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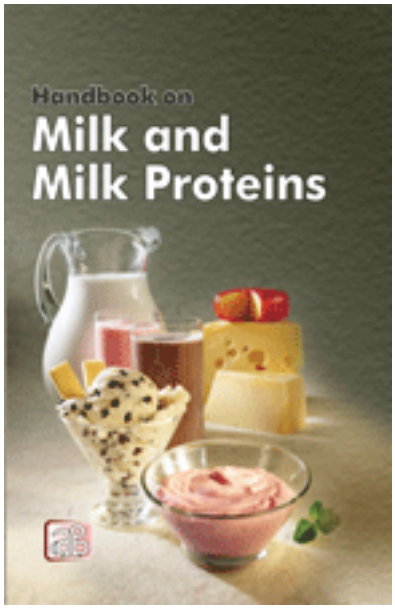
Handbook on Milk and Milk Proteins



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Proteins play an important role in nutrition, taste, allergies, texture, structure, processing and yield performance. In the food industry, proteins are a key element of our diet and an important ingredient for food technologists. The total protein component of milk is composed of numerous specific proteins. Isolated milk protein products represent an important and valuable source of protein ingredients due to their recognized superior nutritional, organoleptic and functional properties. Milk protein is a rich source of essential amino acids and they have been the subject of intensive research for an effort to unravel their molecular structure and interactions, relationship between structure and functional attributes, interactions of proteins during processing and, more recently, their physiological functions. Free fatty acids (FFA) in fresh milk normally amount to less than 1% of the total milk fat, yet they are important because of their effect on milk flavour. Now a day, the processing of milk is part of a highly organized and controlled dairy industry, which produces and markets a multitude of dairy products. Functional milk proteins are perfectly suited for use in the dairy sector of food production and the modern food processing industry is placing more and more emphasis upon the utilization of protein ingredients to provide specific functional properties to a wide range of formulated foods. In recent years, there has been a great deal of progress in the understanding and management of milk proteins across the production chain.

Some of the fundamentals of the book are surface tension of milk, lactose chemistry, milk proteins, phosphorylation of milk proteins, comparative aspects of milk proteins, utilization of milk proteins, heat stability of milks, heat stability of homogenized concentrated milk, lysinoalanine in milk and milk products, heat coagulation of type a milk, syneresis of heated milk, fatty acids in milk, milk gel assembly, mechanical agitation of milk, natural, leucocyte and bacterial milk, grass and legume diets and milk production

This book provides a complete overview and offers insights into topics for more in-depth reading on milk and milk proteins. The book covers chapters on milk proteins, biosynthesis & secretion of milk proteins, utilization, types of milk proteins, phosphorylation, milk glycoproteins and many more. It is hoped that this book will be very helpful to all its readers, students, new entrepreneurs, food technologist, technical institution and scientists.

Tags

Dairy production and products: Milk processing, How milk is made, Dairy Processing, milk processing steps, processing of milk in dairy industry, milk processing pdf, milk processing procedure, Keeping quality of Pasteurized Milk, Milk Production in India, Increasing Milk Production, utilization of milk proteins, Functional Milk Proteins: Production and Utilization, Handbook on Milk and Milk Proteins, Milk Composition, what is the protein in milk, Milk Proteins book, How to Start Food Processing Industry in India, Food Processing Industry in India, Most Profitable Food Processing Business Ideas, Food Processing & Agro Based Profitable Projects, Food Processing Projects, Small Scale Food Processing Projects, Starting a Food or Beverage Processing Business, How to Start a Food Production Business, Agro Based Small Scale Industries Projects

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Sample Chapter:

Surface Tension of Milk

Studies of the surface tension of milk, most of which are not recent, have generally been performed at ambient temperature. Several publications have dealt with determinations at high temperatures, up to 80 °C. Only Watson (1958) has proposed an equation for the dependence of upon temperature based on results obtained at 15.6, 27.2 and 38.9 °C. To our knowledge, no measurements at temperatures greater than 80 °C have been reported and the use of this equation at such high temperatures would be imprudent. A knowledge of the surface tension of milk is of value in designing biphasic milk-steam flows which occur in direct ultra-high-temperature (UHT) milk-steam mixture installations. The present work was thus undertaken to determine the surface tension of milk between 18 and 135 °C. and forms part of a general investigation of the physical properties of milk above 80°C which includes studies of density viscosity and specific heat.

Materials and Methods

Materials and their analysis

A bulk milk from a herd of 146 Holstein-Friesian cows was used. Total milk solids (TS) were determined gravimetrically and fat contents (w/w) by the Gerber method (Kramer & Twigg, 1973). The skim-milk contained 8.99% TS (range 8.89-9.08%).

The whole milk contained 12.94 % TS (range 12.34-13.42 %) and 4.06 % fat (range 3.60-4.37%).

Surface tension measurements

Principle. The milk must undergo a minimum of modifications resulting from the heat treatment during the determinations in order to be closely similar to UHT milk. The residence time of the milk in the apparatus must thus be kept to a minimum (e.g. a few seconds) and evaporation and boiling of the milk must be avoided. Consequently the measuring instrument utilizes continuously flowing milk under pressure. It is impossible in the present circumstances to utilize a method of y measurement requiring equilibration times of several minutes, such as the Du Noüy ring method. A dynamic method was thus chosen, involving the measurement of the mass of a number of drops falling from a stalagmometer contained in a thermostatted chamber at the output of the heating section of an indirect UHT installation. The air in the chamber was kept under pressure to avoid boiling the milk.

Instrumentation

The instrumentation we devised appeared not to be available commercially. The milk was heated with the apparatus previously described (Bertsch *et al.* 1982) and then flowed into the instrument through the horizontal tube A. A fraction of the flow descended the vertical tube B (18/8 gauge stainless steel, o.d. 3.5 ±0.01 mm) whose lower extremity was carefully ground to obtain a section whose surface was circular, horizontal and free from burrs. All angles were 90°.

Tube B opens axially into the vertical glass tube C, the lower end of which is graduated, each graduation corresponding to 0.0284 cm3 at 20 °C. This tube in turn extends to a horizontal metal tube which carries the milk to a flow-rate regulator (model 8800 VD, T1 needle, Brooks, 3 rue des Pyrenees, 94573 Rungis Cedex, France).

Compressed air enters tube C via tube D, a regulator being used (model 8601, Brooks, France) to control its pressure (1% drift in 15 min).

Tube C is housed in a chamber which is thermally insulated with polyurethane foam. A mixture of glycerol and ethylene glycol circulates in the chamber at the same temperatures as the milk (Haake thermostat, Model NBS, set at ±0.1 °C: Roucaire, 20 avenue de 1'Europe, 78140 Velizy-Villacoublay, France).

The graduated tube is surrounded by a glass water jacket in which cold tap water circulates so that the milk is cooled to ambient temperature.

When the drops of milk fall from the tip of tube B they cut a light beam delivered to the wall of tube C by optical fibres (Fort fibers BFS-D-CGP1, Fort, 16 rue Bertin-Poiree, 75001 Paris, France). Fibres in the wall

diametrically opposite conduct the light to a photocell (FOP C 300 D, Fort, France). Each time a drop interrupts the beam, it is recorded on a counter (Picopakt 781-3, Hengstler, 94 rue Blaise Pascal, 93602 Aulnay-sous-Bois Cedex, France).

The temperature *0* (±0.2 °C) of the milk arriving in tube B is measured with a platinum resistance thermometer whose resistance is measured with a multimeter.

Lactose Chemistry

Lactose (4-O-(-D-galactopyranosyl)-D-glucose) is the only disaccharide found in milk, and consequently is sometimes referred to as ‘milk-sugar'. In common with other abundant disaccharides *(e.g.* sucrose, maltose, cellobiose) it is an under-utilized commodity. Moreover, in cheese-producing countries such as Australia and the USA it contributes (as a component of whey) to problems of waste disposal. Clearly there is a need to find more outlets for this product of the dairy industry. One possible strategy is to create new families of products from lactose (and in general - carbohydrates) such as detergents, plastics and resins, pharmaceuticals, agricultural chemicals, *etc.* These derivatives will no doubt derive some special properties from the parent disaccharide. This paper deals with some recent methods for selective reactions of the hydroxyl groups in lactose, in order to determine their relative reactivity, or as synthetic intermediates in the further transformation of lactose. In the latter case, some hydroxyl groups are selectively protected prior to subsequent reaction of those remaining, followed by removal of the protecting groups. This is, of course, a well-known feature of carbohydrate chemistry.

Hydroxyl groups are generally protected in 3 ways - as esters, acetals and ethers, but only the first 2 groups will be dealt with here.

Selective esterifaation reactions

The importance of selective reaction in carbohydrate chemistry is well recognized and arises as a consequence of the relative reactivity of the hydroxyl groups (or their derivatives) towards the reagent used. Selective esterification of carbohydrates is a commonly used method of obtaining valuable synthetic intermediates.

Study of the selective benzoylation of methy -lactoside 2, using benzoyl chloride in pyridine. showed that the order of acylation of the hydroxyl groups in (2) is 6' > 3' > 6 > 2 > 2',4' > 3. This order of reactivity is perhaps at first sight quite unusual, in that the 3'-OH is more reactive than the primary 6-OH. Primary hydroxyls are normally the most reactive towards acylating reagents. The high reactivity of 3'-OH was attributed to an activating effect of the cis-orientated 4'-OH. In contrast, the low reactivity of the 3-OH is thought to arise because of its more hindered location. Furthermore, those other hydroxyl groups in close proximity to the interglycosidie linkage (2'-OH and 6-OH) also display unexpectedly low reactivity. It has been proposed that these inner hydroxyl groups are more hindered than those at the periphery of the molecule, that is, 2-OH, 3'-OH. *4'-OH* and 6'-OH.

By exploiting the low reactivity of the 3-OH towards benzoylation, lactose was converted into its 3-epimer. Selective hexabenzoylation of methyl -lactoside gave the 2,6,2',3',4',6'-hexabenzoate in 33 % yield. Conversion to its monomesylate was achieved in 80 % yield, which was further transformed by nucleophilic displacement of the sulphonyloxy with the benzoate anion, into the 3-epimer. Conventional removal of the protecting groups gave the disaccharide 4-0--D-galactopyranosyl-D-allose a rare sugar, as allose has never been detected in a naturally occurring oligosaccharide. The lack of reactivity of the 3-hydroxyl group towards benzoylation has also been observed in the reaction of lactose with benzoyl chloride in a 20 % NaOH solution. The crystalline hepta-*O*-benzoyl lactose obtained was shown to have the 3-OH free by sequential methylation of the heptabenzoate, deacylation and acid hydrolysis. Isolation of only D-galactose and 3-O-methyl-D-glucose from the hydrolysate indicated a free hydroxyl group at C-3 in the hepta-*O*-benzoyl lactose.

A recent approach to the regioselective acylation of lactose is by use of coordination control. The method

employs the partial stannylation of lactose with tri-t-butyltin (II) oxide and subsequent benzoylation. The effect of stannylation is thought to enhance regioselectively the nucleophilic character of the hydroxyl group with which it bonds and thereby make it more reactive towards electrophiles. As trialkyltin alkoxides form a coordination bond with a neighbouring O2 atom at the tin atom the authors conceived that a similar effect might operate in carbohydrates. The stannylation of lactose using 3.2 molar equivalents of (Bu3Sn)O may be depicted as in Fig. 3 and when benzoylated with 6.6 molar equivalents of benzoyl chloride in toluene gave the 2,6,3',6'-tetra-O-benzoyl lactose in 72% yield.

Tejima and co-workers investigated the reactivities of the secondary hydroxyl groups in 1,6-anhydro-4',6'-*O*-benzylidene--lactose 8 by selective benzoylation. Using various molar proportions of benzoyl chloride in pyridine at -20ºC. they determined the order of reactivity of the secondary hydroxyl groups in 8 to be 3>2>3>2' . The transformation of lactose to its 2',3'-di-epimer was achieved by utilizing these reactions. Selective tribenzoylation of usins 4 molar equivalents of benzoyl chloride in pyridine at -20° gave a 41 % yield of the 2,3,3'-tribenzoate, which upon mesylation gave the 2'-mesylate. Treatment of the 2'-mesylate with 1-1 molar equivalents of Na methoxide in boiling methanol, furnished the*tato*-epoxide. Alkaline cleavage of this epoxide with aqueous KOH, followed by removal of the protecting groups, produced the disaceharide 4-0--D-idopyranosyl-D-glucose.

The relative reactivity of the secondary hydroxyl groups in was also investigated by selective p-toluenesulphonylation. l,6-Anhydro-4',6'-*O*-benzylidene--lactose is of particular interest as it contains 2 pairs of hydroxyl groups. Those in the reducing moiety are (ran-diaxial whereas those in the non-reducing residue are (rarw-diequatorial. In a similar procedure to that used in selective benzoylation, the order of reactivity of the secondary hydroxyl groups in towards tosylation was found to be *2>3'>3>2'.* The 2-tosylate obtained from these studies was further used in the new synthesis of A'-acetyllactosamine via the following sequence of reactions: (a) monoepoxide formation, *(b)* azidolysis of the epoxide, *(c)* acetolysis of the azido sugar, *(d)* reduction of the azido function to the amino group, with subsequent peracetylation and finally *(e)* de-*O*-acetylation.

Milk Proteins

The proteins of bovine milk are the best-characterized food-protein system. While lactoproteins of other species have been less intensively studied, it is apparent that there are many inter-species differences with respect to heterogeneity, the chemistry of individual proteins and the physico-chemistry of the caseinate system.

