# FIBRINOGEN-POLYMER INTERACTION— INFLUENCE OF PLASMA COMPONENTS

A thesis presented
by

**THOMAS CHANDY**

to

The Division of Biosurface Technology
in partial fulfilment of the requirements
for the degree of
Doctor of Philosophy
in the subject of

**BIOMATERIAL SCIENCE AND TECHNOLOGY**

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
TRIVANDRUM

SEPTEMBER 1987

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# C E R T I F I C A T E

I Thomas Chandy hereby certify that I had personally carried out the work depicted in the thesis entitled **Fibrinogen-polymer interaction - Influence of plasma components,** except where external help sought are acknowledged.

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THOMAS CHANDY

Date: 17. 9. 1987

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**(Declaration)**

This is to certify that Sri. Thomas Chandy in the division of Biosurface Technology of this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph. D degree of the Sree Chitra Tirunal Institute for Medical Sciences & Technology, Trivandrum. The work relating to his thesis entitled **FIBRINOGEN - POLYMER INTERACTION - INFLUENCE OF PLASMA COMPONENTS**, was carried out under my direct supervision.

Chandra P Sharma

CHANDRA. P. SHARMA
(Guide)

The thesis

entitled

**Fibrinogen - Polymer interaction - Influence of Plasma Components**

Submitted by

**Thomas Chandy**

for

Doctor of Philosophy

in

**Biomaterial Science & Technology**

of

SREE CHITRA TIRUNAL

INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY

TRIVANDRUM.

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# S Y N O P S I S

Medical devices such as catheters, haemodialysers, oxygenerators, heart valves and blood vessel prostheses are frequently being used in man as a consequence of natural and accidental causes of deterioration of his body. Current research has shown that, upon exposure of synthetic surfaces to blood, most materials become rapidly coated with a layer of blood proteins, in parallel with the adhesion of platelets, leading to thrombus formation. The proteins like albumin, fibrinogen, IgG and γ-globulin have been extensively studied towards their adsorption phenomena to various polymer substrates and have found that albuminated surface adhere less platelets, on the other hand, fibrinogen or γ-globulin coated surfaces show an enhanced platelet adhesion and release of platelet constituents. However, a fundamental understanding of the protein-artificial surface interaction in presence of other trace components of plasma or antiplatelet drugs or other mediators arriving at the interface and their implications to modulate this process is meagre.

This thesis deals with the changes in interfacial phenomena of protein/platelet interaction with polymer substrates due to certain trace components of plasma, antiplatelet drugs and vitamins. The interaction of fibrinogen(Fg) with polycarbonate substrate has been emphasised throughout the study with a comparison to albumin. Thus the Chapter-1 provides a general background with an emphasis to the relevance and objectivity of this work.

Chapter-II presents the experimental techniques, highlighting the use of each for evaluating and correlating various parameters of blood-prosthetic interactions and their relevance to blood compatibility. The kinetics of protein-substrate binding from single protein in buffer, competitive protein adsorption from binary or tertiary mixures or from plasma and their changes with antiplatelet drugs, vitamins, antibiotics, various proteases, platelet inducing agents, blood cells and cAMP etc. have been studied using trace labelled proteins,($^{125}I$ labelled albumin or $^{125}I$ labelled fibrinogen has been used as a tracer in presence or absence of these agents). Polyacrylamide gel electrophoresis has been employed to separate and identify the adsorbed proteins from a mixure of proteins, after desorbing them using Triton X-100. These protein adsorption studies have also been interrelated, in certain cases, with the earlier work from this laboratory, using ellipsometry and infrared attenuated total reflection spectroscopy (IR-ATR) for a better understanding of this interfacial phenomena. Platelet adhesion, using tyrode washed calf platelets, coagulation tests (recalcification time, thrombin time, activated partial thromboplastin time, stypven clotting time, antithrombin-III (AT-III) binding studies) and contact angle studies have also been performed to correlate the complex process occurring at the interface.

In Chapter-III, a discussion related to the fundamental understanding of adsorption kinetics, isotherms, desorption and exchange for the single protein system, with an emphasis to the effect of pH, bulk concentraction, temperature and flow conditions is presented. These findings suggest that the polycarbonate (PC) fibrinogen

interaction is a strong one, since the adsorption is rapid, the surface concentration is high and the adsorption is dependent on pH, flow rates, temperature and bulk concentration of the media.

Chapter III also deals with the competitive adsorption studies of albumin and fibrinogen in binary and ternary mixtures (albumin, fibrinogen and γ-globulin) to polycarbonate surface which shows that all the proteins are adsorbed, but with a preference to Fg. However, the adsorption experiments with plasma indicate that fibrinogen adsorption is less prominent. Thus, it seems that initially adsorbed fibrinogen may be supplanted with other molecular species of complex plasma. The adsorption kinetics of Fg from protein mixture to various other polymers like Teflon, Nylon, Polystyrene etc. have also been performed for better understanding of the interaction process.

Chapter IV provides a fundamental understanding of the effects of Fg variants, certain trace plasma proteins, and proteases to modulate the interfacial phenomena. The three fibrinogen variants have been fractionated and investigated the effect of these fractions towards an artificial surface for surface-protein and surface-platelet binding. It seems that γ'-chain enriched fibrinogen molecule binds less effective to the PC substrate, while the other two fractions behave similarly. The γ'-chain containing Fg, also enhances the surface-albumin concentration resulting in a reduction in platelet-surface binding. The effect of certain trace proteins of plasma, like fibronectin and certain proteases, like thrombin, trypsin and plasmin on $^{125}I$ labelled fibrinogen and the

interaction of the resultant products with the polycarbonate substrate and on washed platelets have also been studied. This indicates that fibronectin significantly modifies the platelet-polymer, protein-polymer interaction, when thrombin is present in the medium. The thrombus development at the interface is highly influenced by the nature and level of proteases present at the vicinity of the implant.

Chapter V presents the studies with certain antiplatelet drugs (like Aspirin, Dipyridamole, and their combinations) vitamins (VitaminA, B6, C,D and E), amino sugars, and certain antibiotics (Neomycin, Gentamycin, Ampicillin, Penicillin-G and Streptomycin) at the polymer interface have also been carried out, which highlights the modulation of surface-protein/platelet interaction. It seems, vitamin B6, C, E, combinations of aspirin- vitamin C, certain antibiotics and galactosamine have inhibited the fibrinogen-surface concentraction or enhanced the albumin surface-binding to variable degrees. This may be one of the parameters for a reduced platelet-surface binding in presence of these agents. It is also evident that, modification of blood with a combination of 0.5 mg% Asprin: 1.5 mg% Vitamin C: 0.15 mM Vitamin B6: 2 mg% Vitamin E, appear to be beneficial towards enhancing the blood compatibility of an artificial surface. From the antibiotics studies, it may be suggested that a controlled antibiotic therapy for patients with artificial implants may limit or avoid the high doses of antiplatelet drugs (to control bleeding problems) in addition it protects from bacterial infection during postoperative period.

In Chapter VI, the role of blood cells like red cells, platelets, whole white blood cells and lymphocytes to modulate the protein-surface binding have been studied from a protein mixture using labelled albumin or fibrinogen. It seems albumin adsorption to polycarbonate is slightly reduced with all blood cells, on the other hand fibrinogen-surface binding is enhanced to variable degrees. So it is possible that the blood cells can cause an augmented fibrinogen adsorption to the surface, which may be a cell membrane related effect, probably via the receptors.

It is believed that an increased level of platelet cyclic AMP can inhibit the platelet response to stimuli. The effect of cAMP towards protein surface binding is not yet understood. The role of this molecule to modulate the surface-protein binding and their inter-relation to platelet adhesion has also been reported in Chapter VI. These studies indicate that the soluble cAMP enhances the surface-albumin binding and inhibits the fibrinogen adsorption to the substrate. The modulation of this interfacial phenomena is dependent of the level of soluble cAMP in the media. So it seems that cAMP may be a common link for most of the antiplatelet agents, which may enhance the albumin to fibrinogen ratio at the polymer interface, resulting in a reduced platelet-surface binding.

Chapter VII gives a critical analysis of the overall results, which enables us to postulate a hypothesis, interrelating for the events at the interface when mediators (eg. antiplatelet drugs) are also present. It is understood that the sequence of proteins displacing each other on contact with plasma starts not with fibrinogen, but with

albumin, followed by IgG, which in turn will be replaced by fibrinogen and fibronectin etc. and ending with high molecular weight kininogen. The sequence is slowed down in more hydrophobic surfaces. Comprehensively, these studies highlight the effect of certain antiplatelet agents to modulate the surface-Fg interaction and the subsequent process of thrombus formation. It seems, those mediators having antiplatelet activity, have caused an increase in surface-albumin concentration in comparison with fibrinogen. This common trend of protein adsorption demonstrated by these agents may be one of the parameters for a reduced surface-platelet binding. Therefore, these two factors, may be beneficial for improving the blood compatibility of materials in in vivo conditions. So it is possible that the mediators arriving at the interface may alter the sequence of proteins deposited or displaced by plasma. The mediators can cause an increase in interfacial tension of the substrate, resulting in an enhanced protein-surface binding favouring towards albumin in several cases. Platelets arrive in the later stage to find fibrinogen to adhere.

An outline has also been given in Chapter VII towards future outlook to search for the missing links, which may occur at the interface to throw light on the events happening in vivo.

# GENERAL INTRODUCTION

# CHAPTER - I

Medical devices like heart valves, haemodialysers, oxygenators, catheters and blood vessel prostheses are frequently being used in man as a consequence of natural and accidental causes of deterioration of his body. The formation of thrombi on the surfaces of artificial materials occurs upon contact with blood, resulting in the failure of the implant. Progress made over the last 25 years in the area of blood-biomaterial compatibility has been impressive. However, we still do not have a satisfactory thromboresistant material to replace a coronary artery, which reminds that many more fundamental problems are yet to be solved. A review of the nature of these problems and possible ways of minimising them is given in this introductory Chapter.

## 1.1. Criteria of Polymers for biomedical use

A biomaterial is any pharmacologically inert material viable or non-viable, natural or manmade, that is a part of or is capable of interacting in a beneficial way with a living organism[31]. Thus the term biomaterials encompasses all materials used for medical applications that are interfaced with living systems or other systems developed for extracorporeal uses[13]. Biomedical materials include metals, ceramics, natural polymers (biopolymers) and synthetic polymers of simple or complex chemical and/or physical structure. In addition to the blood contacting applications

stipulated above, other biomedical applications of polymers include sustained and controlled drug delivery formulations for implantation, biomaterials for skin replacement, reconstruction of vocal cords, opthalmic applications such as therapeutic contact lenses, artificial corneas and intraocular lenses, craniofacial, maxillofacial and related replacements in reconstructive surgery, and neurostimulating and other electrical stimulating electrodes[212,365]. Orthopedic applications include artificial tendons, prostheses, long bone repair and articular cartilage replacement[365]. Finally, dental materials and implants are also considered as biomaterials. Candidate materials for biomedical applications must comply with a variety of requirements characteristic of most biomaterials. These requirements arise either from the specific chemical or physical structure of the materials (chemical, physical and mechanical criteria) or from the physiological environment where they will be used (biological criteria).

The basic criteria for developing biomedical devices from polymers are very rigid[212,298]; which have been indicated as follows:

a) They should be nontoxic, i.e. the material should not produce any undesirable inflammation and/or clinically significant changes in the tissues or the fluids of the body.

b) They should be easily fabricable. So methods must be available to fabricate the implant, possibly on a mass production basis, with accurate dimension and consistency. Such methods must be reproducible and capable of yielding good quality products with improved mechanical properties.

c) They must be sterilizable. The sterilization of these materials is essential to keep it free from bacteria or any other microorganisms, and it must be stable and safe during sterilization, by any of the recognized methods.

d) The material must not undergo significant changes in either mechanical, physical or chemical properties during the period of implantation, such that the implant looses it's efficiency. The mechanical properties should match the nature of the prosthesis, that substitute the body parts.

e) It should not corrode or degrade such that the products of the deterioration are liberated into the environment, where they may induce local or systemic harmful effects.

f) The material should not be carcinogenic. The material in the body environment should not produce mitogenic properties of the tissue as a result of its degradation product, leaching components or corroded materials.

Finally, the cost of the finished product should be as low as possible, consistent with conformity to all other requirements. Currently none of the material existing may be able to satisfy all above requirements, in which case a search for new materials has to be continued. Before introducing a 'new material' very stringent tests are necessary to determine their suitability, especially from compatibility point of view.

## 1.2 Fate of synthetic surfaces in contact with blood

All artificial materials known until now induce the coagulation of blood and/or the adhesion and aggregation of blood platelets, eventually leading to the formation of a thrombus on the material surface[16,40,348]. Current research has shown that, upon exposure of synthetic surfaces to whole, flowing blood, most materials become rapidly coated with a layer of blood proteins[16,40,191,284] in parallel with the adhesion of platelets[190,191,209,257,377] leading to thrombus formation. In addition, it is also reported[190,380] that the decrease of intracellular cyclic AMP (cAMP), the rapid adsorption and desorption of denatured thrombin, haemolysis of red cells, adhered white blood cells, surface activation of intrinsic coagulation factors, secretion of platelet constituents such as ADP, serotonin etc., and/or the release of adsorbed fatty acids may cause the formation of thrombus. Thus, the formation of a thrombus is the end result of a network of complex reactions. Before going in to the details regarding the interaction of materials with blood, some properties of the biological environment will be discussed.

### 1.2.1. The normal vascular endothelium

Blood is a heterogeneous fluid, flowing through the circulatory system at a relatively high pressure (systolic--> 120 and diastolic --> 80 mm mercury) and consisting of formed elements of blood; erythrocytes, leucocytes and platelets suspended in plasma. The plasma contains water, proteins, lipids, carbohydrates, nitrogenous waste products, pigments, inorganic ions, enzymes, hormones and vitamins. The intact

vascular endothelial surface is considered to be 'non-thrombogenic' and blood platelets usually fail to adhere to it[21,141].

Possible factors and mechanisms that may be involved in the non-thrombogenic effect of the endothelium are: (1) electrostatic repulsion, both the intact platelet and endothelial cell have negatively charged membranes at physiological pH and thus are mutually repelled by each other; (2) the surface ADPase associated with endothelial surface can prevent the formation of platelet aggregates near the intact endothelium[259]; (3) the presence of heparans and glycosaminoglycans on the endothelium having anticoagulant activity[243,276]; (4) plasminogen activator, which causes an increased fibrinolysis; (5) the binding of thrombin to the endothelium facilitates the rapid removal of thrombin from the circulation and the formation of thrombin - antithrombin-III complex and (6) release of prostacyclin, a potent inhibitor of platelet aggregation and adherence, by the endothelium in response to various stimuli.

The unique property of normal endothelium is its total compatibility with blood. Endothelial cells are capable of metabolic processes which actively discourage thrombus formation[259,276]. When there is injury to the intima the underlying collagen layer becomes exposed to blood and the contact between platelets and collagen initiates thrombus formation[225,243]. Haemostatic plugs and arterial thrombi are largely composed of platelet aggregates adherent to the injury site, with fibrin in and around the platelets acting to stabilize the plug or thrombus[23,258,325]. Red and white blood cells are also associated with the

thrombus. At a site of repeated injury, fibrin is usually found between the adherent platelets and the damaged vessel wall, indicating that activation of the coagulation pathways and the formation of thrombin have a greater role in the formation of thrombi[123,172].

### 1.2.2. Surface-mediated reactions and coagulation pathways

Little information is available regarding the molecular events that occur when blood clots in vivo. The contact of blood with a foreign surface triggers blood coagulation and it involves a series of coagulation factors that exist as inactive precursors in the plasma and upon activation interact through a cascading series of pathways that lead to the final formation of fibrin threads, which hold the formed elements of blood. The coagulation factors are numbered by Roman numerals from I to XIII, as shown in Table I-I. Current evidence suggests that coagulation is initiated by two processes, both of which are required for normal haemostasis. One, the so-called intrinsic system, is initiated by activation of Hageman factor by collagen, other types of connective tissues and artificial surafaces[274,368]. The other, the extrinsic or tissue factor pathway, is activated by the formation of a complex between tissue factor, also present in the vessel wall[9,180], and factor VII[367], each leading to the activation of factor X and thereafter following a common pathway resulting in the fibrin formation. The reactions are summarised in Fig. I-1.

The initial phase of clotting probably involves the following steps[347,351].

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The initial phase of clotting probably involves the following steps[347,351].

1. Plasma comes in contact with an activating surface (a "hard" surface that is wettable and negatively charged), and deposits factor XII (Hageman factor) in intact form. Factor XII, adsorbed, becomes activated to factor XIIa without cleaving.

2. XIIa can activate other molecules of XII[69].

3. Some molecules of high molecular weight Kininogen (HMWK), carrying factor XI, and other molecules of HMWK that carry Prekallikrein, are adsorbed near the preadsorbed XIIa.

TABLE I - I

**COAGULATION FACTORS**

| Number | Name |
|---|---|
| I | Fibrinogen |
| II | Prothrombin |
| III | Thromboplastin |
| IV | Calcium ion |
| V | Pro-accelerin |
| VI | Not assigned |
| VII | Proconvertin |
| VIII | Antihaemophlic factor |
| IX | Christmas factor |
| X | Stuart factor |
| XI | Plasma thromboplastin antecedent |
| XII | Hageman factor |
| XIII | Fibrin stabilizing factor |

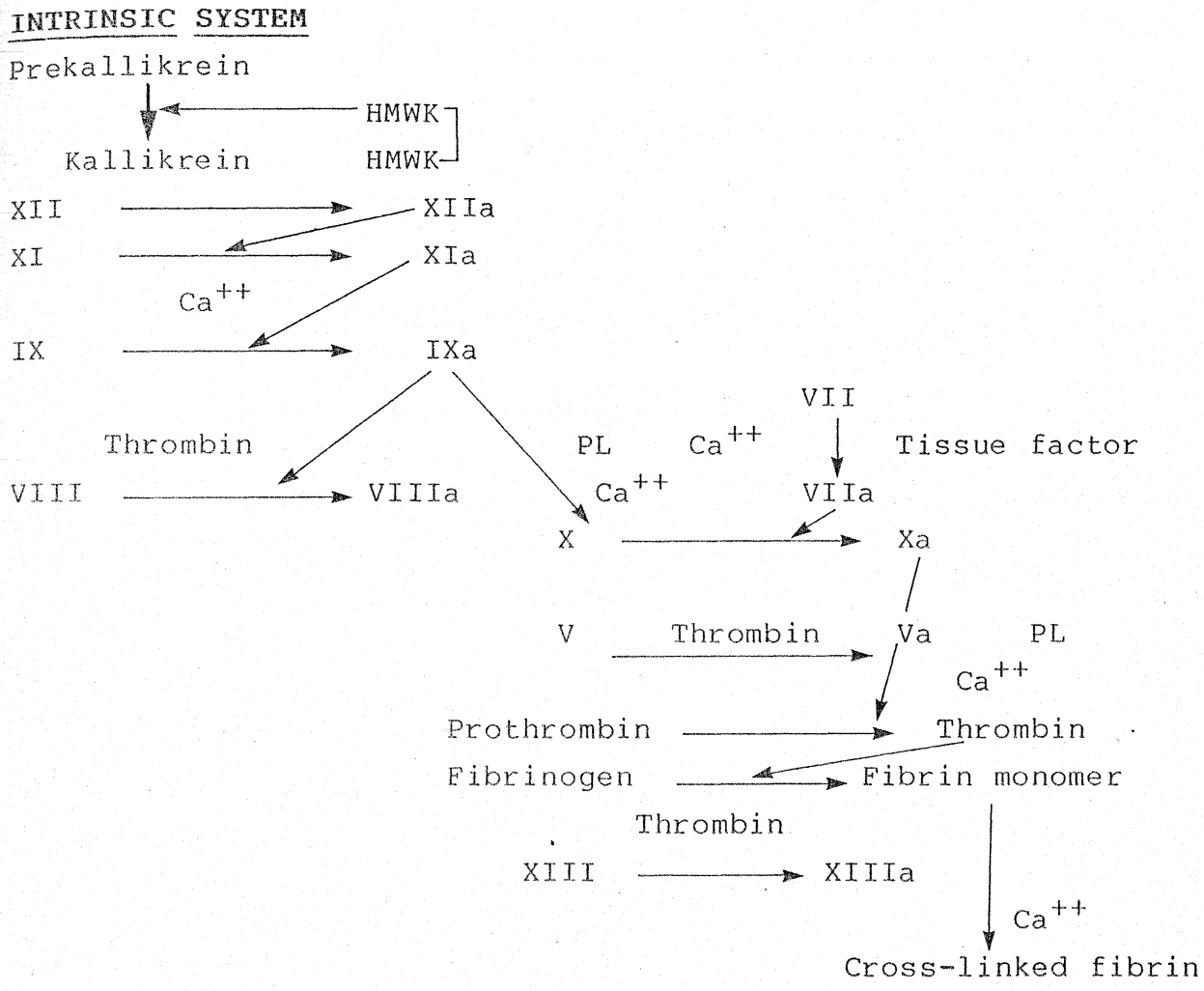


Fig. I-1. Schematic representation of blood coagulation

HMWK : High Molecular Weight Kininogen
PL : Phospholipid
$Ca^{++}$ : Calcium ion

The activated form of the clotting factor is indicated by the letter 'a'.

4. XIIa activates the factor XI, that had been brought to it by HMWK, to XIa.

5. XIIa converts the Prekallikrein (similarly transported to it) in to Kallikrein.

6. A fragment of activated factor XII (XIIf) can activate prekallikrein in solution, converting it to Kallikrein.

7. Kallikrein can activate more factor XII.

These activating reactions do not require $Ca^{++}$ions. Now the "fluid phase" of clotting begins with the participation of $Ca^{++}$ions for the activation of factor IX by XIa. Factor IXa combines with factor VIIIa, phospholipids and $Ca^{++}$ions to form a complex which is able to convert factor X into Xa. Next, factor Xa combines with factor Va, Phospholipids and $Ca^{++}$ ions to form a complex, which converts prothrombin into thrombin, which in trun converts fibrinogen (Fg) molecules into fibrin monomers and finally stabilized by factor XIIIa to fibrin gel. Phospholipids are made available in the intrinsic system by the platelets, whereas in the extrinsic system by the tissue factor. Normal haemostasis requires both pathways and they reinforce each other.

### 1.2.3. Mechanism of Blood-foreign surface interaction

The sequence of processes, that may occur due to contact of an artificial surface with blood is represented in Fig. I-2. The blood deposits proteins upon contact with polymer substrates and the nature of this protein can

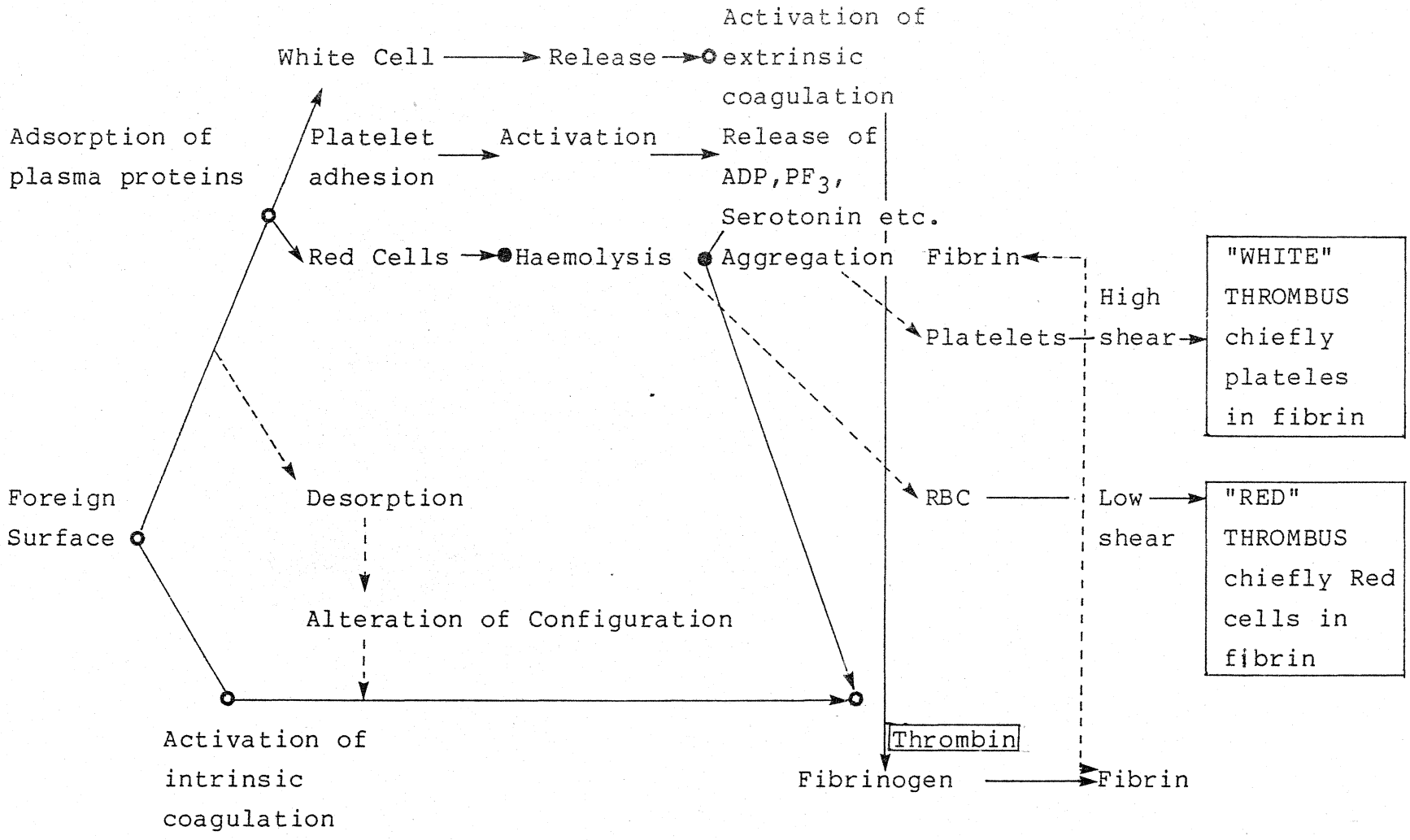


Fig. 1-2: Sequence of processes leading to thrombus formation on artificial surface.

influence the subsequent events of cellular interaction and thrombus formation[143,171,199]. It is observed that albumin coated surfaces do not attract platelets[209,210,257], whereas fibrinogen and γ-globulin coatings cause not only platelet adhesion but also aggregation and the release of platelet constituents[2,378]. The adhered platelets undergo shape change, degranulation and releases it's constituents like ADP, serotonin, fibrinogen, platelet factors etc. These products of degranulation are capable of stimulating adjacent platelets resulting in aggregation and the formation of a platelet plug. Thromboplastin, formed from stimulated monocytes, appears to contribute to the acceleration of thrombin formation.

As represented in Fig. 1-2, the red blood cells (RBC) can also adhere to the surface during this initial phase and can release their consituents like clot promoting factor (erythrocytin) and ADP (Platelet aggregating agent) as a result of haemolysis. Thus the activated platelets, leucocytes and RBC are capable of stimulating both the intrinsic and extrinsic coagulation. Low shear conditions lead to the formation of 'red' thrombus, which is chiefly an interwoven mesh of red cells in fibrin, whereas high shear conditions give rise to 'white' thrombus, which is mainly an interwoven mesh of white cells in fibrin[51].

On many materials, heparinized, citrated or native blood or plasma seems to deposit fibrinogen within a second. On surfaces that are known to activate clotting, the contact system coagulant protein such as factor XII, Prekallikrein and HMWK are adsorbed and induces the activation of factor XII, which triggers blood coagulation[171,347,351]. During

coagulation of blood, thrombin is formed which is a release inducer of platelets, stimulates platelet adhesion, aggregation and intrinsic coagulation. Thus the thrombus formation can occur at the blood-polymer interface, either by platelet adhesion, aggregation and fibrin formation or by the activation of factor XII.

## 1.3. Surface-interface contributions to blood compatibility

The compatibility of a material with blood appears to be influenced by a number of different properties, most of which relates to the surface of the material which contacts blood[208,272,295]. The surface properties like surface free energy, surface molecular motions, surface topography, critical surface tension, electrical conductivity and water content have evolved as important factors[19,211]. Andrade has postulated that material surfaces which tend to have an interfacial energy of zero will be highly thromboresistant[6,7]. Also, the fact that materials with a minimal interfacial energy like hydrogels[145] do possess a low thrombus adherence, enhances the validity of this hypothesis. Similarly, the surface free energies of many polymers have been calculated by contact angle measurements[7,145,376]. It has been also suggested that a polymer having a critical surface tension of around 20-30 dyn/cm is highly blood compatible[15] but exceptions have been reported as in the case of LTI carbons ( $\gamma_c$ = 50 dyn/cm).

Although the contributions of surface-energy parameters to blood compatibility cannot be definitely interpreted as yet, several authors have suggested various

considerations for blood compatible surfaces. It is indicated that[2,305], the initial reactions between the blood components and ploymer surfaces may be expected to involve physical forces such as electrical interactions, hydrogen bonds, dipole-dipole interactions, donor acceptor bonds and Van der Waals forces (Keesom forces, Debye Falkenhagen forces and most important the London-dispersion forces). These forces are manifested in the surface tension of the various surfaces, which in turn determine the surface free energy for the various processes of interest. These free energies provide the driving force for surface-blood interactions.

Further surface characterization has been done by Kaelble and Moacanin[175]. They have indicated that dispersion $\alpha_s = (\gamma_s^d)^{1/2}$ and polar $\beta_s = (\gamma_s^p)^{1/2}$ components of polymer surfaces play an important role in interfacial interactions. They concluded this on the basis of their surface energy analysis of 190 biological and implant surfaces (about 20 different types). It is found that low dispersion - high polar surfaces (typified by surface treated stellite 21) with $\alpha_s = 5.0$ (dyn/cm)$^{1/2}$ $\beta_s \geq 5.0$ (dyn/cm)$^{1/2}$ provide surface energies, which appear to favour weak adsorption and retention of plasma proteins, i.e. a poor surface from the compatibility point of view. Conversely implants with high dispersion and low polar surface energy (e.g. low temperature isotropic (LTI) carbon) with $\alpha_s \geq 6.0$ (dyn/cm)$^{1/2}$, $\beta_s \leq 2.0$ (dyn/cm)$^{1/2}$ provide surface energetics favouring stable plasma protein film retension, and so high blood compatibility, but this does not follow to Andrade's postulate that interfacial energy should be as close as possible to zero.

Although there are limitations in the method of Kaelble[175], in which too much emphasis is given on the $\alpha$ & $\beta$ components of water, recent experimental observations and theoretical calculations have also suggested an optimum $\alpha_s$ (disperson component) value of 4.7 and $\beta_s$ (polar component) value of 3.0 for a possible blood compatible surface[297,304]. It has also been reported[245,262] that such surfaces may adsorb albumin preferentially, which may be one of the reasons for their improved blood compatibility. So the dispersion and polar components of surface free energy of any substrate are important.

Similarly, such components are important for various blood components also on considering their blood material interactions at the interface[310]. Hence the relevance of dispersion-polar components of surface energy for both substrates as well as blood components, in understanding the initiation of cell adhesion (e.g. platelets) process, on to nonbiological substrates has been inter-related[310]. The interfacial tension of any substrate with various blood components will be different, encouraging preferential adsorption/adhesion of some components compared to others. So it seems, the fate of the biomaterial may also be dependent on what is adsorbed preferentially on the basis of dispersion - polar components of substrate and the blood component.

### 1.3.1 Surface charge and Blood Compatibility

The surface charge of artificial surfaces has received considerable attention from those who wish to develop antithrombogenic materials. In 1953, Sawyer and

Pate[290] observed the formation of a thrombus at the anode when a current was passed across a blood vessel. There is still no agreement as to optimal ratio of positive to negative charges desirable or with the density of these charges. It is generally agreed that predominantly positively charged surfaces have poor compatibility with blood[282]. A highly charged negative surface has also shown poor compatibility with blood, in in vitro conditions, whereas a positively charged surface with less total charge per unit area has been slightly more compatible with blood[294]. However, surfaces which contain nearly equal mixtures of positively and negatively charged groups have a fairly high degree of compatibility with blood[223,282,294].

Platelets, cells and proteins tend to have a negative charge as well as the blood vessel wall which has a negative zeta potential of -8 to -13 mv[292]. Thus an act of repulsion between the blood constituents and the blood vessel wall may be the factor which prevents coagulation. So attempts have been made to produce a surface with antithrombotic properties by the incorporation of negative charges, e.g. fixed anionic groups[223], neutral polymers with negative potential[223,292], electrets with a fixed negative charge[223,294]. However, no successful biocompatible polymer with a negatively charged surface has been made so far and no correlation with potential and thrombus formation has been observed[4,223,293]. It is probable that surface charge alters the adsorption of plasma proteins and the subsequent process of thrombus formation.

### 1.3.2. Other surface properties and blood compatibility

In case of polymers, it is essential to understand the chemical nature of the surface with respect to the distribution of chemical groups on it, in correlation with the physical nature of the surface. So surface analysis may provide a certain basis for reasoning out a definite solution to the variety of adverse interactions (such as thrombus and/or embolus formation, haemolysis of red cell and destruction of proteins) existing at an interface, when blood and the polymer surfaces come in contact in vivo. The surfaces of materials are almost always different in structure and chemistry from the bulk or interior of the material. These differences result from surface contamination (a consequence of surface chemistry), molecular orientation and surface reaction.

Multiprobe surface characterization techniques, including contact angle measurements, ESCA, ATR-IR and SEM have been used to obtain surface property information of various polyurethane ureas[195,196]. The blood-contact and surface characterization results indicate[196] that surface concentration and type of hard segment are of importance in determining blood response. The method of fabrication of a polyurethane block polymer, has been shown to markedly affect its surface properties[195,213]. So it appears[196,256] that the relative concentration of hard segment on the polymer surface has been found to affect the observed blood-material interaction, although the extent of this effect depends on the hard and soft segment components of the copolymer system.

Transmission electron microscope studies[296] on the surface analysis of polyether urethane ureas (PEUU) 710, 1025 and 2025 (block copolyether urethane ureas based on polypropylene glycols of molecular weight 710, 1025 and 2025) have indicated changes in domain size depending upon fabrication and processing variables. The glass side and the air side of the cast polymer films are found to be different e.g. PEUU-1025 (10% in dimethyl formamide), the air side domain size is 80-120 Å, but for polymer PEUU-1025 (18%), the glass side domain size is 30-50 Å. It is concluded[296] that PEUU-1025 has shown relatively better blood-compatibility, it seems domain-size of the order of $\sim$ 50 Å may be fairly good for these polymers with $\sim$ 35 domain in a 1000 x 1000 sq Å area.

Apart from this, the ratio of hydrophilic to hydrophobic areas on the polymer surface is known to have a marked influence on the blood compatibility. The protein adsorption[49,296] and platelet adhesion[160] vary with changes in hydrophilicity and hydrophobicity of a polymer surface. The importance of the morphology and texture of the blood contacting surfaces has also been reported by Yui et al[374]. They have synthesized polypropylene oxide segmented polyamides with various polyamide segment lengths and have found that, the platelet adhesion has been strongly dependent on the size of the crystalline and amorphous domains[374]. It is also observed from in vivo experiments[138] that the neointima formation in small diameter (1.5 mm) prosthetic blood vessels has been strongly stimulated by a porous fibrillar inner structure.

The type and amount of polyether segments in copolyether urethanes and their relationship to blood compatibility has been extensively studied[280], and found to have a reduction in platelet adhesion with increasing polyether content. Polyethylene oxide (PEO) has been reported to be most active in suppressing platelet adhesion compared to polypropylene oxide (PPOX) and polytetramethylene oxide (PTMO). Recent studies have also indicated[35] that, a combination of polyethylene oxide and polypropylene oxide in the soft segment region (PPOX/PEO ratio 90/10), of polyurethane urea have very good blood compatibility properties.

Molecular orientation at the surface of biomaterials has received increased attention in recent years. For polymeric systems, chain segments or pendant functional groups migrate to or from the surface in many instances. Thus, for block copolymers containing siloxane chain segments, the tendency is for these low energy blocks to migrate to the surface[269]. For most polymeric systems the casting solvent may significantly affect the outermost surface of the polymers. It is also suggested[275] that there is an interrelationship between molecular motions of polymer segments and thrombogenesis. The initial effect of molecular motions on thrombogenesis occurs independent of morphological order/disorder, crystallinity, and/or associated water (at the 0.01 mg bound water/mg polymer level). At higher levels of bound water, the effect of molecular motions on thrombogenesis is complexed by the presense of the bound water, as evidenced by the increased thrombogenic response[275]. So the nature of the surface (surface characterization) can provide us the knowledge to design

materials rationally for optimized biocompatibility in different implantation sites.

## 1.4. INTERACTION OF MATERIALS WITH PLASMA PROTEINS

Protein adsorption at interfaces is inevitable to all surfaces when exposed to blood and is favoured by the amphipathic character and macromolecular nature of the proteins[8,246,330].

### 1.4.1. Adsorption of proteins to artificial surfaces

Plasma proteins, either in whole blood or in purified form, are easily adsorbed to artificial surfaces in varying degrees[13,67,212]. This process has been shown by various investigators, using a number of physical and chemical methods[14,20,234,291]. Protein adsorption is a competitive process, in which all the plasma proteins may be involved to some extent. It appears that several proteins including albumin, γ-globulin, Fg, fibronectin (Fn), coagulation factors, IgG etc., have been found adsorbed to various artificial surfaces in vitro[16,40,222,349]. This primary adsorption at the interface is one of the most important events to be studied fundamentally, since this initial adsorption brings about changes in the platelets, which may trigger blood coagulation as discussed earlier. The adsorption of proteins is influenced by a number of factors such as material properties, enzymatic influences and blood flow patterns[183,349]. However available evidence suggests[112,250] a single parameter alone cannot explain the fate of proteins at interfaces.

Further, the proteins are intrinsically surface active and tend to concentrate at interfaces, due partly to their polymeric structure and partly to their amphoteric nature[8,169,267]. Thus, multiple contact points with the interface are possible for each protein molecule because of its large size, an effect that greatly increases the tendency for the molecule to remain at the interface. The presence of polar, charged, and nonpolar amino acid side chains in proteins provide the opportunity for multiple modes of binding with many different types of surfaces.

Proteins interact with implant surfaces to form apparent monolayer[41,191,339]. This may be due to the distribution of intermolecular forces developed at the polymer interface; which provides a driving force to attract other molecules, like proteins, to the surface upon contact with blood. When the weak forces alone are involved for attraction, the adsorption is termed physical, and the adsorbed molecules are easily desorbed from the surface. In contrast, chemisorption is used to describe instances, where chemical interactions occur with the surface molecules, stronger forces are involved, and the process is often irreversible. In the case of surface-protein interaction, it has been indicated[44,57] that the adsorption is partly reversible and partly irreversible; which is dependent on the surface properties of the material. In general adsorption is explained in terms of surface tensions (or energy/unit area) of the solid.

A number of studies have dealt with adsorption from solutions of a simple[192,234,358] purified protein or binary mixtures[149,191,331] of purified proteins or from

plasma[178,339]. The differences in affinity of proteins for surfaces is not readily discerned from the extensive literature on adsorption of proteins to surfaces from pure protein solutions[192,234,358]. The typical result of such studies is the adsorption isotherm, but determination of the strength of interaction or surface affinity from such measurements is not possible because the adsorption is essentially irreversible. However, an emperical study of the competition of various plasma proteins from simple mixtures does provide information about the affinity of proteins for surfaces. For example, the adsorption of fibrinogen to several polymers is reduced to half its original value with the addition of albumin or γ-globulin[43,317]. Haemoglobin (in the ferric form) competes much more effectively than any other protein yet studied; at one-tenth the fibrinogen concentration, haemoglobin reduces the adsorption of fibrinogen to polyethylene by 50%[150].

Adsorption studies using whole plasma provide the most relevant in vitro approach to the composition of the adsorbed protein layer. Several observations not predicted from simpler systems have been made in the plasma studies. Examination of the adsorbed protein layer formed from plasma by electrophoretic separation of the proteins in a detergent eluate of the surface has revealed the complexity of the layer[46,47]. As many as nine separate proteins have been detected, including Fg, IgG, albumin, and haemoglobin, as well as several unidentified proteins present in smaller amounts[46]. These studies emphasize the fact that the adsorbed layer is not dominated by any particular protein. The composition of the adsorbed layer appears to reflect

differences in surface activity and bulk concentration of the various proteins in the plasma.

The adsorption of albumin to artificial surfaces is of practical importance, because albumin coated surfaces reduce platelet adhesion and thrombosis[209,257]. In general, proteins interact more strongly with hydrophobic surfaces than with hydrophilic ones[40,151] and hydrophilic surfaces seem to adsorb less protein than hydrophobic surfaces[359]. So it is obvious that the ability of the polymers to adsorb proteins selectively from plasma at their interface may be one of the key factor in the complex process, which determines the compatibility of polymers.

### 1.4.2. Conformation of adsorbed protein

The adsorption, desorption and exchange properties of plasma proteins at the solid/solution interface are important considerations for describing the blood-material interactions. The adsorbed conformation and conformational changes of plasma proteins have, therefore, received attention as a possible means of predicting the effect of interactions with the surface. In the case of adsorbed proteins, numerous internal constraints due to disulfide crosslinks and hydrophobic and electrostatic interactions limit the accessible set of conformations and make a prediction of the adsorbed conformation very difficult[151]. In this context, only experimental measures can provide an insight to the adsorbed protein conformation upon various parameters.

The structure of fibrinogen and albumin molecules have been well studied. It seems, the overall molecular weight of this dimeric, 6 chained fibrinogen molecule is ~340,000 and its total length is ~450 Å. Moreover, from a variety of experimental and other considerations, it has been suggested that the fibrinogen structure occupies some portion of 90x450 Å cylindrical volume[253]. The human serum albumin is a single chain of 585 aminoacids, crosslinked by 17 disulfide bridges, which has a structural dimensions of about 140x40 Å[78]. Conformation changes occuring to these proteins due to substrate contact can alter their dimensions at the interface.

Nyilas and colleagues[253] have developed sensitive microcalorimeters to determine the thermodynamic functions for the adsorption of γ-globulin on glass particles. They have concluded[253] that γ-globulin molecules in the first adsorbed layer undergo substantial conformational changes. Lyklema and Norde[207], have determined the enthalpy of adsorption for serum albumin adsorbed on polystyrene latices as a function of pH and substrate surface charge. At physiological conditions, the enthalpy of adsorption is exothermic and the adsorbance large, despite the fact that both the protein and surface are negatively charged, however; at pH 5, near the isoelectric point where the adsorbance is a maximum, the net enthalpy is highly endothermic. This finding indicates that entropic factors are of dominant importance in the adsorption of serum albumin to polystyrene and suggests that changes in conformation occur, as a function of the pH of the following solution. Kochwa et al[182] also have demonstrated changes in conformation

occurred to γ-globulin molecules, concomitant with adsorption to a polystyrene latex.

Fenstermaker et al[92] and Smith et al[317] have used insitu ellipsometry to determine the adsorbance and extension of Fg, prothrombin and serum albumin adsorbed on a variety of surfaces. The adsorption studies under steady state condition of the adsorbed protein molecule has increased with decreasing surface free energy. The increase in extension with decreasing surface energy would be consistent with flat, tightly held conformations on the low-energy substrates. But Morrissey and Stromberg[234,235], using ellipsometric and IR techniques, have studied the conformation of prothrombin, serum albumin and Fg in adsorbed states. Their results suggest that conformational changes or rearrangements upon adsorption are minimal. However, a random distribution, of conformationally distorted and undistorted protein molecules, is possible on the polymer surface depending on the distribution of chemical groups on the surface[235].

Structural changes in factor XII and Fg upon adsorption to quartz have been detected with circular dichroism[226]. The results indicate that dried, adsorbed films of Fg does not change conformation upon adsorption, however, the spectra for solution and factor XII, both wet and dried, has shown major differences. The authors concluded that the binding of water is conformationally important and may be involved in the activation of factor XII, on the surface. Conformational changes, occurring on adsorption and desorption of albumin, γ-globulin and fibrinogen on copolypeptide and silicone surfaces, have suggested[319] that albumin and fibrinogen sustain a marked

decrease in $\alpha$ helical content, $\gamma$-globulin loses most of its $\beta$-sheet structure.

Recent studies with monoclonal antibodies, using an immunoenzymological assay, have revealed[322] that conformational alterations have occurred to Fg molecule by adsorption to the solid phase, which may have very important applications in the testing of artificial surfaces. Similar studies with polyclonal antihuman - thrombin antibodies produced in rabbits have shown[261] that, prothrombin adsorption to PVC has resulted in some molecular conformational changes, so that immunologically the adsorbed prothrombin resembles that of adsorbed thrombin on the same PVC surface.

In short, proteinated surfaces undergo conformational changes, denaturation, enzymatic degradation and turnover. However, studies so far done are not conclusive for the complicated process of protein adsorption on to various polymers eventhough it is the key event in blood-polymer interactions. The materials by themselves may elicit little or no active cellular response, but they become reactive by selecting and interfacially concentrating specific proteins from the bulk phase onto the surface phase. The cellular reactivity of the proteins in the layer also may be influenced by the accessibility and configurational state of the adsorbed proteins[153]. These factors also may be influenced by surface chemical properties of the polymers underlying the proteins. Thus, both the specific composition of the adsorbed layer and the reactivity of the proteins in the layer may affect the cellular response elicited when materials are used as implants. This concepts constitutes a

major hypothesis about biocompatibility and merits further studies.

## 1.5. Contribution of platelets to thrombus formation

Platelets are structural complex elements of the blood, which circulate in disc form averaging $\sim 3\mu$ in diameter and $1\mu$in thickness, and have an volume of $\sim 6\ \mu m^3$. The contribution of platelets to thrombus formation is known to some extent[117,129,153], which can be overlooked initially. Platelets adhere to damaged subendothelium, they can be stimulated to adhere to each other so that macroscopic platelet aggregates are produced, and they can release mediators that induce more platelets to aggregate and that promote blood coagulation. It is known that through these activities platelets contribute to haemostasis and it is widely believed that these activities are relevant to thrombosis, particularly to arterial thrombosis, where platelet masses form the bulk of the adhesion[22,379]. Whether thrombus formation is akin to haemostasis, where tissue damage initiates platelet deposition, or other factors present in the plasma provide the initial stimulus is unknown.

Haemostatic plugs and arterial thrombi are largely composed of platelet aggregates adherent to the injury site, with fibrin in and around the platelets acting to stabilize the plug or thrombus[129]. Aggregation or clumping of platelets is the sticking of platelets to one another, whereas adhesion can be defined as the sticking of platelets to a non-platelet surface, including subendothelial structures, collagen and artificial surfaces. Platelet that

interact with the subendothelium change from their normal disc shape to a more round form, extend pseudopods, and become adherent to the surface[101,148]. Alternatively, they may release their granule contents in to the surrounding medium, which include ADP, ATP, calcium, serotonin, platelet factors, β-thromboglobulin, fibrinogen, fibronectin etc[148,248].

A great number and variety of agents, including some low molecular weight molecules, proteolytic enzymes, glycoproteins and particulate matter, induce platelets to aggregate[147], but perhaps those that have most relevance to thrombosis are thrombin and collagen. ADP is an aggregating agent that has received special attention, because thrombin, collagen, and many other materials act, at least in part, by releasing ADP from intracellular storage granules[104,132]. It is now known that platelets are also induced to synthesize aggregating agents that are more potent than ADP. These are the prostaglandin endoperoxides, $PGG_2$ and $PGH_2$, and thromboxane $A_2$, the metabolic products formed from arachidonic acid[127,216] on the platelet membranes. Serotonin (5-hydroxy-tryptamine), adrenaline and noradrenaline are aggregating agents that regularly appear in plasma, are taken up by platelets, and are released from them along with ADP[118]. Although the plasma concentrations of these amines are not high enough for including a direct platelet aggregation, they may be important because, they can act synergistically with ADP to enhance its effect[146].

It is well established that fibrinogen is required for ADP induced aggregation of human platelets, binds to the receptors on the platelet surface during aggregation in a specific and saturable manner, and dissociates during

deaggregation[25,219]. When platelets are stimulated with ADP or other aggregating agents, the fibrinogen receptors become available on the surface of the platelets[219]. Thus fibrinogen may be essential for most, if not all, aggregation responses to form a link between adjacent platelets in an aggregate.

There are several ways in which platelets can contribute towards the coagulation process. First, the platelet surface protects active clotting factors from inactivation by their natural inhibitors[165]. Second, platelets contribute through aggregation, during which platelet phospholipids (platelet factor 3) are made available[64] and stimulate activation of factor X and the conversion of prothrombin to thrombin. In the presence of ADP, the coagulation pathway may be activated through factor XII[381], and interaction of platelets with collagen can activate factor XI[354]. Finally, during the release reaction fibrinogen and platelet factor 4 are made available. The amount of released fibrinogen is small in relation to plasma fibrinogen level, but it may provide a high concentration between the platelets in the aggregate[363].

### 1.5.1. Adhesion of platelets to artificial surfaces

The adhesion of platelets to artificial surfaces usually involves the interaction of platelets with a layer of plasma proteins adsorbed[110,179,362] to the surface. It has been suggested that the carbohydrate component of adsorbed proteins are involved in the reaction with platelet receptors in a manner similar to the platelet collagen

reaction[21,190,259] and probably also with surface charged groups such as N-acetyl neuraminic acid[266].

Following exposure of the surface to blood, platelets are seen to adhere rapidly, either as a monolayer or as aggregates and these may also have an associated fibrin meshwork[36,81,230]. The kinetics of platelet adhesion to a range of polymers has been studied using a platelet suspension in a protein-free buffer solution[2]. It is indicated that in a static system platelet adhesion reaches saturation after approximately sixty minutes and that the extent of adhesion increases with increasing substrate surface tension.

It is well known that under certain conditions, platelets can adhere to surface, and via different mechanisms, they may also adhere to each other[347,351]. The most important recent findings related to platelet adhesion and aggregation, is that platelets possess membrane receptors for a wide variety of agents including fibrinogen[219] and that these receptors become available for occupation by Fg when ADP is added to a platelet suspension[25]. Vroman et al[345,347,351] have indicated that large numbers of platelets have been found sticking where fibrinogen had remained adsorbed. Other factors, like the orientation and density of the preadsorbed fibrinogen carpet, help to decide whether the platelets may spread or maintain their original size and whether they can liberate any of the many agents they contain and that may promote aggregation of other platelets, clotting of the plasma, or other events.

It has been seen[100,101] that the platelets upon contact with an artificial surface can undergo shape change, extend pseudopods and become adherent to the surface. Alternatively they may release their granule contents in to the surrounding medium resulting in the formation of platelet aggregates via stimulating the cyclic endoperoxide pathway. Recently Baier et al[17] have studied the effect of surface energetics of substrates towards platelet attachment and spreading and has correlated towards blood compatibility. He has reported[17] that; minimal platelet spread areas have been observed on substrate with critical surface tension between 20 and 30 mN/m (dynes/cm), and increased spreading and morphological changes have occurred on substrate of both higher and lower critical surface tension.

Hence, it is evident that the materials vary in their thrombogenicity, which is dependent on the platelet release reaction and its aggregation. The exact biochemical pathway of this reaction is not clear, but may be associated with the increased synthesis of prostaglandin endoperoxides and thromboxane $A_2$, which in turn inhibits the enzyme adenyl cyclase and leads to a fall in cyclic AMP within the platelets[231,333].

### 1.5.2. The effect of substrate topography and flow on platelet adhesion

It is well known that bubbles and gas nuclei at the solid/liquid interface enhance platelet adhesion[355]. A static platelet adhesion test with smooth and roughened glass surfaces, (The roughness has been produced by using grade 400 sand paper), both in the natural hydrophilic state and after

diminished cell adhesion until some critical shear rate is reached at which erythrocyte haemolysis occurs. The release of haemoglobin then appears to promote increased cell adhesion[326]. As the clinical use of blood-pumping devices has increased, and surgical procedures involving extended extracorporeal circulation have become widespread, the effects of these procedures on formed elements of the blood have become clinically important.

The previous reports of the effects of fluid mechanical trauma, low to moderate levels of shear stress (100 to 300 dyn/cm$^2$) has produced altered polymorphonuclear neutrophil leukocyte morphology and function[327]. Thus the shear-related changes include an increased adhesion; aggregation and cell lysis, moderate decrease in chemiluminescence accompanying phagocytosis, and substantial loss of lysosomal enzymes[327]. These changes also cause an increased platelet-platelet sticking and the formation of microemboli. Thus the surface topography and haemodynamic parameters are highly inter-related to the thrombus formation at the biomaterial-blood interface.

### 1.5.3. The role of erythrocytes and white cells towards protein/platelet adhesion to an artificial surface.

Red cell-surface interactions may play a role in the dynamics of protein and platelet behaviour at the interface. The effect of red cells on platelet sticking has been noted widely, causing an augmentation of the rate of adhesion, probably by a combination of physical and biochemical mechanisms[113,338]. Hellem et al[135] have observed that red cell ghosts restore retention of platelets in columns almost

as effectively as whole red cells, which may be due to the presence of ADP. However; Stormorken[329] has showed that red cell ghosts do not contain ADP, but they may induce platelets to release ADP. So it seems that red blood cells or ghosts at the usual high concentration of 40-45% cause platelets to collide with the walls of a tube or with each other with increased frequency, causing a normal platelet retention.

Vroman et al[345,346,349] have demonstrated that, platelets adhere only where fibrinogen remains adsorbed and in the absence of erythrocytes the ring of platelets deposited is smaller. So it seems, red cells can modulate the protein adsorption and platelet adhesion at the interface, which still need to be better understood.

Recent evidences suggest[201,247] that leukocytes seems to play a role in intravascular clotting and thrombosis. In fact, an attempt has been made to correlate the adhesion of leukocytes to protein covered surfaces with the presence of large thrombi[191]. Several studies have shown that monocytes or polymorphonuclear neutrophils are found basal to the thrombus and large thrombi form over these leukocytes that adhere randomly over the surface[186]. However, how and why fibrin, platelets and leukocytes adhere to a given surface initially is still unclear.

