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ASPECTS OF THE CHEMISTRY OF MILK PROTEIN
FILMS FORMED ON STAINLESS STEEL

by

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ABSTRACT

The literature on the properties of stainless steel surfaces, the physico-chemical properties of milk and the surface chemistry of proteins was reviewed to gain an insight into the formation of protein-mineral films ("milkstone") on the contact surfaces of dairy milk lines. Protein adsorption processes were considered fundamental to the formation of such deposits. Standard methods of protein separation and purification were employed to obtain purified α_{s1} , β , and K-casein.

Radiochemical methods provided a convenient means of studying protein adsorption on to low surface area systems. Of the various methods for labelling proteins, radioiodination by a slightly modified version of Hunter and Greenwood Chloramine T method proved to be the most satisfactory. By this method, stable labelled milk proteins of high specific activity containing not more than one iodine atom per protein molecule and having properties that could not be distinguished from those of unlabelled proteins were prepared.

In the course of the work several further aspects of the iodination reaction and the reactions of iodine species with milk components were investigated. Iodination efficiencies were found to be sensitive to the storage time of the radioactive iodine stock solutions (supplied free of reducing agent at pH 8-11). The drop in iodination efficiency was correlated with an increase in the concentration of oxidised iodine species in the radioactive stock solutions. Studies of the iodination of whole milk showed that

with the exception of K-casein, the tyrosyl residues of all proteins in milk were iodinated with approximately the same iodination efficiency. The iodination efficiencies of K-casein tyrosyls were significantly lower. A theoretical examination of the effect of the hydrophobicity of the molecular environment on tyrosyl iodination efficiency, indicated the likelihood that the effect observed was due to the incorporation of the K-casein in inaccessible regions of the micelle rather than a molecular environment effect.

Iodinated caseins, when added to whole skim milk became bound to the micelle fraction. In the case of β and K-casein, the binding was temperature dependent and at least partially reversible. α_{s1} -casein binding to micelle was largely independent of temperature and irreversible. The stabilising effect of calcium ions on diluted micelle suspensions was demonstrated. The variation in the K-casein content of casein micelles with micelle size was also demonstrated. When inorganic iodine was added to milk, part of this became encapsulated by milk micelles. However, there was virtually no chemical binding of the iodine to the milk proteins of cooled milk.

Techniques were developed to study the surface chemistry of radioactively labelled milk proteins at stainless steel-water interfaces. The amount adsorbed was calculated according to the principle of isotope dilution. Autoradiography and high resolution autoradiography, using nuclear track emulsion and scanning electron microscopy confirmed the uniform distribution of the adsorbed protein. Studies of adsorption from whole skim milk, whey and milk serum, indicated that of the various milk proteins serum caseins

had the greatest affinity for stainless steel surfaces. Adsorption data for purified caseins and mixtures of casein indicated that β -casein was the species preferentially adsorbed. Generally it was observed that adsorption was largely irreversible and providing adequate time was allowed, proceeded to form a relatively constant close packed film of adsorbed protein molecules. The amount of protein in the films ranged from 2.0 mg m^{-2} to 3.0 mg m^{-2} irrespective of protein concentration, except at high concentrations of β -casein and to some extent, α_{s1} -casein, where a second adsorption process seemed to be present. This effect also seemed to be present in studies of adsorption from whole milk.

The kinetics of adsorption and desorption were monitored by tracer techniques. The data for adsorption from unstirred solutions indicated the process was diffusion controlled. A diffusion model was developed which accounted for the rate of adsorption and the amount adsorbed in terms of the size, shape and hydration of the protein molecule, the viscosity of the medium and the effective surface area of the stainless steel surface. The model was used to estimate hydration and axial ratio values for β -casein. Values of 1.9 grams of water per gram of protein for the hydration, and 11 for the axial ratio ($\frac{a}{b}$) were obtained.

Desorption of adsorbed protein is a complex process that does not obey simple rate laws. Desorption was facilitated by hydroxyl ions and anionic surfactants but no simple relationship between rate of desorption and species concentration was obtained.

Simple adsorption process cannot account for the formation of macroscopic milk protein films on milk contact surfaces. After

the surface has been covered by the initial adsorbed layer, no further protein could be adsorbed until the surface of this layer was modified in some way, for example, by the adsorption of positively charged colloids of iron oxide, chromium oxide or calcium phosphate. Deposition by evaporation of solution films, or precipitation from solution are other mechanisms by which macroscopic films may be formed. The film thicknesses of the drained milk films formed after immersions of clean stainless steel surfaces into dilute milk solutions remained independent of dilution at $3.8 \pm 0.6 \times 10^{-4}$ cm. The properties of protein films formed at surfaces can be altered by thermal denaturation, chemical modification and also presumably by bacterial action.

Analytical and microscopic examination of actual milkstone deposits generally supported a previous milkstone classification based upon chemical analysis, i.e. fat deposits, high protein deposits, calcium phosphate milkstone and iron milkstone. As a result of the present investigation the further classification of high protein deposits into chlorine-induced (from sodium hypochlorite) and phosphate-induced (from phosphoric acid) was suggested. It does not seem likely that milkstone deposits are formed by a single mechanism. This conclusion was supported by an analysis of data for deposits formed in simulator trials where evaporation of milk films (in one simulator) and precipitation induced by hypochlorite (in two simulators) were major factors. The variety of possible mechanisms for deposition is due to the range of conditions that can occur in milk lines. The results of the present work will assist in determining the mechanism operative in a particular case and will allow the development of better procedures for the removal and prevention of such deposits.

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LIST OF SYMBOLS

a	length of equatorial semi-axis of ellipsoid
b	length of semi-axis of revolution of ellipsoid
c	concentration of protein solution
D	diffusion coefficient
E	extinction coefficient
G	geometry correction factor
I_R	relative iodination efficiency rate
k_i	rate constant of process i
M	molecular weight
N_r	number of moles of amino acid residues r
r	radius of diffusion molecule
R	ideal gas constant
t	time
T	temperature
$\bar{v}$	partial specific volume
w	hydration of protein
η_T	viscosity of solvent at temperature T
Γ_c	amount adsorbed or retained at concentration c
Γ_{sat}	amount adsorbed at saturation
σ	standard deviation
$H\Phi$	hydrophobicity measurement based on free energy for transfer of amino acid from organic to aqueous environment
NPS	hydrophobicity measurement based on number of hydrophobic amino acid residues divided by total number of amino acid residues
p	hydrophobicity measurement based on volume of amino acid residues that are polar divided by volume of non-polar residues
θ	fraction of surface coverage
k	retention coefficient
ρ	density of solvent

CHAPTER 1

INTRODUCTION

The Dairy Industry is of major importance to the New Zealand economy. Of the total 1976-77 export earnings of \$3228 million (1) the Dairy Industry accounted for \$540 million or 17% of the total. Currently the most important dairy export is butter which accounts for 46% of the dairy export earnings. Other dairy products listed with their percentage of total dairy exports are: cheese (16.9%), skim milk powder (15.5%), casein (11.6%), whole milk powder (7.1%) and other by products such as lactose etc. (2.0%) (2). These exports make New Zealand the world's largest exporter of dairy products.

Over recent years access to international markets has, through a variety of geopolitical events become increasingly tenuous. In addition the industry's ability to sell its products overseas relies on it being able to meet the stringent standards imposed by the importing countries. Maintenance of these standards is made all the more difficult by the large distances between producer and consumer. The continuing viability of the industry in the face of intense competition and political restrictions can be attributed to highly efficient farming techniques combined with efficient and modernised processing facilities, and the fact that large areas of New Zealand have soil and climatic conditions ideal for grassland farming requiring only limited supplementary winter fodder.

One of the major problems involved in the maintenance of high quality dairy produce is the control of deterioration induced by the presence of bacteria. Supervision of dairy hygiene on farms is the responsibility of 72 Farm Dairy Instructors and 15 Dairy Advisory Officers (Farms). A further 32 Dairy Product Officers and Dairy Advisory Officers (Manufacturing) monitor the hygiene in dairy factories and milk tankers. In addition, dairy products for export must satisfy the New Zealand Government, Dairy Division Grade Laboratories' requirements as regards chemical and bacteriological analysis. Finally, in order to encourage farmers to cooperate in maintaining high standards of hygiene, dairy factories offer a pay incentive based on the milk's bacterial count. Over recent years this grading standard has been progressively raised. In spite of these precautions, the down grading and the non acceptance of export dairy produce is estimated to cost New Zealand \$40 million annually in lost export earnings (3). This down grading can be due to any one of a number of product defects originating from the following aspects: animal health, farm practice, processing, packaging, transportation and storage. Very often downgrading follows as a direct consequence of bacterial contamination.

A major source of bacterial contamination of milk and milk products is the bacterial colonies that are associated with protein containing films that form on the contact surfaces of farm milk lines, tankers and factory processing equipment. It is the understanding of the nature of these films that provided the motivation for the investigation reported here.

This thesis is specifically concerned with the study of milk protein interaction stainless steel surfaces and the factors that result in the formation of bacteria supporting deposits. Chapter 2 reviews the literature and background information relevant to the overall investigation. Much of the experimental work relied on the use of radiochemical techniques and the procedures to prepare the labelled milk constituents are described in Chapter 4. The separation of these constituents is described in Chapter 3. A major constituent of milk is the casein micelle. Some investigation of the labelling and exchange reactions undergone by this colloidal species are described in Chapter 5. Studies of adsorption and desorption of milk proteins are described in Chapters 7,8,9 and 10. Attempts to produce macroscopic protein films of sufficient thickness to support bacterial colonisation are reported in Chapters 11 and 12. The results of a study of a limited number of deposits are reported in Chapter 13. Finally the results of all these investigations and their possible relevance to the practical dairy problem are discussed in Chapter 14.

CHAPTER 2

LITERATURE SURVEY AND BACKGROUND TO PROJECT

2.1 Introduction

The interaction of proteins at interfaces is of widespread importance in biological and technological systems and has been the object of scientific study from as early as 1840 when Ascherson (4) observed that a skin of protein formed spontaneously around oil droplets suspended in a solution of egg albumin. Since these early studies the interactions of proteins at liquid-liquid and liquid-air interfaces have been extensively investigated. The denaturation of the adsorbed protein was observed at an early date. For example Devaux (5) in 1903 showed that the collapse of protein films at the air-water interface led to the formation of insoluble artificial fibres. Similarly Ramsden (6) noted that egg albumin was precipitated from solution subsequent to being adsorbed at the air-water interface. Wu and Ling (7), in studies of the phenomena of "surface denaturation", noted that shaking solutions of proteins such as egg albumin oxyhaemoglobin and methaemoglobin resulted in irreversible precipitation.

Unlike the protein adsorption effects at the air-water interface, which can be readily studied by surface tension effects, the monitoring of protein adsorption at the solid-liquid interfaces is technically difficult. Until recently investigation of such systems relied on detecting protein adsorption by analysing the decrease in solution concentration.

The development of methods to monitor adsorbed protein directly has led to renewed interest in the phenomena (see Section 2.6).

A major motivating factor for research into protein interactions at solid-liquid interfaces is the importance of such interaction in science and technology. Everyday examples of the formation of such films include the protein film observed on cell membranes, the thrombogenic protein films formed on foreign surfaces such as artificial cardiovascular systems and kidney machines, the protein incorporated into the pellicles, plaque and calculus formed on teeth, the use of protein in adhesives, paints and plastic coating and the formation of kidney stones. The films that stabilise the dispersed fat in blood and milk are examples of protein adsorption at liquid-liquid interfaces. Examples of protein adsorption at liquid-gas interfaces include protein films formed on the alveolar of lungs (8), the protein films that stabilise foams, e.g. in food technology as in the preparation of ice cream and cooking mixtures. Of particular importance in the Dairy Industry is the formation of protein films on stainless steel and the contact surfaces involved in milk processing. It is the chemistry of such films that forms the topic of this thesis. In the sections that follow (Section 2.2 to Section 2.11), the literature describing the properties of stainless steel surfaces, the composition and physico-chemical properties of milk with special emphasis on the casein constituents and the surface chemistry of proteins will be reviewed to give a background to the project. Also included in this review will be

a summary of the literature on macroscopic protein films and accretions including "milkstone" and other milk deposits. Some mention will also be made of the detergents and cleaning procedures employed to combat the formation of such deposits. Although some of the material contained in this review is not directly relevant to the experimental sections that follow, it was felt that since the present project represented the initiation of a new research interest at the University of Waikato, a general survey was warranted.

2.2 The Properties of Stainless Steel

Since December 1977, New Zealand Government legislation has required that all surfaces that come into contact with milk during processing be of stainless steel or some other approved material. The aim of this legislation was to achieve a better standard of dairy hygiene by allowing the use of more efficient but more corrosive detergent systems. Stainless steel also prevents electro-chemical corrosion that results when milk lines are constructed of a number of different metals such as brass, tin and nickel plated copper (9). Stainless steel is an ideal metal for use in applications requiring a high standard of hygiene, e.g. food processing. It has a hard readily polishable surface of high resistance to corrosion and can be easily extruded, rolled, pressed and welded to the requirements of a given processing design. There are a variety of types of stainless steel available and these have different properties. The type favoured by the food processing industries and in particular the dairy industry, are the austenitic

group, given the American Iron and Steel Institute (AISI) type numbers 302, 304 and 316. Type 316 is used in the dairy industry only where a hard surface is required such as in an evaporator. The compositions of types 302, 304 and 316 are given in Table 2.1.

Table 2.1
Composition of Stainless Steel used in the
Dairy Industry

Type	302	304	316
Percentage Composition			
Chromium	17-19	18-20	16-18
Nickel	8-10	8-12	10-14
Carbon	<0.15	<0.08	<0.08
Manganese	<2.0	<2.0	<2.0
Phosphorus	<0.045	<0.045	<0.045
Sulphur	<0.03	<0.03	<0.03
Silica	<1.0	<1.0	<1.0
Molybdenum	-	-	2-3
Iron	68-72	65-71	62-69

The usefulness of stainless steel is a result of the passivity of its surface. "Passivity" is defined as a condition in which a metal is protected by an impervious layer. Numerous attempts have been made to identify the oxide film on stainless steel. Initial evidence indicated that the film was predominantly chromium oxide (11,12). The chromium appears to be at a much higher concentration on the surface than in the bulk phase. Stainless

steel has a bulk phase chromium concentration of approximately 12% whereas the surface constituents of stainless steel have been reported to be 40% chromium and 60% iron. No nickel was detected at the surface (11). Although no information appears to be available for the isoelectric point of stainless steel, that of chromium oxide is known to be approximately 7.0 (13). Stainless steel could be expected to have a similar value. The isoelectric point of iron has been reported to vary between 5.2 and 8.6.

Recent evidence using Auger electron spectroscopy has indicated that large amounts of iron and carbon are also present on the surface of stainless steel (14). After heat treatment of stainless steel to 300 °C, soft X-ray spectroscopy showed the presence of carbon, oxygen, calcium, chromium, manganese, iron and nickel (15). Such studies indicated that the surface composition of stainless steel depends upon surface treatment (16).

Stainless steel is highly resistant to corrosion by alkaline and weak acid solutions, but is attacked by some strong acids such as hydrochloric and sulphuric acids. Concentrated nitric acid (above 20% by weight), because of its oxidising properties, is well known to render the stainless steel surface passive. Concentrated chloride solutions however, attack stainless steel (17,18,19,20). Various methods have been developed to test the corrosiveness of dairy detergents (21,22).

The surface properties of stainless steel are modified by the method of fabrication. The 2B finish, usually used in the dairy industry, results from temper rolling. In this process the cleaned and annealed sheets of steel are lightly rolled with cold polished

rollers (23). Often the 2B finish is polished to give a number 3, 4 or 7 surface. The numbers 3, 4 and 7 represent degrees of polishing with No. 7 being a pit free surface with almost a mirror polished surface. No significant differences have been found in the relative cleanability of these different surfaces when measured by bacteriological tests (24, 25). Stainless steel with a 2B finish exhibits a contact angle of 91° with water (494) and is thus hydrophobic. The absolute surface area as measured by the adsorption of ethylene, has been reported to be up to three times the geometric area (495). It has a root mean square average roughness height of between 0.25 and 0.40×10^{-5} mm (540).

2.3 The Composition and Physico-Chemical Properties of Bovine Milk

Milk is a complex physico-chemical system consisting principally of protein, fat, carbohydrate, inorganic ions, minor enzymes and vitamin constituents and water which provide an ideally balanced initial food supply for mammalian off-spring (26). The fat and the protein constituents exist in two unique biocolloids: the fat globule and the casein micelle.

The weight percentages of the various components can vary widely among the different bovine species in response to a long list of physiological inherited and environmental factors. Daily composition differences can be observed even from the different quarters of the udder of a cow (depending on how the cow is milked) (26). Data for the composition of milk produced by a typical New Zealand dairy Jersey and a typical New Zealand dairy Friesian cow are summarised in Table 2.2.

Table 2.2

Milk Composition (27) (Weight Percentages)

	Jersey Cow	Friesian Cow
Carbohydrate	5.08	5.08
Fat	5.20	4.86
Protein	3.85	3.65
Ash (Inorganic Ions)	0.70	0.71

The principal carbohydrate constituent of bovine milk is the disaccharide lactose, $C_{12}H_{22}O_{11}$, which is made up from the two monosaccharides D-glucose and D-galactose. It is the bacterial conversion of lactose to lactic acid that is principally responsible for the lowering of the pH that occurs in the initial stages of the souring of milk.

Of the fat constituents of milk, 95-96% are triglycerides of fatty acids, while the remaining 1-1.5% are diglycerides, 0.80-1.0% phospholipids, and also a range of minor lipids such as keta acid glycerides, sterols, carotenoids, vitamins and traces of free fatty acids (29). Apart from the free fatty acids, the fat components are embodied in fat globules. These consist of an inner layer of tightly bound triglycerides, fatty acid, sterols, vitamins, etc., and an outer layer of lipids, proteins and enzymes which stabilise the globule (30). A rancid flavour sometimes observed in milk and milk products is due to the accumulation of free fatty acids that have been cleaved from the fat globule by the lipases or enzymes, that are normally present in milk. Excessive

agitation of milk also appears to accelerate lipolysis and this also results in the appearance of a rancid flavoured milk (31).

When milk is homogenised, the fat globules are broken up into smaller particles and these are stabilised by casein adsorption (28). Sufficient casein is absorbed to increase the density of the newly formed globules to approximately that of the milk plasma. The increase in their density and their smaller size, tends to stabilise the newly formed fat globules against aggregation by clustering and creaming.

The proteins of milk can be classified as caseins or whey proteins. Typically caseins account for approximately 78 to 85% of the total protein. (α_s -casein 45-55%, β -casein 25-35%, K-casein 8-15%, γ -casein 3-7%). The remainder of the protein, generally called whey proteins accounts for between approximately 15 to 22% of the total protein (β -lactoglobulin 7-12%, α lactalbumin 2-5%, serum albumin 0.7-1.3%). Also present are immunoglobulins between 1.9-3.3% and proteose-peptones 2-6% (32). Most of these proteins, along with most other constituents of milk, are synthesised from smaller molecules in the secretory cells of the udder. The serum albumin and immunoglobulins, however, originate principally from the blood. Caseins have a strong tendency to aggregate through hydrophobic, hydrogen bonding, electrostatic and calcium-phosphate bridging interactions to form particles called micelles. The properties of individual caseins and casein micelles will be discussed further in Section 2.4 and Chapter 6 respectively.

Minerals account for less than 1% of the total weight of milk and include the anions: chloride, phosphate, citrate and the cations: potassium, sodium, calcium and magnesium (34). Although in low amounts, they are very important in determining the physico-chemical properties of milk. This is particularly true of the calcium and phosphate. The product of the total calcium and phosphate ion concentration is much in excess of the solubility product of calcium phosphate. Hence much of the calcium and phosphate in milk is likely to be in the form of colloidal calcium phosphate and in some way is associated with the casein micelles.

The binding of the ions to ion exchange sites on the proteins may also be important. For example, it has been shown that phosphate residues provide calcium binding sites on β and κ -casein (36). α_{s1} -casein appears to have calcium binding sites in addition to the phosphate residues (36,37). Ultra filtration and centrifugation studies have indicated that more than 90% of the calcium is either colloidal phosphate or is bound to the casein micelles. Assuming that most of the calcium and phosphate is in the form of colloidal calcium phosphate and making allowances for adsorbed calcium on the protein, calcium:phosphorus molar ratios ranging from 1.63:1 to 1.71:1 have been estimated (63). The exact assignment of calcium to either the casein fraction or the colloidal calcium fraction can cause discrepancies in the calculated ratio. It is interesting to compare this result to the molar ratio of 1.5:1 of calcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ and the molar ratio of

1.66:1 of calcium apatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. It has been reported that in a study of calcium phosphate at pH 7.4 an amorphous calcium phosphate with a molar ratio of 1.5:1 is formed prior to the transition to the crystalline apatite (35). In the presence of casein the stability of the amorphous calcium phosphate is enhanced and the transition to the crystalline phase is retarded (39).

Further evidence for the presence of colloidal calcium phosphate has been obtained by $^{45}\text{Ca}^{++}$ exchange experiments (38). Approximately 40% of the total calcium had not exchanged after 48 hours. This "hard to exchange" calcium was attributed to apatite.

The importance of calcium ions in stabilising micelles has been demonstrated by experiments in which EDTA was added to complex Ca^{++} ions and experiments in which Ca^{++} ions were added to milk to increase the amount of total calcium. In the former experiments an increase in the translucence and the concentrations of serum casein indicated the destruction of micelles, whereas in the latter experiments a decrease in serum caseins indicated the formation of micelles (40). This serum-micelle casein equilibrium will be discussed in more detail in Chapter 6.

In addition to those constituents that occur in relatively large proportions, there are a number of important constituents that are present in low concentration. These include essential elements such as iron, copper, zinc, iodine, aluminium, bromine, silicon, (26), a number of enzymes such as lipase, esterase, alkaline phosphatase (destroyed by pasteurisation), xanthine oxidase, lactoperoxidase, protease, ribonuclease, lysozyme and many

others (26), and the phospholipids, such as lecithin, cephalin and sphingomyelin (41).

In order to study the interaction of milk constituents with surfaces under the varied conditions of milk processing, it is necessary to know something of the effect of these conditions on the properties of milk. The effect of aging, temperature change, addition of rennet and the lowering of pH will thus be outlined below.

Aging of milk results in a number of physical changes. The most noticeable of these is that of the fat globules being lighter than the plasma phase, which rise to form a cream layer within 20-30 minutes of milking. Another well documented effect that occurs when milk is aged, is the lowering of the pH. This is invariably due to bacterial action. It is virtually impossible to store non-sterilised and unsealed milk for periods greater than two or three days at temperatures above its freezing point, without experiencing problems associated with bacterial infection. Even at 7 °C, psychotropic bacteria can be troublesome (42,43) and are first indicated by the formation of para-K-casein, a breakdown product of K-casein (44). Also a decrease in β and α -casein occurs (45). Whey proteins appear to be unaffected by psychrotrophic bacteria.

Milk stored at room temperature is rapidly colonised by bacteria that cause both protein breakdown and lactose fermentation. Both of these effects contribute to the eventual precipitation of the casein constituents. The breakdown of K-casein leads to a destabilisation of the casein micelle since K-casein is an essential

component of the micelle. Lowering of pH due to the formation of lactic acid also induces precipitation. A distinction can be made between the precipitation that results from a lowering of the pH alone and that which results as a consequence of K-casein breakdown with or without an accompanying pH drop. Precipitation by the former effect is reversible whereas the precipitation of caseins that has been accompanied by K-casein breakdown is irreversible. For example acid precipitated caseins are soluble in alkaline solutions whereas the curd formed on soured milk is insoluble in alkali.

Rennin, an extract from calf-gastric enzyme called rennet, also attacks K-casein cleaving it to form the positively charged para-K-casein and a macropeptide (46). The overall effect of this reaction is the coagulation of the casein micelles. The coagulation is not accompanied by an increase in protein bound calcium indicating that calcium bridging is not the mechanism of aggregation.

Mastitis is a bacterial infection of the cow's udder which at the clinical level induces clotting of milk proteins and at a sub-clinical level is manifested by a decrease in the percentage of casein in the form of micelles (47). It is of interest to speculate whether mastitis also affects the K-casein.

When the temperature of milk is lowered below its freezing point of -0.54°C (48), ice is initially formed, leaving the lactose, salts and protein in a highly concentrated solution (49). Eventually the water of solvation is removed from the micelle which then becomes destabilised. Freezing also results in the demulsification of fat in milk due to the

expanding ice crystals (150). Homogenisation prior to freezing however, almost entirely overcomes the demulsification of the fat of milk (161). Apparently the smaller fat globules produced by homogenisation are less easily deformed by freezing. Often frozen homogenised milk exhibits a watery layer after thawing and it is reported that on remixing, it assumes its normal dispersion with no subsequent separation (165).

When milk is heated to temperatures of more than 100 °C for 15 to 20 minutes extensive denaturation and aggregation of the whey proteins and the flocculation of casein micelles by a complex series of reactions results (28). These effects are caused by disulphide interchange between β -lactoglobulin and K-casein (51). The smaller casein micelles in skim milk contain the highest K-casein and also the highest concentration of the heat induced complex (52). Aggregates of more than 100,000 molecular weight are formed (28). This problem is not apparent in pasteurized milk which is subjected to only very short periods at high temperature.

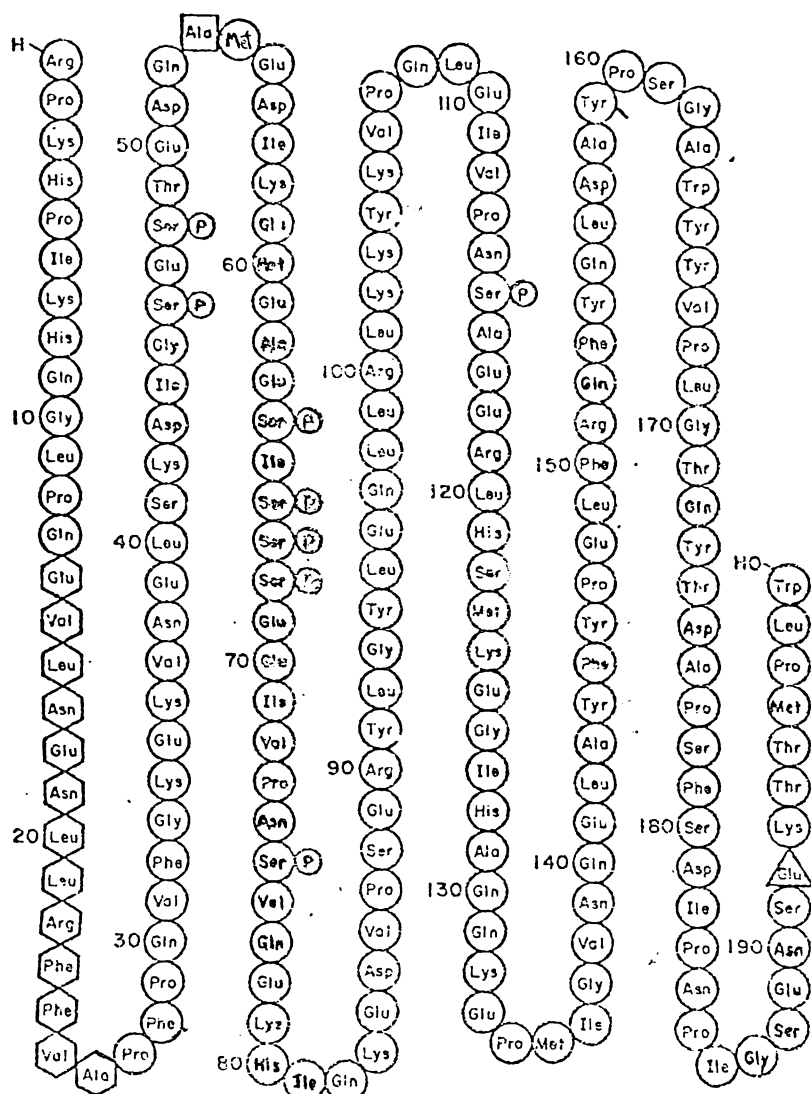
The above review has covered the overall composition of milk and some of the important factors that could affect the stability of milk in adsorption studies. As the object of the present research is not only to study the adsorption of milk on to stainless steel but also individual protein constituents of milk, especially the casein constituents, the next section will review in detail the individual casein fractions and their properties.

2.4 The Casein constituents of Milk

The casein constituents can be separated into three major fractions called: α_s -casein which accounts for about 50% of the total casein and about 17.5 g l^{-1} of milk; β -casein which accounts for about 30% of total casein and about 8.7 g l^{-1} of milk; and κ -casein which accounts for about 10% of total casein and about 3.5 g l^{-1} of milk (32,53). The actual percentage of the individual fractions, varies depending upon the technique used to analyse the individual fraction and the effect of natural biological variations.

α_s -casein can be sub-divided into a major component, α_{s1} -casein, and minor components, α_{s0} -, α_{s2} -, α_{s3} -, α_{s4} -, and α_{s5} -casein (54,55). The primary structure of α_{s1} -casein is known and given in figure 2.1. It consists of a single polypeptide chain of 199 amino acid residues, with a highly uniform distribution of prolyl residues (16.7%). This uniform distribution limits the possibility of a α helix structure and results in a very loose tertiary structure being formed. The primary structure of α_{s0} -, α_{s2} -, α_{s3} -, α_{s4} - and α_{s5} -caseins do not appear to have been investigated in detail. To further complicate matters, the existence of a number of genetic polymorphisms exist among the individual casein fractions. In α_{s1} -casein these are called α_{s1} -casein A, α_{s1} -casein B, α_{s1} -casein C and α_{s1} -casein D (56,57). Friesian cows contain approximately 90% α_{s1} -casein B, 10% α_{s1} -casein C, and 0.05% α_{s1} -casein A, while Jersey cows contain 81% α_{s1} -casein B and 19% α_{s1} -casein C (53). The difference between α_{s1} -casein B and α_{s1} -casein C is the presence of a glutamyl residue

Figure 2.1 Primary Structure of α_{s1} -casein B



Summary of Properties (53)

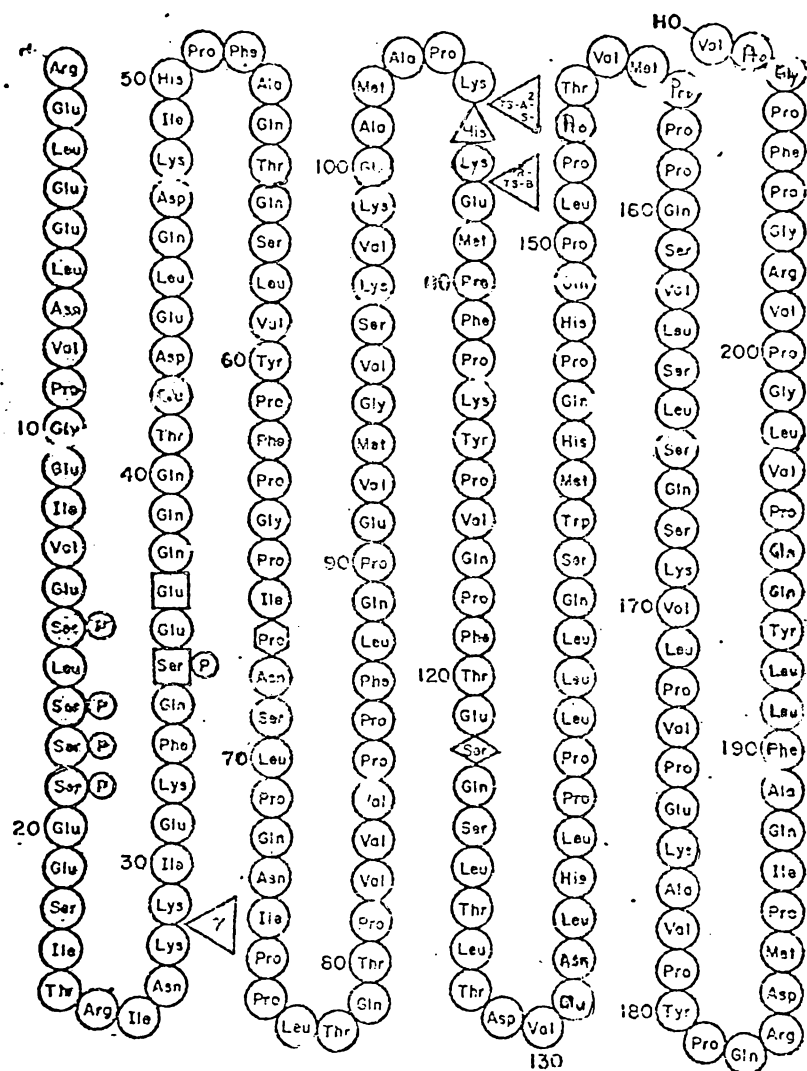
Molecular Weight	= 23,640
Total amino acid residues	= 199
Total tyrosyl residue	= 10
Isoionic point	= 5.0

in α_{s1} -casein B, being replaced by a glycyl residue at the amino acid sequence number 192. It is of interest to note that all the genetic variants of α_{s1} -casein contain ten tyrosyl residues.

The second major casein constituent of milk is β -casein and its primary structure is given in figure 2.2. This protein consists of a single polypeptide chain of 209 amino acid residues and contains 8.5% prolyl residues to give it a loose tertiary structure. A number of minor β -caseins and genetic variants of β -casein are known and these are labelled β -casein A¹, A², A³, B, C, and D. (58,59,60). The β -casein A¹, A² and A³ differ by containing respectively 6, 5, and 4 histidyl residues with β -casein A² containing one extra prolyl residue compared with β -casein A¹. β -casein A³ contains one extra glutamyl and one extra prolyl residue compared with β -casein A¹. The genetic variant β -casein B contains one less seryl residue and one more arginyl residue compared with β -casein A¹, while β -casein C contains one less glutamyl residue and one more lysyl residue compared with β -casein A¹.

Friesian cows contain about 92% β -casein A and 4% β -casein B while Jersey cows contain about 62% β -casein A and 38% β -casein B (53). In all the genetic variants of β -casein the number of tyrosyl residues per molecule is four. Recent studies show that several minor caseins such as γ -casein which makes up about 3% of the total casein in milk consists of amino acid residues 29 to 209 of β -casein. Other minor constituents called R and TS-A² casein represent residues 108 to 209 and 106 to 209 respectively of β -casein A². S and TS- β casein represent residues 106 to 209 and

Figure 2.2

Primary Structure of β -cassin A²

Summary of Properties (53)

Molecular weight	= 23,980
Total amino acid residues	209
Total tyrosyl residues	4
Isoionic point	5.40

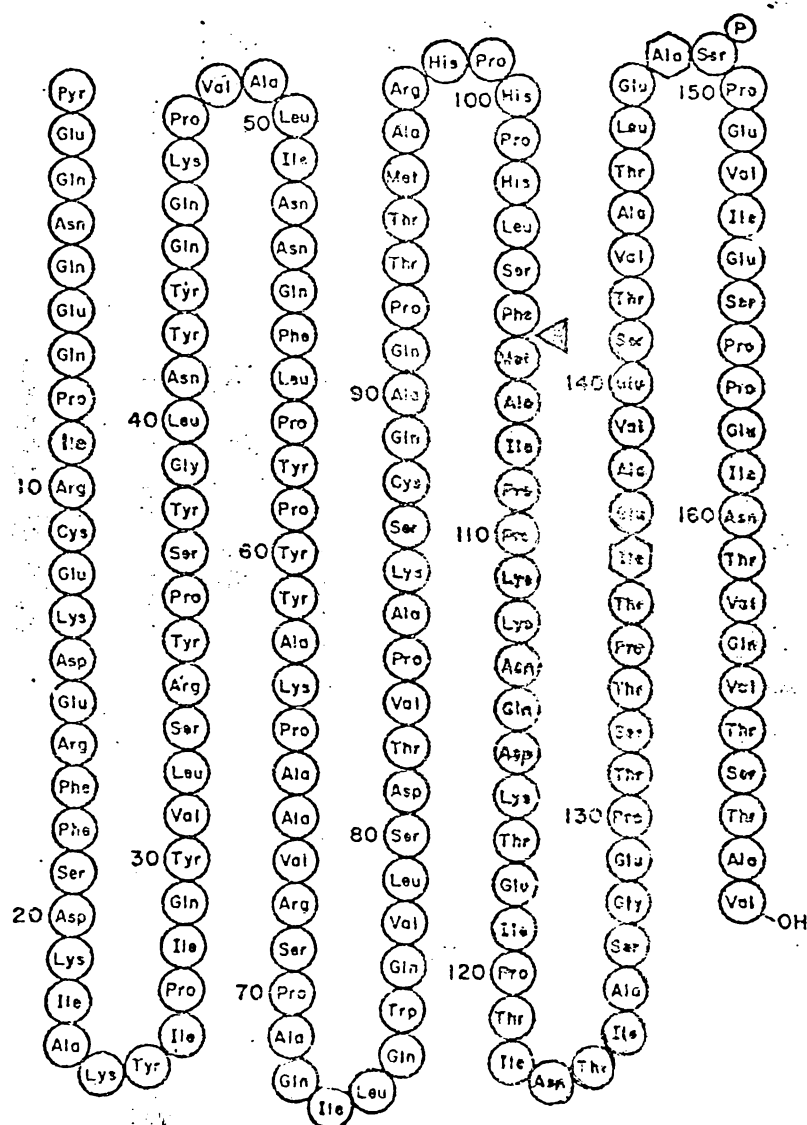
108 to 209 respectively of β -casein B (53).

The third major casein protein present in milk is K-casein and its primary structure is given in figure 2.3. This protein consists of a single polypeptide chain of 170 amino acid residues and like α - and β -casein it contains a high percentage of prolyl residues (9.9%) which are distributed throughout the primary structure causing a very loose tertiary structure. Unlike the other caseins, K-casein has attached to it a carbohydrate residue and a cystinyl residue found respectively at the No.11 and No.88 amino acid residues. Only two genetic variants: K-casein A and K-casein B are known (61,62,32). Both genetic variants are readily cleaved by rennin to form para-K-casein and a macro peptide. In each case the para-K-casein (amino acid residues from 1 to 105) is the same and only the macropeptide (amino acid residues 106 to 170) changes. The macropeptide A contains one more aspartyl and threonyl residue, and one less alanyl and isoleucyl residue, compared with macropeptide B. Of the total K-casein in Friesian milk, approximately 77% is K-casein A and 23% K-casein B. In Jersey milk 38% is K-casein A and 62% is K-casein B. No tyrosyl residues are found in the macropeptide fraction of K-casein but the para-K-casein contains a total of nine tyrosyl residues (53).

The milk used in the present study was from a herd of Jersey cows and therefore could be expected to contain predominantly α_{s1} -casein B, β -casein A and K-casein B. Although slight differences in properties of the genetic variants of some of the

Figure 2.3

Primary Structure of K-casein B



Summary of Properties (53)

Molecular weight	= 19,012
Total amino acid residues	169
Total tyrosyl residues	9
Isoionic point	6.07

individual fractions have been reported (e.g. α_{s1} -casein C is reported to aggregate to a greater extent than α_{s1} -casein B and this is related to a slightly lower net charge of the α_{s1} -casein (65,66)); no effort was made to separate the genetic variants for individual adsorption studies. In most cases the structural differences between the individual caseins are usually small and therefore significant differences in the properties of the genetic variants of a particular casein would not be expected.

Having discussed the primary structure of the main milk caseins, the next section reports on the monomer-polymer equilibria of individual caseins.

2.5 Polymerisation of Casein Constituents

An important property of caseins (and many other proteins) is their tendency to polymerise and aggregate. The precipitation of caseins by lowering of the pH is one manifestation of this tendency. Polymerisation and aggregations effects do not however always lead to precipitation. Stable polymeric species with molecular weights varying with conditions and protein monomer involved can be formed. Forces involved in the intermolecular bonding can include all the forces involved in intramolecular bonding (see section 2.7).

i. α_{s1} -casein

The extent of association of isolated α_{s1} -casein depends upon the concentration of the protein, the pH, the ionic strength, and the type of ions present. Unlike that of β -casein, polymerisation of α_{s1} -casein is not particularly temperature dependent between 8 °C and 30 °C (64) which suggests it to be entropy driven. Studies of the sedimentation coefficient of α_{s1} -casein as a function of pH at a concentration of $7 \times 10^{-3} \text{ g ml}^{-1}$ and at an ionic strength of 0.2 show that only above a pH of 10.6 is the protein completely dissociated (67). This dissociation is thought to be due to the result of strong electrostatic repulsion. The value obtained for the molecular weight of α_{s1} -casein at such pH's correspond to the molecular weight of the unassociated species present at neutral pH's and low protein concentrations (68,69). Apparent molecular weights from light scattering data show that at concentrations above $1 \times 10^{-3} \text{ g ml}^{-1}$ of protein at pH 8.0, a change in the ionic strength from 0.01 to 0.05 caused the apparent molecular weight to triple (70), a result that Schmidt has attributed to hydrophobic bond formation.

ii. β -casein

The association of β -casein differs from that of α_{s1} -casein in several respects. It appears that the association of β -casein is reversible (71) and is dependent on temperature (72,73) and to a lesser extent pH, ionic strength and concentration. A further characteristic of this association is that apparently only the monomers of molecular weight of approximately 24 000 and polymers of molecular weight of approximately 600 000 coexist (72,74) and that the rate of association is low in contrast to that of α_{s1} -casein (73,74). Bovine β -casein has a critical micelle concentration of approximately $0.6 \times 10^{-3} \text{ g ml}^{-1}$ at 21°C . Above this concentration polymerisation occurs (74). Payens et al. (73) and van Hippel et al. (76) have attributed the polymerisation to hydrophobic interaction and have shown it to be endothermic and therefore entropy driven. On increasing the temperature a critical point is reached where rapid aggregation occurs (approx. 28°C). Light scattering measurements at elevated pressures have shown that both α_{s1} - and β -casein polymers dissociate under pressure (74,77), a result most readily explained by the occurrence of hydrophobic bonds. Removal of the first twenty amino acid peptides from the C-terminus of β -casein completely destroyed its ability to form polymers (78), indicating that the C-terminals are responsible for the association behaviour, possibly by the direct participation of their side chains in hydrophobic bond formation. The ability of β -casein to associate in the presence of sodium dodecyl sulphate and urea has

provided evidence that hydrogen bonding is probably not important (79).

iii. K-casein

K-casein is the only casein that contains the readily oxidizable cysteine residue (80) which has a strong tendency to polymerize. The intermolecular disulphide bridges formed give rise to a series of covalent polymers ranging in molecular weight from approximately 60,000 (trimer) to above 150,000. Cleavage of these disulphide linkages can be achieved by raising the pH (81,82) or by the addition of mercaptoethanol, or sodium sulphite (83).

In addition to the disulphide bonds, the possibility of hydrophobic interactions has also been postulated as a cause of aggregation of K-casein (84).

2.6 Techniques for Studying Protein Adsorption and Deposition at Solid-Liquid Interfaces

The surface chemistry of proteins at solid-liquid interfaces has been far less extensively studied than their chemistry at liquid-air interfaces. A major reason for this has been the lack of convenient methods of adequate sensitivity to monitor protein adsorption at solid-liquid interfaces, particularly in low surface area systems.

Early work invariably used large surface area systems and monitored protein adsorption by analysing the decrease in solution protein concentration resulting from adsorption, or analysing the amount of protein adsorbed. These studies employed Kjeldahl

analysis as a method of determination of solution protein concentration or the amount of protein adsorbed (85). In other studies, protein solution concentration was more conveniently monitored by Ultra Violet Spectrophotometry usually at 280 nm wave length (86,88,89,90,91,92,93,94). A more recent technique, but one that again can only be used with large surface area systems, is the technique of micro-calorimetry (87,92). In this method the adsorbate is injected into a carrier solution that flows at a constant rate over a bed of the adsorbant. The method relies on a finite heat of adsorption and can be made quantitative providing that the heat of adsorption is known. This apparatus is expensive, difficult to use and is limited to suitable adsorption beds.

Various Infra Red reflectance methods have been developed to measure the thickness of surface films. Such techniques have been applied to protein adsorption studies (95,96,97,98,99). In these methods the difference between the ratios of the amide ($C=O$ stretching) band of the protein at 1640 cm^{-1} to a standard band for each polymer adsorption surface, before and after adsorption gave a net adsorption value. This net adsorption value was then related by a calibration curve to the actual surface concentration.

Another method involving expensive instrumentation, is that of ellipsometry, which has been used to study protein adsorption (100,101,102). This method is based on the reflectance of polarized light from a film covering an adsorbent surface. The method is limited to mirror surfaces that reflect light and

metallic adsorbents. The assumption of this method for calculating the film thickness is that the film is homogeneous, has a discrete boundary and that it has a constant concentration throughout the thickness of the film. Because these conditions are often not met e.g. the film is often not homogeneous and the refractive index is not constant over the film thickness, this method gives only the "effective" film thickness. No instrument was available in New Zealand to allow this method to be used for the present studies.

The quartz electrostriction method, now under investigation at the University of Waikato, provides a novel means of studying protein adsorption on to low surface area systems. In this method the change in frequency of an oscillatory quartz crystal is directly related to a mass change due to adsorption. The adsorption occurs on to the gold surface of the crystal or else on to a precoated surface on top of the gold. This method is very sensitive and has detected protein adsorption as low as $10^{-8} \text{ g cm}^{-2}$ (104), but is limited to measuring total mass. It cannot be used for continuous monitoring of adsorption.

Transmission electron microscopy of carbon replicas has been used to study the kinetics and conformational changes for the adsorption of fibrinogen on to mica (105,106). This direct method is technically difficult and is limited to large protein molecules. It could not be used to study the adsorption of caseins at monolayer amounts.

A number of electrochemical methods such as capillary electrometry (107,108), cyclic voltammetry (103,109,110) and polarographic techniques (111) have been used to study the adsorption and reversibility of adsorption of proteins at mercury and other metal electrode interfaces.

In the food industry, a wide range of methods have been used to study and detect surface adsorption, deposition and desorption. These methods have recently been reviewed by Kulkarni et al. (113). None of these methods are suitable, however, for low surface area adsorption of proteins. Even the radiochemical methods reported in this review (113) are unsatisfactory as they do not specifically label the protein. Radiochemical methods do offer the advantages of extreme sensitivity, economy and convenience, and can be used both qualitatively and quantitatively. Some precautions must, however, be exercised when using radioisotopes as tracers. It is absolutely essential that the radioactive tracer species behaves identically to the unlabelled species, that it is stable and has the same chemical and physical form so that it obeys the principle of isotope dilution. A high specific activity of the labelled protein is also usually necessary.

Generally β and γ emitters can be conveniently monitored, but isotopes with a half life shorter than a week are of limited use.

In sorption studies involving simple inorganic species, the requirements mentioned above are usually easily met providing a suitable isotope exists for at least one of the atoms. The labelled species can be prepared by general inorganic preparative methods which are adapted for low concentrations of reagents. In the

case of complicated organic molecules, suitable labelling techniques achieving high specific activity can be more troublesome. Biosynthesis generally offers the best method. In order to prepare labelled proteins, several radioactive isotopes could conceivably be used. All proteins contain carbon and hydrogen and these have the convenient isotopes; C-14 and H-3. Many proteins also contain phosphorus and sulphur for which the isotopes P-32 and S-35 would provide suitable labels. Proteins incorporating these isotopes could be prepared by the standard methods of biosynthesis or in some cases, such as S-35 heparin, are available commercially (114,115).

The substitution and acylation reactions of proteins offer another means of incorporating radioactive labels. For example, the use of I-125 and I-131 by substitution into the protein aromatic residues, or C-14 or H-3 labelling by acylation reaction with the protein carboxylic acid groups could be used to label proteins. Iodinated protein labelled with iodine-125 has been used to study the protein associated with fat globules of milk (116) and the interactions of β lactoglobulin with milk micelles on heating milk (117). Protein labelled with tritium by acylation has been used for adsorption studies (118). The adsorption of radioactive atoms such as Au-198 by protein molecules offers a further method of labelling protein, providing that the adsorption process is irreversible (112).

From a consideration of the techniques available for monitoring protein adsorption and deposition, it appears that if a suitably labelled radioactive protein of high specific activity

can be prepared, then this method offers a general and convenient means of performing the protein adsorption studies envisaged in the present project. The technique of radioiodination offers the possibility of preparing such a stable high specific active protein. The two iodine isotopes I-125 and I-131 are cheap and readily available and can be conveniently monitored by γ and /or β counting techniques.

Because of its widespread use in radioimmunological studies, the technique of radioiodination, including the necessary separation procedures for removing the unbound radioactive iodine, has been thoroughly investigated. Radioimmunological studies are likely to be at least as sensitive, if not more sensitive, to slight modifications of protein properties induced by iodination. Generally, providing certain precautions are observed, iodinated proteins can be considered to be sufficiently similar to non-iodinated protein to behave identically in such studies.

A review of protein iodination, the effect of the iodine on the protein and the results of iodination of whole milk and individual milk constituents will be included in Chapters 4 and 5.

2.7 The Surface Chemistry of Proteins

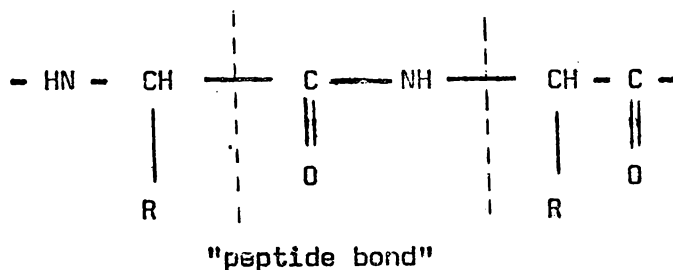
Proteins play a very important role in the structure and function of all living matter (120). Because many proteins are of very complex structure, shape, solubility and chemical composition have been used as a means to classify them.

Fibrous proteins are elongated, insoluble animal proteins, that are highly resistant to digestion by proteolytic enzymes, and are often composed of several coiled peptide chains that are tightly linked. These proteins have been further divided into collagens, elastins and keratins. Collagens are the proteins of connective tissue and are altered to soluble gelatin on boiling in water, dilute acid or alkali. They contain hydroxyproline and hydroxylysine, but lack cysteine, cystine and tryptophan. Elastin are present in tendon, arteries, and other elastic tissue and are not converted to gelatin. Keratins are the proteins of wool, hair, nails, hooves and contain large amounts of cystine.

Globular proteins are those that have spheroidal or ellipsoidal shapes and are generally soluble in water or aqueous media containing salt, acid or base. They include such proteins as enzymes, hormones and oxygen carrying proteins. The whey proteins found in milk are compact, globular proteins.

Conjugated proteins are those that combine with non-amino acid substances. Mucoproteins, for example, are those that contain usually more than 4% carbohydrate (measured as hexosamine) while glycoproteins are restricted to less than 4% carbohydrate such as K-casein. Phosphoproteins are proteins that contain phosphate residues and are usually precipitated by low concentrations of calcium. Examples of this type of protein found in milk are α_{s1} -casein and β -casein. K-casein contains only one phosphate residue and is soluble at most concentrations of calcium, and is sometimes called a glycopospho protein. Lipoproteins are water soluble proteins containing a lipid such as cholesterol.

The basic structure of a protein molecule consists of a long chain (never branched but often cross linked) of amino acids linked together by the α carboxyl group of one amino acid residue and the α -amino group of another residue as follows:



Twenty-one different amino acids are known and these can be linked in various orders to give proteins ranging in molecular weight from 5 000 to many million. The exact sequence is unique for a given protein and is known as its primary structure. A three dimensional arrangement of the polypeptide is known as the secondary structure of the protein. This can be either an α -helix in which the polypeptide exists as a spiral around an axis, or a β structure where the polypeptides are aligned side by side. Non regular three dimensional arrangement of the chain is referred to as tertiary structure and results from the interaction of amino acid residues relatively far apart in the sequence (121,122,123).

Amino acid side chain groups play a very important part in the structure of the protein. Non-polar side chains impart hydrophobicity whereas polar side chains impart hydrophilicity. The hydrophobic side chains are soluble to varying degrees in non-polar solvents, but are only sparingly soluble in water and hence tend to aggregate in water, not because they are attracted to each other but because the water to water interaction is stronger

thermodynamically than the water to hydrocarbon interaction.

This is sometimes termed apolar bonding which refers to the preferential association of macromolecular non polar groups in any polar solvent. This type of interaction can lead to globular shaped proteins.

The polar side chains, which are readily soluble in water, can result in hydrophilic or polar bonding due to ionic or hydrogen bonding. Ionic bonding is due to electrostatic attraction via positive and negative amino acid side chains. The charge of these side chains depends on the pH of the solution. The pH at which all the positive charges are equal to all the negative charges is known as the isoelectric point (pI). Proteins differ both in the total number of positive and negative hydrophilic groups and in the total number of hydrophobic groups.

Hydrogen bonding may also be important. This refers to the interaction of a hydrogen atom attached to an electronegative atom with another electronegative species such as nitrogen or oxygen.

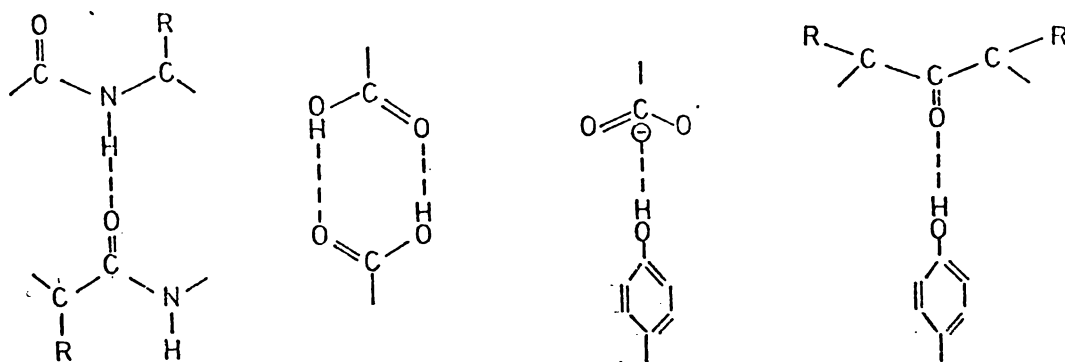
Also occurring in some proteins are amino acids which contain sulphide residues. These can form intermolecular disulphide bonds. Bovine serum albumin for example, contains seventeen disulphide bonds.

The four types of bonding that determine protein structure are summarised in figure 2.4.

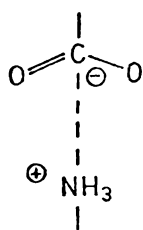
Just as any or all these forces can occur intramolecularly, or possibly intermolecularly with another protein molecule, they could also be the forces responsible for protein adsorption at interfaces. For example, possible adsorption mechanism at solid-

Figure 2.4

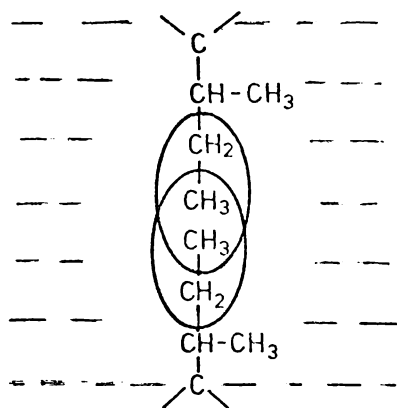
Types of bonding between amino acid residues of proteins



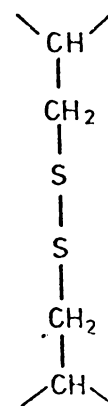
(a) Hydrogen bonds (119)



(b) Ionic bond



(c) Hydrophobic bond



(d) Covalent bonding

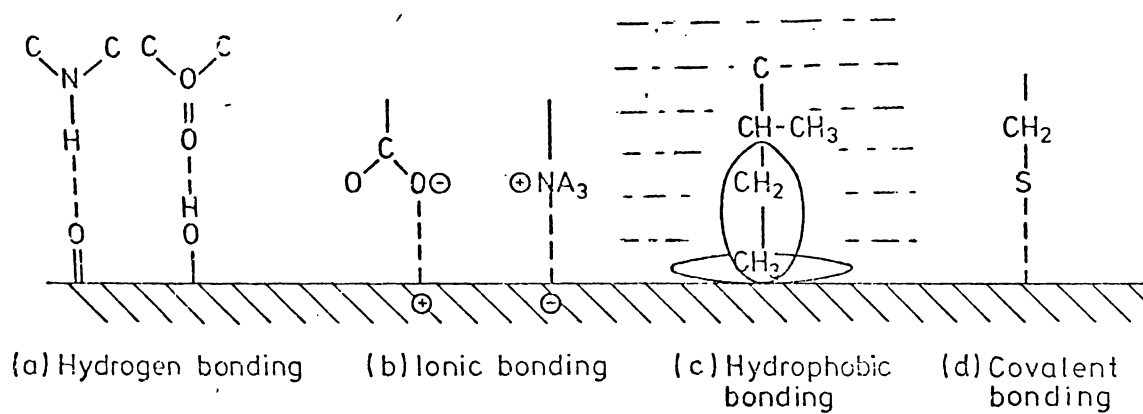
aqueous solution interfaces are illustrated in figure 2.5 .

Hydrophilic or polar bonding could be expected to involve hydrogen bonding (Type a) or ionic bonding (Type b). Although isolated hydrogen bonding is weak, a large number of such bonds could occur between surface and protein leading to a large energy of interaction. Ionic bonding at pH 7, may occur between a negatively charged surface and the end chain amine group, or the positively charged histidyl, lysyl and arginyl residues. Alternatively if the surface is positive, the interaction could be between the surface and the negatively charged groups such as the end chain carboxyl group or the asparagyl or glutamyl residues. At pH's other than 7, the charges on the amino acids will vary according to their pK_a values.

Hydrophobic interaction (Type c) could be expected to occur between hydrophobic surfaces and the side chains of the amino acid residues that contain non-polar residues. The driving force for hydrophobic bonding is not the van der Waals attraction of the non-polar side chains for the surface, but results as a consequence of the hydrogen bonding between the water molecules driving non-polar residues out of solution. Experimentally it has been found that the enthalphy of protein adsorption is usually small and can be positive or negative (166,167,168). In the case of positive enthalphy, the free energy necessary for adsorption to be feasible, must arise from a positive entropy. This positive entropy can be visualised as resulting from a decrease in water structure that occurs when the hydrophobic hydrocarbon residues are removed from solution.

Figure 2.5

Proposed types of bonding of amino residues of
proteins at solid-aqueous solution interfaces



One further possible interaction between protein and interface could be chemical reaction between a sulphide or any other reactive group of the protein molecule and a reactive surface atom, group or "site" forming a covalent bond.

Considerable evidence indicates that proteins can adsorb at many interfaces by a combination of forces (124). For example the adsorption of protein on to nitrocellulose membranes (125) has been attributed to the following forces.

- (a) hydrogen bonding (Type a)
- (b) multielectrostatic attraction between the numerous dipoles of the membrane and the dissociated polar and polarizable groups of the protein molecule (Type b)
- (c) and a general trend of the organised water molecules to force hydrophobic groups to the interface (Type c).

The forces involved in the adsorption between protein and a glass surface (which is hydrophilic) have been interpreted as ionic (amine-silanol ($\text{Si}-\text{O}^{\ominus}$)) bonding and hydrogen bonding (Types a and b) (90,126). Likewise the adsorption of ribonuclease (94), serum albumin, egg albumin (93) adsorption on to glass has been considered to be largely electrostatic (Type b). Numerous other examples of ionic-ionic interaction of protein and surface have been reported (98). An increase in negative potential has been observed to enhance this type of adsorption (98).

The adsorption of albumin, γ globulin and fibrinogen on to polymer membrane has been reported to occur by two mechanisms, one being hydrophilic exothermic and easily reversible, and the other being hydrophobic, endothermic and largely irreversible (148).

Human plasma albumin and ribonuclease are reported to adsorb on to a negative polystyrene surface at pH values greater than the isoelectric point of both the latex and the protein. This adsorption was interpreted as being a hydrophobic interaction (127). On increasing the negative charge by the incorporation of more ionic groups, greater protein adsorption occurred. This was explained by an electrostatic end-on adsorption of the protein molecule arising from local excess of charge at one end of the protein and the hydrophobicity of the polystyrene surface having been decreased by increased surface charge (127).

Frequently it has been assumed that adsorbed protein molecules have structures identical to those of molecules in solution. Early polarographic work assumed that protein structure was essentially unchanged after adsorption (131,132,133). Alternatively structural changes of adsorbed globular proteins resulting in irreversible unfolding have been reported (128). The proteins that are largely non-polar such as insulin, bovine serum albumin and egg albumin are completely unfolded at the air-water interface, and occupy an area of 0.17 nm^2 per amino acid residue. Proteins such as lysozyme and cytochrome C, with high polar character are only partially unfolded at the air-water interface and occupy an area of about 0.04 nm^2 per amino acid residue (129,130). The area occupied by a number of proteins on the dropping mercury electrode was found

to be about 0.19 nm^2 per amino acid residue. This coincides with unfolded protein at the water-air interface (111).

In the case of protein adsorption on to non-polar surfaces, hydrophobic forces should dominate and result in the protein unfolding (127). With adsorption on to polar surfaces the forces of attraction are electrostatic (dipole and ionic) and the protein usually does not unfold (124,89,134).

The existence of adsorption sites could be expected on solid interfaces. Different proteins may adhere with different affinity to such sites. The existence of sites and an irregular surface structure may also explain some of the kinetic effects associated with protein adsorption. Protein molecules may penetrate slowly into the less accessible regions so that adsorption and subsequent rearrangement could be very slow. The presence of sites on solid surfaces has also been used in a statistical theory which has predicted less chain unfolding at solid-liquid interfaces than at liquid-air interfaces (135,136).

The unfolding of proteins does not necessarily result in the total destruction of the secondary structure (137,138). It has been reported that human plasma albumin unfolds when adsorbed by polystyrene lattex (139) though it still retains its immunochemical activity (140). This indicates that either the conformation of human plasma albumin was not very crucial with respect to its immunochemical activity, or the active sites were not impaired appreciably. α Amylase is also found to remain active after adsorption and desorption from quartz, yet it is easily denatured by shaking (141). Ribonuclease, on the other hand, appears to have

a much more rigid structure such that its conformation was not drastically changed on adsorption (94,127), but its enzymatic activity was reduced virtually to zero (140), and it exhibited a more acidic isoelectric point (as determined by microelectrophoresis) (142). This latter result could have been due to distortion of the protein molecules resulting in the ionization of groups of the protein by the charge on the underlying surface, by the close packing of the protein molecule at the surface causing a lowering of the pK_a value, or by structural deformation which results in different ionizable groups being exposed to the surrounding medium (142).

It has been suggested that when proteins are adsorbed on to hydrophobic surfaces, hydrophilic residues are exposed to the aqueous phase, whereas when proteins are adsorbed on to hydrophilic surfaces the non-polar residues will be exposed (143). Protein adsorption decreases the wettability of hydrophilic glass surfaces, but increases the wettability of hydrophobic lucite surfaces (144).

The effect of adsorption on protein structure has been investigated by examining desorbed protein. Using iodinated fibrinogen, albumen, and IgG, adsorbed on to teflon and silicon rubber and then desorbed with sodium dodecyl sulphate and dithiothreitol, electrophoretic analysis revealed complex patterns of up to eight iodinated species as well as the apparently unchanged original protein (145). An inherent problem associated with the investigation of the effect of adsorption on protein structure through examination of desorption products is that the conditions of desorption or the desorption process itself may be

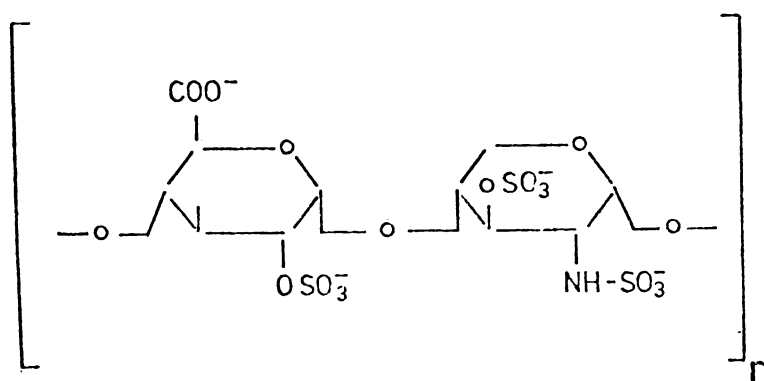
responsible for any changes observed. Usually surfactants such as Triton X-100 (octylphenoxypolyethoxyethanol) and sodium dodecyl sulphate are used to facilitate desorption (146). These surfactants are known to bind strongly with proteins and could be instrumental in causing denaturation. Results have shown that adsorption of thrombin can lead to denaturation and that the denatured molecule is readily desorbed (93).

Evidence for changes in protein properties has been obtained from electrophoretic studies of adsorbed protein (134). For example bovine serum albumin adsorbed on glass particles gave rise to two maximum points in the electrophoretic mobility as a function of concentration. This suggests that the first molecules adsorbed unfold but that increasing surface coverage reverses this unfolding.

It has been shown that the adsorption of albumen on to a hydrophilic silica surface is completely reversible at pH 7.5 whereas at its isoelectric point (pH 4.9) and also when adsorbed on to a hydrophobic surface, reversibility was not observed (88). Likewise the adsorption of protein on to a hydrophilic cuprophane surface has been shown to be reversible (137,146). Generally more albumin can be adsorbed on to a hydrophobic surface than on to a hydrophilic surface (148). Similarly the adsorption of protein on to hydrophobic polymers such as polypropylene and poly(dimethyl siloxane) is greater than adsorption on to a hydroxyethyl methacrylate surface (149).

The adsorption of proteins on to plastic surfaces appears to be related to the abundance of non-polar side chains which suggest the importance of hydrophobic interaction (154). The adsorption of

blood proteins on to the hydrophobic plastic components of artificial cardiovascular systems, including kidney machines, heart lung machines and heart valves, has been implicated in the formation of blood clots. Much effort has been expended to minimise or prevent such adsorption. The use of anticoagulants such as heparin, which has the structure :



Structure of Heparin.

and molecular weight of 17,000 to 20,000, have been used with some success (151). Though the mode of action of heparin is not fully understood, it is unlikely that it is solely electrostatic repulsion of the protein molecule by negative heparinised surface (152). It has been shown that heparinised surfaces can be both positive and negative yet still retain their low affinity for protein adsorption (115). It is most likely that the heparin adsorbs by strong hydrophobic bonding yielding a hydrophilic surface which can be either positively or negatively charged depending on the pH of solution. Hydrophilic bonding, if it occurs, is very weak and readily reversible.

In other experiments the low adsorption affinity of albumin for glass has been reduced by dissolving the protein in glycine and alanine containing buffer solutions (153). Again the mechanism of the effect is not fully understood. It is possible that the relatively hydrophilic amino acids are bound to the hydrophilic surface (possibly by electrostatic interaction) which thus prevents adsorption of the protein.

Polyamino acids such as polylysine, polyglutamic acid and poly-hydroxethyl aspartamide have been investigated but have been found to have limited effectiveness in preventing protein adsorption, for example in the adsorption of iodine-125 labelled insulin on to polystyrene (154).

In some applications the loss of protein at low concentrations can be prevented or minimised by the addition of albumin or polygeline (a polymer of degraded gelatin) (155).

Surfactants such as polyoxethylene sorbitan esters (Tweens) have been used to impregnate nitrocellulose membranes in attempts to minimise protein adsorption. This procedure was effective for the system mentioned above and is likely to be useful where the surfactants are bound to the surface more strongly than the proteins (156,157,158).

Numerous isotherms have been used to fit the adsorption data for proteins studied over a wide range of concentrations and on to various surfaces.

Many workers have claimed that the adsorption process follows a Langmuir-like isotherm (91,96,97,126,148,154,159,160,162).

Langmuir (163) initially derived an adsorption equation by equating the rate of evaporation of a gas from a solid surface to the rate of condensation and obtained the following equation:

$$\Theta = \frac{\Gamma_c}{\Gamma_{sat}} = \frac{bc}{1 + bc}$$

where Θ = degree of coating

Γ_c = amount adsorbed at concentration c .

Γ_{sat} = saturated amount.

b = constant

This equation has since been applied to the adsorption from solution on to solid substrates. To evaluate the experimental data, two types of plots can be chosen:-

$$\frac{1}{\Gamma} \text{ plotted against } \frac{1}{c}$$

or

$$\frac{c}{\Gamma} \text{ plotted against } c$$

Generally in most adsorption studies, an initial plot is made of adsorption against solution concentration. If the adsorption increases and eventually reaches a plateau as the concentration is increased, then the adsorption is spoken of as a Langmuir type. One basic assumption with the Langmuir theory is that the adsorption is assumed to be reversible. This is often not the case in protein adsorption. For example the adsorption of gelatin and egg albumen on to powdered quartz

was reported to follow a Langmuir adsorption isotherm, but the adsorption process was irreversible (164). Because of this irreversibility, application of the Langmuir equation can have little theoretical significance (87). It is of course possible that the interaction may be reversible but that the desorption processes are extremely slow. In adsorption only small segments of the molecule are required to enter the interface. Desorption, however, requires a large number of segments to leave the interface simultaneously (88).

The adsorption of albumen (127), egg albumen, and gastric mucin on to tooth enamel (85) follow S-shape adsorption isotherms. These could be compared with Brunauer, Emmett and Teller (BET) adsorption isotherm initially derived to explain the adsorption of water vapour on to a porous solid where the Langmuir type process was followed by a type of condensation (164). The BET theory applies to examples where more than one layer of molecules are adsorbed. In ellipsometric studies of γ globulin adsorption on to a metallised glass slide, direct evidence for multilayer adsorption was obtained, particularly over the pH range 7.0 to 7.5 and under the influence of an applied electric potential (186).

In studying any adsorption process involving aqueous solutions, a number of variables including temperature, ionic strength, and effect of pH may provide useful information about the adsorption mechanism.

Temperature had no significant effect on the adsorption of ribonuclease to polystyrene lattice over the temperature range from 5 °C to 22 °C (166). On the other hand the adsorption of egg albumin at 25 °C on to collodian membranes was reported to be approximately twice that at 1 °C (167). Others showed that the adsorption of trypsin on to glass depended on the method by which the glass beads were cleaned (168). Both an increase and decrease in the amount adsorbed have been observed on increasing the temperature. The adsorption of human plasma albumin on to polystyrene lattice was sensitive to the temperature of adsorption except when adsorption was monitored at the isoelectric point of the protein. At this pH no change in the amount adsorbed between 5 °C and 22 °C could be detected (166).

The literature contains conflicting reports about the effect of ionic strength on protein adsorption. For adsorption of human plasma albumen on to polystyrene lattices, an increase in adsorption occurred on increasing the ionic strength of the solution. At relatively high pH solutions and at the isoelectric point, the ionic strength appeared to have little effect (166). In contrast to these results, a decrease in the amount of adsorption of ribonuclease on glass powder occurred on increasing the ionic strength from $I = 0.01$ to $I = 0.15$ (94). This effect was also observed for the adsorption of trypsin on to a dialysis membrane (168).

pH is an important variable in protein adsorption studies. Maximum adsorption on both hydrophilic and hydrophobic interfaces

appeared to occur at the isoelectric point of most proteins (88,89,94,95,159,168,169,170,171). A typical example is the adsorption of human plasma albumen on to polystyrene lattice. Maximum adsorption occurred at the isoelectric point of the protein (pH 4.7) with the curve being approximately symmetrical about this pH (166). However in the case of ribonuclease, which has an isoelectric point of 9.3, adsorption appeared to be independent of pH (127). The adsorption of deoxyribonucleic acid (DNA) on to aluminium powder also appeared anomalous in that maximum adsorption occurred at a pH of 2.5, which is far below the isoelectric point. The effect of denaturation on DNA adsorption was studied by taking the pH first to pH 2.5 or 12.5, leaving it for 24 hours, and then readjusting it to pH 6.5 for adsorption studies. Heat denatured DNA was also studied. The results showed that DNA adsorption increased by five to ten-fold after these treatments (91). Anomalously high adsorption at low pH's could thus be due to denaturation.

Before any valid conclusions can be drawn about the thermodynamics of protein adsorption, it is necessary to demonstrate that the adsorption process has gone to completion. Strict thermodynamic treatment is probably precluded in many protein-surface systems because of the apparent irreversibility of the adsorption process. Even for irreversible adsorption, it is desirable that adequate time be allowed for the surface to reach 'saturation' i.e. to adsorb all the protein that is capable of adsorbing under a given set of conditions.

Unfortunately a lot of the work reported in the literature has not included adequate kinetic data. Typically, kinetic investigations have been limited to one or two concentrations, a standard adsorption time has been determined and all other adsorption experiments have been performed using this standard time (92,95,126,148,174). A more detailed example is provided by the study of the adsorption of ovalbumin at the air-water interface (171). The kinetics of adsorption were determined mainly at 2×10^{-6} g ml⁻¹ protein concentration using surface tension and surface potential to measure adsorption as a function of time. The rate of adsorption was measured at various pH's and was found to be greatest at the I.E.P. of the protein. It decreased when the molecule became charged. In view of the relatively little attention that has been devoted to kinetic studies, it is not surprising that the mechanism of protein adsorption is poorly understood.

For any process involving adsorption and/or reaction of a substrate at an interface, it is possible to recognise the following as potential rate limiting steps:

- i) diffusion of substrate to the interface;
- ii) adsorption at the interface;
- iii) reaction of adsorbed species at the interface;
- iv) desorption of the products;
- v) diffusion of the products away from the interface.

The adsorption of proteins at most interfaces is probably less complicated than the above scheme in that although structural rearrangement and denaturation may occur, chemical reaction and the desorption and diffusion of reaction products probably need not be considered. In most instances the kinetic problem will be one of determining whether the rate of adsorption is controlled by the rate of diffusion to the surface or by the rate of adsorption at the surfaces. A further possibility of diffusion into the sorbent must be considered in the case of porous media. In certain instances, where the rates of diffusion and adsorption are similar, it is possible that initial diffusion control at low surface coverage will become adsorption rate controlled as saturation is approached. Diffusion control is also likely to be more important at low protein concentration. There is indeed a developing consensus of opinion that diffusion processes are probably important in determining the rate of protein adsorption. There is much direct and indirect evidence in other polymer-surface systems that indicates diffusion to the surface as being the rate controlling step. Thus it has been found that the rate of adsorption of polystyrene-poly(methyl methacrylate) on to silica is very sensitive to the stirring rate (175). Similar results were obtained for the adsorption of polyvinyl acetate at low concentrations (176). The effect of stirring is to reduce the effective diffusion path length between the species in solution and the adsorbing surface. Surface

structure can also modify this parameter. Thus it can be expected that diffusion path lengths to rough surfaces will be greater than the path length to smooth surfaces. Kinetic data for the adsorption of polyvinyl acetate indicated that adsorption on to irregularly shaped aluminium powder surfaces was slower than adsorption on to smooth and non porous surfaces of iron or tin (177). The increase in adsorption rate with the concentration of polymer (e.g. tritium labelled polystyrene on to chromium (178)) is consistent with diffusion control though such an effect could also be expected if adsorption was rate limiting.

More conclusive evidence for diffusion control is provided by the variation of adsorption kinetics with polymer molecular weight. An increase in molecular weight from 38 000 to 76 000 considerably reduced the rate of adsorption (179). It could be expected that the larger molecules would have smaller diffusion coefficients and hence slower rates of diffusion to the adsorbing surface.