Heterogeneity and fractionation

Bovine milk contains 6 principal proteins: as1-casein, as2,-casein, b-casein, k-casein, b-lactoglobulin (b-lg) and a-lactalbumin (a-la). In addition, there are several proteins e.g. blood serum albumin, immune globulins, lactoferrin, proteose peptone 3. and ceruloplasmin at low or trace levels. Normal milk contains about 30 indigenous enzymes some of which, especially lipoprotein lipase, proteinase, alkaline phosphatase. laotoperoxidase, lysozyme and xanthine oxidase, are technologically important. Further, there are at least 7 derived proteins: g1-, g2-, g3-casems and proteose-peptone 5, 8-fast and 8-slow, all derived from b-casein by post-secretion proteolysis, and l-casein derived from as1-casein. The as2-caseins are also hydrolysed in milk but the products have not been identified.

The caseins (phosphoproteins, insoluble at pH 4-6 at 20 °C) represent ~ 80 % of the N of bovine milk ; as1-, as2-, b-, k- and g-caseins represent approximately 38, 10, 36, 13 and 38º of whole casein respectively although slightly different proportions have been reported by Barry & Donnelly. b-Lg represents about 50% of the whey proteins and a-la about 20%.

The relative ease with which casein and whey protein could be prepared attracted the attention of early biochemists such as Hammersten and Linderstrem-Lang. However, because of a marked tendency to associate, a satisfactory fractionation procedure for the caseins, using selective precipitation from

decreasing concentrations of urea at pH 4.5-4.9, was not developed until 1952. Several chemical fractionation techniques are now available for the preparation of fractions enriched with respect to individual caseins, but some form of chromatography is necessary to obtain ‘homogeneity'.

Chromatography on DEAE cellulose, with or without dissociating agents, has been widely used for many years and hydroxyapatite has recently been used successfully.

Since only k- and as2-caseins contain thiol groups when whole casein is reduced, they can be isolated by covalent chromatography on thiol-Sepharose. A strongly positively charged cluster on k-casein allows its selective adsorption on k-carageenan or cation exchange resins. Several chemical fractionation schemes have been developed for the purification of whey proteins which do not associate strongly. a-La and b-lg were crystallized in the 1950s, but the crystallized proteins are not electrophoretically homogenous. This may be achieved by ion-exchange chromato-graphy.

Microheterogeneity

Electrophoresis in starch or acrylamide gels containing 4-6 M-urea and a reducing agent (usually mercaptoethanol) shows that acid casein contains up to 20 protein zones. Much of the heterogeneity may be regarded as microheterogeneity arising from variation in the degree of phosphorylation or glycosylation and/or genetically-controlled amino acid substitutions.

as1-Casein is a mixture of 2 proteins, as0- and as1-, which have a common polypeptide chain but 9 and 8 mol P/mol protein respectively. as2-Casein is a mixture of 4 proteins, as2,-, as3, as4-, as6-, with a common polypeptide structure, but 13, 12,11 and 10 mol P/mol protein respectively. Apparently b- and k-caseins do not vary with respect to phosphorylation, containing 5 and 1 mol P/mol protein respectively.

k-Casein is the only glycoprotein in the group; its carbohydrate moiety is composed of N-acetyl-neuraminic acid (NANA), galactose and N-acetylgalactos-amine and it exists as a trisaccharide:

Thr----- NAcGal----- Gal----- NANA,

or as a tetrasaccharide:

Thr----- NAcGal----- Gal----- NANA
NANA

k-Casein contains 0-5 mol NANA/mol protein and the number of oligosaccharide chains, which are attached to the polypeptide mainly at threonine 133, but also at Thr 131 and Thr 135, varies from 0 to 3. Reduced k-casein may be resolved into fractions, differing in carbohydrate content, by ion-exchange chromatography.

All the principal milk proteins exhibit genetic polymorphism, usually due to substitution of 1 or 2 amino acids and less frequently to deletion of up to 8 residues, e.g. as1-D and as2-D. The frequency of occurrence of genetic polymorphs is genus-and breed-dependent. With the exception of the very rare as1-D, and possibly k-casein, there are apparently no technologically significant differences between variants, but genetic polymorphism has been useful in tracing the phylogenetic development of the genus Bos and breeds of cattle. Cows homozygous for b-lg A produce milk with a higher protein concentration than those producing the B variant, but casein represents a higher percentage of the N in the latter. Thus, b-lg A-type milk is better suited as fluid milk while b-lg B-type is more suited for cheese or casein manufacture although selection of phenotyped cows may not be feasible.

Milk Protein Biosynthesis and Secretion

This review summarizes the overall process of milk protein biosynthesis and secretion in the mammary gland including detailed aspects of some molecular and cellular mechanisms. Early investigators were concerned with the precursors and blood or mammary origin of the milk proteins - questions settled with the advent of radioactive tracers - characterization of the individual proteins in milk, and elucidation of the general mechanisms of protein synthesis in biological systems. It has become increasingly apparent that the synthesis and secretion of the milk proteins must be considered in context with all of the constituents as components of a delicate interrelated system. Reviews of these aspects with additional background

information include: milk proteins; blood proteins in milk, general mechanisms of milk synthesis and secretion; molecular aspects of milk protein synthesis cellular membrane flow relationships in milk synthesis and secretion.

It is now well understood that the milk proteins arise from several different origins. The majority represent specific proteins unique to lactation, synthesized under genetic control from free amino acids by the highly specialized-mammary secretory cells in structures designed for export proteins. Certain blood proteins also are transferred into the lacteal secretions. Sources of additional proteins present in milk in small amounts include secretory cells and parts derived from them and various other types of whole cells and their products. Proteins from these sources possess a host of immunological, enzymic and other functional properties and collectively contribute to the complex protein system in milk.

Many species have been used to study the mechanisms of milk synthesis and secretion Caution in interpretation is necessary because of the considerable species variations in the types and relative amounts of related proteins in milks derived from the various sources. The milk protein system studied most extensively is that of the ruminant species and the proteins present in skim-milk in particular. Lactation-specific export proteins comprise the majority in bovine milk. Blood proteins amount to only a few percent of normal mid-lactation milk, but are much higher in colostrum and early lactation and tend to rise again in late lactation. Centrifugation of milk removes most of the minor protein constituents associated with the fat globule membrane, intact cells and cellular fragments. Only in recent years has more attention been paid to the minor proteins which to a considerable degree are in milk as by-products of the synthetic and secretory processes.

The Lactating Mammary Gland

The mammary gland and ite constituent cells represent an organ under complex endocrinological control that proceeds through early stages of development into the pregnancy, lactation and regression cycle in a changing hormonal environment. Various hormones including estrogen and progesterone undoubtedly are involved as mitogenic and morphogenic agents with alveolar and lobular development. Working in harmony, particular hormones have been shown to have specific effects. Insulin promotes cell division in the alveolar epithelium, corticosteroids have been implicated in the development and maintenance of the cellular structures for the synthesis of the export proteins, and prolactin is necessary for the initiation and functional maintenance of the cellular apparatus for milk secretion.

The synthesis of the export proteins of milk in the secretory cells is under genetic control with each milk protein being coded for in the deoxyribonucleic acid (DNA) of each cell. Genetic variants of many milk proteins resulting from gene mutations have been described. Amino acid differences between genetic variants of a protein may produce significant changes in their properties.

The secretory tissue of the lactating mammary gland is arranged in lobules with each containing clusters of alveoli. Each alveolus contains a single layer of secretory cells surrounding the central lumen into which the milk is ejected. This layer of cells is, referred to as the mammary barrier. Although blood vessel cells and a basement membrane also lie between the blood stream and the secreted milk, blood components are present in the interstitial spaces below and between the epithelial secretory cell barrier up to. the tight junctional complex.

A typical secretory cell is shown diagrammatically in Fig. Precursors enter the cells through the basal membrane and milk is ejected into the lumen through the apical plasma membrane. There may be notable exchanges of lower-molecular weight materials between adjacent cells through gap junctions. This may contribute to the observation that all secretory, cells in a particular alveolus are synchronized in their periodic ejection of milk into the lumen.

The various cellular organelles noted in Fig. 1 will be referred to in more detail later. Of chief concern at this point are the rough endoplasmie reticulum (RER), rich in bound ribosomes, where the synthesis of the

polypeptide chains of the export proteins occurs; the Golgi apparatus where prosthetic groups are added and the proteins are packaged with other synthesized constituents for export: and the secretory vesicles containing the proteins and other synthesized major non-lipid constituents of milk which move to the apical portion of the cell with discharge of their contents into the lumen.

PREOURSORS

The milk proteins are synthesized from free amino acids most of which are absorbed from the blood stream. The cells have an obligate requirement for all of the usual essential amino acids; significant proportions of most of the non-essential amino acids are also derived from the blood stream. The absorption process of amino acids through the basal membrane into the cell is not well defined. More than one system may be involved and all are likely active transport (energy-requiring) processes based on evidence from studies in other systems. Recent work has been directed towards the gamma-glutamyl peptidase cycle as possibly of significance in mammary amino acid uptake. Little is known about the movement of the milk precursors, including the amino acids, through the cell into the areas where they are utilized for milk constituent synthesis. Studies in the bovine with tracer-labelled essential amino acids have shown a progressive movement of the tracer through the cell. Approximately half of the label is found after : 10 min in the middle portions rich in RER, 30 min in the Golgi and apical regions, 100 min in the lumen as secreted milk proteins. More rapid rates are found in smaller species such as the rat, reflecting their higher metabolic rates.

Precursors for the general metabolism of the secretory cells and from which the other components of milk are synthesized are extracted from the blood stream. The primary substrates utilized are glucose, acetate, -hydroxybutyrate, amino adds, and fatty acids in addition to the mineral constituents. The purine and pyrimidine nucleotides required for DNA and ribonucleic acid (RNA) synthesis, lactose synthesis and other cellular functions, are synthesized in the cells. Certain pathway intermediates and nucleotides may be present in milk in rather high concentration. Bovine milk is high in orotic acid, but low in nucleoides whereas the latter may comprise 1 to 2% of the total solids of ovine milk.

Phosphorylation of Milk Proteins

Phosphorylation of casein in the lactating mammary gland can be postulated to occur by the following mechanism :

$$\text{casein (unphos.)} + \text{ATP} \xrightarrow{Mg^{2+}} \text{casein} + \text{ADP}.$$

The reaction is catalysed by casein kinase, a cyclic nucleotide-independent protein kinase which transfers the terminal phosphoryl group of ATP to specific serine (or threonine) residues in dephos-phorylated caseins.

Casein kinase has been found in participate fractions isolated from mammary gland homogenates of rats and cows and is associated with the Golgi apparatus. Phosphorylation of casein is believed to occur as a post-translational modification of the nascent polypeptide chain.

An intriguing question concerns the specificity of casein kinase, since the phosphate groups always occur on specific serine (or threonine) residues in casein. The 4 major components of bovine casein, s1-, -, k- and s2-caseins, contain 8, 5, 1-2 and 10-13 phosphate groups/monomer. Mercier, observed that glutamic acid or phosphoserine occurs 2 residues to the right of every phosphorylated site in casein. They postulated that casein kinase recognizes a potential phosphorylation site corresponding to the tripeptide sequence Ser/Thr-X-Glu/SerP. Manson, Carolan suggested that aspartic acid could also serve as a recognition site for casein kinase. They showed that the amino acid composition of s0-casein is identical to s1-casein, but s0-casein has an extra phosphate group located in the tripeptide SerP-Lys-Asp. Thus, aspartic and glutamic acids can be regarded as primary recognition sites for casein kinase, while phosphoserine would be a secondary site that becomes available as a result of initial phosphorylation.

While sequence analysis provides convincing evidence that casein kinase specificity is determined by acidic residues in the N + 2 position, it is important to examine the role of casein kinase in the enzymic phosphorylation of caseins. Substrate specificity studies provide insight into the requirements of casein kinase. The proteins listed in Table 1 showed marked improvement as substrates after the phosphate groups were removed. Best substrates were deP (dephosphorylated) s1- and -caseins and deP pepsin. The tripeptide (Ser-X-Glu) occurs 5 times in deP s1-casein, 3 times in deP k-casein A2, but is absent from phosphorylated native proteins. DeP k-casein can be phosphorylated by casein kinase but at a much lower rate than deP s1- and -caseins. Serine 149 (SerP-Pro-Glu), the phosphorylated residue in native k-casein, is probably the site phosphorylated.

These results support the idea that glutamic acid residues are important to casein kinase specificity. Considerable phosphorylation occurs in native s1-casein. Since the phosphorylated protein contains no Ser-X-Glu peptide, serine 41 (the phosphorylated residue in s0-casein) could be the site of casein kinase action. An aspartic acid residue in the N + 2 position would be the determinant.

If glutamic and aspartic acids are recognition sites for casein kinase, pepsin should be an excellent substrate. Three Ser-X-Glu and two Ser-X-Asp sequences occur in the primary structure of pepsin; serine 68 is phosphorylated. Although pepsin can be phosphorylated by casein kinase, the rate increases more than 3-fold for the dephosphorylated pepsin. Therefore, serine 68 in pepsin must exist in a unique environment that makes it particularly available for phosphorylation.

Although the role of acidic residues as determinants of casein kinase specificity is convincing, studies from several laboratories indicate that casein kinase has limited specificity, with some groups in casein more susceptible to phosphorylation than others. For example casein kinase readily catalyses phosphate incorporation into deP phosphopeptide (residues 1-25 of -casein) but not into deP 1-casein (residues 29-209), as shown in Table 2. Therefore, one or more of the serine residues in positions 15, 17, 18 and 19 but not serine 35 are potential sites for phosphate incorporation into deP/-casein. The phosphorylation of -casein C increases 6-fold after the phosphate groups are removed. Since serine 35 in -casein C is not phosphorylated, the high rate obtained with deP -casein C must also involve serine residue 15, 17, 18 or 19.