On many surfaces of dialyzers, where dog blood will deposit platelets, human blood will deposit granulocytes, probably as a result of complement activation[96]. It is also found[3] that granulocytes adhere preferentially where $\gamma$-globulins have been predeposited on to a hydrophobic substrate. It is concluded from these studies[3,351] while

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platelets are in a race with fibrinogen/high molecular weight kininogen interactions, granulocytes are in a race with γ-globulin/plasma protein interactions in a similar way. So the identification of various components deposited from formed elements of blood, to material surface and the knowledge of subsequent interactions at this modified interface towards the formation of thrombi needs to be better understood.

## 1.6. Surface modification and blood compatibility

In order to select a suitable polymer for a particular implant, numerous attempts have been made to understand a relationship between blood compatibility and the surface properties of the polymer, such as charge, surface free energy, etc. which play a key role in the interaction process of proteins and the subsequent cell adhesion. Surface modification of materials have been attempted by various investigators[15,57,107,314,369] to modify a polymer, so that it may not interact adversely with blood components.

### 1.6.1. Surface charge

Recent observations have predicted that a very high negative charge (-40 to -120 mv, zeta potential) on the surface may prove beneficial for developing a nonthrombogenic surface[336]. However, the view that a highly charged surface will not adsorb protein and so will remain nonthrombogenic is dubious because such surfaces will never form passivating layers and so will always remain active. In such cases, even if protein adsorption is not taking place, blood components

may still be suffering damage[336]. This may cause the implant to be unsuccessful in the long term. On the other hand, if the surface is not very highly charged certain proteins may be adsorbed and form a passivating layer, which may ultimately make the material less adverse toward blood components and so relatively more blood compatible[298,336]. Therefore, the surfaces should be modified in such a way that they adsorb certain proteins like albumin preferentially and form a good blood compatible passivating layer.

### 1.6.2. Albumin coatings

It is assumed[209,257] that albuminated surfaces adhere fewer platelets, and it can act as a passive layer at the interface, led to produce albumin coatings to improve blood compatibility of surfaces. The presence of albumin on the surface may be favourable for blood compatibility; however, it may leach out over a prolonged period and the conformationally distorted molecule may cause chemotactic activity. Attempts have been made by various investigators to fix or immobilize albumin on polymeric substrates using different techniques like glow discharge, $^{60}Co$-irradiation, and glutaraldehyde cross-linking[163,302].

Sipehia and Chawla[316] have developed aminogroups on to the surfaces of Celgard 2400 membranes by exposing them to an ammonia plasma. The newly formed amino groups have been utilized for binding excess albumin, which has been further stabilized by crosslinking with glutaraldehyde. Sharma et al[302] has bonded albumin onto polyetherurethane surface using $^{60}Co$ $\gamma$-irradiation. Then a second layer of albumin has been immobilized on this initial layer via glutaraldehyde

crosslinking, which has shown high antiplatelet activity. It is also suggested[173] that first layer of albumin may be bound on the surface of a suitable polymer such as polyetherurethanes using glow discharge or $^{60}Co$-irradiation and a second layer can be adsorbed and then cross-linked via glutaraldehyde. Then the unlinked aldehyde groups can be utilized for immobilizing a 3rd layer of albumin or any biologically active agents like $PGE_1$ for enhancing the blood compatibility of the substrate, where the third layer may possess least conformational variations.

The use of an albumin pre-treatment for porous textile arterial prostheses as an alternative to preclotting has also been investigated[125]. Experiments have been carried out in dogs to evaluate the healing sequences of albuminated grafts compared to preclotted ones following sterilization by either ethylene oxide or $\gamma$-irradiation. It is evident[125] that healing proceeds satisfactorily with albuminated grafts and found to be better in $\gamma$-radiation sterilized cases.

### 1.6.3. Surface grafting of polymers.

Surface grafting of different polymeric materials with 2-hydroxy ethyl methacrylate[7,271], acrylamide (AA)[270], vinyl alcohol or N-vinyl pyrrolidone[206] has helped in enhancing the blood compatibility and antithrombogenicity of conventional plastic materials by surface modifications. Bruck has stated that the nature of the structure of water in hydrogels is critical to biocompatibility[52]. Since hydrogels are polymers which can retain morethan 20% water within their structure[6], they can provide a very low interfacial free energy system with water in blood. Also the

hydrophobic/hydrophilic surfaces may have sufficient bioadhesive potential to form a passivating layer. The active sites at the surface may influence the composition of the adsorbed layer of proteins and possibly the configuration of the adsorbed molecules such that a more inert surface is presented to the blood.

On the basis of these properties, hydrogels have been investigated extensively in a variety of biomaterial applications[52,270]. Hoffman et al[270] have studied the interaction of well characterized grafted polyacrylamide-silastic and silastic surfaces with blood, using an ex vivo, arteriovenous baboon shunt model. The results suggest that the polyacrylamide-silastic surfaces adsorb more proteins overall than silastic, and has a combination of lower adherent to platelets (higher albumin) but higher activity towards platelet aggregation (higher fibrinogen) and subsequent embolization.

The platelet adhesion to HEMA - grafted polyurethane films with a surface hydrophilicity compared with that of HEMA grafted tubes has been measured[170]. Platelet adhesion and protein adsorption found to reduce with increasing surface hydrophilicity (and in these cases also with decreasing interfacial free energy). Further, certain hydrophilic surfaces are found to damage platelets due to their 'never passivated' nature. Hence the selection of substrate polymer and optimization of polar/dispersion components with suitable hydrogels have been advantageous for enhancing substrate blood compatibility.

Aliphatic hydrocarbon chains have been introduced to polymers of biomedical interest and their protein/ platelet interaction studies[83] have been investigated. It seems, polymers treated by covalently binding $C_{18}$ alkyl residues selectively enhances albumin affinity. Simultaneous albumin-fibrinogen exposure to alkylated surfaces has shown that fibrinogen adsorption has reduced in proportion to enhancement of albumin adsorption. This treatment appears promising for general improvement of blood compatibility of a number of polymers.

Diamond polished LTI carbons exhibit excellent overall blood compatibility[52]. The relative smoothness of the substrate and the ultra-smoothness of the coatings are important factors in the biological performance of LTI carbons. The theoretical calculations[304] have also suggested that, these substrates can preferentially adsorb albumin, which may be another parameter for it's antithrombogenic nature. It has been proposed[80] that the biocompatibility of biomaterial surfaces can be improved by mimicking the phospholipid components which are present on the external faces of cellular plasma membranes. The plasma membranes are rich in electrically neutral, but zwitterionic, phosphoryl choline. So, a group of novel reactive species capable of covalently linking phosphoryl choline to a variety of polymers have been synthesised; which can be used as polymers for biomedical applications[134].

### 1.6.4. Modifications using heparin and prostaglandins

Heparin is believed to be the naturally occurring anticoagulant responsible for the fluidity of the blood in

the system. The outstanding anticoagulant activity of heparin is attributed to the high concentration of the sulfamate, sulfamide and carboxylic groups and their steric order and conformation along the chain[30,157]. The methods of bonding heparin to polymers to make non-thrombogenic artificial surfaces are based on achieving ionic attachment of the molecule, such as pre-treatment of polymer with graphite and a cationic surfactant or with quarternary ammonium salt[115,279]. The main problem encountered with heparinized surfaces is slow leaching of heparin from the surface by dissociation of the complex, especially in the case of ionic bonding. The heparinized surfaces appeared to inhibit platelet adhesion and aggregation, upon contact with blood[115,188]. It is interesting that the heparinized surfaces adsorb proteins in a greater extent than do untreated ones[32], and it includes proteins like antithrombin III, and factors IX, X and XI[202].

The heparin releasing materials are assumed to improve the thromboresistance of foreign surfaces by providing a high concentration of heparin at the desired site of action, namely the blood-polymer interface. Heparin releasing polymers have been described by Ebert et al[84] and Heyman et al[139]. In the first study[84] heparin together with the platelet protecting drug $PGE_1$ has been dispersed through poly (hydroxy ethyl methacrylate), where this polymer releases heparin and $PGE_1$ over a 10 hour period and these molecules have retained their biological activities. In the second study[139], low molecular weight with high antithrombin III (AT III) affinity (LMW-HA) heparin has been released through Biomer and PVC. It is observed[139] LMW-HA heparin releases at higher rates from the polymer matrics as

compared to unfractionated heparin and the in vivo studies indicate that the blood compatibility of catheters with a polyurethane/heparin layer has been considerably improved.

Due to the depletion of heparin, the heparin releasing systems lose their efficacy in time. Consequently many investigators have covalently bound heparin to material surfaces, without affecting it's conformation and anticoagulant activity. Salzman et al[287] have covalently coupled heparin to hydroxyl-bearing surfaces via an ethylene imide intermediate. Blood exposed to these surfaces exhibit prolonged clotting times, but platelet adhesion has been substantially higher than untreated, controlled surfaces. The presence of heparin on the surface may have influenced the adsorption of plasma proteins, which subsequently caused platelet adhesion. Cottonaro et al[71] have developed nonthrombogenic surfaces by covalently attaching a bifunctional quarternary ammonium compound to PVC, silicone rubber and latex rubber with subsequent immobilization and stabilization of heparin to specific sites on the cationic chain. The heparin bound surfaces have been more stable to flow conditions, fibrinogen adsorption values have found to be vary low and has less thromboembolism[71,86].

Modifications using prostaglandins

In view of the current interest in prostaglandins and their precursors as agents that reduce platelet adhesion and aggregation to normal endothelium [162,231] these compounds seem to be a good choice for incorporation in to polymer membranes. Grode et al[122] have demonstrated that surface bound prostaglandin $E_1$ is capable of inhibiting platelet

adhesion when compared with control surfaces. This study also suggests that physiologically active coatings may minimize platelet aggregation at the blood surface interface. Prostaglandins such as $PGI_2$, $PGE_1$, and $PGD_2$ etc. are believed to stimulate membrane bound adenyl cyclase and thereby raise intracellular levels of cyclic AMP (cAMP) within platelet; which can inhibit the platelet adhesion and aggregation reactions[33,232].

Earlier observations have shown[227] that the controlled release of prostaglandins from commercially available biomedical polymers greatly reduce platelet adhesion and aggregation. The presence of prostaglandin within the polymer matrix may affect the mechanical properties of the polymer[227], as well it can reduce the biological activity of the prostaglandin molecule itself. Ebert et al[86] have demonstrated the antiplatelet effects with regard to platelet aggregation and adhesion by immobilizing prostaglandin-$F_2$ (precursor of $PGI_2$) and converting it to $PGI_2$ in the polymer matrix. Prostaglandin $E_1$ is also immobilized on albuminated polymer matrix, through glutaraldehyde coupling and such surfaces, then possess a high antiplatelet activity[59,308]. A synthetic $PGI_2$ namely 10, 10-difluoro-13-dehydroprostacyclin[99,174], which has about a tenth of the activity of the natural $PGI_2$, has immobilized on polyetherurethane urea, which showed dramatic reduction in platelet adhesion. Protein adsorption kinetics of $PGI_2$ analog immobilized substrates have demonstrated[309] a reduction in fibrinogen to albumin mole ratio compared to bare polymer, which may be an indication of nonthrombogenicity of the substrate.

### 1.6.5. Drug complexes as coatings for Biomaterials

The possibility of obtaining preparations of enzymatic drugs of multiprofile action has been experimentally confirmed[136,215]. These enzymatic drugs having combined action can be utilized for creating effective coatings for improved blood compatibility. Albumin-heparin conjugates (alb-hep) and high AT-III affinity conjugates (HA-alb-hep) have been prepared, for combining their favourable properties of decreasing the extent of platelet adhesion and inhibiting fibrin formation during thrombogenesis[88,136]. These conjugates have been coated on catheter surfaces to improve the blood compatibility. Alb-hep coated catheters have shown a four to five fold reduction in platelet deposition in comparison with uncoated catheters, whereas HA-alb-hep coating has indicated a ten fold reduction. These modified substrates have also demonstrated their superior anticoagulant properties, compared to the bare substrate.

A covalently bound conjugate of heparin and $PGE_1$ has been synthesized to provide the dual pharmacological role of individual compounds[166]. Bioactivity tests on the conjugates confirm that both the anticoagulant activity of heparin and the inhibitory effect of $PGE_1$ on platelet aggregation have been maintained. Recently an attempt is made to repeatedly modify urokinase with antithrombin III-methyl dopa-prostaglandin $E_1$[312]. The complex is immobilized on albuminated polymer matrix, which showed fibrinolytic, anticoagulant and antiplatelet effects simultaneously. The approaches suggested, indicate the possible new ways of creating nonthrombogenic surfaces with wider applications.

## 1.6.6. Other means of surface modification

Biolized surfaces have also invited considerable attention as candidates for biocompatible surfaces[176]. Natural tissues or proteins can be treated with aldehydes like formaldehyde or glutaraldehyde to produce blood compatible surfaces. Aldehyde treated natural tissue improves their blood compatibility and has been used for arterial substitution, valve replacement and blood access. Polymeric substrates are usually treated with albumin or gelatin followed by a cross-linking treatment with glutaraldehyde to form such a surface[300].

Surface immobilized enzymes and surface grafted polyelectrolytes have been tried to improve the blood compatibility. Sugitachi et al[334] have immobilized a plasminogen activator, urokinase, on polymer surfaces and has demonstrated the fibrinolytic activity of the modified substrate. Polyelectrolytes, having sulfamate and carboxylic groups arranged in a steric manner as that of heparin, have been synthesized from natural rubber[306,341] (Hevea Brasiliencis "para rubber"). This synthetic polyelectrolyte grafted polyurethane grafts have shown good antithrombotic properties[303].

Animal studies have shown[94] that endothelial growth (either by prior seeding or neointimal formation) on the surface of devices implanted in the cardiovascular system enhances their blood compatibility. Ives et al[164] have investigated the growth of human umbilical vein endothelial cells (HUVEC) on a segmented polyether urethane urea, Mitrathane$^{R}$, a relatively hydrophilic, elastomeric polymer

under static, shear stress/strain conditions that simulate the normal physiological range. The results show, the HUVEC grow well on Mitrathane$^{R}$ under stationary conditions; and the cells keep a characteristic polygonal/endothelial appearance. However, under flow conditions, they become more elongated with their long axis aligned with flow. The growth and stability of cells has been improved with proteinated substrates; For eg: albumin alone or added with collagen, have shown good adhesion and migration of the cells, when compared with controls[315]. Moreover, the cell morphology has been better, when albumin, collagen and fibronectin have been combined for seeding the cells.

Thus extensive efforts have been made by various investigators to modify the surface towards improving their blood compatibility; however, still the only alternative for a small vessel substitute is the natural vessel itself. Therefore, more understanding is needed in this area before the challenge of developing a blood compatible surface can be met.

## 1.7. Relevance of this study

When blood plasma comes in to contact with a solid, it will deposit proteins at the interface within seconds followed by the adhesion of platelets to thrombus formation[13,31,40]. The nature of the protein deposited can influence the platelet adhesion and the development of thrombosis. It has been observed[209,257] that albuminated surface adheres less platelets, on the other hand surface bound fibrinogen enhances the platelet adhesion. Thus fibrinogen plays an important role in the clotting systems in

both the plasma phase ("intrinsic") and the cellular phase (platelet aggregation) in normal haemostasis[253], and has also been implicated in thrombosis on foreign surfaces[377]. The relative high concentration of fibrinogen in plasma (Ca 3 mg/ml) and its high affinity for surfaces compared with the major plasma proteins, albumin or immunoglobulin-G also suggests a role for fibrinogen in blood-material interactions[150].

Mason et al[222] have shown that platelet adhesion to glass is strongly inhibited in afibrinogenaemic blood, and Zucker and Vroman[377] have reported that afibrinogenic blood deposits platelets on glass that has been pre-exposed to normal plasma for a few seconds. These results suggest that fibrinogen deposition is required for platelets to stick to glass. Several investigators have found[44,149,192], that fibrinogen can be preferentially adsorbed from two or three protein mixtures. These studies have been done on a variety of surfaces with various mixture compositions and consistently confirm Fg preferential adsorption as a general effect.

However, this preferential adsorption of Fg is not observed from plasma to varous surfaces[44]. It is also indicated, that preadsorbed Fg from buffer has been almost completely desorbed after 5 minutes of contact with plasma, possibly by exchange with other proteins or by enzymatic action[45,47]. Vroman et al[348,352] have indicated that on many surfaces, blood plasma deposits predominantly Fg within a second,but on potentially clot-activating substrates, this Fg appears to be replaced by high molecular weight kininogen and factor XII.

Plasma proteins like albumin, Fg, IgG and $\gamma$-globulin have been extensively studied for their relation to surface induced thrombosis. But, blood contacting devices have to come across a lot of other trace species like vitamins, enzymes, hormones, immunoproteins etc. in the blood. The patients, bearing the implants, have to take antiplatelet drugs, antibiotics, and anticoagulants etc. to reduce the consequences of surface induced thrombosis, and for a normal haemostasis.

Vitamins are esssential for the biochemical functioning of the human body and for maintaining good health. The major portion of them has to be supplied through dietary sources. The dietary deficiencies of these biochemical substances (either because human body could not synthesize at all, or could synthesize only in limited, usually insufficient quantity), can produce conditions such as scurvy, rickets, pellegra, pernicious anaemia[50] etc. It has been suggested that certain vitamins like pyridoxal 5'-phosphate, vitamin E, etc. possess antiplatelet and anticoagulant properties[184,366]. Aspirin, sulfinpyrazone and dipyridamole, the principal drugs tested in a growing number of recent clinical trials, are all efficient inhibitors of platelet aggregation in vitro[72,360]. It is also proposed that, several antibiotics can inhibit platelet function in vivo and in vitro[331]. This seems to induce a bleeding diathesis in patients receiving those antibiotics in large doses.

Thus, it appears that the chemical structure of various agents, inhibiting platelet aggregation may be quite varied. The development of the concept of cAMP as a mediator

of cell function and the observations that many of these agents increase the platelet level of cAMP through the activation of adenyl cyclase and/or the inhibition of phosphodiesterase has led to the unifying concept that these agents are possibly linked by a common effect on platelet-cAMP[217,286]. It has been generally accepted that an increase of platelet cAMP, inhibits the platelet responses to stimuli[133]. Thus, the plasma contains vitamins, antiplatelet drugs, antibiotics, anticoagulants, cAMP etc. as trace components. However, a fundamental understanding of the protein/platelet/surface interaction in presence of these trace components of plasma, or antiplatelet drugs or other mediators arriving at the interface and their implication to modulate this process is meagre.

Earlier studies with vitamin C throw some light in this direction; that vitamin C can modify the surface-protein interaction and surface platelet binding at the interface[299]. It appears that vitamin C can get adsorbed to the surface, which can probably affect protein adsorption and its conformation. The effect of vitamin C in increasing protein adsorption on to an artificial surface has been reported[299]. This may be due to the decreased interfacial tension caused by the molecule in solution, thereby enhances its tendency to reduce the disulfide linkages in protein which will thus induce a change in the conformation of protein. The affinity of vitamin C in reducing the binding of platelets at the polymer interface has been indicated[58]. It has also been reported, using ellipsometric measurements, IR-ATR and PAGE, that the adsorption of albumin is increased, relatively, in the presence of vitamin C, when compared with that of fibrinogen and $\gamma$-globulin from an equal amount of protein

mixture. This increased adsorption of albumin by vitamin C in solution is good from the blood compatibility point of view, since albuminated surfaces adhere less platelets[209,257].

Such interesting observations were the basis of these investigations on the adsorption/desorption of various plasma proteins on to the substrate when the mediators, such as antiplatelet agents or other vitamins, proteases and drugs arrive at the interface. The following chapters provide a fundamental understanding of the results gathered from these studies of protein/platelet- substrate interaction and their modulations at the interface with various mediators. The interaction of fibrinogen/platelet to polycarbonate substrate has been emphasised throughout the study and compared certain cases with albumin. Polycarbonate has numerous applications in the biomedical and dental field[66]. They have high resistance under conditions of sterilization, good mechanical properties over a wide temperature range, complete transparency, physiological inertness, good resistance towards aqueous agents, oils, fats, and alcohols[66]. They have been widely used for preparing disposable syringes, injection moulded dental prostheses and dialysismembranes[66]. Hence, this material has been selected for these studies and compared, in certain observations, with other polymers like, Teflon, Nylon, Polystyrene, Angioflex etc. for better understanding of the interaction process.

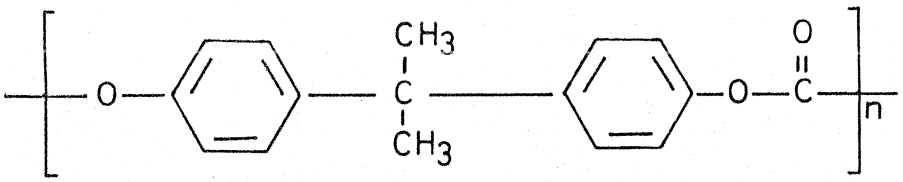
# MATERIALS AND METHODS

# CHAPTER - II

## 2.1. MATERIALS

### Surfaces

Polycarbonate,(poly-4-4'dioxy diphenyl-2-2-propane carbonate)



n is 45 ± 15

Lexan Grade 144R, manufactured by General Electric Co.,USA, to meet the performance requirements of medical,and health related applications was bought in bulk, and the same lot was used for all the studies. The resin was prepared by the manufacturer with controlled colour and contaminants as suggested by FDA and USP class VI compliance. The ingredients like mold release agents ie; silicone fluids and aliphatic esters < 0.5% and colourants, the dyes and pigments < 0.1% had been indicated in the resin (Lexan, Material Safety Data, General Electric, November 1985), Nylon (Nylon 6,6, BDH, England), Polystyrene (pellets, Density 1.047, Aldrich, USA),Teflon (Teflon FEP, Champion, India),and Angioflex cured sheets 10 x 10 x 0.015 inches, Applied

Biomedical Corporation, Massachusetts, USA were used as substrates.

Proteins

Fibrinogen ( Human , fraction I , Over 95 % Protein clottable), Albumin (Human, Fraction V, 96-99% pure), γ-Globulin (Human, Cohn fraction II), Haemoglobin (Human, Type IV, twice crystallized), Thrombin (2000 NIH units per mg protein), Plasmin (4.1 units per mg protein) and Trypsin (Type I, 10,500 BAEE units per mg protein) were obtained from Sigma Co., USA. Fibronectin was a gift from Irvington House Institute, New York and was used as such[260]. The proteins used were pure as indicated by other investigators[57,149,346] and the negligible amount of plasmin existed in the fibrinogen sample as reported elsewhere[289]. All proteins were dissolved in 0.1 M phosphate buffer, pH 7.4, kept frozen in aliquots and used within one month; i.e. always fresh protein solutions were used for each experiment. In some studies fibrinogen was dialyzed overnight at 4°C against 0.1 M phosphate buffer, pH 7.4.

The vitamins, antibiotics and antiplatelet /anticoagulant drugs

Vitamins used were, Vitamin A (Retinol, Type X, Sigma, USA), Vitamin B6 (Pyridoxal Phosphate, SISCO Research Laboratories, India), Vitamin C (Glaxo, India), Vitamin D (Vitamin D2, Ergocalciferol, Sigma, USA), and Vitamin E (α-Tocopherol, Sigma, USA). Antibiotics studied were, Ampicillin injection I.P, (Biocilin, India), Neomycin sulphate (Unichem. Lab. India), Streptomycin sulphate I.P (Indian Drugs and Pharmaceuticals Ltd), Procaine Penicillin,

G.I.P (Alembic Chemicals, India), and Gentamycin I.P (Schering Corporation, USA).

The antiplatelet and anticoagulant agents used were, Heparin and Aspirin (Boots Company, India), Persantine (Dipyridamole, Boehringer Sohn, Germany), the amino sugars like, Glucosamine, Galactosamine, Mannosamine were from Sigma Co, USA and a polyelectrolyte from Natural Rubber, (Hevea Brasiliensis) synthesized in this laboratory.[306] Cyclic AMP (Free acid, 99-100% crystalline) was also a product of Sigma, USA.

## Chemicals required for Polyacrylamide gel electrophoresis

Acrylamide, N-N' Methylene -bis-acrylamide (Bis), Tris (hydroxy methyl) amino methane (Tris), N-N-N'-N'-Tetramethyl ethylene diamine (TEMED), Riboflavin, Ammonium persulphate, glycine, Coomassie brilliant blue R-250, and sodium dodecyl sulphate (SDS). All these chemicals were from Sigma Chemicals, USA.

Platelet aggregating agents like, Epinephrine-HCl (Sigma Co), ADP Disodium Salt (BDH, England), and all other chemicals were of the analytical grade. Radiolabelled proteins used were iodinated $^{125}I$ Human fibrinogen, and iodinated $^{125}I$ Human albumin from Amersham, England.

## Preparation of the Polymer Films

The films of Polycarbonate (PC) and polystyrene (PS) were prepared by casting, solutions of 10% (w/w) in dichloromethane and toluene, respectively, on glass plates.

The solvent was removed in an oven for 4 hours at 60°C. A Nylon film was obtained by casting a 10 % (w/w) solution in m-cresol on a glass plate. The solvent was removed in an oven at 110°C. Teflon and Angioflex were received as films. All films were cut into suitable sizes, washed thoroughly with copious amounts of double distilled water and finally rinsed in ethanol. The films were dried in a vacuum oven at 60°C for 4 hours. Such cleaning procedures had been found adequate[151]. The glass plates used for casting of the polymer solutions, were cleaned in chromic acid for 24 hours and subsequently extensively washed with distilled water before use.

## 2.2. Octane contact angle studies

### Theoretical -Polar interactions across solid/water interface

Here, octane/water method has been selected as a probe for investigating the polar interactions across polymer/water interface[128]. Consider a drop of n-octane at a solid-water interface. From Fig 2-1, we can write (from Young's equation).

$$\gamma_{sw} - \gamma_{so} = \gamma_{ow} \cos\theta \qquad (1)$$

Where $\gamma$ is the interfacial free energy and the subscripts 's' denote solid substrate, 'w' water and 'o' n-octane.

These interfacial tension terms can further be written as[128,145].

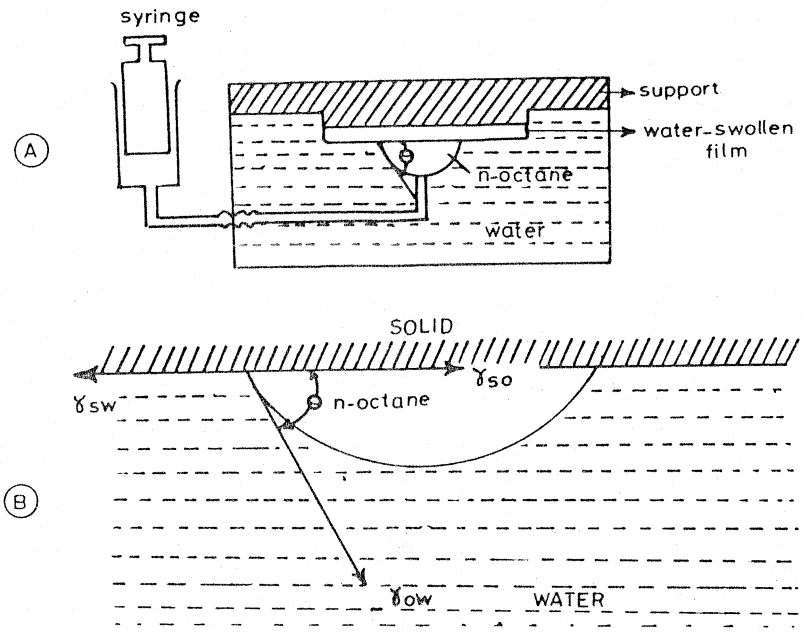



Fig.2-1. Schematic drawing of apparatus for contact angle measurements by the inverted bubble method.
B-Drop of n-octane at the polymer-water interface.

$$\gamma_{so} \simeq \gamma_s + \gamma_o - 2(\gamma_s^d \gamma_o^d)^{1/2} - I_{so} \qquad (2)$$

$$\gamma_{sw} \simeq \gamma_s + \gamma'_w - 2(\gamma_s^d \gamma_w^d)^{1/2} - I_{sw} \qquad (3)$$

Where, $\gamma_s$ and $\gamma'_w$ are the surface tension of polymer and octane saturated water respectively. $\gamma_w^d$, $\gamma_s^d$ and $\gamma_o^d$ are dispersion components of the surface free energies of w, s and 'o' phases, and Iso, Isw are the nondispersive (Polar) interactions at the s-o and s-w interfaces.

n-Octane has a surface tension of 21.6 dynes/cm at 25°C which is also its dispersion component of the surface tension as octane has no polar component.

$$\gamma_o = \gamma_o^d = 21.6 \text{ ergs/cm}^2 \qquad (4)$$

also, at 25°C, $\gamma_w = 72.1$ ergs/cm$^2$,

$\gamma_w^d = 21.6$ ergs/cm$^2$

and $\gamma_w^p = 50.5$ ergs/cm$^2$

So, $\gamma_o = \gamma_o^d = \gamma_w^d = 21.6 \text{ ergs/cm}^2 \qquad (5)$

from equation (1), it follows

$$\cos\theta = \frac{\gamma_{sw} - \gamma_{so}}{\gamma_{ow}} \qquad (6)$$

from equations (2), (3) and taking Iso = o, equation (6) can be written as

$$\cos\theta = \frac{\gamma'_w - \gamma_o - I_{sw}}{\gamma_{ow}} \qquad (7)$$

or $I_{sw} = \gamma'_w - \gamma_o - \gamma_{ow} \cos\theta \qquad (8)$

$\gamma'_w \sim \gamma_w$ (the solubility of octane in water is less

than 1 ppm)[7,145].

$$I_{SW} \simeq 50.5(1 - \cos\theta) \qquad (9)$$

So, thecontact angle $\Theta$, can provide a direct measure of polar interactions across the solid/water interface. For a purely apolar surface in water, $I_{SW} \simeq 0$ and $\theta$ will be small, indicating a high polar component to the interfacial free energy. For a very hydrophilic surface, $\theta$ will approach $180^{\circ}$, indicating a large Isw and a correspondingly low polar component to the interfacial free energy.

## Experimental method

Inorder to develop an understanding of protein-polymer interactions at the solid/liquid interface, which is closest to in vivo conditions, the contact angle of 99.99% pure n-octane on different modified surfaces have been attempted as described elsewhere[12,301]. The modified polymer surfaces, mounted on microscope slides, were supported in an inverted position in a container, as shown in Fig.2-1A. The container was carefully filled with double distilled water until the microscope slide was completely immersed. The goniometer (Kernco Instruments Co. Inc., Texas) was aligned and focused on the polymer-water interface. At this point a microsyringe, containing 99.99% pure n-octane, was lowered in to the water. A drop of $\sim 0.1 - 0.2\,\mu$ litre was formed on the syringe tip, positioned underneath the polymer surface, 'snapped' from the tip, and allowed to rise to the polymer-water interface. The apparent octane polymer contact angle was immediately measured. Angles on both sides of each bubble were measured, assuming symmetry, and at least 30

angles were observed from triplicate experiments. The measurements were averaged and expressed as the mean contact angle with standard deviation.

## 2.3. Adsorption of plasma proteins

### 2.3.1. Trace labelled studies: General principle

Essentially, the isotopic tracer method involves the labelling of a compound with one or more radioactive or stable isotopes, in such a way that the label is firmly attached to the molecule and which acts as a tracer for the studies. The quantity of a radioactive isotope required in a tracer experiments is so small that it is impossible to detect it by any means other than by its radioactivity. This is because, in the detection of radioactive materials, each single emission or particle detected corresponds to the disintegration of one single atom. So, the total radioactivity of a preparation containing a radioactive isotope, is determined by the total number of radioactive atoms present in the sample, or even as a count rate measured under defined conditions.

The measurement of radioactivity depends entirely on the phenomena occurring when radiation passes through matter. $\alpha$ and $\beta$ particles are readily absorbed, depending largely on the specific gravity of the material through which they pass, $\gamma$-radiation is absorbed to a much smaller extent, but produces some secondary $\beta$-particles during its absorption. When a charged particle having an energy above a certain limit passes near a gaseous molecule, it causes an orbital electron to be dragged or pushed out of one of the

atoms in this molecule, producing 'an ion pair' ( ie. a -vely charged electron and a heavy + ve ion) comprising the rest of the gaseous molecule . It is this property of ionizing the materials through which the rays pass, which is utilized in all method of determining radioactive isotopes.

Inthe present studies the protein adsorption kinetics were carried out using $^{125}I$ labelled albumin or $^{125}I$ fibrinogen as a tracer. Radio labelled proteins have been used widely to understand the protein-polymer interaction, mainly for obtaining an accurate measure of the quantity of protein adsorbed. It is also extensively studied and indicated that, the measurement of surface concentration is not affected by tracer level and the labelled and unlabelled protein acts as a tracer of the protein system [44,339]. The radioactivity of the adsorbed proteins were detected using a $\gamma$- counter, which consisted of a miniature halogen quenched Geiger-Muller counter as a $\gamma$-ray detector, connected with a count rate meter. The corrections were made in all cases for background readings and other sources of error like self-absorption and resolving time.

### 2.3.2. Experimental method : Protein adsorption/desorption Kinetics: under static conditions

Adsorption/desorptionexperiments were carried out as described previously[62,303,339]. Briefly, the clean films of size 2x1.5cms, were exposed to albumin or fibrinogen solution (for isolated studies) in 0.1 M phosphate buffer, pH 7.4 at 37$^{o}$C, containing 40% (0.46 $\mu$curie/ml) $^{125}I$ labelled albumin or 7 % (0.51$\mu$ curie/ml) $^{125}I$ labelled fibrinogen respectively[87,260]. Similarly, to protein mixture (25 mg%

albumin, 15 mg% γ-globulin and 7.5mg% fibrinogen) or plasma, any one of the labelled proteins ( either $^{125}I$ albumin or $^{125}I$ fibrinogen) was added as tracer in the concentrations suggested above. The plasma concentrations of albumin: γ-globulin: fibrinogen, is about 25:5:1.5; however higher amounts of γ- globulin and Fg have been used here as reported elsewhere[178].

Thefilms were dipped in buffer for at least one hour and shaken to remove all air bubbles. Then the protein (isolated or mixture), containing a known amount of one of the labelled proteins was added to make respective concentrations of them inside the media to reduce the air/water interface[61]. The experiments were run at 37°C over a period of about 3 hours. The films were removed with time,shaken, and rinsed in three separate beakers filled with approximately 30 ml of phosphate buffer, and a final rinse in flowing buffer (under a flow rate of $\sim$ 150 ml/mt for 60 seconds). A uniform rinsing procedure, necessary for an irreducible level of surface concentration was used for all samples[62,339]. The experiments were repeated in a similar fashion in presence of other blood components, antiplatelet drugs, vitamins, etc. and were compared with controls. The effect of pH, temperature and bulk concentration towards protein - surface binding, were also investigated by varying the pH, temperature and bulk protein concentration of the media.

Thefilms were counted in a γ-counter and are reported as surface concentration of protein $\Gamma$($\mu$g cm$^{-2}$) computed from the equation[339]

$$\Gamma = \frac{C^p R^b}{A R^s}$$ where $C^p$ = bulk concentration

($\mu g\ ml^{-1}$), $R^b$ = count rate of surface, A = area of surface ($cm^2$) and $R^s$ = count rate per ml of protein solution. The values expressed are the average of three separate experiments and the relative error of these determinations have been within 5 to 10%. In most of these studies, the surface counts varied from 1500 to 10,000 depending on the exposure time with a background reading of 500 $\pm$ 50 per minute. The solution count rate per ml (bulk protein) was within 50,000 to 100,000 per minute, which was kept constant for a typical set of experiment.

Theprotein that had been preadsorbed for 3 hours underwent desorption/exchange for 3 hours in an isolated protein solution or protein mixture (without the labelled proteins) under same conditions used for adsorption studies. The films were taken out with time and the surface protein concentration was quantitated as described earlier.

### 2.3.3. Protein adsorption/desorption kinetics: under flow conditions

Adsorption kinetics of Fg in their isolated formto PC and from a protein mixture to different polymer substrates using $^{125}I$ fibrinogen, were also studied; under various flow rates. Fig. 2-2 shows the cross section/layouts of the flow cells, where the material mounted in between the two cavities in constant contact with protein solution. Two acrylic blocks clamped the cell together using stainless steel screws. Fig.2.3 demonstrates a schematic drawing of the flow test system, which consisted of a reservoir (to hold the protein solution), connected with silastic tubes to a perfusion chamber, (to fix the polymer film) and finally to a

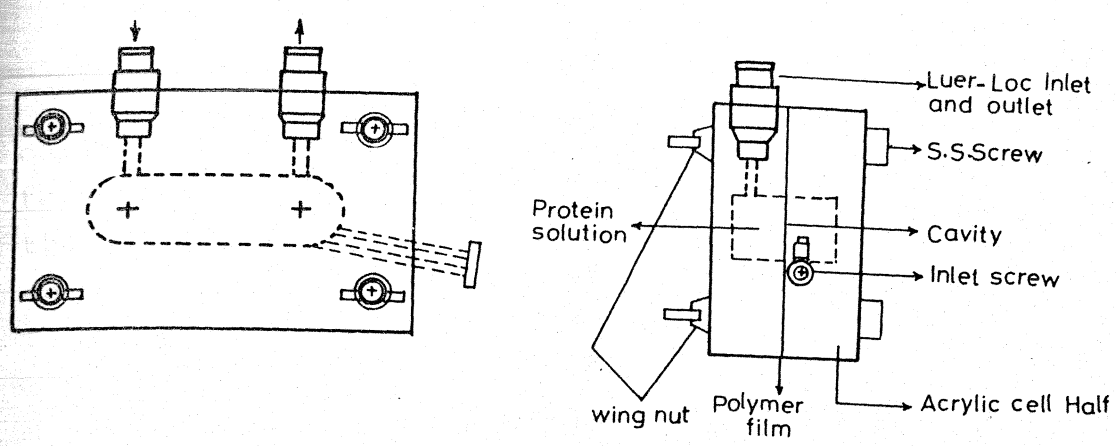
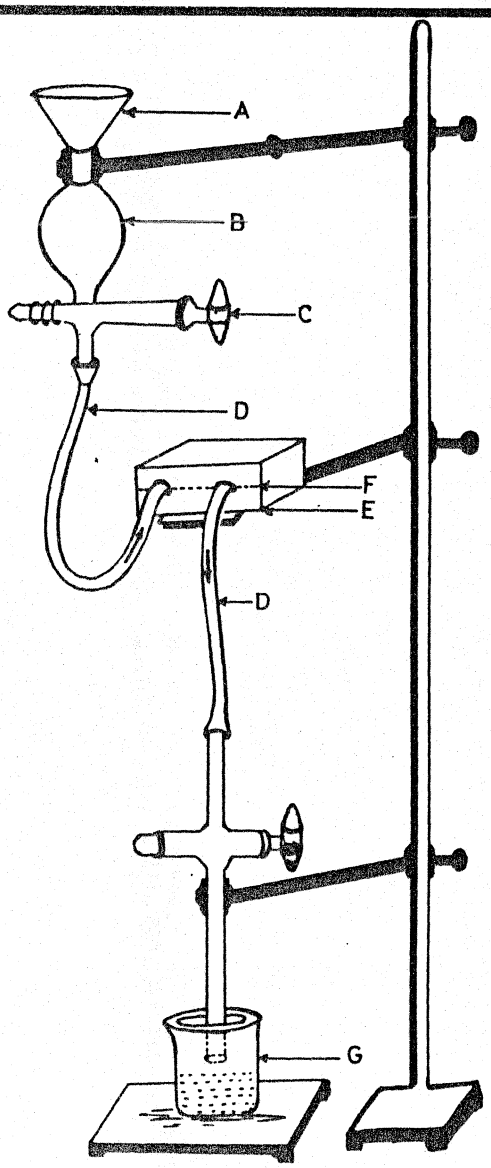



Fig.2-2.Cross/section Layouts of the flow type cell used for the study.




A. FUNNEL
B. RESERVOIR
C. STOP COCK
D. TUBINGS
E. PERFUSION CHAMBER
F. POLYMER FILM
G. COLLECTING VESSEL

Fig. 2-3. Components of the flow test system

collecting vessel. The cells were nearly identical with the test cell used by Mason et al[224]. A circuit was opened with the protein solution from the reservoir to the collecting vessel, which again reached to the reservoir to continue the flow system, for various time intervals.

Thecircuit was primed with 0.1M phosphate buffer, pH 7.4, which was then displaced by the test protein medium containing a known amount of labelled Fg, in a manner to avoid air bubbles for reducing the air-solution-solid interface. Experiments were run at flow rates of 1 ml/mt, 10 ml/mt and 50 ml/mt at room temperature (30°C). To determine the time course of adsorption, films were removed at various times up to 3 hours and were rinsed three times with 30 ml volumes of phosphate buffer, and a final rinse in flowing buffer. The films were counted (2x1.5cms size) in a $\gamma$-counter and the counts of 1 ml of aliquot of the labelled fibrinogen solution, (isolated or protein mixture of known concentration) primed thrqugh the test cell was also counted. The surface concentration of fibrinogen (in $\mu g/cm^{-2}$) was quantitated as described previously.

The desorption kinetics of 3 hours preadsorbed fibrinogen (from above experiments) was also done in a similar fashion under various flow rates and the surface concentration was calculated.

### 2.3.4. Determination of protein concentrations in plasma

In the case of adsorption kinetic studiesusing plasma, the knowledge of the protein concentration in plasma is required. Total protein content in the calf's plasma and

serum was determined spectrophotometrically using the Biuret method[371]. The biuret reagent in presence of cupric ions in alkaline solution produces a violet colour with protein. Albumin was determined after removing all globulins in the serum with sodium sulfate[371] and the globulin content was obtained by deducting the albumin concentration from total proteins. Fibrinogen (in the same plasma) was determined by clotting with thrombin, digestion of the clot[273] and the biuret analysis of the resulting solution[371].

Calfblood was collected in two plastic vials and separated the serum and platelet poor plasma (clotted blood for serum and 0.38% citrated blood for plasma respectively). The protein content of plasma and serum were determined, as follows. Total proteins: To 0.2 ml serum in a test tube added 3.8 ml of normal saline, and mixed well. Albumin: To 3.8 ml of 26% sodium sulphate in the test tube, 0.2. ml of serum was added, mixed by rotating between palms. It was then filtered through whatman No.44 filter paper. Fibrinogen: In a glass centrifuge tube mixed 0.2 ml plasma, 2.8 ml normal saline and 1 ml of thrombin solution (1NIH units). Twirled the tubes gently for 3 minutes and left at room temperature for 10 minutes to form a firm clot. Centrifuged at 700 x g for five minutes and decanted the supernatant. Then washed the clot with normal saline twice, centrifuged each time at 700xg for 3 minutes. Added 1 ml of 10% NaOH to the washed clot and heated in boiling water bath for 10 minutes after covering the tube properly to reduce evaporation and allowed to cool. To 1 ml each of these solutions (Total proteins,albumin and fibrinogen), added 5 ml of biuret reagent, and read after 20 minutes at 540 nm using a Beckman Spectrophotometer, calculated the protein contents by

comparing with a known albumin standard and suitable controls.

### 2.3.5. Polyacrylamide gel electrophoretic analysis of desorbed proteins. (PAGE)

**Theory**

Biological macromolecules like proteins,nucleic acids, polysaccharides etc. in solution possess a certain electrical charge. Under the influence of an electric field, these charged particles will migrate either to the cathode or to the anode, depending on the sign of their net charge, molecular size and shape. The matrix for polyacrylamide gel is formed from acrylamide and a crosslinking agent, N,N'-methylene bisacrylamide (Bis)-in the presence of catalysts and initiators. Polyacrylamide gels also participate in the separation process by molecular sieving effect, which is related to the concentration of acrylamide monomer in the gel. The higher the concentration of acrylamide monomer, the smaller the average pore size in the gel. The diffusion of protein molecules is restricted in the matrix and remarkably sharp zones are consequently obtained in PAGE.

## 2.3.6. Experimental method

### Prepararion of the gel

The following stock solutions were prepared and kept in brown bottles at 4°C[74, 116]

| Solution | Constituents | Weight or Volume | Total Volume | pH |
|---|---|---|---|---|
| A | IN.HCl | 48 ml | | |
| | Tris | 36.6 gms | 100 ml | 8.9 |
| | TEMED | 0.23 ml | | |
| B | IN.HCl | 48 ml | | |
| | Tris | 5.98 gms | 100 ml | 6.7 |
| | TEMED | 0.46 ml | | |
| C | Acrylamide | 28 gms | | |
| | Bis | 0.735 gms | 100 ml | - |
| D | Acrylamide | 10 gms | 100 ml | - |
| | Bis | 2.5 gms | 100 ml | - |
| E | Riboflavin | 4 gms | 100 ml | - |
| F | Sucrose | 40 gms | 100 ml | - |
| G | Amonium- | 14 gms | 100 ml | - |
| | Persulfate | | 100 ml | - |
| H | Tris Buffer | 7 gms | | |
| | Glycine | 28.8 gms | 1 litre | 8.5 |

(the stock buffer was diluted 10 times before use)

I Coomassie brilliant blue R-250 1 gm in methanol: glacial acetic acid: water (450:100:450 ml) and diluted this stock accordingly before use.

A working solution was prepared from the stock as shown below; (eg. for 7% separating gel concentration).

Lower gel : 1.25 ml A + 2.5 ml solution C
+ 1.25 ml double distilled water (DDW) + 5 ml of Soln:No.2 (Solution No 2 was prepared by freshly diluting 0.25 ml of solution G to 25 ml with DDW)
Upper gel : 0.5 ml solution B + 1 ml solution D + 0.5 ml of solution E + 2 ml of solution F.

The analytical disc electrophoresis was carried out in tubes essentially as described by Davis[74]. Glass tubes of size 5 mm internal diameter and 90 mm length were cleaned; well dried and fixed to a support. The lower gel (separating gel) was poured carefully in to the tubes to a height of about 60mm and was overlayed gently with water. Water prevents the formation of curved gel surfaces and excludes atmospheric oxygen, which would inhibit polymerization. Polymerization occured within 30 minutes at $\sim$ 30°C. The water was removed carefully with a needle and syringe and the residual water drops were sucked off with a wick of filter paper. Now the upper gel solution (spacer-gel) was layed on top of the lower gel to a height of 10 mm and covered carefully with water. The spacer-gel solution was irradiated by a fluorescent day-light lamp and the gels were polymerized within 20 minutes. After polymerization, the overlay of water was removed and the surface of the spacer gel was rinsed with buffer solution of the sample. Any desired gel concentration can be prepared in a similar fashion by mixing appropriate amounts of stock solution[102].

## Preparation of the sample

Polyacrylamide gel electrophoresis was used to separate, identify and quantitate the adsorbed proteins[65,211]

from a mixture of proteins, after desorbing them using Triton X-100.

The dried polycarbonate (PC) films of size 4.5 x4.5 cms were exposed to protein mixture for 3 hours containing 25 mg% albumin, 15 mg% ɣ-globulin and 7.5 mg% fibrinogen in presence and absence of various antiplatelet drugs studied. The films were dipped in buffer and shaken, then the protein mixture was added to make respective concentrations of them inside the media to reduce the air/water interface. Similarly other sets of films were prepared by exposing them to protein mixture containing a known amount of antiplatelet agents. Protein adsorption studies using PAGE were done on cyclic AMP modified PC films also. After 3 hours, each film was taken out and rinsed with buffer thoroughly to remove all unadsorbed proteins.

The samples prepared for PAGE were similar to Chieu et al[65] and they had indicated using radiolabelled proteins that the protein lost was insignificant during ultra filtration. It is believed, the desorbed proteins from the polymer substrate by Triton X-100 may be in the denatured form and the insignificant amount of plasminogen even if present as contamination would not cause autodigestion. The PAGE can separate these proteins and reveal the information of adsorbed proteins eventhough the Triton X-100 does denature proteins.

For desorbing,the proteinated films were placed in a beaker containing 10 ml of 0.1 M Tris HCl buffer, pH 8.5 with 1% Triton X-100. The films were incubated at room temperature ($\sim 30^{o}C$) for 16 hours with repeated shaking. It

has been demonstrated[65] using radiolabelled proteins, that all of the protein was removed from the surface within 5 hours, when the films were agitated. The solution was then concentrated to 500 microlitre by Millipore immersible CX-30 ultrafiltration units and the electrophoresis was carried out with 100 microlitre of sample.

## Electrophoresis of desorbed proteins

The electrophoresis was performed on 7% polyacrylamide gels using the method of Davis[74] with a slight modification[79]. When the gels were ready, they were removed carefully from the rubber cap, and the tubes were inserted into the rubber grommet, of the electrophoretic apparatus (Brovica, India), so that all tubes sticked out about 5 mm above the grommets. Cooled electrode buffer ($4^{o}C$) was poured in to the electrode vessels,and eliminated air bubbles from both ends of the tubes, using a pipette with a bend end.

The sample was applied to each tube, where the total sample volume was kept constant for each set of the experiment and no sample gel was used. Suitable staining controls containing the same volume of 0.1 M Tris-HCl (pH 8.5) with 1% Triton X-100 and tracking dye (Bromophenol blue) were also run on separate gels. The electrodes were connected to a DC power supply and the samples were electrophoretically analyzed at a constant voltage of 20 volts per gel at $4^{o}C$. The gels were removed, when the dye moved about 2/3 of the length of the gel and stained with a solution containing 0.05% Coomassie blue,10% acetic acid, and 25% methanol for 16 hours. They were photographed on a fluorescent light box with orange filter or scanned at 600nm

in a Shimadzu UV-240 spectrophotometer using its gel scanner attachment. Repeated at least three times for reproducibility and a typical scan or photographs of the same are presented in the text.

### 2.3.7. Sodium dodecyl sulphate gel electrophoresis(SDS-PAGE)

#### Theory

The proteins can easily be solubilized by the ionic detergent sodium dodecyl sulphate (SDS); and can also be analyzed by polyacrylamide gel electrophoresis in its presence[103]. The migration of proteins complexed with SDS is a function of molecular weight. The following relation has been found between the distance travelled by a protein ($x$) and molecular weight: Molecular weight = $k \times 10^{-bx}$ where k is a constant and b is the slope. The proteins complexed with SDS have the same charge to mass ratio, and that the ratio of effective charge to the frictional coefficient is independent of molecular weight, ie, the free mobility of a large molecule is identical with that of a segment of the same molecule, or of a smaller molecule. The different proteins bind essentially the same amount of SDS per unit protein weight (1.39-1.42 gr of SDS per gm of protein). The protein-SDS complexes have rod-like shapes and an extended conformation. In this case the stoke's radius of proteins depends on the molecular weight, and thus the migration of protein is linearly related to the logarithm of the molecular weight.

## 2.3.8. Experimental method

The effect of proteases like trypsin, plasmin and thrombin to modulate the surface-fibrinogen adsorption was studied, using SDS-PAGE after desorbing the preadsorbed proteins using 1% Triton X-100. The dried PC films were exposed to 220 mg% fibrinogen solution in 0.1 M phosphate buffer, pH 7.4, avoiding the air/water interface as indicated earlier. Similar sets of PC were prepared with fibrinogen in presence of trypsin (25 mg%) and thrombin (0.5 units/ml). After 3 hours of exposure,each film was taken out and rinsed with buffer thoroughly to remove all unadsorbed fibrinogen. The adsorbed proteins were removed from the surface, using 1% Triton X-100 and concentrated as described earlier, similar to that of the preparation for PAGE.

SDS-polyacrylamide gel of desorbed proteins and proteins in media (reduced conditions) was performed on 7% gels containing 1% SDS using the method of Laemmli[187]. The gels were prepared similarly as described earlier, containing 1% SDS in the gel. Protein samples were prepared for electrophoresis by incubating 2 to 5 minutes at 100$^{o}$C in a sample buffer containing 1% SDS, 10% glycerol, 10 mM Tris-HCl, 1mM EDTA and 1% 2-mercaptoethanol (pH 8.0). Bromophenol blue was added to the samples to a final concentration of 10 mg/ml as a tracking dye. Samples were electrophoretically analyzed using 0.1M Tris-HCl (pH 8.5) containing 0.1% SDS, at 4$^{o}$C at a constant voltage of 20 volts per gel. The gels were stained, with a solution containing 0.05% Coomassie blue, 10% acetic acid, and 25% isopropyl alcohol, for 16 hours. They were destained in 10% acetic acid and were photographed.

## 2.4. Platelet adhesion studies with washed platelets

Rationale:

On exposure of any synthetic substrates to whole blood, platelet rich plasma or washed platelets, the platelets adhere within seconds, depending on the nature of the surface, the flow conditions, the nature of the proteins adsorbed on to the substrate, and accordingly the extent of platelet activation. The adhered platelets to the substrate can be quantitated and probably equated with thrombogenic character of the surface.

### 2.4.1 Experimental:Platelet adhesion - Under static conditions

Calf blood platelets were isolated within 2 hours after collection, from citrated blood[58,61] and suspended in tyrode solution (0.055M D-glucose,0.138 M NaCl, 0.012M $NaHCO_3$, 0.0018M $CaCl_2$, 0.0049M, $MgCl_2$, 0.0027M KCl, 0.00036M $NaH_2PO_4$, pH 7.4) for the adhesion studies[58,190]. Briefly, 10 ml of blood was collected in 1 ml of 3.8% sodium citrate, centrifuged at 700xg for 10 minutes. The supernatant having platelet rich plasma (PRP) was centrifuged at 1000xg for 10 minutes and the white blood cell button was removed. The PRP was again centrifuged at 2000xg for 10 minutes to get the platelet button and washed with tyrode solution three times and suspended in the same solution. The concentration of platelets was determined by staining them using trypan blue (one volume of 1% trypan blue to 9 volumes of platelet suspension), and counted the unstained viable cells with a

haemocytometer. Platelet adhesion, to various PC substrate was measured using platelet suspension (approx. $1.0 \times 10^8$ platelets per ml with greater than 90% viability) containing varying concentrations of antiplatelet drugs, cAMP and vitamins. Fibrinogen (50 mg%) and other platelet inducers were also added to platelet suspension, in presence and absence of various antiplatelet drugs investigated, to see their effects to modulate this phenomena with activated platelets. Similarly, platelet adhesion studies were also carried out with other polymers like Nylon, Teflon, Polystyrene etc. in certain cases.