To prove conclusively that a process is diffusion controlled, it is necessary to fit the kinetic data to a diffusion equation derived from Ficks Laws of diffusion using appropriate boundary conditions and a realistic value for the diffusion coefficient. In many instances it is sufficient to show that the amount adsorbed varies with the square root of time during the initial stages of adsorption (180).

The rate of adsorption of a number of fatty acids, amines and alcohols on to steel plates or iron-coated geiger counter

tubes have been studied (181,182,183,184) and the results obtained were very much slower than predicted from an appropriate diffusion equation. These studies, however, were performed by using white oil as the solvent and at solute concentrations sufficiently high (1 to 5×10^{-3} g ml $^{-1}$) that the formation of micelles was a real possibility. Micelle formation could be expected to markedly reduce the effective diffusion coefficient. Another complicating feature of these studies was the very high viscosity of the white oil employed.

To date there has been little in the way of definitive kinetic investigations reported in the literature on protein adsorption kinetics. Adsorption of DNA at the air-water interface appeared to be diffusion controlled in the presence of 1 mol l $^{-1}$ salt solution, giving a straight line plot of amount adsorbed versus square root of time (185). The adsorption of a number of globular proteins on to a dropping mercury electrode gave linear plots when plotted against the square root of time (111). Similar plots were obtained from ellipsometric studies of γ globulin adsorption on to metallized glass slides (186). The dependence on the square root of time has also been observed for the adsorption of ovalbumin at the air-water interface (171). The kinetics of adsorption of this protein were very sensitive to pH, ionic strength and the presence of moderate concentrations of sucrose. If the diffusion coefficient of ovalbumin remains relatively constant for these various conditions then it would appear that agreement of the data with square root of time plot was

largely fortuitous and that factors other than diffusion were rate limiting. For example, the unfolding of the protein molecule in solution which could lead to the exposure of the hydrophobic side chains (187). It is possible also that configurational changes resulting from the different conditions, profoundly affect the hydrodynamic properties of the protein molecule and hence, their diffusion coefficients.

From the above general review of protein adsorption it is clear that considerable uncertainty exists in the literature about the mechanism of adsorption. This is partly due to the large number of proteins with different physical properties, that have been studied in a variety of buffer solutions, at different pH's, different ionic strengths, different temperatures and differing purities. More information for pure protein systems under carefully controlled conditions is required before the mechanism of adsorption can be fully understood. Little information is available for the effect of variables in controlling the kinetics of adsorption and desorption at very low protein concentration.

2.8 The Surface Chemistry of Milk Proteins

Surface Activity of Milk Proteins

The surface tension of milk is approximately 50 dynes cm^{-1} compared with that of water of 72 dynes cm^{-1} at the same temperature. This drop in surface tension could be caused by milk proteins, milk fat, phospholipids or free fatty acids or a combination of these constituents which are present in milk.

It has been reported that the surface tension of skim milk (200) is only slightly higher than that of whole milk. This suggests that the removal of fat constituents has very little effect on the surface tension which must therefore be largely determined by the milk proteins.

Milk proteins, like other proteins, contain amino acids with hydrophobic hydrocarbon side chains and amino acids with hydrophilic ionic and polar groups. As previously discussed in Section 2.7 the surface activity of a molecule in aqueous solution depends upon the incorporation within the structure of that molecule of hydrophilic and hydrophobic groups. Generally it can be expected that the greater the hydrophobicity of a molecule the greater will be the tendency of that molecule to escape from the aqueous phase and hence to concentrate at interfaces, particularly those between the aqueous phase and a hydrophobic phase. The milk proteins are exceptional proteins in that they contain a high proportion of hydrophobic residues such as tryptophan, isoleucine, phenylalanine, proline, leucine, valine, methionine, tyrosine, lysine and threonine. Attempts have been made to quantify the hydrophobicity of proteins, although such attempts are complicated by the lack of agreement about the character of certain residues. For example, Fisher (201) regarded tyrosyl as a polar residue because of its hydroxyl group whereas Waugh (202) regarded it as being non-polar or hydrophobic because of its aromatic ring. One of the simplest methods of expressing hydrophobicity is by means of the non-polar side chains (N.P.S.) method (202). According to this

method the hydrophobicity is determined by counting the number of certain hydrophobic groups (tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine, and valine) and dividing by the total number of amino acid residues in the protein.

Alternatively the method of Fisher (201) takes into account the volume of the various residues. The total volume of the polar residues is divided by the total volume of the non polar residues, which in addition to the residues classified as being hydrophobic by the N.P.S. method, also includes methionine, alanine, glycine and cystine/2, but not tyrosine. This ratio of volumes is called "p".

A more vigorous and thermodynamic approach to the problem has been developed by Bigelow (203) based upon the concept of hydrophobicity, $H\bar{\Phi}$, proposed by Tanford (204) which depicts hydrophobicity as the free energy for the transfer of amino acids from an organic environment to an aqueous environment. Bigelow defined the average hydrophobicity, $H\bar{\Phi}_{Ave}$, as being the total hydrophobicity obtained by summing the $H\bar{\Phi}$ for each amino acid residue and dividing the result by the number of residues. By using the amino acid side chain hydrophobicities and residue volumes (Table 2.3) the values for $H\bar{\Phi}_{Ave}$, N.P.S. and p have been calculated for the known structures of α_{s1} -casein B, β -casein A² and K-casein B (see Table 2.4 and Appendix 1).

Also included in Table 2.4 are the results for albumin, α -lactoalbumin, β -lactoglobulin A, fibrinogen and ribonuclease. Note that $H\bar{\Phi}_{Ave}$ and N.P.S. are a measure of the non-polarity while p is a measure of polarity.

Table 2.3

Amino acid side chain hydrophobicities
and residue volumes (203)

Amino acid	$\frac{H_{\text{D}}}{\text{kJ res}^{-1}}$	Volumes/ $\text{\AA}^3$
Trp	12.55	135.4
Ile	12.34	102.0
Tyr	11.92	116.2
Phe	11.08	113.9
Pro	10.87	73.6
Leu	10.04	102.0
Val	7.11	85.1
Lys	6.27	105.1
Met	5.44	97.7
Cys/2	4.18	68.3
Ala	3.14	52.6
Arg	3.14	109.1
Thr	1.88	71.2
Gly		36.3
Ser		54.9
His		91.9
Asp		68.4
Glu		84.7
Amide		4.0

Table 2.4 Parameters calculated for milk proteins
from their amino acid composition

Protein	$\frac{H_{\text{ave}}}{\text{kJ residue}^{-1}}$	N.P.S.	p
Serum albumin	4.68	0.32	1.22
α -lactalbumin	4.81	0.34	1.11
α_{s1} -casein B	4.89	0.33	1.25
K-casein B	5.12	0.34	1.21
β -lactoglobulin	5.14	0.37	0.96
β -casein A ²	5.58	0.46	0.83
fibrinogen	4.28	0.30	1.35
ribonuclease	3.64	0.23	1.73

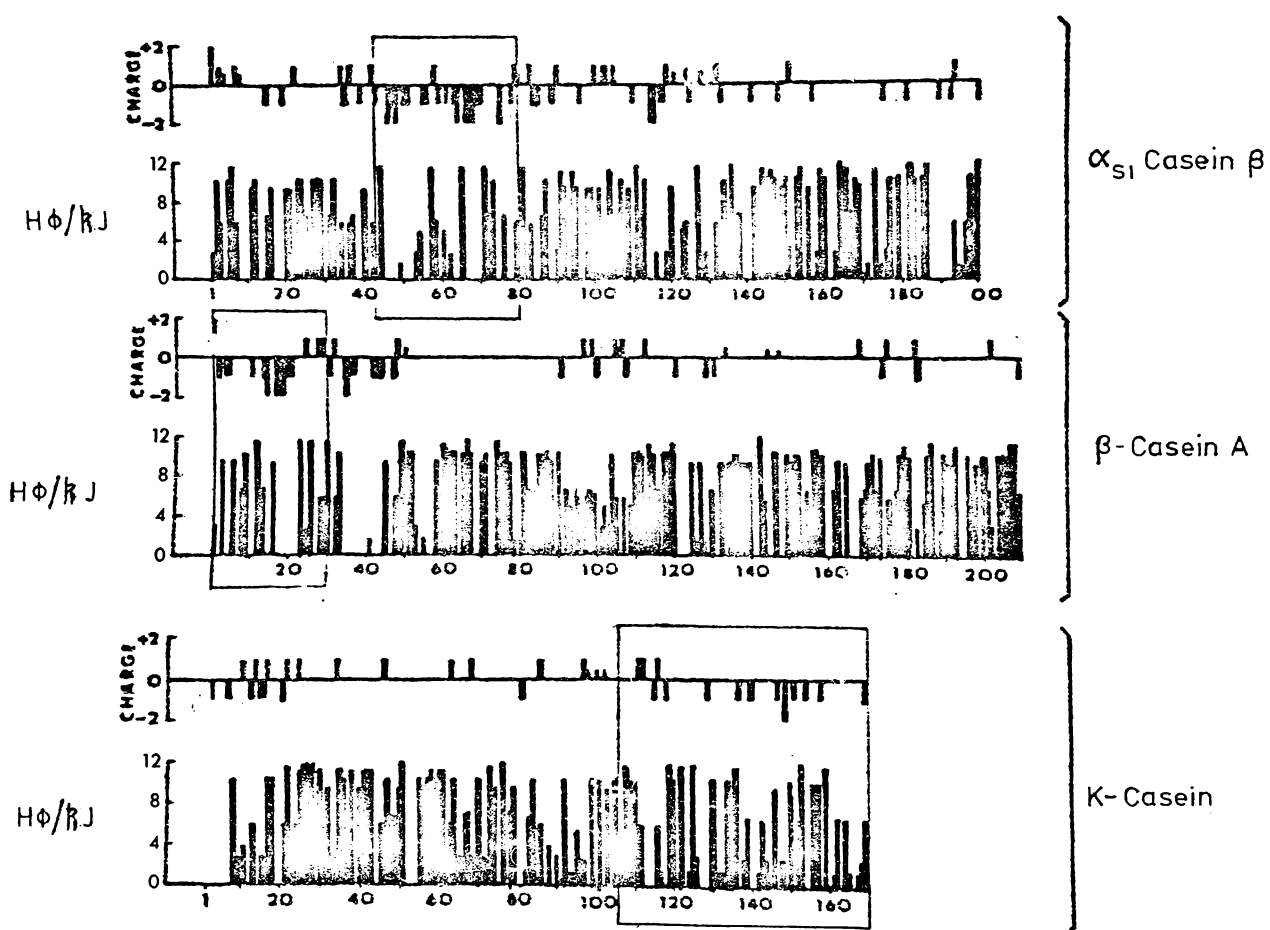
From the results it can be seen that the milk proteins, especially β -casein, have exceptionally high non-polarities. Most proteins have an HQ_{Ave} of between 4.20 and 4.60 kJ/residue (203).

The distribution of the hydrophobicity is of major importance in determining the physicochemical properties of these proteins. Diagrammatic illustration of the distribution of charged and hydrophobic residues for α_{s1} , β and K-casein are given in Figure 2.6. It is of interest to note that α_{s1} -casein B shows three predominantly hydrophobic regions occurring between residues 1 to 40, 81 to 115 and 140 to 170. In contrast, the average hydrophobicity of β -casein is greater than that of α_{s1} -casein B and is located predominantly over two-thirds of the length of the molecule leaving one end very low in hydrophobic character. K-casein also has high hydrophobicity between residues 20 to 60.

The distribution of charge resulting from the amino acid residues and the phosphate groups are also shown in Figure 2.6. In α_{s1} -casein the amino acids with negative charges are concentrated between residues 41 to 80. In β -casein A² a high density of negative charge occurs in the amino acids from residue 1 to 43 which also contain the phosphate groups. In K-casein B the macropeptide fraction from amino acid residue 105 to 169 contains a large percentage of the net charge of the molecule. The phosphate residues of the K-casein are also located in this region.

Figure 2.6 Diagrammatic illustration of the distribution of charged and hydrophobic residues in α_{s1} , β , and K-casein (53)

The phosphopeptides of α_{s1} and β -casein and the macropeptide of K-casein are boxed in.



At pH 6.6 the net charges of α_{s1} -casein B, β -casein A² and K-casein B are approximately -23, -13, and -3 respectively (53).

The preceding paragraphs indicate that the caseins, in common with other proteins, contain regions of both hydrophobic and hydrophilic character. The hydrophilic character in the main, results from the presence of negatively charged carboxyl and phosphate groups and positively charged amine groups. By virtue of their hydrophilic and hydrophobic properties, these proteins are all highly surface active. Thus, it can be expected, for example, that they will be adsorbed at the water-air interface with a concomitant decrease in surface tension. In addition, because they are charged molecules, they can be expected to be adsorbed at the charged sites of ion exchangers. As in the case of most proteins, hydrogen bonding and sulphide bonding are less likely to play an important role in adsorption phenomena.

Review of Milk Protein Adsorption

There has been little detailed study of the adsorption or desorption of milk and milk proteins, although there have been numerous qualitative studies of the effectiveness of various detergents in the removal of milk films. Mills and Creamer (205,206,207) using high surface area systems and standard analytical techniques have studied milk protein

on to clay minerals. Nisbet and Langdon (188,189) developed a radiochemical technique for use in a miniaturised simulator and demonstrated how radiochemical methods could be used in low area systems. This present thesis follows on from this work.

Mills et al. showed that casein bound strongly to clay minerals (halloysite and kaolinite) with bond strengths that varied in the order: γ -casein > β -casein > α_{s1} -casein > K-casein. With the exception of K-casein this is the order of the net negative charge of the individual caseins and although the attractive forces were not known, electrostatic explanations were suggested. It was suggested that the positively charged sites on the casein were attracted to the negatively charged sites on the clay and likewise the negatively charged sites on the casein, such as the phosphopeptides, were attracted to the positively charged alumina sites on the clay. It was also observed that the total amount of casein adsorbed decreased on increasing the pH from 6.0 to 9.5 (205). Later studies involved varying the net charge on β -casein by acetylation to form either acetyl or succinyl β -casein. This resulted in the normally positive lysine residue being replaced by the neutral acetyl lysyl residue or by the negative succinyl lysyl residue. Such replacement of the net protein charge at any given pH resulted in a decrease in the amount of casein adsorbed (206).

Studies of the adsorption of the individual protein fractions of milk showed that the order of the amount adsorbed was:

TS-casein $> K$ plus γ -casein $> \alpha_{s1}$ -casein $B > \alpha_{s1}$ -casein $A > \beta$ -casein. These adsorptions were performed at an ionic strength of 0.1 which may explain the low affinity of β -casein. At this ionic strength the charge may be relatively unimportant (207). Calculations showed that the β -casein molecule could adsorb on to the clay with its long axis nearly perpendicular to the surface, with the acid end of the peptide extending into the water phase.

Initial investigations at Unilever involved the construction of pressure-area isotherms and pressure concentration isotherms at the air-water interface for α_{s1} -casein, β -casein, K-casein, β lactoglobulin and α lactalbumin (208). These results suggested that disordered proteins (α_{s1} -casein and β -casein) are more surface active than globular proteins (whey proteins). At high surface concentrations α_{s1} and β casein showed no detectable viscosity, K-casein and α lactalbumin gave viscous films, while bovine serum albumin and β lactoglobulin were visco elastic (209).

Recent studies (213) have involved the use of C^{14} acyl labelled β and K-casein (which was considered to behave the same as β and K-casein) at the air-water interface. At solution concentrations of $1.0 \times 10^{-6} \text{ g ml}^{-1}$ and $1.5 \times 10^{-6} \text{ g ml}^{-1}$ of β and K-casein respectively, the amount adsorbed at the air-water interface was proportional to the square of the time for which adsorption took place, indicating diffusion control. The diffusion coefficients obtained were, however, higher than the published values. This was possibly due to convection

currents. β -casein adsorption reached a plateau at a solution concentration of 10^{-6} g ml $^{-1}$ to give 2.6 ± 0.1 mg m $^{-2}$ surface adsorption. The adsorption was observed to be irreversible as on removing excess solution protein, desorption did not occur. On further concentration of the solution above 10^{-4} g ml $^{-1}$ an increase in surface concentration occurred. The shape of the isotherm was interpreted as follows:

At low concentration the protein was considered to lie on the plane of the interface. At the concentration where a plateau occurred, loop-tail formation occurred, and at high protein solution concentration the protein was adsorbed as aggregates at the interface. Overall the isotherm was described as fitting a Langmuir adsorption isotherm (210,211) reasonably well. It was observed that the presence of insoluble lecithin monolayers inhibited the adsorption or perhaps led to a layer of relatively restricted lecithin molecules around the periphery of the protein molecule (212).

The adsorption of K-casein at the air-water interface has been studied and compared to β -casein (213). It appeared that with K-casein the surface concentration increased with protein concentration giving no apparent plateau.

The adsorption of milk proteins at liquid-liquid interfaces, in particular, butter-oil and water and butter-oil and protein-free milk plasma has also been studied. A considerable reduction of the interface surface tension resulted in protein adsorption. In the butter oil-water interface β -casein appeared to be most effective in reducing the interfacial tension, while in

the plasma phase little difference was detected between the three caseins. It was also noted that albumin was active in the oil-water interface but not in the oil-protein free milk plasma interface. This result is possibly due to the presence of trace surface active agents in the milk plasma (214).

The uptake of 0.2% casein solution on to powdered bovine enamel was studied by Kjeldahl analysis (85). It was observed that at this concentration of casein about fifteen minutes were required for equilibration. It was also observed that on increasing the pH of the casein solution from 6.0 to 8.0 a slight decrease in casein adsorption occurred.

The collection of milk proteins on an ion-exchange membrane during electro-dialysis has also been studied (215). It was observed that protein collection occurred only at high current densities (30 mA cm^{-2}) and that collection could be avoided by periodic reversal of the polarity.

2.9 Macroscopic Protein Films and Accretions

Bacterial contamination associated with milk protein films can result only when macroscopic deposits are formed, since bacteria cannot multiply on a monolayer of adsorbed protein. The mechanisms by which macroscopic protein films are formed are of interest not only with respect to the present project but also because of the occurrence of fat, protein and protein-mineral deposits in a range of technological and biological systems. In this section the composition of a number of macroscopic protein deposits and the involvement of fat, protein and mineral in their formation will be reviewed.

1. Arteriosclerosis, Thrombosis and the Clotting of Blood

Arteriosclerosis, the thickening and loss of elasticity of artery walls which impedes the flow of blood, is usually caused by lesions and fatty deposits (atherosclerotic) on the inside of the arteries. Abnormal calcification both grossly and microscopically has been detected in the atherosclerotic plaque of aging human aortic intima (190). The calcium deposits associated with atherosclerotic plaque have been identified as crystals of carbonate-apatite $\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$ and hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ by X-ray diffraction analysis (191,192). The elasin component of the heavily calcified aortic plaques also showed an increase in the polar amino acids when compared with young aortae and old aortae free of plaque formation (192). Various theories have been suggested for the mechanism involved in atherosclerotic lesions. One of the first signs of this disease is the accumulation of cholesterol ester on the connective tissue (193). This has been suggested to be due to the results of increased free cholesterol influx, synthesised locally or by increased activity of the esterifying enzymes, either lecithin - cholesterol transacylase or the fatty acid transferase (194,195). The increased production of fatty acids and a decrease in ability to hydrolyse the esterified cholesterol results in its accumulation on the arterial walls. Other hypotheses have considered that the hydrodynamic forces can produce localised deposits of erythrocytes and thrombocytes (platelets) in regions of poor flow and this can result in initial formation of atherosclerosis (196). Alternatively the disease may

be prevented by the existence of a natural plasticizer substance and that deficiency results in atherosclerotic disease (197). Recent investigations have reported that those with heart disease have a reduced level of high density lipoprotein in their blood. These lipoproteins consist of fat, cholesterol and protein tightly bound together into a chemical complex. The lightest of these globules carry fat to various parts of the body, while the heavier globules move the cholesterol from cell to cell. Any excess cholesterol is removed from the system by the liver. These lipoproteins may also help to flush away fatty deposits from plaque on the arterial walls and therefore low levels of the high density lipoprotein are likely to result in atherosclerosis (198). Both drugs and diet appear to be able to raise the level of the high density lipoproteins in blood (198).

E.D.T.A. has been used intravenously in the endeavour to remove the calcium in the atherosclerotic plaque in the coronary arteries (199).

The major chemical defence against blood loss is the formation of the blood clot. Thrombogenesis resulting from the introduction of a foreign surface, is presumably caused by a similar mechanism. Whereas blood clotting is essential to prevent bleeding, thrombogenesis is a considerable problem, for example, in artificial cardio-vascular technology. Various theories have been suggested for the coagulation of blood. The protein mainly responsible for the coagulum is fibrin, which is formed from its precursor fibrinogen. This transformation

is catalysed by the enzyme thrombin. Thrombin, however, is not present in normal blood and its biochemistry is not yet fully understood.

To date, although various materials have shown promise, no technology has yet been found for the implantation of cardiovascular and vascular repair devices that are completely free from thrombogenesis problems. It is possible that the problem is caused by the blood coming into contact with damaged tissue near the implant which triggers the clotting mechanism in a similar way to that of blood coming into contact with the damaged tissue of a bleeding wound. This contact could set in motion the complex biochemistry of the blood clotting mechanism.

It has been demonstrated that the formation of a protein film precedes platelet adhesion during thrombogenesis on foreign surfaces (216). An alternative mechanism of blood clotting is based upon the surface characteristics produced by such a protein surface layer (217,218). Others have suggested that adsorption causes a change in the free energy of the surface and this is responsible for thrombogenicity (219,220). Another possible explanation is that the effect is an electrostatic one. Surfaces containing a positive charge attract the negatively charged blood platelets and proteins (221,222). In vivo studies have indicated that this charge plays an important role in the formation of thrombosis. On metals, fibrinogen is reported to be adsorbed over a wide range of applied potentials (103,107). At positive potentials, fibrinogen undergoes "electropolymerization" to a fibrin-like material (223).

Alternatively adsorbed fibrinogen could induce platelet adhesion via the transferase enzymes. Fibrin formation and clotting results when the platelet is released (224). It seems that the adsorption of fibrinogen is a critical step in leading to clotting and this can be prevented by pretreatment of the surface with albumin. This appeared to give a surface of non-thrombogenicity (224).

2. The Formation of Kidney Stone

Urinary stones sometimes found in the kidneys consist of 95 to 98 percent crystalline material and the remainder is composed of an organic matrix (225). This organic matrix consists of 64% mucoprotein, 9.6% neutral hexoses and 5% hexosamine (226,227) with an abundance of acidic amino acid residues. When the total amino acid residues consist of about 65% aspartic and glutamic acid then the stone contained uric acid (225). If 50% of the amino acid residues are of aspartic and glutamic acid then the stone appeared to contain calcium oxalate, but if 30% of the amino acid residues are of aspartic and glutamic acid, and if glycine is 20% then a mineral stone of apatite-struvite composition resulted. The differences in the crystalline composition could indicate that different organic matrices influence the stone's structure and composition by providing specific templates. This effect has also been postulated as influencing mineral deposition in other matrices such as bone and collagen (228,229). How the stone is formed appears to be unknown. The organic matrix may either be an

"innocent co-precipitant" which may retard the dissolution of the calculi, or it may serve as a specific and essential nucleating substance (225). In bone and dentin the organic matrix may well induce and govern the mineralization of the tissue. The organic matrix does not however, appear to originate from collagen, the supportive and connective tissue of skin and the organic part of bone; as neither hydroxyproline nor hydroxylysine, present in collagen, have been found in kidney stones (227). Serine phosphate found in bone (230), enamel (231) and dentin (232) could play a role in mineralizing tissues that contain hydroxyapatite (229,231). Serine phosphate has been found in calcium oxalate and apatite-struvite kidney stones (225).

3. The Formation of Pellicle, Plaque and Calculus on Teeth

The pellicle which is formed rapidly on the surface of tooth enamel is composed of salivary glycoproteins (233,234), and is the first step leading to plaque and finally to the development of calculus. Plaque contains carbohydrate and bacteria in addition to salivary glycoproteins. Later in an alkaline environment, calcification occurs and the hard mineral deposit formed, high in calcium and phosphorus, is called calculus (235). No evidence exists from X-ray diffraction studies that this deposit originated as crystalline material in saliva (236). It is generally believed that there is a rapid and effective mechanism for concentrating and "fixing" the calcium phosphate (237,238). X-ray diffraction has shown that 40% of the plaque examined contained crystalline material of brushite and apatite (239) as early as twelve hours after the initial formation of the plaque, and by three days up

to 75% of the deposit was apatite. Magnesium, sodium, potassium and chloride have also been found in the deposit and these generally reflect the situation as in saliva at normal flow rates. Up to about 50% of the deposit of the calculus was protein and about 10% was carbohydrate (235,240,241) coming either from the salivary glycoprotein or bacterial cell wall glycoproteins. It is possible that after the interbacterial matrix of the plaque has formed and has been calcified, further salivary glycoproteins could be precipitated (242). It has been shown that the filamentous micro-organisms obtained from dental plaque can, in the presence of calcium salts, lead to the formation of insoluble calcium hydroxyapatite (243,244).

In an attempt to further understand this formation, adsorption of human salivary proteins (245) and streptococcal extracellular polysaccharides (246) on to synthetic hydroxyapatite has been studied. At high polysaccharide concentration the amount of saccharide adsorbed was seen to decrease. This effect has also been observed with the adsorption of dextran on to hydroxyapatite (247). The result could be due to the association of the molecules in solution (248), possibly forming micelles. The effect has also been explained by gel-like clots enmeshing the hydroxyapatite and by the bridging of the hydroxyapatite crystals by the initially adsorbed molecules. In effect this would prevent the access of further molecules (249).

2.10 Milkstone and Other Milk Deposits

Macroscopic deposits formed from milk on metal, rubber and glass surfaces are well known in the dairy industry. This section reviews the types of milk deposits that can occur with: raw milk, as in milking machines and milking machine simulators; processed milk, as in the process of pasteurisation; and coagulated milk as in cheese making and casein production.

Deposits formed from Raw milk

Deposits found in milking machines are usually referred to as "milkstone". Milkstone is a loose term which covers a wide range of deposits resulting from the interaction between the surface, the milk, the chemical constituents of the water supply and the chemicals used in cleaning and sanitising.

Some attempts have been made to classify milkstone deposits according to their physical properties such as hardness and aspects of their visual appearance such as colour and texture. For example Roderig et al. (250) described four classifications, whereas Twomey (251) described three classifications (see Table 2.5).

Table 2.5 Milkstone Classification

Roderig <u>et al.</u> (250)	Twomey (251)
1. Yellow, thin, compact hard	1. Dull yellow, sheet-like hard
2. Brown, thick, soft	2. Yellow, brown, soft
3. Grey, porous, soft	3. Granular, very hard
4. Brown, compact.	

Of the milkstones described by these workers, only the first two types in each classification appear to roughly correspond. Thus according to such a classification system at least five milkstone classifications are different.

It is however, likely that aspects of milkstone appearance are affected by environmental factors and the history of the deposit. In addition, deposits of similar hardness and appearance can vary widely in composition, mode of formation and ease of removal. For example soft yellow deposits can be either predominantly protein (253) or predominantly fat (252) and require quite different methods of prevention and removal.

An alternative method of classification might be based upon the chemical composition of the deposits. Although the compositions of milkstones vary widely (Table 2.6) certain trends in these compositions can be identified. Thus, Nisbet and Langdon (253) proposed a classification based on chemical analysis:

Iron milkstone

Calcium-phosphate milkstone

High Protein milkstone

Fat deposits.

1. Iron milkstone. These deposits contain iron up to 20% or more, probably as hydrous iron III oxide colloid together with phosphate and calcium. Iron is known to bind to tyrosyl groups (254) and histyl groups (255) of the protein. The deposits were produced when water with a high iron concentration was used during cleaning.

Table 2.6

Gross Chemical Composition of Milkstone

Constituent	Percentage of Deposit	
	(250)	(172)
Protein	39.8 - 67.3	4.1 - 43.8
Fat	0 - 10.7	3.6 - 17.7
Ash	19.0 - 57.7	4.2 - 67.3
Ca	7.0 - 28.7	15.0 - 24.7
P	3.2 - 9.6	3.8 - 5.8

2. Calcium-Phosphate milkstone. Deposits of this type were hard and contained large amounts of phosphate and calcium, small amounts of protein and very little iron. It is probable that these deposits are formed when the cleaning water has a high calcium content.

Drying is probably a major factor in the formation of these deposits. This would account for their physical hardness and would be consistent with the deposition of calcium salts by evaporation of cleaning solutions. The calcium in these deposits is believed to occur as calcium phosphate or calcium carbonate, although it is possible that at least some of it is bound as calcium ions to the protein.

3. High protein milkstone. This classification has been proposed to include deposits containing more than 50% protein. Generally the deposits were soft and were often formed in simulator studies (256) where protein contents as high as 80% have been observed.

4. **Fat Deposits.** Excessive turbulence in milklines can result in the separation of free fat as butter-like globules. These have been observed to accumulate in receiving tanks and lead to smeared greasy deposits which melt during the hot cleaning cycles. High turbulence during cleaning could possibly prevent this type of deposition.

The classification based upon chemical composition described above offers the advantage that it gives some insight as to the possible mechanisms by which the deposits were formed and further indicates how the deposits might be removed and how reoccurrence may be prevented. For example the formation of an iron milkstone is indicative of high iron concentration in the water supply and probably the only feasible means of avoiding the problem is to improve the water quality. Calcium phosphate milkstone is induced by hard water containing dissolved minerals and can be removed by acid cleaning and prevented by water softening and more frequent use of acid cleaning.

Some insights into the important variables involved in milkstone formation have been obtained as a by-product of the simulator testing of dairy detergents (see Chapter 14). The deposits formed in simulators, when inadequate detergents or procedures were used, were often quite like the "milkstone" found in milk lines. With few exceptions (189,256) however, no detailed studies of simulator deposits have been reported.

From simulator studies and field trials it appears that the formation of milkstone deposits can be affected by the following conditions:

Topography of surface

Cleaning programme - including detergent, wash cycle,
contact time, temperatures, volumes,
delay times, flow rates, sanitisation.

Water quality

Plant design

Climatic conditions

Milk conditions

Although relatively little is known about the mechanism of milkstone formation, some preliminary observation as to three possible mechanisms have been made (253). These include an adsorption-coagulation mechanism, a colloid-complex mechanism and precipitation-evaporation mechanism.

1. Adsorption-coagulation mechanism

According to this mechanism a monolayer of protein is adsorbed on to the stainless steel, interacting with the surface through electrostatic (257), van der Waals, hydrophobic bonding, covalent bonding, or a combination of such forces. The mineral component of milkstone is then adsorbed in the form of ions such as Ca^{++} , $\text{PO}_4^{=}$, Fe(III) and Al(III) hydroxy cations, Cu^{++} , etc. The adsorption of these ions from either the milk itself or the rinse solutions modify the adsorbed protein in such a way as to facilitate coagulation (or adsorption) of further protein at the surface. In this way an accumulation of deposit can be formed.

2. "Colloid-Complex" Mechanism

It is possible that the mineral component of milkstone is deposited in a colloidal rather than an ionic form. Thus after

the first layer of protein is adsorbed, a layer of colloidal calcium phosphate (from the milk, or precipitate from the phosphate in the detergent) is adsorbed on to the protein layer. It is believed that calcium phosphate plays a major role in the stability of casein micelles. Therefore it can be expected that after the adsorption of the calcium phosphate, more protein will be adsorbed upon subsequent exposure to milk. Other colloids found in the wash solutions could complement or even replace the calcium phosphate in its "complexing" role, e.g. hydrous Fe (III) or Al (III) oxides, silica or clay mineral particles, or the colloidal species, sometimes observed in detergent solutions.

3. Precipitation Mechanism

Where a solution of milk or rinse water is allowed to evaporate in the milk line, precipitation of milk and mineral constituent can occur. This can be accelerated by the warmth of the milk, the vacuum, and the heating of the cleaning solutions. In addition certain detergents such as acid solutions at pH 4.6 could precipitate milk proteins. Mineral precipitation could occur subsequently by evaporation of rinse solutions, or possibly by the action of the detergent with the farm water supply (e.g. high silica containing soft water used with an alkaline detergent is thought to help form milkstone (258)).

Successive layers could be formed by successive cycles. Alternatively the protein and mineral compounds could be precipitated in a single layer and successive layers again formed by successive cycles.

In all cases the deposit formed could be modified by the application of heat, or drying making it more difficult to remove.

Deposits formed during the Heating of Milk

At temperatures involved in pasteurisation (72°C for 15 seconds), in ultra-high temperature milk pasteurisation (UHT), (138°C for 2 seconds) and such milk processing operations as spray drying, the deposition of solid material from the milk on to the plant surfaces is a problem, particularly when thermal gradients are present. For example the deposit formed (259) during the operation of a heat exchanger at 140°C for three hours under conditions of high milk flow, resulted in a 30% decrease of the heat transfer coefficient and necessitated the use of increased pressure to maintain constant flow (260). Also deposits that become detached by milk flow can cause unsightly sediments in the finished milk product and can be a source of contaminating bacteria.

The nature of heat induced milk deposits (261) varied according to the temperature of the surface and were observed to change down the length of a heat exchanger. At the lower temperatures the deposit was a soft voluminous curd-like material, possibly overlaying a harder base formed initially by over heating at the surface. This soft deposit was made up of 50 to 60% protein and 30 to 35% mineral with a low fat content of about 4 to 8%. In the higher temperature part of the heat exchanger, a second type of deposit occurred which was thin, brittle, gritty and grey in colour. Such deposits were often extremely resistant

to chemical cleaning (263). This deposit contained about 70% incombustible solids mainly calcium and phosphate and only 15 to 20% protein. It was also found to contain very small crystals of $\beta\text{-Ca}_3(\text{PO}_4)_2$ with a distorted lattice (261). $\beta\text{-Ca}_3(\text{PO}_4)_2$ has a Ca/P atom ratio of 1.5:1 whereas analysis of the deposit gave an atom ratio of 1.3:1. This difference was attributed to the binding of cations other than calcium at the surface of the crystallites (262). It is likely that the protein components of this deposit were denatured (265,266). It is thought that the soft voluminous curd-like material controlled the interval before cleaning became necessary. The higher temperature deposits, may pose greater removal problems because of their dense nature and greater adhesion (264).

The factors that influence heat induced deposition include preliminary heat treatment, age of milk, pH, flow rate, entrapped air, smoothness of heated surface, seasonal variation of milk and the effect of added species.

It has been suggested that pre-warming milk before treatment causes a reduction in the amount of deposit. In ultra-high temperature milk pasteurisation the preliminary heating of milk to 95 °C for 15 seconds (260) resulted in the amount of deposit being reduced by half, over a given time. Pre-warming for 5 minutes at 85 °C (267) reduced the deposit by about 70%. It could be that the soluble proteins in milk are an important factor in deposit formation and that preliminary heat treatment was sufficient to denature most of the soluble proteins (260),

and so decrease the amount of deposit. Alternatively, preheated milk, compared with raw milk, can form less active calcium phosphate centres and this results in less calcium phosphate being deposited on the heated surfaces (268).

The age of milk has been shown to be important. The amount of deposit formed after twenty-four hours aging was reduced considerably (269,270) and the results for periods of storage indicated an effect that varied approximately exponentially with age (261).

Deposit formation varied non-linearly with pH, increasing as pH was lowered below 6.8, and only slightly decreasing as pH was raised above 6.8 (261).

An increase in the milk flow rate reduced the amount of deposit (264,271). By increasing the Reynolds number from 4 000 to 10 000 the rate of deposition was reduced by approximately 20%. It has been suggested that a Reynolds number greater than 37 000 would give no deposition (259).

The air content of heated milk is an important factor in milk deposition and its reduction can reduce deposition from 50 to 75% (271). Air bubbles on heated surfaces provide a greater area for the denaturation of the protein and induce the formation of a porous deposit (273).

The smoothness of surfaces used in milk processing is very important. Generally less deposition occurs on smooth surfaces (274,275).

Minimum deposition appears to occur during approximately the tenth week of lactation, and a maximum appears to occur

towards the end of lactation (276). This seasonal variation could be related to the phospholipid content (261); however no preferential inclusion of this lipid could be observed in deposits (266).

The amount of deposit was found sensitive to certain added chemicals. For example, the addition of β lactoglobulin decreased the amount of deposit (277,278). It has also been observed that when the fat content, calcium phosphate content and whey protein content of milk was increased the tendency to form deposits was also increased (283). The addition of sodium metaphosphate or casein however, had no effect, although the addition of β -casein by itself increased deposit formation (279). This did not occur when an equal amount of α_{s1} or K-casein was also added.

The addition of small amounts of sodium pyrophosphate (280) reduced the amount of deposit formed from heating milk during the operation of a U.H.T. heat exchanger. A number of other phosphates were studied but the pyrophosphate was the most efficient in reducing the deposit. It was attributed to a reduction in the destabilisation of casein micelles on heating, by providing an additional source of phosphate ions. The amount of colloidal inorganic phosphate increased on heating, while the soluble and micellar phosphate decreased (281).

The deposit does not vary linearly with fat content (282) of milk, and it was still formed even when skim milk was used (261). A suggested mechanism for heat induced milk deposition involves two stages. The first stage was considered to be the

adsorption of the aggregates formed in heated milk on to the surface of the heat exchanger. This was then followed by further adsorption of the aggregates on to the already adsorbed aggregates (261). The exact composition of these aggregates is not known, but it is likely that the reduction in solubility of milk salts, at high temperatures forms an initial first stage and this is followed by the slow formation of crystal nuclei. The growth of the nuclei into crystals then constitute the deposit.

The non-casein proteins of milk are relatively heat-labile, and undergo complete denaturation in 10 minutes at 90 °C, while the caseins are remarkably stable and withstand heating at 140 °C for more than 60 minutes at pH 6.7 before coagulation occurs (285). Interaction between denaturated β -lactoglobulin and precipitated calcium phosphate occurred when a solution of milk salts and β -lactoglobulin was heated to 100 °C (286). This may explain the composition of the aggregates on the surface of the heat exchanger. The result does not however, explain why the addition of β -lactoglobulin decreases deposit formation (287). If the initial adsorption of the aggregates could be prevented, then presumably multilayer deposit formation could also be prevented from forming. Polytetra fluoroethylene (PTFE) which has poor adhesion characteristics, however, was found to build substantial deposits (261,284). When the milk soil dried it could be easily removed from the Teflon surface (284).

Deposits formed from Coagulated Milk

The adhesion of flocculated casein to contact surfaces can be troublesome in continuous cheese making. This is especially so when stale milk, which produces a sticky curd, is used.

Investigations under the conditions of constant flow (288) and under static conditions (289) have shown that adhesion of clotted milk was a serious problem when clotting occurred during circulation.

The coagulation of the milk is based on the reaction of the rennet with K-casein which leads to the formation of para-K-casein and causes the irreversible coagulation of the other caseins. It has not been established whether the formation of deposits results from the adsorption of the curd or by a process in which para-K-casein serves as a bridging species for binding further proteins on to a surface previously saturated with protein.

Prior treatment of surfaces with lecithin (290) considerably reduced the amount of adhesion of clotted milk, as did pretreatment by species such as starch, regenerated cellulose, and polyacrylamide gel, all of which generated hydrophilic surfaces (288). Surfaces coated with cholesterol, producing hydrophobic surfaces gave curd adhesion characteristics similar to untreated stainless steel (288).

2.11 The Role of Detergents and Sanitisers

Dairy detergents are used as aids in the cleaning of milk lines, and sanitisers are used to control the bacterial level that may escape cleaning procedures. It is an unfortunate fact that

many detergent formulations and sanitisers are quite unsuitable for use under certain conditions and often accelerate deposition problems.

A detergent's primary function is to remove any residue that could serve as nutrient for bacteria. A number of authors have reviewed the physical chemistry of cleaning (291,292,293,296), and in particular the cleaning of hard surfaces relevant to the dairy industry (294,295,328,330). The typical formulation of a dairy detergent includes surface active agent, buffer, builder, filling agent, and sometimes anti-redeposition agent. These compounds and their characteristic properties will be discussed briefly.

a. Surface active agents

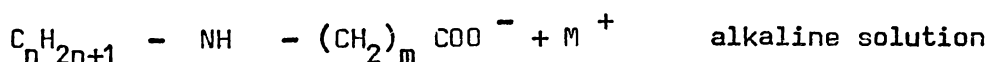
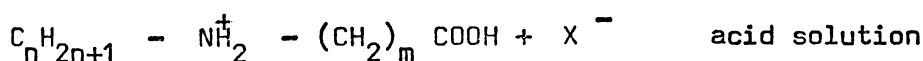
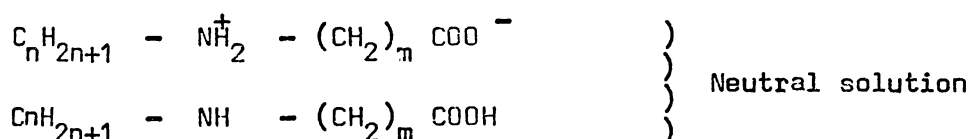
Like proteins, these molecules have both a hydrophobic and a hydrophilic group and their surface activity depends on the balance between these two structures (297,298). As in the case of proteins the association between the water molecules force the hydrophobic groups of the surfactant from solution (299) and therefore good surfactants are concentrated at interfaces. There are five classes of surfactants:

1. Anionic surfactants dissociate in solution to give a negatively charged surface active ion and a small inactive cation. Examples include soap, sodium alkyl aryl sulphonate and sodium fatty alcohol sulphate.
2. Cationic surfactants dissociate in solution to give a positively charged surface active ion and a small inactive anion. The positive charge is localised on a nitrogen atom, sometimes part of a heterocyclic ring as in the case of alkyl pyridinium salt.

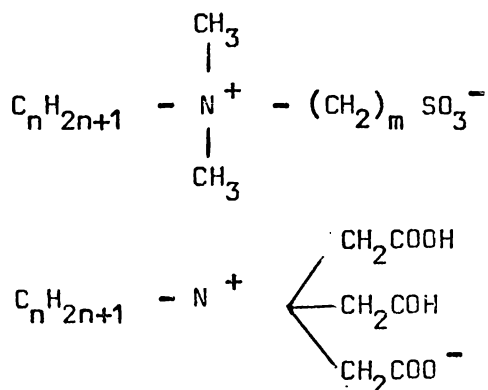
Other common cationic surfactants are cetyl trimethyl ammonium chloride, and cetyl dimethyl benzyl ammonium chloride. Cationic surfactants play an important role as sanitisers and antiseptic agents because of their antibacterial properties.

3. Nonionic surfactants do not ionize in solution and the whole molecule acts as the surface active agent. The most commonly used nonionics are the ethylene oxide condensates of alkyl phenols and fatty alcohols.

4. Ampholytic surfactants have both positive and negative centres on the active molecule and can be either positive or negative in solution depending on the pH of the solution, due to both the acidic and basic functions being weak. For example:



5. Zwitterionic surfactants also have positive and negative centres, but these are internally balanced, and like a nonionic surfactant the whole molecule acts as the active cleaning agent. This occurs when both the acidic and basic functions are strong and can exist over a wide pH range spanning the neutral point.



Ampholytic and zwitteronic surfactants are rarely used in the dairy industry.

During the early 1960's much attention was focussed on the biodegradability of detergents. The branched alkyl benzene sulphonates were not degraded easily and became a biological problem (300). Standard methods of testing detergents for biodegradability are known (301,302).

The interaction of surfactants with proteins will be discussed in Chapter 11. Some surfactants bind very strongly with proteins and could be implicated in the formation of deposits by multilayering effects.

b. Acids, alkaline and buffering agents

Both acid and alkaline detergent formulations are used in the dairy industry. The acid formulations containing: phosphoric, nitric and sulphamic acid are regarded as best for removing mineral deposits. The alkaline formulations containing sodium hydroxide, sodium metasilicate, and sodium carbonate are considered best for removing protein deposits. They are also found good for removing fatty acid deposits but not lipoidal material. The exact mechanism of removing the lipoidal material is not known but recent studies indicate that saponification is only a minor factor (303).

c. Builders

Builders are used in detergent formulations to enhance the cleaning effectiveness of the surfactant when used in water containing dissolved minerals (304). Their principal role is to remove from solution the metal ions that interfere with the action

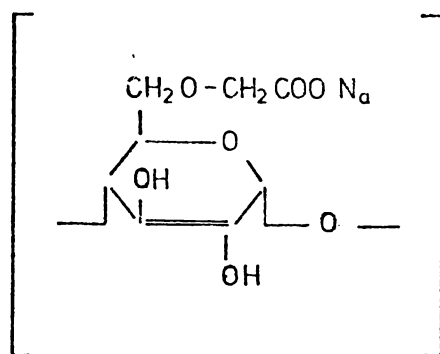
of the surfactant. Calcium and magnesium ions commonly found in farm water supplies are effectively removed as soluble complexes or less effectively as precipitates. The builders also often serve as buffering and anti-redeposition agents. Examples of common builders are sodium tripolyphosphate, sodium hexametaphosphate and disodium ethylenediamine tetraacetic acid. In some countries recently, phosphate builders have been held responsible for contributing to the eutrophication of lakes and considerable research has been undertaken to find a suitable replacement for phosphates in detergent formulations (305).

d. Filling Agents

These are inert agents such as water, sodium chloride, and sodium sulphate that are used to increase the bulk of the formulation for ease of handling.

e. Anti-redeposition agents

Often the redeposition of a soil which has been removed in a cleaning process can be a problem. Sodium metasilicate is often used in dairy detergent formulations partly for its anti-redeposition properties. The silicates are also well known for their anti-redeposition properties in textile detergency (306). Another common anti-redeposition agent, first used about 1940, is carboxymethyl cellulose which acts as a hydrophilic colloid and has the following structure:



Chemical structure of Carboxymethyl Cellulose (307)

It is of interest to note the similarity of this structure to that of heparin (see section 2.7). It seems likely that both species act in a similar way.

The effectiveness of antiredeposition agents in textile cleaning is generally regarded as being due to their adsorption both by the soil and the fabric interface (308). Carboxymethyl cellulose is effective as an anti-redeposition agent for only the fibres with which it can form hydrogen bonds, i.e. fibres that contain cellulose, e.g. cotton, nylon and rayon, but not wool, orlon and acetate (309). Although used extensively in fabric cleaning, this compound is not used in the dairy cleaning, possibly because it interacts with casein causing precipitation (33).

Important Physical Variables

A number of physical-chemical variables are important in determining the effectiveness of a detergent in a cleaning cycle. These include the number of contacts, contact time, concentration, pH, temperature, flow rate past the surface, surface tension effects and water quality.

From a study of the removal of synthetic soil from textiles, it was found that plots of the logarithm of the soil remaining versus the detergent concentration resulted in curves having segments that were linear. For the straight line portions, Bacon and Smith (310) derived an empirical equation relating the effect of time (T), detergent concentration (C) and mechanical force (F) to soil removal (S) as follows:

$$S = K(CFT)^n$$

where n and K are constants characteristic of a given system. The above equation tends to fail after long contact time, after most of the soil has been removed and when soil redeposition effects become significant (293). It also holds over a relatively narrow range of concentration and tends to fail completely at high concentrations. For example in the removal of beef tallow from cotton with sodium dodecylsulphate, maximum soil removal was achieved at 12 mmol l⁻¹. Above this concentration decrease in soil removal occurred (311). In other studies, soil removal reached a maximum and then became independent of further increase in concentration (312,313,314). The failure of the equation to take pH into account is an obvious shortcoming. For example, in the removal of milk films from stainless steel by sodium hydroxide (315), it was found that detergent action was first order with respect to the concentration of the hydroxide ion.

There is considerable difference of opinion as to the effect of temperature. Deviations from the behaviour predicted by Bacon and Smith (310) are frequent (316,317,318,319). This is

partly due to the wide range of conditions studied. For example temperature has little effect on oily soil removal at temperatures between 45 °C and just below 100 °C (313). It could be expected that an oily material would be difficult to remove at temperatures below its melting point. The effect of temperature on the desorbing solution is also important. In a simple system such as sodium hydroxide, the active agent is the hydroxide ion and this is relatively independent of the temperature. Certain surface active detergent formulations however, are very heat-labile and heat causes a decrease in the surfactant concentration (293).

Bacon and Smith's equation (310) also has limitations in respect to the effect of applied mechanical force. It has been reported that the flow rate past the surface is such that until a Reynolds number of 25 000 is achieved, the rate of removal is independent of flow rate (320). Beyond this point increased flow rate and the associated turbulence results in increased cleaning efficiency.

Enhanced detergency through mechanical action brought about by surface tension effects, is known as the Dupre effect. This can result from repeated dipping of the surface into the detergent solution (321,322).

There have been numerous attempts to analyse the plots of soil removal versus time (361,362). Some workers claim that the exponential terms obtained from such plots indicate the presence on the surface of several distinct soils (363,364). No direct evidence for the existence of such species has been obtained (293).

Another important factor that affects detergency is the quality of water used for cleaning. A number of New Zealand dairy farms use water which has a total hardness of between 100 to 200 ppm of soluble calcium and magnesium expressed as calcium carbonate. Unless correctly formulated, detergents can often react with this calcium and magnesium to form insoluble compounds. The most common species likely to react with the soluble calcium, is the carbonate ion either formed from the decomposition of bicarbonate during water heating or introduced as a component of the detergent. The calcium carbonate precipitates form very adherent crystalline scale. Calcium and magnesium silicates precipitated by the silicates in detergents are moderately adherent while the calcium and magnesium phosphates are very much less adherent. Calcium hydroxide is reasonably soluble and seldom causes a scale, but magnesium hydroxide is sometimes deposited when caustic soda is added to water (323). Builders such as E.D.T.A. are added to detergent formulations in an attempt to form soluble complexes with the calcium and magnesium ions and so prevent precipitates from forming. Although reasonably successful in dealing with water hardness problems in textile and industrial cleaning, the use of builders in dairy detergents has not been entirely successful, possibly because economic constraints prevent the use of adequate concentrations. In parts of New Zealand, the water used on dairy farms also contains amounts of iron considerably in excess of 1.0 ppm. Initially in the form of ferrous ions, this iron is eventually oxidised to the ferric oxidation state and forms brown insoluble ferric oxide. If water high in ferrous ion

concentration is used with alkaline detergent, precipitation of a ferrous hydroxide with subsequent oxidation to the very insoluble ferric hydroxide can be expected. The effectiveness of adding builders to dairy detergents to overcome the problem of high iron concentration has not been fully researched. To date the most commonly used means of combating the problem is the oxidation of the iron by aeration and removal of the ferric hydroxide formed by sand filtration.

Cleaning systems used in the Dairy Industry

Milkstone, as previously discussed, contains predominantly protein and minerals and is best removed by solubilising the protein fraction in alkaline solution and the mineral fraction in acid solution (325). Most of the effective cleaning systems used in the dairy industry have incorporated the use of both alkaline and acid cleaning solutions (325). In practice this was satisfied by routine alkaline cleaning followed by a periodic acid clean. The original Ruakura system developed by Whittlestone incorporated the use of an alkaline detergent for six days followed by an acid detergent on the seventh day (326). Although this and other systems proved highly effective in controlling milkstone formation, the development of a procedure to completely eliminate all deposits and their associated bacteria has so far not been achieved. This, in conjunction with the continual demand for a higher standard of milk quality, has necessitated the introduction of sanitisers in cleaning systems. Sanitisers are agents that can eliminate or at least control the bacterial contamination of dairy milk lines. Common sanitisers used in the dairy industry are the cationic

quaternary surfactants (329) and the iodine based iodophors (327), both of which are used in the acid phase. Hypochlorites have also been used but have often resulted in considerable build-up of deposits in the milk lines.

Every year, many new formulations are developed in an attempt to reduce the cleaning cost, increase the effectiveness and convenience of use of the product and also lower energy requirements (331,332). Before new formulations can be accepted for use in the dairy industry, they must satisfy standards of non-toxicity, non-corrosiveness, solubility, stability, non-tainting and absence of objectionable odours, minimal foaming and finally they must be effective detergents. A number of procedures have been developed to evaluate the cleaning effectiveness of a detergent and these include bench tests, simulator trials and eventually farm trials.

Bench tests are usually limited to the testing of a detergent on a single soil load and do not involve the use of a repeated cycle system. They are not usually used for testing a two-stage cleaning system. Optical methods (334,335,336,337,339), chemical analysis (333,338,340,341,346,350) radiochemical methods (315,321,322,342,343,344,345,347,348,349,351) and microbiological methods (352,353,354) are the most common methods used to monitor the film remaining after a standard cleaning treatment. Because the bench tests are of limited usefulness, simulators to test detergents, under conditions more closely resembling those of a milk line, have been developed. These incorporate a repeated soiling-cleaning cycle and the residual soil is monitored after successive cycles or at the end of many cycles. The system is usually automatic with

up to 24 or more complete cycles a day. This considerably accelerates the soiling process when compared with the twice daily cycle of a dairy milk line. Simulators operate either by dipping slides into the various test solutions (355,356) or by flushing them with various solutions (357,358,359,360). Recently a miniaturised simulator (189) incorporating the use of radio iodinated milk was developed. One advantage of this miniaturised simulator was that it required only low volumes of milk in comparison with other simulators and could be used to monitor each step of the cleaning cycle.

All simulators suffer the disadvantage that the artificial deposit formed is not necessarily truly representative of deposits found on the milk lines, and further the bacterial regime will probably not have had sufficient time to fully develop. In addition, all simulator trials are necessarily expensive and time consuming. Because of the large number of new formulations being currently submitted for screening, the National Dairy Laboratory, after having used the simulator method for many years, has recently found it necessary to resort to bench tests as their principal means of evaluating detergent efficiency (365).

The best test for a detergent or cleaning system is by farm trials. However such tests are even more expensive and time consuming than simulator trials and the results are never completely general. It is difficult to do field trials to cover all conditions of farm cleaning. Normally a field trial would not be envisaged until the detergent had been thoroughly tested and the cleaning procedure optimised by use of simulators.

The ultimate test of a product or cleaning system can only be obtained by universal use and monitoring over a long period of time.

One of the important bonuses that has resulted from the testing of detergent formulations has been the better understanding of the mode of detergent action on milk films and the nature of these films. Much is still to be learned of these aspects and in part, the present project was undertaken with the aim of gaining more information about the processes involved.

2.12 Scope of Present Investigation

The preceding sections have described the problems caused in the dairy industry by the formation of protein containing films on milk contact surfaces. Though considerable effort has gone into the formulation of efficient detergents and sanitisers to minimise deposit formation and bacterial colonisation, little is known of the fundamental processes involved in deposit formation. Though the solid-liquid surface chemistry of many proteins, particularly the blood proteins, has been widely researched, there have been few investigations of the solid-liquid surface chemistry of milk proteins and even fewer fundamental studies of the interactions of milk proteins at stainless steel-water interfaces. Even for those protein systems that have been extensively studied, many difficulties remain unresolved. These difficulties have resulted in part from the non-standardisation of experimental conditions employed. Until the advent of radiochemical techniques, investigations of protein adsorption was limited to large area systems (powdered samples) and relatively high protein concentrations. A major aim

of the present work was thus to develop radiochemical techniques that would allow the study of protein adsorption on to low surface area systems at a wider range of concentrations and under reproducible conditions.

Milk is a complex physico-chemical system and in order to understand the adsorption of protein from milk there is a need for further information about the interactions between various milk constituents. In particular it was of interest to study the micelle-serum interaction in order to determine whether it would be possible to prepare labelled micelles for adsorption studies.

In any adsorption study, the effect of experimental variables such as pH, temperature, ionic strength, adsorption time, concentration, are of interest and may give useful information about the mechanism of adsorption. From the point of view of cleaning stainless steel surfaces, the reversibility of the adsorption process is of prime importance. Studies of the kinetics of desorption into various solutions could be of value in understanding the mechanism of desorption and could lead to the more rational development of detergent formulations for use in the dairy industry.

Problems caused by milk protein films only result when macroscopic deposits are formed, since bacteria cannot multiply on a monolayer of adsorbed protein. Three possible mechanisms for the formation of macroscopic protein-mineral films on milk contact surfaces include:

- (i) repeated adsorption of monomers, polymers, micelles or aggregates;

- (ii) multilayer adsorption via a bridging species and/or chemical modification;
- (iii) deposited films caused by evaporation, precipitation and/or thermal modification.

Of the three possibilities, two depend upon adsorption processes either after a single exposure or after repeated exposure of the surface to the milk solution. Even if adsorption is not the principal mechanism of deposit formation, the binding between protein and the surface will be of major importance in determining the durability of any film formed.

The scope of the present investigation, thus includes the radiochemical labelling of milk constituents by iodination, the development of techniques for low area adsorption and desorption studies, the investigation of milk protein interactions at stainless steel interfaces under a wide range of conditions, studies of adsorption after repeated exposure of surfaces to adsorbent solutions and studies of precipitation and evaporation. Finally, an examination will be made of actual deposits formed in milk lines for properties that could be used to classify them, or help to indicate their mode of formation.

CHAPTER 3

THE PREPARATION OF α_{s1} , β , AND K-CASEIN FOR ADSORPTION STUDIES

3.1 Introduction

Although it is possible to prepare labelled individual casein fractions by chromatographic separation of iodinated milk, (see Chapter 5) such procedures result in low specific activity due to the fact that the iodine is distributed over all the proteins present. In addition, the protein fractions are relatively dilute after gel filtration or ion-exchange and therefore require to be concentrated by dialysis or ultrafiltration. Such procedures are made more difficult if the solutions are radioactive and further, they are lengthy procedures which cause undesirable delays between the iodination reaction and the use of the labelled fractions. It is also more convenient to work with one casein fraction at a time in the adsorption-desorption studies.

For the reasons outlined above, it was decided to prepare and store individual casein fractions for use when required. Early procedures for the separation of the individual casein constituents of milk were based upon the differences in their physico-chemical properties such as the effect of temperature, pH and ionic strength (particularly of calcium chloride) and their solubilities (366,367,368,369,370,371). Such procedures were tedious and usually involved many steps. Further, they often gave low yields of impure product. For these reasons, chemical

methods of separation have now almost entirely been superseded by chromatographic methods.

Since the mid-sixties, ion exchange chromatography and gel filtration have made possible the isolation and purification of many previously unrecognised milk proteins. These methods appeared ideal for the preparation of high purity fractions suitable for use in adsorption and tracer studies. Possible limitations of such procedures in preparative work are those of column size, fraction volume and amounts of reagents required to obtain large samples. Thus it was found convenient to combine chemical and chromatographic procedures in the preparation of β -casein.

In any protein separation procedure, the methods used to identify and characterise the various constituents are of high importance. For milk proteins, disc electrophoresis and absorbance measurements sufficed.

3.2 Preparation of Whole Casein

The casein used in all the separation procedures, was obtained from fresh Jersey milk. This was centrifuged for 20 minutes at 3500 rpm in a Sorvall GSA rotor centrifuge to remove the fat, and acidified to pH 4.6 by the addition of HCl at 35 °C. The casein precipitated was separated from the whey proteins dissolved in the supernatant liquid by filtering it through muslin. The precipitate was then washed thoroughly with distilled water and stored frozen at -20 °C.

3.3 Preparation of α_{s1} -casein

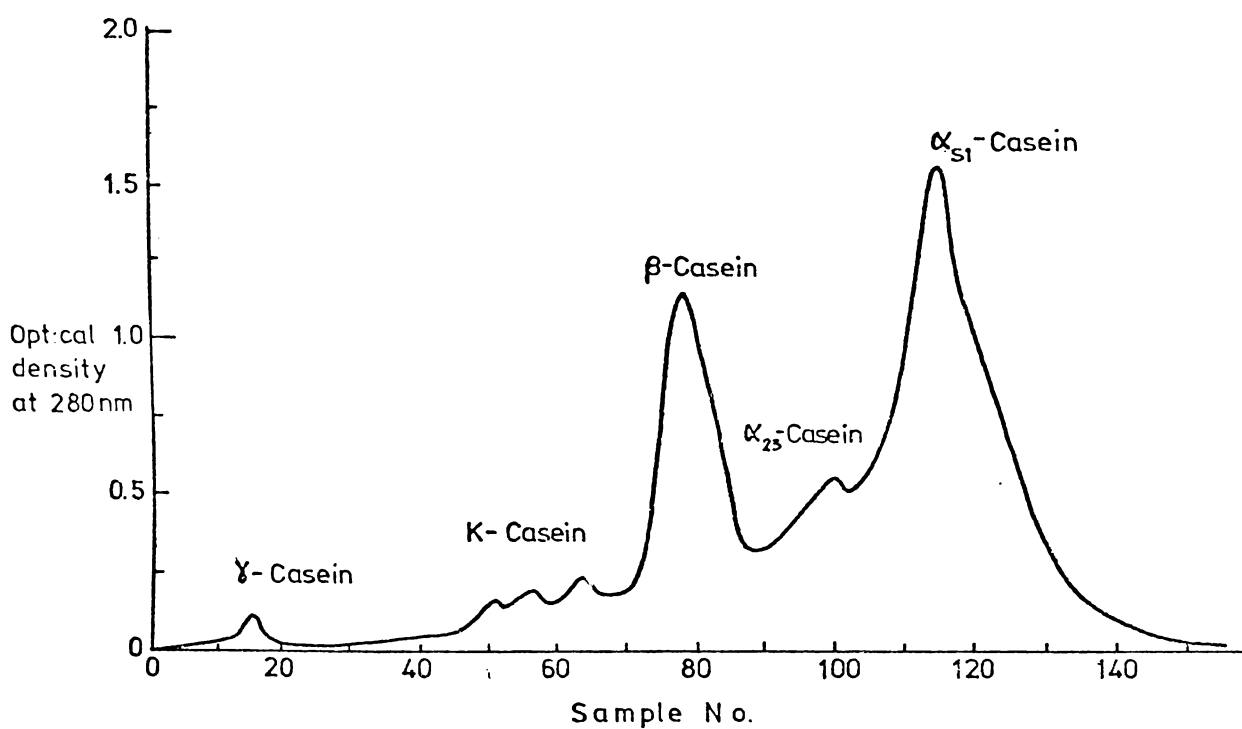
α_{s1} -casein accounts for approximately 50% of the total casein of milk and is readily separated from β , K, and γ -caseins and the other α_s fractions on a preparative scale by ion exchange chromatography (372).

The procedure employed was essentially that of Creamer (373) using a Whatman DE52 DEAE-cellulose with a 2.5 x 40 cm column. The casein was dissolved in 0.01 mol l⁻¹ imidazole-HCl buffer at pH 7.0 containing 4.5 mol l⁻¹ urea. The sample was eluted from the column using the same urea-imidazole-HCl buffer and a salt gradient from 0 to 0.30 mol l⁻¹ NaCl. Fractions were collected with a LKB 7000 Ultrarac Fraction Collector. The absorbance was continually monitored with either a LKB Uvicord II or a Cecil 272 Ultraviolet spectrophotometer at 280 nm. Initially six or more grams of the casein were dissolved and placed on the column in an attempt to prepare large amounts of β and K-casein as well as α_{s1} -casein. It was found that with this quantity of casein, very poor flow rates were obtained. The problem was partly eliminated by using a Peristaltic Pump P3 (Pharmacia Fine Chemicals) and by pumping the solutions through the column at a very slow flow rate. However, it was still found that the casein settled at the top of the column and difficulty was experienced in getting it into the column.

Improved results were obtained when the amount of casein was kept to less than two grams and a peristaltic pump was used to maintain a constant flow rate through the column. The result of a typical run is given in figure 3.1. Samples were collected

Figure 3.1

Chromatographic separation of milk casein



Column: 2.5 x 40 cm Whatman DE52 DEAE - cellulose

Eluent: pH 7.0, 0.01 mol l⁻¹ imidazole-HCl buffer, 4.5 mol l⁻¹ urea.

Salt gradient 0 to 0.30 mol l⁻¹ NaCl

Temperature 4 °C.

every five ml and a total volume of approximately 600 ml was passed through the column at a flow rate of about 30 ml per hour. Samples from the various five ml lots which were expected to contain the α_{s1} -casein fraction were run on the disc electrophoresis (see Appendix 2) and compared with a casein sample to determine their purity. Those containing only the pure α_{s1} -casein were combined together, concentrated by dialysis in a bed of polyethylene glycol flakes. After concentration, the α_{s1} -casein was dialysed with five litres of distilled water which was changed at least three times over a period of about 18 hours. A sample was again checked for protein purity by disc electrophoresis (see Figure 3.5) and a range of solutions of known dilutions were prepared and studied on the Unicam SP 1800 Spectrophotometer at 280 nm. Using the literature extinction coefficients for α_{s1} -casein B (see Table 3.1) and the optical density reading for the various solutions of the α_{s1} -casein (see Figure 3.2) the concentration of these solutions was determined and hence the concentration in the original dialysate estimated. A good correlation was obtained for the estimates made from the various diluted solutions indicating that Beers law was being followed. Generally the protein concentration in the original dialysate ranged up to about 0.03 g ml^{-1} . A spectrum scan from 200 nm to 700 nm was also determined at $14.5 \times 10^{-4} \text{ g ml}^{-1}$ and is given in figure 3.3. Finally, in order to provide an independent check on the optical method, 0.054 grams of the concentrated α_{s1} -casein was placed in a weighing bottle made of borosilicate glass and heated for twelve hours at 105°C . It was then allowed

Table 3.1

Extinction Coefficients of some Milk Proteins
at 278 nm

	$E / \text{cm}^2 \text{g}^{-1}$	Reference
α_{s1} -casein A	1.01	366
α_{s1} -casein B	1.00	366
α_{s1} -casein C	1.00	366
β -casein A	0.46	374
β -casein B	0.47	374
β -casein C	0.45	374
K-casein A	1.05	375
K-casein B	1.05	375
β lactoglobulin	0.95	376
α lactalbumin	2.02	377

E = Extinction Coefficient

Figure 3.2

Concentration Dependence of Optical Density of
casein fractions

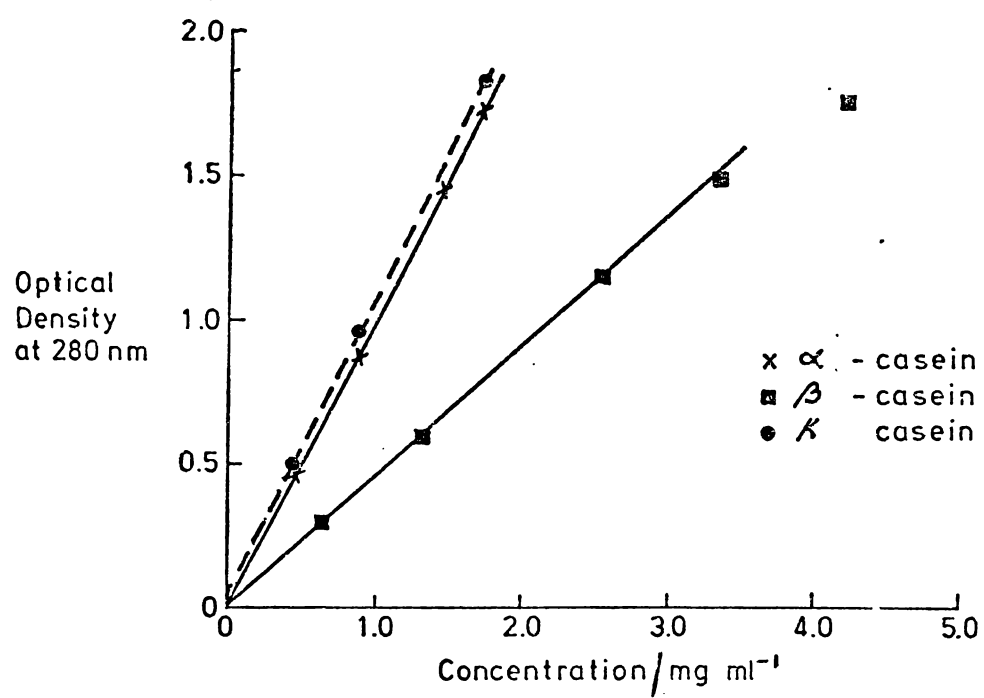
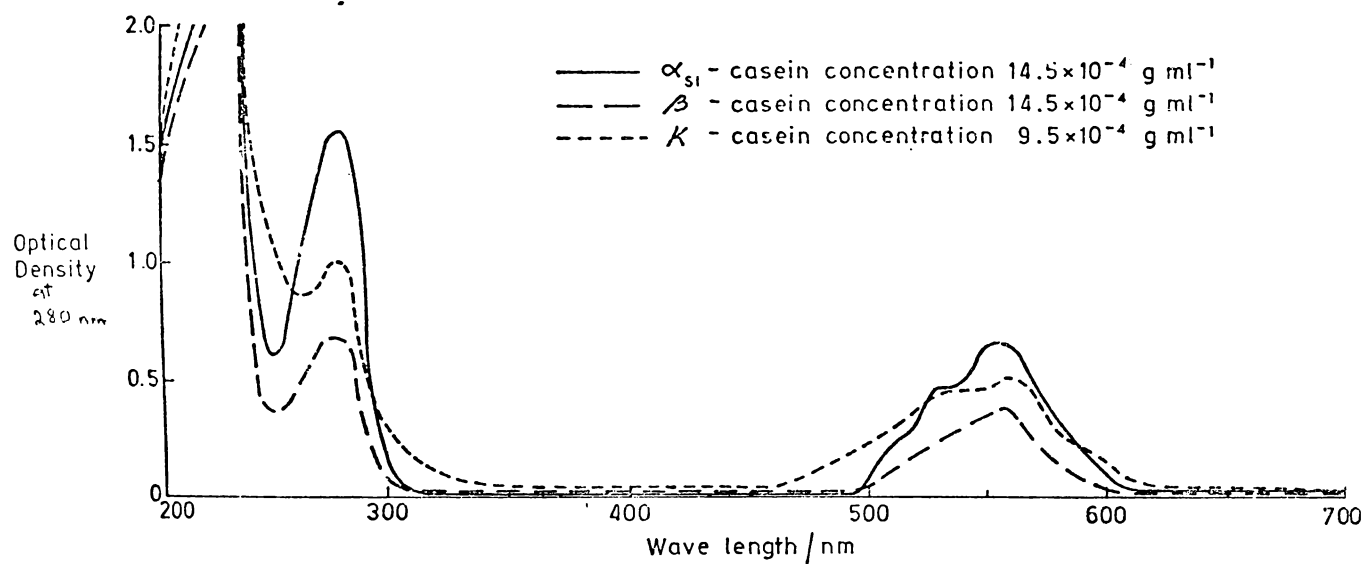


Figure 3.3

U.V.-Visible Spectra of milk caseins



to cool for two hours before reweighing. From this weight, the dialysate concentration was obtained and this agreed within experimental error, with the optical method. This result confirms the value used for the extinction coefficient and shows that the dialysate was free of dissolved material.

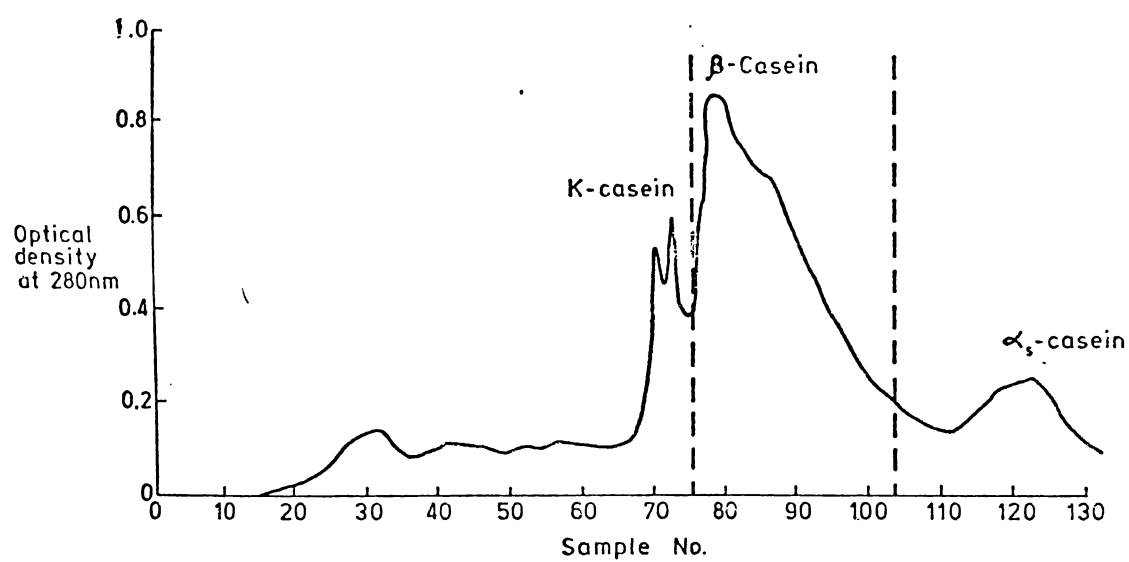
After dialysis the 40 ml of prepared α_{s1} -casein was divided into twenty 2 ml samples. These were deep frozen and stored at -20°C .

3.4 Preparation of β -casein

Approximately 30 to 35% of whole casein is β -casein. Because of the limited capacity of the columns for casein, it was decided that a prior chemical separation, by removing most of the α_s -casein, would facilitate the preparation of the desired amounts of chromatographically purified β -casein. Crude β -casein was prepared by the method of Ashaffenburg (358) (see Appendix 3). A yield of about three grams of crude β -casein was obtained. Disc electrophoresis showed that a significant amount of K-casein and a trace of α_s -casein was present. These components were removed by passing the sample dissolved in urea solution down a DE52 cellulose column. The optical density trace of the eluent from the column is shown in figure 3.4. The fractions were identified using disc electrophoresis, the pure β -casein fractions were combined, concentrated and dialysed as in the method used for α_{s1} -casein. The optical density of a range of diluted solutions were determined (figure 3.2) and using the literature extinction coefficient for β -casein, the concentration of these solutions and that of the

Figure 3.4

Preparative Chromatography of β -casein
from crude β -casein.



concentrated dialysate were determined. A spectrum scan from 200 nm to 700 nm at a β -casein concentration of $14.5 \times 10^{-4} \text{ g ml}^{-1}$ is given in figure 3.3. Finally to provide an independent check on the optical method, 0.060 grams of the concentrated β -casein was dried for twelve hours at 105°C and then weighed, following the same procedure used with α_{s1} -casein. From this weight, the dialysate concentration was obtained which agreed within experimental error, with the optical method.

In order to check the sensitivity of disc electrophoresis in monitoring product purity, a range of β -casein standards from $8 \times 10^{-5} \text{ g ml}^{-1}$ to $5 \times 10^{-6} \text{ g ml}^{-1}$ was prepared and run. The results indicated that the method was sensitive to at least $1 \times 10^{-5} \text{ g ml}^{-1}$ of β -casein. (See figure 3.5).

