ADSORPTION OF BOVINE SERUM ALBUMIN TO POLYETHYLENE TUBING

REVERSIBILITY AND pH-DEPENDENCE

By

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B.Sc., The University of Leeds, 1984

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Chemistry

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1988

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Date 5th October, 1988

DE-6 (2/88)

ABSTRACT

This thesis is concerned with the adsorption of bovine serum albumin to polyethylene tubing. A method using radioiodinated protein was developed to measure the surface concentration taking into account the dilution effect for miscible displacement in a capillary. A steady-state surface concentration was established within 2 hours. Adsorption did not depend on the ratio of radiolabelled to unlabelled protein. The adsorption isotherm was Langmuir-like with a plateau concentration of approximately 0.2 $\mu g/cm^2$.

Two methods were used to calculate the surface concentration in the desorption study. The surface concentration calculated by depletion of the total radioactivity was always higher than that calculated from assaying the radioactivity associated with the tubing. Desorption of at least 5% of the loosely bound protein occurs.

The surface concentration-pH data show two maxima. The first is at the isoelectric point of the albumin while the second is at pH 9.5-10. The second maximum seems to be due to preferential adsorption of the higher molecular weight oligomers in the protein sample.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ACKNOWLEDGEMENTS

I would like to thank Dr. D. E. Brooks, my supervisor, for his help and guidance throughout this study; Johann Janzen for his help with some of the experimental work and invaluable discussion; Charles Ramey for his discussion and suggestions.

A special thank-you goes to all the members of the Brooks' lab for all their help and support.

Finally I wish to thank Terry Jarvis and Tim Haddad for their patience during the preparation of this thesis.

CHAPTER 1

INTRODUCTION

Protein adsorption to solid surfaces is of great biological, medical and technological significance. The blood compatibility or incompatibility with non-biological materials is important especially with the increasing use of prosthetic materials in the body and this is generally considered to be related to protein adsorption.

When a synthetic material is introduced into the cardiovascular system the initial event in a complex series of reactions is the rapid adsorption of a proteinaceous layer (Baier and Dutton, 1969). Subsequent cellular interactions lead to thrombus formation, the entrapment of erthyrocytes and other formed blood elements in a fibrin network, and coagulation as determined by the adsorbed proteins (Baier, 1977; Brash, 1981).

It has been observed that different materials have drastically different thrombogenic activity. This suggests that the characteristics of the protein layers are different for different materials.

It has been shown that by precoating surfaces with plasma proteins, platelet adhesion, a prerequisite to thrombus formation, is greatly altered. An albumin coated surface reduces platelet adhesion (Lyman *et al.*, 1971; Packman *et al.*, 1969), while fibrinogen greatly enhances it and γ-globulin activates the release reaction (Packman *et al.*, 1969; Jenkins *et al.*, 1973).

Protein adsorption is a key event in the blood-surface interaction

that must be understood before we understand the mechanisms of surface-induced thrombosis.

Over the past 30 years many investigators have studied protein adsorption to a variety of non-biological surfaces. Many techniques have been developed providing information regarding the absolute quantities adsorbed from single solutions (Bull, 1956; Brash and Uniyal, 1979), the relative quantities adsorbed from complex solutions (Brash and Davidson, 1976; Lee *et al.*, 1974), the number of surface attachments (Morrissey and Stromberg, 1974), desorption and exchange (Brash and Samak, 1978; Chuang *et al.*, 1978), and measurements of the adsorbed layer thickness (Morrissey *et al.*, 1976; Cuypers *et al.*, 1977).

There is still a lot of controversy regarding protein adsorption at solid/liquid interfaces, particularly concerning the reversibility and the configuration of the adsorbed molecules. The present study was aimed at clarifying the question of reversibility.

Various techniques have been employed to study protein desorption. Solution-depletion methods followed by dilution were used to study albumin desorption from glass (Bull, 1956), and from silica (MacRitchie, 1972). Radiolabelled proteins were used to give a direct measure of the amount of adsorbed protein. Desorption studies usually followed a rinsing period (Brash *et al.*, 1974).

In this study a more direct technique using a radiolabelled protein was developed to measure the amount of protein desorbed and information regarding albumin adsorption to polyethylene and reversibility was obtained.

Many factors contribute to determining the characteristics of protein

adsorption including the nature of the protein, the medium in which it is located and the nature of the solid surface. These properties give an insight into the driving forces and the mechanisms of adsorption.

The information required to give a complete picture of protein adsorption includes the amount of protein adsorbed as a function of solution concentration and time (adsorption kinetics). The orientation and conformation of the protein upon adsorption is important since a conformational change, known as denaturation, alters the properties of the protein. Other desired information includes the capacity of the protein in solution to compete for the surface, and the ability of the protein to desorb or exchange.

1.1 Protein structure

Protein structure is largely determined by the interactions among the amino acids which comprise it, and between the protein molecule and the environment. Because of the structure-function relationships of proteins their three-dimensional structure is of interest.

Proteins are high molecular weight polyamides built up by the specific copolymerization of amino acids for particular functions. Each protein has a unique amino acid sequence known as the primary structure. The secondary structures, ordered three-dimensional regions, are the α-helix and β-sheet resulting from hydrogen bonding in the protein backbone. The protein's tertiary structure is the complete three-dimensional structure and is the result of intramolecular interactions such as ionic or electrostatic interactions, hydrogen bonding, hydrophobic interactions, salt bridges and covalent disulfide bonds. The noncovalent association of independent

tertiary structure gives the quarternary structure.

1.1.1 Bovine serum albumin in solution

Serum albumin is the protein that is present in the largest amount in blood plasma, where approximately half of the protein is albumin. It is one of the most intensely studied proteins.

Globular proteins in aqueous solutions near their isoelectric point have compact configurations with low permeability to water. The nonpolar groups are largely excluded from the surface of the protein while the polar and charged amino acids are at the surface and interact strongly with water. Shifting the pH of a protein solution away from the isoelectric point can decrease the stability of the protein. The protein unfolds exposing the inner part of the molecule. Bovine serum albumin (BSA) expands very readily below the isoelectric point of pH 4.9. This expansion at pH 4.5-3.5 depends on the fact that the carboxyl groups are successively transformed into the uncharged form, leaving the positively charged groups in excess. The repulsion between various segments of the peptide chains increases. This conformational change is usually called the N-F transformation (Foster, 1960). BSA is somewhat more stable on the alkaline side of the isoelectric point. Expansion similar to the conformational change in acid takes place in alkaline pH, but the alteration in size and shape does not begin to occur until pH 10.3 (Tanford *et al.*, 1955). A small conformational change also has been shown to occur in the pH interval 7-8 (Leonard *et al.*, 1963; Harmsen *et al.*, 1971).

The solubility of BSA is a function of pH. As the pH moves away from the isoelectric point the net charge on the protein increases thus

increasing the solubility.

1.1.2 Albumin oligomers

Albumin samples are heterogeneous in the sense that they contain mercaptalbumin, the fraction having a freely reactive sulfhydryl group, and nonmercaptalbumin, the fraction showing no sulfhydryl activity. Most carefully prepared albumin preparations have a sulfhydryl content of 0.65-0.70 sulfhydryl groups per albumin molecule. In most regards mercaptalbumin and nonmercaptalbumin are remarkably similar. Physical chemical studies on whole serum albumin yield results indistinguishable from those on mercaptalbumin.

In virtually all samples of BSA, dimers and higher oligomers, aggregates of monomers, exist. It has been shown that such dimers and oligomers arise as artifacts during and after isolation and are not present in the bloodstream (Andersson, 1966).

Albumin preparations contain variable amounts of dimers and higher polymers depending on the source of plasma (Friedli and Kistler, 1970), the fractionation procedure (Smith *et al.*, 1972; Solli and Bertolini, 1977), the storage conditions (Finlayson *et al.*, 1960) and the length of time of storage (Finlayson *et al.*, 1960; Finlayson, 1965).

The most likely source of dimerization would be the direct formation of disulfide linkages through oxidation reactions involving the sulfhydryl residues of two mercaptalbumin monomers. If this is true then a thiol reagent would break the disulfide linkage, but it is well known that typical albumin samples contain a portion of dimeric forms which are not broken down by reduction with thiol reagents (Hartley *et al.*, 1962;

Janatova *et al.*, 1968). Andersson showed that dimer isolated from serum albumin was heterogeneous (Andersson, 1966). Approximately one-third of the dimer was split into monomer by mercaptoethanol, a reducing agent, at pH 8 or by standing in alkaline solution, pH 11.4, for 2 days. The part of the dimer not split by mercaptoethanol was relatively stable. A small degree of cleavage resulted when the dimer was treated with dioxan or a detergent solution indicating that hydrophobic bonding is of limited importance in holding the dimer together. The stability of the mercaptoethanol resistant dimer at low and high pH values indicates that electrostatic bonds cannot be the explanation. Andersson suggested that both types of dimers are held together by disulfide bonds. In one dimer the disulfide bond is situated in the interior of the molecule and therefore not accessible to react with mercaptoethanol. It was also proposed that hydrogen bonding may be responsible for the stability of the dimer not split by mercaptoethanol (Andersson, 1966).

1.2 Driving forces for adsorption

Before looking at the mechanism of protein adsorption one should look at the driving forces for the process.

For protein adsorption to be spontaneous the change in free energy $\Delta G_{ads} = \Delta H_{ads} - T\Delta S_{ads}$, must be negative, where ΔH_{ads} is the enthalpy of adsorption, T the absolute temperature and ΔS_{ads} is the entropy of adsorption. Calorimetric measurements (Norde and Lyklema, 1978; Nyilas *et al.*, 1974), give a direct measure of enthalpy and the data show that both enthalpically and entropically driven adsorption occur since enthalpy changes ranged from positive to negative depending on pH for albumin

adsorbed on negatively charged polystyrene.

Interactions such as covalent, electrostatic and hydrogen bonding between the protein and the surface are likely to be exothermic while changes in hydrophobic interactions, which are a result of the ordering of water molecules near the surface of the protein or adsorbent, contribute to the changes in entropy.

Proteins have low solubilities which rarely exceed 1% by weight due to high molecular weights. It has been shown (Bull, 1956), that at the isoelectric point proteins usually display minimum solubility and maximum adsorption. The solubility of a protein is determined by the balance of the attraction of the protein molecules for each other, which tends to prevent solution and the attraction of the solvent molecules for the protein, which tends to promote solution. At the isoelectric point the protein has a net neutral charge and the attraction of the protein molecules for each other is maximal. When the pH is shifted away from the isoelectric point the protein molecule becomes charged. This decreases the attraction of the protein molecules for one another and leads to an increase in solubility since the proteins charged groups are more solvated.

The solubility and adsorption of proteins is analogous to some aspects of synthetic polymer adsorption. An increase in adsorption with decreasing solubility has been shown for various polymers on glass (Rowland and Eirich, 1966). For synthetic polymers the amount adsorbed per unit area increases with increasing molecular weight (Gilliland and Gutoff, 1960). From a mixture of polymers of varying molecular weight the larger molecules are adsorbed preferentially since they can form more bonds per molecule with the surface.

Proteins adsorb to non-biological surfaces due to their amphipathic nature, high molecular weight, limited solubility and ability to change configuration at an interface. The decrease in free energy may result from a gain in entropy due to the disorder of water released from the surface or protein but it may also be due to exothermic events.

1.3 Mechanisms for adsorption

Adsorption from an aqueous solution is a competitive process, since, when protein molecules are adsorbed solvent molecules are displaced. When determining the mode of adsorption all the interactions in the system must be taken into account.

When a protein solution flows past a solid surface the protein reaches the surface by a diffusion-convection process, then binds. The initial rate of adsorption depends on the transport and binding. Once protein has been adsorbed onto the surface the surface availability becomes the dominant factor and therefore rate-controlling and now protein-protein interactions may become important.

Proteins may bind to the surface via ionic or electrostatic interactions, hydrophobic interactions, hydrogen bonding and by charge-transfer or partial donor-acceptor interactions. Covalent bonding does not result from adsorption under biological conditions. Figure 1.1 depicts the different types of regions of a protein molecule that may be involved in the adsorption process.

Ionic or electrostatic interactions, due to the attraction or repulsion of two or more groups carrying a net charge, are important in many systems. Proteins may bind to an oppositely charged surface via electrostatic bonds. However, attractive electrostatic bonds may also be

formed between a protein carrying a net charge equal to that of the surface. For instance, it has been shown that the adsorption of negatively charged proteins on a negative polystyrene latex occurs spontaneously and exothermically (Norde and Lyklema, 1978).

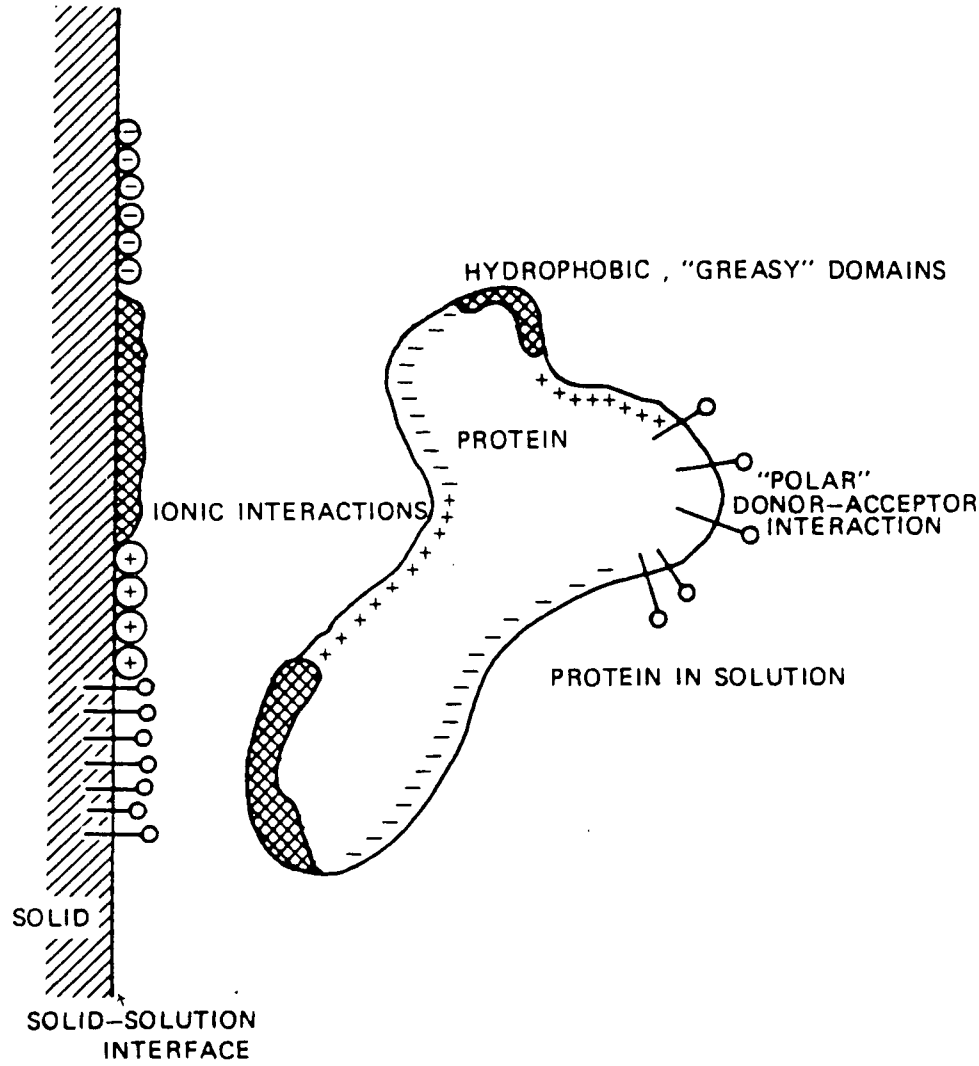

Fig. 1.1. A schematic view of a protein interacting with a well-characterized surface. The protein has a number of surface domains with hydrophobic, charged and polar character. The solid may have a similar domain-like character. (Taken from Andrade, 1985, p.4).

Charges on a protein surface are surrounded by unlike charges in a

diffuse double layer rather than being only solvated by water (Wada and Nakamora, 1981). The stability of a protein depends in some cases on intramolecular electrostatic interactions (Perutz, 1978). If a charged group important for protein stability interacts with a charged surface a conformational change may result due to a change in the electrostatic interactions in the protein.

Proteins adsorbed electrostatically should be sensitive to changes in the ionic composition and pH. One would expect reversible adsorption since continuous exchange of the protein with other ions in the blood ought to occur. Protein adsorption is thought to be irreversible or partially reversible on many surfaces, however, indicating that electrostatic interactions are only of minor importance.

Another protein-surface interaction is the hydrogen bond, a predominantly electrostatic interaction. The dipole/dipole interactions may, in an extreme case, give rise to interaction energies similar to weak covalent bonds and due to the small size of the hydrogen atom a small binding distance results. However, in proteins the binding energies are much smaller and the binding distance larger. Hydrogen bonds are important in proteins and they contribute to the stability of the internal structure and stabilization of the α-helix and β-sheet structures. Again, competition from surface hydrogen bonding groups can cause conformational changes on binding.

The hydrophobic patches on a protein can interact with hydrophobic polymer surfaces such as polyethylene or Teflon. The hydrophobic interaction is an entropically driven interaction resulting from a gain in free energy caused by the loss of structured water at the hydrophobic

interface when two such surfaces come together. This is of great importance in protein adsorption since the stability and interactions of proteins depend on the overall free energy. The ordering of water at air or apolar interfaces is entropically undesirable. To keep these interfacial areas at a minimum the hydrophobic amino acid side chains are excluded from the protein surface. Globular proteins have a hydrophobic core and a relatively hydrophilic shell in aqueous solutions but complete burying of the hydrophobic regions is generally not possible. Intramolecular hydrophobic bonding in dissolved proteins may affect protein adsorption especially when intramolecular hydrophobic bonding is required for the stabilization of the protein structure. Rearrangement of structure upon adsorption is now probable (Birdi, 1973).

Charge transfer interactions in aqueous solutions are due to π-π electron effects and these are important in protein stabilization and surface interaction. Excess electron density can be donated to an electrophilic species or electron density can serve as an acceptor for positive charge.

1.4 Characteristics of protein adsorption

Many investigators have found that the adsorption of proteins from solution to non-biological surfaces is apparently of the Langmuir type (Bull, 1956; Oreskes and Singer, 1961; Cheng *et al.*, 1978; Young *et al.*, 1988). The surface concentration increases asymptotically with an increase in solution concentration until a steady state, plateau value is reached. This is assumed to be associated with the formation of a complete monolayer. The amount adsorbed is not significantly different from that

expected for a close-packed monolayer of native protein in a side-on or end-on conformation depending on the system and conditions. Even though the adsorption isotherm is of the Langmuir type there is no reason to accept the applicability of this model of adsorption because many of the Langmuir assumptions are not satisfied, e.g., adsorption does not take place on sites; adsorption is not fully reversible.

Multilayer adsorption has been demonstrated (Oreskes and Singer, 1961; Pitt and Cooper, 1986; Young *et al.*, 1988). Adsorption experiments were carried out on various surfaces and the data plotted using modified versions of the Langmuir equation (Oreskes and Singer, 1961; Young *et al.*, 1988). Multilayer adsorption was suggested since the data were fitted by two or more regions of different slopes, each slope representing a different binding constant. The first steeper slope was interpreted as representing the initial protein layer bound to the polymer. The second line can be interpreted as a second layer of protein due to protein-protein interactions or to a reorganization of the monolayer from a side-on to an end-on disposition thereby increasing the amount adsorbed.

Protein adsorption has been shown to be pH dependent (Bull, 1956, Morrissey and Stromberg, 1974); see earlier discussion (Section 1.2). Maximum adsorption occurs near the isoelectric point of the protein. A predominantly nonionic, hydrophobic mechanism is suggested since the protein has no net charge at this pH.

Protein adsorption to various surfaces has been shown to depend on the surface. Table 1.1 shows the plateau values, following rinsing with buffer, for the adsorption of albumin onto various hydrophobic and hydrophilic surfaces from a 1 mg/ml solution, pH 7.4 at 23°C.

Table 1.1

The adsorption of human serum albumin (HSA) on various surfaces.

Surface	Plateau surface concentration ($\mu g/cm^2$)	Ref.
Polyurethane 1540 (hydrophilic)	0.02	1
Polyurethane 600 (hydrophilic)	0.04	1
Glass	0.04	2
Silica	0.09†	3
Collagen-coated glass	0.09	1
Poly(vinyl chloride)	0.17	4
Siliconized glass	0.18	1
Polyethylene	0.18	2
Polystyrene	0.20	1
Polyurethane (hydrophobic)	0.57	1

† surface concentration for bovine serum albumin

Key to references:

1 Brash and Uniyal, 1979.
2 Brash and Davidson, 1976.
3 Morrissey and Stromberg, 1974.
4 Young *et al.*, 1988.

The hydrophilic surfaces show low surface concentrations. On these surfaces desorption occurs, therefore there may be some uncertainty with respect to the surface concentration because rinsing may remove adsorbed protein (Chan and Brash, 1981; MacRitchie, 1972). The hydrophobic surfaces show a varying range of plateau values. Polyethylene, polystyrene and siliconized glass show a steady state surface concentration of

approximately 0.2 $\mu g/cm^2$. These are all effectively hydrocarbon polymers. The hydrophobic polyurethane shows a higher plateau surface concentration. This polymer contains a high proportion of ether oxygen and urethane functionalities as well as hydrocarbon. The surface has been shown to posses domains i.e., polyether-rich and urethane-rich regions, which may be responsible for the high albumin adsorption.

Many adsorption studies have been carried out on tubing . Infrared internal reflection techniques were used by Lee and Kim to study the effect of time and flow rate on the adsorption of serum albumin, γ-globulin and prothrombin to silicone rubber (SR), fluorinated ethylene-propylene copolymer (FEP) and segmented polyether urethane (PEUU) (Lee and Kim, 1974). The adsorption was rapid and dependent on the substrate not on the protein. The plateau value concentration was shown to depend on the flow rate with SR but not with PEUU. Increasing the flow rate can delay the plateau time because of shear forces opposing the diffusion of protein molecules to the surface. There was a sixfold increase in the plateau concentration for albumin on SR when the flow rate was increased from 0 to 12 ml/sec. This was explained in terms of surface roughness. A rough surface such as SR for example, has a greater surface area, therefore more anchoring sites are available for the greater number of protein molecules in the vicinity with an increased flow rate. Perhaps this may be due to the formation of a thick adsorption-entanglement layer along the wall analogous to the ones observed in flowing high-molecular weight polymer solutions (Hikmet *et al.*, 1985).

FTIR-ATR (fourier transform infrared spectroscopy coupled with attenuated total reflectance optics) studies on albumin adsorbed onto

polyetherurethaneurea showed that the adsorption kinetics were not significantly affected by the shear rate over a range 0-1800 s^{-1}. However, protein adsorbed at 0 to 200 s^{-1} desorbed more rapidly than that adsorbed at 1800 s^{-1}. This suggests that the protein adsorbed under higher shear rates may bind more tightly (Pitt and Cooper, 1986).

The conformation and conformational changes upon the adsorption of plasma proteins may be a way of predicting the effect of interactions with the surface and have received considerable attention.

Polymers are adsorbed to surfaces by the attachment of various segments along the chain which may occur singly or in runs which have loops extending from the surface into the solution. Statistical mechanics has been applied to independent polymer molecules adsorbed to planar surfaces and one can predict the fraction of bound segments, the distribution of the segments normal to the adsorbing surface and the average number of loops (Cohen Stuart *et al.*, 1986). However, for adsorbed proteins such predictions are not possible due to the numerous intermolecular interactions described earlier.