Platelet suspensions, described above, were exposed to the polymer surface for 15 minutes (it appears from Fig. 5-20, that 5 minutes of surface contact may be sufficient to reach a saturation level of adhesion with washed platelets), at room temperature ($\sim 30^{\circ}C$), and then rinsed with 0.1M phosphate buffer, pH 7.4, under a controlled flow rate of $\sim$150 ml/mt., for two minutes. The platelets were fixed with 2.5% glutaraldehyde and stained with Coomassie blue G. The number of platelets that adhered to the polymer surface was counted using an optical microscope. Different vision fields were read randomly and averaged in an identical fashion for all samples. A minimum of 30 fields were counted, from two to five separate sets of experiments, and the data were expressed as the number of platelets observed per $mm^2$ of the surface with the standard deviation.

### 2.4.2. Platelet adhesion under flow conditions

The adhesion of platelets to PC, Teflon and Nylon surfaces as a function of flow conditions in presence and

absence of aspirin with vitamin combination were also investigated. The same flow cells used for protein adsorption kinetics had been selected here also (Fig.2-3) for such studies.

The polymer film was placed in position and the circuit was opened initially with tyrode solution (pH 7.4), which was then displaced by the tyrode washed platelets ($\sim 1 \times 10^8$ platelets per ml) in the same solution to avoid air-solution-solid interface. The experiment was conducted as indicated earlier[27] for 15 mts at various flow rates (1 ml/mt, 5 ml/mt, 10 ml/mt and 20 ml/mt) at room temperature. The films were removed, and then rinsed with 0.1M phosphate buffer pH 7.4. The adhered platelets were quantitated as described earlier (in the static adhesion studies). Similar experiments were also conducted in presence of vitamin combination infused platelet suspension. The adhesion of platelets to various polymers under 20 ml/mt flow, as a function of time was also studied, in presence and absence of vitamin combination.

## 2.5. Coagulation studies

### 2.5.1. Evaluation of recalcification time : Principle

Citrated plasma is recalcified and clotting takes place via the intrinsic pathway, being initiated by the surface activity of the clotting tube. Surface variation has significant effect on the result.

Experimental

Plasma recalcification time (PRT) was determined using the standard techniques of Austen and Rhymes[10]. Citrated calf blood was collected (9 ml of calf blood with 1 ml of 3.8% sodium citrate solution) and tested for recalcification time under controlled pH (7.4) and temperature (37°C). Glass tubes of 10x75mm were thoroughly cleaned with soap, copious amounts of water, and finally with distilled water and dried. These tubes were coated with 10% polycarbonate solution, dried and were rinsed similar to films, (with distilled water, double distilled water, a final rinse in ethanol and vacuum dried at 60°C for 4 hours). These PC coated tubes were exposed to various antiplatelet agents or vitamins or anticoagulants etc. for 3 hours at room temperature, rinsed with buffer and vacuum dried. Plasma recalcification times were measured as follows. 0.1 ml of calf plasma was pipetted into a tube containing 0.1 ml of 0.9% saline and thermostated at 37°C. After an incubation of exactly 2 minutes, 0.1 ml of 0.025 M $CaCl_2$ was added and the clotting time was registered. The test was repeated at least five times and the recalcification time expressed in seconds with standard deviation.

## 2.5.2. Evaluation of thrombin time : Principle

A known concentration of human thrombin and calcium is added to the test plasma. The clotting time is a direct measure of the amount of fibrinogen present, the function of the fibrinogen,and the presence of antithrombins, eg. heparin. Therefore a prolonged thrombin clotting time is usually considered to be due to either a decrease in

fibrinogen concentration, the presence of a dysfunctional fibrinogen, or the presence of heparin or high concentrations of fibrin-fibrinogen degradation products; which prevent the formation of the fibrin clot[158].

**Procedure**

PC coated glass tubes were prepared as described earlier and thrombin time of certain selected surfaces were carried out. The PC coated tubes were exposed to cAMP or albumin for 3 hours, avoiding the air/water interface as detailed in table 6-III. Thrombin times were measured as described elsewhere[204]. Briefly, 0.2 ml of calf platelet poor plasma was added to 0.2 ml 0.9% saline in a tube and incubated in a water-bath, at 37°C for 30 seconds. Then, 0.1 ml of thrombin -calcium chloride mixture (1NIH unit thrombin and 0.025 M $CaCl_2$) was added and the clotting time recorded on an electronic stop watch. The test was repeated at least five times and the thrombin time expressed in seconds with standard deviation.

### 2.5.3. Activated partial thromboplastin times (APTT)

**Principle**

Platelet substitute, in the form of a partial-thromboplastin prepared usually from rabbit brain, is incubated with a contacting agent to provide optimal activation of the intrinsic coagulation factors. The clotting time is determined after the addition of an excess calcium.

**Procedure**

APTT were also performed to certain PC modified substrates (Table 5-XII), according to the method of Langdell et al[189]. The test system consisted of incubating 0.1 ml citrated plasma and 0.1 ml Kaolin-Cephalin mixture (equal volumes of 4% kaolin in 0.9% NaCl and the working suspension of cephalin, provided by Sigma, U.S.A.) at 37°C, for 6 minutes with regular agitation of the incubation mixture to redisperse the kaolin at 2 minute intervals. At the end of the 6 minute incubation, 0.1 ml $CaCl_2$ (0.025M) was added and the clotting time registered. The test was repeated at least five times and the activated plasma thromboplastin time expressed in seconds with standard deviation.

### 2.5.4. Stypven (Russell's Viper Venom) clotting time

**Principle**

A potent coagulant substance in the venom of Russell's viper (vipera russellii) which, in the presence of calcium ions and phospholipid, accelerated plasma coagulation. Russell's viper venom contained two coagulant proteins and were identified as the factor X activating enzyme and the other activated plasma factor V. Hence stypven activates factor X directly without the need for other coagulation factors[11].

**Procedure**

The test was performed in PC coated glass tubes (bare and modified) with 0.2 ml plasma placed in a test tube

in a water bath at 37°C. 0.2 ml stypven/phospholipid (Sigma, USA) reagent was added and incubated for 30 seconds before the addition of 0.2 ml 0.025M $CaCl_2$. The clotting time was recorded on a stop watch. The experiment was repeated atleast five times and stypven time recorded in seconds, with standard deviation.

### 2.5.5. Antithrombin III (AT-III) binding studies

**Principle**

The defibrinated plasma is diluted in a buffered heparin solution and a specific amount of thrombin is added to the plasma dilution. During a timed incubation, a portion of the added thrombin is neutralized by the AT-III present in the sample. An aliquot of the first stage is then mixed with a fibrinogen solution. The time required for clot formation is proportional to the amount of thrombin remaining after neutralization.

**Procedure**

Antithrombin III activity was studied[1,373] using an antithrombin III kit,from Sigma Co, USA (procedure No.885). The described technique is a modification of the procedure of Bick et al[28] employing snake venom (Bothrops atrox) for plasma defibrination. Citrated calf blood was collected (9 ml of calf blood with 1 ml of 3.8% sodium citrate), centrifuged at 1500xg for 15 minutes and the plasma was separated within 30 minutes of collection. The plasma was defibrinated by adding ATROXIN solution (0.5 ml plasma to 0.01 ml ATROXIN)mixed well and was incubated for 15 minutes

at 37°C. Then, it was centrifuged for 10 minutes at 2000xg and the clear 'defibrinated' plasma was collected and kept in ice bath until assayed.

The clotting time was measured as follows. The defibrinated plasma was diluted by adding 0.1 ml sample to 2.9 ml Heparin reagent. 0.2 ml of fibrinogen solution was dispensed into 'control' and 'test' tubes (12x75 mm glass tubes coated with PC and PC substrates exposed to various substances as shown in table 5-IX respectively) and placed, at 37°C waterbath for 3-5 minutes. Similarly, 0.5 ml of defibrinated diluted plasma was accurately delivered to another set of 'control' and 'test' tubes and kept at 37°C for 3-5 minutes. Then, 0.1 ml of thrombin solution was added to 0.5 ml of the diluted plasma mixed well, and started the first stop watch. Exactly 30 seconds after the addition of thrombin solution, 0.1 ml of the first reaction mixture was removed and rapidly transferred to the prewarmed aliquot of fibrinogen solution. The second stopwatch was started and recorded the time, in seconds, required to form a firm clot. The test was repeated at least three times.

A calibration curve with the same calf plasma was prepared by diluting the defibrinated plasma with Heparin reagent (1:30, 1:40, 1:60, 1:120 and 0 dilutions) and plotted clotting times, in seconds, versus AT-III activity (%) on a semilogarithmic paper. The results for the control and test samples were obtained directly from the calibration curve and expressed in terms of AT-III activity (%).

## 2.6. Preparation of washed cells

The blood cells like red cells, platelets, whole white cells, and lymphocytes were isolated to study their role to modulate the protein-surface binding from a protein mixture using labelled proteins.

### 2.6.1. Isolation of platelets, red blood cells, and white blood cells

These blood cells were isolated within 2 hours after collection from citrated blood as described elsewhere[42,59]. Briefly, freshly drawn calf blood, collected into sodium citrate anticoagulant (1.0 ml of 3.8% sodium citrate for 10 ml of blood), was centrifuged for 15 minutes at 700xg, and the platelet rich plasma, buffy coat (rich in white blood cells) and the red cells were removed to three separate plastic vials. The tyrode washed platelets were prepared from platelet rich plasma as indicated earlier in this section. The other two components obtained were again centrifuged separately at 2000 x g for 15 minutes to settle down the white blood cells and red blood cells. The white blood cells were washed three times using tyrode solution[58,190], centrifuged between each wash at 2000xg for 10 minutes, and finally suspended in the same solution for the studies. The red cells were washed in a similar fashion with 0.1 M phosphate buffered saline, pH 7.4 [340]

### 2.6.2. Isolation of lymphocytes

The lymphocytes were isolated according to the method of Boyum[39] from calf venous blood layered on to FICOLL

type F-P solution, (product No, F8628, Sigma, USA). In brief, 14 ml of 0.1 M phosphate buffered saline (PBS) pH 7.4 was added to 10 ml heparinized blood and mixed gently by inversion. Eight ml of this blood-saline mixture was layered carefully to 3 ml of FICOLL type F-P solution,(contains Ficoll-400$^{R}$, 5.7 gm/100ml and sodium diatrizoate, 9 gm/100 ml in sterile solution; adjusted to a density of 1.077+0.001), in a centrifuge tube and centrifuged at 400xg for 30 minutes at room temperature. After centrifugation, the upper layer was carefully aspirated to within 0.5 cm of the opaque interphase containing mononuclear cells. The upper layer was discarded and the opaque interphase was very carefully transferred to another tube. Then, 10 ml of 0.87% ammonium chloride solution was added to lyse any contaminating erythrocytes and kept at room temperature for 5 mts. The supernatant was aspirated and discarded. The lymphocyte pellet was washed with 5.0 ml phosphate buffered saline and centrifuged at 250xg for 10 minutes. After two washings, the lymphocytes were counted in a haemocytometer. Then, the solution was suspended in PBS to obtain~$2x10^{7}$ lymphocytes /ml with greater than 90 percent viability (trypan blue exclusion).

The adsorption/desorption kinetics of proteins in presence of these blood cells were conducted in 0.1 M phosphate buffer, pH 7.4 at 37$^{o}$C, containing labelled proteins at a specified concentration. To this medium were added, separately washed whole red cells, platelets or white blood cells in sufficient quantity to maintain an approximate normal blood levels in each case(~$4x10^{9}$ red cells/ml, ~$2x10^{8}$ platelets/ml and ~$5x10^{6}$ white cells/ml respectively). In case of lymphocytes, the adsorption experiments were repeated

in a similar fashion by adding lymphocytes inside the media to maintain $\sim 1 \times 10^6$ cells/ml in the presence and absence of various antibiotics, and the surface-bound albumin or fibrinogen were calculated.

## 2.7. Preparation of fibrinogen fractions

In order to study the effect of various fibrinogen heterogeneous forms towards surface protein and surface-platelet binding, three fractions of the molecule had been isolated from crude Fg by standard techniques[131]. A low solubility fraction of human fibrinogen that precipitated between 14% and 20% saturated ammonium sulfate[200] was used as the starting materials for fractionation. The procedure adopted for fractionation was similar to Harfenist et al[131] by ion-exchange chromatography on DEAE cellulose using gradient elution with TRIS-phosphate buffers as described by Finlayson and Mosesson[94].

Briefly, human fibrinogen was dissolved in 0.15 M NaCl to a final concentration of 0.3 percent. A saturated solution of ammonium sulphate was added slowly with constant stirring. The fibrin like material formed at 16% saturation and was collected on a glass rod. The material was dissolved and adjusted to a final concentration of 0.3% and precipitated once more with ammonium sulfate (16% saturation). The final precipitate of fibrin-like material was dissolved in TRIS-buffered-0.15 M NaCl at pH 7.8, and dialyzed against the same solution. The final product was designated as low solubility fibrinogen (LSF). The concentration of LSF was determined by clotting with thrombin, digestion of the clot and biuret analysis of the

resulting solution, as indicated earlier in this chapter for the estimation of Fg in plasma. Approximately 500 mg of low solubility fibrinogen was obtained from 1 gm of Sigma fibrinogen.

A column (2x34 cm) was prepared with DEAEcellulose in TRIS-phosphate buffer No.1 (0.005 mol/L $H_3PO_4$, pH 8.6), and was eluted with TRIS-phosphate buffers containing $H_3PO_4$ at the concentrations indicated and adjusted to the required pH with TRIS. The low solubility fibrinogen was applied to the column and eluted with ~280 ml of buffer No.1, followed by ~660 ml of a linear gradient composed of 400 ml each of buffer No.1 and buffer No.2 (0.0875 mol/L $H_3PO_4$, pH 6.8), followed by buffer No.2. The flow rate was adjusted to 200 ml/hour, the fractions were collected at 4°C and monitored by absorbance at 280 nm. Fractions were pooled as indicated by the hatched rectangle (fractions 1a, 1b and 2a in Fig. 2-4). The second peak to be eluted (fraction 2a, in Fig. 2-4) was rechromatographed on DEAE cellulose column (1.5 x 26 cm). This was eluted in stepwise fashion with ~150 ml of buffer No.1' (0.044 mol/L $H_3PO_4$, pH 7.6), followed by 75 ml of buffer No.2' (0.050 mol/L $H_3PO_4$, pH 6.8) followed by buffer No.3' (0.15 mol/L $H_3PO_4$; pH 5.75). The flow rate was adjusted to 75 ml/hour; at 4°C and monitored the fractions at 280 nm. Fraction 2b was pooled as indicated by the hatched rectangles (Fig. 2-5).

All three fractions (1 a,1b and 2b) were pooled as indicated, dialyzed against 0.15 mol/L NaCl and concentrated. A sample of each fraction was used for thrombin time as well as for percentage of clottability evaluation. The fractions were also characterized by SDS-polyacrylamide gel

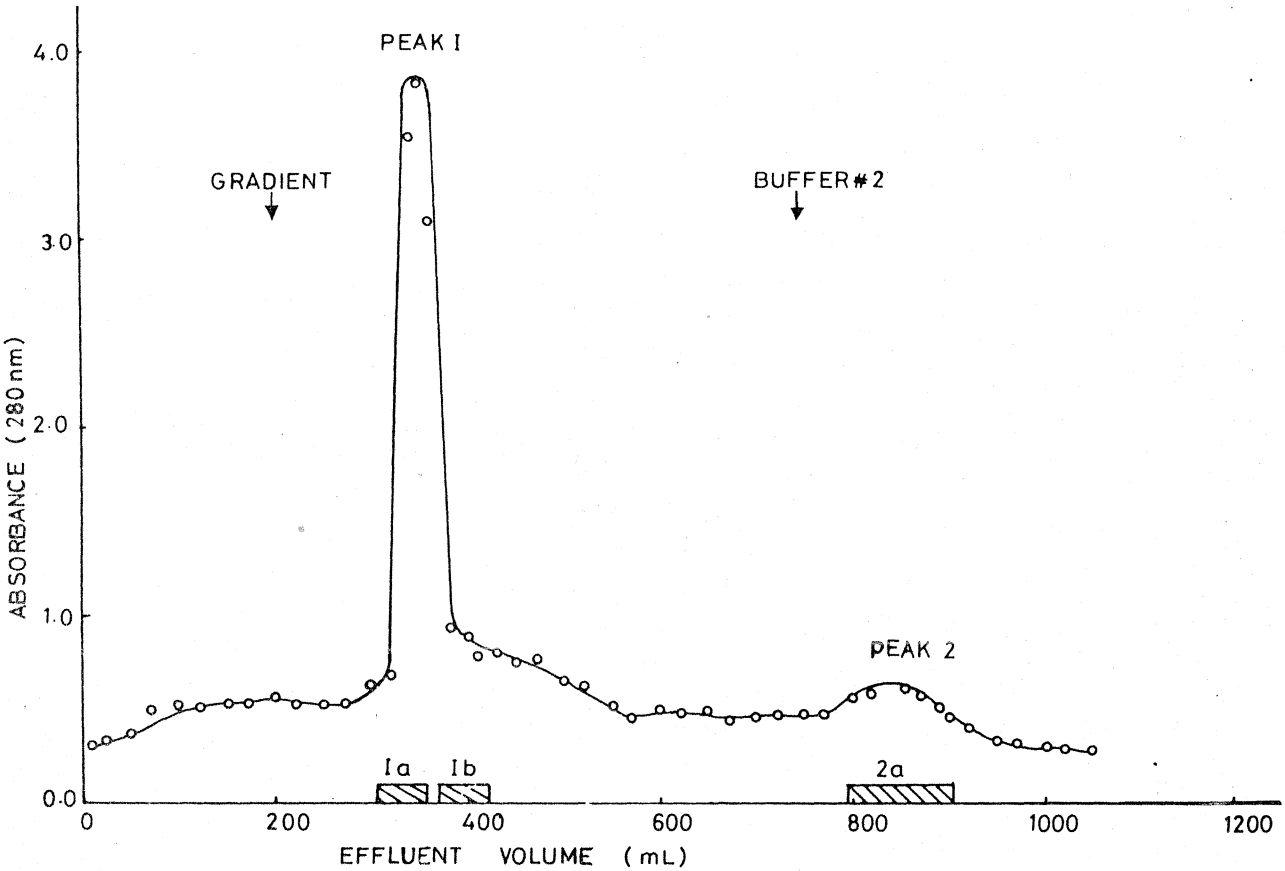



Fig2-4.Chromatograph of 300 mg of low-solubility fibrinogen (14% to 20% ammonium sulfate) on D E A E Cellulose.

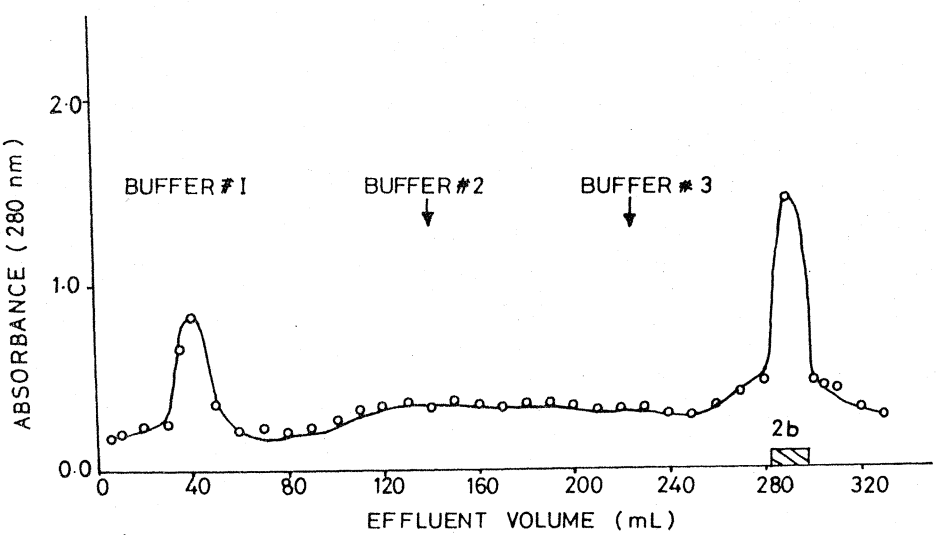



Fig2-5.A second Chromatograph of fraction 2a (Fig.2-4)on D E A E Cellulose.

electrophoresis. The fractions were further used for platelet adhesion studies in presence and absence of ADP towards an artificial surface (PC). Finally, protein binding profile to PC substrate was also investigated from a protein mixture containing 25 mg% albumin, 15 mg% ɣ-globulin and 7.5 mg% fibrinogen (Fraction 1a, or 1b or 2b), using PAGE as described earlier.

**Statistical analysis of data:**

Statistical analysis of important observations were done and probability ('p') for significance were calculated. The standard deviation and 'P' values were provided in most of the tables, for comparing the significance of different observations. All plotted points in the figures were the mean of at least 3 to 4 separate measurements.

# RESULTS AND DISCUSSION

# CHAPTER - III

## 3.1. Kinetics and isotherms of fibrinogen adsorption-single protein system

The adsorption/desorption kinetics of different proteins like albumin, γ-globulin, fibrinogen etc. have been extensively studied by various investigators as reported in Chapter-1. However, different polymeric substrates and conditions; like buffer concentration, pH, varied protein ratios etc. have been used in such studies to follow the adsorption kinetics. Therefore to develop a uniform concept of protein polymer interaction, investigations related to the interaction process of fibrinogen to polycarbonate substrate; and it's competition with other proteins have been performed using trace labelled techniques. These studies can possibly provide an understanding of a unified protein substrate phenomena and can eliminate the influence of substrate factors for the interaction process. In these experiments, fundamental understanding of adsorption kinetics, isotherms, desorption and exchange for the single protein system, giving emphasis to the effect of pH, bulk concentration, temperature and flow conditions are attempted.

### 3.1.1. Effect of bulk concentration

Fibrinogen adsorption to polycarbonate is concentration dependent as shown in figures 3-1 and 3-2. Surface concentration of Fg increases with bulk concentration

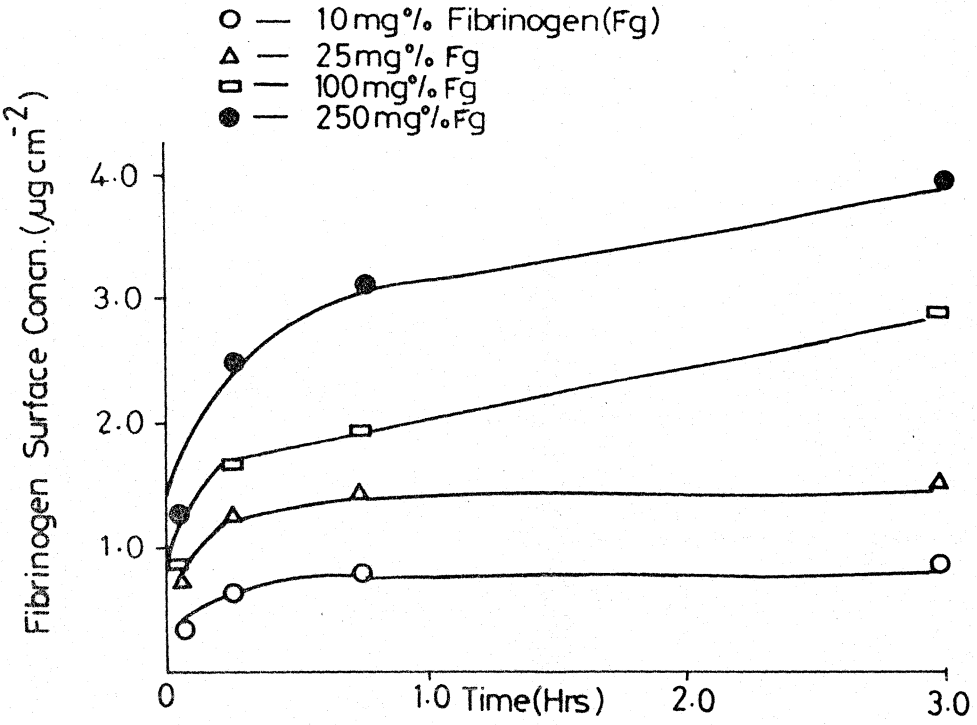



Fig 3-1 Adsorption of fibrinogen on pc as a function of time from various concentrations of fibrinogen.

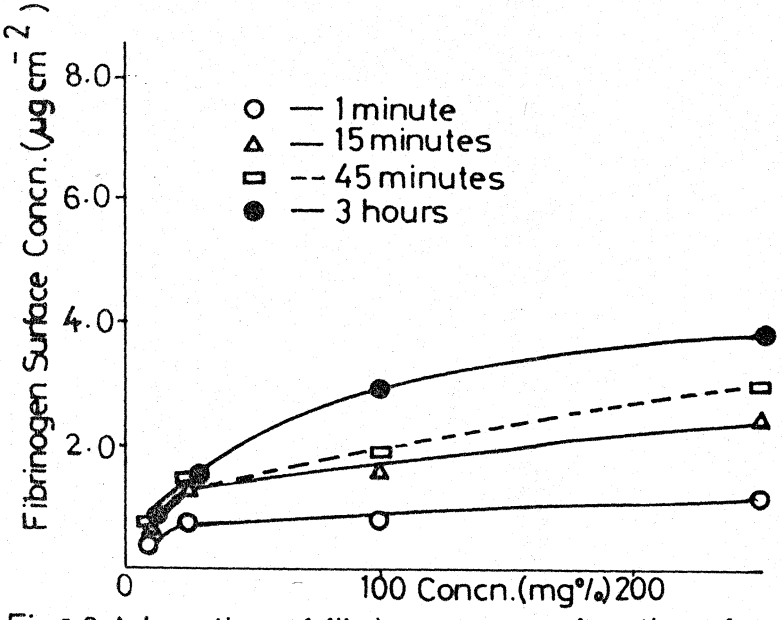



Fig 3-2 Adsorption of fibrinogen as a function of concentration for various time intervals.

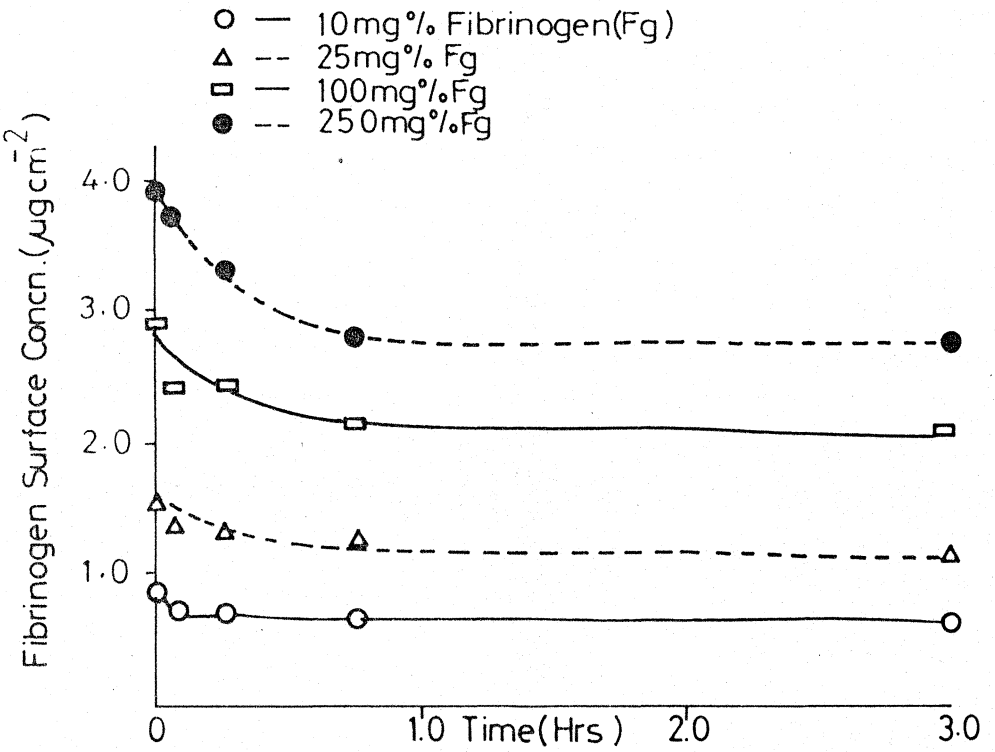



Fig.3-3. Desorption of fibrinogen as a function of time from 3 hr preadsorbed fibrinogenated surfaces (concn: effect).

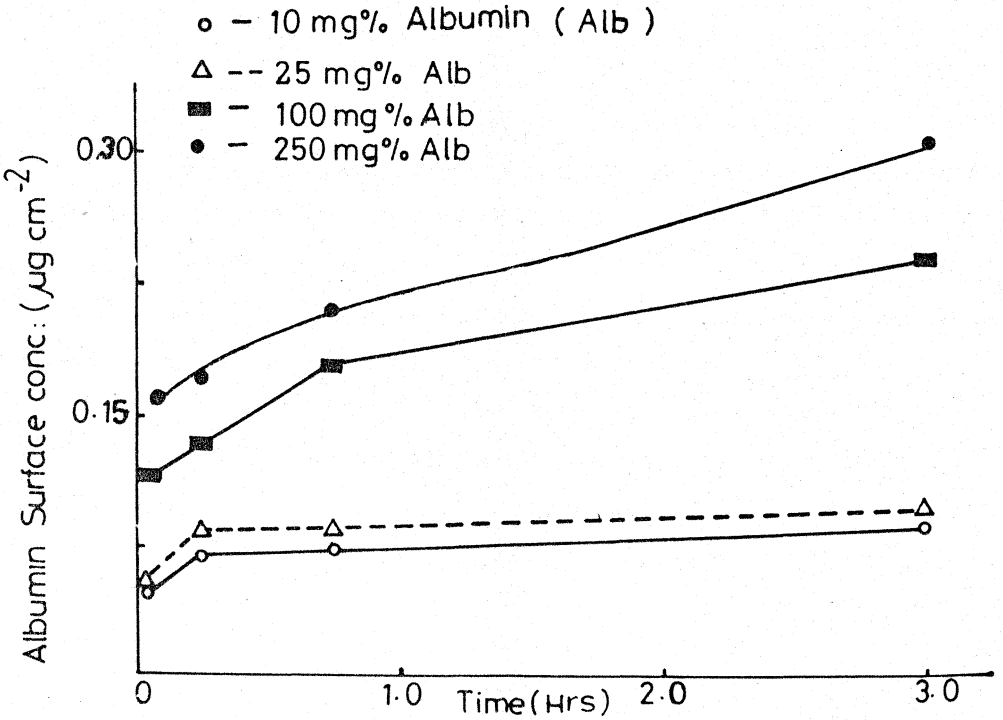



Fig.3-4. Adsorption of albumin on pc as a function of time from various concentrations of albumin

of Fg in the media and the time of contact with the surface. There is no significant difference in surface fibrinogen level, adsorbed from 10 mg% fibrinogen with time, whereas with higher concentrations like 250 mg%, the amount of Fg adsorbed to the substrate has increased with exposure time as is evident from Fig.3-2. (For example, 0.35 $\mu g/cm^2$ of Fg adsorbed to PC, for 1 mt. exposure of 10 mg% Fg, which has reached to 0.87 $\mu g/cm^2$ for 3 hrs, whereas with 250 mg% Fg in the media, the values have been 1.23 and 3.88 $\mu g/cm^2$ respectively). As illustrated in Fig.3-1, it is evident that Fg is adsorbed rapidly to polycarbonate surface and has reached a constant surface concentration within one hour; despite the large range in Fg concentration used.

The pattern of Fg exchange from the polymer substrate with time in an isolated fibrinogen solution is demonstrated in Fig.3-3. This indicates that a part of the preadsorbed Fg is exchanged from the substrate within one hour and has reached to a constant surface level. In other words, after one hour of desorption via exchange, a substantial amount of protein remain 'irreversibly' adsorbed on the PC surface.

Brash et al[44,57] have extensively studied the adsorption kinetics and isotherms of Fg on glass substrate and has shown that the adsorption is 75% complete within 5 minutes and reaches an equilibrium within 2 hours. They have also indicated that the adsorption is irreversible in the sense that one cannot redescend the isotherm by reducing the fibrinogen concentration. The results reported here also correlate with their findings, as well as support the suggestions by Morrissey and Stromberg[233] and by Limber et

al[198] that additional binding may possibly involve several sites on a single protein molecule.

The desorption kinetics of preadsorbed proteins in buffer or protein solution have been indicated by various investigators[34,342]. It has been reported[342] that the initial desorption rate for γ-globulin in protein solution has been very rapid and linear for the first 30 seconds which may be due to the presence of a reversibly adsorbed peripheral layer of γ-globulin. However, after more than an hour of desorption a substantial amount of γ-globulin remains 'irreversibly' adsorbed on the surface. This correlates with the desorption experiments represented here as well, and it may be possible that desorption is facilitated by the participation of protein from solution perhaps via impact collision or complex formation.

For a comparison, the adsorption/desorption isotherms of albumin is also studied as a single protein. The adsorption kinetics of albumin to PC substrate as a function of bulk concentration is depicted in figures 3-4 and 3-5. The isotherms reaches to a constant surface concentration within one hour with low concentrations studied. However, an increase in albumin bulk concentration in the media causes a high surface-albumin binding initially, which slowly increases with time. The desorption kinetics of albumin with time from PC surface is shown in Fig.3-6, which indicates that a part of the preadsorbed albumin is exchangeable with soluble protein. The adsorption isotherms of bovine serum albumin as a function of albumin concentration have been studied on to various latices at pH 5.4 and has shown[335] that the isotherms are rather pseudo-

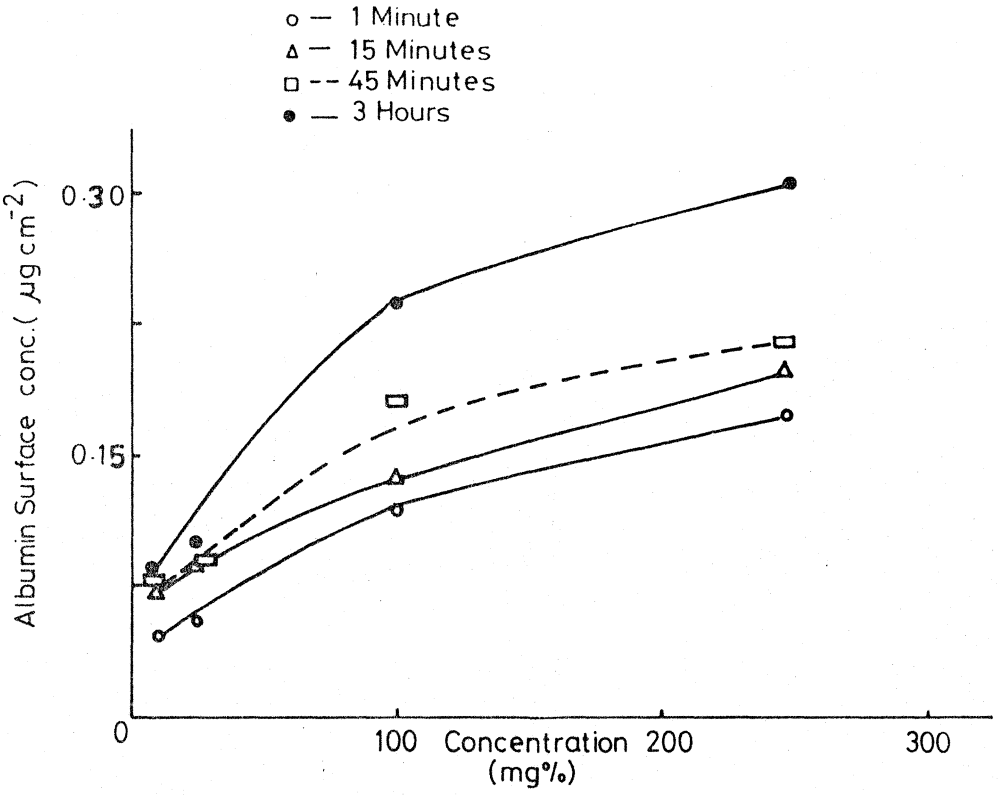



Fig.3-5. Adsorption of Albumin as a function of concentration for various time intervals.

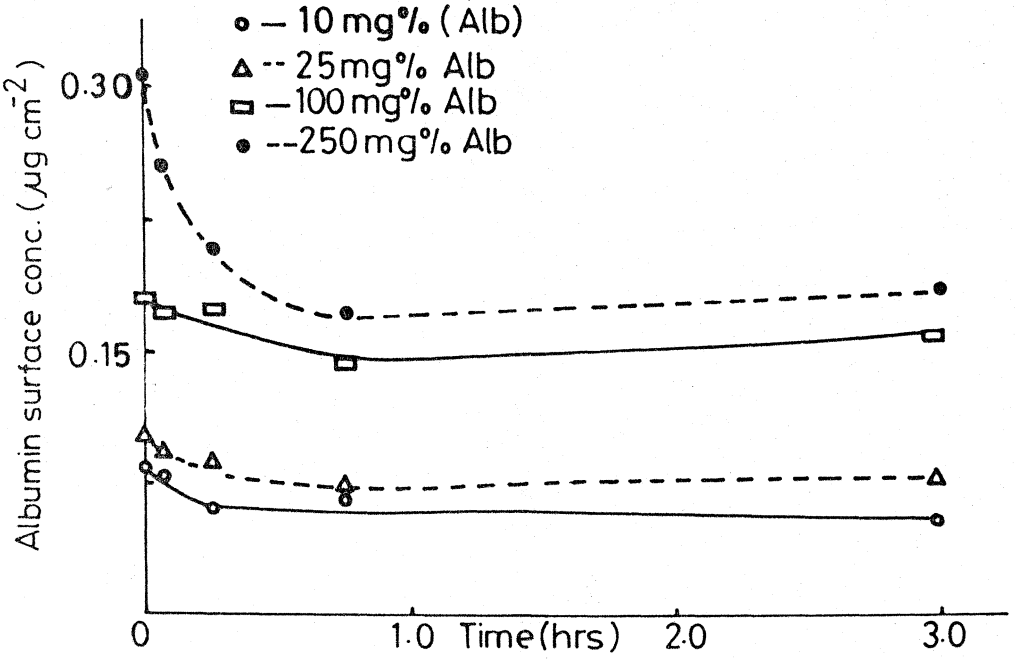



Fig.3-6. Desorption of albumin as a function of time from preadsorbed (3hr) albuminated surfaces.

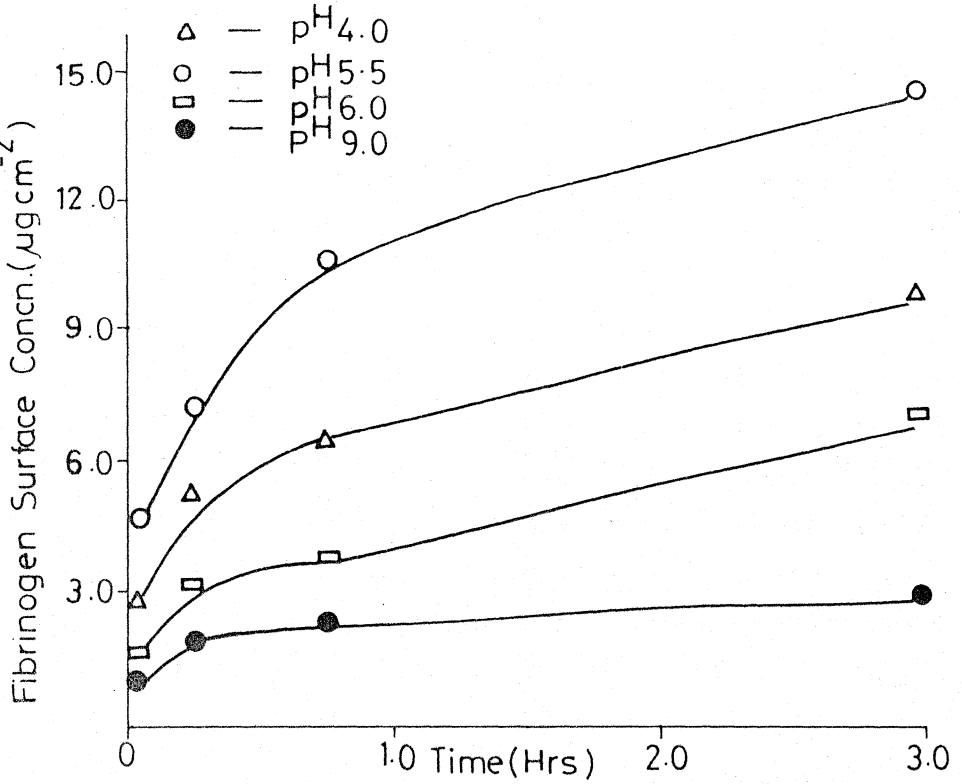



Fig 3-7. Effect of pH towards fibrinogen adsorption on PC from 100 mg% fibrinogen.

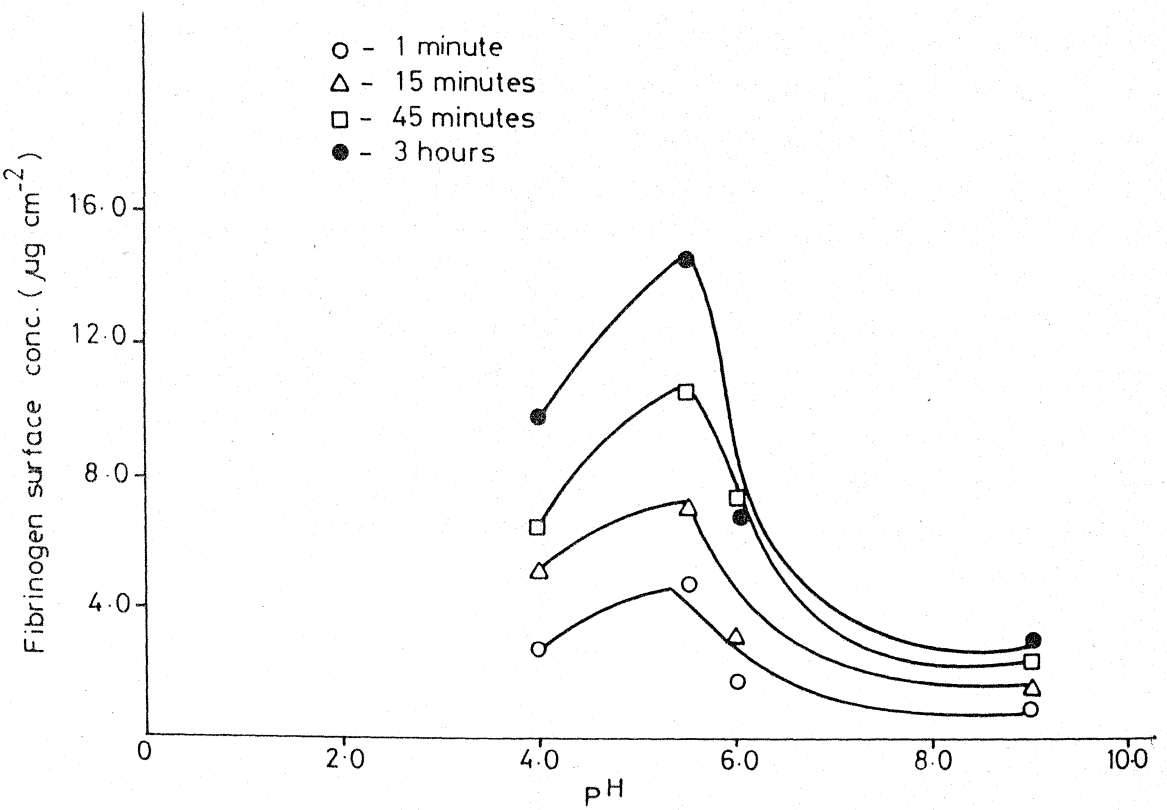



Fig. 3-8. Adsorption of fibrinogen on PC as a function of pH for various time

Langmuir types and show steps at some concentrations of bovine serum albumin. These steps corresponds to the beginning of the conformational arrangement of adsorbed albumin molecules, which become more native and ordered structures[335].

The observations of Suzawa et al[335], Bornzin et al[34] indicate that by increasing bulk protein concentration in the media tends to increase the initial rate of adsorption and the amount adsorbed at later times. These results show an agreement with the present observations, and possibly in the later stages protein-protein interaction may cause an increased surface level, when bulk concentration has increased. Further isolated studies have been done to see the effect of pH, temperature etc. using 100 mg% albumin or Fg concentration; where a relative flat region of the isotherm have been observed.

### 3.1.2. Effect of pH

The effect of pH towards Fg adsorption to PC substrate is illustrated in figures 3-7, 3-8 and their desorption kinetics in Fig.3-9 respectively. It is obvious that at higher pHs' less Fg is getting adsorbed to PC substrate compared to lower pHs. A reversed change of the pH leads to an additional adsorption upto 80%-100% of the amount adsorbed in the direct experiment. It also shows a maximum adsorption at the isoelectric point of fibrinogen (pH 5.5), where a **high** charge density exists for this molecule[149]. It has been reported that the plateau value $\Gamma_m$ of the adsorption isotherm depends on the pH of adsorption (ie. on

the charge of the protein molecule) and on the charge density at the polymer surface[251].

The desorption of Fg from higher pHs' adsorbed cases is very less compared to lower pHs', or on changing the pH away from that value where the adsorption is a maximum, very less or no desorption occurs as is evident from Fig.3-9. It is demonastrated[207] that the $\Gamma_m$ (plateau adsorbed) is to a large extent determined by the net charge of the dissolved protein molecule. The lower surface-protein binding, at increasing distance from the isoelectric point has been ascribed to progressive structural rearrangements in the protein molecule.

Figures 3-10 and 3-11 demonstrate the influence of pH on the adsorption of albumin to PC substrate and it's desorption kinetics in Fig.3-12. It shows a maximum adsorption near the isoelectric point of albumin (pH about 4.9) and demonstrates a similar pattern to that of Fg. The enhanced protein binding to the substrate at it's isoelectric point may also be due to the fact[236,335], protein molecules form compact structures in the zwitter ion state and more molecules can adsorb in the given surface area because the electrostatic repulsions of intramolecules and the lateral interactions between protein molecules are minimized in this state. The effect of ionic strength and pH towards albumin adsorption kinetics have been reported[335]. In the region of acidic pH, the amount adsorbed increases with increasing ionic strength, and it is suggested that the increment of the amount adsorbed has been related to the hydrogen bond formation between protein and the polymer surface[335].

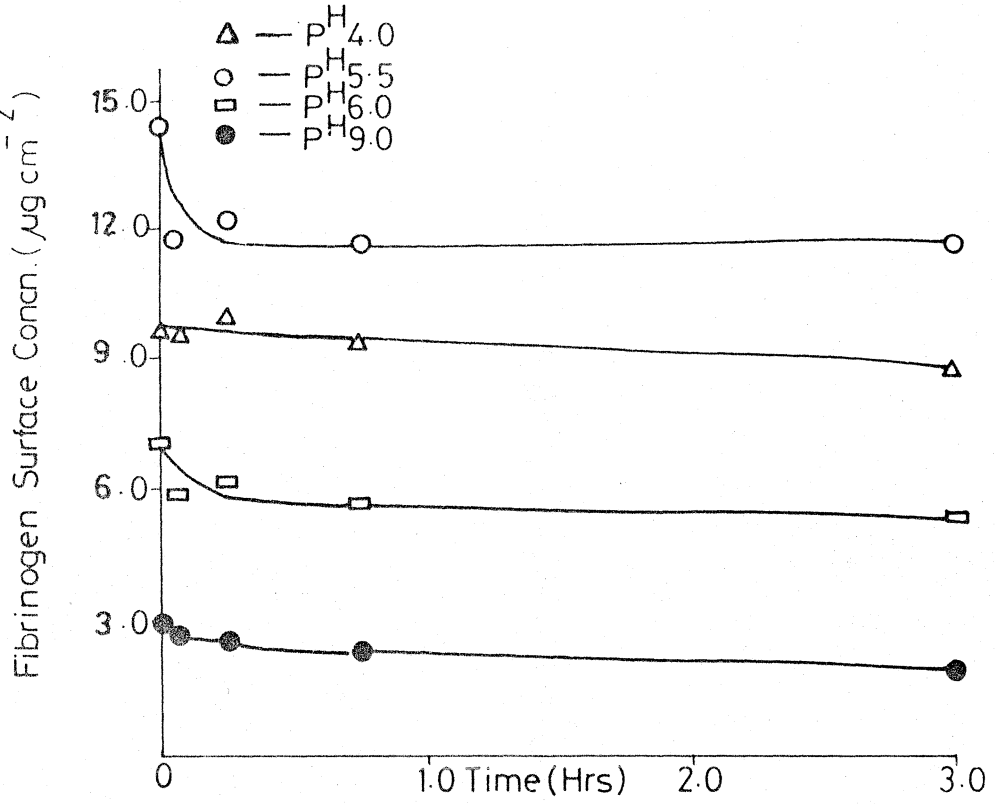



Fig.3-9. Desorption kinetics of 3 hrs preadsorbed fibrinogen from PC surfaces exposed to 100mg% fibrinogen at different pHs.

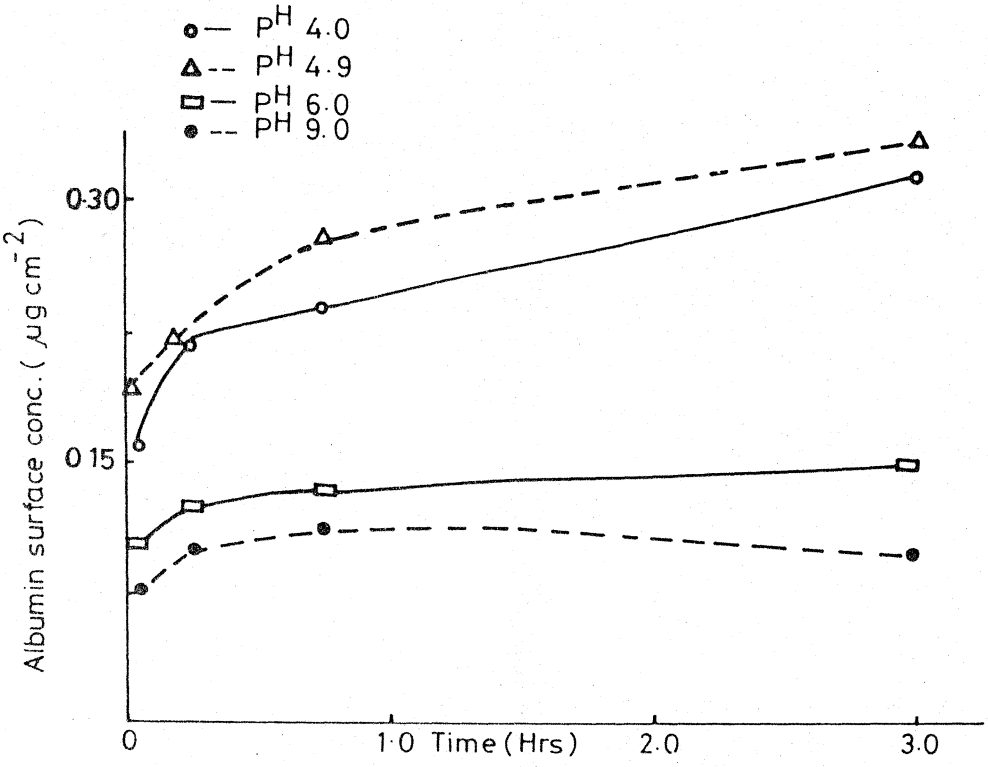



Fig.3-10. Effect of PH towards albumin adsorption on PC from 100mg% albumin.

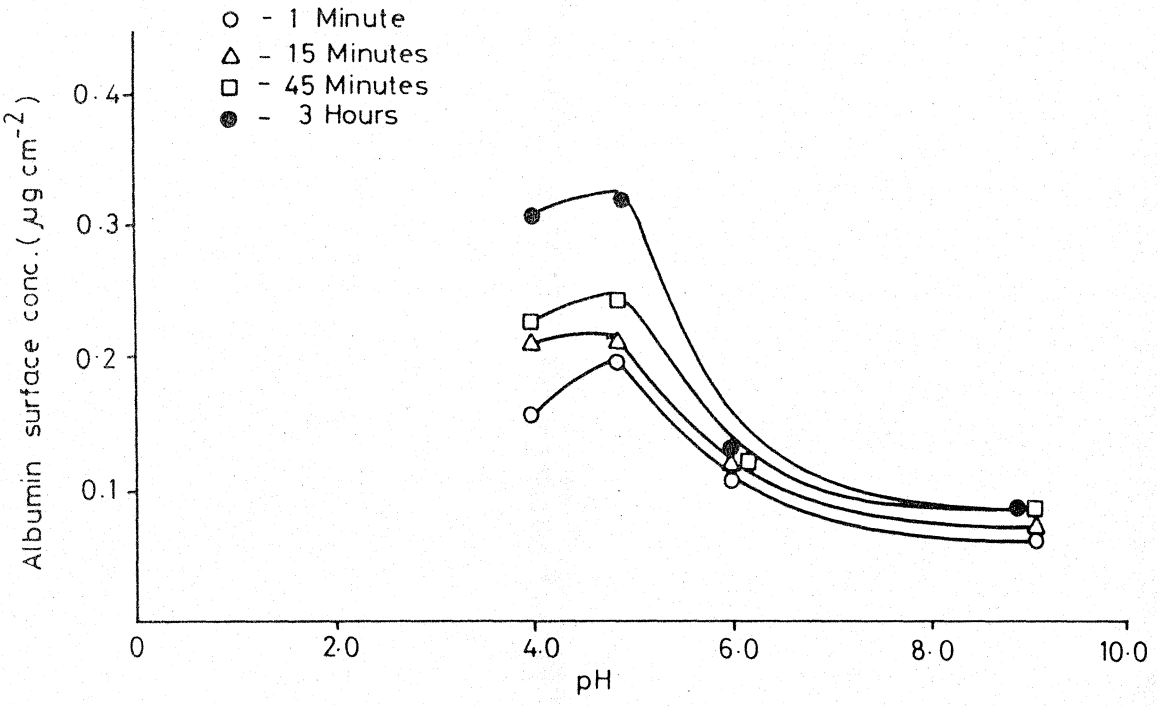



Fig. 3-11. Adsorption of albumin on PC as a function of pH for various time intervals; from 100 mg% albumin.