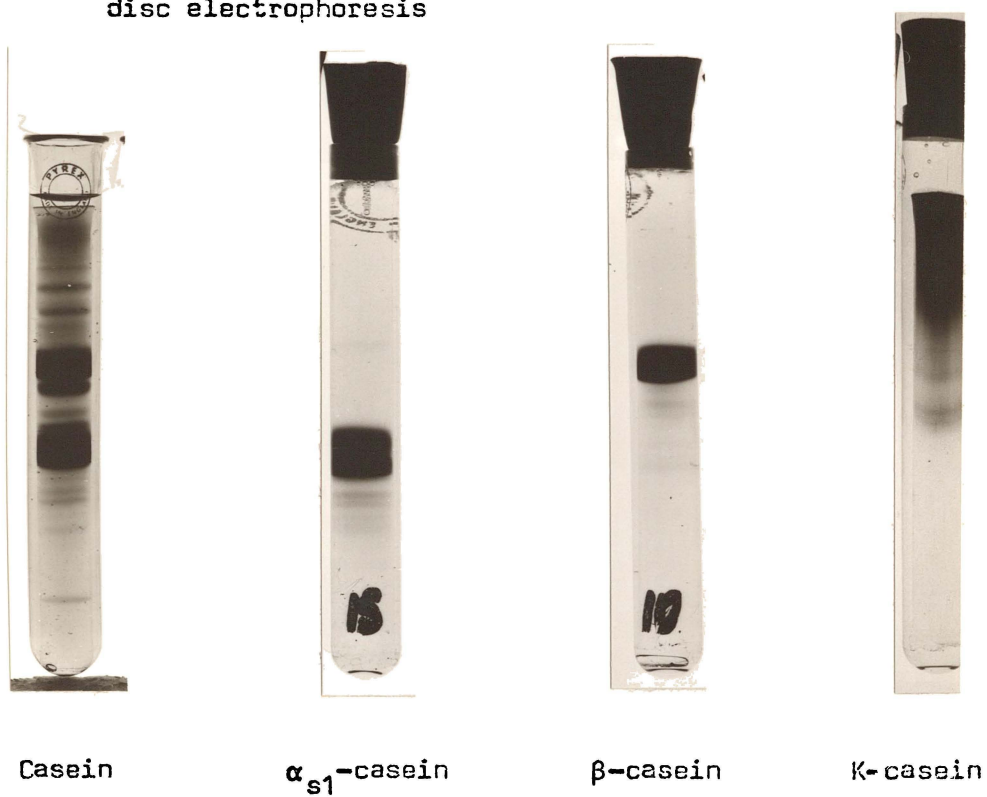
3.5 Preparation of K-casein

K-casein is present in even lesser amounts than β -casein. Only 10-15% of the total casein is K-casein and because of this low concentration it was again considered desirable to prepare crude K-casein by chemical means and to purify it if necessary, by column chromatography. Although the chemical method of McKenzie and Wake (371) was attempted a number of times, the yield obtained was inadequate.

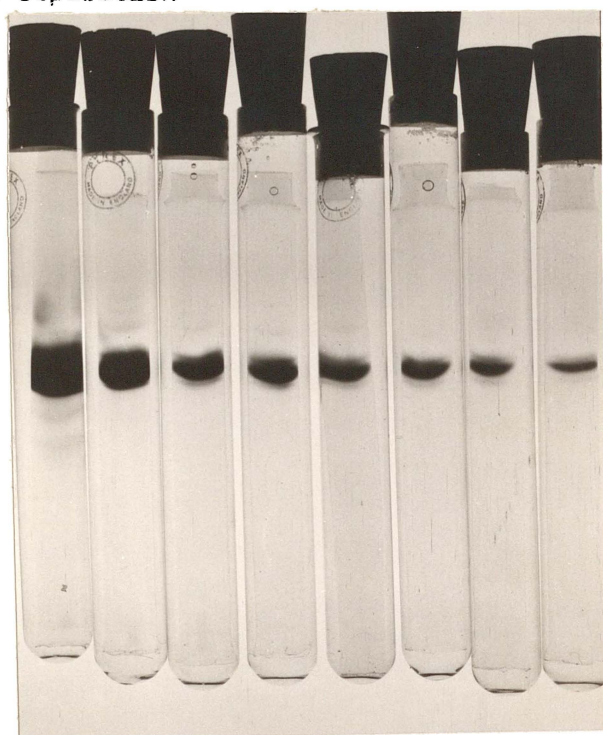
A limited amount of K-casein was therefore prepared by repeated chromatographic separation of whole casein as described previously. The K-casein was identified by disc electrophoresis and the concentrations were obtained from optical density measurements and confirmed gravimetrically. A spectrum scan of K-casein from 200 nm to 700 nm is given in figure 3.3.

Figure 3.5

Purity of α_{s1} -, β and K-casein determined by
disc electrophoresis



a. Casein separation



b. β -casein (concentration range from 8×10^{-5} g to 5×10^{-6} g)

3.6 Conclusion

From the methods described, casein fractions of α_{s1} , β and K-casein were prepared and were shown to be of adequate purity for studies of the adsorption properties of the individual casein constituents of milk.

CHAPTER 4

PREPARATION OF RADIOACTIVE PROTEIN TRACERS BY RADIOIODINATION

4.1 Introduction

The reaction of iodine with milk proteins is of interest for two reasons. Firstly the technique of radioiodination offers the possibility of preparing labelled milk proteins of high specific activity suitable for use as tracers in adsorption studies. Secondly with the increasing use of iodophors as sanitisers in the Dairy Industry, iodine contamination of milk is an ever present possibility. Thus, studies of the interaction of I_2 and I^- with milk constituents are of practical importance. Radioactive iodine provides a powerful tool for investigating the fate of iodine added to milk and studies of this aspect will be discussed in Chapter Five. This chapter will be concerned with the radioiodination reaction as applied to the preparation of radioactive tracers from isolated and purified milk constituents. Theoretical aspects of the iodination reaction and the stability of the iodinated product will also be discussed.

4.2 The Technique of Radioiodination

1. The Iodination Reaction

The technique of radioiodination gained prominence during the early 1960's as a method of preparing the radioactively labelled proteins and hormones required for use in the procedure known as radioimmunoassay, originally introduced to study plasma insulin

In the present discussion it will be convenient to classify the iodination reactions as being high level or low level depending upon the number of iodine atoms incorporated per molecule of protein. Low level iodination will be used in cases where zero or only one iodine atom is incorporated. High level iodination will be used when more than one iodine atom is incorporated. Species with up to 7% by weight of iodine per protein have been prepared by addition of finely ground iodine to an alkaline solution of skim milk (382). A more recent method involved adding large amounts of potassium iodide and an oxidising agent to a protein solution (383). In both preparations the iodinated protein was separated by precipitation at its isoelectric point.

Products of such reactions are likely to have quite different biological, chemical and physical properties because of the large amount of iodine bound to the protein. In addition to the formation of diiodo derivatives, oxidative coupling of tyrosyl residues leading to the formation of small amounts of thyroxine has been observed (384). Iodinated derivatives prepared by methods such as those mentioned above have application in the treatment of myxoedema and in studies of substituent effects on phenolic pKa of the tyrosyl residue.

The actual number of possible iodinated derivatives increases rapidly as the number of tyrosyl residues is increased. With a single tyrosyl derivative there are two possible iodination products corresponding to mono-iodination and di-iodination. For two tyrosyl residues the number of possible derivatives is 8 (see Table 4.1), and generally the number of possible derivatives

is equal to n^3 where n is the number of tyrosyl residues.

Table 4.1

Iodinated Derivatives of a Protein Containing
Two Tyrosyl Residues

		Iodine Atoms on Tyr (I)	Iodine Atoms on Tyr (II)	Total Iodine Atoms
"Low Level"	(	0	1	1
	(			
Iodination	(	1	0	1
	(			
	(	1	1	2
	(			
"High Level"	(	0	2	2
	(			
Iodination	(	2	0	2
	(			
	(	1	2	3
	(			
	(	2	1	3
	(			
	(	2	2	4

2. Mechanism of Iodination

Though extensively studied, the mechanism for iodination of phenolic substituents and the nature of the iodinating species is still open to conjecture. Attack of the ionised and unionised phenol by hypiodous acid (385), the phenoxide ion by acyl hypoiodite (386), the phenoxide ion by iodonium ions (I^+) and the phenoxide ion by molecular iodine (387) have all been suggested. It seems unlikely that the reaction involves the iodate anion (388). Recent studies appear to favour attack by molecular iodine (389,390, 391), although supporting kinetic evidence is sparse.

3. Distribution of Iodine in Iodinated Protein

It is clear that not all tyrosyl residues are iodinated with

equal facility. Thus in the iodination of human growth hormone which has eight tyrosyl residues, only two of these react freely with iodine. Iodination at a level of 3-4 iodine molecules per protein molecule has shown that almost 80% of the radioactivity is in the form of the diiodinated tyrosine (392) despite the fact that only 20-25% of the tyrosyl sites have been saturated.

In the incorporation of iodine into ribonuclease (393) at pH 8.5, it was observed that up to the addition of one mole of iodine per mole of ribonuclease, only moniodo tyrosine was formed. At higher levels of iodination, diiodotyrosine started to be formed and at the level of four moles of iodine per mole of ribonuclease, the amount of diiodotyrosine increased rapidly with a concomitant decrease in the amount of moniodo tyrosine. Also appearing at this stage was moniodohistidine. At the level of eight iodine moles per mole of protein the tyrosyl residues were predominantly diiodotyrosine with a small amount of diiodo and moniodohistidine.

The distribution of iodine after iodination of human K Bence-Jones protein has also been investigated with special emphasis given to the tyrosyl residue 173 and 186 (380). In tyrosyl residue 173, moniodiotyrosine was produced rapidly, reaching a maximum at approximately two moles of iodine per mole of protein. At higher levels of iodination (5-6 moles iodine atoms per mole of protein) the tyrosyl residue 173 was predominantly diiodotyrosine. The tyrosyl residue 186 was very much less active and up to about 5 moles iodine per protein molecule only moniodotyrosine was formed. Above this concentration,

diiodotyrosine commenced to be formed slowly at first and then rapidly with further increases in iodine concentration.

In the iodination of cows' milk, Potter and McIntyre (394) reported that they found only moniodotyrosine whereas Murphy and Campbell (395) found 65-68% of the iodine as mono-iodo tyrosine, 6-7% as diiodotyrosine and 2.9-3.5% as thyroxine. These apparent differences probably arose from variations in the natural concentration of iodine in the milk used.

It appears, then, that under mild conditions and with iodine concentrations such that there is less than one mole of iodine available for each mole of protein, the iodination reaction will in general yield labelled proteins containing one mono-iodo-tyrosyl residue as the predominant derivative. The milk proteins of interest all have several tyrosyl residues so it can be expected that under the appropriate conditions the desired moniodo derivatives can be formed.

A discussion of the effect of environmental factors on the reactivity of the various tyrosyl residues towards iodination is included in Chapter 5.

4. The Effect of Iodination on Protein Properties

In using iodinated protein for adsorption studies, one of the essential criteria to be met is that the iodinated and non-iodinated proteins are identical in chemical and physical properties. This is also a basic assumption in radioimmunoassay studies.

The two problems associated with iodination are the effect of adding iodine to the protein molecule and the effect on the

protein of the different chemicals used during the iodination reaction.

(a) Effect of adding iodine to the protein molecule

Substitution of an atom of iodine for a hydrogen atom results in a negligible change in the molecular weight of a protein molecule. Hence mono-iodination would not be expected to result in appreciable change in the physical properties determined by size and weight. The major chemical effect of iodine substitution is the lowering of the pK_a of the phenolic hydroxyl of the tyrosine. Mono-iodination decreases the pK_a value from 10.1 to 8.2 and di-iodination decreases it further to 6.4 (396). Although the pK_a 's of tyrosyl groups are affected by environments, this same trend is exhibited by moniodinated and di-iodinated tyrosyl residues of proteins. For example, the values for thyroglobulin are 11.35, 9.3, and 7.3 respectively for the pK_a values of tyrosyl, mon-iodotyrosyl and diiodotyrosyl (397). Although the pK_a values are lowered by iodination it is generally agreed that providing no more than one iodine atom per protein molecule becomes labelled, there is no change in the biological activity of the molecule (438).

(b) Effect of different chemicals used during the iodination reaction

The presence of oxidising agents is usually required during the iodination reaction and these could lead to the breakdown of the protein. Generally, evidence for the absence of oxidative breakdown by a particular technique can be obtained by showing that hormones, very susceptible to denaturation, show no loss of immune-activity after low level iodination.

Relatively vigorous conditions are required for the oxidation of tryptophyl, methionyl and histidyl residues (381). These groups are generally more easily oxidised as free amino acids than as components of a protein.

Numerous oxidising agents have been used and largely as a result of the many variables involved, there remain many conflicting opinions as to which is preferable (399,400,401).

Chloramine T method (see next section) does appear to cause aggregation of certain proteins such as fibrinogen (402,403) and albumin (404). These aggregates can be prevented by using low concentrations of Chloramine T or can be removed by adsorption on to a Sephadex column.

Under the same conditions and concentrations, the distribution and labelling percentages of the various tyrosyl residues with iodine-125 and iodine-131 are the same (405), showing that both the radioactive iodine isotopes behave similarly.

5. Review of Radio-Iodination Procedures

Procedures for the radioiodination of proteins by substitution of radioactive iodine into the tyrosyl residues invariably involve the oxidation of added radioactive iodide as an essential step in the reaction. A variety of methods have been used to effect this oxidation and the various iodination procedures are often known by the nature of the oxidation step. In addition to the nature of the oxidising agent, the iodination reaction is critically dependent upon such conditions as pH and the concentration of iodide and protein, as well as that of the oxidising agent.

An early method of radio-iodination was based on the use of iodate (406). This method, however, gave a low efficiency of iodination and when whole casein, β -casein and β -lactoalbumin were iodinated, iodination efficiency of approximately 10% was obtained (407).

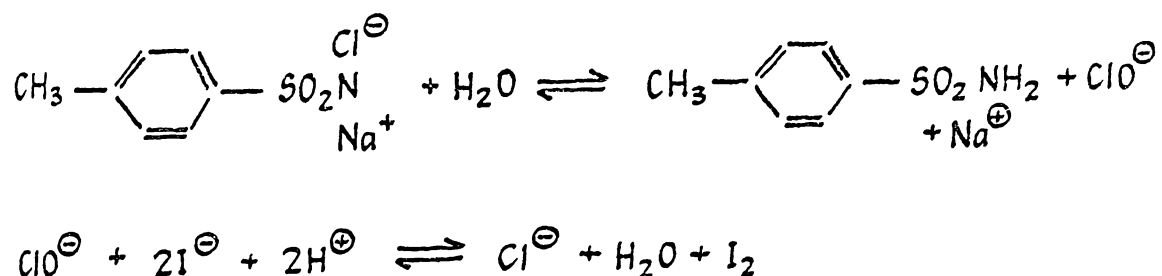
In order to avoid the addition of an oxidising agent to the protein solution, iodine and chlorine vapour have been used in some iodination procedures. Human growth hormone (408) and bovine thyrotropin (409) were labelled by distilling radioactive iodine into the protein solution which in some cases contained sodium iodide (410). Unbound iodine was then removed by dialysis. Iodination efficiencies of up to 66% have been reported. A safer variation of this method employed the use of chlorine vapour to liberate iodine from iodide solution.

Iodination by iodine vapour is of interest in connection with a postulated mechanism for iodination based upon molecular iodine as the reactive species.

Iodine monochloride was introduced as an oxidising agent for the iodination of proteins with radioactive iodide in 1956 (411). A disadvantage of the iodine monochloride method is that it introduces carrier iodide which not only reduces the iodination efficiency, but also the specific activity of the product (412). In a study with albumen at pH 8.0 it was found that preferential oxidation of -SH groups occurred and only 30% of the isotope became substituted in the tyrosyl residue of the protein (413). Recently the efficiency has been increased by using radioactive iodine monochloride and radioactive iodide, but this introduced

the problem of requiring two radioactive sources for every iodination.

A method based upon the use of chloramine T (N-chloro-p-toluenesulphonamide) was developed by Hunter and Greenwood (414) in 1962. Chloramine T in aqueous solution slowly generates hypochlorous acid and this oxidises iodide to iodine.



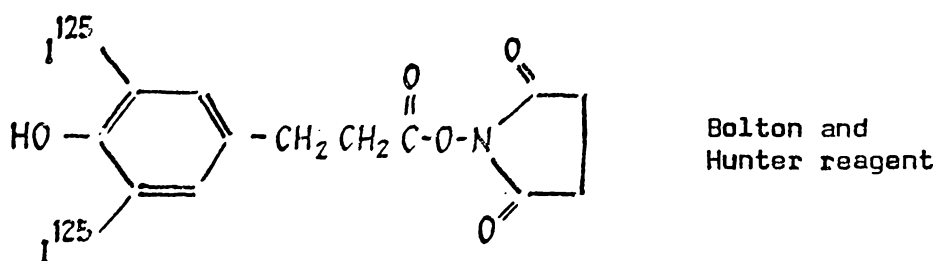
In the presence of protein and carrier-free radioactive iodide, iodination at very high specific activity can be achieved. The chloramine T concentration should be sufficient to oxidise the radioactive iodide without being present in concentrations likely to cause denaturation or aggregation of the protein. The exact amount depends on the efficiency of the iodination desired (415). In order to prevent oxidative damage to the protein, the reaction is terminated by the addition of a reducing agent, usually sodium metabisulphite. An advantage of this chloramine T method is that the low molecular weight reagents are easily removed from the labelled protein product. It has been successfully applied to the iodination of many proteins, including hormones and membranes.

An electrochemical method of radioiodination based upon the electro-oxidation of iodide to iodine (416) has been quite extensively used as an iodination procedure (417,418). Usually the reaction is carried out in a 10 ml platinum vessel which formed

the anode and a platinum wire was used as the cathode. Although the procedure gives high labelling efficiency, with minimum protein damage, the specific activity of the iodinated product is low because of the large volume of protein solution required. More recently a semi-micro technique has been developed (419), but even with this method specific activities obtained are much lower than the chloramine T method.

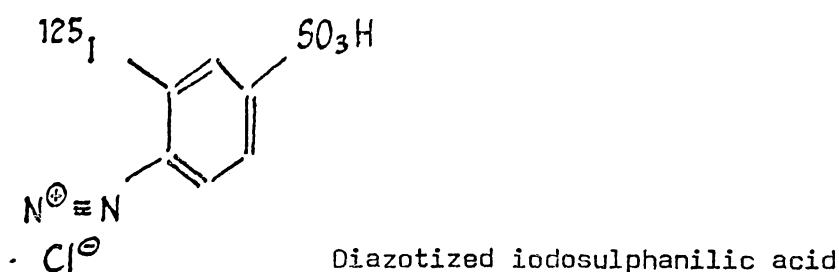
Enzymatic oxidation by peroxidase enzymes such as lactoperoxidase (420) and horse radish peroxidase (421) have been used for iodide to iodine oxidation in the iodination of peptide hormones for radioimmunoassay (422) and of membranes (423). A disadvantage of the enzymatic method is the difficulty of separating the enzyme from the labelled protein at the completion of iodination.

A non oxidative iodination procedure based upon the use of an iodinated acylating agent has been developed by Bolton and Hunter (1973)(424).



Because the acylating reagent reacts with free amino groups, the method is ideally suited to proteins such as secretin which lack tyrosyl groups (425). This method has also been used to label growth hormones (426). The volatility of the acylating reagent however, causes low efficiency of iodination and gives rise to a

possible health hazard. Diazotized iodosulphanilic acid has been used to label cellular membranes and can be used as a substitute for the Bolton-Hunter Reagent when it is undesirable to label terminal amino groups (429,430).



Diazotized (^{35}S) sulphanilic acid has also been used to label the outer surface of cell membranes (431).

Of the various iodination procedures that have been developed, it appears that the requirements of high iodination efficiency, high specific activity of the iodinated product and ease of separation of the iodinated product from the iodination reagents, favour the use of the chloramine-T method. Alternatively the iodine vapour method may be worthy of further investigations if a high efficiency of iodination can be achieved.

6. Separation Procedures

It is of the utmost importance that the radiochemically labelled species to be used in tracer studies is as chemically and physically identical to the non-labelled species as possible. It follows that it is essential that unreacted iodide, iodinating reagents and breakdown products be removed from the protein before its use in adsorption studies. Numerous methods have been employed

to separate the unbound iodine from the labelled protein. These include: acidification, centrifugation, solvent extraction, dialysis and various gel filtration techniques. The efficiency of separation achieved has been found to vary quite widely depending upon the method used and the chemical form of the unbound iodine. For example, extraction with trichloroacetic acid indicated iodine binding to milk protein to be two to four times higher than a separation technique based upon an exchange column (427). Denaturation of the protein during solvent extraction may have been a contributing factor.

Gel filtration is a tried and tested method for protein iodination procedures, making it the obvious choice for the present study.

4.3 Chloramine-T Iodination of Casein

In the previous section it was concluded that the chloramine-T method of iodination would probably provide the most convenient means of iodination of milk proteins for use as tracers in adsorption studies. The application of this method to the iodination of casein is discussed below.

Experimental: The Chloramine-T method which is reported in Appendix 4 was used to iodinate β -casein (prepared in Chapter 3). After the iodination, the reaction mixture was then transferred to a column containing one gram of Sephadex G25 fine resin to separate any free iodide from the iodinated protein. The Sephadex G25 resin had previously been equilibrated for 18 hours in sodium cacodylate buffer at pH 6.8. After placing the iodinated protein mixture on

to the column, it was washed with sodium cacodylate buffer at pH 6.8 and one ml samples were collected. The iodinated protein was collected in the third vial containing one ml and the free iodide in the sixth vial containing one ml of solution.

A number of experiments were performed to investigate the important variables involved in this iodination reaction and the suitability of the iodinated protein for use as a tracer. The following are the results of these investigations.

1. Effect of delay in adding the reducing agent

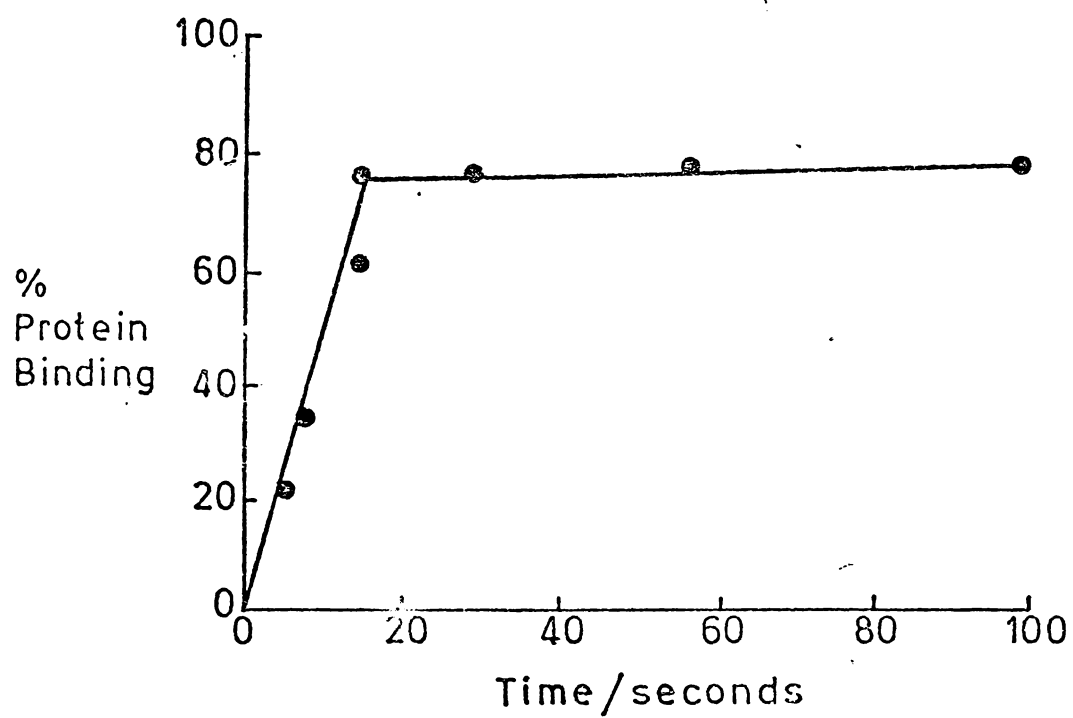
β -casein was iodinated with increasing delays between the addition of the oxidising and reducing agents. Results show (see Figure 4.1) that at least thirty seconds reaction time are required in order to obtain maximum iodination efficiency. In all future experiments the oxidising agent was allowed to react for 45 seconds before addition of the reducing agent.

2. Effect of pH on iodination

Although it has been reported (378) that iodination efficiency is highest at pH 7.4, the efficiency was checked at pH 6.8 to determine if iodination could be carried out at the pH of milk. It was found that the efficiency dropped from approximately 80% to approximately 40% on decreasing the pH. All iodinations were therefore performed at pH 7.4. The pH of the product was subsequently lowered to 6.8 during the sephadex column separation procedure, as this was the pH of milk and adsorption and tracer studies were initially investigated at this pH.

Figure 4.1

Effect of iodination reaction time on iodination efficiency



3. Effect of Oxidising Agent.

The effect of adding the protein to the reaction mixture before and after the addition of the oxidising agent was investigated. No significant difference in the efficiency of iodination was observed. It was concluded that the active species in the iodination reaction is a species present in the oxidised solution rather than a transient formed during the oxidation step.

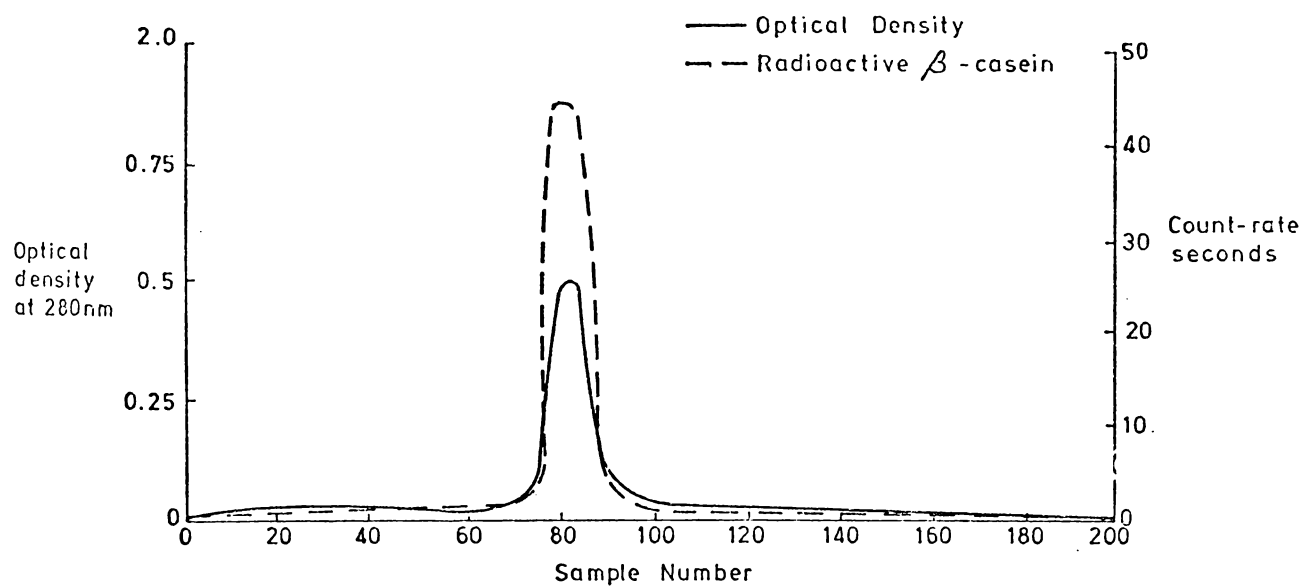
The formation of aggregates during iodination was also investigated using a G175 sephadex gel in a column 30 cm x 2 cm to separate the high aggregates from the rest of the solution. A sample of β -casein in buffer pH 6.8, and spiked with freshly iodinated β -casein, was passed down the column. The β -casein and the radioactively labelled β -casein occurred at the same peak with a very small peak ($\approx 0.5\%$) of radioactive β -casein appearing at the solvent front. This result showed that the labelled and unlabelled β -casein on a Sephadex column behaved the same, there being no significant aggregation of the iodinated protein (see Figure 4.2).

4. Effect of Temperature on Iodination

The iodination of β -casein at 20 °C and 35 °C was compared. At 35 °C, the efficiency of iodination was decreased by twenty percent. It is possible that this decrease was due to the volatilisation of molecular iodine. All future iodinations were therefore carried out at room temperature.

Figure 4.2

Effect of Iodinated and Uniodinated β -casein
on passing down a Gel Column



5. Effect of Age of the Radioactive Iodide

Under identical conditions the efficiency of iodination decreased considerably when sodium iodide-125 that had been standing for one month was used. Fresh iodide typically gave iodination efficiencies of 75%, but on standing the stock sodium-iodide -125 for one month, the efficiency dropped to 20 to 30%. A possible explanation for this effect is that the iodine-125, carrier free and free of reducing agents, is oxidised, possibly to iodate. Paper chromatography was used to investigate the aging effect. Three samples were used: a fresh stock solution, a sample about fifteen weeks old, and a dried sample about twenty-four weeks old. A solution of this third sample was obtained by adding a buffer solution of pH 7.4. The efficiency of iodination of β -casein with these solutions was determined. Samples of the radioactive iodine solutions were chromatographed on a Whatman No.4 paper in a large air-tight glass tank. The solvent used was five parts by volume of ethanol: one part by volume of 1N ammonia. After running until the solvent front was 1 cm from the bottom of the paper, it was removed, dried and autoradiographed. The spots were identified (428) and the activity in each spot is summarised in Table 4.2.

The results show that aging resulted in a decrease in iodide and an increase in iodine, iodate, an unidentified species, and periodate or "colloid bound" iodine immobilised at the origin. In the case of the 24 week old sample the unknown and the periodate spots were considerably more intense than the spots for the other iodine species. The decrease in efficiency of iodination appears

Table 4.2

Chromatographic study of Aged Iodine-125

Age	2 weeks	15 weeks	24 weeks (dried deposit)
Efficiency of iodination	68%	11%	14%
Percentage Distribution			
Origin (Periodate)	6.0	8.0	31.0
Unknown	0.6	3.0	21.0
Iodate	2.4	11.4	5.0
Iodide	78.6	51.1	8.0
Iodine	2.2	12.0	10.0
Streaking	10.0	15.0	23.0

to correlate with the increase in iodate, the unknown, and the periodate. However, the decrease from 78% to 51% in the percentage of iodide does not completely explain the decrease from 68% to 11% in the iodination efficiency. It can be concluded from the above results that the decrease in iodination efficiency was due to formation of a higher oxidation state of iodine. A possible means of regenerating the old stock solution could be treatment with a reducing agent.

Attempts to regenerate the old iodine -125 were made by adding metabisulphite to the radioactive iodine. Sufficient oxidising agent was then added to the solution to oxidise excess reducing agent and the iodination mixture in the usual manner. The oxidised step was monitored on paper chromatography and the

results are shown in Table 4.3. The reducing agent appeared to have reduced all the iodine to iodide, but the higher oxidation states were virtually unaffected. The efficiency of iodination increased only fractionally and was still unacceptably low, even though there was a large amount of iodide in the solution.

Table 4.3

Effect of Reducing Agent on Aged Iodine-125

Percentage Distribution		
		After Addition of metabisulphite
Origin	8.0	7.5
Unknown	3.0	2.8
Iodate	11.3	11.8
Iodide	51.1	66.0
Iodine	12.0	2.0
Streaking	15.0	8.0

It was concluded that the higher oxidation states either prevented the formation of the iodination species during chloramine T oxidation or inhibited the reaction of this species with the protein.

Paper chromatography also offered a convenient means of following the iodination reaction. In an attempt to isolate the active species in iodination, a paper chromatogram was run after chloramine T had been added to the fresh iodide solution and again after the addition of metabisulphite (see Table 4.4). After the addition of chloramine-T, streaking over the length of the

Table 4.4

Iodine Species present during iodination
reaction

Percentage Distribution

	Fresh iodine-125	Added Chloramine T	Addition of metabisulphite after Chloramine T
Origin	6.0	38.0	7.8
Unknown	0.6	5.1	4.1
Iodate	2.4	3.6	13.2
Iodide	78.6	-	62.6
Iodine	2.2	11.0	4.0
Streaking	10.0	41.0	8.1

chromatogram and a considerable increase of the activity at the origin was observed. This streaking could be due to the "bleeding off" of iodine from an insoluble species (such as colloidal or crystalline iodine) localised at the origin.

The effect of adding metabisulphite to the chloramine-T oxidised solution is also shown in Table 4.4. It is clear that most of the species that gave streaking had been reduced to iodide, and that very little change occurred in the amount of activity at the origin. It is of interest to note that the percentage of metabisulphite reducible iodine formed after the chloramine-T treatment correlated closely to the iodination efficiency. This is probably the free iodine formed upon the addition of chloramine-T.

In conclusion it has been observed that the iodination efficiency decreases with the age of the sodium iodide-125 stock

solution. This decrease has been correlated with an increase, in the iodination solution, of species with oxidation states higher than iodine. The use of sodium metabisulphite did not significantly reduce these species or significantly increase the iodination efficiency. Possibly a more effective reducing agent could effect better regeneration of the iodination species.

4.4 Stability of Iodinated Protein

The stability of the iodinated protein with age and in a number of different solutions was investigated.

(a) Stability with age

0.025 ml of β -casein was iodinated, separated from the unbound radio-iodine and stored in a refrigerator at 4 °C. Every second day a sample was removed and passed down a Sephadex G25 column to determine any breakdown. It was found that no breakdown occurred until after between fifteen and twenty days. All radio-iodinated protein tracer solutions were used within seven days of their preparation or fresh samples were prepared.

(b) Stability in the presence of 0.1 mol l^{-1} KI solution

0.025 ml of the iodinated protein was placed in 0.40 ml of 0.1 mol l^{-1} KI solution and left for thirty minutes. The sample was then passed down the Sephadex column and 1 ml samples were collected. No radioactivity was observed in vial No.6 indicating that no exchange between the bound and the unbound iodide had occurred.

(c) Stability in the presence of acid or alkaline solution

0.025 ml of the iodinated protein was placed in 0.40 ml of 0.2 mol l⁻¹ NaOH and left for thirty minutes. The sample was then passed down a Sephadex column and 1 ml samples were collected. No radioactivity was observed in vial No.6 indicating that no breakdown of the radioactive iodine bound to the protein had occurred. This was also repeated using 0.2 mol l⁻¹ HCl and after leaving for thirty minutes, it was neutralised and again passed down a column. Again under these conditions no breakdown was observed to occur.

(d) Stability in hot water

Labelled protein solution was heated up to 90 °C for sixty minutes. It was found that 5% free iodine was formed from the iodinated protein.

These results show that the iodinated protein is stable and thus suitable for use as a radioactive tracer.

4.5 Degree of Protein Labelling

As discussed at the beginning of this chapter it is desirable that no more than one iodine atom react with each protein molecule. Considerable care was taken in all iodinations, to make sure that the number of iodine atoms available for reaction never exceeded the number of protein molecules. Data for a typical iodination were as follows: Approximately 1×10^{-3} g of protein (4×10^{-8} moles) and about 0.2 mCi of carrier free radioactive iodine were used.

Since,

$$N = \frac{dN}{dt} / \lambda$$

and $\lambda = \frac{0.693}{t_{\frac{1}{2}}}$

Therefore in 0.2 mCi I-125, $N = 7.75 \times 10^{13}$ atoms

i.e. 1.28×10^{-10} moles of Iodine atoms were present in the reaction mixture. Thus there was a large excess of protein molecules.

4.6 Some Preliminary Tests of Radioiodinated Proteins as Tracers

A necessary assumption in the use of a radioactively labelled species in tracer studies is that both labelled and unlabelled species behave identically. Chromatography and electrophoresis experiments were performed to test the validity of this assumption for the case of iodinated milk proteins.

A mixture of β -casein and radioiodinated β -casein were passed down a Sephadex G150 column and the eluent monitored spectrophotometrically and radiochemically. The results are shown in figure 4.2, which shows that both species were eluted together.

A more rigorous test was performed using disc electrophoresis. A mixture of α_{s1} , β and K-casein and the corresponding radioiodinated caseins were applied to acrylamide gel columns (see Appendix 2) and a current was applied for fifty minutes. The gel columns were stained to monitor the unlabelled protein and sliced into 0.2 cm thick sections in order to monitor, by radioactive counting, the labelled protein. The results indicated that within the limits of resolution of the technique, both labelled and

unlabelled species behaved identically.

4.7 Conclusion

From the above experiments it was concluded that radioiodinated proteins have potential for use as tracers in the investigation of protein surface chemistry. The validity of their use in isotope dilution studies is demonstrated in Chapter 7.

CHAPTER 5

FURTHER ASPECTS OF THE IODINATION REACTION

5.1 Introduction

Interest in the iodination reaction resulted initially from the fact that it offered a means of preparing radioactively labelled milk protein fraction for use in radio-tracer studies. However the reactions of iodine species with milk are of considerable interest in their own right. Chloramine T iodination of skim milk was of interest because it offered an alternative means of preparing labelled protein fractions. Also labelled casein micelles might be prepared by separation techniques subsequent to the iodination reaction. Direct iodination of skim milk, providing the labelling of all protein constituents in both serum and micelle was to approximately uniform specific activity would provide a convenient means of preparing a radioactive protein tracer for use in general milk studies.

During these investigations it became clear that tyrosyl residues had particular iodination affinities according to their environments within the protein structure. This idea was developed into a model for the prediction of the iodination affinities of the various milk caseins.

5.2 Reaction of Iodine Species with Milk

The reactions of iodine species with whole milk are of interest because radioactive iodine is a significant component of nuclear fall-out and because of the extensive use of iodophors, iodine in the form of molecular I_2 and ionic I^- , is a potential contaminant of milk. In the N.Z. dairy industry it is common practice for iodophor sanitisers containing 25 mg l^{-1} iodine to be used immediately before milking. Residues from such pre-rinses can cause milk iodine concentrations of up to $600 \mu\text{g l}^{-1}$ (433). Under farm conditions, 0.95% of the pre-rinse solution can end up in the milk (434) and this accounts for the elevated levels of iodine in milk when iodophors are used. The total amount and the chemical form of iodine in milk is of interest because of the relationship between the amount of iodine ingested and the incidence of the diseases myxoedema (iodine deficiency) and thyrotoxicosis (iodine excess). Since the introduction of iodophors for use in the dairy industry, iodine in milk has increased from a minor contribution to total iodine ingested, to what is now a significant contribution of iodine in the human diet.

It is known that the amounts of iodine in the various components of milk vary widely according to the nature and concentration of the artificial iodine species inadvertently added to the milk. For example the injection of carrier free, iodine-131 into lactating rats results in up to 86% of the total activity being covalently bound to the protein (435).

In other studies inorganic or unbound iodine has accounted for up to 97% of total iodine in milk (436). The amount of iodine in fat always appears to be low, about 2%, and so need not be considered further (437). The chemical form of milk iodine may give a guide to the fate of the iodine in processed milk products. For example, large proportions of the iodine present in milk would be expected to be found in cheese if the iodine was protein bound. If the iodine was not predominantly protein bound, cheese could be expected to contain lesser amounts of iodine.

Although the enzymatic reaction of iodine added to milk can result in 50% of the iodine becoming bound to the protein, after two hours at 37 °C (80-90% becomes bound after 12 hours at 37 °C), the reaction of iodine with protein is readily blocked by incubation at 8 °C (394). If it could be shown that the 'natural' iodine in milk was largely bound to protein and that the iodine species from iodophor contamination or other artificial residues was not bound, then a convenient means of monitoring iodine contamination would be to measure the unbound inorganic iodine.

Experimental

1. The Reaction of Molecular Iodine with Milk

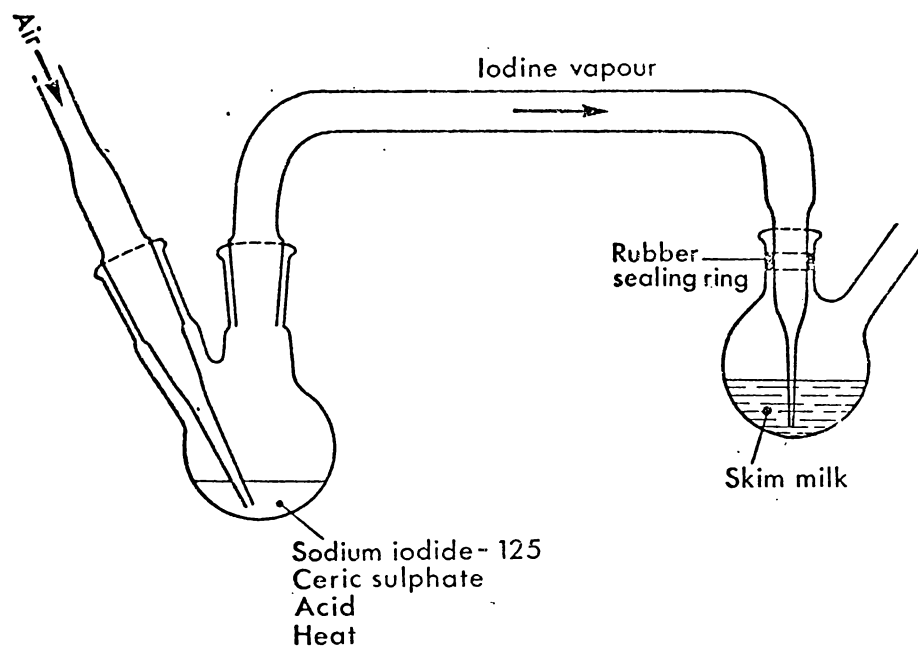
Iodine was generated by reacting radioactive carrier free iodide-131 with ceric sulphate and concentrated sulphuric acid

in the apparatus shown in figure 5.1. The reaction vessel was warmed and the iodine generated was sucked down the tube into one ml of fresh skim milk taken directly from a cow, free of mastitis, at No. 3 Dairy at Ruakura Agricultural Research Centre. Considerable care was taken to obtain milk free of iodine contamination. Before use, the milk was centrifuged for twenty minutes at 3500 r.p.m. to remove the fat. After the radioactive iodine had been bubbled into the skim milk (monitored with a Geiger counter) it was left at room temperature for 15 minutes. 0.5 ml of the milk was then ultracentrifuged using a MSE Superspeed 75 Ultracentrifuge and another 0.20 ml of this same milk was passed down a G25 Sephadex column to separate the protein from the free ions. Sodium cocadylate buffer at pH 6.8 was used as the eluent. Another sample of the milk was stored at 4 °C for two days before being passed down the same column.

Results showed that 50% of the iodine came down with the protein micelles during centrifugation. On passing a sample of the reaction mixture down a G25 Sephadex column (10 cm x 1 cm), less than 1% of the radioactivity was associated with the protein fraction. It was eluted from the column as free or unbound iodine. After two days only 4% of the radioactive iodine appeared to be bound to the milk protein.

The above results showed that molecular iodine did not bind with skim milk proteins at pH 6.8. The iodine associated with the centrifuged micelle fraction is therefore not 'bound' iodine but was presumably molecular iodine physically entrapped or encapsulated within the micelle. The iodination of proteins

Figure 5.1

Reaction vessel used to generate I_2 (g)

by molecular iodine at pH 6.8 did not appear to be a feasible method under the conditions of this experiment.

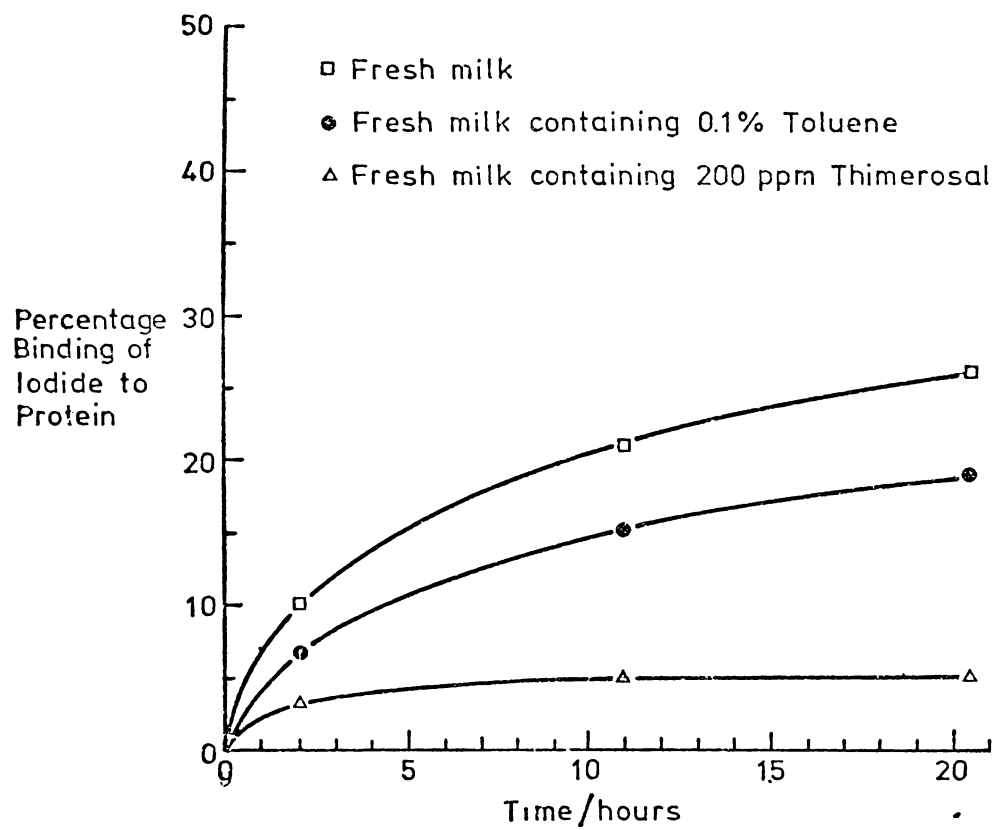
2. The Reaction of Iodide with Milk

Part of the milk sample used in the experiment described above was centrifuged to remove the fat and then warmed to 35 °C. Three, five ml aliquots were then removed. To one was added 0.020 ml of toluene, to the second was added 200 ppm of thimerosal (sodium ethylmercurithiosalicylate) and the third was used as a control. Both toluene (394) and thimerosal (479) have been used to prevent bacterial action in protein studies. To each of these three solutions was added one drop of radioactive iodide-125, and the solutions were incubated for twenty hours at 35 °C. Periodically samples were removed and passed down a G-25 Sephadex column to determine the amount of iodide bound to the milk. The results (see figure 5.2) showed that the maximum amount of binding in any of the three samples was 21%. Toluene appeared to slightly suppress the amount of iodide binding to the protein and thimerosal considerably suppressed the amount of binding.

A sample of the milk equilibrated at 35 °C for four hours was passed down a Sephadex G150 column to observe the distribution of iodide over the casein micelle, serum casein and whey proteins. The protein concentration was monitored continually with a flow cell connected to a Spectrophotometer and the radioactivity was monitored with a well crystal connected to a counting system. The protein and radioactivity peaks eluted from the column were recorded automatically. The radioactivity

Figure 5.2

Binding of carrier free iodide-125 to milk protein



occurred predominantly as free iodide with a small micelle peak. No radioactive peak was observed in the serum caseins or whey fraction. In view of the low level of activity associated with the micelle protein, this result was not surprising (see figure 5.3).

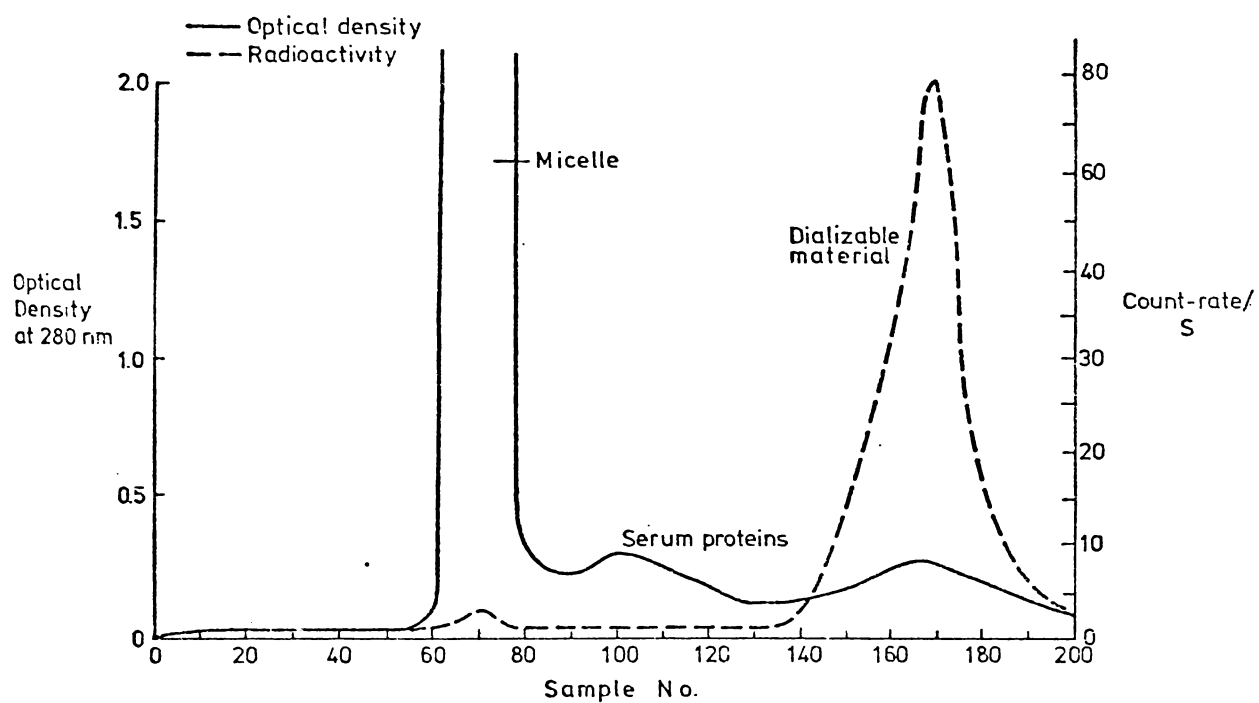
The results above indicated significantly less iodine binding than previously reported. This could have been due to differences in the enzyme activity of the milk used. The difference, however, could not have been caused by suppression of the iodination reaction by the free iodide concentration in the milk as this was well below the 20 mg of iodine per litre of milk that would be required to cause such an effect (394).

Farming practice requires that milk be cooled to approximately 8 °C as soon as possible. Hence the binding of iodide at this temperature was investigated. The results are shown in figure 5.3. Even after prolonged standing less than 2% of the iodine had become bound to the protein. This result agrees with the published result that less than 3% binding of inorganic carrier free iodide-131 occurred on incubation with fresh raw milk at 8 °C (394).

It can be concluded that while iodide does react with milk protein at 35 °C after prolonged standing, the reaction is negligible after short times at this temperature, and after prolonged standing at 8 °C. As in the case of molecular iodine, reaction of iodide with milk does not appear to be a feasible method of iodination for tracer studies. The lack of reactivity of both I_2 and I^- suggested a possible means of monitoring iodine

Figure 5.3

Distribution of iodide-125 bound to milk constituents
as determined by gel filtration



contamination of milk. Providing that the natural milk contained little inorganic iodine, the inorganic iodine detected would be that due to iodine contamination.

Evidence to support this was found in cows and rats on a low iodine intake, which have a much higher fraction of protein bound iodine in their milk (427,432).

5.3 Chloramine T Iodination of Skim Milk

It is clear from the preceding section that iodination of milk by the action of natural enzymes on molecular iodine or iodide ion is slow and inefficient even at 35 °C. Further studies of the iodination of skim milk were thus based on the Chloramine T method of iodination. Iodination of skim milk followed by gel filtration may provide a convenient method of simultaneous labelling of all milk protein constituents. Also, providing appropriate conditions were employed during the separation procedures, it might be possible to prepare and isolate labelled micelles for adsorption and exchange experiments. The labelling efficiencies of the individual casein constituents, both in the serum and in the micelle, is of interest. Providing the labelling efficiency was uniform, or failing that, constant for the various constituents, radioiodination could provide the basis of a simple radiochemical means for monitoring the relative amounts of the various protein components in milk samples. Non-uniform labelling efficiency could conceivably be used to reveal information about the structure of the milk micelle.

Experimental

1. Iodination of Skim Milk

Fresh milk was obtained from a cow at No.3 Dairy, Ruakura Agricultural Research Centre. Considerable care was observed to avoid contamination with iodine from iodophors, teat sprays, or iodine salt lick stick. The milk was centrifuged for 20 minutes at 3 500 rpm to remove the fat. 0.025 ml of the skim milk was iodinated using the Chloramine T method and the efficiency of the iodination was determined by passing the sample down a Sephadex G25 column and collecting the one ml fractions.

An efficiency of iodination of 79% was obtained, indicating that the milk did not contain excessive inorganic iodine.

2. Distribution of Activity between Whey, Serum Casein and Micelle Casein

0.01 ml of the iodinated milk from the above experiment was added to 1 ml of skim milk and equilibrated for thirty minutes at 4 °C. It was then centrifuged at 105 000 g for one hour at 4 °C to separate out the micelles. The supernatant was acidified to separate the serum casein and the whey proteins. The various fractions were counted to obtain the distribution of the iodine activity.

In a second experiment a 0.1 ml sample of the original iodinated milk was added to 3 ml of skim milk, equilibrated at 4 °C for thirty minutes and, using water as an eluent, was passed down a Sephadex G150 column. This procedure has been used to

separate casein micelles from casein serum and whey protein (440). The eluted solution was monitored continually for protein by U.V. spectrophotometry and for radioactivity by scintillation counting. Both results were automatically recorded.

The radioactivity and protein in the peaks corresponding to micelle, serum casein and whey protein are shown in figure 5.4. Unfortunately good separation between the serum casein and whey casein was not obtained. No radioactivity was observed in the non-protein nitrogen peak (N.P.N.) as could be expected since this peak is due to species such as ammonia, urea, creatin, uric acid and trace amounts of amino acids such as alanine, glutamic acid, glycine, aspartic acid, thought to originate from the cow's blood or decomposition of the milk proteins. Results for both experiments are shown in Table 5.1.

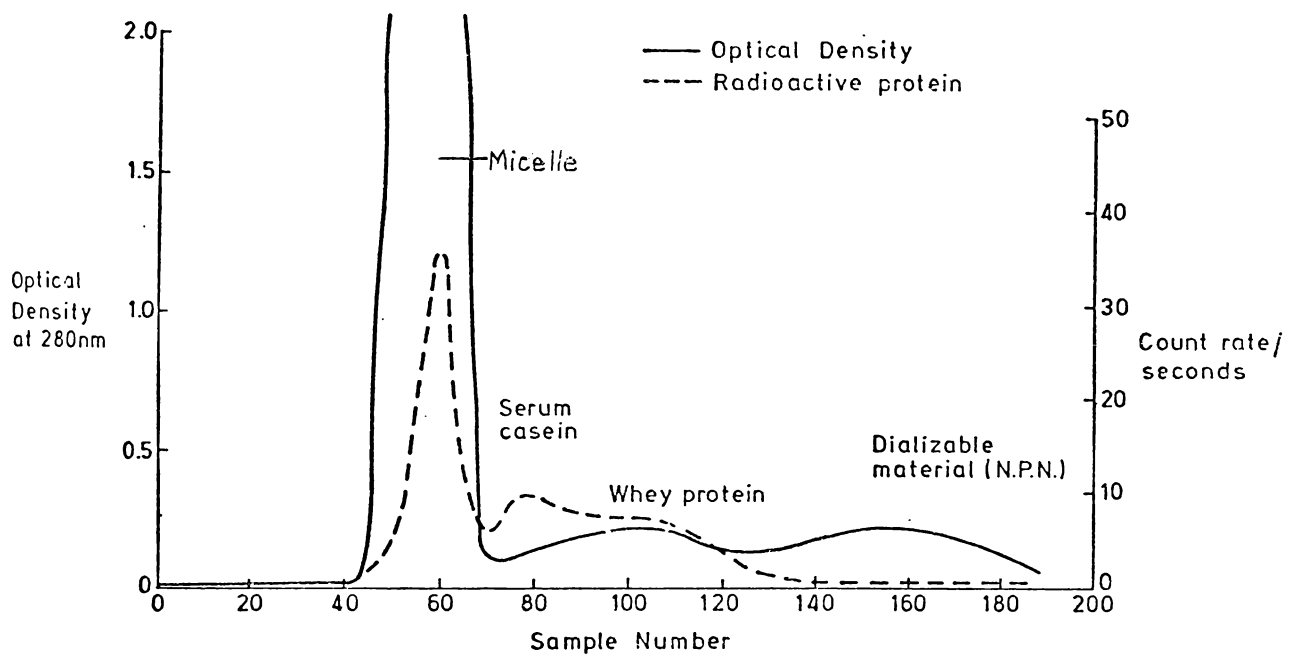
Table 5.1
Distribution of Iodinated Protein between Micelle,
Serum and Whey at 4 °C

	Percentage Radioactivity (Centrifugation and Acidification)	Percentage Radio- activity (Sephadex G150 Column)
Micelle	67	50
Serum Casein	10	22
Whey Protein	23	26

The apparent loss of activity from the micelle constituent upon passing down the Sephadex column could be a dilution effect. Evidence of the dissociation of caseins from the milk micelle during gel chromatography has been reported (441).

Figure 5.4

Distribution of iodinated skim milk added to
skim milk as determined by gel filtration



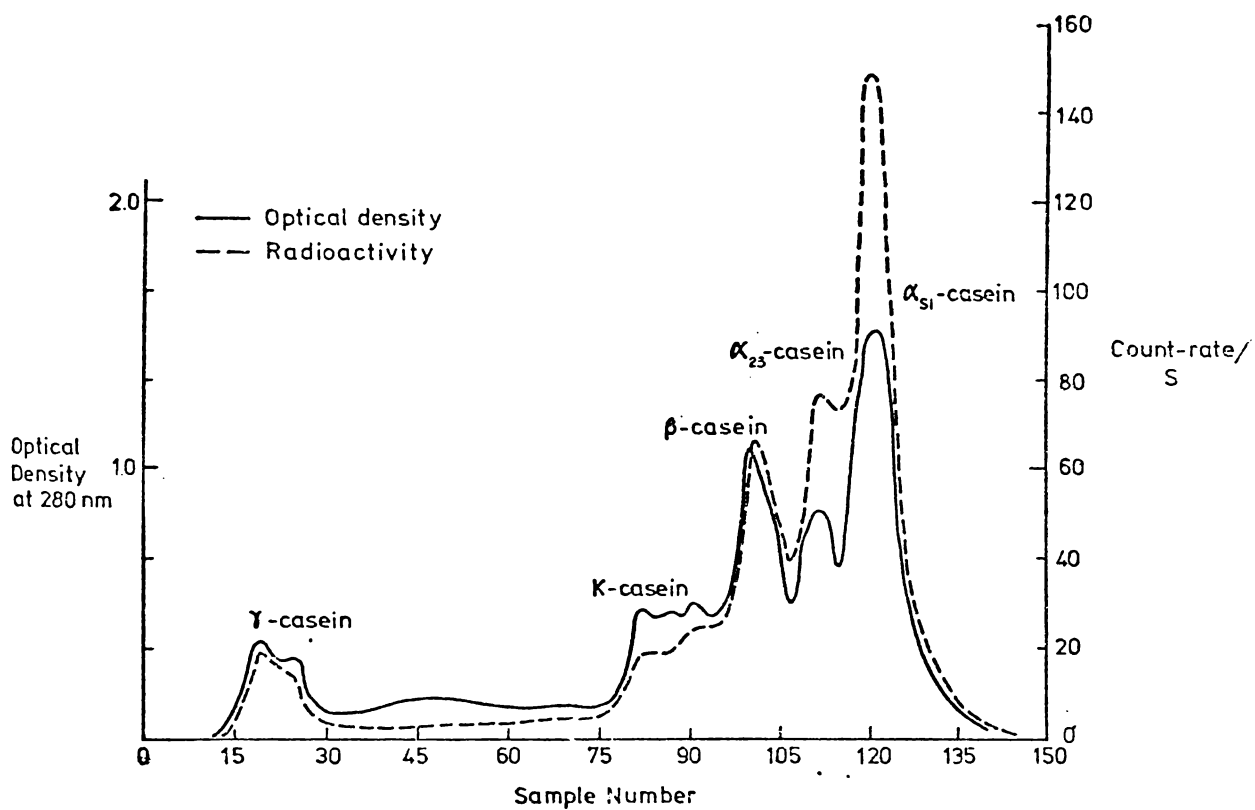
3. Distribution of Activity in the Casein Constituents

In order to separate the labelled caseins, a 0.1 ml sample of iodinated milk was equilibrated with one ml of skim milk for 30 minutes. It was then acidified to pH 4.6 with HCl and centrifuged. The precipitated casein was adjusted to pH 7 and then again acidified to pH 4.6 and centrifuged. The radioactive casein diluted with carrier casein, was further diluted with carrier casein by the addition of approximately one gram of casein prepared by the usual acidification procedure. This radioactive casein mixture was dissolved in 15 ml of 0.01 mol l⁻¹ imidazole-HCl buffer at pH 7 containing 4.5 mol l⁻¹ urea. The resulting solution was passed down a Whatmans DE 52 column according to the procedure for the separation of individual casein fractions outlined in Chapter 3. Optical activity and radioactivity were monitored and recorded continuously. The results are shown in figure 5.5. The optical density pattern obtained is typical of that of whole casein (see Chapter 3). In order to determine the uniformity or otherwise of the iodine distribution in the various casein constituents it was necessary to determine the total amount of protein corresponding to each U.V. adsorption peak monitored at 280 nm and the total amount of radioactivity associated with that protein.

The activity of each fraction was readily obtained by integrating the total activity under each peak. The corresponding amounts of protein for each peak were calculated by assuming Beers law and using literature values for extinction coefficients.

Figure 5.5

Determination of iodination efficiency of milk
caseins using gel filtration



A problem arose in the calculation of the amounts of TS-A/R caseins in that no literature values for the extinction coefficients were available. Thus, the method of Wetlaufer (442) was used to calculate the required extinction coefficients from the extinction coefficients of the constituent amino acids (tyrosine $E = 1340$; tryptophan $E = 5550$; cysteine $E = 150$). For example TS-A² casein contains three tyrosyl and one tryptophan residue and has a molecular weight of 11 830. The extinction coefficient was therefore calculated from:

$$E_{\text{protein}} = \frac{N_{\text{tyr}} E_{\text{tyr}} + N_{\text{trp}} E_{\text{trp}} + N_{\text{cys}} E_{\text{cys}}}{M}$$

where N corresponds with the number of moles of amino acid residues per mole of protein and M is the molecular weight of the protein.

For TS-A² casein

$$E = \frac{3 \times 1\,340 + 1 \times 5\,550}{11\,830} = 0.80 \text{ cm}^2 \text{ g}^{-1}$$

The validity of this procedure, which neglects effects due to the microenvironments at each chromophore, is demonstrated in Table 5.2 in which calculated and literature values of extinction coefficients of milk proteins are compared.

The absence of significant environmental effects was demonstrated by addition of very large amounts of sodium chloride and urea to β -casein solution. There was no change in the absorption spectra even though much structural change could be expected in concentrated solutions of these reagents.

Table 5.2

Experimental and Calculated Extinction

Coefficients (at 260 nm)

Protein	$E/\text{cm}^2 \text{ g}^{-1}$ (Expt. Literature)	$E/\text{cm}^2 \text{ g}^{-1}$ (Calculated from Primary Structure)
α_{s1} -casein B	1.00 (366)	1.03
β -casein A	0.46 (374)	0.45
K-casein A	1.05 (375)	0.93
β -lactoglobulin B	0.95 (376)	0.91
α lactalbumin	2.01 (377)	1.98
γ -casein A	-	0.53
TS-A casein	-	0.80
R-casein	-	0.83

E = Extinction Coefficient

From the optical density data, the amounts and percentages of protein corresponding to each peak were calculated. These are shown in Appendix 10. From these data the percentage of tyrosyl residues occurring with each peak was also calculated and included in the table. Data for the total counts and percentage of counts corresponding to each peak are shown in Appendix 10 and were used to calculate the relative iodination efficiencies summarised in Table 5.3.

When the data for the percentage tyrosyl groups per peak are compared with the data for the percentage radioactivity per peak, it is apparent that tyrosyl residues of α_{s1} , β and TS-A/R caseins constituents are iodinated with essentially the same iodination efficiency. The tyrosyl residues of K-casein, however, appear to iodinate with a much lower efficiency. The lower iodination efficiency of K-casein could be due to either structural effects of the protein itself or it could result as a consequence of K-casein's involvement in micelle formation.

4. Distribution of Activity in the Whey Protein Constituents

50 ml of the whey protein at pH 4.6, prepared during the separation of casein described above, was adjusted to pH 6.0 and concentrated to about 7.0 ml by dialysis in a bed of polyethylene glycol flakes. The sample was then spiked with the radioactively labelled whey protein prepared during the separation of the radioactive casein fraction. The resulting solution was then eluted from a 2.5 cm x 46 cm column of Sephadex G75 with a 0.04 mol l⁻¹ phosphate buffer at pH 6.0 (486).

Table 5.3

Relative Iodination Efficiencies of the Tyrosyl
Residues of Milk Proteins

Protein	Relative Iodination Efficiency *
TS-A/R casein	0.92 $\pm$ 0.2
K-casein	0.57 $\pm$ 0.1
β -casein	0.99 $\pm$ 0.2
α_{s1} -casein	1.18 $\pm$ 0.2
β -lactoglobulin	0.9 $\pm$ 0.2
α -lactalbumin	1.2 $\pm$ 0.2

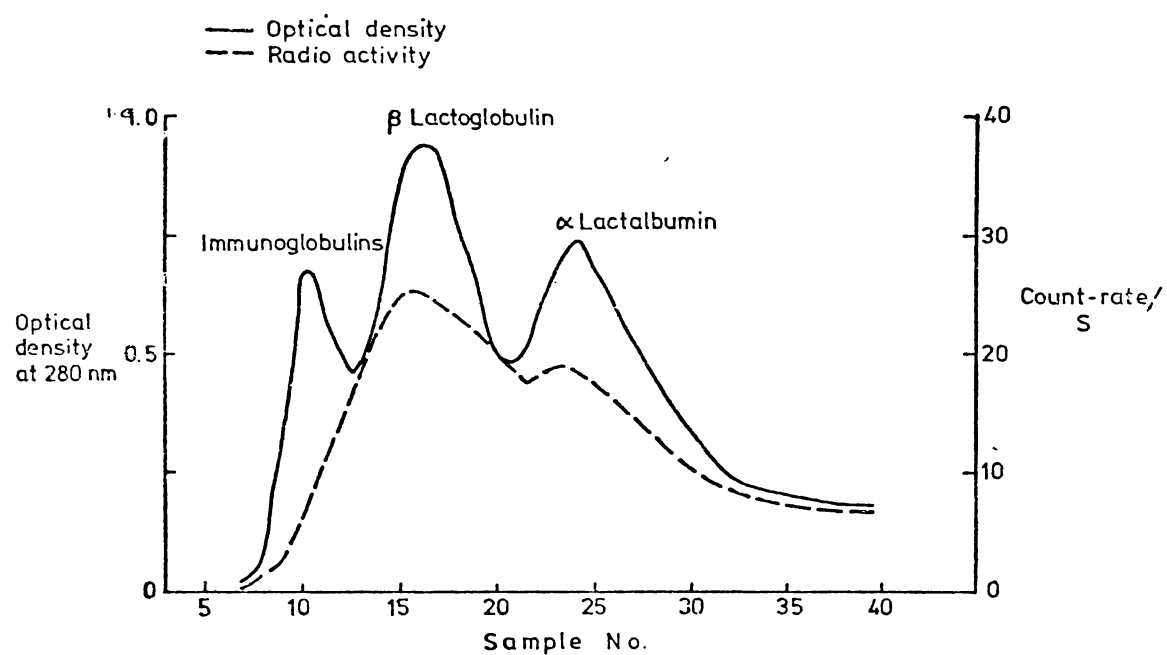
* Relative Iodination Efficiency : $\frac{\% \text{ Radioactive Counts}}{\% \text{ Tyrosyl residues}}$

The optical density and radioactivity were monitored continuously and the results are shown in figure 5.6. No iodination of the immunoglobulins was observed. Approximately 10% of the whey protein can be serum albumin which has a molecular weight of 69 000 and 20 tyrosyl residues. This can contribute up to 12% of the total tyrosyl residues in the whey proteins and could be the reason for the broad radioactive reading at the beginning of the β -lactoglobulin peak. Unfortunately the serum albumin and β -lactoglobulin fractions were not resolved and so were combined for the following discussion. Relatively poor separations were achieved between β -lactoglobulin and α lactalbumin. However the data were processed in a manner similar to that described above for the caseins. The results are tabulated in Appendix 10. The percentage tyrosyl residues per peak was then compared with the percentage activity per peak (Table 5.3). It was concluded that the iodination efficiency of each major whey protein was the same within experimental error.

Overall, the iodination of skim milk by the Chloramine T method gives quite similar mean iodination efficiencies for the tyrosyl residues of all the major milk proteins with the notable exception of K-casein. The low value for K-casein may have been due to environmental factors associated with the K-casein itself or it could have resulted from the effect of incorporating the K-casein into the milk micelles. These two alternatives will be discussed further in section 5.4.

Figure 5.6

Determination of iodination efficiency of whey proteins using gel filtration



From a more practical point of view, the use of radioiodinated milk in tracer studies relies upon the assumption that the specific activities of the various labelled constituents are the same. Specific activities were calculated from the data of Appendix 10, and from a Kjeldahl determination of the total nitrogen in the milk sample. The data are summarised in Table 5.4.

Table 5.4

Specific activity of radioiodinated milk constituents

Protein	Percentage (weight)	Radioactivity/ /cs ⁻¹	Specific Activity /cs ⁻¹ g ⁻¹
TS-A/R casein	6.0	62	10.33
K-casein	11.0	132	12.0
β-casein	27.3	203	7.4
α _s -casein	30.0	668	22.2
β-lactoglobulin	17.25	237	13.7
α-lactalbumin	7.75	135	17.4
		Mean	<u>13.8</u>

Although there is a considerable spread in the data of Table 5.4, the specific activities of the major protein constituents are within $\pm 60\%$ of the mean value. This spread imposes a limitation on the overall accuracy of tracer dilution studies based upon labelled milk (see Chapter 8).

5.4 Analysis of the Effect of Environmental Factors on Iodination Efficiency

The literature on the iodination efficiencies of the various individual tyrosyl residues of proteins and enzymes indicates that quite significant environmental effects may occur. Some tyrosyl residues appear to be much more readily iodinated than others. Generally these effects have been studied at low levels of iodination. The iodination efficiencies have been determined by selective cleavage into polypeptides of known amino acid sequences, each containing one of the tyrosyl residues. The relative activities of the segments gave a measure of the relative iodination efficiencies. It is generally accepted that these efficiencies reflect the kinetics of iodination rather than the thermodynamics of the reaction (405).

An example of the sort of information that can be obtained is provided by results obtained for the iodination of α -chymotrypsin at pH 8.0 (445). When iodinated at a level of 0.33 iodine atoms per molecule of chymotrypsin the relative iodination efficiencies of the four tyrosyl residues at the sequence numbers 146, 94, 171 and 229 were 100 : 40 : 20 : 2 respectively. At lower levels of iodination, a larger proportion of the iodine was found on the tyrosyl residue 146. This result provided evidence that the variation in iodination efficiencies was a kinetic effect. In extreme cases, one residue might become almost completely iodinated before significant reaction occurs at some other residue.

Upon the examination of the amino acid residues adjacent to each of the tyrosyl residues of chymotrypsin, it is apparent that the most reactive residue was adjacent to a basic hydrophilic group on the end of a chain. The least reactive residue was "sandwiched" between two non-polar hydrophobic groups. There is further literature evidence supporting the conclusion that neighbouring hydrophilic groups facilitate iodination and neighbouring hydrophobic groups inhibit the reaction (446). If the tyrosyl residue was surrounded by non polar residues, these would tend to form a hydrophobic bond excluding the aqueous phase and making the tyrosyl residue inaccessible to the iodine. Alternatively, tyrosyl groups in a hydrophilic environment could be expected to occupy exposed surface regions of the molecule and thus be reached easily by dissolved iodine. Iodination efficiencies have been enhanced by incorporation of hydrophilic groups, particularly basic hydrophilic groups, to the tyrosyl residue by amide linkages (447).

In the case of insulin (446), iodination proceeds most readily at tyrosyl residues 14 and 19, which have the hydrophilic neighbouring groups leucyl and glutamyl, and asparagyl and cystinyl residues respectively. The least readily iodinated tyrosyl residues are residues 16 and 26. These have the hydrophobic pairs: leucyl and leucyl, and threonyl and phenylalanyl residues adjacent to these two tyrosyl residues. The importance of hydrophilic-hydrophobic interactions was further demonstrated by iodinations performed in organic solvents

and in 8 mol l⁻¹ urea. These reaction media could be expected to diminish hydrophobic bonding and hence enhance iodination. This was observed.

Classification of neighbouring group pairs according to their hydrophobicity (under the conditions of iodination) might be useful in rationalising the relative efficiencies of iodination at the tyrosyl residues in various molecular environments.

The total hydrophobicity of the neighbouring pairs of amino acids of each tyrosyl residue of chymotrypsin was calculated from the amino acid hydrophobicity ($H_{ij}^{\ddagger}$) data of Table 2.3. A comparison of relative efficiencies of iodination and total hydrophobicity of neighbouring amino acid pairs of all the tyrosyl residues of chymotrypsin is shown in Table 5.5.

Table 5.5
Correlation of Relative Iodination Efficiency with
Total Hydrophobicity of Neighbouring Pairs

Sequence	I_R	$\sum H_{ij}^{\ddagger}/\text{kJ}$	Charge
146 Arg - Tyr	100	3.14	-ve, zero
94 Lys - Tyr - Asn	40	6.27	-ve, zero
171 Lys - Tyr - Trp	20	9.41	-ve, zero
229 Val - Tyr - Ala	2	5.12	-ve, zero

I_R = Relative iodination efficiency

$\sum H_{ij}^{\ddagger}$ = Total hydrophobicity of neighbouring pairs

For the terminal tyrosyl residue 146, the total hydrophobicity was taken as being that of the single neighbouring argininyll residue.

Although there is a general inverse trend in the data for hydrophobicity and relative iodination efficiency, other factors also appear to be important. A major limitation to the use of the hydrophobicity data in the manner described is that such data refer to proteins dissolved in distilled water and hence, in most cases, at pH's significantly different from the pH of the iodination reaction. pH is the major factor that determines the charge of an amino acid residue and this charge could have a profound effect on the iodination efficiency of a neighbouring tyrosyl residue. Charges on the neighbouring amino acid residues at the pH of iodination are included in Table 5.5. Negative charge on a neighbouring residue could facilitate the iodination reaction in two ways. Iodination proceeds by attack by the iodine species at regions of high electron density on the benzene ring of the tyrosine. This could be increased by inductive effects from a neighbouring -ve group. Also, if the species active in iodination is a "I⁺" species, this will be attracted to regions of negative charge.

The presence of a negative charge on lysine might account for the iodination efficiency of the Lys-¹⁷¹Tyr - Trp sequence being greater than that of the Val - ²²⁹Tyr - Ala sequence, even though the former sequence has the greater potential hydrophobicity.

