over the range 5-37°C
Acid stability: stable to pH 2
Indigestibility: resistant to salivary and pancreatic α-amylase and other carbohydrases, and gastric juice. Thus, reduce the glycaemic index of foods
Energy Value: about 50% of that of sucrose
Freezing point: reduces the freezing point of foods
Bacteriostatic properties: through competition for mucosal binding sites and adhesion by pathogens. Some oligosaccharides are also able to absorb lectin-like toxins, e.g., VTEC, ETEC
Bifidogenic ability: able to increase the population of most species of bifidobacteria in mixed populations of bacteria in both *in vitro* and *in vivo* studies in humans and animals
Cariogenicity: acts as an anti-caries agent (anti-cariogenic).

Table 5.13. Physico-chemical properties of oligosaccharides

– Taste – sweetness decreases as molecular size increases
– Mouth-feel – larger-sized oligosaccharides have applications as fat replacers
– Viscosity – increases with increasing molecular size
– Solubility – decreases with increasing molecular weight
– Hygroscopicity – oligosaccharides can be used to control the moisture level in foods
– Colour reactions – decreases as molecular weight increases, because there are relatively fewer available reducing moieties. Thus, reduced Maillard reaction in heat-processed food.
– Freezing point – depression of freezing point is inversely proportional to molecular weight
– Osmolality – decreases as molecular weight increases
– Coarse crystal formation – reduced occurrence with increased molecular weight
– Hydrolysis of oligosaccharides in acid conditions not related to molecular weight, but depends on chemical composition

The susceptibility to hydrolysis of oligosaccharides during passage through the gastrointestinal tract is an important characteristic. This has been determined in a range of *in vitro* model systems of the stomach and small intestine of humans. Different types of oligosaccharides have been subjected to regimes such as (a) hydrolysis by human saliva (50%, v/v, saliva, 30 min exposure, pH 7.0, 37°C); (b) hydrolysis by gastric acid (4 h exposure, pH 2.0, 37°C in acid/pepsin); (c) hydrolysis by pancreatic and brush border carbohydrases (17%, v/v, porcine pancreatic and duodenal homegenates for 1 h exposure, pH 7.0, 37°C). Carbohydrate analysis before and after exposure by HPLC then determines the degree of hydrolysis. Tests such as these have shown that xylo-oligosaccharide mixtures, palatinose condensates, commercial galacto-oligosaccharide mixtures and lactulose are very resistant to hydrolysis, whereas lactosucrose, along with gentio-oligosaccharides, soybean oligosaccharides, fructo-oligosaccharide mixtures and inulin oligosaccharides are hydrolysed slightly by such conditions (Crittenden and Playne, 1997, unpublished data).

5.7.2. Properties of Lactulose

Lactulose solution is a yellowish, odourless clear syrup with a sweet taste. Dry lactulose is a white, odourless crystalline powder. Lactulose is soluble in water, but poorly soluble in methanol and insoluble in ether. Its solubility in water is 76.4% (w/w) at 30°C, rising to 86% at 90°C. The melting point is 168.5–170.0°C. Its sweetness is classified as 0.48–0.62 that of sucrose. It is 1.5 times sweeter than lactose. Acid hydrolysis of lactulose yields galactose and fructose. Unlike lactose, lactulose cannot be hydrolysed by human intestinal enzymes. Lactulose can be fermented by some human colonic bacteria and acts as a prebiotic ingredient (Mizota *et al.*, 1987; Tamura

et al., 1993). Heat sterilization is a common step in food manufacture. Lactulose is stable and little decomposed when heated to 130°C for 10 min at low pH. This relatively high stability makes it a suitable ingredient for normal food processing.

5.7.3. Properties of Lactosucrose

Lactosucrose is very soluble in water (up to 3670 g/L at 25°C). It has a sweetness relative to sucrose of 0.3. It is stable for 1 h at pH 4.5 and 120°C. It is most stable at neutral pH and remains fairly stable at pH 3 at 80°C. As a powder, it has a high moisture-retaining capacity and is highly hygroscopic.

Lactosucrose acts as a prebiotic carbohydrate and is not readily digested by intestinal enzymes in the stomach and small intestine. It is selectively fermented by *Bifidobacterium* in the human colon.

5.7.4. Properties of Lactitol

Lactitol is an odourless, colourless, sweet, non-hygroscopic and stable sugar alcohol. As it lacks a reactive carbonyl group, which is characteristic of sugar molecules, it does not participate in Maillard browning reactions with amino groups. It occurs in both monhydrate and dihydrate crystalline forms as pure products with good flowability. Lactitol is stable under both alkaline and acid conditions and at high temperatures likely to be encountered during food processing. Its melting point is 146°C and it is very soluble in water. The sweetening power of lactitol is about 40% of sucrose and the calorific contribution of lactitol after it is metabolized by colonic bacteria is less than 2.4 cal g^{-1} (10 kJ) compared to 4.0 cal g^{-1} (17 kJ) for sucrose.

Lactitol is not hydrolysed or absorbed in the small intestine and passes through to the colon. Lactitol also acts as a prebiotic carbohydrate and enhances the growth of certain groups of bacteria (e.g., bifidobacteria) in the colon.

5.7.5. Properties of Lactobionic Acid

Lactobionic acid is best described as a natural polyol acid with a formula weight of 358.30 Da and a pK_a of 3.8. It is soluble in water as a free acid or as a salt. The molecule possesses multifunctional groups and thus acts as a metal ion chelator and can sequester calcium (Abbadi *et al.*, 1999). Lactobionic acid inhibits the production of hydroxyl radicals as a result of its iron-chelating capacity and it functions as an antioxidant in tissues. It has a sweet taste despite being a weak acid. Lactobionic acid can be dehydrated to a lactone.

Lactobionic acid is hygroscopic and forms a gel containing about 14% water with atmospheric moisture. Concentrated solutions will form gels and films. Its superior water-retention ability is valuable in cosmetic applications. Its main uses derive from its important chelating and emulsifying properties.

5.7.6. Properties of D-tagatose

D-Tagatose is a monosaccharide with a formula weight of 180.16 Da; it is a white solid with a melting point of 133–135°C. It has a similar texture to sucrose and is 92% as sweet, but its calorific value (1.5 kcal/g) is only 38% that of sucrose since it is metabolized differently from sucrose. Its indigestibility in the stomach and small intestine results in a minimal effect on blood glucose levels in humans. Values for the absorption of tagatose in the small intestine vary widely from 20 to about 80% (Normen *et al.*, 2001). The unabsorbed fraction is then completely fermented in the colon by some intestinal microorganisms. Those genera able to ferment tagatose included *Enterococcus* and *Lactobacillus*, but not *Bifidobacterium*. Many dairy lactic acid bacteria, such as lactococci and streptococci, fermented tagatose readily (Bertelsen *et al.*, 2001). It is claimed that its fermentation in the colon results in an increased production of butyrate relative to that of acetate and propionate (Laerke and Jensen, 1999, Laerke *et al.*, 2000; Bertlesen *et al.*, 1999). It has a glycaemic index of only 3 compared to 100 for glucose. Its glycaemic index is markedly lower than those for other natural sugars and most sugar alcohols. It is claimed to have prebiotic properties (Bertlesen *et al.*, 1999, 2001), but its inability to be fermented by *Bifidobacterium* species limits its application as a prebiotic. However, it is non-cariogenic and non-plaque forming.

5.8. Uses and Applications

5.8.1. Galacto-oligosaccharides

The major uses of galacto-oligosaccharides are in beverages and in infant milk formula, follow-on formula and infant foods. These are usually provided at about 6 g/L of infant formula and are normally supplied as a mixture of nine parts galacto-oligosaccharide and one part fructo-oligosaccharide. A recent major Dutch study with 120 women, half of whom were breast-feeding and half of whom were bottle-feeding, was conducted by Bakker-Zierikzee *et al.* (2005). The data supported the benefit of adding these oligosaccharides, as they were able to mimic the metabolic and microbial effects normally found in breast-fed babies.

Table 5.14. Uses of galacto-oligosaccharides in physico-chemical modification of foodstuffs

– low sweetness (low glycaemic index, low calorie foods)
– taste and transparency
– prevention of hygroscopity (e.g., candies, sweets, chocolates)
– prevention of colouration (e.g., fruits and jams)
– reinforcement agent or thickener (e.g., sauces, creams, jelly)
– glazing agent (e.g., rice crackers, hard lollies/ boilings)
– regulation of freezing point (e.g., ice cream, frozen foods)
– humectant (e.g., cakes, pastries)
– powderizing material (e.g., coffee whitener, soups)

Oligosaccharides are also used widely in confectionery and in bread-making, for their physico-chemical properties, as well as claimed health-enhancing properties. Obviously, oligosaccharides also provide textural and sensory properties to foods (see Table 5.14). They are being used increasingly as ingredients of synbiotic foods, for example, their use in yoghurt and yoghurt drinks.

The use of oligosaccharides in the livestock and pet food industries is also increasing. In the latter case, GOSs are used to improve bowel consistency and to provide firmer and less malodorous faeces. Prebiotic ingredients are finding increased use in the chicken, pig and calf industries for improving health and minimizing the use of antibiotics.

5.8.2. Lactulose

Most lactulose is used in the pharmaceutical industry, but increasingly it is finding application as a prebiotic ingredient in functional foods. Lactulose is generally classified as a drug, the prescription or non-prescription status of which depends on the country. However, it has now been approved for use as a prebiotic in several countries (Italy, Netherlands and Japan) and may be sold as either a food or drink additive.

Lactulose is used to treat constipation and hepatic encephalopathy. It is the principal anti-constipation drug worldwide. Lactulose has exhibited some ability to ameliorate symptoms of idiopathic, as well as infectious, inflammatory bowel disease and this may be due to its prebiotic effects on colonic bacteria. There are some early indications in animal models that it may aid in reducing the incidence of colon tumours and hence colon cancer.

Applications listed by Morinaga are (a) as a prebiotic ingredient in infant foods, (b) as a pharmaceutical for remission of hyperammonemia and chronic constipation, (c) as a sweetener for low-calorie dietary foods and (d) as an

additive for cattle and pet feed. A variety of yoghurts, drinks, ice cream and infant formula have been developed by Morinaga Milk Industry.

5.8.3. Lactosucrose

Lactosucrose has been used as a prebiotic ingredient in a range of food products which have attained FOSHU status in Japan with 15 products approved by 2005. It is used as a sweetener as well as a prebiotic in a range of beverages, including coffee and tea. It is used in confectionaries, desserts, sweets, bakery products and yoghurts. Its use is largely confined to Japan, but marketing into the USA has commenced. It also has a use for improving bowel consistency and faecal odour. Research has also been performed on its use in pet food, particularly for reducing faecal odour (Fujimori, 1992).

5.8.4. Lactitol

The principal use of lactitol is as a low-calorie sweetener in foodstuffs. It is used in a range of low-energy and low-fat foods. Its high stability makes it popular for bakery applications, although it is not able to participate in the Maillard reaction. It is used in sweets, chocolates, biscuits and ice cream where it competes with other sugar alcohols, such as sorbitol, mannitol and xylitol. It is used in glycaemic foods for diabetics and is also recognized as not causing dental caries. Lactitol has also found application in pharmaceuticals as an alternative to lactulose and as a cryoprotectant in surimi. Its low hygroscopicity and high viscosity give it advantages in chocolate and confectionary manufacture. It has an emerging use in pre- and pro-biotic functional foods.

5.8.5. Lactobionic Acid

In the pharmaceutical industry, lactobionic acid is used to deliver erythromycin intravenously and also in calcium supplementation. A major commercial use is its role in organ preservation fluids during transplantation procedures in hospitals. Lactobionic acid is used in the "Wisconsin transplantation solution" because its metal-chelating properties reduce oxidative damage to tissue during storage and preservation of organs caused by some metal ions. Lactobionic acid can also be an ingredient in chlorohexidine-based disinfectants. It is able to suppress tissue damage caused by oxygen radicals and is used to assist wound healing. In skincare, it has a use as a dermal care cosmetic and possesses a number of useful properties for this purpose.

Emerging applications are as an acidulant with a sweet taste; a filler in cheese production; use as a calcium carrier in functional drinks; as a co-builder in detergents; and in corrosion protection. Lactobionic acid amides

have been investigated for use in the surfactant industry. Lactobionic acid *N*-alkylamides have been proposed for use in corrosion inhibition.

5.8.6. Tagatose

The main use of tagatose is as a sweetener with a low calorific content. Its main market appears to be in diabetic foods, as it does not increase blood glucose or insulin levels, and it has a very low GI value. It may also be useful in weight loss diets to help overcome widespread obesity in Western human populations. It has the advantage of being of similar sweetness to sucrose, but with a low contribution to energy supply.

Tagatose has some potential as a prebiotic carbohydrate, but evidence for its effectiveness relative to competing products is not yet available. Its ability to be fermented in the colon to produce enhanced concentrations of butyrate is of interest in relation to colonic cancers, and this requires further investigation (Bertlesen *et al.*, 1999; Topping and Clifton, 2001).

5.9. Physiological and Health Effects

A major driver for the development of lactose derivatives has been the identification of numerous physiological properties beneficial to health. Some of these physiological effects have therapeutic value for specific disorders while other properties are potentially beneficial to the population at large. Hence, lactose derivatives have found applications both as pharmaceuticals and as functional food ingredients. Despite a diversity of chemical structures, the lactose derivates developed to date share a number of common physiological traits important to their health benefits. These molecules are universally

- non-digestible
- non-metabolizable
- poorly fermented by bacteria in the mouth
- fermented by bacteria in the intestinal tract.

These physiological characteristics contribute to health benefits both at specific sites in the body and systemically. The benefits demonstrated, or proposed, include prevention of dental caries, roles in weight management, improved defecation frequency and consistency and a range of other effects stemming from modification of the composition and/or activity of the intestinal microbiota (prebiotic effects). Of course, differences in the chemical structures of the various lactose derivatives also mean that they have differing specific actions and potencies. The following sections outline the known and

purported health benefits of lactose derivates and what we know about their mechanisms of action.

5.9.1. Dental Health

Lactitol is one of a number of sugar alcohols that have found applications in products where sweetness is desired without stimulating cariogenic bacterial activity in the mouth. Other examples include xylitol, sorbitol, mannitol and maltitol, with the first two examples the most widely used. Sugar alcohols are not fermented by bacteria in the mouth and so are non-acidogenic and do not lead to bacterial *exo*-polysaccharide production that contributes to dental plaque (van Loveren, 2004). Since they are small molecules that are not digested, they cannot be consumed in large amounts due to intestinal side effects (discussed later) and so are not used as bulk sweeteners, but rather in products such as chewing gums and toothpaste. The other lactose derivatives discussed in this chapter are also non-cariogenic, but are not widely used specifically for oral health.

5.9.2. Prevention and Treatment of Constipation

Lactulose (10–20 g/day) is used widely as a pharmaceutical to treat constipation and has proven efficacy in a number of placebo-controlled trials (Fernández-Bañares 2006; Quah *et al*., 2006) even in patients with chronic constipation. Since it is a relatively small molecule that is not digested or absorbed, lactulose has an osmotic effect, trapping fluid, accelerating transit in the small bowel and increasing ileocaecal flow. A recent human clinical study has also shown that therapeutic doses of lactulose produce a prolonged tonic contraction in the gut that may also be involved in the laxative effect (Jouët *et al*., 2006). Since any carbohydrate that reaches the large bowel should have a laxative effect (Macfarlane *et al*., 2006), the other lactose derivatives also have benefits in the prevention and treatment of constipation. GOSs too have been demonstrated to improve stool frequency and consistency in infants and adults, though it is used more in functional foods as opposed to pharmaceutical applications. Possible mechanisms of action of lactose derivatives in alleviating constipation are shown in Figure 5.7.

5.9.3. Treatment of Hepatic Encephalopathy

Hepatic encephalopathy (HE) is a neuropsychiatric condition driven by liver dysfunction that includes a spectrum of symptoms ranging from subtle changes in cognition and personality to lethargy, stupor and coma (Dbouk and McGuire, 2006).

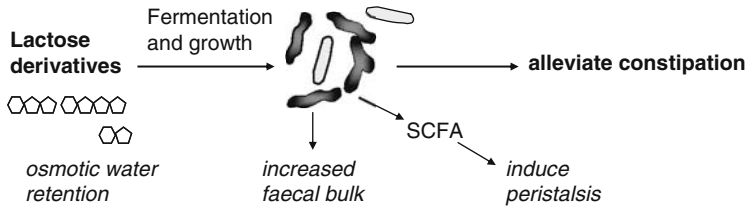

Figure 5.7. Mechanisms by which lactose derivatives prevent and alleviate constipation.

Although the precise underlying mechanisms remain debated, elevated ammonia concentrations in plasma and the central nervous system, caused by the inability of the liver to clear ammonia generated by the gut microbiota, are considered key to its pathogenesis (Prasad *et al*., 2007). Lactulose and lactitol are front-line therapeutic agents in HE because they limit both ammonia production in, and absorption from, the colon. The fermentation of these carbohydrates when dosed at relatively high levels results in a diversion of ammonia to microbial protein production. Additionally, acidification of the colonic lumen inhibits urease-positive and deaminating bacteria (implicated in intestinal ammonia production), increases intestinal transit and reduces absorption of ammonia from the intestinal lumen (Bongaerts *et al*., 2005; Dbouk and McGuire, 2006). Lactulose and lactitol have similar efficacy, although lactitol is more palatable and produces more rapid results with fewer side effects (Dbouk and McGuire, 2006).

5.9.4. Prebiotic Effects

Some of the main physiological effects of lactose derivatives stem from their impact on the composition and activities of the intestinal microbiota. Our intestinal tract is colonized by a complex ecosystem of microorganisms that increase in numbers from 10^2–10^4 per gram of contents in the stomach to 10^6–10^8 per gram in the small intestine and 10^{10}–10^{12} per gram in the colon (McCartney and Gibson, 2006). Far from being inconsequential to our lives, these microorganisms are in a symbiotic relationship with their host and are highly important to our health and well-being. They provide us with a barrier to infection by intestinal pathogens (Bourlioux *et al*., 2003), much of the metabolic fuel for our colonic epithelial cells (Topping and Clifton, 2001), and contribute to normal immune development and function (Blum and Schiffrin, 2003; Tlaskalova-Hogenova *et al*., 2004). Intestinal bacteria have also been implicated in the aetiology of some chronic diseases of the gut such as inflammatory bowel disease (IBD) (Cummings *et al*., 2003; Marteau *et al*.,

2003). As we age, changes occur in the composition of the intestinal microbiota that may contribute to an increased level of undesirable microbial metabolic activity and subsequent degenerative diseases of the intestinal tract (Saunier and Doré, 2002; Guarner and Malagelada, 2003).

Modifying the composition of the intestinal microbiota to restore or maintain a beneficial population of microorganisms would appear to be a reasonable approach in cases where a deleterious or sub-optimal population of microorganisms has colonized the gut. The two genera most often proposed as beneficial bacteria with which to augment the intestinal microbiota are the lactobacilli and bifidobacteria, both of which are numerically common members of the human intestinal microbiota. Two approaches are used to increase the number or proportion of these bacteria in the gut. The first is by directly supplementing the intestinal microbiota by consuming live bacteria, "**pro**biotics", in foods or pharmaceuticals. The second is by consuming dietary components, "**pre**biotics", which selectively stimulate the proliferation and/or activity of these purportedly beneficial organisms that are *already resident* within the intestinal microbiota (Gibson *et al*., 2005). Most prebiotics identified to date are non-digestible, fermentable carbohydrates, particularly oligosaccharides and include the lactose derivatives, GOSs and lactulose.

Despite a diversity of structures, most prebiotics stimulate the proliferation of bifidobacteria in particular and are sometimes referred to as "bifidus factors" or "bifidogenic factors" (Table 5.1). A number of largely prophylactic health targets have been proposed for prebiotics stemming from alterations to the composition or fermentative activity of the microbiota in response to the availability of a selectively utilized carbohydrate source. These include protection against enteric infections, increased mineral absorption, immunomodulation for the prevention of allergies and gut inflammatory conditions (Figure 5.8), and trophic effects of short-chain fatty acids (SCFA) on the colonic epithelium, faecal bulking and reduced toxigenic microbial metabolism that may reduce risk factors for colon cancer (Figure 5.9).

5.9.4.1. Applications of GOSs in Infant Nutrition

The composition of the human intestinal microbiota changes naturally with age, and in early infancy the microbiota is believed to be particularly important in correct functioning of the gut and maturation of the immune system. Indeed, differences have been observed between the composition of the microbiota in allergic and healthy infants including reduced numbers of bifidobacteria and a more adult-like profile of *Bifidobacterium* species (Kirjavainen and Reid, 2006). Bifidobacteria colonize the human intestinal tract during or soon after birth and in breast-fed infants eventually dominate the microbiota (Harmsen *et al*., 2000). The numerical dominance of

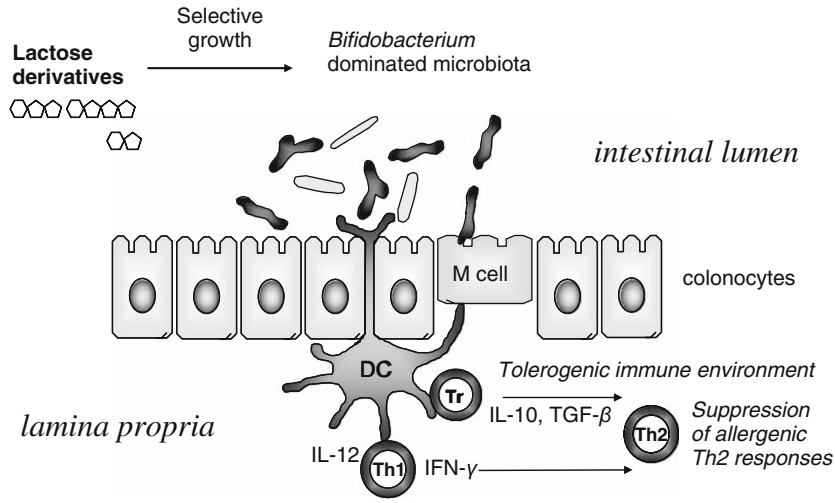

Figure 5.8. Proposed mechanism by which prebiotic lactose derivatives assist in the establishment of a tolerogenic immune environment in the gut leading to correct immune programming to dietary antigens and fewer allergies.

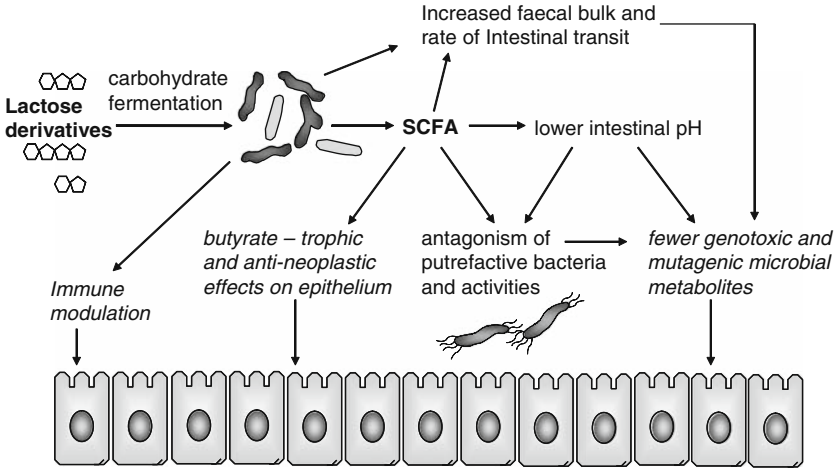

Figure 5.9. Impact of lactose derivatives on reducing purported risk factors for colorectal cancer.

bifidobacteria is induced by bifidogenic components in breast milk, including oligosaccharides (Harmsen *et al.*, 2000; Mountzouris *et al.*, 2002). Indeed, human milk oligosaccharides (HMOs) are the original prebiotics. The concentration of HMOs in breast milk (5–12 g/L) is about 100 times that found in cow's milk (0.03–0.06 g/L) (Kunz *et al.*, 2000; Boehm and Stahl, 2007), representing the third largest solid component behind lactose and fat (Bruzzese *et al.*, 2006). That mothers direct so much energy towards components of breast milk that are not directly metabolized by the infant, but rather selectively feed and direct the composition of the microbiota, shows evolutionarily just how important the infant gut microbiota is to well-being.

In contrast to breast-fed infants, infants fed traditional cow's milk-based formulae develop a more mixed intestinal microbiota, with lower counts of bifidobacteria and higher counts of clostridia and enterococci (Adlerberth, 1999). Formula-fed infants also have been observed to have higher faecal ammonia and other potentially harmful bacterial products (Heavey *et al.*, 2003; Edwards and Parrett, 2002). The bifidogenic effect of HMOs can be emulated using oligosaccharides such as GOSs or fructo-oligosaccharides (FOSs). In fact, most Japanese infant formula manufacturers have been supplementing their products with prebiotic oligosaccharides for many years (Boehm *et al.*, 2005).

GOSs share some structural similarities with the backbone of HMOs, although HMOs are considerably more complex, with over 200 different structures identified based on a variable combination of glucose, galactose, sialic acid, fucose and/or *N*-acetylglucosamine, with varied sizes and linkages accounting for the considerable variety (Kunz *et al.*, 2000). In recent years, a number of studies have investigated the effect of infant formula containing 8 g/L of a mixture of 9:1 GOSs:FOSs on the intestinal microbiota of infants. The GOSs:FOSs mixture was designed to mimic the molecular size distribution of HMOs (Knol *et al.*, 2005). Summarized in Figure 5.10, they show that feeding this formula induced a microbiota similar to that of breast-fed infants, both in terms of composition of the overall microbiota and the proportion and species composition of lactobacilli and bifidobacteria (which tends to be more adult-like in traditional formula-fed infants) (Rinne *et al.*, 2005; Haarman and Knol, 2005, 2006; Knol *et al.*, 2005). The oligosaccharide-containing formula also resulted in a reduced faecal pH and a SCFA profile that mimicked breast milk (dominated by acetate) and was different from that produced by traditional infant formulae (higher in butyrate and propionate) (Boehm *et al.*, 2005; Fanaro *et al.*, 2005; Knol *et al.*, 2005; Bakker-Zierikzee *et al.*, 2005). Feeding the oligosaccharide formula also resulted in an improved stool consistency and frequency, similar to breast-fed infants, while having no adverse impacts on measures of infant growth or other adverse effects (Boehm *et al.*, 2005; Bruzzese *et al.*, 2006;

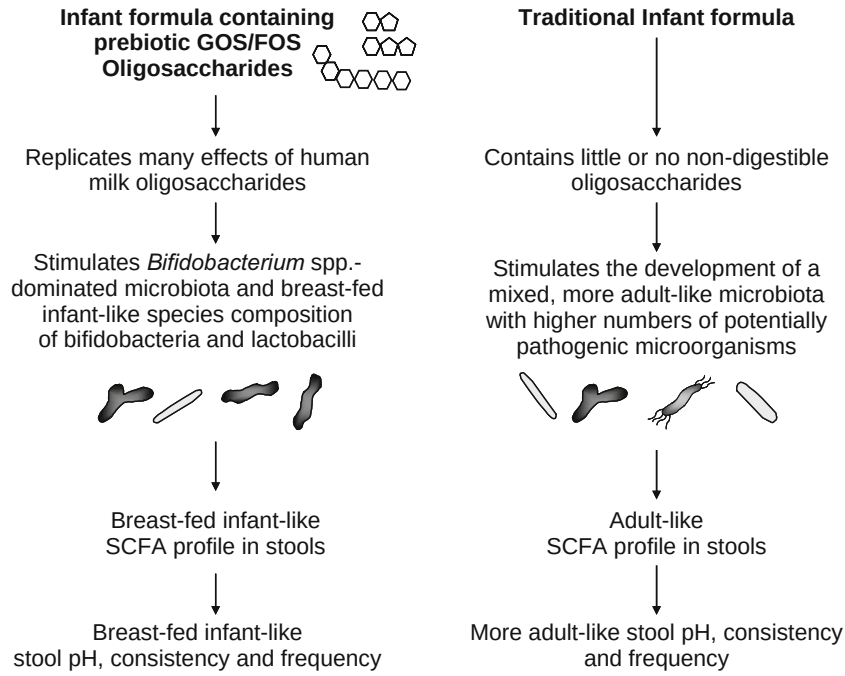

Figure 5.10. The inclusion of a prebiotic mixture of 9:1 galacto-oligosaccharides (GOSs) to fructo-oligosaccharides (FOSs) mimics many of the effects of human milk oligosaccharides (HMOs) on the composition and activity of the human infant intestinal microbiota. In contrast, traditional infant formulae containing no oligosaccharides drive a more adult-like microbiota and stool composition.

Ziegler *et al*., 2007). Finally, the oligosaccharide-supplemented formula showed a positive effect on the level of protective secretory IgA in the gut, increasing concentrations in comparison to standard formula (Bakker-Zierikzee *et al*., 2005). These studies demonstrate that formulae supplemented with the GOSs:FOSs can mimic many of the physiological impacts of HMOs. Now the challenge remains to establish if these translate into benefits for clinical end-points such as incidence of infections or the prevalence of allergy.

5.9.4.2. Impacts on the Microbiota Composition in Adults

Following weaning, the composition of the colonic microbiota becomes increasingly complex. In adulthood, the microbiota consists of more than 500 different species, although it is dominated by 30–40, and its composition becomes quite stable (McCartney and Gibson, 2006).

Bifidobacteria remain numerically important, although their proportion of total microbes declines from 60 to 80% in breast-fed infants to 1–5% in adults. Feeding prebiotics to adults typically induces 10- to 100-fold increases in the size of the intestinal *Bifidobacterium* population (Crittenden, 1999). However, a range of factors may influence the magnitude of any increase in *Bifidobacterium* numbers, the most important being the initial size of the population in the intestinal tract. In individuals colonized by an already large population of bifidobacteria (in the order of 10^8 cfu/g faeces), prebiotic consumption appears not to increase *Bifidobacterium* numbers further (Rao, 1999).

Of the lactose derivatives, lactulose has the best accumulated evidence of prebiotic effects on the adult microbiota. A number of well-controlled feeding trials with lactulose have consistently shown bifidogenic effects, which have been detected using both traditional microbiological culture methods and molecular ecology techniques (Ballongue *et al.*,1997; Tuohy *et al.*, 2002; Bouhnik *et al.*, 2004a; De Preter *et al.*, 2006; Vanhoutte *et al.*, 2006). GOSs have also demonstrated prebiotic effects in adults, but the bifidogenic impact of GOSs in infants has not been as consistently replicated in adult feeding trials (summarized by Rastall, 2006). DNA-based measurements of the composition of the bacterial community in the gut do not necessarily reflect the metabolic activity of specific strains. Using RNA-DGGE, this activity can be monitored (Tannock *et al.*, 2004). Those authors found that feeding adults a dose of GOSs as low as 2.5 g/day did change the metabolic activity of bifidobacteria. Unlike FOSs and inulin, these lactose derivatives also appear to increase the numbers of lactobacilli in faeces. Additionally, these increases have often been accompanied by concomitant reductions in putrefactive and pathogenic bacterial populations. Effective doses have usually been 10–20 g/day, although lactulose has been reported to have bifidogenic effects also at lower doses (Terada *et al.*, 1992; Bouhnik *et al.*, 2004b).

Lactitol also has been shown to have prebiotic effects in adults similar to lactulose (Ballongue *et al.*, 1997; Kummel and Brokx, 2001; Chen *et al.*, 2007) although it is possibly slightly less potent (Ballongue *et al.*, 1997). Additionally, there is some evidence of prebiotic action by lactosucrose from a number of small human feeding trials (Fujita *et al.*, 1991; Yoneyama *et al.*, 1992; Hara *et al.*, 1994b; Ohkusa *et al.*, 1995).

Overall, there is evidence that the consumption of some lactose derivatives can result in changes in the composition of the intestinal microbiota in adults. The observed increases in the numbers of saccharolytic, acidogenic bacteria and reductions in the numbers of putrefactive and potentially pathogenic bacteria could be reasonably hypothesized to benefit health. However, a clear link between these changes in the microbiota composition and improved health clinical end-points in adults is yet to be established.

5.9.4.3. Fermentation of Lactose Derivatives by the Intestinal Microbiota

In addition to modifying population dynamics, prebiotic lactose derivatives also modify the activity of the microbiota by providing a source of readily fermentable carbohydrate. It may be this dietary fibre-like characteristic of modifying the fermentative activity of the existing microbiota that is the important factor in providing a number of health benefits to consumers. The proposed health effects of prebiotics believed to be largely contingent on modifications to the metabolic activity of the microbiota include reductions in risk factors for colon cancer, increased mineral absorption, improved lipid metabolism and increased resistance to intestinal pathogens.

5.9.4.4. Colon Cancer

The intestinal microbiota has a number of biochemical activities relevant to colon cancer risk that relate to the composition and activity of different bacterial populations. Hence, lactose-derivative prebiotics may have a role in reducing risk factors for colon cancer. Proposed mechanisms include supplying the colonic epithelium with SCFA (particularly butyrate); suppression of microbial protein metabolism, bile acid conversion and other mutagenic and toxigenic bacterial reactions; and immunomodulation (Figure 5.8).

A number of studies on humans and animals have shown that the consumption of lactulose (Terada *et al.*, 1992; Ballongue *et al.*, 1997; De Preter *et al.*, 2004) and GOSs (Rowland and Tanaka, 1993; Kawakami *et al.*, 2005) improves the colonic environment in terms of reducing the levels of mutagenic enzyme activities (e.g., β-glucuronidase and azoreductase) and bacterial metabolites (e.g., secondary bile acids, phenols and indoles) that are purportedly associated with risk of colon cancer. However, the quantitative importance of these markers to eventual cancer development remains to be established.

Protection by prebiotics against the development of pre-neoplastic lesions and tumours in rodent models of colon carcinogenesis has also been reported for lactulose (Rowland *et al.*, 1996; Challa *et al.*, 1997), while in a human feeding study, administration of lactulose reduced the recurrence of colonic adenomas in patients who had had surgery to remove adenomas (Ponz de Leon and Roncucci, 1997). However, these results are very preliminary and a great deal more research is required to discern if the consumption of lactulose has a protective function against colorectal cancer.

5.9.4.5. Mineral Absorption

Encouraging results have been reported for the impact of fermentable carbohydrates, including GOSs and lactulose, on increasing mineral absorption from the gut. The precise mechanisms of prebiotic-mediated improvements in

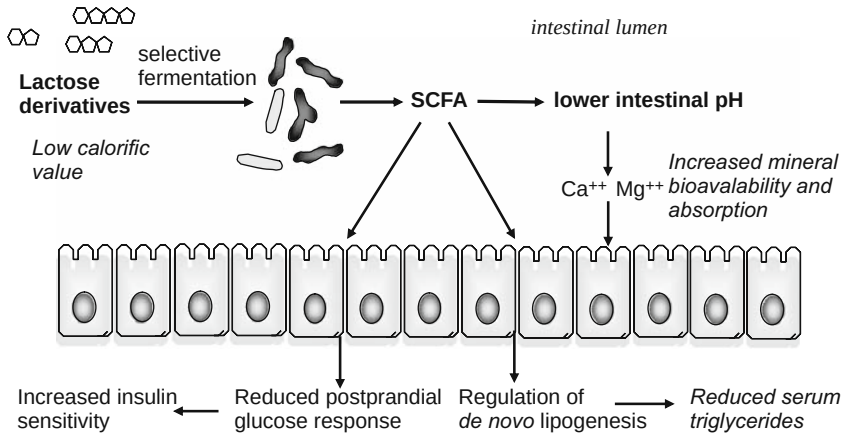

Figure 5.11. Proposed mechanism by which prebiotic lactose derivatives impact on cardiovascular health and mineral absorption.

mineral uptake remain unclear, but fermentative activities of the microbiota, including the production of SCFA and reductions in luminal pH, are believed to be involved in improving mineral solubility and extending the site of mineral absorption further into the colon (Morohashi, 2002) (Figure 5.11). In rat studies, increased calcium uptake has lead to improved bone mineralization for animals fed GOSs (Chonan *et al.*, 1995) or lactulose (Mizota, 1996), and improved calcium uptake has also been reported in human feeding studies using these two lactose derivatives (Van den Heuvel *et al.*, 1999; 2000). Though preliminary, the results to date suggest that the consumption of lactulose or GOSs may improve calcium uptake and further research is warranted to investigate links between long-term consumption and improved bone density in humans at risk of developing osteoporosis.

5.9.4.6. Colonization Resistance

The ability of lactose derivatives to improve colonization resistance and prevent bacterial infections in the gut has not been explored thoroughly, but results so far indicate a potential application for lactulose and NDOs in this function. Özaslan *et al.* (1997) observed lower caecal overgrowth and translocation of *E. coli* in rats with obstructive jaundice when they were fed lactulose, while Bovee-Oudenhoven *et al.* (1997) reported that the consumption of lactulose increased colonization resistance against the invasive pathogen, *Salmonella enteritidis,* in rats. Indeed, the consumption of high doses (up

to 60 g/day) of lactulose is effective in eliminating salmonella from the intestinal tract of chronic human carriers and it is used as a pharmaceutical for this purpose in some countries (Schumann, 2002). The mode of action is speculated to be acidification of the gut that prevents the growth of this acid-sensitive pathogen. In a placebo-controlled study, Chen *et al.* (2007) reported that feeding lactitol significantly reduced plasma levels of endotoxin in a group of patients with chronic viral hepatitis. Endotoxaemia is closely correlated with the disturbance of the gut flora and the decline of colonization resistance in these patients. Consuming lactitol significantly increased populations of bifidobacteria and lactobacilli, perhaps contributing to an improved intestinal barrier.

Another mechanism by which oligosaccharides may provide protection against enteric infections is through competitive inhibition of pathogen adherence to the mucosa. Oligosaccharides can act as structural mimics of the pathogen-binding sites, which are often carbohydrate epitopes. HMOs act in this way to block the initial binding of a range of pathogens to inhibit colonization (Gibson *et al*., 2005; Shoaf *et al*., 2006). *In vitro* experiments using epithelial cell culture models (Tzortzis *et al*., 2005b; Shoaf *et al*., 2006) and *in vivo* monkey challenge experiments (Gibson *et al*., 2005) have shown that GOSs and lactulose have the ability to interfere with the adhesion of enteropathogenic *E. coli.* The ability rapidly to install or restore colonization resistance where the intestinal microbiota has been perturbed may prove to be an effective use of lactose derivatives in the future. Possible mechanisms by which prebiotics may increase colonization resistance are illustrated in Figure 5.12.

5.9.4.7. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) describes a group of chronic, severe, relapsing inflammatory conditions of the gut that includes Crohn's disease and ulcerative colitis. A genetic predisposition to develop an over-zealous inflammatory immune response to components of the intestinal microbiota has been implicated in its aetiology (Schultz *et al*., 2004). Elimination of specific bacterial antigens, immunomodulation and trophic effects of SCFA on the intestinal epithelium have all been proposed as mechanisms by which prebiotics could alleviate symptoms (Figure 5.9). The size of the intestinal *Bifidobacterium* population has been shown to be relatively small (Favier *et al*., 1997; Linskens *et al*., 2001) in subjects afflicted with IBD, although cause and effect links between disease and a diminished intestinal *Bifidobacterium* population remain to be established. Using different rodent models of IBD, Rumi *et al.* (2004) and Camuesco *et al.* (2005) ameliorated inflammation by feeding lactulose. Camuesco *et al*. (2005) noted that the improvement

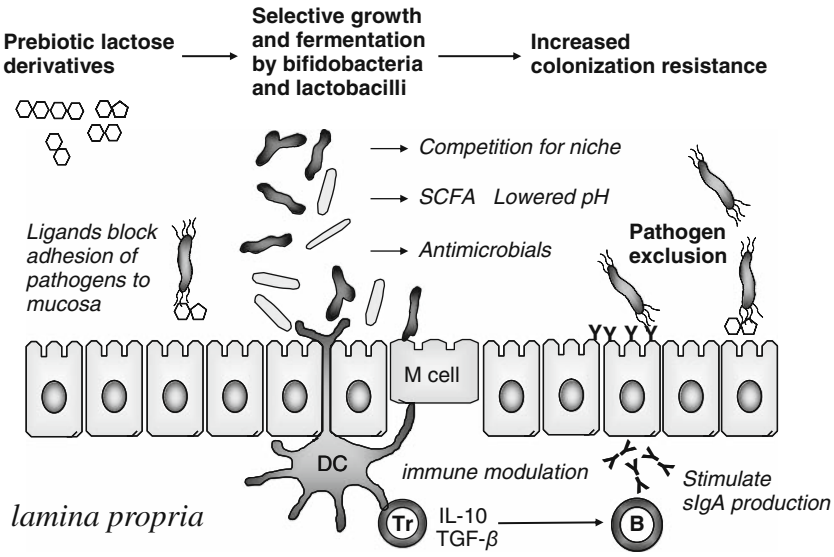

Figure 5.12. Proposed mechanisms by which prebiotic lactose derivatives increase colonisation resistance in the gastrointestinal tract.

was associated with an increase in the numbers of bifidobacteria and lactobacilli. However, using the same model as Camuesco *et al.* (2005), Holma *et al.* (2002) observed no reduction in inflammation by intervention with galacto-oligosaccharides despite an increase in *Bifidobacterium* numbers. While the results with lactulose are encouraging, the scientific literature is unfortunately littered with examples of treatments for IBD that have worked in animal models, only to fail dismally in human studies. Despite this, further research into the efficacy of lactulose in the treatment of IBD is warranted.

5.9.4.8. Glycaemic Index, Weight Management and Serum Lipids

Since lactose derivatives are sweet and not digested, they have a low calorific value and are used as low-energy, low glycaemic index sweeteners that are also suitable for diabetic individuals. Additionally, several other physiological effects resulting from the consumption of non-digestible, fermentable carbohydrates have been reported that impact on weight management and serum lipid profiles. A number of prebiotic carbohydrates, including lactose derivatives (Ferchaud-Roucher *et al.*, 2005; Shimomura *et al.*, 2005; Vogt *et al.*, 2006), have been shown to reduce serum triglyceride level in animal and human

studies. The mechanism by which lowering of serum lipids and cholesterol may occur has been speculated to be regulation of host *de novo* lipogenesis through SCFA (particularly acetate) absorbed from the gut (Williams and Jackson, 2002) or by reduced intestinal fat absorption (Shimomura *et al.*, 2005) (Figure 5.10). Although promising, not all feeding studies in humans using prebiotic carbohydrates have resulted in clinically significant changes in serum lipid profiles, and further research is needed to identify responsive populations, interactions with diet and to show benefits of dietary intervention in clinical end-points such as impact of cardiovascular disease.

Preliminary data have also suggested that lactulose (Ferchaud-Roucher *et al.*, 2005; Brighenti *et al.*, 2006) and lactitol (Juśkiewicz *et al.*, 2006) could improve insulin sensitivity. In a small human feeding trial, Brighenti *et al.* (2006) demonstrated that feeding a meal containing lactulose diminished post-prandial blood glucose response in a subsequent meal ("second meal effect") by reducing competition by non-esterified fatty acids for glucose disposal, and to a minor extent, by affecting intestinal motility. The potential of fermentable carbohydrates in the management of metabolic disorders linked to insulin resistance may warrant further study.

5.9.5. Farm and Companion Animals

Prebiotic oligosaccharides, including GOSs, have been evaluated for use in both farm animal feeds and for companion animals, especially dogs. The advantage of use with dogs is the positive impact of feeding oligosaccharides on reduced odour and improved volume and consistency of faeces (Swanson and Fahey, 2006). With respect to farm animals, prebiotics have been studied for their potential to replace antibiotics in maintaining high-feed conversion efficiencies and also to suppress methane production by ruminants (Mwenya *et al.*, 2004a,b; Sar *et al.*, 2004; Santoso *et al.*, 2004).

5.10. Product Safety, Dose Rates and Regulatory Issues

5.10.1. Galacto-oligosaccharides

The regulatory regimes for non-digestible carbohydrates have been under active review in many countries in recent years. A marked development since 2000 has been the inclusion of most non-digestible oligosaccharides in the category "dietary fibre" (see definition in Section 5.6). This has been a landmark development, because it allows recognition of these products as having some health benefits. Prior recognition in a regulatory sense had been restricted to Japan, where for many years the functional food sector was regulated by the FOSHU system.

In Japan, the occurrence of an ageing population and the likely national health costs were recognized much earlier than in Western countries. So by 1991, a special category of FOSHU regulations was introduced. By December 2007, there were 755 FOSHU-approved products. Those with gastrointestinal claims, which include products containing prebiotics such as FOS, lactosucrose and lactulose, accounted for 51% of sales in 2007 (Bailey, 2008). FOSHU is controlled by the Japan Health Food and Nutrition Food Association. It has produced English-language versions of its guide, brochure and list of approved products.

The use of GOSs as an optional ingredient in infant milk formulae and infant foods has been the subject of intensive regulatory inquiry and its acceptance varies among countries. Revised Codex standards were released in November 2006 (Codex ALINORM 07/30/26). Readers are referred to the Codex website (www.codexalimentarius.net) for current standards (CODEX STAN 72-1981; and 156-1987).

5.10.1.1. Safety Issues and Dose Rates

Safety of use must always be dominant in the development of new food products. Fortunately, it is well established that GOSs and lactulose are safe, even at high doses. Galacto-oligosaccharides are considered safe ingredients as they are constituents of human milk and can also be produced in the gut by intestinal bacteria from ingested lactose. Acute toxicity tests in rats have shown that ingestion of more than 15 g/kg body weight of $\beta 1 \rightarrow 4$ galacto-oligosaccharide was needed for LD_{50}. No adverse symptoms were recorded in a chronic toxicity test when 1.5 g/kg bodyweight were fed for 6 months. Non-mutagenicity was confirmed by the AMES-*Salmonella* and Rec assay (Matsumoto *et al.*, 1993; Sako *et al.*, 1999). However, excessive intakes ($>$ 30 g/day in adults) can lead to flatulence, cramping and osmotic diarrhoea, particularly with the shorter chain oligosaccharides. There is some evidence of adaptation to oligosaccharides by individuals, who can then eat double the normal recommended maximum doses without symptoms (Schoterman, 2001). Furthermore, other non-digestible carbohydrates are present as natural components in many vegetable food sources. Estimates have been published of expected intakes of fructo-oligosaccharides and inulin from such sources. It is likely that an intake around 8 g/day per adult human may be normal.

Recommended effective doses of added oligosaccharides in adult humans usually range from 10 to 20 g/day. With the slower-fermenting inulin or resistant starch, an intake of up to 40 g/day is acceptable. With the short-chain oligosaccharides, few ill-effects have been recorded in adults with intakes of 25–30 g/day, but adaptation was sometimes necessary. These intakes are all additional to what might be derived from other food components in a normal

diet. Bifidogenic responses are not always found when oligosaccharides are added to the diet of humans. This occurs when the populations of bifidobacteria in the colon are already relatively high (Alander *et al.*, 2001).

5.10.2. Lactulose

Most preparations of lactulose contain substantial concentrations of lactose, epi-lactose and galactose. Thus, the use of lactulose is contraindicated in those who require low lactose or low galactose diets. In some countries, such as the USA, lactulose is a prescription drug and its use is limited to prescribed medical use. Those who develop gastrointestinal disturbances, such as bloating, discomfort, flatulence and diarrhoea, with dietary fibre should exercise caution in using lactulose. Acute and sub-acute toxicity data for lactulose have been summarized by Mizota *et al.* (1987) and are similar to that for sucrose. In 1992, the Ministry of Health and Welfare in Japan approved lactulose as a useful ingredient in specified foods currently being sold for health use (FOSHU). Thus, in 1995, a soft drink, "Mai-asa Sohkai", containing 4 g lactulose per carton, was approved as "a beverage made from lactulose which properly supports the proliferation of *Bifidobacterium* in the intestines to maintain good condition of the bowels" (Anon., 1997).

As an anti-constipation drug, the normal daily dose for adults is 20 g lactulose. It is also established that ingestion of lactulose at higher doses can result in diarrhoea.

5.10.2.1. Toxicological Data

Canlac, a subsidiary of Solvay Pharmaceuticals, has measured toxic effects of Lactulose USP concentrate (693 g/L). The results of these studies can be summarized as:

Acute toxicity – by oral route, LD_{50} in rats, 30–45 mg/kg

Chronic toxicity

- oral route (diet), after prolonged exposure, in rats, 15 ml/kg, no observed effect.
- oral route (diet), after prolonged exposure, in dogs, target organ: gastrointestine, 4 ml/kg, no observed effect
- no effect on fertility
- no teratogenic effect.

The lactulose concentrate was not hazardous under normal conditions of handling and use. Ecotoxicity tests were also measured. In summary, no adverse environmental effects were found, and it was not regarded as hazardous for the aquatic environment (Anon., 2003).

5.10.3. Lactosucrose

The minimum effective dose of lactosucrose is 5 g/day for an adult. When it is taken in large amounts, lactosucrose may cause a rise in gastrointestinal osmotic pressure and induce diarrhoea. Its maximum no-effect dose is 0.6 g/kg bodyweight. An optimum dose is considered to be between 5 and 36 g/day for an adult human (Oku and Tsuji, unpublished).

5.10.4. Lactitol

The European Community listed lactitol as a permitted sweetener (E 966) (EC Directive 96/83/EC) and the USA FDA has accepted lactitol for GRAS listing. Lactitol is approved as a sweetener in over 30 countries.

Lactitol was last evaluated in 1983 by the Joint FAO/WHO Expert Committee on Food Additives. A range of short- and long-term studies on its absorption, distribution and excretion in biochemical studies and in animal models were examined. Toxicological studies were examined including studies on carcinogenicity, dermal irritation, eye irritation, mutagenicity, reproduction and teratogenocity (for details, see JECFA, 2008; Inchem, 2008). In humans, lactitol at 24 g/day orally is tolerated well by healthy and diabetic persons. It does not alter blood glucose level. Higher doses (50 g/day) cause diarrhoea. It was concluded that lactitol has only a very low general toxicity following large doses. The Expert Committee did not believe that lactitol at the normal levels of intake likely to be experienced presented a hazard to health. No acceptable daily intake for man (ADI) was specified by the committee, and the establishment of a numerical figure was not deemed necessary.

A biodegradability test based on dissolved organic carbon showed complete degradation within 5 days.

5.10.5. Lactobionic Acid

The regulatory status in the food industry is limited. It is approved as a food additive in the form of its calcium salt. Its use is as a firming agent in dry pudding mixes [Code of Federal Regulations, Title 21, Vol. 3 (21CFR 172.5), US Food and Drug Administration]. However, it has many non-food uses, such as in detergents, chelating agents and in cosmetics. It also is accepted by the US Food and Drug Administration as an inactive ingredient for medical use in organ transplantation solutions.

5.10.6. Tagatose

Tagatose was listed in 2001 as a GRAS ingredient by the US Food and Drug Administration. The Food Standards Agency in the UK recognized tagatose as a novel food ingredient in the EU, and this is expected to be ratified soon. Also, it has been approved for food use in Brazil, Korea, Australia and New Zealand, and approval in Mexico, Japan and Canada is expected shortly.

5.11. Conclusions

5.11.1. Future Directions and Challenges

The manufacture of GOSs and the other lactose-derived compounds is in the process of rapid expansion globally. A major reason for this expansion in production in recent years has been the general regulatory acceptance of the addition of GOSs to infant milk formulae and infant foods. More broadly, the ability to include these ingredients under the heading "dietary fibre" on food labels has enabled some health benefits to be stated. However, as discussed earlier, there is now some doubt whether oligosaccharides will remain classified as dietary fibres.

As further clinical and animal studies are conducted, a range of non-digestible carbohydrates are gaining medical acceptance as prebiotics, which are able to confer some health benefits. This includes GOSs and lactulose. The evidence is less well established for lactosucrose and lactitol. Lactobionic acid and tagatose are not considered to be prebiotics, although unsubstantiated claims have been made for tagatose.

Evidence exists that β-galactosidases obtained from certain microorganisms (e.g., *B. bifidum)* tend to produce higher proportions of the longer chain galacto-oligosaccharides. These mixtures tend to be used more specifically by certain species of *Bifidobacterium* (Rabiu *et al.*, 2001; Tzortzis *et al.*, 2005a). This search for prebiotic carbohydrates with greater selectivity by probiotic bacteria is of high interest to manufacturers, and offers scope for the development of genuine synbiotic products. The ultimate in synbiotic combinations will include oligosaccharides that can not only benefit the proliferation and activity of the specific probiotic strains in the colon, but also protect those bacteria during manufacture, formulation and storage, and during gastrointestinal transit.

The efficiency of oligosaccharide synthesis is also being improved by modifying the β-galactosidase molecule by deletion of amino acid residues in the protein. This has converted the enzyme to being predominantly a trans-galactosylation enzyme rather than a hydrolytic enzyme (Jorgensen *et al.*,

2001). This truncated enzyme performed equally well at lactose concentrations ranging from 10 to 40%. Thus, there is much scope for modifying the chain length of oligosaccharides synthesized and to do this with high yields.

A number of the health benefits of lactose derivatives are well established, including the pharmaceutical applications of lactulose and lactitol as laxatives and treatments for hepatic encephalopathy. It is clear too that ingestion of GOSs and lactulose can modify the composition and activity of the intestinal microbiota, and there is some evidence also for prebiotic effects of lactitol and lactosucrose.

Increasing the numbers of bifidobacteria or lactobacilli in the intestinal microbiota of individuals with an unfavourable intestinal microbial balance appears with our current understanding of the human intestinal microbiota to be a reasonable approach to promoting intestinal health. However, basic research into the composition and role of different microbial populations within the intestinal microbiota in health and disease is an essential prerequisite for the development of appropriate prebiotic strategies. Little is currently known of the sub-genus changes in bifidobacterial populations that can be induced by non-digestible carbohydrates, or if such changes are important in a health context. A better understanding of what constitutes a "healthy" intestinal microbiota composition, and which microbial groups and activities are definitively involved in health and disease, will allow the development of prebiotics with specifically targeted health effects in the future. The recent research showing how infant formulae supplemented with GOSs can emulate many of the effects of human milk oligosaccharides on the infant microbiota and faecal consistency provides encouraging evidence for a useful role in infant nutrition. The challenge remains to link the observed changes in the intestinal microbiota with clinical end-points that clearly demonstrate a health benefit.

The galactose moiety in oligosaccharides is important in mammalian cell biology (Kobata, 1996). There is considerable interest in the development of novel galactosyl structures. This is evidenced by the emergence of a large number of glycoscience companies developing carbohydrate-based drugs. For example, there are opportunities to develop cost-effective manufacturing processes for fucose-containing and *N*-acetylglucosamine-containing galacto-oligosaccharides.

The fact that lactose derivatives are non-digestible and are fermented by the intestinal microbiota to SCFA possibly underpins many of the potential health benefits of these compounds. There is preliminary evidence for a range of health benefits, including improved mineral absorption, protection against colorectal cancer and positive impacts on insulin resistance and serum lipid concentrations. Further studies to elucidate mechanisms of action and to demonstrate clinical benefits in controlled feeding studies are certainly warranted.

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6

Lactose Malabsorption

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This chapter reviews the digestion of lactose by the human enzyme, lactase (β-galactosidase), and explains why many adults cannot digest fresh milk.

6.1. The Small Intestine and Digestion of Lactose

Lactose in milk is digested and the resulting monosaccharides are absorbed in the small intestine. The surface of the small intestine has a specialised structure, composed of hundreds of 'villi', tiny finger-like structures that protrude from the wall of the intestine and have additional extensions called microvilli which make up the apical 'brush border' of the absorptive epithelial cells (enterocytes) lining the villi. This arrangement of the epithelium maximises the surface area through which the body may absorb nutrients. The enzymes, such as lactase, that facilitate digestion and absorption of carbohydrates are anchored to the surface of the brush border.

Small intestinal lactase activity was first demonstrated by Pautz and Vogel (1895). The enzyme lactase is responsible for cleaving lactose into its constituent monosaccharides, glucose and galactose, which are transported across the epithelial cell membranes into the enterocytes and then into the bloodstream via active transport by a sodium-dependent galactose transporter (Wright *et al*., 2007). Lactose itself cannot be transported across the cell membrane, and hence lactase is essential for the nourishment of neonatal mammals, whose sole source of nutrition is milk and in which lactose is the

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major carbohydrate component. However, in adult mammals the enzyme activity usually decreases significantly following weaning (Sebastio *et al.*, 1989; Buller *et al.*, 1990; Lacey *et al.*, 1994; Pie *et al.*, 2004).

Humans differ in this respect, and it has long been noted that whilst some adults are able to digest large quantities of fresh milk, others cannot, and show symptoms of lactose malabsorption following ingestion of a lactose load. This difference is most commonly due to normal genetically determined variation in the quantity of lactase present in the adult small intestine, and individuals with little lactase are described as lactase non-persistent or are said to have primary adult hypolactasia, or be lactose maldigesters, while those who maintain a high level of lactase are said to be lactase persistent. This normal variation is quite distinct from the absence of lactase from birth, which is an extremely rare and potentially fatal inborn error of metabolism and is referred to as congenital alactasia. A number of mutations within the coding region of the lactase gene have been identified in Finnish patients suffering from this condition that affect the primary structure of the protein, resulting in a non-functional lactase enzyme (Kuokkanen *et al.*, 2006).

Lactase level can also be reduced if damage occurs to the brush border due to gastrointestinal disease, and this condition (which is usually reversible) is referred to as secondary or acquired hypolactasia (Villako and Maaroos, 1994).

People who have either primary or secondary lactase deficiency are lactose intolerant, as judged by a lactose tolerance test, and may exhibit symptoms of lactose intolerance. This chapter is concerned primarily with the former scenario, and considers lactose malabsorption in the context of genetically controlled variation in the expression of lactase in adults.

6.2. Lactase and its Structural Gene, *LCT*

The gene *LCT*, which encodes the enzyme lactase, a β-glycosidase capable of hydrolysing a variety of substrates and often known as lactase-phlorizin hydrolase (LPH), maps physically to chromosome 2q21 (Kruse *et al.*, 1988; Spurr and White, 1991; NIH/CEPH Collaborative Mapping Group, 1992; Harvey *et al.*, 1993). The nucleotide sequence exhibits fourfold internal homology, suggesting that two partial gene duplication events occurred in the evolution of this gene. Two of the homologous domains (I and II) occur in the pro-region of the molecule and the others (III and IV) are found in the mature polypeptide (Mantei *et al.*, 1988).

The 5787 bp pre-pro-lactase-phlorizin hydrolase mRNA transcript is encoded by 17 exons (Boll *et al.*, 1991). The pre-pro-protein, composed of 1927 amino acids (Mantei *et al.*, 1988) contains a putative signal sequence of 19 amino acids, and a large 'pro' portion of 847 amino acids, both of which

are proteolytically removed before the protein assumes its mature form. The 19 amino acid pre-sequence is first removed in the ER by a signal peptidase, yielding pro-LPH molecules, which become *N*-glycosylated and pair up to form homodimers (Grunberg and Sterchi, 1995). This dimerisation is essential for the acquisition of transport competence and full enzymic activity of LPH (Naim and Naim, 1996). Further (*O*-linked) glycosylation occurs once the pro-LPH homodimer has been translocated to the Golgi apparatus, which is also the predominant site of proteolytic cleavage of the pro-sequence (Naim *et al*., 1987). The pro-sequence has been shown to play a vital role in the maturation of LPH, being involved in folding, targeting and dimerisation of the molecule (Panzer *et al*., 1998).

Residues from Ala 867 onwards comprise the 160 kDa glycoproteins found anchored to the brush border of the jejunum as mature LPH homodimers (Mantei *et al*., 1988). LPH is an amphiphilic molecule, consisting of a short cytoplasmic domain followed by a membrane-spanning hydrophobic domain (residues 1883–1901) at its C-terminus, orientating the molecule such that the bulky, hydrophilic N-terminal projects into the lumen (Skovbjerg *et al.*, 1981; Wacker *et al.*, 1992). It is within this N-terminal portion that both catalytic activities reside, and it has been demonstrated that the active site for phlorizin hydrolysis is distinct from that for lactose (Leese and Semenza, 1973; Columbo *et al.*, 1973; Skovbjerg *et al.*, 1981). The active sites are situated within the homologous domains III (Glu1271) and IV (Glu1747), respectively (Arribas *et al*., 2000).

The 'phlorizin hydrolase site' is situated closest to the brush border and preferentially catalyses the hydrolysis of β-glycosides with large, hydrophobic aglycones (galactosyl and glucosyl β-ceramides, phlorizin and other aryl- or alkyl-β-glycosides). The 'lactase' catalytic site has been shown to have a preference for β-glycosides with hydrophilic aglycones (e.g. lactose, cellobiose and some β-1,4-linked small glucose oligomers) (Leese and Semenza, 1973; Columbo *et al.*, 1973; Skovbjerg *et al.*, 1981). LPH is also capable of hydrolysing various flavonol and isoflavone glucosides (Day *et al*., 2000), but it is not currently known which active site is used.

6.3. Symptoms of Lactose Intolerance

The symptoms of lactose intolerance due to lactose malabsorption, and caused when milk is consumed by a lactase non-persistent person, vary greatly from person to person, but if they are evident, they usually manifest themselves within 1–2 h of ingestion.

Undigested lactose passing through the small intestine into the colon has two physiological effects. First, an osmotic gradient is set up across the

gut wall, which results in a large influx of water to redress the osmotic imbalance, causing symptoms of diarrhoea. Second, the lactose can be utilised as an energy source by colonic bacteria, which ferment it to produce fatty acids and gaseous by-products, potentially causing discomfort, bloating and flatulence.

Most lactase non-persistent individuals can tolerate small amounts of lactose (as contained in the milk added to tea or coffee). Diarrhoea and discomfort are not seen in all individuals who are diagnosed as lactose maldigesters in a lactose tolerance test, even after consumption of 50 g of lactose, and it has been suggested that variation in the composition of the gut flora between individuals may be responsible for some of this variation (Hertzler and Savaiano, 1996; Hertzler *et al.*, 1997), as well as a psychosomatic component (Briet *et al.*, 1997). In contrast, two separate studies suggest that around 40% of self-diagnosed lactose malabsorbers are actually lactose digesters (Saltzman *et al.*, 1999; Peuhkuri *et al.*, 2000). This effect is perhaps due to prevalence in the public consciousness of 'dairy intolerance' caused by extensive media coverage of the condition, leading individuals to assign any symptoms of gastrointestinal discomfort to this cause.

The symptoms described above were first attributed to a lack of lactase following the observation that a proportion of intestinal tissue samples from healthy adults with histologically normal mucosa had virtually no lactase activity (Auricchio *et al.*, 1963; Dahlqvist *et al.*, 1963). The discovery of this enzyme deficiency or 'abnormality' in humans ignited the interest of researchers into the inter-individual differences in our capacity to tolerate milk and its derived dairy products. In the following years, data on lactase persistence frequencies in other populations began to accumulate and a global picture began to develop that challenged the original perception of lactase non-persistence as the 'abnormal' phenotype.

6.4. Diagnosis of Lactase Non-Persistence/Persistence

In order to collect information on the worldwide frequencies, alternatives to direct quantification via biopsy of the small intestine were used. Biopsies are the most accurate method for establishing lactase activity. However, they are invasive and are not usually a preferred routine diagnostic for lactose intolerance, normally being obtained only when a patient is undergoing endoscopy to exclude another gastrointestinal complaint.

Several indirect methods have been developed for the purpose of diagnosis, all of which utilise lactose digestion to inform on an individual's lactose tolerance status, and by implication lactase persistence status. The general practice is to give a lactose load after an overnight fast. The two most widely used methods are described below.

6.4.1. The Blood Glucose Test

A baseline measurement of blood glucose is taken before ingestion of a lactose load, and then at various time intervals (usually every 30 min) for the following 2 h. An increase in blood glucose indicates lactose digestion (lactase cleaves the lactose molecules into glucose and galactose, allowing absorption into the bloodstream and subsequent detection), and no increase, or a 'flat line', is indicative of a lactose non-digester/maldigester or intolerant phenotype.

6.4.2. The Breath Hydrogen Test

This test measures hydrogen production by colonic bacteria. If a lactose dose meets the enzyme lactase in the small intestine, no changes in breath hydrogen will be observed, and a diagnosis of lactose tolerance is made. Conversely, in lactose maldigesters, the lactose load passes through the small intestine and into the colon where it is digested by bacterial fermentation, one by-product of which is hydrogen. Some of this hydrogen is absorbed into the bloodstream and released into the breath (where it can be detected) as the blood passes through the lungs. A baseline measurement of breath hydrogen is taken, prior to ingestion of the lactose load, and further readings are taken at 30 min intervals from the time of ingestion for the following 3 h.

In both cases, somewhat arbitrary cut-off points have to be set for distinguishing the two phenotypes and both methods inform on the person's ability to digest lactose rather than the given individual's lactase expression. We infer the lactase persistence status of an individual from these tests, and must therefore keep in mind that there will be an error in both directions.

Some of these observed errors could be attributed to test design, particularly the quantity of lactose administered. Non-persistent individuals express a residual amount of lactase, approximately 10% of adult levels (Semenza *et al.*, 1999) and so when only a low-lactose dose is used the quantity passing through to the colon may not be large enough to increase breath hydrogen by the standard $>$20 ppm increment. Using the blood glucose method would, on the other hand, have the opposite effect. Lactase-persistent individuals may show an insufficient rise in blood glucose to cross the nominated threshold (usually 1.1 mmol/L). Also, some studies suggest that even lactase-persistent people fail to digest a proportion of consumed lactose, and therefore an increase in breath hydrogen could be observed even in persistent subjects when a high-dose challenge (such as 50 g) is used (Bond and Levitt, 1976).

Apart from dose, many other factors can impact upon the test result; gastric emptying and intestinal transit times could exert an effect both on blood glucose and breath hydrogen measurements. Diarrhoeal disease is known to reduce lactase expression temporarily as a result of villus flattening and loss of the cells

which express lactase (Villako and Maaroos, 1994) and hence a genetically persistent individual may be classified as a maldigester in this instance. Also, the use of antibiotics may disrupt the gut flora and result in erroneous results. Colonic adaptation to dairy products may affect breath hydrogen production by increasing bacterial populations that have increased metabolic activity for lactose (Hertzler and Savaiano, 1996). Also, some individuals will be 'hydrogen non-producers' (as a result of having a sterile gut or a hostile gut pH with acidity too severe for the existence of hydrogen-producing bacteria), and in this situation the breath hydrogen test would be uninformative.

In the clinical setting, there are ways of improving the quality of the test. These include retesting, and giving a dose of lactulose to test for hydrogen production, and investigation of other causes of the lactose intolerance.

A recent study by members of our group (Mulcare *et al.*, 2004) attempted to estimate the error in both the blood glucose and the breath hydrogen test from published data. Results were pooled from papers that compared either indirect method with each other or with a verified phenotype based on direct enzyme assays from jejunal biopsy. Exact protocols varied between the pooled data set, but all included a minimum 50 g lactose load and measured a change in the parameter one or more times between 30 min and 4 h after ingestion. The error of the blood glucose method was 7% false positive (i.e. non-persistent individuals classified as lactose digesters) and 9% false negative (i.e. persistent individuals classified as lactose malabsorbers). The breath hydrogen method was found to give a slightly more accurate assessment of lactase persistence status, with approximately 5% false positive and 7% false negative error. Thus, the evidence suggests that to obtain the most accurate indirect assessment of lactose tolerance status, a breath hydrogen test should be undertaken. The most accurate method (from our own experience) requires a fast of 12 h to be observed prior to consumption of the lactose dose, 50 g of which is the widely accepted standard (equivalent to approximately 1 L of cow's milk). A baseline breath hydrogen measurement should be taken prior to the lactose dose, and at 30 min intervals afterwards for the following 3 h (Peuhkuri, 2000). Test results for subjects with a H_2 baseline of zero (possible non-producers), or greater than 20 ppm (suggestive of failure to fast or bacterial overgrowth of the colon), should be interpreted with caution and followed up if possible.

6.5. Worldwide Distribution of Lactase Persistence

A number of surveys of lactase persistence phenotype frequencies have been carried out in many populations throughout the years, so that the global distribution of lactase persistence is now fairly well characterised (Figures 6.1a, b)

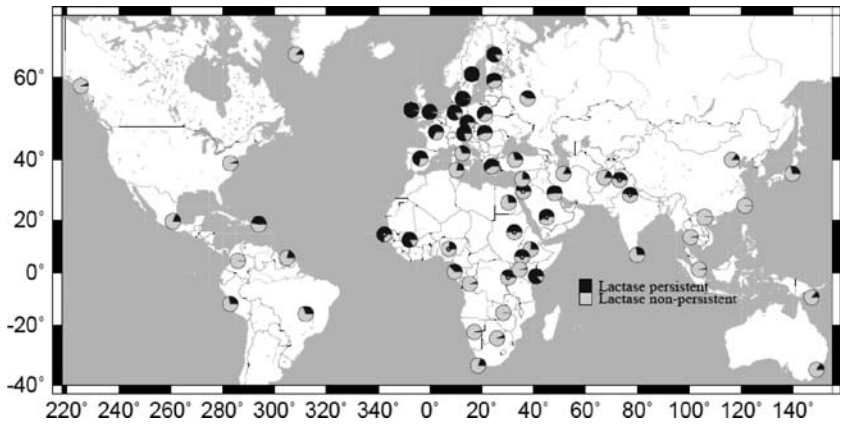

Figure 6.1a. Worldwide distribution of frequency of lactase persistence. Dark grey indicates the proportion of lactose digesters (presumed lactase persistent) in a given population and light grey represents maldigesters (presumed non-persistent). A central circle indicates that the overall frequency for that country is comprised of different ethnic groups with very different phenotype frequencies. For examples of these cases, see Figure 6.1b. (Data taken from Bloom and Sherman, 2005; Mulcare, 2006)

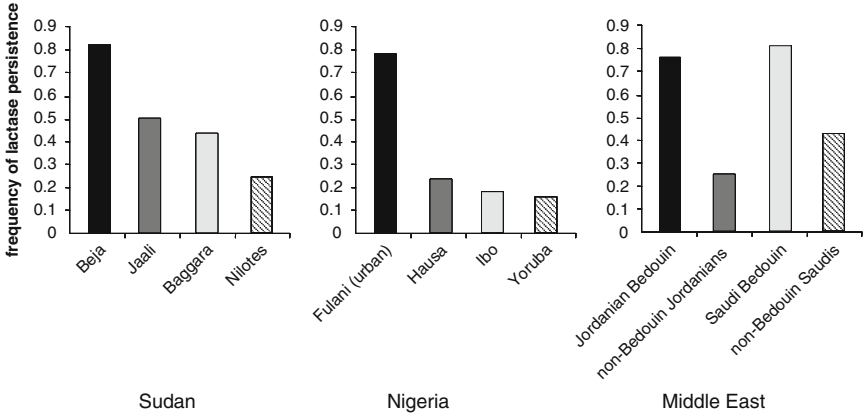

Figure 6.1b. Examples of countries in which individual ethnic groups display large differences in lactose absorption capacity.

(Swallow and Hollox, 1999). These frequencies reveal clearly that lactose intolerance is the most commonly observed phenotype in humans, with lactase persistence being common only in those populations with a long history of pastoralism and where milking has been practised. Lactase persistence is at highest frequency in north-western Europe, with a decreasing cline to the south

and east. In India, the frequency of lactase persistence is higher in the north than the south, and in the rest of the world, lactase persistence frequency is generally low. In Africa, the distribution is patchy, with some pastoralist nomadic tribes having high frequencies of lactase persistence compared with the neighbouring groups inhabiting the same country (Bayoumi *et al.*, 1981), with a similar pattern observed between Bedouin and neighbouring populations in the Middle East (Cook and al-Torki, 1975; Snook *et al.*, 1976; Hijazi *et al.*, 1983; Dissanyake *et al.*, 1990).

The noted correlation of lactase persistence phenotype with the cultural practice of milking engendered the hypothesis that this trait has been subject to strong positive selection.

6.6. Identifying the Causes of Lactase Persistence

As discussed above, many research groups (Sebastio *et al.*, 1989; Buller *et al.*, 1990; Lacey *et al.*, 1994; Pie *et al.*, 2004) reported lactase activity in the intestine of young and suckling mammals, but not in the intestine of the corresponding adult of the species, which provided further evidence that the ancestral state for mammals (and therefore humans) is for lactase expression to be down-regulated following the weaning period.

Many enzymes are regulated via an inducible system whereby expression of an enzyme is activated or increased if the concentration of its substrate is high, for example, the lac operon in *Escherichia coli* is an inducible system in which β-galactosidase is expressed only in the presence of lactose (Jacob and Monod, 1961). Initially, an inducible system was hypothesised to regulate lactase expression in humans (Gilat, 1971; Cook, 1988), and would indeed provide a neat explanation of why lactase is down-regulated following weaning and why lactase persistence is seen more commonly in populations where milk forms an integral part of the diet. However, many animal studies have shown no increase in lactase activity in response to prolonged exposure to lactose (Plimmer, 1906; Leichter, 1973; Gutierrez *et al.*, 2002), and studies in human populations confirm this: a study of 50 adult Thai individuals who voluntarily ingested daily lactose doses for 1 month reported no improvement in lactose absorption or increased lactase activity, thus demonstrating that the enzyme is not inducible (Keusch *et al.*, 1969).

By the early 1970s, it had been established using family studies that the lactase persistence polymorphism in humans had a genetic cause, and was inherited in an autosomal dominant manner (Ferguson and Maxwell, 1967; Sahi, 1974). In another study, monozygotic twins showed 100% concordance of lactase persistence phenotype, and phenotype frequencies in dizygotic twins were found to agree with expectations of an autosomal dominant

pattern of inheritance (Metneki *et al*., 1984). Further evidence that lactase persistence is a genetic trait, and more specifically that it is caused via a *cis*-acting element was produced in the early 1980s. Ho *et al*. (1982) reported a trimodal distribution of lactase:sucrase ratios in British natives. Both lactase and sucrase were extracted from autopsy material (from individuals without gastrointestinal disease) with the sucrase activity serving as an internal standard correcting for non-genetic variation. The trimodal distribution was interpreted to represent individuals homozygous for lactase persistence (highest lactase activity), heterozygotes (mid-level activity) and non-persistent homozygotes (low-lactase activity) (Ho *et al*., 1982). The intermediate lactase activity observed in the heterozygotes indicated that only one copy of the lactase gene was being fully expressed, and concordant results were subsequently obtained for individuals of German ancestry (Flatz, 1984). Confirmatory evidence for the *cis*-acting nature was obtained from mRNA studies. Allelic variants of exonic single nucleotide polymorphisms (SNPs) were used to identify particular transcripts and their expression levels. Europeans of the persistent phenotype who were heterozygous for exonic polymorphisms were used to demonstrate monoallelic expression at the mRNA level (Wang *et al*., 1995).

Studies of the *LCT* immediate promoter show a conserved ~150 bp region that exists in human, rat, pig and mouse, suggesting that key regulatory elements important for lactase expression are encoded within this small region (Troelsen, 2005). This region has been shown to drive low-level expression in an intestinal cell line (Troelsen *et al*., 1992), while transgenic mouse experiments using rat and pig promoter constructs of different sizes show that elements outside this conserved 150-bp region are required for high and tissue-specific expression of lactase (Troelsen *et al*., 1994; Krasinski *et al*., 1997; Lee *et al*., 2002; Wang *et al*., 2006). A 1 kb pig promoter construct is sufficient to mimic endogenous gene expression in transgenic mice (Troelsen *et al*., 1994). However, a 2 kb rat promoter is required to produce the same effect (Lee *et al*., 2002). In addition, the different enhancer sites encoded within the upstream regions are thought to make distinct contributions to the spatial and temporal expressions of *LCT* (Wang *et al*., 2006). The difference in promoter structure outside the proximal region in different species demonstrates the difficulty of finding a suitable model organism in which to replicate the lactase persistence phenotype.

A number of transcription factors have been identified that are important for lactase expression (Figure 6.2) (Troelsen, 2005). The transcription factor Cdx-2 is implicated in the regulation of many intestinally expressed genes (Freund *et al*., 1998; Beck, 2004) and has a number of binding sites upstream of the *LCT* initiation codon, including two within the 150 bp conserved proximal promoter region (Troelsen *et al*., 1997). Cdx-2 has been

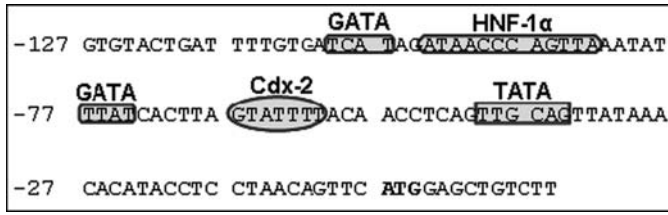

Figure 6.2. Transcription factor-binding sites of *LCT* proximal promoter in humans (adapted from Troelsen, 2005). The transcription initiation codon is indicated in bold.

suggested to be involved in differentiation of the absorptive cells of the intestinal epithelium (Mutoh *et al.*, 2005) and has been shown to up-regulate *LCT* expression in vitro. These observations would appear to support the findings that Cdx-2 may regulate lactase expression at the brush border (Fang *et al.*, 2000).

The transcription factor HNF-1 is also known to modulate the expression of lactase (Spodsberg *et al.*, 1999; Krasinski *et al.*, 2001; Bosse *et al.*, 2006). One HNF-1-binding site occurs in the proximal promoter region, and both human and pig promoters contain distal HNF-1 sites, although only the proximal site shows conservation between the two species (Spodsberg *et al.*, 1999). HNF-1α is probably the main isoform binding the HNF-1 site (Spodsberg *et al.*, 1999; Bosse *et al.*, 2006), and it has been shown that HNF-1α and Cdx-2 act synergistically to activate lactase promoter activity in vitro (Mitchelmore *et al.*, 2000).

The GATA-4/5/6 transcription factors have been shown to play a critical role in the development of a number of endoderm-derived tissues, of which the small intestine is one (reviewed by Burch, 2005), and they are also implicated in the transcriptional regulation of a number of intestinally expressed genes, including sucrase–isomaltase (Krasinski *et al.*, 2001; Boudreau *et al.*, 2002), intestinal fatty acid-binding protein (Gao *et al.*, 1998) and trehelase (Oesterreicher and Henning, 2004), as well as lactase. GATA-4, -5 and -6 all bind to a number of GATA recognition sites which occur upstream of *LCT*, including two within the proximal promoter (Fitzgerald *et al.*, 1998; Fang *et al.*, 2001). It is thought that GATA-4 is the primary GATA factor responsible for modulating *LCT* expression, due to the highly correlated expression pattern of the two genes in small intestinal epithelia, in combination with the observation that GATA-4 binding to the proximal promoter is more evident than binding of either GATA-5 or -6 in EMSAs using nuclear extracts prepared from mouse intestinal epithelia (van Wering *et al.*, 2004). Further to this, it has been shown that transgenic mice producing an inducible mutant form of GATA-4 have significantly reduced *LCT*

expression levels (Bosse *et al.*, 2006). However, in vitro, all GATA factors bind the proximal sites with similar affinity, and it has been found that GATA-5 and HNF-1α are capable of co-operating to stimulate expression from the proximal promoter in this context (Krasinski *et al.*, 2001).

While the identified recognition sites and the corresponding transcription factors of the proximal *LCT* promoter are undoubtedly important in the basal regulation of lactase expression, they are, as discussed above, not sufficient for the correct temporal and spatial expressions of the gene, and hence this region is not thought to be involved in causing lactase persistence. Indeed, sequencing of *LCT* and the immediate promoter region in Europeans showed no nucleotide changes that were absolutely associated with persistence/non-persistence (Boll *et al.*, 1991; Lloyd *et al.*, 1992; Poulter *et al.*, 2003).

Subsequent research has, therefore, focused more intensely on the upstream regions of *LCT*, looking for regulatory elements that influence the lactase persistence phenotype. Interestingly, many enhancer motifs occur upstream of the proximal promoter, which are different depending on the species, and are recognised by various transcriptional regulators (including Cdx-2, HNF-1α and GATA factors) (Troelsen, 2005; Lewinsky *et al.*, 2005). One such highly variable region was identified ~900 bp upstream of the lactase start site (Harvey *et al.*, 1995; Hollox *et al.*, 1999). A nucleotide change, C-958T, was found to affect greatly interaction with an unidentified DNA-binding protein. However, this polymorphism was not considered to be causal of lactase persistence because the ancestral allele, C, was present in both lactase-persistent and non-persistent people. If functional in vivo, this SNP may perhaps affect the timing of down-regulation or spatial expression along the length of the intestine or modulate the effect of other nucleotide changes.

In fact, several polymorphisms exist across the 50 kb *LCT* gene and association studies revealed that very few haplotypes (i.e. a particular combination of alleles at each SNP) occur in most of the human populations tested, although greater diversity was observed in African populations (Hollox *et al.*, 2001). One particular combination of alleles, designated the '**A**' haplotype, is particularly common in northern Europe and is found to associate with lactase persistence (Harvey *et al.*, 1998).

6.7. Identification of Causal Variations

A putative causative single nucleotide polymorphism (*–13910C>T*) has been described 13.9 kb upstream of the *LCT* transcription initiation site (Enattah *et al.*, 2002). It is located in an intron of an adjacent gene, *MCM6*, and occurs

on the background of the **A** haplotype (Poulter *et al.*, 2003). The *-13910*T* allele was found to associate completely with directly ascertained lactase persistence in 196 Finnish individuals, and subsequent studies have confirmed a tight association between *-13910*T* and lactase persistence in populations of northern European ancestry (Poulter *et al.*, 2003). In fact, the **A** haplotype extends far beyond the 50 kb *LCT* gene region, with carriers of the *-13910*T* allele tending to have completely identical chromosomes extending for nearly 1 Mb (Poulter *et al.*, 2003; Bersaglieri *et al.*, 2004).

In vitro studies demonstrated that the *-13910*T* allele increases transcription in promoter–reporter construct assays in a colon carcinoma cell line (Caco-2) (Troelsen *et al.*, 2003; Olds and Sibley, 2003), providing evidence that it may have enhancer activity in vivo. A transcription factor, Oct-1, was identified which bound more strongly to the *-13910*T*-containing motif than to the alternative C allele, providing a possible mechanism for the up-regulation of *LCT* (Lewinsky *et al.*, 2005).

These observations were regarded by many as compelling evidence that the cause of lactase persistence had been identified, and some groups have recommended using the absence of the *-13910*T* allele as a diagnostic test for lactose intolerance (Rasinpera *et al.*, 2004).

However, researchers in our group noted that *-13910*T* was extremely rare in sub-Saharan African populations, even in those populations where lactase persistence frequency had previously been reported to be high. A statistical procedure was designed that enabled a comparison to be made between *-13910*T* allele frequencies and the expected allele frequencies given the previously published lactase persistence frequency for an ethnically matched group. This procedure corrected for genotyping and sampling errors, and despite being conservative, found a highly significant difference between the observed and expected frequency of *-13910*T* if it was assumed to be causal of lactase persistence. The study concluded that *-13910*T* could not be causal of lactase persistence throughout sub-Saharan Africa (Mulcare *et al.*, 2004). The exceptions to this observation, i.e. African populations in which *-13910*T* was able to account for the lactase persistence frequency were in the Fulani and Hausa populations of Cameroon (Mulcare *et al.*, 2004), and Berber populations from Algeria and Morocco (Myles *et al.*, 2005). In both cases, this is thought likely to be a reflection of the demography of those populations, and there is evidence to suggest contacts between these and Eurasian populations (Cruciani *et al.*, 2002; Myles *et al.*, 2005).

The distribution of *-13910*T* could be interpreted in one of the two ways; either *-13910*T* is not truly causal of lactase persistence, but is very strongly associated with the causal element and therefore acts as a marker for lactase persistence. In this case, the apparent rarity of *-13910*T in sub-Saharan Africa could be explained by this variant appearing on the lactase persistence carrying

chromosome after humans had begun to spread out of Africa. The other possibility is that the identification of *-13910*T* as a cause of lactase persistence is correct, but that there is heterogeneity of the trait, and that different causal variations have appeared independently in other human populations.

Subsequent research has sought to distinguish between these two scenarios, and our recent study of Sudanese volunteers concluded that *-13910*T* could not be causal in this group. The test cohort consisted of 94 unrelated individuals of the Jaali ethnic group, from whom buccal DNA was collected and phenotypic information was obtained using the breath hydrogen method. The lactase persistence frequency was 48%, yet only one individual carried a single *-13910*T* allele. Furthermore, it was shown that *-13910*T* was not simply a linked marker on the background of the **A** haplotype chromosome which was carrying the as yet unidentified true cause. The **A** haplotype was not frequent enough to account for, and did not associate with, lactase persistence (Ingram *et al.*, 2007). The interpretation of this was that lactase persistence probably evolved more than once in human history.

Reinforcing this conclusion, we and another research group (Ingram *et al.*, 2007; Tishkoff *et al.*, 2007) reported new sequence variants which were associated with lactase persistence in east Africa and that were located in very close proximity to *-13910*T*. Both groups reported association of a different SNP (*-13915*G* and *-14010*C*, respectively) with lactase persistence, in different populations, although neither association was 100%. The presence of a few individuals who carried an allele but were diagnosed lactase non-persistent could be explained by secondary lactase loss. However, the individuals who were tested persistent but carried no allele at *-13910*T* must carry a variation elsewhere – indicating that there may be more, as yet unidentified, causal variants.

Footprint analysis (empirical determination of DNA–protein-binding sites) of sequence encompassing the intron 13 region reveals transcription factor recognition sequences for Cdx-2, GATA, HNF-3α/Fox and HNF-4α along with Oct-1 (Lewinsky *et al.*, 2005). However, the newly identified SNPs affect only the Oct-1-binding site (Figure 6.3).

Electrophoretic mobility shift assays (EMSAs) were used to ascertain the effect of the new alleles on Oct-1 binding. It was found that only *-13910*T* (and to a much lesser extent *-13907*G*) oligonucleotide probes bound to Oct-1, and that binding of the other alleles (the ancestral sequence as well as *-13915*G* and *-13913*C*) was undetectable. It was therefore concluded that the Oct-1 protein is unlikely to play a critical role in causing lactase persistence (Ingram *et al.*, 2007). The identification of the other associated allele, *-14010*C*, situated 100 bp away from the predicted Oct-1-binding site would appear to confirm this (Tishkoff *et al.*, 2007).

Analysis in vitro of the effects of some of the newly identified intron 13 variant alleles on transcriptional regulation indicates that they affect

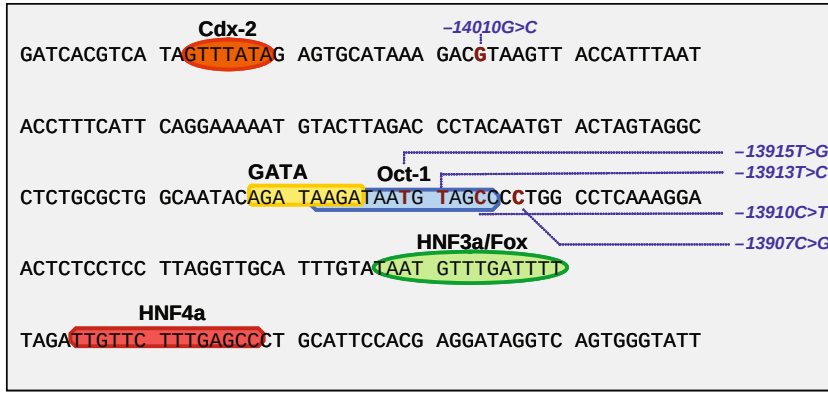

Figure 6.3. Transcription factor-binding sites as identified by Lewinsky *et al.* (2005) with positions of intron 13 sequence variants indicated in bold.

enhancer activity. Transcription activity of the *LCT* core promoter was enhanced twofold by the addition of the ancestral sequence of *MCM6* intron 13, and this activity increased further (by up to 25% more) when one of the variant alleles (*-14010*C*, *-13907*G* or *-13915*G*) was present (Tishkoff *et al.*, 2007). The effect seen was extremely small and the authors did not include *-13910*T* as a positive control (previously shown by Lewinsky *et al.* (2005) to enhance transcription activity a further 80% compared to the ancestral allele). However, the small size of the effect may be attributable to the limitations of the cell line used in the study (a colon carcinoma cell line and the only cell line known to express lactase) (Hauri *et al.*, 1985).

The predictive value of these in vitro functional studies with respect to the effect exerted in vivo is therefore uncertain, but the observations, together with those made previously (Troelsen *et al.*, 2003; Olds and Sibley, 2003; Lewinsky *et al.*, 2005) indicate that this region is important in the regulation of *LCT* expression.

6.8. Evolutionary Considerations

6.8.1. Genetic Adaptation

The original observation of a positive correlation between lactase persistence frequencies and milk drinking led to the widely held notion that lactase persistence has been subject to positive selection. In the intervening years, molecular evidence has accumulated which would appear to corroborate this

hypothesis. Research focussing on the haplotype diversity observed around the lactase gene in various populations suggested that the very different haplotype frequencies observed in northern Europeans compared to other populations are most probably explained by a combination of genetic drift and strong positive selection for lactase persistence (Hollox *et al.*, 2001).

The chromosome carrying *-13910*T* is a real outlier in the context of molecular signatures of selection compared with the rest of the human genome. First, *-13910*T* occurs on an extremely extended haplotype background, which is present in the northern European population at very high frequency. This is consistent with a model of recent positive selection (Bersaglieri *et al.*, 2004), in which alleles surrounding the causal variant 'hitch-hike' rapidly to high frequency due to strong positive selection, and haplotype length is exaggerated, indicating a recent event where recombination has not decayed the allelic associations in the region (reviewed in Sabeti *et al.*, 2006). Diversity of so-called microsatellite polymorphisms (variable simple repetitive sequences that occur throughout the genome) can also be analysed and interpreted in a similar way – reduced microsatellite diversity (as seen on the *-13910*T* carrying chromosomes) indicates that this particular haplotype has risen in frequency quickly and recently (Coelho *et al.*, 2005). These observations are consistent with selection for lactase persistence along with the recent practice of dairying, approximately 9000 years ago in Europe, and date estimates of *-13910*T* place it within this time period (Bersaglieri *et al.*, 2004; Coelho *et al.*, 2005). Recent data confirm the absence of the *-13910*T* allele in ancient DNA samples of the early Neolithic period, consistent with this dating, and supporting the model that the cultural trait of dairying was adopted prior to lactase persistence becoming frequent (Burger *et al.*, 2007). Some of the newly discovered African alleles are also reported to occur as part of an extended haplotype, suggesting that they too carry these signatures of recent positive selection (Tishkoff *et al.*, 2007).

The identification of the newly associated alleles themselves suggests that lactase persistence has been selected for independently in several different human populations, thus the ability to digest milk has been extremely advantageous, at least for some, in the last few thousand years.

6.8.2. Cultural Adaptation

The correlation between pastoralism, milk drinking and lactase persistence is not, however, true for all populations, for example, the Dinka and Nuer in Sudan (Bayoumi *et al.*, 1982) and the Somali in Ethiopia (Ingram, 2008) have a low-lactose persistence frequency despite cows or camels playing a very important role in their lifestyle. These populations are not completely dependent on milk despite its consumption being substantial, and therefore the selective pressure for lactase persistence may have been less strong.

In these populations and in many other peoples, it seems that the lactose concentration is moderated by cultural adaptation. Milk is processed to sour milk, yoghurts and cheeses, which have a reduced lactose content, and individuals also adapt their consumption habits by taking smaller quantities of milk at a time. These cultural adaptations enable non-persistent individuals to benefit from the calorific, mineral and vitamin constituents of milk without inducing the associated symptoms of lactose malabsorption, and are complemented by adaptations of the large intestinal bacterial flora (see Diagnosis of lactase non-persistence/persistence).

6.9. Selective Forces

What were the selective forces that resulted in the elevated lactase persistence frequencies observed in certain populations? A number of theories have attempted to explain the apparent selection for lactase persistence. Because of the worldwide distribution of lactase persistence and the generally coinciding pattern of historically milk-drinking populations, Simoons (1970) and McCracken (1971) independently suggested that the selective force for lactase persistence was milk dependence. This has become known as the 'culture-historical hypothesis', and suggests that the increase in lactase persistence co-evolved alongside the cultural adaptation of milk drinking.

The model works only on the premise that the advantage was conferred specifically by fresh milk, as non-persistent individuals are also able to benefit from the calorific, vitamin and mineral contents of milk by processing it, which reduces the lactose content (e.g. by fermentation to yoghurt or cheese), and therefore selection is most likely to have occurred in populations for whom fresh milk formed an integral part of the diet.

A problem with this hypothesis is the 'non-fit' populations, who have either a high lactase persistence frequency without being milk dependent, or who rely heavily on milk products but who have a low reported frequency of lactase persistence. However, statistical modelling has suggested that an incomplete correlation does not necessarily provide evidence against the culture-historical hypothesis, and non-fits may be expected if some lactase-persistent populations have recently stopped milking or other populations have only recently adopted the habit, therefore allowing insufficient time for lactase persistence to be driven to high frequency (Aoki, 1986). Furthermore, this model does not account for migration, which may provide further explanation for the imperfect correlation.

Other statistical analysis also provides evidence in favour of the culture-historical hypothesis; Holden and Mace (1997) performed an analysis that

revealed lactose digestion capacity had most likely evolved as an adaptation to dairying, and concluded that high-frequency lactose digestion capacity did not evolve in the absence of milking.

More recent research sought to address the question of why some populations and not others had adopted the cultural habit of milk drinking. It was found that frequencies of lactose malabsorption were higher in populations where environmental conditions, such as extremes of climate or high incidence of endemic cattle disease made it impossible to raise livestock, thereby supporting the culture-historical hypothesis by providing further evidence that lactase persistence is selected for only in environments conducive to dairying. The exceptions to the general distribution were a number of African groups who had high lactase persistence and maintained herds despite environmental conditions being unfavourable. The authors suggest that these groups managed to circumvent harsh environmental conditions by becoming nomadic (Bloom and Sherman, 2005).

Other evidence in support of the culture-historical hypothesis has been provided by the observation that high intra-allelic diversity of cattle-milk protein genes in Europe coincides with the geographic incidence of lactase persistence, which is suggested to be consistent with large herd sizes kept for dairying and selection for high milk yields (Beja-Pereira *et al.*, 2003).

The arid climate hypothesis, first suggested by Cook and al-Torki (1975) speculated that in desert climates (i.e. Middle and Near East) where water and food were scarce, nomadic groups could survive by utilising milk as a food source, and in particular, as a source of clean, uncontaminated water. The benefits to persistent individuals may have become even more pronounced during outbreaks of diarrhoeal disease, when non-persistent individuals would be unable to utilise milk as a water source without exacerbating their condition. This scenario could be particularly pertinent to desert nomads who consume camel milk, as these animals continue to lactate for several days in the absence of water.

Obviously, the benefits of drinking milk cannot be explained by the arid climate hypothesis in northern Europe. Here, the calcium absorption hypothesis has been suggested to explain the distribution of the trait (Flatz and Rotthauwe, 1973). The low-light levels experienced in the northern hemisphere are associated with an increased risk of developing rickets and osteomalacia due to a lack of vitamin D (which is synthesised by the skin in the presence of sunlight). Calcium may help to prevent rickets by impairing the breakdown of vitamin D in the liver (Thacher *et al.*, 1999), and is itself an essential mineral required for bone health. Lactase non-persistent individuals could obtain calcium from yoghurt or cheese, dairy foods that contain a reduced level of lactose. However, milk proteins and lactose are believed to facilitate the absorption of calcium (for review, see Gueguen and Pointillart,

2000), and hence the ability to drink fresh milk, which contains both calcium and components that stimulate its uptake (along with small amounts of vitamin D) may have provided an advantage to persistent individuals.

In only one case has selection against lactase persistence been proposed. Anderson and Vullo (1994) suggested that selection had acted in favour of lactase non-persistence in malarial regions because of the observation that individuals with flavin deficiency are at a slightly reduced risk of infection by malaria. The consumption of milk, which is rich in riboflavin was, therefore said to be unfavourable as it would keep the flavin level in the bloodstream high. This explanation is not widely supported, and is thought to be an unlikely mechanism by which to explain the current distribution of lactase persistence.

6.10. The Role of Other Factors that Influence Lactase Expression

While it has been well established that regulation of *LCT* is predominantly under transcriptional control, there is evidence to suggest that additional levels of control over expression of the enzyme exist (Rossi *et al*., 1997). Heterogeneity of the lactase non-persistence phenotype has been reported by a number of research groups. Some have observed individuals who show slower/abnormal processing (Witte *et al.*, 1990; Sterchi *et al.*, 1990) which may imply post-translational controls such as glycosylation and/or transportation, whilst others have made observations suggestive of epigenetic regulation. Although most non-persistent individuals show no immuno-histological staining for lactase in the jejunal biopsies of the small intestine (concordant with low lactase activity and transcriptional regulation of *LCT*), it was found by Maiuri *et al*. (1991) that some individuals show patchy expression of the enzyme in the intestinal epithelia. This mosaic expression pattern may result from somatic cell changes in methylation or histone acetylation.

6.11. Present-Day Health and Medical Considerations

Lactose malabsorption is often confused with other illnesses associated with milk drinking, such as milk protein allergy, which has quite different causes (reviewed by Crittenden and Bennett, 2005), and in recent times lactose intolerance has been blamed for causing a variety of systemic conditions, often without clear evidence (Matthews *et al.*, 2005; Campbell and Matthews, 2005). Nonetheless, it does appear that the consumption of milk and milk products by those who cannot digest lactose is a relatively common cause of

irritable bowel syndrome in Europe and the USA (Vesa *et al*., 2000). Many commercial dairy products and other foods contain very high concentrations of lactose which are introduced in manufacturing, so that lactose is more widespread in the diet than it was for that same person's ancestors. Lactose tolerance testing can be a useful way of detecting lactose malabsorption and enabling avoidance of the cause. In countries such as Finland, which has a high frequency of lactase non-persistence in comparison with the rest of northern Europe, commercial low-lactose products are readily available (Harju, 2003).

Many studies have attempted to demonstrate the health benefits of milk consumption in lactase-persistent people, e.g. by providing protection against osteoporosis as well as contrasting studies that have claimed adverse effects of lactase persistence and associated high milk consumption. This research has so far failed to produce reliable and reproducible results in either direction. It has been suggested that lactase-persistent individuals may be at reduced risk of developing osteoporosis (Obermayer-Pietsch *et al*., 2004); however, a recent study of post-menopausal women found no significant increase of osteoporosis in 'molecularly defined' lactose maldigesters. The maldigesters were found to consume less milk but tended to have an increased intake of calcium supplements (Enattah *et al*., 2005a). The same group found that 'molecularly defined' lactose maldigestion did, however, increase the risk of developing osteoporosis in individuals aged over 85 (Enattah *et al*., 2005b).

Some studies suggest an association of lactase persistence with ovarian cancer, said to be caused by the toxicity of galactose to oocytes (Meloni *et al*., 1999); however a meta analysis combining data from a number of studies showed association only in cohort and not case–control designed research (Larsson *et al*., 2006). Likewise, association studies of lactase persistence with diabetes produce similarly conflicting results (Meloni *et al.*, 2001; Enattah *et al.*, 2004). These often-contradictory findings are difficult to evaluate because of the high risk of confounding effects such as mixed ancestry and dietary intake.

6.12. Genetic Testing

The discovery of the new lactase persistence-associated SNPs in sub-Saharan African and Middle Eastern populations, along with the observations discussed above makes the development of a universal genetic test for lactase persistence currently unfeasible. Furthermore, both research groups who identified new lactase persistence-associated SNPs found that there were many individuals without a –13.9/–14.0 kb variant allele but who were lactase persistent, which indicates that there are other alleles yet to be found, and

they are unlikely to reside within the sequenced region (Ingram *et al.*, 2007; Tishkoff *et al.*, 2007). The currently used genetic test for lactase persistence types only the *-13910*T* allele (Rasinpera *et al.*, 2004) and can therefore reliably predict lactase persistence status only of individuals of northern European descent, and its use should be restricted to members of that population. In addition, recently developed assays relying on probe/template melting curve analysis have been shown to give inaccurate genotype information in the presence of the new alleles (Weiskirchen *et al.*, 2007).

6.13. Summary

Lactose malabsorption refers to the situation in which the milk sugar, lactose, is not hydrolysed and absorbed within the small intestine because of a reduced level of the enzyme lactase. Instead, the lactose is digested by bacteria present in the colon, and this can be associated with symptoms of lactose malabsorption.

In humans, the persistence of lactase into adulthood is a genetically controlled polymorphic trait that varies widely in frequency between different populations. A number of single nucleotide polymorphisms have been identified upstream of the lactase gene that associate with lactase persistence, of which one predominates in Europe (*-13910*T*). These changes are thought to up-regulate lactase expression. However, transcriptional regulation of lactase expression is complex and the precise mechanism underlying lactase persistence remains unknown.

In recent times, the consumption of fresh milk and dairy produce has become somewhat less fashionable, and a number of illnesses and symptoms have been attributed to 'lactase intolerance' without clear evidence. What cannot be disputed is that the ability to digest lactose has clearly been hugely advantageous to some populations in relatively recent human evolutionary history. For individuals who are genetically unable to digest lactose, adapting to taking small quantities or consuming lower-lactose products, such as yoghurts and cheeses, should be sufficient to avoid symptoms of lactose malabsorption. While genetic testing of *-13910*T* in Europeans with irritable bowel syndrome may aid differential diagnosis, this test cannot be considered useful in populations with mixed ancestries such as the urban UK and USA.

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7

Non-Enzymatic Degradation Pathways of Lactose and Their Significance in Dairy Products

John O'Brien*

7.1. Introduction

Milk products are especially sensitive to the effects of heat treatment encountered under conventional process and storage conditions because of an abundance of reactive functional groups: aldehyde group of lactose, ε-amino group of lysine and other reactive N-containing groups (e.g. indolyl group of tryptophan, imidazole group of histidine, guanidino group of arginine and the α-amino group of proteins and free amino acids).

Lactose may isomerize via the Lobrey de Bruyn–Alberda van Ekenstein (LA) transformation, followed by degradation to acids and other sugars. Alternatively, lactose may react with the caseins and whey proteins of milk systems via the Maillard or non-enzymatic browning reaction (also referred to as glycation of proteins, e.g. in the case of lactose, lactosylation). This review examines the chemistry and significance of such reactions in milk products.

Bovine milk contains ~4.8% lactose which is present in the free form (4-*O*-β- D-galactopyranosyl-D-glucopyranose) and lactose-containing oligosaccharides. The concentration of such oligosaccharides is very low and would not be expected to contribute significantly to sugar degradation reactions and Maillard browning in milk systems. In contrast to its LA

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transformation product, lactulose, lactose does not contain furanoid ring structures (Clamp *et al.*, 1961); the formation of such structures is precluded by the (1→4) linkage. In solution, lactose exists as an equilibrium mixture of α- and β-lactose. A third crystalline form, anhydrous α-lactose, may be prepared from crystalline α-lactose hydrate by heating. Although α-lactose hydrate and β-lactose are stable at room temperature, anhydrous α-lactose is unstable and is converted either to α-hydrate (in the presence of water) or to β-lactose (by heating to temperatures greater than 93.5°C). The proportions of α- and β-lactose in an equilibrated solution at room temperature are ~37 and ~63%, respectively. The solubility of lactose in water is low (17.8 g dl^{-1} at 25°C) compared with other sugars, and crystallization is often a problem in concentrated systems.

In addition to its high concentration in dairy products, lactose is still the most widely used excipient in tablet manufacture. Since the majority of drugs have amine functional groups, the role of Maillard reactions between drug molecules and lactose in tablet discoloration is well known. The potential of such reactions in the solid state to limit the shelf-life, efficacy and bioavailability of drug formulations has been studied extensively (Kumar and Banker, 1994; Qiu *et al.*, 2005a). Apart from storage conditions, milling (which influences surface area of reactants) high compression pressure and reactants in the amorphous state have been shown to promote Maillard reactions in tablets (Qiu, *et al.*, 2005b). Whereas browning inhibitors, such as inorganic bisulphites, have been used commonly to stabilize pharmaceutical formulations where Maillard reactions are known to cause a problem, the addition of browning inhibitors is not generally an option for dairy products. However, the detrimental effects of Maillard reactions can be minimized through careful manipulation of processing and storage conditions.

Maillard reactions in dairy products are generally detrimental to the organoleptic, nutritional and functional qualities of the product and are therefore undesirable. However, some dairy processes exploit a controlled Maillard reaction during manufacture. For example, Maillard browning reactions are an integral part of the manufacture of products such as *dulce de leche* in Latin America (also known as *doce de leite* in Portuguese, *confiture de lait* in French or simply 'milk jam' in English) and *khoa* in India in which milk is heated in the presence of sucrose to produce brown products with a pleasant flavour (Pavlovic *et al.*, 1994; Srinivasan and Gopalan, 1994). Maillard reactions also play an important role in the generation of flavour during the manufacture of ghee, or clarified butter, and milk chocolate crumb (Minifie, 1989). Chocolate crumb manufacturing optimally requires high total solids (90–94%), pH 5.5–8.0 and a temperature of 72–77°C for 4–8 h, ideal conditions for Maillard browning.

7.2. Isomerization and Degradation of Sugars

Sugars may undergo five different types of reaction in solution:

- Anomerization
- Enolization/aldo-keto isomerization and epimerization: The Lobrey de Bruyn–Alberda van Ekenstein transformation resulting in the formation of products such as lactulose
- β-Eliminations which may or may not be accompanied by retro-aldol reactions to produce α-dicarbonyl derivatives and compounds such as furfural and hydroxymethylfurfural
- Benzylic acid rearrangements, resulting in the formation of, for example, lactic and saccharinic acids
- Maillard reactions with amino compounds (if present) to give products described above in addition to N-containing products, and S-containing products if sulphur amino acids are present.

Lobrey de Bruyn–Alberda van Ekenstein-type reactions (Figure 7.1) may be catalysed by acid, base or ions of alkaline earth metals (Speck, 1958). In the case of lactulose formation from lactose, the reaction is particularly favoured by increasing pH. In addition to their participation in Maillard reactions, amino acids probably contribute to the catalysis of LA reactions. As in the Maillard reaction (see below), enediols are key intermediates in LA reactions. Enolization is generally accepted to be the rate-limiting step whereas the subsequent β-elimination reactions are rapid in the presence of acid. In the case of 2-ketohexoses, such as fructose, the reaction rate is usually higher than that for aldoses because 1,2-enolization occurs more easily. In addition, the 2-ketoses can form a 2,3-enediol, leading to a wider spectrum of degradation products. Figure 7.1 shows, using lactose as an example, how epimerization among aldoses and ketoses (e.g. glucose $\leftrightarrow$ mannose or fructose $\leftrightarrow$ psicose) may be promoted by alkaline media in addition to aldose–ketose isomerization. At least in the case of [1-^{13}C]-mannose, it appears that epimerization to [1-^{13}C]-glucose and isomerization to [1-^{13}C]-fructose assume equal significance when the system is allowed sufficient time to equilibrate (7 d at 25°C and pH 11.5) (King-Morris and Serianni, 1986).

In the presence of oxygen, the double bond of enediols may be cleaved to produce two corresponding carboxylic acids; for example, formic and arabinonic acids are formed from glucose via this pathway. Retro-aldol cleavage of the double bond is possible at elevated temperatures or in the presence of concentrated alkali to produce hydroxyaldehydes or hydroxyketones. Since enolization is theoretically possible at any part of a molecule, the potential spectrum of products is enormous.

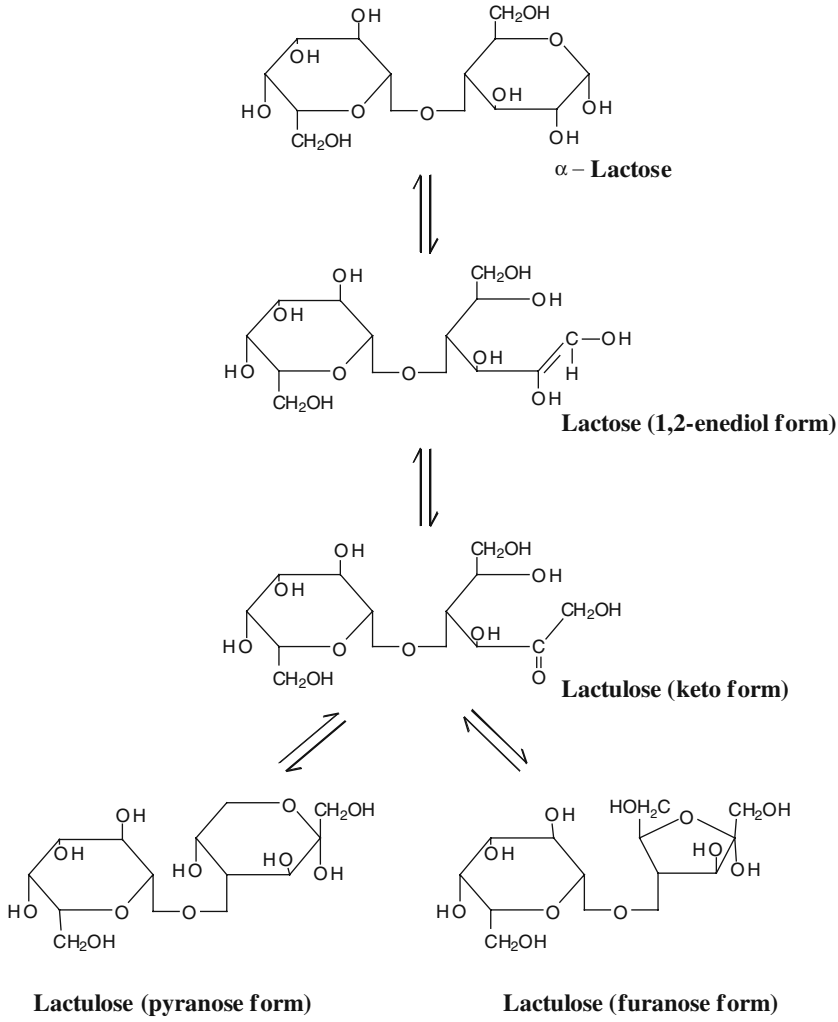

Figure 7.1. Formation of lactulose from lactose via the Lobrey de Bruyn–Alberda van Ekenstein transformation.

Saccharinic acids are products of the benzilic acid rearrangement of the 1,2- and 2,3-dicarbonyl compounds (Figure 7.2) derived from sugar degradation, yielding metasaccharinic acids (from 1,2-dicarbonyls), saccharinic acids (from 1-methyl-2,3-dicarbonyls) and isosaccharinic acids (from 2,3-dicarbonyls). pH has an important effect on such reactions: levulinic acid is characteristic of the oxidation of lactose under acidic conditions

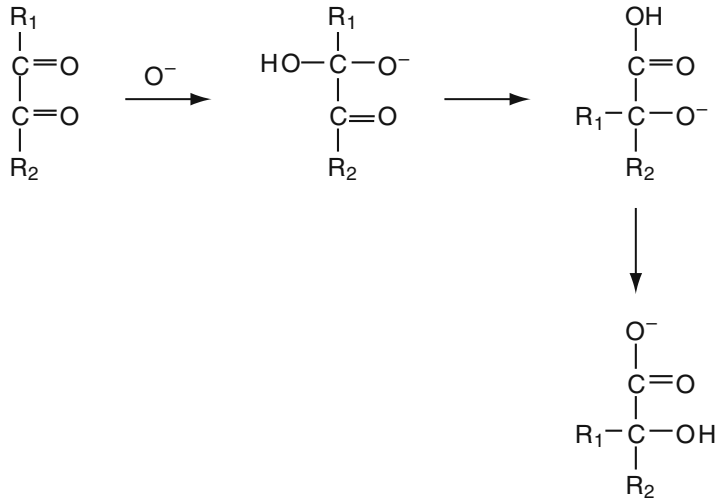

Figure 7.2. Benzilic acid rearrangement of an α-dicarbonyl compound to yield a saccharinic acid.

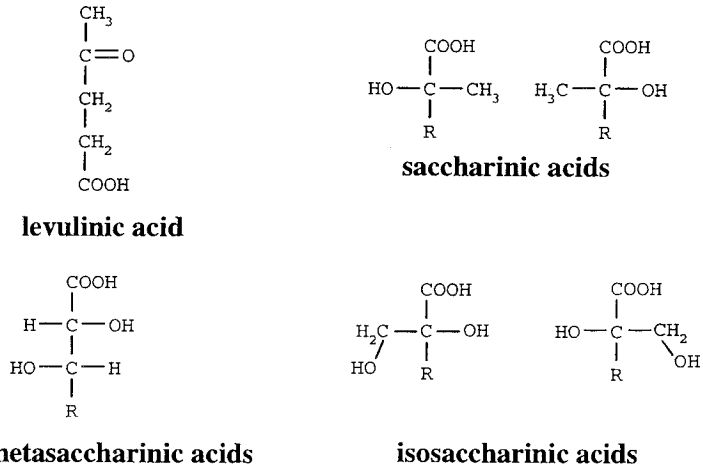

Figure 7.3. Some acid products of sugar degradation.

whereas saccharinic acids are characteristic of oxidation under alkaline conditions (Figure 7.3).

Dehydration of the 1,2-enediol derivatives via a series of β-eliminations is promoted under acidic conditions, resulting in the formation of

2-furaldehyde (e.g. from pentoses or hexoses following cleavage) or 5-hydroxymethyl-2-furaldehyde (e.g. from hexoses). The primary reaction products may, in turn, react via aldol condensations and intramolecular cyclizations (e.g. Cannizaro reactions) to form a range of other products. In the presence of amino acids, α-dicarbonyl compounds may react further in the Strecker reaction which results in the oxidation of the amino acid. In practice, the number of products formed in significant quantities is quite low and depends very much on the severity of heating. However, even a few micrograms of a product with a low odour threshold may be sufficient to have a major impact on the organoleptic quality of a product. In such circumstances, even minor pathways may assume major significance.

7.2.1. Lactulose

Although lactulose was first synthesized in 1929 (Montgomery and Hudson, 1930), its physiological significance was not recognized until 1957 when it was shown to stimulate the growth of bifidobacteria in the intestines of bottle-fed infants. Current figures for the global commercial production of lactulose are difficult to obtain. However, the number of commercial applications of lactulose, lactobionic acid and lactitol is increasing in foods and medical products for use as laxatives and for the modulation of the gut microflora (Mizota *et al.*, 1987; Tamura *et al.*, 1993). Commercial lactulose brand names include 'Cepahalac', 'Constilac', 'Cronolac', 'Constilose', 'Duphalac', 'Evalose', 'Lactogel' and 'Kristalose'. The medical applications of lactulose are now well established and a number of reviews are available (e.g. Als-Nielsen *et al.*, 2004). The production of lactulose for such applications was ~25,000 tonnes in 2006 with a market value of ~US$300m (Horton, 1995; Affertsholt-Allen, 2007). The high lactulose content of some severely heat-treated milks (e.g. in-container-sterilized liquid infant feeds) can lead to a laxative effect which has been shown to require as little as 2 g lactulose fed over 24 h (Andrews, 1986). In addition, some infant formulae have added lactulose for the reasons specified above.

Lactulose has attracted considerable interest as a possible indicator of the temperature/processing history of milk products (International Dairy Federation, IDF, 1993; Pellegrino *et al.*, 1995). The utility of lactulose in this respect was reviewed by Elliott *et al.* (2005). Lactulose content was reported to be on average more than 100-fold higher in sterilized milk samples than in pasteurized milks (Calvo and Olano, 1989). The cut-off currently used by both the IDF and the European Commission is 600 mg l^{-1} lactulose in UHT milk versus a corresponding range for sterilized milk of 600–1400 mg l^{-1}.

Lactulose is formed by the LA transformation of lactose via a 1,2-enediol intermediate. Lactulose is much less stable in solution than lactose and may subsequently degrade via β-elimination to give galactose, tagatose and saccharinic acids and other low molecular weight products (Olano and Martinez-Castro, 1981). Alternatively, lactulose may epimerize via a 2,3-enediol to form epilactose (4-*O*-β-D-galactopyranosyl-D-mannose; a C2 epimer of lactose). Lactulose was reported originally in heated milk by Adachi (1958) but its formation was erroneously attributed to the hydrolysis of the Amadori product, lactulosyl lysine (see below), in addition to the LA transformation (Adachi and Patton, 1961). Heating lactose solutions (pH 6.8) at 120°C for 30 min resulted in the formation of slightly more epilactose than galactose whereas the formation of lactulose was ~50-fold greater than that of epilactose or galactose accounting for up to 20% of the initial lactose content (Martinez-Castro *et al*., 1986). Interestingly, the quantitative differences among lactulose, epilactose and galactose appeared much less in a heated milk ultrafiltrate at pH 6.6. The changing balance in the formation of lactulose, epilactose and galactose in the heated milk ultrafiltrate was attributed by Martinez-Castro *et al.* (1986) to a decrease of pH (from 6.6 to 6.0) as a result of precipitation of calcium phosphate on heating. In the heated milk ultrafiltrate (20 s at 145°C, pH 6.6), the formation of lactulose, epilactose and galactose was reported to be 61.3, 11.3 and 4.4% of total carbohydrate, respectively.

The commercial demand for lactulose has stimulated research into more efficient production methods from lactose or milk ultrafiltrate feedstock. Such research has focused on improving the chemical isomerization of lactose mainly in alkaline media (Montilla *et al.*, 2005a; Aider and de Halleux, 2007). The production of lactulose using enzymatic transgalactosylation using microbial enzymes is also feasible, but may be cost prohibitive (Mayer *et al.*, 2004). Most lactulose sold for medical use is available as concentrated aqueous solutions. Progress has also been made in the manufacture of stable powder forms (Mizota *et al.*, 2004).

More severe treatment of lactose, e.g. acid hydrolysis, may result in the formation of several oligosaccharides by hydrolysis and condensation reactions. The process has been termed 'reversion' in the literature (Clamp *et al.*, 1961). Huh *et al.* (1991) demonstrated the formation of 17 disaccharide derivatives and four anhydro derivatives following acid hydrolysis of a 30% lactose solution. The products included the disaccharides isomaltose, gentiobiose and melibiose, and the anhydro sugar, levogalactosan (Figure 7.4). A number of the disaccharides had (1→4), (1→3), (1→2) and (1→1) glycosidic linkages, but the authors suggested that the preferred linkage appeared to be (1→6), which was present in the majority of the disaccharides characterized.

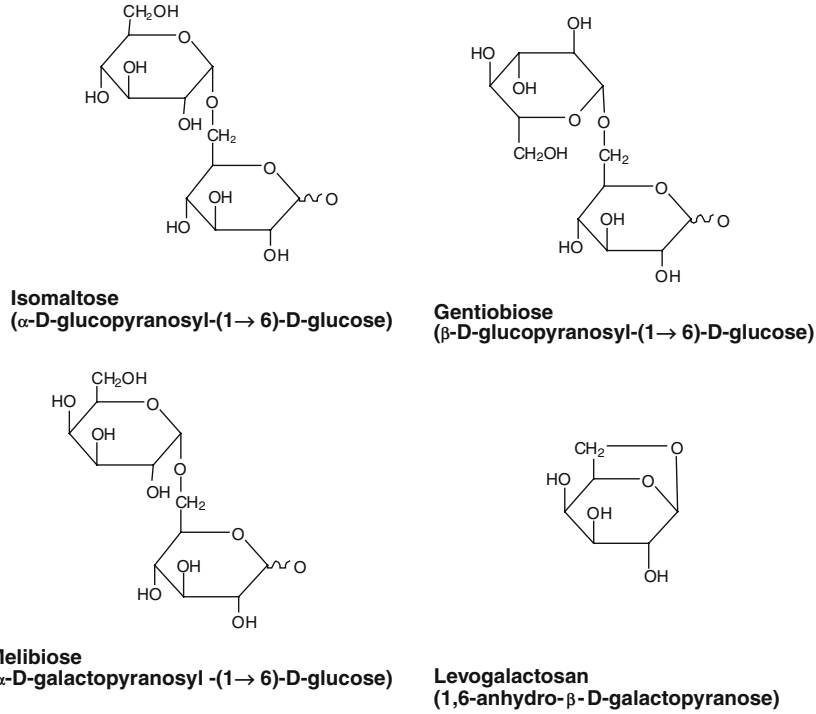

Figure 7.4. Some 'reversion' products of lactose following acid hydrolysis (after Huh *et al.*, 1991).

Although precipitation of tertiary calcium phosphate, hydrolysis of organic casein phosphate and the production of organic acids all contribute to the decrease in pH in heated milk, the most important from a quantitative point of view is the production of organic acids. Of the organic acids, formic is the most important contributor to acidity in heated milks. Based on titratable acidity and HPLC analysis of a heated skim milk (~20 min at 140°C), Berg (1993) concluded that almost all (>95%) of the acid formed is formic acid. The loss of positive charges on protein molecules as a consequence of Maillard reactions is a minor contributor to the decrease in pH under most circumstances. Many workers have noted that the pH decrease in heated milk is strongly influenced by headspace composition (Sweetsur and White, 1975; Fox, 1981). Sweetsur and White (1975) reported that the rate of pH decrease in heated milk was greatest when the headspace contained oxygen, intermediate in the presence of air and least in the presence of nitrogen. It was concluded that oxygen exerts its effect by promoting lactose degradation. In

lactose-free milk, the decrease in pH on heating is dramatically less. pH also decreases during the storage of UHT milk; the extent of the change is proportional to lactose content and to the extent of browning and is higher at elevated temperatures (Venkatachalam *et al.*, 1993). Although formic acid is derived from degradation of sugar moieties, its formation was increased in heated milk at higher casein concentrations, suggesting the involvement of a Maillard reaction (Berg, 1993). Berg (1993) concluded that from a quantitative point of view, direct degradation of lactose is a more important source of heat-induced acidity in milk than the Maillard reaction.

The degradation of lactulose also results in the formation of several C5 compounds. In addition to the formation of furfural and furfuryl alcohol, as discussed earlier, the formation of 3-deoxypentosulose (Troyano *et al.*, 1992a) and deoxyribose (Berg, 1993) has been reported.

The concentration of lactulose in UHT milks has been reported to range from 2 to 25 mg dl^{-1} while the concentration may exceed 75 mg dl^{-1} in sterilized milks (Olano and Calvo, 1989). Interestingly, Klostermeyer and Geier (1983) reported that the lactulose content of UHT milk increased during a production cycle, which they attributed to the build-up of alkaline scale on the heating surfaces. Their observation may offer a partial explanation for the higher lactulose concentration in indirectly heated UHT milks than in those subjected to direct heating. In addition, it is likely that the greater severity of heating during indirect UHT processing contributes to a higher lactulose content (Elliott *et al.*, 2005). Based on the differences between directly and indirectly heat-treated UHT milks, Andrews (1984) suggested that lactulose concentrations could be used to classify such milks according to the processing method.

In UHT milks, the concentration of galactose has been reported to range from 9 to 12 mg dl^{-1} and the concentration exceeds 16 mg dl^{-1} in in-container-sterilized milks, although it is unclear to what extent non-enzymatic degradation of lactose may contribute to such levels. Berg (1993) reported that the amount of free galactose formed in milk at 140°C was more than 60% that of the lactulose formed. Epilactose concentrations up to 5 mg dl^{-1} have been reported for in-container-sterilized milk with values of 1–3 mg dl^{-1} for UHT milks. Tagatose formation appears to require relatively severe heating conditions, such as in-container sterilization (Troyano *et al.*, 1992b, 1994). However, its formation in skim milk powder during storage has been reported; no tagatose was detectable in fresh spray-dried milk powder (Troyano *et al.*, 1994).

Calvo and Olano (1989) reported that the presence of proteins in milk systems reduced the amount of lactulose formed whereas it increased the formation of epilactose and galactose. Furthermore, these authors showed that the galactose did not originate from the degradation of lactose–protein

Amadori products, indicating that the direct degradation of lactose was involved. Studies on the degradation of casein-bound lactose (assumed by the authors to represent the Amadori compound plus Schiff's base) showed the formation of formic acid and galactose, but no HMF or lactulose was detected (Berg, 1993). The yield of galactose appeared to be quite high (~85% of the concentration of the sugar–protein complex after 23 min at 120°C). However, at 140°C, the concentration of galactose began to decrease after 13 min, presumably because of Maillard and degradation reactions of the free sugar. A small amount of lactose was released, presumably from Schiff's base or glycosylamine. Earlier studies (Martinez-Castro *et al.*, 1986; Andrews & Prasad, 1987) suggested that the formation of lactulose in synthetic milk ultrafiltrate was lower than that in milk subjected to similar heat treatment, possibly a result of precipitation of calcium phosphate. Berg (1993) reported that the amount of lactose degraded and the amounts of lactulose and galactose formed increased with increasing casein concentration. The formation of formic acid was also increased at higher protein concentrations. Thus, it is possible that the breakdown of lactulose is promoted at higher protein concentrations. In early papers, this may have led to the erroneous conclusion that protein inhibits lactulose formation. The effect of casein in promoting lactulose formation and degradation is probably related to the buffering capacity of the protein; in the presence of casein the pH decrease on heating is retarded. Furthermore, in the presence of casein, lactulose may be degraded via at least two pathways: via Maillard reaction with casein to form lactosyl lysine adducts and via direct degradation to form galactose, formic acid and other products, the formation of which may be catalysed by the protein.

Protein also affects the levels of HMF and furfural detected. Berg (1993) reported that less free and total HMF were formed in the presence of casein. Since the development of brown colour is predictably greater in the presence of casein, it may be suggested that HMF reacts with casein to produce advanced products of the Maillard reaction. This view is supported by the experiment of Berg (1993) who showed that HMF and furfural were degraded slowly on heating in the presence of casein but appeared to be stable in the absence of protein. Fat content does not appear to influence the kinetics of lactulose formation although the rate of furosine formation appeared to be higher in milk with a higher fat content (Claeys *et al.*, 2003).

Lactulose may react via its keto group with β-lactoglobulin in a Maillard reaction to yield the Heyns rearrangement product, lactosyl lysine (Matsuda *et al.*, 1991). Although lactose is approximately 10 times more reactive in Maillard reactions than lactulose, it appears that the latter is a stronger inducer of protein crosslinking/polymerization on a weight basis than lactose. However, the nature of the actual crosslinking intermediate is

not known. Similarly, the extent of fluorescence development was slightly higher in the lactulose-β-lactoglobulin system than in a lactose-β-lactoglobulin reaction. The release of galactose upon degradation of lactulose is of interest because of its greater reactivity in Maillard reactions than disaccharides or other hexoses (Chavez-Servin *et al.*, 2004). Thus, at least some of the free galactose in heated milks would be expected to react with the ε-NH_2 groups of lysine to form the Amadori product, tagatosyl lysine. The major pathway for the formation of galactose in heated milk is undoubtedly lactulose degradation. The involvement of Amadori product degradation in galactose formation is less clear. Based on studies of the model Amadori product, α-*N*-acetyl-ε-*N*-lactulosyl lysine, Calvo and Olano (1989) concluded that the Amadori product probably did not contribute to the formation of galactose in heated products. However, it is possible that *N*-acetyl-lactulosyl lysine may be more stable than the protein-bound Amadori product. Similarly, Henle *et al.* (1991) could not detect fructosyl lysine in heated milk, suggesting that the disaccharide glycosidic bond is stable.

7.3. Chemistry of the Maillard Reaction

7.3.1. Reaction Mechanisms and Pathways

The first step in the Maillard reaction involves the nucleophilic attack by the nitrogen atom of an amino compound on the electrophilic carbonyl group of an aldehyde or ketone; in food systems, the reactants are predominantly proteins and reducing sugars, although carbonyl products of lipid peroxidation (El Zeany, 1982; Nielsen *et al.*, 1985a; Kaneko *et al.*, 1991), vitamin C (Hayashi *et al.*, 1983), free amino acids and ammonia are also important reactants under some circumstances. The mechanism depends on the ability of the molecule to bear the negative charge on the carbonyl oxygen. Potman and van Wijk (1989) favoured an S_N1 mechanism in which the cyclic pyranose conformation reacts, the leaving group being either the anomeric -OH group (leading to the formation of a cyclic carbocation) or the ring oxygen leading to ring opening. Similarly, the validity of the S_N1 mechanism would depend on the stability of the intermediate carbocation (Isaacs, 1987). The reaction proceeds with the elimination of a molecule of water to form a Schiff's base, which subsequently rearranges to form an *N*-substituted glycosylamine intermediate (Figure 7.5) which has been reported to adopt a β-pyranose structure in the $^4C_1$ conformation (Potman and van Wijk, 1989). The amino acid carboxyl group plays an important role in the catalysis of the Amadori rearrangement. When the carboxyl group is absent (e.g. in aliphatic or aromatic amines) the glycosylamine is more stable and, in many

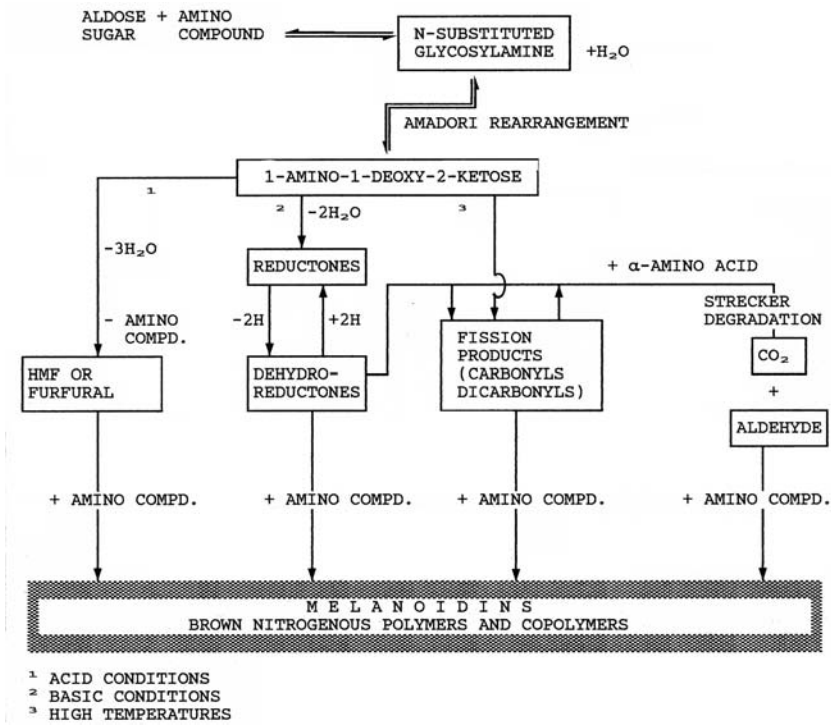

Figure 7.5. Steps in the Maillard reaction.

cases, has been isolated. Thus, *N*-substituted glycosylamines derived from amino acids are inherently unstable and they are either hydrolysed to the parent amino acid and reducing sugar or react via a spontaneous rearrangement to form the corresponding keto-(α-1-amino-1-deoxy-2-ketose) or aldo-(α-2-amino-2-deoxyaldose) derivative, depending on whether the parent sugar is an aldose or a ketose, respectively. The rearrangements are similar chemically to the LA transformation of sugars in heated alkaline solutions. The aldo→keto transformation is referred to as the Amadori rearrangement and the corresponding keto→aldo rearrangement as the Heyns rearrangement. As in the case of LA transformations, such reactions are multistep; for example, in the case of Amadori rearrangements, glycosylamines rearrange first to 1,2-enaminols via a sigmatropic shift followed by ketonization to the Amadori rearrangement product. It appears that the formation of the imminium ion by dehydration during the Amadori rearrangement is rate limiting. Acid catalyses this step, with a maximum rate reported to be between pH 2 and 5 (Yaylayan and Huyghues-Despointes, 1994). Thus, the chemistry of the

reacting carbonyl compound has a major influence on the mechanism of the Maillard reaction because the Amadori and Heyns compounds differ considerably in their reactivity (Pilkova *et al.*, 1990). Heyns products are unstable compared with their corresponding Amadori products, especially in the presence of amino acids. They readily react further to produce Amadori products via an LA-type transformation. However, in spite of their inherent instability, the rate of browning of Heyns products has been reported to be slower than that of corresponding Amadori products.

Although the degradation of the sugar moiety during the early stages of the Maillard reaction is irreversible, some of the amino acids may be recovered by acid hydrolysis of the Amadori product. While Amadori and Heyns products would be expected to brown more readily than mixtures of the corresponding amino acids and sugars (Westphal *et al.*, 1988), such products are relatively stable in food systems and, in many products, are the major or only products of the Maillard reaction.

Glycosylamines may also decompose via a free radical mechanism involving the formation of *N*,*N*′-dialkylpyrazine cation radicals (Hayashi and Namiki, 1981). Such radicals are formed prior to the Amadori rearrangement in a process believed to involve fragmentation of the glycosylamine in a reverse aldol reaction to give 2-carbon enaminols (see review by Rizzi, 2003).

7.3.2. Reactions of Amadori and Hynes Products

The Amadori product of amino compounds is a secondary amine and, as such, may react with a second molecule of sugar to form a diketosyl amine, although this would be expected to be a relatively minor pathway due to both electronic and steric effects. However, once diketosyl Amadori products are formed they are highly reactive and brown more readily than monoketosyl Amadori products. The addition of sugars tends to promote the browning of Amadori products whereas amino acids tend to inhibit browning (Nursten, 2005). The former may be associated with the formation of reactive diketosyl Amadori rearrangement products. The latter is probably due to inhibition of the deamination step (see below).

The extent to which Amadori product degradation contributes to advanced products of the Maillard reaction (including browning) in food and biological systems is contentious and somewhat difficult to quantify. There is evidence that non-Amadori pathways become more significant with increasing pH (>8). Based on observations on the kinetics of Maillard reactions at high and low temperatures, and studies on the fragmentation of sugars by electron impact and Amadori products, Yaylayan (1990) suggested that the direct dehydration of the cyclic forms of Amadori compounds may occur at high temperatures. Similarly, the direct dehydration of the cyclic

forms of the parent sugars may also be possible. It is envisaged that such dehydrations occur via the formation of pyrylium or furylium ions, which may then react with each other to form polymers (Yaylayan, 1990; Yaylayan and Lachambre, 1990). Amadori products may decompose by dehydration reactions or by thermally induced non-hydrolytic scissions (C–C and C–N bond cleavages). The latter become more important at elevated temperatures. Under acidic conditions, the nitrogen atom of the Amadori compound is protonated, and 1,2-enolization (designated the 1,2-E pathway) is promoted by the withdrawal of electrons from C1 of the sugar residue by the positively charged nitrogen atom. As the pH increases, the deprotonation of the nitrogen atom increases the electron density at C1 of the sugar moiety which, in turn, discourages 1,2-enolization. Thus, as pH increases, 2,3-enolization (designated the 2,3-E pathway) becomes more favourable. This effect of pH on the electron density across C1 and C2 is more pronounced with Amadori products derived from basic amino acids. The 2,3-E pathway is a particularly important source of flavour volatiles in food systems, yielding furanones, pyranones and Strecker degradation precursors. The mechanistic similarity between Maillard reactions and caramelization was emphasized by Feather (1981) who used the term 'amine-assisted sugar dehydration reactions'. However, in contrast to caramelization-type reactions, Amadori products can undergo enolization more readily and under milder conditions than the corresponding sugars (as a result of the stabilizing influence of the amino moiety). Thus, the formation of Amadori and Heyns rearrangement products in Maillard reactions can be regarded as low-energy pathways for the decomposition of sugars compared with caramelization.