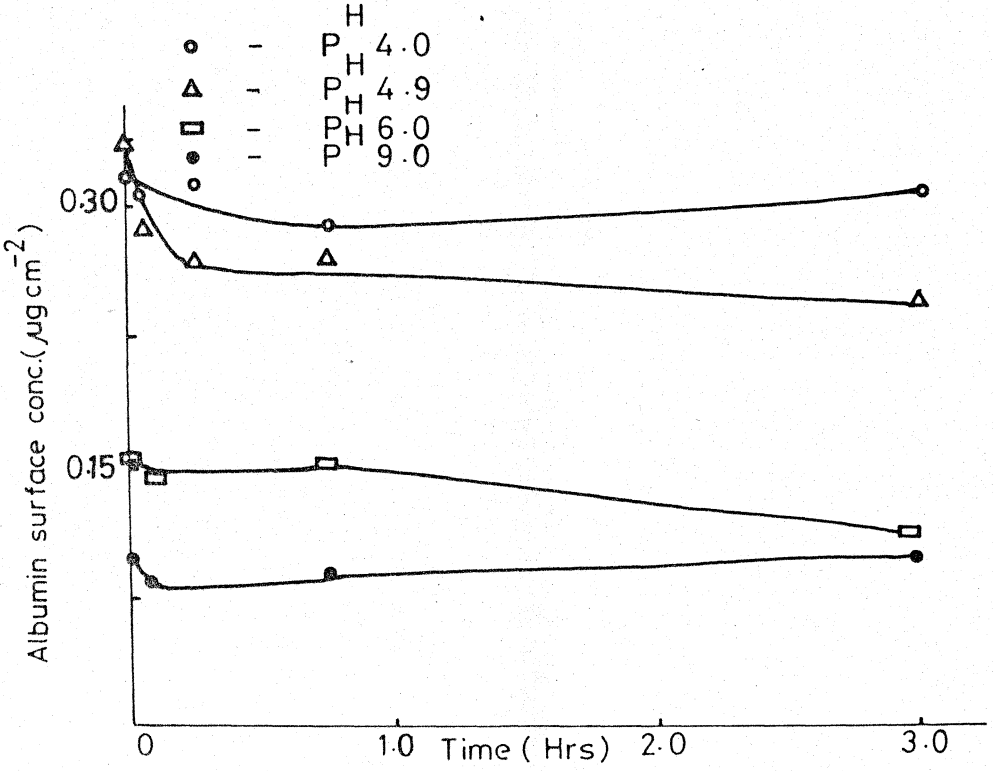



Fig. 3-12. Desorption Kinetics of Preadsorbed (3 hrs) albumin from PC surfaces exposed to 100mg% albumin at different $P^H$s.

### 3.1.3. Effect of temperature

Adsorption isotherms of Fg to PC at different temperatures from 100 mg% fibrinogen are indicated in figures 3-13 and 3-14, and their desorption kinetics in Fig.3-15. Fg adsorption increases with temperature in a time dependent manner, i.e., initially the differences are relatively less compared to 3 hours adsorption at various temperatures. For example, one minute adsorption at 5°C is 1.05 ± 0.1 $\mu$g cm$^{-2}$ compared to 1.92 ± 0.04 $\mu$g cm$^{-2}$ at 43°C. On the other hand after 3 hrs, the adsorbed amount at 5°C is 2.74 ± 0.07 $\mu$g cm$^{-2}$ compared to 11.46 ± 0.27 $\mu$g cm$^{-2}$ at 43°C. Norde and Lyklema[251] have demonstrated a similar behaviour of protein adsorption to polymer substrate with temperature. The influence of temperature towards protein-polymer binding illustrates the complexity of the adsorption process; probably due to the conformational changes happening to protein structure. Bornzin et al[34] has also investigated the protein-surface binding as a function of temperature; and has found that, increasing temperature tended to enhance the amount adsorbed and the initial rate of adsorption.

Desorption and exchange are similarly investigated for the fibrinogen-PC system at various temperatures; as depicted in Fig.3-15. It is interesting to note that, the 3 hours preadsorbed Fg at 5°C is least removed from the surface, while the percent of exchange seems to be similar at 43°C adsorbed substrates. (At 5°C 2.74 ± 0.07 $\mu$g cm$^{-2}$ has reached to 1.81 ± 0.13 $\mu$g cm$^{-2}$ after 3 hours of desorption, and 11.46 ± 0.27 $\mu$g cm$^{-2}$ has desorbed to 7.3 ± 0.51 $\mu$g cm$^{-2}$ at 43°C).

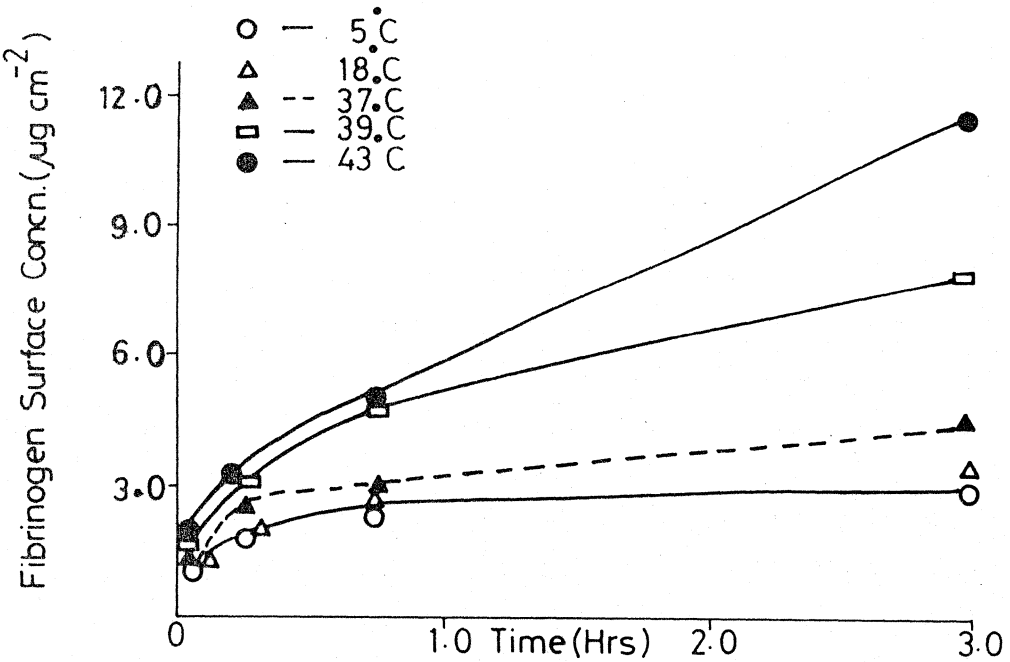



Fig.3-13. Effect of temperature towards fibrinogen adsorption on PC from 100 mg% fibrinogen.

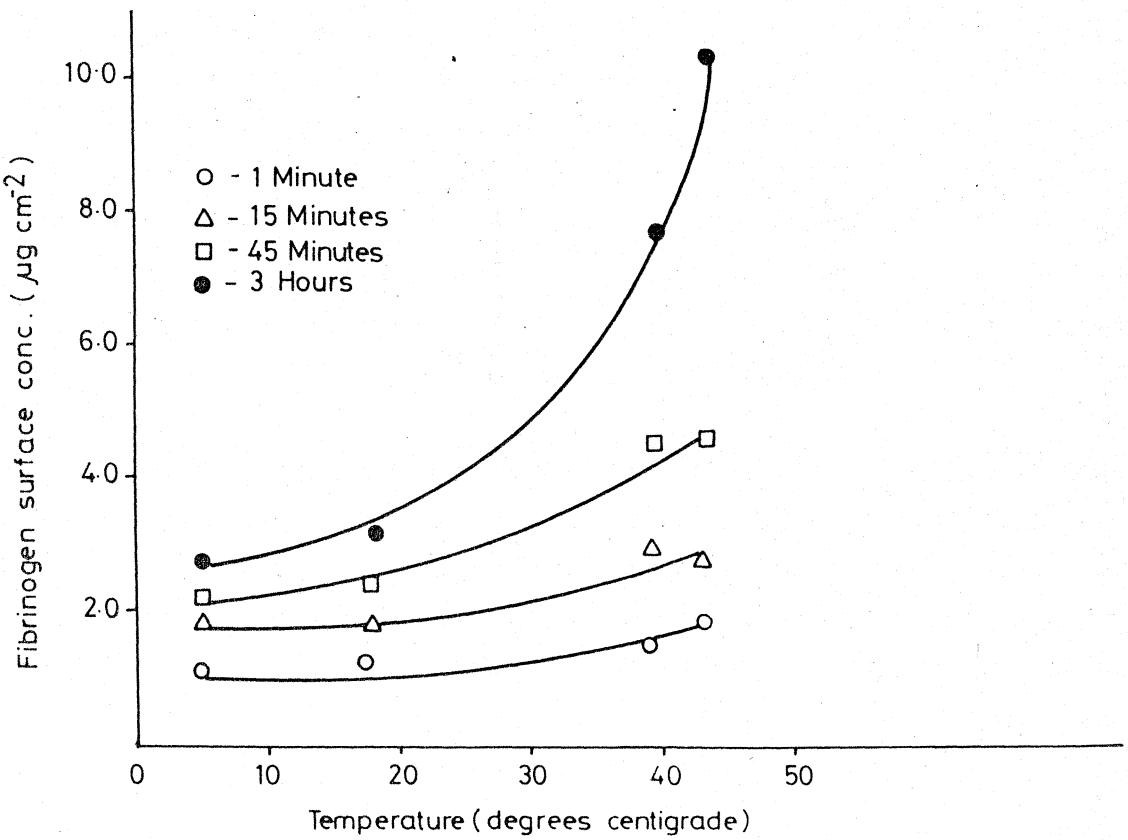



Fig.3-14. Adsorption of fibrinogen on PC as a function of temperature for various time intervals ; from 100 mg% fibrinogen.

It has been proposed[52,236] that under steady state conditions (surface concentration invariant with time), exchange occurs between adsorbed and dissolved fibrinogen, but only a certain fraction of the adsorbed layer (between 30 and 70%, depending on electrolyte concentration and fibrinogen concentration) is exchangeable. Such exchange occurs with relaxation times of the order of one hour. This seems to be true with the desorption studies reported here also (Figures 3-3, 3-9 and 3-15).

These findings suggest that the polycarbonate-fibrinogen interaction seems to be quite strong, since the adsorption is rapid and the surface concentration is high, obviously depending upon pH, temperature and bulk concentration of the media. It is evident that the protein adsorption curves become relatively flat in a concentration range around 60 to 80 mg% (Fig.3-2). Hence, plasma proteins can be considered to be adsorbed as a monolayer in the relatively flat region of the isotherm, where the bulk protein concentration can be near 100 mg%. However, each plasma protein appeared to be adsorbed as a multimolecular layer in a concentration region above 150 mg%. This adsorption isotherm representing a multimolecular layer of plasma proteins has not been reported from observations at low protein concentrations[152,192], but has been at high protein concentrations for albumin, and fibrinogen[152,335].

Thus, it seems that both proteins displayed two independent types of adsorption, one irreversible and the other reversible. The irreversibly bound protein adsorbs rapidly, which does not seem to reach a saturation level, and adsorbs in such large concentrations that it may most likely

a multilayer adsorption process. The reversibly bound protein has tended to reach a saturation level within 40 minutes, though interplay between protein/surface and protein/protein interactions plays a role in the rate and extent of uptake. Such an adsorption mechanism has been proposed by other investigators also[236,342].

The initial adsorption of blood proteins and their subsequent structural changes are likely to affect eventual cell attachment and thrombosis, as discussed in Chapter-I. The changes in pH, temperature and ionic concentration, away from physiological regime can alter the conformation of the protein molecule. So the observed variations related to pH, temperature etc. towards, Fg-surface interaction may be due to the conformational changes occurring to the molecule. Furthermore, it seems that the unfolding of the proteins reveal more of the hydrophobic interior, thus increasing the surface interaction.

It is also suggested[319] the plasma proteins like albumin, $\gamma$-globulin and fibrinogen contain an excess of acidic groups and is thus negatively charged at neutral pH. Further, these proteins do not show any distinct preference for the polypeptide substrates and fully denatured proteins do not appear to adhere to any extent. This may be true with the pH experiments, since the Fg molecule is least adsorbed to PC substrate at pH 9.0, probably due to the extend of denaturation.

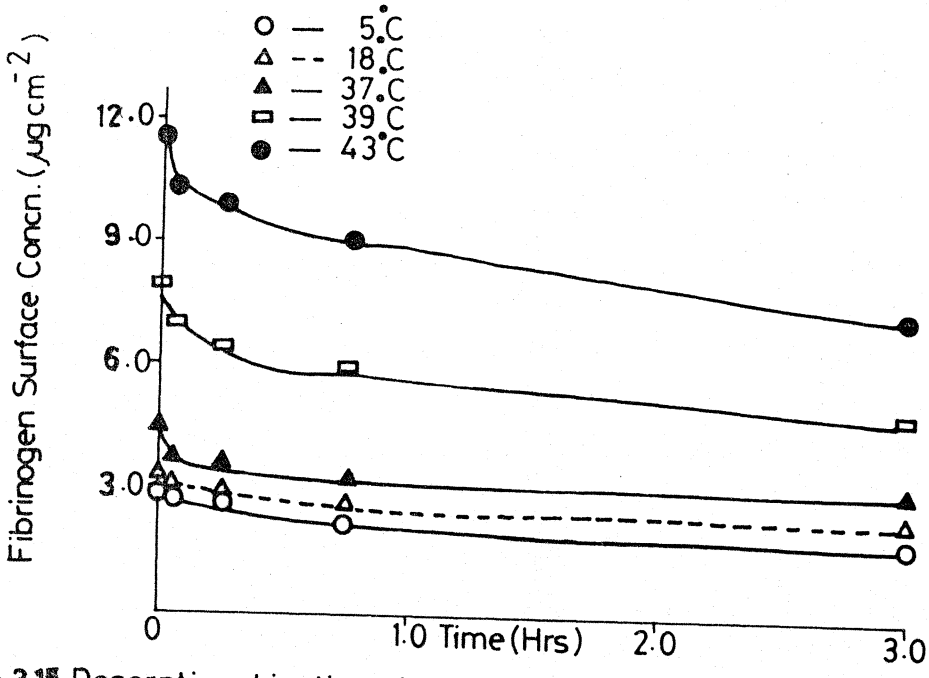



Fig.3-15. Desorption kinetics of 3hrs preadsorbed fibrinogen from PC surfaces exposed to 100 mg% fibrinogen at different temperatures.

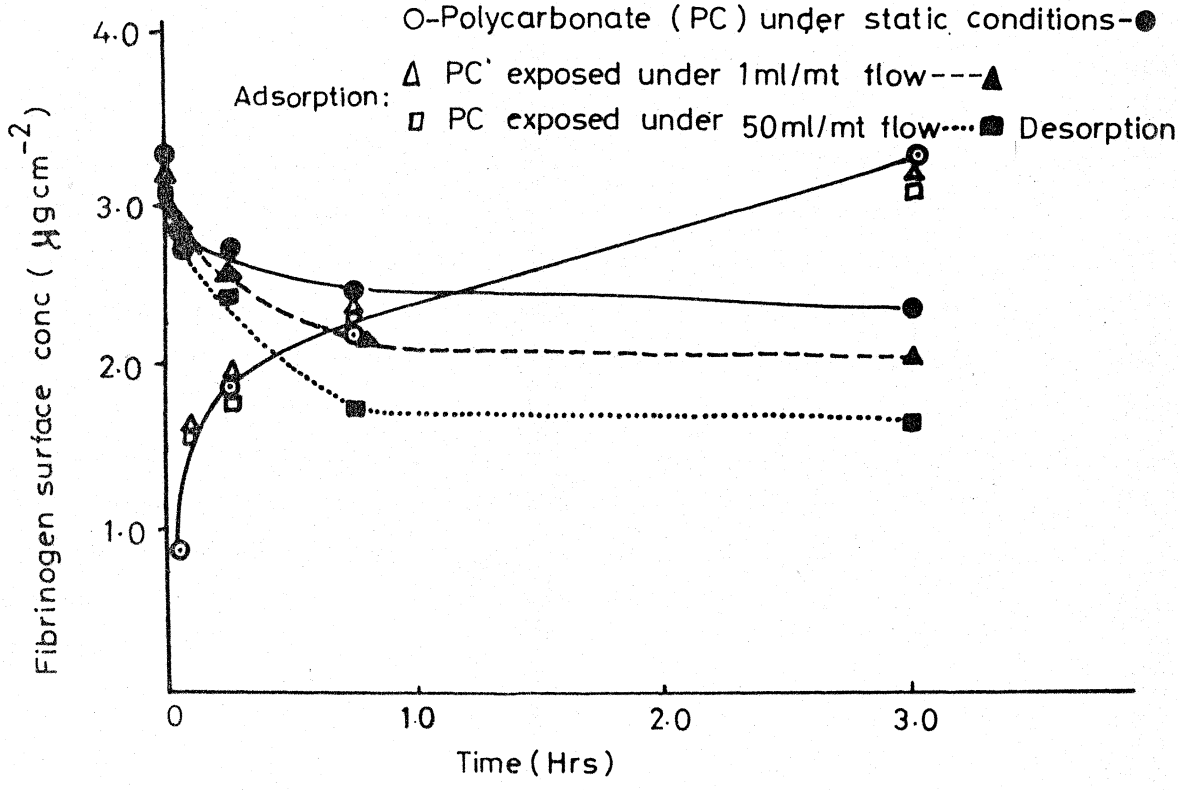



Fig.3-16. Effect of flow rates towards the adsorption/desorption of fibrinogen on PC as a function of time from 100mg% fibrinogen.

### 3.1.4. Effect of flow conditions

The interaction of human Fg with polycarbonate under various flow rates have been studied from 100 mg% Fg using tracer techniques. Figure 3-16 demonstrates the adsorption/desorption pattern of Fg under different flow rates. It seems, the adsorption in this isolated system is not significantly affected by shear rate. However, more Fg gets desorbed off from the substrate with higher flow rates of 50 ml/minute etc. as is evident from Fig.3-16. The lack of dependence of adsorption on various flow rates in this work agrees with previous observations of Brash et al[43] for the albumin-polyethylene system and of Watkins and Robertson[357] for the adsorption of γ-globulin to a silicone polymer substrate. So, it seems that the adsorption kinetics of isolated proteins to a polymer surface is a surface reaction, not transport, which is rate determining.

The effect of wall shear rate upon albumin adsorption on to a polyurethane urea, Biomer, has been studied using FTIR-ATR spectroscopy[264]. It is indicated that increasing wall shear rate during adsorption does not significantly affect the adsorption kinetics, but does decrease the rate of protein desorption. Jacobsen et al[167] have investigated the changes of protein adsorption due to flow rates on to germanium surface. It is reported that for albumin solutions at the same concentration, increasing the flow rate decreases the total amount of protein adsorbed in the 3 hour adsorption period. So it seems, the kinetics of protein adsorption may be dependent of the substrate properties, flow rates and the way in which the initial contact is brought about.

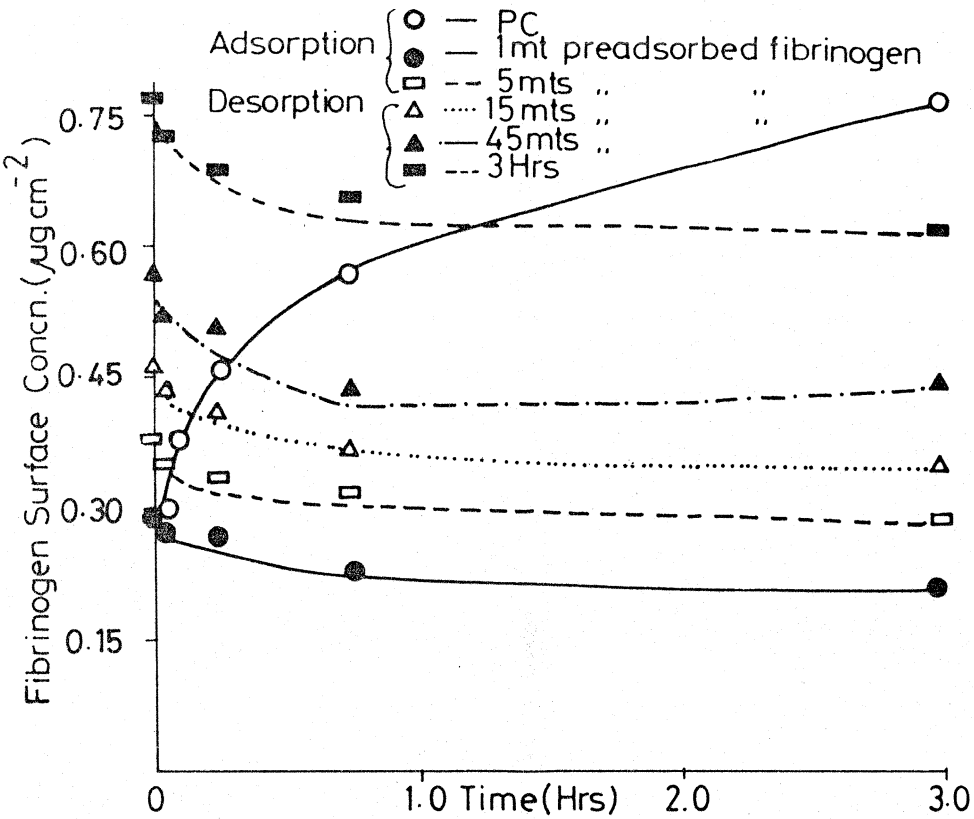



Fig.3-17. Adsorption / Desorption of fibrinogen as a function of time from 7.5 mg% fibrinogen.

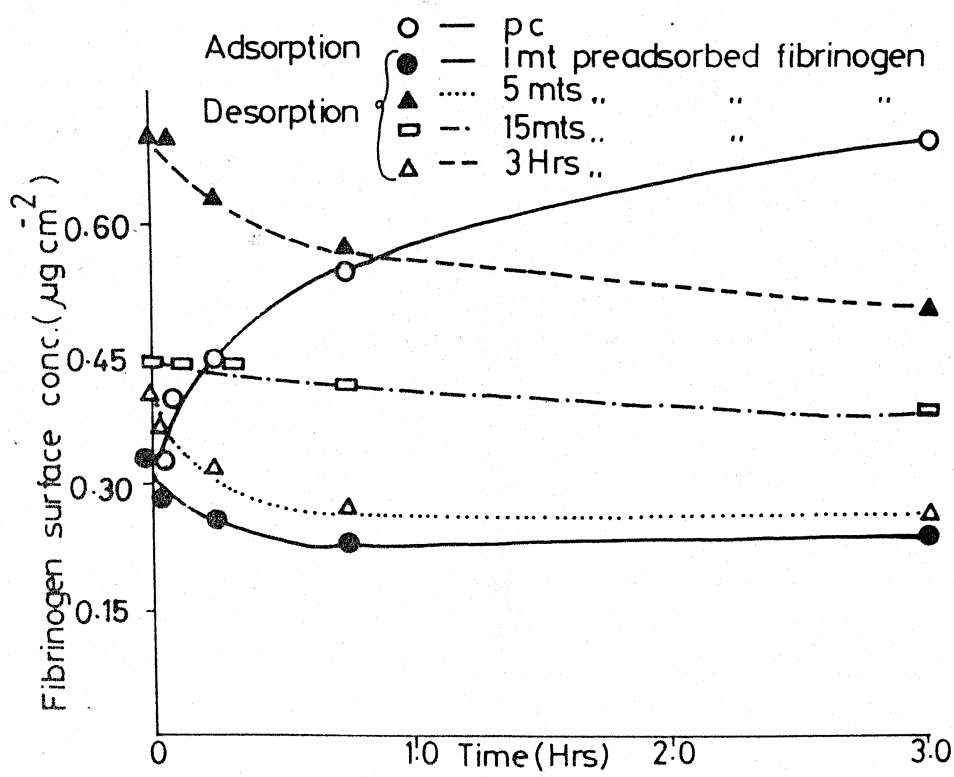



Fig.3-18. Adsorption / Desorption of fibrinogen as a function of time from 25 mg% albumin with 7.5 mg % fibrinogen.

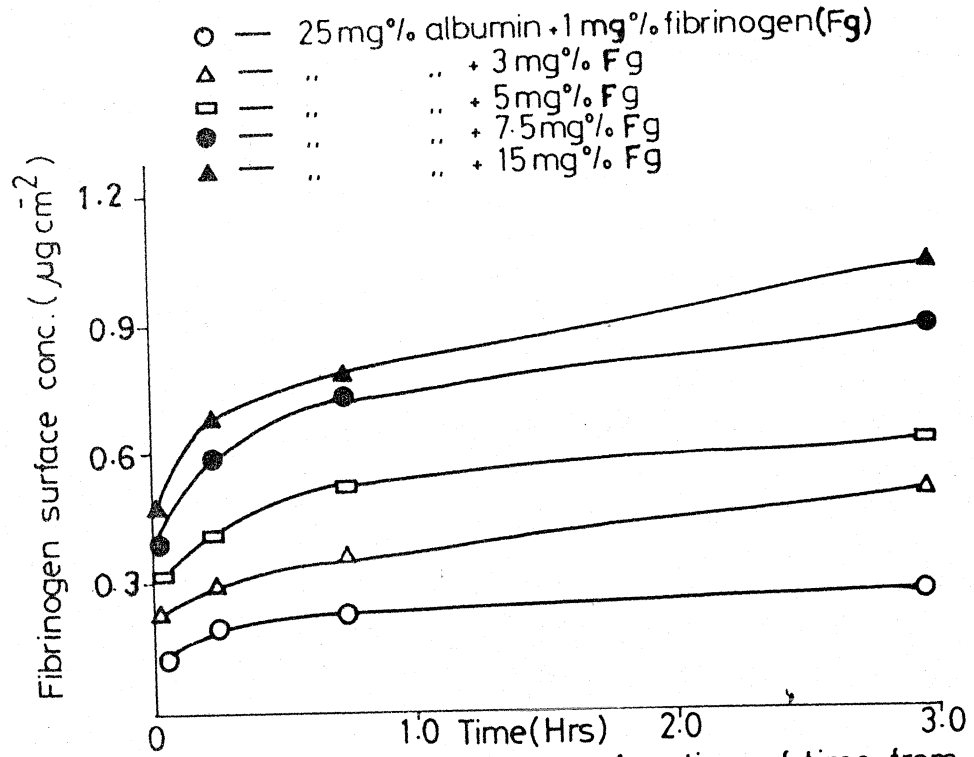



Fig.3-19. Adsorption of fibrinogen as a function of time from 25% albumin with varying conc. of fibrinogen.

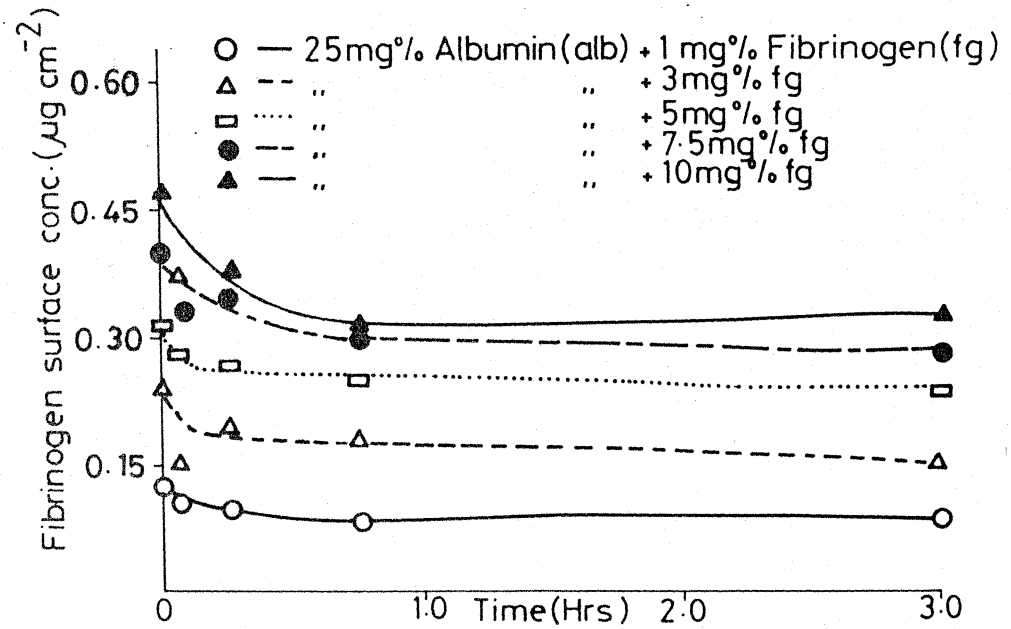



Fig.3-20. Desorption of preadsorbed (1mt) fibrinogen as a function of time from 25mg% alb. with varying conc. of fibrinogen.

## 3.2. Competitive adsorption

### 3.2.1. Addition of albumin to fibrinogen

In order to approach more closely to the blood system itself and to determine the influence of other proteins on fibrinogen-surface binding, studies have been undertaken to evaluate the adsorption between fibrinogen and other plasma proteins. Here $^{125}I$ fibrinogen and $^{125}I$ albumin are followed.

For the binary system, albumin-fibrinogen in 0.1M phosphate buffer is used. Adsorption/desorption kinetics of Fg to PC substrate from 7.5 mg% Fg is depicted in Fig.3-17. Addition of 25 mg% albumin to the above system demonstrates a slight reduction in surface Fg level (Fig.3-18). This may be due to the competition of albumin with Fg for the surface binding. Fig.3-19 shows the adsorption pattern of Fg to PC surface from 25 mg% albumin with low levels of Fg molecule in the system. This indicates that Fg-surface concentration is dependent on the Fg bulk concentration; which is certainly reversible, since addition of Fg to the dilute Fg systems cause further Fg-surface binding (complete depletion is not occurring). A fraction of the preadsorbed Fg is desorbed off from the surface as indicated in figures 3-20 and 3-21. A part of the surface bound Fg is depleted, which may be due to self exchange or competitive exchange though a fraction is retained to the surface.

In another studies, varying concentrations of Fg have been added to 25 mg% albumin and surface albumin binding is monitored. Figures 3-22 to 3-26 demonstrate the

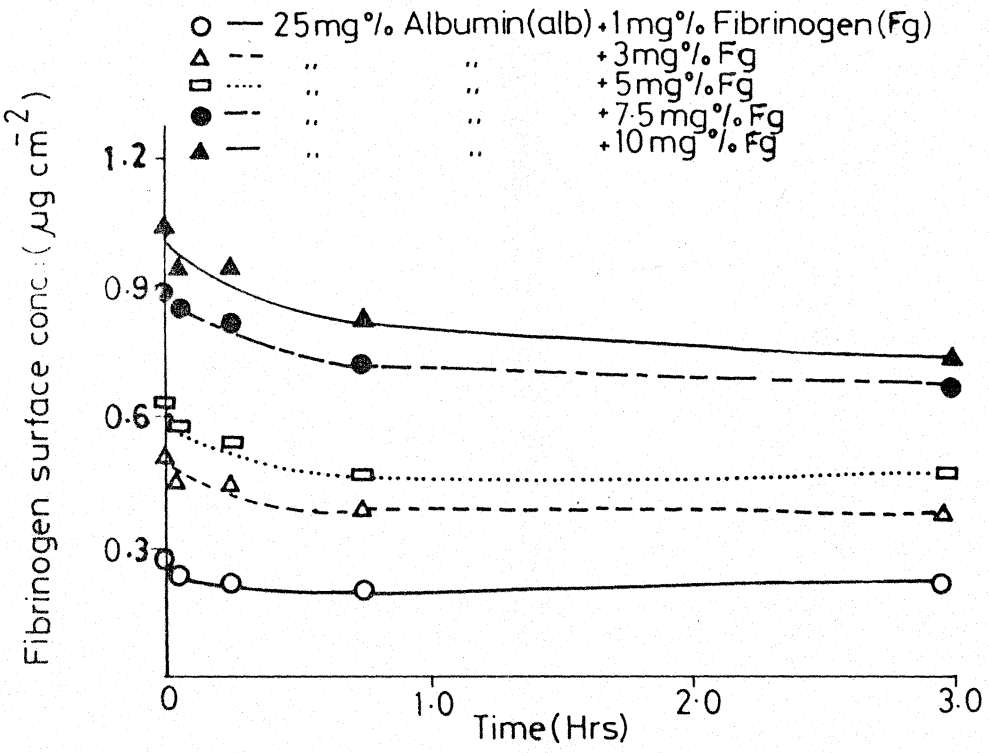



Fig.3-21. Desorption of preadsorbed (3 hours) fibrinogen as a function of time from 25 mg% alb. with varying conc: of fibrinogen.

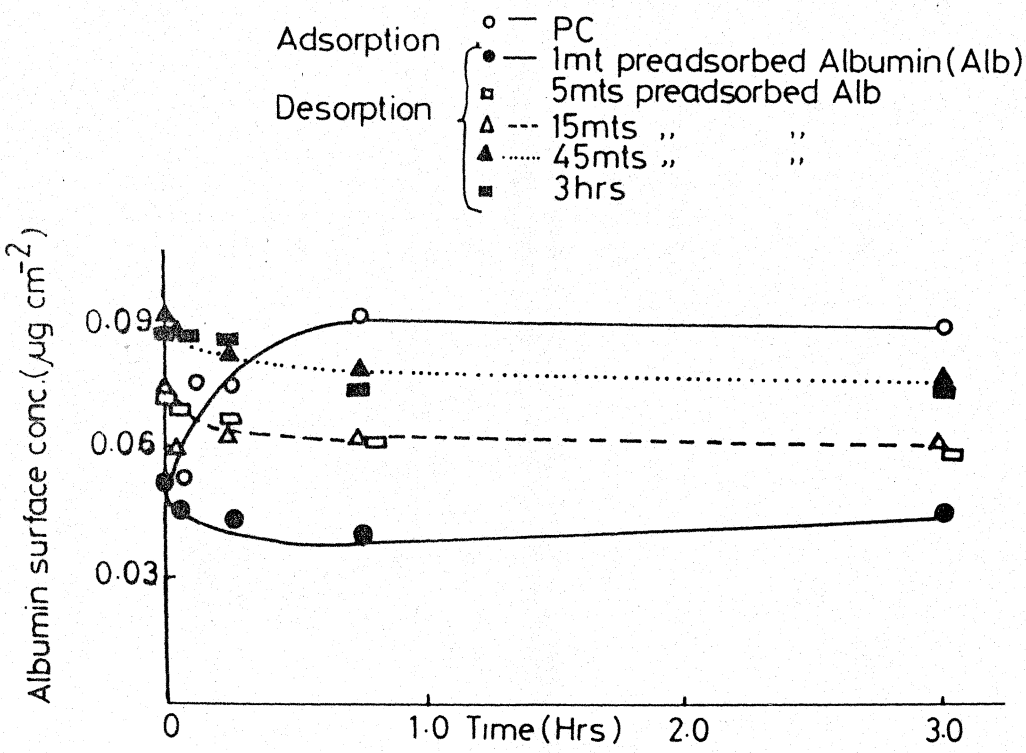



Fig3-22. Adsorption/Desorption of albumin as a function of time from 25mg% albumin.

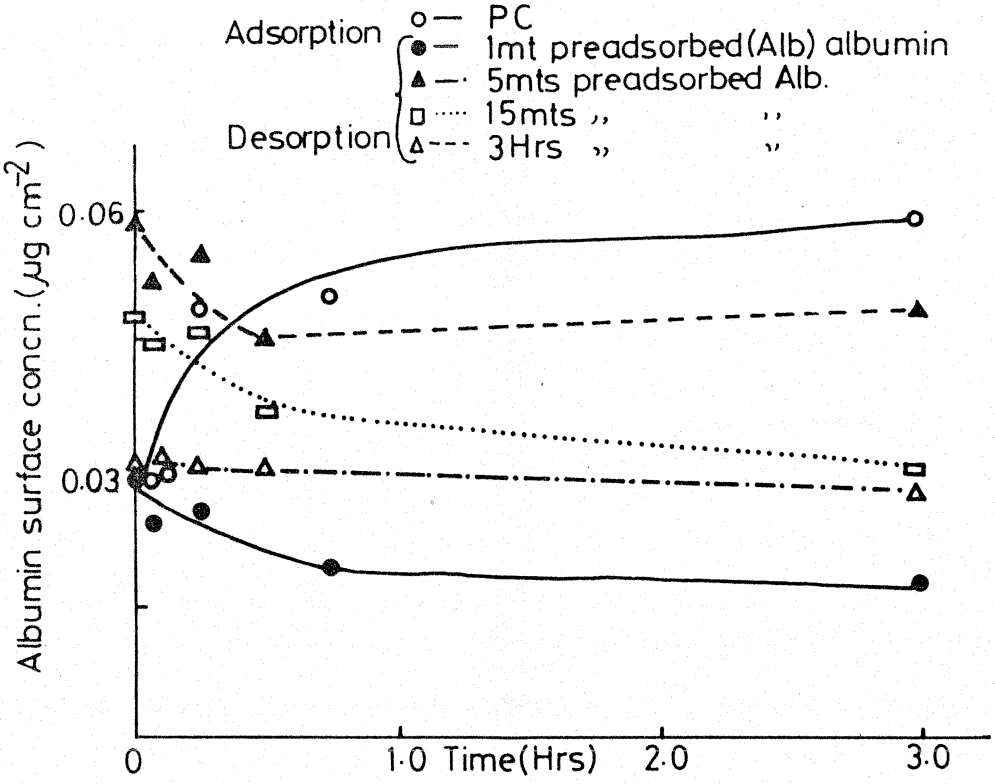



Fig.3-23 . Adsorption/ Desorption of albumin as a function of time from 25mg% albumin with 7·5mg% fibrinogen.

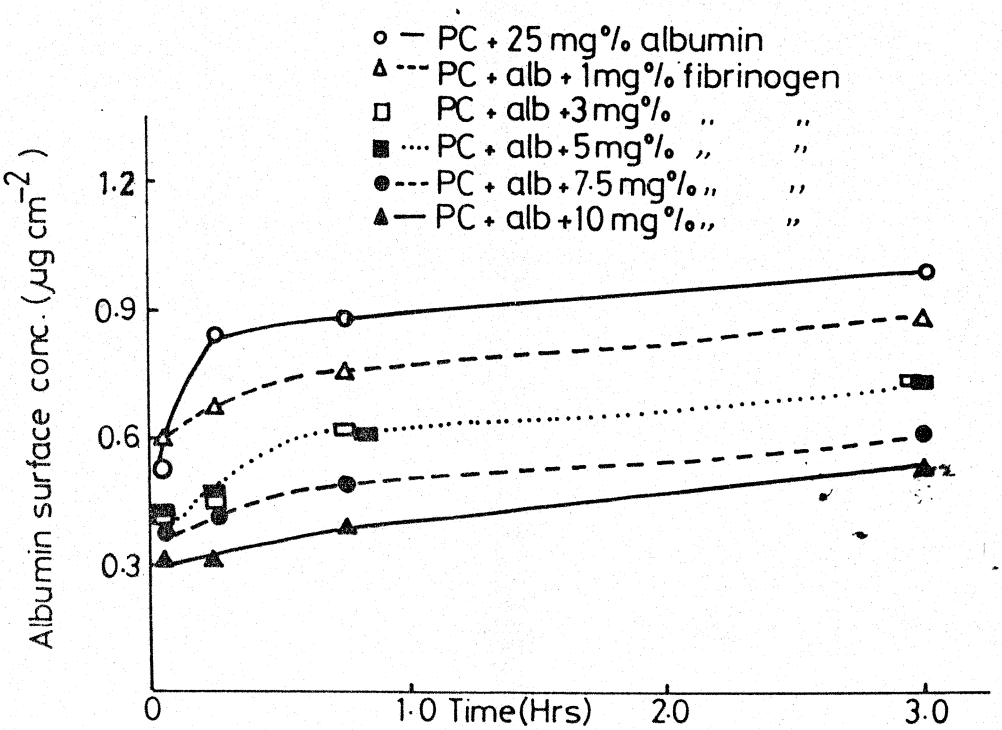



Fig3-24. Adsorption of albumin as a function of time from 25mg% albumin with varying concentrations of fibrinogen.

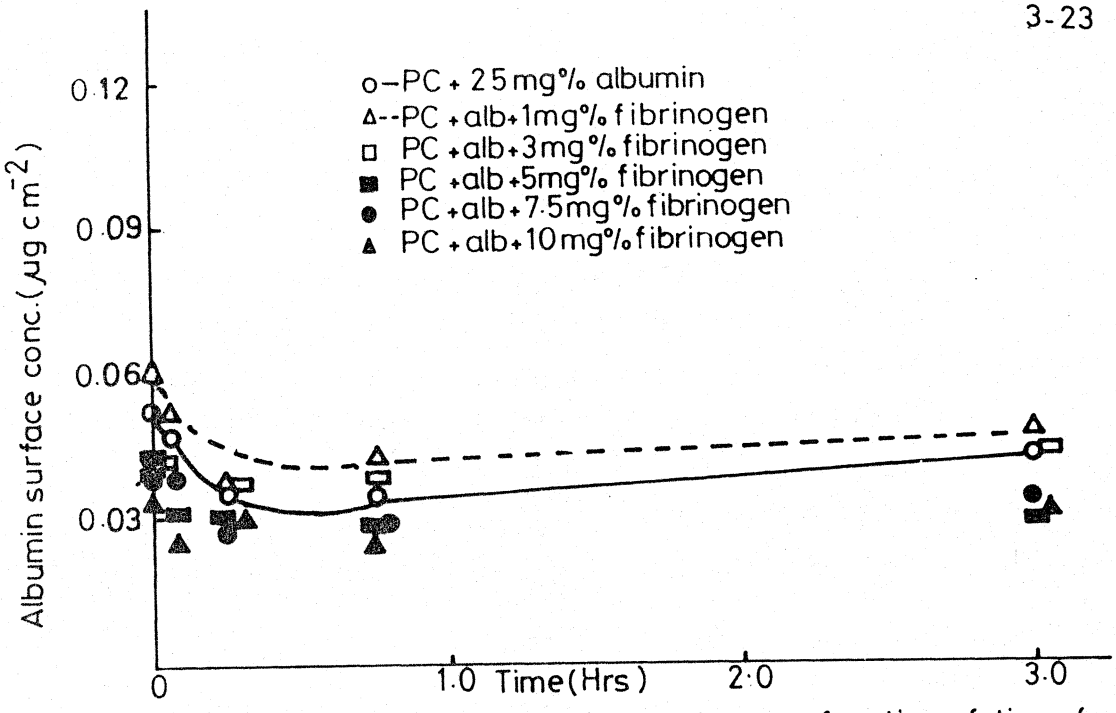



Fig 3-25. Desorption of preadsorbed (1mt) albumin as a function of time from 25mg% albumin with varying concentrations of fibrinogen.

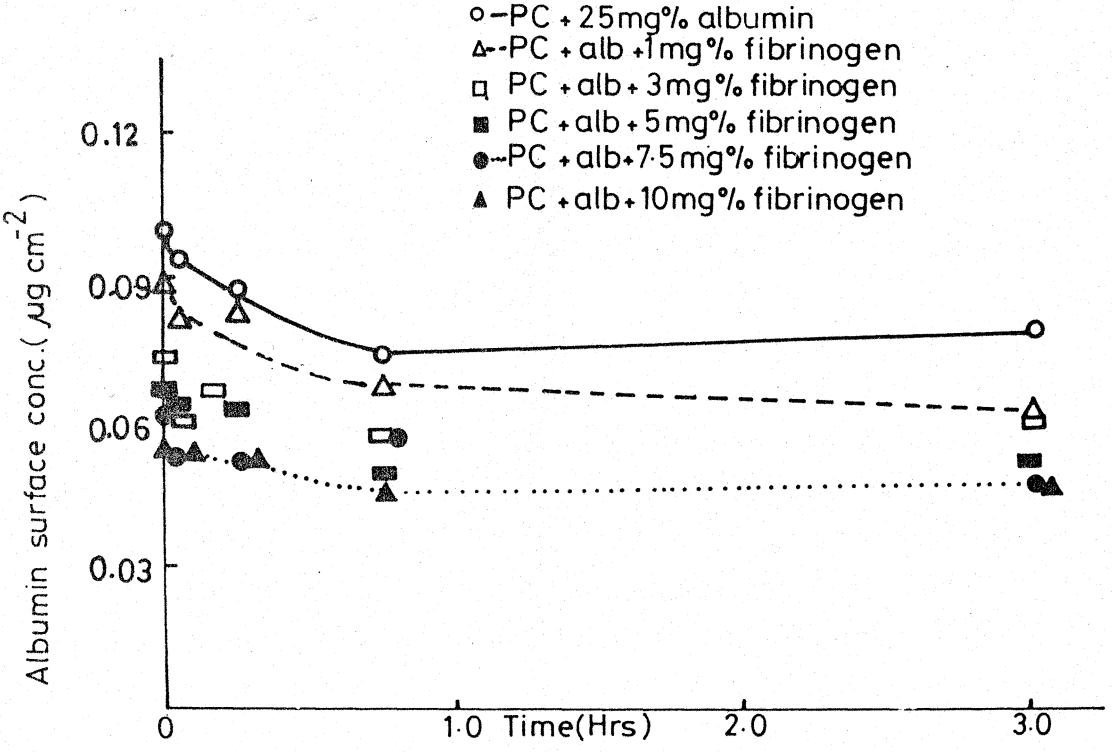



Fig 3-26. Desorption of preadsorbed (3 hrs) albumin as a function of time from 25 mg% alb. with varying cons. of fib.

adsorption/desorption kinetics of such studies. It seems, albumin surface concentration is reduced by the addition of Fg. A fraction ofthe preadsorbed albumin is removed from the substrate as indicated in figures 3-22, 3-23, 3-25 & 3-26. This may be due to self exchange or competitive exchange as demonstrated in the previous cases. It seems, the initial stages of adsorption, at very low concentrations studied, give low surface coverage and in the intermediate and later stages of adsorption, protein-protein interactions may provide the energetics for further interaction processes as suggested by various investigators[152,335].

### 3.2.2. Addition of albumin and γ-globulin

Adsorption kinetics of albumin and Fg from a mixture of albumin, fibrinogen and γ-globulin under static conditions to PC substrate is studied. Fig.3-27 shows the pattern of Fg adsorption to PC and their exchange (desorption) with other proteins from 3 hrs preadsorbed substrates. Similar studies of albumin kinetics is demonstrated in Fig.3-28. A typical steady state adsorption pattern is evident for these proteins with a preferential adsorption for Fg. However, the surface Fg level is drastically reduced to that of isolated Fg case. (The plateau values are, isolated Fg of 7.5 mg% --> 0.76± 0.03 $\mu g\ cm^{-2}$, and 25 mg% albumin, 15 mg% γ-globulin 7.5 mg% Fg system --> 0.63 ± 0.04 $\mu g\ cm^{-2}$). These experiments have been conducted using similar proportions of the proteins, as have been indicated by various investigators[178,191,339]. A part of the preadsorbed Fg or albumin is getting desorbed off from the substrate, probably due to self-exchange or exchange with other proteins in the media.

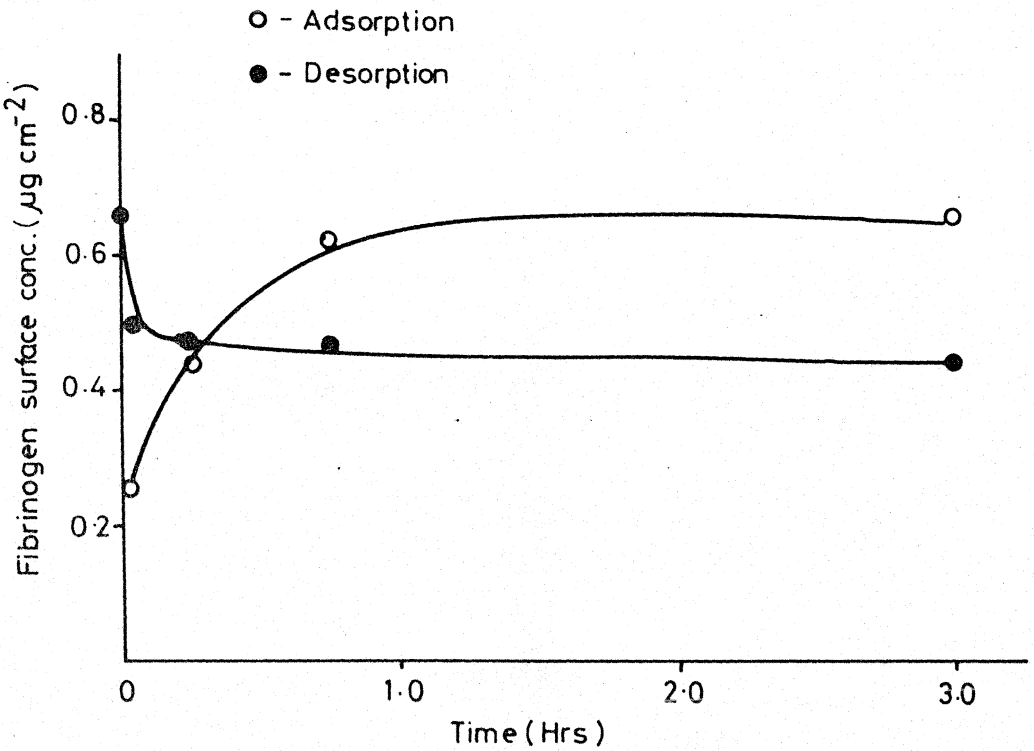



Fig.3-27. Adsorption / desorption of fibrinogen on PC as a function of time from 25 mg% albumin, 15 mg% γ-globulin and 7·5 mg% Fg.

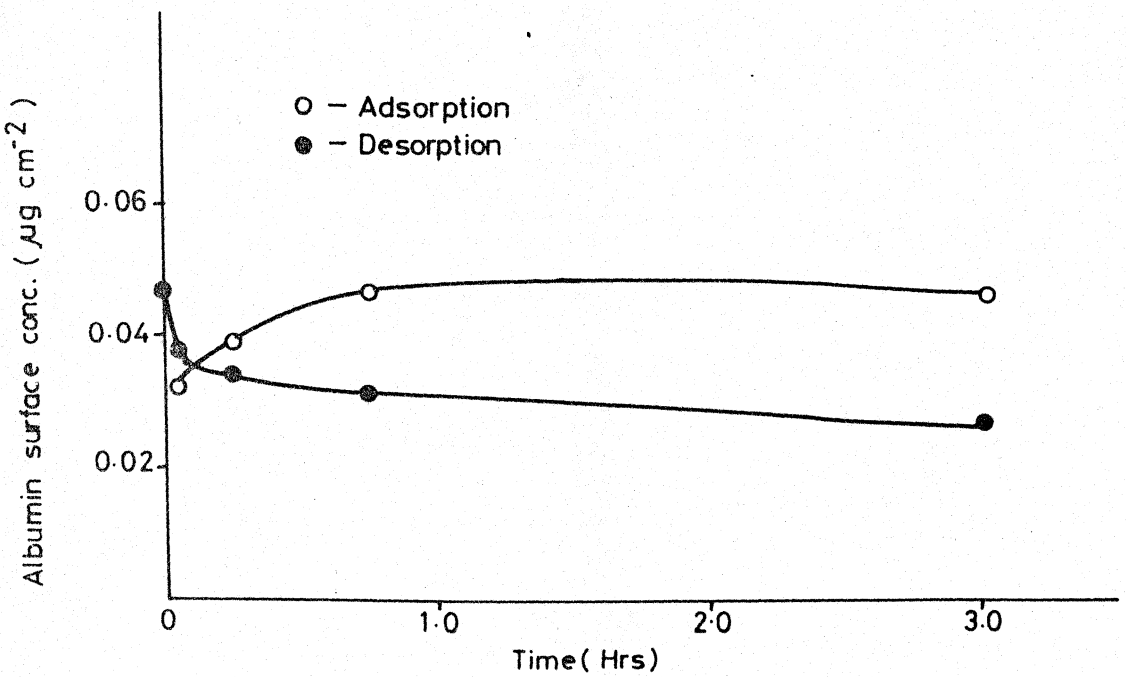



Fig.3-28. Adsorption / Desorption of albumin on PC as a function of time from 25 mg% alb, 15 mg% γ-glo: and 7·5 mg% Fg.