The phosphopeptide of -casein contains 4 phosphoserines, 2 primary sites dependent on glutamic acid in the N + 2 position and 2 secondary sites dependent on phosphoserine in the N + 2 position. Human -casein in the diphospho-form provides an opportunity to examine secondary sites. The N-terminal sequence is: While human -casein in the unphosphorylated form is an excellent substrate for casein kinase with a rate equal to that of deP -casein A2, little phosphate in-corporation occurs in the diphospho-form. Serines 9 and/or 10, therefore, must be available for a high rate of phosphorylation to occur and are likely sites phosphorylated by casein kinase. Although serines 6 and 8 and threonine 3 occur phosphorylated in the pentaphospho-human /-casein, these residues are not phosphorylated by casein kinase. It is necessary to postulate another enzyme or a different environment to explain the complete phosphorylation of human -casein.

Casein kinases with different specificities have been described. Chew & Mackinlay extracted an enzyme from bovine mammary gland homogenates which phosphorylated sites within the phosphate cluster region of deP sl-casein, but did not phosphorylate deP -casein. Mackinlay et al. describe a particulate enzyme which phosphorylates serine residues in the presence of Ca2+; the enzyme phosphorylates serine located at positions 46, 48, 75 and 115 of s1-casein.

Milk Glycoproteins

The main milk glycoproteins are the casein fraction k-casein, lactotransferrin and some globulins. This short review deals mainly with the carbohydrate portions of k-caseins (location, linkage to the peptide chain, structure, microheterogeneity, biological role, phylogenetic aspects) as they have been submitted to

intensive investigations in our laboratory. The structure of the sugar part of human lactotransferrin as established by Montreuil's group will also be discussed.

k-Casein

k-Casein is a Ca-insensitive fraction and is the principal casein component affected by chymosin during the primary phase of the milk-clotting process. k-Casein thus plays a major role in the stabilization of the casein micelle in its natural environment it is also the only casein fraction that contains sugars.

Heterogeneity

The heterogeneity of bovine K-casein from pooled milk can be attributed to the genetic variants, and also to the non-identical composition of the carbohydrate groups present. Indeed, k-casein can be resolved by chromatography on DEAE-cellulose into sugar-free and sugar-rich fractions possessing the same peptide skeleton. Only 3 different sugars have been identified: galactose and 2V-acetylneuraminic acid. Thus, the establishment of their sequence seemed to be a relatively easy problem. However, this was not the case because of microheterogeneity at the sugar level.

N-acetylgalactosamine and N-acetylneuraminic acid. Thus, the establishment of their sequence seemed to be a relatively easy problem. However this was not the case because of microheterogeneity at the sugar level.

Chymosin-sensitive linkage and primary structure

During the primary phase of chymosin action on bovine k-casein, we demonstrated that a Phe Met linkage was specifically split an insoluble part, panne-casein, was formed and a soluble fraction was released, containing the caseinoglycopeptide, when the starting substrate was a sugar-rich k-casein, or the caseinopeptide when the substrate was a sugar-free k-casein. The establishment of the primary structures of bovine and ovine k-caseins was facilitated by this observation.

Structure of the carbohydrate part

By classical enzymic and chemical methods and also by mass spectrometry, the detailed sugar sequences of the 2 main carbo-hydrate portions were established. Table 2 indicates the structure of the tri- and tetrasaccharides. These same sugar sequences have been found in rat brain glycoproteins, in apolipoprotein C-III from human plasma very low density lipoproteins and in bovine milk-fat-globule membrane.

Phylogenetic aspects concerning the sugar part

Ovine *k*-easeinoglycopeptide has a polysaccharide fraction which closely resembles that of bovine *k*-caseinoglycopeptide. Its location and *O*-glycosidic linkage to the protein part have already been mentioned ; it contains not only *N*-acetyl but also *N*-glycolyl neuraminic acid in a terminal position. The primary structure of the human caseirioglycopeptide has also been established. It contains 50% sugars. Thus, the carbohydrate content is about 3 times greater than those of the bovine or ovine peptides and includes 2 additional sugars, GlcNAc and Fuc.

Evolution of the tugar part as a function of development

In bovine colostrum k-casein we observed a change in the sugar part as lactation proceeded. Colostrum k -casein contains about twice as much carbohydrate as normal bovine k-casein with an additional sugar, GlcNAc. The presence of a N-glycosidic linkage was excluded by amino acid sequence studies which did not reveal the code sequence Asn-X-Ser/Thr required for N-glycosidic linkages. GlcNAc seems to contribute to structural differences in the normal prosthetic group which should therefore be more complex. Three days after calving, the sugar content of colostrum k-caseinoglycopeptide decreases to a normal level and at the same time N-acetylglucosamine disappears.

Location of the sugar part in the secondary structure of k-casein

The secondary structures of the peptide segments around the carbohydrate-peptide linkages in k-caseins were recently predicted according to the rules of Chou and Fasman. As previously noted with other

glycoproteins, the O-glycosidic Thr-GalNAc linkages were found to be situated in -turns. These observations suggest that the carbohydrate moiety of a glycoprotein is on the outside of the protein (-turn), that is, in a domain geometrically well denned and therefore allowing an easy approach to the sugars during the biosynthesis of the glycan chains.

Comparative Aspects of Milk Proteins

This paper emphasizes developments in the past 6 years since the chemistry of milk proteins was last reviewed in this journal by Lyster who referenced many publications on non-bovine milk proteins. Several of the chapters in the 2-volume treatise on milk proteins edited by McKenzie include some discussion of proteins of species other than bovine. The comprehensive review of caseins deals only with bovine caseins but Ribadeau Dumas, describe and discuss knowledge of caseins of non-bovine species up to 1973. Woodward has recently reviewed the caseins rather thoroughly and Bezkorovainy has reviewed human milk proteins.

KINDS OF PROTEINS IN MILK

Several well-defined families of proteins occur in milk. These have been most thoroughly delineated in the milk of the domestic cow (Bos taurus). Principal families of proteins in milk of this species are s1-caseins, s2-caseins, -caseins, k-caseins, -lactalbumins (-1a), -lactoglobulins (-lg), serum albumin, and immunoglobulins IgG1, IgG2, IgA, and IgM. Each of these is considered to be coded for by a single gene or in the case of the immunoglobulins by a multigene complex. The nomenclature of these families and of the individual proteins within them has grown somewhat haphazardly and non-systematically, but at present milk proteins of other species are generally assigned names corresponding to those of their homologues in bovine milk.

Dayhoff has organized data for about 500 protein sequences into a hierarchical system of superfamilies (probability of similarity by chance < 0.001), families (< 50% different), sub-families (< 20% different) and entries (< 5% different). On this basis she placed the caseins in 3 superfamilies (s1-, -, and k-), the -la and lysozymes in separate families in the superfamily ‘animal lysozyme related proteins', the immunoglobulins and 2-microglobulin in 2 super families (variable region and constant region) and -lg in a separate superfamily.

A problem in dealing with milk proteins of other species is to assign correct homology. In biological usage homology implies divergence from a common ancestor. It can be inferred from biological function and immunological cross-reactivity, but the most definitive criterion is congruence of amino acid sequence. Ability to perform a given function could conceivably arise by con-vergence from different ancestral lines and thus is not a safe indicator of homology. In any case the biological function of several of the milk proteins is unknown. Immunological cross-reactivity is a reasonable secondary criterion for homology in some cases. Certainly cross-reactivity depends on similarity of sequence and proteins that cross-react are likely to be homologous. The converse is not true however; lack of cross-reactivity does not necessarily imply non-homology. Amino acid composition furnishes useful comparisons among proteins and gives some clues to homologous relations have used this criterion to a considerable extent in this paper.

In recent years, milk proteins of many species have been shown by elect rophoretic techniques to contain several distinct components. These methods do not, however, indicate whether the components are homologous to any of the families of bovine milk proteins. Temptation to name them as members of such families should be resisted and provisional nomenclature used until the individual components are isolated and homology can be established, preferably by sequencing.

Within each family of milk proteins produced by a given species, heterogeneity occurs due to (1) genetic polymorphism and (2) post-translational modification. The first is of course due to mutations that have occurred in the cistrons coding for the particular proteins. The second may be due to many different reactions. recently compiled a list of kinds of naturally occurring post-translational modifications observed or

inferred in proteins. Of these, the ones established as occurring in milk proteins are varying degrees of phosphorylation and grycosylation. Probably proteolytic cleavage and deamidation also occur. N-(-aspartyl)lysine and N-(-glutamyl)lysine have been detected in bovine colostrum after proteolytic hydrolysis, but these crosslinks have not been assigned to any particular protein(s).

Utilization of Milk Proteins

Traditional and newly developed protein products are being extensively utilized as ingredients in an increasing number of formulated foods. They provide the food industry with additional opportunities in research and development of new and improved food products. These proteins are derived from a variety of sources, e.g. milk, meat, eggs, oilseeds, cereals, micro-organisms, by a number of diverse processes. Development and utilization of such protein ingredients represents one of the most important and interesting developments in the food manufacturing industry in recent years. Their importance stems from their nutritional contributions, and also from their ability to provide unique and essential functional properties to the food system.

Due to increasing emphasis by the news media upon govern-mental regulations and nutritional and health topics, consumers are becoming more knowledgeable and concerned about the ingredients in their foods. Since milk, the source material for milk protein products, is associated with wholesomeness and health, and since milk proteins are universally recognized for their excellence in nutritional value, milk protein products are receiving increasing attention as formulation ingredients by the food manufacturing industry.

Although non-fat dry milk and dried whey have been the major sources of milk protein ingredients for the food manu-facturing industry in the past, a number of relatively new and versatile milk protein products are available, which offer special advantages for certain food applications. These milk protein products are generalty produced from low-cost milk and whey, largely in New Zealand, Australia, USA,

The Netherlands and Canada. Milk-pricing policies of the respective governments have a significant effect upon the relative importance of the various milk protein products that are being produced. A most important over-riding factor is the rapidly expanding production of cheese whey, which provides a large supply of high quality protein for human consumption.

This paper presents current information on the amount, production processes, composition, functional properties and major applications of the important milk protein products available today. Recent review articles by Borst and Muller, as well as a personal communication from Dr N. J. Walker of New Zealand Milk Products Inc., and several booklets by Hugunin & Lee and Hugunin & Ewing provided much of the information used in this article.

Milk protein products are complex protein systems that contain a number of major and minor components, all of which possess complex conformational states and undergo important reactions and interactions during their isolation and utilization. Although such information would readily explain many of the major chemical and functional properties of the milk protein products, it is largely outside the scope of this paper.

General properties of milk protein products

A general consideration of milk protein products indicates that they are available in a wide variety of forms, e.g. acid casein curd, rennet casein curd, Na/K/Ca caseinate, casein/whey protein co-precipitate, lactalbumin, partly delactosed whey, partly demineralized whey, whey protein concentrate and as blends of milk proteins with soy and cereal proteins. Each of these milk protein products has its own characteristic properties with respect to composition, nutritional value, flavour, solubility and functionality. For example, caseinates, co-precipitates and lactalbumin contain from 90 to 95 % protein, whereas whey protein concentrates generally contain only from 30 to 50 % protein with correspondingly higher levels of lactose and milk salts. Casein, Ga caseinate, lactalbumin and the co-precipitates are less soluble than Na and K caseinates and whey protein concentrates, and thus would not be selected for those applications for which functionality is dependent upon a high solubility. Although all milk protein products have excellent nutritional

value, those that contain the highest proportions of whey proteins, with their rich supply of S-containina amino acids, exhibit the highest protein efficiency ratios (PER).

Milk protein products are subject to development of off-flavours during storage, which are described as stale, musty or gluey. These flavours are characteristic of dried milk and whey products in general and their origins are believed to be oxidized lipids, degradation of tryptophan, and the Maillard reaction between the proteins and residual lactose.

As mentioned by Muller, major emphasis is being given to production of casein curd, caseinate and other milk protein products by modern sanitary processes that will permit their use in human foods. These and other process modifications, such as the use of charcoal and other adsorbants for removing off-flavour components, are being considered for improving their acceptability for use in formulated foods.

Estimated values for amounts of the major milk protein products produced and utilized in the USA are given in Table 3. Additional details on the applications of milk protein products in processed meats, synthetic meat products, beverages, cereals and pasta products, snack foods and imitation food products are given by Hugunin & Lee.

Preparalion and properties of casein curd and caseinates

Muller reviewed the history and current status of the casein and caseinate manufacturing industry, which is heavily concentrated in New Zealand and Australia. The annual world production of these products has stabilized at about 100-120 thousand metric tons. Although annual USA imports of casein and caseinates have fluctuated around 40-55 thousand metric tons over the last 20 years, there hag been a steady shift from industrial to food uses.

Casein curd is produced from pasteurized skim-milk by either treating with rennet, or direct acidification with acid, or by culturing with micro-organisms to produce acid. All of these treatments precipitate the casein or cause it to form an insoluble clot which can easily be separated from the whey fraction, washed and dried. Acid casein curd contains only about 2 % ash compared to 7.5 % ash for rennet casein curd, which retains most of the colloidal phosphate of the casein micelles. Both of these caseins, unless subjected to enzymic or chemical modification, remain in-soluble and are therefore useful for those functional applications which do not require solubility, e.g. breakfast cereals, protein supplements and baked foods. Rennet casein curd has traditionally been used in the commercial plastics industry, but there is relatively less demand for this product now, due to competition from other cheaper polymer products.

Na, K and Ca caseinates are produced by neutralizing casein curd with the corresponding base to pH 6.8-7.5 and drying the solubilized form. Na caseinate, produced directly from wet casein curd, generally has a better flavour than if produced in-directly from previously dried casein or Ca caseinate. It is important to avoid elevating the pH above the 6.8-7.5 range to minimize development of off-flavours by reaction of alkali with residual lipids, which are presumably bound to the hydrophobic amino acid residues of the casein molecules.