The hydrolysis of the imminium ion has been reported as the possible rate-limiting step in the decomposition of the Amadori compound to form 5-hydroxymethyl-2-furaldehyde (Yaylayan and Forage, 1991). Similarly, cleavage of the C–N bond in the 2,3-E pathway may be a rate- determining step (Yaylayan and Forage, 1991). There is evidence that amino acids may actually inhibit the decomposition of Amadori products to form brown pigments, possibly due to inhibition of the deamination steps of the 1,2- and 2,3-E pathways or because of reaction with the carbonyl groups of the Amadori compound forming less reactive derivatives (Nursten, 2005). The presence and nature of amino acids have a strong influence on the pathway of reaction of Amadori products and the products formed. Yaylayan and Mandeville (1994) showed a marked increase in the formation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, hydroxymaltol and maltol from the Amadori product, fructosyl proline, when an equal weight of alanine was added. Addition of proline to the fructosyl proline led to significant, though less marked, increases in the yield of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one and hydroxymaltol. Simple methods for following the 1,2-E

and 2,3-E pathways have been developed involving, for example, the determination of products such as 2-furaldehyde (1,2-E) or furanone (2,3-E) derivatives (Feather, 1981). However, relatively little is known of the factors that influence such pathways, despite their importance in flavour chemistry. Many papers have focused on the chemistry of deoxy-dicarbonyl compounds (e.g. deoxyglycosuloses) formed in the Maillard reaction (Feather, 1989). The deoxglycosuloses are formed by the deamination and dehydration of Amadori compounds (Hodge, 1967).

7.3.3. Deoxyglycosuloses

Some of the most reactive intermediates in the Maillard reaction are the deoxydiketoses (systematically referred to as deoxyglycodiuloses) and deoxyaldoketoses (systematically referred to as deoxyglycosuloses; formerly designated as 'osones') derived from degradation of Amadori products. Since 3-deoxyglycosulose is a product of the 1,2-E pathway and 1-deoxyglycosulose is a product of the 2,3-E pathway, their formation is influenced by the pH of the system and the basicity of the amino compound as discussed earlier. As in the case of Amadori and Heyns products, compounds such as 3-deoxyglucosulose are stabilized by the existence of several cyclic forms. Similarly, it has been suggested that 1-deoxyglycodiuloses exist as cyclic hydroxyfuranones and hydroxypyranones in solution (Yaylayan and Huyghues-Despointes, 1994).

In contrast to 3-deoxyglycosuloses in Maillard systems, aqueous solutions of pure 3-deoxyglucosuloses are unexpectedly stable. 3-Deoxyglucosulose also appears to be relatively stable under acidic conditions; the major degradation product is 5-hydroxymethyl-2-furaldehyde with smaller amounts of lactic and formic acids. Labelling studies have demonstrated that the carbon atom of formic acid and the aldehyde carbon atom are derived exclusively from C1 of 3-deoxyglucosulose (Weenan and Tjan, 1992). Under mildly basic conditions (0.1 M K_2HPO_4), a high yield of metasaccharinic acid is formed with complete decomposition of 3-deoxyglycosulose (Weenan and Tjan, 1992).

In the presence of amino compounds, the formation of 5-HMF from 3-deoxyglucosulose tends to be suppressed in favour of nitrogen-containing compounds, e.g. pyrroles and pyridinium betaines. The significance of pyrrole formation from 3-deoxyglucosulose derives from the reactivity of the hydroxymethyl carbon giving rise to the potential for protein crosslinking. The pyrrolealdehyde crosslink pyrraline is formed from the reaction of 3-deoxyglucosulose with the lysine Amadori product.

The 1-deoxy-2,3-glucodiulose compound formed via the 2,3-E pathway is considerably less stable than 3-deoxyglucosulose and has never been

isolated in the pure form although its formation as an intermediate has been inferred mechanistically by several workers. A number of studies have examined the formation of reactive osulose intermediates in the Maillard reaction using the trapping reagent *o*-phenylenediamine, and measuring and characterizing the quinoxaline derivatives formed. Such an approach was used to show that the 3-deoxyhexosulose and 1-deoxy-2,3-hexodiulose intermediates are formed from 1-deoxy-1-propylamino-D-fructose in the ratios 1:20 and 5:8 at pH 7 and 4.5, respectively, after 10 h under reflux conditions (Beck *et al.*, 1988).

Deuterium exchange studies suggest that the free C2 keto group is not necessary for the degradation of 3-deoxyglucosulose to hydroxymethylfurfural (Weenan and Tjan, 1992; Yaylayan and Huyghues-Despointes, 1994). A mechanism for the above reaction involving furylium ion formation has been proposed by Yaylayan (1990) and obviates the need for ring opening.

Yet another reaction pathway of glycosulose intermediates is fragmentation. Weenan and Tjan (1992) suggested retro-aldol fragmentation (3,4-scission and 4,5-scission) of glycosulose intermediates to account for pyrazine formation (via the resulting mono- and di-carbonyl species). Fragmentation reactions are discussed in detail below. As in the case of enediols produced from LA transformations, oxidative cleavage may result in the splitting of glycosuloses between the two carbonyls (Ledl and Schleicher, 1990). Such reactions result in the formation of products such as 2-deoxypentaenoic acid lactone and formic acid from 3-deoxyglycosulose.

Both 1- and 3-deoxyosuloses are products of the decomposition of Amadori products. It has been reported that 4-deoxyosuloses are produced by the treatment of sugars with alkali and are thought to be precursors of isosaccharinic acids (Miller & Cantor, 1952). The acid-catalysed degradation of hexoses and pentoses to 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde, respectively, is also believed to proceed via 3-deoxyosulose intermediates (Feather, 1970). It is now widely accepted that both 1- and 3-deoxyosuloses are important intermediates in flavour production in food systems. In particular, 3-deoxyosuloses are extremely reactive intermediates in the Maillard reaction (Glomb *et al.*, 1991). 3-Deoxyosuloses are powerful protein crosslinking agents in laboratory experiments (Igaki *et al.*, 1990)

In addition to the formation of 3-deoxyglycosuloses and 1-deoxyglycodiuloses, the formation of 1-amino-1,4-dideoxy-2,3-hexodiuloses has been suggested by the results of trapping experiments. This product is formed via the 2,3-E pathway following elimination of the C4 hydroxyl group from the enediol. However, it is not clear what role 1-amino-1,4-dideoxy-2,3-glycodiuloses play in the Maillard reaction (Yaylayan and Huyghues-Despointes, 1994).

7.3.4. Formation of Low Molecular Weight Maillard Products in Dairy Products

As might be expected from the mechanistic considerations discussed above, heating acidified skim milk or a neutral or acidic lactose solution results in the formation of HMF in relatively high yield (Patton, 1950a,b). No furfuryl alcohol was detected under such conditions. In concentrated skim milk and weakly alkaline lactose solutions, both furfuryl alcohol and HMF were formed (Patton, 1950b). Furfuryl alcohol is probably formed on reduction of furfural. Furfural and furfuryl alcohol are also formed in stored casein (Ramshaw and Dunstone, 1969). Patton (1950a) suggested that HMF in heated milk systems is formed largely via the Maillard reaction since it could not be detected on heating lactose solutions in the absence of casein or glycine. By contrast, however, Berg (1993) reported that free HMF and furfural levels were slightly higher in lactose-only model systems (at 140°C) than in systems containing casein. Furthermore, Berg (1993) reported that the levels of HMF formed from a lactose–protein complex at 140°C were only ~1% of those formed in normal skim milk heated under similar conditions. No furfural was detected under such conditions. The formation of furfural, HMF and furfuryl alcohol is an order of magnitude less than that of lactulose and galactose. Although HMF is an important product in heated milk, the concentration of lactulose can be more than 20 times that of HMF in UHT milk (75 d storage at 50°C) (Jimenez-Perez *et al*., 1992). However, the concentrations of HMF and lactulose in UHT milk change differently with time. Jimenez-Perez *et al.* (1992) reported that while the concentration of lactulose decreased after 75 d storage at either 40 or 50°C for all of the commercial lots studied, HMF content increased steadily over the 90 d storage period. In UHT milks, the levels of furfural formed may be more than 20 times lower than those of HMF. By comparison, the levels of furfuryl alcohol and HMF in UHT milk have been reported to be similar (380 μmol kg^{-1} after 23 min at 140°C) (Berg, 1993).

Diacetyl is a major volatile fission product of sugars, especially during heating at alkaline pH values (Hayase and Kato, 1986). Shipe *et al.* (1978) suggested that diacetyl is a major contributor to the rich note of heated milk, with minor contributions from maltol and acetophenone. Fission products, such as methylglyoxal and pyruvic acid, have also been identified in heated milk. Such fission products may react readily with amino compounds to form advanced Maillard reaction products.

The pyranone, maltol, has long been known to be formed in heated milks (Patton, 1950a). Maltol has been suggested as a possible contributor to the flavour of heated milk although it is of secondary importance to the contribution of diacetyl (Shipe *et al.*, 1978). The formation of maltol is an

important feature of Maillard and caramelization reactions in milk systems compared with other foods. In the presence of amino compounds, maltol may be formed from disaccharides or monosaccharides. However, in the absence of amino compounds, monosaccharides do not result in maltol formation. The formation of maltol is promoted under alkaline conditions. Although maltol may be formed via the 2,3-E pathway, Yaylayan and Mandeville (1994) reported that the favoured pathway for its formation is via *ortho*-elimination of the Amadori product. The addition of amino acids to the Amadori product, fructosyl proline, resulted in dramatic increases in the yields of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one, hydroxymaltol and maltol (Yaylayan and Mandeville, 1994).

There is evidence that carbonyl intermediates of the Maillard reaction may react with sulphur compounds released in heated milk, leading to a decrease in the latter as the intensity of heat treatment is increased (Jaddou *et al.*, 1978). It appears that oxygen may also play a role in this process (Calvo and de la Hoz, 1992). The reactions of sulphydryl compounds formed in milk with Maillard intermediates have not been investigated in detail. However, many sulphur compounds are inhibitors of the Maillard reaction and such interactions may have an inhibitory effect on the reaction in milk systems by making carbonyl compounds unavailable for further reactions and browning. The reactions are also likely to be of organoleptic significance because of the powerful odour of low molecular weight S-compounds.

Strecker degradation reactions involving oxidative degradation of α-amino acids by dicarbonyl intermediates of the Maillard reaction are among the most important reactions in food systems. Such reactions result in the formation of unstable Schiff's bases which easily decarboxylate to form enamines and CO_2. The CO_2 is derived from the amino acid. The enamines subsequently undergo hydrolysis to form an aldehyde from the amino acid (Strecker aldehyde) and an α-aminoketone from the dicarbonyl compound. Dicarbonyl compounds are formed in both the 1,2-E and 2,3-E pathways. The key Maillard intermediate, 3-deoxyglucosulose, has been shown to react with L-phenylalanine in a Strecker degradation (Ghiron *et al.*, 1988). Strecker degradations have long been known to occur during the processing and storage of dairy products. Patton (1955) considered 3-methylbutanal to be a possible Strecker aldehyde of potential flavour significance in milk. Based on studies using ^{14}C-lactose, Dutra *et al.* (1958) suggested Strecker degradation as the major source of CO_2 produced during the sterilization of milk; only $\sim$4% of total CO_2 was attributable to lactose caramelization. Strecker aldehydes have been detected in pasteurized milk (3-methylbutanal, 2-methylbutanal), UHT milk (2-methylbutanal, isobutanal), sterilized milk (3-methylbutanal, 2-methylbutanal) and milk powder (2-methylbutanal, isobutanal) (Calvo and de la Hoz, 1992). In addition, Calvo and de la Hoz (1992)

suggested that Strecker degradation of phenylalanine and methylglyoxal might contribute to the formation of acetophenone in UHT and sterilized milks.

Metabolic products of dairy cultures have attracted attention because of their possible reactivity in Maillard reactions. Kowalewska *et al.* (1985) showed that the Maillard products, 2,5-dimethyl-4-hydroxy-3[2*H*]-furanone and pyrrolidine, could be produced from metabolic products of *Lactobacillus helveticus*. Griffith and Hammond (1989) attempted to model flavour generation in Swiss cheese by reacting carbonyl products known to be produced by *Lactobacillus delbruechii* subsp. *bulgaricus* with amino acids. The Strecker aldehydes, isovaleraldehyde, 2-methylbutanal, isobutanal, phenylacetaldehyde and methional, derived from leucine, isoleucine, valine, phenylalanine and methionine, respectively, were shown to be formed in reaction systems containing methylglyoxal. In addition, volatile Maillard reaction products of lysine and proline with various carbonyls, such as glyoxal, methylglyoxal and dihydroxyacetone, were detected. These included pyrrolidines, pyrrolizines, pyridines and pyrazines. The results suggested that the final stages of flavour formation in Swiss cheese are dominated by Maillard reactions rather than by enzymatic or microbiological processes. Scanlan *et al.* (1968) reported the presence of furfural, phenylacetaldehyde, maltol, acetophenone and diacetyl in milk heated at 146°C for 4 s. Presumably, the phenylacetaldehyde was derived from the Strecker degradation of phenylalanine.

Extraction of spray-dried skim milk powder using a Likens–Nickerson apparatus and ether as solvent resulted in 196 individual GC peaks (Shiratsuchi *et al.*, 1994). Surprisingly, however, no Strecker aldehydes were detected. The Maillard reaction products, furfural and furfuryl alcohol, were the only furans detected. Shiratsuchi *et al.* (1994) suggested that furfural and furfuryl alcohol could be important flavour molecules in condensed milk but concentrations in skim milk powder were probably too low to be important.

Ferretti and Flanagan (1971) detected many potential products of the Maillard reaction in a lactose–casein mixture allowed to brown for 11 d (75% RH; 75°C): acrolein, 5-hydroxymethyl-2-furaldehyde, 2-furaldehyde, 3-hydroxy-2-butanone, acetol, acetol acetate, 2-furfuryl formate, 2,2'-bifuran, 1-(2'-furyl)'-2-butanone, 1-(2'-furyl)'-3-butanone, maltol, methyl-2-thiofuroate, 5-methyl-2-propionylfuran, 2-furfuryl vinylacrylate, methylethylpyrazine, C-4-alkylpyrazine, tetramethyl pyrazine, trimethylpyrazine, *N*-methyl-2-formyl pyrrole and *N*-methyl-2-acetylpyrrole. The formation of several furanones which were probably products of Maillard reactions was also reported. The volatiles present at high concentrations included several Maillard reaction products: acetic acid, acetol, 2-acetylfuran, 5-methyl-2-furaldehyde, furfuryl acetate, furfuryl alcohol and maltol. Many potential Maillard products were also detected in a 2-year-old skim milk

powder (Ferretti and Flanagan, 1972): 2-acetylfuran, 2-furaldehyde, 2-methylpyrazine, 2,5-/2,6-dimethylpyrazine, 2,3-dimethylpyrazine, 2-methyl-5-ethyl/2-methyl-6-ethylpyrazine, 2,3,5-trimethylpyrazine, 2-formylpyrrole, *N*-methyl-2-formylpyrrole, *N*-ethyl-2-formylpyrrole, 2-acetylpyrrole and maltol. The authors suggested that 2-furaldehyde probably contributes to stale flavour development in stored milk powder, along with some of the pyrazines and several other compounds. Since Maillard reactions contributed at least five of the 12 constituents associated with stale flavour in the stored milk powder, the study illustrated the critical importance of control of such reactions in maintaining organoleptic quality.

Sugar fragmentation has been recognized to play an even more critical role in the Maillard reaction than had hitherto been realized. Sugar fragmentation, *per se,* is not significant at low pH but becomes increasingly important as the pH is increased. Hayashi and Namiki (1986) proposed a new Maillard pathway involving cleavage of the sugar moiety of the Schiff's base, forming C2 and C3 fragments. Such products included glycoaldehyde, glyoxal, methylglyoxal, glyceraldehyde or their imine derivatives.

The significance of sugar fragmentation products is highlighted by their rates of browning compared with their parent sugars. At 80°C, the rates of browning with β-alanine of glyoxal, methylglyoxal, glyceraldehyde and glycoaldehyde were, respectively, $\sim$120, $\sim$650, $\sim$2000 and $\sim$2000 times that of glucose. Thus, even a small amount of sugar fragmentation could make a large contribution to browning. By comparison, a mixture of 3-deoxyglucosone and β-alanine browned $\sim$140 times faster than glucose and β-alanine.

5-Hydroxymethyl-2-furaldehyde is formed by the decomposition of Amadori compounds under acidic conditions via the 3-deoxyosulose intermediate. It has a high molar extinction coefficient at 280 nm and is probably a major contributor to the increase in A_{280} that is a characteristic of Maillard reaction systems (Feather, 1989). Furaldehydes are key intermediates in the formation of high molecular weight melanoidin pigments. 2-Furaldehyde has been shown to react readily with glycine via its aldehyde group (Obretanov *et al.*, 1983) and 5-HMF may react with reducing sugars at high temperatures via both the aldehyde and hydroxymethyl groups (Urashima *et al.*, 1988).

Both the Amadori products, lactulosyl lysine and fructosyl lysine, have been shown in heated milks, the latter ostensibly arising from the hydrolysis of the 1$\rightarrow$4 bond of lactulosyl lysine resulting in the release of galactose (Moller *et al.*, 1977a). However, it is unclear to what extent such a mechanism could account for the evolution of free galactose in heated milks compared with the degradation of lactulose. Even in the presence of extensive Maillard reactions in UHT milk, the concentration of fructosyl lysine is only about 10% that of lactulosyl lysine (Moller *et al.*, 1977b). The ratio of lactulosyl

lysine to fructosyl lysine decreases with increasing temperature and duration of storage suggesting that hydrolysis of the glycosidic bond might be taking place. However, it is also clear that hydrolysis of the glycosidic bond is difficult and probably does not occur at the Amadori stage (Pischetsrieder and Severin, 1994).

Compared with the Amadori products of other disaccharides and of glucose, the Amadori product of lactose and the ε-NH_2 of lysine appears to be relatively stable (Kato *et al.*, 1988, 1989). Kato *et al.* (1988) showed that although the decrease in free amino groups of ovalbumin was similar in systems containing lactose or glucose, the rate of browning and polymerization was higher in the presence of glucose. Kato *et al.* (1989), who studied the reaction of ovalbumin with the disaccharides, maltose, cellobiose, isomaltose, lactose and melibiose, showed that the production of advanced Maillard reaction products (brown colour and fluorescent compounds) was weakest in the maltose, lactose and cellobiose systems. The authors concluded that the effect was due to the nature of the non-reducing pyranoside group and the position (1→4 or 1→6) of the bonds; the reducing moiety is glucose in the case of each of the systems studied. Thus, it appears that substitution at the C4 hydroxy position of glucose has a stabilizing effect on the corresponding Amadori product, as confirmed by experiments using 4-*O*-methyl-D-glucose (Kato *et al.*, 1988, 1990). The conclusions drawn from experiments on model systems appear also to be borne out in practice. Burvall *et al.* (1978) reported that although up to 40% of lysine was unavailable in a stored lactose-hydrolysed dried milk, there was no visible browning in the product, suggesting that the Maillard reaction had not progressed beyond the intermediate stages. The (1→4) linkage in lactose and some other disaccharides also influences the profile of Maillard reaction products formed subsequent to the Amadori rearrangement. For example, Patton (1950b) recognized that the structure of lactose favoured the formation of furfuryl alcohol in heated milk whereas those of glucose, galactose, sucrose or methyl-α-glucopyranoside did not. The influence of sugar structure on reaction mechanism is discussed in more detail below.

A variety of flavour volatiles produced in stored or heated milk systems have been identified as putative products of non-enzymatic browning reactions. These include benzaldehyde, methyl furans, acrolein, maltol, diacetyl and acetaldehyde (Ferretti and Flanagan, 1971, 1972; Calvo and de la Hoz, 1992).

In addition to the formation of brown pigments, fluorescent pigments are also formed in heated and stored milk (Patton, 1955). In general, the activation energy for fluorescence development is less than that for browning (Labuza, 1994), although it is not clear if this applies in the case of dairy products.

7.4. Factors that Influence Maillard Reactions

Both the overall rate and product profile of the Maillard reaction in foods are highly dependent on a number of parameters, the most important of which are reactants, pH and temperature. The impact of moisture content and water activity is mentioned briefly below but the physical state of the food system is beyond the scope of the present review. Readers are referred to the papers by Lievonen and Roos (2002), Miao and Roos (2004) and Thomas *et al.* (2004) for further information.

7.4.1. Reactants

Both the nature and the molar ratio of the reacting species have a considerable influence on the rate and mechanism of the Maillard reaction. In general, low molecular weight reactants tend to react more readily than high molecular weight reactants, partially as a result of steric hindrance in the latter. Thus, glucose is more reactive than lactose and contributes to the increased rate of browning in lactose-hydrolysed milks (Lea, 1948; Evangelisti *et al.*, 1994). Colour intensity produced in model systems may be ranked according to the reacting sugars as follows: xylose>arabinose>fructose>glucose>maltose>lactose (Yang and Shin, 1980). Although glucose, mannose (C2 epimer of glucose) and galactose (C4 epimer of glucose) all resulted in a similar loss of free amino groups on reaction with ovalbumin, the browning that developed in the galactose-containing system was two- to threefold higher than with the other sugars (Kato *et al.*, 1986). In addition, ovalbumin stored in the presence of galactose showed a dramatic decrease in solubility (~40% that in the presence of glucose or mannose). Comparisons with talose, which has the same configuration as galactose at C3 and C4, suggested that the configuration contributed to the high rate of advanced reactions preceding browning in the galactose system. It was suggested that the configurations of galactose and talose contribute to a higher browning rate by stabilizing the cyclic chair conformations of the Amadori products by hydrogen bonding (Kato *et al.*, 1986). It is not known whether sugars are released from glycoproteins in heated milk and participate in Maillard reactions. However, even if such reactions occur, their quantitative significance would be negligible relative to the huge excess of lactose in milk.

Steric effects play an important role in determining the reactivity of amino groups on proteins. Thus, it appears that some internal amino groups on globular proteins, such as β-lactoglobulin, may be inaccessible to sugars and unavailable for Maillard reactions. In addition, the pK_a value of amino groups and acid–base catalysis by adjacent proton donor/acceptor groups contribute to the reactivity of individual amino groups. Although all the

major milk proteins have an abundance of reactive groups, the whey proteins and α_{s2}-casein would be expected to show the highest activity in carbonyl-amine reactions based on amino acid composition. Of the protein-bound amino acids, lysine is usually the most reactive, followed by tryptophan, histidine and arginine. Among other factors, the pK_a of the amino group and the distance between the amino and carboxyl groups of free and protein-bound amino acids influence their reactivity in Maillard reactions. In dipeptides, the presence of hydrophobic residues such as isoleucine, leucine or phenylalanine increased lysine reactivity, whereas the basic amino acids decreased reactivity (Mennella *et al.*, 2006).

The indolyl nitrogen atom of tryptophan is less reactive in Maillard reactions than the ε-NH_2 group of lysine because the lone pair of electrons on the nitrogen atom are delocalized about the ring. In practice, protein-bound tryptophan usually reacts with reducing sugars only to a limited extent. The ε-amino group of lysine is reactive and, because of its availability for reaction, is normally the predominant reacting amino function in proteins. Consequently, milk proteins, which are rich in lysine, tend to brown more readily than proteins low in lysine, such as soy proteins (Wolf *et al.*, 1977). In milk, experiments involving the incorporation of ^{14}C from ^{14}C-lactose during UHT processing suggested that casein micelles incorporated most of the radioactivity (5–6 times that of α-lactalbumin or β-lactoglobulin on a weight basis). Of the caseins, the most reactive appears to be κ-casein (Turner *et al.*, 1978). Clearly, this pattern of reactivity conflicts with what might be expected from the amino acid composition of the proteins. It might be expected that ε-NH_2 groups in micellar κ-casein would be more accessible to reacting sugars than NH_2 groups in the other caseins. Such preferential reactivity of κ-casein may have significant implications for the stability of milk proteins during storage. Similarly, it has been suggested that the compact globular structure of whey proteins makes some of their lysine residues inaccessible (Turner *et al.*, 1978), although a weakness in this argument is that heat-induced denaturation of whey proteins should increase the accessibility of such lysine residues. An alternative explanation for the unexpectedly low reactivity of whey proteins in Maillard reactions is that protein–protein interactions of denatured whey proteins in milk could make lysine residues unavailable for reaction with lactose. The study of Turner *et al.* (1978) did not quantify the release of ^{14}C in the form of low molecular weight products.

The reactivities of free amino acids differ dramatically from those of their protein-bound counterparts (Izzo and Ho, 1992). The reactivity of free amino acids with glucose was ranked as follows: lysine>glycine>tryptophan>tyrosine>histidine>arginine>cysteine (Ashoor and Zent, 1984). Such rankings are dependent on the reacting sugar and the pH of the system under study. In contrast, Labuza (1994) asserted that the assumption that lysine is the most

reactive free amino acid is erroneous and that tryptophan is ~30 times more reactive. In a comparative study on the reactivity of amino acids in total parenteral nutrition solutions, Labuza and Massaro (1990) reported that lysine browned the slowest whereas cysteine had the highest rate of browning (~20 times that of lysine). However, paradoxically, in a mixed system containing lysine, tryptophan and cysteine, there was evidence that cysteine acted as an inhibitor of browning (Labuza and Massaro, 1990). Cysteine, *N*-acetyl-L-cysteine and reduced glutathione have been reported to inhibit non-enzymatic and enzymatic browning in both model and real food systems (Friedman and Molnar-Perl, 1990; Molner-Perl and Friedman, 1990). The strong nucleophilicity of such sulphur compounds probably contributes to their activity as browning inhibitors. Among the possible mechanisms for the activity of sulphur compounds, the suppression of free radical formation and the trapping and inactivation of intermediates of non-enzymatic browning, thus preventing further reactions, have been proposed (Friedman and Molnar-Perl, 1990).

Clearly, many unrelated factors influence the reactivity of amino acids in the Maillard reaction. For example, on the basis of p*K*, arginine might be expected to be more reactive than lysine (the p*K* of the guanidino group is ~12 compared with 9.4–10.6 for the ε-NH_2 of lysine). However, the guanidino group has been reported not to undergo the Maillard reaction (Ledl and Schleicher, 1990). Nevertheless, protein-bound arginine undergoes extensive modification during the Maillard reaction by virtue of reactions involving α- and β-dicarbonyl compounds and lysine resulting in the formation of the imidazopyridinium crosslink, pentosidine.

It is generally agreed that an excess of reducing sugar over the amino compound promotes the rate of Maillard browning (O'Brien and Morrissey, 1989). Quantitatively, increasing sugar concentration at a constant amine concentration has a greater effect on the rate of browning than an increase in amine concentration at a constant sugar concentration (Labuza, 1994). Presumably, the latter results in a mass action inhibitory effect on the deamination steps of the enolization pathways. In addition, because of the recycling of amines in the reaction, the concentration of sugar is more likely to be rate limiting than that of the amino compound. Thus, increasing the lactose–protein ratio in an infant formula was shown to increase lysine blockage due to Maillard reaction (Evangelisti *et al.*, 1993).

There are significant quantitative and qualitative differences in the Maillard reactions of disaccharides compared with those of monosaccharides. Degradation of the 1-deoxyglycodiuloses of disaccharides shows major differences from those of monosaccharides, in part because the second sugar residue is a poor leaving group in the subsequent elimination reactions, forcing the selection of an alternative elimination. Thus, whereas the major products of heated monosaccharides (fructose or glucose) are

2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one and 4-hydroxy-2(hydroxymethyl)-5-methyl-3(2*H*)-furanone, the main products of maltose or lactose are 4-(glycosyloxy)-2-hydroxy-2-methyl-2*H*-pyran-3(6*H*)-ones, 4,5-dihydroxy-2-(glycosyloxy)-5-methyl-2-cyclopenten-1-ones and 4-(glycosyloxy)-5-(hydroxymethyl)-2-methyl-3(2*H*)-furanones (Pischetsrieder and Severin, 1994). One such product, 4-(galactosyloxy)-2-hydroxy-2-methyl-2*H*-pyran-3(6*H*)-one, has been detected in heated milk (Ledl *et al.*, 1986). The elimination of the galactosyl residue from the above molecule results in the formation of maltol (a flavour molecule, flavour enhancer and an antioxidant) in heated milk. Similarly, the disaccharide-specific 4-(glycosyloxy)-5-(hydroxmethyl)-2-methyl-3(2*H*)-furanones are strongly reducing and probably contribute to antioxidative activity. Thus, disaccharides, such as lactose, may be useful reactants in Maillard systems since they generate products not known to arise from monosaccharides.

Under mild processing conditions, non-reducing sugars, such as sucrose, do not react in Maillard reactions with amino compounds. At high temperatures and/or at low pH, sucrose may become a major reactant in Maillard reactions due to hydrolysis of the disaccharide. For example, in a comparative study, Lee and Nagy (1990) reported that the rate of 5-HMF formation from fructose was 31.2 times that from glucose whereas the rate from sucrose was 18.5 times that from glucose at pH 3.5 and 50°C. Moreover, casein–glucose and casein–sucrose systems showed similar losses of lysine and arginine when heated in the dry state at high temperatures (200 or 300°C $\times$ 1 h) (Smith and Friedman, 1984). Similarly, the addition of 8% cane sugar to cows' or buffalo milk resulted in severe browning and a decrease in tryptic hydrolysis when milk was sterilized at 104 kN m^{-2} for 20 min (Gothwal and Bhavadasan, 1991). The extent of browning has been reported to be higher in buffalo milk than in cows' milk (Gothwal and Bhavadasan, 1991; Srinivasan and Gopalan, 1994).

7.4.2. pH

The rate of the Maillard reaction has long been reported to increase with increasing pH up to a maximum at ~pH 9–10 (Ashoor and Zent, 1984; Pokorny *et al.*, 1988), depending on the amino acid involved. Bases can catalyse the initial steps of carbonyl-amine reactions by removing a proton from the nucleophile, increasing its nucleophilicity:

$$NuH + B^- \quad \rightleftharpoons \quad Nu:^- + BH$$

The browning of pure Amadori products is also accelerated at alkaline pH values (Westphal *et al.*, 1988). pH exerts a considerable influence on the

mechanism of the Maillard reaction by determining the type of enolization favoured (1,2- or 2,3-enolization) and hence the pattern of Amadori compound degradation. Consequently, the influence of other factors on reaction rate is frequently pH dependent. For example, the addition of D-alanine or L-lysine to an L-ascorbic acid model system had a small or negligible effect on browning rate at pH 5 or 7, whereas an increase in browning rate occurred when the amino acids were added at pH 8.0 (Löscher *et al*., 1991). In contrast, addition of glycine resulted in an increase in browning at all three pH values. The authors attributed this to a reaction of glycine with furfural produced on degradation of ascorbic acid with a resultant increase in brown pigment formation. By contrast with brown colour formation, the development of fluorescence in an epoxyaldehyde–lysine system appeared to be independent of pH above 6.0; brown colour development showed a maximum at pH 9.0 (Hidalgo and Zamora, 1993). At shorter incubation times, development of fluorescence was linear with increasing pH. Increasing pH may have a major, albeit indirect, effect on the Maillard reaction by increasing the rate of mutarotation of both the parent sugar and of hemiacetal and hemiketal forms of intermediates formed in the reaction. Since the pH of a system decreases during the course of Maillard browning (due to the disappearance of basic amino groups and the formation of formic, saccharinic and other acids), the buffering capacity of the system has an important effect on the rate of reaction. The inhibitory effect of supercritical carbon dioxide treatment on Maillard reactions appears to be due to a reduction in pH due to both high pressure and CO_2 (Casal *et al*., 2006).

7.4.3. Mutarotation

Since it is generally accepted that sugars can react only as their acyclic form (at least at low temperatures), the rate of mutarotation is likely to be a significant rate-limiting step because the amount of sugar in the open-chain form is normally limited to a few percent of total sugar. Vogel *et al.* (1988) reported that the rate of glucose mutarotation was minimal at pH 3. As pH is increased, the rate of mutarotation increased rapidly; the rate of mutarotation of glucose at pH 7 was reported to be approximately 8 times that at pH 3 (Vogel *et al.*, 1988). The rate of mutarotation also increases with increasing a_w and temperature (Angyal, 1984; Vogel *et al.*, 1988). The significance of mutarotation rate and the concentration of acyclic forms were studied by Yaylayan and Forage (1992) who reacted tryptophan with glucose or mannose; since they are C2 epimers, both sugars produce the same Amadori product which enables differences in reaction rate due to mutarotation to be determined. Mannose, which has a higher rate of mutarotation and a higher equilibrium concentration of acyclic form than glucose, reacted

1.3 times faster than the latter at 110°C and 1.8 times faster at 140°C. Glucose has one of the lowest equilibrium concentrations of acyclic form of the monosaccharides and one of the lowest rates in Maillard reactions among monosaccharides. Thus, it has been suggested that glucose may have been selected as the universal metabolic fuel during evolution because of its stability (Kaanane and Labuza, 1989). For example, fructose reacted ~7 times faster than glucose with haemoglobin at 37°C (Kaanane and Labuza, 1989)

Little is known of the influence of factors other than pH, temperature and a_w on mutarotation rate. Amino acids have been reported to be weak catalysts for the mutarotation of reducing sugars (Shallenberger, 1984). Depending on the presence of complex-forming configurations, the presence of ions such as Ca^{2+}, Mg^{2+}, Na^+ and K^+ can influence the tautomeric equilibrium of a sugar. It has been reported that Ca^{2+} shifts the tautomeric equilibrium of D-glucose towards the α-anomer. Addition of 1% $CaCl_2$ at a constant initial pH to hydrolysed concentrated whey (50% solids), caused a slight decrease in browning rate (Buera *et al.*, 1990). The authors suggested that a complexation reaction between Ca^{2+} and the sugar resulted in a shift in anomeric equilibrium. The effect was greater in the absence of protein. In contrast, Kaanane and Labuza (1989) reported that $FeCl_2$, $CaCl_2$, $CoCl_2$, KCl or NaCl had no significant effect on the mutarotation of glucose or fructose. However, there was a highly significant difference between systems studied in D_2O or H_2O, illustrating that solvent has an important influence on the kinetics of mutarotation. Addition of KCl, $CaCl_2$ or $MgCl_2$ had no effect on the formation of 5-HMF from glucose, fructose or sucrose in the presence of citric acid (Lee and Nagy, 1990). NaCl and NH_4Cl have been shown to have a significant inhibitory effect on the rate of browning in model food systems, including casein–glucose, when added to a concentration of 0.5% (w/w) (Pham and Cheftel, 1990).

7.4.4. Moisture Content and a_w

Both the moisture content and the a_w of a food system exert a major influence on the Maillard reaction. Water may influence the rate of reactions by controlling the viscosity of the liquid phase and by dissolution, concentration or dilution of reactants (Warmbier *et al.*, 1976; Labuza, 1980). At very low a_w values, the proportion of total reactants in solution is negligible and, therefore, the reaction rate is minimal. As the a_w increases, the concentration of reactants remains constant provided excess solute is available to maintain a saturated solution. However, the total volume in which the reaction takes place increases. Eventually, an a_w value is reached when the solution of reactants is diluted and the reaction rate decreases again. Since water is a product of the Maillard reaction, the a_w may increase with the development of

Maillard reactions, further destabilizing a food system. Conversely, as a product of the reaction, water may inhibit the reaction via a mass action effect (Eichner and Karel, 1972). Indeed where diffusion/dissolution are not limiting factors, as in liquid model systems containing organic solvents, the addition of water can lead to a dramatic decrease in browning rate (Peterson *et al.*, 1994). In studies on liquid cows' and buffalo milk systems, Gothwal and Bhavadasan (1991) reported that a 40% decrease in the concentration of total solids led to a 25.6 and 36.5% decrease in browning rate, respectively. It is generally accepted that rates of Maillard reactions are maximal at intermediate a_w values (0.5–0.7) (Labuza *et al.*, 1970). In milk powder, browning reactions are maximal at $a_w$values of 0.6–0.7 (Loncin *et al.*, 1968; Ben-Gera and Zimmerman, 1972), whereas in whey powders, the maximum occurs at an a_w of 0.44. However, the relative increase in reaction rate with a_w is less as temperature is increased (Malec *et al.*, 2002). The difference is related to the higher concentration of lactose in the latter and differences in physicochemical properties between the dried milk and dried whey systems (Huss, 1974a,b). The moisture content corresponding to maximum browning rates in a spray-dried skim milk powder were 6.5 and 7.5% at 35 and 130°C, respectively (corresponding to a_w values of 0.44 and 0.86, respectively, at those temperatures) (Franzen *et al.*, 1990). In addition, the induction period was shorter in high-moisture systems although the ultimate extent of browning was lower than in the low-moisture systems. In contrast, the addition of water to a liquid model system containing L-proline and D-glucose in propylene glycol led to an increase in the induction period which was more pronounced at lower temperatures (Peterson *et al.*, 1994).

Moisture content also appears to have a significant qualitative effect on the development of Maillard reactions in food systems. For example, in a reaction of casein and glucose in the dry state, the reaction involved mainly basic amino acids, whereas all amino acids were decomposed during solution state reactions (Kato *et al.*, 1981a). In addition, reactions of solution systems led to greater amounts of free amino acids and low molecular weight peptides, suggesting hydrolysis of peptide bonds. The influence of a_w on the mechanism of Amadori product degradation is poorly understood. However, Eichner and Ciner-Doruk (1979) proposed that the 1,2-E pathway assumes increasing importance as the water content increases. Significantly, a_w may influence the site specificity of Maillard reactions involving proteins (Wu *et al.*, 1990) and also the relative reactivity of individual amino acids (Leahy and Warthesen, 1983). Ideally, a_w should be used as a predictor of browning rate and other spoilage reactions in foods only in the absence of moisture sorption–desorption hysteresis (i.e. in fully equilibrated systems) (Franzen *et al.*, 1990; Franks, 1991). In non-equilibrium systems, it has been proposed that the moisture content is a more useful/valid predictor of browning. In addition,

moisture content has an advantage in that it avoids difficulties caused by the dependency of a_w on temperature. However, moisture content as a predictor of browning may be equally flawed if a phase change occurs in the system, which may be possible at constant moisture content.

7.4.5. Miscellaneous Factors

Phosphate, citrate and phthalate buffers have been shown to accelerate the Maillard reaction (Bobbio *et al.*, 1973). However, it is unclear if such anions had a catalytic effect *per se* or if the effect was simply pH related due to differences in buffering capacity. At constant pH in the range 5–7, phosphate has a dramatic effect on reaction rate which increases up to 15-fold over that of a phosphate-free system (Potman and van Wijk, 1989). Chan and Reineccius (1994) showed that the activation energy for the formation of 2-acetylfuran and di(H)di(OH)-6-methylpyranone in a model system was approximately halved by conducting the reaction in phosphate buffer. Potman and van Wijk (1989) concluded that phosphate acts as an acid–base catalyst during the Amadori rearrangement and does not react directly in the Maillard reaction. Increasing the concentration of phosphate or citrate increased the rate of browning in cows' or buffalo milk on sterilization (Gothwal and Bhavadasan, 1991). When citrate or phosphate levels were increased by 50%, there was an increase in the browning index of ~17–23% in buffalo or cows' milk, the increase being slightly greater in the latter. Although the pH values following sterilization were similar, the addition of phosphate or citrate salts led to a change in the initial pH (up to ~0.2 pH units for a 50% increase). It is likely that the increase in initial pH was responsible for the increase in browning. The study of Gothwal and Bhavadasan (1991) illustrates clearly the extreme sensitivity of browning reactions in milk systems to even small changes in pH. Citric acid has a marked catalytic effect on the rate of 5-HMF formation from fructose at constant pH (3.5) (Lee and Nagy, 1990).

The overall Maillard reaction and the rate of browning of Amadori products are accelerated by Fe(II), Fe(III) and Cu(II) (Patton, 1955; Kato *et al.*, 1981b; Pilkova *et al.*, 1990). Some of the effects of metals on the Maillard reaction may be due to pH effects, which is the case for aluminium and zirconium compounds (Powell and Spark, 1971). There is evidence that complex formation between intermediates in the Maillard reaction and the metal ion is a prerequisite for the promoting activity of Fe and Cu on the rate of browning (Kato *et al.*, 1981a). The presence of copper ions (as $CuCl_2$) promoted the rate of browning in glucose–glycine, glyoxal–glycine and 5-HMF–glycine systems (Rendleman and Inglett, 1990). The activity of copper in this study also appeared to be associated with its ability to form

strong complexes with melanoidins. UV absorbance and fluorescence intensity were also increased in the presence of copper. The formation of a range of metal ion complexes with Maillard reaction intermediates was described by O'Brien and Morrissey (1997).

It might be expected that the presence of metal ions would also accelerate the oxidation of Amadori products to form carboxymethyl amino acids which have been used as indicators of the progress of the Maillard reaction in foods (see below). Similarly, the concentration of oxygen may influence both the overall rate of browning and the formation of low molecular weight oxidation products, such as carboxymethyl lysine. Pokorny *et al.* (1988) reported that the replacement of air by nitrogen in model systems containing Amadori products reduced the reaction rate by half.

A variety of other factors, such as tertiary amine salts and acetic acid, have been shown to promote the Maillard reaction in model systems (Yoshimura *et al.*, 1969). However, the effect of all such miscellaneous factors on the rates of Maillard reactions in food systems is likely to be negligible compared with the effect of temperature, moisture and the nature of the reacting species.

Non-thermal energy sources may also influence browning reactions in food systems. For example, treatment of milk with ionizing radiation has been reported to lead to browning on subsequent storage at 4.4°C (Wertheim *et al.*, 1956). Browning was attributed to radiation-induced degradation of lactose, since the removal of carbonyl compounds from irradiated milk as their hydrazone derivatives following reaction with 2,4-dinitrophenylhydrazine markedly reduced the extent of browning. Significantly, the paper by Wertheim *et al.* (1956) was also the first to suggest that browning may be catalysed by free radicals.

The consequences of ultrasound treatment is similar in many respects to that of irradiation treatment. Heusinger (1986) identified malondialdehyde as a product of the ultrasound treatment of glucose or lactose solutions. The effects of such ultrasound treatment appear to be similar to the effect of high-speed stirring operations which have become more common in food manufacturing.

UV irradiation of sugar–amino acid solutions has been reported to produce volatile compounds similar to those found in heated systems, although it appears to be possible to distinguish UV-treated from thermally processed systems based on the volatile profile (Sheldon *et al.*, 1986).

7.5. Interaction of Oxidation and Maillard Reactions

The interaction between oxidation reactions (of sugars and proteins in addition to lipids) and Maillard reactions is now accepted as being of more importance than previously recognized (Zamora and Hidalgo, 2005). The

term 'glycoxidation' recognizes our growing understanding of the Maillard reactions of sugar oxidation products and the oxidation of Maillard reaction products (Goldberg *et al.*, 2004).

Amadori products are more easily oxidized than glucose. The enediol of fructosyl lysine can be oxidatively cleaved to carboxymethyl lysine and erythronic acid under neutral to basic conditions and under slightly acid conditions to 3-(*N*-ε-lysine)-lactic acid and glyceric acid (Figure 7.6). Thus, the rate of disappearance of ε-fructosyl lysine increases in the presence of air. Experiments on model systems have shown that pH has a strong influence on the extent of carboxymethyl lysine formation from fructosyl lysine (Hartkopf and Erbersdobler, 1994); on increasing the pH from 4.0 to 9.0, the yield of carboxymethyl lysine in a lysine–glucose model system heated at 100°C for 3 h increased from 70 to 3170 mg kg^{-1} lysine. The presence of iron, phosphate or nitrate also promotes the formation of carboxymethyl lysine. Zyzak *et al.* (1994) suggested that autoxidative glycosylation may be the major pathway of the Maillard reaction under simulated physiological conditions in vitro. The major product of glucose oxidation was considered to be glyoxal which in

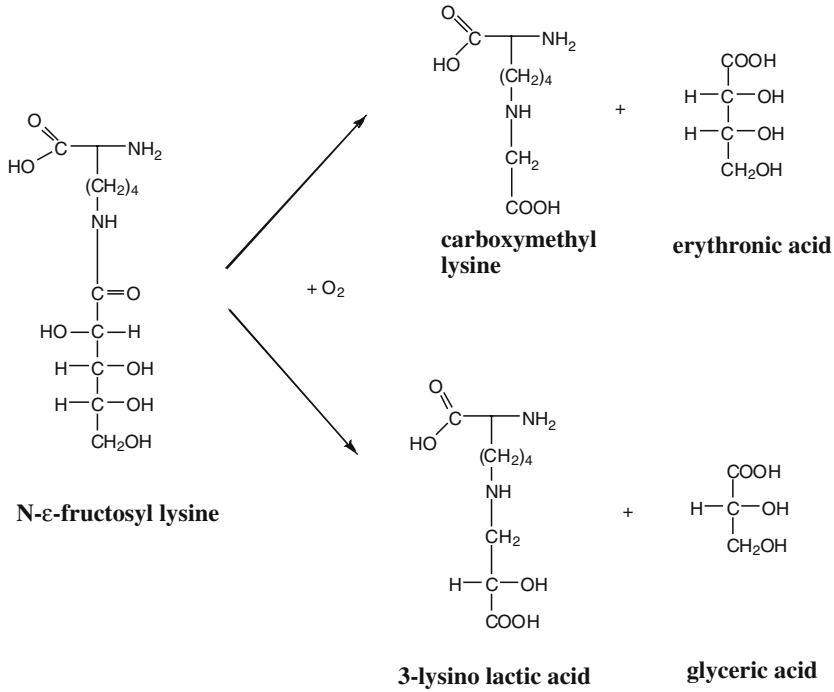

Figure 7.6. Major oxidation pathways of the Amadori product fructosyl lysine.

turn resulted in the formation of carboxymethyl lysine from lysine via an intramolecular Cannizaro reaction. It has been reported that oxidized ascorbic acid reacts rapidly with casein to produce a red coloration (Namiki *et al.*, 1986; Gopalan *et al.*, 1994). Namiki *et al.* (1986) showed that incubation of ascorbic acid in the presence of an air stream was necessary to produce red coloration in the presence of casein. Thus, it appears that the reactivity of ascorbic acid *per se* in Maillard reactions is negligible compared with that of dehydroascorbic acid. It is recognized that volatile aldehyde products of lipid peroxidation react readily with amino acids and proteins in Maillard-type reactions (Okitani *et al.*, 1986; Shibamoto and Yeo, 1992). Hexanal, a major volatile product of lipid peroxidation, reacts with proteins resulting in blocking of lysine and tryptophan residues, and polymerization (Okitani *et al.*, 1986). Aliphatic aldehydes produced on UHT processing of milk have long been known to decrease on storage, presumably due to such reactions (Earley and Hansen, 1982). The major lipid peroxidation product of butterfat, 4,5-epoxy-2-heptenal, has been shown to develop a brown colour and fluorescence on reaction with lysine (Hidalgo and Zamora, 1993). Interestingly, the activation energies for browning and fluorescence development on reaction of 4,5-epoxy-2-heptenal with lysine were comparatively low (66.5 and 50 kJ mol^{-1}, respectively). Products of the Maillard reaction have been shown to have a pro-oxidant effect in food systems (Miyazawa *et al.*, 2005) (see also Section 7.8.1).

Maillard reactions are also responsible for the formation of a host of powerful antioxidant compounds, many of which have not been characterized (Manzocco *et al.*, 2001; Morales and Jimenez-Perez, 2001; Cejpek *et al.*, 2004). The formation of such compounds is now increasingly being exploited industrially to improve the oxidative stability of foods. The inhibitory effect of pasteurization on the oxidation of milk lipids has been attributed to the release of sulphydryl groups during heating (Calvo and de la Hoz, 1992). However, it is possible that products of the Maillard reaction may also have a positive role in this respect. For example, maltol, an important flavour molecule in heated milk, is a powerful antioxidant.

The influence of oxygen on the formation of maltol and other products in a model system was studied by Yaylayan and Mandeville (1994). Although the decomposition of glucose and fructose did not appear to be sensitive to the presence of oxygen, the exclusion of oxygen seemed to promote the formation of maltol and HMF from maltose. In contrast, exclusion of oxygen from a tagatose system shifted the profile of products in favour of HMF, minimizing the formation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one. Oxygen is unlikely to have an important influence on lactose degradation during the heating of milk because of decreasing O_2 solubility at high temperatures. However, the presence of

oxygen may play a significant role in such reactions in stored dairy products, especially in dry systems.

The organoleptic acceptability of stored milk powder is closely related to the formation of volatile lipid peroxidation products and the oxidative stability and shelf-life of whole milk powders may be enhanced by the addition of Maillard reaction products (McGookin, 1991). The addition of Maillard reaction products as an antioxidant to skim milk powder has been reported to have a beneficial effect on the flavour profile (Hall and Andersson, 1985). Such applications are, however, limited by the effect of Maillard reaction products on the organoleptic acceptability of the products.

7.6. Methods for the Analysis of LA-Transformation Products and Degradation Products of Lactose

Although early methods for the identification of lactulose and its degradation products in milk products employed paper or thin layer chromatography or spectrophotometry, the methods most commonly used at present are based on HPLC, GLC or enzymatic reactions. The enzymatic methods of Geier and Klostermeyer (1980) and Andrews (1984) assume that lactulose is the only source of fructose in heated milks. Following the precipitation of fat and protein from milk samples, lactose and lactulose are enzymatically hydrolysed and the fructose is determined enzymatically. This approach is simple and inexpensive but is applicable only to products that have no added source of fructose (Luzzana *et al.*, 2003; Marconi *et al.*, 2004).

The use of capillary gas chromatography has been used to measure lactulose, galactose, epilactose and tagatose in heated milks. Sugars are separated as trimethylsilyl derivatives on a fused silica capillary column coated with, e.g. OV-17 or SPB-17 (Martinez- Castro *et al.*, 1986; Montilla *et al.*, 2005b). Tagatose has been separated from galactose using a similar method where the stationary liquid phase is replaced with AT-1000 (Troyano *et al.*, 1992b).

Perhaps the most straightforward method for the analysis of lactose isomerization products is ion exchange chromatography since no derivatization is required (Verhaar *et al.*, 1979). The international standard method adopted by the International Dairy Federation (1991) for the determination of lactulose in milk samples is based on HPLC separation on an ion exchange column following precipitation of proteins and fat. Lactose, lactulose and galactose may be determined simultaneously by this method using a refractive index detector.

7.7. Methods for Monitoring Maillard Reactions in Milk Products

Many methods are available for evaluating the extent of Maillard reactions in foods (Table 7.1). The objective of such measurements is to determine the nutritional value of the product or the impact of such reactions on the organoleptic quality and functional properties of products (Fayle and Gerrard, 2002; Silvan *et al.*, 2006). Although still of limited value for monitoring the quality of dairy products, the measurement of brown colour, A_{280}, fluorescence and reducing power are crude approaches to monitoring Mail-

Table 7.1. Methods for monitoring the Maillard reaction in foods

Based on chemically reactive lysine
Dye-binding methods (e.g. Acid Orange 12 or Remazol Brilliant Blue R)
Fluorodinitrobenzene (FDNB)
Guanidation
Trinitrobenzene sulphonic acid (TNBS)
Reduction by borohydride
Amino acid analysis, HPLC
Lysine oxidase electrode
Based on biologically available lysine
Rat bioassay (e.g. protein efficiency ratio, net protein utilization)
Microbiological bioassays based
Based on measurement of Amadori products
Direct measurement of Amadori products using HPLC or amino acid analysis following enzymatic hydrolysis
Separation of glycated proteins using capillary electrophoresis or LC–electrospray MS
Measurement of furosine and pyridosine following acid hydrolysis using HPLC, GC or amino acid analysis
Immunoassay of the protein Amadori adduct
Measurement of HMF (colorimetric or HPLC) following its formation from Amadori products by mild acid treatment
Based on the measurement of low molecular weight advanced products of the reaction
Measurement of *N*-ε-carboxymethyl lysine
Determination of hydroxymethylfurfural and/or 2-furaldehyde (HPLC or colorimetric)
Headspace CO_2 measurement
Measurement of volatile products of the reaction
Determination of ε-pyrrole lysine
Miscellaneous methods
Measurement of brown colour (transmission or reflectance spectrophotometry)
A_{280} measurement
Fluorescence measurement
Measurement of reducing power

lard reactions and will not be considered here. In particular, the measurement of advanced products of the Maillard reaction provides limited information about the progress of the reaction because of the existence of induction phases as a result of the formation of intermediates (Fayle and Gerrard, 2002; Nursten, 2005). Similarly, the use of microbiological or rat bioassays has declined as chemical methods have become more refined. Consequently, this review will concentrate on the chemical methods for monitoring the Maillard reaction.

7.7.1. Determination of Available Lysine

Perhaps, the most direct approach to monitoring the Maillard reaction in food systems is to measure the destruction of reactive amino acids. Clearly, because of its abundance and reactivity, lysine is likely to be the most important reactant in milk proteins although the destruction of arginine, tryptophan and histidine may also become significant, depending on the processing and storage conditions. A variety of methods are available for the determination of available lysine in proteins and much experience has been gained on the use of most of the chemical methods. A disadvantage of such an approach is lack of knowledge of the amount of 'blocked' or destroyed lysine obtained by measuring 'available' or reactive lysine. The simplest approach for the estimation of available lysine in proteins is probably the dye-binding methods. A variety of anionic dyes combine at low pH with the basic ε-NH_2, imidazole and guanidino groups of lysine, histidine and arginine residues, respectively, and with the terminal α-NH_2 of proteins. Binding of the dyes, Orange 12 and Remazol Brilliant Blue R, has been reported to correlate well with the fluorodinitrobenzene (FDNB) method for quantifying available ε-NH_2 lysine groups (Hurrell and Carpenter, 1975). However, although the dye-binding methods are very rapid (Hurrell *et al.*, 1979), they suffer from lack of specificity and may also underestimate the damage to lysine due to binding of dyes to some products of the Maillard reaction (e.g. Amadori adducts).

Several methods are available for the chemical estimation of reactive ε-NH_2 groups in proteins, including reaction with trinitrobenzene sulphonic acid (TNBS; Kakade and Liener, 1969), *O*-methylisourea (Mauron and Bujard, 1964), borohydride (Hurrell and Carpenter, 1974), [^{14}C]succinic anhydride (Anderson and Quicke, 1984) and fluorodinitrobenzene (FDNB; Carpenter, 1960; Mottu and Mauron, 1967). Although still used occasionally to measure the destruction of lysine in food systems, the TNBS method is very unsatisfactory for this purpose due to reaction of the reagent with the Amadori adduct (Hurrell and Carpenter, 1974). Of the methods listed above, the FDNB procedure is probably the most satisfactory, although it

is more time-consuming than the TNBS method. In addition, FDNB reacts weakly with the lysine ε-NH_2 Amadori adduct, leading to a slight overestimation of available lysine ($\sim$10%). The original FDNB method has been modified by including a shorter hydrolysis time (4 h), followed by HPLC separation and detection at 254 nm of the *N*-ε-dinitrophenol lysine derivative (Rabasseda *et al.*, 1988). The modified procedure may offer an attractive intermediate-length, intermediate-cost assay for quantifying the extent of Maillard reactions in heat-processed proteins.

Although expensive and time-consuming, acid hydrolysis (in 6 M HCl) and amino acid analysis, followed by reaction with ninhydrin, are still used by some laboratories to determine the nutritional quality of proteins. An advantage of the technique is that amino acid analysis and furosine determination (see below) can be conducted simultaneously. Measurement of lysine in the absence of furosine is of more limited value because of the release of some blocked lysine during acid hydrolysis. Thus, conventional amino acid analysis has long been known to overestimate the amount of unreacted lysine in food products (Moller, 1981). In addition, digestion conditions have a significant effect on the yields of amino acids detected. Rowan *et al.* (1992) suggested that hydrolysis time should, ideally, be optimized for the samples under study because of such variations in amino acid yield.

An addition to the range of methods for the determination of available lysine is the lysine oxidase electrode (Assoumani *et al.*, 1990). However, the method requires acid hydrolysis of samples, which normally releases some of the bound lysine. The authors also reported interference from levulinic acid formed via the degradation of glucose.

7.7.2. Determination of Amadori Products: The Furosine Assay

Acid hydrolysis of the protein-bound or -free Amadori product, ε-*N*-(deoxy-1-D-fructosyl)-L-lysine, results in the release of lysine (50%) and the production of two unique amino acids, furosine (20%) and pyridosine (10%). The Amadori derivative of lactose, ε-*N*-(deoxy-1-D-lactulosyl)-L-lysine, is hydrolysed to 40% lysine and 32% furosine. Over the past decade or so, determination of furosine in acid hydrolysates has been used increasingly to measure the amount of 'blocked' lysine in food proteins, particularly milk proteins. The major disadvantage of the early method for furosine determination was that it required an amino acid analyser. The determination of furosine may now be conducted successfully and reproducibly by GC (Buser and Erbersdobler, 1985) or HPLC (Chiang, 1983; Resmini *et al.*, 1990). Before commercially available furosine standards were available, Resmini *et al.* (1990) described the use of 2-acetylfuran as an external standard in the HPLC separation and detection of furosine at 280 nm.

The study of Hartkopf and Erbersdobler (1994) highlighted the problem of furosine instability during analysis. For example, during ion exchange chromatography, the use of a buffer at pH 6.4 with a temperature programme of 30 min at 60°C followed by 40 min at 80°C, resulted in a 60% decrease in recovery compared with a similar temperature programme at pH 4.7. Temperature alone does not appear to affect recovery but has a dramatic effect at higher pH values. The use of 2-acetylfuran, as proposed by Resmini *et al.* (1990), may also lead to errors; due to differences in the peak areas of equimolar concentrations of furosine and 2-acetylfuran, the use of the latter may overestimate furosine content by as much as 25% (Hartkopf and Erbersdobler, 1994). Hartkopf and Erbersdobler (1994) advocated the use of pure furosine as standard (available from Neosystem, Strasbourg, France) using either HPLC or IEC. Optimum conditions for IEC were reported to be pH 4.0 at a column temperature of 60°C. The advantage of the furosine method over other approaches to monitoring the Maillard reaction in milk systems is that it directly quantifies the concentration of lactose–protein Amadori products. A possible criticism of such an approach is that the furosine method does not detect later products of the Maillard reaction (e.g. 5-hydroxymethyl-2-furaldehyde, melanoidins), a factor that might lead to under-estimation of lysine destruction. However, as outlined above, the stability of the lactose–protein Amadori adduct is such that it would be expected to be the major or only product of Maillard reactions in most milk systems. A serious shortcoming of the early furosine method was lack of sensitivity. Most of the early studies could not detect furosine in conventional UHT or spray-dried milks (Moller *et al.*, 1977a,b; Moller, 1981; Finot *et al.*, 1981). The modified furosine assay used by Erbersdobler *et al.* (1987) gave furosine values between 0.5 and 1.5 mg dl^{-1} in milk samples processed at 135–145°C for 4–16 s. In addition, Erbersdobler *et al.* (1987) observed that UHT milk processed by direct heating contained lower furosine levels (1.5–2.5 mg dl^{-1}) than milk processed by indirect heating (2.6–5.3 mg dl^{-1}), which compares with the trend reported for the production of lactulose in UHT milks. A modification of the furosine procedure involves the enzymatic digestion and dialysis of milk proteins prior to acid hydrolysis and furosine determination, to distinguish between enzymatically available and chemically available lysine (Desrosiers *et al.*, 1989). The authors reported that, of the original lysine present in whey protein concentrate at an a_w of 0.97 heated at 121°C for 5000 s, 93% was chemically available, whereas only 76% was available enzymatically. Differences probably arose due to heat-induced conformational changes and crosslinking, which would have limited the enzymatic digestion without necessarily destroying lysine.

7.7.3. Determination of Amadori Products: Direct Determination of Lactulosyl Lysine

An alternative approach is the direct measurement of Amadori adducts of proteins, for example, the determination of lactulosyl lysine in dairy products. Antibodies have been raised against lactulosyl derivatives of β-lactoglobulin (Matsuda *et al.*, 1985a) and ovalbumin (Matsuda *et al.*, 1985b, 1986). A more promising approach is the determination of lactulosyl lysine in milk products by LC-MS following complete enzymatic hydrolysis. The availability of a standard makes this approach more attractive as a method of detecting low levels of Amadori products in dairy products (Vinale *et al.*, 1999). However, the limitation of this technique is the time-consuming enzymatic hydrolysis step (Henle *et al.*, 1991). In view of the success of the many HPLC approaches for the separation and determination of amino acid–sugar Amadori products in model systems and foods (Reutter and Eichner, 1989; Moll and Gross, 1981), the application of a similar approach to enzymatically hydrolysed proteins offers promise and merits further investigation. In addition, the replacement of the traditional amino acid analysis approach by HPLC, as employed by Henle *et al.* (1991), would greatly reduce the cost of the analysis.

7.7.4. Determination of 5-hydroxymethyl-2-furaldehyde

Determination of HMF, as used in the original method of Keeney and Bassette (1959), is one of the classic methods for monitoring the Maillard reaction in dairy products. It could be classified as an indirect method for the determination of Amadori adducts plus intermediates of the 1,2-E pathway, including preformed HMF. The determination of HMF has long been used as an indicator of temperature abuse of fruit juices, especially citrus juices. Because of the acidic nature of such products, the 1,2-E reaction pathway would be expected to predominate with the result that HMF levels would be a valid and logical indicator of the extent of the Maillard reaction. Both HMF and 2-furaldehyde react with 2-thiobarbituric acid (TBA) under acidic conditions to produce chromophores with absorption maxima at 436 and 414 nm, respectively. Several analytical procedures are available for HMF and 2-furaldehyde in fruit juices, based on the TBA reaction, HPLC methods and combinations of both (Mijares *et al.*, 1986; Poretta and Sandei, 1991; Tu *et al.*, 1992). In the case of milk products, the 1,2-E pathway would not be as predominant as in fruit juices due to the higher pH of the former. Thus, preformed HMF would not be expected to represent as reliable an indicator of the heat treatment of milk products as it is in fruit juices. Keeney and Bassette (1959) attempted to overcome the inherent shortcomings of the

method by including a pre-treatment involving heating samples in 0.3 N oxalic acid at 100°C for 1 h to convert Amadori products and 1,2-E intermediates to HMF. Although the measurement of HMF in dairy products has been criticized on the grounds of lack of specificity (Burton, 1984), it is a cheap and relatively simple method for monitoring Maillard reactions and is still widely in use (De Block *et al.*, 2003). HPLC methods are now available for the determination of free and 'bound' HMF in dairy products exploiting the strong absorption of HMF at 280 nm (van Boekel and Rehman, 1987; Morales *et al.*, 1997).

7.7.5. Fluorescence Spectroscopy

The generation of fluorescent products in Maillard reactions is well described and fluorescence spectroscopy has been used to monitor the development of Maillard reactions in dairy products (Morales *et al.*, 1996; Leclère and Birlouez-Aragon, 2001; Bosch *et al.*, 2007). However, the limitation of classical methods is that they cannot be applied to turbid systems such as dairy products without a sample preparation step. More recently, the application of front-face (surface) fluorescence spectroscopy to dairy products offers potential as a rapid non-destructive means of assessing product quality (Kulmyrzaev and Dufour, 2002; Birlouez-Aragon *et al.*, 2004). The coupling of chemometric methods with fluorescence spectroscopy has been reported to determine accurately the levels of both lactulose and furosine in milk samples (Kulmyrzaev and Dufour, 2002).

7.7.6. ε-Pyrrole Lysine

The advanced Maillard reaction product, ε-pyrrole lysine, has been proposed as a useful indicator of reactions in stored foods because Amadori products tend to decompose over time to other products, limiting their usefulness as indicators of Maillard reactions (Chiang, 1988). Chiang (1988) found that ε-pyrrole lysine was readily detectable in skim milk powder heated at 80°C for 1 or 2 h (6.25 or 20.32 mg kg^{-1}, respectively).

7.7.7. Carboxymethyl Lysine

An alternative to the furosine assay is the measurement of *N*-ε-carboxymethyl lysine (CML; Figure 7.6) formed by oxidation of the protein-bound Amadori product (Erbersdobler and Dehn-Müller, 1989; Badoud *et al.*, 1990; Lüdemann and Erbersdobler, 1990). In the case of milk products, the method involves the oxidation of lactulosyl lysine using periodic acid, followed by acid hydrolysis. The method is reported to have the

advantage that the same oxidation product is formed regardless of the nature of the sugar moiety of the Amadori product. In addition, the method can be extended to the oxidation products of the Amadori products of several other amino acids in addition to lysine; fluorescent detection of carboxymethyl amino acids following HPLC separation affords high sensitivity (Badoud *et al.*, 1990). CML is also formed from fructosyl lysine and lactulosyl lysine in food systems by the action of oxygen on the Amadori product. Its formation is promoted at low a_w values, at high pH and by replacing glucose by maltose or lactose in the food system (Lüdemann and Erbersdobler, 1990). Lüdemann and Erbersdobler (1990) proposed that since CML is more heat resistant than fructosyl lysine, its formation in foods could be a useful indicator of advanced heat damage.

7.7.8. Pyrraline

The determination of the pyrrolealdehyde crosslink, pyrraline, may be a useful marker of heat treatment in that it is independent of the degradation of lactulosyl lysine and increases linearly with heating time of milk powder samples (Henle *et al.*, 1994). However, a dramatic decrease in pyrraline is observed on prolonged heating (100–110°C for >6 h). Levels of pyrraline in food samples varied dramatically. Whereas levels in raw, pasteurized and UHT milks did not exceed 2 mg kg^{-1} protein, levels in skim milk powder and whey powder were as high as 1150 and 3150 mg kg^{-1} protein, respectively. Concentrations in sterilized (<2–260 mg kg^{-1} protein) and evaporated (110–130 mg kg^{-1} protein) milks appeared to be intermediate. In addition to pyrraline, the authors also identified low levels of maltosine (~30-fold less than pyrraline) in samples of severely heated milk powder. It is assumed that maltosine is formed via the 2,3-E pathway while pyrraline is a product of the 1,2-E pathway.

7.7.9. Immunoassays

The use of immunoassays to detect advanced Maillard products has been the subject of several studies. However, a possible disadvantage of most of these assays is that the antigens have not been subjected to full chemical characterization. Kato *et al.* (1994) used monoclonal antibodies for a lactose–protein Maillard reaction product to characterize a number of milk samples. Skim milk powders showed reactivity to the antibody in all milk protein fractions except the κ-casein fraction. Lactoferrin, serum albumin and the immunoglobulins were only weakly reactive in the assay whereas α_{s1}-casein, β-casein, β-lactoglobulin and α-lactalbumin were highly reactive. Packaging of the skim milk powder in N_2 appeared to reduce the formation of the

antigen, suggesting the involvement of an oxidation reaction. Furthermore, the antigen may have been lactose specific as a lactose-free, glucose-containing milk powder was non-reactive.

7.8. Consequences of Undesirable Maillard Reactions in Milk Systems

7.8.1. Nutritional Implications

Maillard reactions may compromise the nutritional value of foods through the destruction of essential amino acids and by limiting the bioavailability of amino acids and other essential nutrients. Clearly, in dairy products, lysine is the most vulnerable amino acid to the Maillard reaction because of its abundance and the reactivity of the ε-NH_2 group. While the free Amadori products, lactulosyl lysine and fructosyl lysine, are absorbed from the intestine, they appear to be excreted rapidly, largely unchanged, in the urine and do not accumulate in the body (Hultsch *et al.*, 2006; Schwenger *et al.*, 2005).

In the developed world, however, the destruction of even 10–20% of lysine in dairy products does not assume great nutritional significance because of the excess of lysine in milk proteins and because of the high intake of proteins by most individuals. In view of the fact that tryptophan, arginine and histidine are not as abundant in milk proteins as lysine, their possible reactivity in Maillard reactions is of nutritional interest. In particular, it has been reported that tryptophan may be even less stable than lysine under some conditions (Leahy and Warthesen, 1983). Dworschak and Hegedüs (1974) reported the loss of 97% of the tryptophan in a milk powder containing 5.7% moisture that was heated at 100°C for 4 h. This conflicts with the results of Nielsen *et al.* (1985b,c), who could not detect a decrease in tryptophan (by chemical analysis or rat bioassays) in a browned milk powder. Nielsen *et al.* (1985b,c) suggested that such differences among studies may be attributable to the different analytical methods used. A study of the nutritional consequences of storage of a dried whey protein concentrate (35% lactose; 52% protein) reported a lysine loss of 23% after 3 months at 40°C and an a_w of 0.41, whereas tryptophan and methionine levels were not significantly affected (Lindemann-Schneider and Fennema, 1989). Nitrogen packing appeared not to affect lysine loss. Based on plasma amino acid levels in adult dogs after a test meal, Longenecker and Hause (1959) reported that arginine was the first limiting amino acid in casein.

Processes such as HTST pasteurization, UHT treatment and spray drying of milk usually result in only slight losses of available lysine. Drying of infant formulae and whey, however, usually results in higher losses due to

the higher concentration of lactose. Roller-dried dairy products are invariably inferior to spray-dried products (see El-Shafei *et al.*, 1988). Roller drying has been reported to lead to available lysine losses of 10–75%, depending on the process (Mauron, 1981; Finot *et al.*, 1981). Lysine losses in fresh UHT-treated milks are generally accepted to be comparable or slightly higher than those in conventionally pasteurized samples. Park and Hong (1991) reported lysine losses of 6.3 and 4.9% for UHT-pasteurized (130°C, 2–3 s; 135°C, 2 s) and UHT-sterilized (135°C, 2 s; 140°C, 2–3 s) milks, respectively, using the TNBS method. Such values are higher than those reported previously. In addition, since the TNBS method has been criticized as underestimating lysine losses, it is possible that the actual losses in the samples of Park and Hong (1991) may be higher. However, ideally, any future conclusions as to the effect of thermal processes on lysine availability should be based on more reliable direct methods of measuring lysine destruction, such as amino acid analysis in combination with the furosine assay. In UHT milks stored at 30–37°C for 6–36 months, 10–30% of lysine residues were blocked as lactulosyl lysine (Moller *et al.*, 1977b).

In contrast to freshly processed UHT milk, stored UHT milk may exhibit relatively high losses of available lysine. Moller (1981) reported lysine losses of 10–30% in UHT milk samples that had been stored for 14 months at 4–30°C. Freeze drying is the most acceptable process for the drying of milk from a nutritional point of view although in practical terms there is a negligible difference between most spray drying processes and freeze drying. Lactose-hydrolysed formulae have the greatest propensity to lysine loss because of the increased number of reactive sugar moieties and because glucose and galactose are individually more reactive than lactose. Burvall *et al.* (1978) reported available lysine losses of 40% for a spray-dried lactose-hydrolysed milk after 6 months storage. Thermal processing of milk in the presence of added sucrose to produce *doce de leite* has been reported to cause significant decreases in lysine (33%), arginine (11%) and histidine (10%) levels (Pavlovic *et al.*, 1994). The available lysine levels, as measured by the FDNB method, were reduced by 50%; the discrepancy between the FDNB method for measuring lysine availability and the results of amino acid analysis highlights the need for extreme care in estimating amino acid bioavailability from heated proteins.

Hypoallergenic infant formulae prepared from protein hydrolysates are more vulnerable to the nutritional consequences of Maillard reactions due to the increased availability of free amino groups for reaction (Finot, 2005). This results in the loss of essential amino acids other than lysine, such as alanine, valine, leucine and isoleucine (Penndorf *et al.*, 2007). Penndorf *et al.* (2007) reported losses of N-terminal amino acids of up to 8.4% in such hydroylsed products which is similar to the losses reported for lysine in conventional

infant formulae. Added iron and ascorbic acid appear to have a particularly destructive effect on tryptophan in dairy systems. It is hypothesized that the main pathway for tryptophan destruction under mild heating conditions is not direct glycation of the indole N but a consequence of oxidation due either to the activity of the Fe-ascorbate system or the pro-oxidant activity of Maillard reaction products following ascorbylation of lysine ε-NH_2 groups (Puscasu and Birlouez-Aragon, 2002; Leclère *et al.*, 2002). The presence of oxygen is particularly detrimental under such conditions (Gliguem and Birlouez-Aragon, 2005). The high levels of blocked lysine in some fortified milks clearly limit their usefulness as dietary supplements (Evangelisti *et al.*, 1999; Rada-Mendoza *et al.*, 2005).

Similarly, the higher extent of the Maillard reaction in liquid-sterilized infant formulae, compared with powdered and UHT counterparts, is well described (Guerra-Hernandez *et al.*, 2002; Puig *et al.*, 2003). The considerably higher lysine losses and furosine levels in follow-on formulae may be related to the processing method but are also related likely to the impact of added nutrients, such as maltodextrin or iron, on the Maillard reaction (Ferrer *et al.*, 2003; Pereyra Gonzales *et al.*, 2003).

Maillard reactions may limit the bioavailability of undamaged amino acids by inhibiting digestive enzymes and/or by inhibiting amino acid transport at the intestinal level. In addition, there is evidence that severely browned proteins are less digestible than native proteins (Dalsgaard *et al.*, 2007). Gothwal and Bhavadasan (1991) reported that although slight browning of milk was accompanied by an increase in the rate of trypsin proteolysis, severely browned milk samples are less readily hydrolysed by trypsin. A stored dried casein–glucose mixture showed a loss of digestibility of 14–88%, depending on conditions (Culver and Swaisgood, 1989). The available chemical evidence on the effect of Maillard reactions on the nutritional value of food proteins is supported by clinical evidence that diets rich in Maillard reaction products have a negative impact on protein digestibility (Seiquer *et al.*, 2006). Moreover, protein-bound lactose is resistant to hydrolysis by β-galactosidase (Morgan *et al.*, 1999a)

Maillard reactions may also contribute to the destruction of vitamins in food systems. Vitamin C can participate directly in the reaction and it has been proposed that vitamins B1, B6, B12 and pantothenic acid may react with pre-melanoidins (Hurrell, 1990). Although vitamin B1 contains an amino group and, therefore, could participate in Maillard reactions, it is unclear to what extent this mechanism contributes to its destruction in food systems. Folic acid is now added in the free form to many manufactured foods and through its free amino group is believed to participate in Maillard reactions.

There is evidence that dietary Maillard reaction products may disrupt mineral homoeostasis in vivo, reducing the bioavailability of some minerals.

Maillard reaction products have been shown to increase the intestinal absorption and urinary excretion of calcium and magnesium (O'Brien and Morrissey, 1989), which resemble the action of poorly digestible carbohydrates, such as lactose and polyols. However, while calcium absorption has been shown to be higher in a bottle-sterilized liquid infant formula, calcium retention was significantly lower in a study on suckling rats (Sarriá *et al.*, 2001). Casein subjected to a Maillard reaction with reducing sugars impairs the uptake of zinc by Caco-2 cells in culture, suggesting a reduction in bioavailability compared with untreated casein (Seiquer *et al.*, 2000). By contrast, Sarriá and Vaquero (2006) reported higher percentage copper absorption and erythrocyte copper concentrations despite lower food intake and body weight in in-bottle-sterilized formula compared with a reconstituted powder formula.

Dietary Maillard reaction products of a glucose–glutamate system have been reported to reduce zinc retention in rats as a result of increases in both faecal and urinary zinc losses (O'Brien and Morrissey, 1989). Furniss *et al.* (1989) reported a large increase in urinary zinc in rats fed a heated casein–glucose mixture. Urinary zinc was also increased in animals fed a reacted casein–lactose mixture, although the effect was not as pronounced as for the casein–glucose system. However, there were no changes in faecal zinc or in zinc retention and the authors concluded that an increased loss of urinary zinc would have little significance for individuals consuming a diet adequate in zinc.

7.8.2. Milk Protein Allergy

There is an increasing volume of scientific literature on the influence of processing, in particular thermal processing, on food protein allergy (Davis *et al.*, 2001). This research has been driven by increased awareness and better risk management of food allergens. However, an additional consideration has been to ensure that novel foods and processes do not increase allergenic risk for sensitized consumers. It was long known that the allergenicity of β-lactoglobulin could be enhanced by the Maillard reaction. Matsuda *et al.* (1985b) found higher antibody titres in mice immunized with a β-lactoglobulin–lactose adduct than animals injected with native β-lactoglobulin. The increased allergenicity of lactosylated β-lactoglobulin was demonstrated by Bleumink and Berrens (1996). Matsuda *et al.* (1990) reported that the lactose-ovalbumin adduct induced a stronger antibody response when injected into mice than the adducts of other sugars (glucose, galactose, melibiose, maltose or cellobiose). The authors suggested that the immunodominancy of the lactose adduct was due to some feature of its stereochemical configuration. The effect of Maillard reactions on the epitopes of native proteins was

illustrated by the work of Öste *et al.* (1990) who showed a decrease in the antigenicity of Kunitz soybean trypsin inhibitor which had been heated with glucose, lactose or maltose.

Recent research on the allergenicity of the major peanut allergens has corroborated the above findings and has suggested a mechanism of action that involved both increased IgE binding and a fourfold increase in trypsin inhibitory activity (Maleki and Hurlburt, 2004), which would potentially allow intact antigens to reach the systemic circulation. As Maillard reactions have the potential to produce trypsin inhibitory compounds and to modify the structure of food proteins, this area is in need of further research. Meanwhile, the effect of processing on a number of allergens has been studied (Berrens, 1996; Wolff *et al.*, 2004).

Research on the lactosylation of milk proteins during heating or storage may help define the structural changes that influence milk protein allergenicity. Electrospray ionization MS and capillary electrophoresis have been used to study the non-enzymatic glycosylation of milk proteins (Leonil *et al.*, 1997; Siciliano *et al.*, 2000; French *et al.*, 2002). Jones *et al.* (1998) demonstrated that capillary electrophoresis can be used to monitor several glycated forms of β-lactoglobulin in skimmed milk powder. Guyomarc'h *et al.* (2000) were able to show several new peaks for β-lactoglobulin, α-lactoglobulin, α_{s1}-casein, β-casein and κ-casein, in capillary electropherograms. Additional analysis by mass spectrometry suggested the increases in mass for the whey protein peaks were approximately multiples of 320 Da consistent with incremental lactosylation reactions. Other studies have demonstrated that lactosylation of β-lactoglobulin can proceed under relatively mild conditions in the absence of browning is site specific (appearing to prefer Lys_{47}), and results in conformational changes and ultimately aggregation via non-covalent interactions (Leonil *et al.*, 1997; Morgan *et al.*, 1999b; Chevalier *et al.*, 2002; French *et al.*, 2002; Czerwenka *et al.*, 2006).

7.8.3. Changes in Functional Properties

In both food systems and biological systems in vivo, there is evidence that Maillard reactions may result in substantial changes in the functional properties of proteins. Such changes include reduced enzyme activity, altered receptor binding (Amaya *et al.*, 1976), altered drug binding to plasma proteins (Tsuchiya *et al.*, 1984) and changes in protein stability (Kato *et al.*, 1983). Kato *et al.* (1981b,c) reported that Maillard reaction products of ovalbumin and glucose were more soluble and heat stable than the native protein. Electrophoretic studies confirmed that such protein–glucose adducts had fewer positive charges. Circular dichroism spectroscopy revealed a more ordered structure in the reacted protein than expected: even though 62% of

the ε-amino groups were blocked, the helix content was 27% compared with 33% in the native protein. The authors suggested that the structure of the reacted protein may be stabilized by intrachain non-covalent interactions between the protein and bound sugar moieties. The ratio of arginine to lysine has been reported to be directly related to the stability of proteins (Kinsella *et al.*, 1989). Since lysine is generally reported to react more readily than arginine (Kaanane and Labuza, 1989) and since there are many more lysyl than argininyl residues, it might be expected that the Maillard reaction would result in increases in the effective arginine: lysine ratio in food proteins, possibly resulting in an increase in protein stability. The selective chemical modification of ε-NH_2 groups has been reported to change the activity, pH optimum and heat stability of several enzymes (Smith *et al.*, 1991; Smith and Yada, 1991). The Maillard reaction of glucose-6-phosphate with β-lactoglobulin has been used as a means of attaching more than 1% phosphorus to the protein (Aoki *et al.*, 1994). The reaction was reported to result in a significant increase in the heat stability and emulsification capacity of the protein. Similar results were reported by Chevalier *et al.* (2001) who used Maillard reactions with several monsaccharides to modify the solubility, heat stability, emulsifying capacity and foaming properties of β-lactoglobulin. The well-described increase in heat stability of milk following preheating may be related to the effect of the Maillard reaction. Such an increase in heat stability might be a consequence of the lowering of isoelectric point which follows Maillard reactions of proteins.

Conjugation of ovalbumin, lysozyme and α-lactalbumin with galactomannan in Maillard reactions resulted in dramatic increases in the emulsifying capacity and emulsion stability of the proteins (Nakamura *et al.*, 1992, 1994). Experiments involving the acetylation of free amino groups of lysozyme showed that the positive charge on the protein (contributing to electrostatic repulsion) is necessary for its emulsifying properties. On the basis of electrophoretic studies and free amino group measurement, the authors suggested that $\sim$3 and $\sim$2 mol of galactomannan bound to α-lactalbumin and lysozyme, respectively (Nakamura *et al.*, 1994). The emulsifying capacity of β-casein was reported to be increased following a Maillard reaction with dextran (Mu *et al.*, 2006).

Studies on the globular proteins, bovine serum albumin, pea protein and soy protein isolate have shown that Maillard reactions increase the gel strength (possibly related to an increased level of crosslinking) on heating and reduce the threshold level of protein required for gelation (Armstrong *et al.*, 1994; Cabodevila *et al.*, 1994). Experiments on glucono-δ-lactone-induced gels showed that the properties of gels formed from proteins subjected to the Maillard reaction could not be explained simply by the pH decrease that accompanies browning.

The presence of lactose during the heating of whole casein has been shown to reduce calcium binding, presumably due to reduced accessibility of

binding sites due to Maillard reactions (Pappas and Rothwell, 1991). The functional consequences, if any, of such a reduction in calcium binding have not been investigated. In view of the reactivity of κ-casein in Maillard reactions, it is possible that such reactions may reduce the stability of casein micelles such as occurs during storage and age gelation of UHT milk. Available evidence suggests that Maillard browning reactions do not contribute to age gelation (Venkatachalam *et al.*, 1993). However, the development of Maillard crosslinks has not yet been examined in UHT milk and, therefore, the possible role of Maillard reactions in age gelation cannot be discounted.

7.8.4. Formation of Toxic Compounds

7.8.4.1. Furan

The formation of furanic derivatives in Maillard reactions is well described and the toxicology of furfural and HMF in particular is well described (see Section 7.3.4). In early 2004, the US Food and Drug Administration (FDA) expressed concern at the presence of furan, a possible human carcinogen, in a number of heat-treated foods (FDA, 2007). The presence of furan and hundreds of furanic molecules in foods has been recognized for at least 30 years. Most of these molecules derive from Maillard reactions (Maga, 1979; Crews and Castle, 2007).

The level of furan found in heated dairy products is relatively low (generally <20 μg kg^{-1} in infant formulas and evaporated milks compared with products such as some soups and canned foods where levels >200 μg kg^{-1} have been reported) (Märk *et al.*, 2006; FDA, 2007).

Maillard products such as furfural and furoic acid have been proposed as furan precursors in heated foods (Crews and Castle, 2007). Systems containing ascorbic acid yield the highest levels of furan, especially in the presence of oxygen (Perez Locas and Yayalayan, 2004; Hasnip *et al.*, 2006). Removal of oxygen or addition of a reducing agent, such as sulphite, results in lower furan levels (Märk *et al.*, 2006). Märk *et al.* (2006) also noted that more complex systems resulted in less furan formation, presumably due to competing reaction pathways; the intermediates 2-deoxyaldotetrose and 4-hydroxy-2-butenal were proposed to yield furan from ascorbic acid/carbohydrate pathways or polyunsaturated fatty acid oxidation, respectively.

7.8.4.2. Acrylamide and Other Vinylogous Maillard Reaction Products

The discovery, in 2002, of high levels of the carcinogen, acrylamide, in some thermally processed plant foods revealed a hitherto unknown Maillard pathway with significant food safety implications (Friedman and Mottram, 2004; Skog and

Alexander, 2006). The most important pathway for acrylamide formation appears to be the decarboxylation of the Schiff's base of asparagine and a carbonyl reactant. Thus, the level of acrylamide in the finished product correlates highly with the level of asparagine, the sugar level and the severity of heat treatment. The levels of acrylamide reported in dairy products are low relative to other foods ($<$10–100 μg hg^{-1}) (Petersen and Tran, 2005). Although this pathway is of little significance for dairy products, the chemistry is of interest as it gives rise to the possibility that vinylogous reaction products may be formed from the decarboxylated Amadori compounds of other amino acids (e.g. styrene from phenylalanine) (Blank *et al.*, 2004; Stadler *et al.*, 2004; Blank, 2005).

7.8.4.3. Other Toxic Compounds

It is now well accepted that the Maillard reaction plays an important role in the formation of the aminoimidazoazarene (AIA) group of food mutagens in muscle foods. Such mutagens are products of the reaction of the Maillard reaction products 2-methylpyridine or 2,5-dimethylpyrazine with creatinine and glycine or alanine (Lee and Shibamoto, 2002). The presence of such mutagens has not been reported in dairy products. Although mutagenic activity has been reported in extracts of severely heated dairy products (Rogers and Shibamoto, 1982; Yen and Lee, 1986), dairy products are not unique in this respect since mutagenic activity develops in a host of severely heat-treated foods (Kato, 1986; O'Brien and Morrissey, 1989). In pure form, HMF and its metabolite 5-sulphooxymethylfurfural have genotoxic properties (Lee and Shibamoto, 2002), but there is no evidence that the levels of HMF found in dairy products pose a health risk. In fact the levels of HMF in dairy products is very low compared with other food categories (Glatt and Sommer, 2006). There is no evidence that dairy products subjected to conventional heat treatments, such as HTST pasteurization, UHT treatment or in-bottle sterilization, are mutagenic (Sekizawa and Shibamoto, 1986; Berg *et al.*, 1990). In fact, there is evidence that mutagen binding to casein may reduce the mutagenicity of food systems (Berg *et al.*, 1990).

7.9. The Future

The discovery of high levels of acrylamide in heated plant-derived food systems in 2002 revealed that there is much yet to be discovered about Maillard reactions in foods. Consequently, Maillard reaction chemistry remains an active area of research aimed at minimizing the detrimental effects of such reactions while enabling preservation of unstable food systems and delivering the organoleptic benefits of thermally processed foods. The

development of functional foods with demonstrated health benefits is an active area of food product development, especially in the dairy industry. Many such products contain unstable active ingredients that may participate in Maillard reactions (e.g. bioactive peptides, vitamins, oligosaccharides, botanical extracts) with consequences for product efficacy (Roscic and Horvat, 2006). There is, therefore, a strong stimulus for continuing research into better understanding and controlling Maillard reactions in dairy products.

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8

Milk Oligosaccharides

T. Urashima, M. Kitaoka, S. Asakuma, and M. Messer

8.1. Introduction

Mammalian milk contains up to 10% carbohydrate, of which the disaccharide, lactose (Gal(β1-4)Glc), is usually a prominent component. Milk and colostrum also contain lesser amounts of other saccharides, referred to as milk oligosaccharides, nearly all of which have a lactose unit at their reducing end to which GlcNAc, Gal, Fuc and/or Neu5Ac or Neu5Gc residues can be attached (Jenness *et al*., 1964; Newburg and Neubauer, 1995; Boehm and Stahl, 2003; Urashima *et al*., 2001; Messer and Urashima, 2002). Pronounced heterogeneity as well as homology of milk oligosaccharide structures among different mammalian species has been documented (Urashima *et al*., 2001; Messer and Urashima, 2002).

It is well known that free lactose is a significant energy source for human infants, but the exact biological significance of the milk oligosaccharides remains to be clarified. The study of milk oligosaccharides was greatly stimulated by the discovery of a *Bifidobacterium* growth factor in human milk and colostrum, but there is still no unequivocal answer to the question of which oligosaccharides in human milk are responsible for the bifidus factor, even though it is acknowledged that the oligosaccharide fraction is responsible for promoting the growth of bifidobacteria within the infant colon.

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Another critical problem to be solved has been the metabolic fate of human milk oligosaccharides. Recently, it has been concluded that most of these oligosaccharides are neither digested nor absorbed within the infant small intestine (Engfer *et al.*, 2000) and that instead, they can act as prebiotics and soluble receptor analogues, inhibiting the attachment of pathogenic bacteria or viruses to receptors in the infant colon, as discussed below. There is evidence, however, that a minor proportion of human milk oligosaccharides can be absorbed intact (Gnoth *et al.*, 2001), acting as immunomodulators within the circulation, and that the sialic acid of sialyl milk oligosaccharides can be used as a precursor for the formation of brain gangliosides or sialoglycoproteins.

The problem of the *Bifidobacterium* growth factor is at present still open to speculation. The genome libraries of a few species of bifidobacteria that have become publicly available enable one to focus on a series of enzymes involved in the metabolism of carbohydrates by these bacteria. From this theoretical approach, using the DNA database, one can speculate on which oligosaccharide structure might be used for the metabolism of each strain of *Bifidobacterium*. Kitaoka *et al.* (2005) have recently hypothesized that lacto-*N*-biose I (Gal(β1-3)GlcNAc) is the genuine, i.e., specific, bifidus factor (see below).

In this chapter, we describe the structures of human and bovine milk oligosaccharides as well as those of some other species, discuss their biosynthesis, intestinal digestion/absorption and significance as prebiotics, soluble receptor analogues and immunomodulators, and describe the present and likely future industrial utilization of milk oligosaccharide-like materials.

8.2. The Chemical Structures of Human Milk Oligosaccharides: Analytical Methods

The structures of at least 93 oligosaccharides of human milk have been determined to date (Table 8.1), while mass spectra (MS) data have suggested the presence of almost 130 oligosaccharides in human milk or colostrum (Newburg and Neubauer, 1995). Moreover, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) analyses suggest that polysaccharides, consisting of more than 50 monosaccharide residues, as indicated by size exclusion chromatography, are also present in human milk. Therefore, considerably more than 130 different saccharides are probably present in human milk (Boehm and Stahl, 2003). Recently, as many as 200 different human milk oligosaccharides have been separated and studied by microfluidic HPLC – chip MS (Ninonuevo *et al.*, 2006).

It has been shown that human milk and colostrum are rich in oligosaccharides, in concentration as well as in variety, compared with the milk/colostrum of the cow and of many other species (Gopal and Gill, 2000). As

Table 8.1 The structures of human milk oligosaccharides

	Abbreviation	Oligosaccharide	Reference
1. Lactose series			
1	2'-FL	Fuc(α 1-2)Gal(β1-4)Glc	Kuhn *et al.* (1956b)
2	3-FL	Gal(β1-4)Glc | Fuc(α1-3)	Montreuil (1956)
3	DF-L	Fuc(α1-2)Gal(β1-4)Glc | Fuc(α1-3)	Kuhn and Gauhe (1958b)
4	LN triose II	GlcNAc(β 1-3)Gal(β 1-4)Glc	Dabrowski *et al.* (1983)
5	3'-GL	Gal(β 1-3)Gal(β 1-4)Glc	Donald and Feeney (1958)
6	4'-GL	Gal(β 1-4)Gal(β 1-4)Glc	Sugawara and ldota (1995)
7	6'-GL	Gal(β 1-6)Gal(β 1-4)Glc	Yamashita and Kobata (1974)
8	3'-SL	Neu5Ac(α 2-3)Gal(β 1-4)Glc	Kuhn and Brossmer (1959)
9	F-SL	Neu5Ac(α2-3)Gal(β1-4)Glc | Fuc(α1-3)	Gronberg *et al.* (1989)
2. Lacto-*N*-tetraose series			
1	LNT	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn and Baer (1956a)
2	LNFP I	Fuc(α 1-2)Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn *et al.* (1956c)
3	LNFP II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)	Kuhn *et al.* (1958a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
4	LNFP V	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-3)	Ginsberg *et al.* (1976)
5	LNDFH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-4)	Kuhn and Gauhe (1958b)
6	LNDFH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc \| \| Fuc(α1-4) Fuc(α1-3)	Kuhn and Gauhe (1960)
7	LSTa	Neu5Ac(α 2-3)Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn and Gauhe (1962)
8	LSTb	Neu5Ac(α2-6) \| Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Kuhn and Gauhe (1962)
9	F-LSTa	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-4)	Wieruszeski *et al.* (1985)
10	F-LSTb	Neu5Ac(α2-6) \| Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Wieruszeski *et al.* (1985)
11	DS-LNT	Neu5Ac(α2-6) \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Grimmonorez and Montreuil (1968)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
12	FDS-LNT I	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)	Kitagawa *et al.* (1991b)
13	FDS-LNT II	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)	Gronberg *et al.* (1990)
3. Lacto-*N*-neotetraose series			
1	LNnT	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn and Gauhe (1962)
2	LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)	Kobata and Ginsburg (1969)
3	LSTc	Neu5Ac(α 2-6)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc	Kuhn and Gauhe (1962)
4	F-LSTc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)	Smith *et al.* (1987)
4. Lacto-*N*-hexaose series			
1	LNH	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)	Kobata and Ginsburg (1972a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
2	F-LNH I	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)	Yamashita *et al.* (1977a)
3	F-LNH II	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)	Dua *et al.* (1985)
4	DF-LNH	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3) | Fuc(α1-4)	Dua *et al.* (1985)
5	DF-LNHa	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)	Yamashita *et al.* (1977a)

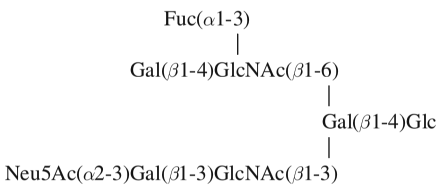
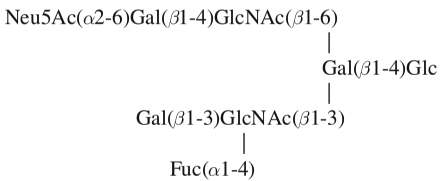
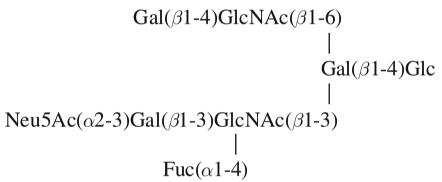
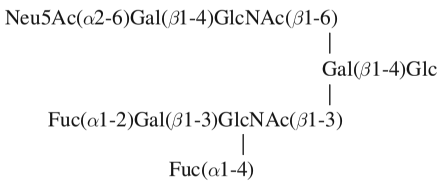
(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
6	TF-LNH	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3) | Fuc(α1-4)	Sabharwal *et al.* (1988a)
7	S-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)	Kobata and Ginsburg (1972a)
8	FS-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)	Yamashita *et al.* (1977a)
9	FS-LNH I	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Gronberg *et al.* (1992)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
10	FS-LNH II		Gronberg *et al.* (1992)
11	FS-LNH III		Gronberg *et al.* (1992)
12	FS-LNH IV		Kitagawa *et al.* (1989)
13	DFS-LNH I		Gronberg *et al.* (1992)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
14	DFS-LNH II	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Fuc(α1-4)	Kitagawa *et al.* (1989)
15	DS-LNH I	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)	Kitagawa *et al.* (1991b)
16	DS-LNH II	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Kitagawa *et al.* (1991b)
17	FDS-LNH I	Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Yamashita *et al.* (1976a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
18	FDS-LNH II	Fuc(α1-3) | Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Yamashita *et al.* (1976a)
19	FDS-LNH III	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Fuc(α1-4)	Kitagawa *et al.* (1989)
20	TS-LNH	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) | Neu5Ac(α2-6)	Fievre *et al.* (1991)
5. Lacto-*N*-neohexaose series			
1	LNnH	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-4)GlcNAc(β1-3)	Kobata and Ginsburg (1972b)

(*Continued*)

Table 8.1 (*Continued*)

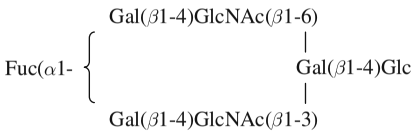
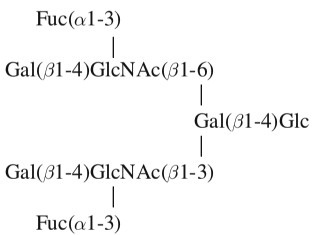
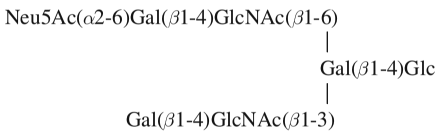
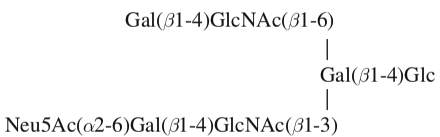
	Abbreviation	Oligosaccharide	Reference
2	F-LNnH		Kobata and Ginsburg (1972b)
3	DF-LNnH		Haeuw-Fievre *et al.* (1993)
4	S-LNnH I		Kobata and Ginsburg (1972b)
5	S-LNnH II		Tarrago et al. (1988)

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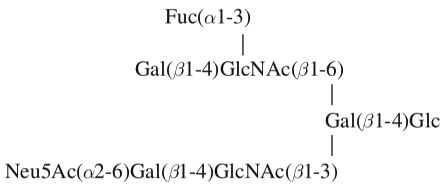
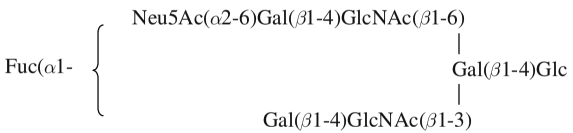
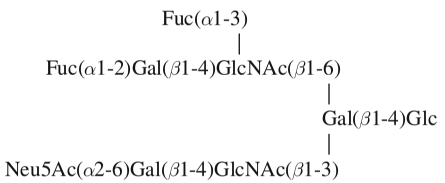
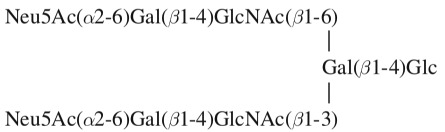
Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
6	FS-LNnH I		Gronberg *et al.* (1989)
7	FS-LNnH II		Kobata and Ginsburg (1972b)
8	DFS-LNnH		Gronberg *et al.* (1992)
9	DS-LNnH		Gronberg *et al.* (1992)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
10	FDS-LNnH	Neu5Ac(α2-3/6)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3/6)Gal(β1-4)GlcNAc(β1-3) \| Fuc(α1-3)	Yamashita *et al.*(1976a)
6. *para*-Lacto-*N*-hexaose series			
1	F-paraLNH I	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-3)	Sabharwal *et al.* (1988b)
2	F-paraLNH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-4)	Bruntz *et al.* (1988)
3	DF-paraLNH	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| \| Fuc(α1-4) Fuc(α1-3)	Yamashita *et al.* (1977b)
4	TF-paraLNH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| \| Fuc(α1-4) Fuc(α1-3)	Strecker *et al.* (1988)
5	TF-paraLNH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| \| \| Fuc(α1-4) Fuc(α1-3) Fuc(α1-3)	Bruntz *et al.* (1988)
6	DF-paraLNH sulfate I Fuc (α1-2)	6S | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-3)	Guerardel *et al.* (1999)

(*Continued*)

Table 8.1 (*Continued*)

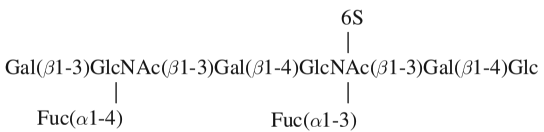
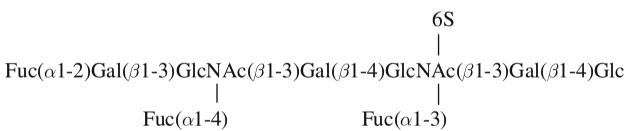
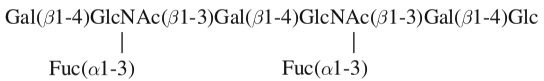
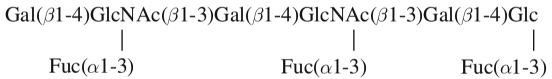
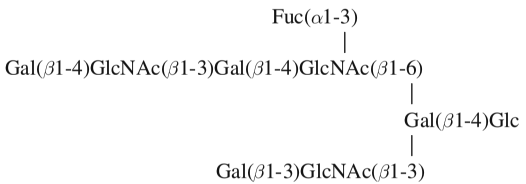
	Abbreviation	Oligosaccharide	Reference
7	DF-paraLNH sulfate II		Gruerardel *et al.* (1999)
8	TF-paraLNH sulfate		Gruerardel *et al.* (1999)
7.*Para*-Lacto-*N*-neohexaose series			
1	DF-paraLNnH		Yamashita *et al.* (1977b)
2	TF-paraLNnH		Bruntz *et al.* (1988)
8. Lacto-*N*-octaose series			
1	F-LNO		Yamashita *et al.* (1976b)

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Table 8.1 (*Continued*)

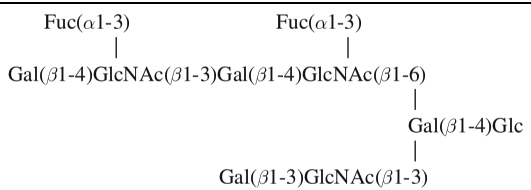
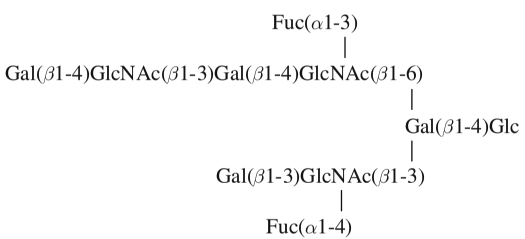
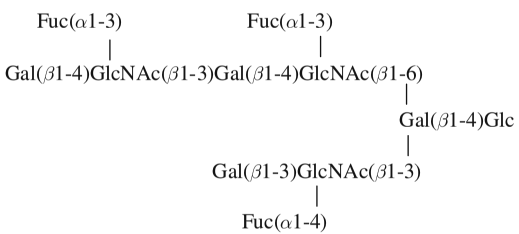
	Abbreviation	Oligosaccharide	Reference
2	DF-LNO I		Tachibana *et al.* (1978)
3	DF-LNO II		Tachibana *et al.* (1978)
4	TF-LNO		Tachibana *et al.* (1978)

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Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
5	FS-LNO	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(β2-3)Gal(β1-3)GlcNAc(β1-3) \| Fuc(α1-4)	Kitagawa *et al.* (1993)
6	DFS-LNO	Fuc(α1-3) \| Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) \| Fuc(α1-4)	Kitagawa *et al.* (1991a)
9. Lacto-*N*-neooctaose series			
1	F-LNnO	Fuc(α1-3) \| Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-4)GlcNAc(β1-3)	Yamashita *et al.* (1976b)

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Table 8.1 (*Continued*)

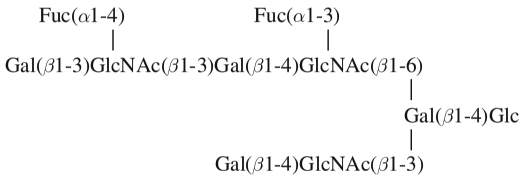
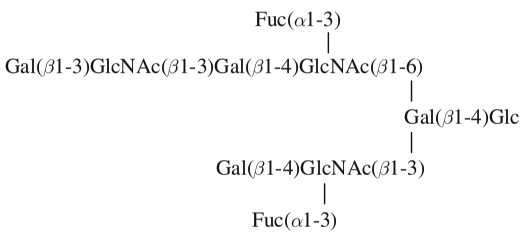
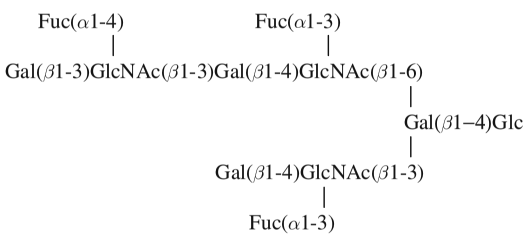
	Abbreviation	Oligosaccharide	Reference
2	DF-LNnO I		Tachibana *et al.* (1978)
3	DF-LNnO II		Tachibana *et al.* (1978)
4	TF-LNnO		Tachibana *et al.* (1978)

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Table 8.1 (*Continued*)

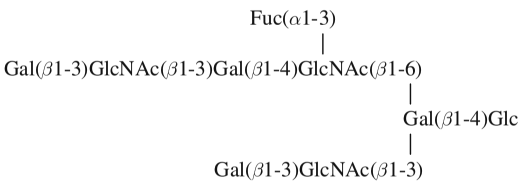
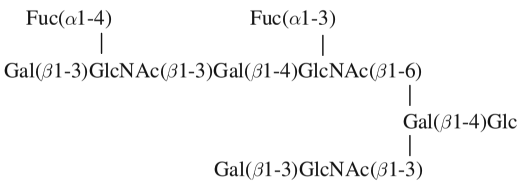
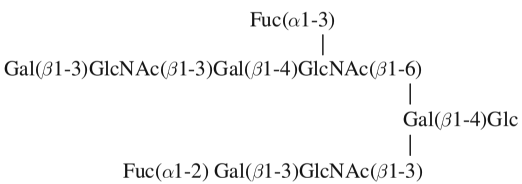
	Abbreviation	Oligosaccharide	Reference
10. iso-Lacto-*N*-octaose series			
1	F-isoLNO		Kogclberg *et al.* (2004)
2	DF-isoLNO I		Strecker *et al.* (1989)
3	DF-isoLNO II		Strecker *et al.* (1989)

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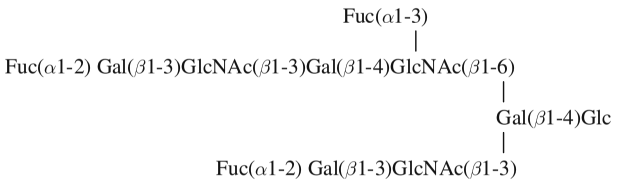
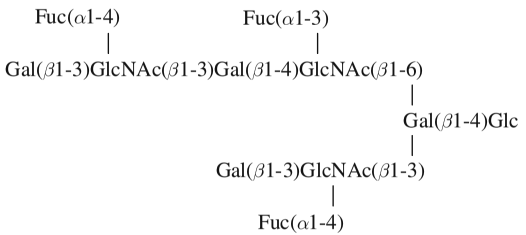
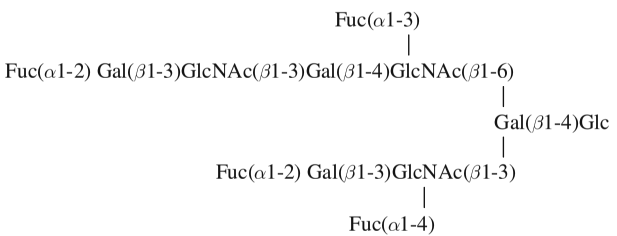
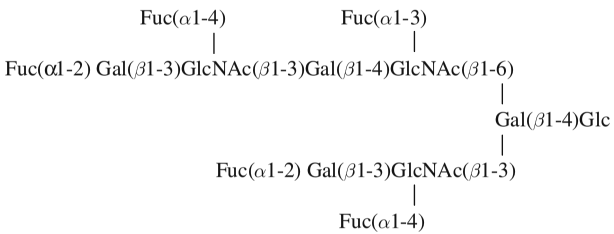
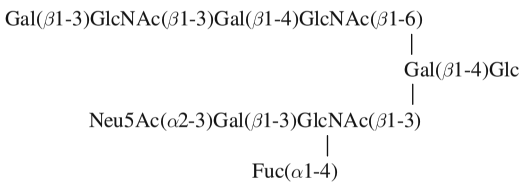
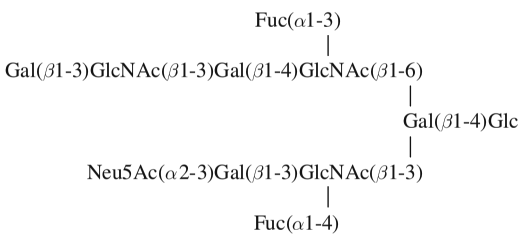
Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
4	TF-isoLNO I		Strecker *et al.* (1991)
5	TF-isoLNO II		Kogelberg *et al.* (2009)
6	TetraF-isoLNO		Hacuw-Fievre *et al.* (1993)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
7	PF-isoLNO		Haeuw-Fievre *et al.* (1993)
8	FS-isoLNO		Kitagawa *et al.* (1991a)
9	DFS-isoLNO I		Kitagawa *et al.* (1991a)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
10	DFS-isoLNO II	Fuc(α1-4) \| Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) \| Fuc(α1-4)	Kitagawa *et al.* (1991a)
11	TFS-isoLNO	Fuc(α1-3) \| Fuc(α1-2) Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3) \| Fuc(α1-4)	Kitagawa et al. (1993)
12. *para*-Lacto-*N*-octaose series, Lacto-*N*-decaose series and others			
1	Tetra F-paraLNO	Fuc(α1-2)Gal(β1-3) GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)- \| Fuc(α1-4) \| Fuc(α1-3) –Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc \| Fuc(α1-3)	Haeuw-Fievre *et al.* (1993)

(*Continued*)

Table 8.1 (*Continued*)

	Abbreviation	Oligosaccharide	Reference
2	F-LND	Fuc(α1-3) \| Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc NAc(β1-6) \| \| Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc \| Gal(β1-3)GlcNAc(β1-3)	Chai *et al.* (2005)
3		Fuc(α1-4) \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc	Kitagawa *et al.* (1990)
4		Fuc(α1-4) \| Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal	Kitagawa *et al.* (1990)
5		Fuc(α1-3) \| Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Neu5Ac(α2-3)Gal(β1-3)	Gronberg *et al.*(1992)

described below, these oligosaccharides are considered to play a significant role in the protection of human neonates against infection by pathogenic microorganisms. It is likely that, because of their immaturity, human neonates are relatively susceptible to infection by pathogenic microorganisms; the presence of significant amounts and a large variety of milk oligosaccharides may therefore be advantageous insofar as they supplement the other, known, anti-infection properties of the milk/colostrum. In this connection, it is notable that the milk of species, such as monotremes, marsupials and a few species of eutherians, including Ursidae, that produce very altricial neonates, also contain relatively high concentrations of oligosaccharides, with lesser amounts of free lactose (Messer and Urashima, 2002).

The 93 human milk oligosaccharides, the structures of which have been determined to date, can be grouped into 12 series based on their core units as in Table 8.2 (Haeuw-Fievre *et al.*, 1993). The many variations of the oligosaccharides are constructed by the addition of a Neu5Acα 2-3/2-6 residue to Gal or GlcNAc, and of Fucα1-2/1-3/1-4 to Gal, GlcNAc or a reducing Glc of the core units.

Table 8.2 The 12 core structures of human milk oligosaccharides

	Abbreviation	Oligosaccharide
1	Lactose	Gal(β 1-4)Glc
2	Lacto-*N*-tetraose	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)Glc
3	Lacto-*N*-neotetraose	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
4	Lacto-*N*-hexaose	Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-3)GlcNAc(β1-3)
5	Lacto-*N*-neohexaose	Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-4)GlcNAc(β1-3)
6	*para*-Lacto-*N*-hexaose	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
7	*para*-Lacto-*N*-neohexaose	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
8	Lacto-*N*-octaose	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-3)GlcNAc(β1-3)

(*Continued*)

Table 8.2 (*Continued*)

	Abbreviation	Oligosaccharide
9	Lacto-*N*-neooctaose	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-4)GlcNAc(β1-3)
10	Iso-Lacto-*N*-octaose	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)
11	*para*-Lacto-*N*-octaose	Gal(β 1-3)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
12	Lacto-*N*-decaose	Gal(β1-4)GlcNAc(β1-6) | Gal(β1-4)GlcNAc(β1-6) | | Gal(β1-3)GlcNAc(β1-3) Gal(β1-4)Glc | Gal(β1-3)GlcNAc(β1-3)

The main structural features of human milk oligosaccharides are the presence of oligosaccharides containing the type I unit (Gal(β1-3)GlcNAc), as well as those containing the type II unit (Gal(β1-4)GlcNAc), and that oligosaccharides containing the type I predominate over those containing the type II unit. The milk oligosaccharides of other species investigated to date mostly have the type II but not the type I unit. The many varieties of oligosaccharides in human milk and colostrum are produced by the addition of Neu5Ac and/or Fuc residues to these two units.

Isolation of the milk oligosaccharides has more recently been accomplished by normal-phase or reverse-phase high-performance liquid chromatography (HPLC) or by high pH anion exchange chromatography with pulsed amperometric detection (HPEAC-PAD). It is noteworthy, for example, that Gronberg *et al.* (1989, 1990, 1992) isolated several novel minor oligosaccharides by HPLC using triethylamine as an ion-pair reagent. On the other hand, the selective separation of specific epitopes has been carried out by affinity chromatography using a monoclonal antibody or lectin. For example, Kitagawa *et al.* (1988, 1989, 1990, 1991a,b, 1993) selectively separated oligosaccharides containing sialyl Le^a, a tumor-related carbohydrate epitope, by affinity column chromatography in which MSW113, the monoclonal antibody specific for this unit, was bound to Protein A-Sepharose GL4B as a ligand: the oligosaccharides containing sialyl Le^a were adsorbed by the column and were then eluted by raising the pH of the eluent.

At present, oligosaccharide structures are usually characterized by resonance assignments of the reporter groups in one-dimensional ^{1}H-NMR, of each chemical shift in one-dimensional ^{13}C-NMR and two-dimensional NMR such as ^{1}H–^{13}C correlated spectroscopy (^{1}H–^{13}C COSY), heteronuclear single quantum coherence experiments (HSQC), ^{1}H–^{1}H homonuclear Hartmann–Hahn experiments (^{1}H–^{1}H HOHAHA), heteronuclear multiple bond correlation experiments (HMBC), with the aid of MALDI-TOFMS or fast atom bombardment mass experiments spectrometry (FAB-MS) analysis.

Further detailed information on structural and technological aspects of human and bovine milk oligosaccharides can be obtained from a recent review by Mehra and Kelly (2006).

8.3. Human Milk Oligosaccharides: Quantitative Aspects

Milk oligosaccharides can be quantified using reverse-phase or normal-phase HPLC subsequent to pre- or post-column labeling techniques. Derivatizations are often performed by condensation of 2-aminopyridine, 2-aminobenzamide, 2-aminobenzoic acid or 1-phenyl-3-methyl-5-pyrazolone to the reducing end of the sugar aldehyde (Hase *et al*., 1978; Honda *et al*., 1989; Bigge *et al*., 1995; Fun *et al*., 1995; Tokugawa *et al*., 1996; Fu and Zopf, 1999; Sumiyoshi *et al*., 2003a; Sumiyoshi *et al*., 2003b). Sialyl oligosaccharides can be quantified by capillary electrophoresis, with detection at 205 nm (Bao *et al*., 2007).

Mature human milk and colostrum contain 12–13 and 22–24 g/L of oligosaccharides, respectively (Newburg and Neubauer, 1995). Oligosaccharides constitute the third quantitatively largest component, after lactose and lipids, of the dry matter of human milk.

The concentration of neutral oligosaccharides in human milk is greater than that of acidic oligosaccharides. The neutral fraction contains many fucosyl oligosaccharides. For example, Thurl *et al*. (1996) showed that human milk contains significant amounts of 2′-fucosyllactose (2′-FL: Fuc(α1-2)Gal(β1-4)Glc), lacto-*N*-fucopentaose I (LNFP I: Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc), lacto-*N*-difucohexaose I (LNDFH I: Fuc(α1-2)Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)Glc) and lacto-*N*-tetraose (LNT: Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc).

Representative acidic oligosaccharides of human milk are sialyl lacto-*N*-neotetraose c (LST c: Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc), disialyl lacto-*N*-tetraose (DSLNT: Neu5Ac(α2-3)Gal(β1-3)[Neu5Ac(α2-6)]Gal(β1-4)Glc), 3′-*N*-acetylneuraminyllactose (3′-SL: Neu5Ac(α2-3)Gal(β1-4)Glc) and 6′-*N*-acetylneuraminyllactose (6′-SL: Neu5Ac(α2-6) Gal(β1-4)Glc) (Thurl *et al*., 1996; Shen *et al*., 2000). Bao *et al*., (2007) stated that the most prominent acidic oligosaccharide in the milk/colostrum is DSLNT, whereas Asakuma *et al*. (2007) found that in colostrum it is LST c.

Changes in the concentrations of the following saccharides during the course of lactation may be crucial with respect to the biological significance of human milk/colostrum for infants: 2′-FL, 3-fucosyllactose (3-FL: Gal(β1-4)[Fuc(α1-3)]Glc), LNT, lacto-*N*-neotetraose (LNnT: Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc), LNFP I, lacto-*N*-fucopentaose II (LNFP II: Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Glc(β1-4)Glc), lacto-*N*-fucopentaose III (LNFP III: Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc), LNDFH I, lacto-*N*-difucohexaose II (LNDFH II: Gal(β1-3)[Fuc(α1-4)]GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)Glc], lacto-*N*-hexaose (LNH: Gal(β1-3)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc), lacto-*N*-neohexaose (LNnH: Gal(β1-4)GlcNAc (β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc), 6′-SL, 3′-SL, sialyl lacto-*N*-tetraose a (LST a: Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc), sialyl lacto-*N*-tetraose b (LST b: Gal(β1-3)[Neu5Ac(α2-6)]GlcNAc(β1-3)Gal(β1-4)Glc), LST c and DSLNT (Coppa *et al*., 1999; Chaturdevi *et al*., 2001).

The concentrations of oligosaccharides in milk or colostrum, as well as changes in their concentrations during the course of lactation, are likely to be significant with respect to their ability to act as anti-infection agents and to stimulate the growth of bifidobacteria in the infant's colon, as described below.

It is of interest that among mammals whose milk oligosaccharides have been investigated, humans are the only species in which oligosaccharides containing lacto-*N*-biose I units dominate over those containing *N*-acetyllactosamine (Gal(β1-4)GlcNAc) units (Urashima *et al*., 2001). The high expression of lacto-*N*-biose I-containing oligosaccharides in human lactating mammary glands, relative to that of other milk oligosaccharides, may be the key to the acquisition of a bifidus flora in the colon of the human infant.

8.4. Biosynthesis of Milk Oligosaccharides

As most milk oligosaccharides, including those of humans, contain a lactose unit at their reducing end, it is generally considered that they are synthesized by the action of a variety of glycosyltransferases acting on free lactose as the acceptor. Lactose is synthesized within lactating mammary glands from UDP-Gal (donor) and glucose (acceptor) by a transgalactosylation catalyzed by lactose synthase, an enzyme that is a complex of a β-4-galactosyltransferase I and α-lactalbumin. Other tissues do not contain α-lactalbumin but do contain β-4-galactosyltransferase which transfers galactose from UDP-Gal to non-reducing GlcNAc residues in glycoconjugates to synthesize *N*-acetyllactosamine (Gal(β1-4)GlcNAc) units. In lactating mammary glands, the presence of α-lactalbumin changes the preferred acceptor of β-4-galactosyltransferase from GlcNAc to glucose (Rajput *et al*., 1996). Thus, the expression of α-lactalbumin in the lactating mammary gland is the key to the presence of lactose in milk.

Since lactose is an obligatory acceptor for the mammary glycosyltransferases, α-lactalbumin is also essential for the presence of milk oligosaccharides. The exact number of glycosyltransferases involved in the biosynthesis of the oligosaccharides is still uncertain. These enzymes are known to be very specific, their specificity being directed toward both the type of linkage and the acceptor molecule. For example, in addition to the above-mentioned β-galactosyltransferase, there are probably at least two other human mammary β-galactosyltransferases, the actions of which are independent of α-lactalbumin, that catalyze the synthesis of Gal(β1-3)GlcNAc-R and Gal(β1-4)GlcNAc-R structures such as those shown in Table 8.1. Although human milk contains traces of three different galactosyllactoses, it is possible that these trisaccharides are formed by the transferase actions of β-galactosidase rather than by three specific galactosyltransferases. Judging from the variety of structures among the human milk oligosaccharides (Table 8.1) one can assume that the human mammary gland probably contains at least three β-*N*-acetylglucosaminyltransferases, three α-fucosyltransferases and two sialyltransferases.

Additional glycosyltransferases are found in lactating mammary glands of non-human species. An α-galactosyltransferase that synthesizes α-3′-galactosyllactose (isoglobotriose), a trisaccharide present in bovine, ovine and caprine colostrum and the milk or colostrum of several other species but not in human milk or colostrum (Urashima *et al*., 2001). The synthesis of α-4′-galactosyllactose (globotriose), which has been found in colostrum of the bottlenosed dolphin (Uemura *et al*., 2005), is presumably catalyzed by a different α-galactosyltransferase. Lactating mammary glands of the tammar wallaby contain a very active β-galactosyltransferase that is involved in the synthesis of a series of β(1-3)-linked galactosyllactoses that are unique to the milk of marsupials (Messer and Nicholas, 1991). The mammary glands of this species also contain an unusual β-*N*-acetylglucosaminyltransferase that attaches a *N*-acetylglucosaminyl residue to the trisaccharide β-3′-galactosyllactose and to the tetrasaccharide β-3′, 3″-digalactosyllactose (Urashima *et al*., 1992).

Although the milk/colostrum of most mammalian species contains lactose as the dominant saccharide, constituting more than 80% of the carbohydrate, milk oligosaccharides are found at higher concentrations than lactose in the milk of monotremes, marsupials and a few species of eutherians such as bears, giant panda, mink and white-nosed coati (Urashima *et al*., 2001; Messer and Urashima, 2002). It has been suggested that the ratio of oligosaccharides to lactose in milk is based on the ratio of expression of the glycosyltransferases to α-lactalbumin within the lactating mammary gland (Messer and Urashima, 2002). In those species whose mammary glands are characterized by a low expression of α-lactalbumin, the biosynthesis of lactose is likely to be relatively slow; therefore, their mammary glycosyltransferases would tend to utilize almost all the available free lactose for the synthesis of oligosaccharides. In

the milk of such species, e.g., monotremes, very little lactose would accumulate and the ratio of milk oligosaccharides to lactose would be relatively high.

8.5. Gastrointestinal Digestion and Absorption of Milk Oligosaccharides

When infants consume milk, the free lactose contained therein is split into galactose and glucose by intestinal lactase (neutral β-galactosidase, lactose-phlorizin hydrolase), an enzyme that is located in the membrane of the microvilli of the brush border of the small intestine. The two monosaccharides are transported into the enterocytes by a specific mechanism, whereupon the glucose enters the circulation and is used as an energy source while most of the galactose is converted to glucose in the liver, to be used as an energy source as well.

Much less is known about the exact fate of human milk oligosaccharides. These are resistant to enzymatic hydrolysis by the intestinal lactase of the brush border (Engfer *et al.*, 2000) and there is evidence that the major part survives passage through the small intestine and enters the colon where they are fermentated by colonic bacteria (Brand-Miller *et al.*, 1998; Newburg, 2000). Evidently, the brush border of the small intestine does not contain enzymes, such as sialidase, fucosidase or *N*-acetylglucosaminidase, that can remove sialic acid, fucose or *N*-acetylglucosamine residues, respectively, from the lactose units of the milk oligosaccharides. A small fraction of human milk oligosaccharides is absorbed intact, perhaps by receptor-mediated endocytosis (Gnoth *et al.*, 2001), some of which are excreted in the urine. It is unclear what proportion and exactly which of the ingested milk oligosaccharides are absorbed, but there is evidence suggesting that circulating oligosaccharides may have immunological effects on endothelial cells (Rudloff *et al.*, 1996).

There has been interest to investigate whether the sialic acid of sialyl milk oligosaccharides can be absorbed and utilized as precursors for the biosynthesis of brain gangliosides and sialoglycoproteins. Rat milk contains significant amounts of sialyllactose (Kuhn, 1972) which can be hydrolyzed to sialic acid and lactose by a very active small intestinal neuraminidase that is present in suckling rats. Since this enzyme has a low pH optimum and is absent from the brush border, it is probably of lysosomal, i.e., intracellular origin (Dickson and Messer, 1978). An intracellular location for this neuraminidase implies that the ingested sialyllactose has to be transferred into the enterocytes before it can be digested within lysosomes or supranuclear vacuoles; the most likely mechanism for this transfer is pinocytosis or endocytosis. It has been found that when adult rats were fed sialyllactose, there was an increase in brain ganglioside, including GM3, content and an improvement in the ability to learn to swim (Sakai *et al.*, 2006). It has also been reported that the amount of sialic acid bound to brain

gangliosides and sialoglycoproteins is significantly higher in breast-fed than in formula -fed infants; this may be due to a difference between human milk and infant formula with respect to their content of sialic acid (Wang *et al*., 2003). When piglets were supplemented with increasing amounts of sialic acid as casein glycomacropeptides for 35 days, there were proportionate increases in protein-bound sialic acid concentrations in the frontal cortex and improvements in learning (Wang *et al*., 2007). These observations support the notion that the sialic acid of milk oligosaccharides and also of sialoglycopeptides can be absorbed and utilized despite the fact that most milk oligosaccharides are considered to be indigestible within the small intestine.

It would be of interest to investigate whether, and to what extent, other monosaccharides, such as fucose, that are constituents of the neutral milk oligosaccharides, can similarly be absorbed and used as biosynthetic precursors. Rudloff *et al*. (2006) recently fed ^{13}C galactose to breast-feeding women and determined the amount of ^{13}C incorporated into lactose and both neutral and acidic oligosaccharides, notably LNT and fucosyl LNT. Incorporation of ^{13}C was also observed in the fraction containing difucosyl LNT, fucosyl LNH and difucosyl LNH (Rudloff *et al*., 2006). These results should make it possible to feed ^{13}C-enriched milk oligosaccharides to infants, thus facilitating studies on their metabolic fate.

8.6. *Bifidobacterium* Growth Stimulation by Human Milk Oligosaccharides

As noted above, although a small fraction of human milk oligosaccharides is known to be absorbed intact, it is now generally accepted that the major part survives passage through the small intestine and enters the colon. At this location, they are believed to act as prebiotics, stimulating the growth of bifidobacteria, and as soluble receptor analogues that inhibit the attachment of pathogenic microorganisms to the colonic epithelial cells. *Bifidobacterium longum* ssp. *infantis* has recently been shown to ferment purified human milk oligosaccharides *in vitro* as a sole carbon source, whereas another gut commensal, *Lactobacillus gasseri*, did not; this supports the hypothesis that human milk oligosaccharides selectively amplify specific bacterial populations in the infant intestine (Ward *et al*., 2006; Ninonuevo *et al*., 2007). There is evidence that the metabolic activity of the bifidobacteria reduces the colonic pH, which has the additional effect of inhibiting the proliferation of pathogenic organisms such as *Shigella flexneri* and *Escherichia coli* (Gopal and Gill, 2000). It has recently been shown that this strain is able to grow on human milk oligosaccharides as the only carbon source (Ward *et al*., 2007). The degradation of these oligosaccharides was studied during the growth of *B. longum* ssp. *infantis*

ATCC 15697 and it was found that tri- to hepta-saccharides were completely degraded during the growth over 25 and 50 h (LoCascio *et al.*, 2007).

Intestinal colonization by bifidobacteria is especially important to the health of infants because there is evidence that it prevents infection by some pathogenic organisms and reduces the incidence of diarrhea (Bezkorovainy, 1989). In breast-fed infants, bifidobacteria usually dominate the intestinal flora within 1 week after birth, constituting 95–99.9% of the bacterial population over time (Rotimi and Duerden, 1981; Benno and Mitsuoka, 1986). By contrast, intestinal colonization by bifidobacteria is not as rapid or as predominant in bottle-fed infants, who, prior to the early 20th Century, often experienced infection by pathogenic bacteria (Bezkorovainy, 1989). To improve the growth of intestinal bifidobacteria, saccharides such as lactulose (Petuely, 1957) have been used as supplements to formula milk, resulting in bottle-fed infants being healthier. However, the intestinal flora of bottle-fed infants consists of about 90% bifidobacteria and 10% Enterobacteriaceae; this ratio is smaller than that in breast-fed infants (Benno *et al.*, 1984).

Growth factors for bifidobacteria in human milk, so-called bifidus factors, have been investigated for many years. It was initially thought that, in this regard, nitrogen-containing sugars represented the main difference between breast milk and formula, but this was shown to be due to the use of a strain of *Bifidobacterium bifidum* that requires GlcNAc for growth (Gyorgy *et al.*, 1954; Veerkamp, 1969). Further studies revealed that oligosaccharides in human milk are candidate bifidus factors (Bezkorovainy, 1989), but a specific oligosaccharide responsible for stimulating the growth of bifidobacteria was not identified, mainly because of the complexity caused by the fact that human milk contains more than 100 kinds of oligosaccharides. Recently, however, Kitaoka *et al.* (2005) presented a new hypothesis based on a novel metabolic pathway for galactose in bifidobacteria, which proposed that Gal(β1-3)GlcNAc (lacto-*N*-biose I, LNB) structures, which are found in type I human milk oligosaccharides, act as specific bifidus factors.

Derensy-Dron *et al.* (1999) reported the presence of an enzyme in cell-free extracts of *B. bifidum* that reversibly converted LNB to α- D-galactopyranose-1-phosphate (Gal-1-P) and GlcNAc. This enzyme, which also converts Gal(β1-3)GalNAc (galacto-*N*-biose, GNB) to Gal-1-P and GalNAc, was named β-1,3-galactosyl-*N*-acetylhexosamine phosphorylase (EC 2.4.1.211) (Derensy-Dron *et al.*, 1999). Subsequently, a shorter name, lacto-*N*-biose phosphorylase (LNBP), was used for this enzyme (Kitaoka *et al.*, 2005). Derensy-Dron *et al.* (1999) assumed that the enzyme played a role in the metabolism of mucin sugars by bifidobacteria during colonization of the intestine. This assumption has been supported by the presence of a bifidobacterial endo-α-*N*-acetylgalactosaminidase that hydrolyzes the linkage between galacto-*N*-bioside and serine or threonine in *O*-linked glycoproteins of mucins (Fujita *et al.*, 2005).

Kitaoka *et al*. (2005) purified LNBP from a cell-free extract of *B. bifidum* and found that its partial amino acid sequence was homologous with that of the BL1641 protein of *Bifidobacterium longum* ssp. *longum* NCC2705, the complete genomic sequence of which was available (Schell *et al*., 2002). A homologous gene was then cloned from the type strain of *B. longum* ssp. *longum* JCM1217 and the expressed protein showed LNBP activity. The LNBP showed no significant identity with any other proteins of known functions. Thus, LNBP appears to belong to a new family of enzymes.

The LNBP gene, BL 1641, seems to be located in a gene cluster in the *B. longum* genome (Schell *et al*., 2002) as shown in Figure 8.1. The members of the cluster were annotated as follows: BL1638-1640, component proteins of ATP-binding cassette (ABC)-type sugar transporter; BL1642, mucin desulfatase; BL1643, galactose-1-phosphate uridylyltransferase (EC 2.7.7.10, GalT); BL1644, UDP-glucose 4-epimerase (EC 5.1.3.2, GalE). Judging from the members of the cluster, LNBP seems to be related to the metabolism of mucin sugars because GNB, one of the substrates of LNBP, is the core structure of mucin type I sugars. The proteins coded by BL1643 and BL1644 genes are well-known members of the Leloir pathway for galactose metabolism. In this pathway, Gal-1-P formed by a galactokinase (EC 2.7.1.6, GalK) is converted to α-glucose-1-phosphate by the action of GalT and GalE, to enter the glycolytic pathway. The bifidobacterial gene cluster does not include GalK; this is consistent with the fact that Gal-1-P is formed directly by LNBP, without

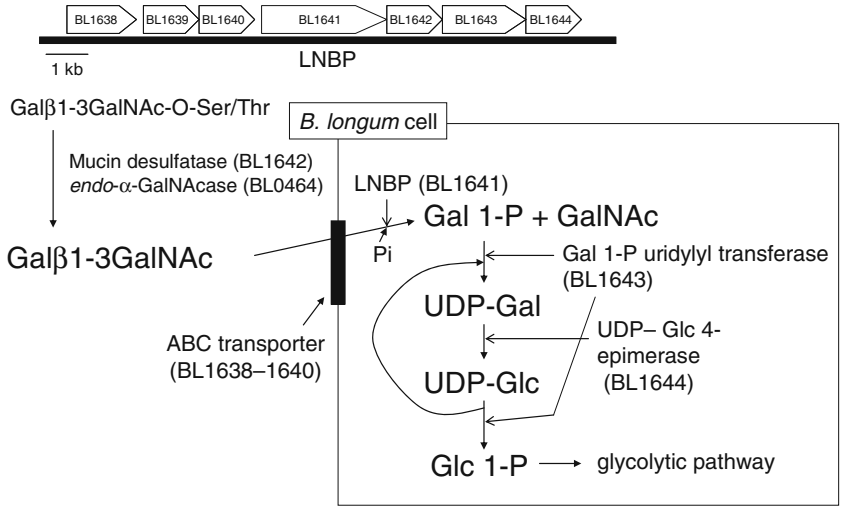


Figure 8.1. The gene cluster of *Bifidobacterium longum* ssp. *longum* containing the lacto-*N*-biose phosphorylase gene. Modified Figs. 2 and 3 of Kitaoka *et al*. (2005).

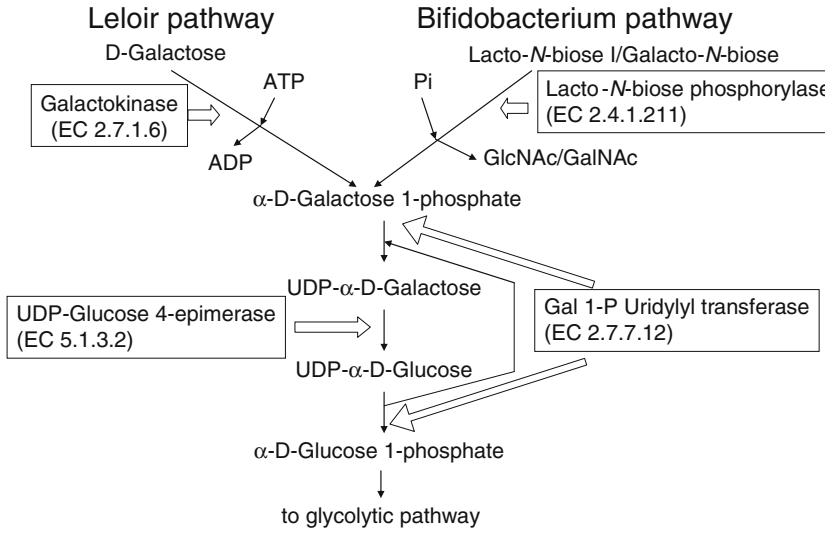


Figure 8.2. Comparison of the novel *Bifidobacterium* pathway with the Leloir pathway.

the consumption of ATP (Figure 8.2). On the other hand, it is suggested that N-acetylglucosamine is converted to N-acetylglucosamine-1-phosphate by a novel enzyme, N-acetylhexosamine 1-kinase, and then to UDP-N-acetylglucosamine by UDP glucose hexose 1-phosphate uridyltransferase, finally entering to metabolic pathway of amino sugars (Nishimoto and Kitaoka, 2007a).