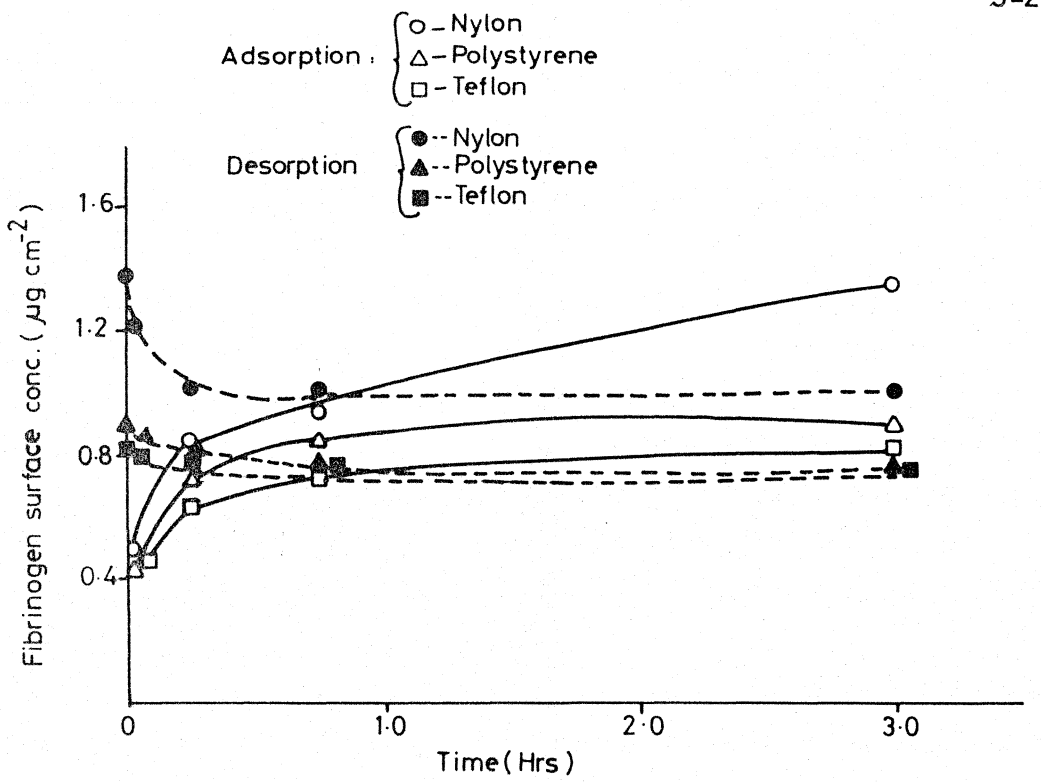



Fig.3-29. Adsorption / desorption of fibrinogen on various polymers as a function of time from protein mixture

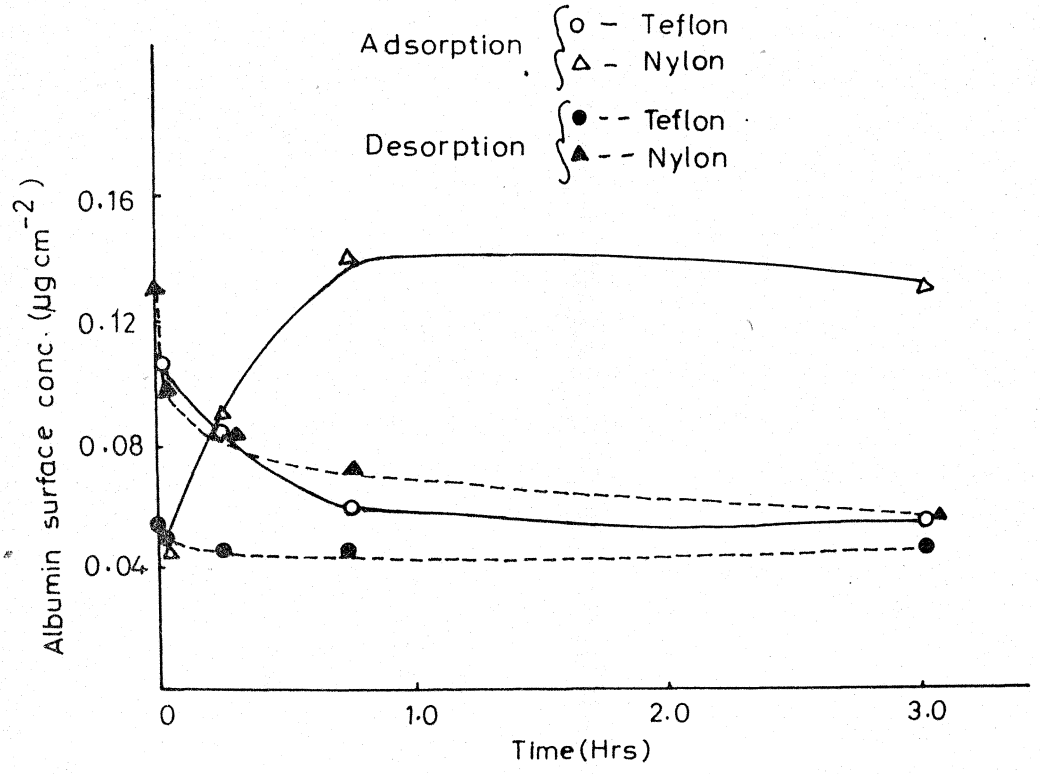



Fig.3-30. Adsorption/desorption of albumin on various polymers as a function of time from proten mixture.

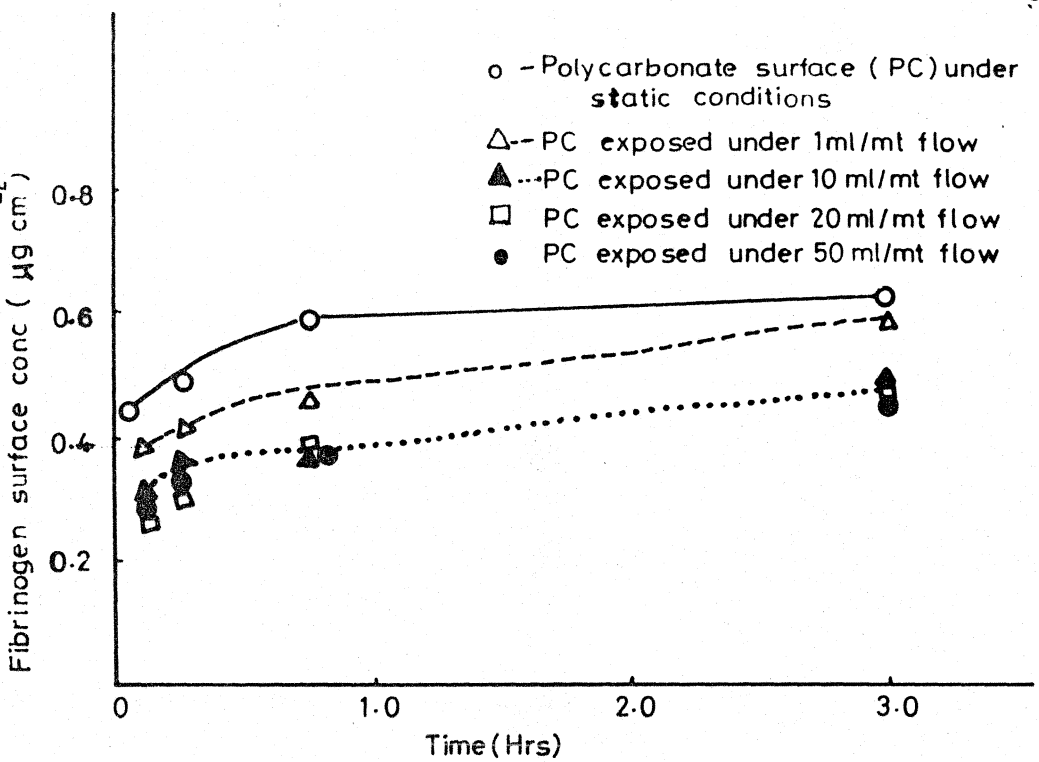
The adsorption kinetics of fibrinogen and albumin from protein mixture to various other polymers like Teflon, Nylon and Polystyrene have also been performed for better understanding of the interaction process. Fig.3-29 shows the pattern of Fg adsorption/desorption from these substrates under static conditions and the same for albumin in Fig.3-30. It is evident from Fig.3-29 that the Fg adsorption kinetics vary with substrates and a part of the preadsorbed Fg is being exchanged, depending on the surface properties. In the case of albumin adsorption to these surfaces, Teflon substrate shows a maximum adsorption initially, which tend to reduce with time (Fig.3-30). However the desorption from the 3 hours preadsorbed Teflon is negligible due to its hydrophobic nature compared to Nylon, where sufficient albumin is desorbed off with time in the protein mixture.

Lee et al[191] have studied the competition between albumin, γ-globulin and fibrinogen from their mixture in buffer and from their isolated forms towards surface binding. They have observed that Fg adsorption on to silastic from the mixture has been found to be 67% of that for the Fg adsorption from an isolated fibrinogen solution. Several other groups have also found preferential adsorption of Fg from 2- and 3- protein mixtures[57,109,150]. These studies have been done on a variety of surfaces with various mixture compositions and total concentrations of proteins. Such experiments consistently confirm Fg preferential adsorption as a general effect, which also confirms the present observations.

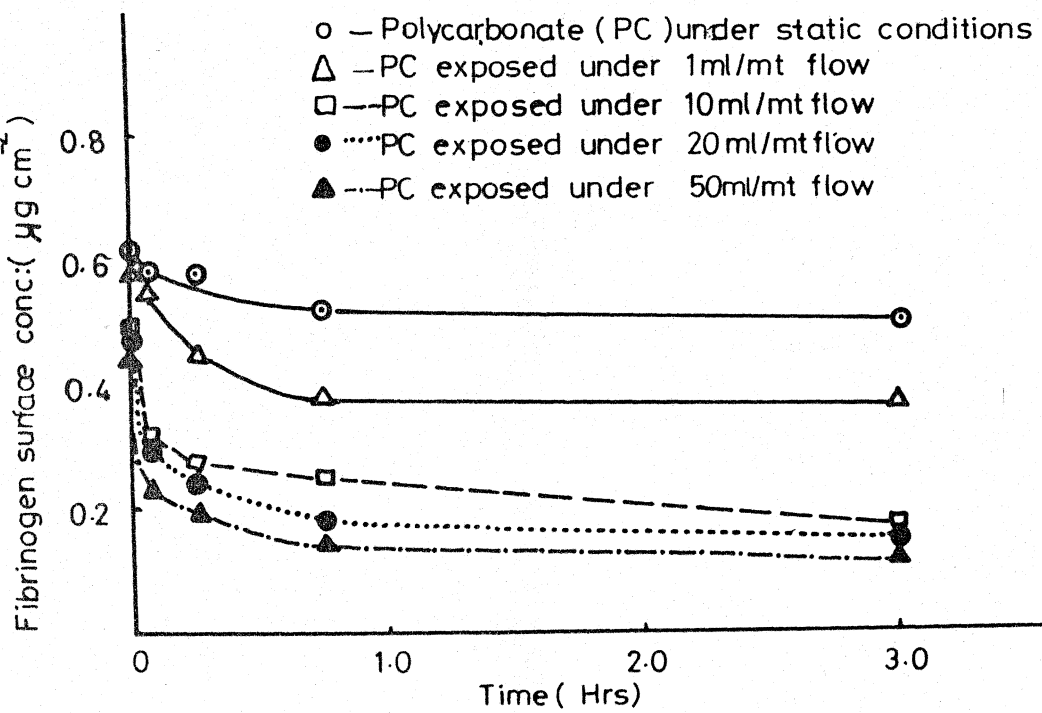
### 3.2.3. Changes of protein adsorption with flow conditions

Adsorption kinetics of Fg from a mixture of albumin, fibrinogen and γ-globulin under various flow rates to different polymers are also investigated. Figures 3-31 to 3-34 show the pattern of Fg adsorption to PC, Teflon and Nylon surfaces under various flow rates. It is evident from Fig.3-31, that the lower flow rate (1 ml/minute) does not alter the Fg surface concentration, whereas the increased flow rate has inhibited the Fg-surface binding. However, further increase of flow rates from 10 ml/minute to 20 or 50 ml/minute do not seem to cause any significant change to the pattern of Fg adsorption to the polymer substrates. (Figures 3-33 and 3-34).

The desorption kinetics of 3 hours preadsorbed Fg from PC substrate to protein mixture is depicted in Fig.3-32. It indicates, as flow rate increase, more Fg gets desorbed off the surface. Earlier in this Chapter, it has been indicated that the flow-rate has no significant effect to alter the Fg adsorption to PC surface from isolated Fg (Fig.3-16). However, the Fg level has reduced with increase of flow rate from a protein mixture (Figures 3-31, 3-33 and 3-34). The amount of Fg adsorbed for 3 hours from 100 mg% fibrinogen, under static conditions is $2.90 \pm 0.24 \mu g\ cm^{-2}$ and the same under 50 ml/mt flow is $2.70 \pm 0.20 \mu g\ cm^{-2}$, while from a protein mixture of 25 mg% albumin, 15 mg% γ-globulin and 7.5 mg% Fg, under static conditions, the Fg concentration is $0.645 \pm 0.025 \mu g\ cm^{-2}$, which has come down to $0.45 \pm 0.03 \mu g\ cm^{-2}$, under 50 ml/mt flow rate. So, it seems that the adsorption kinetics of Fg from a protein mixture under flow conditions is also dependent on the




Fig·3-31. Effect of flow rates towards the adsorption of fibrinogen on PC as a function of time from protein mixture




Fig·3-32. Effect of flow rates towards the desorption of 3 hrs preadsorbed fibrinogen from PC as a function of time to protein mixture.

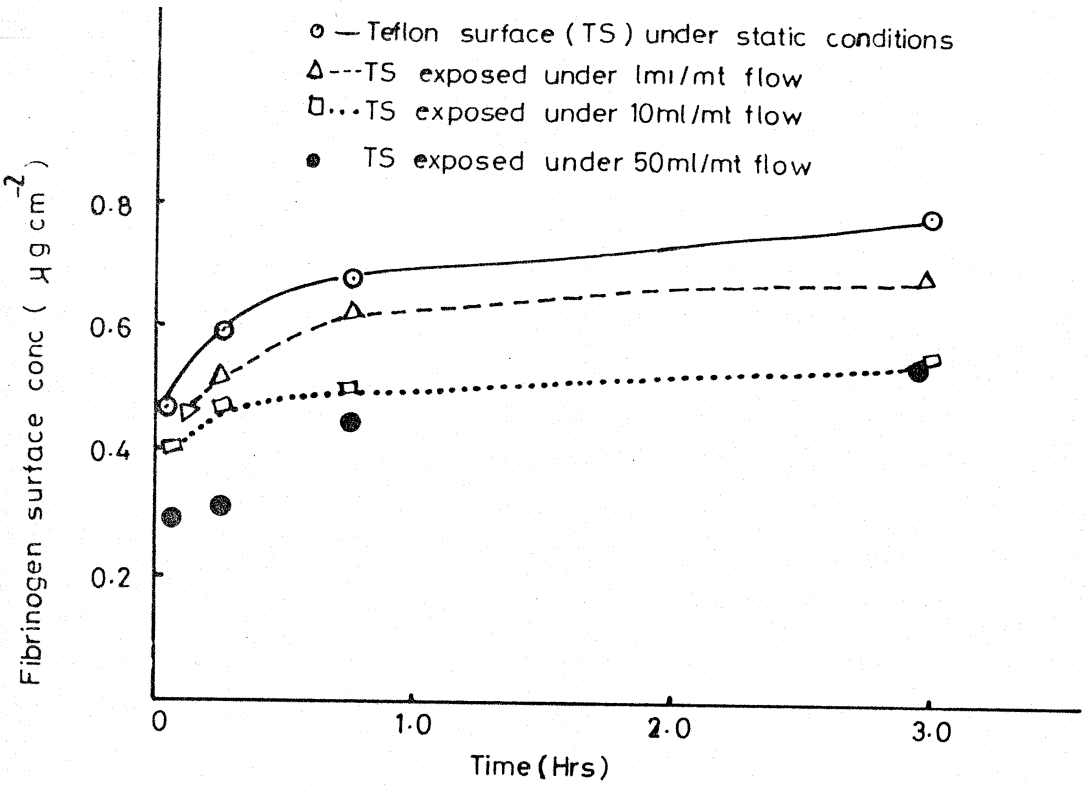



Fig 3-33. Effect of flow rates towards the adsorption of fibrinogen on Teflon as a function of time from protein mixture.

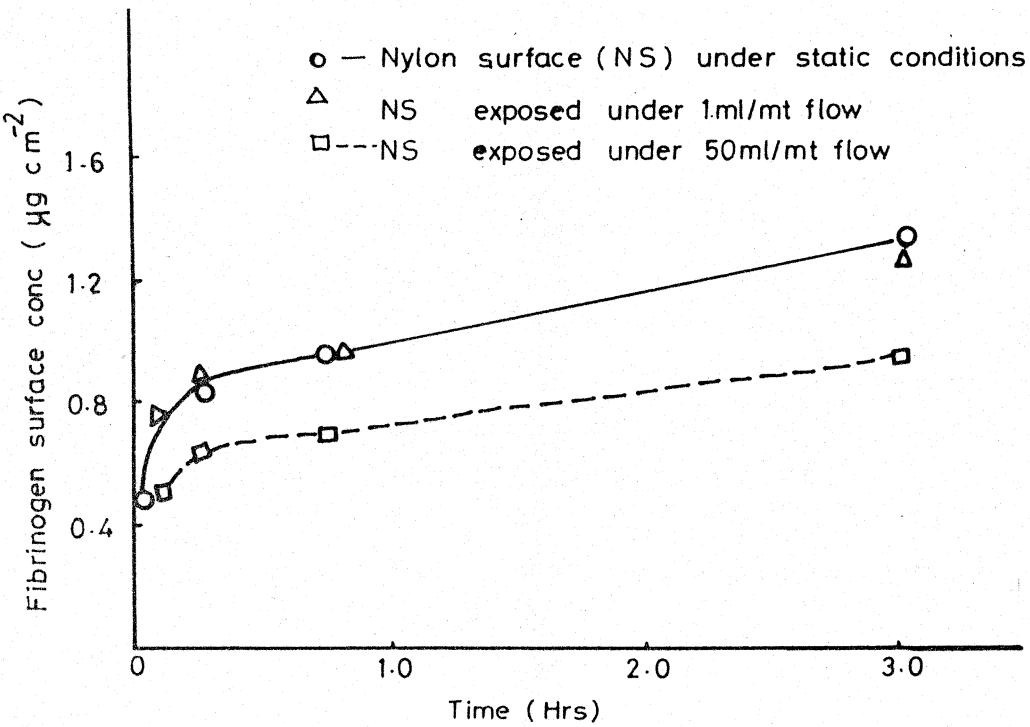



Fig 3-34. Effect of flow rates towards the adsorption of fibrinogen on Nylon as a function of time from protein mixture.

transport properties of the species in the media, which may vary with molecular size and the exchange properties.

Jacobsen et al[167] have studied the changes of protein adsorption from a mixture of 1:1 albumin and fibrinogen, under flow conditions using spectroscopic techniques on to germanium surfaces. It is indicated that at the same flow, and concentration of protein, more Fg is adsorbed than albumin and less Fg is desorbed than albumin. They have also shown[167], that from the mixture, the protein adsorption is less, the fact that the amount of albumin adsorbed is very less than in pure Fg solutions; which may suggest that albumin adsorbs intially and thus modifies the surface onto which the Fg molecules adsorb. Thus it is possible that the amount and nature of protein adsorption from the mixture can also be affected by the time for replacement of albumin by Fg, which may cause a reduction in protein-surface concentration, as is evident from these experiments.

### 3.2.4. Adsorption from plasma

The competitive adsorption of Fg from plasma to polycarbonate substrate have also been evaluated using polyacrylamide gel electrophoresis of desorbed proteins and their kinetics with labelled fibrinogen. The effect of plasma to modulate the surface-protein binding have been investigated using calf plasma (Albumin --> 4.02 gm%, Globulins --> 2.22 gm% and Fibrinogen --> 0.34 gm%), exposed to PC substrate for 15 minutes and 3 hours. The proteins, desorbed from the substrate, are applied to PAGE along with the bulk plasma in separate gels. Fig.3-35 shows the typical

PAGE of desorbed proteins from PC substrate. It is obvious that most of the proteins seen in plasma can be adsorbed to the substrate, however, the Fg concentration is negligible. This is not the case with tertiary mixtures of proteins adsorbed from buffer, as indicated earlier in this Chapter.

The kinetics of Fg adsorption as a function of plasma dilution and time have been also studied using $^{125}I$ fibrinogen added to the plasma as a tracer. Calf plasma containing 0.34 gm% Fg has been used either as undiluted plasma or diluted to various concentrations and the amounts of labelled Fg have been always less than 10% of the total amount of fibrinogen in the plasma. The Fg adsorption as a function of time with varying plasma dilutions are depicted in figures 3-36 and 3-37. The Fg adsorbs to the substrate within seconds, reaches to a plateau value and remains constant. It is also evident that less amount of Fg is adsorbed from plasma in comparison to the adsorption from single protein or simple mixtures and the increase of plasma concentrations from 10% do not demonstrate further adsorption of Fg to the substrate. The desorption kinetics of 3 hours preadsorbed Fg from PC substrate in plasma is shown in Fig.3-38. The preadsorbed Fg is drastically removed within seconds (less than 2 minutes) from the substrate, and it reaches to a constant value for all plasma dilutions studied. In other words the Fg deposited with diluted plasma is not further increased with higher plasma concentrations.

An overview of Fg-polycarbonate, interaction from isolated cases, simple mixtures and plasma are demonstrated in Table 3-I.

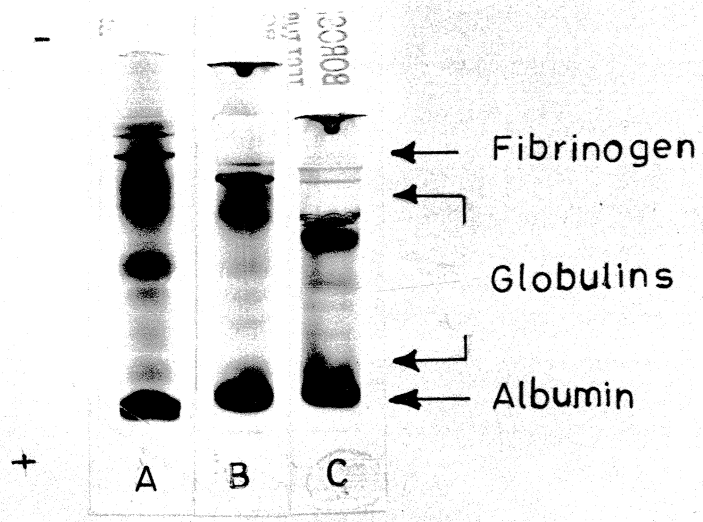



Fig 3-35. Polyacrylamide gel (7%) electrophoresis of calf plasma (A), desorbed proteins from PC exposed to calf plasma for 15mts (B), and desorbed proteins from PC exposed to calf plasma for 3 hours (C) respectively. Stained with Coomassie blue.

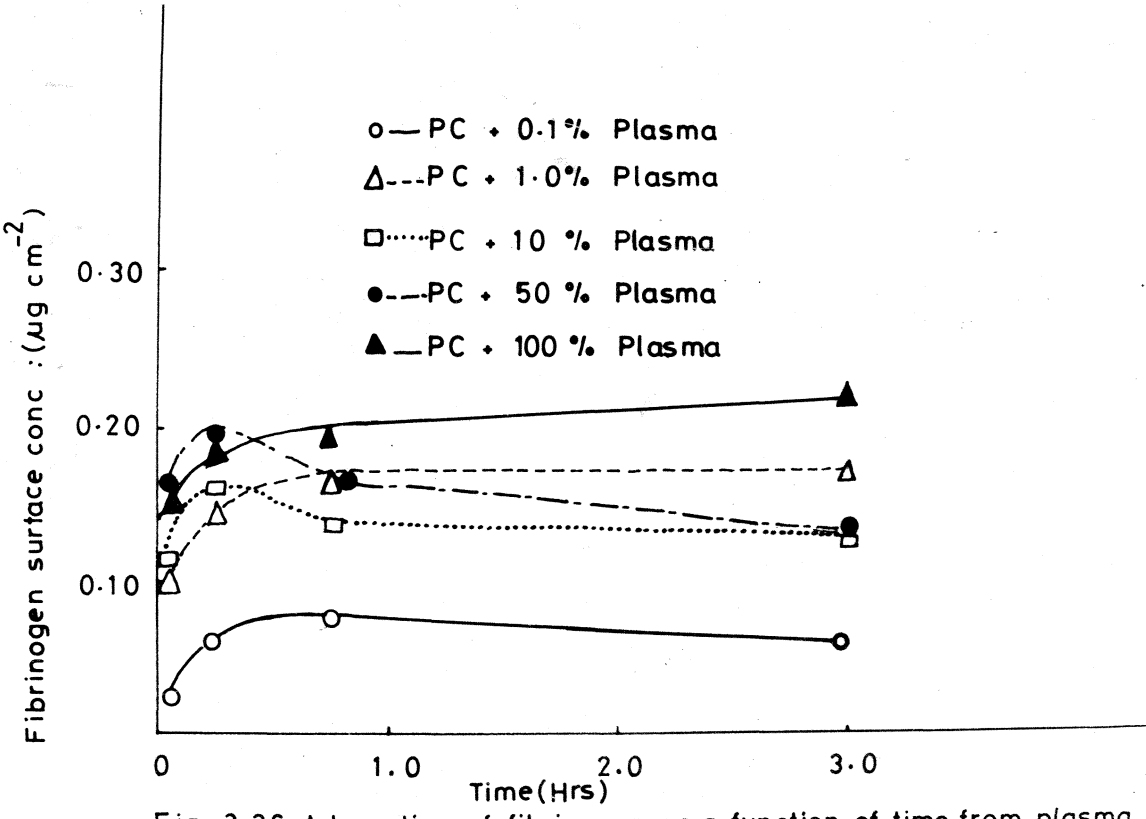



Fig. 3-36. Adsorption of fibrinogen as a function of time from plasma

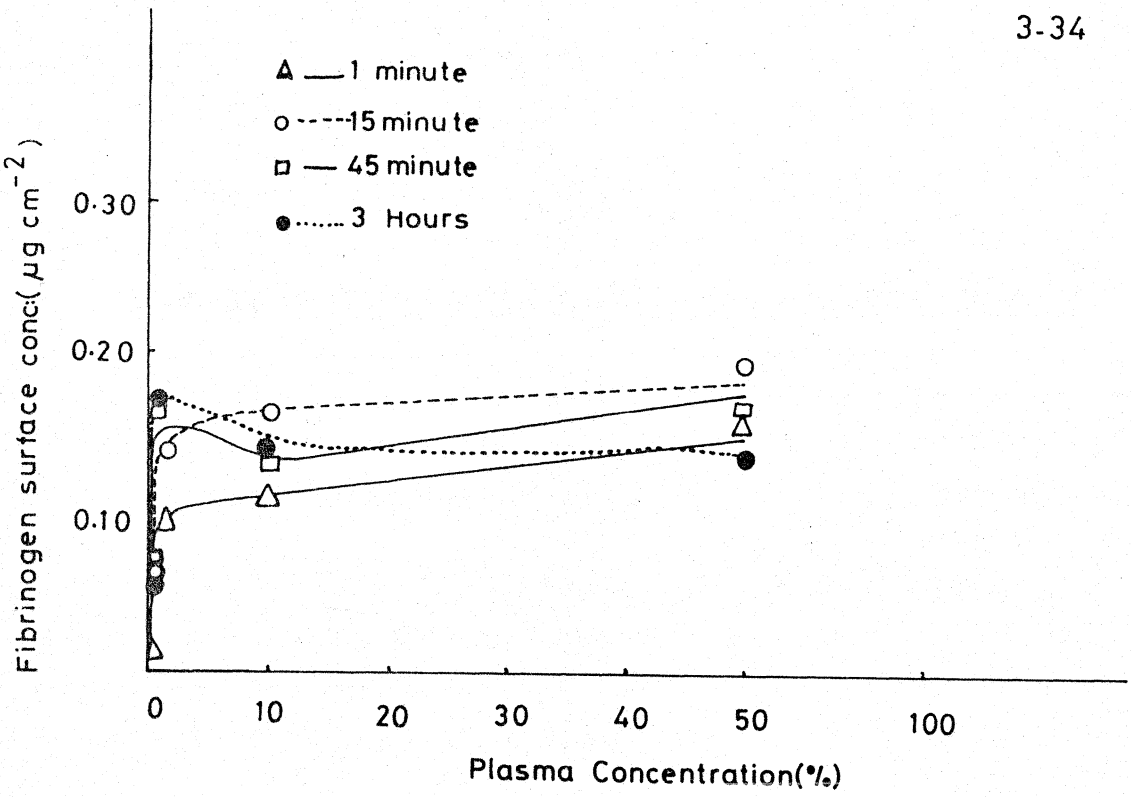



Fig. 3-37. Adsorption of fibrinogen as a function of plasma concentration for various time intervals.

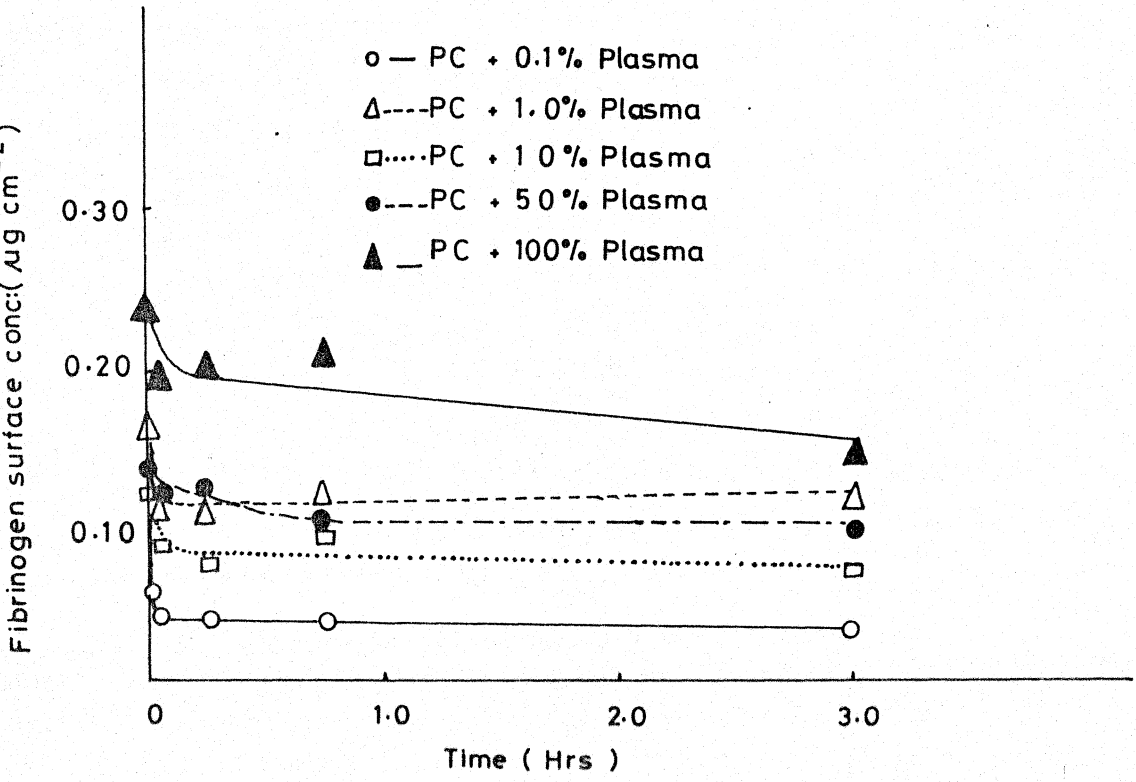



Fig. 3-38. Desorption kinetics of 3hours preadsorbed fibrinogen to plasma.

TABLE 3-1

SUMMARY OF FIBRINOGEN ADSORPTION TO POLYCARBONATE

| Details of the media | Fibrinogen concentration $\mu g\ cm^{-2}$ $\pm SD^*$ |
|---|---|
| 1. 100 mg% fibrinogen (Fg) in phosphate buffer | 2.90 ± 0.24 |
| 2. 7.5 mg% fibrinogen in buffer | 0.76 ± 0.01 |
| 3. 7.5mg% Fg + 25 mg% albumin in buffer | 0.69 ± 0.03 |
| 4. 7.5 mg% Fg+25 mg% albumin+15 mg% $\gamma$ -globulin | 0.645 ± 0.025 |
| 5. 7.5 mg% Fg+25 mg% albumin+15 mg% $\gamma$ -globulin+35 mg% fibronectin | 0.465 ± 0.015 |
| 6. 1.0% diluted calf plasma (contains 3.4 mg% Fg) | 0.169 ± 0.004 |
| 7. 100% Calf plasma (contains 340 mg% Fg) | 0.225 ± 0.07 |

* Values expressed as the average of Fg adsorbed to the substrate with standard deviation, from at least 3 separate experiments, 'p' $\leq$ 0.05.

It clearly indicates that addition of other proteins to the isolated Fg causes substantial reduction of surface bound fibrinogen and from plasma very small amount of Fg gets adsorbed in comparison to the isolated system. These observations suggest that Fg may well adsorb to polycarbonate at very short times from plasma and then be substancially reduced. Vroman et al[348,351] have indicated that on many surfaces, blood plasma or blood deposits predominantly Fg within a second, but on potentially clot-activating substrates, this Fg appears to be replaced by high molecular weight kininogen and factor XII. Brash et al[44] using undiluted plasma or plasma diluted 1:4 have demonstrated that essentially no Fg has been adsorbed to a pyrex glass surface over a period of 4 hours. These observations support the plasma effect presented here and it is possible that Fg can be adsorbed substantially at shorter times, but can get desorbed off almost completely over a subsequent exposure times, possibly by exchange with other proteins or by enzymatic action.

It seems obvious to consider a possible connection between Fg turn over from plasma or from protein mixture and it's fate at the interface (eg: platelet adhesion). It is established[25,219] that human platelets possess membrane receptors for a wide variety of agents including fibrinogen and platelets adhere where they find adsorbed Fg[3,352]. However, the amount of fibrinogen receptors available for the subsequent adhesion of platelets are important, rather than the amount of molecular fibrinogen deposited alone (possibly in multiple layers). Since it is possible the receptor sites can be modulated by various other plasma factors or by protein-protein or protein-polymer interactions, which

require a better knowledge to correlate with the interfacial phenomenon. The proceeding Chapters provide an insight to the changes, which can happen to the protein/platelet interactions with polymer substrates when other trace proteins or mediators arrive at the interface.

# FIBRINOGEN - POLYMER INTERACTION - INFLUENCE OF Fg HETEROGENEITY, CERTAIN PROTEASES AND FIBRONECTIN

# CHAPTER-IV

## 4.1. Effect of fibrinogen heterogeneity

The human fibrinogen molecule consists of three pairs of peptide chains, $(A\alpha, B\beta, \gamma)_2$, joined by disulfide bridges as represented in Fig.4-1. The Fg molecule can have atleast three major sources of heterogeneity due to several criteria, in pooled or single donor plasma.

Initially these three Fg fractions have been separated and purified as described in the experimental part. The characteristics of the three Fg fractions, 1a, 1b, 2b, are summarised in Table 4-1. Thrombin clotting times and percentage of clottabilities among the different fractions, are quite agreeable with those of previous investigators[94,98]. SDS gel electrophoresis patterns of these fractions are shown in Fig.4-2. This suggests that fractions 1a and 1b consist of $A\alpha$, $B\beta$ and $\gamma$ chains, whereas fraction 2b contains; in addition to $A\alpha$, and $B\beta$ chains, approximately equal amounts of two types of $\gamma$-chain, one is having slightly higher molecular weight than the normal $\gamma$-chain. Thus, the composition of fraction 2b is $(A\alpha, B\beta)_2\gamma\gamma'$ as indicated elsewhere[131]. These three Fg fractions have been used for the subsequent platelet experiments and protein binding studies with the PC substrate.

The adhesion of platelets to polycarbonate has been increased by the addition of all three fibrinogen

fractions, when compared with the bare substrate, as demonstrated in Table 4-II. However, the Fg fraction 2b has demonstrated a reduction in platelet-surface attachment, in comparison with, fractions 1a and 1b.

It appears that ADP induced platelet binding to PC substrate has also been decreased in presence of fraction 2b. Further studies have been carried out to investigate the affinity of these fractions to PC substrate, from a protein mixture, containing 25 mg% albumin, 15 mg% $\gamma$-globulin and 7.5 mg% fibrinogen (Fraction 1a, or 1b or 2b respectively) using PAGE, after desorbing them using Triton

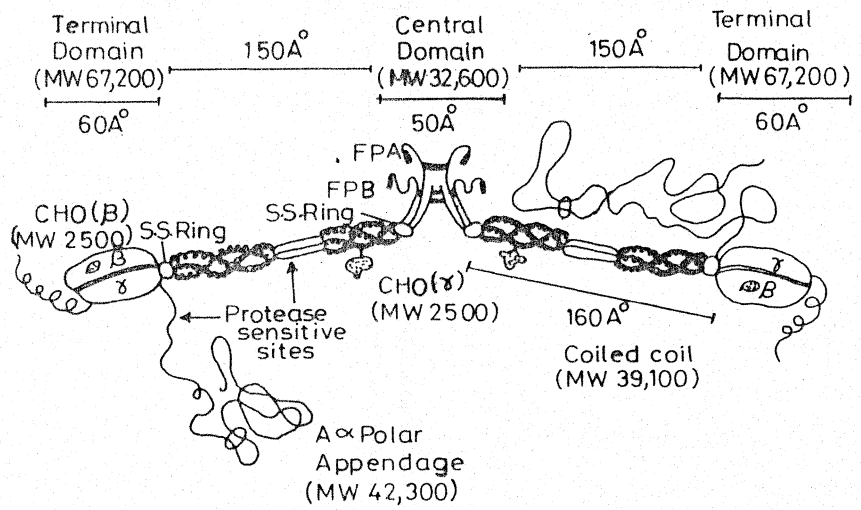



Fig 4-1 Schematic drawing of fibrinogen (Doolittle, et al-1978). The molecule is shown with four major protein regions: the central domain which contains aminoterminal ends of all six chains, the thin connecting $\alpha$-helical coiled-coils, the two carboxy terminal domains and the $\alpha$-chain polar appendages. Fibrinopeptides A and B (FPA and FPB) are noted as slightly thickened parts of the aminoterminal ends of the A$\alpha$ and B$\beta$ chains. There are four carboxyl clusters (CHO). SSRING-Disulfide bridges.

X-100. The electrophoresis pattern as demonstrated in Fig. 4-3, shows variations in the composition of the protein layer removed from the PC surface. It seems the Fg, containing $\gamma'$ chains (Fraction 2b), binds less effectively to PC substrate, while the other two fractions behave similarly. The $\gamma'$ chain containing Fg system also enhances the surface albumin binding (Fig.4-3).

TABLE 4-I

**CHARACTERISTICS OF THE THREE FIBRINOGEN FRACTIONS USED IN PLATELET/PROTEIN BINDING STUDIES WITH POLYCARBONATE SURFACE.**

| Fibrinogen Fraction | Clotting time (Seconds) | Clottability (%) | Chain Composition |
|---|---|---|---|
| Fraction 1a | 25.0 | 93.7 | $(A\alpha, B\beta\gamma)_2$ |
| Fraction 1b | 20.9 | 93.0 | $(A\alpha, B\beta\gamma)_2$ |
| Fraction 2b | 22.3 | 92.0 | $(A\alpha, B\beta)_2\gamma\gamma'$ |

The binding of Fg to surface or to platelets have been extensively studied[21,258,377]; however the part played by the $\gamma'$ extension in Fg molecule for the modulation of interfacial phenomena is meagre. Harfenist et al[131] have separated Fg in to three fractions by DEAE cellulose chromatography and have shown that $\gamma'$ chains bind less effectively to ADP stimulated platelets, and thus reduced platelet aggregation. The present studies report the

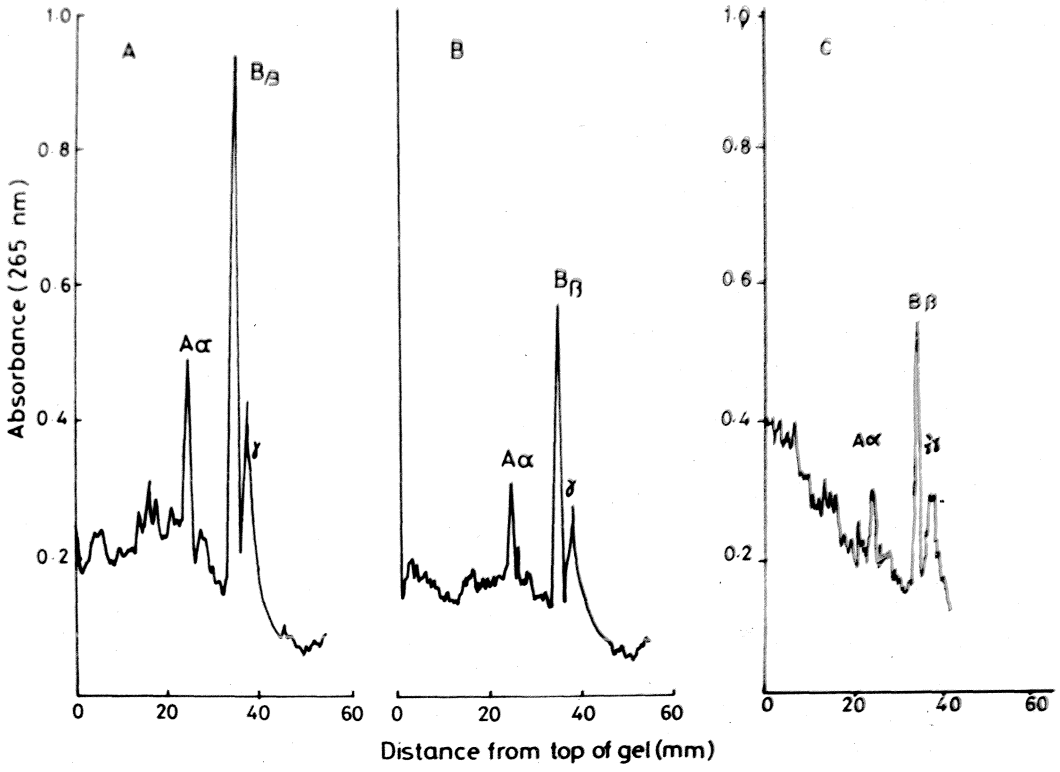



Fig 4-2. Profiles of 7% SDS polyacrylamide gels of three sample of reduced Fg. Gels were stained with Coomassie blue and scanned at 265 nm. (A) Fg fraction 1a, (B) Fg fraction 1b and (C) Fg fraction 2b

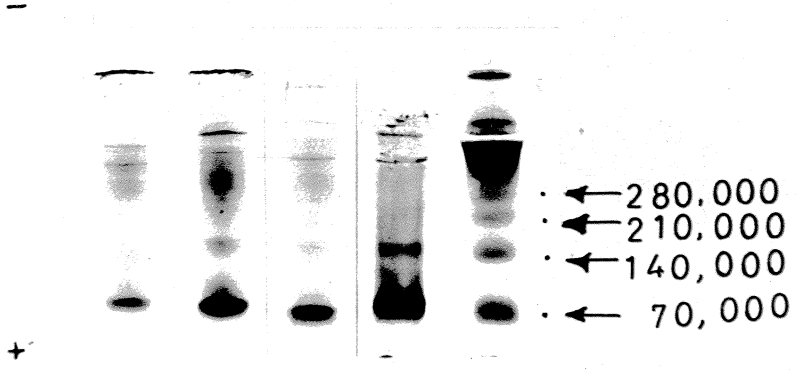



Fig 4-3. PAGE (7%) of desorbed proteins from PC stained with Coomassie blue. Mixture of Proteins containing 25 mg% albumin, 15 mg% γ- globulin and 7.5 mg% whole fibrinogen (A) or Fg fraction 1a (B) or Fg fraction 1b (C) or Fg fraction 2b (D) exposed to PC and Molecular weight marker proteins (E) respectively

TABLE 4-II

**EFFECTS OF DIFFERENT FIBRINOGEN FRACTIONS IN PLATELET ADHESION TO POLYCARBONATE WITH NORMAL AND ADP STIMULATED CALF PLATELETS**

| Fibrinogen Fraction* | Mean ‡platelets ± SD | Fibrinogen+ fraction in presence of ADP | Mean ‡platelets ± SD |
|---|---|---|---|
| 1. Bare PC | 18.0 ± 2.0 | PC+5 μM ADP | 22.2 ± 3.0 |
| 2. PC+Fraction 1a(100μg/ml) | 39.3 ± 4.1 | PC+100 μg/ml Fr:1a+5μM ADP | 47.6 ± 4.7 |
| 3. PC+Fraction 1b(100μg/ml | 37.7 ± 3.8 | PC+100 μg/ml Fr:1b+5 μM ADP | 46.8 ± 3.4 |
| 4. PC+Fraction 2b(100μg/ml | 25.2 ± 3.1 | PC+100 μg/ml Fr:2b+5 μM ADP | 34.9 ± 3.2 |

* - Platelet suspension containing Fibrinogen fractions exposed to polycarbonate (PC)

\+ - and the same exposed in presence of ADP to PC and

‡ - Values expressed as the average of the number of platelets adhered per $mm^2$ to the surface with standard deviation (at least 25 observations from duplicate experiments).

inhibition of platelet-surface attachment due to γ chains in Fg molecule. Thus an extension in the Fg γ-chain has demonstrated a different behaviour with platelets. It has been indicated[221] that γ-chain of Fg molecule is also involved for binding the molecule with platelets. Recently Kloczewiak et al[181] have further localized a γ-chain binding site by demonstrating that Fg binding,to ADP stimulated platelets is inhibited by a peptide corresponding to the sequence of the COOH-terminal 15 residues in the normal γ-chain. So, the reduced platelet-surface or Fg-surface binding in presence of γ'chain Fg may be due to unavailability of binding site on this molecule; or because of a conformational change in the COOH-terminus of the γ'chain or γ' chain could cause a change in Fg conformation itself as proposed earlier[131].

It is also possible to suggest from the present protein adsorption studies that the γ'chain enriched Fg molecule is less effective in binding to an artificial surface, in comparison with normal composition. This may have caused the increase in surface albumin binding to the available free sites on the substrate. This itself may be one of the parameters for a reduced platelet-surface binding as well; since albuminated substrates adhere less platelets[209,257]. So it seems, the slight modulation in the fibrinogen molecule itself can cause drastic changes in surface-protein/platelet interactions at the interface. Further studies are needed to elucidate the structural or conformational contributions to the observed reduction of γ'Fg to surface/platelets and the clinical significance of this fragment in normal blood, though negligible amount is present.

## 4.2. Fibrinogen polymer interaction - changes with platelet inducing agents and proteases

The initially adsorbed Fg to an artifical substrate appears to be supplanted with high molecular weight kininogen or factor XII, and it is also possible to degrade the Fg molecule due to surface induced fibrinolysis, as discussed in Chapter III. Thus it seems, platelet inducers and proteases have definite role to play at the interface and it may be interesting to investigate the effect of them towards Fg binding to an artificial surface and/or to platelets. This may provide an insight to the nature of the hydrolytic Fg fragments, and their role to modulate the Fg-surface/Fg-platelet interactions, leading to the fundamental understanding of the haemostatic mechanism. Hence, the effect of three proteases, plasmin, trypsin and thrombin on Fg molecule, (220 mg% Fg used for these studies, which is closest to normal plasma concentration[371]) and the interaction of the resultant products, fibrinogen degradation products (FDP) or fibrin(ogen); with an artificial surface and subsequently with washed platelets have been investigated using labelled Fg and SDS-PAGE. Further studies related to the modulation of platelet-surface interaction by these proteolytic products of Fg have also been attempted.

The platelet inducing agents, ADP, thrombin and epinephrine demonstrated an enhanced platelet-surface binding as is evident from Table.4-III. It seems, human platelets possess membrane receptors for a wide variety of materials. These include (a) agents involved in physiologic platelet activators such as ADP[224], thrombin[26,106], collagen and fibrinogen[219], (b) hormones such as epinephrine[25], and (c)

pathologic or pharmacologic agents including endotoxin and heparin. Thus it appears that, these inducing agents may activate the platelets and the activated platelets may adhere more to the substrate, or the platelet aggregates formed may have more affinity towards the substrate.

The effect of platelet inducing agents, ADP, thrombin and epinephrine towards Fg surface interaction has been demonstrated in Fig.4-4 from a protein mixture. Thrombin infused system shows an enhanced surface radioactivity, which may be due to an increase in fibrin monomer deposition formed from fibrinogen. ADP and epinephrine do not modify the Fg-substrate interaction; however, adenosine (a platelet adenyl cyclase activator and phosphodiesterase inhibitor) reduces the surface Fg binding. The desorption kinetics of 3 hours preadsorbed Fg in presence or absence of these agents are depicted in Fig.4-5, which indicates an initial desorption of about 30-40% in all cases and reaches to an equilibrium state with time.

Concentration effect of plasmin and trypsin towards Fg/FDP adsorption to PC is demonstrated in Fig.4-6. Fibrinogen/FDP binding to surface is dramatically reduced by very low concentrations of plasmin or trypsin, but higher concentrations do not show significant effect. On the other hand, the amount of fibrin(ogen) adsorbed to PC has increased with thrombin concentration in the medium as shown in Fig. 4.7. It is obvious from Table 4-IV, that Fg induced platelet density on the surface is considerably inhibited by trypsin, but thrombin system has enhanced the effect.

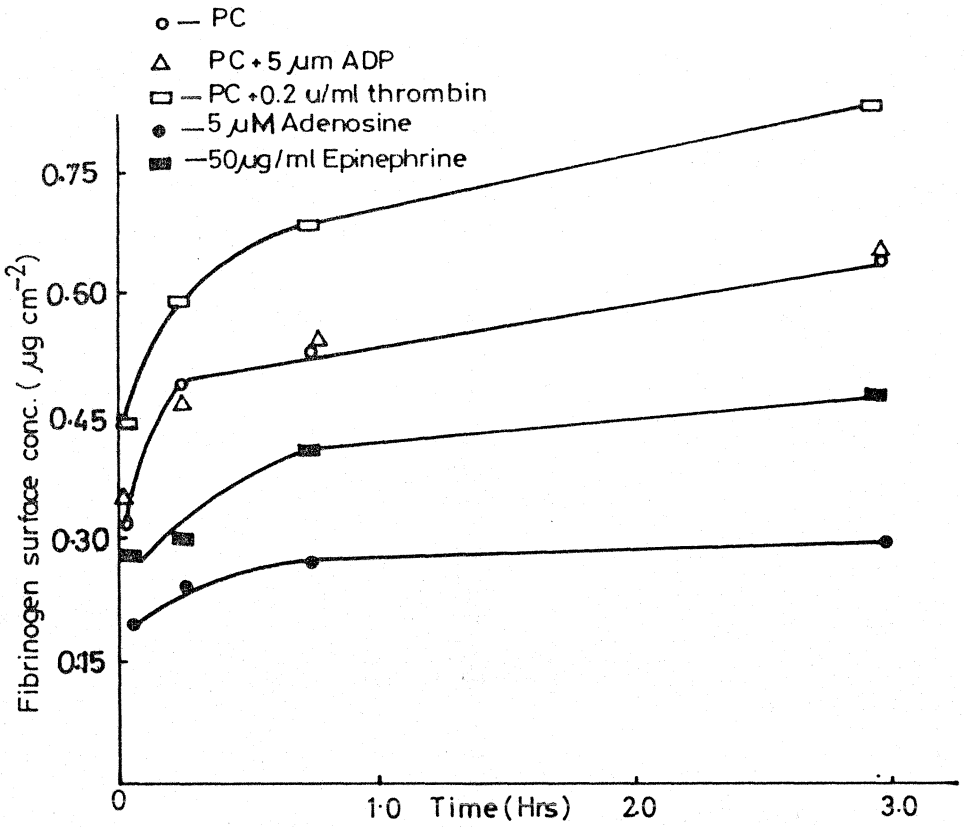



Fig. 4-4. Adsorption of fibrinogen as a function of time from protein mixture with platelet inducing agents & adenosine.

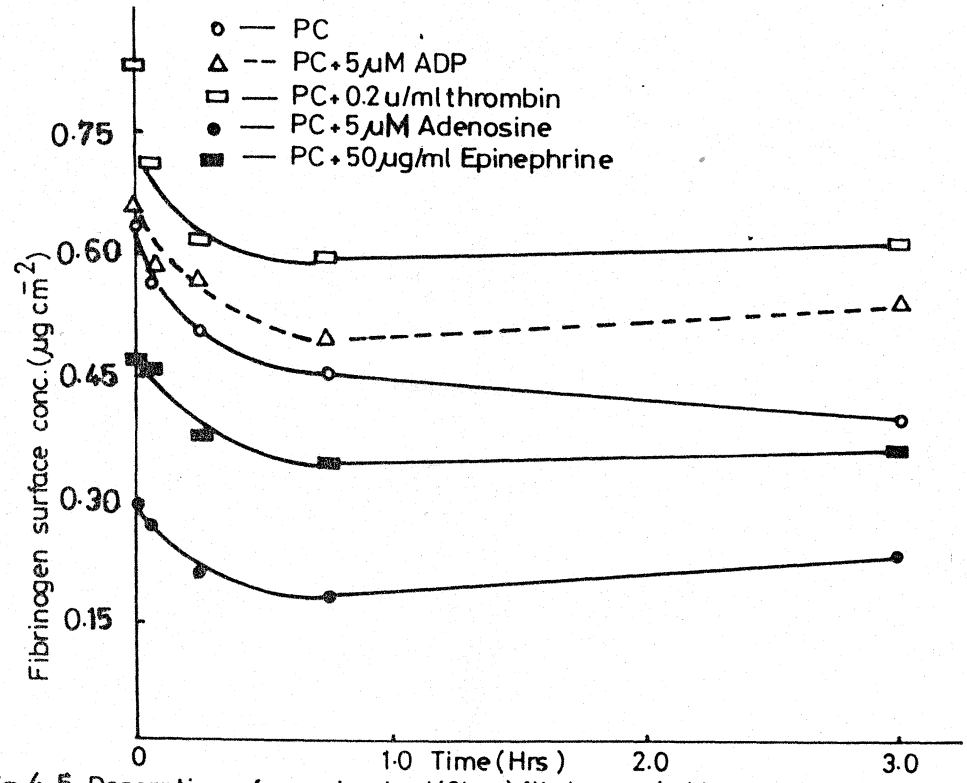



Fig. 4-5. Desorption of preadsorbed (3hrs) fibrinogen (with & without platelet inducing agents & adenosine) to protein mixture.