Morrissey and Stromberg used infrared difference spectroscopy to study protein adsorption on silica particles. By observing a shift of 20 cm^{-1} in the amide I band upon adsorption, the fraction of adsorbed protein carbonyl groups bound to the surface could be measured and used to calculate the number of surface attachments (Morrissey and Stromberg, 1974). The conformation of serum albumin, fibrinogen and prothrombin was studied as a function of surface concentration, time of adsorption, pD and ionic strength. There was no change in the bound fraction of albumin or prothrombin along the isotherm. Both these proteins have an average bound

fraction of 0.11 indicating about 80 carbonyl attachments to the surface but most of the molecule is in solution and away from the solid surface. The bound fraction does not change with the time of adsorption. This data suggests that conformational changes do not occur or are minimal upon adsorption, therefore it may be said that the internal bonding in these proteins is sufficient to prevent structural changes. The bound fraction of fibrinogen was found to increase with increasing adsorption which may suggest interfacial aggregation.

Studies on cross-linked and denatured albumin showed that cross-linked albumin gave a bound fraction similar to that of the native protein, while unfolded albumin gave an increase of 55 contacts and aggregated albumin resulted in a decrease of 50 surface contacts. It is concluded that no aggregation or conformational changes occur upon adsorption of the native serum albumin.

Other investigators have also concluded by infrared spectroscopic techniques that in general plasma proteins were not dimensionally denatured, i.e., no change in conformation occurred on adsorption to the surfaces studied (Brash and Lyman, 1969).

It has been shown that adsorbed fibrinogen and γ-globulin are required for platelet adhesion and are therefore important for surface induced thrombosis (Zucker and Vroman, 1969; Kim *et al.*, 1974). A study of these from a protein mixture or plasma may help predict biocompatibility. Radiolabelled proteins have to be used since spectroscopic techniques do not discriminate between different proteins.

Lee and coworkers studied the competitive effects of plasma proteins adsorbed to hydrophobic polymer surfaces (Lee *et al.*, 1974). The rates of

adsorption of ^{125}I labelled albumin, γ-globulin and fibrinogen were measured separately and from a mixed solution. The composition of the adsorbed layer, following rinsing, at the plateau value was determined. The amount of each protein adsorbed from a mixed solution was less than that compared to the adsorption from a single protein solution. The time to reach the plateau concentration for albumin was doubled while that for fibrinogen and γ-globulin decreased by a factor of ten.

Lyman and coworkers tried to establish a relationship between the adsorbed protein composition and the extent of surface-induced thrombosis on PEUU, SR and FEP (Lyman *et al.*, 1974). Recirculation tubes were implanted in dogs for varying periods of time. The tubes were rinsed and then soaked in a detergent to remove the adsorbed protein. The amount of albumin, γ-globulin and other globulins was determined using acrylamide gel electrophoresis. The results indicated that low thrombogenic surfaces adsorbed mainly albumin while thrombogenic materials adsorbed largely globulins.

1.4.1 Protein desorption and exchange

The reversibility of protein adsorbed has to be established in order to justify using thermodynamic equations to describe the adsorbed phase. It is also of interest to establish any changes in the composition of the protein films adsorbed from mixtures.

If protein adsorption is assumed to occur at multiple sites on the protein, desorption results only when all these sites simultaneously detach. This would be expected to be a very improbable event and therefore one would expect significant desorption not to occur.

The reversibility of protein adsorption is somewhat controversial. Adsorption data has frequently been found to be in good agreement with the Langmuir model (Bull, 1956; Brash and Lyman, 1969; Chuang *et al.*, 1978; Young *et al.*, 1988). This might be interpreted as supporting reversibility in terms of a dynamic equilibrium. However, there is little evidence that supports the Langmuir assumptions of reversible binding to single sites per protein. Generally it has been observed that significant desorption from a hydrophobic surface does not occur, while for hydrophilic surfaces both reversibility and irreversibility of the adsorbed protein has been found.

Various methods have been employed in order to study the desorption of proteins from solid surfaces and care must be taken to ensure that one measures the amount of protein actually desorbed.

Bull used solution-depletion methods to study the adsorption of bovine serum albumin on glass (Bull, 1956). The surface concentration is determined by monitoring a change in the bulk solution concentration. In this way the adsorption at equilibrium is measured. The absorbance near 278 nm is measured following equilibration and centrifugation of the suspended solid. A large surface area to solution volume ratio was required to produce a significant decrease in solution concentration. Desorption was studied by allowing two samples of pyrex glass to adsorb protein at pH 5.05. The amount of protein adsorbed from a 0.0140% protein solution was 0.78 mg per gram of glass. One of the samples was then diluted with buffer to give an equilibrium protein concentration of 0.0065% and the amount of protein adsorbed was found to be 0.77 mg per gram of glass. The amount of protein adsorbed in each case was found to be approximately the same. It was concluded that no protein was removed from

the glass surface by diluting the protein solution.

Bull carried out an experiment to determine if protein could be removed from the surface by extensive washing. Glass powder was suspended in a protein solution and allowed to equilibrate. The protein solution was removed and the glass was resuspended in buffer at selected pH values. The resuspension procedure was repeated five times. The protein was removed from the glass by suspending it in one molar sodium acetate and its concentration determined. The results show that a considerable amount of the adsorbed protein is removed by extensive washing. The removal is pH dependent and it is more difficult to remove the protein at pH values near the isoelectric point of albumin.

MacRitchie used similar solution-depletion techniques and dilution with buffer to study the desorption of bovine serum albumin from hydrophobic and hydrophilic silica particles (MacRitchie, 1972). It was shown that at pH 7.5 albumin adsorption to hydrophilic silica was completely reversible, but at the albumin isoelectric point, pH 4.9, adsorption was not reversible. Reversibility was not observed with the hydrophobic silica surface.

Brash and his coworkers studied the desorption and exchange of serum albumin on polyethylene and cuprophane (Brash *et al*, 1974). The polymers were in the form of tubes. Radiolabelled serum albumin was pumped through the tubes. After a 24 hour rinsing period the tube was assayed via gamma counting (gamma counted) to give the surface concentration. The adsorption and exchange of albumin adsorbed from a 0.1 mg/ml solution on polyethylene was studied. No desorption into water was detected and exchange of the radiolabelled with nonlabelled albumin at 0.1 mg/ml solution concentration

did not occur under static conditions. Rapid desorption would not be detected in this way since the tubing is washed before the "equilibrium" adsorption is determined by gamma counting. Exchange of radiolabelled protein was detected at a solution concentration of 3.7 mg/ml. The turnover was 10% in the first hour and 85% in 220 hours.

Grant *et al.* and Stromberg *et al.* used rapid rinsing techniques to study desorption (Grant *et al.*, 1977; Stromberg *et al.*, 1975). Adsorption was carried out using radiolabelled albumin. The substrate was removed and immersed in a rinse vessel and continuously washed with water. Desorption from polyethylene was not detected. Albumin adsorbed on chromium showed reversibility with up to 25% of the protein being removed in the first minute.

Chuang *et al.* used polymer discs to study desorption and exchange (Chuang *et al.*, 1978). Cuprophane and poly(vinyl chloride), (PVC), discs were precoated with ^{125}I-protein by incubating the disc in a protein solution for 30 minutes. The discs were washed by dipping in Tyrode's buffer. Desorption and exchange studies were carried out by incubating the precoated discs in Tyrode's buffer or in homologous unlabelled proteins for 24 hours at room temperature. The residual radioactivity was gamma counted.

It was demonstrated that desorption and exchange was dependent on both the specific protein species and the type of polymer surface. Albumin adsorbed to cuprophane did not desorb into Tyrode's buffer but 38% of the radiolabelled albumin was found to exchange with unlabelled albumin from a 1 mg/ml solution. For fibrinogen adsorbed on cuprophane 23% desorbed into Tyrode's buffer and 42% exchanged with unlabelled fibrinogen at a faster

rate than for albumin under similar conditions. ^{125}I-IgG adsorbed on cuprophane showed a 19% desorption and gave an exchange of 30% with unlabelled IgG, whereas for ^{125}I-IgG adsorbed on PVC the desorption was 4% and exchange was 6%, both ocurring at a much slower rate.

Double isotope labelling experiments have shown exchange of the adsorbed protein with the protein in solution even though the quantity of protein adsorbed remains the same (Brash and Samak, 1978; Chan and Brash, 1981). In these experiments the polymer surface was rinsed with buffer before exchange runs were carried out. The results indicate a constant exchange of protein between the surface and solution. The levelling off of the protein loss and gain curves suggest that there is a fraction of the adsorbed protein that is exchangeable and a fraction that is not and that this varies with conditions.

The rates and extent of exchange have been shown to be greater for glass and hydrophilic surfaces (Chan and Brash, 1981) than for hydrophobic surfaces (Brash and Samak, 1978; Cheng *et al.*, 1987). This would suggest stronger binding for hydrophobic surfaces.

1.5 Objectives and methods

In many studies on protein adsorption, and in all studies on adsorption to tubing, the protein layer is washed before determining the "equilibrium" adsorbed protein concentration. Desorption studies are then carried out on the remaining adsorbed layer. The problem with this protocol is that any weakly bound protein will be washed off. It is this weakly bound protein with which this thesis is concerned. Such material is of interest because weakly adsorbed macromolecules are known to play a

central role in some blood cell adherence phenomena (Brooks *et al.*, 1980). The tubing form for the substrate is important because it is this form that is utilized in many blood compatibility applications.

Desorption studies have also been carried out using solution-depletion techniques. A solid is placed in a protein solution and a protein layer is adsorbed to the surface. The protein solution is then diluted. A decrease in surface concentration following dilution would indicate desorption. Solution-depletion techniques have had limited application since a finely divided substrate has to be used and there may be some uncertainty in determining the available surface area per gram of material. Also, the geometry of the substrate has been shown to affect the amount of protein adsorbed (Oreskes and Singer, 1961) and it is not possible, in general, to produce dispersions and tubular geometries of the same material with the same surface properties.

In the adsorption experiments of this study, BSA was adsorbed to a length of polyethylene tubing. Following equilibration of the radiolabelled BSA with the surface the protein solution was displaced with buffer. All the fractions collected were gamma counted. In an experiment of this type the surface concentration can be calculated by a variety of methods. Firstly, a minimum surface concentration is determined by cutting up the polyethylene tubing following rinsing and gamma counting. In this case any reversibly adsorbed protein will have been washed off. The amount of protein displaced from the tube, taking into account the dilution effect, can be calculated from the activity of the collected samples. From the total activity added and the activity of the protein displaced the surface concentration in the tube can be calculated. This solution

depletion value gives the maximum amount of protein adsorbed at equilibrium and the difference between these two values, if any, represents the loosely bound protein.

CHAPTER 2

EXPERIMENTAL

2.1 Radiolabelling

Radiolabelling of bovine serum albumin (BSA) was carried out using iodo-beads, a commercial solid state reagent (Markwell, 1982). The iodo-beads have N-chloro-benzenesulfonamide, an oxidant, immobilized on 2.8 mm diameter non-porous polystyrene spheres. The iodination involves the oxidation of the radioiodide which then reacts with tyrosine (4-hydroxyphenylalanine) residues of the protein by the electrophilic substitution of the ortho hydrogens on the phenolic ring (Regoeczi, 1984).

Bovine serum albumin (Fraction V, code no. 81-003, Miles Scientific, Rexdale, Ont.), the protein to be iodinated, was dissolved in phosphate buffered saline (PBS)/azide, pH 7.4, to give a concentration of 1 to 4 mg/ml. Isotonic PBS/azide pH 7.4 consisted of Na_2HPO_4 2.367 g/l, NaH_2PO_4 0.400 g/l, NaN_3 0.195 g/l and NaCl 7.621 g/l. The iodo-beads (Pierce Chemical Company, Rockford, Ill.) with an oxidative capacity of 0.45 μmol/bead for tyrosine-containing peptides, were washed twice in PBS/azide and blotted dry on filter paper. To a 1.5 ml Eppendorf micro test tube 2-4 iodo-beads and 0.5 ml of the protein solution were added. The reaction was initiated by the addition of 10-20 μl (200-400 μCi) of carrier free $Na^{125}I$ (Amersham, Arlington Heights, Ill.). The capped tube was rotated for 30 minutes at room temperature (19°C) after which the reaction was monitered by a trichloroacetic acid (TCA) precipitation assay to determine the amount

of free label hence the completeness of the reaction.

2.1.1 TCA precipitation assay

A small sample, 1 µl, of the reaction mixture was added to 1 ml of a 1 mg/ml BSA solution in a polypropylene test tube. To this 1 ml of 0.5 M trichloroacetic acid (TCA) solution was added to precipitate the protein. The sample was centrifuged at 4500 × g for 10 minutes and 1 ml of the supernatant was pipetted into a second tube and both samples were counted in a LKB-Wallac 1282 Compu Gamma gamma counter. The radiolabelled protein pelleted with the albumin while the free ^{125}I was distributed evenly between the pellet and the supernatant. The percent of protein bound was calculated using the following equation

$$\% \text{ Bound} = \frac{\text{Pellet} - \text{Supernatant}}{\text{Pellet} + \text{Supernatant}}$$

2.1.2 Gel filtration

Following iodination the free label was separated from the radiolabelled protein by gel filtration. A Bio-Rad column (1 × 20 cm) was packed with Sephadex G-25 (Pharmacia, Uppsala, Sweeden) and equilibrated with PBS/azide at pH 7.4 . Radiolabel free BSA, (2 ml at 1 mg/ml) was put on the column first to reduce binding of the labelled protein to the gel. The radiolabelled protein was loaded onto the column and eluted with PBS/azide buffer pH 7.4 and the eluate was collected in 20, 25 or 30 drop fractions. The fractions were sampled with 1 µl Drummond micro capillaries (Fisher Scientific) and gamma counted. The appropriate fractions were

pooled and before use the samples were either put through a second Sephadex G-25 column or dialysed against PBS/azide using an ultrafiltration unit (molecular weight cut off = 10,000, Millipore Ltd., Mississauga, Ont.) to remove more free label. The sample was split into convenient aliquots and stored at -20°C. The amount of free label was checked by TCA precipitation and instant thin layer chromatography (TLC).

2.1.3 Thin-layer chromatography

A small amount, 1 µl, of the radiolabelled protein was added near the base of a 10 × 1.5 cm strip of polysilicic acid gel chromatography media (Gelman Sciences Inc., Ann Arbor, Mi.). This was put into a chamber and developed with 1:1 (v/v) acetone:methanol. The strip was air-dried, cut up into 1 cm sections perpendicular to the direction of migration and placed in gamma tubes containing 2 ml of 10 mM NaOH and counted. The amount of free label can be calculated since the free label migrates up the strip and the radiolabelled protein remains where spotted.

2.2 Protein Electrophoresis

The purity of the radiolabelled BSA was determined using polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), (Ornstein 1964, Davis 1964). The mobility of a protein in a polyacrylamide gel is governed mainly by the protein molecular weight (Sharpiro *et al.*, 1967; Weber and Osborn, 1969). In the presence of SDS, all proteins whatever their original charge, are converted to complexes having strong negative charges. This causes them to behave as rods of constant diameter. Electrophoresis carried out in gels with pores small enough to restrict

mobility shows that the observed mobility is related very nearly linearly to the log of the molecular weight of the protein. This is unaffected by the proteins original charge.

The protein sample is layered on the polyacrylamide gel and a voltage gradient is applied. The macromolecules migrate at different (constant) rates in the gel and their location in the gel is determined after the experiment by staining with Coomassie Blue, a cationic dye that binds mainly to amines (Fazekas et al., 1963), or by gel slicing and counting the slices for gamma radiation.

2.2.1 Materials

The following were of electrophoresis purity from Bio-Rad laboratories, Richmond, CA.: acrylamide, N,N-methylene-bis-acrylamide (BIS), sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethylenediamine (TEMED) and ammonium persulphate. The disodium ethylenediamine-tetraacetate (EDTA) was from Fisher Scientific Company, Fair Lawn, N.J.. Tris(hydroxymethyl)aminomethane (tris-base) and Tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl) were obtained from Sigma Chemical Company, St.Louis Mo.. Sucrose was from Baker and Adamson, Morristown, N.J.. Pyronin Y (C.I. 45005) was from J.T. Baker Chemical Company, Phillipsburg N.J.. Coomassie Brilliant Blue G-250 (42655) and Photo-Flo 200 solution were obtained from Eastman Kodak Co. Rochester N.Y..

2.2.2 Sample preparation

The samples for electrophoresis contained 1 mg/ml of protein or had approximately 2000 cpm/µl.

Following an adsorption experiment the tubing was placed in a hot water bath (80°C) for 5 minutes. The solution in the tube was displaced with a hot alkaline solution of SDS (4%). The hot SDS solution was allowed to sit for 5 minutes and was then displaced. This procedure was repeated until the counts coming off the tube were negligible. The displaced solutions were pooled and concentrated using a Millipore ultrafiltration unit (Millipore Corporation, Bedford, Mass.). The ultrafiltration unit (10,000 molecular weight cut off) connected via silicone tubing to a 50 ml syringe was lowered into the sample to be concentrated in a 15 ml polypropylene tube. A vacuum was applied using the syringe which allowed the filtrate to pass through the membrane into the syringe. The radiolabelled BSA remained in the tube.The sample was then used for SDS-PAGE.

2.2.3 Method

The procedure was a modification of that used by Fairbanks (Fairbanks et al., 1971). The solutions and concentrated stock solutions were mixed in the order and proportions given in Table 2.1.

The 3.75% gel system was prepared and cast as rod gels in acid cleaned 125 × 7 mm inside diameter glass tubes, 2 ml gel solution was used per tube. The gels were overlayed with buffer to produce a flat surface on the gel on which to layer the sample. When polymerization was complete, 40 minutes at room temperature or overnight, the gels were mounted in a Bio-Rad model ISOA electrophoresis chamber and the overlay solution was flushed away with fresh reservoir buffer.

The samples were mixed 1:1 (v/v) with the sample reagent and 20 µl

Table 2.1

Composition of buffers and 3.75% gels for SDS-PAGE.

10 X Acrylamide-Bis

Acrylamide	40.0 g
Bis	1.5 g
Water to 100 ml	

Fairbanks Reservoir Buffer

10 X Buffer	200 ml
4 % SDS	100 ml
Water	1700 ml

Fairbanks Gel System

10 X Acrylamide-bis	3.0 ml
10 X Buffer	3.0 ml
Water	18.0 ml
4 % SDS	1.5 ml
0.5 % TEMED	1.5 ml
1.5 % Ammonium persulphate	3.0 ml

Fairbanks Sample Reagent

Tris-HCl	0.1211 g
EDTA	0.0372 g
SDS	1.0 g
Sucrose	7.0 g
Pyronin Y	
Water to 50 ml	

Overlay Solution

The same as the gel solution with the acrylamide-bis replaced with water

Fairbanks 10 X Buffer

Tris-base	24.23 g
Sodium acetate	13.61 g
EDTA	3.72 g
Water to 500 ml	
pH to 7.4 with glacial acetic acid	

aliquots were layered on the gel surface with the displacement pipettor. For the protein washed off the tubing 60 µl was used to provide sufficient radioactivity. A voltage was applied across the gels at a constant current of 0.5 mA per tube until the sample entered the gel and then the current was increased to 6 mA per tube. The gels were run at 4°C for approximately 2 hours until the tracer dye was near the bottom of the gels.

A syringe was filled with water and a few drops of glycerin and a hypodermic needle was attached. The gels were removed from the glass tubes which were rotated as the hypodermic needle was pushed between the tube and the gel while expressing a fine stream of the glycerin solution. The

position of the tracking dye was marked in each gel by pricking it with a needle dipped in Indian ink.

2.2.4 Gel staining

The gels were stained for protein with coomassie blue for 1-2 hours. Coomassie blue stain consisted of coomassie blue G-250 0.20 g, methanol 28 ml, glacial acetic acid 5 ml, perchloric acid (70%) 25 ml, water to 500 ml. Destained with methanol fix (methanol 300 ml, glacial acetic acid 50 ml, water 650 ml) for one hour and the final clearing in 7% glacial acetic acid overnight until the background was clear. The staining was repeated if necessary. Densitometry was performed with a Auto Scanner Flur-Vis equipped with a 595 nm filter and a zig-zag time base integrator.

Gels run with radiolabelled samples were sliced using a Bio-Rad model 195 electric gel slicer with a 1 mm blade-to-blade separation. The slices were transferred to gamma tubes and counted for radiation.

2.3 Protein Concentration

Proteins show strong absorption at a wavelength of approximately 280 nm due to the residues of phenylalanine, tyrosine and tryptophane. The protein concentration can be determined by measuring the optical density (OD) at about 280 nm if the molar extinction coefficient is known.

The extinction coefficient of the BSA sample used at 278 nm was determined. BSA (5 g) was freeze dried for 24 hours and then dried over phosporous pentoxide for 3 days until no change in weight was observed. A 10 mg/ml stock solution of BSA in PBS/azide was prepared. A series of serial dilutions were carried out and the OD of the solutions at 278 nm

were measured and the extinction coefficient calculated.

The concentrations of radiolabelled protein solutions were determined by measuring the OD at 278 nm. This was found to be more reliable and consistent than the colourimetric method used by Smith et al., 1985.

2.4. Adsorption Experiments

The aim of the work for this thesis was to develop a technique that would detect, if any, the reversibly adsorbed protein bound to a plastic surface.

The technique developed was to use a long tube of small diameter that would give a large surface area. The radiolabelled protein was pumped into the tube, left to equilibrate and pumped out. By collecting all fractions, the amount of protein is calculated. The experiments were performed to obtain the surface concentration as a function of time of contact, specific activity, pH and concentration.

2.4.1 Methods and Materials

The BSA was labelled with ^{125}I using iodo-beads; the amount of free label was less than 1% as checked by TLC. The average degree of iodination was less than one ^{125}I atom per molecule of protein and this degree of substitution has been shown to leave the protein properties biologically unaltered, (McFarlane, 1963; Harwig *et al.*, 1975).

The polyethylene tubing of inside diameter 0.038 cm was obtained from Intramedic. This material is intended for clinical use and is made from low density polyethylene. It allegedly contains no additives or plasticizers. The tubing was prepared for adsorption experiments by

pumping methanol and then distilled water through the tubing.

Solutions of BSA were made up in PBS/azide, pH 7.4, to the desired specific activity and concentration. Protein concentrations were calculated by measuring the OD at 278 nm or from a known specific activity.

A 5 m length of tubing was connected via a three-way valve to a syringe on a Harvard pump. The valve allowed the removal of air bubbles through the side arm before introducing protein solution or buffer. Adsorption runs were carried out by filling the entire system with PBS/azide buffer, displacing with protein solution and collecting all fractions using a Gilson micro-fraction collector. The protein solution was left to equilibrate for 4 hours at room temperature (23°C), except in the case of the time dependence experiment. The contents of the tube were displaced and the tube rinsed with PBS/azide and 3-drop fractions were collected. A chart recorder was connected to the fraction collector to provide a time base and enable the volume of the samples to be determined. The tubing was finally cut into 20 cm segments. All the fractions and tubing were counted for gamma radiation.

2.4.2 Surface concentration as a function of time

To determine the time for the system to reach steady state the equilibration time was varied from 0.5 to 24 hours. One stock protein solution was used for the series of experiments to ensure a constant concentration.

