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ASPECTS OF THE SURFACE CHEMISTRY
OF PROTEINS AT THE
HYDROXYAPATITE/AQUEOUS SOLUTION INTERFACE

by

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A thesis submitted in partial fulfilment
of the requirements of the degree
of
Doctor of Philosophy in Chemistry
at the
University of Waikato, Hamilton, New Zealand
February, 1984

To My Parents and Friends

ABSTRACT

An investigation into some aspects of the surface chemistry of proteins at the hydroxyapatite/aqueous solution interface has been undertaken. A stoichiometric hydroxyapatite, fluoride and carbonate substituted hydroxyapatites and an amorphous calcium phosphate were prepared and characterised for use as adsorbents.

Adsorption studies were performed using, mainly, systems of suspended adsorbent particles and radiochemical methods. Bovine and Human Serum Albumin, β -Casein, and a salivary glycoprotein, isolated from whole saliva, were radioiodinated to high specific activity but low degrees of labelling using modifications of the Chloramine-T or Iodo-Gen methods of iodination that minimised damage to the protein during labelling. The adsorption of Haemoglobin and Myoglobin was monitored by spectrophotometric methods.

Care was taken to characterise the labelled and unlabelled preparations to ensure that the labelled protein behaved identically to unlabelled protein in adsorption experiments. Similarly, the necessary preliminary experiments were performed to adequately characterise the experimental system in use and so avoid the spurious results and interpretations that have characterised some earlier work. Special attention was paid to the adsorbent surface area under various experimental conditions, the effect of polymers and polymerisation behaviour of the protein on its adsorption and the effect of time, particularly at low protein concentrations. Care was taken to ensure that sufficient protein was available to achieve maximal adsorption.

All the proteins studied showed a high affinity for the hydroxyapatite adsorbents i.e. the concentration adsorption isotherms were always parallel to the concentration axis at low concentrations and

adsorption proceeded to saturation even at the lowest concentrations. Dilution studies showed that the concentration required to maintain saturation adsorption was negligibly small but desorption and exchange studies indicated that proteins can have different affinities for hydroxyapatite. Two types of adsorption behaviour were observed at higher concentrations. The majority of the proteins studied displayed an isotherm that remained parallel to the concentration axis with increasing concentration but β -Casein displayed a stepped isotherm which led to higher plateau level of adsorption. The incorporation of fluoride or carbonate in the hydroxyapatite lattice had no effect on the saturation levels of adsorption of any of the proteins at any concentration.

The saturation amounts of adsorption of BSA corresponded to monolayer coverage of the adsorbent surface by molecules close packed in a side-on configuration with little, if any, structural alteration upon adsorption. The effect of the variables of pH, ionic strength and temperature were consistent with the known effects of these variables on the size of the BSA molecule. The saturation amounts of adsorption of β -Casein could similarly be accounted for in terms of monolayer coverage of the adsorbent surface. The low plateau level of adsorption corresponded to coverage by molecules lying prone on the surface in a close-packed configuration but the high plateau corresponded to molecules packed in a more vertical configuration. The stepping effect appeared to be related to the micellisation of this protein in solution. The effects of temperature and calcium ion concentration on adsorption were consistent with the effects of these variables on the solution micellisation.

Although it is generally accepted that ionic forces are responsible for the binding of proteins by hydroxyapatites, the present work has indicated that non-ionic and entropic effects can also operate and

contribute to the driving force for adsorption. In addition, protein carboxyl or phospho group exchange with species in the hydroxyapatite surface cannot be precluded.

The effect of some multivalent metal ions on adsorption was studied. Mercury enhanced the adsorption of a second layer of protein with properties different from the layer initially adsorbed. Although calcium enhanced the levels of adsorption of BSA and β -Casein, the increased amounts adsorbed and the non-reversibility of the adsorption did not provide evidence of a second layer and were more consistent with denser packing in the original monolayer.

Multilayer adsorption of β -Casein was promoted by the use of colloidal hydroxyapatite and amorphous calcium phosphate particulate species. Stable multilayers were formed, with consequent accretion formation, when the protein could bridge directly between the particulates i.e. at degrees of surface coverage less than saturation. BSA did not display such behaviour. The phenomenon appears to be related to the protein induced stabilisation/destabilisation of suspensions of colloidal species.

The interfacial behaviour of the salivary glycoprotein at the hydroxyapatite/aqueous solution interface is similar to that observed for β -Casein. Many other salivary components also destabilised suspensions of colloidal calcium phosphate species and it is postulated that pellicle formation in the oral environment may be related to the phenomenon.

ACKNOWLEDGEMENTS

I sincerely wish to thank my supervisor Dr. A.G. Langdon for the time spent in discussion and assistance received in writing this thesis. The contributions of Dr. P. Molan and Mrs. Mary Smith of the Biochemistry Department, University of Waikato, toward the more biochemical aspects of the thesis and the offer of assistance in the clinical aspects by Professor A.C. Smillie of the Dental School, University of Otago, is gratefully acknowledged.

I also wish to thank:

Mr G. Leet of the Meat Industry Research Institute, Hamilton, for assistance with the transmission electron microscopy;

The technical staff of the University of Waikato, particularly the Photography and Electronics Departments;

Mr. Warr of Judd Dental Surgery for the donation of dental restorative materials;

The Wrigley Chewing Gum Company for the donation of gum;

"In-Tone Electronics" for the production of the thesis manuscript; and

The Medical Research Council of New Zealand for the award of a Post Graduate Scholarship and financial assistance during the study.

Special heartfelt thanks are due to my parents and friends for their continual moral support and almost simultaneous distraction, and whom I neglected at various stages of the work.

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LIST OF ABBREVIATIONS AND SYMBOLS

BSA	Bovine Serum Albumin
HSA	Human Serum Albumin
OHAp	Hydroxyapatite (Stoichiometric)
F-OHAp	Fluoride Substituted Hydroxyapatite
CO ₃ -OHAp	Carbonate Substituted Hydroxyapatite
ACP	Amorphous Calcium Phosphate
A	total geometric surface area
A _n	nominal surface area
A _s	specific surface area
C _i	initial protein concentration
C _f	final protein concentration
CMC	critical micelle concentration
M _a	apparent mass
M _{ads}	mass of adsorbed protein
M _{non-ads}	mass of non-adsorbed protein
M _{salts}	mass of buffer salts
M _{solid}	mass of adsorbent
M _{soln}	mass of solution
r _b	background count
r _{ret}	count from labelled species in solution
	retained or adhering to adsorbent
r _{solid}	count of adsorbent
r _{soln}	count of solution
RP	activity adsorbed per unit nominal surface area of adsorbent
R _i ^S	initial activity of solution per unit volume
R _f ^S	final activity of solution per unit volume
V	solution volume
V _A	interparticle attraction energy arising from van der Waal's force
v _R ^{elec}	electrostatic repulsion energy arising between particles of like charge

v_R^{steric}	repulsion energy arising from entropic or osmotic effects
V_T	total energy of interaction
x	surface roughness factor
α	fraction of protein depleted by adsorption
Γ	mass of protein adsorbed per unit area of adsorbent
λ	retention coefficient
σ	solution density

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CHAPTER 1

GENERAL INTRODUCTION

Attention was first drawn to the unique properties of matter at an interface when, in 1795, Benjamin Franklin noted that a teaspoon of oil calmed the ripples on as much as a half-acre of the surface of a pond (Franklin, 1931). Early research on interfacial phenomena concentrated on the liquid/liquid and liquid/gas interface. By the early 1950's, the importance of the phenomenon in foam production, coatings and dispersions was well established. Yet, the topic was infrequently treated in basic research as it was neither mainstream chemistry nor engineering. However the last two decades has seen an increasing interest and involvement of researchers in fields that could be designated as interfacial phenomena. This is particularly due to an increased awareness of their applicability to problems facing the world as a whole: pollution control (e.g. water purification), energy supply (e.g. through oil recovery by surfactant flooding, and aspects of coal treatment), food supply (e.g. protein recovery), and by the search for applications in non-traditional areas such as the ocean, clouds and in physiological systems.

Studies of solid/liquid interfaces had begun by the mid 1800's and the many current examples of their application testify to their ubiquity and importance: in biology, chromatographic and electrophoretic techniques and the modification and immobilisation of enzyme activity; in engineering, the fields of adhesion, coatings, lubrication and the modification of surface properties; and, in food technology, the manufacture of food and cooking mixture preparations. The importance of the application of the interfacial chemistry of proteins in solid/liquid systems was not realised until after 1905 when it was first reported that inorganic powders removed proteins from solution (Landsteiner and Uhlirz, 1905). Since this time, the application of protein solid/liquid interfacial phenomena to biomedical aspects of human health has seen a

spectacular growth that has shown remarkable consequences. In particular, the manufacture and use of biomedical grade materials and devices for implantation has become a multi-million dollar enterprise: surgical adhesives, cosmetics and wound healing dressings, dental restoratives, prosthetic implants such as pacemakers and artificial joints, intrauterine devices, and extra-corporeal circuits such as artificial cardiovascular and kidney systems.

The use of biomedical materials and devices necessitates biocompatibility i.e. a passive, immuno and thromboresistant surface. Fundamental to these requirements is the observation that whenever a synthetic or biological solid confronts biological tissue, it is constantly in contact with biofluids rich in surface active biological macromolecules e.g. proteins, and the surface spontaneously acquires an adsorbed proteinaceous film. This bioadhesion has also been found to be fundamental to interfacial phenomena observed in thrombogenesis, marine fouling in both fresh and saline waters, tissue culture and the oral environment (Baier, 1975). It is in the oral environment that the adsorbed film affects the practical factors of human health and disease to a great extent.

In contrast to the size and volume of the remainder of the human body, the mouth is quite small and yet it constitutes a massive disease and problem area: cold sores, cankerous sores, cancer, bone resorption, malocclusion (faulty bite), tooth ache, bad breath, tooth decay (caries), diseases of the gums (gingival disease) and diseases of the gums and tooth supporting structures (periodontal disease). Gingival disease is usually regarded as reversible whereas periodontal disease is not. Both dental decay and gingival and periodontal disease are multifactorial diseases related to the production of organic acids during the metabolism of dietary carbohydrates by oral bacteria. A mandatory prerequisite to

the attachment of these bacteria to the tooth surface is the adsorption of protein to tooth enamel to form a thin film, known as the "acquired pellicle" (Dawes, et al., 1963). Colonies are linked together and a soft, tenacious, macroscopic deposit, known as plaque is formed. This may, under suitable conditions, evolve into a hard, calcified material known as calculus.

Plaque has been indisputably linked to both caries and periodontal disease. Acids produced by the plaque bacteria penetrate the surface of the tooth enamel and cause it to slowly dissolve. Over a period of 12 to 24 months, this results in the formation of caries. Periodontal disease appears to begin at the plaque/gum interface. Bacterial byproducts cause inflammation of the gums and this results in the initiation of the body's immune response systems. Unfortunately, the response of the immune system, over a period of years, also destroys the gum tissue and tooth supporting structures. As a result, the tooth becomes loose and may eventually be lost. Plate 1.1 shows a case of chronic periodontal disease and calcification of plaque.

It has been estimated that tooth decay is more prevalent than the common cold (Sanders, 1980). New Zealand teenagers, for example, have on average 13.5 decayed, missing or filled teeth, and gingival disease is common (N.Z. Dept. of Health, 1978). Generally, up to the age of 35, tooth decay is the major contributing factor to tooth damage and loss. After this, the main cause of tooth loss is periodontal disease.

The extent of gingival and periodontal disease in the adult New Zealand population (i.e. those aged 15 years or older) is shown in Figure 1.1. It is seen that fewer than 5% of the population have completely healthy gums. Data on the extent of edentulousness (Table 1.1) suggests that tooth extraction and loss is a common occurrence. These figures,

PLATE 1.1

CHRONIC PERIODONTAL DISEASE
AND CALCIFICATION OF PLAQUE
ON TEETH



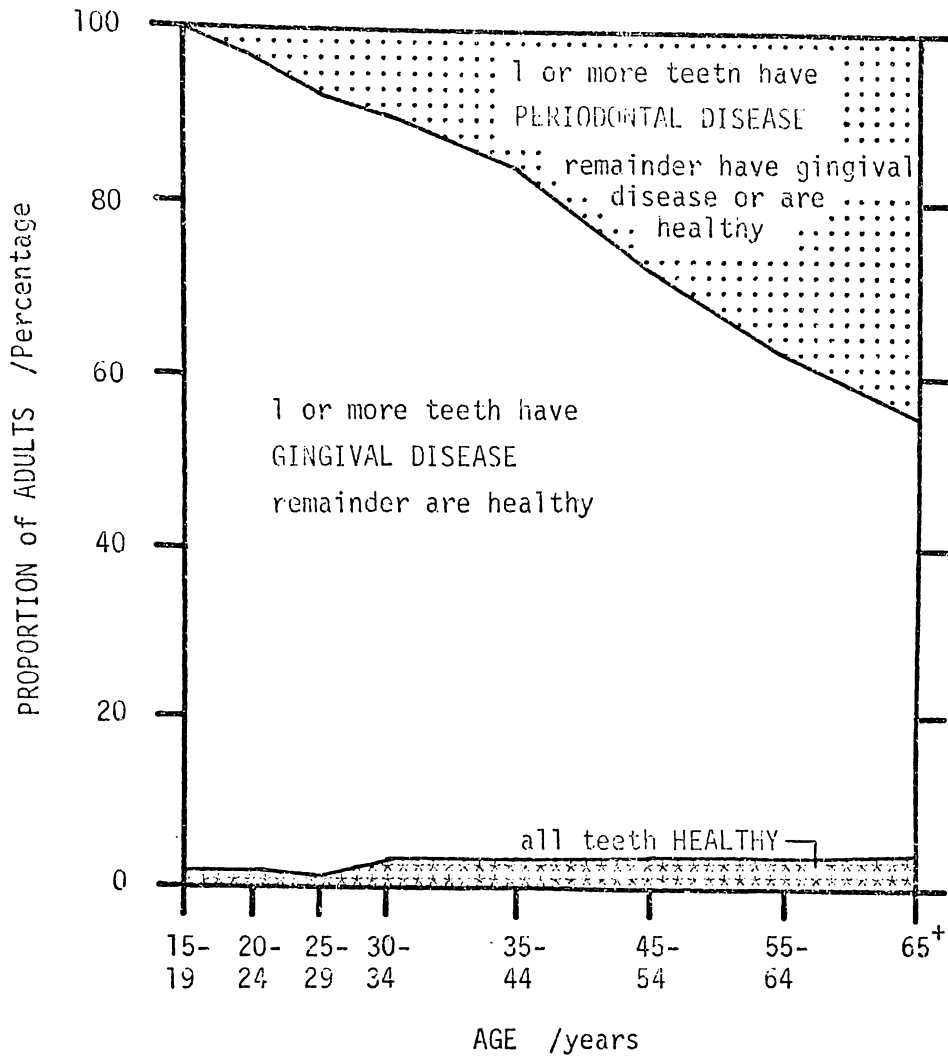


Figure 1.1: The Extent of Dental Diseases in the New Zealand Adult Population (taken from Cutress et al., 1979)

Table 1.1: Edentulousness in the New Zealand Population

<u>Age Group</u>	<u>% Edentulous</u>
15-19	0.3
20-24	4.2
25-29	12.2
30-34	20.7
35-44	28.4
45-54	37.5
55-64	55.1
65+	73.6

(Data taken from Cutress, 1977)

when compared to the population distribution (N.Z. Dept. of Statistics, 1978) show that more than 20% of the New Zealand population wear full upper and lower dentures. The total expenditure on dental care in New Zealand (ca. 3 000 000 pop.) exceeds \$50 million annually.

A similarly bleak picture of the state of oral health is apparent in many western countries. The average 15 year old American has 10 decayed, filled or missing teeth. More than 95% of Americans have decayed teeth by the time they become adults (Sanders, 1980). It is estimated that more than 10% of the population wear full upper and lower dentures (Beck, 1972).

Approximately \$(US) 6 billion is spent annually to repair damage caused by decay, and the total cost of all forms of dental care exceeds \$(US) 13 billion annually (Sanders, 1980).

Dental problems are not entirely a monetary matter however, for often associated with them are excruciating pain, lost working hours, the inability to chew or speak correctly, and a general loss of appearance and well being. Comparisons of oral survey data of previous years suggests that there has been an improvement in oral health. For example, in New Zealand, 22% of the 20-29 age group were edentulous in 1950, but by 1968 this had fallen to 13%, and by 1976, further decreased to 8% (Cutress et al., 1979). This probably reflects the introduction of free dental services for school children, public education and a changed emphasis from restorative to preventative dentistry. There is still room for a considerable improvement in oral health however. The continued promotion of public education in matters of dental health e.g. attitudes, diet, dental care and preventative dentistry, and the removal of the 'painful and expensive' image associated with dental treatment will undoubtedly prove beneficial. Fundamental studies pertaining to dental health, plaque formation and dental decay are also necessary.

Plaque formation, and the ensuing dental problem associated with its presence on teeth, is preceded by the formation of the dental pellicle. The initial step in pellicle formation is the interaction of proteins with tooth enamel. It is the physical chemistry of this process, particularly protein adsorption on the calcium phosphates of tooth enamel, that is the concern of this thesis.

CHAPTER 2

AN OUTLINE OF THE ORAL ENVIRONMENT AND OBJECTIVES OF THE THESIS

2.1 INTRODUCTION

For all its faults, the mouth is a remarkable engineering feat. The salivary glands can produce and spill as much as 1 litre of saliva, a highly complex fluid, into the oral cavity per day; and, the teeth are able to withstand a lifetime of grinding and tearing and the shocks from a biting pressure of over 50 kg force.

The following sections of this Chapter provide an outline of the oral environment and a general background and perspective to this thesis. Pertinent regions and properties of the oral cavity are defined and illustrated generally. Those aspects of direct importance to the topic are noted and the Chapter concluded with a description of the intended experimental procedure.

2.2 TOOTH STRUCTURE AND COMPOSITION

Figure 2.1 shows a simplified cross section of a tooth and its supporting structures. The tooth can be grossly divided into two basic areas: the crown, that portion that extends above the gum (gingiva) which consists of an enamel exterior overlying dentin and the inner pulp cavity; and the root, that portion which extends below the gingiva into the socket, and is coated with cementum.

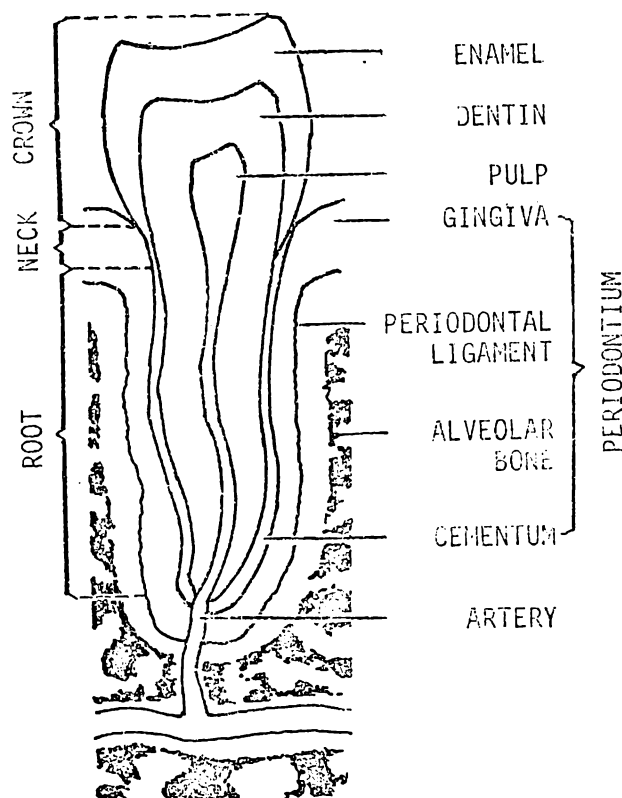


Figure 2.1 : Anatomy of the Tooth and its Environment

The enamel is a light yellow coloured material which is about 99% (w/w) inorganic matter (including water) and 1% organic matter. It is the hardest substance produced by the body and, as such, forms a physically hard protective surface about 1 mm thick over the remainder of

the tooth. The inorganic component is composed of a calcium phosphate salt. It is susceptible to acid attack however, and it is a localised demineralisation of the enamel by organic acids of bacterial origin that leads eventually to the formation of dental caries. The vulnerability of teeth to decay depends on their composition and structure. These are extremely heterogeneous, varying from person to person, geographical position within the mouth and even differing locations on the same tooth. Many of the chemical elements are not evenly incorporated into dental enamel. Concentration versus depth profiles often show a marked variation, so that the surface enamel can be very different from the subsurface enamel. Coupled to these factors are the influences of dietary factors (e.g. the mineral composition of the diet before and after tooth eruption), dental health factors (e.g. the types of toothpastes and/or prophylactic solutions used, the types of dental restoratives), tooth age and wear, and the continual demineralisation and remineralisation of the enamel. The tooth surface is not entirely smooth and, in addition, contains pits and fissures. These are often inaccessible to the toothbrush. Because the enamel is directly exposed to food particles, bacteria and saliva, and is the surface on which pellicle and plaque form, it is the region of particular interest to the present study. A comprehensive study of the chemical and physical properties of hydroxyapatite and enamel is given in Section 3.6.5 and 3.6.6 respectively.

Encapsulated between the enamel and cementum is another calcium and phosphate containing material called dentin. It is a more pronounced yellow colour and forms the bulk of the tooth structure. Dentin is somewhat softer than enamel but is the second hardest material produced by the body. It has a 70% inorganic content and 30% organic content.

The dentin forms a cushioning material that resists the stresses and strains placed upon the tooth by mastication. It is quite porous and readily attacked chemically.

The cementum, a material softer than human bone, is of major importance as it is the material to which the fibres of the periodontal ligament attach and thereby hold the tooth in place. The cementum constantly regenerates and any fibres which detach during normal chewing (or as the result of injury or orthodontic treatment) will reattach. The root of the tooth is not directly attached to the alveolar bone of the jaw which forms a tailored socket for the tooth. If inflamed, the alveolar bone withdraws and rejects the tooth.

The innermost part of the tooth is called dental pulp. It is a fleshy material composed mainly of nerve endings, lymphatics, blood vessels and connective tissue.

The gingiva, in their healthy state, extend above the point where the cementum ends and the enamel begins. The cementum, alveolar bone, periodontal ligament, the gingiva and the dento-gingival junction are collectively referred to as the periodontium (hence periodontal disease).

2.3 ORAL FLUIDS

The fluid found in the oral cavity is derived from several sources: salivary glands, crevicular fluid, serous exudates, the oral mucosa, leucocytes and other blood cells, microorganisms and ingested matter. The fluid is rich in both inorganic and biological material. It is extremely diverse in composition, varying from the inorganic ions and molecules of relative atomic or molecular mass of less than 100, to the complex macromolecules of relative molecular mass approaching 10^6 or greater. All of these components may exhibit surface activity and participate in interactions at any of the interfaces available in the oral cavity.

The components of the oral fluids can be divided arbitrarily into three basic categories: the macromolecules, smaller molecules and inorganic ions. The macromolecules can be further divided to distinguish the complex carbohydrates, immune system components, enzymes, and other macromolecules. Some of the components of these categories have been listed in Table 2.1. The ionic composition of the oral fluids varies widely. The list of ions is therefore not comprehensive but includes those which may play a role in interactions at the tooth/oral fluid interface. Dietary factors are primarily responsible for the variations in the ionic composition. These factors affect the smaller molecules in the oral fluids similarly.

Many of the components listed in Table 2.1 are contributed by saliva formed by the salivary glands. There are three pairs of large salivary glands (the parotid, sublingual and submandibular (also known as submaxillary)) and four smaller glands (the labial, lingual palatal and buccal) in the oral cavity. Saliva keeps the mouth moist, aids taste sensation and digestion, facilitates swallowing, holds certain microbes

TABLE 2.1: SOME COMPONENTS OF THE ORAL FLUIDS.

<u>ENZYMES</u>	<u>COMPLEX CARBOHYDRATES</u>
amylases	bacterial agglutinins
carbonic anhydrase	blood group substances
dehydrogenases	glycoproteins
esterases	polysaccharides
fucosidase	
galactosidase	<u>IMMUNE SYSTEM COMPONENTS</u>
glucosaminidase	C3, C3c
glucosidase	Immunoglobulins
glucuronidase	IgA, IgG, IgM
kallikrein	secretory piece
lactoperoxidase	
lipase	<u>OTHER MACROMOLECULES</u>
lysozyme	albumin
mannosidase	ceruloplasmin
neuraminidase	fibrinogen
phosphatases	lactoferrin
pseudo cholinesterase	lipoproteins
ribonucleases	orosomucoid
sulphatase	phosphoproteins
transaminases	sulphoproteins
<u>SMALLER MOLECULES</u>	<u>INORGANIC IONS</u>
amino acids	H ⁺ , OH ⁻
ammonia, urea	Na ⁺ , K ⁺
cholesterol	Ca ²⁺ , Mg ²⁺
creatinine	F ⁻ , Cl ⁻ , Br ⁻ , I ⁻
glucose	phosphates
lactate, citrate	carbonates
polypeptides	
uric acid	

(Compiled from Mandel (1974); Mason and Chisholm (1975))

in check and rinses the mouth. These latter two functions provide a critical role for saliva in tooth decay. The removal of salivary glands from rats substantially increases their susceptibility to tooth decay. Similarly, people who suffer a markedly reduced formation of saliva, as a result of radiation damage in cancer treatments for example, usually develop rampant tooth decay.

The organic and inorganic composition of saliva is highly variable,

depending on many factors. These include: the nature, intensity and duration of flow stimulating factors; flow rate; genetic patterns; age, sex and hormonal influences; circadian rhythm; pharmacologic agents; environmental and dietary influences; and disease. A minimum of 100 protein components have now been identified in whole saliva by electrophoretic techniques (Giometti and Anderson, 1980). Few have been otherwise separated and/or characterised and little is known about them unless they also belong to the generally more well characterised serum proteins. Often the names (or numbers) given to separated components are only significant in the context of the separation procedures used. The state of the ionisation and form of the inorganic components is also dependent on the organic composition (e.g. the concentrations of calcium binding materials) and the pH of the fluid (e.g. the carbonates and phosphates). The fluids of the individual gland secretions can also differ considerably. Generally, saliva is composed of about 99.5% water. Of the remaining 0.5% of dissolved material, some of the macromolecular components are highly surface active.

A tooth will, within minutes of having been scrupulously cleaned, have a pellicle formed from these components that extends over the entire exposed surface. This pellicle is remarkably constant in composition despite large differences between the individual glandular secretions (e.g. Mandel 1974 and 1977) and the locations of their outlets in the oral cavity, and the complex nature of the salivary proteins. The pellicle is the subject of increasing research as it is now thought to play a double role in tooth decay: the pellicle can facilitate the adherence of bacteria to the tooth and the consequent formation of dental plaque, but can also retard the demineralisation of tooth enamel by acting as a diffusion barrier or modifier. Its action as a diffusion barrier/modifier is two fold, as it influences not only the transport of

organic acids to the enamel surface, but also the transport of calcium and phosphate ions out of the enamel after acid attack.

More detailed descriptions of relevant properties of saliva, and the nature and postulated mechanisms of formation of the salivary pellicle are given in Sections 3.6.4 and 3.7.2 respectively.

2.4 ORAL MICROBIOLOGY

The microflora of the oral cavity continues to be the subject of extensive research. Although the oral cavity is sterile immediately after birth, staphylococci and other organisms are present within 6 to 10 hours. The varieties and types of microorganisms present at any time after this are dependent on many factors e.g. whether the mouth is edentulous, has natural or synthetic teeth, the arrangement of the teeth and their history (cariou, filled etc.), diet, oral hygiene, and the nature and flow of saliva. The oral cavity provides a complex continuous culture vessel and ecosystem. The microflora is biochemically active in the fermentation of carbohydrates and the production of proteolytic enzymes. As many as 30 different genera of bacteria have now been isolated and saliva contains as many as 10^9 of these microorganisms per millilitre. A compilation of some of the genera of microorganisms present in the oral cavity is given in Table 2.2. Of those listed, the streptococci, veillonellae, neisseriae and haemophili comprise the bulk of the salivary organisms.

It was indisputably shown in the 1950's that oral bacteria play an important role in dental caries and germ-free rats, fed a cariogenic (i.e. caries supportive) diet, did not develop caries unless inoculated with microorganisms (Orland et al., 1954). The streptococci are the earliest colonisers of the tooth surface after pellicle formation. S. Mutans is now implicated as one of the most virulent causative agents in dental decay and S. Sanguis, S. Mitis, lactobacillus casei and actinomyces viscosus may also be involved. All of these microorganisms have special sites of action in or on the teeth, and are capable of producing enamel destroying acids i.e. are acidogenic.

Table 2.2: Some Organisms Present in Saliva

Actinomyces	Mycoplasma
Bacillus	Neisseria
Bacteriodes	Nocardia
Bacterionema	Pasturella
Borrelia	Propionibacterium
Camphylobacter	Proteus
Clostridium	Pseudomonas
Corynebacterium	Rothia
Escherichia	Selenomonas
Fusobacterium	Spirillum
Haemophili	Staphylococcus
Klebsiella	Streptococcus
Lactobacillus	Treponema
Leptotrichia	Veillonella
Micrococcus	Vibrio

(Compiled from Russel and Melville, 1978)

Bacteria comprise about 70% of dental plaque i.e. up to 10^{12} cells per gram wet weight, and 30% bacterial exudates and salivary components. It will form upon any tooth surface not subject to heavy abrasion. Plaque is an insidious material as not only is it the home of the acidogenic microorganisms, but its structure may also hold the acids produced in close proximity to the tooth surface and prevent their neutralisation or dilution by saliva. The reader is referred to reviews by Gibbons and van Houte (1973) and Bowen (1976) for information on the nature and formation of plaque, and to the review by Theilade and Theilade (1976) for the role of plaque in the etiology of caries and periodontal disease.

2.5 CONCLUSION

Dental decay is not due solely to the presence of acidogenic bacteria as the simultaneous existence of the bacteria, a cariogenic diet and teeth susceptible to decay is required. The complexity of the oral environment and the many causative and contributory factors that lead to poor oral health, has led to the development, in recent years, of research on antidecay vaccines, antibiotics, antiseptics, mutant non-acidogenic strains of oral bacteria, sugar substitutes and plastic sealants for teeth.

Fundamental to this research however, is a better understanding of the nature of the protein interaction with tooth enamel and the subsequent pellicle build-up. The adsorption of proteins at an interface is in itself a complex phenomenon (Section 3.3) and the preceding sections of this Chapter have outlined the complexity of the oral environment. It was therefore decided to perform experimental work to investigate the adsorption of some well characterised proteins on some characterised calcium phosphates under controlled conditions. These studies could then be extended, again under controlled conditions, to include some salivary protein components. Such studies should allow a better understanding of the events that occur in the oral environment.

CHAPTER 3

LITERATURE SURVEY AND OBJECTIVES

3.1 INTRODUCTION

The aim of this chapter is to describe the literature pertinent to the present study and to formulate the objectives of the thesis.

3.2 TECHNIQUES FOR THE STUDY OF ADSORPTION AT SOLID/LIQUID INTERFACES

The interactions of proteins at solid/liquid interfaces have been determined by techniques which monitor changes in some property of the solution and/or the interface itself. The techniques which are available have previously been reviewed (van Enckevort, 1978; Nisbet, 1979; Dass, 1982) and these include various optical techniques e.g. Ultra Violet Spectrophotometry, Infra Red Spectroscopy, Ellipsometry, Optical Interference methods, Circular Dichroism and Electron Microscopy, various electrochemical techniques e.g. Capillary Electrometry, Cyclic Voltammetry and Polarography, various electrical techniques e.g. Electrostriction (using quartz crystal mass detectors) and Electrophoresis, various tracer techniques e.g. Radiochemical and Fluorescent markers, and surface tension, contact angle and calorimetric methods. Using such techniques, information on one or more of the kinetics, amount, mechanism and reversibility of adsorption, the thickness of the adsorbed film, the degree and type of contact, the extent of structural alteration in adsorbed molecules, electrical properties and the thermodynamics of adsorption can be obtained.

In previous studies at this University, extensive use has been made of a radiochemical method, using radioiodinated proteins, which has been found to be suitable for the measurement of protein adsorption in both low and high surface area systems (van Enckevort, 1978; Nisbet 1979; Dass, 1982). This method was chosen as most suitable for the studies envisaged as it offered the required sensitivity, allowed the direct measurement of adsorption, and was adaptable to both qualitative and quantitative studies. Detailed descriptions of the factors which must be considered for its application and the methodology are given in Chapters 5 and 6 respectively.

3.3 PROTEIN ADSORPTION AT SOLID/LIQUID INTERFACES

3.3.1 INTRODUCTION

Although the interactions of proteins at solid/liquid interfaces have now been the subject of much study, little detailed information is available on the adsorption of proteins on calcium phosphate materials such as hydroxyapatite or tooth enamel. Some indication of the surface chemistry of proteins at such surfaces can be obtained, however, from the literature on adsorption at other interfaces. A large and diverse literature now exists on protein adsorption at solid/liquid interfaces and many variables are known to be important. These will be described before proceeding to other aspects of adsorption and models for adsorption.

3.3.2 EFFECT OF EXPERIMENTAL VARIABLES

(A) Effect of Time - Adsorption Kinetics

The following may be recognised as potential rate determining steps in any surface process:

- (a) Transport to the interface
- (b) Adsorption
- (c) Rearrangement and/or reaction
- (d) Desorption
- (e) Transport away from the interface

If desorption is absent or a very slow process, steps (d) and (e) will not be rate limiting. The kinetics of adsorption will then be determined by any of steps (a) - (c). Which of these is rate limiting will be determined by a balance between the diffusion coefficient of the diffusing species, the boundary conditions existing in the system i.e. the diffusion path length as determined by solution conditions such as concentration of the adsorbing species and convection or stirring, factors which may enhance or decrease the rate of adsorption e.g. charge effects and energy barriers, and the actual rate of adsorption. At sufficiently low concentrations of the adsorbing species, diffusion of the species to the interface will be the rate determining step for at least the initial stages of adsorption. At higher levels of surface coverage or solution concentration, steps (b) and (c) may become important.

It is well established that adsorption is time dependent until saturation is reached but there is still considerable confusion in the literature as regards the actual kinetics of adsorption. There is now a growing consensus that the rate limiting step in low surface area systems

is one of diffusion controlled transport to the adsorbent surface, particularly in solutions of low concentration that are not stirred and at low degrees of surface coverage (Mathot and Rothen, 1969; Brynda et al., 1978b; van Enkevort, 1978; Bagchi and Birnbaum, 1981; Bornzin and Miller, 1982; Dass, 1982; Lok et al., 1983b) or at low rates of stirring (Dass, 1982). At higher degrees of surface coverage, the rate of adsorption may decrease because of a decreased area available for adsorption (e.g. Azzam et al., 1977; Penners et al., 1981). Adsorption at high degrees of surface coverage or high rates of stirring might also be slower than expected from diffusion control if a second rate limiting step is operative. Three processes might be envisaged to contribute to this step: surface migration from regions of saturation to regions accessible for adsorption, rearrangement of adsorbed protein to make partially covered regions more accessible and/or enable higher adsorption and the rate of binding to the surface.

In high surface area systems there is often no diffusion path length along which depletion occurs to enable diffusion controlled kinetics. The kinetics in such systems will be determined by either of steps (b) or (c).

In systems where the adsorbed protein is able to desorb (i.e. reversible adsorption), the kinetics may be determined by steps (d) or (e).

(B) Effect of Concentration - Adsorption Isotherms

The adsorption isotherm most commonly reported for protein adsorption at solid/liquid interfaces is that of an initial rise in the amount of adsorption with increasing concentration until a saturation value is attained. The shape of the resulting isotherm is typically that

of the Langmuir type, and the saturation level of adsorption interpreted as monolayer coverage.

Many authors have analysed their apparently Langmuir type isotherms in terms of Langmuir theory of adsorption. Such an analysis is not valid and misleading if the requirements of the Langmuir theory are not met. One of the basic assumptions of the Langmuir theory is that of reversible adsorption. Many workers who have assumed Langmuir adsorption have not tested the reversibility of adsorption (e.g. Hitchcock, 1925; Antoni et al., 1973; Leal and Finlayson, 1977; Lavrent'ev and Sorokin, 1979; Grant and Dehl, 1979) or have shown the adsorption to be only partly reversible or completely irreversible (e.g. Dillman and Miller, 1973; Lee and Kim, 1974; Chuang et al., 1978; Ton et al., 1977; Grant and Dehl, 1979). Norde (1976) observed that the only premise of the Langmuir theory that was met for Human Serum Albumin (HSA) adsorption on polystyrene latex was that the adsorption was "mostly physical".

Stepped isotherms have been observed for α_s - and β -casein adsorption on stainless steel (Nisbet, 1979). The step was suggested to arise through the re-orientation of the adsorbed molecules to enable increased adsorption.

Several interesting phenomena have now been observed in the adsorption of Serum and Egg Albumins. While early studies typically reported Langmuir type isotherms, there have been several reports in which the rising part of the isotherm, rather than being a smooth curve, displayed distinct steps (Bull, 1956 and 1957; Norde, 1976; Hlady and Füredi-Milhofer, 1979; Fair and Jamieson, 1980; Valerio et al., 1980; Walton and Soderquist, 1980; Koutsoukos et al., 1982; Suzawa et al., 1982) but the amounts adsorbed did not correspond to more than monolayer coverage. Many of these workers have suggested the steps arise through changes in molecular orientation or conformation. Norde observed that

the behaviour of HSA in their systems was not always consistent and although a stepped isotherm was observed in some experiments, in other experiments it was smooth. There have now also been several reports of Serum Albumin adsorption in which saturation adsorption was attained down to the lowest concentrations studied (Brynda et al., 1978b, van Enkevort, 1978 and in press; Dass, 1982) even though these concentrations were within or below the range in which increasing adsorption was usually reported. The Langmuir type isotherms were apparently the result of experimental artifacts. Several other authors have suggested that the apparent Langmuir type behaviour observed in their studies with Serum Albumin or other proteins might be due to experimental artifacts (Bagchi and Birnbaum, 1981; Penners et al., 1981). Such artifacts are discussed further in Section 3.4.

Although increases in concentration generally do not promote increased adsorption once monolayer adsorption has been attained, 'S' shaped isotherms and isotherms showing increased adsorption with increasing concentration have been reported (Williams and Choppin, 1950; Brash et al., 1974; Nyilas et al., 1974; Horbett and Hoffman, 1975; Burghardt and Axelrod, 1981). Again it is probable that at least some of these effects are attributable to experimental artifacts discussed further in Section 3.4.

(C) Effect of pH and Ionic Strength

For the majority of systems studied, adsorption is maximal in the region of the IEP of the protein, with a decrease on either side of this pH. In some studies the adsorption has been observed to increase at one (Hitchcock, 1925) or both (Williams and Choppin, 1950) extremes of pH, to decrease on one side of the IEP but increase on the other (Koutsoukos

et al 1982) and little or no variation with pH (Norde, 1976) has also been observed.

Reports of the effect of ionic strength on adsorption have also shown varied results. Increases in ionic strength have been observed to increase (Dow, 1936; Norde, 1976), decrease (Hummel and Anderson, 1965) and to have no effect (Norde, 1976) on adsorption.

The effects of pH and ionic strength on adsorption are often not easily evaluated. Many proteins and surfaces interact with various ionic and non-ionic species and hence, as has been observed, adsorption may be dependent on buffer types (Hitchcock, 1925; McLaren, 1954; Mizutani, 1981; van Dulm et al., 1981). The interaction of the protein or surface with these species is often dependent on the concentration of the species and the solution pH. The effect of pH and ionic strength may be interdependent therefore. This has been observed, for example, in the adsorption behaviour of BSA (Norde, 1976; Suzawa and Murakami, 1980; Dass, 1982; Suzawa et al., 1982), Ribonuclease (Hummel and Anderson, 1965; Norde, 1976) and Egg Albumin (Dow, 1936), and different effects may be observed on different surfaces (Koutsoukos et al., 1982).

There is now evidence to suggest that electrolyte co-adsorption may play a crucial role in the adsorption of proteins on surfaces, particularly if the protein and surface are of similar charge sign (Norde, 1976; van Dulm et al., 1981; Koutsoukos et al., 1982). It is thought that ion co-adsorption occurs to prevent excessive charge buildup at an interface.

Generally, Serum Albumin adsorption shows maximal adsorption at the IEP, with a sharp decrease on either side, on a wide variety of hydrophobic and hydrophilic surfaces and in a wide variety of types and concentration of electrolytes (c/f Bull, 1956; Dillman and Miller, 1973;

Norde, 1976; van Enckevort, 1978; Hlady and Füredi-Milhofer, 1979; Grant and Dehl, 1980; Matuszewska et al., 1980; Suzawa and Murakami, 1980; Dass 1982; Suzawa et al., 1982). The behaviour therefore appears to be independent of the nature of the surface and solution and to reflect a property of the protein. Norde (1976) has suggested that the decreased adsorption on either side of the IEP could arise from either (i) a less densely packed layer due to increased intermolecular electrostatic repulsion between like charged molecules, or (ii) progressive structural alterations of the protein molecule as the pH was shifted away from the IEP. The reduction in adsorption by intermolecular electrostatic repulsion is consistent with increasing net charge on the protein as the pH is shifted away from the IEP. It is also supported by the observation that although ionic strength does not usually affect adsorption at the IEP, at pH's away from the IEP increasing ionic strength results in increased adsorption of Serum Albumins (e.g. Norde, 1976; Suzawa and Murakami, 1980; Dass, 1982). However, this proposal is not supported by data for the adsorption of Ribonuclease, a protein whose structure is essentially unaffected by pH, even at pH's far away from the IEP. The lack of a pH effect is contrary to that expected from increasing intermolecular charge repulsion and supports the postulation that molecular structure is important. This is further supported by the known effect of pH and ionic strength on the size and shape of the HSA molecule (Section 3.6.2) i.e. maximal adsorption occurs at the IEP where the protein is in its native and most stable conformation and decreased adsorption at lower or higher pH's is due to progressive unfolding and expansion. Increased ionic strength at pH's away from the IEP reduces the susceptibility of Serum Albumin to the pH induced structural changes and, hence, leads to increased adsorption. van Enckevort (1978) and Bagchi and Birnbaum (1981) have also suggested, from the study of Bovine

Serum Albumin (BSA) adsorption on Stainless Steel and Immunoglobulin A on poly (vinyl toluene) respectively, that maximal adsorption is governed by the size of the protein molecule in solution. Shastri and Roe (1979) concluded, however, that the effect of pH on adsorption of BSA on polyoxymethylene crystals was due to intermolecular electrostatic repulsion. The authors observed that the amount of adsorption was a unique function of the second virial coefficient (or interaction parameter), B , which was suggested to be a measure of the degree of repulsion between solute molecules in solution.

There have been several reports where the adsorption behaviour of Serum Albumins has been contrary to that usually observed or expected from the above considerations (Brynda et al., 1978b; Mitra and Chattoraj, 1978; Suzawa et al., 1982; Koutsoukos et al., 1982). The specific reasons for such behaviour are not apparent. Assuming that artifacts have been excluded, these may reflect the effects of particular ions at a particular protein interface, the contribution of a significant protein-surface interaction, changed molecular orientation in the adsorbed layer or entropic effects.

(D) Effect of Temperature

The amount of adsorption is dependent on temperature but the nature of this dependence is variable. There have been reports of increasing (Dow, 1936; Kobamoto et al., 1966; van Enkevort, 1978; Dass, 1982) and decreasing (Hummel and Anderson, 1965; Mitra and Chattoraj, 1978) adsorption with increasing temperature.

The effect of temperature in Serum Albumin adsorption on β -ferric oxyhydroxide has been attributed by Dass (1982) to be due to conformational effects. Changes in the levels of adsorption were

consistent with the expected effect of temperature on the conformation of the protein molecule at both the IEP and at pH's away from the IEP. At the IEP, the protein molecule is in its most stable form and, as expected, temperatures up to 40°C have little effect on the levels of adsorption. At pH's away from the IEP, increasing temperature should compress the protein to a more stable form if hydrophobic forces are important in contributing to the determination of the protein structure. Increasing temperature up to 40°C increased the level of adsorption. Further increases in temperature resulted in greatly increased levels of adsorption and this was attributed to the self-association of this protein above 40°C (Section 3.6.2).

However, there have also been reports of Serum Albumin adsorption at pH's away from the IEP (and at low ionic strength) where there has been very little change with increasing temperature (Norde, 1976) or adsorption has decreased (Mitra and Chattoraj, 1978).

3.3.3 THE NATURE OF THE SURFACE-ADSORBED PROTEIN INTERACTION

Intermolecular and intramolecular interactions between amino acid side chain groups play an important part in the formation of the secondary, tertiary and quaternary structures of proteins. It is possible to visualise that the interactions of a protein with an adsorbent surface may involve any or all of the forces responsible for protein structure. Both physical adsorption and chemisorption interactions would be possible.

Electrostatic interactions could arise between oppositely charged ion-ion or ion-dipole groups. The charges on protein side chains or surface groups are dependent on the acid dissociation constant values of the amino acids or groups and hence on the solution pH, ionic strength and temperature. The interactions of these groups may result in the formation of an actual chemical bond (i.e. chemisorption). Protein molecules provide many potential electron donor groups and hence could behave as ligands in surface coordination reactions.

Non-ionic interactions such as interactions between dipoles, induced dipoles and instantaneous dipoles are also possible. These interactions include dispersion or van der Waal's forces and hydrogen bonding. Although a single non-ionic interaction might be weak, a large number of such interactions would give rise to a large net energy of interaction.

Some studies have suggested that carbonyl groups are involved in the interaction of proteins with some adsorbents (Morrissey and Stromberg, 1974; Morrissey, 1977). However, the specific interactions involved between an adsorbed protein and the adsorbing surface generally remain unresolved and speculative. Thus, the adsorption of proteins on glass has been suggested to occur mainly by ionic and electrostatic interactions (Barnett and Bull, 1959; Chatteraj and Bull, 1959; Hummel

and Anderson, 1965; Messing, 1969; Bresler et al., 1974; Nyilas et al., 1974; Chan and Brash, 1981) but that on silicone treated (and hence hydrophobic) glass by non-ionic interactions (Ogino et al., 1979; Mizutani, 1981). The adsorption of proteins on kalonite clay appeared to be similarly governed mainly by electrostatic interactions (McLaren, 1954), but that on halloysite clay (Mills and Creamer, 1973) and hematite (Koutsoukos et al., 1982) was governed by both non-ionic and electrostatic interactions. A similar combination of mechanisms has been suggested for polymer surfaces containing both charged and/or polar and non-polar groups (Pristoupil et al., 1969; Dillman and Miller, 1973; Norde, 1976; Paul and Sharma, 1981; Baszkin and Lyman, 1980; Koutsoukos et al., 1982). Norde (1976) and Bagchi and Birnbaum (1981) have suggested that hydrophobic interactions may be more important than electrostatic interactions on hydrophobic surfaces even though these have a high degree of surface charge. Hence adsorption proceeds even if the protein and adsorbent are of the same net charge. Some authors suggest the mode of interaction is determined by the surface (Baszkin and Lyman, 1980) but other authors suggest this is determined by the protein (Ohno et al., 1981; Paul and Sharma, 1981). Although it may seem reasonable to suggest that ionic or electrostatic interactions will predominate at hydrophilic surfaces and non-ionic interactions at hydrophobic surfaces, proteins and surfaces can be expected to have both hydrophilic and hydrophobic regions. In any system both hydrophilic and hydrophobic interactions could therefore occur. Furthermore, the contribution of each may change with changing conditions which affect the hydrophilicity or hydrophobicity of the protein or surface.

Brash and Lyman (1971) have suggested that maximal adsorption of the IEP indicated a predominantly non-ionic mechanism. These authors seem to have assumed that the protein is not charged at the IEP.

However, it should be noted that at the IEP there are many charged groups present in a protein molecule and it is only the net charge that is zero.

In some studies, the adsorption of proteins on hydrophobic or hydrophilic surfaces has been observed to render them respectively hydrophilic or hydrophobic (Vroman, 1962, Kim and Lyman, 1973; Pearce and Bibby, 1966a; van Enkevort, 1978). Some authors have suggested that this is indicative of the mechanism of adsorption (e.g. Horbett and Hoffman, 1975). Adsorption via a hydrophobic interaction would expose polar sites and render the surface hydrophilic and conversely, an initially hydrophilic surface would become hydrophobic. Such a simplistic interpretation does not allow for the fact that overall hydrophobicity and hydrophilicity are net effects and that a protein is likely to adsorb through both ionic and non-ionic interactions. As there has been no evidence of extensive unfolding upon adsorption at solid/liquid interfaces, it would be expected that adsorption of a hydrophilic protein to a hydrophobic surface would render that surface hydrophilic and vice versa.

3.3.4 REVERSIBILITY

Protein adsorption is a spontaneous process that usually occurs even if the sign of the net charge of the protein and adsorbent surface are the same. For any process to occur spontaneously, the change in the Gibbs free energy of the process (ΔG_{ads}) must be negative. At a given temperature, T , ΔG_{ads} may be expressed as

$$\Delta G_{\text{ads}} = \Delta H_{\text{ads}} - T\Delta S_{\text{ads}}$$

where ΔH_{ads} and ΔS_{ads} are respectively the net changes in enthalpy and entropy for the adsorption process. An increase in entropy, if sufficiently large, could provide the necessary negative value for ΔG_{ads} and the driving force for adsorption, even when ΔH_{ads} is positive.

Non-polar residues are sparingly soluble in water and tend to spontaneously aggregate in an aqueous environment. This type of interaction, although commonly called "hydrophobic bonding", occurs because water-water interactions are thermodynamically favoured over those between non-polar groups or water and non-polar groups. Tanford (1973) has suggested that a higher degree of local order exists in water around non-polar solutes than is usually found in pure (liquid) water. Such solutes are therefore structure promoters (i.e. lyotropic) and an increase in entropy should result from a breakdown of this increased order if the solutes are removed from solution. The effect is thus one of expulsion, rather than bonding. The phenomenon is characterised by a small enthalpy of adsorption, positive or negative, and a large positive entropy increase in the order of $6 - 50 \text{ JK}^{-1}\text{mole}^{-1}$. In solution, many proteins adopt a globular form with a hydrophobic core and a relatively hydrophilic shell. Due to various constraints, complete separation of hydrophobic residues or regions from water is not usually possible and other proteins have very little globular structure at all. Hydrophobic

forces might therefore provide the driving force for adsorption. This suggestion is supported by many literature studies in which various combinations of hydrophilic and hydrophobic adsorbents and proteins have been studied, and in which the authors have suggested that entropy changes provide the largest contribution to the driving force of a reaction (e.g. Pristoupil et al., 1969; Mills and Creamer, 1972; Brash et al., 1974; Norde, 1976; van Enckevort, 1978; Nisbet, 1979; Soderquist and Walton, 1980; Bagchi and Birnbaum, 1981). Norde (1976) has suggested the hydrophobic effects arise from changes in protein structure and dehydration of the adsorbent surface and/or protein upon adsorption. If such effects are not important in a particular system, adsorption might be expected to be determined by charge effects.

If the affinity of a protein for a adsorbent, measured as ΔG_{ads} , decreases with decreasing adsorbent hydrophobicity, and specific interactions between the protein and surface are absent, then relatively weak adsorption would be expected at hydrophilic surfaces. Adsorption at hydrophilic surfaces might therefore be expected to be reversible, while that at hydrophobic surfaces would probably be irreversible (Norde, 1979). Literature studies tend to support this conclusion (e.g. Brash and Lyman, 1969; Norde, 1976; Shastri and Roe, 1979; Edwards and Rutter, 1980; van Wagenen, 1980). Similarly, the adsorption of proteins on some surfaces has been characterised as partially reversible i.e. having both a hydrophilic component which was reversible and a hydrophobic component which was irreversible (e.g. Dillman and Miller, 1973).

However, several curious phenomena have been reported. The irreversible adsorption of BSA to hydrophilic surfaces has been observed (Hlady and Füredi-Milhofer, 1979; Chuang et al., 1978). Bull (1956) and MacRitchie (1972) noted that Serum Albumin adsorption on some hydrophilic surfaces was reversible at pH's away from the IEP but not at this pH.

The adsorption of Collagen on both a hydrophilic and hydrophobic adsorbent was irreversible (Penners et al., 1981). Reversible adsorption has been reported for some hydrophobic surfaces (Grant et al., 1977; Baszkin and Lyman, 1980; Grant and Dehl, 1980). Horbett (1980) observed that Haemoglobin adsorption was irreversible on a series of hydrophobic - hydrophilic adsorbents but that of BSA was partially reversible. Fibrinogen adsorption on hydrophobic and hydrophilic surfaces was partially reversible (Brynda et al., 1980; Chuang et al., 1978) but that of BSA and Immunoglobulin G was not (Chuang et al., 1978). The adsorption of several proteins onto a variety of hydrophobic surfaces, although irreversible at high degrees of surface coverage, was suggested to be reversible at low degrees of coverage (Soderquist and Walton, 1980). Other authors have suggested that the initial adsorption is irreversible (Fair and Jamieson, 1980). These apparent anomalies suggest that hydrophobic forces may not always provide the driving force for adsorption and/or that specific interactions with the surface may be important. Norde and Lyklema (1979) have suggested that ΔG_{ads} can also be determined enthalpically or both enthalpically and entropically depending on the conditions and nature of the system in study.

Unfortunately, a thermodynamic treatment of adsorption is further complicated by a general lack of reversibility when tested by dilution methods. Some authors have suggested that this irreversibility may be essentially a statistical effect as the number of contacts of a protein with a surface is probably large and it is unlikely that sufficient of these would break simultaneously to allow desorption to occur. It could be envisaged however, that regions of a molecule could make and break contacts with the surface where these are reversible. This may explain the surface mobility of adsorbed protein recently reported for BSA adsorption on glass (Michaeli et al., 1980). Irreversibility might also

arise through the formation of chemisorptive interactions.

The reversibility of protein adsorption is a particularly important consideration in the techniques of adsorption chromatography. The desorption of proteins from the hydrophilic adsorbents usually used in these techniques appears to be a competitive or preferential adsorption mechanism where the protein molecules are displaced by either solvent or other solute molecules (Morris and Morris, 1976). Desorption often requires changes in pH, ionic strength or solvent composition. Chan and Brash (1981) observed that although Fibrinogen adsorption on glass was irreversible to dilution, increasing ionic strength caused some desorption and also affected the exchange behaviour. The authors attributed this to ion exchange type behaviour and this was consistent with their suggestion that electrostatic interactions were responsible for binding the protein to this surface. Quantitative desorption of proteins from glass was achieved by the use of acidified urea solutions which were reported to specifically disrupt the postulated ionic amine-silanol and hydrogen bond interaction (Messing, 1969 and 1975). Adsorption chromatography using hydrophobic adsorbents is a relatively new biochemical technique and is not very well characterised (Yon, 1978). The adsorbents consist of hydrophobic groups immobilised on hydrophilic substrates. Desorption from these materials also involves changes in solution conditions (e.g. pH, ionic strength, temperature, the use of detergents, lyotropic salts, chaotropic (i.e. solvent structure breaking) salts and polarity reducing agents such as alcohols. The effects of pH and ionic strength on desorption are suggested to occur via hydrophobic and/or electrostatic effects. Reversibility will therefore depend on whether interactions with the surface can be broken and the driving force for adsorption overcome.

Irreversible binding is observed in some cases or, at best, only partial reversibility. Some authors have suggested that partial reversibility can occur through physical effects. van Enkevort (1978) noted that although the adsorption of BSA on stainless steel was generally irreversible except at very high pH, a small amount of desorption was observed at lower pH's. The amount desorbed increased symmetrically with increasing pH shifts away from the IEP. He suggested that the partial reversibility observed occurred through the pH induced expansion of adsorbed molecules which either forced molecules from the surface by a steric crowding phenomenon, or reduced the degree of protein-surface contact thereby facilitating desorption. Desorption was not enhanced by electrostatic repulsion due to increasing net charge on the protein molecules or like charged protein molecules and adsorbing surface. The mechanism was consistent with the postulate that the amount of adsorption was determined only by the size of the protein molecule in solution (van Enkevort, 1978). The amount of desorption could not be quantitatively predicted however as some hysteresis was observed. (This might be expected if the protein molecule is stabilised to some degree by protein-surface contact). Bagchi and Birnbaum (1981) have suggested similar mechanisms for adsorption and desorption in the Immunoglobulin G/poly (vinyltoluene) system. These authors also observed in their adsorption studies at pH's away from the IEP that a change in pH towards the IEP allowed increased adsorption. This was ascribed to decreases in the size of the adsorbed molecules allowing increased adsorption through reduced steric crowding.

The exchange of adsorbed protein with solution protein has been observed in some studies even where adsorption was previously shown to be irreversible to dilution (Brash et al., 1974; Brask and Samak, 1978; Chan and Brash, 1981). Brash and Samak suggested exchange, rather than

reflecting a spontaneous desorption-replacement mechanism, occurred as a result of collision and dislodgement by solution protein. However, it can also be envisaged that a co-operative effect might occur where as a region on one molecule was breaking contact with the surface an adjacent solution molecule would form contact. Eventually this could result in the newly arrived molecule replacing the previously adsorbed molecule. Similarly, exchange of one adsorbed protein with a different solution protein has been observed (e.g. Vroman and Adams, 1969; Lee et al, 1974; Cuypers et al., 1977; Horbett et al., 1977; Chuang et al., 1978; Waugh et al., 1978; Horbett, 1980; Vroman et al., 1980). Such behaviour can also be inferred from reported competitive adsorption experiments (e.g. Vroman et al., 1975; Brash and Davidson, 1976; Horbett et al., 1977). Waugh et al. (1978) observed that Thrombin was readily displaced by BSA but that BSA was not significantly displaced by Thrombin. The authors attributed this to a higher binding constant for BSA on the Lucite (poly (methyl methacrylate)) surface. Paul and Sharma (1981) have suggested a similar effect is responsible for the preferential adsorption of Albumin on some surfaces. This may well explain the observations of other workers that one component of a mixture of several proteins is often preferentially adsorbed.

3.3.5 THE ADSORBED LAYER

It is now generally accepted that the maximum or plateau values of adsorption correspond to saturation of the adsorbent surface with a close-packed monolayer of molecules. This has been suggested to account for the adsorption behaviour of a wide variety of proteins e.g. β -Casein (Nisbet, 1979); Collagen (Penners et al., 1981), Fibrinogen (e.g. Chau and Brash, 1981), γ -Globulin (e.g. Oreskes and Singer, 1961), various Immunoglobulins (e.g. Cantarero et al., 1980), Bovine and Human Serum Albumin (e.g. Bull, 1956; Norde, 1976; van Enkevort, 1978) and Ribonuclease (e.g. Norde, 1976). Many authors have postulated that this maximum is related to the size and shape of the protein molecule in solution. Provided these are known, the expected amount of adsorption on a known surface can be calculated by assuming close-packing. As the protein molecule has no significant net charge at the IEP, it should suffer minimal intermolecular electrostatic repulsion at this pH and hence adsorb as a close-packed monolayer. Information about the mode of packing can also be obtained by comparison of the dimensions of the protein molecule with the thickness of the adsorbed layer.

Ellipsoidal molecules such as Serum Albumins can be envisaged as adsorbing in a side-on or an end-on configuration. Generally, Serum Albumins are regarded as adsorbing side-on (Bull, 1956; Norde 1976; Mitra and Chattoraj, 1978; van Enkevort, 1978; Grant and Dehl, 1979; Lavrent'ev and Sorokin, 1979; Fair and Jamieson, 1980). This configuration would allow maximal protein-surface interaction. Some authors have, however, suggested a mixture of side-on and end-on adsorption (Dass, 1982), a configuration tending from side-on to end-on (Koutsoukos et al., 1982) or only end-on adsorption (Quinn, 1979; Baszkin and Lyman, 1980). Quinn and Dass have suggested surface morphology and

topography may be important in determining the mode of packing and hence facilitate or stabilise otherwise unusual packing.

Immunoglobulin G is a 'Y' or 'T' shaped molecule with hingelike flexible arms. Bagchi and Birnbaum (1981) have suggested this molecule adsorbs in an upright fashion with the arms away from the surface. Penners et al. (1981) suggested that collagen adsorbed in an end-on rather than a side-on configuration and Uzgiris and Fromageot (1976) have also suggested end-on adsorption for a human red blood cell glycoprotein. Collagen and many glycoproteins undergo self-association in solution and hence their tendency to stable end-on adsorption may be related to this phenomenon. Similarly, Nisbet (1979) has suggested the stepped plateau adsorption isotherm observed for β -Casein, also a self-associating protein, is related to changes in the packing from a side-on to a more end-on configuration.

The concept of a close packed monolayer of adsorbed molecules has been extended by many authors to suggest the adsorption of dimensionally unaltered molecules, or molecules that have suffered changes as a result of adsorption and whether this is related to the surface concentration of the adsorbing species. The phenomenon is particularly important in many biological studies, e.g. the retention or loss of enzyme or immunologic activity upon adsorption. However, it is often not clear in these studies whether these losses arise by denaturation as the result of an altered conformation, or through a masking of active sites. Similarly, in studies of surface thrombogenicity and biocompatibility, it is not known why some surfaces are less thrombogenic than others or invoke some other systemic reaction. Many studies now suggest that the nature of the protein (e.g. Morrissey and Stromberg, 1974; Kim et al., 1975; Morrissey and Fenstermaker, 1976; Akaike et al., 1980; Horbett, 1981) and the nature of the surface (Walton and Soderquist, 1980; Akaike et al., 1980;

Ohno et al., 1980; Horbett, 1981; Suzawa et al., 1982) and hence the nature of the interaction between them are important. This may explain the often reported observation that adsorption at hydrophobic surfaces is usually greater than at hydrophilic surfaces (e.g. MacRitchie, 1972; Edwards and Rutter, 1980; Ikada et al., 1981) but that the converse has also been observed (e.g. Kobamoto et al., 1966; Norde, 1976; Suzawa et al., 1982) as has equal levels of adsorption on both types of surfaces (Grant and Dehl, 1980). The nature of the protein might be important, for example, in the strength of the intramolecular bonds responsible for its structural integrity, and the nature of the surface in its surface energy, hydrophobicity or hydrophilicity. Solution composition and conditions may also be important in determining the stability of the protein molecule under the conditions of the experiment. Although such factors may account for some of the anomalous behaviour, the anomalies in many cases can be ascribed to experimental artifacts e.g. surface preparation procedures, the use of incorrect surface area data and rinsing procedures. These are discussed further in Section 3.4.

Some discussion of adsorption and the adsorbed layer at pH's away from the IEP was given earlier in Section 3.3.1(B).

3.3.6 MODELS FOR ADSORPTION

Several models for protein adsorption have been proposed. These usually attempt to account for the shape of the adsorption isotherm, the amounts adsorbed and the nature of the interactions.

Reversible (i.e. equilibrium) or irreversible (i.e. non-equilibrium) modes of adsorption behaviour can be envisaged. Equilibrium processes could give rise to Langmuir, Freundlich or BET adsorption isotherms. If the adsorption was by a non-equilibrium process, adsorption would proceed to saturation providing that sufficient time was allowed and that the protein solution was not depleted.

The majority of workers have either explicitly or implicitly assumed equilibrium models after obtaining apparent agreement with Langmuir type adsorption behaviour. Often however, adsorption was irreversible or only partially reversible (Section 3.3.2 (B)).

Dillman and Miller (1973) proposed a site model for protein adsorption based on Langmuir theory. The adsorption isotherm was resolvable as the sum of two separate Langmuir isotherms, one reflecting hydrophilic and reversible adsorption, the other reflecting hydrophobic and very slowly reversible adsorption. The authors suggested that many literature reports on reversibility, partial reversibility or irreversibility could be explained by consideration of the relative amounts of both types of adsorption and the effect of experimental variables on these. However, a Langmuir shaped isotherm to describe an essentially irreversible adsorption process is incongruous. Similarly, as a protein molecule is quite large and might well cover several types of adsorption site, binding by both types of binding could be expected. The validity of the desorption studies is also questionable as no steps were taken to prevent microbial attack of adsorbed material.

A combined reversible - irreversible model to describe adsorption on surfaces has also been described by Bornzin and Miller (1982). The model improves on that of Dillman and Miller as the irreversible adsorption is modelled by a diffusion controlled process with saturation instead of an "irreversible Langmuir" process.

An adsorption process where the maximum amount of protein adsorbed is limited solely by the size of the protein molecule has been inferred by some workers since the early days of protein adsorption studies. For example, Hitchcock (1925), Lindau and Rhodius (1935) and Bull (1956) have all suggested the amount of adsorption was limited by the size of the protein molecules. Similarly, Norde (1976) suggested such an effect accounted for the adsorption of HSA and Ribonuclease on polystyrene latex. He further suggested that structural alterations might occur upon adsorption and hence a direct relationship between the solution protein size and the amount of adsorption might not be observed. The co-adsorption of ions could also affect the process. Upon seeing that the adsorption of HSA was irreversible upon dilution, he concluded that the amount of protein adsorbed must be influenced by the kinetics of the adsorption process.

Generally, the models have received little detailed attention. However, van Enkevort (1978, in press) has observed that BSA adsorbs to stainless steel to saturation coverages from solutions of final concentrations as low as 2 g m^{-3} . The adsorption was irreversible to both dilution and exchange. The effect of experimental variables on the rate of adsorption under conditions of diffusion control was consistent with the known effect of these variables on the size, and hence diffusion coefficient, of the protein molecule. Similarly, the saturation amounts of adsorption showed excellent quantitative agreement with those calculated from the hydrodynamic dimensions of the molecule over the

range for which such data was available (pH 3.5 - 5.0). There was no evidence, within experimental error, to suggest any significant adsorption induced structural alteration, nor any significant void space due to intermolecular repulsion. The effects of ionic strength and temperature on adsorption were consistent with the known effects of these variables on the size of the protein molecule in solution.

Bagchi and Birnbaum (1981) have shown that the effect of pH on the adsorption of Immunoglobulin-G on polystyrene latex is also determined by the size of the protein molecule in solution. The levels of adsorption were not affected by changes in sign of the protein relative to the surface. At pH's far removed from the IEP (about 4 pH units), intermolecular electrostatic repulsion did appear to affect the level of adsorption however.

Fair and Jamieson (1980) used a non-equilibrium model to account for the stepped isotherm for BSA adsorption on polystyrene. The authors proposed that at low concentrations adsorption proceeds via a random, uncorrelated, irreversible mode. This is followed by a transition at higher concentrations to a co-operative adsorption mode, forming a close-packed high density ordered surface phase described as a "2-dimensional crystal". Other authors studying Serum Albumin adsorption under similar conditions have not observed this phenomenon however.

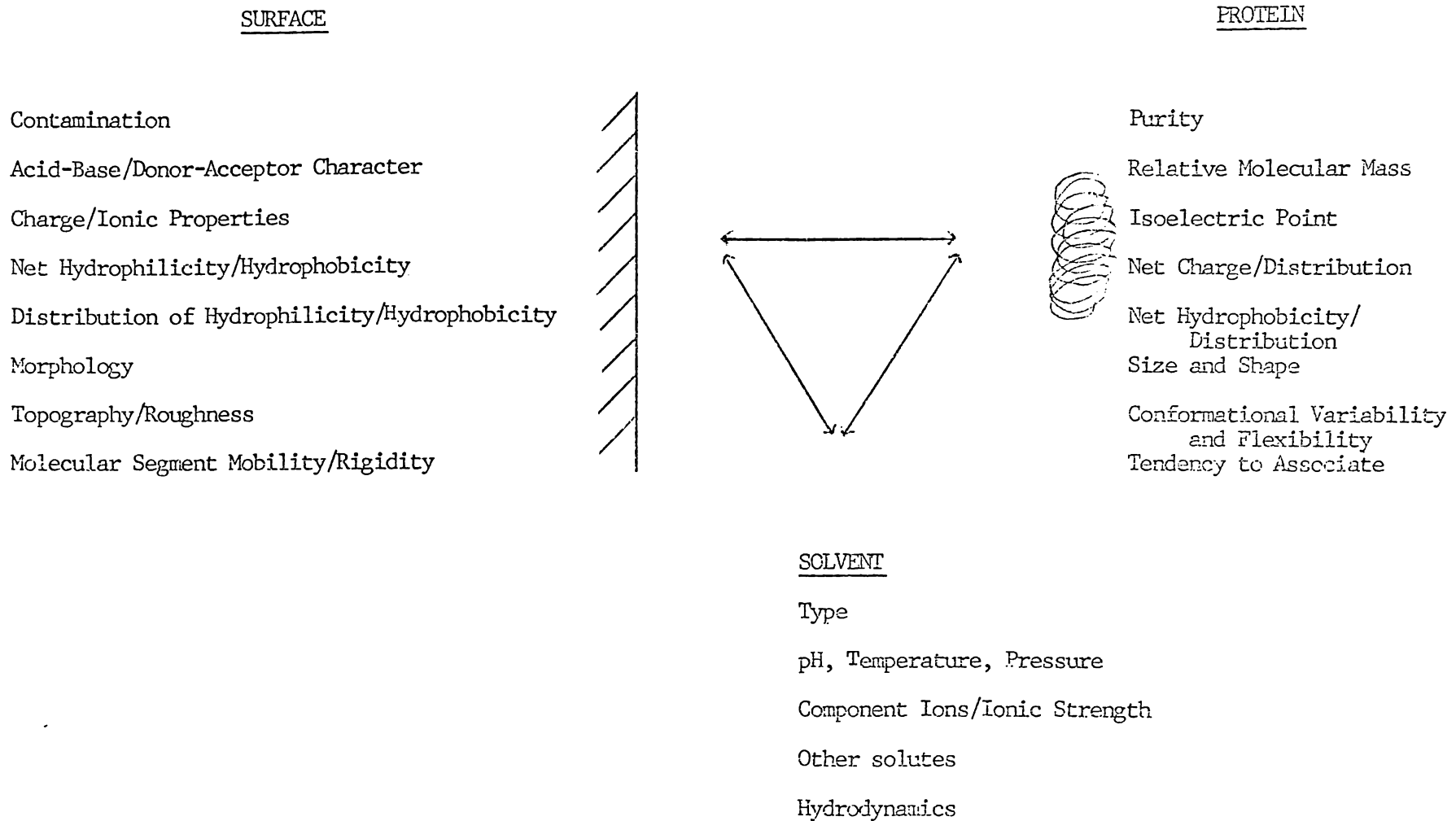
3.3.7 CONCLUSION

Protein adsorption at solid/liquid interfaces is a process affected by many variables. These are often difficult to assess as they represent an interaction between the surface, the protein molecule, other solutes and the solvent, and can also be affected by external parameters such as temperature. The solvent is important in mediating all these interactions. This complex phenomenon and some of the factors that could affect it are summarised in Figure 3.1.