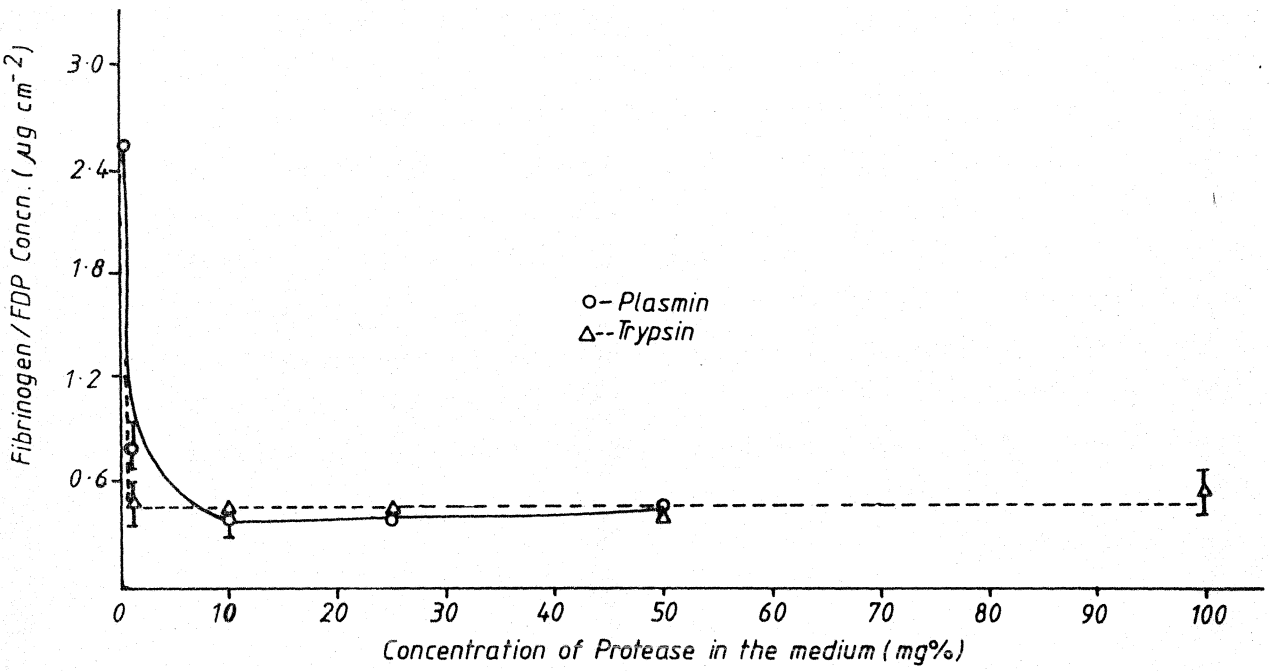



Fig.4-6. Effect of plasmin and trypsin on fibrinogen/FDP adsorption from 220 mg% Fg to polycarbonate

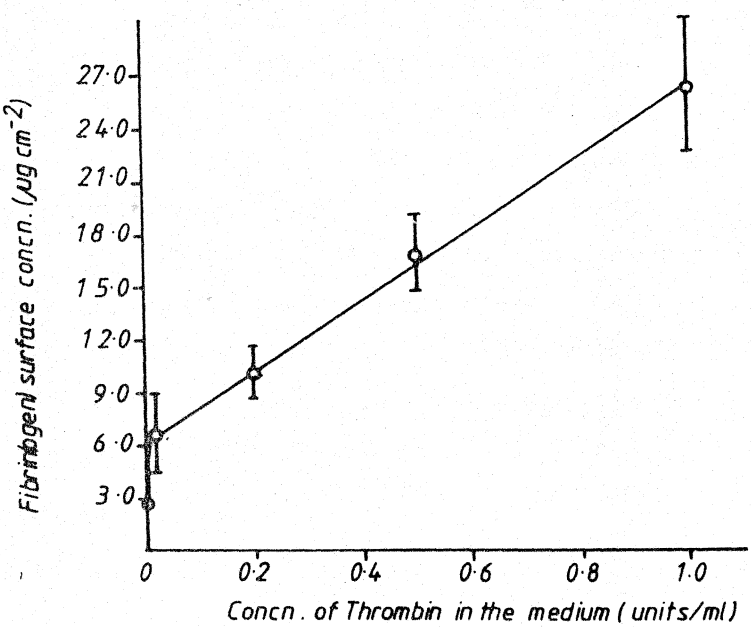



Fig.4-7. Effect of Thrombin on fibrin(ogen) adsorption from 220 mg% Fg to Polycarbonate. Error limits are standard deviations from three experiments

Figures 4-8 and 4-9 indicate the patterns of Fg/FDP adsorption from Fg in presence of plasmin, trypsin and platelets to PC substrates. This clearly indicates a plateau of the isotherm, (i.e, the maximum possible FDP concentration) reaches within 45minutes and attains an equilibrium surface concentration. Fig. 4-8 also suggests that plasmin and trypsin have dramatically reduced the surface concentration of the hydrolytic products, but with platelets and these proteases,an initial enhanced Fg/FDP surface binding is observed as shown in Fig. 4-9. A part of the preadsorbed molecules are also desorbed off from the substrate (about 20-40%) as is evident from figures 4-10 and 4-11.

Thrombin enhances the surface bound fibrin, which has been extended with platelet-thrombin system as depicted in Fig. 4-12. The maximum fibrin-surface concentration reaches within one hour and gradually reduces with prolonged incubation time, which may be due to the detachment of the preadsorbed fibrin molecule. The Fig.4-12 also demonstrates that a part of the preadsorbed Fg/fibrin is desorbed off from the surface. This may be due to the partial removal or exchange of surface bound fibrin(ogen) with unlabelled species in the medium.The preadsorbed fibrin; formed in the presence of thrombin and platelets with Fg; are not removed to the extent as in the case of platelets free system; which may be due to the cumulative effect of the fibrin bound platelets adhering to the surface, compared to fibrin surface binding.

Concentration effect of trypsin and thrombin towards fibrin(ogen)/FDP adsorption and platelet adhesion to

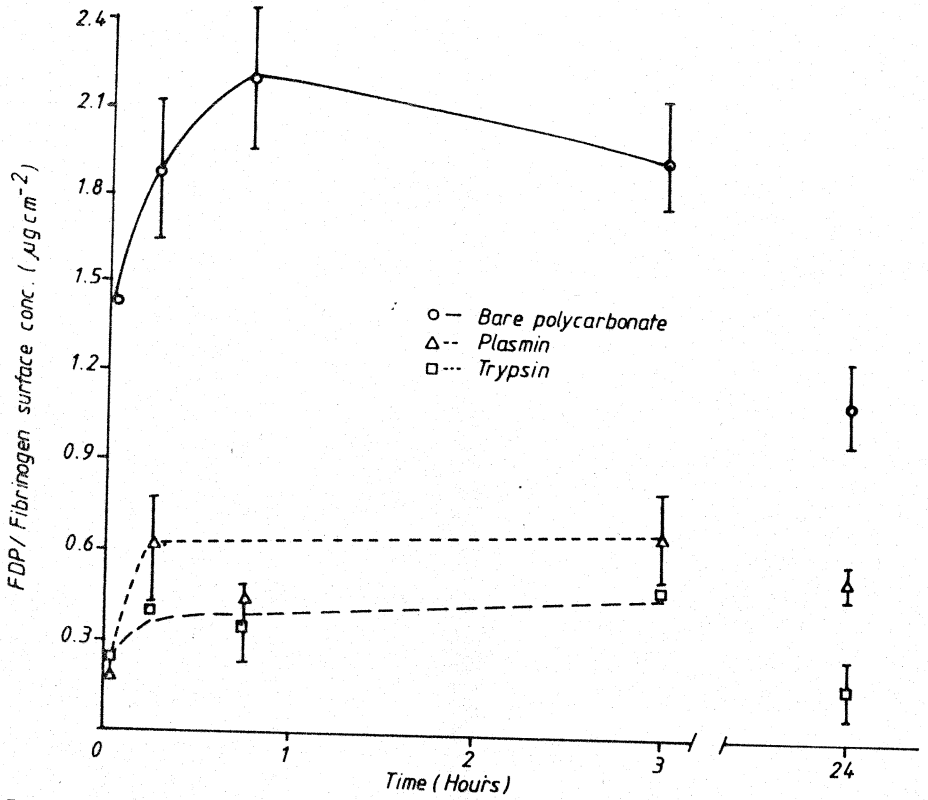



Fig.4-8. Adsorption of fibrinogen/FDP on Polycarbonate as a function of time from 220 mg% Fg & in presence of proteases. Error limits are standard deviations from 3 experiments.

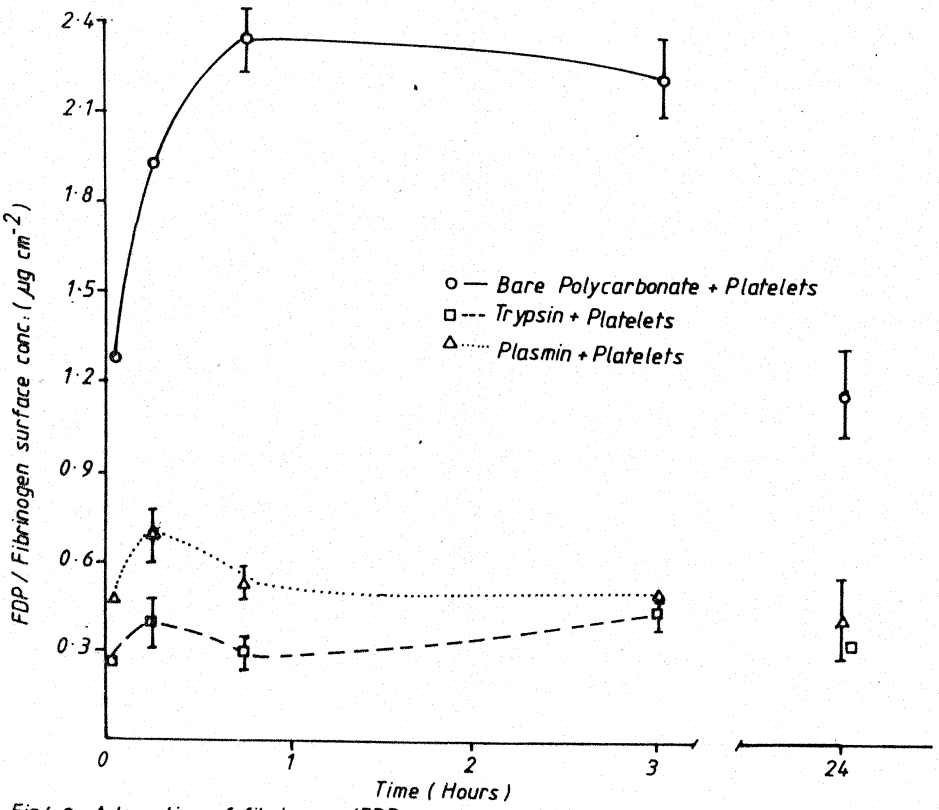



Fig.4-9. Adsorption of fibrinogen /FDP on polycarbonate as a function of time from 220 mg% Fg & in presence of protease and platelets. Error limits are standard deviations from three experiments

PC is depicted in the Table 4-IV. This provides the inter-relation between surface fibrin(ogen)/FDP concentration and subsequent adhering platelets to them. It has been suggested[352,357] that platelets, at least in vitro, adhere preferentially where surfaces have adsorbed Fg. The fibrinolytic effects and proteolytic action of plasmin and trypsin are well studied[105,229]. Thus from protein and platelet binding studies it is evident that the observed reduction in surface radioactivity with trypsin or plasmin system may be due to the fibrinolysis of preadsorbed Fg or less FDP (formed in solution) may be getting adsorbed to the substrate. However fibrin formed as a result of thrombin action on Fg, has drastically deposited to PC surface, causing an enhanced surface level of it. It has been indicated that fibrin fibrils can augment the platelet aggregation[320], whereas FDP can inhibit platelet aggregation[321]. Hence present results agree with the surface fibrin(ogen)/FDP concentration and platelet adhesion.

Figures 4-13 and 4-14 demonstrate, the SDS-Polyacrylamide gel electrophoresis (reduced) of fibrinogen, trypsin-Fg system, and thrombin-Fg system, after elution from PC substrate (3 hours incubated to PC) and from their bulk solution respectively. $A\alpha$, $B\beta$ and $\gamma$-chains of fibrinogen bands are obtained on PC substrate eluted case (Fig.4-13A), along with their degradation products in their solution form (Fig.4-14A). Trypsin treated Fg system shows only one band in both cases (Fig.4-13B and Fig.4-14B), but more dense in their solution form. In case of thrombin-treated Fg system, the fibrinopeptide is more adsorbed to the PC substrate (Fig. 4-13C), though $A\alpha$, $B\beta$, $\gamma$ bands and degradation products (Fibrinopeptides A and B) are also seen in solution.

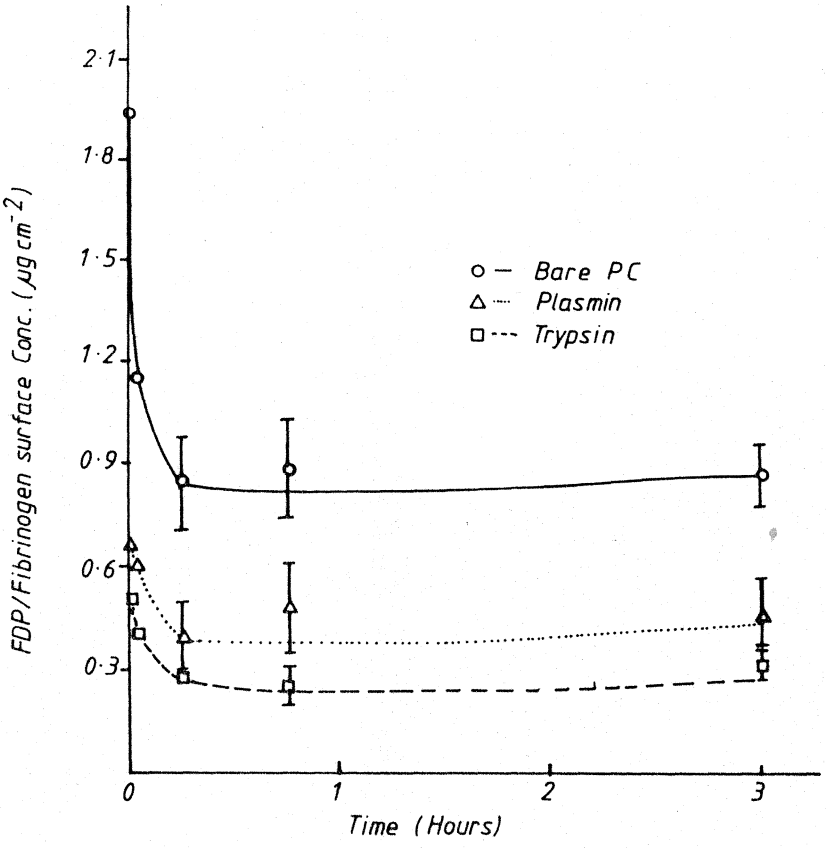



Fig.4-10. Desorption of fibrinogen from PC as a function of time. Error limits are standard deviations from three experiments

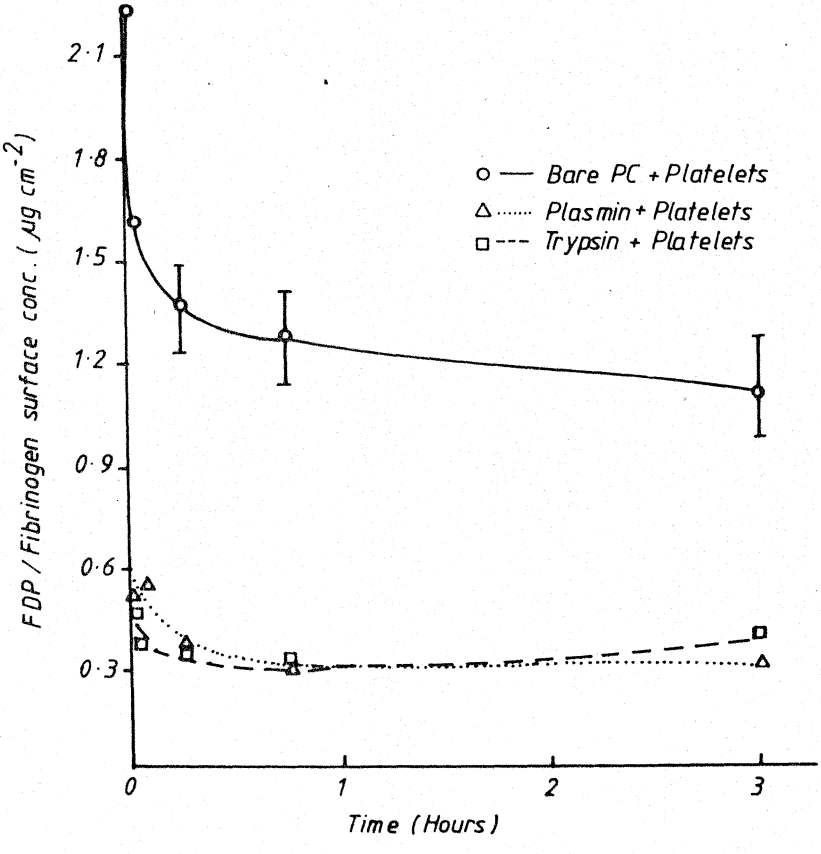



Fig.4-11. Desorption of fibrinogen on PC as a function of time. Error limits are standard deviations from three experiments

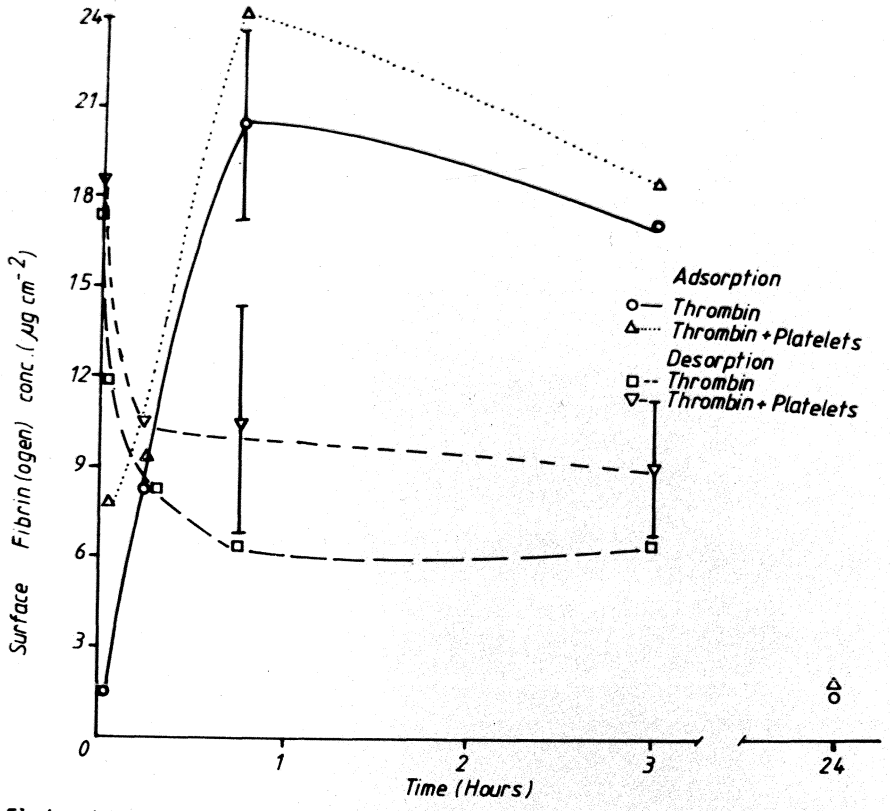



Fig:4-12. Adsorption & Desorption of fibrin(ogen) on PC as a function of time from 220 mg% Fg & in presence of thrombin & platelets. Error limits are standard deviations from three experiments

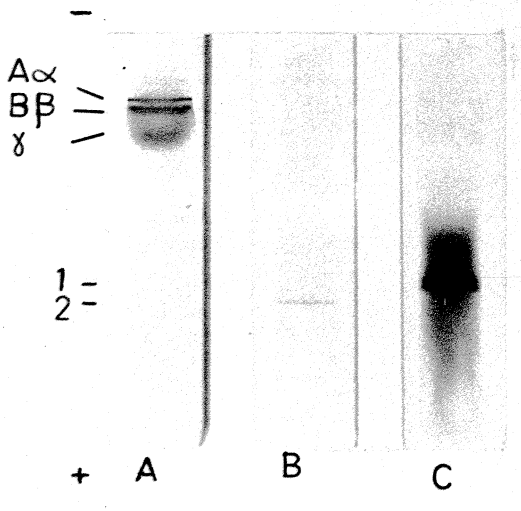



Fig. 4-13. SDS-PAGE (reduced) of fibrinogen (A), trypsin-Fg system (B), and thrombin-Fg system (C), after elution from PC substrate (3 hours incubated to PC)

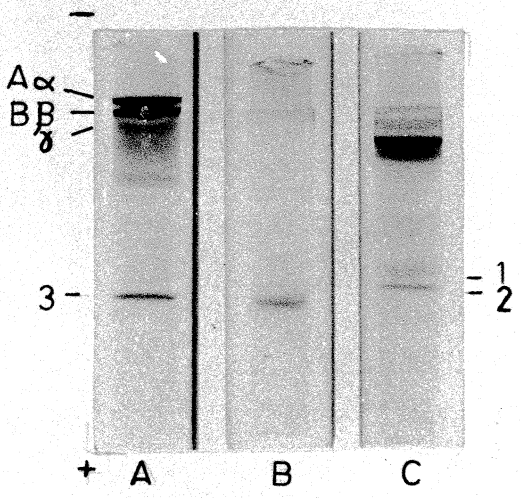



Fig: 4-14. The same in their bulk so (see results and discussion for de 1,2 and 3 various Fg degradation products.

TABLE-4-III

**CONCENTRATION EFFECT OF TRYPSIN & THROMBIN ON FIBRIN(OGEN)/ FDP ADSORPTION AND PLATELET ADHESION TOWARDS POLYCARBONATE**

| | Surfaces* | Mean platelets+ ±SD | ‡Fibrin(ogen)/ FDP surface conc. ($\mu g\ cm^{-2}$) |
|---|---|---|---|
| 1. | Bare polycarbonate (PC) | 18.0 ± 1.9 | - |
| 2. | PC+220 mg%Fibrinogen (Fg) | 43.0 ± 1.0 | 2.52 ± 0.57 |
| 3. | PC+5 μM ADP | 28.75 ± 3.75 | - |
| 4. | PC+50μg/ml Epinephrine | 24.37 ± 4.75 | - |
| 5. | PC+0.2u/ml Thrombin | 37.12 ± 3.75 | - |
| 6. | PC+Fg+0.1 mg% Trypsin | 40.2 ± 3.8 | 0.46 ± 0.1 |
| 7. | PC+Fg+1 mg% Trypsin | 38.4 ± 3.5 | 0.43 ± 0.2 |
| 8. | PC+Fg+10 mg% Trypsin | 35.2 ± 3.7 | 0.40 ± 0.13 |
| 9. | PC+Fg+25 mg% Trypsin | 30.7 ± 4.6 | 0.37 ± 0.045 |
| 10. | PC+Fg+50 mg% Trypsin | 28.4 ± 5.2 | 0.52 ± 0.09 |
| 11. | PC+Fg+0.02u/ml Thrombin | 48.8 ± 4.4 | 6.46 ± 2.2 |
| 12. | PC+Fg+0.2u/ml Thrombin | 49.6 ± 3.8 | 10.11 ± 1.35 |
| 13. | PC+Fg+0.5u/ml Thrombin | 55.0 ± 6.0 | 16.83 ± 2.34 |
| 14. | PC+Fg+1u/ml Thrombin | 55.2 ± 6.0 | 26.22 ± 3.88 |

* - Platelet suspension containing fibrinogen, trypsin, thrombin and other platelet inducing agents were exposed to PC (as demonstrated in surfaces 1 to 14).

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation. (at least 30 observations from duplicate experiments)

‡ - Surface concentration of fibrin (ogen)/FDP in $\mu g\ cm^{-2}$, when PC substrate was incubated with 220 mg% Fg and proteases system for 3 hours at 37°C.

Brash et al[45,47] have also studied the plasmin effect on fibrinogen molecule on glass bead columns and have shown that plasmin degraded Fg fragments are adsorbed to the substrate.

These Fg-protease interaction studies towards an artificial surface clearly indicate that (a) the initial events of protease-Fg modification or Fg-platelet or Fg-surface binding is important and (b) all the proteases do not behave similarly towards Fg-surface, Fg-platelet interaction and ultimately, the surface initiated thrombosis may also be different. Hence, it seems that several populations of molecules are formed in solution as a result of protease action, but the nature of the products adsorbed to the substrate may vary with surface properties and the extent of protease activation by the substrate, resulting in changes in subsequent thrombotic process at the interface.

## 4.3. The effect of plasma fibronectin on protein/platelet interaction to polymer substrates

Fibronectin (Fn) is a glycoprotein present in plasma and in extracellular matrices, which seems to participate in cell adhesion and in the formation of haemostatic plug. Thus the influence of this trace component of plasma towards platelet adhesion to an artificial surface with normal and thrombin stimulated platelets have been investigated. This also provides information related to the role of Fn to modulate the kinetics of polymer-Fg/fibrin interaction in presence and absence of thrombin.

### 4.3.1. Role of fibronectin on platelet adhesion to PC substrate

The role of Fn to modulate the platelet adhesion to PC substrate in presence of platelet inducing agents are depicted in Table-4-IV. Addition to PC, of platelet suspended in tyrode solution containing fibronectin, has been slightly higher than that observed for platelets in the absence of this molecule (Table-4-IV). Thrombin induced platelet density on the surface has considerably increased compared to ADP and epinephrine, however in presence of ADP and Fg the surface bound platelets have been dramatically enhanced. The concentration effect of Fn on platelet adhesion to PC surface in presence and absence of 0.2u/ml thrombin is indicated in Fig.4-15. The addition of Fn to tyrode suspended platelets do not, significantly, modify the initial surface-platelet binding, whereas, incubation of thrombin with platelets causes a substantial increase in platelet adhesion.

Thrombin induced platelet adhesion to PC is concentration dependent, and the infusion of 350$\mu$g/ml Fn to the thrombin-platelet suspension system have demonstrated (Fig.4-16) a profound platelet surface binding. 350 $\mu$g/ml fibronectin exposed to PC surface for varying time intervals (1 mt to 24 hrs) have also demonstrated a slight increase in surface-platelet attachment, compared to bare PC substrate as indicated in Table 4-V.

The behaviour of Fn towards platelet-collagen interaction has shown contrasting results. In fact, several authors,[89,288] using different experimental approaches, have

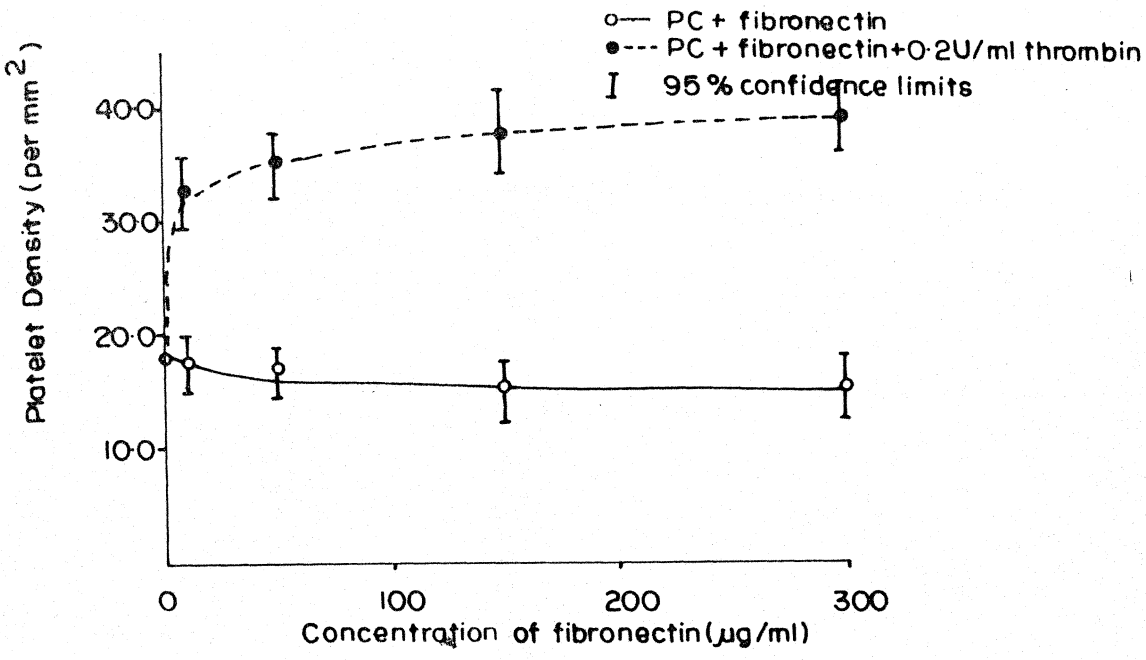
TABLE-4-IV

**EFFECT OF FIBRONECTIN ON PLATELET ADHESION IN PRESENCE OF VARIOUS INDUCING AGENTS**

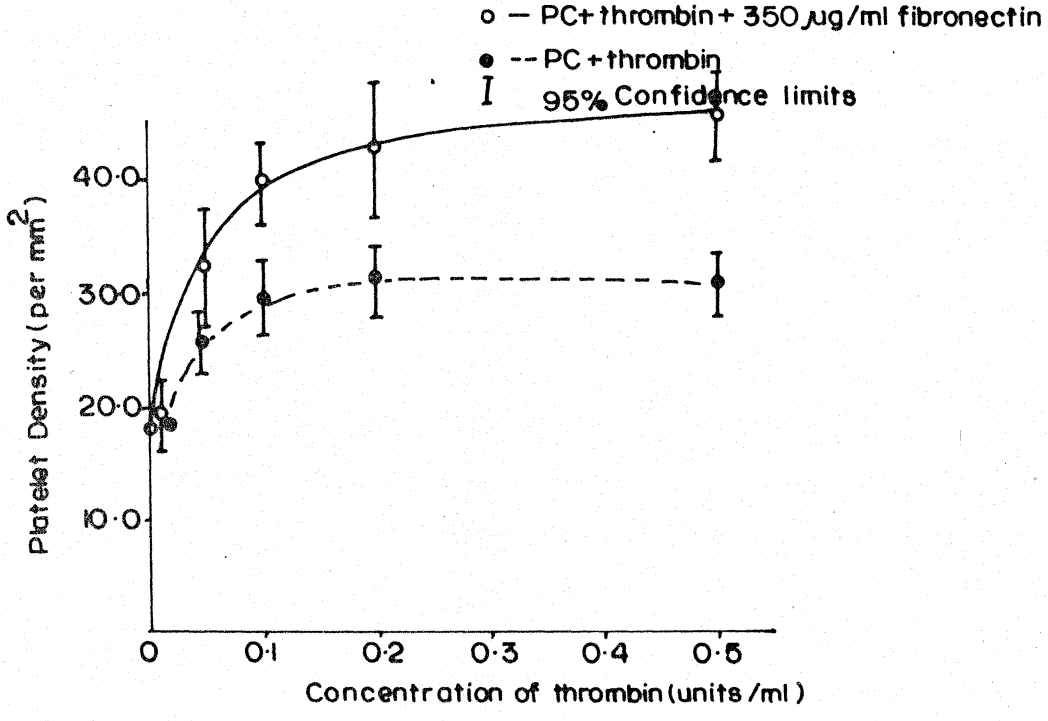
| Surfaces* | Mean platelets±SD+ |
|---|---|
| 1. Bare Polycarbonate (PC) | 17.8 ± 2.0 |
| 2. PC+350 μg/ml Fn | 19.5 ± 2.5 |
| 3. PC+5 μM ADP | 22.2 ± 3.0 |
| 4. PC+5 μM ADP+350 μg/ml Fn | 25.5 ± 2.7 |
| 5. PC+5 μM ADP+30 mg% Fg | 33.5 ± 3.2 |
| 6. PC+5 μM ADP+30 mg% Fg+350μg/ml Fn | 42.1 ± 3.5 |
| 7. PC+0.2u/ml Thrombin | 31.2 ± 2.7 |
| 8. PC+0.2u/ml Thrombin+350 μg/ml Fn | 42.6 ± 5.2 |
| 9. PC+50 μg/ml Epinephrine | 25.5 ± 2.8 |
| 10. PC+50 μg/ml Epinephrine+350 μg/ml Fn | 35.0 ± 2.8 |

* - Platelet suspension containing fibronectin (Fn) in presence and absence of inducing agents exposed to PC (as shown in surfaces 1 to 10).

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 25 observations from duplicate experiments).




Fig·4-15. Platelet adhesion to PC surface and the effect of thrombin with varying concentrations of fibronectin.




Fig·4-16. Platelet adhesion to PC surface and the effect of fibronectin with varying cons. of thrombin

TABLE-4-V

**PLATELET ADHESION TO FIBRONECTIN ADSORBED PC SURFACES**

| Time of Fibronectin exposure to PC * | Mean platelets±SD+ |
|---|---|
| 1. Bare polycarbonate (PC) FIBRONECTIN 350 $\mu$g/ml. | 17.8 ± 2.0 |
| 2. 1 mt. exposed PC | 19.9 ± 2.4 |
| 3. 15 mts. exposed PC | 23.8 ± 2.6 |
| 4. 45 mts exposed PC | 27.8 ± 3.4 |
| 5. 3 hrs exposed PC | 27.2 ± 2.7 |
| 6. 24 hrs exposed PC | 28.7 ± 3.4 |

* - Tyrode solution washed platelets exposed to fibronectin preadsorbed PC surfaces.

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 30 observations from duplicate experiments).

come to the conclusion that Fn significantly modify the extent of platelet-collagen binding, while others[18,318] support the opinion that this molecule does not play any role in platelet adhesion. However, it has been suggested that Fn has an acceptor site for blood coagulation factor XIIa [241], and can be crosslinked to collagen[89], fibrin[155] and to cell surfaces [265]. All these properties propose a possible role for Fn towards protein-surface, protein-thrombus and/or protein-platelet interactions.

It has been proposed that platelets bind to Fn through their cell surface fibronectin receptors[265]. Grinnell et al[121] have reported, Fn precoated polystyrene surfaces can promote both platelet attachment and spreading. This correlates with the present observations that Fn precoated PC (Table 4-VI) promotes the platelet attachment, though soluble Fn effect is not so evident. Hence it seems, that soluble Fn may have only a limited role in platelet-polycarbonate binding using tyrode washed platelets, however; activated platelets do significantly promote the platelet adhesion. It is possible that the activated platelets dramatically modify the platelet membrane structure and it's binding capacities. This has been supported by the results indicated elsewhere[265] that the membrane of thrombin stimulated platelets can link a large number of Fn molecules.

The contribution of soluble Fn to enhance platelet-surface attachment with platelet activators may be due to the influence of these stimulants to promote platelet aggregation itself. Further, the mode of behaviour of nonactivated platelets and platelet aggregates (caused by aggregating agents) adhering to an artificial surface is still unclear. However, it is conceivable that platelet aggregation which occurs after adhesion may get magnified by aggregating agents in presence of Fn or that aggregates adhere more to the substrate than nonactivated platelets.

### 4.3.2. Effect of fibronectin towards protein adsorption to PC substrate from protein mixture

The patterns of albumin adsorption and desorption from protein mixture to PC substrate, and changes with 350

μg/ml Fn infusion to the medium is indicated in Fig.4-17. Protein mixture (albumin-25 mg%, γ-globulin-15 mg% and fibrinogen-7.5 mg%) containing Fn has reduced the surface albumin binding up to 3 hours of study. A part of the preadsorbed albumin get desorbs off in unlabelled protein mixture. As shown in Table 4-VI, the desorption of albumin has been more when Fn has been included in the desorbing media. For example, the maximum desorption or exchange (for 3 hours) of albumin appears to be 41.91% from PC in the protein mixture, compared to 68% when soluble Fn has also been present in the media.

TABLE-4-VI

**PERCENT DESORBED FROM VARIOUS SUBSTRATES OF 3 HOURS PREADSORBED PROTEINS**

| Time of desorption | Albumin | | | | Fibrinogen | | | |
|---|---|---|---|---|---|---|---|---|
| | PC* | PC+ + Fn | AF‡ | AF§ + Fn | PC* | PC+ + Fn | AF‡ | AF§ + Fn |
| 1 mt. | 19.3 | 16 | 23.8 | 1.0 | 11.6 | 22.6 | 22.8 | 26.5 |
| 15 mts. | 25.8 | 48 | 21.4 | 3.1 | 20.9 | 19.4 | 37.1 | 40.2 |
| 45 mts. | 32.2 | 68 | 11.9 | 18.2 | 25.6 | 29.0 | 48.6 | 50.9 |
| 3 hrs. | 41.9 | 68 | 21.4 | 33.3 | 34.9 | 41.9 | 54.3 | 50.1 |

* - polycarbonate (PC) and

‡ - Angioflex (AF) exposed to protein mixture, (albumin, γ-globulin and fibrinogen ) for 3 hours. The preadsorbed films exposed to protein mixture for desorption, + - PC and §-Angioflex exposed to protein mixture, containing fibronectin for 3 hours, and then their desorption kinetics in protein mixture containing Fn.

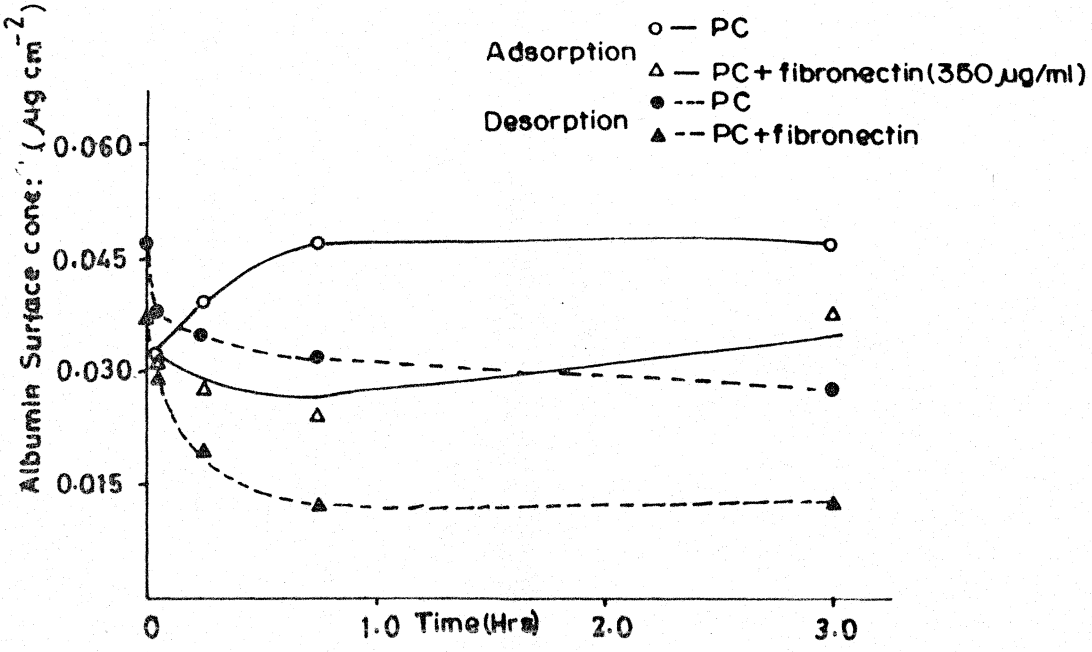



Fig 4-17. Adsorption / Desorption of albumin on PC as a function of time from protein mixture and fibronection

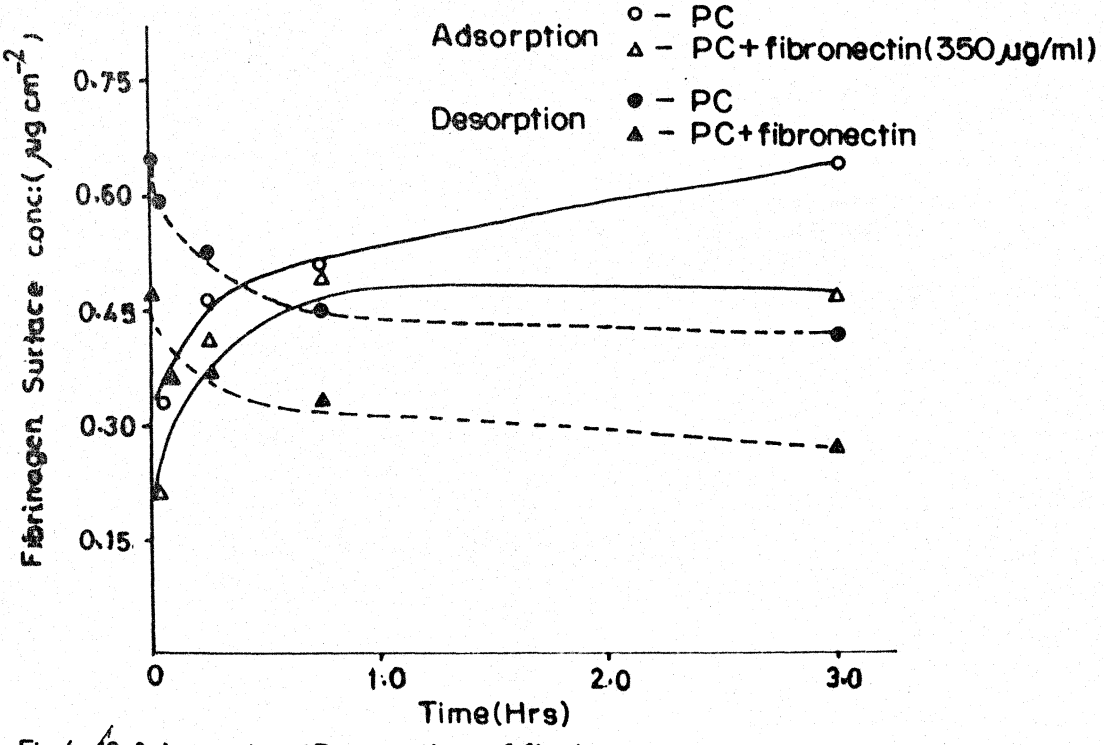



Fig 4-18. Adsorption / Desorption of fibrinogen on polycarbonate as a function of time from protein mixture and fibronectin

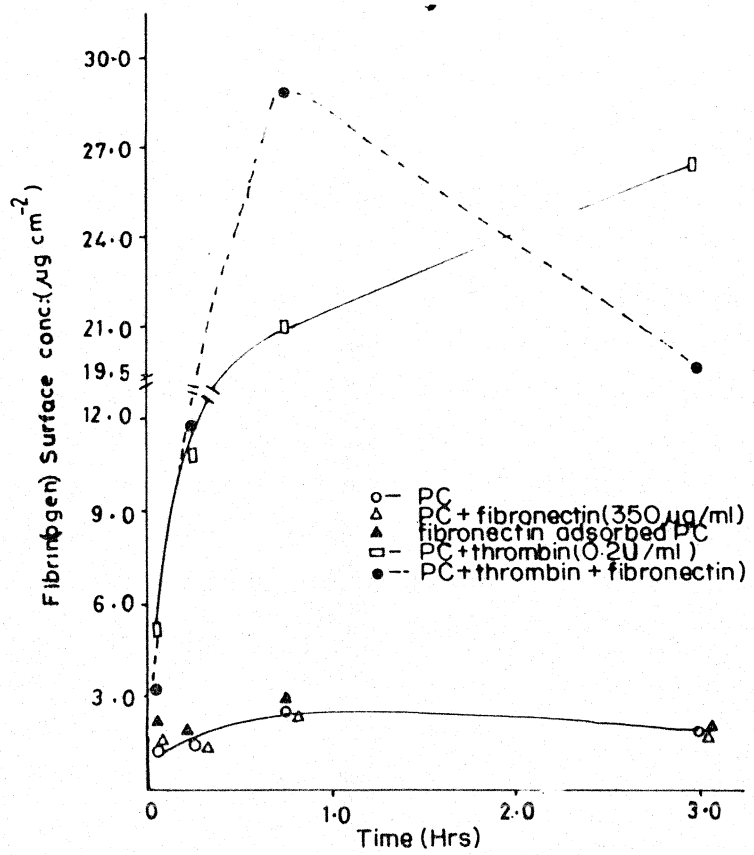



Fig.4-19. Adsorption of fibrin(ogen) on PC as a function of time from 250 mg% fibrinogen.

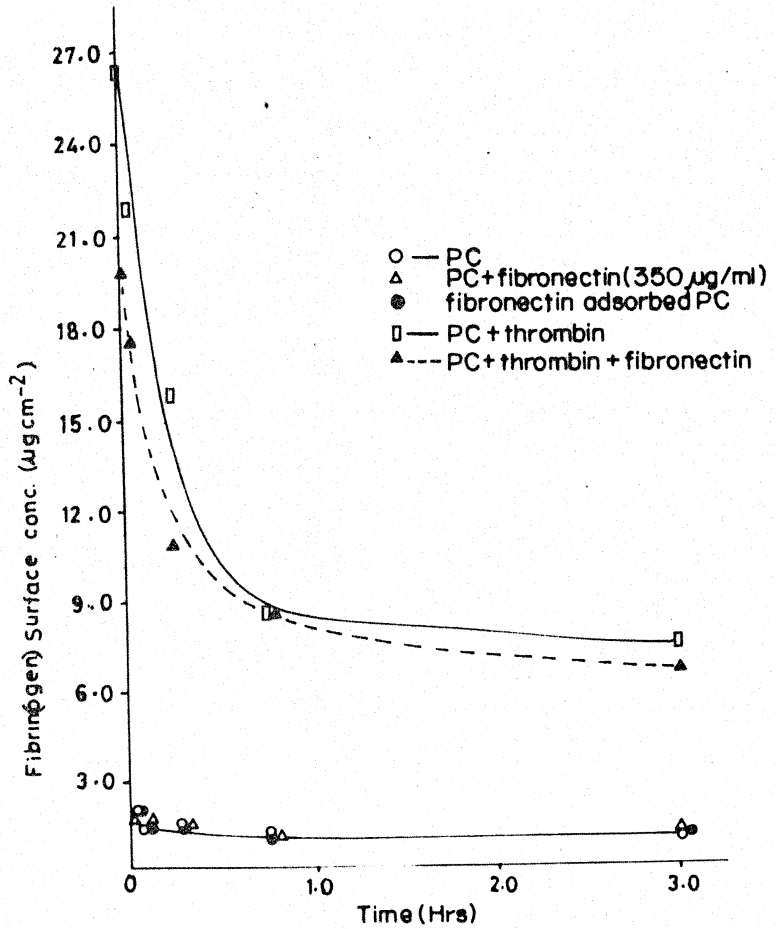



Fig4-20. Desorption of fibrin(ogen) from PC as a function of time in 250 mg% fibrinogen

Kinetics of Fg adsorption to PC substrate from protein mixture with and without Fn is indicated in Fig.4-18. The surface concentration of Fg is also reduced with Fn addition (28% inhibition) to the protein mixture, but a constant Fg level is reached within one hour compared to Fn free system. Figure 4-18 also reveals the rapid exchange of 3 hours preadsorbed Fg from the substrate in the protein mixture. The desorption pattern also suggests a removal (for 3 hours) of 34.9% and 41.9% fibrinogen from PC to protein mixture and Fn included system respectively, as demonstrated in Table.4-VI.

Fibrinogen/Fibrin deposition to PC substrate have been higher initially with thrombin-Fn system($28.8\pm4.2 \mu gcm^{-2}$) from 250 mg% Fg as depicted in Fig.4-18, compared to thrombin (0.2u/ml) -fibrinogen system alone ($20.83\pm4.8 \mu gcm^{-2}$) for 45 mts exposure. However, the preadsorbed fibrin detaches off with time (3 hours period) from Fn included thrombin-fibrinogen system and causes a reduction in surface fibrin(ogen) level. This is not observed with thrombin-fibrinogen case as shown in Fig. 4-19. Young et al[372] have also suggested a similar phenomenon with Fn coated PVC substrate in an ex vivo shunt experiment. It appears that 97% of the predeposited thrombus (both platelets and fibrinogen) sheds away from Fn-coated PVC substrate, after 120 minutes of blood contact.

The desorption pattern of 3 hours preadsorbed fibrin(ogen) from PC, in presence of thrombin and Fn, is demonstrated in Fig.4-20, which indicates a tremendous detachment of fibrin from the PC substrate in both cases.

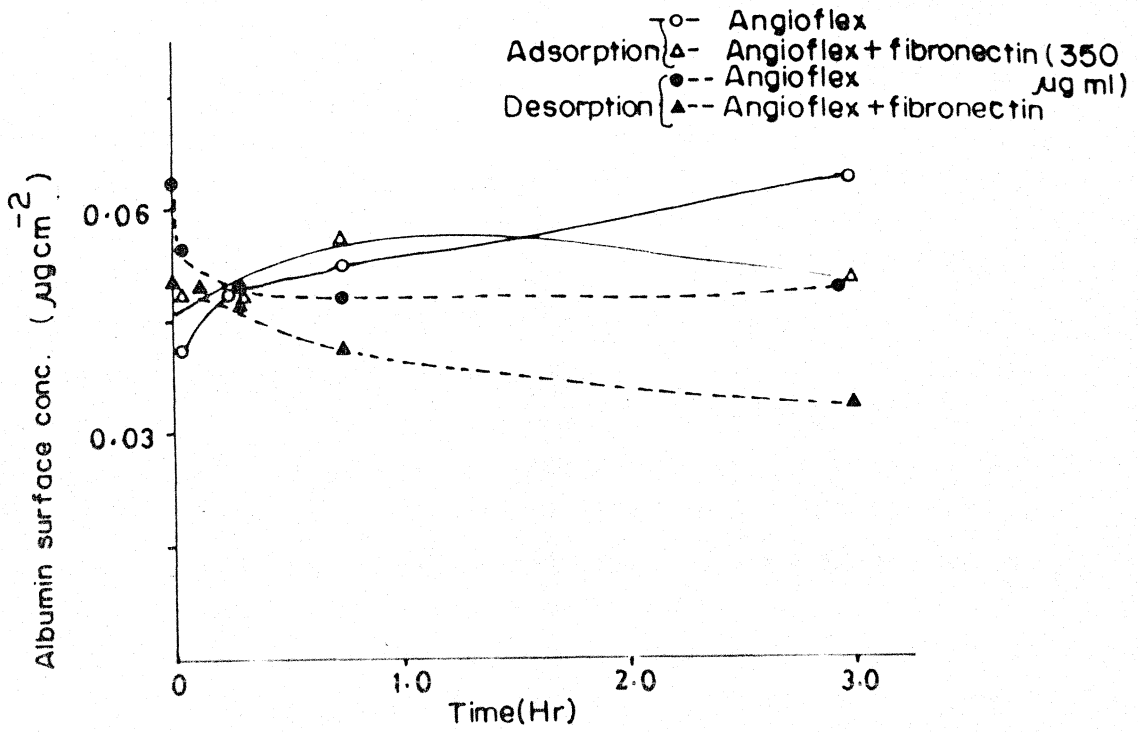



Fig. 4-21 Adsorption / Desorption of albumin on Angioflex as a function of time from Protein mixture and fibronectin

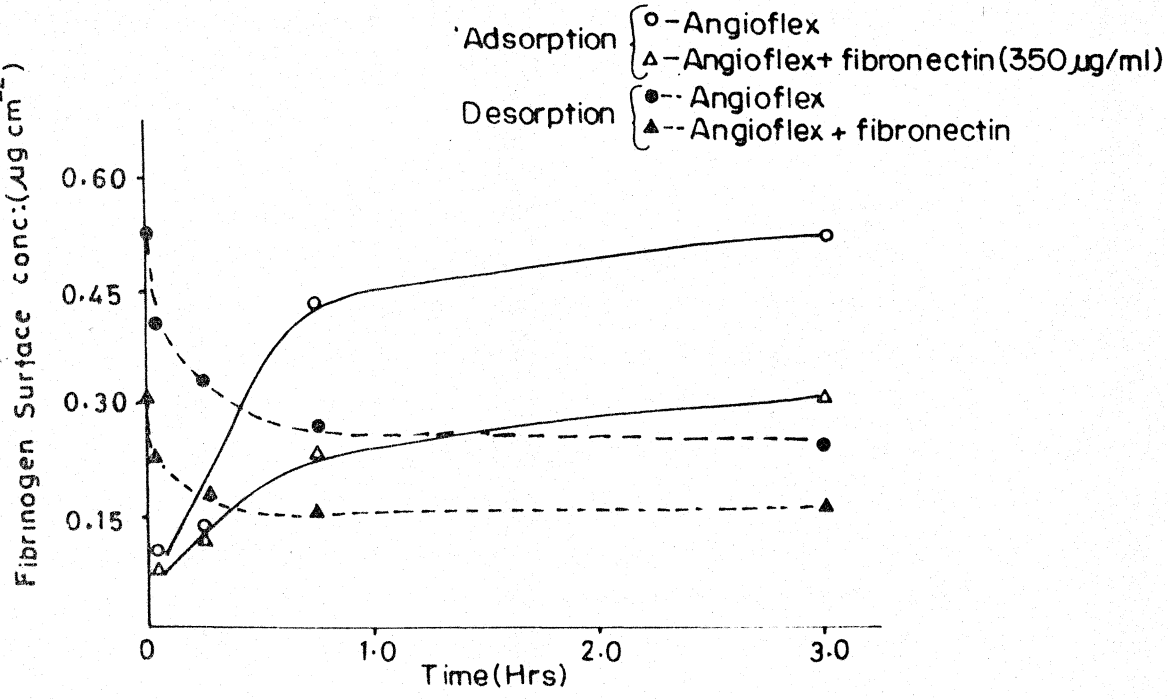



Fig. 4-22 Adsorption/Desorption of fibrinogen on Angioflex as a function of time from protein mixture and fibronectin

The patterns of albumin and fibrinogen adsorption/desorption as a function of time to Angiflex substrate and changes with 350 $\mu$g/ml Fn infusion to the medium is demonstrated in figures 4-21 and 4-22 respectively. Albumin adsorption to Angioflex does not show any changes initially with Fn addition, but has reduced slowly up to 3 hours of incubation. The desorption pattern (Fig.4-21) of preadsorbed albumin from Angioflex to protein mixture has been similar to that of PC substrate. The initial desorption in Fn included system is very less, but with time (3 hrs.) it removes 33.3% of preadsorbed albumin in comparison to 21.4% for Fn excluded case as shown in Table-4-VI.

Fibrinogen-Angioflex binding has been substantially modified by infusion of Fn to the protein mixture as indicated in Fig.4-22, in comparison to PC substrate. Fn inhibits 41% of Fg binding to Angioflex, which may be due to it's competition for the adsorption sites. But the desorption demonstrates a similar pattern to that of PC substrate.

These studies suggest the importance of protein turnover for the blood-material interface problem, in that, it provides a mechanism whereby the composition of the adsorbed protein layer, known to be important for platelet-surface interactions; can change with time. It also seems that the extent of protein adsorption is not a simple function of protein concentration when surfaces are exposed to complex protein mixtures. So it appears[120] that protein adsorption patterns are likely to be even more complex when chemical interactions (eg. blood clotting) occur on the surface.

It is concluded that Fn significantly modifies the platelet-polymer, protein-polymer interactions, when thrombin is present in the medium. Therefore, it seems, the role of Fn in platelet/ protein and surface interaction becomes fundamental, once the haemostatic process has been initiated. The physiological significance of these observations still remains to be determined.