Two types of galactose metabolism are known (de Vos and Vaughan, 1994). One is the Leloir pathway and the other is initiated by the phosphoenol pyruvate-dependent phosphotransferase system (PTS) transporter, in which lactose is transported into the cell across its membrane by a lactose-specific PTS transporter forming lactose-6-phosphate. *Bifidobacterium longum* ssp. *longum* does not possess the PTS pathway as evidenced by its genomic sequence (Schell *et al*., 2002; Kitaoka *et al*., 2005). A GalK gene is found in the *B. longum* genome (BL1210) accompanied by a GalT gene (BL1211), but no GalE gene is found near these genes. *Bifidobacterium longum* ssp. *longum* possesses a different GalE gene (BL1671), which is not accompanied by genes for enzymes related to the Leloir pathway. Based on the above observations, it can be hypothesized that the gene cluster containing the LNBP gene encodes the major metabolic pathway for galactose in *B. longum* ssp. *longum* and that the substrate of LNBP plays an important role for this organism. Since LNBP has been found in several strains of *B. longum* ssp. *longum* and *B. bifidum*, this pathway might be present in many bifidobacteria. LNB might also be an important sugar source for bifidobacteria. In light of the above, Kitaoka *et al.*

(2005) formulated the hypothesis that the LNB structure which occurs at the non-reducing end of several human milk oligosaccharides is a specific bifidus factor (Figure 8.3). Since LNB-containing oligosaccharides predominate over those containing *N*-acetyllactosamine in human milk (see above), this LNB hypothesis seems attractive.

It should be noted that free LNB is not found among the human milk oligosaccharides. For the hypothesis to be valid, bifidobacteria would have to have enzymes that liberate LNB from human milk oligosaccharides, since LNBP cannot act on β-glycosides of LNB (Derensy-Dron *et al.*, 1999; Kitaoka *et al.*, 2005). Considering the fact that lacto-*N*-tetraose and lacto-*N*-fucopentaose I are the major components of human milk oligosaccharides along with 2′-FL, lacto-*N*-biosidase and α-fucosidase enzymes would be required for the entry of these oligosaccharides into the LNB pathway (Figure 8.3). The gene encoding an α-fucosidase that hydrolyzes lacto-*N*-fucopentaose I and 2′-FL has been already cloned from *B. bifidum* (Katayama *et al.*, 2004). This activity was found in the culture supernatant of *B. bifidum* but not of *B. breve* or *B. longum*. As a lacto-*N*-biosidase that hydrolyzes lacto-*N*-tetraose into lacto-*N*-biose I and lactose has recently been cloned from *B. bifidum* JCM1254

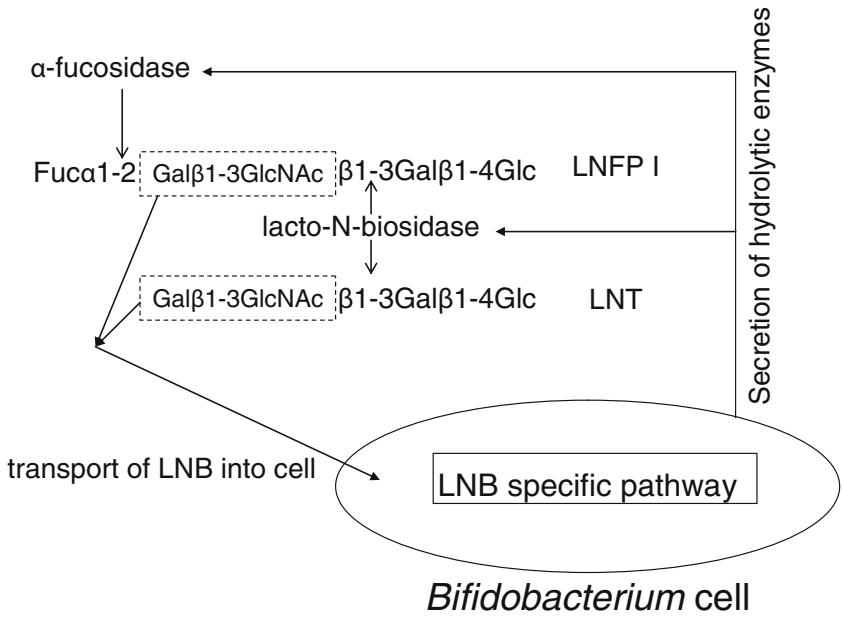


Figure 8.3. The lacto-*N*-biose hypothesis. *Bifidobacteria* secrete hydrolytic enzymes that act on milk oligosaccharides to form lacto-*N*-biose, which is selectively transported into bifidobacterial cells to be metabolized by lacto-*N*-biose phosphorylase.

(Wada *et al.*, 2008), the finding of this enzyme activity, as well as of α-fucosidase, should mean that *B. bifidum* and possibly other bifidobacterial strains can colonize the colon of breast-fed infants. In addition, an ABC type transporter, which delivers LNB through the cell membrane, has been purified from *B. longum* JCM1217 and crystallized (Wada *et al.*, 2007).

To validate the LNB hypothesis, it may be necessary to develop a practical method for the production of LNB on a large scale. Should LNB become easily available, the enduring enigma of the bifidus factor of human milk may be solved. Recently Nishimoto and Kitaoka (2007b) succeeded in the production of this disaccharide on a large scale using four different enzymes that were incubated in a medium containing sucrose, GlcNAc, phosphate, UDP-Glc and $MgCl_2$.

Changes in the concentrations of specific colostrum oligosaccharides may be significant with respect to the development of the colonic flora of breast-fed infants. The concentrations in colostrum of LNDFH I and LNFP I, which contain the lacto-*N*-biose I unit, have been found to be maximal at 1.9 and 2.1 g/L, respectively, on day 2 of lactation, while that of LNT, which also contains this unit, is higher on days 2 and 3 than at the beginning of lactation (Asakuma *et al.*, 2008). The relatively high concentrations of these oligosaccharides and the increase in LNT content during the early lactation period may significantly affect the formation of the colonic bifidus flora. The decrease in 2′-FL and increase in LNT during the first 3 days of lactation may mean that at the beginning of lactation the anti-infection properties of milk oligosaccharides are based mainly on their action as receptor analogues, inhibiting the adhesion of pathogens; thereafter, these properties may depend mainly on their prebiotic effect on the formation of the bifidus flora.

8.7. Milk Oligosaccharides as Anti-Pathogenic Agents

Pathogenic bacteria and viruses, to begin their infection, need to attach to the colonic mucosa, which they do by adhering to specific carbohydrate structures of glycoconjugates on the surface of the colonic epithelial cells. Because many milk oligosaccharides contain structural units that are homologous to these carbohydrate structures, it has been suggested that they act as soluble receptor analogues, inhibiting the adhesion of the pathogens, thus preventing infection.

The following anti-adhesion phenomena have been observed for human or other milk oligosaccharides or glycoconjugates. A trisaccharide unit, Gal(β1-4)GlcNAc(β1-3)Gal(β1-, which is found in lacto-*N*-neotetraose, etc., inhibits the adherence of *Streptococcus pneumoniae* to buccal epithelial cells (Andersson *et al.*, 1986). The bacteria that are inhibited from binding to enterocytes by fucosylated oligosaccharides include *Campylobacter jejuni*,

strains of *E. coli* and their heat-stable toxin, and enteropathogenic *E. coli* (Newburg *et al.*, 1990; Cravioto *et al.*, 1991; Cervantes *et al.*, 1995).

The observation that 2′-FL inhibits the binding of *C. jejuni* to H(O) antigen (Fuc(α1-2)Gal(β1-4)GlcNAc) in the infant colon is noteworthy (Ruiz-Palacios *et al.*, 2003), because this trisaccharide is the most abundant oligosaccharide in human milk (Thurl *et al.*, 1996). Intestinal infection by *C. jejuni* is one of the most common causes of diarrhea worldwide (Ruiz-Palacios, 1997). An inverse correlation has been observed between the concentration of 2′-FL in breast milk and the frequency of diarrhea in breast-fed infants, supporting the view that 2′-FL reduces the pathogenicity of *C. jejuni* (Morrow *et al.*, 2004). Asakuma *et al.* (2008) recently observed that the concentration of 2′-FL in human colostrum was 2.5 g/L at the start of lactation and decreased during the 2 subsequent days, suggesting that inhibition by 2′-FL of the adhesion of *C. jejuni* to the colonic mucosa is most significant immediately after birth. However, a recent study found that the concentration of 2′-FL in the milk of Italian and Burkinabe women was 1.0 and 1.8 g/L, respectively, on the first day of lactation, and 4.2 and 8.4 g/L on the third day (Musumeci *et al.*, 2006).

Sialylated oligosaccharides, at physiologic concentrations, strongly inhibit the binding of influenza A virus and S-fimbriated enteropathogenic *E. coli* to their respective host target cells (Zopf and Roth, 1996). It is recognized that sialyl Le^x (Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc) or Le^b (Fuc(α1-2)Gal(β1-3)[Fuc(α1-4)]GlcNAc) epitopes have affinity for a lectin, a carbohydrate-binding protein, that is found on the surface of *Helicobacter pylori*, a Gram-negative bacterium, the main host of which is man. It resides in the gastric mucosa and adheres to the epithelial cells lining the stomach. Some 50% of the world population is infected by this organism, with a higher incidence in developing countries. *Helicobacter pylori* is associated with the development of peptic ulcers, mucosa-associated lymphoid-tissue (MALT) lymphoma and gastric adenocarcinoma (Blaser, 1996; Crespo and Suh, 2001).

The binding of *H. pylori* to various carbohydrate structures is mediated by two adhesins, Bab A and Sab A, which are expressed on its surface. As Bab A and Sab A recognize and bind Le^b and sialyl Le^x, respectively, it is likely that oligosaccharides or glycoconjugates containing Le^b or sialyl Le^x inhibit the attachment of *H. pylori* to gastric epithelial cells, thus preventing its colonization within the stomach. Recently, an adhesion assay was used to investigate the capacity of pig milk to inhibit *H. pylori* binding to a neoglycoprotein that has Le^b or sialyl-di-Le^xunits conjugated to human serum albumin. α1,3/4Fucosyltransferase transgenic FVB/N mice, known to express Le^b and sialyl Le^x in their gastric epithelium, were colonized by *H. pylori* and subsequently treated with porcine milk or water. The expression of the Le^b and sialyl Le^x carbohydrate epitopes on pig milk proteins was breed- and individual-specific and correlated with the ability of porcine milk to inhibit *H. pylori* adhesion to the gastric

mucosa (Gusteffsson *et al*., 2006). As human milk oligosaccharides such as LNDFH I or 3-fucosyl-3′-*N*-acetylneuraminyllactose (Neu5Ac(α2-3) Gal(β1-4) [Fuc(α1-3)]Glc) contain Le^b or sialyl Le^x, it seems likely that these effects may be achieved also by human milk oligosaccharides.

Other researchers have reported on the interaction of *H. pylori* with sialylated glycans. The preferred interaction is with α3-linked sialic acid; glycans having α6-linked Neu5Ac are non-binding. For example, 50% inhibition by *H. pylori* of hemagglutination of human erythrocytes was observed at a low concentration of some sialylated saccharides. The data show that S-3-PG (Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc-Cer), Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)GlcNAc, 3′-*N*-acetylneuraminyl-*N*-acetyllactosamine (Neu5Ac(α2-3)Gal(β1-4)GlcNAc) as well as 3-*N*-acetylneuraminyl-lacto-*N*-neotetraose (Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc) all bound to *H. pylori* CCUG17874 at similar strength. 3′-SL also bound to this organism but its binding ability was somewhat weaker than that of the above saccharides. It has also been reported that LST a, a human milk oligosaccharide, was able to bind to another strain, *H. pylori* J99 (Johansson *et al*., 2005).

The binding of 3′-SL to *H. pylori* CCUG17874 is noteworthy because this saccharide is found in human milk and bovine colostrum. Asakuma *et al*. (2007) found that at the start of lactation the concentration of 3′-SL in human colostrum is 360 mg/L, similar to that of 6′-SL. However, the concentration of 3′-SL decreased during the 2 subsequent days of lactation, whereas that of 6′-SL did not. This suggests that, very early in lactation, 3′-SL may be more significant in the prevention of transmission of *H. pylori* from mother to infant than later on.

Recent studies on the ability of various fractions of human milk oligosaccharides to inhibit the adhesion of three intestinal microorganisms (enteropathogenic *E. coli* serotype O119, *Vibrio cholerae* and *Salmonella fyris*) to differentiated Caco-2 cells have shown that the acidic fraction had an anti-adhesive effect on all three pathogenic strains. The neutral high molecular weight fraction significantly inhibited the adhesion of *E. coli* O119 and *V. cholerae*, but not that of *S. fyris*; the neutral low molecular weight fraction was effective toward *E. coli* O119 and *S. fyris* but not *V. cholerae* (Coppa *et al*., 2006). This demonstrated that human milk oligosaccharides inhibit the adhesion to epithelial cells not only of common pathogens such as *E. coli* but also of other aggressive bacteria such as *V. cholerae* and *S. fyris*. Thus, oligosaccharides may be important factors in human milk that defend against acute diarrhea in breast-fed infants.

It is thought that adhesion of *Neisseria meningitidis*, a human-specific pathogen causing meningitis and septicemia, is mediated by type IV pili (Hakkarainen *et al*., 2005). A microtiter well pili-binding assay was used to investigate the binding of type IV pili isolated from *N. meningitidis* to different glycoproteins. Inhibition of pili binding to bovine thyroglobulin and human salivary agglutinin by fractionated human and bovine milk

oligosaccharides was demonstrated. The binding of *Neisseria pili* to bovine thyroglobulin was most effective and was clearly inhibited by neutral human or acidic bovine milk oligosaccharides at concentrations of 1–2 g/L, suggesting that these fractions had the potential ability to inhibit the attachment of this bacterium to the colonic mucosa (Hakkarainen *et al*., 2005).

There is evidence that oligosaccharides from milks other than human can act as receptor analogues, inhibiting the adhesion of pathogenic microorganisms. Fractions containing milk oligosaccharides, in the form of supernatants that had been separated from colostrum and from transitional, mature and late lactation milk of Spanish brown cows by ethanol precipitation and subsequent centrifugation, were used to investigate the inhibition of hemagglutination by seven enterotoxigenic *E. coli* strains (K99, FK, F41, F17, B16, B23 and B64). These strains had been isolated from diarrheal calves. The fractions from the transitional and late lactation milk inhibited hemagglutination by all of these strains, whereas those from colostrum and late lactation milk produced weaker inhibition (Martin *et al*., 2002). It was assumed that this inhibition was due to 3′-SL, 6′-SL, 6′-*N*-acetylneuraminyl-*N*-acetyllactosamine (6′-SLN: Neu5Ac(α2-6)Gal(β1-4)GlcNAc) and disialyl lactose (DSL: Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc). The fractions from transitional and mature milk, in which the ratio of 6′-SL to 3′-SL was higher than in the fractions from colostrum and late lactation milk, had a stronger effect than the others.

It has been suggested that Neu5Gc(α2-3)Gal(β1-4)Glc, which is found in ovine (Nakamura *et al*., 1998) and caprine (Urashima *et al*., 1997) colostrum, inhibits the attachment of enterotoxigenic *E. coli* K99 to the infant's colon, Neu5Gc(α2-3)Gal being the receptor recognized by *E. coli* K99 adhesins (Kyogashima *et al*., 1989). Globotriose (Gal(α1-4)Gal(β1-4)Glc), which is present in bottle-nosed dolphin colostrum (Uemura *et al*., 2005), is suggested as a possible inhibitor of the binding of Shigella toxin and Shiga-like toxin produced by pathogenic *E. coli* (*Lindberg et al*., 1987; Samael *et al*., 1990). Gal(α1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc, which has been found in the milk of the white-nosed coati (Urashima *et al*., 1999) and mink (Urashima *et al*., 2005), has been suggested to be a possible inhibitor of the binding of toxin A produced by *Clostridium difficile* (Clark *et al*., 1987).

8.8. Immuno-Modulating Effect of Milk Oligosaccharides

Although direct detection of human milk oligosaccharides in the blood of infants has not yet been reported (Bode, 2006), it nevertheless seems very likely that small amounts of intact human milk oligosaccharides are normally absorbed from the gastrointestinal tract, and that they are transported into the blood, on the basis of their observed urinary excretion (Rudloff *et al*.,

1996; Obermeier *et al*., 1999). It follows that they may alter protein–carbohydrate interactions also at a systemic level. For example, recent studies suggest that human milk oligosaccharides interfere with the adhesion of neutrophils to vascular endothelial cells (Klein *et al*., 2000) and platelets (Bode *et al*., 2004). These effects appear to be based on the structural resemblance of some human milk oligosaccharides to the glycoprotein ligands of selectins. Selectins are trans-membrane proteins that are involved in cell–cell interactions in the immune system. P-selectin mediates leukocyte deceleration (rolling) on activated endothelial cells and initiates leukocyte extravasation at sites of inflammation. P-selectin is also involved in the formation of platelet–neutrophil complexes (PNC), a sub-population of highly activated neutrophils primed for adhesion, phagocytosis and enhanced production of reactive oxygen species. Recent studies suggest that oligosaccharides containing sialyl Le^x or its stereoisomer sialyl Le^a, which resemble the P-selectin ligand, inhibit the binding of selectin ligands to the surface of endothelial cells and platelets; this interferes with the formation of PNC, the effect of which is anti-inflammatory. The following oligosaccharide fractions were tested *in vitro* to establish whether they reduce leukocyte deceleration on U937 cells, which express the P-selectin ligand: total human milk oligosaccharides, neutral oligosaccharides, total acidic oligosaccharides, neutral oligosaccharides with a polymerization degree of 4, fucosylated oligosaccharides and disialyl lacto-*N*-tetraose. The acidic oligosaccharides fraction produced a slight but definite reduction of P-selectin ligand binding, similar to that of standard sialyl Le^x, whereas the total neutral oligosaccharides and neutral fucosylated oligosaccharides fractions did not (Schumacher *et al*., 2006). These results support the notion of anti-inflammatory effects of acidic human milk oligosaccharides.

However, Klein *et al*. (2000) showed that, *in vitro*, the neutral milk oligosaccharide fraction can inhibit the binding of neutrophils to TNF-stimulated endothelium and that the whole milk oligosaccharides fraction enhanced the formation of platelet–neutrophil complexes (Klein *et al*., 2000).

It has been reported that the incidence of necrotizing enterocolitis, a condition which is considered to be an exaggerated immune response, is about 85% lower in breast-fed than in formula-fed infants. This is consistent with an anti-inflammatory effect of absorbed human milk oligosaccharides (Lucas and Cole, 1990).

Another potential human milk oligosaccharide target could be DC-SIGN (dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin). This is expressed on dendritic cells (DC) in the intestine and other tissues and is involved in the capture of different pathogens, including HIV-1, hepatitis C, cytomegalovirus, Dengue virus, *Mycobacterium* and *Candida albicans*. Unidentified components in human milk bind to DC-SIGN and inhibit HIV-1 transfer to CD4+ T lymphocytes. DC-SIGN has high affinity

for Le^x, which is a unit contained in some human milk oligosaccharides, suggesting that the unknown inhibitory milk components could be milk oligosaccharides. The putative complex oligosaccharides with multiple Le^x determinants may inhibit DC-SIGN-mediated interactions similar to the multivalent binding hypothesis for selectins (Bode, 2006; Naaling, 2005).

The question of whether human milk oligosaccharides influence cytokine production and activation of cord blood T cells has recently been investigated (Eiwegger *et al.*, 2004). Cord blood mononuclear cells from randomly chosen healthy newborns were co-cultured for 20 days with acidic or neutral oligosaccharides, and intracellular cytokine production and surface marker expression of T cells were studied using flow cytometry. The authors used concentrations of oligosaccharides (neutral human milk oligosaccharides, 10 μg/ml; acidic human milk oligosaccharides, 1 μg/ml) that were considered by them to mimic physiologic conditions, although these concentrations are considerably lower than the calculated values of 100–200 mg/L for circulating human milk oligosaccharides mentioned by Bode (2006). The acidic, but not the neutral oligosaccharide fraction, increased the percentage of interferon-γ-producing CD3+CD4+ and CD3+CD8+ cells, of IL-13 production in CD3+CD8+ cells and significantly elevated CD25+ expression in CD3+CD4+ cells. These results showed that human milk oligosaccharides affect cytokine production and activation of cord blood-derived T cells *in vitro*. Oligosaccharides and, in particular, acidic milk oligosaccharides may therefore influence lymphocyte maturation in breast-fed newborns. The authors concluded that human milk oligosaccharides can modulate the immune system of the maturing infant.

A recent study on rats showed that goat milk oligosaccharides have an anti-inflammatory effect in the colon (Daddaowa *et al.*, 2006). In this study, colitis was induced by the hapten, trinitrobenzenesulfonic acid (TNBS). The experimental rats (OS) were fed a diet containing 500 mg/kg per day of goat milk oligosaccharides, from 2 days prior to the induction until day 6, after which all the rats were killed, the entire colon was removed, opened and scored for visible damage and then divided into several pieces for biochemical determinations. When the OS rats were compared with control rats in which colitis had been induced by TNBS but that had not been treated with oligosaccharides, it was found that the OS rats showed decreased anorexia, reduced loss of body weight, reduced bowel wall thickening and less necrosis of the colon. Biochemically, the colon of the rats had lower levels of inducible oxide nitric synthase (iNOS), cyclooxygenase 2 (COX2), interleukin-1β and mucin 3, as well as increased trefoil factor 3. These results showed that goat milk oligosaccharides are anti-inflammatory when administered as a pretreatment in the TNBS model of rat colitis, most likely due to their action as prebiotics resulting in favorable changes in the colonic bacterial flora. Since TNBS-induced colitis is widely used as a preclinical model of

inflammatory bowel disease in humans, it was suggested that goat milk oligosaccharides may be useful in the management of this disease.

Another study was performed to evaluate the effect of oligosaccharides from goat milk in a rat model of dextran sodium sulfate (DSS)-induced colitis (Lara-Villoslada *et al.*, 2006). DSS treatment produced a decrease in body weight that was not observed in rats fed the goat milk oligosaccharides. DSS also caused an acute colonic inflammatory process that was weaker in rats fed the goat milk oligosaccharides, as shown by colon myeloperoxidase activity, as well as clinical symptoms measured by a scoring system. The rats that had been fed goat milk oligosaccharides also showed less severe lesions. It was concluded that the goat milk oligosaccharides reduced intestinal inflammation and contributed to the recovery of damaged colonic mucosa.

8.9. Chemical Structures and Features of Bovine Milk Oligosaccharides: Milk Oligosaccharides of Other Domestic Farm Animals

The structures of 11 acidic and 10 neutral bovine milk oligosaccharides that have been discovered to date are shown in Table 8.3 (Gopal and Gill, 2000; Urashima *et al.*, 2001; Nakamura and Urashima, 2004). Most of these oligosaccharides had been isolated from colostrum (Gopal and Gill, 2000). Bovine colostrum contains more than 1 g/L of oligosaccharides (Nakamura *et al.*, 2003), the majority of which are acidic, whereas the mature milk contains only trace amounts (Gopal and Gill, 2000). The low concentration of oligosaccharides in mature bovine milk makes it difficult to use such milk in the production of human infant formulae designed to provide prebiotics and receptor analogues (see above). This means that certain chemically produced oligosaccharides that are not normally found in milk, but which have functions similar to those of human milk oligosaccharides, could with advantage be incorporated into infant formulae. Recently, 39 oligosaccharides have been found in bovine colostrum by employing microchip liquid chromatography separation and high-performance mass spectroscopy including Fourier transform ion cyclotron resonance (FTICR) and time-of- flight (TOF) analysis. The presence of LNnT, LNnH, lacto-N-novopentaose I (Gal(β 1-3)[Gal(β 1-4)GlcNAc(β 1-6)]Gal(β 1-4)Glc) and their N-acetylneuraminyl or N-glycolylneuraminyl derivatives was suggested by this method (Tao, *et al.*, 2008).

Most of the oligosaccharide fraction of bovine colostrum consists of 3′-SL, 6′-SL, 6′-SLN and DSL, with 3′-SL constituting 70% of the total oligosaccharide content. Changes in the levels of 3′-SL, 6′-SL and 6′-SLN in Holstein colostrum *pre-partum* to 1 week *post-partum* are shown in Figure 8.4. The levels were maximal immediately after parturition, rapidly

Table 8.3 The structures of bovine milk oligosaccharides

	Abbreviation	Oligosaccharide	Reference
1		GalNAc(β 1-4)Glc	Saito *et al.* (1984)
2	LacNAc	Gal(β 1-4)GlcNAc	Saito *et al.* (1984)
3	F-LacNAc	Gal(β1-4)GlcNAc \| Fuc(α1-3)	Saito *et al.* (1984)
4	α3'GalNAcL	GalNAc(α 1-3)Gal(β1-4)Glc	Urashima *et al*. (1991)
5	α3'-GL	Gal(α 1-3)Gal(β 1-4)Glc	Urashima *et al*. (1991)
6	3'-GL	Gal(β 1-3)Gal(β 1-4)Glc	Saito *et al.* (1987)
7	4'-GL	Gal(β 1-4)Gal(β 1-4)Glc	Kimura *et al.* (1997)
8	6'-GL	Gal(β 1-6)Gal(β 1-4)Glc	Saito *et al.* (1987)
9	3'-GalNAcL	GalNAc(β 1-3)Gal(β1-4)Glc	Watanabe *et al.* (2006)
10	novo LNPI	Gal(β1-4)GlcNAc(β1-6) \| Gal(β1-4)Glc \| Gal(β1-3)	Urashima *et al.* (1991)
11		Gal(β 1-4)Glc-3'-PO_4	Cumar *et al.* (1965)
12		Neu5Ac(α 2-3)Gal	Kuhn and Gauhe. (1965)
13	3'-SL	Neu5Ac(α 2-3)Gal(β 1-4)Glc	Schneir and Rafelson (1966)
14	6'-SL	Neu5Ac(α 2-6)Gal(β 1-4)Glc	Kuhn and Gauhe. (1965)
15	3'-Neu5GcL	Neu5Gc(α 2-3)Gal(β 1-4)Glc	Kuhn and Gauhe. (1965)
16	6'-Neu5GcL	Neu5Gc(α 2-6)Gal(β 1-4)Glc	Veh *et al.* (1981)
17	6'-SLacNAc	Neu5Ac(α 2-6)Gal(β 1-4)GlcNAc	Kuhn and Gauhe. (1965)
18	6'-Neu5GcLacNAc	Neu5Gc(α 2-6)Gal(β 1-4)GlcNAc	Veh *et al.* (1981)
19		Neu5Ac(α 2-3)Gall-3Gall-4Glc	Parkkinen and Jinne (1987)
20	DSL	Neu5Ac(2-8)Neu5Ac(α 2-3) Gal(β1-4)Glc	Kuhn and Gauhe. (1965)
21	6'-SLacNAc-1-phosphate	Neu5Ac(α 2-6)Gal(β1-4)GlcNAc-1-PO_4	Parkkinen and Jinne (1987)
22	6'-SLacNAc-6-phosphate	Neu5Ac(α 2-6)Gal(β1-4)GlcNAc-6-PO_4	Parkkinen and Jinne (1987)

decreasing by 48 h *post-partum* (Nakamura *et al*., 2003). In another study, the concentrations of 3′-SL, 6′-SL, 6′-SLN and DSL were found to be 681, 243, 239 and 201 mg/L, respectively, in Holstein colostrum and 867, 136, 220 and 283 mg/L, respectively, in Jersey colostrum immediately after parturition (McJarrow and van Amelsfort-Schoonbeek, 2004).

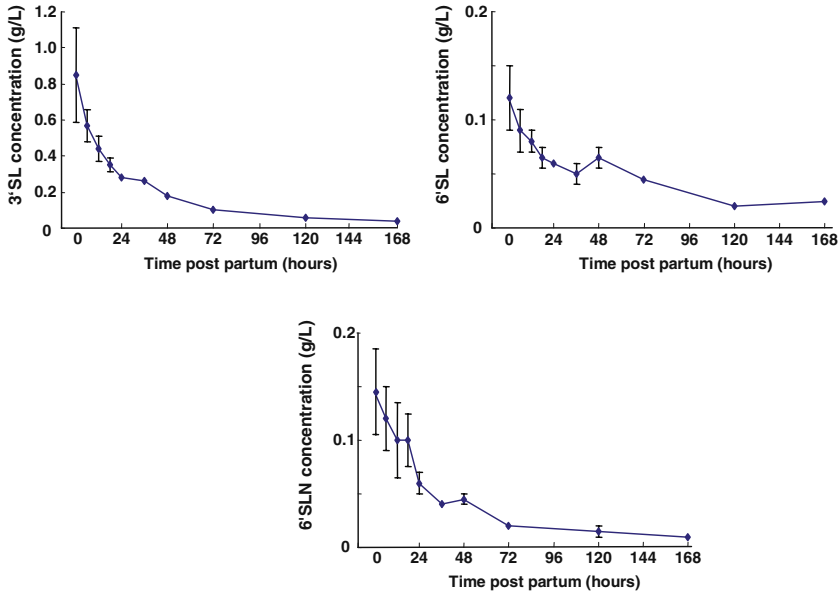


Figure 8.4. Changes in the concentrations of 3′-SL, 6′-SL and 6′-SLN in Holstein bovine colostrum during early lactation. Values are indicated as means ± SD ($n = 4$). Reproduced from Nakamura *et al.* (2003) with permission.

Among the neutral oligosaccharides, the following are characteristic of bovine colostrum, insofar as they have not been found in the milk or colostrum of other mammals: GalNAc(β1-3)Gal(β1-4)Glc (Watanabe *et al.*, 2006), GalNAc(α1-3)Gal(β1-4)Glc and GalNAc(β1-4)Glc. It is noteworthy that both α(1-3)- and β(1-3)-linked galactosyllactose and *N*-acetylgalactosaminyllactose have been found among the bovine oligosaccharides

As shown in Table 8.3, some bovine milk oligosaccharides have a *N*-acetyllactosamine (Gal(β1-4)GlcNAc) unit at their reducing end, in contrast to human milk oligosaccharides, almost all of which have a lactose residue in that position. The core units of most bovine milk oligosaccharides are lactose or *N*-acetyllactosamine, unlike human milk oligosaccharides the core units of which are LNT, LNnT, LNH and LNnH, etc. Both the variety and the concentration of fucosylated oligosaccharides in bovine colostrum and milk are very low; this is in contrast to human milk oligosaccharides, many of which are fucosylated.

It can be expected that milk oligosaccharides of other domestic farm animals, such as goats and sheep, will also be used as biofunctional materials. The content of milk oligosaccharides in goat milk is 0.25–0.30 g/L; this is higher than that of bovine (0.03–0.06 g/L) or ovine (0.02–0.04 g/L) milk. In addition, the variety of oligosaccharides in goat milk is greater than that in bovine or ovine

milk, as shown by the profiles of HPEAC analysis (Martinez-Ferez *et al.*, 2006; Mehra and Kelly, 2006). Colostrum from Japanese Saanen breed goats contains more 6′-SL than 3′-SL; it also contains 6′-*N*-glycolylneuraminyllactose, 6′-SLN, Gal(α1-3)Gal(β1-4)Glc, Gal(β1-3) Gal(β1-4)Glc, Gal(β1-6)Gal(β1-4)Glc and 2′-FL (Urashima *et al.*, 1994; Urashima *et al.*, 1997). Another study has shown that mature milk from Spanish goats contains 6′-SL, 3′-SL, DSL, *N*-glycolylneuraminyllactose, 3′-galactosyllactose, *N*-acetylglucosaminyllactose, LNH and additional high molecular oligosaccharides, as demonstrated by analysis with FAB-MS (Martinez-Ferez *et al.*, 2006). Ovine colostrum contains more 3′-*N*-glycolylneuraminyllactose than 3′-SL and 6′-SL (Nakamura *et al.*, 1998) and, notably, contains Neu5Gc in preference to Neu5Ac.

8.10. Milk Oligosaccharides of Other Mammals

Milk oligosaccharides of the following species, other than human and cow, have been studied and characterized (see also Urashima *et al.*, 2001); brown capuchin, buffalo, horse, goat, sheep, Ezo brown bear, Japanese black bear, polar bear, white-nosed coati, elephant, rat, dog, beluga, Minke whale, giant panda, crabeater seal, hooded seal, bearded seal, harbor seal, mink, bottlenose dolphin, echidna, platypus, and tammar wallaby (Urashima *et al.*, 2001). These oligosaccharides contain mainly lactose, lacto-*N*-neotetraose, lacto-*N*-neohexaose or para-lacto-N-neohexaose as core units, and there are species-specific features of the structures with respect to the presence or absence of Lewis x (Gal(β1-4)[Fuc(α1-3)]GlcNAc), H antigen (Fuc(α1-2)Gal), A antigen (GalNAc(α1-3)[Fuc(α1-2)]Gal), B antigen (Gal(α1-3)[Fuc(α1-2)]Gal) and α-Gal epitope (Gal(α1-3)Gal(β1-4)GlcNAc). As described above, the milk or colostrum oligosaccharides of non-human mammals either contain only the type II (Gal(β1-4)GlcNAc) but not the type I (Gal(β1-3)GlcNAc) unit, or else the former saccharides dominate over the latter.

The tammar wallaby is unique insofar as the neutral milk oligosaccharides of this species, and probably of most or all other marsupials, consist of at least two series, a major series, which can be described as $[\text{Gal}(\beta1\text{-}3)]_{n=1\sim5}$ Gal(β1-4)Glc, and a minor one, the members of which contain a branched unit of GlcNAc(β1-6) (Messer *et al.*, 1980, 1982; Collins *et al.*, 1981; Bradbury *et al.*, 1983). Of the major series, only the trisaccharide Gal(β1-3)Gal(β1-4)Glc has been found also in the milk/colostrum of some eutherian species, but higher members have not been detected (Urashima *et al.*, 2001; Messer and Urashima, 2002). It is notable that no fucosyl oligosaccharides have so far been detected in the milk of any marsupial (Messer and Urashima, 2002). In this respect the milk oligosaccharides of marsupials differ from those of both eutherians and monotremes since the major neutral milk oligosaccharides of the echidna and

platypus (monotremes) are fucosyllactose and difucosyllactose, respectively (Messer and Urashima, 2002). Furthermore, the higher milk oligosaccharides of the platypus contain lacto-*N*-neotetraose or lacto-*N*-neohexaose as core units (Amano *et al.*, 1985), which is a feature that is shared by many eutherian milk oligosaccharides. In these respects, also, eutherian milk oligosaccharides are more similar to those of monotremes than to those of marsupials.

The milk of all three infraclasses of mammals (eutherians, marsupials and monotremes) contains acidic (sialyl) in addition to neutral oligosaccharides (Urashima *et al.*, 2001). Milk of the echidna, a monotreme, uniquely contains, as its major oligosaccharide, a sialyllactose in which the sialic acid residue has a 4-*O*-acetyl substituent (Kamerling *et al.*, 1982).

It is worth noting that oligosaccharides dominate over free lactose in the milk of monotremes, marsupials and a few species of eutherians such as the Canoidea (other than the dog, *Canis familiaris*); the biological significance of this phenomenon, which appears to be found mainly in species whose neonates are altricial, is open to speculation (Messer and Urashima, 2002).

8.11. Future Aspects of Milk Oligosaccharides

In the above discussion, the main focus has been on the chemical structures and biological significance of oligosaccharides of human milk and colostrum, rather than of other species. Among more than 100 human milk oligosaccharides, each oligosaccharide may have its own specific role as a prebiotic and/or receptor analogue for different pathogenic microorganisms. Based on these functions, the possible utilization of artificial human milk oligosaccharide-like components on an industrial scale will be discussed below.

2′-FL is significant in the prevention of diarrhea caused by *C. jejuni* (Ruiz-Palacios *et al.*, 2003; Morrow *et al.*, 2004). As bovine milk does not contain this saccharide, there is a need for the development of techniques for its preparation and also incorporation into infant formulae produced from this milk. Murata *et al.* (1999a) prepared Fuc(α1-2)Gal(β1-4)GlcNAc, a saccharide that is similar to 2′-FL, using *p*-nitrophenyl-α-L-fucopyranoside as a donor and *N*-acetyllactosamine as an acceptor, by reverse hydrolysis with fucosidase from porcine liver. However, the yields were low and Fuc(α1-3) Gal(β1-4)GlcNAc and Fuc(α1-6)Gal(β1-4)GlcNAc were formed as undesirable by-products. It may be that one could use a different, more suitable, fucosidase that would give a higher yield of 2′-FL, and one could develop a method that uses fucose instead of *p*-nitrophenyl-α-L-fucopyranoside as a donor and lactose as an acceptor.

As shown by Kitaoka *et al.* (2005), LNT has the most potential as a candidate for prebiotics. As this saccharide has not been detected in bovine milk, there is a need for development of a method for its preparation, so that it

can be incorporated into infant formulae. Murata *et al*. (1999b) prepared lacto-*N*-triose II (GlcNAc(β1-3)Gal(β1-4)Glc) by the action of β-3-*N*-acetylglucosaminyltransferase from bovine serum, using lactose as the acceptor and UDP-GlcNAc as the donor. They then prepared LNT by reverse hydrolysis using β-galactosidase from recombinant *Bacillus circulans* ATCC31882, with lacto-*N*-triose II as the acceptor and lactose as a donor. However, bovine serum β-3-*N*-acetylglucosaminyltransferase is not available on an industrial scale, and the cost of UDP-GlcNAc is still very high. Because the LNB structure in LNT is considered to act as a specific bifidus factor in human milk (Kitaoka *et al*., 2005), LNB is also a possible candidate as a prebiotic. LNB, a disaccharide, should be much easier to produce than LNT, a tetrasaccharide. Nishimoto and Kitaoka (2007b) succeeded in the mass production of LNB using LNBP.

It can be expected that, in future, milk oligosaccharides will be isolated from the colostrum or milk of cows, or of other domestic farm animals, on an industrial scale for use in the production of infant formulae. Bovine colostrum contains more than 1 g/L of sialyl oligosaccharides, of which 3′-SL constitutes 70% (Nakamura *et al*., 2003). As noted above, there is evidence that 3′-SL prevents the adhesion of *H. pylori* to the gastric mucosa (Karlsson, 1998; Mysore, 1999; Sharon and Ofek, 2000). It can therefore be expected that 3′-SL isolated from bovine colostrum will be incorporated into infant formulae and biofunctional foods to prevent this adhesion.

Furthermore, the oral administration of goat milk oligosaccharides may be useful as a treatment for inflammatory bowel disease (Daddaowa *et al*., 2006; Lara-Villoslada *et al*., 2006) and it is possible that oligosaccharides from goat milk or colostrum or bovine colostrum will be used for this purpose. It has been noted, however, that the presence of Neu5Gc in caprine and ovine milk (see above) and the milk of other non-humans may be a significant drawback, since this type of sialic acid is not normally found in human milk oligosaccharides. There appears to be evidence that circulating anti-Neu5Gc-antibodies can be found in humans, probably as a result of dietary ingestion of Neu5Gc (Bode, 2006).

Nevertheless, oligosaccharides isolated from milk or colostrum of domestic farm animals, as well as milk oligosaccharide-like components prepared by synthetic methods, the functions of which are similar to those of milk oligosaccharides, can in future be expected to be used in industry as biofunctional materials.

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9

Milk Salts: Technological Significance

J.A. Lucey and D.S. Horne

9.1. Introduction

Mammalian milk contains all the essential components to sustain the growth and development of the newborn suckling. Usually, this is taken to mean the protein, fat and carbohydrate, but it must also apply to the mineral components, the milk salts, including the citrates, phosphates and chlorides of H^+, K^+, Na^+, Mg^{2+} and Ca^{2+}, whether as ions in solution or as colloidal species complexed with the caseins. These minerals are essential for bone growth and development, for efficient cellular function or for maintaining osmolality in the wake of carbohydrate (lactose) synthesis. Like the other components, all these mineral species are there for a purpose and until weaning, milk may often be the only source of these essential elements.

There have been a number of reviews on the topic of milk salts (Allen, 1931; Pyne, 1962; Jenness and Patton, 1976; Walstra and Jenness, 1984; Holt, 1985, 1997; Fox and McSweeney, 1998; Gaucheron, 2005). In this chapter, the term salts will be used to represent substances that are, or can be, present in milk as low molecular weight ions. This group includes both inorganic and organic (e.g. citrate) substances. We can distinguish between the major salt constituents and trace elements and the latter will not be considered in this chapter. The approximate concentration of milk salts is shown in Table 9.1. The milks salts have a crucially important impact on many properties of milk, including the formation and stability of the casein micelles, acid–base buffering and various colligative properties, as well as its key biological role (i.e. providing nutrition for the newborn). In addition, these salts have a powerful

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Table 9.1. Approximate salt composition in milk (from various sources)

	Concentration			Concentration	
Cationic	mg l^{-1}	mmol kg^{-1}	Anionic	mg l^{-1}	mmol kg^{-1}
Calcium	1040–1280	26–32	Carbonate (including CO_2)	~200	~2
Magnesium	100–150	4–6	Chloride	780–1200	22–34
Potassium	1210–1680	31–43	Citrate	1320–2080	7–11
Sodium	350–600	17–28	Total phosphorus (PO_4) (all forms)	930–1000	30–32
			Inorganic phosphorus (as PO_4)	1800–2180	19–23
			Sulphate	~100	~1

influence on protein stability during processing (e.g. rennet coagulation, heat and alcohol stability), the texture of various types of milk protein gels, cheese texture and functionality and emulsion stability.

Milk is saturated with respect to calcium and phosphate ions, and these ions exist in a *dynamic equilibrium* with undissolved or colloidal forms (there is no true equilibrium for the Ca phosphates but some type of *pseudoequilibrium* that is influenced by several factors, including the presence of caseins). It has been recognized since Hammarsten (1879) that this insoluble Ca phosphate fraction is held in solution in the casein micelles (at that time the micelles were called the Ca caseinogenate). The partition of salts between the colloidal (micellar) and serum (soluble) phases is shown in Table 9.2 (the distribution between these two phases depends on the environmental conditions, including pH, temperature, concentration, etc.). In the serum phase, milk salts may be present as ion pairs (e.g. anions with cations). The Ca and Mg in milk are present at low concentrations as free ions, some as complexes with citrate and phosphate as well as significant amounts associated with

Table 9.2. Approximate distribution of salts between the colloidal and serum phases in milk (from various sources)

	Colloidal (micellar) (%)	Serum (soluble) (%)
Calcium	69	31
Chloride	5	95
Citrate	14	86
Inorganic phosphate	53	47
Magnesium	47	53
Potassium	6	94
Sodium	5	95

Table 9.3. Calculated values for the major forms of calcium and magnesium in milk (mainly adapted from Neville, 2005)

Binding species	Concentration (mmol l^{-1})
Calcium	
$[Ca^{2+}]$	2.0
$[CaCit^{-}]$	6.9
$[CaPO_4^{-}]$	0.6
Casein	19.4
α-lactalbumin	0.5
Magnesium	
$[Mg^{2+}]$	0.8
$[MgCit^{-}]$	2.0
$[MgPO_4^{-}]$	0.3
Casein	1.9

casein micelles (Table 9.3). Both Mg and citrate are present in the colloidal phase, which is remarkable since their concentrations (or activities) are not in excess of solubility (Walstra and Jenness, 1984). The Ca and phosphate contents vary in proportion to the casein content of milk since much of the Ca and phosphate are associated with the casein micelles.

9.2. Methods of Analysis

Ashing (heating in a muffle furnace at ~550°C) of milk is an approximate method of quantifying the inorganic elements (0.7–0.8% in normal milk but values >1.3% can be found in colostrum). However, organic salts are lost during ashing. Some carbonates are lost during ashing (as CO_2) and some carbonates are formed from organic compounds. Phosphates from lipids (i.e. phospholipids) also appear in the ash. The sulphur of proteins is oxidized during incineration and appears as sulphate. Oxidation also results in the formation of metal oxides. Ashing is routinely used as a pre-treatment (by oxidizing organic matter) step for elemental analysis as the ash can be dissolved with acid and used for quantification of Ca, Fe, etc. by atomic absorption spectroscopy. The various techniques used for the analysis of milk salts were described by Fox and McSweeney (1998). Partition of salts between the colloidal and dissolved forms can be achieved by dialysis, ultrafiltration and the preparation of rennet whey (Davies and White, 1960; de la Fuente *et al.*, 1996), although some adjustments (e.g. to account for excluded volume effects) must be made with these techniques to calculate the serum concentration.

9.3. Secretion of Milk Salts

The biosynthesis of components in milk and milk secretion have been reviewed many times (e.g. Blackwood and Stirling, 1932; Petersen, 1944; Linzell and Peaker, 1971; Larson, 1985; McManaman and Neville, 2003). The cytoplasm of lactating alveolar cells is filled with numerous mitochondria and an extensive rough endoplasmic reticulum network. In addition, there is a well-developed Golgi apparatus, and secretory vesicles containing casein micelles are present in the apical region of the cell. Epithelial cells are connected to each other through an apical junctional complex composed of adherens and tight junctional elements that function to inhibit direct paracellular exchange of substances between vascular and milk compartments during lactation (McManaman and Neville, 2003).

The secretion of milk salts has been reviewed by Holt (1981, 1985) and Neville (2005). Lactating mammals must supply large amounts of Ca to the mammary gland where it is transported across mammary epithelial cells and into milk. Calcium is pumped from the cytoplasm into the Golgi compartment and enters milk via exocytosis of secretory vesicles from the Golgi compartment with a membrane-associated Ca ATPase mediating the transport (Bingham *et al.*, 1993). Circulating Ca concentration must remain relatively constant, i.e. Ca homeostasis; a number of diseases/conditions occur when this is not the case. Such stability relies on cooperation between several organs, principally the parathyroid glands, the kidneys, the skeleton and the gut. Several important entities are involved in the feedback loop that regulates Ca fluxes to the mammary gland. These control features include an extracellular Ca-sensing receptor (CaR) and parathyroid hormone-related protein (PTHrP) (VanHouten, 2005). Very high concentrations of Ca are transferred from the cytoplasm although the cytoplasmic Ca concentration remains relatively constant (in the μM range). This demand for Ca is associated with transient loss of bone mass (in humans), triggered, in part, by the secretion of PTHrP from the mammary gland into the circulation (Ardeshirpour *et al.*, 2006). The CaR is a G-protein-coupled receptor that signals in response to extracellular Ca^{2+} (Ardeshirpour *et al.*, 2006). It is responsible for coordinating Ca homeostasis by regulating parathyroid hormone secretion and by regulating Ca handling in the renal tubules. Calcium activates basolateral CaRs to stimulate its own transport into milk (VanHouten *et al.*, 2004). The intracellular Na and K concentrations are established by a Na/K-activated ATPase on the basolateral surface of the secretory cell, and there is a dynamic electrochemical equilibrium of these ions across the apical membrane (Holt, 1985).

It has been known for a long time that milk is in osmotic equilibrium with blood, i.e. milk is isotonic with blood (van der Laan, 1915). Taylor and

Husband (1922) were probably the first to suggest that the quantity of lactose produced by the mammary gland controls the daily volume of the milk. Koestler (1920) used the ratio of lactose and chloride as a method to indicate normal and mastitic (abnormal) milk. The Koestler number is given by (100 × chlorine %)/lactose %. Normal milk has a Koestler number less than 3 while mastitic milk is considerably higher (e.g. 15). One of the first studies of the possible mechanisms involved in the secretion of Ca and phosphate in milk was reported by Wright (1928).

The large amounts of phosphate required by the suckling for normal growth and development are also supplied through the milk in at least three chemical forms, namely free inorganic orthophosphate in solution, colloidal phosphate associated with Ca in micellar Ca phosphate and the ester phosphate of the caseins. The major pathway for phosphate secretion into milk is believed to be the Golgi vesicle route by a Na^+-P_i co-transport mechanism (Shennan and Peaker, 2000). Holt (1985) described another possible mechanism by which phosphate is generated in the Golgi lumen by hydrolysis of UDP during lactose synthesis (Kuhn and White, 1977). This uridine nucleotide cycle involves UDP-galactose and glucose. Within the vesicle, these precursors form UDP and lactose. The UDP cannot cross the vesicle membrane unless hydrolysed to UMP and inorganic phosphate, both of which can re-enter the cytosol, avoiding product inhibition of lactose synthetase. However, the widely varying concentrations of lactose found in milks of different mammalian groups suggest that other routes for P_i transport across the Golgi membrane may exist conjointly with the UDP hydrolysis mechanism. These may involve the ATP-driven Ca^{2+} pump discussed above, driving the accumulation of Ca^{2+} and actively participating in the phosphorylation of casein. Here, P_i is a by-product of the hydrolysis of ADP produced in that phosphorylation reaction (Shennan and Peaker, 2000).

Citrate concentration in milk varies widely throughout lactation (Banks *et al*., 1984). In general, citrate levels are higher during the grazing season (Holt and Muir, 1979) and during early lactation (Braunschweig and Puhan, 1999; Garnsworthy *et al*., 2006). In studies on the goat, Linzell *et al*. (1976) found that the mammary epithelium is impermeable to citrate in both directions, suggesting that citrate is synthesized within the secretory cells and released into milk after exocytosis of Golgi vesicles. Citrate has an indirect role in fat synthesis by providing reducing equivalents in the form of NADPH, which are required for de novo synthesis of fatty acids (Faulkner and Peaker, 1982). Citrate is in equilibrium with iso-citrate which is converted to α-ketoglutarate in the production of NADPH. Thus, increased de novo synthesis of fatty acids is predicted to lead to a decrease in citrate concentration. Such a correlation was found in the studies of Banks *et al*. (1984) who

used fat supplements to reduce de novo synthesis of fatty acids in the mammary gland and induce increases in milk citrate concentration and is confirmed in the more recent lactational studies of Garnsworthy *et al.* (2006). The latter authors found a significant correlation between milk citrate and the amounts of acetate required for chain elongation in de novo fatty acid synthesis. Any change in the citrate concentration of milk would therefore directly influence the Ca^{2+} concentration (as citrate readily binds Ca^{2+}), which could influence the functionality of milk, e.g. its rennet coagulation time. This type of mechanism could account for at least some of the observed seasonal or diet-related changes in milk functionality.

It has recently been proposed that casein-derived phosphopeptides disrupt tight junction integrity and precipitously cause milk secretion to dry up, i.e. they may help trigger the involution process (Shamay *et al.*, 2002). It is known that plasmin activity increases near the end of lactation (Politis *et al.*, 1989) and it is possible that some phosphopeptides are produced by this mechanism.

9.4. Factors Influencing the Milk Salts Equilibria

There are numerous dynamic equilibria between the salts in milk, and changes in many environmental conditions influence these equilibria. Some of these changes occur relatively quickly but those involving colloidal Ca phosphate (CCP) can be slow. Mastitic infections of the udder result in a decrease in the concentrations of Ca^{2+} and K^+ in milk but an increase in the concentrations of Na^+ and Cl^- (due to leakage of these ions into milk from blood where their concentrations are much higher than in milk). It should be noted that during milking and processing most CO_2 is lost. The impact of various processing techniques on the milk salts equilibria has been reviewed (Holt, 1985; de la Fuente, 1998; Gaucheron, 2005).

9.4.1. Temperature

Milk as secreted by the cows probably contains about 20 mg of CO_2 per 100 ml (Jenness and Patton, 1976). This gas is lost rapidly and heating and agitation accelerate this loss. The pH of milk decreases as its temperature increases, although few measurements of pH have or can be made at very high temperatures.

The solubility of Ca phosphates decreases at high temperature and during heating heat-induced CCP is formed, which re-solubilizes when milk is cooled subsequently. Jenness and Patton (1976) roughly gave that reaction as

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

The release of H^+ contributes to the decrease in milk pH observed on heating (with extreme heating there is also the production of organic acids, principally formic from lactose) (Dalgleish, 1989). This heat-induced CCP appears to associate with the existing CCP in casein micelles, possibly by increasing the size of the nanoclusters (Holt, 1995). The original equilibrium is mostly restored after cooling but there is some hysteresis. Cooling and holding milk at low temperatures result in an increase in the solubility of Ca phosphate and thus a decrease in the concentration of CCP. The Ca^{2+} activity is also restored if sufficient time is allowed for equilibration (Geerts *et al.*, 1983; Augustin and Clarke, 1991). At temperatures $\geq 40^\circ C$, artificial milk serum buffers (or ultrafiltrate) are prone to precipitation. Caseins are effective stabilizers of CCP and usually prevent precipitation of these salts in milk. The absence of casein from these buffers alters the behaviour of salts during heating and irreversible precipitation of Ca phosphate occurs (Holt, 1995). The deposits found on the surfaces of ultra-high-temperature heat exchangers are rich in Ca phosphate. There are indications that very severe heat treatments (e.g. 120°C for 15 min) cause a change in the nature of CCP, as indicated by an altered acid–base buffering profile (Lucey *et al.*, 1993a), e.g. to form hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; Visser *et al.*, 1986); under these severe heating conditions caseins are unable to prevent the precipitation of Ca phosphate (Holt, 1995).

Holding milk at low temperatures causes dissociation of some caseins, especially β-casein (<20% of total β-casein) (Downey and Murphy, 1970; Creamer *et al.,* 1977) and some dissolution of CCP (Qvist, 1979; Ali *et al.*, 1980). Most of these changes are reversed readily by mild heating, e.g. pasteurization (Qvist, 1979).

Freezing of milk is sometimes practiced where milk production is seasonal, e.g. goat and ewe milk for cheese making (Wendorff, 2001). Freezing and thawing for a sufficient time result in the reversal of most of the changes in the salt equilibrium that may have been caused during freezing. Long-term storage (after several months) of milk at $\leq -15^\circ C$ can result in protein precipitation (see Chapter 1). Ovine and caprine milk stored frozen for a few months had similar levels of soluble Ca, Mg and P after thawing as in the unfrozen milk (de la Fuente *et al.*, 1997).

9.4.2. pH

Acidification solubilizes CCP which is an integral part of casein micelles. The extent of solubilization increases markedly below pH 5.6 and is complete at approximately pH 5.0 (Pyne and McGann, 1960; Brule *et al.*,

1974; Pierre *et al.,* 1983; van Hooydonk *et al*., 1986; Dalgleish and Law, 1989; Mariette *et al*., 1993). The pH at which CCP is completely solubilized presumably varies with the conditions (e.g. rate and temperature) of acidification. At pH values <5 milk is unsaturated with respect to most types of calcium phosphate (Lyster, 1979). At high pH values (<6), concentrated milk products (e.g. processed cheeses) have an increased likelihood of precipitation of some types of Ca phosphate. Increasing the pH of milk results in the formation of additional CCP. McGann and Pyne (1960) described a method for increasing the CCP content of milk (by up to 200%). Milk pH was increased by the addition of NaOH at about 0°C followed by exhaustive dialysis against a large excess of the original milk.

Lucey *et al*. (1996) studied the impact of (cold) acidification and neutralization of milk on the properties of casein micelles. Acidification of milk to pH 5.0 or 4.6, followed by neutralization to pH 6.6, resulted in a reduction in the buffering maximum of milk at pH ~5.1; this buffering peak is caused by the solubilization of CCP. The reduced buffering in reformed milk suggests that little reformation of CCP occurs on neutralization; reformed milks also had an elevated Ca^{2+} activity. Acidification of milk to pH > 5.5, followed by neutralization to pH 6.6, hardly reduced buffering (at pH ~5.1), suggesting that either little CCP dissolved on acidification in that pH range or reformation of CCP occurred on neutralization. Canabady-Rochelle *et al*. (2007) also reported that milk had a higher soluble Ca level after acidification and neutralization.

Gevaudan *et al*. (1996) used high-pressure CO_2 to acidify milk reversibly (pH was restored to the original value after depressurization). Acidification to pH ~5 with high-pressure CO_2 resulted in a reduction in the buffering peak at pH ~5.1 but this peak increased during chilled storage of this milk (Raouche *et al*., 2007).

Heat treatment has little impact on the pH-dependent release of Ca and phosphate from micelles during acidification (Law, 1996; Singh *et al*., 1996).

9.4.3. Concentration of Milk

Concentrating milk by evaporation results in a decrease in milk pH, e.g. a decrease of ~0.3 and 0.5 pH units for 2:1 and 3:1 concentrations, respectively (Walstra and Jenness, 1984). The [Ca^{2+}] increases with concentration but less than the concentration factor (Walstra and Jenness, 1984). Presumably, the smaller increase in Ca^{2+} is at least partly due to the formation of additional CCP (even though the pH decreases in evaporated milk). Membrane filtration of milk using either ultrafiltration or microfiltration results in retentates where CCP is a greater proportion of the total Ca content as some soluble Ca is lost in the permeate during processing (Lelievre and Lawrence

1988; Srilaorkul *et al*., 1989; Solanki and Rizvi, 2001). In the production of highly concentrated (casein content $\geq$70%) milk protein powders (e.g. milk protein concentrates, MPC), extensive diafiltration or washing is required to reduce the lactose content. This extensive washing removes most soluble Ca, and partly reduces the CCP content in micelles and causes some casein dissociation. It is well known that extensive dialysis of casein micelles against water causes dissociation of caseins due to the loss of CCP.

9.4.4. Ca Sequestrants or Chelating Agents

Sequestrants (e.g. citrates and phosphates) combine with polyvalent metal ions (e.g. Ca^{2+} or Mg^{2+}) to form a soluble metal complex. Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), are complexes in which the metal ion is bound to two or more atoms in the chelating agent, usually in the form of a ring-type structure. The addition of sequestrants or chelating agents to milk disrupts casein micelles by reducing the [Ca^{2+}] and CCP content (Munyua and Larsson-Raznikiewicz, 1980; Visser *et al*., 1986; Udabage *et al*., 2000), which causes casein micelle dissociation (Morr, 1967; Gaucheron, 2005). Several studies have reported that some of the CCP cross-links can be removed from micelles without causing a lot of protein dissociation; higher levels of Ca removal caused micellar disintegration (Lin *et al*., 1972; Griffin *et al*., 1988). Udabage *et al*. (2000) found that adding high concentrations of sequestrants or chelating agents resulted in a significant reduction in micelle diameter and a very large reduction in particle light scattering.

Removal of Ca from milk using an ion exchange resin resulted in an increase in pH, a reduction in Ca^{2+}, an increase in ethanol stability and an increase in the rennet coagulation time (Lin *et al*., 2006).

When comparing the various types of phosphates, the orthophosphates are relatively poor at complexing Ca. Comparing the ability to complex Ca, phosphates and citrates can be ranked in the following order: long-chain phosphates > tripolyphosphate > pyrophosphate > citrate > orthophosphate (Van Wazer and Callis, 1958). Figure 9.1 shows a comparison of the [Ca^{2+}] remaining in solution in equilibrium with a 0.01 M solution of a number of sequestering agents (Van Wazer and Callis, 1958). This figure demonstrates the relative complexing abilities of the orthophosphates (weak, more free Ca left in solution) with long-chain polyphosphates (strong, little free Ca left in solution).

In well-defined systems, the relative efficiency of sequestrants can be compared by looking at the stability constants (formation constant, equilibrium constant) for a given metal (Furia, 1972). In general terms the

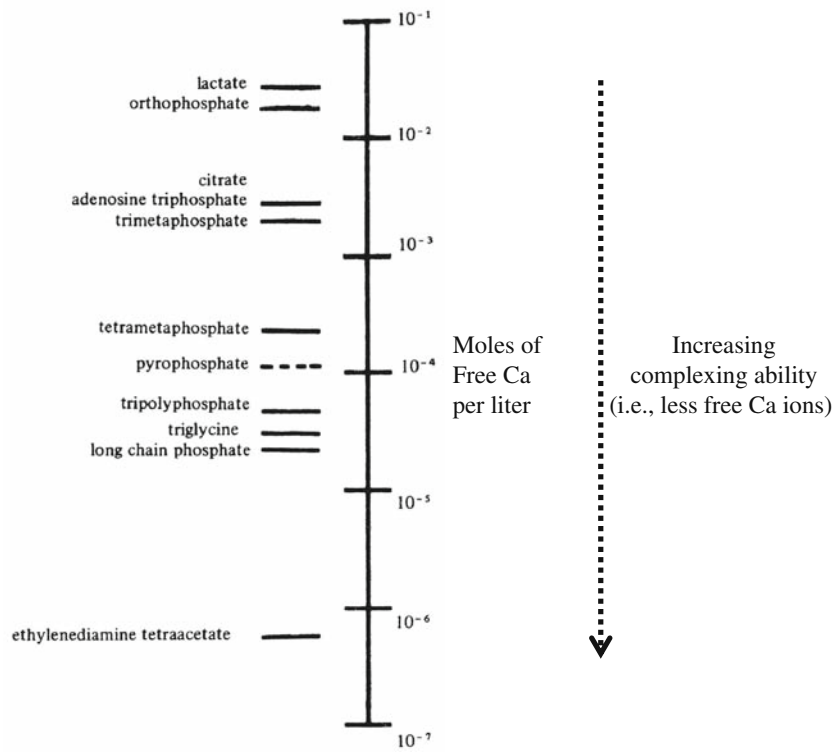

Figure 9.1. The free calcium concentration (i.e. not chelated or sequestered) for various types of complexing agents are estimated for the dissociation of a 0.01 M solution of the 1:1 Ca complex. Complexing agents that are lower on the scale (e.g. EDTA) are stronger chelators for calcium (adapted from Van Wazer and Callis, 1958).

stability constant of a metal (e.g. Ca^{2+}) complex can be calculated as follows (Furia, 1972):

$$K = \frac{[ML]}{[M]\,[L]}$$

where M is the metal ion, L is the ligand (sequestrant, chelating agent) and ML is the metal complex.

The (log K) stability constants for Ca-chelates with citrate, pyrophosphate and EDTA are 3.5, 5.0 and 10.7, respectively (Furia, 1972). Higher values indicate a stronger tendency to form a complex.

The highly charged anionic nature of polyphosphates causes them to be attracted to, and to orient themselves along, the charged sites of other long-chain polyelectrolytes such as proteins (Van Wazer and Callis, 1958). This should increase the charge repulsion between caseins at pH values above their isoelectric point (as in most dairy processing situations). At pH values below the isoelectric point, polyphosphates can induce protein precipitation by cation–anion interactions (Van Wazer and Callis, 1958). Casein has been reported to precipitate or aggregate in the presence of phosphates (Fox *et al*., 1965). Some types of phosphates can crosslink caseins, e.g. pyrophosphates, and they can even induce casein gelation (Mizuno and Lucey, 2005, 2007). Phosphate salts have also been used to cause heat-induced aggregation of caseins (Panouillé *et al*., 2003).

9.4.5. High Pressure

High hydrostatic pressure (HHP) reduces the light scattering of milk due to the disruption of casein micelles (Schmidt and Buchheim, 1970). HHP influences various properties of milk, including a reduction in the size of casein micelles, denaturation of β-lactoglobulin and a reduction in CCP content (see reviews by Huppertz *et al*., 2002; López-Fandiño, 2006). HHP treatment influences the functional properties of proteins through the disruption of hydrogen bonds and hydrophobic interactions and the separation of ion pairs. The impact on the properties of casein depends not only on the pressure applied but also on factors such as the application time, pH and temperature. It is well known that very high hydrostatic pressure $\geq$300 MPa causes the disintegration of the casein micelles as observed by a reduction in particle size (Needs *et al*., 2000; Garca-Risco *et al*., 2003). Micelle size is hardly unaffected, or is slightly increased, by pressures up to 250 MPa (Needs *et al*., 2000; Huppertz *et al*., 2004). Concomitantly with these size changes there is dissociation or aggregation (when there is an increase in size) of caseins. Huppertz and de Kruif (2006) proposed that the unfavourable exposure of hydrophobic surfaces at a pressure >200 MPa leads to the formation of larger casein particles from fragments of disrupted casein micelles during prolonged HHP treatment. The interactions responsible for this re-association were likely to include van der Waals or hydrophobic interactions.

HHP treatment solubilizes some of the CCP of raw (Schrader *et al.,* 1997; López-Fandiño *et al*., 1998) and heat-treated milk (Gaucheron *et al*., 1997; Schrader *et al*., 1997). Some or nearly all of the CCP is restored during subsequent storage of HHP-treated milk (Gaucheron *et al*., 1997: Schrader *et al*., 1997; Huppertz *et al*., 2006). Similar trends have been observed for milk of various species although the magnitude of the changes in the state of the CCP varied (López-Fandiño *et al*., 1998; Huppertz *et al*., 2006). Some studies

have found hardly any change in the concentration of soluble Ca after HHP treatment (Law *et al*., 1998). It is presumed that during HHP the solubilization of some of the CCP helps to cause casein micelle disintegration by disrupting one of the key crosslinking agents within micelles. Although pressure release helps to reverse the increase in soluble Ca during pressurization, the original micelle structure is not reformed (Law *et al*., 1998).

High-pressure CO_2 has been used as a recyclable acid for the isoelectric precipitation of casein (Hofland *et al*., 1999). After the pressure is released precipitation of Ca phosphate occurs (as the pH is also restored to the original value).

9.5. Impact of Milk Salts on the Buffering Properties of Milk and Dairy Products

The buffering properties of dairy products have been reviewed by Singh *et al*. (1997) and Salaün *et al*. (2005). The affinity of acids and bases for H^+ may be expressed in terms of titration curves and dissociation constants. An acid–base titration curve is a plot of pH versus the amount of acid or base neutralized in the titration. The buffering value (index) at any pH may be determined graphically from the slope of the tangent to the titration curve at that pH. If the added alkali or acid is *dB* and the resulting change in pH is *dpH,* then the average buffering value, i.e. the amount of acid or base required to cause a predetermined change in pH (dpH), is the differential ratio, *dB*/*dpH* (Van Slyke, 1922), where

$$\frac{dB}{dpH} = \frac{(\text{ml of acid or base added}) \times (\text{normality of acid or base})}{(\text{average volume of sample}) \times (\text{pH change produced})}$$

Apart from casein, the principal buffering components in milk are soluble phosphate, CCP, citrate and bicarbonate. Srilaorkul *et al*. (1989) estimated that the contribution of casein, whey proteins and milk salts to the buffering of skim milk was 36.0, 5.4 and 58.6%, respectively. Lucey *et al*. (1993b) reported that in the pH range 6.7–4.0, soluble salts and whey proteins (i.e. the substances in rennet whey), CCP and casein contributed approximately 47, 21 and 32%, respectively, to buffering in milk.

When milk is acidified (Figure 9.2a), maximum buffering occurs at approximately pH 5.1 but when acidified milk is back-titrated with base, there is low buffering at pH 5.1 and maximum buffering occurs at pH ~6.3. The maximum in the buffering curve at pH ~5.1 is due to the solubilization of CCP, which results in the formation of phosphate ions that combine with H^+

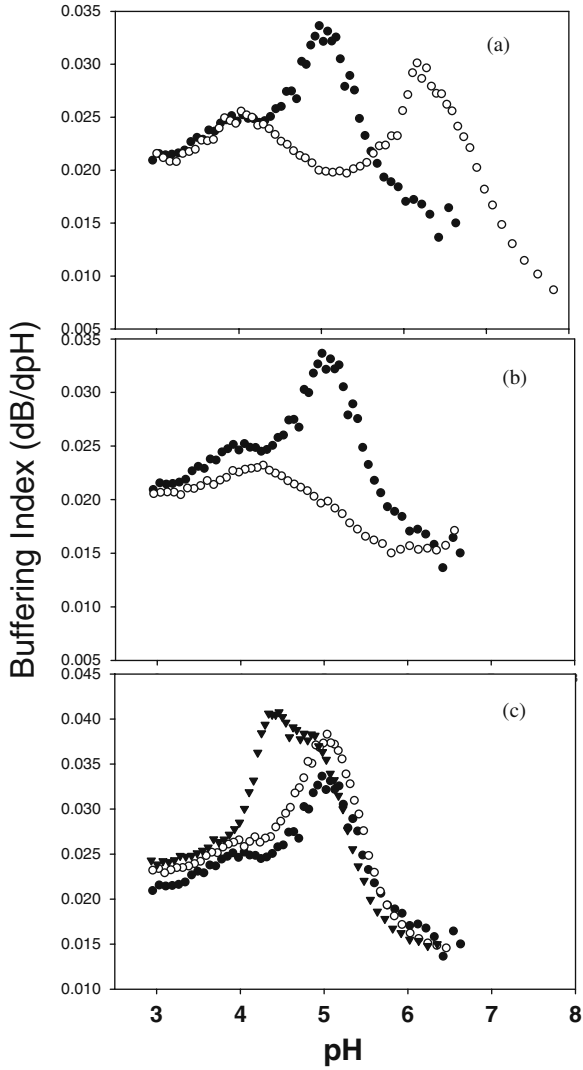

Figure 9.2. Acid–base buffering curves of (**a**) milk titrated from its initial pH to pH 3.0 with 0.5 N HCl (•) and back-titrated to pH 8.0 with 0.5 N NaOH (○); (**b**) milk (•) and colloidal calcium phosphate-free milk (○) titrated from the initial pH to pH 3.0 with 0.5 N HCl; (c) titration of unheated milk (•), milk heated at 100°C for 10 min (○), milk heated at 120°C for 15 min from the initial pH to pH 3.0 with 0.5 N HCl (▼) (adapted from Lucey *et al.*, 1993a,b).

(to form HPO_4^{2-} and $H_2PO_4^-$), resulting in pH buffering. The removal of the CCP from milk, as in CCP-free milk made by the method of Pyne and McGann (1960), results in the absence of the buffering peak at pH 5.1 during acid titration (Figure 9.2b). When the acidified milk sample is back-titrated with base, buffering is low at pH 5.1, because CCP is already solubilized, but maximum buffering occurs at pH 6.3, due to the formation of Ca phosphate (precipitation) with the release of H^+ (from HPO_4^{2-} and $H_2PO_4^-$) which can combine with OH^-.

High heat treatments cause an increase in CCP due to the formation of heat-induced CCP (Figure 9.2c). Some of the heat-induced CCP solubilizes on cooling (depending on the equilibration time allowed) but there is a substantial shift in the type of buffering curve observed during the acidification of very severely heated milk (e.g. 120°C for 15 min) (Figure 9.2c).

A strong buffering effect in the pH range 6–7 arises from the formation of Ca phosphate as can be seen in the titration of phosphoric acid in the presence of Ca (Figure 9.3). This buffering effect due to precipitation of Ca phosphate has been reported by many investigators, e.g. Visser (1962). Due to the precipitation of Ca phosphate around pH 6, the titration behaviour of phosphoric acid in the presence of Ca is completely different from that when this titration is performed in the absence of Ca (Figure 9.3). In milk, both Ca and phosphate are present which suggests that this behaviour would occur in dairy products. As is shown in Figure 9.3, the onset of precipitation of Ca phosphate results in the release of H^+. We can speculate that this release of H^+ could also occur during the formation of CCP in the mammary gland and may contribute to the lower pH of milk compared to that of blood.

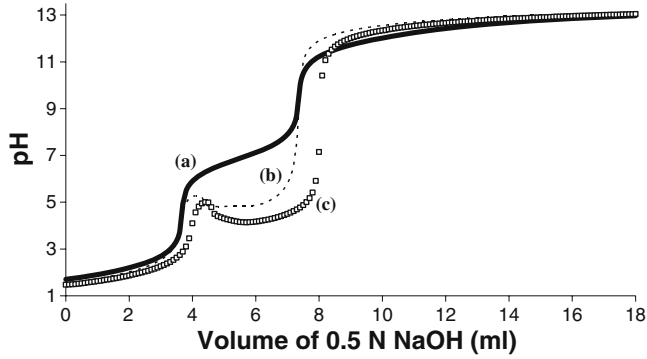

Figure 9.3. Potentiometric titrations of 400 mg phosphoric acid with 0.5 N NaOH in the presence of (**a**) no calcium, (**b**) 325 mg $CaCl_2$, (**c**) 650 mg $CaCl_2$; the method reported by Visser (1962) was used for these titrations (Salim and Lucey, unpublished data).

Acid–base buffering analysis is now widely used to indicate changes in the amount and type of CCP in milk as influenced by various technological treatments (e.g. Mizuno and Lucey, 2005).

9.6. Interactions Between Milk Salts and Casein

9.6.1. Introduction

Caseins constitute approximately 80% of the protein in bovine milk, with four main types (α_{s1}-, α_{s2}-, β- and κ-caseins) (casein fragments can be produced as a result of proteolysis). Caseins are found in combination with appreciable quantities of micellar or CCP, sometimes called CCP nanoclusters, in the form of aggregates called casein micelles (Holt, 1992). Casein plays a critical role in making milk super-saturated with Ca phosphate. As a packaging system, the micelles convert the milk into a free-flowing, low-viscosity fluid and provide the means to transport the high levels of Ca and phosphate at concentrations which would normally precipitate in the mammary gland in the absence of the caseins. The CCP is completely soluble at pH values < 5 (Pyne and McGann, 1960; Lyster, 1979) and the released Ca and phosphate are then available for absorption by the digestive system.

The caseins are a family of phosphoproteins found in the milk of all mammals. They are members of the group of Ca-phosphate-sequestering proteins which include dentine, bone matrix proteins and salivary proteins, amongst others (Kawasaki and Weiss, 2003). Phosphorylation is a post-translational modification of the caseins and it occurs at the serine groups, or rarely threonine, following a recognized template sequence Ser-X-Y, where X is any amino acid and Y = Glu, Ser-P or Asp. Due to the placing of the serine residues along the molecular sequences of α_{s1}-, α_{s2}- and β-caseins, most of the phosphorylated residues are found in clusters. Thus, four of the five phosphorylated serine residues in bovine β-casein are found in the sequence of residues 15–19, with the fifth at position 35. Four of the eight serines in bovine α_{s1}-casein are located in the sequence 64–68 with two more downstream at positions 46 and 48 and one upstream at position 75. Bovine α_{s2}-casein can have a variable level of phosphorylation from 10 to 13 moles P per mole of protein. The most abundant of these, α_{S2}-casein-11P, has three groupings of phosphorylated residues, one cluster of three from residues 8 to 10, four SerP spread as a group of three from 56 to 58 with the fourth member at 61, with the third cluster of two at positions 129 and 131. The remaining two single Ser-P residues of the total eleven are located at positions 16 and 135 (Horne, 2002). κ-Casein is unique amongst

the caseins in the absence of phosphoseryl clusters. Most molecules of κ-casein contain only one phosphoserine residue, rarely two or three, and all singlets located in the hydrophilic C-terminal region. The caseins are therefore sensitive to coagulation or precipitation by Ca. Horne and Dalgleish (1980) demonstrated that the logarithm of this critical coagulation time is a linear function of Q^2, where Q is the net negative charge on the protein, taking into account the binding of Ca to the casein. This linear correlation was also maintained when protein charge was changed following chemical modification of charged residues along the protein chain (Horne, 1979, 1983; Horne and Moir, 1984).

9.6.2. Casein Micelle Formation

The caseins are sensitive to precipitation due to the presence of approximately 30 mM Ca in milk. However, a key biological purpose of milk is to provide high concentrations of the essential Ca and phosphate required for the growth of the newborn mammal. So how are these two conflicting factors resolved? The solution involves casein micelle formation, the formation of an insoluble CCP phase within the micelles and the requirement for one of the caseins (usually κ-casein) to be insensitive to Ca and provide stability against Ca-induced precipitation to the other caseins. The details of how this occurs have been the subject of much debate and intensive study. For a discussion of the various casein micelle models, the reader is referred to various reviews (Farrell, 1973; Slattery, 1976; Rollema, 1992; De Kruif and Holt, 2003; Farrell *et al*., 2006; Qi, 2007). We will focus our explanations on the dual-binding approach for micelle formation as described by Horne (1998, 2002, 2006, 2008).

In the dual-binding model (Figure 9.4), micellar assembly and growth take place by a polymerization process involving two distinct forms of bonding/interactions, namely association through clustering of hydrophobic regions/patches of the caseins and second, linking of several phosphopeptides into the Ca phosphate nanoclusters. Central to the model is the concept that bond formation is facilitated, and hence micellar integrity and stability are maintained, by a local excess of hydrophobic attraction over electrostatic repulsion (otherwise if the repulsive interactions were too large, little association of casein would occur and micelle formation would not be observed in milk). It should be noted that there are quite different ranges for these interaction components. Compared to hydrophobic interactions, electrostatic repulsion is a long-range force. Clustering of charged groups in specific regions of the protein molecule means that electric dipole moments may be

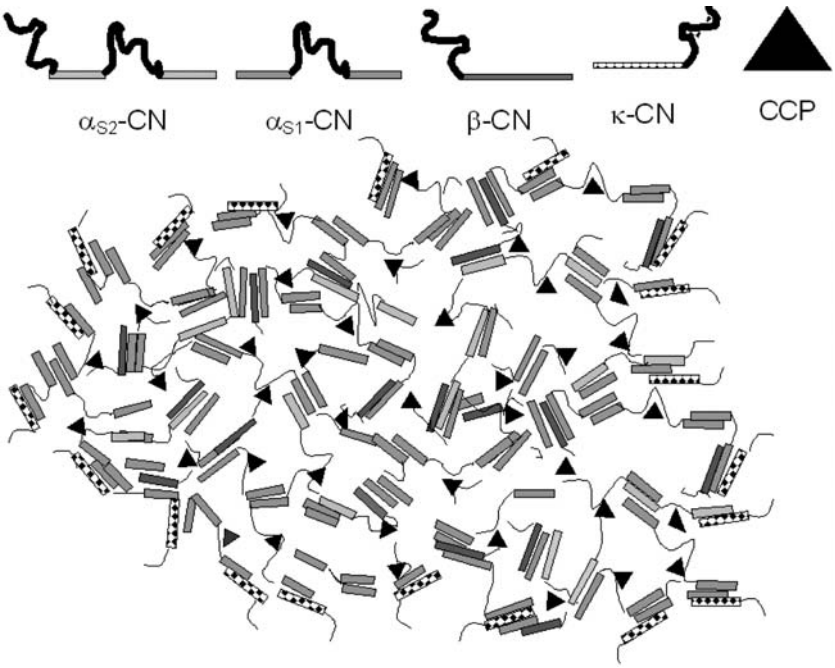

Figure 9.4. Dual-binding model for the casein micelle. CN is casein and CCP is colloidal calcium phosphate (Horne, 1998).

large, so that their effects on interparticle interactions may be rather strong (Piazza, 2004).

Each casein molecule effectively functions as a block copolymer, with the hydrophobic region(s) offering the opportunity for a multitude of individual, weak, hydrophobic interactions (driven by the thermodynamically favourable exclusion of water by this type of association). The hydrophilic regions of the casein molecules contain the phosphoserine cluster (or clusters), with the exception of κ-casein which has no such cluster, each offering multiple functionality for crosslinking. α_{s1}-Casein can polymerize (self-associate) through the hydrophobic blocks, forming a worm-like chain. Further growth is limited by the strong electrostatic repulsion of the hydrophilic regions, but in the casein micelle the negative charges of the phosphoserine clusters are neutralized by intercalating their phosphate groups into a facet of the Ca phosphate nanoclusters. This has two very important implications for the micelle. First, by removal of a major electrostatic repulsion component, it increases the propensity for hydrophobic bonding upstream

and downstream of the nanocluster link. It effectively permits and strengthens those bonds. Second, it allows for multiple protein binding to each nanocluster (on different facets), allowing a network to be built up. β-Casein, with only two blocks, a hydrophilic region containing its phosphoserine cluster and the hydrophobic C-terminal tail, can form polymer links into the network through both, allowing further chain extension through both. α_{s2}-Casein is envisaged in this model as having two of each block, two (possibly three, see below) phosphoserine clusters and two hydrophobic regions. It is only a small fraction of the total bovine casein but, by being able to sustain growth through all its blocks, it is likely to be bound tightly into the network. α_s-Caseins cannot be essential to micelle formation as human milk contains only trace amounts ($\sim$0.06% of total protein) of α_s-caseins (Lönnerdal, 2004) and yet micelles are formed. κ-Casein is the most important of the caseins in the dual-binding model of micellar assembly and structure. It can link into the growing chains through its hydrophobic N-terminal block but its C-terminal block is hydrophilic and cannot sustain growth by linking hydrophobically to another casein molecule. Nor does κ-casein possess a phosphoserine cluster and therefore it cannot extend the polymer cluster through a nanocluster link. Thus, chain and network growth are terminated wherever κ-casein joins the chain.

This polymerization process leaves the network with an outer layer dominated by κ-casein although other caseins are also present at, or close to, the surface (Dalgleish, 1998). Srinivasan and Lucey (2002) studied the impact of plasmin on the rennet coagulation of skim milk. They found that even partial hydrolysis of β- and α_s-caseins accelerated the rennet coagulation of milk. Plasmin hardly degrades κ-casein. Srinivasan and Lucey (2002) hypothesized that plasmin could have degraded β-casein "hairs" present on the surface of micelles and that this could have reduced the repulsive barrier to aggregation of rennet-altered micelles such that aggregation could occur at a lower degree of κ-casein hydrolysis.

Other evidence that some β-casein may be on or close to the micelle surface is that a considerable proportion (up to about 20%) of β-casein can dissociate from the micelle at low temperatures (this also occurs in ovine micelles but to a much lesser extent in porcine milk due to its high CCP content; Umeda, 2005; Umeda and Aoki, 2005; Umeda *et al.,* 2005). Some CCP also dissolves at low temperatures and that occurrence might also weaken the interactions between β-casein molecules and the rest of the micelle. How can a considerable proportion of β-casein dissociate at low temperatures while little κ-casein dissociates even though κ-casein is mainly on the surface? It is possible that κ-casein becomes polymerized by S–S bridging between κ-casein molecules (Farrell *et al*., 1996) after it has terminated the growth of the casein chains. If κ-casein polymers are formed

(in vivo), it is likely that these polymers would have greater attachment/linkage to the rest of the micelle structure, making them more difficult to remove. Also, the attractive balance in κ-casein is not very sensitive to changes in phosphoserine involvement in CCP nanoclusters (so the loss of some CCP crosslinks at low temperatures does not have a major impact on its dissociation from the micelle) as they do not interact through CCP crosslinks. In contrast, for β-casein, if some of the CCP crosslinks are dissolved at low temperatures then the exposed negative charge on the phosphoserine residues would make the binding of β-casein to other casein molecules unfavourable. This type of process could allow some of the β-casein to dissociate as the temperature is lowered. It is likely that the β-casein that dissociates is closer to the micelle surface or if not, then the β-casein freed by this process would have some potential chances to re-attach/associate with other caseins as it diffuses through the inner micelle network out to the bulk solution.

κ-Casein-deficient mice, produced by genetic modification, were unable to lactate because of destabilization of the micelles in the lumina of the mammary gland (Shekar *et al.*, 2006). The milk of various species appears to have a κ-casein or Ca-stabilizing casein (i.e. a casein that does not have a phosphate cluster), whereas some milks contain little or no α_s-caseins (human milk) and various ratios of α_s- to β-caseins.

9.6.3. Nature of Colloidal Calcium Phosphate

The nature of CCP or micellar Ca phosphate (as it is sometimes called) has been the subject of intense study and debate over the years. There have been several reviews of the nature of CCP (Pyne, 1934; McGann and Pyne, 1960; Schmidt, 1980; van Dijk, 1990; Holt, 1992, 1995; De Kruif and Holt, 2003). Schmidt (1980, 1982) considered the CCP to be a ubiquitous coating or "cement" that bonded many casein molecules together. McGann *et al.* (1983a,b) reported that the CCP depositions in milk systems consist of spherical granules (other later names for these granules include nanoclusters) 2–3 nm in diameter. Such a large entity is incompatible with the small type of CCP structures proposed by van Dijk (1990) or Schmidt (1980).

For many years, CCP was believed to be a basic Ca phosphate salt (e.g. Pyne and McGann, 1960). Pyne and McGann (1960) and McGann *et al.* (1983a) reported that in CCP, the Ca/P_i ratio is >1.5, which would make it some type of basic salt, like apatite or tricalcium phosphate (e.g. $Ca_3(PO_4)_2$). Citrate and magnesium are also associated with the CCP phase. Various other studies have suggested that CCP is a more acidic phase, such as some brushite-type structure ($CaHPO_4$) (e.g. Holt *et al.*, 1982). Various groups have considered CCP to be amorphous, i.e. lacking a crystalline structure (Pyne and

McGann, 1960; Knoop *et al.*, 1979; McGann *et al.*, 1983b; Lyster *et al.*, 1984), although we believe that it is also possible that CCP forms some type of crystalline structure (Horne *et al.*, 2007). Various researchers have included casein (serine) phosphate groups in the calculation of the ratio of Ca/P in CCP and have obtained a value <1.3. It is now realized that phosphoserine groups are not part of CCP but a small number of these peptide residues (around four) can terminate the growth of CCP crystals (Horne *et al.*, 2007).

The pK_a values for phosphate reported in chemistry textbooks are 2.1, 6.9 and 12.0, and Walstra and Jenness (1984) suggested that comparing the pH of milk (~6.7) with the pK_{a2} of phosphoric acid, one would expect $CaHPO_4$ to be the form of CCP. However, in the presence of Ca, all phosphates (pK_{a2} and pK_{a3}) are titrated around pH 6–7 due to the precipitation of Ca phosphate (Figure 9.3). That observation and the strong buffering at pH ~5.1 during acid titration of milk (Figure 9.2a) caused by the protonation of the released phosphate ions (from CCP) suggest that the form of CCP in milk is less likely to be an acidic form (like $CaHPO.2H_2O$) and may be a more basic form (e.g. tricalcium phosphate). Other titration studies, including those with oxalate (Pyne and Ryan, 1950; Jenness, 1973) have indicated that most P_i in CCP is in the form of PO_4^{3-} (i.e. tricalcium phosphate). Holt (1985) suggested that there may be a difficulty in titration studies if it is assumed that the exposed phosphoserine groups, after the dissolution of CCP, do not contribute to the titration. This suggestion by Holt (1985) does not explain the acid–base buffering behaviour shown in Figure 9.2a as the back-titration with base indicated that CCP does indeed contribute to the buffering at pH ~5. Removal of CCP resulted in the elimination of the buffering peak at pH ~5 (Figure 9.2b). Any calculation of milk salts equilibria needs to take into account the unexpected pK_{a2} and pK_{a3} values of phosphate in milk/serum although this does not appear to have always been the case. In summary, evidence from the various types of titration, the real pK_a values for phosphate in milk and the distinctive acid–base buffering properties of milk all suggest that the form of CCP is a basic form (e.g. tricalcium phosphate).

9.7. Functional Properties of Milk Products

Milk salts greatly influence the functional properties of milk and various dairy products primarily by influencing the structural integrity of the casein micelles or the sensitivity to aggregation of caseins. There have been a number of reviews on the effects of salts on the functionality of milk products (e.g. Augustin, 2000).

9.7.1. Rennet-Induced Gels

Generally, it is thought that Ca does not directly affect the enzymatic phase, although addition of $CaCl_2$ does reduce milk pH, which accelerates the hydrolysis reaction (Lucey and Fox, 1993). Rennet-altered micelles will aggregate only in the presence of Ca^{2+}, and gelation occurs only if there is sufficient CCP present (i.e. needs some type of casein micellar structure, as sodium caseinate does not form a rennet-induced gel, even though there is release of the macropeptide). Addition of ($<$50 mM) Ca reduces the rennet coagulation time, even at a constant milk pH, and flocculation occurs at a lower degree of κ-casein hydrolysis. Addition of Ca increases the rate of firming of renneted milk gels, mainly by neutralization of the negatively charged groups on the micelle surface and possibly by the formation of Ca bridges. Addition of high concentrations of Ca (e.g. $>$0.1 M) reduces the rate of gel firming, probably by increasing the effective (positive) surface charge on the micelles. Addition of up to 10 mM Ca increases the strength of rennet-induced gels (Lucey and Fox, 1993). Low levels ($\leq$0.02%) of $CaCl_2$ are often added by cheesemakers to help standardize the coagulation process (e.g. cutting time).

Reduction of the CCP content of casein micelles by $\sim$30% prevents coagulation unless [Ca^{2+}] is increased (Shalabi and Fox, 1982). Udabage *et al.* (2001) investigated the effects of mineral salts and Ca sequestrants or chelating agents on the gelation of renneted skim milk. They found that depending on the level of chelating agent, addition of citrate or ethylenediaminetetraacetic acid (EDTA) reduced the storage modulus (G′) of rennet-induced gels and above a certain concentration rennet gelation was inhibited completely (10 mmol/kg milk).

Choi *et al.* (2007) demonstrated that the concentration of insoluble Ca phosphate (CCP) associated with the casein micelles had an important influence on the properties of rennet-induced gels. Removal of some CCP from milk prior to gelation using a Ca-chelator lowered the storage modulus of rennet-induced gels due to the reduction in the amount of CCP crosslinking in casein micelles. Reduction in the CCP content prior to rennet-induced gelation resulted in gels with higher loss tangent values, indicating greater bond mobility.

The swelling, hydration and solubility of casein micelles in renneted milk are greatly increased in the presence of NaCl but markedly reduced if the brine solution contains Ca (Lucey and Fox, 1993). The addition of high concentrations of NaCl causes a reduction in rennet coagulation time. In some cheese varieties, salt is added to the cheesemilk (e.g. Domiati) resulting in a slower set and weaker curd (Fahmi and Shahara, 1950). These changes are largely reversible on removal of the excess NaCl by exhaustive dialysis against bulk milk (Huppertz, 2007).

9.7.2. Acid-Induced Milk Gels

Acid-induced casein gels can be made from sodium caseinate, indicating that the presence of CCP is not a requirement for the formation of acid milk gels (Lucey *et al.*, 1997). Since CCP is completely soluble at pH $\leq$ 5, CCP crosslinks do not contribute to the (final, or at least at pH values <5) stiffness of acid milk gels. The rate and extent of CCP solubilization during the gelation process is an important variable influencing acid milk gel properties.

The addition of Ca-chelating agents to milk has been reported to increase the firmness of acid milk gels made with glucono-δ-lactone (GDL) (Johnston and Murphy, 1992). Addition of EDTA also caused an increase in the loss tangent (tan δ) value in acid-heat-induced skim milk gels (Goddard and Augustin, 1995). Recently, Ozcan-Yilsay *et al.* (2007) studied the effect of trisodium citrate (TSC) on the rheological and physical properties and microstructure of yogurt. The storage modulus of gels increased significantly on addition of low levels of TSC and highest values were observed in samples with 10–20 mM TSC; higher (>20 mM) concentrations of TSC resulted in a large decrease in stiffness. No maximum in tan δ was observed in yogurts made with $\geq$25 mM of TSC as CCP was dissolved completely prior to gelation. Partial removal of CCP resulted in an increase in the tan δ at pH 5.1. Ozcan-Yilsay *et al.* (2007) suggested that at low TSC levels, the removal of CCP crosslinks may have facilitated greater rearrangement and molecular mobility of the micelle structure, which may have helped to increase the storage modulus and tan δ of gels by increasing the formation of crosslinks between strands. Ozcan-Yilsay *et al.* (2007) also concluded that the tan δ maximum observed in yogurts made from heated milk was due to the presence of CCP, as modification of the CCP content altered this peak and the removal of CCP eliminated this feature in the tan δ profiles.

Roefs and van Vliet (1990) reported that increasing the concentration of NaCl added to cold-acidified skim milk samples resulted in a decrease in the dynamic moduli of the gels formed when these samples were warmed. This indicated that electrostatic interactions are important for particle interactions. At high ionic strength, charged groups on casein particles would be screened, thereby weakening interactions between particles, which would result in a slower rate of increase of the storage moduli. In preparing Na caseinate gels by cold acidification, the addition of at least 0.1 M NaCl (to the acidified sample) was necessary to prevent precipitation during the warming up procedure (Roefs and van Vliet, 1990). Possibly, the primary effect of NaCl was to reduce rearrangement during the aggregation stage of gel formation. The addition of a high concentration of NaCl (>0.24 mol L^{-1}) to cold-acidified milk prevented gel formation when it was subsequently heated to a higher temperature for gelation (Roefs and van Vliet, 1990). Lucey *et al.*

(1997) studied the impact of NaCl on the properties of acid casein gels. They found that the pH at gelation was lower, $\leq$5.0, in gels made with added NaCl than in gels made without added NaCl, pH $\sim$ 5.1.

Low-methoxyl pectin is often used as a stabilizer in acid milk gel systems. Harte *et al.* (2007) proposed that during the acidification of milk, the release of Ca^{2+} arising from the solubilization of CCP induces the formation of pectin–pectin complexes, and at lower pH values these complexes interact with the casein particles. For acid casein gels made in the absence of Ca ions, a substantial reduction in the storage modulus was detected at pectin concentrations as low as 0.01–0.02% (w/v) and a significant increase in gelation time at pectin concentrations $\geq$0.05% (w/v) (Matia-Merino *et al.*, 2004). Complete inhibition of acid-induced gelation of casein was noted at $\geq$0.8% (w/v) pectin. Addition of Ca at low pectin contents (<0.2%) reduced the modulus of acid milk gels but there was a large increase in the storage modulus at higher levels of pectin ($\geq$0.2%, w/v).

9.7.3. Heat-Induced Whey Protein Gels

Salts have a major effect on the type, as well as the mechanical/sensory properties, of whey protein gels formed as a result of heat treatment. It is generally recognized that the addition of $CaCl_2$ to dialysed samples of whey protein concentrate (WPC) or whey protein isolate (WPI) results in an increase in gel strength. Above a level of 10–20 mM $CaCl_2$ gel firmness starts to decrease (Schmidt *et al.*, 1979; Kuhn and Foegeding, 1991). It has been speculated that excessive Ca causes rapid protein aggregation (due to decreased protein stability), which limits protein unfolding and network formation (Mangino, 1992). Caussin *et al.* (2003) reported that the addition of Ca to whey proteins resulted in the formation of very large protein aggregates during heating. Most commercially available WPC products probably have a Ca content that is greater than that required for optimal gel strength (Mangino, 1992). There is considerable variability in the thermal aggregation behaviour of commercial whey products and some of these differences could be removed by dialysis of these samples to a common ionic strength (McPhail and Holt, 1999). The concentrations of divalent cations are higher in WPC made from cheese whey than in WPC made from acid whey and these cations are not easily removed by dialysis, suggesting some binding by the whey proteins (Havea *et al.,* 2001). Presumably, membrane filtration of acid whey WPC at low pH values resulted in its greater demineralization. WPC made from acid whey has superior heat gelling properties than WPC made from rennet-coagulated cheese whey (Veith and Reynolds, 2004). These differences could be due to absence of GMP and the low Ca concentration in acid whey WPC.

9.7.4. Cold-Set Whey Protein Gels

Whey protein gels can also be produced using a two-step process that involves heat treatment at low ionic strength and/or far from the isoelectric point, followed by an increase in ionic strength and/or adjustment of pH (Barbut and Foegeding, 1993; Britten and Giroux, 2001). These gels are called cold-set gels, as the initial heat treatment produces a polymerized solution and gelation can occur at low temperatures ($\leq$ambient) if the repulsive forces are screened by the addition of mono- or polyvalent cations (e.g. Ca^{2+}) or a decrease in pH (e.g. through the addition of GDL or by bacterial fermentation). To obtain gels via the cold-set gelation method, it is necessary to first prepare a solution of heat-denatured proteins, with a protein concentration below the critical gelation concentration. Heating (e.g. 80°C for 30 min) results in the formation of soluble, denatured whey protein aggregates. Whey protein fibril-type gels are formed at very low pH values (e.g. 2) and cold-set fibril gels can also be made by the addition of Ca^{2+} (Bolder *et al.*, 2006).

9.7.5. Emulsions

Caseins, especially caseinates, are widely used as emulsifiers (Dickinson, 1997). The aggregation state of casein greatly influences surface activity with sodium caseinate (non-micellar), having greater surface activity than micellar or Ca caseinate (Mulvihill and Murphy, 1991). Dalgleish (1987) reported that emulsions prepared with α_s- or β-casein were sensitive to precipitation by Ca but emulsions prepared with κ-casein did not aggregate on Ca addition. The phosphoserine residues in β-casein helped that molecule maintain a thick steric stabilizing monolayer on emulsion interfaces (Dickinson, 1997). Increasing ionic strength by the addition of electrolytes screens out the double-layer repulsion and therefore reduces the electrostatic stabilization of proteins. Therefore, emulsions prepared with commercial milk protein ingredients of high salt content may be more flocculated than model systems prepared with pure proteins dissolved in low ionic strength buffer solutions (Dickinson, 1997). Calcium ions influence the stability of sodium caseinate-stabilized emulsions (Ye and Singh, 2001). Addition of $CaCl_2$ before or after homogenization caused a decrease in the creaming stability of emulsions made with 0.5% caseinate. In contrast, addition of $CaCl_2$ up to $\sim$10 mM increased the creaming stability of emulsions made with 3% caseinate, although the stability decreased again $>$20 mM $CaCl_2$. There was an increase in the surface protein concentration with an increase in the level of $CaCl_2$, which was due to enhanced adsorption of the α_s-caseins (Ye and Singh, 2001).

9.7.6. Foaming and Rehydration Properties After Spray Drying

Milk exhibits improved foam expansion when treated with EDTA (Ward *et al.*, 1997), probably due to disruption of the micellar structure following the chelation of CCP. The Ca concentration influences the interactions of β-casein at the air–water interface; in the absence of Ca, a weak interfacial gel forms whereas with Ca addition, a strong interfacial gel forms quickly (Vessely *et al.*, 2005). The foamability of reconstituted skim milk powder increased as the NaCl concentration was increased from 0 to 0.8 M due to the gradually increasing dissociation of casein micelles (Zhang *et al.*, 2004). The foamability of whey protein isolate increased when NaCl concentration was increased from 0 to 0.1 M, but decreased at higher NaCl concentrations (Zhang *et al.*, 2004).

The addition of citrate or phosphate solutions to micellar casein suspensions before drying considerably increased rehydration rates and this was related to the destruction of the micelle structure (Schuck *et al.*, 2002); this also alters the calcium equilibrium as extensive washing removes most soluble calcium. Water uptake in casein suspensions was improved by adding NaCl during rehydration. The addition of $CaCl_2$ considerably affected micelle organization and led to the formation of insoluble structures during spray drying.

9.7.7. Stability of Caseins

9.7.7.1. Ethanol

The stability of milk to various concentrations of added ethanol has been used as a milk quality index and is important in the production of drinks, such as cream liqueurs. Figure 9.5 is an attempt to illustrate the impact of pH on the ethanol and heat stability of caseins. Low pH values reduce stability and stability increases sigmoidally with pH. The inflection point (pK) depends on the properties of individual milks. For a more complete description of this profile/behaviour see Horne (2003). Horne and Parker (1981) found that the addition of Ca or Mg to milk samples caused a shift of the ethanol stability (ES)/pH profile to more alkaline pH. The addition of phosphate or citrate had little or no effect on the ES/pH profile, although addition of the stronger sequestrant, EDTA, caused a shift in the profile to more acidic pH. The studies of Horne and Parker on ethanol stability, reviewed by Horne (2003), emphasized the role of the inorganic components of the milk serum, reinforcing the conclusions of Sommer and Binney (1923) that salt balance, the excess of Ca and Mg over citrate and phosphate in milk serum, was critical in alcohol-induced coagulation. Decreasing the salt balance ratio thus caused a shift in the ES/pH profile to acidic pH, whereas increasing the salt balance ratio shifted the profile to more alkaline values. The mechanism, proposed by Horne (1987) to explain these observations, suggests that ethanol has two

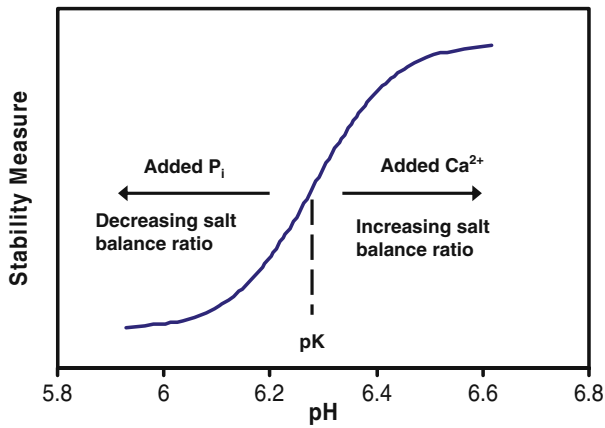

Figure 9.5. Stability phase diagram of milk as a function of pH or other treatments; schematic, meant to indicate trends for both ethanol and heat stability behaviour of milk (adapted from Horne, 2003).

competing effects on the micellar system, destabilization through loss of the hairy layer and shifts in the Ca phosphate equilibria, first noted by Pierre (1985). If the ethanol promotes the precipitation of Ca phosphate external to the micelle, it would first reduce the concentration of free Ca, reduce the level of caseinate-bound Ca and disrupt the binding through Ca phosphate nanoclusters. Moderate losses would increase the negative charge on the caseins and increase the thickness of the steric stabilizing layer. The higher the alcohol concentration, the faster and more extensive would be the precipitation of Ca phosphate. The ensuing adjustment in protein charge and conformation, although relatively rapid, still requires a finite response time. Countering these changes are the effects of ethanol as a non-solvent for the proteins, promoting crosslinking and collapse of the hairy layer. When the coagulation reaction occurs faster than the adjustment of charge and conformation resulting from shifts in Ca phosphate equilibria or the extent of the latter is limited by insufficient ethanol, the aggregation reaction dominates and precipitation of micelles follows.

The origin of the sigmoidal ethanol stability/pH profile (Figure 9.5) can also be explained through the effect of pH on Ca phosphate precipitation. Increasing pH brings about increased Ca phosphate precipitation, possibly further enhanced by the ethanol, which means that more ethanol is required to precipitate the protein, i.e. to overcome the increased energy barrier being erected following the transfer of Ca phosphate from the nanoclusters. Conversely, decreasing pH acts to diminish the influence of ethanol-induced

precipitation of Ca phosphate by titrating away negative charge and reducing electrostatic repulsion between protein species. Other effects of milk serum composition, of forewarming the milk and of modifying milk concentration and ionic strength, can all be explained in a similar fashion (Horne, 2003).

Tsioulpas *et al.* (2007) reported that there is an inverse non-linear relationship between free Ca ion concentration and ethanol stability ($r = 0.84$), confirming the earlier observations of Davies and White (1958). Citrate in natural samples acts as a stabilizing factor, as it slightly improved milk stability (Tsioulpas *et al.*, 2007). Ethanol stability values for milks during lactation were reported to have a mean of 83.2 ± 12.6% (range 62–100%) (Tsioulpas *et al.*, 2007). Chavez *et al.* (2004) found a (positive) correlation between the concentrations of chloride, potassium, ionic Ca and somatic cell count and ethanol stability. Horne and Parker (1983) reported that the addition of NaCl reduced the ethanol stability of unconcentrated milk, primarily at pH > 6.5, which they suggested is a result of increased ionic strength. O'Kennedy *et al.* (2001) demonstrated that α_{s1}- and β-caseins were only minor components of the ethanol-induced precipitate whereas α_{s2}- and κ-casein were the main proteins susceptible to aggregation.

9.7.7.2. Heat

Heat stability of milk has been reviewed (Fox and Morrissey, 1977; Singh and Creamer, 1992; O'Connell and Fox, 2003; Singh, 2004). Older literature was reviewed by Pyne (1962). The effects of raising temperature on the status of the various Ca phosphate species have been discussed earlier (Section 9.4.1). Heat stability is the ability of milk or concentrates to resist severe heat treatments without thickening, gelation or coagulation (Augustin, 2000).

Discussions on heat stability are complicated by the knowledge that heat-induced coagulation as a function of pH can follow two distinct profiles. In the Type A heat coagulation time (HCT) versus pH profile, the time to induce coagulation at a fixed temperature first increases with pH, then enters a minimum before stability increases again at more alkaline pH values. In the Type B profile, heat coagulation time increases progressively with pH. Individual milks which follow Type A behaviour predominate in most countries, while all bulk milks show Type A behaviour (O'Connell and Fox, 2003). When milk is heated, several competitive and often interdependent reactions occur, not all of them directly involving the milk salts. Fox (1981) listed a selection of these but it is now generally agreed that the presence of the minimum in a Type A profile is associated with the heat-induced formation of a complex between β-lactoglobulin and κ-casein. Such chemical reactions

are outside the scope of this chapter and are covered in the reviews of Fox and Morrissey (1977), Singh and Creamer (1992), O'Connell and Fox (2003) and Singh (2004). However, milks showing Type A characteristics can be converted into Type B profiles and vice versa. For a list of methods and a discussion of these observations, see Horne and Muir (1990). Interestingly, several of these methods involve manipulating the levels of milk salts, particularly Ca and phosphate. For many years, it was considered that differences in the heat stability of milk were due to variations in the composition of milk salts and this led Sommer and Hart (1919) to propose the salt balance theory referred to above in our discussion of ethanol stability. O'Connell and Fox (2003) have suggested that subsequent attempts to correlate heat stability with natural variations in the composition of milk salts failed because the original studies were based on deliberate additions of salts to milk at levels outside natural variability. This overlooks the fact that the experiments of Sommer and co-workers employed a different protocol for the heat stability assay, namely a measurement of the heat coagulation temperature, the temperature at which milk instantaneously coagulates (i.e. effectively coagulates within a short time, <2 min). Because it is a measure of instantaneous coagulation, it is unaffected by changes that occur on prolonged heating. Instead, the response to changing pH, as observed by Miller and Sommer (1940), is remarkably similar to the sigmoidal ethanol stability/pH profile. Moreover, the addition of Ca shifts this profile to more alkaline values while the addition of phosphate has the opposite effect of producing an acidic shift, just like the response of ethanol stability profiles. Horne and Muir (1990) suggested that such behaviour indicated that heat-induced coagulation as measured by this assay might follow a similar, if not identical, pathway to alcohol-induced coagulation as described above, involving the precipitation of Ca phosphate and a decrease in Ca activity with increasing pH. Such a scenario also ties in with the observation that the amount of free Ca^{2+} has been associated by various authors with the heat stability of milk, powdered milk and recombined milk (Augustin and Clarke, 1990; Singh and Creamer, 1992; Williams *et al.*, 2005). Addition of Ca to milk results in a decrease in heat stability due to the increase in free [Ca^{2+}] (Philippe *et al.*, 2004). Seasonal changes in milk salts (soluble Ca) have been correlated with the heat stability of milk (Kelly *et al.*, 1982). Salts, such as orthophosphates, are often added to milk concentrates (or ultra-high-temperature sterilized milks) during processing to improve heat stability. Orthophosphates reduce the Ca^{2+} activity, which is mainly responsible for the improved heat stability (Augustin and Clarke, 1990). O'Connell and Fox (2001) suggested that heat-induced precipitation of CCP is involved in the thermal coagulation of milk and that the specific effect of β-lactoglobulin at the pH of maximum stability may be related to its ability to chelate Ca.

9.7.8. Cheese Texture and Functionality

The importance of calcium and phosphate interactions for cheese manufacturing properties, as well as the textural properties, has been reviewed (Lucey and Fox, 1993; McMahon and Oberg, 1998; Lucey *et al*., 2003; Johnson and Lucey, 2006). The process cheese industry is based on the use of citrate or phosphate salts to sequester some of the Ca from the residual CCP, which solubilizes caseins that can then emulsify fat globules. The acidity of whey at drainage and rate of acid development are recognized as important parameters that determine the mineral content, acidity and quality of cheese. Schulz (1952) developed a classification of cheese varieties based on their Ca contents. Monib (1962) was one of the first investigators to study the Ca phosphate–casein complex in cheese and he concluded that very dilute cheese extracts did not represent cheese-like conditions and their use would lead to incorrect conclusions about serum Ca concentrations (i.e. excessive dilution resulted in the dissolution of more insoluble Ca). By the 1980s, it was recognized that acid development during manufacture determines the loss of Ca, which determines the basic structure of cheese (e.g. Lawrence *et al*., 1983). By the early 1990s, there was the realization that much of the residual Ca in cheese is associated with casein and that much of the CCP was not dissolved during cheesemaking (Lucey and Fox, 1993). It was also recognized that the residual insoluble Ca component is an important structural unit influencing cheese texture (Lucey and Fox, 1993). Many studies have demonstrated the importance of pH and Ca content on the functional properties of cheese (e.g. Yun *et al*., 1993; McMahon and Oberg, 1998; Guinee *et al*., 2002; Joshi *et al*., 2002). It is now accepted that during ripening there are important changes in the amount of insoluble Ca (e.g. Guo and Kindstedt, 1995; Hassan *et al*., 2004) and that these shifts in the Ca equilibrium contribute to textural changes during ripening (Lucey *et al*., 2005; O'Mahony *et al*., 2005). The proportion of insoluble Ca in cheese has been estimated by the expression of some of the aqueous phase ("juice") under high hydraulic pressure (Morris *et al*., 1988; Lucey and Fox, 1993), centrifugation to extract some expressible serum in young, high-moisture cheeses (Guo and Kindstedt, 1995), acid–base buffering (Lucey and Fox, 1993; Hassan *et al*., 2004) and water extraction methods (Metzger *et al*., 2001).

9.8. Other Uses/Applications of Milk Salts

Milk minerals (typical composition: <5% protein, <9% lactose, >70% ash, 25% Ca, 14% phosphorus) are produced by concentrating and drying deproteinized delactosed whey. This ingredient is often used for mineral

fortification purposes in a range of food products. A number of biologically active peptides are released during digestive breakdown of caseins and they play a physiological role in newborn mammals (Kitts, 2006). Casein phosphopeptides (CPP) are resistant to further hydrolysis by mammalian digestive enzymes and accumulate in the small intestine. CPP renders Ca^{2+} in a relatively soluble form for a potential enhanced bioavailability by paracellular (passive) mechanisms. CPPs are produced commercially by a number of dairy companies and used as a nutritional ingredient to enhance mineral absorption as well as provide anticariogenic benefits (Reynolds, 1999; Tsuchita *et al.*, 2001).

9.9. Concluding Remarks

Milk salts play a critical role in the formation and stability of casein micelles. Milk salts influence many of the important functional properties of milk products including gelation, protein stability, emulsification, foaming and cheese texture. The concentration of milk salts can be varied by processing conditions including acidification or the addition of metal chelators/sequestrants. The nature and structure of CCP is still under debate. The manipulation of the amount of insoluble Ca in cheese is the major focus of ongoing studies related to controlling cheese performance. There is growing awareness of the nutritional benefits of Ca and P, which has resulted in the fortification of many dairy products like cheese with Ca.

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10

Nutritional Aspects of Minerals in Bovine and Human Milks

C.D. Hunt and F.H. Nielsen

10.1. Introduction

This review summarizes the nutritional aspects of the 21 mineral elements present in bovine and human milks and considered essential or beneficial for human health (Table 10.1). This includes discussion of their respective physiological roles, signs of deficiency and toxicity, current recommended intakes, chemical presence as compounds that affect bioavailability and utilization and known enhancers and/or inhibitors of their absorption. The term "mineral" is not an accurate descriptor for the chemical nature of some of these elements but is a widely accepted terminology in the field of nutrition. The mineral elements occur in the body in one or more chemical forms, including inorganic ions and salts, complexes or constituents of organic molecules.

Fourteen of the minerals present in bovine and human milks (calcium, chloride, cobalt, copper, iodine, iron, magnesium, manganese, molybdenum, sodium, phosphorus, potassium, selenium and zinc) have well-established essential physiological functions that range from structural components of body tissues to essential components of many enzymes and other biologically important molecules. Another seven minerals (arsenic, boron, chromium, fluorine [as fluoride], nickel, silicon and vanadium) are not considered essential but may be beneficial, based on the evidence that they have a role in some physiological processes in one or more mammalian species.

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Table 10.1. Mean and/or range of concentration (per L) of minerals in mature human milk, bovine milk and humanized infant formulae

Mineral	Mature human milk, term pregnancy; ≤12 wk postpartum	Reference	Bovine milk	Reference	Humanized infant formula	Reference
Arsenic (μg)	0.2–0.6	(Anke, 1986)	20–60	(Anke, 1986)		
Boron (μg)	28–30	(Hunt *et al.*, 2004)	280	(Hunt and Meacham, 2001)	120[a]	(Hunt and Meacham, 2001)
	30–50	(Hunt *et al.*, 2005)				
Calcium (mg)	279	(Fransson and Lonnerdal, 1983)	1060	(Pennington *et al.*, 1987)	560[a]	(Hunt and Meacham, 2001)
	294	(Butte *et al.*, 1987)	1130	(Hunt and Meacham, 2001)	510[c]	(US Department of Agriculture Agricultural Research Service, 2007)
	250	(Yamawaki *et al.*, 2005)	1043–1283	(Gaucheron, 2005)		
	268–281	(Hunt *et al.*, 2005)				
	250	(Mastroeni *et al.*, 2006)				
Chloride (mg)	359	(Yamawaki *et al.*, 2005)	772–1207	(Gaucheron, 2005)		
Chromium (ng)	250	(Casey *et al.*, 1985)	<500	(Anderson *et al.*, 1992)		
Cobalt (μg)	0–6	(Friel *et al.*, 1999)				

(*Continued*)

Table 10.1. (*Continued*)

Mineral	Mature human milk, term pregnancy; ≤12 wk postpartum	Reference	Bovine milk	Reference	Humanized infant formula	Reference
Copper (μg)	443	(Alkanani *et al.*, 1994)	100	(Fransson and Lonnerdal, 1983)	620[a]	(Hunt and Meacham, 2001)
	400–710	(Al-Awadi and Srikumar, 2000)	90	(Pennington *et al.*, 1987)	500	(US Department of Agriculture Agricultural Research Service, 2007)
	360–650	(Hunt *et al.*, 2004)	90	(Hunt and Meacham, 2001)		
	120–160	(Domellof *et al.*, 2004)				
	350	(Yamawaki *et al.*, 2005)				
Fluoride (μg)	19	(Koparal *et al.*, 2000)	22	(Koparal *et al.*, 2000)	76–1053	(Buzalaf *et al.*, 2004)
	17	(Chuckpaiwong *et al.*, 2000)	80	(Atac *et al.*, 2001)	21–118	(Koparal *et al.*, 2000)
					101	(Atac *et al.*, 2001)
Iodine (μg)	142; 21–281	(Bruhn and Franke, 1983)	178; 48–661	(Bader *et al.*, 2005)		
	50–60	(Parr *et al.*, 1991)	140–195	(Li *et al.*, 2006)		
	94	(Bazrafshan *et al.*, 2005)				

(*Continued*)

Table 10.1. (*Continued*)

Mineral	Mature human milk, term pregnancy; ≤12 wk postpartum	Reference	Bovine milk	Reference	Humanized infant formula	Reference
	22	(Skeaff *et al.*, 2005)				
	169; 33–348	(Bader *et al.*, 2005)				
	155 (median); 27–1968	(Pearce *et al.*, 2007)				
Iron (μg)	308; 242–160	(Butte *et al.*, 1987)	290	(Fransson and Lonnerdal, 1983)	11700[a]	(Hunt and Meacham, 2001)
	270–430	(Al-Awadi and Srikumar, 2000)	700	(Pennington *et al.*, 1987)	4300[b]	(Hunt and Meacham, 2001)
	225–355	(Hunt *et al.*, 2004)	200	(Hunt and Meacham, 2001)	11800[c]	(US Department of Agriculture Agricultural Research Service, 2007)
	210–290	(Domellof *et al.*, 2004)				
	900	(Mastroeni *et al.*, 2006)				
Manganese (μg)	4–6	(Al-Awadi and Srikumar, 2000)	40	(Pennington *et al.*, 1987)	70[a]	(Hunt and Meacham, 2001)
	6		20		100[c]	

(*Continued*)

Table 10.1. (*Continued*)

Mineral	Mature human milk, term pregnancy; ≤12 wk postpartum	Reference	Bovine milk	Reference	Humanized infant formula	Reference
		(Alkanani *et al.*, 1994)		(Hunt and Meacham, 2001)		(US Department of Agriculture Agricultural Research Service, 2007)
	11	(Yamawaki *et al.*, 2005)				
Magnesium (mg)	35	(Fransson and Lonnerdal, 1983)	117	(Fransson and Lonnerdal, 1983)	50[a]	(Hunt and Meacham, 2001)
	30	(Butte *et al.*, 1987)	98	(Pennington *et al.*, 1987)	50[c]	(US Department of Agriculture Agricultural Research Service, 2007)
	27	(Yamawaki *et al.*, 2005)	97–146	(Gaucheron, 2005)		
	29–33	(Hunt *et al.*, 2005)				
	30	(Mastroeni *et al.*, 2006)				
Molybdenum (μg)	5	(Alkanani *et al.*, 1994)	97	(Hunt and Meacham, 2001)	55[a]	(Hunt and Meacham, 2001)
	0–4	(Friel *et al.*, 1999)				
Nickel (μg)	16	(Alkanani *et al.*, 1994)				

(*Continued*)

Table 10.1. (*Continued*)

Mineral	Mature human milk, term pregnancy; ≤12 wk postpartum	Reference	Bovine milk	Reference	Humanized infant formula	Reference
	0–28	(Friel *et al.*, 1999)				
	11–16	(Parr *et al.*, 1991)				
Phosphorus (mg)	153	(Butte *et al.*, 1984)	830	(Pennington *et al.*, 1987)	440[a]	(Hunt and Meacham, 2001)
	150	(Yamawaki *et al.*, 2005)	880	(Hunt and Meacham, 2001)	350[c]	(US Department of Agriculture Agricultural Research Service, 2007)
	137	(Mastroeni *et al.*, 2006)	930–992	(Gaucheron, 2005)		
Potassium (mg)	430–527	(Dewey and Lonnerdal, 1983)	1340	(Pennington *et al.*, 1987)	820[a]	(Hunt and Meacham, 2001)
	443	(Butte *et al.*, 1987)	1480	(Hunt and Meacham, 2001)	710[c]	(US Department of Agriculture Agricultural Research Service, 2007)
	470	(Yamawaki *et al.*, 2005)				
	462	(Mastroeni *et al.*, 2006)				
Selenium (μg)	12–20	(Arnaud *et al.*, 1993)	17–23	(al-Saleh *et al.*, 1997)	18[c]	(US Department of Agriculture Agricultural Research Service, 2007)

(*Continued*)

Table 10.1. (*Continued*)

Mineral	Mature human milk, term pregnancy; ≤12 wk postpartum	Reference	Bovine milk	Reference	Humanized infant formula	Reference
	112 (seleniferous region)	(Bratter *et al.*, 1997)	22	(Muniz-Naveiro *et al.*, 2005)		
	14	(Bianchi *et al.*, 1999)	19	(Juniper *et al.*, 2006)		
	19–27	(Hunt *et al.*, 2004)				
	17	(Yamawaki *et al.*, 2005)				
Sodium (mg)	134–264	(Dewey and Lonnerdal, 1983)	420	(Pennington *et al.*, 1987)	170[a]	(Hunt and Meacham, 2001)
	168	(Butte *et al.*, 1984)	350	(Hunt and Meacham, 2001)	200[c]	(US Department of Agriculture Agricultural Research Service, 2007)
	112	(Butte *et al.*, 1987)	391–644	(Gaucheron, 2005)		
	135	(Yamawaki *et al.*, 2005)				
	205	(Mastroeni *et al.*, 2006)				
Zinc (mg)	0.46–0.70	(Domellof *et al.*, 2004)	3.50	(Fransson and Lonnerdal, 1983)	5.70[a]	(Hunt and Meacham, 2001)

(*Continued*)

Table 10.1. (*Continued*)

Mineral	Mature human milk, term pregnancy; ≤12 wk postpartum	Reference	Bovine milk	Reference	Humanized infant formula	Reference
	1.19–4.06	(Hunt *et al.*, 2004)	3.70	(Pennington *et al.*, 1987)	6.6[c]	(US Department of Agriculture Agricultural Research Service, 2007)
	1.45	(Yamawaki *et al.*, 2005)	3.90	(Hunt and Meacham, 2001)		
	1.00–2.30	(Hunt *et al.*, 2005)				
	1.50	(Mastroeni *et al.*, 2006)				

[a] Infant formula, Gerber, with iron, ready-to-feed
[b] Infant formula, Gerber, iron low, ready-to-feed
[c] Infant formula, Mead Johnson, Enfamil Lipil, with iron, ready-to-feed, arachidonic acid (ARA) and docosahexaenoic (DHA) acid.

Several other mineral elements (aluminum, bromine, cadmium, cesium, lead, lithium, strontium, mercury, silver, rubidium and tin) are present normally in very low amounts in bovine and human milks. They are considered important because of their high orders of toxicity but are not reviewed here because of the lack of compelling evidence for their beneficial functions. All essential or beneficial minerals are toxic when ingested in excess. Therefore, an upper limit (UL) of intake has been established for each (Table 10.2). Because individual ULs are generally well above the total amounts supplied by typical diets, the toxicological aspects of each mineral are discussed only when they are considered relevant.

Human milk and bovine milk are markedly different in several aspects, including mineral content (Table 10.1). Furthermore, the bioavailability of most minerals in human milk is much higher than from bovine milk or infant formula (Emmett and Rogers, 1997). Frequent revision of humanized infant formulae to match the nutritive value of human milk complicates comparisons and notable current differences in content or bioavailability are identified in the discussion. This discussion focuses on mature human milk which has a mineral content considerably different from that of early and transitional human milks. In human milk, the concentrations of most minerals remain fairly constant throughout the course of lactation. Notable exceptions are zinc, copper and iron, but even these elements have predictable patterns of change over time, falling for several months after parturition (Emmett and Rogers, 1997).

Mineral concentrations in human milk are resistant to changes in maternal dietary mineral intakes (except for selenium; Kumpulainen *et al*., 1985), age, parity or lactational history (Butte *et al*., 1987). Differences in mineral composition of milk between term and preterm mothers are not universal and the lack of agreement among investigators may be due to differences in sample collection, a wide range of gestational ages and greater individual variation in the composition of milk from mothers of premature infants (Friel *et al*., 1999). Reported values for the concentration of a given mineral in milk among laboratories are remarkably consistent (Table 10.1). Although each mineral is discussed separately, interactions of minerals with each other, with other constituents of milk and with other food constituents are considered to be of paramount importance.

10.2. Sodium, Chloride and Potassium

The cation sodium and the anion chloride are required to maintain extracellular volume and plasma osmolarity. Human populations have the capacity to survive at extremes of sodium intake from less than 0.2 g (10 mmol)/d by the

Table 10.2. Dietary reference intake values by life-stage group for the United States and Canada for arsenic, boron, calcium, chloride, chromium, cobalt, copper, fluoride, iodine, iron, magnesium, manganese, molybdenum, nickel, sodium, phosphorus, potassium, selenium, vanadium and zinc

Age/sex	As (mg/d)	B (mg/d)	Ca (mg/d)	Cl^- (g/d)	Cr (μg/d)	Cu (μg/d)	F^- (mg/d)	I (μg/d)	Fe (mg/d)	Mg (mg/d)	Mn (mg/d)	Mo (μg/d)	Ni (mg/d)	Na (g/d)	P (mg/d)	K (g/d)	Se (μg/d)	V (mg/d)	Zn (mg/d)
Recommended dietary allowance (RDA)																			
0–6 month	ND[1]	ND	210, AI[2]	0.18, AI	0.2, AI	200, AI	0.01, AI	110, AI	0.27, AI	30, AI	0.003, AI	2, AI	ND	0.12, AI	100, AI	0.4, AI	15, AI	ND	2, AI
7–12 month	ND	ND	270, AI	0.57, AI	5.5, AI	220, AI	0.5, AI	130, AI	11	75, AI	0.6, AI	3, AI	ND	0.37, AI	275, AI	0.7, AI	20, AI	ND	3
1–3 yr	ND	ND	500, AI	1.5, AI	11, AI	340	0.7, AI	90	7	80	1.2, AI	17	ND	1.0, AI	460	3.0, AI	20	ND	3
4–8 yr	ND	ND	800, AI	1.9, AI	15, AI	440	1, AI	90	10	130	1.5, AI	22	ND	1.2, AI	500	3.8, AI	30	ND	5
9–13 yr; M	ND	ND	1300, AI	2.3, AI	25, AI	700	2, AI	120	8	240	1.9, AI	34	ND	1.5, AI	1250	4.5, AI	40	ND	8
9–13 yr; F	ND	ND	1300, AI	2.3, AI	21, AI	700	2, AI	120	8	240	1.6, AI	34	ND	1.5, AI	1250	4.5, AI	40	ND	8
14–18 yr; M	ND	ND	1300, AI	2.3, AI	35, AI	890	3, AI	150	11	410	2.2, AI	43	ND	1.5, AI	1250	4.7, AI	55	ND	11
14–18 yr; F	ND	ND	1300, AI	2.3, AI	24, AI	890	3, AI	150	15	360	1.6, AI	43	ND	1.5, AI	1250	4.7, AI	55	ND	9
19–30 yr; M	ND	ND	1000, AI	2.3, AI	35, AI	900	4, AI	150	8	400	2.3, AI	45	ND	1.5, AI	700	4.7, AI	55	ND	11
19–30 yr; F	ND	ND	1000, AI	2.3, AI	25, AI	900	3, AI	150	18	310	1.8, AI	45	ND	1.5, AI	700	4.7, AI	55	ND	8
31–50 yr; M	ND	ND	1000, AI	2.3, AI	35, AI	900	4, AI	150	8	420	2.3, AI	45	ND	1.5, AI	700	4.7, AI	55	ND	11
31–50 yr; F	ND	ND	1000, AI	2.3, AI	25, AI	900	3, AI	150	18	320	1.8, AI	45	ND	1.5, AI	700	4.7, AI	55	ND	8
51–70 yr; M	ND	ND	1200, AI	2.0, AI	30, AI	900	4, AI	150	8	420	2.3, AI	45	ND	1.3, AI	700	4.7, AI	55	ND	11

(*Continued*)

Table 10.2. (*Continued*)

Age/sex	As (mg/d)	B (mg/d)	Ca (mg/d)	Cl^- (g/d)	Cr (μg/d)	Cu (μg/d)	F^- (mg/d)	I (μg/d)	Fe (mg/d)	Mg (mg/d)	Mn (mg/d)	Mo (μg/d)	Ni (mg/d)	Na (g/d)	P (mg/d)	K (g/d)	Se (μg/d)	V (mg/d)	Zn (mg/d)
51–70 yr; F	ND	ND	1200, AI	2.0, AI	20, AI	900	3, AI	150	8	320	1.8, AI	45	ND	1.3, AI	700	4.7, AI	55	ND	8
>70 yr; M	ND	ND	1200, AI	1.8, AI	30, AI	900	4, AI	150	8	420	2.3, AI	45	ND	1.2, AI	700	4.7, AI	55	ND	11
>70 yr; M	ND	ND	1200, AI	1.8, AI	20, AI	900	3, AI	150	8	320	1.8, AI	45	ND	1.2, AI	700	4.7, AI	55	ND	8
Pregnancy																			
14–18 yr	ND	ND	1300, AI	2.3, AI	29, AI	1000	3, AI	220	27	400	2.0, AI	50	ND	1.5, AI	1250	4.7, AI	60	ND	13
19–30 yr	ND	ND	1000, AI	2.3, AI	30, AI	1000	3, AI	220	27	350	2.0, AI	50	ND	1.5, AI	700	4.7, AI	60	ND	11
31–50 yr	ND	ND	1000, AI	2.3, AI	30, AI	1000	3, AI	220	27	360	2.0, AI	50	ND	1.5, AI	700	4.7, AI	60	ND	11
Lactation																			
14–18 yr	ND	ND	1300, AI	2.3, AI	44, AI	1300	3, AI	290	10	360	2.6, AI	50	ND	1.5, AI	1250	5.1, AI	70	ND	14
19–30 yr	ND	ND	1000, AI	2.3, AI	45, AI	1300	3, AI	290	9	310	2.6, AI	50	ND	1.5, AI	700	5.1, AI	70	ND	12
31–50 yr	ND	ND	1000, AI	2.3, AI	45, AI	1300	3, AI	290	9	320	2.6, AI	50	ND	1.5, AI	700	5.1, AI	70	ND	12
Upper limit (UL)																			
0–6 month	ND	ND	ND	ND	ND	ND	0.7	ND	40	ND	ND	ND	ND	ND	ND	ND	45	ND	4
7–12 month	ND	ND	ND	ND	ND	ND	0.9	ND	40	ND	ND	ND	ND	ND	ND	ND	60	ND	5
1–3 yr	ND	3	2.5	2.3	ND	1000	1.3	200	40	65	2	300	0.2	1.5	3000	ND	90	ND	7
4–8 yr	ND	6	2.5	2.9	ND	3000	2.2	300	40	110	3	600	0.3	1.9	3000	ND	150	ND	12
9–13 yr	ND	11	2.5	3.4	ND	5000	10	600	40	350	6	1100	0.6	2.2	4000	ND	280	ND	23
14–18 yr	ND	17	2.5	3.6	ND	8000	10	900	45	350	9	1700	1.0	2.3	4000	ND	400	ND	34
19–70 yr	ND	20	2.5	3.6	ND	10000	10	1100	45	350	11	2000	1.0	2.3	4000	ND	400	1.8	40
>70 yr	ND	20	2.5	3.6	ND	10000	10	1100	45	350	11	2000	1.0	2.3	3000	ND	400	1.8	40

(*Continued*)

Table 10.2. (*Continued*)

Age/sex	As (mg/d)	B (mg/d)	Ca (mg/d)	Cl^- (g/d)	Cr (μg/d)	Cu (μg/d)	F^- (mg/d)	I (μg/d)	Fe (mg/d)	Mg (mg/d)	Mn (mg/d)	Mo (μg/d)	Ni (mg/d)	Na (g/d)	P (mg/d)	K (g/d)	Se (μg/d)	V (mg/d)	Zn (mg/d)
Pregnancy																			
14–18 yr	ND	17	2.5	3.6	ND	8000	10	900	45	350	9	1700	1.0	2.3	3500	ND	400	ND	34
19–50 yr	ND	20	2.5	3.6	ND	10000	10	1100	45	350	11	2000	1.0	2.3	3500	ND	400	ND	40
Lactation																			
14–18 yr	ND	17	2.5	3.6	ND	8000	10	900	45	350	9	1700	1.0	2.3	4000	ND	400	ND	34
19–50 yr	ND	20	2.5	3.6	ND	10000	10	1100	45	350	11	2000	1.0	2.3	4000	ND	400	ND	40

[1] ND, not determinable because of lack of data of adverse effects in this age group and concern with regard to lack of ability to handle excess amounts. Source of intake should be from food only to prevent high levels of intake.

[2] AI, adequate intake was established because of insufficient data to establish an RDA.

Yanomamo Indians of Brazil to over 10.3 g (450 mmol)/d in Northern Japan (Food and Nutrition Board: Institute of Medicine, 2005). Under normal circumstances, dietary deficiency of sodium or chloride does not occur, but the body can be depleted of sodium and chloride under extreme conditions, e.g., heavy perspiration, chronic diarrhea or renal disease. Chlorine deficiency in breast-fed infants is rare, and it is generally accepted that the chlorine level of breast milk is not affected by maternal diet (Lonnerdal, 1986). The ability to survive at extremely low levels of sodium intake reflects the capacity of the normal human body to conserve sodium by markedly reducing losses of sodium in the urine and sweat. Under conditions of maximal adaptation and without sweating, the minimal amount of sodium required to replace losses is around 0.18 g (8 mmol)/d (Food and Nutrition Board: Institute of Medicine, 2005). Still, it is unlikely that a diet providing this level of sodium is sufficient to meet dietary requirements for other nutrients.

Because of insufficient data from dose–response trials, an Estimated Average Requirement (EAR) for sodium could not be established by the U.S. Food and Nutrition Board (FNB). Thus, a Recommended Dietary Allowance (RDA) could not be derived for any sex–age group. Hence, only Adequate Intakes (AIs) are provided (Table 10.2). For example, the AI for sodium for infants aged 0–6 months is based on the average amount of sodium in human milk. For young adults, it is the amount that ensures that the overall diet provides an adequate intake of other important nutrients and to cover sweat losses of sodium in unacclimatized individuals who are exposed to high temperatures or who become physically active. This AI does not apply to individuals who lose large volumes of sodium in sweat. The AI for chloride is set at a level equivalent to that of sodium on a molar basis.

Bovine milk contributes only 7% of dietary sodium in the United Kingdom, but some dairy products, such as cheese and butter, contain added salt and can be significant sources of sodium in some countries (e.g., about 13% of total sodium intake in the United Kingdom; Hazell, 1985). It has been estimated that milk and dairy products provide 20% of total sodium in the diet in Ireland and the United Kingdom (Flynn *et al.*, 1990). Mature human milk contains considerably less sodium and chloride than bovine milk (Table 10.1). No relationship has been demonstrated between maternal dietary sodium or chloride intakes and the concentrations of these electrolytes in milk (Ereman *et al.*, 1987). The chloride concentration in bovine milk increases sharply toward the end of lactation and is independent of dietary intake. Sodium and chloride are believed to be present in milk almost entirely as free ions (Holt, 1993). Almost all sodium and chloride in milk are absorbed in the gastrointestinal tract, although much of what is absorbed is not retained.

In the young infant, clinical problems, including dehydration, may arise if there are excessive intakes of sodium and chloride. In these individuals, the

capacity to concentrate solids is limited and the renal solute load exerts a major effect on water balance. Renal solute load is determined mainly by sodium, chloride, potassium, phosphorus and protein. Bovine milk has a much higher potential renal solute load (~300 mOsmol/l) than human milk (~93 mOsmol/l) (Ziegler and Fomon, 1989). The high renal solute load resulting from the ingestion of bovine milk may be of relatively little significance in healthy growing infants without increased evaporative water losses, because the kidney excretes a more concentrated urine. However, this reduces the margin of safety against dehydration that can occur in conditions of diarrhea, fever or low water intake. For this reason, it is recommended that the upper limit of potential renal solute load in formulae for young infants should be about 220 mOsmol/l (Ziegler and Fomon, 1989).

Potassium plays many roles in the body (Committee on Minerals and Toxic Substances in Diets and Water for Animals, 2005; Preuss, 2006), including acid–base balance, maintenance of osmotic pressure and blood pressure, cellular uptake of amino acids and as a co-factor or activator in many enzyme systems. Potassium has a critical role in membrane transport and carbohydrate and energy metabolism. Cellular membrane polarization depends upon the internal and external concentrations of potassium. As a result, the major clinical disturbances of severe abnormal potassium status usually are associated with an altered membrane function, especially in neuromuscular and cardiac conduction systems. Both deficient (hypokalemia) and excess (hyperkalemia) circulating potassium results in disorders in cardiac, muscle and neurological function (Preuss, 2006). Adverse effects of hypokalemia include cardiac arrhythmias, muscle weakness and glucose intolerance. Adverse effects of moderate potassium deficiency without hypokalemia include increased blood pressure, salt sensitivity and bone turnover. Cardiac arrest caused by abnormal electrical conduction is the most serious clinical manifestation of hyperkalemia. Neuromuscular symptoms of potassium excess include tingling, paresthesia, weakness and flaccid paralysis.

The FNB determined that an EAR could not be established for potassium, so a RDA could not be derived (Food and Nutrition Board: Institute of Medicine, 2001). The FNB also stated that the health effects of potassium intake in infants and children are uncertain. Thus, only an AI was set that reflected a calculated mean potassium intake through human milk (age 0–6 months) or a combination of human milk and complementary foods (age 7–12 months). The AI for children was derived by extrapolating from the adult AI of 4.7 g (120 mmol) potassium/d (Table 10.2).