Although the complexity of the interactions of proteins at solid/liquid interfaces may result in inconsistent behaviour in some situations, several generalisations appear to be valid:

- (i) adsorption proceeds to monolayer coverage of the adsorbent,
- (ii) the level of saturation adsorption is usually determined only by the size of the protein molecule and is maximal at the IEP, and
- (iii) adsorption is usually irreversible to dilution.

Figure 3.1: Factors That Could Affect Protein Adsorption at Solid/Liquid Interfaces.



3.4 EXPERIMENTAL ANOMALIES IN ADSORPTION STUDIES

3.4.1 INTRODUCTION

Previous studies of the adsorption of proteins at solid/liquid interfaces at this University have reinforced the necessity for careful characterisation of the system in use and rigorous control of all experimental variables if spurious results and/or incorrect interpretations are to be avoided (van Enckevort, 1978; Dass, 1982). Much confusion exists in the literature where:

- (i) there is a lack of sufficient detail to enable useful comparison or comment;
- (ii) inadequate control or attention has been paid to experimental conditions and/or variables e.g. insufficient time allowed to achieve maximal adsorption, insufficient protein in solution to enable saturation adsorption, the effect of the presence of contaminants, methods of surface preparation;
- (iii) the system in use has been insufficiently characterised e.g. in respect of surface area, the nature of the protein in solution, reversibility and the validity of the use of tracers.

3.4.2 KINETIC STUDIES

In many adsorption studies, information on saturation adsorption limits, adsorbate-adsorbent affinity, surface packing and adsorbate-adsorbate interaction is sought. Such information can only be obtained if adequate time has been allowed for the adsorption process to go to completion.

Some consideration of the possible rate limiting steps in protein adsorption phenomena was given in Section 3.3.2.(A). From this discussion, it is obvious that any rigorous treatment of adsorption kinetics initially requires establishment of whether the adsorption process is reaction rate or transport controlled. Literature studies suggested the latter was often important in protein adsorption studies, particularly at low concentration.

Kinetic processes can often be modelled by the use of mathematical equations. The use of such equations, particularly in diffusion studies, requires some knowledge of the boundary conditions and certain aspects of the adsorption behaviour for the system in use (e.g. Crank, 1957). There are several examples apparent in the literature where authors have tested their data with equations inappropriate to their system and, consequently, drawn invalid inconclusions. For example, Morrissey and Stromberg (1974) tested their adsorption data for Fibrinogen adsorption on finely divided silica by an analysis developed by Crank (1957) to fit diffusion into a sphere. The authors concluded the adsorption was controlled by other than diffusion kinetics. However, the equation of Crank is not suitable to test the kinetics in this experimental system as it is unrealistic to consider the interactions of a large Fibrinogen molecule (~ 45 nm in length) and the smaller silica particles (nominal diameter 12 nm) as diffusion of Fibrinogen into silica. Similarly, the

studies of Gorman et al. (1971) showed that although the adsorption of Fibrinogen on mica appeared to be diffusion controlled when tested by a diffusion equation described by Goodrich (1954), the adsorption on evaporated carbon could not be modelled by this equation. The authors concluded that adsorption on this surface was therefore not diffusion controlled. However, adsorption on the carbon appeared to be reversible and the equation of Goodrich does not apply to a process in which an equilibrium is established. A diffusion equation which allows for simultaneous desorption, such as that developed by Ward and Tordai (1946), would be more suitable.

There have been several examples of reports in which an inadequate control of convection may have contributed to anomalous kinetic behaviour. Recently, De Feijter et al. (1978) and Graham and Phillips (1979) used the technique of ellipsometry and the diffusion equation of Ward and Tordai (1946) to show that the adsorption of Caseins at the air/water interface was diffusion controlled. The calculated diffusion coefficients were greater than the known literature values by up to an order of magnitude however. Similarly, MacRitchie and Alexander (1963) observed that the diffusion coefficient of BSA for adsorption at the air/water interface became faster than predicted with decreasing bulk concentration. In all of the above cases, no precautions were taken to minimise convection. The onset of any convection in these systems would enhance the rate of reaction by reduction of the diffusion path length, a situation not allowed for by the treatment of Ward and Tordai.

The results of these studies contrast with those of van Enckevort (1978) and Dass (1982). van Enckevort studied the rate of adsorption of BSA at the stainless steel/aqueous solution interface in narrow walled adsorption vessels designed to minimise natural convection. Adsorption data was analysed by the Ward and Tordai equation and good agreement

between the calculated and literature value of the diffusion coefficient was obtained. Dass studied the rate of adsorption of BSA to a surface layer of β -ferric oxyhydroxide. He obtained a diffusion coefficient greater than the accepted literature value from adsorption studies in which the large vessels and large volumes of solution subject to convection were used. However, excellent agreement was obtained using a rotating disc apparatus, which allowed a rigorous control of boundary conditions, and consequent analysis by the theory of Levich (1962).

Many proteins display a high degree of conformational variability and adaptability in solution as induced by the solution conditions. The value of the diffusion coefficient may therefore vary with changes in these conditions. This is particularly so for Serum Albumins (c/f Creeth, 1952; Champagne, 1957a; Harrington et al., 1956) but does not seem to have been taken into account by van Dulm and Norde (1983) in their study of HSA adsorption to some solid surfaces.

A significant proportion of the literature reports do not include adequate kinetic data, if any at all. Typically, kinetic studies are limited to one or two moderately high concentrations and the time taken to reach maximum adsorption at these concentrations is then used routinely for all other concentrations. As diffusion control becomes important at low concentrations, the rate of adsorption may be orders of magnitude slower at such concentrations. van Enkevort observed that the adsorption of BSA on stainless steel at a concentration of 8 g m^{-3} apparently reached a maximum after 30 minutes. Consequently, an adsorption time of 35 minutes was used to obtain the concentration adsorption isotherm and a Langmuir shaped isotherm was obtained. Subsequently it was found that the adsorption at low concentrations was diffusion controlled and the adsorption from a solution of concentration 2.1 g m^{-3} required about 11 hours to reach saturation. The same level of

adsorption was attained as at the higher concentrations (Figure 3.2) and hence the Langmuir shaped isotherm was an artifact of the experimental procedure. Adsorption at less than saturation coverages reflected the diffusion of protein to the surface.

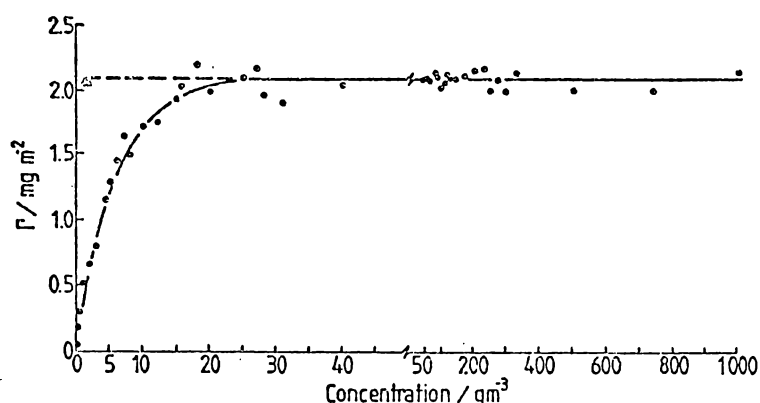


Figure 3.2: Adsorption Isotherm of BSA on Stainless Steel.
 Adsorption times: (—•—) 35 minutes
 (—Δ—) 11 hours.

A similar result was observed for HSA adsorption to polyethylene by Brynda et al. (1978a). At concentrations less than 200 g m^{-3} and under static conditions, less than saturation coverage was attained after 1 hour adsorption time. The amount of adsorption was observed to be flow rate dependent however, and the same level of saturation could be achieved from solutions of any concentration provided sufficient time was given. An adsorption time of 4 hours was required at a flow rate of 115 ml hr^{-1} and a concentration of 1 g m^{-3} . The resulting straight line isotherm contrasts markedly to that of Grant and Dehl (1980) who also studied the adsorption of HSA on polyethylene. These authors found that

at a concentration of 560 g m^{-3} , saturation adsorption was reached within 5 minutes. The subsequent adsorption showed less than saturation adsorption in the concentration range 0 to 300 g m^{-3} . This was regarded as evidence for Langmuir type adsorption whereas it more probably reflects the kinetics of adsorption.

3.4.3 INCOMPLETE ADSORPTION DUE TO DEPLETION

van Enckevort (1978) observed that the use of small solution volumes of low protein concentration could be significantly depleted by adsorption to container walls and to the adsorbent itself. The system used was one of a low surface area : volume ratio (stainless steel plate) and suggested that depletion problems could be highly significant in systems of high surface area : volume ratios e.g. studies using colloidal or suspended particles. Dass (1982) observed that the adsorption isotherm obtained in such systems when there was insufficient protein to allow saturation adsorption was of the Langmuir shape. If sufficient protein was available, adsorption proceeded to the same saturation level at all concentrations studied. The resulting isotherms were similar to those shown previously in Figure 3.2.

The study of Ton et al. (1977) seems to have suffered from this problem but this does not appear to have been recognised by the authors. Examination of their data shows that the solution protein was completely depleted in the low concentration region of the isotherm. The authors used 50 ml of 100 mg ml^{-1} HSA mixed with 10 g of XAD-7 resin, having a specific surface area of $450 \text{ m}^2 \text{ g}^{-1}$, to study the kinetics. Maximal adsorption of $\approx 1.1 \text{ mg m}^{-2}$ was observed after some 40 hours. This was suggested to correspond to equilibrium adsorption and experimental work for the adsorption isotherm was carried out in a similar manner except that 48 hours was allowed for adsorption. The resulting isotherm when initial concentration was plotted against adsorption was Langmuir in shape. A simple calculation shows that the level of adsorption observed in the kinetic study, for example, corresponds to total depletion of solution protein. This suggests the isotherm reflects the maximum adsorption at each concentration limited only by the total amount of protein in solution.

3.4.4 CONTAMINATION

The contamination of experimental systems may arise from chemical materials e.g. reagents or by species such as air or solution borne particulate matter.

Many chemical species are surface active. These may concentrate at an interface and influence adsorption behaviour. The problem is of particular relevance to the preparation of polymers and polymer lattices. The difficulties associated with the removal of emulsifiers, initiators, monomers and buffers has previously been discussed by Norde (1976). Baszkin and Lyman (1980) have observed that the surface tension of solutions used to store various polymer films may also decrease with time as different polymer constituents such as solvents and plasticizers are leached out.

Contamination may also result from dusts or other solid particulates either suspended in solution or in direct contact with a surface. These contaminants may adsorb proteins themselves and/or physically block adsorption on surface sites.

Although many different methods of surface preparation are used, there have been few studies to assess their effect on adsorption. Several authors have observed that different cleaning procedures used to prepare surfaces for adsorption studies were significant in affecting the levels of adsorption. van Enckevort (1978) observed that the preparation of stainless steel slides by detergent or acetone washing resulted in anomalous adsorption behaviour compared to those cleaned by an acid/alkali treatment procedure. This was attributed to chemical contamination of the surface. Similarly, Baszkin and Lyman (1980) observed that polymers cleaned by the often reported technique of immersion in a dilute detergent, followed by rinsing with water, methyl alcohol and ether showed up to 10 times greater levels of adsorption.

3.4.5 SURFACE AREA CHARACTERISATION

It is well established that the amount of adsorption is dependent on the surface area available. Probably one of the more useful parameters obtained from adsorption studies is the amount of adsorption and this is most usefully expressed as a mass adsorbed per unit surface area. Solid/liquid systems can be broadly classified as those employing adsorbents of relatively low specific surface area e.g. plate or sheet materials, and those of relatively high specific surface area e.g. suspended particles or colloidal materials.

Many low specific surface area materials do not have a plane surface. The true surface area is then not the apparent or nominal area but this area multiplied by a surface roughness factor. For example, the true surface area of AISI Type 304 Stainless Steel with a 2B surface finish may be up to 3 times the nominal area (O'Connor and Uhlig, 1957). The roughness factor may vary from sample to sample. van Enkevort (1978) observed that the stainless steel surface used in this study had a roughness factor of 2.6 but that used by Dass in a later study (Dass, 1982: unpublished data) had a roughness factor of 2.3. Many sheet polymer materials also display a high degree of surface roughness (Ratner and Hoffman, 1980) and different samples may display different degrees of roughness (Watkins and Robertson, 1977). Few studies appear to take this into consideration however.

Surface modification methods may result in changes in the true surface area available for adsorption. There is now an increasing amount of literature on adsorption studies involving the use of polymer materials grafted to or deposited on a substrate (e.g. Ikada et al., 1981). Consideration of the true surface area of these adsorbents is notably lacking however. Ratner and Hoffman (1980) have shown that many

of these materials have a high degree of surface roughness. Further, the polymers may not always uniformly or completely cover the substrate material nor be stable to the methanol/acetone extraction methods routinely used on them. The use of materials known as hydrogels for protein adsorbents is also increasing (e.g. Horbett and Hoffman, 1975). The materials are distinguished from other polymers by their capacity to imbibe up to 90% by weight of water in their structure. The gel structure is therefore highly porous to water at least. King et al. (1979) have described the hydrogel as a highly mobile interface in which the surface polymers adopt a configuration which minimises their interfacial free energy. Adsorption might therefore be dependent not only on the degree of grafting, as has been observed by Horbett and Hoffman (1975), but also dependent on the nature of solution and be altered by protein adsorption itself. The porosity and/or mobility of the interface may explain the continued slow increases in adsorption observed over several days from solutions of relatively high concentration by some workers (Horbett and Hoffman, 1975; Brynda et al., 1978b).

The stability of colloidal or particulate suspensions may be profoundly affected by the presence of surface active materials. These may stabilise or destabilise the suspension. If the latter occurs, the resultant floc formation may result in a decreased surface area. The phenomenon is complex in that the stability of the suspension is affected by electrostatic, entropic, osmotic, steric and/or bridging mechanisms. Electrolytes, the ratio of the amount of surfactant : amount of particulate matter, the rate of adsorption, method of mixing and amount of agitation may all be important.

Dass (1982) observed irreversible flocculation of colloidal β -ferric oxyhydroxide in adsorption studies, particularly at low concentrations of

BSA. The levels of adsorption were decreased in solutions showing flocculation and Dass suggested this occurred through flocculation causing a reduced surface area available for adsorption. When the colloidal material was stabilised by pre-adsorption to a solid surface the same saturation level of adsorption was attained at all concentrations. The resulting isotherms were again similar to those shown in Figure 3.2. Norde (1976) observed a Langmuir shaped isotherm in his studies of HSA adsorption on polystyrene latex even though the adsorption was shown to be irreversible to dilution. The results seemed to show that "bare" polystyrene latex could co-exist with dissolved protein molecules. However, polystyrene latex particles have been observed to be flocculated by γ -Globulin (Singer et al., 1973) and HSA and Fibrinogen (van der Scheer, 1978a). This suggests that flocculation may have been the cause of the anomalous behaviour observed by Norde.

Ultimately, the surface area available for adsorption in any system will be determined by the relative size of the protein molecules with respect to the morphological and topographical variations of the adsorbent. This will be of particular importance in the use of porous materials as adsorbents where the availability of the pore to protein adsorption will determine the level of adsorption.

3.4.6 NATURE OF THE ADSORBING SPECIES

Valid interpretation of the results of any adsorption study requires some general knowledge of the nature of the adsorbing species and its state under the conditions of the experiment.

Lyklema (1980) has suggested that polydispersity may result in preferential adsorption phenomena with consequent changes in the shape of isotherms. Koopal (1981) has suggested that in polydisperse polymer systems, the shape of the isotherm and the amount adsorbed depends on the area : volume ratio used in experiments. These effects have received little attention in protein adsorption studies. Recently Grieg and Brooks (1981) have voiced some concern over this issue as regards the rate and amount of adsorption and reversibility effects in protein systems.

Serum Albumins, which are a much used protein in adsorption studies, can form polymeric species. Many commercial preparations of this protein contain high levels of various polymers though a study of BSA adsorption on stainless steel did not reveal different levels of adsorption for several preparations containing varying proportions of monomeric and polymeric material (van Enkevort, 1978). Chuang et al. (1978) likewise did not observe any preferential adsorption of polymeric material on Cuprophane. However, Grieg et al. (1981) observed that the dimer : monomer ratio of polystyrene adsorbed protein was enhanced compared to the bulk protein.

A further complication arising from the use of Serum Albumin is that the protein binds fatty acids. Horbett and Hoffman (1975) observed that the defatted sample gave a Langmuir shaped isotherm for adsorption on hydrogels but the non-defatted sample gave a Freundlich type isotherm over the same concentration range. Chuang et al. (1978) and van

Enkevort (1978) observed no differences in behaviour, however, for the adsorption of these preparations on PVC, Cuprophane or Stainless Steel. The reason for the anomalous behaviour is not apparent but may reflect on interaction of the protein and/or fatty acids with the hydrogel adsorbents used in the former study.

3.4.7 REVERSIBILITY OF ADSORPTION

Many of the methods used in protein adsorption studies require some rinsing of the adsorbent prior to determination of the amount adsorbed or use in another experiment. Rinsing must remove any carry-out, i.e. adherent solution containing non-adsorbed material, without desorbing significant amounts of adsorbed material. If any desorption occurs within the rinsing time, uncertainty will exist in the experimental results. It is therefore desirable to keep rinsing to a minimum and consistent with avoiding carry-out from the solution. Typically, the adsorbent is rinsed until a constant activity is obtained. It is particularly important to determine the effect of rinsing but such data is notably lacking in many reports.

A short rinsing time might generally be expected. van Enckevort (1978) and Nisbet (1979) observed that carry-out on stainless steel was effectively removed within about 10 seconds of rinsing. Further rinsing had no effect on the level of surface activity. Kim and Lee (1975) observed that one polymer used in their study required a two minute rinsing time whereas two other polymers both only required one minute. The level of adsorption on the first polymer was always lower than on the other two. This may therefore reflect some degree of reversibility. Chuang et al. (1978) observed that significant counts were observed in the rinse solutions for adsorbed Fibrinogen and to a lesser extent for Immunoglobulin G on Cuprophane and PVC. This was attributed to a slow continual desorption of adsorbed material. Horbett (1980) observed a significant degree of reversibility for BSA adsorbed on several polymers on standing overnight in adsorption buffer but did not ascertain the effect of his 1/2 minute high flow rate rinse. Eberhart et al. (1977 and 1980) used a 1 hour rinse time for HSA adsorbed on Teflon. The authors

did not report the effect of rinsing but did observe that HSA only adsorbed to partial coverage. Kim and Lee (1975) observed in their study that a one minute rinse was sufficient to remove carry-out from this material when using BSA and several other proteins. This suggests the results of Eberhart et al. reflect a degree reversibility rather than an inability to achieve saturation coverage. Brash and Uniyal (1979) have suggested that lower levels of adsorption on hydrophilic surfaces may often be due to rinsing effects. However, as mentioned previously, surface area effects may also be a contributing factor.

The observation of rounded isotherms i.e. isotherms that show curvature at low concentrations in systems where adsorption is shown to be irreversible, is particularly anomalous. The disparity may be due to several factors: Lyklema (1980) has described how the presence of polydispersity in synthetic polymer preparations may give rise to such isotherms and suggested the effect may also apply to biopolymers such as proteins. The larger polymeric species displace the smaller species and hence give rise to a higher mass adsorbed. The greater the polydispersity, the more rounded the isotherm. Adsorption must be reversible, or at least exchangeable, for this effect to occur. Such an effect may account for the non-smooth or stepped isotherms observed in some studies, especially those using Serum Albumin as commercial preparations of this material often contain much polymeric material. Many studies using this material do not report such isotherms however.

The discussion in previous sections has shown that rounded isotherms may arise from kinetic, solution depletion and/or flocculation effects.

Irreversibility to dilution may occur as a result of experimental procedures in desorption studies. It is important to have a large volume of desorption solution so that if adsorption is reversible, the solution protein concentration will be below that required to maintain saturation

and significant desorption can be achieved. For surfaces of high affinity this will be particularly difficult as the solution concentration required to maintain saturation adsorption will be very low. Solution or surface concentration changes could be insignificant within the sensitivity of the measuring technique.

3.4.8 THE USE OF TRACERS

Some discussion of the factors relevant to the production of valid tracer preparations is given in Chapter 5. There are now many reports in the literature of adsorption studies which have used proteins labelled with radiochemical or fluorescent markers. The use of such tracers is invalidated if the labelled and non-labelled species do not behave identically. Several reports have now appeared which question the validity of the use of radiochemical tracers in adsorption studies (Reed and Rossall, 1958; Grant et al., 1977; van der Scheer et al., 1978b; Klein Elhorst et al., 1978). The early study of Reed and Rossall used Iodine-131 labelled HSA to monitor the adsorption of this protein on glass. These authors observed that the labelled material behaved differently to the non-labelled. This is not surprising however, as at the time of this study, many of the criteria for the preparation or use of labelled proteins as suitable tracers were not known. Grant et al. observed that the iodine labelled material was preferentially adsorbed to some materials e.g. platinum, but not on others e.g. chromium or silicon. The effect in this study appeared to be a preferential binding of Iodine to the platinum surfaces, as the presence of free iodide in solution caused reduced levels of adsorption. The levels of adsorption in the 'absence' of free iodide are about 30 times the maximum observed in other studies of this protein however. This provides some evidence for the presence of free iodide in their protein preparations. van der Scheer et al. observed that Iodine labelled species might be preferentially adsorbed to polystyrene but the accuracy of their analytical method did not allow a positive conclusion to be made. Studies at this University have shown that Iodine-125 labelled BSA was not preferentially adsorbed to polystyrene (van Enkevort, unpublished results).

There are now several reports where the validity of the use of labelled proteins as tracers has been demonstrated (Brash et al., 1974; Horbett et al., 1977; Brash and Samak, 1978; Chuang et al., 1978; van Enkevort, 1978; Hlady and Furedi-Milhofer, 1979; Nisbet, 1979; Horbett, 1981; Dass, 1982). However, some anomalous behaviour can be observed if care is not taken. van Enkevort (1978) observed several anomalous isotherms for BSA adsorption on stainless steel and these were attributed to the use of tracer solutions which had suffered a breaking of the protein-iodine bond. The isotherms obtained in these situations were of the Freundlich type. A saturation value for adsorption was not observed and the levels of adsorption were far above that usually observed in the system. Such phenomenon may also account for anomalous adsorption in studies by Brash and co-workers. In contrast to an early study in which Brash and Lyman (1969) observed a Langmuir type isotherm for the adsorption of HSA on polyethylene, the results of a later study showed a similar anomalous Freundlich behaviour (Brash et al., 1974). Brynda et al. (1978a) have also noted that free radioiodide should be rigorously excluded from experiments of protein adsorption on hydrogels as the label can penetrate and accumulate in the hydrogel structure.

The use of fluorescent markers as tracers in adsorption studies is now becoming more widespread. Watkins and Robertson (1977) who used fluorescein labelled γ -Globulin to monitor the adsorption of this protein on silicone rubber demonstrated the validity of the use of this material as a tracer in their system. Lok et al. (1983b) observed that similarly labelled BSA and Fibrinogen were also valid tracers in adsorption studies using this substrate. Brynda et al. (1978a) have observed however, that the fluorescence of the labelled protein was not always constant, the label was not particularly stable, that the stability was dependent on the marker used as well as the surface in study, and that the labelled

protein could be preferentially adsorbed on some surfaces. These authors used two fluorescein derivatives and a naphthalene derivative as markers to monitor BSA adsorption. Burghardt and Axelrod (1981) used a rhodamine dye labelled BSA as a tracer in adsorption studies. The authors observed multilayer adsorption of BSA on the quartz glass substrate. This result contrasts markedly to that observed in other adsorption studies using this protein and a wide variety of surfaces including glass. The authors could not establish the validity of the use of this tracer nor did they characterise the tracer preparation. Typically the degree of labelling was 2 - 5 rhodamine groups per protein molecule. This is far above the level generally accepted as avoiding alteration of the physiochemical properties of a protein molecule by the incorporation of the much smaller Iodine atom (Section 5.2).

3.4.9 CONCLUSION

The literature on protein adsorption provides ample evidence that the adsorption process is a complex phenomenon involving the interplay of many variables. Meaningful results, conclusions and comparisons can only be obtained from studies in which experimental artifacts and anomalies have been rigorously precluded. The present survey shows that it is important to fully characterise the system in use, specifically the nature of the protein e.g. polydispersity; the nature of the adsorbent e.g. surface area and the effect of experimental conditions on this; and the experimental procedures used e.g. avoidance of contamination, the validity of the use of tracer preparations, the kinetics of adsorption, depletion effects, flocculation effects, reversibility studies.

Any studies engaged in in the present study will be designed to give such information and to avoid pitfalls. A primary requirement will be characterisation of the experimental system and procedures used prior to further studies.

3.5 PROTEIN ADSORPTION ON CALCIUM PHOSPHATE MATERIALS

3.5.1 INTRODUCTION

There are now many reports in the literature on protein adsorption on various calcium phosphate materials such as hydroxyapatite and tooth enamel, and the use of these materials in chromatographic studies. Generally, the emphasis of these studies has been on qualitative and clinical aspects with little attempt to provide a rigorous quantitative study. It is for this reason that the literature on protein adsorption on calcium phosphates is described separately from that at other solid/liquid interfaces.

3.5.2 EFFECT OF EXPERIMENTAL VARIABLES

(A) Effect Of Time - Adsorption Kinetics

There do not appear to be any detailed studies of the kinetics of adsorption. Typically, researchers use adsorption times varying from 1 minute to 4 hours, with the majority of workers using times in the range of 5 - 20 minutes, as after such times there is apparently no further increase in adsorption. However, Kozin and McCarty (1977) who used an adsorption time of 20 minutes in their study, noted that longer times allowed more adsorption. Similarly, Pearce and Bibby (1966 a, b) who used an adsorption time of 1 hour in their studies noted that this was insufficient to allow maximum adsorption. Further, the procedural details of the various isotherm experiments e.g. protein concentrations and area : volume ratios were often different from those used in the kinetic studies.

(B) Effect of Concentration - Adsorption Isotherms

Some attempts have been made to do quantitative studies of the effect of concentration on adsorption. As in the studies of adsorption in other systems (Section 3.3), the most commonly reported isotherm is of the Langmuir shape. Many authors have analysed their isotherms in terms of the Langmuir theory although the irreversibility of adsorption had not been tested (Moreno et al., 1977 and 1978; Pruitt, 1977; Hay and Moreno 1979b; Roukema et al., 1980). The results of some workers suggest that adsorption is generally irreversible to dilution (Pruitt et al., 1969; Knutilla and Paunio, 1978). Hlady and Füredi-Milhofer (1979) observed that the adsorption of HSA on hydroxyapatite was irreversible but still observed a rounded isotherm similar to the Langmuir shape. The isotherm was not smooth and displayed small steps over the whole concentration range.

Pearce and Bibby (1966a) have measured isotherms for the adsorption of various proteins on tooth enamel. The authors observed Langmuir shaped isotherms for Gelatin, γ -Globulin, β -Lactoglobulin and Serum Albumin, isotherms showing increasing adsorption with increasing concentration for Casein, α -Globulin, Haemoglobin and β -Lipoprotein, and roughly 'S'-shaped isotherms for Egg Albumin and Gastric Mucin. However, the authors' acknowledgement that their adsorption time was insufficient to allow saturation adsorption (Section 3.5.2(A)) suggests the isotherms may reflect the kinetics of adsorption.

The effect of the presence of fluoride in the hydroxyapatite lattice remains confused despite many studies. Fluoride treated apatites have been observed to adsorb decreased (e.g. Ericson and Ericsson, 1967; Rølla et al., 1977; Belcourt and Voegel, 1978; Voegel et al., 1981) or increased (Moreno et al., 1977; Rølla et al., 1977) amounts of protein,

and that this could also depend on the protein under investigation (Rølla et al., 1977). Some workers have taken the initial slopes of their isotherms as a measure of the affinity of the protein for the adsorbent and the number of adsorption sites (e.g. Moreno et al., 1977 and 1978; Hay and Moreno, 1979b). Such an analysis is meaningless if the initial slopes are kinetically controlled.

In many studies, adsorption results are often reported as the amount of adsorption per mass of adsorbent added and have a notable lack of surface area data (e.g. Krögstad and Rølla, 1977; Bennick et al., 1981).

(C) Effect of pH

Studies of the effect of pH on adsorption are limited by the increasing solubility of calcium phosphate salts at acid pH's. In a study of the effect of pH at pH's greater than 5.5, Sörbo (1961) observed that adsorption was maximal at the IEP for those proteins with IEP's higher than this pH, or increased with decreasing pH for those proteins with IEP's lower than this value. Later studies have observed similar behaviour (e.g. Pearce and Bibby, 1966b; Hlady and Füredi-Milhofer, 1979).

(D) Effect of Ionic Strength

Sörbo (1961) observed that the effect of ionic strength on adsorption was dependent on the protein, the electrolyte used, and the pH of the solution. He suggested the effects were due to anion-protein and anion-adsorbent interactions. Generally, phosphate ions decreased adsorption whereas calcium ions increased adsorption. McGaughey and Stowell (1971b) observed that calcium ions enhanced the adsorption of salivary proteins by hydroxyapatite. The effect was pH dependent and

appeared to arise only if the calcium was bound to the proteins.

Some authors have purported to study the effect on adsorption of the presence of fluoride and/or monofluorophosphate ions in solution. However, due to the rapid exchange observed for these ions of surface hydroxyl in hydroxyapatites (Section 3.6.5), it is not clear whether the effects are due to the experimental conditions or adsorption and/or exchange of the fluoride on the surface.

(E) Effect of Temperature

Salivary protein adsorption has been observed to increase as the temperature was increased from 4°C to 37°C (Ericson, 1968) though little effect occurred between 26°C and 37°C (McGaughey and Stowell, 1971b).

3.5.3 THE NATURE OF THE SURFACE-ADSORBED PROTEIN INTERACTION

Current concepts on the nature of the interactions between proteins and calcium phosphate materials have largely been formulated as a result of the chromatographic studies of Bernardi et al. (1972). The workers suggested the electrostatic interactions form the basis of the interaction : calcium binds the acidic (i.e. anionic) protein side chain groups; phosphate sites have a high affinity for basic (i.e. cationic) side chain groups. These studies have since received support from electron spin resonance studies (Peckauskas et al., 1976). Although hydroxyl groups did not appear to be involved in the binding, a recent report by Musha and Takaesu, Y. (1976) suggests that interactions with this group do occur. Polar forces (Glantz, 1977) and non-ionic forces (Kozin and McCarty, 1977) have also been suggested to be involved.

Phosphoproteins appear to be exceptionally strongly bound to

hydroxyapatite and to tooth enamel (Bernardi and Cook, 1960; Bennick et al., 1979; Sleight et al., 1979; Juriaanse et al., 1981). In a study of several low molecular weight salivary proteins, Juriaanse et al. suggested that this arises through an exchange reaction between protein and adsorbent phosphate groups.

3.5.4 REVERSIBILITY

As observed in studies at other solid/liquid interfaces, protein adsorption on hydroxyapatite is generally irreversible to dilution (Section 3.5.2(i)). Some authors have observed that Amylase (Pruitt et al., 1969) and Lysozyme (Steifel, 1976) may be desorbed from tooth enamel using water. These reports are contradictory however, as Pruitt et al. did not observe desorption of Lysozyme using water, and are also inconsistent with the observed behaviour of proteins in chromatographic procedures. Juriaanse et al. (1981) have observed that the strength of adsorption, as determined by elution molarities, is the same for tooth enamel and hydroxyapatite, and Tiselius et al. (1956) observed that elution of Lysozyme from hydroxyapatite required ionic strengths higher than those used in the adsorption solution. The general mechanism thought to be involved in the chromatography of proteins on hydroxyapatites is ion exchange and competition for binding sites i.e. desorption of anionic proteins is induced by phosphate or other anions and desorption of cationic proteins is induced by cations. The concentration gradients employed in chromatographic procedures are usually low if calcium or phosphate salts are used to desorb oppositely charged proteins but much higher for proteins of the same net charge. However, unexpectedly strong, and irreversible binding is observed for

some proteins (Bernardi et al., 1972) even though these are not phosphoproteins.

Exchange of hydroxyapatite or tooth enamel adsorbed protein by other solution protein molecules does not appear to have been studied. If such a phenomenon does occur, as has been reported for some solid/liquid systems and mixtures of proteins (Section 3.3.4), this may explain reports of the preferential or specific adsorption of some proteins from saliva (e.g. Hay, 1967; Pruitt et al., 1969; Armstrong, 1971; Hay, 1973a).

3.5.5 CONCLUSION

The adsorption of proteins on calcium phosphates is generally poorly characterised, particularly as to the nature of the adsorption isotherm and the effect of the incorporation of fluoride in the crystal lattice. This is not altogether surprising as many studies have been of a qualitative nature without any rigorous control of experimental variables. Further, the usefulness of those studies in which a more rigorous and quantitative approach has been adopted is limited as many of these appear to suffer from the lack of a careful determination of the kinetics of adsorption, and those studies using whole saliva deal with a complex mixture of proteins in a complex solution.

It is therefore the concern of present study to establish the adsorption isotherms of several proteins and the effect of the incorporation of fluoride in the hydroxyapatite lattice on the levels of adsorption. The effect of experimental variables on adsorption, under rigorously controlled conditions, can then be determined and should provide further information on the nature of the adsorption isotherm e.g. reversibility and exchange, and the nature of the adsorbed layer.

3.6 PHYSICO-CHEMICAL PROPERTIES OF SOME PROTEINS AND ADSORBENTS USED

3.6.1 INTRODUCTION

The importance of the use of well characterised proteins and adsorbents has been noted (Section 3.4). The complexity and variability of the salivary fluid and the general poor characterisation of many of the components has been noted (Section 2.3). Further, due to envisaged difficulties associated with the isolation of individual components in a pure form and in sufficient quantity for the studies envisaged, it was decided to initially use proteins commonly found in the diet. The proteins chosen were Bovine Serum Albumin and β -Casein as these are readily available commercially in a pure form and much is known of their physico-chemical properties. Both proteins have been previously used in adsorption studies at this University (van Enkevort, 1978; Nisbet, 1979; Dass, 1982) and Serum Albumins are commonly used by other researchers in adsorption studies in solid/liquid systems.

Two choices of adsorbent seemed reasonable for the present study: either tooth enamel or model systems such as various synthetic hydroxyapatites. Due to the difficulties in obtaining tooth enamel of uniform composition and in a reasonable and suitable condition for the studies envisaged, it seemed likely that the synthetic materials would offer the better system for study.

The following Section is therefore used to provide a detailed review of the physico-chemical properties of the major proteins and adsorbents used.

3.6.2 SERUM ALBUMINS

Serum Albumin is the most plentiful and most familiar plasma protein. It has been assigned many physiological roles e.g: transport of fatty acids, sequestration and transport of bilirubin, and maintenance of the osmotic pressure of blood. Several comprehensive reviews on Serum Albumin are available (Hughes, 1954; Foster, 1960; Peters, 1970, 1975 and 1977; Franglen, 1974; Janatova, 1974; Rosenoer et al., 1977; Peters and Reed, 1978).

Some of the physico-chemical properties of Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) are listed in Table 3.1. The properties are very similar for both species, as are the amino acid compositions (Brown, 1977), and it may not be unreasonable to assume therefore that the surface activity of the two proteins will also be very similar.

The (monomeric) molecule exists as a globular, tightly coiled single polypeptide chain. The precise structure and size of the molecule is still in dispute (e.g. Champagne 1957 (b) and (c), Luzzati et al., 1961; Slayter, 1965; Riddiford and Jennings, 1966; Squire et al., 1968). Many of the observed physico-chemical properties of the native molecule are satisfactorily accounted for by assuming that the molecule is a prolate ellipsoid of revolution, with major and minor axes of 14 nm and 4 nm respectively (Squire, et al. 1968). There is no conclusive evidence to suggest that polymeric fractions co-exist with the monomer in an equilibrium process.

Polymeric fractions do occur however, and they can be easily separated by electrophoretic and gel filtration chromatography techniques (e.g. Pedersen, 1962; Saifer and Paolo, 1969; Fuller Noel and Hunter, 1972). The polymers appear to form as a result of isolation,

TABLE 3.1 PHYSICO-CHEMICAL PROPERTIES OF SERUM ALBUMINS (a)

PROPERTY	BOVINE	VALUE	HUMAN
Amino Acid Residues	581		584
Relative Molecular Mass	66 210		66 240
Hydrophobicity/kJ residue ⁻¹ ($H\phi_{ave}$)	4.7(b)		
Isoionic Point (pH)	5.3		5.2
Isoelectric Point (pH, I 0.15 mole l ⁻¹)	4.7		4.7
Charge at pH 7	-18		-18
Dimensions/nm (Major axis x minor axis)	14 x 14		15 x 4
Hydration/g g ⁻¹	0.2-0.5(c)		
Diffusion Coefficient/m ² s ⁻¹ ($D_{20,w} \times 10^{11}$)	5.9		6.1
Sedimentation Coefficient/S ($S_{20,w} \times 10^{13}$)	Monomer 4.5 Dimer 6.7		4.6 6.5
Frictional Ratio (f/f_0)	1.30		1.28
Intrinsic Viscosity/m ³ kg ⁻¹ ($\eta \times 10^3$)	4.13	4.2	
Partial Specific Volume/m ³ kg ⁻¹ ($V_{20} \times 10^3$)	0.733	0.733	

a Data from Peters (1975)

b Bigelow (1967)

c Oakes (1976)

fractionation, purification and lyophilisation steps (e.g. Andersson, 1966; Janatova, 1974) and as a result of heating above 40°C (Neet and Putnam, 1965; Wallevik, 1973; Khalimov et al., 1976). Some of the dimeric material (and presumably also some of the higher polymeric material) can be depolymerised using reducing agents (see, for example, Janatova (1974) and Foster (1977), and refs. therein). Few studies have reported on the structure of the higher polymers. It has been suggested that the dimer exists as a side-by-side association of monomers (Bloomfield, 1966; Squire et al., 1968).

The structure of the Serum Albumin molecule is dependent on solution conditions. At the IEP, the molecule is in its most compact and globular form. As the pH is shifted from this point, a continuous expansion occurs until, at pH's greater than 10 and less than 4, electrostatic forces cause an unfolding of the molecule and it no longer exists in a compact and globular form. The expansion above the IEP is not as well characterised as that below this pH, but the process and resultant forms appear to be similar. The effect of pH is reversible, and results in a longer and more asymmetric molecule (Yang and Foster, 1954; Harrington et al., 1956; Champagne, 1957(c); Luzzati et al., 1961). The structure of the expanded molecule is in dispute. Models retaining a compact configuration (Luzzati et al., 1961) or expanded into two or more subunits (Harrington et al., 1956; Foster, 1960; Weber and Young, 1964; Bloomfield, 1966) have been proposed. Increasing ionic strength confers an increased stability on the molecule to pH induced changes (Tanford et al., 1955; Tanford and Buzzell, 1956; Foster, 1960; Peters, 1975).

Serum Albumin is not a homogeneous protein. The non-homogeneity is observed in various chromatographic and electrophoretic experiments and heat denaturation studies, and occurs through differences in sulphhydryl reactivity. Albumin molecules may exist with a free sulphhydryl group in

an exposed position on the surface of the molecule, or in a state in which the sulphydryl group has been blocked by cysteine or glutathione. The two types are distinguished as 'mercaptalbumin' and 'non-mercaptalbumin' respectively. The situation is further complicated however, by a multiplicity of non-mercaptalbumin forms and a more subtle heterogeneity that is evident in some experiments. It has been suggested that this 'micro-heterogeneity' probably arises through differences in amino acid side chains and disulphide isomerisation (Foster, 1977). Critical discussions of the evidence for and source of the non-homogeneity of Serum Albumin can be found in the reviews of Janatova (1974) and Foster (1977).

The Serum Albumin molecule contains 17 disulphide bonds. Their accessibility to reduction or oxidation is dependent on pH. Between pH 5 and 7 no disulphide bonds react, but as the pH is decreased or increased, these bonds gradually become more exposed and reactive.

There are more than 200 positive and negative charges distributed over the molecule (Peters, 1975) and hence the protein is very hydrophilic. The amino acid distribution is rather uniform over the polypeptide chain (Braam, 1972) and this suggests a similarly uniform charge distribution over the molecule. A diagrammatic representation of the distribution of charged groups at pH 7 is shown in Figure 3.3 and shows that at this pH, there are charged groups spread over the whole molecule. The C-terminal region is less highly charged however.

The binding of ions may affect the properties of the molecule by altering the net charge and/or by altering the charge distribution and, consequently, affecting the conformation of the molecule. At neutral pH's, there is no significant binding of sodium or potassium ions (Carr, 1956). Anion and divalent cation binding is much more pronounced and, as expected, an interaction with some buffers and electrolytes has been

observed (Cann, 1966; Wallevik, 1973; McMenamy, 1977). The binding of calcium ions is ionic in nature, relatively weak ($K_a \approx 100 \text{ l mole}^{-1}$), appears to occur at carboxyl groups (Carr, 1953; Pederson, 1971; Fogh-Anderson, 1977) and decreases with increasing ionic strength (Pederson, 1972). Binding increases with pH at pH's higher than the IEP until, at pH 8.6, about 40 ions are bound. At pH 6.8 and an ionic strength of about 0.15 mole l^{-1} , 17 ions are bound.

The interaction of Serum Albumins with the divalent ions of copper and mercury is unusual in that it causes a polymerisation of the protein molecules (Everett, 1963; Hughes and Dintzis, 1964; Sogami et al., 1972). It is thought that polymerisation occurs by cross linking of the sulphydryl groups to produce dimers, although copper is also known to bind to an amino group in a complex site (Shearer et al., 1967) and there are also about 5 other mercury binding sites (Foster, 1960). The binding is much stronger than that with calcium, and association constants of $\sim 10^7 \text{ l mole}^{-1}$ for copper (Peters, 1975) and for mercury binding at the sulphydryl group (Saroff and Mark, 1953) have been calculated. The association constants for mercury at the other binding sites are of the order of $\sim 10^4 \text{ l mole}^{-1}$.



Figure 3.3: Diagrammatic Representation of the Distribution of Charged Groups Over BSA at pH7. It is assumed no carboxyl or imidazole groups are protonated. The shaded portions represent portions of the molecule bonded by disulphide bonds. (From Peters, 1975.)

3.6.3 β -CASEIN

Caseins comprise approximately 78-85% of the total protein of milk and β -Casein contributes about 25-35% of this total protein. β -Casein is not homogeneous however and seven genetic variants, labelled β -Casein A¹, A², A³, b, B₂, C and D, are known. The A and B variants together comprise 96-100% of the total β -Casein content of milk. Several review articles on milk proteins and Caseins are available (McKenzie, 1971a; Swaisgood, 1973; Schmidt and Payens, 1976).

Some of the physico-chemical properties of β -Casein are listed in Table 3.2. The protein contains 209 amino acid residues but no sulphhydryl groups or disulphide bonds. A significant aspect of the primary structure is the uneven distribution of the amino acids along the polypeptide chain. This uneven distribution leads to separate highly charged polar and apolar (hydrophobic) regions. The molecule has about 50 charged groups. A diagrammatic representation of the distribution of charged and hydrophobic residues is shown in Figure 3.4. The N-terminal

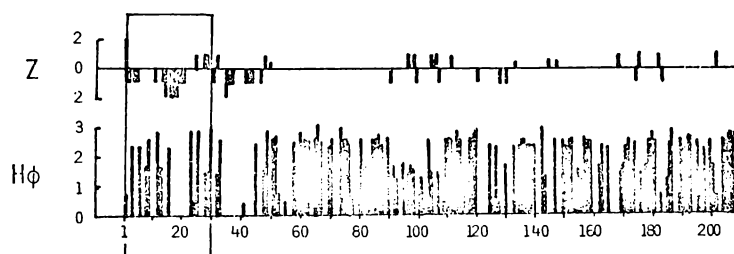


Figure 3.4: Diagrammatic Representation of the Distribution of Charged and Hydrophobic Residues in β -Casein A². The Hydrophobicity of each residue is expressed relative to that of cysteine (4.184 kJ residue⁻¹). The phosphopeptide residue is boxed in. (Illustration taken from Ribadeau-Dumas et al., (1972)).

region of the molecule, comprising about one-tenth of the total amino acids, carries about one-third of the charge. Roughly two-thirds of the C-terminal region is highly hydrophobic and consequently the molecule has a high average hydrophobicity (Table 3.2).

β -Casein is unusual in that the protein displays a temperature dependent self-association such that at temperatures above 4°C, the protein exists as a micellar species. The structure of the monomer is still in dispute. Recent studies suggest that the molecule is expanded with many of its residues in contact with water but more compact than a completely random coil (Andrews et al., 1979; Arima et al., 1979; Creamer et al., 1981). Andrews et al. have suggested that about one-third of the molecule, i.e. about one-half of the apolar section, is ordered. Waugh et al. (1970) have suggested the structure is that of a compact body, stabilised by hydrophobic interactions, with an exterior and flexible acidic peptide which is solvent accessible.

As the temperature of β -Casein solution is raised above 4°C, the monomeric units associate and two well separated peaks can be observed in the sedimentation (e.g. Schmidt and Payens, 1976) and gel filtration (Niki et al., 1977; Andrews et al., 1979) patterns. An unusual feature of the association is the narrow size distribution of the micelles formed. It appears that only the monomer and highest order polymer coexist (Payens and van Marwijk, 1963; Schmidt and Buchheim, 1975; Buchheim and Schmidt, 1979). The formation of the micelles is a reversible process that is dependent on solution conditions. Although ionic strength only affects the position of the equilibrium (Takase et al., 1980), temperature and concentration also affect the size of the polymeric species and the degree of polymerisation increases with increases in these variables (Payens and van Marwijk, 1963; Schmidt and

TABLE 3.2: PHYSICO-CHEMICAL PROPERTIES OF β -CASEIN^(a)

PROPERTY	VALUE
Amino Acid Residues	209
Relative Molecular Mass	24 000
% Phosphorous	0.52 - 0.65
Hydrophobicity/kJ residue ⁻¹ ($H\phi_{ave}$)	5.6 ^(b)
Isoionic Point (pH)	5.20 - 5.85
Monomer Net Charge at pH 6.6	-8 - -13
Diffusion Coefficient/m ² s ⁻¹ ($D_{20,W} \times 10^{11}$)	6.05 ^(c)
Sedimentation Coefficient (at 4°C)/S ($S_{20,W} \times 10^{13}$)	1.5
Frictional Ratio (f/f_0)	1.83 ^(c)
Intrinsic Viscosity (at 4°C)/m ³ kg ⁻¹ ($\eta \times 10^3$)	23
Partial Specific Volume/m ³ kg ⁻¹ ($V_{20} \times 10^3$)	0.749

^a Data from Swaisgood (1973), taken over all variants

^b Calculated according to the method of Bigelow (1967)

^c Sullivan et al. (1955)

Payens, 1972; Niki et al., 1977; Arima et al., 1979; Evans et al., 1979). The concentration dependence is unusual in that often a critical micelle concentration (CMC) can be observed (Payens and van Marwijk, 1963; Niki et al., 1977; Evans et al., 1979). There is a lack of consistency among experimental reports as to the size of the micelles formed or the value of the CMC. Similarly, the rate of association has been reported by Payens and van Marwijk to be slow but Evans et al. observed that equilibrium was rapidly established. The most likely explanation of the differences in behaviour is that the properties of a particular β -Casein sample are not only dependent on the exact solution conditions, but also on the amino acid composition and method of preparation of the sample. Several methods are available for the isolation of β -Casein and these have previously been observed to affect the degree of association (Payens and Heremans, 1969).

The shape of the micelles has been suggested to be rod-like or as interlinked flexible coils (Sullivan et al., 1955; Payens and van Marwijk, 1963; Niki et al., 1977). More recent evidence suggests that the rod-like configuration is not correct (Schmidt and Payens, 1976) and that the micelle is spherical (Waugh et al., 1970; Andrews et al., 1979; Buchheim and Schmidt, 1979).

The association of the β -Casein monomers occurs through hydrophobic interactions and is an entropy driven process (Woodhead et al., 1977; Evans et al., 1979; Takase et al., 1980). The entropy change is in the range of $200\text{--}300 \text{ J K}^{-1} \text{ mole}^{-1}$ at 20°C . Protein modification studies (McKenzie and Wake, 1959; Thompson et al., 1967; Hoagland, 1968; Berry and Creamer, 1975) and experiments involving changes in solution conditions e.g. temperature, ionic strength and solvent (Evans et al., 1979), all support this conclusion. The results are consistent with a micellisation involving a delicate balance of hydrophobic forces

favouring association and electrostatic forces opposing it. This further suggests that the ionic strength dependence of association will also be affected by the nature of the buffer materials and salts used to adjust the ionic strength.

β -Casein binds calcium ions. The binding is reversible, increases with increasing pH and decreases with increasing ionic strength (Dickson and Perkins, 1971; Parker and Dalgleish, 1981). Parker and Dalgleish showed that the average binding constant at 20°C, pH 7.0 and an ionic strength of 0.05 mole⁻¹ was 886 l mole⁻¹. Binding was dependent on the number of calcium ions already bound however, and a value of the binding constant of 2100 l mole⁻¹ was obtained for the first ion bound. Binding has been suggested to result in an unfolding of the peptide chain (Imade et al., 1977). The solubility of the calcium-casein complex was highly temperature dependent (Parker and Dalgleish, 1981). At 4°C, the complex was completely soluble, but as the temperature was increased, precipitation was induced. A critical calcium concentration could be defined for precipitation at a given temperature. It appeared that hydrophobic interactions were more important than charge characteristics in determining precipitability.

3.6.4 SALIVA

The composition and properties of saliva have been the subject of much study. The pre-1961 literature has been compiled and reviewed by Afonsky (1961). Some more recent reviews are those of McCann (1968), Klinkhamer (1968), Mukherjee (1968b), Mason and Chisholm (1975), Beeley (1977) and Arglebe (1981).

The organic components of saliva are mainly of proteinic nature, extremely diverse and generally poorly characterised (Section 2.3). Many of the salivary proteins are known to bind to calcium phosphate salts such as hydroxyapatite or tooth enamel (Section 3.5) and to inhibit or modify calcium phosphate salt precipitation and/or crystallisation (Section 3.7.4). The mean total protein content of unstimulated and stimulated saliva is 1.55 mg ml^{-1} and 1.34 mg ml^{-1} respectively (Arglebe, 1981). The submandibular secretion contributes 50-70% of the total salivary protein. When combined with the parotid secretion, this fluid provides 90% of the total protein. The glycoproteins form the major part of this material. Saliva contains at least 40 glycoprotein components (Ellison, 1979). The detailed structure of very few of these is known however. The glycoproteins are highly surface active and form the 'glue' on cell surface membranes, act as lubricants and are responsible for the viscosity of saliva.

The glycoproteins are made up essentially of a long protein chain to which are attached the much shorter chains of carbohydrates. Carbohydrate contents of 10 - 25% are typical, but this can extend to 40% or more. The isoelectric points vary from as low as pH 2 to as high as pH 11 (e.g. Beeley, 1977), and hence the glycoproteins are either cationic, neutral or anionic in the saliva secretion. The submandibular secretion has a (relatively) high concentration of anionic glycoproteins

but low concentration of cationic glycoproteins, and also includes most of the parotid proteins. The parotid secretion has a relatively high concentration of cationic glycoproteins but a low concentration of anionic glycoproteins (Mandel, 1974). Relative molecular masses vary from about 1×10^4 to as high as 2×10^6 .

The amino acid composition of the protein chain is very similar in all the glycoproteins. It is characterised by a large proportion of the simpler aliphatic amino acids and a relative deficiency of aromatic and sulphur-containing amino acids (Arglebe, 1981). Hydrophobic amino acids comprise more than 30% of the total amino acid residues (Leach et al., 1967; Leach, 1979). The properties of the glycoproteins appear to depend mainly on the composition and relative arrangement of the carbohydrate varieties. These are strongly hydrophilic, and make the protein molecule take up a threadlike configuration in solution but with a degree of flexibility that can vary from that of a rigid rod to a contorted threadlike molecule which assumes a roughly spherical configuration (Gibbons, 1966). The carbohydrates help maintain solubility and precipitation occurs if the majority of these are lost. The release of the carbohydrates by extracellular enzymes, of bacterial origin, occurs quite rapidly and the half life of the sugars in whole saliva is of the order of 1 hour.

A more comprehensive discussion of salivary glycoproteins can be found in the reviews by Mandel (1977) and Arglebe (1981).

Saliva contains many inorganic species (Section 2.3). Calcium and phosphorous are particularly of interest because of their relevance to enamel demineralisation/remineralisation phenomena and calculus formation. Some indication of the mean calcium and phosphorous concentrations and their physical state in glandular and whole saliva is presented in Table 3.3 and Table 3.4 respectively. Although a great deal

of data has been collected, much of this has been obtained using few subjects and usually under non-defined and non-standardised conditions. Definition is particularly important for filtration, ultrafiltration and dialysis techniques, commonly used in studies of saliva, where one researcher's filter or dialysis membrane may be another's sieve. The loss of organic material in dialysis and ultrafiltration is well known (e.g. Lamberts and Mayer, 1979).

TABLE 3.3: MEAN CONCENTRATIONS OF SOME ELECTROLYTES IN SALIVA⁽¹⁾

SALIVA		CALCIUM	PHOSPHATE		pH
			Inorganic	Total	
Whole	Unstimulated	1.56±0.06	6.14±0.61	6.2	
	Stimulated	1.48±0.04			7.08±0.31
Parotid	Unstimulated	0.75±0.25	3.4±1.4		5.92±0.51
	Stimulated	0.82±0.55	3.3±2.6	5.6±2.6	7.67±0.18
Submandibular	Unstimulated	1.56±0.45	3.6±0.7	7.9±6.5	6.73±0.45
	Stimulated	1.57±0.32			
Sublingual	Unstimulated	2.29±0.47	0.62±0.23		
	Stimulated	2.03±0.38	0.45±0.11		

(1) Compiled and adapted from Mason and Chisholm (1975). Concentrations reported as millimoles per litre. Errors reported for whole saliva as the standard error of the mean, and for glandular secretions as the standard deviation.

TABLE 3.4 THE STATE OF CALCIUM AND PHOSPHATE IN STIMULATED WHOLE
SALIVA⁽¹⁾

STATE	METHOD	%CALCIUM	%PHOSPHATE
Protein bound	Dialysis	15-30	15(est.)
Ultrafilterable	Ultrafiltration	65-70	67-94
Ionised	Specific Ion Electrode	50	
	Murexide Dye	50	
	Dialysis, Ultrafiltration		67-94
Ion pairs		13	2

(1) Compiled from Grøn (1973b) and Mandel (1974)

The mechanisms by which calcium and phosphorous are rendered non-dialysable or non-ultrafilterable are not known in detail. Saliva is known to contain metallo-proteins e.g. the calcium-metallo-enzyme α -Amylase (e.g. Hsiu et al., 1964) and various phospho-proteins (e.g. Bennick, 1977b). Calcium binding by salivary proteins is also well known (e.g. Bennick, 1977b). The observed lack of agreement between inorganic and non-organic calcium and phosphorous as determined by various techniques (Table 3.4) is not surprising however: calcium-binding by salivary proteins has been observed to be highly pH dependent (e.g. Rølla and Jonsen, 1967; Bennick, 1977b); and, saliva contains many proteins and peptides, known to bind calcium or contain phosphorous, and to be of low relative molecular mass (e.g. Bettelheim, 1971; Oppenheim et al., 1971; Hay, 1973b; Arneberg, 1974; Hay and Oppenheim, 1974; Bennick 1975, 1977b; Hay, 1975; Baum et al., 1976; Hay and Grøn, 1976; Schlesinger and Hay, 1977; Kanai, 1980; Li and Levine, 1980) which might easily pass through

some commonly used dialysis tubing and/or filtration membranes. Some workers have suggested that the formation of calcium phosphate precipitates results in non-ultrafiltrability (Dreisbach, 1960; Sullivan and McCoombe, 1960; Gow, 1965; Mukherjee, 1968b).

Ionic calcium and phosphate activity products have been calculated in attempts to determine the degree of saturation of saliva with respect to various calcium phosphate salts (e.g. Vogel et al., 1965; McCann, 1968 and refs. therein; Grøn, 1973c) and the relationship to salivary calculi and calculus formation. The results of such calculations show that stimulated saliva is generally more saturated than resting saliva. The stimulated secretions are saturated or supersaturated with respect to tricalcium phosphate (TCP), octacalcium phosphate (OCP), hydroxyapatite (OHAP) and in some cases also with respect to dicalcium phosphate dihydrate (DCPD). Submandibular saliva is more saturated than parotid saliva and closely resembles the state of saturation of whole saliva. The spontaneous precipitation of whole saliva or the individual secretions is not usually observed however. Precipitation can be seen if whole saliva or the submandibular secretion is shaken, left to stand, or dialysed to lower ionic strength and/or pH (Edgar and Jenkins, 1971; Jenkins, 1971; Leach, 1980; Edgar et al., 1980). Leach (1980) observed that the precipitates contain very small, poorly defined crystals that have salivary components attached.

Precipitation in saliva can also be induced by pH titration (Chatterjee and Kleinberg, 1979), the addition of calcium (e.g. Mandel, 1974 and 1977, and references therein; Edgar and Jenkins, 1971; Hay, 1973b; Belcourt, 1975; Bennick, 1976 & 1977a) and other polyvalent metal ions (Voegel and Belcourt, 1980). Several authors have shown that the inorganic component present in these precipitates is an hydroxyapatite-like material (Warton and Blomfield, 1971; Mandel, 1974; Chatterjee and

Kleinberg, 1979; Edgar et al., 1980). Chatterjee and Kleinberg (1979) showed that the precipitates formed at pH's higher than neutrality tend to have higher amounts of calcium and phosphorous than those formed at lower pH's. Voegel and Belcourt (1980) described the precipitates obtained with the polyvalent metal ions studied (Ca^{2+} , Cu^{2+} , Fe^{3+} , Zn^{2+} , Ba^{2+} , Sr^{2+} , Mg^{2+} , Al^{3+}) as X-ray amorphous, but noted that the calcium and strontium precipitates also contained some crystalline material. X-ray diffraction showed the crystalline materials corresponded to poorly crystalline calcium and strontium phosphates of the apatite type. The calcium : phosphate ratio of the calcium precipitate was determined to be 1.39 (after standing overnight at 4°C). Similar calcium: phosphate ratios are commonly observed for amorphous calcium phosphates precipitated in the presence of organic material (Section 3.7.4). X-ray fluorescence studies of precipitates formed by Sn^{2+} , Fe^{3+} and Zn^{2+} show that these contain the relevant cation together with calcium and phosphate (Leach and Appleton, 1981).

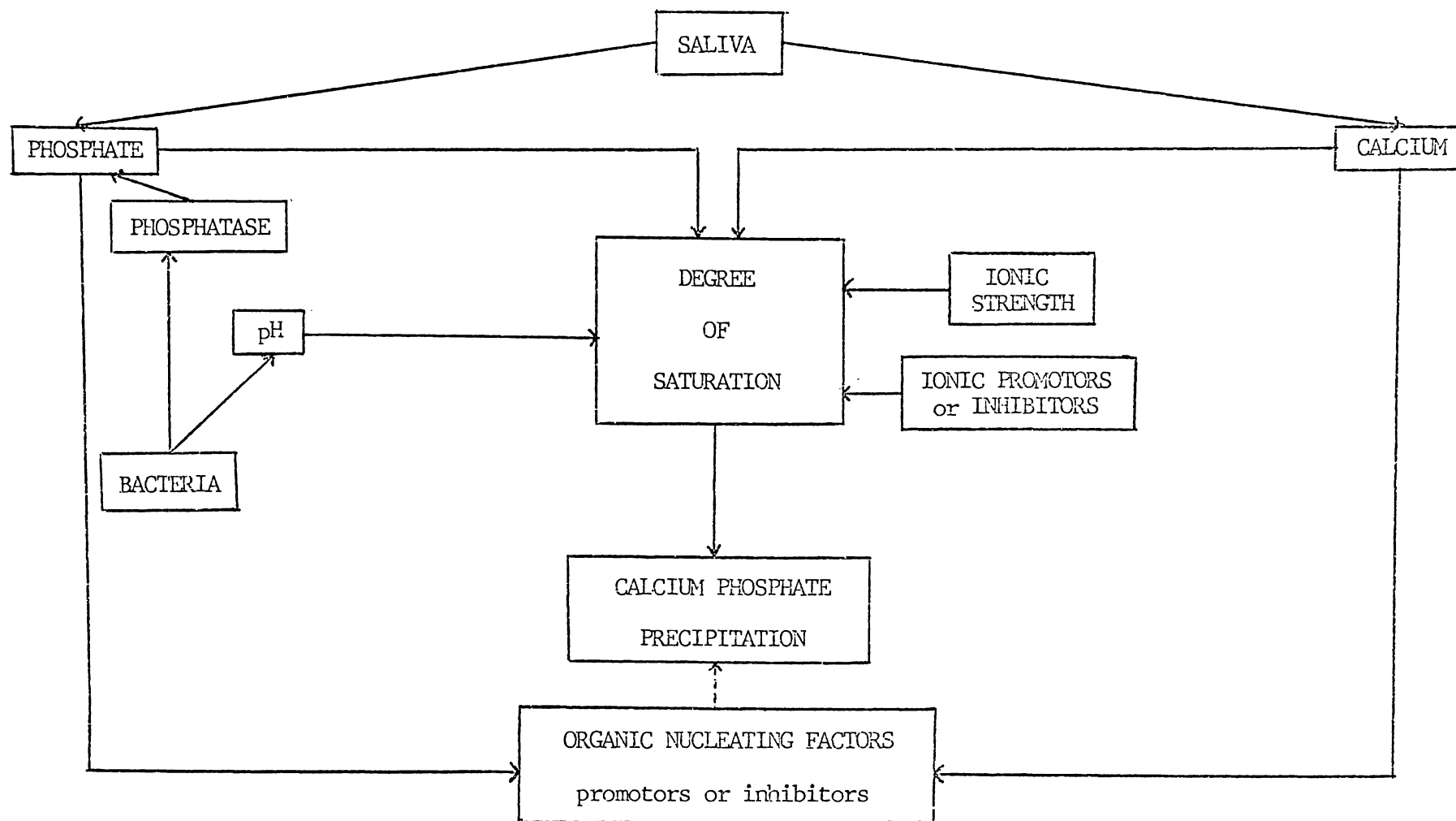
The role of phosphate in the precipitation by calcium is controversial however. Although several authors have noted that while saliva did not precipitate upon the addition of small amounts of calcium unless phosphorous was also present (Embery, 1971; Leach, 1980), Mandel (1974) observed that dialysis of parotid saliva, "to remove inorganic phosphate", did not prevent precipitation upon the addition of calcium. However Edgar et al. (1980) observed that precipitates formed upon the addition of calcium to parotid saliva after dialysis for 4 hours, but not after dialysis for 24 hours. It was shown further that if the pre-dialysis calcium and phosphorous levels were restored, precipitation occurred upon further addition of calcium. Although dialysis of submandibular saliva for 24 hours did not prevent precipitation by added calcium, the residual inorganic phosphate in this case was almost 3 x

higher than that of the similarly dialysed parotid secretion. These authors suggested that precipitation by calcium might be due to the formation of insoluble calcium phosphates to which protein is adsorbed, or of an insoluble calcium-protein complex together with co-precipitated calcium phosphate material and favoured the latter interpretation. However, studies of amorphous calcium phosphate precipitation and conversion to more crystalline forms in the presence of synthetic or biological polymer material suggest the former interpretation is correct.

A summary of some of the factors which affect the calcium and phosphate concentrations in saliva is given in Figure 3.5.

In conclusion, saliva is seen to be a complex fluid containing many protein components. The physico-chemical properties of these components are generally not well characterised and can be modified by external factors such as the oral bacteria. Thus the glycoproteins, although initially hydrophilic immediately upon secretion into the oral cavity, become more hydrophobic with increasing residence time and show consequent conformation changes. Although calcium and phosphorous play an important role in dental phenomena, the role of these components in saliva is generally poorly characterised and confused. A complex interaction between the protein components, other inorganic components and external factors is likely. This further supports the use of the well characterised proteins BSA and β -Casein. Further, these two proteins are representatives of, respectively, hydrophilic and hydrophobic proteins and β -Casein is also a phosphoprotein.

Figure 3.5: SOME FACTORS AFFECTING CALCIUM PHOSPHATE PRECIPITATION IN SALIVA



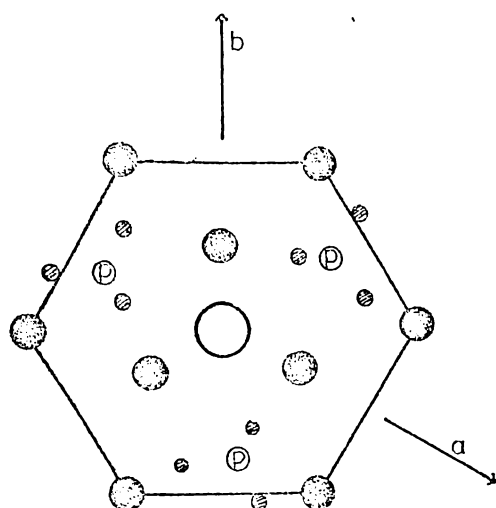
3.6.5 HYDROXYAPATITES

Calcium phosphate minerals form an important group of compounds and those of the apatitic form are of particular interest. The mineral apatites are a major source of fertilizers, catalysts and phosphors. They are also the prototypes of the major inorganic constituent of bones and teeth. Several comprehensive review articles on apatitic calcium phosphate minerals are available (Young and Elliott, 1966; Elliott, 1969; Posner, 1969; Eanes and Posner, 1970; Kibby and Hall, 1972; Simpson, 1972; Elliott, 1973; Young 1975; Le Geros, 1981). Calcium phosphates of the hydroxyapatite type, in particular stoichiometric hydroxyapatite, and fluoride and carbonate substituted hydroxyapatites are discussed in this Section.

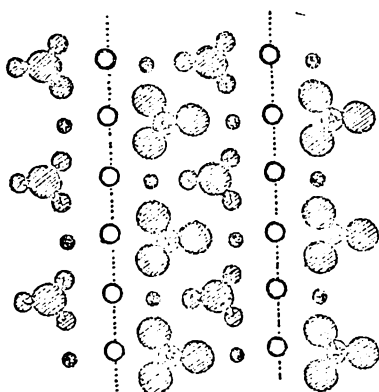
(A) Stoichiometric Hydroxyapatite

Stoichiometric hydroxyapatite is a crystalline calcium phosphate mineral of general formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. It is found in both an hexagonal and a monoclinic form. These differ only in the orientation of the hydroxyl group within the crystal. The monoclinic form is produced in syntheses involving the use of high temperature methods (e.g. Mackie et al., 1973), whereas synthesis involving aqueous hydrolysis or precipitation methods produces the hexagonal form (Young, 1975). No evidence of the occurrence of the monoclinic form in tooth enamel has yet been found.

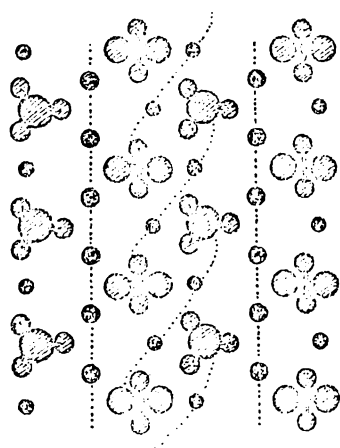
The unit cell of hydroxyapatite is a right rhombic prism of dimensions $a = b = 0.942 \text{ nm}$ and $c = 0.688 \text{ nm}$. These form a simple hexagonal lattice when stacked together (Figure 3.6(a)). The atomic contents of the cell are expressed by the formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ whereas the chemical composition can be described by half this number of



3(a)
ab face



3(b)
ac face



3(c)
ac face

Figure 3.6: Exposed Crystal Faces of the Hydroxyapatite Lattice.

Key :

	Ca	Calcium	} of Phosphate radicals
	O	Oxygen	
	P	Phosphorous	
	OH	Hydroxide	

atoms. The calcium : phosphate ratio (Ca/P) is 1.667. The long axis of the crystal is parallel to the c-axis and hence most of the crystal surface is made up of a-c faces. Either the hydroxyl ions (Figure 3.6(b)) or calcium ions (Figure 3.6(c)) are exposed depending on where the crystal lattice is terminated. In comparison, the diagram of Figure 3.6(a) approximates the surfaces of the ends of the crystals. In all cases, calcium and phosphate ions are always exposed on the crystal surface, whereas the hydroxyl ions are only exposed in the arrangement of Figures 3.6(a) and (b)

The apparent surface area of hydroxyapatite, as measured by nitrogen adsorption, is dependent on the water content of the sample (Dry and Beebe, 1960; Holmes and Beebe, 1971; Rootare and Craig, 1977). Higher outgassing temperatures result in increased specific surface areas. This is thought to be due to the removal of chemisorbed layers of water (Rootare and Craig, 1977), or the removal of water at higher temperatures from micropores and grain boundaries (Füredi-Milhofer et al., 1979). Bett et al. (1967) report that the specific surface areas determined by electron microscopy and nitrogen adsorption are in good agreement. They do not however quote an outgassing temperature. Rootare and Craig suggest that outgassing temperatures of about 300°C cause the surface layer of chemisorbed water to be removed. The surface areas measured by nitrogen adsorption and by water adsorption are then in good agreement.

Studies of the vapour phase adsorption of water by hydroxyapatite (Rootare and Craig, 1977) suggest that the initial adsorption is chemisorptive. Additional water is then taken up until the surface is covered with a bilayer of water molecules after which the liquid-like surface is similar to bulk water. About 8 layers were physisorbed to the surface of apatites with a specific surface area of about $70 \text{ m}^2\text{g}^{-1}$. The studies indicated the presence of hydrophilic and hydrophobic areas on

the surface. Repeated wetting and drying cycles led to a more homogeneous hydrophilic surface. Thermodynamic calculations indicated a large decrease in entropy accompanied adsorption.

Upon suspension in aqueous solutions, the hydroxyapatite crystals take on a solvent layer or hydration shell. Centrifugation data suggests that the volume of the hydration layer is almost twice the volume of the crystal (Neuman and Neuman, 1958). Further, the calcium and phosphate ion concentrations in the hydration layer may be 3 000 x greater than those observed for the bulk solution. The calcium is in higher concentration than phosphate (Table 3.6; see also Pak et al., 1968).

