

ALBUMIN - POLYETHYLENE SURFACE  
INTERACTIONS

by  
Qussay Samak.

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QUSSAY SAMAK

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ALBUMIN - POLYETHYLENE SURFACE

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AUTHOR : Qussay Samak.  
Cairo University.

SUPERVISOR : Dr. J.L. Brash.

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ABSTRACT

This thesis reports on experimental investigation of the surface reactions between human albumin and polyethylene (medical grade). Radioactive tracer technique was used in investigating these reactions. Albumin adsorption and turnover kinetics to polyethylene were studied under both static and flow conditions. Shear rate was not found to affect the kinetics and steady state surface concentration levels of protein adsorption within the range of shear studied ( $0 - 2500 \text{ Sec.}^{-1}$ ). Turnover showed dependence on shear rate. Compartmental analysis and non-linear parameter estimation theory were used to estimate the kinetic parameters of the turnover process. The study deals with the relation between these findings and the problem of thrombogenesis induced by blood-foreign surface contact.

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## CHAPTER 1

### INTRODUCTION

#### 1.1 PROBLEM OUTLINE

There is a growing use of prostheses as well as temporary support devices in the medical field. Such devices are of a wide variety: hemodialyzers, blood oxygenators, man-made heart valves, artificial organs in general. On using these devices in the vascular system their contact with blood causes traumatic effects like clotting, hemolysis and thrombus formation. This is due mainly to the difference in surface structure and properties of these devices from those of the endothelial lining of the circulatory system.

The present understanding of the complete sequence of events that follow when a "foreign" surface comes in contact with blood is by no means complete; however, some phenomenologically significant events can be described such as hemolysis, platelet adhesion and protein denaturation, all of which may be interrelated<sup>(1)</sup>.

The interaction between plasma proteins and polymer surfaces is believed to be of importance in understanding and analyzing those events mentioned above. It is established that adsorption of proteins onto the exposed surfaces of these materials is intimately related to the compatibility of these surfaces with blood<sup>(2), (3), (4), (5)</sup>. Therefore investigation of plasma protein-polymer surfaces interaction is believed to give important information about that problem.

It has been shown that surfaces have different effects on blood

constituents depending on the composition of the layer of protein originally adsorbed on them. Surfaces coated with albumin tend to reduce platelet adhesion<sup>(6), (7)</sup>, while fibrinogen and  $\gamma$ -globulin tend to enhance it<sup>(7), (8)</sup>. It is also reported that the layers of proteins adsorbed from simple solutions on hydrophobic polymer surfaces consist of close-packed monolayers<sup>(9), (10)</sup>. Brash et al. put forward the possibility that in blood or plasma the adsorbed layer, while remaining indefinitely at the equilibrium level of a close-packed monolayer, could be exchanging material with the fluid and that protein released from the surface could be altered to an activated form. This form may be decisive in triggering the subsequent traumatic events that take place at the blood-"foreign" material interface.

The present investigation is directed by the above mentioned information and possibilities. It deals with plasma protein-polymer surface interactions and is aimed at investigating principally two aspects:

- i) studying kinetics of plasma protein adsorption under both static and flow conditions with particular emphasis on the effects of wall shear rate, solution concentration and time on the protein-surface equilibrium state;
- ii) investigating the possibilities of turnover and exchange between proteins adsorbed on the polymer surface and proteins in solution under both static and flow conditions. The effects of solution concentration, wall shear rates and time on these processes are also of particular interest.

Since the problem studied here is of physiological importance, the range of experimental variables is mainly limited to

those conditions met in physiological instances.

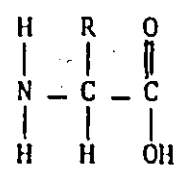
1.2 PROTEIN DESCRIPTION

Proteins are usually classified into two major groups (10):

- i) Fibrous proteins. These are generally insoluble and have a structural function. Examples of this group are keratin, myosin and collagen.
- ii) Globular proteins. These tend to be soluble and have compact molecules of ellipsoidal average shape. This group includes the physiologically important plasma proteins. Fig.1 shows a classification of proteins. Most of the plasma proteins are included within the dashed lines.

Globular proteins are further divided into simple and conjugated types. Simple proteins are wholly peptidic while conjugated proteins are more complex and possess associations between protein and non-protein material. Plasma proteins generally fall into one of these groups.

Plasma proteins are large molecules of 50,000 - 400,000 molecular weight with very complex structures. The primary structure is a polymer of amino acids (10), (11). The general formula of an amino acid may be written as



where R represents a side chain specific for each amino acid. About twenty-two or more than eighty occurring naturally are found in common proteins (10). Proteins can be considered to be formed by the following reaction (10):

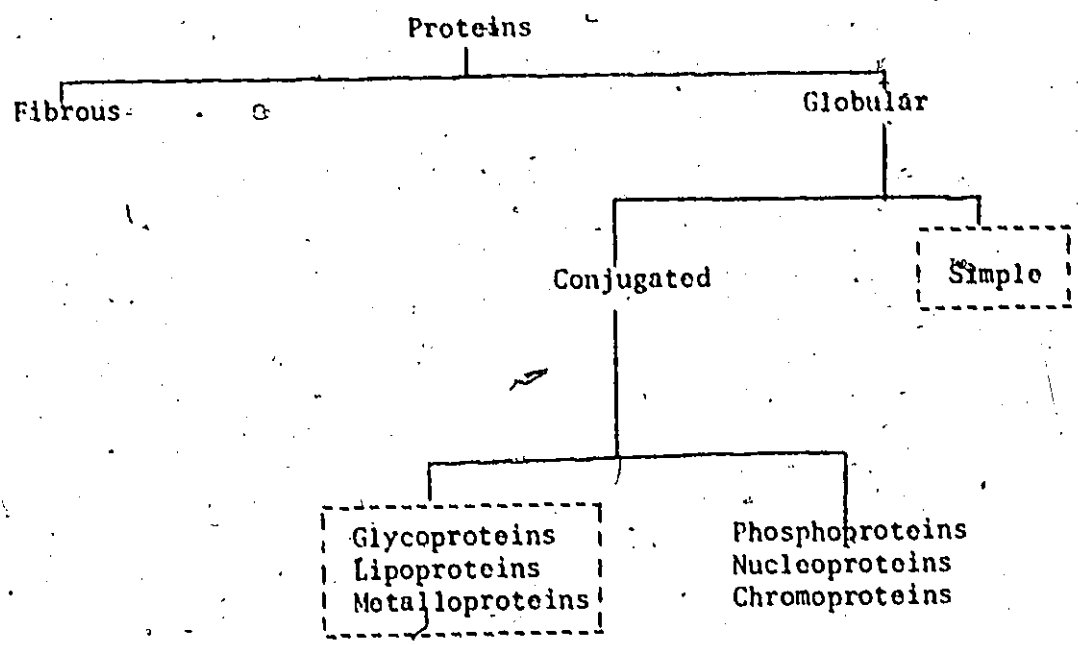
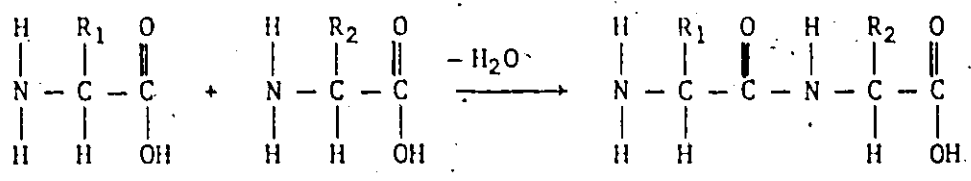
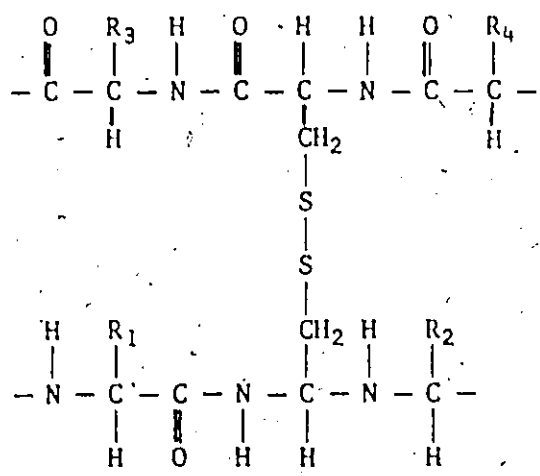


FIGURE 1

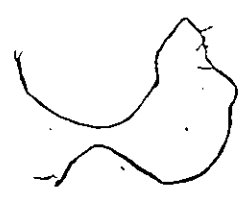
PROTEIN CLASSIFICATION (10)



in which -CO-NH- or peptide bonds are formed. The polymer chains thus formed are folded back on each other and in some places chemically cross-linked by disulphide bridges, thus:



According to Scheraga<sup>(12)</sup> the structure of a protein molecule can be considered on three levels: primary, secondary and tertiary. The primary structure is concerned with the covalent linkage and the sequence of amino acid residues in the polypeptide chains of which the protein is constituted; the secondary structure involves the folding of the polypeptide chains, and the tertiary structure the interaction between the side chain R groups. Scheraga<sup>(12)</sup> points out that hydrodynamic studies show that the fully extended form of chains in the secondary structure is rarely, if ever, encountered. The secondary structure consists of the chains taking the  $\alpha$ -helix structure with some short segments being in fully extended form. Interactions between side-chain groups, which can



determine tertiary structure can be of several kinds. They can involve formation of disulphide bonds which serve as cross-links between two different polypeptide chains or between two parts of the same chain. There can also be weaker interaction involved in the tertiary structure such as hydrophobic bonds between nonpolar residues, and hydrogen bonds between polar side-chain groups.

The internal structure of the native protein molecule and the kind of bonds involved in stabilizing its secondary and tertiary structure are expected to affect protein-surface interactions, since they will determine the reactivity and availability of the end groups involved in the surface reaction.

Two of the more abundant plasma proteins are now described in more detail.

#### Albumin:-

Albumin is quantitatively the most significant plasma protein, accounting for 50-60% of the total plasma pool<sup>(10)</sup>. According to Putnam<sup>(13)</sup> it is the major plasma protein involved in the transport of anions, cations, dyes and drugs in the blood. This activity is of considerable pharmacological significance. Some of the physicochemical characteristics of albumin are found in Table 1.

Turner<sup>(10)</sup> reports that little is known of the structure of the molecule. Generally it appears to consist of a single polypeptide chain of 610 amino acid residues held together by numerous disulphide bridges.

9  
7

TABLE 1

PHYSICOCHEMICAL CHARACTERISTICS OF ALBUMIN<sup>(10,13)</sup>

Concentration in normal serum (mg/100 ml)	3500 - 4500
Molecular weight	69000
Electrophoretic mobility (cm <sup>2</sup> /vol <sup>-1</sup> sec.)	6.1 x 10 <sup>-5</sup>
Total Carbohydrate	0.08%
Total lipid	0.2%
Partial specific volume at 20°C	0.733
No. of peptide chains	1
Intrinsic viscosity (100 ml/gm)	0.042
Diffusion coefficient (cm <sup>2</sup> /sec)	6.1 x 10 <sup>-7</sup>
Isoelectric point	4.7

The shape and size of different plasma proteins have been determined by physicochemical studies. On the basis of viscosity data, an ellipsoidal shape has been proposed for albumin<sup>(11)</sup>. However, sedimentation, diffusion and molecular weight studies suggest a spherical shape<sup>(14)</sup>.

Fibrinogen:-

Fibrinogen is considered a key protein in blood coagulation<sup>(1,8, 10,13)</sup>. It is known that the blood clotting enzyme, thrombin, converts fibrinogen to fibrin, thus forming the supporting matrix for clots.

Some physicochemical characteristics of fibrinogen are shown in Table 2.

TABLE 2

PHYSICOCHEMICAL CHARACTERISTICS OF FIBRINOGEN<sup>(10,12,13)</sup>

Concentration in normal plasma (mg/100 ml)	200 - 600
Molecular weight	341,000
Electrophoretic mobility (cm <sup>2</sup> /volt.sec)	2.1 x 10 <sup>-5</sup>
Total Carbohydrate	2.5%
Partial specific volume at 20°C	0.723
Diffusion coefficient (cm <sup>2</sup> /sec)	1.97 x 10 <sup>-7</sup>
Isoelectric point	5.8
Intrinsic viscosity (100 ml/g)	0.25

Scheraga<sup>(12)</sup> reports that electron micrographs show the fibrinogen molecules as consisting of a linear array of 3 modules held together by a very thin thread. According to Ratnoff and Davie<sup>(15)</sup> these connecting thin threads contain most of the helical structure present in the molecule.

Table 3 shows the dimensional data for albumin,  $\gamma$ -globulin and fibrinogen reported by Lyman et al.<sup>(9)</sup> after Oncley et al.<sup>(11)</sup>

TABLE 3

DIMENSIONAL DATA OF PLASMA PROTEINS<sup>(9)</sup>

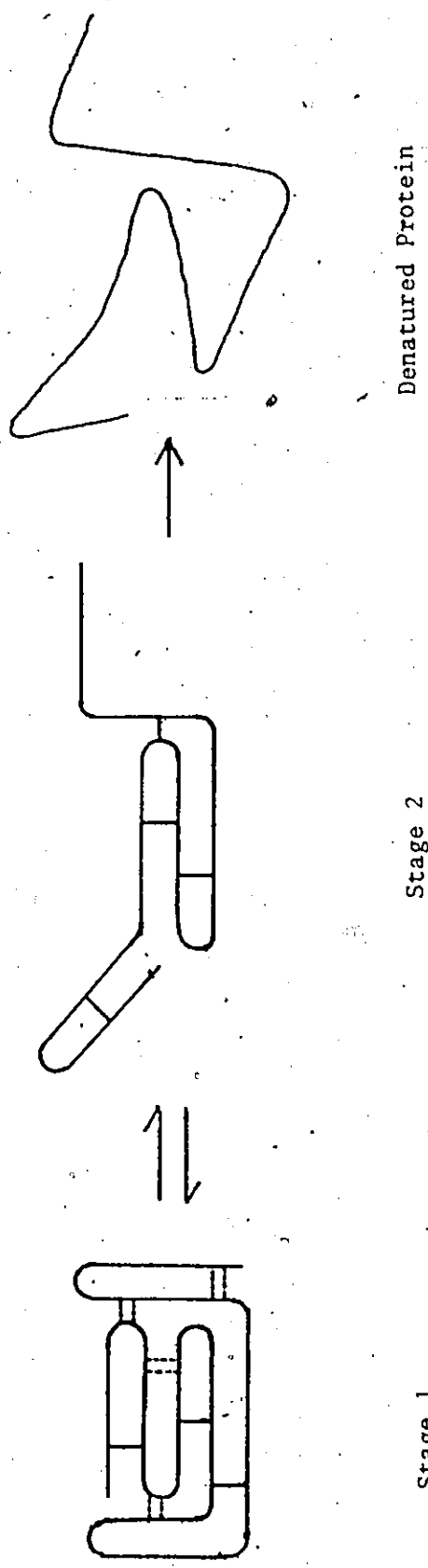
Protein	Diameter in Angstroms	Projected Area; end-on A <sup>2</sup>	Length A	Projected Area; side-on A <sup>2</sup>
Albumin	40	1700	115	4600
$\gamma$ -Globulin	44	2000	235	10300
Fibrinogen	65	4200	475	13000-30000



### Protein Denaturation:

A denatured protein is one that has lost its three-dimensional native molecular structure. This can be brought about by a number of physical and chemical agents, e.g. heat, high and low pH, shaking, detergents, etc.. Scheraga<sup>(12)</sup> considers denaturation of protein as a phase transition from a crystalline to an amorphous state. Here the orderly  $\alpha$ -helix and extended chain in the secondary structure of the native protein are referred to as crystalline while the disorganized structure referred to as amorphous is the randomly coiled form the protein molecule takes after denaturation. According to a model of denaturation cited by Turner<sup>(10)</sup>, it is the hydrogen bonds and disulphide bridges that are most vulnerable to the denaturing agents mentioned above. The first stage of denaturation is, according to this model, reversible, and consists mainly of rupturing the hydrogen bonds which stabilize the tertiary structure (See Fig.2). This leads to random coiling of the polypeptide chain with an accompanying increase in viscosity and decrease in solubility. This stage is also characterized by either molecular association or dissociation with globular proteins. Continuation of the effect of the denaturing agent leads to the second stage, which, according to the model, is usually irreversible. In this stage, the disulphide bonds stabilizing the secondary structure are ruptured with new -SH groups appearing. At this stage the protein turns into an amorphous insoluble form and is usually biologically inactive<sup>(10)</sup>.

Denaturation also takes place at the protein solution - air interface. Brash and Lyman<sup>(17)</sup> report partial denaturation at air-water interface with the degree of denaturation depending on the film pressure. However they mention that denaturation induced exclusively by spreading at



Stage 1

Stage 2

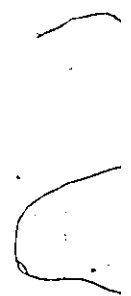
Denatured Protein

FIGURE 2

SCHEMATIC DIAGRAM ILLUSTRATING A MODEL OF PROTEIN DENATURATION (10)

----- Hydrogen bonds

| Disulphide bonds



the air-water interface is not always equivalent to total destruction of the secondary and tertiary structure.

Another aspect of the behaviour of protein films at the air-water interface, described by Brash and Lyman<sup>(17)</sup>, is Langmuir-Blodgett transfer. It was observed that on dipping a glass or metal slide into (or withdrawing it from) a liquid with a protein film at its air interface, the protein is deposited on the slide. It was also found, according to Brash and Lyman, that the number of molecular thicknesses deposited is directly related to the number of passages through the interface.

Based on these observations, air-solution-solid contact during experimental runs is avoided since it can lead to transfer of a partially denatured layer to the test surface. Also the presence of air bubbles inside a flow system would give rise to Langmuir-Blodgett transfer and could lead to erroneous results in measuring the amount of protein adsorbed on the solid surface.

### 1.3 BLOOD COAGULATION

The mechanism of coagulation of blood is now known to involve the participation of several blood proteins (factors) which according to Olsen et al.<sup>(18)</sup> act alternatively as substrates in the inactive form and as enzymes in the active form. Coagulation involves a complex reaction sequence usually represented by the cascade or waterfall mechanism shown in Fig.3.