It is also important to avoid exposure of the caseinate to elevated pH because of the likelihood of catalysing various cross-linking reactions that might produce potentially toxic amino acid derivatives. Na and K caseinates both have excellent solubility, heat stability, water absorption and, due to their unique amphiphilic conformation perform without equal in stabilizing oil-in-water emulsions and aqueous foam systems.

Heat Stability of Milks

The factors which contribute to the heat stability of milk and its variation with season and environment are not fully understood. The highly sensitive and complex influence of pH is well recognized. Other factors which have been studied are the levels of different proteins, particularly -lactoglobulin (lg), and interactions between the latter and -casein. More recently, urea concentrations in the milk have also been studied. However, only few attempts have been made to relate data on non-concentrated milks to those on

concentrated (evaporated) milk.

In the present study, heat stability-pH curves for simplified milk systems have been examined at casein levels equivalent to both concentrated and non-concentrated skim-milks, and the influence of preheating and of levels of -lg and urea have been investigated.

EXPERIMENTAL METHODS

Casein micelles were separated from skim-milk by centrifugation and, when resuspended in the supernatant, yielded heat stability behaviour comparable to the original milk, indicating that the mechanical operations did not affect the heat stability of the system. For all the studies on simplified milk systems described below, micelles were resuspended in skim-milk ultrafiltrate at a casein level of 2.5 % (non-concentrated suspension). Suspensions were concentrated, when desired, to 20% total solids (concentrated suspension) by rotary evaporation, either with or without prior preheating (85 °C for 30 min). The concentrated suspensions showed very high heat stability over a broad pH range. The only effect of preheating was to change the pH of maximum stability from about 6.6 to 6.5.

RESULTS AND DISCUSSION

Effects of -lg

Addition of -lg to non-concentrated suspensions produced small increases in heat stability at pH 6.5 and below, and induced a trough between pH 6.8 and 7.2 in the heat stability-pH curve the depth of the trough increasing with -lg concentration. This behaviour suggests that the main effect of -lg is to sensitize the coagulation of casein between pH 6.5 and 7.2. The trough is broadened by preheating so that the sensitizing effect of -lg is thereby increased.

When -lg was added before concentrating, the concentrated suspensions displayed a considerable narrowing of the broad peak in the heat stability-pH curve and a dramatic reduction in maximum heat stability (e.g. < 1 min at 2-0 mg/ml -lg). With prior preheating, the reduction in heat stability was not as great but still considerable, and the peak in the heat stability-pH curve remained fairly broad.

Effects of urea

The addition of urea to non-concentrated suspensions produced considerable increases in the magnitude of the maximum in the heat stability-pH curve, supporting previous work on skim-milk. However, the -lg-induced coagulation between pH 6.8 and 7.2 was little affected, suggesting that the effect of urea is on the heat-induced casein coagulation.

In contrast to the effect of urea on skim-milk and non-concentrated simplified milk systems, increasing levels of urea produced small decreases in the heat stability when concentrated non-fat milks were tested. The enhanced heat stability effected by preheating was similarly slightly diminished by increasing urea concentrations and this effect was independent of whether the urea was added before or after preheating.

Heat Stability of Homogenized Concentrated Milk

Practical difficulties with protein stability are still encountered in milk products as such as full-cream evaporated milk, sterilized cream and ultra-high temperature sterilized milks. The common ingredient in these products is the milk fat which is finely dispersed as a result of homogenization. A series of investigations was therefore made into the effect of homogenization on protein stability.

Marked seasonal differences were found to exist in the effects of homogenization of subsequently concentrated milk: ‘summer' milks were found to be much more stable than ‘winter' milks after homogenization and to be more readily stabilized by added Na phosphates and citrates. Also, milk serum rather than milk fat was shown to be the major determinant in the heat stability of concentrated homogenized milk and all such milks were found to be very sensitive to small changes in their level of soluble Ca. Although homogenization per se generally reduces the heat stability of subsequently concentrated milk, it was found that the heat stability of these products could be enhanced by a number of processing techniques such as high temperature forewarming (145 °C, 5 s) and 2-stage homogenization.

These investigations emphasized the marked influence that seasonal or dietary induced changes in the concentrations of the milk salts have on heat stability. However, the heat stability of concentrated homogenized milk was not highly correlated with the absolute levels of any of its milk salts (either in the colloidal or in the soluble form) and clearly other factors also affect stability. It has been found that sulphydryl group interactions between native milk proteins play an important role in determining the heat stability of unconcentrated homogenized milk. This investigation has consequently been extended to homogenized milk subsequently concentrated to 31 % total solids (TS), which is equivalent to commercial full cream evaporated milk.

Heat Stability of Evoporated Milk

It has long been known that heat treatment of milk before its manufacture into evaporated milk (EM), i.e. forewarming or preheating, increases the resistance of the EM to heat-induced coagulation during subsequent in-tin sterilization. However, the mechanism by which preheating protects the EM against heat coagulation is little understood. It has been suggested that the heat stability of the milk is dependent upon heat-induced interactions involving the whey proteins, particularly -lactoglobulin. It has also been shown that EM made from milk from which the whey proteins had been largely removed was heat stable whether the milk was preheated or not; EM made from the original milk was heat stable only if the milk had been preheated. A destabilizing effect of the whey proteins which can be overcome by preheat treatment is thus indicated. However, the question as to how preheating milk reduces its tendency to coagulate during subsequent heat treatment, often at a similar temperature, remains unanswered.

In the normal process for the manufacture of EM, the milk is evaporated and homogenized after preheating and before sterilization, and for recombined EM the unconcentrated skim-milk is preheated before evaporation, drying, recombination with fat and homogeni-zation. Thus, the obvious factors for consideration are the effects of concentration and homogenization. Considering the previously reported effects of whey proteins it seemed possible that these could play a role in the homogenization process.

The object of the work reported here was to investigate the effects on the heat stability of EM of homogenization of the milk before or after preheating at different total solids (TS) concentrations and at different whey protein concentrations.

MATERIALS AND METHODS

Bulk milk was obtained from the Massey University mixed herd and skimmed as described previously. To inhibit bacterial growth during the necessarily prolonged processing at room temperature and above, a mixture of antibiotics was added to the complete batch of skim-milk at the following concentrations per ml of skim-milk: Penicillin G, 0.2 i.u.; cephaloridine, 0.2 g; streptomycin sulphate, 1g; neomycin, 1 g; chlortetracycline, 1g.

All processing and analytical methods were carried out as described previously. Preheat treatment of the milks was at 120 °C for a nominal 120 s and homogenization was carried out in 2 stages at first- and second-stage pressures of 15.9 and 3.5 MPa, respectively.

Data were evaluated by analysis of variance. The data were not normally distributed and required logarithmic transformation. The means given in the results are therefore geometric means.

The following procedure was repeated on the 12 and 19 September, 6 and 12 December 1977, and the 1 and 8 March 1978; differences between the months were taken as indicating seasonal effects while the repeats within each month were treated as replicates for estimation of the error variance. From 801 of bulked raw skim-milk the following milks were prepared: (1) milk with a low whey protein content (low whey protein) made by suspending ultracentrifugally sedimented casein micelles in the ultrafiltrate from the same milk, as described previously (2) Becombined ultracentrifuged milk (recomb. UC) as a control for (1), made by resuspending ultracentrifugally sedimented casein micelles in their own supernatant serum. (3) Concentrated (cone.) milk prepared by evaporation at 60-55 °C of a portion of the raw skim-milk to 20-

22 % TS. (4) Original skim-milk.

Each of the above 4 milks was combined with anhydrous milk fat to give a fat: non-fat-solids ratio of 8:18 and each was then divided into 2 portions. One portion, A, was preheated and then homo-genized. The other, B, was homogenized and then preheated. Three additional control milks were prepared as follows: (5) to determine the effect of homogenization on skim-milk in the absence of fat, one batch of the original skim-milk was homogenized, preheated, combined with fat and again homogenized. (6) To determine the effect of unhomogenized fat during preheating portion of the original skim-milk was preheated, then combined with fat and homogenized. (7) In order to establish the basic heat stability of the milk a further portion of the original skim-milk was evaporated to 20-22% TS, combined with fat and homogenized without any preheat treatment.

Cationic Detergent on Heat Stability of Milk

The heat stability of the caseinate micelle system dispersed in milk diffusate or in a synthetic milk salts buffer increases progressively with increasing pH. However, a maximum-minimum can be introduced into the heat coagulation time (HOT) - pH profile by addition of -lactoglobulin (-lg) -lactalbumin. The HCT-pH profile of a caseinate system containing -lg (e.g. milk) is of course modified by the colloidal and soluble milk salts, k-casein and many other factors.

SDS increases the maximum heat stability of milk and shifts the pH of maximum stability to more acidic values; tetramethyl ammonium bromide has no effect on maximum heat stability but destabilizes the caseinate system at pH values > ~ 7.0. Pearce reported that cationic detergents displace the HCT-pH curve to higher pH values while anionic detergents have the opposite effects: unfortunately, additional information on the nature of the detergents was not provided. Both Pox & Hearn concluded that surface charge is important in determining the heat stability of milk and that the charge is offset by an appropriate pH change resulting in low stability such as in the pH range of minimum stability.

Treatment of milk with rennet reduces the zeta potential of casein micelles which permits micelle interaction via hydrophobic bonding leading to gel formation. Rennet coagulation is facilitated by cationic detergents and other polyvalent cations and inhibited by anionic detergents.

In the present work the influence of cationic detergents, other cationic species and -lg on the heat stability of milk and caseinate systems was investigated.

MATERIALS AND METHODS

Milk supply

Bulk herd milk from the University herd, defatted by centrifugation at 2000 g for 20 min at 30 °C, was generally used, but in some experiments 10% low heat skim-milk powder reconstituted in distilled water was used.

Caseinate systems

Dispersions of serum protein-free casein micelles (SPFCM) were prepared as described by Fox & Hoynes. Milk partly depleted of soluble salts was prepared by dialysing skim-milk against 100 vol. distilled water for 4 h.

Detergents, salmine sulphate or -lg, as appropriate, were added to milk or to casemate systems as indicated, with equilibrium at 2°C overnight with stirring.

Materials

-Lg, lysolecithin, SDS and proline were purchased from Sigma London Chemical Co., Poole, Dorset, UK; tetramethyl- (TMAB), tetrabutyl (TBAB)-, cetyl trimethyl-(CTMAB) ammonium bromide and salmine sulphate were obtained from BDH Chemicals Ltd, Poole, Dorset, UK.

Arginine Residue and Heat Stability of milk

Formaldehyde and other low molecular weight aldehydes, including sugars, considerably increase the heat stability of milk and concentrated milk. The efficacy of a range of carbonyl compounds is increased in the

presence of low levels of urea which appears to stabilize milk only in the presence of a carbonyl compound, including lactose.

Aldehydes are assumed to exercise their stabilizing influence by modifying e-amino groups of lysine. However, in seeking to explain the synergic effect of urea one must consider also the possible modification of arginine. This is structurally very similar to homocitrulline which is produced from lysine on heating with urea.

The influence of reagents with a high degree of specificity for arginine, such as glyoxal derivatives, or for lysine, on the heat stability of milk was investigated and the results are reported here.

MATERIALS AND METHODS

Milk supply

Bulk herd milk from the University herd, defatted by centrifugation at 3000 g for 20 min at 30 °C, was used.

Lactose-free milk

Skim-milk samples were dialysed against two 20 vol. changes of synthetic milk salt buffer at 4 °C for 3 d. Dialysis against the lactose-free buffer caused an increase in volume of ~ 35% which did not alter the shape of the HCT-pH curve and caused only a slight change in maximum stability.

Reagents

Glyoxal, glyoxal derivatives and dansyl chloride were purchased from Sigma London Chemical Co., Poote, Dorset, UK; diacetyl, acetyl acetone and acetic anhydride were obtained from BDH Chemicals Ltd, Poole. Dorset, UK; o-methylisourea hydrogen sulphate and 1,2-cyclohexadione were purchased from Aldrich Chemical Co., Gillingham, Dorset, UK and acetoin was a product of Hopkin & Williams Ltd, Chadwell Heath, Essex, UK.

Reagents, as appropriate, were added directly to lactose-free milk or milk samples at the natural pH as indicated in the Results section; the reaction was continued for 6 h and in some cases overnight at 2°C. Methylisourea was added to lactose-free milk at pH values 7.5, 8.0 and 10.5. The pH was kept constant and the reaction was continued overnight at 2°C.

Determination of heat stability

The pH of samples of milk or lactose-free milk were adjusted to pH values in the range 6.4-7.4 with 2 M-NaOH or 2 M-HCl and held for 1 h before assay. Heat coagulation times (HCT) were determined in a thermostatically controlled oil bath at 140 °C according to the method of Davies & White.

Keeping Quality of Pasteurized Milk

Commercial pasteurized milk generally becomes recontaminated with microorganisms, especially from non-sterile plant surfaces. Post-pasteurization contamination (PPC) with heat-sensitive, psychro-trophic Gram-negative rods (GNR) is particularly important. Owing to their comparatively rapid growth in refrigerated pasteurized milk, they usually predominate in the microflora of such milk at spoilage.

In this paper, we present results on the keeping quality of HTST-pasteurized milk stored at 11 and 5 °C, with and without psychrotrophic PPC.

EXPERIMENTAL

Filled retail containers of non-homogenized pasteurized milk and a sample of the corresponding silo raw milk were obtained from each of 5 dairies within 50 miles of the NIRD. The raw milk was pasteurized at the laboratory and samples of the laboratory pasteurized milk and the corresponding commercial pasteurized milk stored alongside each other. They were examined bacteriologically and organoleptically during storage.