2.4.3 Surface concentration as a function of the ratio of radiolabelled to unlabelled BSA

The effect of the ratio of radiolabelled:unlabelled protein on the surface concentration was determined. Using a saturating concentration of BSA, 500 μg/ml, the ratio of ^{125}I-BSA to unlabelled BSA was varied, a series of experiments were carried out and the surface concentrations calculated.

2.4.4 Adsorption Isotherm

Adsorption was studied as a function of concentration. Protein solutions were prepared at various concentrations with a specific activity of approximately 2×10^4 cpm/μg. Protein concentrations were determined from the OD or from a known specific activity.

2.4.5 Surface concentration as a function of pH

A series of protein solutions was prepared at different pH values by mixing radiolabelled and unlabelled BSA with NaOH or HCl at a final protein concentration of 500 μg/ml. A stock solution of BSA was adjusted to the appropriate pH using a pHM63 Digital pH meter. Approximately 1.5 ml of the stock BSA was weighed and a small amount of radiolabelled BSA was added. The pH was checked using pH paper since the solution contained radiolabelled BSA.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Radiolabelling

A plot of the radioactivity (counts per minute (cpm)) from a 1 μl sample of each fraction against the fraction collected from a Sephadex G-25 column (June 23, 1987) following radiolabelling is shown in Figure 3.1. The first peak corresponds to the radiolabelled protein. The plot shows that the radiolabelled BSA and the free iodide fractions are well separated. In this example a TCA precipitation assay of the pooled fractions (12 to 14) showed that 6% of the activity was due to the free label. The amount of free label was reduced to less than 1%, as tested by TLC, by a second column or by dialysing against PBS/azide when a more concentrated solution was required.

3.1.1 Degree of Radiolabelling

The degree of radiolabelling was found to be less than one molecule of ^{125}I per molecule of BSA and this has been reported to have no effect on the biological activity, (McFarlane, 1963; Harwig *et al.*, 1975).

For a typical labelling experiment the degree of radiolabelling is calculated as follows. The $Na^{125}I$ on June 1, 1987 had an activity of 16.6 mCi/μg of iodide. The atomic weight of ^{125}I is 126.9 g/mole and 1 mCi = 2.2×10^9 dpm, therefore the activity of the ^{125}I was 4.63×10^{18} dpm/mole. The dpm of the ^{125}I on June 24, 1987 is calculated from the

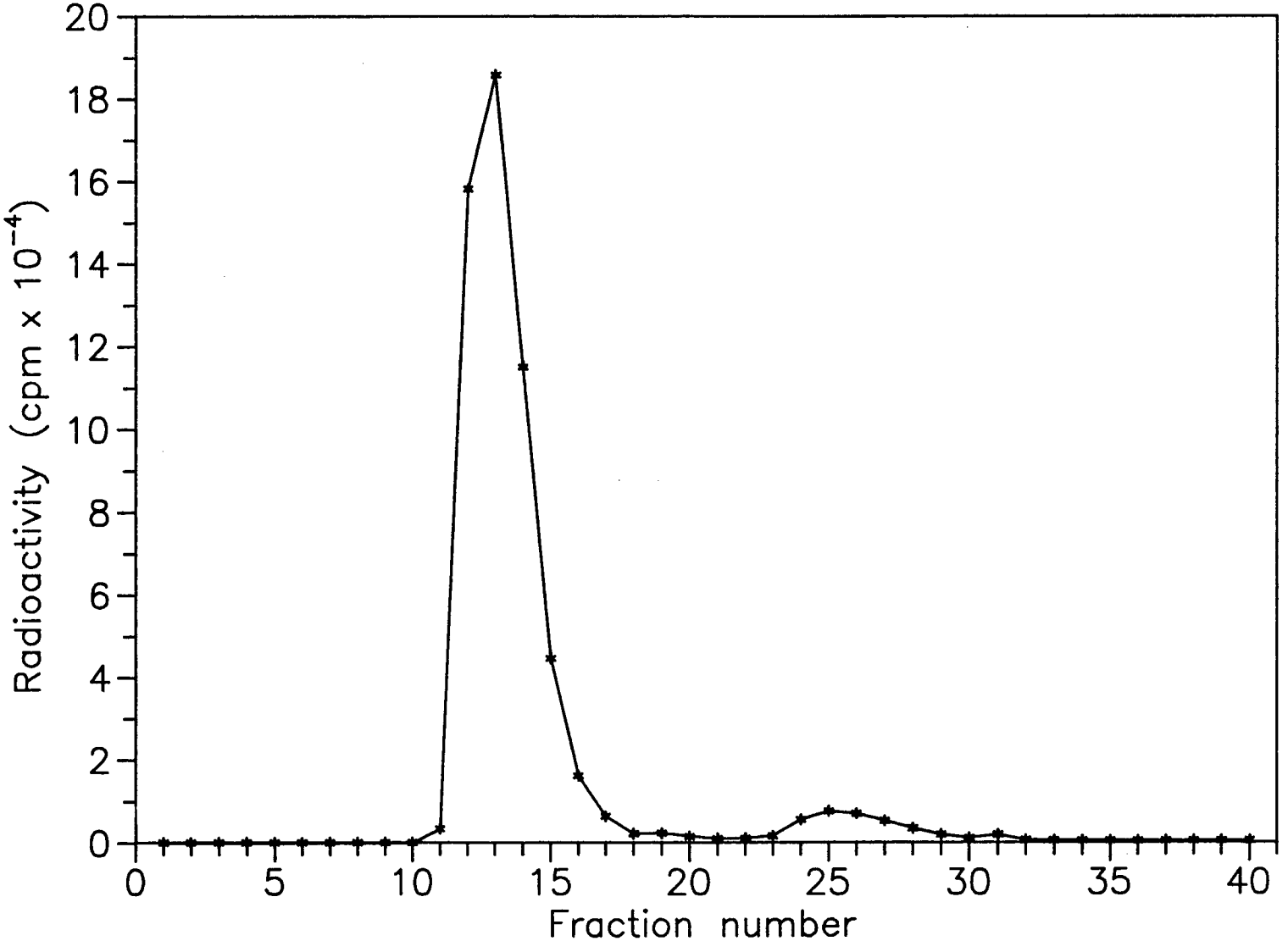

Fig. 3.1. Radioactivity vs fraction number for the samples collected from a Sephadex G–25 column following radiolabelling.

following equation

$$A = A_0 e^{-\lambda t} \qquad (3.1)$$

where A_0 and A are the activities at times t_0 and t respectively and λ is the decay constant which is given by

$$\lambda = \ln 2 (t/2)^{-1} \qquad (3.2)$$

where t/2 is the half-life. Substituting into equations 3.2 and 3.3 using 60 days as the half life of ^{125}I, on June 24, 1987 the activity of ^{125}I was 3.55×10^{18} dpm/mole.

The activity of the radiolabelled BSA on June 24, 1987 was 196,000 cpm/μg or assuming 77% efficiency of the gamma counter (Janzen, 1985) 255,000 dpm/μg. Using 66,000 as the molecular weight for BSA its activity was about 1.68×10^{16} dpm/mole. Dividing the two activities gives a degree of radiolabelling of 211 moles of BSA per mole of ^{125}I.

3.2 Extinction coefficient

The optical densities of a series of solutions of known BSA concentration were measured. A plot of BSA concentration against OD at 278 nm is given in Figure 3.2. The plot indicates a linear relationship between the OD and a concentration of 0-1.63 mg/ml. The extinction coefficient is given by the slope and is calculated to be 0.641 ± 0.002. This value is similar to the BSA extinction coefficient of 0.66 given in the literature (Cohn *et al.*, 1947).

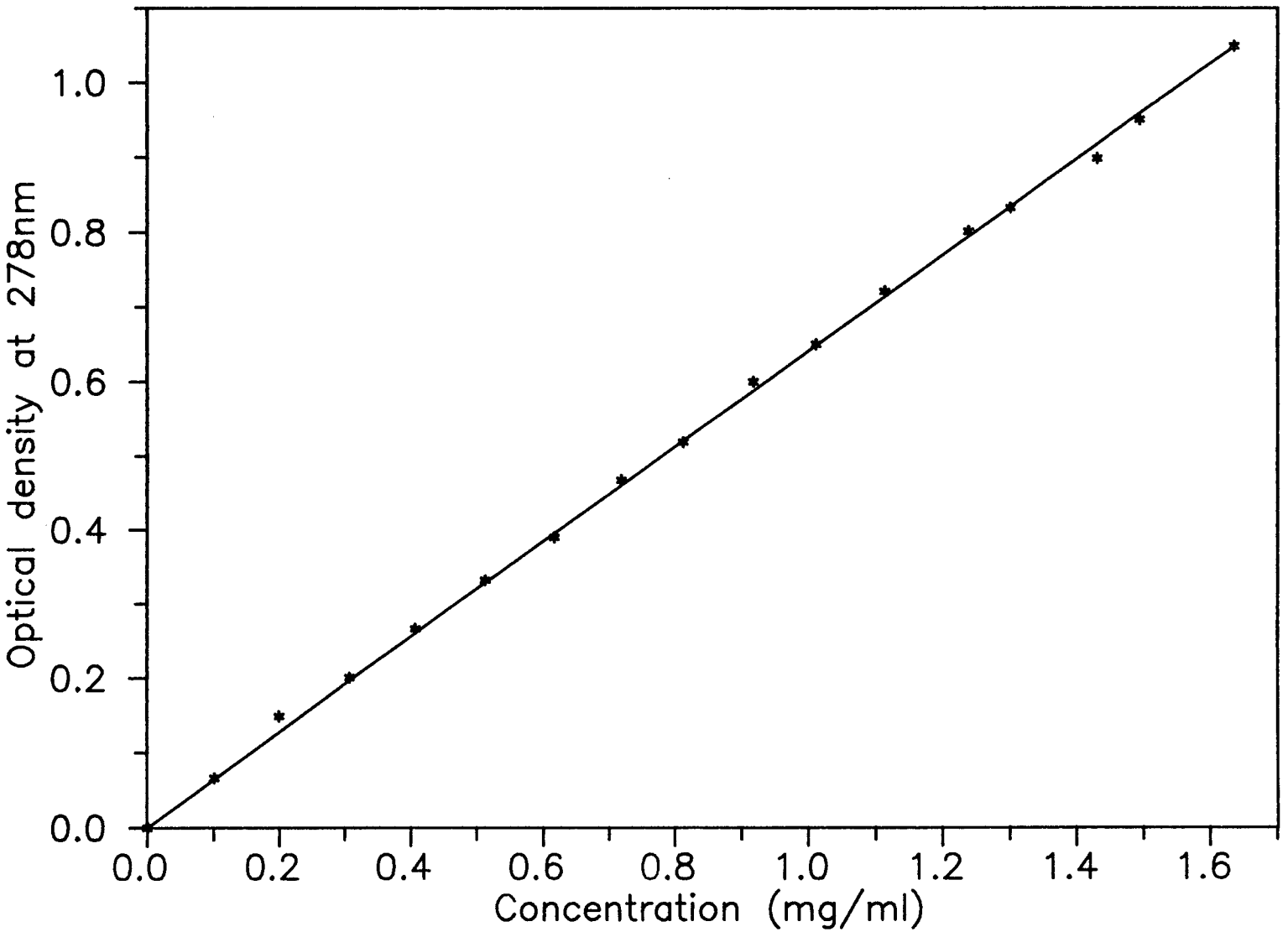

Fig. 3.2. Calibration curve of BSA. Optical density vs BSA concentration at 278nm.

3.3 SDS-PAGE

The purity of the BSA used in the adsorption experiments was determined using SDS-PAGE. The gels were calibrated using Pharmacia electrophoresis calibration kits. In both the high molecular weight kit (HMWK) and the low molecular weight kit (LMWK) several SDS-denatured proteins were run on the same gel. The relative mobility (R_f) of a protein is calculated as

$$\text{Relative mobility} = \frac{\text{distance of protein migration}}{\text{distance travelled by tracking dye}}$$

The R_f values for the proteins of known molecular weights are given in Table 3.1. The relative mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale. The plot in Figure 3.3 indicates a linear relationship and provides the molecular weight calibration for the gels.

The SDS-PAGE rod gels of the stock unlabelled BSA and radiolabelled BSA along with the molecular weight standards are shown in Figure 3.4. A densitometric scan of the unlabelled BSA and the relative mobility versus cpm for radiolabelled BSA are given in Figure 3.5. The unlabelled BSA showed bands with apparent molecular weights of 60,000, 133,000 and 214,000 corresponding to R_f values of 0.540, 0.371 and 0.270. The R_f values of the radiolabelled BSA at 0.539, 0.371 and 0.264 likewise imply molecular weights of approximately 60,000, 134,000 and 220,000. The more intense band with a molecular weight of approximately 60,000 corresponds to the BSA monomer while the less intense bands at 134K and 220K presumably correspond

Table 3.1

Molecular weight assignments for the protein standards used on the 3.75% SDS-PAGE gels.

Protein	Molecular weight	R_f
Thyroglobulin	330,000	0.165
Ferritin (half unit)	220,000	0.311
Phosphorylase b	94,000	0.420
Albumin (BSA, HMWK)	67,000	0.495
Albumin (BSA, LMWK)	67,000	0.506
Catalase	60,000	0.540
Ovalbumin	43,000	0.607
Lactate dehydrogenase	36,000	0.653
Carbonic anhydrase	30,000	0.702
Trypsin inhibitor	20,000	0.771
Ferritin	18,500	0.786
α-Lactalbumin	14,400	0.831

to the dimer and trimer. The 220K band is less intense than the 134K band. The BSA molecular weight determined from the gels is lower than the actual value of 66,000. This may have been due to the wide bands of stained protein in the standards. Since the R_f values for both the labelled and the radiolabelled BSA are similar it is concluded that radiolabelling does not effect the mobility of the protein.

The specific activity for the oligomeric species have been calculated to be approximately the same as the monomer.

The nature of the BSA dissolved in solutions at a variety of pH values

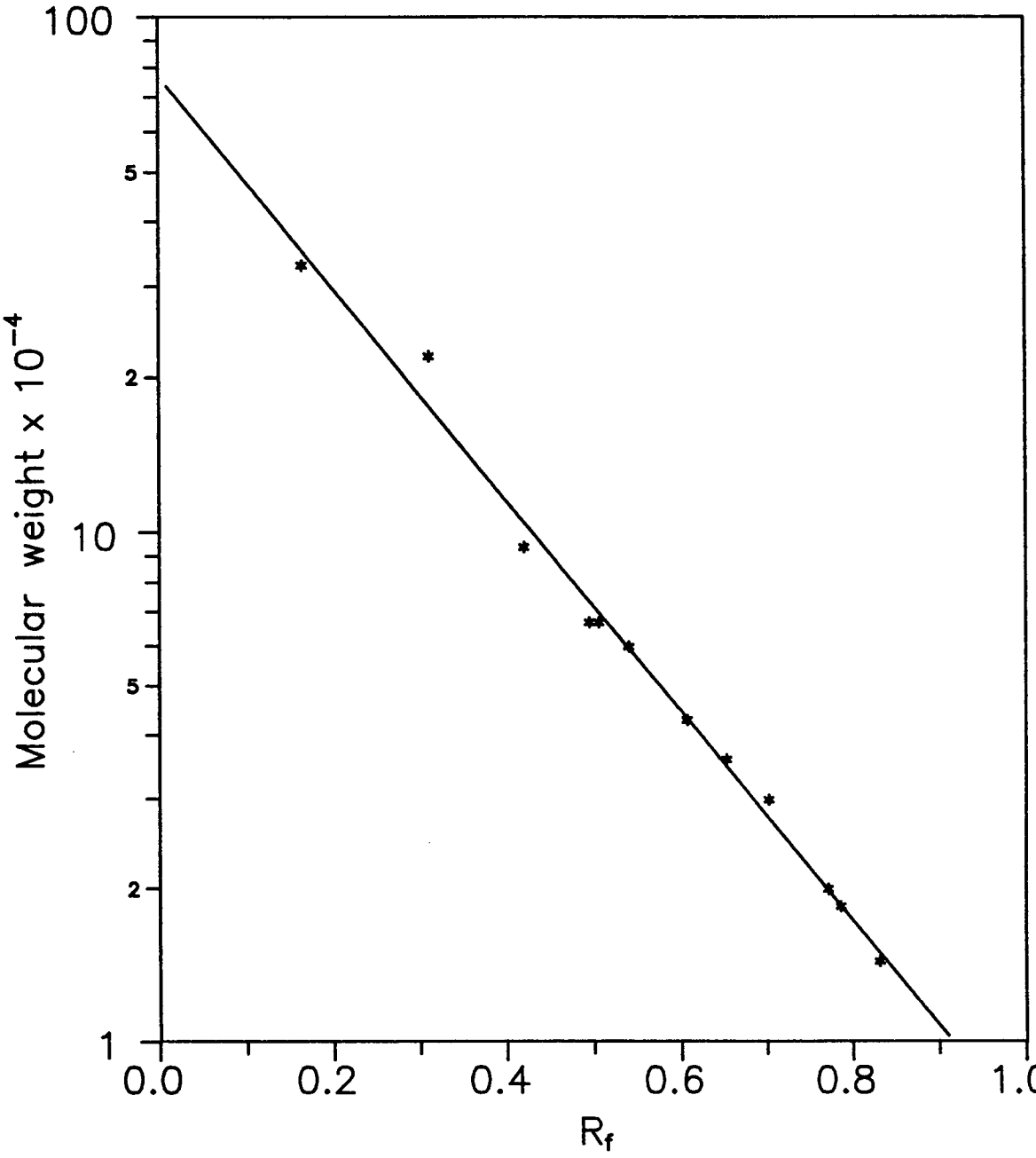

Fig. 3.3. The molecular weight on a semi–log scale is plotted against the relative mobility (R_f) for a variety of SDS–protein complexes run on 3.75% gels.

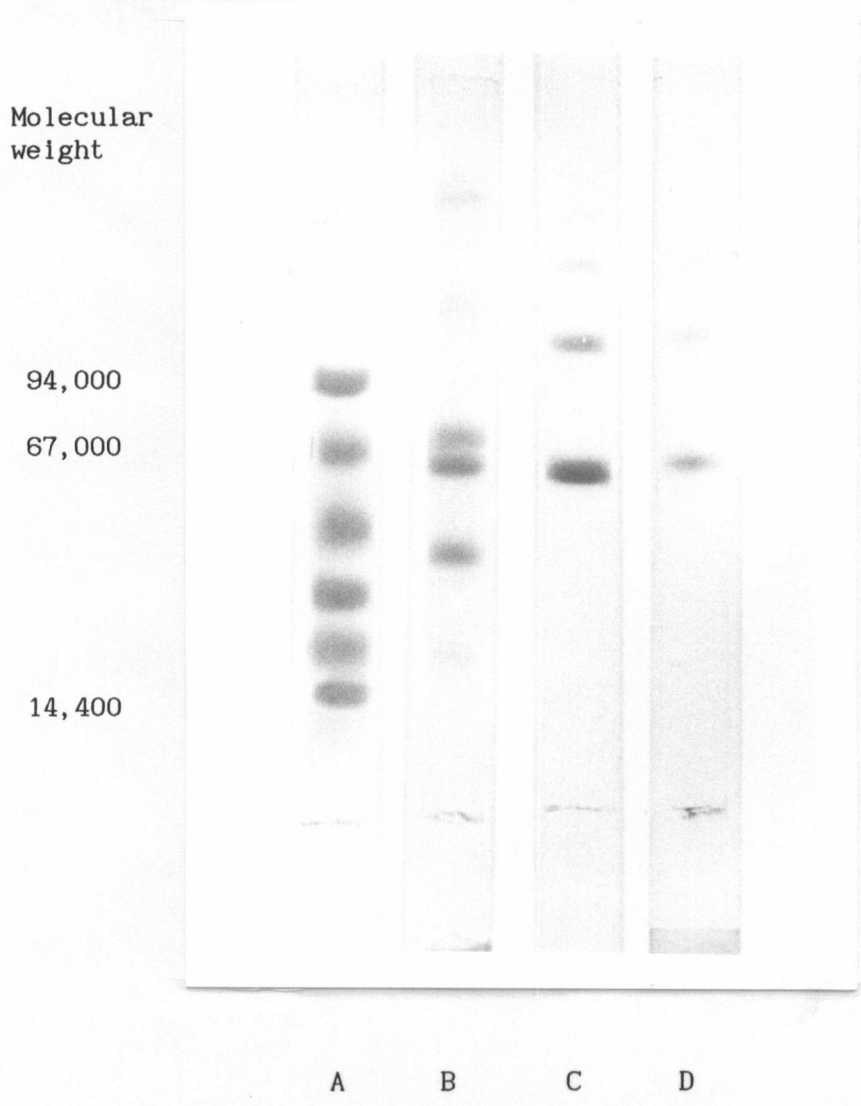

Fig. 3.4. SDS-PAGE rod gels stained with coomassie blue

A low molecular weight standards
B high molecular weight standards
C unlabelled stock BSA
D radiolabelled BSA

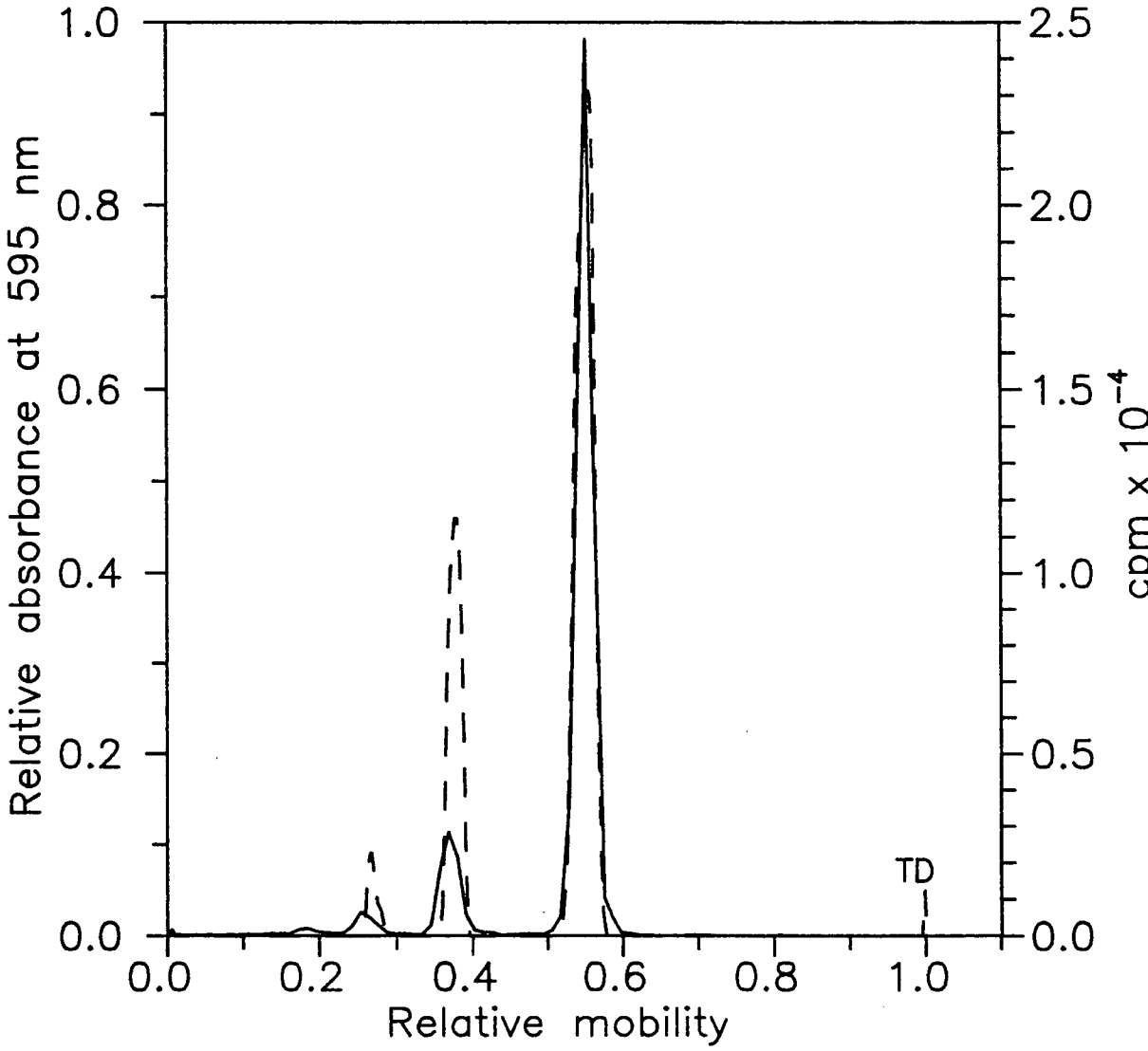

Fig. 3.5. The mobility of BSA in 3.75% SDS–PAGE gels: (———) densitometric scan of BSA. The protein was coomassie blue stained and scanned at 595 nm. TD = tracking dye. (– – –) relative mobility vs cpm for radiolabelled BSA.

was investigated by electrophoresis. The samples of the gels plotted in Figures 3.6a-d were from the stock solutions in the adsorption experiments. The amounts of monomer and polymer in the BSA samples were calculated from the radioactivity of the gel slices and the data is presented in Table 3.2. It is apparent from the plots that as the pH increases there is a significant decrease in the amount of monomer present.