Table 3.5: MOLAR CONCENTRATIONS OF SOME BULK SOLUTION SPECIES IN
EQUILIBRIUM WITH HYDROXYAPATITE

pH	H ⁺	Ca ²⁺	OH ⁻	H ₂ PO ₄ ⁻	HP ₄ ²⁻
4	10 ⁻⁴	1.92x10 ⁻²	very small	1.07x10 ⁻²	1.05x10 ⁻⁵
5	10 ⁻⁵	2.51x10 ⁻³	"	1.47x10 ⁻³	1.47x10 ⁻⁵
6	10 ⁻⁶	3.45x10 ⁻⁴	"	1.86x10 ⁻⁴	1.86x10 ⁻⁵
7	10 ⁻⁷	6.03x10 ⁻⁵	10 ⁻⁷	1.58x10 ⁻⁵	1.58x10 ⁻⁵
8	very small	1.45x10 ⁻⁵	10 ⁻⁶	7.88x10 ⁻⁷	7.88x10 ⁻⁶
9	"	4.43x10 ⁻⁶	10 ⁻⁵	very small	2.64x10 ⁻⁶
10	"	1.41x10 ⁻⁶	10 ⁻⁴	"	8.63x10 ⁻⁷
11	"	4.59x10 ⁻⁷	10 ⁻³	"	6.22x10 ⁻⁸

(from Chander and Fuerstenau, 1979)

In aqueous solutions, hydroxyapatite is slightly soluble and the surface ions are in equilibrium with those in solution. The equilibrium is sensitive to pH, the ionic composition and ionic strength of the solution, the presence of impurity ions in the crystal lattice, and the solid : solution ratio. For dilute aqueous solutions in which a large fraction is dissolved, the solubility product $K_{sp} = 10^{-115}$ at 25°C. The solubility is greater in acid solutions, especially below pH 6 (Chander and Fuerstenau, 1979).

Table 3.6: SOME POSSIBLE DISSOLUTION AND HYDROLYTIC REACTIONS FOR HYDROXYAPATITE

Reaction	Equilibrium Constant
$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 = 10\text{Ca}^{2+} + 6\text{PO}_4^{3-} + 2\text{OH}^-$	10^{-115}
$\text{Ca}^{2+} + \text{OH}^- = \text{CaOH}^+$	25.1
$\text{CaOH}^+ + \text{OH}^- = \text{Ca}(\text{OH})_2(\text{aq})$	23.4
$\text{PO}_4^{3-} + \text{H}^+ = \text{HPO}_4^{2-}$	2.1×10^{12}
$\text{HPO}_4^{2-} + \text{H}^+ = \text{H}_2\text{PO}_4^-$	1.6×10^7
$\text{H}_2\text{PO}_4^- + \text{H}^+ = \text{H}_3\text{PO}_4$	1.4×10^2
$\text{Ca}^{2+} + \text{PO}_4^{3-} = \text{CaPO}_4^-$	2.9×10^6
$\text{Ca}^{2+} + \text{HPO}_4^{2-} = \text{CaHPO}_4$	2.4×10^2
$\text{Ca}^{2+} + \text{H}_2\text{PO}_4^- = \text{CaH}_2\text{PO}_4^+$	8.2

(from Bell et al., 1978)

The hydroxyapatite surface is highly charged due to the exposed calcium, phosphate and hydroxyl ions (Figure 3.7) and can interact

strongly with ions and polar species. Both acid and base functions are present and the surface can function as an electron-pair donor or proton-acceptor. In aqueous solutions, the surface charge will be a function of any dissolution and hydrolytic reactions that occur upon suspension. Some of the various reactions possible are shown in Table 3.6 and, as expected, the surface charge is highly pH dependent (Figure 3.7). In the wet state, the surface phosphate ions will exist mostly as HPO_4^{2-} and/or H_2PO_4^- ions at pH's near neutrality. The point of zero charge (pzc) of hydroxyapatite, calculated from solubility data, is suggested to be at pH 8.7 (Figure 3.7). Titrimetric determinations, using KCl as an electrolyte, suggest the pzc lies in the range pH 8.3 - pH 8.6 (Bell et al., 1973).

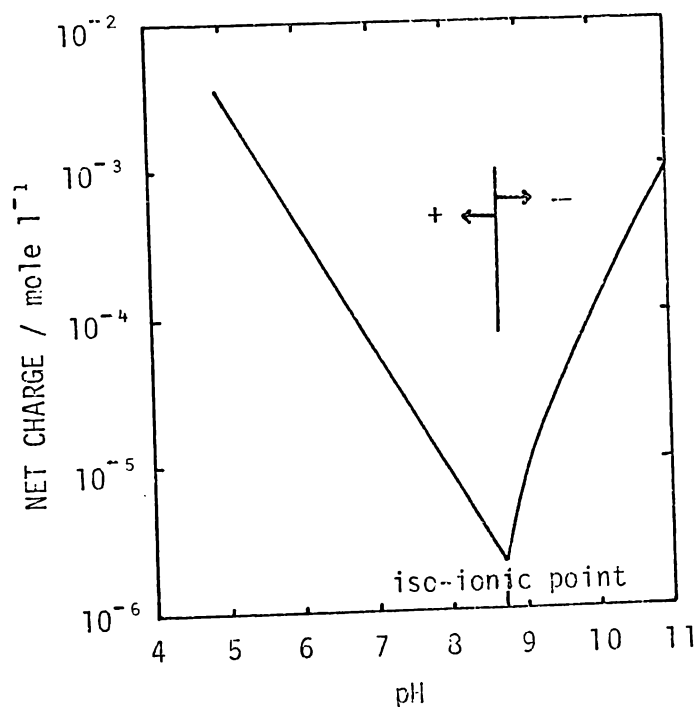


Figure 3.7: Net Lattice Ion Contribution to Charge in Solution in Equilibrium with Hydroxyapatite as a Function of Solution pH.
(from Chander and Fuerstenau, 1979)

Many electrolyte species other than hydronium or hydroxyl ions interact with hydroxyapatite and are potential determining e.g. calcium, phosphates, sodium, potassium, chloride, fluoride and carbonates. The mechanism of interaction involves one or more of: concentration in the hydration layer, surface adsorption, reaction, and exchange. These may be isoionic or heteroionic effects and are often pH and ionic strength dependent. (The reader is referred to the reviews by Neuman and Neuman (1958) and Kibby and Hall (1972) for further discussion.) The nature of the hydroxyapatite surface may therefore change with changes in solution composition and these will be reflected in changes in the electrophoretic behaviour e.g. Table 3.10 (Section 3.6.6) see also Doss, 1976).

(B) Fluoride and Carbonate Substituted Hydroxyapatites

The hydroxyapatite lattice is very accommodating toward isomorphic substitution. About one-third of all the ions in the periodic system can be incorporated into the crystal lattice as substitutes at appreciable concentrations. Some of these substitutions produce only small changes in the morphology and properties of the material whereas other substitutions produce more dramatic effects. Often, little or no detailed information about the location or role of the substitution has been developed and the manner of charge compensation, if required, is often obscure. The incorporation of fluoride and carbonate into hydroxyapatite is very important from a biological point of view, particularly as regards the caries process and dental health.

The hydroxide in hydroxyapatite can be replaced completely by fluoride to form the mineral fluorapatite $\text{Ca}_{10}(\text{PO}_4)_6 \text{F}_2$. Hydroxyapatites of varying degrees of fluoride substitution have been prepared by aqueous precipitation methods (Moreno et al., 1977). These can be represented by

the chemical formula $\text{Ca}_5(\text{PO}_4)_3 \text{OH}_{1-x} \text{F}_x$ where the degree of fluoridation, x , varies from 0 (hydroxyapatite) to 1 (fluorapatite). The calcium:phosphate ratio remains unchanged. The effect of increasing substitution is to progressively decrease the length of the a -axis to 0.936 nm, while the c -axis remains relatively fixed (e.g. Moreno et al., 1977). It has been suggested that this may account for the greater chemical stability of fluoride substituted hydroxyapatite when compared with pure hydroxyapatite (Young and Elliott, 1966). Surface hydroxyl groups of hydroxyapatite can be substituted for fluoride by surface exchange reactions in fluoride containing solutions. The exchange proceeds readily, even at low temperatures, but is not readily reversed (Kibby and Hall, 1972).

The enhanced chemical stability of the fluoride substituted material is particularly noted in the increased resistance to acid dissolution and dental caries formation. The effect is dependent on the degree of substitution however: the resistance to acid dissolution increases with increasing fluoride substitution until at about a level of 50% substitution, maximum stability is achieved. Higher degrees of substitution then result in a gradually decreasing stability to acid dissolution (Moreno et al., 1977). The incorporation of fluoride also affects the surface charge characteristics. Fluorapatite has a pzc, measured titrimetrically, in KCl electrolyte, at about pH 6.8 (Bell et al., 1973). Increasing fluoride substitution would be expected to gradually lower the pzc of hydroxyapatite ($\approx$ pH 8.5) to the value for fluorapatite.

Hydroxyapatites have been synthesised with as much as a 22% (w/w) incorporation of carbonate (Le Geros et al., 1968). There is now general agreement that the carbonate is present as CO_3^{2-} ions and substitutes in some manner for hydroxyl and/or phosphate ions. These are termed the A site and B site respectively (Montel, 1968) and the chemical formulae may

be written as $\text{Ca}_{10}(\text{PO}_4)_6(\text{CO}_3)_y(\text{OH})_{2-2y}$ for type A, and as $\text{Ca}_{10-y}(\text{PO}_4)_{6-y}(\text{CO}_3)_y(\text{OH})_{2-y}$ for type B (Arends and Jongebloed, 1981). Type A is formed by solid state reaction at high temperature whereas precipitation from aqueous solution produces Type B (Montel, 1968). The type B is hexagonal (Montel, 1977), and shows an a-axis decrease and c-axis increase in length with increasing carbonate content (Le Geros et al., 1968). The carbonate at the B site is always exposed on the crystal surface. The presence of carbonate decreases the stability of the crystal lattice (Le Geros et al., 1968) and increases the solubility of the hydroxyapatite (Grøn et al., 1963). Consideration of the corresponding 2nd acid dissociation constants of o-phosphoric and 'carbonic' acids (6.23×10^{-8} and 5.61×10^{-11} respectively) suggests that a type B substitution would also reduce the pzc of hydroxyapatite.

3.6.6 TOOTH ENAMEL VS HYDROXYAPATITE

A great deal of literature is now available on the structure and composition of tooth enamel. Table 3.7 shows the average enamel composition. The inorganic component is present in the form of long needle-like and roughly hexagonal crystals, 20 - 60 nm in diameter, that run from the outer tooth surface to the dentine and are nearly perpendicular to the surface (Orams et al., 1976). All teeth contain two distinct types of enamel which differ in the arrangement of the enamel crystallites (Ripa et al., 1966). In prismatic enamel, the crystals are grouped together to form key-hole shaped enamel prisms about 4 nm in diameter (See, for example, Arends and Jongebloed, 1977). The crystals within the prisms are tightly packed. Adjoining prisms and crystals are bonded together by a matrix comprising water and approximately equal amounts of lipid and protein (Odutuga and Prout, 1974). A schematic representation of the dental enamel and crystallite arrangement in such areas is shown in Figure 3.8. Surface area calculations based on this diagram (Appendix I) suggest that the organic matrix comprises about 2% of the total surface area of these areas of teeth. In aprismatic enamel the crystals are placed closely side by side without any evidence of an organic matrix sheath. The aprismatic layer is most predominant on deciduous teeth but is also found on 70% of all permanent teeth (Ripa et al., 1966).

Dental enamel is microporous. The micropores appear to be mainly associated with the organic interprismatic matrix however (Zahradnik and Moreno, 1975), and range from 1 - 10 nm in diameter (see also Orams et al., 1976).

Much of what is known about the inorganic crystallites of tooth enamel has been inferred by comparison with synthetic standards. Some

excellent reviews are available e.g. Posner (1969); Elliott (1969 and 1973); Simpson (1972); Kibby and Hall (1972); Young (1974 and 1975); Eanes (1979). The crystallites are well represented by materials with the crystalline apatite mineral structure. Mineral phases other than apatites are present only to a very small extent, if at all (Eanes, 1979). Hydroxyapatite, of the hexagonal form, is regarded as the best synthetic analogue of inorganic enamel.

Table 3.7: AVERAGE ENAMEL COMPOSITION

MATERIAL	DENSITY/ g cm^{-3}	WEIGHT/%	VOLUME/%
Inorganic	23.15	96 - 97	≈ 86
Water	1	≈ 1	≈ 12
Organic	≈ 1.1	≈ 2	≈ 2

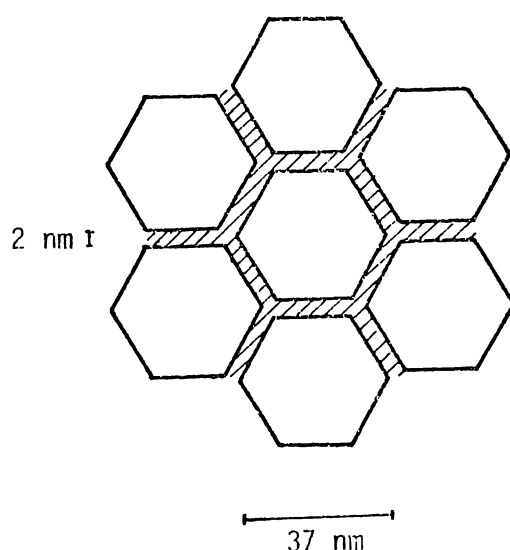


Figure 3.8 : Schematic Diagram of Enamel with (hexagonal) Hydroxyapatite Crystallites Embedded in the Organic Matrix (hatched).
(from Arends and Jongebloed, 1977)

The ultrastructural arrangement of the crystallites in tooth enamel suggests that the enamel surface is composed mostly of ab crystal faces, however the ac crystal faces do contribute significantly to the total surface area (Plate 3.1 (Section 3.7.2)). Although the ac crystal face contributes a greater proportion of the total surface area in synthetic hydroxyapatite, the relative proportions of the two areas will not be too different from that of tooth enamel.

Tooth enamel contains significant amounts of species not present in stoichiometric hydroxyapatite and differing relative amounts of those species that are in common (Table 3.8). There is also an enormous variation in the number, concentration and distribution of these species within the enamel (Section 2.2).

Obviously, stoichiometric hydroxyapatite is not a good model for tooth enamel in terms of the chemical composition. Many of the observed differences can be accounted for in terms of crystalline apatite models. Nevertheless, some of the differences reported may be due to species that are surface adsorbed, trapped in lattice defects or concentrated in the organic matrix. A summary of some of the physical and chemical characteristics of tooth enamel and synthetic hydroxyapatites is shown in Table 3.9. Surface enamel, with higher calcium and phosphorous content, would seem to be more similar to hydroxyapatite than the subsurface enamel. It is reasonable therefore to use a synthetic hydroxyapatite as a model for tooth enamel in the studies envisaged. The relatively high concentrations of fluoride and carbonate in tooth enamel, coupled with their particular relevance to dental health, mean that the incorporation of these species into a synthetic hydroxyapatite will provide other useful model systems for the study of protein adsorption. Further, this may help to resolve the question of whether the incorporation of such species, particularly fluoride, has any effect on protein adsorption.

Table 3.8: COMPARISON OF THE COMPOSITION OF HUMAN TOOTH ENAMEL^(1,2)
AND STOICHIOMETRIC HYDROXYAPATITE

SPECIES ⁽²⁾	HYDROXYAPATITE	WHOLE ENAMEL	REGION ⁽³⁾ OF HIGHEST CONCENTRATION
	<u>(Wt%)</u>	<u>(Wt%)</u>	
Ca	39.90	33.6-39.4	surface
P	18.50	16.1-18.0	surface
OH	3.38		
H ₂ O		0.9-7	subsurface
Organic		0.39-2.0	subsurface
CO ₂		1.95-3.66	EDJ ⁽³⁾
	<u>(molar ratio)</u>	<u>(molar ratio)</u>	
Ca/P		1.4 - 1.69	
	<u>(ppm)</u>	<u>(ppm)</u>	
Na		2500-9000	subsurface
Cl		<1000-6500	surface
Mg		730-5600	EDJ
F		26-3370	surface
K		<10-3000	even
Zn		<180-2100	surface
Si		3-1000	surface
Sr		<25-600	even
Pb		<10-550	surface

¹ Data compiled from Little and Casciani, 1966; Brudevold and Soremark, 1967; Lazzari, 1968; Zipkin, 1970; Weatherell and Robinson, 1973; Losee et al., 1974; Craig and Hercules, 1975; Young, 1975.

² Trace elements reported (<500ppm) include: Ag, Al, As, Au, B, Ba, Bi, Br, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hg, I, In, La, Li, Mn, Mo, Nb, Ni, Pd, Pr, Pt, Rb, Ru, S, Sb, Se, Sn, Ta, Ti, V, W, Y.

³ EDJ signifies enamel-dentine junction. Data from Zipkin, 1970; Weatherell and Robinson, 1973; Peterson et al., 1978.

Table 3.9: SOME PHYSICO-CHEMICAL PROPERTIES OF HUMAN TOOTH ENAMEL AND
SYNTHETIC HYDROXYAPATITES

PROPERTY	PERMANENT WHOLE		SYNTHETIC HYDROXYAPATITE		
	TOOTH ENAMEL	Stoichiometric	Fluoridated	Carbonated	
Exposed	ab, ac	ab, ac	ab,ac	ab,ac	
Surface Area: BET (N ₂)	4 m ² g ⁻¹ (1)	60-100m ² g ⁻¹ (2)			
Dye chemisorption	4 m ² g ⁻¹ (3)				
Electron microscopy		60-100m ² g ⁻¹			
Organic Phase	yes	no	no	no	
Relative Surface Area:	<2%				
Porosity	microporous				
Pore Diameter	1-10nm				
Crystalline Phases	one	one	one	one	
Phase	hexagonal	hexagonal	hexagonal	hexagonal	
Surface Species	variable ⁽⁴⁾	Ca,P,OH	Ca,P,OH,F	Ca,P,CO ₃ ,OH	
Ca/P molar ratio: whole	1.4-1.67	1.67	1.67	>1.67	
ab face	variable	3	3	>3	
ac face	variable	2 or 1	2 or 1	>2or>1	
CO ₃ : wt% surface	1.6			≤22	
wt% whole	2 - 4				
% Type A	5 (4)				
% Type B	95 (4)			100	
F: wt% surface	0.9 (5)				
wt% whole	<0.3		<3.8		
%OH Deficiency	<30 (6)		<100	variable ⁽⁷⁾	
Isoelectric Point:					
KCl 10 ⁻³ mole ⁻¹	3.9 (8)				
0.1-1.0 mole ⁻¹		8.5 (9)	8.5-6.8 ^(7,10)	<8.5 ⁽¹⁰⁾	
NaCl 0.1 mole ⁻¹	7.6 (9)				
PO ₄ solution 10 ⁻⁴ mole ⁻¹			8.2 (9)		
10 ⁻³ mole ⁻¹			6.8 (9)		
0.3 mole ⁻¹	3.8 (11)				
Ca solution 10 ⁻⁴ mole ⁻¹			9.0 (9)		
10 ⁻³ mole ⁻¹			10.0 (9)		
10 ⁻² mole ⁻¹			>11.0 (9)		

Continued Over

- 1 Moreno and Zahradnik, 1973
- 2 Kibby and Hall, 1972.
- 3 Misra et al., 1978
- 4 Doi (1880) and
Touchon-Darguy (1980)
- 5 Petersson et al., 1978
- 6 Young and Spooner, 1969
- 7 Dependent on degree and/or type of
substitution
- 8 Parreira, 1981
- 9 Bell et al, 1973
- 10 Estimated
- 11 Kambara et al., 1978

3.7 MACROSCOPIC PROTEINACEOUS ACCRETIONS

3.7.1 INTRODUCTION

The results of protein adsorption studies in pure systems show that generally adsorption proceeds only as far as saturation of the adsorbent surface with a monolayer of protein molecules. However, macroscopic proteinaceous films occur widely in natural and technological systems e.g. on surfaces in dairy and food processing industries, on surfaces exposed to marine and fresh waters and pellicle and calculus formation on teeth. The common mechanism for these processes can be summarised as one of rapid protein adsorption to monolayer coverage and consequent thickening and hardening of adsorbed layers. The phenomenon may also be important in the formation of urinary and salivary stones (i.e. calculi). Bacterial colonisation is not a prerequisite for the formation of these deposits although they are often associated with them e.g. plaque formation on pellicle and the contamination of food and dairy lines. The current state of knowledge concerning the formation and structure of dental pellicle and the consequent hardening to form calculus is reviewed in this Section.

Amorphous calcium phosphates may play a role in the formation of bones and dental calculus as crystallisation of this material can provide the crystalline calcium phosphates found in such deposits. A possibility not considered in the literature is that amorphous calcium phosphate also plays a role in pellicle formation. The material was of sufficient interest therefore to be investigated in some detail.

3.7.2 THE ACQUIRED PELLICLE

The integuments on teeth (Plate 3.1) have been the subject of much study in dental research. However those studies undertaken prior to the early 1970's have often shown conflicting results and conclusions. This may have resulted through the use of scraping techniques, and dilute acids and/or EDTA to demineralise the tooth surface and float off the integuments. It was not realised that the integuments themselves were often demineralised and that some of their components were also acid-soluble (Mayhall, 1970; Sonju and Rølla, 1973). Further, the importance and degree of bacterial colonisation and metabolism of the pellicle was not known or overlooked.

More recent studies have shown that if any clean surface is exposed to saliva, either in vitro or in vivo, an acellular and bacterial-free layer is deposited on that surface within a few minutes. This material is defined as the 'acquired pellicle' (Dawes et al., 1963) and is formed independently of whether oral bacteria and/or food stuffs are present or not. The in vitro and in vivo pellicles are composed essentially of the same materials (Hay, 1967). The amino acid composition suggests that primarily salivary glycoproteins form the pellicle (e.g. Hay, 1967; Leach et al., 1967; McGaughey and Stowell, 1967; Sonju and Rølla, 1973). All the glycoprotein carbohydrates are found in the pellicle, but the specific glycoproteins involved are not known. Electrophoretic experiments show the presence of several anionic and cationic species (Armstrong, 1970, 1971; Stiefel, 1976) and also neutral proteins (Juriaanse and Booiij, 1979). Some of these components have mobilities similar to those of some parotid and submandibular protein components, the amino acid analyses also show some similarity, and the components are also known to bind calcium (Mandel, 1974 and 1977). In addition, traces

of Albumin, Amylase, blood group proteins, components of the complement system, Immunoglobulins and Lysozyme have also been detected in 2 hour pellicle (Kraus et al., 1973; Orstavik and Kraus, 1973; Stiefel, 1976). The organic composition of the 2 hour pellicle has been observed to be dependent on the forming surface (Sonju and Rølla, 1972; Orstavik et al., 1974; Sonju and Glantz, 1975; Sonju and Skjorland, 1976; Baier and Glantz, 1978). However, the composition of early pellicle is independent of the location in the mouth (Mayhall, 1970; Sonju and Rølla, 1973; Mandel, 1974).

The thickness of pellicle varies between $0.03 - 0.08\mu\text{m}$ on tooth areas subject to abrasion and up to $6\mu\text{m}$ in protected areas. Lie (1977) has made a comprehensive scanning and transmission electron microscope study of pellicle morphogenesis over the period of formation from 2 - 48 hours. The initial deposits appeared as either irregularly or more distinctly outlined globules of amorphous or granular aggregates of material. The dimensions ranged between 20 - 350 nm in size. The contents of the aggregates were either densely granular or more loosely arranged exhibiting small electron translucent areas. With increasing time, the morphology of the pellicle changed and it became fibrillar and then granular. Transitional and composit forms were usually observed but the globular form predominated at all time periods studied. Aging of the pellicle was also indicated by changes in the globules themselves. The outer edge often appeared to condense or remain unchanged while the interior became less electron dense, even extending to the point where the inner content seemed lost.

The mechanism of pellicle formation is not known but several theories have been proposed. These include:

(i) Precipitation of salivary proteins by some physical and/or chemical processes such as:

PLATE 3.1.

ULTRASTRUCTURAL DETAIL OF PELLICLE AND
PLAQUE ON TEETH



Electron micrograph shows the dental plaque (Bacteria (B) and Organic Matrix (O) overlying the Pellicle (SP) and Enamel Surface (E).

(Reproduced from Frank and Brendel (1966) by permission of the publisher).

Alternate wetting and drying of teeth;

Isoelectric precipitation or precipitation by other mechanisms as a result of pH changes in saliva brought about by bacterial metabolic processes;

Precipitation through the decreased solubility of salivary glycoproteins as a consequence of the removal of sialic acid or carbohydrate groups by bacterial enzymes; and,

Precipitation by higher concentrations of calcium ions than usually found in saliva.

The sequence of events in the formation of deposits by these mechanisms have been discussed by Dawes (1968), Jenkins (1968a and b, 1971), Kleinberg (1970) and Edgar et al. (1980).

(ii) Adsorption of salivary proteins at the tooth surface resulting in denaturation and/or aggregation (Winkler and Dirks, 1958; Jenkins, 1966).

(iii) Electrostatic interactions between the net negatively charged salivary protein molecules and the net positively charged tooth enamel surface, or more specifically, the positively charged calcium ions in the enamel surface (e.g. McGaughey and Stowell, 1971a; Pruitt, 1977; Rølla, 1977). Einfeldt (1978) suggested that the pellicle built up to a thickness of about 1 μm as it was only beyond this point that the positive electrostatic potential of the surface calcium ions was neutralised. Hay (1973 a,b) and Rølla (1977) envisaged a macroscopic pellicle build up as the adsorbed anionic proteins, in turn, bound cationic proteins or calcium ions. The pellicle was thus formed by a bridging mechanism resulting from layering and surface denaturation/aggregation. Leach (1980) has presented a comprehensive discussion of the evidence for and against the calcium bridging mechanism and other possible calcium mediated aggregating mechanisms.

(iv) Aggregation at the tooth enamel surface through the co-operative

effect of many weak bonds, particularly hydrophobic interactions (Leach, 1980).

Of the four groups of mechanism, those listed under groups (i) and (ii) are now generally little favoured by workers in the field for several reasons:

Tooth surfaces do not usually undergo alternate wetting and drying sequences except possible in mouth-breathers;

Bacteria are not necessary to pellicle formation; and,

Proteins generally do not denature and aggregate upon adsorption.

3.7.3 CALCIFIED PELLICLE AND/OR PLAQUE: DENTAL CALCULUS

Dental calculus is a hard, mineral-like material embedded within the organic matrix of pellicle and plaque, and can form a macroscopic deposit if not removed (Chapter 1, Plate 1.1). The macroscopic deposit is conveniently classified according to its location on teeth i.e. above the gums (supragingival) or beneath the gums (subgingival). Subgingival calculus is randomly distributed in the mouth whereas supragingival calculus is found near the outlet ducts of the salivary glands. The following reviews have been used to provide the general information given in this section and the reader is referred to them for a more in-depth discussion: Leung and Jensen, 1958; Leung, 1960; Rowles, 1964 a and b; Leach, 1973).

Calculus contains on average about 80% mineral matter, the principle components of which are calcium and phosphate (Table 3.10). This mineral is composed of an X-ray amorphous phase and up to four crystalline phases. The exact composition of calculus is dependent on the age of the sample and its location in the mouth. Brushite, the least stable of the crystalline calcium phosphate phases, is found more frequently in young calculus. The calcium : phosphate ratio of subgingival calculus is higher than that of supragingival calculus.

The factors affecting the formation, survival and rate of change of all these phases as they exist in mineralising pellicle and plaque are not known. This is not surprising considering the complexity of the oral environment (Chapter 2) and biological mineralisation (e.g. Zipkin, 1970; Krampitz and Witt, 1979). However, it is known that pellicle and plaque are formed first and, within a few days, hardening through the formation of crystalline calcium phosphates (i.e. calcification) in the organic matrix is clinically obvious. The bacteria of plaque are not

necessary to the calcification of either the pellicle or plaque matrix.

In calcified pellicle (Plate 3.2), the calcification may be distributed throughout the pellicle while in others it appears as a calcifying front originating from, and running parallel to, the enamel surface. The calculus is made up of very small randomly oriented crystals. A calcifying front is seen particularly in cases where calcification of the pellicle is not extensive (refer Plate 3.3 showing calcifying front in plaque) and the deposition of the calcified material follows a laminated pattern, also running approximately parallel to the enamel surface. Pellicle often stains in a similar manner if it is free of crystalline mineral matter. Salivary calculi are known to contain an organic and an inorganic phase (Vignoles et al., 1980), and display similar laminations to those observed in pellicle except that these surround a central spheroid core (Taniguchi et al., 1979). The inorganic component comprises hydroxyapatite, octacalcium phosphate, whitlockite and brushite (rarely), and displays variable crystallinity (Burstein et al., 1979).

In calcified plaque (Plate 3.3) the calculus is also made up of very small crystals. These originate at localised sites in the organic matrix and progress as a calcifying front throughout the remainder of the matrix. Plaque bacteria are initially engulfed by the calcifying front which progresses some considerable distance before the bacteria themselves also calcify. In some areas there is often no indication of any organic matrix between the calculus crystals.

Early theories of calculus formation suggested that calcium phosphate salts spontaneously precipitated in the organic matrix due to conditions which caused a local supersaturation of calcium and phosphate.

Table 3.10: THE COMPOSITION⁽¹⁾ OF MACROSCOPIC DENTAL CALCULUS

COMPONENT ⁽²⁾	%ABUNDANCE	%INCIDENCE	Ca/P
Inorganic	≈80		1.64
Organic	≈20		
Calcium	31.7		
Phosphorous	14.9		
Hydroxyapatite $\text{Ca}_5 (\text{PO}_4)_3 \text{OH}$	55.3	99.5	1.67
Whitlockite $\text{Ca}_3 (\text{PO}_4)_2$	24.2	80.7	1.50
Octacalcium Phosphate $\text{Ca}_8 \text{H}_2 (\text{PO}_4)_6$	20.8	94.8	1.33
Brushite CaHPO_4	8.9	43.6	1.00

¹ Data taken from Leung, 1960; Rowles 1964a, 1964b.

² Other species reported (<2% w/w) include: Na, Mg, Cl, CO_3 . Trace elements reported include Al, Au, Br, Cu, F, Fe, Mn, Si, Sr, W, Zn.

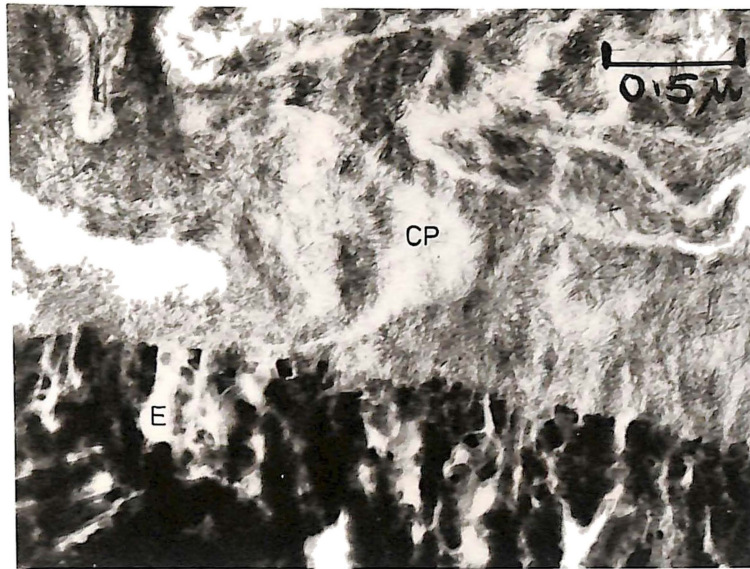
Contemporary opinion favours an epitactic concept of formation. It is not known whether this is a homonuclear process, a heteronuclear process involving substances present in the organic matrix of pellicle and plaque, or a combination of both of these processes. (See Posner (1969) for some discussion of these as regards osteoid tissue mineralisation). Either mechanism probably involves clusters of the constituent ions (Bachra, 1973). The initial material formed is not known. Physiochemical studies of calcifying bone and cartilage indicate an amorphous calcium phosphate is probably the first mineral salt to be deposited (for discussions see Posner, 1969; Eanes and Posner, 1970; Eanes, 1973; Elliott, 1973; Krampitz and Witt, 1979).

PLATE 3.2

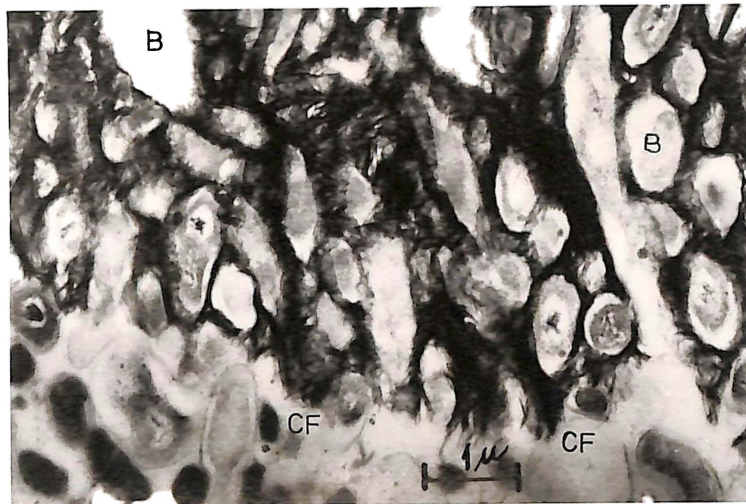
CALCIFIED PELLICLE

PLATE 3.3

CALCIFIFYING PLAQUE



Electron micrograph showing the small and randomly oriented crystals of calcified pellicle (CP) overlying the enamel (E).



Electron micrograph showing calcification of the interbacterial matrix preceding calcification of the bacteria (B) along a calcifying front (CF). (Both Plate 3.2 and 3.3 reproduced from Leach (1973) by permission of the publisher).

3.7.4 AMORPHOUS CALCIUM PHOSPHATES

The solid phase that forms initially in the precipitation of calcium phosphates from aqueous solution at pH's of about 7 and above is an X-ray amorphous material containing calcium and phosphate. Some review articles on biomineralisation which incorporate discussions of amorphous calcium phosphates are those of Posner (1969), Eanes and Posner (1970), Elliott (1973), and Krampitz and Witt (1979).

Under observation by electron microscopy, the amorphous calcium phosphates appear as discoidal or spherical particles often with an electron translucent centre (e.g. Eanes, 1973). It is now known that the electron translucent centre is an artifact of any or all of aging, drying and electron irradiation of the sample. Particle sizes have been observed to range from 20 - 200 nm (Nylen et al., 1972) and to have a surface area of about $40 \text{ m}^2 \text{g}^{-1}$. It is suggested that the particles are formed from highly hydrated solution aggregates (Eanes et al., 1973).

Amorphous calcium phosphates are unstable in solution and hydrolyse autocatalytically over a period of several hours to a crystalline apatite material. The mechanism is thought to involve dissolution, nucleation and crystal growth. The solution calcium concentration decreases during this time (Eanes et al. 1965; Termine and Posner, 1970; Meyer and Weatherall, 1982). The initial product formed during precipitation often has a calcium : phosphorous ratio of about 1.44 - 1.55, the exact value of which is dependent on the initial solution composition and conditions. However, ratios as low as 1.15 have been observed (Termine and Eanes, 1972). Those products formed near physiological pH values show a rather constant ratio of near 1.5 and this material shows the greatest solution stability. The calcium : phosphorous ratio of 1.5 suggests the material is a tricalcium phosphate and the chemical formula $\text{Ca}_3 (\text{PO}_4)_2$ for the

basic chemical unit has been proposed (Eanes, 1970). Several researchers have suggested $\text{Ca}_9 (\text{PO}_4)_6$ clusters (Eanes et al., 1965; Betts and Posner, 1974; Blumenthal et al., 1977). It has also been suggested that the amorphous phase is an agglomeration of hydrated calcium and phosphate ions with little or no three dimensional order (Holmes and Beebe, 1971). Certainly, it seems that the amorphous phase does not have any long range atomic order as occurs even in very small crystals. Although the chemical composition is not known definitely, it is likely to include hydrogen and hydroxyl ions and water. Carbonate ions may also be present if the initial reactant solutions contain this species (Greenfield and Eanes, 1972). With increasing contact time with the mother solution, the calcium : phosphate ratio approaches the value of 1.67 observed for crystalline hydroxyapatite (e.g. Eanes et al., 1965 and Barton and Harrison, 1976). The conversion is thought to occur through one or more transient calcium phosphate phases: dicalcium phosphate dihydrate $\text{Ca HPO}_4 \cdot 2\text{H}_2\text{O}$ (DCPD, brushite), octacalcium phosphate $\text{Ca}_8 \text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$ (OCP), and tricalcium phosphate $\text{Ca}_3 (\text{PO}_4)_2$ (TCP, whitlockite) have been suggested (Nancollas et al., 1979; Meyer and Weatherell, 1982). The solubility products for these phases are shown in Table 3.11. It appears that the transient phases formed are dependent on pH, ionic strength, the degree of supersaturation and the chemical composition of the precipitating solution, and also the rate at which the conversion proceeds. There is a concomittant increase in crystallinity as observed by X-ray diffraction (Holmes and Beebe, 1971) and an increase in surface area as measured by gas adsorption (Holmes and Beebe, 1971; Barton and Harrison, 1976). The conversion appears to proceed by the formation of crystals on the surface of the amorphous spherical particles until the spherules are enveloped (Eanes et al., 1973). The crystals do not occupy the space previously occupied by the amorphous spherules which

seem to lose mass to the exterior. The spherules thus act essentially as a reservoir for the steady state condition of supersaturation during the conversion (Eanes et al., 1967).

Table 3.11: SOLUBILITY PRODUCTS OF SOME CALCIUM PHOSPHATE PHASES

PHASE	MOLECULAR FORMULA	K _{sp} (at 25°C)
Dicalcium Phosphate Dihydrate	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	1.26×10^{-7}
Tricalcium Phosphate	$\text{Ca}_3(\text{PO}_4)_2$	1.20×10^{-29}
Octacalcium Phosphate	$\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$	1.56×10^{-94}
Hydroxyapatite	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	2.2×10^{-117}

(from Nancollas et al., 1979).

Amorphous calcium phosphates can be stabilised to some extent by various physical and chemical means. Temperature, pH, ionic strength, solution viscosity, and the initial calcium : phosphate mixing ratio and millimolar product affect the initial phase separation (i.e. metastability of the precipitating solution) and consequent conversion (Eanes et al., 1967; Termine and Posner, 1970; Meyer and Weatherell, 1982). Similarly, the metastability, the product calcium : phosphate ratio, and the rate of conversion can be altered by a variety of foreign substances (e.g. Termine et al., 1970; Blumenthal et al., 1972, 1977). These include magnesium, fluoride, carbonate, pyrophosphate, phosphonates, poly amino acids, synthetic polyacrylate, and various

biological macromolecules such as peptides, proteins, protein-polysaccharide complexes (glycoproteins), phospholipids, sRNA and ATP. Stabilisation appears to be related to calcium binding ability for Wuthier and Eanes (1975) observed that only calcium binding phospholipids stabilised the amorphous material. Any of these materials may be added prior to or after the initial phase separation (Termine and Posner, 1970; Termine et al., 1970). In the former case, the onset of precipitation may be either enhanced or delayed. In the latter case, the conversion to hydroxyapatite is delayed. Stabilisation of the amorphous phase has been observed for periods of less than one hour up to several days (c/f Termine et al., 1970; Blumenthal et al., 1977). Also, the particle morphology and aggregation is greatly affected (Eanes et al., 1973, Wuthier and Eanes, 1975). Wuthier and Eanes (1975) observed that the final crystal phase formed was one of thin sheets of crystals about 1.7nm thick which frequently stacked in multilayered membrane like structures of alternating leaves of electron dense (i.e. the inorganic crystals) and electron-lucent (i.e. the organic layer) material. Calcium : phosphate ratios varying from 1.31 - 1.58 were observed for precipitates aged for periods up to 30 minutes.

Many of the types of substances mentioned above are found in saliva (Table 2.1) and the pellicle and plaque of teeth. Some have been shown to inhibit precipitation (Hay and Grøn, 1975a, 1976; Hay and Belenz, 1977; Schlesinger and Hay, 1977; Hay and Moreno, 1979a or stabilise otherwise unstable calcium phosphate phases (Grøn, 1973a).

3.7.5 CONCLUSION

The survey of the literature on pellicle and calculus formation showed that the mechanism of formation of these deposits was not known. Recently, studies at this University by Dass (1982) have shown that macroscopic proteinaceous accretions can be formed employing colloidal particulate bridging species. As amorphous calcium phosphate almost certainly exists in the oral environment, this species could provide a bridging mechanism for the formation of dental pellicle. It is therefore proposed to investigate the phenomenon.

3.8 OBJECTIVES OF THE THESIS

The aim of the present study is to gain a better understanding of the films that form on teeth. This requires a good understanding of the formation of the initial protein layer. Crystalline hydroxyapatite has been chosen as a model system for these studies since tooth enamel is composed predominantly of this material.

Studies of protein adsorption are characterised by many problems. These include the lack of adequate characterisation of the adsorbents, proteins and the experimental systems in use. This can subsequently lead to experimental artifacts and incorrect conclusions being drawn from results obtained. Typically, surface area effects, changes in protein composition and kinetic and preferential adsorption effects account for many of the problems. A major part of the present work is to adequately characterise the materials and experimental system in use and to devise procedures to reduce problems to a minimum.

Once suitable experimental procedures were established to study adsorption, it was sought to answer several specific questions. These include the nature of the adsorption isotherm and the adsorbed layer, particularly with respect to solution protein concentrations required to achieve maximal adsorption, what the corresponding amount of adsorption is and the effect of ionic substitutions in the hydroxyapatite lattice on these, the reversibility of adsorption, the exchange and stability properties of the adsorbed layer and factors that contribute to the formation of more than one adsorbed layer. Information on these aspects provide the major direction of this thesis.

Initially it is intended to use well characterised proteins and adsorbents as model systems although some experiments using salivary protein and tooth enamel are also contemplated. The source of all commercially available materials used in the course of study is given in Appendix II.

CHAPTER 4

PREPARATION AND CHARACTERISATION OF ADSORBENTS

4.1 INTRODUCTION

Literature studies indicated it was possible to prepare a pure and stoichiometric hydroxyapatite of the hexagonal form relatively simply. Although several 'hydroxyapatite' preparations are available commercially, the calcium : phosphate ratio of these materials is low and suggests the presence of calcium phosphate phases other than hydroxyapatite (Spencer and Grynpas, 1978). Preparations of fluoride substituted and carbonate substituted hydroxyapatites have also been described in the literature. It was thought that these materials would provide useful model surfaces for the studies envisaged. The existence of amorphous calcium phosphates is well known and their preparation has also been well described.

The syntheses and characterisation of these materials are described in this Chapter.

4.2 PREPARATION OF ADSORBENTS

4.2.1 INTRODUCTION

Hydroxyapatites are formed by a direct slow precipitation using relatively high temperatures and pH to avoid the formation of unwanted calcium phosphate salts. However, pH values greater than 10 are not used as these result in the formation of very finely divided non-stoichiometric precipitates. The method and materials used to prepare the crystalline non-substituted and carbonate substituted hydroxyapatites (OHAp and $\text{CO}_3\text{-OHAp}$ respectively) were similar to those used by Moreno et al. (1968). A fluoride substituted hydroxyapatite (F-OHAP) was then prepared from the non-substituted hydroxyapatite. A modification of the method of Belcourt and Voegel (1978) was used in which OHAp is exposed to a neutral 10 ppm solution of sodium fluoride. The mechanism of fluoride uptake by high surface area hydroxyapatites in such systems is mainly by surface adsorption or exchange with hydroxyl groups (Spinelli et al., 1971). The use of low concentrations of fluoride avoids the formation of calcium fluoride. This method was chosen in preference to a precipitation method (Moreno et al., 1977) because it provided a simpler method of producing a fluoridated surface structure.

Amorphous calcium phosphate phases are formed in preference to crystalline phases by rapid precipitation at low temperature. The use of high pH's (Eanes et al. 1967; Termine and Posner, 1970; Barton and Harrison, 1976) and low temperatures (Termine and Posner, 1970) have been observed to produce the most solution stable product. The method and materials used to prepare an amorphous calcium phosphate (ACP) were similar to those described by Eanes (1970).

All glassware was carefully cleaned by the procedures detailed in Appendix III.

4.2.2 NON-SUBSTITUTED HYDROXYAPATITE

A non-substituted hydroxyapatite was prepared by taking precautions, at all stages of the preparation, to avoid contamination by CO₂. Fresh doubly distilled water was degassed under vacuum and then saturated with CO₂ free nitrogen gas. This water was then used to prepare 8 l solutions of 0.246 mole l⁻¹ (NH₄)₃ PO₄·3H₂O and 0.423 mole l⁻¹ Ca(NO₃)₂·4H₂O. The solutions were filtered through Whatman No.6 filter paper and their pH adjusted to 8.5 - 9.0, using concentrated ammonia solution, prior to storage in 2 l volumetric flasks. The air spaces above these solutions were flushed with nitrogen gas before the flasks were stoppered.

The reaction vessel was a 20 l, 5-neck, glass flask set in a boiling water bath, the water coming approximately half way up the flask (this maintained the temperature of liquid in the flask at 93 - 95°C). A 1m condenser was attached to one neck; vigorous stirring was maintained using an overhead stirrer passing through the second neck; nitrogen gas was continuously bubbled through the reaction mix via the third neck; transfer of reagents and materials was done through the fourth neck; the fifth neck was kept sealed and used as required for pH determination and adjustment of the reaction mix.

The calcium nitrate solution was added to the flask and brought to temperature. The phosphate solution was added, using a 5 l separating funnel (vented through a Carbosorb Column) at a rate of 0.31ml hr⁻¹. Concentrated ammonia solution was used to maintain the pH between 8.5 and 9.5 (measured at room temperature) during the addition of the phosphate solution. The reaction mix was then refluxed for 36 hours.

The solid was allowed to settle, the supernatant withdrawn, 10 l of boiling, degassed, double distilled water added and the mixture left to reflux for a further 2 hours. This washing operation was repeated 5

times. Further washing (by decantation, using boiled doubly distilled water and short settling periods) was done to remove as much as possible of the finest fraction of the precipitate. During these washing and settling operations, the flow of nitrogen gas was maintained.

The suspension was filtered and the solid dried overnight at 105°C. A yield of 328 g (~99% of theoretical yield based on stoichiometric OHAp) was obtained.

4.2.3 CARBONATED HYDROXYAPATITE

A carbonate substituted hydroxyapatite was prepared as per OHAp (Section 4.2.2) but with the inclusion of a carbonate in the reactant solutions (Featherstone, 1979). No precautions were taken to avoid contamination by atmospheric CO₂.

Fresh doubly distilled water was used to prepare 1 l of 1.0 mole l⁻¹ NH₄HCO₃ solution. The solution was filtered (Whatman No.6) and adjusted to pH 8.5 - 9.0 as for the preparation of OHAp. 400 ml of this solution was then added to each of the calcium and phosphate solutions prior to reaction. The temperature of the reaction mix, during addition of the phosphate, was maintained at 90 - 92°C to avoid loss of CO₂ at higher temperatures.

The yield obtained was 337g.

4.2.4 FLUORIDATED HYDROXYAPATITE

A fluoride substituted hydroxyapatite was prepared by modification of the previously prepared OHAp (Section 4.2.2). Precautions, as described for the preparation of OHAp, were taken to avoid CO₂ contamination. 20.0 g of OHAp was suspended in 200 ml of distilled water and ultrasonicated for 1 hour with periodic vigorous shaking to ensure dispersion. The suspension was added to 200 ml of 1.052×10^{-3} mole l⁻¹ NaF solution (20 ppm), with rapid stirring, and allowed to react for 24 hours in a sealed plastic bottle. (A preliminary experiment, using a 2 ppm solution of NaF, showed no detectable loss of fluoride within a 24 hour period when the solution was stored in the plastic bottle.) The precipitate was allowed to settle and a sample of the supernatant withdrawn for fluoride analysis.

The suspension was filtered and washed extensively with doubly distilled water until no fluoride emerged. The solid was dried overnight at 105°C.

Fluoride concentrations in the solutions were determined using an Orion Model 96-09 Combination fluoride Electrode and Radiometer pH Meter 26. The electrode was calibrated using NaF solutions of 10^{-1} - 10^{-5} mole l⁻¹ (0.2 - 2 000 ppm; see Appendix IV). All standard and test solutions were adjusted to a total ionic strength of 0.1 mole l⁻¹ using a solution of 1.0 mole l⁻¹ NaCl.

4.2.5 AMORPHOUS CALCIUM PHOSPHATE

An amorphous calcium phosphate was prepared as described below. The material was made fresh as required as it has been reported that significant crystallisation occurs upon storage (Holmes and Beebe, 1971).

No precautions were taken to avoid CO₂ contamination during the mixing, precipitation and centrifugation steps.

Equal volumes of 0.246 mole l⁻¹ (NH₄)₂ HPO₄ and 0.423 mole l⁻¹ Ca(NO₃)₂·4H₂O were prepared, filtered (Whatman No.6) and the pH adjusted to pH 9.6 using concentrated ammonia solution. The phosphate solution was dumped into the calcium solution, with rapid stirring. The precipitate was centrifuged (6 000g) at 0°C for 5 minutes, the supernatant decanted and the solid washed with ice-cold ammoniated distilled water of pH 9.6. The centrifugation and washing procedure was repeated three more times before the solid was freeze dried. Care was taken to ensure that the wet solid was spread out on the inner walls of the freeze drier vessels to avoid continuation of the ACP to OHAp crystallisation process.

4.3 CHARACTERISATION OF ADSORBENTS

4.3.1 INTRODUCTION

Information on the crystallinity, particle morphology, surface area, and chemical composition was sought. The techniques of X-ray diffraction, transmission electron microscopy and BET (N₂) adsorption provided physical characterisation. Chemical characterisation was performed by X-ray fluorescence, carbonate and nitrogen analysis, solution depletion techniques (in the case of F-OHAp) and Infrared analysis.

4.3.2 X-RAY DIFFRACTION (XRD) STUDIES

XRD analysis of the prepared adsorbents was obtained using a Philips X-ray Diffractometer and copper K α radiation. The samples were scanned (Fig.4.1) and the 2θ and corresponding I/I_0 values for the crystalline materials recorded (Table 4.1).

The XRD patterns obtained for the crystalline materials (Figs 4.1 a, b, c) were typical of well-crystallised hydroxyapatites (Posner, 1969). The Powder Diffraction Files and Fink Index (Smith, 1967) confirmed the samples were hydroxyapatites (Table 4.1). There was no indication in the XRD patterns of the presence of non-apatitic phases. The small shifts in peak positions and line broadening effects observed for some of the CO₃-OHAp lattice reflections, relative to OHAp, are typical of those observed for precipitated apatites containing carbonate substitutions (Le Geros et al., 1968). These reflect changes in lattice parameters due to carbonate substitution. Although the incorporation of fluoride into the hydroxyapatite lattice also alters the lattice parameters (Young and

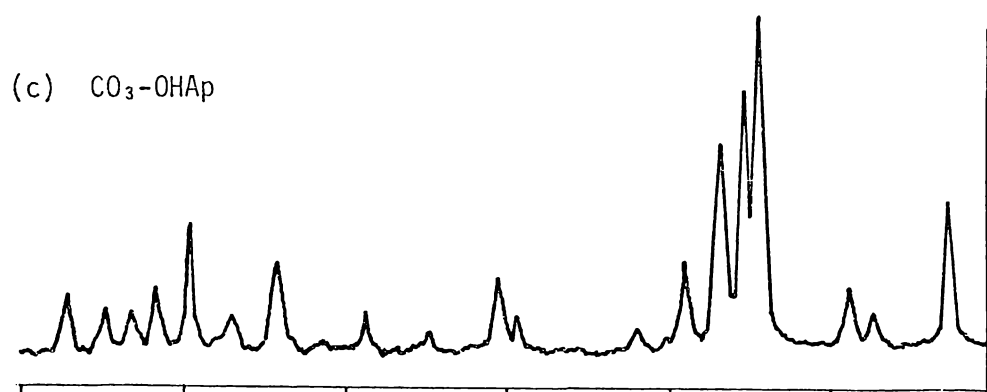
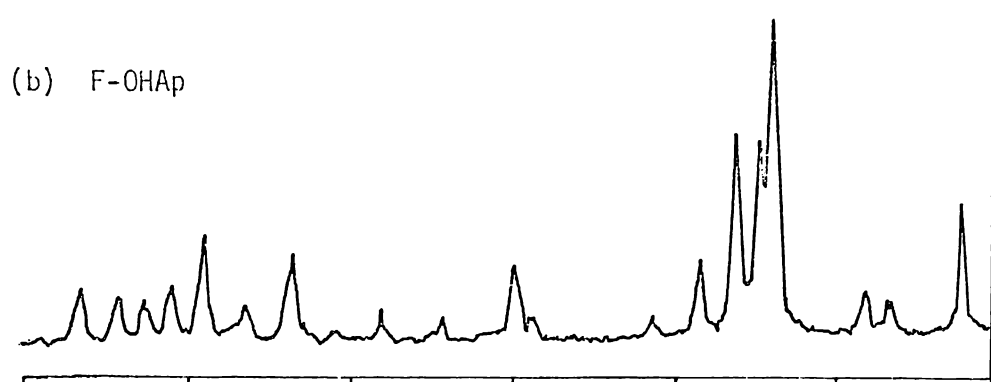
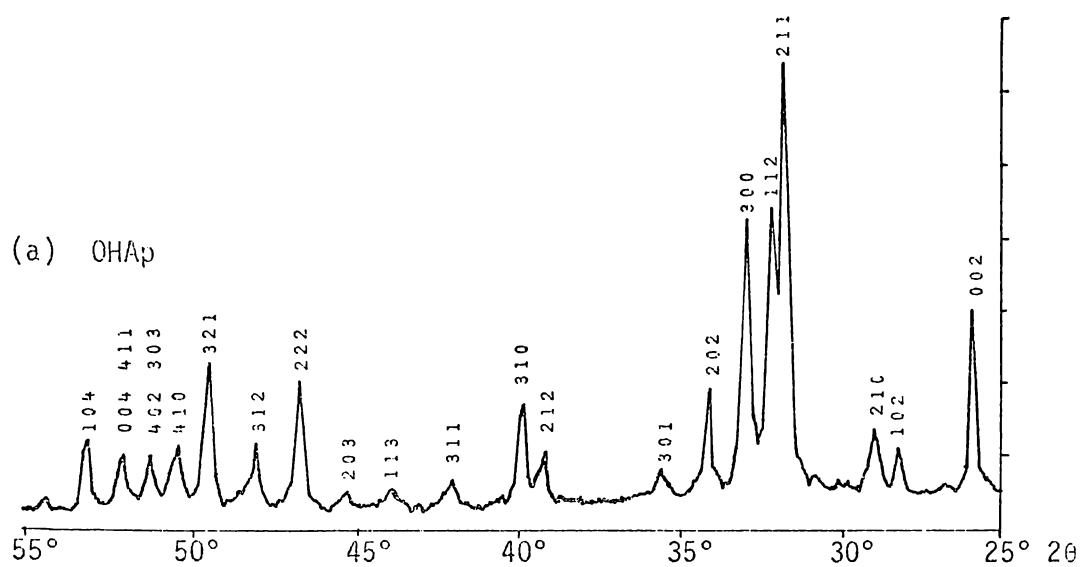


Figure 4.1 : X-Ray Diffraction Analysis of Adsorbents

TABLE 4.1: X-RAY DIFFRACTION ANALYSIS OF ADSORBENTS

Powder Diffraction File				Experimental		
hkl	2 θ^a	I/I ₂₁₁	2 θ^b	OHAp I/I ₂₁₁	CO ₃ -OHAp I/I ₂₁₁	F-OHAp I/I ₂₁₁
002	25.9	40	25.9	42	48	41
102	28.1	12	28.2	11	10	10
210	29.0	18	28.9	15	18	13
211	31.8	100	31.8 (31.9)	100	100	100
112	32.2	60	32.2	67	78	62
300	32.9	60	32.9 (33.0)	63	30	64
202	34.0	25	34.1	26	27	23
301	35.5	6	35.5	7	5	8
212	39.2	8	39.2	7	8	8
310	39.8	20	39.8	22	21	23
221	40.5	2				
311	42.0	10	42.0 (42.1)	7	5	8
302	42.3	4				
113	43.8	8	43.7	6	8	13
400	44.4	2				
203	45.3	6	45.3	4	4	3
222	46.7	30	46.7	30	29	28
312	48.1	16	48.1	15	14	10
320	48.6	6	48.6	6	8	5
213	49.5	40	49.5	36	39	33
321	50.5	20	50.4 (50.5)	17	16	18
410	51.3	12	51.3	15	14	13
402,303	52.1	16	52.1	15	14	13
004,411	53.1	20	53.2 (53.1)	19	19	18

^a Calculated from Bragg equation given d values

^b Bracketed values refer to the 2 θ values as observed for the carbonate substituted hydroxyapatite

Elliott, 1966), surface fluoridation would not affect the XRD pattern.

The XRD pattern of the amorphous material was typical of that observed for amorphous calcium phosphates (e.g. Holmes and Beebe, 1971). There was no clear evidence of any crystallinity within the sensitivity of the technique.

4.3.3 TRANSMISSION ELECTRON MICROSCOPE (TEM) STUDIES

The morphology of the material in the adsorbent preparations was established using a Philips EM400 Transmission Electron Microscope and formvar coated 200 mesh copper grids. Suspensions of the crystalline materials (5×10^{-3} g ml⁻¹, in water) were prepared by ultrasonication for 1 hour. The ACP was prepared immediately prior to examination. One drop of the suspension was placed on each grid. After approximately 15 minutes, the grids were gently rinsed and allowed to air dry at room temperature. The micrographs obtained are shown in Plate 4.1.

Both the OHAp and F-OHAp samples contained needle shaped crystals typical of those observed for well crystallised hydroxyapatites. The crystals of the CO₃-OHAp were much smaller and more irregular in shape. A large proportion of the crystals were plate-like. This change in crystal morphology is typical of that observed upon the inclusion of carbonate into the hydroxyapatite lattice (Le Geros et al., 1968).

The ACP sample appeared to consist of approximately spherical globules, about 16 nm in diameter, and was typical of preparations observed by electron microscopy (Section 3.7.4). The appearance of some granularity and electron translucent centres in the particles is consistent with some electron beam damage during observation.

PLATE 4.1

TRANSMISSION ELECTRON MICROGRAPHS
OF ADSORBENTS



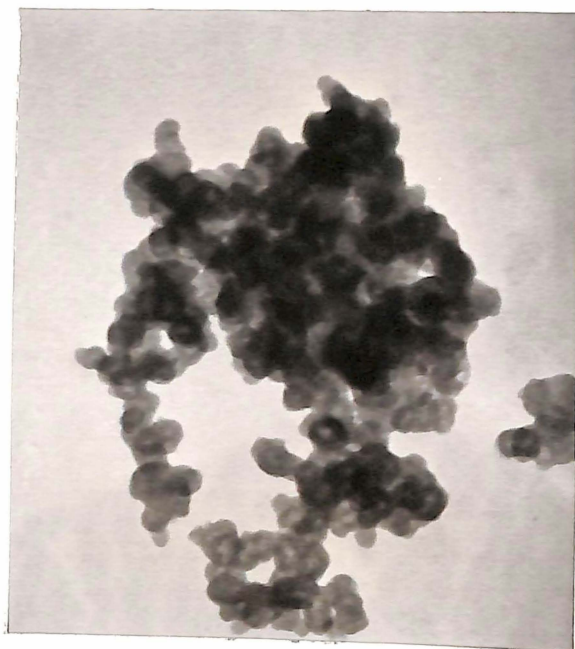
OHAp, 46 000 x



F-OHAp, 46 000 x



CO₃-OHAp, 46 000 x



ACP, 125 000 x

4.3.4 SURFACE AREA DETERMINATIONS

The BET nitrogen adsorption method (Brunauer et al., 1938) was used to determine surface areas of the prepared adsorbents. Samples of 0.2 – 0.5 g weight were degassed for 18 hours at $\approx 10^{-5}$ Torr and 300°C immediately prior to the determination. An outgassing temperature of 300°C does not cause a significant change in the measured surface areas of ACP or crystalline hydroxyapatite (Section 3.6.5). The results of the surface area determinations of the adsorbents are shown in Table 4.2.

The greatly increased surface area observed for CO₃-OHAp, relative to OHAp, reflects the decreased crystal size of this preparation as observed in the TEM studies. Although the ACP particles were much smaller than those of the crystalline preparations, a larger specific surface area was not observed. This is probably due to the aggregation of the particles as observed in the TEM studies (Plate 4.1d).

4.3.5 INFRARED (IR) ABSORPTION SPECTROSCOPY STUDIES

IR spectra of prepared adsorbents were obtained using a Shimadzu IR-27G Spectrometer and KBr discs (~200 mg KBr, ~2 mg adsorbent). The spectra in which the major absorption peaks have been identified (Bhatnagar et al., 1968; Termine, 1973) are shown in Figure 4.2.

The spectra for OHAp, F-OHAp and ACP are typical of those found for crystalline and amorphous calcium phosphates of Ca/P ratios of 1.67 or less (Stutman et al., 1965). The spectrum for ACP showed an absorption band at $\sim 1650\text{ cm}^{-1}$. This has been assigned to the presence of water as analysis failed to indicate the presence of nitrogen (micro analysis performed by Chemistry Department, University of Otago, New Zealand).

The spectrum for CO₃-OHAp was typical of that seen for carbonated apatites in which carbonate has substituted for phosphate in the crystal lattice (Montel, 1977).

Table 4.2: SURFACE AREAS OF ADSORBENTS

ADSORBENT	SURFACE AREA/m ² g ⁻¹
OHAp	40 ± 2 ^(a)
F-OHAp	44 ± 2
CO ₃ -OHAp	60 ± 3
ACP	35 ± 3

^a Errors are reported as the standard deviation.

4.3.6 X-RAY FLUORESCENCE (XRF) STUDIES

Standards of known Ca/P ratios were prepared using mixtures of CaHPO₄·2H₂O and Ca₃ (PO₄)₂ salts. XRF analysis of the standards and prepared hydroxyapatites was obtained using an Ortec X-Ray Analytical System and Ortec Model 6200 Multichannel Analyser. A calibration curve obtained for the Ca/P ratios is given in Appendix V. Results of the XRF analysis are shown in Table 4.3.

The Ca/P ratios for OHAp and F-OHAp compare with the theoretical value of 1.66 for hydroxyapatites of the form Ca₅ (OH)_{1-x} F_x (PO₄)₃ where x = 0 is the non-substituted hydroxyapatite
0 < x < 1 is the fluorine substituted hydroxyapatite.

The high Ca/P value of 1.76 for CO₃-OHAp is consistent with a calcium rich hydroxyapatite due to carbonate substitution of phosphate and/or hydroxide species. Although both types of substitution are possible, the carbonate in substituted hydroxyapatites prepared by precipitation at low temperatures is usually found in a phosphate site (Montel, 1968).

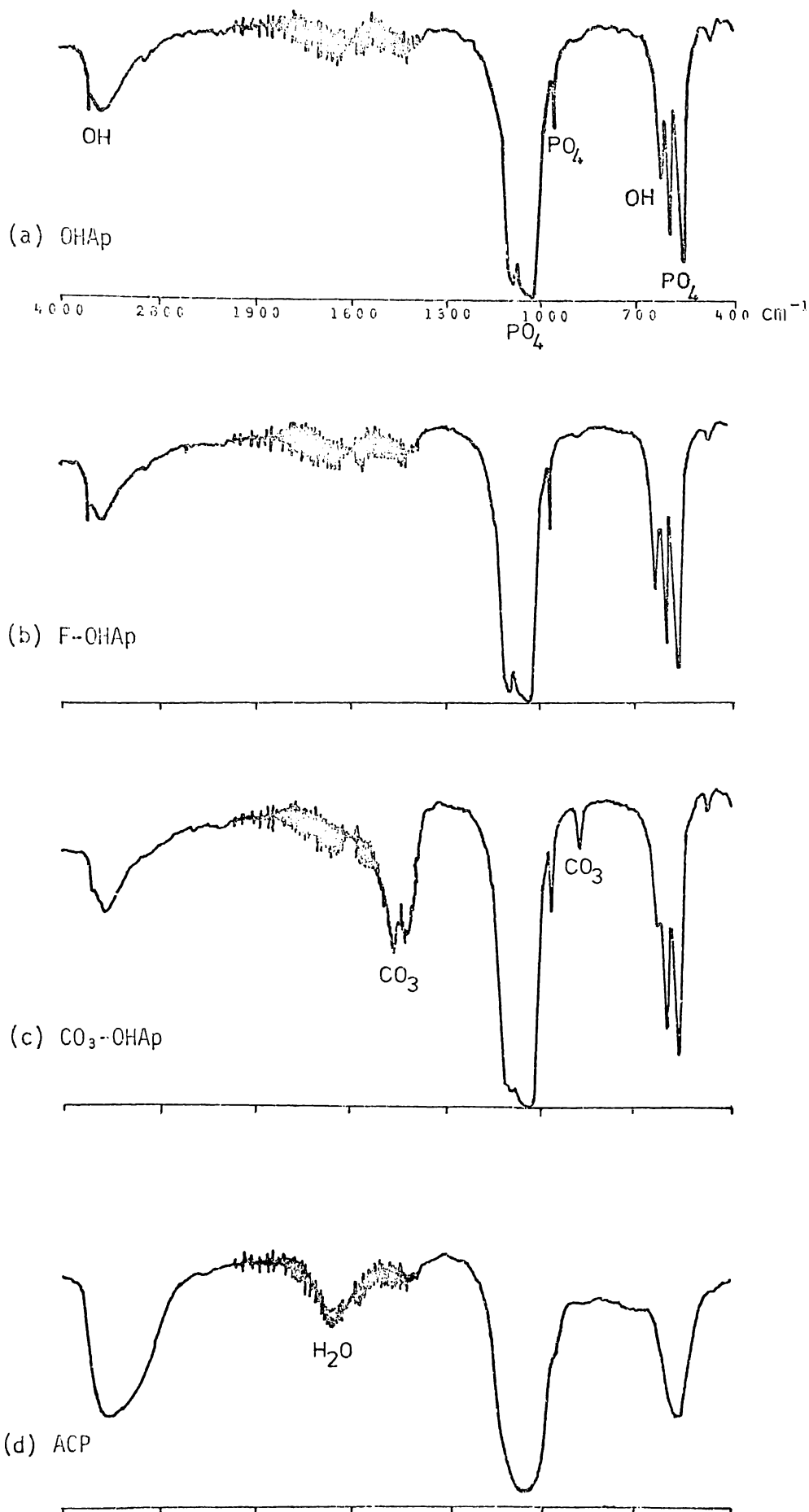


Figure 4.2 : Infrared Spectra of Adsorbents

The Ca/P ratio of 1.47 for ACP is typical of that found for amorphous calcium phosphates (Section 3.7.4).

Table 4.3: XRF ANALYSIS OF ADSORBENTS

ADSORBENT	Ca/P
OHAp	$1.66 \pm 0.01^{(a)}$
F-OHAp	1.66 ± 0.01
CO ₃ -OHAp	1.76 ± 0.01
ACP	1.47 ± 0.01

^a Errors are reported as the standard deviation.

4.3.7 CARBONATE ANALYSIS

The carbonate content of the prepared adsorbents was obtained using a modification of the method of McCrea (1950). The method involves the acidimetric evolution of carbon dioxide from carbonate followed by determination of the carbon dioxide using vacuum line techniques. A schematic representation of the vacuum line used is shown in Figure 4.3.

Samples of 0.2 - 0.5 g of ACP and the finely ground crystalline hydroxyapatites were weighed into separate reaction vessels. About 10 ml of concentrated phosphoric acid was carefully pipetted into the side arm of each reaction vessel. The vessels were attached to a separate vacuum line and outgassed at $\sim 10^{-6}$ Torr for 30 minutes prior to mixing, under vacuum, of the acid and adsorbent. The reaction vessels were then

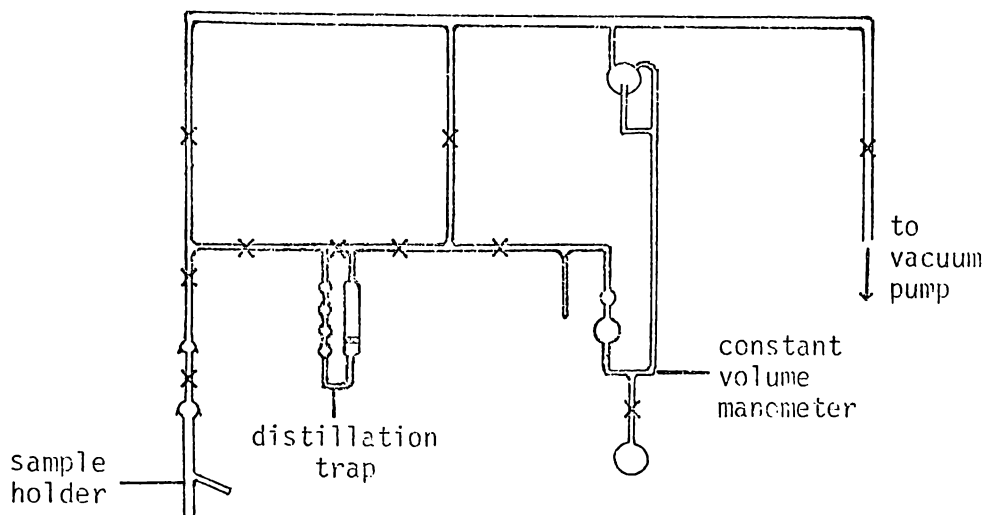


Figure 4.3: Schematic Representation of Vacuum Line Used for Carbonate Analysis.

attached to the vacuum line used for carbon dioxide determination and the reaction allowed to proceed for 1 hour.

The quantity of carbon dioxide evolved was determined in the following manner:

The vacuum line was evacuated to a pressure of $\sim 10^{-6}$ Torr and isolated. Gases evolved during the acid reaction were frozen into the liquid nitrogen cooled distillation trap by opening the tap on the reaction vessel. After about 10 minutes, the reaction vessel was isolated and non-condensables removed from the vacuum line system by re-opening the

line to the vacuum pump. Water was removed by distillation of the CO₂ into the cold finger of the constant volume manometer using an ethanol/n-propanol (1:1) and liquid nitrogen slush bath at -125°C on the distillation trap. The pressure of CO₂ was measured after the return of the manometer to room temperature. Results of the carbonate analysis are reported in Table 4.4.

Table 4.4: CARBONATE ANALYSIS OF ADSORBENTS

ADSORBENT	CARBONATE CONTENT/wt%
OHAp	0.03 ± 0.01
F-OHAp	0.03 ± 0.01
CO ₃ -OHAp	2.91 ± 0.01
ACP	0.95 ± 0.01

The carbonate analysis confirmed the effectiveness of procedures to minimise CO₂ contamination during the preparation of the non-substituted hydroxyapatite and its later conversion to a fluoride substituted product. The carbonate substituted material was observed to contain almost 3%, by weight, of carbonate. This approximates that commonly observed for the surface of tooth enamel, and is within the range observed for whole tooth enamel (Table 3.9).