The commercial HTST-pasteurized milk had been filled as follows: in dairy A, into glass bottles or polyethylene-coated cartons (pre-formed type); in dairy B, into glass or plastics bottles; in dairies C and D, into glass bottles; and in dairy E, into cartons (pre-formed or formed ‘off-the-reel').

Sampling at the dairies

For each experiment, 12 (dairies, A, B, C) or 6 (dairies D, E) 1 pint retail containers of commercial pasteurized milk were collected from the fillers. Raw milk for laboratory pasteurization was filled from the silo directly before transport to the laboratory into a steamed (2 x 1 h on consecutive d) 5 gal milk can. Approximately 3 gal raw milk was drawn from the bottom outlet of the silo after 1-2 gal had been run to waste.

During 3 additional sampling visits each to dairies A and D, pasteurized milk was sampled from the finished milk tanks supplying the fillers as well as from filled retail containers to determine the most probable number (MPN) of psychrotrophs in milk from both sources. Two autoclaved 1-pint screw-cap bottles were filled from each of the tanks after swabbing the sampling cock with 95% ethanol (EtOH) and then running 0.5-1 gal of milk to waste.

Pasteurized milk was taken to the laboratory in insulated boxes with ice packs and the raw milk in a can wrapped in a wet cloth with journey times varying from 20 min to 1.5 h.

Preparation of the milks for storage

At the laboratory, while the raw milk was being pasteurized, the commercial pasteurized milk was stored at 7 °C (for 1-2 h). Laboratory pasteurization was carried out at 72 °C with a holding time of 20 s and the milk cooled to 5 °C in a plate pasteurizer with a capacity of 82 1/h.

From the pasteurizer, the milk was transferred to an autoclaved aspirator jar by inserting the outlet tube into the neck of the jar and replugging with autoclaved, non-absorbent cotton-wool. Before insertion, the outlet tube was rinsed with EtOH. From the jar, the milk was filled into autoclaved storage bottles using conventional aseptic procedure.

The commercial pasteurized milk from each filler was bulked, mixed in an autoclaved aspirator jar and filled aseptically into autoclaved storage bottles in order to eliminate any potential differences in the level of PPC for different containers from one filler. Cartons and plastics bottles were swabbed with ethanol and opened with a pair of scissors which had been cleaned with EtOH and flamed. The tops of glass bottles were also wiped with EtOH and the caps removed with flamed forceps. The milk was then poured into the autoclaved aspirator jars.

The milks from dairy A were stored in 100 ml quantities and 2 bottles were examined on each sampling day. There were marked differences between the level of psychrotrophic growth in duplicate samples of milk taken from the bulk contents of the cartons, indicating low PPC. Subsequently, the storage volume was therefore increased to 300 ml as being closer to the usual commercial size of pasteurized milk packages of 1 pint. However, with the larger volume only 1 sample was examined on each sampling day (dairies B-E).

Subsamples for the determination of the MPN of psychrotrophs in commercial pasteurized milk were taken from finished milk tanks and after filling into bottles or cartons were prepared as soon as the milk arrived at the laboratory. From each sample, five 50 ml, five 10 ml and five 1 ml quantities of milk were transferred aseptically by pipetting into autoclaved screw-cap bottles or test tubes.

Lysinoalanine in Milk and Milk Products

During the past half century dietary trends have gradually changed. Scientific studies of the nutritional value of food ingredients and medical advice on dieting have led many individuals to decrease their consumption of fat. At the moment more attention is given to the value of proteins as a necessary food ingredient. In this respect it is important to note that milk contains the best balanced and least expensive animal protein.

Nowadays, the processing of milk is part of a highly organized and controlled dairy industry, which produces and markets a multitude of dairy products. In modern food technology heat and/or alkali treatment of food proteins is firmly estabished for the production of protein isolates with special functional properties.

Owing to processing, a number of changes take place in the chemical, physical and nutritional properties of proteins. Heat and/or alkali treatment of proteins may result in the decomposition of cystine, serine, phosphoserine and threonine residues and formation of amino acids not normally found in nature. These

‘unnatural' amino acids include lysinoalanine (LAL lanthionine and ornithinoalanine. The special interest in these amino acids was the discovery, some years ago, that LAL was toxic when fed to rats. From experiments with rats which, for some months, were fed diets containing an industrial-grade soya protein which had been subjected to severe treatments with heat and alkali, Newberne & Young observed a unique histological lesion of the kidney in the rats, called nephrocytomegaly. This lesion consisted of an increase in the size of the nucleus and the cytoplasm of the epithelial cells of the straight portion (pars recta) of the proximal renal tubules. The relation between this lesion and the presence of LAL in the diet of rats was demonstrated by Woodard & Short.

This discovery has initiated considerable research into the conditions in which LAL is formed and the examination of foods and common food protein ingredients for their content of LAL. Since the toxic effect on rats of free synthetic LAL was shown to be much higher than that of protein-bound LAL special attention was given to the form in which it was present.

In this paper the conditions necessary for the formation of LAL are reviewed, methods of analysis are described, and the LAL content of a number of milk and milk products mentioned in the literature are compared with the results of our own investigations. The biological effects and implications for human health are also discussed.

Conditions for the formation of LAL

In the modern food industry heat treatments under mild acid or alkaline conditions are common practice. During these processes the lysyl residues of proteins maybe lost by reaction with sugar components as occurs in the well-known Maillard reaction. It was recognized by Bohak that losses of lysine also occurred by alkali treatment of proteins in the absence of carbohydrates. After alkali treatment of ribonuclease a decrease of cysteine and lysine content was observed while a new cross-linked amino acid was isolated named lysinoalanine (N-(DL-2-amino-2-carboxyethyl)-L-lysine). It was suggested by Bohak that the formation of LAL should be attributed to the nucleophilic addition of a lysyl residue to an intermediate dehydroalanyl residue formed by -elimination of a cystine disuhphide bond on attack by hydroxyl ions. Later, Whitaker & Feeney also isolated LAL from the alkali-treated phosphoprotein, phosvitin, which contains no cystine. Another possibility was demonstrated by Whiting who isolated LAL from the protein-polysaccharide complex of cartilage in a quantity in excess of that which could be accounted for by its cystine content.

From these observations it was concluded that the intermediate dehydroalanyl residue necessary for the formation of LAL may be produced by -elimination of a phosphoseryl, glycosylated seryl or a cystyl residue. Three different ways for the formation of dehydroalanine followed by the formation of LAL are shown in Fig. 1.

The reactive double bond of dehydroalanine may also react with the side chains of other amino acids or with ammonia. Products of addition reactions between dehydroalanine and the -amino group of ornithine which can be formed from arginine by alkali treatment as demonstrated by Ziegler et al. or with the thiol group of cysteine lead to the formation of ornithinoalanine and lanthionine respectively. Asquith et al. reported that -aminoalanine was probably derived from the reaction of ammonia with dehydroalanine. These reactions are summarized in Fig. 2.

4-Caseins in Raw Milk

The storage of bulk milk at 2-6 °C is the most suitable procedure besides heat treatment to limit undesirable quality changes by contaminating micro-organisms. Its introduction was necessary for economic and practical reasons related to centralization in the dairy industry. However, the introduction of cold storage of bulk milk has brought about some unexpected problems. The fermentation properties of cooled raw milk are significantly different from fresh raw milk. This has been recognized to be due to a change from mesotrophic to psychrotrophic micro-organisms. The resulting problems during fermentation (proteolytic

and lipolytic activities instead of lactic acid formation) have been solved by pre-ripening with mesotrophic starter cultures.

Changes in the properties of bulk milk stored at 2-8 °C may require important changes to be made in the normal processing schedules of cheese-making. Coagulation time and curd formation especially are influenced by cold storage, with the times involved sometimes increasing by as much as 100%. Systematic investigations have shown that these differences are not due to changes in the contaminating flora but to changes in the milk itself.

The effects of cold storage on the processing parameters of raw milk are related to the miceller character of the casein fraction of the milk proteins. Several authors have described the dissociation of micelle components, especially of the highly hydrophobic -casein during cooling of milk. While the s-casein equilibrium between milk serum and the micelles is almost unaffected by temperature, the -casein is dissolved from the micelles into milk serum to a large extent at low temperatures.

The isolation of milk serine proteinases revealed a resem-blance between the temperature-dependent association with micelles exhibited by the proteolytic enzymes and -casein. The micelles were isolated at the appropriate temperature by ultracentrifugation and the enzymes were obtained by extraction of the protein pellet at pH 2. At low centrifngation temperatures only low activities were obtained, which means that, as in the case of -casein, the enzyme equilibrium is shifted towards the milk serum.

The transfer of the trypsin-like milk proteinases and -casein into the milk serum during cooling suggests a mechanism for -casein formation. From sequence analyses it is well established that the -caseins are proteolytic fragments of -casein, and the micelle-associated proteinases show a very specific cleavage of -casein. Therefore an increase in -casein content during cooling of raw milk may be expected, and estimations of -casein content could be useful for evaluating pre-treatmonts of raw milks in relation to their subsequent processing properties.

The -casein content was estimated by a simple extraction procedure with organic solvents. -Caseins are soluble in organic solvents because they are oven more hydrophobic than -casein, having lost the hydrophilic N-terminal phosphopeptides by proteolytic degradation. From a series of solvent systems the combination of 1-propanol and diethylether (2:1, v/v) was found to give the best results. After incubating the milk at a given temperature, the casein fraction was precipitated at pH 4-6, isolated by centrifugation, resuspended in the above solvent system for 30 min, filtered and the soluble fraction recovered by evaporation. The residual proteins were determined by Kjeldahl analysis and identified by polyacrylamide-gel electrophoresis. Electrophoresis showed that only -caseins were extracted by this procedure. Fig. 3 shows typical results for -casein formation in raw skim-milk at 4, 11 and 26 ºC. The pattern for storage at 26 °C shows a significant decrease in the concentration of the -caseins, which could be due to further degradation by amino peptidases which are either originally in the milk serum or might be derived from contaminating micro-organisms which show a pronounced growth at this temperature. During cold storage there is a significant increase in -casein formation which is greater at 4 than 11 °C, the opposite of what would be expected on the basis of normal enzyme kinetics. This can be explained by the dissociation of the substrate (-casein) and enzyme (milk proteinases) from the casein micelles at low temperatures, with resulting proteolysis, whereas both these components are partly immobilized by hydrophobic association with the micelles at room temperature, allowing only limited proteolysis. However, this is only a partial explanation since -casein may account for up to 10% of total casein in some fresh raw milks, and this is presumably formed at 35 ºC.

Proteolysis in UHT Milk

A residual proteolytic activity, measured as liberation of non-protein nitrogen (NPN), has recently been found both in milk after ultra-rapid sterilization by friction and in UHT-processed milk. This activity decreases when the sterilization temperature or the amount of heat applied to the milk increases.

Our results indicate particularly that the time before gelation in UHT milk is inversely related to proteolytic activity and assist in identifying gel formation, at least after dissociation of the casein-whey protein complex, as an enzymic process due to the action of residual protein-ases, which survive the heat treatment or are reactivated during storage.

The present paper reports the effect on proteolytic activity in UHT-sterilized milk of a proteinase-containing fraction isolated from UHT milk samples, stored at room temperature.

MATERIALS AND METHODS

Materials

The UHT skim-milk samples, used both for the separation of the proteinase-containing fraction and experimental trials, were obtained from a commercial direct heating plant. The milk was preheated to 80 °C, and after 30 s at this temperature, rapidly heated by steam injection to 148 °C for 6 s. After cooling it was aseptically packaged in aluminium-lined Tetrapacks. The samples were then stored at room temperature.

Isolation of a proteinase-containing fraction

A proteinase-containing fraction was isolated from casein micelles from each of 2 UHT milks (milks A and B, Table 1) after 4 months storage, as described by Reimerdes & Klostermeyer.

The casein micelles were separated from 300 ml milk by centrifugation (1 h, 4 °C, 120000g), resuspended in 100 ml of water and extracted at pH 2.0 with 7 N-H_2SO_4. A proteinase-containing precipitate was obtained from the acid extract by adding ammonium sulphate to 40%(w/v) saturation. This precipitate was suspended in 15 ml 0-1 M-Tris-HCl buffer and, after dialysis, diluted to 25 ml with the Tris-HCl buffer. This suspension was stored frozen at -20 °C until used.

Determination of proteolytic activity

Proteolytic activity in samples was estimated by a modification of Noomen's method. It involved the determination of the percentage increase in NPN in milk which was incubated anaerobically for 6 d at 37 °C, and which contained thiomersal as preservative.

The total N (TN) and NPN in the samples were determined by a Kjeldahl method, before and after incubation.

Experimental trials

Proteolytic activity was determined, with and without addition of the proteinase-containing fraction, in 2 UHT milks which had previously been stored for 3 months. The proteolytic effect of the fraction isolated from UHT milk was evaluated from the NPN increase after incubation of samples in which 1 ml proteinase-containing suspension and 10 ml milk were mixed before incubation at 37 °C.

RESULTS AND DISCUSSION

The extent to which proteolysis had occurred during storage of the UHT milks from which the proteinase-containing fraction was isolated was not particularly high considering the age of the milks. This fact indicated that the proteinases were not produced by psychrotrophic bacteria, which is in agreement with Adams et al. and Driessen who reported that heat resistant Pseudomonas proteinases caused rapid spoiling of sterile milk. However, the proteinase-containing fraction isolated from these milks is capable of producing extensive proteolysis of milk.