The adult human body contains about 45 mmol K/kg body weight or about 3150 mmol (1230 g) for a 70 kg adult (Preuss, 2006). Intracellular potassium accounts for 98% of the potassium and for 75% of total intracellular cations. Because extracellular fluid contains only 2% of the potassium in the

body, plasma potassium is a poor indicator of tissue concentrations. The average concentration of potassium in mature human milk is 0.5 g/l, which is considerably less than that of bovine milk (Food and Nutrition Board: Institute of Medicine, 2001). Lactating dairy cows have a very high dietary potassium requirement (10 g/kg dry matter), and potassium is the mineral element found in the highest concentration in milk (1.5 g/l) (Hunt and Meacham, 2001; Committee on Minerals and Toxic Substances in Diets and Water for Animals, 2005). Potassium concentration in bovine colostrum is higher than that in mature milk (Ontsouka *et al.*, 2003). No relationship between maternal dietary potassium and its concentration in milk has been demonstrated. Almost all potassium in milk is absorbed from the digestive tract (Flynn, 1992).

Bovine milk and dairy products can be major contributors to the total dietary intake of potassium (24–29% in Ireland and the United Kingdom; Flynn *et al.*, 1990), because the richest sources, vegetables and fruits, often are not consumed in recommended amounts. Potassium deficiency is unlikely unless excessive alimentary (e.g., diarrhea) or renal (e.g., diuretic use) losses occur because many foods contain significant amounts of potassium (Preuss, 2006). The dietary intake of potassium in the United States and Canada, however, is considerably lower than the AI, which was based on the need to blunt the severe salt sensitivity prevalent in African-American men, and decreasing the risk of kidney stones (Food and Nutrition Board: Institute of Medicine, 2001). A diet rich in fruits, vegetables and milk would assure a healthy intake of potassium.

10.3. Calcium

Calcium is the most abundant mineral in the body (~1000 g adults) (Favus *et al.*, 2006). Approximately 1% of total body calcium is found in extracellular fluids, intracellular structures and cell membranes where it serves as a second messenger, coupling intracellular responses to extracellular signals (Awumey and Bukoski, 2006; Kirchhoff and Geibel, 2006). As such, calcium mediates muscle contraction, nerve transmission and glandular secretion. The remaining 99% of total body calcium is found in bones and teeth where its chemical properties are indispensable for skeletal function and dental structure and function.

Bone crystals (an analogue of the geological mineral, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]) have a significant (~39%) calcium content. These crystals, with the ability to resist compression, are arrayed in a protein matrix that has the ability to withstand tensile loads. Changes in either the inorganic (e.g., hydroxyapatite) or the organic (e.g., collagen) matrix components can alter

bone strength (Rubin and Rubin, 2006). The bone calcium deposition rate *in vivo* (V_o+) during early prepuberty (8.3 yr) was estimated at 1504 mg/d; puberty (10.2 yr), 1952 mg/d (Abrams *et al*., 2000); menarche, 3000 mg/d; and postmenarche (60 months), 1000 mg/d (Abrams *et al*., 1996). Accordingly, the rate of calcium deposition is much higher than either calcium consumption (~1200 mg/d) or the gut absorption (~400 mg/d) observed in human studies (Bronner and Abrams, 1998; Abrams *et al*., 2000). Skeletal tissue is replaced every 10–12 yr, on average (Heaney, 2006), because the skeleton is a metabolically active organ and must undergo continuous remodeling throughout life to adapt its internal microstructure to changes in the mechanical and physiological environment. Furthermore, bone is renewed continuously to repair micro-damage to minimize the risk of fracture. If the bone repairing function is slower than micro-damage accumulation, a "stress fracture" (very small, incomplete fracture) finally occurs (Garcia-Aznar *et al*., 2005).

Factors that contribute to skeletal fragility include suboptimal calcium intake, genetics, lifestyle, smoking, decrease in sex hormone production and certain medications. Cases of rickets caused by calcium deficiency have been reported in developed (Davidovits *et al*., 2006) and developing (Thacher *et al*., 2006) countries. Age-related osteoporosis is a major metabolic bone disease of unknown but apparent multifactorial etiology with nutritional, lifestyle, genetic and endocrine components. It is characterized by compromised bone strength that predisposes a person to increased fracture risk. A diagnosis of osteoporosis requires both low bone mass and a preexisting loss of bone tissue (Harvey *et al*., 2006). A distinguishing characteristic of this disease, compared to osteomalacia, is a normal mineral/collagen ratio (Looker *et al*., 1993). One in two women and one in five men who are 50 yr of age will have an osteoporotic fracture in their remaining lifetime (Harvey *et al*., 2006).

Calcium intake recommendations vary widely worldwide, with that of the US National Academy of Sciences (NAS) among the highest. The NAS established AIs, rather than EARs or RDAs, for Ca (Table 10.2). This decision was based on several concerns, including uncertainties in the precision and significance of balance studies needed to determine a desirable retention model and lack of concordance between mean Ca intake and experimentally derived values predicted to achieve a desirable level of Ca retention (Food and Nutrition Board: Institute of Medicine, 1997). NAS recommendations for pregnancy and lactation are specific for the age of the mother. The dietary reference nutrient intake values for calcium in the United Kingdom are lower than in the United States: ages 0–12 months, 525 mg (17.1 mmol); 1–3 yr, 350 mg (11.4 mmol); 4–6 yr, 450 mg (14.6 mmol); 7–10 yr, 550 mg (17.9 mmol); 11–18 yr (male), 1000 mg (32.5 mmol);

11–18 yr (female), 800 mg (26.0 mmol); and 19+ yr, 700 mg (22.8 mmol)/d (Francis, 2008). Dietary allowance of calcium recommended by the Indian Council of Medical Research is 400 mg/d for adults (Harinarayan *et al*., 2007). A recent analysis of primary calcium balance data from a group of separate, but tightly controlled metabolic feeding studies, indicates a balance point of 741 mg/d and a presumptive RDA of 1035 mg/d for calcium for men and women (Hunt and Johnson, 2007). Several countries and organizations, including the United States and the European Community, have identified an upper limit for calcium (2500 mg/d) (Looker, 2006).

Calcium intakes vary considerable among countries. In the United States, mean usual calcium intakes from food calculated for males from NHANES 2001–2002 data (Moshfegh *et al*., 2005) were as follows: ages 1–3 yr, 972 mg; 4–8 yr, 960 mg; 9–13 yr, 1139 mg; 19–30 yr, 1098 mg; 31–50 yr, 1021 mg; 51–70 yr, 874; and 51+ yr, 817 mg/d. For females: ages 1–3 yr, 972 mg; 4–8 yr, 960 mg; 9–13 yr, 865 mg; 19–30 yr, 784 mg; 31–50 yr, 755 mg; 51–70 yr, 701; and 51+ yr, 666 mg/d. Calcium intake data from the UK Women's Cohort Study (Cade *et al*., 2004) with 35,372 women, aged 35–69 yr, indicate that British women consume a mean of 1141 mg calcium per day. Average calcium intake was 558 mg/d in 647 subjects from Reus, Spain (Garcia-Lorda *et al*., 2007). Calcium intake data (four standardized 24-h dietary recalls collected 3–6 weeks apart) for 4680 men and women, aged 40–59 yr, in Japan, China, the United Kingdom and the United States indicated mean daily calcium intakes of 605, 356, 1013 and 882 mg in those countries, respectively (Zhou *et al*., 2003). Healthy urban and rural men of Tirupati, southern Andhra Pradesh, India, consumed on average 323 and 271 mg calcium per day, respectively, similar to women living in the same urban and rural areas (306 and 262 mg/d, respectively) (Harinarayan *et al*., 2007).

In the United States, the current calcium AI for infants 0–6 months of age is based on the reported intake of milk and on the reported average calcium concentration in human milk after 1 month of lactation. For infants aged 7–12 months, the AI is based on the average calcium intake from human milk and solid foods. For females aged 1–8 yr, calcium accretion in the range of 60–200 mg/d has been predicted. Therefore, the AI for children aged 4–8 yr reflects data from balance studies with girls (and findings extrapolated to boys) that indicate that a calcium intake of 800–900 mg/d would result in mean calcium retention of up to 174 mg/d. These data were extrapolated to set the AI for younger children aged 1–3 yr.

Adequate dietary calcium intake is required to achieve full accretion of bone mass prescribed by genetic potential. On the other hand, the efficacy of calcium supplementation in healthy children as a public health intervention remains highly controversial. Catch-up mineralization later in puberty

appears likely if calcium intake is consistent with usual average intakes in the United States (Abrams, 2005). For example, a recent meta-analysis of 19 randomized controlled trials involving 2859 children (aged 3–18 yr) examined the effects of the addition of 300–1200 mg calcium per day from supplementation or dairy products (Winzenberg *et al*., 2006). This analysis concluded that there was no significant persistent effect of calcium supplementation on femoral neck or lumbar spine bone mineral density (BMD). A small long-term effect of supplementation on BMD in the upper limb seemed unlikely to reduce the risk of fracture, either in childhood or in later life, to a degree of major public health importance. During sexual maturation (~9–18 yr), calcium retention increases to a peak and then declines, a physiological phenomenon that complicates derivation of estimates of calcium needs. The AI for this age group has been estimated from three major lines of evidence: (1) a factorial approach (summation of calcium needs for growth plus calcium losses with adjustments for absorption), (2) calcium retention to meet peak bone mineral accretion and (3) clinical trials in which bone mineral content was measured in response to variable calcium intakes. There is no consensus as to when peak bone mass occurs (Nilas, 1993) and there may be an overall increase, not decrease, in total skeletal mass of about 4% between 18 and 50 yr (Matkovic *et al*., 1994). The AI for calcium for men and women aged 19–50 yr is based on balance studies and bone mineral density data.

It is well recognized that calcium has a limited role in maintaining bone health because calcium adequacy alone does not provide full protection against bone loss (Shapiro and Heaney, 2003) associated with age and menopause (Heaney, 2006). In perimenopausal women, significant metacarpal and lumbar bone loss occurs even with supplements of 1000 or 2000 mg calcium per day (Elders *et al*., 1991). Spinal BMD lost at menopause can be attributed completely to estrogen deprivation, and femoral neck losses are a composite of estrogen deprivation and age-related loss (Recker *et al*., 2000). The AI for calcium for men and women aged $\geq$51 is based on the assumption that individuals in this age range would have calcium needs somewhat higher than that of the 19- through 30-yr age group (Food and Nutrition Board: Institute of Medicine, 1997). However, among the several studies of calcium intake and fracture risk, no consistent association has been demonstrated between reported calcium intake over periods of up to 10 yr and fractures (Cumming and Nevitt, 1997; Dawson-Hughes, 2006; Jackson *et al*., 2006), the only sequela of importance in osteoporosis. Also, most (Kochersberger *et al*., 1990; Chapuy *et al*., 1992; Chevalley *et al*., 1994; Dawson-Hughes *et al*., 1997; 2000; Hitz *et al*., 2007), but not all (Phang *et al*., 1969), studies on adults indicating a positive influence of high dietary Ca in reducing the rate of bone remodeling were confounded by the presence of vitamin D as an experimental co-variable.

Evidence to support a role for dietary calcium in the prevention of a number of diseases other than osteoporosis (Heaney, 1996; Food and Nutrition Board: Institute of Medicine, 1997; Weaver, 2006) remains controversial. Higher calcium intake (>1250 mg/d versus ≤500 mg/d) is associated with a reduced risk of distal, but not proximal, colon cancer, but calcium intakes beyond moderate levels were not associated with a further risk reduction (Wu *et al*., 2002). Calcium supplementation (1200 mg/d) was associated with a significant, though moderate, reduction (31 versus 38%) in the risk of recurrent colorectal adenomas (Baron *et al*., 1999). On the other hand, elevated calcium intake (> 2000 mg/d) was related positively to advanced prostate cancer (Giovannucci *et al*., 1998), a finding not in agreement with a similar prospective study (Schuurman *et al*., 1999) that found no positive association between calcium intake and prostate cancer risk. Dietary calcium was associated with reduced incidence of kidney stones in an observational study on men (Curhan *et al*., 1993). However, in a separate study on women, moderate intake of supplemental calcium appeared to increase risk for symptomatic kidney stones whereas high intake of dietary calcium appeared to reduce that risk (Curhan *et al*., 1997). In a separate study with healthy postmenopausal women supplemented with 1000 mg calcium per day and 400 IU vitamin D per day, the subjects had increased risk of kidney stones and did not have significantly reduced hip fracture (Jackson *et al*., 2006). A meta-analysis of randomized, controlled calcium supplementation trials indicated that calcium supplementation leads to a small reduction in systolic, but not diastolic, blood pressure (Bucher *et al*., 1996). Even so, increasing attention has been given to the influence of dietary patterns rather than individual nutrients on disease prevention (Appel *et al*., 1997).

Milk products, the most calcium-dense foods in Western diets, contain about 300 mg (7.5 mmol) calcium per serving (244 g of milk or yogurt or 42.5 g of Cheddar cheese) (Food and Nutrition Board: Institute of Medicine, 1997). Bovine milk and other dairy products, such as cheese and yoghurt, provided 84% of the dietary calcium in the United States in 1989–1993. However, milk is being replaced by sweetened soft drinks and juices such that Americans drank nearly 2.5 times more soda than milk in 2001 (Weaver and Heaney, 2006). Grains are not particularly rich in calcium, but because they may be consumed in large quantities, they can account for a substantial proportion of dietary calcium. Among Mexican American adults, corn tortillas are the second most important food source of calcium, after milk (Looker *et al*., 1993).

The concentration of calcium in mature human milk is 250–300 mg/l (Butte *et al*., 1984; Hunt, 1988; Lonnerdal, 1997), and findings from several longitudinal studies indicate that the calcium concentration in breast milk is

stable (Kirksey *et al.*, 1979; Lonnerdal, 1997), exhibits a transient decrease (Friel *et al.*, 1999), or decreases slightly but significantly (Hunt *et al.*, 2005) during the first 4 months of lactation. Generally, the maternal diet is not believed to affect the concentration of calcium in breast milk (Lonnerdal, 1986). However, there is some evidence that calcium levels in breast milk are lower than normal during extended lactation (Laskey *et al.*, 1990) and that breast milk of lactating young teenage mothers may have lower calcium concentrations than that of older women (Lipsman *et al.*, 1985). The calcium content of human milk is considerably lower than that of bovine milk (~1100 mg/l) (Table 10.1) and also that of milk-based (~500 mg/l) (Hunt and Meacham, 2001) or soy-based (~600 mg/l) formulae. Because formula-fed infants usually consume higher volumes than breast-fed infants, they receive at least twice the amount of calcium that breast-fed infants ingest (Lonnerdal, 1997). The fundamental concept that the body composition of exclusively breast-fed infants in the first 6 months of life is the ideal standard for all full-term infants is now in conflict with the reality that it is technologically possible with infant formulae to increase calcium absorption and bone calcium accretion to levels above those achieved by human milk-fed, full-term infants. However, there are no data to support such a goal or suggest that it is beneficial for short- or long-term bone health (Abrams, 2006). Regardless, in the United States, the mandate of the Infant Formula Act and certain policy statements have led to the marketing of infant formulae with much higher concentrations of calcium than is present in human milk (Abrams, 2006).

Calcium sources should be evaluated on the basis of both content and bioavailability of calcium (Weaver *et al.*, 1999). About 60–70% of calcium is absorbed from human milk, leading to a net retention of about 90–100 mg calcium per day during the first 6 months for babies fed exclusively human milk (Abrams, 2006). Calcium absorption from bovine milk (~32%) is similar to that from other dairy products even though the lactose content and the chemical form of calcium in cheese or yogurt are altered during processing (Weaver *et al.*, 1999). Fractional calcium absorption from infant formulae tends to range between 40 and 60% in most studies (Abrams, 2006). Calcium salts, regardless of solubility, have fractional calcium absorption values similar to that for bovine milk, with the exception of calcium citrate malate, from which absorption is slightly higher (Weaver *et al.*, 1999). Fractional absorption of calcium from low-oxalate vegetables, i.e., broccoli (61%), bok choy (54%) or kale (49%) is actually higher than that from bovine milk (Weaver *et al.*, 1999). Calcium bioavailability is typically greatly reduced in foods containing high level of oxalate and/or phytate (e.g., common dried beans) but foods produced from soybeans, rich in both oxalate and phytate, have relatively high calcium bioavailability. However, increasing dietary

protein from omnivorous sources increases intestinal calcium absorption and substitution of soy protein for meat protein causes an acute decline in dietary calcium bioavailability (Kerstetter *et al.*, 2006). Regardless of the source of calcium, calcium absorption efficiency decreases with increasing intake but total calcium absorbed continues to increase with load (Weaver and Heaney, 2006). Earlier concern that purified proteins in the diet decrease calcium balance (Weaver *et al.*, 1999) was muted by the finding that calcium retention was not reduced when subjects consumed a high-protein diet from common dietary sources such as meat (Roughead *et al.*, 2003). Calcium absorption efficiency is upregulated during puberty and during the third trimester of pregnancy. On the other hand, calcium absorption efficiency declines with age (Weaver and Heaney, 2006). Active calcium absorption is compromised by hyperparathyroidism and diseases of the kidney (Weaver and Heaney, 2006).

In bovine milk, 99% of the calcium is in the skim milk fraction, whereas, in human milk, 16% of calcium is present in the lipid fraction (bound to the fat globule membrane), 47% is protein bound (6% to casein and the remainder to whey proteins), about 38% is soluble (mainly as calcium ions) (Fransson and Lonnerdal, 1983) and 1% is bound to α-lactalbumin (Lonnerdal and Glazier, 1985). Casein micelles are colloidal protein–calcium-transport complexes with the colloidal calcium phosphate present in nanoclusters with a diameter of $\sim$2.5 nm (Marchin *et al.*, 2007). Therefore, calcium levels are higher in milks rich in caseins (Gaucheron, 2005). Micellar calcium is not exclusively associated with the colloidal inorganic phosphate; part is bound directly to the phosphoserine residues of casein, which are organic phosphate. Thus, the colloidal calcium in milk is a mixture of calcium caseinate (containing organic phosphate) and calcium phosphate (an inorganic phosphate) (Gaucheron, 2005). In bovine milk, two-thirds and one-third of the total calcium are in the micellar and soluble forms, respectively (Gaucheron, 2005). Micellar calcium phosphate is exchangeable with the diffusible fraction, and the calcium bound to phosphoserine residues is more exchangeable than that associated with colloidal phosphate (Gaucheron, 2005). Ionized calcium in the soluble phase accounts for about 10% of the total calcium, with the remaining soluble portion as calcium citrate. Micellar calcium phosphate plays a key role in the maintenance of the structure of the casein micelle, and the physico-chemical properties are exploited for the manufacture of dairy products (Gaucheron, 2005). Casein micelles of human and bovine milk differ in composition such that when clotted by proteolytic enzymes under conditions simulating those found in the infant stomach, either no clot or an almost undetectable very fine curd forms as opposed to the large curds in bovine milk. The small clots may be involved in the proper absorption of the milk constituents into the body for optimal utilization (Sood *et al.*, 1997).

10.4. Phosphorus

Phosphorus is an essential nutrient for humans, and the adult human body contains about 850 g of elemental phosphorus with about 85% in the skeleton; 14% in the soft tissues; and 1% in the extracellular fluids, intracellular structures and cell membranes (Anderson *et al*., 2006). Food phosphorus is a mixture of inorganic phosphate (P_i) and various organic phosphates. Most phosphorus absorption occurs as P_i because intestinal phosphatases hydrolyze the organic forms in foods (Food and Nutrition Board: Institute of Medicine, 1997) and the predominant form of inorganic phosphate in all biological fluids and tissues is the hydrogen phosphate ion (HPO_4^{2-}), a divalent anion. The dietary phosphorus present as phytate, the storage form of phosphorus in food plant seeds (beans, cereals, nuts, peas), is not available directly. Phosphorus bioavailability from these foods depends on the hydrolysis of phytate by phytase produced by yeasts and colonic bacteria and the natural phytase content of all foods. Leavening breads with yeasts that produce phytase improves phosphorus bioavailability from those foods (Anderson *et al*., 2006). Because there is a multitude of factors that influence the presence of phytase, there is considerable flux in phosphorus bioavailability from food phytate in the gut over a given period of time (Food and Nutrition Board: Institute of Medicine, 1997).

The organic phosphates are major components of phospholipids, nucleotides and nucleic acids. Also, the hydroxyapatite-like bone crystals contain a constant ratio of calcium-to-phosphate of approximately 2:1. On the other hand, the whole body P_i compartment comprises a minute fraction of total body phosphorus and is located mainly in the blood and extracellular fluid (ECF). However, the P_i compartment is a critical pool because it accepts phosphate absorbed from the diet and the phosphorus resorbed from bone and is the source of most bone fluid phosphorus and most urinary phosphorus. The normal very high fluxes of P_i between bone and the bone fluid compartment each day (5000 mg) occur by ionic exchange and active bone resorption (Anderson *et al*., 2006). Bone turnover rates are relatively slow, so that dynamic ionic exchange is critical for maintenance of blood P_i concentration.

Phosphorus is found in nearly all foods, with most food sources having high phosphorus bioavailability (55–70%) (Food and Nutrition Board: Institute of Medicine, 1997). The phosphorus content of the US food supply is increasing as phosphate salts are added to processed foods for non-nutrient functions such as moisture retention, smoothness, and binding. As a result, near total starvation is required to produce dietary phosphorus deficiency which manifests as hypophosphatemia (Food and

Nutrition Board: Institute of Medicine, 1997). However, there are several situations where hypophosphatemia may be induced and becomes life-threatening. In intensive care units, hypophosphatemia may be expected in the presence of risk factors including alcohol withdrawal, parenteral nutrition and/or glucose loading, insulin infusion during ketoacidosis treatment and sepsis, dialysis and treatment with antiacids, diuretics and dialysis (Palmese *et al.*, 2005). When ECF P_i levels are low, cellular dysfunction follows. Thus, the effects of hypophosphatemia include anorexia, anemia, muscle weakness, bone pain, rickets and osteomalacia, general debility, increased susceptibility to infection, paresthesias, ataxia, confusion and even death (Lotz *et al.*, 1968). In growing skeletal tissue, the supply of P_i from dietary sources becomes potentially limiting but the typical abundance of phosphorus in the diet minimizes this rate-limiting risk (Anderson *et al.*, 2006).

Excess phosphorus intake from any source is expressed as hyperphosphatemia, an abnormally high level of plasma P_i (Food and Nutrition Board: Institute of Medicine, 1997). There are several potential problems of P_i metabolism related to excessive phosphorus consumption. Most clinical studies of acute and longer exposure to phosphorus loading show an increase in PTH levels. Thus, a chronic high phosphorus intake may impair the adaptive mechanism needed for adequate calcium absorption and optimal bone accretion (Anderson *et al.*, 2006). Even so, the FNB concluded that current phosphorus intakes thought to be experienced by the US population are unlikely to adversely affect bone health (Food and Nutrition Board: Institute of Medicine, 1997). Calcification of non-skeletal tissues (metastatic calcification) is a serious and harmful effect of hyperphosphatemia, a situation that occurs when calcium and phosphorus concentrations of the extracellular fluid exceed limits of calcium phosphate solubility. Metastatic calcification of the kidney is not known to occur from dietary sources alone in persons with adequate renal function but hyperphosphatemia is an almost universal finding in patients with end-stage renal disease and is associated with increased all-cause mortality, cardiovascular mortality and vascular calcification (Kooienga, 2007).

Dietary phosphorus is derived mainly from foods high in protein or from foods where phosphate salts are added to improve non-nutritive properties including moisture retention, smoothness, or binding. In adults, total dietary phosphorus intake varies more with total food intake than with food composition. Even so, increased consumption of soft drinks that use phosphoric acid as the acidulant or dairy products will increase phosphorus density values; the phosphorus densities of either cola or bovine milk are higher than those of most other foods in a typical diet (Food and Nutrition

Board: Institute of Medicine, 1997). In the United States, median phosphorus intakes exceed DRIs for both genders by 300–600 mg/d. Furthermore, it is not difficult for adults aged 19–70 yr to exceed the UL for phosphorus (4000 mg/d). For example, consumption of certain high-energy bars and shakes or creatinine monophosphate supplements at the manufacturer's recommended daily dose alone will provide up to 3000 mg/d of phosphorus (Anderson *et al.*, 2006). In the United States, intakes of calcium are consistently lower than those of phosphorus in the absence of calcium supplementation, the significance of which remains controversial.

Infants, toddlers and adolescents derive 32–48% of their dietary phosphorus from milk and milk products, whereas dairy foods provide only 20–30% of phosphorus for most adult age and sex groups (Anderson *et al.*, 2006). Particular attention must be paid to increased risk of hyperphosphatemia in neonates because renal handling of phosphorus is developmentally immature in this age group. Persistent hyperphosphatemia during early infancy may cause parathyroid hyperplasia, ectopic calcifications, low serum calcitriol, and hypocalcaemia severe enough to induce neonatal tetany. This condition is more prevalent in artificially fed than in breast-fed infants (Food and Nutrition Board: Institute of Medicine, 1997) because bovine milk or formulae may contain, respectively, six or three times as much phosphorus as human milk (see Table 10.1). The phosphorylation level of caseins is believed to have major implications for the formation of micelles that are involved in delivering valuable calcium phosphate and other minerals to the newborn (Kjeldsen *et al.*, 2007). Even so, human milk with its low phosphorus content is both safer and better suited to the growth needs of the infant than bovine milk. Casein micelle structure is not characterized fully, but in all compositional models, micellar calcium phosphate is an integral part of the casein micelle and is responsible for the structure and stability of these particles. In bovine milk, about 54% of P_i is soluble and about 46% is associated with casein micelles as calcium phosphate, probably by binding mainly to casein phosphoserine residues (organic phosphate) and glutamate and aspartate residues (Gaucheron, 2005). In human milk, about 15% of phosphorus occurs in an inorganic form, 23% is protein bound and about 62% is present with lipids (Renner, 1983).

10.5. Magnesium

Magnesium is the most prevalent intracellular divalent cation and is needed for enzymatic reactions vital to every metabolic pathway (Food and Nutrition Board: Institute of Medicine, 1997; Committee on Minerals and Toxic Substances in Diets and Water for Animals, 2005; Volpe, 2006). These

reactions involve synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), protein and, adenylate cyclase glycolysis, cellular energy production and storage and preservation of cellular electrolyte composition. Magnesium regulates intracellular calcium and potassium at the cell membrane level, and thus is a controlling factor in nerve transmission, skeletal and smooth muscle contraction, cardiac excitability, vasomotor tone, blood pressure and bone turnover.

Severe magnesium deficiency, usually the result of dysfunctional states causing malabsorption or excessive excretion, results in numerous signs and symptoms including loss of appetite, nausea, vomiting, fatigue and weakness. As the deficiency becomes more severe, numbness, tingling, muscle contractions and cramps, seizures, personality changes and coronary spasms (angina pectoris) occur. A chronic low intake of magnesium may result in cardiovascular dysfunctions such as hypertension and dysrhythmias, bone loss leading osteoporosis, and insulin resistance and impaired insulin secretion leading to diabetes mellitus. The major effect of excessive magnesium intake is diarrhea (Food and Nutrition Board: Institute of Medicine, 1997; Volpe, 2006), but nausea and abdominal cramping may also occur. High serum magnesium (hypermagnesmia) causing more severe magnesium toxicity signs is most commonly associated with the combination of impaired renal function and high intakes of non-food sources of magnesium such as magnesium-containing laxatives and antacids. Signs of hypermagnesmia include muscle weakness, extremely low blood pressure, difficulty in breathing, irregular heartbeat and change in mental status.

The FNB (Food and Nutrition Board: Institute of Medicine, 1997) found no functional criteria on which to base an RDA for infants. Thus, they set an AI for magnesium that reflects the mean intake of infants fed principally with human milk (Table 10.2). An RDA for magnesium was set for children aged 1–3 yr by interpolating data from other age groups. Data from the US National Health and Nutrition Examination Survey (NHANES) 2001–2002 indicated that the majority of people consumed less than the EAR for magnesium (Moshfegh *et al.*, 2005). For example, 64% of women aged 51–70 yr did not attain the magnesium EAR. A recent analysis of primary magnesium balance data from tightly controlled metabolic feeding studies indicates a presumptive EAR of 165 mg/d and a presumptive RDA of 237 mg/d for magnesium for men and women (Hunt and Johnson, 2006).

The adult human body contains about 25 g (1028 mmol) of magnesium, which is about equally divided between bone and soft tissue (Volpe, 2006). Less than 1% of the total body magnesium is in blood. Approximately one-third of skeletal magnesium is exchangeable, and acts as a pool for maintaining normal concentrations of extracellular magnesium. Thus, extracellular magnesium stays relatively constant, even if skeletal and intracellular levels

are decreasing. This results in plasma or serum magnesium being a poor indicator of tissue magnesium status. The concentration of magnesium in human colostrum is about 30% higher than in mature human milk (Rajalakshmi and Srikantia, 1980), which is about 34 mg (1.4 mmol)/l (Food and Nutrition Board: Institute of Medicine, 1997; Hunt *et al*., 2005). Mature bovine milk contains about 100 mg (4.1 mmol)/l (Hunt and Meacham, 2001; Volpe, 2006). Magnesium concentration in bovine colostrum is two to three times that in mature milk, but decreases to the mature milk value within the first 1–3 d of lactation (Hidiroglou and Proulx, 1982). The concentration of magnesium in human milk increases slightly between months 1 and 4 (Hunt *et al*., 2005) but remains relatively constant in bovine mature milk (Hidiroglou and Proulx, 1982) over the first year of lactation. Milk magnesium concentration is unaffected by dietary magnesium intake. Because bovine milk contains over three times as much magnesium as human milk, commercial formulae that are based on bovine milks are generally higher in magnesium concentration (40–50 mg or 1.7–2.1 mmol/l) than human milk (Food and Nutrition Board: Institute of Medicine, 1997).

In human milk, 2% of magnesium is in the fat fraction, 6% in casein, 36% associated with whey proteins and 58% as low-molecular-weight forms (Fransson and Lonnerdal, 1983). In bovine milk, 98–100% of the magnesium is in the skim milk phase (Fransson and Lonnerdal, 1983), with 65% in a soluble form (40% as magnesium citrate, 7% as magnesium phosphate, and 16% as free magnesium ions). The other 35% of magnesium is colloidal and associated with casein micelles (Holt, 1985). Premature and full-term infants absorb 45% of the magnesium in human milk (Food and Nutrition Board: Institute of Medicine, 1997) and absorption varies from 40 to 71% in bovine milk-based infant formulae (Food and Nutrition Board: Institute of Medicine, 1997).

Green leafy vegetables, whole grains and nuts are the richest sources of magnesium (Volpe, 2006). However, because milk and milk products are moderate sources of magnesium, increasing their intake would be a reasonable recommendation for assuring a healthy intake of this element.

10.6. Iron

Dietary iron is required for a wide variety of biochemical processes. Biological systems utilize the chemical reactivity of iron and interconvert iron oxidation states. This interconversion allows iron to participate in electron transfer as well as the reversible binding to ligands (Food and Nutrition Board: Institute of Medicine, 2001). Iron is suited to participate in oxygen transport and storage, electron transfer and substrate oxidation–reduction.

For example, one of the main functions of iron is the movement of oxygen from the environment to body tissues (Beard, 2006). As a result, approximately 66% of iron in the body is found in hemoglobin of circulating erythrocytes. Another 25% is in the readily mobilizable iron store and the remaining 15% is contained in the myoglobin of muscle tissue and a variety of enzymes required for oxidative metabolism and other functions in cells. In humans, there are four major classes of iron-containing proteins: heme proteins (hemoglobin, myoglobin, cytochromes), iron–sulfur enzymes (flavoproteins, heme-flavoproteins), iron transport and storage proteins (lactoferrin, transferrin, ferritin, hemosiderin) and other iron-containing or activated enzymes (sulfur, non-heme enzymes). Iron is critical for the regulation of genes, cell growth and differentiation, hematopoiesis and cognitive development during infancy (Kelleher and Lonnerdal, 2005).

Iron-deficiency anemia is the late stage of negative iron balance and is diagnosed as low serum transferrin saturation (<15%), a low serum ferritin concentration (<12 μg/l) and an elevated soluble transferrin receptor (>6 mg/dL) in a background of microcytic anemia. The physical symptoms of iron-deficiency anemia are tiredness, listlessness, apathy and general feelings of lack of energy. Endurance exercise is markedly impaired. These symptoms represent true reductions in muscle functioning but may also reflect pathological changes in central nervous system functioning (Beard, 2001). Iron-deficiency anemia affects an estimated 20–25% of infants worldwide and appears to cause irreversible developmental delays (Lozoff *et al.*, 2003). Other clinical signs of iron deficiency include inflammation or infection of the tongue (glossitis), fissuring in the corners of the lips (angular stomatitis), spoon nails (koilonchyia), blue sclera, esophageal webbing and behavior disturbances (pica, the abnormal consumption of non-food items) (Beard, 2006).

Ferritin and hemosiderin (a water-insoluble degradation product of ferritin) comprise 95 and 5%, respectively, of the storage forms of iron in the liver (Beard, 2006). Because both the human host and most pathogens require iron, the host must meet cellular demands while simultaneously preventing excess accumulation. Iron overload can be induced by several factors including blood transfusions, hereditary hemochromatosis or particular forms of food and their preparation (i.e., consuming beer cooked in iron pots). In iron overload, there is gross cellular accumulation of both ferritin and hemosiderin in several tissues but particularly in the liver where the rate of increase of hemosiderin is 10 times that of ferritin (Miyazaki *et al.*, 2002).

Iron requirements are highest during pregnancy (Table 10.2). On the other hand, the requirement for exogenous iron is virtually zero for the normal full-term infant at birth because of the normally very high

hemoglobin concentration and high iron stores in the newborn (Food and Nutrition Board: Institute of Medicine, 2001). Maternal iron deficiency is not associated with neonatal iron-deficiency anemia but neonatal iron stores are reduced (Kelleher and Lonnerdal, 2005), which puts the newborn at increased risk for iron deficiency if they do not consume adequate amounts of iron-rich complementary foods after 4–6 months of age (Domellof *et al*., 2002). Regulation of iron absorption changes between 6 and 9 months of age that apparently enhances the ability of the infant to adapt to a low-iron diet. That is, fractional iron absorption from breast milk can double between 6 and 9 months of age in infants not supplemented with iron. Thus, some infants can avoid iron deficiency despite low-iron intake in late infancy (Domellof *et al*., 2002). Men have higher iron stores than women and the requirement for iron is much higher for reproductive-age females than any other sex–age group. This is a reflection of the additional iron requirement associated with menstrual blood loss (Food and Nutrition Board: Institute of Medicine, 2001). The iron requirement increases considerably for lactoovovegetarians compared to non-vegetarians because non-heme iron absorption is considerably lower in the former (Hunt and Roughead, 1999).

Heme iron is highly bioavailable, is soluble in the alkaline luminal environment (such that binding proteins are not necessary for luminal absorption) and enters the intestinal cell intact. On the other hand, non-heme iron absorption depends on the solubilization of predominately ferric food iron in the acid milieu of the stomach and subsequent reduction to the ferrous form (Beard, 2006). This reduction is dependent upon interaction with endogenous compounds such as a ferrireductase (present at the mucosal surface of duodenal enterocytes) (Han *et al*., 1995) and dietary ascorbic acid (Food and Nutrition Board: Institute of Medicine, 2001). Because mammals lack a regulated pathway for iron excretion, iron homeostasis is regulated primarily by iron absorption by duodenal mucosal cells. The hepatic antimicrobial peptide, hepcidin, plays a key role in the negative feedback regulation of intestinal iron absorption (Ward and Conneely, 2004).

The iron concentration in breast milk is probably under homeostatic control because anemic women as well as women taking iron supplements have levels of iron in their milk that are similar to those of non-anemic women (Lonnerdal, 1997). Prematurity does not affect human milk iron concentrations (Hunt *et al*., 2004). Maternal dietary iron supplementation with up to 30 mg of iron per day does not affect its concentration in milk (Siimes *et al*., 1984). Apparent homeostatic control of iron in breast milk is probably achieved by up- and down-regulation of transferrin receptors in the mammary gland such that in a situation of maternal iron deficiency, a higher number of mammary transferrin receptors facilitates the uptake of iron into the gland and ensures a normal iron level in the milk (Lonnerdal, 1997).

Debate continues as to whether the iron content of human milk is sufficient for the breast-fed infant and whether these infants should be supplemented with iron. The concentration of iron in human milk is normally very low and decreases significantly further during the first 12 weeks of lactation (Butte *et al.*, 1987; Davidsson *et al.*, 1994; Hunt *et al.*, 2004; Kelleher and Lonnerdal, 2005). Even so, several studies have demonstrated that the iron status of exclusively breast-fed infants is satisfactory up to 6 months of age and, in some studies, up to 9 or 12 months of age (Lonnerdal, 1997). A panel of experts (American Academy of Pediatrics, 2005) concluded that exclusive breast-feeding is sufficient to support optimal growth and development for approximately the first 6 months of life but complementary foods rich in iron should be introduced gradually beginning around 6 months of age. Preterm and low-birth-weight infants have high iron requirements (Griffin and Abrams, 2001). More recently, a report suggested that iron supplementation from 1 to 6 months of age results in higher hemoglobin and mean corpuscular volume at 6 months of age and significantly higher visual acuity and psychomotor developmental indices at 13 months of age (Friel *et al.*, 2003). On the other hand, new evidence (Sazawal *et al.*, 2006) suggests that current guidelines for universal supplementation with iron and folic acid in areas of high malaria transmission should be revised. Children in those areas who received iron and folic acid were more likely to die or need treatment in hospital for an adverse event. In general, iron-deficient and anemic children can benefit from supplementation but supplementation of those who are not iron deficient might be harmful.

Earlier estimates (Lonnerdal, 1989) of typical iron absorption from human milk (50%) are considerably higher than newer estimates (~20%) obtained with stable isotope technology (Fomon *et al.*, 1993; Lonnerdal, 1997). Also, formula-fed infants absorb considerably less iron (~7%) than breast-fed infants (Fomon *et al.*, 1993). Reasons for the higher bioavailability of iron in human milk are not understood. Lactoferrin is a highly efficient mammalian iron-scavenging defense protein (~80 kDa) (Weinberg, 2007), a glycoprotein that has a very high association constant for iron (K_a, 1.0×10^{30} M^{-1}) (Lonnerdal, 1997) and is found at high concentrations in all human milk, including colostrum (~9.8 g/l) and mature breast milk (~2.4 g/l) (Velona *et al.*, 1999). Despite a very high binding affinity, there is still no good evidence that lactoferrin plays any role as an iron transporter or is involved in "mainstream" iron metabolism (Brock, 2002). In fact, in the murine model, lactoferrin ablation did not result in iron-deficiency anemia (Ward and Conneely, 2004) and, in infants older than 4 months of age, iron absorption was found to be slightly higher from lactoferrin-free milk than from intact breast milk (Lonnerdal, 1997).

Human milk, as well as bovine milk and milk products, are poor sources of iron (Table 10.1) (Pennington *et al.*, 1987). To prevent iron deficiency and

anemia by 6–9 months of age, most infant formulae are supplemented with iron but at various concentrations (Lonnerdal, 1997). In the United States, infants fed typical iron-fortified formula (11.8 mg/l) (Hunt and Meacham, 2001) in typical quantities (0.78 l/d) may have an iron intake of 9.2 mg/d, a level of iron that corresponds to as much as 34 times more iron per day than that consumed by breast-fed infants (0.27 mg Fe/d). Because most grain products are fortified with non-heme iron, approximately 50% of ingested iron comes from bread and other grain products, including cereals and breakfast bars. Heme iron represents only 7–10% of dietary iron for girls and women and 8–12% for boys and men.

Distribution of iron among compartments in human milk is complex and distribution within a compartment differs with species (Fransson and Lonnerdal, 1983). In bovine and human milks, the lipid fraction contains ~14 and ~33% of total milk iron, respectively. Within the lipid fraction of human milk, iron is thought to bind to xanthine oxidoreductase (an enzyme located in the fat globule membrane), one molecule of which contains eight atoms of iron. In both bovine and human milks, whey proteins bind similar amounts of iron (~26 to ~26%) but lactoferrin, a whey protein, has a much more prominent role in binding iron in human milk than in bovine milk. Another ~24% of total bovine milk iron is present in casein, considerably more than the amount of total iron present in human casein (~9%). Finally, in both bone and human milks, ~32% of total iron is associated with low-molecular-weight compounds, most likely citrate.

10.7. Zinc

Because of its stability and coordination flexibility, zinc performs catalytic, structural and regulatory functions in protein, nucleic acid, carbohydrate and lipid metabolism (Food and Nutrition Board: Institute of Medicine, 2001; Cousins, 2006). Approximately 300 enzymes contain zinc. Zinc has a catalytic role in enzymes of all six classes. Zinc also has a structural role in some enzymes and in zinc finger proteins of transcription factors. Through binding to trans-acting factors, zinc regulates the expression of some genes. Mild signs of zinc deficiency described for infants and young children include suboptimal growth, poor appetite and impaired taste acuity. Zinc is a relatively non-toxic element, but chronic zinc supplementation greater than 100 mg/d may result in impaired immune function, decreased HDL cholesterol and gastric distress (Food and Nutrition Board: Institute of Medicine, 2001; Cousins, 2006).

The FNB (Food and Nutrition Board: Institute of Medicine, 2001) found no functional criteria on which to base a zinc RDA for early infancy. Thus, they set an AI of 2.0 mg/d for infants aged 0–6 months based on the

mean intake exclusively from human milk. RDAs for zinc for all other sex–age groups are listed in Table 10.2.

The adult human body contains between 1.5 and 2.5 g of zinc (Cousins, 2006), 85% is in skeletal muscle and bone (Food and Nutrition Board: Institute of Medicine, 2001). About 95% of body zinc is intracellular, with 40% found in the nucleus. Plasma contains about 0.1% of the body's zinc, which responds markedly to external stimuli including zinc intake, fasting and acute stressors such as infection (Cousins, 2006). Thus, using plasma zinc to asses zinc status should be done with caution. In humans, plasma zinc is reduced in severe zinc deficiency, but not with moderate zinc deprivation (Food and Nutrition Board: Institute of Medicine, 2001). Zinc concentrations in human milk decline rapidly during the first 6 months of lactation (Food and Nutrition Board: Institute of Medicine, 2001; Hunt *et al*., 2005). They decline from approximately 4 mg/l at 2 weeks to 3 mg/l at 1 month, 2 mg/l at 2 months, 1.5 mg/l at 3 months and 1.2 mg/l at 6 months postpartum. No significant correlation was observed between dietary zinc intake and zinc concentration in human milk, and zinc supplementation does not appreciably affect its zinc concentration (Lonnerdal, 1997). In contrast to human milk, the zinc concentration in mature bovine milk changes little after the first 3 d of lactation when the concentration decreases by 50% in colostrum (de Maria, 1978). Mature bovine milk contains between 3 and 5 mg zinc/l (Lonnerdal *et al*., 1981; Hunt and Meacham, 2001).

The reported mean distribution of zinc in human milk is 29% in the lipid fraction (bound to the fat globule membrane), 14% associated with casein, 28% associated with whey proteins and 29% in the form of a low-molecular-weight compound (probably as citrate) (Lonnerdal *et al*., 1982; Fransson and Lonnerdal, 1983). The distribution of zinc in bovine milk is significantly different; most is found in the skim milk fraction, with only 1–3% in the lipid fraction (Fransson and Lonnerdal, 1983). Approximately 32% of the zinc is bound to casein and most of the remaining zinc is bound to colloidal calcium phosphate; only a small amount (~5%) is associated with a low-molecular-weight compound, which was identified as citrate (Blakeborough *et al*., 1983; Singh *et al*., 1989). Proteins in human milk, which bind most of the zinc, are believed to be more easily digestible than casein, the major protein in bovine milk. This difference may explain the higher zinc bioavailability from human milk (Cousins, 2006). Pasteurization of human milk decreases zinc bioavailability to the point of affecting zinc balance in preterm infants, a situation probably caused by redistribution and alterations in the zinc-binding pattern (Goes *et al*., 2002).

Because increasing zinc concentration from 1.8 to 5.8 mg/l in infant formulae resulted in increased growth in male infants (Walravens and Hambidge, 1976), zinc supplementation of formulae is practiced widely.

The zinc concentration of bovine milk-based infant formulae in the United States is in the range of 4.0–7.4 mg/l (Hamill *et al.*, 1989; Hunt and Meacham, 2001). Dairy products such as milk, cheese and yoghurt are moderate sources of zinc for adults. It has been estimated that milk and dairy products contribute between 19 and 31% of the total zinc intake in Western countries (Hazell, 1985; Renner *et al.*, 1989).

10.8. Copper

Copper functions in a metal center of (co-factor) about 10 enzymes that bind oxygen and produce water, superoxide or hydrogen peroxide in addition to producing molecules involved in energy production, iron absorption and utilization, extracellular matrix maturation, neuropeptide activation and neurotransmitter synthesis (Food and Nutrition Board: Institute of Medicine, 2001; Prohaska, 2006). Clinically evident (Frank) copper deficiency has been reported for infants fed milk formulae and bovine milk, malnutrition associated with chronic diarrhea and patients nourished with prolonged total parenteral nutrition. Signs of copper deficiency include normocytic hypochromic anemia, leukopenia, neutropenia and osteoporosis. Severe copper deficiency caused by the genetic disorder, Menke's syndrome, and animal deprivation studies suggest that a chronic low intake of copper may have pathological consequences for cardiovascular health, immune response and neuronal function.

Acute excessive copper intake (e.g., >3–4 mg/l drinking water) induces gastrointestinal distress including abdominal pain, cramps, nausea, diarrhea and vomiting (Food and Nutrition Board: Institute of Medicine, 2001; Prohaska, 2006). Chronic copper toxicity is rare unless some hereditary defect in copper homeostasis is present (Food and Nutrition Board: Institute of Medicine, 2001). Liver damage in childhood cirrhosis in India has been associated with the use of copper-contaminated water to prepare infant formula and the consumption of other foods that were stored or prepared in brass and/or copper vessels (Tanner, 1998).

The FNB (Food and Nutrition Board: Institute of Medicine, 2001) found no functional criteria of copper status to use for establishing a copper RDA for infants. Thus, they set AIs that reflected the mean intake of infants principally fed with human milk; these AIs were 200 μg/d for age 0–6 months and 220 μg/d for age 7–12 months. Extrapolation from the RDA for adults was the basis for setting RDAs for children (Table 10.2)

The human body contains about 1 mg copper/kg body weight (Prohaska, 2006). While liver, brain and kidney have the highest concentrations, muscle and bone, because of their overall greater mass, account for 50–70 % of total body copper (Harris, 1997). Low serum copper can indicate copper deficiency

(Food and Nutrition Board: Institute of Medicine, 2001). However, using serum copper to assess copper status requires caution because concentrations increase during pregnancy and with a number of diseases. The concentration of copper in human milk is highest during early lactation and declines with time (Food and Nutrition Board: Institute of Medicine, 2001; Hunt *et al.*, 2004). The mean copper concentration in human milk is about 250 μg/l during the first 6 months of lactation and declines to between 100 and 200 μg/l between 7 and 12 months postpartum (Food and Nutrition Board: Institute of Medicine, 2001). No correlation was found between dietary copper intake and human milk copper concentrations. The mean concentration of copper is higher in human milk than in bovine milk (60–90 μg/l) (Fransson and Lonnerdal, 1983; Hunt and Meacham, 2001). The concentration of copper in bovine milk decreases by up to 50% during the first 3 d of lactation (de Maria, 1978) but can be increased by dietary copper supplementation (Murthy and Thomas, 1974) or by contact with copper-containing containers and processing equipment (Roh *et al.*, 1976). The copper content of commercial infant formulae is often similar to that in human milk (Table 10.1).

In human milk, 9–15% of copper is in the fat fraction, 39–56% bound to whey proteins (mainly the albumin fraction of milk), 7–28% bound to casein and 21–24% in a low-molecular-weight form (bound mainly to citrate) (Lonnerdal *et al.*, 1982; Lonnerdal, 1985). In bovine milk, 2% of copper is in the fat fraction, 8% bound to whey proteins, 44% bound to casein and 47% is in a low-molecular-weight fraction (Lonnerdal, 1985). It has been reported that infants absorb much more copper from human milk than from a bovine milk-based formula (Food and Nutrition Board: Institute of Medicine, 2001). However, suckling rats absorbed about 80% of copper from human or bovine milks (Lonnerdal, 1985).

Milk and milk products are considered poor sources of copper (Pennington *et al.*, 1987; Renner *et al.*, 1989; Hunt and Meacham, 2001). Bovine milk contributes very little to the total dietary intake of copper.

10.9. Manganese

Manganese functions as an enzyme activator and is a constituent of several metalloenzymes (Leach and Harris, 1997; Food and Nutrition Board: Institute of Medicine, 2001; Nielsen, 2006). The numerous enzymes that can be activated by manganese include oxidoreductases, lyases, ligases, hydrolases, kinases, decarboxylases and transferases. Most enzymes activated by manganese in higher animals and humans can also be activated by other metals, especially magnesium; exceptions are the manganese-specific activation of glycosyltransferases, glutamine synthetase, farnesyl pyrophosphate synthetase and

phosphoenolpyruvate carboxykinase. The few manganese enzymes in higher animals and humans include arginase, pyruvate carboxylase and manganese superoxide dismutase. Manganese deficiency has been difficult to induce or identify in humans, and thus is generally not considered to be of much nutritional concern. The most likely case of manganese deficiency is that of a child on long-term parenteral nutrition who exhibited diffuse bone demineralization and poor growth that were corrected by manganese supplementation. Other probable signs of manganese deficiency are a finely scaling, minimally erythematous rash, decreased plasma cholesterol concentration and increased serum alkaline phosphatase activity. Low dietary manganese or low blood and tissue manganese have been associated with osteoporosis, diabetes, epilepsy, atherosclerosis, impaired wound healing and cataracts (Wedler, 1994). Findings from animal experiments have provided some support for these associations. In the past, manganese was considered to be one of the least toxic of the essential mineral elements (Food and Nutrition Board: Institute of Medicine, 2001; Nielsen, 2006). Recently, however, magnetic resonance imaging has shown that signals for manganese in brain are strongly associated with neurological symptoms (e.g., sleep disturbances) exhibited by patients with chronic liver disease. Such findings suggest that high intakes of manganese are ill-advised because of potential neurotoxicological effects, especially in people with compromised homeostatic mechanisms or infants whose homeostatic control of manganese is not fully developed.

The FNB (Food and Nutrition Board: Institute of Medicine, 2001) found no functional criteria of manganese status to use for establishing an RDA for manganese for infants. Thus, they set AIs for manganese that reflected the mean intake of infants fed principally human milk; these AIs were 0.003 mg/d for age 0–6 months and 0.6 mg/d for age 6–12 months. Intake data were used to determine AIs for other age groups (Table 10.2).

Manganese is fairly uniformly distributed in soft tissues at concentrations generally less than 3 μg/g (Leach and Harris, 1997; Committee on Minerals and Toxic Substances in Diets and Water for Animals, 2005). Bone contains a substantial amount of manganese but should not be considered a reservoir. Serum or plasma manganese concentrations apparently are somewhat sensitive to large variations in manganese intake, with normal values near 1 μg/l (Food and Nutrition Board: Institute of Medicine, 2001). Mean concentrations of manganese in human milk were found to be highest during the first few days postpartum (6–12 μg/l); at 1 month it was found to be approximately 4.0 μg/l, and apparently declined to near 2.0 μg/l by 3 months postpartum (Food and Nutrition Board: Institute of Medicine, 2001). A correlation between dietary intake and human milk concentration has been reported (Vuori *et al.*, 1980). The mean concentration of manganese in mature bovine milk is 30 μg/l (Lonnerdal *et al.*, 1981). The concentration in

bovine colostrum is 100–160 μg/l but declines by over 50% during the first 3 d of lactation (de Maria, 1978). Oral supplementation of large doses of manganese for an extended time can increase the manganese content of milk from bovine milk (Archibald, 1958).

In human milk, 67% of manganese is bound to lactoferrin, 11% to casein, 18% to the fat globule membrane, and 4% in a low-molecular-weight form (Lonnerdal *et al*., 1985). In bovine milk, 67% of manganese is bound to casein, 1% in the fat globule membrane, 14% to whey proteins and 18% in a low-molecular-weight form (Lonnerdal *et al*., 1985). Very little is known about the availability of manganese in milk to infants. Manganese absorption by human adults was higher (8.4%) from human milk than from bovine milk (2.4%) or bovine milk-based infant formula (3.1%) (Davidsson *et al*., 1989). However, suckling rats absorbed about the same percentage (between 80 and 89%) of manganese from human milk, bovine milk and bovine milk-based formula (Keen *et al*., 1986); this finding suggests that infants absorb a greater percentage of manganese from milk than adults do.

Based on the fact that infants do not have fully developed homeostatic control of manganese, its content in formulae has raised concerns. In the early 1980s, supplementation of formulae resulted in some having 100–1000 times higher amounts of manganese than in human milk (Lonnerdal *et al*., 1983; Stastny *et al*., 1984). Even without manganese supplementation, milk-based infant formulae contain more manganese than human milk (2–4 μg/l) because of the higher concentration of this element in bovine milk (30 μg/l). Manganese supplementation of formulae has now ceased, so they now contain manganese in the range of 34–300 μg/l (Lonnerdal, 1985; Johnson *et al*., 1998), which is lower than the recommended UL of 335–600 μg/l in infant formula (Lonnerdal, 1985; Hambidge and Krebs, 1989). The FNB did not set a manganese UL for infants, but set 2 mg/d as an UL for children aged 1–3 yr (Food and Nutrition Board: Institute of Medicine, 2001).

For adults, milk and milk products are considered poor sources of manganese. Bovine milk has been estimated to contribute 1–3% of the dietary intake of manganese in Western countries (Hazell, 1985).

10.10. Selenium

Selenium is involved in many biological functions, including thyroid, neural and immune function and protection against oxidative stress and gastrointestinal disease (Food and Nutrition Board: Institute of Medicine, 2000; Burk and Hill, 2005; Esworthy *et al*., 2005; Sunde, 2006). Research with Coxsackievirus indicates that oxidative stress in a selenium-deficient host leads to genomic changes of an RNA virus that can increase virulence (Beck *et al*.,

2003). This finding has proven important for understanding Keshan disease, an endemic cardiomyopathy that appears to involve a viral co-factor along with severe selenium deficiency (Beck *et al*., 2003).

Most selenium in animal tissues is present as selenomethionine or selenocysteine. In plants, selenium is metabolized as though it was sulfur to form the amino acid selenomethionine, which is incorporated randomly in place of methionine into proteins. Selenomethionine cannot be synthesized by humans, and in plants and animals, this compound does not have a physiological function separate from that of methionine. The metabolite selenide ($H_2Se/HSe^-/Se^{2-}$) is derived from reductive metabolism of inorganic selenium (selenite or selenate) or from catabolism of selenocysteine derived from food selenoproteins or trans-selenation of food selenomethionine. As such, selenide occupies a central role in selenium metabolism; it is the obligate selenium donor in the co-translational synthesis of selenocysteine, in selenoproteins, accounting for the nutritional activity of the element.

Selenoproteins have been identified in all phylogenetic kingdoms, and, with one known exception (a structural role in a sperm selenoprotein), selenium serves as a catalytic component in selenoproteins (Sunde, 2006). Selenium is best known for its role as an antioxidant, especially as a component of the glutathione peroxidases. Extracellular glutathione peroxidase (GPX1) was the first of the 25 selenoproteins discovered to date. Even so, GPX1 *per se* does not appear to have a critical role under normal conditions and has been postulated to function as a biological selenium buffer than can be used to expand and store selenium before excretion mechanisms are activated (Sunde, 2006). However, knockout of GPX1 renders experimental animals susceptible to severe acute oxidative stress and overexpression confers extra protection against the insult (Lei *et al*., 2007).

Implementation of dietary selenium supplementation in the human population must be done with caution because selenium has a narrow therapeutic range and may be toxic (Sunde, 2006). For example, indiscriminate supplementation of AIDS patients with selenium may aid the human skin poxvirus, *Molluscum contagiosum*, during infection. This poxvirus has acquired in its genome a cDNA sequence for GPX1. Excess selenium for this pox GPX, with antioxidant activity, may block full activation of antivirus mechanisms, which include peroxidative stimulation of programmed cell death (Sunde, 2006). In addition, selenium and iodine deficiencies exacerbate each other to contribute to the etiology of endemic myxedematous cretinism. Administration of selenium alone apparently aggravates this disease by restoring selenium-dependent deiodinase activity, which in turn exacerbates iodine deficiency through increased use of iodine (Vanderpas *et al*., 1993). Finally, supranutritional selenium supplementation (200 μg/d) increased the incidence of type 2 diabetes (Stranges *et al*., 2007) in a

randomized trial with patients with a history of skin cancer. In a separate study, a probability sample of the US population also indicated that high serum selenium levels (>130 ng/ml) were positively associated with the prevalence of diabetes (Bleys *et al*., 2007). The NHANES III survey found that the mean serum selenium level for all Americans aged >4 yr is 125 ng/ml (Food and Nutrition Board: Institute of Medicine, 2000). Increased risk for diabetes suggests asymptomatic changes related to chronic selenium toxicity that could take place at intake levels lower than the currently defined UL (400 μg/d for adults). There are some contrary data; a substudy of the Health Professionals Follow-up Study found an inverse association between toenail selenium levels and the prevalence of diabetes at baseline (Rajpathak *et al*., 2005).

Additional findings from the supranutritional selenium supplementation study discussed above (Stranges *et al*., 2007) indicated that selenium supplementation (200 μg/d) increased the risk of squamous cell carcinoma (25%) and total non-melanoma skin cancer (17%) (Duffield-Lillico *et al*., 2003) despite reductions in total cancer incidence and mortality (Duffield-Lillico *et al*., 2002). Also, participants with baseline plasma selenium concentrations in the lowest two tertiles (<121.6 ng/ml) experienced reductions in total cancer incidence, whereas those in the highest tertile (>121.6 ng/ml) showed a possible small increase in total cancer risk.

The FNB found no functional criteria on which to base an RDA for selenium for infants. Thus, they set AIs for selenium (Table 10.2) that reflected the mean intake of infants fed principally human milk. In the age group 7–12 months, an amount was added for the contribution obtained from weaning foods. For children and adolescents aged 1–18 yr and all adults aged 19–50 yr, the selenium EARs were based on selenium intakes expected to maximize plasma extracellular glutathione peroxidase (GSHPx-3) activity. Because the aging process does not appear to impair selenium absorption or utilization, selenium EARs for adults aged >50 yr are also based on maximal GSHPx-3 activity. Data from the available human studies are generally compatible with the possibility that intakes of selenium above those needed to maximize selenoproteins have an anticancer effect in humans. However, confounding factors, small sample sizes and the potential for excess selenium supplementation to raise cancer risk collectively do not allow use of these studies as the basis for determining dietary selenium requirements at this time (Food and Nutrition Board: Institute of Medicine, 2000).

Dietary selenium is consumed principally in the form of selenomethionine (plant, animal and supplements sources), selenocysteine (mainly animal sources) or selenate and selenite (from supplements). Significant sources of dietary selenium vary widely because the wide range in soil selenium content is reflected in the selenium content in foods of plant origin as well as that of

grazing livestock. Accordingly, handbook values for selenium in foods should be considered unreliable unless confirmed by actual analysis. Drinking water usually has negligible selenium content (Sunde, 2006). In the United Kingdom, meat, poultry and fish make up the largest contribution to selenium intake. In North America, wheat-derived foods make a substantial contribution to selenium intake (Rayman, 2000). Average selenium intake ranges from $\leq$11 μg/d (in areas of endemic Keshan disease in China), to 25 μg/d (in New Zealand and Finland with no reported occurrence of Keshan disease), to 79–104 μg/d (across the United States), to 113–220 μg/d (across Canada)

Selenomethionine is a less available metabolic source of selenium than selenite or selenate but is more effective at increasing apparent selenium status because of its non-specific incorporation and retention in body proteins in place of methionine, a characteristic that increases its toxicity during long-term consumption. More than 90% of selenomethionine, the major dietary form of the element, is absorbed. Selenate and selenite are inorganic forms of selenium commonly used to fortify foods. Selenate is absorbed almost completely but a significant fraction is lost in the urine before tissue incorporation. Selenite has a more variable absorption (generally $>$50%) but once absorbed is retained better than selenate (Food and Nutrition Board: Institute of Medicine, 2000).

Human milk selenium apparently is not under homeostatic control because the concentration of selenium in breast milk is strongly dependent on the maternal intake of selenium (McGuire *et al.*, 1993; Bratter *et al.*, 1997; Lonnerdal, 1997). For example, the mean concentration of selenium in breast milk varies from ~10 μg/l (collected in Finland with low soil selenium) (Kumpulainen *et al.*, 1985), to ~22 μg/l (collected in St John's, Canada) (Hunt *et al.*, 2004), to 112 μg/l (collected in a seleniferous region in Venezuela; Bratter *et al.*, 1997). Selenium concentration in human milk typically ranges from ~12 to 30 μg/l (Arnaud *et al.*, 1993; al-Saleh *et al.*, 1997; Bianchi *et al.*, 1999; Hunt *et al.*, 2004; Yamawaki *et al.*, 2005). Ready-to-feed infant formulae manufactured by American companies have imputed selenium concentrations that range from 13 to 18 μg/l (US Department of Agriculture Agricultural Research Service, 2006). Compared to infants fed humanized formulae not supplemented with selenium, infants fed human milk or humanized formula supplemented with selenium have increased plasma and serum selenium concentrations and higher plasma glutathione peroxidase activity (Carver, 2003). The concentration of selenium in human milk decreases during the first 12 weeks of lactation (Levander *et al.*, 1987; Hunt *et al.*, 2004). Selenium speciation studies suggest that there is a minimum of five selenium-binding compounds in human milk (Bratter *et al.*, 1998). In bovine milk whey, most selenium is

incorporated non-specifically as selenomethionine into the two major whey proteins, β-lactoglobulin and α-lactalbumin. Another approximately 25–33% of bovine whey selenium is found in the low-molecular fraction (Hoac *et al.*, 2007). There are also distinctive selenium isotope distribution profiles in human milk whey. For example, there is considerably more ^{78}Se than either ^{82}Se or ^{77}Se in human milk whey (Bratter *et al.*, 1998).

10.11. Iodine

Iodine is an essential nutrient but has only one clearly established function, a component of the thyroid hormones. The thyroid gland selectively concentrates iodine and stores 70–80% of total body iodine (15–20 mg). In the thyroid gland, iodine is attached to the glycoprotein thyroglobulin to produce monoiodotyrosine (MIT) and diiodotyrosine (DIT), the precursors of thyroid hormone. Through complex biochemical reactions, linkage of two DIT molecules produces thyroxine (T_4; tetraiodothyronine) and linkage of a MIT and DIT produces triiodothyronine (T_3). Enzymatic degradation of thyroglobulin releases T_4 and T_3 into circulation. In target tissues, T_4 is deiodinated to T_3, the main physiologically active form of thyroid hormone (Zimmermann, 2006). Other than the lactating breast, the significance of iodine concentration in extra-thyroidal tissues, such as the gastric mucosa, is less well understood (Josefsson *et al.*, 2002).

The thyroid hormones are important in regulating the basal metabolic rate and brain development. Iodine deficiency disorders (IDD) are characterized by loss of energy, mental retardation, hypothyroidism, goiter, cretinism and varying degrees of other growth and developmental abnormalities. The most serious adverse effect of iodine deficiency is damage to reproduction. For example, thyroid hormones are required for neuronal cell migration and myelination of the central nervous system. Maternal thyroid is the only source of T_4 and T_3 for the brain of the fetus because its thyroid gland does not start contributing to fetal requirements until mid-gestation in humans. Therefore, severe iodine deficiency during pregnancy increases the incidence of stillbirths, abortions and congenital abnormalities. Cretinism, the most severe form of neurological damage from foetal hypothyroidism, is characterized by gross mental retardation along with various degrees of deaf mutism and spasticity (Auso *et al.*, 2004; Perez-Lopez, 2007).

Many geographical regions inhabited by significant human populations have iodine-deficient soils as a result of soil leaching which depletes surface soils of iodide. Areas of frequent flooding and erosion (especially in south and southeast Asia), high alpine areas (e.g., the Alps, Andes, Atlas and Himalaya ranges) and several inland areas (including central Asia and Africa and central

and eastern Europe) are typically iodine deficient (Zimmermann, 2006). In goitrous children, iodine deficiency is exacerbated by inadequate intakes of selenium, iron or vitamin A, or by consumption of goitrogens. Some goitrogens are chemical agents (e.g., polychlorinated biphenyls, organophosphate pesticides and dioxin) that can permanently alter the pituitary–thyroid axis if exposure occurs during the perinatal period. Thyroid disruptor properties have also been attributed to several plant-derived substances. Examples are flavonoids, C-glycosylflavones (in millet), linamarin (from insufficiently processed cassava leaves) and thioglucosides (from cabbage, Brussel sprouts, broccoli and sorghum) (Fountoulakis *et al*., 2007).

The newest estimates of the iodine requirements for several age–sex groups have been revised upward. For example, the iodine requirement for infants aged 0–12 months are based on the current mean iodine intake of American infants exclusively fed human milk (Table 10.2). The AI iodine values established in 2001 for infants aged 0–6 months (110 μg/l) are much higher than the RDA values established earlier for the same age group (40 μg/d) (Food and Nutrition Board: Institute of Medicine, 2001). Likewise, the iodine AI for infants aged 7–12 months was increased also (130 μg versus 50 μg/d). The estimate average requirement for iodine during lactation is based on the average requirement of adolescent girls and non-pregnant women (95 μg/d) plus the average daily loss of iodine in human milk (114 μg/d). The RDA for iodine during lactation is 290 μg/d (Food and Nutrition Board: Institute of Medicine, 2001). Data appear insufficient to assess whether providing preterm infants with supplemental iodine (to match fetal accretion rates) prevents morbidity and mortality in preterm infants (Ibrahim *et al*., 2006).

Since 1990, the widespread introduction of iodized salt has greatly reduced the global prevalence of iodine deficiency. However, the World Health Organization recently estimated the worldwide prevalence of iodine deficiency at nearly 2 billion individuals, of whom 285 million are school-aged children (Zimmermann, 2006). Furthermore, the median iodine urine concentration (a standard indicator of iodine nutrition) has fallen in the United States from 321 μg/l in the 1970s to 145 μg/l currently (Zimmermann, 2006). The current average urine iodine concentration indicates adequate iodine nutriture but a reminder of the importance of regular monitoring. Socioeconomic status is not necessarily a predictor of iodine sufficiency for the general population. Despite the generally high standard of living in New Zealand, the mean iodine concentration in breast milk of mothers living in the South Island as late as 1998 and 1999 was 22 μg/l, a value only 15% of the average US value (Skeaff *et al*., 2005). The prevalence of iodine deficiency is the lowest (10.1%) in North and South Americas where the proportion of households consuming iodized salt is highest (90%). Europe has the highest

prevalence of iodine deficiency (59.9%), and the proportion of households consuming iodized salt is the lowest (27%). Most Europeans countries have weak or non-existent national programmes for iodine supplementation (Zimmermann, 2006). In Spain, almost half of pregnant women may have iodine deficiency (Perez-Lopez, 2007). In some countries, "silent iodine prophylaxis" occurs as improvements made in socioeconomic status allowed easier access to imported food products richer in iodine. Ironically, this approach sometimes has resulted in change of phenotypic expression of thyroid disease from endemic goiter to goiter associated with autoimmune thyroiditis from excessive iodine intakes (Fountoulakis *et al*., 2007).

Dietary iodine in the United States has generally been adequate since the 1920s but median urinary iodine level, a biomarker for dietary iodine, has decreased by over 50% from 1971 to 1994 among American women of childbearing age. However, The NHANES 2001–2002 data confirm the current stability of the US iodine intake and continued adequate iodine nutrition for the country (Caldwell *et al*., 2005).

Evidence supporting the protective effect of breast-feeding on the infant's thyroid metabolism is overwhelming, and breast milk is the only source of organic I, mostly thyroid hormones (T-4, T-3 and metabolites), in early infancy (Dorea, 2007). The iodine concentration in both human and bovine milks reflects maternal intake and thus are highly variable (see Table 10.1). In the United States, average breast milk iodine concentration of mothers is 146 μg/l. In countries with endemic IDD, breast milk iodine concentration is typically lower than 50 μg/l. Bovine milk is an important source of dietary iodine. The iodine content in bovine milk varies linearly with intake in the normal dietary range if other conditions are equal (Miller *et al*., 1975). Iodine content also varies with the season (Dahl *et al*., 2003) to the point that the incidence of thyrotoxicosis in Britain may be causally related to the high milk iodine levels in winter–spring (Phillips *et al*., 1988). In the United States, supplementation of animal feed with organic iodine has increased the amount of iodine in bovine milk by 300–500% between 1980 and 1986 (Food and Nutrition Board: Institute of Medicine, 2001). On the other hand, replacement of iodophors by other sanitizers in the dairy industry is associated with a decrease in iodine content of bovine milk and a re-emergence of iodine deficiency (Li *et al*., 2006).

Iodine enters bovine milk primarily as inorganic iodide, and iodine in bovine milk, as naturally secreted, is only about 10% bound. About 16% of total bovine milk iodine is in the cream but nearly all of the iodine in cream is in the non-fat (serum) portion (Miller *et al*., 1975). Cream separation, pasteurization and spray-drying of milk appear not to affect the concentration on a dry weight basis of either natural or iodophor-derived iodine in bovine milk. Boiling apparently does not induce iodine volatilization significantly

(0.02%) (Wheeler *et al*., 1983). The concentration of iodine is higher in whey cheeses than in casein cheeses (Dahl *et al*., 2003).

10.12. Molybdenum

Molybdenum is essential for the synthesis of a molybdenum co-factor containing a pterin nucleus that is required for the activity of sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase in higher animals and humans (Nielsen, 2006). These enzymes catalyze the conversion of sulfite to sulfate, the transformation of hypoxanthine to xanthine and the oxidation and detoxification of various pyrimidines, purines and pteridines. Nutritional molybdenum deficiency has not been identified unequivocally in humans other than in one individual nourished by total parenteral nutrition (Abumrad *et al*., 1981). Thus, molybdenum generally is considered to be of no practical nutritional concern for humans. The individual fed by parenteral nutrition and individuals with genetic molybdoenzyme deficiencies (Johnson, 1997) have enabled the description of signs and symptoms of molybdenum deficiency; these include hypermethioninemia, hypouricemia, hyperoxypurinemia, hypouricosuria, low sulfate excretion and mental disturbances. Inadequate data exist to identify any adverse health outcomes caused by excessive molybdenum intake by normal, apparently healthy individuals.

The FNB (Food and Nutrition Board: Institute of Medicine, 2001) found no functional criteria on which to base a RDA for infants. Thus, they set AIs for molybdenum that reflected the mean intake of infants fed principally human milk; these AIs were 2 μg/d for age 0–6 months and 3 μg/d for age 7–12 months. Extrapolation from the RDA for adults was the basis for setting RDAs for children (Table 10.2).

Concentrations of molybdenum in tissues, blood and milk vary with molybdenum intake (Committee on Minerals and Toxic Substances in Diets and Water for Animals, 2005). Highest concentrations of molybdenum are found in liver, kidney and bone (normally >1 mg/kg dry weight) (Johnson, 1997). The concentration of molybdenum in other tissues usually is between 0.14 and 0.20 mg/kg dry weight (Committee on Minerals and Toxic Substances in Diets and Water for Animals, 2005). Molybdenum concentration decreases rapidly in human milk with stage of lactation; for example, in a study of full-term infants, the median concentration of molybdenum in mothers' milk fell from 4 μg/l at 1 week postpartum to nearly undectable levels by 12 weeks postpartum (Friel *et al*., 1999). Another study found postpartum concentrations (μg/l) of 15 on day 1, 2.8 at 7–10 d and 2.6 by

1 month (Bougle *et al*., 1988). In addition, a study found that human milk contained 1.42 μg/l at 42–60 d postpartum and 1.78 μg/l at 293 d (Rossipal and Krachler, 1998). The FNB (Food and Nutrition Board: Institute of Medicine, 2001) used an average value of 2 μg molybdenum/l of human milk to calculate the AI for infants aged 0–6 months. The concentration of molybdenum is much higher in bovine milk and humanized infant formulae, which reports indicate ranges from 50 to 100 μg/l (Archibald, 1958; Tsongas *et al*., 1980; Hunt and Meacham, 2001). The concentration in milk can be increased substantially by ammonium molybdate supplementation (Archibald, 1951). Much of the molybdenum in human (Zeise and Zikakis, 1987) and bovine (Hart *et al*., 1967) milks is associated with xanthine oxidase. Xanthine oxidase activity of human colostrum is about 10% that of bovine milk (Oliver *et al*., 1971). Milk and milk products are considered rich sources of molybdenum for humans (Nielsen, 2006).

10.13. Cobalt

The only known function of cobalt in humans relates to its role in the structure of the cobalamins, a group of cobalt-containing compounds (corrinoids) (Stabler, 2006). The cobolamins are required as a co-factor for only two, but very important, enzymes in humans: L-methylmalonyl CoA mutase and methionine synthase. Only microorganisms retain the ability to synthesize cobalamins such that the source of cobalamins in all higher animals is the product of microbial synthesis. Characteristic of these compounds, cobalt is the central metal ion with four of the six coordination sites provided by a corrin ring (similar to the porphyrin ring found in heme). The fifth cobalt coordination site is through 5,6-dimethylbenzimidazole but the sixth coordination site, the center of reactivity, is variable: cyano, hydroxyl, glutathione or coenzyme forms (methyl or adenosyl). Thus, cyanocobalamin (the scientific term for "vitamin B_{12}") can be converted to either of the two cobalamin coenzymes that are active in human metabolism: methylcobalamin and 5-deoxyadenosylcobalamin. Ironically, cyanocobalamin is an artifact formed as a result of the use of cyanide in the purification procedures. Furthermore, cyanocobalamin, compared to hydroxocobalamin, binds to serum proteins less well and is excreted more rapidly (Tudhope *et al*., 1967).

Unless cobalt is absorbed as an integral part of vitamin B_{12}, the amount of cobalt in milk or other foods is not relevant *per se*. However, B_{12} deficiency is a serious problem in human populations and is caused either by an inadequate dietary intake of vitamin B_{12} or by malabsorption (e.g., lack of intrinsic

factor critical for B_{12} internalization and absorption in the ileal mucosa). Untreated vitamin B_{12} deficiency results in potentially irreversible neurological damage and life-threatening anemia. Infants fed predominantly human milk usually demonstrate clinical signs of B_{12} deficiency if the mother has been a strict vegetarian for at least 3 yr or has untreated pernicious anemia (Food and Nutrition Board: Institute of Medicine, 1998). Humans ordinarily obtain vitamin B_{12} from animal foods, mainly meat, fish and poultry. Milk and milk drinks are the second category contributing the most B_{12} to the diets of women. However, there is some evidence that boiling milk for 10 min reduces B_{12} content by $\sim$50%. This practice may of particular concern for certain lactovegetarians. For example, boiling milk was described as a common cooking practice among Hindu women in the United Kingdom (Stewart *et al.*, 1970).

10.14. Fluoride

Unequivocal or specific signs of fluorine deficiency have not been described for higher animals or humans (Nielsen, 2006). Fluoride, the ionic form of fluorine, has a well-established beneficial function in humans; it protects against pathological demineralization of calcified tissues as a pharmacological agent (Food and Nutrition Board: Institute of Medicine, 1997). Fluoride inhibits tooth enamel degradation by two separate mechanisms. Uptake of fluoride by tooth enamel crystallites and the formation of fluorhydroxyapatite during pre-eruptive tooth development reduce the risk of dental caries; the fluorhydroxyapatite is less acid-soluble than hydroxyapatite. After eruption, fluoride protects tooth enamel by reducing acid production by plaque bacteria and increasing the rate of enamel re-mineralization during an acidogenic challenge (Sieck *et al.*, 1990). Sodium fluoride (NaF) has been shown repeatedly and reproducibly to increase spinal bone mass in a dose-dependent manner. Yet, despite numerous studies, NaF has never been convincingly demonstrated to reduce the vertebral fracture rate in established spinal osteoporosis (Kleerekoper and Mendlovic, 1993).

Although fluoride intakes by fully breast-fed infants is low, fluoride intakes by partially breast-fed infants and by formula-fed infants is highly variable, depending primarily on the fluoride content of the water used to dilute concentrated liquid or powdered infant formula products. Prolonged exposure to high intakes of fluoride during infancy is much more common now than in the past because of a trend toward more extended feeding of formula (Fomon and Ekstrand, 1999). Infants fed human milk consume about 0.01 mg/d fluoride but those fed a formula reconstituted with fluoridated water may receive as much as 1.0 mg/d. For infants aged 0–6 months,

the current recommendation for adequate intake of fluoride is based on fluoride normally received from human milk, 0.01 mg/d, an amount 10–50 times less than the estimated safe and adequate intake established in 1989 (0.1–0.5 mg/d) (Food and Nutrition Board: Institute of Medicine, 1997).

Excessive intake of fluoride causes fluorosis, characterized by mottling of teeth (with hypomineralized and porous enamel), and sometimes the more severe skeletal fluorosis with lower extremity pain and microfracture (Hallanger Johnson *et al.*, 2007). For infants, the lowest-observed-adverse-effect level is 0.10 mg/kg/d for moderate enamel fluorosis. On this basis, the upper limit of fluorine is 0.7 and 0.9 mg fluoride/d for infants aged 0–6 months and 7–12 months, respectively.

The mean concentration of fluoride in mature human milk is approximately 18 μg/l (Table 10.1). The fluoride content of bovine milk is variable (Table 10.1). About 46–64% of the fluoride in bovine milk occurs as free fluoride ions with the remainder bound to proteins (Esala *et al.*, 1982). Essentially 100% of fluoride ingested in the fasted state as fluoridated water and 50–80% of fluoride ingested with food is absorbed from the gastrointestinal tract (Nielsen, 2006).

10.15. Boron

Boron is a bioactive element of low molecular weight (atomic weight = 10.81 $g \cdot mol^{-1}$) that is essential for all vascular plants (Loomis and Durst, 1992). There are four lines of evidence, derived in large part from research in animal models, that dietary boron can have beneficial effects on humans: (1) In amounts typically found in human and animal diets, boron improved bone health (independent of vitamin D status) by increasing bone development in frogs (Fort *et al.*, 2000), bone breaking strength in pigs (Armstrong *et al.*, 2000), broilers (Rossi *et al.*, 1993) and growing pullets (Wilson and Ruzler, 1997) and bone calcium concentration in chicks (Hunt *et al.*, 1994). (2) Boron interacts with specific steroid hormones; it counteracts the deleterious effects of dietary vitamin D deficiency on body growth in chicks (Bai and Hunt, 1996) and growth plate morphology in embryonic (King *et al.*, 1991) or hatched chicks (Hunt, 1989; Hunt *et al.*, 1994). In addition, boron increased the circulating concentration of 17β-estradiol in humans (Nielsen *et al.*, 1992; Naghii and Samman, 1997) and, together with injections of 17β-estradiol, increased trabecular bone surfaces in ovariectomized rats (Sheng *et al.*, 2001). (3) Physiologic amounts of boron apparently reduce the amount of insulin required to maintain plasma glucose in rats (Bakken and Hunt, 2003). (4) Borate or borate analogs can inhibit the *in vitro* activity of several enzymes in the eicosanoid pathway related to inflammation and

immune function (Spielberg *et al.*, 1979; Belver and Donaire, 1983; Rajendran *et al.*, 1994).

Evidence suggests that boron may be under metabolic control, possibly through the function of a boron transporter localized recently in tissues with excretory functions (kidney, parotid gland, submandibular gland, pancreas and liver) (Park *et al.*, 2004). Human blood boron concentrations are insensitive to changes in dietary boron intake (Hunt *et al.*, 1997), and the concentration of boron in milk from mothers of full-term healthy infants is highly conserved across time despite the fact that dietary intake of boron typically varies widely with food intake patterns and drinking water sources (Hunt *et al.*, 2004).

Because no biological function has been identified for boron in humans, neither an EAR nor an RDA nor an AI has been established for the element. The DRI UL for boron is 20 mg/d for adults; boron is considered to have a low order of toxicity (Table 10.2).

Boron is present naturally in all foods (Hunt and Meacham, 2001). Recent findings indicate that the mean concentration of boron in human milk from full-term mothers over the first 12 weeks of lactation (28 μg/l) is much less than that present in ready-to-eat infant formulae (~120 μg B/l) or in bovine whole milk (280 μg B/l; fluid, 3.3% milkfat) (Hunt and Meacham, 2001). Assuming that breast-fed full-term healthy infants consume milk at a daily rate of ~0.74 l during the first 4 months of lactation (Butte *et al.*, 1987), their total daily boron intake during the first 12 weeks of lactation is estimated at 0.022 mg/d. Even so, at a slightly older age (6–11 months), American infants are estimated to consume 0.55 mg B/d from all dietary sources (Hunt and Meacham, 2001). For males aged 51–70 yr, boron intake was estimated at 1.34 mg/d (1st percentile, 0.39; 99th percentile, 3.34 mg/d); for lactating females, 1.39 mg/d (1st percentile, 0.38; 99th percentile, 3.49 mg/d) (Food and Nutrition Board: Institute of Medicine, 2001). In postmenopausal women, diets that provide 0.36 mg B/2000 kcal (and otherwise nutritionally replete with minor supplements) result in negative boron balance (Hunt *et al.*, 1997).

The bioavailability of boron in human milk is likely to be high. It is known that low amounts of naturally occurring dietary boron (0.36 mg B/d) as well as supplemental inorganic forms (2.87 mg B/d; as orthoboric acid) are absorbed almost completely and excreted in the urine (Hunt *et al.*, 1997) of post-menopausal women. Little is known about the speciation of boron in natural foodstuffs. However, boron transport molecules in breast milk are probably associated with the soluble, instead of fat, fraction because the boron content of bovine whole milk (3.3% milkfat) and skim milk (0.08% milkfat) are not significantly different: 280 and 310 μg/l, respectively (Hunt and Meacham, 2001).

10.16. Chromium

Trivalent chromium was described as the glucose tolerance factor that alleviated impaired glucose tolerance in rats fed torula yeast-sucrose diets by Schwarz and Mertz (1959). Shortly thereafter, the glucose tolerance factor, or GTF, evolved into a speculated organic form of chromium. In 1977, it was reported that chromium supplementation overcame what was considered to be signs of chromium deficiency in a patient receiving total parenteral nutrition (Jeejeebhoy *et al*., 1977). This report occurred during the time when a mineral element often was accepted as essential based simply on evidence that dietary deprivation consistently induced a change in a biological function that was preventable or reversible by physiological amounts of the element. As a result, chromium was widely regarded as essential with a role in glucose metabolism. Thus, an estimated safe and adequate daily dietary intake was established for chromium by the US Food and Nutrition Board in 1980 (National Research Council, 1980). However, doubts about the nutritional essentiality of chromium arose after it was found that chromium analyses before 1980 were not valid and repeated efforts to characterize definitively a chromium-containing GTF were not successful. Additionally, studies on chromium essentiality subsequent to 1985 were not successful in showing that chromium deprivation consistently impaired a biological function that was prevented by physiological or nutritional amounts of chromium. Furthermore, early studies of chromium essentiality provided supplements to controls that resulted in chromium intakes over 100 times that of normal nutritional intakes. Thus, the early reports of chromium essentiality may have been describing pharmacologic or supranutritional actions of chromium.

Although chromium apparently is losing its designation as an established essential nutrient, there is much evidence showing that chromium is a bioactive and beneficial element for higher animals and humans. Numerous studies show that chromium beneficially affects circulating glucose, insulin and lipids in humans and a variety of animal species (Stoecker, 2006). A study that has received much attention found that 1000 μg chromium per day as chromium picolinate for 4 months markedly reduced blood glucose and glycated hemoglobin in diabetic Chinese subjects (Anderson *et al*., 1997b). The basis for this finding may be chromodulin, a naturally occurring oligopeptide composed of glycine, cysteine, aspartate and glutamate that tightly binds four chromium ions (Vincent and Bennett, 2007). Chromodulin apparently amplifies the tyrosine kinase activity of insulin-activated insulin receptor; the amplification is directly dependent upon the chromium content of chromodulin (Vincent and Bennett, 2007).

Methods of administration have resulted in controversy about the toxicity of chromium. Rats intravenously injected daily with 5 μg of chromium as a picolinate complex for 60 d exhibited signs of oxidative stress and DNA damage (Hepburn *et al*., 2003). In contrast, mice fed 5000 μg Cr/l in drinking water as chromium acetate for 17 months (Schroeder *et al*., 1963), and rats fed 100,000 μg Cr/kg diet as chromium picolinate for 24 weeks (Anderson *et al*., 1997a), exhibited no apparent signs of toxicity. However, a single acute oral dose of 895,000 μg of chromium as aqueous $CrCl_3$ induced increases in several markers of oxidative stress (Bagchi *et al*., 2002). Ingested trivalent chromium probably has a low order of toxicity because its complexes with oxygen-based ligands are usually electrochemically inactive and have poor ability to cross cell membranes.

The FNB did not find sufficient evidence to set EARs, so AIs were set for chromium-based estimated mean intakes (Food and Nutrition Board: Institute of Medicine, 2001). An AI of 0.2 μg/d for infants aged 0–6 months was based on a mean chromium intake principally from human milk. Intake data were used to determine AIs for other age groups (Table 10.2), including 5.5 μg/d for infants aged 7–12 months. An UL was not established for chromium because few serious adverse effects have been associated with excess intake of chromium.

Chromium is present in biological tissues at very low concentrations with picomolar concentrations occurring in liver, kidney, testis, bone and spleen (Stoecker, 2006). The average concentration of chromium in human milk has been estimated to be 0.25 μg/l (Casey *et al*., 1985). Bovine milk contains very little chromium. One reliable analysis indicated that chromium concentration in whole or skim milk was less than 0.5 μg/l (Anderson *et al*., 1992). Thus, milk and milk products are poor dietary sources of chromium. The chemical form of chromium in milk is unknown, but chromium in foods is generally in the trivalent state.

10.17. Arsenic

Arsenic can be toxic and carcinogenic, but some evidence also suggests that it may have beneficial actions in low amounts. Arsenic affects the utilization of labile methyl groups arising from methionine in higher animals. Thus, arsenic may beneficially affect the methylation of metabolically or genetically important molecules such as DNA and *S*-adenosylmethionine, the functions of which are dependent on or influenced by methyl incorporation. Most adverse effects associated with arsenic occur upon ingestion of inorganic arsenic present at high amounts in drinking water (Nielsen, 2006). The classical symptoms of arsenic toxicosis include numbness; tingling and paresthesia in

the extremities; decreased touch, pain and temperature sensation; and muscular tenderness. Chronic consumption of high amounts of inorganic arsenic in drinking water results in hyperkeratosis of the hands and feet, symmetrical pigmentation, conjunctivitis, tracheitis, acrocyanosis and polyneuritis and skin cancer.

The FNB (Food and Nutrition Board: Institute of Medicine, 2001) set no dietary reference intakes (DRI) for arsenic. Animal data would suggest that intakes of 12–25 μg/d may be beneficial (Nielsen, 2006). Recent surveys indicate that arsenic intakes from food are less than this; the median intakes of adult men and women in the United States are approximately 2.0–2.9 and 1.7–2.1 μg/d, respectively (Food and Nutrition Board: Institute of Medicine, 2001). Earlier surveys indicated higher mean intakes of arsenic ranging from 23 to 72 μg/d (Food and Nutrition Board: Institute of Medicine, 2001).

The mean arsenic concentration in healthy adult human tissues was reported to be highly variable and between 40 and 90 μg/kg dry weight (Anke *et al*., 1997). Human milk was found to contain 0.2–6 μg arsenic/l, with no differences between colostrum and mature milk (Anke *et al*., 1997). Normal bovine milk was found to contain 15–60 μg arsenic/l (Anke *et al*., 1997). Increasing dietary arsenic did not increase the arsenic concentration in bovine milk. Reported arsenic concentrations in milk indicate that dairy products can contribute a substantial portion of the total daily dietary intake of arsenic.

10.18. Nickel

Nickel is not generally regarded as an essential nutrient for higher animals and humans, apparently because of the lack of a clearly defined specific biochemical function (Committee on Minerals and Toxic Substances in Diets and Water for Animals, 2005; Nielsen, 2006). However, nickel has been identified as an essential component of seven different enzymes involved in hydrolysis and redox reactions in some lower forms of life (plants and some bacteria). Interestingly, in microbes, the substrates or metabolites of the enzymatic reactions are dissolved gases of hydrogen, carbon monoxide, carbon dioxide, methane, oxygen and ammonia. Additionally, nickel deprivation studies show that it has beneficial actions in higher animals (Nielsen, 2006). Nickel deprivation detrimentally affects reproductive function and bone strength. Nickel deprivation also has been shown to increase sensitivity to salt, increase triacylglycerol levels in serum and liver and reduce the activity of enzymes that degrade glucose. Nickel might have a function that is associated with vitamin B_{12}, because lack of this vitamin inhibits the response to

nickel supplementation when dietary nickel is low, and nickel can alleviate vitamin B_{12} deficiency in higher animals. No chronic nickel toxicosis signs caused by oral intake have been reported for humans (Food and Nutrition Board: Institute of Medicine, 2001; Nielsen, 2006). Toxicosis through oral intake is limited to a few case reports of acute affects resulting from the ingestion of high doses of soluble nickel salts; this resulted in nausea, abdominal pain, diarrhea, vomiting and shortness in breath. Nickel-sensitive individuals may show a contact dermatitis-like reaction after a high intake (i.e., 0.6 mg) of soluble nickel after fasting.

The FNB (Food and Nutrition Board: Institute of Medicine, 2001) set no RDA or AI for nickel, or an UL for infants; however, an UL of 0.2 mg/d was set for children aged 1–3 yr. Animal data suggest that intakes near 100 μg nickel per day may be beneficial (Nielsen, 2006). Typical daily dietary intakes for nickel are 70–400 μg/d (Food and Nutrition Board: Institute of Medicine, 2001).

Nickel is widely distributed in tissues at concentrations generally between 0.01 and 0.2 mg/kg wet weight (Eder and Kirchgessner, 1997; Nielsen, 2006). At 38 d postpartum, the mean nickel concentration in human milk was reported to be 1.2 μg/l (Casey and Neville, 1987). However, another report indicated that human milk contained a much higher concentration of nickel of 41 μg/l (Anke *et al.*, 1993). Mature bovine milk was found to contain 10–30 μg nickel/l (Casey, 1977; Anke *et al.*, 1993). Thus, dairy products could supply a significant proportion of the daily intake of nickel. This suggestion is supported by the finding that Canadian infants (age 0–12 months) had a nickel intake of 38 μg/d supplied by both human milk and formula consumption (Dabeka, 1989).

10.19. Silicon

Although silicon deprivation has been reported to produce aberrant metabolism of connective tissue and impaired immune function, silicon is still not generally accepted as an essential nutrient for higher animals. Recently, however, dietary silicon correlated positively and significantly with bone mineral density at all hip sites in men and pre-menopausal women in a large cross-sectional, population-based study (Jugdaohsingh *et al.*, 2004). No acute oral silicon toxicity signs have been identified for humans. Extremely high amounts of silicon are needed to have just relatively minor effects on growth in experimental animals (Committee on Minerals and Toxic Substances in Diets and Water for Animals, 2005). Silica stones have been found in people on long-term antacid therapy with magnesium trisilicate (Food and Nutrition Board: Institute of Medicine, 2001). The FNB did not set an UL for silicon

because of the lack of data showing adverse effects of silicon (Food and Nutrition Board: Institute of Medicine, 2001).

The FNB (Food and Nutrition Board: Institute of Medicine, 2001) set no RDA or AI for silicon. Based on the US Total Diet Study (1991–1997), the median intake of silicon was 14 and 21 mg/d for women and men, respectively (Food and Nutrition Board: Institute of Medicine, 2001). The range of intakes (1st and 99th percentiles for all individuals) was 3.5–80 mg/d.

Animal studies indicate that the silicon content of connective tissue (e.g., aorta and tendon) is four to five times richer in silicon than soft tissues (e.g., liver, heart and muscle), which contain 2–10 μg/g dry weight (Carlisle, 1997). The mean concentration of silicon in human milk has been reported to be 0.47 mg/l up to 5 months postpartum (Anderson, 1992). Bovine milk apparently contains a similar amount of silicon (Jugdaohsingh *et al*., 2002). Thus, dairy products contribute very little to the dietary intake of silicon.

10.20. Vanadium

Vanadium is not generally accepted as an essential nutrient; however, it can be bioactive. Its ability to inhibit selectively protein tyrosine phosphatases at submicromolar concentrations probably explains a broad range of effects found for vanadium, most notably insulin-mimetic action. Additionally, limited animal deprivation studies suggested that vanadium deprivation alters thyroid hormone metabolism, impairs reproduction and induces bone and joint abnormalities. In lower forms of life, some haloperoxidases require vanadium. Vanadium is a relatively toxic element for humans (Nielsen, 1997; Food and Nutrition Board: Institute of Medicine, 2001). The threshold for toxicity through ingestion apparently is between 10 and 20 mg/d. Signs of toxicity include abdominal pain, anorexia, nausea and diarrhea.

The FNB (Food and Nutrition Board: Institute of Medicine, 2001) set no RDA or AI for vanadium, or an UL for infants. An UL of 1.8 mg/d was set for adults older than 18 yr. Typical intakes of vanadium are 6–18 μg/d for adults (Pennington and Jones, 1987).

Vanadium concentrations in tissues normally are <10 ng/g fresh weight (Nielsen, 1997). Bone apparently is a major sink for excessive retained vanadium. Human colostrum, transitional and mature milk were found to generally contain <1 ng vanadium/g dry weight (Kosta *et al*., 1983). Bovine milk may be a significant dietary source of vanadium because it reportedly contains about 3 ng vanadium/g (Myron *et al*., 1977). However, a fivefold variation in the vanadium content of milk, depending upon geographic location, has been described (Soremark, 1967). Apparently, the vanadium in milk

is water soluble, because it is relatively more abundant in skim milk than in butter (Myron *et al*., 1977).

10.21. Summary and Conclusions

Defining the roles of dietary minerals in human health has advanced remarkably in recent years. This new knowledge has led to significant revision of dietary recommendations and guidelines worldwide. Even so, there are still considerable deficits in our understanding of mineral nutrition. A primary challenge for current human mineral nutrition research is more complete characterizations of interactions among mineral salts and with other nutrients and environmental (e.g., infectious agents and exercise) and genetic factors to maintain health.

Progress in analytical methodologies has greatly improved the confidence in reported macromineral and trace element content in human and bovine milks and humanized infant formulae. With few exceptions (e.g., iodine and selenium), the values for human and bovine milks are remarkably similar across a wide range of geographical locations. Milk and milk products are significant dietary sources of arsenic, boron, calcium, cobalt (as vitamin B_{12}), iodine, molybdenum, nickel, phosphorus, potassium, sodium and zinc.

The mineral content of human breast milk remains the "gold standard" for establishing dietary mineral requirements of infants for the first 6 months of life. The concentrations of minerals in human milk are generally lower than those in bovine or humanized infant formulae. Thus, approaches for infant nutrition other than exclusive breast feeding should be taken with caution. Attempts to provide universal iron and folic acid supplementation in areas of high malaria transmission have caused increased morbidity and mortality. The high phosphorus content of bovine milk compared to human milk increases the risk of hyperphosphatemia in neonates fed bovine milk-derived formulae. Infants fed formulae may consume amounts of fluoride above the lowest-observed level known to induce moderate enamel fluorosis, a level nearly 100 times that consumed by exclusively breast-fed infants.

Recommendations for mineral nutrition pertinent to the consumption of milk and milk products must remain vigilant of changes in marketplace activities and changes in food choices that affect mineral intakes and bioavailability. Increased consumption of sweetened soft drinks and juices reduces milk consumption, a shift in dietary practice that tends to reduce calcium intake. Increased use of phosphate salts in processed foods for nonnutrient functions and increased consumption of those products increase the phosphorus content of the US food supply. Replacement of iodophors by other sanitizers in the dairy industry has contributed to the re-emergence of

iodine deficiency in some countries while increases in "silent iodine prophylaxis" has increased the incidence of iodine toxicity in other countries. Because zinc and iron requirements are not met by exclusive breast-feeding after approximately 6 months of age, proper selection of complementary foods with low phytate-to-mineral ratios becomes critical for normal growth and development.

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11

Water in Dairy Products

D. Simatos, D. Champion, D. Lorient, C. Loupiac and G. Roudaut

Abbreviations

BET:	Brunauer, Emmett, Teller, 1938
DMTA:	dynamic thermal analysis
DS:	dielectric spectroscopy
DSE:	Debye-Stokes-Einstein
DSC:	differential scanning calorimetry
ERH:	equilibrium relative humidity
ESR:	electron spin resonance
GAB:	Guggenheim, 1966, Anderson, 1946, De Boer, 1953
GLT:	glass liquid transition
NEB:	non-enzymatic browning
NMR:	nuclear magnetic resonance
PALS:	positron annihilation lifetime spectroscopy
RH:	relative humidity
SI:	sorption isotherm
SMP:	skim milk powder
WMP:	whole milk powder
WPI:	whey protein isolate

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11.1. Introduction

During the last 50 years, our knowledge of the properties and roles of water in foods has progressed very significantly; at the beginning of this period, the emphasis was on the binding of water to other constituents, which was supposed to impart to it special properties, different from those of bulk water. These concepts of free and bound water were used widely, although most often poorly defined. They can now be supplemented by much more precise descriptions of the properties of water present in food products, in terms of thermodynamics and molecular mobility. The concept of bound water in foods (as well as in biological systems) originated in various observations, such as increasing difficulty to dehydrate the materials and increasing irreversibility of the dehydration. The concept was backed up by the knowledge of the unique properties of the water molecule. The dipolar structure of the molecule and its ability to interact with various chemical groups of the other constituents actually are at the basis of the most important role of water in some sensory properties of foods and in many of the changes that occur during processing and storage.

To relate the properties of food materials to the properties of water, thermodynamical approaches were first used. For long, the freezability of water was considered as an index of its binding state. Then, water activity became the favoured parameter to characterize the availability of water to control physical, chemical or biological evolutions in foods. The mobility of water itself, as well as the mobility it confers on other constituents, had also been considered early; this approach, however, experienced an explosive development with the awareness that food products most often are in a non-equilibrium state (and then that the water activity concept should not be used with them). The glass transition was proposed as a unifying concept and became very popular among food scientists.

The respective relevance of both approaches, thermodynamical properties and glass transition, has been discussed thoroughly. Currently, consensus appears to be established, to recognize that both may have an essential role, in a particular domain, depending on the type of product or the objectives (ISOPOW, 2000). As an example, both concepts contribute to the construction of state diagrams.

11.2. Water Activity

11.2.1. Definitions

Water activity of a system is a way of characterizing the potential energy of the contained water, which would be related to the difficulty of removing it, e.g. on drying, and to its availability to allow the functioning of living cells. It can be viewed, for instance, as the difference between water vapour pressure

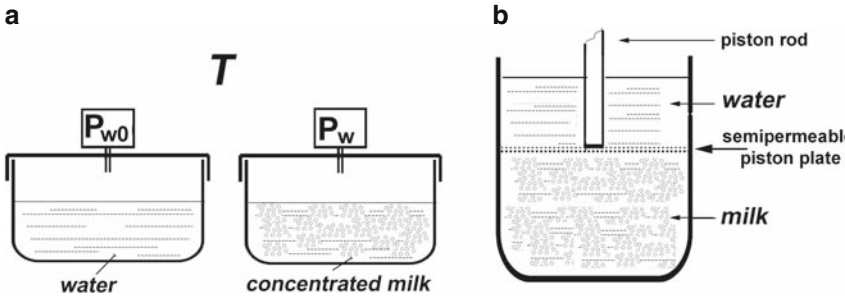

Figure 11.1a. (**a**) Schematic representation of the difference in water vapour pressure above pure water (P_{w0}) and above concentrated milk (P_w), measured at the same temperature (T). (**b**) Since the chemical potential of water in the concentrated milk is less than in pure water, there is a tendency for water to move into the milk through the semi-permeable plate. A downward pressure, Π, on the piston is required to maintain it in place.

measured over pure water and over a food product (Figure 11.1a), or in the energy necessary to compensate for the osmotic pressure of a solution (Figure 11.1b). Water activity (a_w) is defined by the following relation:

$$\mu_w - \mu_{w0} = RT \ln a_w \tag{1}$$

where μ_w and μ_{w0} are, respectively, the chemical potential of water in the material and of pure water at the same temperature, T, and pressure. From the definition of chemical potential (Emschwiller, 1951), the difference (μ_w–μ_{w0}) is equal to the variation of free energy (ΔG) of the system when the water chemical potential changes from μ_w to μ_{w0}. Calculation of ΔG, e.g. the work corresponding to the upward motion of the piston if water is allowed to enter the concentrated solution (Figure 11.1b), allows derivation of relations between a_w and physical properties, which lead to measurement methods and technological applications (calculations can be found in, e.g., Griffin, 1981):

- Vapour pressure, equilibrium relative humidity (ERH):

$$a_w = \frac{p_w}{p_{w0}} \qquad a_w = \frac{ERH}{100} \tag{2}$$

p_w and p_{w0} are the water vapour pressures at equilibrium, respectively, of the sample and pure water at the same temperature.

- Osmotic pressure:

$$\ln a_w = -\Pi \frac{V_w}{RT} \tag{3}$$

Π is the osmotic pressure that would be exerted by the solution of water activity, a_w, at temperature T.

- Freezing point:

$$\ln a_w = \frac{\Delta H_m}{R}\frac{T - T_0}{TT_0} + \frac{\Delta C(T - T_0)^2}{2RT^2} \tag{4}$$

T_m and T_0 are, respectively, the temperature of freezing of the sample and of pure water ((T_m–T_0) being the cryoscopic lowering); ΔH_m is the melting enthalpy of ice; and ΔC is the difference in the specific heat of ice and liquid water.

In ideal aqueous solutions (dilute solutions or/and where solutes are of molecular size similar to water), it is observed that a_w is equal to the mole fraction of water (X_w) within the solution (Raoult's law). A parameter, γ (activity coefficient), was introduced to represent the deviation from ideality:

$$a_w = \gamma\ X_w \tag{5}$$

when interactions occur between water and solutes or when the size of the solute is much larger than the size of the water molecule ($\gamma < 1$ in both cases), or when there are solute–solute interactions ($\gamma > 1$). The presence of solutes induces a disorder in the structure of water, i.e. an increase in entropy, which results in a reduced chemical potential. With small solutes, a_w is controlled mainly by X_w, i.e. by the number of solute molecules. In the presence of macromolecules or hydrophilic surfaces (cell membranes), the mixing entropy plays a minor role in the depression of a_w (X_w remains high); low values of μ_w (and then of a_w) are attributed to interactions of the water molecules with the other constituents. If pure water is contained in a cylindrical capillary space (radius r), the wall of which is perfectly wetted by water, the difference in chemical potential defined as in (1) can be shown to be

$$\mu_w - \mu_{w0} \cong -V_w\sigma\frac{2}{r} \tag{6}$$

V_w and σ are, respectively, the molar volume and the superficial tension of water. If water is held within a system of elastic walls (living cell or a gel) exerting a pressure, P, then

$$\mu_w - \mu_{w0} = V_w\ P \tag{7}$$

All in all, for a solution being exposed to these various effects,

$$\ln a_w = \frac{V_w}{RT}\left(P - \frac{2\sigma}{r}\right) + \ln(\gamma_w X_w) \quad (8)$$

With food products, a_w is more commonly considered to be controlled by the solutes only, probably because capillary and pressure effects are quantitatively small; they should not be ignored, however.

11.2.2. Relevance

It must be remembered that the definition of a_w and the derived expressions assume that the system is in a state of thermodynamic equilibrium. It is sensible to question the validity of their application to food products (ISOPOW, 2000).

Actually, solutions of small solutes, even if concentrated, can be considered to be at thermodynamic equilibrium, whereby the a_w concept is valid. Microbial cells cannot be considered to behave as true osmometers; other parameters, such as pH, nature of the solutes in the medium and mobility of the metabolites, must be taken into account. Water activity, however, is an essential parameter for all aspects of their activity: germination and growth, production of toxins and of aroma. a_w is therefore an important parameter of processing and storage to obtain the desired organoleptic properties and safety standard of the product especially for cheeses (Figure 11.2). Some studies have demonstrated a relationship between molecular mobility and some aspects of microbiological activity (Kou *et al.,* 1999). However, from an extensive review (Chirife and Buera, 1996) of the then available knowledge, it was concluded that, although mobility factors, in addition to a_w, may be useful for a better prediction of microbial behaviour in foods, glass transition concepts do not provide any better alternatives than a_w as a predictor for this. The texture of cheese was found to be better correlated to water content (coefficient of correlation with an extrusion force = –0.867) and fat content, than with a_w (coefficient of correlation = –0.548) (Ruegg, 1985). Water activity, however, will control the exchanges of water between cheese and the environment and is thus an important parameter controlling texture.

For solid low-moisture or semi-moist food products, being closer to their glass transition, it must be recognized that what is measured is the relative humidity of the atmosphere in contact with the product, at best in a pseudo-equilibrium state. The term "apparent water activity" should be used (ISOPOW, 2000). As will be discussed later, the current sorption models provide no reliable information about the physical state and properties of water. From a practical point of view, however, the a_w concept is very useful, because it allows description of the gradient that will determine the transfer of

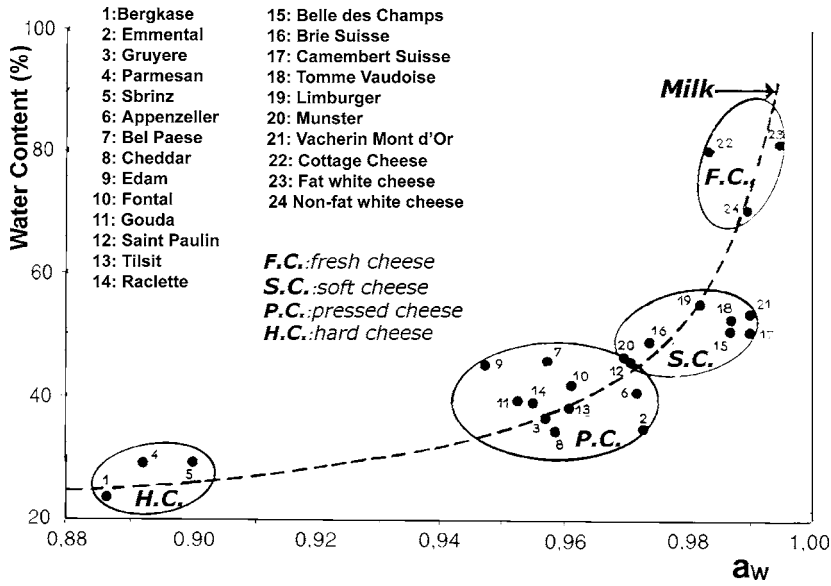

Figure 11.2. Water content and a_w of cheeses (Banon and Hardy, 2002).

water between two compartments having different initial relative vapour pressures in a multi-domain food system or between a food product and its environment during drying or osmotic dehydration or during storage (ISOPOW, 2000). The rate of these exchanges is controlled by the diffusivity of water, or possibly by the presence of a barrier against moisture transfer. Sorption isotherms can also be used to estimate the local water content, and then the local glass transition temperature in multi-component products (Mousia *et al.*, 2000).

11.2.3. Models

For systems where the a_w concept is valid, numerous models, of a theoretical or empirical nature, are available to describe (sometimes predict) a_w in non-electrolyte or electrolyte solutions (Sereno *et al.*, 2001). The Norrish equation (Norrish, 1966)

$$\log \gamma = -k_2 \, X_2^2 \tag{9}$$

allows calculation of the water activity coefficient, γ, for solutions of low molecular weight non-electrolytes; values for the k_2 coefficient can be found

in the literature (Chirife *et al.*, 1980, 1982; Chirife and Ferro Fontan, 1980; Miracco *et al.*, 1981); and X_2 is the solute molar fraction.

The Flory–Huggins equation (Flory, 1953) is based on the statistical calculation of the number of possible arrangements for a polymer molecule in solution. From the calculation of the mixing enthalpy and entropy, the following equation was derived:

$$\ln a_w = \ln(1 - \Phi_2) + \left(1 - \frac{1}{n}\right)\Phi_2 + \chi\Phi_2^2 \qquad (10)$$

where Φ_2 is the volume fraction of the polymer; n the number of polymeric segments, which is generally assumed to be equal to the ratio of molar volumes of the polymer and water; and χ is a fitting interaction parameter ($\chi>$ or <0.5 depending on the solute–solvent affinity being, respectively, weak or strong). The Flory–Huggins equation generally describes satisfactorily the water content-a_w relationship for $a_w > 0.9$.

The UNIQUAC method (Universal Quasi-Chemical) (Abrams and Prausnitz, 1975) derives activity coefficients from the excess Gibbs function by estimating the interactions between molecules. The UNIFAC model (Universal Functional Activity Coefficient) (Fredenslund *et al.*, 1977) is similar, but the molecules are replaced by functional groups. Although UNIFAC interaction parameters are obtained by fitting, the model can be considered as fully predictive, since its parameters are derived from experimental data concerning a great number of various molecules.

For electrolytic solutions, the activity coefficient can be predicted from the Pitzer model, based on the calculation of electrostatic interactions between ions according to the Debye–Hückel theory (Pitzer and Kim, 1974; Pitzer, 1980). The model was used to calculate the a_w of solutions of organic and inorganic strong electrolytes (Ferro Fontan *et al.*, 1980).

For cheese, empirical equations can be used to predict a_w or to explain measured changes based on chemical characteristics (Ruegg, 1985; Esteban and Marcos, 1990). From a multiple regression analysis using data from 82 different cheeses ($0.87<a_w<0.994$), a predictive equation was established, relating a_w to NaCl, non-protein nitrogen and ash contents and pH, with the first two characteristics being the most influential (Ruegg, 1985). Simpler expressions are proposed for particular cheese varieties (Esteban and Marcos, 1990; Saurel *et al.*, 2004), e.g. a linear expression relating a_w to the NaCl molality, which can be used for soft cheeses, with a water content >40% and a low degree of proteolysis.

Sorption isotherms, which represent the relationships between water content and the relative vapour pressure of water at "equilibrium" with the product at a given temperature, have been determined for a great number of

food products and materials, as most important data for technological applications. A variety of mathematical models have been proposed to describe these curves, the most popular of which being the BET and GAB isotherms. According to the adsorption BET model, the vapour molecules are first adsorbed on the solid as a monolayer, with a constant energy; further layers are then deposited with the liquefaction heat. The following expression is derived:

$$\frac{\mathrm{m}}{\mathrm{m}_1} = \frac{(\mathrm{c}-1)a_w}{1+(\mathrm{c}-1)a_w} + \frac{a_w}{1-a_w} \tag{11}$$

m being the water content, m_1 the water content corresponding to the monolayer and c a constant related to the adsorption energy of the first layer. This model correctly describes the sorption isotherms of food materials for $0.2 < a_w < 0.5$. Many other expressions can be found in the literature: Van den Berg and Bruin (1981) counted 75 expressions, comprising one to seven parameters. The GAB expression, which is currently the most often used, can be satisfactorily fitted to experimental data up to $a_w \approx 0.90$, with three parameters:

$$\frac{\mathrm{m}}{\mathrm{m}_1} = \frac{(\mathrm{c}-1)\mathrm{k}a_w}{1+(\mathrm{c}-1)\mathrm{k}a_w} + \frac{\mathrm{k}a_w}{1-\mathrm{k}a_w} \tag{12}$$

It must be noted that the parameters m_1 and c do not necessarily have the same values in the BET and GAB expressions for the same product (Timmermann *et al.,* 2001). A better fitting, however, can be obtained with a four-parameter expression, even for non-sigmoid curves, which cannot be fitted to GAB (Peleg, 1993):

$$\mathrm{m} = \mathrm{k}_1 {a_w}^{\mathrm{n}1} + \mathrm{k}_2 {a_w}^{\mathrm{n}2} \tag{13}$$

In the end, a two-parameter expression was claimed to have the advantage over GAB of allowing water content to infinitely rise for a_w approaching 1 (Lewicki, 2000):

$$\mathrm{m} = A\left(\frac{1}{a_w} - 1\right)^{b-1} \tag{14}$$

The mathematical description of sorption isotherms is extremely useful in many circumstances of food technology: It provides the necessary information for food formulation, i.e. the water content of the different ingredients,

which will be in equilibrium with each other, or the formulation allowing the desired a_w to be obtained; it is an indispensable tool for designing drying operations, or packaging. On the other hand, the use of the BET and GAB models to obtain information on the interactions of water with the other constituents is very questionable. First, the hysteresis observed between sorption and desorption points to the non-equilibrium character of the isotherms. Although correlations could be found between the chemical structures of solutes and BET or GAB parameters, for instance between the "monolayer" GAB values and the number of polar groups in proteins (Timmermann *et al*., 2001), it is increasingly admitted that the basic assumptions of the BET model are not fulfilled in the case of water sorbed on polar materials (energetic equivalence of all sites on the sorbing solid surface). Moreover, the plastifying action of water on the solid certainly plays a role in the form of the sorption curves. It would most likely be more justified to consider low-water-content food materials as solid solutions (Kuntz and Kauzmann, 1974). Models taking into account the plastifying effect of water on the sorption process have been proposed recently. While these models do not allow more accurate mathematical description of isotherms than the previously reported ones, they advance our understanding of the processes that occur as water vapour is taken up into amorphous solids. Zografi and co-workers (Hancock and Zografi, 1993; Shamblin *et al*., 1998; Zhang and Zografi, 2000) combined the Flory–Huggins solution model with the Vrentas model based on the free volume changes resulting from the water sorption to describe sorption isotherms of water on poly(vinyl pyrrolidone) (PVP), sugars and their mixtures. Benczedi *et al.* (1998a,b) analysed the sorption isotherms of water on starch as a combination of the Freundlich adsorption model (a monolayer adsorption model with independent, different sorption sites) and the Flory–Huggins model of polymer solution. The approach has been further refined by Ubbink *et al.* (2007), considering that the composition of carbohydrate systems influences the sorption of water differently in the glassy and rubbery states and that the two models of Freundlich adsorption and of Flory–Huggins solution therefore apply *successively* during the sorption process. The authors satisfactorily described the isotherms of mixtures of a maltopolymer with various concentrations of maltose.

Sorption energies are often calculated from sorption isotherms obtained at various temperatures using a relationship derived from the Clausius–Clapeyron law. As discussed for the BET/GAB monolayer values, the "net isosteric heat of sorption", Q_{nst}, thus obtained cannot be used to characterize the interaction of water with the sorbing solid. A maximum in the Q_{nst} values for a given water content has often been reported. This suggests a

biasing by entropy variations resulting from changes in the organization of the solid. Direct measurements of the desorption heat by calorimetry, with plant products (Mulet *et al.*, 1999) and various products, including whole and skim milk powders (Ruckold *et al.*, 2003), while consistent with Q_{nst} values at high water contents, confirm the expectation of continuously increasing heat when water content is decreasing.

A great number of studies have been devoted to water sorption by dairy powders. Skim and whole milk powders have the same sorption behaviour (i.e. the same "equilibrium" water content at the same a_w), when the water content is calculated by a non-fat basis (Jouppila and Roos, 1994; Lin *et al.*, 2005) (Figure 11.3). The isotherm for water sorbed by freeze or spray-dried lactose shows a break for a relative humidity around 40%, depending on the storage temperature and time, which is assigned to the desorption of water associated with lactose crystallization (Jouppila and Roos, 1994). This break can be

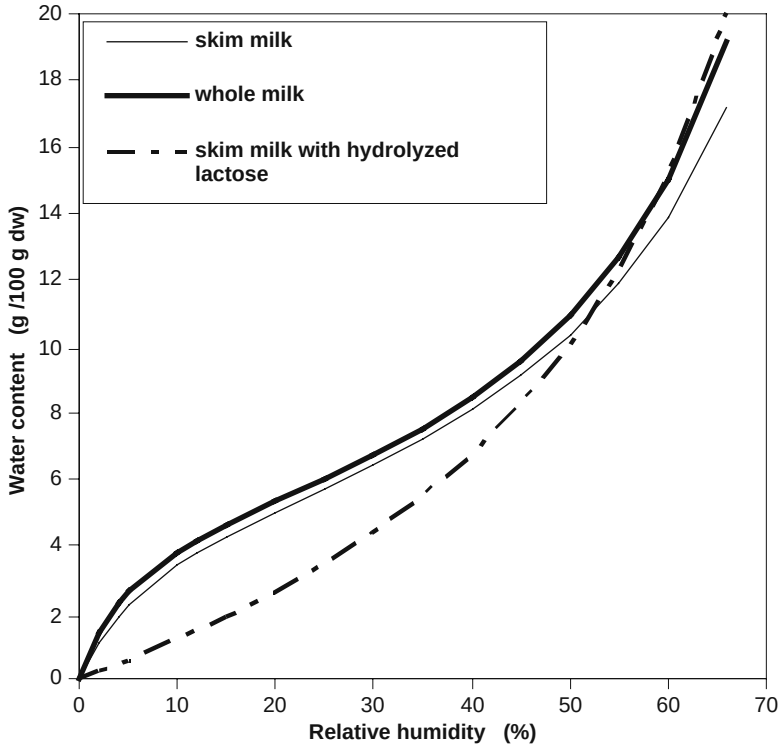

Figure 11.3. Water sorption isotherms for skim milk, whole milk and skim milk with hydrolysed lactose (Jouppila and Roos, 1994). The lines are drawn from the GAB model.

observed also with milk and whey powders (Berlin *et al.*, 1968). In order to obtain isotherms for truly amorphous lactose, particularly for the temperature and RH range relevant to spray-drying conditions, dynamic methods were proposed, allowing samples to reach equilibrium with the air in a short time [3 h with a fluidized bed in sorption conditions (Kockel *et al.*, 2002) or 50 min with a thin film, in desorption (Lin *et al.*, 2005)]. At low RH, amounts of water sorbed by crystalline α-lactose monohydrate are only 100 times lower than by amorphous lactose, but strongly increase for RH >80%. The water is then retained as capillary water between lactose particles, the water content being dependent on particle size (Bronlund and Paterson, 2004).

An exhaustive review of studies on water sorbed on caseins and whey proteins can be found in Kinsella and Fox (1986). Water sorption by caseins or whey proteins yields sigmoidal SI, as for biopolymers in general (Figure 11.4). The shape of the curves and the sorption rates vary with the composition of powders and the method of preparation, although denaturation of the proteins

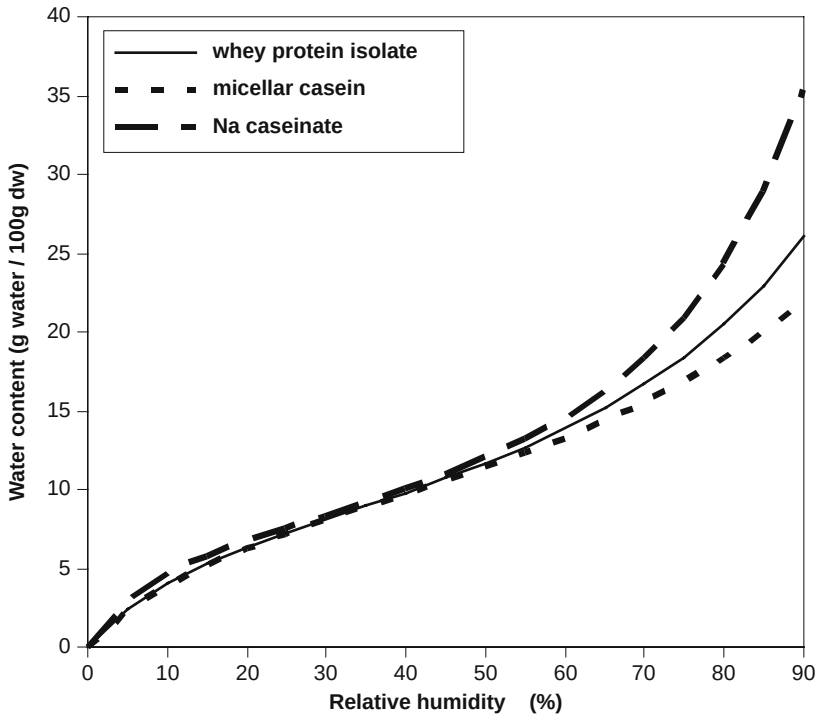

Figure 11.4. Water sorption isotherms for micellar casein and whey protein isolate (sorption 4–37°C, Foster *et al.*, 2005) and Na-caseinate (sorption 25°C, Weisser, 1985). The lines are from the GAB model.

was generally reported to cause only little change in the sorption behaviour. From a study of dried yogurt, for instance, it was concluded that the sorption capacity of powder might be influenced more by differences in raw material than by the drying methods (Kim and Bhowmik, 1994). The amounts of water sorbed by caseins and caseinate are strongly dependent on pH, particularly at high a_w (above ≈ 0.5). SI show typical hysteresis loops, water contents retained during the initial desorption being higher than the amount of water sorbed during subsequent sorption.

The isotherm for a multi-component dairy product can be predicted using a simple additive model, in which the amount of water sorbed at any given a_w is the weighted addition of the water that the components would sorb alone, assuming that no interactions between components occur (Berlin *et al.*, 1968; Foster *et al.*, 2005). Isotherms for commercial dairy powders could be predicted based on the isotherms for amorphous lactose, micellar casein and whey proteins, with the difference between predicted and measured water contents being mostly less than $\pm 10\%$, i.e. within the measure error limit. The predicted water contents, however, were in general slightly greater than those measured, which could suggest some interactions between components (Foster *et al.*, 2005).

The amounts of water sorbed by various dairy powders (lactose, whey protein isolate, milk protein concentrate and micellar casein) showed no obvious temperature dependence between 4 and 38°C, but were lower at 50°C (Foster *et al.*, 2005). Desorption isotherms for skim and whole milk powders also showed slightly lower residual water contents as temperature increased between 53 and 90°C, at low RH, but increasing with temperature at RH>30–40%. This phenomenon, which has been reported previously for various sugar-containing foods, is explained by the increase in solubility of lactose (Lin *et al.*, 2005). The a_w measured for six different cheeses between 5 and 30°C showed no significant temperature dependence. SI, however, revealed that a slightly greater amount of water sorbed at 5°C than at 25°C in the intermediate moisture range (Ruegg, 1985).

Variability in published sorption data results not only from variations in composition and methods of sample preparation but also from variations in water content determination, equilibration time, etc. Literature SI, therefore, even taken from careful compilations (Iglesias and Chirife, 1982; Wolf *et al.*, 1985) should be used only as a first approximation.

11.3. State Diagrams

State diagrams are now commonly used to represent the interactions of water with food materials, as they can help to design many processing operations or storage conditions. Figure 11.4 represents the state diagram for milk, with

indication of the processing steps from fresh milk to milk powder (Vuattaz, 1999, 2002). Similar state diagrams have been published for complex food products and biological systems (Rasmussen, 1969; Simatos *et al.*, 1975; Levine and Slade, 1989, 1990; Sa *et al.*, 1999).

11.3.1. Freezing and Solubility Lines

The T_m curve (freezing or cryoscopic line) (Figures 11.5, 11.6) represents the temperature at which ice begins to separate when the system having the indicated concentration is cooled under equilibrium conditions. This curve also represents the concentration of the remaining liquid phase, as the temperature continues to decrease and more ice is separated from it. Similarly, the solubility line (T_s) represents the concentration of the liquid phase at equilibrium with crystals. With some solutions, e.g. those containing high concentrations of mineral salts, the freezing process ends when the residual liquid phase, having reached a specific concentration (at the intersection with the T_s line), fully crystallizes as a eutectic mixture of ice crystals and crystals of solute. With most food-component solutions, however, the solute does not crystallize during cooling. Because of the difference in kinetics of crystallization between solute and water, only ice crystallization occurs. The concentrated liquid phase is then solidified as a glassy material. The water in this glassy phase has been observed to remain unfrozen whatever low temperature

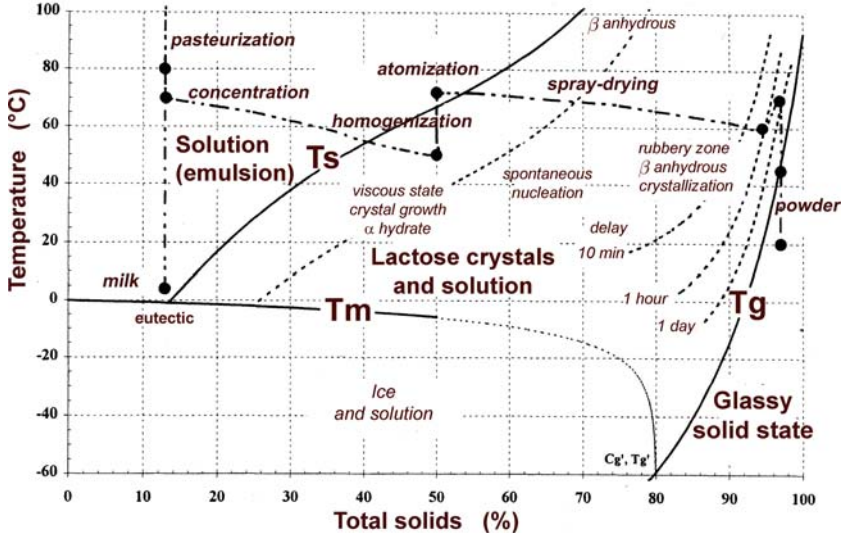

Figure 11.5. State diagram for milk, based on lactose (Vuattaz, 1999, 2002).

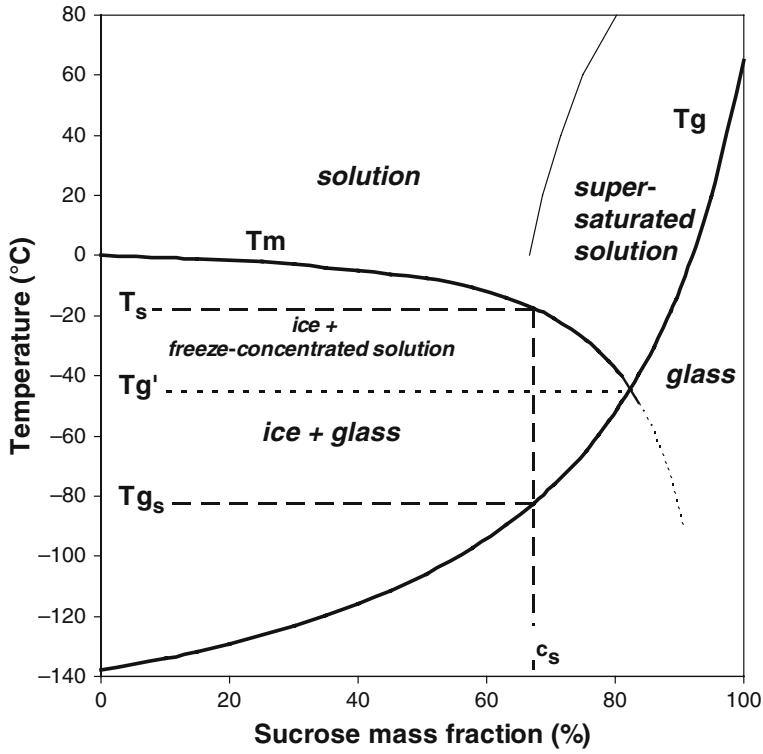

Figure 11.6. State diagram of the sucrose–water system. T_g and T_m indicate the temperatures of glass transition and of "equilibrium" freezing/melting, respectively, versus sucrose mass fraction. T_g', glass transition temperature of the maximally freeze-concentrated phase (here defined as the intersection of curve T_g (DSC T_{gonset}) and curve T_m (UNIQUAC model)). C_s and T_{gs}, concentration and glass transition temperature of the partially freeze-concentrated liquid when the product is stored at a temperature $T_s > T_g'$ (from Blond *et al.*, 1997).

and long cooling time may be used. This "unfreezable water", which was thought to represent a fixed quantity proportional to the dry matter content, has long been believed to be representative of "bound water". Since the first claim by Franks (1985) that the energy of interaction between water and any solute cannot be stronger than that of the hydrogen bond between two water molecules in ice and that the crystallization of water is arrested when the viscosity of the freeze-concentrated solution reaches 10^{12} Pa s (which corresponds to the point of intersection of the T_m and T_g curves), the view has become widely accepted that the state of "unfreezable water" is determined by kinetic factors rather than by energetic ones. It may be noted, however, that the maximal cryo-concentration reported for sucrose solutions (which have

been the subject of the greatest number of studies, with various approaches) stands in the range 0.79–0.82 g sucrose/g solution (Blond *et al.*, 1997). This range includes the value (0.805 g/g) corresponding to five molecules of water associated with one molecule of sucrose. As pointed out by Schawe (2006), this is consistent with the existence of two intramolecular H-bonds in a molecule of sucrose in concentrated solutions (Mathlouthi, 1981), the stability of which would be increased by the low temperature.

The freezing line is most often determined by DSC. These measurements, however, are subject to question because of kinetic problems, particularly with concentrated solutions (beyond ca. 40% for sucrose). The T_g' value determined from the intersection of the T_m and T_g lines, which is important because it is supposed to represent a stability threshold for frozen products, may then be questionable. Using UNIQUAC models allowed reliable prediction of T_m values for sucrose solutions up to 85% sucrose (Blond *et al.*, 1997). The UNIQUAC prediction was consistent with DSC T_m values (up to 65% sucrose). Although verification with other systems would be desirable, we suggest that the freeze-concentration described by curve T_m might correspond to a pseudo-equilibrium process governed by the activity of water (see Eq. 4) – provided appropriate precautions are taken (e.g. small sample size, slow cooling, annealing treatments). Only the arrest of the process (i.e. departure from equilibrium) is controlled by the viscosity of the freeze-concentrated phase. Extrapolation of the peak (or, better, of the offset) temperature of the melting peak to a zero scan rate was claimed to yield a correct value of the equilibrium melting temperature (Bhatnagar *et al.*, 2005).

Freezing point depression and freezing curves of ice cream systems, obtained from measurements and calculation with various models, were compared by Livney *et al.* (2003). Freezing temperature calculations and measurements were found similar and were independent of stabilizer addition. Freezing curves, however, were found to deviate beyond 50–60% of water frozen, and stabilizers induced lower values of frozen water.

11.3.2. Glass Transition Line

The glass transition has been given special attention in the field of polymer science, since this phenomenon is associated with several important changes in physical properties. The glass transition temperature (T_g) is a major characteristic in applications of polymeric materials. However, the phenomenon may be observed with similar features in any kind of amorphous material. Indeed, many low-moisture foods (and frozen products) are partly or entirely amorphous. The glass transition is the main event affecting these amorphous regions. Since, on the one hand, a characteristic feature of the glass transition is a drastic change in transport properties and, on the

other hand, the phenomenon is strongly dependent on the water content of food products, it offers a rational basis for understanding the role played by water in food processing operations such as drying, freezing, extrusion, baking or during storage. The basic knowledge may be found in many books and reviews (Ferry, 1980; Sperling, 1986; Perez, 1994). Recently, the relevance of the glass transition to food technology has been emphasized strongly (Levine and Slade, 1988; Simatos and Karel, 1988; Slade and Levine, 1985, 1993, 1994; Noel *et al.*, 1990; Roos and Karel, 1991a; Karel *et al.*, 1993; Champion *et al.*, 2000b; LeMeste *et al.*, 2002).

Glass transition denotes all phenomena observed when a glass is transformed in a supercooled material when heated, or the reverse when cooled (Figure 11.7). A glassy material is a rigid solid, but its microstructure is non-crystallized (amorphous), with only short-distance order, like a liquid. The supercooled state is the state of the material between the glass transition

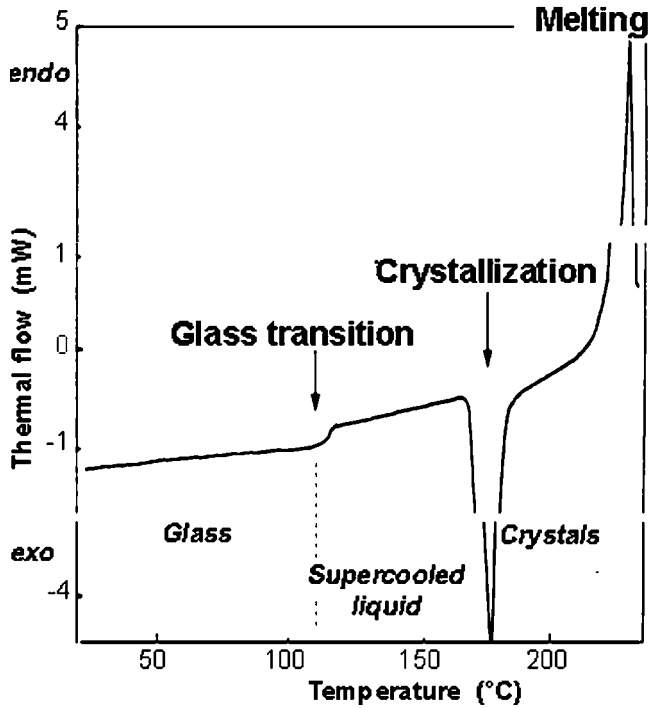

Figure 11.7. DSC thermogram of amorphous lactose, to show the successive changes in physical state in the course of rewarming. The amorphous lactose was prepared by freeze-drying and dried over P_2O_5 for at least 7 days. Scanning rate for DSC was 5°C/min. (Redrawn from Roos and Karel, 1991b.).

temperature (T_g) and its melting temperature (T_m). The mechanical behaviour may be viscous, for small molecular weight substances, or viscoelastic, "rubbery", for macromolecular compounds. Glass transition is associated with changes in thermodynamical properties (volume and enthalpy) within a narrow temperature interval, resulting in a detectable heat capacity jump (Figures 11.7, 11.8). Glass transition, however, is not a phase transition: the supercooled material is in a *metastable state*, with a tendency to crystallize; the glassy state is an *out-of-equilibrium state* of the liquid where an excess of volume and enthalpy is "frozen". Glass transition is therefore a kinetic and relaxation process.

The relaxation time, i.e. the time necessary for the structure to reorganize as a response to a perturbation such as temperature change, increases rapidly when the system is cooled below T_m. The T_g range is the temperature domain where the relaxation time becomes similar to the experimental time; below T_g, the structure relaxation cannot be completed within the observation time; the structure is "frozen" in the glass. The kinetic character of the glass transition is revealed, e.g. by the fact that T_g depends on the rate of change in temperature.