4.3.8 FLUORIDE ANALYSIS

Beebe and Frankel (1968) have calculated the quantity of surface hydroxyl ions of the hydroxyapatite crystal to be about 2.2×10^{-6} mole (OH) m^{-2} for a crystal of mainly ac faces. The authors, however, did not take into account the fact that the ac crystal face may be terminated in either of two positions, only one of which has exposed hydroxyl ions (Section 3.6.5). If it is assumed that either of these faces is equally likely to be exposed, the average surface hydroxyl population will be about 2.6×10^{-6} mole (OH) m^{-2} . The figures for the surface hydroxyl population then agree well with the same authors' alternative calculation based on a random orientation of the units of the actual crystal surface. The surfaces of F-OHAp particles were seen to be comprised mainly of ac crystal faces (Plate 4.1(b)). An estimate of the fluoride content of the F-OHAp can therefore be made from the decrease in fluoride concentration of the NaF fluoridating solution, and the value of the surface hydroxyl population (2.6×10^{-6} moles (OH) m^{-2}).

A control experiment (Section 4.2.4) showed that the loss of fluoride from the NaF solution was due solely to fluoridation of the OHAp. The fluoride taken up is then given by the difference in initial and fluoride concentrations. A total of 2.09×10^{-4} mole was adsorbed. Taking into account the weight of OHAp (20.0g) and the specific surface area ($40 \text{ m}^2\text{g}^{-1}$), the fluoride uptake per mole of surface hydroxyl is calculated as 0.1. This corresponds to a surface fluoride concentration of 0.8% w/w which is slightly below the value of 0.9% w/w commonly observed for the surface of tooth enamel (Table 3.9).

4.4 SUMMARY

Three crystalline hydroxyapatite materials were prepared and characterised. These materials provide large surface area systems of a well defined structure similar to that of the mineral component of tooth enamel. The fluoride and carbonate substituted forms will allow the effect of substitution on protein adsorption to be determined. Amorphous calcium phosphate was prepared to investigate its role in the oral environment.

CHAPTER 5

PREPARATION AND CHARACTERISATION OF RADIOIODINATED PROTEINS

5.1 INTRODUCTION

Radiolabelled materials have found widespread use in medical and biological/biochemical sciences as tracers, particularly in radioimmunoassay work. Radionuclides of bromine, carbon, chlorine, cobalt, hydrogen, iodine, mercury, phosphorous, selenium, sulphur and technetium have been used. Two isotopes of iodine, Iodine-125 and Iodine-131, have found general acceptance as convenient labels for protein studies. Some of the factors pertinent to the production of a suitable tracer are discussed.

In any adsorption studies it is important to know the nature of the adsorbing species. Initially, only proteins which were readily available commercially and whose physico-chemical properties are reasonably well characterised were used. The preparation and characterisation of these proteins is described. Studies using a glycoprotein component obtained from saliva are described in Chapter 10.

5.2 THE USE OF RADIOIODINATED PROTEINS AS TRACERS

The use of any radiolabelled species as a tracer requires that the label is stable under the conditions it is used, and that the labelled and non-labelled species should be identical in nature and behaviour. For the purposes of the following discussion, a low degree of iodination will be taken to mean the case where, on average, less than one iodine atom is incorporated per protein molecule and a high degree of iodination when, on average, more than one iodine atom is incorporated.

Iodine can be incorporated into several of the amino acid residues of proteins e.g. tyrosine, methionine, tryptophan, phenylalanine, histidine and cysteine (Roche and Michel, 1948; Hughes and Straessle, 1950; Alexander, 1973; Krohn et al., 1977; Knight and Welch, 1978). Stable iodine labels result from the substitution of iodine into aromatic rings such as found in tyrosine, phenylalanine and tryptophan and the most stable of these is the tyrosine-iodine bond (Krohn et al., 1977). No exchange of the iodine label with free iodine has been observed (Eisen and Keston, 1949; Hughes, 1957) and hydrolysis only occurs at very low or high pH (Harrison and Garratt, 1970; Krohn et al., 1977). The label has been observed to be stable to storage at 4°C for periods of upwards of 2 weeks (van Enkevort, 1978; Nisbet, 1979), but storage at higher temperatures results in a more rapid release of the free iodide (Crandall et al., 1981). Generally, the predominant iodination reaction at low degrees of iodination and under conditions of neutral pH, room temperature, and mild oxidising agents involves the tyrosyl residues. The suggested mechanism for iodination is the substitution of an active electrophile, "I⁺", for hydrogen to form the mono-iodo derivative (Hughes, 1957). At high degrees of iodination a di-iodo derivative is formed and, particularly under harsh iodinating conditions or high pH,

other residues can be iodinated (Hughes and Straessle, 1950; Koshland et al., 1963; Perlman and Edelhoch, 1967; Seon et al., 1970; Krohn et al., 1977). A pH of 7.5 has been suggested to be optimal for the substitution of iodine into the tyrosyl residue (Freedlander, 1969a; Hunter, 1971).

The labelled species must be able to be separated from non-reacted label and other reaction products. Numerous methods are available e.g. acidification, precipitation, centrifugation, solvent extraction, dialysis, electrophoresis and gel filtration. The efficiency of separation can vary widely depending on the method used and the chemical form of the unbound iodine. Generally gel filtration methods provide excellent separation and are convenient to use.

It is important that the label be evenly distributed, or, at least, the distribution be known. The efficiency of labelling may be affected by physical factors which lead to a decreased reactivity through a masking of the group to be labelled, or affected by molecular factors which lead to differences in chemical reactivity. Physical factors have been suggested to account for observations that some monomeric proteins or protein sub-fractions are more easily iodinated than the polymerised species (Springell, 1962; Covelli and Wolff, 1966; Bocci, 1967; Marchalonis, 1969; van Enkevort, 1978). Similarly, Nisbet (1979) observed that κ -Casein was less readily iodinated than other casein components in mixtures of TS-A/R, α_s , β and κ -Caseins. The effect appeared to be due to incorporation of κ -Casein into the casein micelle in such a position that either the molecule itself was almost inaccessible, or that the tyrosine residues were not readily accessible. The chemical reactivity of the tyrosine residue may be affected by the nature of the amino acid groups it is attached to or surrounded by. Further, the incorporation of iodine into the protein molecule is also a statistical problem. This becomes important at tyrosines already

iodinated as the di-iodo derivative is more readily formed.

The incorporation of iodine into a protein molecule may affect its physico-chemical properties by direct changes in the mass or by changes in the charge or charge distribution. The latter effect may be observable solely as a charge effect, or through changes in the conformation of a molecule. The binding of an atom or molecule to an amino acid group will alter the pK_a value. Mono-substitution of tyrosine decreases the pK_a from 10.1 to 8.2 and disubstitution decreases this further to 6.4 (Edelhoch, 1962). Although the pK_a 's are affected by environments, the same trend is observed for the tyrosyl groups in proteins. For example, the respective pK_a values for tyrosyl, moniodotyrosyl and diiodotyrosyl of Thyroglobulin are 11.4, 9.3 and 7.3 (Wolf and Covelli, 1966). Hence, for the mono-substituted protein, any charge effects may only be apparent at higher pH's. Alterations of the physico-chemical properties will therefore be a function of the relative molecular mass, the nature of the protein, the extent of substitution and the solution conditions. Smaller labelled and unlabelled peptides have been separated solely by difference in charge (Freychet et al., 1971; Bolton and Hunter, 1973) but larger proteins, for example Caseins and Serum Albumin, often do not show these differences (Nisbet, 1979; Crandall et al., 1981). The titration behaviour of moniodinated Serum Albumin is only affected at high pH (Tanford, 1950). Generally, changes will become more apparent as the degree of substitution increases. For example, progressive increases in the degree of iodination of Serum Albumin increasingly affect the sedimentation and solubility behaviour and the ESR and UV spectra (Hughes and Straessle, 1950; Hughes, 1957; Perlman and Edelhoch, 1967; Kulakov et al., 1976), but the incorporation of a single iodine atom has little affect on the protein.

An increased degree of iodination also usually results in an increased rate of catabolism (McFarlane, 1956; Freeman, 1959; Bocci, 1967; Harwig et al., 1975; Knight et al., 1977) and an alteration of the immune response (Eisen and Keston, 1949; Johnson et al., 1960; Morrison et al., 1971). Providing that the degree of iodination is no greater than one, there is generally no change in the biological activity of the molecule (Rosa, 1976) or of the stability of the protein (Tanford, 1950; Perlman and Edelhoch, 1957).

An alteration of the physiochemical properties of a protein may also arise through the effect(s) of different chemicals used in the iodination reaction. Immunoreactivity has often been used as a criterion to judge the extent of denaturation incurred by iodination and the iodination procedure. In some cases however, immunoreactivity can be specific towards iodine (Bolton and Hunter, 1973). It has been suggested that exposure to oxidising and reducing agents is responsible for much of the denaturation observed in preparations of radioiodinated proteins (Hunter, 1970). Some methods of iodination produce more denaturation than others (e.g. Krohn et al., 1972; Reuter et al., 1976; Weech et al., 1978) and some proteins, especially hormones and enzymes, are also more susceptible to denaturation. Denaturation is often most obvious as aggregation or dissociation of a protein and a changed polymer distribution (Bocci, 1967; Sherman et al., 1974; Krohn et al., 1977; van Enckevort, 1978; Jackson, 1980), and this may be affected by the relative concentrations of the protein and these reagents. The sulphhydryl group of the cysteinyl residues are the first species to react with iodine, even during the mildest iodination reaction (Hung et al., 1974). The resultant oxidisation may affect the properties and biological activities of some proteins, not only by induced polymer formation, but also by subtle conformational changes. For example, the chromatographic behaviour of Serum Albumin on some gel filtration media is dependent on the presence of the sulphhydryl group and its nature (Section 3.6.2), and this behaviour is sensitive to the iodination conditions (Crandall et al., 1981). Similarly, the pore gradient gel electrophoresis patterns are

also affected (van Enkevort, 1978). Tryptophan residues may also be oxidised during iodination procedures and the resulting cleavage of the residue may cause denaturation. Oxidation can occur not only by added oxidising agents but also by iodine itself (Roche and Michel, 1948; Hughes and Straessle, 1950; Koshland et al., 1963). However, iodination at alkaline pH results in little or no oxidation of tryptophan (Alexander, 1973). Denaturation can be minimised therefore by the use of less harsh or reduced quantities of reagents, and by different iodination conditions.

Proteins may be damaged by radiolysis. This is probably not important however until 30 - 80 mCi of radioiodide is used in the labelling procedure.

It has been also suggested that protein molecules can be damaged by exposure to 'noxious' impurities in isotope solutions (Hunter, 1971; Rosa and Malvano, 1973).

The production of a suitable tracer is therefore favoured by the use of a labelling procedure in which the degree of iodination is kept as low as possible (favoured by the use of carrier-free methods), in which exposure of the protein to radioiodine solutions and chemical reagents is avoided or at least minimised, and in slightly alkaline conditions. Denaturation will be dependent on the sensitivity and nature of the protein, particularly as regards the presence and location of 'active' residues. Also, what constitutes a change in behaviour, and how deviant that behaviour must be before experimental results lose their validity, will be dependent on how important any property is to the phenomenon under investigation.

5.3 SURVEY OF METHODS AVAILABLE FOR IODINATION

5.3.1 INTRODUCTION

There are now many methods available for the iodination of proteins. These have been developed in the search for simple, efficient and reliable methods that also cause the least possible damage to the protein molecule during the labelling procedure. Labelling may be achieved either by the direct incorporation of the radioiodine or by the indirect method of incorporation of a previously labelled molecule.

5.3.2 METHODS

Methods of indirect substitution offer the advantages that they avoid contact between the protein and the radioiodine, and provide a method for the iodination of proteins without tyrosine groups or that are particularly sensitive to sulphydryl oxidation. Iodination is accomplished using previously labelled molecules containing an aromatic ring as this is most stable (Hughes, 1957; Bolton and Hunter, 1973; Hayes and Goldstein, 1975; Wood et al., 1975). However, the process involves many steps to give the labelled molecule before conjugation to the protein is possible, the labelled molecule is often not very stable under the conditions required for conjugation, bonding is often not confined to a specific amino acid or very stable, the efficiency of labelling is usually low, and a molecular species even larger than iodine is introduced into the protein.

A number of methods for direct radioiodination are available:

The iodide-iodate method of Hughes and Straessle (1950) and its various modifications. The protein is exposed to chemical oxidants,

maximum efficiencies of iodination of only 40% are observed, and it is not a carrier-free method.

The iodine monochloride method of McFarlane (1958) and later improved by Helmkamp et al. (1967). Although iodination efficiencies of greater than 70% can be obtained, these decrease as the amount of protein used falls below 2.5 mg. The method requires the use of carrier iodide and this results in the production of a lower specific activity product. Exposure of the protein to oxidants is not avoided.

The electrolytic method of Rosa et al. (1964) avoids exposure of the protein to chemical oxidants as it involves the electro-oxidation of iodide to iodine. The method is particularly gentle and high iodination efficiencies can be obtained. However, amino acid residues other than tyrosine are labelled even at low degrees of substitution (Jovanovic et al., 1972), and the method is not suitable as a microtechnique as large volumes of solution are required. The product is of low specific activity. Although longer iodination times can be used to give a product of higher specific activity, these usually result in some denaturation.

Enzymic oxidation of iodide by peroxidase enzymes (Marchalonis, 1969; Bayse et al., 1972) is a gentle technique. Although iodination efficiencies of up to 90% have been obtained, protein susceptibility to iodination by this method is very variable. Further, the enzymes are prone to self-iodination and contaminate the final labelled product. Methods using enzymes bound to solid substrates are now available and these avoid contamination of the sample by foreign protein (David, 1972; David and Reisfeld, 1974). However, the iodination efficiency is low in carrier-free iodinations, significant self-iodination (up to 20%) is still a problem, and the optimum pH of iodination (pH 6.5 - 7.0) is below that preferred for tyrosine (pH 7.5).

The Iodo-Gen method of Fraker and Speck (1978) employs a solid phase

oxidant. Iodo-Gen is suggested to be a less harsh oxidising agent than Chloramine-T and the reaction is terminated by removal of the protein solution from the oxidant rather than by the addition of a reducing agent. Iodination efficiencies of up to 60% can be achieved, but the rate of reaction is slow and reaction times of 10 minutes or more are required.

The Chloramine-T method of Greenwood et al. (1963) and its modifications allows iodination at efficiencies of greater than 70% and to high specific activity at low degrees of labelling. The method was developed as a microtechnique, allows the use of carrier free radioiodine and is convenient and rapid to use. Exposure of the protein to oxidants is not avoided. The technique, in a modified form, was regarded as the most suitable method for routine iodinations of BSA (van Enckevort, 1978; Dass, 1982) and Caseins (Nisbet, 1979) for use as tracers in adsorption studies on various surfaces. A labelled product of high specific activity but low degree of labelling could be produced quickly, at high iodination efficiencies, and at a pH close to that considered optimal for incorporation of radioiodine in the tyrosyl residues of the proteins. This method was therefore chosen for the studies of this thesis.

5.3.3 ASPECTS OF THE CHLORAMINE-T METHOD OF IODINATION

The original Chloramine-T method of Greenwood et al. (1963) used a high ratio of Iodine : protein. The iodinated protein molecule consequently contained about 17 atoms of iodine on average (for the iodinations at 80% efficiency), a level of iodination far above that suggested to avoid significant alteration of the physico-chemical properties of proteins. Successful iodinations of high efficiency can be

achieved at lower iodine : protein ratios however (Bocci, 1964 and 1969; van Enkevort, 1978; Nisbet, 1979), and this also minimises the possibility of radiolysis damage during the iodination reaction or in the radioiodinated product.

Many proteins are denatured or altered by exposure to oxidising and reducing agents. The use of these in iodination reactions therefore requires closer examination. Much criticism has been levelled at the use of Chloramine-T as an oxidant as it has been suggested to be a particularly harsh oxidant, and some proteins form macromolecular complexes as a result of exposure to it (e.g. Bocci, 1967; Krohn et al., 1972) while others are dissociated (Jackson, 1980). However, the susceptibility to complex formation appears to be dependent on the protein type and the Chloramine-T : protein ratio. Serum Albumins are less easily aggregated than Fibrinogen (Lee et al., 1977) and even different Fibrinogens display different degrees of denaturation (Harwig et al., 1975). Lipoproteins are more aggregated by the Iodine monochloride method than by Chloramine-T (Weech et al., 1978). The original iodination method of Greenwood et al. also used a high Chloramine-T : protein ratio of 20 : 1. Reductions of this ratio still allow successful iodinations however (e.g. Bocci, 1964 & 1969; Sherman et al., 1974; van Enkevort, 1978; Nisbet, 1979). Although Chloramine-T has an adverse effect on the sulphhydryl group of Serum Albumins, this effect is associated with all methods of iodination and does not appear to affect the validity of the use of the labelled product as a tracer in adsorption studies (van Enkevort, 1978; Dass, 1982).

The Chloramine-T method requires the use of a reducing agent to halt the iodination reaction. Although exposure of a protein may cause damage to the protein molecule, the effect of exposure to oxidants might also be reversed by this treatment.

5.4 MATERIALS AND METHODS

5.4.1 INTRODUCTION

The radiodination of the proteins used in this work was achieved either by a modified version of the Chloramine-T method of Greenwood et al. (1963) in which a Chloramine-T : protein ratio of 1:1 is used, or by the Iodo-Gen method of Fraker and Speck (1978) at an Iodo-Gen : protein ratio of 0.1:1 when the Chloramine-T method proved too harsh. The detailed compositions of all buffers used in the iodination reactions are given in Appendix VI, and the cleaning procedures used for all glassware in Appendix II.

5.4.2 CHOICE OF ISOTOPE

Radiochemical properties of the isotopes Iodine-125 and Iodine-131 are given in Appendix VII. Iodine-125 was the preferred isotope for the work envisaged as it offered the following advantages:

- (i) materials labelled with this isotope are generally more stable than those labelled with Iodine-131 (Evans, 1976).
- (ii) The higher isotope abundance allows the preparation of tracers of high specific activity at lower degrees of labelling.
- (iii) The longer half-life is more convenient as it allows repeated use of stock solutions and makes decay corrections unnecessary.
- (iv) The γ -radiation of this isotope can be efficiently detected by both solid and liquid scintillation counting techniques.
- (v) The softer radiation requires less shielding for radiation safety reasons and when counting defined areas on solid surfaces.

5.4.3 MATERIALS

All solutions and materials were prepared immediately prior to use. Buffers for protein solutions were filtered through millipore filters (0.05 μm pore size). During iodination reactions, solutions were dispensed using Eppendorf micro-pipettors. All iodinated protein preparations were stored at 4°C and generally used within 7 days to prevent possible hydrolysis or bacterial attack.

(i) Buffers: Cacodylate - HCl Buffer : pH 6.8, I 0.06 mole l^{-1}

Barbital - HCl Buffer : pH 7.4, I 0.07 mole l^{-1}

As the need to increase buffering capacity when labelling with solutions of radioiodine has been reported (Freedlender, 1969b), a separate iodination buffer was used. This was a barbital buffer of pH 7.4, as above, but composed of twice the concentration of its component solutes.

(ii) Chloramine-T: 0.10 g Chloramine-T dissolved in 25 ml barbital buffer.

(iii) Sodium Metabisulphite: 0.12 g sodium metabisulphite dissolved in 25 ml barbital buffer.

(iv) Protein Solution: 0.1 g protein dissolved in 25 ml cacodylate buffer (unless stated otherwise).

(v) Iodo-Gen Solution: 0.0100 g of Iodo-Gen dissolved in 10 ml of methylene chloride (CH_2Cl_2).

(vi) Radioiodine: usually less than 0.2 mCi (74 MBq) Iodine-125 used per iodination.

(vii) Gel Filtration Column: 1.0 g Sephadex-G25 (fine) equilibrated overnight in cacodylate buffer was washed into a glass column previously plugged with a small amount of glass wool. The final gel column dimensions were about 100 mm x 10 mm.

5.4.4 THE CHLORAMINE-T METHOD OF IODINATION

(i) To a small test tube (0.4 ml) containing the Iodine-125 was added 0.100 ml of the barbital iodination buffer and the solution thoroughly mixed using a fine glass stirring rod.

(ii) 0.025 ml of protein solution was added to the Iodine-125 solution and thoroughly mixed. 0.025 ml of the Chloramine-T solution was then added and the reaction allowed to proceed for a pre-determined time with constant gentle stirring. During this time the iodine is oxidised by the Chloramine-T to a higher oxidation state and reacts with the protein. The reaction was halted by the addition of 0.100 ml of the sodium metabisulphite solution which reacts with the Chloramine-T and reduces any unreacted iodine back to iodide.

(iii) Separation of the iodinated protein from the unreacted iodide was achieved by gel filtration using the Sephadex-G25 column. The iodination reaction mixture (0.250 ml) was transferred to the column and the reaction test tube rinsed with 3 x 0.25 ml washings of cacodylate buffer (total 1.0 ml). Each successive addition was allowed to drain to the top of the gel column prior to application of the next aliquot. In this manner, repeated additions of 1.0 ml volumes of buffer were added to and collected from the column until 8 x 1 ml fractions were eluted. The protein fraction was eluted as the third fraction and the unreacted iodide as the sixth fraction. The efficiency of iodination was calculated as the percentage of radioiodine bound to the protein to the total radioiodine eluted in the 8 fractions.

5.4.5 THE IODO-GEN METHOD OF IODINATION

(i) 0.010 ml Iodo-Gen stock solution was added to a small test tube (0.4 ml) containing 0.040 ml methylene chloride, the solution mixed and then evaporated to dryness under vacuum. A thin film of Iodo-Gen is thus plated in the bottom of the reaction vessel and acts as a solid phase oxidant.

(ii) 0.025 ml of a barbital iodination buffer containing Iodine-125 and 0.025 ml of protein solution were added to the reaction vessel. This initiates the iodination reaction. The reaction was allowed to proceed for 10 - 15 minutes with periodic gentle mixing using an Eppendorf micropipettor. The reaction was terminated by removal of the sample from the reaction vessel.

(iii) Separation of the iodinated protein from the unreacted iodide was achieved by gel filtration in a manner similar to that used in the Chloramine-T iodination procedure (Section 5.4.4)

5.4.6 RESULTS AND DISCUSSION

(A) CHLORAMINE-T IODINATIONS

(i) Bovine Serum Albumin and β -Casein

The routine iodination of Bovine Serum Albumin (BSA) and β -Casein by the modified Chloramine-T method has been well described previously (van Enkevort, 1978; Nisbet, 1979; Dass, 1982). Generally, the results obtained were similar to those obtained in these studies i.e. iodination efficiencies of 60 - 80% after a reaction time of 45 seconds. This corresponds to an average substitution of one atom Iodine per 20 molecules BSA and one atom Iodine per 50 molecules β -Casein, assuming the iodination of 100 μ g protein (M_r (BSA) = 66 210, M_r (β -Casein) = 23 983) and the use of 0.2 mCi Iodine-125 (~ 16 mCi/ μ g Iodine) at 80% efficiency.

(ii) Human Serum Albumin

A preliminary experiment was performed to test the kinetics of iodination of Human Serum albumin (HSA). The results are shown in Figure 5.1 where it can be seen that the degree of labelling increased rapidly up to about 20 seconds after the addition of Chloramine-T. This was then followed by a slow increase in the degree of labelling. These results parallel those observed for the iodination of BSA (van Enkevort, 1968) and, consequently, the 45 second reaction period used routinely in the iodination of BSA and β -Casein was also used for HSA. An average substitution of one atom Iodine per 20 molecules HSA ($M_r = 66\,248$) is calculated.

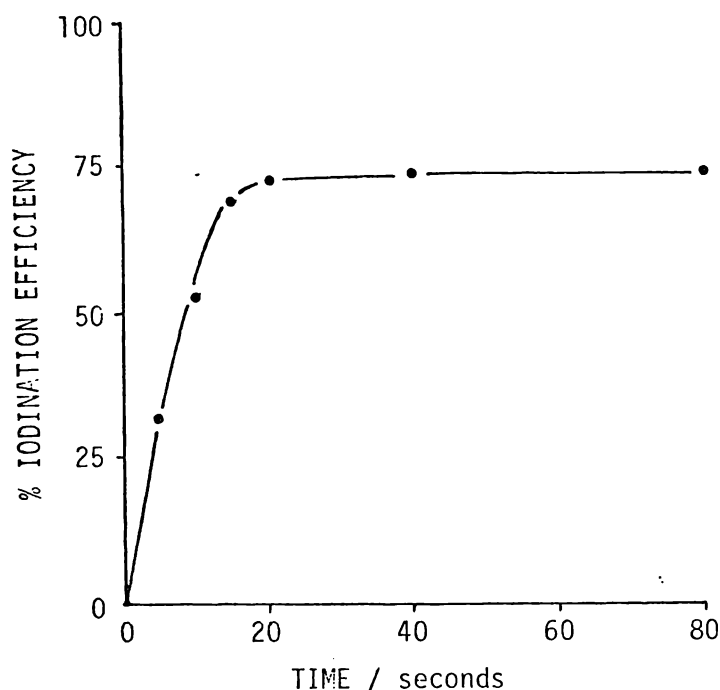


Figure 5.1: Kinetics of Iodination of HSA: The Effect of Reaction Time on Iodination Efficiency.

(iii) Other Proteins

The proteins Haemoglobin, Myoglobin, Ovalbumin, Lysozyme and β -Lactoglobulin are found in most diets and are readily available commercially. These proteins all contain tyrosine groups and are therefore able to be iodinated by direct methods.

Upon addition of Chloramine-T to the solutions of Haemoglobin, Myoglobin and Lysozyme, the solutions rapidly became turbid. This suggested oxidation of the protein and consequent denaturation and aggregation had occurred. The addition of the reducing agent sodium metabisulphite to the Myoglobin and Lysozyme solutions reversed the effect of Chloramine-T addition to some extent. The Haemoglobin solution became even more turbid however. Particularly low levels of radioactivity were observed in the 3rd fractions collected from the gel-filtration columns used for these proteins. Those columns used for Haemoglobin and Myoglobin, both strongly coloured proteins, were noticeably discoloured at the top of the gel column. A scan of each column with a hand-held geiger counter showed intense levels of activity for all three proteins at this position. Those columns used for Ovalbumin and β -Lactoglobulin showed no positions of intense activity and less retained activity overall. This was reflected by the higher levels of activity in the 3rd fractions collected from these columns.

A semiquantitative, non-radioactive experiment was therefore performed to assess the loss of protein through denaturation and aggregation in the iodination procedure. Equal amounts (and volumes) of protein before and after subjection to the iodination procedure were passed down a gel filtration column. The column effluent was monitored by spectrophotometry at 280 nm and the peak heights observed for the 3rd ml fractions compared to estimate the percentage loss of protein.

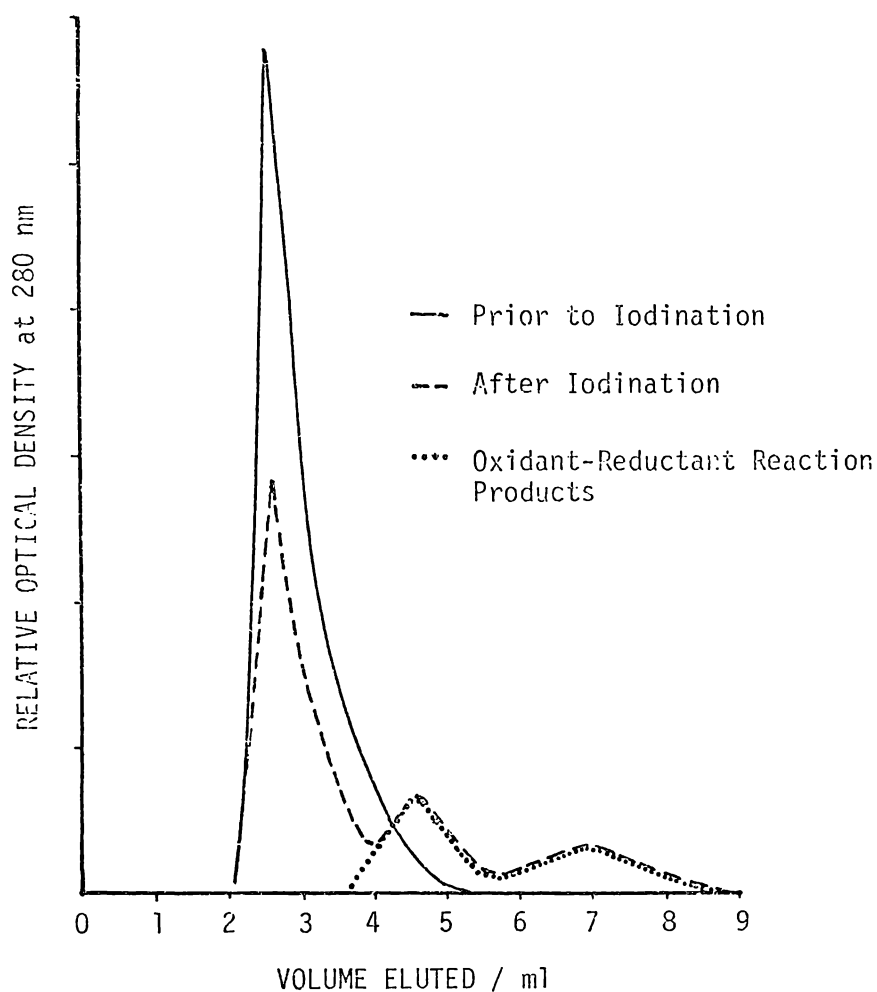


Figure 5.2: Loss of Protein by Aggregation During Iodination:
The Effect of Chloramine-T on Haemoglobin Recovery.

TABLE 5.1: LOSS OF PROTEIN BY AGGREGATION DURING IODINATION BY
CHLORAMINE-T

Protein	%Loss
Haemoglobin	51
Myoglobin	25
Lysozyme	51
Ovalbumin	12
β -Lactoglobulin	10

A typical spectrum and the results obtained are shown in Figure 5.2 and Table 5.1 respectively. All the proteins studied were irreversibly aggregated and suffered some loss of material upon gel filtration. Ovalbumin and β -Lactoglobulin were least affected and showed about 10% loss of protein, whereas Haemoglobin and Lysozyme suffered a greater than 50% loss of material. It was therefore decided to attempt iodinations of all these proteins by the Iodo-Gen method.

(B) Iodo-Gen Iodinations

The Iodo-Gen method of protein iodination utilises an oxidant which is supposedly more gentle than Chloramine-T and is used in the solid phase. Exposure of protein to the oxidant can be avoided, therefore, by iodinations in which the iodination buffer/Iodine-125 mixture is exposed to the oxidant and then transferred to the protein/buffer solution. The following preliminary experiment was carried out using the proteins BSA and β -Casein as these could be successfully iodinated by the Chloramine-T procedure.

The iodination buffer/Iodine-125 mixture was exposed to Iodo-Gen in a prepared reaction vessel for 1 minute with gentle mixing (to avoid displacement of solid Iodo-Gen from the vessel walls). The contents were transferred to another test tube containing the protein solution and gently mixed for 2 minutes. The mixture was then applied to the gel filtration column and eluted in the usual manner. An experiment in which the iodination buffer/Iodine-125 and protein were simultaneously exposed to Iodo-Gen provided a suitable control. Whereas iodination efficiencies of 65% and 60% were achieved for BSA and β -Casein, respectively, in the control iodination, iodination efficiencies of less than 5% were obtained in the experiment in which exposure of the protein to the Iodo-Gen was

avoided. Iodination appeared to require the simultaneous presence of oxidant, iodide and protein. An attempt was therefore made to iodinate the proteins Haemoglobin, Myoglobin, Lysozyme, Ovalbumin and β -Lactoglobulin in this manner (i.e. the method as described in Section 5.4.5). The reaction mixtures of Haemoglobin, Myoglobin and Lysozyme were again observed to become turbid during the iodination reaction. The Ovalbumin and β -Lactoglobulin reaction mixtures remained clear and were subsequently passed down gel-filtration columns. A geiger counter scan of both columns revealed a higher residual activity in that used for Ovalbumin and it was therefore decided to continue work with β -Lactoglobulin.

(i) β -Lactoglobulin

Iodination efficiencies of about 15% were obtained for β -Lactoglobulin after an iodination reaction time of 10 minutes. β -Lactoglobulin exists in solution as a dimeric species in dynamic equilibrium with the monomer. The dimer predominates in the pH range 3.5 - 7.5 (McKenzie, 1971b). Polymerisation may result in the blocking of tyrosine residues normally available for iodination and reduce iodination efficiencies. Such an effect has been observed to account for the ease of iodination of γ -G Immunoglobulin, which exists as a monomeric species, relative to the γ -M and γ -A Immunoglobulins which exist as polymeric species (Marchalonis, 1969). However, the tyrosine residues of β -Lactoglobulin appear to be generally less reactive for reasons other than masking by polymerisation. It was therefore decided to increase the reaction time to 1 hour, at 4°C, after which time an increase in the iodination efficiency to 50% was observed. An average substitution of one atom Iodine per 120 molecules β -Lactoglobulin ($M_r = 18\ 000$) is calculated.

5.4.7 CONCLUSION

The Chloramine-T method of iodination in which the Chloramine-T : protein ratio was decreased to 1:1 has previously been used in studies at this University to label Bovine Serum Albumin (van Enkevort, 1978; Dass, 1982) and β -Casein (Nisbet, 1979). The method provided a suitable tracer for adsorption studies in several systems and hence was chosen as most suitable for the routine iodinations of these proteins and Human Serum Albumin for use in this study. The labelled material could be prepared at high levels of iodination efficiency and to high specific activity at low degrees of labelling. An average substitution of 0.05 atom Iodine per molecule Serum Albumin and 0.02 atom Iodine per molecule β -Casein was obtained and this was well below that which has been suggested to avoid alteration of the physico-chemical properties and biological activity of the proteins. Further, at this level of substitution, all the label should exist as the mono-iodo tyrosine derivative and provide the most stable label.

Attempts were also made to iodinate the proteins Haemoglobin, Myoglobin, Lysozyme, Ovalbumin and β -Lactoglobulin by the modified Chloramine-T method. These proteins were extensively damaged by the procedure, however, and labelling by the potentially less harmful Iodo-Gen method was attempted. There was some evidence that the method was in fact less damaging and that β -Lactoglobulin suffered the least damage. Consequently, studies with this protein were continued in the hope that the labelled preparation might still provide a useful tracer. β -Lactoglobulin was less efficiently iodinated than the Serum Albumins or β -Casein. This appeared to be a kinetic effect. Extension of the reaction time to 1 hour increased the efficiency to about 50%. An average substitution of less than 0.01 atom Iodine per molecule protein

was obtained.

Successful iodination of the proteins Ovalbumin, Haemoglobin, Myoglobin and Lysozyme might be achieved using a technique which uses a more gentle oxidising agent e.g. the enzymatic method or by a method which avoids the use of an oxidising agent e.g. the electrolytic method. However, neither method gives a successful product suitable for adsorption studies. Both methods result in the iodination of amino acids other than the tyrosine residue. The electrolytic method gives a product of too low specific activity, and the possibility of protein exchange phenomena in the use of enzymes bound to solid substrates in the enzymic method has not been excluded.

5.5 CHARACTERISATION OF LABELLED AND NON-LABELLED PROTEINS

5.5.1 INTRODUCTION

A critical level of iodination known to avoid any significant alteration of the physico-chemical properties of protein molecules and to ensure the most stable protein-iodine bond has been shown in Section 5.2 to be one atom Iodine per protein molecule. Protein iodinated to less than this level (Section 5.4) was characterised to determine the effect of labelling on the polymer distribution, the uniformity of labelling over the polymers, and the stability of the label to storage.

Electrophoretic and column chromatographic techniques are useful in the characterisation of proteins. Electrophoretic techniques offer the advantages of high sensitivity and resolution, and are relatively inexpensive and simple to use. Polyacrylamide gels, as used in polyacrylamide gel electrophoresis, can be conveniently sectioned and dissolved in liquid scintillation cocktails if required. Protein bands can be fixed and stained for visualisation, and subsequently quantified by densitometry.

The stability of the labelled protein to storage is simply tested by gel-filtration in which the protein is separated from the free iodide. The procedure used was that described for the separation of iodinated protein from non-reacted iodide after iodination (Section 5.4.4).

5.5.2 BOVINE AND HUMAN SERUM ALBUMIN

It has previously been shown that significant dissociation of the carbon-iodine bond of radioiodinated BSA, labelled by the modified Chloramine-T method and subsequently stored in cacodylate buffer at 4°C, does not occur until after about 14 days storage (van Enkevort, 1978).

Serum Albumin polymers do not exist in a state of dynamic equilibrium with the monomeric species but are non-reversibly formed as a result of sample preparation techniques and storage (Section 3.6.2). BSA can be readily separated into its polymeric fractions by pore gradient gel electrophoresis (van Enkevort, 1978). As BSA and HSA are very similar proteins (Section 3.6.2), the technique should also prove useful in the characterisation of HSA. Pore gradient gel electrophoresis employs a polyacrylamide gel having a continuous gradient of decreasing pore size. The molecules are forced to migrate through the gel until they attain a position of quasi-equilibrium. This position is determined by the dimensions of the molecule relative to the local pore size (Morris and Morris, 1976). The polyacrylamide gels can be sectioned and subsequently solubilised in a liquid scintillation cocktail to enable the location of radioactivity.

MATERIALS

- (i) Electrophoresis Buffer: Cacodylate buffer, pH 6.8, I 0.06mole l⁻¹.
- (ii) Pore Gradient Electrophoresis Gels: Premade gels are available commercially. These were rinsed with distilled water after removal from the storage solution.
- (iii) Fixing and Staining Solution: Samples were fixed and stained after electrophoresis using a 0.025% Coomassie Blue Dye in a methanol : acetic acid : water solution of ratio (v/v) 25 : 7 : 68.
- (iv) Destaining Solution: A solution of methanol : acetic acid : water

in ratio (v/v) 25 : 7 : 68.

METHODS

- (i) Protein Samples: Four samples of each protein were required:
- (a) the standard protein
 - (b) sample (a) after gel filtration
 - (c) sample (a) after subjection to the iodination procedure (non-radioactive)
 - (d) sample (a) after radioiodination.

Samples a, b, and c provided information on the polymer distribution of the protein and the effects of gel-filtration and exposure to oxidising and reducing agents. These were stained after electrophoresis to enable visualisation and quantitation of bands. Sample (d) provided information on label distribution. It was left unfixed to avoid loss of the radioiodine label in highly acidic solutions, and unstained to avoid dye quenching effects during liquid scintillation counting. The samples were prepared in cacodylate buffer (pH 6.8, I 0.06 mole l⁻¹) as follows and 'weighted' with the addition of glycerol (10% v/v) prior to electrophoresis. Samples containing about 10⁻⁴g Serum Albumin were known to give good resolution without overloading (van Enkevort, 1978). Sample a: 0.010 g protein in 1 ml buffer. Samples containing 1 x 10⁻⁴ and 2 x 10⁻⁴g protein were applied to the gel.

Sample b: 0.050 g protein was dissolved in 1 ml buffer. 0.1 ml of this solution was then passed down a gel filtration column, as used during the iodination procedure (Section 5.4.4), and the 3rd 1 ml fraction collected. 20 µl (1 x 10⁻⁴g protein) of this solution was applied to the gel.

Sample c: 0.015 g protein was dissolved in 1 ml buffer and 0.025 ml of a 5.9 x 10⁻⁴ mole l⁻¹ NaI solution added. This gave a protein : iodide ratio similar to that used during the normal iodination procedure.

0.025 ml of a Chloramine-T solution ($1.5 \times 10^{-2} \text{g ml}^{-1}$) was added to 0.025 ml of the protein/iodide solution and allowed to react, with gentle mixing, for 45 seconds. 0.025 ml of a sodium metabisulphite solution ($7.2 \times 10^{-2} \text{g ml}^{-1}$) was then added. Although the reaction volumes are different from those used in the normal labelling procedure, the oxidant : protein : reductant ratio has been maintained. 20 μl ($1 \times 10^{-4} \text{g}$ protein) of this solution was applied to the gel.

Sample d: as for sample c except that 1 μCi Iodine-125 in 5 μl buffer was added to the 0.025 ml volume of protein solution prior to the addition of Chloramine-T. 20 μl ($1 \times 10^{-4} \text{g}$ protein) of this solution was applied to the gel.

(ii) Electrophoresis: Electrophoresis was conducted at an ambient temperature of 4°C . The gel was pre-run at 15 mA for 30 minutes prior to sample application. Samples were applied to the gel via a sample spacer inserted to a depth of 1 mm into the gel. A constant current of 15 mA was applied for 48 hours as this was known to give good resolution of BSA (van Enckevort, 1978). After electrophoresis, the gel was cut to separate samples a, b, and c for each protein from sample d.

(iii) Determination of Polymer Distribution: The gel containing samples a, b, and c was fixed and stained for at least 3 hours. It was destained by immersion in destaining solution, at 4°C and with several changes of solution, until the areas of gel surrounding the bands were free of stain. The polymer distribution was determined by the analysis of a densitometer scan of the gel after destaining. An integrating facility was incorporated in the scanning densitometer and this allowed an approximate quantitative determination of the amounts of material in each electrophoretically resolved fraction.

(iv) Determination of Label Distribution: The gel containing sample d was immediately sectioned into 2 mm strips across the gel. Sections were

placed in liquid scintillation vials and 10.0 ml of Aquasol-2 added. The vials were tightly sealed and placed in an oven at 35°C until the sections had dissolved. The solutions were allowed to cool to room temperature before counting.

RESULTS AND DISCUSSION

Radioiodinated HSA, like radioiodinated BSA, was observed to be stable to storage at 4°C in cacodylate buffer for about 14 days before significant breakdown of the carbon-iodine bond occurred.

A photograph of the electrophoresis gel after staining and destaining is shown in Plate 5.1, and the polymer and label distribution reported in Table 5.2. Both BSA and HSA contained a high polymer content. The monomeric species contributed about 50% of the total protein. Although gel filtration did not alter the polymer distribution, this was altered during the iodination procedure. The amount of tetrameric (M_4) and pentameric (M_5) material was reduced by about 50% for both proteins, and there was a resultant 10% overall increase in the amount of monomer (M_1) and dimer (M_2). The differences appear to be due to association and dissociation of the polymeric fractions by the use of the oxidising and reducing agents in the iodination procedure as gel filtration did not affect the polymer distribution. Protein aggregation through the use of Chloramine-T is well known (see Section 5.3.3) and reducing agents are known to cause some dissociation of polymeric BSA (Hartley et al., 1962; Andersson, 1966; Franglen, 1974). Presumably a similar effect might also apply to the higher polymeric fractions and to HSA, and therefore account for the changes in polymer distribution of both these proteins.

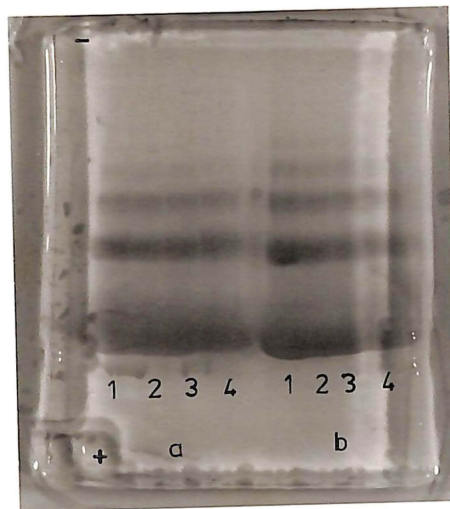
The label appeared to be evenly distributed, within experimental

error, over the various polymeric fractions. However, there did appear to be a slight decrease in labelling efficiency of the polymers of higher order than the dimer. A similar effect has been reported in previous studies of the iodination of BSA (Bocci, 1967; van Enkevort, 1978). Aggregation of monomeric molecules presumably results in a blocking of the tyrosine residues normally available for iodination.

It has previously been observed that some separation of various BSA fractions according to the nature of their sulphydryl group can be achieved by pore gradient gel electrophoresis (van Enkevort, 1978). Although this separation is not readily apparent in Plate 5.1, it was observed for both BSA and HSA in this work and is more apparent in the densitometer scans of the gel. A comparison of the scans obtained for HSA before and after subjection to the iodination procedure is shown in Figure 5.3. The shoulders observed on the monomeric and dimeric fractions of the protein sample before iodination were absent after this procedure. Similar results were observed for BSA. Their disappearance is consistent with the expected alteration of the sulphydryl group during iodination. Analysis of the integrator outputs associated with the densitometer scans showed that less than 12% of the BSA and 9% of the HSA preparations were affected.

PLATE 5.1

PORE GRADIENT GEL ELECTROPHORESIS OF
BOVINE AND HUMAN SERUM ALBUMIN



Key: (a) Bovine Serum Albumin

(b) Human Serum Albumin

Sample 1: Standard, 0.2 mg

2: Standard, 0.1 mg

3: Standard after gel filtration, 0.1 mg

4: Iodine labelled, 0.1 mg

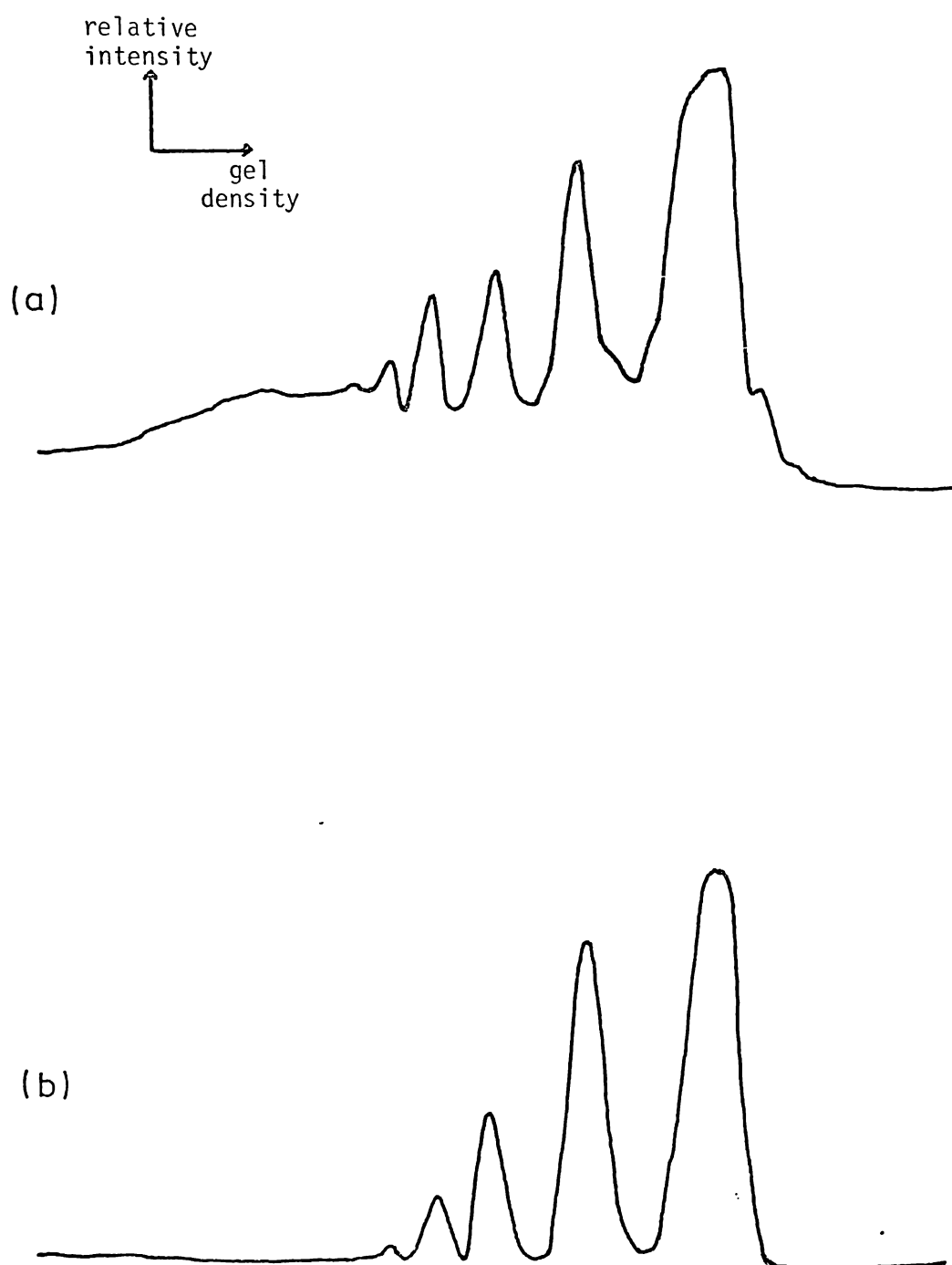


Figure 5.3: Densitometer Scans of HSA after Pore Gradient Gel Electrophoresis: (a) Before Iodination
(b) After Iodination.

TABLE 5.2: POLYMER AND LABEL DISTRIBUTIONS OF SERUM ALBUMINS

POLYMER FRACTION	POLYMER DISTRIBUTION/%			LABEL DISTRIBUTION/%	RATIO ^(a)
	Control	After Gel Filtration	After Iodination		
<u>BOVINE SERUM ALBUMIN</u>					
M ₁	47	48	54	55	1.0
M ₂	27	27	28	30	1.1
M ₃	16	16	14	12	0.9
M ₄	8	7	4	3	0.8
M ₅	2	2			
<u>HUMAN SERUM ALBUMIN</u>					
M ₁	51	49	54	56	1.0
M ₂	22	23	27	28	1.0
M ₃	13	14	12	10	0.8
M ₄	10	10	6	5	0.8
M ₅	4	4	1	1	(1)

^a Calculated as %Label/%Polymer

5.5.3 β -CASEIN

β -Casein can exist in solution as a monomeric species or as a polymeric (micellar) species in dynamic equilibrium with the monomer. Whether the polymeric species are formed is determined by various chemical and physical factors (Section 3.6.3). β -Casein has previously been used in protein adsorption studies, using a radiochemical tracer method similar to that used in this work, by Nisbet (1979). He showed by

column chromatography that iodination by the Chloramine-T method at the low Chloramine-T : protein ratio also used in this thesis did not cause irreversible aggregation of β -Casein. Further chromatographic and electrophoretic tests did not distinguish between the non-labelled and labelled species. The label was observed to be stable to storage, at 4°C in cacodylate buffer, for 15 - 20 days. Further characterisation for the purposes of this thesis was not felt necessary.

5.5.4 β -LACTOGLOBULIN

β -Lactoglobulin, like β -Casein, exists in solution as an equilibrium mixture of monomeric and polymeric species and the position of the equilibrium is determined by several factors (McKenzie, 1971b). Iodination by the Chloramine-T procedure produced some irreversible polymerisation and aggregation and it was of interest to know if iodination by the Iodo-Gen method did likewise.

The equilibrium between monomeric and polymeric species complicates separation procedures. However, polymer formation in some equilibrium situations can be prevented by the use of detergent molecules such as sodium dodecyl sulphate (SDS). SDS also causes dissociation of polymers held together by physical interactions. The binding of SDS by proteins is reversible but, in the presence of excess SDS, the amount taken up is sufficiently large to swamp the intrinsic charge of the protein. These observations form the basis of SDS-gel electrophoresis. Electrophoresis is conducted using polyacrylamide gels containing SDS, and provided that the gel has sufficient molecular-sieving properties, the monomeric and irreversibly formed polymeric protein fractions can be separated.

CHOICE OF GEL

Polyacrylamide gels are prepared by co-polymerisation of acrylamide ($\text{CH}_2\text{CHCONH}_2$) with a cross-linking agent such as N.N'-methylene-bisacrylamide ($(\text{CH}_2\text{CHCONH})_2\text{CH}_2$). Gel compositions are conveniently described by the notation T x C where T denotes the total weight of acrylamide monomer per 100 ml solution, and C denotes the amount of bisacrylamide expressed as a percentage (w/w) of the total amount of monomers. Both T and C can be varied to obtain a wide range of gel porosities. It was thought a 10 x 2.6 gel would be suitable for this study. (A detailed description of the theory and practice of gel electrophoresis can be found in Morris and Morris (1976)).

MATERIALS

- (i) Electrophoresis Buffer: Cacodylate buffer, pH 6.8 (I 0.06 mole l^{-1}) containing 1 g l^{-1} SDS.
- (ii) SDS-Polyacrylamide Gels: Gels of composition 10 x 2.6 were prepared as described in Appendix VIII.
- (iii) Fixing/Staining and Destaining Solutions: Samples were fixed and stained after electrophoresis using the solutions prepared as for Serum Albumin electrophoresis (Section 5.5.2).

METHODS

- (i) Protein Samples: Protein samples were prepared similarly as described for Serum Albumin (Section 5.5.2). The Iodo-Gen method of iodination was used to prepare samples to investigate the effects of contact with the oxidising agent and labelling. SDS was added to each sample to a concentration of 0.01 g ml^{-1} and incubated at 37°C for 2 hours prior to weighting with glycerol and application to the gel. A 4th sample, incorporating ~2 μl of a 0.05% aqueous Bromophenol Blue solution, was used as a band marker.

(ii) Electrophoresis: Electrophoresis was conducted at an ambient temperature of 20°C. A current of 7 mA was applied until all the marker dye had entered the gel and the sample spacer was removed. The current was then increased to 20 mA. Electrophoresis was conducted until the marker was about 1.5 cm from the bottom of the gel.

(iii) Determination of Polymer and Label Distribution: The polymer and label distribution in samples was determined by the procedures described for Serum Albumin (Section 5.5.2).

RESULTS AND DISCUSSION

Preliminary experiments using non-labelled protein showed that the β -Lactoglobulin used in this work contained a small quantity of irreversibly formed polymeric material. A minimum sample protein content of $\sim 8 \times 10^{-5}$ g was necessary for this material to be visible upon electrophoresis. Exposure of the protein to Iodo-Gen caused a pronounced increase in the amount of polymeric material present. Polymers of 2nd and 3rd order were observed. Electrophoresis was therefore repeated with the following modification to the iodination procedure.

The deleterious effect of protein exposure to oxidising agents had been noted earlier although subsequent exposure to reducing agents seemed to reverse some of the damage (Section 5.5.2). The compounds β -mercaptoethanol and dithiothreitol are commonly used in biochemical systems as reductants. They function as protective agents for the easily oxidised sulphydryl (-SH) groups, and also cause dissociation of polymers or aggregates of proteins formed as a result of sulphydryl oxidation and consequent disulphide bridge formation. β -mercaptoethanol is specific for disulphide bonds whereas dithiothreitol is a non-specific reductant. It was decided to investigate the effect of these compounds on the

polymers formed by the iodination procedure. After subjection to the iodinating reaction at various Iodo-Gen : protein ratios, the reaction mixtures were transferred to clean micro test tubes and dithiothreitol or β -mercaptoethanol added to a final concentration of $0.007 \text{ mole l}^{-1}$. The solutions were incubated at 37°C for 1 hour prior to the addition of SDS, further incubation and electrophoresis.

A photograph of the electrophoresis gel after staining and destaining is shown in Plate 5.2, and the polymer and label distribution reported in Table 5.3. The results show that the standard β -Lactoglobulin contained a monomeric fraction (M_1), and a polymeric fraction (M_n) which comprised about 1% of the total protein. Exposure to Iodo-Gen during iodination increased this fraction to 16%, and this was further increased to 23% when an Iodo-Gen : protein ratio four times that of the standard iodination procedure was used. Both dithiothreitol and β -mercaptoethanol reversed some of the aggregation caused by Iodo-Gen oxidation. The use of the non-specific reductant dthiothreitol did not cause more reversal of aggregation than the disulphide bond specific reductant β -mercaptoethanol. The iodinated and non-iodinated fractions were not grossly different however and the radioiodine label was evenly distributed over the monomeric and polymeric fractions.

Radioiodinated β -Lactoglobulin was observed to be stable to storage for up to 14 days, at 4°C .



SAMPLE KEY:

- 1: β -Lactoglobulin standard
 (a) 0.2 mg
 (b) 0.1 mg
- 2: After Iodo-Gen iodination at varying
 Iodo-Gen : protein ratio (IG/P)
 (c) 0.2 mg, IG/P = 0.1
 (d) 0.1 mg, IG/P = 0.1
 (e) 0.1 mg, IG/P = 0.4
- 3: After Iodo-Gen iodination and incubation
 with dithiothreitol
 (f) 0.2 mg, IG/P = 0.1
 (g) 0.1 mg, IG/P = 0.1
 (h) 0.1 mg, IG/P = 0.4
- 4: After Iodo-Gen iodination and incubation with
 β -mercaptoethanol
 (i) 0.1 mg, IG/P = 0.4

PLATE 5.2

SDS - POLYACRYLAMIDE GEL
ELECTROPHORESIS OF β -LACTOGLOBULIN

TABLE 5.3: POLYMER AND LABEL DISTRIBUTION OF β -LACTOGLOBULIN^(a)

TREATMENT	POLYMER DISTRIBUTION/%		LABEL DISTRIBUTION/%	
	M ₁	M _n	M ₁	M _n
None	99	1		
Iodination IG/P=0.1	84	16		
+ dithiothreitol	90	10	89	11
Iodination IG/P=0.4	77	23		
+ dithiothreitol	84	16		
+ β -mercaptoethanol	84	16		

^a Results are reported for samples containing 0.1 mg protein
 IG/P = Iodo-Gen : protein ratio

5.5.5 CONCLUSION

Pore gradient gel electrophoresis was used to separate the polymeric fractions of Serum Albumins. The monomeric and dimeric species contributed about 75% of the total protein in both the Bovine and Human types. Iodination caused a small change in the label distribution. There was a net increase in the amount of the monomeric and dimeric material such that these fractions now contributed more than 80% of the total protein. The change in polymer distribution was caused by exposure of the protein to the oxidising and reducing agents in the iodination reaction and was not affected by gel filtration. There was some evidence to indicate that iodination also resulted in a change in the nature of the sulphydryl group of the protein. Less than 12% of the BSA and 9% of the HSA was affected. The polymers were essentially uniformly labelled but there was some indication that the %polymer : %label ratio was decreased as the order of the polymer increased.

β -Lactoglobulin was characterised by SDS-gel electrophoresis and observed to contain about 1% of an irreversibly formed polymeric material. Iodination by the standard Iodo-Gen method, in which exposure of protein to reducing agents is avoided, resulted in an increase in the irreversibly formed polymer to 16% of the total protein. Modification of the procedure to incorporate a reducing agent reversed some of the aggregation and provided a preparation with 10% of irreversibly formed material. The label was evenly distributed over the monomeric and polymeric fractions.

All labelled proteins were observed to be stable to storage in cacodylate buffer and at 4°C for up to 2 weeks. Breakdown of the carbon-iodine bond only became significant after this time.

5.6 SUMMARY

Electrophoretic techniques enabled characterisation of the protein samples with regard to their irreversibly formed polymer content. The monomeric and dimeric material provided a high proportion of the total protein in the Bovine and Human Serum Albumins. β -Lactoglobulin was observed to consist of almost entirely monomeric material.

The iodination of Serum Albumins and β -Casein was performed using a modification of the original Chloramine-T technique of Greenwood et al. This allowed iodination at high efficiency and to high specific activity at a low level of labelling.

The iodination of β -Lactoglobulin was performed by the Iodo-Gen method of Fraker and Speck as even the modified Chloramine-T method produced extensive aggregation. Iodo-Gen was a less harsh oxidant than Chloramine-T but still produced some aggregation. This could be reversed to some extent by modification of the original Iodo-Gen method to incorporate the use of a reducing agent.

The degree of labelling of the various proteins was such that alteration of the physico-chemical properties of the molecules was minimised and such that the most stable protein-label bond would be obtained. The labelled proteins were stable to storage for up to 2 weeks at 4°C before significant release of the label from the protein occurred. The samples were not greatly different from the unlabelled protein in regard to their polymeric content. It appears that the labelled proteins, prepared as described, will be suitable as tracers for the studies envisaged in this thesis.

CHAPTER 6

PROTEIN ADSORPTION: METHODS AND MATERIALS

6.1 INTRODUCTION

The purpose of the work described in this Chapter was to establish suitable methods for the study of protein adsorption at the hydroxyapatite/aqueous solution interface. There appeared to be two possible systems for studying adsorption on the prepared synthetic hydroxyapatites. Provided the material could be pressed into satisfactory discs, it would be possible to use low surface area systems. These offer the advantages of ease of handling and the economical use of protein. Alternatively, if satisfactory discs could not be formed, a method using suspended particles could be used.

The presence of polymeric material in the protein materials needs to be investigated with respect to such aspects as preferential adsorption and changes in the amounts of adsorption. In any study using tracer techniques, it is necessary to demonstrate that the tracer behaves identically to the non-labelled material. In the case of proteins, spectrophotometric methods provide an alternative means of monitoring adsorption.

6.2 RADIOCHEMICAL METHODS FOR MEASURING ADSORPTION

6.2.1 INTRODUCTION

Radiotracers have been used extensively in quantitative and qualitative studies of adsorption at solid/liquid interfaces. Quantitative studies employ the principle of isotope dilution. In the present work, a direct method involving the counting of adsorbed activity (Iodine-125 labelled proteins), and a differential method, involving solution depletion measurements, were developed to quantitate adsorption. Although most of the work reported in this thesis was obtained using high surface area systems, it was found convenient at times to use a low surface area system. Both solid and liquid scintillation counting techniques were used.

6.2.2 THE DIFFERENTIAL METHOD: DETERMINATION BY SOLUTION DEPLETION

The depletion of protein from solution after contact with the solid adsorbent can be expressed as a fraction, α , defined by the equation:

$$\alpha = \frac{R_i^S - R_f^S}{R_i^S} \quad - - 6.1$$

where R_i^S = initial solution activity per unit volume solution
(counts $s^{-1}m^{-3}$)

R_f^S = final solution activity per unit volume solution
(counts $s^{-1}m^{-3}$).

Provided that the relationship between the measured activity and the solution concentration of the labelled species is linear, the mass of protein adsorbed per unit surface area of adsorbent, Γ , can be calculated from the equation:

$$\Gamma = \frac{\alpha C_i V}{A} \quad - - 6.2$$

where C_i = initial protein concentration (g m^{-3})

V = volume of solution (m^3)

A = total geometric surface area adsorbent (m^2).

Note:

(i) The minimum change in activity that can be measured easily and accurately is limited to approximately 5%. The technique is therefore suited to studies which have a high surface area : volume ratio.

(ii) In high surface area systems, the geometric surface area is given by the product of the mass of adsorbent (m_{solid}) in grams, and the specific surface area (A_s) in area (m^2) per gram.

(iii) In low surface area systems, the geometric surface area is given by the product of the nominal surface (A_n) and the surface roughness factor (x).

(iv) The use of equation 6.2 presumes that the protein depleted from solution is adsorbed only on the adsorbent. At low protein concentrations, corrections to allow for depletion by adsorption to reaction vessel walls may be necessary.

The determination of solution activities is discussed in Section 6.2.4.

6.2.3 THE DIRECT METHOD: DETERMINATION BY SOLID ACTIVITY

The mass of protein adsorbed per unit surface area of adsorbent can be calculated from the equation:

$$\Gamma = \frac{\lambda C_i}{x} \quad - - 6.3$$

where the retention coefficient, λ , is evaluated from the relation:

$$\lambda = \frac{R^P}{R_i^S} \quad - - 6.4$$

where R^P = activity adsorbed per unit (nominal) surface area of adsorbent (counts $s^{-1}m^{-2}$).

The determination of solid activities is discussed in Section 6.2.5.

6.2.4 DETERMINATION OF SOLUTION ACTIVITY

Solution can be calculated by the equation:

$$R_{i \text{ or } f}^S = \frac{r_{\text{soln}} - r_b \times \rho}{m_{\text{soln}}} \quad - - 6.5$$

where

r_{soln} = solution count (counts s^{-1})

r_b = background count (counts s^{-1})

m_{soln} = mass of solution (g)

ρ = density of solution ($g\ m^{-3}$)

Note: The solution density was usually taken as $10^6\ g\ m^{-3}$ (i.e. equivalent to $1\ g\ cm^{-3}$) except when density corrections were required due to high solute concentrations e.g. in high ionic strength experiments.

The mass adsorbed was then calculated from equations 6.1 and 6.2.

Determination by Solid Scintillation

Samples for the determination of solution activities by solid scintillation were obtained by placing 0.02 - 0.05 ml aliquots on previously weighed planchettes and immediately reweighing. Care was taken to ensure the samples were placed in the centre of the planchettes. Samples were evaporated to dryness under a heat lamp before counting.

Determination by Liquid Scintillation

Samples for the determination of solution activities by liquid scintillation were obtained by placing known aliquots into weighed scintillation vials and immediately reweighing. The volume was adjusted to 1.0 ml with water, and after the addition of 10.0 ml of Aquasol-2 liquid scintillation cocktail, the solution was vigorously shaken. Preliminary experiments showed that the presence of protein did not cause quenching.

6.2.5 DETERMINATION OF ADSORBED ACTIVITY

High Surface Area Systems

The adsorbent was removed from solution by centrifugation. Samples for the determination of solid activities were obtained by placing small amounts ($\leq 0.02g$) of solid on previously weighed planchettes (weighings to $\pm 0.00001g$) and immediately reweighing. Care was taken to ensure the sample was placed in a thin even layer in the centre of the planchette. This maintained the counting geometry and avoided self-absorption effects. Samples were evaporated to dryness under a heat lamp and weighed again.

The activity adsorbed can be calculated from the equation:

$$RP = \frac{r_{\text{solid}} - r_b}{m_{\text{solid}} A_s} \quad - - 6.6$$

where r_{solid} = solid count (counts s^{-1})

Note:

(i) The activity of the separated solid includes an activity contribution from the labelled species in the adhering solution. The necessary correction factor may be determined from the product of the

solution activity after adsorption and the volume of adhering solution. This is determined simply by weighing after sample application and again after evaporation to dryness.

(ii) The mass of separated solid includes mass contributions from buffer salts, non-adsorbed protein and also from the adsorbed protein. Any or all of these may be significant under various conditions. The true mass of adsorbent, m_{solid} , can be calculated from the relation:

$$m_a = m_{\text{solid}} + m_{\text{salts}} + m_{\text{non-ads}} + m_{\text{ads}} \quad - - 6.7$$

where m_a = apparent mass of adsorbent (g), and is determined from the weight of the planchette before and after sample application and evaporation to dryness

m_{salts} = mass of buffer salts (g), and is determined from the volume of adherent solution and buffer salt concentration

$m_{\text{non-ads}}$ = mass of non-adsorbed protein (g), and is determined from the volume of adherent solution and the final protein concentration as determined by the solution depletion measurements

m_{ads} = mass of adsorbed protein (g), and is given by the relation:

$$m_{\text{ads}} = m_{\text{solid}} \times A_s \times \Gamma \quad - - 6.8$$

Substitution of equation 6.8 into equation 6.7 and rearrangement gives:

$$m_{\text{solid}} = \frac{m_a - m_{\text{salts}} - m_{\text{non-ads}}}{1 + A_s \Gamma} \quad - - 6.9$$

where the value of Γ used is that determined from solution depletion.

The mass adsorbed was then calculated from equations 6.3 and 6.4. If this value did not agree to within 10% of that calculated from solution depletion measurements, both points were discarded.

Low Surface Area Systems

The solid was removed from solution, rinsed (if feasible or necessary to remove adherent solution) and a nominal area counted. The activity adsorbed can be calculated from the equation:

$$R_P = \frac{r_{\text{solid}} - r_{\text{ret}} - r_b}{x A_n} \quad - - 6.10$$

where r_{ret} = count from the labelled species in the retained or adhering solution

Note: Rinsing of the adsorbent is an acceptable and accurate practice if adsorption is known to be irreversible to dilution. Alternatively:

- (i) the activity contribution of the adhering solution may be determined from the solution activity and the volume of the adhering layer which may be determined by measuring the weight decrease upon evaporation to dryness; or,
- (ii) 'carry out' can be minimised by working with sufficiently dilute solutions such that the amount of active material in the adherent layer

6.3 SPECTROPHOTOMETRIC METHODS FOR MEASURING ADSORPTION

6.3.1 INTRODUCTION

Spectrophotometric methods provide an independent method for monitoring behaviour in systems using labelled material. They can also be useful for determining adsorption where proteins cannot be labelled or are extensively damaged by labelling procedures. The sensitivity of the method is dependent on the protein in study having a sufficiently high extinction coefficient but can be improved, when required, by the use of protein-dye binding methods e.g. the Lowry procedure (Lowry et al., 1951), Amidoscharwz 10-B binding (Schaffner and Weissman, 1973), Orange-G binding (Kaul et al., 1970), and binding with Coomassie Brilliant Blue G-250 (Bradford, 1976). The method of Bradford offers high sensitivity (micrograms of protein), short analysis times, little or no interference from other solution components and was chosen as most suitable for this study. Amounts of adsorption are determined most easily by a differential method involving solution depletion measurements. The method is therefore most suitable for studies in high surface area systems.

6.3.2 DETERMINATION BY SOLUTION DEPLETION

The depletion of protein from solution after contact with the solid adsorbent can be expressed as a fraction α , defined by the equation:

$$\alpha = \frac{C_i - C_f}{C_i} \quad - - 6.11$$

where C_f = final protein concentration (g m^{-3}).

In cases where the optical absorbance varies linearly with concentration,

the concentration terms of equation 6.11 can be replaced by the measured optical absorbance. The mass of protein adsorbed per unit surface area, Γ , is then calculated from equation 6.2.

6.3.3 EXPERIMENTAL METHODS AND RESULTS

Direct Determination

The proteins Haemoglobin and Myoglobin suffered extensive denaturation during labelling (Section 5.4.6). These proteins are strongly coloured and are easily monitored by direct spectrophotometric measurements.

Spectrum scans from 250 - 600 nm wavelength were made to determine the absorption maxima. Maximal absorption was observed at 407 nm for both Haemoglobin and Myoglobin and the absorbance calibrated for each protein at this wavelength. A concentration range of 5 - 200 g m⁻³ was used. The calibration curves are presented in Appendix IX.

Determination by Dye Binding

The wavelength of maximum absorbance for the protein-Coomassie Brilliant Blue G-250 dye complex is 595 nm. The absorbance of each protein to be monitored was calibrated at this wavelength over the concentration range 5 - 100 g m⁻³. The calibration curves obtained are presented in Appendix IX.

6.4 ADSORPTION IN LOW SURFACE AREA SYSTEMS: PLATE OR SHEET MATERIALS

6.4.1 INTRODUCTION

Low surface area systems, such as plate or sheet surfaces, offer an advantage over high surface area systems, such as powders or colloids, because of the relatively simple experimental methods involved and ease of manipulation of the adsorbent. Significant depletion of adsorption solutions is not observed except when using small volumes of very low protein concentration. The amounts of adsorption are therefore most easily determined by radiochemical methods and direct counting of the adsorbent.