Table 3.2

The effect of pH on the amount of BSA polymer present in the stock solutions. The amount of polymer is represented as a percentage of the total protein ± error. The error was calculated from the activity; see Appendix 2.

pH	% monomer	% dimer	% trimer	% tetramer	% pentamer
4.4	83.36 ± 0.72	11.42 ± 0.21	2.68 ± 0.13	1.31 ± 0.08	
7.4	83.16 ± 0.48	11.87 ± 0.14	2.93 ± 0.08	1.54 ± 0.08	
9.4	71.61 ± 0.45	19.42 ± 0.20	5.17 ± 0.10	1.94 ± 0.06	1.05 ± 0.06
12.0	52.96 ± 0.31	25.53 ± 0.22	10.92 ± 0.14	4.95 ± 0.10	2.01 ± 0.05

It is of interest to establish if the BSA is altered upon adsorption to the polyethylene tubing. An adsorption experiment was run at pH 7.4 and SDS-PAGE was carried out on, (i) the stock radiolabelled BSA i.e., the radiolabelled BSA before adsorption, (ii) the radiolabelled BSA pumped out of the tubing during input of the BSA, (iii) the BSA pumped out of the tubing after the four hour equilibration time and (iv) the BSA washed off the tubing using hot basic SDS. Plots of the gels are shown in Figures

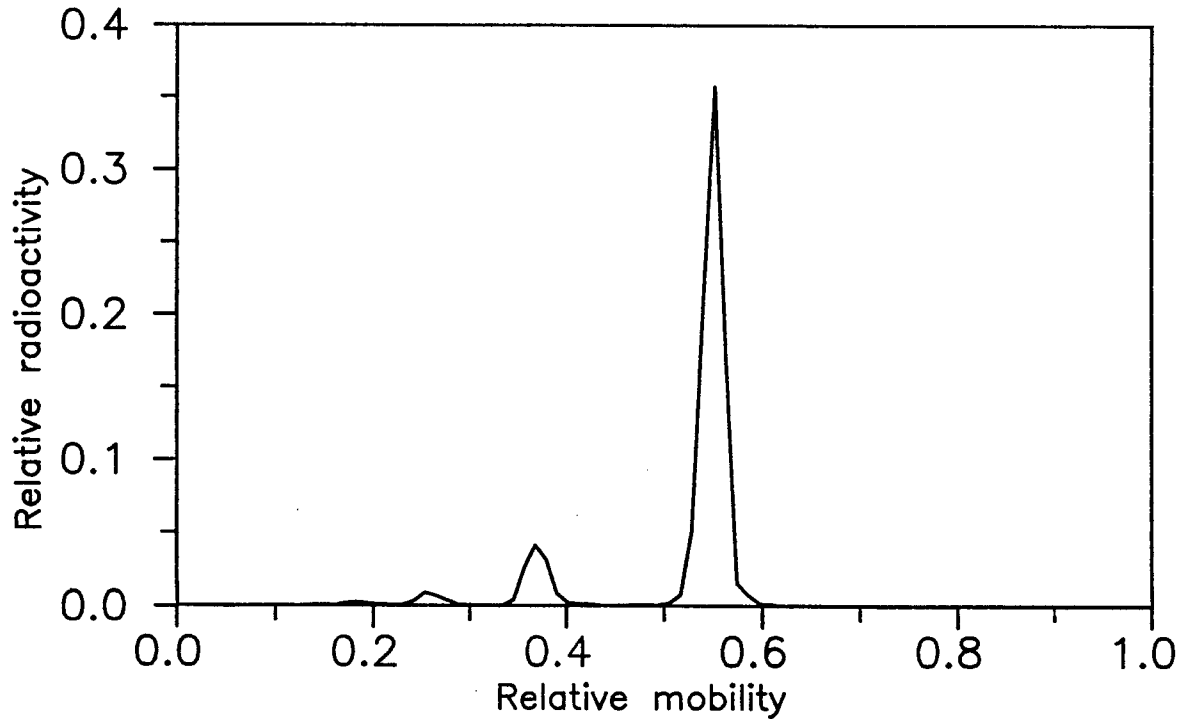

Fig. 3.6a. Relative mobility vs relative radioactivity of BSA, pH 7.4.

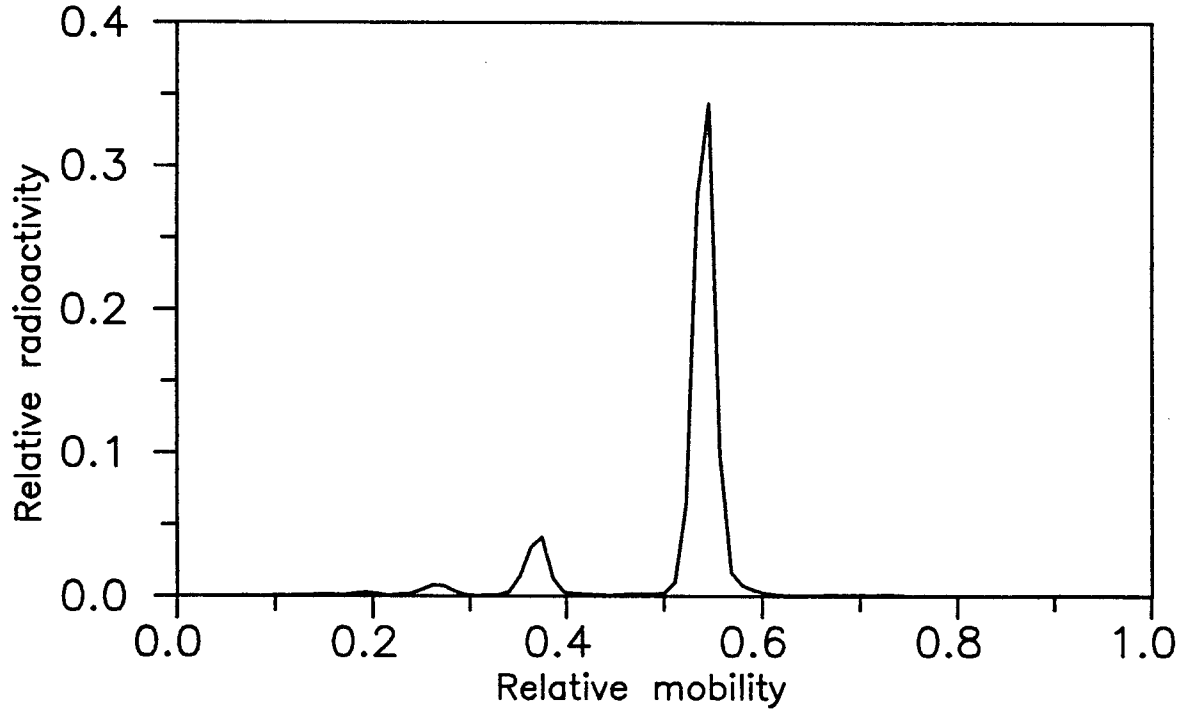

Fig. 3.6b. Relative mobility vs relative radioactivity of BSA, pH 4.4.

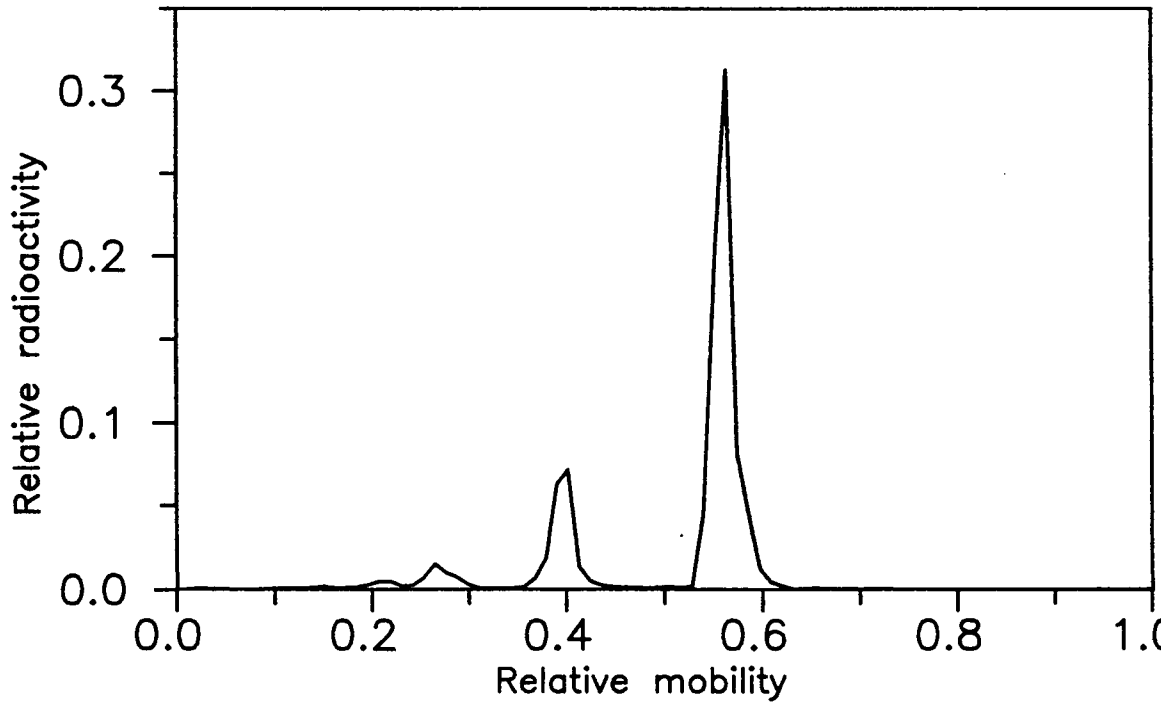

Fig. 3.6c. Relative mobility vs relative radioactivity of BSA, pH 9.4.

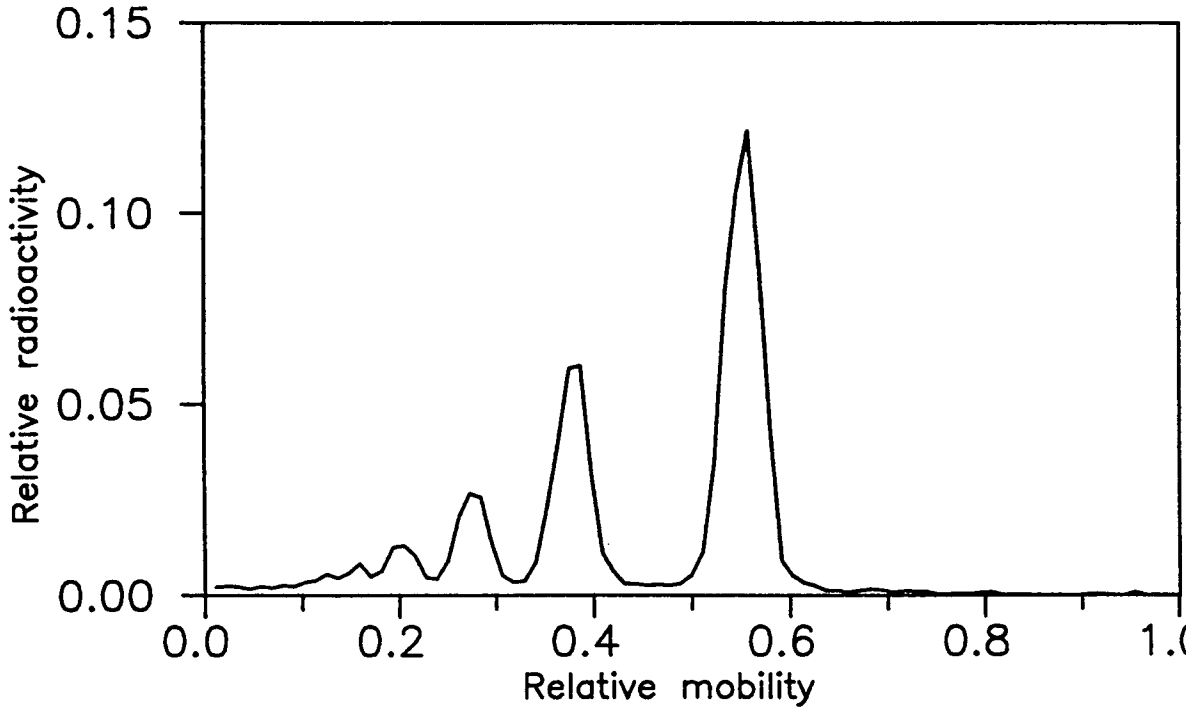

Fig. 3.6d. Relative mobility vs relative radioactivity of BSA, pH 12.0.

3.7a-d. The amount of monomer and polymer in each sample is given in Table 3.3.

Table 3.3

The amount of BSA polymer present in various samples represented as a percentage of the total protein ± error.

Sample	% monomer	% dimer	% trimer	% tetramer
(i)	77.91 ± 0.54	14.83 ± 0.19	3.47 ± 0.10	1.86 ± 0.08
(ii)	78.21 ± 0.66	14.21 ± 0.23	3.51 ± 0.12	1.54 ± 0.10
(iii)	79.87 ± 0.48	14.15 ± 0.16	3.34 ± 0.08	1.41 ± 0.06
(iv)	65.91 ± 0.60	17.68 ± 0.26	9.45 ± 0.19	

The data in Table 3.3 shows that there is no difference between samples i, ii and iii but that the BSA washed off the tubing (iv) contains less monomer. The bands in Figure 3.7d are wider since a larger sample volume (60 μl) was used to obtain a significant level of radioactivity. 20 μl samples were loaded on the other gels. Assuming that the BSA washed off the tubing is representative of the BSA adsorbed then one may conclude that less monomer is adsorbed. It is seen that more of the higher molecular weight dimer and trimer are preferentially bound. The radioactivity at higher R_f values may be due to low molecular weight fragments resulting from the harsh treatment with hot basic SDS. It was possible to remove more than 90% of the adsorbed BSA with hot basic SDS as indicated by counting the tubing for radioactivity following the washing procedure.

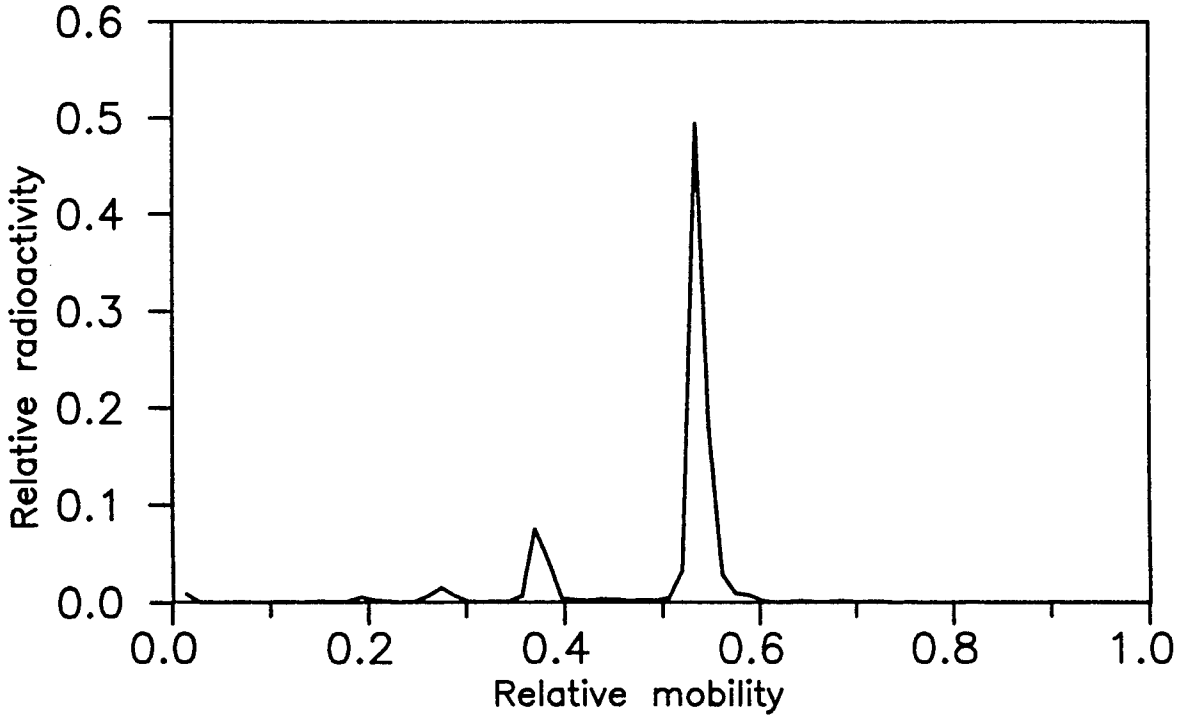

Fig. 3.7a. Relative mobility vs relative radioactivity of sample (i).

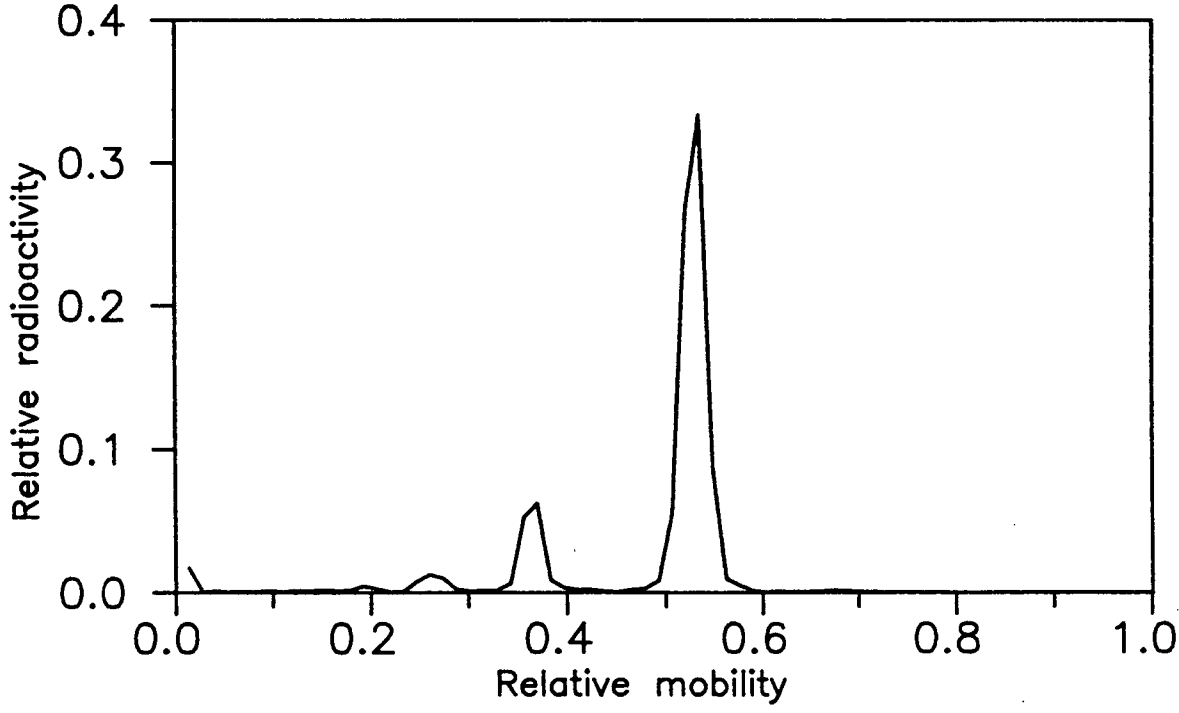

Fig. 3.7b. Relative mobility vs relative radioactivity of sample (ii).

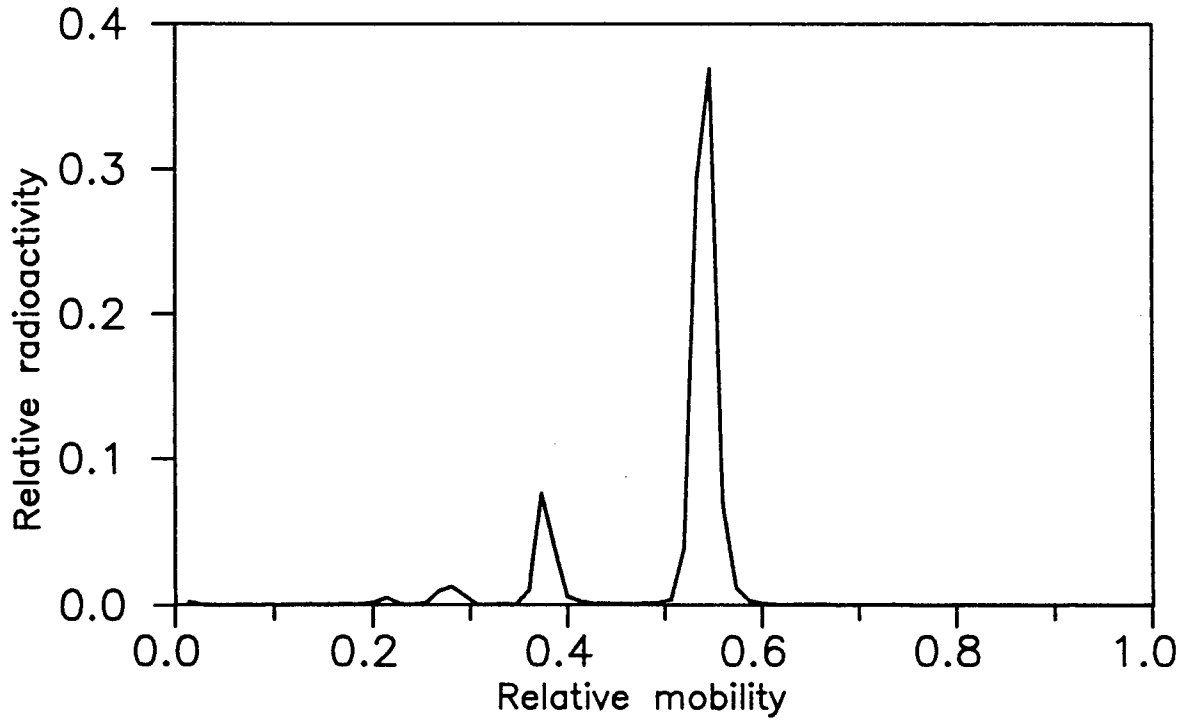

Fig. 3.7c. Relative mobility vs relative radioactivity of sample (iii).