With the limited information available it does not seem possible at present to correlate iodination efficiency with any single parameter relating to the molecular environment of a given tyrosyl residue. The evidence pointing to the importance of environmental factors however, is so well established that in an attempt to rationalise the data that do exist, a table of relative iodination efficiencies of tyrosyl residues, classified according to the pairs of neighbouring amino acids, was constructed. Table 5.6 is based on the relative efficiencies of iodination of chymotrypsin tyrosyl residues which serve to provide a scale of efficiencies ranging from 0 to 100. Unfortunately chymotrypsin is the only protein for which numerical data were available. For amino acid neighbouring pair combinations not represented by chymotrypsin, interpolated values have been entered. These were based upon the relative rates of iodination of the tyrosyl residues of insulin (446) and K - Bence-Jones protein (448). While the resulting table is clearly an oversimplification, suffering the disadvantage of incorporating interpolated data and taking no account of the effect of experimental parameters such as pH and concentration; it does provide a model on which to base predictions about the iodination of tyrosyl residues in particular environments. It can also be used to get an estimate of the overall iodination efficiencies of protein molecules.

Table 5.6

Relative Rates of Iodination

Amino Acid Combination	I_R
Basic hydrophilic - Tyr residue	100 ^x
Basic hydrophilic - Tyr - Basic hydrophilic residue residue	60 ⁺
Basic hydrophilic - Tyr - Neutral amine residue residue	40 ^x
Basic hydrophilic - Tyr - Non-polar residue residue	20 ^x
Neutral amine - Tyr - Non-polar residue residue	15 [*]
Neutral amine - Tyr - Steric residue residue (cysteine)	10 [*]
Non-polar - Tyr - Non-polar residue residue	2 ^x

^x Data from iodination of chymotrypsin

^{*} Interpolated data estimated from iodination of insulin

⁺ Interpolated data estimated from iodination of K-Bence-Jones

I_R relative iodination efficiency rate

5.5 Estimation of Relative Iodination Efficiencies of Tyrosyl

Residues of Milk Proteins

The amino acid residues neighbouring each tyrosyl residue of the various milk proteins were obtained from the amino acid sequences of the proteins. The neighbouring residues were then classified according to the type and the relative iodination efficiency, I_R for each tyrosyl residue was then obtained from Table 5.6. Results are summarised in Table 5.7. The ranking of the tyrosyl residues in order of their iodination efficiencies can be obtained directly from the table and is as follows:

α_1 -casein	104 Tyr	>	91 Tyr	>	154 Tyr	,	173 Tyr	>	94 Tyr	,	144 Tyr	,	146 Tyr	,	159 Tyr	,	165 Tyr	,	166 Tyr
β -casein	114 Tyr	>	193 Tyr	>	60 Tyr	,	180 Tyr												
K-casein	25 Tyr	,	35 Tyr	>	30 Tyr	>	42 Tyr	,	43 Tyr	>	38 Tyr	,	58 Tyr	,	60 Tyr				

The relative efficiencies for the two whey proteins are less easily resolved because no data were available for neighbouring acidic hydrophilic polar residues. It was assumed that such groups would not favour iodination (as previously discussed in section 5.4), and so a value of 2 was interpolated. Existing data do allow the prediction that the most active tyrosyl residue in β -lactoglobulin A is tyrosyl residue 102 and that of α -lactalbumin is tyrosyl residue 103.

Table 5.7

Relative rate of Iodination of Tyrosyl Residues present in different Milk Proteins estimated from adjacent residues (I_R = Relative Iodination Efficiency)

Casein: (53)	α_{s1} -casein B	I_R	β -casein B	I_R	K-casein B	I_R
	91		60		25	
	Arg - Tyr - Leu	20	Val - Tyr - Pro	2	Lys - Tyr - Ile	20
	94		114		30	
	Gly - Tyr - Leu	2	Lys - Tyr - Pro	20	Gln - Tyr - Val	15
	104		180		35	
	Lys - Tyr - Lys	60	Pro - Tyr - Pro	2	Arg - Tyr - Pro	20
	144		193		38	
	Ala - Tyr - Phe	2	Leu - Tyr - Gln	<u>15</u>	Ser - Tyr - Gly	2
	146		Total	<u>39</u>	42	
	Phe - Tyr - Pro	2			Asn - Tyr - Tyr - Gln	20
	154				58	
	Phe - Tyr - Gln	15			Pro - Tyr - Pro	2
	159				60 61	
	Ala - Tyr - Pro	2			Pro - Tyr - Tyr - Ala	2
	165 166				Total	<u>81</u>
	Trp - Tyr - Tyr - Val	4				
	173					
	Gln - Tyr - Thr	<u>15</u>				
	Total	<u>122</u>				

Whey Proteins (32)	β -lactoglobulin A	I_R	α -lactalbumin C	I_R
	20		18	
	Trp - Tyr - Ser	2	Gly - Tyr - Gly	2
	4		36	
	Val - Tyr - Val	2	Gly - Tyr - Asp	2
	99		50	
	Asp - Tyr - Lys	?	Asp - Tyr - Gly	2
	102		103	
	Lys - Tyr - Leu	<u>20</u>	Asn - Tyr - Trp	<u>15</u>
	Total	<u>24 +</u>	Total	<u>21</u>

The sum of all the tyrosyl iodination efficiencies for each protein ($\sum I_R$) should give a measure of the overall tendency of a given protein molecule to become labelled during the iodination reaction. This information is summarised in Table 5.8 along with the mean iodination efficiencies of the tyrosyl residues of a given protein. This latter value was obtained by dividing the sum of the relative tyrosyl iodination efficiencies $\sum I_R$, by the number, N_{Tyr} , of tyrosyl residues in the protein.

Table 5.8

Tyrosyl Iodination Efficiency Sums and Means
for Milk Proteins

Protein	$\sum I_R$	$\frac{\sum I_R}{N_{\text{Tyr}}}$
α_{s1} -casein	122	12.2
β -casein	39	9.75
K-casein	81	9.0
β -lactoglobulin	24	6.0
α -lactalbumin	21	5.3

The latter figure for the caseins indicates that average iodination efficiencies are quite similar. The low experimental value for K-casein obtained in Section 5.3 thus does not appear to be due to a molecular environment effect.

5.6 Discussion and Conclusion

The investigations reported in this chapter have examined aspects of the iodination reaction of general relevance to the use of iodine in the dairy industry.

It has been shown that iodine, added to cooled milk, either as iodine or as iodide ions, reacts slowly if at all, with the protein constituents. Although much of the added iodine appeared to be spun out with the micelles during centrifugation, gel filtration experiments demonstrated that the iodine associated with the micelles was not chemically bound to the micelle caseins. The determination of the level of free inorganic iodine in milk could thus be of use in the monitoring of iodine adulteration resulting from sanitisation with iodophors.

The Chloramine T iodination of skim milk was investigated as a means of simultaneous labelling of all protein constituents of milk, including the milk micelles. Quantitative studies indicated that while all major constituents were labelled, the specific activities varied quite considerably. This variation was shown to be more of a reflection of the differing number of tyrosyl residues contained in the various proteins than of the mean iodination efficiencies of the tyrosyl residues of each protein. However, the mean iodination efficiency of K-casein tyrosyl residues appeared to be significantly lower than that for all the other proteins investigated. The low mean iodination efficiency of the tyrosyl residues of K-casein was considered in terms of either a molecular environment effect or

a micelle effect. A table of the relative iodination efficiencies of tyrosyl residues for different categories of neighbouring amino acid pairs was constructed from literature data and used to estimate relative mean tyrosyl iodination efficiencies for the various milk proteins. Such calculations did not indicate a low value for K-casein. Thus it seemed likely that molecular environmental effects were not responsible for the lower experimental efficiencies observed.

The casein micelle is currently regarded as an open web-like structure, being very porous and highly solvated (449,454). It is envisaged that K-casein is held well within the micelle structure, especially in the Garnier and Ribadeau-Dumas models (461). It could therefore be much less readily accessible to iodination. Tentatively, then, it is concluded that the low mean iodination efficiencies of K-casein tyrosyls are a consequence of the micelle structure.

Further discussion of molecular environment and micelle effects on the iodination efficiencies of tyrosyl groups of milk proteins is largely beyond the scope of the present work. Further study is required to establish the nature of the environmental effects so that the data of Table 5.8 can be more firmly established. Iodination of appropriate tripeptides at appropriate pH's and other experimental conditions would be of value.

CHAPTER 6

MILK MICELLES AND MICELLE-SERUM INTERACTIONS

6.1 Introduction

In the previous chapters the radioiodination of both skim milk and the separated protein fractions of skim milk were discussed. Iodination of skim milk gave results that suggested the possibility of either a micelle effect or a molecular environmental effect on the iodination of K-casein (see Section 5.3). From a comparison of the mean iodination efficiencies of the tyrosyl residues for different molecular environments, it appeared unlikely that the observed result was due to an environmental effect. This led to the conclusion that the low iodination efficiency was due to the K-casein being embedded in the micelles in such a way as to be less readily accessible to iodination. A basic requirement for this explanation to be valid, is that the micelle K-casein be non-exchangeable with serum K casein over the time scale of the iodination reaction. For this reason, and also because labelled micelles and the exchange of tracer caseins with micelles were of interest with respect to adsorption studies, studies of micelle-serum exchange reactions were initiated.

From consideration of the various models that have been suggested for the milk micelle (see Section 6.2), (along with the data used to set up these models) it was initially reasoned that the serum and micelle protein constituents would not be

exchangeable. This result would be consistent with the non-exchangeability required for the non-uniform labelling to result from a micelle effect. Non-exchangeability would make it possible for micelles to be labelled and used as tracers in adsorption studies.

Before proceeding further it was considered necessary to briefly review theoretical models for micelle structure and the literature on micelle-serum interactions. At the time this work was initiated no studies of tracer exchange had been reported and so an experimental programme was devised to elucidate any exchange and/or dissociation processes involving the micelle and casein monomers.

6.2 The Casein Micelle - Literature Survey

Physical Properties and Models

In the presence of calcium, the caseins found in milk have a strong tendency to form aggregates called micelles. These casein micelles are hydrophilic colloids stabilized by electrostatic charge and hydration and have an apparent zeta potential of approximately -18 mV (450,451). They are of spherical shape and range in size from 20 nm to 300 nm in diameter and in volume from $4.1 \times 10^3 \text{ nm}^3$ to $14.1 \times 10^6 \text{ nm}^3$. Inelastic laser light scattering of skim milk, diluted with simulated milk ultrafiltrate at a concentration of $5 \times 10^{-5} \text{ g ml}^{-1}$ of casein, gave a micelle diffusion coefficient of $2.18 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$ (443). The molecular weight of the micelle ranges from 10^7 to 10^9 .

Approximately 21 000 monomers of an average casein molecular weight of 23 000, would be required to yield a typical micelle of 'molecular weight' of 5×10^8 . Although the composition of casein micelles is known (see Table 6.1), the actual assembly of the various monomers in the micelle structure has not been fully elucidated. Currently, the most generally accepted structure for the micelle is of a porous spherical aggregate composed of smaller spherical sub-units. Numerous other models however, have been proposed in recent years. These have been discussed in detail in the following reviews (453,455,457,462,463,464).

According to Thompson and Farrell (453) the milk micelle models can be grouped into three classes. The first group were based on a coated core system where K-casein was spread over the surface of the micelle (see figure 6.1) (458), or where the K-casein served as the point of nucleation (see figure 6.2) about which the other caseins were clustered (459).

The second class of models were based on a porous internal structure. For example, depending upon the method used (449,454), it has been shown that the casein micelle is a highly solvated structure with between 1.9 to 3.7 grams of water per gram of protein. Supporting evidence shows that the casein micelle can be permeated by large molecular weight polymers such as carboxypeptidase A, dansyl chloride and myoglobin (476). Porous internal structures have been proposed by Garnier and Ribadeau Dumas (461) who placed considerable emphasis on K-casein as the keystone of the micelle structure (see figure 6.3);

Table 6.1

Composition of a casein micelle found in bovine
milk (452)

(Note: Considerable variations occur in the analytical data for
casein micelle).

Component	Weight Percentage
α_{s1} -casein	42
β -casein	28
K-casein	14
Minor casein components	10
Calcium	2.9
Magnesium	0.1
Organic Phosphorus	0.8
Inorganic Phosphorus	1.4
Citrate	0.5

Figure 6.1

Waugh model (458)

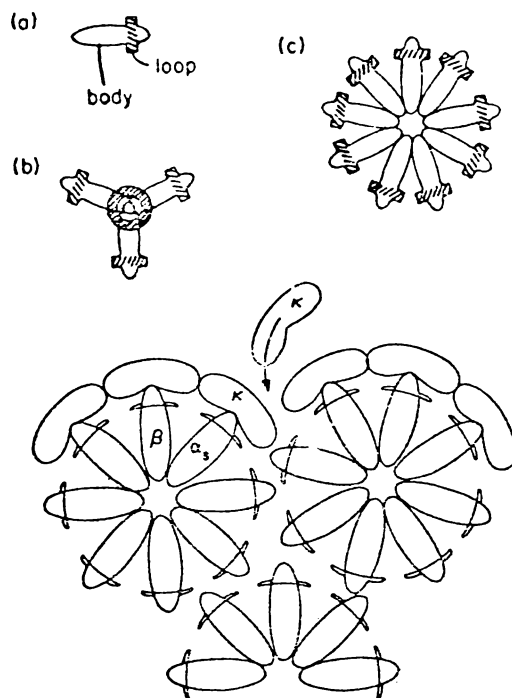


Figure 6.2

Parry and Carroll model (459)

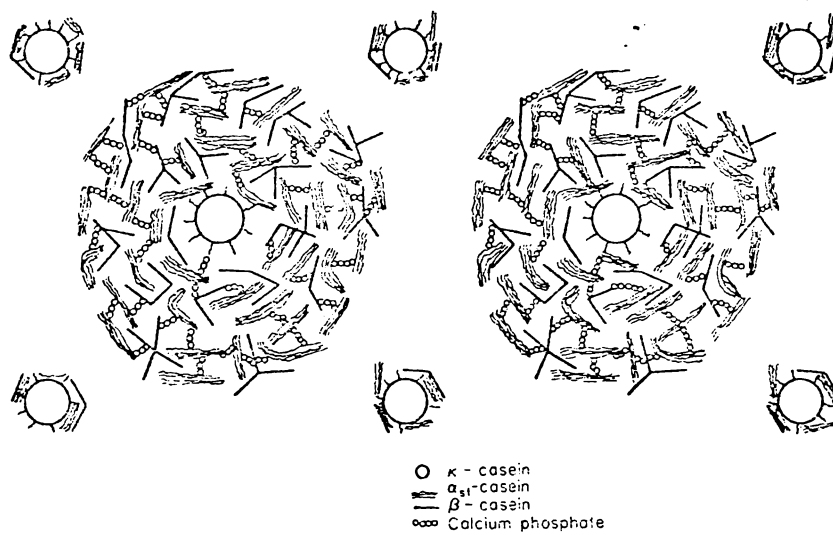


Figure 6.3
Garnier and Ribadeau-Dumas model (461)

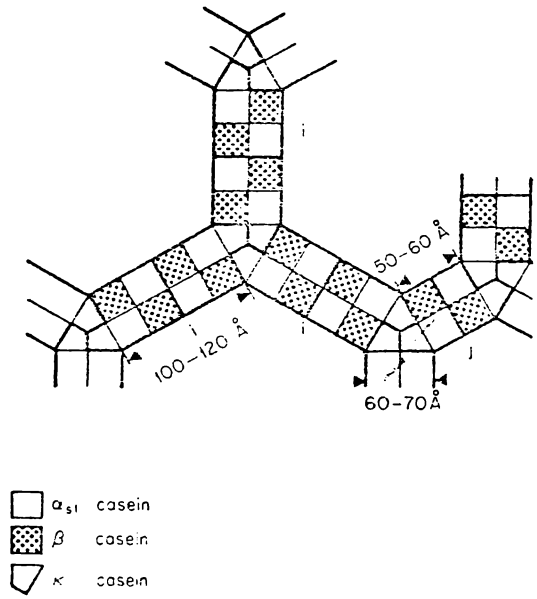
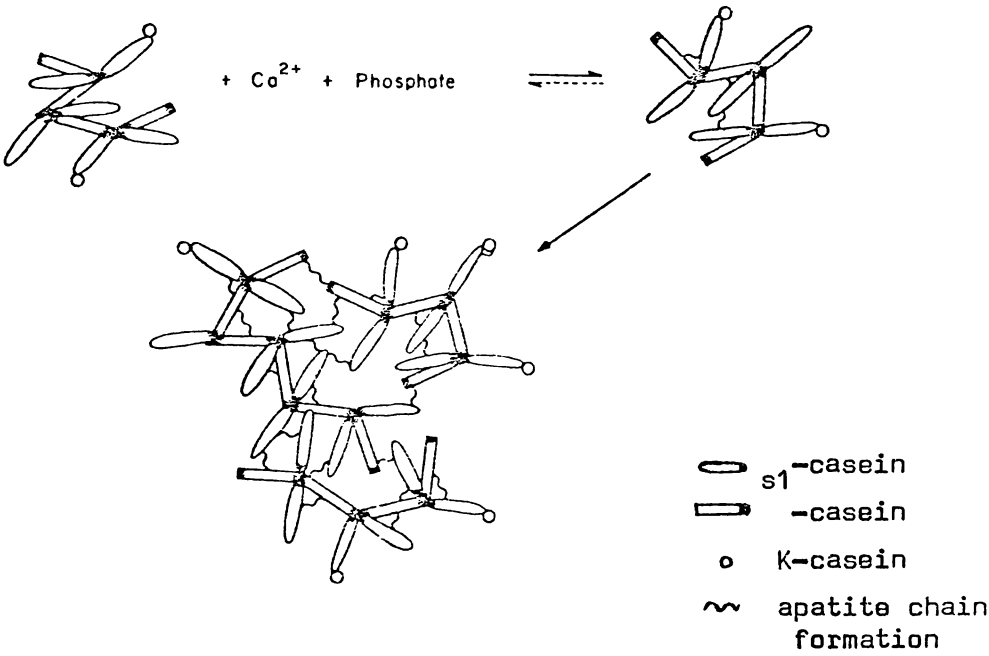


Figure 6.4
Rose model (457)

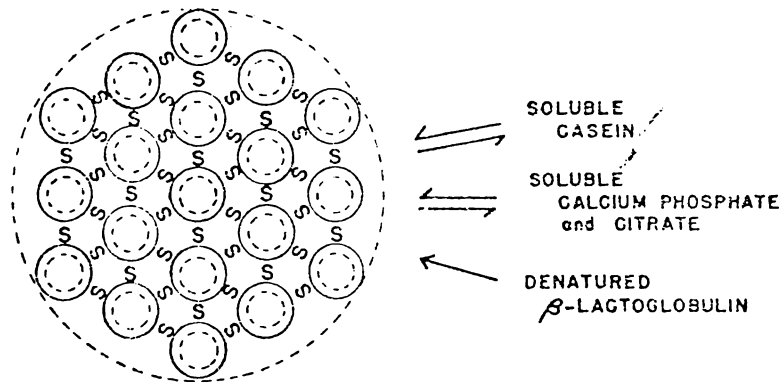


and by Rose (457), who used the polymerisation of β -casein as the building blocks for his micelle (see figure 6.4). Considerable evidence for and against these models has been presented (452,462,463,464).

Accumulated evidence now favours a third class of models based on a subunit micelle as the building block of casein micelles. This subunit structure was first proposed by Shimmin and Hill (465) after their electron microscope studies. They predicted a diameter of 10 nm for the subunit of the casein micelle. This theory was modified by Morr (466) who suggested that the subunits were stabilized by hydrophobic bonding and calcium caseinate bridges and were aggregated into micellar structure by colloidal calcium phosphate (see figure 6.5). The initial formation of the sub micelle unit has been demonstrated by the electron microscopic observations of particles of about 10 nm diameter taken from the Golgi vacuoles of lactating rat mammary glands (460,467,468). These particles were uniform in size and may be the precursors of casein micelles. These subunits have also been reported in bovine mammary tissue (469). The milk proteins are initially synthesised on the ribosomes of the rough endoplasmic reticulum. It is thought that the caseins interact to form subunits composed of several monomers which are then expelled into the vacuoles of the Golgi apparatus. In these Golgi vacuoles, the submicelles aggregate to micelles under the influence of calcium ions and calcium phosphate and are finally secreted into the alveolar lumen. Disruption of casein micelles by agents (467) such as ethylene diamine tetra-

Figure 6.5

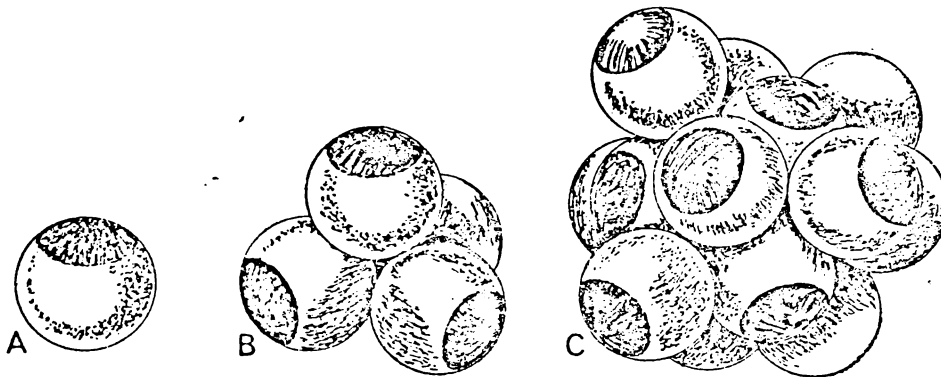
Morr model (465)



calcium phosphate
linkages
spherical complexes

Figure 6.6

Slattery and Evard model (470)



- A Submicelle with part of its surface hydrophilic (dark) and part hydrophobic (light)
- B Tetrahedral aggregate of four submicelles
- C Large aggregate of submicelles

acetic acid, sodium dodecyl sulphate and urea, causes the dissociation of the subunits from the micelle. Gel filtration chromatography and the removal of the soluble milk salts have also been used to detect and characterise these small casein aggregates (470). In order to explain why smaller casein micelles contained more κ -casein than the larger casein micelles, Slattery and Evard (470) considered casein micelles as being composed of subunits of constant size, but containing differing proportions of κ -casein. They considered that the κ -casein was segregated into a hydrophilic portion of the sub micelle surface and the α_{s1} - and β -casein was concentrated in a hydrophobic region of the sub micelle (see figure 6.6). They predicted that a minimum of fourteen sub micelles would be required to make a micelle (470), and the subunits containing lower percentages of κ -casein would aggregate to form larger micelles. The micelle could be stabilized by salt bridges of calcium and phosphate ions between the sub micelles. The total calcium content of skim milk has been estimated to be 30 mmol l^{-1} (473), and on ultra filtration or centrifugation, the resulting serum part of the skim milk was observed to contain only about 2.9 mmol l^{-1} of calcium ions (472,474).

6.3 The Chemistry of Micelle-Serum Interactions

Serum casein is an often neglected casein fraction which may play an important role in milk stability and should be considered, when considering micelle structure. This non-micellar casein, accounting for approximately 10% of the total casein in milk, at 25°C , does not sediment with the micelles

when ultracentrifuged at 78 000 g for 60 minutes (476,477). The distribution of the individual casein fractions is given in table 6.2. It should be noted that not only does the casein distribution vary from one individual milk sample to another, but the amount in the micelle fraction also varies. The percentage of serum casein increases to between 20 and 40% of the total casein upon the cooling of milk (477). This increase in serum casein is primarily β -casein, to a lesser extent, K-casein, and to an even lesser extent, α_{s1} -casein. The amount of β -casein in the serum at 4 °C varies from 15% (478) to 30% (477) of the total β -casein.

The use of EDTA to remove subcritical amounts of Ca^{++} from casein micelles resulted in a progressive decrease in the sedimentation velocity of the micelles without altering their hydrodynamic radii (40). Only after the calcium phosphate was removed by dialysis or by gel filtration did submicelles break away from micelles (480). It has been suggested recently that the submicelles are in equilibrium with their component caseins and that their size distribution is not constant as previously suggested (470) but depends on the protein concentration, pH, temperature and ionic strength (481).

It is clear from the above review, that while there is a general consensus of opinion that milk micelles are composed of subunits, much of the detailed structure of the micelle and the sub-micelles is still not understood. The literature appears to contain many contradictions.

Table 6.2

Distribution of the Casein Proteins in Milk
at 25 °C (477°).

	Percentage Protein in Milk	Percentage of Total casein
α_{s1} -casein -		
Micelle	1.60	48.0
Serum	0.15	4.4
β -casein -		
Micelle	0.64	19.0
Serum	0.23	6.8
K-casein -		
Micelle	0.25	7.4
Serum	0.10	2.9

The application of isotopes offers a method for gaining a better understanding of these particles and their interactions with serum constituents.

6.4 Methods for Preparing Radioactively labelled Micelles

As a large percentage of the proteins in milk are in the form of micelles, it is of interest to label these radioactively for adsorption studies. Properties required of labelled micelles are stability, non-exchangeability and high specific activity for ease in monitoring their adsorption. Four possible ways of labelling micelles are as follows:

1. Iodination of whole skim milk

The iodination and separation of the radioactively labelled casein micelles have been discussed in Chapter 5. A review of the advantages and disadvantages of this method led to the conclusion that it was not an ideal method for preparing labelled micelles.

2. In vitro labelling of Milk

In principle (if not in practice) the casein micelles could be labelled by injecting a radioactively labelled casein fraction into the endoplasmic reticulum of the cow. It is here that the casein polypeptide synthesis occurs (482). The polypeptide then goes into the Golgi apparatus where the micelle bioassembly takes place (483,484) and eventually leads to the secretion of the fully formed micelle. Ideally, by this method, it should be possible to label the

micelles with radioactively labelled α , β or κ -casein. Technical problems however, make this procedure of questionable feasibility. Alternatively the injection of a radioactive amino acid into the jugular vein of a lactating mammal has resulted in the preparation of radioactively labelled casein (485,486). This appears to label all the caseins in the micelle and thus offers no specific advantage over the iodination of skim milk.

3. The labelling of Micelles with Iodinated Casein Monomers

Radioactively labelled micelles could conceivably be prepared by attaching labelled proteins to the micelle by exchange, adsorption or encapsulation. The addition of tracer amounts of labelled individual casein fractions should not disturb casein-micelle interactions. Although less convenient than direct iodination of skim milk, the addition of labelled casein monomers is of interest, because such experiments could provide information about micelle-serum interactions. The usefulness of the micelles so labelled would depend very much on the kinetics of these interactions. Rapid kinetics could mean that the label would be lost before any tracer experiment could be completed, whereas slow rates of exchange could mean that insufficient activity was incorporated into the micelle. This need not be too much of a problem providing exchange took place before bacterial deterioration.

4. The Preparation of Artificial Micelles

The preparation of artificial micelles using labelled casein monomers provides an alternative approach to the preparation of

labelled micelles. The preparation of micelles from casein monomers has been well documented (456,487). This possibility was not investigated because it might be argued that artificial micelles do not necessarily have the same structure or properties as natural micelles. A study of the exchange reactions of artificial micelles and a comparison with natural micelles would be of interest.

6.5 Experimental

1. Addition of labelled Casein Tracers to Skim Milk

Experiments were performed to test whether there was any interaction between the serum casein and milk micelles. The procedure adopted was to add tracer amounts (10^{-5} g) of iodinated α_{s1} , β , κ , and γ caseins to 1 ml of skim milk such that the concentration of the casein constituent added to the serum was never more than 1% of the total serum casein. The milk was equilibrated for 1 hour at 25 °C and centrifuged overnight at 36 000 g using a Sorvall RC2-B centrifuge. In order to eliminate the possibility of bacterial action, the experiments were repeated by using 0.01% thimerosal. No difference in the effect was observed. To ensure that there was no colloidal material in the tracer casein solution, a sample of the tracer was centrifuged under the same conditions and shown to be free of particulate matter.

The results for this experiment, summarized in Table 6.3, consistently showed that the majority of the labelled tracer became associated with the micelles. This surprising result

Table 6.3

Radioactive labelling of the milk micelle by
incorporation of the individual casein fractions
at 24 °C

% Radioactivity in the Micelle							
Milk Sample							
Labelled with	(a)	(b)	(c)	(d)	(e)	(f)	(g)
α_{s1} -casein	90	80	83	90	81	90	84
β -casein	76	63	84	52	69	84	78
K-casein	82	73	46	34	21	81	35
γ -casein	14	-	-				

was reproduced many times. Up to the date of performing the experiment, literature reports had indicated that the constituents of the micelle were largely non-exchangeable with the constituents of the serum. At this stage of the investigations, the work was seriously handicapped because a high speed centrifuge was not available. The uncertainties introduced by the necessity for 12 hour centrifugations made desirable an alternative means of separating the micelle and serum caseins. Gel filtration experiments were performed.

2. Separation of Tracer labelled Micelles by Gel Filtration

A tracer amount (5×10^{-5} g) of labelled β -casein in sodium cacodylate buffer at pH 6.8 was added to 5 ml of skim milk. This was equilibrated for 30 minutes and then passed down a Sephadex G150 column (2.5 cm x 30 cm) with distilled water at 4 °C.

The total protein and radioactive tracer protein were monitored in the usual way (see Section 6.3).

The plots for total protein and the radioactive tracer are shown in figure 6.7. Forty-two percent of the total added β -casein tracer was incorporated in the casein micelle. This result showed a considerably lower fraction of the total activity associated with the casein micelle than was obtained in the centrifugation experiments. Such a result could be due to a temperature effect or a dilution-dissolution effect. It is known that milk micelles lose β -casein upon cooling (see Section 6.3). Little is known about the leaching of caseins from micelles by dilution with distilled water.

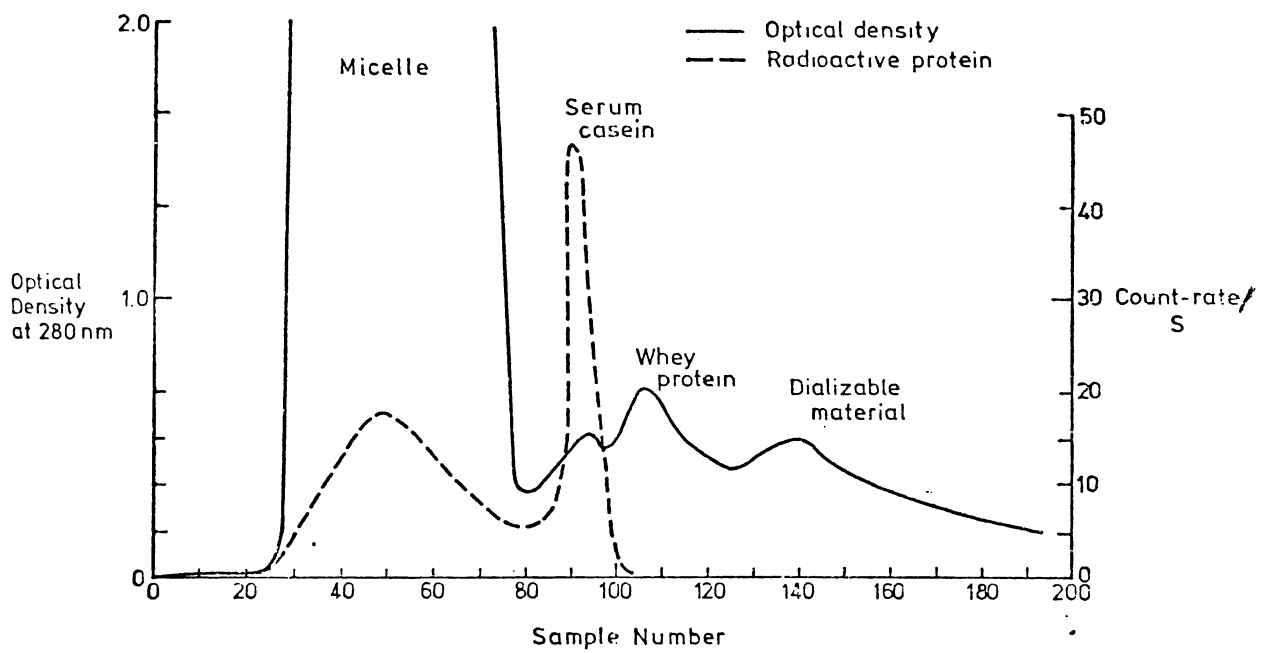
Further experiments were devised to test whether the tracer labelled micelles showed similar properties to those expected for the unlabelled micelles. If it could be shown that tracer labelled micelles behaved identically to unlabelled micelles, then the technique of tracer labelling could provide a useful means of investigating micelle properties.

3. Effect of Temperature on β -casein exchange

It is well established that β -casein diffuses out of micelles upon cooling (477,478). This effect seemed to be present in the previous experiment. It was considered desirable to check this result under more carefully controlled conditions and also to test the effect of temperature on the "exchange" of the other casein constituents.

Figure 6.7

Distribution of β -casein between micelle and serum as determined by gel filtration (Temperature 4 °C)



Fresh milk samples were labelled with the individual casein fractions at 24 °C and at 6 °C. The labelled solutions were equilibrated for one hour and then centrifuged. The results are tabulated in Table 6.4.

Table 6.4
Effect of Temperature on Serum-Micelle "exchange"

	Percentage Uptake (24 °C)	Percentage Uptake (6 °C)
α_{s1} -casein	90	88
β -casein	76	45
K-casein	82	60
γ -casein	14	14

These results showed that the amount of radioactively labelled micelles formed depended upon the temperature of equilibration and centrifugation, and that on cooling milk, β -casein diffused out of the micelle. This result is consistent with published data which indicated that on cooling milk, large amounts of β -casein diffused out of the micelle. A smaller effect has been reported for K-casein, but very little α_{s1} -casein was lost after cooling. In this experiment, therefore, the labelled monomers appeared to be behaving in the same way as the unlabelled monomers.

4. Micelle Composition as a function of Size

Another well established literature result is that smaller micelles contain considerably more K-casein (488). This property provides a test that can be applied to the labelled K-casein of the micelle.

Fresh skim milk samples were labelled with individual casein fractions, equilibrated for one hour, and then centrifuged for two and twelve hours at 36 000 g and 25 °C. The results are tabulated in Table 6.5.

Table 6.5

Effect of Size on Composition of Micelle

	% Activity of Large Micelles after two hours centrifugation	% Activity of Total Micelles after twelve hours centrifugation
α_{s1} -casein	68	90
β -casein	70	84
K-casein	18	81

From the above table it can be concluded that most of the radioactive K-casein is associated with small micelles.

At this stage of the work an MSE Superspeed 75 ultracentrifuge became available. This greatly facilitated the study of micelle-serum interactions.

5. Hysteresis effects in the labelled Casein-Micelle Interaction

In simple systems such as ion exchange resins the reversibility of tracer exchange experiments can be easily tested by removing the exchange phase from the radioactive solution and equilibrating it with non-radioactive carrier solution. If the exchange was reversible and if the tracer and

carrier species were chemically identical, the ratio of solution activity to ion exchange activity should in both the instances described above, reflect the relative amounts of the species in the two phases. If the ratios were not the same then some irreversibility or hysteresis effects would be indicated.

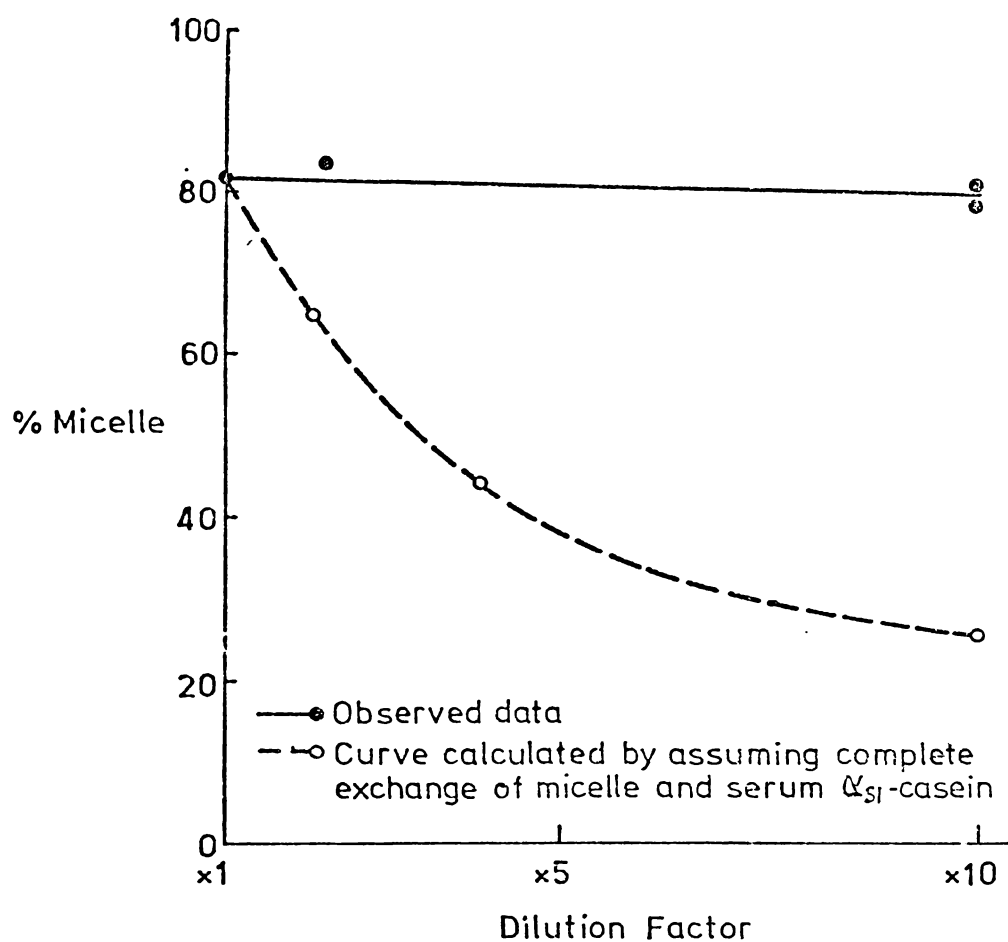
A similar procedure should be appropriate to test the reversibility of the interaction of labelled caseins with milk micelles. In this case, however, the situation is complicated by the difficulty of separating the micelle phase from the serum without the risk of causing structural damage. Centrifuged micelles are difficult to resuspend. Column separation techniques lead unavoidably to dilution.

In order to test the reversibility of the micelle - tracer interaction under conditions that could be expected to cause minimal micelle structural disruption, the following procedure was used. After initial treatment of skim milk with labelled α_{s1} -casein as in section 6.5.1, varying quantities of milk serum prepared by ultracentrifugation were added in order to increase the amount of serum α_{s1} -casein in the system. The effect of increasing the amount of α_{s1} -casein in the serum phase should lead to a redistribution of the activity between the serum and the micelle if interaction between serum and micelle was reversible.

Results are shown in figure 6.8 where it is clear that the activity taken up by the micelle in the initial treatment with labelled α_{s1} -casein in buffer did not exchange with the increased amounts of α_{s1} -casein in the serum phase.

Figure 6.8

Redistribution of labelled α_{s1} -casein from
micelles in milk diluted with milk serum
(Temperature 25 °C)



It appeared, therefore, that the interaction of the α_{s1} -casein with the micelle was irreversible. This means that the micelles labelled with α_{s1} -casein could be used as radioactive tracers. One possible explanation of the results described above is that labelled α_{s1} -casein interacts with the components of the serum to form new sub-micelles in which the α_{s1} -casein was non exchangeable. Alternatively, the α_{s1} -casein could have been adsorbed on to milk micelles by an irreversible adsorption process. The latter possibility did not seem likely in view of the non-labelled α_{s1} -casein already present in the serum.

The loss of radioactive β and K-casein from micelle into serum after a tenfold dilution with serum was also studied. In these particular cases, 52% and 34% of the original β and K-casein activity dropped down to 27% and 13% respectively. It appears that these caseins are not strongly bound to the micelle making them unsuitable for labelling the micelle.

6. Effect of Dilution on Micelle Stability

In order to separate milk micelles from serum with minimal risk of structural damage, column techniques are preferable to centrifugation or ultra-filtration. It was thus necessary to show that the micelles would not be affected by the unavoidable dilution that would result. This effect was studied by adding 0.10 ml of the individually labelled radioiodinated α_{s1} , β and K-casein to 5 ml samples of skim milk. The solutions were equilibrated for one hour at room temperature and then a range of

dilutions with distilled water were prepared (see Table 6.6). The resulting solutions were again allowed to equilibrate for one hour before ultracentrifugation at 98 000 g for one hour. The results are summarised in Table 6.6.

Table 6.6
Percentage of Labelled Casein Fraction in the
Micelle on diluting with Distilled Water

Dilution Factor	1	3	10	30	60
α_{s1} -casein	80	76	56	19	3
β -casein	63	27	9	3	6
K-casein	73	17	10	3	3

This experiment was repeated a number of times and although some variability was obtained with different milk samples, it was observed in all cases that on diluting the milk less radioactive protein was observed in the micelle. More β and K-casein than α_{s1} -casein appeared to dissociate from the micelle. This experiment was repeated by using a calcium chloride solution that contained 10 mmol l^{-1} calcium ions in place of the distilled water. The results are shown in Table 5.7.

Table 6.7

Percentage of Labelled Casein Fraction in the
Micelle on dilution with 10 mmol l^{-1} calcium ions

Dilution Factor	1	3	6
α_{s1} -casein	87	94	93
β -casein	71	85	73
K-casein	78	78	61

In the dilution range studied, the results showed that in the presence of calcium ions, no dissociation of radioactive protein from the micelle occurred. It is apparent that the calcium helped to hold the micelle together.

Dilution with distilled water, would cause the calcium ions to become diluted and result in the micelle dissociating. An alternative explanation is that the free calcium ions caused precipitation of the individual casein fractions. This possibility seemed to be eliminated by the fact that most of the casein remained in the form of micelles.

The dilution results showed that α_{s1} -casein was relatively strongly associated with the micelle and did not dissociate as easily as β and K-casein. On diluting the milk with distilled water containing calcium ions the radioactively labelled α_{s1} , β and K-casein are all very much more strongly bound to the micelle.

6.6 Conclusion

It has been shown in this chapter that it is relatively easy to prepare radioactive micelles by the addition of α_{s1} -casein to milk, the micelles being separated from the serum and

whey proteins by chromatography. The addition of calcium ions to the distilled water (provided an adequate concentration was maintained) appeared to increase the stability of the α_{s1} -labelled micelles and also to produce stable β and K labelled micelles.

It was observed that the radioactively labelled micelles exhibited the well documented properties upon cooling (partial dissociation of the radioactively labelled β -, and to a lesser extent, K-casein) and it was shown that larger micelles contained less radioactive K-casein than smaller micelles.

The use of radioactively labelled proteins together with isotopes such as calcium-45 offer much to the study of micelle structure. The results for the distribution of radioactive iodine on iodination of milk (see Chapter 5.3) indicated that the micelle structure probably affected the iodination efficiency of the K-casein. The results tend to support the model proposed by Garnier and Ribadeau-Dumas where the trimers of K-casein are at the apex of the micelle structure (see figure 6.3) and hence are less accessible for iodination.

Experimental results from the present chapter indicated that radioactively labelled β and K-casein can exchange more readily than α_{s1} -casein. It is possible that some of the K-casein is labile while the remainder is buried deeply within the micelle and is non-labile. It is interesting to note that radioactively labelled α_{s1} -casein added to milk became bound irreversibly to micelles whereas the binding of the K and

β -caseins to micelles was much more reversible. It appears from the present investigation that models proposing either complete exchangeability or non-exchangeability of casein between serum and micelle have serious inadequacies and must be modified accordingly.

Since this work was completed, some of the results discussed above have been confirmed by Creamer et al. (489) who added Carbon-14 labelled β -casein to milk and studied its exchange with the micelle.

CHAPTER 7

THE DEVELOPMENT OF RADIOCHEMICAL TECHNIQUES FOR PROTEIN ADSORPTION

7.1 Introduction

Previous chapters have described the preparation of radioiodinated proteins for use as tracers in adsorption studies. Such tracers must be free of unbound iodine and the labelled molecules must behave identically to the unlabelled molecules. In order for the latter condition to be satisfied it is necessary to ensure that the degree of labelling is at most one iodine atom per protein molecule. Adequate specific activity, however, is required in order to be able to monitor the small amounts of protein adsorbed by the low surface area systems that are to be studied. In addition, the labelled species must be stable and free of problems associated with bacterial action.

A slightly modified version of the Hunter and Greenwood method of iodination has been used to prepare iodinated proteins that should satisfy the requirements outlined above.

The iodine isotope chosen was I-125. I-125 is a weak γ emitter and can only be effectively counted by crystal scintillation counting. It has the major advantage of having a more convenient half-life than I-131 and is also more easily shielded. (For further physical data on I-125 and I-131 see Appendix 5).

Much care was given to the preparation of the stainless steel surfaces, the performing of the adsorption experiments and to the counting activity adsorbed.

The principle of isotope dilution was developed to calculate the amounts of protein adsorbed. Autoradiography was routinely employed to examine the uniformity of the adsorption.

7.2 Preparation of Protein Solutions

The preparation of the protein fractions from milk has been described in Chapter 3. All protein solutions were made up with double distilled water and were routinely filtered by using a millipore filter code VC of filter size $0.10\mu\text{m}$.

All glassware was cleaned before use with hot concentrated nitric/sulphuric acid for one hour and then thoroughly rinsed with double distilled water.

Protein stock solutions were stored at -20°C and immediately prior to use were warmed to the required adsorption temperature. During and/or between use, solutions were stored at 4°C and were generally never used after more than two days storage at this temperature. Bactericides were generally avoided because of the complicating effects they could have on protein structure and adsorptive properties. For example diethylpyrocarbonate, which is known to have good bactericidal properties, interacts chemically with proteins (490). Likewise the binding of azide to crystalline horse methemoglobin has been reported (505).

Concentrations of protein solutions were determined by ultraviolet spectrophotometry using literature values of extinction coefficients. The protein concentration of skim milk was determined by the Kjeldahl method using a conversion factor of 6.38 for total nitrogen to total protein.

7.3 Preparation of Stainless Steel

A major objective of the present study was to gain information about the adsorption of the various protein constituents of milk on to the stainless steel surfaces of milk processing equipment. In order to meet this objective the steel used was commercial stainless steel of type 304 with a 2B surface as is used in the dairy industry (see Section 2.2.). All the samples used for adsorption studies were cut from a single sheet of the stainless steel.

Immediately before adsorption experiments, the surfaces of samples were polished lightly with silicon carbide Grade 400 to remove grease and dirt. This was achieved by fifty strokes of a silicon carbide impregnated polishing cloth. After polishing the surfaces were washed with distilled water, boiled in 2% phosphoric acid for ten minutes, rinsed with distilled water, boiled in 2% potassium hydroxide solution for ten minutes and finally rinsed three times in boiling double distilled water before oven drying at 105 °C and storing in a dust free chamber until required. In all the adsorption experiments the stainless steel was used on the day it was cleaned. Considerable care was taken not to contaminate or touch the surfaces at any stage before or during an adsorption study.

Electron micrographs of the unpolished and polished surfaces are shown in figure 7.1. Also shown is a highly polished surface obtained by using a range of silicon carbide powders followed by a final polishing with tin oxide. Such highly polished surfaces were not used in the present study because the technological surfaces of interest are not nearly as smooth. Also preliminary adsorption studies indicated no significant surface roughness effect. This has recently been confirmed for surfaces polished with Grade 120 carborundum paper, Grade 360 carborundum paper, Grade 400 silicon carbide powder and tin oxide powder (491).

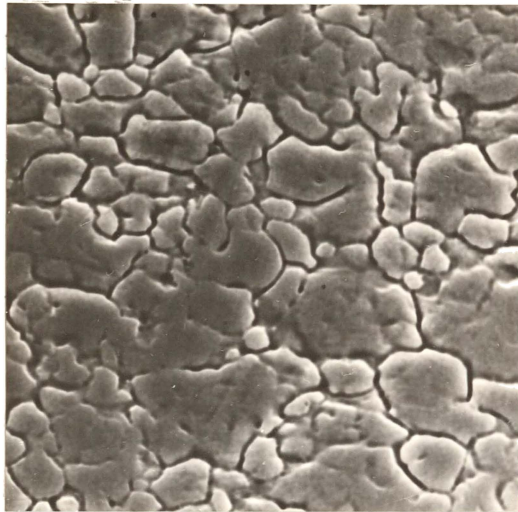
All manipulations involving the cleaned surfaces and the protein solutions were performed in a dust-free laminar flow box. During the adsorption experiments, surfaces and solutions were enclosed in a desiccator at controlled relative humidity and temperature.

7.4 Autoradiography

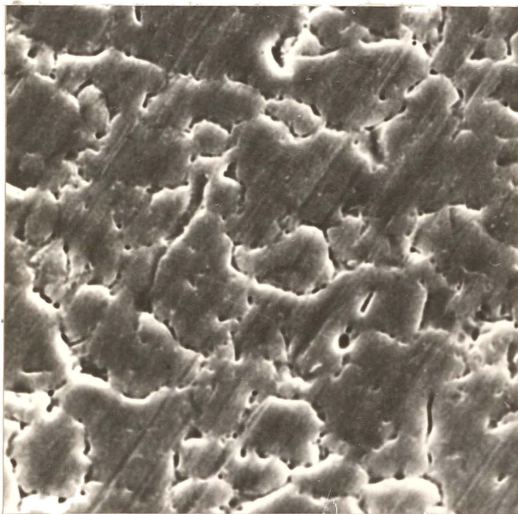
Autoradiography using Kodak X-ray film was routinely used to monitor the uniformity of adsorption of labelled protein. The surfaces to be studied were mounted on a sheet of paper with double sided cellotape, covered with glad wrap and X-ray films were placed to cover the side(s) containing adsorbed radioactivity. Exposure times were varied according to the amount of adsorbed activity. The exposed films were then developed in the normal manner.

Figure 7.1

Electron Micrograph of AISI Type 304 Stainless Steel
surfaces (X 1000)



a. Unpolished
2B surface



b. Polished with
silican carbide
Grade 400



c. Polished with tin
oxide powder

High resolution autoradiography using a technique based upon the use of a scanning electron microscope and Kodak Nuclear Track Emulsion (N.T.E.) was developed to determine the microscopic distribution of the adsorbed protein (543). In particular it was of interest to determine whether the adsorption reflected the surface morphology of cracks and crevices of the stainless steel surface. The procedure used was as follows:

After the protein had been adsorbed at monolayer coverage, the stainless steel slide was dipped into a 20% aqueous emulsion of N.T.E. at 20 °C. It was allowed to drain dry and was then stored in a dark box for ten days before developing and fixing in the normal manner. Radiochemical counting indicated no loss of activity due to this treatment. The developed slide was then examined with the S.E.M.

7.5 Development of Techniques for Studying Adsorption

Adsorption experiments using flat sheet metal surfaces involve low surface area systems where very small amounts of material are adsorbed. Two important problems associated with such experiments are the problem of contamination and the problem of analysing very small quantities of adsorbed material. In addition, adsorption experiments with surface active species such as proteins could be influenced by processes occurring at the water-air interface as well as the water-solid interface.

"Spot" Technique

Initially a spot adsorption technique as developed by Langdon et al. (492) was used. The radioactively labelled protein solution was placed in the adsorption cups (see figure 7.2a) and the air-water interface was 'swept' clean by overflow immediately before applying the clean stainless steel sample. This was in the form of a disc and was placed in such a manner that a restricted area of one face of the disc was in contact with the solution. The spot container and disc were enclosed in a vessel in which a 100% relative humidity was maintained in order to avoid solution evaporation.

After treatment for periods of 45 minutes they were removed, rinsed for 20 seconds with distilled water from a wash bottle, dried under a heat lamp and the activity of a defined area of the adsorbed protein was determined.

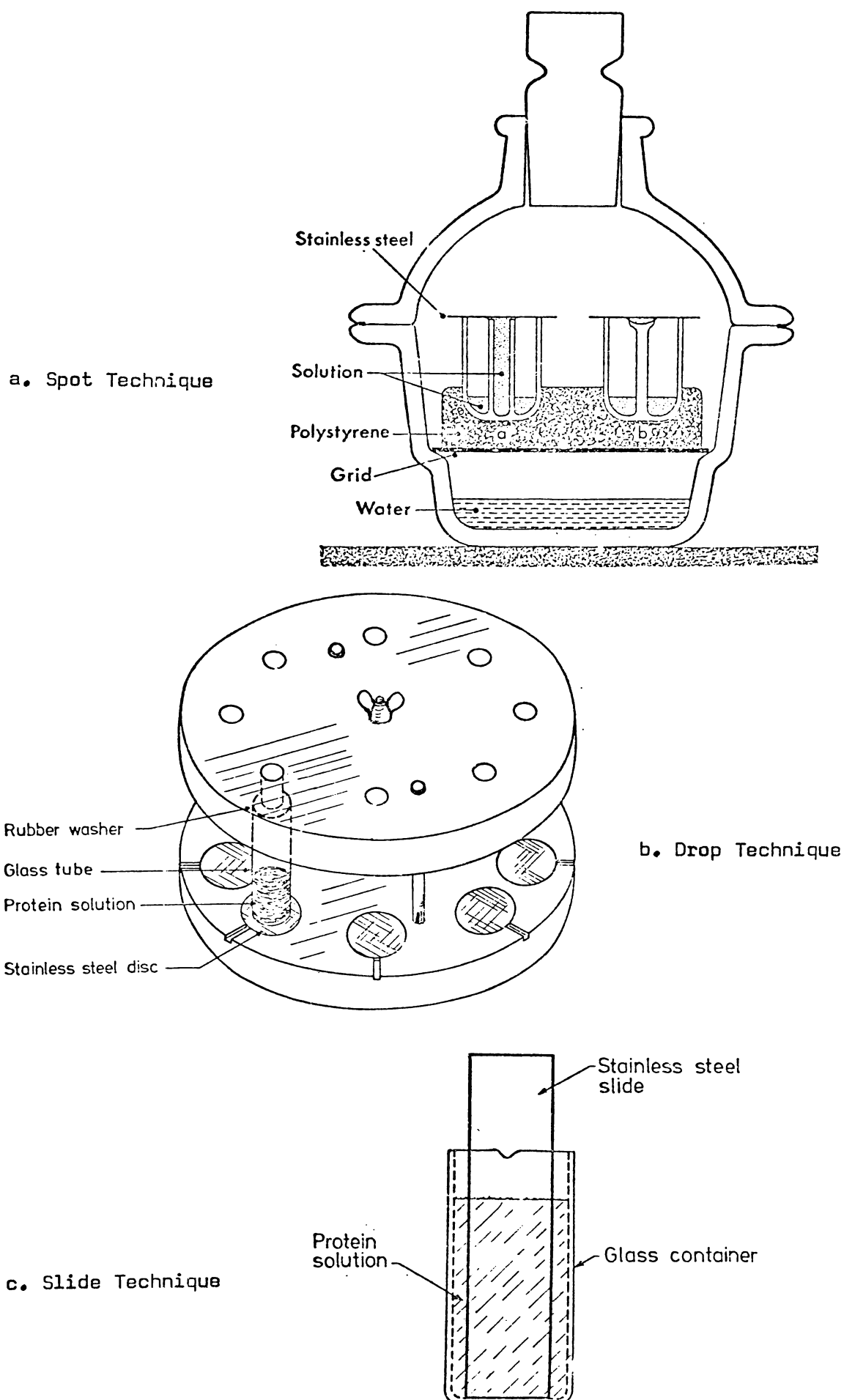
Autoradiography of the adsorbed radioactive protein was used to check the uniformity of adsorption (figure 7.3a). Repeatedly it was found that the adsorption was not uniform. Regions of high activity were often located around the edge of the 'spot' and could have been due to evaporation. Regions of low activity were usually centrally located and could have been due to the formation of air bubbles during the placing of the disc. The method was originally used to study the adsorption of ions on to mica which was considerably less rigid than stainless steel and also had the advantage of being transparent so that bubbles could easily be seen and avoided.

"Drop" Technique

A preliminary version of the "drop" technique was performed by dropping about five drops of the radioactive protein on to the centre of a flat disc of about 2.5 cm diameter. After a given period of time the radioactive protein was rinsed from the surface and treated as described for the 'spot' technique. Although the problem of bubbles was overcome, evaporation effects were still apparent in many of the experiments (see figure 7.3b). Reproducibility was also a problem using this technique.

In order to improve the reproducibility and avoid the effects of evaporation, the drop method was modified by using the apparatus as shown in figure 7.2b. The cleaned glass tubes were clamped in position over the stainless steel disc and up to 0.5 ml of the radioactive protein solution "dropped" on to the disc. The glass rings had the advantage of allowing a greater volume of solution to be in contact with the stainless steel to minimise drying effects. The large solution volumes also had the advantage of providing a larger reservoir of protein, and hence there was less depletion of the solution during adsorption. Again at the beginning of each run, the apparatus was sealed in a desiccator containing water to maintain a 100% relative humidity. At the end of each adsorption run, the radioactive protein was removed with a Pasteur pipette and the apparatus rinsed with distilled water. This method had a number of potential advantages. It avoided the problem of passing the stainless steel disc through an air-liquid interface, used larger solution volume, and avoided the problems associated with air bubbles. However,

Figure 7.2 Techniques used to study protein adsorption



leaking between the stainless steel disc and the glass ring often resulted in the radioactive protein solution forming a thin film over the disc. This thin film often dried and once again gave rise to evaporation-deposition effects. Another disadvantage of the method was its unsuitability for kinetic investigations.

"Slide" Technique

Because of problems associated with the two techniques described above, an alternative method based on dipping stainless steel slides into radioactive protein solutions was investigated. The stainless steel slides measured four cm by one cm and were 0.05 cm thick. Special flat walled 1 ml glass vials were used to minimise the volume of radioactive solution required. (see figure 7.2c). The vials were pinched at the top to ensure that the slide remained vertical and in a central position in the vial during the experiment.

One major disadvantage of the slide technique was the possibility of effects caused by passing the slide through an air-water interface either during the initial dipping of the slides into the radioactive solution, or upon withdrawing them from the solution at the completion of an adsorption experiment. The deposition of monolayers of a surface-active species from the air-water interface is a well known phenomenon (493). Initially special precautions were taken to minimise the possibility of such effects. These included placing the slides into empty vials and adding freshly agitated protein solution rather than dipping the slides into the protein solution. Before the slides were removed the solutions were again thoroughly

agitated in order to disrupt any layer formed at the air-water interface. Subsequently it was shown that effects due to adsorption at the air-water interface were negligible (see section 7.8).

Autoradiography of the adsorbed protein (see figure 7.3c) indicated uniform adsorption and an absence of the artifacts characteristic of the "Spot" and "Drop" procedures described previously. The slide method proved to be a convenient economical and reproducible (see section 7.9) method for studying adsorption under a variety of conditions including a range of temperatures and protein concentrations. The method could also be easily adapted to follow adsorption kinetics. Unless otherwise stated all further adsorption studies reported in this thesis were performed using this technique.

7.6 Calculation of Amount of Protein Retained

The amount of protein retained by the surface, either by adsorption or deposition, was calculated from the surface radioactive count using the principle of isotope dilution. For convenience, adsorption experiments were characterised by a retention coefficient, k , defined as:

$$k = \frac{\Gamma}{C} \quad \dots \dots \dots 7.1$$

where Γ = amount retained by the surface (mg m^{-2}) and
 C = concentration of protein solution (mg m^{-3}).

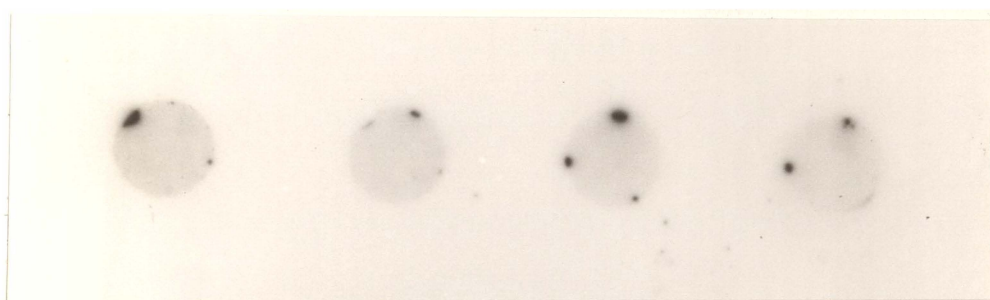
Providing that all the protein species present are labelled at the same specific activity, the retention coefficient for both single

Figure 7.3

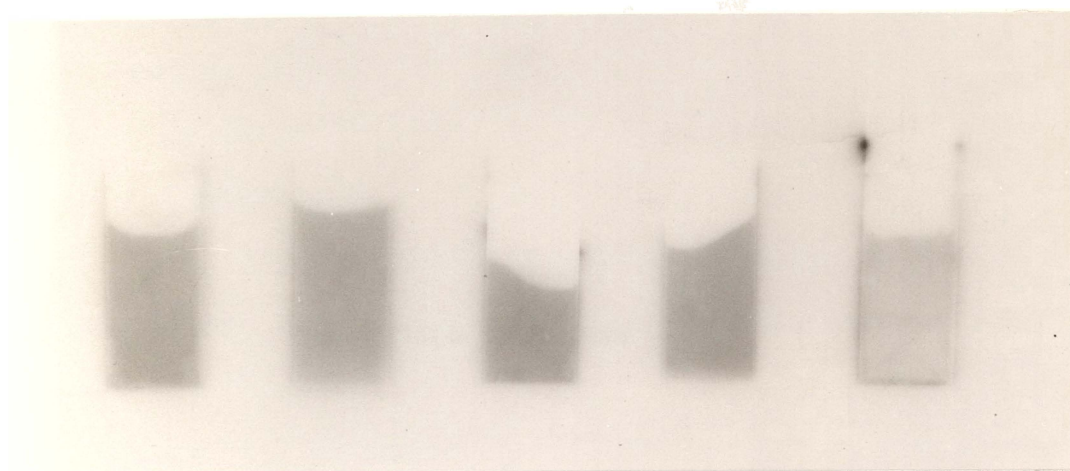
Autoradiography of the Adsorbed Protein



a. "Spot" Technique



b. "Drop" Technique



c. Slide Technique

and multiple component protein solutions (e.g. milk) can be determined by the equation:

$$k = \frac{\text{Surface activity}}{\text{Solution activity}} \quad \dots \quad 7.2$$

where : surface activity = measured radioactivity adsorbed

or retained by surface

(disintegration $\text{s}^{-1} \text{m}^{-2}$)

solution activity = radioactivity of solution

(disintegration $\text{s}^{-1} \text{m}^{-3}$)

If counting efficiency and counting geometry is maintained constant, then the ratio k can simply be obtained from:

$$k = \frac{\text{Surface count}}{\text{Solution count}} \quad \dots \quad 7.3$$

where : surface count = count-rate per unit area of surface

(counts $\text{s}^{-1} \text{m}^{-2}$)

solution count = count-rate per unit volume of solution

(counts $\text{s}^{-1} \text{m}^{-3}$)

The amount retained Γ can then be calculated from k and C using equation 7.1.

7.7 Counting Procedures

As indicated in Section 7.6, in quantitative adsorption (or deposition) experiments, the counting problem resolved into one of counting:

- (a) the activity adsorbed or deposited on a defined area in order to determine the surface count;
- (b) the activity contained in a defined volume of solution in order to count the solution count.

Surface Count

The main difficulty in determining the surface count was the arrangement of shielding in such a way that only a known area of the radioactive surface was counted. This was achieved by completely covering the radioactive area with a slab of stainless steel 0.160 cm thick in which a hole of 0.5 cm² area defined the region counted. The thickness of the stainless steel was chosen so that less than 0.3% of the shielded activity was counted. Autoradiography was routinely employed to ensure the area of activity counted was of uniform distribution. Care was taken to avoid counting regions near the edges of the strip or near the position at which the air/water interface had been formed during the adsorption experiment. A suitable counting configuration was ensured by designing a slide holder that held the shielding slab so that the hole was located centrally over the adsorption region. The shielding slab was held in position by two stubbs fixed to the tray of the slide holder. Glad wrap was used to separate the active surface and the shielding slab and it was replaced as soon as any contamination was observed. The position for maximum counting efficiency was determined and marked to ensure reproducibility of counting geometry.

Solution Count

A drop of the radioactive protein solution was carefully transferred to the centre of a counting planchette and weighed immediately. The planchette was then placed under a heat lamp planchette drier that slowly rotated the planchette during drying.

The radioactivity on the planchette was then counted with as near as possible, the same geometry as used to count the surface activity.

The Effect of Horizontal Displacement on Counting Efficiency

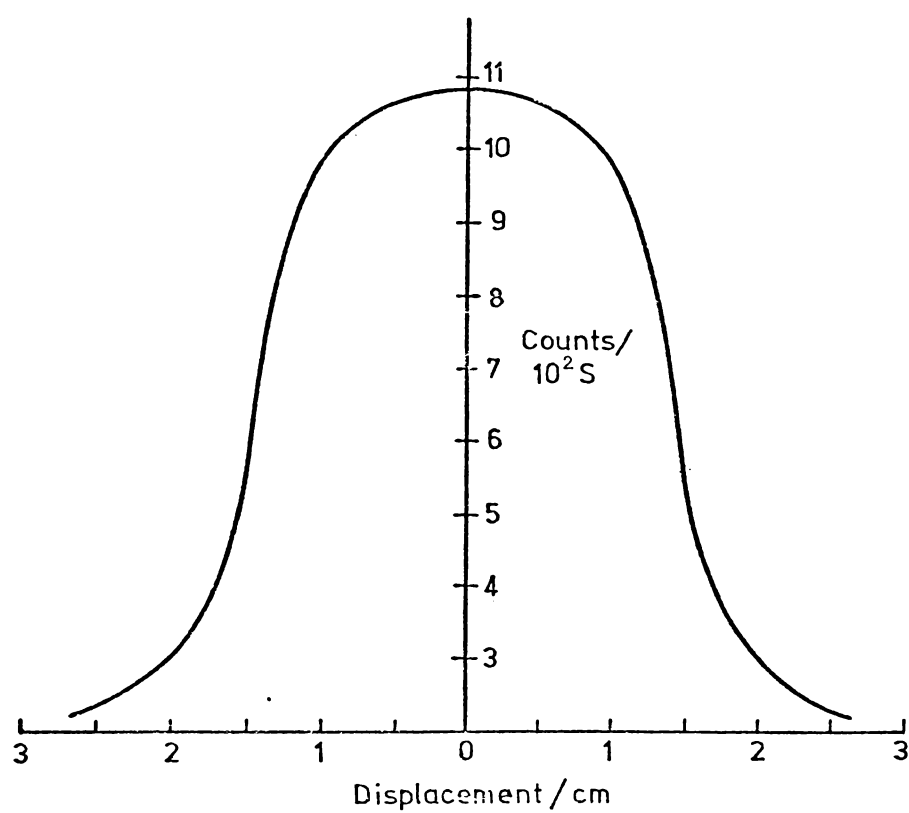
The variation of count rate due to the horizontal displacement of the source from the centre of the counting window was determined by displacing a spot source to the left and to the right. The results (see figure 7.4) showed a plateau in which the source can be displaced by ± 0.5 cm from the centre of the counting window without affecting the count obtained by more than $\pm 1\%$. In all experiments care was taken to make sure that the area to be counted was located to within ± 0.5 cm of the position for maximum efficiency. In the case of planchette counting of the solution count, special care was taken to ensure that the drop of solution was centrally positioned and that spreading was kept to less than 0.5 cm.

Relative Counting Efficiencies of Surface and Solution Activities

The method described for the calculation of amount adsorbed relies on identical counting efficiency for the counting of the surface activity and the solution activity. Because these activities were counted with less than 2π geometry using a solid crystal detector and because such counting is quite susceptible to small unavoidable geometry changes, it was felt necessary to check the relative counting efficiencies of surface and solution activities by an independent method that used the rigorously identical counting geometry and counting efficiency of a well type

Figure 7.4

Effect of horizontal displacement on counting efficiency



counter for both types of activity. In this way a geometry correction factor G was obtained, where:

$$G = \left(\frac{\text{surface activity}}{\text{solution activity}} \right)_{\text{well}} / \left(\frac{\text{surface counts}}{\text{solution counts}} \right)_{\text{solid crystal}}$$

The procedure used was as follows:

- (1) A solution of I-125 labelled protein of suitable activity was prepared. One sample of this solution was applied to a planchette, weighed and counted according to the procedure previously described. A second sample of the liquid was weighed into a vial and the volume made up to one ml. The activity was counted with a well crystal scintillation counter.
- (2) A strip of filter paper was saturated with the radioactive solution and allowed to dry. Autoradiography indicated uniform distribution of activity. An area of the strip the same size as the counting hole in the stainless steel shielding slab was carefully punched out and counted using the solid crystal counting system and also by the well counter. For the well count the paper disc was broken up by agitation with 0.5 ml of 0.1 mol l⁻¹ NaOH. The volume was made up to 1 ml with distilled water and counted.

From the results of these experiments, the geometry correction factor was calculated from:

$$G = \frac{\left(\frac{C_{\text{crystal}}}{C_{\text{soln count}}} \right) / \left(\frac{W_{\text{crystal}}}{W_{\text{well}}} \right)}{\left(\frac{C_{\text{crystal}}}{C_{\text{surface count}}} \right) / \left(\frac{C_{\text{well}}}{C_{\text{surface count}}} \right)}$$

where:

$C_{\text{crystal soln count}}$ = count rate of weight W^{crystal} of solution dried on planchette and counted with solid crystal counter

$C_{\text{well soln count}}$ = count rate of weight W^{well} of solution transferred to counting vial and counted with a well counter

$C_{\text{crystal surface count}}$ = count rate of paper disc counted with solid crystal counter

$C_{\text{well surface count}}$ = count rate of paper disc dispersed in counting vial and counted with well counter

This experiment was repeated twice using the same radioactive protein solution and the results are summarised in Table 7.1.

Table 7.1

Determination of Geometry correction factor G

Sample	$C_{\text{crystal sol.cnt.}}$	W^{crystal}	$C_{\text{well soln.cnt.}}$	W^{well}	$C_{\text{crystal surf.cnt.}}$	$C_{\text{well surf.cnt.}}$	G
1	405 900	0.0630	242 500	0.1376	28 200	7238	0.94
2	192 400	0.0285	497 000	0.2961	28 000	7138	1.02

(Note: The counts are for 100 second time period and the weight in grams).

The average value of G obtained, $G = 0.98 \pm 0.04$, shows that no correction for counting geometry is needed.

The background count-rate was kept to a minimum by surrounding the detector with lead. This background count rate was checked regularly and was subtracted from all count rates.

Back scattering effects were checked by counting I-125 adsorbed on to a piece of filter paper suspended on Glad wrap. A stainless steel plate was then placed behind the sample and it was again counted. No backscattering was detected.

Counting Equipment

Initially, preliminary experiments were performed using Geiger counters to monitor adsorbed activity. This counting method imposed major limitations in that counting efficiency for the more convenient I-125 was very low. Use of I-131, which could be counted with high efficiencies, was made difficult by the very short half life of this isotope.

The delivery of a Panax crystal scintillation counting and print out system greatly facilitated the counting of radioactive samples containing I-125. The major part of the work reported in this thesis used this counting system. It consisted of the following components:

PSU - 13	Power Supply
EHT - 10	Voltage regulator
AAU - 10	Amplifier/Analyser
TIM - 10	Timer
SON - 10	Scaler
RTM - 12	Ratemeter
PNI - 6	Print out

and a photomultiplier tube with a 2 inch NaI crystal.

Optimum counting efficiency was obtained by using upper level and lower level window setting of 5.50 and 3.00 respectively, an

attenuation of 1.0, and a photomultiplier voltage of 630 volts.

7.8 Effects at the Air-Water Interface

Surface active species such as proteins are known to concentrate at air-water interfaces and can be transferred to the solid surfaces during immersion or removal. It is possible to devise procedures that avoid the necessity of passing the solid surface through the air-water interface. Thus a series of experiments were performed to test for effects associated with such interfaces.

In one set of experiments the possibility of air-water interface effects during immersion was eliminated by placing the stainless steel slides in empty adsorption vessels and adding the labelled protein solution from a pipette. α_{s1} -casein at 4×10^{-6} g ml⁻¹ was used. After a predetermined adsorption period, adsorption vessel and slide were plunged into 5 litres of distilled water. After 10 seconds, the slide was removed and immersed in a further litre of distilled water for thirty seconds. This was followed by a 10 second rinse from a wash bottle.

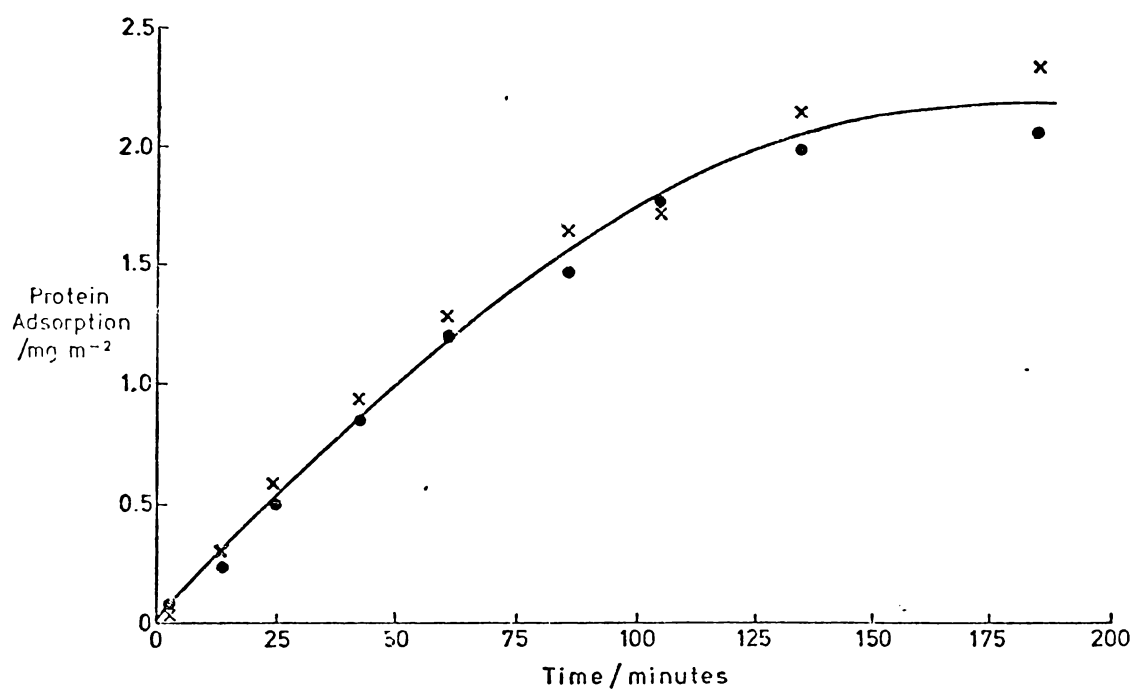
In a second set of experiments the same protein stock solution was used, but the procedure was such that the slides were dipped through the air-water interface to initiate adsorption and withdrawn through the air-water interface at the end of the predetermined adsorption time.

The results for both sets of experiments are shown in figure 7.5. There is no significant difference in the amount of adsorption for the two procedures so it can be concluded that

Figure 7.5

Air-water interface effects on protein adsorption
on to stainless steel

Adsorption of α_{s1} -casein at 4×10^{-6} g ml $^{-1}$ on to stainless steel



- Slide removed through air-water interface before rinsing
- x Slide rinsed without being withdrawn through air-water interface

effects due to air-water interfaces are negligible under the conditions described. This result was further substantiated by the results of autoradiography which indicated the absence of boundary effects at the air-water interface. Thus in subsequent work the possibility of such effects was discarded, though, as a precaution the surfaces of the protein solutions were always agitated before slide immersion or withdrawal.