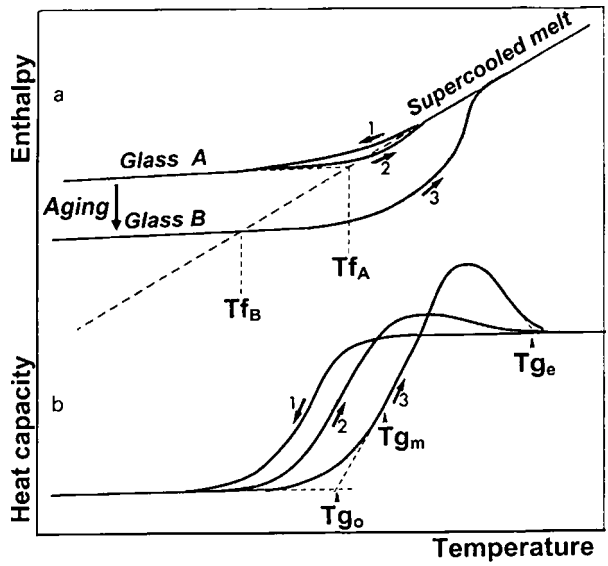

Figure 11.8. Evolution of enthalpy (or specific volume) (**a**) and of heat capacity (or of thermal expansion coefficient) (**b**) versus temperature near glass transition, during cooling (1), rewarming (2) or after physical ageing during time t (3). T_{go} and T_{gm} are the two points (onset, middle) that are commonly used to define T_g. T_{fA} and T_{fB} are the fictive temperatures characteristic of the glass structure in states A and B.

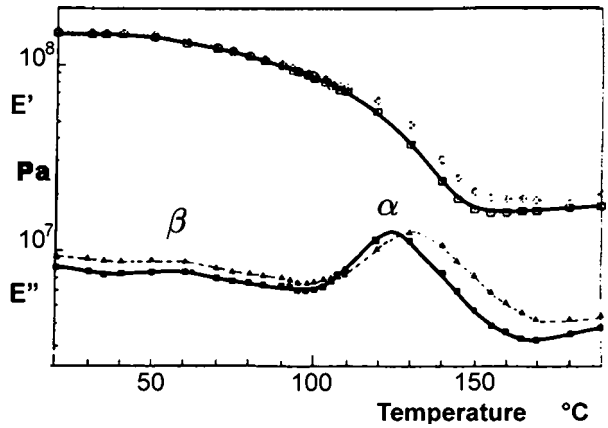

Figure 11.9. DMTA curves for white bread (5% water content) during rewarming (1°C/min), illustrating the changes in mechanical properties in the glass and at the glass transition for food systems. The α relaxation associated with glass transition is characterized by an abrupt decrease of the storage modulus (E′), from a high value in the glass (typically 10^9 Pa) to the rubbery plateau (about 10^7 Pa) and a maximum of the loss modulus (E″). Measurement frequencies were 5 Hz (*solid lines*) and 20 Hz (*dotted lines*). The temperature of the α relaxation increased with the frequency, i.e. when the characteristic observation time decreased. A β relaxation is also visible in the glassy state (LeMeste *et al.*, 1992).

Another manifestation of the kinetic character is the fact that the temperature, T_α, at which the mechanical behaviour shows the structural relaxation, is dependent on the measurement frequency (Figure 11.9). Moreover, below T_g, the relaxation processes continue, the slower the lower the temperature. This "physical ageing" is accompanied by a reduction in volume and enthalpy, as the system comes closer to the metastable equilibrium (Figure 11.8).

As a rule, the temperature of the glass transition is an increasing function of the molecular weight (M) of the material. The expression

$$\frac{1}{T_g} = \frac{1}{T_{g\infty}} + \frac{K}{DP} \tag{15}$$

is used to describe the molecular weight dependence of T_g in a homogeneous polymer series. DP is the degree of polymerization, K a constant and $T_{g\infty}$ is the high molecular weight limit of T_g. It has been proved to apply to carbohydrates (Orford *et al.*, 1989). This is explained by the free volume increase when M decreases, at a given temperature (Ferry, 1980; Sperling, 1986). For a blend of two or more compatible components, the temperature of the glass transition has a value intermediate between the T_g of the components.

When a small molecular weight compound is added to a polymer, the resulting increase in free volume, which is equivalent to a temperature increase (Sears and Darby, 1982), induces a decrease in T_g; this effect is called plasticization. Water is certainly the most important plasticizer for food components, as previously noted by Karel (1985) and strongly emphasized by Slade and Levine in their various publications (Slade and Levine, 1985, 1991, 1993, 1994; Levine and Slade, 1988). The literature is replete with examples of the strong T_g-depressing effect of water on biopolymers, as well as on sugars and other small molecules. An exhaustive list of references can be found in Slade and Levine (1993).

The water content dependence of the glass transition temperature is most commonly described by the empirical Gordon–Taylor expression:

$$T_g = \frac{m_2 T_{g2} + k m_1 T_{g1}}{m_2 + k m_1} \tag{16}$$

where m_2 and m_1 are the mass fractions of solid and water, respectively, T_{g2} and T_{g1} their glass transition temperatures and k a fitting parameter. Because of the uncertainty on the T_g value for water, the latter can be taken as a fitting parameter. Values of 135 K ($T_{g\ onset}$) or 138 K ($T_{g\ midpoint}$) were observed to give better fitting than higher values for the sucrose–water system (Blond *et al*., 1997). For intimately mixed, flexible polymers, the semi-theoretical Couchman and Karasz expression

$$\ln T_g = \frac{\sum_1^n m_i\ \Delta Cp_i \ln T_{gi}}{\sum_1^n m_i\ \Delta Cp_i} \tag{17}$$

should allow the prediction of T_g from m_i, the mass fraction, T_{gi}, the glass transition temperature and ΔCp_i, the increment in heat capacity at Tg_i for each component i (Couchman, 1978). Whereas this expression was found to predict accurately the T_g values for anhydrous binary sugar mixtures (Finegold *et al*., 1989), important deviations are observed between calculated and measured values, when it is tested for binary aqueous solutions of sugars (Orford *et al.*, 1989). Although the uncertainty in the T_g and ΔCp values for water may be partly responsible for this lack of agreement, the contribution of "specific interactions" is also envisaged (Franks, 1993).

Pure water may be obtained in a glassy state, by hyperquenching or vapour deposition. An onset value, $T_g \approx 136$ K, had been widely accepted by the scientific community, based on DSC observations (Johari *et al*., 1987; Hallbrucker *et al.*, 1989) and on the extrapolation to pure water of the T_g measured for various binary aqueous solutions (Angell and Tucker, 1980; MacFarlane and Angell, 1984). Such extrapolations, however, may be

misleading in the case of systems, such as aqueous solutions, where one component is a network former (Angell, 2002). Although the controversy continues, several arguments, including the annealing behaviour of hyperquenched water, are presented in support of a water $T_g \approx 165$ K (Velikov *et al.,* 2001; Angell, 2002). From the study of dielectric properties of water "confined" in vermiculite clay or bread, a state allowing the study of supercooled water in a temperature range where crystallization normally precludes observation, it was suggested that what is observed by DSC at 136 K is a local β-like process (Cerveny *et al.*, 2004). In mixtures of water and solutes breaking up the H-bond network of supercooled water, the T_g should be determined by this β-like relaxation process. On the contrary, extrapolation of the T_g values obtained by DSC for mixtures of water with n-propylene glycols, which expand the network instead of breaking it, gives a $T_g \approx 162$ K (Cerveny *et al.*, 2004; Jansson *et al.*, 2005a).

The heat capacity increment at T_g for water may be even more influential, when the Couchman and Karasz expression is used to predict T_g for a mixture, or to determine the $(\Delta Cp)_{Tg}$ of the dry solute (Borde *et al.*, 2002), because very different values were reported. For pure water (conventional T_g=136 K), reported values range between 0.09 (hyperquenched liquid) (Hallbrucker *et al.*, 1989) and 1.94 J/g//K (vapour-deposited liquid) (Sugisaki *et al.*, 1968). Whereas the latter value may be falsely high due to the presence of a sharp relaxation overshoot (Angell and Tucker, 1980), the first one is only 10% of the value extrapolated to pure water with various salt–water mixtures, which are in the range 1.06–1.39 J/g/K (Angell and Tucker, 1980). Extrapolation to pure water of the values obtained with galactose solutions gave 0.93 J/g/K (Blond and Simatos, 1991).

The question of glass transition in proteins deserves some clarification. The observation of glass transition on DSC thermograms is most often difficult with proteins. The Cp jump, which is usually considered as the signature of glass transition, is more clearly observed, in a given range of water content, with fibrous proteins (gelatine: Marshall and Petrie, 1980; gluten: Kalichevsky *et al.*, 1992; Noel *et al.*, 1995; Morales and Kokini, 1997) or polypeptides (poly-L-asparagine: Green *et al.*, 1994) or after denaturation with globular proteins (legumin: Sochava and Smirnova, 1993; bovine serum albumin: Farahnaky *et al.*, 2005). Due to the low value of the Cp jump, compression of the powder submitted to DSC is most useful (casein: Kalichevsky *et al.*, 1993). Identification of this feature with glass transition is confirmed by thermomechanical spectroscopy, showing the characteristic drop in E′ and maximum in E″ in a similar temperature range (Kalichevsky *et al.*, 1992–1993, De Graaf *et al.*, 1993). The variation of the T_g or T_α thus determined as a function of water content exhibits the expected plasticization behaviour, with values ranging from 80–100°C to 0–40°C for a water content

varying from 8 to 20%. Experimental study of glass transition in proteins is all the more difficult because it occurs in a temperature range where denaturation can occur.

Based on a description of the plasticizing effect of water in terms of shielding of intra- and intermolecular hydrogen bonds and dipole–dipole interactions, a method was recently proposed to calculate the glass transition temperature of biopolymers as a function of water content. The T_g values for 13 proteins and polysaccharides at different water contents between 5 and 25%, calculated using information about their chemical structure, were found to differ from experimental values by less than 10–30°C (Matveev *et al.*, 2000). According to these authors, over the three main chemical characteristics of a protein, i.e. amino acid composition, disulphide cross-links and molecular weight of the polypeptide, amino acid composition alone is sufficient to calculate T_g. The T_g of α-casein, however, was shown to increase (by some 40°C at a water content ≈20%) after cross-linking with transglutaminase (Mizuno *et al.*, 1999).

The glass transition behaviour of fully hydrated proteins is currently a matter of intensive debate, as it is most important for the understanding of water–protein interactions. However, the issue is also of interest for the stability of frozen products. Very different values have been reported for the glass transition temperature of the maximally freeze-concentrated phase (T_g') of animal products: on the one hand, in the range –20 to –12°C for various fish and meat products (Levine and Slade, 1989; Brake and Fennema, 1999), and on the other hand, –84 and –85°C for beef and egg white, respectively (Simatos *et al.*, 1975), –65°C for tuna (Orlien *et al.*, 2003), –90°C (T_α) for salmon (Champion, unpublished) and –75°C for blood plasma (Simatos *et al.*, 1975). It may be argued that the low level of these values is due to the lowering effect of salts and other small molecular weight molecules. It is consistent, however, with the values reported for fully hydrated BSA and several other globular proteins, which all fall within the range –67 to –87°C (Chang and Randall, 1992; Inoue and Ishikawa, 2000). The values reported in this section are the temperature of the middle of the transition considered as the glass transition.

A dynamic transition is evidenced for fully hydrated proteins at –90 to –50°C from the variation with temperature of the mean-squared displacement of atoms, as determined by a variety of spectroscopic techniques and dynamic computer studies (see Gregory, 1995, for review). According to protein scientists, this low temperature transition and the low water content at higher temperature appear to be manifestations of a common hydration-dependent transition, in line with the behaviour expected for a glass transition in which water acts as a plasticizer (Gregory, 1995). Objections have been raised, however, that the low temperature transition cannot be what is usually

understood as a glass transition, because it is observed in extremely short timescale studies (of the order of ps or ns), very different from the timescale of molecular diffusion or reorientation (≈100 s) that is considered to be characteristic of glass transition (Green *et al*., 1994; Angell, 1995). It was proposed, instead, that the low temperature transition is actually a strong water-sensitized β relaxation. When the temperature is raised, the progressive evolution of heat capacity, mechanical relaxation studies and dielectric measurements suggest a continuing build-up of relaxation processes, eventually merging with the α process at some higher temperature, consistent with the glass transition temperature observed with low-moisture proteins and polypeptides (Green *et al*., 1994). Dielectric measurements in wide frequency and temperature ranges on haemoglobin (0.8 g water/g protein) showed several relaxation processes with different temperature dependencies. One of them, a "β-like" process (Arrhenius temperature dependence) corresponding to a $\tau \approx$ 100 s at –103°C, was attributed to local motions of polar side groups of the protein (possibly together with a small amount of bound water molecules). This process changed at ca. –63°C to a more cooperative "α-like" relaxation (VTF temperature dependence) (Jansson *et al*., 2005b, 2006). Similar results were obtained with myoglobin, although with somewhat lower temperature levels (Swenson *et al.,* 2006, 2007). There seems to exist a consensus, however, on the idea that compact proteins will not undergo a true glass transition, the term "protein dynamic transition" being preferred (Doster and Settles, 2005). This view has much in common with the model proposed by Gregory (1995, 1998). According to the knot-matrix model, knots are regions of the native structure with efficient packing of residues, higher density and low conformational mobility, embedded in more flexible matrices. Only the surface and matrix regions should undergo the low temperature transition, while the knots remain glassy and rigid, cooperative disruption of the knots occurring only at denaturation. Protein motions appear to be coupled to the β and α relaxation processes in water (Doster and Settles, 2005; Jansson *et al*., 2005b, 2006; Swenson *et al*., 2006, 2007).

The state diagram in Figure 11.5 is based on data for the lactose–water system, because it has been recognized widely that the glass transition behaviour of milk powders is very close to that of pure lactose (e.g. Jouppila and Roos, 1994; Vuattaz, 1999, 2002; Fernandez *et al*., 2003). This is true whatever the fat content of milk is, provided the solid non-fat is considered (Figure 11.10). A unique line is also obtained when T_g is represented against a_w for amorphous lactose, milk powder with varying fat content and whey powders with various degrees of lactose precrystallized in the α form. This curve is correctly described by a linear expression for $0.12 < a_w < 0.65$: T_g (°C) $= -143.6\, a_w + 77.8$ (Vuattaz, 2002).

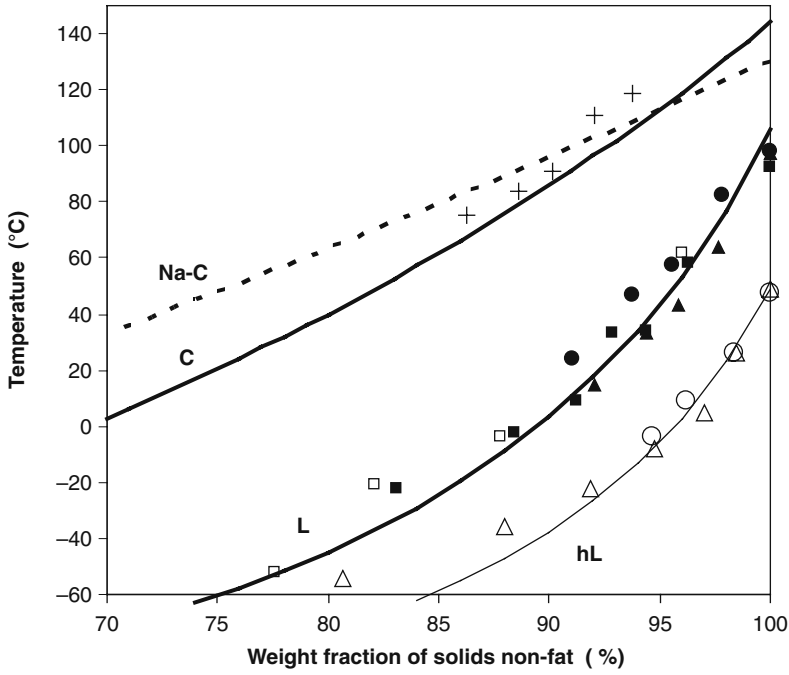

Figure 11.10. Glass transition temperature for milk constituents:
C and **Na-C**: Gordon–Taylor fit for casein and Na-caseinate, respectively (Kalichevsky *et al.*, 1993),
+: whey proteins isolate (Zhou and Labuza, 2007),
□, ■, ▲, Δ: whole milk, skim milk, lactose and skim milk with hydrolysed lactose (Jouppila and Roos, 1994),
●, ○: lactose and skim milk with hydrolysed lactose (Shrestha *et al.*, 2007),
L, hL: Gordon–Taylor fits for lactose and skim milk with hydrolysed lactose (Jouppila and Roos, 1994).

Since casein and whey proteins are high molecular weight compounds, the T_g value for milk could be expected to be higher than those for lactose. Likewise, addition of sugars to casein (casein/lactose: 10/1) was reported not to affect significantly its T_g (Kalichevsky *et al.*, 1993). It is suggested that casein and lactose behave as immiscible components, the T_g of carbohydrate-rich systems (milk) being governed by lactose T_g and in casein-rich systems by casein T_g (Burin *et al.*, 2002). For mixtures of whey proteins, albumin or gelatine (lactose/protein: 3/1), T_g was reported to be slightly higher than for pure lactose in the dry state, but slightly lower and then again higher with increasing humidity (Haque and Roos, 2004). As could be expected,

hydrolysis of lactose induces an important decrease of the glass transition temperature of milk (Figure 11.10) (Jouppila and Roos, 1994; Fernandez *et al.*, 2003; Shrestha *et al.*, 2007).

11.4. Water Retention

As was nicely summarized by Van Vliet and Walstra (1994) "Water in Casein Gels; How to Get It out or Keep It in", an important problem regarding high water content dairy products is to prevent syneresis of liquid from products such as yoghurt and gelified dairy desserts, or to remove just the required amount of water in the case of cheese production. Gels may hold considerable amounts of water, part of it being difficult to remove by pressing, centrifuging or drying. This observation has led to the belief that part of the water is bound to the molecules (particularly proteins) forming the gel network. It may be observed, first, that this holding ability is not connected to a low water activity. From Figure 11.11, one can see that lowering the water content of

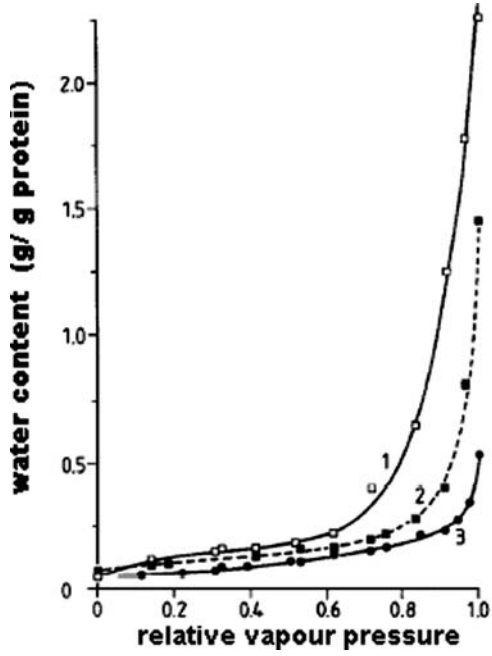

Figure 11.11. Water sorption isotherm for paracasein (3) and desorption isotherms for salted (1, 12.6 g NaCl/l00 g protein) and unsalted (2, 0.4 g NaCl/l00 g protein) cheese (Van Vliet and Walstra, 1994).

unsalted curd to 50% (i.e. removing 88% of the initial water) will maintain a_w very close to 1.

Characterizing water molecular mobility by NMR seems to be the method of choice to elucidate the retention mechanisms and to reveal the possible influence of product formulation and processing parameters. NMR relaxation times of protons (T_1, called longitudinal or spin-lattice relaxation time, and T_2, transversal or spin–spin relaxation time) can be used to monitor the rotational motion of water molecules. From them (particularly T_2, which depends more univocally on mobility), a rotational correlation time, τ_c (the average time taken for a molecule to rotate through one radian), can be deduced. Transverse relaxation in food systems is often found to be multi-exponential and always faster than relaxation in bulk water (i.e. water molecules are less mobile). The common interpretation has been for long that water exists in a number of populations that are "bound" in various ways to the solutes.

Water molecules in close contact with a solute molecule have a reduced mobility, which induces an increase in relaxation rate (their T_2 is decreased). Via rapid exchange of energy between spins, the observed T_2 of the aqueous bulk phase is a weighted average of those of the unmodified water and of the small motionally modified fraction. The mono–multi-exponential behaviour of relaxation is a consequence of the spatial heterogeneity of the sample (Lillford *et al*., 1980). If a water molecule can reach a macromolecule surface within a diffusion time shorter than its intrinsic relaxation time, its relaxation will be hastened. The diversity of distances will result in a diversity of relaxation times. The occurrence of multi-exponential relaxation was shown to be predictable on the basis of the diffusion coefficient of water molecules, the characteristic dimension in the sample heterogeneity and the difference in relaxation rates in the two sites allowing chemical exchange of protons (Hills *et al*., 1990; Belton, 1990). Fat-free milk powder dispersed in water showed a single exponential relaxation, as predicted for a casein micelle of diameter ≈ 0.1 μm, which is small enough for the fast exchange limit to apply. On the contrary, with a skim milk powder paste with particles of aggregated casein of the order of 400–500 μm in radius, multi-exponential relaxation was observed, as expected on the basis of the diffusive exchange model. As stressed by the authors, "the transition from single to multiexponential behaviour is explained on the basis of morphology without recourse to any speculation as to changes in the nature or state of water present".

The dramatic enhancement of the relaxation rate of protons in solutions must also be discussed further. ^{17}O relaxation, which does not suffer from complications such as hydrogen exchange between water and protein, allows a reasonable picture of water dynamics in protein solutions to be described, together with other methods, e.g. molecular dynamics simulation. Only water

molecules in direct contact with the protein surface are significantly perturbed, although they are still highly mobile, with an average $\tau_c \approx 20$ ps, i.e. approximately 10 times that of the bulk water (Halle *et al.*, 1981; Hills *et al.*, 1990). This "hydration water" is estimated to be ca. 500 molecules for a 15 kDa protein (0.6 g water/g protein) (Halle, 2004). ^{17}O relaxation measurements as a function of resonance frequency have shown that only water molecules that are buried inside a globular protein have a τ_c exceeding 1 ns at room temperature, i.e. they reorient (along with the protein) about 2000 times more slowly than in bulk water, and they exchange with bulk water in the 10–1000 ns range (Denisov and Halle, 1995). The authors pointed out that this dramatic reduction in their mobility is not due "to the interaction with the protein per se (most external water molecules are also extensively hydrogen bonded), but rather to their physical entrapment within the protein matrix". Whereas the rotational retardation factor $\langle\tau_{hyd}\rangle/\tau_{bulk} \approx 5.4$ for water in contact with proteins, it is usually found in the range 1.0–2.5 for small organic molecules. This difference has been attributed to the presence of strongly retarded water molecules at special locations on protein surfaces (Halle, 2004). Indeed molecular dynamics simulation as well as NMR studies have confirmed the importance of surface topography and failed to establish correlation between water residence times and the chemical structure, charge or polarity of the contacting groups (Halle, 2004).

For milk, the enhanced relaxation rate of water could be attributed to chemical exchange of protons with labile protons of whey proteins and lactose, while the micellar casein contribution was due mainly to the exchange between bulk and internal water molecules (Hills *et al.*, 1990; Mariette *et al.*, 1993). Water trapped in bovine casein sub-micelles and micelles was estimated to be ≈ 0.004 and 0.010 g/g, respectively, at 21°C and pH 6.98 (Mora-Gutierrez *et al.*, 1995, 1996).

In addition to T_1 and T_2, which reflect the rotational motion of water, NMR can be used to determine its translational mobility. The water self-diffusion coefficient, D_w, was found to decrease when the casein concentration increased, but to be insensitive to the structure of the casein, whether in solution (micellar casein or Na-caseinate) or in a gelled state (acid gel or rennet gel) (Figure 11.12) (Mariette *et al.*, 2002). The reduction in the mobility of water was demonstrated to result from an obstruction effect from the casein molecules (with no significant contribution of any specific water–protein "binding"). The D_w inside a casein micelle, in the low-concentration range (less than 0.10 g/g, where the water concentration inside the micelle is assumed to be constant), was estimated to be 1.45×10^{-9} m^2/s (compared to the value for pure water at the same temperature of 2.29×10^{-9} m^2/s).

From T_2 measurements, Van Vliet and Walstra (1994) concluded that all the water present in casein gels is mobile on the timescale of processes

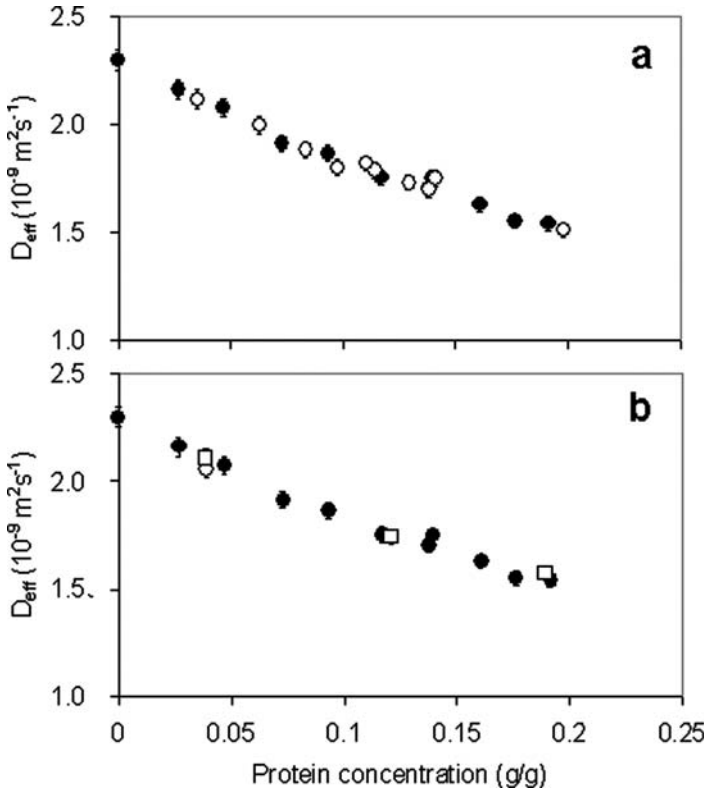

Figure 11.12. Observed water self-diffusion coefficients as a function of protein concentration (g/g) for **(a)** Na-caseinate solutions (○) and micellar casein dispersions (●) and **(b)** micellar casein dispersions (●), acid gels (○) and rennet gels (□) (Mariette *et al.*, 2002).

used in practice to remove water from the gels. Differences in mobility of the hydrogen nuclei could not explain the difference in syneresis behaviour of gels at high (>5.15) and low (<5.1) pH, syneresis rate being higher and water content after syneresis lower in the first case. The microscopic structure of casein gels, characterized by a fractal dimension, was found to be about the same for gels prepared by acidification or by rennet action. The permeability coefficient was also very similar, in line with the D_w results reported above (Figure 11.12). The best explanation to the difference in the ease of water removal appeared to be the dynamics of structure rearrangements of the gel. Actually, the pH and temperature dependence of the syneresis rate were found to parallel the tan δ obtained in rheological measurements (Van Vliet and Walstra, 1994). The authors then concluded,

"The answer to the question 'how to get water out of a casein gel or keep it in' has nothing to do with the state of the water but everything to do with the dynamics of the casein network".

NMR proton relaxation and diffusometry are currently used in many studies on complex dairy products, with the objective of relating the water mobility with the product composition and the processing parameters. T_1 T_2 measurements are sometimes interpreted based on the existence of several water phases with varying mobility, e.g. 3–4 water fractions in yoghurt (Hinrichs *et al*., 2003), whey protein concentrates (Hinrichs *et al*., 2004a) and fresh cheese (Hinrichs *et al*., 2004b). Such a description does not appear truly realistic. By contrast, the NMR results obtained with acidified milk drinks were interpreted as representing a continuous distribution of relaxation times. Samples with low (0.0%, 0.1%) or high (0.3%, 0.5%) pectin concentrations which were defined as unstable and stable, respectively, were shown to differ significantly by their relaxation behaviour. The "unstable" samples exhibited a longer T_2 mean value (corresponding to an irreversible whey separation during a heating–cooling treatment 5→25→5°C) and a broader T_2 distribution (likely due to the more inhomogeneous structure of the gel (Figure 11.13) (Salomonsen *et al*., 2007).

Voluminosity has been considered for long as providing information on water–protein interactions. For casein micelles, methods based on hydrodynamic volume (intrinsic viscosity, diffusion coefficient) yield a value of roughly 3.9 ml/g dry casein, while other methods (microscopy, light scattering) indicate about 2.2 ml/g (Walstra, 1979). The hydration values deduced from voluminosity data (e.g. 3.7 g water/g casein for bovine casein micelles at 25°C), however, should not be interpreted as "bound water", implying that some several hundred water molecules migrate with the protein, i.e. they have residence times longer than the rotational correlation time of the protein, which is incompatible with the ps dynamics in the hydration layer of proteins, as deduced from NMR experiments (Halle, 2004). Actually, it was recently demonstrated that the discrepancy between experimental and calculated rotational diffusion coefficients (which is inversely proportional to viscosity) for globular proteins (e.g. $D_{rot(calc)}/D_{rot(exp)} \approx 2$ for egg lysozyme in water at 20°C) is more satisfactorily explained by allowing the viscosity in the first hydration layer to differ from the bulk water viscosity than by a rigidly bound water shell (Halle and Davidovic, 2003; Halle, 2004). According to the authors, an important factor reducing D_{rot} is the presence of large-scale surface irregularities. Voluminosity, therefore, appears more as a tool to characterize the rheological behaviour of dairy constituents than their hydration properties. Their various interactions with water, of course, play a major role in voluminosity, because they control their conformation in solution and their dispersion/aggregation.

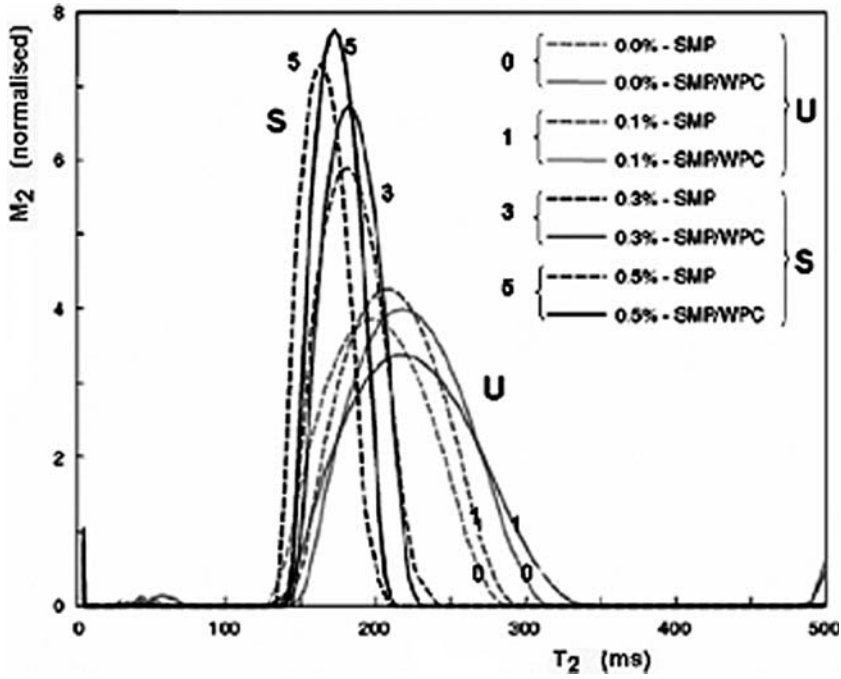

Figure 11.13. Distribution of T_2 relaxation times estimated by distributed exponential fitting of low-field nuclear magnetic resonance relaxation curves obtained on acidified milk drinks (AMDs) at 5°C. The AMDs vary with respect to the combination of pectin concentration (0.0, 0.1, 0.3 and 0.5%) and protein type (skim milk powder (SMP) or SMP with added whey protein concentrate (SMP/WPC)). Each distribution is a mean of six measurements (Salomonsen *et al.,* 2007).

As illustrated in Table 11.1, voluminosity (V_e) (or intrinsic viscosity [η]) varies with the nature of milk protein, temperature and physico-chemical characteristics of the dispersing medium. For casein monomers, the looser the conformation, the higher the [η]; see, e.g. β-casein. Structure-loosening with guanidine chlorohydrate also induces an increase in [η]. On the contrary, despite a very hydrophilic surface, β-lactoglobulin and α-lactalbumin show low [η] values because of their compact structure. For casein micelles, high V_e values (up to 10 ml/g and above) are attributed to water entrapped within loose aggregates and to the "hairy" surface layer composed of the hydrophilic κ-casein. Renneting skim milk, before increasing [η] through aggregation of micelles, is thought to reduce it through the (part) removal of the "hairy" layer, as a consequence of the splitting off of the glycomacropeptide from κ-casein (Walstra, 1979).

Table 11.1. Intrinsic viscosity of milk proteins (voluminosity, V_e, can be calculated from $V_e = [\eta] / 2.5$ where $[\eta]$ is intrinsic viscosity (Mulvihill and Fox, 1989))

Proteins	Temperature °C	pH	Minerals		Intrinsic viscosity, ml/g
α_{s1}-Casein	37	7.02	0.01 M KCl		10.2
	4	7.02	0.01 M KCl		11.6
	20	7.08	0.1 M KCl		7.7
	4	7.08	0.1 M KCl		9.3
	20	7	0.1 M KCl	6 M Gu HCl	19
	25	12			19.2
α_{s2}-Casein		7	0.1 M NaCl		11.3
β-Casein	4	7	I=0.2		23
κ-Casein	25	7	0.1 M NaCl		9.5
		12			15.1
		7		5 M Gu HCl	31
β- Lactoglobulin		5.2	0.1 M NaCl		2.9
α- Lactoglobulin		6	I=0.2		3

The increase of V_e with decreasing temperature (Table 11.1) is assigned to the release of β-casein from the micelle, due to a direct effect of the high $[\eta]$ of β-casein, a loosening of the micelle structure (Walstra, 1979) and a decrease in hydrophobic interactions (Snoeren *et al.*, 1984). Changes in pH induce strong variations in V_e, particularly for micellar casein. As expected from the minimum in electrostatic repulsion, V_e was found to be minimal at pH 4.6, the isoelectric point of casein, for proteins in bulk milk. A maximum in V_e was found at pH 5.4, which may be due partly to the release of β-casein (Snoeren *et al.*, 1984). With isoelectric-precipitated casein, however, V_e was reported to be maximal ($[\eta] \approx 71$ ml/g) close to the isoelectric point. This very high V_e value was interpreted as representing water occluded in aggregates (Colas *et al.*, 1988a). An inverse relationship between V_e of casein and whey proteins and ionic strength (0.005–0.07 M NaCl, pH 6–8) (see also Table 11.1) was explained by the shielding effect of neutral salts on the electrostatic charges of the proteins (Boulet *et al.*, 1998).

Table 11.2 illustrates the voluminosity increase for casein modified by reductive alkylation with reducing sugars. The decrease in the parameter k′ of the Huggins equation ($\eta_{red}=[\eta]+k'.[\eta]^2.c$ where η_{red} and $[\eta]$ are reduced and intrinsic viscosity, respectively, and c the protein concentration) confirmed that the modification had resulted in a better dispersion of the casein molecules (Colas *et al.*, 1988b). Moreover, ESR measurements showed that the

Table 11.2. Voluminosity of glycosylated caseins (Colas et al., 1988)

Attached sugar	Level of modification, % of ε amino groups	Voluminosity, ml/g
Control	0	4.32
Fructose	16	5.56
Glucose	23	7.20
Galactose	19	6.08
	32	6.48
	39	6.04
	78	7.36
Lactose	20	6.24
Maltose	27	7.88

mobility of casein side chains was increased by alkylation. Consistent with this increase in flexibility and voluminosity, the foaming capacity and foam stability were increased (Colas *et al.*, 1988a).

11.5. Water and Molecular Mobility

The influence of water on molecular properties is particularly effective in systems with low water content or in which a major fraction of water is immobilized as ice, i.e. in systems close to their glass transition.

11.5.1. Glass Transition and Mechanical Properties

Changes in mechanical properties are major characteristics of glass transition. Glasses have high elastic moduli (Young's modulus or shear modulus) and they are rigid (possibly brittle). As temperature is increased, the elastic moduli drop over a narrow temperature range around T_g while the loss moduli show a maximum, characteristic of the α relaxation associated with glass transition (Figure 11.9). Polymeric materials have then the viscoelastic behaviour typical of lightly cross-linked rubbers or physically entangled long chains. Depending on whether the material is cross-linked or not, the modulus exhibits a "rubbery plateau" or drops further as the temperature continues to increase.

At temperatures above T_m, the variation in viscosity as a function of temperature is generally Arrhenius-like. The viscosity shows an important change in the vicinity of the glass transition. For most materials, in the temperature range between T_m and T_g, Arrhenius plots show an increase in slope (equivalent to an increase in activation energy) as the temperature decreases, the apparent activation energy (E_a) commonly attaining

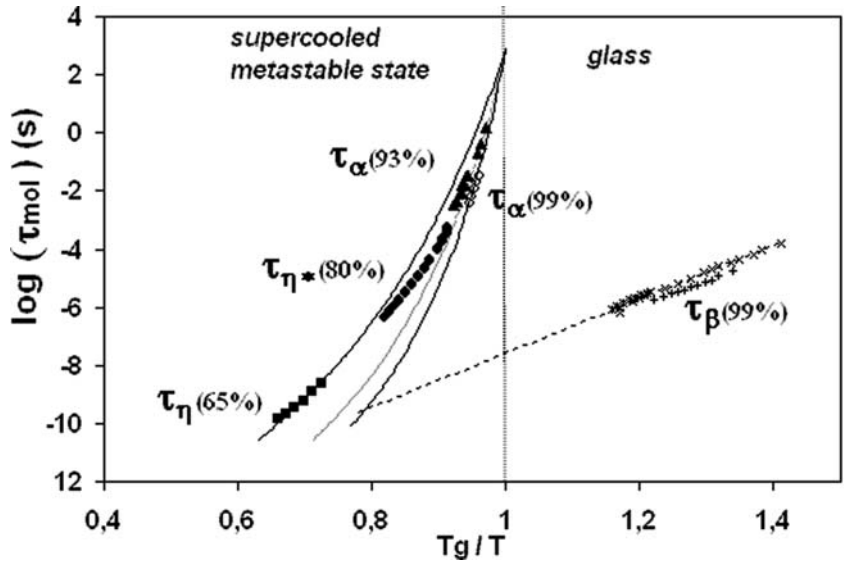

Figure 11.14. Arrhenius representation of characteristic relaxation times (τ_{mol}) for amorphous sucrose–water samples, as derived from various techniques: ○ and ▲, α relaxation in DMTA (99 and 93% sucrose); ●, complex viscosity (80% sucrose); ■, viscosity (65% sucrose); + and x, β relaxation in DS (99% sucrose).
All the α relaxation and viscosity data could be fitted to the WLF expression with the coefficients C_{g1}=–19.8 and C_{g2}=51.6 K (i.e. the fragility did not vary significantly with water content). The continuous lines correspond to WLF with these coefficients (but with the different T_g varying with water content) (Champion *et al.*, 1997b). The activation energy for the α relaxation near T_g was ≈375 kJ/mol and ≈ 60 kJ/mol for the β relaxation (Champion *et al.*, 2003).

200–400 kJ/mol near T_g (Figure 11.14). This strong influence of temperature on viscosity is due to the fact that molecules no longer move individually, as they do above T_m, but in a coordinated manner, e.g. due to physical entanglements, which are not able to relax during experiments with too short a timescale. Above T_g, the variation of viscosity is satisfactorily described by the so-called Vogel, Tamman and Fulcher expression (VTF)

$$\eta_T = \eta_0 \exp\left(\frac{B}{T - T_0}\right) \tag{18}$$

or by the Williams, Landel and Ferry expression (WLF; Williams *et al.*, 1955):

$$\log\frac{\eta_T}{\eta_{T_g}} = \frac{-C_{1\,g}(T - T_g)}{C_{2g} + T - T_g} \tag{19}$$

where η_T and η_{Tg} are viscosities at T and T_g, respectively, and η_0, B, T_0, $C_{1\,g}$ and $C_{2\,g}$ are constants. Both expressions (VTF and WLF) can be interconverted. They were originally phenomenological expressions, although theoretical interpretations have been given.

In the glass, long-range cooperative motions are restricted. Motions (vibrations of atoms, reorientation of small groups of atoms) are mainly local, not involving the surrounding atoms or molecules. The temperature dependence of dynamic properties in glasses is generally considered to obey Arrhenius law, with an apparent activation energy that is lower than at $T>T_g$ but still rather high (Perez, 1994). Mobility in the glass will be described more extensively in the next section.

The most popular method used to determine T_g is based on the change in heat capacity monitored by DSC (Figure 11.7). For the study of food systems where the glass transition is smeared out over a broad temperature range, the method may not be sensitive enough; the glass transition range is then determined from the α relaxation, monitored by mechanical or dielectric spectroscopy (Figure 11.9). The points that are chosen on the curves to define T_g (onset, midpoint) or T_α (maximum of the loss moduli E'' or G'', maximum of the loss factor tan δ or drop in E' or G') must be specified, as well as the measurement conditions (cooling/heating rates, frequency, thermal history).

Equations (18) and (19) can be written with viscosity replaced by a relaxation time (τ). Significantly different values of τ may be obtained with different techniques because of varying response of the material to the imposed perturbations (Angell *et al.*, 1991). The relaxation time most characteristic of glass transition is considered the relaxation time of the system at the calorimetric T_g, i.e. ≈ 200 s (Angell *et al.*, 1991).

Parameters $C_{1\,g}$ and $C_{2\,g}$ are frequently considered as universal constants (17.4 and 51.6, respectively). This should be true for $C_{1\,g}$ (Angell *et al.*, 1994). On the contrary, $C_{2\,g}$ is now recognized to show large variations. The variations in $C_{2\,g}$ and B are related to the classification scheme for glass-forming liquids that has been proposed based on the dependence of η or τ on temperature (Angell, 1985; Angell *et al.*, 1994). "Strong" liquids are those with Arrhenius or almost-Arrhenius behaviour (above T_g) and show only a small change in heat capacity at T_g. These are liquids with a three-dimensional network of bonds, such as SiO_2. In contrast, "fragile" liquids show VTF/WLF behaviour and a large change in heat capacity at T_g. Various approaches have been taken to quantify the fragility (Angell *et al.*, 1994; Simatos *et al.*, 1995b; Angell 2002). One of them is the m fragility parameter, defined as the slope at T_g in an Arrhenius plot of viscosity or relaxation time, where the abscissa is scaled to T_g; m is then given by

$$m = \frac{E_a}{RT_g \ln 10} \tag{20}$$

E_a being the apparent activation energy at T_g. Since "fragility" is a measure of the sensitivity of amorphous materials to temperature and water content, it seems to be a relevant parameter to evaluate the efficiency of ingredients in imparting stability during storage or processability in operations such as extrusion, flaking or drying. A discussion of the various methods used to estimate m for food materials can be found in Simatos *et al.* (1995b).

As is well known, liquid water possesses unusual physical properties compared to most other liquids. These "anomalies" become even more pronounced for supercooled water. Moreover, unlike most other substances, glassy water can apparently exist in at least two distinct forms (see Angell, 2002, and Debenedetti, 2003, for reviews). Some arguments had been used to suggest that water undergoes a transition from extreme fragility, in the range 240–273 K, to strong character close to its glass transition (Angell, 1993); although this view is still a matter of controversy (Debenedetti, 2003), newer studies have been presented to support the occurrence of this transition in the range 220–240 K (Angell, 2002; Starr *et al.*, 2003). All low molecular weight sugars for which data have been published (glucose, fructose, sucrose, maltose, trehalose) can be classified as rather fragile materials; they seem to be located in a narrow domain of the fragility diagram, with low or no influence of water content (LeMeste *et al.*, 2002) (Figure 11.14). One may assume that this is true also for lactose. For proteins, the scarce experimental data seem to indicate a strong behaviour: $m \approx 40.5$ for poly-L-asparagine (15–25% water) (Angell *et al.,* 1994) and similar values for elastin and gluten (Simatos *et al.*, 1995b). Pullulan–starch blends were found to show strong behaviour ($m \approx 42$–51) increasing with water content (Biliaderis *et al.*, 1999). Fragility was reported to increase in the order: pullulan<dextran and phytoglycogen <amylopectin and to decrease for amylopectin with increasing water content (Borde *et al.*, 2002).

11.5.2. Transport Properties

The drastic changes in mechanical properties that occur at the glass transition led to the assumption of a parallel evolution of the translational diffusion of water and solutes, resulting in important applications for food processing and storage. These expectations have been fulfilled only partially. In liquid systems well above T_g, the translational diffusion (D_{trans}) and rotational diffusion (D_{rot}) of molecules can be predicted from the so-called Debye–Stokes–Einstein relations (DSE):

$$D_{trans} = \frac{kT}{6\pi\eta rc} \qquad D_{rot} = \frac{kT}{8\pi\eta r^3 c} \tag{21}$$

where k is the Boltzmann constant, T the temperature, η the viscosity, r the hydrodynamic radius of the diffusant and c the coupling factor between the molecule and the matrix. First, the respective sizes of the matrix and the diffusant must be taken into account; second, when the T_g of the matrix is approached, decoupling of diffusion from viscosity may be observed. Third, diffusion may not be the controlling factor of the physical or chemical changes in the considered food system. Finally, the assumption of a large increase in stability for storage below T_g may not be always verified. During the last 10 years, it was increasingly recognized that some molecular mobility persists in the glassy state, which may result in significant quality changes in food if the timescale of observation is long enough

11.5.3. Mobility in the Glass

According to the free volume theory, first developed for diffusion in liquids (Cohen and Turnbull, 1959), then extended to glasses (Vrentas and Duda, 1978), translational diffusion of molecules is due to jumps between structural defects (i.e. low-density domains) of a size large enough to accommodate them. Infrared microscopic studies suggest that, when obtained under similar conditions, glasses of larger molecules are more loosely packed than those made of smaller ones (Wolkers *et al.*, 1998). Molecular mobility could then be expected to be lower in small-molecule glasses. Similar conclusions were drawn from specific volume measurements of maltodextrin–water powders ($0.11 < a_w < 0.75$) (Kilburn *et al.*, 2005). While in the rubber state, the specific volume of the matrix was independent of the molecular weight of the carbohydrates, in the glassy state, the density of the matrix increased with decreasing molecular weight. The average size of the interstitial holes between the constituent molecules was measured by PALS. The hole volume in the rubbery state was independent of the matrix molecular weight. In contrast, it increased with molecular weight in the glassy state (from 37 to 70 $Å^3$ at T_g for DE decreasing from 33 to 6 with $a_w = 0.22$).

The mobility of water remains high, even in a glass. The rotational mobility of water does not show a break in the GLT range in concentrated solutions of maltose (Hemminga *et al.*, 1999) or sucrose (Hills *et al.*, 2001). In these solutions around T_g, the rotational correlation time is $\approx 10^{-7}$ s, i.e. about 10^5 times higher than in pure liquid water, whereas the viscosity is about 10^{15} times greater in the sugar solutions than in water. Figure 11.15 illustrates the difference in mobility for water and for a maltodextrin matrix:

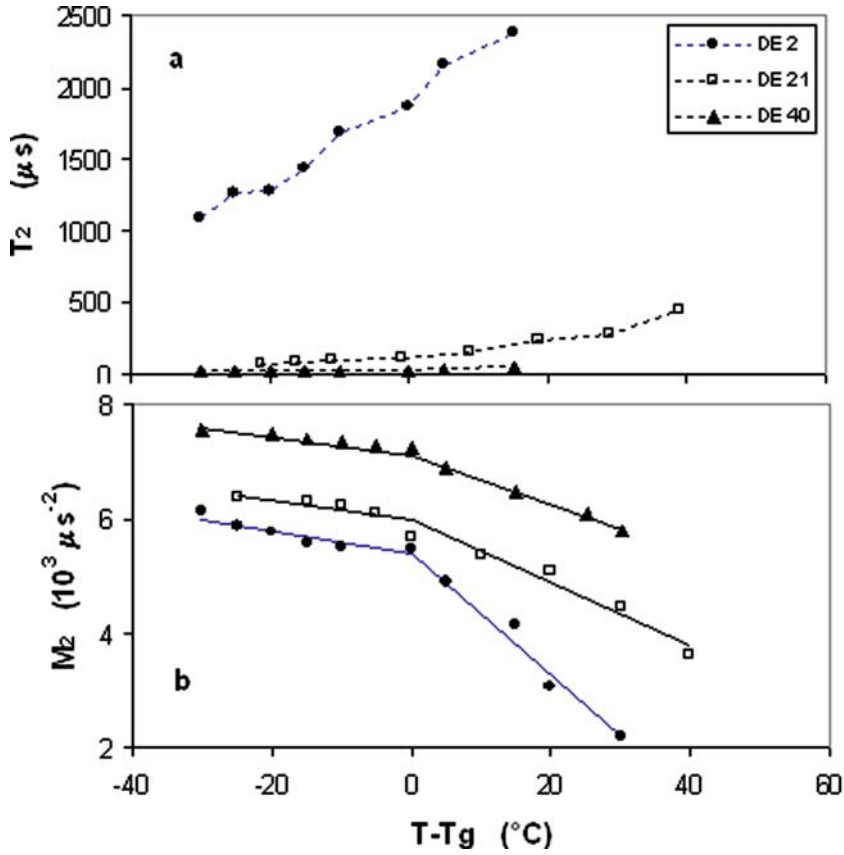

Figure 11.15. NMR relaxation time T_2 (**a**) and second moment M_2 (**b**) versus ($T–T_g$) for freeze-dried maltodextrins (dextrose equivalents, DE 2, 21, 40) equilibrated at $a_w = 0.4$.
T_2 represents the rotational mobility of the more mobile protons (mainly water). M_2 is considered to be inversely related to the mobility of the less mobile protons (matrix protons + possibly some water protons) (Data from Grattard *et al.,* 2002).

While the matrix mobility showed a significant enhancement of the temperature dependence near T_g, for mobility of water, a continuous increase in mobility with temperature was observed; moreover, the mobility of water increased with the molecular weight of the matrix material (Grattard *et al.*, 2002). Similar conclusions can be derived from studies on water translational diffusion (D_{trans} or D_{app}) (Ablett *et al.*, 1993; Tromp *et al.*, 1997; Aldous *et al.*, 1997).

PALS, together with density measurements, also provide information concerning the changes in nanostructure when water is sorbed on carbohydrates (Kilburn *et al.*, 2004, 2005). The mean hole volume was found to increase strongly, and linearly, with water content ($0.1 < a_w < 0.75$ at 25°C). The partial molar volume of water in the carbohydrate glassy matrix was estimated to be close to the van der Waals' volume of water (7.1 cm^3/mol), i.e. much lower than its value in the liquid state (18 cm^3/mol). Water influences the average hole size, on the one hand, by filling the smallest voids in the glassy matrix and, on the other hand, by increasing the mobility of the polymer chains, which allows the coalescence of the smallest voids under the surface tension driving force (Kilburn *et al.*, 2004). Whereas water acts as a plasticizer mainly by interacting with the polymer H-bonding, conversely, the plastifying action of low molecular weight sugars is suggested to be due to a reduction of the number of molecular entanglements because of their small size (Kilburn *et al.*, 2005). The differences in the glass nanostructure observed as a function of the molecular weight have important technological implications, as the barrier properties of carbohydrates in the glassy state increase with decreasing molecular weight, although their glass transition temperature decreases (Kilburn *et al.*, 2005).

Several sub-T_g relaxations can be observed in biopolymers and low molecular weight sugars (β and γ relaxations). Their origin is still being discussed. As observed for polysaccharides, they could correspond to the rotation of lateral groups (γ relaxation at low temperature) or to local conformational changes of the main chain (β relaxation closer to T_α) (Montès *et al.*, 1998; Einfeldt *et al.*, 2004; Bidault *et al.*, 2005). The E_a value ranges between 40 and 70 kJ/mol for β relaxation in sucrose (Figure 11.14), maltose and glucose. Sub-T_g relaxations are also observed in complex food products, such as bread (Roudaut *et al.*, 1999a,b) (Figure 11.9). The influence of water on these processes is less well known than its plastifying action on T_g. For polysaccharides, T_β was found to decrease for increasing water content (Borde, 1999; Lievonen and Roos, 2003; Poirier-Brulez *et al.*, 2006).

Water at low concentration may induce an increase in rigidity, although T_g is decreased. This hardening effect, observed with starch and cereal products under high deformation conditions (Attenburrow *et al.*, 1992; Nicholls *et al.*, 1995; Fontanet *et al.*, 1997; Roudaut *et al.*, 1998; Li *et al.*, 1998; Chang *et al.*, 2000; Konopacka *et al.*, 2002), is known as antiplastification for polymers (Vrentas *et al.*, 1988). This antiplastifying effect of water in starch systems was attributed to a short-range reorganization (Fontanet *et al.*, 1997), a density increase by filling the defects in the glass structure (Benczedi, 1999; Chang *et al.*, 2000).

When stored below T_g, a glass is subject to a slow evolution of many of its properties. This "physical ageing" is the result of a microstructure

evolution, by which the material is approaching closer to the metastable equilibrium, by reduction of the excess volume, enthalpy and entropy, which had been "frozen-in" at the glass transition (Figure 11.8). Since the timescale for equilibrium becomes very long as the storage temperature is reduced, this evolution can be observed only in a narrow temperature interval below T_g (Hutchinson, 1995). In the field of material science, physical ageing is well known for its technological significance, as it manifests itself as an increase in rigidity and brittleness and a decrease in dimensions and permeability. It is expected to be of importance for the stability of low-moisture food and pharmaceutical products and is currently receiving a lot of attention. The extent of structure relaxation is generally characterized by the amplitude of the enthalpy recovery (commonly denoted as "relaxation enthalpy") as a function of storage time. To characterize the kinetics of enthalpy relaxation, several models can be used, the most popular being the Cowie–Ferguson semi-empirical model (Cameron *et al.*, 2001), which is based on the stretched exponential (KWW) expression:

$$\Phi(t) = \exp[-(t/\tau)^{\beta}] \tag{22}$$

where Φ is the property studied as a function of time (t), e.g. the proportion of glass that has not yet relaxed at time t and that is calculated as $\Phi(t) = 1 - \Delta H_t / \Delta H_\infty$, ΔH_t being the enthalpy recovery measured with DSC. The parameter β is close to 1 for strong liquids (nearly exponential relaxation). For fragile liquids, it changes from near 1 at high temperature to a value close to 0.3–0.5 near T_g. It must be remembered, however, that the KWW and Cowie–Ferguson expressions are only phenomenological models, whose parameters lack real physical meaning. In particular, the Cowie–Ferguson expression is an oversimplified model because the characteristic relaxation time varies with ageing time (non-linearity) (Hodge, 1994).

Since the rate of structure relaxation depends on the molecular mobility, it may be expected to increase with the water content and to be higher in glasses of lower molecular weight. The rate of enthalpy relaxation of gelatine was indeed found to increase with water content (7–14%). The effect of water could be fully explained in terms of decreasing T_g (Badii *et al.*, 2005). To judge the specific influence of the constituents, enthalpy relaxation must be measured for a same increment (T_a–T_g) of the ageing temperature from T_g. The rate of enthalpy relaxation was reduced, as expected, upon addition of a substance with a higher T_g, for instance dextran in sucrose (Blond, 1994; Shamblin et al., 1998). In contrast, increasing the weight fraction of fructose in glucose–fructose mixtures resulted in a decrease in the ageing rate, although T_g was depressed (Wungtanagorn and Schmidt, 2001a,b). In

starch–sucrose mixtures, the relaxation extent was found to decrease when the sucrose content increased (0–20%) (water content ≈12%) and to be linearly correlated with the distance from the β relaxation temperature, suggesting that the relaxation rate was actually related to the mobility induced by the β relaxation (mobility decreasing upon addition of sucrose to starch) (Poirier-Brulez *et al.*, 2006).

The consequences of physical ageing for the physical properties of biopolymer glasses are similar to what was described for other materials. The storage modulus of gelatine films increased with ageing time, in agreement with the rate of increase of the relaxation enthalpy (Badii *et al.*, 2006). The same result was obtained for the breaking strength of corn starch bars (Chung *et al.*, 2005). The water permeability of potato starch films was shown to decrease with ageing time (Kim *et al.*, 2003). Ageing also reduced the water sorption ability of dried fish meat (Hashimoto *et al.*, 2004). Although no crystallization was observed during the prolonged storage of a trehalose glass, ageing was claimed to induce an increase in the tendency to crystallize on subsequent heating (Surana *et al.*, 2004). The temperature dependence of changes associated with physical ageing seems to be Arrhenius-like (at least over a small temperature range) with an apparent activation energy around 250 kJ/mol (Kim *et al.*, 2003; Green *et al.*, 2007; Liu *et al.*, 2007).

To conclude this section, let us say that a significant molecular mobility may be observed in glasses, for temperature and time conditions in use, e.g. with low-moisture foods. T_g, therefore, cannot be taken as a safety limit. It has been proposed that a more meaningful temperature could be the Kauzmann temperature (T_k or T_o), which is the temperature where the configuration entropy of glasses would be zero if the material could be kept at this temperature until equilibrium. This is not realistic. First, the decrease in entropy to zero is only hypothetical (Perez, 1994; Ediger *et al.*, 1996); instead of the linear extrapolation of the entropy curve below T_g, it is quite possible to consider a decrease in entropy to zero at a much lower temperature. Second, it cannot even be taken as a reference to predict the stability of a product from the distance of the storage temperature to T_k, since T_k would be meaningful only for a product having attained the equilibrium structure, thanks to an infinitely long storage time (Figure 11.8).

11.5.4. Mobility Above T_g

In the "WLF temperature domain" (between T_g and $\approx T_g+100$) translational diffusion may be decoupled from viscosity because of various phenomena. We will first consider systems where the diffusing probe is of

a size similar to that of the matrix material. D_{trans} of fluorescein was measured in concentrated sucrose solutions in a temperature range from –15 to 20°C using the FRAP method (Champion *et al.*, 1997b). The evolution of the fluorescein D_{trans} with temperature followed Eq. (21) when the temperature was higher than 1.2 T_g ($T_g/T < 0.86$). As shown in Figure 11.16, values obtained with solutions of various concentration are all on the same line, which demonstrates that D_{trans} is indeed controlled by viscosity. When the temperature was closer to T_g, the diffusion of fluorescein was faster than predicted from Eq. (21).

The decoupling of translational diffusion and viscosity in the same temperature range (between T_g and 1.2 T_g) was first reported for probes dispersed in various organic liquids or polymers. By contrast, rotational diffusion data, obtained by a variety of techniques, always appear to scale with the matrix α relaxation dynamics (except when probes dispersed in a polymer are smaller than a minimum size). The decoupling between translational and rotational diffusions has been explained by the spatial heterogeneity of supercooled materials, comprising domains with varying mobility, both types of motions having different sensitivity to the local variations of

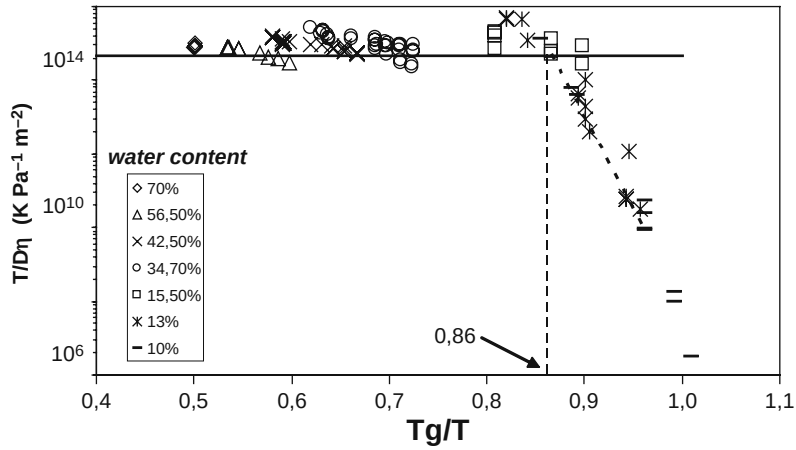

Figure 11.16. Variation of translational diffusion of fluoerescein (corrected for temperature and viscosity) in concentrated sucrose solutions, with the inverse of temperature.
This shows the change in diffusion behaviour for T=1.2 T_g. At T >1.2 T_g, T/ D_{trans} η is constant and independent of the water content, equation 21 is obeyed and D_{trans} is actually controlled by viscosity. On the contrary, at T < 1.2 T_g, D_{trans} remains higher than predicted from Eq. (20). The different symbols correspond to water contents varying from 10 to 70% (Champion *et al.*, 1997b).

relaxation time (Fujara *et al.*, 1992; Blackburn *et al.*, 1996; Hall *et al.*, 1998). Since the decoupling temperature was in the range where α and β relaxations merge (Perez and Cavaillé 1994), it was suggested that the translational diffusion of the probe could be facilitated by local motions of the matrix when the temperature approaches T_g (Champion *et al.*, 1997b). The practical importance of this decoupling has to be emphasized, with regard to the stability of amorphous products: In the vicinity of T_g, the translational diffusivity may be 2–5 orders of magnitude higher than that predicted from the viscosity and the Stokes–Einstein equation. The temperature dependence also is much weaker (apparent activation energy = 59 kJ/mol for the D_{trans} of fluorescein in sucrose below 1.2 T_g).

For food systems composed of polymers, small molecules and water, DSE expressions may not be valid to predict diffusion, because the "macroscopic viscosity" (commonly measured) does not reflect the local environment of the diffusing species and is not the factor that controls diffusion. The translational and rotational mobilities of small probes dispersed in concentrated sucrose solutions (57.5%) were not significantly affected, or were reduced only slightly, upon addition of, respectively, 1 or 10% polysaccharides, in spite of the large increase in viscosity (Contreras-Lopez *et al.*, 2000).

A study on caseinates provides a good illustration of the mobilization process induced by water (Figure 11.17). The mobility of a small probe (monosaccharide analogue) dispersed in the protein matrix was monitored by ESR. Below a critical water content (0.25 g H_2O/g dw), all the small solutes exhibited very slow motions (rotational correlation time, $\tau_c > 10^{-7}$ s). Then, between the above critical value and approximately 1 g H_2O/g dry weight, a progressive dissolution of the small solutes was observed. Once dissolved, solute diffusivity was controlled by the hydrodynamic radius of the solute, the water content and the changes in plasticity of the surrounding protein (LeMeste *et al.*, 1991). Although the glass transition for caseinate occurs at room temperature for a water content close to 20% (wet basis), it seems that the glass transition of the polymeric network and the mobilization process of small solutes, as also that of the protein side chains, are not closely related: "(1) the mobilization process of spin-labelled protein side chains (lysyl residues) started at a water content (0.5 g H_2O/g dry weight) higher than that for small solutes (data not shown); (2) the critical water content necessary for the first solute molecule to become more mobile (i.e. to be dissolved) increases with the size of the solute (Figure 11.17); (3) internal plasticization of caseinates (resulting from the glycation of lysyl residues) did not modify the water content where, on the one hand small solutes (Figure 11.17), and on the other hand, the spin-labelled side chains, became mobile (LeMeste *et al.,* 1990)".

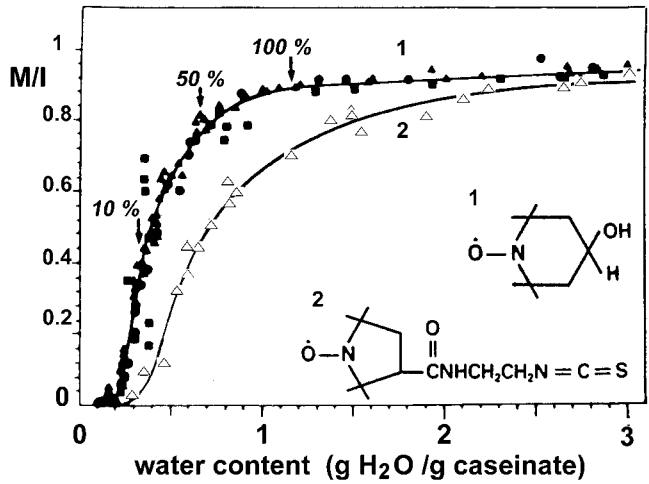

Figure 11.17. Solubilization process for probes 1 and 2 (of different hydrodynamic radius) dispersed in caseinate and modified caseinates, evaluated from ESR spectra. 1: Probe 1 in caseinate (■) and in maltosylated (●) or galactosylated (▲) caseinates. 2: Probe 2 in caseinate (Δ). Some values of the proportions in mobile probes are indicated on the graph (LeMeste *et al.*, 1991).

11.5.5. Influence on the Quality of Low-Moisture Foods

The influence of molecular mobility and of glass transition on the quality of low-moisture food products was reviewed by LeMeste *et al.* (2002). Here, we will therefore recall the main conclusions and review the newer literature, particularly when dealing with dairy products.

11.5.5.1. Collapse, Caking, Agglomeration

Stickiness and caking are important phenomena to be taken into account upon drying of dairy products and in handling and storage of their powders. Structure collapse, which occurs during freeze- or air-drying, or during the storage of dried products, hinges on the same process as stickiness. While these phenomena are responsible for deterioration in the quality of dairy powders, agglomeration – a controlled caking process – is used to improve the appearance and handling of powders and their dispersibility in water. From the evidence available in 2002, mainly based on studies on carbohydrate model systems, we concluded (LeMeste *et al.*, 2002) that structural collapse, stickiness and caking/agglomeration could be explained successfully using the glass transition concept. The formation of bridges between

adjacent particles, and then aggregation, was shown to take place when surface viscosity decreases because of an increase in temperature or in water content, and reaches a critical value depending on the particle size and the characteristic time of the method used to monitor the changes. The sticky point (T_s) and glass transition temperature were observed to be affected similarly by increasing moisture content, with T_s close to the $T_{g\ end}$ values, that is about 20°C above $T_{g\ onset}$ (Roos and Karel, 1991b). Caking and collapse were modelled according to WLF kinetics. For a ($T–T_g$) range between 15 and 30°C, the relaxation time for collapse of a freeze-dried sucrose–raffinose model could be fitted to the WLF expression with the "universal" coefficients (Levi and Karel, 1995). Because of the rather narrow ($T–T_g$) range, the C_1 and C_2 values may not be meaningful. What is most important, however, is the high level of the mean apparent activation energy (>200–400 kJ/mol), which points to the large temperature dependence of the phenomenon. Caking of a spray-dried fish protein hydrolysate was also characterized by a WLF relationship, with adjustable C_1 and C_2 coefficients, for ($T–T_g$) between about 20 and 80°C (Aguilera and del Valle, 1995). Recently, a test was developed to monitor the stickiness progression with time (Paterson *et al*., 2005). The ($T–T_g$) value was confirmed to be the main factor determining the rate of stickiness development in amorphous lactose, regardless of which combination of temperature and relative humidity was used to achieve it. The rate of sticking was fitted to the WLF expression with adjustable C_1 and C_2 coefficients (respectively, 1.6 and 3.5°C), for a ($T–T_g$) range 1–25°C (Paterson *et al*., 2005). Given that the viscosity of various sugars [e.g. sucrose (Champion *et al*., 1997a,b), glucose (Williams *et al*., 1955)] was observed to show WLF behaviour with coefficients close to the "universal" values, we must recognize that the reported values may suggest viscosity is not the only phenomenon at work in stickiness. Based on this inconsistency of the reported coefficients, Chen (2007) showed that the lactose results could be modelled using an Arrhenius-based expression. The "driving force" for the stickiness development as a result of glass transition in amorphous materials would then be ($1/T–1/T_g$) rather than ($T–T_g$), which would indicate an independent action of temperature and water content.

For spray-dried milk powders, stickiness and caking are quite different depending on their fat content. In skim milk powder (SMP), cohesiveness appears to be dependent on the glass–rubber transition of lactose, as a result of increasing temperature or water content [with a mean activation energy ≈270 kJ/mol in the temperature range T_g +5°C to +15°C (Ozkan *et al*., 2003)]. In whole milk powder (WMP), the loss of flowability is observed at a temperature below the glass transition and is related to melting of fat. As the surface composition of WMP is mainly fats, liquid fats are supposed to form liquid inter-particle bridges, or to soften the

particle surface, increasing the contact area (Rennie *et al.*, 1999; Ozkan *et al.*, 2002; Fitzpatrick *et al.*, 2004, 2007).

11.5.5.2. Crystallization

Crystallization is a most important process regarding various aspects of the quality of dairy food powders. At an advanced stage of caking, crystallization of lactose can reinforce the already formed liquid bridges and then consolidate the caking (Chen, 2007). Controlled crystallization of lactose can be used to reduce the hygroscopicity and caking tendency of dairy powders. Crystallization of lactose impairs the solubility of milk powder and accelerates damaging chemical changes (see Buera *et al.*, 2005, for a review). Crystallization of lactose is responsible for the undesirable sandy texture in ice cream and condensed milk, while it is considered desirable in milk chocolate, where the free fat content is proportional to the fraction of crystallized lactose in the concentrate (Vuattaz, 1999). The size of ice crystals in ice cream is also a major factor in consumer acceptability.

Crystallization comprises two steps: nucleation and growth. According to classical crystallization theories, both processes are controlled by thermodynamic effects (formation of nuclei of the critical size, incorporation of molecules into the crystal lattice) and kinetic ones (transport of molecules from the bulk solution to the nucleus or to the growing crystal interface). Both types of effect have opposite temperature dependence, resulting in nucleation and growth rates with bell-shaped curves in a temperature range between T_g and T_m. The overall crystallization rate is therefore expected to show a similar behaviour. The (time-dependent) crystallization temperature (T_{cr}) in amorphous sugars determined by DSC was reported in a number of studies to be approximately halfway between their respective T_g and T_m values (Roos and Karel, 1991b,c, 1992; Saleki-Gerhardt and Zografi, 1994; Gabarra and Hartel, 1998). The effect of water was found to be about the same on T_{cr} and T_g as indicated by a fairly constant value of ($T_{cr} - T_g$) (Roos and Karel, 1991b,c). The time to complete crystallization (t_{cr}) in samples of lactose with a water content between about 1 and 8% could be fitted to a WLF-type equation: This is the behaviour to be anticipated for experimental temperature ranges near T_g, where the crystallization rate is controlled by kinetic effects. For broader temperature ranges, extending to smaller undercooling (T close to T_m), bell-shaped curves were reported for sucrose, lactose (Kedward *et al.*, 1998, 2000) and starch (Marsh and Blanshard, 1988; Farhat *et al.,* 2000) (Figure 11.18). The shift in the maximum crystallization rate with water content could be explained by changes in T_g and T_m (Kedward *et al.*, 2000).

Lactose crystallization can be retarded, not only by maintaining low-temperature/low-water-content conditions but also by adapting product

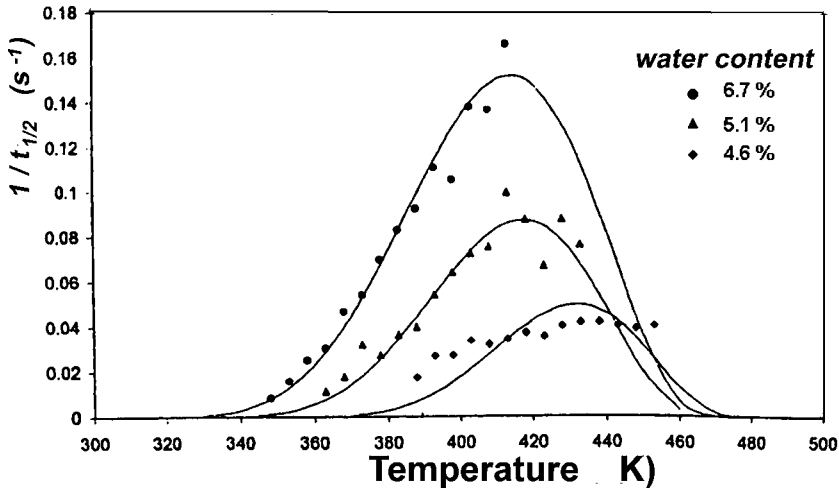

Figure 11.18. Crystallization rate of amorphous lactose at three water levels.
The lines are the fitting of data to the semi-empirical Lauritsen-Hoffman model (Kedward *et al.*, 2000).

formulation (see Buera *et al.*, 2005, for a review). β-Lactoglobulin was shown to retard the crystallization of lactose in whey powder, when co-lyophilized. This effect was ascribed to non-covalent or covalent interactions between lactose and β-lactoglobulin resulting in modifications of the water sorption behaviour (Thomas *et al.*, 2004).

11.5.5.3. Non-enzymatic Browning and Other Chemical Reactions

In our earlier review (LeMeste *et al.*, 2002), the provisional conclusion was that the temperature of glass transition does not constitute an absolute stability threshold and that, above this temperature, the reaction kinetics do not obey WLF kinetics. Almost all of the quoted studies reported finite reaction rates in glassy products. The reaction rate increased with the difference $(T–T_g)$. However, when the variation of T_g was induced by changes in water content, the expected single relationship between the rate and $(T–T_g)$ was not observed. The reaction rate evolution with temperature was most often described as uniform, of Arrhenius type, even within the glass transition temperature range. The apparent activation energy remained low, even at $T > T_g$, in the range 100–150 kJ/mol. These values are much smaller than the activation energies commonly observed for dynamical properties in the glass

transition range. Newer studies on non-enzymatic browning confirm these conclusions. The reaction is observable for temperatures as low as a T_g −40°C, in matrices of maltodextrins, PVP or trehalose (Lievonen and Roos, 2002, 2003; Kawai *et al*., 2004, 2005). The reaction rate does not even appear to be correlated with mobility, as it is higher in the PVP matrix than in the maltodextrins, although dielectric measurements (Lievonen and Roos, 2003) or enthalpy relaxation times suggest the opposite order for mobility. The large difference in the reaction rate observed when the water content varies, but for the same value of $(T–T_g)$, is again emphasized (Miao and Roos, 2004). The temperature dependence is of Arrhenius type, with no consistent break in the glass transition range, apparent activation energy being between 100 and 200 kJ/mol for NEB in matrices of lactose-trehalose (Miao and Roos, 2004; Kawai *et al*., 2004, 2005), casein-lactose (Malec *et al*., 2002) and milk (Thomsen *et al*., 2005). These newer studies appear to confirm the previously proposed interpretation (Karel and Saguy, 1991; Craig *et al*., 2001). For bimolecular reactions that are influenced by diffusion of reactants, the reaction rate constant, k_{app}, may be modelled by the expression

$$k_{app} = k_{act}/(1 + k_{act}/\alpha D) \tag{23}$$

where k_{act} is the reaction constant observed in well-stirred solutions, which recognizes the fact that only a fraction of potential reactants coming into contact are activated and can react. D is the diffusivity of the reactants (equal to the sum of the individual diffusion coefficients) and α is a coefficient depending on a collision distance. Both parameters, k_{act} and D, are temperature dependent. The reaction can be fully controlled by diffusion if the reaction constant, k_{act}, is much larger than α D (then $k_{app} \cong \alpha$ D); the temperature dependence is the same as for D (possibly WLF kinetics). In the case of reactions with a high activation energy (as it could be for NEB), k_{act} remains low as long as the temperature is not high enough, and consequently k_{app} is controlled by the activation of reactants rather than by diffusion ($k_{app} \approx k_{act}$). The temperature dependence is of Arrhenius type.

A study on a caseinate-based model system, where a_w and T_g were modified through addition of sorbitol or glycerol, confirmed that plasticization of the matrix cannot be viewed as the critical effect on NEB reaction rate, whether caused by the addition of glycerol or the increase in water content (Sherwin and Labuza, 2003). Instead, it was suggested that the reaction rate was controlled by the dissolution of reactants, as first proposed by Duckworth *et al*. (1976). This mechanism is illustrated in Figure 11.19 by expressing the NEB rate against the mass of solvent, as determined by water or glycerol content (reactant glucose being soluble in glycerol, but not in sorbitol).

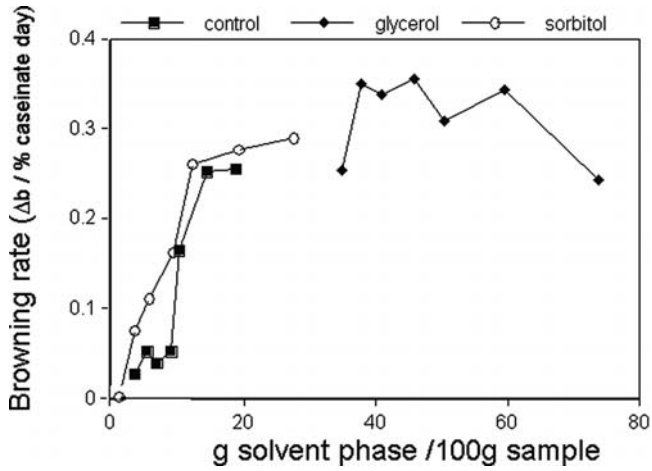

Figure 11.19. Browning rate in a caseinate-based model system (Na-caseinate/glucose ~74/25% (dry basis) and Na-caseinate/glucose/humectant ~50/17/33% dry basis) at $0.11 < a_w < 0.75$ (Sherwin and Labuza, 2003).

Colour formation resulting from the thermal degradation of maltodextrins was also reported to show an Arrhenius-type temperature dependence across the glass transition range (with an apparent activation energy ~ 83 kJ/mol for $a_w = 0.15$) (Claude and Ubbink, 2006). The thermal inactivation of β-galactosidase in dairy model systems was found to occur in the glassy state. The reaction rate showed no sharp increase above T_g (Burin *et al.*, 2002). As shown in Figure 11.20, the rate of thermal inactivation in various systems was more dependent on water content than on the $(T–T_g)$ value.

To be able to carry out their specific activity, proteins need a minimum amount of water. The water content threshold for activity was shown to coincide with the water content for incipient mobility. In more complex food systems at low water content, however, glass transition did not appear to be the main factor controlling the enzymatic activity. For instance, the rate of sucrose hydrolysis by invertase, in maltodextrins–sucrose–lactose systems, significantly increased only for water contents corresponding to $(T–T_g)$ values as high as 40°C (Kouassi and Roos, 2001). For β-galactosidase in whey powder or dextrans, the small change in reaction rate for increasing $(T–T_g)$ suggests that T_g is not a significant factor; reduction of pH upon dehydration seems to be the main cause of the low activity (Burin and Buera, 2002). As will be seen in the next section,

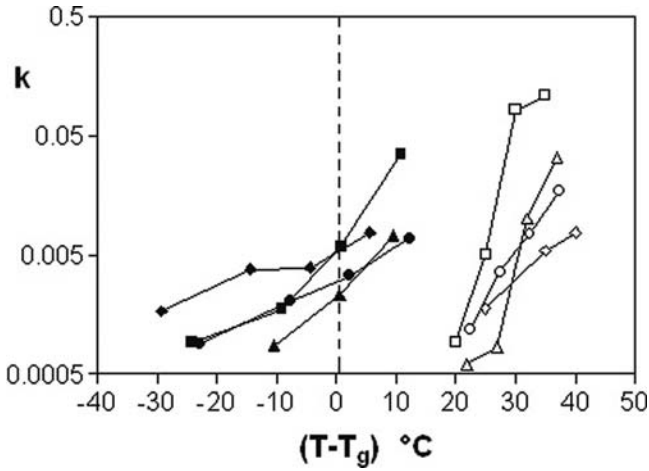

Figure 11.20. Rate of thermal inactivation of β-galactosidase in dairy model systems, either anhydrous (*filled symbols*) or exposed to 22% RH (*open symbols*) (●, ○: lactose, ■, □: sweet whey powder, ▲, △: skim milk powder, : lactose+caseinate 1/1) (Burin *et al.*, 2002).

in contrast, modelling enzymatic activity according to WLF kinetics may be applicable in some situations.

11.5.6. Stability of Frozen Food Products

As is well known, the rate of physical, chemical and biochemical changes in frozen products is strongly affected by the storage temperature. This is shown for instance by the high values of the empirical coefficient, Q_{10}, which can be determined from temperature–time tolerance diagrams (Jul, 1984). These values, which range from 2 to 30 in the usual temperature range of frozen storage, are often much higher than the Q_{10} values observed for common chemical reactions, particularly in food systems at temperatures above 0°C; i.e. the Q_{10} is around 2 for most chemical or biochemical reactions and 3–4 for Maillard reactions. The slowing down of changes with decreasing temperature has been attributed to the greatly increased viscosity of the freeze-concentrated phase, resulting from the combined effects of concentration and temperature. Levine and Slade (1988, 1989, 1990) have promoted the idea that the glass transition temperature of the maximally freeze-concentrated phase (T_g') is a threshold of instability in frozen food systems and that kinetics above this temperature are controlled by the difference between the temperature of storage and the specific T_g' of the product, according to WLF kinetics.

11.5.6.1. Where Is T_g'

In order to evaluate the validity of this concept, and to make practical use of it, a primary requisite is reliable values of T_g'. DSC thermograms of frozen aqueous solutions usually display a complex feature before the ice melting peak, which can be seen for instance in the DSC curve of ice cream in Figure 11.21. According to Levine and Slade (1988, 1989, 1990), T_g' (defined as the glass transition temperature of the maximally freeze-concentrated phase) should be represented by the inflection point in the second step of the heat capacity increase (T_2 in Figure 11.21). Despite numerous studies involving conventional and modulated DSC, mechanical spectroscopy, etc., the nature of events corresponding to transitions T_1 and T_2 is still highly controversial (Goff *et al.*, 2002, 2003; Schawe 2006; Chang *et al.*, 2006). The current trend, however, seems to regard the heat capacity jump at T_1 as the glass transition in the freeze-concentrated phase. Whatever the true nature of transitions T_1 and T_2, the important issue is the evolution of mobility in this temperature range. Mechanical spectroscopy gave evidence for the increase in

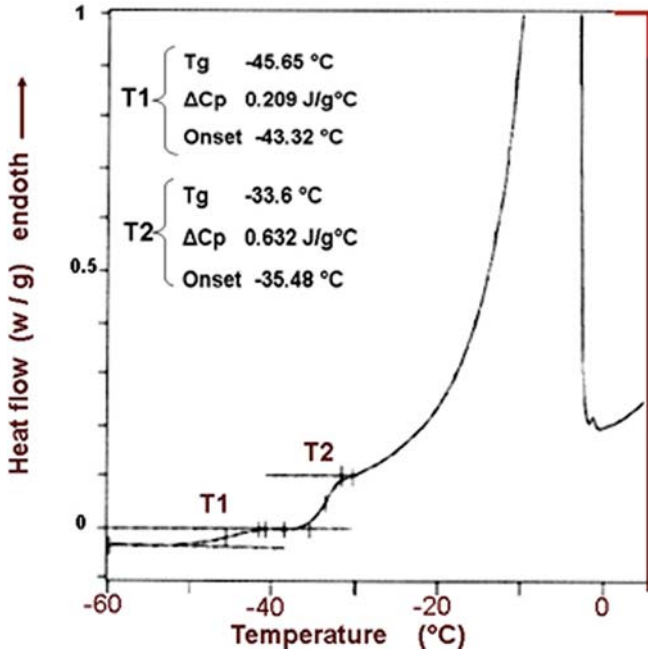

Figure 11.21. DSC curve for ice cream. This curve is very similar to thermograms of sucrose solutions at a sucrose concentration below 80 % (Blond, unpublished).

mobility induced by glass transition beginning at a temperature as low as T_0 (the onset of transition T_1) (Simatos and Blond, 1993). ESR showed that transition T_2 corresponded to an important increase in mobility, suggesting that ice melting was beginning at that temperature (LeMeste and Simatos, 1980). Recently, in the case of gluten, the minor endothermic peak observed just before the major ice melting peak was assigned to the melting of ice confined in capillaries (Kontogiorgios and Goff, 2006). Although the authors related the existence of confined water to the porous microstructure of gluten, one could envisage the same explanation for other food systems. Water confined between the ice crystals formed during the first freezing step could freeze as small crystals, the melting of which would give the endothermic feature T_2. The latter has been shown to develop as a peak during an annealing treatment in sugar solutions (Simatos and Blond, 1993; Champion, pers. comn.).

11.5.6.2. Can T_g' Be Raised? Effect of "Stabilizers" in Ice Cream

As stability of frozen products at a given temperature was hypothesized to increase with a raise in T_g' (Levine and Slade, 1988), the possibility of raising T_g' through the addition of high molecular weight compounds was explored in many studies. The influence of macromolecules on the glass transition temperature of frozen solutions was reviewed, e.g. by Simatos *et al.* (1995a), and can be summarized as follows. When added at a rather high concentration to solutions of low molecular weight solutes, polymers induce an increase in the value of T_2. The values of T_0 and T_1, however, do not increase, which indicates that the temperature at which some mobility appears is not raised on addition of the polymer. The T_1 temperature of frozen sucrose or trehalose solutions, to which egg albumin, gelatin, cornstarch or a mixture of the three was added (at the solids content ratio 1/1), was even reported to decrease (by 2–7°C), although the glass transition temperatures of the dry mixtures were strongly raised (by 9–59°C) and the temperatures T_m' (i.e. T_2) were raised by 3–11°C (Singh and Roos, 2005, 2006).

Macromolecules (various polysaccharides and gelatine) are commonly added to ice cream mixes as "stabilizers" as they are known to exert a beneficial effect on the texture of the final product. At the very low concentrations that are used in the ice cream industry, these stabilizers do not induce a visible effect on the DSC T_g' (T_2) values (Levine and Slade 1988; Goff *et al.*, 1993; Blond 1994). The main mechanism of the favourable effect of these stabilizers on the sensory properties of texture may relate to the perception in the mouth, either through a change in the viscoelastic properties of the unfrozen phase or any other sensory effect. Stabilizers, however, have been shown to reduce the recrystallization rate in ice creams stored at temperatures above T_g' (Caldwell *et al.*, 1992; Hagiwara and Hartel, 1996). In frozen

products, all the "freezable" water has generally been transformed to ice during the freezing stage. The issue is then the coarsening of the ice structure during frozen storage, which is considered to occur through several mechanisms, including accretion, migratory recrystallization (or Ostwald ripening), where differences in the stability of crystals, based on size differences, cause small crystals to disappear and large crystals to grow, and finally isomass rounding, which is also based on differences in stability for surfaces with different curvature radii (Hartel, 1998). The potential mechanisms suggested to explain the inhibitory effects of stabilizers include restriction of mobility around T_g' (Blond, 1994); mechanical limitation of the crystal growth related to the rigidity of the freeze-concentrated phase (Muhr and Blanshard, 1986; Blond, 1988); and adsorption of the macromolecules on the crystal surface (Sutton and Wilcox, 1998). Recent studies support these various mechanisms, emphasizing the diversity of the stabilizers' functionalities. Some non-gelling polysaccharides (xanthan) can effectively retard recrystallization through an increase in microviscosity of the unfrozen phase (Regand and Goff, 2003). The NMR results in which an increase in viscosity due to polysaccharides is not accompanied by a decrease in the mobility of water molecules seem to be contrary to this mechanism. The authors, however, point out the difference in scales involved in NMR measurements (translational diffusion in the 10 nm range) and in water migration from one crystal to another (longer than 10 μm). Antifreeze proteins (now named ice-structuring proteins: ISP) extracted from cold-acclimated winter wheat grass proved to be effective for retarding ice recrystallization at a very low concentration in sucrose solutions and ice creams. A synergistic effect between ISP and stabilizer was observed. These proteins are thought to adsorb at the ice-solution interface, thereby modifying the surface curvature of the ice crystal and then the difference in freezing temperature of neighbouring crystals, which is the driving force for migratory recrystallization (Regand and Goff, 2005, 2006).

11.5.6.3. Kinetics of Changes Above T_g'

The recrystallization rate of ice in ice cream was shown to be well described by the expression $r = r_0 + R\ t^{1/n}$, where r is the mean crystal radius at time t, r_0 the initial value and R the crystallization rate. Since the value of $n = 3$ fitted the data best, the mechanism for recrystallization was suggested to be dependent on non-convective diffusion processes (Hartel, 1998). The temperature dependence of R was checked for WLF kinetics, using a constant temperature T_g' (T_2). The authors reported that the fit of R to WLF kinetics was reasonably good, although recognizing that the range and the number of tested storage temperatures (–20 to –5°C, i.e. 14–29°C above the DSC T_g'), were too small to allow exact determination of the best model for temperature

dependence. Moreover, based on the "universal coefficients", WLF kinetics predicted a more rapid increase in crystallization rate with temperature than was actually observed (Hartel, 1998). The same observation had been reported (Simatos and Blond, 1991, 1993) concerning the crystal growth rate in frozen beef, as well as other processes of deterioration in frozen foods. The discrepancy was particularly striking, when one considered that the decrease in viscosity as temperature increased above T_g', while initially induced by the glass transition, was strongly amplified by dissolution (melting) of ice in the freeze-concentrated phase. It was further shown that the dilution of reactants resulting from the dissolution of ice in the freeze-concentrated phase could partly compensate the effect of decreasing viscosity on reaction kinetics, but that this effect was not significant enough to explain the observed discrepancy. It was then suggested that the discrepancy could be solved using T_g values much lower than T_g' (Simatos and Blond, 1991).

These observations have been confirmed by recent studies. The temperature dependence of various chemical and enzymatic reactions in the temperature range around or just above T_g' (or T_2) can be characterized by apparent activation energies between 50 and 150 kJ/mol: oxidation rate of ascorbic acid in starch hydrolysates (Biliaderis *et al.*, 1999), tyrosinase activity (Manzocco *et al.,* 1999), formaldehyde production in fish extracts with maltodextrins (Herrera and Roos, 2001), colour changes and chlorophyll degradation in green beans (Martins and Silva, 2002), colour and ascorbic acid changes in peas (Giannakourou and Taoukis, 2003). Although fairly high for chemical reactions in food systems, these figures are lower than those usually observed for dynamic properties immediately above the glass transition range. Apparent activation energy for recrystallization in ice creams was in the same range (120 kJ/mol) (Hartel, 1998). Actually, WLF behaviour should be considered only for systems the chemical composition of which is constant over the period of time and temperature range considered. For food system applications in the temperature range where melting of ice occurs, the reference temperature should not be T_g', but rather the T_g values relevant to the actual concentration of the freeze-concentrated phase at the storage temperature T_s (Figure 11.6).

This approach was used to model the alkaline phosphatase activity in frozen sucrose solutions (Champion *et al.*, 1997a). The rate constant was assumed to vary with viscosity according to the expression derived by Atkins (1998) for diffusion-controlled bimolecular reactions:

$$k_{app} = \frac{8RT}{3\eta} \tag{24}$$

The viscosity of the freeze-concentrated phase at T was predicted from the WLF equation, with C_1 and C_2 determined from viscosity data for sucrose

solutions. The concentration of the freeze-concentrated phase and the variable T_g at the storage temperature, T, were deduced from the state diagram (Blond *et al.*, 1997). As shown in Figure 11.22 the predicted temperature dependence of the reaction rate constant was very similar to that of the experimental data. The chosen system represented a favourable situation for modelling according to WLF kinetics, probably because the enzyme exhibits a relatively high k_{cat}, even in concentrated sucrose solutions and at low temperature (Champion *et al.*, 2000). However, the temperature dependence of the reaction rate of pectin methylesterase in sucrose solutions was also found to be consistent with the same model (Figure 11.23) (Champion *et al.*, 2004). In contrast, reaction rates for tyrosinase and peroxidase activities showed the same temperature dependence in sucrose, fructose and glycerol frozen aqueous solutions, despite the different viscosities of the respective concentrated liquid phases (Manzocco *et al.*, 1999), giving evidence of the failure of WLF kinetics in these systems.

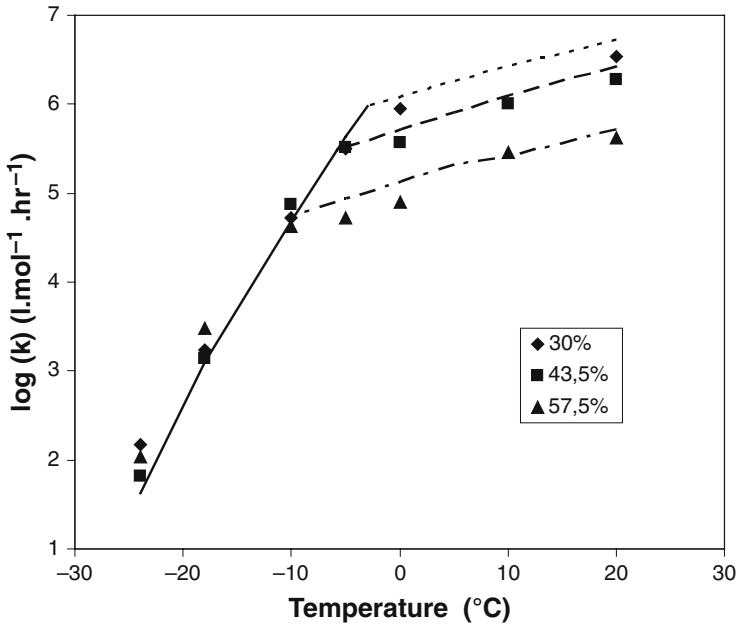

Figure 11.22. Initial reaction rate constant, k, of alkaline phosphatase as a function of temperature. Symbols: experimental values in different sucrose solutions. Solid line: k values predicted from the viscosity of the freeze-concentrated phase calculated from the state diagram. Dashed lines: k predicted from the measured viscosity (samples without ice) (Champion *et al.*, 1997a).

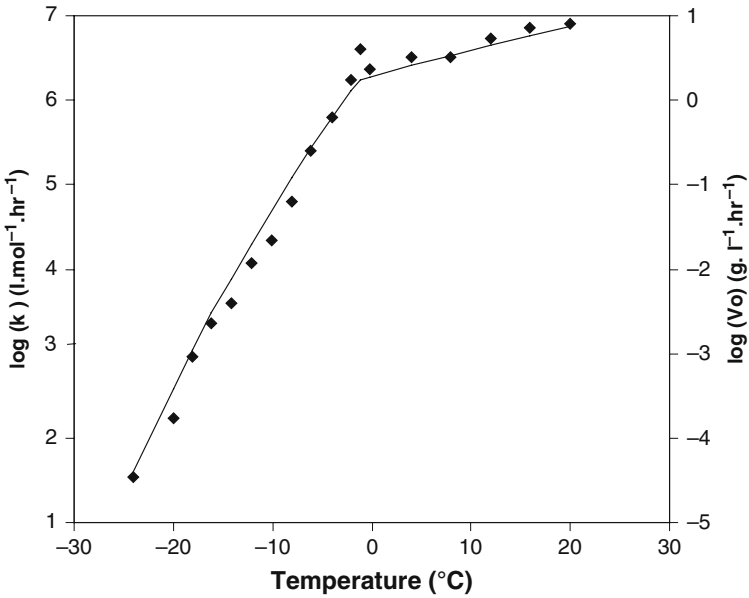

Figure 11.23. Pectin methyl esterase activity in 20% sucrose solutions. Symbols: experimental values of the initial rate from (Terefe and Hendrickx, 2002) (corrected for the variations in concentration in frozen solutions). Solid line: k values predicted from the viscosity of the freeze-concentrated phase calculated from the state diagram (Champion *et al.,* 2004).

11.6. Rehydration of Powders

The rate and extent at which a powder can be redispersed in water is an essential quality attribute of milk powder as well as of milk components used as ingredients in the food industry; this process is commonly considered as comprising several steps: wetting, sinking, dispersion and dissolution (Ennis *et al*., 1998). These steps were characterized for native phosphocaseinate by a combination of viscosity and particle size measurements (Figure 11.24) (Gaiani *et al*., 2006). The behaviour of dairy powders during these successive steps is dependent on composition, processing parameters before and during drying, storage conditions, pH and mineral composition of the dispersion medium. We will review here only the effects of processing and storage events that are water dependent.

Wettability and sinkability are influenced mainly by collapse, caking and crystallization of lactose. The strong decrease in the dispersibility of skim milk powder after 6 months' storage was attributed to the collapse of particle structure. The decrease in the particle surface area results in a lower contact

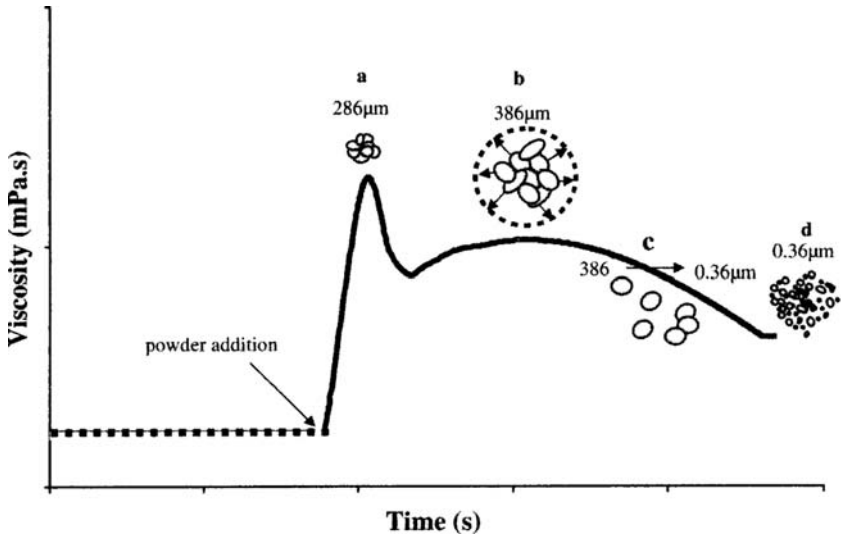

Figure 11.24. Model proposed for the dissolution of native phosphocaseinate based on viscosity and particle size distributions: a: powder wetting, b: swelling, c: powder disintegration, d: dispersion of casein micelles (size 0.36 μm) (Gaiani *et al.*, 2006).

area with water and the decrease in porosity restricts the penetration of water (Yetismeyen and Deveci, 2000). On the contrary, controlled agglomeration was found to improve significantly the rehydration time of whey protein powder and to slow down the rehydration of casein powder, these opposite effects being related to the rate-controlling stage: i.e. wetting step for whey proteins and dispersion step for casein (Gaiani *et al.*, 2007a).

Crystallization of the lactose results in the release of encapsulated fat, which is then spread on the particle surface and impairs the wettability of milk powders (Faldt and Bergenstahl, 1996). The wetting time of native phosphocaseinate powder increased markedly due to the migration of lipids, including polar lipids, to the particle surface during storage (Gaiani *et al.*, 2007b). Solubility also appears to be impaired by lactose crystallization; protein structure is maintained during drying, thanks to the protective action of lactose. Crystallization of the latter during storage, however, induces the destabilization of proteins and their aggregation (Schebor *et al.*, 1997; Yetismeyen and Deveci, 2000). Solubility seems to be particularly affected during storage by non-enzymatic browning and oxidation reactions. Contrasting with studies on model systems (Labuza, 1971), which had shown an oxidation rate minimum at $a_w \approx 0.30$–0.35, the rate of autoxidation in whole milk powder was reported to increase steadily with increasing a_w between 0.11 and 0.33. The concomitant

increase in cross-linking of whey proteins and development of non-enzymatic browning were attributed to the reaction of autoxidation products (free radicals) with amino groups in proteins, explaining the insolubility arising in milk powders during long-term storage (Stapelfeldt *et al.*, 1997). The decrease in the solubility of proteins in whey protein powders was shown to be caused mainly by the formation of intermolecular disulphide bonds, under conditions where a decrease in T_g results from hydrolysis of the proteins and increase of water content of the powder during storage (Zhou and Labuza, 2007).

11.7. Conclusion

In dairy products, as in foods in general, the implications of water are multiple. Water activity and glass transition are the two concepts that are most commonly referred to for explaining the role of water in many aspects of the product characteristics and behaviour during processing and storage. The relevance of both approaches, thermodynamics and material science, has been strongly questioned. They currently appear, however, as forming a coherent knowledge base in "Food Material Science" (Karel, 1999). According to this author, "Perhaps the only concept in food technology with equal or greater impact was the development of heat processing criteria based on heat transfer parameters combined with kinetic equations relating rates of thermal inactivation of microorganisms to temperature". The applicability and limits of both approaches are being recognized. Their respective relevance is limited to water content/temperature domains depending on the product and on the objectives (ISOPOW, 2000). Moreover, whereas the usefulness of these concepts is to provide guidelines for product and processing design, abusive generalization must be avoided. Examples may be, concerning a_w, the need to consider specific solute effects or additional effects due to molecular mobility. Concerning the glass transition-associated concepts, WLF kinetics should be applied only when conditions are fulfilled (i.e. constant concentration). It should also be remembered that the molecular mobility of all constituents does not always obey WLF kinetics or that other parameters than mobility may play a role in reaction kinetics. One must remark that water retention, which is of particular importance for dairy products, cannot be predicted based on a_w or water dynamics. Water interactions with other constituents have only an indirect role on this characteristic, which is controlled mainly by the product structure (ISOPOW, 2000).

Many questions remain to be answered. Properties of water itself are not fully understood, even though the "anomalies" in its physical properties do not mean that water behaviour is unique when compared to other liquids (Angell *et al.*, 2000). The situations in which the properties of water are the

less well known and the most controversial are low temperature and low dimensionality, two domains that are of much interest to food scientists. It seems that water in reduced spaces "may be an even more extraordinary substance than it is under normal conditions" (Angell, 2001). This should be relevant to water in living cells, but may be important also for water in dairy products in any situation, as water may be confined in reduced space in structured macromolecular systems (gels, emulsions), as well as in frozen or low-water-content products.

The manufacture and ageing of cheese provide plenty of examples of the importance of water relationships in food and of the problems resulting from the heterogeneity of food structures and composition. The structures established by the coagulation/gelation step, which will control the process of water syneresis/retention, depend on the complex interactions of water with macromolecular constituents, as influenced by pH, minerals, temperature, etc. In the salting step, the diffusion of salt and water implies complex modelling operation taking into account the influence of temperature, chemical composition of solids and fat content (Geurts *et al*., 1980; Hardy, 1983). Water relationships still play a very important role during the ripening period and storage until consumption. a_w changes as influenced by evaporation, salt migration and microbial/biochemical actions. It also shows the persisting distribution heterogeneity (Hardy, 1983; Ruegg, 1985; Desobry and Hardy, 1994; Saurel *et al*., 2004). The distribution of water, on various scales, was recognized as a most important issue in many food technology situations during the ISOPOW 2000 (Karel and Reid, 2000). This is particularly true for cheese. The lack of stability of low-moisture products, however, might also result from the existence of higher water content regions on the microscopic or sub-microscopic scale. Thanks to the rapidly developing of micro- and nano-scale techniques for the study of food structures (ISOPOW, 2006), the distribution of water in dairy products in connection with stability and texture will probably receive a great deal of attention in the near future.

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12

Fat-Soluble Vitamins and Vitamin C in Milk and Milk Products

P.A. Morrissey and T.R. Hill

12.1. Introduction

Vitamins are a heterogeneous group of organic substances that are present in our natural foods, that are of very high biological potency and that are required in extremely small concentrations for growth and maintenance of normal cells and body function. They need to be supplied in the diet, because the body either cannot make them or cannot do so in amounts that are essential for growth, maintenance and normal and body function. Vitamin families are chemically heterogeneous and are generally classified according to their physical properties, i.e. as being either fat soluble, or water soluble. The fat-soluble vitamins tend to have predominately aromatic or aliphatic character, whereas the water-soluble vitamins tend to have one or more polar or ionizable groups (carboxyl, keto, hydroxyl, amino or phosphate).

The first half of the twentieth century saw the identification, extraction and purification of many vitamins. The synthesis and production of these essential nutrients soon followed and cures for many classical nutritional diseases, including scurvy, beriberi, pellagra and rickets, were demonstrated. More recently, several epidemiological studies have examined the association between vitamin intake/status and various chronic diseases, such as cancer, cardiovascular diseases and osteoporosis. Recognition of the prominent role of some micronutrients as antioxidants in preventing free radical-mediated tissue damage has awakened a re-evaluation of vitamin status and has shed new light on the importance of some vitamins in the prevention of some

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chronic diseases and the influence of these nutrients at the cellular and molecular levels. For example, the discovery of vitamin D receptors in over 30 different body tissues suggests that this vitamin has important regulatory effects in tissues outside its classical role in the skeleton. In relation to vitamin intake and vitamin status, emphasis is now increasingly placed on determining dietary recommendations to take account of the amounts of vitamin needed to optimize physiological and mental functions and to minimize the development of degenerative diseases. Milk makes a significant contribution to the intake of vitamins A and D in American children and adults, with a minor contribution to the intake of vitamins C, E and K. For example, milk contributes between 10–30% to vitamin A intake in children and adults. Fortified milk contributes 40–60% of vitamin D intake by toddlers and adults and is thus the most important source of this vitamin in the American diet.

12.2. Vitamin A; Retinol and β-Carotene

12.2.1. Chemistry and Analysis

Vitamin A is a generic term used to designate any compound possessing the biological activity of retinol (Figure 12.1). The term 'retinoids' was designated to include compounds consisting of four isoprenoid units joined in a head-to-tail manner. All retinoids may be derived from a monocyclic parent compound containing five carbon–carbon double bonds and a functional terminal group at the terminus of the acyclic portion. All three basic forms (retinol, retinal and retinoic acid) are found in two variants: with the β-ionone nucleus (vitamin A_1) or the dehydrogenated β-ionone nucleus (vitamin A_2) with about half the vitamin A activity. The parent retinoid compound, all-*trans*-retinol, is a primary alcohol with a molecular mass of 286 Da. In most animal tissues, the predominant retinoid is retinyl palmitate but other fatty acid esters, such as retinyl oleate and retinyl stearate, are also found. Most of these metabolites occur in the all-*trans* configuration. The 11-*cis*-aldehyde form, 11-*cis*-retinal, is present in the retina of the eye, and several acid forms, such as all-*trans* and 13-*cis*-retinoic acid, are metabolites of retinol found in many tissues. Carotenoids may contribute significant vitamin A activity to foods of both animal and plant origins. Of the estimated 500 known carotenoids, ~50 exhibit some pro-vitamin activity (i.e. are partially converted to vitamin A in vivo).

For a compound to have vitamin A or pro-vitamin A activity, it must exhibit certain structural similarities to retinol, including (a) at least one intact monooxygenated β-ionone ring and (b) an isoprenoid side chain terminating in an alcohol, aldehyde or carboxyl functional group (Figure 12.1).

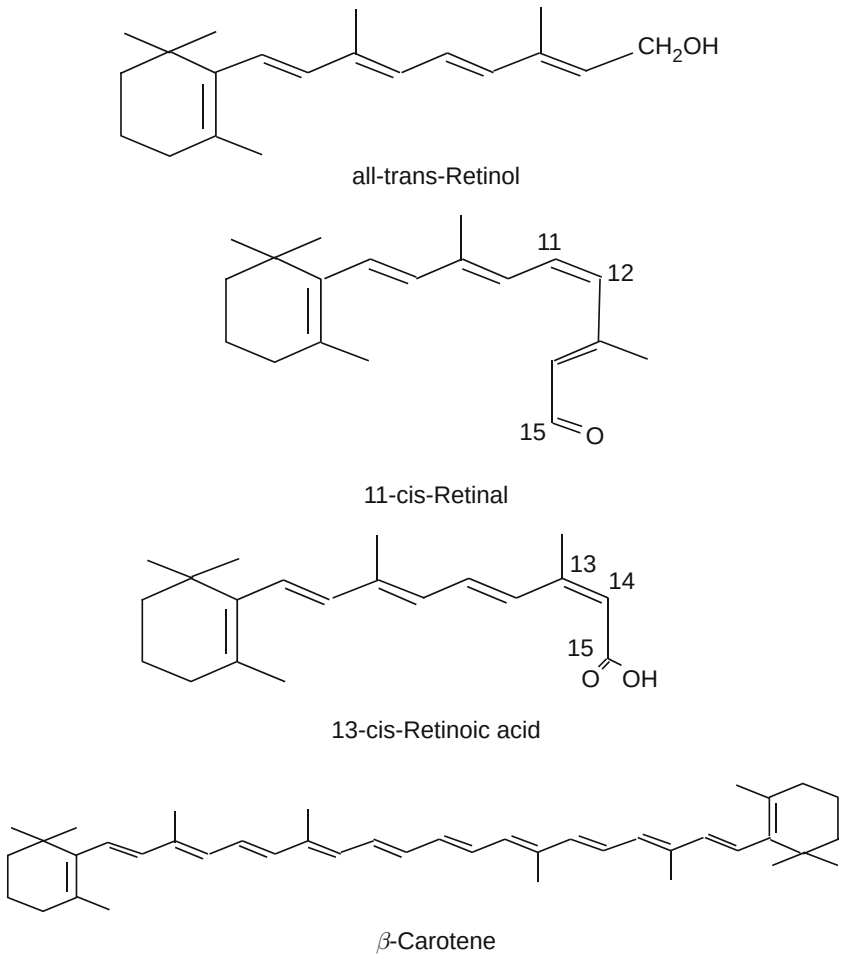


Figure 12.1. Structures of some common retenoids and β-carotene.

The vitamin A-active carotenoids such as β-carotene (Figure 12.1) are considered to have pro-vitamin A activity until they undergo oxidative enzymatic cleavage of the central C15:C15' bond in the intestinal mucosa to yield two molecules of retinal, which can either be reduced to retinol or oxidized to retinoic acid. Carotenoids with ring hydroxylation or the presence of a carbonyl group exhibit less pro-vitamin A activity than β-carotene if only one ring is affected and have no activity if both rings are oxygenated.

Until recently, the term 'retinol equivalents' (REs) was used to convert all sources of preformed retinol and pro-vitamin A carotenoids in the diet into

a single unit (NRC, 1989). Nutritionally, 1 μg RE = 1 μg of all-*trans*-retinol = 2 μg of supplemental (in oil) all-*trans*-β-carotene = 6 μg of dietary all-*trans*-β-carotene = 12 μg of other dietary pro-vitamin A carotenoids. When defining RE, it was assumed that the efficiency of absorption of pro-vitamin A carotenoids was relatively good. Recent studies document, however, that absorption of carotenoids is much lower and appears to be quite variable. In addition, a number of factors such as protein-energy malnutrition, zinc deficiency, dietary fat, alcohol, infections and degree of food processing/food matrix affect the bioavailability and bioconversion of retinol and carotenoids (Parker *et al.*,1999; van het Hoff *et al.*,1999). Based on these and other studies, it is estimated that 1 retinol activity equivalent (μg RAE) is equal to 1 μg of all-*trans*-retinol, 2 μg of supplemental all-*trans*-β-carotene, 12 μg of dietary all-*trans*-β-carotene or 24 μg of other dietary pro-vitamin A carotenoids (e.g. α-carotene, β-cryptoxanthin) (Parker *et al.*, 1999; Food and Nutrition Board, 2001; Trumbo *et al.*, 2001).

Most retinoid and carotenoid analyses have been performed by partition RP-HPLC on octadecylsilane (C18) columns, but C30 columns have proven useful for certain demanding separations of carotenoids (Sander *et al.*, 1994). The most commonly used detectors are UV and visible light absorbance detectors, although fluorescence detectors (retinol and retinyl esters are fluorescent but other retinoids and most carotenoids are not), electrochemical detectors and mass spectrometers are used also (Furr, 2004). Detailed reviews of HPLC analysis of retinoids and carotenoids, including sample preparation techniques, have been published (Barua *et al.*, 2000; Song *et al.*, 2000). A number of analytical methods have been described for the determination of vitamin A in milk and milk-based infant formulae either alone (Strobel *et al.*, 2000; Miyagi *et al.*, 2001) or with other fat-soluble vitamins (see vitamin E section for details).

12.2.2. Absorption, Metabolism and Excretion

Higher animals must obtain vitamin A from the diet, either as the preformed vitamin or as a pro-vitamin carotenoid such as β-carotene. The major dietary forms of preformed vitamin A are long-chain fatty esters of retinol (Plack, 1965). In Western countries, the intake of preformed retinol or retinyl esters typically accounts for 25–75% of the total vitamin A intake; the rest is provided by pro-vitamin A carotenoids (Harrison, 2005). The retinyl esters must be hydrolysed prior to absorption. The presence of dietary fat in the intestine can promote retinyl ester digestion by stimulating the secretion of pancreatic enzymes and bile salts, which serve to form mixed micelles of lipids, and by providing products of lipid digestion (i.e. lysophospholipids, monoglycerides and free fatty acids),

which serve as components of micelles. The dietary retinyl esters are hydrolysed in the intestine by pancreatic triglyceride lipase, and the intestinal brush border enzyme, phospholipase B (Harrison and Hussein, 2001). The unesterified retinol, at physiologic and pharmacologic concentrations, is taken up by the enterocytes, perhaps involving both diffusion and protein-mediated facilitated transport systems (Harrison and Hussein, 2001; Harrison, 2005). Once in the cell, retinol is complexed with cellular retinol-binding protein type II (CRBPII) and the complex serves as a substrate for reesterification of the retinol by the enzyme lechitin:retinol acyltransferase (LRAT) and the retinyl esters are incorporated into chylomicrons (Harrison, 2005; Blomhoff and Blomhoff, 2006). The absorption efficiency of dietary vitamin A in healthy persons who ingest significant amounts of fat (>10 g/day) is normally >80%. After the consumption of carotenoid-containing foods, carotenoids are released from their food matrix and, like retinol, are incorporated into mixed micelles (Yeum and Russell, 2002). The amount of carotenoid incorporated into micelles depends on the polarity of the carotenoid and on micellar fatty acid composition and saturation. The carotenoids, carried in mixed micelles, are absorbed by the mucosa of the small intestine, mainly in the duodenum, via passive diffusion in parallel with fat digestion and absorption, and become packaged into triacylglycerol-rich chylomicrons (Parker, 1996). The absorption efficiency, or bioavailability, of dietary carotenoids can vary from approximately 60 to <5% (Bender, 2003), and it appears that the human intestine has only a limited capacity to take up carotenoids from the diet (Thurnham and Northrop-Clewes, 1999). A number of factors affect carotenoid absorption, including the specific carotenoid, its isomeric form, the amount ingested, the presence of accompanying fat, the particle size of the ingested food and the integrity of the gut (Castenmiller and West, 1998; Yeum and Russell, 2002).

Pro-vitamin A carotenoids, β-carotene, α-carotene and cryptoxanthin are partly converted to vitamin A in the intestinal mucosa. The key step is the oxidative central cleavage of β-carotene at the central 15:15' double bond by β-carotene 15,15'-monooxygenase (Nagao, 2004). The retinal formed from carotenoids is reduced to retinol and further converted to retinyl ester by retinal reductase and LRAT with the aid of CRBP-II and thereafter incorporated into chylomicrons (Ong, 1993). The extent of conversion of a highly bioavailable source of β-carotene to vitamin A in humans is considered to be between 60 and 75%, with an additional 15% of β-carotene absorbed intact.

The majority of the retinyl esters formed from vitamin A and carotenoids and small amounts of retinol and carotenoids are incorporated into chylomicrons which consist of molecules of triacylglycerols and

phospholipids packed together in a characteristic manner with cholesteryl esters and some specific apolipoproteins (Harrison, 2005; Blomhoff and Blomhoff, 2006). These lipoprotein complexes are secreted into the lymph where much of the triacylglycerols are hydrolysed by plasma lipoprotein lipase, leaving remnants that contain most of the newly formed retinyl ester, retinol and carotenoids (Harrison, 2005). The chylomicron remnants are taken up rapidly by the liver and almost quantitatively (50–80%), which is the main site of storage of total body retinol (retinol plus retinyl esters) (Biesalski and Nohr, 2004; Blomhoff and Blomhoff, 2006). The carotenoids do not accumulate in liver cells, but are mobilized as components of very low density lipoprotein (VLDL) particles that are converted to VLDL remnants and low-density lipoprotein (LDL) in the circulation (Parker, 1996).

To meet tissue needs for retinoids, the liver secretes retinol bound to an α-globulin, retinol-binding protein (RBP); this serves to maintain the vitamin in aqueous solution, protects it against oxidation and delivers the vitamin to target tissues. Approximately 95% of the retinol-RBP is associated (1:1) with the thyroxine-binding prealbumin, transthyretin (TTR). This complex is important to prevent urinary loss of retinol bound to the relatively small RBP (Zanotti and Berni, 2004).

The plasma concentration of retinol-RBP is strictly regulated and maintained at about 2 μmol/l, despite fluctuations (varies by >15-fold) in daily intake of vitamin A (Olson, 1984). Only in severe vitamin A deficiency, i.e. when liver retinyl esters are depleted, is the concentration of plasma retinol-RBPreduced (Blomhoff and Blomhoff, 2006). Kinetic studies show that each molecule of retinol circulates several times between plasma and liver before undergoing irreversible disposal (Green and Green, 2003; Cifelli *et al*., 2005). In humans, ~50 μmol/day (14.3 mg/day) of retinol passes through plasma, compared with an estimated disposal rate of 4 μmol/day (1.14 mg/day) (Von Reinersdorff *et al*., 1998). Therefore, a large portion of retinol taken up by most cell types in the body is recycled to plasma and only a minor portion is converted to active metabolites or is degraded. Ross and Zolfaghari (2004) concluded that recycling provides an ideal means for the liver to sample constantly and adjust the concentration of retinol available in plasma for peripheral tissues.

The main excretory product of both retinol and retinoic acid is retinoyl glucuronide, which is secreted in the bile (Bender, 2003). Some products of side-chain oxidation of retinoic acid are also excreted in the urine. As the concentration of retinol intake increases, a microsomal cytochrome P_{450}-dependent oxidation process is activated, leading to a number of polar metabolites, including 4-hydroxyretinol, which is excreted in the urine and bile. At high intakes, this pathway becomes saturated and excess retinol is toxic since there is no further capacity for its catabolism and excretion (Bender, 2003).

12.2.3. Functions of Vitamin A and β-Carotene

Vitamin A, its analogues and its metabolites function in vision, cell differentiation, embryogenesis, the immune response, reproduction and growth. Carotenoids also have a variety of different actions, including possible antioxidant activity, immunoenhancement, inhibition of mutagenesis and transformation and reduced risk of age-related macular degeneration and cataracts, decreased risks of some cancers and decreased risk of cardiovascular events.

12.2.3.1. Vitamin A and Vision

The pathway for the delivery of vitamin A to the eye involves isomerization of all-*trans*-retinol to 11-*cis*-retinol and oxidation to 11-*cis*-retinal, which then reacts with a specific lysine group in the membrane-bound protein, opsin, forming the holoprotein, rhodopsin (Saari, 1994). Vision is initiated by the photochemical *cis*-to-*trans* isomerization of the 11-*cis*-retinal Schiff base chromophore of rhodopsin to all-*trans*-retinal and a conformational change in opsin (Rando, 1994). This results in the release of retinal from the protein and the initiation of a nerve impulse. The all-*trans*-retinal released from rhodopsin is reduced to all-*trans*-retinol and joins the pool of retinol in the pigment epithelium for isomerization to 11-*cis*-retinol and regeneration of rhodopsin. Overall, the key to initiation of the visual cycle is the availability of 11-*cis*-retinal, and hence vitamin A.

12.2.3.2. Physiological Functions of Retinoic Acid

A second major function of vitamin A in the body is in the control of cell differentiation and turnover. The discovery of two sets of retinoic acid receptors, RAR and RXR, has clarified in large part the molecular action of vitamin A in the above processes (Chambon, 1996, 2004). The RAR receptors bind either all-*trans*- or 9-*cis*-retinoic acid, whereas the RXR receptors bind only 9-*cis*-retinoic acid (Soprano *et al*., 2004; Balmer and Blomhoff, 2005). The RXR receptors form homodimers with themselves as well as heterodimers with RAR, the vitamin D receptor, thyroid hormone, long-chain polyunsaturated fatty acid derivatives and several other transcription factors (Blomhoff and Blomhoff, 2006). Retinoic acid is a key factor in the development of different vertebrate tissues and organs, due to its ability to promote differentiation (Spinella *et al*., 2003) and regulate apoptosis (Jimenez-Lara *et al*., 2004). The overall result is that a large number of genes are sensitive to control by retinoic acid in different tissues and at different stages of development. Numerous studies have elucidated the important role of

retinoic acid in many physiological processes, including lens development and regeneration, lung development and regeneration, development of the central nervous system, reproduction, as well as conditions such as cancer, skin disease, premature birth, rheumatoid arthritis and osteoporosis (Blomhoff and Blomhoff, 2006). Retinoic acid also has a number of functions in the developing and mature central nervous system (Vegara *et al.*, 2005).

12.2.3.3. Antioxidant Activity of Vitamin A and Carotenoids

The antioxidant activity of vitamin A and carotenoids is conferred by the hydrophobic chain of polyene units that can quench singlet oxygen, neutralize thiyl radicals and combine with and stabilize peroxyl radicals (Palace *et al.*, 1999). Retinol is an effective peroxyl radical scavenger only when the initiating radical species are presented from within the lipid bilayer and not when they originate from the aqueous environment. Carotenoids are the most efficient naturally occurring quenchers of singlet oxygen. The rate constant for singlet oxygen scavenging by carotenoids is in the order of 10^{10} $mol^{-1}s^{-1}$ and is even greater than that of α-tocopherol (10^8 $mol^{-1}s^{-1}$) (Ojima *et al.*, 1993). It has been proposed that β-carotene scavenges peroxyl ($LOO^{\bullet}$) radicals and forms a complex between β-carotene and $LOO^{\cdot}$, yielding a resonance-stabilized structure or carotenoid radical that effectively terminates the lipid peroxidation process (Burton and Ingold, 1984). There is increasing evidence that both antioxidant and/or prooxidant mechanisms are exerted by β-carotene in biological systems. The balance between prooxidant and antioxidant behaviour is very delicate and the antioxidant potential is more pronounced at low oxygen partial pressure ($<$150 Torr O_2) (Burton, 1989). On the other hand, at high, non-physiologic oxygen tension and relatively high concentrations ($>$500 μM), β-carotene loses its antioxidant potency and exhibits prooxidant activity (Zhang and Omaya, 2000, 2001). Krinsky (2001) concluded that the antioxidant activity of β-carotene and other carotenoids may shift to a prooxidant mode depending on the redox potential of the carotenoid and on the biological environment.

12.2.4. Vitamin A and Carotenoids in Milk

Vitamin A is present in cow's milk as retinol, retinyl esters and carotenes. The mean content of vitamin A and β-carotene in cow's milk is 40 μg/100 g (range 10–100) and 20 μg (range 3–50), respectively (Walstra and Jenness, 1984; Renner *et al.*, 1989). The retinol content averages 16.3, 32.6 and 52.2 and that of β-carotene 9.6, 16.7 and 3.0 μg/100 g in milk (1.9% fat), milk (3.9% fat) and human milk, respectively (Ollilainen *et al.*, 1989). The

levels of the fat-soluble vitamins and β-carotene in milk are highly dependent on the amount consumed in the feed (Jensen *et al*., 1999). Vitamin A and β-carotene concentrations of milk follow a seasonal trend with higher values obtained during the outdoor grazing period (O'Brien *et al*.,1999). Higher concentrations of vitamin A and β-carotene are present in retail milk during both the outdoor grazing (June–October) and indoor feeding (December to March) periods compared to manufacturing milk, which reflected the higher feed concentrate input in retail milk production (O'Brien *et al*., 1999). Seasonal variation has also been observed by Hulshof *et al*. (2006), who observed that winter milk contains 20% less retinol and β-carotene than summer milk. Higher amounts of retinyl esters are found in colostrum (233–369 μg/100 ml) than in mature milk (33–57 μg/100 ml) (Debier *et al*., 2005). The age of the cow also appears to exert an effect on the concentration of vitamin A in colostrum and milk. In cows, primiparous females exhibit significantly higher vitamin A concentrations in plasma, colostrum and milk than multiparous females (Kumagai *et al*., 2001).

Kim *et al*. (1990) reported that the retinol concentration (mean $\pm$ SD) in human milk is 57 ± 25 μg/100 g, and carotenoid concentrations (μg/100 g) are 4.6 ± 1.6 for β-carotene, 3.2 ± 0.9 for α-carotene, 3.8 ± 2.1 for lycopene and 11.5 ± 3.4 for lutein. Meneses and Trugo (2005) determined the concentrations of retinol, β-carotene and non-pro-vitamin A (lutein + zeaxanthin) carotenoids in mature human milk. Nutrient concentrations (μm/l, mean $\pm$ SE) in milk were as follows: retinol, 1.4 ± 0.1; β-carotene, 0.018 ± 0.002; lutein + zeaxanthin, 0.006 ± 0.001. Similar retinol levels have been described for the milk of well-nourished lactating women (Roy *et al*., 1997; Canfield *et al*., 1998; Rice *et al*., 1999). The milk of multiparous women contained higher levels of retinol than milk of primiparous women. Colostrum samples from human donors showed considerable variation in total carotenoid concentration (34–757 μg/100 ml) (Patton *et al*., 1990). Multiparous mothers had higher mean colostrum carotene concentrations than did primiparae, 218 ± 194 vs 114 ± 132 μg/100 ml, respectively.

Loss of vitamin A activity of retinoids and carotenoids in foods occurs mainly through reactions involving the unsaturated isoprenoid side chain, by either autoxidation or geometric isomerization. The 9-*cis* and 13-*cis* isomers, resulting from all-*trans*-retinol isomerization reactions, have been found in many types of food, including cheese, UHT milk and butter, at different concentrations, depending on the processing and/or storage conditions (Woolard and Indyk, 1986; Fellman *et al*., 1991). Pasteurized milk heated at temperatures ranging from 72 to 76°C for 15 s had an average 13-*cis*: all-*trans* ratio of 6:100 (Panifili *et al*., 1998). Milk subjected to a more severe heat treatment had a higher degree of isomerization (UHT milk, 15.7%; sterilized milk, 33.5%), consistent with increased thermal conversion

of the retinol isomers. Photochemical isomerization of vitamin A compounds occurs both directly and indirectly via a photosensitizer (Pesek and Warthesen, 1990). The ratios and quantities of *cis* isomers produced differ depending on the photoisomerization. The type of packaging material has a significant effect on net retention of vitamin A activity in food exposed to light during storage. Vassila *et al.* (2002) reported vitamin A losses ranging from 15.1 to 73.6% in whole milk stored in various flexible monolayer and multilayer co-extruded pouches held under fluorescent light at 4°C for up to 7 days. Zygoura *et al.* (2004) reported losses of 8.8–50.9%, depending on the packaging material, when pasteurized milk was stored under fluorescent light at 4°C for 7 days.

12.2.5. Vitamin A Status and Requirements

Worldwide, vitamin A deficiency is a major public health problem and the most important preventable cause of blindness. The earliest signs of deficiency are loss of sensitivity to green light, followed by inability to adapt to dim light and finally inability to see in dim light (night blindness). More prolonged or severe deficiency leads to xerophthalmia, which involves keratinization of the cornea, followed by ulceration, which leads to irreversible damage to the eye that causes blindness. In addition to clinical deficiency, more than 200 million children suffer from vitamin A inadequacy in the absence of clinical signs of acute deficiency (WHO, 1996). These children generally show a higher mortality rate and a greater incidence of severe infection than do vitamin A-sufficient children. Symptoms of vitamin A deficiency are associated with a low intake of fat and lipid malabsorption syndrome (Olson *et al.*, 2000). Vitamin A deficiency symptoms also occur in protein-energy deficiency, regardless of whether or not the intake of vitamin A is adequate. This is due to impairment of the synthesis of plasma RBP. Zinc is also required for the synthesis and secretion of RBP and TTR; therefore, zinc deficiency influences the mobilization of vitamin A from the liver and its transport into the circulation (Food and Nutrition Board, 2001).

When adequate retinol is provided in the diet, there are no known clinical effects of consuming diets low in carotenes over the short term (Food and Nutrition Board, 2000). However, carotene-deficient diets were reported to increase various measures of oxidative susceptibility (Dixon *et al.*, 1998; Lin *et al.*, 1998), but this is of uncertain relevance with regard to clinical outcomes.

Current estimates of vitamin A requirements are based on the intake required to maintain a reserve concentration of at least 20 μg retinol/g of liver tissue (Olson, 1987; Food and Nutrition Board, 2001). This concentration is

adequate to maintain normal plasma concentrations of retinol and protect against a vitamin A deficiency for approximately 4 months while the person consumes a vitamin A-deficient diet. The estimated average requirement (EAR) of preformed vitamin A required to assume an adequate body reserve in men >19 years is 625 μg RAE/day, and for women the EAR is 500 μg RAE/day. The recommended dietary allowance (RDA) for vitamin A is set using a coefficient of variation (CV) of 20% and the EAR for adequate stores of vitamin A. The RDA is defined as equal to the EAR plus twice the CV to cover the needs of 97–98% of the individuals in the group. The RDAs for men and women are 900 and 700 μg RAE/day, respectively. In Europe, the Scientific Committee for Food (1993) set a population reference intake (PRI) of 700 and 600 μg retinol equivalent/day for men and women, respectively.

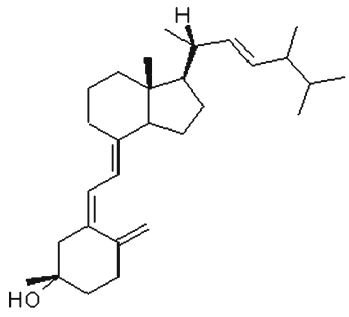
When ingested in large doses, vitamin A can be toxic (Ross, 1999a). Acute toxicity is produced by one or several closely spaced, very large doses of vitamin A, usually >100 times the recommended intake in adults and >20 times the recommended intake for children (Olson *et al.*, 2000). Chronic toxicity, which arises from ingestion of doses greater than or equal to 30,000 μg/day for months or years, is a major cause for concern (Food and Nutrition Board, 2001). The symptoms of toxicity include headache, vomiting, alopecia, cracking of lips, ataxia and anorexia. Permanent damage to liver, bone and vision, as well as chronic muscular and skeletal pain, may result (Ross, 1999a). Chronic excessive vitamin A intake has been shown to lead to bone mineral loss in animals (Rohde *et al.*, 1999) and may also be associated with osteoporosis and increased risk of hip fracture in humans with intakes of 1.5–2.0 mg of retinol/day (Promislow *et al.*, 2002; Penniston and Tanumihardjo, 2003, 2006). Concern for the possible teratogenicity of high vitamin A intake in humans is based on the unequivocal demonstration of human teratogenicity of 13-*cis*-retinoic acid (Eckhoff *et al.*, 1991; Armstrong *et al.*, 1994). The most serious teratogenic effects of vitamin A include fetal resorption, abortion, birth defects and permanent learning disabilities in the progeny.

No adverse effects other than carotenodermia, which is characterized by a yellowish discolouration of the skin, have been reported from the consumption of β-carotene or other carotenoids in foods (Food and Nutrition Board, 2000). In addition, long-term supplementation with β-carotene to persons with adequate vitamin A status does not increase the concentration of serum retinol (Nierenberg *et al.*, 1997). The tolerable upper limit (UL) for vitamin A for men and women $\geq$19 years is set at 3,000 μg/day of preformed vitamin A (Food and Nutrition Board, 2001). A UL has not been set for β-carotene or other carotenoids.

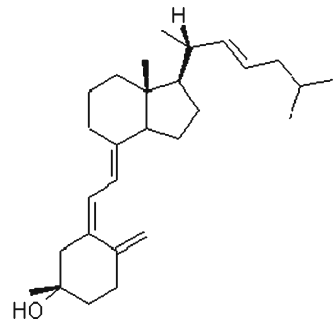
12.3. Vitamin D

12.3.1. Chemistry and Analysis

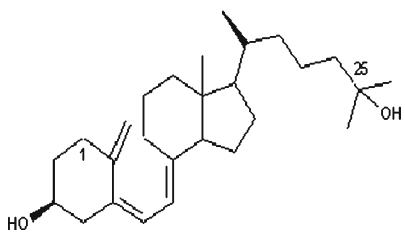
The term 'vitamin D' was given during the early 1920s to a group of closely related secosteroids with antirachitic properties. The two major dietary forms of vitamin D in foods are chole-calciferol (vitamin D_3, derived from animals) and ergo-calciferol (vitamin D_2, derived from plants) (Figure 12.2). Both chole- and ergo-calciferol are also formed by photoirradiation from their precursors 7-dehydrocholesterol and ergosterol in vetebrates and some fungi, respectively. The chemical structures of vitamin D_2 and vitamin D_3 differ only in their side chain at C-17 which in vitamin D_2 has a double bond and an additional methyl group.



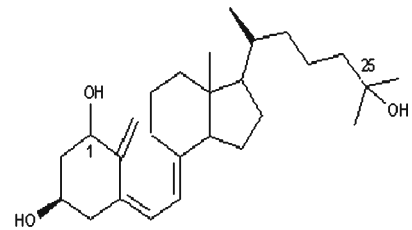
Ergocalciferol (Vitamin D_2)



Cholecalciferol (Vitamin D_3)



25-hydroxyvitamin D_3 (Calcidiol)



1,25-dihydroxyvitamin D_3 (Calcitriol)

Figure 12.2. Structures of the major metabolites of vitamin D.

Unlike the precursors of vitamin D compounds (i.e. ergosterol and 7-dehydrocholesterol) which have four intact rings, vitamin D compounds have only three intact rings; one ring has undergone fission resulting in the conjugated *cis*-triene system of double bonds, thus forming a group of 9,10-secosteroids. This system gives rise to the characteristic UV-absorption spectrum of vitamin D compounds (molar extinction coefficient, $\varepsilon = 18300$ M/cm, $\lambda_{max} = 265$ and $\lambda_{min} = 288$ nm). It is noteworthy that unlike the vitamin D compounds, the pro-vitamins, ergosterol and 7-dehydrocholesterol show no antirachitic activity.

About 50 different metabolites of vitamin D_2 and D_3 have been isolated and identified (Singh, 1985; Ikekawa and Ishizuka, 1992). Nomenclature of the various vitamin D metabolites is by indicating the number and location of the additional hydroxyl groups added to the parent chole- or ergo-calciferol structure: e.g. 25-hydroxycholecalciferol, abbreviated as $25(OH)D_3$, indicates that one additional hydroxyl group has been added to chole-calciferol at the C-25 position and 1,25-dihydroxycholecalciferol, abbreviated as $1,25(OH)_2D_3$, indicates that two hydroxyl groups have been added to chole-calciferol at the C-1 and C-25 positions. The various vitamin D compounds found in foods include chole- and ergo-calciferol, their pro- and pre-vitamins and their hydroxylated metabolites. The determination of these in foods provides a good estimate of the total vitamin D content of foods (Mattila *et al.,* 1996). Previous analytical methods based on biological assays were unable to distinguish between different vitamin D compounds and were laborious and very costly. High-performance liquid chromatography (HPLC) with UV detection is now the most widely used analytical method for measuring vitamin D compounds in foods because of its reliability, ease and ability to separate the various vitamin D compounds (Takeuchi *et al.,* 1993; Mattila, 1995).