The proteolysis, after 6-d incubation, in control samples and samples containing added proteinase-fraction from milks A and B,

Proteolysis in Mastitic Milk

The consequences of mastitis on milk composition have been reviewed recently. Some of the effects of mastitis on protein components have been described. The latter authors reported the presence of para-k-casein in the milk of animals with clinical mastitis and suggested that many of the changes observed could be attributed to the presence of proteolytic enzymes. Barry have recently confirmed that relatively high levels of proteinase are present and they indicated that the predominant enzyme responsible was plasmin,

the proteinase responsible for most of the much lower levels of activity present in normal bovine milk. Only milks with comparatively modest elevations in cell count from animals with subclinical mastitis were studied in detail however, and their findings did not agree in all respects with results on milk of much higher cell count from animals with clinical mastitis in which several proteinases appeared to contribute significantly to the overall level of activity.

In mastitic milk, proteinase levels may be so high that substantial proteolysis probably occurs in the udder between synthesis and milking, and subsequently a few hours of further incubation at 37 °C may be sufficient for complete hydrolysis of the caseins. The present paper seeks to quantify the separate contributions of plasmin (and its inactive precursor plasminogen) and other proteinases to protein breakdown in mastitic milk, to study the distribution of proteinase(s) between the micellar and soluble phases and to examine the casein fragmentation patterns to enable a 1 comparison to be made with proteolysis in normal milk.

MATERIALS AND METHODS

Intramammary infusions of Escherichia coli endotoxin (2 cows) or of Streptococcus agalactiae (2 cows) were performed as described by Anderson. Reference milk was collected immediately before infusion and samples then collected twice daily for the next 3 d. Milks were immediately cooled and, when appropriate, centrifuged at 1500 g for 10 min, the cream layer and cellular pellet discarded and the skim-milk frozen until required.

RESULTS

When cows were infused with E. coli endotoxin the cell count increased sharply within 15 h of the infusion, and in these experiments, this coincided with an increase in proteolytic activity. Reaction towards infusions of Sir. agalactiae was less rapid and at least 24 h elapsed before the onset of mastitis, when both somatic cell count and proteolytic activity began to increase rapidly. We have previously reported that the peak of proteolytic activity may either coincide with or just precede maximum cell count, and in this study the 2 maxima again coincided following Str. agalactiae infusion, as with the endotoxin experiments. Pooling the data from all 4 cows (2 endotoxin and 2 Str. agalactiae infusions) the relationship shown in Fig. 2 was found between cell count and proteolytic activity.

The contribution of plasmin to the overall level of proteolytic activity was determined by measurements before and after the addition of soyabean trypsin inhibitor (SBTI). The amount of plasminogen present was calculated from measurements of proteolytic activity before and after activation with urokinase. From Table 1, it can be seen that the level of potential plasmin activity (i.e. active plasmin + its inactive zymogen, plasminogen) varied considerably from about 8 units in the milk before mastitis to 17 units in mastitic milk with a cell count of 2.0 x 107/ml.

Most of this was clearly due to plasminogen, however, and the levels of active plasmin were only 1-2 units before mastitis rising to 5-7 units at cell counts above 107/ml. This was in agreement with our hypothesis that plasminogen alone is present in freshly secreted milk. A low rate of activation of plasminogen to plasmin then occurred in normal (before mastitis) samples, but a much higher rate of activation was apparent in high cell count milks.

When the same high cell count milks were centrifuged at 30000 g for 1 h it was found that most of the proteinase activity (10-15 units) was associated with the micellar fraction. Rather more than half of this was probably attributable to plasmin or a similar enzyme and was inhibited by SBTI, but 4-8 units still remained active after SBTI addition and these were clearly due to a different proteinase(s). A further 6-7 units of proteinase activity remained in the serum phase and most of this was not inhibited by SBTI.

Some of this serum activity may have been due to enzymes similar to those in the micellar fraction remaining associated with the small amounts of submicellar and soluble caseins not sedimented by the comparatively mild centrifugation conditions used. This was probably the case with the small amount of

SBTI-inhibitable activity for example, which may have been the same as the plasmin in the micellar fraction. The relatively large proportion of SBTI-insensitive activity, however, suggested the presence of a third proteinase present predominantly in the serum phase. It can also be seen from Table 2 that the sura of the activity in the micellar fraction and that in the serum phase did not together equal the activity in the parent skim-milk. This may be a consequence of a non-linearity in the assay procedure, or may be due to the action of proteinase inhibitors present in the serum phase (Andrews, unpublished observations). Such inhibitors would no longer affect proteinases separated out into the micellar phase, which would therefore show an enhanced activity.

Polyacrylamide gel electrophoresis (PAGE) patterns of proteins in high somatic cell count milks reflected 3 types of change, i.e. those due directly to the influence of the disease or endotoxin (such as increased membrane permeability leading to elevated levels of blood constituents in the milk), those resulting from proteolysis before milking within the udder itself and those occurring after milking during storage or incubations. Comparison of high cell count milks with normal milk from the same animal revealed that a number of minor bands characteristic of casein breakdown by proteinases were present even in freshly secreted milk. It was therefore clear that considerable casein modification by proteinases had occurred in the udder prior to milking.

The most striking change in the proteins of fresh unincubated high cell count milk is the pronounced increase in bovine serum albumin from the blood, but already a number of minor bands, such as the y-caseins and some components of the proteose-peptone fraction, which can be attributed to proteolysis of the caseins, were apparent. After incubation at 37 °C for 24 h, some of the fragments increased, other bands due to further degradation appeared, and particularly in milks with very high cell counts, some bands were subjected to further hydrolysis and decreased in intensity. Generally fn such milks no intact s1- or -casein remained after the incubation.

Peptide fragments produced by proteinase action on caseins during the incubation of high cell count milks could be classified in 2 main categories.

Separation of mastitic skim-milk by high speed centrifugation followed by incubation of the fractions obtained with added heated normal milk to provide casein substrate and PAGE analysis showed that proteinase activity was confined to the micellar fraction. To judge from the casein fragmentation patterns produced during incubation, both groups of proteinases referred to above and present in the initial skim-milk were sedimented with the casein micelles. As would be expected if both groups of proteinases were present, the micelle-associated activity was partly inhibited by SBTI but EDTA had no effect. As in normal bovine milk. when the serum phase of mastitic milk was incubated with an equal
volume of boiled milk at 37 °C for 24 h, the serum phase enzymes caused little change in PAGE band patterns in spite of the fact that quantitatively levels were much higher in serum from mastitic milk (~7-0 unite/ml) than normal milk (~1-5 units/ml). This may indicate that the serum phase enzyme(s) are endo- or exopeptidases or other amino group producing enzymes and not true proteinases. The contributions of the major whey proteins to band patterns in serum fractions were of course reduced by the dilution with heated whole milk.

Immunoreactive b-Casomorphin in Milk

Recently, bovine -casein has been shown to contain amino acid sequences which may give rise to peptides having opiate properties; these have been termed -casomorphins and represent the sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile (-casomorphin-7) or fragments thereof, which are shortened at the C-terminus by one, two or three residues (-casomorphin-6, -5 or -4) -Casomorphins have been shown to behave like opioids of the -type in opioid receptor binding assays or in isolated organ preparations; they are able to elicit analgesia after intracerebroventricular administration in rats. Moreover, the modulation of postprandial insulin or somatostatin release on oral administration of -casomorphins to dogs has been reported.

Evidence has been presented by our group that -casomorphin immunoreactive materials are released from -casein on ingestion of cows milk in the gastrointestinal tract of adult humans; this indicates the possibility of a -casomorphin-induced hormone release after milk ingestion.

To obtain information concerning the possibility that release of -casomorphins might occur in milk on its way from the cow to the consumer, caused by proteolytic action of microorganisms, cows' milk was incubated with various bacterial species frequently found therein and samples were drawn after various incubation periods and assayed for the presence of -casomorphin immunoreactive materials. Some species which also occur in the human intestine were included in the study.

MATERIALS AND METHODS

Incubation of milk with bacteria

Samples (10 ml) of raw milk from a single cow were sterilized (98 °C for 20 min) and inoculated with 13 strains of bacteria belonging to 11 different species (~105 viable bacterial cells/ml milk), viable cells being determined by plate count. Pseudomonas fluorescens subsp. haemolytica (I), Ps. fluorescent subsp. anhaemolytica (II) and Pa. putida (IV) were incubated at 25 °C for 24 d and Ps. aeruginosa (III), Aeromonas hydrophila (V), Esclierichia coli (VI), Enterobacter liquefacien-s (VII), Streptococcus faecalis subsp. faecalis (VIII), Str. faecalis subsp. liquefaciens (IX), Str. faecium subsp. durum (X) Staphylococcus aureus (XI), Bacillus cereus (XII), and Micrococcus sp. (XIII) were incubated at 37 °C for the same time. Milk samples without added microorganisms were incubated for 12 d as controls. When tested in our laboratory, strains I, II, HI, V, VII, IX, XI and XII showed caseolytic activity; the other strains did not. Some strains have been reported to play a role in human infections while developing resistance to antibiotics.

Samples were taken after 1, 2, 3, 6, 12 and 24 d, sterilized by steam (20 min at 98 °C) and stored at - 30°C until further processing. When samples of authentic -casomorphins, heated under analogous conditions or frozen in milk, or samples from various extraction stages as described below were examined no loss of immuno-reactivity was observed.

Extraction procedure

All extraction steps were performed at 0-4 °C. Milk samples (1 ml) were stirred and added to 400 mg charcoal and 2 ml 0-5 M-Tris-HCl buffer, pH 7.4. The suspension was shaken on a ‘press to mix' shaker (Cenco, Breda, The Netherlands) for 30 s and subsequently for 10 min on a high frequency shaker; it was then centrifuged at 8000 g for 5 min and the supernatant discarded. The pellet was resuspended in 2.5 ml methanol, shaken and centrifuged as described above, and the supernatant again discarded. This pellet was resuspended in 5 ml chloroform/methanol (3/1; v/v); after shaking and centrifugation as described above, the supernatant was removed and centrifuged again. The supernatant obtained was evaporated at 56 °C under N2 and the residue dissolved in 0.5 ml 0.1 m-bis-Tris-propane HCl buffer, pH 7.0, and stored at -30°C for subsequent radioimmunoassay or chromatography.

The recovery rates of -casomorphins extracted from non-incubated and non-inoculated milk samples were about 10% ; recovery rates from incubated milks could not be determined exactly and varied considerably, possibly owing to variations in composition and consistency (usually viscid or lumpy) of the incubated milk compared with the non-incubated milk samples. Pilot experiments showed that the recovery could vary depending on the pH of the medium from which the -casomorphins were extracted; however, no difference in recoveries was found between defatted and non-defatted milk. It must be emphasized that only extraction recoveries for authentic -casomorphins could be measured; however, extraction recoveries for structurally unidentified -casomorphin immunoreactive compounds, as found in the incubated and inoculated milk samples, could not be determined per ee.

Syneresis of Heated Milk

It is clearly established that -lactoglobulin (-lg) and k-casein form a complex by thiol-disulphide exchange when the isolated proteins are heated together in solution. It has been suggested that this complex

formation is responsible for the increased clotting time the reduction in total amount of peptides released by chymosin (EC 3.4.4.3) and changes in heat stability when milk is heated. However, because of the complex nature of milk the occurrence of heat induced interaction between -lg and -casein has been more difficult to demonstrate. Tessier et al. failed to separate a -lg/k-casein complex from heated milk and concluded that either complex formation does not occur or its structure must differ from the interaction product obtained when -lg and -casein are heated together in solution. More recent investigations, however, support the existence of such an associated in heated milk.

The effect of -lg/k-casein complex formation on rennej. Coagulation, particularly the primary phase, has been extensively investigated but little attention has been given to the effect of preheating on the process of syneresis. In this work we use artificial micelle milk (AMM) prepared from isolated components (native or modified) to examine this latter effect.

MATERIALS AND METHODS

Low heat skim milk powder and bulk fresh milk were obtained from Dairy Farmers Pty Ltd, Sydney, Australia. Fresh milk was skimmed by centrifugation at 400 g for 25 min (4 °C). a-Lactalbumin (-la) and -Mg, obtained from Sigma Chemical Co., St Louis, MO 63178, USA, were demonstrated to be pure by polyacrylamide gel electrophorcsis. Gels were 7.5% acrylamide and the running buffer was 5 mM-Tris. 40 mM-glycine, pH 8-4. k-Casein was prepared by the procedure of McKenzie & Wake.

Carboxymethylation of whole casein

S-carboxymethyl-whole casein (SCM-whole casein) was prepared by the procedure of Crestfield et al. using guanidine HC1 (6 m) as the disaggregating agent.

Artificial micelle milk

AMM was prepared according to the procedure of Schmidt et al. When required, -la and -lg were added to AMM in solid form and the mixture stirred until complete solution of the added protein had occurred (15-20 min). Milk samples were then held at 30 °C for 30 min before further treatment.

Heat treatment

In all heating experiments milk samples (15-20 ml) were brought to temperature in a boiling water bath and then transferred to a bath at the appropriate experimental temperature. After heating, milk samples were cooled to 30 °C and held at this temperature for 1 h, and if necessary the pH was adjusted to 6.7, before coagulation and syneresis measurements were performed.

Syneresis

Syneresis measurements were performed on 4 ml portions of milk using the microscale optical density dilution assay described by Pearse et al.

Rennet coagulation time (RCT)

Coagulation times were performed in duplicate at 30 °C and judged visually as the time after rennet addition when the first signs of coagulation were detected.

Results

Effect of preheating on syneresis

The effect of neat treatment (80 °C, 10 min) on the syneresis properties of fresh skim milk, reconstituted skim milk (RSM) and AMM are compared in Fig. 1. This relatively mild heat treatment cauaed a significant reduction in the syneresis of fresh and reconstituted skim milk curds (30%). The effect on the syneresis of AMM curd was far less pronounced: 80 °C for 10 min caused only a 7 % reduction to the final extent of syneresis at 120 min. Heating at 80 °C for 10 min caused the RCT of fresh skim milk, RSM and AMM to increase by 75, 60 and 12% respectively. It is known that the RCT of preheated milk can vary considerably during the initial stages of storage, owing to the phenomenon of rennet hysteresis. However, our results showed that there was little change in syneresis or RCT after storage for up to 2 d at 4 °C, so all estimates of syneresis and RCT on heated milk were performed after 1 h equilibration at 30 °C.