7.9 Reproducibility

As a final check on the reliability of the slide procedure for determining protein adsorption, two checks on the reproducibility of the techniques were made.

In the first experiment a labelled solution of α_{s1} casein was added to six glass vials each containing a freshly cleaned stainless steel slide. After an adsorption time of 45 minutes and a rinse time of 20 seconds, the surface count of each slide was determined as previously described. The results are tabulated in Table 7.2.

Although the scatter of the data is slightly greater than can be accounted for by counting statistics, all the results are within 10% of the mean. This is a very satisfactory result in view of the inherent lack of reproducibility often associated with low area solid-liquid systems.

The reproducibility of the adsorption experiment was further tested using separate samples of four-fold diluted milk containing a wide range of solution activities. Results tabulated in Table 7.3 show very satisfactory reproducibility in the amount adsorbed for the different milk solutions.

Table 7.2

Reproducibility of Technique

Surface count/s ⁻¹	σ /s ⁻¹
9.88	0.31
10.21	0.32
9.20	0.30
9.91	0.32
10.61	0.33
9.51	0.31
11.05	0.33
10.18	0.32
10.07	0.32
10.88	0.33
10.26	0.32
10.38	0.32

Mean surface count = 1018

Counting time = 100 s

σ = Standard deviation

Table 7.3

Effect of Specific Activity on the Amount

Absorbed (T = 20 °C)

Protein Concentration /g ml ⁻¹	Specific Activity /count s ⁻¹ g ⁻¹	Activity Adsorbed /count s ⁻¹	Amount Adsorbed /mg m ⁻²
7.52 × 10 ⁻³	11.7 × 10 ³	.700	8.97
7.93 × 10 ⁻³	13.5 × 10 ³	.738	8.65
8.25 × 10 ⁻³	69.5 × 10 ³	3.66	8.70
8.25 × 10 ⁻³	80.5 × 10 ³	4.27	8.75
7.74 × 10 ⁻³	113.3 × 10 ³	6.941	9.48
8.31 × 10 ⁻³	118.0 × 10 ³	5.656	8.00
8.25 × 10 ⁻³	138.0 × 10 ³	6.97	8.33

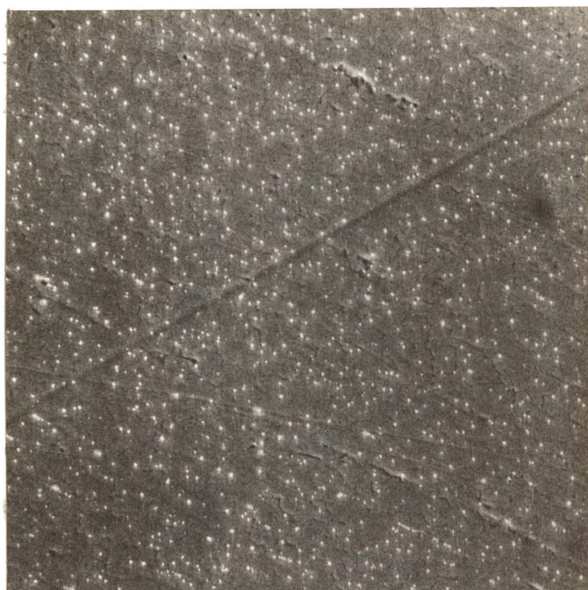
7.10 Distribution of Adsorbed Protein

Conventional autoradiography indicated (see figure 7.3) that within the limits of resolution of the X-ray film used, the adsorption was uniform. Results from high resolution autoradiography using Kodak Nuclear Track Emulsion are shown in figure 7.6. The random distribution of the developed silver spots indicated an absence of any accumulation in the microscopic defects of the stainless steel surfaces still visible beneath the emulsion film.

It can be concluded that the adsorption of protein by stainless steel is uniform down to the resolving power of the SEM autoradiography technique. From a consideration of the size of the developed silver particles and also their separation, this was estimated to be of the order of a few microns.

Figure 7.6

Electron Micrograph of N.T.E. Autoradiograph
of Adsorbed Protein:



(X 200)



(X 1000)

7.11 Conclusion

The results reported in this chapter established that the 'Slide' technique based upon the use of I-125 iodinated protein and the principle of isotope dilution provided a convenient and reproducible method for the study of qualitative and quantitative aspects of milk protein interactions at solid-liquid interfaces. The absence of effects due to the air-water interface was demonstrated. The way was now clear for detailed studies of adsorption and deposition from milk and milk protein solutions. These form the topics of succeeding chapters.

CHAPTER 8

ADSORPTION FROM SKIM MILK, MILK SERUM AND WHEY PROTEIN SOLUTIONS

8.1 Introduction

In previous chapters, methods for preparing iodinated protein for tracer studies, for labelling milk with such proteins and for calculating the amount of material adsorbed on to a surface from a radioactive solution, have been described. In this chapter the use of these techniques to study the adsorption of milk proteins on to stainless steel surfaces will be discussed. Initial experiments were performed using labelled skim milk. In later experiments milk serum and solutions of whey proteins were used.

Much of the work reported in this chapter was of a preliminary nature and in some instances served largely as a guide for further investigations. Initially, experiments were performed to determine how much protein could be adsorbed by stainless steel after a single exposure to a milk solution. Also of interest was the determinations of which protein constituents of milk were adsorbed.

8.2 Material and Method

The method used for the adsorption studies was the 'Slide' technique described in section 7.5. All adsorption experiments were performed in a dust free laminar flow box at room temperature, or in a desiccator in a cold room maintained at 4 °C or in an

incubator maintained at 35 °C. Before each experiment all glassware and stainless steel samples were cleaned according to procedures already described (sections 7.2 and 7.3) and equilibrated at the appropriate temperature for at least 30 minutes.

All milk used was obtained as fresh Jersey milk from No.3 Dairy at Ruakura. It was centrifuged at 3 500 rpm for 20 minutes to remove the fat. A sample of this milk was iodinated with iodine-125 and used to spike the milk samples used in the adsorption studies. Initial protein concentration of the milk was determined by Kjeldahl nitrogen. When necessary, samples were diluted with distilled water. The amount of protein adsorbed was determined using the procedure described in section 7.6.

8.3 Adsorption of Protein from Skim Milk

Kinetics of Adsorption

A preliminary experiment indicated that significant amounts of protein were adsorbed on to stainless steel and that a 20 second rinse from a wash bottle was adequate to remove unadsorbed activity. Little adsorbed radioactivity was removed with prolonged rinsing.

The kinetics of adsorption at 20 °C from skim milk diluted four-fold with distilled water were determined in separate experiments. Results for adsorption times ranging from 5 seconds to 2 hours are summarised in Table 8.1. It is evident that at the concentration employed, the surfaces were effectively 'saturated' after even the shortest adsorption time employed. In further

Table 8.1

Kinetics of Adsorption from Skim Milk

(Protein concentration = 8.3 mg ml^{-1} , $T = 20^\circ\text{C}$)

Time/min	Amount adsorbed/ mg m^{-2} ($\pm 0.5 \text{ mg m}^{-2}$)
0.0833	7.2
0.25	7.4
0.50	7.2
1.0	8.1
20	8.1
40	7.9
60	8.2
120	7.5

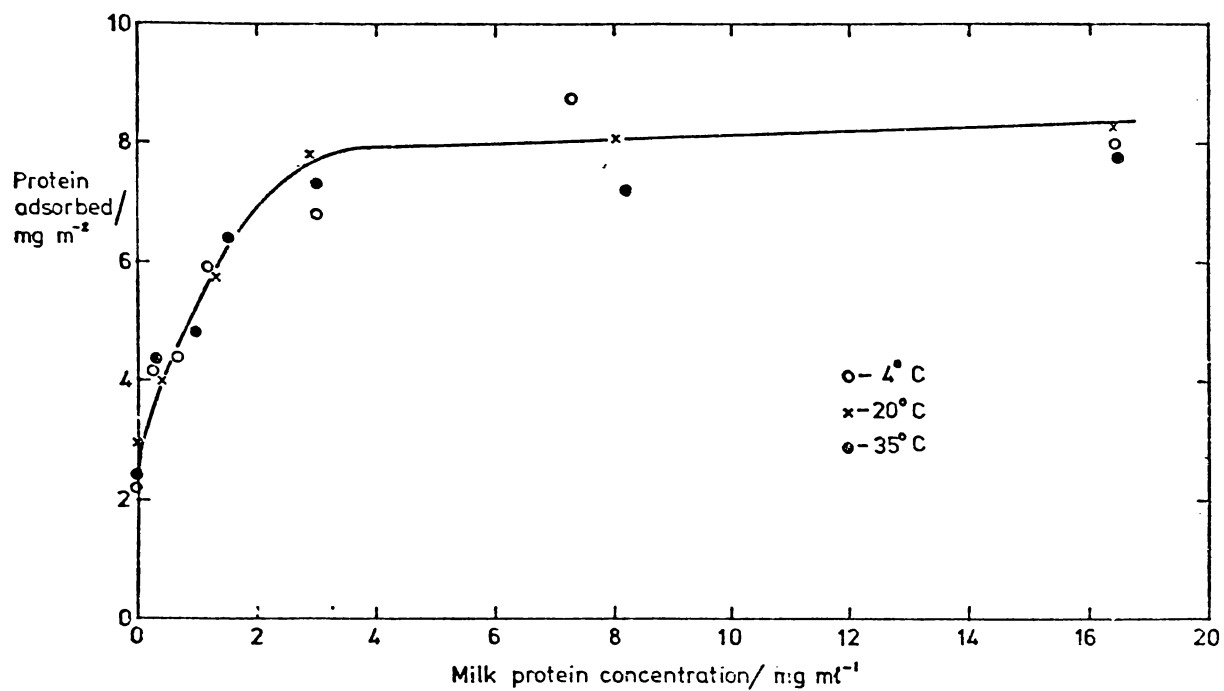
adsorption studies using skim milk, an adsorption time of 20 minutes should be adequate to ensure maximum adsorption.

The Effect of Protein Concentration and Temperature

Radioactively labelled skim milk diluted two-fold with distilled water was prepared as previously described. This solution was then further diluted by approximate dilution factors of 2, 4, 10, 20 and 100, the exact protein concentration being determined by radioactive counting. Adsorption experiments at each concentration and at 4°C , 20°C , and 35°C were then performed. The results for all experiments are summarised in figure 8.1. It is apparent from these results that under the conditions of the experiments described surface saturation was achieved at total protein concentrations greater than 3 mg ml^{-1}

Figure 8.1

Adsorption of protein on to stainless steel from
skim milk



(i.e. at milk dilutions less than 10-fold dilution). However significant protein is adsorbed even at 100-fold dilution. This illustrates a point of practical significance, i.e. adsorption of milk protein could still occur even after milk had been diluted many times.

The shape of the adsorption isotherms for the various temperatures were essentially identical and conform to the general shape of a Langmuir adsorption isotherm. Attempts were made to fit the data to the Langmuir equation using the standard graphical procedure of plotting $\frac{C}{P}$ versus C (see figure 8.2). Values of $\Gamma_{\text{saturation}} = 8.6 \text{ mg m}^{-2}$ and $b = 1923$ were obtained and a reasonably good fit of the data with the Langmuir equation was indicated. Attempts to fit the data to other adsorption equations such as the Frenlich equation gave unsatisfactory results.

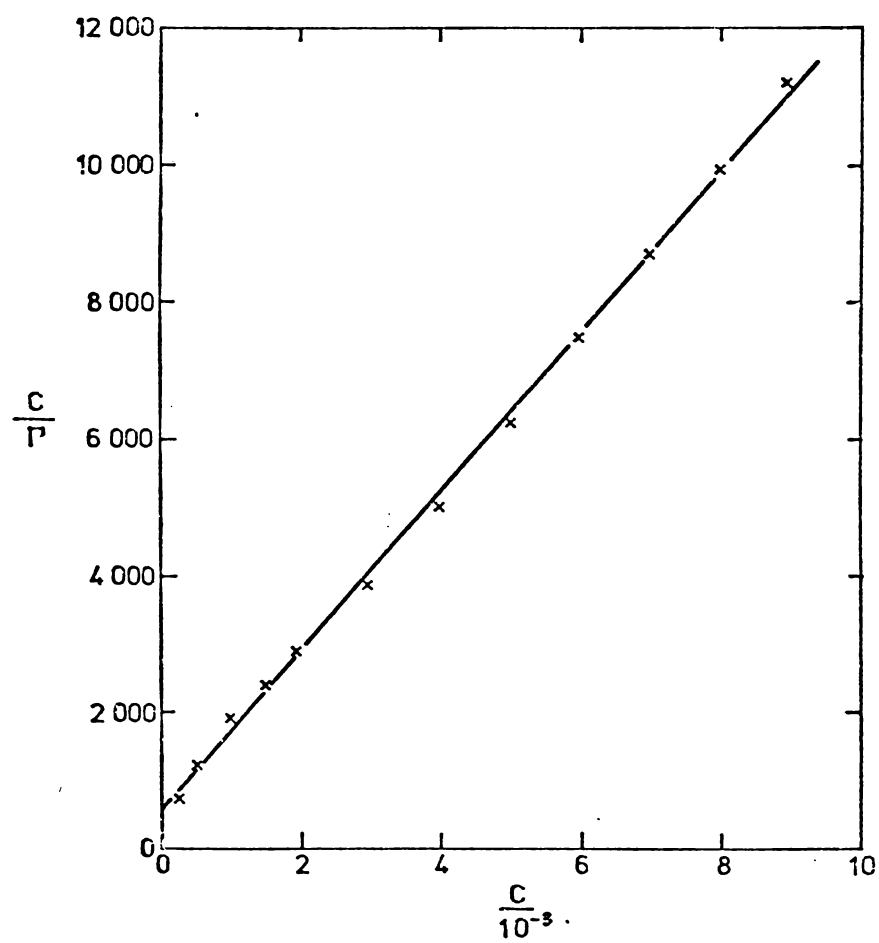
Temperature variation over the range from 4°C to 35°C appeared to have very little effect on the amount of adsorption at the protein concentrations investigated.

S.E.M. Investigation of the Adsorbed Film

In an attempt to obtain direct evidence for the presence or absence of adsorbed micelles on surfaces treated with a milk solution, a S.E.M. study was made of the adsorbed film after fixation with gluteraldehyde (see section 13.2). A control was prepared by mounting a sample of micelles fixed according to the procedure of Carroll et al. (444) on a stainless steel slide.

Figure 8.2

Langmuir plot for protein adsorption from
skim milk



At sufficiently high magnification (12 000 X) micelles could be identified on the control sample but not on the adsorbed film. This provided good evidence that micelles are not adsorbed as discrete particles (see figure 8.3).

Discussion

The adsorption isotherms indicated that stainless steel surfaces exposed to slightly diluted skim milk become saturated with a layer of approximately 8.0 mg m^{-2} of milk proteins. Assuming the adsorbed protein to be uniformly distributed and to have a density similar to that of anhydrous protein (approximately 1.3 g ml^{-1}), the adsorbed film of protein would be about 6.2 nm thick. Taking into consideration the reported dimensions of the milk micelle (average diameter from 20 nm to 300 nm) and of the various milk protein molecules (13 nm x 2 nm approximately) (206) it appears that if the protein is adsorbed as micelles then these must be widely scattered over the surface. Alternatively the close packed unfoiled side-on adsorption of monomers would give rise to a surface film of approximately 2.0 mg m^{-2} . Close packed end-on adsorption of monomers would give a surface film of about 8.2 mg m^{-2} (see section 9.4). These calculations, which take into account a surface roughness factor of 1.7, (see section 9.4) indicate the likelihood of surface saturation by a coherent film of monomers rather than a scattered layer of micelles and this result is supported by S.E.M. studies. This conclusion is not significantly affected by the unavoidable uncertainty in the specific activity of the labelled milk (see section 5.4).

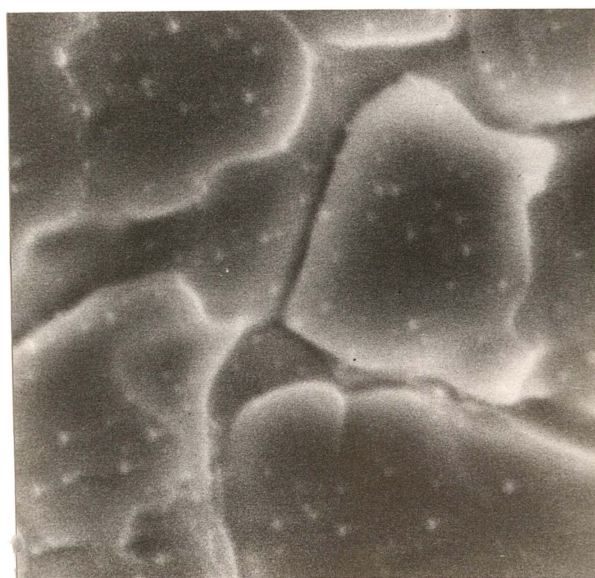
Figure 8.3

S.E.M. studies of adsorbed milk film and deposited
casein micelles

- a. Stainless steel surface after treatment with skim milk
(X 12 000)



- b. Stainless steel surface showing deposited micelles
(X 12 000)



In order to further investigate which species were adsorbed, experiments were designed to study casein adsorption, whey protein adsorption and adsorption from serum free of micelles.

8.4 Adsorption of Protein from Whey Solution

Preparation of Whey

The whey protein was separated from milk by cooling the milk to 2 °C and adding 1 mol l⁻¹ HCl until the pH of solution was pH 4.6. It was then warmed to 30 °C to precipitate the casein which was separated by centrifugation. The resulting whey solution was decanted through a filter paper and desalted by dialysis against two changes of dilute acetate buffer at pH 4.6 (using 10 ml of 10% acetic acid and 10 ml of 1 mol l⁻¹ sodium acetate made up to one litre) for one hour followed by dialysis over night at 4 °C with tris-HCl buffer at pH 7.5. The protein nitrogen determined by Kjeldahl nitrogen. A sample of this whey solution was iodinated for use in the tracer studies.

Adsorption Isotherm

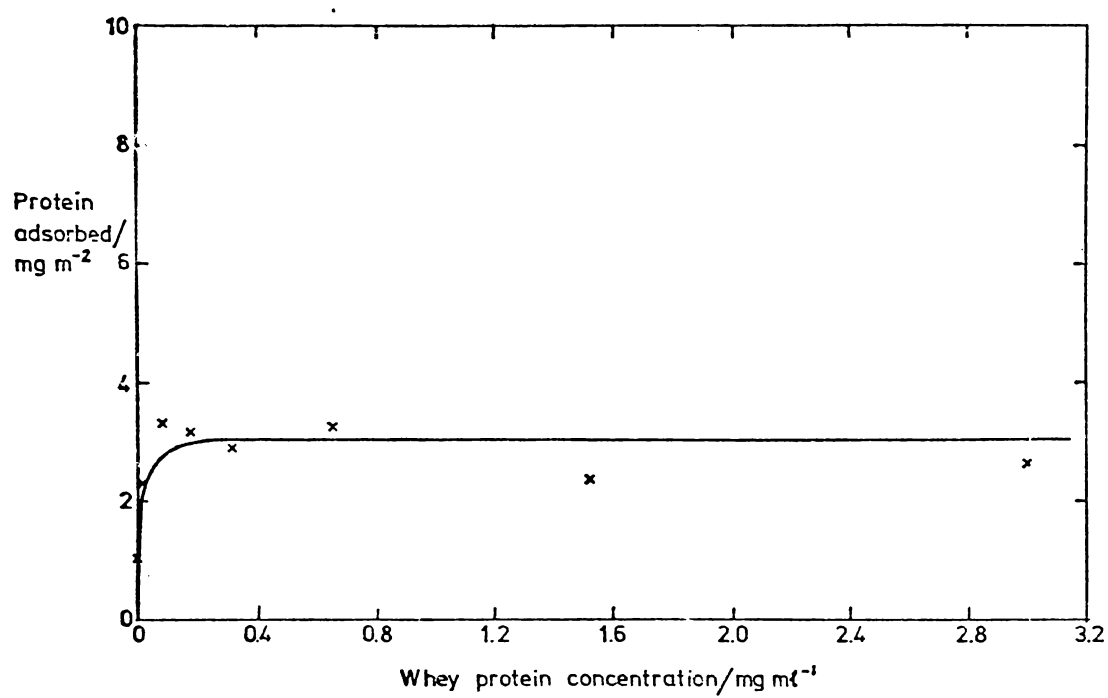
The adsorption isotherm for the adsorption of protein from whey protein solutions at 20 °C is shown in figure 8.4. The isotherm appears to be of a Langmuir type with a $\Gamma_{\text{saturation}}$ value of approximately 3 mg m⁻² (much lower than that for skim milk).

Adsorption of Whey Protein from Skim Milk

In order to test for the effects of cooperative or competitive effects in the adsorption of whey protein, a portion

Figure 8.4

Adsorption of protein on to stainless steel from
whey



of labelled whey protein was added to skim milk and adsorption experiments performed. Results for skim milk with known whey concentrations diluted with distilled water to give a range of concentrations are summarised in figure 8.5. Data for the temperatures: 20 °C, 35 °C, 50 °C and 80 °C are shown. It is clear that except for the experiments at 80 °C, the amount of whey protein adsorbed is considerably suppressed by the presence of the other constituents of skim milk. Apparently these other constituents are more strongly adsorbed than the whey proteins. As in the case of skim milk, there does not appear to be a significant temperature effect over the range 20 °C to 50 °C. The result at 80 °C is probably anomalous and could be a result of the known tendency of whey proteins to aggregate with the caseins at high temperature.

8.5 Adsorption of Protein from Serum

The serum obtained when the micelles of skim milk are removed by centrifugation consists principally of whey protein and serum caseins. A sample of labelled milk serum was prepared by spiking milk with iodinated milk, centrifugating it at 102 000 g for one hour, separating off the serum and centrifuging it again at 102 000 g. As no further precipitation, radioactive or non-radioactive, was observed it was presumed to be free of micelles. Again the protein concentration was determined by Kjeldahl nitrogen.

Adsorption data were obtained at 20 °C for a range of serum concentrations. The results are plotted in figure 8.6. It appears that approximately twice as much protein is adsorbed from

Figure 8.5

Adsorption of whey protein on to stainless steel
from skim milk

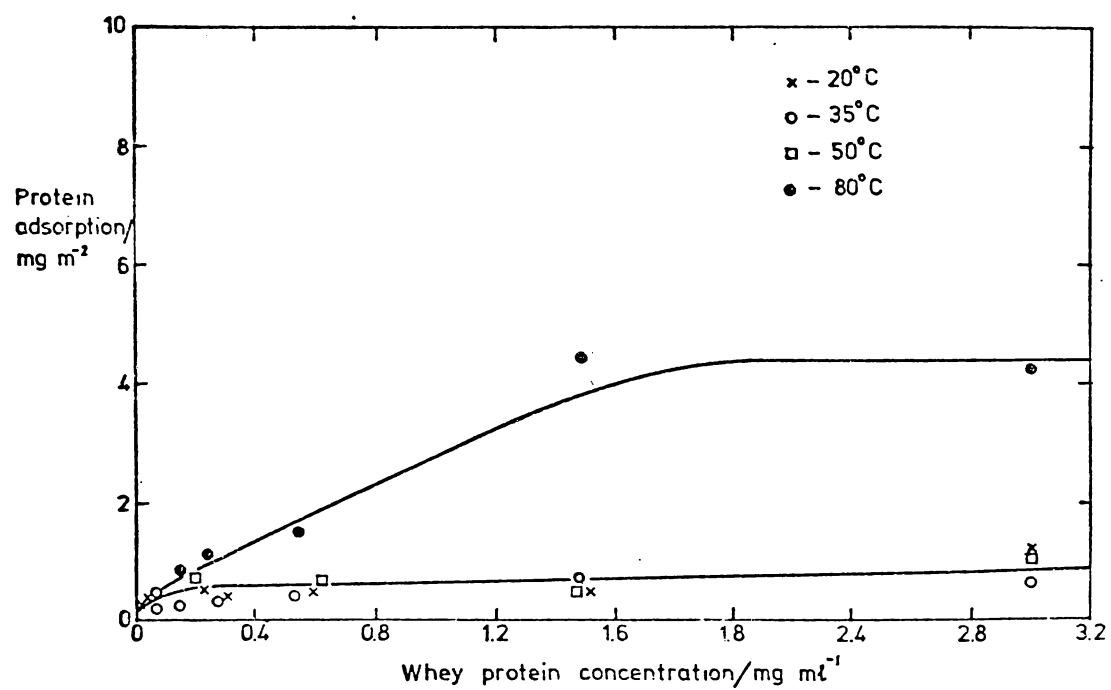
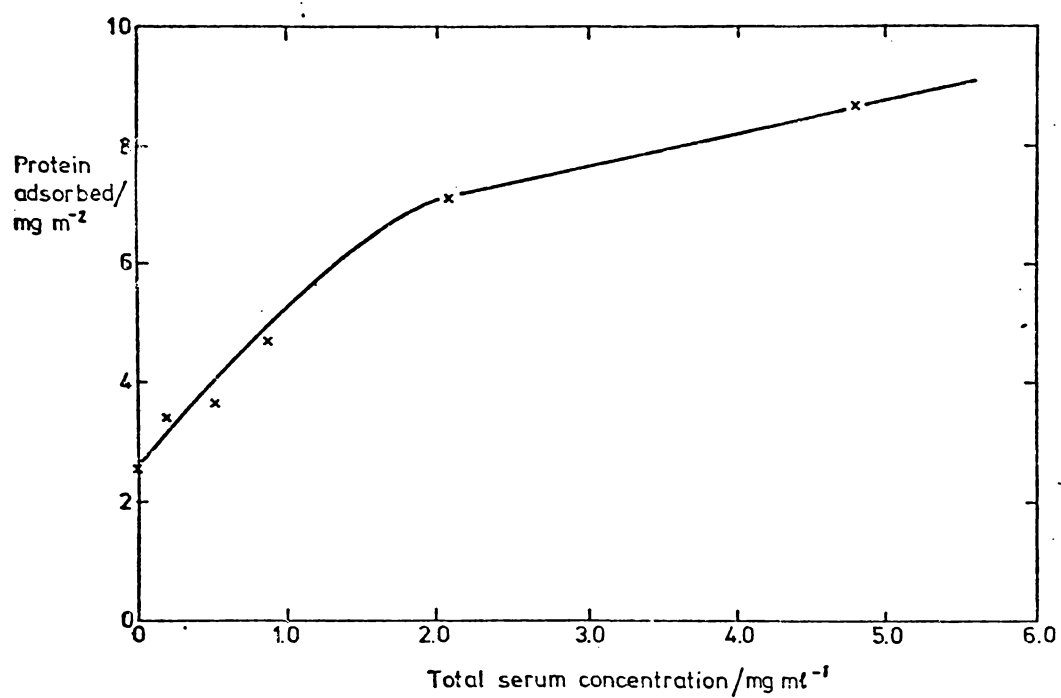


Figure 8.6

Adsorption of protein on to stainless steel from
milk serum



the serum solution (containing whey proteins and serum casein) as is adsorbed from solutions of whey proteins alone. Also, since it was shown in the previous experiment that the adsorption of whey protein was suppressed by the presence of the other constituents of milk, it is most likely that the protein adsorbed from the milk serum mixture of whey protein and serum casein is predominantly the serum caseins.

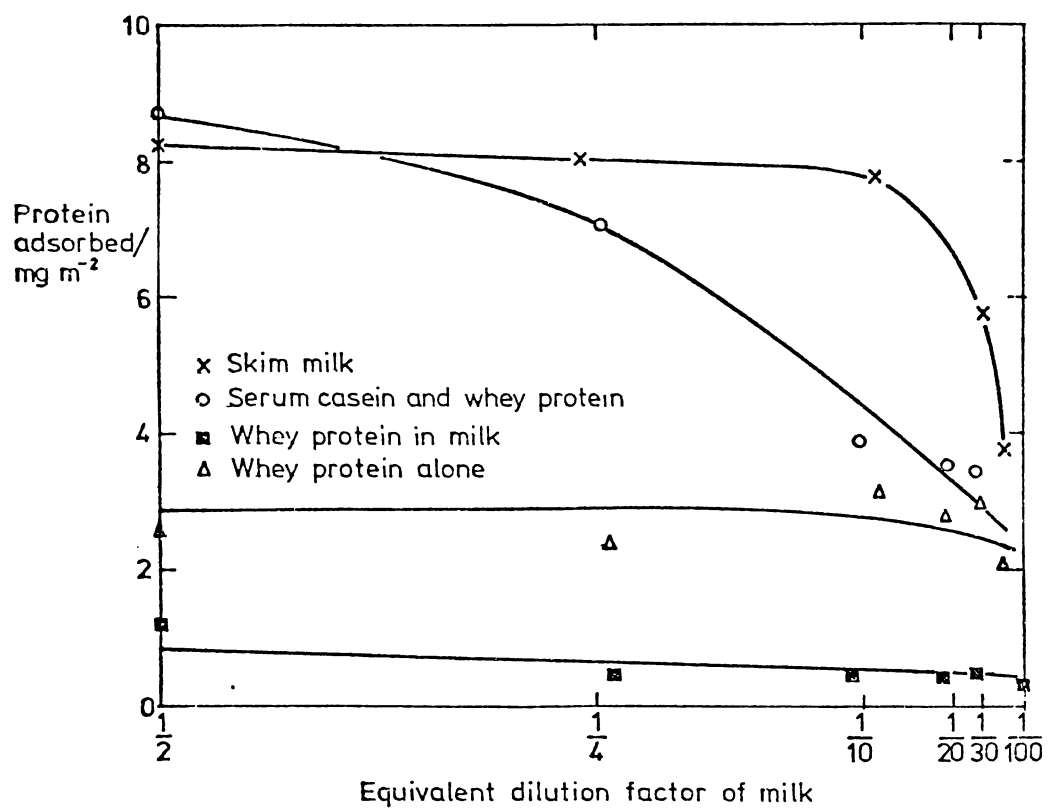
8.6 Discussion and Conclusion

The protein adsorption results described above, provided circumstantial evidence that the protein adsorbed when stainless steel came into contact with milk, was predominantly the casein constituents of the milk serum. The amount of protein adsorbed from skim milk was approximately 8.0 mg m^{-2} at saturation. This amount of protein could be adsorbed as scattered micelles, or as a monomolecular layer of protein molecules. The latter possibility seems more likely. This view is reinforced by the similar value for $\Gamma_{\text{saturation}}$ obtained from whole serum free of micelles. The fact that the adsorption of the whey protein constituent of serum is suppressed by the presence of the other serum constituents (mainly caseins) suggests that the serum caseins were adsorbed preferentially.

An interesting insight into the adsorption behaviour of milk upon dilution can be obtained by replotting the data obtained in this chapter according to the effective dilution factors i.e. where the concentrations are expressed as the fraction of their values in milk.

Figure 8.7

The effect of other milk constituents on the adsorption of whey protein and serum protein



It is apparent from figure 8.7 that 'saturation' adsorption at 8.0 mg m^{-2} is maintained at a much higher dilution factor in the case of adsorption from diluted skim milk than from diluted serum casein. This indicates that the micelle constituents may be playing some role in the adsorption effect. Given that it is unlikely that the micelles are adsorbed, it seems reasonable that the micelles act as a reservoir of caseins, that by dissociating from the micelles help to maintain the casein concentration of the serum. This would ensure 'saturation' adsorption over a wide range of dilutions.

CHAPTER 9

SOLID-LIQUID SURFACE CHEMISTRY OF THE MILK CASEINS

9.1 Introduction

The results of Chapter 8 indicated that the adsorption of caseins from milk serum was the most significant adsorption phenomenon occurring within the first few hours of contact of a clean stainless steel surface with skim milk. It was thus decided to study the solid-liquid surface chemistry of α_{s1} , β and κ -caseins at the stainless steel-water interface in more detail with the view of determining adsorption characteristics of the different caseins which might suggest factors involved in the mechanism of adsorption.

In preliminary experiments the adsorption of α_{s1} , β and κ -casein was studied over a concentration range of 0.01 to 1.6 mg ml⁻¹. At these concentrations the adsorption process was found to be very rapid. Detailed studies were made of the adsorption of β -casein and α_{s1} -casein. β -casein was studied because of its high affinity for stainless steel and α_{s1} -casein was included in similar studies because it is the major casein constituent of milk. The kinetics and thermodynamics of adsorption were of initial interest. In addition the effect of the variables : pH, ionic strength and temperature will provide further information on which to base models for the adsorption process. Casein adsorption effects at stainless steel-aqueous interface, apart from having relevance to milkstone formation, will also be of more general interest in providing additional data on the solid-liquid surface chemistry of proteins.

9.2 Material and Method

The procedure for the preparation of individual casein constituents was described in Chapter 3. The procedure for protein iodination used, was that described in Chapter 4.

Adsorption experiments were carried out using the 'slide technique' as discussed in Chapter 7. Protein concentrations were determined spectrophotometrically using extinction coefficient data given in Chapter 3.

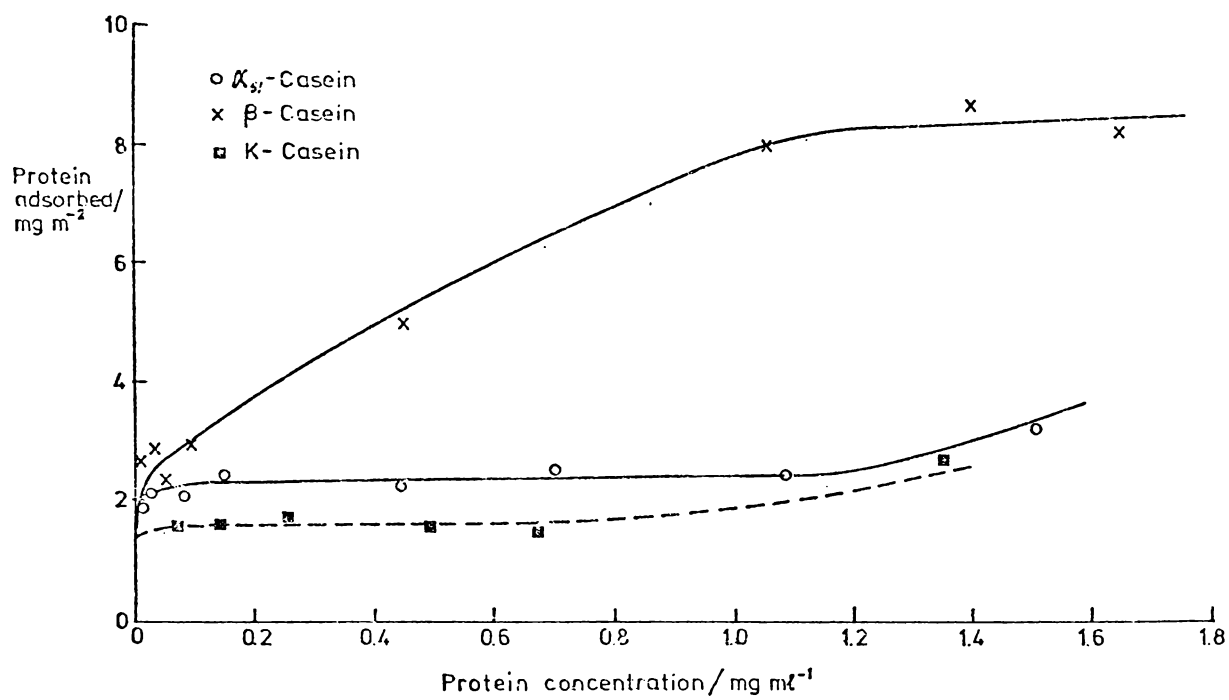
9.3 Preliminary Experiments

The adsorption of α_{s1} , β , and K caseins was studied as a function of concentration at 20 °C, pH 6.8 (sodium cacodylate buffer) and at an ionic strength of 0.06, (adjusted with NaCl). A standard adsorption time of forty minutes was employed for each adsorption.

The results are summarised in figure 9.1, where it is clear that β -casein is adsorbed to a significantly greater extent than either α_{s1} or K-casein, particularly at the higher protein concentrations investigated. There is little variation in the amount of α_{s1} and K-casein adsorbed over the concentration range studied. It appears that the surfaces are "saturated" with respect to the individual proteins at concentrations as low as 0.01 mg ml⁻¹ with approximately 2.5 mg m² of protein being adsorbed. The adsorption of β -casein is similar to that of α_{s1} -casein at the lower concentrations, but at higher protein concentrations the β -casein is adsorbed to a greater extent.

Figure 9.1

Adsorption Isotherms for adsorption of α_{s1} , β
and K-casein at high concentration



With β -casein, continued adsorption occurred until a maximum of about 8 mg m^{-2} at a concentration of 1.4 mg ml^{-1} .

In order to check that adsorption had reached completion in these experiments, a kinetic study was undertaken. The results of figure 9.2 show that in all cases, completion of adsorption was obtained after at most, a few minutes. In particular the data of figure 9.2 established that the continued adsorption of β -casein at the higher concentration was not a kinetically controlled effect.

From the preliminary experiments described above, the importance of β -casein adsorption in the formation of protein films at stainless steel-aqueous interfaces exposed to milk was indicated. It is of interest to note that the "saturation" coverages of surfaces exposed to high β -casein concentrations were approximately the same as those obtained due to adsorption from skim milk.

At this stage it was decided to extend the adsorption studies by investigating the kinetics and thermodynamics of the adsorption of β -casein (because of its high affinity for the surface) and α_{s1} -casein (because of its abundance in milk) over a greatly extended range of concentrations.

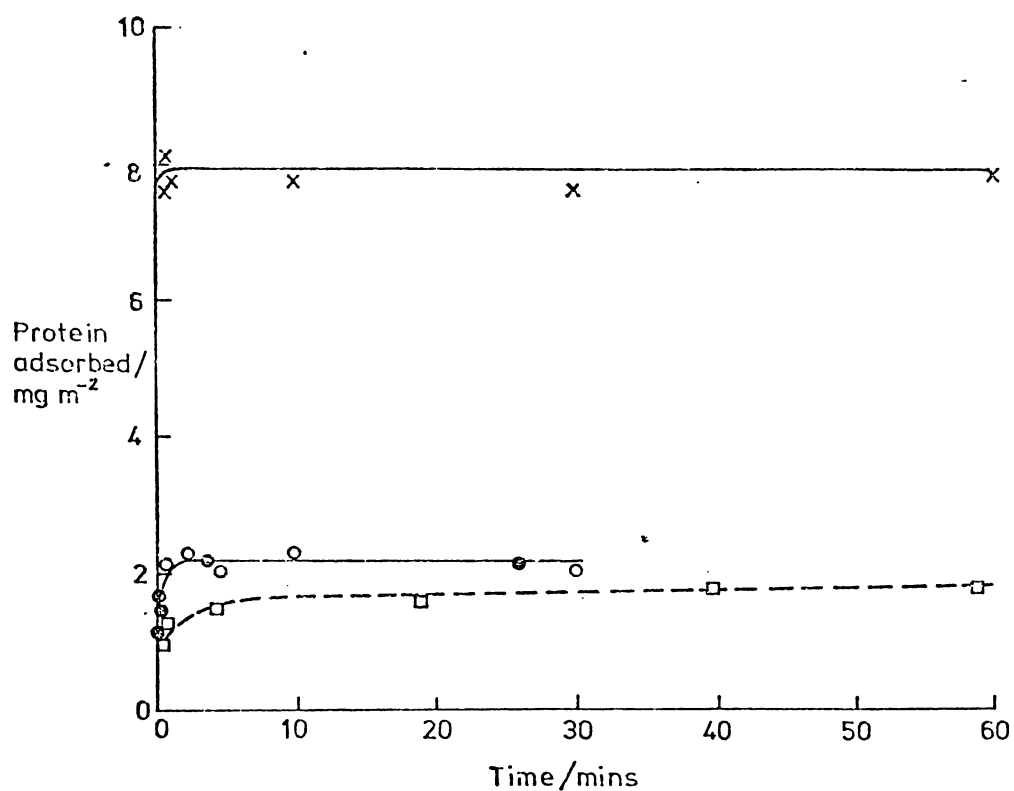
9.4 Kinetics and Thermodynamics of α_{s1} and β -casein adsorption

Adsorption Isotherms

Adsorption isotherms for α_{s1} and β -casein adsorption were determined for concentrations ranging over several orders of magnitude from 10^{-4} to 3 mg ml^{-1} . A constant adsorption time

Figure 9.2

Kinetics of Adsorption of α_{s1} , β , and K-casein
at high concentration



α_{s1} -casein concentration 0.84 mg ml⁻¹
 β -casein concentration 1.2 mg ml⁻¹
 β -casein concentration 0.05 mg ml⁻¹
 K-casein concentration 0.42 mg ml⁻¹

of 40 minutes was used in all of these experiments. Other experimental conditions were adjusted in such a way as to illustrate the effect of temperature and ionic strength on the amount of adsorption (see figures 9.3 and 9.4). The data are plotted on a semi-log scale in order that all the data can be included on a single plot. The shapes do not correspond to recognisable isotherms.

Amount Adsorbed

The semi-log plots reveal that in the case of β -casein, there are two concentration regions over which protein adsorption is independent of concentration. The lower concentration region extends from $6 \times 10^{-3} \text{ mg ml}^{-1}$ to $10^{-1} \text{ mg ml}^{-1}$ over which the surface appears to be saturated with approximately 2.8 mg m^{-2} of protein. Above 1 mg ml^{-1} a second plateau region is observed corresponding to a protein adsorption of approximately 8.4 mg m^{-2} .

In the case of α_{s1} -casein the first plateau occurs at concentrations above $7 \times 10^{-3} \text{ mg ml}^{-1}$ with protein adsorption of approximately 2.5 mg m^{-2} . The high concentration behaviour of α_{s1} -casein differs from that of β -casein. Above $7 \times 10^{-1} \text{ mg ml}^{-1}$ there is a slight increase in the amount of protein adsorbed but this is much less than the increased adsorption observed with β -casein at the equivalent concentration.

Effect of Temperature

Figures 9.3 and 9.4 show that over the temperature range of 4°C to 20°C the amount adsorbed did not vary significantly with temperature at the various solution concentrations. Studies at

Figure 9.3

Extended Adsorption Isotherm for adsorption of

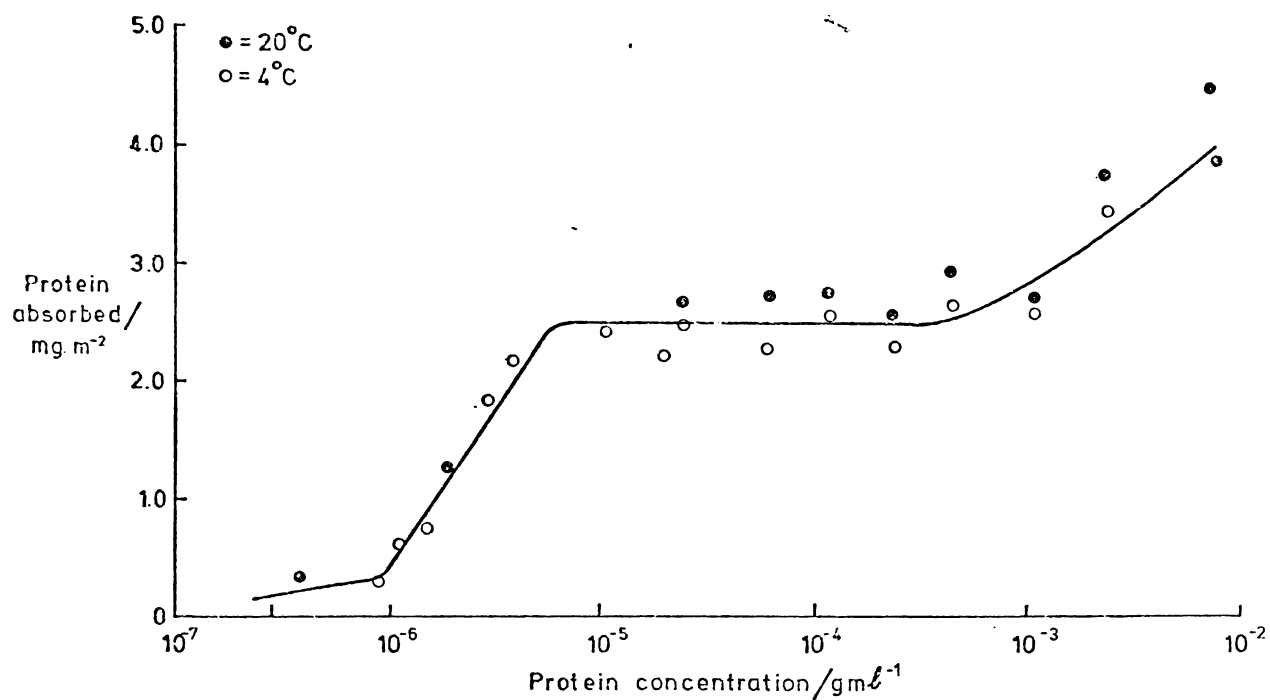
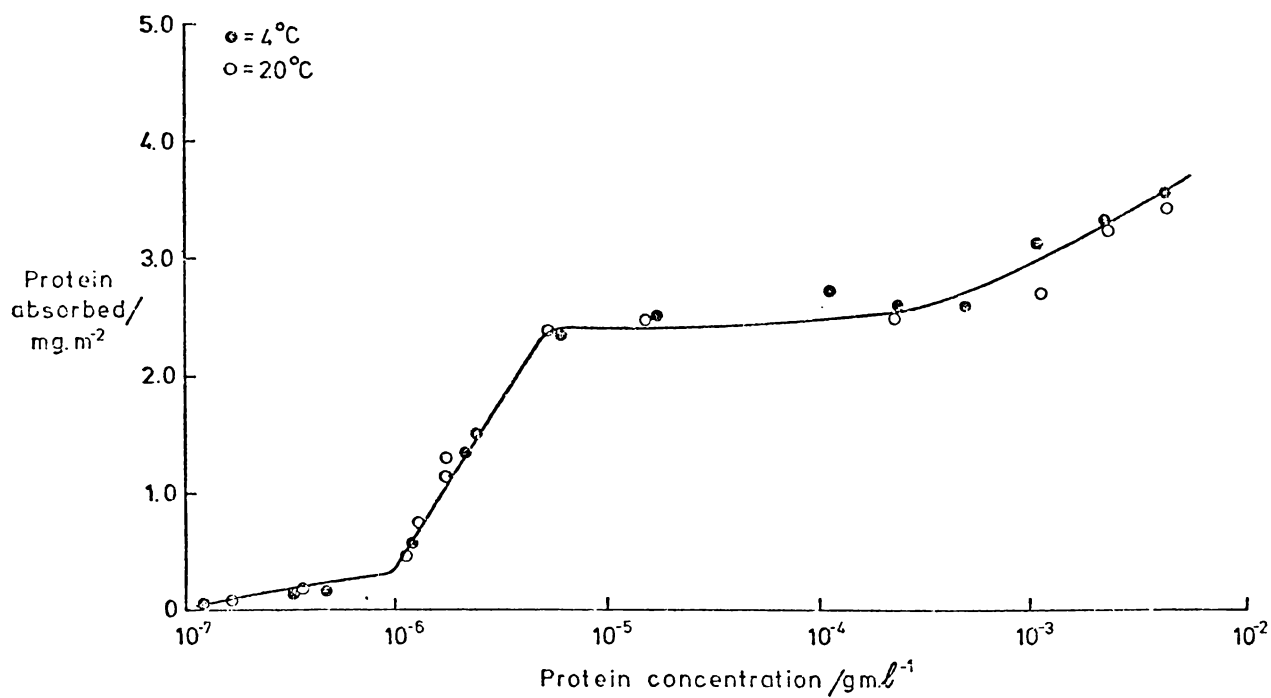
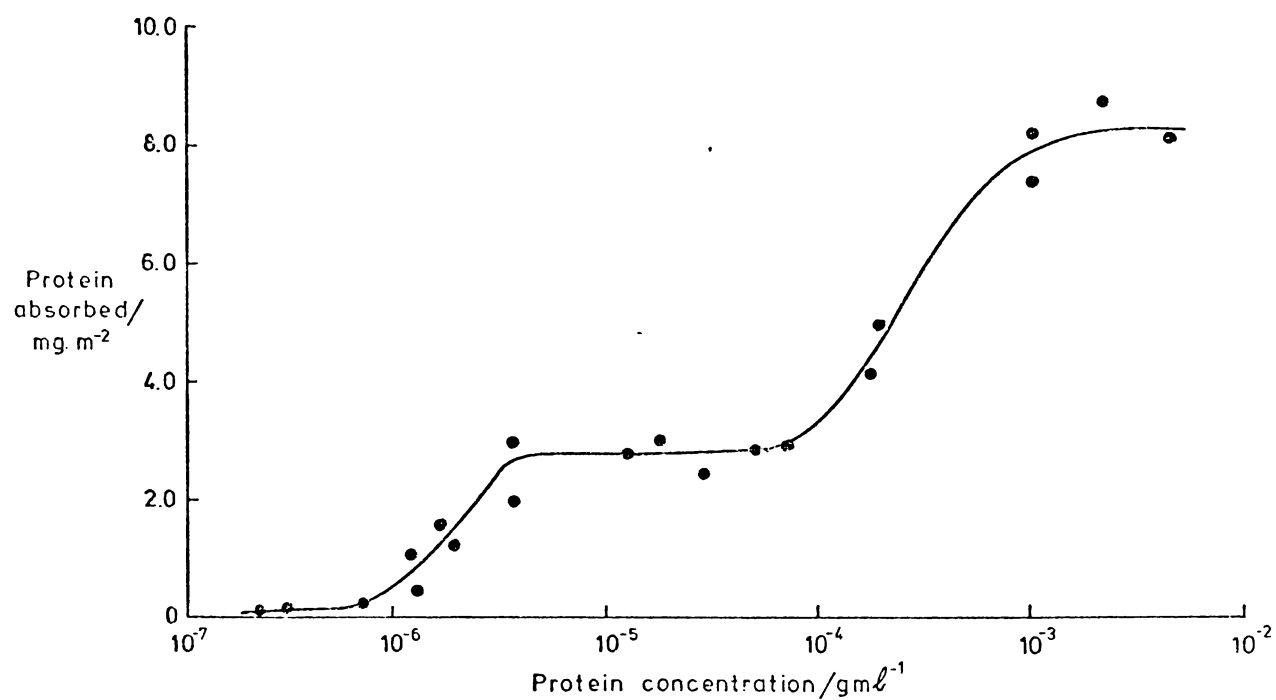
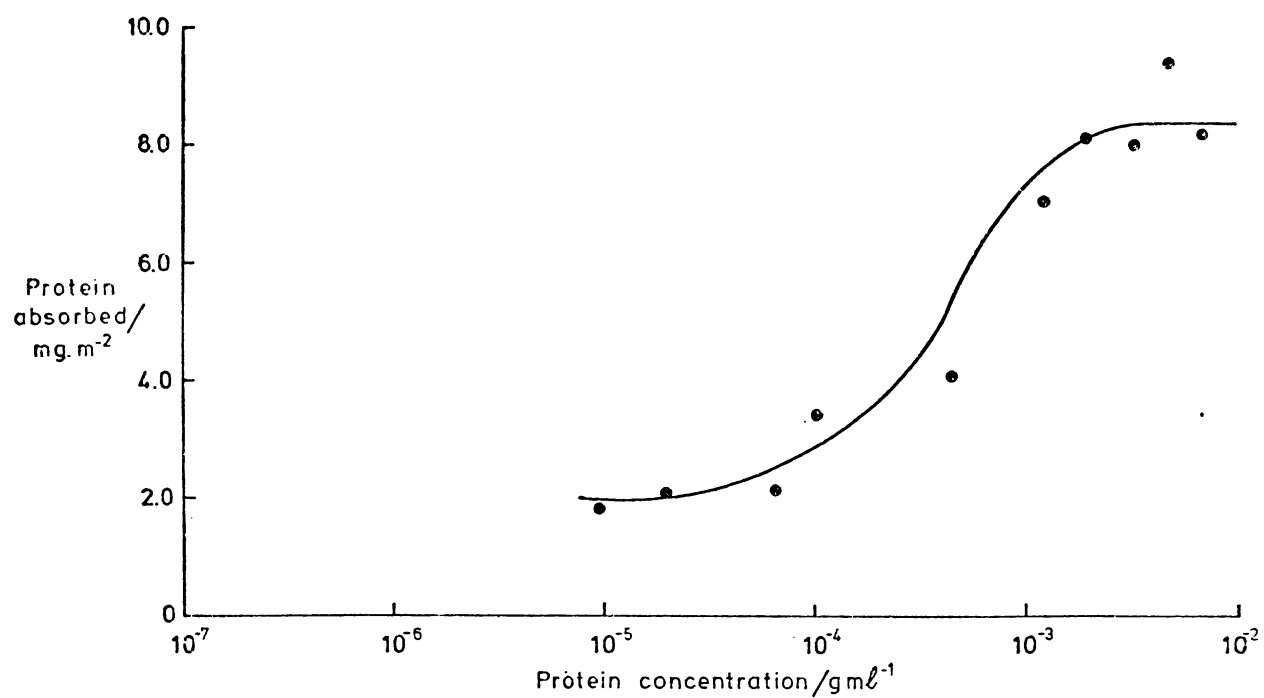
 α_{s1} caseina. $I = 0.02$, pH 6.8b. $I = 0.06$, pH 6.8

Figure 9.4

Extended Adsorption Isotherm for adsorption
of β -casein



a. $I = 0.02$, pH 6.8, 20°C



b. $I = 0.06$, pH 6.8, 4°C

temperatures greater than 20 °C were omitted because of evaporation and possible denaturation problems.

Effect of Ionic Strength

The figures 9.3 and 9.4 show that lowering the ionic strength from $I = 0.06$ to $I = 0.01$ has a negligible effect on the adsorption of α_{s1} - and β -casein. The effect of high ionic strengths on β -casein adsorption, however, suppresses the adsorption at low protein concentration but enhances the adsorption at the higher concentration (see figure 9.5). In fact at the higher protein concentrations, the amount adsorbed increased asymptotically with increasing concentration.

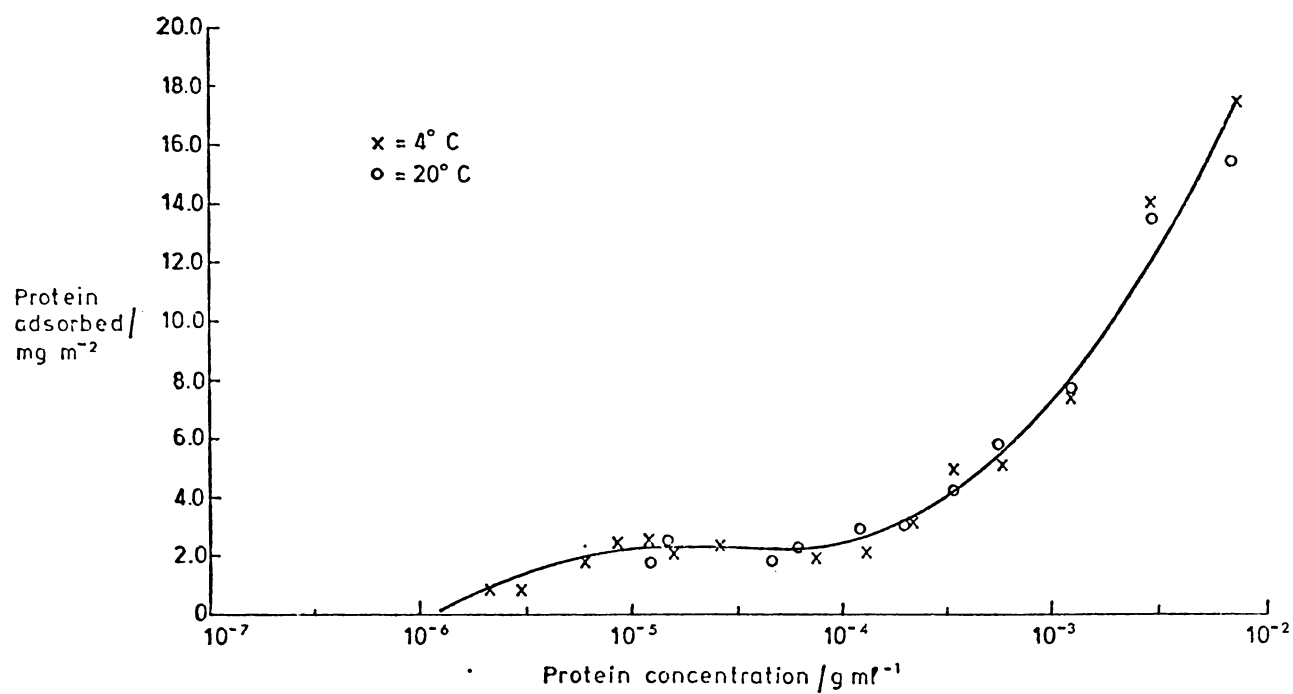
Effect of pH

The effect of pH on the adsorption of α_{s1} -casein at a concentration of 0.2 mg ml^{-1} is shown in figure 9.6. An initial slight increase in adsorption as the pH was lowered from pH 9 to pH 7.0 was followed by a steep increase in the vicinity of the isoelectric point as the pH was further lowered. At pH's below the isoelectric point down to pH 2.5, the amount adsorbed remained approximately constant at a maximum value of approximately 7.0 mg m^{-2} . Below pH 2.5 the amount adsorbed decreased rapidly.

A similar trend (see figure 9.6) was indicated by studies with β -casein at the much lower concentration of $5.4 \times 10^{-3} \text{ mg ml}^{-1}$. The increase in adsorption in the vicinity of the isoelectric point was again observed. At high concentrations of β -casein (3.5 mg ml^{-1}) increased adsorption in the vicinity of the isoelectric point was again observed. In this case precipitation of the casein

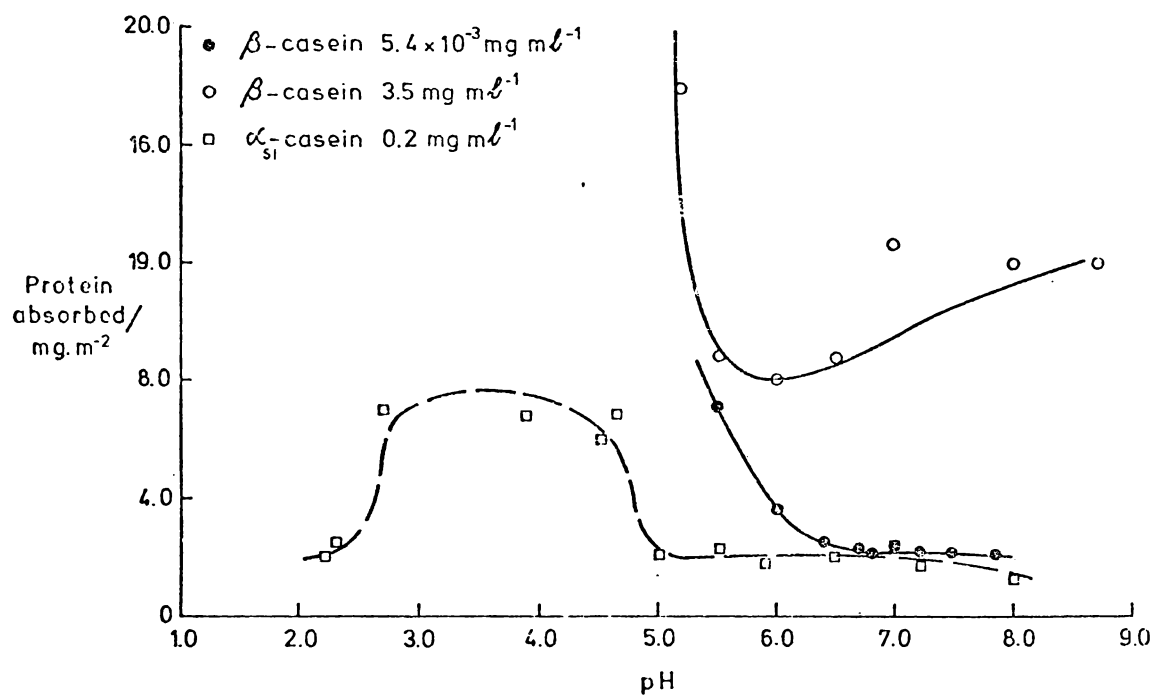
Figure 9.5

Extended Adsorption Isotherm for β -casein at
high ionic strength



$I = 1.2, \text{ pH } 6.8$

Figure 9.6

Effect of pH on α_{s1} and β -casein adsorption

from solution was also noticed. Thus the effect observed at the isoelectric point could well have been associated with precipitation.

Competitive Effect

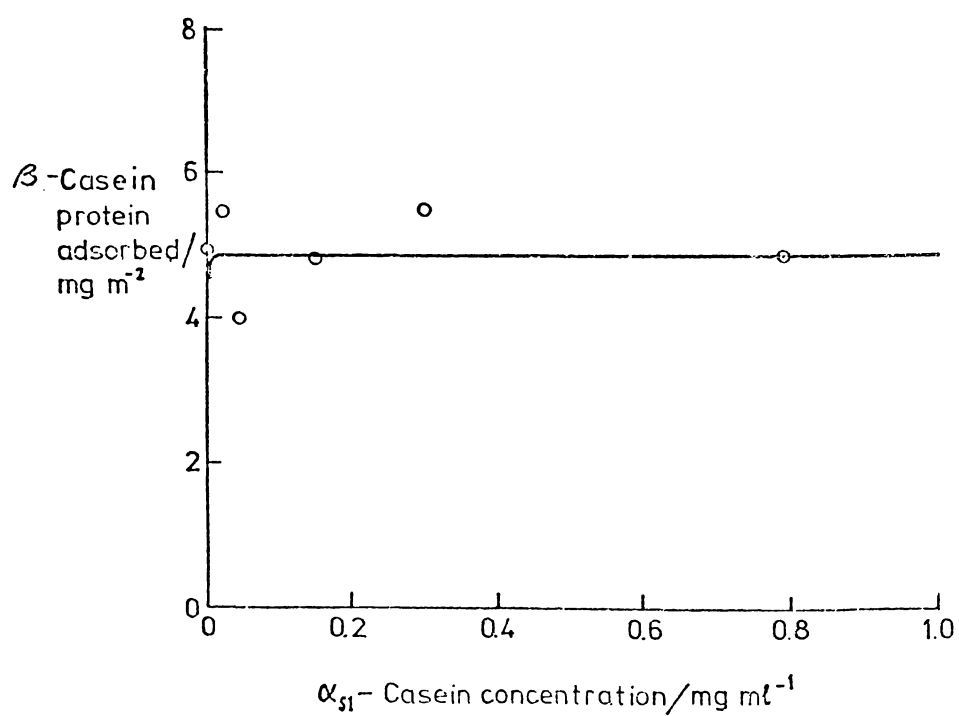
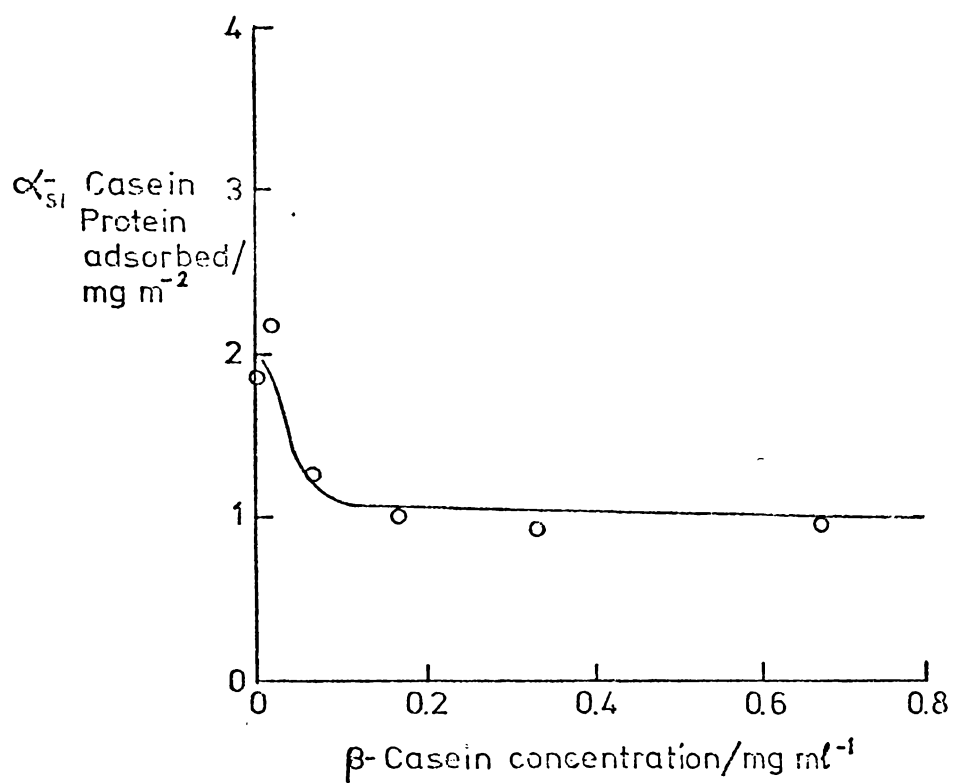
The adsorption of mixtures of α_{s1} and β -casein were studied by monitoring the adsorption of one of these proteins in various concentrations of the other protein (see figure 9.7). It was observed that in studying the adsorption of β -casein at 0.3 mg ml^{-1} concentration in the presence of various amounts of α_{s1} -casein, there was no significant difference in the amount of β -casein adsorbed. In monitoring the amount of α_{s1} -casein adsorbed at 0.3 mg ml^{-1} concentration, with various amounts of β -casein, it was observed that the initial α_{s1} -casein adsorption of about 2.3 mg m^{-2} dropped rapidly to less than 1.0 mg m^{-2} on increasing the concentration of β -casein above 0.2 mg ml^{-1} . This result indicated that above this concentration, β -casein was being adsorbed in preference to α_{s1} -casein.

The Kinetics of Adsorption

Preliminary experiments were carried out to determine the adsorption time required to reach "maximum" adsorption. The concentrations used in these experiments, although quite low ($8.4 \times 10^{-2} \text{ mg ml}^{-1}$), were considerably higher than some of the concentrations used in the extended adsorption isotherm experiments. Thus it was necessary to check that the standard adsorption time of forty-five minutes used in these experiments was in fact adequate to allow maximum adsorption at the lowest concentration. A detailed kinetic study over the range of concentrations from

Figure 9.7

Adsorption from mixed solutions of α_{s1} and β -casein



$1 \times 10^{-2} \text{ mg ml}^{-1}$ to $9 \times 10^{-4} \text{ mg ml}^{-1}$ was initiated. The adsorption curves (plots of Γ versus t) are shown in figure 9.8 for α_{s1} - and β -casein. From these plots it is clear that maximum adsorption was not obtained after forty-five minutes for concentrations below about $1.1 \times 10^{-2} \text{ mg ml}^{-1}$. Hence the decrease in the adsorption isotherms data at concentrations below this value as shown in figures 9.4 and 9.5 reflect the kinetics rather than the thermodynamics of adsorption. Given sufficient time and an adequate volume of solution, it appears that even at the lowest concentrations, an essentially constant amount of protein would be adsorbed at all α_{s1} - and β -casein concentrations below approximately $6 \times 10^{-3} \text{ mg ml}^{-1}$.

Analysis of Adsorption Curves

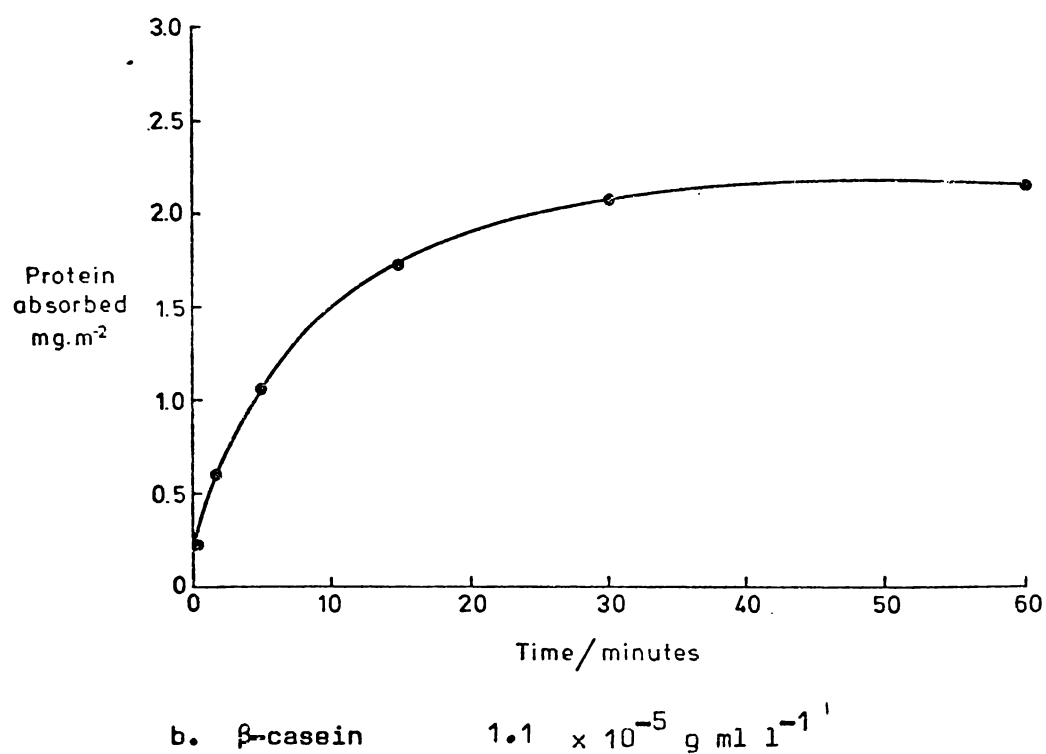
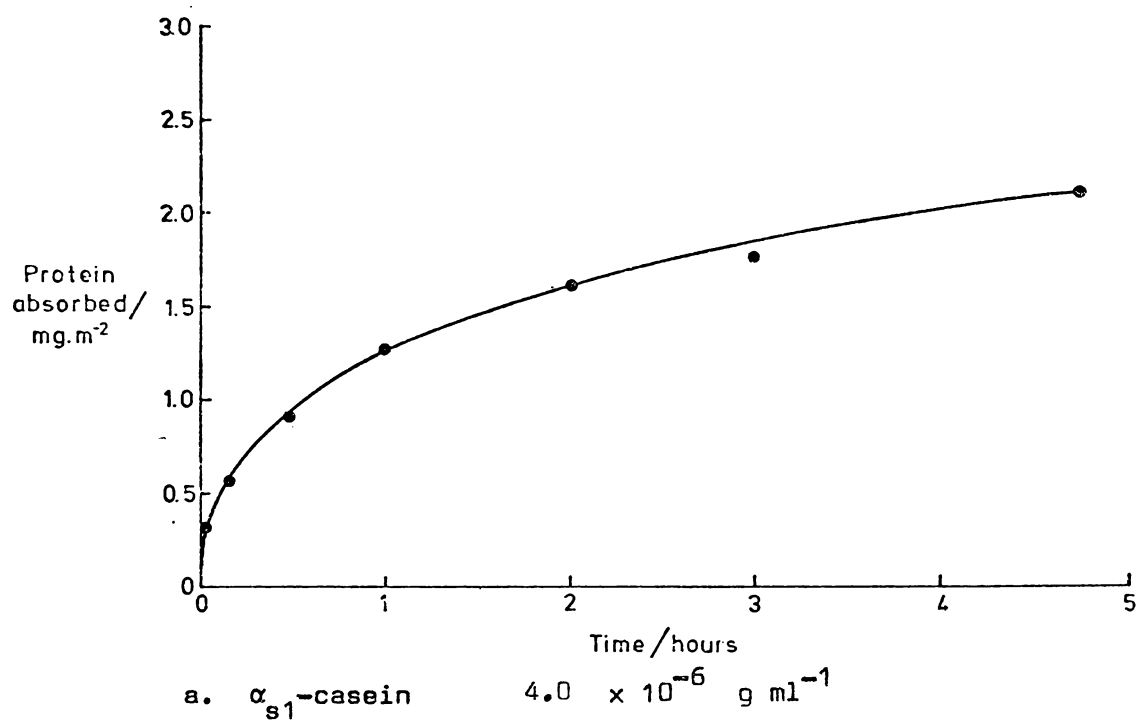
For processes involving a solid-liquid interface the overall rate could be limited by any one (or more) of the following steps: (see Chapter 2:)

1. diffusion of the substrate to the interface;
2. adsorption at the interface;
3. reaction of adsorbed species at the interface;
4. desorption of reaction products;
5. diffusion of products away from interface.

In the case of protein adsorption on to clean stainless steel the two most likely rate limiting steps are diffusion to the interface and adsorption at the interface. It is possible that the adsorption step could be limited by the desorption of a species already occupying surface sites though this is not particularly

Figure 9.8

Kinetics of Adsorption of α_{s1} and β -casein
at low concentrations



likely. Assuming a simple first order adsorption process, the kinetics of adsorption should be exponential. If diffusion was rate limiting, then the amount adsorbed could be expected to vary with the square of time at least during the initial stages of adsorption. This is a standard test for diffusion controlled processes.

Plots of log (amount adsorbed) versus time, and amount adsorbed versus the square root of time are shown in figure

9.9 for both α_{s1} - and β -casein. The best fit of the data was obtained when the amount adsorbed is plotted against the square root of time suggesting that the rate of adsorption at low concentrations of protein was diffusion controlled (see Figure 9.10).