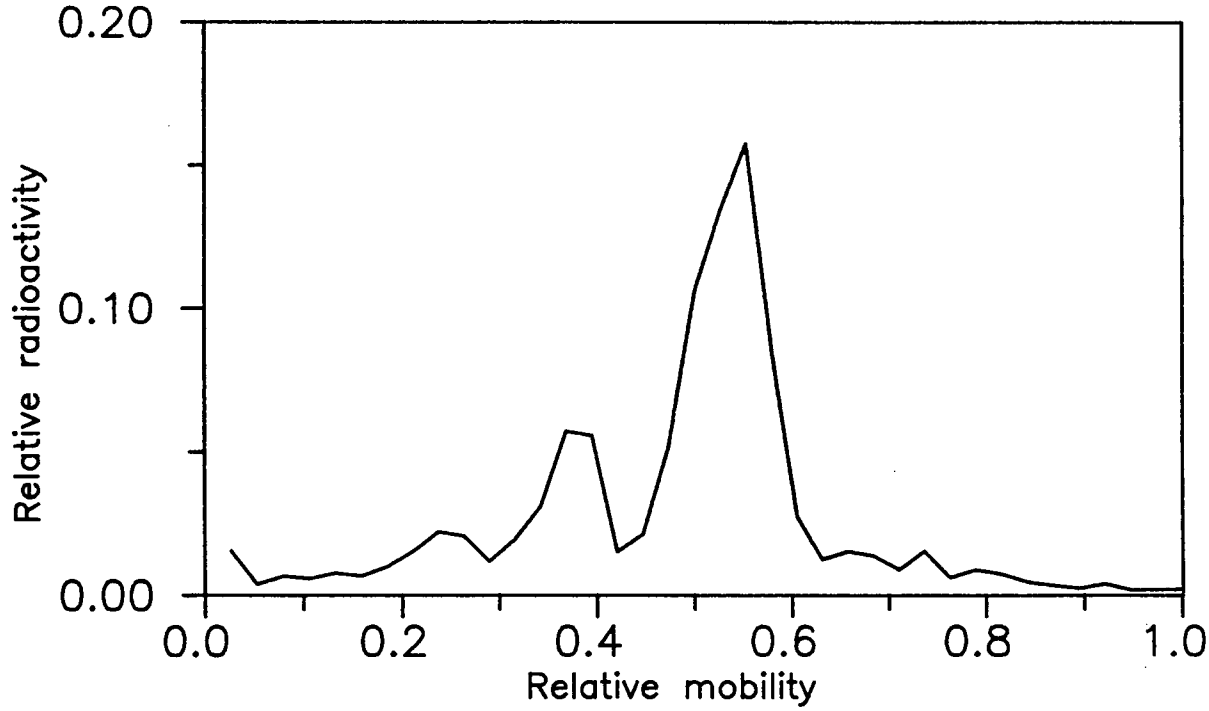

Fig. 3.7d. Relative mobility vs relative radioactivity of sample (iv).

3.4 Adsorption Experiments

The surface concentration (weight/unit area) of albumin was calculated by comparing the specific activity (cpm/μg) of BSA with the activity of a known surface area and is computed using equation 3.3

$$\Gamma = \frac{C}{SA\ \mathcal{A}} \tag{3.3}$$

where

Γ = surface concentration ($\mu g/cm^2$)

C = counts per minute from the tubing (cpm)

SA = specific activity (cpm/μg)

$\mathcal{A}$ = surface area (cm^2)

3.4.1 Surface concentration as a function of the ratio of radiolabelled to unlabelled BSA

As a preliminary to studying adsorption it was necessary to establish whether the relative amounts of labelled and unlabelled protein had any effect on the adsorption. The adsorption experiments were carried out using a series of solutions with a concentration of 0.5 mg/ml but the ratio of labelled to unlabelled protein was varied. The equilibration time was 4 hours at 23°C. The results are given in Table 3.4, (assuming the molecular weight of BSA to be 66,000). The results indicate that the surface concentration is essentially independent of the ratio of labelled to unlabelled protein. Therefore one can conclude that iodination at a tracer level of less than one ^{125}I atom per protein molecule does not affect the proteins' affinity for the polyethylene surface.

TABLE 3.4

The effect of labelled BSA content on the adsorption to polyethylene from a 0.5 mg/ml solution, (see Appendix 2 for the calculation of $\Delta\Gamma$).

Moles of unlabelled BSA per mole of ^{125}I-BSA	Surface concentration (Γ) ($\mu g/cm^2 \pm \Delta\Gamma$)
668	0.197 ± 0.019
804	0.223 ± 0.021
1352	0.215 ± 0.022
1682	0.215 ± 0.022
1721	0.196 ± 0.023
2350	0.192 ± 0.018
2470	0.202 ± 0.020
mean ± error	0.206 ± 0.050

3.4.2 Surface concentration as a function of time

It was important to determine the time for the surface concentration to reach a steady state so that a suitable time could be chosen for the adsorption study.

Using the technique from the literature of counting the tubing following washout of protein by buffer, adsorption runs were carried out using various equilibration times. The time curve for a 0.19 mg/ml BSA solution at 23°C is given in Figure 3.8. The kinetics of adsorption was in agreement with earlier reports (Brash and Davidson, 1976). It was found that the surface concentration reaches a value of approximately 0.14 $\mu g/cm^2$ within 2 hours and remains constant over a period of up to 8 hours.

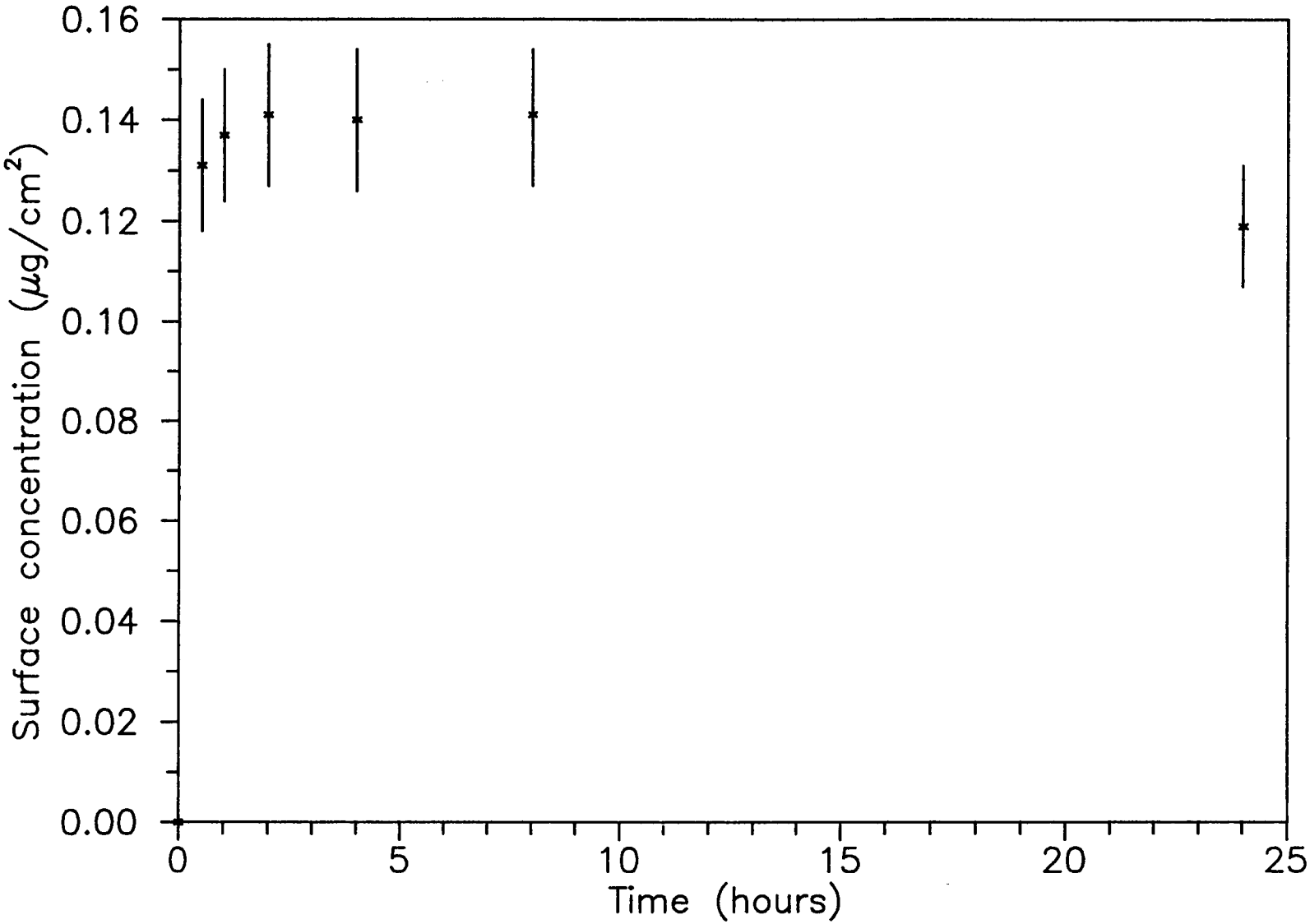

Fig. 3.9. Time dependence for the adsorption of BSA on polyethylene at a solution concentration of 0.19 mg/ml.

3.4.3 Adsorption isotherm

The BSA-polyethylene system was investigated by determining the quantity of protein adsorbed as a function of solution concentration. The surface concentration was calculated directly by cutting up the tubing and gamma counting. Since the tubing had been rinsed prior to counting, this value must represent a minimum amount adsorbed. Any loosely bound protein would have been removed during the rinsing period. The adsorption isotherm for albumin at 23°C over a concentration range of 0 to 2.7 mg/ml is shown in Figure 3.9, the upper limit being about 6% of the value in blood. The error is calculated from the various components in equation 3.3, see Appendix 2.

The surface concentration increases with concentration asymptotically until a plateau value of approximately 0.20 $\mu g/cm^2$, ($3 \times 10^{-12} M/cm^2$). The surface concentration is similar to the values reported previously (Brash and Davidson, 1976; Morrisey and Stromberg, 1974). This behaviour indicates a limited capacity of the surface for adsorption. This general behaviour has been observed with other types of macromolecules at a solid-solution interface (Silberberg, 1962) and is in general typical of all protein-plastic systems.

The surface concentration quoted is an average taken from the 5 m tube length. The plots in Figures 3.10a-h show the surface concentration against tube section for BSA adsorbed from radiolabelled BSA solutions of varying concentrations. Tube section 1 is the end of the tube at the fraction collector while tube section 25 is the end of the tubing connected to the syringe. Adsorption from a solution concentration of 8 $\mu g/ml$ is not uniform along the tube. This may be due to a concentration gradient along

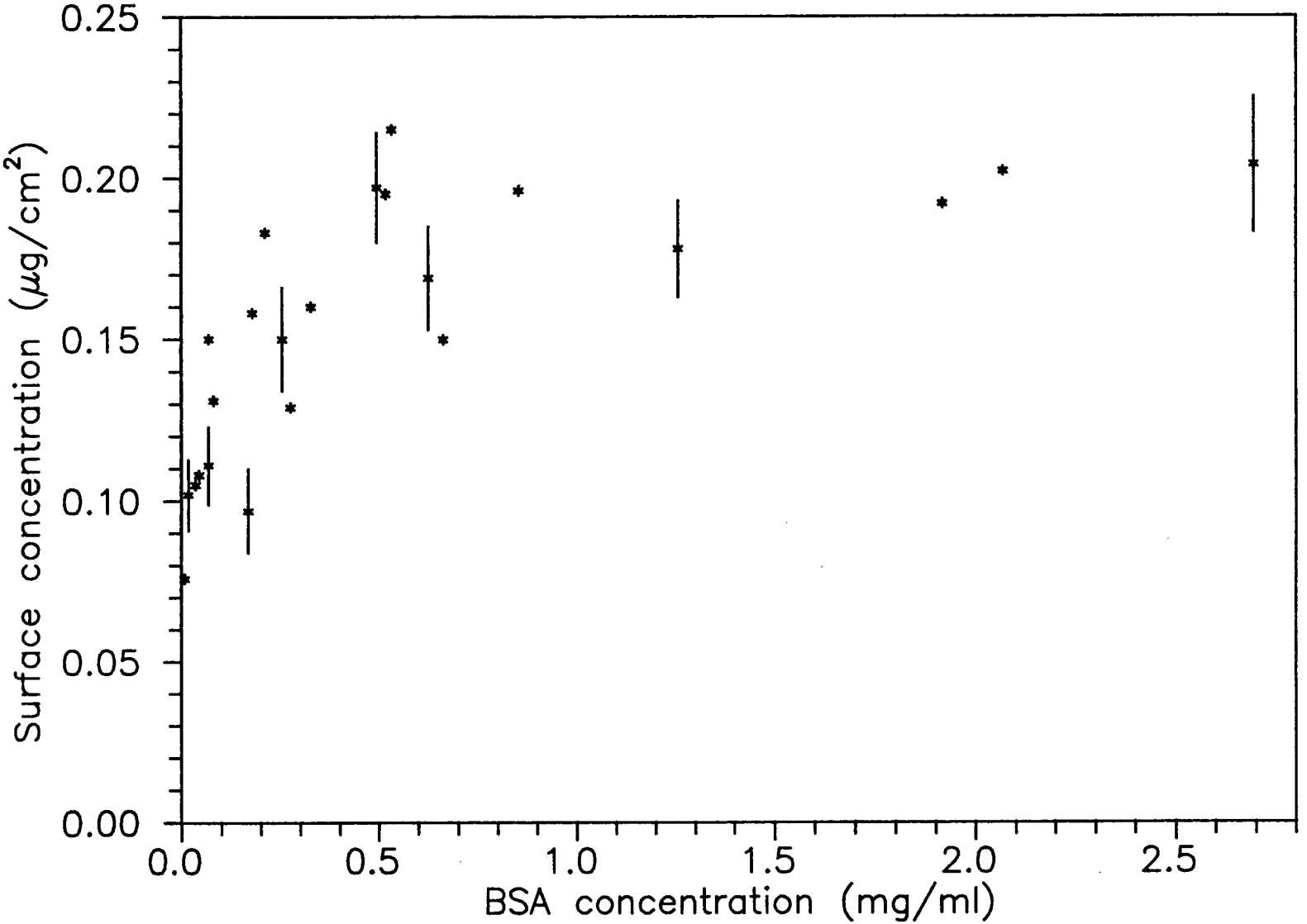

Fig. 3.9. Adsorption isotherm for albumin on polyethylene at 23°C.

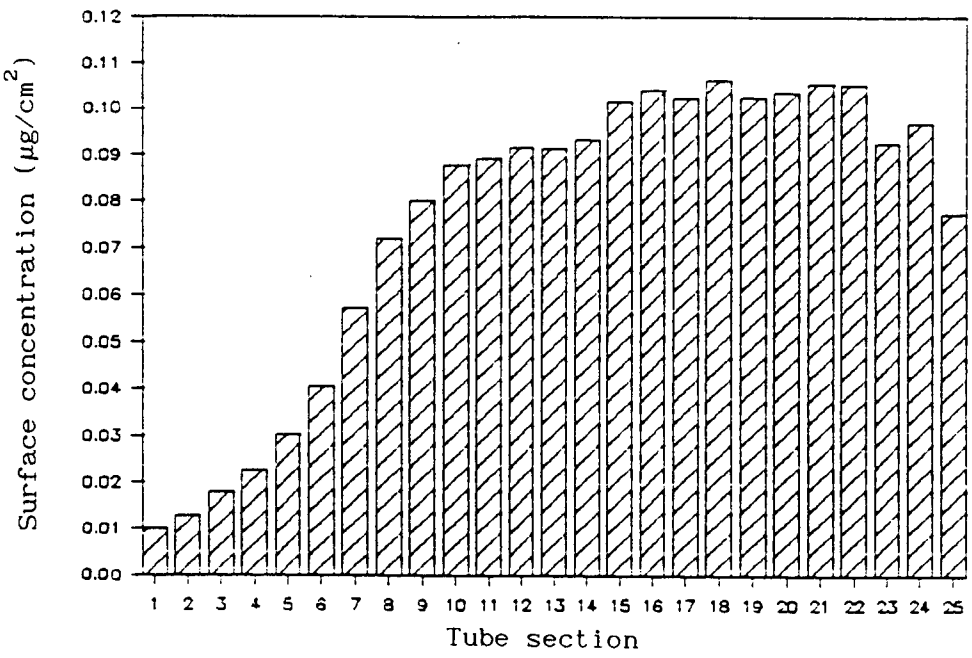

Fig. 3.10a. Surface concentration vs tube section for BSA adsorbed from a 8 $\mu g/ml$ solution. In all cases tube section 1 is the fraction collector end i.e. output end, and tube section 25 is the syringe end i.e. input end.

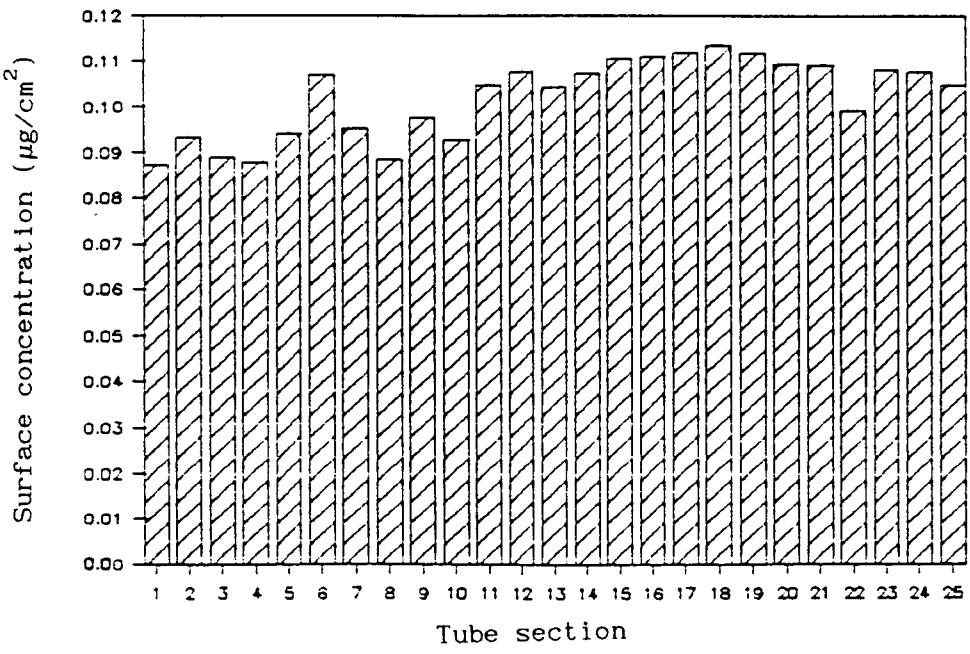

Fig. 3.10b. Surface concentration vs tube section for BSA adsorbed from a 17 $\mu g/ml$ solution.

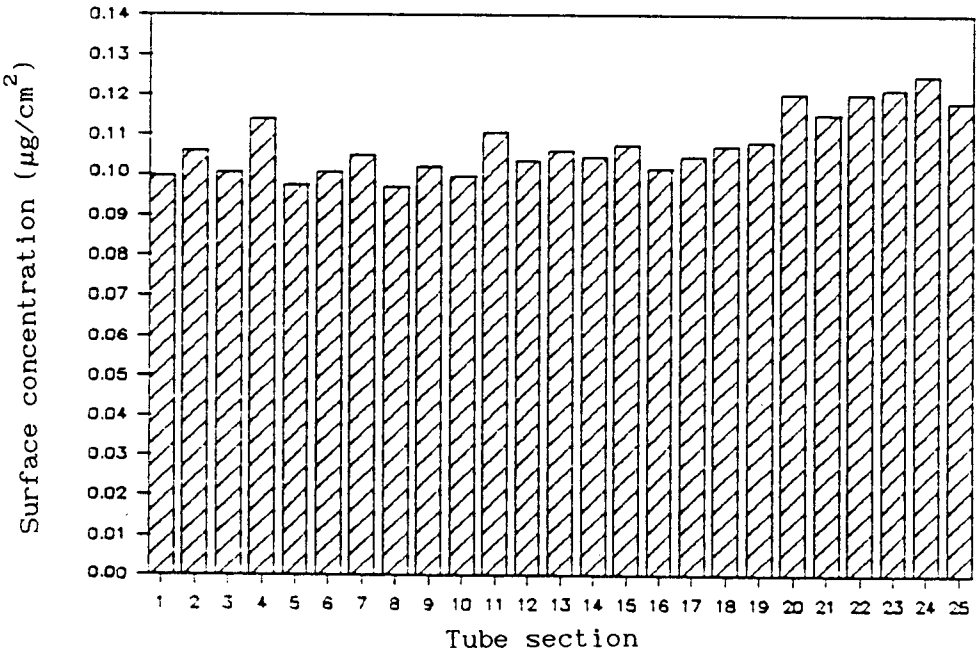

Fig. 3.10c. Surface concentration vs tube section for BSA adsorbed from a 44 μg/ml solution.

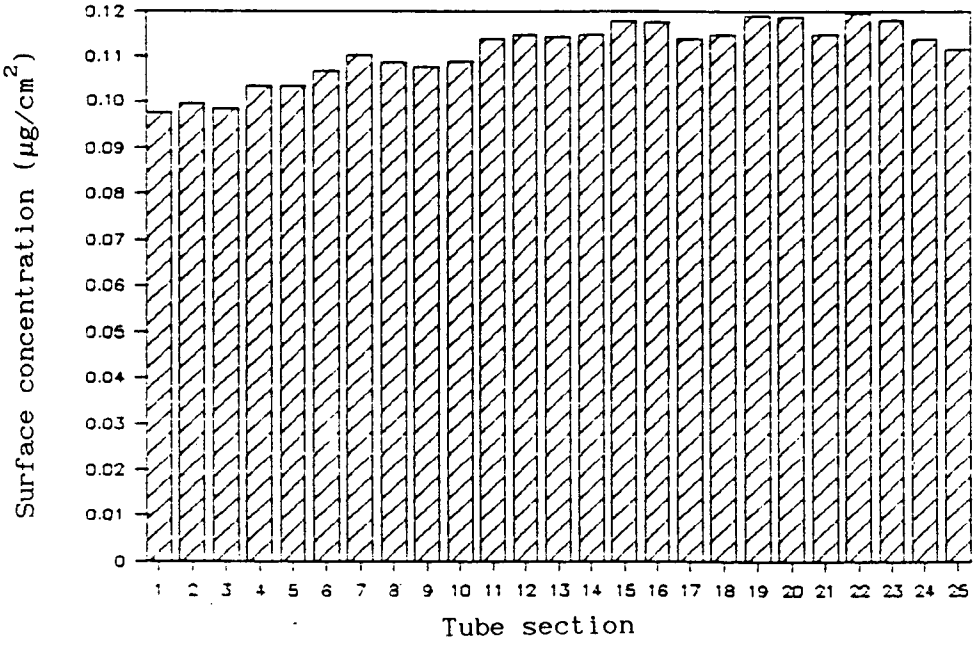

Fig. 3.10d. Surface concentration vs tube section for BSA adsorbed from a 67 μg/ml solution.