6.4.2 THE HYDROXYAPATITE COMPACT SYSTEM

Powder compaction sintering techniques that produce a firm ceramic material from hydroxyapatite powders have been described in literature (e.g. Rao and Boehm, 1974; Rootare and Craig, 1974; Jarcho et al., 1976, 1977; Rootare et al., 1978).

Method and Materials

0.4 g of hydroxyapatite was lightly ground, using a mortar and pestle, before transfer to a 13 mm die. The die was evacuated for 2 minutes using a rotary vacuum pump before application of a 10 tonne load for 3 minutes. After removal from the die, the compacted disc was sintered at 350°C for 24 hours. Higher temperatures were not used to avoid changes in the chemical composition of the hydroxyapatite (Kibby and Hall, 1972). Non-sintered and sintered discs were immersed in water to observe their stability. Disc surfaces were observed by transmission

electron microscopy using carbon/platinum replicas. To avoid disruption of the replicas, the sintered discs were removed by dissolution in 18% HCl.

Results and Discussion

Non-sintered discs were observed to be quite porous and to crumble upon immersion. The sintered discs maintained their integrity for about 30 minutes. Small bubbles of gas were released, particularly from around the side of the disc. Few bubbles were released from the disc surface however, and this indicated that a 'spot' or 'drop' radiochemical technique for monitoring adsorption (Langdon et al., 1973; Nisbet, 1979) might be feasible. Observation of disc surfaces by electron microscopy showed the surfaces to be rough and to contain many very small pores (Plate 6.1). This suggested that the compacted and sintered discs would not prove useful in adsorption studies however.

An alternative low surface area solid/liquid interface that might be useful is the stainless steel/aqueous solution system. The adsorption of BSA and β -Casein at this interface has been characterised by van Enkevort (1978) and Nisbet (1979). The preliminary experiments with suspended hydroxyapatite particles suggested that the levels of adsorption observed for these proteins were similar to those observed in the studies of van Enkevort and Nisbet. Rather than continue studies with the compaction and sintering of hydroxyapatite discs, it was decided that the stainless steel system might be useful in some experiments.

PLATE 6.1

SURFACE MORPHOLOGY OF A COMPACTED
AND SINTERED HYDROXYAPATITE DISC

(Magnification: 16 000 x)



6.4.3 THE STAINLESS STEEL SLIDE SYSTEM

Slides of size 42 mm x 12 mm were cut from AISI type 304 sheet stainless steel with a 2B surface. All slides for adsorption studies were polished and cleaned immediately prior to use by the following procedure:

- (i) Lightly polished with Grade 400 silicon carbide grit and thoroughly rinsed with distilled water.
- (ii) Successive immersion in boiling 2% phosphoric acid (5 min.), 2% potassium hydroxide (5 min.) and three changes of boiling distilled water (5 min. each)
- (iii) Dried by heat lamp in dust free conditions.

The cleaned slides were handled by similarly cleaned stainless steel tweezers only by the end not immersed during adsorption experiments.

All glassware used in the preparation of protein solutions and for adsorption experiments was cleaned by the procedures outlined in Appendix II. Adsorption experiments were carried out at the conditions envisaged for the hydroxyapatite experiment unless specified otherwise. Specially constructed narrow rectangular glass adsorption vessels (30mm x 15mm x 5mm) of approximately 2 ml capacity were used in adsorption experiments. These were pinched at the top to hold slides in an upright central position. In the typical experiment, a batch of freshly polished and cleaned slides was allowed to equilibrate at 4°C and 100% relative humidity for one hour before being partially immersed in 1.0 ml volumes of protein solution of known concentration at the conditions of the experiment. At the end of a pre-determined time the slides were rinsed for 20 seconds in running distilled water if preliminary experiments showed that this did not cause significant desorption. (The previous studies of van Enkevort and Nisbet showed that monolayer adsorption of

BSA and β -Casein on stainless steel was irreversible to dilution. (Non-reversibility was also observed in this thesis for β -Lactoglobulin and salivary glycoprotein adsorption on stainless steel.) Solution activities were measured before the adsorption and the amount adsorbed calculated from the count rate obtained from a well defined area of the exposed surface. Counting times were chosen such that the overall standard deviation arising from counting was less than 1%. The roughness factor of the abraded and cleaned stainless steel surface has been suggested to be 1.7 (van Enkevort, 1978).

6.5 ADSORPTION IN HIGH SURFACE AREA SYSTEMS: SUSPENDED PARTICLES

6.5.1 INTRODUCTION

Several technical difficulties can be encountered in studying interfacial phenomena e.g. adsorption, desorption and exchange in suspensions. These include homogeneous dispersion of the suspended particles and separation of the suspended phase from the suspending medium. Ultrasonics can often be used to facilitate dispersion of particle aggregates. Filtration and centrifugation methods are the most convenient for achieving phase separation in order that appropriate analyses can be made. Filtration has the disadvantage, however, that it is less convenient to use and requires expensive filters, many of which also show significant protein retention (e.g. Dass, 1982). Experimental procedures, based on ultrasonication and centrifugation, that enabled the determination of protein adsorption in hydroxyapatite particle suspensions are described in this Section.

6.5.2 METHOD AND MATERIALS

All glassware was carefully cleaned prior to use in adsorption experiments. Details of the cleaning procedures are given in Appendix II. All adsorption experiments were carried out at 4°C using a cacodylate buffer, pH 6.8 and I 0.06 mole l⁻¹ (Appendix VI) unless stated otherwise. The buffer was pre-equilibrated over hydroxyapatite for at least 1 month and filtered using a Millipore filter (0.05 µm pore size) prior to use.

Protein solutions, usually of concentration range 10 - 20 000 g m⁻³, were prepared in glass vials or bottles. In radiochemical experiments,

the solutions were spiked with Iodine-125 labelled protein to give solution and solid activities such that the standard deviation of counting was better than 1%. The volumes of protein solution chosen were such that sufficient solid adsorbent could be collected for a reasonably accurate determination of solid activity at about 50% depletion. This usually corresponded to a minimum total solution protein content of $\sim 1 \times 10^{-3}$ g. Below solution concentrations of about 10 g m^{-3} this method was generally not feasible because of the difficulty of separating small masses of adsorbent dispersed in large volumes (~ 1 litre) of solution. Adsorption in these cases was determined solely by solution depletion measurements.

Initial solution activity or optical absorbance measurements were made immediately after mixing. Solutions were equilibrated at 4°C by gentle rotation on a turning wheel, or using magnetic stirrers for larger volumes. A period of 24 hours was generally found sufficient and convenient for equilibration for solutions of concentration greater than 10 g m^{-3} . Solution activities or optical absorbance measurements were taken again immediately prior to adsorption to obtain the correction factor for protein depletion to the walls of the reaction vessels. For solutions of concentration less than 10 g m^{-3} , the period of equilibration was that time necessary for activity or absorbance measurements to reach a constant value.

In radiochemical experiments, an indication of the saturation level of adsorption for each protein was readily obtained using the low surface area stainless steel slide system. In non-radiochemical experiments, the mass of hydroxyapatite required was determined by the following preliminary experiment: a sufficient volume of a hydroxyapatite suspension, containing a known mass of hydroxyapatite, was added to a protein solution of known concentration to cause a measureable depletion.

The mass of hydroxyapatite required for 50% depletion of the protein from the adsorption solutions was calculated from these preliminary experiments. Adsorption experiments were then set up such that the amount of hydroxyapatite introduced into each solution would be sufficient to achieve a level of depletion of 50% - 60%.

A stock suspension of hydroxyapatite was prepared by placing a known mass of hydroxyapatite in buffer. This was subjected to ultrasonics for 1 hour with periodic vigorous shaking to ensure thorough dispersion. A minimal volume of suspending buffer was used so that addition of the hydroxyapatite to the protein solution gave minimal dilution. (This would minimise changes in the nature of any protein-protein interactions, e.g. polymerisation equilibria, in solution). Generally, hydroxyapatite concentrations of less than 0.1 g ml^{-1} were used as higher concentrations were difficult to disperse. When necessary, the level of solution depletion was decreased accordingly as suspensions of high concentration usually gave lower values of adsorption (up to 10% less). The stock suspension was diluted as required to allow the introduction of convenient volumes of suspension to the adsorption solutions. The suspensions were allowed to equilibrate at 4°C for 24 hours prior to the addition of known volumes, i.e. mass of hydroxyapatite, to the protein solutions.

After the required adsorption time, the hydroxyapatite was removed from solution by centrifugation (Sorval RC-5B Refrigerated Superspeed Centrifuge), at $48\,000g$ and at the temperature of the experiment, for 10 - 15 minutes. Preliminary experiments showed that bench centrifuges did not satisfactorily remove the hydroxyapatite from solution. They also did not offer the advantage of temperature controlled centrifugation.

For experiments investigating the effect of pH, ionic strength and

temperature, the buffers and (ultrasonicated) hydroxyapatite were pre-equilibrated for about 2 months at the conditions of the experiment envisaged. As the solubility of hydroxyapatite is strongly influenced by the properties of the suspending medium, the effect of these variables on the BET surface area was monitored (Figure 6.1). Although temperature and ionic strength did not significantly affect the measured surface area, a change in pH from 6.5 to 4.9 resulted in a progressive decrease of specific surface area from $40 \text{ m}^2\text{g}^{-1}$ to $32 \text{ m}^2\text{g}^{-1}$. The measured surface area was not affected by pH changes above pH 6.5.

In adsorption experiments at extended times, all adsorption solutions were monitored daily for the presence of free radioiodine label.

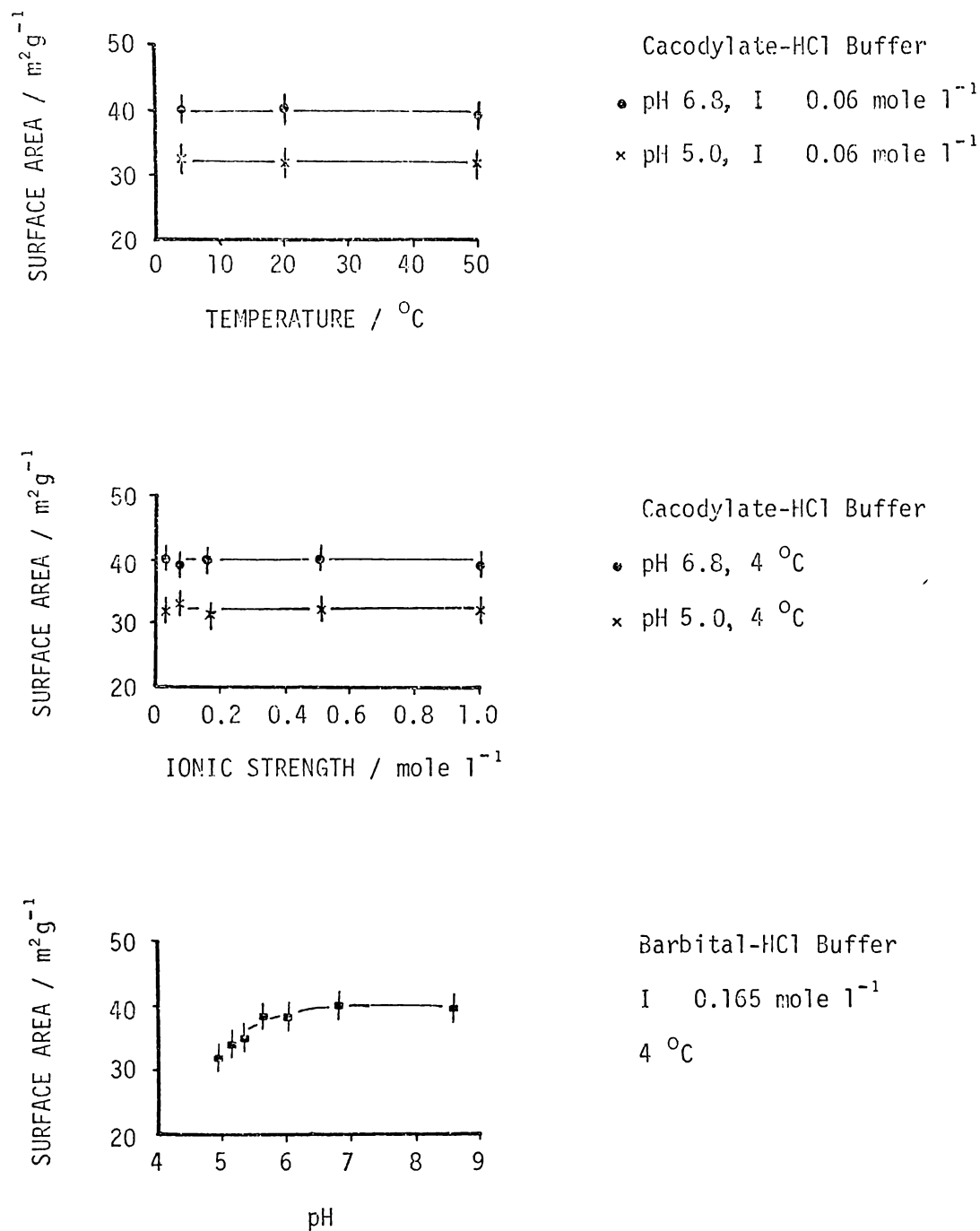


Figure 6.1: The Effect of Solution Conditions on the Surface Area of Hydroxyapatite.

6.6 KINETICS OF ADSORPTION

6.6.1 INTRODUCTION

Ideally, the kinetics of adsorption is best studied using a continuous monitoring process. This is difficult in high surface area systems of suspended particles as protein adsorption proceeds at a fast rate at most solution concentrations, and solution depletion effects are troublesome. In order to design valid experiments to test the effects of variables on the adsorption process however, some qualitative knowledge of the rate of adsorption is necessary. It was envisaged a batchwise sampling technique would provide this information.

6.6.2 METHOD AND MATERIALS

Adsorption experiments were set up at BSA concentrations of 10, 100 and 1 000 g m⁻³ to give approximately 80% depletion. 1 ml aliquots were removed periodically, centrifuged immediately and the supernate used for activity determinations. Adsorption was monitored for 3 days after which the final solution and solid activities were determined.

6.6.3 RESULTS AND DISCUSSION

The adsorption kinetics were characterised by an initial rapid adsorption up to a level of about 1 mg m⁻² and then became progressively slower (Figures 6.2 A, B, C). Maximal adsorption was achieved only after about 24 hours in the case of adsorption from a protein solution of

initial and final concentrations of 10 g m^{-3} and 3 g m^{-3} respectively. In all cases, the same level of adsorption was attained.

The data suggested that adsorption experiments at initial protein concentrations of greater than 10 g m^{-3} , and at less than 70% depletion, be left for 24 hours to attain saturation adsorption. This time was subsequently used for all experiments except those at lower final concentration. The solution activities of these solutions were monitored until they attained a constant value.

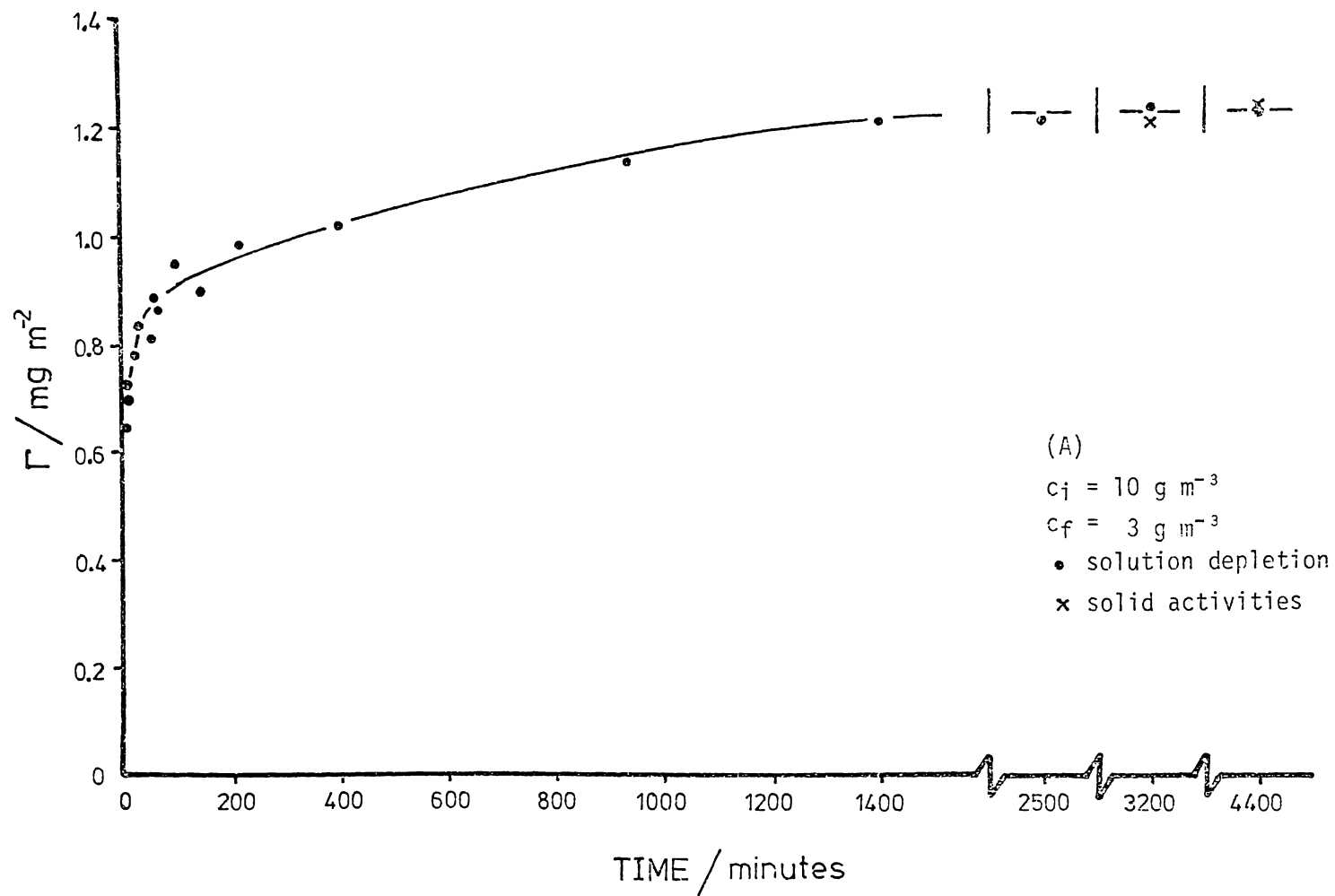


Figure 6.2: The Kinetics of Adsorption of BSA to Suspended Hydroxyapatite: The Effect of Concentration.

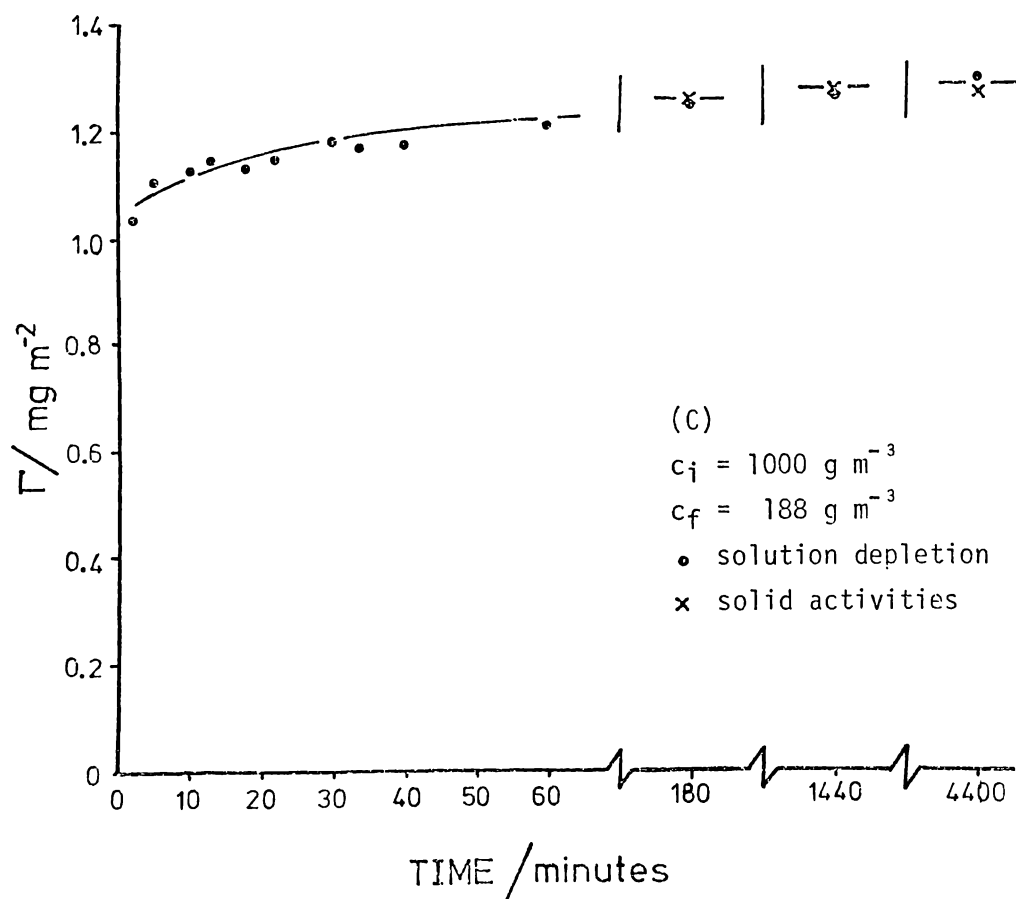
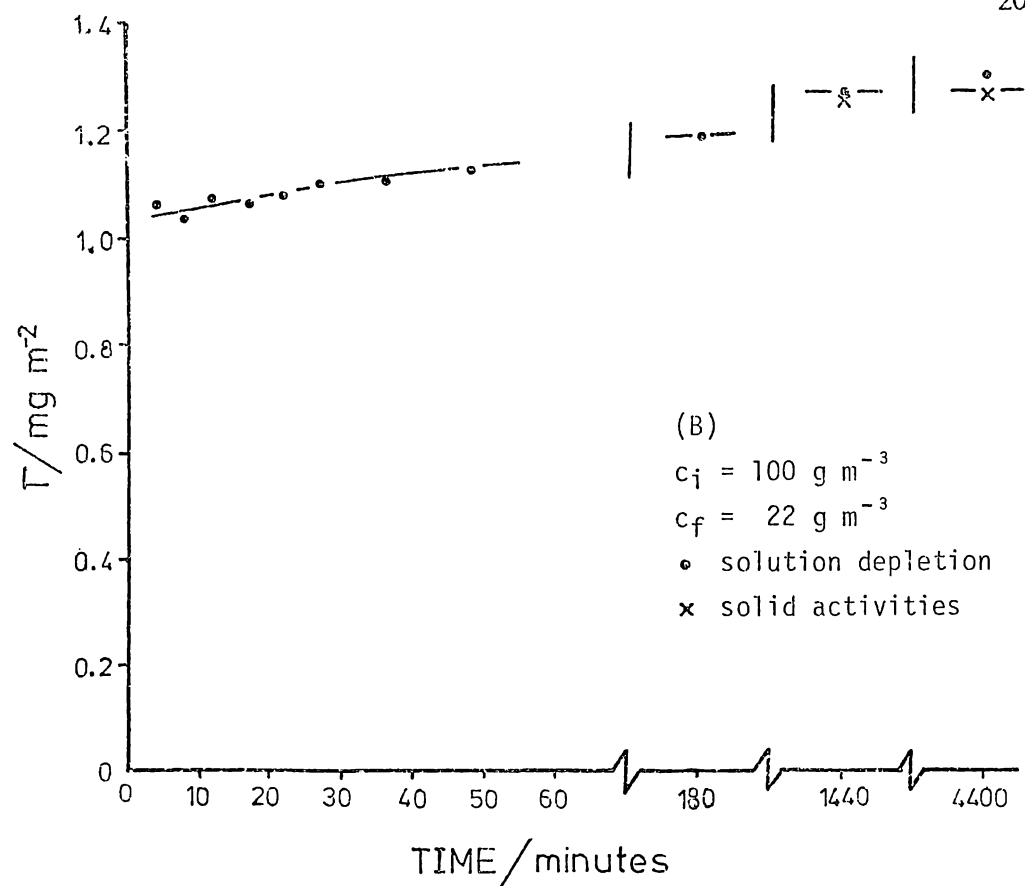


Figure 6.2 contd..

6.7 THE EFFECT OF POLYMERS ON ADSORPTION BEHAVIOUR

6.7.1 INTRODUCTION

The effect of polymeric species on adsorption is ideally tested using solutions which contain only the individual monomeric or polymeric species. Although this is possible with proteins such as BSA where the protein molecule shows no tendency to associate except as a result of physical damage, this is not possible with proteins such as β -Casein or β -Lactoglobulin where polymer formation is an equilibrium process. BSA can be separated into its individual polymeric fractions by column chromatography (Pederson, 1962; Saifer and Palo, 1969). However, monomeric BSA is available commercially. β -Casein exists as only the monomeric species at low temperatures ($<5^{\circ}\text{C}$) and the polymeric (micellar) species is formed at higher temperatures (Section 3.6.3). β -Lactoglobulin can be induced to partially depolymerise by pH changes (McKenzie, 1971b), it was of particular interest to determine the effect on adsorption of the polymer species formed irreversibly by the iodination procedure (Section 5.5). Experiments were therefore performed to determine and compare the levels of adsorption from various solutions and to determine if the polymeric species were preferentially adsorbed.

6.7.2 METHODS AND MATERIALS

Adsorption was monitored by spectrophotometric methods in non-radiochemical systems. The experimental procedures used for adsorption and separation of the suspended hydroxyapatite were similar to those described in Section 6.5. Adsorption was allowed to proceed for 72 hours

so that any slow processes might attain a steady state.

BSA: the polymeric content of the BSA solutions used was determined by pore gradient gel electrophoresis (Section 5.5.2) prior to and after adsorption. Adsorption was performed at 4°C and a concentration of 8 000 g m⁻³. Electrophoresis samples contained 1 x 10⁻⁴g protein.

β-Casein: Adsorption was performed at a concentration of 4 000 g m⁻³ at temperatures of 4°C and 20°C. The concentration and temperatures chosen were such that, under the solution conditions (pH 6.8, I 0.06 mole l⁻¹), the protein was entirely monomeric (4°C) or highly aggregated (20°C; Schmidt and Payens, 1972).

β-Lactoglobulin: Adsorption was performed at 4°C and a concentration of 8 000 g m⁻³ using the standard β-Lactoglobulin of known polymer distribution (Section 5.5.4) and a second sample that had been exposed to Iodo-Gen at an Iodo-Gen : protein ratio of 0.4:1. This sample was prepared in the following manner:

(i) 0.1 ml of an Iodo-Gen stock solution (9.6 x 10⁻² g ml⁻¹ in CH₂Cl₂) was added to 0.4 ml CH₂Cl₂ in a 75 mm x 10 mm test tube. The solution was mixed and then evaporated to dryness under vacuum while continuously rotating the test tube, on its side, to ensure the walls were evenly covered by the deposited Iodo-Gen. The tube was rinsed with distilled water to remove any loose flakes of Iodo-Gen.

(ii) 3.0 ml of β-Lactoglobulin (8 x 10⁻³g ml⁻¹) in cacodylate buffer was added to the test tube and allowed to react for 1 hour, at 4°C, with periodic mixing using a pasteur pipette. The protein solution was then transferred to a clean glass vial.

The polymeric content of the β-Lactoglobulin solutions was determined by SDS-gel electrophoresis prior to and after adsorption. (Section 5.5.4).

6.7.3 RESULTS AND DISCUSSION

The results of the electrophoretic and adsorption experiments are presented in Table 6.1. The 'monomeric' albumin was found to contain only 62% monomer. Although this is much less than the specified 99% monomer at the date of manufacture and prior to lyophilisation, this process, and subsequent storage in the dry state, are known to cause some polymerisation. The degree of polymerisation was such, however, that an adequate comparison of the amounts of adsorption for Serum Albumin preparations containing (i) mainly monomeric and (ii) monomeric and polymeric species could not be made. The levels of adsorption observed for the two samples of differing polymer distribution were not significantly different. The polymer distribution before and after adsorption did not show any particular species was preferentially adsorbed.

No significant difference in the amounts of adsorption from β -Casein solutions containing (i) only monomeric and (ii) mainly micellar species was observed. The design of the experiment did not preclude the effect of temperature on the level of adsorption. However, given that the relative molecular masses of the monomeric and micellar species differ by a factor of about 25, the adsorption data show that the level of adsorption, at the concentration studied, was independent of temperature and the presence of micelles.

The level of adsorption observed from a β -Lactoglobulin solution containing over 20% of an irreversibly formed polymeric fraction was not significantly different from that observed for adsorption from a solution containing 99% monomer. The polymer distribution before and after adsorption did not indicate either species was preferentially adsorbed.

TABLE 6.1: THE EFFECT OF POLYMERIC SPECIES ON ADSORPTION

PROTEIN	%POLYMER DISTRIBUTION					$\Gamma/\text{mg m}^{-2}(\text{a})$
	M ₁	M ₂	M ₃	M ₄	M ₅	
<u>Bovine Serum Albumin</u>						
'Monomeric':						
before adsorption	62	20	10	8	-	
after adsorption	64	19	10	7	-	1.26
Polymeric:						
before adsorption	47	27	16	8	2	
after adsorption	48	26	17	7	2	1.28
 <u>β-Casein</u> ^(b)						
Monomeric	100					3.35
Polymeric		micellar				3.30
 <u>β-Lactoglobulin</u> ^(c)						
Monomeric	99	1				1.03
Polymeric:						
before adsorption	79	21				
after adsorption	78	22				1.02

^a Data represent average of 3 separate experiments.

^b Adsorption performed at 4°C (monomeric) and 20°C (polymeric)

^c Polymeric content refers to irreversibly formed polymers

6.8 VALIDITY OF THE USE OF RADIOIODINATED PROTEINS

6.8.1 INTRODUCTION

The ultimate test of the validity of the use of labelled proteins as tracers in adsorption studies is that the labelled and non-labelled protein behave identically in the system in use. Experiments were therefore performed to establish that each radioiodinated protein used had the same adsorption characteristics as the non-iodinated protein at the hydroxyapatite/solution interface. Adsorption data for the three hydroxyapatite adsorbents used in this work was obtained radiometrically, using the labelled protein, and compared with data obtained spectrophotometrically for solutions of varying labelled : non-labelled protein concentration ratio.

6.8.2 METHOD

The concentration of the stock solution of radioiodinated protein was determined by spectrophotometry. Measured aliquots of this solution were added to aliquots of non-labelled protein solutions of the same concentration to give solutions containing from 0 - 100% labelled protein of the same final total protein (i.e. labelled and non-labelled) concentration. This concentration was usually about 100 g m^{-3} . The experimental procedures used for adsorption and separation of the suspended hydroxyapatite were similar to those described in Section 6.5. All initial and final solution protein concentrations were measured spectrophotometrically as well as radiometrically in those solutions containing labelled material. The mass of hydroxyapatite added was sufficient to obtain about 60% depletion of solution protein. Adsorption

was allowed to proceed for 72 hours so that any slow processes might attain a steady state.

6.8.3 RESULTS AND DISCUSSION

The amounts of adsorption from solutions of varying labelled : non-labelled protein concentration ratios have been presented in Table 6.2. The mean values of BSA adsorption as determined by spectrophotometric and radiometric measurements were in good agreement. This showed that the hydroxyapatite surface did not discriminate between the iodinated and non-iodinated species. There were no apparent trends in the amounts of adsorption as the amount of labelled protein was increased. This result showed that the iodinated and non-iodinated BSA had the same level of saturation adsorption and behaved similarly in adsorption studies.

Consequently, tests of the validity of the use of radioiodinated HSA, β -Casein and β -Lactoglobulin were only performed on the non-substituted hydroxyapatite and restricted to 3 points. The results of these tests showed that, like BSA, the labelled and non-labelled species behaved identically in the adsorption studies.

A comparison of the amounts of adsorption for 0% and 100% labelled BSA, HSA and β -Lactoglobulin supported the previous observation that small changes in the polymer distribution of a protein did not noticeably alter the levels of adsorption (Section 6.7).

TABLE 6.2: ADSORPTION FROM SOLUTIONS OF VARYING LABELLED : NON-LABELLED
 PROTEIN CONCENTRATION RATIO AS DETERMINED BY
 SPECTROPHOTOMETRIC AND RADIOMETRIC MEASUREMENTS

%LABELLED PROTEIN	$\Gamma/\text{mg m}^{-2}$					
	Adsorbent: OHAp		F-OHAp		CO ₃ -OHAp	
	Method ⁽¹⁾ : (a)	(b)	(a)	(b)	(a)	(b)
<u>Bovine Serum Albumin</u>						
$C_i=91 \text{ g m}^{-3}$						
0	1.34	-	1.30	-	1.29	-
20	1.27	1.27	1.30	1.36	1.30	1.28
40	1.41	1.31	1.41	1.34	1.37	1.33
60	1.19	1.25	1.26	1.26	1.19	1.23
80	1.36	1.34	1.22	1.28	1.21	1.26
100	1.28	1.25	1.33	1.30	1.29	1.29
	mean = 1.31	1.30	1.30	1.31	1.28	1.28
	$\delta = 0.08$	0.04	0.06	0.04	0.07	0.04
<u>Human Serum Albumin</u>						
$C_i=86 \text{ g m}^{-3}$						
0	1.24	-				
5	1.18	1.22				
100	1.21	1.17				
<u>β-Casein</u>						
$C_i=250 \text{ g m}^{-3}$						
0	3.10	-				
5	2.94	3.05				
100	3.03	2.95				
<u>β-Lactoglobulin</u>						
$C_i=85 \text{ g m}^{-3}$						
0	0.99	-				
5	1.00	0.95				
100	1.04	1.02				

(1) Method (a): Spectrophotometric Measurements
 Method (b): Radiometric Measurements.

6.9 SUMMARY

Two methods for the study of protein adsorption on hydroxyapatites were investigated. The compaction and sintering of the powders to produce a low surface area disc suffered from disruption upon immersion and porosity problems that could not be overcome by sintering at the low temperatures necessary to avoid changes in surface properties. A method using the powders in suspended form was consequently developed.

Preliminary studies to characterise the adsorbents, protein and system used were performed. It was observed that the specific surface area of the suspended hydroxyapatite was dependent on pH below about pH 6.5. Although BSA adsorption was a rapid process up to about 70% saturation coverage, the rate of adsorption decreased dramatically above this point. Solutions of low final concentrations required at least 1 day to achieve maximal adsorption. The proteins BSA and HSA contained a significant polymeric fraction. However, there was no indication that any particular fraction was preferentially adsorbed, and a 40% increase in the amount of BSA polymer did not significantly alter the level of adsorption of that protein. Similarly, the presence of reversibly formed micelles in β -Casein solutions and irreversibly formed polymers in β -Lactoglobulin solutions (as a result of labelling procedures) did not affect the levels of adsorption. Extensive tests using the non-labelled and labelled proteins showed that the hydroxyapatite adsorbents prepared did not discriminate between the iodinated and non-iodinated protein, and that the iodinated and non-iodinated proteins behaved identically. This showed that the labelled proteins were valid tracers for the studies envisaged.

CHAPTER 7

PROTEIN ADSORPTION AT THE HYDROXYAPATITE / AQUEOUS SOLUTION INTERFACE

7.1 INTRODUCTION

In order to gain information that could lead to a better understanding of the adsorption of protein by tooth enamel, it was felt necessary to characterise the more important aspects of the adsorption of several proteins on the non-substituted and substituted hydroxyapatites OHAp, F-OHAp and CO₃-OHAp. The proteins studied included the Serum Albumins BSA and HSA, β -Casein, β -Lactoglobulin and the Haem proteins Haemoglobin and Myoglobin. Data are, in general, presented graphically. The main source of error in determining the amounts of adsorption using spectrophotometric techniques was in reading the optical density. This error is shown as error bars in data plots obtained using these techniques. The main source of error in radiochemical experiments was, however, a statistical error and this is not shown in data plots obtained using this technique.

Previous studies at this University on the adsorption on stainless steel of two of these proteins, BSA and β -Casein, have suggested that the levels of adsorption are determined by the size and shape of the protein molecules and their configuration in the adsorbed layer. The adsorption of these proteins was therefore studied in greater detail.

The results of the adsorption studies reported in this Chapter will be discussed in Chapter 11.

7.2 SERUM ALBUMINS

7.2.1 INTRODUCTION

Much of the literature on protein adsorption at solid/liquid interfaces concerns the use of either Bovine or Human Serum Albumin. Their adsorption behaviour appears to be very similar as might be expected by little difference in their physico-chemical properties (Section 3.6.2). Most of the work reported in this Section describes data obtained using radiochemical systems and BSA.

It was of particular interest to establish the concentration adsorption isotherm. Further, literature studies showed that the size of the Serum Albumin molecule was affected by changes in pH, ionic strength and temperature and that the levels of adsorption observed in some solid/liquid systems might be solely determined by this parameter. It was also of interest therefore to study the effect of variations in pH, ionic strength and temperature as well. Data have been plotted as the amount of adsorption versus the second variable and were obtained from experiments designed to test the effect of the variable at about 50% depletion i.e. the initial concentration was twice that of the final value. The concentrations quoted refer to final concentrations and show that there was always some protein left in solution.

It was also of interest to directly compare the behaviour of BSA at the hydroxyapatite/aqueous solution interface with that observed previously under the same solution conditions, with similar protein samples, at the stainless steel/aqueous solution interface (van Enkevort, 1978). The results of this study (after correction for surface roughness) have therefore been included, where appropriate, with the results obtained using the hydroxyapatite system.

7.2.2 THE EFFECT OF EXPERIMENTAL VARIABLES ON ADSORPTION

(i) Concentration

The effect of concentration on the adsorption of BSA on the non-substituted, fluoride substituted and carbonate substituted hydroxyapatites (OHAp, F-OHAp and CO₃-OHAp respectively) is shown in Figure 7.1. The data were obtained mainly from experiments designed to test the effect of concentration on adsorption but supplementary data from any adsorption studies performed under correct conditions have been included. Data points at final concentrations greater than about 5 g m⁻³ represent mean values of adsorption calculated from solid and liquid activities. Those data below this concentration were determined from solution activity measurements.

Least squares analyses of the data points confirmed that the isotherms were parallel to the concentration axis within experimental error i.e. the saturation limit was observed even at very low concentrations. The mean values and standard deviations for the saturation limits obtained from these analyses are reported in Table 7.1. The same level of saturation adsorption was observed for BSA on OHAp, F-OHAp and CO₃-OHAp. The confusion as to the effect of the incorporation of fluoride into hydroxyapatite is due to a surface area effect. Some workers have treated their hydroxyapatite preparations by exposure to NaF solutions and appear to have assumed no change in the specific surface area of the material (e.g. Belcourt and Voegel, 1978; Voegel et al., 1981). It was observed in the present study that such a treatment increased the effective surface area available for adsorption (Table 4.2). When this change is accounted for in adsorption studies, the same level of adsorption is observed for the non-substituted hydroxyapatite. Similarly, essentially the same level of adsorption was observed for BSA adsorption under the same experimental conditions.

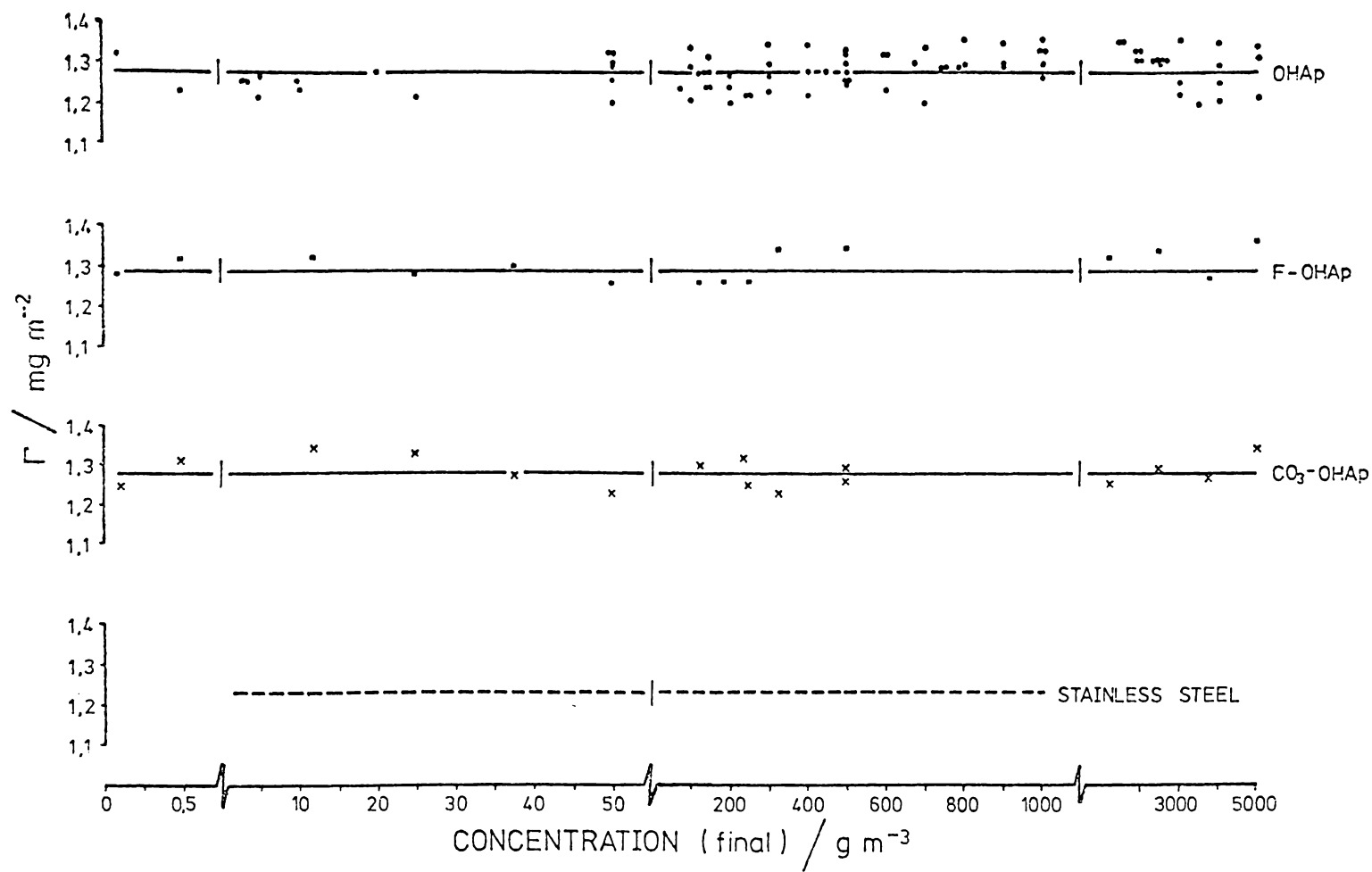


Figure 7.1: The Effect of Concentration on BSA Adsorption on Hydroxyapatites.

TABLE 7.1: SATURATION LIMITS OF SERUM ALBUMIN ADSORPTION ON
HYDROXYAPATITES AND STAINLESS STEEL

SERUM ALBUMIN	ADSORBENT	$\Gamma/\text{mg m}^{-2}$		n
		Mean	δ	
Bovine	OHAp	1.28	0.01	87
	F-OHAp	1.29	0.01	15
	CO ₃ -OHAp	1.28	0.01	16
	Stainless Steel ^(a)	1.23	0.01	146
Human	OHAp	1.19	0.01	19

(a) from van Enkevort, 1978

The shapes of the isotherms suggest that BSA has a high affinity for hydroxyapatites. They are not consistent with the Langmuir or other round shaped isotherms generally reported for these proteins at solid/liquid interfaces. However, values for the saturation limits are of the same order of magnitude as observed in these studies.

It has recently been reported that HSA adsorption on a precipitated stoichiometric hydroxyapatite follows an approximately Langmuirian isotherm (Hlady and Füredi-Milhofer, 1979). Saturation adsorption was attained only at "equilibrium" concentrations of 200 - 300 g m⁻³ or higher. Further, the isotherm was not continuous but showed steps in the entire concentration range studies viz. equilibrium concentrations of 5-400 gm⁻³. These results are contrary to what might be expected for HSA given the similarities between this protein and BSA, and the high affinity isotherms observed for BSA adsorption on the stoichiometric and

substituted hydroxyapatites. The apparent differences in behaviour may reflect a kinetic effect. Hlady and Füredi-Milhofer noted that adsorption was complete after 30 minutes (although no protein concentration or data was presented) and consequently used an adsorption time of 4 hours. The kinetic studies of BSA adsorption on hydroxyapatite reported in this thesis (Section 6.6), using a similar experimental procedure to that used by these authors, showed that a minimum of about 3 hours was required to achieve saturation at an equilibrium concentration of about 200 g m^{-3} (Figure 6.2(C)). Adsorption at lower concentrations required much longer however, e.g. at least 24 hours at 3 g m^{-3} (Figure 6.2(A)), and, provided sufficient time was allowed, adsorption always proceeded to the same saturation coverage at all concentrations. Consequently, an adsorption experiment was performed using HSA and the stoichiometric hydroxyapatite OHAp. At all concentrations sufficient time was allowed to attain maximal adsorption. A high affinity isotherm was observed (Figure 7.2) and a least squares analysis verified that isotherm was parallel to the concentration axis over a concentration range extending from final concentrations of $0.05 - 5\,000 \text{ g m}^{-3}$. It seems likely that the data of Hlady and Füredi-Milhofer, at less than saturation coverages, reflects the adsorption times used. The mean value for the saturation limit was similar to that observed for BSA adsorption on the various hydroxyapatites and stainless steel.

(ii) pH

The effect of pH on BSA adsorption on OHAp at two ionic strengths is shown in Figure 7.3. Data were obtained from experiments designed to test the effect of pH on adsorption in cacodylate and barbital buffers (Appendix VI), at ionic strengths of 0.06 mole l^{-1} and 0.16 mole l^{-1} , and at final solution protein concentrations of $400 - 600 \text{ g m}^{-3}$. No significant differences in adsorption between the two buffers were

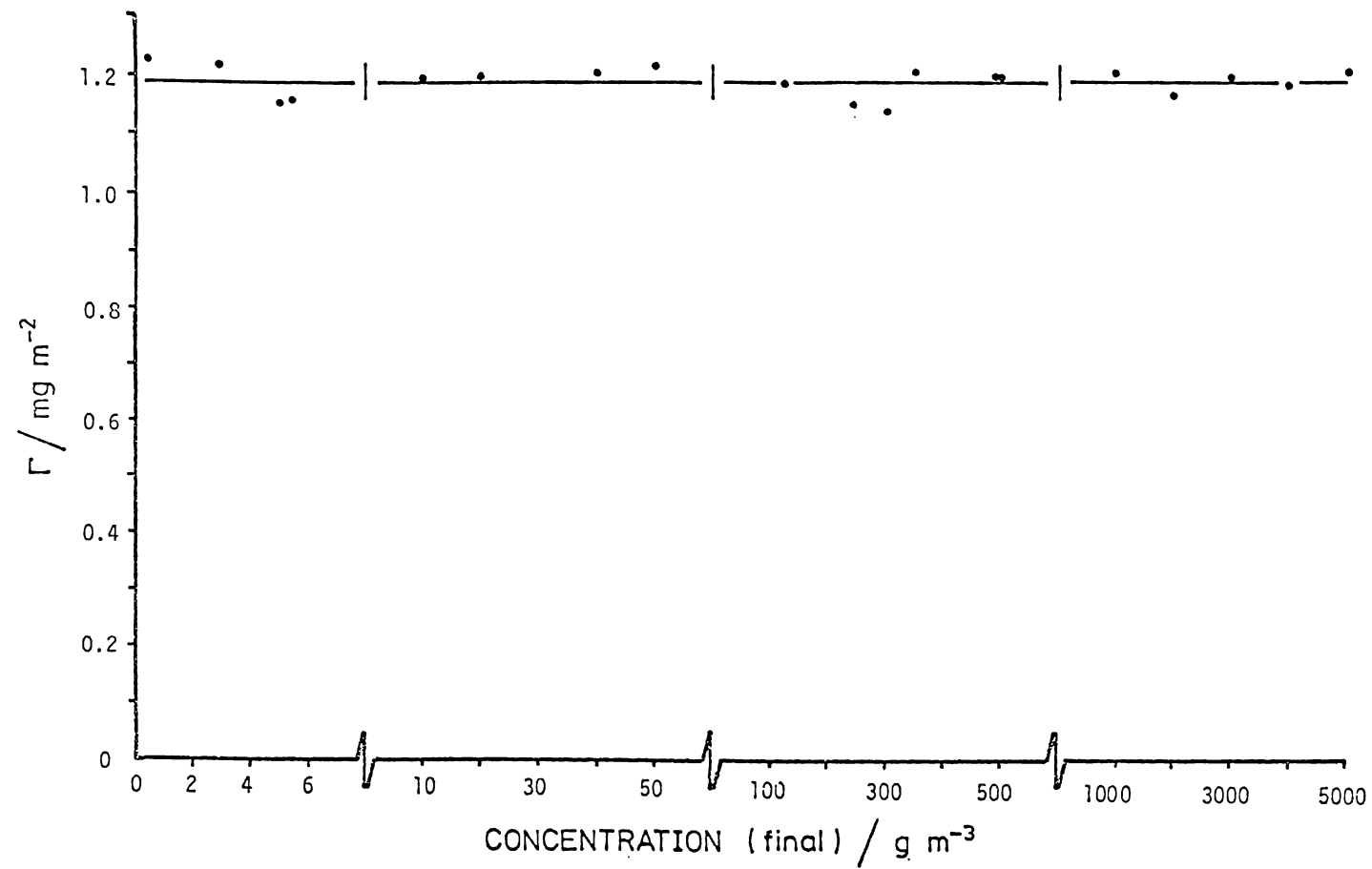


Figure 7.2: The Effect of Concentration on HSA Adsorption on Hydroxyapatite (OHAp)

observed. Supplementary data at an ionic strength of 0.2 mole l^{-1} , obtained from experiments designed to test the effect of ionic strength on adsorption, have also been included.

The amount of adsorption decreased as the pH was shifted away from the region of the IEP of the protein ($\sim \text{pH } 4.8 - 5.0$). This behaviour is typical of that observed for Serum Albumins and other proteins at many solid/liquid interfaces. The level of adsorption appeared to be independent of ionic strength in the region of the IEP. As the pH was shifted from this region, a decrease in ionic strength caused a more marked decrease in the level of adsorption.

Although only two datum points were available for direct comparison of the levels of adsorption observed on hydroxyapatite and stainless steel (0.2 mole l^{-1} , pH 5.1 and pH 6.8), the corresponding points were in good agreement. Further, the shape of curve for the effect of adsorption on stainless steel is similar to that observed for the effect of this variable on adsorption on hydroxyapatite at the lower ionic strengths.

(iii) Ionic Strength

The effect of ionic strength on BSA adsorption on OHAp at pH 5.0 and pH 6.8 is shown in Figure 7.4. Adsorption was studied at a final solution concentration of about $400 - 600 \text{ g m}^{-3}$ in cacodylate buffer. In the region of the IEP, a change in ionic strength from 0.03 mole l^{-1} to 1.0 mole l^{-1} had virtually no effect on the level of adsorption. This confirmed the previous observation that the level of adsorption at the IEP appeared to be independent of ionic strength. At pH 6.8, changes in the ionic strength above about $\sim 0.2 \text{ mole l}^{-1}$ had no effect on the level of adsorption. Below 0.2 mole l^{-1} there was a decrease in the level of adsorption with decreasing ionic strength.

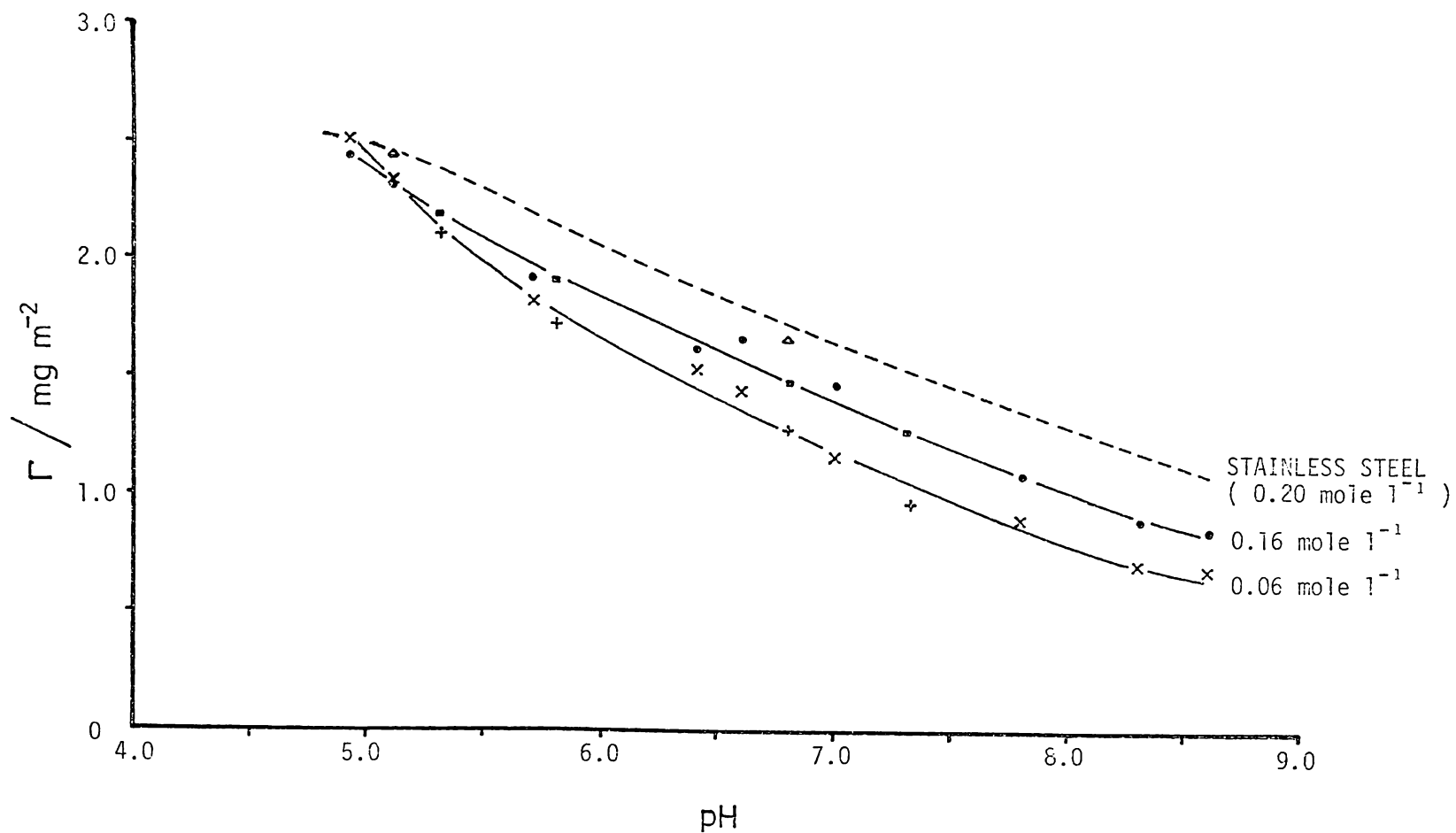


Figure 7.3: The Effect of pH on BSA Adsorption on Hydroxyapatite (CHAp):

Ionic Strength 0.06 mole l⁻¹: Barbital - HCl buffer (x); Cacodylate - HCl buffer (+).
 Ionic Strength 0.16 mole l⁻¹: Barbital - HCl buffer (•); Cacodylate - HCl buffer (◻).
 Ionic Strength 0.20 mole l⁻¹: Cacodylate - HCl buffer (Δ).

The effect of ionic strength (adjusted using NaCl) on adsorption on hydroxyapatite at pH 6.8 closely followed that observed for stainless steel at the same pH.

(iv) Temperature

The effect of temperature on BSA adsorption on OHAp at pH 4.9 and pH 6.8 is shown in Figure 7.5. While little change in the level of adsorption was observed at pH 4.9 between 0°C and 30°C, a significant increase resulted when the temperature was raised to 50°C. However, at pH 6.8, increasing levels of adsorption were observed when the temperature was raised above 4°C. A dramatic increase in adsorption was observed at both pH's when the temperature was increased from 40°C to 50°C.

The effect of temperature on BSA adsorption on stainless steel showed a good quantitative agreement with that observed for adsorption on hydroxyapatite at the same pH at 0°C and 4°C. As the temperature was increased, an increasing divergence between the levels of adsorption was observed although the overall behaviour was similar. The less pronounced effect of temperature on adsorption on stainless steel reflects on artifact due to the use of an adsorption time which was insufficient to allow saturation adsorption.

7.2.3 SUMMARY

Both BSA and HSA had a high affinity for the non-substituted and substituted hydroxyapatites. The adsorption process proceeded to saturation at all concentrations studied. The same level of adsorption was achieved on all three adsorbents. The effects of the variables pH, ionic strength and temperature were studied. It was observed that the

effect of ionic strength and temperature was related to the pH of the adsorption solution. At the IEP, changes in these variables generally did not alter the level of adsorption but at pH's away from the IEP, significant effects were observed. Increases in temperature above 40°C caused a large increase in adsorption.

The adsorption behaviour of BSA on hydroxyapatite generally paralleled that observed for adsorption on stainless steel and also showed a good quantitative agreement.

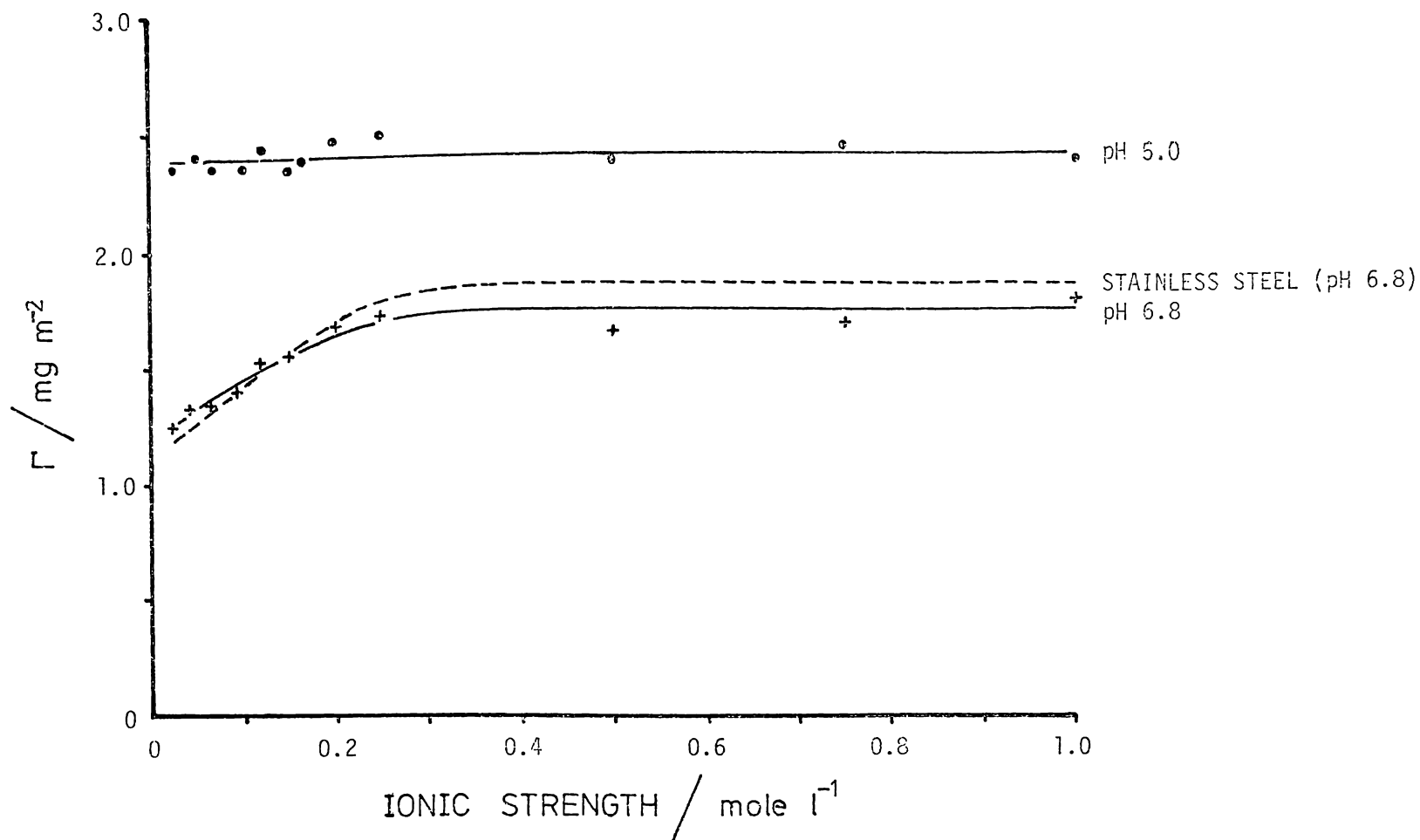


Figure 7.4: The Effect of Ionic Strength on BSA Adsorption on Hydroxyapatite (OHAp).

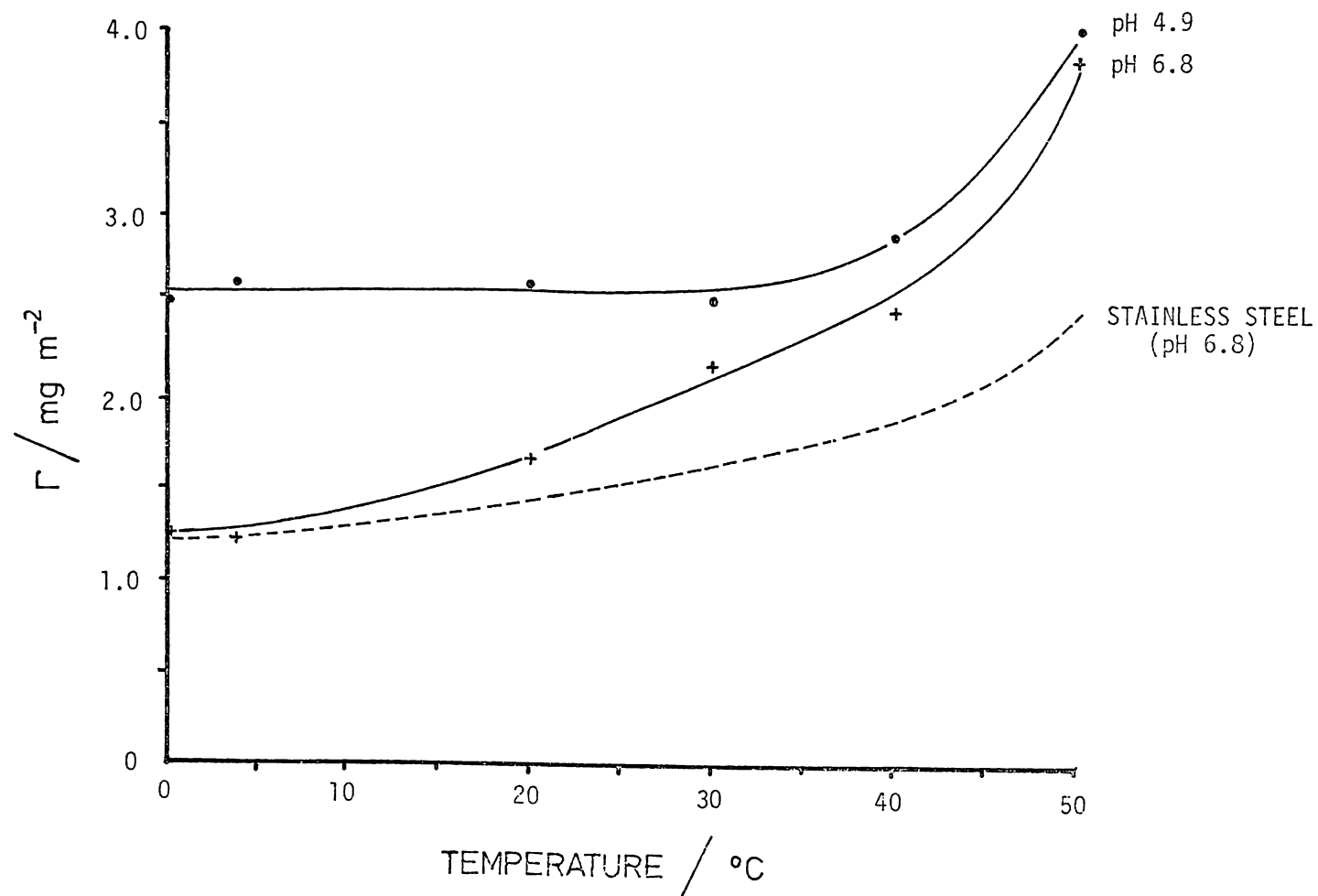


Figure 7.5: The Effect of Temperature Adsorption on Hydroxyapatite (OHAp).

7.3 β -CASEIN

7.3.1 INTRODUCTION

There is very little work in the literature describing the adsorption of β -Casein at solid/liquid interfaces. Nisbet (1979) has studied the adsorption of this protein on stainless steel and observed a stepped isotherm. This appeared to be related to a change in the configuration of the adsorbed molecules. It was of interest to ascertain if this might be related to the known (reversible) self-association of this protein. This phenomenon is particularly affected by changes in concentration, temperature and the presence of calcium ions. Therefore the effects of these variables on adsorption (at about 50% solution depletion) were studied using radiochemical techniques. Protein concentrations quoted are the initial concentrations as this determines the form of the protein initially present in solution. Care was taken to ensure that the β -Casein solutions would be at equilibrium prior to adsorption studies. An equilibrium period of 24 hours was used for all experiments at 4°C. Solutions to be used in studies at 20°C were initially allowed to stand at 4°C for 24 hours, to disrupt any micelles, before being brought to temperature and again allowed to stand for 24 hours to come to equilibrium. This procedure was adopted as it has been suggested that the equilibrium of β -Casein solutions might be a slow process (Payens and van Marwijk, 1963).

7.3.2 THE EFFECT OF EXPERIMENTAL VARIABLES ON ADSORPTION

(i) Concentration

The effect of concentration on the adsorption of β -Casein (Batch 1, refer Appendix II) on the non-substituted hydroxyapatite OHAp is shown in Figure 7.6. Data were obtained from two experiments designed to test the effect of concentration: the first experiment covering the range $5\ 000 - 20\ \text{g m}^{-3}$, the second extending the isotherm from $20 - 0.1\ \text{g m}^{-3}$. This also confirmed the presence of the steep rise in adsorption from $\sim 0.8\ \text{mg m}^{-2}$, at concentrations below $400\ \text{g m}^{-3}$, to more than $3.2\ \text{mg m}^{-2}$ at concentrations of $450\ \text{g m}^{-3}$ and higher. The adsorption isotherm for a different β -Casein sample (Batch 2, refer Appendix II), purchased later in this study, is also shown in Figure 7.6. The rise in adsorption of β -Casein from Batch 2 occurred at the lower concentration of $\sim 1\ \text{g m}^{-3}$ but over a similarly narrow concentration range. The corresponding values of the plateau levels of adsorption were essentially equal. No significant differences were observed for adsorption on the non-substituted, the fluoride substituted and the carbonate substituted adsorbents. The observation that the plateau region at low concentrations of β -Casein from Batch 1 extended over a wide concentration range and to the lowest concentration studied (and that β -Casein from Batch 2 also displayed this plateau but from a lower concentration) suggests that β -Casein has a high affinity for hydroxyapatites.

The occurrence of a stepped isotherm for β -Casein adsorption has previously been observed at the stainless steel/aqueous solution interface by Nisbet (1979). The step occurred over a wider concentration (about an order of magnitude) and was little affected by a temperature increase from 4°C to 20°C . Although the absolute values of the plateau levels of adsorption are of the same order of magnitude in both studies,

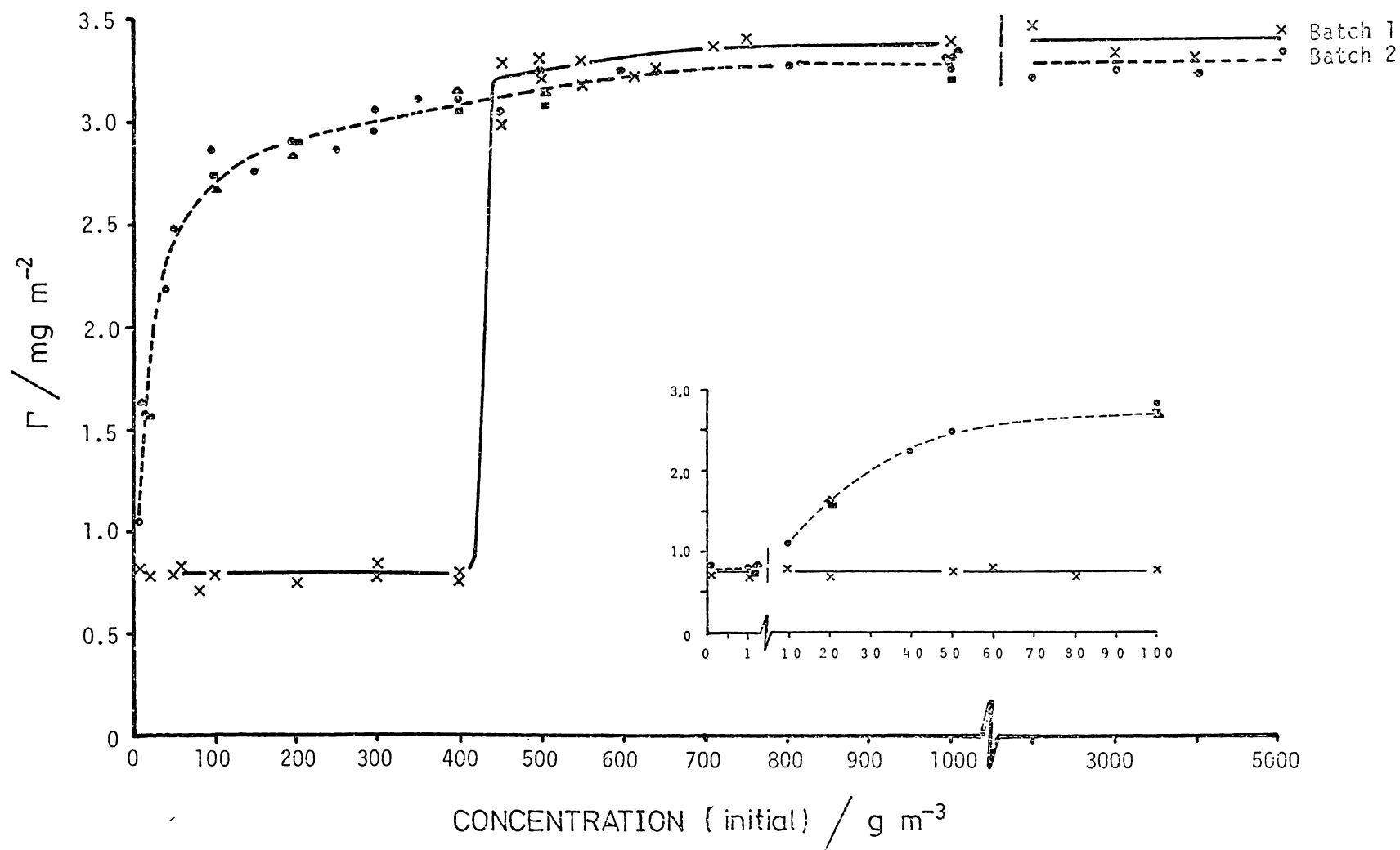


Figure 7.6: The Effect of Concentration on β -Casein Adsorption on Hydroxyapatites: OHAp (x, •), F-OHAp (■), CO₃-OHAp (Δ).

some quantitative difference (given the same solution conditions and surface roughness of stainless steel) was apparent. The plateau levels of adsorption in the hydroxyapatite system are some 25 - 30% lower than those observed by Nisbet. This may be attributed to the use of different β -Casein preparations and/or to floc formation. It was observed in the adsorption experiments with β -Casein that the protein-hydroxyapatite suspensions settled rapidly due to the formation of large flocs at all protein concentrations, levels of adsorption and solid : volume ratios. These did not form provided sufficient agitation of the adsorption solution was maintained. End over end rotation of the adsorption vials every 2 seconds was sufficient to prevent macroscopic floc formation.