9.5 Discussion

1. Kinetics of Adsorption - The Importance of Diffusion

The solution of Fick's first Law for diffusion from an unstirred liquid solution to an interface is:

$$\Gamma = 2C \sqrt{\frac{Dt}{\pi}}$$

where Γ = amount adsorbed

C = solution concentration

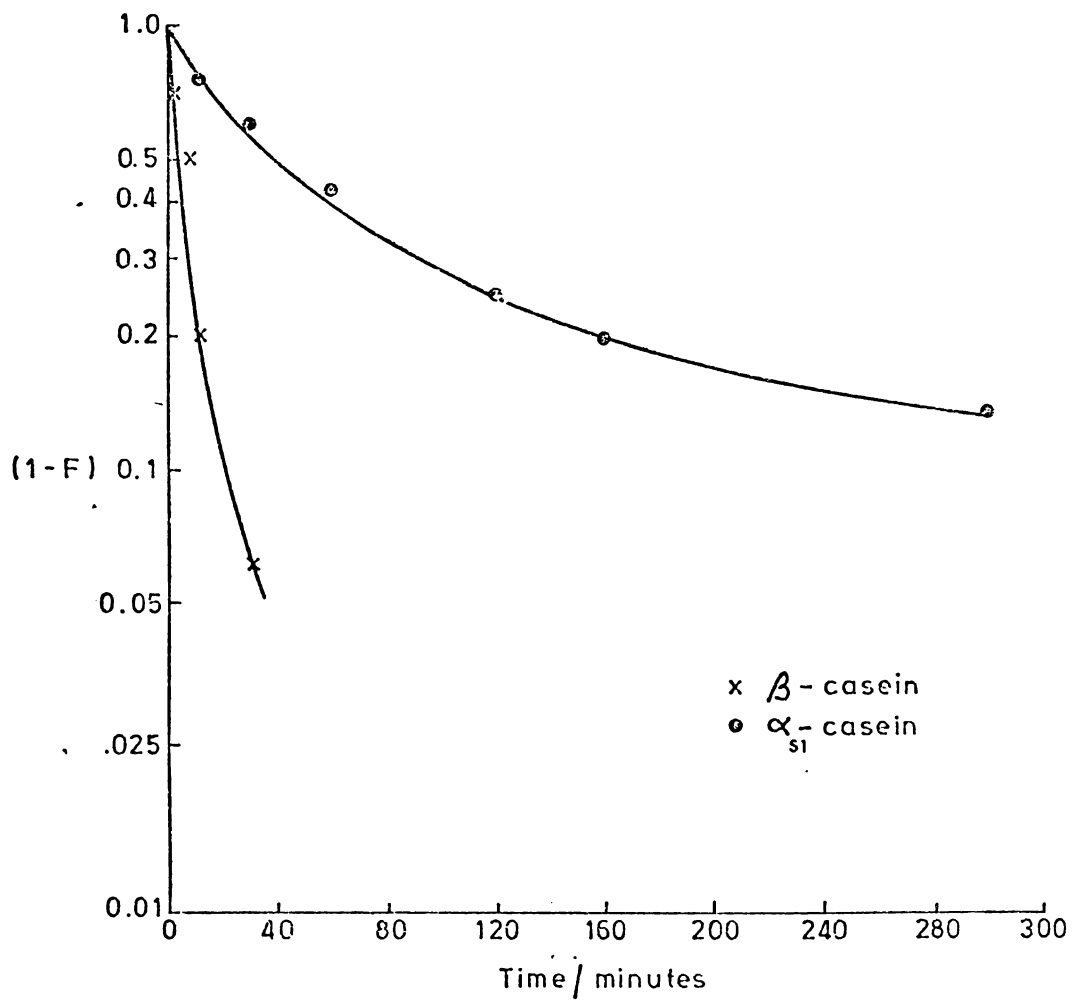
D = Diffusion coefficient

t = amount adsorbed in time t

This equation was first used by Ward and Tordai (496) to describe the rate of arrival of molecules at an air-water interface in the absence of stirring, desorption, or an energy barrier.

Figure 9.9

Logarithmic plot of data for adsorption kinetics



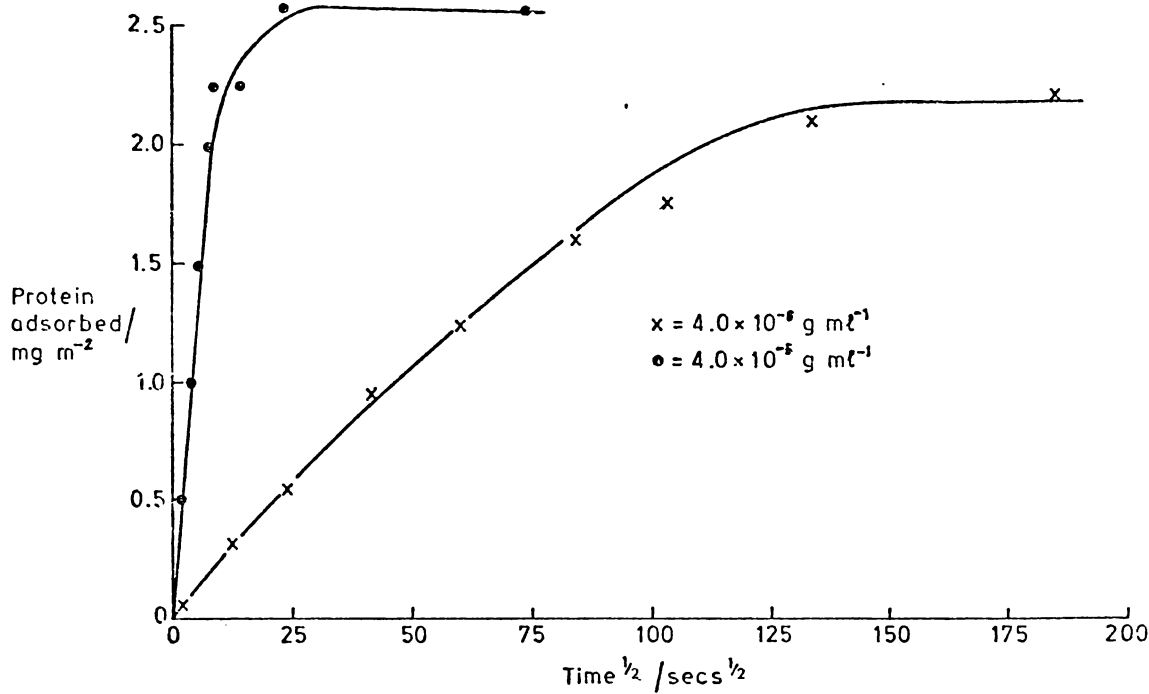
$$F = \frac{\text{Count-rate at } t = t}{\text{Count-rate at } t = 00}$$

$$\beta\text{-casein} = 1.1 \times 10^{-5} \text{ g ml}^{-1}$$

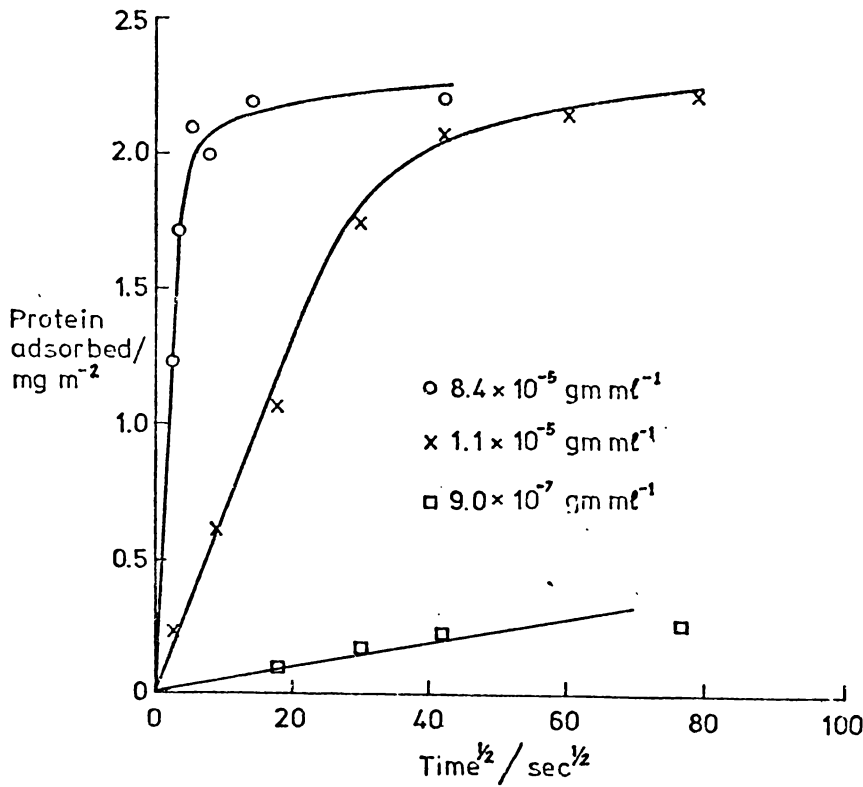
$$\alpha_{s1}\text{-casein} = 4.0 \times 10^{-6} \text{ g ml}^{-1}$$

Figure 9.10

Square-root plot of data for adsorption kinetics



a. α_{s1} -casein



b. β -casein

Rearrangement of this equation allows the diffusion coefficient to be estimated as follows:

$$D = \frac{\pi}{4 C^2} \left(\frac{\Gamma}{t^{\frac{1}{2}}} \right)^2$$

where $\frac{\Gamma}{t^{\frac{1}{2}}}$ is the slope obtained from the plot of Γ against $t^{\frac{1}{2}}$.

Using this equation values (Table 9.1) for the diffusion coefficients of α_{s1} - and β -casein were calculated.

Literature data for the diffusion coefficients of caseins are sparse. No values have been reported for α_{s1} -casein. The value for α -casein, a mixture of α_{s1} -casein and K-casein, was found to be $2.90 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ (497) at pH 7.8 and ionic strength of 0.1. The value for α_s -casein at pH 7.08 and 0.1 mol l^{-1} KCl of $3.7 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ (498) was reported.

Table 9.1

Diffusion Coefficient of α_{s1} - and β -casein

Protein	Concentration g ml^{-1}	D_4 (Expt) $\text{cm}^2 \text{ sec}^{-1}$	D_{20} (Literature) $\text{cm}^2 \text{ sec}^{-1}$	D_4 (Cal. Literature) $\text{cm}^2 \text{ sec}^{-1}$
α_{s1} -casein	4×10^{-6}	2.1×10^{-7}	2.90×10^{-7} (497) (α casein)	1.7×10^{-7}
			3.7×10^{-7} (498) (α casein)	2.2×10^{-7}
β -casein	4×10^{-5}	1.9×10^{-7}		
	9.0×10^{-7}	3.5×10^{-7}	6.05×10^{-7} (497)	3.63×10^{-7}
	1.1×10^{-5}	2.6×10^{-7}		
	8.4×10^{-5}	1.9×10^{-7}		

All the literature values for the diffusion coefficients were reported at 20 °C. In order to compare these data with the experimental results they were adjusted to 4 °C using the following equation (499):

$$D_{T1} = D_{T2} \frac{T_1}{T_2} \frac{\eta_{T_2}}{\eta_{T_1}}$$

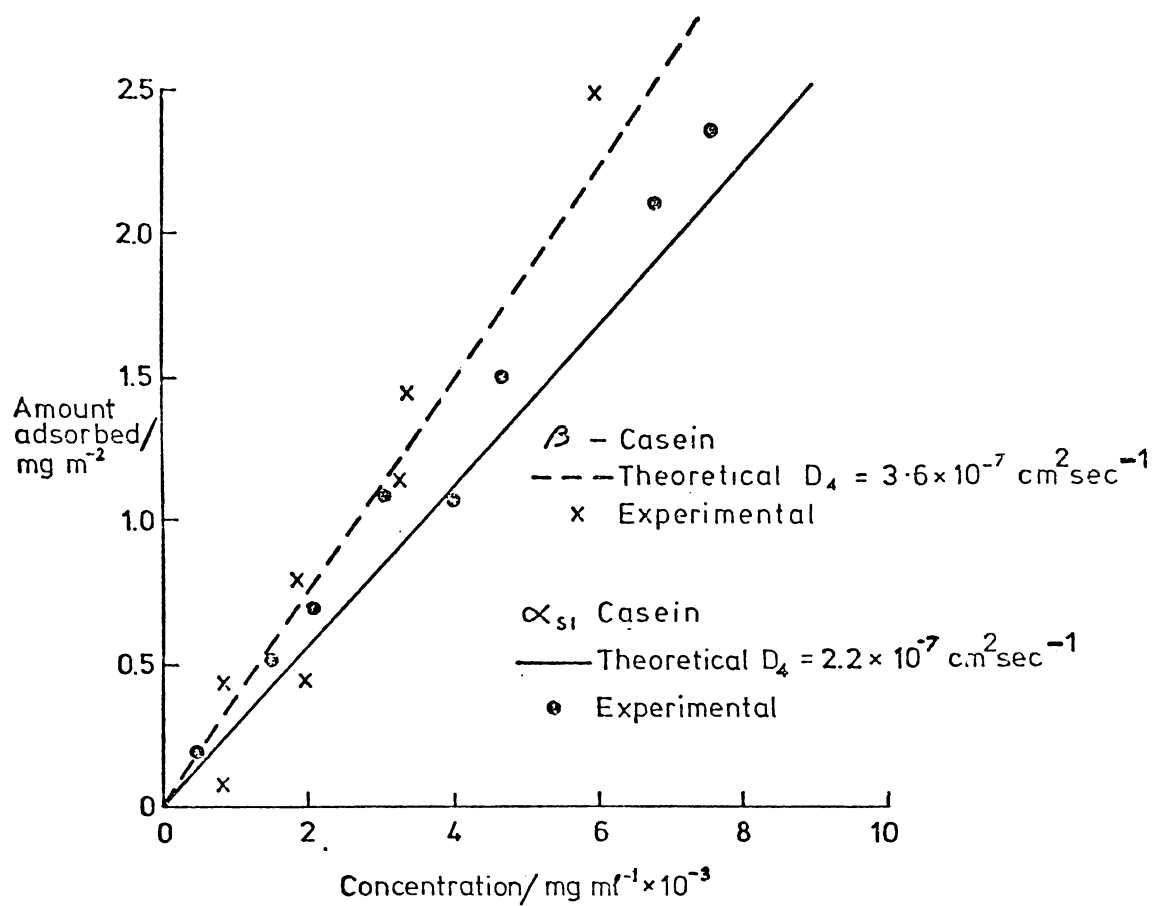
where η_T is the viscosity of the solvent at the temperature T. (For water $\eta_{25} = 0.8904$ cp, $\eta_{20} = 1.002$ cp, $\eta_4 = 1.567$ cp (500)). The adjusted data are tabulated in the last column of Table 9.1. The experimental data and the adjusted literature data agree within the experimental error.

Further evidence for the diffusion control of adsorption at low concentration is provided by the adsorption isotherm data of figures 9.11. In these adsorption experiments, a constant adsorption time of forty-five minutes was used in all cases. At concentrations below about 6×10^{-3} mg ml⁻¹, this time was inadequate to allow maximum adsorption to occur for either α_{s1} - or β -casein. By assuming diffusion control and using the adjusted literature value of the diffusion coefficient at 4 °C given in Table 9.1, the amount of protein adsorbed at various solution concentrations was calculated. A comparison of the calculated value with the experimentally determined amount is also shown on figure 9.11. Again a diffusion model gives a very good fit of the experimental data.

The apparent fit of all low concentration adsorption data to a diffusion mechanism could possibly have been due to the fortuitous cancelling of opposing effects: convection causing

Figure 9.11

Effect of concentration on the amount adsorbed after
a fixed adsorption time



an increase in the rate of adsorption and of depletion of the solution due to adsorption on to the walls of the container causing a decrease in the rate of adsorption.

The possibility of convection was eliminated by an experiment in which a few crystals of potassium permanganate were placed in the bottom of the adsorption vessel containing 1 ml of distilled water and a stainless steel slide. Other conditions were exactly the same as those used in the adsorption studies. The diffusion of the permanganate from the crystals were observed for 48 hours. There appeared to be no evidence for convection.

The adsorption of protein from dilute solution on to the glass walls of the vial was also investigated. Only in the case of the very dilute solutions and then only after a very long time was there any appreciable depletion of the solution by adsorption on to the glass. In any case the plot of Γ versus $t^{\frac{1}{2}}$ was fitted to the data at relatively short periods of time. Hence solution depletion due to adsorption on to glass would have had minimal effect on the estimation of the initial slope and hence the diffusion coefficient.

An important conclusion that can be drawn from the kinetic studies at low protein concentration is that protein adsorption appears to be independent of protein concentration. Provided sufficient time is allowed, a monolayer of protein is adsorbed from even the most dilute protein solution. Previous investigations that have shown a decrease in adsorption at low concentration and typical Langmuir type isotherms must therefore

be regarded with caution. It seems possible that the fall-off at low concentration could have been due to insufficient time being allowed for maximum adsorption to be attained.

2. The Structure of the Adsorbed Protein Layer

(a) Low Concentrations

A simple explanation for the amount of protein adsorbed over the wide range of concentrations studied, could be that protein adsorption at the stainless steel-water interface proceeds until the surface becomes covered with a single layer of protein molecules and that the adsorption process is irreversible. The amount of protein adsorbed on the surface layer could be expected to reflect the effective surface area of the interface and the area occupied per protein molecule.

The effective surface area of the 2B stainless steel surfaces used in the present work has been estimated to be 1.7 times the geometric area (491). Although this value is difficult to check absolutely, such a value seems reasonable and the agreement observed between results from gas adsorption (495) and serum albumin adsorption (491) allows this value to be used with some confidence.

Literature data on the size and shape of casein molecules have been obtained mainly from viscosity studies and studies of protein adsorption at the air-water interface. β -casein, for example, has been reported to be a flexible coil linear polymer of dimensions $13 \text{ nm} \times 2 \text{ nm}$ with cross section areas of 20.41 nm^2 (long axis) by 3.14 nm^2 (short axis) (206). α_{s1} -casein, because of its similar molecular weight and amino acid sequence, could

be expected to have very similar structural properties. A monomer model of α_{s1} -casein was reported as a compact body about 10 nm x 2.5 nm. (464).

Adsorption of caseins at the air-water interface is thought to be accompanied by the unfolding of the polymer chain. From the amount adsorbed it has been calculated that each amino acid residue of non polar proteins such as insulin and albumin (and also presumably the caseins) occupies an area of 0.17 nm^2 (129). An unfolded α_{s1} - or β -casein could thus be expected to occupy an area of 33.8 nm^2 or 35.5 nm^2 respectively. If the protein was predominantly polar in nature then only partially unfolding is reported to occur and this would occupy an area of 0.04 nm^2 per amino acid. For α_{s1} - and β -casein this would correspond to 7.96 nm^2 and 8.36 nm^2 respectively. The area calculated for each of the spherical coils was obtained from the radius calculated from the literature data for partial specific volumes. From the partial specific volumes : 0.728 ml g^{-1} , 0.739 ml g^{-1} , and 0.736 ml g^{-1} for α_{s1} -, β , and K-casein respectively (53), the following areas: 11.26 nm^2 , 11.60 nm^2 and 9.6 nm^2 were calculated.

Working on the assumption of a roughness factor of 1.7 and using the cross sectional areas given above, estimates of the amount of adsorption can be calculated according to the different possible surface configurations of the adsorbed protein. These are summarized in Table 9.2, where the experimental data are also included.

Table 9.2

Molecular Configuration of α_{s1} , β and K-casein

Protein	Unfolded coil		Flexible coil		Spherical coil		Folded coil		Experimental
	nm ²	mg m ⁻²	nm ²	mg m ⁻²	nm ²	mg m ⁻²	nm ²	mg m ⁻²	
α_{s1} -casein	33.8	2.0	19.6	3.4	11.2	5.9	7.9	8.4	2.4
β -casein	35.5	1.9	20.4	3.3	11.6	5.8	8.3	8.1	2.8
K-casein	27.7	1.7	-	-	9.6	5.4	6.7	7.7	1.6

The amounts adsorbed were calculated on the assumption that the molecules deformed to cover all of the available area. If the molecules were taken to be rigid spheres or spheroids in close packed arrangements, the calculated amount of adsorption could be expected to be slightly reduced. For example with β -casein the close packed sphere value is reduced from 5.8 mg m⁻² to 4.9 mg m⁻² and for the flexible coil spheroid the value of 3.31 mg m⁻² is reduced to 2.9 mg m⁻².

Although the errors in the experimental data and the experimental error in the value for the roughness factor make it impossible to come to any firm conclusion about the mode of packing, the results of these calculations are consistent with little change in the flexible coils on adsorption.

(b) High Concentrations

At concentrations greater than 0.6 mg ml⁻¹ for α_{s1} -casein and 0.1 mg ml⁻¹ for β -casein, increased protein adsorption on to the 'saturated' surface was observed. The explanation for this behaviour is not clear. In the case of β -casein the increased adsorption reached a maximum of approximately 8.2 mg m⁻² at which

no further increase in adsorption with increasing protein concentration was observed. Although α_{s1} -casein exhibited a similar trend no maximum was obtained at the highest concentration used in these studies. This result is consistent with the previously observed greater affinity of β -casein for the stainless steel surface.

The amount adsorbed at high concentration could reflect a change in the conformation of the adsorbed protein film. The higher concentration could conceivably induce folding of the protein molecule or end-on rather than side-on adsorption. Calculated amounts of adsorption based upon these adsorption configurations for β -casein are given in Table 9.3.

Table 9.3

Molecular Configuration of β -casein at high
Protein Concentration (1 mg ml^{-1})

	Folded Coil	End-on Coil	Experimental
β -casein	8.1 mg m^{-2}	21.5 mg m^{-2}	8.3 mg m^{-2}

The agreement of the folded coil calculation with the experimental result could be fortuitous. If folding of β -casein occurs at high concentration then it would be expected that the hydrophobic groups would be innermost, exposing a surface that consisted predominantly of hydrophilic groups. Such a species could interact with surfaces only by weak and reversible hydrophilic interactions (see Section 2.7). There was no experimental evidence that protein adsorbed at high concentration was any less strongly held than that adsorbed at low concentration.

This is evidence against the folded coil model for adsorption at high concentration.

Another explanation for the adsorption data at high concentration could be based upon the displacement of segments of the protein molecules already adsorbed. It is well known that casein has detergent properties. It therefore seems possible that provided the concentrations are high enough, the hydrophobic parts of the solution casein molecules could displace the more hydrophilic parts of the adsorbed molecule, without causing desorption. Thus, more than one molecule could occupy a space on a surface previously occupied by only one molecule. In order to account for the amount adsorbed at high concentration, it would be necessary to envisage two solution protein molecules being involved with each partial displacement of an adsorbed molecule so that one third of the effective surface area of the protein remained in contact with the surface. The partial displacement could be from one end of the molecule (β -casein has one end that is mainly hydrophilic and would be expected to be more readily displaced) or it could occur through the buckling of the molecule. Unfortunately it does not appear possible to test this model by any simple means.

One further model that may explain the adsorption data could be based upon the observation that caseins polymerise at high concentrations (see Section 2.5). Therefore at high concentrations, the increased amount of adsorption could be a result of the adsorption of polymerised aggregates or formation of a polymerised surface film. In solution, the polymerisation

reactions are generally reversible, and therefore it could be expected that the additional adsorbed protein would be readily desorbed into distilled water. This result was not observed. If polymerisation does occur, the influence of the surface must be to make the process irreversible.

3. Effect of Experimental Conditions on the Protein Film

(a) Temperature

As previously discussed, temperature variation over the range 4 °C to 20 °C had little effect on the amount of protein adsorbed indicating that enthalpy effects were probably negligible. This result is similar to those obtained with other protein systems at solid-liquid and solid-gas interfaces (see Section 2.7). At temperatures greater than 50 °C significant changes in the adsorbed film can be induced. Such effects are discussed in Chapter 12.

(b) Ionic Strength

The effect of increased ionic strength was to slightly inhibit monolayer adsorption at low protein concentration but to significantly enhance the "multilayer" adsorption of β -casein at high protein concentration.

The effect at low concentrations can be most readily explained in terms of the effect of ionic strength on the size of the protein molecules. Although casein molecules are largely unfolded, the tertiary structure they possess is determined partially by electrostatic interactions between oppositely charged residues. Increased ionic strength will diminish such

interaction and lead to further unfolding. The molecule, being larger, would be expected to diffuse more slowly and the adsorbed species would occupy more surface area. Thus less adsorption over the "monolayer" region and slower adsorption below the "threshold" concentration for the time scale of the adsorption experiment would be expected. The "threshold" concentration, i.e. the lowest concentration for a given adsorption time at which a "monolayer" is formed in the adsorption experiments would be expected to be raised as the ionic strength was increased. At high protein concentrations increased ionic strength results in polymerisation and aggregation and may lead to precipitation through "salting out" effects. The enhanced adsorption caused by high ionic strengths probably reflects these phenomena. It seems unlikely that the result could be a molecular size effect as all evidence indicates that molecular size increases with ionic strength.

(c) pH

In section 9.3 the maximum adsorption observed occurred in the vicinity of the isoelectric point and was attributed to precipitation effects. The precipitation of caseins at the isoelectric point results from aggregation of the molecules that occurs when their stabilising charge has been neutralised. At pH's slightly displaced from the isoelectric point, the formation of stable polymeric species could account for the enhanced adsorption observed.

Unfortunately there is no information on the effect of pH on casein molecule size. However extrapolating from the data

for serum albumin in which the size decreases with pH through the isoelectric point (501) it does seem possible to interpret the increased adsorption in terms of decreased size.

4. Competitive Effects

In a mixture of α_{s1} - and β -casein the rate of diffusion of the individual protein to the surface and the strength of the bonding between the stainless steel and the adsorbed protein could both contribute to the competitive effects observed. As β -casein has a higher diffusion coefficient than α_{s1} -casein, a solution containing an equal concentration of both these proteins (0.3 mg ml - see figure 9.7) would result in the preferential adsorption of β -casein. If more α_{s1} -casein reached stainless steel because of a higher α_{s1} -casein concentration, β -casein may still be able to replace the adsorbed α_{s1} -casein due to its higher hydrophobicity (see Table 2.4). The preferential adsorption of β -casein in the presence of an excess of α_{s1} -casein indicated that such replacement must have been occurring (see figure 9.7).

5. A Limiting Case Model for Protein Adsorption

Although the data for high protein concentrations, high ionic strengths and pH's near the isoelectric point indicate that protein adsorption, under these conditions, is a complex process which possibly involves aggregation, polymerisation or precipitation effects, the data at low concentrations and pH's away from the isoelectric point can be explained quite well by a limiting case model. This model assumes that the rate of adsorption (from

unstirred solution) is determined solely by the rate of diffusion of the protein to the interface and the maximum amount of adsorption after "infinite" adsorption time is controlled solely by the close packing of protein molecules at the interface. Since both the diffusion coefficient and the number of molecules that can be packed on to an interface are controlled by size and shape alone, this model provides an interpretation of the rate of adsorption and the amounts of adsorption in terms of these molecular parameters. Thus factors that reduce the size of the molecule should increase the rate of adsorption and also the amount adsorbed and vice versa for factors that cause an increase in size. In general the mobility of protein molecules decreases as the axial ratio increases. Thus factors that cause unfolding of the protein chain can be expected to decrease the rate of adsorption and also decrease the amount adsorbed at infinite time.

It is possible to express these ideas quantitatively. The influence of size, shape and hydration on protein mobility has been extensively studied. The diffusion coefficient of a large spherical particle moving in a medium of small particles was derived from Stokes Law by Sutherland (502) and is:

$$D_{\text{Stokes}} = \frac{RT}{6\pi r\eta N} \dots\dots\dots 9.1$$

where R = ideal gas constant
 T = temperature in degrees absolute
 r = radius of the diffusing molecule
 η = viscosity of the solvent
 N = Avogadro's number

Similarly the equation for the frictional coefficient is given by:

$$f = 6\pi r \eta$$

and therefore the diffusion coefficient is inversely proportional to the frictional coefficient. Assuming a molecule to be spherical, its radius can be obtained from the partial specific volume $\bar{V}$ which is the increment in volume when one gram of dry protein is added to a large amount of solvent:

$$\frac{1}{r} = \left(\frac{4\pi N}{3M\bar{V}} \right)^{1/3} \dots\dots\dots 9.2$$

where M is the molecular weight of the protein.

The diffusion coefficients calculated using this value of r (and assuming spherical molecules) are obtained from the equation:

$$D_{\text{Stokes}} = \frac{RT}{N6\pi\eta} \left(\frac{4\pi N}{3M\bar{V}} \right)^{1/3} \dots\dots\dots 9.3$$

Two factors will cause actual diffusion coefficients to differ from the D_{Stokes} values calculated as above. Protein molecules are extensively hydrated in solution and the effect of this hydration is to increase effective size and hence reduce the diffusion coefficient. Secondly most proteins have a shape that more closely resembles a spheroid than a sphere. Both factors can be allowed for, providing the hydration and axial ratios are known.

For proteins the effect of hydration on diffusion of spherical particles can be described by the equation:

$$\frac{D_{\text{anhydrous}}}{D_{\text{hydrated}}} = \left(1 + \frac{w}{\bar{V}\rho} \right)^{1/3} \dots\dots\dots 9.4$$

where W = the hydration, expressed in grams of water bound
by one gram of protein

ρ = density of solvent at the temperature of the
experiment

The effect of the shape on the diffusion coefficient is described
by the following equations where "b" is the length of the
equatorial semi-axis of the ellipsoid and "a" the length of the
semi-axis of revolution (503).

$$\frac{D_{\text{sphere}}}{D_{\text{ellipsoid}}} = \frac{2 \left(1 - \frac{b^2}{a^2}\right)^{\frac{1}{2}}}{\left(\frac{b}{a}\right)^{\frac{2}{3}} \ln \frac{1 + \left(1 - \frac{b^2}{a^2}\right)^{\frac{1}{2}}}{1 - \left(1 - \frac{b^2}{a^2}\right)^{\frac{1}{2}}}} \dots \dots \dots 9.5$$

prolate ellipsoid ($b < a$)

$$\frac{D_{\text{sphere}}}{D_{\text{ellipsoid}}} = \frac{\left(\frac{b^2}{a^2} - 1\right)^{\frac{1}{2}}}{\left(\frac{b}{a}\right)^{\frac{2}{3}} \tan^{-1} \left(\frac{b^2}{a^2} - 1\right)^{\frac{1}{2}}} \dots \dots \dots 9.6$$

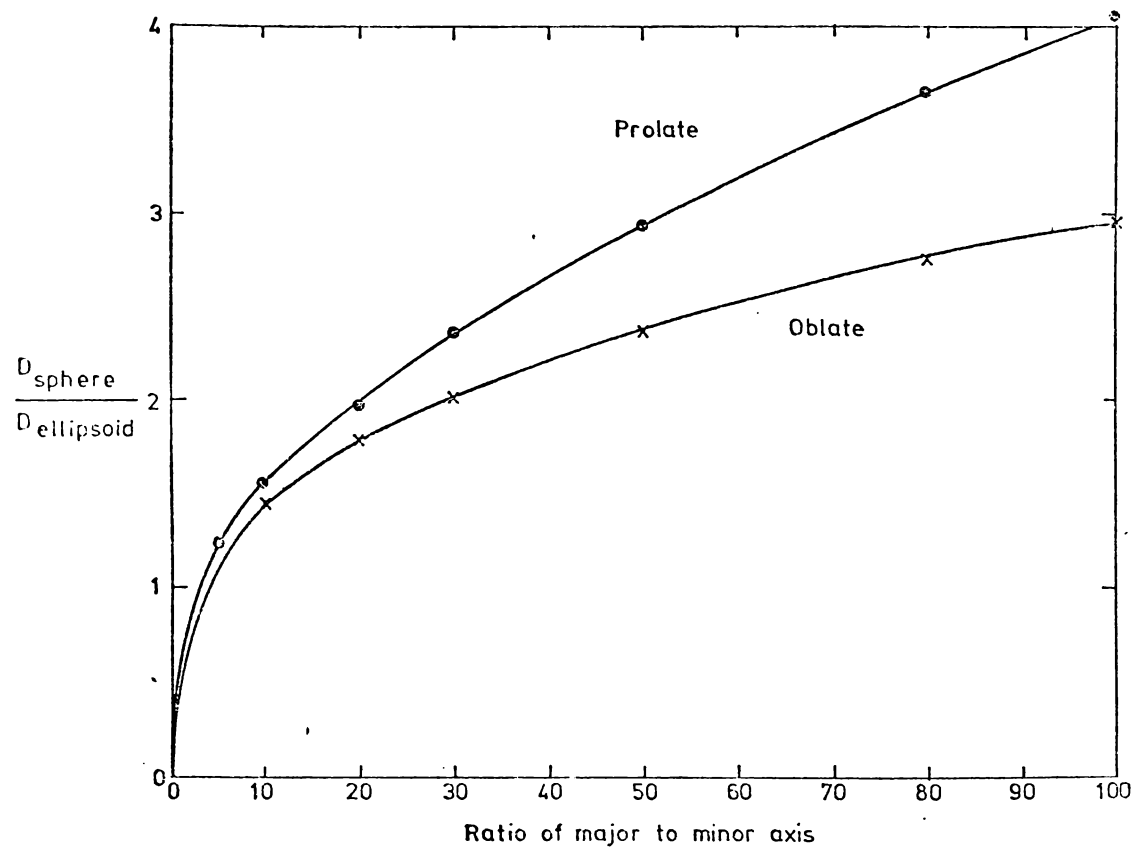
oblate ellipsoid ($b > a$)

The effect of axial ratio on the diffusion coefficients for
prolate and oblate ellipsoids is shown in figure 9.12.

Combining equations 9.3, 9.4 and 9.5 or 9.6 it is possible
to calculate the axial ratio i.e. the shape of the protein
molecule (assuming it to be ellipsoidal) from the observed diffusion
coefficient, the Stokes Law diffusion coefficient (calculated
from molecular weight and partial specific volume of solute and

Figure 9.12

Effect of axial ratio on diffusion coefficients
for prolate and oblate ellipsoids



viscosity of solvent) and the hydration factor (equation 9.4),
i.e.

For spherical anhydrous molecules

$$D_{\text{obs}} = D_{\text{Stokes}}$$

For ellipsoidal anhydrous molecule

$$D_{\text{obs}} = D_{\text{Stokes}} \frac{D_{\text{ellipsoid}}}{D_{\text{sphere}}}$$

For ellipsoidal, hydrated molecules

$$D_{\text{obs}} = D_{\text{Stokes}} \frac{D_{\text{ellipsoid}}}{D_{\text{sphere}}} \times \frac{D_{\text{hydrated}}}{D_{\text{anhydrous}}}$$

$$\therefore \frac{D_{\text{sphere}}}{D_{\text{ellipsoid}}} = \frac{D_{\text{Stokes}}}{D_{\text{obs.}}} \cdot \left(\frac{D_{\text{hydrated}}}{D_{\text{anhydrous}}} \right) \dots\dots\dots 9.7$$

From the value of $\frac{D_{\text{sphere}}}{D_{\text{ellipsoid}}}$ the value of $\frac{a}{b}$ can be obtained from figure 9.12. D_{obs} can be obtained from the kinetics of adsorption, although independent methods could also be used.

An illustration of the use of these equations can be provided from the data for β -casein adsorption at $8.4 \times 10^{-5} \text{ g ml}^{-1}$. The diffusion coefficient calculated from the adsorption kinetics was found to be: $D = 2.8 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. Using the value of 0.739 ml g^{-1} (53) for the partial specific volume, the value of D_{Stokes} was calculated ($6.84 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$). Unfortunately no data were available for β -casein hydration. Therefore calculations were performed for a range of hydration data. Literature data for the hydration of casein micelles varies from 1.9 to 3.67 grams of water per gram of protein (449,454).

It is likely that the degree of hydration of individual β -casein is less than that of the casein micelle which probably contains encapsulated water as well as bound water. Thus as a first approximation degrees of hydration of 3.97, 1.9, 0.5 and 0 grams of water per gram of protein were assumed for β -casein. The values for the axial ratio assuming these degrees of hydration are shown in Table 9.4.

Table 9.4

Effect of Hydration of β -casein Adsorption

Hydration *	$\frac{a}{b}$ ratio (prolate)	Molecular area/nm ²	Calculated Amount Adsorbed mg m ⁻²
0	32	36	1.8
0.5	20	31	2.2
1.9	11	26	2.6
3.67	6	19	3.4

* grams of water per gram of protein

Also shown in the table are the corresponding amounts of protein that would be adsorbed. These values were calculated from the estimated area occupied by each molecule. The relevant area was obtained from the axial ratio and the partial specific volume. When the calculated amounts of adsorption are compared with the actual value adsorbed (2.6 mg m⁻²) it can be seen that the experimental value corresponds to value calculated for a hydration of 1.9 g H₂O per g of protein and an axial ratio of 11.

This model will only apply when adsorption takes place with close packing and without denaturation or change in size, shape or hydration. Any effect such as dehydration, polymerisation or denaturation will cause deviation from this limiting case model. A further difficulty with the model is the number of parameters that cannot be checked independently. These parameters include: the roughness factor of the stainless steel, the degree of hydration, and the ratio of the protein molecule axes.

The degree of surface roughness of the stainless steel could be studied indirectly by studying the adsorption of protein on to a cleaved mica surface. Freshly cleaved mica can be obtained smooth down to a molecular level. It would be necessary to assume that the protein adsorbed on to mica in the same way it adsorbed on to stainless steel and that close packing occurred.

Hydration of the protein and axial ratio values could possibly be obtained from X-ray studies, dielectric measurements or from the viscosity coefficient (506). The hydration value could also be obtained from the crystal density.

9.6 Conclusion

The aim of the present chapter was to carry out an investigation of the solid-liquid surface chemistry of the major casein constituents of milk: α_{s1} -casein, β -casein and K-casein. Most attention was focused on α_{s1} -casein and β -casein. From detailed studies of the kinetics and thermodynamics of the

adsorption of these species it was concluded that casein adsorption from low concentrations is diffusion controlled and probably irreversible and that adsorption ceases after a single layer of protein molecules has been formed. The amount adsorbed is thus limited by the size and packing arrangement at the interface. A limiting case model that illustrated how all the adsorption characteristics at low concentration could be interpreted in terms of size, shape and hydration of the protein molecule was developed. This model is expected to have relevance to proteins other than caseins.

At high concentrations, high ionic strength and under conditions of varying pH, adsorption behaviour is complex and cannot be so readily explained.

The lack of a significant temperature effect indicated that the driving force for adsorption was primarily entropic and is consistent with the formation of hydrophobic bonds.

Results reported in this chapter have demonstrated that adsorption of casein could be an important factor in the formation of milkstone. However, since adsorption does not appear to go beyond a single layer under most conditions, it is likely that other factors are also involved.

CHAPTER 10

DESORPTION OF ADSORBED PROTEIN FILMS

10.1 Introduction

The adsorption of protein on to stainless steel, as described in the previous chapter, could be the first step leading to the formation of macroscopic films observed on milk contact surfaces. Prevention of the initial adsorption, or the removal of the first layer once formed, would presumably prevent the formation of accumulated films.

This chapter therefore investigates the relative ease with which this film can be removed and factors that might affect its removal. Such factors include: the effect of time of contact between the adsorbed protein and the desorbing solution, the effect of flow rate, the effect of pH, type of surfactant and concentration of the desorbing solution. At a more fundamental level, an understanding of these desorbing conditions should also give further information on the interaction of protein with stainless steel. Literature studies indicate that protein adsorption on to a hydrophobic surface is generally irreversible, while on to a hydrophilic surface the protein adsorption is reversible at neutral pH. This suggests that the adsorption of protein on to the hydrophobic stainless steel surfaces would not be reversible. A further aim of the present work was also to investigate the exchangeability of the adsorbed protein.

10.2 Materials and Methods

The cleaning of glassware and the preparation of the protein solutions and the stainless steel surfaces were conducted as described in previous chapters. The surfactant solutions were made up in distilled water to the concentration stated and the pH was adjusted using hydrochloric acid or sodium hydroxide unless otherwise stated.

The studies were conducted at 20 ± 2 °C unless stated otherwise and degassed water was used for higher temperature studies to prevent bubble formation.

In experiments where the radioactivity had to pass through the stainless steel before being counted it was necessary to use I-131 in order to achieve adequate counting efficiency. The β radiation of I-131 was counted with Geiger counters mounted against the rear side of the stainless steel sample. Appropriate coincidence backscatter and decay correction were made where necessary (see Appendix 6 and 7). Where the activity was monitored directly it was found more convenient to use I-125 and the counting system described previously (see section 7.7).

Three different methods were developed to study the various aspects of interest.

(a) Experiments to Determine Effect of Flow Rate

In this system the radioiodinated protein was adsorbed on to the inside walls of the stainless steel tubing. This stainless steel tubing had been made from a rod of stainless steel. It had an inner diameter of about 0.6 cm and the wall thickness was about 0.1 mm thick. The radioactive I-131 labelled protein was adsorbed

on to the inner walls by placing a stopper at one end of the tube and by filling the container with radioiodinated protein solution and equilibrating for thirty minutes in a desiccator containing distilled water. The water was put in the desiccator to help prevent the protein solution from evaporating. The protein solution was then sucked out of the tube, the stopper removed and the tube was connected up as shown in figure 10.1a. The flow rates were controlled by a combination of varying the pressure of the solution, using either gravity or compressed air and various orifices or, in the case of desorption with tap water, by using the main supply of cold tap water. Lead shielding was placed at the end of the stainless steel tubing to avoid monitoring any edge effect or any protein that could be washed on to the polystyrene tubing.

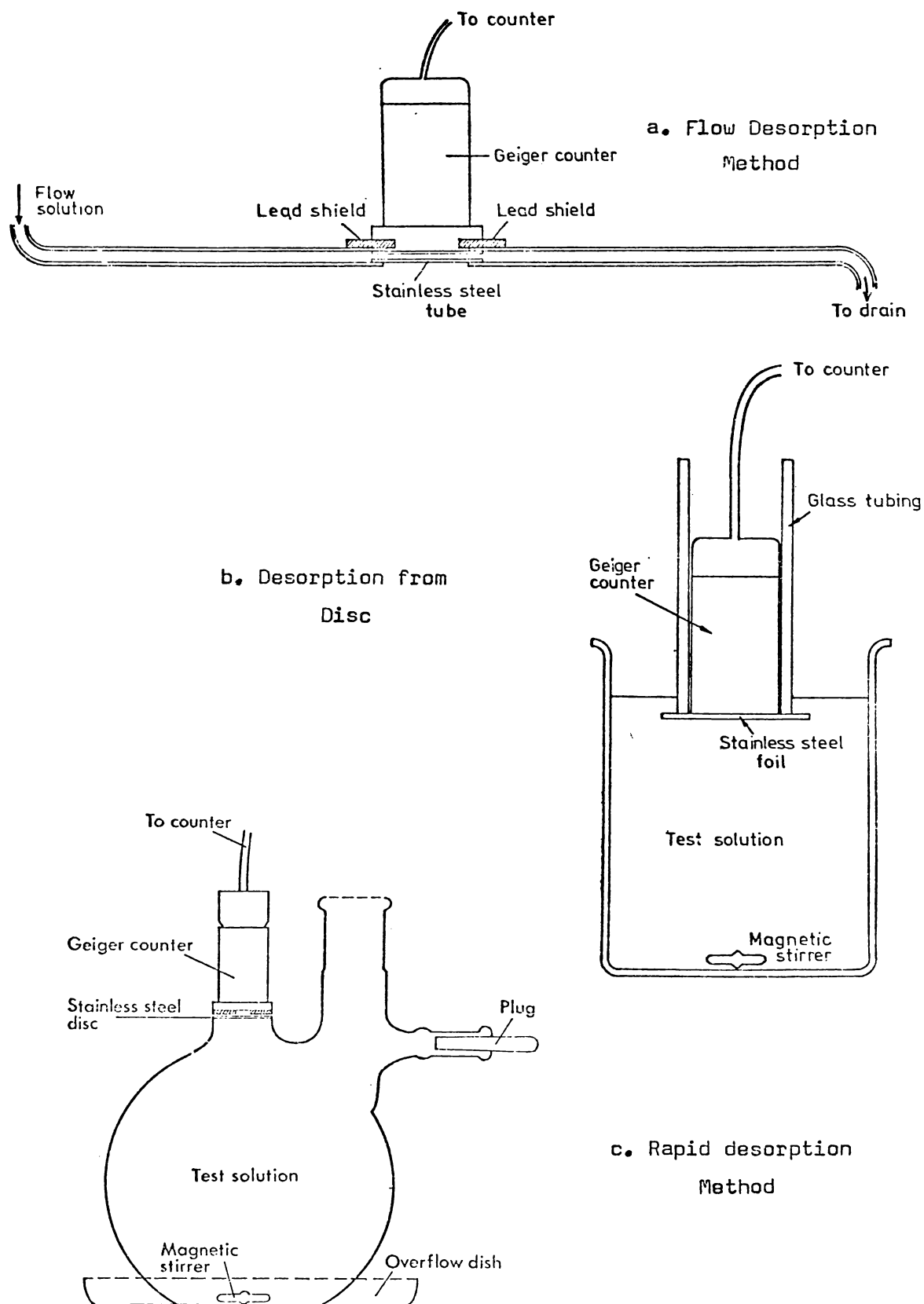
This method allowed the continuous monitoring of desorption, but because of the large volumes consumed was not suitable as a rapid method of screening the effect of different chemical conditions on removing protein.

(b) Desorption from Discs into Stirred Solutions

A procedure was developed to investigate the desorption of the I-131 labelled protein adsorbed on to the underside of a stainless steel disc by the continuous monitoring of the opposite face with a Geiger counter. The apparatus used in the initial studies is shown in figure 10.1b. The stainless steel foil disc of 3.0 cm diameter and 0.1 mm thick was cut from AISI Type 304 stainless steel and glued using a thin film of "Silastic"

Figure 10.1

Apparatus for Studying Desorption



silicone rubber glue applied to the end of the glass tube. The radio-iodinated protein was adsorbed on to the stainless steel by the 'spot' apparatus discussed in section 7.5. After adsorbing the protein solution on to the stainless steel, a Geiger counting tube was placed inside the glass tube and the system was immersed into the test solution. This method was ideal for studying the initial desorption into distilled water, but was unsatisfactory with detergents at high pH's because a reliable seal between the stainless steel foil and the glass rod could not be obtained.

The technique was modified for studies involving long desorption periods as is shown in figure 10.1c. The protein was again adsorbed on to the clean stainless steel disc (0.1 mm thick) by the spot technique (see Section 7.5) before placing it on the apparatus and testing it with various test solutions. This method was relatively rapid and was found suitable for testing protein desorption over a wide variety of conditions.

(c) Batch procedure using Slides

To monitor the desorption of low levels of activity a batch procedure using slides was developed. After adsorbing ^{125}I -labelled protein on to the stainless steel slide, they were removed from solution and lowered immediately into a beaker containing the test solution. At no time was the adsorbed protein allowed to dry out. At the end of a set period the slides were removed and the amount of desorbed protein determined in the usual manner. At low levels of adsorbed activity the direct monitoring of the adsorbed protein is very much more sensitive than monitoring through the stainless steel foil as used in the other two methods.

A disadvantage of the technique was that it was not possible to continuously monitor the desorption.

10.3 Results

a. The Effect of Flow Rates

These experiments were designed to study the effect of flow rates on the removal of adsorbed protein. Labelled iodinated milk was added to milk and diluted four times before being adsorbed on to the stainless steel tube. The desorption solution was passed through the system for various times at different flow rates. The results showed (see figure 10.2) no significant effect for the rate of flow of sodium hydroxide and tap water on the rate of desorption.

The effect of increased flow rates on the desorption of β -casein was also investigated. Results are shown in figure 10.2. Again flow rate had no significant effect on the rate of desorption.

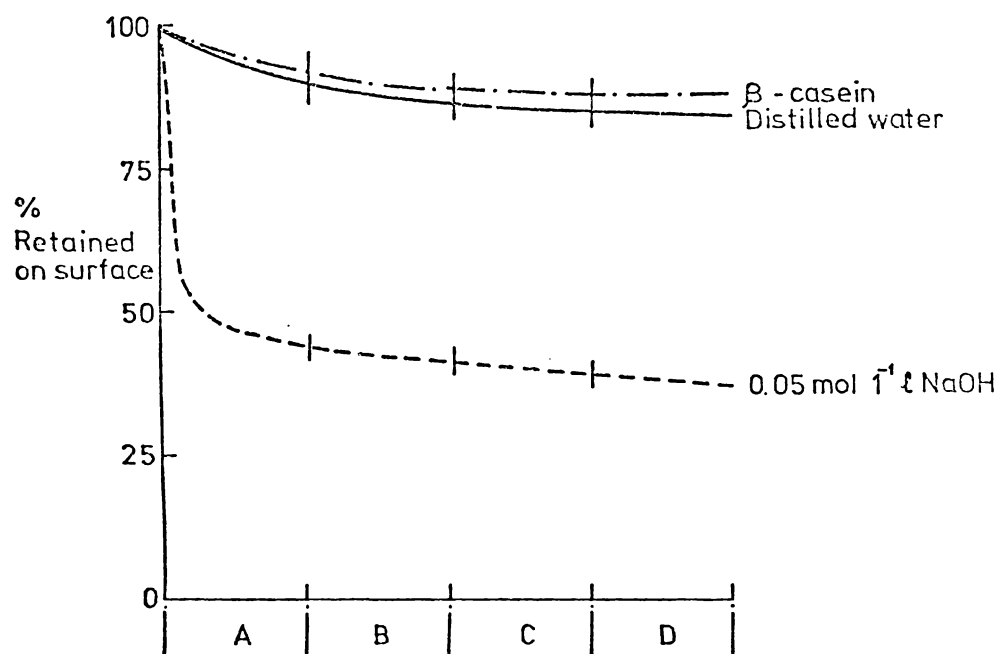
These results indicate that over the range investigated flow rate has no effect on the desorption of adsorbed protein. Thus in further desorption studies magnetic stirring of the desorption solutions would be adequate.

b. Initial rinsing of the adsorbed protein monolayer

The initial desorption of protein into distilled water and tap water rinse solutions was compared using the apparatus as shown in figure 10.1c. The protein used in these studies was labelled β -casein at a concentration of 0.4 mg ml^{-1} . The results showed that after the initial desorption had removed all the

Figure 10.2

Effect of flow rate on protein desorption



	Time/min	Flow Rate/ml min ⁻¹		
		0.05 mol l ⁻¹ NaOH	Distilled water	β-casein
A	20	30	90	16
B	20	77	490	125
C	20	420	800	270
D	20	1000	1146	1350

unbound and weakly adsorbed protein, further rinsing did not remove significant further protein even after sixteen hours. Twenty seconds was observed to be sufficient time to remove the excess unbound protein. The experiment was repeated using tap water instead of the distilled water with similar results.

c. Desorption Kinetics

After checking the reproducibility of the continuously monitored desorption method (see figure 10.1c), the desorption of protein under a range of conditions was studied. In all the following experiments the protein treated surface was first rinsed with distilled water for 20 seconds so that only adsorbed protein was studied.

(1) Exchange of Adsorbed Protein

The results for the desorption into distilled water and buffer solution at pH 7.0 indicated that the adsorption process was virtually irreversible under these conditions (see figure 10.3). It was of interest to test the exchangeability of the adsorbed protein with dissolved protein under similar conditions. Exchange of adsorbed β -casein with carrier β -casein at $4 \times 10^{-4} \text{ g ml}^{-1}$, four-fold diluted skim milk and whole skim milk was monitored. The results (see figure 10.4) show that approximately 20% of the adsorbed β -casein exchanged within the first few hours of the experiment. Some further exchange, particularly at the higher protein concentrations of skim milk, was observed for the next five hours, after which time more than 50% of the adsorbed protein had exchanged.

Figure 10.3

Effect of distilled water, buffer pH 7.0 and urea
on desorption kinetics (β -casein)

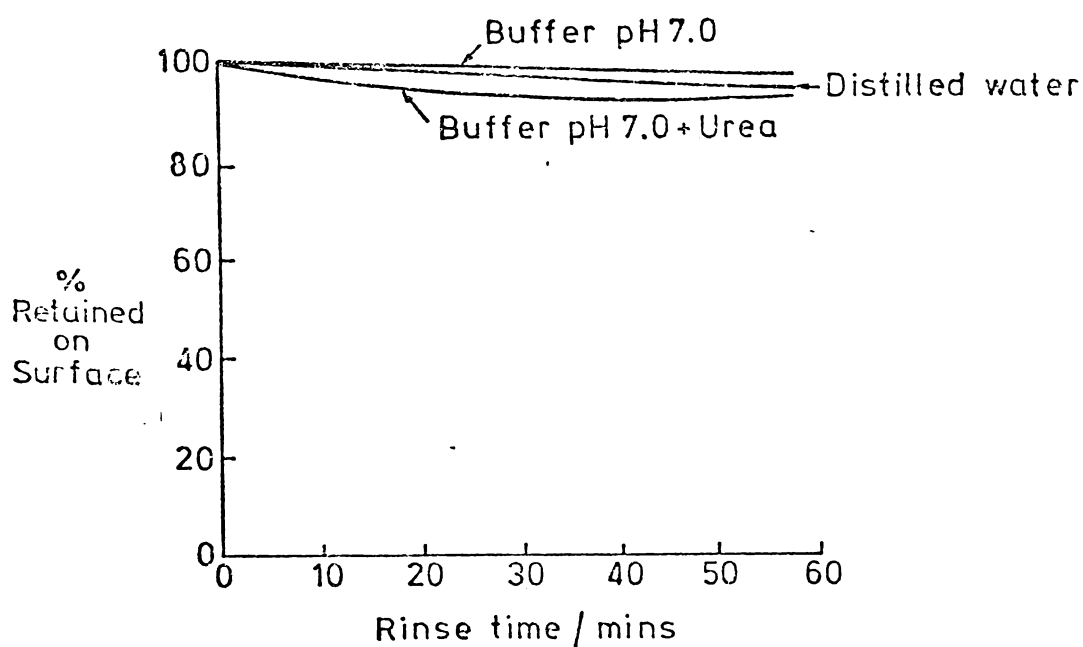
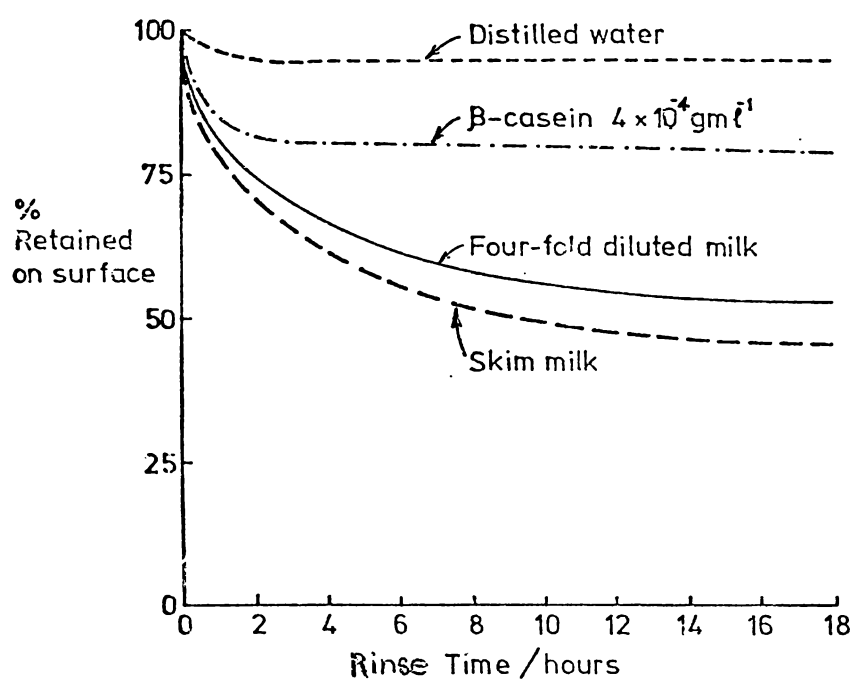


Figure 10.4

Effect of protein solutions on desorption kinetics
(β -casein)



(2) Effect of pH

The results for the effect of pH are shown in figure 10.5 where it is apparent that the rate of desorption increased with increased pH at pH's above pH 7.0. Very little desorption was observed in acid solutions. The removal of protein at pH 1.0 and at a temperature of 80 °C was also studied. High temperatures did not significantly increase the amount of protein removed at this low pH.

(3) Effect of Ionic Strength

The effect of ionic strength ($1.0 \text{ mol l}^{-1} \text{ NaCl}$) on the desorption of β -casein was investigated at pH 7.0 and at pH 12.4 (NaOH). At pH 7.0 the salt solution did not remove the adsorbed protein any more effectively than the distilled water. At pH 12.4 (NaOH) the high, ionic strength appeared to increase the efficiency of removal (see figure 10.6).

(4) Effect of Anionic Surfactants

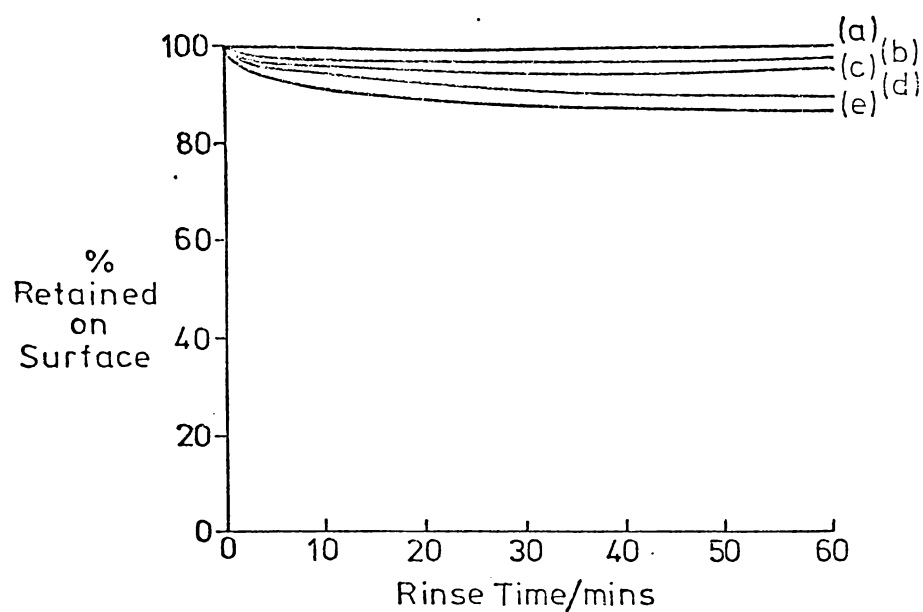
The effect of adding increasing amounts of sodium alkyl aryl sulphonate to a $0.05 \text{ mol l}^{-1} \text{ NaOH}$ solution was studied and the results are shown in figure 10.7. On increasing the surfactant concentration, but keeping it below the C.M.C. value of 0.5 g l^{-1} (504), an increase in the rate of desorption was observed.

(5) Effect of Nonionic Surfactant

The effect of 0.1% nonyl phenol - 9 moles of ethoxylate (Teric GN9) in pH 7.0 buffer solution (sodium barbitone) and in pH 12.4 (sodium hydroxide) was studied. The results showed that the nonionic had no effect at pH 7.0 but increased the

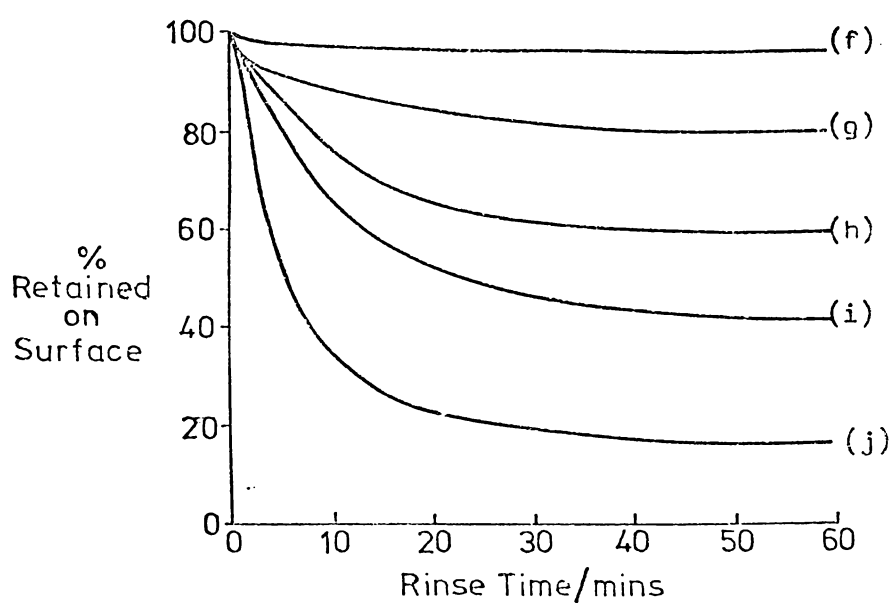
Figure 10.5

Effect of pH on desorption kinetics (β -casein)
(20 °C)



pH of solution:

- | | |
|------------|-------------|
| (a) pH 7.0 | (d) pH 5.0 |
| (b) pH 3.0 | (e) pH 0.44 |
| (c) pH 1.0 | |



pH of solution:

- | | |
|-------------|-------------|
| (f) pH 7.0 | (i) pH 12.4 |
| (g) pH 8.3 | (j) pH 12.8 |
| (h) pH 11.0 | |

Figure 10.6

Effect of ionic strength on desorption kinetics
(β -casein)

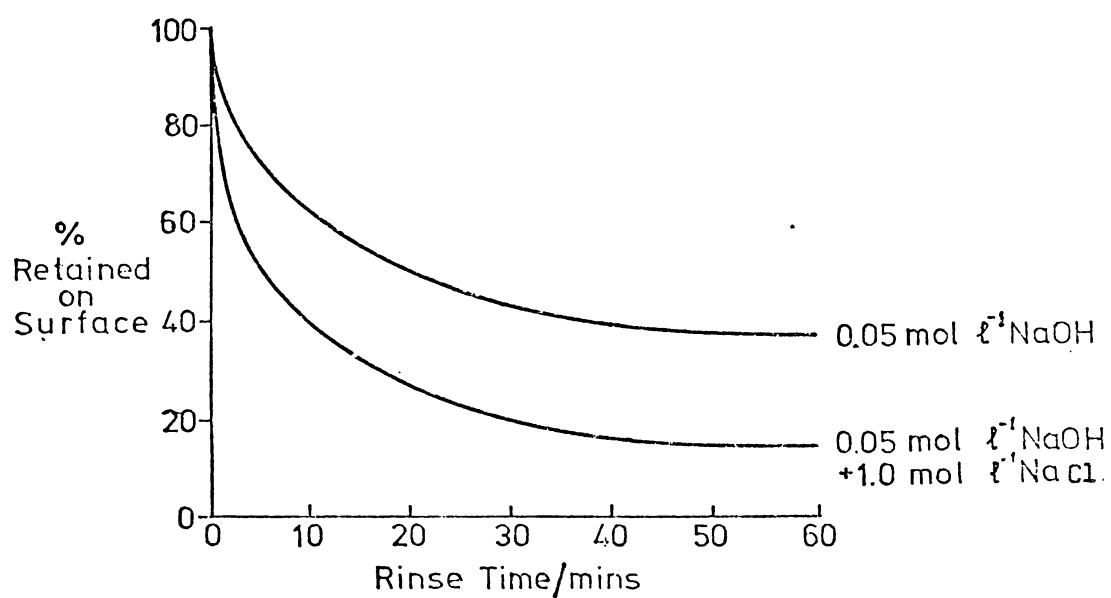
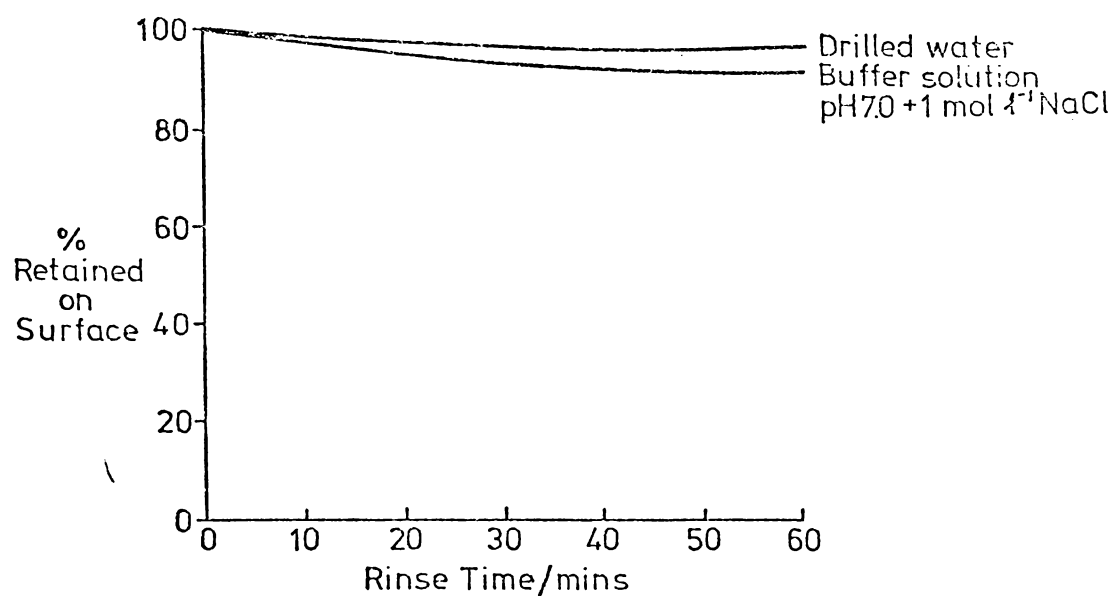
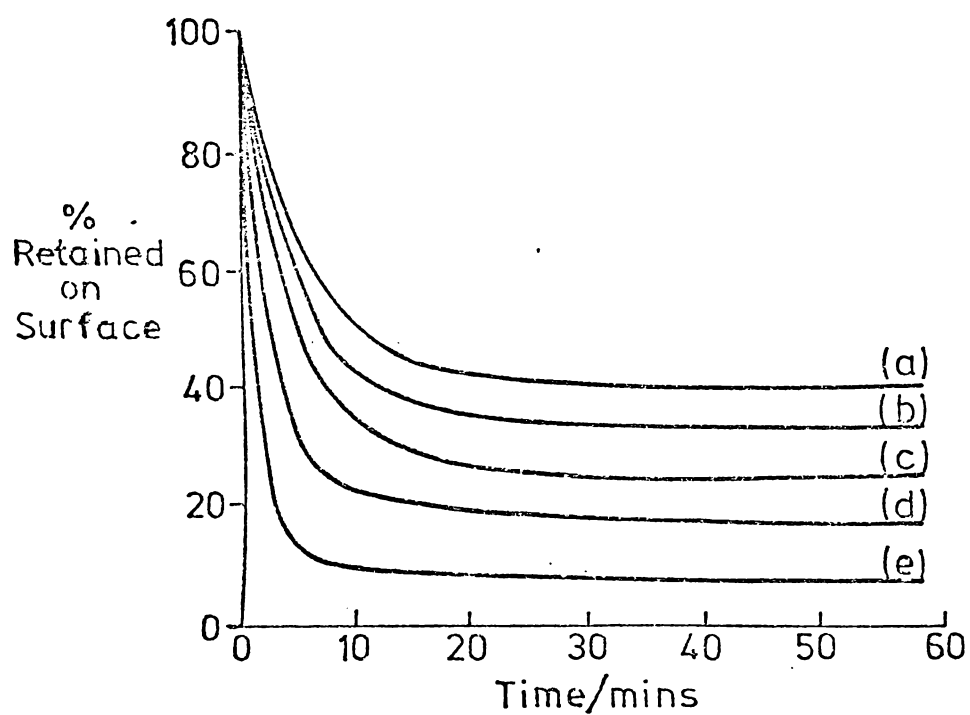


Figure 10.7

Effect of Anionic Surfactant (sodium alkyl aryl sulphonate) on desorption kinetics (β -casein)



- (a) 0.05 mol l⁻¹ NaOH
- (b) 0.05 mol l⁻¹ NaOH + 0.0025 % anionic surfactant
- (c) 0.05 mol l⁻¹ NaOH + 0.0050 % anionic surfactant
- (d) 0.05 mol l⁻¹ NaOH + 0.010 % anionic surfactant
- (e) 0.05 mol l⁻¹ NaOH + 0.015 % anionic surfactant

desorption when added to the 0.05 mol l^{-1} sodium hydroxide solution (see figure 10.8). These results are similar to those obtained for the anionic surfactant.

(6) Effect of Urea

The effect of 6 mol l^{-1} urea (in pH 7.0 buffer solution) on removing adsorbed protein was studied. The results showed that urea had no effect on the desorption of β -casein at pH 7.0 (see figure 10.3).

(7) Effect of Acid Pretreatment

A comparison was made of the effectiveness of an acid detergent and an alkaline detergent in removing adsorbed protein. The formulated alkaline detergent was found to be very much better than the acid detergent. Further experiments (see figure 10.9) showed that when the adsorbed protein was first exposed to an acid detergent and then exposed to an alkaline detergent, the acid detergent modified the adsorbed protein in such a way as to render it more resistant to removal by the alkaline detergent.

d. Effect of Adsorption Time

Stainless steel surfaces were treated with protein solution at low concentration ($4 \times 10^{-6} \text{ g ml}^{-1}$ α_{s1} -casein) for various times. The protein was then desorbed into either distilled water or 0.05 mol l^{-1} NaOH for thirty minutes. The results show that the proportion of protein removable by NaOH after thirty minutes decreases with adsorption time (see figure 10.10).

Figure 10.8

Effect of nonionic surfactant (Nonyl phenol-9 moles of ethoxylate) on the desorption kinetics (β -casein)

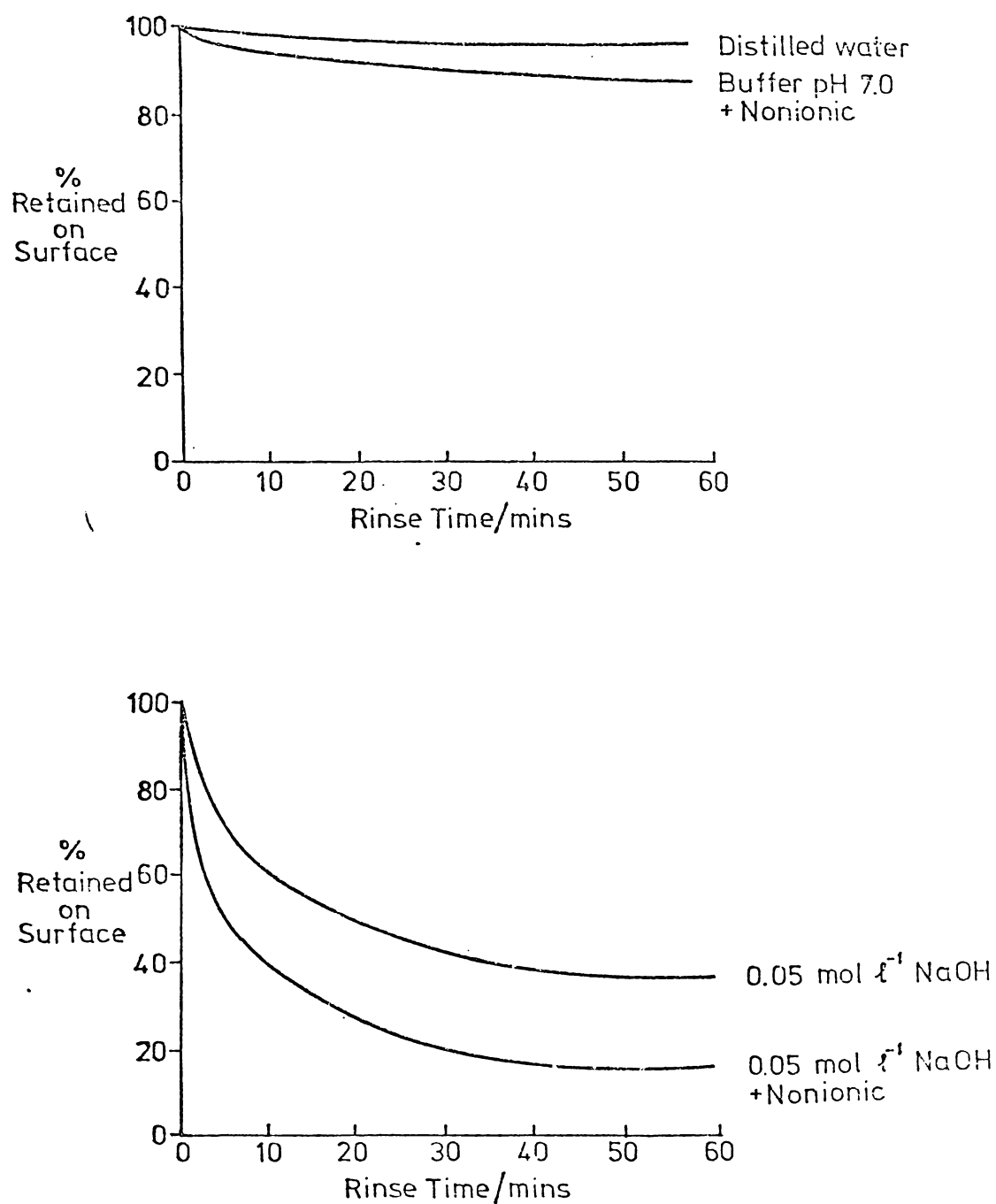
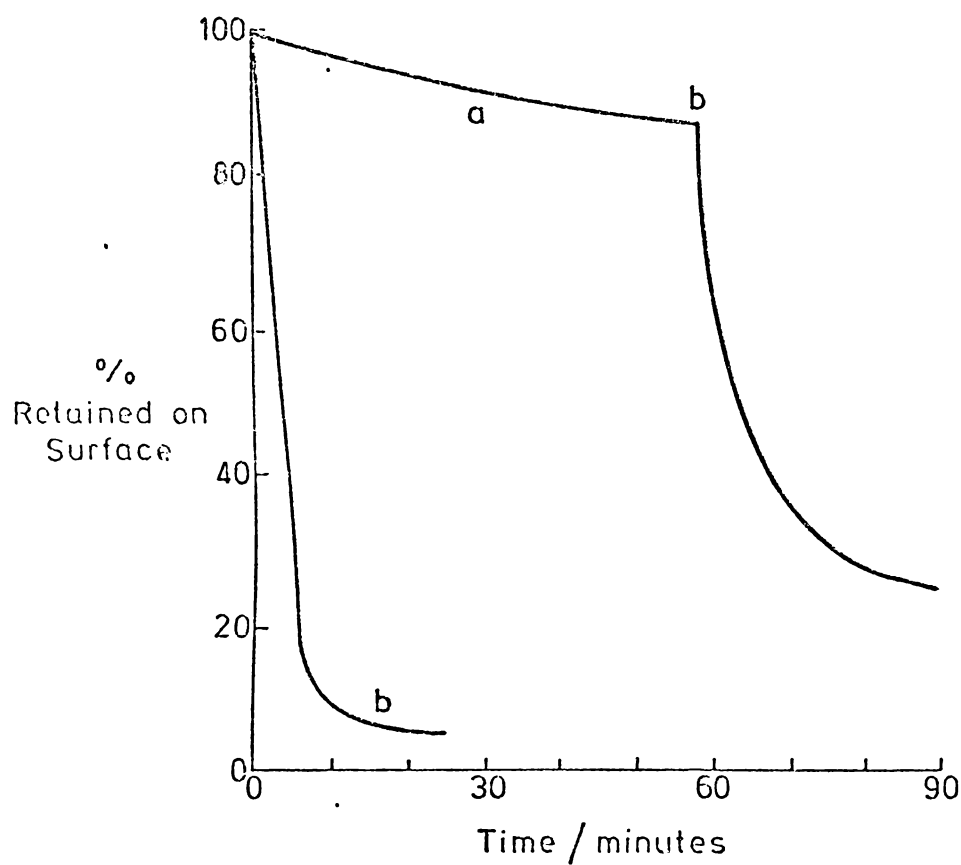


Figure 10.9

Modification of Adsorbed Protein by Acid Detergents

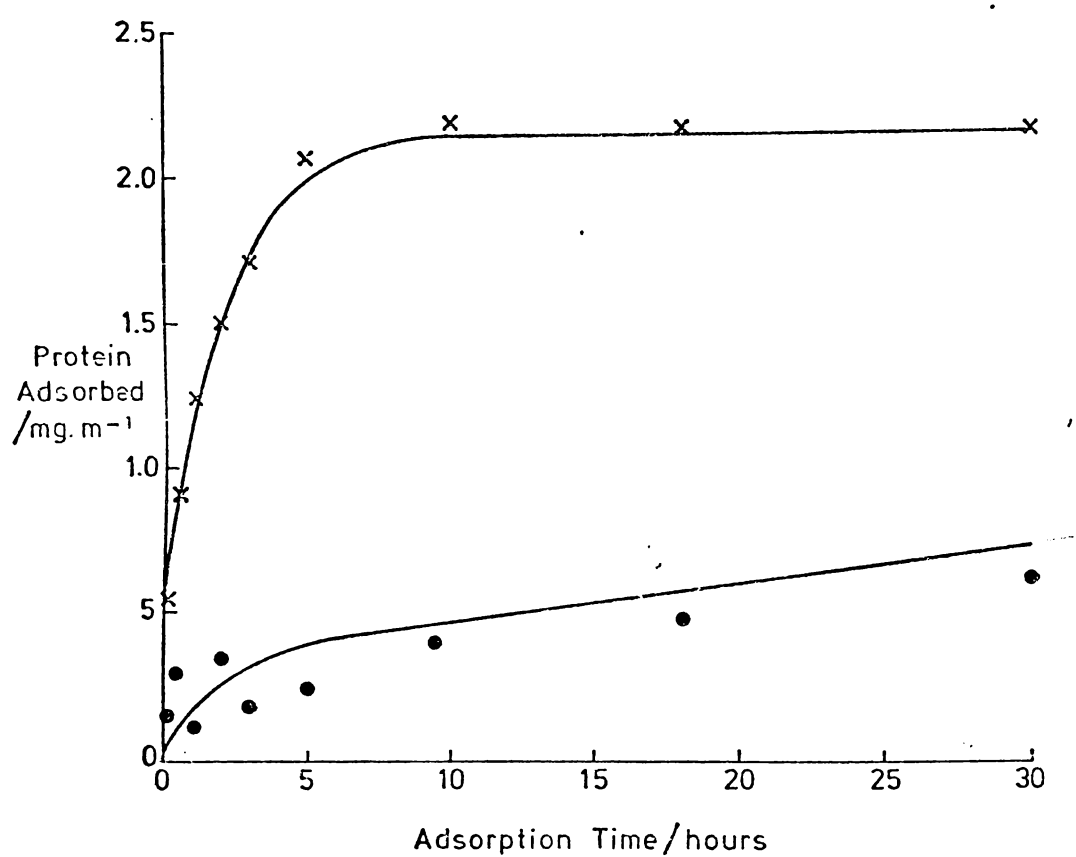


a. Desorption with acid detergent

b. Desorption with alkaline detergent

Figure 10.10

Variation of alkali resistance of the adsorbed protein with adsorption time



α_{s1} -casein at 4×10^{-6} g ml⁻¹ adsorbed on to stainless steel at 4 °C

x = adsorbed protein washed with distilled water

• = adsorbed protein washed with 0.05 mol l⁻¹ NaOH for thirty minutes

10.4 Discussion

1. Analysis of Desorption Curves

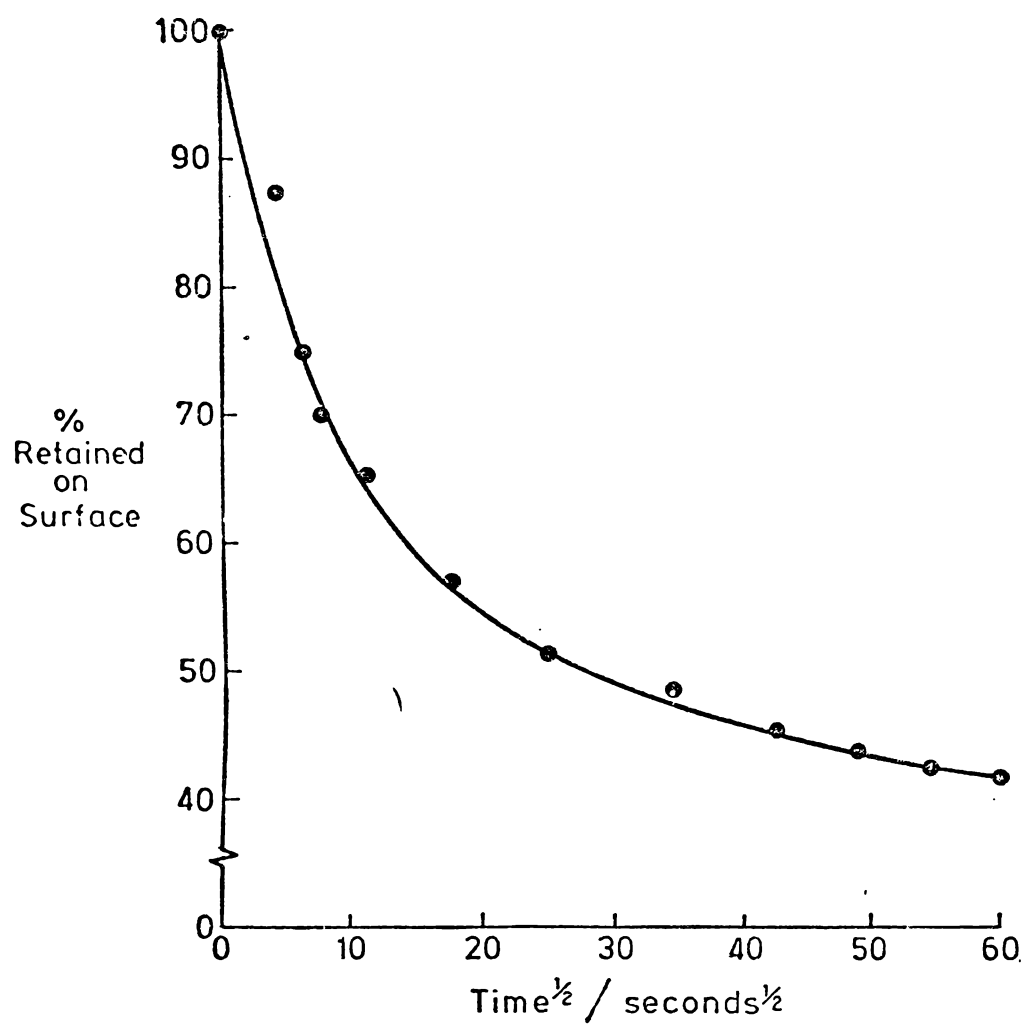
Attempts were made to analyse the kinetic data for the desorption processes in terms of the various possible rate limiting steps of a surface reaction summarised in Chapter 9.