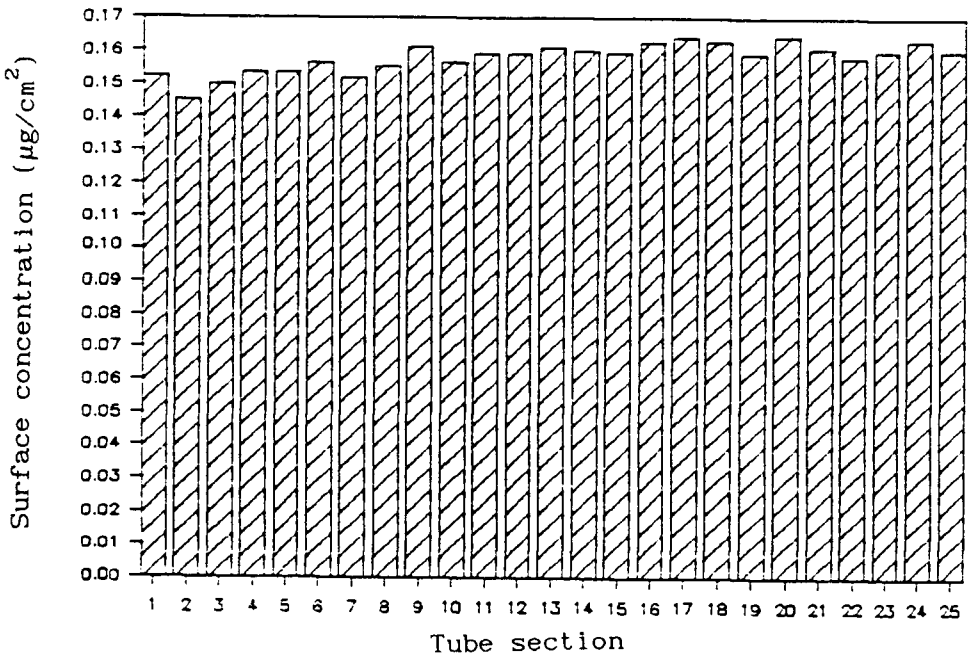

Fig. 3.10e. Surface concentration vs tube section for BSA adsorbed from a 178 μg/ml solution.

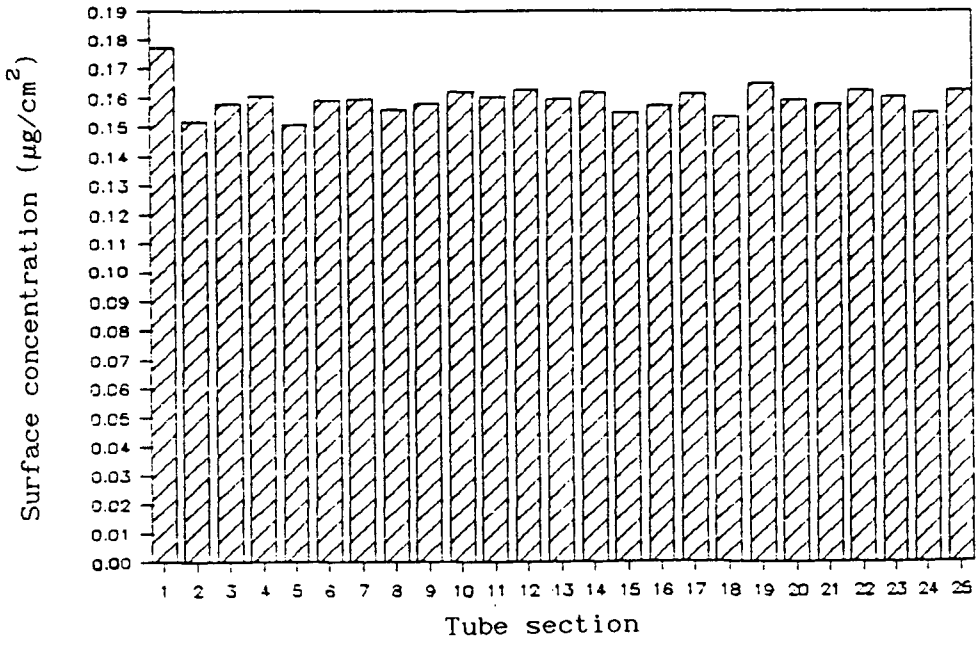

Fig. 3.10f. Surface concentration vs tube section for BSA adsorbed from a 326 μg/ml solution.

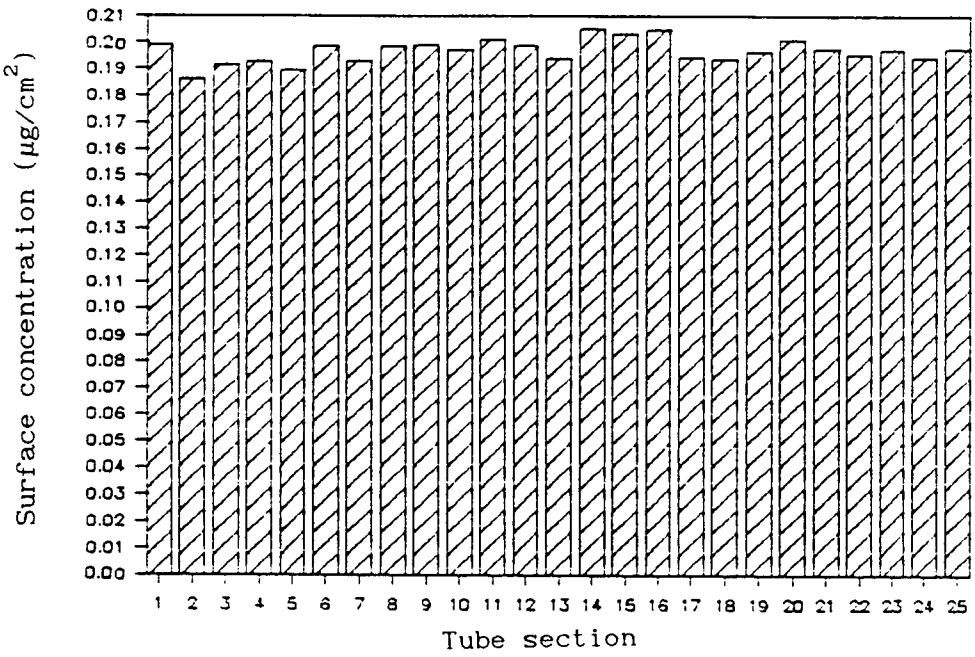

Fig. 3.10g. Surface concentration vs tube section for BSA adsorbed from a 493 μg/ml solution.

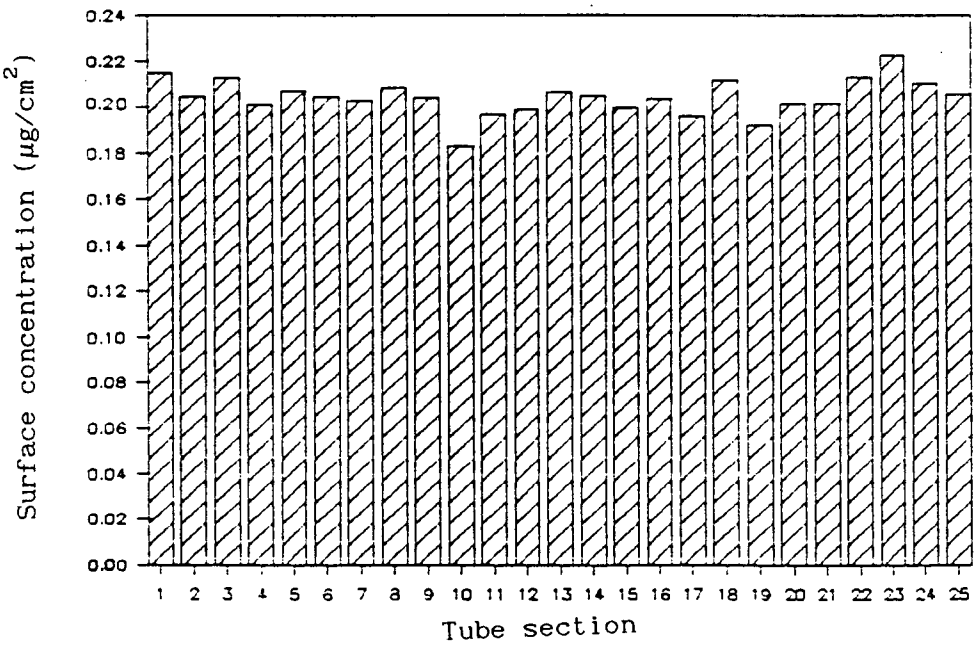

Fig. 3.10h. Surface concentration vs tube section for BSA adsorbed from a 2.694 mg/ml solution.

the tube and to BSA being adsorbed. The BSA molecules first entering the tube will be adsorbed thus decreasing the concentration of the solution as it flows through the tube. This will be significant at low BSA concentrations since virtually all of the protein is adsorbed. If most of the protein is adsorbed before the solution reaches the end of the tube the concentration at the end is reduced significantly. Adsorption from a solution of bulk concentration 8 μg/ml shows a large change in surface concentration over the length of the tube, probably due to the above effect. A uniform surface concentration is obtained when BSA is adsorbed from solutions with a concentration of 17 μg/ml or greater.

The layer thickness and the average area per BSA molecule were calculated using a surface concentration of 0.20 $\mu g/cm^2$ and assuming the protein density to be 1.3 g/cm^3 and a protein molecular weight of 66,000. The calculated values are given in Table 3.5 along with the values for an end-on and a side-on molecule using reported dimensions of the native globular protein (Squire *et al.*, 1968).

Table 3.5

Dimensions of bovine serum albumin.

Molecular weight	66,000
Overall dimensions	40 × 140 Å
Average area per molecule, side-on	5600 $Å^2$
Average area per molecule, end-on	1260 $Å^2$
Average area per molecule for BSA surface concentration of 0.2 $\mu g/cm^2$	5480 $Å^2$
Calculated layer thickness	15 Å

The calculated average area per molecule indicates that a surface concentration of 0.20 $\mu g/cm^2$ ($3 \times 10^{-12} M/cm^2$) is at the lower end of the range for a close packed monolayer configuration suggesting the molecules may be in a side-on configuration. A close packed monomolecular layer of BSA is calculated to have a surface concentration of 0.2 - 0.7 $\mu g/cm^2$. Assuming a uniform layer, the experimental layer thickness is less than the diameter of the protein molecule, perhaps indicating the adsorbed layer consists of a single layer of slightly uncoiled protein strongly bound to the surface. The molecular weight is probably more accurate than the assumption that the protein density is the same as in the crystalline form. The layer density at the surface would probably be less than that of the crystal and the layer thickness would be greater, thus, corresponding to a side-on configuration as suggested from the area. However, it is probable that the BSA does not form a complete monolayer and that gaps exist between the adsorbed molecules so little can be said about the geometry of the layer from these measurements.

3.4.4 Reversibility of the adsorbed BSA

One object of this work was to look at the reversibility of BSA adsorption, to determine if there is any loosely bound protein or desorption of BSA, i.e., the movement of protein from the surface into pure buffer during the initial rinsing of the tube. This means that the rinsing procedure for the removal of the radioactive protein solution prior to counting the tubing is critical. Hence, all the fractions coming from the tubing were collected and counted. The surface concentration was calculated two ways, first directly by cutting up the tubing after washing,

Figure 3.9. Secondly it was calculated by depletion of the total activity using the following equation

$$\Gamma = \frac{C_T - C_0}{SA\ \mathcal{A}} \qquad (3.4)$$

where Γ is the surface concentration, C_T is the total counts in the tube during equilibration, C_0 the counts output during the collection of the first fractions after equilibration, SA is the specific activity and $\mathcal{A}$ the surface area. It was possible to calculate C_0 by Taylor's analysis; see Appendix 3. The output concentration from the tube stays constant near the bulk concentration for the first fractions coming out of the tube and then decreases rapidly to nearly zero. An example of the concentration change is given in Figure 3.11.

The data in Table 3.6 shows the calculated surface concentrations and the % protein adsorbed from solution. At a solution concentration of 8 μg/ml all the protein is adsorbed from solution and there is no detectable desorption on washing the tube with PBS/azide, pH 7.4. At higher concentrations less of the protein was adsorbed and a suggestion of desorption was observed.

The adsorption isotherm for the surface concentration calculated from the tubing is given in Figure 3.9 and by depletion from the tubing and total counts in Figure 3.12. The isotherms show that the depletion values are always higher than those determined from the rinsed tubing. However, the data points are close enough, and the uncertainties large enough, to require a stastical analysis of the results to determine their probable significance.

A statistical comparison between the two sets of data for the adsorption isotherm, was carried out to determine if there was any significant difference between them. There is no unequivocal way to test the significance of the difference between two non-linear plots. Therefore, the data was linearized by applying an equation with the form of a Langmuir isotherm.

The Langmuir isotherm is given by

$$\Gamma = \frac{MW}{\mathcal{A}_S N_A} \times \left(\frac{KC_b}{1 + KC_b} \right) \qquad (3.5)$$

where

Γ = weight of protein adsorbed per unit area of surface
MW = molecular weight of the adsorbing protein
$\mathcal{A}_S$ = surface area per site
N_A = Avogadro's number
K = adsorption constant
C_b = bulk protein concentration

At the plateau surface concentration the monolayer concentration is $\Gamma_m = MW/\mathcal{A}_S N_A$. Rearranging equation 3.5 gives

$$\frac{C_b}{\Gamma} = \frac{1}{K\Gamma_m} + \frac{C_b}{\Gamma_m} \qquad (3.6)$$

Plotting C_b/Γ against C_b gives a straight line if K and Γ_m are constant. The slope is equal to $1/\Gamma_m$ and the adsorption constant is given by the slope/intercept.

A plot of C_b/Γ versus C_b for the isotherm data is given in Figure 3.13. Each set of data appears to be fit by a straight line, the least squares regression lines are shown.

Table 3.6

Comparison of the surface concentration ($\mu g/cm^2$) of BSA at pH 7.4 calculated from cutting up the rinsed tubing, Γ (tube), and by depletion of the total counts, Γ (cpm); see Appendix 2 for error analysis.

Concentration (C_b) (mg/ml)	Γ (tube) ($\mu g/cm^2$)	Γ (cpm) ($\mu g/cm^2$)	% Protein adsorbed from solution
0.008	0.076 ± 0.010	0.076 ± 0.001	100
0.017	0.102 ± 0.010	0.102 ± 0.001	63
0.035	0.105 ± 0.011	0.106 ± 0.001	32
0.044	0.108 ± 0.011	0.108 ± 0.002	26
0.067	0.111 ± 0.012	0.112 ± 0.002	17
0.068	0.150 ± 0.018	0.151 ± 0.003	23
0.080	0.131 ± 0.013	0.131 ± 0.002	17
0.167	0.097 ± 0.013	0.104 ± 0.003	6
0.178	0.158 ± 0.015	0.159 ± 0.003	9
0.210	0.183 ± 0.027	0.216 ± 0.003	10
0.253	0.150 ± 0.017	0.151 ± 0.003	6
0.274	0.129 ± 0.015	0.131 ± 0.003	5
0.326	0.160 ± 0.022	0.160 ± 0.006	5
0.493	0.197 ± 0.017	0.201 ± 0.003	4
0.516	0.195 ± 0.019	0.199 ± 0.004	4
0.531	0.215 ± 0.022	0.220 ± 0.004	4
0.623	0.169 ± 0.016	0.175 ± 0.004	3
0.761	0.173 ± 0.020	0.178 ± 0.005	2
0.851	0.196 ± 0.020	0.201 ± 0.005	2
1.255	0.178 ± 0.015	0.193 ± 0.007	1
1.916	0.192 ± 0.018	0.202 ± 0.007	1
2.067	0.202 ± 0.020	0.216 ± 0.008	1
2.694	0.204 ± 0.021	0.214 ± 0.008	1

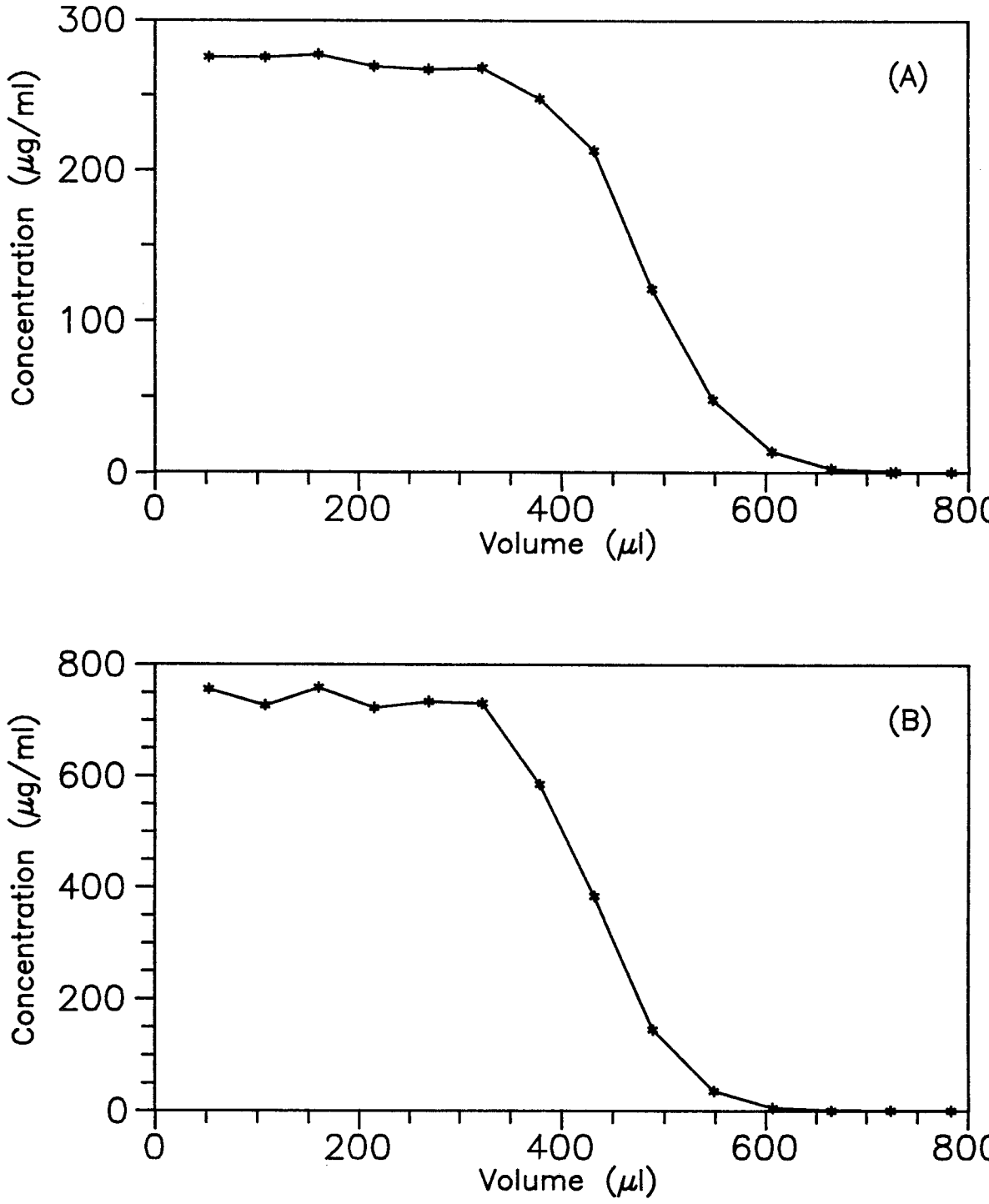

Fig. 3.11. The concentration of the BSA solution displaced from an adsorption experiment vs the volume collected. The initial bulk concentrations of the BSA solutions were (A) 274 and (B) 761 μg/ml.

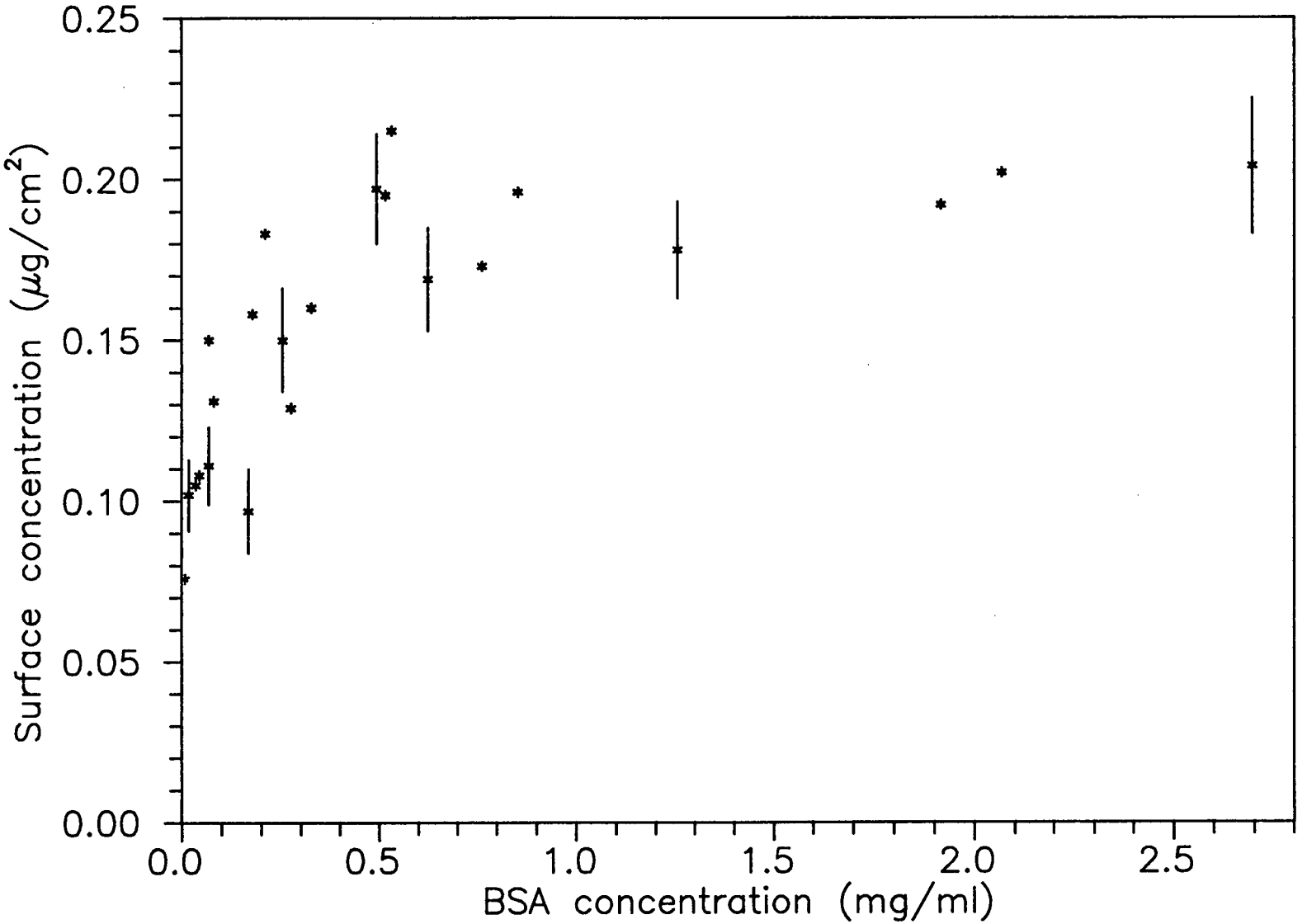

Fig. 3.10. Adsorption isotherm for albumin on polyethylene at 23°C.