The coagulation reaction can be initiated extrinsically by extracts of damaged tissue that gain access to the blood and activate factor X. Subsequent reactions lead to fibrin formation.

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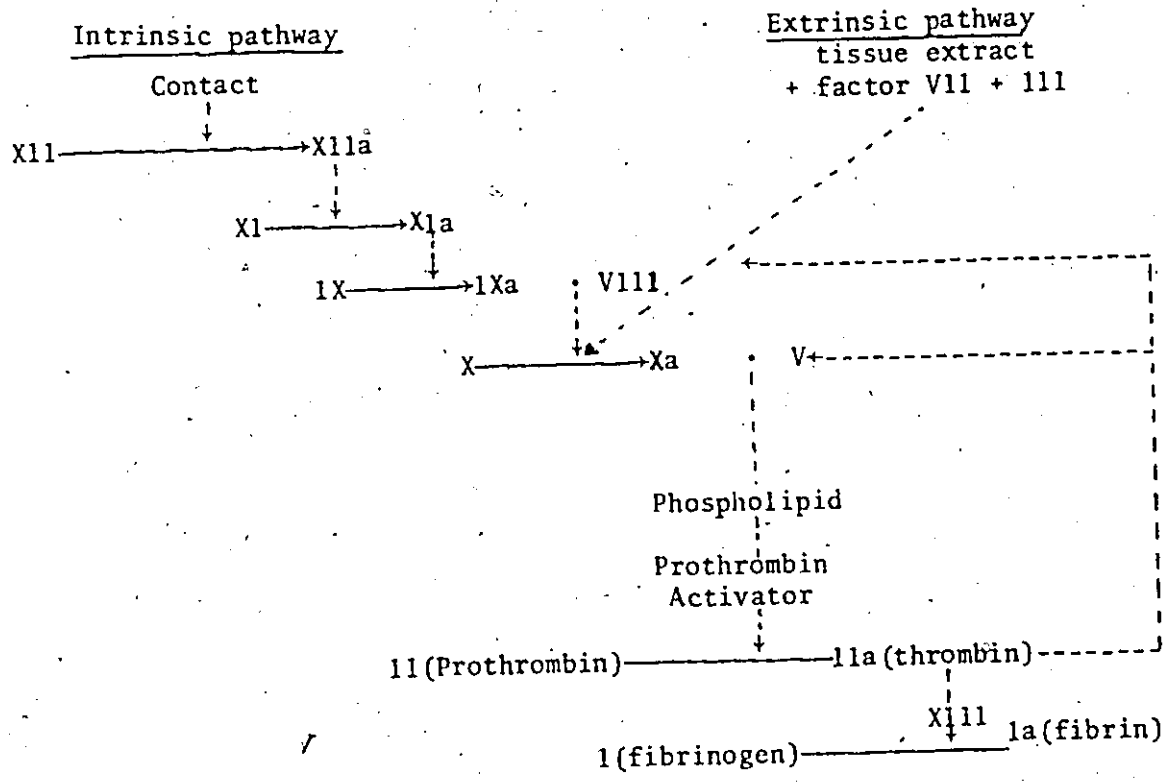


FIGURE 3

CASCADE MECHANISM OF BLOOD COAGULATION

The activation of the intrinsic pathway can take place via contact of blood with a foreign surface like glass. This contact gives rise to the activation of factor XII (Hageman factor) and the subsequent enzyme-substrate reactions that lead to formation of fibrin.

By examining the cascade model in Fig.3., it can be seen that the tissue damage that initiates the extrinsic pathway of coagulation is actually short-circuiting the first steps of the intrinsic pathway.

In Fig.3, suffix "a" indicates the active forms of the factors. According to Olsen et al.<sup>(18)</sup>, phospholipid, possibly of platelet origin, may be required at the reactions noted, while calcium ion is required in some stages. Solid lines in the figure represent a transformation process while dashed lines represent an activation process.

1.4 BLOOD - SYNTHETIC POLYMER INTERACTIONS AND SURFACE PROPERTIES

Early studies of blood clotting showed that paraffin-coated tubes increased the clotting time of blood in vitro. This observation, together with the large number of polymers that became available in the last three decades, stimulated interest in examining the effect of polymers on blood clotting. The problem acquired growing importance with the increasing prospects of using these materials in life assist devices and artificial organs.

A primary problem is the preparation and characterization of the surface. The problem of preparation is essentially that of producing a "pure" surface. Normal processing makes it inevitable that the polymer produced will contain traces of other materials that may affect the blood-surface interactions in one way or another. These materials could be plasticizers and stabilizers, anti-oxidants or catalyst residues used as

processing aids or in tailoring the material according to some physical properties required. According to Clark et al. <sup>(19)</sup> these substances may be leached out in varying degrees from the material in contact with blood. The effect of these substances on blood is unknown or incompletely known.

In this connection, it is noted that a substantial part of the work involving interactions of blood and polymers until a few years ago, did not consider the complex nature of polymeric materials. Clark et al. <sup>(19)</sup> stress the fact that many authors use materials described by generic names like "polyurethane" or "silicone" without considering the fact that these names cover families of materials the members of which may be different in chemical composition.

As mentioned above protein adsorption appears to be a primary factor in blood coagulation and consequently in surface thrombogenicity. Usually the polymer surface on which adsorption takes place consists of crystalline and amorphous areas. The crystalline part can be either oriented or unoriented depending on the nature of the production process. Also, high energy sites at lattice defects in crystals, faces, edges, or strain points in the material, may be present. The effect of these factors upon the extent and kind of protein adsorption remains to be thoroughly studied.

With respect to surface morphology, Peterlin and Olf <sup>(20)</sup> have studied the effect of drawing on sorption of water vapour into polyethylene. They report that sorption occurs preferentially into the amorphous areas. They also report that sorption decreased upon drawing the polymer, and related this to the change in the ratio of the crystalline portion to the amorphous as well as the changes in the orientation of the crystalline

portion upon drawing. Clark et al. (19) put forward the possibility that such preferential sorption could cause local changes in osmolality and be responsible for damage to formed elements of the blood, such as erythrocytes (red blood cells).

#### Properties of Polyethylene

In the laboratory in which this research was carried out, a variety of polymer surfaces of interest with regard to surface thrombogenesis has been and will be studied. However, this study deals only with polyethylene surface. Polyethylene is essentially an uncharged, hydrophobic material. It is a polyolefin polymer having a milky translucent and wax-like surface. Three densities are commercially available (21) with tensile strength, hardness and chemical resistance increasing as the density increases. This class of material is resistant to all acids, alkalis and inorganic chemicals; however, it will dissolve in a number of common solvents at 150°F (21). Table 4 shows some typical properties of commercially available low, medium and high density polyethylene.

A description of the particular polyethylene used in this study will follow in the experimental section.

TABLE 4

## TYPICAL PROPERTIES OF LOW, MEDIUM AND HIGH DENSITY POLYETHYLENE

Property	low density	medium density	high density
Molecular weight	3-40000	100-200,000	500,000
Specific gravity (g/cc)	0.912-0.925	0.926-0.940	0.941-0.965
Tensile strength (psi)	1100-1600	1700-1800	3400-37000
Ultimate elongation (%)	150	125-150	400-500
Impact strength (ft.lb/in)	no break	-	0.7-20
Resistance to heat (F)	180	230	250
Dielectric strength, (short time(v/mil))	460-700	460-650	450-500
Hardness, Shore	D41-D46	D50-D60	D60-D70
Compressive strength (psi)	-	-	2700-3600
Modulus of elasticity (x 10 <sup>-5</sup> psi)	0.14-0.38	0.25-0.55	0.6-1.8



## CHAPTER II

### LITERATURE SURVEY ON PROTEIN ADSORPTION

Studies of protein surface activity in general and the adsorption of proteins on solid surfaces in particular can be traced back as far as 1905. A good review of these early studies is given by Neurath and Bull<sup>(22)</sup>. Extensive and recent reviews are given by Brash and Lyman<sup>(17)</sup> and by Dillman<sup>(16)</sup>.

An attempt will be made here to delineate aspects of recent studies significant to the problem at hand.

There is agreement that protein adsorption isotherms on different solid surfaces fit the Langmuir model<sup>(16,17,23)</sup>. Brash and Lyman<sup>(23)</sup> reported that adsorption of plasma proteins from solutions on uncharged hydrophobic polymer surfaces (polyethylene, polystyrene, etc..) was identical for different surfaces. Adsorbed protein was reported to form monolayers from solutions of concentration ranges up to normal plasma levels. Measurements in these experiments used Infrared Internal Reflection Spectroscopy (IRS).

Similar results were reported by Dillman<sup>(16)</sup>, however maximum adsorbed quantity in the work of Brash and Lyman was about 6 times larger than that reported by Dillman. Dillman explains the difference in terms of the electrostatic charges of polyethylene used by Brash and Lyman, compared with those of the membranes used by himself (polyethylene backbone with polystyrene sulphonic acid grafted to the matrix). Also the

pH of the protein solutions used in these two studies was different. Many authors report that the pH has a large effect on the extent of adsorption<sup>(24,25,26)</sup>. Lee et al.<sup>(27)</sup> studied protein adsorption on a variety of surfaces from simple and multiple solutions. They found the rate of adsorption and the amount adsorbed to depend on both the protein and the surface.

There is agreement that equilibrium between protein solutions and surfaces is established in a relatively short time. Leininger<sup>(28,29)</sup> from measurements of zeta-potential as a function of time, suggests adsorption reaches equilibrium within one hour. Pearce and Bibby<sup>(30)</sup> report that casein,  $\gamma$ -globulin,  $\beta$ -globulin, serum and egg albumin adsorbed on bovine enamel all reached equilibrium in less than two hours. In mixture systems Lee et al.<sup>(27)</sup>, found that the amount adsorbed is less than that adsorbed from simple solutions. They also found that the plateau (equilibrium) time for fibrinogen and  $\gamma$ -globulin adsorption is less than one minute, while for albumin it extends up to 150 minutes.

Lee and Kim<sup>(31)</sup> using I.R.S. for studying protein adsorption on hydrophobic surfaces found results consistent with a Langmuir model. Their time curves showed equilibrium plateau concentrations depending on the polymer substrate. Plateau times ranged from 10 to 60 minutes under static condition and 10 to 250 minutes under flow condition with the highest plateau time corresponding to the highest flow rate used (12 ml/sec). However, they do not mention the shear rates or shear stresses corresponding to these flow rates. Obviously wall shear rate is the pertinent parameter in correlation of flow effects with adsorption on the surface.

Brash and Lyman<sup>(17)</sup> report results of adsorption under flow expressed in terms of Reynolds number. Although they used I.R.S. for measurement, it is difficult to compare their findings with the findings of Lee and Kim because aside from the unknown shear range of the latter's work, their studies use different solution concentration as well as different surfaces. Nerem et al.<sup>(32)</sup> studied <sup>131</sup>I-Albumin uptake from blood to the arterial wall both in vitro and in vivo. Their experiments were done under both steady flow and oscillatory flow with zero average flow rate. Their results demonstrate a weak correlation between albumin uptake and wall shear stress, rule out a diffusion controlled model for adsorption and suggest an interfacial control mechanism.

Dependence of protein transport to the wall on the shear stress is also reported by Caro<sup>(33)</sup>, greater shear stress corresponding to greater uptake

An interfacial control mechanism, with the fluid-wall interface uptake as the controlling step is also found by Caro and Nerem<sup>(34)</sup>. However the latter study was done on cholesterol (a lipid) while the work of Nerem et al.<sup>(32)</sup> was done on albumin. Evidence is available from both studies that albumin and cholesterol have virtually the same level of uptake for similar conditions, which is interesting considering the gross differences in size and structure of the molecules. In the study of Nerem et al., measurements were taken during the initial transient phase and prior to equilibration of the transport process.

Vroman and Adams<sup>(35)</sup> studied adsorption of plasma proteins from plasma by ellipsometry. Polymers, silicon oxide and tantalum oxide were among the surfaces studied. They found that fibrinogen adsorbs on sili-

con oxide surface within 2 seconds; they also found the fibrinogen adsorbed undergoes "conversion", i.e. exchanges with factor XII and other constituents when exposed to intact plasma. The process was found to be rapid. Flow seems to be an important factor in plasma protein-surface interaction. Thrombus formation was found to be affected markedly by shear rate, and since protein adsorption is a step believed to affect thrombus formation and structure, flow effects appear to be relevant in analyzing the protein-surface interaction problem.

Dintenfass<sup>(36)</sup> from rotational viscosity found thrombus structure to vary markedly with shear rate. On the other hand, Downie et al.<sup>(37)</sup> found thrombus formation in a Y-shaped chamber in which blood is flowing towards a bifurcation to be relatively independent of shear rate.

Grabowski<sup>(38)</sup> suggests the necessity of convective diffusion for the transport of clotting proteins as well as platelets to the vicinity of an artificial surface, if initial interactions leading to thrombus growth are to take place. He further suggests that the initiation of adsorption of "surface active" protein, as well as the subsequent release of surface active "proteins" may depend on flow rate. However, such a flow rate dependence may not have been previously observed because the characteristic time for protein surface interaction is very short, i.e. on the order of seconds. Several studies have been done to investigate the effect of chemical structure and surface properties on adsorption as well as the type of bond that is formed between the protein and the surface. Sawyer<sup>(39)</sup> and Falb et al.<sup>(40)</sup> studied many surface characteristics in connection with thrombus formation and protein adsorption.

Lyman et al.<sup>(9)</sup> found adsorption of plasma proteins on hydrophobic

polymer surfaces, of low surface energy, to be irreversible. They also found critical surface tension which is related to surface free energy to correlate with clotting time. Dillman and Miller<sup>(41)</sup> studied the adsorption and desorption of plasma proteins on a variety of polymer membranes. They report that adsorption takes place in two separate and distinct ways simultaneously. Both types of adsorption are reported to follow the Langmuir isotherm. They represent separate membrane sites and are non-interacting.

Studying protein adsorption to hydrogels from water, buffered saline and protein mixtures and plasma, Hoffman<sup>(42)</sup> observed that the monomer purity has a large effect on protein adsorption to hydrogels. He reports different adsorption behaviour between buffered solutions and plasma, relating this to unknown plasma factors that modify fibrinogen adsorption of some of the hydrogel surfaces studied.

Morrissey and Stromberg<sup>(43)</sup> studied conformation and conformational changes of adsorbed plasma protein on a silica surface. They report that the internal bonding of these proteins is sufficient to maintain their native structure while adsorbed. They also report that the conformation of adsorbed protein was found to be independent of the time of adsorption.

Lumber et al<sup>(44)</sup> studied elution of proteins adsorbed from plasma onto glass, silicone-coated glass and cuprophane. They found that most of the adsorbed proteins are loosely adherent to all three surfaces with certain plasma proteins strongly adsorbed to glass, less strongly to silicone-coated glass and least strongly to cuprophane.

Many types of bond have been suggested to describe the adsorption behaviour of proteins to surfaces. They include hydrogen bonds and

hydrophobic bonds, electrostatic bonds and combinations of these<sup>(16)</sup>.

## CHAPTER III

### EXPERIMENTAL

#### 3.1 ANALYTICAL TECHNIQUE

Protein adsorption has been studied by several methods such as as: reduction of solution protein concentration, measurement of nitrogen content, internal reflection spectrometry (IRS), and ellipsometry, as well as counting the individual molecules with the use of an electron microscope<sup>(16)</sup>.

Of these methods, IRS and ellipsometry are most frequently used. However, there are some limitations on their use. For example, ellipsometry would not be feasible if the adsorbed film and the substrate have comparable refractive indices. On the other hand, IRS cannot be used for competitive adsorption studies because of the similarity in the infrared spectra of different proteins.

In this study the radio tracer method is used. When experimental conditions are designed adequately to assure the proportionality between the mass and the fraction that is labelled<sup>(45)</sup>, this method provides data related directly to the mass of labelled substance with high accuracy.

In the present work protein fractions are labelled with  $^{125}\text{I}$  or  $^{131}\text{I}$ , and by monitoring radio activity on the surface, full information can be obtained about adsorption to, or release from the surface.

Advantages of this method over the others mentioned above are:

1 - It gives direct measurement of the mass on the surface with

accuracy comparable to that of a micro-balance<sup>(45)</sup>.

- 2 - It is not based on measuring certain physical properties of proteins. This is an important advantage, since the properties of proteins may be undistinguishable from those of the substrate.
- 3 - It is an ideal method for studying competitive protein adsorption from multi-component solutions or plasma.
- 4 - It is the only way to study turnover and exchange between adsorbed and dissolved protein.