The possibility of the desorption process being dependent upon the rate of diffusion, either of a displacing species to the surface or the displaced protein away from the surface, can be eliminated because the desorption processes were not affected by flow rate. Further, the desorption curves did not give linear $t^{\frac{1}{2}}$ plots and could not be fitted to diffusion equations derived from appropriate boundary conditions (see figure 10.11).

Attempts were made to fit the desorption data to the equations for simple first order or pseudo first order desorption processes. This method has been used for the characterisation and kinetic analysis of desorption curves by the resolution of semi-logarithmic plots into a number of independent first order processes (361). Such an analysis requires the half-life (and hence the rate constant) of each of the various processes to differ by about an order of magnitude. It has been suggested that the exponential terms obtained from such an analysis correspond to first order desorption processes that can be attributed to "surface complexes" or different types of soil on the surface (507,508). All the curves were resolvable into the sum of at least two exponential terms and some were resolved into as many as five such terms. An example

Figure 10.11

Square-root Plot of Data for Desorption Kinetics
(Desorption into $0.05 \text{ mol l}^{-1} \text{ NaOH}$)



of such an analysis is shown in figure 10.12 for the desorption of β -casein into 0.05 mol l^{-1} NaOH. This result is typical of the type of result obtained for the other desorption experiments. Values for the "rate constants" and percentages of each "desorption process" are reported in the Appendix 8. It should be noted that the "rate constants" did not always show an order of magnitude difference and the errors in the "rate constants" for the fast "processes" were likely to be large, through the accumulation of successive errors. It was observed that the percentages of each "process" were not consistent and there were no apparent trends in the effect of the variables studied on the "rate constants". This suggested that the analysis of desorption curves into exponential desorption "processes" was of limited usefulness in this study. Further discussion will therefore be limited to the qualitative effect of the variables studied and how these effects can be used to deduce information about the mechanism of the desorption.

2. The Mechanism of Desorption

As shown in the previous section it does not seem possible to analyse the kinetic data from protein desorption experiments in terms of rate equations derived from simple physical models.

Desorption appears to be a complex process, complicated perhaps by multiple surface-substrate interactions. Some insight, however, as to the factors involved can be gained from a consideration of the effects of various experimental variables.

In previous sections the forces that could be important in the protein-surface interaction were discussed. These included:

- (i) electrostatic interaction
- (ii) hydrophobic bonding
- (iii) hydrogen bonding
- (iv) covalent bonding (e.g. by disulphide bridging).

While proteins may well interact with surfaces by more than one of these bonding mechanisms, it seems unlikely that hydrogen bonding, because of the weakness of the bonds, plays any important role. Disulphide bridging could occur only with K-casein and hence can be eliminated from the other casein adsorption mechanisms.

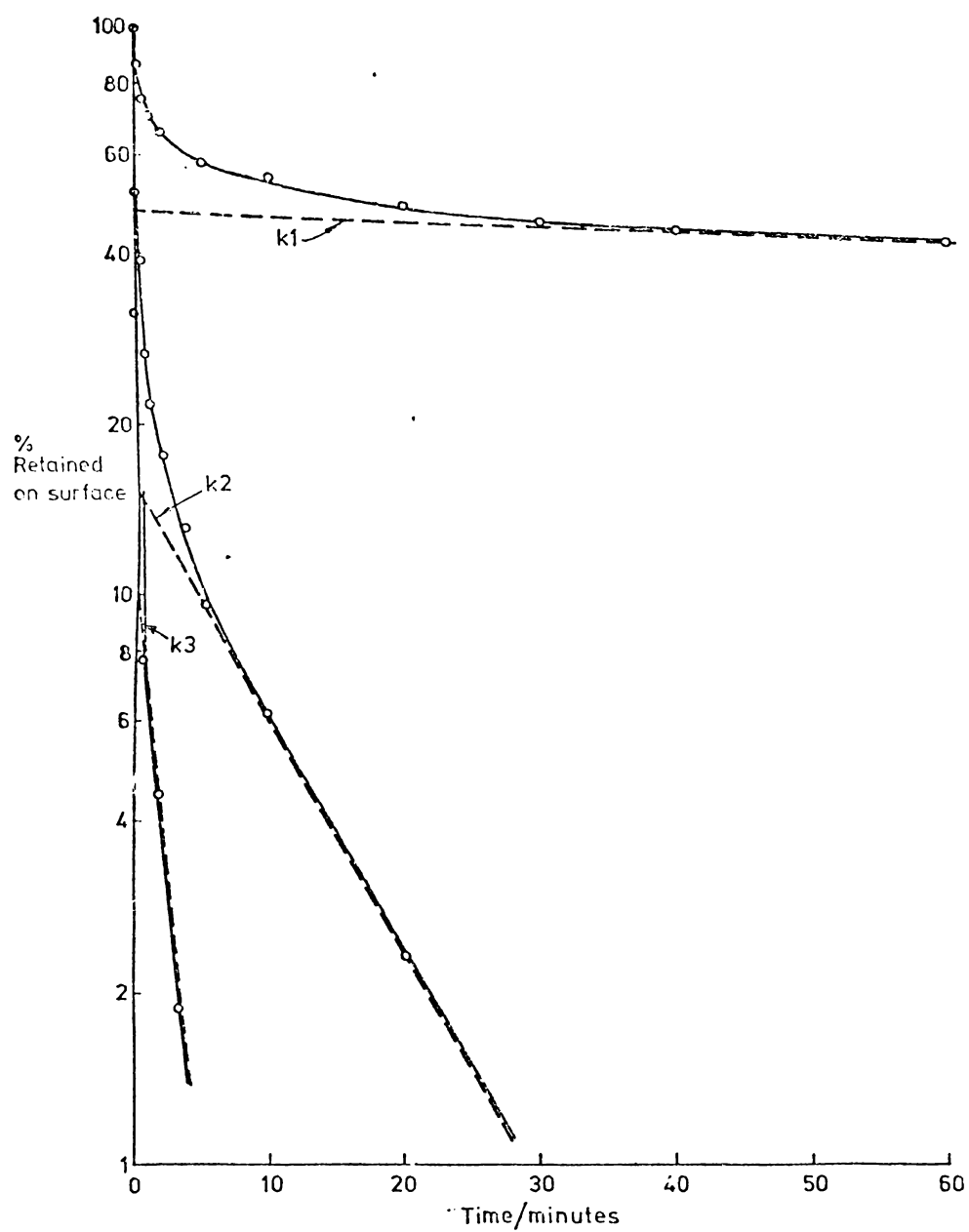
Desorption studies may offer some information on protein-surface interaction, however interpretation of the results is complicated by the fact that various reagents may act in more than one way and that, in addition to desorption being caused by breaking the surface bond, it could also result from:

- (i) chemical denaturation - e.g. by hydrolysis
- (ii) physical denaturation - e.g. displacement by chain
uncoiling

The importance of simple electrostatic interactions can best be judged by the effect of ionic strength and pH change in the vicinity of the isoelectric point. Changes in the ionic strength have virtually no effect on desorption kinetics at neutral pH's and only a slight effect at strongly alkaline pH's. This is good evidence that protein binding to stainless steel surfaces is not by means of a simple "ion exchange" interaction. Changing the pH so that the adsorbed protein could be expected to undergo reversal

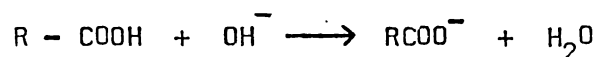
Figure 10.12

Log Plot of Data for Desorption Kinetics

(Desorption into $0.05 \text{ mol l}^{-1} \text{ NaOH}$)

of charge has virtually no effect on the desorption kinetics. Although it was demonstrated earlier that the amount of protein adsorbed was quite sensitive to pH in the vicinity of the isoelectric point, the lack of an effect of net charge on desorption kinetics indicated that forces other than simple electrostatic forces involving net charges were responsible for holding the protein at the surface.

The lack of significant evidence for electrostatic binding confirms earlier conclusions that the interaction between protein and stainless steel is predominantly a result of hydrophobic bonding. Thus reagents that facilitate desorption without actual denaturation of the adsorbed protein must be species that can modify the hydrophobicity of the adsorbed protein. From the desorption studies it is apparent that of the species investigated, those most effective in the causing of desorption of adsorbed protein are the OH^- ions and anionic surfactants. The data do not allow their mode of action to be determined. However it is likely that the surfactants enhance desorption by binding hydrophobically with the protein with hydrophilic ends outermost. This would have the overall effect of solubilising the protein and causing it to leave the surface. In the case of the OH^- ions, a similar effect could be brought about by ionisation of the free-COOH groups of the protein chains:



At very high and very low pH's, the desorption kinetics are almost certainly affected by chemical denaturation of the adsorbed protein.

Hydrolysis of casein into peptides and amino acid residues has been observed to commence at pH's as low as pH 10 at 60 °C. At pH 12 as much as 70% of casein is denatured after one hour at 60 °C (509). While in section 4.4 it was shown that iodinated casein did not lose its label after contact with 0.2 mol l⁻¹ NaOH for thirty minutes at room temperature, this experiment would not have detected partial hydrolysis to large polypeptide fragments.

That some denaturation of the adsorbed protein occurs after acid treatment was clearly apparent from results of the experiment in which a surface treated with acid detergent was subsequently desorbed into an alkaline detergent solution previously used in a direct desorption experiment. After exposure to the acid solution, the adsorbed protein became very much more resistant to desorption in an alkaline solution. This experiment, incidentally, illustrated an important practical aspect. Clearly, freshly adsorbed protein films should not be exposed to acid detergents of this type. When an acid formulation is used in a sequence with an alkaline formulation, the alkaline detergent formulation should be used first.

Many reagents are known to modify protein structure without causing cleavage of polypeptide or other bonds. These could lead to desorption by physical denaturation. For example at high and low pH's deprotonation of amines and protonation of carboxyl groups respectively, can cause a diminution of intramolecular electrostatic interaction. The associated coil unfolding could lead to an over-crowding of the surface and displacement desorption. The fact, that no such effect was observed

at low pH's suggested that the increased desorption observed at high pH's resulted from other factors. Increase in ionic strength should also lead to similar effects but again this was not observed. In addition, urea is known to break hydrogen bonding and this could cause the coil to unfold, or alternatively, if hydrogen bonding between the protein and the surface is important, to break this bond and cause desorption. Again no experimental evidence for this was obtained.

10.5 Conclusion

The overall conclusion of this work is that adsorbed protein is held to stainless steel surfaces mainly by hydrophobic interactions. From the results of studies of desorption kinetics it can be concluded that the adsorption process is only partially reversible and only with solutions of fairly high protein concentration. This result together with the non exchangeability of the adsorbed protein with solutions of high ionic strength and resistance to desorption into distilled water, provides more evidence that the adsorption mechanism is by hydrophobic bonding.

Of the various species investigated, OH^- proved to be the most effective in removing adsorbed protein. The effectiveness of alkaline solutions could be enhanced by the addition of anionic surfactant. It has not been proved how the OH^- operates in causing desorption. It is likely that it solubilises the molecules by deprotonation of the free carboxyl groups. However partial hydrolysis of the adsorbed protein could be a contributing factor.

A feature of the desorption experiments was that of all the solutions tested, none was completely effective in removing adsorbed protein in the first five minutes of the desorption experiment. It is possible that the accumulation of this residual protein is involved in the formation of "milkstone" on milk contact surfaces.

CHAPTER 11

FORMATION OF MACROSCOPIC PROTEIN FILMS BY REPEATED ADSORPTION

11.1 Introduction

Previous chapters of this thesis have been concerned with the adsorption of protein films after a single contact with a protein or milk solution. It has been shown that a film of protein up to 8 mg m^{-2} can be formed by a simple adsorption process. However the thickness of a protein film required to support bacterial colonisation is much thicker than this. It was therefore considered necessary to investigate the possible ways in which thicker films could be formed. This information is needed in order to better understand the properties of such films and so devise better methods of preventing and/or removing them.

Preliminary experiments established that successive layers of protein could not be accumulated by simple repeated exposures of stainless steel surfaces to milk or protein solution. Stainless steel surfaces freshly "saturated" with protein showed no tendency to adsorb further protein. Thus it was of interest to determine whether such a freshly "saturated" surface could be rendered adsorptive to further protein by modification under controlled conditions.

11.2 Review of Possible 'Multilayer' Bonding Mechanisms

The two most obvious ways in which the adsorbed protein could be modified as to promote further adsorption are:

- 1) physical and/or chemical modification including reactions involving surface functional groups, dehydration and denaturation;
- 2) modification of surface properties by adsorption (or deposition) of a bridging species that interacts strongly with protein and on to which further protein can be adsorbed.

Physical and Chemical Modification of Adsorbed Protein

Protein films adsorbed on to milk contact surfaces of farm and factory milk lines and on to the test surfaces of detergent testing simulators are subjected to a variety of chemical and physical conditions during cleaning cycles that could conceivably modify the adsorbed protein in such a way that further protein could be adsorbed.

Of the physical variables involved, heating, particularly under reduced pressure, seems the condition most likely to bring about denaturation of the adsorbed film.

A variety of chemical conditions are commonly employed deliberately or occur inadvertently in the various stages of milk processing. Thus the surface molecules may be subjected to bond cleavage, polymerisation, oxidation, reduction (less likely), halogenation, nitration, sulphonation and other changes brought about by enzymatic action, bacterial colonisation, pH change, variations in ionic strength and treatment with various cleaning and sanitising agents.

(i) Chemical cleavage

Alkaline phosphatase and rennin are examples of agents that cause cleavage of phosphate groups and peptide bonds respectively. In the case of rennin, cleavage of K-casein results in the formation of para-K-casein, an insoluble species which, as previously discussed, is known to cause aggregation of protein molecules. Likewise β -casein can be cleaved by protease to yield γ -casein and a small peptide fraction.

(ii) Oxidation and Reduction

Sulphur containing amino acids of proteins are susceptible to oxidation-reduction effects. Thus disulphide bonds are susceptible to mild reducing conditions and polymerisation of surface film is possible on reoxidation.

(iii) Halogenation

The reaction of iodine with proteins and the changes in properties that result have been discussed in earlier chapters. In milk lines where hypochlorite is used, the formation of insoluble chloro-proteins is a possibility. Such species have been implicated in deposit formation (510).

(iv) The Effect of pH and Ionic Strength

Since proteins are polyelectrolytes containing both cationic and anionic groups derived from weak acids and bases, many intermolecular and intramolecular interactions are electrostatic and are critically dependent upon pH and ionic strength. It has already been shown in Section 10.3 that treatment of adsorbed protein with acid solution causes an irreversible inhibition of

desorption. Aggregation and precipitation of caseins as their pH's are lowered to their isoelectric points is commonly used in their preparation. Here however, the effect is reversible.

Increase in ionic strength, especially for concentrated protein solutions, is also known to cause aggregation. The molecular weight of α_{s1} -casein increases to more than 60 000 at $I = 0.05$ and β -casein at $I = 0.20$ has a molecular weight of over 400 000 (see section 2.5). If these aggregation effects were irreversible they could lead to the formation of macroscopic films.

(v) Reactions with Functional Groups

Protein molecules undergo a wide range of reactions involving the $-COOH$, $-NH_2$, $-OH$, $-SH$, and tyrosyl groups. These include acylation (e.g. with acetic anhydride, acetic acid, N-acetylimidazole and succinic anhydride), esterification (e.g. with methanol/HCl), reductive alkylation (e.g. reaction of amino groups with aldehydes and ketones followed by reduction), reaction with maleimides (e.g. reacts with sulphhydryl groups and to a lesser extent, amino groups) and nitration (e.g. tetranitromethane nitrates tyrosyl residues). By such chemical modification it seems possible that the surface properties of an adsorbed protein layer could be modified in such a way as to promote further adsorption. In particular, reactions that convert hydrophilic ionic groups into hydrophobic hydrocarbon chains could be expected to promote further protein adsorption.

Bridging or 'Sandwich' Mechanisms

Many species are known to interact strongly with proteins and in some instances to act as bridging species binding together two protein molecules. This effect, operating between an adsorbed protein molecule and a solution protein molecule, could provide a mechanism for multilayer adsorption. How the bridging species is applied to the adsorbed protein is immaterial to the mechanism. In dairy milk-lines adsorption and evaporation-deposition effects during and between cleaning cycles could be involved. Although as in the previous section, the main interest of the present discussion is in species that may be present in milk-line systems, some reference will also be made to other species which seem to be of interest and relevance.

(i) Multivalent Cations and Anions

Multivalent ions, either by electrostatic or coordination interactions, could act as a bridging species. The implication of calcium ions in the formation of milkstone is suggested by its known ability to bind with casein (36) and bovine fibrinogen (512), the increased problems associated with hard water and the fact that exchangeable calcium ions are thought to play an essential role in stabilising milk micelles. In milk micelles and possibly also in milkstone deposits, calcium ions are considered to act as a bridging species between carboxyl groups.



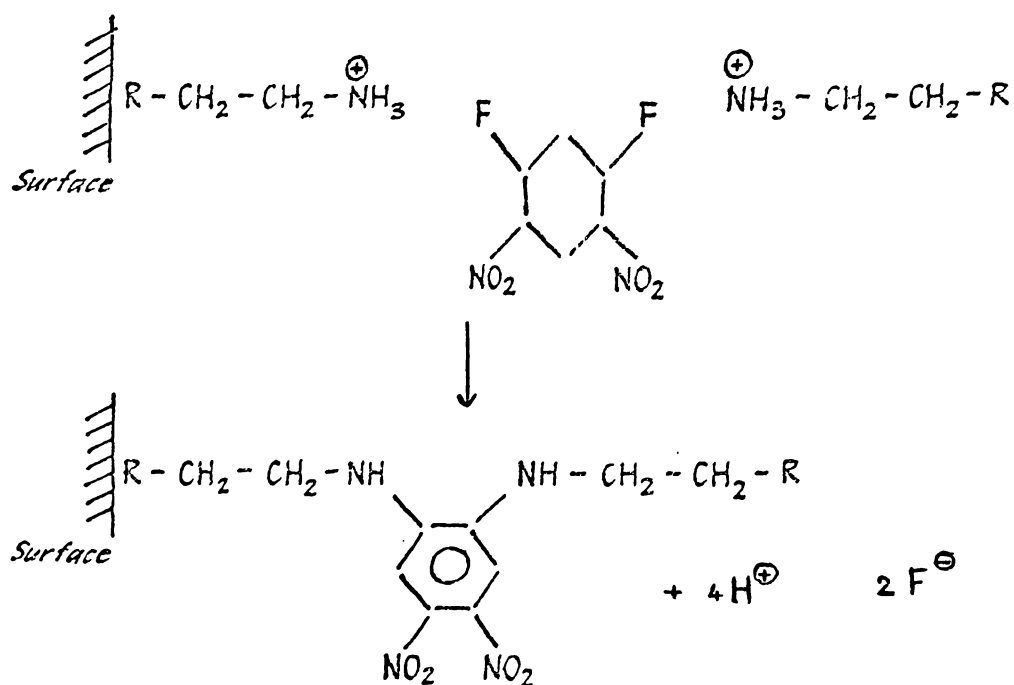
Numerous other multivalent cations are known to bind strongly to proteins and so could act as bridging species. Examples include

Fe(III) (440), Mg (II) and Mn(II)(514), Cu(II)(515), Ni(II), Hg(II), Pb(II), Cd(II).

Multivalent anions such as phosphate and polyphosphate could also conceivably act in the same general manner.

(ii) Specific Protein Binding Agents

A number of species including many drugs are known to bind specifically to proteins. Examples include the binding of corticosteroids to plasma protein (516), acetyl salicylic acid to albumin (517) the association between progesterone and di acid glycoprotein (518) and the numerous agents that have been developed for the biochemical separation technique of affinity chromatography (e.g. cyanogin bromide). These interactions are often quite sensitive to experimental conditions, involve a single primary binding site and can be easily inhibited by the presence of ions such as lead ions or silver ions. It would seem likely that a prerequisite of any bridging species is that the species has at least two reactive groups able to introduce intermolecular cross links. Bifunctional reagents such as 1,5 difluoro 2-4 dinitrobenzene which react primarily with amino groups may serve this function between adsorbed protein and solution protein molecules. e.g.



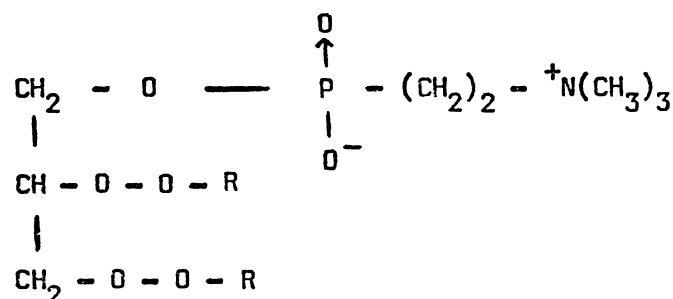
In solution this reaction has been used to cross link derivatives of pancreatic ribonuclease (519). Other species effective in inter and intramolecular cross linking include p p¹ - difluoro-mm¹ dinitrodiphenyl sulfone (520,521) and iodoacetamide (522).

(iii) Surfactants

The interactions of surfactants both synthetic and biological are sufficiently important to justify a separate section. In many cases their interactions are very similar (523).

(a) Biological Surfactants

Phospholipids of the general structure



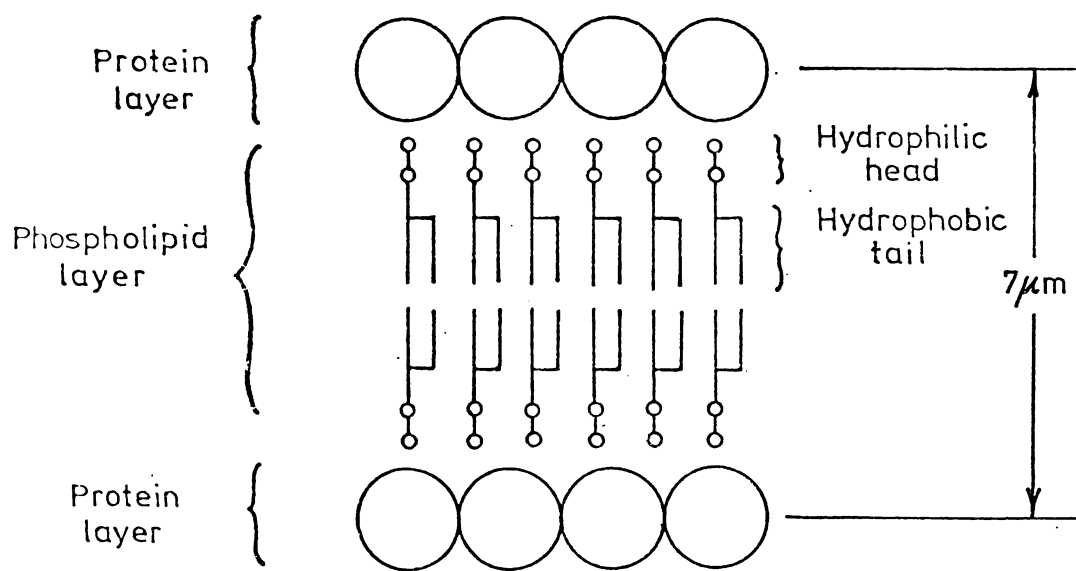
are biological surfactants that play important roles in cellular structure particularly in the membranes of cells where the phospholipids tend to align themselves in a bilayer of about $7\mu\text{m}$ thick with the hydrophobic hydrocarbon chains facing one another (see figure 11.1) and with the hydrophilic ends of the molecules pointing outwards. In cellular membranes this phospholipid layer is thought to be sandwiched between outer layers of protein and polysaccharide, though more recent models allow the protein to be located within the bilayer as well. If conditions were correct it is conceivable that multiple layers of this type could be formed. Relevant to the possibility of adsorption of milk protein by such a mechanism are the studies of the interaction between casein fractions and synthetic lecithins. The lipid alkyl chain length, the casein fraction and the method employed all seem important in determining whether a complex will be formed (524,525,526).

(b) Synthetic Surfactants

Synthetic surfactants such as Triton X-100 (octyl phenoxy polyethoxy ethanol) and sodium dodecyl sulphonate bind to proteins

Figure 11.1

A Postulated Structure of Lipid Bilayer



at the biologically active lipid binding areas (527,528,529,530) and have been used to determine the lipid binding behaviour of some proteins. Typical binding of sodium dodecyl sulphate with hydrophilic proteins occurs at concentrations about one quarter of that of the critical micelle concentration (528). Triton X-100 does not interact with hydrophilic proteins (528) but both Triton X-100 and sodium dodecyl sulphate interact with hydrophobic proteins.

There is no evidence that interactions between protein and surfactant leads to aggregation. In fact with casein, synthetic surfactants cause the dissociation of the high molecular weight casein aggregates presumably by preferential binding with the hydrophobic regions of the casein molecule (531,532).

The binding of synthetic cationic surfactants to proteins is similar to anionic surfactants except that the surfactant monomer must be about ten times more concentrated (533).

(iv) Colloidal Particles and Macromolecules

The formation of colloidal multilayers by the alternated application of different colloids that have a high affinity for one another (usually but not necessarily of opposite charge) is a phenomenon that has been well established (534). Proteins are known to bind strongly to a number of species that can be prepared in colloidal form including allophane (535), halloysite and kaolinite (207) and calcium hydroxyapatite (536,538). The sorptive properties of hydrous oxides are also well known.

The possibility of building up protein multilayers via bridging species of colloidal iron oxide is particularly relevant to the present work in that many farm water supplies in the Waikato district, particularly those farms located in peat soils, contain high levels of iron.

Scope of Present Investigations

From the above review it appears that macroscopic protein films could be formed either by repeated adsorption after chemical modification of the adsorbed layer or by repeated adsorption via a bridging species. It is beyond the scope of the present investigation to test all these mechanisms thoroughly under all the conditions that could be important in order to achieve the maximum effects. Instead the experimental section that follows was designed principally to demonstrate that such multilayering effects can occur under laboratory conditions and thus demonstrate that such a mechanism could provide a means of protein accumulation at surfaces in technological systems. In order that the work have relevance to the dairy industry the systems chosen for the laboratory studies were ones that could exist in some form in farm and factory milk lines. For this reason no attempt was made to study protein bridging through specific binding agents not likely to occur in milk.

11.3 Materials and Method

The radiochemical methods developed to study protein adsorption after a single exposure of the surface to the radioactively labelled solutions offered a simple means of studying

adsorption after repeated exposures. For these experiments the "slide" method (see Chapter 7) seemed most appropriate. It offered a method of much greater sensitivity than simulator methods previously used to study the effect of repeated milk contact-cleaning cycles (199). In order to demonstrate a multilayering effect it was decided that a four-dip sequence should be adequate. Of course even if a monolayer (8 mg m^{-2}) was adsorbed after each dip, many more than four cycles would be necessary to form a macroscopic deposit capable of sustaining bacterial colonisation.

One disadvantage of the radiochemical method based upon iodinated protein is that only the adsorption of protein can be monitored. This problem could be overcome in those instances where suitable isotopes are available for double labelling experiments. Alternatively a mass sensitive technique based upon a vibrating quartz crystal currently being investigated at the University of Waikato may eventually find application in these studies.

As with the adsorption experiments described in Chapter 9, stringent precautions were taken to avoid contamination. All experiments were performed in the dust free environment provided by a lamina flow work bench. Protein solutions were prepared from freshly iodinated skim milk at four-fold dilution. Clean stainless steel slides were initially exposed to labelled skim milk for 20 minutes. They were then rinsed with distilled water for 20 seconds, air dried and counted. After applying conditions to induce modification, either on the adsorbed layer

or by adsorbing a bridging species, the slides were again rinsed and dried. Following this the slides were again dipped in protein solution, rinsed and monitored for increase in activity. The results are plotted as bar diagrams showing successive adsorption steps using a relative adsorption factor which is the ratio of counts after N cycles compared with counts after the first cycle.

11.4 Results

1. Successive dipping without Intermediate Treatments

Preliminary experiments demonstrated that surfaces freshly saturated with protein showed no tendency to adsorb further protein on repeated further exposures to milk solutions.

2. Effect of Drying Time and Drying Temperature

The effect of prolonged drying periods (30 minutes) at room temperature (in lamina flow work bench) and at 140 °C (in an oven) was tested over a four cycle adsorption experiment. Results shown in figure 11.2 indicated that after the initial adsorption no further protein adsorption resulted in successive cycles even with the drying and heat treatment.

3. Treatment with Phosphoric Acid, Sodium Hypochlorite and Sodium Chloride

The effect of 2% phosphoric acid, 1 mol l⁻¹ sodium chloride and 28 ppm sodium hypochlorite was tested. After the protein adsorption step the slides were rinsed, dried, counted and then immersed for ten minutes in the solution under study. The slides were rinsed, dried and counted again before being reimmersed in

Figure 11.2

Effect of drying temperature

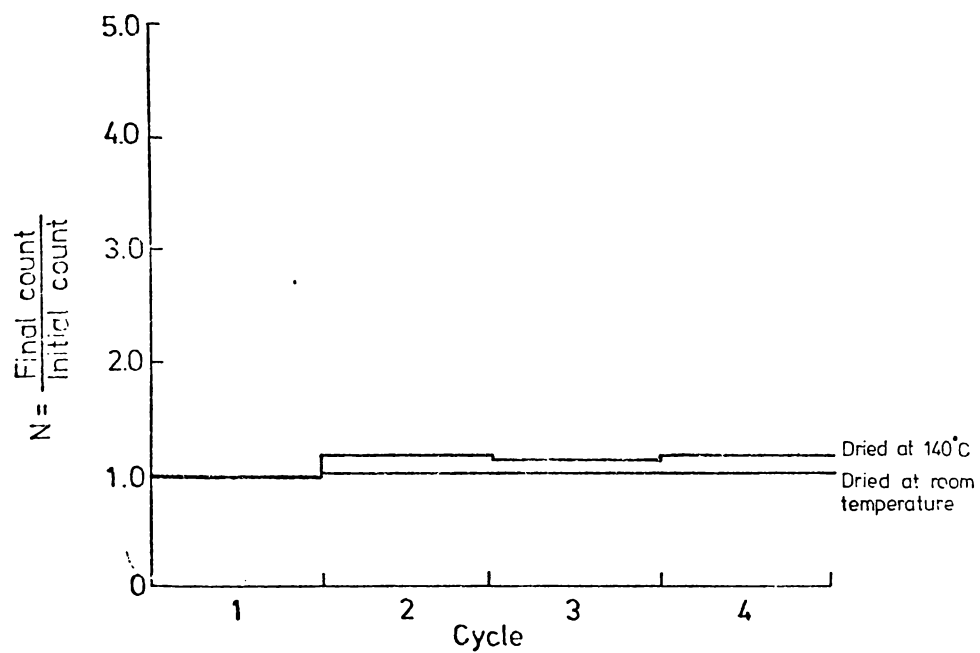
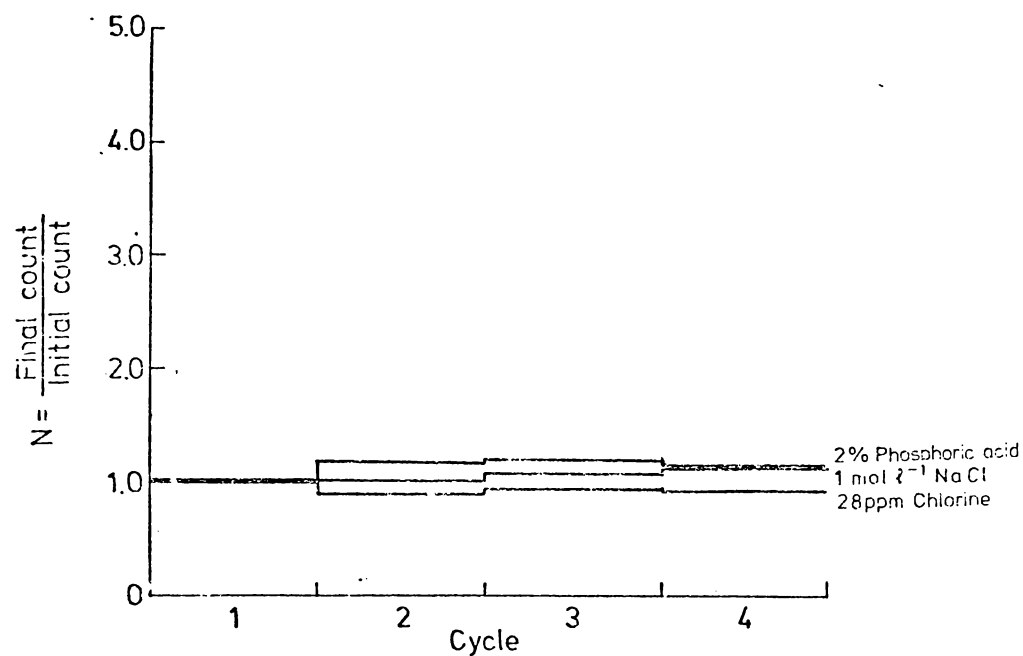


Figure 11.3

Effect of phosphoric acid, sodium hypochlorite and sodium chloride rinses



the protein solution. The results (see figure 11.3) indicated that none of the reagents tested had any effect. The negative result for hypochlorite was surprising in view of the published data for the effect of this species in milk line studies (510) in which hypochlorite at 28 ppm chlorine was implicated as being a milkstone promoter.

4. Effect of Multivalent Cations and Anions

Species investigated included CaCl_2 , MgNO_3 , FeCl_3 , and Na_3PO_4 . The CaCl_2 solution was made up to 0.01 mol l^{-1} which corresponded to a water hardness of 600 ppm expressed as CaCO_3 . This would correspond to a very hard water. The other solutions were used at concentrations of 0.2 mol l^{-1} , 0.01 mol l^{-1} and 0.1 mol l^{-1} respectively.

The results for all the above species were negative as can be seen from the data in figure 11.4. Presumably this experiment means that the involvement of species such as the calcium ion and the ferric ion in milkstone formation is other than as the simple cations acting as electrostatic bridging species.

5. Effect of Natural and Synthetic Surfactants

As an example of a naturally occurring surfactant, the phospholipid DL - α -phosphatidyl dipalmitoyl, a constituent of milk, was applied to the stainless steel strip after an initial protein adsorption experiment. The surfactant was applied to the strip via the air-liquid interface by dropping the phospholipid dissolved in benzene on to the surface of a beaker of distilled water.

Figure 11.4

Effect of multivalent cation and multivalent
anion salt rinses

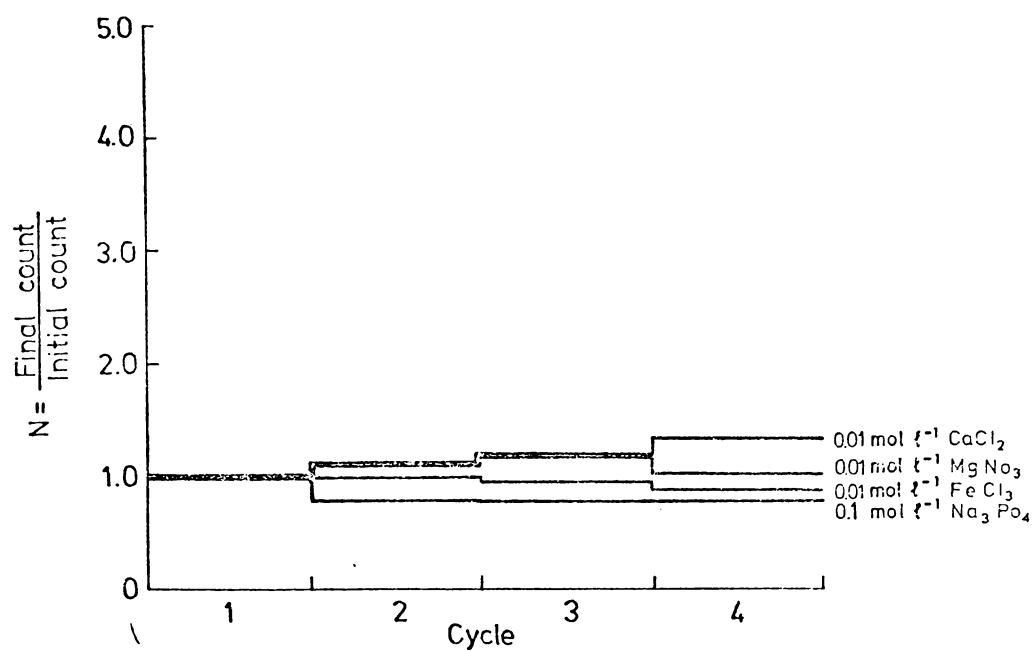
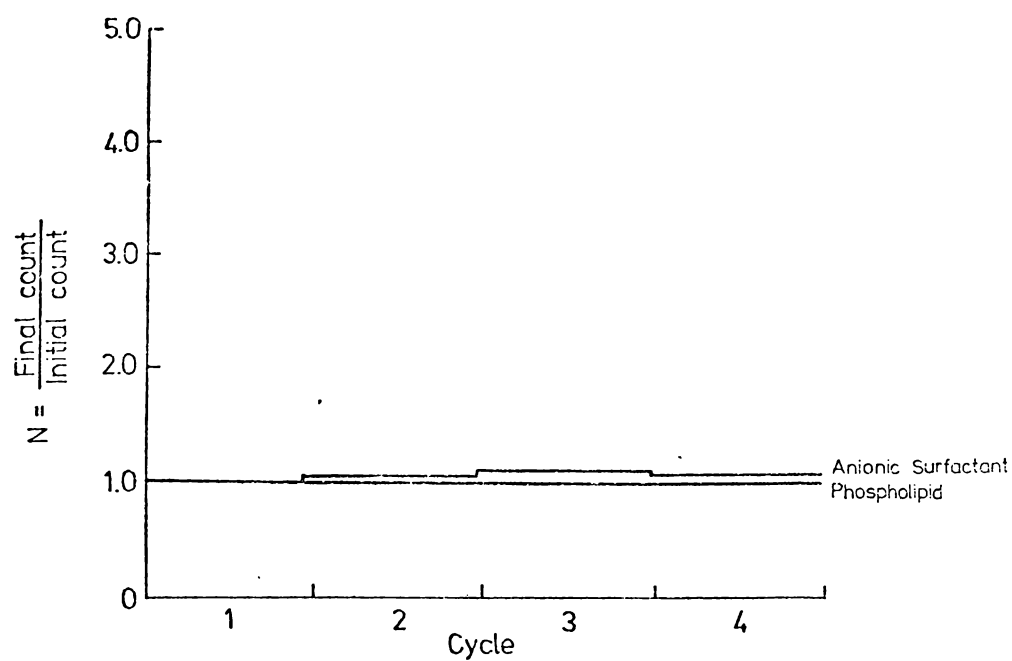


Figure 11.5

Effect of surfactant rinses



The synthetic surfactant, sodium alkyl aryl sulphonate was applied to a protein covered surface from a 0.01% solution at pH 8.6.

The results for both experiments involving surfactants as potential bridging species were negative (see figure 11.5).

6. Colloidal Hydrous Oxide Bridging Species

Four colloidal species were chosen for these studies. They included allophane, colloidal silica, chromium hydroxide colloid, and iron oxide colloid. The first three of these species have relevance to the milkstone problem in that they are possible colloidal constituents of farm water supplies and chromium hydroxide colloid is of interest because the surface of stainless steel is predominantly oxides of chromium. Details of the preparation of the colloidal species were as follows:

Allophane : purified allophane (an amorphous aluminosilicate) which had an isoelectric point of pH 6.0 was obtained from the University of Waikato (539).

Silica Colloid : this colloid was obtained from B.D.H. Laboratory Chemicals. It has an isoelectric point of pH 2 (13).

Chromium hydroxide Colloid : 4×10^{-4} mol l⁻¹ chromium sulphate was heated at 75 °C for 26 hours. The colloid prepared by this procedure is reported to have an isoelectric point of 7.7 (541).

Iron Oxide Colloid : 5 ml of 10% w/w aqueous solution of ferric chloride was added to 300 ml of distilled water and refluxed

for one hour. The resulting colloid was dialysed with distilled water until the pH rose to pH 5. Iron colloid prepared under these conditions has an isoelectric point of 6-7.

The pH at which each experiment was performed was adjusted to ensure that the colloidal species would have a well defined charge. The experiments with allophane and silica colloid were performed at pH 7.2 and 7.0 respectively at which pH the colloids would be negatively charged. Ferric oxide and chromic hydroxide colloids were studied at pH 5 and so would have been positively charged.

The results after four cycles using these species are summarised in figure 11.6.

It appeared that under the conditions employed neither of the negatively charged colloids (allophane and silica) caused enhanced adsorption after the first cycle. However, in the case of the positively charged iron and chromium colloids, a definite increase in surface protein after each adsorption cycle was observed.

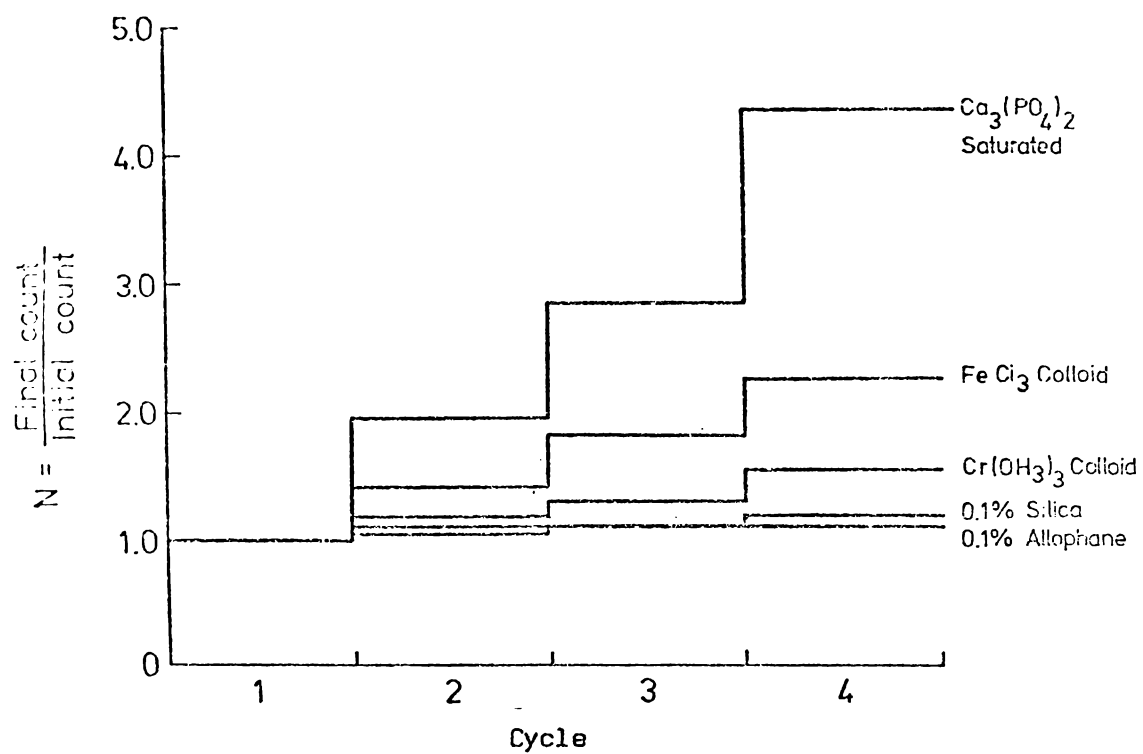
"Colloidal" Calcium Phosphate Bridging Species

The possibility of calcium phosphate species acting as a bridging agent was of interest both because calcium phosphate is thought to be involved in stabilising the structure of milk micelles and also because the formation of calcium phosphate species is a real possibility in hard water where high phosphate detergents are employed.

Tricalcium orthophosphate is only very slightly soluble in cold water (0.002% w/w) at neutral pH. A suspension was therefore

Figure 11.6

Effect of Colloidal Suspension Rinses



prepared by dissolving the salt in acid solution to form a 0.1% solution. The pH was then raised slowly to pH 6.4. Tricalcium orthophosphate has an isoelectric point of approximately 7.6 and therefore is positively charged at pH 6.4.

The results shown in figure 11.6 indicated that treatment with the calcium phosphate suspension induced marked enhancement of protein adsorption after each adsorption cycle.

Electron Microscope Examination

The adsorption process induced by treatment with tricalcium orthophosphate was monitored at 2000 X magnification using a Scanning Electron Microscope. The results (see figure 11.7) show that the particles of calcium phosphate range in size from $0.5\text{ }\mu\text{m}$ to $1\text{ }\mu\text{m}$ and were adsorbed only after the surfaces were first treated with the milk protein solution. The density of the particles increased upon successive adsorption cycles but only after treatment with the protein solutions.

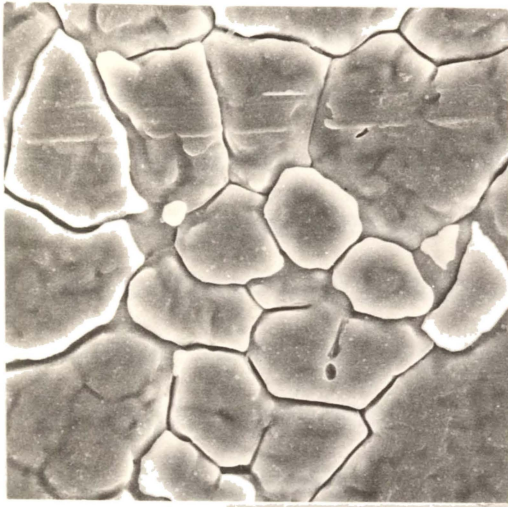
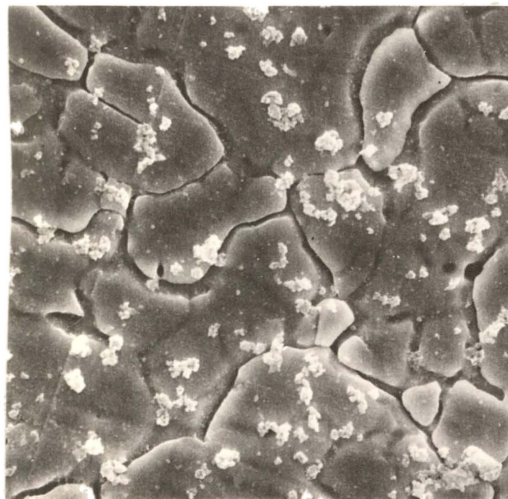
11.5 Discussion and Conclusion

(a) Possible Mechanism for Milkstone Formation

Although not exhaustive in their scope, the experiments of this section have demonstrated that the formation of protein multilayers by repeated adsorption is a process that requires special conditions. Most of the experiments described were chosen in order to have some relevance to the formation of protein films at milk contact surfaces. It appeared from these experiments that the conditions most likely to lead to the

Figure 11.7

Protein Mineral Aggregation by Repeated Adsorption

 $\text{Ca}_3(\text{PO}_4)_2$ (four cycles)Protein/ $\text{Ca}_3(\text{PO}_4)_2$ (one cycle)Protein/ $\text{Ca}_3(\text{PO}_4)_2$ (four cycles)

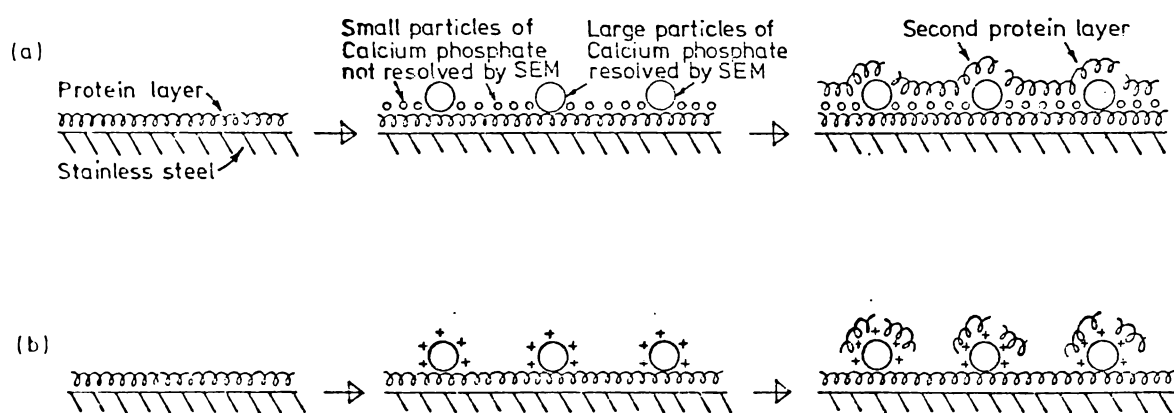
formation of macroscopic films are those in which the surfaces come in contact with positively charged colloidal species after initial protein adsorption. The colloidal particles are adsorbed on top of the first protein layer on the surface, in such a way that further protein can be adsorbed when the surfaces next come in contact with protein containing solution.

The electron micrographs for the calcium phosphate treated surfaces suggested a possible mechanism for the 'multilayer' build up. This is represented diagrammatically in figure 11.8. Depending upon whether the area between the large particles is covered by smaller colloidal particles not resolved by the S.E.M. the two alternatives (a) and (b) could be important. According to alternative (a) the large and small colloidal particles are closely packed on to the protein surface in such a way that the properties of the solid-liquid interface are essentially those of $\text{Ca}_3(\text{PO}_4)_2$, a strong adsorber of protein. On the other hand the wide distribution of the large particles adsorbed initially could result from the effect of electrostatic repulsion so that the adsorption pattern would be better represented by figure 11.8(b).

The absence of an effect for surfaces treated with negatively charged colloids is not surprising in view of electrostatic effects. The adsorbed protein layer could be expected to have a -ve charge at the pH of the experiment and hence would repel negatively charged colloidal particles.

Figure 11.8

Possible Mechanism for Multilayer Adsorption



(b) Relevance to Formation of Mineralised Deposits in
Biological Systems

The demonstration that successive adsorption of protein and of mineral components provides a mechanism for the formation of macroscopic deposits has relevance beyond the problem of milkstone formation in the dairy industry. The formation of other mineralised deposits such as calculus on teeth, and kidney stones have similar features to milkstone. The presence of calcium and phosphate in all these deposits suggests the possibility of a common mechanism of formation. In the case of kidney stones, the consensus of opinion at present is that the stones form initially as a protein gel that is progressively mineralised by a calcification process (see Section 2.9). It is unlikely that such a mechanism could account for milkstone formation. The question therefore arises as to whether a colloidal multilayering effect could provide a feasible alternative mechanism for the formation of all of these deposits.

(c) Scope for further Investigation

The results of this chapter have demonstrated that although multilayering of protein can be achieved by repeated exposure to milk solutions, a stepwise enhancement of adsorption can be achieved by alternative treatment of the surfaces with protein solution and a suspension of a positively charged colloid. Further studies of this effect to illustrate the effect of pH and surface charge would be of interest.

The experiments reported above were intended primarily to demonstrate the likelihood of protein multilayering being induced by species likely to be found in dairy milk lines. There is much scope for further investigation of artificially induced multilayering using other protein bridging species of the type described in section 11.2.

CHAPTER 12

FORMATION OF MACROSCOPIC PROTEIN FILMS BY EVAPORATION AND PRECIPITATION

12.1 Introduction

Any surface wetted by a film of solution containing a non-volatile solute must become covered by that solute when the solvent is allowed to evaporate. Depending upon the nature of the solute, either discrete crystals, amorphous particles, or a coherent amorphous film (solid or liquid) may be formed. The ease of removal of this deposited film will depend upon such factors as the size of the crystals, thickness and mechanical properties of film formed and the nature of the interaction between the deposited material and the surface.

Films may also be formed on surfaces during the precipitation or aggregation of dissolved species. The ease of removal of this type of deposit will also depend upon factors similar to those of deposits formed by evaporation. Both the mechanisms: evaporation and precipitation, could be important in milkstone formation.

12.2 Deposits formed by Evaporation

In dairy milk lines, conditions conducive to evaporation of milk result as a consequence of the use of partial vacuum, warm temperatures, and the repeated wetting and drying of surfaces. Experiments were therefore performed to determine the thickness of the liquid film left after exposure of freshly cleaned stainless

steel to various concentrations of skim milk. These results were compared with film thicknesses left adhering to surfaces previously saturated with adsorbed protein. The effect of age and temperature on dried protein films was also studied briefly.

a. Materials and Methods

The method used to study the formation of milk deposits by evaporation was based on the "slide" method used previously to study adsorption (see section 7.4). The only modifications of the method described previously were that on removal from solution, the stainless steel slides were not rinsed and were subjected to a variety of drying, heating and aging treatments. Iodinated milk protein was used for all experiments apart from the experiment used to determine the liquid film thicknesses. In this experiment I-125 iodide was added to skim milk.

b. Results

1. Protein solution film thickness

The thicknesses of the films of protein solution left adhering to clean stainless steel slides immersed for thirty seconds in a range of diluted solutions of I-125 iodide labelled skim milk at 25 °C were determined by counting the activity left on the drained surface after evaporation. Draining and evaporation were achieved by suspending the slide vertically. The excess solution that drained to the bottom of the slide was carefully removed with filter paper. The small amount of iodide adsorbed under these conditions was estimated by repeating the experiment except that

each side was washed with distilled water for twenty seconds immediately upon removal from the milk solution. The adsorbed activity was subtracted from the radioactive count obtained due to the evaporated film and the film thickness was calculated. The amount of protein deposited was then estimated from the protein concentration of the milk solution (see Table 12.1).

Table 12.1
Film Thickness formed on Stainless Steel from Diluted
Milk Solutions (25 °C)

Protein Concen- tration /mg ml ⁻¹	Film Thickness /10 ⁻⁴ cm	Amount Deposited /mg m ⁻²	Film Thickness (milk treated) /10 ⁻⁴ cm	Amount Deposited (milk treated) /mg m ⁻²
38	3.7	140	0.6	23
23	4.4	101	1.5	34
20	3.9	78	2.3	46
11	3.2	35	2.0	22
5	4.0	20	1.9	9.5

The solution film thickness was found to be approximately constant at $3.8 \pm 0.6 \times 10^{-4}$ cm. This experiment was repeated to study the effect of milk pretreatment on the thickness of the solution film. The pretreatment involved dipping the clean stainless steel slide into skim milk for thirty seconds, removing and rinsing in distilled water for twenty seconds and finally allowing it to dry for one hour at room temperature. The film thickness experiment was then repeated as described previously. The solution film thickness varied with milk dilution and was

observed to plateau at $2.0 \pm 0.5 \times 10^{-4}$ cm, approximately half the film thickness of the untreated surface (see Table 12.1).

2. The Effect of Drying Time at 35 °C

Clean stainless steel slides were dipped into fourfold diluted skim milk at 35 °C. The stainless steel slides were withdrawn from solution after ten minutes and held vertically for varying drying periods at 35 °C. They were then rinsed with distilled water for twenty seconds, dried and counted to determine the protein left adhering to the surface.

The surfaces were observed to be visually dry after three to four minutes drying at 35 °C. It appeared that the effect of drying was most significant over this period. However the results also indicated that even after the surface had dried, prolonged periods of drying caused more protein to be retained (see figure 12.1).

3. Thermal Modification of Evaporated Milk Films

The results of the experiments described in section 12.2.2 above, suggested that modification of evaporated milk films occurs after prolonged drying periods. It was decided to examine the effect of temperature on these films. Clean stainless steel slides were dipped into fourfold diluted milk at 4 °C for ten minutes. The slides were then removed and dried for thirty minutes at temperatures ranging from 4 °C to 120 °C and then counted. After rinsing with distilled water for twenty seconds (at 20 °C), the slides were again counted and the

Figure 12.1

Variation of Protein Retained with Drying Time (35 °C)

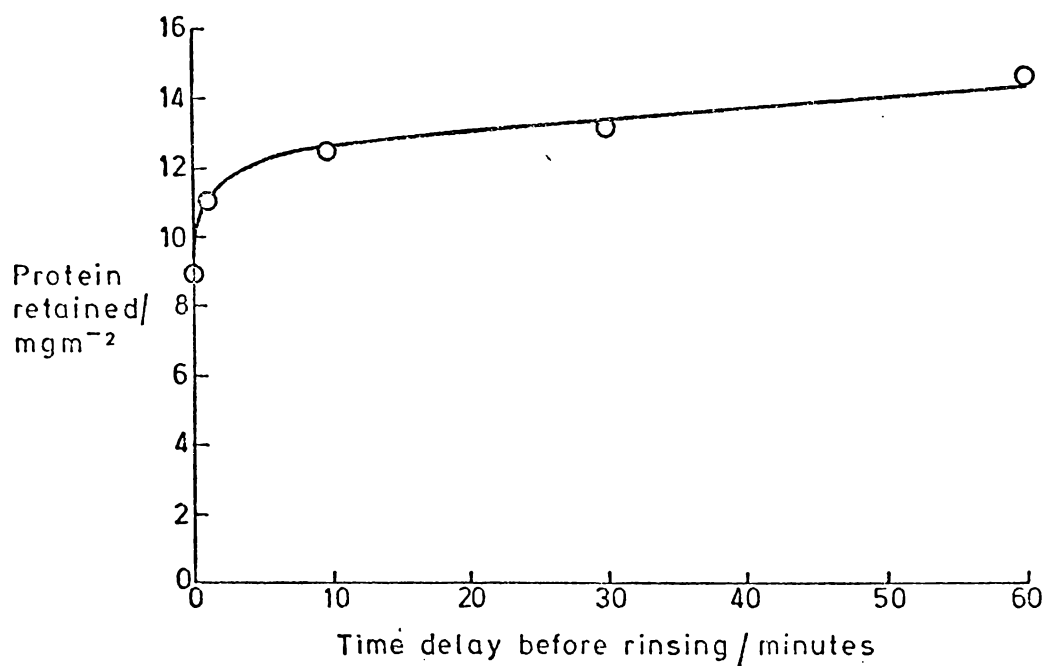
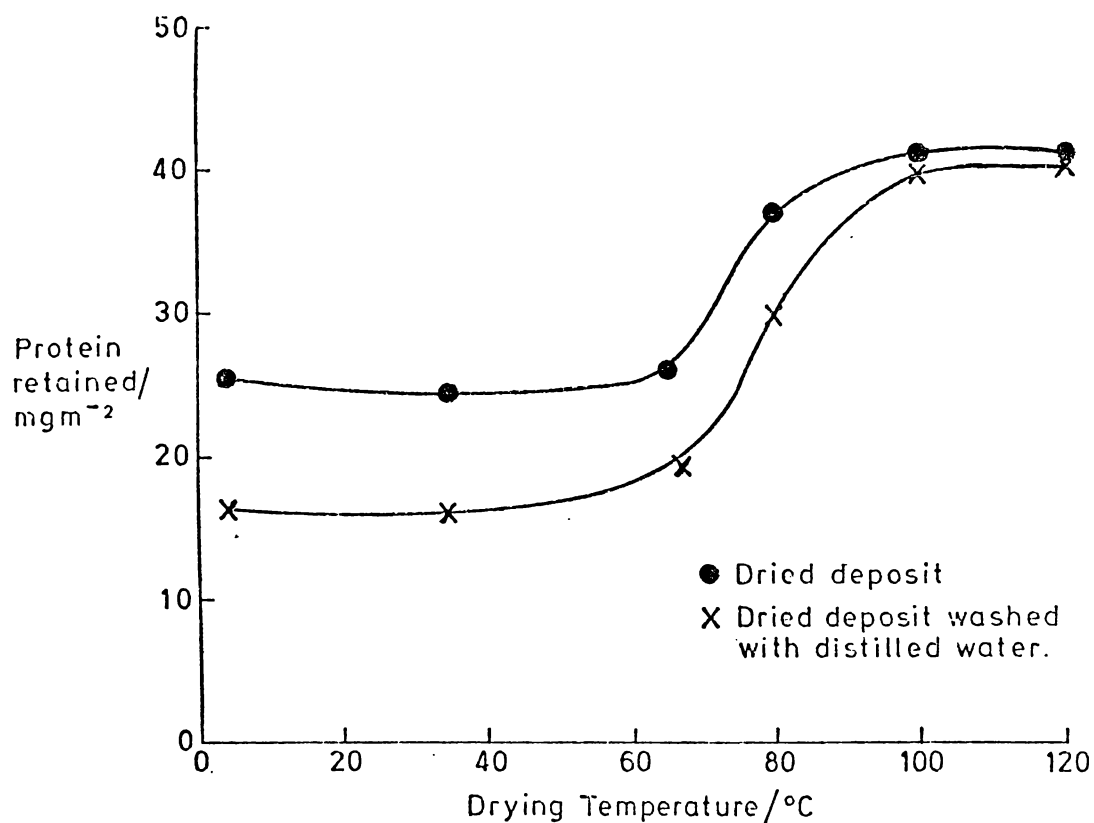


Figure 12.2

Variation of Protein Retained with Drying Temperature
(30 minutes drying time)

percentage of the deposited protein removed was calculated.

The results are shown in figure 12.2 where it is apparent that significant modification of the deposited protein occurred when heated above 100 °C. In this experiment it was observed that above 65 °C, there was a significant increase in the total activity deposited on the surfaces. This, presumably, was due to evaporation before the surfaces had adequate time to drain completely.

12.3 Deposits formed by Precipitation

Although precipitation effects were not investigated in detail, the importance of this mechanism could be inferred from the large amount of casein that was observed adhering to glass surfaces after the precipitation of caseins from milk at pH 4.5 during separation of the individual casein fractions. Any agent that caused the precipitation of milk constituents could be expected to enhance milkstone formation. In particular acid cleaners that for any reason were not used at sufficiently low pH and could thus be troublesome. Also the acid conditions produced by bacterial action could be expected to enhance milkstone formation.

12.4 Discussion and Conclusion

Clean stainless steel surfaces when dipped into milk or protein solutions and then allowed to drain are covered with a liquid film approximately $3.8 \pm 0.6 \times 10^{-4}$ cm thick. These liquid films dry rapidly to leave a layer of deposited protein, the amount depending upon the protein concentration of the solution. The effect of prior exposure to milk solutions is to reduce the thickness of the film.

A brief study was made of the thermal modification of deposited films. It was found that significant modification of the deposited protein occurred when heated above 65 °C. These thermally modified films were much more difficult to remove.

It follows from the results described above that evaporation of whole milk films, and precipitation by inappropriate detergents and sanitisers or by the acid condition produced by bacterial action, could result in significant protein deposition. Evaporation of protein containing films of rinse solutions is probably less important. Assuming the solution film left adhering to the contact surface was 4×10^{-4} cm, a protein concentration of approximately 2.0 mg ml^{-1} would be required to leave a protein film equivalent to a monolayer of adsorbed protein. This protein concentration corresponds to a 10-fold dilution of whole milk. Generally it could be expected that the milk concentration in rinse solutions would be very much less than this.

CHAPTER 13

STUDIES OF MILKSTONES FORMED IN DAIRY MILK LINES

13.1 Introduction

Throughout the duration of the project described in this thesis, samples of milkstone were collected and examined with a view to accumulating information about such deposits that might eventually lead to a better understanding of their formation and properties. Generally the deposits were collected from dairy farms in the Waikato area although some samples were obtained from other localities around New Zealand. One sample was obtained from a dairy factory. Wherever possible the history and condition of formation (e.g. cleaning system, water supply) were compiled for each sample but in many cases such information was not available.

13.2 Materials and Methods

Samples were collected from milk contact surfaces. Often the milkstone film was so thin that the amount of deposit obtained was less than 0.05 grams. The samples were stored in stoppered bottles and refrigerated. After visual inspection, a small sample was removed for electron microscopic examination. This sample was also used for qualitative and semi-quantitative trace element analysis by the electron probe technique. Detailed chemical analysis was then performed by conventional methods if sufficient sample was available. Finally samples

containing significant amounts of mineral were examined for crystallinity by X-ray diffraction.

1. Sample Preparation for Electron Microscopy and Electron Probe Analysis

Approximately 0.05 grams of the deposit was placed in 10 ml of 2% gluteraldehyde solution and equilibrated for twelve hours. The sample was then washed with distilled water to remove excess gluteraldehyde. It was then washed with increasing amounts of alcohol-water mixture to remove the water from the sample. An initial rinse for five minutes with 5% alcohol solution was followed by five minute rinses with 25%, 50%, 75%, 95% and finally absolute alcohol. The alcohol was then replaced by Freon TF and the sample was then critical point dried. The sample was mounted on to a brass shield using double sided adhesive tape and coated with gold palladium before examination with a Jeol JSM-35 Scanning Electron Microscope.

The same sample was used for an electron probe analysis to determine the main mineral constituents and their distribution throughout the sample. The electron probe analysis was made semi-qualitative by considering the ratio of peak heights using appropriate standards. This procedure proved particularly valuable for the investigation of the calcium:phosphorous ratio of light and dark bands of a deposit consisting of quite distinct layers (see Section 13.3).

2. Chemical Analysis

Analysis for moisture, protein, total ash, calcium, phosphorus and iron were carried out according to the procedures described in the appendix 9.

3. X-ray Diffraction

Samples containing minerals were dried at 35 °C where necessary, ground to a fine powder and examined with a Philips 1130 X-ray Diffraction Unit. Where crystallinity was observed, X-ray diffraction files (439) were used, in an attempt to identify the crystalline components.

13.3 Results

Preliminary Studies

Preliminary Studies to develop procedures for handling milkstone deposits, particularly for electron microscopy studies, were centred on an old milkstone sample (sample x) formed in a tin-nickel plated milk line. This sample had been stored for several years at the National Dairy Laboratory, but unfortunately, relatively little was known about its history. The visual appearance of the deposit was of a thick, crusty, white deposit with a rough surface texture (see figure 13.1).

An electron microscopic examination (see figure 13.2) revealed a mixture of an apparently amorphous material interlaced with well formed needle shaped crystals. Electron probe analysis (see figure 13.3) indicated the presence of calcium, phosphorus and sulphur in the crystals. X-ray diffraction confirmed the presence

Figure 13.1

Milkstone Deposit x



Table 13.1

X-ray Powder pattern of Milkstone Sample x

Sample		$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$			$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$		
$d/\text{\AA}^\circ$	Intensity	$d/\text{\AA}^\circ$	Intensity	hkl	$d/\text{\AA}^\circ$	Intensity	hkl
7.59	70	7.56	100	020	7.57	100	020
4.27	100	4.27	50	12T	4.24	100	021
3.05	70	3.06	55	14T	3.05	75	111,041
2.86	44	2.86	25	002	2.92	50	22T
2.68	30	2.68	28	022,051	2.62	50	220,15T

Figure 13.2

Electron micrograph of Milkstone Deposit x
(X 900)

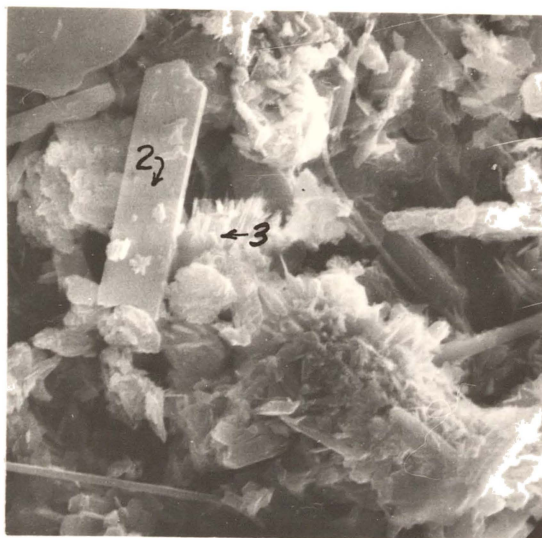
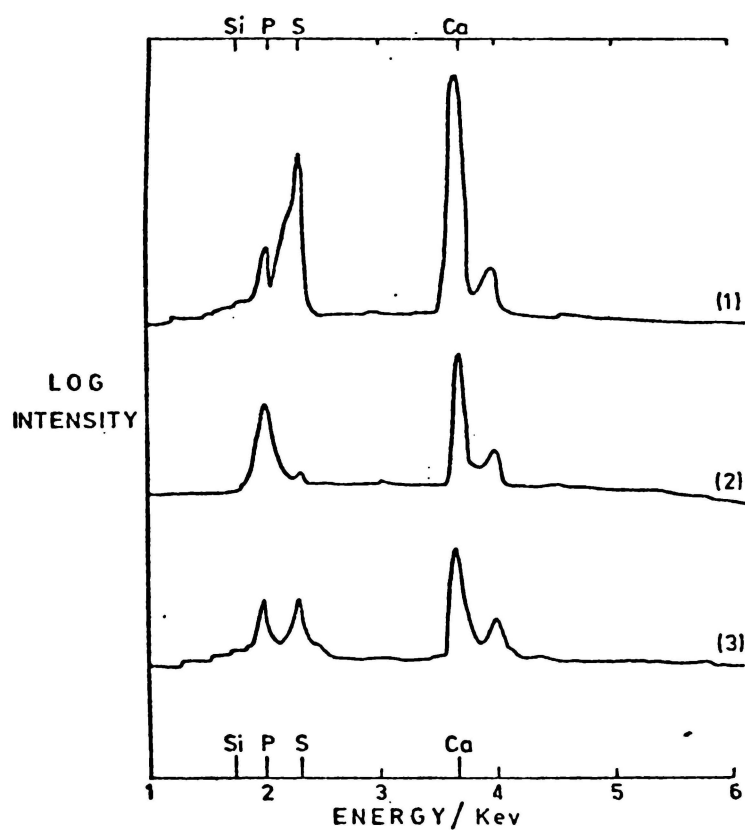


Figure 13.3

Energy Dispersive XRF Spectra of Milkstone
Deposit x



Note: (1) Complete scan analysis of figure 13.2
(2) and (3) One spot analysis on figure 13.2.