Nitrogen Content of Human Milk

In recent years there have been major developments in the field of human milk banking in order to cater for the nutritional requirements of sick and low birth weight infants. Since the quality control and storage of human milk pose problems similar to those faced by the dairy industry, paediatricians have turned for advice to dairy scientists, who are now involved increasingly in research on human milk. Therefore, the extent to which analytical methods designed for studies on bovine milk can be applied to human milk is an important area for investigation.

Nakai have published a simple micro-method for estimating the protein content of bovine milk based on the close correlation of absorbance at 280 nm (A280) and the total N content as determined by the Kjeldahl method. We considered that if this method could be shown to be applicable to human milk, it would provide a simple, cheap and rapid screening procedure for breast milk protein content, a factor that is known to vary considerably e.g. in relation to the postnatal and postconceptual age of the donor. We present here the results of an investigation of the value of this method for estimating the protein concentration of human milk, and compare our results with estimates made on bovine milk and 2 modified, bovine milk-based infant formulae.

MATERIALS AND METHODS

Sample material

Pasteurized bovine milk, samples from 24 h collections of human expressed milk from individual donors and samples from banks of human milk were examined. Many banks are constituted entirely from drip milk which, in about 20% of mothers, drips spontaneously in significant amounts from the contralateral breast during feeding

Protein, g/100 ml

Fig. 1. Portions of bovine milk (x), human expressed milk (O) and 2 bovine mil*k*-baaed formulae („) of known protein content were added to 5 ml 97% (v/v) acetic acid. Individual data points and the calculated line of best fit (*r* > 0.99 in all cases) for the correlation of *A280* with protein content are shown.

We also included 2 bovine milk-based infant formulae that have qualitatively identical protein content but differ greatly in their gross composition.

Sampling procedure

All materials were heated to 40°C in a water bath and mixed thoroughly by repeated inversion before samples were taken.

Absorbance measurements

Following the method of Nakai 50l samples were mixed thoroughly with 5 ml 97% (v/v) acetic acid and A280 was determined with a Pye Unicam SP30 spectrophotometer (Pye-Unicam Ltd, Cambridge, UK) in a 1 cm light path. Measurements were alao made on a series of dilutions of the bovine milk and formulae, and of a single Mmple of expressed milk and banked drip milk to establish the linearity of the method.

N content

Total N and non-protein N (material not precipitated in the presence of 10 % (w/v) trichloroacetic acid) were determined on 0.5 ml samples of human milk and 1 ml samples of the other materials by the Kjeldahl procedure.

RESULTS AND DISCUSSION

Nakai have demonstrated a close correlation between the total N content and the A280 of milk samples diluted in 97% acetic acid. In this study the method has been applied to the determination of the protein content of human milk.

Linearity of the method

We first examined the linearity of the method in our hands. Absorbance measurements were made at 280

nm on a series of dilutions of samples with known protein content (determined by the Kjeldahl procedure). The regression equation obtained for bovine milk (y = 8.48x; -0.058; r = 0.99) agrees well with that reported by Nakai & Chi Le (y = 8.12a; + 0.104; r = 0.99). However, as may be seen in Fig. 1, although the method yielded results that were strictly linear for all the materials studied, each sample had a characteristic slope for the line of best fit.

Applicability of the method to milk stored in human milk banks

Bearing in mind the caveat established above, that the identity of the sample determined the slope of the regression line, we examined 120 samples collected from 3 hospital banks of human milk (40 from each bank). The correlation between A280 and total N was significant; however, at the mean nx6-38 concentration of this population of samples, 1.06 g/100 ml, the error in predicting total N content from absorbance measurements was ± 27 % (95 % con-fidence), which is unacceptably high for most analytical purposes.

Much of the scatter around the regression line is attributable to samples from only one of the 3 centres, and treatment of the data from the other 2 centres as a single group resulted in a reduction of the error of the method in predicting N content to ±17% (95% confidence). At this level of error (±0-18 g/100 ml), the method might have some value as a crude screening test for milk N content.

The milk banks at the 2 centres that contributed to the results shown in Fig. 26 were constituted entirely from drip milk, whereas the other centre actively sought to include expressed milk in its bank. Comparison of the absorbance/N content relationship for the samples from this centre, which had the higher absorbance values, with those obtained for expressed milk samples suggested that the difference in origin of the milk may account for the large scatter around the regression line in Fig. 2a.

Stimulation of Milk Lipolysis

The accumulation of free fatty acids (FFA) in milk results from the hydrolysis of milk fat by lipase. This process, known as lipolysis, may cause the development of off-flavours in milk, a potentially important problem in the dairy industry. Although a number of factors have been identified which affect FFA levels in milk the relative importance of these factors has not been determined. Milk lipase is activated by blood serum lipoproteins and the addition of blood serum to milk enhances lipolysis. The presence of blood serum high density lipoprotein in normal milk has been demonstrated immunologically. Therefore, it appears as originally proposed by Driessen that variation in the amount of lipase activator from blood serum could play an important role in promoting spontaneous lipolysis. Furthermore, it is possible that the primary effect of other factors known to enhance lipolysis such as diet, oestrus, mastitis and stage of lactation is to influence the amount of lipase activator which reaches the milk. However, diet and mastitis may exert their influence on lipolysis by causing changes in the stability of the fat globule. Lipolysis may also be enhanced by milk glycosaminoglycans since the addition of heparin to milk increases lipolysis. The mechanism of heparin-stimulated lipolysis has not been clearly established, but at least 3 factors could be involved. Heparin releases lipase from the casein micelle overcomes inhibition by anions and may directly activate the enzyme. Of these, potentially the most significant effect, in terms of milk lipolysis, is that concerning inhibition. Although the proportion of milk serum lipase increases after heparin treatment this does not appear to be directly related to the accompanying increase of milk FFA levels. In addition a stimulatory effect of heparin on lipase activity in milk has not been demonstrated.

The above observations cast gome doubt on the relative importance of serum activator in promoting lipolysis and this assertion was evaluated in the present work.

EXPERIMENTAL

Methods

Unless specified, all milk samples were obtained at afternoon milking from individual cows in the Institute herd, and immediately cooled in crushed ice. FFA values were determined after storage at 4 °C for 18 h,

unless specified otherwise, by the Cu soap method of Koops as modified by Shipe et. al.
Somatic cell counts were determined by the epifluorescent microscopic technique of Pettipher.
Lipoprotein lipase activity was measured by the method of Nilsson-Ehle & Schotz as previously described.
The response of bulk tank milk lipoprotein lipase to increasing amounts of bovine blood serum in the assay system is shown in Fig. 1. Optimum response occurred at about 60 l serum/ml assay and was linear between 10 and 32 l/ml. The amount of blood serum used subsequently to enhance lipolyria in milk samples was 15 l/ml milk.

Variation between cows

To determine whether the effect of increasing levels of enzyme activator on lipolysis in milk was similar to that in vitro and to investigate possible variation between individuals, blood serum was added to afternoon milk obtained from 10 randomly selected cows and a bulk tank sample at levels between 1.5 and 5.5% (v/v).
The effect of adding 1.5% (v/v) blood serum to afternoon milks obtained from 2 groups of cows during the first 6 months of lactation was also investigated. Group 1 consisted of 16 animals and samples were taken 8, 12,16 and 24 weeks post partum. Group 2 consisted of 13 animals and samples were taken 4, 8, 12, 16 and 24 weeks post partum.

Effect of high cell count

The influence of added blood serum or heparin (porcine intestinal mucosa, Sigma London Chemical Co., Poole, Dorset, UK) on lipolysis was examined in milk samples with elevated somatic cell counts induced by intramammary infusion of Escherichia coli endotoxin or Staphylococcus aureus.
Experiment 1. Endotoxin (Lipopolysaccharide B E. coli, Difco Laboratories. Detroit, Michigan, USA) was administered (1 g in 1 ml isotonic saline) to both left side quarters of 2 cows. The animals were milked with a half-udder machine and samples obtained from 4 milkings before and 8 milkings after the infusion. FFA levels were determined both immediately after milking (0 h) and after storage for 18 h at 4°C.
Experiment 2. S. aureua (25 colony forming units) was infused into diagonally opposite quarters of 2 cows which were quarter milked. Samples were collected directly before, 24 h and 48 h after the infusion. FFA levels were determined both immediately after milking (0 h) and after storage for 18 h at 4 °C. Blood serum 1.6 % (v/v) and heparin 5 g/ml were added to portions of the stored samples.

Analysis of Individual Free Fatty Acids in Milk

Free fatty acids (FFA) in fresh milk normally amount to less than 1 % of the total milk fat. yet they are important, because of their effect on milk flavour. Short chain FFA are responsible for producing off or rancid flavours in milk. The normal measure of milk FFA, the acid degree value (ADV) expressed in mequiv./100 g fat, does not take into account the composition of the FFA. Connolly el al. suggested that as short chain FFA are partly distributed in the skim-milk phase they are not completely detected by the ADV determination. Randolph pointed to the difficulties in relating ADV to organoleptic flavour tests since a milk with a high proportion of short chain FFA but low ADV could have a more rancid flavour than one containing a high proportion of long chain FFA and a high ADV.
The methods available for the determination of FFA composition in milk and other dairy products involve silicic acid - KOH column chromatography or thin-layer chromatography. Both require concentration, by evaporation, of the extract. These methods are time consuming particularly with large numbers of samples and may lead to loss of volatile fatty acids during evaporation. Where ion exchange resins have been used the type of resin is not ideal for use in non-aqueous and non-polar media. Quantitative recovery of FFA from Amberlitc IRA 400 and Dowex 3 is reported to be very difficult particularly for short chain acids.
A satisfactory method for the quantitative determination of milk FFA should fulfil the following requirements: the presence of the other lipids of milk should not interfere with extraction or estimation of the FFA; quantitation of individual acids should not be influenced by the chain length (molecular weight) of each acid;

and the range of FFA concentrations found in milk should be within the scope of the method. The procedure described in the present paper meets these requirements. The method involves a modification of the lipid extraction described by Salih et al. followed by FFA extraction and methylation adapted from the method of Edwards-Webb.

MATERIALS AND METHODS

Preparation of Amberlyst 26 ion exchange resin

Amberlyst 26 (BDH Chemicals Ltd, Poole, Dorset, UK) is a strongly basic anion exchanger especially suited for use in non-aqueous media, and described as having a macroreticular structure. Before use 20 g resin were shaken for 15 min with 200 ml M-NaOH, and washed 3 times with 300 ml CO2-free distilled water. The resin was shaken for 10 min between each wash. Finally it was washed 3 times with 150 ml methanol, shaken for 10 min between each wash and stored ready for use under methanol.

Each preparation of resin was checked by extraction and gas-liquid chramatography (GLC) as some batches gave rise to spurious peaks when examined by GLC.

Extraction of lipid

Milk (30 ml) was added to an ice-cold mixture consisting of 10 ml HCl (35% v/v aqueous HCl), 64 ml diethyl ether and internal standards of n-valeric acid (C5:0) (BDH Chemicals Ltd) and tridecanoic acid (C13:0) (Fluka AG, Buchs, Switzerland). A 100 ml screw-capped bottle, having a PTFE insert to seal the cap and containing the extraction mixture, was shaken by hand for 3min and centrifuged at 2000; for 10 min at 4 °C. A sample (30-45 ml) of the ether layer was transferred to a bottle containing 1 g anhydrous Na2SO4 (BDH Chemicals Ltd). After drying for a minimum of 20 min the ether was decanted into a measuring cylinder and the Na2SO4 was rinsed with further portions of ether. The rinsings were transferred to the measuring cylinder until the volume of ether had reached 50 ml.

Adsorption of FFA

The ether extract of milk was transferred to another 100 ml screw-capped bottle and 25 ml methanol were added followed by 200 mg Amberlyst 26 resin. The bottle was shaken for 1 h on a horizontaflWicnanical shaker (120 shakes/min). The solvent was removed and the resin washed 5 times with 15 ml ether/methanol mixture (2:1 v/v) after which it was dried at room temperature under a gentle stream of N2.

Preparation of methyl esters

The dried resin was transferred to a 1.5 ml GLC sample vial and 300 l 5% (v/v) HO in methanol were added. The methylating reagent was prepared as described by Carroll care is required as these constituents may react violently. The vial was capped and left in the dark overnight at room temperature. Methyl heptadecanoate (Me C17:0) (Fluka AG), was included as a recovery indicator. An appropriate volume of a standard solution of Me C17:0 was evaporated to dryness in the vial before addition of the resin. Following overnight methylation 150 l ether were injected through the cap and the vial gently shaken for 2 min. The cap was removed and 1 ml saturated NaCl which had been previously mixed with and stored under ether was added to partition the ether from the methanol and resin. The vial was resealed and gently shaken for 20 s. Between 1 and 4 l of the ether phase were removed for chromatography.

Gas chromatography conditions

Samples were chromatographed on a Dual FID Hewlett Packard 7620A Research Chromatograph (Hewlett Packard, Winnersh, Wokingham, Berks, UK). Two stainless steel (2.07 m 1/8" o.d.) columns were packed with 20% stabilized ethylene glycol adipate (Analabs Inc., North Haven, Conn. 06473, USA) on Diatomite C-AAW 100-120 mesh (JJS Chromatography Ltd, King's Lynn, Norfolk, UK). The N, carrier gas flow rate was 31.5 ml min-1 and the temperature was programmed from 80 °C, with a 4 min hold, rising at 10 °C/min to 200 °C held for 40 min. The instrument was linked ‘on-line' to a PDP8 chromatographic data processor (Digital Equipment Co. Ltd, Reading, Berks., UK) The output of the processor gave the percentage contribution by weight of the individual fatty acids and, by reference to the internal standards, the

concentrations (mg/100 ml milk) of the individual fatty acids. For C4:0 and 06:0 reference was made to the 05:0 standard and for acids of greater chain length the C13:0 standard was used. The percentage recovery of these internal standards was calculated by reference to the Me C17:0 recovery indicator.