### 3.2 MATERIALS

Human albumin, 100% electrophoretically pure, and in dried form, was obtained from Canadian Höchst Ltd., Montreal; it was used with no further purification. Na. <sup>125</sup>I and <sup>131</sup>I was obtained from New England Nuclear, Boston, U.S.A. Albumin (10 to 50 mg portions) was labelled with <sup>125</sup>I and <sup>131</sup>I using the iodine monochloride method of McFarlane<sup>(46)</sup>. In this reaction iodine substitutes mainly in the aromatic rings of tyrosine residues<sup>(47)</sup>, and the degrees of substitution can be carefully controlled by regulation of stoichiometry and mixing technique. At a level of substitution not exceeding one iodine atom per molecule of protein, it has been shown that biological activity is not altered<sup>(48)</sup>. Solutions of albumin were made up to the proper concentration in a solution of 0.2 Molar of tris (hydroxymethyl) amino methane of pH 7.4 in distilled deionized water. Polyethylene was in the form of tubing (Intramedic PE 320) manufactured by Clay Adams, New Jersey, U.S.A. and supplied in a pure form containing no additives or plasticizers. It has a specific gravity of 0.92 (low density, Table 4). Figure 4 shows a transmission electron micrograph of



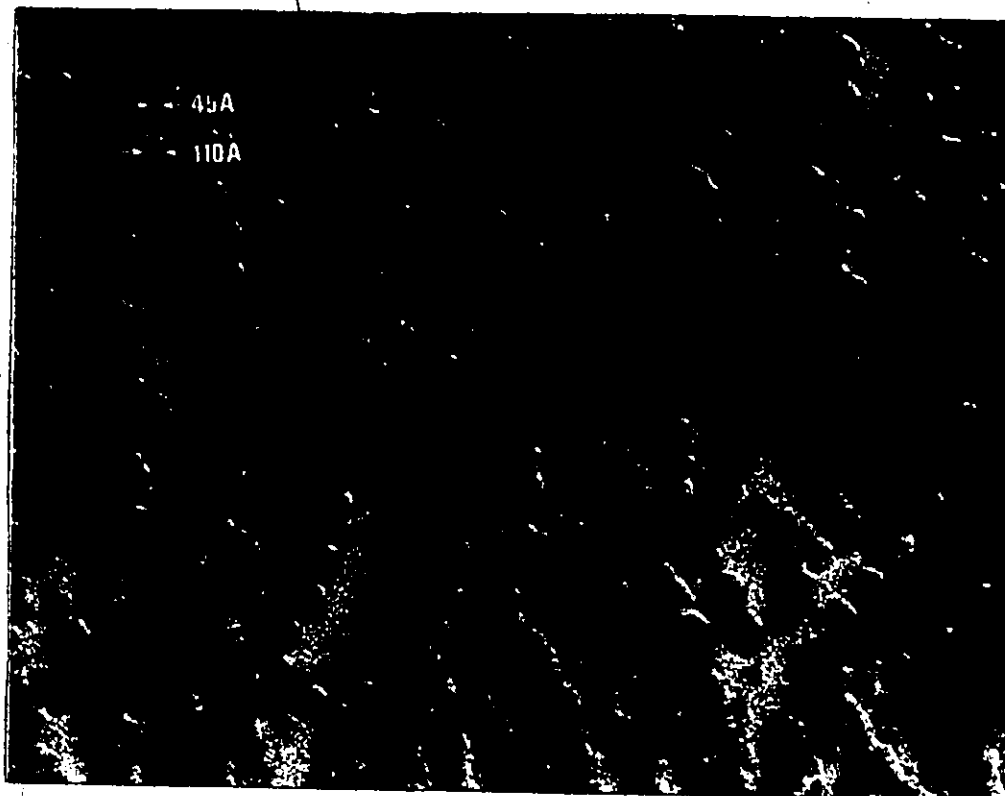


FIGURE 4

TRANSMISSION ELECTRON MICROGRAPH OF THE POLYETHYLENE SURFACE  
SHOWING THE MOLECULAR DIMENSIONS OF NATIVE ALBUMIN.

45 Å : Projection of end-on disposition  
115 Å : Projection of side-on disposition

of the polyethylene surface. The electron micrograph shows the surface is rough when compared with the molecular dimensions of albumin. The tubes, of 0.106 inch I.D. were cleaned immediately before use by rinsing with methanol for 10 minutes followed by two changes of distilled, de-ionized water each for 10 minutes.

### 3.3 PROCEDURE AND METHODOLOGY

Experiments were performed under both static and flow conditions. Segments of tubing 45 cm long were filled initially with solvent and the solvent was displaced with protein solution using a syringe. With three-way valves at each end of the tube intervening air bubbles could be flushed out through a sidearm and contact of the test surface with the air-solution interface avoided. For the flow experiments three pumps were used: a Harvard automatic infusion pump (model 600950), a Redy roller pump (Brader-John Inc., model 6-500) and a Watson-Marlow (model MHRE 200). The polyethylene tubing was connected to the pump using silastic tubing. A schematic diagram of the flow system is shown in Fig. 5. In general an experimental "run" involved about 15 lengths of tubing in series. Each tubing length has a 3-way valve at both ends. After an appropriate contact time with either adsorbate or exchange solution, a length of tubing was disconnected from the circuit, rinsed and counted while the remainder of the run continued. In general running time per experiment, for both adsorption and exchange was 150-200 hours. For the flow experiments turnover was carried out at the same flow rate as adsorption so as to eliminate "apparent" turnover due to shear-induced desorption. Rinsing procedure was established by determining the treatment required to obtain a constant surface concentration. In general three aliquots of solvent were used. The total rinse time was 24 hours.

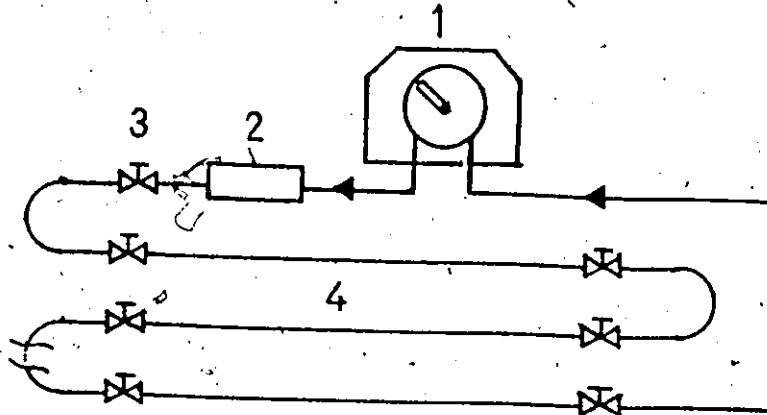


FIGURE 5

SCHEMATIC DIAGRAM OF THE FLOW CIRCUIT.

- 1 - Roller Pump
- 2 - Bubble Trap
- 3 - Three-way Valve
- 4 - Polyethylene Test Sections

An exchange experiment consisted of adsorption of labelled protein (either  $^{125}\text{I}$ -Albumin or  $^{131}\text{I}$ -Albumin) to steady state. Adsorption time ranged between 24 to 40 hours, this was followed by rinsing and filling the system with protein solution of the same concentration as that of the adsorbate solution and labelled with the other isotope ( $^{125}\text{I}$  or  $^{131}\text{I}$ ). With test sections of 45 cm length an entrance of 17 cm, about 8.5 cm length of the tube at each end was allowed for flow development. Samples for counting were about 14 cm long ( $11.4\text{ cm}^2$ ) and at the levels of adsorption experienced, initial counting rates of 2000 to 4500 CPM were usually obtained. For both  $^{125}\text{I}$  and  $^{131}\text{I}$  sample counts per minute encountered were usually at least 4-5 times the background noise level. There was no limitation on precision from this source. All experiments were performed at room temperature ( $22^\circ\text{C}$ ). Samples were counted in a well type solid scintillation counter (Beckman Biogamma T.M. Counting System). This is a three-channel gamma counter of 200-sample capacity. The detector consists of a three-inch sodium iodide crystal doped with thallium iodide encased in an aluminum housing with a thin aluminum window which permits efficient counting of gamma emitters. The counting system includes a teletypewriter.

The half-life of the isotopes used are 60 days for  $^{125}\text{I}$  and 8 days for  $^{131}\text{I}$ . Duration of the kinetics and turnover experiments (100 - 150 hours) was within these half-life periods. To avoid correction for decay in counting the radioactivity level in the samples, all the samples were collected in counting vials and counted at once at the end of the experimental run. Counting time per run never exceeded 150 minutes, this period is short enough to exclude radioactive decay as a

variable affecting the recorded readings in the course of counting.

In the turnover experiments, the two isotopes were simultaneously used, and counting was carried out by adjusting two channels of the gamma counter on the peak radiation level of the two isotopes. In this situation there is usually a count spill-over from the channel set with a wider range (in this case the  $^{131}\text{I}$ -channel) to the channel set with a narrower range. This was corrected, after effective counts were obtained, by subtracting background counts from recorded counts, by means of a formula supplied by the manufacturer.

## CHAPTER IV

### ANALYSIS

#### 4.1 THEORY

##### 4.1.1 Introduction

Biological studies have been greatly facilitated by the use of radioactive tracer materials. One of the mathematical concepts used to describe biological and biochemical processes by means of tracers is called compartment analysis. Mathematically speaking, compartment theory is the representation of the processes mentioned above by a system of first-order linear differential equations with constant coefficients; these equations are called compartment equations. The subject has a much wider range of applicability in chemical engineering, electrical engineering, economics, etc. With respect to application in biology, there is now an extensive literature concerned with it (49,50).

According to Jacquez<sup>(51)</sup>, a compartmental system is made up of a finite number of macroscopic subsystems, called compartments or pools, each of which is homogeneous and well mixed, and the compartments interact by exchanging material. There may be inputs from the environment into one or more compartments and there may be outputs from one or more compartments into the environment. If there are no exchanges with the environment the system is said to be closed, otherwise it is an open system.

In this study, during exchange, material (albumin) moves from the

solution to the polyethylene surface, while adsorbed albumin leaves the surface to the bulk solution. The surface may have crystalline and amorphous regions, with possibly different kinetic behaviour vis-a-vis protein adsorption and exchange. It may have high energy sites or strain points. In other words, the surface may not be kinetically uniform. In this study, these "types" of surface sites will be treated as distinct, non-interacting and homogeneous compartments interacting with the solution compartment. The first order differential equations describing labelled protein transport between the surface and the solution will be the compartment equations of the system. Description of these equations and their solution follows.

#### 4.1.2 Mathematical description of a system of compartments.

Consider a system of  $n$  distinct, homogeneous compartments. It is assumed that the transport of a labelled species between these compartment may be described as a first order process. Consequently the  $n$  compartments are governed by a system of linear ordinary differential equations with constant coefficients that is written in matrix notation as

$$\frac{d\underline{Y}}{dt} = \underline{A} \underline{Y} \quad [1]$$

where:  $\underline{Y}$  is a column vector whose generic elements  $Y_i$  represent

the total amount of labelled material in the  $i$ th compartment.

$\underline{A}$  is an  $n \times n$  matrix, the elements of which are  $a_{ij}$ . For  $i \neq j$ ,

$a_{ij}$  is the material transport rate from compartment  $j$  to compartment  $i$  per unit amount of material in the  $j$ th compartment.

$a_{ij}$  is also referred to as the fractional turnover rate of com-

partment  $j$  with respect to  $i$ .  $(-a_{ii})$  is the rate at which the total amount of material in the  $i$ th compartment is replaced.

Implicit in equation [1] is the assumption that the transit time for material flux between any two compartments is negligible. Accordingly, only  $\underline{Y}$  is time dependent and  $\underline{A}$  is constant.

In view of their physical significance, the elements of the matrix  $\underline{A}$  must satisfy the conditions

$$a_{ij} \geq 0 \quad i, j = 1, 2, \dots, n, \quad i \neq j \quad [2]$$

$$\sum_{i=1}^n a_{ij} \leq 0 \quad j = 1, 2, \dots, n, \quad [3]$$

Equation [3] follows from the definition of  $a_{ij}$  and the requirement that no compartment is a material sink.

From equation [2] and [3] it can be deduced that:

$$a_{ii} \leq 0 \quad i = 1, 2, \dots, n, \quad [4]$$

and the equality sign holds only if  $a_{ij} = 0$  for all  $j$  with  $i$  fixed.

To differentiate between closed and open systems, the excretion rate per unit volume for the  $i$ th compartment is defined as

$$a_{oi} = \sum_{j=1}^n a_{ji} \quad i = 1, 2, \dots, n, \quad [5]$$

From equation [3] it follows that  $a_{oi} \geq 0$ .  $a_{oi}$  represents the fractional rate at which material is lost from compartment  $i$  to the exterior but not to other compartments. If  $a_{oi} = 0$ ,  $i = 1, 2, \dots, n$ , the system of  $n$  compartments is said to be closed, if not it is said to be open.

Solution of the set of ordinary differential equation represented vectorially by equation [1] was obtained using Laplace transforms (52)



Another more concise method of solution is that of matrix diagonalization<sup>(53,55)</sup>, the solution is

$$\underline{Y} = \underline{G} [e^{B_i t}]$$

[6]

and  $\underline{A} = \underline{G} \underline{\Lambda} \underline{G}^{-1}$

where  $\underline{\Lambda}$  is the diagonal matrix of the  $n$  eigenvalues  $B_i$  of the matrix  $\underline{A}$  and  $\underline{G}$  is a matrix function of fractional turnover rates and initial distribution of tracer.

It was shown that conditions (2) and (3) guarantee that the  $B_i$  have negative or zero real parts and that no pure imaginary  $B_i$  exists<sup>(54)</sup>.

When the set of equations describes a closed system, such as the one dealt with in this study,  $\underline{A}$  is a singular matrix and thus one of its eigenvalues is zero<sup>(55)</sup>, such a system is unconditionally stable, all eigenvalues having non-positive real parts.

The concepts of compartment analysis outlined above will be used to analyse the data collected in this study. The purpose of the analysis is to find the fractional turnover rates that describe the exchange between a species (protein) adsorbed on the test surface (polyethylene) and the same species in the bulk solution. Investigation is extended to study the effect of both shear rate and solution concentration on these fractional turnover rates under steady state (surface-solution) equilibrium conditions.

Experimental evidence in this study suggests the presence of more than one "type" of surface involved in adsorption. Turnover curves show that there is a fraction of the adsorbed protein that is unexchangeable with the bulk solution. In other words a fraction of the adsorbed protein is

bound to the surface in such a way as to allow it to exchange position with the free bulk solution protein, while another fraction is bound to the surface in such a way that this exchange process at equilibrium conditions is now allowed.

#### 4.2 STATISTICS

As shown above, the solution of a set of linear ordinary differential equations with constant coefficients describing mass transport among  $n$  compartment can be expressed in the vectorial form [6].

$$\begin{aligned} \underline{Y} &= \underline{G} \cdot (e^{B_i t}) \\ \text{and } \underline{A} &= \underline{G} \underline{\Lambda} \underline{G}^{-1} \end{aligned} \quad [6]$$

with the elements of  $\underline{G}$  and  $B_i$  being related to the fractional turnover rates or the rate constants of the system. The relation depends on the arrangement of the compartments in the system and their pair-wise interaction.

To solve the problem estimates of  $B_i$ 's and elements of  $\underline{G}$  are required from the data collected. In other words the problem is to fit the data to a function of the form

$$f(t) = y = \sum_{i=1}^n g_i e^{-B_i t} \quad [7]$$

to determine the  $g_i$ 's, the  $B_i$ 's and the number of compartments  $n$ . In systems that tend to reach an equilibrium state equation [7] appears with an exponential free term. In this study the exponential free term would account for the part of the surface with non-exchangeable adsorbed protein.

Several methods have been described for the analysis of multi-

exponential decay curves such as:

- 1 - The graphical technique, which may be performed by hand by curve-peeling on semi-logarithmic coordinates<sup>(56)</sup>. It can also be done on a digital computer<sup>(57)</sup>.
- 2 - An algebraic method in which the exponential identification problem is formulated in the form of a set of linear equations. The solution of these equations determines a polynomial whose roots indicate the value of the exponents<sup>(58)</sup>.
- 3 - Transform methods such as solutions based on deconvolution for Fourier transforms<sup>(52)</sup>.
- 4 - Iterative methods based on non-linear least squares methods<sup>(59,60)</sup>.
- 5 - The Bayesian estimation of parameters by multivariate analysis<sup>(55,61)</sup>.

In this study a non-linear least squares program is used to fit the data to the proposed model and estimate the parameters of the model (the  $B_i$ 's and  $g_i$ 's in equation [7]). The program is based on Marquardt's method<sup>(60)</sup> which is a compromise between Gauss-Seidel and the steepest descent method. According to Himmelblau<sup>(60)</sup> Marquardt's method combines the best features of both methods.

A problem usually encountered in models involving sums of exponentials is that the parametric covariance matrix is ill-conditioned. This leads to large parameter estimation confidence limits<sup>(55)</sup>. However, to be able to assess the significance of any change where conditions are varied, precise parameter values are important.

It has been reported that obtaining more than one response from the system studied would diminish the size of the confidence regions of parameter estimates<sup>(55)</sup>.

In compartment analysis one response is provided by the measurement of the amount of one tracer in one compartment over time. Further responses can be obtained by measurement of the same tracer in a different compartment. When other compartments are inaccessible another tracer can be used and the measurement of both tracers from the same compartment made independently.

There are different approaches to the simultaneous analysis of multiple responses depending on the kind of criteria used in the analysis. One approach is the least sum of squares method discussed by Beauchamp and Connell<sup>(62)</sup>. In this method, the sum of squares of deviations of model from data for each response must be weighted by the inverse of the variance of the observations of that response<sup>(55)</sup>.

When replicates are not available to give estimates of the variance of observations, least squares analysis of individual responses could be used for parameter estimation. According to Allen et al.<sup>(55)</sup> the Bayesian multivariate analysis technique of Box & Draper<sup>(61)</sup> could also be used when replicates are not available. This method is reported to possess superior properties with respect to tightening the confidence limits of parameter estimates.

In this study, the experiments dealing with the turnover kinetics are designed to produce two responses obtained from monitoring the polyethylene surface with time. This is achieved through the use of two isotopes as described in the experimental part above. In the course of the

process, the tracer used originally in adsorption shows the multi-exponential decay pattern described above, while the surface concentration of the other tracer (in the exchange solution) builds up, until both reach an equilibrium value. Both responses are obtained by independent radioactivity measurements from the polyethylene surface and thus lend themselves to the multiresponse analysis discussed above. This method will be briefly outlined here.

Details of the theory for this method are given by Box & Draper (61). The method relaxes the assumptions on which the ordinary least squares method is based. These assumptions are:

- (a) Errors are all uncorrelated
- (b) Errors all have equal variance.

Even an improved least squares method like simultaneous least squares analysis of multiresponse problems cannot account for correlations between responses.

The technique of Box & Draper provides the Bayesian estimate of parameters which have the highest posterior probability. It assumes that the errors are distributed according to a multivariate normal distribution with unknown variance-covariance matrix. By using a "non-informative" prior distribution, it was found that estimates of parameters  $\underline{B}$  yielding maximum posterior density are those obtained by minimizing the determinant.

$$|V| = (V_{ij}) = \begin{vmatrix} \sum (y_{1u} - \hat{y}_{1u})^2 & \sum (y_{2u} - \hat{y}_{2u})(y_{1u} - \hat{y}_{1u}) & \dots & \sum (y_{vu} - \hat{y}_{vu})(y_{1u} - \hat{y}_{1u}) \\ \sum (y_{1u} - \hat{y}_{1u})(y_{2u} - \hat{y}_{2u}) & \sum (y_{2u} - \hat{y}_{2u})^2 & \dots & \sum (y_{vu} - \hat{y}_{vu})(y_{2u} - \hat{y}_{2u}) \\ \vdots & \vdots & \ddots & \vdots \\ \sum (y_{1u} - \hat{y}_{1u})(y_{vu} - \hat{y}_{vu}) & \dots & \sum (y_{2u} - \hat{y}_{2u})(y_{vu} - \hat{y}_{vu}) & \dots & \sum (y_{vu} - \hat{y}_{vu})^2 \end{vmatrix} \quad [8]$$

where all sums range from  $u = 1$  to  $u = n$  observations,  $y_{vu}$  is the observation for the  $v$ th response at the  $u$ th set of conditions and  $\hat{y}_{vu}$  is the corresponding value from the model to be fitted. A more concise form of this objective function is

$$|V| = \{v_{ij}\} \text{ where } v_{ij} = \sum_{u=1}^n (y_{iu} - \hat{y}_{iu})(y_{ju} - \hat{y}_{ju}) \quad [9]$$

for the  $i$ th and  $j$ th response.