Table 13.2

Composition of Milkstone Deposits

Sample	x	a	b	c	e	f	h	i	m
Moisture	10	19.5	8.0	5.1	22	34	86	48	16
Protein	13.3	62	12.5	1.8	76	91	72.8	89.5	40.5
Ash	69.0	8.5	61.0	61.5	3.4	7.5	17.1	<2.0	35
Calcium	21	2.6	21.7	12.6	<1.0	<0.1	2.1	<0.1	9.5
Phosphorus	11.8	1.4	11.8	6.9	<1.0	<0.1	1.7	<0.1	7.2
Iron	<0.5	<0.1	<0.1	17.4	<0.1	<0.1	8.6	<0.1	<0.1
Sulphur	4.8	<0.5	<0.1	<0.1	<0.5	<0.5	<0.5	<0.5	
Fat	-	-	-	-	-	-	-	9.6	

All percentages expressed as percentages of dry weight

of calcium hydrogen orthophosphate and calcium sulphate (see Table 13.1). Chemical analysis showed that the deposit contained only a small percentage of protein (see Table 13.2) and very little water.

The results of the above study indicated the scope of the physical methods used in determining detailed data on the composition and structure of amounts of deposits much smaller than can be handled by traditional 'wet' chemical procedures. The existence of crystallinity was surprising in view of the fact that there do not appear to be any reports in the literature of crystallinity in milkstone deposits. Further samples were examined by the same general procedures.

Survey of Milkstone Deposits

1. Deposits studied

The following is a short description of the deposits studied (see figure 13.4).

Deposit a: brown/white flaky deposit collected March 1974 from a farm in the Te Awamutu area. The history relating to the formation of the deposit is unknown.

Deposit b: hard brown/white flaky deposit scraped from the claw of a milking machine at the National Dairy Laboratory. Its history is unknown.

Deposit c: brown flaky deposit collected in January 1974 from a farm milk line near Whatawhata. The farmer was using a water supply which had a total hardness of 25 ppm, an iron content of 30 ppm and dissolved minerals of about 150 ppm. A hot alkaline

detergent solution was used. Although much milkstone was evident, the milk quality had not been affected.

Deposit d: brown flaky deposit collected from a farm at Westport, South Island. The history related to the formation of this deposit is unknown.

Deposit e: brown soft stringy deposit collected in January 1975 from a Waikato farm. The farmer was using a good quality water low in dissolved minerals and hot chlorinated alkaline based detergent to clean his milking machine.

Deposit f: brown soft stringy deposit obtained from a Waikato farm in March 1975. This farmer had good quality water and was using hot chlorinated alkaline based detergent to clean his milking machine.

Deposit g: brown soft deposit collected in January 1976 from a farm at Gardonton. The farmer had a good water supply and was using a hot chlorinated alkaline detergent to clean his milking machine.

Deposit h: brown soft watery deposit collected in February 1976 from a farm plate cooler on Holland Road, Hamilton. The farmer was using a water supply low in calcium and magnesium but containing 2 ppm iron. A cleaning procedure based on a hot alkaline detergent with a periodic acid rinse was used.

Deposit i: white soft deposit collected in March 1976 from a milk line of a milking machine near Morrinsville. No information about the water used to clean the milking machine is available. The cleaning system was based on a hot alkaline detergent.

Figure 13.4 Milkstone Deposits



Milkstone Deposit a (4 X)



Milkstone Deposit b (4 X)

Figure 13.4

Milkstone Deposits



Milkstone Deposit c (6 X)



Milkstone Deposit d (4 X)

Figure 13.4

Milkstone Deposits



Milkstone Deposit e (3 x)



Milkstone Deposit f (4 x)

Figure 13.4

Milkstone Deposits



Milkstone Deposit g (4 X)



Milkstone Deposit h (7 X)

Figure 13.4

Milkstone Deposits



Milkstone Deposit i (4 X)



Milkstone Deposit j (2 X)

Figure 13.4

Milkstone Deposits



Milkstone Deposit k (1 X)

Deposit j: brown flaky deposit received in January 1977. It was from the rubber tubing used in the pipeline of a milking machine. Its history is not known.

Deposit k: brown flaky deposit collected in February 1977 from a milk line of a milking machine at No.5 Dairy, Ruakura. The water used to clean this milking machine was low in dissolved minerals. The cleaning system used on this farm was an alkaline detergent followed by an acid iodophor.

Deposit l: brown soft deposit collected in June 1978 from a milk line of a milking machine near Matangi. The water supply was good apart from containing colloidal silica. The cleaning system used on this farm was an acid detergent sanitiser. No photograph of this deposit was taken.

Deposit m: brown hard deposit obtained from an evaporator in a milk processing dairy factory. The history of the deposit was largely unknown though it was estimated to have formed over several months and was known to have been subjected to temperatures greater than 100 °C.

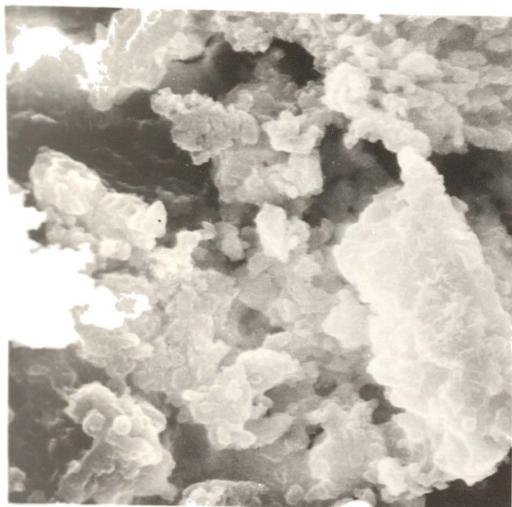
2. Scanning Electron Microscope Studies

All the deposits collected were examined under the scanning electron microscope to see if any characteristic features were present (see figure 13.5). Although each deposit appeared to have unique features, four broad classifications could be recognised. These were:

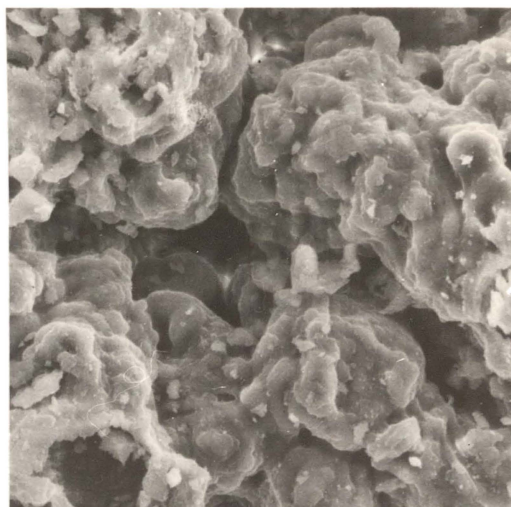
Figure 13.5

Electron Micrographs of Milkstone Deposits

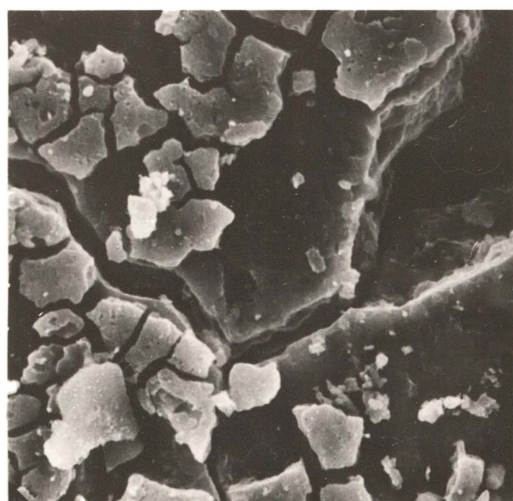
(x 900)



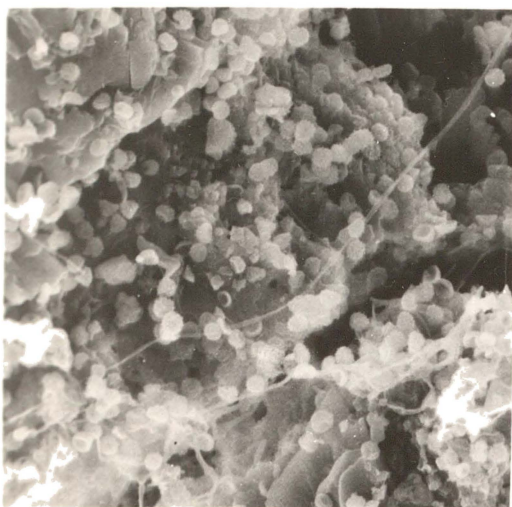
(a)



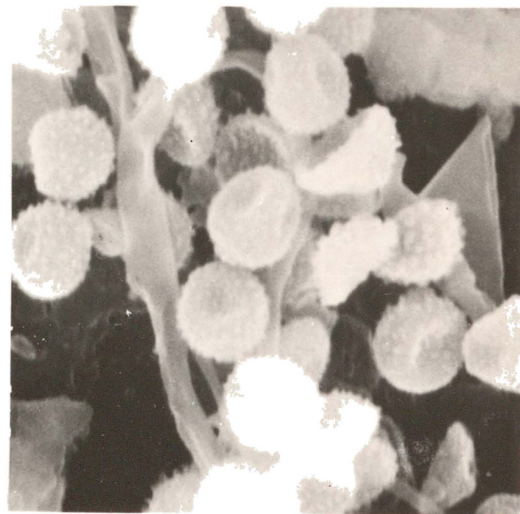
(b)



(c)



(d)



(x 5000)

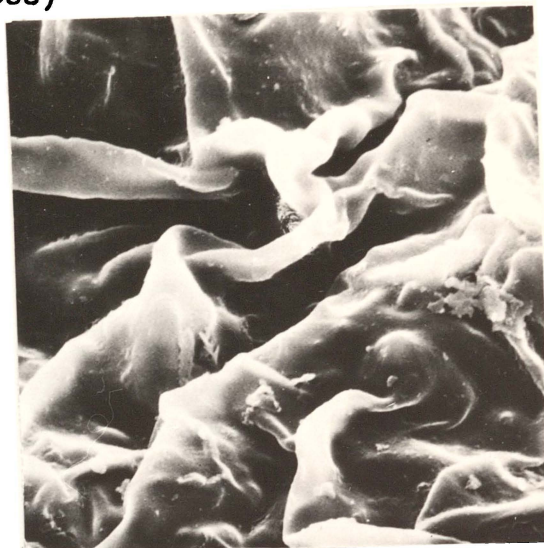
Figure 13.5

Electron Micrographs of Milkstone Deposits

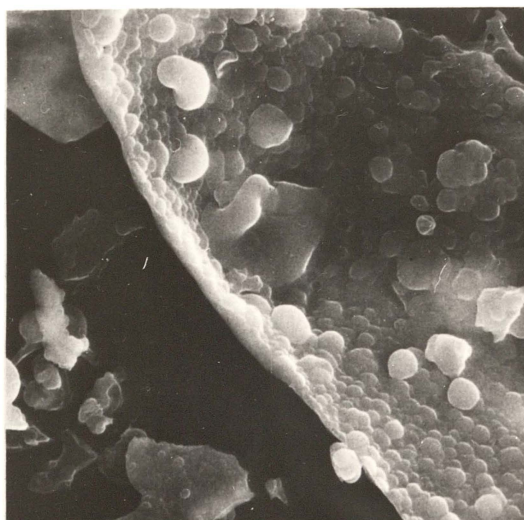
(x 900)



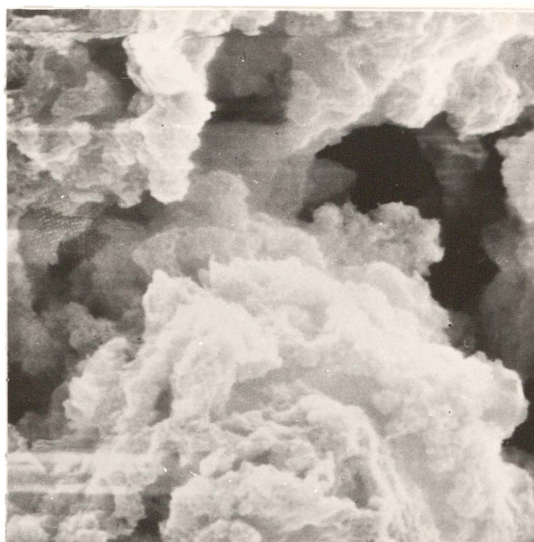
(e)



(f)



(g)



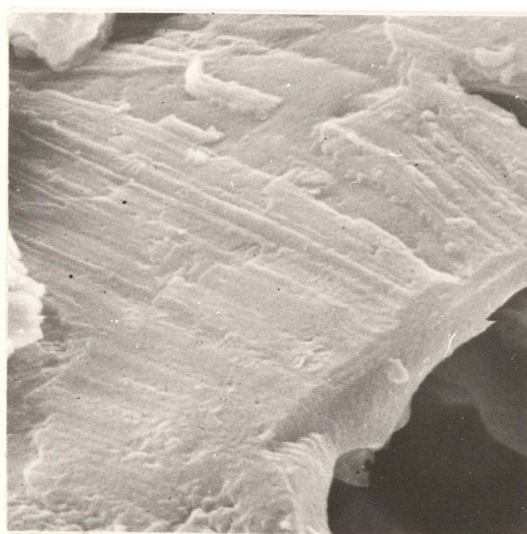
(h)

Figure 13.5

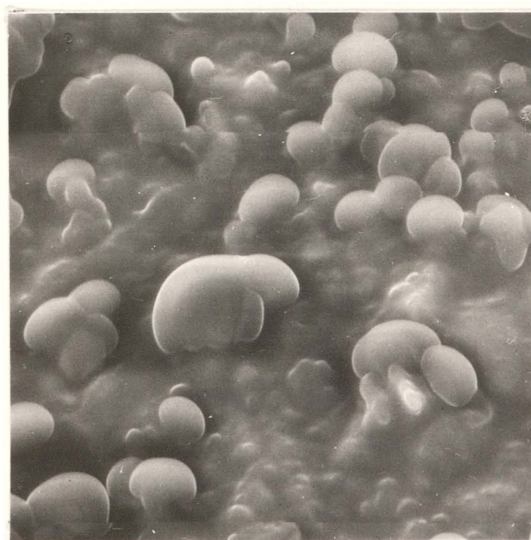
Electron Micrographs of Milkstone Deposits (X 900)



(j)



(k)



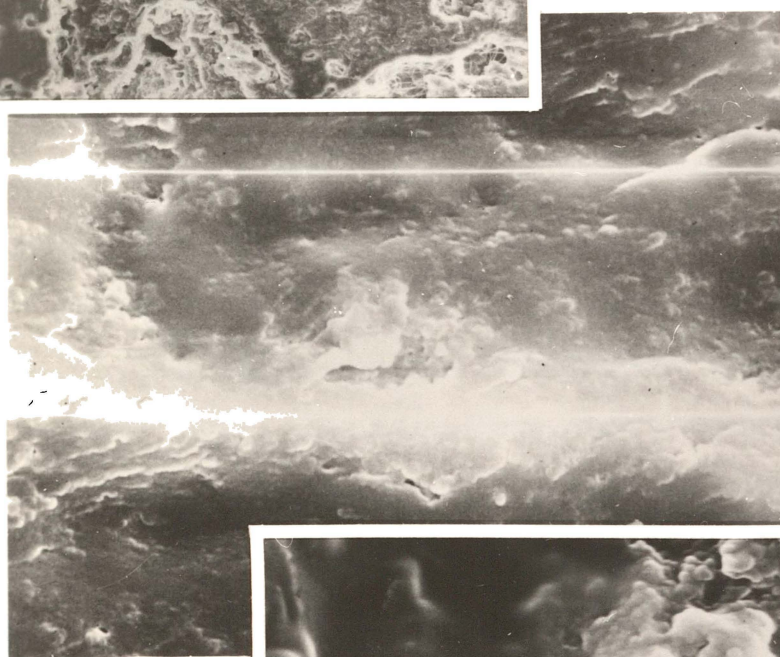
(l)

Figure 13.5

Electron micrograph of Heated Induced Milk Deposit m



a
(X 300)



b
(X 2000)



c
(X 2000)

1. Flaky Deposits - a, b, c, d, h, j, k
2. Globular Deposits - g, l.
3. Stringy Deposits - e, f.
4. Layered Deposits - m

Considerable difficulty occurred in trying to obtain a clean image of Deposit i and eventually no micrograph was taken of this deposit. Numerous bacterial spores were observed in Deposit d.

3. Analysis of Deposits

Deposit composition was determined by wet chemical analysis, together with the electron probe analysis. The electron probe was used mainly as a qualitative tool, but semi-quantitative information was obtained by considering peak heights for those samples that could not be analysed chemically due to insufficient sample. The analysis of the main components of each deposit are shown in Table 13.2. The energy dispersive X-ray spectra of each deposit is shown in figure 13.6. Apart from the analysis reported in Table 13.2, copper was observed in deposit c, silica in deposit f, l, chlorine in deposit e, f, g, and i and iodine in deposit k. The electron probe was also used to monitor the layer formation in deposit m.

The energy dispersive X-ray spectra for each region in the heat induced milk deposit m are shown in figure 13.6. Using the same acceleration voltage, standards of known calcium: phosphorus ratios were examined and a calibration curve using the

Figure 13.6
Energy dispersive XRF Spectra of Milkstone Deposits

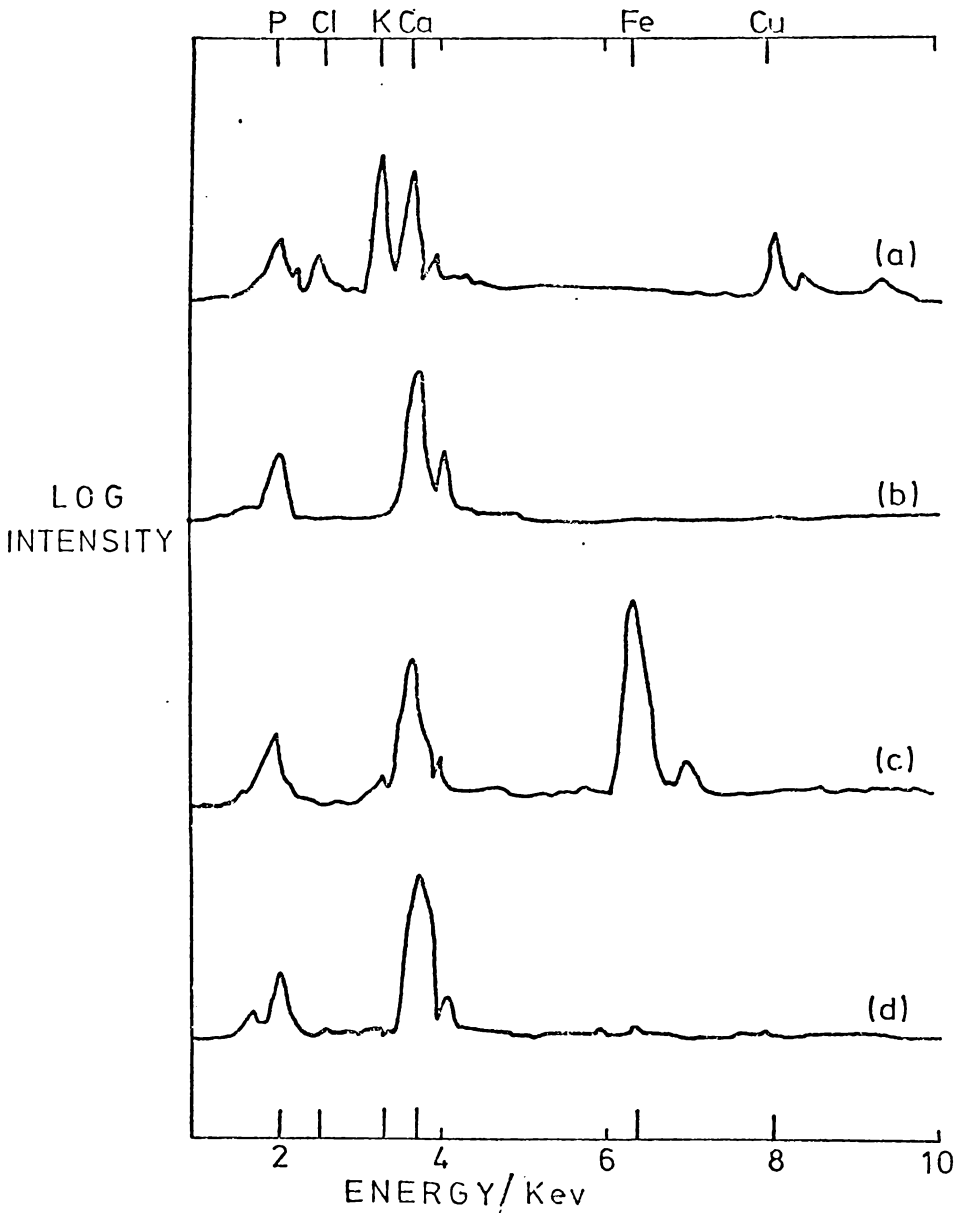


Figure 13.6

Energy dispersive XRF Spectra of Milkstone Deposits

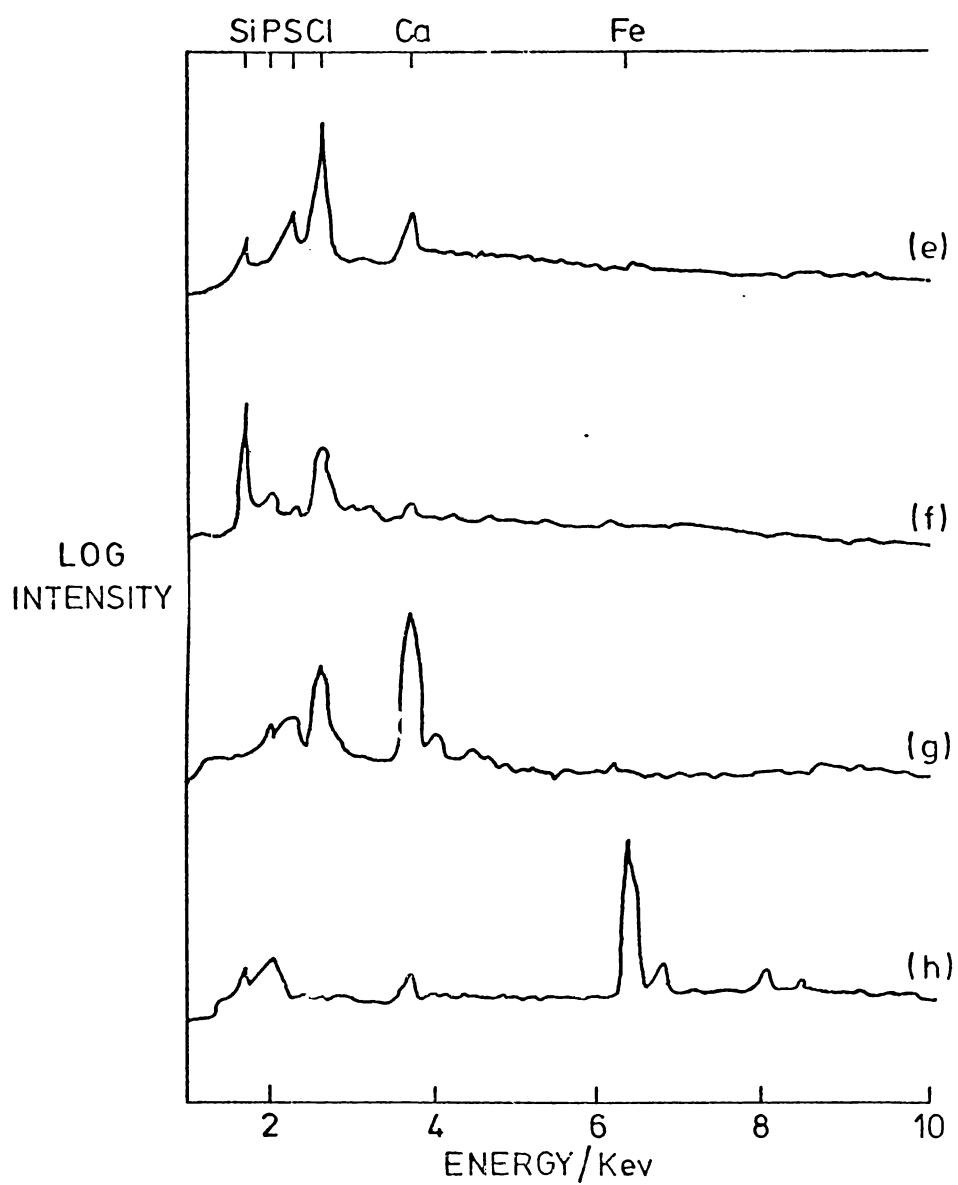


Figure 13.6

Energy dispersive XRF Spectra of Milkstone Deposits

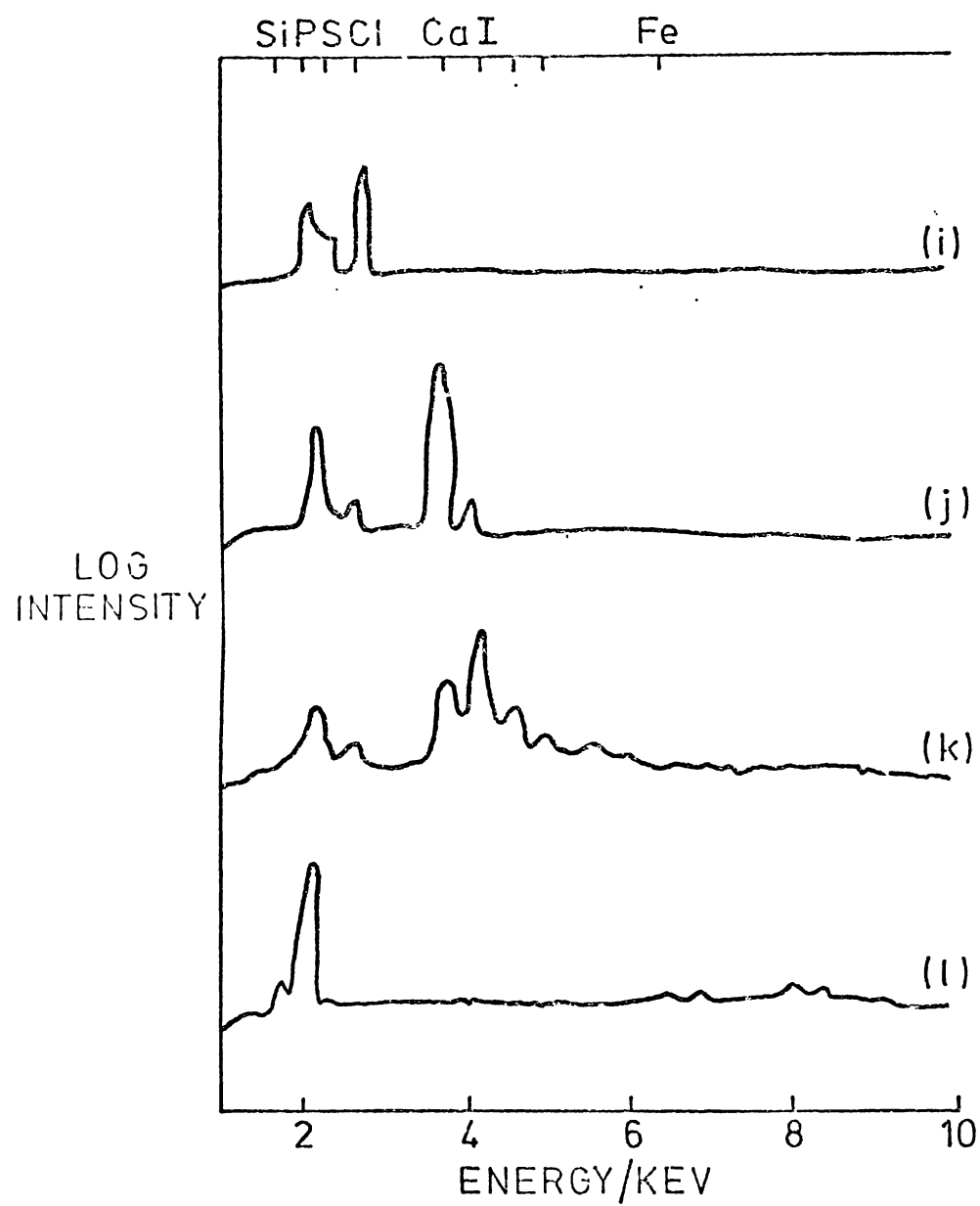
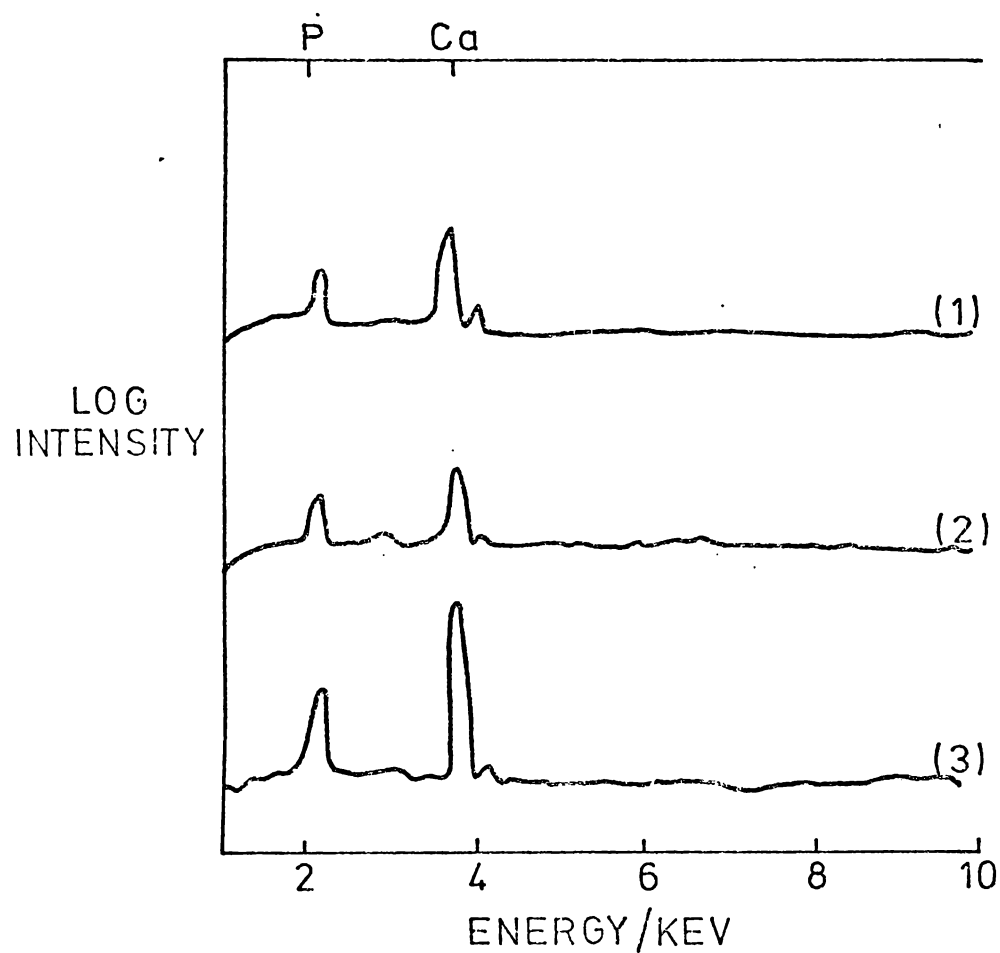


Figure 13.6

Energy dispersive XRF Spectra for heat induced
milk deposit m



1. General Scan
2. Dark Band
3. Light Band

various peak heights was then obtained (see figure 13.7).

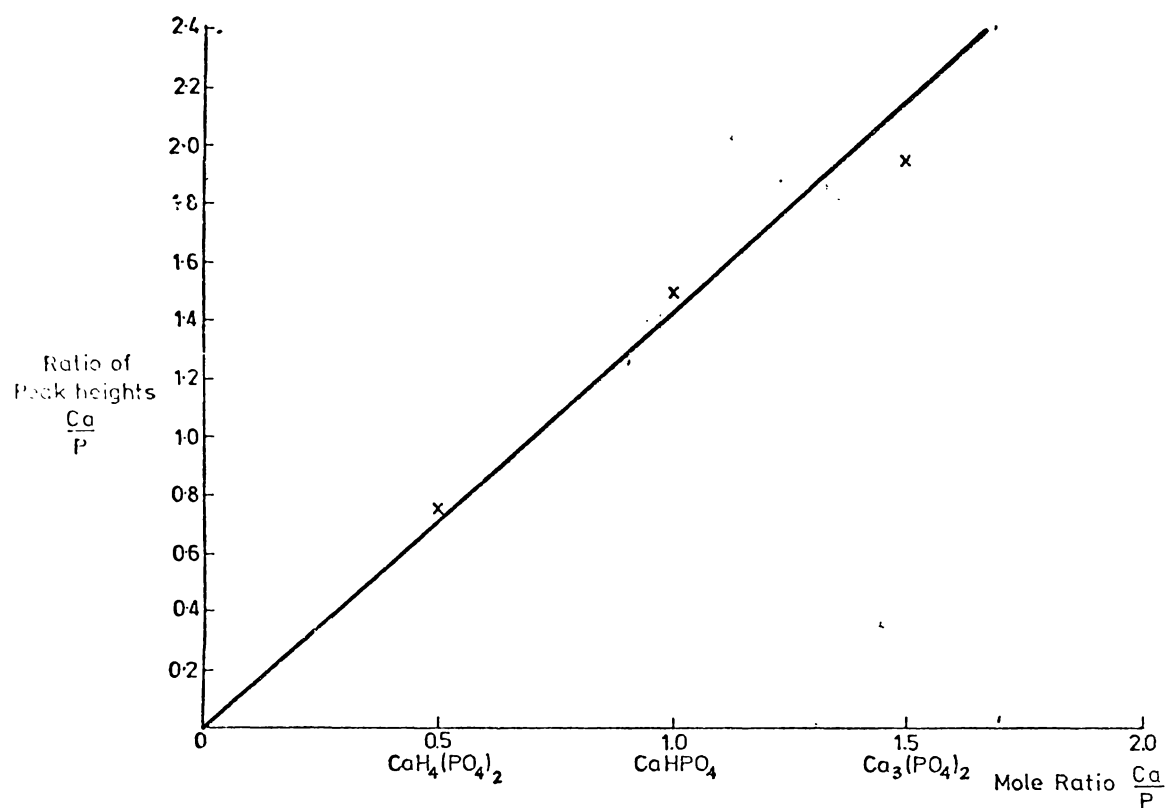
Using this calibration curve it was found that the dark bands had a calcium:phosphorus mole ratio of 0.95:1 and the light band, a calcium:phosphorus mole ratio of 1.7:1. A general scan gave a calcium:phosphorus mole ratio of 1.3:1 which agrees closely with chemical analysis (see Table 13.2) and with other published results (261). Skim milk has a calcium:phosphorus mole ratio of 1.0:1 (34).

It was observed that under the same counting conditions the peak heights of calcium and phosphorus in the dark bands were approximately one third of the peak heights of calcium and phosphorus in the light bands. This result indicated that there was more mineral in the light bands than in the dark bands. X-ray diffraction of the deposit indicated the presence of small amounts of calcium apatite.

One explanation for the layering effect might be differential precipitation of calcium phosphate species during evaporation. This possibility was eliminated by evaporation of a solution of milk and the examination of a vertical section of the deposit with the electron probe. No variation in the calcium:phosphorus ratio was observed.

A more likely explanation can be based on the formation of an initial film (either by evaporation or heat induced deposition) of whole milk. This initial film could then be modified during an inefficient cleaning cycle which removed relatively more protein than mineral and more phosphorus than calcium. The increase in the calcium:phosphorus ratio might also have been brought about by the use of hard water in the cleaning cycle.

Figure 13.7

 $\frac{\text{Ca}}{\text{P}}$ mole ratio calibration curve

13.4 Discussion

The visual, analytical and microscopic examination of the various deposits indicated that generally each deposit had unique characteristics. Some broad generalisations may be applied although the number of samples examined was too small to allow definite classifications. In most cases samples high in mineral content were hard and appeared flaky under microscopic examination, whereas samples high in protein were soft and either stringy or globular. Samples h and a appeared to be exceptions in that they were high protein deposits and had a flaky appearance. However sample h was unusual in that it contained a very high percentage of water so that its flakiness could have resulted during drying for the scanning electron microscopic study. Sample a also had a high water content though not exceeding the water content of some of the other high protein deposits which appeared stringy and globular under electron microscopic examination.

All deposits contained some protein ranging from as low as 1.8% up to 91%. They all had compositions different from that of dried milk films. The chemical compositions of the deposits indicated the importance of inorganic constituents in their formation. The high mineral deposits were probably derived from water high in calcium or iron (III). The presence of phosphorus or chlorine in the high protein deposits suggested that in the former case the use of phosphoric acid (possibly at inadequate concentrations) may have contributed to the formation of deposits. Likewise the presence of chlorine in the deposits indicated the use of chlorine

based detergents, which possibly induced the formation of insoluble chloro-protein.

The results of the survey discussed in this chapter tend to support the milkstone classification suggested as a result of the earlier and more restricted survey described in Chapter two. This survey classified milkstone deposits in terms of high protein milkstone, calcium phosphate milkstone, iron (III) milkstone and fat deposits. It seems that as a result of the present survey, the high protein milkstones could be further classified according to the detergent or sanitiser constituents that probably contribute to their formation. Thus phosphoric acid milkstone and chlorine milkstone might be appropriate classifications. It is likely that in the formation of both types of deposit, these constituents cause precipitation of the milk proteins.

CHAPTER 14

GENERAL DISCUSSION

14.1 Introduction

One of the principal objectives of the present study was to investigate factors that could be involved in the formation of milkstone, the protein containing films formed on milk contact surfaces. In order to undertake this project it was necessary to develop procedures for the radiochemical labelling of milk constituents and techniques for investigating aspects of the chemistry of these constituents at stainless steel surfaces. To a large extent the results of these studies have been discussed in the chapters in which they were presented. Some aspects using the results of several different sections of the work have yet to be considered. These include the kinetics and thermodynamics of protein adsorption, the mechanism of milkstone formation, some practical consequences of the investigation and suggestions for further work.

14.2 Kinetics and Thermodynamics of Protein Adsorption

The adsorption studies of Chapters 8 and 9 showed that monolayers of protein were formed on stainless steel surfaces within very short times upon contact with milk solutions. The affinity of protein for stainless steel surfaces was such that protein even at low concentrations eventually became adsorbed

providing the surface had not already become "saturated". From the rate studies of Chapter 9 a diffusion model was developed to account for the kinetics of adsorption from unstirred solutions. While this model is probably an over simplification and starts to breakdown at high concentrations, it provides a useful picture of the adsorption process. According to the model protein adsorption is limited by size, shape and hydration of the protein and the available area of the surface. Irreversibility of the adsorption process was assumed. Further it was assumed that adsorption proceeds without denaturation to form a close-packed surface film. Adsorption from stirred solutions should also be diffusion controlled. However, because of the much shorter diffusion path lengths the rate of adsorption should be much faster. Slow desorption kinetics (at least at neutral pH's) provided evidence of the irreversibility of the adsorption process over short time intervals. However some slow exchange did occur. The kinetics of desorption of these processes could not be analysed in terms of any simple rate law.

Adsorption studies indicated that stainless steel surfaces generally "saturated" after monolayer coverage had been achieved, although evidence for two adsorption plateaux was obtained in the case of β -casein. Once surfaces had become saturated they showed little tendency to adsorb further protein.

14.3 Mechanism of Milkstone Formation

The studies of adsorption effects after repeated exposures of the surfaces to protein solutions eliminated the possibility of simple adsorption being an important mechanism in the accumulation stage of milkstone formation. Other factors must be involved. Treatment by positively charged colloidal species of ferric oxide and calcium phosphate caused successive layers of protein to be adsorbed. A sandwich mechanism of this type could explain the formation of milkstone. It is unlikely, however, that the special conditions required are sufficiently common for this type of mechanism to be generally valid. In the light of the results of the investigations described above, it seems more likely that precipitation and/or evaporation effects are implicated in deposit formation. Effects contributing to the formation of deposits include:

1. protein deposition by evaporation of milk films followed by protein denaturation and mineralisation;
2. protein precipitation induced by cleaning conditions;
3. mineral deposition (from hard water) by evaporation followed by protein adsorption upon the next contact of the surface with milk.

It is difficult to test the mechanism of milkstone formation in actual dairy milk lines because of the many variables that can exist. However, data from simulator studies used to evaluate dairy detergents may allow assessment of the mechanism of deposit formation under closely controlled conditions that prevail in these devices. Though simulators are designed to reproduce

farm milk line cleaning conditions as accurately as possible, the time factor alone prevents conclusions from their use being applied directly to dairy milk lines. Also in many instances deposit formation in simulators is induced deliberately in order to maximise the capability of the device to discriminate between different detergents.

14.4 Mechanism of Deposit Formation in Simulators

Results from studies involving three simulators, A(189), B(256) and C(356), were analysed to determine average values for the total deposit formed per cycle and the amount of protein deposited per cycle. In order to simplify the analysis a linear increase in deposit formation was assumed so that mean per-cycle-increments of the deposit could be calculated. Also in order to calculate the amount of protein deposited after each cycle it was necessary to take a value of 30% as the protein content of the deposits formed in two of the simulators (simulator B and simulator C). This value was the mean of the percentage protein composition of the deposits formed in simulator A. Data for the mean per cycle protein increments and the mean per cycle mineral increments, for the three simulators using various detergents are summarised in Table 14.1.

Table 14.1

Amount of Protein and Mineral Deposited on Stainless
Steel from the various Detergent Simulators

		Total Deposit /cycle mg m^{-2}	Mineral Deposit /cycle mg m^{-2}	Protein Deposit /cycle mg m^{-2}
Simulator A (189)				
Detergent	1	71	58	13
Detergent	2	160	92	68
Detergent	3	100	78	22
Simulator B (256)				
Detergent	X	29	20.3	8.7
Detergent	Y	89	63	26
Detergent	Z	290	203	87
Simulator C (356)				
Detergent	S	45	32	13
Detergent	T	100	70	30

It is clear from these data that the amount of mineral deposited per cycle is much in excess of that which could be expected if the mineral was derived from the evaporation of rinse solutions. For example a liquid film 4×10^{-4} cm thick containing 300 ppm of CaCO_3 would give rise to a mineral deposit of 1.2 mg m^{-2} . In comparison the values of Table 14.1 range from 32 to 203 mg m^{-2} .

It is possible that the liquid films left adhering to porous deposits would have an effective thickness much in excess of the assumed value of 4×10^{-4} cm. However, it is unlikely that this could account for the order of magnitude difference in the above data.

The data of Table 14.1 can also be used to eliminate the possibility of protein deposition from evaporation of liquid film of final rinse solutions. Assuming a 10% carry-over from one solution to the next for three rinses, would give a final rinse solution containing approximately 0.04 mg ml^{-1} . This could be expected to give an evaporated protein film of 1 mg m^{-2} . Again this is much lower than the mean per cycle protein increment. Again, however, the result could be increased by the porosity of the deposit.

It does not seem possible to come to any conclusion about the relative importance of precipitation of protein from whole milk or deposition due to evaporation of whole milk liquid films. Simulators A and B relied on the use of sodium hypochlorite to enhance deposit formation. This was probably effected through precipitation. Simulator C involved a dipping procedure in which milk films were dried on to the surface so that in this case, at least, the deposit originated from the evaporation of milk.

It is of interest to note that in none of the simulator studies was the mean per cycle increment of protein less than the amount that would correspond to a monolayer of adsorbed protein: (8 mg m^{-2}). In many cases the amount was much in excess of this figure.

Simulator trials do not provide a complete representation of "milkstone" formation which occurs over the longer time scales of the dairy milk-line situation. Bacterial action and the mineralisation of protein gels may be important. In addition deposits formed in milk lines may be very much more subtle than

those formed in simulators. Effects appearing insignificant in simulator studies may be significant in milk lines.

14.5 Suggestions for Further Studies

The present project has, of necessity, covered many aspects of milk science and surface chemistry. It has not been possible in the time available to investigate all of these aspects in depth. In many instances, interesting preliminary results await further investigation. Some aspects that could provide the basis of future work are summarised below.

Much has still to be learned of protein adsorption at solid-liquid interfaces. In order to rigorously test the limiting case model proposed in the present work, studies of adsorption kinetics for proteins other than caseins on to a variety of surfaces and under a variety of experimental conditions (pH, concentration, temperature, ionic strength and flow rate) are required. The assumptions of irreversibility of adsorption and surface saturation need to be tested for these systems.

The problem of accounting for protein desorption has yet to be satisfactorily resolved. Before this can be done it will be necessary to determine whether the adsorbed species is denatured during desorption. Gradient pore gel electrophoresis could be used to determine whether the adsorbed species has undergone any change upon desorption. Analysis of kinetic data from desorption experiments presents a difficult problem. Their much poorer reproducibility compared with the adsorption curves compounds the difficulty. Further work on the related problem of determining

the mechanism of detergent action on adsorbed protein films would also be of interest.

An important conclusion of the present investigation is that casein adsorption on to stainless steel is thermodynamically favoured because of the entropy term associated with the increased randomisation of the water structure that results when protein molecules are adsorbed from solution. This effect is sometimes known as "hydrophobic bonding". A measure of protein hydrophobicity can be obtained by measuring the distribution of protein between aqueous and non-aqueous phases. The variation of hydrophobicity with experimental conditions such as pH and investigations to determine whether hydrophobicity can be correlated with adsorption affinity and displacing power would give valuable insight into the nature of the surface-protein interaction. Preliminary results of the present work suggest that the more hydrophobic β -casein has the ability to displace less hydrophobic α_{s1} -casein from stainless steel.

As an interesting aside, the effect of hydrophobicity on functional group reactivity in proteins could be studied. Evidence from work on iodination suggests that iodination occurs most readily at the tyrosyl residues with the most hydrophilic environment and negatively charged neighbouring residues. Iodination reactions on selected tripeptides at controlled pH's could be undertaken to establish this result. In the present work a model was developed to predict the sequence of preferential labelling of tyrosyl residues of a given protein. This model remains to be tested experimentally.

The problem of "milkstone" formation in milk lines seems qualitatively quite similar to effects such as the formation of calculus on dental enamel, the formation of kidney stones and other mineralised protein deposits. Further studies of protein aggregation at surfaces by bridged multilayer or other mechanisms, and the mineralisation of such deposits could provide the topic for extended studies.

In undertaking the present project, some attention to the physico-chemical properties of milk was inevitable. Considerable information is still to be learned about the structure of the milk micelles and the nature of micelle-serum interactions. The use of iodinated caseins and other labelled species (P-32, Ca-45) could be of use in such studies.

The use of iodinated proteins during the project illustrated how these species could be of use in studies of the general chemistry of iodine in milk and milk products. This is an area of renewed interest because of the widespread use of iodine containing iodophors as dairy sanitisers.

Finally, returning to the problem of milkstone, continued monitoring of actual deposits formed, along with details of the conditions prevailing during deposit formation, will be necessary to ensure that the many conditions that can lead to deposit formation can be catalogued. In this connection there is a need for continued studies into the efficiency and mode of action of detergents and sanitisers, the optimisation of dairy procedures, the surface properties of bacteria and the chemistry of protein films.

CHAPTER 15

GENERAL CONCLUSION

The studies described in this thesis have been directed towards gaining a better understanding of the factors that are important in the formation of protein films on the stainless steel contact surfaces of dairy milk lines. Of basic importance in determining the formation and stability of organic films on solid surfaces is the nature of the surface-substrate interaction. A suitable starting point for a study of protein films on stainless steel was thus determined to be an investigation of the surface chemistry of the various protein constituents of milk.

When freshly cleaned stainless steel surfaces are exposed to milk, they are rapidly covered with a layer of adsorbed protein molecules. Most of this protein is casein and because of its greater affinity for stainless steel surfaces, a large proportion of this casein is likely to be β -casein. Micelles are not adsorbed but they may play a role in the formation of the adsorbed films by maintaining the serum casein concentration.

The surface chemistry of bovine milk caseins at stainless steel-water interfaces is determined largely by molecular size, shape, hydration and hydrophobicity. Data for the adsorption of caseins at low concentration agreed well with a model which pictured the adsorption process from unstirred and non-convecting solutions as being diffusion controlled, irreversible and proceeding, at all concentrations, to the formation of a layer

of close packed molecules. Thus both the rate of adsorption and the amount adsorbed at saturation, depend on molecular size and shape since these parameters determine diffusion coefficients and surface packing. This model provides a valuable limiting case description of protein adsorption. Some deviations from the limiting case behaviour were observed at high concentration. These could have been due to adsorption to form different packing arrangements, or to the effects of polymerisation. It is likely that the rate of adsorption is diffusion controlled at all concentrations and under all conditions, but this could not be tested at the higher concentration because the process was too fast to follow by the methods available. Some reversibility of the adsorption process was observed at high protein concentrations. The effects of reversibility could be accommodated in the model by incorporating a surface-solution equilibrium term, but this was not included in the present work.

Casein adsorption at stainless steel-water interfaces results from hydrophobic bonding. Though the evidence for this conclusion is largely circumstantial (hydrophobicity of the 2B stainless steel surface and regions of the protein molecule, absence of temperature effects on adsorption, absence of urea effects, absence of ion exchange effects, absence of metal-sulphide linkages), this mechanism provides the best explanation of the data obtained and is consistent with the conclusions drawn from other protein adsorption studies. In addition a hydrophobic interaction could account for the difficulty experienced in removing adsorbed protein from stainless steel.

The desorption of protein adsorbed on to stainless steel is a complicated process that cannot be explained in terms of simple diffusion or reaction rate controlled processes. The slowness of the process under mild conditions and the likelihood of protein denaturation under the more vigorous conditions required for faster rates, are additional problems. Also it appears that the desorption process is inherently less reproducible than the adsorption process. Generally the hydroxyl ion and anionic surfactants are the most effective species for enhancing desorption. There does not appear to be any simple relationship between the kinetics of desorption and the concentration of these species. A feature of all desorption experiments was the result that some of the adsorbed protein adhered very tenaciously and could not be desorbed even after prolonged periods.

An important result established by the adsorption studies was that macroscopic casein films cannot be formed by simple adsorption processes. A variety of mechanisms by which accumulation of further protein on the initially adsorbed layer could occur were investigated. Adsorption of multilayers via bridging colloidal species may occur under special circumstances. More likely effects are deposit formation by precipitation or evaporation. Any condition that leads to the precipitation of casein from milk, or rinse solutions, can produce surface deposits, since precipitated casein aggregates are adsorbed by stainless steel. Other precipitated aggregates may similarly be adsorbed. Deposit formation by evaporation of liquid films is inevitable if

the liquid films contain nonvolatile solutes. It is possible that a combination of deposition (evaporation or precipitation) and adsorption effects are involved in the formation of actual milkstone deposits.

Actual milkstone deposits vary widely in macroscopic and microscopic appearance and in chemical and physical properties. These may be significantly modified by the effects of bacterial action and temperature. In the present work a classification based upon chemical composition has been found useful. In favourable circumstances the composition of the deposit provides a good indication of the mechanism by which the deposit was formed. Thus high mineral milkstones can be formed either by protein multilayering via iron oxide or precipitated calcium phosphate or by the deposition from evaporated milk films followed by leaching of the protein with alkaline detergents. Alternatively the mineral could have been deposited by precipitation from high mineral water (i.e. high calcium or high iron content) possibly by use of inappropriate detergents. High protein deposits could be formed by protein deposition induced by casein precipitation or milk film evaporation. The presence of chlorine or inorganic phosphorus in such deposits is a good indication that precipitation (by use of sodium hypochlorites or an inadequate concentration of phosphoric acid) was involved.

In spite of the stringent hygiene requirements of modern dairy practice, problems associated with bacterial contamination originating from protein films on milk contact surfaces continue to occur.

The results of the present investigation go some way towards furthering the understanding of these deposits, their properties, modes of formation and hence possible means of prevention and/or removal. It has revealed many areas for more detailed investigation, and indeed, further research has already been initiated in this field.

APPENDICES

APPENDIX 1. NPS, p and $H\ddot{I}$ data for α_{s1} , β and K-casein(a) α_{s1} -casein B

Amino acid	Number of residues	Volume/ $\frac{\text{Å}^3}{\text{residue}}$	$H\ddot{I}$ / kJ
Non-polar			
Tryptophan	2	270.8	25.1
Isoleucine	11	1122.0	135.74
Phenylalanine	8	911.2	95.36
Proline	17	1251.2	184.79
Leucine	17	1734.0	170.68
Valine	11	936.1	72.21
Methionine	5	488.5	27.2
Alanine	9	473.4	28.26
Glycine	9	<u>326.7</u>	
		<u>7513.9</u>	
Polar			
Tyrosine	10	1162.0	119.2
Lysine	14	1471.4	87.78
Arginine	6	654.6	18.84
Threonine	5	356.0	9.4
Serine	16	878.4	
Histidine	5	459.5	
Aspartic	15	1026.0	
Glutamic	39	3303.3	
Amide	(22)	<u>88.0</u>	
		<u>9399.2</u>	
Total	199		<u>974.56</u>

(b) β -casein A²

Amino acid	Number of residues	Volume / Å ³	H ₀ / kJ
Non-polar			
Tryptophan	1	135.4	12.55
Isoleucine	10	1020.0	123.4
Phenylalanine	9	1025.1	99.72
Proline	35	2576.0	380.45
Leucine	22	2244.0	220.88
Valine	19	1619.9	135.09
Methionine	6	586.2	32.64
Alanine	5	263.0	15.7
Glycine	5	181.5	
		<u>9651.1</u>	
Polar			
Tyrosine	4	464.8	47.68
Lysine	11	1156.1	68.97
Arginine	4	436.4	12.56
Threonine	9	640.8	16.92
Serine	16	878.4	
Histidine	5	459.5	
Aspartic	9	615.6	
Glutamic	39	3303.3	
Amide	(27)	108.0	
		<u>8062.9</u>	
Total	<u>209</u>		<u>1166.46</u>

(c) K-casein B

Amino acid	Number of residues	Volume/ $\frac{\text{g}}{\text{A}}^3$	H ₂ O/ kJ
Non-polar			
Tryptophan	1	135.4	12.55
Isoleucine	13	1326.0	160.42
Phenylalanine	4	455.6	44.32
Proline	20	1472.0	217.4
Leucine	8	816.0	80.32
Valine	11	936.1	78.21
Methionine	2	195.4	10.88
Alanine	15	789.0	47.1
Glycine	2	72.6	.
Cystine/2	2	136.6	8.36
		<u>6334.7</u>	
Polar			
Tyrosine	9	1045.8	107.28
Lysine	9	945.9	50.43
Arginine	5	545.5	15.7
Threonine	14	996.8	26.32
Serine	13	713.7	
Histidine	3	275.7	
Aspartic	11	752.4	
Glutamic	27	2286.9	
Amide	21	84.0	
		<u>7646.7</u>	
Total	<u>169</u>		<u>865.28</u>

APPENDIX 2. Identification of protein fractions by Disc Electrophoresis (511)

Stock solutions for gels

A (pH 8.9)	1 mol l ⁻¹ HCl	48 ml
	Tris (hydroxymethylaminomethane	36.6 g
	Temed (N,N,N ¹ ,N ¹ -Tetramethylethylene-diamine)	0.23 ml
	Distilled water to	100 ml
B (pH 6.7)	1 mol l ⁻¹ HCl	48 ml
	Tris	5.98 g
	Temed	0.46 ml
	Distilled water to	100 ml
C	Acrylamide	28 ml
	Bis (N,N ¹ -methylene acrylamide)	0.735 g
	Distilled water to	100 ml
D	Acrylamide	10 g
	Bis	2.5 g
	Distilled water to	100 ml
E	Riboflavin	4 mg
	Distilled water to	100 ml

(All solutions were stored in a refrigerator)

Buffer solution for electrode chambers

Tris	3 g
Glycine	14.4 g
Bromo-phenol blue 0.1%	0.25 ml
Distilled water to	1 litre

Solutions for Preparing Gels

1. Small Pore I

1 part of stock solution A
2 parts of stock solution C
1 part of 9 mol l⁻¹ urea

2. Small Pore II

Ammonium persulphate 0.14 g
9 mol l⁻¹ urea to 100 ml

3. Large Pore

1 part of stock solution B
2 parts of stock solution D
1 part of stock solution E
6 parts of 9 mol l⁻¹ urea

Staining Solution

0.1% amido black in 7% acetic acid

Destaining and Storage solutions

10% acetic acid for washing
3% acetic acid for storage

Method

1. Preparation of the Gels

Equal volumes of a mixture of small pore I and small pore II were poured into the dry tubes standing upright in a drilled rubber block. Each tube was filled to 2 cm from the top and a layer of distilled water was carefully placed over the gel which was left for approximately thirty minutes for the gel to set. The water was removed from the gel and after rinsing with the large pore solution, a layer of approximately 1 cm thick of the large pore gel was left in the tubes and again a layer of distilled water was placed on the gel. The tubes were then left for twenty minutes under the U.V. light for the large pore gel to polymerize.

2. Electrophoresis of Samples

After removing the distilled water from the top of the gels and rinsing with buffer solution the gels were placed in the electrophoresis apparatus. Buffer was put into both chambers, the sample placed on the top of each gel, and the top chamber was connected to the negative electrode and the bottom chamber to the positive electrode. The apparatus was switched on for 8 minutes at 15 mA followed by 50 minutes on 30 mA. When the bromo-phenol blue marker bands reached 1 cm from the bottom of each tube the run was stopped.

3. Removal and Staining of the Gels

The apparatus was disconnected, and by using a hypodermic needle, the gels were removed from the tubes and placed in the staining solution for one hour. They were then removed, destained overnight and stored in 3% acetic acid solution.

APPENDIX 3. Method used to prepare crude β -casein (368)

Sufficient 1 mol l^{-1} HCl was added to 1 litre of skim milk to lower the pH to 4.6. This resulted in the precipitation of the milk protein. After filtering through muslin and washing with warm water, the precipitate was redispersed with NaOH and again precipitated by lowering the pH to pH 4.6 with acid. The filtered precipitate was then dispersed with 700 ml of distilled water containing 180 g of urea and the pH was adjusted with NaOH to pH 7.5. The volume was made up to 900 ml and reprecipitated at pH 4.6 by adding HCl.

The supernatant which contained the β -casein was filtered through a Whatman's No.4 filter paper and the cloudy filtrate was adjusted to pH 4.9, diluted to 2 litres and warmed to 30°C . The flocculation formed containing crude β -casein was allowed to settle overnight, filtered and dispersed in 400 ml of 3.3 mol l^{-1} urea at pH 7.5. Again the pH was lowered to pH 4.6 and after filtration, the filtrate was adjusted to pH 4.90, diluted to 800 ml with distilled water and warmed to 30°C . The precipitate formed containing crude β -casein was centrifuged down and further purified by dispersing in 300 ml of distilled water at pH 7.5. Finally it was reprecipitated by lowering the pH to 4.90. The gummy precipitate was washed successively with ether, absolute alcohol and acetone, pre-dried in air and then crushed to a fine powder and vacuum dried.

A yield of about 3 grams of crude β -casein was obtained.

APPENDIX 4. Chloramine T method of Iodination (544)

Solutions:

1. Barbital - HCl buffer

A buffer of pH 7.4, $I = 0.07 \text{ mol l}^{-1}$ was made up as follows:

Solution A: 14.714 grams of sodium 5,5-diethylbarbiturate
9.714 grams sodium acetate trihydrate
made up to 1 litre with distilled water.

Solution B: 0.1 mol l^{-1} HCl

The buffer solution contained 200 ml of solution A
and 200 ml of solution B and was diluted to
1 litre with distilled water.

2. Cacodylate - HCl buffer

A buffer of pH 6.8, $I = 0.06 \text{ mol l}^{-1}$ was made up as follows:

Solution X 0.2 mol l^{-1} sodium cacodylate trihydrate

Solution Y 0.1 mol l^{-1} HCl

250 ml of solution A and 93 ml solution B were diluted to
1 litre with distilled water to give the cacodylate buffer.

3. Chloramine T solution

1 g of chloramine T was dissolved in 250 ml of barbital buffer.

4. Metabisulphite solution

1.2 g of sodium metabisulphite was dissolved in 250 ml of
barbital buffer.

5. Barbitol Iodination buffer

20 ml of Solution A (see Barbitol-HCl buffer) and
20 ml of Solution B (see Barbitol-HCl buffer) were made up to
50 ml with distilled water.

Gel Filtration Column

1.0 g of Sephadex G25 (fine), after equilibration overnight
in cacodylate buffer was poured into a glass column previously
plugged with a small amount of glass wool. The final column
dimension was 100 nm x 10 nm.

Glassware

All glassware used in the iodination was cleaned by standing
for 18-24 hours in a 1:1 solution of concentrated nitric:sulphuric
acid heated on a sand bath. These were then oven dried and stored
in a dry glass desiccator until required.

Method of Iodination

All solutions were made up immediately before iodination.

1. To a small test tube containing 0.2 mCi of iodide-125 (or
iodide-131) was added 0.100 ml of barbitol iodination buffer and
the solution was thoroughly mixed using a fine glass stirring rod.
2. 0.025 ml of the protein solution was added to the radioactive
solution and after thorough mixing, 0.025 ml of the Chloramine-T
solution was added. The Chloramine T, which oxidised to iodide,
was allowed to react for 45 seconds before the addition of 0.100 ml
of the sodium metabisulphite. This halted any further reaction by
the deactivation of the Chloramine T and the reduction of any
iodine back to iodide. The reaction mixture was constantly stirred
during the reaction.

3. Separation of the iodinated protein from the unreactive iodide was achieved by gel filtration using a Sephadex G25 column.

The iodination reaction mixture was immediately transferred to the column after the addition of the metabisulphite and the test tube was rinsed with three separate 0.15 ml of cacodylate buffer. The solution was allowed to drain to the gel level at the top of the column before each addition. The volume of buffer added to the column was made up to one ml, allowed to drain and further one ml volumes of buffer were repeatedly added to and collected from the column. In this manner eight one ml fractions were eluted from the column, collected in glass sample vials and counted.

The protein fraction (third ml), after counting, was stored at 4 °C.

The efficiency of iodination was calculated as the percentage of radioactively labelled protein to the total radioactivity eluted from the column in the eight one ml vials.

Cacodylate buffer was used in the elution sequence to minimise the possibility of protein-buffer interactions that have been reported for other commonly used buffering agents. It was also desirable to use a buffer that would not interfere in Kjeldahl nitrogen determination.

APPENDIX 5. Radiochemical Properties of Iodine-125 and
Iodine-131

	I-125	I-131	Reference
Half life $t_{\frac{1}{2}}$ (days)	60	8.06	513
Isotope Abundance (1%)	30-100	15-20	537,542
Principal Decay			
Mode and Energy (MeV)	γ 0.035	β 0.60 γ 0.364	513
Decay Product	Te-125	Xe-131	513
Half Value Thickness for Gamma Radiation (Lead)	<0.011 cm	0.25 cm	547
Tenth Value Thickness for Gamma Radiation (Lead)	<0.035 cm	0.8 cm	547

APPENDIX 6. Determination of Geiger Counter Dead Time

$$\text{Dead Time } T = \frac{m_1^2 + m_2^2 - m_{12}^2}{2 m_1 m_2} - m_b$$

m_1 = observed count rates No.1

m_2 = observed count rates No.2

m_{12} = observed count rates of No.1 and No.2 together

m_b = background

Experiment No. 1

(count - rate/second)

m_1	96.98
m_2	89.10
m_{12}	180.66
m_b	0.45

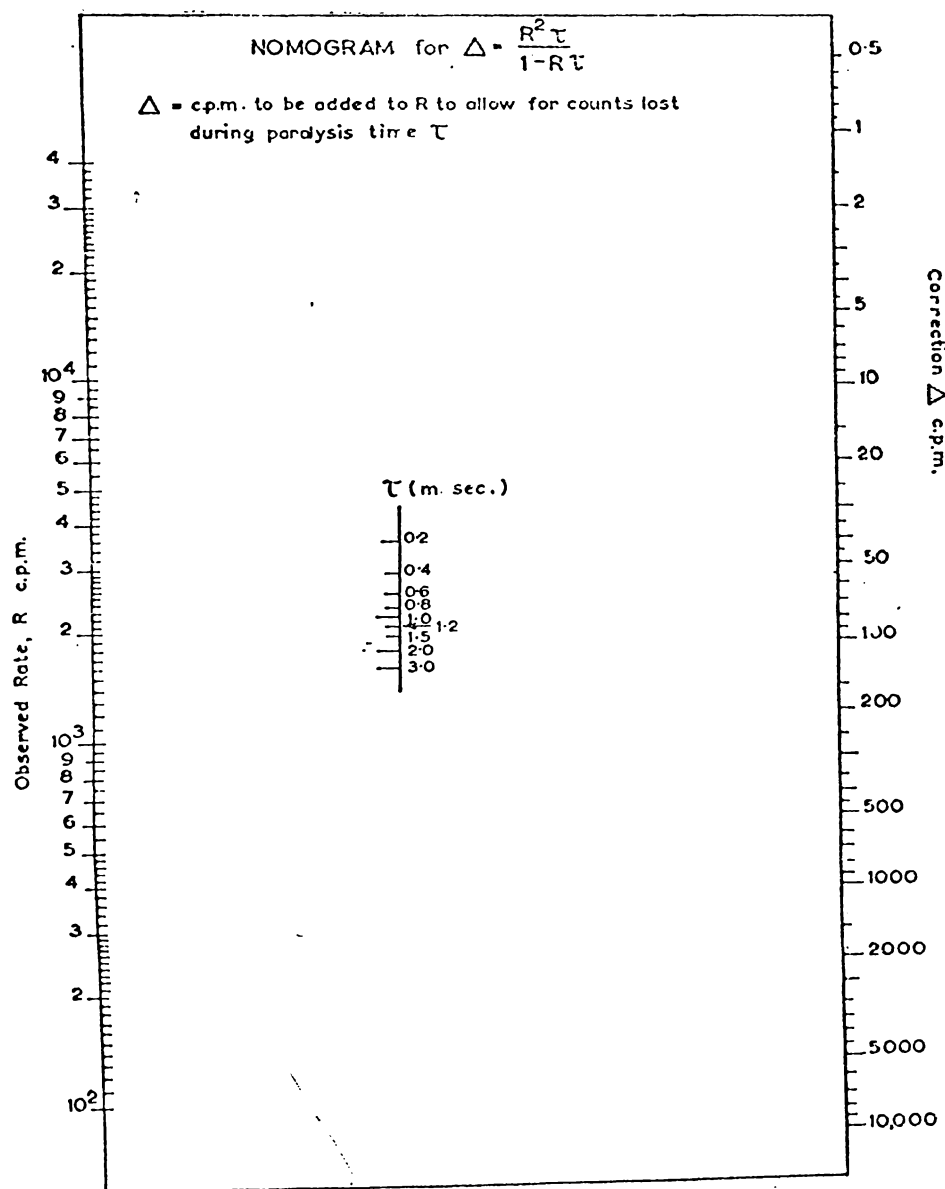
Dead Time = 0.324 ms.

Correction Factor for Backscattering in Desorption Studies

It was found necessary to correct for the backscattering that occurred when the disc containing the adsorbed protein was placed in the various solutions used in the desorption studies. On monitoring the activity adsorbed on to the stainless steel just above the test solution, a five percent increase in count-rate due to backscattering was observed. All initial count rates were corrected for this effect.

Nomogram for correcting counts lost during dead time

(545)



APPENDIX 7. Decay correction factors for I-131

$$\text{Decay Correction} = \frac{C_o}{C_t} = \exp \left(\frac{0.6931 t}{t_{\frac{1}{2}}} \right)$$

where C_o = count rate at time zero

C_t = count rate at time, t (hour)

$t_{\frac{1}{2}}$ = 193.44 hours (half-life of I-131)

Time/h	$t/t_{\frac{1}{2}}$	Decay Correction	Time/h	$t/t_{\frac{1}{2}}$	Decay Correction
0.50	2.58×10^{-3}	1.0017	30.0	0.155	1.113
1.0	5.16×10^{-3}	1.003	32.0	0.165	1.121
2.0	1.03×10^{-2}	1.007	34.0	0.175	1.129
3.0	1.55×10^{-2}	1.010	36.0	0.186	1.137
4.0	2.06×10^{-2}	1.014	38.0	0.196	1.145
5.0	2.58×10^{-2}	1.018	40.0	0.206	1.154
6.0	3.10×10^{-2}	1.021	42.0	0.217	1.162
7.0	3.61×10^{-2}	1.025	44.0	0.227	1.170
8.0	4.13×10^{-2}	1.029	46.0	0.237	1.179
9.0	4.65×10^{-2}	1.032	48.0	0.248	1.187
10.0	5.16×10^{-2}	1.036	50.0	0.258	1.196
12.0	6.20×10^{-2}	1.044	100.0	0.516	1.43
14.0	7.23×10^{-2}	1.051	193.4	1.00	2.00
16.0	8.27×10^{-2}	1.059	300	1.55	2.93
18.0	9.30×10^{-2}	1.066	400	2.06	4.19
20.0	0.103	1.074	500	2.58	5.99
22.0	0.113	1.082	600	3.10	8.58
24.0	0.124	1.089	700	3.62	12.28
26.0	0.134	1.097	800	4.13	17.57
28.0	0.145	1.105	1000	5.17	35.98

t = time (hours)

$t_{\frac{1}{2}}$ = half-life of isotope (hours)

APPENDIX 8 Desorption kinetic data

In the following data, k_i represents the rate constants for the processes resolved from the desorption curves and have been numbered consecutively i.e. $i = 1, 2, 3, \dots$ from the slowest to the fastest process. The figures in brackets represent the percentage of each 'type' of protein.

1. Effect of pH

	pH			
k_i/min	5.0	8.3	11.0	12.4
k_1	0.001 (95)	0.003 (84)	0.001 (64)	0.002 (48)
k_2	— (5)	0.25 (6)	0.03 (32)	0.10 (15)
k_3		2.1 (6)	— (4)	0.46 (35)
k_4				— (25)

2. Effect of 1 mol l^{-1} NaCl

k_i/min	pH 7.0	0.05 mol l^{-1} NaOH
k_1	0.005 (90)	0.003 (19)
k_2	0.2 (10)	0.07 (42)
k_3		8.32 (32)
k_4		(7)

3. Effect of Anionic Surfactant in $0.05 \text{ mol l}^{-1} \text{ NaOH}$

k_i/min	0.0025% surfactant	0.010% surfactant	0.015% surfactant
k_1	0.003 (38)	0.003 (31)	0.0006 (7.8)
k_2	0.11 (33)	0.14 (53)	0.002 (11)
k_3	0.47 (28)	— (14)	0.48 (35)
k_4			— (46)

APPENDIX 9. Procedure for Analysis of Milkstone Deposits (546)

The milkstone deposits were analysed for moisture, protein, phosphate, calcium and in some samples, iron and fat.

The method used was as follows:

1. Moisture

The moisture was determined by measuring the weight change on drying the sample for 4 hours at 102-105 °C.

2. Protein

The protein was determined by micro Kjeldahl as follows:

1. Weigh 0.05 to 0.20 g into a clean dry boiling tube
2. Add 0.5 g of anhydrous sodium sulphate, 2 ml of concentrated sulphuric acid, 1 ml of 1% mercurous oxide in 1 mol l⁻¹ H₂SO₄, 1 glass bead
3. The tube was heated gently until colourless then for an extra 20 minutes
4. After cooling the solution was made up to 100 ml and 1 ml was added to a 50 ml Nessler Tube
5. 0.5 ml of 5% NaOH was added to the Nessler Tube and the volume made up to 50 ml
6. 2 ml of Nessler's reagent was then added, stirred and allowed to stand for 10 minutes
7. The intensity was compared to a range of standards and the concentration of protein calculated

3. Phosphorus

The phosphorus was determined from the protein solution using a spectrophotometer at 420 nm or 440 nm as follows:

1. To 10 ml of the protein digestion solution in a 25 ml volumetric flask was added 1 drop of phenolphthalein and 5% NaOH solution until the solution turned red.
2. 1 mol l⁻¹ HCl was added dropwise until the solution turned clear.
3. After adding 5 ml of reagent A the solution was made up to volume with distilled water and allowed to stand for ten minutes before the absorbance was measured at 420 nm or 440 nm.
4. The amount of phosphorus present was determined from a calibration curve based on potassium dihydrogen phosphate.

Reagent A: Solution 1. Dissolve 12.5 g ammonium molybdate in 180 ml water by gently warming
Solution 2. Dissolve 0.63 g ammonium meta-vanadate in 150 ml water by warming followed by adding 165 ml conc. HCl.

Mix solution 2 into solution 1 and make up to 500 ml.

4. Calcium and Iron

The calcium and iron concentrations were determined using an atomic adsorption spectrophotometer with a calcium and iron lamp.

Both these ions were determined after the digestion of protein as in the method used for protein determination, against treated standard, allowing for sulphate ions or inclusion of lanthimum chloride to both standards and the solution examined.

5. Fat

The fat was determined by the Rose-Gottlieb method.

APPENDIX 10 Determination of Tyrosyl residues labelled in the
individual casein proteins

	Area (Arbitrary Unit)	Extinction Coefficient/ $\text{cm}^2 \text{g}^{-1}$	Total Protein (Arbitrary Unit)	% Protein ($\pm 10\%$)
TS-A/R casein	2.95	0.80	3.68	8.1
K-casein	6.23	0.96	6.49	14.7
β -casein	7.56	0.46	16.43	37.2
α_{s23} -casein	4.98	1.00	4.98	40.0
α_{s1} -casein	12.50	1.00	<u>12.50</u>	
		Total	<u>44.08</u>	

	M.Wt	N _{Tyr}	Factor 10^{-3}	% Tyrosyl ($\pm 10\%$)
TS-A/R casein	11 800	3	2.03	6.3
K-casein	19 000	9	6.96	21.6
β -casein	24 000	4	6.20	19.2
α_s -casein	23 500	10	<u>17.02</u>	52.8
		Total	<u>32.21</u>	

Percentage radioactivity in the various casein fractions

	Radioactivity/ cs^{-1}	Percentage radioactivity
TS-A/R casein	62 ± 6	5.8
K-casein	132 ± 13	12.4
β -casein	203 ± 20	19.0
α_s -casein	668 ± 66	62.5
	<u>1065</u>	

Determination of Tyrosyl residues labelled in whey proteins

	Area (Arbitrary Unit)	Extinction Coefficient/ $\text{cm}^2 \text{g}^{-1}$	Total Protein (Arbitrary Unit)	% Protein ($\pm 10\%$)
β -lactoglobulin	1.164	0.95	1.22	69
α -lactalbumin	1.094	2.01	0.54	31
		Total	<u>1.76</u>	

	M.Wt	N _{Tyr}	Factor 10^{-3}	% Tyrosyl
β -lactoglobulin	18 400	4	15.0	63
α -lactalbumin	14 000	4	8.8	37

Percentage radioactivity in the various casein fractions

	Radioactivity/ cs^{-1}	Percentage radioactivity
β -lactoglobulin	237 ± 23	63
α -lactalbumin	135 ± 13	36

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