# FIBRINOGEN/PLATELET INTERACTION WITH ARTIFICIAL SUBSTRATES: EFFECT OF CERTAIN VITAMINS, ANTI-PLATELET DRUGS AND ANTIBIOTICS

# CHAPTER - V

## 5.1. Effect of vitamins and antiplatelet drugs towards platelet adhesion

The effect of vitamin C to modulate the protein/platelet binding towards an artificial surface has been described in Chapter-I. This provided an insight to go into the depth of such studies with other vitamins and antiplatelet drugs and their behaviour towards an artificial substrate-blood interfaces. Hence an attempt has been made here to investigate the effect of persantine, aspirin-persantine, certain vitamins like vitamin A, $B_6$, C, E and D and their various combinations towards protein adsorption to an artificial surface after desorbing and analyzing these proteins electrophoretically. The kinetics of protein/platelet interactions with various polymer substrates have also been investigated under static and flow conditions to strengthen these observations.

The reduction of platelets adhered to the polymer surface by persantine is demonstrated in Table 5-1. Persantine modification of platelet-polymer interaction varies with concentration and 5 mg% of persantine considerably blocks the platelet adhesion to substrate. A combination of 0.5 mg% aspirin, 2 mg% persantine has demonstrated similar reduction to that of 5 mg% persantine alone. The adhesion of Fg induced platelets to PC is

inhibited by the addition of 2 mg% persantine; which is further reduced with aspirin-persantine combination as shown in Table-5-1.

TABLE-5-1.

CONCENTRATION EFFECT OF ASPIRIN AND PERSANTINE COMBINATION TOWARDS PLATELET ADHESION

| PER. concentration* | Mean+ platelets ± SD | PER.concentration* +0.5 mg% ASP | Mean+ platelets ± SD |
|---|---|---|---|
| 1. Bare Polycarbonate (PC) | 18.00±2.0 | 10.PC+0.5mg%ASP | 12.00±4.0 |
| 2. PC+0.1mg% PER | 14.70±3.0 | 11.PC+0.5mg%ASP +0.1 mg% PER | 10.80±2.5 |
| 3. PC+0.5mg% PER | 13.00±2.5 | 12.PC+ASP+O.5mg% PER | 9.80±3.0 |
| 4. PC+1.0mg% PER | 11.60±4.0 | 13.PC+ASP+1mg%PER | 8.20±3.5 |
| 5. PC+2.0mg% PER | 10.20±3.0 | 14.PC+ASP+2mg%PER | 7.25±3.2 |
| 6. PC+5.0mg% PER | 7.25±3.0 | 15.PC+ASP+5mg%PER | 6.50±3.0 |
| 7. PC+10.0mg% PER | 7.20±3.3 | 16.PC+ASP+10mg%PER | 6.36±3.0 |
| 8. PC+50mg% Fibrinogen | 38.40±6.0 | 17.PC+50mg% Fg+ASP | 24.60±5.6 |
| 9. PC+Fg+2mg% PER | 19.10±3.8 | 18. PC+Fg+ASP+2mg%PER | 15.5±4.3 |

* - Platelet suspension containing persantine (PER), aspirin (ASP), in presence and absence of fibrinogen (Fg) exposed to PC (as demonstrated in surfaces 1 to 18).

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 30 observations from triplicate experiments; 'p' $\leq$ 0.05).

The adhesion of platelets to PC is inhibited by addition of vitamin C and aspirin-vitamin C combination as depicted in Table-5-II. A combination of 1.5 mg% vitamin C and 0.4 mg% aspirin has demonstrated similar reduction to that of 1.6 mg% aspirin alone. A similar blocking effect to PC surface with Fg induced platelets is observed with 1.6 mg% aspirin and is further decreased with the combination of aspirin-vitamin C. However, from tables 5-I and 5-II it seems that, very low doses of aspirin (0.5 mg%) with persantine combination has drastically inhibited the surface-platelet attachment, which is not attainable to the same extent with aspirin-vitamin C concentration alone.

It is known recently that aspirin inhibits the platelet release reaction induced by ADP and collagen in vitro[360]. Additionally aspirin prolongs the bleeding time[142]. Therefore, it may be expected that aspirin can exert an antithrombotic effect in those clinical situations where platelets can play a significant role in the pathogenesis of thrombosis. It has been suggested[126,231] that both platelets and endothelial cells have the capacity to generate prostaglandin products, (thromboxane-$A_2$, a potent platelet aggregating agent in platelets and $PGI_2$, a potent antiplatelet agent in vessel wall) via the arachidonic acid pathway by the action of the enzyme, cyclo-oxygenase. Aspirin acts by acetylating a serine residue at the active site of the cyclo-oxygenase, consequently the inhibition caused by aspirin in this case is irreversible[168].

However, the platelet cyclo-oxygenase has been found to be more sensitive to aspirin than the endothelial cyclo-oxygenase and the endothelial cyclo-oxygenase activity

TABLE-5-II

## CONCENTRATION EFFECT OF ASPIRIN & VITAMIN-C COMBINATION TOWARDS PLATELET ADHESION

| ASP.concentration* | Mean+ platelets+ ±SD | ASP. concentration* 1.5 mg% Vit.C | Mean+ platelets ±SD |
|---|---|---|---|
| 1.Bare polycarbonate (PC) | 17.50±2.0 | 11.PC+1.5mg%Vit.C | 13.00±2.25 |
| 2.PC+0.2mg% ASP | 15.00±2.0 | 12.PC+Vit.C+0.2mg% ASP | 11.00±2.00 |
| 3.PC+0.4mg% ASP | 12.20±1.8 | 13.PC+Vit.C+0.4mg% ASP | 8.90±2.00 |
| 4.PC+0.8mg% ASP | 10.75±2.0 | 14.PC+Vit.C+0.8mg% ASP | 8.00±2.00 |
| 5.PC+1.2mg% ASP | 9.40±2.0 | 15.PC+Vit.C+1.2mg% ASP | 7.70±1.95 |
| 6.PC+1.6mg% ASP | 8.90±2.2 | 16.PC+Vit.C+1.6mg% ASP | 8.20±1.90 |
| 7.PC+2.5mg% ASP | 8.75±2.2 | 17.PC+Vit.C+2.5mg% ASP | 7.80±2.30 |
| 8.PC+5mg% ASP | 8.5±2.4 | 18.PC+Vit.C+5mg% ASP | 7.90±2.30 |
| 9.PC+50mg% Fg | 40.8±5.3 | 19.PC+Fg+1.5mg% Vit.C | 26.50±4.20 |
| 10.PC+Fg+1.6mg% ASP | 20.9±2.6 | 20.PC+Fg+Vit.C+ 1.6mg% ASP | 17.70±2.50 |

* - Platelet suspension containing aspirin (ASP), vitamin C (Vit.C),in presence and absence of fibrinogen (Fg) exposed to PC (as demonstrated in surfaces 1 to 20)

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$, with standard deviation (at least 30 observations from duplicate experiments).

can be recovered by removal of aspirin from the system[168]. Buchanan and co-workers[54] have provided recent information to suggest that doses of aspirin, in excess of that required to inhibit thromboxane $A_2$ synthesis, produce alterations of platelet function in vivo and in vitro, which is usually not seen when a low dose of aspirin, sufficient to inhibit thromboxane $A_2$, is employed. So it seems, the use of low doses of aspirin to prevent thrombosis certainly warrant further consideration.

Persantine inhibits primary and secondary ADP induced platelet aggregation and in high concentrations inhibits the platelet release reaction induced by collagen, adrenaline and thrombin[72]. Persantine appears to act in vivo by synergistically modifying seveal biochemical pathways including; (a) inhibition of platelet cAMP phosphodiesterase (b) potentiation of adenosine inhibition of platelet function by blocking reuptake by vascular and blood cells and subsequent degradation of adenosine, and possibly, (c) potentiation of $PGI_2$ antiaggregatory activity and enhancement of $PGI_2$ biosynthesis. The antithrombotic effects of persantine in a baboon model of arterial thromboembolism has been optimally potentiated by the simultaneous addition of aspirin in doses of 20 mg/kg/day; which has no detectable antithrombotic effects, when used alone[53,130]. These observations clearly support the present results shown by these agents to potentiate the inhibition of platelet surface-binding in their combinations. Therefore, further studies have been proposed to investigate the effect of low doses of aspirin (0.5 mg%) with the addition of other vitamins, which can have antiplatelet activity, to search for a combination, which may be competent to aspirin-persantine.

Initially, vitamin $B_6$ has been chosen for such studies due to it's antiplatelet activity. The adhesion of platelets to PC is modified by the addition of vitamin $B_6$

TABLE-5-III

**CONCENTRATION EFFECT OF PLP ON PLATELET ADHESION TOWARDS PC SURFACE WITH AND WITHOUT FIBRINOGEN OR RED BLOOD CELLS**

| PLP concentration | Mean platelets* ± SD | |
|---|---|---|
| | A | B |
| 1. Bare polycarbonate (PC) | 18.0±2.0 | 24.0±4.5 |
| 2. PC + 0.5 mM Pyridoxal phosphate (PLP) | 15.6±3.0 | 23.6±4.5 |
| 3. PC + 1 mM PLP | 12.6±3.2 | 23.0±4.3 |
| 4. PC + 2 mM PLP | 9.9±2.7 | 21.4±5.3 |
| 5. PC + 4 mM PLP | 6.9±2.5 | 18.7±4.7 |
| 6. PC + 6 mM PLP | 6.8±2.4 | 19.9±2.8 |
| 7. PC + 50 mg% Fibrinogen | 39.2±5.0 | 44.0±5.3 |
| 8. PC + 50mg% Fg + 5 mM PLP | 17.4±5.2 | 37.1±5.6 |

A - Platelet suspension containing varying concentrations of PLP in solution were exposed to PC in presence and absence of fibrinogen (as shown in 1 to 8)

B - Similar sets of experiments as indicated in 'A' in presence of red blood cells.

* - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 30 observations from three separate experiments; 'p' $\leq$ 0.05).

TABLE-5-IV

CONCENTRATION EFFECT OF VITAMIN B6 & ASPIRIN-VITAMIN C COMBINATION TOWARDS RECALCIFICATION TIME & PLATELET ADHESION

| ASP-Vit.C+ B6 concentration* | Mean+ platelets±SD | Mean time‡ in seconds±SD |
|---|---|---|
| 1. Bare polycarbonate (PC) | 17.9±3.3 | 155.4±4.3 |
| 2. PC+ASP-Vit.C(A) (0.5 mg%: 1.5 mg%) | 9.5±2.7 | 268.8±7.5 |
| 3. A + 0.1 mM $B_6$ | 8.7±1.9 | 313.2±5.6 |
| 4. A + 0.25 mM $B_6$ | 7.5±1.8 | 331.0±6.3 |
| 5. A + 0.5 mM $B_6$ | 6.9±1.8 | 351.5±7.7 |
| 6. A + o.75 mM $B_6$ | 6.8±1.8 | 431.6±11.3 |
| 7. A + 1.0 mM $B_6$ | 6.2±1.9 | > 10 mts. |
| 8. A + 2.0 mM $B_6$ | 5.7±2.4 | > 15 mts. |

* - Platelet suspension containing aspirin-vitamin C (A) and varying concentrations of vitamin $B_6$ exposed to PC (as demonstrated in surfaces 1 to 8)

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 30 observations from triplicate experiments; 'p' $\leq$ 0.05).

‡ - Values expressed as the average of the recalcification time in seconds with standard deviation (from 5 experiments, 'p' $\geq$ 0.05).

(PLP) as can be seen from Table-5-III, and seems to be concentration dependent. Fibrinogen induced platelet density on the surface has also been inhibited by 5 mM PLP as is evident from Table-5-III. Addition of RBC in the platelet system has enhanced the surface platelet binding, however, higher PLP concentrations has modified the platelet adhesion at the interface. But no significant changes are observed with low concentrations of PLP in solution with the RBC inducecd platelet adhesion. So an optimum concentration of 0.5 mg% aspirin and 1.5 mg% vitamin C obtained earlier is chosen for further studies with PLP.

The adhesion of platelets to PC is inhibited by addition of aspirin-vitamin C combination and is further modified by vitamin $B_6$ as depicted in Table-5-IV. The lower concentrations of PLP do not show significant changes in adhering platelets to PC substrate, when added to aspirin-vitamin C system. But higher amounts of PLP added to aspirin-vitamin C combination do demonstrate drastic inhibition of platelet attachment to the substrate. However, from the observations from Table-5-IV, it may be possible to select a combination of ASP-Vit.C-Vit.$B_6$ (0.5 mg% : 1.5 mg% : 0.15 mM) for reducing the platelet adhesion to PC considering a low dosage of Vit. $B_6$.

Several workers have proposed that PLP inhibits ADP-induced shape change, aggregation and release reaction and have demonstrated a competitive inhibition of ADP aggregation by PLP[184,332]. It seems, the results presented here with PLP to modulate the platelet-surface binding correlate with their observations.

The effect of red cells on platelet sticking has been noted widely, causing an augmentation of the rate of adhesion, probably by a combination of physical and biochemical mechanisms[113,338]. The studies of RBC indicated here suggests that RBC may enhance the surface-platelet binding, however, high concentrations of soluble PLP have modified the platelet adhesion at the interface. It appears that[328] the inhibition of ADP and adrenaline induced aggregation and thromboxane $B_2$ generation by PLP may be mediated via schiff base formation with platelet membrane aminogroups. So it seems, the PLP may bind with platelets or polymer surface, which modifies or masks the platelet receptor sites for the fibrinogen molecule or the polymer substrate itself and cause the reduction in platelet density on the surface.

Vitamin E ($\alpha$-tocopherol) first identified by Evans and Bishop[91] in 1922 is a required nutrient which has been the focus of new interest, since clinical trials has suggested, that it may be effective in reducing the incidence of certain thrombotic disorders, including intermittent claudication[366], cerebral arteriosclerosis, and coronary artery disease[90]. These findings concerning vitamin E shed light to include this also in the combination of 0.5 mg% ASP: 1.5 mg% Vit.C: 0.15 mM Vit.$B_6$, to investigate further their effects in combination.

The inhibition of platelet adhesion to the polymer surface by vitamin E and the combination of ASP-Vit.C-$B_6$ is demonstrated in Table-5-VI. The platelet adhesion to PC is considerably blocked by higher concentrations of Vit.E in presence of the combination used,

TABLE 5-V

**CONCENTRATION EFFECT OF ASPIRIN AND VITAMIN C COMBINATION ON RECALCIFICATION TIME**

| ASP.concentrations | Mean* time in sec.±SD | ASP. concentration +1.5mg% Vit.C | Mean time* in sec.±SD |
|---|---|---|---|
| 1. Bare glass | 145.0±5.0 | 9. PC+1.5mg% Vit.C | 235.0±5.0 |
| 2. PC coated glass (PC) | 225.0±4.7 | 10. PC+0.2mg%ASP+ Vit.C | 246.0±3.5 |
| 3. PC+0.2mg% ASP | 239.0±3.5 | 11. PC+0.4mg%ASP+ Vit.C | 255.0±2.4 |
| 4. PC+0.4mg% ASP | 250.0±3.5 | 12. PC+0.8mg%ASP+ Vit.C | 259.0±3.8 |
| 5. PC+0.8mg% ASP | 251.0±2.7 | 13. PC+1.2mg%ASP+ Vit.C | 254.5±3.5 |
| 6. PC+1.2mg% ASP | 254.0±2.4 | 14. PC+1.6mg%ASP+ Vit.C | 254.0±3.0 |
| 7. PC+1.6mg% ASP | 253.0±2.7 | 15. PC+2.5mg%ASP+ Vit.C | 252.5±3.0 |
| 8. PC+2.5mg% ASP | 255.0±3.0 | | |

* - Values expressed as the average of the recalcification time in seconds with standard deviation (from 5 experiments 'p' $\leq$ 0.05)

but an optimum reduction in platelet affinity to PC can be suggested by 2 mg% Vit.E with combination (2 mg% Vit.E with combination has reduced the platelets to 6.0±2.4, from 8.2±2.0 of the combination alone).

## 5.2. Effect of vitamins and antiplatelet drugs upon recalcification time (RT)

Plasma recalcification time with aspirin, and aspirin-vitamin C in varying concentrations in contact with PC are depicted in Table-5-V. Recalcification time clearly reveals the anticoagulant effect of aspirin, which has been prolonged with aspirin-vitamin C combination. A combination of 0.4 mg% aspirin, 1.5 mg% vitamin C has shown a similar anticoagulant effect to that of higher concentrations of aspirin alone. The anticoagulant property of these antiplatelet drugs may be due to the effects of them on platelets itself.

Combination of ASP-Vit.C with vitamin $B_6$ demonstrates an enhanced RT compared to ASP-Vit.C alone (A) as shown in Table-5-IV. The infusion of vitamin $B_6$ to the ASP-Vit.C combination dramatically increases the recalcification time and is concentration dependent. A combination of ASP-Vit.C with 0.15 mM vitamin $B_6$ has been chosen for further studies. Concentration effect of Vit.E towards RT is not significant in their lower ranges with the ASP-Vit-C combination used (as demonstrated in Table 5-VI). However, 2 mg% Vit.E with the ASP-Vit-C-$B_6$ combination demonstrates a recalcification time of 357.2±10.0 compared to 307.2±8.7 seconds of the ASP-Vit-C-$B_6$ combination alone. Hence from antiplatelet and anticoagulant point of view a

TABLE-5-VI

**CONCENTRATION EFFECT OF VITAMIN E & ASPIRIN-VITAMIN C-VITAMIN B6 COMBINATION TOWARDS RECALCIFICATION TIME & PLATELET ADHESION**

| ASP-Vit.C-$B_6$+Vit.E concentrations* | Mean+ platelets±SD | Mean time ++ in seconds±SD |
|---|---|---|
| 1. Bare polycarbonate (PC) | 17.90±3.0 | 166.2± 5.0 |
| 2. PC+ASP-Vit.C-B6 (B) (0.5mg%:1.5mg%:0.15mM) | 8.20±2.0 | 307.2± 8.7 |
| 3. B+0.5mg% Vit.E | 7.50±1.9 | 311.2± 8.3 |
| 4. B+1.0mg% Vit.E | 6.90±2.0 | 339.4± 7.6 |
| 5. B+2.0mg% Vit.E | 6.00±2.4 | 357.2±10.0 |
| 6. B+5.0mg% Vit.E | 6.25±2.3 | 369.4± 7.9 |
| 7. B+7.5mg% Vit.E | 5.90±2.4 | 411.8± 8.4 |
| 8. B+10.0mg% Vit.E | 5.30±2.2 | 419.0±11.4 |

* - Platelet suspension containing Aspirin-Vitamin C-Vitamin $B_6$ (B) and varying concentrations of vitamin E exposed to PC (as demonstrated in surfaces 1 to 8)

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation. (at least 30 observations from four separate experiments' 'p' $\leq$ 0.05).

\+ - Values expressed as the average of the recalcification time in seconds with standard deviation (from 5 experiments' 'p' $\sim$ 0.05).

combined dosage of ASP-Vit.C-$B_6$-Vit.E (0.5 mg%: 1.5 mg% :0.15 mM : 2 mg%) can be suggested for better performance.

The effect of these vitamins and antiplatelet drugs on adsorbed PC substrates are also investigated to see the role of these modified substrates to modulate the interfacial phenomena. Table-5-VII provides the information related to octane contact angle, platelet adhesion and recalcification time of various vitamins and antiplatelet drugs adsorbed PC substrates. It is evident that, except PLP adsorbed PC, all other substrates demonstrate a reduction in octane contact angle, compared to bare PC. Octane contact angle technique is widely used to study the nature of the surface by various investigators[48,257] and have been correlated with blood compatibility.

This can provide information related to the hydrophilic or hydrophobic nature of the surface via the surface properties like surface energy and interfacial tension at the solid- liquid interface. All the modified substrates (except PLP) show (Table-5-VII) hydrophobic character in variable degrees compared to bare PC.

Vitamin D indicates an enhanced platelet adhesion but there is no significant difference with vitamin A, compared to bare PC (PC = 18.0$\pm$2.0, Vit. A adsorbed PC = 16.7$\pm$2.1 and Vitamin D adsorbed PC = 20.0$\pm$2.5 platelets per $mm^2$). However the recalcification time of these vitamins coated PC surfaces is reduced compared to bare surface. (PC = 119.6$\pm$4.0, Vitamin A = 97.2$\pm$2.0; and Vitamin D = 97.8$\pm$2.0 seconds respectively). All other vitamins and antiplatelet drugs adsorbed substrates demonstrate (Table-5-VII)

TABLE-5-VII

**OCTANE CONTACT ANGLE, PLATELET ADHESION AND RECALCIFICATION TIME ON VITAMINS AND DRUGS ADSORBED PC FILMS**

| Surfaces | Octane* contact angle±SD | Mean+ platelets ±SD(per $mm^2$) | Mean‡ time in seconds±SD |
|---|---|---|---|
| 1. Bare polycarbonate(PC) | 115.5±2.9 | 17.8±1.9 | 119.6±3.9 |
| 2. Vit.A adsorbed PC | 73.8±5.2 | 16.7±2.1 | 97.2±1.7 |
| 3. Vit.$B_6$ adsorbed PC | 121.8±3.3 | 11.1±1.7 | 135.2±4.0 |
| 4. Vit.C adsorbed PC | 103.8±3.2 | 11.9±2.1 | 124.2±2.7 |
| 5. Vit.D adsorbed PC | 87.2±6.6 | 20.0±2.5 | 97.7±1.7 |
| 6. Vit.E adsorbed PC | 71.9±4.2 | 11.6±1.9 | 122.8±5.8 |
| 7. Aspirin adsorbed PC | 109.3±3.9 | 11.4±1.8 | 129.1±4.1 |
| 8. Dypiridamole adsorbed PC | 87.0±3.5 | 10.2±3.2 | 127.2±3.8 |

* - 30 Contact angles were observed from 3 separate experiments and the data expressed as the mean angle with standard deviation.

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation. (at least 35 observations from four experiments, 'p' $\leq$ 0.05).

‡ - Values expressed as the average of the recalcification time in seconds with standard deviation (from 5 experiments, 'p' $\sim$ 0.05).

anticoagulant and antiplatelet activities, to variable degrees. Therefore, vitamin A and vitamin D are not included in the combinations studied towards blood compatibility.

Davidson et al.[73] have proposed that freshly prepared ascorbate (10$\mu$ M) increased platelet cyclic GMP about 8-fold, and a small increase in cylic AMP ultimately inhibits platelet function. It has been hypothesized[156] that the production of prostaglandin $E_1$ is dependent upon dietary factors, with linolenic acid, $\gamma$-linolenic acid, zinc, pyridoxine and vitamin C all playing key roles. Prostaglandins such as $PGI_2$, $PGE_1$ and $PGD_2$ are believed to stimulate membrane-bound adenyl cyclase and thereby raise the intracellular levels of cAMP within platelets[33,232], which ultimately inhibits platelet aggregation and adhesion.

There is a close relationship between vitamin E and the coagulation system. Inhibition of platelet aggregation by vitamin E has been observed by several investigators[90,97]. Prolongation of plasma clotting time has been reported with vitamin E therapy, together with a decrease in the fibrinolytic activity[185]. The mechanism of action of $\alpha$-tocopherol on platelet aggregation and release has not yet been clearly established, but the concept of a direct physicochemical interaction between $\alpha$-tocopherol and polyunsaturated fatty acids is an attractive one. The unsaturated fatty substances present in the membranes of the cells in the walls of blood vessels and other tissues are damaged by oxidation. These important substances are protected against this damage by vitamin C and vitamin E, which are natural antioxidants[55].

These findings clearly support the present observations with these vitamin combinations towards inhibiting the platelet surface attachment. The combined use of these vitamins with low doses of aspirin appears to reduce, on the one hand, the accessible arachidonic acid and the other, decrease its oxidative conversion via the cyclo-oxygenase pathway. Steiner[324] has also studied the effect of vitamin E and aspirin-vitamin E combination via clinical trials with humans and their platelet response to collagen substrates. It seems that adhesiveness to collagen has not been affected by aspirin ingestion, but has shown a highly significant reduction in vitamin E and vitamin E + aspirin treated individuals.

The search for an appropriate agent or combination of agents that can effectively reduce the incidence of thrombosis in the arterial circulation continues to be a field of extensive investigation. The combination of an oral anticoagulant with dipyridamole has proved beneficial in patients with artificial heart values, although the combination of aspirin with an oral anticoagulant led to unacceptable bleeding problems[53]. Hence, the combination proposed here, (0.5 mg% Aspirin, 1.5 mg% Vitamin C; 0.15 mM Vitamin $B_6$, 2 mg% Vitamin E) seems to be highly competent or even better in comparison to aspirin-dypiridamole combination; or any other combinations yet studied on antiplatelet and anticoagulant points of view. Now the effect of these vitamins and their combinations to modulate the protein-polymer interactions may be interesting, to investigate, for inter-relating the interfacial phenomenon.

## 5.3. Effect of vitamins and antiplatelet drugs towards fibrinogen adsorption

The electrophoresis pattern as demonstrated in Fig-5-I, shows variations in the composition of the protein layer removed from the PC surface exposed in presence and absence of vitamins. PC films were exposed to a mixture of proteins containing 25 mg% albumin, 15 mg% γ-globulin and 7.5 mg% fibrinogen for 3 hours in presence and absence of various vitamins. The preadsorbed proteins, were desorbed using 1% Triton X-100, concentrated and carried out the PAGE in all cases reported here. The experiments were repeated at least three times for reproducibility and their typical representations are shown with standard molecular weight markers. From Fig-5-1, it is evident that vitamin A and D indicate an enhanced Fg and γ-globulin surface concentration and diminished albumin adsorption. However, the Fg and γ-globulin surface concentrations have been decreased, significantly with vitamin E adsorbed PC or with $B_6$ (PLP) in solution. On the other hand an encouraged albumin adsorption has been observed in both cases. The solution of $B_6$-protein mixture exposed samples reveal a new band between albumin and γ-globulin region, which may be PLP-albumin complex or Fg degradation product. This is evident with PLP adsorbed films as well.

The combinations of aspirin-persantine and aspirin-vitamin C demonstrate an enhanced albumin and decreased γ-globulin and Fg bands in the PAGE system as shown in Fig 5-2. Aspirin or persantine alone do not indicate this change, however persantine shows a decrease in fibrinogen surface concentration.

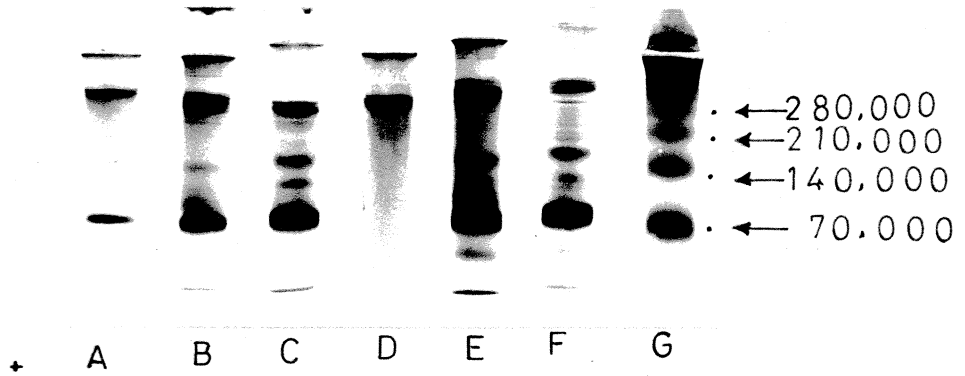



Fig 5-1. Polyacrylamide gel (7%) electrophoresis of desorbed proteins from PC stained with Coomassie blue. Mixture of proteins containing 25 mg% albumin, 15 mg% γ-globulin and 7.5 mg% Fg exposed to PC (A) and vitamin adsorbed surfaces. 50 mg% vitamin A (B), vitamin $B_6$ (C), vitamin D (D vitamin E (E), $B_6$ in solution (F) and molecular weight mar proteins (G) respectively

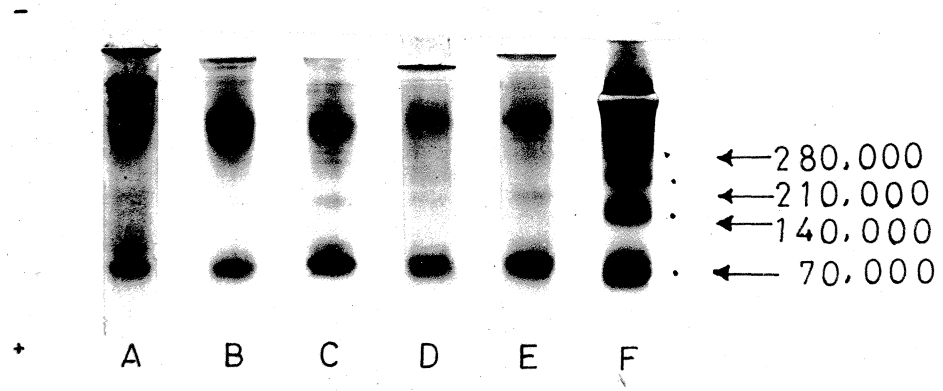



Fig 5-2 Polyacrylamide gel (7%) electrophoresis of desorbed proteins from PC stained with Coomassie blue. Mixture of proteins exposed to PC (A), 2 mg% asprin (B), 0.5 mg asprin: 2 mg% persantine (C), 0.5 mg% aspirin: 1.5 mg% vit. (D), 5 mg% persantine (E) and molecular weight marker proteins (F) respectively

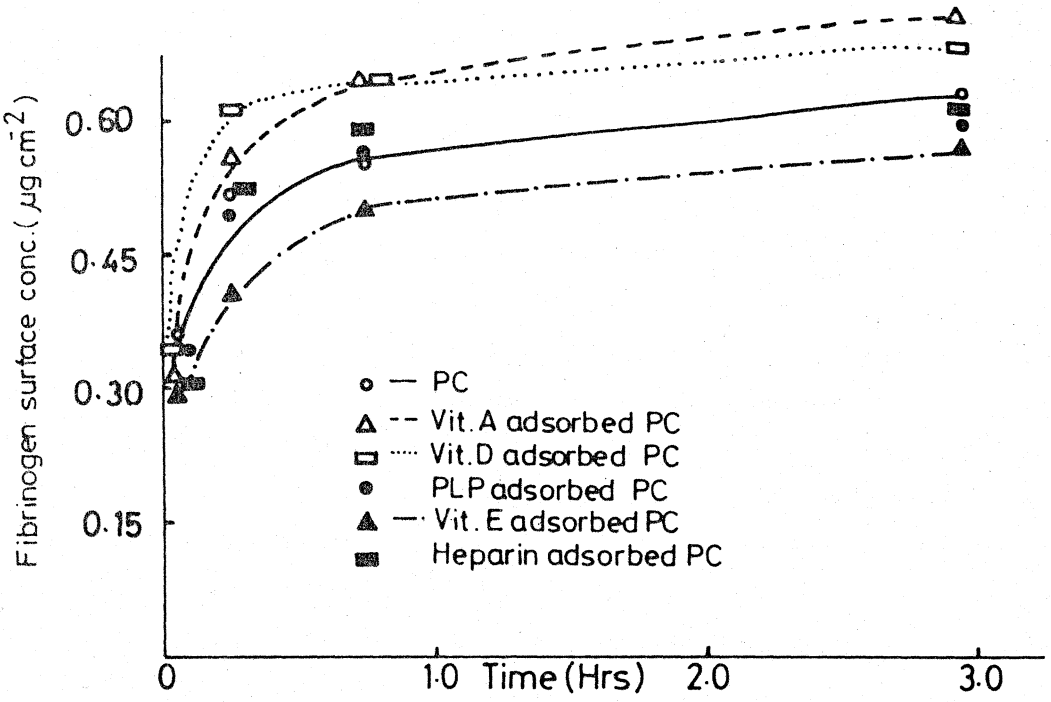



Fig.5-3. Adsorption of fibrinogen as a function of time on Vitamins or Heparin preadsorbed PC surfaces from protein mixture.

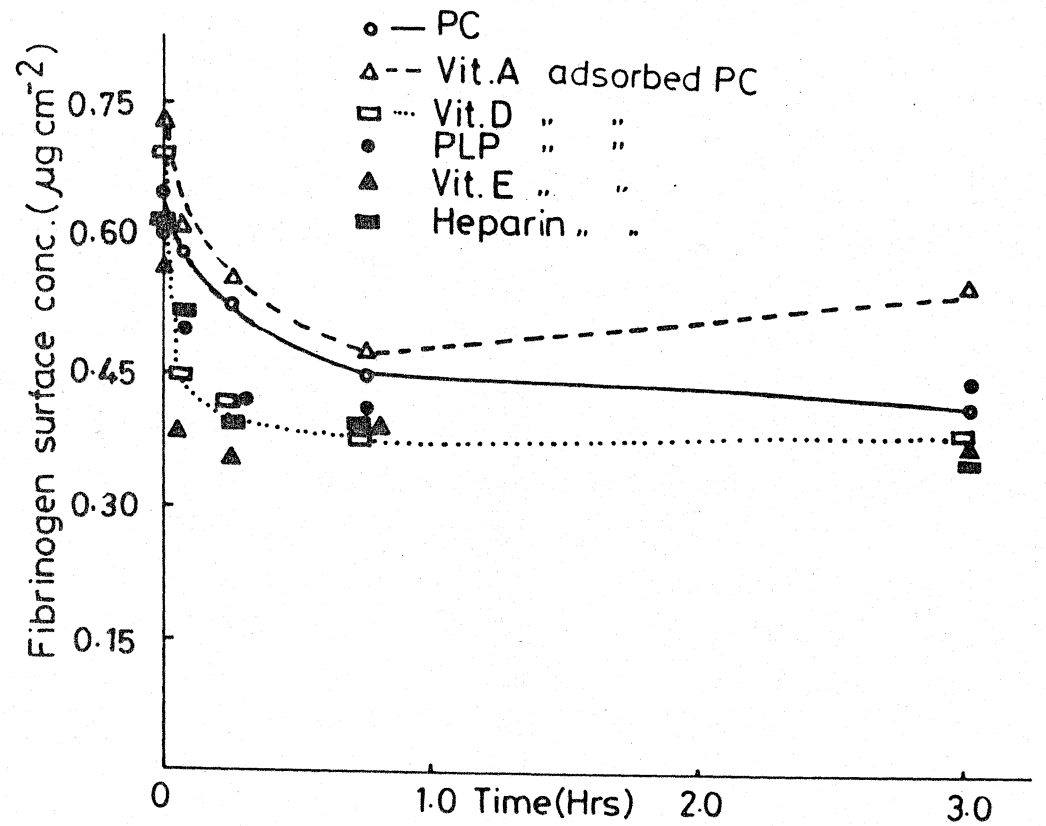



Fig.5-4. Desorption of fibrinogen as a function of time from 3hrs preadsorbed (Vitamins or Heparin) surfaces.

The pattern of protein interaction with the polycarbonate substrate as a function of time using labelled proteins in the presence of various vitamins and antiplatelet drugs have been also investigated. The kinetics of Fg adsorption to vitamins and heparin preadsorbed PC surfaces from protein mixture is shown in Fig.5-3. Vitamin A and D preadsorbed substrtates have enhanced the Fg binding, whereas PLP and heparin have been without significant effect, but vitamin E has reversed the Fg surface binding, in comparison with bare substrate. The 3 hours preadsorbed Fg is readily suplanted with other proteins or with Fg itself, when placed in a protein mixture from all those surfaces (Fig.5-4). The initial desorption is drastic for 15 minutes, which reaches to a minimal value within 45 minutes.

Adsorption/Desorption kinetics of fibrinogen to the PC surface from protein mixture in the presence of 0.2 mM vitamin $B_6$ or 2 mg% vitamin E is demonstrated in Fig.5-5. It seems, vitamin $B_6$ and vitamin E have reduced the surface-fibrinogen binding, as well they removed a part of the preadsorbed Fg from the surface when placed in a protein mixture. Adsorption of albumin has been dramatically increased with $B_6$ adsorbed PC, while all other vitamins (A,D&E) preadsorbed substrates have shown a slight reduction in albumin surface attachment (Fig.5-6). The desorption kinetics via exchange of 3 hours preadsorbed albumin from these substrates also reveal that vitamin $B_6$ preadsorbed substrate can retain maximum albumin (Fig.5-7). Adsorption/Desorption kinetics of albumin from protein mixture in presence of vitamin $B_6$ or vitamin E in their solutions are indicated in Fig.5-8. This further strengthen

the earlier observations (PAGE of desorbed proteins) that soluble vitamin E and vitamin $B_6$ do enhance the surface-albumin binding.

The platelet adhesion and recalcification studies reported earlier in this Chapter has suggested an optimum drug, vitamin combinations of 0.5 mg% Aspirin: 1.5 mg% Vitamin C: 0.15 mM vitamin $B_6$: 2 mg% vitamin E for improved blood compatibility. The same combination has also been studied towards their protein surface behaviour. The Fig.5-9 demonstrates the adsorption and desorption kinetics of albumin as a function of time to PC surface in presence and absence of ASP-Vitamin C-Vitamin $B_6$-Vitamin E combination. The adsorption of albumin on to the surface appears to increase initially with the drug combination, no significant change is observed after one hour adsorption. The Fig.5-9 also reveals that less albumin is desorbed from the preadsorbed PC substrate in presence of the drug vitamin combination. In other words, it appears that more albumin is retained on the substrate compared to the bare surface, throughout the study.

The adsorption/desorption pattern of fibrinogen as a function of time to PC surface in presence and absence of vitamin combination is depicted in Fig.5-10. The drug-vitamin combination has reduced the surface-Fg binding. It seems the protein exchange phenomenon in both cases is similar, though relatively more surface bound Fg is observed throughout the study in absence of drug-vitamin combination. Table 5-VIII, provides the mole ratio of albumin to fibrinogen, when exposed to PC as a function of time. The albumin to Fg mole ratios are higher with aspirin and vitamin

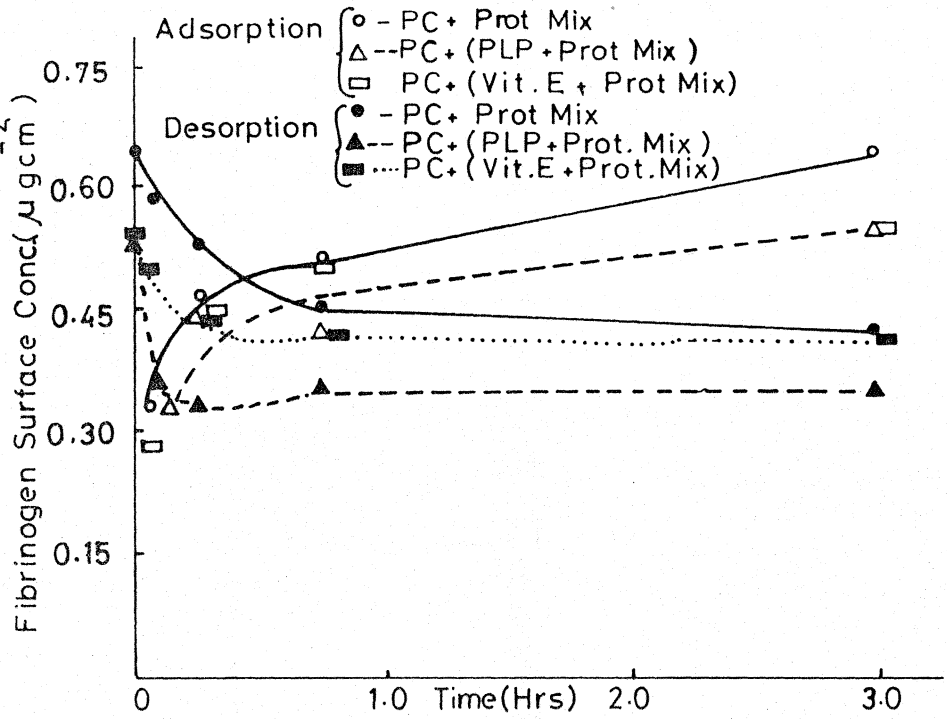



Fig.5-5. Adsorption/Desorption of fibrinogen as a function of time from protein mixture with PLP or Vit. E in solution.

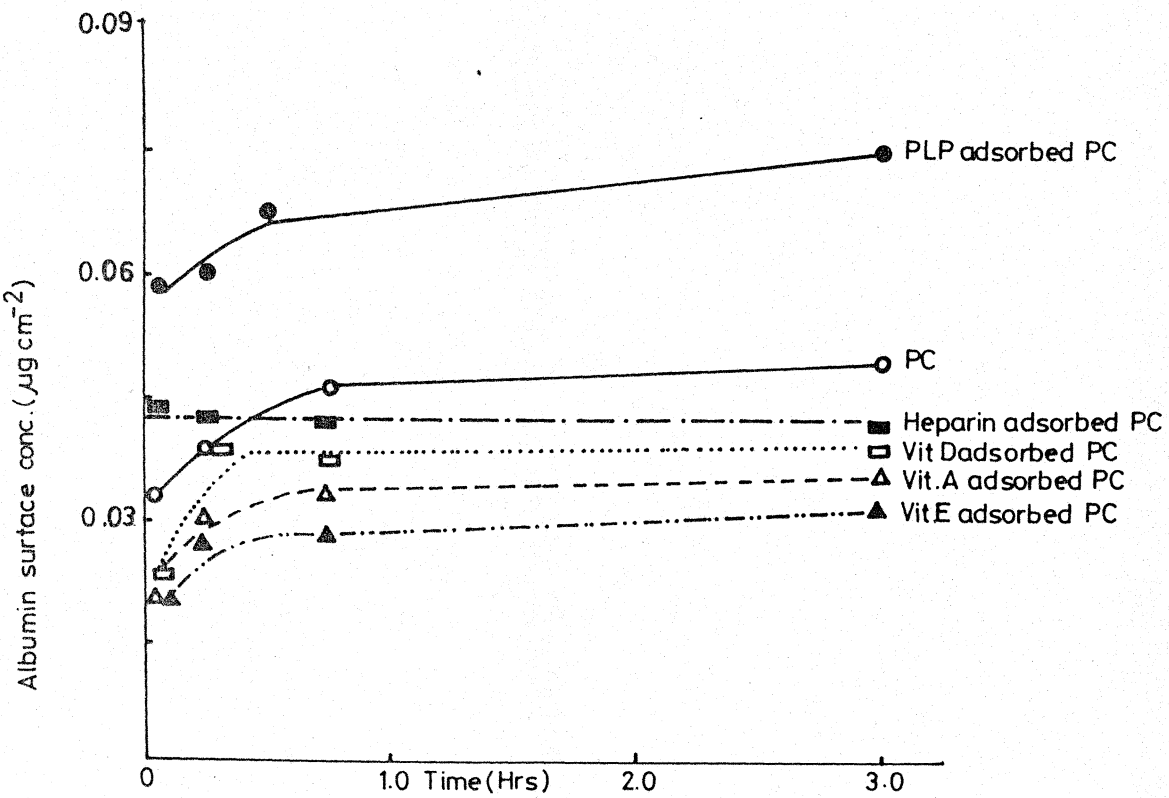



Fig.5-6. Adsorption of albumin as a function of time on Vitamins preadsorbed PC surfaces from protein mixture.

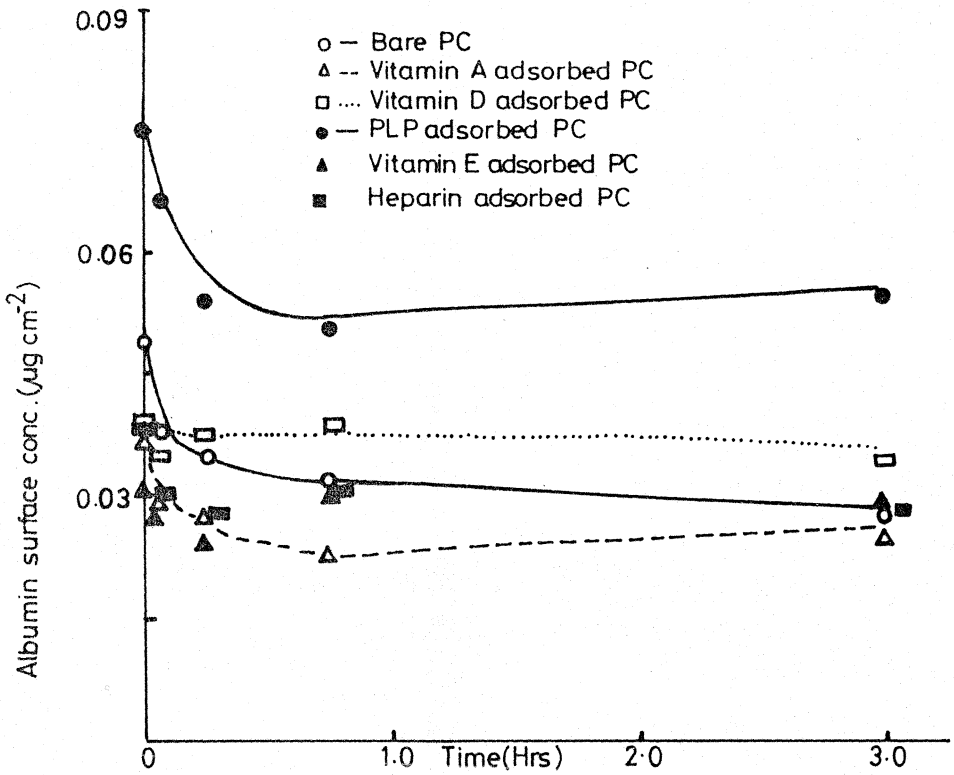



Fig.5-7. Desorption of albumin as a function of time from various 3hrs preadsorbed surfaces.

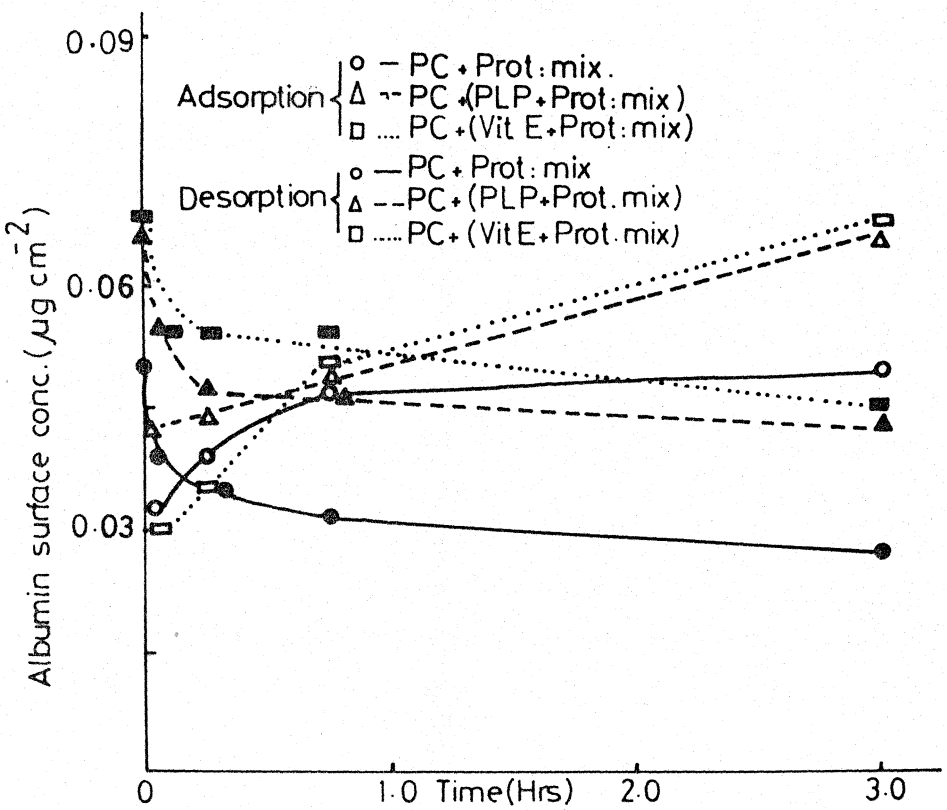



Fig.5-8. Adsorption/Desorption of albumin as a function of time from protein mixture with PLP or Vit. E in solution.

TABLE 5-VIII

**MOLAR RATIO OF ALBUMIN/FIBRINOGEN ON BARE PC AND ASPIRIN-VITAMIN COMPLEX**

| Time of exposure | Bare PC (Alb/Fg) | ASP-Vit.C-$B_6$-E Combination (Alb/Fg) |
|---|---|---|
| 1 minute | 0.50 | 0.69 |
| 15 mts. | 0.35 | 0.52 |
| 45 mts. | 0.40 | 0.47 |
| 3 hours | 0.41 | 0.51 |

combination, which may improve the substrate blood compatibility as discussed earlier[43,46].

The adsorption/desorption kinetics of Fg to PC surface under a flow of 1 ml/mt, in presence and absence of aspirin-vitamin combination, from a protein mixture is indicated in Fig.5-11. It is evident that the combination has significantly inhibited the Fg adsorption to the substrate. A part of the preadsorbed Fg is readily getting desorbed from the substrate which is more in the combination infused system. Further studies of protein/platelet interactions with these combinations on other polymer substrates like Teflon, Nylon have also been attempted under static and flow conditions for better understanding of the interfacial phenomena.