Preparation of FFA mixture

The level of FFA in the majority of normal milks has been reported to lie within the range 0.1-2.5 mequiv. palmitic acid/1. A mixture of FFA was made, which had a composition similar to that found in normal milks, from authentic standards. From a stock solution, containing a total weight of mixed FFA of 28 mg ml-1, samples were taken for methylation, by the method outlined by Storry et al. GLC was used to confirm the composition of the mixture. Portions of the stock solution were diluted 50-fold in ether and samples of the diluted mixture, increasing in FFA content from 0.08 to 2.5 mequiv./l, were included in the extraction procedure. Water (30 ml) replaced the milk and the volume of ether was adjusted to accommodate the volume of the FFA mixture.

Milk Treatment and Curd Structure

It is well established that milk composition and treatment affect cheese body and texture. This appears to be due to effects on both cheese composition and the microstructure of the coagulum. The latter seems to be a major factor determining the structure and texture of cheese.

Both concentration by ultrafiltration and homogenization influence the cheese-making properties of milk and cheese texture. Cheese made with concentrated milk tends to be hard and granular, because of excessive segregation of the fat and protein resulting from a coarser than normal protein network, with high fat losses occurring during the manufacture. Homogenization of milk reduces both curd firming and syneresis, so more moisture than normal is retained in the cheese. It also results in increased fat retention and a softer, smoother and more elastic cheese.

The aim of this work was to investigate whether the adverse effects of using concentrated milk for cheesemaking might be overcome by introducing a homogenization step. The first part of the paper describes the effects on the milk of processing in 2 ultrafiltration (UF)-plants and the effects of concentration with or without homogenization, and also conventional homogenization, on the formation, structure and properties of curds. The second part describes the cheesemaking properties of milks processed in the 2 UF-plants, with and without much concentration.

Experimental

Preparation of processed milks

Fresh milk was obtained from Institute herd of Friesian cows and was pasteurized at 72 ºC for 17 a before further treatment. Homogenization was carried out at 72 °C in a 3 piston homogenizer (Manton-Gaulin Mfg Co. Inc., Everett, Mass., USA) in 2 stages at 14 and 3.4 MPa. Treatment in the UF-plants was of about 1.2 h duration at 50 °C, and control milk for cheesemaking was held at 50 °C for 1.2 h. During the UF-processing the permeate was either removed continuously to produce concentrated milk or returned immediately to the feed to produce minimally concentrated milk. Plant A (Abcor Inc., Wilmington, Mass. USA) had a spirally wound membrane, designated HFK 131, area 12 m8, nominal mol. wt cut-off 3000. Plant B used a tubular membrane, area 7 m', specified as capable of retaining 98 % of the protein from cheese whey. In the context of this paper, the relevant differences between the 2 plants were in the rates of flow of the milk, the degrees of turbulence and the modes if operation. Only in the pumps, pipes and valves were there factors of any consequence, for it had been shown that no reduction of fat globule size occurred in the modules. Both plants had the same size pipework, but Plant B had the larger pump. Bates of flow were 150 and 3751 min-1 giving Reynolds ‘numbers for the connecting pipes of 32000 and 80000 in Plants A and B respectively. Both exceeded the critical values of Reynolds' number with the greater turbulence occurring in Plant B. Whereas the operation in Plant B was a batch process in which the milk pressure dropped to atmospheric on each passage through the pressure-retaining valve, Plant A had an inner

recirculation loop which avoided such repeated pressure reductions.

Analyses of milk

The concentration factors of milk were taken as the mean of those for fat and casein, determined by standard methods. The fat content of the aqueous phase of milk was determined after separation of the phases by centrifugation for 15 min at 400 g at room temperature.

Fat globule diameters were measured with the Coulter Counter Model A using a 50 m orifice and a volume of 0.05 ml. The dilution rate was clone to 104 in 0.9% NaCl (w/v). Counts were obtained over the size range 1.25-10.25 m. The results are given in terms of the median size within the range covered by the counter. Thus, fat globules too small to be detected have been ignored.

Four electron micrographs at x 37500 final magnification were prepared from each milk as described by Green et al. Measurements of profiles of fat globules and casein micelles were made on 2 prints of each sample taken at random (total area 42.2 m2), using a semi-automatic image analyser.

Measurement of formation, properties and structures of curds

Milks were adjusted to pH 6.55 with HCl and held at 5 °C overnight. They were then treated at 30 °C with cheesemaking rennet (Chr. Hansen Ltd, Reading, Berks. UK), 0.26 ml/1 for control and minimally concentrated milks and 0.23 ml/1 for milks concentrated about 2-fold. The rennet clotting time (RCT) was determined visually. The curd firmness was followed over 40 min with an Ultra-Viscoson 1800, as described by Marshall et al., the final value being corrected by subtraction of the initial value. Syneresis of curd cut at 40 min was determined over 40 min as described by Marshall and expressed as the first order rate constant, assuming that the partial specific volumes of fat and casein are 1 ml/g.

Transmission electron micrographs of curd samples were prepared and the frequency distributions of aggregates containing denned numbers of casein micelles were determined as described by Green et al. Scanning electron micrographs (stereo pairs) and light micrographs of curd samples were prepared, and the degrees of coarseness of the protein networksin the light micrographs were determined using a grid as described by Green et al.

Cheese-making and analyses of cheeses

Cheddar cheeses were made in 3 matched vats using 150-190 milk and rennet at 0.26 ml/l for control and minimally concentrated milks and 0.23 ml/1 for milks concentrated about 2-fold. Otherwise, the cheesemaking procedure of Chapman et al. was followed, starter NCDO 924 being used and titratable acidity and pH measurements being made on milks and wheys. The cheeses were ripened at 12-13 °C. Fats in wheys and cheeses and moisture in curds and cheeses were determined by standard methods. The extent of lipolysis in the milk added to the cheese vat was determined as the acid degree value (ADV) by the method of Koops & Klomp. Cheeses were assessed at 26 weeks of age for the total free fatty acid (FFA) contents by a modification of the method of Harper el al. and for texture by a sensory method using a trained panel.

Fermentation of Goats Milk

In Norway interest has increased recently in the use of goat's milk and goat's milk products. In order to develop such products, more information is required concerning goat's milk as a medium for different starters. The most common starters used in the dairy industry in Scandinavia and several other countries in Western Europe for production of most cheese types, butter and cultured buttermilk are mixed cultures of DL-type. Cogan showed that Leucotwstoc cremoris and Streptococcus lactis subsp. diacetylactis behaved entirely differently in pure cultures from mixed cultures, and in our laboratory this has been confirmed for Leus. cremoris. Our aim was to obtain information of scientific as well as practical value, and this investigation compares the bacterial growth of and production of some important aroma compounds by 2 commercial DL-type starters grown in cow's and goat's milk. Citrate is the precursor for many of these compounds in starters. As preliminary investigations showed that the content of citrate in goat's milk

decreased during lactation, samples of goats-milk from the early and late lactation periods were examined.

MATERIALS AND METHODS

Starter cultures

Two different DL-type mixed strain starters were used: (1) Christian Hansen 01 (CH01), and (2) Flora Danica normal (FDn) lyophilized starter cultures of the redi-set type both obtained from Chr. Hansen Laboratories A/S, Copenhagen, Denmark. In mixed commercial cultures the ratio between species and strain composition may vary, and the declared composition of CH 01 was *Streptococcus cremoris* (70-75%), *Str. lactia* (1-5%), *Str. loctis* subsp. *diocetyloctis* (15-20%), *Leuc. cremoris* (5-10%), and of PDn was Str. *cremoris* (75-85%), *Str. loctis* subsp. *diocetyloctw* (10-15%) and *Leuc. cremoris* (5-10%). The starter cultures were received in pouches and stored at 20 °C until used. One pouch was sufficient to make 200 1 bulk starter. To prevent variations in the cultures during the experiment, pouches for the whole investigation were ordered from the same production batch at Chr. Harisen laboratory for both cultures.

Propagation. Starters were grown at 21-5°C, and 1% inocula were used in experiments.

Milks

Skim-milk from bulk collected cow's milk and 2 samples of skimmed goat's milk from the University herd with 70 goats in the same stage of lactation were used in the trials. Goat's milk I was from the first part of lactation (2-3 weeks after parturition) and goat's milk II was from the latter part of lactation (8 months after parturition). All milks were spray-dried in a Niro laboratory spray drier (Niro Atomizer, Copenhagen, Denmark) and reconstituted before use.

Experimental procedure. At the start of every experiment, one pouch which was intended for 200 1 bulk starter was mixed in 1 1 reconstituted sample of either goat's milk I, goat's milk II or cow's milk. Twenty five ml of this mixture was transferred to 5 1 heat-treated (115 °C for 5 min) reconstituted milk of the corresponding type. The inoculated milk was thoroughly mixed, and 9 portions of about 450 ml distributed into sterile bottles which again were incubated in a water bath at 21.5 °C. Single bottles were removed every 2.5 h for determination of pH, volatile aroma compounds, acetic acid, and bacteriological composition.

Two additional 100 ml flasks were filled with inoculated milk and closed with rubber stoppers. Electrodes for measuring dissolved O, and redox potential were placed in the milk through holes in the stoppers and kept there during the whole incubation period.

Chemical analyses

The content of dissolved O2 was measured with a YSI O2 meter (Model 57, Yellow Springs Instruments Co., Yellow Springs, Ohio, USA) and the redox potential registered with a platinum plate electrode P101 v. standard calomel electrode (SCE) K401 connected to a pH/MV meter 28 (Radiometer A/S, Copenhagen, Denmark). Citric acid wm determined according to the method of Marier & Boulet. The content of acetic acid was measured by weighing 5 g of the milk culture in a 50 ml flask, adding 10 drops of 50 % H2SO4, 2 ml valeric acid (5 mg/ml) as internal standard, 25 ml diethylether and 25 g of Na2SO4. After shaking for 30 min, the sample was analysed by gas chromatography. The concentrations of volatile aroma compounds were measured by the method of Svensen modified by Jonsson and Pettersson.

Production of CO2 was determined by Warburg technique according to the IDF method except that 3 ml milk and 0.3 ml NaHCO3 were used. Both 10 and 1% inocula were used in the Warburg flasks and the samples were incubated at 21.5 °C.

Bacteriological analyses

Total counts of bacteria were made by plating on Eugon agar and numbers of citrate fermenting bacteria were estimated according to the modified method of Nickels & Leesment. Leuconostocs and *Str. lactis* subsp. *diacetylactis* were differentiated by transferring those colonies giving a clear zone in the calcium citrate agar to litmus milk reduction of litmus milk was considered as indicating *Str.*

tactis subsp. *diacetylactis.*

Lipolysis in Deep Frozen Raw Sheep's Milk

The population of milking ewes was estimated in 1988 to be around 10000 in the British Isles and this figure was expected to rise. The sheep dairy industry is much larger in some other EEC countries such as Greece, Spain and France. Typically, lactation lasts for 6 months with yields averaging between 70 and 150 kg per head, depending on breed. As much as 70% of ewes' milk is used in cheese production.

In order to obtain a sufficient quantity of milk for processing it may be necessary to store raw milk until a large enough bulk has accumulated. Freezing the fresh milk may allow the milk to be stored for the required length of time. However, such a process could have adverse effects on product stability. The aim of the present work was to examine the effect of storage temperature on the stability of the emulsified fat. Lipolytic breakdown of the fat was measured in milks held at three different temperatures at intervals during 6 months storage. Rates of lipolysis were also determined in the samples after they had been removed from the deep freeze and had thawed to 4 °C. Lipase activity was measured in the fresh milk and again at the end of 6 months storage. By measuring the concentration of solvent-extractable fat it was hoped that the damage sustained by the fat globules as a result of the storage conditions could be assessed.

MATERIALS AND METHODS

Fresh raw sheep's milk, from three separate farms, was chilled to 4 °C and transported to the Institute. Within 3 h of milking the milk was divided into portions for imim diate assay and for freezing. The portions were placed in three separate freezers with nominal temperatures of -12, -20 and -27 °C. Further analyses of the samples were carried out after storage for 1, 4, 12 and 26 weeks.

Free fatty acid (FFA) concentration was measured by the Cu soap method of Koops & Klomp as modified by Shipe et al. FFA concentration in fresh milk was assayed on arrival at the Institute (FFAFM) and again after 24 h at 4 °C. The extent of FFA development in the frozen milks was determined at the end of the deep-freeze storage period (DFFA), frozen samples being thawed rapidly to 4 °C in a water bath at 20 °C for immediate DFFA determination. DFFA values were corrected for the initial value (FFAFM). The potential for lipolysis in the thawed samples was recorded as the concentration of FFA released after a further incubation (1FFA) at 4 °C for 24 h (the DFFA + FFAFM values were subtracted from the values measured at the end of the incubation to give IFFA values). Results were subjected to analysis of variance and linear regression. DFFA and IFFA values were analysed for the main effects of herd, storage time and temperature and for interactions between these factors.

Lipoprotein lipase (LPL) activity was measured in the fresh milk and again after 26 weeks storage at the three different temperatures. 3H-triolein was used as the substrate and incubation was for 10 min at 20 °C according to the method of Nilsson-Ehle & Schotz.

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