For a two-response problem,  $v = 2$  and the parameters  $\underline{B}$  would best be estimated by minimizing the  $2 \times 2$  determinant of equation [8]. It is noted here that for the particular case of a single response, this procedure leads to the method of least squares.

## CHAPTER V

### RESULTS

#### 5.1 REPRODUCIBILITY OF SURFACE CONCENTRATION MEASUREMENTS

Surface concentration (weight/unit area) of albumin was calculated by comparing the radioactivity of an aliquot of solution of known concentration with that of a known area of surface. The surface concentration thus obtained was independent of the relative amount of labeled and unlabeled protein in solution, as shown by the data in Table 5. This indicates that there was no preferential selection of labeled or unlabeled molecules by the surface. The reproducibility of the surface concentration measurement was about  $\pm 5\%$  at the level of radioactivity most frequently used.

TABLE-5

Relative content of labeled protein in adsorbate solution. 10 mg% concentration	Surface Concentration $\mu\text{g}/\text{cm}^2 \pm \text{S.D.}$
1.0	$0.170 \pm 0.004$
7.1	$0.188 \pm 0.005$
12.1	$0.161 \pm 0.004$
28.0	$0.151 \pm 0.004$
38.4	$0.179 \pm 0.004$

## 5.2 ALBUMIN ADSORPTION KINETICS AND ISOTHERMS

### 5.2.1 Adsorption Kinetics

Figures 6 and 7 show surface concentration-time curves for two solution concentrations and four shear rates. Table 6 shows the plateau surface concentrations for all conditions studied. Adsorption times for these plateaux are 24 hours.

The adsorption kinetics experiments show that steady state surface concentration is reached in a short time with respect to the duration of the kinetic runs. Kinetic runs were extended to beyond a hundred hours.

Results show that the steady state surface concentration is reached within 2 hours and is maintained thereafter. This behaviour holds for both static and flow experiments. Steady state levels of the time-concentration data show the expected dependence on solution concentration, i.e. these levels increase with increase in solution concentration. However, these steady state levels do not show a dependence on shear rate within the range investigated (0 to 2500 sec<sup>-1</sup>).

### 5.2.2 Adsorption Isotherms

Data for the adsorption isotherms at various shear rates are shown in Table 6. The isotherms constructed for the albumin-polyethylene system are shown in Figs. 8, 9, 10, 11. They appear to have a shape typical of the Langmuir isotherm which can be represented by the relation:

$$\frac{y}{y_m} = \frac{bc}{1 + bc} \quad [10]$$

$y$  = amount of protein in the adsorbed monolayer in equilibrium with solution of concentration  $c$ ,



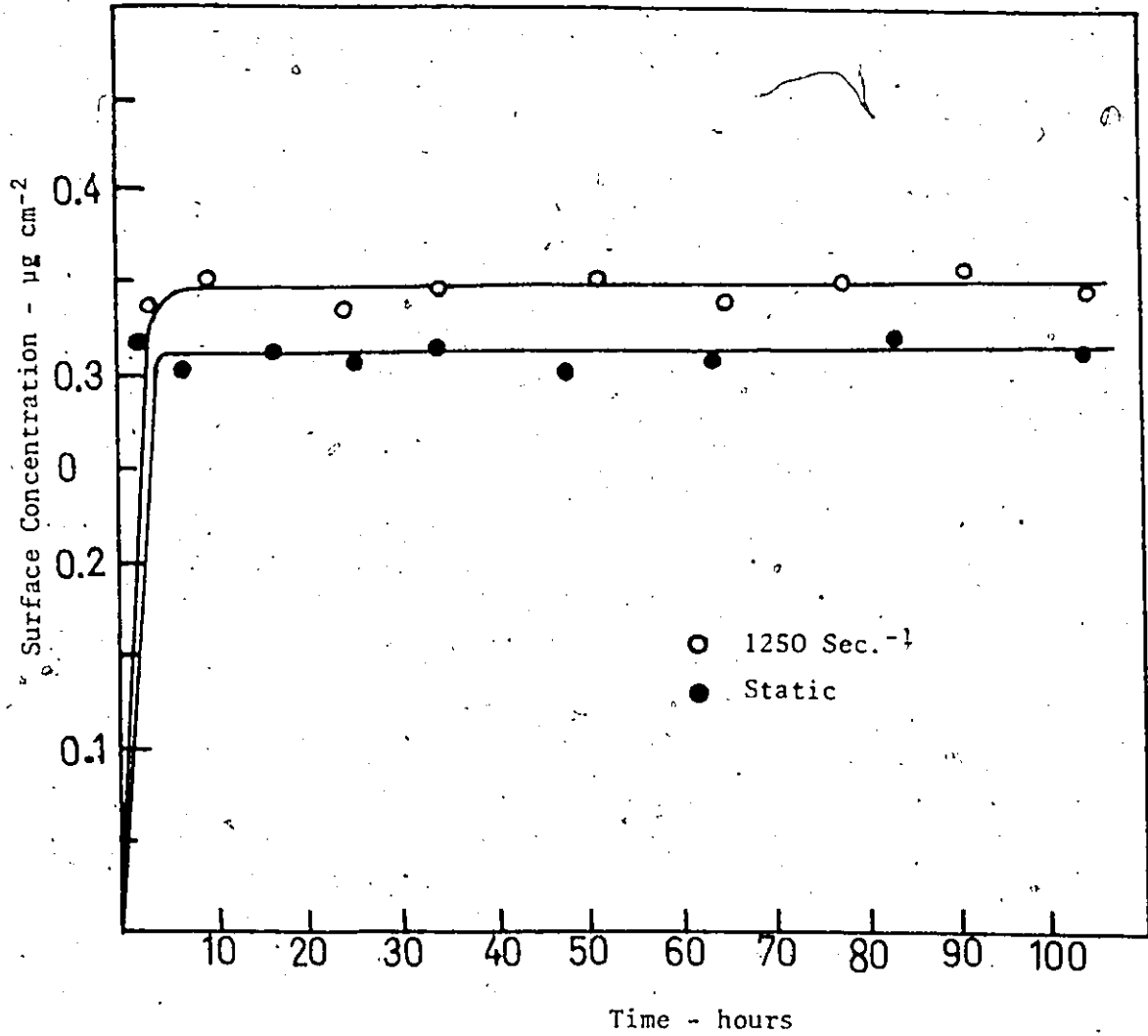


FIGURE 6

SURFACE CONCENTRATION - TIME CURVES 380 mg%  
ALBUMIN CONCENTRATION.

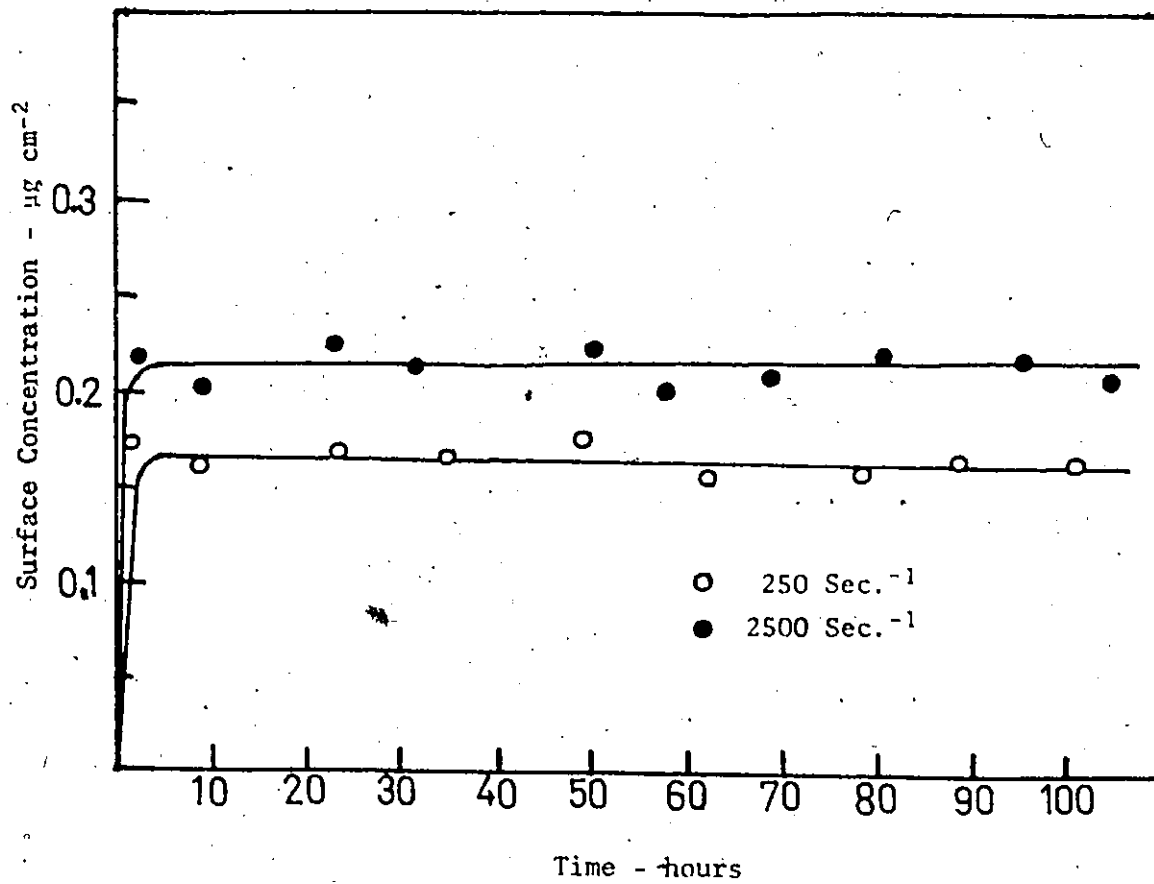


FIGURE 7

SURFACE CONCENTRATION - TIME CURVES 80 mg%  
ALBUMIN SOLUTION

TABLE 6

ADSORPTION OF ALBUMIN TO POLYETHYLENE - DEPENDENCE  
ON SHEAR RATE

Solution Concentration mg%	Equilibrium Surface Concentration $\mu\text{g}/\text{cm}^2$			
	0. Sec. <sup>-1</sup>	250 Sec. <sup>-1</sup>	1250 Sec. <sup>-1</sup>	2500 Sec. <sup>-1</sup>
5	0.07	0.047	0.04	0.045
10	0.11	0.09	0.085	0.095
20	0.135	0.135	0.1	0.13
30	0.145	0.145	0.14	0.15
60	0.17	0.175	0.19	0.18
80	0.175	0.17	0.22	0.225
140	0.21	0.27	0.24	0.285
200	0.215	0.26	0.30	0.34
290	0.265	0.29	0.285	0.31
380	0.32	0.39	0.34	0.375

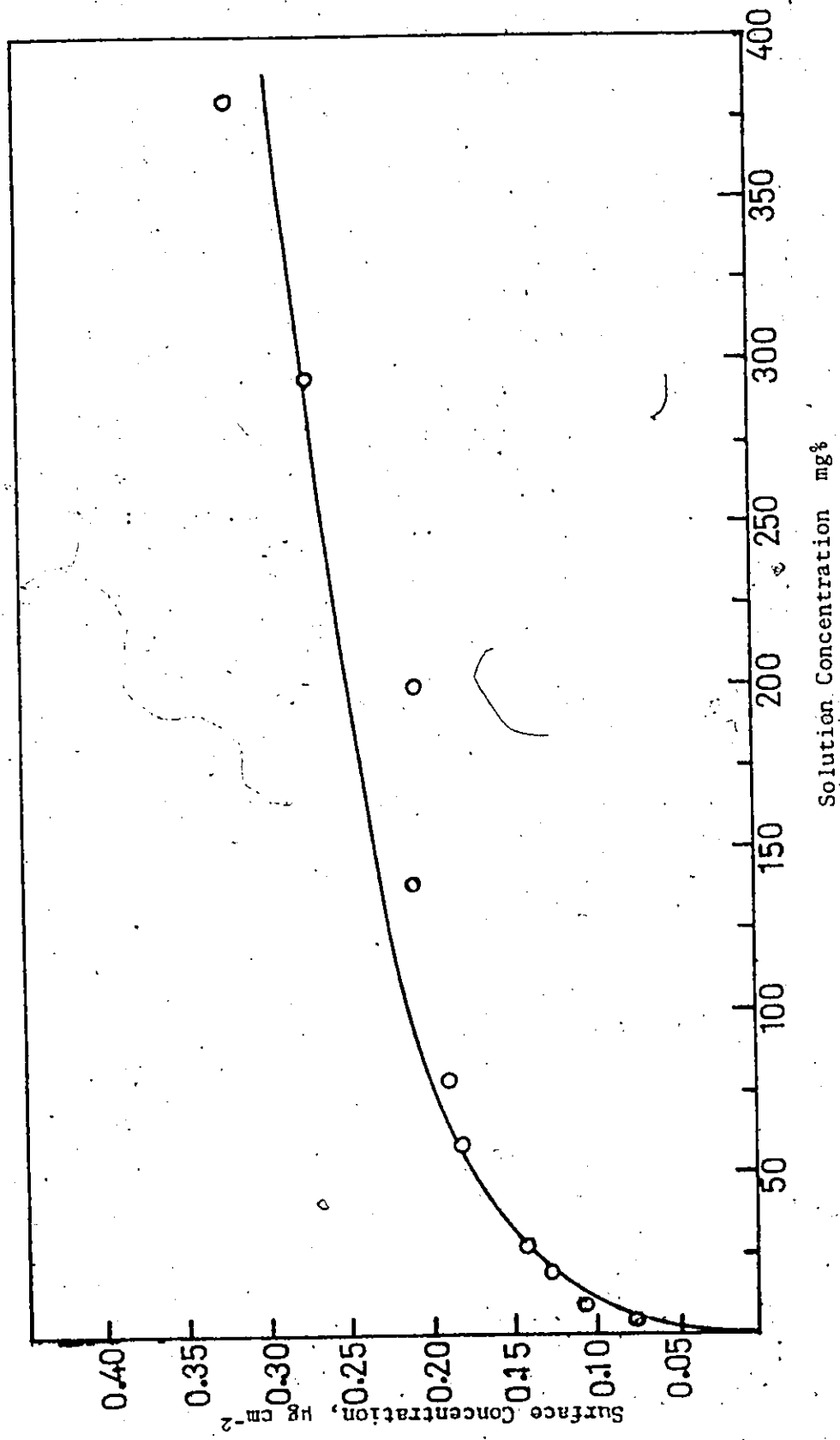


FIGURE 8

ISOTHERM OF ALBUMIN ADSORPTION TO POLYETHYLENE STATIC CONDITIONS  
AT 22° C

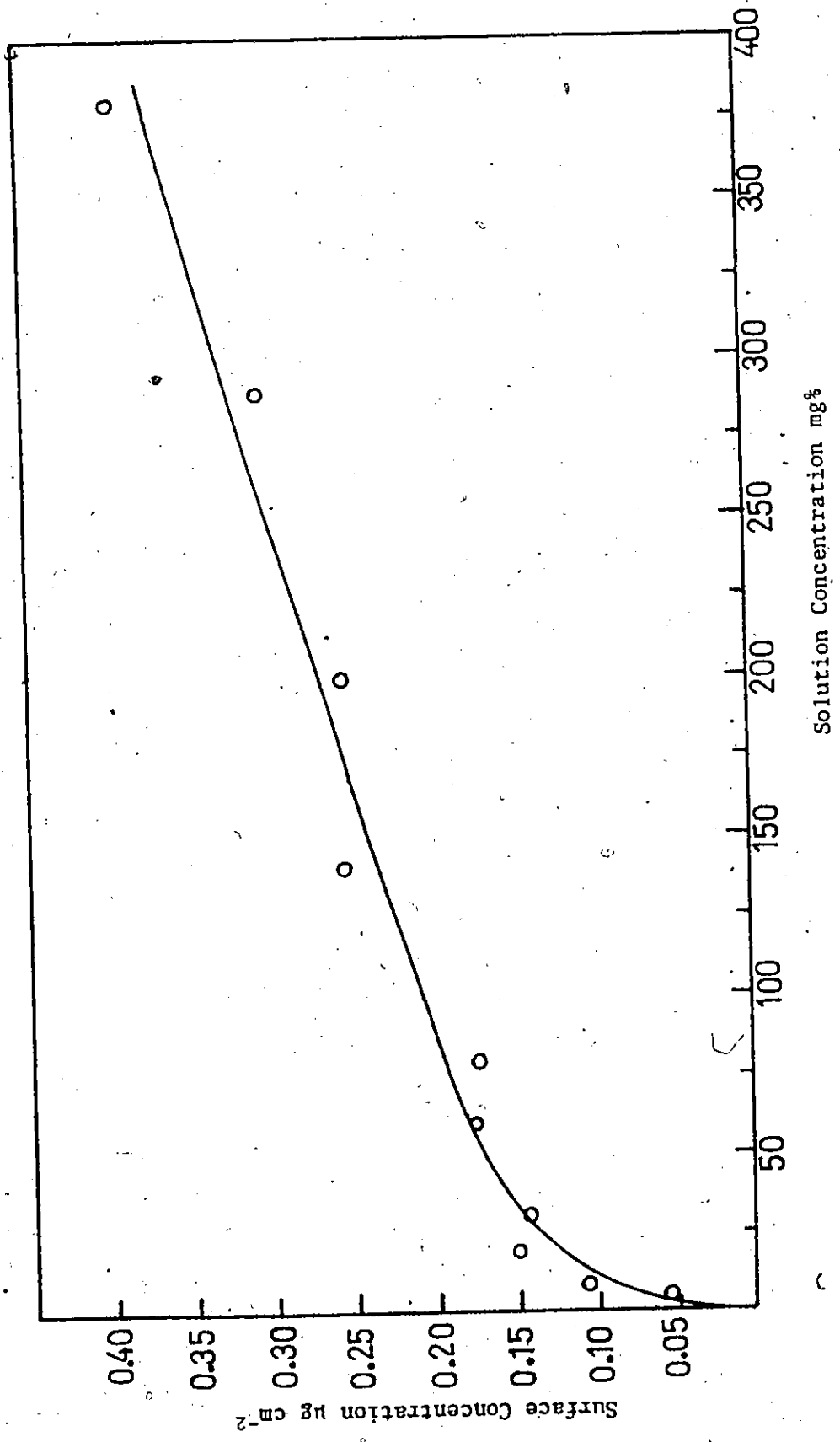


FIGURE 9

ISOTHERM OF ALBUMIN ADSORPTION TO POLYETHYLENE 250 Sec<sup>-1</sup>  
AT 22° C.