Older HPLC methods only characterized ergo- and chole-calciferol but recent advances in analytical methodology can separate and characterize the 25-hydroxyvitamin D compounds in addition to ergo- and chole-calciferol. For example, the ability to detect $25(OH)D_3$, especially in meat products, from the mid-1990s produced large amounts of data on the vitamin D content of meat which was previously unavailable (Mawer and Gomes, 1994; Mattila, 1995; Clausen *et al.,* 2002; Ovesen *et al.,* 2003a). This is of importance because $25(OH)D_3$ is absorbed better and more rapidly than native vitamin D_3 and it may be up to 5 times more active (Ovesen *et al.,* 2003a). Unfortunately, to date, no reliable HPLC method has been developed which is capable of quantifying 1,25-dihydroxyvitamin D compounds due to the fact that these compounds are present in very low amounts. When estimating the total vitamin D content of foods, it is important to note the varying antirachitic activity of the various vitamin D compounds. For example, it is assumed that vitamin D_2 and D_3 are equal, whereas 25(OH)D is assumed to have 5 times

the antirachitic activity of either vitamin D_2 or D_3 (Ovesen *et al.*, 2003a). The vitamin D content of foods is usually described in μg or international units (IU), where 1 μg = 40 IU.

12.3.2. Vitamin D Synthesis in the Skin

In vertebrates, the cholesterol-like precursor, 7-dehydrocholesterol, present in the skin epidermis, undergoes photolysis when exposed to UVB light of wavelength 290–315 nm to yield a variety of photoirradiation products, including tachysterol, lumisterol and pre-vitamin D_3. Pre-vitamin D_3 then undergoes spontaneous thermal rearrangement to vitamin D_3. The fate of ergo-calciferol in plants and lower organisms such as fungi follows a similar pathway to chole-calciferol. Because of the skin's ability to synthesize the vitamin upon exposure to appropriate sunlight, vitamin D is an essential nutrient only when sunlight is limited. For this reason, some scientists consider vitamin D to be as much a hormone as a nutrient (Singh, 1985; Ball, 1992; Park, 1997).

As a consequence of dermal synthesis upon exposure to UVB radiation, serum levels of vitamin D in Northern populations exhibit a clearly evident seasonal variation, with highest levels at the end of summer (August–September) and lowest levels at the end of winter (February–March) (Stamp and Round, 1974; van der Wielen *et al.,* 1995; and for reviews, see Lips, 2001; Ovesen *et al.,* 2003b; Zittermann, 2003). It has been suggested that in northern regions at a latitude of 40° N such as Boston, the skin is incapable of synthesizing vitamin D from November to March (Webb *et al.,* 1988). For regions >50° N, such as Edmonton, this period extends from October to March. At higher latitudes during winter, sunlight must pass a much longer distance through the atmosphere and most UVB light is absorbed, thus preventing any effective UV radiation from reaching the skin (Holick, 2004).

12.3.3. Absorption, Metabolism and Excretion

Dietary vitamin D is absorbed from the jejunum and ileum and, like other fat-soluble vitamins, is transported in chylomicrons and lipoproteins via the lymphatic system (Endres and Rude, 1994). Absorption is by non-saturable passive diffusion and the efficiency of absorption is estimated to be 60–90% (Van den Berg, 1997). Vitamin D absorption efficiency is unaffected by vitamin D status (Fraser, 1983). Vitamin D absorption has been suggested to be facilitated by long-chain fatty acids (Holmberg *et al.,* 1990), while others suggest that the elimination of vitamin D from the gastrointestinal lumen is increased by a high fibre diet (Batchelor and Compston, 1983).

Vitamin D_3 (obtained from dermal synthesis or from dietary sources), which is biologically inactive, is transported to the liver where it is hydroxylated at the C-25 position to yield 25-hydroxyvitamin D_3 [25(OH)D_3 or calcidiol], the major circulating form of vitamin D and the most commonly used index of vitamin D status (van der Wielen *et al.,* 1995). The enzyme, 25-hydroxylase, regulates the 25-hydroxylation of vitamin D_3 to produce 25(OH)D_3. This enzymatic conversion is dependent on the concentration of vitamin D_3 in serum/plasma. From the liver, 25(OH)D_3 is returned to the circulation, bound to vitamin D-binding protein (DBP) and transported to the kidneys where the enzyme 1-α-hydroxylase converts it to 1,25-dihydroxycholecalciferol (1,25$(OH)_2D_3$ or calcitriol), which is the major active metabolite of vitamin D. 1,25$(OH)_2D_3$ is estimated to be 500–1000 times more active than 25(OH)D_3 (Henry and Norman, 1984; Reichel *et al.,* 1989). It has been estimated that the biological half-lives of 25(OH)D_3 and 1,25$(OH)_2D_3$ are 10–20 days (Vicchio *et al.,* 1993) and 4–6 h (Haddad and Rojanasathit, 1976; Clements *et al.,* 1992), respectively, which partly explains why 25(OH)D_3 is a better indicator of vitamin D status than 1,25$(OH)_2D_3$. Furthermore, the plasma level of 1,25$(OH)_2D_3$ is under homeostatic control, which further limits its value as a status marker (Holick, 1995). When 1,25$(OH)_2D_3$ is in excess, the enzyme 24-hydroxylase in the kidney converts 25(OH)D_3 to 24,25-dihydroxycholecalciferol, which is biologically inactive. Furthermore, 25(OH)D_3 can be converted to other inactive metabolites such as 23,25-dihydroxycholecalciferol, 25,26-dihydroxycholecalciferol and 1,24,25-trihydroxycholecalciferol and excreted mainly in faeces, but the biological roles of these metabolites are not well understood (for reviews, see Horst and Reinhardt, 1997, and Holick, 2003).

12.3.4. Role of Vitamin D in Bone Metabolism

The major biological role of 1,25$(OH)_2D_3$ is to promote intestinal calcium absorption. In addition, 1,25$(OH)_2D_3$ increases the absorption of other essential minerals across the intestine, such as phosphorus, zinc and manganese (Biehl *et al.,* 1995), and enhances the net renal reabsorption of calcium and phosphorus (Singh and Dash, 1997). Thus, 1,25$(OH)_2D_3$ is a major regulator of calcium homeostasis. The classical target organs for 1,25 $(OH)_2D_3$ are the intestine, bone, the kidneys and the parathyroid glands. Normal physiological concentrations of calcium are required for proper neuromuscular and cellular functions. Low serum calcium (hypocalcaemia) stimulates the secretion of parathyroid hormone (PTH) from the parathyroid gland, which, in turn, enhances the conversion of 25(OH)D_3 to 1,25$(OH)_2D_3$. 1,25$(OH)_2D_3$ acts on the intestine, kidneys and bone to restore normal serum calcium concentrations. In addition to PTH, it is also well recognized that

other hormones, such as calcitonin, glucocorticoids, growth hormones and sex steroids, regulate the production of $1,25(OH)_2D_3$ (Lal *et al.,* 1999). The biological actions of $1,25(OH)_2D_3$ in target tissues are mediated through either (i) a nuclear vitamin D receptor (VDR), which, once complexed with $1,25(OH)_2D_3$ and retinoic acid receptors (RXR), can regulate gene expression (genomic effects) or (ii) intra-cellular signalling pathways activated through putative plasma membrane receptors (non-genomic effects).

12.3.5. Vitamin D and Bone Disease

It is well known that severe vitamin D deficiency causes rickets in children and osteomalacia in adults, processes in which the bone matrix (osteoid) fails to mineralize (Parfitt *et al.,* 1982). In addition, prolonged vitamin D deficiency has been implicated in the pathogenesis of senile osteoporosis (Parfitt *et al.,* 1982). Osteoporosis has been defined as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Concensus Development Conference, 1993). While the aetiology of bone loss is multifactorial, it is believed that secondary hyperparathyroidism as a result of mild vitamin D deficiency is a significant contributing factor (Dawson-Hughes *et al.,* 1991; Lips, 2001).

Seasonal changes in bone mineral content (BMC) (Hyldstrup *et al.,* 1986) and bone mineral density (BMD) (Rosen *et al.,* 1994; Rapuri *et al.,* 2002; Meier *et al.,* 2004) have been reported in both adults and elderly subjects in some studies, but not others (Overgaard *et al.,* 1988; Thomsen *et al.,* 1989; Tsai *et al.,* 1997). Some studies appear to demonstrate a positive association between serum 25(OH)D and BMD (Ooms *et al.,* 1995; Scharla *et al.,* 1996; Sahota *et al.,* 1999; Mezquita-Raya *et al.,* 2001; Rapuri *et al.,* 2002). Furthermore, the association between serum 25(OH)D and BMD appears to be stronger for cortical-rich sites, such as the femoral neck, than for trabecular-rich sites such as the trochanter (Ooms *et al.,* 1995). In German women, aged 50–80 years, significant positive correlations were found between serum 25(OH)D and BMD of the proximal femur during both winter and summer (Scharla *et al.,* 1996). BMD of the lumbar spine has also been positively associated with serum 25(OH)D in healthy elderly (Rapuri *et al.,* 2002) and osteoporotic subjects (Mezquita-Raya *et al.,* 2001). In studies of healthy elderly women, a serum 25(OH)D level <30 nmol/l was associated with significantly lower BMD at the hip (Sahota *et al.,* 1999) and the femoral neck (Ooms *et al.,* 1995) compared to that of women with serum 25(OH)D levels >30 nmol/l. In adolescent girls, Outila *et al.* (2001) showed that serum 25(OH)D levels ≥ 40 nmol/l are associated with lower radius forearm BMD.

12.3.6. Extra-Skeletal Roles of Vitamin D

Over 30 different tissues possess receptors for $1,25(OH)_2D_3$ (Lal *et al.,* 1999). In addition to the classical role of $1,25(OH)_2D_3$ on calcium homeostasis, it has important modulatory roles in other organ systems, including the endocrine glands, the immune system, the cardiovascular system and the reproductive and nervous systems (Lal *et al.,* 1999). Therefore, it is not surprising that evidence is accumulating to suggest a role for vitamin D in the pathogenesis of certain chronic diseases such as type 1 diabetes, immune disorders, hypertension, certain cancers and cardiovascular disease (Zittermann, 2003; Holick, 2004). Moreover, a serum 25(OH)D level <50 nmol/l has been associated with the development of these chronic diseases (Zittermann, 2003). Some intervention trials have shown that vitamin D supplementation may (i) reduce blood pressure in hypertensive patients, (ii) improve glucose tolerance in diabetics and (iii) improve symptoms of rheumatoid arthritis and multiple sclerosis (for review, see Zittermann, 2003).

12.3.7. Vitamin D in Milk

Vitamin D is naturally present (mainly as vitamin D_3) in only a few foods, such as oily fish, meat, egg yolk and milk, and the level present in such foods can be highly variable. Since animals can synthesize vitamin D and obtain it from their feed; both ergo- and chole-calciferol-based compounds can be found in milk. In general, bovine and human milk are not good sources of vitamin D, containing 0.1–1.5 μg/l (Søndergaard and Leerbeck, 1982; Reeve *et al*., 1982). Both vitamin D_3 and $25(OH)D_3$ have been identified in milk (Mattila, 1995). Milk also contains significant amounts of its water-soluble analogue, vitamin D sulfate, although the biological activity of this compound is low (Hollis *et al*., 1981). The most important determinant of the vitamin D content of milk is sunlight exposure, and seasonal variation in the vitamin D content of cow's milk has been reported, with higher values in summer ($\sim$0.35 μg/l) than in winter ($\sim$0.25 μg/l) (Scott *et al*., 1984b). The antirachitic activity of human milk is variable and is predominately affected by season and maternal vitamin D intake. In addition, the circulating 25(OH)D concentrations in breast-fed infants are related directly to the vitamin D content of the mothers' milk (Cancela *et al*., 1986).

In the United States, nearly all milk is fortified with vitamin D_3 to a level of approximately 10 μg/l (400 IU). In such countries, fortified milk makes a substantial contribution to the mean daily intake of vitamin D. Furthermore, the effect of vitamin D-fortified milk on the serum 25(OH)D level shows marked increases. For example, in a study of healthy adults, aged between 17 and 54 years, the consumption of fortified milk (12 μg vitamin D_3/l) reduced

the seasonal decline in serum 25(OH)D by >50% (McKenna *et al.,* 1995). However, in countries where the fortification of milk is not mandatory, such as the United Kingdom and Ireland, milk contributes <10% of the mean daily intake of vitamin D (Hill *et al.,* 2004). Stability studies show that on exposure to light, there is slight loss of vitamin D_3 from fortified milk (Renken and Warthesen, 1993), although this is unlikely to be caused during normal handling and storage of milk. Air exposure does not affect stability in milk (Renken and Warthesen, 1993).

12.3.8. Vitamin D Status and Requirements

12.3.8.1. Measurement of Vitamin D Status

Serum or plasma 25(OH)D [including 25(OH)D_2 and 25(OH)D_3] is considered to be the best index of vitamin D nutritional status because it closely reflects the amount produced in the skin and ingested in the diet (Holick, 1990a; Parfitt, 1998). In addition, measurement of 25(OH)D is used routinely for the detection of vitamin D deficiency (Preece *et al.,* 1975; Holick, 1996). Several analytical techniques have been used to measure 25(OH)D in serum or plasma, such as HPLC (Shepard *et al.,* 1979; Bouillon *et al.,* 1984; Hollis and Frank, 1985), radioimmunoassay (RIA) (Hummer *et al.,* 1984; Hollis and Napoli, 1985; Hollis *et al.,* 1993), competitive protein binding (CPB) assay (Belsey *et al.,* 1971; Bouillon *et al.,* 1976) and recently developed enzyme immunoassays (EIA) (Lind *et al.,* 1997). However, many of these techniques can give quite different results when compared with each other (Lips *et al.,* 1999). There is a need for standardization of laboratory methodology for serum 25(OH)D so that results from various populations can be compared. The establishment of a vitamin D external quality assessment scheme (DEQAS) operated by Charing Cross Hospital, London, UK, is designed to ensure analytical reliability of vitamin D assays within laboratories worldwide.

Although serum 25(OH)D is dependent on a variety of factors including season, latitude and race, a typical normal range for serum 25(OH)D in adults living in northern Europe is between 25 and 200 nmol/l (Keane *et al.,* 1998). Defining adequacy of vitamin D status has proved difficult and there is no consensus among researchers as to what cut-off level of 25(OH)D should be applied (McKenna and Freaney, 1998). According to Scharla (1998), bone disease such as rickets in children and osteomalacia in adults are reported with serum 25(OH)D levels below 12.5 nmol/l, although some studies demonstrate that these conditions also develop at higher serum 25(OH)D concentrations (~25 nmol/l) (Basha *et al.,* 2000). Serum 25(OH)D levels <50 nmol/l, which are characteristic of vitamin D insufficiency, result in secondary

hyperparathyroidism and a marked increase in serum PTH, potentially resulting in increased bone calcium mobilization and accompanying bone loss (Malabanan *et al.,* 1998; Lips, 2001). In addition, calcium absorption is compromised at serum 25(OH)D levels $\geq$50 nmol/l (Heaney *et al.,* 2003). Even when serum 25(OH)D is as high as 80–100 nmol/l, slight elevations in PTH can be observed, although levels remain within the normal range (McKenna and Freaney, 1998; Lamberg-Allardt *et al.,* 2001). In elderly subjects, it is only when serum 25(OH)D exceeds 100 nmol/l that serum PTH concentration becomes minimal (Dawson-Hughes *et al.,* 1997; Kinyamu *et al.,* 1998). Furthermore, at serum 25(OH)D concentrations $>$100 nmol/l, no disturbances in vitamin D-dependent physiological functions are observed (Peacock, 1995). Therefore, serum 25(OH)D $>$100 nmol/l, but $<$250 nmol/l, above which toxicity may occur (see above), is considered 'adequate' (Zittermann, 2003).

12.3.8.2. Dietary Vitamin D Requirements

Establishing dietary requirements for vitamin D is difficult because sunlight makes a highly significant contribution to the functional marker of vitamin D [serum 25(OH)D]. In the United States, the adequate intake (AI) is 5 μg/day for children aged 4–17 years, between 0 and 10 μg/day for adults aged 18–50 years, 10 μg/day for adults aged 51–70 years and 15 μg/day for adults over 70 years (Food and Nutrition Board, 1997). The difference in requirements between children and older adults relates to the reduced ability for dermal synthesis in older subjects. In the United Kingdom, there is no reference nutrient intake (RNI) for vitamin D for people aged 4–64 years based on a lack of evidence that individuals in this age group rely on dietary intake for adequate vitamin D status (Department of Health, 1998). However, the RNI for people aged 65 and over is 10 μg/day, which was considered to help safeguard against vitamin D deficiency and its adverse effects on bone health (Department of Health, 1998).

12.3.8.3. Vitamin D Toxicity

It has been suggested that the concentration of pre-vitamin D in the skin of Caucasians reaches an equilibrium within 20 min of UVB exposure (Holick, 1995), with any excess 7-dehydrocholesterol being converted to inactive metabolites such as tachysterol and lumisterol. Therefore, UVB exposure beyond the minimal erythemal dose does not increase vitamin D production further (Vieth, 1999). This explains why it is impossible to get toxicity from UVB radiation, even after prolonged exposure. Such inherent feedback regulatory control mechanisms do not exist for oral exposure to vitamin D. There are,

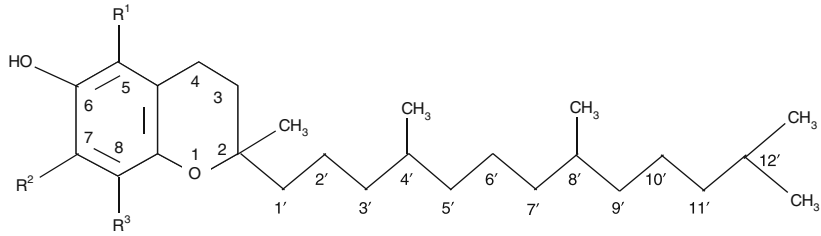
however, no reports in the literature about vitamin D intoxication with foods that are consumed traditionally (Chesney, 1989). All reports of vitamin D intoxication involve either highly fortified foods (Jacobus *et al.,* 1992) or pharmacological doses of vitamin D supplements (for review, see Vieth, 1999). In almost every instance of vitamin D intoxication, serum 25(OH)D levels were >200 nmol/l (Jacobus *et al.,* 1992; Vieth, 1999; Zittermann, 2003), but were usually well above this level (Vieth, 1999). Vitamin D intoxication is characterized by hypercalcaemia and hypercalciuria and can lead to the calcification of soft tissues (Vieth, 1990), with minimal or no change in $1,25(OH)_2D_3$ levels (Hughes *et al.,* 1976). In vitamin D intoxication, a serum 25(OH)D level of ~1000 nmol/l is not uncommon (Heaney *et al.,* 1997; Vieth, 1999). In one study, a serum 25(OH)D level of 3700 nmol/l was reported in a subject exhibiting vitamin D intoxication after the consumption of table sugar to which pure crystalline vitamin D_3 had been added either accidentally or intentionally (Vieth *et al.,* 2002). The subject in question reported extreme pain, nausea, dehydration and severe nephrocalcinosis.

The supplemental dose of vitamin D that may lead to toxicity is unclear. One study showed that 30 μg of vitamin D per day was sufficient to cause hypercalciuria in one subject (Adams and Lee, 1997), yet the same vitamin D intake was associated with vitamin D deficiency in two other studies (Chapuy *et al.,* 1997; Trang *et al.,* 1998). It has been suggested that the differences between the outcomes of these studies can be explained by sunlight exposure (Vieth, 1999). In addition, in nearly all cases of vitamin D intoxicity except for the study by Adams and Lee (1997), the reported vitamin D intake was above 100 μg/day (Vieth, 1999). The current No Observed Adverse Effect Level (NOAEL) for vitamin D is set at 100 μg/day (Scientific Committee on Food, 2002). This figure is based on the consensus that at a vitamin D intake of 100 μg/day, the risk of hypercalcaemia/hypercalciuria starts to increase (Tjellesen *et al.,* 1986; Vieth *et al.,* 2001). The upper limit (UL) for vitamin D stands at 50 μg/day (Food and Nutrition Board, 1998; Scientific Committee on Food, 2002), which incorporates a safety factor of 2 from the NOAEL.

12.4. Vitamin E

12.4.1. Chemistry and Analysis

Tocochromanols are a group of four tocopherols (α-, β-, γ- and δ-) and four tocotrienols (α-, β-, γ- and δ-) produced at various levels and in different combinations by all plant tissues and some cyanobacteria. All tocochromanols are amphipathic molecules, with the general structures shown in Figure 12.3. The polar head group is derived from aromatic amino acid



Compound	Formula	R^1	R^2	R^3
α-Tocopherol	5,7,8-Trimethyl	CH_3	CH_3	CH_3
β-Tocopherol	5,8-Dimethyl	CH_3	H	CH_3
γ-Tocopherol	7,8-Dimethyl	H	CH_3	CH_3
δ-Tocopherol	8-Methyl	H	H	CH_3

Figure 12.3 The four major forms of vitamin E (α-, β-, γ- and δ-tocopherol) differ by the number and position of methyl groups on the chromanol ring. In α-tocopherol, the most biologically active form, the chromanol ring is fully methylated. In β- and γ-tocopherol, the ring contains two methyl groups, while in δ-tocopherol it is methylated in one position. The corresponding tocotrienols have the same structural arrangement except for the presence of double bonds on the isoprenoid side chain at C3′, C7′ and C11′.

metabolism and the hydrophobic tail from phytyl-diphosphate (phytyl-DP) or geranygeranyl diphosphate (GGDP) for tocopherols and tocotrienols, respectively (DellaPenna and Pogson, 2006). The term 'vitamin E' is used to describe all tocopherol and tocotrienols that qualitatively exhibit the biological activity of α-tocopherol. Tocopherol is methylated at C-5, C-7 and C-8 on the chromonal ring, whereas the other homologues (β-, γ- and δ-) differ in the number and positions of the methyl groups on the ring (Figure 12.3). Tocopherols have a fully saturated 20-carbon phytyl side chain attached at C-2 and have three chiral centres in the R configuration at positions C-2, C-4^1 and C-8^1 in the naturally occurring form, which are given the prefix 2R, 4^1R and 8^1R (designated RRR). They are more biologically active than their synthetic counterparts, which are mixtures of all eight possible stereoisomers and are given the prefix all-*rac*. Tocotrienols differ from the corresponding tocopherols in that the 20-carbon isoprenoid side chain is unsaturated at C-3^1, C-7^1 and C-11^1 and possess one chiral centre at C-2 in addition to two sites of geometric isomerism at C-3^1 and C-7^1. Natural tocotrienols have the 2R, 3^1-*trans*, 7^1-*trans* configuration. The phenolic hydroxyl group is critical for the antioxidant activity of vitamin E, as donation of hydrogen from this

group stabilizes free radicals. The presence of at least one methyl group on the aromatic ring is also critical. Vitamin E biological activity is defined in terms of α-tocopherol equivalents (α-TE) whenever possible. RRR-α-tocopherol has an activity of 1 mg α-tocopherol equivalent (α-TE/mg compound). The activities of RRR-β, RRR-γ and RRR-δ-tocopherol are 0.5, 0.1 and 0.03, respectively. Synthetic all-*rac*-α-tocopheryl acetate has an activity of 0.74 mg α-TE/mg. Of the tocotrienols, only α-tocotrienol has significant biological activity (0.3 mg α-TE/mg). Lengthening or shortening the side chain results in a progressive loss of vitamin E activity (Ingold *et al*., 1990).

In the determination of vitamin E, solid-phase extraction (Luque-Garcia and Luque de Castro, 2001) and supercritical fluid extraction (Turner and Mathiasson, 2000; Turner *et al*., 2001) are now used extensively for sample extraction and clean-up. Methods used for the determination and quantitation of vitamin E include normal-phase high-performance liquid chromatography (NP-HPLC), reversed-phase high-performance liquid chromatography (RP-HPLC), gas chromatography (GC) and supercritical fluid chromatography (SFC) (Pyka and Sliwiok, 2001; Ruperez *et al*., 2001; Turner *et al*., 2001). Detection of fat-soluble vitamins after HPLC resolution can be accomplished by UV (using diode array detection), fluorescence (FLD), electrochemical (ED) or evaporative light scattering (ELSD) detection methods (Ruperez *et al*., 2001). The most commonly used detector for vitamin E analysis is FLD, which is considerably more sensitive and selective than UV, but less sensitive than ED.

A number of analytical methods have been described for the determination of vitamin E in milk and milk-based infant formulae, either alone (Rodrigo *et al*., 2002; Romeu-Nadal *et al*., 2006a) or simultaneously with other fat-soluble vitamins (Turner and Mathiasson, 2000; Rodas Mendoza *et al*., 2003; Heudi *et al*., 2004; Chavez-Servin *et al*., 2006).

12.4.2. Absorption, Metabolism and Excretion

Following ingestion, fats are emulsified into smaller particles, first in the stomach and then in the small intestine, where they are mixed with pancreatic and bilary secretions. Pancreatic esterases convert triglycerides to monoglycerides and free fatty acids, and together with bile acids, these products form micelles into which vitamin E and other hydrophobic molecules become solubilized (Traber and Sies, 1996). Vitamin E is absorbed in the proximal part of the small intestine, where transport across the brush border is thought to occur by passive diffusion (Traber and Sies, 1996). There are no selective differences in absorption between α- and γ-tocopherols (Jiang *et al.*, 2001), but β- and δ-tocopherols are absorbed poorly and are excreted in the faeces. Together with triglycerides, phospholipids and apolipoproteins, the

tocopherols (α- and γ-) are reassembled to chylomicrons by the Golgi of the mucosal cells (Brigelius-Flohe and Traber, 1999). The chylomicrons are stored as secretory granula and eventually excreted by exocytosis to the lymphatic compartment from which they reach the bloodstream via the *ductus thoracicus*.

The transfer of vitamin E from chylomicrons is regulated by complex mechanisms that control lipid and lipoprotein metabolism (Traber and Sies, 1996; Traber and Arai, 1999). Chylomicrons are degraded to remnants by lipoprotein lipase (LPL) and some α- and γ-tocopherols are transferred to peripheral tissues such as muscle, adipose tissue, skin and brain by this enzyme-mediated mechanism (Traber and Sies, 1996; Jiang *et al.*, 2001; Morrissey and Kiely, 2005). The resulting chylomicron remnants are then taken up by the liver, where most of the remaining α- and only small amounts of γ-tocopherols are reincorporated into nascent very low density lipoproteins (VLDLs) by a specific 32-kDa α-tocopherol transfer protein (α-TTP), which enables further distribution of α-tocopherol throughout the body.

Approximately 50% of the dietary intake of γ-tocopherol appears to be degraded in the liver by a cytochrome P450-dependent process (Parker *et al.*, 2000) and is then excreted, primarily in the urine (Swanson *et al.*, 1999). Catabolism of α-tocopherol by this route occurs only when the daily intake of α-tocopherol exceeds 150 mg (Schultz *et al.*, 1997) or the plasma concentration of α-tocopherol is above a threshold of 30–40 μmol/l (Schuelke *et al.*, 2000). Plasma or serum concentrations of α-tocopherol are typically around 20–35 μmol/l. γ-Tocopherol concentrations are approximately 5–15% of those of α-tocopherol and generally remain around 1 μmol/l even after supplementation (Hayes *et al.*, 1993; O'Byrne *et al.*, 2000). The highest concentrations of α-tocopherol in the body are in the adipose tissues and adrenal glands (Drevon, 1991). Adipose tissues are also the major stores of the vitamin, followed by the liver and skeletal muscle. The rate of uptake and turnover of α-tocopherol by different tissues varies greatly (Burton *et al.*, 1990). Uptake is most rapid into lungs, liver, spleen, kidney and red blood cells (in rats $t_{½} < 15$ days) and slowest in brain, adipose tissues and spinal cord ($t_{½} < 30$ days). Likewise, depletion of α-tocopherol from plasma and liver during times of dietary deficiency is rapid, whereas adipose tissues, brain, spinal cord and neural tissues are much more difficult to deplete (Bourne and Clement, 1991).

A considerable, but variable, proportion (typically 30–70%) of ingested vitamin E is unabsorbed and therefore excreted in the faeces, making this the main route of elimination (Kayden and Traber, 1993), and is influenced by experimental conditions and a variety of luminal and physiological factors (Cohn, 1997). When large doses of vitamin E are administered, much of it is secreted in the bile, which may account for its relative safety compared to

vitamins A and D (Bramley *et al*., 2000). Both bile acids and pancreatic juices are important for the absorption of vitamin E (Traber and Sies, 1996). This has been established clearly in patients where secretion of either, or both, is severely diminished as in patients with cystic fibrosis or pancreatitis. The simultaneous intake of fat is necessary to stimulate bile flow and the secretion of pancreatic enzymes to allow micelle formation. However, the amount of fat necessary to ensure absorption may be small (Roodenburg *et al*., 2000).

12.4.3. Tocopherols and Prevention of Lipid Oxidation

Vitamin E (as α-tocopherol, α-TOH) is an indispensable component of biological membranes with membrane-stabilizing properties and high antioxidant activity. The overall mechanisms of lipid peroxidation and antioxidant protection in biological and food systems have been reviewed extensively (Buettner, 1993; Frankel, 1998; Morrissey *et al*., 1998; Morrissey and Kerry, 2004). The antioxidant activity of chain-breaking antioxidants is determined by how rapidly they scavenge free radicals, the ease of hydrogen transfer from an antioxidant to a free radical and the difference in the standard one-electron reduction potentials (Buettner, 1993). A compound, the reduction potential of which is lower than that of the reduction potential of a free radical or oxidized species, can donate a hydrogen to that free radical if the reaction is kinetically feasible. The peroxyl radical ($LOO^{\cdot}$) ($E^{o'} = +1000$ mV) has a reduction potential greater than α-TOH ($E^{o'} = +500$ mV), meaning that hydrogen transfer between the antioxidant and $LOO^{\cdot}$ is energetically favourable (Buettner, 1993; Morrissey *et al*., 1994). When the chromanol phenolic group of α-TOH encounters an $LOO^{\cdot}$, a hydroperoxide (LOOH) is formed and in the process an α-tocopheroxyl radical (α-$TO^{\cdot}$) is generated:

$$\alpha - \mathrm{TOH} + \mathrm{LOO}^{\cdot} \xrightarrow{\Delta E^{o'} = +500\,\mathrm{mv}} \mathrm{LOOH} + \alpha - \mathrm{TO}^{\cdot}$$

The rate constant (k_1) for this chain-inhibition reaction is 8×10^4 l/mol/s (Buettner,1993) or 2.35×10^6 l/mol/s (Azzi and Stocker, 2000), which is considerably higher than that for the other tocopherols and related phenols. Because the rate constant (k_2) for the chain propagation reaction between $LOO^{\cdot}$ and an unsaturated fatty acid (LH) ($LOO^{\cdot} + LH \rightarrow LOOH$) is much lower than k_1, at approximately 10^2 l/mol/s, α-tocopherol outcompetes the propagation reaction and scavenges the $LOO^{\cdot}$ $\sim 10^4$ times faster than LH reacts with $LOO^{\cdot}$. Thus, the kinetics of antioxidants, and particularly α-TOH, require that only a relatively small amount be present for them to act as effective antioxidants (Buettner, 1993; Morrissey *et al*.,

1994). The concentration of α-TOH in biological membranes is approximately 1 mol/1000–2000 mol phospholipids (i.e. $\sim 1{:}10^3$) (Burton *et al.*, 1983). This effectively means that about 90% of the $LOO^{\cdot}$ are scavenged by α-TOH before they can attack another LH (Buettner, 1993; Niki and Matsuo, 1993; Morrissey *et al.*, 1994). The overall antioxidant activity of α-TOH depends also on the fate of the α-$TO^{\cdot}$ generated when α-TOH scavenges lipid $LOO^{\cdot}$. The α-$TO^{\cdot}$ is relatively stable due to resonance delocalization (Nawar, 1996), the radical energy is low ($E^{o'} = +500$ mV), and it is less likely to promote the oxidation of other biomolecules. In biological systems, ascorbate can reduce α-$TO^{\cdot}$ back to α-TOH with a fairly high rate constant (1.5×10^6 l/mol/s). Because γ-tocopherol lacks one of the electron-donating methyl groups on the chromonal ring, it is less hydrophobic and is somewhat less potent in donating electrons than α-tocopherol, and is, thus, a slightly less powerful antioxidant. However, the unsubstituted C5 position on γ-tocopherol allows it to trap lipophilic electrophiles, such as reactive nitrogen species (RSN) (Christen *et al.*, 1997; Jiang *et al.*, 2001). Excessive generation of RSN is associated with chronic inflammation in humans and animals.

12.4.4. Tocopherols and Chronic Diseases

It is generally accepted that low-density lipoprotein (LDL) undergoes oxidation in vivo when challenged by a variety of ROS and RNS species and that oxidized LDL is the component central to the initiation and/or progression of atherogenesis at the molecular and cellular levels (Parthasarathy *et al.*, 1999; Ross, 1999b; Pryor, 2000; Lapointe *et al.*, 2006). Vitamin E, mainly as α-tocopherol, is quantitatively the most important lipophilic antioxidant present in LDL particles. On average, each LDL particle is protected by $\sim$10 molecules of α-tocopherol (range 3–15 mol/l), 1 mol/l γ-tocopherol and smaller amounts of carotenoids (Esterbauer *et al.*, 1992; Carroll *et al.*, 2000). Vitamin E in the LDL particles acts as a chain-breaking antioxidant and prevents peroxidation of LH and modification of proteins by ROS (Carr *et al.*, 2000a).

In vitro studies have indicated that increasing the vitamin E content of LDL particles increases LDL resistance to oxidation (Esterbauer *et al.*, 1992). The oxidative resistance of LDL is increased in vitamin E-supplemented individuals and the rate of oxidation was significantly decreased at 269 and 537 mg α-tocopherol/day (Princen *et al.*, 1995). The authors concluded that vitamin E was the most important variable that determined the oxidative resistance of LDL. Reactive nitrogen species are also implicated in aortic oxidation of LDL, and therefore potentially in atherosclerosis (Jiang *et al.*, 2001). This mechanism of protecting LDL may be significant when

γ-tocopherol constitutes a major portion of vitamin E intake, as in the United States (Jiang *et al*., 2001; Wagner *et al*., 2004). It is worth noting that the ability of γ-tocopherol to attenuate oxidative damage produced by RNS may prevent or delay the progression of other diseases, as well as cardiovascular disease (CVD), in which inflammation plays a role, such as cancer, rheumatoid arthritis, inflammatory bowel disease and neurodegenerative disorders.

The effects of vitamin E have been examined in several studies, many of which have reported a clear association of the reduction in the relative risk of CVD with high intake from foods or supplements of vitamin E, although some have shown no such association (Pryor, 2000; Neuzil *et al*., 2001; Brigelius-Flohe *et al*., 2002; Knekt *et al*., 2004). Cross-cultural studies in Europe reported that high plasma levels of vitamin E are associated with lower mortality rates from CVD (Gey *et al*., 1995). Prospective cohort studies on men and women also reported a lower risk of CVD with long-term vitamin E supplementation (Rimm *et al*., 1993; Stampfer *et al*., 1993). Stampfer *et al*. (1993) reported a significantly reduced risk of CVD associated with high α-tocopherol intake from supplements, but not from dietary sources. The Health Professional Study (Rimm *et al*., 1993) also showed that dietary intake of vitamin E was not significantly correlated with reduced risk of CVD or death. In contrast, Kushi *et al*. (1996) concluded that the intake of dietary vitamin E (mainly γ-tocopherol), but not of supplemental vitamin E (mainly α-tocopherol), was significantly inversely associated with increased risk of death from CVD. Other studies point to the potential importance of γ-tocopherol in preventing heart disease (Jiang *et al*., 2001; Wagner *et al*., 2004). γ-Tocopherol may also function by up-regulation of endothelial nitric oxide synthase and nitric oxide formation which suppresses the expression of pro-inflammatory cytokines and maintains the integrity of the arterial wall (Carr and Frei, 2000; Jiang *et al*., 2001). Several other human clinical trials have shown an improvement in markers of atherosclerosis by vitamin E supplementation. These findings have been contradicted by several vitamin E supplementation trials (Brigelius-Flohe *et al*., 2002; Knekt *et al*., 2004; Meydani, 2004). However, overall evidence from cell culture, as well as animal and human clinical and observational studies, strongly supports the contribution of dietary vitamin E in the maintenance of vascular function and health, in particular when used in combination with other foods containing antioxidants.

The capacity of vitamin E, particularly α-tocopherol, to quench free radical damage, induces apoptosis and impact expression of oncogenes makes it a strong candidate for chemotherapeutic strategies (Tucker and Townsend, 2005). Evidence of a role for vitamin E in cancer prevention was derived from a study conducted in Linxian, China, in a population with persistently low micronutrient intakes and one of the world's highest incidences

of oesophageal/stomach cancer (Knekt, 1993), where an overall reduction in cancer mortality, particularly mortality from stomach and oesophageal cancer, was observed. The efficacy of supplementation with α-tocopherol on the prevention of certain cancers in male smokers was investigated in the Finnish ATBC study. There was no decrease in the incidence of lung cancer among men supplemented with synthetic α-tocopherol compared to those who were not fed supplements. In contrast, prostate cancer incidence and mortality were reduced by 32 and 41%, respectively, among the vitamin E-supplemented group (Heinonen *et al.*, 1998). Overall results of epidemiological studies relating to vitamin E and colon cancer have been inconsistent and mixed.

Recent epidemiological experiments and mechanistic evidence suggest that γ-tocopherol may be a more potent cancer chemopreventative agent than α-tocopherol. A nested case-control study examined the association of α-tocopherol, γ-tocopherol and selenium with the incidence of prostate cancer (Helzlsouer *et al.*, 2000). The most striking finding was that men in the highest quintile of plasma γ-tocopherol concentration had a fivefold reduction in the risk of prostate cancer compared with those in the lowest quintile. Wagner *et al.* (2004) concluded that only the plasma level of γ-tocopherol served as a biomarker of CVD and cancer. The differences in chemical reactivity, metabolism and biological activity that may contribute to the differences in the effects of γ-tocopherol compared to α-tocopherol are discussed by Jiang *et al.* (2001) and Campbell *et al.* (2003).

In summary, inverse associations between dietary and supplemental vitamin E intakes and the incidence of several common chronic diseases have been noted in many observational studies, whereas the results of studies using blood concentration of vitamin E have been limited and inconsistent (Sung *et al.*, 2003; Gaziano, 2004). Randomized trials using supplemental vitamin E have not shown substantial effects on mortality end-points (Sung *et al.*, 2003; Jialal and Devaraj, 2005). However, Wright *et al.* (2006) reported the findings of a prospective cohort study of 29092 Finnish male smokers, aged 50–69 years who participated in the ATBC Study. They evaluated the prospective association between the circulating concentration of α-tocopherol and total and cause-specific mortality in the group. Significantly lower total and cause-specific mortality in older male smokers were observed as the serum α-tocopherol value increased from 9.1 mg/l (21 μmol/l) to $\sim$13–14 mg/l ($\sim$30–33 μmol/l), after which no further benefit was noted.

12.4.5. Vitamin E in Milk

The concentration of vitamin E in animal products is usually low but they may be significant sources of the vitamin because of their high level of consumption. Different authors have reported concentrations of

α-tocopherol between 0.2 and 0.7 mg/l in bovine milk (Renner *et al*., 1989; Jensen, 1995). γ-Tocopherol has also been found and trace amounts of some other vitamers. Barrefors *et al*. (1995) reported α-tocopherol levels of 7.4–10.0 mg/g lipid for different herds and also observed the presence of low levels of γ-tocopherol and α-tocotrienol. Colostrum contains about 1.9 mg/l of α-tocopherol and the level decreased in approximately 4 days to the level in fresh milk (0.3 mg/l) (Hidiroglou, 1989). γ-Tocopherol is also present in small amounts in colostrum. The transfer of vitamin E into colostrum does not appear to occur through a passive mechanism following the transfer of lipid (Debier *et al*., 2005). A mechanism involving low-density lipoproteins (LDL) may be responsible for the high vitamin E concentration in colostrum compared to mature milk (Schweigert, 1990). Also, tissue delivery of α-tocopherol into milk may be promoted by the action of lipoprotein lipase on triglyceride-rich lipoproteins (Martinez *et al*., 2002). The quantitative secretion of α-tocopherol (also β-carotene) from plasma into cows' milk appears to follow Michaelis–Menten kinetics for active transport across membranes (Jensen *et al*., 1999). According to these authors the daily secretion of α-tocopherol and β-carotene is limited in quantity and is independent of the yield of milk and fat content. The concentration of vitamin E in milk appears to be dependent principally on the amount consumed by the cow. Indyk *et al*. (1993) observed that the vitamin E content of cow's milk exhibits a significant seasonal pattern that is largely independent of fat content, with pasture maturity and quality the dominant factors. However, this seasonal effect was not observed in an Irish study, probably because farm management practices include supplementation of the diets of spring-calved cows with silage or concentrates from mid-September onwards (O'Brien *et al*., 1999).

The vitamin E concentration in human milk is also much higher in colostrum than in mature milk. Boersma *et al*. (1991) observed that human colostrum contained 22 $\pm$14 mg/l as α-tocopherol equivalents compared with transitional milk (14$\pm$8) and mature milk (8$\pm$5). Barbas and Herrera (1998) also observed significantly higher levels of vitamin E in human colostrum (14.4$\pm$2.3 mg/l) compared with 3.1$\pm$0.5 mg/l in mature milk. The vitamin E/linoleic acid ratio in human colostrum is higher (89.8$\pm$14.7 μg/g) compared with 25.9$\pm$3.49 μg/g in mature milk. Vitamin E does not consistently cross the placental barrier (Quigley and Drewey, 1998), and as a consequence, plasma vitamin E concentration in pre-suckled newborn infants (Sinha and Chiswick, 1993) and calves (Nonnecke *et al*., 1999) is very low. Colostrum ingestion is therefore important to provide mammalian neonates with an adequate source of vitamin E to protect against oxidative stress and enhance the immune response. Following birth, colostrum intake induces a sharp increase in the circulating and tissue levels of vitamin E in the young (Hidiroglou *et al*., 1993)

The stability of vitamin E in foods is affected by environmental factors and food-related factors such as water activity, the degree of unsaturation of biomembranes and the presence of trace elements such as copper and iron (Frankel, 1998). Vidal-Valverde *et al.* (1993) observed that α-tocopherol in UHT milk stored at 30°C decreased by 3–14% at 1 month and by 9–30% at 2 months. On storage of UHT milk at –20°C, α-tocopherol levels were stable for 2 months, but decreased by 10–20% after 4–8 months. Supplementation of animal feed with vitamin E increases the oxidative stability of milk (Barrefors *et al.*, 1995; Focant *et al.*, 1998). Elevated α-tocopherol levels contribute to lower lipid and cholesterol oxidation in whole milk powders during storage at elevated temperatures (McCluskey *et al.*, 1997; Morrissey and Kiely, 2006).

12.4.6. Vitamin E Status and Requirements

Vitamin E deficiency occurs only rarely in humans, and overt deficiency symptoms in normal individuals consuming diets low in vitamin E have never been described. However, it may occur in premature and some newborn infants (Godell, 1989). When it occurs in older children and adults, it usually arises as a consequence of malabsorption complicating cholestasis, abetalipoproteinaemia, coeliac disease or cystic fibrosis (Sokol, 1993). There is also an extremely rare disorder in which primary vitamin E deficiency occurs in the absence of lipid malabsorption. Ataxia with isolated vitamin E deficiency (AVED) is a rare autosomal recessive neurodegenerative disease characterized clinically by slowly progressive development of peripheral neuropathy, spinocerebellar ataxia, dysarthria, the absence of deep tendon reflexes and loss of proprioceptive and vibration sense (Mariotti *et al.*, 2004). AVED is caused by mutation in the α-TTP gene, which is located at chromosome 8q13 (Federico, 2004). Patients with AVED have serum vitamin E levels well below the normal range [$<$2.5 mg/l, often $<$1 mg/l (normal range is 6–15 mg/l)] (Koeing, 2003).

In general, there is no consensus as to the threshold concentration of plasma or serum α-tocopherol at which people can be defined as having either an inadequate or an acceptable vitamin E status (Morrissey and Sheehy, 1999). A recent prospective cohort study by Wright *et al.* (2006) showed that increasing the circulating concentration of α-tocopherol up to $\sim$13–14 mg/l (30–33 μmol/l) was associated with significantly lower total and cause-specific mortality in older male smokers, after which no further benefit was noted. The estimated dietary vitamin E intake to achieve this serum concentration may be as high as 15 mg/day (Traber, 2006), an amount that could be obtained from dietary sources if a concerted effort was made to eat foods rich in vitamin E (Gao *et al.*, 2006; Traber, 2006). The Food and

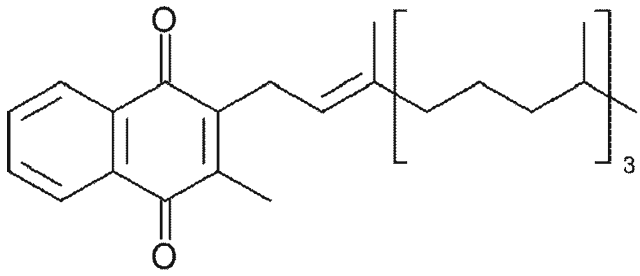
Nutrition Board (2000) set an estimated average requirement (EAR) of 12 mg (27.9 μmol)/day of α-tocopherol for adults >19 years. The same value was set for men and women on the basis that although body weight is smaller on average for women than for men, fat mass as a percentage of body weight is higher for women than for men. The recommended dietary allowance (RDA) was established for men and women as the EAR (12 mg) plus twice the coefficient of variation (CV) (assumed to be 10%), rounded up, giving a value of 15 mg (34.9 μmol)/day. In Europe, the Scientific Committee for Food (1993) did not set a population reference intake (PRI) for vitamin E on the basis that there is no evidence of deficiency from a low intake. The tolerable upper intake level (UL) is 1000 mg/day, based on studies showing haemorrhagic toxicity in rats, in the absence of human dose–response data (Food and Nutrition Board, 2000). The Scientific Committee for Food (1993) proposed that the intake should not exceed 2000 mg α-tocopherol equivalents per day.

12.5. Vitamin K

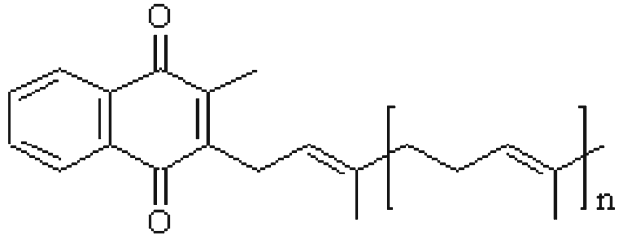
12.5.1. Chemistry and Analysis

Vitamin K (the coagulation vitamin) was discovered in the 1940s as a result of investigations into the cause of an excessive bleeding disorder in chickens fed on a fat-free diet. The term 'vitamin K' is a group name for a number of related compounds, which have in common a 2-methyl-1,4-napthoquinone ring system, but differ in the length and degree of saturation of their isoprenoid side chain at the 3-position. Three vitamin K compounds have biological activity (Figure 12.4).

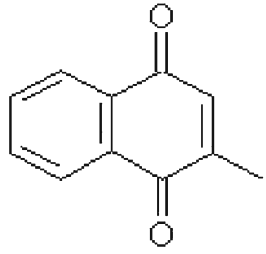
Phylloquinone, vitamin K_1 (2-methyl-3-phytyl-1,4-napthoquinone), is found in green leafy vegetables and represents the main dietary source of vitamin K in Western diets (Bolton-Smith *et al.,* 2000). Menaquinones (MKs), vitamin K_2 (2-methyl-3-1,4-napthoquinone), are synthesized by the gut microflora, with fully or partially unsaturated isoprenoid side chains of various length at the 3-position. The predominant forms of the MK compounds contain between 6 and 10 isoprenoid units, but MKs containing up to 13 units have been isolated (Suttie, 1985). The parent structure of the vitamin K group of compounds is 2-methyl-1,4-naphthoquinone, commonly called menadione (vitamin K_3), is not found in nature but is a synthetic form which can be metabolized to phylloquinone or menaquinone and thus may be regarded as a pro-vitamin. Menadione is also used as an animal feed supplement and in this way may indirectly enter the human food chain as preformed MK-4 (Shearer *et al*., 1996).



(i) Phylloquinone (Vitamin K_1)



(ii) Menaquinone (Vitamin K_2)



(iii) Menadione (Vitamin K_3)

Figure 12.4. Structures of the vitamin K compounds (phylloquinone, menaquinone and menadione).

Previous analytical techniques to measure vitamin K compounds, such as the chick bioassay, were cumbersome and tended to overestimate the vitamin K content of foods. However, at present, the method of choice for vitamin K analysis in foodstuffs is HPLC separation after lipid extraction (Booth *et al.,* 1993, 1995). Electrochemical or fluorescence detection (after reduction to the hydroquinone form) offers the sensitivity and selectivity needed for quantification of the small amounts of the vitamin K compounds.

Food composition data for vitamin K derived from HPLC are generally lower than earlier data derived from the chick bioassay (Booth *et al.,* 1993). The use of these HPLC-derived data on the vitamin K content of foods allows for a more accurate determination of the phylloquinone content of a typical Western diet.

12.5.2. Absorption, Metabolism and Excretion

Dietary vitamin K, mainly as phylloquinone, is absorbed into the lymphatic system from the proximal intestine after solubilization into mixed micelles composed of bile salts and the products of pancreatic lipolysis (Shearer *et al.,* 1974). In healthy adults, the efficiency of absorption of phylloquinone is about 80% (Shearer *et al*., 1974). Intestinal bacteria can synthesize a variety of menaquinones which are absorbed to a limited extent from the large intestine, transported into the lymphatic system, cleared by the liver and released in VLDL. However, it is not fully clear to what extent intestinal menaquinone contributes to the vitamin K requirement. Approximately 50% of vitamin K is carried in the plasma in the form of VLDL, about 25% in LDL (low-density lipoprotein) and about 25% in HDL (high-density lipoprotein). Once in the circulation, phylloquinone is cleared rapidly at a rate consistent with its continuing association with chylomicrons (Kohlmeier, 1996). Vitamin K is extensively metabolized in the liver and excreted in the urine and bile. It has been demonstrated in tracer experiments that about 20% of an injected dose of phylloquinone is recovered in urine whereas about 40–50% is excreted in the faeces via the bile (Shearer *et al*., 1974) and the proportion excreted was the same regardless of whether the injected dose was 1000 or 45 μg. It seems likely, therefore, that about 60–70% of the amount of phylloquinone absorbed from each meal will ultimately be lost to the body by excretion. These results suggest that the body's stores of phylloquinone are constantly replenished. Vitamin K itself is too lipophilic to be excreted in the bile and is excreted as side chain-shortened carboxylic acid metabolites.

There is no evidence that phylloquinone or menaquinone are toxic. However, high intakes of phylloquinone can negate the effects of the anticoagulant warfarin. The synthetic form of vitamin K, menadione, can interfere with the function of glutathione, one of the body's natural antioxidants, resulting in oxidative damage to cell membranes. Menadione given by injection has induced liver toxicity, jaundice and haemolytic anemia (due to the rupture of red blood cells) in infants and is no longer used for treatment of vitamin K deficiency (Suttie, 1996). No tolerable upper level (UL) of intake has been established for vitamin K.

12.5.3. Function of Vitamin K

Vitamin K acts as a cofactor for a specific carboxylation reaction that transforms selective glutamate (Glu) residues to γ-carboxyglutamate (Gla) residues (Suttie, 1995). The reaction is catalysed by a microsomal enzyme, γ-glutamyl, or vitamin K-dependent, carboxylase, which, in turn, is linked to a cyclic pathway known as the vitamin K epoxide cycle. The resultant Gla residues are common to all vitamin K-dependent proteins and these have increased affinity for calcium (Furie and Furie, 1990). Prothrombin and other proteins of the blood clotting system, as well as certain bone matrix proteins, contain γ-carboxyglutamate.

12.5.4. Vitamin K-Dependent Proteins

12.5.4.1. Vitamin K-Dependent Coagulation Proteins

There are seven vitamin K-dependent proteins involved in blood coagulation, namely, prothrombin (Factor II), Factors VII, IX, X and proteins C, S and Z, all of which are synthesized in the liver and contain between 10 and 12 Gla residues (Suttie, 1985). The Gla residues enable Ca^{2+}-mediated binding of the proteins to the negatively charged phospholipid surfaces provided by blood platelets and endothelial cells at the site of injury. Prothrombin and Factors VII, IX and X possess pro-coagulant activity and participate in the cascade that results in the formation of the fibrin clot. A key element in the formation of fibrin is the conversion of prothrombin to thrombin by activated Factor X (which is, in turn, activated by activated Factors VII and IX) (Suttie, 1985). In contrast, proteins C and S act as anticoagulants. Protein C inhibits coagulation by inactivating activated Factors V and VIII and enhancing fibrinolysis, with protein S as a cofactor.

12.5.4.2. Bone Vitamin K-Dependent Proteins

There are two bone matrix proteins which contain γ-carboxyglutamate: osteocalcin (OC) and matrix Gla protein (MGP). Osteocalcin is an osteoblast-derived, specific vitamin K-dependent protein which also contains hydroxyproline and is the most abundant of all the non-collagenous bone matrix-bound proteins. It has a molecular mass of 5700 Da and contains three Gla residues, which give this protein a high affinity for hydroxyapatite, much higher, in fact, than its affinity for calcium (Hauschka *et al*., 1989). The synthesis of osteocalcin is under the regulatory control of vitamin D (Hauschka et al., 1989) and its release into the circulation provides a sensitive index of vitamin D action. While a

high proportion of newly synthesized osteocalcin is incorporated into bone, approximately 30% of it is released into the circulation and serum levels of the protein are used widely as an indicator of the rate of bone formation (Brown *et al.*, 1984). The precise physiologic function of osteocalcin remains unclear. The less well characterized GMP has a molecular mass of 9600 Da and contains 5 Gla residues and in contrast to osteocalcin, which is exclusively associated to mineralized tissues, MGP is present in cartilage and is expressed at a high rate in many soft tissues (heart, kidney, lungs), in addition to bone (Fraser *et al.*, 1988).

12.5.5. Vitamin K Deficiency and Chronic Disease

Newborn infants are at serious risk of haemorrhaging because of poor placental transfer of vitamin K, lack of intestinal bacteria and the low vitamin K content in breast milk. For this reason, they receive intramuscular vitamin K at birth. In children and adults, 'clinical' vitamin K deficiency in terms of blood coagulation is rare. However, 'subclinical' vitamin K deficiency in extrahepatic tissues, particularly in bone, is not uncommon in the adult population. The multitude of proteins which require carboxylation of Glu to Gla residues for proper functioning suggests that poor vitamin K status may contribute to certain chronic vascular and skeletal diseases. For example, post-menopausal loss of bone mass is associated with a long-lasting poor vitamin K status and may contribute to osteoporosis (Weber, 2001). Furthermore, it has been reported that bone mineral density is lower and fracture rates higher among patients with lower circulating vitamin K levels (Kanai *et al.*, 1997). In two prospective cohorts, women with low-dietary vitamin K levels appear to be at increased risk of hip fracture (Feskanich *et al.*, 1999; Booth *et al.*, 2000). Increasing evidence is emerging, suggesting a role for vitamin K in the calcification of arteries and atherogenesis (Kaneki *et al.*, 2006). Moreover, the therapeutic potential of vitamin K_2 as an antihepatoma drug has been recently highlighted (for review see Kaneki *et al.*, 2006).

12.5.6. Vitamin K in Milk

Milk is not a good dietary source of vitamin K, containing between 3.5–18 μg/l as phylloquinone (Haroon *et al.*, 1982) and contributes minimally to vitamin K intake in adults (Booth *et al.*, 1996). Mature human milk contains less phylloquinone than cow's milk (~0.25 μg/l) (Haroon *et al.*, 1982). However, vitamin K levels are higher in colostrum than in mature milk (von Kries *et al.*, 1987). The menaquinone concentration in human milk has not been accurately determined but appears to be much lower than that of

phylloquinone. Phylloquinone concentration in infant formulae milk ranges from 3 to 16 μg/l in unsupplemented formulae and up to 100 μg/l in fortified formulae.

The average intake of phylloquinone by infants fed human milk during the first 6 months of life has been reported to be less than 1 μg/day, which is approximately 100-fold lower than the intake by infants fed a typical supplemented formula (Greer *et al*., 1991). A study in Germany showed that a minimum daily intake of about 100 ml of colostrum milk (which supplies about 0.2–0.3 μg of phylloquinone) is sufficient for normal vitamin K homeostasis in a baby of about 3 kg during the first week of life (von Kries *et al.,* 1987). Similar conclusions were reached in a Japanese study which showed a linear correlation between the prevalence of undercarboxylated coagulation protein and the volume of breast milk ingested over 3 days (Motohara *et al*., 1989).

12.5.7. Vitamin K Status and Requirements

12.5.7.1. Measurement of Vitamin K Status

Defining reliable indicators of vitamin K status has proven to be a difficult task. The serum concentration of undercarboxylated osteocalcin (ucOC, a protein secreted by osteoblasts in bone tissue) is a more sensitive indicator of vitamin K status than the traditional blood coagulation tests and a high serum level of ucOC is indicative of low vitamin K status and vice versa (Vermeer *et al*., 1995; Sokoll *et al*., 1997). Undercarboxylated osteocalcin has been reported to have a negative association with plasma phylloquinone concentrations (Ferland *et al*., 1993). The difference between the vitamin K-dependent coagulation factors (all synthesized in the liver) and the bone Gla protein osteocalcin suggests that different tissues (at least bone and liver) may have different vitamin K requirements: hence bone tissue may be more prone to vitamin K deficiency than liver (Vermeer *et al*., 1995). If this is the case, impaired synthesis of some vitamin K-dependent proteins may be far more prevalent in the human population than coagulation assays previously indicated (Price, 1993), potentially resulting in an increase in dietary recommendations for vitamin K, especially for the elderly. A number of clinical trials have shown that high circulating ucOC levels are common in post-menopausal women as well as in healthy young and elderly adults but levels are reduced significantly with vitamin K supplementation (Szulc *et al*., 1993; Binkley *et al*., 2000). Even in healthy newborns, whose vitamin K status is known to be precarious, very low levels of undercarboxylated prothrombin are detectable, whereas all babies tested exhibited high concentrations

of serum undercarboxylated osteocalcin (Jie *et al*., 1992). These data together with other evidence suggest that circulating osteocalcin is the most sensitive known marker for vitamin K status (Vermeer *et al*., 1995).

12.5.7.2. Dietary Requirements for Vitamin K

Until recently, the only widely accepted criterion for vitamin K sufficiency was the maintenance of plasma prothrombin concentration. Frick *et al*. (1967) estimated that 0.5–1.0 μg/kg/day was required to correct induced clotting changes. In adults, primary vitamin K-deficient states that resulted in bleeding were almost unheard of, except in a hospital setting (Shearer, 1995). This is due to the widespread distribution of vitamin K in foods, the ability of the vitamin K cycle to conserve vitamin K and endogenous bacterial syntheses of menaquinones. Therefore, a healthy population is not at risk of dietary vitamin K deficiency as the recommendation for optimal blood clotting is readily achievable (Suttie, 1992). However, recent attention has focused on the importance of vitamin K for optimizing bone health and it has been proposed that vitamin K supplies believed to be sufficient to maintain normal blood coagulation may be sub-optimal for bone (Kohlmeier *et al*., 1996; Weber, 2001).

The Food and Nutrition Board (2001) has recently established an adequate intake (AI) value for vitamin K. The recent development of indicators sensitive to vitamin K intake, though useful to describe relative diet-induced changes in vitamin K status, was not used for establishing an estimated average requirement (EAR) because of the uncertainty surrounding their true physiological significance and the lack of sufficient dose–response data. Therefore, the AI for adults was based on reported vitamin K dietary intake in apparently healthy populations. Booth and Suttie (1998) reviewed 11 studies in which phylloquinone intake ranged from 60 to 210 μg/day, with an average intake of approximately 80 μg/day for younger adults (<45 years old) and approximately 150 μg/day for older adult (>55 years old).

Healthy individuals with a phylloquinone intake approaching 80 μg/day have been investigated and showed no signs of deficiency, suggesting that this level is probably adequate for the majority of the adult population (Ferland *et al*., 1993; Bach *et al*., 1996). Because dietary assessment methods tend to underestimate the actual daily intake of foods, the highest intake value reported for four adult age groups was used to set the AI for each gender rounding up to the nearest 5 μg (Food and Nutrition Board, 2001). Therefore, the most recent guideline (AI) for vitamin K intake in the United States for adults (aged 19 years and older) is 120 and 90 μg/day for men and women, respectively.

12.6. Vitamin C

12.6.1. Chemistry and Analysis

Ascorbate, also known as ascorbic acid (AA) or vitamin C, is synthesized from guanosine diphosphate (GDP)-mannose and the pathway shares GDP-sugar intermediates with the synthesis of cell wall polysaccharides and those glycoproteins that contain D-mannose, L-fucose and L-galactose (Smirnoff, 2000). Humans and non-human primates, guinea pigs, the Indian fruit bat, several birds and some fish have lost the ability to synthesize ascorbate de novo as a result of a gene mutation that has rendered inactive a key ascorbate biosynthetic enzyme, L-gulonolactone oxidase, which is required to catalyse the last step in the biosynthesis of ascorbate (Woodall and Ames, 1997). Ascorbate is quantitatively the predominant antioxidant in plant cells; it is found in all subcellular compartments, including the apoplast, and has an average cellular concentration of 2–25 mM or more in the chloroplast stroma (Smirnoff, 2000).

Ascorbic acid is the enolic form of an α-ketolactone, 2,3-didehydro-L-threo-hexano-1,4-lactone. The molecular structure (Figure 12.5) contains two ionizable –OH groups at C_2 and C_3 that give the compound its acidic character,

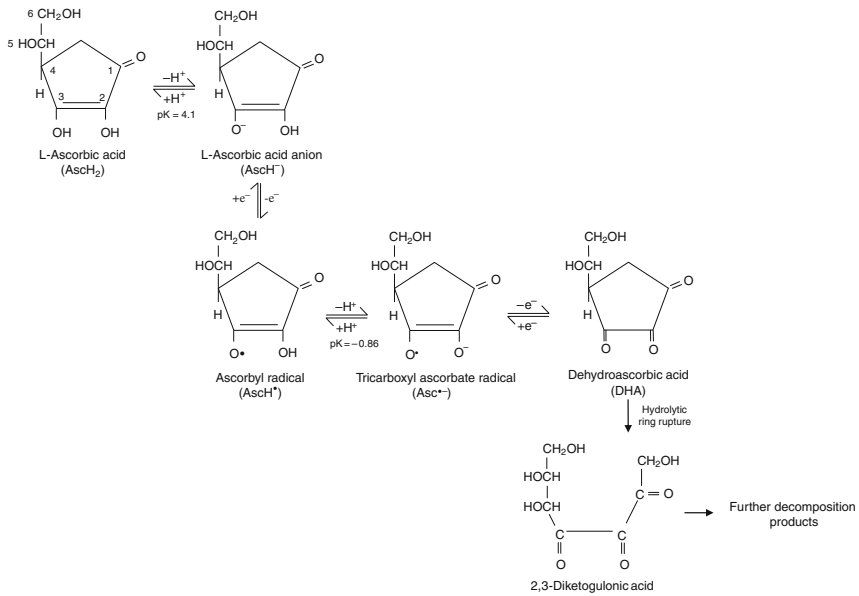


Figure 12.5. L-Ascorbic acid and its sequential oxidation.

and since pK_{a1} at C_3 is 4.17 and pK_{a2} at C_2 is 11.79, a monoanion is the favoured form at physiological pH. For example, at pH 7.4, 99.95% of AA is present as ascorbate monoanion ($AscH^-$), 0.05% as AA ($AscH_2$) and 0.004% as ascorbate dianion (Asc^{2-}) (Bors and Buettner, 1997). Thus, the antioxidant chemistry of vitamin C is the chemistry of $AscH^-$. The asymmetric carbon 5 atom allows two enantiomeric forms, of which the L-form is naturally occurring. Oxidation of AA takes place as either two one-electron transfer processes or a single two-electron reaction without detection of the intermediate ascorbyl radical. In the two one-electron oxidation process, the first step involves loss of one electron from $AscH^-$ to form the neutral ascorbyl radical ($AscH^{\cdot}$) which is not protonated in biological systems, and will be present as the resonance-stabilized tricarbonyl ascorbate free radical ($Asc^{-\cdot}$), which is relevant in biology (Bors and Buettner, 1997). $Asc^{-\cdot}$ is a weakly reactive radical, and in vivo it is likely that reducing enzymes are involved in its removal, resulting in the recycling of ascorbate. Loss of an additional electron yields L-dehydroascorbic acid (DHA). The oxidation of AA to DHA is reversible via the same intermediate radical process and for this reason DHA also exhibits biological activity, since it can be easily converted to AA in the human body. However, DHA is highly unstable because of the susceptibility to hydrolysis of the lactone bridge. DHA has a half-life in aqueous solutions at 37°C of approximately 6–20 min as a function of concentration and catabolism beyond DHA is enhanced by alkaline pH and metals, especially copper and iron (Washko *et al.*, 1993). Hydrolysis of DHA irreversibly forms 2,3-diketogulonic acid and leads to loss of vitamin C activity (Figure 12.5). Further catabolism leads to the formation of a wide array of other nutritionally inactive products such as L-xylonic acid, L-lyxonic acid, L-xylose, oxalic acid and L-threonic acid (Bors and Buettner, 1997). The rate of oxidative degradation of the vitamin is a non-linear function of pH because the various ionic forms of the AA differ in their susceptibility to oxidation: fully protonated $AscH_2 < AscH^- < Asc^{2-}$ (Buettner, 1988). Under conditions relevant to most biological systems, the pH dependence of oxidation is governed mainly by the relative concentrations of $AscH_2$ and $AscH^-$ species and this, in turn, is governed by pH (pK_{a1} 4.17).

Several methods have been developed for determining the amount of vitamin C in biological specimens, foods and pharmaceutical products in which spectrophotometric, fluorometric, chromatographic and electrochemical techniques are used. High-performance liquid chromatography (HPLC), using a UV detector, is currently the most commonly used technique for the analysis of ascorbic acid in foods (Esteve *et al.*, 1995; Perez-Vicente *et al.*, 2002; Sanchez-Moreno *et al.*, 2003; Romeu-Nadal *et al.*, 2006b). Some HPLC methods require electrochemical (Margolis and Schapira, 1997) or fluorometric detection (Nielsen *et al.*, 2001) because of the low absorptivity of DHA

in the ultraviolet range of the spectrum. A HPLC/UV method for determining total vitamin C (AA and DHA) and ascorbic acid in human milk has recently been described (Romeu-Nadal *et al*., 2006b). The National Institute of Standards and Technology (NIST) has developed food matrix reference materials and methods for the measurement of naturally occurring levels of vitamins C, A and E (Sharpless *et al*., 2000).

12.6.2. Absorption, Metabolism and Excretion

In rats and hamsters (for which AA is not a vitamin), intestinal absorption is passive (Bender, 1999). In the case of guinea pigs and humans, both of which have an absolute requirement for exogenous AA in their diet, there is a sensitive sodium-dependent saturable active transport system for AA in the brush border of the duodenum and upper ileum and another sodium-independent transfer process in the basolateral membrane (Bates and Heseker, 1994; Rumsey and Levine, 1998). There is also a passive transport mechanism, which in humans is only predominant at high intake levels. Ascorbate transport has been specifically shown to require metabolic energy, with a stoichiometry for Na^+ from 1.1 to 2.1 (Rumsey and Levine, 1998). Intestinal absorption of AA and its entry into cells are facilitated by conversion to DHA, which is transported across cell membranes more rapidly than AA (Jacob, 1999). Transport of DHA is primarily Na^+-independent in tissues from animals and humans and does not require metabolic energy (Rumsey and Levine, 1998). Upon cell entry, DHA is reduced immediately to AA, which produces an effective gradient of DHA across the membrane (Washko *et al*., 1993; Welch *et al*., 1995). Intracellular reduction of DHA to AA is mediated by two major pathways: chemical reduction by glutathione and enzymatic reduction (Rumsey and Levine, 1998) .

Information on the bioavailability of vitamin C in foods is limited. It is generally agreed that at relatively low intakes (less than 30 mg/day), AA is nearly completely absorbed, and 70–90% of the usual dietary intake of ascorbate (30–180 mg/day) is absorbed (Jacob, 1999). Similar levels of absorption (~80%) have been reported for pure ascorbate, ascorbate in orange juice and ascorbate in cooked broccoli, which suggests that the absorption of vitamin C is almost complete. Based on a pharmacokinetic model, ascorbate bioavailability from a liquid solution given in the fasted state was 90% for $\leq$200 mg, 73% for 500 mg and 40% for 1250 mg (Graumlich *et al*., 1997). Following absorption, ascorbic acid circulates freely in plasma, leukocytes and red cells and enters all tissues, with maximum concentrations of 68–86 μmol/l plasma being achieved with an oral intake of 90–150 mg/day (Thurnham, 2000). Excess is excreted by the kidney, which conserves the vitamin at plasma levels of up to 46–86 μmol/l by a saturable, sodium-dependent reabsorption process

(Thurnham, 2000). The upper limit of plasma ascorbic acid concentration is controlled by the gastrointestinal absorption and renal reabsorption mechanisms, and fasting plasma concentration rarely exceeds 100 μmol/l, even with dietary supplementation (Graumlich *et al*., 1997). However, following a large (>500 mg) oral dose, plasma level can increase several fold, and may approach 200 μmol/l (Benzie and Strain, 1997).

Vitamin C concentration varies widely in different blood cell types. About 70% of blood-borne ascorbate is in plasma and erythrocytes (Bender, 1999). The remainder is in white cells, which have a marked ability to concentrate ascorbate; mononuclear leucocytes achieve 80-fold concentration, platelets 40-fold and granulocytes 25-fold, compared with plasma concentration. Many tissues also appear to have an increased need to conserve ascorbate (Bates and Heseker, 1994). Tissue-specific cellular mechanisms of transport and metabolism allow for wide variation of tissue ascorbate concentration in order to enhance its function as an enzyme cofactor and antioxidant (Wilson, 2005). Specific proteins mediate the entry and exit of vitamin C in cells by facilitated diffusion or active transport. These cellular transport systems are responsible for high inter-tissue ascorbate levels found in the pituitary and adrenal glands (30–400 mg/100 g tissue), followed by the brain, spleen, pancreas, kidney, liver and in the tissues of the eye with between 10 and 50 mg/100 g of tissue. Intracellularly, and in plasma, vitamin C exists predominantly in the free form as $AscH^-$. DHA is either not detectable or found only at very low levels in the circulation of healthy people (Rumsey and Levine, 1998).

In all species studied, ascorbate is filtered at the glomerulus and is actively reabsorbed at the proximal tubules, in a concentration-dependent manner, by an ascorbate transport protein. When the transport protein is saturated, the remaining vitamin C is not transported, but excreted in the urine. Ascorbate is excreted in the urine at an ingested dose above 100 mg/day (i.e. at plasma concentrations above ~60 μmol/l, with plasma saturated at 70% and white blood cells saturated at 100%). At 100 mg/day, approximately 25% of AA is excreted, while above 500 mg/day almost all AA is excreted (Levine *et al*., 1999).

12.6.3. Antioxidant Activity of Ascorbic Acid

Ascorbate is often called the outstanding antioxidant. In chemical terms, this is simply a reflection of its redox properties as a reducing agent. Ascorbate is a reversible biological reductant and as such provides reducing equivalents for a variety of biochemical reactions and is considered the most important antioxidant in extracellular fluids (Sies *et al.*, 1992). Ascorbate is thermodynamically close to the bottom of the list of one-electron reducing

potentials of oxidizing free radicals ($E^{o'} = +282$ mV (Buettner, 1993). For this reason, ascorbate is the first line of defence against ROS and RNS in plasma (Briviba and Sies, 1994). It efficiently scavenges all oxidizing species with a greater one-electron potential (higher $E^{o'}$ values), which includes $O_2^{\cdot -}$ (rate constant 2.7×10^5 l/mol/s), $HO^{\cdot}$ (rate constant $\sim 10^9$ l/mol/s), $^1O_2$ (rate constant 10^7 l/mol/s), water-soluble $LOO^{\cdot}$ (rate constant $= 2 \times 10^8$ l/mol/s), ^-OCl and thiyl radical (Briviba and Sies, 1994). Overall, ascorbate is reactive enough to affectively interrupt oxidants in the aqueous phase before they can attack and cause detectable oxidative damage to DNA and lipids. In aqueous solutions, ascorbate also scavenges RNS efficiently, preventing nitrosation of target molecules.

Ascorbate may also restore α-TOH by re-reducing $TO^{\cdot}$ to its native state. Ascorbate has a lower redox potential ($E^{o'} = +282$ mV) than α-TOH ($E^{o'} = +500$ mV) and, in addition, the α-$TO^{\cdot}$ is at the membrane–water interface, thereby allowing water-soluble ascorbate access to membrane-bound α-$TO^{\cdot}$ for the repair reaction and recycling of α-tocopherol (Frei *et al.*, 1989; Buettner, 1993; May *et al.*, 1999):

$$AscH^- + \alpha - TO \xrightarrow{\Delta E^{o'} = +220\,mv} Asc^{\bullet -} + \alpha - TOH$$

The rate constant for the reaction is 1.5×10^6 l/mol/s.

The resulting radical ($Asc^{\cdot -}$) dismutates to dehydroascorbate (Buethner, 1993) and is then regenerated to ascorbate at the expense of glutathione, dihydrolipoate, thioredoxin and enzyme systems. This process allows for the transportation of a radical load from a lipophilic compartment to an aqueous compartment where it is taken care of by efficient enzymatic defences (Diplock *et al.*, 1998).

It should be noted that as a reducing agent, ascorbate has the ability to reduce Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^+, thereby increasing the prooxidant activity of the metals and generating $HO^{\cdot}$, $O_2^{\cdot -}$ and H_2O_2 that initiate lipid peroxidation in biological systems (Buettner and Jurkiewicz, 1996). It is considered unlikely that ascorbate shows prooxidant properties in vivo since the concentrations of 'free' transition metals in healthy biological systems are very small because they are effectively bound by metal ion storage and transport proteins.

12.6.4. Biological Functions

Many of the biological functions of ascorbic acid are based on its ability to provide reducing equivalents for a variety of biochemical reactions (Buettner, 1993). The vitamin can reduce most physiologically relevant

reactive species and, as such, functions primarily as a cofactor for reactions requiring a reduced iron or copper metalloenzyme and as a protective antioxidant that operates in the aqueous phase both intra- and extracellularly (Halliwell and Whiteman, 1997). Ascorbate is known to be a specific electron donor for eight human enzymes (Levine *et al*., 1999); three enzymes participate in collagen hydroxylation, two in carnitine biosynthesis and three in hormone and amino acid biosynthesis. Evidence also suggests that ascorbate plays a role in or influences collagen gene expression, cellular procollagen secretion and the biosynthesis of other connective tissue components besides collagen (Ronchetti *et al*., 1996).

In the body, ascorbate acts as a cofactor for several metal-dependent oxidation reactions, catalysed by both monooxygenases and dioxygenases (Englard and Seifter, 1986). Other cofactors required by the dioxygenases are Fe^{2+}, α-ketoglutarate and O_2, whereas the monoxygenases require Cu^+ and O_2 for activity (Padh, 1991; Phillips and Yeowell, 1997). Ascorbate functions as a reductive cofactor for post-translational hydroxylation of peptide-bound proline and lysine residues during the formation of collagen. The hydroxyproline is required for normal triple-helical backbone structure and the hydroxylysine cross-linkages are needed for normal collagen fibre formation (Burri and Jacob, 1997). In these reactions, ascorbate reactivates the enzymes by reducing the metal sites of prolyl (iron) and lysyl (copper) hydroxylases (Tsao, 1997). A deficiency of ascorbate results in a weakening of collagenous structures, causing tooth loss, joint pains, bone and connective tissue disorders and poor wound healing, all of which are characteristic of scurvy (Burri and Jacob, 1997). Ascorbic acid is required as a cofactor for the copper-containing dopamine-β-monooxygenase enzyme which catalyses hydroxylation of the dopamine side chain to form norepinephrine (Burri and Jacob, 1997). Ascorbate provided electrons for reduction of molecular oxygen, transferred by copper to dopamine, and hydrogen atoms to reduce the other oxygen to water. The active enzyme contains Cu^+, which is oxidized to Cu^{2+} during hydroxylation of the substrate: reduction back to Cu^+ specifically requires ascorbate, which is oxidized to $AscH^{\cdot}$. Depression, hypochondria and mood changes frequently occur during scurvy and could be related to deficient dopamine hydroxylation. Ascorbic acid also appears to be involved in the hydroxylation of tryptophan to form serotonin in the brain and in the degradation of tyrosine by *p*-hydroxyphenylpyruvate hydroxylase.

Carnitine plays a central role in transporting long-chain fatty acids across the mitochondrial membrane wherein β-oxidation provides energy to cells, especially for cardiac and skeletal muscles (Burri and Jacob, 1997). The biosynthesis of carnitine involves the methylation of lysine with methionine as methyl donor and requires ascorbate, ferrous iron, vitamin B_6 and niacin as cofactors for various enzymes of the pathway. The loss of fatty acid-based

energy production because of limited carnitine biosynthesis may explain the fatigue and muscle weakness observed in humans with ascorbic acid deficiency.

Ascorbate is also involved in the hepatic microsomal hydroxylation of cholesterol in the conversion and excretion of cholesterol as bile acids via 7α-hydroxycholesterol (Tsao, 1997). These reactions require the microsomal enzymatic system containing cytochrome P-450 hydroxylase. Impaired cholesterol transformation to bile acids causes cholesterol accumulation in the liver and blood and atherosclerotic changes in coronary arteries. Hydroxylation and demethylation of aromatic drugs and carcinogens by hepatic cytochrome P-450 appears to be enhanced by reducing agents such as ascorbate (Tsao, 1997). Ascorbic acid is the most potent enhancer of non-haem iron absorption, both in its natural form in fruits and vegetables and when added as the free compound (Hallberg *et al*., 1987; Hurrell, 1992). In addition, ascorbic acid increases the bioavailability of all fortification compounds.

12.6.5. Ascorbate and Chronic Disease

The oxidation of LDL particles and the accumulation of oxidized LDL in the vessel wall are key early events in the progression of atherosclerosis (Parthasarathy *et al*., 1999; Binder *et al*., 2002). High plasma concentrations of ascorbate not only correlate with lower concentrations of oxidized LDL (Carr *et al*., 2000b) but also function to protect endothelial cells against the detrimental effects of oxidized LDL once this is formed (Siow *et al*.,1999). Since ascorbate is water soluble and is not incorporated in LDL particles, it has been proposed that it may prevent the oxidation of LDL particles by scavenging free radical species in the aqueous milieu, and it is also capable of regenerating α-TOH from α-TO$^{\cdot}$, which is formed on inhibition of lipid peroxidation by vitamin E (Carr *et al*., 2000c). Ascorbyl radicals formed in this process may be reduced to ascorbate by dismutation, chemical reduction or enzymatic reduction.

Increased risk of chronic disease, including coronary heart disease, cancer and cataracts, is associated with low intake or plasma concentrations of vitamin C (Riemiersma, 1994; Gey, 1995). Plasma ascorbate levels were inversely related to mortality from all causes, and from cardiovascular disease and ischaemic heart disease in men and women in the EPIC-Norfolk prospective study (Khaw *et al*., 2001). A 20% fall in risk of all-cause mortality, independent of other risk factors, was associated with a 20 μmol/l rise in ascorbate, approximately equivalent to a 50 g/day increase in fruit and vegetable intake. Boekholdt *et al*. (2006) observed that a high plasma concentration of ascorbate was inversely related to various cardiovascular

risk factors. Compared with people in the lowest quartile of the ascorbate distribution, those in the highest quartile had a 33% lower risk of coronary artery disease, independent of other known risk factors, including age, blood pressure, plasma lipids, cigarette smoking, body mass index and diabetes.

Consistent evidence from epidemiological studies indicates that high intakes of fruits and vegetables are associated with reduced risk of certain cancers (Block *et al*., 1992; Steinmetz and Potter, 1996). Recent reports from the United States (Loria *et al*., 2000) and the EPIC-Norfolk prospective study (Khaw *et al*., 2001) suggest that men with a low serum ascorbate concentration may have an increased risk of mortality, probably because of an increased risk of dying from cancer. In contrast, serum ascorbate concentration was not related to mortality among women.

12.6.6. Vitamin C in Milk and Milk Products

The mean content of vitamin C in cow's milk is 2.11 mg/100 g (range 1.65–2.75 mg/100 g) (Walstra and Jenness, 1984), 5.48 mg/100 ml in goat's milk (Kondyli *et al*., 2007), 2.49 mg/100 ml in camel's milk (Mehaia, 1994) and 3.9 ($\pm$ 1.05) mg/100 ml (summer milk) and 3.02 ($\pm$ 2.01) mg/100 ml (winter milk) in humans (Tawfeek *et al*., 2002). The mean concentration of vitamin C in human milk is also affected by the stage of lactation and declined from 6.18$\pm$0.09 mg/100 ml in colostrum to 4.68$\pm$0.1 mg/100 ml at 9 months (Salmenpera, 1984). The influence of maternal vitamin C intake and its effect on the vitamin C content of human milk has not been defined clearly. Byerley and Kirksey (1985) observed that the vitamin C level in human milk did not increase significantly in response to increasing maternal intake (up to tenfold) of the vitamin. The authors proposed that a regulatory mechanism may be present in mammary cells to prevent an elevation in the concentration of vitamin C in milk beyond a certain saturation level. On the other hand, when the intake of vitamin C is low, the level in breast milk is sensitive to supplementation. Ortega *et al*. (1998) observed a relationship between ascorbic acid intake and serum and milk levels. However, the changes in breast milk ascorbate level were less pronounced than those in serum. The breast milk ascorbate to serum ascorbate ratio decreased with increasing intake, and with serum vitamin C level, which suggests that mammary tissue becomes saturated with the vitamin when intake is high. The plasma concentration of vitamin C in breast-fed infants was generally in the normal range, indicating that exclusively breast-fed infants are well protected against vitamin C deficiency (Salmenpera, 1984). Breast-fed infants appear to be capable of maintaining a high plasma concentration of vitamin C independently of maternal and milk concentrations.

There is some evidence that the concentration of vitamin C in cow's milk (Andersson and Oste 1994; Lindmark-Mansson and Akesson, 2000) and goat's milk (Kondyli *et al.*, 2007) changes with season. Andersson and Oste (1994) found that in raw milk sampled in March or August, the concentration of vitamin C was higher (2.0–2.7 mg/100 ml) than in samples collected in October (1.2 mg/100 ml). Lindmark-Mansson and Akesson (2000) reported that mean values for vitamin C were higher in July and September than in January and March.

Ascorbic acid is considered to be the first line of defence against oxidizing species and consequently appears to be the vitamin lost most readily in milk and cooking of foods (Bates, 1997). Loss of vitamin C is determined primarily by the concentration of O_2 dissolved in milk and is greatly accelerated by exposure to light and the presence of heavy metals such as Cu and Fe (Scott *et al.*, 1984). Andersson and Oste (1992a, b) observed that at a low oxygen content (0.6 ppm), ascorbic acid decreased by about 50% after 1–2 weeks' storage with somewhat higher stability at 7°C than at 23 or 35°C. At oxygen contents of 3.5–5.4 ppm the loss was much greater.

12.6.7. Vitamin C Status and Requirements

In setting values for average population requirements and individual nutrient intakes, the important question is, how do we differentiate between preventing deficiency symptoms, ensuring an adequate intaike and promoting optimal intake for the prevention of disease? The recommended dietary allowance (RDA) of 60 mg/day in the United States (Food and Nutrition Board, 1989), the reference nutrient intake (RNI) of 40 mg/day in the United Kingdom (Department of Health, 1991) and the population reference intake (PRI) of 45 mg/day in the EU were aimed at prevention of the clinical deficiency state, scurvy. The Food and Nutrition Board (2000) established an estimate average requirement (EAR) for vitamin C, which is the nutrient intake value that is estimated to meet the requirements of half of a specific gender and life-stage group, and was based on evidence that 75 mg/day vitamin C can maintain near-maximal neutrophil concentration with minimal urinary loss (Levine *et al.*, 1996). Thus, the EAR for men aged 19–50 years is 75 mg/day, with a value of 60 mg/day for women, based on women having less lean body mass and body water and a smaller body size than men. There are no data on the distribution of vitamin C requirements in healthy adults; therefore the US Food and Nutrition Board (2000) recommended daily allowance (RDA) for vitamin C, which is the intake value considered to meet the requirements of 97.5% of the relevant life-stage and gender population group, be set at 90 mg/day for men and 75 mg/day for women (RDA = EAR + 2CV), assuming a coefficient of variation (CV) of 10%. There is

evidence to show that an average intake of 90 mg/day of vitamin C can maintain a plasma ascorbate concentration at 50 μmol/l (Jialal *et al*., 1990). A threshold 'potential protective plasma level' of 50 μmol/l has also been proposed by Gey (1995). This concentration has been shown to inhibit plasma LDL oxidation in vitro and may have relevance for the prevention of heart disease in vivo (Jialal *et al*., 1990). Smokers have been recommended by the Food and Nutrition Board (2000) to consume an additional 35 mg over and above the RDA value, but this recommendation has not been made explicit in other countries. Excessive consumption of vitamin C is unusual, and the upper intake level (UL) set by the Food and Nutrition Board (2000) is 2000 mg/day, which is achievable only by using chronic mega doses of concentrated vitamin C supplements.

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13

Vitamins in Milk and Dairy Products: B-Group Vitamins

D. Nohr and H.K. Biesalski

13.1. Introduction

The B-group of vitamins is composed of up to eight vitamins; however, in some cases pantothenic acid (B5), biotin (B7; vitamin H) and folates are described without mentioning them as of the members B-group.

In this chapter, all eight B-group vitamins will be described in detail (e.g. synonyms, structure and function, food sources and recommended daily uptake) and their concentrations in the various dairy products will be discussed. At the end of the chapter, a few selected and actual meta-analyses on the function of more than one B-vitamin in epidemiological or clinical studies will be presented. It must be kept in mind that, under normal conditions in developed countries, only marginal risks for deficiencies of B-vitamins exist; however, there are special risk groups (e.g. critically ill patients, long-term tube-fed patients, breast-fed babies and children, pregnant women and elderly people) who may develop such deficiencies. Therefore, clinicians, especially pediatricians, should develop a basic understanding of the clinical manifestations of deficiencies which often exhibit first symptoms on the skin (Heath and Sidbury, 2006).

The B-group vitamins are

B1 Thiamine
B2 Riboflavin
B3 Niacin
B5 Pantothenic acid

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B6 Pyridoxine
B7 Biotin
B9 Folate
B12 Cobalamin

13.2. Thiamine (Vitamin B1)

Thiamine is a water-soluble vitamin, is unstable and thus loses its biological activity in alkaline solutions (pH >7) as well as in the presence of oxidants and radiation. The chemical name of thiamine is 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium; its co-enzyme form is thiaminepyrophosphate (TPP; Figure 13.1).

In pharmaceutical and other preparations, thiamine is used in the form of water-soluble thiazolium salts (thiamine chloride hydrochloride, thiamine mononitrate); there are also synthetic lipophilic derivatives (the so-called allithiamins). The latter can pass through biological membranes more easily and in an almost dose-related manner, thus offering a possibility to develop thiamine stores by supplementation, which under normal circumstances are low and sufficient for only 4–10 days (Biesalski and Back, 2002a).

In the presence of oxidizing agents and in strongly alkaline solutions, thiamine is converted to thiochrome, a fluorescent substance used to determine the thiamine content of feeds, foods or pharmaceutical preparations (Alonso *et al.,* 2006).

13.2.1. Thiamine Function

A number of enzymes involved in intermediary metabolism and playing a role in the oxidative decarboxylation of alpha-keto acids require TPP as co-enzyme (pyruvate-dehydrogenase complex; alpha-ketoglutarate dehydrogenase complex; branched chain alpha-keto acid dehydrogenase complex). Thus, metabolites from carbohydrate metabolism and keto analogues from amino

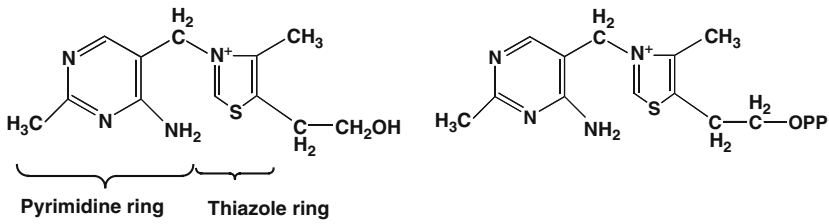


Figure 13.1. Structure of thiamine (*left*) and thiaminepyrophosphate (TPP, *right*).

and fatty acid metabolism are made available for energy metabolism. In addition, the TPP-dependent transketolase is involved in the formation of NADPH and pentose in the pentose phosphate pathway; both metabolites play important roles in several other synthetic pathways. There are hints that the above-mentioned enzymes are also involved in neural functions; however, the exact mechanisms of action need to be elucidated further. Interestingly, Carvalho *et al*. (2006) described a decrease of glutamate uptake in the prefrontal cortex of thiamine-deficient mice.

13.2.2. Sources of Thiamine

Tables 13.5 and 13.6 show the thiamine content of a number of foods. Table 13.6 focuses on dairy products, including milk from different species that are consumed by humans. It has to be taken into account that heat treatment, as well as storage conditions, can lead to losses of the thiamine content of the foods: low pasteurization leads to a loss of 3–4%; boiling 4–8%; spray-drying 10%; roller drying 15%; pasteurization 9–20%; condensed milk 3–75%; sterilization 20–45%; and evaporation of milk 20–60%.

Fresh milk in dark bottles loses 24% of its initial thiamine content on storage for 24 h at 4°C,14% on storage at 12°C and 16% on storage at 20°C. Evaporated milk looses 15–50% over periods >12 months; spray-dried whole milk shows no change after up to 12 months. Thiamine is lost during cheese manufacture, mainly during drawing of the first whey; no significant changes are found during maturation (cf. Biesalski and Back, 2002a).

UV light-induced inactivation of thiamine can, under certain conditions (cheese, fresh milk), be counterbalanced by thiamine-synthesizing microorganisms (Biesalski and Back, 2002a). Modern high-pressure-assisted thermal sterilization methods result in almost stable vitamins, although the decay in model solutions (acetate-buffered, pH 5.5) was about 30 times higher than in minced pork (Butz *et al*., 2007). Thus, a general extrapolation of the test results to routine food preparation needs further investigations.

The recommended daily uptake of thiamine given by the DGE (Deutsche Gesellschaft für Ernährung, 2007) is shown in Table 13.1.

13.2.3. Thiamine Deficiencies

Due to the relatively small and short-lasting thiamine stores, marginal deficiencies are quite common, but early symptoms are rarely recognized.

Symptoms of thiamine deficiency are cardiac failure, muscle weakness, peripheral and central neuropathy and gastrointestinal malfunction (Biesalski, 2004). Reasons for deficiency, besides a thiamine-free diet (e.g. parenteral nutrition; Attard *et al*., 2006), might be reduced absorption

Table 13.1. Recommended daily uptake of thiamine

	Thiamine (mg day^{-1})	
Age	Male	Female
Sucklings <4 months	0.2	
Sucklings 4–12 months	0.4	
Children 1–4 years	0.6	
Children 4–7 years	0.8	
Children 7–10 years	1.0	
Children 10–13 years	1.2	1.0
Children 13–15 years	1.4	1.1
Adults 15–25 years	1.3	1.0
Adults 25–51 years	1.2	1.0
Adults 51–65 years	1.1	1.0
Adults >65 years	1.0	1.0
Pregnant		1.2
Breast feeding		1.4

From DGE (2007)

(gastrointestinal diseases), impaired transport, increased requirements (pregnancy, lactation, infancy, childhood, adolescence, increased physical activity, infections, trauma and surgery) or increased losses and impaired biosynthesis of TPP.

Clinically manifest deficiency appears in several forms of an illness called beriberi (cf. Lonsdale, 2006; Nohr, 2009c), which is nowadays mostly a problem in some regions of southeast Asia, mainly due to the consumption of thiamine-free rice, raw fish (contains thiaminase) or chewing of betel-nut or fermented tea leaves (which contain "anti-thiaminic" tannins). Another risk group comprised chronic alcoholics who often consume low-quality meals, have poor appetite and suffer from gastrointestinal problems and malabsorption. One can differentiate infantile beriberi (often lethal in sucklings fed by thiamine-deficient mothers) from two forms of adult beriberi: dry beriberi is characterized by peripheral neuropathy ("burning feet syndrome", exaggerated reflexes, diminished sensation and weakness in all limbs, muscle pain, problems rising from squatting position and, in severe cases, eventually seizures). Wet beriberi is characterized by cardiovascular symptoms (rapid heart rate, enlargement of the heart, oedema, breathing problems and ultimately congestive heart failure). "Cerebral" beriberi mostly leads to Wernicke's encephalopathy and to Korsakoff's psychosis, both together appearing as Wernicke–Korsakoff syndrome which, however, is not easily diagnosed but can be treated by thiamine supplementation (Meier and Daeppen, 2005).

13.2.4. Thiamine Supplementation

For the therapeutic treatment of diseases of the central (CNS) and the peripheral nervous system (PNS), as well as of exhaustion and during cytostatic treatment, doses of 50–200 mg thiamine day^{-1} are administered orally (Harper, 2006). Clinically manifest beriberi is treated by 50–100 mg day^{-1} subcutaneously or intravenously for several days, followed by the same dose orally for several weeks. Besides single cases of anaphylactic shock after intravenous application, no side effects of higher doses of thiamine (e.g. up to 200 mg day^{-1}) are known (Bitsch, 2002).

13.3. Riboflavin (Vitamin B2)

The chemical name for riboflavin is 7,8-dimethyl-10-(1′- D-ribityl)isoalloxazine; riboflavin exists in an oxidized and a reduced form (Figure 13.2) and from which two co-enzymes are formed, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD; Figure 13.3).

The ending "flavin" refers to its yellowish colour (latin "flavus" = yellow).

Free, as well as protein-bound riboflavin, occurs in the diet, and milk in general is the best source. In cow's milk, the free form, with the higher bioavailability, is the major one (61% riboflavin, 26% FAD, 11%

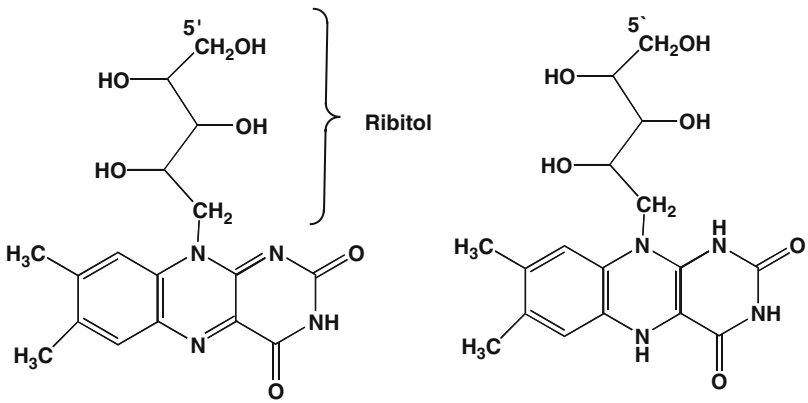


Figure 13.2. Structures of oxidized (flavoquinone, *left*) and reduced (flavohydroquinone, *right*) forms of riboflavin (vitamin B2).

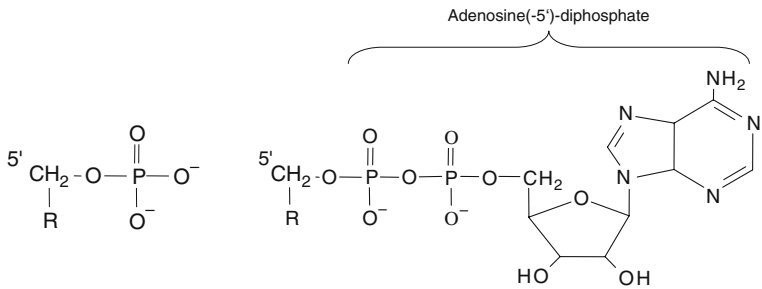


Figure 13.3. Structure of flavinmononucleotide (FMN, *left*) and flavin adenine dinucleotide (FAD, *right*). R: riboflavin + ribose.

hydroxyethyl form and others) whereas the protein-bound, and thus the less bioavailable, form predominates in other foods. In human breast milk, approximately one to two thirds of riboflavin occurs as FAD.

Riboflavin is very heat stable but it is extremely photosensitive. It is photodegraded to lumiflavin (under alkaline conditions) or lumichrome (under acidic conditions) (Ahmad *et al*., 2006), both of which are biologically inactive. UV light excites riboflavin to a high degree of natural fluorescence that is used for its detection and determination in yogurt or non-fat dry milk (Liu and Metzger, 2007).

13.3.1. Functions of Riboflavin

Riboflavin-dependent enzymes are called flavoproteins or flavoenzymes because of their yellowish appearance. They catalyse hydroxylations, oxidative decarboxylations, dioxygenations and reduction of oxygen to hydrogen peroxide, serving as electron carriers, mediators of electron transfer from pyridine nucleotides to cytochrome c or to other one-electron acceptors and as catalysts of electron transfer from a metabolite to molecular oxygen (Biesalski and Back, 2002b). The two flavin-containing co-enzymes, FMN and FAD, play major roles in the metabolism of glucose, fatty acids, amino acids, purines, drugs and steroids, folic acid, pyridoxine, vitamin K, niacin and vitamin D.

The FAD-dependent enzyme, glutathione reductase, plays a major role in the antioxidant system by restoring reduced glutathione (GSH) from oxidized glutathione (GSSH). GSH is important in protecting lipids from peroxidation and in stabilizing the structure and function of red blood cells; it is the most important antioxidant in erythrocytes and in keeping lens proteins in solution (thus preventing cataracts; Kohlhaas *et al*., 2006).

The formation of FMN and FAD is ATP-dependent and takes place mainly in the liver, kidney and heart. All enzymatic steps are under the control of thyroid hormones:

- Flavokinase: Riboflavin + ATP → flavin mononucleotide + ADP
- FAD pyrophosphorylase: FMN + ATP → flavin adenine dinucleotide + PP
- FAD + apoenzyme/protein → covalently bound flavins

13.3.2. Sources of Riboflavin

Tables 13.5 and 13.6 summarize dietary sources of riboflavin and its concentration, especially in the milk of various species and dairy products.

Heat treatment has only negligible effects on riboflavin concentrations, whereas exposure of milk to sunlight results in a loss of 20–80% of riboflavin. Thus, storage in dark bottles, light-tight wax cartons or special polyethylene terephthalate (PET) bottles is recommended (Mestdagh *et al.*, 2005).

The photodegradation of riboflavin catalyses photochemical oxidation and a loss of ascorbic acid. Gamma radiation of 10 Gy destroys about 75% of riboflavin in liquid milk; while milk powder shows no losses even at higher doses.

Storage influences riboflavin concentrations as follows: condensed milk looses 28 or 33% of its initial riboflavin content when stored at 8–12°C for 2 years or 10–15°C for 4 years, respectively; ice-cream looses 5% when stored at –23°C for 7 months. No losses were found in fresh milk stored at 4–8°C for 24 h or in dried milk powder stored for 16 months.

In cheese, most of the losses (66–88%) of the original riboflavin of the milk appear to occur during whey drainage, while ripening has almost no effects. However, in some cheese varieties, the concentration is higher in the outer layers due to microbial synthesis. High-pressure tests for thermal sterilization processes led to different results for the decay of the vitamin dependent on the matrix of the respective food tested.

13.3.3. Riboflavin Deficiency

Riboflavin is essential for humans, animals and some microorganisms. Among humans, seniors and adolescents seem to be at particular risk of deficiency (Powers, 2003); the recommended uptake is given in Table 13.2. In some cases, recommended uptake is related to energy intake and 0.6 mg riboflavin per 1000 kcal is considered adequate. Milk and milk products (excluding butter) can contribute about 30% of total riboflavin supply (Scholz-Arens, 2004).

Table 13.2. Recommended daily uptake of riboflavin

	Riboflavin (mg day^{-1})	
Age	Male	Female
Sucklings <4 months	0.3	
Sucklings 4–12 months	0.4	
Children 1–4 years	0.7	
Children 4–7 years	0.9	
Children 7–10 years	1.1	
Children 10–13 years	1.4	1.2
Children 13–15 years	1.6	1.3
Adults 15–25 years	1.5	1.2
Adults 25–51 years	1.4	1.2
Adults 51–65 years	1.3	1.2
Adults >65 years	1.2	1.2
Pregnant		1.5
Breast feeding		1.6

From DGE (2007)

The major portion of riboflavin is bound to proteins and these flavoproteins have to be hydrolysed before absorption by specialized transporters in the upper gastrointestinal tract. The amount that can be stored depends on the availability of proteins providing binding sites. Although a limited uptake makes sense in preventing accumulation in tissues, it increases the body's dependence on dietary supply. Under normal conditions, riboflavin stores last for 2–6 weeks, but in cases of protein deficiency, they last significantly shorter.

Symptoms of a marginal deficiency are often non-specific: weakness, fatigue, mouth pain, glossitis, stomatitis, burning and itching of the eyes, personality changes. Signs of increased deficiency are cheilosis, angular stomatitis, seborrheic dermatitis at the mouth, nasolabial sulcus and ears (later extending to the trunk and extremities), desquamative dermatitis with itching in genital regions, opacity of the cornea, cataract and brain dysfunction (cf. Bitsch, 2002; Biesalski, 2004). Major reasons for riboflavin deficiency are

- Insufficient dietary intake by seniors and adolescents (mainly girls)
- Endocrine abnormalities, insufficient adrenal and thyroid hormones
- Drugs (psychotropic, antidepressant, cancer therapeutics, antimalarial)
- Alcohol intake, which interferes with digestion of food flavins and absorption
- Agents that chelate or form complexes with riboflavin or FMN affecting their bioavailability: copper, zinc, iron, caffeine, theophylline, saccharine, nicotinamide, ascorbic acid, tryptophane, urea.

As riboflavin (via FAD-dependent glutathione reductase) is involved in antioxidant mechanisms, riboflavin deficiency may considerably affect erythrocyte metabolism. However, several studies reported protective effects of a deficiency against malaria infections. A study in the USA showed, that the intake of yogurt, milk, cereals and also riboflavin was inversely correlated with the homocysteine level in plasma which in turn seems to be positively correlated with a higher risk of developing atherosclerosis (Ganji and Kafai, 2004).

Assessment of the riboflavin (mainly by HPLC methods) status uses the following parameters:

- Erythrocyte glutathione reductase activity coefficient
- Excretion in urine (mg g^{-1} creatinine to assess short-term effects)
- Riboflavin in erythrocytes (mg g^{-1} haemoglobin)

Concerning supplementation, no case of intoxication has been described. Thus, riboflavin is regarded as safe even at high doses. Supplements are usually given to reverse deficiency symptoms or to support high-risk groups:

- Regular intake of drugs (e.g. antidepressants, oral contraceptive)
- Malnutrition
- Patients after trauma
- Malabsorption
- Chronic alcoholics

Hyperbilirubinaemia can be treated much quicker by phototherapy when 0.5 mg riboflavin kg^{-1} bodyweight is given. Finally, persons with congenital methemoglobinaemia might benefit from 20 to 40 mg day^{-1}.

13.4. Niacin (Vitamin B3)

Niacin is the common name for a group of vitamers with a biological activity associated with nicotinamide. These are nicotinamide itself (pyridine-3-carboxamide), nicotinic acid (pyridine-3-carboxylic acid) and a number of pyridine nucleotide structures. Nicotinamide and nicotinic acid are white, crystalline compounds, soluble in water, with a maximum UV absorbance at 263 nm. Both vitamers can be interconverted; their structures are shown in Figure 13.4. Two co-enzymes exist, nicotinamide-adenine dinucleotide (NAD^+) and nicotinamide-adenine dinucleotide phosphate ($NADP^+$), the structures of which are shown in Figure 13.5. These co-enzymes are found at highest concentrations and at high bioavailability in animal food sources, in contrast to nicotinic acid which is found, at lower bioavalability and lower concentrations, mainly in plants. Niacin in cereals is found mainly in the outer layer, bound to the protein,

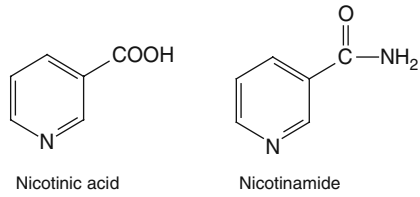


Figure 13.4. Structures of nicotinic acid and nicotinamide.

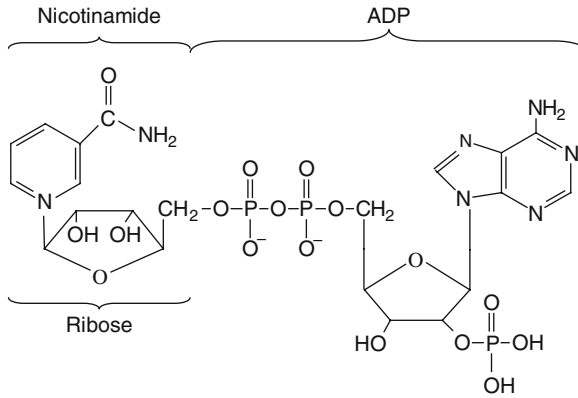


Figure 13.5. Structure of nicotinamide-adenine dinucleotide (NAD^+) and nicotinamide-adenine dinucleotide phosphate ($NADP^+$).

niacytin, and thus having a bioavailability of only 30%. In milk, almost all niacin is in the free form.

Niacin is stable to sunlight, various storage conditions and heat treatments as used in dairy processing. Exposing liquid milk to gamma radiation (10 Gy) leads to a loss of about 30%, while niacin in milk powder survives higher doses of radiation. During cheese production, most niacin passes to the whey which can be compensated partly by an increase of the niacin content in the outer layers due to microbial action during maturation (~10- to 25-fold).

13.4.1. Function of Niacin

The most important functions of niacin are the roles of NAD and NADP as co-enzymes of dehydrogenases (cf. Biesalski and Back, 2002c). The C4 position on the pyridine ring of the nicotinamide part of the molecule

participates in oxidation and reduction reactions by electron transfer. Thus, NADH and NADPH are intermediate hydrogen and electron carriers.

NADP-dependent co-enzymes are involved in cytosolic syntheses (reductive biosynthetic processes), while NAD-dependent enzymes are located in the mitochondria, delivering H_2 to the respiratory chain for oxidation and energy metabolism. NADH is used in the respiratory chain and can be derived from the tricarboxylic acid cycle, glycolysis, the β-oxidation of fatty acids and the degradation of amino acids. NADPH is used for the synthesis of fatty acids, cholesterol and steroids and for hydroxylations and can be derived from the pentose phosphate pathway, photosynthesis, malic enzyme and extramitochondrial isocitrate dehydrogenase.

In addition, NAD can have non-redox functions: the energy provided by breaking the high-energy bond of the glycosidic linkage between nicotinamide and ribose allows the addition of ADP-ribose to a number of nucleophilic acceptors. NAD serves also as a substrate in poly(ADP-ribose) synthesis (important for DNA repair processes) and in mono(ADP-ribosyl)ation reactions (involved in endogenous regulation of various aspects of signal transduction and membrane trafficking in eukaryotic cells).

NADP is the substrate for the formation of nicotinic acid adenine dinucleotide phosphate (NAADP) which is involved in the regulation of intracellular calcium stores. Recent publications describe additional functions of nicotinic acid, exhibiting vasodilatory and antilipolytic effects (Bolander, 2006), and of niacin which lowers plasma levels of C-reactive protein (CRP), a general marker for inflammation that is also involved in cardiovascular diseases (Prasad, 2006). The nicotinic acid receptor is also considered to be a target for the development of dyslipidemic drugs for the prevention and treatment of cardiovascular diseases (Offermanns, 2006).

13.4.2. Niacin Sources

Tables 13.5 and 13.6 give an overview of dietary sources of niacin, especially in milk of various species and dairy products.

13.4.3. Niacin Deficiencies

Due to the major role of the co-enzymes in energy metabolism, their requirement is related to energy intake. The recommended dietary allowance in the USA has been set to 6.6 niacin equivalents (NE; see below) 1000 $kcal^{-1}$ day^{-1}; requirements have been established experimentally for adults and were set at a minimum of 8 mg NE day^{-1}. However, the overall calculation is difficult because the intestinal utilization rates are unknown and, in addition, NAD can be

Table 13.3. Recommended daily uptake of niacin

	Niacin (mg equivalent day^{-1}) 1 mg niacin equivalent = 60 mg tryptophan	
Age	Male	Female
Sucklings <4 months	2 (estimated)	
Sucklings 4–12 months	5	
Children 1–4 years	7	
Children 4–7 years	10	
Children 7–10 years	12	
Children 10–13 years	15	13
Children 13–15 years	18	15
Adults 15–25 years	17	13
Adults 25–51 years	16	13
Adults 51–65 years	15	13
Adults >65 years	13	13
Pregnant (>4 month)		15
Breast feeding		17

From DGE (2007)

synthesized from tryptophan when the latter is sufficiently available. Therefore, the niacin content of the diet is often expressed as niacin equivalents, i.e. 1 NE = 1 mg niacin = 60 mg tryptophan. The tryptophan content of some selected foods as a percentage of total protein is as follows: eggs (1.5%), milk (1.4%), meat products (>1.1%), cereals, fruits, vegetables (~1%) and maize (~0.6%). A "normal, mixed diet" in industrial countries includes about 13 mg NE day^{-1}, thus meeting more than the requirements (see Table 13.3). Milk, as an example, is regarded as a good pellagra-preventing food (see below), not because of its relatively low, niacin concentration but because of its high concentration of tryptophan (see above). Other good sources for niacin are shown in Table 13.5, concerning milk and dairy products in Table 13.6. Side effects due to niacin uptake have been observed only after excess uptake over at least months (Nohr, 2009b).

The main disease resulting from niacin deficiency is called pellagra (Pellagrosis; maidism; mal de la rosa, Saint Ignatius Itch, erythema endemicum; Jolliffe syndrome; Hegyi, 2004; Kumaravel, 2006; Nohr, 2009a). It is characterized by the three Ds: dermatitis, diarrhoea, dementia. The name "pellagra" is derived from "pelle agra" = "rough skin", i.e. the prominent appearance is a rough skin in light-exposed areas with a symmetric appearance with a distinct border to normal skin (glove-like appearance of the hands). Disorders of the gastrointestinal tract (GIT) can include nausea, abdominal pain, increased salivation, soreness of the mouth, inflammation of the mucosa and diarrhoea. Early neurologic disorders are depression,

anxiety and poor concentration; prolonged symptoms are disorientation, confusion and delirium (Biesalski, 2004). The prevalence of pellagra is sporadic in the USA and Europe but higher in poor and malnourished people, alcoholics, during long-time parenteral nutrition with insufficient niacin supplementation, and also in some psychiatric patients. Pellagra is endemic in a few regions of Africa and Asia where maize (corn) represents a major part of the diet, together with very low amounts of meat, fruits and vegetables (Pitche, 2005). Pellagra-like symptoms can also appear during tryptophan deficiency (cf. Hartnup syndrome).

The clinical diagnosis of pellagra is that of the (rough) skin, most reliable in context with GIT symptoms. The laboratory diagnosis is still unsatisfactory in terms of a specific measurement to estimate niacin. Thus, fluorometric assays for the urinary metabolites, *N′*-methyl-2-pyridone-5-corboxamide (2-pyridone) and *Ń*-methyl-nicotinamide (NMN) are used; their quotient should be between 1.3 and 4.0. A decrease indicates deficiency as nowadays also do blood serum levels of niacin below 30 μmol/l.

Acute therapy for pellagra is by oral application of 100–300 mg day^{-1} niacinamide or niacin in three doses; niacinamide causes less side effects (flushing). Mental changes disappear within 24–48 h, skin lesions within several weeks. Concomitant administration of riboflavin and pyridoxine and a diet rich in calories and protein are reasonable.

Nicotinic acid is used as a supplement in the treatment of hyperlipidemia: high doses of up to 5 g day^{-1} resulted in the reduction of total serum cholesterol (–20%), serum triglycerides (–40%) and high-density lipoprotein (HDL; +15%). Such high doses ingested over long periods can lead to some side effects: besides the immediate symptoms (mainly flushing and itching), hepatotoxicity, including elevated liver enzymes and jaundice mainly appear after long-term treatment (3–9 g day^{-1}) for months or years, but have also been described with 750 mg day^{-1} for 2 months. Nicotinamide potentiates the effects of chemotherapy and radiation treatment in fighting tumor cells, which may be attributable to increased blood flow and oxygenation. Interestingly, supplementation with 2 g day^{-1} led to a reduced insulin sensitivity in adults at high risk of insulin-dependent diabetes (Greenbaum *et al*., 1996).

13.5. Pantothenic Acid (Vitamin B5)

The natural form of pantothenic acid (Figure 13.6) and the only stereoisomer with biological activity is D(+)-pantothenic acid. However, the alcohol (D)-panthenol can be converted to pantothenic acid, thus having, although indirectly, also biological activity. The major portion of pantothenic acid in

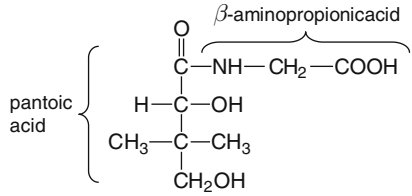


Figure 13.6. Structure of pantothenic acid.

the diet occurs as co-enzyme A (CoA) or pantetheine (cf. Biesalski and Back, 2002d) (Figure 13.7).

Pantothenic acid is a highly hygroscopic, light-yellowish viscous oil that is soluble in water as well as in ethanol. It is stable to heat and light, but otherwise is unstable; thus in pharmaceutical preparations the Na^+ or Ca^{2+} salts of panthenol are usually used (cf. Plesofsky, 2001).

About 50–95% of pantothenic acid occurs as CoA or pantetheine (fatty acid synthetase complex) in the overall diet. In milk, about 25% of pantothenic acid is protein-bound but this value rises to 40–60% in cheese, depending on the type of cheese.

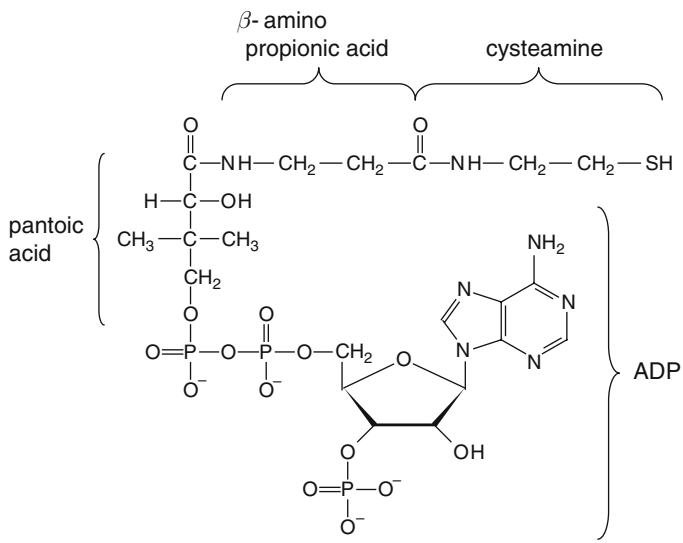


Figure 13.7. Structure of co-enzyme A (CoA) (ADP = adenosine diphosphate).

13.5.1. Function of Pantothenic Acid

The main physiological activity of pantothenic acid is related to that of CoA and pantetheine, being a part of both. The SH group of cysteamine in CoA and pantetheine represents the active site for the binding of acyl or acetyl residues. Furthermore, there is a pantothenate-dependent step in the synthesis of arginine, leucine and methionine.

For the detailed synthesis of pantetheine and CoA and their numerous functions in intermediate metabolism of animal cells (carbohydrates, fatty acids, nitrogenous compounds), the reader is referred to relevant textbooks on biochemistry or physiology or a recent review by Leonardi *et al.* (2005). In brief, CoA plays a role in

- Acylation of proteins
- Internal acetylation of proteins
- N-terminal acetylation of proteins
- Transfer of C2 units released in β-oxidation of fatty acids and oxidative degradation of amino acids
- Transfer of C2 units required for fatty acid synthesis
- Introduction of C2 units in the tricarboxylic acid cycle
- Transfer of fatty acids required for triglyceride and phospholipid synthesis
- Synthesis of isoprenoid-derived compounds
- Synthesis of haemoglobin, cytochromes, acetylcholine, taurine and acetylated sugars

In summary, as a part, especially of CoA, pantothenic acid plays numerous roles in animal and human cellular metabolism.

13.5.2. Pantothenic Acid Sources

Tables 13.5 and 13.6 give an overview of dietary sources of pantothenic acid, especially in the milk of various species and in dairy products; the recommended daily intake is given in Table 13.4. In cheese, the amount of pantothenic acid depends on the level of proteolysis. While Cheddar and Cottage cheese loose large amounts (mainly of the free form) during manufacture, the concentration in some types of cheese (e.g. Limburger, Camembert, Brie) increases due to microbiological synthesis.

13.5.3. Pantothenic Acid Deficiencies

When suffering from a deficiency of pantothenic acid, chickens develop keratitis, dermatitis, degenerations of the spinal cord and a fatty liver, while rats show retarded growth. For humans, only effects that

Table 13.4. Recommended daily uptake of pantothenic acid

Age	Pantothenic acid (mg day^{-1})
Sucklings <4 months	2
Sucklings 4–12 months	3
Children 1–4 years	4
Children 4–7 years	4
Children 7–10 years	5
Children 10–13 years	5
Children 13–15 years	6
Adults 15–25 years	6
Adults 25–51 years	6
Adults 51–65 years	6
Adults >65 years	6
Pregnant	6
Breast feeding	6

From DGE (2007)

occur under experimental conditions are known (e.g. application of an antagonist (ω-methylpantothenic acid); severe malnutrition), as pantothenic acid occurs in almost all kinds of food and true requirements are hard to assess. The first symptoms of a deficiency are headache, fatigue, GIT disturbances, palpitation of the heart, burning feet syndrome (first described in prisoners during World War II in Burma, Japan and the Philippines); prolonged deficiency leads to retarded wound healing, hypotonia and uncoordinated movements (Biesalski, 2004). In general, all symptoms are reversible.

Due to large individual variations, both the levels in blood and the amount in urine are not good indicators for the vitamin status. However, a blood level between 1 and 4 mg l^{-1} is regarded as sufficient. A daily uptake of 4–6 mg day^{-1} is recommended by several nutritional societies because of epidemiological data: 1 mg day^{-1} did not lead to deficiency symptoms in humans. Interestingly, some intestinal bacteria can synthesize pantothenic acid, but this source seems to be ineffective in humans.

Pantothenic acid is used therapeutically in doses up to 5 mg day^{-1} to treat burns (sunburn), anal fissures, rhagades or conjunctival inflammation without any signs of hypervitaminosis. It is required as a supplement in patients on total parenteral nutrition or those who regularly undergo dialysis. In addition, administration of pantothenic acid is used to counteract the inhibitory effects of some drugs on respiratory metabolism (e.g. valproic acid). During surgery, the vitamin leads to improved wound healing (Liepa *et al.*, 2007).

Table 13.5. Concentrations of vitamins B1, 2, 3, 5 in selected foods (in alphabetical order)

Food	Thiamine (μg 100 g^{-1})	Riboflavin (μg 100 g^{-1})	Niacin (μg 100 g^{-1})	Pantothenic acid (μg 100 g^{-1})
Almonds		620		
Beef		260	7500	
Brewers' yeast	12000	3800		
Broccoli	99	178		1300
Chicken			6800	
Coffee, roast			14000	
Eel	180	320		
Egg		408		1600
Halibut			5900	
Herring		220	3800	940
Lentil, seed dry		262		1600
Mackerel		360		
Maize		200		
Mushroom		436	5200	
Oat flakes	590	150	1000	1090
Oats, whole grain	520	170	2370	710
Ox heart			7200	
Ox kidney	3900			
Ox liver	310	2900	15000	7300
Pea seed, dry	765			2000
Peanuts			15000	
Pig's heart			6600	
Pig's liver	325	3200	16000	6800
Pork	900	230	5000	700
Potato	110			
Rice, unpolished			5200	1700
Rye, whole grain				1500
Salmon			7500	
Soya bean, seed dry	999	460		1900
Sunflower seeds, dry	1900		4100	
Trout			3400	
Watermelon				1600
Wheat bran		510	18000	
Wheat flour, wholemeal	470		5000	1200
Wheat germ	2000	720		

From Souci *et al*. (2000)

Table 13.6. Concentrations of vitamins B1, 2, 3, 5 in milk, dairy products and cheese (in alphabetical order)

Food	Thiamine (μg 100 g^{-1})	Riboflavin (μg 100 g^{-1})	Niacin (μg 100 g^{-1})	Pantothenic acid (μg 100 g^{-1})
Blue cheese (50% fat in dry matter)		500	870	2000
Brie (50% in dry matter)			1100	690
Buttermilk	34	160	100	300
Camembert (45% fat in dry matter)	45	600	1100	800
Condensed milk (min. 10% fat)	88	480	260	840
Consumer milk (3.5% fat)	37	180	90	350
Cottage cheese	29			
Cream (min. 30% fat)	25	150	80	300
Cream cheese (min 60% fat in dry matter)	45	230	110	440
Dried whole milk	270	1400	700	2700
Gouda	30			
Limburger (40% fat in dry matter)		350	1200	1200
Parmesan cheese	20	620		530
Quark/fresh cheese (from skim milk)	43	300		740
Skim milk	38	170	95	280
Sterilized milk	24	140	90	350
Sweet whey	37	150	190	340
UHT milk	33	180	90	350
Yoghurt (min. 3.5% fat)	37	180	90	350
Milk from				
Buffalo	50	100	80	370
Cow	37	180	90	350
Donkey	41	64	74	
Goat	49	150	320	310
Horse	30		140	300
Human	15	38	170	270
Sheep	48	230	450	350

From Souci *et al.* (2000)

13.6. Vitamin B6

The term, vitamin B6, represents a group of substances that express vitamin-B activity and which are derivatives of 3-hydroxy-2-methylpyridine: pyridoxine (PN, alcohol), pyridoxal (PL, aldehyde), pyridoxamine (PM, amine; Figure 13.8) and their 5′-phosphorylated forms (Figure 13.9).

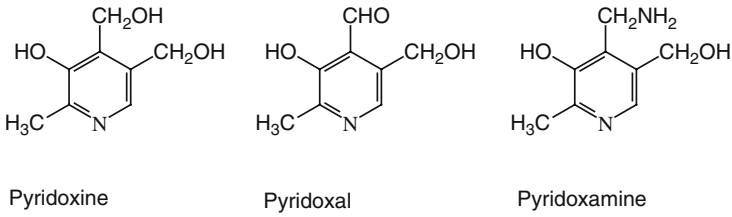


Figure 13.8 Structures of pyridoxine, pyridoxal and pyridoxamine.

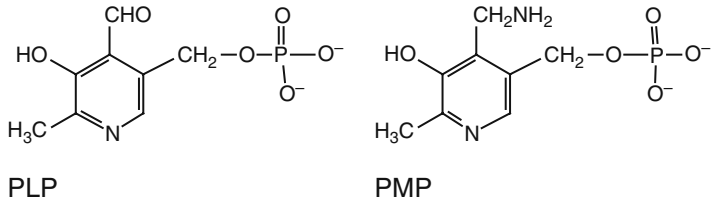


Figure 13.9. Structure of the co-enzymes pyridoxal-5′-phosphate (PLP) and pyridoxamine-5′-phosphate (PMP).

PN and PM and their phosphorylated forms are the predominant forms in plant-derived foods, while in animal-derived foods PL and PLP predominate (Bitsch, 1993). In cow's milk, 14% of vitamin B6 is in the bound form and 86% in the free form (Jensen, 1995). It is sensitive to light and heat, partly depending on the pH of the medium. PM and PN are more stable than PL, particularly to light. In neutral and alkaline solutions, B6 is destroyed by UV light. PL, PM and PN are generally stable to heat in acidic surroundings but heat sensitive in an alkaline medium. The amount of reported loss differs in different studies, maybe due to different experimental procedures. However, some approximations can be given (cf. Biesalski and Back, 2002e).

Heat treatment leads to a loss (in %):

- Insignificant effects in dried milk products
- Pasteurization: 0–8%
- UHT milk: <10%
- Boiling: 10%
- Sterilization: 20–50%
- Evaporated milk: 35–50%

UV light leads to a reduction of 10–45%, depending on the intensity and duration of exposure; gamma radiation (10 Gy) causes a loss of ~90%.

13.6.1. Functions of Vitamin B6

In general, PLP serves mainly as a co-enzyme for about 100 enzymes in amino acid metabolism (Bitsch, 1993). It is bound covalently to its enzyme by a Schiff base with an ε-amino group of lysine in the enzyme. During the enzymatic reaction, the amino group of the substrate and the aldehyde group of PLP form a Schiff base. All subsequent reactions can occur at the α-, β- or γ-C of the respective substrate. In the following, a brief list of common reaction types is given; for more details the reader is referred to specific literature (e.g. Leklem, 2001; Frank, 2002a):

- Transamination (transfer of the amino group of one amino acid to the keto analogue of another amino acid)
- Decarboxylation (resulting in biogenic amines)
- β- or γ-elimination

PMP acts exclusively as a co-enzyme for transaminases:

1. Transferase
    a. Serine hydroxymethyl transferase (C1 metabolism)
    b. D-aminolevolunate synthase (porphyrin biosynthesis)
    c. Glycogen phosphorylase (glycogen mobilization)
    d. Aspartate aminotransferase (transamination)
    e. Alanine aminotransferase (transamination)
2. Oxidoreductase
    a. Lysyl oxidase (collagen biosynthesis)
3. Hydrolase
    a. Kynureninase (niacin biosynthesis)
4. Lyase
    a. Glutamate decarboxylase (GABA synthesis)
    b. Tyrosine decarboxylase (tyramine biosynthesis)
    c. Serine dehydratase (β-elimination)
    d. Cystathionine β-synthase (methionine metabolism)
    e. Cystathionine γ-lyase (γ-elimination)

Due to its role as a co-enzyme in amino acid metabolism (see below), vitamin B6 has a broad range of functions in many systems of the body, including the immune system, the nervous system, gluconeogenesis, lipid metabolism, erythrocyte function, hormone modulation, gene expression and niacin formation. In the immune system, as an example, vitamin B6 increases the immune response of critically ill patients (Cheng *et al.*, 2006)

and suppresses the nuclear factor kappa B-(NFκB-)-reaction in lipopolysaccharide-(LPS-)stimulated mouse macrophages (Yanaka *et al.*, 2005), a well-established laboratory method to challenge the immune system. In the nervous system, PLP may cause neurological dysfunction, particularly epilepsy (Clayton, 2006). On the other hand, there is no direct evidence for an influence on cognitive functions in people with either normal or impaired cognitive functions (Balk *et al.*, 2007). An intensively investigated role is that of vitamin B6 (with B12 and folate) in regulating homocysteine levels, the latter playing a major role in cardiovascular diseases like atherosclerosis or endothelial dysfunction (Cook and Hess, 2005), and also correlations with the risk for hip fractures in the elderly could be shown (McLean *et al.*, 2004). Associations of vitamin B6 benefits during pregnancy (e.g. higher birthweight or reduced incidence of preclampsia and preterm birth) could not be confirmed by meta analysis (Thaver *et al.*, 2006). Bolander (2006) summarizes additional effects of B6, including decarboxylation and transamination, inhibition of DNA polymerases and a couple of steroid receptors and a usefulness as an adjunct in cancer chemotherapy.

13.6.2. Sources of Vitamin B6

Tables 13.11 and 13.12 give an overview of the concentrations of vitamin B6 in selected foods and dairy products, including the milk of different species which is used for human consumption. Regarding vitamin B6 in cheese, most of the vitamin passes into the whey and the concentration decreases further during early maturation, while in later phases the concentration increases, especially on the surface of some varieties (due to synthesis by yeasts and moulds). Cheese types like Camembert and Brie have the highest vitamin B6 concentration, followed by very hard, hard, semi-hard and soft unripened cheese. Table 13.7 shows recommended dietary uptakes.

13.6.3. Vitamin B6 Deficiencies

The vitamin is essential for humans, most animals and some microorganisms. Some recommendations concerning the uptake relate the concentration of B6 to protein uptake; the German Society for Nutrition, for example, recommends a minimum intake, as shown in Table 13.7 based on a quotient of 20 $\mu g\ g^{-1}$ recommended protein uptake (DGE, 2007; see also Table 13.7). Bioavailability is negatively correlated with the amount of glycosylated forms of vitamin B6 in the respective food. The glycosylated form mainly appears in plants but not in animal-derived foods. As an estimation, the bioavailability of vitamin B6 in a "normal, mixed diet" is about 75%.

Table 13.7. Recommended daily uptake of vitamin B6

	Vitamin B6 (mg day^{-1})	
Age	Male	Female
Sucklings <4 months	0.1 (estimated)	
Sucklings 4–12 months	0.3	
Children 1–4 years	0.4	
Children 4–7 years	0.5	
Children 7–10 years	0.7	
Children 10–13 years	1	
Children 13–15 years	1.4	
Adults 15–25 years	1.6	1.2
Adults 25–51 years	1.5	1.2
Adults 51–65 years	1.5	1.2
Adults >65 years	1.4	1.2
Pregnant (>4 month)		1.9
Breast feeding		1.9

From DGE (2007)

A specific vitamin B6 deficiency in humans can hardly be detected, as the first symptoms resemble niacin and riboflavin deficiency (stomatitis, dermatitis-like pellagra). Sometimes in children, neurological problems occur, maybe due to changes in neurotransmitter metabolism (PLP functions as a co-enzyme of an amino acid decarboxylase). Longer-lasting deficiency (but also a megadose of vitamin B6, at least in rats; Arkaravichien *et al.*, 2003) might lead to peripheral neuropathy (nerve demyelinization; Dellon *et al.,* 2001; Head, 2006) and hypochromic anaemia that cannot be cured by iron supplementation (B6 functions in haem synthesis).

Some drugs reduce vitamin B6 concentration, especially when they are taken chronically (then vitamin B6 status should be monitored): hydrazines, chelators, antibiotics, oral contraceptives, L-DOPA, alcohol.

Vitamin B6 status is normally measured by

- PLP in plasma
- 4-pyridoxic acid excretion in 24 h urine (short term)
- assessment of the activation coefficient of erythrocyte transaminase (long term)

Pyridoxine is highly toxic when taken chronically. A dose of 150 mg day^{-1} over several months leads to a (reversible) peripheral neuropathy with dysreflexia and insensibility. However, therapies with megadoses of vitamin B6 showed high positive potential in the treatment of pyridoxine dependency (2–11 mg day^{-1}), cystathioninuria (400 mg day^{-1}), homocystinuria (250–1250 mg day^{-1}), primary oxalosis type I ("spine syndrome", 150 mg

day^{-1}) and also isoniazid intoxication (1 g PN g^{-1} isoniazid). In some cases, beneficial effects have been described for carpal tunnel syndrome (Aufiero *et al.*, 2004), pre-menstrual syndrome (Kashanian *et al.*, 2007) and rheumatic diseases, although the latter is still unclear (Chiang *et al.*, 2003, 2005).

13.7. Biotin (Vitamin B7)

The chemical structure of biotin (hexahydro-2-oxo-1H-thieno (3,4-D) imidazol-4-valeric acid) is shown in Figure 13.10. Although eight isomers exist, only D-(+)-biotin is biologically active and occurs naturally. While in milk (cow's as well as human), biotin occurs in the free form, most foods from animal sources or cereals contain it in an enzyme-bound form named biocytin (*ε-N*-biotinyl-L-lysine).

Biotin is essential for many microorganisms, numerous animals and humans but can be synthesized by the colonic microflora. Although recent findings demonstrate an uptake of water-soluble vitamins (including biotin) by colonocytes, it remains unclear whether the amount of this production is sufficient to fulfil all physiological functions or may be only a kind of fine-tuning of body homeostasis (Said and Mohammed, 2006). The loss of biotin during processing or storage of food is generally small or negligible. There was no loss of biotin in milk in the frozen state for some weeks, in dried milk at room temperature for 1 year or after 2 h of sunlight or 10 Gy gamma radiation. Even UHT sterilization caused no losses, while pasteurization and in-container sterilization caused <10%. In evaporated, condensed dried whole milk less than 15% loss could be detected (Biesalski and Back, 2002f).

13.7.1. Functions of Biotin

Carboxylation and decarboxylation processes are the main reactions in which biotin is involved. It is linked to the enzymes by an amide bond between the amino group of a specific lysil residue in the active centre of the respective

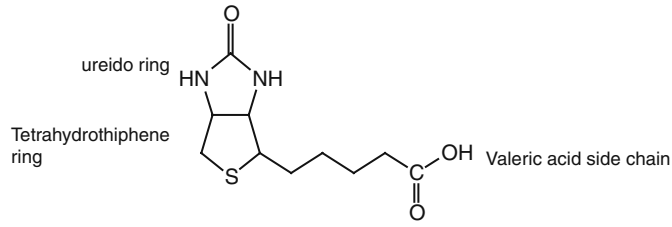


Figure 13.10. Structure of biotin.

apocarboxylase and its valeric acid side chain. During the (ATP-dependent) carboxylase reaction, a CO_2 molecule is attached to biotin at the ureido nitrogen which is opposite to the side chain. The activated CO is then transferred from carboxybiotin to the substrate. The four representative biotin-dependent enzymes (as a prosthetic group) of intermediary metabolism are

- Acetyl-CoA-carboxylase (catalyses the first step in fatty acid synthesis)
- Propionyl-CoA-carboxylase (catalyses the carboxylation of propionyl-CoA to form methyl-malonyl-CoA)
- Pyruvate carboxylase (catalyses the carboxylation of pyruvate to form oxaloacetate)
- 3-Methylcrotonyl-CoA-carboxylase (plays an important role in the catabolism of leucine which, however, can be metabolized via alternative pathways. Such metabolites detected in the urine suggest biotin depletion at the tissue level in individuals without congenital metabolic disorders).

Recently, some additional functions of biotin have been found: it induces dermal differentiation and has been used to treat lameness in animals and brittle nails in humans (see Bolander, 2006).

13.7.2. Sources of Biotin

Tables 13.11 and 13.12 give an overview of dietary sources of biotin and its presence especially in the milk of various species and dairy products; the recommended daily intake is given in Table 13.8. In cheese, changes in the concentration of biotin depend on processing procedures or maturation (microbiological synthesis; e.g. Limburger or Brie); the highest concentrations are often found in the outer layers but can also extend throughout the cheese.

13.7.3. Biotin Deficiencies

One outstanding biotin deficiency is egg-white injury, caused by the extensive consumption of raw egg white which contains the glycoprotein, avidin, which binds biotin and is resistant to intestinal digestion. Symptoms of biotin deficiency are severe dermatitis (cf. Krol and Krafchik, 2006), hair loss and neuromuscular dysfunction. In several other species (mouse, rat, hamster), sub-clinical biotin deficiency has been shown to be teratogenic (Watanabe and Endo, 1989), and this may be the case also in humans (Mock, 2005).