(ii) Temperature

The effect of temperature on the adsorption of β -Casein was studied by comparison of adsorption data, at 4°C and 20°C (Figure 7.7). Data were obtained for both batches of β -Casein at 20°C at concentrations above 10 g m^{-3} . Adsorption data below this concentration could not be obtained as the time required to reach saturation was such that saturation adsorption could not be obtained before significant breakdown of the protein-radioiodine bond occurred. Although the radiolabelled protein was stable for up to 14 days at 4°C, the use of higher temperatures led to faster breakdown. β -Casein from both Batch 1 and Batch 2 gave the same level of adsorption at 20°C as observed for the adsorption of Batch 2 at 4°C. Thus, while the temperature change from 4°C to 20°C had no significant affect on the adsorption of β -Casein from Batch 2, that of Batch 1 was markedly affected: the step in the adsorption isotherm was displaced to a lower concentration at the higher temperature.

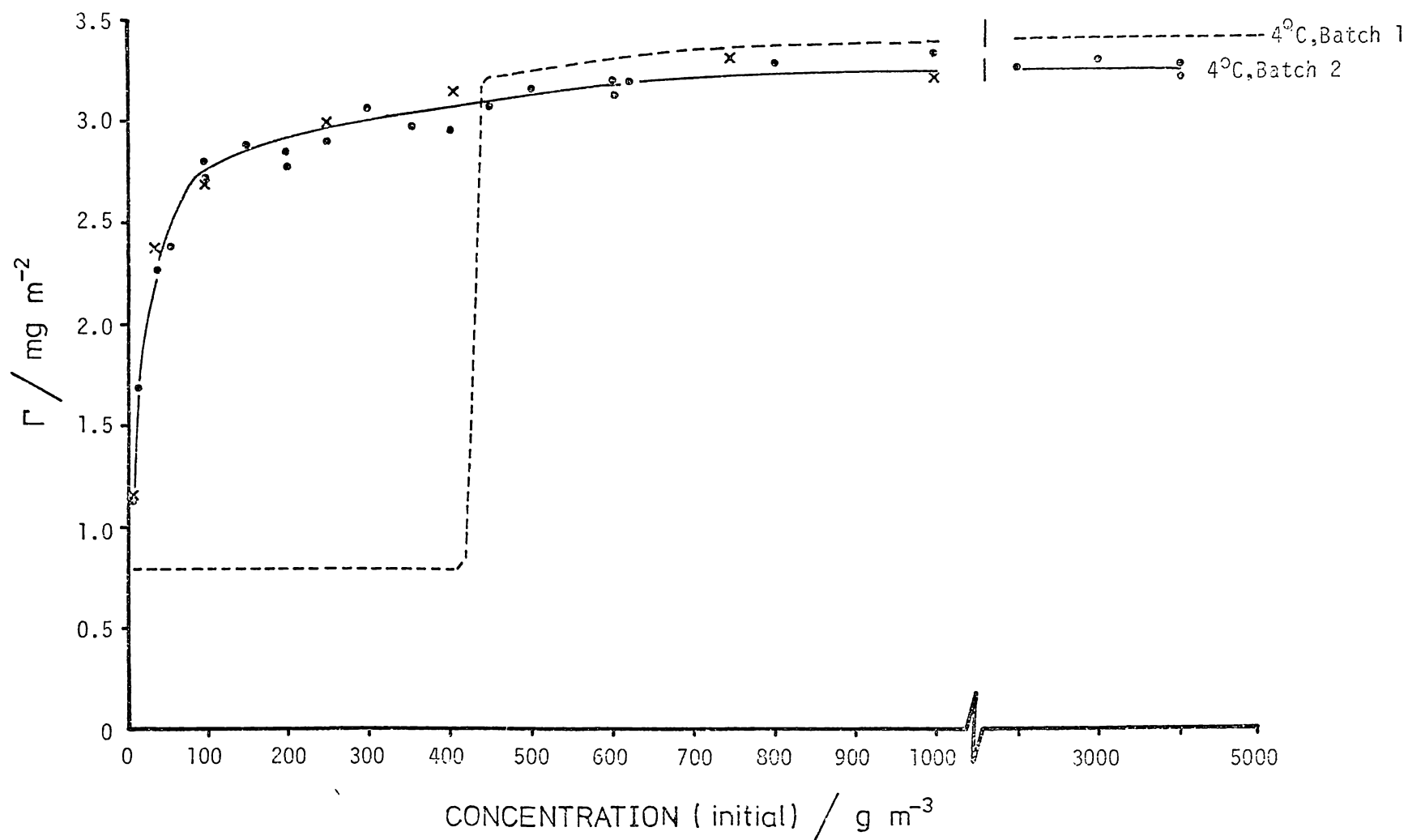


Figure 7.7: The Effect of Temperature on β -Casein Adsorption on Hydroxyapatite (OHAp):
20°C, Batch 1 (•), Batch 2 (x)

(iii) Effect Of Calcium Ions

The effect of calcium ions on the adsorption of β -Casein (Batch 2) at 4°C and 20°C is shown in Figure 7.8. The calcium concentration was 0.005 mole l⁻¹ and the total ionic strength maintained at 0.06 mole l⁻¹ by appropriate dilution of the adsorption buffer. Data for adsorption at 20°C and concentrations below 25 g m⁻³ could not be obtained as significant breakdown of the protein-radioiodine bond again occurred before saturation adsorption was attained (see (ii) above). Least squares analyses of the data points at concentrations above 200 g m⁻³ confirmed that the plateau regions of the isotherms were parallel to the concentration axis.

The presence of calcium ions enhanced the level of adsorption at 4°C and an increase in temperature to 20°C further enhanced adsorption. The step in the adsorption isotherm observed previously in solutions without calcium ions was shifted to a lower concentration in their presence.

The observed effect of calcium raised the question of whether calcium contamination caused the differences in adsorption behaviour of β -Casein from Batch 1 and Batch 2. However, experiments to determine the calcium concentration by Atomic Absorption showed that this was negligible in both samples.

7.3.3 SUMMARY

β -Casein has a high affinity for hydroxyapatite. The concentration adsorption isotherm at low concentrations was parallel to the concentration axis and saturation adsorption was observed even at very low concentrations. With increasing concentration a step was observed in the adsorption isotherm. This gave rise to a second and higher

saturation level of adsorption and hence the adsorption isotherm consisted of two plateau regions. The saturation levels of adsorption were not affected by the presence of fluoride or carbonate substitutions in the hydroxyapatite lattice.

Two β -Casein samples were used during the course of the experiments and these displayed differences in their adsorption behaviour. Although the saturation levels of adsorption were the same, the step in the adsorption isotherm occurred at a lower concentration for β -Casein of Batch 2 than for the original sample, Batch 1. The two samples also displayed differences to the effect of temperature on adsorption. The adsorption isotherm for Batch 2 was not affected by a temperature increase from 4°C to 20°C. The isotherm for Batch 1 was shifted to a lower concentration. The resulting isotherm was indistinguishable from that of the Batch 2 sample.

The presence of calcium ions enhances adsorption, at least for adsorption above the lower plateau, at both 4°C and 20°C. The step in the adsorption isotherm was again shifted to a lower concentration.

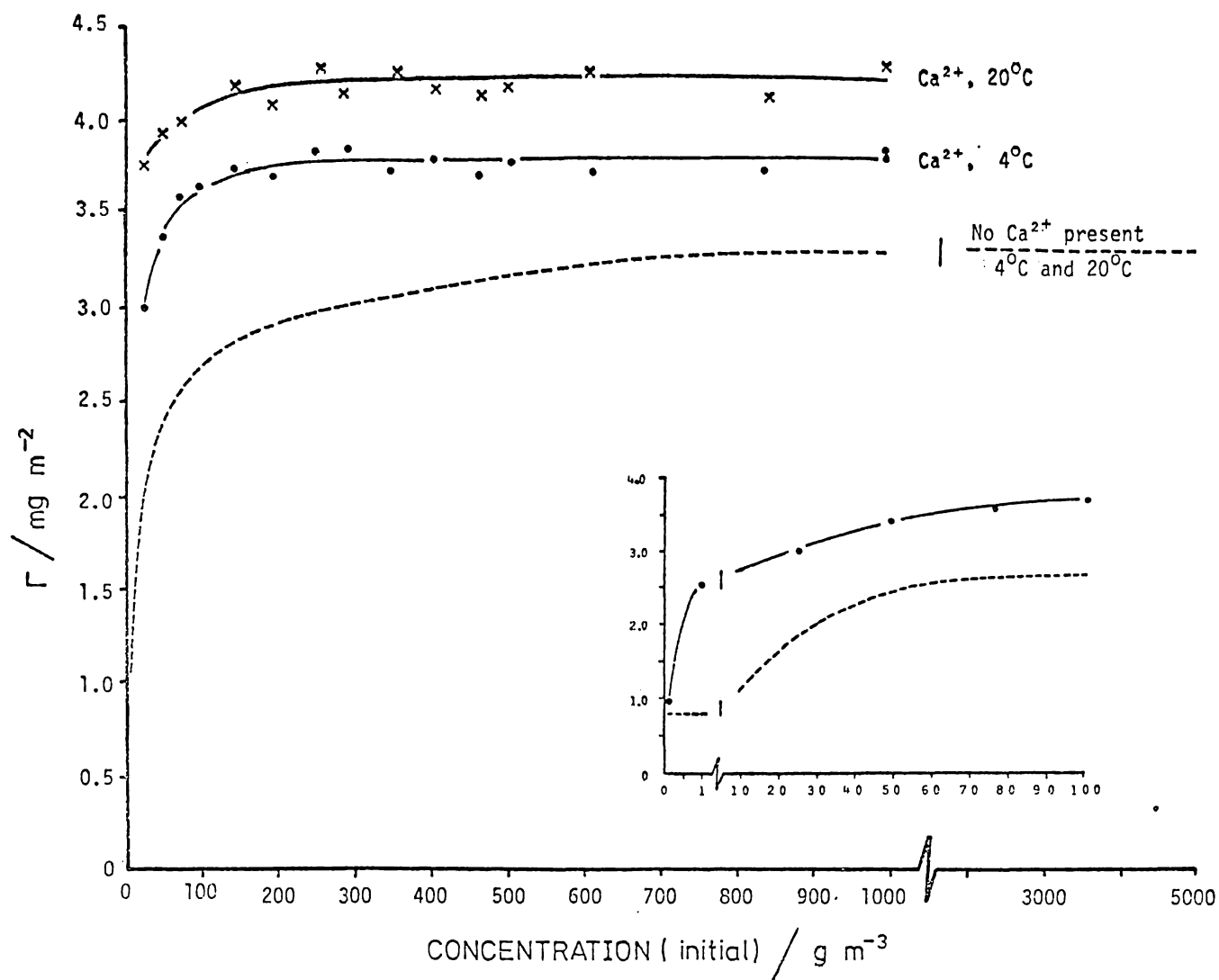


Figure 7.8: The Effect of Calcium Ions on β -Casein Adsorption on OHAp at 4°C and 20°C .

7.4 β -LACTOGLOBULIN, HAEMOGLOBIN, MYOGLOBIN

7.4.1 INTRODUCTION

It was of interest to determine whether other proteins would display the high affinity type adsorption behaviour observed for BSA, HSA and β -Casein. The effect of concentration on the adsorption of β -Lactoglobulin, Haemoglobin and Myoglobin was studied. Data for β -Lactoglobulin were obtained from radiochemical experiments, and from non-radiochemical experiments using spectrophotometric methods for the haem proteins. The experiments were designed to test the effect of concentration at about 50% depletion and data have been plotted as the amount of adsorption versus final concentration.

7.4.2 CONCENTRATION ADSORPTION ISOTHERMS

The effect of concentration on the adsorption of β -Lactoglobulin, Haemoglobin and Myoglobin is shown in Figures 7.9, 7.10 and 7.11 respectively. Least squares analyses of the data confirmed that the isotherms for all three proteins were parallel to the concentration axis within experimental error. The mean values and standard deviations for the saturation limits obtained from these analyses are reported in Table 7.2. The isotherm for Haemoglobin contrasts to the 'S' shaped isotherm observed by Pearce and Bibby (1966b) for the adsorption of this protein on tooth enamel.

The shapes of the isotherms suggest that the proteins have a high affinity for hydroxyapatites. The levels of adsorption for each protein were the same for the non-substituted and substituted hydroxyapatites.

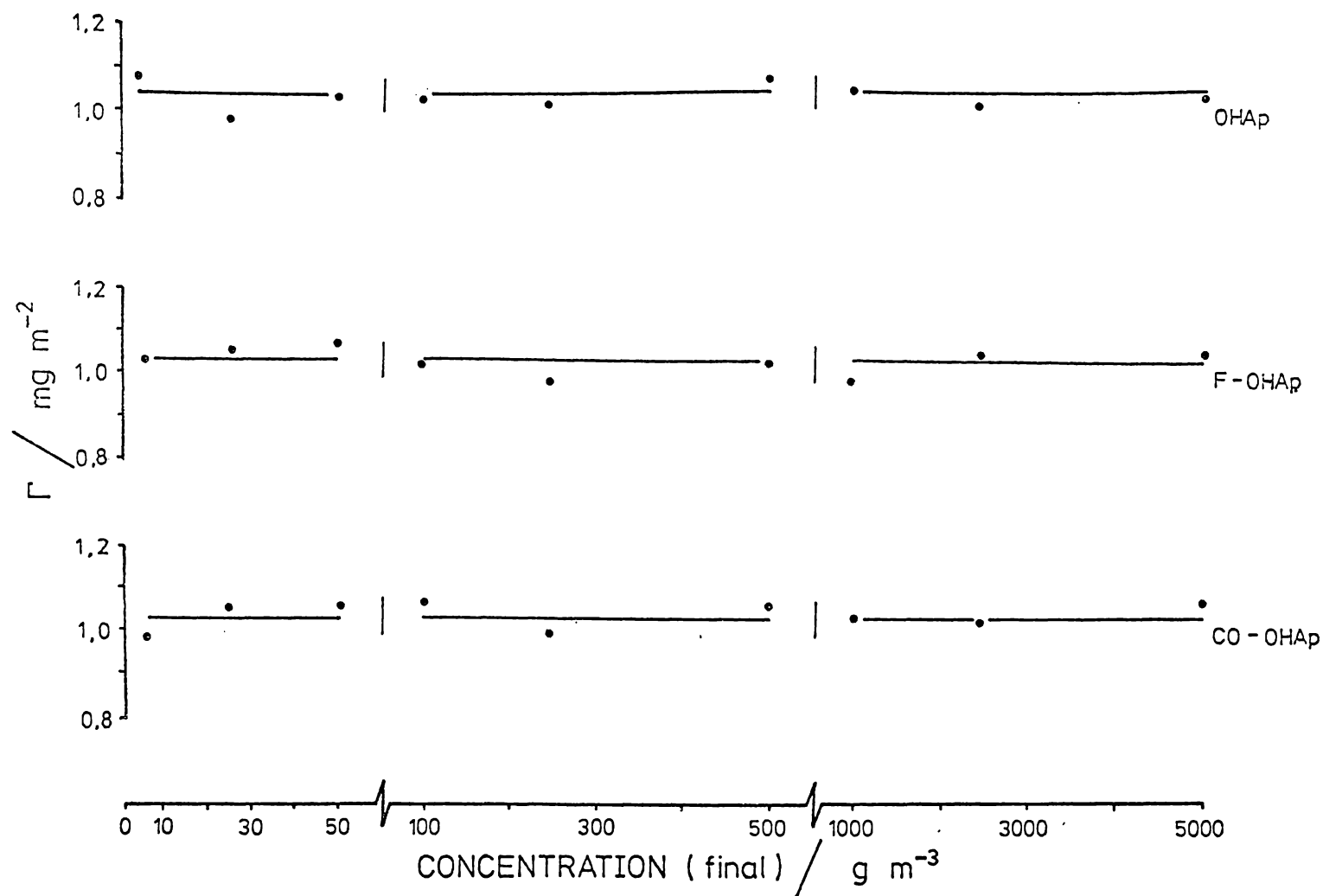


Figure 7.9: The Effect of Concentration on β -Lactoglobulin Adsorption on Hydroxyapatites.

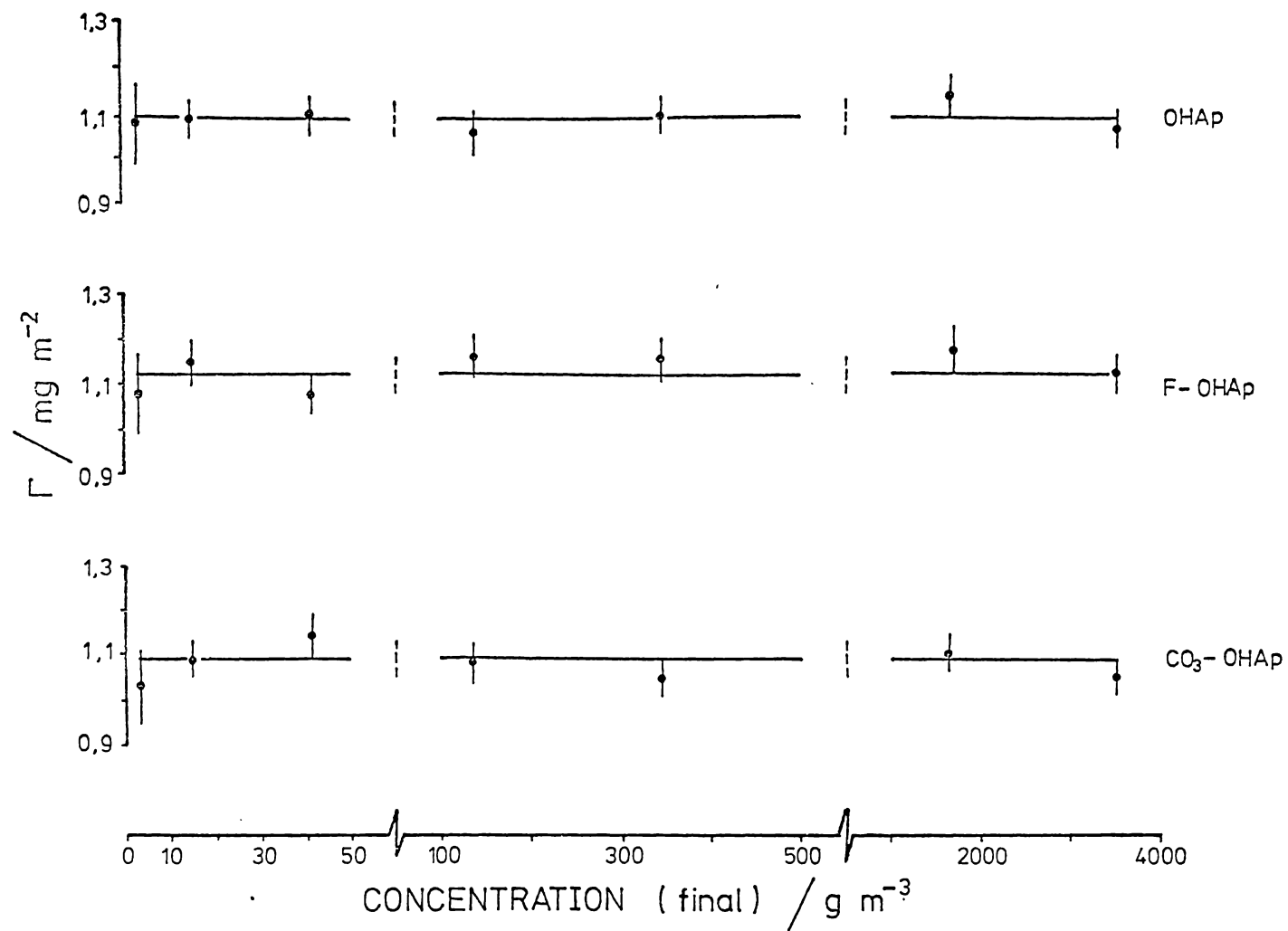


Figure 7.10: The Effect of Concentration on Haemoglobin Adsorption on Hydroxyapatites.

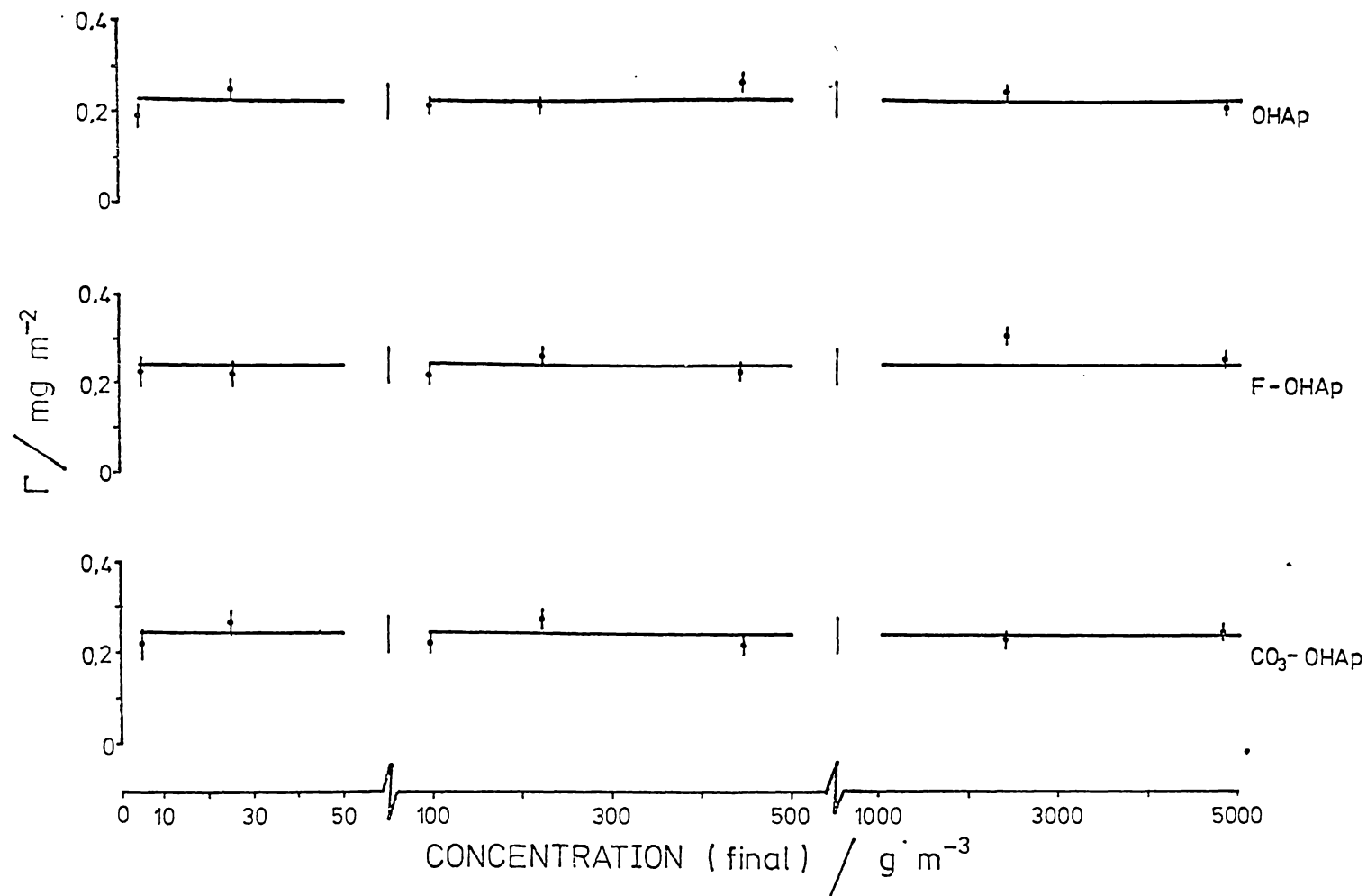


Figure 7.11: The Effect of Concentration on Myoglobin Adsorption on Hydroxyapatites.

TABLE 7.2: SATURATION LIMITS OF β -LACTOGLOBULIN, HAEMOGLOBIN AND
MYOGLOBIN ON HYDROXYAPATITES

PROTEIN	ADSORBENT	$\Gamma/\text{mg m}^{-2}$		n
		Mean	δ	
β -Lactoglobulin	OHAp	1.03	0.02	9
	F-OHAp	1.02	0.01	9
	CO ₃ -OHAp	1.02	0.02	9
Haemoglobin	OHAp	1.09	0.01	7
	F-OHAp	1.12	0.02	7
	CO ₃ -OHAp	1.08	0.02	7
Myoglobin	OHAp	0.23	0.01	7
	F-OHAp	0.24	0.01	7
	CO ₃ -OHAp	0.24	0.01	7

7.4.3 SUMMARY

Although suitable radiochemical tracers of Haemoglobin and Myoglobin could not be prepared by the iodination methods used in this study, their adsorption could be successfully monitored by spectrophotometric methods. It was observed that these proteins and β -Lactoglobulin showed a high affinity for all three hydroxyapatites and adsorption proceeded to saturation even at the lowest concentrations studied. The presence of fluoride or carbonate in the hydroxyapatite lattice did not affect the level of adsorption.

7.5 SUMMARY

An important observation in the adsorption studies of BSA, HSA, β -Lactoglobulin, Haemoglobin and Myoglobin was that the proteins had a high affinity for the non-substituted and substituted hydroxyapatites. Adsorption proceeded to saturation coverage at all concentrations provided sufficient protein was present in solution and adequate time was allowed. The rounded adsorption isotherm observed previously by some authors for HSA adsorption on hydroxyapatite was shown to arise from an inadequate allowance of time for adsorption to proceed to saturation. At relatively low protein concentrations, adsorption on suspended hydroxyapatites is a slow process and this accounts for the isotherm reported in the literature i.e. the isotherm reflects the kinetics of adsorption.

The adsorption of BSA was studied in greater detail and was modified by changes in pH, ionic strength and temperature. The effects of ionic strength and temperature were observed to be pH dependent. Generally, the results paralleled those observed in a previous study of the adsorption of this protein on stainless steel under similar conditions (van Enkevort, 1978).

The adsorption of β -Casein was similarly studied in some detail. β -Casein also displayed a high affinity for hydroxyapatite but a pronounced step in the adsorption isotherm, giving an increased level of adsorption, was observed with increasing concentration. The isotherm therefore consisted of two distinct plateau levels of adsorption. The transition from the lower to the higher level occurred over a limited concentration range. The stepping effect varied with the particular batch of β -Casein in use in the first instance. The effect was temperature dependent and sensitive to the presence of calcium ions, and also

varied between the different batches. Generally, the results were similar to those observed in a previous study of the adsorption of this protein on stainless steel (Nisbet, 1979). The adsorption of β -Casein caused massive flocculation of the hydroxyapatite suspensions if these were not sufficiently agitated.

The incorporation of fluoride and carbonate into the hydroxyapatite lattice did not affect the saturation levels of adsorption of any of the proteins studied. It appeared that some of the confusion in the literature regarding the effect of the presence of fluoride in hydroxyapatite was due to the lack of characterisation of surface area changes upon modification of the adsorbent.

CHAPTER 8

EXCHANGE AND DESORPTION STUDIES

8.1 INTRODUCTION

As part of the study of protein adsorption, it was of interest to determine the exchangeability and desorbability of the adsorbed film. Such experiments can provide information on the reversibility of adsorption, the nature of the interaction between the adsorbate and adsorbent and further information on the nature of the adsorbed film. The proteins films used were those of BSA and β -Casein, and the results of previous studies at this University using these proteins and stainless steel (van Enkevort, 1978; Nisbet, 1979) have been noted for comparison.

The results of the studies reported in this Chapter will be discussed in Chapter 11.

8.2 METHODS AND MATERIALS

8.2.1 ADSORPTION

The adsorption of protein to hydroxyapatite was achieved as described in Section 6.5 except that the amount of hydroxyapatite used was sufficient to obtain ~95% depletion of solution protein. The adsorption solution concentration and volume was such that this corresponded to about 4×10^{-3} g protein adsorbed. A higher specific activity of solution protein (up to 3x) than typically used in adsorption studies was usually employed to facilitate the monitoring of solution activities during the dilution and exchange experiments. After adsorption, the suspended hydroxyapatite was allowed to settle for 24 hours and as much as possible of the suspending medium removed. The volume of the supernate was determined and the solution then centrifuged prior to determination of the residual solution activity. A 0.025 ml aliquot of the remaining hydroxyapatite suspension (of known volume and mass of hydroxyapatite) was removed to determine the solid activity.

8.2.2 EXCHANGE AND DESORPTION

Although exchange and desorption studies are often facilitated by continuous monitoring techniques, the small size of the hydroxyapatite particulates made it difficult to set up such systems and maintain reasonable flow rates. Consequently, a batchwise method of periodic sampling was used.

(i) Exchange

A known quantity of the adsorbed protein/hydroxyapatite suspension was added to 50 ml of a protein solution containing a 100 fold mole

excess of protein. Exchange was carried out under the same conditions as those used for adsorption. Solutions were stirred continuously by a magnetic stirrer and follower.

(ii) Desorption

Desorption was tested by the addition of a quantity of an adsorbed protein/hydroxyapatite suspension (containing a known amount of solution and adsorbed protein) to 1 litre volumes of the desorption solution. Desorption upon dilution was tested at the temperature of the adsorption experiment (i.e. 4°C) but all other experiments were performed at 20°C. The desorption solutions were continuously stirred by magnetic stirrer and follower.

The desorption upon dilution experiments were designed so that non-reversibility was not an experimental artifact within the limits of the experiment. The total amount of protein in the hydroxyapatite suspension (solution and adsorbed) was sufficiently low so that even if all the adsorbed protein was desorbed, the final solution protein concentration was lower than the lowest used in the adsorption studies.

8.2.3 DETERMINATION OF EXCHANGE AND DESORPTION

In order to keep the amounts of activity used to a minimum, exchange and desorption solution activities were monitored by the more sensitive liquid scintillation counting technique (Section 5.3.4). Solution activities as determined by this method were 2.6 x higher than those determined by solid scintillation. 2 ml aliquots of the exchange or desorption solutions were removed periodically and centrifuged immediately. 1.0 ml aliquots of the supernate were used for activity determinations. Initial activities were taken immediately upon mixing.

Quenching effects, if any, were determined by preliminary experiments. Generally, exchange and desorption were allowed to proceed for up to 6 days. The hydroxyapatite suspension was allowed to settle and as much of the supernate removed as was necessary to leave an easily handled volume of solution. The remainder was centrifuged to obtain the final solution activity (by liquid scintillation) and, if sufficient solid was present, also the solid activity (by solid scintillation). The percentage exchange or desorption was calculated from the gain in solution activity and loss of solid activity.

8.3 EXCHANGE AND DESORPTION STUDIES USING BSA

8.3.1 EXCHANGE

The results of experiments to test the exchangeability of adsorbed BSA are shown in Figure 8.1. A rapid initial exchange of more than 35% of the adsorbed protein was observed within the first 10 minutes. This had increased to more than 50% after 1 hour and a constant level of exchange of almost 90% was observed after 3 days. Given that the exchange solutions contained a 100 fold excess of protein over that adsorbed, this level of exchange indicated that the adsorbed BSA was almost totally exchangeable. The exchange behaviour of the protein on all three hydroxyapatite adsorbents was essentially the same. However, BSA adsorbed on stainless steel was not exchanged (van Enkevort, 1978).

8.3.2 DESORPTION UPON DILUTION

The results of experiments to test the effect of dilution upon adsorption of BSA on all three hydroxyapatite adsorbents are shown in Figure 8.2. In all cases less than 3% desorption was observed immediately upon mixing of the suspended hydroxyapatites with either the adsorption buffer or distilled water. No further desorption occurred over the next 5 days. BSA adsorption on stainless steel was also observed to be irreversible to dilution (van Enkevort, 1978).

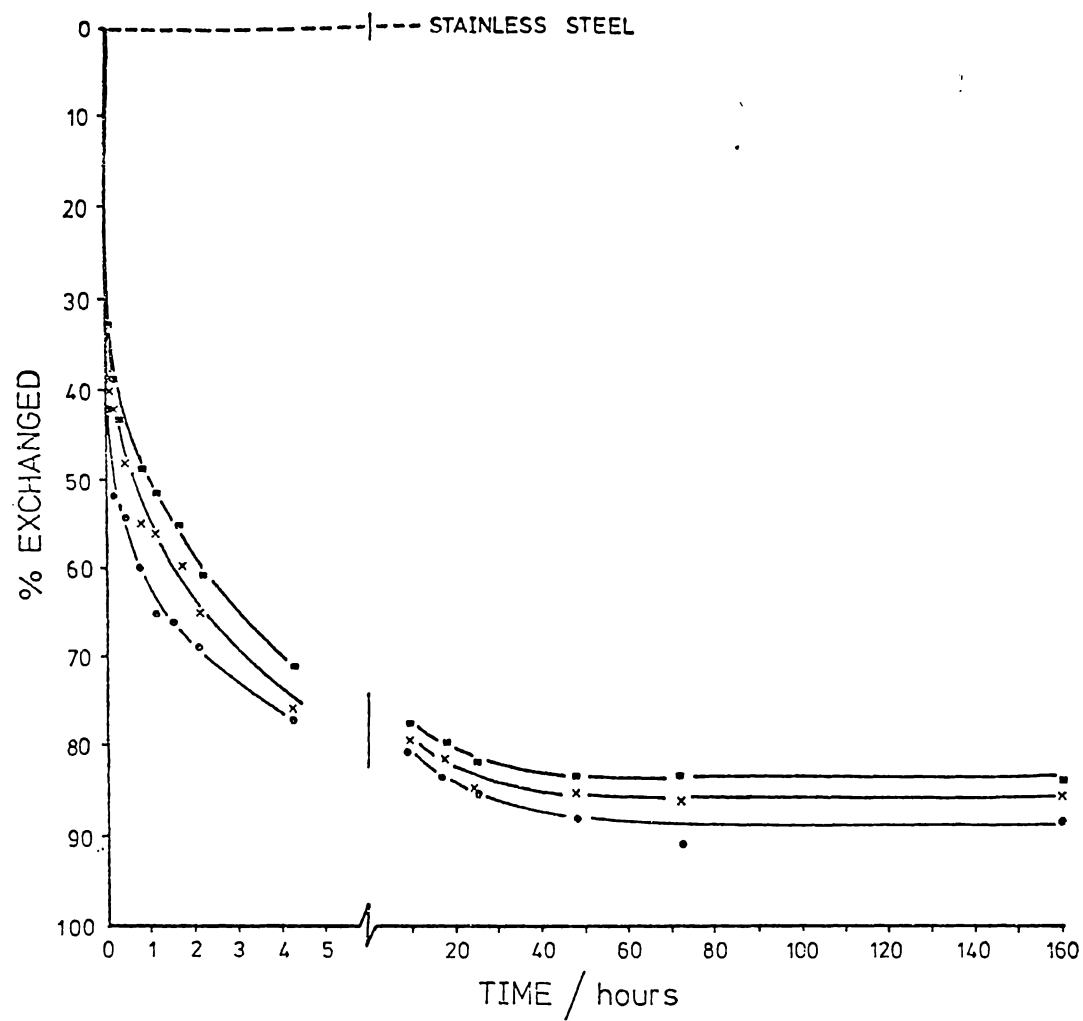


Figure 8.1: The Kinetics of Exchange of BSA Adsorbed on Hydroxyapatites:
OHAp (x); F-OHAp (■); CO₃-OHAp (●).

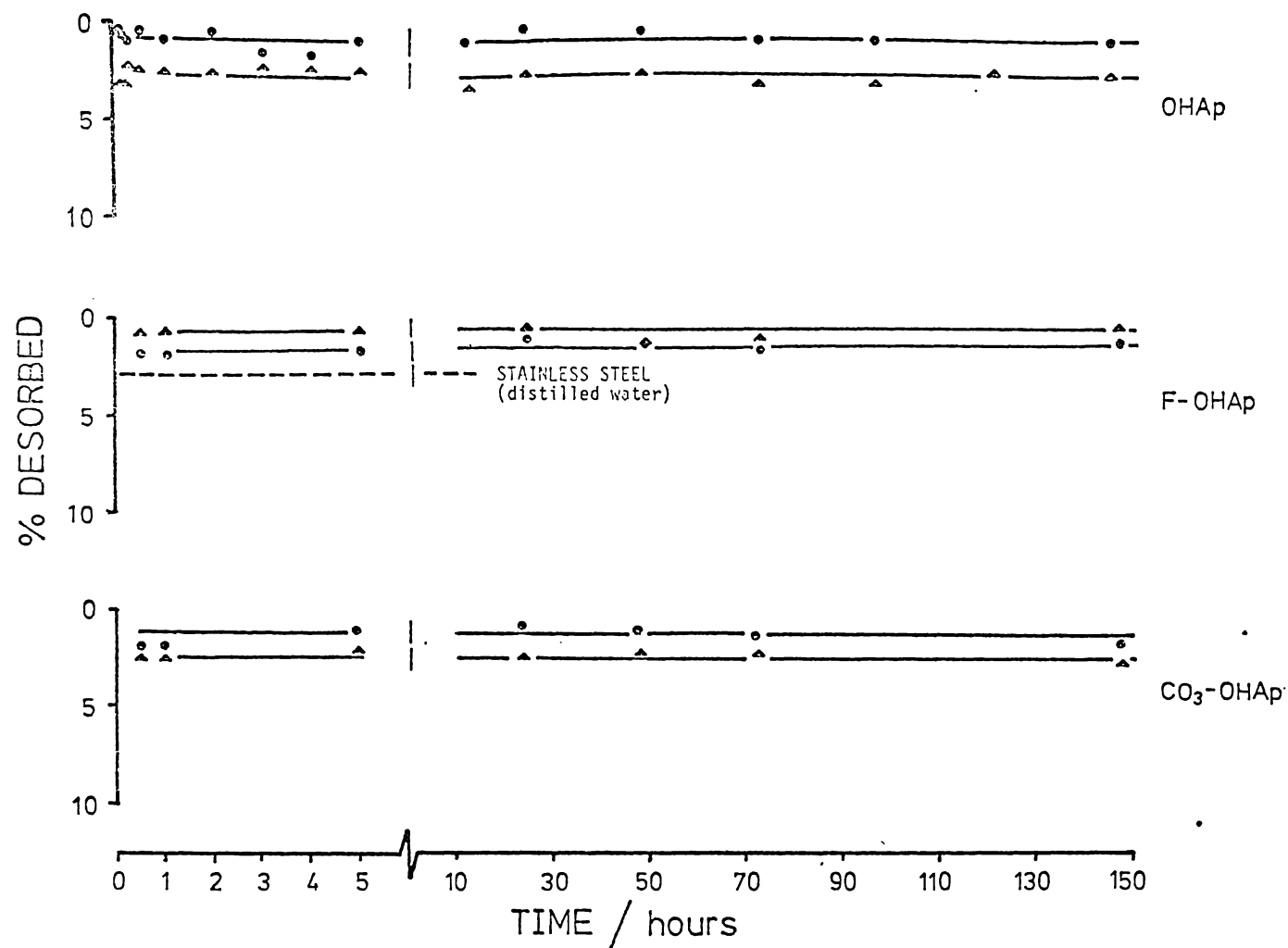


Figure 8.2: The Kinetics of Desorption of BSA Adsorbed on Hydroxyapatites:
Distilled Water (•); Cacodylate Buffer (▲).

8.3.3 INDUCED DESORPTION

The effectiveness of some reagents in causing desorption has been tabulated in Table 8.1. Both calcium chloride and sodium chloride were ineffective in causing desorption at concentrations of 4 mole l^{-1} at pH 6.8. These results are similar to those reported by Bernardi et al., (1972), using concentrations of 3 mole l^{-1} , and support his postulate that anionic proteins are not desorbed by anions that have a low affinity for calcium. However, even though phosphate does have a high affinity for calcium ions, it appeared to have little effect on desorption at the pH studied and the amount of desorption was not greatly enhanced over that observed for solutions of equivalent pH but no phosphate. A similar effect was observed for the detergent sodium dodecyl sulphate (SDS) although urea did enhance desorption more markedly. No reagent caused 100% desorption.

TABLE 8.1: EFFECTIVENESS OF SOME REAGENTS IN CAUSING DESORPTION OF ADSORBED BSA

DESORPTION SOLUTION	1 Day	% DESORBED 2 Days	3 Days
NaCl, 4 mole l^{-1} , pH 6.8	6	5	7
CaCl ₂ , 4 mole l^{-1} , pH 6.8	4	5	5
Na ₂ HPO ₄ , 2 mole l^{-1} , pH 10.8	55	59	60
NaOH, pH 10.8	52	53	51
SDS, 50 g l^{-1} , pH 10.8	50	52	53
Urea, 6 mole l^{-1} , pH 10.8	54	64	76

8.4 EXCHANGE AND DESORPTION STUDIES USING β -CASEIN

Two plateau regions were observed in the concentration adsorption isotherm of β -Casein. It was of interest to determine whether the adsorbed material behaved similarly in exchange and dilution experiments. Although experiments at the lower plateau would have been facilitated using β -Casein of Batch 1 (refer Appendix II), the original sample of this material was exhausted. A further sample of the batch was obtained after communication with the supplying company (United States Biochemical Corporation) but the material was quite different in appearance (pale yellow granules as opposed to the original white powder) and did not display the same adsorption behaviour. The dilution and exchange experiments were therefore conducted using β -Casein from Batch 2.

In view of the low protein concentration and very small amounts of hydroxyapatite used in the initial adsorption step at the low plateau, it was fortunate that the β -Casein/hydroxyapatite suspension tended to flocculate and make separation easier. Even so, quantitative recovery of the adsorbed protein/hydroxyapatite material was difficult and provided the major source of error in these experiments. This is reflected in the apparently higher degrees of exchange and desorption calculated for studies at the low plateau level.

Induced desorption studies were only conducted using β -Casein adsorbed at the high plateau level of adsorption.

8.4.1 EXCHANGE

The results of experiments to test the exchangeability of β -Casein adsorbed at both the high and low plateau levels are shown in Figure 8.3. No exchange of adsorbed protein was observed even after 5 days.

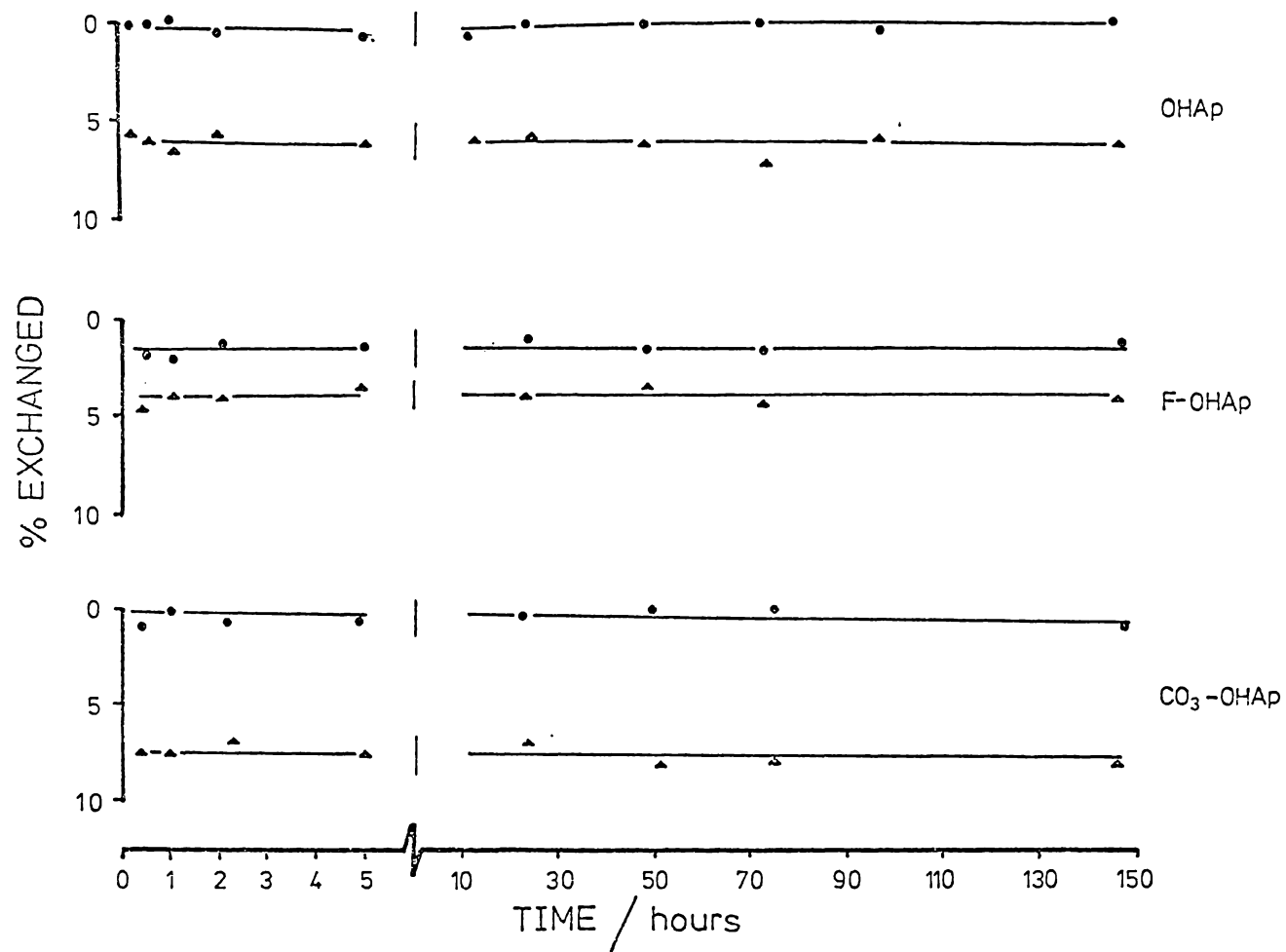


Figure 8.3: The Kinetics of Exchange of β -Casein Adsorbed on Hydroxyapatites:
 - High Plateau Level of Adsorption (°)
 - Low Plateau Level of Adsorption (▲).

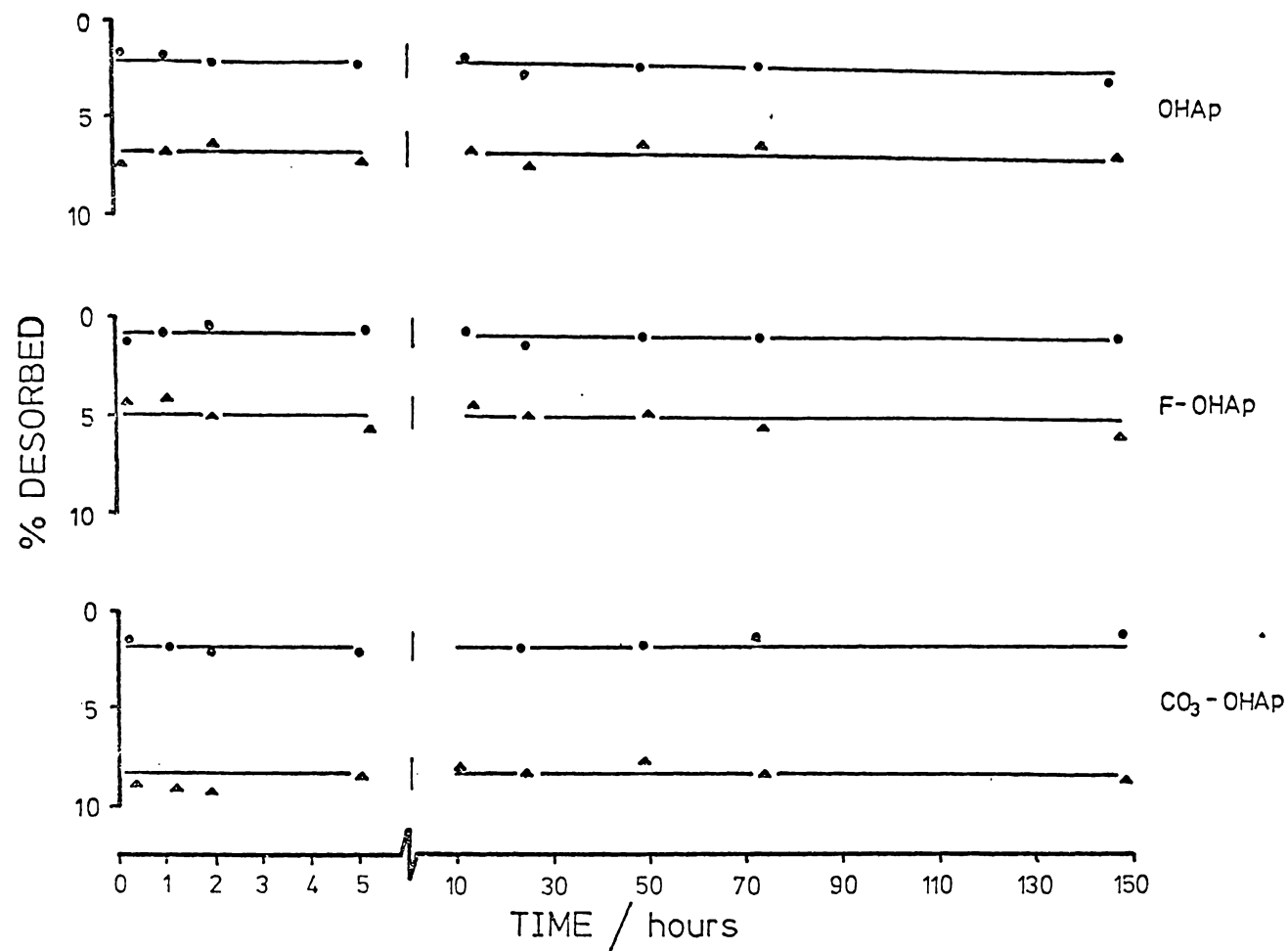


Figure 8.4: The Kinetics of Desorption of β -Casein Adsorbed on Hydroxyapatites - Desorption Into Cacodylate Buffer:
 High Plateau Level of Adsorption (●)
 Low Plateau Level of Adsorption (▲).

Similar results (not shown) were obtained for β -Casein adsorbed at both 4°C and 20°C at the high plateau level in the presence of calcium ions.

The results for the hydroxyapatite system compare with those of Nisbet for β -Casein adsorbed on stainless steel (Nisbet, 1979). He observed that partial exchange of the adsorbed protein.

8.4.2 DESORPTION UPON DILUTION

The results of experiments to test the effect of dilution on β -Casein adsorption using the adsorption buffer are shown in Figure 8.4.

The results at the low plateau level of adsorption show an immediate desorption of up to 8% upon mixing of the suspended hydroxyapatites with the adsorption buffer. No further desorption occurred over the next 5 days. A similar result was observed for desorption from the high plateau level of adsorption except that no more than 2% desorption was observed. Nisbet (1979) observed that no significant desorption of β -Casein from stainless steel occurred upon dilution.

The experiments to test the effect of dilution on β -Casein adsorbed in the presence of calcium were similar to those shown in Figure 8.4 and have therefore not been shown.

8.4.3 INDUCED DESORPTION

The effectiveness of various agents in causing desorption of β -Casein is tabulated in Table 8.2. Both sodium chloride and calcium chloride did not induce any significant desorption. About 20% desorption was induced in solutions of high pH and this was increased slightly by the incorporation of urea, SDS and phosphate into the solution. No solution induced more than about 30% desorption, however, even after 3 days.

TABLE 8.2: EFFECTIVENESS OF SOME REAGENTS IN CAUSING DESORPTION OF
ADSORBED β -CASEIN

DESORPTION SOLUTION	1 Day	% DESORBED 2 Days	3 Days
NaCl, 4 mole l^{-1} , pH 6.8	4	5	3
CaCl ₂ , 4 mole l^{-1} , pH 6.8	3	4	3
Na ₂ HPO ₄ , 2 mole l^{-1} , pH 10.8	26	28	31
NaOH, pH 10.8	20	19	20
SDS, 50 g l^{-1} , pH 10.8	27	26	28
Urea, 6 mole l^{-1} , pH 10.8	22	23	23

8.5 COMPETITIVE ADSORPTION IN THE β -CASEIN/BSA SYSTEM

The observation that BSA adsorption on hydroxyapatite was almost completely exchangeable but that β -Casein adsorption was non-exchangeable suggested that competitive adsorption experiments might prove interesting. Experiments were conducted in which adsorbed BSA was exposed to a β -Casein solution and adsorbed β -Casein (Batch 2, high plateau) exposed to a BSA solution. The results are presented in Figure 8.5 along with the self-exchange patterns observed earlier.

Although adsorbed β -Casein did not display any self-exchange with solution protein, a very slow exchange was observed with solution BSA. This resulted in less than 8% exchange even after 80 hours of contact time. Adsorbed BSA was readily exchanged by solution β -Casein however. More than 60% exchange occurred within 10 minutes and the adsorbed BSA was almost totally replaced after 80 hours of contact.

8.5 COMPETITIVE ADSORPTION IN THE β -CASEIN/BSA SYSTEM

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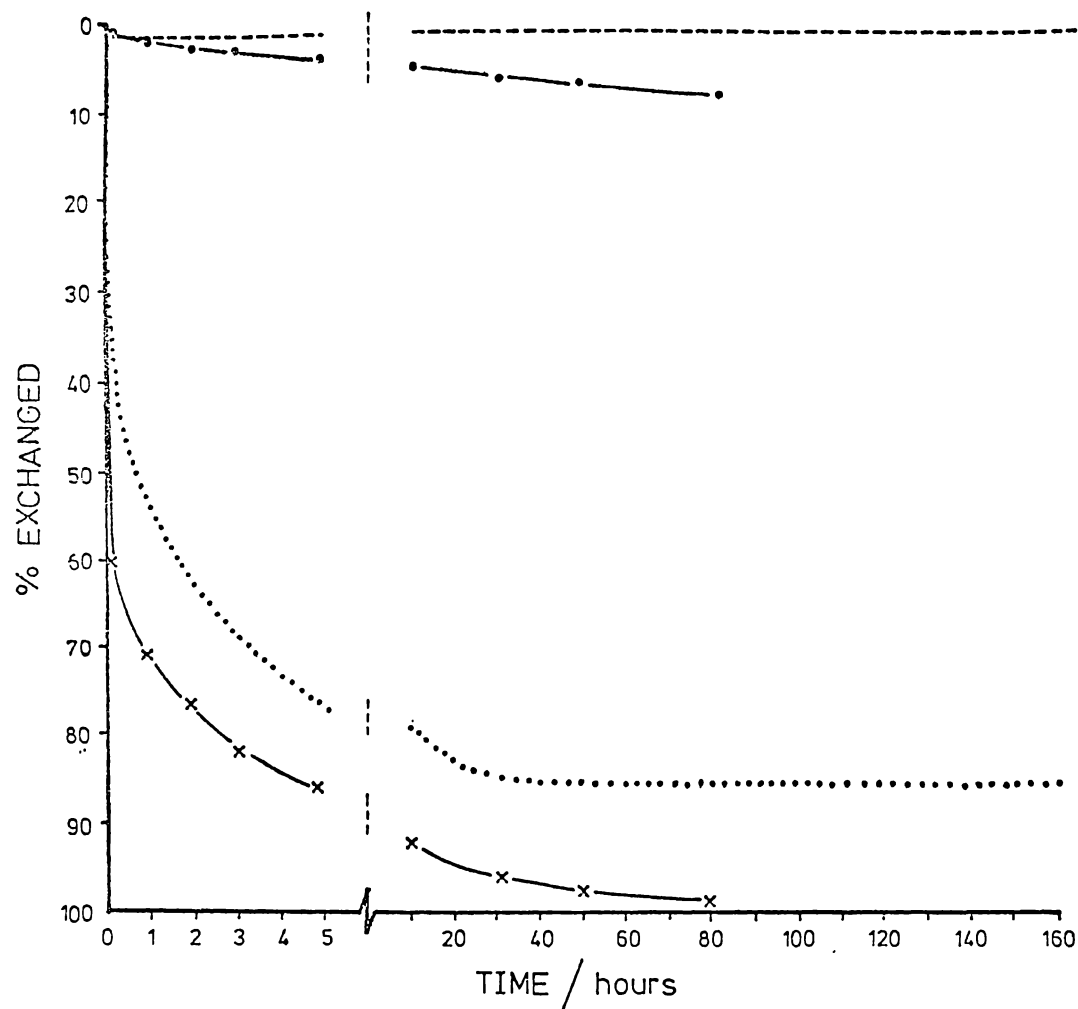


Figure 8.5: The Kinetics of Exchange Between BSA and β -Casein Adsorbed on Hydroxyapatite (OHAp). Adsorbed protein denoted by (ads), solution protein by (sol).

8.6 SUMMARY

BSA and β -Casein adsorbed on hydroxyapatite showed no detectable desorption upon dilution within the limits of experimental error. If adsorption is an equilibrium process, the solution protein concentration required to maintain saturation must be very low.

The degree of self-exchange of adsorbed protein was dependent on the protein used but independent of whether the adsorbent was OHAp, F-OHAp or CO_3 -OHAp. Adsorbed BSA was almost totally exchangeable but adsorbed β -Casein was observed to be completely non-exchangeable. These results contrast to those found in earlier studies of these proteins on stainless steel (van Enkevort, 1978; Nisbet, 1979) where it was observed that BSA adsorption was non-exchangeable and β -Casein adsorption was partially exchangeable.

The results of the competitive adsorption experiments of BSA and β -Casein were consistent with the observed self-exchange properties. Adsorbed BSA was almost totally displaced by solution β -Casein whereas less than 10% of adsorbed β -Casein could be displaced by solution BSA.

Neither adsorbed BSA and β -Casein were easily desorbed. Solutions containing NaCl, CaCl_2 , Na_2HPO_4 , NaOH, SDS and urea were tested for their efficacy. Generally, desorption was only induced by solutions of high pH and was facilitated to some extent by the presence of phosphate, SDS or urea. Complete desorption was not achieved in any of the solutions studied and BSA was always desorbed to a greater extent than β -Casein.

CHAPTER 9

ASSOCIATION EFFECTS IN PROTEIN / HYDROXYAPATITE SOLUTIONS

9.1 INTRODUCTION

Dental pellicle and calculus are examples of macroscopic proteinaceous films. The survey of the literature on protein adsorption showed that such films are not formed by simple adsorption as this proceeds only to monolayer coverage. Polyvalent metal ions, in particular calcium ions, have been postulated to be important in pellicle formation (Section 3.7.2). Previous studies at this University (Nisbet, 1979) suggested that the adsorption of multilayers could not be induced by simple ions. However, the experimental method employed in this study viz. the exposure of a protein layer, adsorbed on stainless steel, to a solution of the ions to be tested followed by rinsing and re-exposure to the protein solution, is such that this result may have been a consequence of the technique used as the binding of ions by protein species is often a reversible process. Hence, experiments were performed under conditions where multilayer formation, if feasible, could occur and would be retained on the surface studied.

The studies of Nisbet did suggest, however, that multilayer adsorption leading to accretion formation could be facilitated by particulate bridging species. It was observed during the progress of the present studies that BSA stabilised hydroxyapatite suspensions, but that β -Casein destabilised the suspensions. Destabilising by β -Casein resulted in massive flocculation of the suspended particles. Further, in some instances, a large build-up of material occurred on the walls of the adsorption vials. In view of the fact that dental pellicle is a macroscopic proteinaceous film on tooth enamel, it was of interest to characterise these effects further. Experiments were performed to investigate various aspects of the stability of calcium phosphate suspensions and factors that might be important in accretion formation.

The results presented in this Chapter will be discussed in Chapter 11.

9.2 MULTILAYER ADSORPTION BY ION PARTICIPATION

9.2.1 INTRODUCTION

Calcium ions have been suggested to promote pellicle formation by a bridging mechanism between layers of adsorbed protein molecules. β -Casein and BSA both bind calcium ions, presumably by an electrostatic interaction, and the binding promotes the self-association of β -Casein. BSA polymerisation can be induced by the covalent binding of mercuric ions.

It has already been shown that the effect of the presence of calcium ions on β -Casein adsorption was to lower the concentration at which the step in the adsorption isotherm, leading to a higher level of adsorption, was observed and to give increased adsorption at this plateau (Section 7.3.2(iii)). Further, the enhanced adsorption was not exchangeable (Section 8.4.1) and not reversed upon dilution (Section 8.4.2). The effect of calcium ions on BSA adsorption was therefore tested. As the possible role of mercury seemed to warrant further study, the effect of this ion on BSA adsorption was also studied.

9.2.2 METHOD AND MATERIALS

For experiments designed to test the role of calcium and mercuric ions on desorption, the use of a low surface area stainless steel slide system offered the following advantages:

- (i) the levels of adsorption were similar
- (ii) the technique was more convenient
- (iii) adsorption occurred at a faster rate (van Enckevort, 1978)
- (iv) solution depletion problems would be avoided; and
- (v) a continuous monitoring system could be used in desorption studies

to monitor effects due solely to any multilayers as monolayer adsorption on this surface was both irreversible to dilution and non-exchangeable (van Enkevort, 1978).

A continuous monitoring system similar to that described by van Enkevort (1978) was used. This consisted of a solid scintillation probe mounted directly over the back of a thin stainless steel foil disc on which protein had been adsorbed by a spot technique (Langdon et al., 1973). The face of the foil disc was exposed to a rapidly stirred 1 litre volume of the desorption solution contained in a narrow necked flask. Desorption and exchange were monitored by strip chart recorder. Experiments were performed at HgCl_2 and CaCl_2 concentrations of $0.001 \text{ mole l}^{-1}$ and $0.005 \text{ mole l}^{-1}$ respectively, in a cacodylate buffer (diluted to maintain the ionic strength at 0.06 mole l^{-1}) at pH 6.8 and at 4°C . Slides were typically allowed to stand in protein and CaCl_2 or HgCl_2 solutions for 2 hours. Rinsing procedures were avoided by the use of solutions of sufficiently low protein concentration such that, after removal from the protein solution and a fixed period of draining, solution carry-over (as determined by preliminary experiments) contributed about 10% to the total surface activity.

Once the levels of adsorption had been determined in the stainless steel system, an experiment was performed using hydroxyapatite to confirm that these were equal.

9.2.3 RESULTS

The results of the adsorption studies and the procedures used are presented in Figure 9.1. Those for the adsorption on stainless steel have been corrected for solution carry-over and surface roughness, and

represent the average amount of adsorption as determined from both sides of 2 slides (i.e. 4 points). Adsorption of BSA was enhanced by the presence of either calcium or mercuric ions. The mercuric ions caused a 100% increase in the level of adsorption whereas calcium only promoted an increase of about 25%. Exposure of an adsorbed BSA layer to a solution containing both BSA and Hg^{2+} also led to enhanced adsorption. None of the experiments in which the cations were not present with the protein gave the effect.

The results of experiments to test the desorbability of the metal ion enhanced protein adsorption are shown in Figure 9.2. Although the calcium enhanced adsorption was not reversed upon dilution, all of the increased adsorption promoted by the mercury was desorbable within 5 minutes. The level of protein remaining after desorption had ceased corresponded to that expected for adsorption in the absence of this ion.

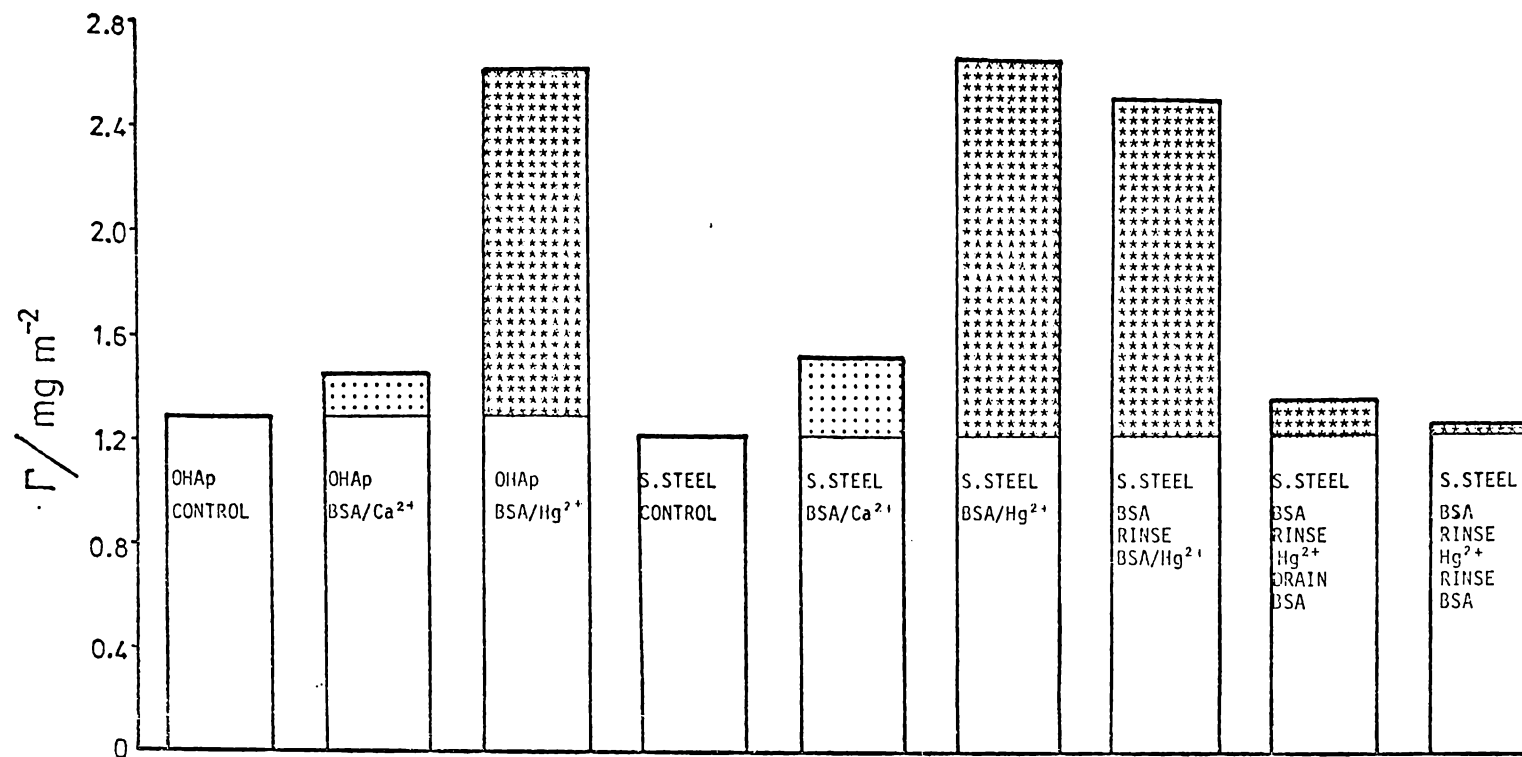


Figure 9.1: The Effectiveness of Calcium and Mercuric Ions in Promoting Multilayer Adsorption.
Blank Bar: Monolayer Adsorption; Stippled Bar: Additional Adsorption Induced by Ions.

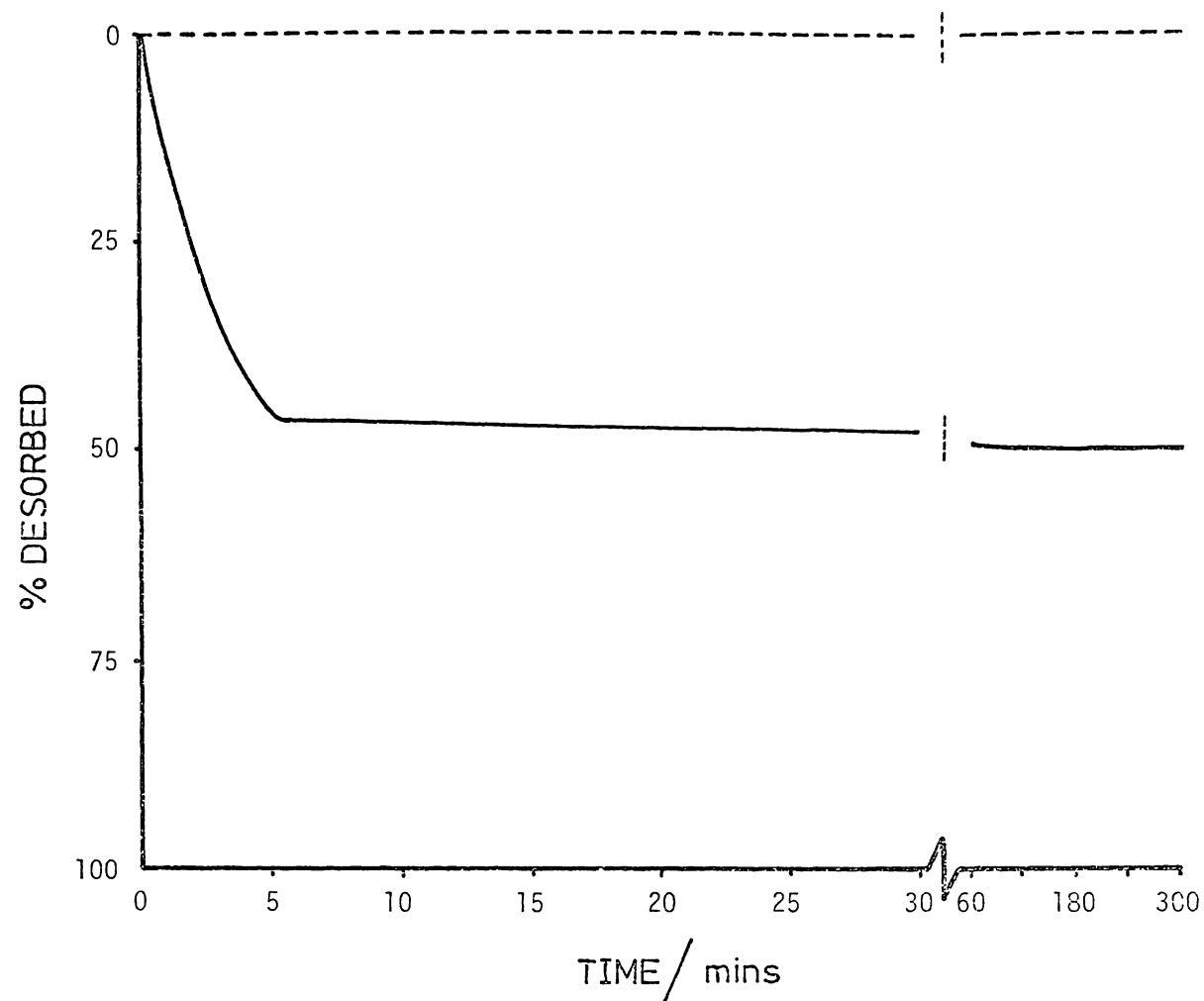


Figure 9.2: The Kinetics of Desorption of Ion Induced Adsorption of BSA on Stainless Steel: Calcium Induced (---); Mercuric Induced(—).