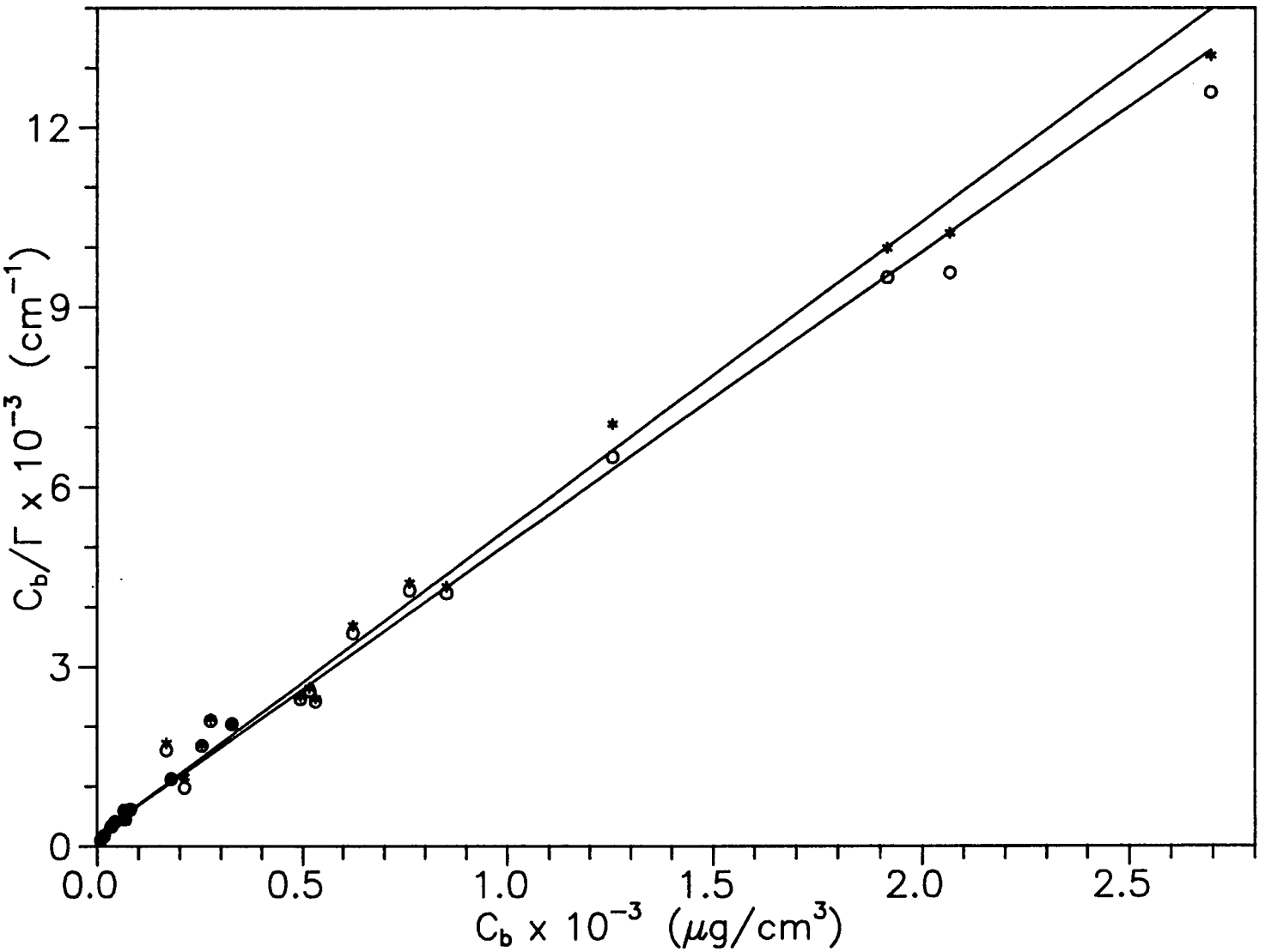

Fig. 3.13. C_b/Γ versus C_b for adsorption data calculated from the rinsed tubing (*) and by depletion of the total radioactivity (o).

Statistical analysis was carried out to determine if there is any significant difference between the regression lines; see Appendix 4. The student's t test was used. A t value is calculated which is the difference between the slopes divided by the standard error of the difference between the slopes. If the value for t is below a critical value the null hypothesis, a statement of no difference, is assumed i.e., there is no significant difference between the slopes. From the analysis it was concluded that there was a significant difference between the regression lines. This implies that a small amount of desorption occurs.

Statistical analysis was also carried out using a paired-sample t test; see Appendix 4. This test is used when two sets of results are obtained under the same conditions. The test uses the difference between the two surface concentrations calculated by the two methods to determine the mean, variance, standard deviation, the standard error and a t value. Again the analysis shows that there is a significant difference between the surface concentration calculated from the rinsed tubing and from the depletion of total radioactivity.

3.4.5 Effect of pH on BSA adsorption

The solubility and structural stability of protein macromolecules are a result of many interactions. If the electrostatic interactions are modified by a change in the pH a conformational change may be anticipated, potentially resulting in a change in the surface concentration of the adsorbed protein.

A series of adsorption runs was carried out at various pH values and a concentration of 0.5 mg/ml. The results from counting the rinsed tube

are plotted in Figure 3.14. The plot shows that as the pH increases the surface concentration increases to a maximum at around the isoelectric point of BSA, pH 4.9, and then decreases to a minimum going through pH 7.4. As the pH becomes more basic the surface concentration increases again and then decreases under very basic conditions. This behaviour differs from the adsorption of BSA on silica with pH, (Morrisey and Stromberg, 1974), where there is a maximum around the isoelectric point but then the adsorption decreases.

Polyethylene is an example of an inert hydrophobic surface in that it does not contain any reactive groups and is capable of binding proteins only by dispersion forces and hydrophobic interactions. Surface contaminants may allow other types of bonding, however. It is important to determine whether the alkaline solution had any effect on the surface. The tube was filled with PBS/azide, pH 9.0, left for 4 hours and then an adsorption experiment was conducted at pH 7.4. The surface concentration along with others at pH 7.4 are given in Table 3.7.The surface

Table 3.7

Surface concentration (Γ) of BSA at pH 7.4 from a 0.5 mg/ml solution

Γ tube ± s.d.
0.215 ± 0.022
0.223 ± 0.022
0.223 ± 0.023†

† the tube was exposed to pH 9.0 before the adsorption run

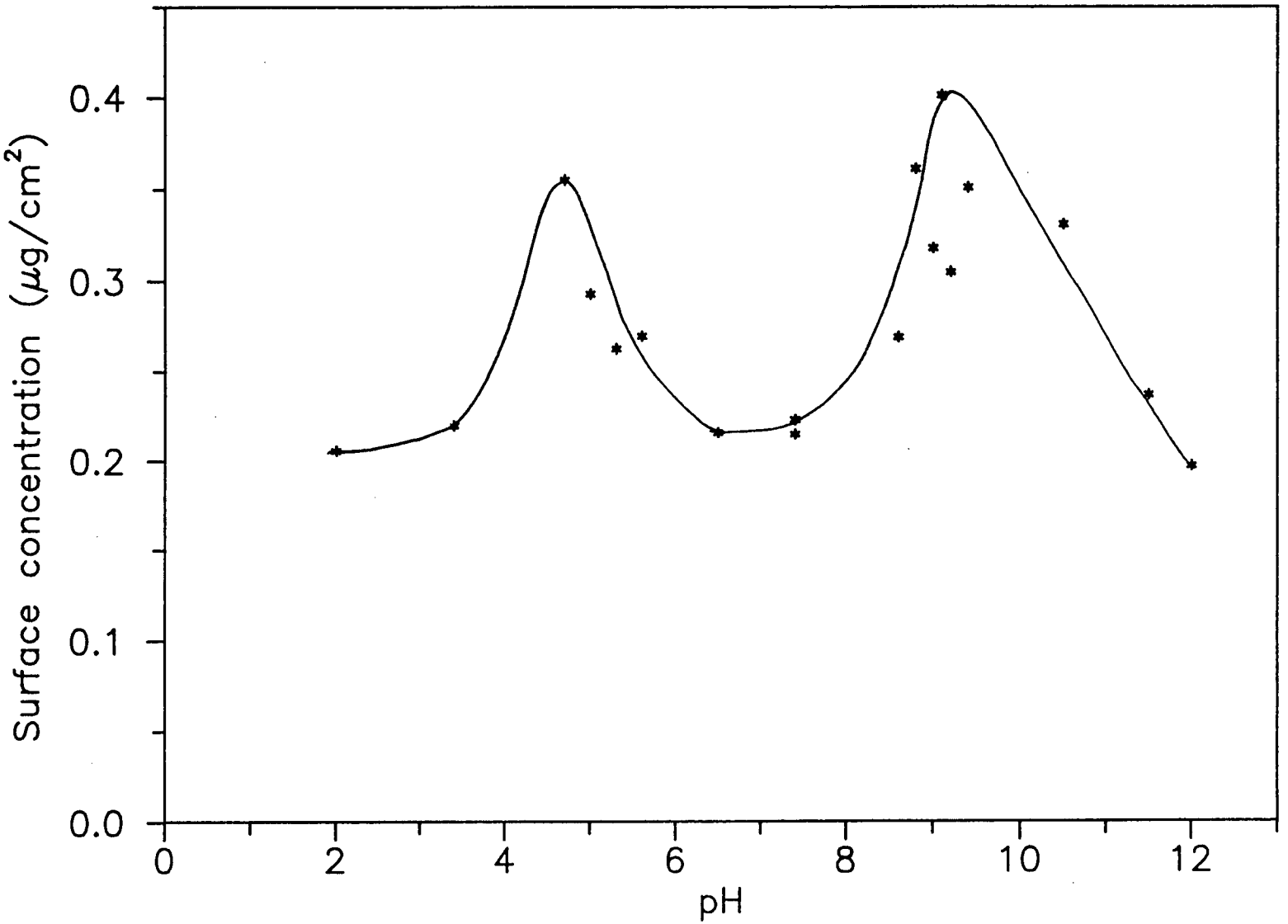

Fig. 3.14. The surface concentration of BSA adsorbed to polyethylene tubing plotted against the pH. The bulk concentration in each case was 0.5 mg/ml.

concentrations show that there is no change on exposing the tubing to pH 9.0 before an adsorption run therefore one can conclude that the pH does not irreversibly effect the solid polyethylene surface. Reversible changes could not be detected by this approach, however.

To test for desorption a concentration of 100 μg/ml was used and adsorption experiments were carried out. The displacement of the protein solution was with PBS at the same pH as the protein solution. The results are given in Table 3.8.

Table 3.8

Surface concentrtations (Γ) calculated from the rinsed tube and from depletion of total cpm. The pH was adjusted with borax[1] or NaOH[2] .

pH	Concentration (μg/ml)	Γ tube ± s.d. ($\mu g/cm^2$)	Γ cpm ± s.d. ($\mu g/cm^2$)
5.3	89	0.172 ± 0.017	0.176 ± 0.002
8.8[1]	95	0.267 ± 0.025	0.270 ± 0.003
7.4	80	0.131 ± 0.018	0.135 ± 0.002
5.0	488	0.293 ± 0.029	0.306 ± 0.005
5.3	511	0.263 ± 0.025	0.319 ± 0.004
8.6[1]	452	0.269 ± 0.031	0.301 ± 0.004
9.0[2]	517	0.318 ± 0.031	0.320 ± 0.005
7.4	516	0.195 ± 0.019	0.200 ± 0.004

At a low protein concentration the surface concentrations calculated from the rinsed tube and from the depletion of total counts are similar indicating that negligible or no desorption is detected on washing the tubing. The amount of BSA adsorbed at pH 5.3 and pH 8.8 is increased from

that at a pH of 7.4 at this low concentration as well. To determine if the NaOH had any effect on the adsorption experimental runs were carried out using borax to alter the pH. The results given in Table 3.8 show that the adsorption increases at pH 9 did not depend on the agent used to make the protein solution alkaline. Again, the equilibrium values (depletion) are slightly higher than the values following rinsing, Figure 3.15.

Statistical analysis was carried out using the paired-sample t test on the surface concentration-pH data; see Appendix 4. Again it was concluded that the surface concentrations calculated by the depletion of the total radioactivity were higher than those calculated from the rinsed tubing, suggesting protein is desorbed.

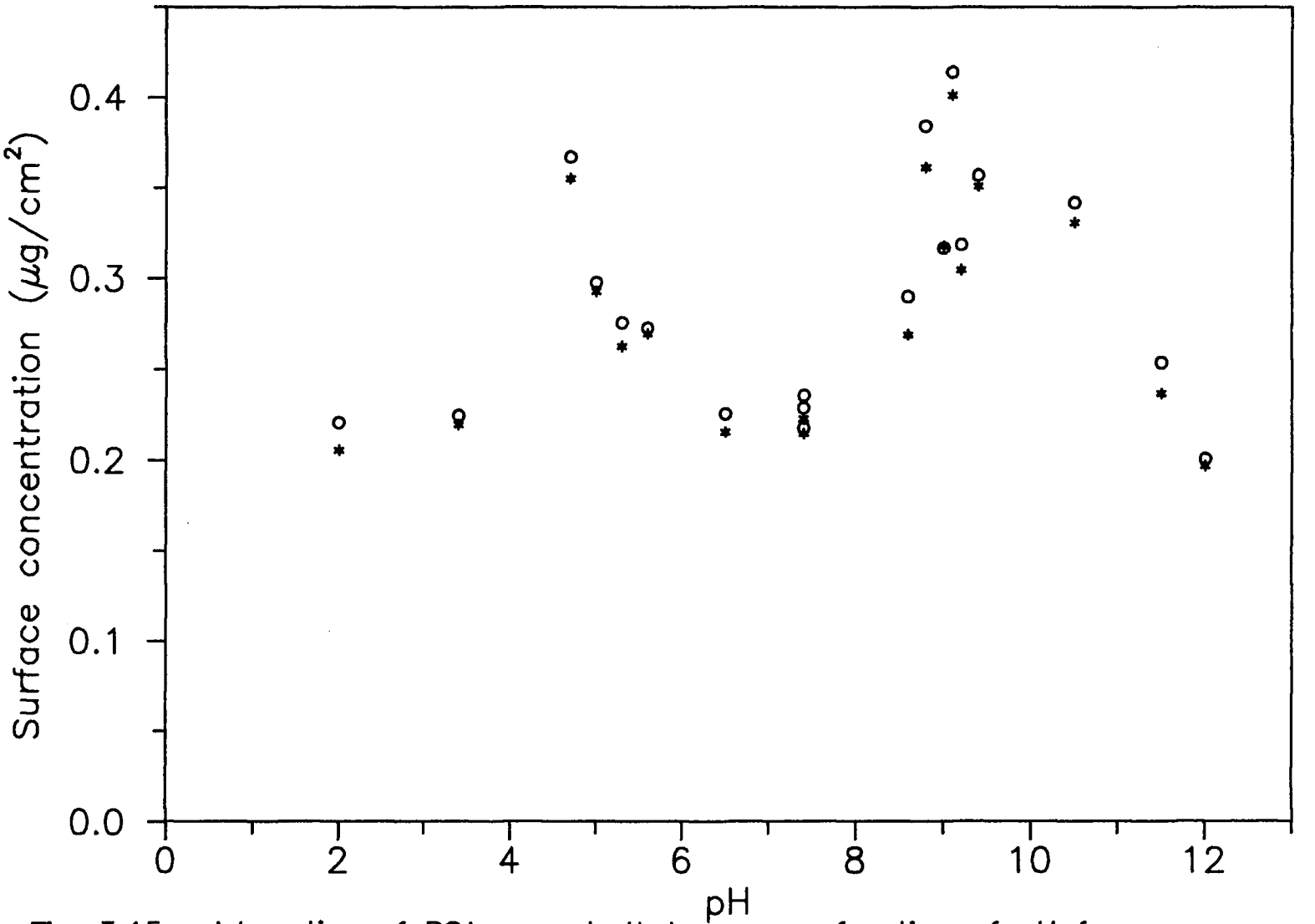

Fig. 3.15. Adsorption of BSA on polyethylene as a function of pH from a 0.5 mg/ml solution. The surface concentrations were calculated from the rinsed tubing (*) and from depletion of the total radioactivity (o).

CHAPTER 4

CONCLUSIONS

Measurements of adsorption of radiolabelled BSA on hydrophobic polyethylene tubing at 23°C showed that a steady-state surface concentration was established in 2 hours and remained constant over a period of 8 hours. The adsorption isotherm was apparently Langmuir-like even though the Langmuir assumptions are not obeyed.

The question of reversibility was investigated in this thesis. The statistical analysis showed a significant difference between the surface concentration calculated from the rinsed tubing and that from depletion of the total radioactivity. This suggests a small amount of desorption occurs on washing the tubing with buffer, estimated from Figure 3.13 to be approximately 5%. This is a lower limit to the amount desorbing, however, since the surface concentration calculated by the depletion method is probably lower than the actual value due to the loosely bound protein being released as the protein concentration decreases during displacement by buffer. Hence C_0 is likely higher than the equilibrium value, implying that the surface concentration is underestimated.

The surface concentration-pH curve shows two maxima. The maximum at pH 5 occurs at the isoelectric point of the protein, this has been observed by other workers; (Bull, 1956; Morrissey and Stromberg, 1974). This shows the importance of protein-protein electroststic interactions. At the isoelectric point the protein has a net zero charge and maximum adsorption

occurs. Away from the isoelectric point the charge on the protein increases, causing repulsion between protein molecules and the surface concentration decreases. The second maximum at pH 9.5-10 can be explained by an increase in oligomer adsorption. From the SDS-PAGE analysis it was shown that as the pH increases a greater percentage of BSA oligomers is present in solution. The increase in surface concentration can be explained by an increase in BSA dimer and higher oligomeric molecules being adsorbed. It was shown by releasing the adsorbed protein from the tube with hot SDS and running a gel that the larger molecular weight oligomers were adsorbed preferentially. Preferential adsorption of larger molecular weight species has been reported by other workers (Gilliland and Guttoff, 1960). As the pH increases the BSA molecule uncoils and an expansion similar to the one under acidic conditions occurs around pH 10.3 (Tanford *et al.*, 1955). A large increase in the net negative charge on the BSA molecules could therefore account for the decrease in surface concentration seen above pH 9.5 due to electrostatic repulsion.

Statistical analysis carried out on the surface concentration-pH data showed a significant difference between the surface concentration calculated from cutting up the tubing and that calculated by the depletion of the total radioactivity and desorption was concluded.

The reversibility of protein adsorption is important in developing thromoresistant materials. It has been shown that by precoating a surface with albumin the thrombogenic character of the material is increased, since platelet adhesion is reduced (Lyman *et al.*, 1971). If albumin adsorption is reversible, as indicated here, an initially thromboresistant albumin precoated surface would be expected to become less so with time. Hence,

the results obtained here bear relevance to the development of non-thrombogenic surfaces.

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APPENDIX 1

ABBREVIATIONS

A	activity
$\mathcal{A}$	surface area
$\mathcal{A}_s$	surface area per site
a	equilibrium solution concentration
BIS	N,N-methylene-bis-acrylamide
BSA	bovine serum albumin
C_b	bulk protein concentration
C_m	monolayer concentration
C_s	weight of protein adsorbed per unit area of surface
cpm	counts per minute
dpm	disintigrations per minute
EDTA	ethylenediamine tetraacetate
FEP	fluorinated ethylene-propylene copolymer
FTIR-ATR	fourier transform infrared spectroscopy coupled with attenuated total reflectance optics
HMWk	high molecular weight kit
HSA	human serum albumin
K	adsorption constant
LMWK	low molecular weight kit
MW	molecular weight
N_A	Avogadro's number
OD	optical density

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEUU	polyether urethane
PVC	poly(vinyl chloride)
R_f	relative mobility
SA	specific activity
SDS	sodium dodecyl sulphate
SR	silicone rubber
t	time
TCA	trichloroacetic acid
TD	tracking dye
TEMED	N,N,N',N'-tetramethylenediamine
TLC	thin layer chromatography
tris	Tris(hydroxymethyl)aminomethane
v/v	volume per volume

ΔG_{ads}	free energy of adsorption
ΔH_{ads}	enthalpy of adsorption
ΔS_{ads}	entropy of adsorption
Γ	surface concentration ($\mu g/cm^2$)
λ	decay constant (s^{-1})

APPENDIX 2

CALCULATIONS AND ERROR ANALYSIS

A2.1 Error analysis

An equation can be represented by the formula

$$F = f(x_1, x_2, \ldots . x_n) \qquad (A2.1)$$

The value of F is calculated by substituting experimentally determined values of x_i into the formula (f). An infinitesimal change in F is calculated by considering the infinitisimal change in dx_i and

$$dF = \frac{\partial F}{\partial x_1} dx_1 + \frac{\partial F}{\partial x_2} dx_2 + \ldots . + \frac{\partial F}{\partial x_n} dx_n \qquad (A2.2)$$

For finite changes small enough not to effect the partial derivative

$$\Delta F = \frac{\partial F}{\partial x_1} \Delta x_1 + \frac{\partial F}{\partial x_2} \Delta x_2 + \ldots . + \frac{\partial F}{\partial x_n} \Delta x_n \qquad (A2.3)$$

This formula provides the most conservative estimate of the uncertainty in F propogated by the uncertainties Δx_i in the independent variables. However, in reality there is a high probability that some errors in x_i will cancel each other out. To allow for this effect, square both side of equation A2.3:

$$(\Delta F)^2 = \left(\frac{\partial F}{\partial x_1}\right)^2 (\Delta x_1)^2 + \left(\frac{\partial F}{\partial x_2}\right)^2 (\Delta x_2)^2 + \ldots\ldots + 2\left(\frac{\partial F}{\partial x_1}\right)\left(\frac{\partial F}{\partial x_2}\right)\Delta x_1 \Delta x_2 + \ldots\ldots \quad (A2.4)$$

If the average is taken over all values of Δx_1 and Δx_2, each Δx_i has an average of zero and the cross terms vanish but the average of the squared terms are positive and remain. Taking the square root of each side the propogated uncertainty in F is given by (Shoemaker and Garland, 1962)

$$\Delta F = \left[\left(\frac{\partial F}{\partial x_1}\right)^2 (\Delta x_1)^2 + \left(\frac{\partial F}{\partial x_2}\right)^2 (\Delta x_2)^2 + \ldots\ldots + \left(\frac{\partial F}{\partial x_n}\right)^2 (\Delta x_n)^2 \right]^{1/2} \quad (A2.5)$$

For the sum Y = A + B the imprecision in Y calculated from equation A2.5 is given by

$$\Delta Y = \left[\left(\frac{\partial Y}{\partial A}\right)^2 \Delta A^2 + \left(\frac{\partial Y}{\partial B}\right)^2 \Delta B^2 \right]^{1/2} \quad (A2.6)$$

therefore

$$\Delta Y = \left[\Delta A^2 + \Delta B^2 \right]^{1/2} \quad (A2.7)$$

For $Y = \frac{AB}{C}$ the error in Y from equation A2.5 is given by

$$\Delta Y = \left[\left(\frac{B}{C}\right)^2 \Delta A^2 + \left(\frac{A}{C}\right)^2 \Delta B^2 + \left(\frac{AB}{C^2}\right)^2 \Delta C^2 \right]^{1/2} \quad (A2.8)$$

taking the square of each side

$$(\Delta Y)^2 = \left(\frac{B}{C}\right)^2 \Delta A^2 + \left(\frac{A}{C}\right)^2 \Delta B^2 + \left(\frac{AB}{C^2}\right)^2 \Delta C^2 \quad (A2.9)$$

multiplying each side by $\left(\frac{C}{AB}\right)^2$

$$\left(\frac{C}{AB}\right)^2 \Delta Y^2 = \left(\frac{\Delta A}{A}\right)^2 + \left(\frac{\Delta B}{B}\right)^2 + \left(\frac{\Delta C}{C}\right)^2 \qquad (A2.10)$$

Taking the square root and multiplying by AB/C, (Y), gives the imprecision in Y as

$$\Delta Y = \left[\left(\frac{\Delta A}{A}\right)^2 + \left(\frac{\Delta B}{B}\right)^2 + \left(\frac{\Delta C}{C}\right)^2 \right]^{1/2} Y \qquad (A2.11)$$

The following error analysis will use the equations in the form of A2.7 and A2.11.