Table 13.8. Recommended daily uptake of biotin

Age	Biotin (μg day^{-1})
Sucklings <4 months	5
Sucklings 4–12 months	5–10
Children 1–4 years	10–15
Children 4–7 years	10–15
Children 7–10 years	15–20
Children 10–13 years	20–30
Children 13–15 years	25–35
Adults 15–25 years	30–60
Adults 25–51 years	30–60
Adults 51–65 years	30–60
Adults >65 years	30–60
Pregnant	30–60
Breast feeding	30–60

From DGE (2007)

In humans, biotin deficiency is very rarely detected in industrial countries with an average biotin intake of 35–70 μg day^{-1}, which exceeds the recommended daily intake (see Table 13.8). However, it has to be taken into account that such recommendations are difficult to calculate, as the sources of biotin (diet, microorganisms, bioavailability) are quite variable. In general, biotin uptake seems to be adaptively regulated and the sodium-dependent multivitamin transporter (hSMVT) is involved in this regulation (Reidling *et al.*, 2007).

Besides the excessive uptake of raw egg white, persons on long-term parenteral nutrition with insufficient biotin supplementation, people suffering from congenital biotinidase deficiency ("secondary biotin deficiency"; see below) or patients on a long-term anti-convulsant therapy are at risk of developing biotin deficiency symptoms, although only after longer periods of biotin deprivation of months or even years. Symptoms are scaly/seborrhoeic and red/eczematous skin rash around the eyes, nose and mouth, anorexia, and also neurological symptoms like depression, lethargy, hallucinations and paraesthesiasis of the extremities have been described. In children, generally comparable symptoms appear under parenteral nutrition, but earlier (within 3–6 months).

Biotinidase is an enzyme which catalyses the hydrolysis of biocytine to biotin and lysine in the intestine, making biotin bioavailable. In addition, biotinidase plays a role in biotin recycling; a deficiency of this enzyme is therapeutically treated by a daily supplementation with 50–150 μg of free biotin. In this context, it is interesting that in these patients intestinal biotin production is insufficient.

13.8. Folates (Vitamin B9)

"Folates" are a group of compounds with a chemical structure and nutritional activity similar to that of folic acid (pteroyl-L-glutamic acid; Figure 13.11). Native folates occur as di- or tetrahydrofolates with different substituents (with only one carbon) at the N5 or N10 position (Figure 13.12). The side chain normally has five to seven glutamate residues in dietary folates, but theoretically more than 100 are possible (Frank, 2002b; Witthöft and Jägerstad, 2002).

Folates are the most vulnerable vitamins as regards losses during processing and storage, as they are very susceptible to oxidation, leakage or enzyme activities. Pasteurization of milk leads to a loss of 0–10%, UHT processing to 0–20%, blanching of spinach or broccoli to 40–90%; oven

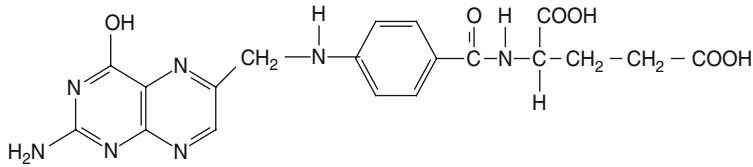
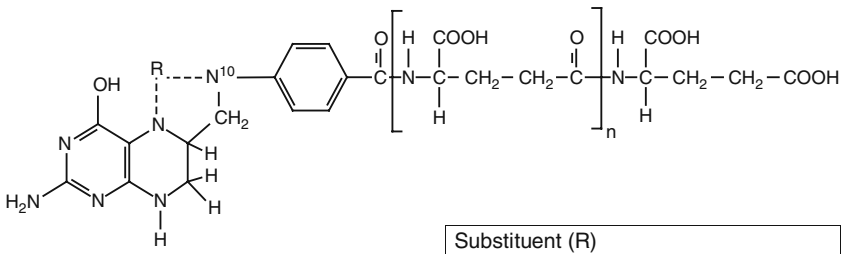


Figure 13.11. Structure of folic acid (pteroylmonoglutamic acid).



Substituent (R)	
$-CH_3$	methyl-; 5-position
-CHO	formyl-; 5 or 10 position
-CH-NH	formimino-; 5-position
$-CH_2-$	methylene-; 5 and 10 position
-CH-	methenyl-; 5 and 10 position

Figure 13.12. Structure of tetrahydropteroylpolyglutamate.

baking (trout, chicken breast) to 30–45%, cooking, chilling/reheating of various vegetables, 25%; soaking and cooking of pulses, 30–70%; ionized radiation of vegetables, 10–60%; storage of spinach, liver or strawberries in the freezer over 6–8 months led to a loss of 17–40%; chilled storage of yoghurt or fermented milk for 2 weeks had no effect. Storage of UHT milk at room temperature for 8 weeks led to losses between 0 and 100%, dependent on the degree of oxidation and enzyme activity (cf. Witthöft and Jägerstad, 2002).

13.8.1. Functions of Folate

Folate serves as an enzyme cofactor; its principal function is to carry one-carbon units, e.g. methyl-, formyl-, formimino-, methylene- or methenyl- (see Figure 13.12). The origin of these carbon units is the catabolism of amino acids like glycine, serine and histidine. Folates also serve as coenzymes providing the above-mentioned carbon-containing radicals for the synthesis of purines and pyrimidine bases for DNA and RNA synthesis, i.e. folates are very important for cell replication and also repair mechanisms involving DNA and RNA. Another role is the regeneration of methionine from homocysteine by the enzyme, methionine synthase. Vitamins B6 and B12 are also involved in this mechanism. Recently, homocysteine has been recognized as an independent risk factor for the development of atherosclerosis, and a sufficient folate level can help reduce this risk by avoiding elevated homocysteine plasma levels (see Herrmann *et al.*, 2007).

13.8.2. Sources of Folate

Tables 13.11 and 13.12 give an overview on the content of folate in several nutrients, including milk, dairy products and cheese. Table 13.9 shows the recommended daily uptake of folate published by the German Society for Nutrition. It must be taken into account that the bioavailability of folates from different sources can be very different, e.g. $<5\%$ from vegetables, 55% from liver, 70–75% from egg yolk or up to 100% from pharmaceutical preparations taken as supplements. The overall situation in humans is still unclear, as only few studies, with contradicting results, have been performed. Interestingly, some nations (e.g. Canada and the USA) add folate to basic nutrients (in the USA to wheat flours, rice, corn meals, bread, pasta at a concentration of 1.4 mg kg^{-1} product) which has led to a reduction of neural tube defects (see below). To improve the bioavailability of folate – and also other secondary plant components – the uptake of dairy products was shown to be helpful (Forssén *et al.*, 2000; Pfeuffer and Schrezenmeir, 2007). Another important factor concerning bioavailability seems to be the folate-binding

Table 13.9. Recommended daily uptake of folate

	Folate		
		μg MJ^{-1} (nutrient density)[b]	
Age	μg equivalent[a] day^{-1}	Male	Female
Sucklings <4 months[c]	60	30	32
Sucklings 4–12 months	80	27	28
Children 1–4 years	200	43	45
Children 4–7 years	300	47	52
Children 7–10 years	300	38	42
Children 10–13 years	400	43	47
Children 13–15 years	400	36	43
Adults 15–25 years[d]	400	38	48
Adults 25–51 years[d]	400	39	51
Adults 51–65 years	400	43	54
Adults >65 years	400	48	58
Pregnant[d]	600		65
Breast feeding	600		56
From DGE (2007)			

[a]Calculated for the sum of folates in normal nutrition; [b]calculated for adolescents and adults mostly sedentary (PAL-value 1.4); [c]estimated value; [d]women planning pregnancy should take additional 400 μg day^{-1} synthetic folic acid (pteroylmonoglutamic acid; PGA) at least 4 weeks before conception and for the first trimester to avoid neural tube defects.

protein (FBP) in milk (Achanta *et al.,* 2007), which has been suggested to promote folate uptake by the intestinal mucosa, at least in animals. The opinion that breast-fed human babies had a better folate status than bottle-fed babies due to an undenatured FBP in human milk seems to be outdated, as babies fed with formula food had better folate levels at 2 and 6 months after birth than breast-fed ones (Karademir *et al*., 2007).

13.8.3. Folate Deficiencies

One of the intensively discussed effects of (maternal) folate deficiency is premature birth, low birth weight and an increased risk of neural tube defects (NTD) in the offspring (Pitkin, 2007). Therefore, since the late 1990s, in some countries (the USA, Canada, Chile), selected basic nutrients have been fortified with folate, leading to a reduction of NTDs of 50–78% (Liu *et al*., 2004; Bockelbrink *et al*., 2005). However, in Germany there are still no such general fortifications and here the mean daily folate uptake is around 100 μg which is about one fourth of the recommended uptake of 400 μg day^{-1} for adults and even less compared to 600 μg day^{-1} for pregnant women or women planning pregnancy. The latter group, especially, should take care of an optimal folate

intake, as folate status has to be in an optimal range already 4–6 weeks before conception, a fact that cannot always be planned. Women with an NTD anamnesis should take up 4000 μg day^{-1}, which is the tenfold of the normal dose. An actual meta-analysis described that folic acid combined with a multivitamin supplementation was as effective at NTD reduction as folic acid alone but additionally reduced a number of other congenital anomalies (Goh *et al.*, 2006). Anyhow, the exact mechanisms by which sufficient folate levels prevent NTDs are still unclear; an actual "methylation hypothesis" suggests that folic acid stimulates cellular methylation reactions (Blom *et al.,* 2006).

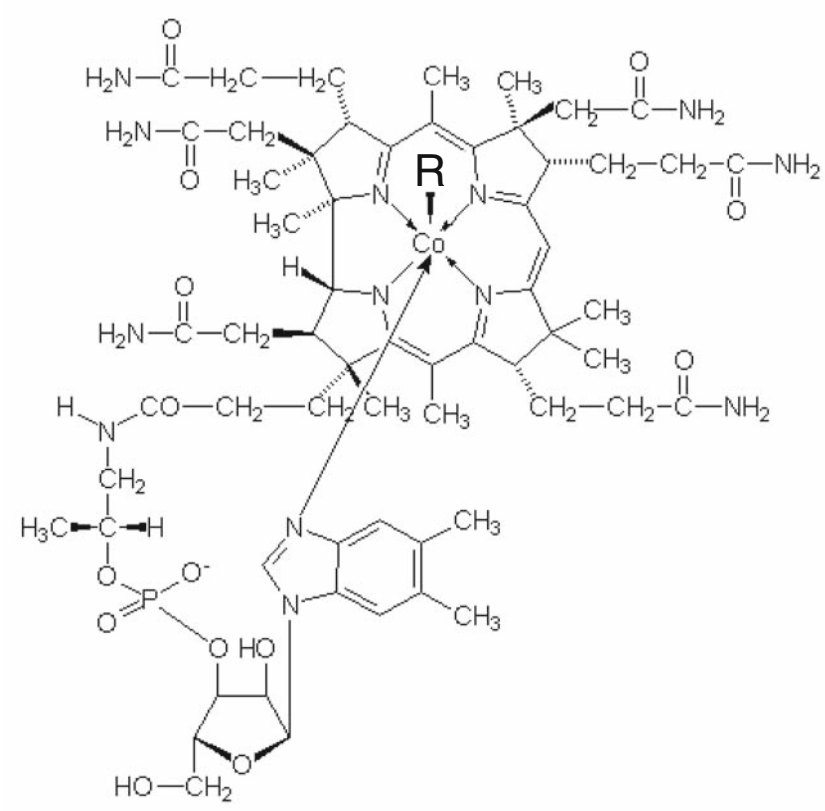
A number of epidemiological and clinical studies showed an inverse correlation of folate status/intake and coronary heart disease, based on the observation that (1) elevated plasma homocysteine levels are a strong and independent risk factor for the development of atherosclerosis and (2) homocysteine plasma levels increase during folate deficiency. As for riboflavin, the uptake of selected nutrients (e.g. cereals, milk, yogurt and those containing riboflavin and folate) was inversely related to serum homocysteine level (Ganji and Kafai, 2004). In addition, high plasma homocysteine levels are linked to cognitive dysfunction and dementia in connection with reduced folate, but also vitamin B12 levels; the exact mechanisms are still unclear and have to be elucidated further.

A long-term folate-free diet (4 months) leads to a change in the erythrocyte population, i.e. the total number decreases with a parallel increase of oversized, haemoglobin-rich erythrocytes (macrocytic hyperchromatic anaemia).

An overdosage of folate must be mentioned, especially concerning the synthetic vitamin folic acid, as natural sources are regarded as safe over a wide range. The safe upper intake level has been set to 1 mg day^{-1} by the US Food and Nutritional Board. Overdosage can lead to epileptogenic and neurotoxic effects (in animals); in humans there is a risk of masking an unrecognized vitamin B12 deficiency (pernicious anaemia). However, the numerous tests for vitamin status available strongly reduce the risk of such undiagnosed B12 deficiencies. There seems to be evidence that supplementation with folate (>or = 400 μg day^{-1}) increases the risk of developing breast cancer by 20–30% (Kim, 2006).

13.9. Cobalamin (Vitamin B12)

The terms vitamin B12 or cobalamin represent a group of several cobalt-containing corroids. A corrin ring with four reduced pyrrole rings and cobalt as central atom, a nucleotide-like compound and an additional variable compound are their common features (Figure 13.13). B12 is the only vitamin containing a metallic ion. In biological systems, hydroxo-, aquo-, methyl- and 5′-deoxyadenosylcobalamin occur, while cyanocobalamin is a decomposition



R	Vitamer
–CN	Cyanocobalamin
–OH	Hydroxocobalamin
–5'-Deoxyadenosyl	5'-Deoxyadenosylcobalamin
$-CH_3$	Methylcobalamin
$-H_2O$	Aquacobalamin
$-NO_2$	Nitritocobalamin

Figure 13.13. Structure of the different vitamin B12 vitamers.

product which, however, is used for therapeutic purposes as also is hydroxycobalamin.

Only microorganisms are able to synthesize vitamin B12. Thus, some animal species have a sufficient supply from their intestinal microorganisms. In humans, however, the synthesizing organisms are localized in the colonic part of the intestine which is too distal from the small intestine (ileum), where vitamin B12 must be taken up. Consequently, humans obtain B12 exclusively from their diet and only animal-derived foods contain sufficient amounts of vitamin B12 (Tables 13.11 and 13.12). Another prerequisite for the uptake of vitamin B12 is the intrinsic factor which is secreted by gastric parietal cells and facilitates ileal uptake of cobalamin (Said and Kumar, 1999).

While storage has only minor effects on cobalamin concentration in milk (~30–40% loss in sterilized milk after 90 days at room temperature) and radiation also has small effects, heat destruction plays a major role. Losses in cow's milk caused by heat treatment are sterilization: 20–100%; evaporated milk: ~50%; boiling: 20%; pasteurization: <10%; UHT: 5–10%. In cheese, an overall loss of 10–50% can be assumed, although there are differences between cheese types, e.g. in Gruyère cobalamin concentration even increases due to vitamin B12-synthesizing microorganisms (Biesalski and Back, 2002 g).

13.9.1. Functions of Cobalamin

Adenosylcobalamin (in the cytosol) and methylcobalamin (in the mitochondria) are the co-enzyme forms of cobalamin. In humans, these co-enzymes are involved in three metabolic reactions (for details see relevant textbooks on biochemistry):

1. Leucine 2,3-amino-mutase reversibly changes α-leucine to β-leucine (3-aminocaproic acid), thus starting the degradation of this amino acid.
2. Methionine synthetase (N5-methyltetrahydrofolate homocysteine methyltransferase) reaction. Methionine synthetase needs methylcobalamin as a co-factor for the re-methylation of homocysteine to methionine. During this process, the methyl group from 5-methyltetrahydrofolate is transferred to homocysteine resulting in methionine and tetrahydrofolate. The latter is converted to N5,10-methylenetetrahydrofolate, a co-factor of thymidylate synthetase, finally ending up in DNA synthesis.
3. Adenosylcobalamin is needed by methylmalonyl-CoA-mutase for the isomerization of methylmalonyl-CoA to succinyl-CoA during the degradation of propionic acid, thus offering the entrance to the citric acid cycle.

13.9.2. Sources of Cobalamin

Cobalamin can be synthesized only by microorganisms and does not occur in plant-derived food. Therefore, animal-derived foods containing cobalamin are essential for humans; more or less good sources are listed in Tables 13.11 and 13.12. In cow's milk, the cobalamin content is very constant regarding feed, breed, season or stage of lactation, except colostrum which has a very high level. In contrast, concentrations in human milk are markedly lower than in cow's milk and vary dependent on the above-mentioned parameters. Concentrations in the milk of cow, human and other species and in some dairy products are given in Table 13.12.

13.9.3. Cobalamin Deficiencies

Although normally in developed countries the vitamin B12 uptake meets the recommendations (Table 13.10), cobalamin deficiencies are the most numerous ones in overall vitamin deficiencies to be clinically treated.

Table 13.10. Recommended daily uptake of vitamin B12

	Vitamin B12		
		μg MJ^{-1} (nutrient density)[a]	
Age	μg day^{-1}	Male	Female
Sucklings <4 months[b]	0.4	0.20	0.21
Sucklings 4–12 months	0.8	0.27	0.28
Children 1–4 years	1.0	0.21	0.23
Children 4–7 years	1.5	0.23	0.26
Children 7–10 years	1.8	0.22	0.25
Children 10–13 years	2.0	0.21	0.24
Children 13–15 years	3.0	0.27	0.32
Adults 15–25 years	3.0	0.28	0.36
Adults 25–51 years	3.0	0.29	0.38
Adults 51–65 years	3.0	0.33	0.41
Adults >65 years	3.0	0.36	0.43
Pregnant[c]	3.5		0.38
Breast feeding[d]	4.0		0.37

[a]Calculated for adolescents and adults mostly sedentary (PAL value 1.4); [b]estimated value; [c]0.5 μg more for filling up body stores and to maintain nutrient density; [d]approximately. 0.13 μg vitamin B12 added per 100 ml secreted milk.
From DGE (2007)

This is due mainly to a reduced uptake in the intestine that can have various causes (Sahid and Mohammed, 2006). One major prerequisite is a sufficient amount of intrinsic factor which is produced by parietal cells of the stomach and binds to vitamin B12. This molecule is resistant to intestinal proteolysis and binds under neutral pH conditions to specific mucosal receptors on the microvilli of the enterocytes, mostly in the distal ileum to be taken up either as a whole complex or as cobalamin alone. After absorption, cobalamin is bound to transcobalamin and transported to the liver in the portal blood. Reasons for vitamin B12 deficiency can be

- Defects of the gastric mucosa (loss of parietal cells)
- Atrophic gastritis (chronic)
- Gastrectomy (partial or total)
- Malabsorption in the ileum
- Intestinal stasis
- Sucklings when mothers suffer from vitamin B12 deficiency
- Congenital disturbances of cobalamin metabolism
- Intestinal parasites
- Age

If normal stores in humans are sufficiently filled (2–5 mg) and enterohepatic recirculation takes place, it takes 10–15 years until deficiency symptoms appear after stopping any B12 intake.

Characteristic vitamin B12 symptoms are macrocytic hyperchromic anaemia (CAVE: this can also appear due to folic acid deficiency) and funicular myelitis, i.e. neurological disorders like symmetrical paraesthesias in feet and fingers, disturbances of proprioception and vibratory senses, spastic ataxia and degeneration of the spinal cord. Vitamin B12 delays the onset of signs of dementia (and blood abnormalities), provided it is administered before the onset of the first symptoms (Bourre, 2006; Smith, 2006). Supplementation with cobalamin improves cerebral and cognitive functions in the elderly; it frequently improves the functioning of factors related to the frontal lobe, as well as the language function of those with cognitive disorders. Adolescents who have a borderline level of vitamin B12 develop signs of cognitive changes (Reynolds, 2006; Balk *et al.*, 2007). Although the homocysteine-lowering effect of vitamin B12 and folate supplementation is well known, a protective effect on the development of vascular diseases by this supplementation can be seen as a tendency; however, the final results of some ongoing trials are required before making a final recommendation (Clarke *et al.*, 2007).

While in former times the ingestion of about 1 kg of raw liver per week was used to treat vitamin B12 deficiency, nowadays pernicious

anaemia is treated by a multi-step therapy: 1 mg day^{-1} hydroxycobalamin intramuscularly for 7 days followed by the same dose applied weekly for up to 6 weeks, followed by the same dose each 2 months for life. Funicular myelitis is treated by a 2-week therapy with 250 μg day^{-1} to replenish stores followed by lifelong monthly injections of 100 μg if a disturbed absorption was the cause of cobalamin deficiency. Elderly

Table 13.11. Concentrations of vitamins B6, 7,9,12 in selected foods (in alphabetical order)

B-vitamin food	Pyridoxine (μg 100 g^{-1})	Biotin (μg 100 g^{-1})	Folsäure (μg 100 g^{-1})	Cobalamin (μg 100 g^{-1})
Bean, white, seed dry			187	
Beef				5
Brewers' yeast		115	3170	
Broccoli			48	
Calf liver		75		
Chick pea, seed dry	550			
Egg		25	184	2
Herring	450			9
Kipper				10
Lamb's lettuce			145	
Lentil, seed dry	575		168	
Mackerel	630			9
Maize	400			
Millet	519			
Oat flakes		20		
Oats, whole grain	960			
Ox kidney			170	33
Ox liver	877	100	592	65
Pea seed, dry			151	
Peanuts	34			
Pig's liver	590		136	39
Pork	565			
Rice, unpolished	275	12	16	
Salmon	980			3
Soya bean, seed dry	1000	60	240	
Soya flour			190	
Wheat flour, wholemeal	460	8		
Wheat germ	492	17	520	

From Souci *et al.* (2000)

Table 13.12. Concentrations of vitamins B6, 7, 9, 12 in milk, dairy products and cheese (in alphabetical order)

Food	Pyridoxine (μg 100 g^{-1})	Biotin (μg 100 g^{-1})	Folic acid (μg 100 g^{-1})	Cobalamin (μg 100 g^{-1})
Blue cheese (50% fat in dry matter)			40	
Brie (50% in dry matter)	230	6	65	2
Buttermilk	40	2	5	<1
Camembert (45% fat in dry matter)	250	5	44	3
Condensed milk (min. 10% fat)	77	8	6	<1
Consumer milk (3.5% fat)	36	4	6	<1
Cream (min. 30% fat)	36	3	4	<1
Cream cheese (min 60% fat in dry matter)	60	4		<1
Dried whole milk	200	24	40	1
Emmental cheese	111			3
Limburger (40% fat in dry matter)		9	60	
Quark/fresh cheese (from skim milk)		7	16	
Skim milk	50	2	5	<1
Sterilized milk	23	4	3	<1
Sweet whey	42	1		<1
UHT milk	41	4	5	<1
Yoghurt (min. 3.5% fat)	46	4	13	<1
Milk from				
Buffalo	25	11		<1
Cow	36	4	7	<1
Donkey				<1
Goat	27	4	1	<1
Horse	30			<1
Human	14	1	8	<1
Sheep		9		<1

From Souci *et al*. (2000)

people with reduced absorption due to gastric problems (reduced IF) should be supplemented with 500 μg of crystalline vitamin B12 daily (Park and Johnson, 2006). Side effects have not been observed, even with pharmacological doses up to 5 mg. An overview of the varying strategies and the discussion of the different methods in different countries are given (besides numerous others) by Hvas and Nexo (2006), Butler *et al*. (2006) and Carmel and Sarrai (2006).

13.10. Selected Actual Publications of Meta-analyses Concerning More Than One of the B-Vitamins

To evaluate the disappearance of supplementary B-vitamins before and from the small intestine, four lactating Holstein cows were equipped with ruminal, duodenal and ileal cannulas and supplemented with a number of B-vitamins either with the food 5 days before and during a 4-day collection period or by post-ruminal infusion 1 day before and during a 4-day collecting period; the concentrations of the vitamins were (in mg day^{-1}) thiamine: 300 and 10; riboflavin: 1600 and 2; niacin: 12,000 and 600; vitamin B6: 800 and 34; biotin: 20 and 0.02; folic acid: 2600 and 111; vitamin B12: 500 and 0.4. Apparent intestinal absorption values differed greatly among vitamins, but the proportion of vitamins disappearing from the small intestine was not negatively influenced by supplementation. Except for riboflavin and niacin, absolute amounts disappearing from the small intestine were greater during the treatment than the control periods, suggesting that B-vitamin supply in dairy cows is increased by supplementation, although losses in the rumen are extensive (Santschi *et al.*, 2005).

Meta-analyses on the influence of the vitamins B6, B12 and folic acid on cognitive functions gave no clear or even contradictory results for an evidence of an impact of these vitamins. One analysis postulated no impact, independent from a single or a combined application or if normal or cognitively impaired persons were investigated, while another meta-analysis postulated an association between these vitamins (low vitamin level and high homocysteine levels) and cognitive dysfunction as well as dementia; however, the biological pathways and possible therapeutic interventions remain unclear, i.e. large-scale randomized trials are demanded by the authors (Balk *et al.*, 2007). Anyhow, an important role of these vitamins in the nervous system seems to be generally accepted, but the details and separated functions of the respective vitamins need to be further elucidated (Bourre, 2006; Reynolds, 2006).

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14

Flavours and Off-Flavours in Milk and Dairy Products

K.R. Cadwallader and T.K. Singh

14.1. Introduction

Milk and milk products are an important part of daily nutrition in many regions of the world. Besides fulfilling nutritional requirements, the flavour of milk and milk products is a key parameter for consumer acceptance and marketing (Drake *et al*., 2007a). The market for dairy products in more traditional dairying countries has been growing steadily; most of this growth can be attributed directly to the introduction of novel product options and increasing application of milk constituents in other food formulations. Due to the importance of dairy products in daily life, especially for consumers in traditional dairying countries, they are being used increasingly as delivery systems for biologically active/nutraceutical preparations. Even higher growth in the consumption of milk and milk products is now coming from countries which did not have any tradition of dairying; such countries offer tremendous opportunity for further enhanced sales. At the same time this increased consumption also challenges researchers and manufacturers to create new product solutions to better suit the palette of consumers recently introduced to dairy products.

During the last two decades, considerable progress has been made towards understanding the flavour chemistry/biochemistry of dairy products, but defining fresh dairy or dairy flavour continues to challenge researchers working in sensory/flavour area. Fresh milk (raw or pasteurized) of overall

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good flavour quality has a bland but characteristic flavour (Badings, 1991). Products made from this basic raw material vary in flavour from mild/weak (e.g., yoghurt, Mozzarella cheese) to intensely flavoured (e.g., Blue cheeses, hard Italian cheeses). For dairy products, three elements comprise the overall sensory experience:

(1) The **mouthfeel** from the constituents of the milk, especially milk fat and proteins which are essential for viscosity and/or texture
(2) T**aste** components, e.g., slight sweet/salty taste from lactose, milk salts and added salt (NaCl) to bitterness in cheese caused by peptides
(3) A**roma** caused by a proper balance of numerous volatile organic compounds

In the past, the stability of milk and milk products was the primary consideration but is no longer the principal objective due to the evolution of modern sanitary practices, as well as pasteurization. Today, the manufacture of dairy products of consistently good flavour and texture is crucial. In early flavour studies, researchers identified hundreds of volatile compounds, with little or no attention paid to their sensory contribution to the overall flavour of the products. The availability of powerful chromatographic separation techniques, like high-resolution capillary gas chromatography in combination with mass spectrometry and olfactory detection techniques (use of olfactory detection ports), has revolutionized work on the characterization of flavour compounds. Advances in instrumental/chemical analysis have paralleled the developments in sensory methods for the analysis of flavour compounds. Recently, published reviews by Parliment and McGorrin (2000), McGorrin (2001), Singh *et al*. (2003a, 2007), Cadwallader (2007) and Drake *et al.* (2007a) described various sensory-directed analytical flavour techniques used in the evaluation of key aroma compounds in milk and dairy products. A recently published text, '*Sensory-Directed Flavour Analysis'* edited by Marsili (2007a), is highly recommended to readers who may be interested in more details on flavour analysis techniques.

This chapter presents a discussion on the aroma/taste compounds in various dairy products and reactions involved with their production. Mechanisms involved in the production of odorants responsible for the specific flavour notes and off-flavours in dairy products are also discussed.

14.2. Reactions Involved in the Formation of Flavour Compounds in Milk and Milk Products

The volatile flavour and taste compounds in milk products originate from the degradation of the major milk constituents, namely lactose, citrate, milk lipids and milk proteins (particularly the caseins). The physico-chemical

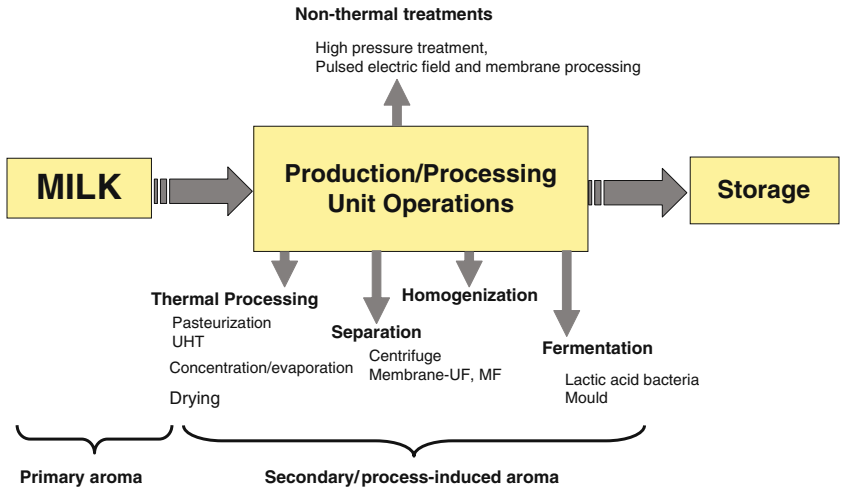

Figure 14.1. Main sources of aroma components in dairy products.

parameters, i.e., heat treatment, pH, water activity, salt concentration and ripening temperature, necessary for the right balance of biochemical changes are set during manufacturing (Figure 14.1). In case of deviation of any of these parameters, cheeses or other dairy products could potentially develop texture and/or flavour inconsistencies or defects. The degradation of milk constituents during the manufacture of dairy products involves a concerted series of chemical and biochemical reactions. Reactions and/or pathways involved in the production milk and milk products can be sub-divided into three major categories:

(1) Lipid oxidation
(2) Process-induced changes

   a. Heat-induced changes
   b. Changes induced by non-thermal processing technologies

(3) Fermentation by lactic acid bacteria and other cultures.

14.2.1. Lipid Oxidation

Auto-oxidation of fat in milk and milk products occurs initially in the polyunsaturated phospholipids fraction of the milk fat globule membrane, followed by the main triacylglycerol fraction. It has been reported that different milks vary widely in susceptibility to oxidation. The oxidative

deterioration of milks is classified empirically as (a) spontaneous (developing oxidized flavours within 2 days without added metal catalyst), (b) susceptible (developing oxidized flavours within 2 days after the addition of copper ions) and (c) resistant or non-susceptible (not developing oxidized flavours after 2 days even after addition of copper or iron ions) (Frankel, 1980). Unlike fluid milk, in dry milk products it is the triacylglycerol fraction which is more susceptible to oxidation and the phospholipids act as antioxidants (Frankel, 1980). From this it appears that the susceptibility of milk phospholipids to oxidation depends on whether they are suspended in water or a continuous fat phase. This difference in oxidative stability influences the development of different flavour or off-flavour compounds in various dairy products.

A simplified scheme of lipid oxidation involving three distinct steps is shown in Figure 14.2 (for further details see Frankel, 1980; Walstra *et al.*, 1999). Some of these oxidation products can be perceived at exceptionally low concentrations and thereby cause off-flavours, described as fatty, fried, plastic, tallowy, fishy, metallic or cardboard-like. Off-flavour development can cause problems in fluid milk, sour-cream butter milk, cream, butter, butter oil, a variety of milk powders, cheeses and casein and whey protein products.

Some volatile compounds produced by the auto-oxidation of specific unsaturated fatty acids are listed below:

- **Oleic acid**—*Octanal, nonanal, decanal, 2-decenal, 2-undecenal*
- **Linoleic acid**—*Hexanal, 2-octenal, 3-nonenal, 2,4-decadienal*
- **Linolenic acid**—*Propanal, 3-hexenal, 2,4-heptadienal, 3,6-nonadienal, 2,4,7-decatrienal*

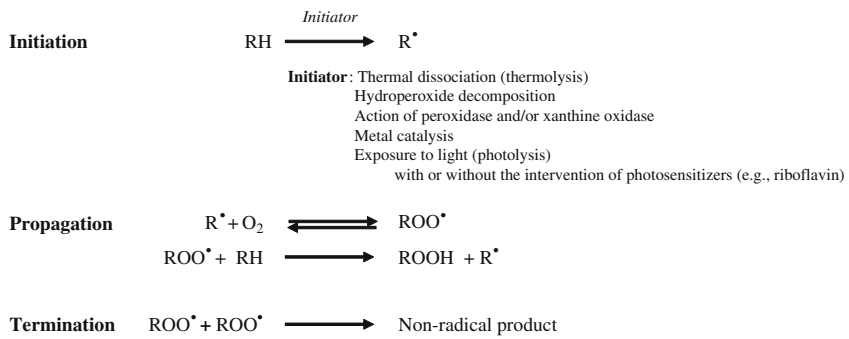

Figure 14.2. General pathway way for the autoxidation of unsaturated lipid (modified from Singh *et al.*, 2007).

14.2.2. Processing-Induced Changes

14.2.2.1. Heat-Induced Changes

Thermal treatment of fluid milk, such as pasteurization or the production of various concentrated/dried milk products, leads to a whole series of desirable, and undesirable, chemical changes which can have major consequence for the texture, taste and flavour. Selected changes, which directly influence the flavour/off-flavour of milk and milk products, are

- Thermal degradation of lipids (e.g., accelerated degradation of hydroperoxides resulting in the formation of 2-alkanones). (For more information on the thermal degradation of lipids, see Nawar, 1989; Yoo *et al.*, 1989).
- Thermal reactions involving amino acid side chains (e.g., generation H_2S and other S-compounds).
- Maillard reactions: e.g., reaction of lactose, a reducing sugar, with ε-amino groups on the side chain of lysine residues in milk proteins (e.g., resulting in the formation of a wide variety of odorants, namely furfurals, 3-/4-furanones, Strecker aldehydes, pyrazines, pyrroles, thiazoles maltol, furaneol, etc., see Chapter 7), (Figure 14.3).
- Limited information is available on the interaction of lipid oxidation and Maillard reaction products on the flavour and off-flavour chemistry of milk and milk products. These reactions also contribute to non-enzymatic browning in food systems, which may or may not be desirable (see details in review by Zamora and Hidalgo, 2005; see also Chapter 7).

This is not a complete list of thermally induced reactions in milk and milk products. Other reactions such as hydrolysis of peptide bonds, dephosphorylation of proteins, etc., also occur but are not important from a flavour point of view.

14.2.2.2. Changes Induced by Non-Thermal Processing Technologies

New technologies are needed to process milk without compromising its flavour. Several non-thermal processing technologies have been explored to achieve microbial safety and minimize the formation of off-flavour. Some promising technologies are

- Membrane filtration
- High pressure processing
- Pulsed electric field treatment

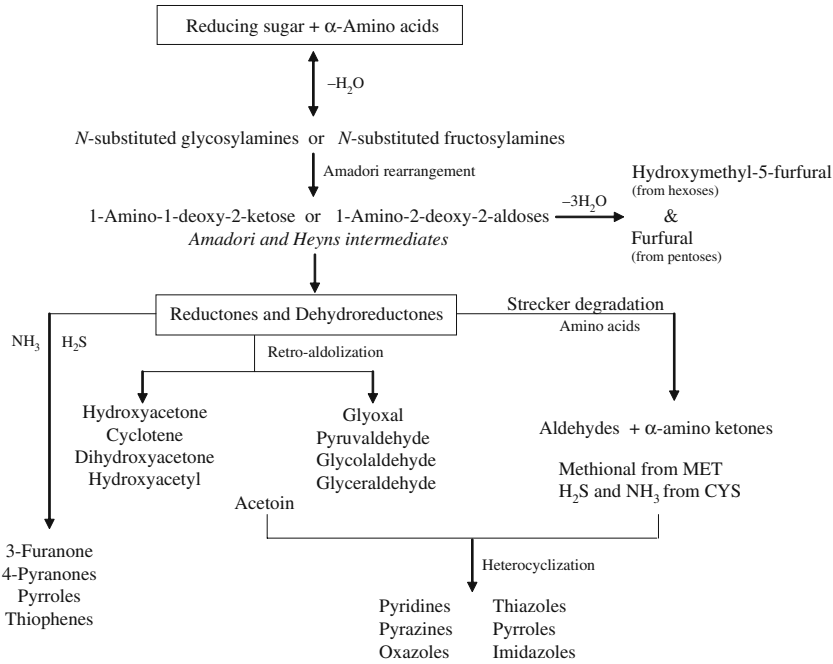

Figure 14.3. Formation of aroma compounds *via* Maillard reaction/non-enzymatic browning (modified from Singh *et al*., 2007).

Microfiltration (MF) using cross-flow membrane separation has shown promising results in eliminating bacteria from milk and increasing shelf-life without the development of off-flavours (Elwell and Barbano, 2006; Rysstar and Kolstad, 2006).

High hydrostatic pressure processing (HPP), a new technology to the food industry (Torres and Velazquez, 2004), can destroy microorganisms by high hydrostatic pressure without heat (Berlin *et al*., 1999; Velazquez *et al*., 2002). This technology has been gaining commercial acceptance in the manufacture of food products with 'fresh' flavour that are not possible with other preservation technologies. To retain the 'fresh' milk flavour, HPP has been studied as a potential alternative to the pasteurization of milk. A microbiological reduction and significant extension in the shelf-life of milk compared to that of pasteurized milk has been achieved using pressure treatments. HPP can reduce the size of casein micelles in milk at pressures above 230 MPa, resulting in a decrease in whiteness and turbidity and an increase in the viscosity of milk (Buchheim and El-Nour, 1996). High pressure can also affect the crystallization properties of milk fat. It is generally assumed that

HPP at low temperature will retain the flavour of the product; however, Hofmann *et al*. (2005) reported that HPP could potentially change the formation of Maillard-derived compounds at high temperature.

Recent work by Vazquez-Landaverde *et al*. (2006a) showed that HPP at a low temperature causes minimum change of the volatile composition of milk. However, under extreme pressure and temperature conditions, volatile compound formation is different from that under atmospheric pressure conditions. Heat treatment at high temperature promotes the formation of both aldehydes and methyl ketones, whereas high pressure at high temperature favours the formation of aldehydes. The formation of sulphur compounds was also different under high pressure.

Further work is required to understand the mechanism/kinetics of volatile formation under high hydrostatic pressure, pulsed electric field and/or their combination with heat treatment.

14.2.3. Fermentation by Lactic Acid Bacteria

Considerable knowledge on the principal changes and pathways involved in manufacture of fermented milk and cheese ripening has been accumulated over the last several decades. The three primary biochemical processes are

- Metabolism of lactose, lactate and citrate ('Glycolysis')
- Lipolysis
- Proteolysis

The relative importance of each of these processes depends on the type of fermented dairy product. These primary changes are followed and overlapped by many secondary catabolic changes, including deamination, transamination, decarboxylation and desulfurylation of amino acids, β-oxidation of fatty acids and even purely synthetic chemical changes, e.g., formation of thioesters. The above-mentioned primary reactions are mainly responsible for the basic textural changes and are also largely responsible for the basic flavour of fermented dairy products. However, secondary transformations are mainly responsible for the finer aspects of cheese flavour and for modification of cheese texture. Glycolysis, lipolysis, proteolysis and related reactions are further discussed in the next few sections.

14.2.3.1. Glycolysis and Related Reactions

During the manufacture of fermented milks and cheese, starter lactic acid bacteria ferment lactose to (mainly L) lactic acid (Figure 14.4). In the case of Cheddar-type cheeses, most of the lactic acid is produced in the vat before

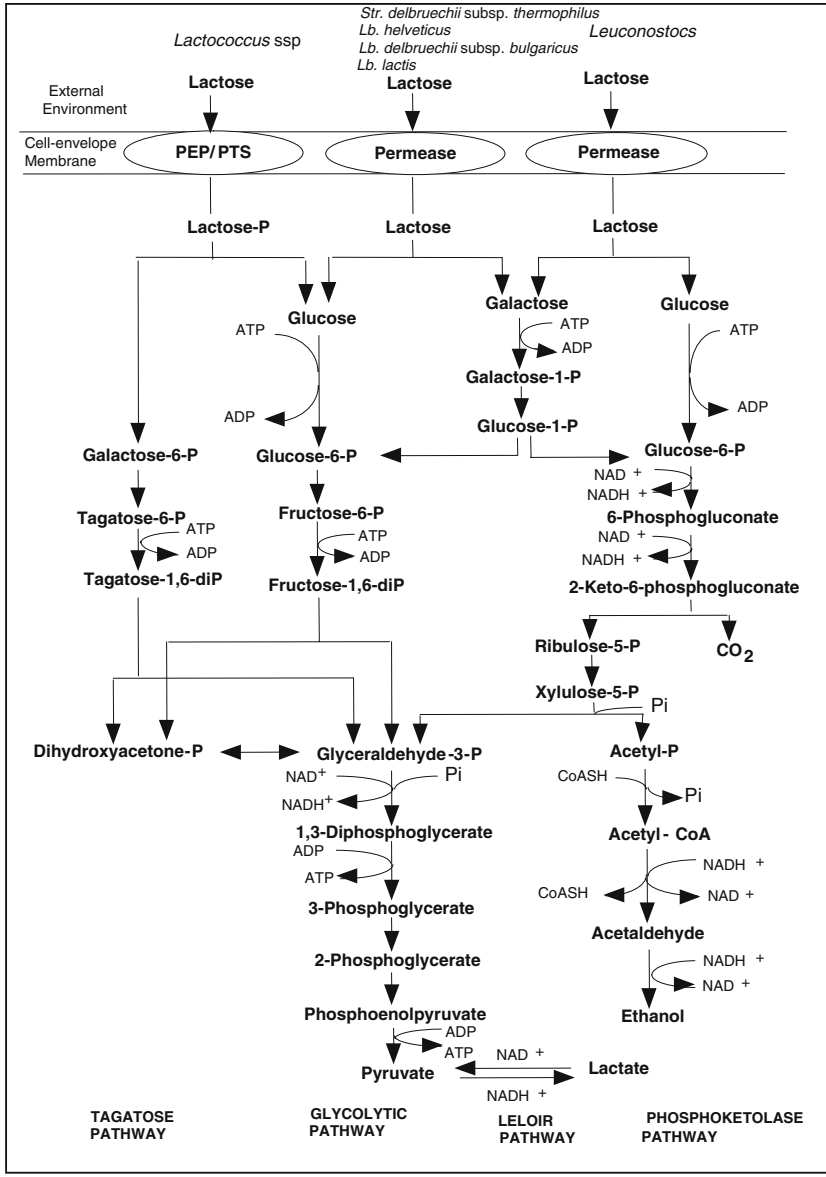

Figure 14.4. Pathways for the metabolism of lactose by mesophilic and thermophilic lactic acid bacteria (reproduced with permission from Singh *et al.,* 2003; © Blackwell Publishing, Inc.).

salting and moulding. During manufacture or shortly thereafter, curd pH reaches ~5.0, but the rate is characteristic of the cheese variety (6–24 h). Even after losing ~98% of the total milk lactose in the whey as lactose or lactate, the cheese curd still contains 0.8–1.5% lactose at the end of manufacture (Huffman and Kristoffersen, 1984).

The pH at whey drainage largely determines the mineral content of a cheese. The loss of Ca^{2+} and phosphate from casein micelles determines the extent to which the micelles are disrupted and this largely determines the basic structure and texture of a cheese (Lawrence *et al.,* 1983). In general, curds with a low pH at drainage have a crumbly texture, e.g., Cheshire, while high pH curds tend to be more elastic, e.g., Emmental.

The racemization of L-lactate is probably not significant from a flavour viewpoint, but D-lactate may have undesirable nutritional consequences in infants. Calcium-D-lactate is believed to be less soluble than calcium-L-lactate and may crystallize in cheese, especially on cut surfaces (Dybing *et al.,* 1988). Consumers may mistake the crystals as spoilage, and crystal formation is generally considered negative.

Lactic acid is metabolized by propionic acid bacteria, e.g., in the production of Swiss-type cheeses, to propanoic and acetic acids, H_2O and CO_2. The production of CO_2 is responsible for the eye formation which is a characteristic of Swiss-type cheeses (Fox *et al.,* 1995). Oxidation of lactate may also occur in cheese to a very limited extent. During this process, lactate is converted to acetate and CO_2. Acetate is present at fairly high concentrations in Cheddar and is considered to contribute to cheese flavour, although a high concentration may cause off-flavour (Aston and Dulley, 1982).

14.2.3.2. Citrate Metabolism

Bovine milk contains relatively low levels of citrate (~8 mM). Approximately 90% of the citrate in milk is soluble and most is lost in the whey; however, the concentration of citrate in the aqueous phase of cheese is ~3 times that in whey (Fryer *et al.,* 1970), presumably reflecting the concentration of colloidal citrate. Cheddar cheese contains 0.2–0.5 % (w/w) citrate, which is not metabolized by *L. lactis* ssp. *lactis* or *L. lactis* ssp. *cremoris*, but is metabolized by citrate-positive lactococci and *Leuconostoc* sp. with the production of diacetyl and CO_2. Due to CO_2 production, citrate metabolism is responsible for the characteristic eyes in Dutch-type cheeses. Diacetyl and acetate produced from citrate contribute to the flavour of Dutch-type and Cheddar cheeses (Aston and Dulley, 1982). Several species of mesophilic lactobacilli metabolize citrate with the production of diacetyl and formate (Fryer, 1970); the presence of lactose influences the amount of formate formed.

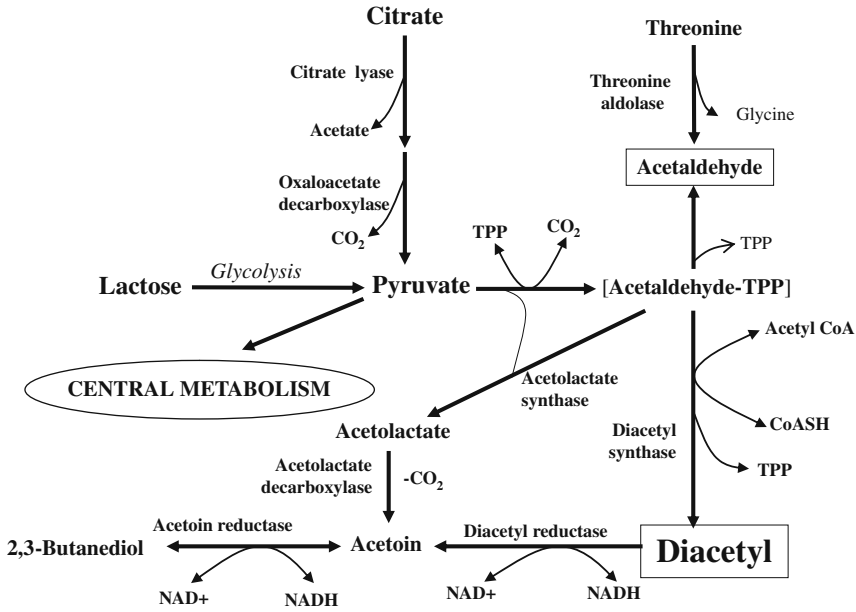

Figure 14.5. Metabolism of citrate by lactic acid bacteria (modified from Singh *et al*., 2003).

The principal flavour compounds produced from the metabolism of citrate are acetate, diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanone) and 2,3-butandiol (Cogan, 1995) (Figure 14.5). Diacetyl is usually produced in small amounts but acetoin is generally produced at much higher concentration (10–50 fold higher than diacetyl).

14.2.3.3. Lipolysis and Related Reactions

Like all types of food with a high fat content, lipolytic (enzymatic hydrolysis by lipases and esterases) and oxidative (chemical) changes are likely to occur in dairy products. Lipases and esterases in cheese originate from milk, starter, secondary starter and non-starter bacteria. A number of psychrotrophic organisms, which can dominate the microflora of refrigerated milk, produce heat-stable lipases. The hydrolysis of triglycerides, which constitute more than 98% of milk fat, is the principal biochemical transformation of fat, which leads to the production of free fatty acids (FFAs), di- and mono-glycerides and possibly glycerol (Figure 14.6). FFAs contribute to the aroma of cheese. Individual FFAs, particularly

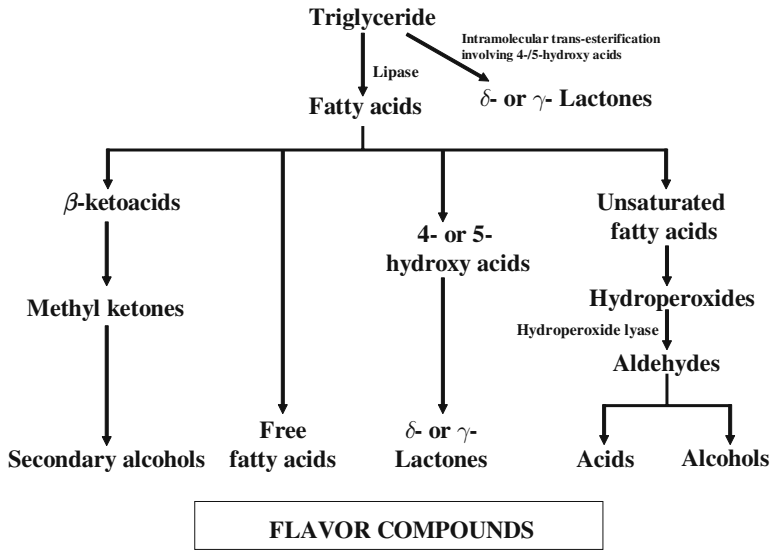

Figure 14.6. General pathways for the metabolism of milk triglycerides and fatty acids (reproduced with permission from Singh *et al.,* 2003; © Blackwell Publishing, Inc.).

acids between $C_{4:0}$ and $C_{12:0}$, have specific flavours (rancid, sharp, goaty, soapy, coconut-like). The flavour intensity of FFAs depends not only on the concentration but also on the distribution between aqueous and fat phases, the pH of the medium, the presence of certain cations (e.g., Na^+, Ca^{2+}) and protein degradation products (Adda *et al.,* 1982). The pH has a major influence on the flavour impact of FFAs. At the pH of Cheddar cheese (pH ~5.2), a considerable portion of the FFAs are present as salts, which are non-volatile, thus reducing their flavour impact. In most cheese varieties, relatively little lipolysis occurs during ripening and too much is considered undesirable. Most consumers would consider Cheddar, Dutch and Swiss-type cheeses containing even moderate levels of free fatty acids to be rancid. Even lesser amounts of FFAs would make fermented milk such as yogurt rancid. However, extensive lipolysis is desirable as part of overall flavour development in certain cheeses, such as hard Italian cheeses (Romano, Provolone), Blue and Feta.

The fat fraction of dairy products is also important for the development of typical flavour. Cheddar cheese made from non-fat milk does not develop full aroma, even after 12 months (Ohern and Tuckey, 1969). Foda *et al.* (1974) suggested that the fatty acid composition and natural emulsion of milk fat are important for flavour development.

14.2.3.4. Fatty Acid Metabolism

FFAs are involved in several types of reactions which vary in importance with the type of dairy product involved (Figure 14.6). Methyl ketones are produced from fatty acids by oxidative degradation. The production of methyl ketones involves oxidation of fatty acids (mainly from $C_{6:0}$ to $C_{12:0}$) to β-ketoacids, which are then decarboxylated to corresponding methyl ketones with one carbon atom less (Hawke, 1966). Methyl ketones are responsible for the characteristic aroma of blue-veined cheeses (Gripon *et al.,* 1991). However, they play a limited role in Cheddar cheese flavour. Ultimately, methyl ketones can be reduced to secondary alcohols, which do not contribute to cheese aroma. Another reaction in which polyunsaturated and, perhaps, monounsaturated fatty acids can be involved, is oxidation. The extent of oxidation in cheese is, however, rather limited, possibly due to a low redox potential. This, together with the presence of natural antioxidants, could prevent the initiation of oxidation mechanisms, or create conditions under which the primary oxidation products are reduced (Adda *et al.,* 1982).

Aliphatic and aromatic esters play an important part in the flavour and, sometimes, the off-flavour of cheese. This synthesis mainly concerns the above-mentioned short- or medium-chain fatty acids and an aliphatic (ethanol) or aromatic (phenylethanol) alcohol or thiol (methanethiol) may be involved. Esters generally contribute a fruity flavour to dairy products which is desirable and characteristic in many cheeses (Parmesan, Parrano) but undesirable in others (Cheddar).

The mechanism of synthesis of esters in cheese is still largely unknown (Liu *et al*., 2004b). It is generally accepted that the enzymes of the cheese microflora are involved in the formation of cheese esters (Hosono *et al*., 1974; Harper *et al*., 1980; Cristiani and Monnet, 2001; Liu *et al*., 2004b), but some authors also suggest that cheese esters are not of enzymatic origin (Forss, 1972; Adda *et al*., 1982). Most *S*-methyl thioesters can be formed spontaneously in cheese from the reaction of acylCoA with methanethiol (Helinck *et al*., 2000). Two ester-producing reactions could be involved in ester formation in cheese, esterification and alcoholysis. Until recently, ester synthesis in cheese was regarded as resulting from the esterification of an alcohol and an acid. The acid or acyl CoA moieties of esters are formed from the action of the cheese microflora and their enzymes on lactose, lactate, lipids and proteins of cheese curd (Urbach, 1997a). It has been shown recently that cheese esters could also be synthesized directly from glycerides and alcohols *via* an alcoholysis reaction. Esterases of lactic acid bacteria can catalyse this reaction, involving the transfer of a fatty acyl group from triglycerides (and, preferably, mono- and di-glycerides) to an alcohol, without the direct involvement of water (Liu *et al*., 2003, 2004a). Alcoholysis could be a more common route for

esters synthesis in aqueous environments than the esterification reaction, which is favoured under low water activity conditions (Liu *et al*., 2004b). The rate-limiting factors of ester synthesis in cheese are unknown. Substrates, enzymes and the environment may all determine the rate of ester formation. In Cheddar and Swiss cheeses, however, ethanol is regarded as the limiting factor for ester synthesis (Liu *et al*., 2004b; Thierry *et al.*, 2006). The 'fruitiness' defect of Cheddar cheese, which results from the formation of ethyl esters (Bills *et al*., 1965), was found significantly correlated to the concentration of ethanol (Manning, 1979). It was reported that the addition of ethanol to Cheddar curd produced increased levels of ethyl esters (Urbach, 1993). Ethanol is the main alcohol detected in Cheddar cheese (McGugan *et al*., 1975). Its formation in Cheddar results mainly from the activity of heterofermentative lactobacilli and/or yeasts. A recent study by Thierry *et al.* (2006) demonstrated that ethanol is the limiting factor for ethyl ester synthesis in Swiss cheese.

Lactones (γ- and δ-) are potent fat-derived flavour compounds that play an important role in the overall cheese flavour profile, particularly in Cheddar (Wong *et al.,* 1973; Drake *et al.,* 2001). Data from a recently published work (Alewijn *et al.,* 2006) showed that lactic acid bacterial enzymatic activities played no role in the formation of lactones from milk triglycerides or free fatty acids. The same study also demonstrated that the mechanism of lactone formation in cheese is a one-step, non-enzymatic reaction, where a hydroxyl fatty acid esterified in a triglyceride undergoes trans-esterification to release the lactone directly.

14.2.3.5. Proteolysis and Related Reactions

Proteolysis is the most widely studied biochemical change in dairy products. During the manufacture of fermented milks and cheese and during the ripening of cheese, a gradual decomposition of caseins occurs due to the combined action of various proteolytic enzymes. These generally include enzymes from the following sources:

- Coagulant
  a) Chymosin (genetically engineered-Maxiren™, Chymax™)
  b) Chymosin/pepsin (from calf stomach)
  c) Rennet substitutes microbial (enzymes from *Rhizomucor pusillus*, *R. miehei*, *Cryphonectria parasitica*, *Aspergillus oryza*e and *Irpex lactis*), and
  d) Plant rennet (e.g., *Cynara cardunculus*)
- Indigenous milk enzymes (e.g., plasmin, cathepsins)

- Starter and non-starter bacterial enzymes
  a) Cell envelope-associated proteinases (lactocepins)
  b) Peptidases (including endopeptidases, aminopeptidases, di-/tri-peptidases and proline-specific peptidases)
- Secondary starter enzymes
- Exogenous enzymes used as ripening aids

Enzymes from the first three sources are active in most ripened cheeses. The secondary starters (microorganisms added to cheese milk or curd for purposes other than acidification, e.g., surface smear organisms, blue/white moulds) exert considerable influence on the maturation of cheese varieties in which they are used. Exogenous enzymes, when present can be very influential.

The correct pattern of proteolysis is generally considered to be a pre-requisite for the development of the correct flavour of cheese. Products of proteolysis *per se* (i.e., peptides and free amino acids) probably are significant in cheese taste, at least to 'background' flavour, and some off-flavours, e.g., bitterness, but are unlikely to contribute aroma. Compounds arising from the catabolism of amino acids contribute directly to cheese taste and aroma.

14.2.3.5.1. Hydrolysis of α_{s1}-Casein

In the cheese environment, with a high ionic strength and a low a_w, rennet-induced breakdown of α_{s1}-casein proceeds much faster than that of β-casein (α_{s2}- and *para*-κ-caseins are quite resistant to hydrolysis by the enzymes of rennet) (Visser, 1993). The residual chymosin rapidly hydrolyses α_{s1}-casein at the Phe_{23}–Phe_{24} bond during the initial stages of ripening (Creamer and Richardson, 1974). Hydrolysis of this single bond of α_{s1}-casein causes a rapid change in the rubbery texture of young Cheddar curd into a smoother, more homogeneous product (Lawrence *et al.*, 1987). However, more recent work of O'Mahony *et al.* (2005) showed that the initial softening of texture is more highly correlated with the level of insoluble calcium than with the level of intact α_{S1}-casein in cheeses early in ripening. The authors of this study concluded that the hydrolysis of α_{S1}-casein at Phe_{23}–Phe_{24} is not a prerequisite for softening which is due principally to the partial solubilization of colloidal calcium phosphate associated with the *para*-κ-casein matrix of Cheddar cheese during the early stages of ripening. The peptide α_{s1}-casein f1-23, produced by chymosin action on the bond Phe_{23}–Phe_{24} of α_{s1}-casein, is further hydrolyzed in Cheddar cheese (Singh *et al.*, 1994) by the CEP from starter *L. lactis* ssp. *cremoris,* resulting in the production of a range of small molecular weight peptides, representing N-terminal (α_{s1}-casein f1–7, 1–9,

1–13 and 1–14) and C-terminal (α_{s1}-casein f14–17, 17–21) sequences, which were found to be bitter in taste (Richardson and Creamer, 1973; Lee *et al.*, 1996). The large peptide produced by chymosin, α_{s1}-casein f24–199, is further hydrolyzed by chymosin and CEP (for further details see McSweeney *et al.*, 1994; Singh *et al.*, 1995, 1997).

14.2.3.5.2. Hydrolysis of β-Casein

Chymosin has limited action on β-casein in Cheddar, although some activity is indicated by the presence of the peptide β-casein f1–192 (McSweeney *et al.*, 1994). Hydrolysis of the bond Leu_{192}–Tyr_{193} of β-casein by chymosin releases a small C-terminal fragment, β-casein f193–209, which is extremely bitter (Singh *et al.*, 2004b). Nearly half of the β-casein in Cheddar cheese is hydrolyzed during ripening by plasmin, an indigenous milk proteinase. Plasmin hydrolysis of β-casein results in the formation of three γ-caseins [γ_1- (β-casein f29–209), γ_2- (β-casein f106–209) and γ_3- (β-casein f108–209)], representing the C-terminal region, and five proteose-peptones (β-casein f1–28, β- casein 1–105/107 and β-casein f29–105/107), representing the corresponding N-terminal region. The γ-caseins seem to accumulate in Cheddar over the ripening period. The proteose-peptones are extensively hydrolyzed by the starter bacterial CEP and peptidases to produce small peptides and free amino acids (Singh *et al.*, 1995, 1997).

Proteolysis in cheese seems to be a sequential process involving enzymes from rennet, milk proteinases (particularly plasmin), the starter culture, secondary microorganisms and non-starter lactic acid bacteria (NSLAB):

- The hydrolysis of casein to high molecular weight peptides is thought to be primarily the result of chymosin and plasmin.
- The subsequent hydrolysis of high molecular weight peptides into small peptides and free amino acids is primarily the result of proteolytic enzymes from lactic acid bacteria (Singh *et al.*, 2003a).

Proteolytic degradation of caseins into peptides in Cheddar (Singh *et al.*, 1994, 1995, 1997; Fernandez *et al.*, 1998), Emmental (Gagnaire *et al.*, 2001) and Parmigiano-Reggiano (Addeo *et al.*, 1992, 1994) cheeses has been characterized in detail.

14.2.3.6. Catabolism of Amino Acids

In lactococci, the first step in the degradation of amino acids is transamination (Figure 14.7; Gao *et al.,* 1997), leading to the formation of α-keto acids (α-KAs). Aromatic aminotransferases have been characterized from *L. lactis* ssp *cremoris* (Yvon *et al.,* 1997; Rijnen *et al.,* 1999a) and *L. lactis* ssp

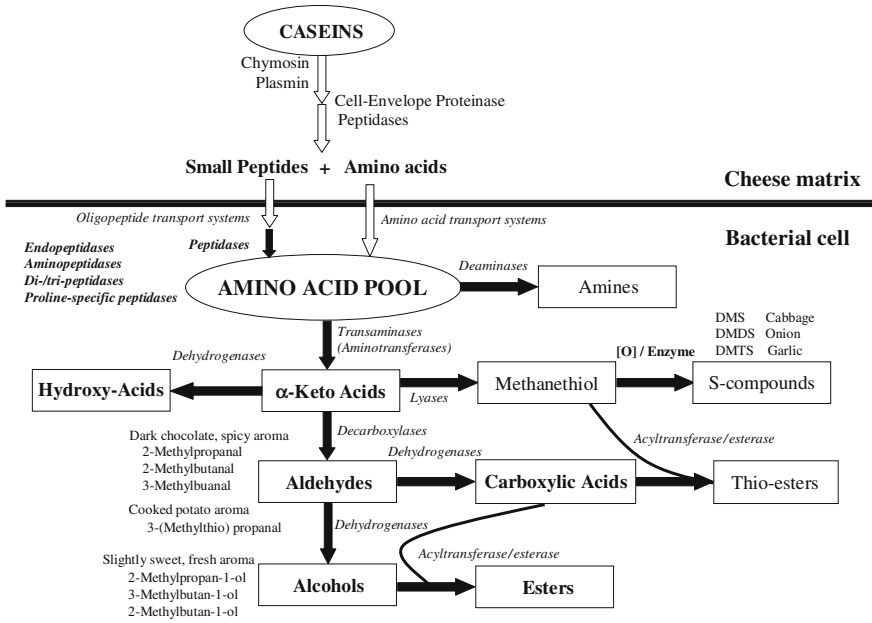

Figure 14.7. Generation of flavour compounds from milk protein degradation. DMS, dimethyl sulphide; DMDS, dimethyl disulphide; DMTS, dimethyl trisulphide (modified from Singh *et al*., 2003).

lactis (Gao and Steele, 1998). These enzymes initiate the degradation of Val, Leu, Ile, Phe, Tyr, Trp and Met, all of which are known precursors of cheese flavour compounds. Inactivation of aminotransferases involved in the breakdown of amino acids by lactococci has been shown to reduce aroma formation during cheese ripening (Rijnen *et al.,* 1999b).

α-KAs are central intermediates and can be converted to hydroxyl acids, aldehydes and CoA-esters (Figure 14.7). These reactions are mostly enzymatic, but some chemical conversion steps have also been described, like the formation of benzaldehyde from phenylpyruvic acid (Smit *et al.,* 2004). The aldehydes formed can generally be dehydrogenated or hydrogenated to their corresponding alcohols or organic acids, which are, in their turn, substrates for esterases and acyltransferases leading to (thio)esters (Figure 14.7).

Other enzymatic routes for the conversion of amino acids involve lyases (e.g., cystathionine β-lyase, threonine aldolase) and deamination/decarboxylation (resulting in the formation of amines). For further details see Smit *et al.* (2005).

The volatile fraction of cheese contains several sulfur-containing compounds such as methanethiol, methional, dimethyl sulphide, dimethyldisulphide, dimethyltrisulphide, dimethyltetrasulphide, carbonyl sulphide and hydrogen

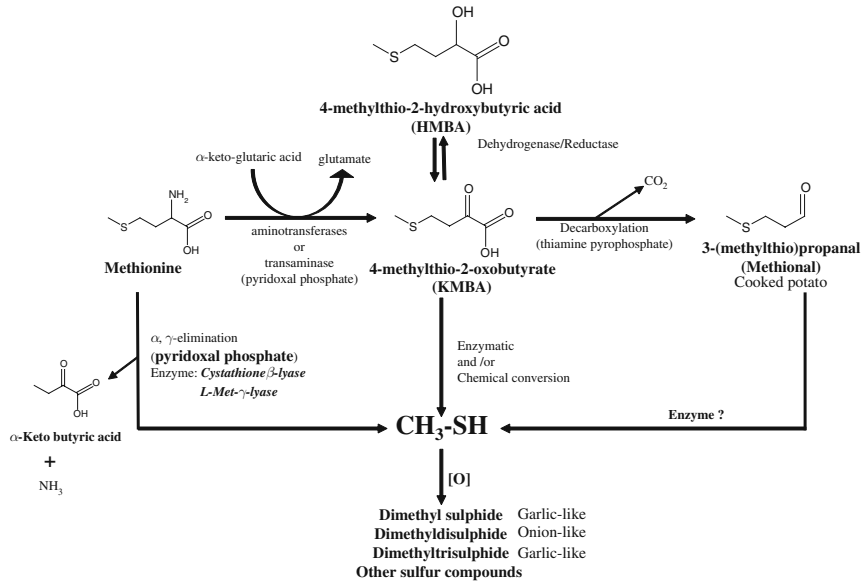

Figure 14.8. Degradation of methionine to potent sulphur-containing odorants (reproduced with permission from Singh *et al.,* 2003; © Blackwell Publishing, Inc.).

sulphide (Lindsay and Rippe, 1986; Urbach, 1995; Weimer *et al.,* 1999) which contribute to the aroma of cheese (Milo and Reineccius, 1997). These compounds are known to originate from Met (Figure 14.8). Methanethiol has been associated with desirable Cheddar-type sulfur notes in good-quality Cheddar cheese (Manning and Price, 1977; Manning and Moore 1979; Price and Manning 1983). However, alone or in excess, methanethiol does not produce typical Cheddar cheese flavour (Weimer *et al.,* 1999).

Methanethiol is readily oxidized to dimethyl disulphide and dimethyl trisulphide (Parliment *et al.,* 1982; Chin and Lindsay, 1994). The occurrence of these compounds is a direct result of the methanethiol content and is modulated by the low redox potential of cheese. Methanethiol can potentially oxidize during analysis to form these compounds, and this may account for some reports of dimethyl disulphide and dimethyl trisulphide in cheese. Dimethyl sulphide (Milo and Reineccius, 1997) and dimethyl trisulphide were noted recently as important odorants in aged Cheddar cheese (Milo and Reineccius, 1997; Suriyaphan *et al.,* 2001b; Zehentbauer and Reineccius, 2002). Further work is needed to define the mechanism and cheese conditions needed for the production of these compounds.

The volatile sulphur compounds from Met can also be produced non-enzymatically. Wolle *et al.* (2006) reported that incubation of Met in the

presence of pyridoxal-5'-phosphate (PLP) under cheese-like conditions (pH 5.2 + 4% salt at 7°C) resulted in the production of methanethiol, dimethyl disulphide and dimethyl trisulphide. A 1000-fold increase in the production of volatile sulphur compounds was observed when the reaction temperature was raised from 7 to 37°C. This reaction in conjunction with an adjunct culture with higher peptidolytic activity may offer an improved method to control and enhance the production of volatile sulphur compounds in cheeses or enzyme-modified cheeses.

Amino acid degradation plays a vital role in flavour development in cheese. A number of researchers have attempted to enhance the free amino acid content of Cheddar cheese by direct addition of amino acids (Wallace and Fox, 1997) or genetic modification of lactococci with increased amino-peptidase N activity (McGarry *et al.,* 1994; Christensen *et al.,* 1995). However, an increased amino acid content in Cheddar did not affect flavour development, which led Yvon *et al.* (1998) to hypothesize that the rate-limiting factor in flavour biogenesis was not the release of amino acids but their subsequent conversion to aroma compounds. Yvon *et al.* (1998) identified the transaminase acceptor, α-ketoglutarate, as the first limiting factor in the degradation of amino acids. Addition of α-ketoglutarate to Cheddar curd resulted in increased volatile components originating from branched-chain and aromatic amino acids (Banks *et al.,* 2001). Results of a recent study showed that Cheddar cheese made using an adjunct starter *Lactobacillus casei* (genetically modified to enhance the expression of hydroxyl acid dehydrogenase, HADH) retarded flavour development (Broadbent *et al.,* 2004). HADH catalyses the conversion of α-KAs to α-hydroxy acids, which have little or no importance from a flavour point of view. It may still be possible to selectively suppress aromatic amino acid (Phe, Tyr, Trp)-derived off-flavour compounds by overexpression of an alternative HADH with more narrow specificity for aromatic amino acid-derived α-KAs.

The effect of membrane permeablisation, by treatment with the bacteriocin lacticin 3147, on the branched-chain amino acid transamination by *L. lactis* IFPL359 was investigated by Martinez-Cuesta *et al.* (2002). Membrane permeabilisation of the cells made them non-viable, but they remained metabolically active and facilitated free amino acids diffusion into the cell. These changes made intracellular enzymes more accessible to their substrates and hence increased branched-chain amino acid transamination.

The detailed understanding of the mechanistic pathways involved in the degradation of amino acids to cheese flavour compounds and the capacity of starter/non-starter bacteria to generate volatiles will not only result in enhanced control/acceleration of cheese flavour development but also in minimizing the occurrence of off-flavours.

14.3. Characteristic Flavours of Milk and Milk Products

According to the 'Component Balance Theory', the flavour of cheese is produced by the correct balance and concentrations of a wide range of sapid and aromatic compounds (Mulder, 1952; Kosikowski and Mocquot, 1958). If a proper balance of components is not achieved, then undesirable or defective flavour occurs. Decades of flavour research on dairy products have confirmed the accuracy of this concept not only for cheese but also for various other milk and milk products. See Tables 14.1, 14.2, 14.3, 14.4, 14.5, 14.6, 14.7, 14.8 and 14.9 for a list of odorants found in milk and milk products.

14.3.1. Aroma of Fluid Milk

The delicate but weak flavour of milk is caused by very low concentrations of numerous odorants (Badings, 1991; Bendall, 2001). Thermal processing is a commonly employed method to achieve microbial safety and shelf-life stability of milk, e.g., high–temperature short-time pasteurization (typically at 72°C for 15 s; the shelf-life is only 20 days at refrigeration temperatures) and ultrahigh-temperature (UHT) processing (135–150°C for 3–5 s; UHT-treated milk is stable at room temperature for up to 6 months). When milk is severely heated, both flavour and colour are affected. Varying levels of heat treatment during processing of raw milk, e.g., pasteurization, UHT-treatment or sterilization, have been shown to increase lipid oxidation products (Table 14.1). The UHT process can induce a chalky taste and strong 'cooked' off-aroma notes in milk (Shipe, 1980). Numerous studies have linked the 'cooked' off-aroma defect to the production of volatile sulphur compounds, aldehydes and methyl ketones (Scanlan *et al.*, 1968; Jeon *et al.*, 1978; Moio *et al.*, 1994; Contarini *et al.*, 1997). Moio *et al.* (1994) compared the aroma-impact compounds in raw milk to those in pasteurized and UHT milks using gas chromatography–olfactometry–mass spectrometry (GCO–MS) and aroma extract dilution analysis (AEDA) (Table 14.1). Recently published work by Czerny and Schieberle (2007) demonstrates a remarkably different set of odorants in UHT milk (see Table 14.1 for details). Such differences may be attributed to different breeds of cow, the cow feeding or milk processing, although 2-alkanones have rather high odour thresholds and may, thus, not contribute much to the overall milk aroma. Czerny and Schieberle (2007) also reported the influence of the packaging material on the odorant profiles of UHT milk (Table 14.1).

Volatile compounds that contribute particularly to sterilized milk are lipid oxidation products (mainly 2-alkanones: $C_{5,\,7,\,9,\,11}$) and several heterocyclic compounds such as pyrazines, furans, lactones and other products of

Table 14.1. Aroma compounds identified in fluid milk

Milk (Fresh)[a]	Milk (Pasteurized)[a]	Milk (UHT)[a]	Milk (UHT)[b] Glass bottles	Milk (UHT)[b] PE bottles
Ethyl hexanoate	Dimethyl sulfone	2-Heptanone	δ-Decalactone (1024)	δ-Decalactone (>4096)
Ethyl butyrate	Hexanal	2-Nonanone	Vanillin (128)	γ-Dodecalactone (>4096)
Dimethyl sulfone	Nonanal	2-Undecanone	γ-(*Z*)-6-dodecenolactone (128)	Vanillin (256)
Nonanal	1-Octen-3-ol	δ-Decalactone	γ-Dodecalactone (128)	(*Z*)-4-Heptenal (64)
1-Octen-3-ol	Indole	2-Tridecanone	*trans*-4,5-epoxy-(*E*)-2-decenal (128)	3-(Methylthio) propanal (64)
Indole	Benzothizole	Dimethyl sulfone	δ-Octalactone (128)	Hexanoic acid (64)
	δ-Decalactone	Benzothiazole	2-Acetyl-2-thiazoline (64)	δ-Octalactone (64)
	2-Tridecanone	Hexanal	3-(Methylthio) propanal (64)	*trans*-4,5-epoxy-(*E*)-2-decenal (64)
		Indole	2-Acetyl-1-pyrroline (64)	γ-Nonalactone (64)
				δ-Nonalactone (64)
				γ-(*Z*)-6-Dodecenolactone (64)

[a]Raw, pasteurized and ultra-high temperature (UHT) treated milks analysed by gas chromatography-olfactometry/aroma extract dilution analysis/GC-mass spectrometry (GCO/AEDA/GC-MS) (Moio *et al.*, 1994).

[b]UHT treated milks stored in glass and polyethylene (PE) bottles analysed by GCO/AEDA/GC-MS (Czerny and Schieberle, 2007). Values in parenthesis represent flavour dilution factors

Table 14.2. Aroma compounds identified in cream

Cream[a]	Pasteurized cream (15% Fat)[b]	Sterilized cream (25% Fat)[c]
γ-Decalactone	2,3-Butanedione (diacetyl)	Dimethyl trisulphide
δ-Decalactone	3-Hydroxy-2-butanone (acetoin)	2-Alkanones (C_5, C_7 and C_9)
δ-Dodecalactone	Dimethyl trisulphide	2-Furfural
(*Z*)-4-Heptenal	2-Nonanone	2-Furanmethanol
(*E,E*)-2,4-Nonadienal	Butanoic acid	
	Acetic acid	
	Dimethyl sulphide	
	2-Butanone	

[a]McGorrin (2001).
[b,c]Pionnier and Hugelshofer (2006).

non-enzymatic browning/Maillard reactions [e.g., maltol, 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (HDMF), 2,5-dimethylpyrazine and *o*-aminoacetophenone] (Badings, 1991). Sweetened condensed milk has a similar volatile flavour profile (Shimoda *et al*., 2001).

14.3.2. Aroma of Fat-Enriched Milk Products

The flavour of cream is due mainly to the contributions from the aqueous phase of milk and from the fat globule membrane (Bading and Neeter, 1980), while butter aroma is derived primarily from the volatile compounds present in the fat fraction (Mallia *et al*., 2008). Whipping of cream may increase slightly the concentrations of oxidation products and, provided that it is not overdone, the flavour is improved. Begemann and Koster (1964) found (*Z*)-4-heptenal to be important in cream flavour. Pionnier and Hugelshofer (2006) analysed cream that had been subjected to different processes (pasteurisation, sterilization, UHT) and having different fat levels by GCO (Table 14.2). They identified 32 aroma compounds, which included ketones, acids, lactones and sulphur compounds. The aroma profile of sterilized cream was dominated by compounds such sulphides, ketones and Maillard products.

The key aroma compounds in sweet-cream butter were studied using AEDA by Budin *et al.* (2001) and Peterson and Reineccius (2003a). In the first study, lactones, ketones and aldehydes were found to have high aroma dilution factors (Table 14.3). Interestingly, skatole was also found as a key odorant of sweet-cream butter, showing an aroma dilution factor of 128. Peterson and Reineccius (2003a) identified additional aroma compounds in the headspace of sweet-cream butter, namely hydrogen sulphide, acetaldehyde, dimethyl sulphide, diacetyl, hexanal, 2-methylbutanal,

Table 14.3. Aroma compounds identified in butter and butter oil

Sweet cream butter[a]	Sweet cream butter[b]	Heated butter[a]	Sour cream butter[c]	Butter oil[d]
δ-Decalactone	Dimethyl trisulphide	1-Hexen-3-one	δ-Decalactone	1-Octen-3-one
δ-Dodecalactone	1-Acetyl-2-pyrroline	1-Octen-3-one	(*Z*)-6-Dodecen-γ-lactone	(*Z*)-3-Hexenal
(*Z*)-6-Dodecen-γ-lactone	2,3-Butanedione	(*E*)-2-Nonenal	Skatole	(*Z*)-2-Nonenal
1-Hexen-3-one	1-Octen-3-one	(*Z*)-2-Nonenal	Butanoic acid	(*E*)-2-Nonenal
1-Octen-3-one	δ-Decalactone	(*E,E*)-2,4-Decadienal	2,3-Butanedione	(*E,E*)-2,4-Decadienal
(*E*)-2-Nonenal	γ-Nonalactone	*trans*-4,5-Epoxy-(*E*)-2-decenal	(*Z*)-2-Nonenal	
(*E,E*)-2,4-Decadienal	Butanoic acid	2,5-Dimethyl-4-hydroxy-3(*2H*)-furanone (HDMF)	Acetic acid	
trans-4,5-Epoxy-(*E*)-2-decenal	Ethyl butanoate	Methional	(*E*)-2-Nonenal	
(*Z*)-2-Nonenal		δ-Octalactone	(*Z,Z*)-3,6-Nonadienal	
Skatole		δ-Decalactone	γ-Octalactone	
		δ-Dodecalactone	Hexanoic acid	
		Skatole	1-Penten-3-one	
			Unknown compound (fatty/nutty odour)	
			(*E,E*)-2,4-Decadienal	
			trans-4,5-Epoxy-(*E*)-2-decenal	
			(*E,E*)-2,4-Nonadienal	
			Hexanal	
			1-Octen-3-one	

[a]Budin *et al.* (2001).
[b]Lozano *et al.* (2007)-Commercial samples of fresh sweet-cream butter obtained within the 24 h of production.
[c]Schieberle *et al.* (1993).
[d]Widder *et al.* (1991).

3-methylbutanal, butanoic acid, dimethyl trisulphide, hexanoic acid, δ-hexalactone, nonanal, δ-octalactone and γ-dodecalactone. Aroma recombination studies, followed by sensory analysis, indicated that the synthetic aroma model mixture was significantly different from the reference (direct from manufacturing plant) but ranked the same (similarity index) as the aroma of a commercial margarine or an unsalted fresh butter. According to Peterson and Reineccius (2003a), sweet-cream butter was characterised by δ-octalactone, δ-hexalactone and γ-dodecalactone. In particular, δ-hexalactone and γ-dodecalactone had creamy and peach-like odours, respectively, but were identified in sweet-cream butter only by Peterson and Reineccius (2003a). Day *et al.* (1964) identified DMS in butter and considered it to be a desirable component, which smoothes the strong flavour of diacetyl. In a study by Schieberle *et al.* (1993), the overall odour impression of sweet-cream butter was evaluated by a trained sensory panel and compared with the odorants of different types of sour-cream butter. The results showed that the concentration of diacetyl is lower without a fermentation process as in sweet-cream butter, resulting in an overall mild and sweet odour impression.

Schieberle *et al.* (1993) studied different kinds of butter: sour-cream butter, Irish sour-cream butter, German farm sour-cream butter and cultured butter and compared them with sweet-cream butter. AEDA on Irish sour-cream butter, showing the most intense odour during a preliminary sensory analysis, revealed 18 odour-active compounds (Table 14.3). Sunflower oil was spiked with diacetyl, δ-decalactone and butanoic acid at the same concentrations occurring in cultured butter that was chosen as standard for the most typical butter odour. The results indicated that sunflower oil containing the three odorants exhibited an aroma note, which in quality and intensity was very similar to the odour of the cultured butter.

Butter generates potent odorants during heating (Grosch, 1986). Budin *et al.* (2001) studied odorants in heated sweet-cream butter using AEDA. The volatile fraction of butter, heated to 105–110°C for 15 min, was isolated by high-vacuum distillation. The odorants with the highest aroma dilution factors are listed in Table 14.3. The key aroma compounds of heated butter were compared with those of fresh butter: δ-decalactone, skatole, 1-octen-3-one, (*E*)- and (*Z*)-2-nonenal, (*E,E*)-2,4-decadienal and *trans*-4,5-epoxy-(*E*)-2-decenal had higher aroma dilution values in heated butter. Schieberle *et al.* (1993) determined the sensory threshold of δ-decalactone as 120 μg/kg sunflower oil. It is present in heated butter approximately 50 times above its threshold, which suggests that it is the most important odorant in heated butter (Budin *et al.*, 2001). Due to their high odour activity values (OAVs), 1-octen-3-one, methional, HDMF and *trans*-

4,5-epoxy-(*E*)-2-decenal were found to contribute to heated butter aroma. Peterson and Reineccius (2003b) studied the key aroma compounds of heated butter, using static headspace analysis, and confirmed methional, (*E*)-2-nonenal, 1-hexen-3-one, 1-octen-3-one, δ-octalactone, δ-decalactone, HDMF and skatole as potent odorants. In addition, they found hydrogen sulphide, methanethiol, acetaldehyde, diacetyl, 2-heptanone, dimethyl trisulphide, nonanal, butanoic acid, 3-methylbutanoic acid, δ-hexalactone and hexanoic acid. According to these authors, 3-methylbutanoic acid (cheese-like odour), methional (potato-like), HDMF (caramel-like) and 2-heptanone (blue cheese-like) characterize the odour of heated butter. These compounds were not detected in fresh sweet-cream butter. On the other hand, odorants such as 2- and 3-methylbutanal, hexanal, γ-dodecalactone and dimethyl sulphide, found in fresh sweet-cream butter, were not detected in heated butter. Concentrations of ketones and especially lactones increased significantly in heated butter.

Widder *et al.* (1991) investigated the key odorants of butter oil, using vacuum distillation for isolation and GCO-MS for the identification of the volatile compounds. Sixteen potent odorants were identified by AEDA; the most potent odorants are listed in Table 14.3. Vanillin was reported by the authors in butter oil for the first time. The most important aroma compounds with the highest flavour dilution (FD) factors are listed in Table 14.3 (Widder *et al.,* 1991). In the same study, the odour-active compounds in fresh butter oil were compared with those in butter oil which had been stored for 42 days at room temperature. The FD factors of the carbonyl compounds formed by lipid peroxidation increased.

Volatile compounds in traditional sour-cream butter (Wadodkar *et al.*, 2002) and in particular of Smen, a fermented butter produced in Morocco and in other Arab countries, were studied by GCO (Triqui and Guth, 2001). The results of an AEDA indicate butanoic and hexanoic acids as potent odorants. The primary mechanism of aroma development in this product is lipolysis.

14.3.3. Aroma of Dried Milk Products

Milk powder is used widely as a raw material in food formulation. Therefore, flavours and off-flavours originating from milk powders could appear in the final products (Shiratsuchi *et al.*, 1994a). Consequently, flavour monitoring is a part of the research, development, production and quality control of milk powders. The volatile flavour compounds of commercial skim milk powder were studied by extraction of volatiles using simultaneous steam distillation–extraction under reduced pressure (SDE) followed by analysis of the extracts by GCMS. The major compounds were

hydrocarbons, aldehydes, ketones, alcohols, fatty acids, esters, furans, phenolic compounds, lactones and nitrogenous compounds, which constituted over 99.5% of the total volatiles recovered. Results obtained showed that the levels of flavour compounds in the skim milk powder were very low and that their compositions were extremely complicated. Among them, free fatty acids and lactones, which were present at relatively high levels, were considered to be fundamental contributors to the flavour of skim milk. Moreover, aldehydes, aromatic hydrocarbons and some heterocyclic compounds, such as indoles or thiazole, seem to participate indirectly in the flavour of skim milk.

The aroma of dried milk products, such as non-fat dry milk, with varying heat treatment, involves very similar compounds as shown in Table 14.4, but the high heat-treated non-fat dried milk particularly has a strong aroma intensity caused by HDMF, butanoic acid, methional, *o*-aminoacetophenone, *trans*-4,5-epoxy-(*E*)-2-decenal, sotolon and vanillin (Karagul-Yuceer *et al*., 2001, 2003a). A stored sample of non-fat dried milk had particularly high odour intensities for methional and *o*-aminoacetophenone (Table 14.4; Karagul-Yuceer *et al*., 2002). The odorant profile of whole milk powder showed a significant effect of the season, as monitored by the levels of compounds such as dimethyl sulphide, pentanal, hexanal and butanoic acid (Biolatto *et al*., 2007). Whole milk powder manufactured in summer had significantly higher levels of hexanal, pentanal and dimethyl sulphide as compared to autumn and winter samples. On the other hand, butanoic acid showed significant differences between autumn and spring.

Other dried products such as rennet casein, an important food ingredient, particularly suffer from off-flavour. Results of AEDA indicated *o*-aminoacetophenone to be a potent odorant; however, descriptive sensory analysis of model aroma systems revealed that the typical odour of rennet casein was caused principally by hexanoic acid, indole, guaiacol and *p*-cresol (Karagul-Yuceer *et al.,* 2003c). It is essential for rennet casein to be bland and free of any off-flavour for its use as a food ingredient.

Dried whey and dried whey products are important ingredients in the food industry. Liquid whey is further processed into dried whey powder, whey protein concentrates (WPC; 35–80% protein) and whey protein isolates (WPI; >90% protein). Dried whey proteins are commonly used as ingredients due to their exceptional functional characteristics (Morr and Foegeding, 1990) and nutritional value (Quach *et al*., 1999). From the literature, a somewhat vague description of dried and liquid whey flavour can be obtained. Based on US standards for dry whey (USDA, 2000), reconstituted whey should have a normal liquid whey flavour, free from undesirable flavours. Characteristically, cheese whey has a slightly

Table 14.4. Aroma compounds identified in non-fat dried milk manufactured with varying heat treatments and storage

Non-fat dry milk (Low heat)[a]	Non-fat dry milk (Medium heat)[a]	Non-fat dry milk (High heat)[a]	Non-fat dry milk (stored)[b]
2,5-Dimethyl-4-hydroxy-3(*2H*)-furanone (HDMF)	δ-Decalactone	HDMF	3-(Methylthio) propanal
Butanoic acid	HDMF	Butanoic acid	*o*-Aminoacetophenone
3-(Methylthio) propanal	Butanoic acid	3-(Methylthio) propanal	HDMF
o-Aminoacetophenone	3-(Methylthio) propanal	*o*-Aminoacetophenone	2-Methyl-3-hydroxy-*4H*-pyran-4-one (Maltol)
δ-Decalactone	*o*-Aminoacetophenone	(*E*)-4,5-Epoxy-(*E*)-2-decenal	Butanoic acid
(*E*)-4,5-Epoxy-(*E*)-2-decenal	(*E*)-4,5-Epoxy-(*E*)-2-decenal	δ-Decalactone	Pentanoic acid
Pentanoic acid	Vanillin	Pentanoic acid	Acetic acid
4,5-Dimethyl-3-hydroxy-2(*5H*)-furanone (Sotolon)	2-Acetyl-1-pyrroline	Sotolon	Hexanoic acid
3-Methoxy-4-hydroxybenzaldehyde (Vanillin)	2-Acetyl-2-thiazoline	Vanillin	Octanoic acid
2-Acetyl-1-pyrroline	Sotolon	Phenyl acetic acid	Decanoic acid
2-Acetyl-2-thiazoline	Hexanoic acid	Nonanal	Dodecanoic acid
Hexanoic acid	Phenyl acetic acid	1-Octen-3-one	*p*-Cresol
Phenyl acetic acid	γ-Dodecalactone	2-Acetyl-1-pyrroline	Skatole
Octanoic acid	(E)-2-Undecenal	Hexanoic acid	Dimethyl trisulphide
Nonanal	(*E,E*)-2,4-Decadienal	Octanoic acid	(*E,E*)-2,4-Decadienal
1-Octen-3-one		(*E*)-2-Nonenal	Furfuryl alcohol
			Phenyl acetic acid
			1-Octen-3-one

[a]Non-fat dried milks analyzed by gas chromatography-olfactometry/aroma extract dilution analysis/GC-mass spectrometry (GCO/AEDA/GC-MS)(Karagul-Yuceer *et al.*, 2001, 2003a).

[b]Stored nonfat dried milks analyzed by GCO/AEDA/GC-MS (Karagul-Yuceer *et al.*, 2002).

dirty-sweet/acidic taste and odour (Bodyfelt *et al.,* 1988). The flavour quality of whey depends on

- The quality of the milk from which the cheese was made
- The type of cheese manufactured
- The method of whey handling immediately after curd draining
- The elapsed time between whey draining and pasteurization (Bodyfelt *et al.,* 1988)

The presence of strong flavours and flavour variability can limit dried whey ingredient applications. Flavour variability may be inherent in the liquid whey itself, or may be an outcome of downstream processing techniques. A better understanding of flavour variability in liquid whey may lead to methods to minimize flavour variability in dried whey ingredients. Volatiles from fresh Cheddar cheese whey batches from different processing plants/starter culture rotations were extracted with diethyl ether followed by isolation of volatiles by high-vacuum distillation (Karagul-Yuceer *et al.*, 2003b). GCO-AEDA analysis showed that 2,3-butanedione (buttery), hexanal (green), 2-acetyl-1-pyrroline (popcorn), methional (potato), (*E,E*)-2,4-decadienal (frying oil) and (*E,E*)-2,4-nonadienal (frying oil) were potent neutral/basic aroma-active compounds identified in all whey samples. Odour intensities of hexanal, (*E,E*)-2,4-nonadienal, 2,3-butanedione and (*E,E*)-2,4-decadienal were variable. Short-chain volatile acids were predominant in acidic fractions and their intensities differed among the whey samples. Results obtained by GCO agreed with quantitation results. Liquid whey aroma components are influenced by starter culture rotation (Karagul-Yuceer *et al.*, 2003b).

Few studies have specifically addressed the flavour of dried whey or whey protein products (Stevenson and Chen, 1996; Quach *et al.*, 1999; Mahajan *et al.,* 2004). Recently, aroma-impact compounds in WPC and WPI were studied using descriptive sensory analysis in conjunction with instrumental volatile aroma compound analysis studies (Carunchia-Whetstine *et al.*, 2005b). Sensory analysis of whey protein preparations showed that the samples exhibited sweet aromatic, cardboard/wet paper, animal/wet dog, soapy, brothy, cucumber and cooked/milky flavours, along with a bitter taste and an astringent mouthfeel. Key volatile flavour compounds in WPC (80% protein) and WPI are listed in Table 14.5. According to authors, these baseline data on flavour chemicals in whey proteins will help to identify the most appropriate whey ingredients to use to control or minimize flavour variability in whey-enhanced products.

Table 14.5. Aroma compounds identified in dairy ingredients

Liquid cheese whey[a]	Whey protein concentrate/ isolate[b]	Rennet casein[c]
2,3-Butanedione	Butanoic acid	*o*-Aminoacetophenone
Hexanal	2-Acetyl-1-pyrroline	2-Methoxyphenol
2-Acetyl-1-pyrroline	2-Methyl-3-furanthiol	Hexanoic acid
3-(Methylthio) propanal	2,5-Dimethyl-4-hydroxy-3-(2*H*)-furanone (HDMF)	Maltol
(*E,E*)-2,4-Decadienal	2-Nonenal	HDMF
(*E,E*)-2,4-Nonadienal	(*E,Z*)-2,6-Nonadienal	Sotolon
Short chain acids	(*E,Z*)-2,4-Decadienal	Decanoic acid
		γ-(*Z*)-6-Dodecenolactone
		Skatole
		Dodecanoic acid
		Vanillin

[a]Liquid Cheddar whey analysed by dynamic headspace/gas chromatography-mass spectrometry (DHS/GC-MS) (Karagul-Yuceer *et al*., 2003b; Carunchia-Whetstine *et al.,* 2003a).
[b]Whey protein concentrate (80% protein) and isolates were analyzed by gas chromatography-olfactometry/ aroma extract dilution analysis/GC-mass spectrometry (GCO/AEDA/GC-MS)(Carunchia-Whetstine *et al*., 2005b).
[c]Rennet casein analysed by GCO/AEDA/GC-MS (Karagul-Yuceer *et al*., 2003c).

14.3.4. Aroma of Fermented Milk Products

Diacetyl and acetaldehyde are important odorants in yoghurt and are produced by lactic acid bacteria used as starter cultures (Table 14.6). Numerous other fermented milks are produced around the world involving lactic acid bacteria and/or in some cases fairly complicated sets of microflora in which a host of volatile compounds play a crucial role in flavour. Fat content has a profound effect on flavour release, and the extent of this effect is determined to some extent by the physical chemistry of the compound concerned. Low-fat yogurts (0.2%) were found to release volatiles more quickly and at a higher intensity but with less persistence than yogurts containing fat at 3.5 or 10% (Brauss *et al.,* 1999). Sensory assessment of the yogurts showed significant differences in intensity and speed of onset of flavour, but not the overall duration of perception. Because different compounds are affected differently, the physical chemistry of flavour molecules should be considered when formulations designed to accommodate changes in fat content are created.

There has been extensive research on the flavour of cheeses, but despite this effort, only limited information is available on the chemistry of the flavour of most cheese varieties and the flavour of none is characterized

Table 14.6. Aroma compounds identified in yoghurt and Mozzarella cheese

Yoghurt[a]	Mozzarella (Bovine milk)[b]	Mozzarella (Water buffalo milk)[b]
2,3-Butanedione	Ethyl-3-methyl butanoate	1-Octen-3-ol
Acetaldehyde	Ethyl isobutanoate	Nonanal
Dimethyl sulphide	2- and 3-Methyl-1-butanol	Indole
Benzaldehyde	Phenyl acetaldehyde	3-Hydroxy-2-butanone
2,3-Pentanedione	Ethyl hexanoate	3-Methyl-2-buten-1-ol
	Ethyl butanoate	2-Octanone
	Nonanal	2-Hydroxy-3-pentanone
	1-Octen-3-ol	Heptanal

[a]Yoghurt data adapted from McGorrin (2001).
[b]Mozzarella cheese analyzed by gas chromatography-olfactometry/aroma extract dilution analysis/GC-mass spectrometry (GCO/AEDA/GC-MS) (Moio *et al*., 1993).

sufficiently to permit its reproduction by mixtures of pure compounds in a cheese model (Fox *et al.,* 1995; McGorrin, 2001; Parliment and McGorrin, 2000; Singh *et al.,* 2003a). In many previous studies, cheeses were simply analyzed for flavour by cheese graders. Such qualitative sensory data have limited use. More defined and analytical information using descriptive sensory and instrumental analysis is required. In the last couple of decades, a number of published works attempted to characterize the mechanism/enzymology of various reactions involved in the generation of volatiles in cheese. Only recent work in the last decade has attempted to study cheese flavour in detail.

In recent years, key odorants in a number of cheese varieties have been characterized by GCO and AEDA. Mozzarella cheeses produced using buffalo or cow milk were found to have very different volatiles compounds in their aroma profiles (Table 14.6).

Odorants in surface mould-ripened cheeses, like Camembert, were studied in detail by Kubickova and Grosch (1997, 1998a,b) (Table 14.7). Aroma compounds were analyzed by GCO using both AEDA and aroma extract concentration analysis (AECA). Compounds like 1-octen-3-ol and the corresponding ketone were found to be responsible for the mushroom/musty aroma note of Camembert. Kubickova and Grosch (1998a) incorporated key odorants identified in Camembert in a model cheese, which was found to be close to the genuine Camembert. The origins and properties of compounds involved in the flavour of surface-ripened cheeses were reviewed by Molimard and Spinnler (1996). Gorgonzola is an Italian soft blue-veined cheese made from whole cows milk inoculated with spores of *Penicillium roqueforti* var. *weidemanni*. There are two commercial types of Gorgonzola cheese: a traditional variety, also termed 'natural', and a creamy type also called 'sweet',

with a more delicate taste and less pungent flavour. 1-Octen-3-ol, ethyl hexanoate, 2-nonanone, 2-heptanone, 2-heptanol, ethyl butanoate, 2-nonanol and 4-methylanisole were the key odorants of the natural cheese, whereas 2-heptanone, 2-heptanol, ethyl butanoate, 3-(methylthio)propanal and an unidentified constituent with a fruity odour were characteristic of the creamy Gorgonzola cheese (Moio *et al*., 2000) (Table 14.7). On the basis of high odour activity values, 2-nonanone, 1-octen-3-ol, 2-heptanol, ethyl hexanoate, methylanisole and 2-heptanone were the most important odorants in natural and creamy Gorgonzola cheese aroma (Moio *et al*., 2000).

Key odorants in the aroma profile of Swiss Emmental were methional, HDMF, ethyl furaneol, diacetyl, 3-methyl butanal and esters (Preininger and Grosch, 1994). Cheese models composed of methional, HDMF, ethyl furaneol, acetic acid, propanoic acid, lactic acid, succinic acid, glutamic acid, sodium, potassium, calcium, magnesium, ammonium, phosphate and chloride were judged to match the flavour of Swiss Emmental cheese very well (Preininger *et al.,* 1996). The flavour of typical Swiss Gruyère cheese and a Gruyère sample exhibiting a potato-like off-flavour were characterized by Rychlik and Bosset (2001a,b). Odorants like methional, 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine were the probable source of potato-like off-flavour in Gruyere.

Münster cheese made in the United States, using *Streptococcus thermophilus* as culture and no surface smear, was described by cooked/milky, whey, milk fat/lactone, sour and salty notes using descriptive sensory analysis (Singh *et al.,* 2003b). The use of dynamic headspace dilution analysis (DHDA) methodology, previously described by Cadwallader and Baek (1998), showed that the most aromatic compounds in the headspace of Müenster were 2,3-butanedione, dimethyl sulphide, dimethyl disulphide, 2/3-methylbutanal and 2-acetyl-2-thiazoline (Singh *et al.,* 2003b).

Numerous (>80) odour-active compounds were identified in fresh Chèvre-style goat cheese and assessed by sensory analysis of model cheeses for their specific role in the overall aroma. Overall, the flavour was found to be dominated by 2,3-butanedione, 1-octen-3-one, *o*-aminoacetophenone, lactones and octanoic acid. In addition, 4-methyl octanoic and 4-ethyl octanoic acids were found to impart waxy/crayon odour to fresh goat cheese (Carunchia-Whetstine *et al.,* 2003b).

The flavour of Cheddar cheese is by far the most widely studied. It is generally accepted that the flavour quality of Cheddar cheese in the marketplace today differs considerably from that manufactured before the widespread use of pasteurization, microbial rennets and other modern manufacturing practices (Dunn and Lindsay, 1985). Much of the differences between traditional and contemporary Cheddar flavours probably should be attributed to current

Table 14.7. Aroma compounds identified in mould ripened and Swiss Gruyère cheeses

Blue-type[a]	Gorgonzola (Normal)[b]	Gorgonzola (Sweet or creamy)[b]	Camembert[c]	Swiss Gruyère[d]
2,3-Butanedione	1-Octen-3-ol	2-Heptanone	2,3-Butanedione	2- and 3-Methyl butanal
2-Methyl propanal	Ethyl hexanoate	2-Heptanol	3-Methyl butanal	3-(Methylthio)propanal
3-Methyl butanal	2-Nonanone	Ethyl butanoate	3-(Methylthio)propanal	Dimethyl trisulphide
Ethyl butanoate	2-Heptanone	3-(Methylthio)propanal	1-Octen-3-ol	Phenyl acetaldehyde
Ethyl hexanoate	2-Heptanol	Unknown (fruity odour)	1-Octen-3-one	2-Ethyl-3,5-dimethylpyrazine
3-(Methylthio) propanal	Ethyl butanoate		Phenyl ethyl acetate	2,3-diethyl-5-methylpyrazine
Dimethyl trisulphide	2-Nonanol		2-Undecanone	Methanethiol
2-Heptanone	4-Methylanisole		δ-Decalactone	Acetic acid
2-Nonanone			Methanethiol	Propanoic acid
			Dimethyl sulphide	Butanoic acid
			Acetaldehyde	3-Methyl butanoic acid
			Hexanal	Phenyl acetic acid
			Dimethyl trisulphide	
			Butanoic acid	
			Isovaleric acid	

[a]Blue cheese analyzed by gas chromatography-olfactometry/aroma extract dilution analysis/GC-mass spectrometry (GCO/AEDA/GC-MS) (Qian *et al.*, 2002).
[b]Gorgonzola cheese analyzed by GCO/AEDA/GC-MS (Moio *et al.*, 2000).
[c]Camembert cheese analyzed by GCO/AEDA/aroma concentration analysis (AECA)/GC-MS and GCO of decreasing headspace samples (GCO-H) (Kubickova and Grosch, 1997).
[d]Swiss Gruyère cheese analyzed by GCO/AEDA/GC-MS and dynamic headspace (DH)-GC-MS (Rychlik and Bosset, 2001a,b).

marketing of bland-flavoured young cheeses. However, even longer-aged cheeses are frequently criticized for a lack of adequate Cheddar-type flavour.

The significance of sulfur compounds, such as H_2S, methanethiol and dimethyl sulphide, in Cheddar was shown by Manning and Robinson (1973). The compounds with low vapor pressure/high boiling points in the distillate, such as 2,3-butanedione, methyl ketones and volatile fatty acids were also considered to play an important role in Cheddar flavour. Analysis of Cheddar headspace volatiles also reconfirmed the important role played by H_2S, methanethiol and dimethyl sulphide in flavour (Manning and Price 1977; Manning and Moore 1979; Price and Manning 1983).

In order to evaluate important odorants, GCO/AEDA was first applied to Cheddar cheese by Christensen and Reineccius (1995). The components found to have the highest potency (dilution factor) in a 3-year-old Cheddar cheese were ethyl acetate, 2-methylbutanal, 3-methylbutanal, 2,3-butanedione, α-pinene, ethyl butyrate, ethyl caproate, 1-octen-3-one, acetic acid, methional, propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, decanoic acid and dodecanoic acid. The authors pointed out that the technique did not allow the determination of the most volatile odour fraction, which included hydrogen sulphide, acetaldehyde and methanethiol. Descriptive sensory analysis was not conducted on the cheese used in the study, which limited conclusions about the role of individual compounds on specific cheese flavours. Based on these results, a subsequent sensory study using a concept matching technique was conducted. Dacremont and Vickers (1994) found that a recognizable Cheddar aroma was produced by a mixture of 2,3-butanedione, methional and butanoic acid. However, the authors also indicated a possible contribution of other aroma compounds that were not commercially available at that time.

Milo and Reineccius (1997) applied both traditional high-vacuum isolation-aroma extract dilution analysis (AEDA) and GCO of decreasing headspace samples (GCO-H) to further study the aroma of a regular and a low-fat Cheddar cheese (Table 14.8). After quantification and calculation of the respective odour activity values, based on sensory thresholds in oil and water, they suggested that acetic acid, butyric acid, methional, 2,3-butanedione and homofuraneol are the primary odorants responsible for the pleasant mild aroma of Cheddar cheese. In addition to these compounds, the contribution of highly volatile sulfur compounds such as methanethiol and dimethyl sulphide to nasal perception of Cheddar cheese was quite obvious on the basis of GCO analysis of static headspace samples. The authors further hypothesized that the meaty–brothy odour characteristic of low-fat Cheddar was caused by high concentrations of methional, HDMF and especially homofuraneol. The HDMF-type odorants are known to be produced by certain strains of lactobacilli (Preininger, 1995). While the mixture of these

volatile compounds in a model cheese matrix had Cheddar aroma, attribute profiling described it as lacking in sour, mouldy and sulfurous notes relative to the real cheese. Also, the overall odour was described as weak. This discrepancy in sensory character between the aromatized model and the real cheese was caused partially by aroma–matrix interactions, which resulted in quantitative errors (Wang and Reineccius, 1998).

A comparison of the volatile components of full- and reduced-fat Cheddar showed that the level of methanethiol in the cheese is highly correlated with the flavour grade. This observation may indicate that the lack of aroma in reduced-fat Cheddar is due to the lack of methanethiol. However, a combination of methanethiol and decanoic acid or butanoic acid in all cheeses gave a better correlation with Cheddar flavour than methanethiol alone (Dimos *et al.,* 1996). Addition of methanethiol to a bland slurry of reduced-fat Cheddar produced a strong Cheddar aroma (Urbach, 1997b).

The use of dynamic headspace dilution analysis (DHDA) methodology has suggested additional volatiles as being important to Cheddar cheese aroma as compared to the aforementioned results from GCO-H and solvent extraction/AEDA (Zehentbauer and Reineccius, 2002) (Table 14.8). Results of DHDA showed that in addition to the odorants previously identified by AEDA and GCO-H, (*Z*)-4-heptenal, 2-acetyl-1-pyrroline, dimethyl trisulphide, 1-octen-3-one, (*Z*)-1,5-octadiene-3-one and (*E*)/(*Z*)-2-nonenal, which have been underestimated or not even perceived during AEDA, may also contribute to the overall aroma of Cheddar cheese.

The volatile aroma components of two sharp Cheddar cheeses of British Farmhouse origin, made using raw milk and ripened for at least 1 year, were analyzed by AEDA (Suriyaphan *et al.,* 2001b, Table 14.8). Descriptive sensory analysis of these cheeses was also conducted. Key flavours in sharp Cheddar cheeses were barnyard and earthy. Following instrumental analysis, model system addition was used to confirm compounds responsible for specific flavour notes. *p*-Cresol was mainly responsible for a 'cowy-barny' note, whereas an intense 'soil-like' note was due to 2-isopropyl-3-methoxypyrazine. At much lower odour intensity, 2-isobutyl-3-methoxypyrazine contributed a 'bell pepper-like' note. Direct addition of *p*-cresol (>100 μg/kg) or 2-isopropyl-3-methoxypyrazine (>3 μg/kg) to a mild domestic Cheddar cheese resulted in increased intensities of cowy/phenolic and earthy/bell pepper aroma notes. Additionally, within the same wedge of cheese, the concentrations of *p*-cresol and 2-isopropyl-3-methoxypyrazine were lower at the center than at the rind.

Avsar *et al.* (2004) determined that the aldehydes, 2- and 3-methyl butanal and methyl propanal, which are derived from leucine, isoleucine and valine, respectively, have central roles in the nutty flavour of Cheddar

Table 14.8. Aroma compounds identified in Cheddar and Parmigiano-Reggiano cheeses

Mild Cheddar[a]	Sharp Cheddar (British Farmhouse)[b]	Parmigiano Reggiano[c]
2,5-Dimethyl-4-hydroxy-3-(*2H*)furanone (HDMF)	2-Isopropyl-3-methoxypyrazine	2-Methylpropanal
(*E*)-2-Nonenal	3-(Methylthio) propanal	2-Methylbutanal
2,3-Butanedione	*p*-Cresol	3-Methylbutanal
(*Z*)-4-Heptenal	δ-Dodecalactone	Dimethyl trisulphide
3-(Methylthio) propanal	Butanoic acid	2,3-Butanedione
1-Octen-3-one	3-methyl butanoic acid	3-(Methylthio) propanal
2-Acetyl-2-thiazoline	2-Phenylethanol	Phenyl acetaldehyde
Dimethyl trisulphide	Ethyl octanoate	Ethyl butanoate
(*Z*)-1,5-Octadien-3-one	Acetic acid	Ethyl hexanoate
(*Z*)-2-Nonenal	β-Damascenone	Ethyl octanoate
Ethyl butanoate	Octanoic acid	Acetic acid
Hexanal	Sotolon	Butanoic acid
2-Isobutyl-3-methoxypyrazine	Phenyl acetic acid	Hexanoic acid
trans-4,5-Epoxy-2-(*E*)-decenal	Ethyl butanoate	Octanoic acid
2-Nonanone	Ethyl hexanoate	
2-Isopropyl-3-methoxypyrazine	Dimethyl trisulphide	
Decanal	Phenyl acetaldehyde	
2/3-Methyl butanal	Pentanoic acid	
Ethyl octanoate	Guaiacol	
1-Hexen-3-one	γ-Decalactone	
Methyl propanal	δ-Decalactone	
Ethyl hexanoate	1-Octen-3-one	
Homofuraneol	2-Acetylpyrazine	
Butanoic acid	2-Isobutyl-3-methoxypyrazine	
	Linalool	
	(*E,Z*)-2,6-Nonadienal	
	Geosmin	
	HDMF	

[a]Mild Cheddar cheese analyzed by gas chromatography-olfactometry/aroma extract dilution analysis/GC-mass spectrometry (GCO/AEDA/GC-MS) and GCO/dynamic headspace dilution analysis (DHDA)/GC-MS (Zehentbauer and Reineccius, 2002).
[b]British farmhouse Cheddar cheese analyzed by GCO/AEDA/GC-MS (Suriyaphan *et al.,* 2001b).
[c]Parmigiano Reggiano cheese analyzed by dynamic headspace analysis-GC-MS (Qian and Reineccius, 2002).

cheese. Nutty flavours are desirable, but they are generally found only in very mature Cheddar cheeses (>9 mo; Avsar *et al*., 2004). After the identification of volatiles responsible for the nutty flavour in Cheddar, Carunchia-Whetstine *et al.* (2006) attempted to create this specific aged cheese flavour note by employing specific adjunct cultures in pilot-scale cheese-making trials. Adding *L. lactis* ATCC 29146 at the level of 10^4 or 10^5 cfu/mL of milk as an

adjunct culture during manufacture of Cheddar cheese resulted in an increase in nutty flavour perception in the cheese. Cheeses ripened at 13°C developed aged flavours (including nutty) more rapidly than cheeses ripened at 5°C. Panellists described the 1-week old cheeses as nutty/malty, whereas the 4- and 8-mo cheeses were described only as nutty. The concentrations of 2- and 3-methyl butanal and methyl propanal increased during aging and were higher in the cheeses with the adjunct culture added and in the cheeses ripened at 13°C. This study demonstrates the advantages of linking descriptive sensory analysis, flavour chemistry and starter culture biochemistry to control cheese flavour. These results allow cheese manufacturers the opportunity to optimize the cheese-making procedure to produce a consistent nutty-flavoured Cheddar cheese.

It is important to note that in each of the studies mentioned previously, different Cheddar cheeses of different ages, microflora, and biochemistry were studied. Cheddar cheese encompasses a wide category and there are numerous potential flavour profiles. Thus, to elucidate Cheddar cheese flavour is a large task and descriptive sensory analysis should be conducted in conjunction with any instrumental study to provide clarification.

14.4. Specific Flavours or Off-Flavours in Milk and Milk Products

In addition to the characteristic desirable flavours, dairy products frequently suffer from specific flavour defects. While desirable flavour has been difficult to define in chemical and sensory terms since consumers vary in preference and definition of dairy product flavour, the specific cause(s) of many of these specific flavour or off-flavour notes have been established more or less definitively. This section presents an overview on chemicals responsible for the specific flavour/off-flavour notes in dairy products.

It is important to note that at the beginning of the development of flavour defects, or before the aroma detection threshold of certain off-flavours is reached, the fluid milk may show a poor/flat flavour that lacks freshness (Badings, 1991). GCO analysis performed on five milk samples (one of good flavour and four samples tainted with off-flavour characterized as 'feed') revealed approximately 75 aroma-active compounds (Mounchili *et al.*, 2005). Nearly all those odorants were common to all the milk extracts analysed (both good-quality and off-flavoured samples), suggesting that off-flavour originated from the concentration differences of a common set of compounds rather than from the absence or presence of specific compounds. Off-flavours in milk may develop *via* the different

mechanisms outlined below (see Table 14.9 for possible causative chemicals/mechanisms involved):

- Off-flavour transferred to milk during lactation
  - Feed off-flavours (e.g., from green forages, silage, etc., consumed by the animal a few hour before milking)
  - Weed off-flavour (e.g., volatiles formed from certain weeds during digestion)
- Cowy flavours (related to ketosis and acetonemia in cattle resulting in an increased concentration of acetone)
- Stable off-flavours (e.g., transferred via the respiratory system from stable/feed to cow)
- Lipid oxidation—most widely implicated in the defects in milk and milk products
- Microbial action
- Contamination (e.g., compounds from sanitizers)
- Thermal abuse
- Adsorption/permeation of aroma compounds with/through packaging material

Some of the mechanisms of off-flavour development, in particular lipid oxidation and microbial metabolites, have also been responsible for the development of a whole variety of off-flavours in milk and milk products (Table 14.9). Specific flavours like nutty or brothy flavours were also characterized in Cheddar cheese (Table 14.9), which may or may not be considered as an off-flavour depending on the consumer preference.

14.5. Taste Compounds in Milk and Milk Products

Research on taste compounds in dairy products is fairly limited but there seems to be an increasing interest in this area in recent years. In food systems such as milk and milk products, study of taste compounds in isolation or in matrices devoid of contribution from volatile aroma compounds is difficult due to complex nature, in terms of both number of food constituents and their competing/synergistic effects on taste and/or aroma. Compounds which contribute to the taste of milk and milk products can originate from three possible sources:

(1) Naturally found in milk, e.g., lactose
(2) Added/produced during the manufacturing process (e.g., NaCl, lactic acid)
(3) Produced by many biochemical reactions occurring during fermentation.

Table 14.9. Specific flavour and off-flavour compounds in cheeses (modified from Singh *et al.*, 2007)

Defect	Chemicals	Mechanism of Formation	Reference
Processed milk			
Oxidized	Heptanal, octanal, nonanal, 2-octenal, 2-nonenal	Lipid oxidation, light abuse, Cu oxidation	Forss *et al.* (1955a, b)
Malty	2- and 3-methylbutanal	Microbial, enzymatic, Strecker degradation	Morgan (1970a,b)
Metallic	1-Octen-3-one	Lipid oxidation	Day *et al.* (1963)
Fishy	Trimethylamine	Microbial	Corfield (1955), Humphriss (1953) Lunden *et al.* (2002)
Phenolic	*p*-Cresol	Enzymatic, microbial	Badings and Neeter (1980)
Fruity	Ethyl butanoate, ethyl hexanoate	Microbial, enzymatic	Wellnitz-Ruen *et al.* (1982) Whitfield *et al.* (2000) Marsili (2003)
Rancid	FFAs (C4-10)	Microbial, enzymatic, sanitizer	Azzara and Campbell (1992), Marsili (2000)
Oxidized	Hexanal, 1-octen-3-one	Lipid oxidation, light abuse, Cu oxidation	Cadwallader and Howard (1998) Marsili and Miller (1998)
Oxidized (cardboard)	1-Octen-3-one, octanal	Lipid oxidation	Hammond and Seals (1972)
Oxidized (general)	Aldehydes and ketones	Lipid oxidation	Badings (1991)
Oxidized (fatty/fried)	2-Alkenals (C7-C10), 2,4-alkadienals (C7, C10)	Lipid oxidation	Badings (1991)
Oxidized (green/cucumber)	(*Z*)-3-Hexenal, (*E,Z*)-2,6-nonadienal	Lipid oxidation	Badings (1991)
Oxidized (tallowy)	2-Alkenals (C7-C10), alkanals	Lipid oxidation	Badings (1991)
Oxidized (fishy)	2,4,7-Decatrienal	Lipid oxidation	

(*Continued*)

Table 14.9. (*Continued*)

Defect	Chemicals	Mechanism of Formation	Reference
Cooked flavour	H_2S, methanethiol, dimethylsulphide and other sulphides	Thermally induced	Boelrijk and de Jong (2003)
	Maltol, furans, pyrazines and other Maillard reaction products	Thermally induced	Badings (1991)
Weed taints	Indole, skatole, mercaptans, sulphides, nitriles, Thiocyanates	Metabolites of weed	Badings (1991)
	Skatole (fecal odour)	Metabolites of weed	Park (1969)
	Benzylthiol, benzyl methyl sulphide (burnt odour)	Metabolites of weed	Park *et al.* (1969)
Feed flavours	Dimethyl sulphide, acetone, butanone, isopropanol,ethanol, propanal	Metabolites of feed and silage	Badings (1991)
Feed flavours	(*E*)-2- and (Z)-3-hexenal	Freshly cut alfalfa	Marsili (2003)
Feed flavours	Dimethyl sulphide, 2-butanone, hexanal	Grass silage	Mounchili *et al.* (2005)
Light induced	Dimethyl disulphide	Light-induced degradation (singlet oxygen oxidation) of methionine	Jung *et al.* (1998)
Heat abuse, cooked	2-Pentanone, 2-heptanone, 2-nonanone	Thermally induced, microbial	Marsili and Miller (2003)
	Methanethiol, dimethyl sulphide, dimethyl trisulphide, hydrogen sulphide	Thermally induced	Vazquez-Landaverde *et al.* (2005, 2006b)
Stale	C3-11, 13 2-alkanones / C4-10 saturated aldehydes	Thermally induced	Perkins *et al.* (2005)
Cooked/stale/sulphury	2,3-Butanedione, 2-alkanones, 2-methylpropanal, 3-methylbutanal, nonanal, decanal, dimethyl sulphide	Thermally induced	Vazquez-Landaverde *et al.* (2005)

(*Continued*)

Table 14.9. (*Continued*)

Defect	Chemicals	Mechanism of Formation	Reference
Chemical, solvent-like	Propylacetate	Solvent residue from packaging material	Marsili (2003)
Butter			
Green or yogurt-like	Acetaldehyde	Microbial, enzymatic	Lindsay *et al.* (1965)
Metallic	1,5-Octadiene-2-one	Lipid oxidation	Swodoba and Peers (1977)
Stale	Styrene	Residue from packaging material	Lozano *et al.* (2007)
Burnt/rubbery/sulphur	Benzyl methyl sulphide	Unknown	Drake *et al.* (2007b)
Butter oil			
Cardboard	(*E*)-/(*Z*)-2-Nonenal	Lipid oxidation	Grosch *et al.* (1994) Widder and Grosch (1997)
Sterilized concentrated milk			
Stale	*o*-Aminoacetophenone	–	Arnold *et al.* (1966)
Ice cream			
Putrid flavour	Dimethyl disulphide, hexanal	Light-induced degradation (singlet oxygen oxidation) of methionine / lipid oxidation	Marsili (2003)
Sour cream buttermilk			
Metallic	(*E,Z*)-2,6-Nonadien-1-ol	Lipid oxidation, microbial, enzymatic	Helier and Schieberle (1997a, b)
Yogurt			
Smoky or phenolic aroma	Guaiacol (defect found in vanilla flavoured products)	Microbial	Whitfield (1998)
Cheese-like off-note	Short-chain fatty acids	Microbial, enzymatic	Rychlik *et al.* (2006)

(*Continued*)

Table 14.9. (*Continued*)

Defect	Chemicals	Mechanism of Formation	Reference
Rancid, unclean	Short-chain fatty acids	Enzymatic, microbial	Cadwallader (unpublished)
Swiss Gruyère			
Potato-like aroma	Methional	Microbial, enzymatic	Rychlik and Bosset (2001a,b)
Gruyère de Comtè			
Potato-like aroma	3-Methoxy-2-propylpyridine	Microbial	Dumont *et al.* (1975)
Smear-coated cheese			
Potato-like aroma	2-Methoxy-3-isopropylpyrazine	Microbial	Dumont *et al.* (1983)
Feta			
Kerosene-like	*trans*-1,3-Pentadiene	Microbial, enzymatic	Horwood *et al.* (1981)
Goat cheese			
Waxy/crayon	4-Methyl octanoic acid, 4-ethyl octanoic acid	Milk, enzymatic	Carunchia-Whetstine *et al.* (2003b)
Oxidized	1-Heptanol, heptanal, nonanal, 2-decenal	Lipid oxidation, light-induced	Kim *et al.* (2003)
Brie / Camembert			
Musty/earthy	Methylisoborneol	Microbial	Karahadian *et al.* (1985)
Cheddar cheese			
Unclean/off-flavour	2-Methyl propanoic acid, 3-methyl butanoic acid	Microbial, enzymatic	Nakae and Elliot (1965a,b)
Catty flavour	2-mercapto-2-methylpentan-4-one and sulphide	Reaction of mesityl oxide (contaminant)	Badings (1967), Spencer (1969a,b) Drake *et al.* (2001, 2002)
Fruitiness	Ethyl butanoate, ethyl hexanoate, ethyl octanoate	Microbial, enzymatic	Bills *et al.* (1965), Morgan (1970b)

(*Continued*)

Table 14.9. (*Continued*)

Defect	Chemicals	Mechanism of Formation	Reference
Floral/rose-like	Phenyl ethanol, Phenyl acetaldehyde	Microbial, enzymatic	Dunn and Lindsay (1985)
Unclean, utensil-like	*p*-Cresol (Off-flavour enhanced by FFAs)	Microbial, enzymatic	Dunn and Lindsay (1985)
Unclean (dull harsh)	2- and 3-Methylbutanal, 2-methylpropanal	Microbial, enzymatic	Dunn and Lindsay (1985)
Rosy/floral	Phenylacetaldehyde, phenylacetic acid	Microbial, enzymatic	Carunchia-Whetstine *et al.* (2005a)
Yeasty flavour	Ethanol, ethyl acetate, ethyl butanoate	Microbial, enzymatic	Horwood *et al.* (1987)
Phenolic	*p*-Cresol	Microbial, enzymatic	Ramshaw *et al.* (1990)
Mayonnaise/bread-like	(*E,E*)/(*E,Z*)-2,4-Decadienal	Microbial, lipid oxidation, enzymatic	Suriyaphan *et al.* (1999, 2001a)
Cowy / barny-flavours	*p*-Cresol	Microbial, enzymatic	Suriyaphan *et al.* (2001b)
Earthy / bell pepper	2-isopropyl-3-methoxypyrazine	Microbial, Maillard reaction	Suriyaphan *et al.* (2001b)
Brothy flavour	3-(Methylthio) propanal, 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone, Ethyl furaneol, 2-Methyl-3-furanthiol (and its dimerized form)	Microbial, enzymatic, Maillard reaction	Singh *et al.* (2004a), Cadwallader *et al.* (2006)
Nutty flavour	2-Methylpropanal, 2- and 3-methylbutanal	Microbial, enzymatic	Avsar *et al.* (2004) Carunchia-Whetstine *et al.* (2006)
Mothball/grassy	Acetic acid, 2-methyl butanoic acid, skatole	Microbial, nzymatic	Drake *et al.* (2007b)
Italian cheeses (Provolone, Pecorino, Romano and Parmesan)			
Medicinal/cowy	Phenol, *m*- and *p*-cresol, ethyl phenol, 3,4-dimethyl phenol, 2-isopropyl phenol, thymol and carvacrol	Microbial, enzymatic, feed	Ha and Linsay (1991), Ney (1973)

(*Continued*)

Table 14.9. (*Continued*)

Defect	Chemicals	Mechanism of Formation	Reference
Club cheese			
Musty/dirty	2-Alkanone (C7,9,11), 8-nonen-2-one	Mould growth/ metabolites	Marsili (2007b)
Skim milk powder			
Stale	*o*-Aminoacetophenone	–	Parks *et al.* (1964)
Stale	2-Furaldehyde, 2-furfuryl butyrate, alkylpyrazine, *N*-ethyl-2-formylpyrrole	Non-enzymatic browning	Ferrenti and Flanagan (1972)
Cow house-like	β-Ionone, benzothiazole, tetradecanal	–	Shiratsuchi *et al.* (1994b)
Papery/cardboard-like	(*E,E*)-2,4-Nonadienal, (*E,E*)-2,4-decadienal	Lipid oxidation	Karagul-Yuceer *et al.* (2003a)
Infant formula			
Rancid	Propanal, pentanal, hexanal	Lipid oxidation	Romeu-Nadal *et al.* (2007)
Whey protein concentrates/isolates			
Cabbage off-flavour	Dimethyl trisulphide	Degradation of Met	Wright *et al.* (2006)
Sour cream powder			
Melon/ripened kiwi-like	2,4,5-Trimethyloxazole	Reaction of diacetyl and Arg/Cys	Marsili (2007c)

Table 14.10. Taste compounds in milk and milk products (modified from Singh *et al.*, 2007)

Compounds	Taste/other complex sensation	Products
Lactose	Sweet	Milk, concentrated / evaporated milks, dried milk powders
Sucrose	Sweet	Sweetened yogurt, ice creams, sweetened condensed/evaporated milks
Lactic acid	Sour	Fermented milks / cream, cheeses
Acetic/propanoic acid	Sour	Fermented milks, cheeses
Propionic acid	Sour, umami	Umami taste in Swiss cheese
Succinic acid	Umami	Umami taste in Swiss cheese
Ca/Mg salt of propanoic acid	Sweet	Cheeses
Peptides	Mostly bland, some can be bitter, sour or umami	Milk, Fermented milks, cheeses
γ-Glutamyl dipeptides	Sour, salty, brothy metallic	Comté Cheese
Amino acids		
Gly, Ala, Ser, Thr	Sweet	Cheeses
Glu, Asp, Gln, Asn	Sour, umami	Cheeses
His	Sour (?)	Cheeses
Pro, Lys	Sweet, bitter	
Leu, Val, Ile, Arg, Phe, Tyr	Bitter	Cheeses
Trp		
NaCl	Salty	Cheeses
Ethanol	Slightly sweet, cooling/ drying sensation	Fermented milks (e.g., Kefir, Koumiss)
CO_2	Sour, fresh, cooling sensation	Fermented milks (e.g., Kefir, Koumiss)

Table 14.10 lists a number of compounds, and their taste contribution, present in dairy products. Important details available in the literature on taste compounds are summarized below:

- The main taste compounds in milk are lactose (approximately 0.3 times as sweet as sucrose) and the dissolved salts, which cause a sweet and salty taste, respectively. The sweet taste dominates, whereas salty taste is prevalent if the Na/lactose ratio is high, as in the case of mastitic milk. The casein reportedly somewhat masks the sweet taste of lactose in milk (Walstra *et al.,* 1999). Lactose-hydrolyzed milk and whey have a sweeter taste than regular pasteurized milk.

- A chalky taste is noticed in high heat-treated or UHT-treated milks. This may be the result of precipitation of colloidal calcium phosphate.
- Sodium chloride is an important contributor to the taste of cheeses. The apparent saltiness of cheese increases with maturity, increased NaCl concentration and decreasing pH (McSweeney, 1997).
- The principal acid in fermented milk and cheese is lactic acid. The concentration of lactic acid, and also the pH, varies considerably with:
  - The type of fermented dairy product
  - Initial production by the starter culture
  - Extent of loss in whey
  - Its metabolism by the non-starter microflora

Several other acids, e.g., acetic, propanoic, and $C_{4–10}$, also contribute to sour/soapy taste but they contribute mostly towards the aroma. Some of the characteristic taste (sour, sweet, salty) compounds of Emmental (Swiss) cheese are acetic acid, propanoic acid, lactic acid, succinic acid glutamic acid, each in free form and/or as ammonium, sodium, potassium, magnesium and calcium salts, as well as corresponding chlorides and phosphates (Warmke *et al.,* 1996). Magnesium and calcium propionate mainly cause the sweetish note in the taste profile of Emmental cheese.

- Casein is hydrolyzed to varying degrees depending on the type of fermented milk and cheese, resulting in the production of peptides and free amino acids. The precise role of the intermediate to small molecular weight peptides is not clear, but they are generally accepted to play an important role in the background taste of cheese (Fox *et al.,* 1994). Several peptides have been identified in different types of cheeses as bitter (see Table 14.11 for a list of bitter peptides). An interesting relationship was established by Ney (1981) between the average hydrophobicity (Q) of a peptide, as measured by the hydrophobicity of amino acid side chains determined by Tanford (1962), and bitterness. Peptides with a Q value >1400 cal mol^{-1} $residue^{-1}$ and a molecular weight up to 6000 Da (molecules >6000 Da are likely to be too large to interact with the taste receptors) taste bitter, and no bitterness occurs when Q is <1300 cal mol^{-1} $residue^{-1}$. Peptide β-CN f193–209, identified in both Cheddar and Gouda, was shown to be bitter by detailed sensory analysis (Singh *et al.,* 2004b). This peptide with a Q value of 1762 cal mol^{-1} $residue^{-1}$ and molecular weight of 1882.51 Da was

Table 14.11. Bitter peptides identified in cheeses

Peptide	Sequence	Hydrophobicity cal mol^{-1} $residue^{-1}$	Type of cheese and Reference
α_{s1}-CN f1-7	H.Arg.Pro.Lys.His.Pro.Ile.Lys.OH	1771.0	**Cheddar**; Lee *et al.* (1996)
α_{s1}-CN f1-13	H.Arg.Pro.Lys.His.Pro.Ile.Lys. His.Gln.Gly.Leu.Pro.Gln.OH	1363.0	**Cheddar**; Lee *et al.* (1996)
α_{s1}-CN f11-14	H.Leu.Pro.Gln.Glu.OH	1367.0	**Cheddar**; Lee *et al.* (1996)
α_{s1}-CN f14-17	H.Glu.Val.Leu.Asn.OH	1162.5	**Cheddar**; Hodges *et al.* (1972), Richardson and Creamer (1973), Hamilton *et al.* (1974)
α_{s1}-CN f17-21	H.Asn.Glu.Asn.Leu.Leu.OH	1074.0	**Cheddar**; Hodges *et al.* (1972), Richardson and Creamer (1973), Hamilton *et al.* (1974)
α_{s1}-CN f26-32	H.Ala.Pro.Phe.Pro.Glu.Val.Phe.OH	1930.0	**Cheddar**; Richardson and Creamer (1973)
α_{s1}-CN f26-33	H.Ala.Pro.Phe.Pro.Glu.Val. Phe.Gly.OH	1688.8	**Cheddar**; Hodges *et al.* (1972), Hamilton *et al.* (1974)
α_{s1}-CN f198-199	H.Leu.Trp.OH	2710.0	**Alpkäse;** Guigoz and Solms (1974)
α_{s2}-CN f191-197	H.Lys.Pro.Trp.Ile.Gln.Pro.Lys.OH	2010.0	**Cheddar**; Lee *et al.* (1996)
β-CN f8-16	H.Val.Pro.Gly.Glu.Ile.Val. Glu.Ser(P).Leu.OH	1390.0	**Cheddar**; Lee *et al.* (1996)
β-CN f 46-67	H.Gln.Asp.Lys.Ile.His.Pro.Phe. Ala.Gln.Thr.Gln.Ser.Leu. Val.Tyr.Pro.Phe.Pro.Gly.Pro.Ile. (Pro/His).OH	1580.5	**Cheddar**; Richardson and Creamer (1973), Hamilton *et al.* (1974)
β-CN f 61-69	H.Pro.Phe.Pro.Gly.Pro.Ile. Pro.Asn.Ser.OH	1792.2	**Butterkäse;** Huber and Klostermeyer (1974)
β-CN f46-84	H.Gln.Asp.Lys.Ile.His.Pro.Phe.Ala. Gln.Thr.Gln.Ser.Leu.Val.Tyr.Pro. Phe.Pro.Gly.Pro.Ile.(Pro/ His).Asn.Ser.Leu.Pro.Gln.Asn.Ile. Pro.Pro.Leu.Thr.Gln.Thr.Pro.Val. Val.Val.OH	1508.5	**Cheddar**; Hamilton *et al.* (1974)

(*Continued*)

Table 14.11. (*Continued*)

Peptide	Sequence	Hydrophobicity cal mol^{-1} $residue^{-1}$	Type of cheese and Reference
β-CN f84-89	H.Val.Pro.Pro.Phe.Leu.Gln.OH	1983.3	**Gouda**; Visser *et al.* (1983)
β-CN f193-209	H.Tyr.Gln.Glu.Pro.Val.Leu.Gly.Pro. Val.Arg.Gly.Pro.Phe.Pro.Ile.Ile. Val.OH	1762.4	**Cheddar**; Kelly (1993), Broadbent *et al.* (1998), Soeryapranata *et al.* (2002a, b), Singh *et al.* (2004b, 2005) **Gouda**; Visser *et al.* (1983)
β-CN f193-208	H.Tyr.Gln.Glu.Pro.Val.Leu.Gly. Pro.Val.Arg.Gly.Pro.Phe.Pro.Ile. Ile.OH	1766.9	**Gouda**; Visser *et al.* (1983)
β-CN f193-207	H.Tyr.Gln.Glu.Pro.Val.Leu.Gly. Pro.Val.Arg.Gly.Pro.Phe. Pro.Ile.OH	1686.7	**Gouda**; Visser *et al.* (1983)

also classified as potentially bitter in the Q value model proposed by Ney (1981). Amino acids are also known to elicit different taste (see Table 14.10).

- Several small peptides were identified in Comté cheese. Cyclic dipeptides were described as bitter (Roudot-Algaron *et al.,* 1993) and dipeptides with a gamma-glutamyl residue were found to be sour (e.g., γ-Glu-Tyr), apart from γ-Glu-Phe described to have a complex taste, which was brothy and slightly sour, salty and metallic (Roudot-Algaron *et al.,* 1994). However, because of their low concentration compared to their taste threshold value, they could not be directly responsible of cheese taste.

A number of workers have studied taste-active compounds in Camembert (Engel *et al.,* 2001a,b,c), Cheddar (Yang and Vickers, 2004), Comté (Salles *et al.,* 1995), Goat (Engel *et al.,* 2000a,b; Engel *et al.,* 2002; Salles *et al.,* 2002) and Emmentaler cheeses (Warmke *et al.,* 1996). Drake *et al.* (2007c) studied compounds responsible for the umami taste in Cheddar and Swiss cheeses. Low and high intensity umami-tasting cheeses were selected using a trained sensory panel. Some compounds, namely monosodium glutamate (MSG), disodium-5'-inosine monophosphate (IMP), disodium-5'-guanosine monophosphate (GMP), sodium chloride, lactic acid, propionic acid and succinic acid, were quantified in both types of cheese with and without umami taste. Comparison of analytical data and sensory thresholds indicated that IMP and GMP thresholds were 100-fold higher than their concentrations in cheese. All other compounds contributed some umami taste within their concentration range in umami cheeses. Sensory analysis of model cheeses clearly demonstrated that Glu played a major role in the umami taste of both Cheddar and Swiss cheese, while succinic and propionic acids contributed in Swiss cheese. The knowledge of umami-tasting components of cheeses will be useful in developing technologies to control and regulate the level of this specific taste attribute in cheeses.