9.3 THE STABILISATION AND DESTABILISATION OF CALCIUM PHOSPHATE SUSPENSIONS

9.3.1 INTRODUCTION

Electron microscope characterisation of the calcium phosphate materials prepared for use in the present studies showed that the particles were of submicron size (Section 4.3.3) and well within colloidal dimensions. Although stable suspensions of the particulates would therefore be expected, this was not observed as the suspensions were observed to gradually settle. Further, the presence of protein could either increase or decrease the rate of settling. The literature on the stability of colloidal species suggested electrostatic effects and, in the presence of macromolecules, surface coverage effects might be important in determining suspension stability. Experiments were performed to assess the contributions of these phenomena. Due to the chemical instability of amorphous calcium phosphates in solution, only more qualitative experiments were performed with this material.

9.3.2 METHODS AND MATERIALS

Suspensions of hydroxyapatite (OHAp) were prepared in barbital buffers of various pH and I 0.06 mole l^{-1} (Appendix VI) as described for the adsorption studies (Section 6.5).

For experiments to determine the effect of protein adsorption on suspension stability, a known aliquot of an OHAp suspension (cacodylate buffer, pH 6.8, I 0.06 mole l^{-1}) was added to constant volumes of protein solutions containing sufficient protein to give nominal degrees of surface coverage, based upon complete depletion, varying from 0% - 100%.

Adsorption was conducted at 20°C and usually allowed to proceed for 2 hours before the adsorbed protein/OHAp suspensions were used in the experiments.

A fresh ACP sample was prepared as described in Section 4.2.5 with the modifications that rinsing was conducted using cacodylate buffer (pH 6.8, I 0.06 mole l^{-1} , T 4°C) and the product was not freeze dried.

9.3.3 PROPERTIES OF PURE CALCIUM PHOSPHATE SUSPENSIONS

OHAp suspensions were prepared in the pH range 4.9 - 8.8. The suspensions were observed to settle in the entire pH range studied, including in the region of the IEP ($\approx$ pH 7 - 8). Further, once the suspensions had settled, the pH of the solution and hence the charge on the particles did not appear to affect the volume of the sediment formed. This suggested factors other than electrostatic repulsion were dominant in determining suspension stability.

Suspensions of ACP also settled. The rate of settling was very rapid, however, due to the formation of macroscopic flocs of the particulates.

9.3.4 THE EFFECT OF PROTEIN ADSORPTION

The addition of BSA to suspended OHAp was observed to stabilise the suspension at all degrees of surface coverage from 1% - 100%. Settling was obvious in the pure suspension within about 40 seconds but no settling was apparent at 100% coverage even after 4 minutes. The degree of stabilisation increased with increasing surface coverage. A significant difference in turbidity was apparent after 2.5 hours.

Suspensions at 60% - 100% coverage were still quite turbid after this time and complete settling of these usually required about 24 hours. The packing of the adsorbed BSA/OHAp sediments was always less dense than that observed for the pure OHAp. Once allowed to settle, all the sediments could always be easily redispersed by gentle rotation of the sample vials.

The addition of β -Casein to suspended OHAp caused massive flocculation, at all degrees of surface coverage, within 20 seconds. The suspensions could be redispersed but only by methods more vigorous than those used in the BSA system. Further, those suspensions at degrees of surface coverage of 60% or less were still more difficult to completely redisperse if they were allowed to stand for a period after settling.

The results of experiments with ACP paralleled those using OHAp. The presence of BSA, but not β -Casein, was observed to stabilise the ACP suspension to flocculation.

9.4 ACCRETION FORMATION

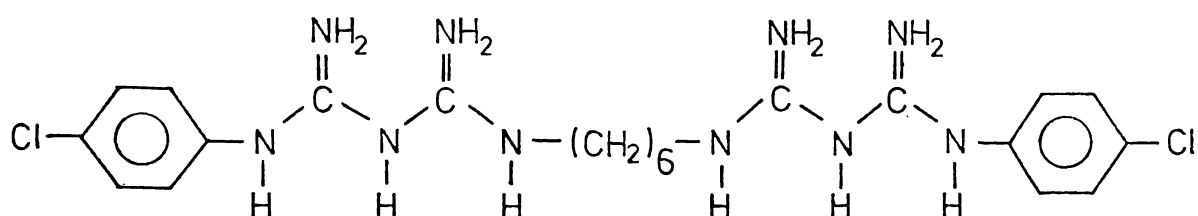
9.4.1 INTRODUCTION

Preliminary observations in the experimental studies suggested that accretion formation might be dependent on the degree of surface coverage by adsorbed molecules. In order to study this effect, experiments were performed with BSA and β -Casein. It was intended to investigate the generality of the effect by using a range of surfaces including various resins, cements and synthetic polymers commonly used in dental procedures, and silanised and protein covered glass. The effect of the surface active chemicals sodium dodecyl sulphate (SDS), commonly used in toothpaste preparations, and chlorhexidine, an effective anti-plaque agent, on accretion formation was also investigated.

9.4.2 METHOD AND MATERIALS

Hydroxyapatite/adsorbed protein suspensions of known degrees of surface coverage were prepared as described in Section 9.3.2. The glass vials containing the suspension were rotated end-over-end on a rotating wheel for 24 hours unless specified otherwise. The rate of inversion was such that macroscopic floc formation in the β -Casein/hydroxyapatite solutions was prevented and was kept as constant as possible throughout the various experiments. The effect of various pretreatments and coatings of the glass vials on accretion formation was tested using β -Casein/hydroxyapatite at 40% surface coverage as this suspension was observed to give the most dramatic and stable accretion. The dental materials tested included a photo-cured resin composit, epoxy and methacrylate resins, a cyanoacrylate cement, a glass ionomer filling

material and a polyurethane tooth sealant. (These materials were kindly donated by Mr. Warr of Judd Dental Surgery, Hamilton). The dental literature showed that chlorhexidine was an effective anti-plaque agent (e.g. Quintana, 1975). Although this is partially attributed to the antimicrobial behaviour of this agent, it may also disrupt plaque. Chlorhexidine is suggested to behave similarly to surface-active quaternary ammonium compounds and has the structure:



It was used as the digluconate preparation.

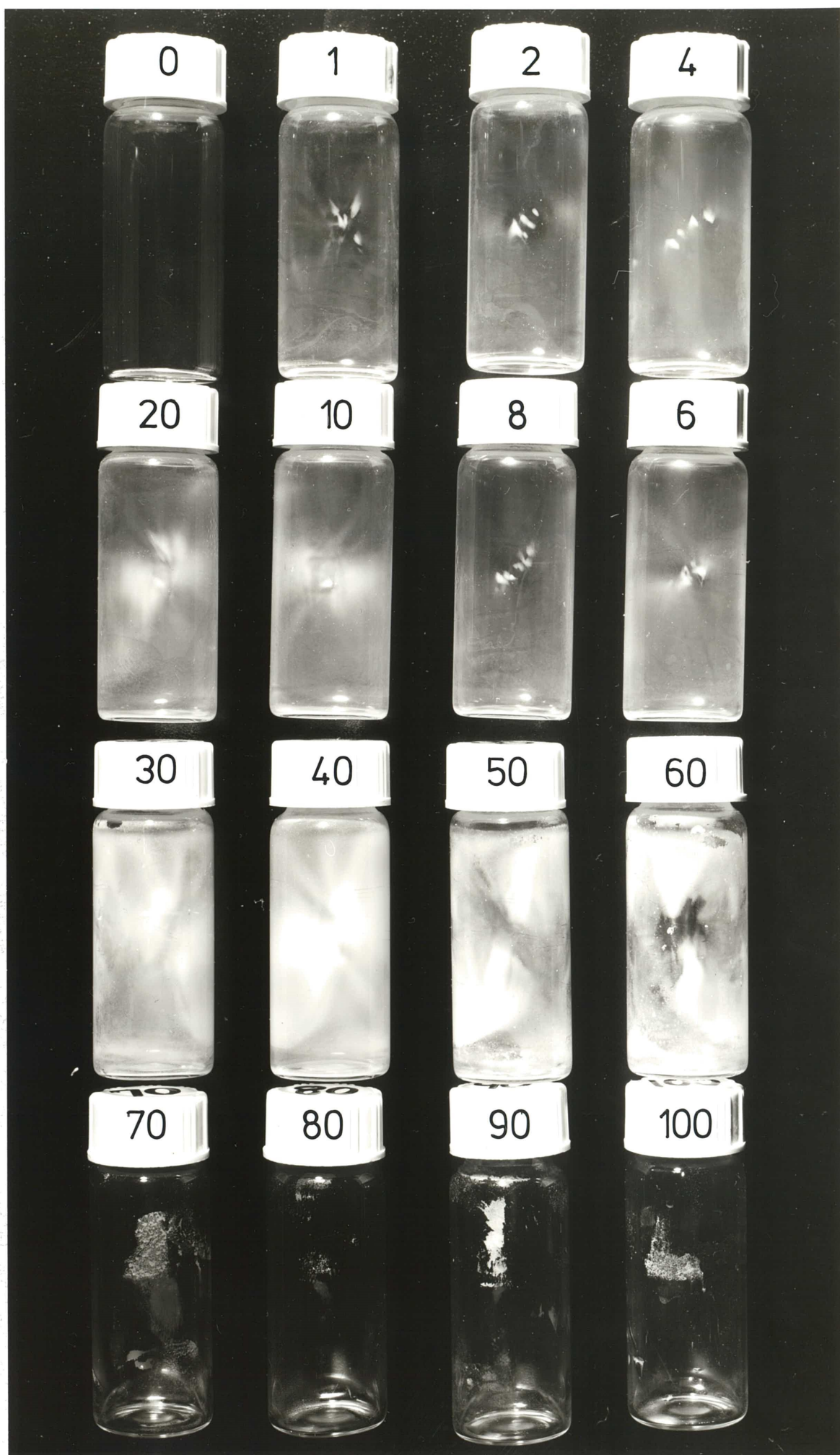
9.4.3 EFFECT OF DEGREE OF SURFACE COVERAGE

Rotation of the vials containing β -Casein/OHAp suspensions resulted in the formation of a white film on the walls of the glass vials at all degrees of surface coverage and particularly at the "hinge" points of the solution (Plate 9.1). The thickness of the film increased as the degree of surface coverage increased from 1% - 40%. The coherence of the film decreased upon further increases in the degree of coverage until, at coverages of 70% and higher, very little build-up was apparent and this was quite granular in appearance. The adsorbed film was apparent, again more noticeably at the solution hinge points, within 1 hour of the commencement of rotation. Macroscopic films similar to those observed in Plate 9.1 were formed for β -Casein adsorbed on F-OHAp, CO_3 -OHAp and ACP.

In contrast to the behaviour observed above, BSA/OHAp suspensions did not give a macroscopic build-up at any degree of surface coverage.

PLATE 9.1

MACROSCOPIC ACCRETION FORMATION IN THE
 β -CASEIN / HYDROXYAPATITE SYSTEM



There was some evidence of a slight build-up at the hinge points of the solutions at surface coverages of 30% and 40%. The films were barely visible but became more apparent after freezing (presumably through disruption of the film). However, the films were much less apparent than those observed for β -Casein at 1% surface coverage even after this treatment.

9.4.4 EFFECT OF SURFACE TYPE

(A) Formation on Teeth

A human incisor and molar were fixed to the caps of the glass vials prior to exposure to the β -Casein/OHAp suspension. The teeth were in good sound condition and non-carious, and had previously been cleaned by boiling in distilled water. A thick coherent film was formed over the entire tooth surface (Plate 9.2 - the film has been removed from the root and part of the crown of each tooth to provide some contrast).

(B) Formation on Some Synthetic and Pretreated Surfaces

The effect of different surfaces was tested by applying a thin smear of some dental restorative and protective resin materials to the inside wall of the glass vials. The materials were then allowed to cure and set before addition of the protein and hydroxyapatite suspension. The effect of pretreatment of the glass by silanisation and protein adsorption was also tested. Silanisation was achieved by the addition of 30 μ l N-trimethyl silyl Imidazole to a vial full of toluene. The vial was sealed and allowed to stand for 2 days prior to removal of the toluene by evaporation with gentle heating. Saturation of the glass by protein was

PLATE 9.2

MACROSCOPIC ACCRETION FORMATION ON TEETH
USING THE β -CASEIN / HYDROXYAPATITE SYSTEM



achieved by adsorption from standard protein solutions ($1\ 000\ \text{g m}^{-3}$, 20°C 12 hr).

The results obtained are summarised in Table 9.1. A macroscopic continuous film was formed on all the materials tested. Visual observation did not suggest that any decreased levels of build-up occurred on any particular material.

TABLE 9.1: ACCRETION BUILD-UP ON SOME SYNTHETIC SURFACES

SURFACE	BUILD-UP
Resin Composit (photo-cured)	yes
Methylmethacrylate Resin (cold-cured)	yes
Cyanoacrylate Cement	yes
Glass Ionomer (filling material)	yes
Epoxy Resin	yes
Polyurethane	yes
Polyvinylacetate	yes
Silanised Glass	yes
Glass: β -Casein covered	yes
BSA covered	yes

The studies using different proteins showed that a BSA/hydroxyapatite accretion appeared to form only to a very slight extent at protein surface coverages corresponding to 30% and 40% and then only at the hinge points of the solution. An experiment was performed therefore to determine if the pretreatment of glass by β -Casein would enhance build-up. It was observed, in contrast to the previous experiment, that a thin film was formed over the entire surface of the glass. The film was very faint, even after freezing, and of about the same density as observed at the hinge points in the previous experiment.

9.4.5 EFFECT OF CHEMICAL REAGENTS

The effect of SDS and chlorhexidine was tested in two series of experiments:

- (i) the effect on accretion formation, and
- (ii) the effect on pre-formed accretion.

Chlorhexidine was used at a concentration of 0.1% (v/v) and SDS was used at a concentration of 0.05% (w/v).

Both SDS and chlorhexidine prevented accretion formation when added simultaneously with the adsorbed protein/hydroxyapatite suspensions to clean glass vials. However, only SDS was effective in breaking down the pre-formed accretion. The breakdown was slow and 4 - 5 days of continual rotation was required to clean the glass.

9.4.6 SUMMARY

Accretion formation can be modified by a number of variables. Build-up (on glass) was most significant when the degree of surface coverage of OHAp was in the range of 1% - 60% and was always thickest in regions of minimum turbulence. The thickest and most coherent accretion was formed at 40% surface coverage. Little build-up occurred at surface coverages of 70% and higher. This appeared to be due to a loss of mechanical cohesiveness. Similar results were observed for the calcium phosphates F-OHAp, CO₃-OHAp and ACP.

A dependence on the nature of the protein was observed. Significant accretion formation could not be achieved using BSA. Pre-adsorption of β -Casein to a surface prior to exposure to a BSA/OHAp suspension enhanced film formation slightly.

The formation of β -Casein/OHAp accretions was prevented by the

presence of SDS or chlorhexidine. However, only SDS was effective in disrupting the accretion once formed.

Accretion formation in the β -Casein system was not affected by the nature of the surfaces tested. These included whole teeth, various resins, cements and synthetic polymer sealants, hydrophilic and hydrophobic (silanised) glass, and β -Casein or BSA saturated glass.

9.5 SUMMARY

Experiments to test the efficacy of polyvalent metal ions in promoting multilayer adsorption showed that the presence of mercuric ions in solutions of BSA led to a doubling of the amount of adsorption. The additional amount adsorbed was exchangeable and readily desorbed upon dilution. The result contrasted to that observed for the presence of calcium ions in adsorption solutions of BSA and β -Casein in that this ion only promoted a small increase in the level of adsorption and this was not desorbed upon dilution.

Hydroxyapatite and ACP suspensions were observed to be unstable i.e. even though the particulates were of colloidal dimensions and hence expected to remain dispersed in solution, settling occurred relatively quickly and suggested some form of association was occurring. The association appeared to over-ride electrostatic repulsion effects. The adsorption of BSA stabilised the suspensions, the degree of stabilisation increasing with increasing degree of surface coverage of the particulates by the protein. However, β -Casein adsorption, at any degree of surface coverage, caused destabilisation and resulted in the rapid formation of massive flocs. These could be dispersed by agitation but eventually films of hydroxyapatite formed on walls of the glass adsorption vials. The thickness and mechanical stability of the film was dependent on the degree of surface coverage of the particulates by adsorbed protein. Films were formed using OHAp, F-OHAp, CO₃-OHAp and ACP and on a wide variety of surfaces including whole teeth, several types of dental resin and restorative materials, glass and on silanised and protein pretreated glass. SDS and chlorhexidine prevented film formation but only SDS could break down a pre-formed film.

Film formation was dependent on protein type. BSA did not form

significant accretions. There was some evidence to indicate this was partially due to a lack of mechanical cohesiveness. Pretreatment of the glass vial by β -Casein led to an enhancement of film formation.

CHAPTER 10

SALIVARY GLYCOPROTEIN ADSORPTION AT THE HYDROXYAPATITE / AQUEOUS SOLUTION INTERFACE

10.1 INTRODUCTION

There is considerable evidence that glycoproteins constitute a significant proportion of the protein material in saliva and dental pellicle, and are strongly adsorbed by tooth enamel and hydroxyapatite (refer Sections 3.6.4, 3.7.2). The isolation of the principal glycoprotein of whole saliva has been reported by Schrager and Oates (1971a and b) who characterised it as being basically of homogeneous structure and composition, but of varying degrees of sulphation and charge. The amino acid tyrosine was present and hence the molecule should be able to be radioiodinated.

The studies reported in this Chapter describe the collection and fractionation of saliva to isolate this component, some preliminary characterisation studies to determine the electrophoretic properties and size of the protein molecule, and the results of experiments to determine the adsorption isotherm and exchange and desorbability properties of the adsorbed layer. The effect of calcium ions on adsorption was of interest in relation to their postulated role as a bridging species between glycoprotein layers in dental pellicle (Section 3.7.2). Due to the problems associated with obtaining large amounts of the protein, the adsorption isotherms were constructed using stainless steel in order to conserve the material collected. A limited number of experiments were performed using hydroxyapatite for comparison.

Data was obtained using radiochemical methods. The results of the adsorption studies will be discussed in Chapter 11.

10.2 PRELIMINARY EXPERIMENTS

10.2.1 COLLECTION OF SALIVA

Whole saliva (1.7 l) was collected from about 200 1st year University students into ice-chilled sterile plastic cups. Chewing gum base (supplied courtesy of the Wrigley Company (New Zealand) Ltd.) was used as a stimulant. The saliva was centrifuged (25 000 g, 4°C) for 30 minutes and the deposit discarded. The supernatant was passed through a sterilising filter (Millipore, 0.45 μ m), concentrated by ultrafiltration (4°C, Amicon UM2 membrane Mr cut off > 1 000) under nitrogen (350 kPa), dialysed for 2 days against 5 changes of doubly distilled water (4°C, Mr cut off > 8 000) and finally freeze dried.

10.2.2 FRACTIONATION OF SALIVA

Sephacryl S-200 Superfine (specified Mr fractionation range, assuming globular proteins, 5 000 - 250 000) was used instead of BioGel P150 (as used by Schragger and Oates, equivalent Mr fractionation range 15 000 - 150 000) as the material was already available at this University.

Sephacryl S-200 Superfine gel (pre-swollen) was washed three times with distilled water and twice with cacodylate buffer (pH 6.8, I 0.06 mole l⁻¹). After cooling to 4°C, the gel was packed into a Pharmacia K 26/1 000 chromatography column. The gel column was equilibrated using 2 column volumes of cacodylate buffer, at a flow rate of 17 ml hr⁻¹. The final bed height was 950 mm.

One-third of the lyophilised saliva was reconstituted in cacodylate buffer (15 ml) and added to the column. The sample was fractionated

using cacodylate buffer, at a flow rate of 17 ml hr^{-1} , and 15 ml fractions, were collected. The elution pattern obtained (monitored at 280 nm) is shown in Figure 10.1. The non-retarded fraction (fraction no's 38 - 52) has been characterised as containing mainly amylase and the third peak (fraction no's 60 - 72) as polypeptides (Schrager and Oates, 1971a). The remaining saliva samples produced similar profiles upon fractionation.

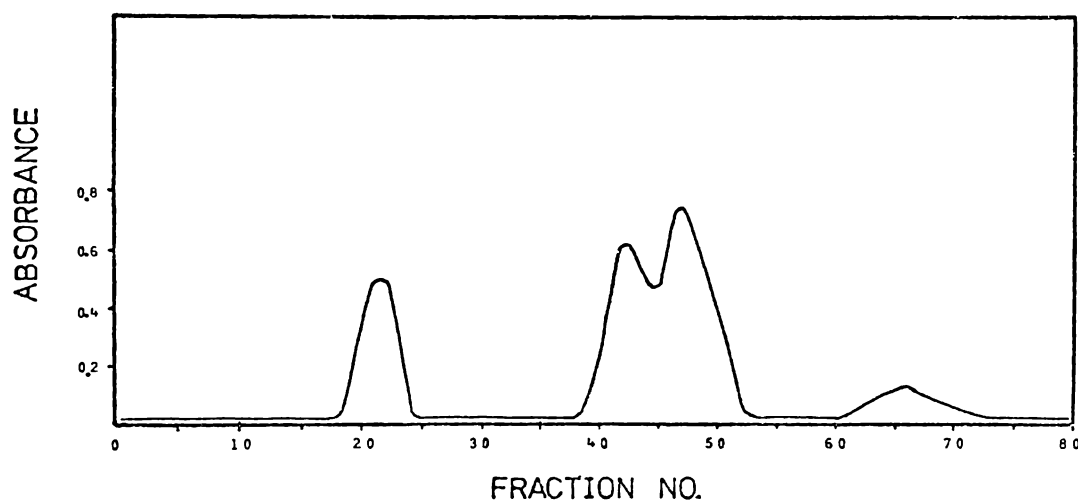


Figure 10.1: Elution Profile of Whole Saliva from Sephacryl S-200 Superfine (Elution conditions given in text).

The glycoprotein fraction was concentrated by dialysis (M_r cut off $> 1\,000$) against polyvinylpyrrolidone (a hygroscopic polymer, $M_r \sim 44\,000$). The final solution volume was 40 ml.

10.2.3 CHARACTERISATION OF GLYCOPROTEIN FRACTION

(A) Test For Carbohydrate

The presence of carbohydrate in the glycoprotein fraction was confirmed by a ninhydrin spot test, an alkaline silver nitrate test and periodate oxidation/Schiff's reagent reaction.

(B) Concentration:

The concentration of the glycoprotein fraction was determined by the dye binding method, described previously in Section 6.3, using β -Casein as the standard as recommended by Arglebe (1981). The protein concentration was determined to be 400 g m^{-3} .

(C) Electrophoretic Characterisation:

It was of interest to determine the nature of the protein fraction under the conditions of the adsorption experiments, particularly whether the sample contained various fractions of net positive or net negative charge. A polyacrylamide electrophoresis gel was therefore prepared such that the gel consisted of two 10×2.6 gels sandwiched together by a 5×2.6 gel which contained the protein sample. The gels were prepared by the method described in Appendix VIII except that: (i) SDS was omitted, and (ii) barbital buffer of the same pH and ionic strength was substituted for the cacodylate buffer as this did not react in the periodic acid/Schiff's Reagent staining procedure used for the determination of carbohydrate. The sample gel was prepared by dilution of the 10% gel using an equal volume of protein solution. Electrophoresis was run for 2.5 hr at 20°C and a constant current of 30 mA. A small sample of a tracker dye/glycerol solution was added to the top left hand corner of the gel and electrophoresis was halted when this was 0.5 cm from the top of the sample gel. After electrophoresis

the gel was bisected. One half was stained by the general protein staining and destaining procedure, using Coomassie Brilliant Blue, described previously (Section 5.5.2). The second half was stained specifically for carbohydrate using the following procedure:

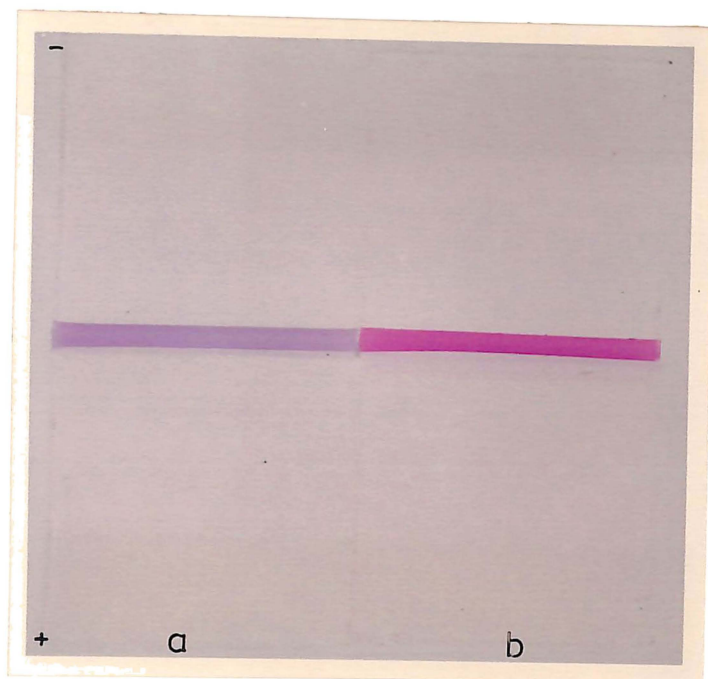
- (i) Immersion in a 1% Periodic Acid/3% Acetic Acid (v/v) solution for 1.5 hours.
- (ii) Immersion in distilled water for 1 hour with 3 changes of solution.
- (iii) Immersion in Schiff's Reagent for 1 hour.
- (iv) Storage in Sodium Metabisulphite solution (1% w/v).

The result of the electrophoresis experiment is shown in Plate 10.1. With the exception of one very faint anodically migrating band, no electrophoretic separation of the protein fraction was observed. This suggested that either the material was too large to migrate through the gel or that it did not have significant net charge at the pH of the experiment. Treatment of the protein sample with 0.2 mole l^{-1} EDTA, followed by dialysis, did not enable separation. This showed that an aggregation of the protein through calcium binding effects was not responsible for the lack of separation. Even the use of polyacrylamide gels of composition 3 x 5 and 1.5 x 5 (both stabilised in 0.5% agarose), which should allow the migration of molecules of relative molecular mass up to $\sim 10^7$ (Morris and Morris, 1976), did not enable any separation. This indicates that the size of the protein molecule was not the reason for the lack of mobility but rather that the protein was of neutral charge at pH 6.8.

Gel filtration methods can often be used successfully for the determination of the relative molecular mass of proteins if these are of a globular shape. Non-globular proteins such as glycoproteins display anomalous behaviour in that they are eluted more quickly from filtration columns than predicted because of their more expanded structure (e.g.

PLATE 10.1

POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE PRINCIPAL
GLYCOPROTEIN FRACTION OF WHOLE HUMAN SALIVA



Key: (a) Gel section stained for protein
(b) Gel section stained for carbohydrate.

Running gel : 10 x 2.6
Sample gel : 5 x 2.6

Spiro, 1973). Thus, although the chromatography of the glycoprotein on Sephacryl S-200 Superfine indicated the relative molecular mass was greater than 250 000, this will probably be an overestimate. There is much to indicate the relative molecular mass of the molecules is no more than 150 000. Schragar and Oates observed in their study that the glycoprotein appeared homogeneous in an ultracentrifugation study but of varying degrees of charge. The migration of a small band in the 10% polyacrylamide gel indicates the relative molecular mass must have been less than 150 000 (Morris and Morris, 1976). Further, the exclusion limit of Sephacryl S-200 Superfine for straight chain dextrans is 88 000. Hence the relative molecular mass must lie between this value and 150 000.

10.2.4 RADIOIODINATION

Radioiodination of salivary protein was achieved using the Iodo-Gen method of iodination (Section 5.4.5) with the modification that the Iodo-Gen was plated over a wider surface area of the glass vial to enable the iodination of a larger volume of protein solution (due to its lower concentration). Typically, an iodination efficiency of about 30% was obtained after a 15 minute reaction time. A scan of the gel filtration column using a hand-held geiger counter revealed little residual activity. This provided good evidence that the iodination had been achieved without significant aggregation.

10.2.5 STABILITY OF THE TRACER TO STORAGE

The tracer preparation was observed to be stable to storage in cacodylate buffer at 4°C for up to at least 11 days.

10.2.6 VALIDITY OF THE USE OF THE RADIOLABELLED TRACER

The validity of the use of the radioiodinated protein as a tracer for adsorption studies on hydroxyapatite and stainless steel was tested by comparisons of the amounts of adsorption determined by radiochemical and non-radiochemical methods as described previously (Section 6.8).

The results of the validity experiments are presented in Table 10.1. Good agreement was observed between the levels of adsorption on hydroxyapatite and stainless steel and there was no evidence to indicate the preferential adsorption of the labelled or unlabelled species on either adsorbent.

TABLE 10.1: VALIDITY OF THE USE OF RADIOIODINATED SALIVARY PROTEIN AS A TRACER IN ADSORPTION STUDIES^(a)

ADSORBENT	% LABEL	$\Gamma/\text{mg m}^{-2}$		
		RADIOCHEMICAL Solution	Solid	SPECTROPHOTOMETRIC (Solution)
Hydroxyapatite	0			1.26
	20	1.31	1.28	1.28
Stainless Steel	0.25		1.34	
	0.50		1.37	
	0.63		1.30	
	0.83		1.29	
	1.25		1.38	

^a Adsorptions conducted at protein concentration of 60 g m^{-3} .

10.3 ADSORPTION EXPERIMENTS

Adsorption studies were performed as described previously in Sections 6.4.3 and 6.5. The effects of concentration and the presence of calcium ions on adsorption was tested and the results of these studies are presented in Figure 10.2.

Least squares analysis of the data points of the concentration adsorption isotherm showed that the isotherm was parallel to the concentration axis, within experimental error, over the concentration range $12 - 400 \text{ g m}^{-3}$. The amounts of adsorption on the three hydroxyapatite adsorbents OHAp, F-OHAp and CO_3 -OHAp were in good agreement with each other although, as observed with BSA, they did appear to be slightly lower than that observed for stainless steel. The shape of the isotherm shows that the glycoprotein has a high affinity for hydroxyapatite and stainless steel and contrasts to those usually reported for the adsorption of salivary proteins on hydroxyapatite (Section 3.5.2(B)). Similarly, the presence of fluoride in the hydroxyapatite lattice neither decreased nor increased the amounts of adsorption as has been reported in the literature.

The presence of calcium ions ($0.005 \text{ mole l}^{-1}$) in the adsorption solutions was observed to affect the levels of adsorption. At concentrations above $\sim 50 \text{ g m}^{-3}$, the amount of adsorption increased with increasing concentration from $\sim 1.3 \text{ mg m}^{-2}$ until the level of adsorption appeared to attain another plateau at $\sim 2.9 \text{ mg m}^{-2}$ at a concentration of $\sim 350 \text{ g m}^{-3}$. This behaviour is similar to that observed previously for β -Casein except that the step in the adsorption isotherm occurred at a lower concentration for β -Casein.

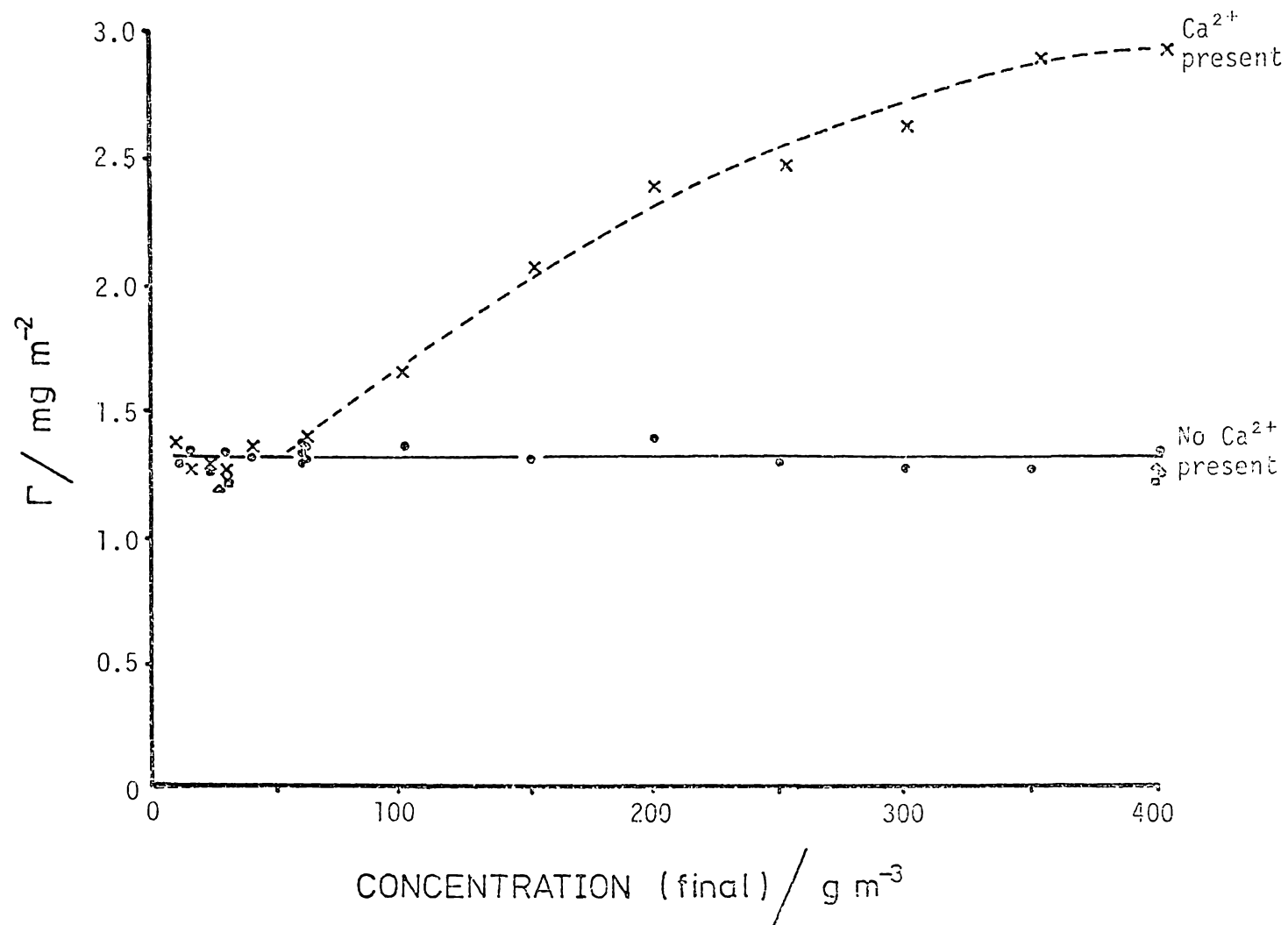


Figure 10.2: The Effect of Concentration and Calcium Ions on the Adsorption of Salivary Glycoprotein. OHAp (◐), F-OHAp (◻), CO₃-OHAp (Δ), stainless Steel (◐, x).

10.4 EXCHANGE AND DESORPTION EXPERIMENTS

Experiments to test the exchange of adsorbed protein and desorption upon dilution were performed for the adsorption on hydroxyapatites (in the absence of calcium ions) and for the adsorption on stainless steel (in the presence of calcium ions) at the high plateau level of adsorption. The procedures used were those described previously for such experiments i.e. a batchwise periodic sampling for experiments using the hydroxyapatite adsorbents, and a continuous monitoring technique for experiments using stainless steel. The exchange experiments were conducted using equal quantities of adsorbed and solution protein.

The results were typical of those observed previously for β -Casein in that the protein adsorbed on the hydroxyapatites was not exchangeable nor desorbed upon dilution and have therefore not been shown (refer Figures 8.3 and 8.4 for the β -Casein results).

Similarly, the calcium induced adsorption on stainless steel was neither exchangeable nor desorbed upon dilution. The result is similar to that observed previously for β -Casein adsorption on OHAp involving calcium ions and calcium enhanced BSA adsorption on stainless steel (refer Figure 9.2).

10.5 PROTEIN INDUCED FLOCCULATION AND ACCRETION FORMATION

It was observed during the adsorption experiments with hydroxyapatites that the adsorption of glycoprotein produced massive flocculation of the suspended particulates. An experiment was therefore performed to test if macroscopic accretions, such as those observed in the β -Casein/hydroxyapatite system, could be formed. The method used was as described previously (Section 9.5.2). In order to conserve the protein available, accretion formation was tested at a level of adsorption corresponding to a 40% surface coverage by adsorbed protein.

A dense white film formed over the walls of the glass adsorption vial within 24 hours and was similar in appearance to that observed for β -Casein at the same degree of surface coverage (Plate 9.1).

10.6 SUMMARY

The major glycoprotein component of saliva was fractionated from whole saliva. Characterisation studies indicated the protein had a relative molecular mass in the range 88 000 - 150 000 and had an IEP of pH 6.8. Radiolabelling was achieved using the Iodo-Gen method. The tracer preparation was stable to storage for up to 11 days at 4°C and was shown to be suitable for use in adsorption studies.

The glycoprotein adsorption on stainless steel and hydroxyapatite was characterised as of the high affinity type, no desorption upon dilution was detected and the adsorbed protein was non-exchangeable. The presence of fluoride or carbonate in the hydroxyapatite lattice did not affect the amounts of adsorption.

The presence of calcium ions induced a change in adsorption behaviour and the adsorption isotherm now appeared to consist of 2 plateau levels. The additional adsorption was characterised as non-exchangeable and not desorbed upon dilution.

Adsorption of the glycoprotein to hydroxyapatite destabilised the suspension. Large flocs were formed and, at a level of adsorption corresponding to about 40% surface coverage, a thick accretion could be formed on the walls of adsorption vials.

Thus, in its interactions at the hydroxyapatite/aqueous solution interface, the behaviour of the salivary glycoprotein appeared more similar to β -Casein than BSA.

CHAPTER 11

DISCUSSION OF ADSORPTION AND DESORPTION BEHAVIOUR

11.1 INTRODUCTION

The preceeding Chapters have given information on some aspects of protein adsorption at the hydroxyapatite/aqueous solution interface. The results of these studies, in light of what is known about the proteins and adsorbents used, allows certain conclusions to be drawn about the adsorption isotherms, the adsorbed layers, the interaction between the protein and adsorbent, the stability of hydroxyapatite suspensions and multilayer adsorption. These can then be extended to the oral environment.

11.2 IMPLICATIONS OF THE ADSORPTION ISOTHERM AND THE DESORPTION AND EXCHANGE BEHAVIOUR

The concentration adsorption isotherms obtained for BSA (Figure 7.1), HSA (Figure 7.2), β -Casein (Figure 7.6), β -Lactoglobulin (Figure 7.9), Haemoglobin (Figure 7.10), Myoglobin (Figure 7.11) and salivary glycoprotein (Figure 10.2) adsorption on hydroxyapatites at low protein concentrations were parallel to the concentration axis, even at concentrations considerably lower than those usually reported. This behaviour is typical of an adsorption process in which there is a high affinity between the adsorbent and adsorbate: the amounts adsorbed are always independent of the final solution protein concentration provided sufficient time is allowed for adsorption to proceed to the saturation limit. At higher concentrations, two types of behaviour were observed. The majority of proteins displayed an isotherm that remained parallel to the concentration axis with increasing concentration. However, β -Casein (Figure 7.6) and salivary glycoprotein (Figure 10.2) displayed stepped isotherms although in the case of salivary glycoprotein the effect was observed only in the presence of calcium.

The effect of concentration for a simple adsorption process can usually be represented by adsorption isotherms represented below in Figure 11.1. If adsorption is of very high affinity i.e. follows isotherm (a), any molecule striking the surface will remain permanently adsorbed. Given sufficient time and total solution protein, adsorption will always proceed to saturation. Adsorption will be irreversible to dilution. For adsorption of lower affinities, the adsorption isotherm will be of type (b) or (c) i.e. an increase in the amount of adsorption with increasing concentration until a saturation limit is obtained, further increases in concentration having no effect. Whether isotherm

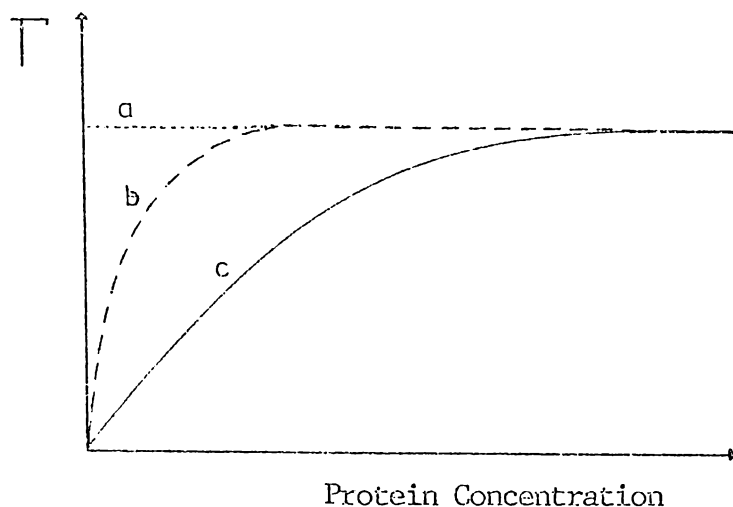


Figure 11.1: Typical Adsorption Isotherms

(b) or (c) is observed will depend on the relative affinity constants and the concentration range studied. If the adsorption is of relatively high affinity, i.e. follows isotherm (b), then the rising part of the isotherm might not be observed. The solution concentration required to maintain saturation may be below the range studied, be too small to be measurable in the experimental system in use or even be too small for the design of practicable adsorption experiments. Dilution will cause a negligibly small amount of desorption as the solution concentration required to maintain saturation is negligibly small. If the adsorption is of relatively low affinity the adsorption isotherm will follow isotherm (c). Dilution will lead to desorption provided the resultant solution concentration is below that required to maintain saturation. At sufficiently high dilution almost 100% desorption will be observed.

No significant desorption into distilled water or buffered solutions was observed for BSA (Figure 8.2), β -Casein (Figure 8.4) or salivary glycoprotein (Section 10.4). The lack of desorption implies that the

concentration of solution protein required to maintain saturation adsorption is extremely low and probably several orders of magnitude lower than could be meaningfully studied. The final solution protein concentrations calculated for the experiments testing desorption upon dilution were calculated to be respectively for BSA, β -Casein and salivary glycoprotein 0.007 g m^{-3} , 0.0002 g m^{-3} and 0.02 g m^{-3} . These are at least two orders of magnitude lower than the concentrations studied in the adsorption experiments using high surface area hydroxyapatite suspensions and indicate that the adsorption would proceed to saturation from concentrations at least as low as these values. In desorption experiments with low surface area systems, e.g. the salivary glycoprotein studies of Section 10.4 where the amount of adsorbed protein and solution carry-out is very much less and the final solution protein concentration was calculated to be $\sim 10^{-6} \text{ g m}^{-3}$, it is implied that the concentration required to maintain saturation is ~ 4 orders of magnitude lower still.

Adsorbed BSA was observed to be exchangeable (Figure 8.1) but adsorbed β -Casein and salivary glycoprotein (respectively Figure 8.3 and Section 10.4) was not exchangeable. The exchangeability of adsorbed protein does not necessarily imply the reversibility of adsorption, however, and hence does not allow distinction of the type of adsorption isotherm either. Exchange by solution protein can be envisaged to occur through any or all of the three following mechanisms (Section 3.3.4):

- (i) spontaneous desorption and displacement
- (ii) collision and dislodgement
- (iii) co-operative desorption and adsorption.

If adsorption follows isotherm (a) of Figure 11.1, exchange by mechanism (i) will not occur. However, mechanisms (ii) and (iii) may be feasible and these do not result in desorption of protein. In addition to the

three mechanisms for exchange described above, a fourth mechanism in which adsorbed protein is exchanged by non-protein species could also be envisaged. Although desorption would be observed in this case, the adsorbent surface will remain saturated by adsorbed species.

In the limit of those concentrations which are experimentally inaccessible, it is therefore impossible to discriminate between isotherms (a) or (b) shown in Figure 11.1 and such is the case for the adsorption on hydroxyapatite of the proteins studied. It can only be said that BSA, HSA, β -Casein, β -Lactoglobulin, Haemoglobin and Myoglobin have a high affinity for hydroxyapatites. The fact that β -Casein was preferentially adsorbed over BSA in experiments in which both proteins were present in solution (Figure 8.5), implies that β -Casein has a higher affinity for hydroxyapatite than BSA.

11.3 MODELS FOR THE ADSORBED LAYER

11.3.1 THE ADSORBED LAYER OF BSA

A saturation limit for BSA adsorption on hydroxyapatite was attained that was independent of the presence of fluoride or carbonate in the lattice and specific surface area (Table 7.1), concentration (Figure 7.1) and time (Figure 6.2), but dependent on pH, ionic strength and temperature (Figures 7.3, 7.4, 7.5 respectively). The effects of ionic strength and temperature were pH dependent.

Two basic modes of adsorption are those of monolayer or multilayer adsorption. The observed adsorption behaviour, the size and conformation of the adsorbed molecule and the nature of the interactions that exist between it, the adsorbent surface and other adsorbed molecules must be accounted for in any elaboration of these models. Multilayer formation is neither consistent with the generally observed monolayer adsorption in solid/liquid systems nor with BSA monomer molecule interactions. BSA solutions of high concentration (25%) have been kept refrigerated for 16 years with little change in polymer content (Pedersen, 1962). Polymeric fractions are only formed as a result of handling procedures, e.g. isolation, fractionation, purification and lyophilisation. Adsorbed BSA (Dass, 1977 and 1982) and Casein (Nisbet, 1979) monolayers require bridging species to adsorb further layers.

Calculations, based upon the relative molecular mass and dimensions of the dissolved native BSA (van Enckevort, 1978) and HSA (Norde, 1976) molecules, indicate that a complete close-packed monolayer of these proteins would correspond to adsorption levels of $\sim 2.5 \text{ mg m}^{-2}$ for adsorption in a side-on configuration and a level of $\sim 8.0 \text{ mg m}^{-2}$ for an end-on configuration of the ellipsoidal molecule. Further, adsorption in

a close-packed but completely unfolded conformation would result in a surface coverage of $\sim 0.6 \text{ mg m}^{-2}$ (Norde, 1976). The levels of adsorption observed for BSA, on hydroxyapatite, with the exception of those observed at temperatures greater than $\sim 40^\circ\text{C}$, are within the limits for a monolayer of close-packed molecules in a side-on configuration. The presence of a significant polymeric content in the BSA solutions requires some discussion. It can be envisaged that polymers could occupy an area between that occupied by one monomeric unit and n times this area (where n is the number of monomeric units in the polymer). This could result in increased levels of adsorption in the former case but would have no effect in the latter. The situation is further complicated by the exchangeability of the adsorbed protein. Experimentally, it was observed for BSA and β -Lactoglobulin (which also contained some irreversible polymeric material) that, within the time scale and sensitivity of the experiments, the amounts of adsorption were not significantly different for samples of differing polymer content and that no species was preferentially adsorbed (Section 6.7). These results provide good evidence that the average area occupied per monomer unit is little affected by polymerisation.

At the IEP, the BSA protein molecule is in its most stable conformation and there is excellent agreement between the amount calculated for monolayer adsorption and experimentally observed amounts of adsorption. As the protein molecule has no net charge at the IEP, intermolecular repulsion between like-charged molecules is not expected to be important in determining packing. A closely packed arrangement with maximum total protein-surface interaction and little intermolecular void space seems probable. This is supported by the lack of ionic strength effects on adsorption at this pH. Further, the constant amount adsorbed over the range of ionic strengths studied provides good evidence

that the size of the adsorbed molecule is not affected by NaCl concentration at the IEP. Similarly, changes in temperature from 0°C to 30°C had no effect on the amount of adsorption. A small rise in the amount of adsorption was observed between 30°C to 40°C and a further 10°C rise caused a dramatic increase in adsorption. There is little evidence available on the effect of temperature on conformational changes in BSA below 40°C but heating above this temperature is known to cause denaturation and aggregation of the protein (Khailov et al., 1976). This may account for the large increase in adsorption upon heating to 50°C as observed at both the IEP and pH 6.8.

As the pH was shifted away from the IEP there was a decrease in the level of adsorption, and the levels of adsorption were affected by changes in ionic strength and temperature. At pH's away from the IEP, protein molecules acquire a net charge. For BSA, the net charge increases over several pH units away from the IEP. It is possible that at the ionic strengths of the adsorption versus pH experiments (I 0.06 mole l⁻¹ and 0.16 mole l⁻¹), electrostatic repulsion at pH's away from the IEP affects the packing of the adsorbing molecules. Although the amounts of adsorption at pH 6.8 remained constant for ionic strengths greater than about 0.2 mole l⁻¹, there was a steady decrease in the amount adsorbed with decreasing ionic strength below this value. Diffuse double layer effects will be significant when the diffuse layer extends for distances approaching the dimensions of the protein molecule (14 nm x 4 nm). The diffuse double layer decreases in thickness from ≈2.5 nm to ≈1.0 nm as the ionic strength is increased from 0.03 mole l⁻¹ to 0.2 mole l⁻¹ and further decreases to ≈0.6 nm at 0.5 mole l⁻¹ (Appendix X). The observed adsorption behaviour shows a much slower increase with changing ionic strength than that predicted by the above consideration. There is evidence to suggest that it is the progressive intramolecular structural

rearrangements of the protein molecule, rather than the intermolecular interactions, that determines the varying amounts of adsorption with changing pH.

It has been demonstrated (van Enkevort, 1978) that, in the pH range 3.5 - 5.0, there was a quantitative agreement between the observed amounts of adsorption on stainless steel and those calculated from the hydrodynamic dimensions of the BSA molecule (based on data from the hydrodynamic studies of Champagne (1957a,b,c)). Corrections for void space between adsorbed molecules or structural changes in the adsorbed molecules as might be induced by adsorption were not required. The symmetry of the adsorption data about the IEP, and the similarity of the progressive structural alterations that accompany changes in pH on both sides of this point, suggested a similar agreement would be observed at pH's alkaline to the IEP. The continuity of the pH isotherm curve was not affected by a change in net charge of the surface. Similar behaviour and a good qualitative agreement between the effect of pH on adsorption on stainless steel and hydroxyapatite at pH's above the IEP was observed. (Below pH 5 the increasing solubility of hydroxyapatite precludes its use in experiments). An excellent quantitative agreement was observed at pH 6.8 and the same ionic strength. Increasing ionic strengths confer an increased stability to pH induced changes (e.g. Peters, 1975). At pH 6.8, an increased amount of adsorption was observed as the ionic strength was increased. Unfolding of the BSA molecule occurs above pH 10 (Peters, 1975). As the pH was shifted toward this value, the amount of adsorption approached the expected limiting value of 0.6 mg m^{-2} for close-packed but unfolded BSA molecules. It appears, therefore, that the level of BSA adsorption on hydroxyapatite is also determined solely by the size of the protein molecule in solution.

The effect of temperature on adsorption may be explained by

intramolecular conformational changes. Studies using hydrocarbon model systems show that the extent of phobia for water and the strength of hydrophobic effects for non-polar groups increases with increasing temperature. An increase in temperature should stabilise the native form if hydrophobic interactions are a major factor in the determination of temperature related conformational changes (Ben-Naim, 1980). At the IEP, BSA is in its most stable and compressed form and temperature changes would not be expected to alter its conformation. Consequently, changes in the level of adsorption are not expected and this was observed. At pH 6.8 however, BSA should gradually compress to a more stable form with increasing temperature at this pH and the amount of adsorption at 40°C was almost equal to that observed at the IEP at this and lower temperatures.

11.3.2 THE ADSORBED LAYER OF β -CASEIN

The concentration adsorption isotherm of β -Casein was unusual in that the isotherm consisted of two plateau regions. At low concentrations, a saturation limit of 0.8 mg m^{-2} was observed and with increasing concentration the adsorption isotherm displayed a step which gave rise to the higher plateau level of adsorption of 3.4 mg m^{-2} (Figure 7.6). The concentration range over which the step occurred was affected by temperature and the presence of calcium ions. (Figures 7.7 and 7.8). The amount of adsorption at the plateau levels was independent of the type of hydroxyapatite (Figure 7.6), the specific surface area and time.

Information about the size and shape of β -Casein has been obtained mainly from studies of viscosity and protein adsorption at the air/water

interface. Viscosity data has suggested the molecule to be an ellipsoid of dimensions 13 nm x 2 nm (Waugh et al., 1970) and hence to have cross sectional areas of 20.41 nm^2 and 3.14 nm^2 for the long and short axes respectively (Mills and Creamer, 1973). Adsorption at the air/water interface is thought to be accompanied by unfolding of the polymer chain and this follows some estimate of the area occupied by the molecule. Birdi and Fasman (1972) have suggested that each amino acid residue of non-polar proteins such as Insulin and Albumin (and presumably also the Caseins) occupies on average an area of 0.17 nm^2 . If the protein is predominantly polar in nature, then only partial unfolding occurs and this area is reduced to 0.04 nm^2 . These average residue areas correspond to molecular areas of 35.5 nm^2 and 8.4 nm^2 respectively. Area values, assuming a spherical shape, can also be calculated from partial specific volume data. This method gives a molecular area of 11.53 nm^2 . Results of calculations of the expected amounts of adsorption, assuming the different shapes and sizes mentioned above and different surface configurations, are presented in Table 11.1. The figures in column (a) have been calculated assuming the molecules deform to cover the entire surface area available. If the molecules are taken to be rigid and to pack in a close-packed arrangement, the calculated amounts of adsorption will be reduced. For example, the values for the spherical coil and both the side-on and end-on ellipse are reduced by 20% to 2.7 mg m^{-2} , 1.5 mg m^{-2} and 10.0 mg m^{-2} respectively. If a similar effect applies to the other calculated values (column (b)), it is seen that the experimental data at the low and high plateau levels (for adsorption at 20°C) are in good agreement with those calculated for the unfolded and folded coil.

TABLE 11.1: CALCULATED AND EXPERIMENTAL AMOUNTS OF ADSORPTION FOR
 β -CASEIN

CONFIGURATION	$\Gamma/\text{mg m}^{-2}$	
	(a)	(b)
Unfolded Coil	1.1	0.9
Side-on Ellipse	1.9	1.5
Spherical Coil	3.4	2.7
Folded Coil	4.8	3.8
End-on Ellipse	12.6	10.0
- - - - -		
Experimentally observed: Low plateau	0.8	
(20°C) High plateau	3.4	

^a Molecules assumed to deform and cover entire surface area available
^b Molecules assumed to be rigid and pack in close-packed configuration

The adsorption data therefore suggests that the step in the adsorption isotherm occurs because of a rearrangement in the configuration of adsorbed molecules. This process could be seen as similar to the process of micelle formation in β -Casein solutions. Micellisation is enhanced by increasing temperature and the presence of calcium ions, and the decreased concentrations at which the step in the adsorption isotherm occurred as a result of temperature increases and/or the presence of calcium ions is consistent with this postulate.

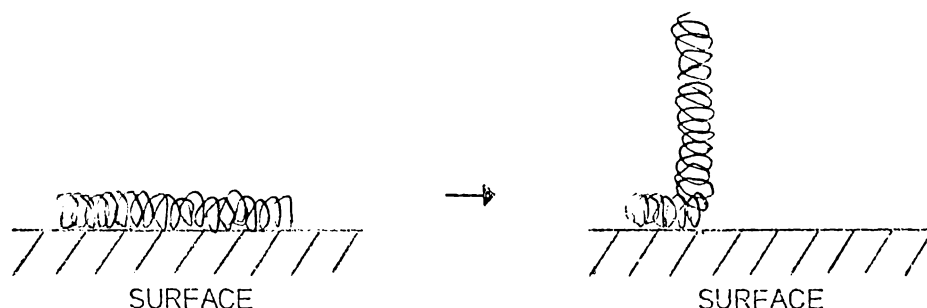
Critical micelle concentrations of about 600 g m^{-3} (Schmidt and Payens, 1972) and $2\,400 \text{ g m}^{-2}$ (Evans et al., 1979) have been observed for some samples of β -Casein. The step in the adsorption isotherm at 20°C occurred at lower concentrations ($\sim 10 \text{ g m}^{-3}$) and suggests adsorption may

facilitate and stabilise the association. This is not unreasonable if adsorption occurs in a configuration that could promote significant protein-protein interaction in the adsorbed layer and/or (further) reduce unfavourable protein-solvent interactions. The avoidance of these is known to be responsible for the self-association of β -Casein. A step in the adsorption isotherm at 4°C, even though β -Casein does not usually undergo self-association at this temperature, is therefore reasonable. The concentration at which the step occurs, at least for β -Casein of Batch 1, is higher than that observed at 20°C as might be expected. The specific reasons for the difference in behaviour of the two β -Casein samples used are not known. Tests for calcium content (performed as the adsorption was observed to be sensitive to the presence of calcium) did not show significant differences. The effect did not appear to be due to experimental procedures either as these were always constant between the two samples. Apparently the differences in adsorption behaviour are due to inherent differences in the samples, either as genetic variants of the source material, possible contamination e.g. from unhealthy animals or other sources, and/or property modification by the separation and purification methods (Section 3.6.3).

β -Casein binds calcium ions. The binding enhances self-association and induces unfolding of the protein molecule. These effects might be expected to further decrease the concentration at which the step in the adsorption isotherm occurs and, at the high plateau level, to lead to increased levels of adsorption. The observed experimental behaviour (Figure 7.8) is in accord with this explanation: the amounts of adsorption were increased slightly at both 4°C and 20°C, and the step in the isotherm now occurred at 0.1 g m^{-3} at 4°C. Unfortunately, the equivalent concentration for the data at 20°C could not be obtained. The experiment was not feasible by the methods available as, at the low

protein concentrations, the long times necessary to reach saturation adsorption were such that the radiochemical method was no longer reliable. However, the shape of the isotherm suggests that the concentration at which the step might occur would be lower still. The amount of adsorption at the lower plateau level might also be expected to be decreased due to the unfolding of the protein molecule induced by calcium binding. Again, the data for this region could not be obtained for the reasons outlined above.

The exchange and desorption data showed that the adsorbed β -Casein was non-exchangeable and not desorbed upon dilution. Any mechanism for the surface rearrangement must account for these phenomena. A suitable mechanism could be the displacement of segments of the protein molecules already adsorbed. It is well known that β -Casein displays detergent-like properties and structure (Section 3.6.3). It could be envisaged therefore that a solution molecule could displace part of the adsorbed molecule without causing desorption. More than one molecule could occupy the area on the surface previously occupied by only one molecule. In order to account for the increased level of adsorption, the adsorption of a further 4 - 5 solution protein molecules would be necessary. The partial displacement could occur either by a buckling mechanism or displacement of one end of the adsorbed molecule. Consideration of the mechanisms for protein adsorption on hydroxyapatite (Section 3.5.3) suggests that for β -Casein, specific surface-protein interactions would occur mainly through the hydrophilic and phosphate containing end of the molecule. The hydrophobic region, comprising 70% of the molecule, might therefore be buckled or displaced into a more coiled and/or vertical position and allow an enhanced adsorption without necessitating desorption or exchange. This is illustrated schematically below:



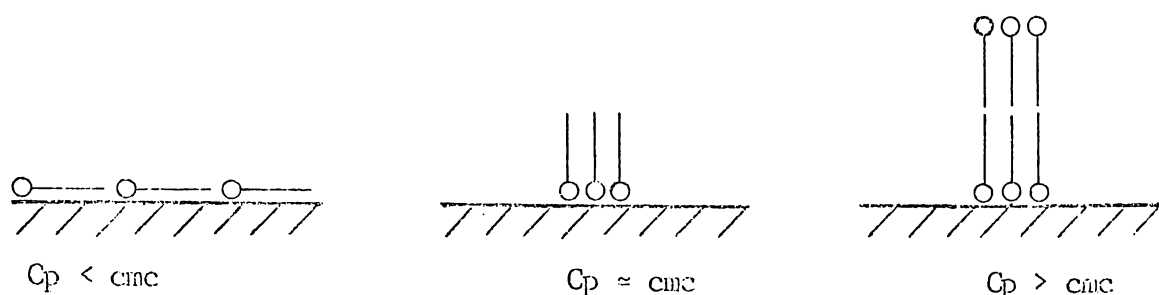
Key:  hydrophilic region of β -Casein
  hydrophobic region of β -Casein

The observation that the adsorption levels on hydroxyapatite were consistently lower than those observed by Nisbet for adsorption on stainless steel, under the same conditions, requires some discussion. As the β -Casein/hydroxyapatite suspensions were observed to flocculate, the surface area available for adsorption might be reduced. The kinetic studies (Section 6.6) showed that in most of the adsorption studies the protein concentrations were such that there was an immediate adsorption corresponding to >60% coverage of the adsorbent surface. Under such conditions the irreversible loss of surface area through flocculation is not likely to occur. The time scale of this experiment is determined by the collision number of the hydroxyapatite particles rather than the particle and protein. The particle concentration was always much less than the protein concentration and hence adsorption could be expected to proceed at a higher rate than flocculation. The shape of the adsorption isotherm at low concentrations did not suggest a loss of surface area occurred (compare Figures 3.2 and 7.6). Also, the observation that the

formation of stable accretions only occurred at surface coverages of less than 60% (Section 9.4.3) and that flocculation at lower degrees of surface coverage was initially reversible (Section 9.3.4) further indicated that flocculation effects were not responsible for the lower level of adsorption in the hydroxyapatite versus the stainless steel system. (Accretion formation is discussed further in Section 11.6).

In the absence of loss of specific surface area in the colloidal system by flocculation, the decreased level of adsorption could be explained by specific protein-hydroxyapatite interactions that induce structural alterations in the adsorbed protein molecule. There is evidence of such specific β -Casein-hydroxyapatite interactions (Section 11.4). On the other hand, Nisbet observed that adsorption on stainless steel, in contrast to hydroxyapatite, was at least partially exchangeable and concluded that adsorption resulted from non-specific hydrophobic forces.

Several authors have suggested some surfactant molecules may change their configuration in an adsorbed layer to allow increased adsorption in a manner similar to that postulated above for β -Casein (Bisio et al., 1980; Scamehorn et al., 1982; and references therein). Bisio et al. envisaged that at concentrations below the cmc the molecules lay prone on the surface, at concentrations near the cmc the molecules adopted a vertical configuration that allowed significant lateral tail-tail interaction and at concentrations just above the cmc, the postulated bilayer formation occurred through the same forces responsible for micellisation of the molecules. This is shown schematically below:



Key: C_p = solution protein concentration
 cmc = critical micelle concentration

The possibility of bilayer formation in β -Casein adsorption therefore requires some discussion.

The experimental data obtained for β -Casein adsorption argues against bilayer adsorption. The level of adsorption at the high plateau i.e. 3.4 mg m^{-2} , is far below that expected for bilayers of spherical coils, folded coils or end-on ellipses, respectively 5.4 mg m^{-2} , 7.6 mg m^{-2} and 20.0 mg m^{-2} (refer Table 11.1). Bilayer formation of molecules lying prone e.g. side-on ellipses does not seem reasonable intuitively as no advantage in the screening of regions of molecules from the solvent is gained and, if such layers did form, it is difficult to envisage why the number of layers should not progress beyond two. The self-association of β -Casein is reversible and solution protein can exchange with micellar protein but the adsorption, even at the highest levels, was observed to be both non-exchangeable and not reversed upon dilution.

11.3.3 THE ADSORBED LAYER OF SALIVARY GLYCOPROTEIN

It was of interest to determine if the glycoprotein of saliva behaved in a similar manner to BSA and β -Casein. Data on the size and shape of the glycoprotein material as separated by Schrage and Oates (1971a) and used in this study is not available. However, it is reasonable to assume that the levels of adsorption observed reflect monolayer adsorption as the adsorption was non-exchangeable and no desorption occurred upon dilution (Section 10.4), and the shape of the adsorption isotherm (Figure 10.2) and amount of saturation adsorption (given the probable relative molecular mass) do not suggest multilayer adsorption.

The presence of calcium ions in the adsorption solutions led to a stepped adsorption isotherm (Figure 10.2) i.e. a plateau level at low protein concentrations followed by a step in the amount of adsorption giving rise to a second and much higher plateau level with increasing concentration. The role of calcium in enhancing the adsorption of the glycoprotein is therefore more similar to its role in the case of β -Casein (Figure 7.8) than BSA (Figure 9.1). The increased adsorption was not desorbed upon dilution and not exchangeable for the reasons discussed as in the case of β -Casein (Section 11.2.2). The step in the adsorption isotherm may have been caused by a configuration change of molecules in the adsorbed layer in a manner similar to that argued for β -Casein. It may not necessarily mean a buckling however. This explanation receives some support from a comparison of the composition and properties of the salivary glycoproteins (Section 3.6.4) and β -Casein (Section 3.6.3) as described below.

Although freshly secreted salivary glycoproteins are highly hydrophilic, bacterial activity can cause the rapid release of the

carbohydrate moieties to expose the protein molecule itself. Schrager and Oates (1971b) have also noted that the conditions under which they collected saliva and isolated the glycoprotein component, and as used in the present study, could also lead to significant release of charged sialic acid groups (acyl derivatives of the amino sugar acid neuraminic acid) from the molecules. The lack of significant electrophoretic polydispersity of the glycoprotein fraction used in the present study (Section 10.2.3(C)) suggests much of the carbohydrate may have been lost. The amino acid composition shows a high proportion of relatively hydrophobic residues, similar to that of β -Casein. Further, both proteins contain few or none of the sulphur containing amino acids which frequently contribute to the formation of specific tertiary (or quaternary) structures of proteins through the formation of disulphide bonds. The salivary glycoprotein polypeptide chain may therefore have a high degree of flexibility and hydrophobic forces may play a significant role in determining the physicochemical behaviour of the protein molecule in a manner similar to that for β -Casein. Many salivary glycoproteins and other proteins are known to interact with calcium ions. This is known, at least for some components, to result in changed conformations and/or some degree of association (e.g. Bettelheim, 1971; Bennick, 1977b). The association has been suggested to occur through hydrophobic forces and/or conformational changes in the protein core rather than electrostatic bridging by the calcium ions (Bettelheim, 1971; Boat et al., 1974). This behaviour is also typical of that observed for β -Casein.

11.4 A RECONSIDERATION OF THE ADSORPTION MECHANISM

It is generally considered that electrostatic interactions are responsible for protein adsorption on hydroxyapatite (Section 3.5.3). Both BSA and β -Casein contain positively and negatively charged groups and these could interact with the charged species on the hydroxyapatite surface. Although the salivary glycoprotein was of zero net charge under the conditions of the experiment, positively and negatively charged groups will still co-exist in the protein and allow interaction. However, there is evidence in the literature which is supported by the present work that non-ionic and entropic effects also contribute to the driving force for adsorption. Arguments pertaining to BSA and β -Casein are discussed below. The physico-chemical similarities between β -Casein and the salivary glycoprotein component suggests that arguments proposed for β -Casein should also apply to this protein.

The hydroxyapatite surface has been suggested to have both hydrophilic and hydrophobic regions (Rootare and Craig, 1977). Some interaction between the hydrophobic regions of the protein and the surface might therefore be expected. The hydroxyapatite surface binds many layers of water and this binding is accompanied by a large and negative entropy change (Rootare and Craig, 1977). If an adsorbing protein molecule were to displace some of this water from the crystal surface, the process would be accompanied by a positive entropy change that would contribute to the driving force for adsorption.

According to the literature on protein adsorption, hydrophobic forces can provide a driving force for adsorption for both hydrophobic and hydrophilic adsorbents as proteins generally cannot adopt a conformation which removes all hydrophobic residues from an aqueous solvent (Section 3.3.4). The desorption studies showed that high

concentrations of salt and/or high pH did not induce complete desorption of adsorbed protein. Apparently adsorption is not governed entirely by electrostatic interactions for either BSA or β -Casein. Further, the hydrophobic effect appears to be more important for β -Casein (at least at the high plateau level of adsorption) than for BSA. β -Casein was less readily desorbed than BSA under all conditions, and the incorporation of SDS into the desorption solution caused increased desorption of β -Casein but had little effect on BSA. This trend follows the respective degree of hydrophobicity of these proteins.

Similarly, structural rearrangements in the adsorbed/adsorbing molecules may play an important role in the adsorption process as hydrophobic forces also contribute to the determination of protein structure. This has previously been suggested by Norde and co-workers, particularly in reference to the adsorption of HSA and Ribonuclease (e.g. Norde and Lyklema, 1979). Such an effect is consistent with the postulated change in conformation of the adsorbed β -Casein molecules at the step in the adsorption isotherm and the effects of temperature and calcium concentration on the phenomenon. The spontaneous association of β -Casein molecules in aqueous solution is an endothermic process, driven by hydrophobic forces, with a consequent large positive entropy change (Section 3.6.3). Increasing temperature would favour the association process. This was manifested in the adsorption behaviour (Figure 7.7) by a shift in the position of the step to a lower protein concentration at a higher temperature. The effect of the presence of calcium ions in β -Casein solutions is to enhance the hydrophobic interactions that lead to micellisation. If hydrophobic forces are important in the stepping effect, the observed enhancement of the effect by calcium is predictable.