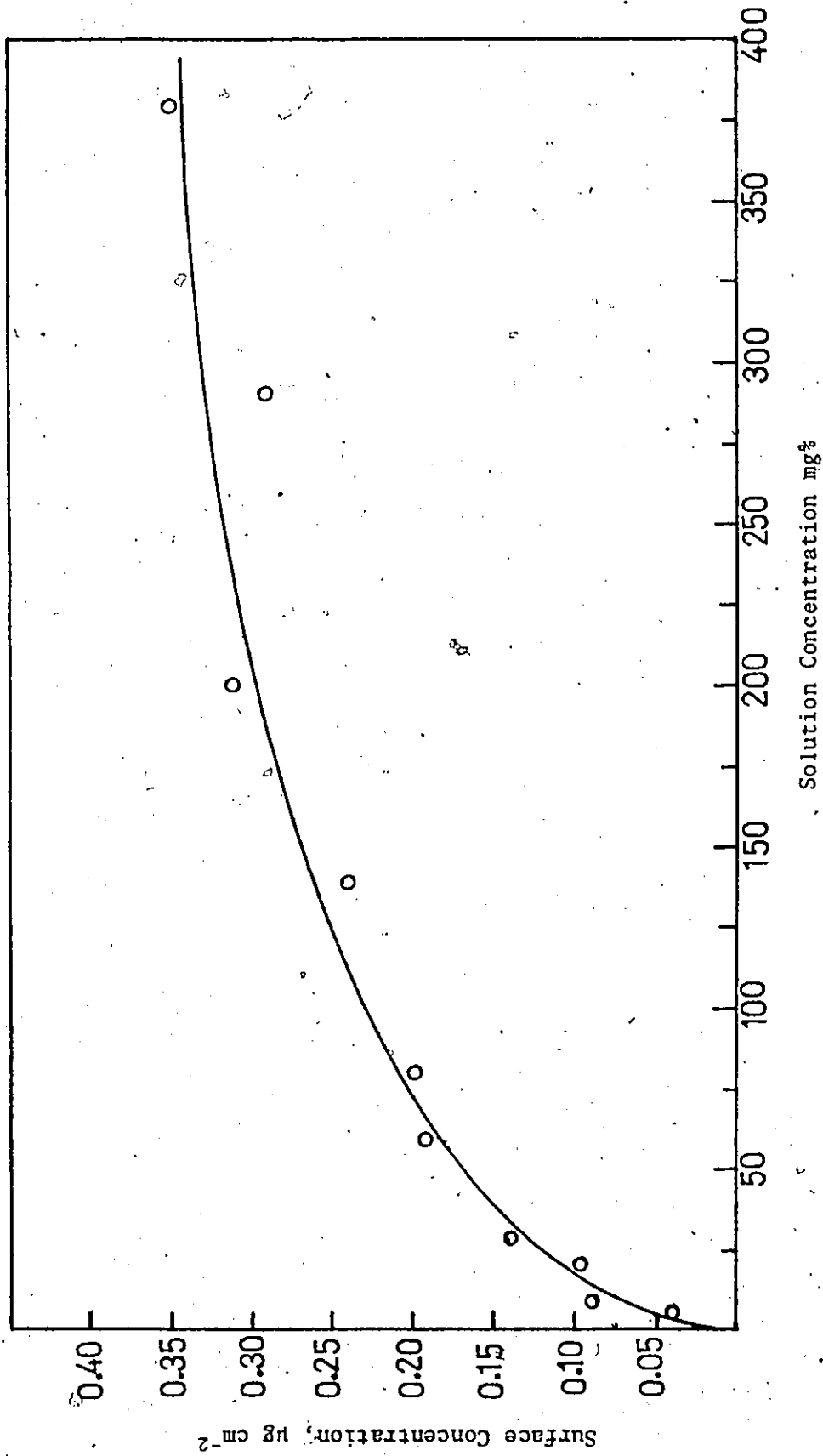


FIGURE 10

ISOTHERM OF ALBUMIN ADSORPTION TO POLYETHYLENE 1250 Sec. <sup>-1</sup>  
 AT 22° C

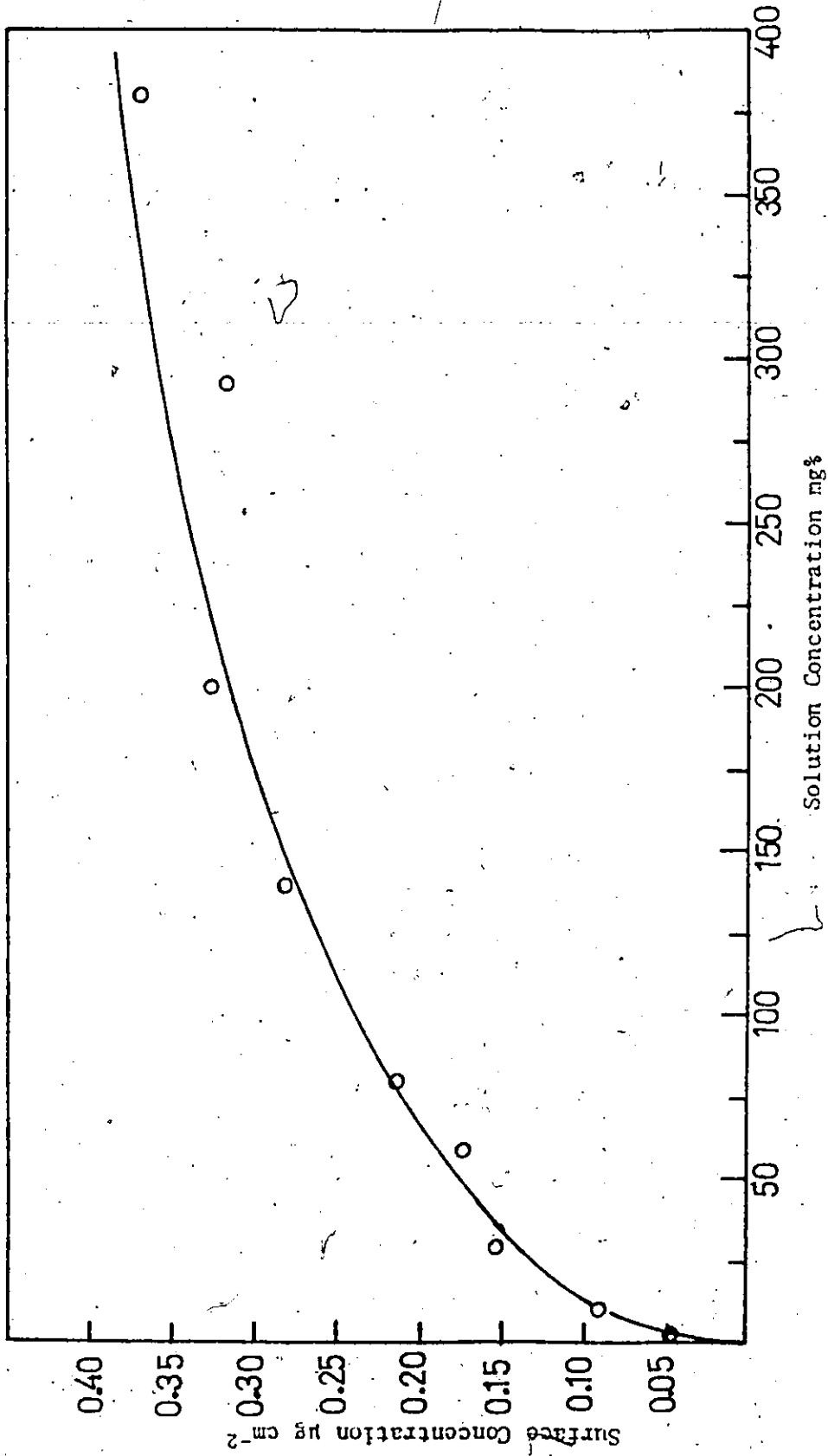


FIGURE 11  
ISOTHERM OF ALBUMIN ADSORPTION TO POLYETHYLENE } 2500 Sec.<sup>-1</sup>  
AT 22° C

$y_m$  = amount of protein in complete monolayer.

Strictly speaking the activity rather than the concentration should be used. However, in the case of dilute solutions we can use the concentration in the above expression with reasonable accuracy.

The term  $b$  according to Langmuir includes various constants and is dependent upon temperature (63,64).

$$b = \frac{N e^{E/RT}}{y_m \nu \sqrt{2\pi MRT}} \quad [11]$$

$N$  = Avogadro's number

$E$  = Activation energy of removal of the solute from the substrate  
(On the assumption that the adsorption has zero activation energy at low coverage for solutes or gases).

$y_m$  = The number of adsorbate molecules per square centimeter of complete monolayer.

$\nu$  = The frequency of oscillation of the adsorbate molecules perpendicular to the surface.

$R$  = The gas constant.

$T$  = Absolute temperature

Langmuir equation can be expressed in the following alternate form (63)

$$\frac{1}{y} = \frac{1}{bcy_m} + \frac{1}{y_m} \quad [12]$$

$$b = \frac{k_1}{k_2} = K_a \quad [13]$$

where:

$k_1$  and  $k_2$  are rate constants of adsorption and desorption respectively.

This relation is linear with respect to  $\frac{1}{y}$  and  $\frac{1}{c}$ .



Figures 12 and 13 show plots of  $\frac{1}{y}$  v.s.  $\frac{1}{c}$  for the isotherms corresponding to no flow and  $2500 \text{ sec}^{-1}$  shear rate respectively. The plots show the linear relation expected in Langmuir adsorption systems.

### 5.3 TURNOVER RESULTS

The results of the turnover study conducted appear to confirm the possibility put forward by Brash et al. (65) that while the protein layer adsorbed on a polymer surface may remain indefinitely at the steady state level of a close-packed monolayer, it may be exchanging material with the fluid in contact with the surface. It was observed, as shown in Figs. 14 - 17, that the albumin adsorbed on the surface, tagged with one radioactive isotope ( $^{125}\text{I}$ ) is released from the surface in an exponential decay pattern, while albumin present in the bulk solution tagged with another isotope ( $^{131}\text{I}$ ) moves on to the surface to replace the released protein. The process continues with time until the two species (with respect to radio-labeling) reach an equilibrium value on the surface.

It was observed that within the range of time studied, up to 100 - 120 hours exchange time, the turnover took place within the steady state condition of a constant surface concentration, as reported in the adsorption kinetics results above.

The two responses collected for different combinations of shear rate and solution concentration were used to estimate the invariants of the turnover process. These invariants are used to estimate the specific rate constants of the surface reaction and to examine the dependence of these rate constants on both the shear rate and solution concentration.

Four solution concentrations were used in the turnover study:

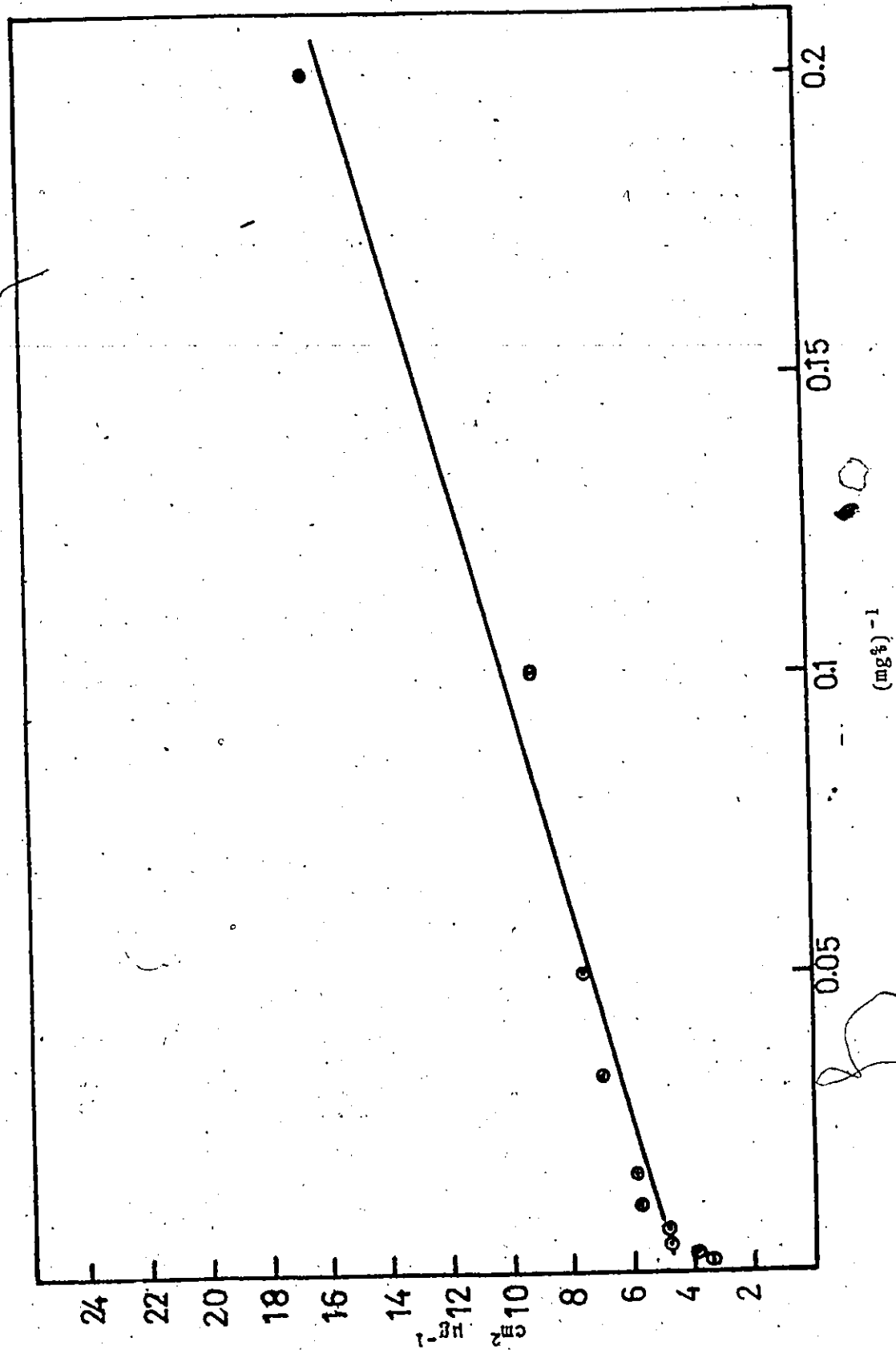
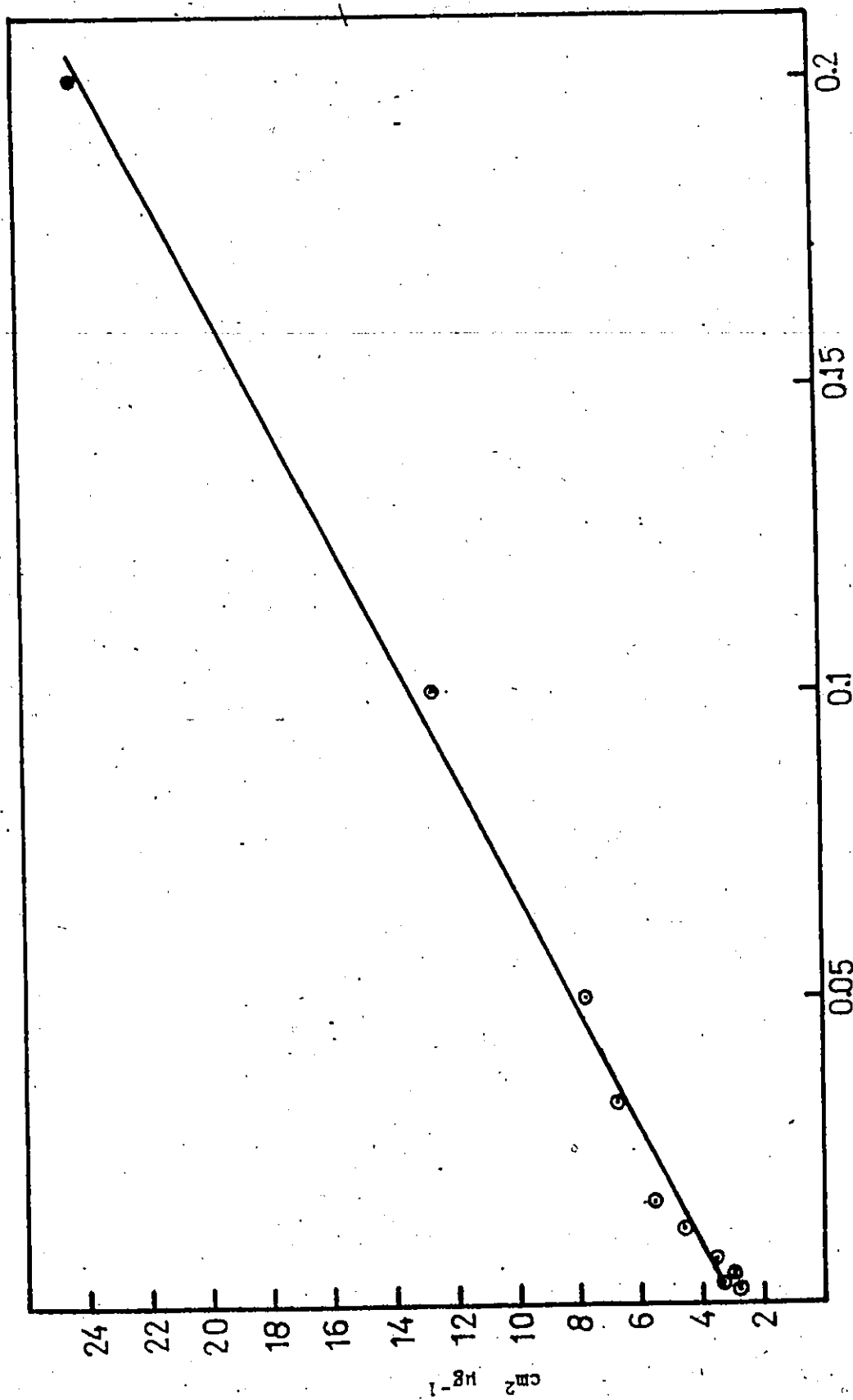