14.6. Conclusions

A concerted series of chemical and biochemical reactions are involved in the formation of dairy flavour and off-flavour compounds. The general chemical/biochemical pathways, i.e., (1) heat-induced changes, (2) lipid oxidation, (3) glycolysis, (4) lipolysis and (5) proteolysis, involved in the degradation of milk constituents are now fairly well characterized. Recent works on the enzymology and genetic manipulation of the starter and non-starter lactic acid bacteria have helped in the better understanding of further catabolic modification of the products of primary degradation pathways. This has lead

to immense progress in understanding the flavour chemistry of fermented dairy products. So far, a large number of volatile compounds have been identified from various types of cheese but still it is not possible to duplicate cheese flavour by pure chemicals in model systems. However, there is now a good understanding of the causes of bitterness and specific flavours/off-flavours in dairy products.

Growing consumer awareness and demand for minimally processed fresh food products has lead to the application of non-thermal processing technologies in the processing of milk and milk products. The influence of new processing technologies like high pressure and pulsed electric field on sensory and flavour chemistry will need to be studied further. In addition, there is a demand for rapid and high-throughput processes that create dairy foods with traits (in particular, flavour and texture) identical to those of traditional dairy products that may take months or even years to develop.

Recent developments in sensory and instrumental methodologies in flavour analysis have been of immense help in furthering our understanding of the flavour chemistry of dairy products . Further work on the characterization of flavour (both aroma- and taste-active) compounds, flavour–matrix interaction mechanisms and flavour release mechanisms are needed to elucidate fully the complex nature of dairy flavours. The better understanding of flavour chemistry will be useful in the development of new technologies/mechanisms for the effective control and acceleration of the ripening process in cheese.

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15

Physico-chemical Properties of Milk

O.J. McCarthy and H. Singh

15.1. Introduction

Milk is a complex colloidal dispersion containing fat globules, casein micelles and whey proteins in an aqueous solution of lactose, minerals and a few other minor compounds. Its physical and chemical properties depend on intrinsic compositional and structural factors, extrinsic factors such as temperature and post-milking treatments. An understanding of these properties is important in the technological and engineering design and operation of milk processes and processing equipment, the design of modern methods of milk analysis, the determination of milk microstructures and the elucidation of complex chemical reactions that occur in milk. Measurement of some of the physico-chemical properties is used to assess milk quality. Various physical and chemical properties of milk have been reviewed previously (Jenness and Patton, 1959; Jenness *et al.*, 1974; Walstra and Jenness, 1984; Lewis, 1987; Sherbon, 1988; Singh *et al.*, 1997).

This chapter is intended to provide a general survey of some physical and chemical properties of milk, with an emphasis on general principles, the contributions of various milk components and the effects of compositional and processing factors. Compilations of numerical data on many of the properties considered may be found in Anon (1996) and Houška *et al.* (1994).

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15.2. Acid–Base Equilibria

The buffering of milk influences many of its physico-chemical properties (by controlling pH) during processing, e.g., heat and ethanol stability, rennet coagulation time, firmness and syneresis of renneted milk gels, rate of pH change in cheese, the pH of cheese at the end of manufacture and the subsequent pH changes during ripening. The buffering properties of milk and milk products have been reviewed thoroughly by Singh *et al.* (1997), Salaün *et al.* (2005) and Lucey and Horne (Chapter 9) and are described briefly here.

15.2.1. The pH and Buffering Properties of Milk

The pH of bovine milk at 25°C is between 6.5 and 6.7, with 6.6 being the most common value. Differences in pH and buffering between individual lots of fresh milk reflect compositional variations. The pH of colostrum is lower, e.g., pH 6.0 (McIntyre *et al.*, 1952), and that of mastitic milk or end-of-lactation milk is higher, up to pH 7.5 (Prouty, 1940), than the pH of normal mid-lactation milk. This increase in pH is presumably due to increased permeability of the mammary cells, with an increase in $[Na^+]$ and $[Cl^-]$ and possibly other ions and a reduction in the lactose content, as well as a reduction in the concentration of soluble inorganic P (White and Davies, 1958).

Milk contains many acidic and basic groups that result in buffering action over a wide pH range. The principal buffer components in milk are soluble phosphate, colloidal calcium phosphate (CCP), citrate, bicarbonate and casein (Jenness and Patton, 1959; Kirchmeier, 1980; Walstra and Jenness, 1984; Srilaorkul *et al.*, 1989; Lucey, 1992; Lucey *et al.*, 1993) (see Table 15.1). The pK_a values of many milk constituents are uncertain since the ionization of a group is affected by adjacent groups and electrostatic effects are long-range effects (Tanford, 1962).

Quantitative assignment of the buffering of each of these individual components is rather difficult. Three approaches have been used in attempts to account for the buffering behaviour of milk in terms of the properties of its constituents: calculation (Whittier, 1929, 1933), titration of artificial mixtures (Wiley, 1935a, b) and fractionation (Srilaorkul *et al.*, 1989; Lucey *et al.*, 1993). Srilaorkul *et al.* (1989) estimated that the contribution of casein, whey proteins and milk salts to the buffering of skim milk was 36.0, 5.4 and 58.6%, respectively. Lucey *et al.* (1993) reported that in the pH range 6.7–4.0, soluble salts and whey proteins (rennet whey), CCP and casein contributed approximately 47, 21 and 32%, respectively, to buffering in milk.

Table 15.1. Principal buffering groups in milk

Group	Approximate concentration (mM)[a, b]	Expected pK_a[c,d]	pK_a (in milk)[a]
Salts			
Inorganic phosphate	21.0[e]	2.1, 7.2, 12.3	3, 5.8, 6.6[f]
Citrate	9.0–9.2	3.1, 4.7, 5.4	3, 4.1, 4.8[f]
Organic phosphate esters	2.5–3.5	1.4, 6.6	1.7, 5.9[f]
Carbonate	2.0	6.4, 10.1	6.4, 10.1
Lactic acid	<0.4	3.9	3.9
Formic acid	0.2–1.8	3.6	3.6
Acetic acid	0.05–0.8	4.7	4.8
Various amines	1.5	~7.6	7.6
Protein-bound residues	Titratable group[c]	Expected pK_a[c,d]	pK_a (in milk)[a]
Aspartic acid	β-COOH	4.6	4.1
Glutamic acid	γ-COOH	4.6	4.6
Histidine	Imidazole	6.1	6.5
Tyrosine	Phenol	9.7	–
Lysine	ε-NH_3^+	10.4	10.5
Phosphoserine	Phosphate	1.5, 6.5	2, 6
N-acetylneuraminic acid	COOH	2.6	5.0
Terminal carboxyl	α-COOH	3.6	3.7
Terminal amino	α-NH3+	7.9	7.9

[a]Data from Walstra and Jenness (1984).
[b]Data from Jenness (1988).
[c]Data from Tanford (1962).
[d]Data from Edsall and Wyman (1958).
[e]About 10 mM colloidal phosphate, 11 mM in solution (at pH 6.6).
[f]pK_a values from titration with $Ca(OH)_2$.

There are many reports on the buffering properties of milk (Buchanan and Peterson, 1927; Whittier, 1933; Wiley, 1935a, b; Jenness and Patton, 1959; Kirchmeier, 1979, 1980; Unnikrishnan and Doss, 1982; Walstra and Jenness, 1984; Miyagawa and Namba, 1988; Sherbon, 1988; Srilaorkul *et al.*, 1989; Lucey, 1992; Lucey *et al.*, 1993) calculated from titration curves. However, the titration curve obtained depends on the methodology used, e.g., the forward and back-titration curves of milk do not coincide (Lucey, 1992; Lucey *et al.*, 1993). For instance, when milk was acidified, maximum buffering occurred at approximately pH 5.1 but when acidified milk was back-titrated with base, there was low buffering at pH 5.1 and maximum buffering occurred at pH 6.3 (Lucey *et al.*, 1993). The removal of CCP by acidification to pH 4.9 at 2°C and dialysis against an excess of normal milk (Pyne and McGann, 1960) resulted in the disappearance of the buffering maximum at

pH ~5.1 (during acidification of milk) and at pH 6.3 (during back-titration of acidified milk) (Lucey *et al.*, 1993). Acidification of milk to a low pH value (5.0–4.6) and neutralization resulted in a reduction in the buffering maximum at pH 5.1. This suggests that little reformation of CCP occurs on neutralization; presumably, other calcium phosphates are formed that have different buffering properties compared with normal milk.

The pH and buffering properties of milk are influenced by a number of compositional and processing factors, including temperature, pH, heat treatments, concentration, presence of CO_2 and concentrations of proteins and salts. These factors are discussed fully in Chapter 9.

15.2.2. Titratable Acidity

Titratable acidity is determined in the dairy industry mainly for two reasons: (a) to check the freshness of milk and milk products and (b) to control the manufacture of cultured (fermented) dairy products. The main advantage of using titratable acidity as a quality index is its simplicity and speed of measurement. Titratable acidity is a measure of the buffering of milk between pH 6.6 and 8.3 (phenolphthalein end-point) and is due mainly to phosphates, proteins, citrate and CO_2 (van der Have *et al.*, 1979).

The titratable acidity of milk is usually expressed as percentage lactic acid, although lactic acid (pK_a 3.95) does not buffer in the pH range 6.6–8.3. The acidity of milk and dairy products is usually determined by titration with NaOH to the phenolphthalein end-point (pH ~ 8.3). Titration of fresh milk from pH 6.6 to 8.3 requires between 13 and 20 ml of 0.1 M NaOH per 100 ml (1.3–2.0 meq/l00 ml). Various methods exist, prescribing titration with NaOH of various strengths: M/10, M/9 (Dornic) or M/4 (Soxhlet-Henkel) (Jenness and Patton, 1959; Walstra and Jenness, 1984).

A high initial acidity (in the absence of lactic acid development) suggests a milk that is rich in proteins and other indigenous buffering constituents. The initial acidity of milks from individual cows varies within the range 0.08–0.25% lactic acid but the titratable acidity of fresh bulk milk seldom falls outside the range of 0.14–0.16%. The titratable acidity of milks varies only slightly with the breed of cow (Herrington *et al.*, 1972). The liberation of fatty acids from milk fat by lipase action can result in some acidity being developed in cream and high-fat products (Walstra and Jenness, 1984).

The titratable acidity of milk is affected by the speed of titration, mainly due to the precipitation of calcium phosphate which causes the release of H^+ and therefore a decrease in pH. This phenomenon is described as 'fading of the phenolphthalein end-point'.

15.3. Oxidation–Reduction Equilibria

Oxidation is defined as the loss of electrons by a substance while reduction is defined as the gain of electrons. A substance that causes the oxidation of another is called an oxidizing agent and, since it must accept electrons from a substance it oxidizes, it is reduced during the reaction. A substance that causes reduction is called a reducing agent and is itself oxidized during the reaction.

The relative tendency of a substance to lose or gain electrons can be measured using galvanic or voltaic cells that separate the reactants and cause the electron transfer to occur along a wire, producing an electric current. The galvanic cell is composed of two half-cells, each of which contains the two forms of an oxidation–reduction pair and an electrode, usually platinum. In practice, the potential is measured by connecting the two half-cells *via* a salt bridge through a potentiometer. The reference standard is the hydrogen half-cell, which is assigned a potential of zero, i.e., the hydrogen ion is at unit activity.

The actual potential of any given half-cell, called the redox potential, depends not only on the components of the half-cell reaction system but also on the concentrations of all the ions involved in the half-cell reactions. The following equation, known as the Nernst equation, shows the relationship between the concentrations of the oxidized and reduced forms of a substance and redox potential:

$$E_h = E_0 + \frac{2.3RT}{nF}\log\left(\frac{[\mathrm{Ox}]}{[\mathrm{Red}]}\right) \tag{1}$$

where E_h is the redox potential (V), E_0 is the standard redox potential (i.e., the potential when the reactant and product are at unit activity) (V), R is the universal gas constant (8.314472 J K^{-1} mol^{-1}), F is the Faraday constant (96 500 C mol^{-1}), T is the absolute temperature (K), n is the number of electrons transferred per molecule and [Ox] and [Red] are the molar concentrations of the oxidized and reduced forms, respectively.

At 25°C, the value of $2.3RT/F$ is 0.059 V, and, for a one-electron transfer, the equation becomes

$$E_h = E_0 + 0.059\log\left(\frac{[\mathrm{Ox}]}{[\mathrm{Red}]}\right) \tag{2}$$

It should be noted that the molar concentration expression in the logarithm term is identical to the reciprocal of the equilibrium constant for the reaction, so that Equation 2 may be written as

$$E_h = E_0 + 0.059\log\frac{1}{K_{\mathrm{eq}}} \tag{3}$$

The redox potential of certain half-cells is affected by a variation in the pH, which can be represented as follows when a one-electron transfer is involved:

$$E_h = E_0 + 0.059 \log\left(\frac{[\text{Ox}]}{[\text{Red}]}\right) - 0.059\text{pH} \quad (4)$$

The E_h of individual milk samples in equilibrium with air falls within the range +0.25 to +0.35 V at 25°C and at milk's normal pH (Jackson, 1936; Eilers *et al.*, 1947; Harland *et al.*, 1952; Bhandari and Singh, 2003). Milk is essentially oxygen-free when secreted but about 0.3 mmol L^{-1} O_2 is present after equilibrium with air is established. The removal of oxygen by nitrogen sweeping lowers the redox potential to about −0.12 V.

The major components of milk other than water, i.e., fat, lactose and protein, have no effect on its redox potential. The redox systems in milk involve lactate-pyruvate, ascorbate and riboflavin. The ascorbic acid content of fresh milk is about 11.2–17.2 mg L^{-1}. As milk is drawn from the udder, all ascorbate is in the reduced form, but reversible oxidation to dehydroascorbate occurs at a rate dependent on temperature and on the concentrations of Cu, Fe and O_2 (Walstra and Jenness, 1984).

The ratio of ascorbate to dehydroascorbate remains high until the system disappears from the milk, and this system stabilizes the E_h of oxygen-free milk at ~0.0 V. Ascorbate is preserved in milk by preventing contamination with Cu and by deaeration. The riboflavin system in milk is active, but its concentration in milk is too low to influence the E_h value of milk significantly.

The E_h of milk is strongly influenced by heat treatment, bacterial activity, contamination with metal ions such as Cu^{2+}, concentration of O_2 and exposure to light. The effects of heat treatment of milk on oxidation–reduction have been studied (Josephson and Doan, 1939; Eilers *et al.*, 1947; Greenbank and Wright, 1951; Harland *et al.*, 1952; Higginbottom and Taylor, 1960). The E_h of milk decreases during heating; this is largely related to the liberation of the active sulphydryl group of β-lactoglobulin, which is oxidized by atmospheric oxygen. Reducing substances produced in milk as a result of the Maillard reaction between lactose and protein also influence the redox potential of milk, particularly concentrated and dried milks (Harland *et al.*, 1952). High-temperature short-time heating of milk results in lower E_h values, less oxidation of ascorbic acid and greater retention of disulphide-reducing substances in liquid milk and milk powders. The removal of oxygen from the system before heat treatment results in lower E_h values and better retention of various reducing substances than when the heating is done in air or in equilibrium with oxygen (Higginbottom and Taylor, 1960).

Bacterial activity reduces the E_h, largely through the consumption of available oxygen in the medium in the course of bacterial metabolism

(Jenness and Patton, 1959). The decrease in potential can be identified by the change in colour of certain dyes added to milk, e.g., methylene blue. These dyes are reduced to colourless forms with time as the bacteria grow in milk. Therefore, such changes in colour can be used as an index of microbial contamination and form the basis of the methylene blue and resazurin reduction tests for the bacterial quality of milk.

Copper and iron have strong catalytic effects on oxidative processes. The catalytic effect of copper is up to 100 times greater than that of iron (King and Dunkley, 1959). Consequently, contamination of milk with copper or iron increases the E_h. The initial stage of the reaction involves the oxidation of ascorbic acid. Vahcic *et al.* (1992) derived mathematical relationships between E_h and the copper, iron and ascorbic acid contents of 72 milk samples. A discussion of redox reactions and photo-oxidation was presented by Walstra and Jenness (1984).

15.4. Surface and Interfacial Tension

At an interface between liquid and air, the behaviour of molecules of the liquid is different from that of similar molecules in the bulk phase. Molecules in the bulk phase are subjected to attractive forces equally in all directions by other molecules of the liquid whereas molecules at the surface or interface experience a net attractive force directed towards the bulk phase. This inward attraction reduces the number of molecules at the surface, resulting in a decrease in interfacial area. The forces causing a reduction in surface or interfacial area are referred to as surface tension or interfacial tension (γ), respectively. The surface tension acting on a liquid can be considered in two ways, either as a force per unit length acting on a given length of surface (N m^{-1}) or as the work required to increase the area of a surface by a unit amount, referred to as interfacial free energy (J m^{-2} or N m^{-1}). Surface tension is a characteristic property of a liquid and varies greatly for different liquids, or for the same liquid but at different degrees of purity or at different temperatures. With an increase in temperature, the kinetic energy of the molecules increases and cohesive forces between them decrease, resulting in a decrease in interfacial tension. For example, the surface tension of water decreases from 72.6 to 58.9 mN m^{-1}, with an increase in temperature from 20 to 100°C.

The presence of surface-active agents, or surfactants, influences the surface tension of water. Surfactants contain both hydrophobic and hydrophilic groups and adsorb at the interface with their hydrophobic groups oriented towards the non-aqueous phase and their hydrophilic groups pointed towards the aqueous phase. The surfactant molecules form a monomolecular

layer on the surface until the surface is fully covered. Usually, this results in a reduction in interfacial tension that can be expressed by the Gibbs equation

$$d\gamma = -RT\,\Gamma d\ln a \tag{5}$$

where Γ is the excess concentration of solute at the interface over that in the bulk solution and a is the activity of the solute in the bulk phase. Many proteins are good surfactants and may be adsorbed at the interface to form either a monolayer or a multilayer, more or less irreversibly, depending on the individual protein (Walstra and de Roos, 1993). When protein molecules are adsorbed at the interface, they may unfold and rearrange their conformation or even denature to suit the new environment; Gibbs' equation no longer applies (Walstra and Jenness, 1984).

Methods for determining surface tension were described and reviewed by Harkins (1952) and Whitnah (1959). These are based on the following principles: (1) the increase in the height of liquid in a capillary, (2) the weight or volume of drops formed when a given amount of liquid is allowed to flow from a capillary tip, (3) the pressure required to force a bubble of gas through a nozzle immersed in the liquid and (4) the force required to pull a ring or plate free from the surface of a liquid. Methods involving a ring or plate have been used most commonly for milk and milk products.

The surface tension of milk is a fundamental physical property that relates to the stability of foams, emulsions and films; it affects fractionation, concentration and drying processes. Milk contains several surface-active components (casein micelles, phospholipids, whey proteins and fatty acids) that can readily adsorb at an air–water interface and reduce surface tension; salts and lactose do not contribute to surface tension (Walstra and Jenness, 1984). Jenness and Patton (1959) reported surface tension values for several fractions from milk, with rennet whey, skim milk, whole milk, 25% fat cream and sweet-cream buttermilk having values of 51–52, 52–52.5, 46–47.5, 42–45 and 39–40 mN m^{-1}, respectively.

The surface tension of milk decreases during the time required for measurement until it reaches a base value, although the real equilibrium is never reached or is reached very slowly (Michalski and Briard, 2003). Values in the range 40–60 mN m^{-1} (average ~52) at 20°C have been reported (Aschaffenburg, 1945; Dunkley, 1951; Watson, 1958; Parkash, 1963; Sharma, 1963; Calandron and Grillet, 1964; Bertsch, 1983; Kristensen *et al.*, 1997; Michalski and Briard, 2003), depending on the measurement technique. The surface tension decreases with increase in temperature for both skim milk and whole milk (Table 15.2).

Other factors that influence the surface tension of milk include fat content, homogenization and temperature history. Surface tension decreases with increasing fat content up to ~4% but no further decrease occurs at higher

Table 15.2 Surface tension of skim milk and whole milk (3.5% fat) at 10, 25 and 40°C after a 300 s measuring time[a]

	Surface tension (mN m^{-1})	
Product temperature, °C	Mean	Standard deviation
Skim milk		
10	49.72	0.86
25	47.29	1.21
40	42.22	2.87
Whole milk (3.5% fat)		
10	49.87	1.32
25	41.89	1.05
40	41.56	1.01

[a]Adapted from Kristensen *et al*. (1997).

fat contents (Watson, 1958; Michalski and Briard, 2003). The effect of homogenization is dependent on the pressure; at low homogenization pressure (5 MPa), the surface tension of homogenized milk has been found to be lower than that of whole milk, which is due to the release of surface-active components of the milk fat globule membrane as a consequence of fat globule disruption (Michalski and Briard, 2003) or could be due to the release of free fatty acids because of activation of lipase during homogenization (Whitnah, 1959). Homogenization of raw milk at higher pressures, however, can lead to an increase in surface tension (Michalski and Briard, 2003). The surface tension of homogenized milk is higher than that of unhomogenized milk, if the milk has been pasteurized before homogenization (Webb, 1933; Watson, 1958). The surface tension of milk held at 5°C and brought to 20°C is lower than that of milk cooled to 20°C and measured immediately (Mohr and Brockmann, 1930; Sharp and Krukovsky, 1939). Heat treatment of milk at sterilization temperatures can cause a small increase in surface tension (Nelson, 1949), which is probably related to the denaturation of whey proteins, which reduces their surface activity.

15.5. Optical Properties

15.5.1. Light Absorption and Scattering

When electromagnetic radiation encounters chemical species (atoms, ions or molecules), it can be absorbed (i.e., its energy is transferred to the chemical species), scattered (i.e., its direction of propagation changes) or can excite fluorescence. Absorption occurs when specific frequencies of a beam of

electromagnetic radiation are absorbed by chemical species in the sample. In the process, the energy is transferred to the atom or molecule and causes it to be promoted to an excited state from the ground state. Absorption of energy occurs only if the energy of the photon exactly matches the energy difference between the ground state and excited states of the atom or molecule. With transmission, the velocity at which the radiation is transmitted through the medium containing atoms or molecules decreases compared to the velocity in a vacuum. The lifetime of an atom or molecule in an excited state is limited and it will rapidly relax back to the ground state. The energy released may be transformed into electrical or thermal energy or re-emitted as radiation of lower energy (therefore longer wavelength), known as fluorescence. Scattering of electromagnetic radiation does not involve transfer of energy, but rather the radiation is totally re-emitted randomly in all directions. The intensity of scattering is dependent on the number and size of particles and the difference in refractive index between the particles and the surrounding medium.

Milk is a complex colloidal dispersion of fat globules, casein micelles and whey proteins in an aqueous solution of lactose, salts and other compounds. Thus, milk not only absorbs light at several wavelengths but also scatters light because of the presence of large particles, e.g., casein micelles and fat globules.

In the visible region, riboflavin in milk absorbs strongly near 470 nm and emits fluorescent radiation with a maximum at 530 nm. Milk fat contains β-carotene, which absorbs near 460 nm. In the ultraviolet region, aromatic amino acid residues of proteins (tyrosine and tryptophan) strongly absorb near 280 nm and a part of the ultraviolet radiation energy is re-emitted as fluorescence at 340 nm. Measurement of the intensity of ultraviolet fluorescence has been used to quantify the protein content of milk (Konev and Kozunin, 1961; Fox *et al.*,1963; Bakalor, 1965; Porter, 1965).

In the infrared region, absorptions are due primarily to amide (II) groups (CONH) of proteins at 6465 nm, hydroxyl groups (OH) of lactose at 9610 nm and ester carbonyl groups (C═O) of lipids at 5723 nm. These absorption principles have been used to estimate the protein, fat and lactose content of milk simultaneously (Goulden *et al.*, 1964; Biggs, 1964, 1979a, b; Gillickson, 1983) and are the basis of official methods of the Association of Official Analytical Chemists (AOAC, 1995a) and the International Dairy Federation (lDF, 1990). Several custom-built infrared analysers are available commercially for this purpose. The use, additionally, of measurement of the methylene (CH) stretch has been proposed for the measurement of fat (Clemmensen, 1980; Mills and van de Voort, 1982). The milk is homogenized before measurement to reduce the size of fat globules to below 1 μm where light scattering is negligible (Rudzik and Wöbbecke, 1982). An extremely narrow sample thickness, typically 0.037 mm, is used, and under these conditions, a linear Beer–Lambert plot is obtained for each milk component in

the range 0–6%. Infrared methods correlate well with chemical methods (Adda *et al*., 1968; Biggs, 1964, 1979a), although a repeatable procedure must be established for homogenization and dilution, and the instrument must be calibrated using several samples analysed by standard methods to cover the range of each component expected.

Both fat globules and casein micelles scatter light, and it is possible to measure both the scattered intensity at some angle to the incident beam and the intensity of light transmitted through the sample. Both transmitted and scattered light measurements form the basis of the measurement of the fat content of milk and the size distributions of fat globules and casein micelles. Haugaard and Pettinati (1959) and Goulden and Sherman (1962) described methods for the determination of fat content, average fat globule size and homogenization efficiency in dairy products based on light scattering by fat globules. Instruments for fat determination are based on measurement of turbidity (i.e., the attenuation of the incident beam caused by scattering), usually expressed as optical density or absorbance in a way similar to that in normal absorption photometry. Milk is first homogenized to achieve a uniform fat globule size distribution and the interfering scattering due to protein is eliminated by using an appropriate dissociating agent, e.g., EDTA or detergents. The commercial version of this method is known as the Milko-Tester, the results of which compare favourably with other methods for fat analysis, provided the instrument is frequently checked and calibrated against standard methods (Grappin and Jeunet, 1970; Shipe and Senyk, 1973, 1975, 1980). Methods for measuring the average size of casein micelles or fat globules in milk using the wavelength dependence of turbidity have been described (Walstra, 1965, 1967; Holt, 1975).

Nakai and Le (1970) described a simple method for the determination of protein and fat in milk simultaneously. Acetic acid was used to dissolve both protein and fat and the protein content determined from the absorbance at 280 nm. Turbidity due to fat was developed by adding a solution of urea and imidazole and measured at 400 nm.

Light scattering and absorption properties of milk greatly affect the appearance of milk (Walstra and Jenness, 1984). The creamy colour of milk is due to β-carotene in the fat. Casein micelles scatter blue light (short wavelength) more effectively than red, giving the bluish colour of skim milk. The most common methods for the determination of colour involve transmission and reflectance techniques. Light is directed at the sample and the signal transmitted through, or reflected from, the sample is measured either in a tristimulus colorimeter or over the visible spectrum, 380–759 nm, using a spectrophotometer. Generally the results are expressed using the L*, a*, b* (Hunter) systems. In this system, L* denotes the position of sample on the dark–light axis, a* on the green–red axis and b* on the blue–yellow axis.

Liquid milk shows slight 'green' and 'yellow' components (Kneifel *et al*., 1992). Homogenization of whole milk causes colour changes, making the milk whiter. Heat treatment causes milk to appear whiter initially; this has been attributed to an increase in casein micelle size and denaturation of whey proteins causing an increase in refractance (Singh and Patil, 1990). More severe heating causes the development of brown colour due to the Maillard reaction. The factors affecting browning include time, temperature, pH and water activity (O'Brien, 1995; Chapter 7). Milk colour and its measurement were reviewed by Chatelain *et al*. (2003).

15.5.2. Refractive Index

The refractive index of a transparent medium is defined as the ratio of the velocity of light in air to the velocity of light in the medium. When a ray of light passes at an angle through the interface between two transparent media of different densities, an abrupt change in direction, i.e., refraction, of the ray is observed. When the ray of light passes from a less dense to a more dense medium, the bending is towards the normal to the interface, while when the densities are reversed, bending away from the normal is observed. The refractive index (n) is then given by

$$n = \frac{\sin i}{\sin R} \tag{6}$$

where i is the angle between the ray in the first medium and the normal to the dividing surface (incidence) and R is the exit angle (refraction). The refractive index varies with temperature and the wavelength of light. At a constant temperature and wavelength, the refractive index is a characteristic constant for a particular pure medium and can be used to determine the purity of a substance and to determine the composition of mixtures of known constituents. Refractive index values are usually measured using a light of constant wavelength, 589.3 nm (i.e., the D line of the sodium spectrum), at a constant temperature, normally 20°C.

The refractive index of water at 20°C (n_D^{20}) is 1.3330 and the value for bovine milk is in the range 1.3440–1.3485 (Walstra and Jenness, 1984; Sherbon, 1988). The difference between the values of n for water and milk reflects the presence of dissolved substances, such as minerals, lactose, whey proteins and casein micelles in milk. Large colloidal substances, such as fat globules and air bubbles, do not contribute to the refractive index of milk.

The increase in refractive index of a solution over that of the pure solvent is directly proportional to the volume concentration of solute (Walstra and Jenness, 1984). Because the volume concentration of solute is the product of

solution density (ρ) and solute concentration (c_w) (w/w), the specific refraction increment (r) can be defined as

$$r = \frac{n - n_0}{\rho c_w} = \frac{\Delta n}{\rho c_w} \tag{7}$$

where n and n_0 are the refractive indices of the solution and the solvent, respectively, measured under the same conditions. Since refraction is an additive property, the refractive index increment (Δn) for a multicomponent system is given by

$$\Delta n = n_{solution} - n_{solvent} = \rho \Sigma (mr) \tag{8}$$

where m is the mass fraction of a solute, r is the specific refraction increment and ρ is the density of the solution. Goulden (1963) reported the following specific refraction increments (ml g^{-1}): casein micelles, 0.207; whey proteins, 0.187; lactose, 0.140. Refractive index measurements can be used satisfactorily to estimate the solids-not-fat (SNF) content of milk and condensed milk. Generally, there is a linear relationship between solids content (based on weight per unit volume) and refractive index, but this relation varies between lots of milk, owing mainly to variations in the lactose/protein ratio. The refractive index can be determined quickly and accurately and can be used as a rapid method of checking the SNF content and purity of butter fat.

15.6. Freezing Point

The freezing point of water is directly related to the concentrations of water-soluble constituents. The addition of solute to water lowers the freezing point, the degree of depression of the freezing point being proportional to the molality of the solution. The Raoult (1884) equation reflects the relationship between the depression of the freezing point (T_f) and the molality of an aqueous solution (M):

$$T_f = K_f M \tag{9}$$

where K_f is the molar depression constant (1.86°C for an ideal aqueous solution). This relationship applies only to very dilute solutions.

Milk is a complex aqueous solution and Raoult's law can therefore be used only for a rough calculation of the relation between the concentration of milk constituents and freezing point. The freezing point of milk is lower than that of water and is proportional to the concentration of milk's water-soluble

constituents. Fat globules, casein micelles and whey proteins make negligible contributions to the freezing point depression (Sherbon, 1988). Lactose, chloride salts and other water-soluble constituents (such as calcium, potassium, magnesium, lactate, phosphate, citrate, etc.) contribute about 55, 25 and 20%, respectively, to the freezing point depression of milk (Jenness and Patton, 1959).

The vast majority of individual milks have a freezing point in the range −0.512 to −0.550°C and few milks fall outside the range −0.520 to −0.530°C (Davis and MacDonald, 1953; Shipe, 1959; Henningson, 1963; Eisses and Zee, 1980). Although the concentration of individual solute constituents in milk may vary, the total molality of the soluble constituents remains fairly constant; since the milk secretion process dictates that the osmotic pressure of milk is kept in equilibrium with that of blood, a constant osmolality is maintained in milk by the passage of blood constituents into the mammary gland. Any decrease or increase in the concentration of lactose is compensated for by a proportional change in the concentrations of sodium and chloride. The constant osmolality of milk is reflected in the relative constancy of the freezing point depression.

Environmental and nutritional factors, such as season, climate, feed, stage of lactation, breed of cow, time of day, water intake and clinical mastitis, have only relatively small effects on the freezing point of milk, although substantial variations are observed in extreme circumstances (see reviews by Shipe, 1959; Brathen, 1983).

The effect of processing on the freezing point of milk has been reviewed by Shipe (1959) and Harding (1983). Heat treatment of milk causes the transfer of soluble salts to the colloidal state and changes in lactose that include its interaction with proteins and its conversion into formic acid. Such effects would be expected to increase the freezing point of milk. However, reports on the effects of heat treatment of milk on its freezing point are inconclusive; some workers reported increases in the freezing point while others reported no effect. Kessler (1984) reported that pasteurization, UHT heat treatment or preheating at 95°C for 303 s did not affect the freezing point of milk. However, the freezing point increased by almost 0.010°C when milk was UHT treated by direct steam injection, apparently owing to the degassing caused by flash cooling. Similarly, vacuum treatment of milk has been shown to increase its freezing point, presumably due to the removal of dissolved gases from the milk (Shipe, 1964; Demott, 1967). Freezing and subsequent thawing of milk does not affect its freezing point provided the thawed milk is well mixed before samples are taken. Any processing that involves fermentation of lactose to lactic acid (e.g., in the production of yoghurt) results in a lowering of the freezing point, as one mole of lactose is converted into four moles of lactic acid.

The determination of freezing point is used extensively to assess whether milk has been adulterated by the addition of water and to quantify

the addition. Milk with a freezing point of −0.525°C or below is usually presumed to be unadulterated. The selection of −0.525°C as the upper limit is based on the statistical evaluation of data from a 1968 North American survey of the freezing point of authentic samples (Henningson, 1969). The freezing point is generally determined with thermistor or Hortvet-type cryoscopes; the apparatus and techniques have been described by AOAC (1995b).

15.7. Density

Density is defined as mass per unit volume. It has the units kg m^{-3} and is commonly indicated by the symbol ρ (rho). A related dimensionless quantity, specific gravity (SG), is defined as $\rho_{product}/\rho_{water}$, densities being measured at specified temperatures. Specific gravity has the advantage that its numerical value is independent of the units used for density, and its temperature dependence is much lower than that of density. Specific gravity is a particular form of relative density, the ratio of the absolute density of a material to the absolute density of a reference material. As water is almost always chosen as the reference material, the terms 'specific gravity' and 'relative density' can be considered synonymous (Figura and Teixeira, 2007).

Density is identified for a given measurement temperature as, for example, ρ^{20}, where the superscript is the temperature in °C (Walstra and Jenness, 1984). Specific gravity is identified in a similar way, e.g., SG_{20}^{20} or SG_{4}^{20}, where the superscript is the temperature at which milk density was measured and the subscript the temperature at which the density of water was determined (Walstra and Jenness, 1984). Density and specific gravity are related by the equation

$$\rho^{\theta_1} = SG_{\theta_2}^{\theta_1} \times \rho_{water}^{\theta_2} \tag{10}$$

where θ is temperature (°C).

ρ^{20} *of* whole milk is about 1030 kg m^{-3} and usually ranges from 1027 to l033 kg m^{-3}, depending mainly on fat content (Walstra and Jenness, 1984). $SG_{15.5}^{15.5}$ ranges from 1.030 to 1.035 for mixed herd milk, and a commonly quoted average value is 1.032 (Jenness and Patton, 1959). The equivalent range and average value for skim milk are 1.0320–1.0365 (Jenness and Patton, 1959) and 1.036 (Sherbon, 1988), respectively. Inter-breed variations in density and specific gravity are small.

Density data are needed for converting mass to volume and *vice versa* and for calculating values of physical properties such as kinematic viscosity and thermal diffusivity (Walstra and Jenness, 1984). Measurement of the

density of whole milk is one way of checking for extraneous water, and it provides a rapid means of indirectly measuring total solids (TS) (Jenness and Patton, 1959). Toenjes *et al.* (1991) suggested that temperature-corrected specific gravity measurement by a lactometer is a dependable means of evaluating the concentration of immunoglobulins in colostrum.

The principal methods of determining the density of milk involve weighing a known volume (by pycnometer or hydrostatic balance) or measuring the volume of a known weight (by hydrometer or dilatometer) (Jenness and Patton, 1959; Sherbon, 1988). Other methods include measurement of the distance a drop of product falls in a density gradient column (Sherbon, 1988). Hydrometry is the most common method, employing beads of graded densities, or, more usually, hydrometers – called lactometers when applied to milk (Sherbon, 1988). A lactometer scale may be graduated directly in terms of specific gravity. Detailed specifications for lactometers have been published (International Organization for Standardization, 1974). The choice of density measurement method depends on consideration of the requirements for precision and speed.

The density and specific gravity of milk depend on composition, temperature and temperature history. Other factors, such as stage of lactation and nutritional status of the cow, affect density only in so far as they affect composition (Sherbon, 1988). The effects of composition, temperature and temperature history on the density of whole milk interact (Jenness and Patton, 1959; Walstra and Jenness, 1984). Density at a given temperature is a resultant of the densities and mass fractions of the individual components of milk, but these depend on temperature history. Fat, the component with the lowest density, has the greatest influence. This influence is complicated by the fact that within the melting point range of milk fat (−35 to 38°C), the density of the fat depends on the ratio of liquid fat (lower density) to solid fat (higher density). This ratio, in turn, depends on both the temperature and temperature history of the milk, because considerable supercooling of milk fat can occur when the temperature of milk is lowered and because milk fat crystallization can be slow. It follows that density should be measured and reported for conditions under which the state of the fat is known (Jenness and Patton, 1959). The best approach is to pre-warm the milk to 40–45°C, hold for a few minutes to completely melt the fat, cool to 20°C and measure density. Provided cooling to 20°C is done rapidly and density measured immediately, the milk fat will not have time to crystallize to any great extent, i.e., those triglycerides that would crystallize at 20°C given time will still be liquid and therefore in a supercooled state (Walstra and Jenness, 1984). Liquid fat at 20°C has a density of about 915 kg m^{-3} but if crystallization equilibrium has been reached, the density will be about 928 kg m^{-3} (Walstra and Jenness, 1984). Differences caused by temperature history become more pronounced the higher the fat content; 20% fat cream, for example, has a density of about 1009 kg m^{-3} if

the fat is in a supercooled liquid state, but a density of about 1019 kg m^{-3} if kept for some time at 4°C and then warmed to 20°C (Walstra and Jenness, 1984).

The density of milks and creams may be calculated if their proximate compositions are known using the equation

$$\frac{1}{\rho} = \Sigma\left(\frac{x_i}{\rho_i}\right) \tag{11}$$

where x_i and ρ_i are the mass fraction and density, respectively, of the ith component, and $\Sigma(x_i) = 1$. Equation 11 can be derived readily from first principles. ρ_i is unlikely to be equal to the density of the pure component, because a contraction usually occurs when two components are mixed. However, Equation 11 can be used to predict the ρ^{20} of fluid milk products (skim milk, whole milk, cream) using the values of component densities given in Table 15.3 (Walstra and Jenness, 1984). The value of 918 kg m^{-3} for milk fat given in Table 15.3 assumes that the fat is a supercooled liquid, but allows for the higher density of the milk fat globule membrane (Walstra and Jenness, 1984).

As fat content has a major influence on density, ρ^{20} of fluid milk products can be calculated approximately using the equation

$$\rho^{20} \approx \frac{1000}{0.123x_{fat} + 0.9665}(\text{kg m}^{-3}) \tag{12}$$

where x_{fat} is the mass fraction of supercooled liquid fat (Walstra and Jenness, 1984).

Concentration of fluid milk products by the removal of water results in an increase in density. Equation 11 can be written for both the initial and concentrated product. Combination of the two forms of the equation, followed by suitable algebraic rearrangement, yields Equation 13:

$$\frac{1}{\rho_c^{20}} = \frac{R}{\rho_0^{20}} + \frac{1-R}{\rho_{water}^{20}} \tag{13}$$

Table 15.3 Density of milk components at 20°C[a]

Component	ρ^{20} (kg m^{-3})
Water	998.2
Fat	918
Protein	1400
Lactose	1780
Other components	1850

[a]Data from Walstra and Jenness (1984).

where ρ_c^{20} is the density of the concentrate (kg m^{-3}), ρ_0^{20} is the density of the initial product (kg m^{-3}), ρ_{water}^{20} is the density of water (kg m^{-3}) and R is the concentration factor (= the ratio of the dry solids content of the concentrate to that of the initial product). It is assumed implicitly in formulating Equation 13 that concentration causes no changes in physical state or chemical activity coefficients (for example, hydration of proteins or insolubilization of salts) (Sherbon, 1988).

If concentrated or unconcentrated milk or other fluid contains dispersed gas, such as air, the actual density will be lower than that predicted by Equations 11, 12 or 13:

$$\rho_{actual}^{\theta} = (1 - \varepsilon_a)\rho_{predicted}^{\theta} \tag{14}$$

where ε_a is the volume fraction of dispersed gas. This equation is easily derived from first principles.

Dispersed gas can considerably lower density, which then becomes appreciably pressure dependent (Walstra and Jenness, 1984). Density measurement at a controlled pressure is one way of determining the gas content of fluid milk products (Lindqvist, 1976).

Equation 11 can be transformed to the following expression:

$$SG_{milk} = \frac{F + SNF + (100 - TS)}{\dfrac{F}{SG_{fat}} + \dfrac{SNF}{SG_{SNF}} + \dfrac{100 - TS}{1}} \tag{15}$$

where F is the % fat (w/w), SNF is the % solids-not-fat (w/w) and TS is the % total solids (w/w). Equation 15 may be solved for TS to give

$$TS = \frac{(SG_{SNF} - SG_{fat})F}{SG_{fat}(SG_{SNF} - 1)} + \frac{SG_{SNF}(100SG_{milk} - 100)}{SG_{milk}(SG_{SNF} - 1)} \tag{16}$$

This equation forms the basis of the rapid determination of TS by measuring SG_{milk} using a lactometer. F must be measured separately and suitable values assumed for SG_{fat} and SG_{SNF}. Modern analytical techniques have now largely superseded this method of measuring TS.

Whitnah *et al.* (1957) found that the temperature of maximum density of whole milk (which is several degrees lower than 0°C) approached that of water (4°C) linearly as milk was diluted with water. They suggested that measurement of this temperature could be used for detecting adulteration of milk by water, but acknowledged that differences between milk samples, and changes with age in a given sample, were serious problems in developing such an application.

The density of milk and fluid milk products decreases with increasing temperature because of thermal expansion. The effect is due mainly to thermal

expansion of the water (Jenness and Patton, 1959). However, the fat content has a significant simultaneous effect, for two reasons. First, the coefficient of cubic expansion of milk fat (0.0008 $m^3\ m^{-3}$ per °C in the temperature range 0–60°C) is much greater than that of water (0.00015 $m^3\ m^{-3}$ per °C at 10–20°C; 0.00055 $m^3\ m^{-3}$ per °C at 60–70°C) (Jenness and Patton, 1959). Second, as milk fat expands by 0.045 $m^3\ m^{-3}$ when it melts (Jenness and Patton, 1959), the value of density at a given temperature will depend on temperature history and on the temperature itself.

The density of supercooled milk measured at a temperature close to 20°C may be corrected to 20°C using the correction factor 0.3 kg m^{-3} per °C recommended by Ruegg and Moor (1985).

There have been many attempts to develop empirical equations relating milk density to fat content and temperature, the two most important determining variables. Bertsch *et al.* (1982) obtained the following such equation:

$$\begin{aligned}\rho = &- 0.2307 \times 10^{-2}\theta^2 - 0.2655\theta + 1040.51 \\ &- F(-0.478 \times 10^{-4}\theta^2 + 0.969 \times 10^{-2}\theta + 0.967)(\text{kgm}^{-3})\end{aligned} \tag{17}$$

where θ is the temperature (°C) and F is the % fat (w/w). Equation 17 is valid for milks and creams with a fat content in the range 0–15% (w/w), at a temperature in the range 65–140°C. The relative mean error of the equation was ±0.1 % at the 95% level of confidence (88 experimental points).

Bertsch *et al.* (1982) compared Equation 17 with the density data published previously by other workers. They found that the validity of the equation could be extended to cover the fat content range 0–50% and the temperature range 20–40°C, although with a relative mean error as high as (but not exceeding) ±0.4%. This higher error was probably due to variations in SNF and to the effects of temperature history (within the milk fat melting point range), as well as to experimental error (Bertsch *et al.*, 1982).

Watson and Tittsler (1961) determined the following empirical relationship for the dependence of whole milk density on fat content and SNF content in the low-temperature range 0.95–9.85°C:

$$\rho = 1003.073 - 1.79\theta - 3.68F + 37.44SNF\ (\text{kgm}^{-3}) \tag{18}$$

The slight inverse temperature dependence of milk density was ignored in establishing Equation 18. Fernández-Martin (1975) deliberately studied this temperature dependence by measuring the coefficient of volumetric thermal expansion (β_V) of milks and creams of a range of fat and total solids contents over the temperature range 0–80°C. He derived from his data the following relationships:

$$\beta_V = 1.04 \times 10^{-2} + 1.2 \times 10^{-2} r + (0.0046 - 0.0023r)TS \times 10^{-3} + [0.0074 - 0.0015r - (0.00011 - 0.00013r)TS]\theta_m \times 10^{-3} (\mathrm{m^3 kg^{-1} K^{-1}}) \quad (19)$$

$$\Delta V_{0 \rightarrow \theta^\circ C} = \{[23.3 + (6.71 + 12.80r)TS]\theta + [4.43 - (0.071 + 0.064r)TS]\theta^2\} \times 10^{-6} (\mathrm{m^{-3} kg^{-1}}) \quad (20)$$

where r is the ratio of % fat (w/w) to %TS (w/w), θ_m is the mean temperature (°C) of any one degree temperature increment and $\Delta V_{0 \rightarrow \theta^\circ C}$ is the increase in volume per unit mass between 0 and θ°C.

The specific gravity of whole milk decreases slightly with increasing temperature, partly because of the effect of temperature on milk fat but also because the contraction of the other solids that occurs on mixing with water decreases slightly with increasing temperature (Walstra and Jenness, 1984). The rate of decrease in specific gravity is about 5×10^{-5} per °C in the temperature range 10–40°C (Sherbon, 1988), the precise value depending on temperature and temperature history. There is some evidence that at temperatures >40°C, specific gravity increases to the same extent (Sherbon, 1988).

The specific gravity of skim milk, like that of whole milk, decreases somewhat with increasing temperature, especially in the range 5–40°C. Since the specific gravity of a 5% lactose solution remains constant over this range, the observed decrease may be the result of changes in protein hydration (Jenness and Patton, 1959).

Processing operations such as homogenization (of whole milk) and sterilization (of both whole and skim milks) have negligible effects on density (Rutz *et al.*, 1955; Bertsch *et al.*, 1982; Sherbon, 1988). The in-line measurement of density by automatic instruments is commonly used for process control purposes; one example is the indirect measurement and control of TS in milk concentrates from evaporators.

15.8. Rheological Properties

15.8.1. Newtonian Behaviour

Fresh skim milk, whole milk and cream are for most practical purposes Newtonian liquids under the following conditions: fat content <40% (w/w), temperature >40°C (milk fat completely molten, no cold agglutination of fat globules) and moderate to high shear rates (Mulder and Walstra, 1974; Randhahn, 1974; Prentice, 1992). Rheological behaviour is completely

characterized by a temperature-dependent coefficient of viscosity, which is defined by Newton's law of viscosity:

$$\tau = \eta\dot{\gamma} \tag{21}$$

where τ is shear stress (Pa), $\dot{\gamma}$ is shear rate (s^{-1}) and η is the coefficient of viscosity (Pa s). The term 'coefficient of viscosity' is strictly the proportionality constant in Equation 21. The term 'viscosity' is a more general one, meaning the extent to which a fluid resists being sheared. The distinction between the two is irrelevant for Newtonian liquids, though not for non-Newtonian liquids.

Whole milk at temperatures <40°C exhibits close to Newtonian behaviour provided cold agglutination is absent. For the latter condition, representative values of the viscosity of whole milk and fractions derived from it, at 20°C, are shown in Table 15.4.

Several points can be made on the basis of these data. First, lactose, the major low-molecular-weight milk constituent, and even the whey proteins influence viscosity to a relatively small extent. Second, the fat content has a relatively large influence. Third, the casein content has by far the greatest influence. Indeed, the viscosity and rheological behaviour of milk and creams depend largely on the state and concentration of the fat and of the casein, and thus on factors that affect these (Jenness and Patton, 1959; Sherbon, 1988).

The viscosity of whole milk, skim milk, cream and some milk concentrates, for conditions under which Newtonian behaviour occurs, can be predicted at a given temperature by Eilers's semi-empirical equation (van Vliet and Walstra, 1980; Walstra and Jenness, 1984):

$$\eta = \eta_0\left(1 + \frac{1.25\Sigma(\phi_i)}{1 - \Sigma(\phi_i)/\phi_{\max}}\right)^2 \tag{22}$$

Table 15.4. Representative values of the viscosity at 20°C of whole milk and fractions thereof[a]

Milk or milk component	Viscosity (mPa s)
Water	1.005
5% (w/w) lactose solution	1.150
Rennet whey	1.250
Skim milk	1.790
Whole milk	2.127

[a]Data from Jenness and Patton (1959)

where η is the coefficient of viscosity of the milk product (Pa s), η_0 is the coefficient of viscosity of the portion of the product consisting of water and low-molecular-weight substances other than lactose (Pa s), ϕ_i is the volume fraction of a dispersed component with a particle size at least an order of magnitude greater than the size of the water molecule, $\Sigma(\phi_i) = \phi_{fat} + \phi_{cas} + \phi_{wp} + \phi_l$, where fat = milk fat, cas = casein, wp = whey proteins and l = lactose, and ϕ_{max} is the assumed value of $\Sigma(\phi_i)$ for maximum packing of all dispersed particles (0.9 for fluid milk products). ϕ_{max} may be somewhat higher than 0.9 for evaporated milk and somewhat lower for high-fat cream (Walstra and Jenness, 1984).

The volume fraction of an individual component is given by

$$\phi_i = V_i c_{v,i} \tag{23}$$

where V_i is the voluminosity of component i ($m^3\ kg^{-1}$ of dry component) and $c_{v,i}$ is the volume concentration of the component in the product ($kg\ m^{-3}$ of product). Walstra and Jenness (1984) give the following values of voluminosity (V):

Fat globules	$\sim 1.11 \times 10^{-3}\ m^3\ kg^{-1}$ of lipid in fat globules
Casein	$\sim 3.9 \times 10^{-3}\ m^3\ kg^{-1}$ of dry casein
Whey proteins	$\sim 1.5 \times 10^{-3}\ m^3\ kg^{-1}$ of dry protein
Lactose	$\sim 1.0 \times 10^{-3}\ m^3\ kg^{-1}$ of lactose

Voluminosity and volume fraction pertain to hydrodynamic volume and thus account for particle shape and water of hydration as well as volume *per se* (Walstra and Jenness, 1984).

Equation 22, for $\Sigma(\phi_i) \rightarrow 0$, reduces to the well-known Einstein equation for the viscosity of a very dilute solution of hard spheres:

$$\eta = \eta_0(1 + 2.5\phi) \tag{24}$$

Einstein's equation assumes no particle–particle interaction ($\phi < 0.05$; Hinrichs, 1999). Eilers's equation accounts both for the presence of the dispersed phase and for hydrodynamic interaction between particles during flow.

Van Vliet and Walstra (1980) showed that the volume fractions of all the dispersed species and dissolved lactose must be included in the $\Sigma(\phi_i)$ term in Eilers's equation (Equation 22); the equation will, for example, underestimate the viscosity of cream if ϕ_{fat} is substituted for $\Sigma(\phi_i)$ and η_{skim} (taken as being the same as η_{plasma}) for η_0.

The volume fraction of the casein (ϕ_{cas}) in milk is the main determinant of $\Sigma(\phi_i)$ and thus of viscosity. Large differences in viscosity between different milks are almost certainly attributable to differences in ϕ_{cas}, which depend, in

turn, on such factors as the concentration of colloidal calcium phosphate, Ca^{2+} activity and pH (Walstra and Jenness, 1984). The viscosity of milk and cream increases with age owing partly to changes in ionic equilibria (Jenness and Patton, 1959; Prentice, 1992). The viscosity of milk increases slightly with increasing pH, perhaps owing to swelling of casein micelles. Conversely, a small decrease in pH reduces viscosity, while a large decrease causes micelle aggregation (Walstra and Jenness, 1984).

The viscosity of Newtonian milk products depends on several factors besides those mentioned above. These include composition, concentration, temperature, thermal history and processing operations. The effects of composition and concentration of milks and creams can be predicted using Equation 22. Viscosity increases with %TS, but, for a given TS, is inversely related to % fat content (F) because of the lower voluminosity of fat compared with that of casein in particular. When $\Sigma(\phi_i)$ exceeds 0.6 (which corresponds to $\eta/\eta_0 \approx 10$), viscosity increases steeply with $\Sigma(\phi_i)$, and rheological behaviour becomes non-Newtonian (Walstra and Jenness, 1984).

Viscosity is inversely related to temperature *per se*. Cooling milk results in an increase in viscosity that is partly the result of an increase in η_0 and partly the result of a sharp increase in V_{cas} and thus in ϕ_{cas} (Walstra and Jenness, 1984; Sherbon, 1988). Furthermore, some caseins, especially β-casein, dissociate from the micelles at low temperatures; this contributes to an increase in viscosity because dissociated protein molecules have higher hydrodynamic volumes. Rheological behaviour becomes non-Newtonian (shear thinning) at low temperatures (Randhahn, 1973; Walstra and Jenness, 1984). The heating of skim milk or whole milk to 65°C, followed by rapid cooling, results in a temporary decrease in viscosity because of the increased association of β-casein with the micelles that would have occurred at the higher temperature. Viscosity recovers exponentially with time as some β-casein gradually dissociates again at the lower temperature (Sherbon, 1988).

Warming milk to temperatures above ambient causes viscosity to decrease, because η_0 decreases and because ϕ_{cas} decreases moderately. The change in ϕ_{cas} is reflected by a decrease in the ratio η_{skim}/η_{whey} when temperature is increased from 5 to 30°C. The decrease is less marked at temperatures >30°C (Sherbon, 1988).

The viscosity of milk increases with age. Whitnah (1996) showed that the viscosity of both unhomogenized and homogenized pasteurized milk, measured at temperatures ranging from 4 to 44°C, increased during storage at 4°C as approximately a linear function of log_{10} *(storage time)*. The rate of increase in viscosity with age decreased as the temperature of measurement increased.

Janal and Blahovec (1974) found that the viscosity of whole milk during dynamic heating and cooling depended on both temperature and temperature history (in the temperature range 5–85°C): there was hysteresis in plots of viscosity versus temperature obtained while heating whole milk (previously stored for a time at a relatively low temperature) and then cooling it. The hysteretic pattern for fresh milk usually showed higher viscosities during heating than during cooling. Janal and Blahovec (1974) attributed this phenomenon to the reversible melting/crystallization behaviour of the milk triacylglycerols. Different hysteretic patterns were found with aged milk; these were considered to reflect decreases in the heat stability of proteins with age owing to increasing milk acidity.

Pasteurization, a relatively mild heat treatment, results in no noticeable change in the rheological properties of whole milk (Prentice, 1992).

15.8.2. Non-Newtonian Behaviour in Milks and Creams

Non-Newtonian behaviour becomes manifest in raw milks and creams under conditions that favour cold agglutination of fat globules (temperatures <40°C and low shear rate) (Randhahn, 1973, 1974; Randhahn and Reuter, 1978; Reuter and Randhahn, 1978; Walstra and Jenness, 1984).

Shear thinning is the predominant rheological behaviour. Clusters of fat globules contain trapped interstitial plasma, and thus together have a high effective volume fraction, especially at low shear rates. If the shear rate is increased, shearing forces cause aggregates to become more regular in shape or to break down; the volume fraction and thus the apparent viscosity (defined as $(\eta_{app})_{\dot{\gamma}} = \tau/\dot{\gamma}$) then decreases. As shearing forces become larger compared with the attractive forces holding globules together, successive increases in shear rate have smaller and smaller effects on apparent viscosity. At sufficiently high shear rates, behaviour becomes Newtonian (Walstra and Jenness, 1984).

Kyazze and Starov (2004) studied the influence of fat globule cluster formation at 20°C on the viscosity of model emulsions (of $\phi_{fat} \leq 0.4$) made by mixing pasteurized double cream (47.5 g fat per 100 ml) with pasteurized skimmed milk, in the low shear rate range of 0.06–30 s^{-1}. Their aim was to test experimentally the theoretical equations proposed by Starov and Zhdanov (2003) for describing the viscosity of concentrated emulsions. They found that while the model emulsions showed shear thinning at shear rates of up to about 10 s^{-1}, relative viscosity ($\eta_{emulsion}/\eta_{skim}$) was independent of shear rate, but increased with ϕ_{fat}. This means that the shear rate dependency was due only to the skim milk. (The data of Randhahn (1973) demonstrate that skim milk would have undergone shear thinning under the temperature and shear rate conditions existing in the experiments of Starov and Zhdanov (2003)). Kyazze

and Starov (2004) found, further, that the packing density of fat globules inside clusters, which increased with increasing ϕ_{fat}, was also independent of shear rate. This means that, in the shear rate range investigated, fat globule clustering was influenced more by factors such as partial coalescence, inter-globule pair potential, depletion interactions and agglutination than by shear rate. The experimental data were not fully modelled by the theoretical equations of Starov and Zhdanov (2003). Kyazze and Starov (2004) explained this by pointing out that the presence of the 50 nm thick milk fat globule membrane, and the fact that a proportion of the fat inside the globules would have been solid at 20°C, meant that the contents of the globules would not have been completely mobile, as demanded by theory.

At a high fat content, non-Newtonian behaviour in milk and cream is more pronounced and persists to higher shear rates. For example, a 40% fat cream at a temperature of >40°C might be Newtonian at $\dot{\gamma} > 10\,s^{-1}$, while a 49% fat cream might be Newtonian only at $\dot{\gamma} > 100\,s^{-1}$ (Walstra and Jenness, 1984).

Lower temperatures enhance cold agglutination, resulting in stronger fat globule clusters; this increases both apparent viscosity and deviation from Newtonian behaviour, the latter persisting to higher shear rates (Walstra and Jenness, 1984). Separation of milk at a temperature of ~40°C gives cream in which cold agglutination is greatly reduced owing to the loss of agglutinin in the skim milk. Conversely, cold separation enhances cold agglutination (Mulder and Walstra, 1974). Thus, separation conditions influence the rheology of cream. Cold agglutination in raw cream, and the presence of homogenization clusters in homogenized cream, can result in thixotropy (time-dependent shear thinning) (Walstra and Jenness, 1984).

Homogenization of raw milk increases viscosity but reduces deviation from Newtonian behaviour, the latter effect probably being the result of a reduction in globule–globule attraction, resulting from the destruction of agglutinin (Randhahn, 1973, 1974); Reuter and Randhahn, 1978; Walstra and Jenness, 1984).

Randhahn (1974) and Reuter and Randhahn (1978) investigated the pseudoplastic behaviour exhibited by raw milk at temperatures <40°C. Apparent viscosity at an infinite shear rate ($\eta_{\dot{\gamma}=\infty}$) was related to ϕ_{fat} according to the equation

$$\eta_{\dot{\gamma}=\infty} = \eta_{skim}(1 + 3.25\phi_{fat}) \tag{25}$$

Evaluation of apparent viscosity at $\eta = \infty$, by extrapolation, allowed the effects of fat content to be separated from the effects of shear rate (Randhahn and Reuter, 1978). Randhahn and Reuter (1978) reported that the pseudoplastic nature of raw cream containing ≤40% fat appeared to be

due only to cold agglutination. Relative viscosity at $\dot{\gamma} = \infty$ was related to fat content in this range by the equation

$$\ln \eta_{rel,\dot{\gamma}=\infty} = \ln \frac{\eta_{\dot{\gamma}=\infty}}{\eta_{skim}} = \frac{2.5\phi_{fat}}{1 - 1.165\phi_{fat}} \tag{26}$$

The rheological properties of cream containing >40% fat were influenced also by the tendency of fat globules to become orientated by shearing. The relationship between relative viscosity and fat content, for fat contents in the range 40–70% (w/w), was

$$\eta_{rel,\dot{\gamma}=\infty} = 1 + 2.5\phi_{fat} + 4.55\phi_{fat}^2 + 13.45\phi_{fat}^3 + 100.6\phi_{fat}^4 \tag{27}$$

The temperature dependence of η_{skim} in the temperature range 5–40°C was accounted for by the equation

$$\eta_{skim} = 5.0213 \times 10^{-7} \exp(2398/T) \tag{28}$$

where T is absolute temperature (K). Randhahn and Reuter (1978) pointed out that the viscosity of raw cream depends to some extent also on temperature history, possibly owing to temperature-dependent characteristics of milk proteins.

Randhahn and Reuter (1978) found that the Cross equation (29), which relates apparent viscosity to shear rate, exactly described the pseudoplastic nature of raw cream:

$$\eta_{app} = \eta_{\dot{\gamma}=\infty} + \frac{\eta_{\dot{\gamma}=0} - \eta_{\dot{\gamma}=\infty}}{1 + b\dot{\gamma}^m} \tag{29}$$

where η_{app} is the apparent viscosity at $\dot{\gamma}$ s^{-1}, $\eta_{\dot{\gamma}=0}$ and $\eta_{\dot{\gamma}=\infty}$ are the apparent viscosities at $\dot{\gamma} = 0\,s^{-1}$ and $\dot{\gamma} = \infty$, respectively, found by extrapolation, and b, m are constants. The factors $\eta_{\dot{\gamma}=0}$, $\eta_{\dot{\gamma}=\infty}$, b and m are each dependent on temperature and fat content. The applicability of the Cross equation implies that a state of equilibrium exists during flow between the size and number of fat globule clusters and shear rate.

Homogenization of milk results in an increase in viscosity at high shear rates. The increase is inversely related to fat globule size (Reuter and Randhahn, 1978). Reuter and Randhahn (1978) found that for raw milk the relative viscosity at $\dot{\gamma} = \infty$ depends on the mean distance between fat globules:

$$\ln \eta_{rel} = (4.7\phi_{fat} - 0.1)\left(\frac{1}{a} - \frac{1}{a_R}\right) + \ln \eta_{rel,\mathrm{R}} \tag{30}$$

where a and a_R are the mean inter-globule distances (μm) in homogenized milk and in raw milk, respectively. The value of a was given by

$$a = d_{vs}\left(\left(\frac{\phi_{fat,\max}}{\phi_{fat}}\right)^{1/3} - 1\right) \qquad (\mu\text{m}) \tag{31}$$

where d_{vs} is the volume-surface (Sauter) mean diameter (μm) and $\phi_{fat,\ max}$ is the maximum possible ϕ_{fat} (value not given).

For raw creams, with a fat content of up to 15%, Randhahn and Reuter (1978) presented the following, similar, equations:

$$\ln \eta_{rel} = 4.673\phi_{fat}\left(\frac{1}{a} - \frac{1}{a_R}\right) + \ln \eta_{rel,\mathrm{R}} \tag{32}$$

where

$$a = 0.225 d_{vs}\left(\frac{\phi_{fat,\max}}{\phi_{\mathrm{fat}}}\right) - 1 \tag{33}$$

Randhahn and Reuter (1978) concluded that the rheological behaviour of milk and cream is in accord with that of emulsions and suspensions in general. Cream, a concentrated emulsion, can exhibit most possible types of non-Newtonian behaviour depending on conditions and treatment (Mulder and Walstra, 1974). Raw cream containing cold-agglutinated fat globules and homogenized cream containing homogenization clusters can exhibit time-dependent shear thinning (as distinct from time-independent pseudoplasticity). Recovery of viscosity with time after shearing has ceased is slow and incomplete in raw cream (Walstra and Jenness, 1984; Prentice, 1992) and virtually non-existent in homogenized cream (Walstra and Jenness, 1984).

Shear thickening may be observed in high-fat creams; shearing induces partial coalescence of globules, thereby increasing the effective ϕ_{fat} by entrapment of plasma (Walstra and Jenness, 1984). Partial coalescence appears to explain the rebodying of cream resulting from temperature fluctuation (Walstra and Jenness, 1984). Total coalescence causes little change in viscosity because ϕ_{fat} remains almost constant (Walstra and Jenness, 1984).

The ageing of homogenized cream results in an increase in viscosity, probably owing to irreversible flocculation of casein micelles (Mulder and Walstra, 1974).

Cream is viscoelastic; its rheological behaviour may be represented by the generalized Maxwell body (a mechanical analogue) whose relaxation time is nearly independent of fat content (Randhahn and Reuter, 1978).

Viscoelasticity manifests itself by, for example, causing a stress overshoot in a rotational viscometer at very low shear rates (e.g. 0.005–0.15 s^{-1}) or as a phase difference between shear stress and shear strain in dynamic (oscillatory) rheological measurements (Prentice, 1992).

15.8.3. Effects of Technological Treatments on the Viscosity of Milk

The effects of homogenization on the rheology of milk and cream have been discussed above. Here, the effects of some further technological treatments are considered.

The effects of heat treatment, renneting and acidification on the viscosity of milk, under conditions where coagulation has not (yet) occurred, can be modelled using the so-called adhesive hard sphere (AHS) theory (de Kruif *et al*., 1992; Jeurnink and de Kruif, 1993; de Kruif, 1998).

It has been shown that untreated skim milk behaves rheologically as a semi-dilute colloidal suspension of (non-adhesive) hard spheres – the casein micelles – in a medium comprising all of the other milk components (de Kruif *et al*, 1992; Jeurnink and de Kruif, 1993). For such a suspension, the viscosity (strictly speaking the coefficient of viscosity at $\dot{\gamma} \rightarrow 0$) is given by the following theoretical equation (which is a modification of Einstein's equation (Equation 24):

$$\eta = \eta_0(1 + 2.5\phi + k_H\phi^2) \tag{34}$$

k_H (the Huggins constant) accounts for hydrodynamic interactions, which occur in other than very dilute dispersions. For $\phi \leq 0.2$ (semi-dilute dispersions), in the absence of mutual attraction or repulsion between the micelles (non-adhesive hard spheres), k_H is equal to 5.913. Viscosity is then given by

$$\eta = \eta_0(1 + 2.5\phi + 5.913\phi^2) \tag{35}$$

Untreated skim milk closely follows this equation (de Kruif, 1998).

A series development of the semi-empirical Eilers equation (with $\phi_{max} = 0.74$) gives the similar expression

$$\eta = \eta_0(1 + 2.5\phi + 4.94\phi^2 + 8.78\phi^3 + \cdots) \tag{36}$$

Equations 35 and 36 both reduce to Einstein's equation (24) when ϕ is small enough to preclude interactions of any kind between the suspended spheres.

The Huggins constant is given by the following equation:

$$k_H = 5.913 + \frac{1.9}{\tau_B} \tag{37}$$

τ_B is the Baxter interaction parameter, which is a measure of adhesive interactions between the spheres (stickiness). It is related to the second osmotic virial coefficient, B_2, a property of the casein micelle suspension:

$$\frac{B_2}{V_{HS}} = 4 - \frac{1}{\tau_B} \tag{38}$$

where V_{HS} ($= \frac{4}{3}\pi a^2$) is the volume of a hard sphere of radius a.

For non-adhesive hard spheres (no mutual attraction), $\tau_B = +\infty$ and $k_H =$ constant $= 5.913$), as indicated above (Equation 35). When mutual attraction does exist (adhesive hard spheres), τ_B has a positive finite value. k_H is then >5.913, and viscosity has a value greater than that predicted by Equation 35. The adhesive hard sphere theory is embodied in Equations 34, 37 and 38.

15.8.3.1. Heat Treatment

Jeurnink and de Kruif (1993) showed using viscosity and other measurements that heat treatment of skim milk (85°C for various holding times), severe enough to cause denaturation of β-lactoglobulin and its subsequent association with casein micelles, caused an increase in viscosity resulting from both an increase in ϕ and a decrease in τ_B. The decrease in τ_B corresponded to an increase in the value of k_H to 7.1 (Equation 37). This indicated the development of mutual attraction between the casein micelles upon heating; measured viscosities of heated milk were greater than those predicted by Equation 35 (even when using the measured, higher, heat-induced values of ϕ) because of this mutual attraction (adhesiveness), for which this equation does not account.

Eilers's equation (Equation 22) also predicts too low a viscosity for heated skim milk (Langley and Temple, 1985; Jeurnink and de Kruif, 1993). Jeurnink and de Kruif (1993) reported that using a value of ϕ_{max} lower than 0.74 in the equation, to take into account the change in the size distribution of casein micelles upon heating, did not result in better prediction for any reasonable value of ϕ_{max}. This again indicated that heating increased adhesiveness between micelles, a phenomenon for which the Eilers equation, like Equation 35, does not account.

The viscosity of dispersions of hard spheres (at $\dot{\gamma} > 0$) depends on both volume fraction and shear rate (Jeurnink and de Kruif, 1993). Jeurnink and de Kruif (1993) found that skim milk showed shear thinning in the shear rate range 5–800 s^{-1} and that mild heat treatment increased the degree of shear thinning. This provided further evidence that interactions between micelles had increased upon heating (i.e., that B_2/V_{hs} and thus τ_B had decreased).

A computer-based model utilizing the AHS theory, the denaturation/aggregation kinetics of β-lactoglobulin and the kinetics of the reaction between denatured and aggregated β-lactoglobulin and casein micelles has been developed that allows the prediction of the viscosity of skim milk resulting from any combination of heating temperature and heating time (de Jong and van der Linden, 1998). The model shows that the viscosity of heated milk not only depends on the extent of β-lactoglobulin denaturation but, for a given extent, is higher for a higher heating temperature.

15.8.3.2. Renneting

The addition of chymosin to milk at the start of renneting initiates two sequential changes. First, there is a fall in viscosity caused by the enzyme cutting κ-casein hairs from the surface of the casein micelles, with a consequent reduction in ϕ_{cas}. Second, the loss of the stabilizing κ-casein layer causes the micelles to become mutually attractive (adhesive hard spheres). This leads to an increasingly rapid rise in viscosity that culminates in micelle flocculation. de Kruif *et al*. (1992) showed that these two effects are together qualitatively and quantitatively well modelled by the AHS theory as expressed by Equations 34 and 37. The theory can be used to predict coagulation time, defined as the time after chymosin addition at which the viscosity recovers its initial value after its temporary fall.

15.8.3.3. Acidification

Acidification of skim milk, e.g., that resulting from the activity of starter bacteria during yoghurt manufacture, causes micelle flocculation (eventually leading to coagulation) owing to a loss of the stearic stabilization of micelles. Loss of stabilization is caused by loss of the extended conformation of the κ-casein hairs on the micelle surfaces and the hairs' eventual collapse. The AHS theory accurately quantifies the relationship between viscosity (increasing) and pH (decreasing) during acidification (de Kruif, 1998).

The AHS theory has been applied mainly to skim milk. The effects of other variables such as fat content and calcium concentration on the applicability of the theory have apparently yet to be investigated.

15.8.3.4. Storage of UHT Milk

The viscosity of UHT (ultra high-temperature sterilized) milk can increase gradually during storage. This phenomenon – age thickening – can lead eventually to gelation. It is thought to be the result of the gradual release

from the casein micelles of β-lactoglobulin/κ-casein complexes formed during the high-temperature heat treatment, and the subsequent cross-linking of these complexes to form a gel network (McMahon, 1996; Datta and Deeth, 2001).

15.8.4. Technologically Useful Phenomenological Relationships for Predicting the Rheological Properties of Milks and Creams

Successful attempts have been made to establish empirical relationships useful for technological and engineering purposes between the viscosity of Newtonian fluid milk products and their temperature and composition. A number of these, whose development ignored any observed (slight) non-Newtonian behaviour, are given in Table 15.5. Details of milk treatment prior to viscosity measurement may be found in the papers cited.

Table 15.5. Technologically useful relationships between the viscosity of Newtonian milk products and temperature and composition (η and η_0 = viscosity in mPa s, θ = temperature in °C, TS = % total solids, F = % fat and P = % protein.)

Product specifications	Relationship
Milk, 8–30% TS, 0.07–7.4% fat, Fat to solids-not-fat ratio: 0.01–0.4, 0–80°C	$\log \eta = 0.249 - 1.3 \times 10^{-2}\theta + 5.2 \times 10^{-5}\theta^2 + (2.549 \times 10^{-2} - 9.8 \times 10^{-5}\theta + 4 \times 10^{-7}\theta^2)(TS) + (5.43 \times 10^{-4} - 1.39 \times 10^{-5}\theta + 1.117 \times 10^{-7}\theta^2)(TS)^2$ (Fernández-Martin, 1972b)
Milk, 0.03–15% fat, 70–135°C	$\ell n\ \eta = 3.92 \times 10^{-5}\theta^2 - 1.951 \times 10^{-2}\theta + 0.666 + F(-9.53 \times 10^{-6}\theta^2 + 1.674 \times 10^{-3}\theta - 4.37 \times 10^{-2}) + F^2(9.75 \times 10^{-7}\theta^2 - 1.739 \times 10^{-4}\theta + 9.83 \times 10^{-3})$ (Bertsch and Cerf, 1983)
Milk of normal composition, 25°C	$\eta = 0.96 + 0.058F + 0.156P$ (Rohm *et al.*, 1996)
Milk and cream, 0.1–30% fat, 0–30°C	$\ell n\ \eta = \left(\frac{2731.5}{(273+\theta)}\right) + 0.1F - 8.9$ (Bakshi and Smith, 1984)
Milk and cream, 0–40% fat, 40–80°C	$\log \eta = A(F + F^{5/3}) + \log \eta_0$ where $A = 1.2876 + 11.07 \times 10^{-4}\theta$ and $\eta_0 = 0.7687\left(\frac{10^3}{273+\theta}\right) - 2.437$ (Phipps, 1969)

In the case of non-Newtonian milks and creams, phenomenological relationships between shear stress and shear rate have been developed for practical purposes. These contrast with expressions involving viscosity at zero shear rate and viscosity at infinite shear rate, which are valuable for relating rheological behaviour to structure and composition in a fundamental way.

The shear thinning behaviour of raw whole milk in the shear rate range 1-1500 s^{-1} can be well modelled by the power law (Ostwald and de Waele) equation (Randhahn, 1973; Rohm *et al.*, 1996):

$$\tau = k\dot{\gamma}^n \tag{39}$$

Rohm *et al.* (1996) showed that, at 25°C, as milk fat content increased from 3.3 to 4.95%, k increased from 6 to 10.5 Pa s while n decreased from 0.8 to 0.75 (the latter change indicating a slight increase in non-Newtonian behaviour). The equation of Rohm *et al.* (1996) for predicting the coefficient of viscosity of raw milk displayed in Table 15.5 was developed from measurements of kinematic viscosities at (indeterminate) low shear rates.

Randhahn (1973) found that the rheological behaviour of raw milk at 15°C becomes essentially Newtonian at shear rates >800 s^{-1}, n increasing from 0.54–0.58 at $\dot{\gamma} = 50s^{-1}$ to 1.0 at $\dot{\gamma} = 1000s^{-1}$.

The data of Wayne and Shoemaker (1988) were recalculated by Rohm *et al.* (1996) to show an n value of about 0.88 for pasteurized milk.

The shear thinning behaviour of pasteurized skim milk, whole milk and cream at temperatures <40°C can also be modelled satisfactorily by the Bingham equation (Kristensen *et al.*, 1997):

$$\tau = \eta_{pl}\dot{\gamma} + \tau_0 \tag{40}$$

Values of η_{pl} (the plastic viscosity) for skim milk and whole milk (3.5% fat) at 25°C were 1.53×10^{-3} and 1.81×10^{-3} Pa s, respectively. The corresponding values of τ_0 (the yield stress) were 1.98×10^{-2} and 4.6×10^{-2} Pa. For cream containing 38% fat at 25°C, the values of η_{pl} and τ_0 were 9.44×10^{-3} Pa s and 0.381 Pa, respectively.

Sadowska (1990) found that raw cream containing 60% fat obeyed the Bingham equation at constant temperatures in the range 15–80°C. When subjected to dynamic heating and cooling in this temperature range, the rheological behaviour of the same cream became better modelled by the power law equation (Equation 39). The values of k and n at a given temperature depended on thermal history, a phenomenon attributed to interaction between heating/cooling rates and rates of melting and crystallization of milk triacylglycerols.

Grikshtas (1990) found that cream containing 40–55% fat, at 5–20°C, obeyed the Herschel–Bulkley equation:

$$\tau = k\dot{\gamma}^n + \tau_0 \tag{41}$$

This equation is a generalized form of the Bingham equation (Equation 40). Values of n were not reported. (The power law equation (Equation 39) and Newton's equation (Equation 21), as well as the Bingham equation, are special cases of Equation 41).

As stated above, the Cross equation (Equation 29) accurately models the shear dependence of the apparent viscosity of cream.

The numerical values of the parameters in these phenomenological equations depend on composition, concentration and temperature.

Published rheological studies of milk and cream are comprehensively reviewed by Vélez-Ruiz and Barbosa-Cánovas (1997).

15.8.5. Rheology of Concentrated Milks

When milk is concentrated by heat evaporation or by membrane processes (ultrafiltration, reverse osmosis), $\Sigma(\phi_i)$ increases because of the concentration effect *per se* and because particle–particle interactions (especially micelle–micelle interactions) increase owing to smaller inter-particle distances. These interactions lead to aggregate formation. The effects on rheological behaviour of aggregate formation are essentially the same as the effects of concentrating only the fat globules, as in cream: apparent viscosity increases, shear thinning becomes more pronounced and deviation from Newtonian behaviour persists to higher shear rates (Walstra and Jenness, 1984). Thixotropy (time-dependent shear thinning) appears at a sufficiently high concentration, or after a sufficiently long storage time (at concentrations and temperatures above certain minima) during which structure development occurs. Such structure development, and the consequent steady viscosity increase, is known as age thickening.

Rheologically, there are no fundamental differences between concentrates produced by heat, by reverse osmosis or by ultrafiltration, or between whole milk and cream concentrates on the one hand and skim milk concentrates on the other. Rather, the differences are ones of degree. For given conditions, non-Newtonian behaviour tends to appear at lower %TS in skim milk concentrates produced by ultrafiltration than in evaporated skim milk (because of the preferential concentration of proteins in the former) and at lower %TS in skim milk concentrates than in whole milk concentrates (because the proportion of protein in the dry solids is higher in the former). For the same reasons, viscosity at a given %TS is higher in ultrafiltration skim milk concentrates than in evaporated skim milk, and in skim milk concentrates than in whole milk concentrates (Culioli *et al.*, 1974; Hinrichs, 1999).

The viscosity of any concentrate under given conditions of %TS, shear rate, temperature, dry solids composition, time after preparation and other variables such as preheat treatment and pH is directly related to $\Sigma(\phi_i)$. This is discussed first below. Then, phenomenological relationships that have been found useful in describing the non-Newtonian behaviour of concentrates as a function of shear rate are reviewed. Lastly, the phenomenological description of age thickening is described.

15.8.5.1. Dependence of Concentrate Viscosity on Total Volume Fraction

Snoeren *et al*. (1982, 1983) showed that the apparent viscosity (at a specified shear rate) of heat-concentrated whole and skim milks is poorly related to %TS but closely related to total volume fraction calculated as

$$\Sigma(\phi_i) = \phi_{cas} + \phi_{nwp} + \phi_{dwp} + \phi_{fat} \tag{42}$$

where ϕ_{nwp} and ϕ_{dwp} are the volume fractions of native and denatured whey proteins, respectively. Experimentally determined values of voluminosity were 3.57×10^{-3} m^3 kg^{-1} of dry casein, 1.07×10^{-3} m^3 kg^{-1} of dry native whey protein and 3.09×10^{-3} m^3 kg^{-1} of dry denatured whey protein; preheat treatments caused whey protein denaturation, which increased $\Sigma(\phi_i)$.

Volume fractions (ϕ_i) of individual components were calculated as

$$\phi_i = \phi_{i,\text{milk}} \times \frac{\text{TS}_{\text{conc}}}{\text{TS}_{\text{milk}}} \times \frac{\rho_{\text{conc}}}{\rho_{\text{milk}}} \tag{43}$$

where ρ is density (kg m^{-3}) and the subscripts 'milk' and 'conc' refer to the milk prior to evaporation and to the concentrated milk, respectively.

Concentrates showed shear thinning; $\eta_{app,\dot{\gamma}=\infty}$ of freshly prepared concentrates (no age thickening) was closely predicted by Eilers's equation (22). ϕ_{max} in the equation was taken as 0.79, which was determined by extrapolating curves of skim milk concentrate gelling time versus ϕ_p (the total protein volume fraction), at two temperatures, to zero time (Snoeren *et al.*, 1982).

η_0 in Eilers's equation was calculated as

$$\eta_0 = \eta_{water} + (\Delta\eta_s + \Delta\eta_{ls})\left(\frac{TS_{conc}}{TS_{milk}}\right) \tag{44}$$

where

$$\Delta\eta_S = \eta_{water+salts} - \eta_{water} = 0.02\,\eta_{water}$$

$$\Delta\eta_{ls} = \Delta\eta_{water+5\%lactose} - \eta_{water}$$

Temperature dependence of viscosity was allowed for by evaluating $\Delta\eta_s$ and $\Delta\eta_{ls}$ at required temperatures using literature data.

Hallström and Dejmek (1988b) used viscosity measurement and Eilers's equation, with $\phi_{max} = 0.79$ (Snoeren *et al.*, 1982) and $\eta_0 = \eta_{permeate}$, to calculate the ϕ_p of skim milk concentrates prepared using ultrafiltration. (ϕ_p was equivalent to $\Sigma(\phi_i)$ because of the loss of lactose during ultrafiltration and the absence of fat). Overall protein voluminosity (dominated by casein voluminosity) was then calculated from ϕ_p for various temperatures, pH values and skim milk preheat treatments. Comparison with voluminosities measured by sedimentation showed that the latter were 1.3 times smaller. This was attributed to compression during sedimentation of the hairy κ-casein outer layer of casein micelles, and thus underestimation of voluminosity by this method. Hallström and Dejmek (1988b) tried without success to find a constant value of ϕ_{max} applicable to concentrates with different protein contents. This indicated that the casein micelles in skim milk cannot be regarded as hard spheres when the milk is (ultrafiltration) concentrated.

Snoeren *et al.* (1984) investigated the age thickening of skim milk concentrates at 50°C. The apparent viscosity, the rate of age thickening and the degree of shear thinning all increased with both time and %TS. Even $\eta_{app,\dot{\gamma}=\infty}$ increased slightly with storage time (at a given %TS), suggesting that holding at 50°C induced some permanent change in the structure of the concentrate that could not be reversed by shearing. Binh Trinh *et al.* (2007a) found that concentrated reconstituted whole milk (48% TS, held at 65°C) showed the same phenomenon.

Snoeren *et al.* (1983, 1984) showed that the increase with time in the apparent viscosity of both skim milk and unhomogenized whole milk concentrates during age thickening, which is the result of an increase in $\Sigma(\phi_i)$ owing to aggregation, could be predicted by a modified Eilers equation:

$$\eta_{app,t} = \eta_0 \left(1 + \frac{1.25\phi_p(1 + t.k_{\phi_p}/\phi_p)}{1 - \phi_p(1 + t.k_{\phi_p}/\phi_p)\phi_{\max}} \right)^2 \tag{45}$$

where $\eta_{app,\ t}$ is the apparent viscosity at a specified shear rate and temperature, t is time, $\phi_p = \phi_p$ at $t = 0$ and $k_{\phi_p} = d\phi_p/dt$. For a given shear rate and temperature, k_{ϕ_p}/ϕ_p, called the age thickening constant, was independent of %TS over the ranges studied (TS = 45.4–52.5% for skim milk concentrates and TS = 46.7–58.9% for whole milk concentrates). The average values of the constant at $\dot{\gamma} \to \infty$ and 50°C were 2.82 and 2.60 for skim milk concentrates and whole milk concentrates, respectively. The rate of age thickening is somewhat lower for whole milk concentrates than for skim milk concentrates

because the fat in the former, while contributing to the overall volume fraction, is inert with respect to the age thickening process.

Snoeren *et al.* (1984) proposed the following explanation of age thickening. Concentration of milk causes an increase in ionic strength and a reduction in pH. These changes reduce the amount of calcium bound to protein, especially to β-casein. This in turn leads to increased solubility of β-casein, the consequence of which is a loosening of the casein micelle structure, increasing ϕ_{cas} and thus ϕ_p. Snoeren *et al.* (1984) pointed out that only a very small increase in casein voluminosity is required to cause a substantial increase in viscosity. The effective size of the loosened micelles is reduced by higher shear rate, leading to shear thinning behaviour.

15.8.5.2. Phenomenological Relationships for Describing the Non-Newtonian Behaviour of Concentrates

As %TS increases, the rheological behaviour of freshly prepared milk concentrates changes from Newtonian to time-independent shear thinning to time-dependent shear thinning (Binh Trinh *et al.*, 2007b).

Under conditions where time dependency is absent, flow behaviour changes, as %TS increases, from Newtonian (Equation 21) to shear thinning with no yield stress (power law behaviour; Equation 39) to shear thinning with a yield stress (Bingham plastic behaviour; Equation 40) or Herschel–Bulkley behaviour (Equation 41) (Randhahn, 1973, 1976; Hallström and Dejmek, 1988a; Stepp and Smith, 1991; Horne, 1993); Sierzant and Smith, 1993; Vélez-Ruiz and Barbosa-Cánovas, 1997, 1998; Hinrichs, 1999; Bienvenue *et al.*, 2003a, b; Binh Trinh *et al.*, 2007a, b).

In fact, because (as pointed out above) the Newtonian, power law and Bingham equations are all special cases of the Herschel–Bulkley equation, this last equation can be said to describe adequately the flow curves of time-independent milk concentrates, or the instantaneous flow curves of time-dependent concentrates, under all conditions of temperature, shear rate and other variables. When $\tau_0 = 0$ and $n = 1$ in the Herschel–Bulkley equation, $k = \eta$, the Newtonian coefficient of viscosity.

For example, Vélez-Ruiz and Barbosa-Cánovas (1997) demonstrated that whole milk concentrates prepared by evaporation were Newtonian up to approximately 20% TS, obeyed the power law between about 20 and 34% TS and obeyed the Herschel–Bulkley equation >37% TS. Newtonian behaviour persisted to higher %TS at higher temperature. For milk concentrates in general, the precise %TS ranges over which Newtonian, power law and Herschel–Bulkley behaviours are seen will depend on numerous factors

such as dry solids composition, temperature, shear rate range, pre-concentration milk treatment, concentration method and conditions.

Generally, k (directly related to viscosity) increases with increasing %TS and/or decreasing temperature, while n (the degree of deviation from Newtonian or from Bingham plastic behaviour) does the opposite (Randhahn, 1976; Stepp and Smith,1991; Sierzant and Smith, 1993; Hinrichs, 1999). k becomes more sensitive to %TS as temperature decreases (Vélez-Ruiz and Barbosa-Cánovas, 1997).

Vélez-Ruiz and Barbosa-Cánovas (1997) were able to relate n and k of their whole milk concentrates to %TS by equations of the form

$$n, k = Ae^{b(\%TS)} \tag{46}$$

where A and b are empirical constants. Further, they were able to relate k to both temperature and %TS by the equation

$$\ell n\ k = \beta_0 + \beta_1\left(\frac{1}{T}\right) + \beta_2(\%TS) \tag{47}$$

where T is absolute temperature, and β_0, β_1 and β_2 are empirical constants.

Randhahn (1976), Hallström and Dejmek (1988a), Stepp and Smith (1991) and Hinrichs (1999) found that the power law described well the flow behaviour of ultrafiltration skim milk concentrates. Randhahn (1976) demonstrated a unique temperature-independent inverse relationship between n and k, while Hallström and Dejmek (1988a) demonstrated a similar relationship between n and $\log_{10} k$ that was independent not only of temperature but also of pH, protein content and whole milk preheat treatment prior to separation. These relationships reflect the fact that structure development in concentrates that results in greater deviation from Newtonian behaviour also results in increased viscosity.

The power law has also been found to adequately to characterize the flow behaviour of ultrafiltration whole milk and cream concentrates (Sierzant and Smith, 1993; Hinrichs, 1999). Hinrichs found that the dependence of k on %TS itself depended on the fat content of the original, unconcentrated milk or cream. However, there was a unique direct relationship between $\log_{10} k$ (at 20°C) and concentrate $\Sigma(\phi_i)$, showing that rheological behaviour at finite shear rates is, like $\eta_{\dot{\gamma}=0}$, largely determined by the total volume fraction. This relationship was linear up to $\Sigma(\phi_i) = 0.5$ and also linear, but with a higher slope, at $\Sigma(\phi_i) > 0.5$. Hinrichs (1999) found that at $\Sigma(\phi_i) < 0.5$, n $\approx$ 1 (Newtonian behaviour), and derived the following relationship from his data:

$$\log_{10} k = \log \eta = 3.82\Sigma(\phi_i) - 3.35 \tag{48}$$

where the units of k and η are Pa s^n and $n = 1$.

Hinrichs (1999) found that shear thinning behaviour occurs at $\Sigma(\phi_i) > 0.5$. This critical value of ϕ is close to the theoretical maximum value of 0.52 that could exist in an ideal monodisperse suspension in which the suspended particles are in contact but able to move in straight lines in the direction of flow. The dependence of k (at 20°C) on $\Sigma(\phi_i)$ at $\phi > 0.5$ is greater than that expressed by Equation 48 and can be estimated approximately from Hinrichs' data to be

$$\log_{10} k = 10.15\Sigma(\phi_i) - 6.51 \tag{49}$$

Interestingly, Hallström and Dejmek (1988a) found that for ultrafiltration skim milk concentrates, a similar double linear relationship existed between $k_{relative}$ and $\Sigma(\phi_i)$ (expressed as ϕ_p), also with a critical value of $\Sigma(\phi_i)$ of about 0.5, at which the slope changed to a higher value. $k_{relative}$ was defined by the equation

$$k_{relative} = \frac{k}{\eta_{permeate}} \tag{50}$$

where $\eta_{permeate}$ was taken as being the viscosity of the continuous phase of the concentrates. The relationship between $k_{relative}$ and $\Sigma(\phi_i)$ was independent of temperature, pH and preheat treatment of the original whole milk prior to separation.

Horne (1993) found that the shear thinning behaviour of fresh skim milk concentrates (made by thermal evaporation and concentrated up to 6.22-fold) could be described by the Bingham equation. Suitable manipulation of η_{pl} data for low concentrations (<1.5-fold) gave a value of ϕ_{cas} of 3.85×10^{-3} m^3 kg^{-1}, a figure close to the values of 3.57×10^{-3} and 3.9×10^{-3} given by Walstra and Jenness (1984) and Snoeren *et al.* (1982), respectively.

Horne (1993) treated skim milk concentrates as dispersions of casein micelles in a continuous phase. He found by further manipulation of his data that the viscosity of the continuous phase was numerically the same as that of a 4% lactose solution. η_{pl} expressed as viscosity relative to this continuous phase viscosity was uniquely related to ϕ_{cas} when ϕ_{max} was taken as 0.66 ±0.02. (The volume fraction of the whey proteins was apparently not considered explicitly.)

Yield stress (τ_0) was found to increase steeply with ϕ_{cas} above 0.3 and to scale with ϕ_{cas} to the power 4.1. The value of this exponent was close to the value of 3.85 exhibited by weakly flocculated latex suspensions, implying that concentrated milks are themselves weakly flocculated suspensions. Horne

(1993) showed that the strength of inter-micellar attractions can be calculated from the measured yield stress.

15.8.5.3. Phenomenological Description of Time-Dependent Behaviour

Milk concentrates can exhibit two forms of time-dependent rheological behaviour: thixotropy (the decrease in viscosity with time at constant shear rate) and age thickening (the increase in viscosity with storage time). These can be directly related: age thickening is caused by gradual structure development which at a certain point starts to result in thixotropic behaviour, the time-dependent breakdown of developed structure when the age-thickened concentrate is sheared.

When concentrates age thicken, the Herschel–Bulkley equation *per se* or the Bingham equation has been found to describe adequately instantaneous flow curves (Bienvenue *et al*., 2003a, b; Binh Trinh *et al*., 2007a, b). Bienvenue *et al*. (2003a, b) found that the Bingham equation described the flow behaviour of 45 %TS evaporated skim milk concentrates containing different levels of soluble minerals or different genetic variants of β-lactoglobulin, while Binh Trinh *et al*. (2007a, b) successfully applied the Herschel–Bulkley equation to reconstituted whole milks of $\geq$40 %TS (the rheological properties of which they had demonstrated to be essentially the same as those of fresh concentrates).

The rate of viscosity increase during age thickening can be quantified in terms of the rate of evolution with time of the flow equation constants, or the rate of increase with time in apparent viscosity, which at a given time is a function of these constants (Vélez-Ruiz and Barbosa-Cánovas, 1997; Bienvenue *et al*., 2003a, b; Binh Trinh *et al*., 2007a).

Binh Trinh *et al*. (2007a) developed a novel way of analysing the so-called hysteresis loop area (the area between the increasing shear rate curve and the subsequent decreasing shear rate curve on a plot of shear stress versus shear rate, obtained using the shear sweep function on a controlled shear stress rheometer). This enabled the evolution of thixotropy with time in reconstituted whole milks during age thickening to be quantitatively modelled in a way that was independent of experimental limitations.

15.8.6. Rheometry

Rheological properties can be measured with a wide range of proprietary viscometers and rheometers, varying in sophistication and versatility (McCarthy, 2006). Many instruments are equipped with computerized control, data acquisition and data analysis, allowing rapid and accurate measurement of rheological properties.

15.9. Thermal Properties

15.9.1. Specific Heat Capacity and Enthalpy

The specific heat capacity of a material is defined as the quantity of thermal energy required to raise the temperature of unit mass of the material by 1°C; its units are J kg^{-1} K^{-1}. In what follows, 'specific heat capacity' means specific heat capacity at constant pressure, rather than at constant volume; the distinction between the two is not of practical importance for solids, or for liquids outside the critical region, and in most practical situations isobaric conditions exist.

As the specific heat capacity of fluid milk products is temperature dependent, the amount of heat energy to be added or removed to effect a given temperature change is given by the equation

$$q = \int_{\theta_1}^{\theta_2} c(\theta)\mathrm{d}\theta \qquad (\mathrm{Jkg}^{-1}) \tag{51}$$

where q is the quantity of heat energy added or removed per kg, θ is temperature (°C), $\theta_2 - \theta_1$ is temperature change (θ_1 to θ_2 or θ_2 to θ_1) brought about by adding or removing q and $c(\theta)$ is specific heat capacity expressed as some function of temperature (J kg^{-1} K^{-1}).

Under isobaric conditions and in the absence of phase transitions, chemical reactions, changes in composition and forms of work other than displacement work, the heat added to or removed from a material is equal to the change in enthalpy of the material (Smith and Van Ness, 1987; Figura and Teixeira, 2007). Thus

$$\Delta h = \int_{\theta_1}^{\theta_2} c(\theta)\mathrm{d}\theta \qquad (\mathrm{Jkg}^{-1}) \tag{52}$$

where Δh is the enthalpy change per unit mass (J kg^{-1}).

Absolute values of enthalpy cannot be determined. However, Equation 52 may be used to calculate enthalpy, h, at any temperature, θ_2, relative to an arbitrarily assumed value of $h = 0$ at temperature θ_1 (the datum temperature in this case).

Differentiation of Equation 52 gives

$$c(\theta) = \frac{dh}{d\theta}(\theta) \qquad (\mathrm{Jkg}^{-1}\mathrm{K}^{-1}) \tag{53}$$

Thus, the specific heat capacity at a given temperature is equal to the rate of change of enthalpy with temperature, at that temperature (Smith and Van Ness, 1987; Figura and Teixeira, 2007).

Specific heat is sensible heat, the addition or removal of which results in temperature change but no phase change. Latent heat, on the other hand, has to do with phase change but not temperature change; it is defined as the quantity of heat absorbed or released per unit mass when a pure material changes phase (solid↔liquid or liquid↔vapour) at constant temperature. For the conditions (apart from the absence of phase changes) under which Equation 52 is valid, the latent heat of fusion, L, is equal to the enthalpy change accompanying the phase change solid↔liquid:

$$L = \Delta h \tag{54}$$

When a mixture of substances with different melting points (e.g., milk fat, a mixture of triacylglycerols) is heated or cooled within its melting point range, both sensible and latent heat must be added or removed to effect changes in temperature. A measured specific heat capacity thus includes both latent and sensible heat requirements and must, therefore, be termed an apparent specific heat capacity. In this case, Equation 52 is written as

$$\Delta h = \int_{\theta_1}^{\theta_2} c_{app}(\theta)\mathrm{d}\theta \qquad (\mathrm{Jkg}^{-1}) \tag{55}$$

where $c_{app}(\theta)$ is apparent specific heat capacity expressed as some function of temperature (J kg^{-1} K^{-1}). This equation is valid for any temperature interval lying above the freezing point and any interval whose lower limit is the freezing point.

The apparent specific heat capacity of milk fat is markedly temperature dependent at temperatures <40°C because of latent heat of fusion effects. A pronounced maximum is exhibited in the range 15–20°C, and a secondary maximum or inflection at about 30°C. The apparent specific heat capacity decreases to relatively low values at temperatures <0 and >40°C (Riedel, 1955); Phipps, 1957). The precise shape of the apparent specific heat capacity–temperature curve depends on the milk fat solid–liquid ratio that existed at each temperature during measurement. This, in turn, depends on fat composition (proportions of different triacylglycerols) and on the thermal history of the fat sample (Riedel, 1955; Phipps, 1957; Sherbon, 1988). Representative values of c_{app} for summer milk fat are shown in Table 15.6.

Table 15.6. Effect of temperature on the apparent specific heat of summer milk fat[a]

Temperature (°C)	c_{app} (J kg^{-1} K^{-1})
−20	~2090
0	~3340
15	~5430
25	~2930
30	~3760
40	~2090

[a]Data from Riedel (1955).

In contrast to that of milk fat, the specific heat capacity of the non-fat part of whole milk and cream at temperatures greater than the product's freezing point includes no latent heat requirement. The composition of this non-fat part is approximately that of skim milk, the specific heat capacity of which increases essentially linearly but slowly with temperature over a wide range (Phipps, 1957; Fernández-Martin, 1972a). Representative values for skim milk are 3899 and 3988 J kg^{-1} K^{-1} at 0 and 50°C, respectively (Phipps, 1957).

It follows from the above that the specific heat capacity of whole milk and cream must be termed apparent specific heat capacity at temperatures <40°C. The fat content and thermal history of the product, and fat composition, determine precisely how the apparent specific heat capacity varies with temperature. The apparent specific heat capacity of whole milk exhibits a maximum in the temperature range 15–20°C (Phipps, 1957; Fernández-Martin, 1972a), a minimum at ~40°C and a gradual increase with temperature above 40°C. The picture is similar for creams, but the maximum for high-fat creams is higher than that for milk because of the large latent heat contribution from the milk fat. At temperatures away from the temperature of the maximum, apparent specific heat capacity is inversely related to fat content, and, for concentrated milks and creams, to %TS (Phipps, 1957; Rambke and Konrad, 1970; Fernández-Martin, 1972a).

Phipps (1957) showed that the apparent specific heat capacity of cream measured under otherwise standardized conditions depends on the degree of dispersion of the milk fat (as resulting from homogenization treatments), as this influences the crystallization/melting behaviour of the fat.

At temperatures >40°C, milk fat is completely molten and no latent heat effects occur during heating or cooling of milk and cream. Fernández-Martin (1972a) measured the specific heat capacity of skim milk, half-skim milk and whole milk, and concentrates (≤30% TS) of these, over the

temperature range 5–70°C. He developed from his data the following empirical equation relating specific heat capacity to composition and temperature for the temperature range $40 < \theta < 70$°C:

$$c = 41.8\,W + (13.71 + 0.1129\theta)TS \qquad (\text{Jkg}^{-1}\text{K}^{-1}) \qquad (56)$$

The equation is valid for the %TS range $8 < \text{TS} < 40\%$ and the fat/SNF ratio range $0.01 < \text{fat/SNF} < 0.04$. Fernández-Martin (1972a) pointed out uncertainties in the results of a similar study carried out by Rambke and Konrad (1970).

For each of his milk preparations, Fernández-Martin (1972a) calculated enthalpy relative to an arbitrarily assumed value of $h = 0$ J kg^{-1} at 5°C, at each of several temperatures in the overall range $5 < \theta < 70$°C, by graphical integration of the experimentally determined specific heat capacity–temperature plot. (This operation was equivalent to integrating the right-hand side of Equation 55 with θ_1 and θ_2 equal to 5 and 80°C, respectively, and with $c_{app}(\theta)$ in graphical rather than algebraic form.) Linear regression of the enthalpy–temperature data yielded an average apparent specific heat capacity ($= dh/d\theta$ of Equation 53) for each preparation for the temperature range 5–70°C. The results were presented as a nomogram (stated to be valid for the temperature range 0–80°C) allowing average apparent specific heat capacity to be found for milks and concentrated milks with fat and SNF contents in the ranges $0 < \text{F} < 12\%$ and $0 < \text{SNF} < 43\%$, respectively.

Bertsch (1982) measured the specific heat capacity of skim milk and whole milk (4% fat) over the temperature ranges 52–134°C and 53–143°C, respectively. Specific heat was empirically related to temperature by the following equations:

$$\text{Skim milk}: \quad c = 2.814\theta + 3942 \qquad (\text{Jkg}^{-1}\text{K}^{-1}) \qquad (57)$$

$$\text{Whole milk}: \quad c = 2.976\theta + 3692 \qquad (\text{Jkg}^{-1}\text{K}^{-1}) \qquad (58)$$

Both equations had mean relative errors of about 4% ($p = 0.05$). Bertsch (1982) showed that his data were in good agreement with those of a number of previous workers (including those of Fernández-Martin, 1972a) in the temperature range 52–80°C.

The ability to predict the enthalpy change between two chosen temperatures is, from a technological or engineering point of view, more useful than the ability to predict specific heat capacity at specific temperatures. An equation for predicting enthalpy change can be derived easily by inserting an appropriate form of $c(\theta)$ into Equation 52, or of $c_{app}(\theta)$ into Equation 55, and integrating. For example, taking the right-hand side of Equation 57 as

$c(\theta)$ in Equation 52 gives the following expression for enthalpy change in skim milk (valid for the temperature range 52–134°C):

$$\Delta h_{\theta_1 \leftrightarrow \theta_2} = 1.407\theta_2^2 - 1.407\theta_1^2 + 3824(\theta_2 - \theta_1) \ (\mathrm{Jkg}^{-1}) \tag{59}$$

Similar expressions (valid for temperatures >40°C) could be obtained by taking the right-hand sides of Equations 56 and 58 as $c(\theta)$.

For whole milk and cream (unconcentrated or concentrated, and at temperatures above or below 40°C, but above the product's freezing point), enthalpy change can be predicted using the following general expression (Miles *et al*., 1983):

$$\Delta h_{\theta_1 \leftrightarrow \theta_2} = \Delta h_{fat} x_{fat} + \Delta h_{non-fat} x_{non-fat} \tag{60}$$

where Δh_{fat} is the enthalpy change (J kg^{-1}) in milk fat between θ_1 and θ_2, $\Delta h_{non\text{-}fat}$ is the enthalpy change in the non-fat portion of the product between θ_1 and θ_2, x_{fat} is the fraction of milk fat in the product and $x_{non\text{-}fat}$ is the mass fraction of the non-fat portion. Δh_{fat} must be found from empirical data; Riedel (1955), for example, tabulated the enthalpies of summer and winter milk fat at temperatures in the range −50 ($h = 0$) to 55°C.

$\Delta h_{non\text{-}fat}$ can be predicted from

$$\Delta h_{milk\ plasma} = \int_{\theta_1}^{\theta_2} \Sigma(c_i x_i) d\theta = \Sigma(c_i x_i)(\theta_1 - \theta_2) \tag{61}$$

where c_i is the specific heat of the ith component, x_i is the mass fraction of the ith component and $\Sigma(c_i x_i)$ is the specific heat of milk plasma. Suitable values of c_i for water, lactose, protein and ash are 4200, 1400, 1600 and 800 J kg^{-1} K^{-1}, respectively (Kessler, 1981). The slight temperature dependence of the specific heat capacities of the non-fat components may, for practical purposes, usually be ignored.

The calculation of specific heat as $\Sigma(c_i\ x_i)$, which embodies the principle of additivity of specific heats, can be applied to whole milk and cream at temperatures >40°C (milk fat completely liquid; no latent heat effects) by including the term $c_{fat}x_{fat}$ in $\Sigma(c_i\ x_i)$. A suitable value of c_{fat} is 2174 J kg^{-1} K^{-1} (Sherbon, 1988). In applying the principle of additivity it is assumed implicitly that there are no interactions between the different components of the mixture that affect the components' individual specific heat capacities; this assumption is considered valid for the non-fat part of milk or cream (Phipps, 1957) and is thus almost certainly valid for the whole product.

In calculating enthalpy changes over a temperature range that straddles or lies below the freezing point of milk or cream, it is necessary to account for latent heat effects caused by phase change in the water substance (water or ice) as well as in the milk fat. Miles *et al.* (1983) describe methods for doing this.

Enthalpy data obtained by direct measurement are available. Riedel (1976) presented extensive carefully measured data on the enthalpy (over the temperature range −60 to 80°C, relative to $h = 0$ at −60°C) of mixtures of dried whole milk (10% (w/w) fat in the dry matter) and water, mixtures of dried skim milk and water, and mixtures of skim milk and milk fat. He also presented data on the temperature dependence of the apparent specific heat capacity of selected mixtures, obtained by differentiating his enthalpy data at temperatures over the measurement range (as in Equation 53, but with $c(\theta) = c_{app}(\theta)$).

15.9.2. Thermal Conductivity

The property thermal conductivity (λ) is a measure of a material's ability to conduct heat unidirectionally. It is defined by the equation

$$\lambda = \frac{\phi d}{A\Delta\theta} \qquad (\mathrm{W m^{-1} K^{-1}}) \tag{62}$$

where ϕ is the rate of heat transfer (W), d is the distance (m) in the direction of heat flow, A is the area (m^2) for heat transfer perpendicular to the direction of heat flow and $\Delta\theta$ is the temperature drop, in the direction of heat flow, over the distance d(K). The thermal conductivity of skim milk, whole milk and cream increases slowly with increasing temperature. It decreases with increasing %TS or increasing fat content, the effects being generally greater at higher temperature (Konrad and Rambke, 1971; Fernández-Martin and Montes, 1972, 1977). Representative values of the thermal conductivity of water and fluid milk products at 20°C are shown in Table 15.7.

Table 15.7. Representative values of the thermal conductivity of water and fluid milk products at 20°C[a]

Water or milk product	Thermal conductivity ($\mathrm{W\ m^{-1}\ K^{-1}}$)
Water	~0.603
Skim milk (0.1% fat)	~0.568
Whole milk (2.9% fat)	~0.559
Cream (30.7% fat)	~0.384

[a]Data from Fernández-Martin and Montes (1972, 1977) and Cooper and Le Fevre (1979).

Gustavsson and Gustafsson (2006) described a thermal conductivity probe which they showed had potential for rapidly and accurately indicating the fat content of milk; their measurements showed that the thermal conductivity of retail milk samples with a fat content in the range 0.5–3.0% fat was linearly (and inversely) dependent on fat content. The thermal conductivity of a milk sample with less than 1% fat was, however, greater than indicated by this linear relationship.

Fernández-Martin and Montes (1972) established an empirical quadratic relationship between thermal conductivity and temperature for each of the unconcentrated and concentrated skim milks, half-skim milks and whole milks they studied. They established six-parameter relationships between thermal conductivity and both temperature and %TS for each of the three milk types. They were able to correlate all of their experimental data to an accuracy of ±3% by a single 18-parameter equation relating thermal conductivity to temperature, percentage fat content and percentage SNF content.

Cuevas and Cheryan (1978) give the following empirical relationship for predicting the thermal conductivity of foods:

$$\lambda = (0.565 + 0.00180\theta - 0.0000058\theta^2)(1 - 0.005TS) \qquad (\mathrm{Wm^{-1}K^{-1}}) \quad (63)$$

For milk products, TS may be calculated as

$$TS = (10 + 2F)R \qquad (64)$$

where F is the fat content (%, w/w) and R is the concentration factor. Equations 63 and 64 were developed by Riedel (1949). Cuevas and Cheryan (1978) examined the prediction accuracy of Equation 63 and three other temperature-dependent equations for food thermal conductivity (including the 18-parameter equation of Fernández-Martin and Montes, 1972) by comparing predicted values with experimental data available in the literature. The data used related to 158 food samples, about 70% of which were dairy products. Equation 63 gave the lowest standard error, while the equation of Fernández-Martin and Montes (1972) gave the next lowest. Interestingly, the prediction accuracy of Equation 63 did not improve when TS in the equation was substituted by Equation 64 and the equation applied only to dairy products.

More and Prasad (1988) measured the thermal conductivity of thermally concentrated whole milk and developed the following form of Equation 63 from their data:

$$\lambda = (0.59 + 0.0012\theta)(1 - 0.0078TS) \qquad (65)$$

The equation is valid for the temperature range $40 < \theta < 90°C$ and the %TS range $37 < TS < 72\%$.

The equations so far mentioned for predicting the thermal conductivity of milk products are semi-empirical (Equations 63 and 65) or empirical. Fernández-Martin and Montes (1977) attempted to take a fundamental approach. They compared their experimentally determined thermal conductivities of creams with nine different theoretical equations for predicting the thermal conductivity of two-phase systems in which the dispersed phase consists of spherical particles. Agreement between experimental and predicted values was good for $\phi_{fat} < 0.2$ for all but one of the theoretical models. Agreement was more variable for $\phi_{fat} > 0.2$. Fernández-Martin and Montes (1977) chose one of the theoretical equations and, by using their data and suitable regression methods, adjusted its constants to yield the following semi-empirical relationship:

$$\lambda = (0.5279 + 0.0021\theta - 0.00000732\theta^2)(1 - (0.843 + 0.0019\theta)\phi_{fat}) \quad (\mathrm{Wm^{-1}K^{-1}}) \tag{66}$$

This equation, which is valid for fat contents in the range $20 < F < 40\%$ and the temperature range $5 < \theta < 75°C$, fitted the experimental data to within 10%. It is not dissimilar to Equation 63. When $\phi_{fat} = 0$, Equation 66 reduces to the empirical quadratic equation relating the thermal conductivity of skim milk to temperature that was developed by Fernández-Martin and Montes (1972).

15.9.3. Thermal Diffusivity

The property thermal diffusivity is the ratio of thermal conductivity to volumetric specific heat. It is defined by the equation

$$\alpha = \frac{\lambda}{\rho c} \tag{67}$$

where α is thermal diffusivity ($m^2\ s^{-1}$), λ is thermal conductivity ($W\ m^{-1}\ K^{-1}$), ρ is density ($kg\ m^{-3}$), c is specific heat capacity ($J\ kg^{-1}\ K^{-1}$) and ρc is volumetric specific heat ($J\ m^{-3}\ K^{-1}$). The thermal diffusivity of a material is a measure of the rate of temperature propagation that occurs in the material during unsteady state heating or cooling; it is a measure of the ability of the material to dissipate temperature gradients within itself.

Kostaropoulos *et al.* (1975) gave the following representative values of the thermal diffusivity of fluid milk products:

Milks in general: 1.25×10^{-7} m^2 s^{-1} in the temperature range 15–20°C.
Milk and cream: 9.7×10^{-8} m^2 s^{-1} in the temperature range 0–22°C.

Thermal diffusivity can be measured directly; alternatively, a value can be calculated readily using Equation 67 and appropriate values of thermal conductivity, density and specific heat capacity.

15.9.4. Measurement of Thermal Properties

The accurate measurement of the thermal properties of foods requires careful design and operation of suitable equipment, which is often purpose-built. Proprietary equipment is available for some types of measurement; differential scanning calorimetry (DSC) is an example. DSC, a form of differential thermal analysis (DTA), is used to investigate phase changes in foods. The measurements obtained can be converted to enthalpy–temperature data (Lewis, 1987). DTA and calorimetry are discussed in detail by Wunderlich (1990). Measurement of the thermal properties of foods is discussed and reviewed by Ohlsson (1983), Lewis (1987), Rahman (1995), Sahin and Sumnu (2006) and Figura and Teixeira (2007).

15.10. Electrical and Dielectric Properties

15.10.1. Electrical Conductivity

Electrical conductivity (EC), σ, is a measure of a material's ability to carry an electrical current. It ranges in value from 10^{-18} to 10^{7} S m^{-1}, depending on the material. The conductivity of aqueous systems such as milk lies between these extremes (Mabrook and Petty, 2003). The EC of normal whole milk is about 0.460 S m^{-1} (Hogeveen and Ouweltjes, 2003).

EC is easily measured by applying a known voltage across a pair of parallel electrodes immersed in the sample, measuring the current produced, and calculating the resistance of the specimen (the volume bounded by the electrodes):

$$\sigma = \frac{1}{R}.\frac{l}{A} = G.\frac{l}{A} \qquad (\mathrm{Sm}^{-1}) \tag{68}$$

where R = resistance (Ω);

G = conductance (S);
l = distance between the electrodes (m);
A = electrode area (m^2).

Equation 68 shows that EC and electrical conductance (the reciprocal of resistance) are related *via* the specimen dimensions. In the dairy science literature, values of conductance rather than conductivity are sometimes reported. For a fixed measurement system, these two properties vary in an identical way with influencing factors such as temperature and milk composition.

In EC measurements, an AC rather than a DC voltage is generally applied to the sample in order to avoid electrolysis and consequent polarization of the electrodes. Both the in-phase current (which is related to the conductance, G) and the out-of-phase current (which is related to the capacitance) are measured over a range of frequencies using an impedance analyser. This technique is known as impedance (or admittance) spectroscopy (Mabrook and Petty, 2003). Impedance and admittance (the reciprocal of impedance) are complex properties whose real parts are, respectively, resistance and conductance.

At frequencies lower than 10 kHz, the conductance of milk and cream as measured increases rapidly with frequency, owing to electrode polarization effects, but reaches a constant saturation value at a frequency of 10 kHz. At and above this frequency, the measured value is a property of the bulk milk and not of the milk–electrode interface (Lawton and Pethig, 1993; Mabrook and Petty, 2003).

Mabrook and Petty (2003) used impedance spectroscopy to measure the conductance of raw milk and pasteurized retail samples of full-fat homogenized milk, semi-skim milk, skim milk and lactose-reduced milk. They reported the values of conductance at 100 kHz and 8°C (and for compositions as declared on product labels) shown in Table 15.8.

While these values of conductance are specific to the electrode system of Mabrook and Petty (2003), they show exactly the same dependence on composition as would the corresponding values of EC *per se*. The data for

Table 15.8. Electrical conductance of various milk products at 100 kHz, 8°C and their composition as declared on product labels[a]

Milk	Conductance (mS)
Raw full-fat milk (3.6% fat, lactose content unknown)	4.85 mS
Pasteurized full-fat milk (3.6% fat, 4.9% lactose)	5.05
Semi-skim milk (1.6% fat, 4.9% lactose)	5.23
Skim milk (0.1% fat, 4.9% lactose)	5.4
Lactose-reduced milk (3.6% fat, 0% lactose	5

[a]Data from Mabrook and Petty (2003)

the full-fat milks show that pasteurization and homogenization appeared to cause an increase in conductance. This result may have been due to milk batch to batch variation; homogenization has been shown not to change EC (Prentice, 1962), and it has been shown that variation in the fat globule size distribution in milks and creams with a fat content ranging from 1 to 35% (w/w) has no effect on EC (Robin *et al.*, 1994).

By measuring also the conductance of NaCl, KCl, MgCl and sodium caseinate solutions, Mabrook and Petty (2003) showed that milk EC is determined mainly by the charged species present in milk, particularly the salts. There is very little contribution from lactose. Casein, also, makes a much smaller contribution than the milk salts; the main effect of milk proteins in general is to hinder the migration of ions and thus depress EC (Haman and Zecconi, 1998). However, under certain conditions the release of calcium ions from the casein micelles can result in an increase in EC. Mabrook and Petty (2003) found that the conductance of pasteurized full-fat milk increased by about 15% when the milk was left at room temperature for 48 h. A similar increase occurred when the milk was acidified to a pH of 4.9–5.0, suggesting that the first effect could have been due to a decrease in pH caused by microbial activity. Żywica and Budny (2000) showed by measurements at 20 kHz that the decrease in the impedance (which is related to a decrease in the resistance and thus an increase in the conductance) of whole milk and skim milk during storage at 20°C was closely paralleled by decreases in pH. For both milk types, impedance dropped to a steady value at the final steady pH of about 4.4.

A decrease in milk pH to about 5 causes all of the colloidal calcium phosphate to dissolve and the equilibria of milk buffer systems to change, resulting in saturation of the conductance to a constant maximum value (Mabrook and Petty, 2003). This phenomenon is the basis of the automatic monitoring of the growth of lactic acid bacteria by conductimetric methods (Mucchetti *et al.*, 1994).

Mastitis in a quarter of a cow's udder results in decreases in the concentrations of lactose and K^+ in the milk secreted and corresponding increases in the concentrations of Na^+ and Cl^- that preserve the milk's iso-osmolality with the cow's blood (Haman and Zecconi, 1998). The net effect is an increase in the EC of milk. This phenomenon has led to much research over the last 60 years aimed at finding a reliable way of using in-line EC measurement at milking to detect both subclinical and clinical mastitis in the quarters of individual cows (Norberg, 2005). Although the EC of milk from individual quarters can be monitored easily and accurately, achieving this aim has been hampered by the fact that variation in EC depends not only on the level of infection but also on numerous other factors such as breed, parity,

oestrus, lactation stage, the presence of other diseases, milking interval, time of day and milk composition (Norberg *et al.*, 2004; Norberg, 2005). In 1998, an extensive analysis of published data carried out by the International Dairy Federation (Haman and Zecconi, 1998) concluded that EC measurement could not identify mastitic quarters or cows, or detect subclinical mastitis, with sufficient accuracy to be useful. Currently, most automatic milking systems and some manual systems incorporate sensors for measuring the EC of quarter milk and software for processing the data generated (Norberg, 2005). The development of sophisticated statistical modelling of the data, which involves comparisons between the quarters of the individual cow over successive milkings, has led to an improvement in the sensitivity of detection of subclinical and clinical mastitis. In the case of automatic milking systems the farmer is dependent on EC records for detecting mastitic cows (Norberg, 2005).

Several studies have indicated that there is a correlation between the EC of milk and the genetic susceptibility of cows to mastitis. Much further investigation will be required to determine whether or not this correlation will be useful in breeding programmes that include selection for resistance to mastitis (Norberg, 2005).

The EC of fresh milk and cream decreases with increasing fat content because the fat globules (themselves non-conducting) occupy volume that would otherwise be filled with the conducting aqueous phase of the product, impeding the mobility of the conducting ions and increasing the distance that migrating ions have to travel (Prentice, 1962; Fernández-Martin and Sanz, 1985; Mabrook and Petty, 2003). Prentice (1962) determined the following empirical equation relating EC to the volume fraction of fat in milk, for fat contents of 2–6.1% (w/w) at room temperature (unspecified):

$$\sigma = \sigma_s(1 - \phi_{fat})^{1.5} \tag{69}$$

where

σ_s = electrical conductivity of skim milk prepared from the same batch of milk;
ϕ_{fat} = volume fraction of fat in the milk.

A more recent study of the EC of milks, creams and model dairy emulsions (Robin *et al.*, 1994) found that 'the presence of ... fat globules reduced the conductivity of the milks and creams approximately by a power of 3/2 [=1.5] of the dispersed fat fraction present', thus confirming the result of Prentice (1962).

Lawton and Pethig (1993) developed a relationship similar to Equation 69 for fat contents, F, of up to 7% (w/w) at 20°C:

$$\sigma = \sigma_s(1 - F)^{1.74} \tag{70}$$

The data of Mabrook and Petty (2003) are in good agreement with this equation.

Lawton and Pethig (1993) developed the following more complex relationship for the F range of 0.15–51% (w/w):

$$\sigma = \sigma_s(1 - F)^{\beta}(1 + pF^2)^{\beta} \tag{71}$$

Over the 12 month period during which Lawton and Pethig (1993) conducted their study, the value of β varied between 1.67 and 1.75, while p varied between 0.3 and 0.35, depending on the batch of milk from which samples of different fat content were prepared. β tended to be directly proportional to the general EC level of the batch, probably owing to variability among batches in the levels of mineral and solids-not-fat.

Using appropriate values of density, Lawton and Pethig (1993) converted Equation 71 into the following form to allow comparison with Prentice's equation (Equation 69):

$$\sigma = \sigma_{skim}(1 - \phi_{fat})^{\delta}(1 + q\phi_{fat}^2)^{\delta} \tag{72}$$

They found δ to be 1.56 ± 0.04, and q to vary between 3.0 and 3.5 depending on the batch of milk. This value of δ suggests that Prentice's value of 1.5 requires a small but significant correction to account for variability in the ion and solids-not-fat contents of milk (Lawton and Pethig, 1993).

Mabrook and Petty (2002) showed that measurement of the admittance of milk has the potential to allow estimation of milk fat content if the water content of milk is known, and *vice versa*. The latter utility of the measurement could provide a means of detecting the adulteration of milk with water.

The EC of stored whole milk reaches a higher saturation value than that of stored skim milk, because for whole milk the EC-depressing effect of the presence of fat is more than compensated for by the production of free fatty acids and the release of phosphate ions from the milk fat globule membrane (Mabrook and Petty, 2003).

Fernández-Martin and Sanz (1985) developed the following empirical equation for predicting the electrical conductivity of a range of milks and milk concentrates containing various levels of fat and total solids:

$$\sigma = \{(B_0 + B_1 r) + (C_0 + C_1 r)TS + [(B_2 + C_2 s) + (B_3 + C_3 TS)r + (B_4 + C_4 TS)r^2]\theta\}TS \quad (\mathrm{S\ m^{-1}}) \tag{73}$$

where

r = ratio of fat content to SNF content (dimensionless);
TS = % total solids;
θ = temperature (°C);
B_i and C_i are regression coefficients.

Fernández-Martin and Sanz (1985) provided numerical values of B_i and C_i. They used the technique of impedance measurement to obtain their data and were aware of the high dependence of EC on frequency at frequencies of less than 10 kHz. However, they chose to carry out their measurements at a single frequency of 1 kHz, which is within the range in which electrode polarization effects could be expected to occur.

Fernández-Martin and Sanz (1985) used Equation 73 to construct a nomogram for predicting EC as a function of total solids and temperature.

The EC of milk is markedly dependent on temperature. Mabrook and Petty (2002, 2003) found that the conductance of whole milk increased linearly with temperature at a rate of about 5% per °C over the narrow temperature range of 4–9°C. Sharma and Roy (1976) determined the following empirical relationship between conductivity and temperature for buffalo milk, valid for the temperature range 5–70°C:

$$\sigma = 1.71 \times 10^{-1} + 6.32 \times 10^{-3}\theta + 9.01 \times 10^{-6}\theta^2 \quad (\mathrm{Sm^{-1}}) \tag{74}$$

This equation is a polynomial of the form stated by Prentice (1972) to be satisfactory for modelling the temperature dependence of milk EC. Prentice found that the temperature coefficient, α, of EC at a given temperature, defined as $\alpha = \frac{1}{\sigma}\frac{d\sigma}{d\theta}$, decreased with temperature; the decrease was 28.2% between 15 and 40°C. The temperature coefficient for cow's milk differs quantitatively but not qualitatively from that of buffalo milk Sharma and Roy (1976).

The EC and viscosity of milk have been shown to be related. This relationship is thought to be due to the fact that the ion content of milk affects both the EC and the conformation of milk proteins, the latter influencing viscosity (Rao *et al.*, 1989). Rao *et al.* (1989) found that the EC of whole buffalo milk was linearly related to the ratio of absolute temperature to relative viscosity (where relative viscosity was measured as the ratio of milk viscosity to the viscosity of water). The linear regression constants were found to depend on breed and time of milking (morning or evening). Fernández-

Martin and Sanz (1985) found that the following equation modelled the relationship between conductivity and viscosity for the range of milks and milk concentrates they investigated:

$$\sigma \times \eta^{(1-0.011TS)} = 2.038 - 0.230TS + 0.0127(TS)^2 \quad (\mathrm{Sm}^{-1}) \qquad (75)$$

where

η = coefficient of viscosity ((Pa s) $\times 10^{-3}$);
TS = percentage total solids (w/w).

The equation allowed EC to be calculated at a given temperature (in the range 15–60°C) when the viscosity at that temperature was known, for TS of up to about 30%. Presumably, the effect of fat content on conductivity was accounted for by its effect on viscosity. Fernández-Martin and Sanz (1985) discuss earlier attempts to relate EC to milk viscosity.

15.10.2. Dielectric Properties

The dielectric properties of a material, the permittivity (ε') and the dielectric loss factor (ε''), are the real and imaginary parts, respectively, of the complex permittivity (ε) (Nunes *et al.*, 2006):

$$\varepsilon(\omega) = \varepsilon'(\omega) - j\varepsilon''(\omega) \qquad (76)$$

where ω = frequency (Hz);

$j = (-1)^{1/2}$.

As Equation 76 indicates, these properties are frequency dependent. ε' is a measure of the ability of the material to store electromagnetic energy. ε'' is a measure of the material's ability to dissipate electromagnetic energy as heat. The latter phenomenon is exploited in microwave and radio frequency heating.

Representative values of the dielectric properties of milks with various fat contents are shown in Table 15.9, which includes values for pure water. At microwave-heating frequencies, the permittivity of skim milk is lower than that of water while the loss factor of skim milk is higher. Both ε' and ε'' decrease with increasing temperature at these frequencies (Mudgett *et al.*, 1974).

Nunes *et al.* (2006) showed that at room temperature (17–20°C) the ratio of the permittivity of UHT skim milk to water permittivity dropped from ~0.93 at 1 GHz to ~0.83 at 20 GHz, while the corresponding values for loss factor were ~2.8 and ~0.8. For UHT whole milk, the decreases were from 0.91 to 0.77 for permittivity and from 3.5 to 0.8 for loss factor.

Table 15.9. Representative values of the dielectric properties of milks with various fat content, and of water. ε' is the permittivity and ε'' is the dielectric loss factor

Sample	Temp (°C)	Frequency (GHz)	ε'	ε''	Source
Skim milk	25	3	*68	*18	Mudgett *et al.* (1974)
Skim milk	55	3	59	16	
1% fat milk	20	**2.45	70.6	17.6	Kudra *et al.* (1992)
2% fat milk	20	2.45	69.4	17.8	
3.25% fat milk	20	2.45	67.9	17.6	
Water	20	2.45	80.2	13.4	Lide and Frederikse (1996)

*ε' and ε'' are expressed as ratios of the actual values to the corresponding values for free space. The values shown are thus dimensionless quantities.
**This frequency is an internationally permitted ISM (industrial, scientific and medical) frequency. It is the frequency at which domestic microwave ovens normally operate.

Mudgett *et al.* (1974) found that, over the temperature range 25–55°C, experimentally determined values of the dielectric properties of skim milk agreed to within 5% with the theoretically predicted values for a 0.1 M solution of sodium chloride, provided that, in the case of the permittivity (ε'), a correction was made for the combined depressive effect of the non-ionic milk components casein and lactose. The required correction at 3 GHz and 25°C was −4.9.

The data of Kudra *et al.* (1992) in Table 15.9 show that for whole milk, at 2.45 GHz and 20°C, ε' was inversely related to fat content, while ε'' was insensitive to fat content. Thus, fat, an additional non-ionic component in whole milk, depresses the permittivity further.

Nunes *et al.* (2006) measured the frequency dependence of the permittivity and loss factor of retail samples of UHT skim, low-fat and whole milk over the frequency range 1–20 GHz at 20°C. At given frequencies, values read from the ε' versus frequency and ε'' versus frequency spectra presented by Nunes *et al.* (2006) are in accord with those shown in Table 15.9.

For all three milk types, permittivity decreased with increasing frequency. The loss factor, on the other hand, exhibited a net increase with frequency over the frequency range studied, but went through a minimum near the lower end and a maximum near the higher end. For the whole milk, permittivity dropped by ∼57%, and loss factor increased by ∼100%, between 1 and 20 GHz.

Nunes *et al.* (2006) also measured the dielectric property spectra of mixtures of whole milk and deionized distilled water, and of deionized distilled water itself. They modelled these spectra, and those of the three UHT milks, using the following theoretical equations:

$$\varepsilon'(\omega) = \varepsilon'_{\infty} + \frac{\varepsilon'_S - \varepsilon'_{\infty}}{1 + \omega^2\tau^2} \tag{77}$$

$$\varepsilon''(\omega) = \frac{(\varepsilon'_S - \varepsilon'_{\infty})(\omega t)}{1 + \omega^2\tau^2} + \frac{\sigma}{\omega\varepsilon_0} \tag{78}$$

where

ε'_{∞} = the real permittivity at very high frequency;
ε'_S = the real static (low-frequency) permittivity;
$\tau = 1/2\pi f_R$, where f_R is the relaxation frequency;
σ = electrical conductivity;
ε_0=the permittivity of free space=8.854×10^{-12} F m^{-1};
t = time.

Modelling showed that the complex permittivity of milk is essentially that of water, with perturbations arising from the ionic and non-ionic milk components. Analysis of the spectral data relating to permittivity (ε'_S, ε'_{∞} and τ) and those relating to loss factor ($(\varepsilon'_S - \varepsilon'_{\infty})$, τ and σ), for water and for all of the milks and diluted milks investigated, showed that perturbations are sensitive to the presence of fat, carbohydrate, protein and ionic species. For example, Nunes *et al.* (2006) state that the measurement of ε'_{∞} and ε'_S alone should be enough to allow an estimate of the fat to protein-plus-lactose ratio. They suggested that measurement of dielectric property spectra could potentially be useful in monitoring the gross composition of large quantities of milk in real time.

15.11. Acoustic Properties

Acoustics is the science of sound (mechanical vibrations at frequencies in the range 1.6×10^{-2} to 20 kHz, which are detectable by the human ear), infrasound (frequencies of <0.016 kHz) and ultrasound (frequencies of >20 kHz) (Kuttruff, 2007). Only ultrasound is of significance in dairy science and technology.

The following account of acoustic properties in the ultrasound region, and their significance with respect to milk and cream, is based on McClements (1995, 1997, 1998) and Povey (1998). Other sources of information are cited appropriately.

Ultrasound can be applied to milk and other foods in two ways: as low-intensity ultrasound and as high-intensity ultrasound. The former, which involves power levels of <1 W cm^{-2} is non-destructive and is used principally as an analytical tool for indirectly measuring food characteristics and as an

in-line method of measuring flow rate. The latter, with power levels in the range 10–1000 W cm^{-2}, is used as a processing technique deliberately to cause changes in food materials. Only low-intensity ultrasound is considered here, as high-intensity ultrasound does not at present appear to be of relevance to the processing of milk. The potential in food processing generally of high-intensity ultrasound is reviewed by Knorr *et al.* (2004) and Mason *et al.* (2005).

The ultrasonic properties of a material most frequently measured are the ultrasonic velocity, the attenuation coefficient and the acoustic impedance. These depend on other physical properties of the material.

The ultrasonic velocity (the distance travelled by an ultrasonic wave in unit time) is related to the elastic modulus, E, and density, ρ:

$$\left(\frac{k}{\omega}\right) = \frac{\rho}{E} \tag{79}$$

where

ω = angular frequency (rad s^{-1});
k = the complex wave number $= \frac{\omega}{c} + i\alpha$;
c = ultrasonic velocity (m s^{-1});
α = attenuation coefficient (Np m^{-1});
$i = \sqrt{-1}$.

For low-attenuating materials (i.e., $\alpha << \omega/c$), Equation 79 can be written as

$$\frac{1}{c^2} = \frac{\rho}{E} \tag{80}$$

where

ρ = density (kg m^{-3});
E = modulus of elasticity (Pa). In the case of milk, E is the bulk modulus, as liquids are normally subjected to a compressive (as opposed to a shearing) ultrasonic wave.

The ultrasonic velocity is measured either by measuring the wavelength at a known frequency ($c = \lambda f$, where λ = wavelength and f = frequency) or by measuring the time for a wave to travel a known distance d ($c = d/t$).

The attenuation coefficient, α, is a measure of the decrease in amplitude of an ultrasound wave as it travels through the material. It is given by the equation

$$A = A_0 e^{-\alpha x} \tag{81}$$

where A_0 is the initial amplitude of the wave and A is the amplitude after the wave has travelled a distance x through the material. Attenuation is the result of absorption and scattering.

The acoustic impedance is a measure of the fraction of an ultrasonic wave that is reflected from a material's surface. The (complex) specific acoustic impedance, Z, is defined as

$$Z = \frac{\omega\rho}{k} \tag{82}$$

where

$Z = R_Z + iX_Z$;
R_Z = the resistive (real) part of the complex impedance;
X_Z = the reactive (imaginary) part.

For low-attenuation materials (i.e., $\alpha << \omega/c$), Equation 82 can be written as

$$Z = R_Z = \rho c \tag{83}$$

Values of the three ultrasonic properties for skim milk (at 1 mHz and 28°C) are

Velocity, $c = 1522$ m s^{-1};
Attenuation coefficient, $\alpha = 23$ Np m^{-1};
Impedance, $Z = 1.5 \times 10^{-6}$ kg m s^{-1}.

Ultrasonic properties are most commonly measured using the pulse-echo technique. An ultrasonic pulse of set frequency and amplitude is applied to one side of the sample. This propagates through the sample and is partly reflected from the opposite side of the sample-holding cell. Successive echoes are detected and recorded. The velocity and attenuation coefficient (and hence the impedance) are determined by measuring the time interval between echoes and the echoes' amplitudes. These properties are frequency dependent; enhanced information about the characteristics of the sample can be gained by carrying out measurements over a range of frequencies and also over a range of temperatures.

The ultrasonic properties of milk are fundamental properties, but they are not of interest in themselves. Their measurement is useful only in so far as it is possible to establish relationships between them and other physico-chemical characteristics such as composition, structure and state – characteristics which determine how ultrasound interacts with the sample. Such relationships can be developed empirically by means of calibration experiments, or theoretically. The theoretical approach is conceptually better for complex multiphase materials such as milk, but is made difficult by the fact that a large amount of data is

needed on the thermophysical properties (e.g., density, viscosity, specific heat capacity, thermal conductivity and coefficient of thermal expansion) of the component phases.

Ultrasound has been used to measure the composition of milk in terms of fat and solids-not-fat (Fitzgerald *et al.*, 1961; Winder *et al.*, 1961; Moy and Winder, 1971), the milk fat globule size distribution, the rate of coagulation of milk by chymosin, and the non-invasive and non-destructive detection of microbial spoilage of packaged milk. Savaroglu and Aral (2007) showed that measurement of ultrasonic velocity and density can be used to detect the presence in milk of chemical additives such as sodium bicarbonate and hydrogen peroxide, which are used in some parts of the world to prolong the shelf life of informally sold milk. The measurement of creaming profiles, the principle of which is the measurement of the ultrasound velocity and/or attenuation coefficient as a function of sample height and time, has obvious potential for milk and other liquid dairy products.

As well as being non-invasive and non-destructive, ultrasound can be used to analyse opaque and concentrated systems and is inexpensive, rapid and accurate.

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