The use of urea in the desorption solutions caused an increase in the level of desorption. As urea is known to be a hydrogen bond

disrupting agent, this suggests that these interactions also contribute to the protein-hydroxyapatite binding. The effect was observed to be greater for BSA than for β -Casein.

The results of the competitive adsorption experiments suggest that β -Casein adsorption, at least at the high plateau level, is energetically more favoured than that of BSA. From the above discussion, it seems reasonable to suggest that at least part of this is due to a more favourable entropic contribution to adsorption. Other factors may also be important as discussed below.

Although adsorbed BSA could be exchanged by solution BSA (Figure 8.1), adsorbed β -Casein could not be exchanged by solution β -Casein (Figure 8.3). The model proposed for the adsorbed layer of β -Casein (Section 11.3.2) is such that it could be envisaged that at the high level of adsorption, where the hydrophilic part of the molecules lies prone on the surface and the hydrophobic end is buckled upwards and away from the surface, an intermolecular interaction between the protruding hydrophobic tails and/or steric effects could prevent exchange. However, intermolecular interactions in the adsorbed layer are probably not important in preventing exchange as micellar β -Casein can exchange with solution β -Casein. Similarly, steric effects are probably also not important in preventing exchange as even at the low level of adsorption, where the molecules are postulated to lie prone on the surface, the adsorbed molecules were not exchangeable. It therefore appears that the non-exchangeability of adsorbed β -casein may be due to specific protein-hydroxyapatite interactions.

BSA and β -Casein both bind calcium ions and hence specific protein-hydroxyapatite interactions probably involve these ions. The binding in β -Casein is much stronger, the average binding constants being $\sim 100 \text{ l mole}^{-1}$ and 900 l mole^{-1} respectively (Sections 3.6.2 and 3.6.3). Further, the

binding constant for each site in BSA is relatively constant (Fogh-Andersen, 1977) but that for β -Casein is much greater for the first ion bound: $\sim 2\,400\text{ l mole}^{-1}$ (Parker and Dalglish, 1981). The binding of calcium by phosphoproteins is highly dependent on the presence of the phospho group in the protein (e.g. Bettelheim, 1971; Bennick, 1977b). This suggests that any specific calcium-phospho group interactions involving β -Casein will be stronger than the equivalent calcium-carboxyl interaction expected for BSA.

11.5 STABILITY OF HYDROXYAPATITE SUSPENSIONS

Suspensions of non-aggregating particulates are usually stable to settling if the dimensions of the particulates are in the range of those of colloidal species i.e. from 1 nm to 1 μ m. Although the particulates of the various hydroxyapatite and amorphous calcium phosphate preparations were of colloidal dimensions (Section 4.3.3), suspensions of these materials were observed to settle. The presence of protein altered the stability of the suspensions to settling, BSA enhancing stability and β -Casein (Section 9.3) and salivary glycoprotein (Section 10.5) decreasing stability. Prior to discussion of these phenomena, a brief description of some of the theoretical aspects of the stability of colloidal suspensions seems warranted. Various comprehensive reviews are available and the reader is referred to these for a more in-depth discussion (La Mer and Healy, 1963; Healy, 1970; Vincent 1974; Sato and Ruch, 1980).

The total energy of interaction, V_T , between two like charged particles on collision is taken as the sum of the attractive and repulsive potential energies:

$$V_T = V_A + v_R^{\text{elec}} \quad - - 11.1$$

where V_A = interparticle attraction energy arising from van der Waal's forces

v_R^{elec} = electrostatic repulsion between particles of like charge.

At short and large distances the attractive energy may dominate but at intermediate distances there may be electrostatic repulsion (Figure 11.2).

At small and large distances the attractive forces may predominate but at intermediate distances there may be net electrostatic repulsion. Although the coulombic term is sensitive to changes in the nature and

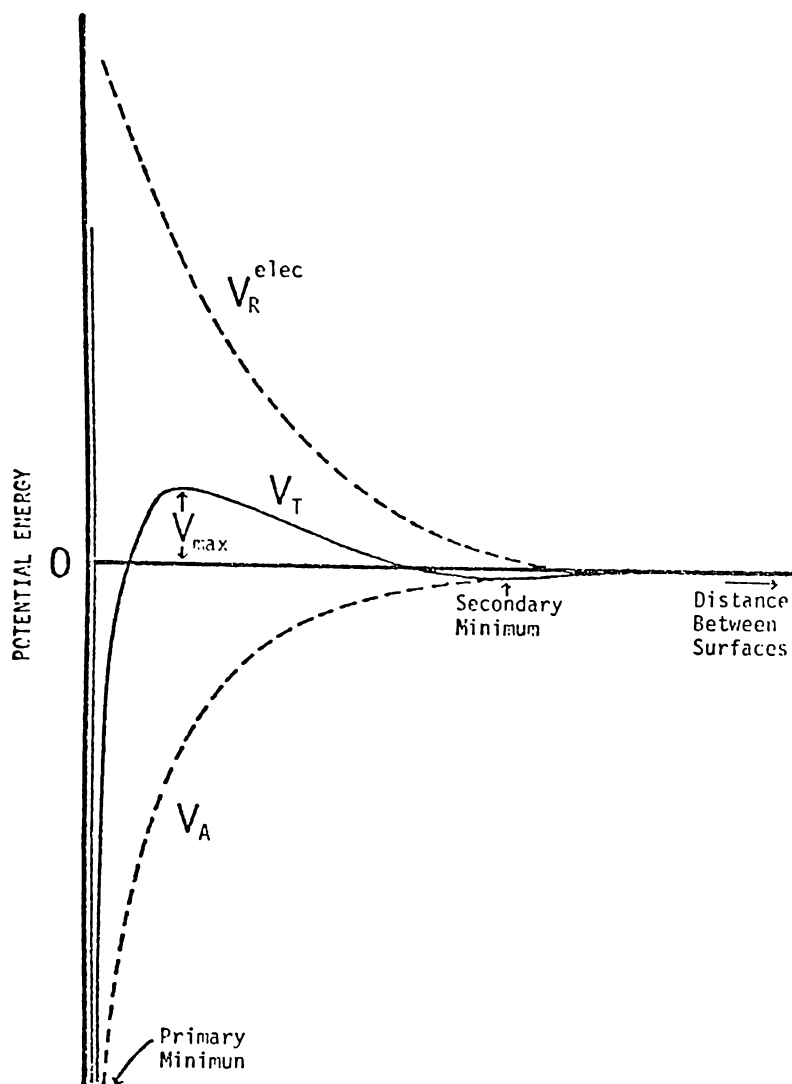


Figure 11.2: Potential Energy Curves for the Interaction of Two Charged Surfaces

concentration of electrolytes present, the van der Waal's term is only dependent on temperature. For particles charged by the adsorption of H^+ and OH^- e.g. hydroxyapatite, changes in the pH will have a profound influence on the surface potential. This may result in the coagulation of the colloidal particles, particularly at the IEP.

The adsorption of electrically neutral macromolecules was observed

in many early studies to result in the stabilisation of colloidal dispersions. This was attributed to a steric stabilisation effect where the adsorbed polymer prevented contact and coalescence of approaching particles (see Sato and Ruch, 1980 and refs. therein). In the presence of polymer, equation 11.1 must be modified to include an additional repulsive energy term, V_R^{steric} :

$$V_T = V_A + V_R^{\text{elec}} + V_R^{\text{steric}} \quad - - 11.2$$

V_R^{steric} is suggested to arise via an entropic or osmotic effect (see review by Sato and Ruch, 1980):

(i) Entropic Effects: The adsorbed layer is assumed to be impenetrable. Compression of the adsorbed layer by the approach of a second particle results in a loss of configurational entropy of the polymer segments in the region of interaction.

(ii) Osmotic Effects: If the adsorbed layers overlap, a high polymer segment concentration would arise in the region between the particles. This would cause an "osmotic" repulsion effect.

A third mechanism may arise if the adsorbing polymer is an ionic substance and if complete coverage of the surface occurs. In this case the colloidal dispersion may be stabilised by increased coulombic repulsion as well as by a steric effect (Taylor, 1977). The complete coating by the polymer would impart to the colloid, to some extent, the aggregation properties of the dispersed polymer (van der Scheer et al., 1978a).

The destabilisation and flocculation of colloidal dispersions by polymer adsorption is generally interpreted in terms of two mechanisms: surface charge neutralisation and via bridging mechanisms.

Neutralisation of surface charge may result in dispersions stabilised by coulombic repulsion when an oppositely charged polymer is

adsorbed onto a particle. Flocculation then results through a lowering of V_R^{elec} .

Polymer bridging may result in flocculation via two mechanisms:

Type 1: bridging of two or more particles by one polymer molecule when the polymer has more than one adsorption site and the surface coverage is low. This type of bridging could be expected to occur at low polymer concentrations and to be dependent on the relative polymer and particle concentrations.

Type 2: bridging between adsorbed polymer chains may occur if the chains are sufficiently long and the association of the interacting chains is favoured over the repulsion due to electrostatic, entropic and/or osmotic effects.

Flocculation is a complex phenomenon and may be affected by agitation, the method of mixing, the relative rates of adsorption and flocculation and the nature of the polymer.

The settling of pure hydroxyapatite and amorphous calcium phosphate suspensions indicates that interparticle attraction forces, V_A , are stronger than the electrostatic repulsion of the like charged particles, V_R^{elec} . The surface of hydroxyapatite surface has hydrophobic regions (Section 5.6.5) and the effect could be a characteristic of this. However, as described in the discussion of the mechanism of adsorption (Section 11.4), the adsorption of a protein molecule to hydroxyapatite could result in dehydration of the surface with a consequent entropy gain. A similar effect in which two or more particulates approach each other with some consequent dehydration of the surface and coalescence may explain the instability of the suspensions. The effect is similar to "hydrophobic bonding" and could also account for the massive flocs formed in ACP suspensions.

The suspensions are stabilised by adsorption of BSA. Increasing

stabilisation with an increasing degree of protein surface coverage is attributable to a decrease in the contribution of the attractive force, V_A , and/or increasing contributions from either or both the repulsive forces V_R^{elec} and V_R^{steric} .

In contrast to the behaviour for BSA, the adsorption of β -Casein and the salivary glycoprotein caused destabilisation and flocculation of hydroxyapatite suspensions. The effect occurred at all degrees of surface coverage by β -Casein. As the electrical properties of BSA and β -Casein are quite similar, the difference in behaviour seems to rule against both the stabilisation by BSA and destabilisation by β -Casein as attributable to electrostatic effects. It seems likely that stabilisation by BSA would be at least partly due to electrostatic and steric effects whereas in the case of β -Casein, destabilisation is probably due to a bridging mechanism. The Type 2 bridging mechanism is in some ways similar to the self-association that occurs in solution. At the higher degrees of surface coverage, the tendency of β -Casein to self-associate is likely to promote a Type 2 bridging mechanism. However, at the lower degrees of surface coverage, such a mechanism is unlikely and a Type 1 bridging mechanism, involving specific protein-hydroxyapatite interactions, is probable. The mechanism of Type 1 bridging can be expected to involve both ionic and non-ionic interactions whereas Type 2 bridging will be predominantly non-ionic.

11.6 MULTILAYER ADSORPTION

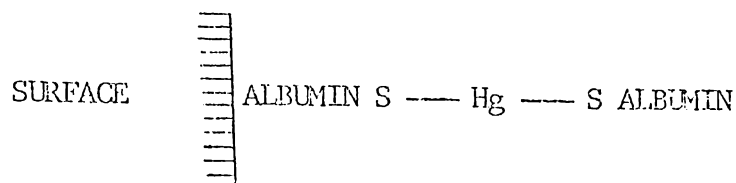
In the discussion on the nature of the adsorbed layer of BSA, β -Casein and salivary glycoprotein, it was concluded that adsorption is limited to monolayer coverage, even for proteins that tend to self-associate (Section 11.3). The multivalent metal ions Ca^{2+} and Hg^{2+} were observed to enhance the adsorption of BSA (Section 9.2) and Ca^{2+} also enhanced β -Casein adsorption (Section 7.3.2(iii)).

It can be envisaged that multivalent ions could act as bridging species by electrostatic or site-specific reactions and thereby enable/facilitate multilayer adsorption. Although calcium ions enhanced BSA and β -Casein adsorption, the non-exchangeability and non-reversibility of the protein so adsorbed (Section 9.2 and Section 8.4 respectively) was inconsistent with micelle-like multilayers. In contrast, mercuric ions enhanced the adsorption of BSA and the enhanced adsorption was reversible (Section 9.2).

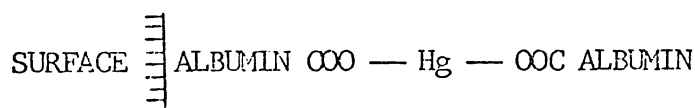
The effect appears to be attributable to differences in the binding of the ions: calcium is bound essentially by weak electrostatic interactions but mercury is bound by both these and a site-specific covalent interaction involving the free sulphydryl group (Section 3.6.2). The binding constants for the mercury interaction are of the order of 10^2 and 10^5 times greater, respectively, than those for calcium. Further, the mercury ion can interact simultaneously with the free sulphydryl groups of two adjacent molecules to form a dimeric species. Multilayer formation by ion bridging therefore seems to require covalent bonding.

The amount of additional adsorption induced by the mercuric ion was about equal to that of the first adsorbed layer (Figure 9.1). This indicates that the enhanced adsorption probably arises through a dimerisation reaction in which the second layer is equivalent to the

first:



However, this may be fortuitous. The free sulphydryl content of commercial BSA preparations is usually only about 0.65 mole/mole Albumin (e.g. Peters, 1975). This might be increased by the radioiodination procedure and the mercuric ion (the ion can disrupt disulphide bonds (Brown and Edwards, 1969) which are known to be responsible for the decreased free sulphydryl content of monomeric forms of BSA and to be involved in the polymerisation of the protein). However, the presence of excess mercuric ion, as under the conditions of the experiment, causes dissociation of the dimer (Foster, 1960). Also, it was observed that mercuric ion concentrations above $\sim 0.005 \text{ mole l}^{-1}$ caused precipitation of BSA in the adsorption solutions. Other forms of intermolecular binding might therefore also contribute e.g. bridging via carboxyl groups:



Such an effect presumably does not occur with calcium as the binding of this ion by the carboxyl group is much weaker (and the protein cannot be precipitated by low concentrations of calcium).

The results of the flocculation and accretion studies showed that multilayer formation could also be promoted by particulate bridging species (Section 9.4). The destabilisation of hydroxyapatite suspensions upon adsorption of β -Casein was attributed to either of two bridging mechanisms (Section 11.5) and both of these result in flocculation of the suspension. Significant accretion formation occurs only at levels of

adsorption corresponding to less than 60% saturation. Type 1 bridging, which involves specific particle-protein-particle interactions and is more probable at low degrees of surface coverage, is expected to lead to stable multilayer and accretion formation as the adsorbed layer of β -Casein is non-exchangeable and non-reversible. Type 2 bridging, which arises through interactions between the adsorbed molecules themselves and hence will predominate at high degrees of surface, is expected to be less stable as the region of interaction between the particles now more closely approximates that of the β -casein micelle i.e. a reversible interaction. This suggests that beyond 60% saturation of the adsorbent surface, the major mechanism of flocculation and accretion formation changes from a Type 1 to a Type 2 bridging mechanism and the possibility of stable multilayer adsorption is reduced.

Macroscopic accretion formation did not occur in the BSA/hydroxyapatite system although a weak effect was noted at 30% - 40% surface coverage (Section 9.4.3). Dass (1982) observed that BSA destabilised suspensions of β -ferric oxyhydroxide and promoted multilayer adsorption and macroscopic accretion formation. This was attributed to a Type 1 bridging mechanism as BSA does not usually undergo self-association and multilayers could be formed even when the protein and colloid species were of the same charge. It appears therefore that the lack of significant multilayer adsorption and accretion formation in the hydroxyapatite system is not due to an inherent inability of BSA to act as a bridging species. The effect may be due to physical shearing effects and/or the exchangeability of the adsorbed protein giving rise to unstable multilayers or to the presence of a sufficiently large energy barrier that inhibits the close approach of adsorbed BSA/hydroxyapatite particles under the conditions of the experiment. Shearing effects are unlikely to be a major contributory factor, however, as the size of the

hydroxyapatite particulates (≈ 150 nm x 22 nm) is similar to the β -ferric oxyhydroxide particulates used by Dass (150 nm x 38 nm) and thick accretions could be formed with β -Casein.

Dass (1982) has suggested that the main requirement for multilayer adsorption and accretion formation by particulate bridging species is that the particulate have a high affinity for the protein. The results of the present study show that while a high affinity type adsorption isotherm may be necessary, it is not sufficient to enable such phenomena for all systems. It is postulated that colloid/protein multilayer adsorption occurs when protein adsorption leads to bridging between the particulates. Accretion build-up will occur if the protein can bridge between particulates and the support surface or if the particulate can bridge between the support surface and protein molecules. The stability of the multilayers and accretion might be affected by any of the variables which affect protein adsorption, the stability of particulate and protein/particulate suspensions and the support-protein-particulate or support-particulate-protein interaction.

11.7 PROTEIN ADSORPTION EFFECTS IN THE ORAL ENVIRONMENT

One of the aims of the studies conducted in the present work was to gain a better understanding of phenomena that occur in the oral environment. An important conclusion reached is that the hydroxyapatites studied have high affinities for both hydrophilic and hydrophobic proteins. Adsorption in pure systems proceeds to a saturation level which corresponds to complete coverage of the available surface by a monolayer of protein molecules. The amount of adsorption is determined by the available surface area, the size of the protein molecule in solution and the configuration of the molecules in the adsorbed layer and, hence, also by variables which affect these factors. The presence of fluoride and carbonate as ionic substitutions in the hydroxyapatite lattice does not affect the amount of adsorption. The solution protein concentration required to maintain saturation is very low and the adsorption is essentially irreversible to dilution. However, in some instances the adsorbed protein may be exchangeable with solution protein. Non-exchangeability appears to be related to the presence of phosphate groups in the protein.

The adsorption of protein to the hydroxyapatite surface proceeded irrespective of the sign or magnitude of the net charge of the proteins or adsorbent studied. The complete removal of adsorbed protein was not achieved even under conditions expected to lead to strong electrostatic repulsion between the protein and surface. Although the current view is that protein adsorption on calcium phosphates results through ionic interactions, the present studies indicate that non-ionic interactions can also be important. In general, the contribution of ionic and non-ionic interactions is determined by the nature of the adsorbent surface, the protein and the solvent. The role of the solvent is important in

that changes in the nature of the solvent can affect the hydrophilicity/hydrophobicity of the adsorbent and/or the protein. Substitutions in the hydroxyapatite lattice may alter the relative contribution of ionic and non-ionic interactions similarly and could also affect specific interactions between protein and surface. If the affinity between the protein and surface is altered by the incorporation of other species in the hydroxyapatite lattice, changes in the composition of films adsorbed from solutions of mixed proteins might also be expected. Studies of early pellicle formation reported in the literature show that the composition is uniform throughout the mouth, suggesting that the overall effect of impurities in the hydroxyapatite is very small. Studies of early pellicle on widely different surfaces, e.g. various plastics materials and glass, do show differences however (Section 3.7.2).

Selective or preferential adsorption of proteins from saliva has often been reported and, until recently, the implication was that some proteins might not be adsorbed. Studies using various isolated salivary peptides and proteins show that these are all adsorbed by hydroxyapatite. Further, the proven usefulness of hydroxyapatite in chromatographic procedures for a wide variety of proteins and the lack of evidence for non-binding proteins suggests the presence of non-adsorbing proteins in saliva is unlikely. The chromatographic studies and those of the present work have shown that some proteins are more tightly bound than others, particularly the phosphoproteins. Juriaanse et al. (1981) have recently suggested that phosphoproteins might therefore displace other (phospho)proteins from the hydroxyapatite and enamel/pellicle interface. A comparison of the adsorption behaviour of BSA (a non-phosphorylated protein) and β -Casein (a phosphoprotein) in pure and mixed systems shows that an adsorbed protein can exchange with solution protein, and, the

more strongly bound protein (β -Casein) could displace the less strongly bound protein (BSA) but not vice versa. These results provide direct experimental evidence of the preferential or selective adsorption of some proteins by hydroxyapatites.

A brief overview of some of the theories of pellicle formation was presented in Section 3.7.2. Some of these theories have been discounted on clinical grounds. The review of the literature on protein adsorption reveals that some of the theories are also not in accord with what is known about protein adsorption. For example, it has been reported that pellicle formation results from repeated adsorption, leading to denaturation and aggregation. This is not consistent with the fact that protein adsorption ceases at monolayer coverage. Further, there is no evidence to indicate that adsorption at electrically neutral solid/liquid interfaces causes gross changes in the structure or unfolding of the protein polypeptide chain. It has been suggested (Einfeld, 1978) that pellicle builds up to layers of $\sim 1 \mu\text{m}$ because it is only beyond this distance that the positive electrostatic potential of surface calcium ions is neutralised by the adsorption of anionic proteins. This is not consistent with the fact that protein adsorption often proceeds even when the protein and adsorbent are of the same (net) charge, and that electrophoretic experiments show that when adsorbed protein molecules cover an adsorbent surface, the electrical properties of the adsorbent tend to those of protein itself (e.g. Chatteraj and Bull, 1959, Norde 1976; Bagchi and Birnbaum, 1981; Dass, 1982).

A great deal of experimental evidence indicates the importance of calcium ions in aggregation phenomena in the mouth. The evidence supports the hypothesis that this ion may bridge between negatively charged groups to form a three dimensional network of aggregated material such as the dental pellicle. Recently, authors have postulated that

inorganic ions are not essential to the structural integrity of pellicle. Glantz (1980) studied, by dispersive energy X-ray analysis, the film formation on silica or germanium prisms worn intra orally by human subjects for 15 minutes. If the prisms were rinsed with distilled water no appreciable amounts of calcium were observed in the film. Those prisms which were not rinsed did contain significant calcium. Glantz therefore suggested that the presence of calcium in oral films might be due solely to the permeation of calcium into the pellicle. He did not establish that the film remained on the prism surface after rinsing however, nor did he show that the films could be formed in the absence of calcium. In a more recent study using titanium surfaces and a similar detection method and experimental procedure (10 minute exposure time, followed by draining or rinsing), Leach and Appleton (1981) report similar findings for calcium and various other inorganic species (K, Na, Mg, Cl, P). The authors state that the presence of the pellicle could easily be demonstrated by gently scratching the titanium surface with a sharp metal point. Similarly, Neuman (1980) has shown that pellicle can be removed, seemingly physically intact, from an extracted tooth by immersion in HCl (0.1 mole l^{-1}) and under these conditions, the pellicle can be shown to be free of calcium while still retaining its structural integrity as observed by electron microscopy. However, neither of these studies preclude the involvement of inorganic ions in pellicle formation.

The present studies show that bilayer formation by ion bridging, at least under conditions in which precipitation is not induced, requires a covalent interaction between protein molecules and a bridging ion. The formation of larger numbers of layers would require multiple binding sites i.e. at least one on the "top" and the "bottom" of the adsorbed protein molecule. Multilayer formation would require a relatively non-labile interaction between the protein molecules and the bridging ion.

The ability of a protein to bind a multivalent ion does not indicate multilayer formation will occur, even when the binding is relatively strong e.g. compare calcium binding by β -Casein and BSA. It is difficult to make conclusive statements about the salivary proteins as these are still generally poorly characterised. As yet there is no evidence to indicate that the proteins in saliva, under normal conditions, are irreversibly associated through intermolecular interactions with calcium or other polyvalent ions. The precipitation of salivary material by added calcium does not occur in the absence of inorganic phosphate. Further, studies of calcium binding by various isolated salivary components which associate to some extent as a result of binding indicate that association does not occur via a calcium ion bridging mechanism.

The most recently proposed theory of aggregation phenomena in the mouth suggests hydrophobic interactions between proteins, resulting in association, may be important in pellicle and plaque formation (Leach, 1980). Individual molecules could first associate in solution and adsorb or else attach to an already present matrix. Although whole protein flocs can adsorb and adhere to surfaces (Berridge and Scurlock, 1976), the present studies, using proteins, and other studies using surfactants, show that molecular associates formed by hydrophobic forces generally do not adsorb. Rather, the monomeric species adsorbs. Although bilayer formation may occur in the adsorption of straight chain detergent molecules as a result of hydrophobic forces, protein molecules generally do not have this linear backbone, nor the sharp separation between hydrophobic and hydrophilic regions which would contribute to such behaviour.

The present studies allow an alternative explanation for pellicle formation to be developed. It has been shown that stable multilayers can be formed by some proteins using amorphous calcium phosphates as a

bridging species. The phenomenon was observed for the salivary glycoprotein isolated, and the observation that hydroxyapatite suspensions were destabilised and flocculated by all the fractions collected during gel filtration of saliva is a good indication that accretion formation by this mechanism will be possible with other saliva components. Such a phenomenon may therefore be important in pellicle formation in the mouth. The fundamental requirement of the hypothesis is a mechanism for the formation of insoluble calcium phosphate salts, either in saliva, in situ at the (adsorbed) protein/saliva interface or in a pellicle formed by other mechanisms. The chemical form of calcium and phosphorous in saliva is still controversial. There is, however, much to suggest the hypothesis is reasonable.

Although saliva contains many protein factors which inhibit calcium phosphate precipitation, their activity need not be confined to blocking nucleating activity.

Saliva is saturated with respect to many calcium phosphate phases and although precipitation is not usually observed, this can be induced by small changes in chemical conditions or by physical effects. The precipitates formed are an intimate association of protein and a calcium phosphate mineral of very small and poorly defined crystals or amorphous colloidal particles.

Pellicle that appears to be free of crystalline mineral matter often stains differentially in a laminated pattern. In cases where calcification is not extensive, the deposition of calcified material also follows a laminated pattern, running approximately parallel to the tooth surface. The lamination suggests a controlled deposition of both inorganic and organic components of the pellicle (Leach, 1973).

The mechanism of pellicle formation by multilayer adsorption via a colloidal calcium phosphate bridging species is consistent with the

mineralisation of pellicle and the formation of calculus. The crystalline phases observed in calculus (Section 3.7.3) are all observed in the conversion of amorphous calcium phosphate to crystalline phases (Section 3.7.4). Studies of calcium phosphate precipitation show that the amorphous product is formed first under most conditions both in pure calcium and phosphorous systems and in impure systems containing organic material. The fact that electron microscope studies have not shown any direct evidence of amorphous calcium phosphate particles in pellicle, plaque or calculus is not altogether surprising. The mechanism does not seem to have previously been considered nor amorphous calcium phosphate particles sought. In addition, the primary non-crystalline material which has so far been used as a reference material has not been precipitated in physiological systems and the inherent instability of the material, problems associated with the observation and characterisation of calcium phosphates by electron microscopy or electron and X-ray diffraction, and the use of decalcification techniques in sample preparation make location and characterisation difficult.

There is some indirect evidence for the presence of the amorphous calcium phosphate particles however. In a study of pellicle morphogenesis, Lie (1977) observed small globules which resemble the aggregates formed upon the precipitation of amorphous calcium phosphate in the presence of biopolymers described by Eanes et al. (1973). The aggregates were either densely granular or more loosely arranged exhibiting small electron translucent areas. Lie describes the aggregates as those of organic substances and does not suggest the possibility of an inorganic component. However, the aging mechanism observed for these globules is also very similar to that reported by Eanes et al. for the conversion of amorphous calcium phosphate to a crystalline material. The time scale of the process (up to 2 days)

compares favourably with the known stabilisation of amorphous calcium phosphate by biological macromolecules (section 3.7.4) and the observation that the calcification of dental integuments is quite obvious within this time period. Indeed, crystalline material was apparent 2 hours after the commencement of pellicle formation. Non bacterial structures resembling globules in pellicle, beneath plaque, are also apparent but left unexplained in some previous studies of plaque morphology (Frank and Houver, 1970; Berthold et al., 1971). Structures similar to the basic particles of amorphous calcium phosphate have also been described in studies of calcifying bone and tissue (Molnar, 1959).

11.8 SOME SUGGESTIONS FOR FURTHER WORK

An important and unexpected observation of the present work was that all the proteins studied displayed high affinity type adsorption isotherms. In the case of BSA and β -Casein, detailed reversibility and exchange experiments showed that although high, the affinities were not the same. It would be of interest to ascertain the adsorption isotherms of other proteins e.g. food, salivary and serum proteins, and to determine the relative affinity of each for hydroxyapatite. It could not be evaluated from the experimental results whether the preferential adsorption and greater tenacity of adsorbed β -Casein was due to the presence of phospho groups in β -Casein or to the greater hydrophobicity of this protein. Adsorption studies with a relatively hydrophobic non-phosphoprotein would initially prove useful in determination of the effect of these variables. Such studies could be combined with adsorption and desorption experiments in which the effects of substitutions in the hydroxyapatite lattice and different solvents were investigated.

The results of the desorption studies indicate that quantitative recovery of adsorbed protein is not possible. This is surprising in view of the use of calcium phosphates in protein chromatographic procedures and the implied quantitative recovery of the adsorbed protein. It was observed in the studies of β -Casein induced flocculation of hydroxyapatite that the flocs at low degrees of surface coverage were initially readily dispersed but were irreversibly formed after standing for about 1 hour. This suggests that the protein molecules in the adsorbed layer may undergo changes such that adsorption becomes less readily desorbed with time and experiments to test this phenomenon can be envisaged.

Jurjaanse et al. (1981) have recently proposed that a protein phosphate-surface phosphate exchange may occur. Such an effect is reasonable as the phosphate groups in the hydroxyapatite surface are exchangeable (see review by Kibby and Hall, 1972). It could also be envisaged that the carboxyl groups of the protein might exchange with either the phosphate and/or carbonate groups of the hydroxyapatite surface. Experiments to determine whether such exchange reactions occur between hydroxyapatites and adsorbed protein may be possible.

The adsorption behaviour of β -Casein was unusual in that this protein displayed a stepped adsorption isotherm. Experiments to further characterise the nature of the adsorbed layer and to investigate whether other self-associating proteins display similar behaviour are of interest. The adsorption behaviour of the salivary glycoprotein at concentrations above those used in the present study would be of particular interest as literature and preliminary experimental observations have suggested this protein might behave similarly to β -Casein.

Protein adsorption in systems of suspended particles (i.e. high surface area : volume ratios) is often a rapid process and not easily followed. Recently, Dass (1982) adopted a technique based on the principles of the rotating disc electrode that allowed determination of the kinetics of adsorption in such systems. The adsorbent particles are fixed to a rotating disc which is placed in the protein solution and the kinetics of adsorption determined by the principles described by Levich (1962). Similar experiments using hydroxyapatite particles should also be possible.

Although Serum Albumin adsorption generally proceeds only to monolayer coverage, the presence of mercuric ions led to an enhanced adsorption that was consistent with bilayer formation. The second layer

was reversible to dilution and exchangeable. The protein β -Lactoglobulin exists in slightly acidic/neutral solution as a dimeric species in equilibrium with a monomeric species but the presence of cupric ions leads to irreversible dimerisation (McKenzie, 1971b). This suggests that this protein may also show bilayer adsorption but, unlike Serum Albumin, might be expected to be stable to dilution or solution protein effects. Experiments to investigate the possibility would be useful to further the understanding of the effects of polyvalent metal ions in modifying adsorption behaviour and could be extended to include other proteins which may display similar behaviour.

The phenomenon of macroscopic proteinaceous film formation through the use of colloidal bridging species requires further investigation. It was observed in the present study that whereas several colloidal calcium phosphates promoted β -Casein multilayer formation on glass, multilayer formation was not observed with BSA. Dass (1982) has observed that various silicas, clays, iron oxides and polycation species and a polystyrene latex all enabled multilayer formation of BSA on stainless steel, and Nisbet (1979) observed that although a colloidal calcium phosphate also promoted multilayer formation of β -Casein on this surface, silica and allophane (a clay) did not. The specific properties of a particular surface, protein and colloid that determine multilayer formation are not clear. Presumably the properties of the solution in use are also important. Experiments to clarify the phenomenon would be of considerable scientific and technological interest.

Attempts to radioiodinate the proteins Haemoglobin, Myoglobin, Ovalbumin and Lysozyme by the Chloramine-T and Iodo-Gen methods resulted in an unacceptable degree of aggregation of these proteins. Although a different radioiodination technique (Section 5.2) may provide useful tracers for these proteins, proteins are often stabilised against

denaturing conditions when adsorbed to a surface and, recently, Horbett (1981) and Horbett and Weathersby (1981) have reported the in situ iodination of adsorbed protein. Such a modification to radioiodination methods may provide a useful technique to yield information on the surface chemistry of easily denatured proteins where this cannot be obtained by other than radiochemical methods.

CHAPTER 12

GENERAL SUMMARY

An attempt is made in this thesis to gain a better understanding of the interactions of proteins at the hydroxyapatite/aqueous solution interface, as a fundamental process in pellicle formation on teeth, and to resolve some of the confusion and contradiction in the literature. An investigation of various aspects of the surface chemistry of some common dietary proteins at the hydroxyapatite/aqueous solution interface has been undertaken.

Three crystalline hydroxyapatites and an amorphous calcium phosphate were prepared and characterised for the studies envisaged. The crystalline materials included a pure stoichiometric hydroxyapatite and fluoride and carbonate substituted hydroxyapatites. The degree of fluoride and carbonate substitution was such to closely approximate those levels commonly found in tooth enamel.

Radiochemical techniques were mainly used in the adsorption studies and were found to provide a reliable and sensitive method in solid/liquid systems, they were readily adaptable to systems of both high or low surface area : volume ratios. Literature reports had suggested that a low degree of labelling was essential to avoid significant alteration of the physico-chemical properties of the labelled molecule. The use of labelled proteins as tracers in studies of interfacial phenomena requires several criteria to be met. A significant part of the work of this thesis was directed towards establishing the validity of the use of radioiodine labelled proteins as tracers for the studies envisaged. It was observed that the labelled proteins did provide a useful and sensitive means of monitoring interfacial behaviour providing certain precautions were observed:

(i) The radioiodine label was stable for up to two weeks when stored and used at low temperatures. However, exposure to higher temperatures resulted in a more rapid release of the label from the protein; and

(ii) The labelling procedure may alter the polymer distribution of the species to be labelled. It was observed that proteins varied in their susceptibility to aggregation during labelling.

A Chloramine-T method of radioiodination, modified by using a lower Chloramine-T : protein ratio to avoid aggregation, was initially used. This allowed the rapid and reliable routine labelling of Serum Albumins and β -Casein at high efficiency but resulted in massive aggregation of β -Lactoglobulin, Ovalbumin, Lysozyme, Haemoglobin and Myoglobin. This was diminished to some extent by the use of the Iodo-Gen method of labelling but was still unacceptable for Ovalbumin, Lysozyme, Haemoglobin and Myoglobin. Further studies with β -Lactoglobulin showed that modification of the Iodo-Gen method to incorporate a reducing agent reversed some of the aggregation and allowed the preparation of a tracer more similar to the unlabelled material.

A survey of the literature on protein adsorption showed that some of the confusion and apparent contradiction as regards the phenomenon could be ascribed to an inadequate characterisation of the materials and experimental systems used. A major part of the preliminary work was concerned with the characterisation of the labelled and non-labelled proteins and the determination of the effect of surface modification and solution conditions on the surface area of adsorbent available for adsorption, the kinetics of adsorption at several concentrations i.e. surface area : protein ratios and the effect of varying degrees of solution depletion and protein polymeric species on adsorption.

Labelled and unlabelled proteins were characterised by electrophoretic techniques. There was evidence of only modest changes in the polymer distribution of Serum Albumin and β -Lactoglobulin tracer preparations and the degree of labelling over the various polymeric fractions was essentially uniform. There was also some evidence to

indicate that the free sulphhydryl group of the Serum Albumins was altered by the labelling procedure but only about 10% of the protein was affected. In all cases experiments were performed in order to test that the labelled and non-labelled proteins behaved identically in adsorption studies.

The fluoridation of hydroxyapatite and exposure to solution pH's below about pH 6.5 resulted in changes in the specific surface area. The kinetics of adsorption were characterised by an initial rapid process followed by a much slower adsorption to saturation. An important observation was that at concentrations below 10 g m^{-3} at least 24 hours was required to achieve saturation adsorption. The degree of depletion of solution protein and the presence of polymeric protein species did not affect the level of saturation adsorption.

Using solution depletion techniques and suspended hydroxyapatite particulates, the effect of several variables on the adsorption of protein was studied. The effect of concentration on the amount of adsorption was determined for Bovine and Human Serum Albumin, β -Casein, β -Lactoglobulin and salivary glycoprotein using radiochemical tracer techniques. Although a suitable tracer preparation for the proteins Haemoglobin and Myoglobin was not achieved using either the Chloramine-T or Iodo-Gen method of radioiodination, the concentration adsorption isotherm of these proteins was obtained using spectrophotometric methods. The isotherms at low protein concentrations were always parallel to the concentration axis, even at concentrations below those usually studied, and showed that all the proteins had a high affinity for hydroxyapatite. Indeed, dilution studies showed that the solution protein concentration required to maintain saturation was negligibly small and hence adsorption always proceeded to saturation provided sufficient time was allowed and the solution was not depleted of protein. Desorption and exchange

studies showed that protein could have different affinities for the adsorbent, however, and hence a protein of high affinity could displace an adsorbed protein of lower affinity.

Calculations based upon the molecular size of the more well characterised proteins BSA and β -Casein indicated that adsorption proceeds to monolayer coverage of the adsorbent surface by adsorbed molecules in a close-packed, side-on configuration. The effect of the variables pH, ionic strength and temperature on BSA adsorption were consistent with this model. Hydrophobic forces were observed to be able to markedly modify the adsorption behaviour of relatively hydrophobic proteins however. The concentration isotherm of such a protein, viz. β -Casein, was unusual and unlike those for relatively hydrophilic proteins in that, at higher concentrations, a step occurred which gave rise to a higher plateau level of adsorption. The effect appeared to be similar to the self-association of the protein in solution attributed to hydrophobic forces, and the effects of temperature and calcium ions on adsorption were consistent with the effects of these variables on the association. The solution process was dissimilar to the surface process, however, in that the hydrophobic effect did not appear to give rise to the formation of multiple layers of protein molecules on the adsorbent surface. Rather, the effect was more consistent with an altered configuration of the adsorbed molecules that allowed significant lateral intermolecular interaction.

Although the mechanism of interaction between proteins and hydroxyapatite is generally considered to involve only ionic interactions, the present work has shown that non-ionic forces can also contribute significantly. Evidence was obtained to suggest hydrophobic forces and hydrogen bonding were involved and, further, that the contribution of any of the interactions possible between the protein and

the adsorbent is dependent on the nature of the protein, the adsorbent and the solvent in use. Thus hydrophobic forces could be more important than electrostatic forces, particularly for hydrophobic proteins, in providing the driving force for adsorption. It was not clear from the studies performed, however, whether the observed greater affinity of β -Casein over BSA for hydroxyapatite was due to specific interactions between this protein and the adsorbent or to a greater driving force for adsorption, as provided by a greater positive entropic contribution, due to the increased importance of hydrophobic effects for this relatively hydrophobic protein.

Although protein adsorption in solid/liquid systems generally proceeds only to monolayer coverage of the adsorbent by protein molecules in pure systems, multilayer adsorption can be observed under special conditions. Multivalent ions can promote multilayer adsorption if the ion can bridge between protein molecules via a covalent binding interaction. The phenomenon was observed in this study in the BSA/Hg²⁺ system where the Hg²⁺ ion binds between two BSA molecules via the sulphhydryl group. It appears that the number of layers formed is probably determined by the number and location of groups available for binding. Stable multilayer formation will only occur, however, if the ion-protein binding is non labile. Colloidal particulate species can also promote multilayer formation in some systems. The hydroxyapatite particulates and an amorphous calcium phosphate were observed to facilitate multilayer adsorption of β -Casein but not of BSA. The effect appeared to be related to the stability of the particulate suspensions in the presence of the particular protein as although BSA stabilised the particulate suspension and no multilayering was observed, β -Casein destabilised the suspension, causing flocculation, and multilayer adsorption occurred. However, stable multilayer adsorption, resulting in

accretion formation, occurred only if the protein could bind directly to two particulates. Hydrophobic forces, although important in the micellisation of β -Casein, did not enable multilayer adsorption.

The studies of this thesis have been extended to include a glycoprotein component isolated from whole human saliva. Characterisation studies reported in literature indicated the protein might have physico-chemical properties similar to β -Casein and this was supported by results of initial adsorption and accretion formation experiments. Further, the observed instability of hydroxyapatite suspensions in the presence of other salivary component fractions isolated during the separation of the glycoprotein component have allowed an alternative explanation for pellicle formation in the oral environment to be developed. It is proposed that pellicle formation may occur as a result of a multilayer adsorption process in which an amorphous calcium phosphate material acts as a bridging species. The calcification of pellicle to form dental calculus would result from mineralisation of the amorphous material to more crystalline forms.

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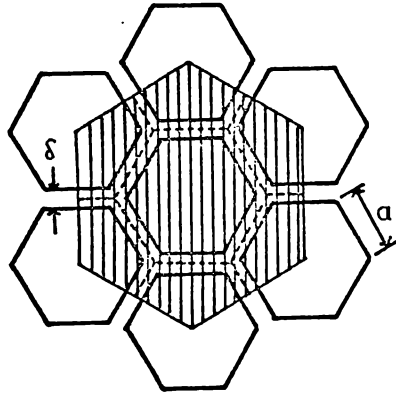
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APPENDICES

APPENDIX I: Surface Area Calculations On Prismatic Dental Enamel



(Schematic model of crystallite arrangement in enamel
From Flim et al., 1978)

Diameter of hexagons: 37 nm

Hexagonal sides have length $a + \frac{37}{2}$ nm

Distance between adjacent hexagons = 2 nm

Hence, for shaded region:

Total interprismatic length = $9a$

Total interprismatic surface area is $9a\delta = 3.3 \times 10^{-16} \text{ m}^2$

Length of side b is $(2a + 2) \text{ nm} = 39 \text{ nm}$

Diameter of shaded region is $2b = 78 \text{ nm}$

Total surface area is $2 \times \frac{1}{2}(b + 2b)b = 1.825 \times 10^{-14} \text{ m}^2$

Therefore the contribution of interprismatic surface area to
total area = 1.8%.

APPENDIX II: Materials Available Commercially

BOVINE SERUM ALBUMIN: Sigma Chemical Co. Samples Supplied as Albumin, Bovine. Fraction V powder, 96-99% albumin, remainder mostly globulins. Cat. No. A-4503.

BOVINE SERUM ALBUMIN MONOMER: Miles Research Products. Sample supplied as Albumin, Bovine powder, 99% pure. Code No: 81-028, Lot No: P338.

HUMAN SERUM ALBUMIN: Sigma Chemical Co. Samples supplied as Albumin, Human. Fraction V powder. Cat. No. A-2386.

β -CASEIN: United States Biochemical Corp. Samples supplied as fraction from whole Casein, separation by repeated fractionation using aqueous urea. Cat. No. 12825. Lot No. 17941 (designated as Batch 1 in text of thesis) Lot No. 27244 (designated as Batch 2 in text of thesis).

HAEMOGLOBIN: Calbiochem. Samples supplied as powder, A grade, 98%+ purity. Cat. No. 3745.

β -LACTOGLOBULIN: ICN Biochemicals. Sample supplied as powder, 99%+ purity. Cat. No. 100363.

LYSOZYME: Miles Research Products. Sample supplied as chloride, from egg white. Cat. No. 36-324.

MYOGLOBIN: United States Biochemical Corp. Sample supplied as powder, Horse, 98%+ purity. Cat. No. 19285.

OVALBUMIN: ICN Biochemicals. Sample supplied as powder, 99%+ purity. Cat. No. 100126.

RADIOIODINE SOLUTIONS: Radiochemical Centre, Amersham, England. Samples supplied as Iodine-125, as iodide, carrier free in NaOH, pH 8-11, free from reducing agent, for protein iodination. Cat. Code IMS-30.

SEPHADEX-G25 (fine): Pharmacia, Uppsala, Sweden. Sample supplied as particle size 20-80 μ m, bed volume per gram dry gel 5 ml, water regain value 2.5 ± 0.2 g/g.

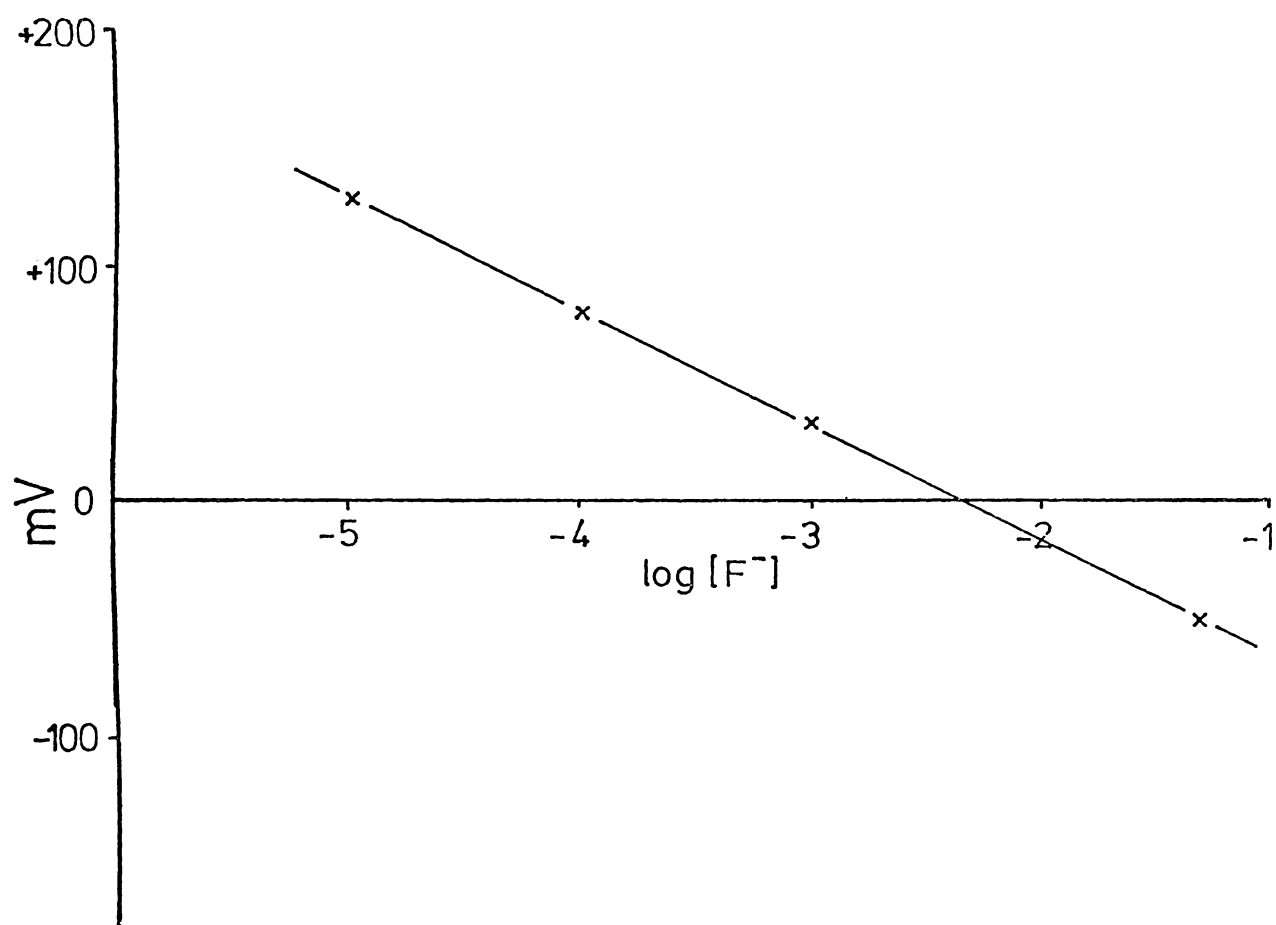
AQUASOL-2: New England Nuclear, Boston, Massachusetts, United States of America. Universal Liquid Scintillation Cocktail, Cat. No. NEF 952.

GRADIPORE GEL SLABS: 2.5-27% Concentration Gradeint. Gradient Laboratories Pty Ltd., Pymont, Australia.

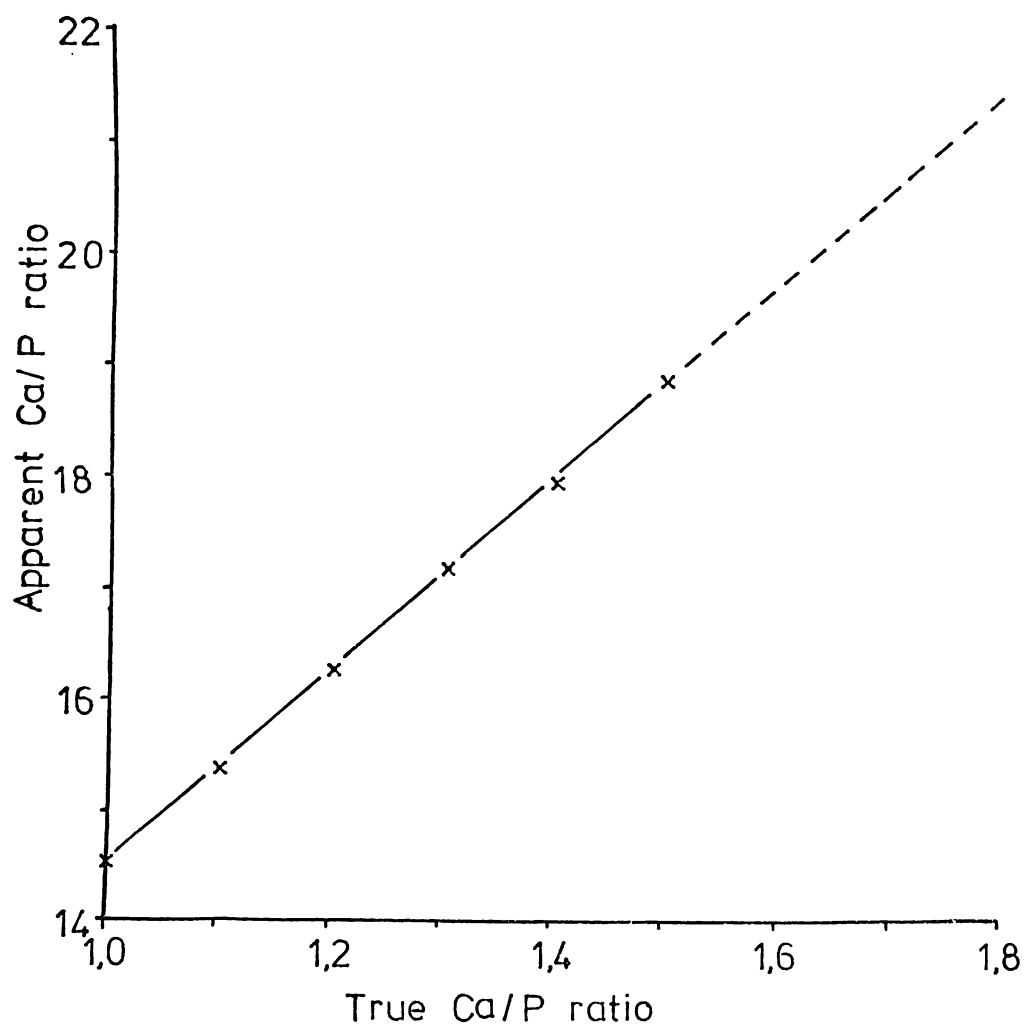
APPENDIX III: Methods of Cleaning Glassware

General glassware was allowed to stand in chromic acid for at least 48 hours, thoroughly rinsed four times with distilled water and twice with doubly distilled water, and allowed to dry at room temperature under dust free conditions.

Glassware used in the iodination reaction and for the collection of fractions from the gel filtration column was treated as for general glassware except that it was oven dried before use. Iodination reaction vessels were allowed to stand for 18-24 hours in a solution of concentrated nitric and sulphuric acids (1 : 1) that was heated on a sand bath. These were rinsed as per general glassware, oven dried and kept in a clean, dry glass dessicator until required.

APPENDIX IV: Calibration Curve for Orion Combination Fluoride Electrode

APPENDIX V: Calibration Curve for X-Ray Fluorescence Analysis
of Adsorbents



Apparent Ca/P ratio calculated as:

$\frac{\text{counts s}^{-1} \text{ for Calcium} - \text{background}}{\text{counts s}^{-1} \text{ for Phosphorous} - \text{background}}$

APPENDIX VI: Composition of Buffers(A) Barbital - Hydrochloric Acid

pH range 2.62 - 9.16

Solution A: 9.714g sodium acetate trihydrate + 14.714g sodium barbitone in 500 ml.

Solution B: 0.1 mol l⁻¹ hydrochloric acid.

<u>Solution A/ml</u>	<u>Solution B/ml</u>	<u>Dilute to/ml</u>	<u>Final pH</u>
5.0	16.0	25.0	2.62
"	15.0	"	3.20
"	14.0	"	3.62
"	13.0	"	3.88
"	12.0	"	4.13
"	11.0	"	4.33
"	10.0	"	4.46
"	9.0	"	4.66
"	8.0	"	5.32
"	7.0	"	6.12
"	6.5	"	6.75
"	6.0	"	6.99
"	5.5	"	7.25
"	5.0	"	7.43
"	4.0	"	7.66
"	3.0	"	8.00
"	2.0	"	8.18
"	1.0	"	8.55
"	0.5	"	8.85
"	0.25	"	9.16

(B) Cacodylate - Hydrochloric Acid

pH range 5.0 - 7.4

Solution A: 0.2 mol l⁻¹ sodium cacodylate
(42.8g Na(CH₃)₂ AsO₂·3H₂O per litre).

Solution B: 0.2 mol l⁻¹ hydrochloric acid

<u>Solution A/ml</u>	<u>Solution B/ml</u>	<u>Dilute to/ml</u>	<u>Final pH</u>
50	47.0	500	5.0
"	45.0	"	5.2
"	43.0	"	5.4
"	39.2	"	5.6
"	34.8	"	5.8
"	29.6	"	6.0
"	23.8	"	6.2
"	18.3	"	6.4
"	13.3	"	6.6
"	9.3	"	6.8
"	6.3	"	7.0
"	4.2	"	7.2
"	2.7	"	7.4

APPENDIX VII: Radiochemical Properties of
Iodine-125 and Iodine-131.

	I-125	I-131
Half Life $t_{1/2}$ /(days)	60	8.06
Isotope Abundance/(%)	80-100	15-20
Principal Decay Mode and Energy/(MEV)	γ 0.035	β 0.60 γ 0.364
Decay Product	Te-125	Xe-131
Half Value Thickness for Gamma Radiation (Lead)	<0.001 cm	0.25 cm
Tenth Value Thickness for Gamma Radiation (Lead)	<0.035 cm	0.8 cm

APPENDIX VIII: Preparation of SDS-Electrophoresis Gels

Stock Acrylamide Solution:

22.2 g acrylamide (BDH, specially purified for electrophoresis)
0.6 g N.N' methylene bisacrylamide (BDH, specially purified for electrophoresis)

made up to 100 ml with doubly distilled water, filtered and stored in a dark bottle at 4°C.

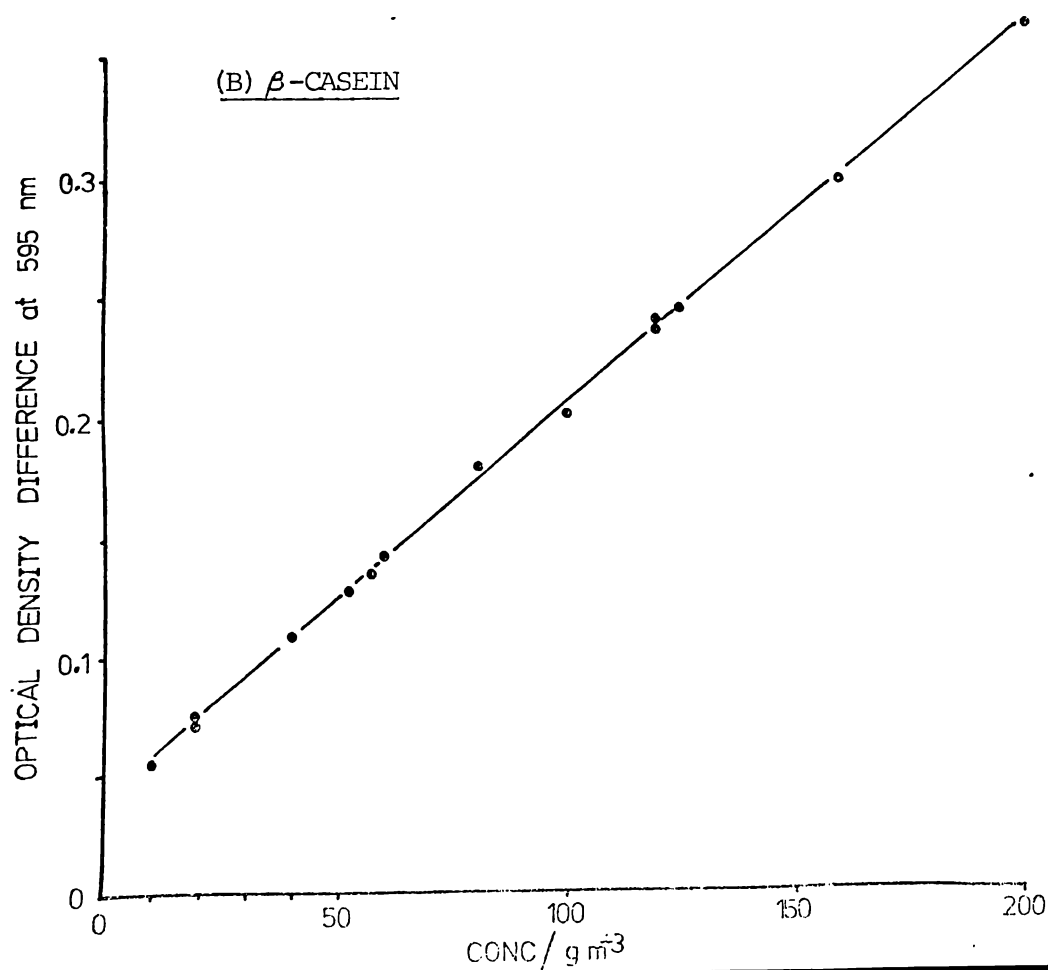
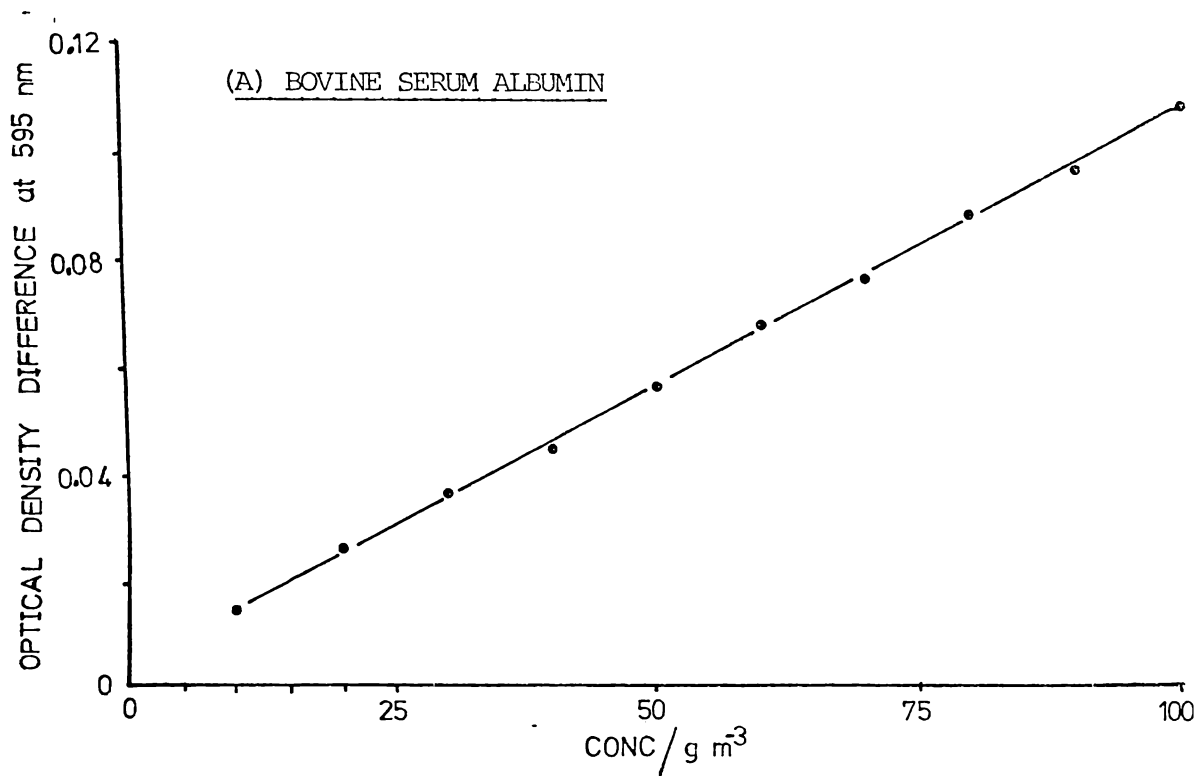
Gel Buffer:

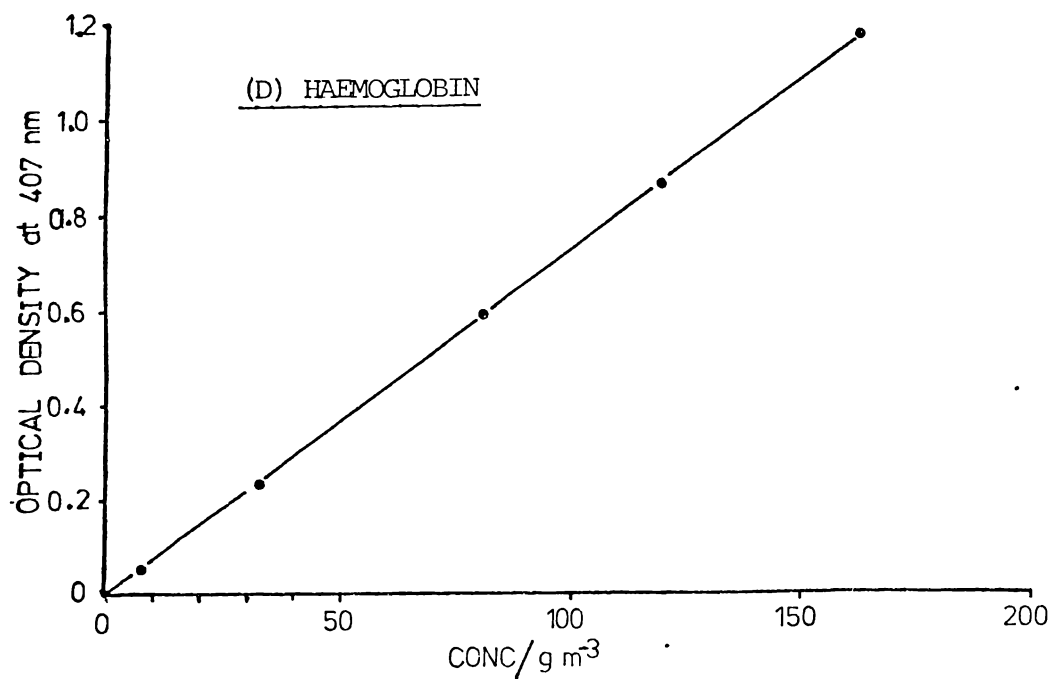
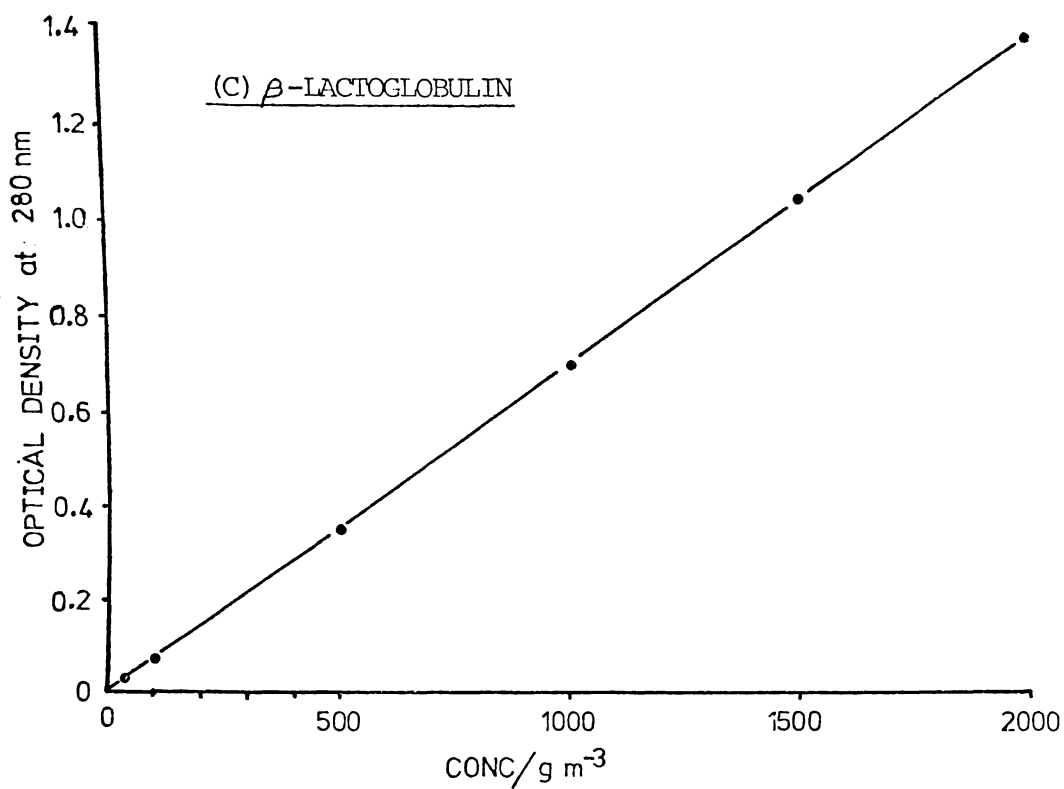
Cacodylate-HCl Buffer containing 2.0 g sodium dodecylsulphate (SDS; Serva, Cat. No. 20760, AR grade, specially purified for electrophoresis) per litre solution.

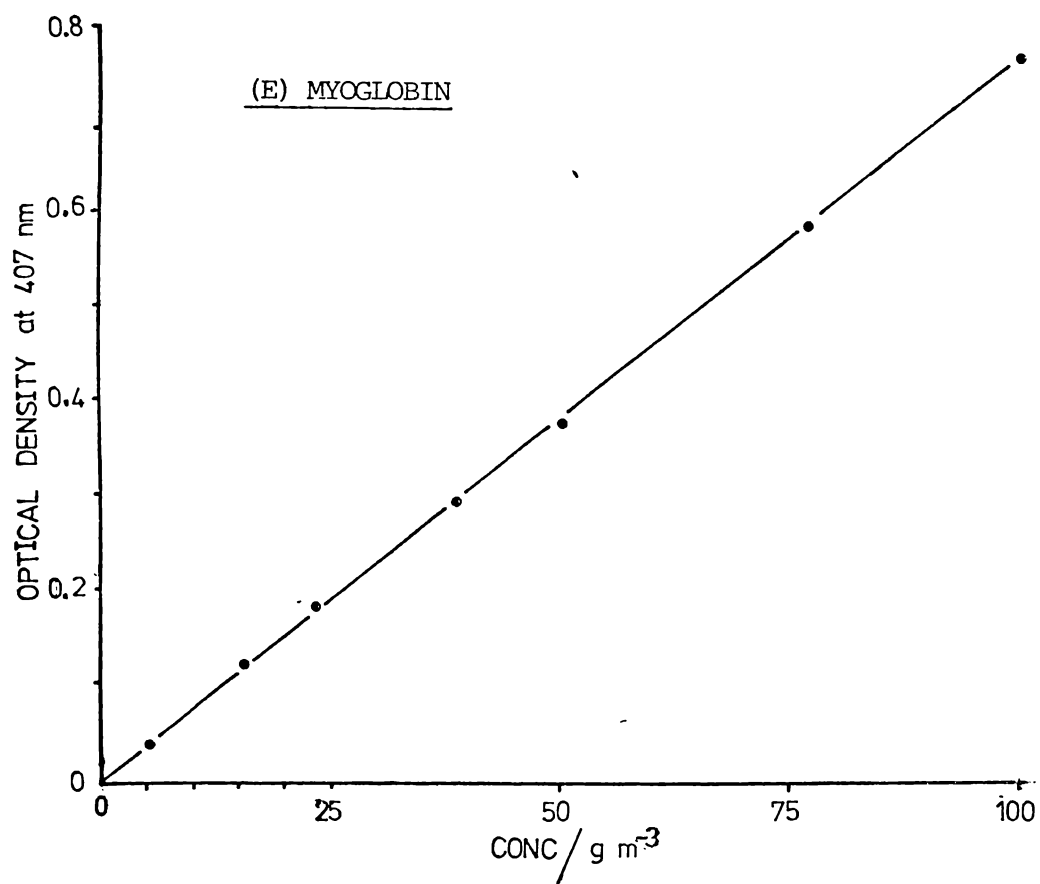
Gel Mix:

10 ml gel buffer
9 ml stock acrylamide solution
0.1 ml 20% TEMED (Tetramethylethylenediamine; BDH)
1 ml ammonium persulphate solution (0.1 g per 15 ml solution, freshly prepared).

APPENDIX IX: Calibration Curves for the Determination of
Protein Concentration by Spectrophotometry.







APPENDIX X: Effect of NaCl Concentration on the Diffuse DoubleLayer Thickness $\left(\frac{1}{\kappa}\right)$

$$\frac{1}{\kappa} = \left[\frac{\epsilon KT}{8\pi n e^2} \right]^{\frac{1}{2}}$$

where: $n = (\text{molarity} \times 10^3 \times \text{Avogadro's number}) = \frac{M \times 10^3 \times 6.02 \times 10^{23}}{\text{ions/m}^3}$

$$KT = 0.4 \times 10^{-20} \text{J}$$

$$e = 1.602 \times 10^{-19} \text{C}$$

$$\epsilon = (\text{static permittivity}) = 80 \times 8.85 \times 10^{-12} \text{J}^{-1} \text{C}^{-2} \text{m}^{-1} (\text{or } \text{Fm}^{-1})$$

The values for $\frac{1}{\kappa}$ for a range of NaCl concentration are tabulated below.

NaCl concentration/mol l ⁻¹	$\frac{1}{\kappa}/\text{nm}$
0.001	13.0
0.01	4.4
0.03	2.5
0.06	1.8
0.10	1.4
0.15	1.1
0.20	1.0
0.30	0.8
0.50	0.62
0.60	0.57