FIGURE 12  
LANGMUIR CURVE FOR ALBUMIN ADSORPTION  
TO POLYETHYLENE, STATIC CONDITIONS



$(\text{mg}\%)^{-1}$

FIGURE 13

LANGMUIR CURVE FOR ALBUMIN ADSORPTION  
TO POLYETHYLENE, 2500 Sec.<sup>-1</sup>

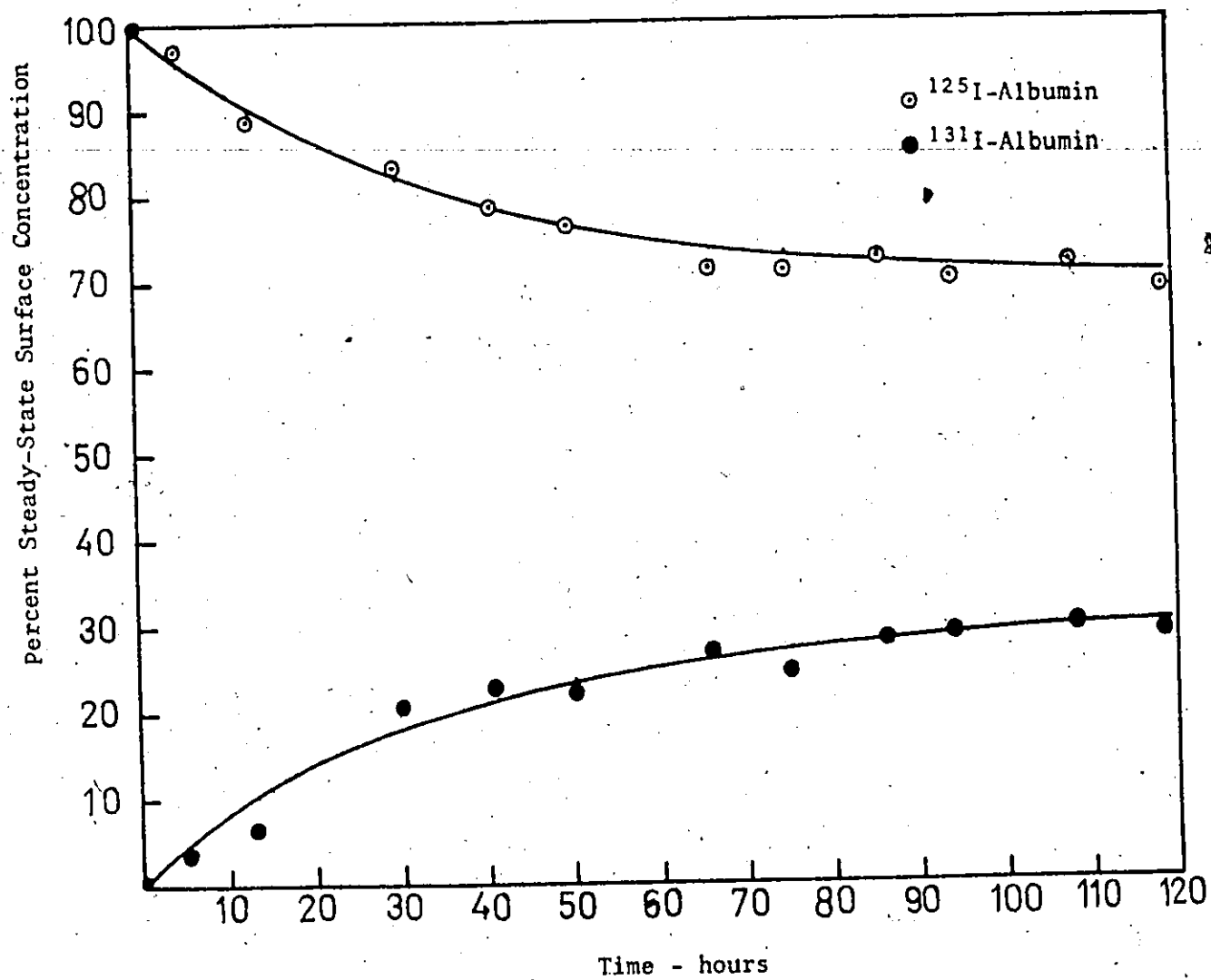


FIGURE 14

TURNOVER CURVES FOR 80 mg% ALBUMIN SOLUTION 250 Sec.<sup>-1</sup>

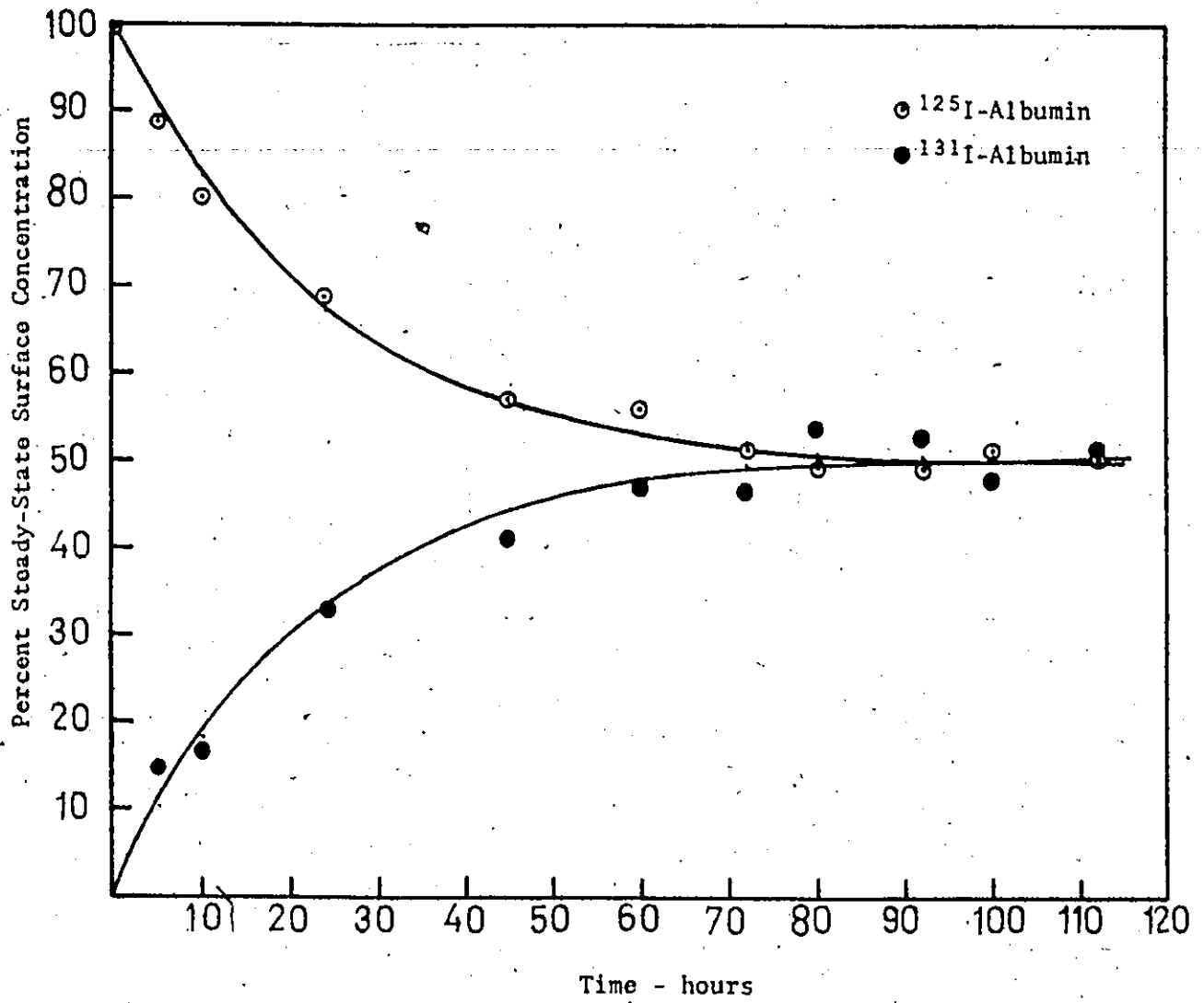


FIGURE 15

TURNOVER CURVES FOR 200 mg% ALBUMIN SOLUTION  $2500 \text{ Sec.}^{-1}$

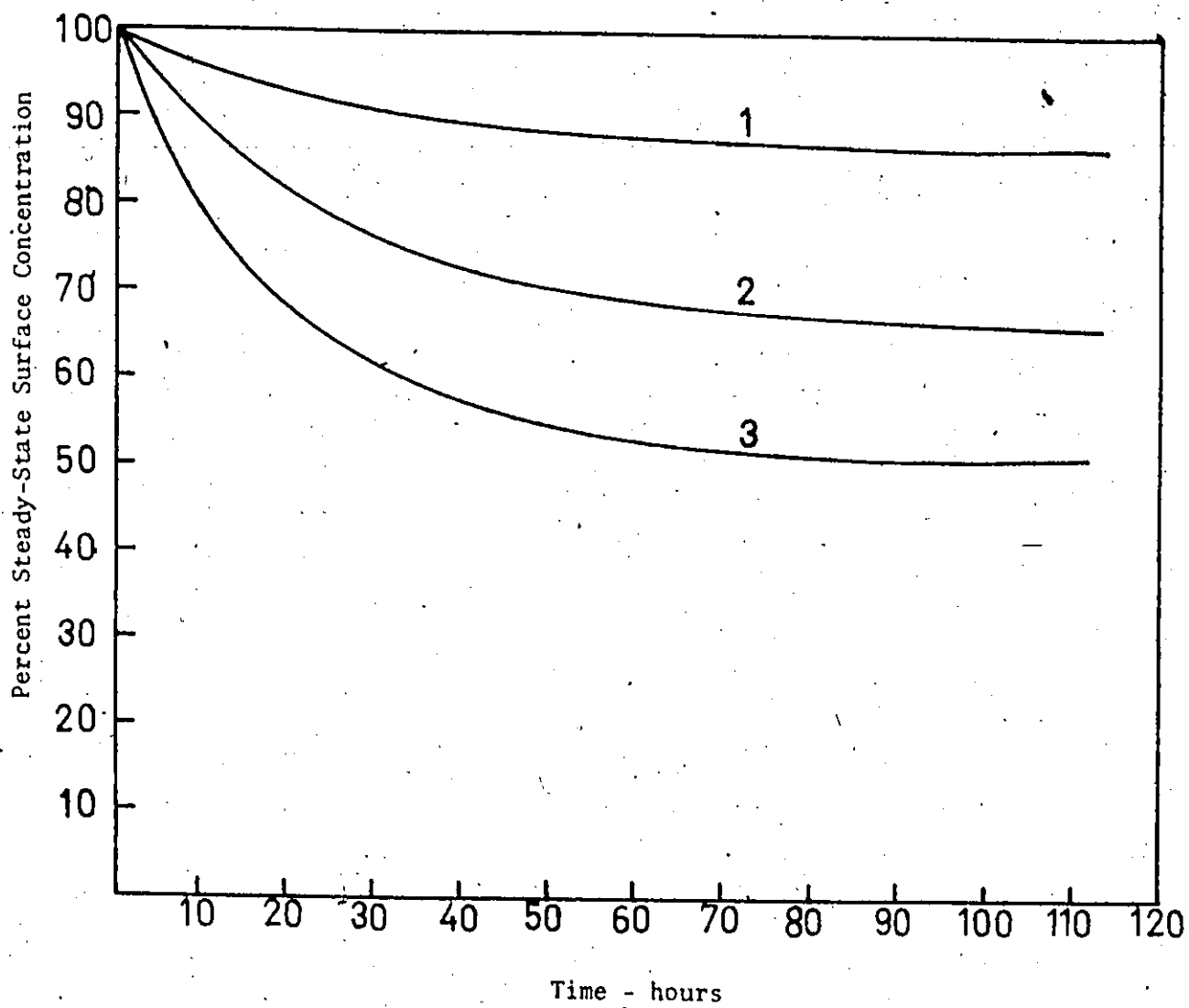


FIGURE 16

FITTED TURNOVER DECAY CURVES FOR 10 mg% ALBUMIN SOLUTION.

1. 250 Sec.<sup>-1</sup>
2. 1250 Sec.<sup>-1</sup>
3. 2500 Sec.<sup>-1</sup>

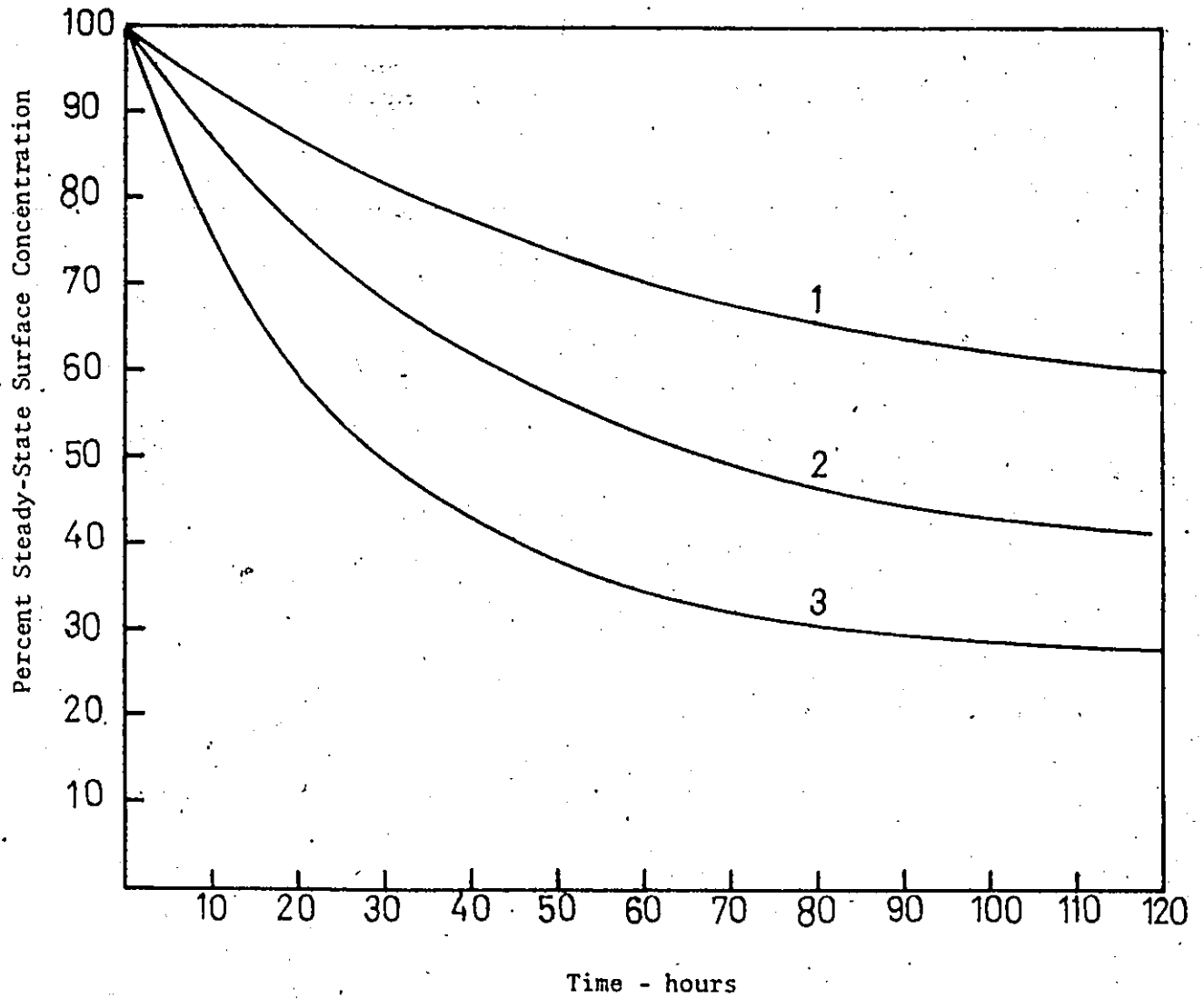


FIGURE 17

FITTED TURNOVER DECAY CURVES FOR 380 mg% ALBUMIN SOLUTION.

1. 250 Sec.<sup>-1</sup>
2. 1250 Sec.<sup>-1</sup>
3. 2500 Sec.<sup>-1</sup>

10, 80, 200 and 380 mg/100 ml under static conditions as well as under flow rates corresponding to 250, 1250, 2500 reciprocal seconds wall shear rate.

The static runs did not show detectable turnover between the surface albumin and the solution albumin for the 10 and 80 mg% solution. Slight turnover was observed with the 200 mg% solution where the extent of exchange was about 5% of the steady state surface concentration in a span of 120 hours. 15% turnover was observed under static conditions with the 380 mg% solution after 120 hours. When flow was introduced, much higher turnover was observed at comparable exchange time (Figs.14, 17).

Non-linear parameter estimation was used to find estimates of the parameters of the proposed model to describe the turnover process under flow conditions. For the decay curve describing the species originally on the surface the model proposed is:

$$y_1 = \sum_{i=1}^{n-1} g_i e^{-B_i t} + g_0 \quad [14]$$

With the steady state condition of constant surface concentration holding and with the concentration of the species present on the surface during the turnover process expressed as a fraction of the steady state concentration, the expected model of the other response is

$$y_2 = 1 - y_1 = 1 - \left( \sum_{i=1}^n g_i e^{-B_i t} + g_0 \right) \quad [15]$$

where  $y_1$  = fraction of the steady state concentration attributed to original species on the surface.



$y_2$  = fraction of the plateau concentration attributed to protein species in solution.

$t$  = time.