A2.2 Imprecision in the activity

The net activity, (A_N), (observed counts per unit time), of a radioactive sample is the difference between the total activity, (A), and the background activity, (B).

$$A_N = A - B \qquad (A2.12)$$

When determining the imprecision in the counts generally more than one activity is added together. For more than one sample the net activity would be

$$A_N = \sum_i \left(A_i - B \right) = \sum A_i - iB \qquad (A2.13)$$

where A_i is the activity of the ith sample and i is the number of samples counted. The imprecision can be espressed as

$$\Delta A_N = \left[\sum_i \left(\Delta A_i \right)^2 + i \left(\Delta B \right)^2 \right]^{1/2} \qquad \text{(A2.14)}$$

since the activity is the observed counts, (C), per unit time, (t),

$$A_i = \frac{C_i}{t_i} \qquad \text{and} \qquad B = \frac{C_B}{t_B}$$

the imprecision can now be written as

$$\Delta A_N = \left[\sum_i \left\{ \Delta \left(\frac{C_i}{t_i} \right) \right\}^2 + i \Delta \left(\frac{C_B}{t_B} \right)^2 \right]^{1/2} \qquad \text{(A2.15)}$$

and

$$\Delta A_i = \Delta \left(\frac{C_i}{t_i} \right) = \left[\left(\frac{\Delta C_i}{C_i} \right)^2 + \left(\frac{\Delta t_i}{t_i} \right)^2 \right]^{1/2} A_i \qquad \text{(A2.16)}$$

$$\Delta B_i = \Delta \left(\frac{C_B}{t_B} \right) = \left[\left(\frac{\Delta C_B}{C_B} \right)^2 + \left(\frac{\Delta t_B}{t_B} \right)^2 \right]^{1/2} B \qquad \text{(A2.17)}$$

from Poisson statistics $\Delta X = \sqrt{X}$

substituting equations A2.16 and A2.17 into equation A2.15 and assuming $t_1 = t_2 = t_i = t_0$

$$\Delta A_N = \left[\sum_i \left\{ \frac{1}{C_i} + \left(\frac{\Delta t_0}{t_0} \right)^2 \right\} A_i^2 + iB^2 \left\{ \frac{1}{C_B} + \left(\frac{\Delta t_0}{t_0} \right)^2 \right\} \right]^{1/2} \qquad \text{(A2.18)}$$

since $\left(\frac{\Delta t_0}{t_0} \right)$ is very small the imprecision can be written as

$$\Delta A_N = \left[\sum_i \left[\frac{A_i^2}{C_i} \right] + \frac{iB^2}{C_B} \right]^{1/2} \qquad (A2.19)$$

when, t = 1 minute, $B = C_B$ and $A_i = C_i$

$$\Delta A_N = \left[\sum_i A_i + iB \right]^{1/2} \qquad (A2.20)$$

A2.3 SDS-PAGE GELS, % monomer or polymer ± error

The BSA monomer and polymers appear as separate bands on a SDS-PAGE gel. The amount of BSA in each band is calculated by taking the sum of the activity in a particular band (ΣA_i) and dividing by the total activity (A_T). For example, the % monomer in a sample is given by

$$\%\ \text{monomer}\ (\%M) = \frac{\sum A_i}{A_T} \times 100 \qquad (A2.21)$$

and the error is calculated from

$$\Delta\%M = \left[\left(\frac{\Delta \sum A_i}{A_T} \right)^2 + \left(\frac{\Delta A_T}{A_T} \right)^2 \right]^{1/2} \%M \qquad (A2.22)$$

using the imprecision in the counts from equation A2.20 to calculate $\Delta\Sigma A_i$ and ΔA_T.

A2.4 Surface concentration from cutting up the tubing

1. The tubing was cut into twenty-five 20 cm sections and gamma counted.

2. The background count was subtracted from each section.

3. The surface concentration was calculated for each section using the following equation

$$\Gamma = \frac{C}{SA\ \mathcal{A}} \qquad (A2.23)$$

where

Γ = surface concentration
C = tube section cpm
$\mathcal{A}$ = surface area
SA = specific activity

4. The error was calculated using

$$\Delta\Gamma = \left[\left(\frac{\Delta C}{C}\right)^2 + \left(\frac{\Delta SA}{SA}\right)^2 + \left(\frac{\Delta \mathcal{A}}{\mathcal{A}}\right)^2 \right]^{1/2} \Gamma \qquad (A2.24)$$

where ΔC was calculated from equation 2.23
and ΔSA was obtained from the deviation in determining the specific activity.

5. The value plotted is an average of the twenty-five sections.

A2.5 Surface concentration calculated from the total counts

The surface concentration was calculated using the equation

$$\Gamma = \frac{C_T - C_0}{SA\ \mathcal{A}} \qquad (A2.25)$$

where

Γ = surface concentration

$\mathcal{A}$ = surface area

C_T = tube cpm

C_0 = output cpm from the displaced radiolabelled BSA

and

$$C_T = C - O \qquad (A2.26)$$

where

C = total counts

O = output counts during the input of labelled protein.

The error in calculating the surface concentration is given by:

$$\Delta\Gamma = \left[\left(\frac{\Delta\left(C_T - C_0 \right)}{C_T - C_0} \right)^2 + \left(\frac{\Delta SA}{SA} \right)^2 + \left(\frac{\Delta\mathcal{A}}{\mathcal{A}} \right)^2 \right]^{1/2} \Gamma \qquad (A2.27)$$

since

$$\Delta\left(C_T - C_0 \right) = \left(\Delta C_T^{\,2} + \Delta C_0^{\,2} \right)^{1/2} \qquad (A2.28)$$

the imprecision can now be written as

$$\Delta\Gamma = \left[\frac{\Delta C_T^{\,2} + \Delta C_0^{\,2}}{\left(C_T - C_0 \right)^2} + \left(\frac{\Delta SA}{SA} \right)^2 + \left(\frac{\Delta\mathcal{A}}{\mathcal{A}} \right)^2 \right]^{1/2} \Gamma \qquad (A2.29)$$

APPENDIX 3

MISCIBLE DISPLACEMENT IN A CAPILLARY

Taylor's analysis was used to determine the length of the zone of mixing i.e., the displacement front for miscible displacement in a tube (Taylor, 1953).

When a single liquid flows through a cylindrical tube, assuming laminar flow, the velocity distribution is parabolic. The maximum velocity at the axis of the tube is twice the average velocity. When a solution is displacing another of the same viscosity and density the centre of the invading solution flows much faster than the solution near the edge of the tube. In the absence of radial diffusion this results in an ever-lengthening needle of the invading solution down the tube. The tip of this needle will reach the end of the tube when half of the solution in the tube has been displaced. This is the breakthrough point and always occurs when half a tube volume has been injected.

When displacement occurs the invading solution sets up a large radial concentration gradient. The two solutions will interdiffuse radially thus blunting the needle-like profile of the invading solution. If the invading solution is spread over a length of tube L, the time required for convection to make an appreciable change in the concentration is of the order L/u_0, where u_0 is the maximum velocity. If the time for molecular diffusion to minimize the radial concentration gradient is much shorter than the time for an appreciable gradient to be established by the velocity

distribution no needle will occur and

$$\frac{L}{u_0} \gg \frac{a^2}{3.8^2 D} \qquad (A3.1)$$

where a is the tube radius and D is the relevant diffusion coefficient. The length of the front refers to the distance over which the concentration ranges from 0 to 100% of the invading solution. The position of the front from the entrance of the tube is a function of the tube diameter, the flow rate and the diffusion coefficient.

Substituting into equation A3.1 the relevant values from the adsorption experiments; L = 500 cm, a = 0.038 cm, D = 5.9×10^{-7} cm^2/s (Wagner and Scheraga, 1956) and u_0 = 0.933 cm/s

$$\frac{500}{0.933} > \frac{0.038}{3.8^2 \; 5.9\times10^{-7}}$$

$$536 > 169$$

and radial diffusion predominates.

Taylor has obtained an approximate solution to the problem where longitudinal molecular diffusion has been neglected and where the radial diffusion is rapid. The longitudinal transfer is due to convection. This will be the case when a dissolved material of uniform concentration C_0 is allowed to enter a pipe at a uniform rate. At time t = 0 the position of the invading material is given by x = 0, where x represents the distance from the entrance of the tube. The pipe is filled with solvent only, concentration C = 0. The solution to this problem is given by

$$C/C_0 = \frac{1}{2} + \frac{1}{2}\,\mathrm{erf}\left[\frac{x_1}{2\sqrt{kt}}\right] \qquad (x_1<0) \qquad \text{(A3.2)}$$

$$C/C_0 = \frac{1}{2} - \frac{1}{2}\,\mathrm{erf}\left[\frac{x_1}{2\sqrt{kt}}\right] \qquad (x_1>0) \qquad \text{(A3.3)}$$

where

$$\mathrm{erfz} = \frac{2}{\sqrt{\pi}}\int_0^z e^{-z^2}\,dz \qquad \text{(A3.4)}$$

$$x_1 = x - \frac{1}{2}u_0 t \qquad \text{(A3.5)}$$

and

$$k = \frac{a^2 u_0^2}{192D} \qquad \text{(A3.6)}$$

In this limit the concentration is constant across any cross section due to rapid radial diffusion.

Using Taylor's approximation it was possible to calculate the length of the mixing zone for the displacement of a protein solution by a buffer in a polyethylene tube. The relevant constants used in the calculation were listed on page 91.

The theoretical distribution of the concentration was calculated for the displacement of BSA in a polyethylene tube. The plot of C/C_0 against distance is shown in Figure A3.1. The plot shows that at $C/C_0 = 0.5$, $x = 500$ cm, i.e., the tube has been filled with one tube volume of the displacing buffer. When x is 700 cm, $C/C_0 = 0.004$ and 99.6% of the BSA has been displaced by the buffer. This length of 700 cm can be used to calculate the amount of protein displaced and finally the amount of protein adsorbed.

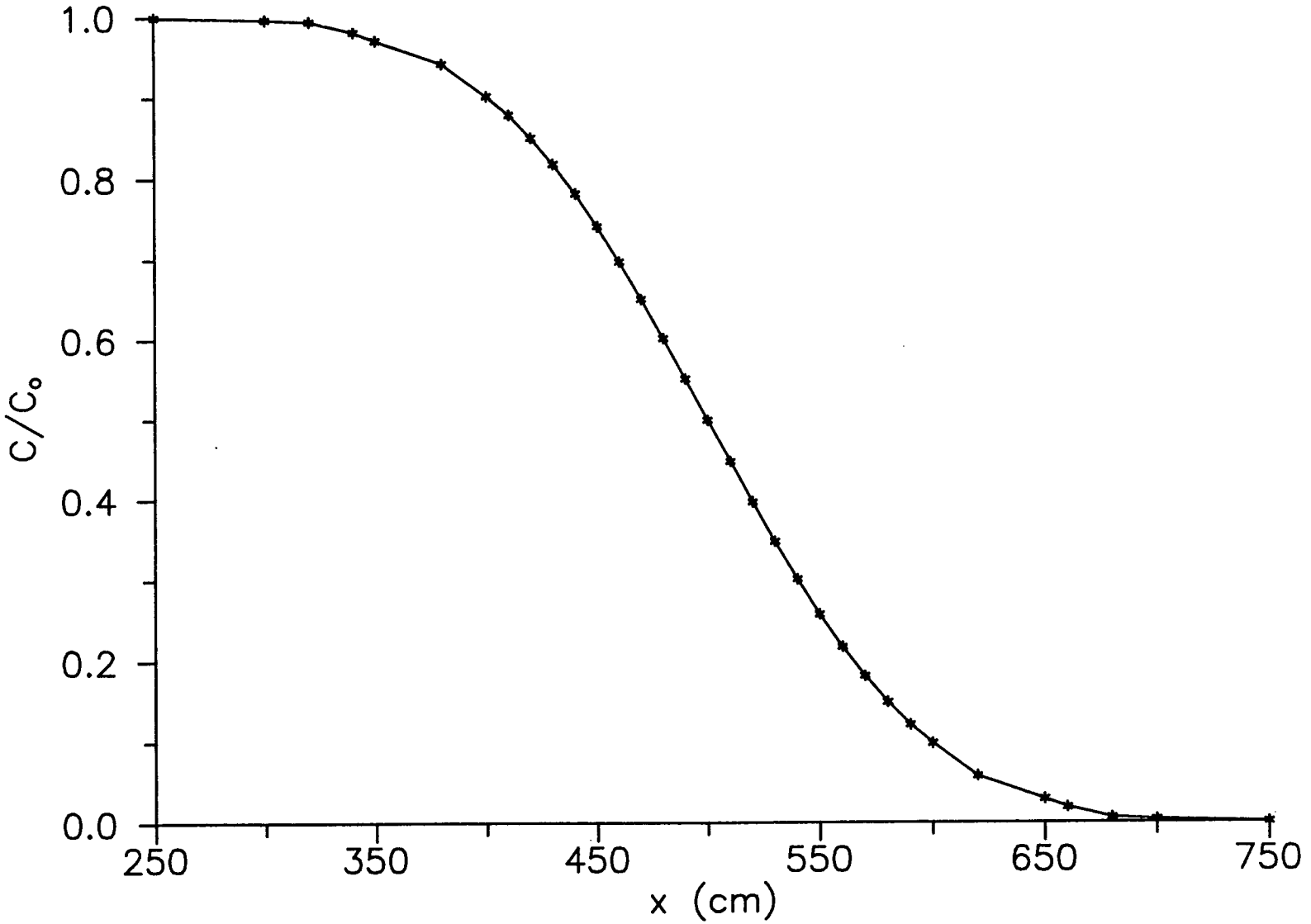

Fig. A3.1. C/C_o vs x for the miscible displacement of BSA with buffer in a capillary tube.

The volume contained in a tube of diameter 0.038 cm and length 700 cm i.e., volume used to displace 99.6% of the protein, is 794 μl. From the displacement of the protein in an adsorption experiment the number of counts, hence the amount of BSA, in 794 μl is determined. The surface concentration is calculated from the following equation

$$\Gamma = \frac{C_T - C_0}{SA\ \mathcal{A}} \qquad (A3.7)$$

where Γ = surface concentration

C_T = total counts in tube during the adsorption experiment

C_0 = cpm output in a volume of 794 μl

SA = specific activity

$\mathcal{A}$ = total surface area

The surface concentration calculated in this way will represent the amount adsorbed in equilibrium with the bathing solution. If anything it will underestimate the true equilibrium value since some rapodly desorbing material may appear in the displaced desorbing solution, thus increasing C_0 and reducing $(C_T - C_0)$.

APPENDIX 4

STATISTICAL ANALYSIS

A plot of C_b (bulk concentration) against C_b/Γ (bulk concentration/surface concentration per unit area,) appears to be best fit by two linear regions of different slopes. A linear regression equation was calculated for each set of data. The question to ask is, are the slopes of these lines significantly different or are they estimating the same population?

The null hypothesis H_0: $l_1 = l_2$ will be tested. The null hypothesis is a statement of no difference. In this case we are testing the equality of two linear regression lines (Zar, 1984).

For a simple linear regression

$$Y = a + bX \qquad (A4.1)$$

using the method of least squares the slope or regression coefficient is given by

$$b = \frac{\Sigma xy}{\Sigma x^2} \qquad (A4.2)$$

and

$$a = \bar{Y} - b\bar{X} \qquad (A4.3)$$

where the crossproducts sum of the deviations from the mean is given as

$$\Sigma xy = \Sigma(X - \bar{X})(Y - \bar{Y}) = \Sigma XY - \frac{\Sigma X\ \Sigma Y}{n} \qquad (A4.4)$$

$\bar{X}$ and $\bar{Y}$ are the mean values of X and Y respectively, and n is the number of samples.

x is the deviation of a X value from the mean of all X's and the sum of squares is given as

$$\Sigma x^2 = \Sigma(X - \bar{X}) = \Sigma X^2 - \frac{(\Sigma X)^2}{n} \qquad (A4.5)$$

The student's t test was used to test the equality of two regression lines. The test statistic is

$$t = \frac{\text{mean difference}}{\text{standard error of mean difference}} = \frac{\bar{b}_1 - \bar{b}_2}{s_{\bar{b}_1 - \bar{b}_2}} \qquad (A4.6)$$

If the test statistic, t, is greater than some critical value the hypothesis H_0 is rejected and the alternate hypothesis, H_A, $l_1 \neq l_2$ accepted. The critical value of t depends on the degrees of freedom (ν) and the level of significance (α). For a two-tailed t test H_0 will be rejected

$$\text{if} \quad |t| \geq t_{\alpha(2),\nu} \qquad (A4.7)$$

The standard error of the difference between the regression coefficients of sample 1 and sample 2 is

$$s_{\bar{b}_1-\bar{b}_2} = \sqrt{\frac{\left(s^2_{Y.X}\right)_p}{(\Sigma x^2)_1} + \frac{\left(s^2_{Y.X}\right)_p}{(\Sigma x^2)_2}} \qquad (A4.8)$$

$\left(s^2_{Y.X}\right)_p$ is the pooled residual mean square and denotes the varience of the Y coordinate after taking into account the dependence of Y on the X coordinate.

The pooled residual mean square is given by

$$\left(s^2_{Y.X}\right)_p = \frac{(\text{residual SS})_1 + (\text{residual SS})_2}{(\text{residual DF})_1 + (\text{residual DF})_2} \qquad (A4.9)$$

where SS ≡ sum of squares
DF ≡ degree of freedom

$$\text{residual SS} = \Sigma y^2 - \frac{(\Sigma xy)^2}{\Sigma x^2} \qquad (A4.10)$$

$$\text{residual DF} = n - 2 \qquad (A4.11)$$

Σy^2 is the sum of squares of the differnce between Y and the mean $\bar{Y}$, and is given as

$$\Sigma y^2 = \Sigma(Y - \bar{Y}) = \Sigma Y^2 - \frac{(\Sigma Y)^2}{n} \qquad (A4.12)$$

In the comparison of the linear regression lines sample 1 refers to the line obtained from cutting up the tubing and sample 2 is that calculated from the depletion of total counts.

A4.1 Testing the difference between the two regression lines

H_0: $l_1 = l_2$

H_A: $l_1 \neq l_2$

For sample 1:

$\Sigma X = 13433.03$

$\Sigma Y = 73031.48$

For sample 2:

$\Sigma X = 13433.03$

$\Sigma Y = 69946.35$

The X and Y values were first divided by 1000.

$\Sigma x^2 = 19.62382$

$\Sigma y^2 = 517.7767$

$\Sigma xy = 100.4844$

$n = 23$

$a = 184.661$

$b = \text{slope} = 5.1205$

residual SS = 3.2426

residual DF = 21

$\Sigma x^2 = 19.62382$

$\Sigma y^2 = 467.0201$

$\Sigma xy = 95.40137$

$n = 23$

$a = 197.134$

$b = \text{slope} = 4.8614$

residual SS = 3.2255

residual DF = 21

$\left(s^2_{Y.X}\right)_p = 0.1540$

$s_{\bar{b}_1 - \bar{b}_2} = 0.1253$

$t = 2.0675$

Reject H_0 if $|t| \geq t_{\alpha(2),\nu}$

The critical value of t for $\nu = 42$ and a significance level $\alpha = 0.05$ (i.e., 5%) is taken from tables and

$$t_{0.05(2),42} = 2.018$$

H_0 is rejected, therefore one can conclude that there is a significant difference between the two regression lines.

If there is a correlation between sample 1 and sample 2 as in the case when the surface concentration is calculated by cutting up the tubing and by depletion of the total radioactivity, a paired-sample t test can be used. This two-tailed t test calculates a t value by using the difference, d_i, between the two samples. The mean, variance, standard deviation and standard error are calculated using the difference betwwen the samples and n is the number of differences.

A4.2 The paired-sample test for the adsorption isotherm data

H_0: $\mu_1 = \mu_2$

H_A: $\mu_1 \neq \mu_2$

In this case the alternate hypothesis is given by H_A $\mu_1 > \mu_2$ when sample 1 refers to the surface concentration (Γ) calculated from the depletion of the total radioactivity and sample 2 to that from the rinsed tubing.

From the resullts in Table 3.6

$\Sigma d = 0.125$

$\Sigma d^2 = 1.911 \times 10^{-3}$

$n = 23$

$\bar{d} = \Sigma d/n = 5.343 \times 10^{-3}$

$\nu = 23 - 1 = 22$

$SS = \Sigma d^2 - \frac{(\Sigma d)^2}{n} = 1.232 \times 10^{-3}$

variance $= s_d^2 = \frac{SS}{\nu} = 5.60 \times 10^{-5}$

standard deviation $= s_d = \sqrt{s_d^2} = 7.482 \times 10^{-3}$

standard error $= s_{\bar{d}} = \frac{s}{\sqrt{n}} = 1.560 \times 10^{-3}$

$$t = \frac{\bar{d}}{s_{\bar{d}}} = 3.483$$

A one tail distribution is used to determine the critical t value since we are testing the difference in one direction i.e., the surface concentration calculated from the depletion of total radioactivity is always higher than that calculated by cutting up the tubing.

$$t_{0.05(1),22} = 1.717$$

Therefore, reject H_0. The two sets of Γ are significantly different.

A4.3 The paired-sample test for the pH data.

H_0: $\mu_1 = \mu_2$

H_A: $\mu_1 \neq \mu_2$

	Sample 1	Sample 2		
pH	Γ (cpm)	Γ (tube)	d	
2.0	0.221	0.206	0.015	$\Sigma d = 0.195$
3.4	0.225	0.220	0.005	
4.7	0.367	0.355	0.012	$\Sigma d^2 = 2.709 \times 10^{-3}$
5.0	0.298	0.293	0.005	
5.3	0.276	0.263	0.013	$n = 19$
5.6	0.273	0.270	0.003	
6.5	0.226	0.216	0.010	$\bar{d} = 1.026 \times 10^{-2}$
7.4	0.236	0.223	0.013	
7.4	0.218	0.215	0.003	$\nu = 19 - 1 = 18$
7.4	0.229	0.223	0.006	
8.6	0.290	0.269	0.021	$SS = 7.08 \times 10^{-4}$
8.8	0.384	0.361	0.023	
9.0	0.319	0.318	0.001	$s^2 = 3.93 \times 10^{-5}$
9.1	0.414	0.401	0.013	
9.2	0.319	0.305	0.014	$s = 6.270 \times 10^{-3}$
9.4	0.357	0.351	0.006	
10.5	0.342	0.331	0.011	$s_{\bar{d}} = 1.438 \times 10^{-3}$
11.5	0.254	0.237	0.017	
12.0	0.201	0.197	0.004	$t = 7.135$

$$t_{0.05(1),18} = 1.734$$

Therefore, reject H_0.

The two sets of Γ are significantly different.