The data were fitted to the models with a digital computer subroutine based on Marquardt's non-linear least squares parameter estimation method (55).

Forms of the model with different numbers of exponential terms were used for fitting the data. The goodness of fit for parameter estimation for the different model forms was checked by examining the ratio of residual sum of squares of each form fit according to the formula:

$$\frac{S_k}{S_{k+1}} > 1 + \frac{\Delta P}{n - P_{k+1}} F_{\Delta P, n - P_{k+1}} \quad [16]$$

where  $S_k$  = residual sum of squares corresponding to a model form of  $k$  exponential terms.

$S_{k+1}$  = residual sum of squares corresponding to a model form of  $k+1$  exponential terms.

$n$  = number of points fitted.

$\Delta P$  = increase in the number of parameters due to the inclusion of the extra exponential term (=2).

$P_{k+1}$  = number of parameters corresponding to  $k+1$  exponential terms.

$F_{\Delta P, n - P_{k+1}}$  = The value of the F-distribution function with  $\Delta P$  and  $n - P_{k+1}$  degrees of freedom at 95% confidence level obtained from F-distribution table.

If the addition of one more exponential term significantly improves the goodness of fit, the inequality above holds.

Using the above criterion for each response independently, it was found that the data fit reasonably well to the model form having one exponential term:

$$y_1 = g e^{-Bt} + g_0 \quad [17]$$

$$y_2 = g(1 - e^{-Bt}) \quad [18]$$

where  $g_0 = 1.0 - g$  [19]

when  $y_1$  and  $y_2$  are expressed as fractions of the steady state surface concentration.

Estimates of the parameters obtained from fitting data to this model for different solution concentrations and shear rates for each response are shown in Tables 7 and 8. Figures 14 - 17 show the two fitted responses as well as the data for the turnover process at different shear rates and solution concentrations.

Some experimental runs were performed in which albumin was adsorbed on the polyethylene surface until steady state surface concentration was reached for a certain shear rate. Pure solvent was then circulated in the system at the same shear rate. It was observed that the surface concentration remained constant and that "pure shear" removal of adsorbed albumin from the surface within the range of shear rates used in this study did not take place.

After it was found that the data fit reasonably well to the proposed model for each response by the ordinary least squares method, and that the individual confidence intervals for each parameter estimate overlaps for both responses (see Tables 7 and 8), the Bayesian multivariate analysis technique of Box and Draper<sup>(55,61)</sup> was used to obtain

TABLE 7

TURNOVER OF ADSORBED ALBUMIN AT A POLYETHYLENE SURFACE  
 ESTIMATES OF THE PARAMETER B IN  $y = ge^{-Bt} + g_0$

Solution Concentra- tion mg%	250 Sec. $^{-1}$		1250 Sec. $^{-1}$		2500 Sec. $^{-1}$	
	125I	131I	125I	131I	125I	131I
10	0.018 (0.012, 0.0200)	0.017 (0.011, 0.021)	0.042 (0.039, 0.046)	0.048 (0.034, 0.0618)	0.0378 (0.028, 0.0468)	0.0353 (0.02, 0.05)
80	0.03 (0.021, 0.055)	0.0317 (0.024, 0.038)	0.024 (0.018, 0.030)	0.032 (0.020, 0.043)	0.0396 (0.025, 0.053)	0.038 (0.022, 0.054)
200	0.036 (0.018, 0.043)	0.032 (0.028, 0.046)	0.0334 (0.027, 0.039)	0.0366 (0.025, 0.0479)	0.044 (0.038, 0.049)	0.043 (0.033, 0.055)
380	0.017 (0.014, 0.0201)	0.0201 (0.016, 0.024)	0.0268 (0.024, 0.029)	0.0264 (0.021, 0.0311)	0.0404 (0.035, 0.044)	0.0448 (0.035, 0.053)

TABLE 8

TURNOVER OF ADSORBED ALBUMIN AT A POLYETHYLENE SURFACE  
 ESTIMATES OF PARAMETER  $k$  NORMALIZED TO EQUILIBRIUM  
 SURFACE CONCENTRATION OF 1.0.  
 (95% confidence interval in parentheses)

Solution Concentration mg%	250 sec. <sup>-1</sup>		1250 sec. <sup>-1</sup>		2500 sec. <sup>-1</sup>	
	125I	131I	125I	131I	125I	131I
10	0.225 (0.198, 0.24)	0.213 (0.188, 0.256)	0.33 (0.324, 0.336)	0.32 (0.3, 0.341)	0.517 (0.483, 0.551)	0.555 (0.487, 0.622)
80	0.307 (0.285, 0.316)	0.301 (0.282, 0.319)	0.666 (0.598, 0.733)	0.665 (0.584, 0.746)	0.802 (0.71, 0.891)	0.812 (0.703, 0.925)
200	0.262 (0.246, 0.278)	0.266 (0.244, 0.28)	0.413 (0.389, 0.438)	0.414 (0.376, 0.452)	0.505 (0.29, 0.521)	0.515 (0.484, 0.545)
380	0.44 (0.409, 0.471)	0.471 (0.437, 0.504)	0.598 (0.577, 0.62)	0.606 (0.568, 0.644)	0.713 (0.653, 0.834)	0.723 (0.666, 0.770)

better estimates of the parameters. According to that technique, the Bayesian estimate of parameters for two responses is that which minimizes the determinant of the matrix  $V$  where  $V_{ij}$ , the element of that matrix resulting from the two responses is

$$V_{ij} = \sum_{u=1}^n (y_{1u} - \hat{y}_{1u}) (y_{2u} - \hat{y}_{2u})$$

where  $n$  = number of observations

$y_{1u}$  = the  $u$ th observation of the first response

$\hat{y}_{1u}$  = the corresponding value of the Model's response

$y_{2u}$  = the  $u$ th observation of the second response

$\hat{y}_{2u}$  = the corresponding value of the Model's response.

A grid search method was used to minimize the determinant of  $V$  being the objective function, and find estimates for the parameters. Estimates of parameters by this method are shown in Tables 9 and 10.

TABLE 9

ESTIMATES OF PARAMETER B BY MULTIVARIATE ANALYSIS

Solution Concentration mg%	Shear Rate		
	250 sec <sup>-1</sup>	1250 sec <sup>-1</sup>	2500 sec <sup>-1</sup>
10	0.0176 (0.017-0.0179)	0.0435 (0.0426-0.044)	0.0369 (0.036-0.0372)
80	0.028 (0.0273-0.029)	0.03 (0.029-0.0313)	0.0383 (0.0378-0.039)
200	0.0325 (0.031-0.0328)	0.035 (0.0328-0.0358)	0.0435 (0.043-0.0441)
380	0.019 <sup>s</sup> (0.018-0.0201)	0.0267 (0.025-0.028)	0.0403 (0.0385-0.041)

TABLE 10

ESTIMATES OF PARAMETER  $g$  BY MULTIVARIATE ANALYSIS

Solution Concentration mg%	Shear Rate		
	250 sec <sup>-1</sup>	1250 sec <sup>-1</sup>	2500 sec <sup>-1</sup>
10	0.223 (0.203-0.231)	0.331 (0.315-0.342)	0.522 (0.517-0.533)
80	0.304 (0.285-0.315)	0.664 (0.649-0.678)	0.810 (0.801-0.821)
200	0.263 (0.249-0.274)	0.413 (0.039-0.0425 )	0.508 (0.493-0.517)
380	0.456 (0.435-0.47 )	0.601 (0.596-0.612)	0.721 (0.717-0.733)

## CHAPTER VI

## DISCUSSION

6.1 KINETICS OF ADSORPTION AND ISOTHERMS

In agreement with previous studies<sup>(27,28,29,31)</sup>, it was found that a steady state surface concentration is reached within a short time of exposing the polymer surface to the albumin solutions, usually in the first two hours. Similar behaviour has been observed for mixtures of proteins by Lee and Kim<sup>(31)</sup>. These authors studying protein adsorption on hydrophobic surfaces, found times required to reach steady state ranging from 10 to 60 minutes under static conditions and 10 to 250 minutes under flow conditions with the highest time corresponding to the highest flow rate. In the present study, steady state concentration was reached within the first two hours at all shear rates used from zero to 2500 sec<sup>-1</sup>. However, the time scale of the kinetic runs here was much longer than that of Lee and Kim. On the other hand they report the flow in terms of milliliters per second without mentioning the wall shear rates, which makes the comparison difficult.

The dependence of steady state concentration on flow rate found by Lee and Kim suggests that protein adsorption is diffusion dependent. Of the surfaces used in their study, only segmented copolyether-urethane-urea did not show this flow dependence. Lee and Kim explain this in terms of the roughness of the polyurethane surface compared with other surfaces that do show flow dependence.

In the present study steady state surface concentration was not found to show regular dependence on wall shear rate and there was no spatial change of albumin concentration along the tube axis. This suggests that, with respect to polyethylene, convective transport does not play a major role in albumin adsorption, contrary to the findings of Lee and Kim. Nerem et al. (32) did not find albumin uptake by an arterial wall both in vivo and in vitro to be diffusion dependent, which is in agreement with the findings reported here.

The adsorption isotherms were found to fit the Langmuir model of adsorption. This is in agreement with the findings of many authors (16,17,23,27,31). The Langmuir equation describes well a lot of gas-solid adsorption data and can be derived from the kinetic theory as well as from thermodynamics (63). However, it was also shown to fit some solute adsorption processes on solid substrate, since solute adsorption is very similar to that in gas adsorption, the difference being in the free path of the molecules (64,66).

The Langmuir treatment takes into account only the forces between adsorbate and substrate and is confined to monolayer coverage. In other words the Langmuir model implies that there is no lateral interaction between the adsorbate molecules. Furthermore, the Langmuir model assumes an energetically homogeneous surface. The shape of the isotherms obtained at different flow conditions, as well as the fact that it can be shown that a monolayer has not been exceeded (implying no solute-solute interaction that could lead to multilayer formation) provides additional confirmation that the system can be described fairly well by a Langmuir model. However, there are other factors to be considered, e.g. observations by Peterlin and Olf (20) regarding the surface morphology of polyethylene.



These authors have shown that the ratio and spatial distribution of crystalline and amorphous regions depend on factors such as drawing and annealing conditions. The amorphous-crystalline balance in turn was found to affect the sorption of water vapour. These findings plus the possibility of having some chemical inhomogeneity in the polyethylene surface, for example the presence of carbonyl groups ( $\text{C} = \text{O}$ ) in some oxidized spots on the surface capable of forming hydrogen bonds, make the assumption of an energetically homogeneous surface somewhat simplistic. The presence of a part of the adsorbed protein that is unexchangeable with the bulk protein in the turnover experiments may be seen as an indication of the presence of such factors. Since the Langmuir model ideally implies complete reversibility, which was not found to hold in this study.

Observed surface concentration levels of the isotherms are within the monolayer adsorption range, values for a close-packed monolayer being in the range of 0.25 to 0.68  $\mu\text{g}/\text{cm}^2$  for globular molecules based on the data of Oncley et al<sup>(11)</sup> and using a molecular weight of 69,000. These data are in agreement with infra red results obtained by Brash and Lyman (23). The data obtained here suggest that the adsorbed layer is at the lower end of the range for a close-packed configuration indicating a side-on disposition. However, the transmission electron micrograph shown in Fig.4 shows that the molecular dimensions of albumin are smaller than the roughness scale of the surface. This implies that the adsorption area is larger than the geometric surface area of the test tubes used for obtaining the observed surface concentration. So the real surface

concentration is expected to be lower than that reported in the isotherm data. This difference does not affect the quantitative analysis of the data since the error is constant for all the experimental results reported.

The concentration isotherms for static and flow conditions are constructed over the concentration range 0 - 380 mg%, the upper limit being about 10% from the albumin concentration in plasma. Adsorption time was 24 hours.

The results found here are different from those previously reported by Brash and Lyman<sup>(23)</sup>. Under static conditions it was found that the isotherm rises continuously through the concentration range used with a change of slope between 10 and 50 mg%. The slope at concentrations greater than 50 mg% is much smaller than the initial slope and even if extrapolated to physiological concentration, the surface layer is only about twice the upper limit for close-packed monolayer<sup>(65)</sup>.

While Brash et al<sup>(65)</sup> suggest that it is probable that the isotherm would decrease in slope and become asymptotic to the upper limit of the range for a close-packed monolayer, Hoffman<sup>(42)</sup> reports that the isotherm continues to have an upward trend until physiological concentration range, the corresponding plateau concentration being more than  $1.5 \mu\text{g}/\text{cm}^2$  which is about twice the upper limit of monolayer range. Hoffman attributes this to the presence of traces of fatty acids in albumin, he reports that complete defatting of albumin appears to normalize the appearance with a plateau level attained at a concentration of about 500 mg%. The form of the isotherms in this work is more similar to that

expected for physical adsorption than for chemical adsorption<sup>(67)</sup> showing a small but significant increase with solution concentration.

## 6.2 TURNOVER KINETICS

Results of the turnover process reported in Tables 9 and 10, and shown in Figs.14-17, show dependence of the parameters describing the process on shear rate. Results also show that a part of the protein adsorbed on the surface is non-exchangeable with the bulk solution protein. It is also found that the decay data can be described adequately by a model of the form:

$$y = g e^{-Bt} + g_0 \quad [17]$$

when surface concentrations are normalized to a value of 1.0. This model can be derived from first order kinetics as follows. During the turnover process there are two reactions taking place simultaneously: desorption, in which albumin originally adsorbed on the surface moves out to the solution with a rate constant  $k_1$ , and adsorption onto the sites vacated by the desorbed albumin with a rate constant  $k_2$ . Because of the small surface to volume ratio of the system which makes the total amount of protein on the surface very small compared to the protein content of the solution, it can be assumed that the solution concentration remains constant during the experiment. This implies that

$$k_1 \gg k_2$$

If we assume that:

$N_1$  = The number of originally adsorbed albumin molecules remaining on the surface per  $\text{cm}^2$  at time  $t$  during exchange.

$N_0$  = The number of albumin molecules incapable of exchanging with

the solution albumin per  $\text{cm}^2$ .

$N_T$  = The total number of albumin molecules on the surface at steady state.

Assuming first order kinetics we can express the desorption process by the following equation

$$\frac{d(N_i - N_o)}{dt} = -k_1(N_i - N_o) \quad [20]$$

The solution of this differential equation is

$$\ln(N_i - N_o) = -k_1 t + \text{const.}$$

since at  $t = 0$ ,  $N_i = N_T$

$$\frac{N_i - N_o}{N_T - N_o} = e^{-k_1 t} \quad [21]$$

Normalizing the number of molecules to steady state level

$$\frac{N_i}{N_T} = \left(1 - \frac{N_o}{N_T}\right) e^{-k_1 t} + \frac{N_o}{N_T} \quad [22]$$

Comparing this result with the model equation [17] we find that

$B = k_1$  = the desorption rate constant  $\text{hr}^{-1}$

= the fractional turnover rate

$(1.0 - g) = \frac{N_o}{N_T}$  = the unexchangeable part of albumin as a fraction of steady state =  $g_o$

$g = \left(1 - \frac{N_o}{N_T}\right)$  = the exchangeable fraction of the steady state surface concentration.

Examining Table 9 it can be seen that the fractional turnover rate  $B$  does not show a correlation with solution concentration. This may be explained by the fact that even at the lowest solution concentration (10  $\text{mg}\%$ ) the solution albumin content is much larger than the total albumin ad-

sorbed on the surface. Therefore, increase in solution concentration is not expected to affect the turnover rate. Increase in shear rate, on the other hand, seems to increase the fractional turnover rate. It is also noted that shear forces of pure solvent do not bring about detectable removal of adsorbed albumin from the surface. These two observations suggest that a main factor behind desorption, and subsequently exchange with the bulk solution protein, is the continuous "bombardment" of the adsorbed albumin with solution albumin under flow conditions. The energy acquired by solution albumin molecules at higher shear rates would increase the rate of molecular collisions, giving rise to faster removal of exchangeable adsorbed albumin and consequently higher fractional turnover rate as observed.

It is also noted that while there is no detectable exchange under static conditions at the low range of solution concentrations studied, very slight exchange is observed with the 200 mg% solutions, and slightly greater exchange with the 380 mg%. This may suggest that as the solution concentration increases, protein-protein interaction plays a larger role in the turnover process. So it is probable that with albumin concentration reaching physiological level, substantial turnover would be observed under static conditions. The parameter  $g$  (Table 10) which is a measure of the exchangeable part of the adsorbed albumin, appears to be dependent, solely, on the shear rate, with no apparent dependence on solution concentration under flow conditions. However, the effect of solution concentration on the unexchangeable part of the adsorbed protein may be more apparent at concentrations approaching the

physiological level when exchange is expected to be more substantial under static conditions

There is more than one possibility to account for the non-exchangeable part of adsorbed albumin represented by parameter  $g_0$ . A possibility is the presence of sites on the polyethylene surface capable of forming bonds with albumin stronger than the usual Van der Waal's bonds involved in physical adsorption (e.g. hydrogen bonds). The presence of carbonyl groups ( $\text{C} = \text{O}$ ) at oxidized spots on the surface would provide sites for possible hydrogen bonding.

The idea that adsorbed protein is bound to a solid substrate in more than one way is not new. Vroman<sup>(35)</sup> has suggested that hydrophobic and hydrophilic bonding occur in protein adsorption. This concept was used to explain why fibrinogen adsorption renders glass more, and lucite less hydrophobic. Earlier Lindau and Rhodius<sup>(68)</sup> noticed that quartz acquired hydrophobic character by adsorbing albumin, indicating that the polar side chains of the protein are directed toward the quartz with the hydrophobic side chains pointing toward water.

Dillman and Miller<sup>(41)</sup>, studying protein adsorption on cation exchange and non-ion exchange polymer membranes, found evidence to support the presence of two types of bonding sites giving rise to two types of protein bonding to the surface. However, they do not provide a physical explanation for the presence of these different sites. The presence of more than one type of protein bonding is relevant in explaining reports that protein adsorption can be irreversible, partly irreversible or reversible, since reversibility can then be dependent on the ratios

of the different types of bonding present.

Another possibility that could account for the presence of a non-exchangeable part of adsorbed albumin is that part of the protein in the early stages of adsorption is denatured in the process of binding with the surface. This would lead to uncoiling of the albumin molecule on the surface exposing additional side chains which could provide hydrogen bonding sites for attachment. The average binding forces would then be greater than for native protein.

A third possibility is the difference in behaviour of the crystalline and amorphous parts of the polyethylene surface as described by Peterlin and Olf<sup>(20)</sup>. They found that sorption of water vapour takes place preferentially into the amorphous part. It is possible that a similar preference may exist in protein adsorption.

One of these possibilities or a combination of them may be responsible for the presence of a non-exchangeable portion of the albumin adsorbed to polyethylene surfaces.

### 6.3 RELATION TO THROMBOGENICITY

There is almost unanimous agreement that plasma protein-polymer surface interactions are intimately linked to traumatic effects such as thrombus formation that take place when blood comes in contact with these surfaces. In this study interactions between albumin and polyethylene were examined. Albumin is the most abundant protein in plasma while polyethylene is one of a wide group of uncharged hydrophobic polymers extensively used clinically and investigated in biomedical research. Previous studies of protein adsorption on hydrophobic surfaces<sup>(9,23)</sup>, showed that the layers formed from simple solutions consisted of close packed mono-

layers, while no alteration in shape occurred, suggesting no denaturation. If this mode of interaction could be taken as characteristic of proteins in general, including clotting factors, then it was felt that a contact activation mechanism of clotting could not apply to these surfaces. It seems possible, however, that in blood or plasma the adsorbed layer, while remaining at the steady state level of close-packed monolayer, could be exchanging material with the fluid and that protein released from the surface could be altered. If the protein were a clotting factor the alteration might take the form of activation. If exchange is occurring to any extent, as proved true in this study, the composition of the layer then may alter with time. Thus, a layer of albumin, formed initially and furnishing protection against platelet adhesion, might exchange with fibrinogen and become highly adhesive to platelets.

The present study shows that exchange of adsorbed protein with dissolved protein does take place. A relation between shear rate in a flow situation (normally met in clinical assist devices and artificial organs), and protein turnover rate was also found. However, exchange was not found to be complete for the surface studied and the shear rates used.

In a recent study of in vivo adsorption of proteins on polymer surfaces, Lyman et al<sup>(69)</sup> concluded that for a hydrophobic polymer to be non-thrombogenic, it must preferentially adsorb albumin in vivo. In their study, surface specimens were examined for protein adsorption after maximum exposure time of 45 minutes. This duration was chosen as



a suitable time interval for reaching steady state surface concentration. The present study suggests that this hypothesis is not tenable as a criterion of non-thrombogenicity for hydrophobic polymer surfaces. The exchange process observed here suggests that an albumin coated surface may become thrombogenic by exchanging its albumin with fibrinogen or  $\gamma$ -globulin. Moreover a 45-minute exposure time is not enough to detect this exchange, since it was found to be a slow process extending over a span of over 40 hours.

Thus differences in the thrombogenicity of polymers must relate to changes that may occur in the composition of the adsorbed protein layer after long exposure time, as well as to the overall composition.

#### 6.4 DATA FITTING AND PARAMETER ESTIMATION

Two methods were used to data fitting and parameter estimation in this study. The ordinary least sum of squares method<sup>(59,60)</sup>, and the Bayesian multivariate method<sup>(55,61)</sup>. According to Beauchamp and Cornell<sup>(62)</sup>, the method of least sum of squares can be used for the analysis and fitting of multiresponse data. However, it is only valid if the sum of squares of deviations of the model from the data for each response is weighted by the inverse of the variance of the measurements of that response.

When replicates of experiments are not available and the covariance matrix of the data is unknown, then least squares analysis of individual responses or the Bayesian multivariate analysis should be resorted to. According to Allen et al<sup>(55)</sup>, it is essential that the model should give a qualitative fit to each response individually

before the Bayesian multivariate analysis is used for parameter estimation. The Bayesian multivariate method is superior to the least squares method in two respects:

- 1) While the weighted least squares method can be used for simultaneous analysis of multiple response problems, with the weighting factor being the inverse of the variance of observations, it does not account for correlations between responses. The multivariate technique recognizes and accounts for the correlation of error between responses. This situation might arise from sampling when responses are measured from the same sample which is the case in this study.
- 2) Multivariate technique greatly reduces the size of the joint confidence regions of parameter estimates.

In this study, the two responses obtained from monitoring the movement of albumin from and onto the surface were fitted individually by the least squares method, to check the goodness of fit and adequacy of the model. Estimates of parameters by this method as well as individual confidence limits for each parameter for each response are found in Tables 7 and 8. After the model was found adequate and confidence intervals of parameter estimates were found to overlap from each response individually, the method of multivariate analysis was used. Results of the estimation of parameters by this method are found in Tables 9 and 10.

It was found that the multivariate technique improves estimation of parameters by greatly reducing the joint confidence regions of

parameter estimates. Fig. 18 shows the joint confidence regions of  $g_0$  and B obtained from multivariate analysis as well as those obtained from each response individually by the ordinary least squares method. For the least squares method, the joint confidence region of parameter estimates is obtained from the following equation: (70)

$$S_{(1-\alpha)} = S_{\min} + \frac{S_{\min} P}{(n-P)} F_{\alpha, [P, (n-p)]} \quad [23]$$

where  $S_{1-\alpha}$  = the sum of squares on the 100(1- $\alpha$ )% confidence contour

$S_{\min}$  = the minimum value of the residual sum of squares of deviations of data from model after data are adequately fit to the model

P = number of parameters

n = number of observations

$F_{\alpha, [P, (n-p)]}$  = value of the F-distribution function at confidence level  $\alpha$ , and degrees of freedom P, (n-p) respectively.

This equation gives the rigorous confidence contour for nonlinear models. Only the confidence level,  $\alpha$ , associated with this contour is approximated. The confidence region for the parameters obtained by multivariate analysis can be computed, according to Box and Draper (61), from the general formula.

$$|V|_{(1-\alpha)} = |V|_{\min} \exp \left\{ \chi^2_{p(1-\alpha)} / n \right\} \quad \text{where} \quad [24]$$

$|V|_{(1-\alpha)}$  = the value of the determinant of Eqn. [ 8] on the 100(1- $\alpha$ )% confidence contour.

$|V|_{\min}$  = the minimum value of  $|V|$

$\chi^2_{p(1-\alpha)}$  = Chi-square value for p degrees of freedom and  
100 (1- $\alpha$ )% probability level.

Joint confidence regions plotted in Fig. 18 are those for the 380 mg% -  
2500 sec<sup>-1</sup> exchange results.

Since the effect of shear rate on the fractional turnover rate is of interest, multivariate analysis provides a powerful tool to examine this effect by yielding more precise estimates for the parameters determined. Precise parameter estimates are necessary also because parameter values do not change over a wide range. Precise parameter estimation by multivariate analysis is also necessary because the confidence intervals of parameter estimate obtained by the least square method shown in Table 7 sometimes overlap for two different shear rates, thus making the effect of shear on the process parameter uncertain. Figure 19 shows the joint confidence intervals at the three shear rates used for 380 mg % solution obtained by multivariate analysis. Examining this figure shows that the overlap of individual confidence intervals for parameter estimates that is seen in Table 7 is eliminated, thus the certainty about the significance of the change of parameter estimates with shear rates is increased.

Thus, it can more confidently be concluded that the fractional turnover rate and exchangeable fraction of the surface both increase with increasing shear rate.

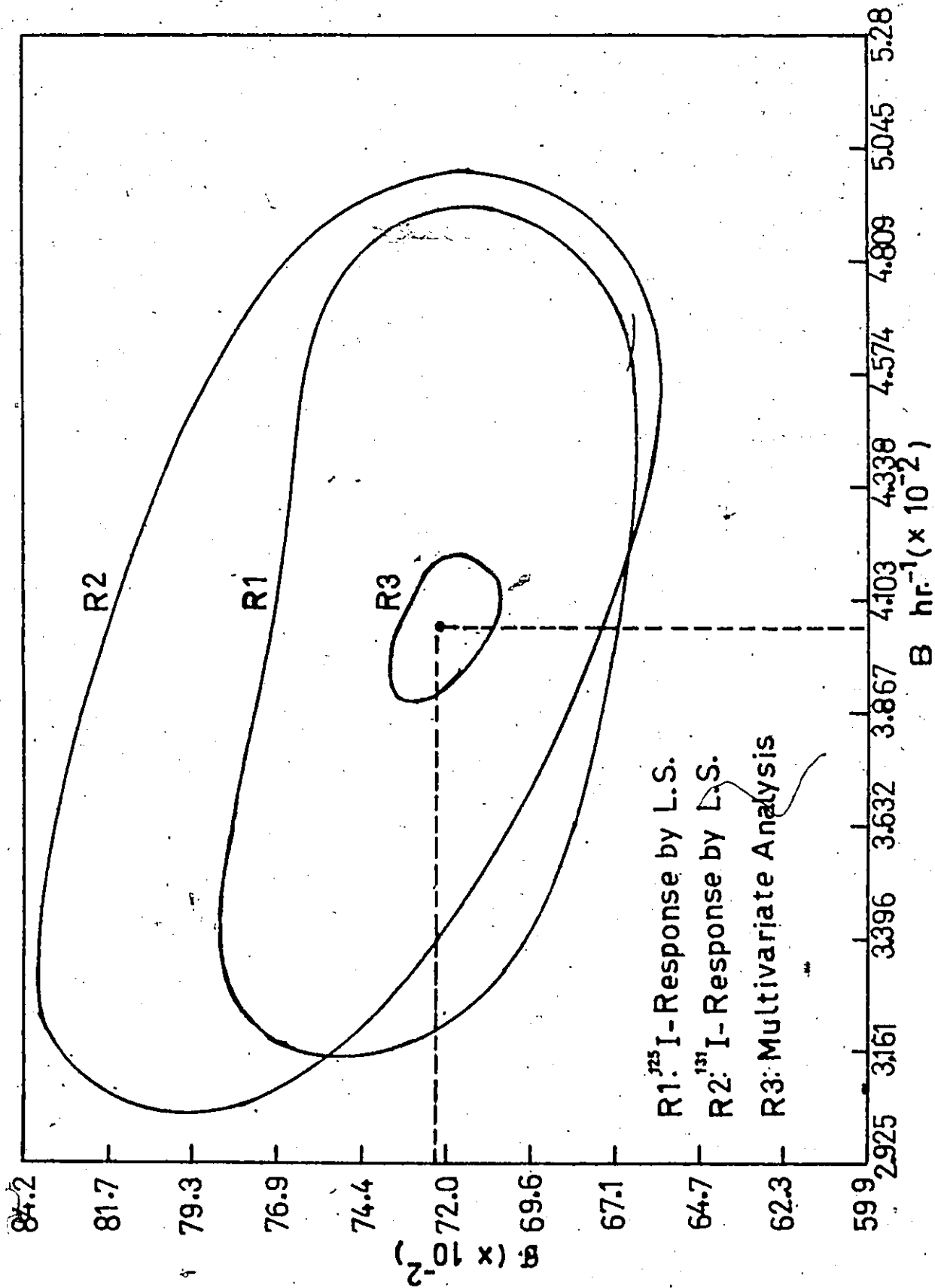


FIGURE 18

JOINT CONFIDENCE REGIONS FOR PARAMETERS' ESTIMATES AT 95% CONFIDENCE LEVEL.  
38 mg % solution, 2500 sec<sup>-1</sup> shear rate

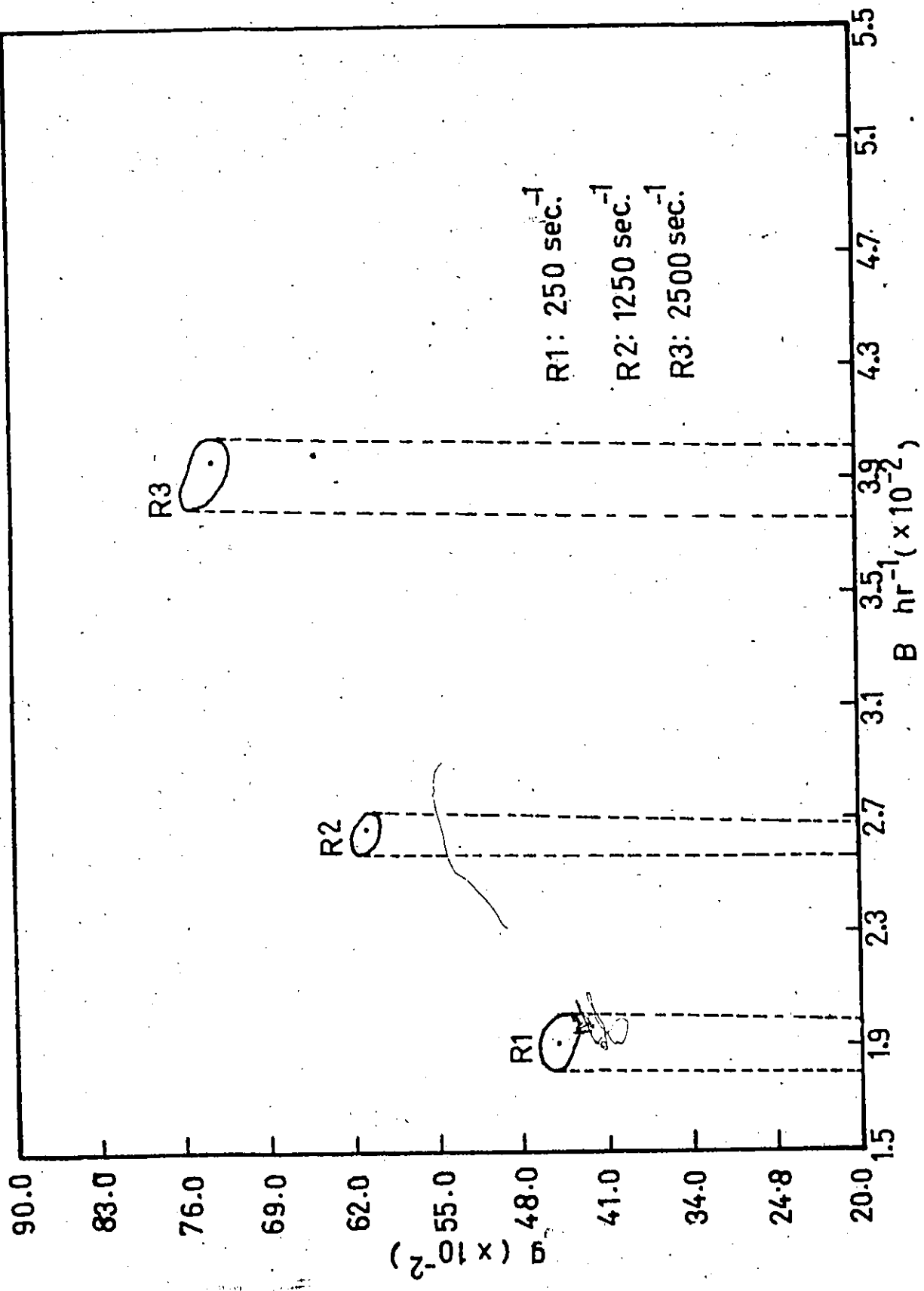


FIGURE 19

JOINT CONFIDENCE REGIONS FOR PARAMETER ESTIMATES AT 95% CONFIDENCE LEVELS BY MULTIVARIATE ANALYSIS 380 mg %

## CHAPTER VII

### CONCLUSIONS

- A. Surface reaction between human plasma albumin and polyethylene surface is fast. Steady state surface concentration is reached within two hours exposure time under both static and flow conditions of shear rate up to  $2500 \text{ sec}^{-1}$ .
- B. Plateau surface concentration does not show dependence on flow within the range of shear studied. It was also found that the time to reach steady state concentration did not show dependence on flow, which supports the view of other investigators that protein adsorption is not diffusion-controlled.
- C. Albumin adsorption isotherms for both static and flow conditions show that albumin adsorption follows a Langmuir model.
- D. While equilibrium between the surface and solution is maintained with concentration remaining constant, adsorbed protein is exchanged with protein in the bulk solution. This turnover process shows dependence on flow conditions with fractional turnover rate increasing with increase in shear rate.
- E. While part of the adsorbed albumin exchanges with the solution, another part is unexchangeable. This suggests that there is more than one type of bond involved in albumin adsorption on polyethylene surfaces, or that the unexchangeable part of albumin is

denatured and spread on the surface thus exposing more side chains for binding than the native globular exchangeable molecules of albumin.

- F. Under static conditions no exchange was observed at the lower end of the solution concentrations studied. However, at the upper end of the range (380 mg%) some exchange was observed. This suggests that at higher protein concentrations approaching the physiological level, turnover will be greater and faster.
- G. While a hydrophobic surface coated with albumin may show resistance to thrombus formation, it can in the course of time exchange its albumin with a protein that catalyzes thrombus formation like fibrinogen and thus could become eventually thrombogenic.
- H. The exponential kinetic model

$$y = g e^{-Bt} + g_0$$

was found to describe reasonably well the exchange process with:

$y$  = surface concentration

$B$  = fractional turnover rate,  $\text{hr}^{-1}$ .

$g+g_0 = y_0$  = steady state surface concentration

$t$  = time

With regard to parameter estimation for kinetic models, the Bayesian multivariate analysis was found superior to the minimum least square method with respect to precision of estimates and tightening of the joint confidence region of parameter estimates.



## CHAPTER VIII

### SUGGESTIONS FOR FUTURE RESEARCH

- A. Kinetics of adsorption from complex solutions of plasma proteins should be investigated under both static and flow conditions. This would bring the model of experimentation closer to the real situation of interactions between plasma or whole blood and foreign surfaces.
- B. Kinetics of adsorption of proteins should be studied in the presence of formed elements of blood, like red blood cells. Since the presence of red cells in a shear field has been found to augment platelet diffusion and possibly energy of interaction with the surface<sup>(38)</sup>, it is possible that their presence may have comparable effects on protein adsorption especially with protein molecules larger than albumin, like fibrinogen and  $\gamma$ -globulin.
- C. Kinetics of exchange in solutions of mixtures of plasma proteins should be investigated. This should give more information about the changes that occur in the composition of the surface layer of adsorbed protein, and consequently about the ability of the surface to cause or resist thrombus formation in the course of time. Kinetics of exchange in complex solutions should also be studied in the presence of the formed elements of blood.

- D. The protein released from the surface during the exchange process should be investigated for possible alteration that may have occurred to it. This is relevant to the initiation of blood coagulation reaction and thrombus formation if the protein is a clotting factor.

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