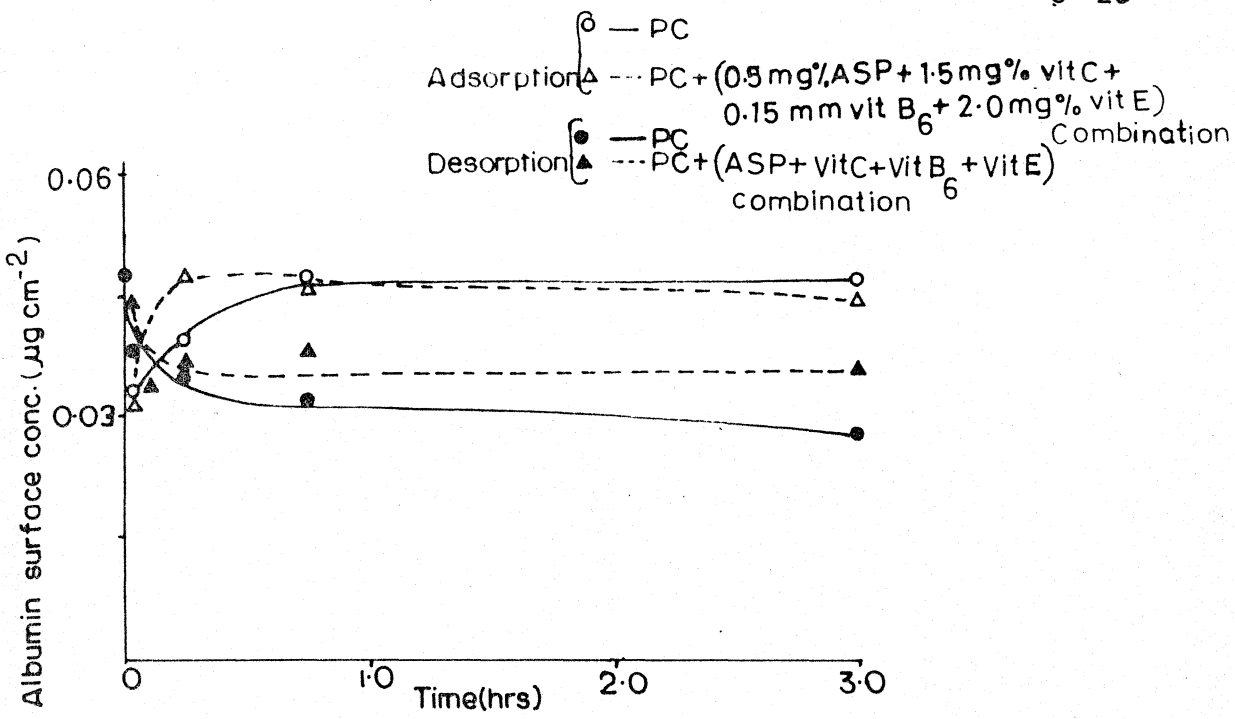



Fig 5-9 . Adsorption/Desorption of albumin on Polycarbonate as a function of time Effect of Vitamin combination

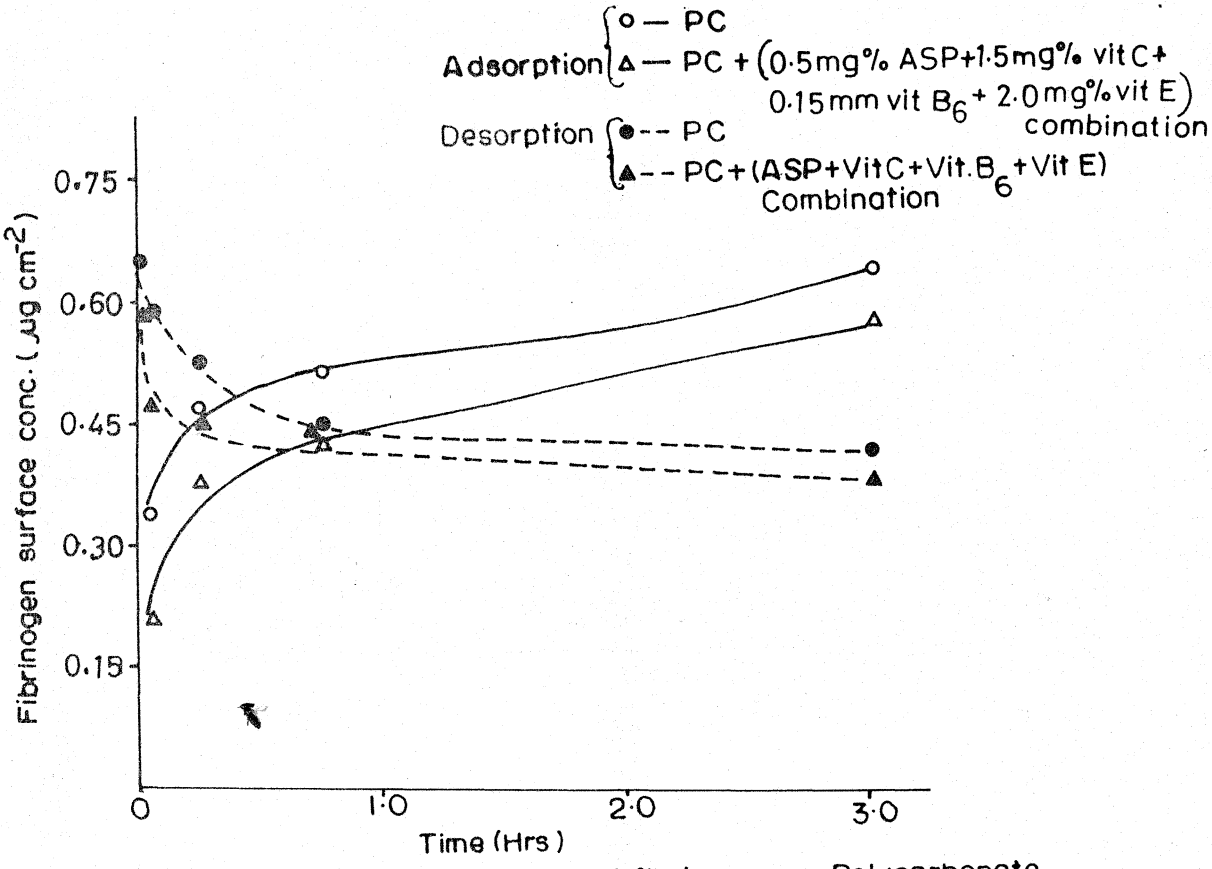



Fig.5-10 Adsorption / Desorption of fibrinogen on Polycarbonate as a function of time. Effect of Vit . combination

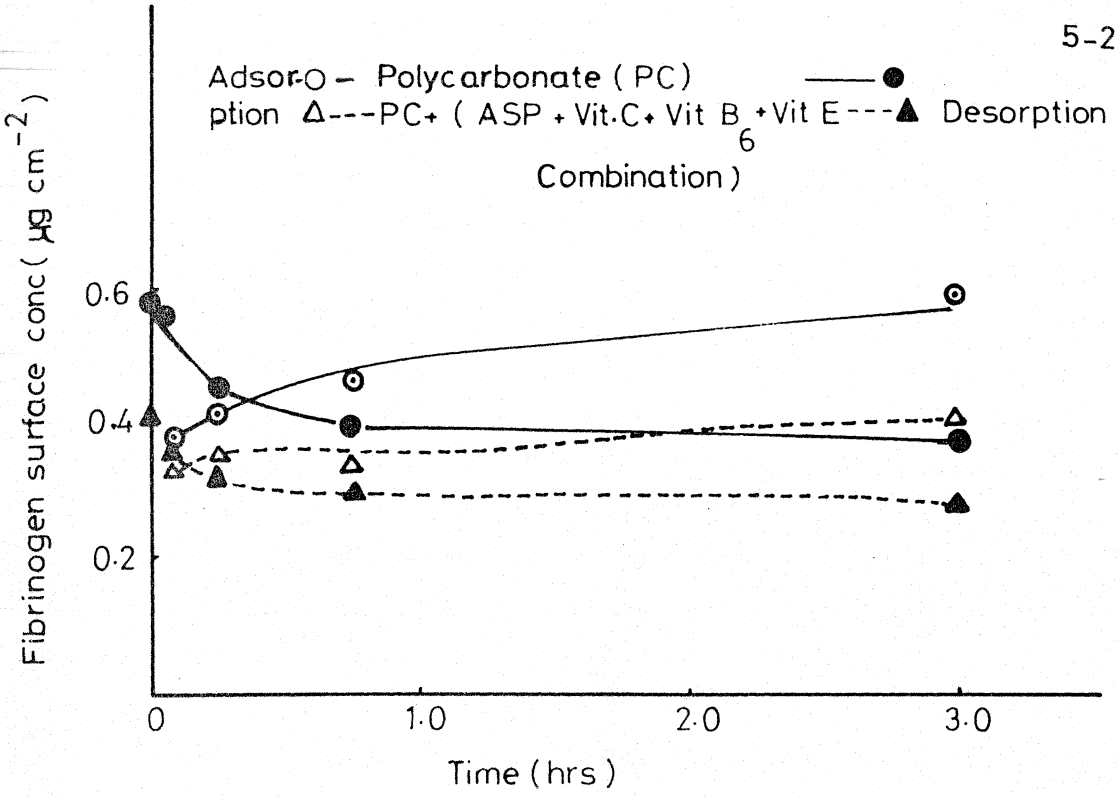



Fig.5-11 . Effect of Aspirin with vitamin combination towards the adsorption/desorption of fibrinogen on PC as a function of time from protein mixture; under 1 ml/mt flow

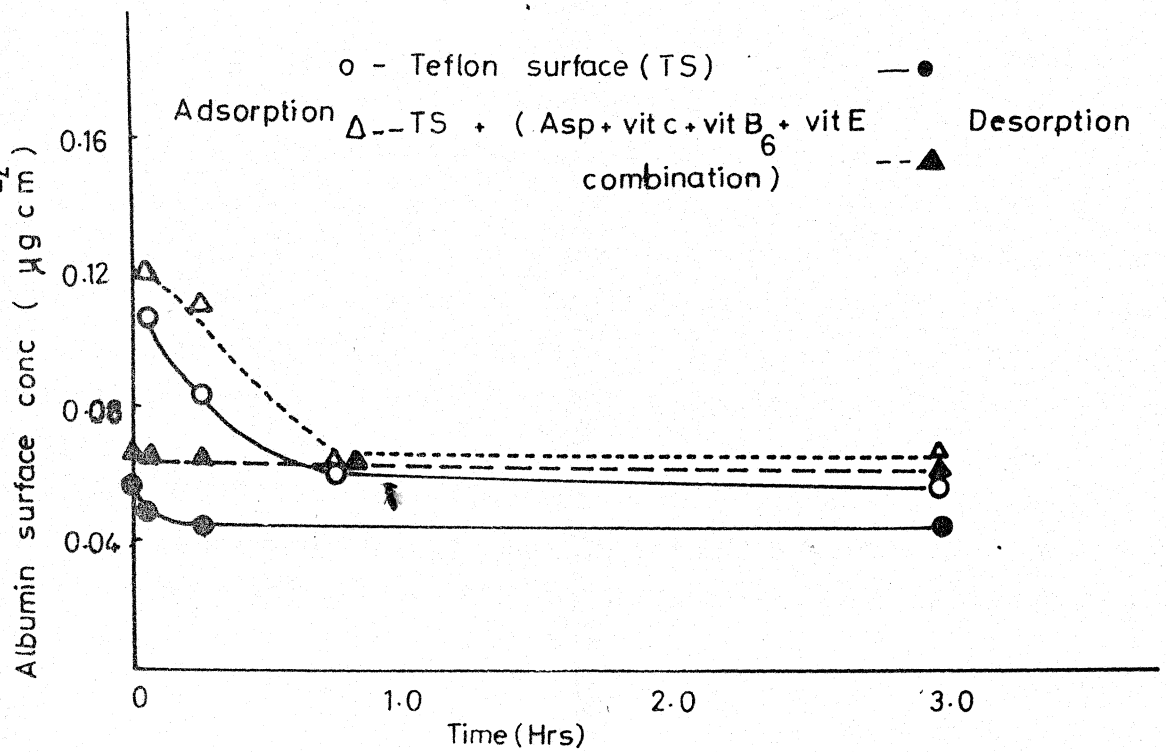



Fig.5-12 . Effect of Aspirin with vitamin combination towards the adsorption/ desorption of Albumin on Teflon as a function of time from protein mixture.

Effect of aspirin and vitamins, in their combinations, towards albumin adsorption/desorption to Teflon and Nylon surfaces are shown in figures 5-12, 5-13 and the Fg adsorption/desorption kinetics in figures 5-14, 5-15 respectively. Adsorption kinetics of albumin to Teflon surface seems to be quite interesting. It appears that maximum surface-albumin level is evident initially, within one minute, which reduces with time as shown in Fig.5-12. This is true with vitamin combination infused protein system as well, however the amount of albumin binding in this case is higher compared to bare Teflon substrate. On the other hand, the albumin adsorption has increased with exposure time to Nylon surface, which has been further elevated in the vitamin infused system as indicated in Fig.5-13. A part of the preadsorbed albumin gets desorbed from both substrates as shown in figures 5-12 and 5-13, though it is not significant to Teflon substrate in presence of vitamin-combination.

Combinations of aspirin with dipyridamole,or aspirin with vitamins, dramatically reverses the surface-Fg level to Teflon and Nylon substrates as depicted in figures 5-14 and 5-15. In the case of Nylon substrate, aspirin with vitamin combination has shown a drastic reduction of Fg-surface level compared to aspirin-dipyridamole and the bare substrate (Fig.5-15). However, Teflon indicates the least Fg adsorption with aspirin-dipyridamole combination. These changes in Fg adsorption kinetics may be due to the variations in surface properties of the material. A part of the preadsorbed Fg from all these substrates get exchanged with time, and is more with drug-vitamins infused system compared to the bare substrate.

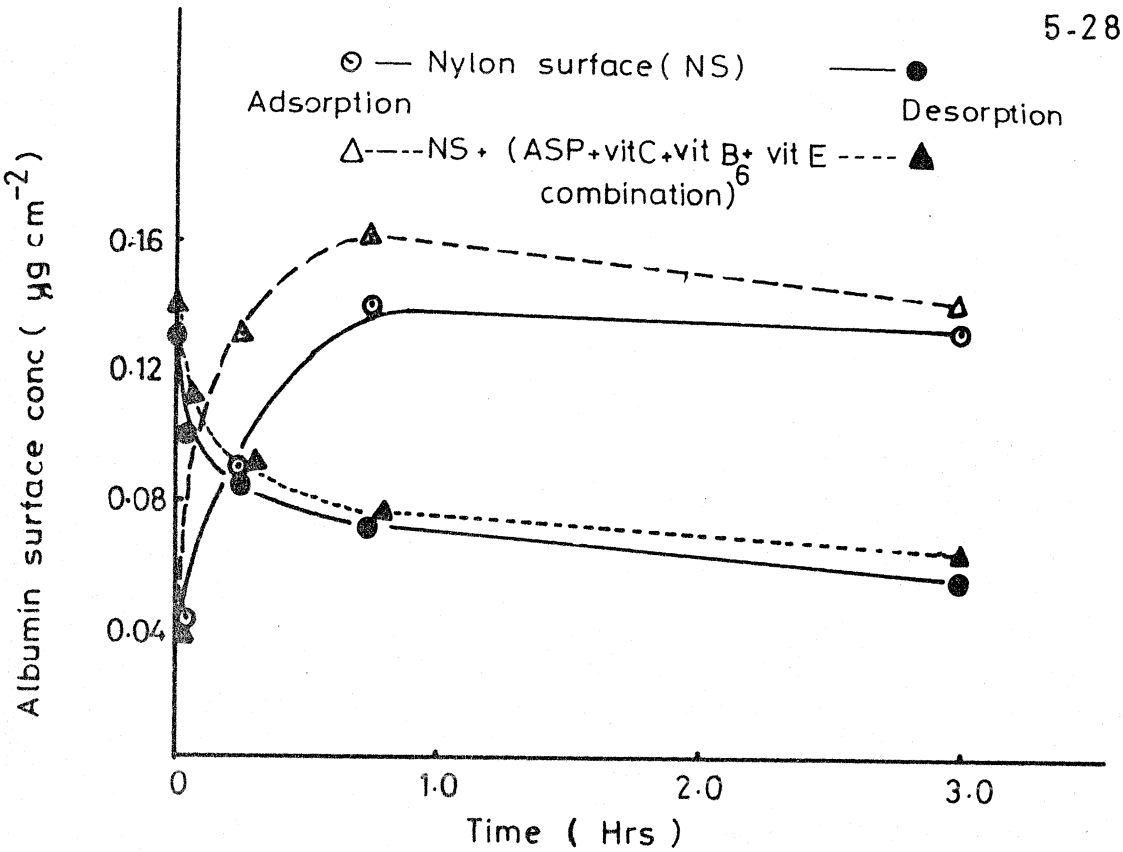



Fig.5-13 · Effect of aspirin with vitamin combination towards the adsorption / desorption of Albumin as a function of time from protein mixture on Nylon surfaces.

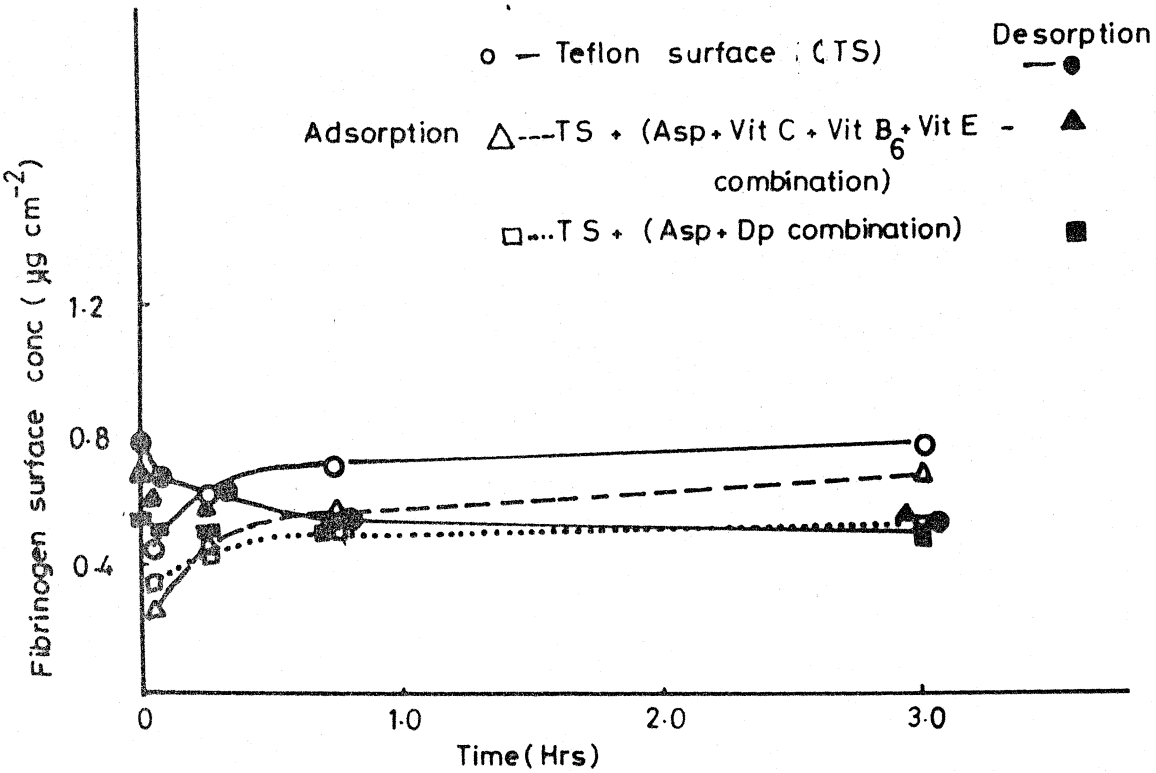



Fig.5-14 · Effect of drug and vitamin combination towards the adsorption/desorption of fibrinogen on Teflon as a function of time from protein mixture.

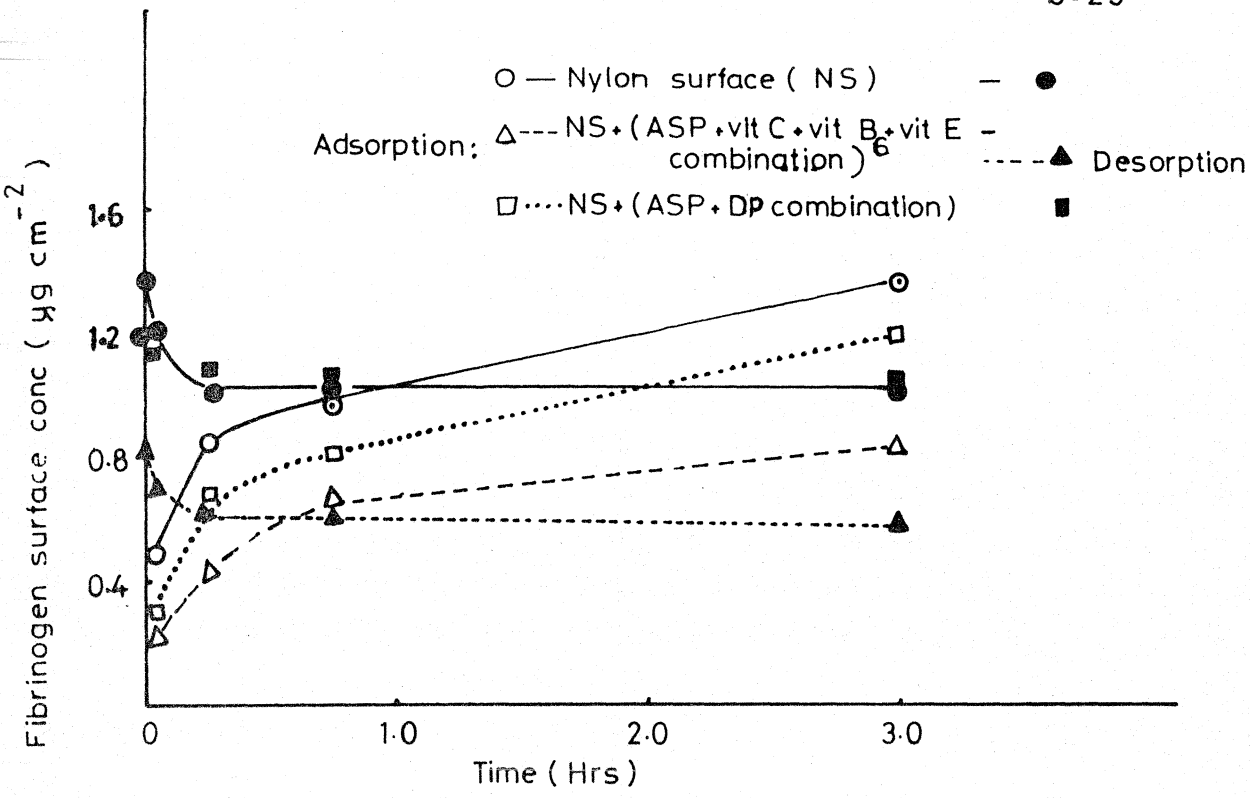



Fig. 5-15. Effect of drug and vitamin combination towards the adsorption/ desorption of fibrinogen on Nylon as a function of time from protein mixture.

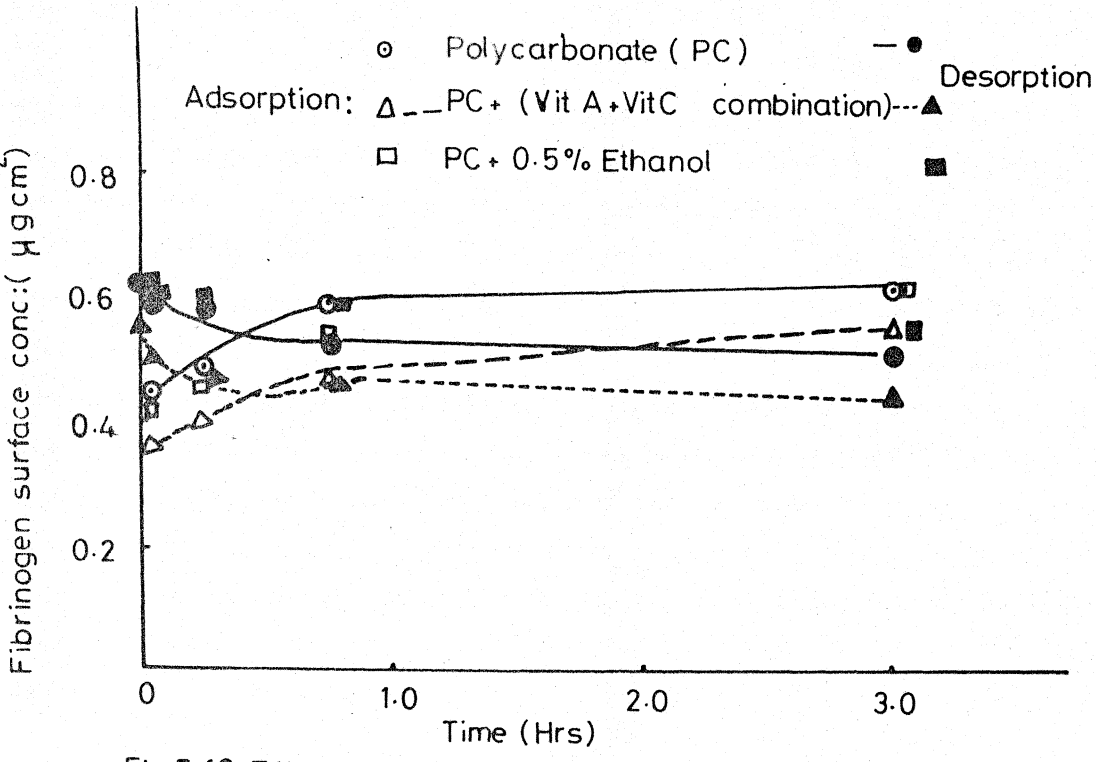



Fig. 5-16. Effect of vitamin A and C combination towards the adsorption/ desorption of fibrinogen on PC as a function of time from protein mixture.

It has been reported[161] that a combination of 10 μM vitamin A and 50 μM vitamin C demonstrated a synergistic effect to enhance the extracellular and intracellular activities of plasminogen activator by cultured endothelial cells. This resulted in an increased fibrinolysis and may be effective in preventing thrombosis. The synergistic effect of these vitamins may modulate the Fg adsorption/desorption kinetics, which have been attempted to various substrates like PC and Teflon. The adsorption/ desorption kinetics of Fg to PC and Teflon surfaces in presence and absence of 10 μM vitamin A and 50 μM vitamin C combination from a protein mixture are demonstrated in figures 5-16 and 5-17 respectively.

The adsorption kinetics of Fg to both substrates (PC and Teflon) are inhibited by vitamin A and C combination. It is also evident from figures 5-16 and 5-17 that a part of the preadsorbed Fg is getting exchanged from the substrates, in the protein mixture. The fat soluble vitamins like vitamin A, vitamin D and vitamin E have been dissolved in ethanol and infused to the adsorption media. Hence a same amount of ethanol has also been incubated with the control media (0.5% v/v) to check the ethanol effect towards Fg-surface interaction. However, the figures 5-16 and 5-17 indicate that the ethanol do not have much significant effect towards Fg-surface binding. Thus the changes in protein-surface binding observed by these drugs and vitamins may obviously be due to their effects itself.

Surface concentration of Fg, in presence of certain antiplatelet drugs is depicted in Fig.5-18. It is evident from Fig.5-18 that, the combinations of aspirin with

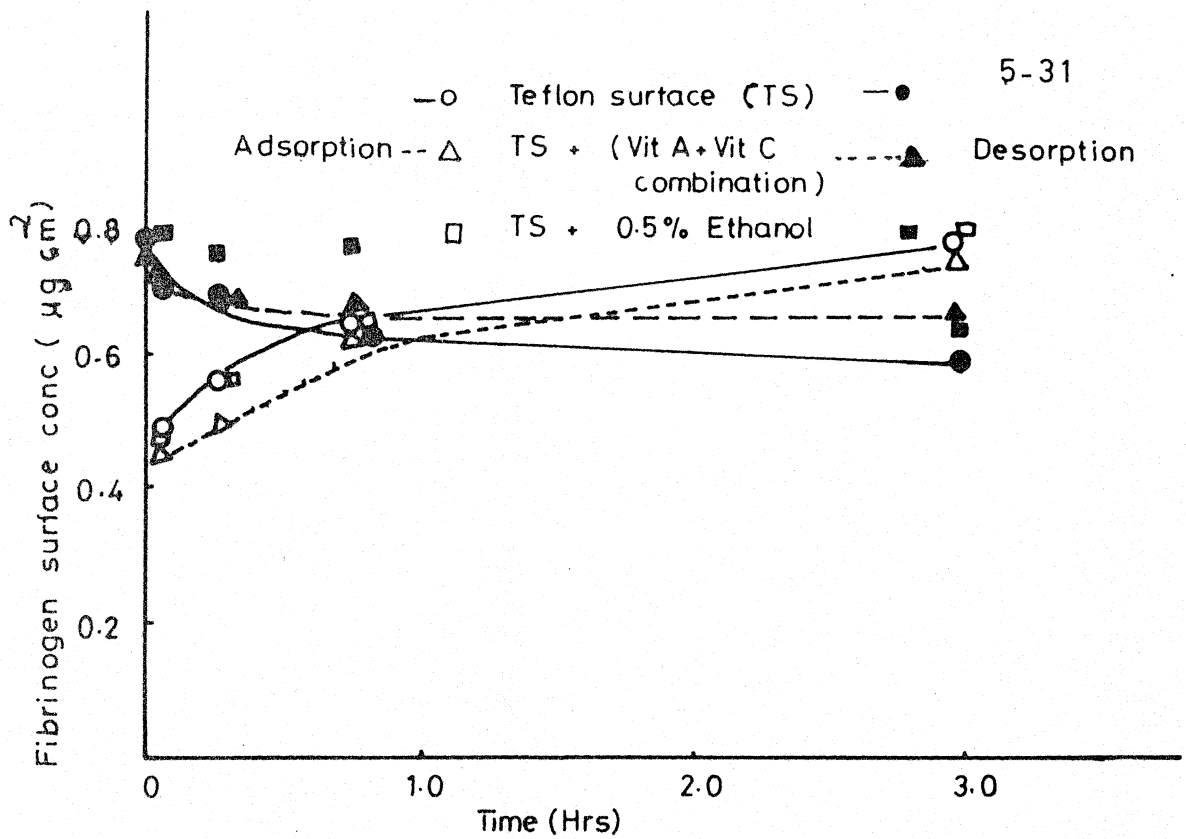



Fig. 5-17. Effect of vitamin A and C combination towards the adsorption/desorption of fibrinogen on Teflon as a function of time from proten mixture.

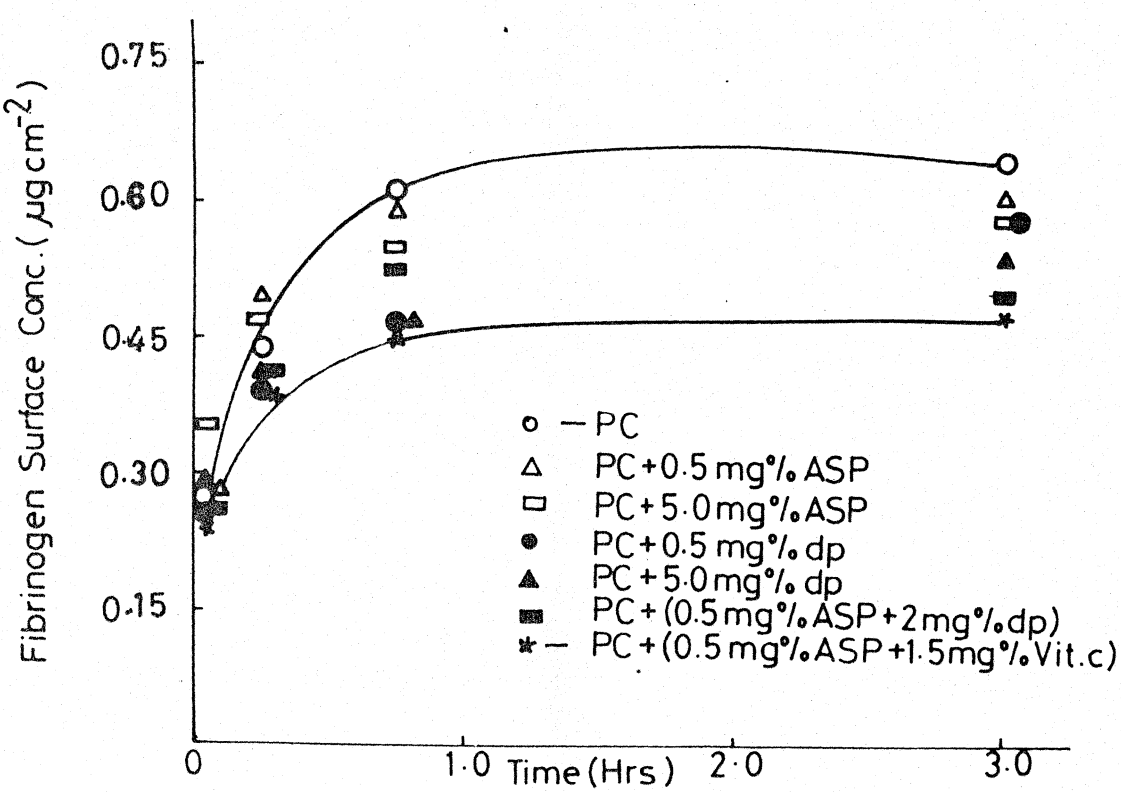



Fig5-18. Adsorption of fibrinogen on PC as a function of time from protein mixture. Effect of antiplatelet drugs.

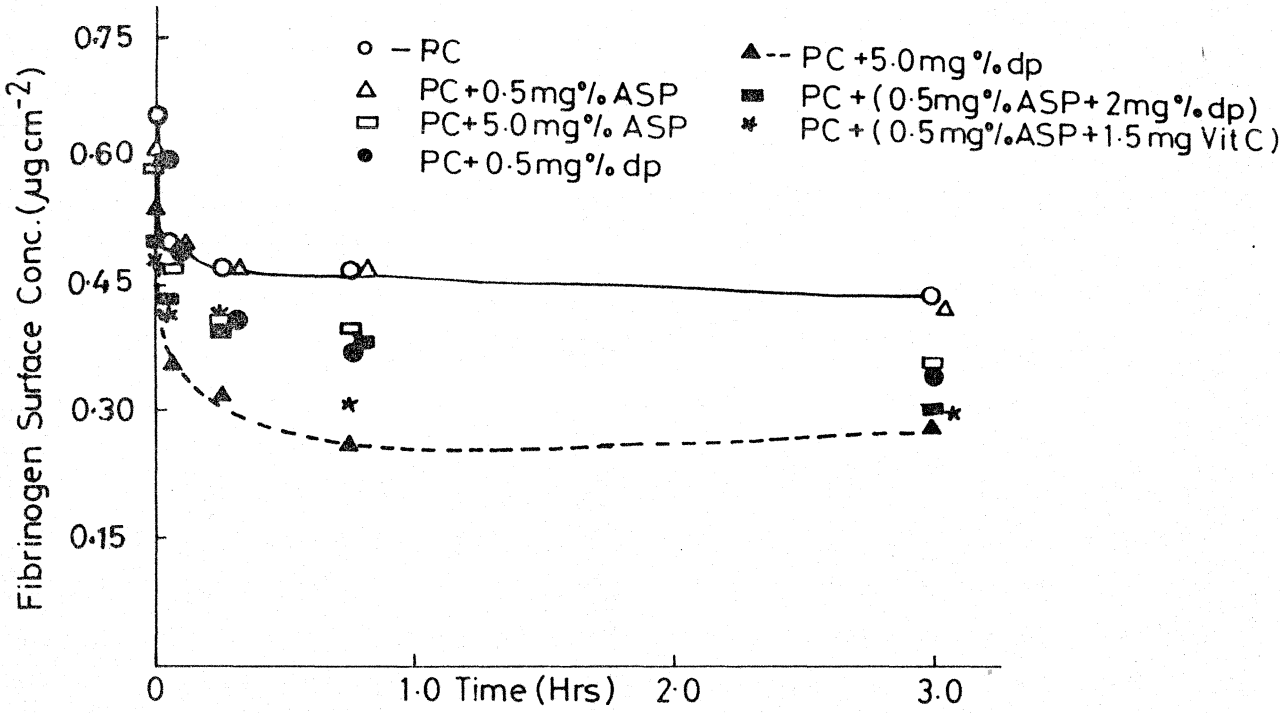



Fig.5-19. Desorption of preadsorbed fibrinogen from PC as a function of time in to protein mixture.

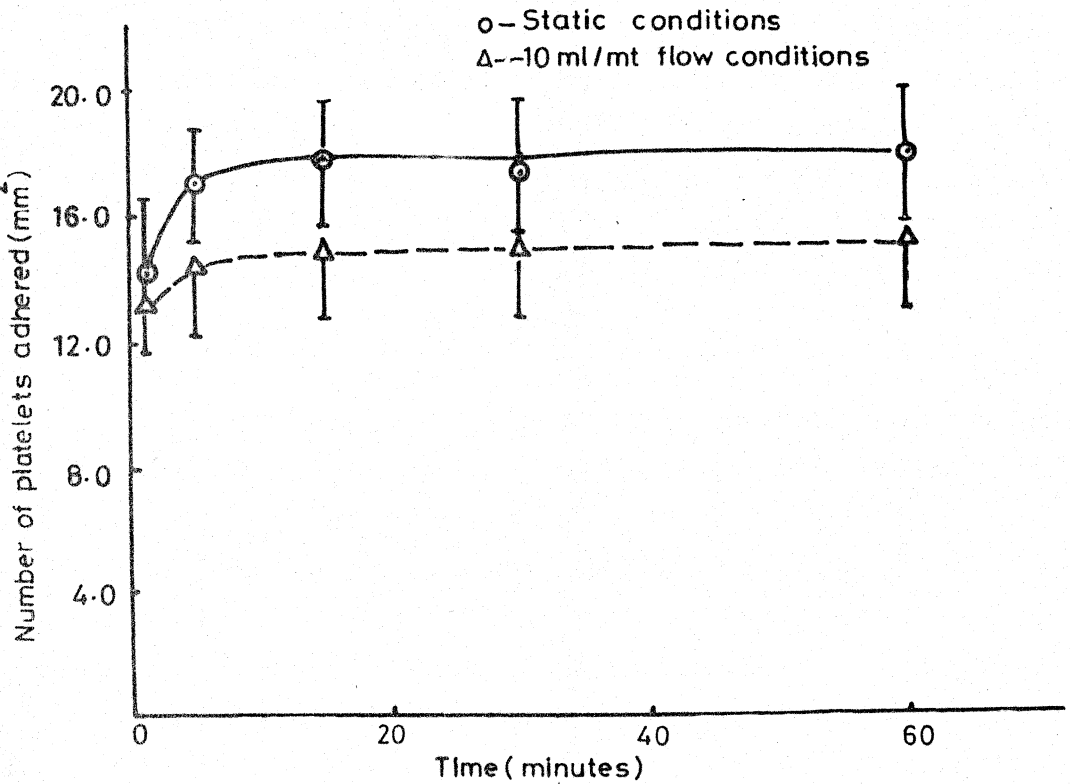



Fig.5-20. Adhesion of platelets to PC surface as a function of exposure time to platelet suspension. I 95% confidence limits.

dipyridamole or vitamin C, have significantly reduced the Fg level on the substrate, rather than single agent alone. The initially adsorbed Fg is subsequently removed in part from the surface as shown in Fig.5-19 with all drug infused protein systems.

The role of flow rates to alter the platelet-surface attachment in presence and absence of aspirin-vitamin combinations have been studied. Figure 5-20 reveals that in static or under flow conditions, the platelet density reaches to an optimum value within 15 minutes of platelet contact to polycarbonate substrate, though the number of platelets adhering to the substrate has been relatively low under the flow rates investigated.

As indicated in Fig.5-21, the platelet adhesion on the bare substrates of Nylon have been maximum, followed by polycarbonate and least in Teflon surfaces. It is also evident that the amount of platelets adhering to PC, Teflon and Nylon substrates have been dramatically inhibited by aspirin with vitamin combination under static and flow conditions.

Antithrombin III (AT-III) neutralises thrombin in physiological milieu to reduce the thrombus formation. Hence the effect of certain modified substrates towards AT-III activity at the polymer interface is also studied. Antithrombin III activity (%) of calf plasma on exposure to various modified PC substrates are shown in Table-5-IX. It clearly indicates that albumin, polyelectrolyte (synthesised from natural rubber" Hevea Brasiliensis"), vitamin $B_6$ and

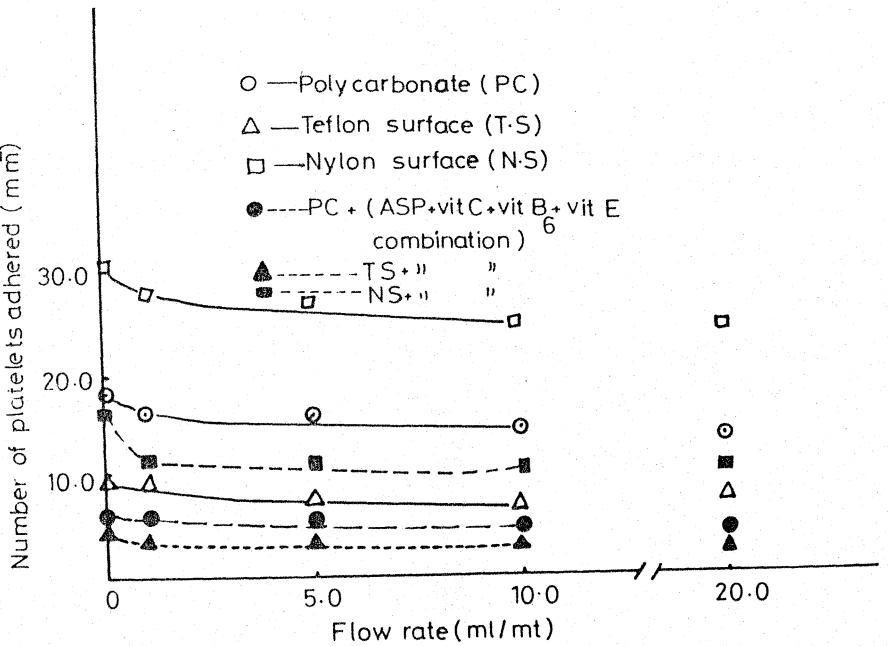



Fig.5-21. Platelet adhesion to various polymer surfacesas a function of flow rate. Changes with Aspirin-vitamin combination

heparin has shown variable degrees of reduction in AT-III activity in the same calf plasma. It has been suggested that, heaprin[157] binds to numerous plasma proteins, such as proteases inhibitors, clotting factors, and proteins of the complement system. Hennink et al.[136] have demonstrated that the different albumin-heparin conjugates can bind with AT-III, which is dependent on the type of conjugate used. It appears[341] that the polyelectrolyte is also an antithrombin and that its action, like heparin, depends on a factor (presumably antithrombin III), present in the blood plasma. PLP seems to bind with proteins such as albumin[75] and thrombin[119].

The observations suggest, that these surfaces may possibly bind with AT-III and cause a reduction in it's activity in the plasma. These agents may be acting via AT-III for an enhanced anticoagulant property, which need further research to confirm. However, it seems surface bound, heparin, PLP, albumin, polyelectrolyte and ASP-Vit combination may bind AT-III from plasma, which may probably neutralize the activity of thrombin that have been generated at the blood (plasma) - material interface.

From these studies, it appears that certain vitamins and antiplatelet drugs can block fibrinogen-surface binding from a mixture of proteins. These antiplatelet agents have also enhancecd the albumin surface concentration. Not all antiplatelet agents studied block Fg binding to artificial surface. Thus heparin and aspirin have been without significant effect towards Fg-surface interaction, whereas vitamin $B_6$, E, combinations of aspirin-persantine, aspirin-vitamin C and aspirin with vitamin combinations, have caused variable degrees of inhibition (PAGE patterns). It has been suggested that inhibitors of aggregation do not necessary impede platelet Fg interaction[5]. Marguerie et al.[219] proposed that platelet -Fg binding is independent of platelet aggregation and the release reaction. The failure of heparin and aspirin to inhibit Fg-surface interaction may be due to their high concentration; which need further studies with respect to concentration effect. This can be supported by the in vivo observation in rats:high dose heparin treatment in combination with thrombocytopenia failed to prevent the intra-aortical fibrin deposition[203].

TABLE-5-IX

ANTITHROMBIN-III ACTIVITY OF VARIOUS PREADSORBED SUBSTRATES

| Surfaces* | AT-III+ activity (%) |
|---|---|
| 1. Bare polycarbonate (PC) | 97.5±1.6 |
| 2. Albumin adsorbed PC | 87.0±2.0 |
| 3. Fg adsorbed PC | 101.0±3.3 |
| 4. Polyelectrolyte adsorbed PC | 83.0±0.7 |
| 5. Vitamin C adsorbed PC | 99.0±2.5 |
| 6. Vitamin E adsorbed PC | 94.0±2.3 |
| 7. PLP adsorbed PC | 88.0±2.9 |
| 8. Aspirin adsorbed PC | 97.0±2.3 |
| 9. ASP-Dipyridamole (0.5 mg%:2mg%) adsorbed PC | 89.5±3.8 |
| 10. ASP-Vit.C-Vit.E-$B_6$(0.5mg%:1.5mg%:0.15mM: 2 mg%) combination adsorbed PC | 89.0±2.0 |
| 11. Heparin adsorbed PC | 69.0±1.4 |
| 12. cAMP adsorbed PC | 96.5±1.6 |

* - PC surfaces exposed to 50 mg% of each substances in their respective solutions for 3 hours, avoiding the air/water interface.

+ - Results are obtained directly from the calibration curve (prepared from calf plasma) and expressed in terms of antithrombin-III (AT-III) activity (%) from 4 experiments; 'p' $\leq$ 0.05.

Polyacrylamide gel electrophoresis was used by various investigators[65,211] to separate, identify and quantitate the adsorbed proteins from a protein mixture, after desorbing them using Triton X-100. Chiu et al.[211] throughly investigated the possibility of any real selectivity in protein loss through adsorption onto the ultrafiltration system, but found to be negligible. they found that the method of protein desorption with Triton X-100, concentration of the desorbate and electrophoretic analysis is useful in determining the composition of the adsorbed protein layer which forms on a polymer surface exposed in vitro or ex vivo to plasma or protein mixtures. In the present studies, all the proteinated surfaces have been kept in Triton X-100 for atleast 16 hours with repeated shaking, for complete protein removal. It has been indicated using labelled proteins and ATR-IR techniques, that essentially all of the protein was removed from the surface within 5 hr, when the films were agitated[211]. Thus the PAGE pattern reported here are quite interesting with respect to the enhancement of albumin binding to surface or decreased Fg concentration with certain antiplatelet drugs or vitamins. The effect of the two drugs combined is promising like aspirin-persantine or aspirin-vitamin C, to inhibit the Fg-surface binding.

A panoramic view of the protein adsorption data to PC substrate in presence and absence of vitamins or antiplatelet drugs and the molar ratio of albumin to Fg are presented in Table-5-X. This clearly indicates that vitamin E, vitamin $B_6$, persantine, and combinations of aspirin-vitamin C or aspirin-persantine have dramatically reduced the surface-Fg binding, which are obviously significant from the

standard deviations presented in various tables of this Chapter. The molar ratio of albumin to Fg also demonstrated an increase due to vitamin $B_6$ and E, which is certainly good from blood compatibility point of view[43].

The relation of protein adsorption and platelet adhesion to various polymer substrates and their changes due to aspirin-vitamin combination is summarised in Table-5-XI. It seems, the amount of Fg adsorbed to the substrate and the adhesion of platelets to them, vary with the nature of polymer surface and there does not seem to be a simple interrelation between the amount of Fg deposited and subsequent platelet attachment. However; aspirin-vitamin combination drastically inhibited the surface-Fg binding in all cases.

It has been proposed[349,351] that platelets adhere, where they find adsorbed Fg; however , it appears that it is not the amount of adsorbed Fg for platelet attachment, rather, the receptor sites available for the adhesion is important. Ward and Stanga[356], recently suggested that platelet adhesion increases with increasing concentration of preadsorbed Fg until a first plateau is obtained, but higher surface concentration of Fg do not promote further platelet adhesion. So the conformational status of the preadsorbed Fg on adsorption to various substrates and the availability of platelet receptors is most important factor for the subsequent platelet attachment; which is dependent upon the surface properties of the material and the nature of the contacting media.

TABLE 5-X

**PROTEIN ADSORPTION TO PC SUBSTRATES-EFFECT OF VITAMINS AND ANTIPLATELET DRUGS**

| Surfaces* | Protein 3 hours Adsorbed+ ($\mu g\ cm^{-2}$) ±SD | | Molar Ratio Alb/Fg |
|---|---|---|---|
| | Albumin | Fg | |
| 1. Bare polycarbonate(PC) | 0.051±0.006 | 0.645±0.04 | 0.41 |
| 2. Vitamin A adsorbed PC | 0.035±0.004 | 0.720±0.015 | 0.25 |
| 3. Vitamin $B_6$ adsorbed PC | 0.075±0.007 | 0.600±0.025 | 0.65 |
| 4. Vitamin D adsorbed PC | 0.042±0.004 | 0.690±0.02 | 0.32 |
| 5. Vitamin E adsorbed PC | 0.068±0.004 | 0.560±0.03 | 0.63 |
| 6. 0.2 mm Vit.$B_6$ in solution | 0.066±0.006 | 0.540±0.03 | 0.64 |
| 7. 2 mg% Vit.E in solution | 0.067±0.004 | 0.540±0.06 | 0.65 |
| 8. 0.5 mg% Aspirin in solution | - | 0.606±0.03 | - |
| 9. 0.5 mg% Persantine | - | 0.580±0.034 | |
| 10. 0.5 mg% ASP+2 mg% Persatine | - | 0.500±0.018 | |
| 11. 0.5 mg% ASP+1.5 mg% Vit.C | - | 0.480±0.02 | |
| 12. 0.5% Ethyl alcohol | 0.053±0.007 | 0.630±0.02 | 0.43 |

* - PC surfaces exposed to 50 mg% of each substances in their respective solvents for 3 hours, avoiding the air/ water interface (2 to 5) and their soluble forms (6 to 12).

+ - protein mixture containing 25 mg% albumin, 15 mg% ɣ-globulin and 7.5 mg% Fg in 0.1 M phosphate buffer, exposed to all substrates for 3 hours, taking care of the air/water interface. Represented the surface concentration in $\mu g\ cm^{-2}$, with standard deviation from at least three separate experiments, 'p' < 0.05.

TABLE 5-XI

**PLATELET ADHESION,AND PROTEIN ADSORPTION ON VARIOUS POLYMER SUBSTRATES EFFECT OF ASPIRIN-VITAMIN COMBINATION**

| Surfaces | platelet adhesion* per $mm^2 \pm SD$ | 3 Hrs.protein adsorbed in $\mu g\ cm^{-2} \pm SD^+$ Albumin | Fibrinogen |
|---|---|---|---|
| Bare polymers (A) | | | |
| 1. Polycarbonate (PC) | 17.90±3.0 | 0.0514±0.006 | 0.645±0.04 |
| 2. Nylon 6,6 | 31.07±3.2 | 0.13±0.02 | 1.370±0.03 |
| 3. Polystyrene | 15.00±1.9 | - | 0.900±0.04 |
| 4. Teflon FEP | 9.83±1.7 | 0.056±0.003 | 0.810±0.07 |
| ASP-vit.combination (A-Vit.) (0.5mg%ASP: 1.5mg% vit.C:0.15mM vit.$B_6$:2mg% Vit.E) | | | |
| 5. PC+(A-Vit.) | 6.0±2.4 | 0.0565±0.003 | 0.575±0.075 |
| 6. Nylon+(A-Vit.) | 17.3±2.4 | 0.139±0.01 | 0.820±0.030 |
| 7. Polystyrene+(A-vit.) | 8.8±2.3 | - | 0.660±0.070 |
| 8. Teflon+(A-vit.) | 4.7±2.0 | 0.065±0.005 | 0.690±0.015 |

* - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 30 observations from 4 separate experiments)

+ - A protein mixture containing 25 mg% albumin 15mg% γ-globulin and 7.5 mg% Fg exposed to various polymer substrates for 3 hrs, taking care of the air/water interface. Represented the surface concentration in $\mu g\ c\ m^{-2}$ with standard deviation, from at least 3 separate experiments, 'p' < 0.05.

So the modification of blood with a combined vitamin thereapy with low doses of aspirin is proposed from these in vitro observations. The beneficial role of aspirin to inhibit the cyclo-oxygenase as an oral anticoagulant by limiting the unacceptable bleeding problems[53] has been utilized with a combination of vitamin therapy towards reducing the antithrombotic character with least side effects. Kinetics of protein adsorption to various substrates have indicated that combination of aspirin-vitamin C-vitamin $B_6$-vitamin E appeared to reduce the surface-Fg binding, which, itself, may be one of the parameters for blocking platelet-surface interaction. The purpose of combined drugs is to utilise the beneficial antiplatelet effect of each and keeping each within reasonable dosage. The antiplatelet drugs in their combinations can also extend their action probably due to the competitive drug effect for the receptor modulation and the inhibition. The in vitro experiments presented here are not sufficient to speculate the interactions and more clinical trials are needed to explain the complex in vivo system.

In brief, these studies demonstrate theinter-relation of Fg surface concentration and platelet adhesion at the interface, which may be related to Fg receptor sites available for the interaction. The modification of an artificial surface along with the modification of blood itself with a combination of drug therapy, may be beneficial towards enhancing the blood compatibility of an artificial surface.

## 5.4. Effect of aminosugars, heparin and polyelectrolyte towards Fg-surface interaction

Glucosamine, galactosamine and a polyelectrolyte (synthesized from natural rubber)have shown anticoagulant and antiplatelet activities[60.306]. Further studies related to other coagulation assays and Fg adsorption kinetics are attempted to correlate their anticoagulant and antiplatelet activity with Fg surface binding. Coagulation times of various substances pre-adsorbed PC substrates are shown in Table-5-XII. Plasma thromboplastin time indicates that galactosamine, albumin and vitamin $B_6$ adsorbed surfaces have

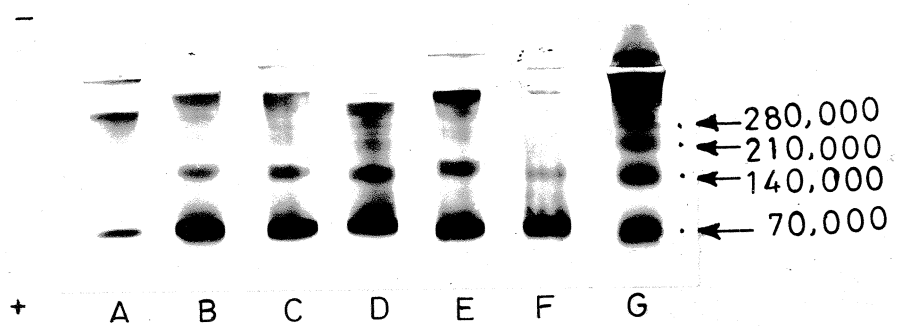



Fig:5-22 PAGE(7%) of desorbed proteins from PC stained with Coomassie blue. Mixture of proteins exposed to PC(A) and in presence of 50 mg% glucosamine(B), galactosamine(C), mannosamine(D), heparin(E), polyelectrolyte(F), and molecular weight marker proteins(G), respectively.

prolonged the clotting tendency, while glucosamine and mannosamine do not have any significant effect. Heparin and polyelectrolyte preadsorbed PC substrates have demonstrated an enhanced PTT or stypven clotting time.

The protein-surface binding in presence of these aminosugars, polyelectrolyte and heparin have also been investigated, from a protein mixture, using polyacrylamide gel electrophoresis. Glucosamine and galactosamine demonstrate an enhanced albumin surface concentration with a slight decrease in fibrinogen surface level in the later case as depicted in Fig.5-22. Heparin and mannosamine effect towards protein-surface interaction is not significant. The synthetic polyelectrolyte has enhanced the albumin and has reduced the fibrinogen and γ-globulin bands tremendously. Further, the kinetics of fibrinogen binding to PC as a function of time has been presented in Fig.5-23. It suggests that galactosamine inhibits the surface-Fg adsorption significantly, while other aminosugars do not have much effect. A part of the initially adsorbed Fg is removed from the substrate in all cases as depicted in Fig.5-24, due to exchange/replacement with the proteins in the media.

It has been suggested[60] that glucosamine and galactosamine inhibit the platelet-surface binding and enhance the coagulation time, but in case of mannosamine none of the above effects have been found to be significant. The polyelectrolyte has also shown antiplatelet and anticoagulant activity[306]. This may be due to the presence of both sulfamate and carboxylate groups arranged in a steric manner in the molecule as that of heparin.

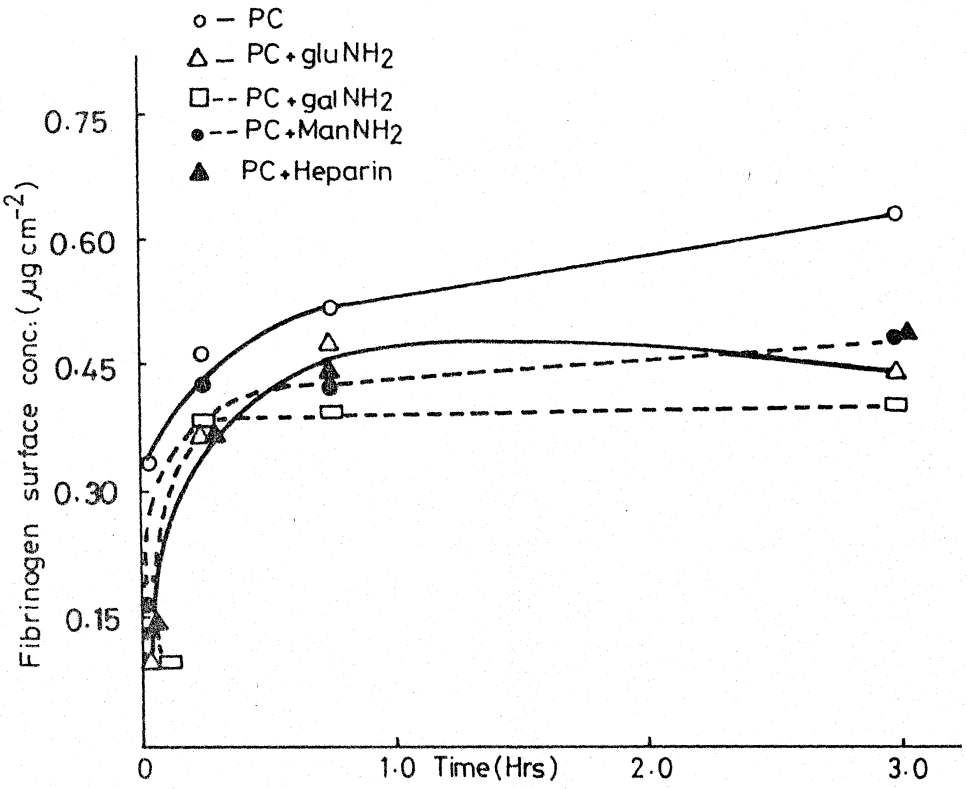



Fig. 5-23. Adsorption of fibrinogen as a function of time from protein mixture with aminosugars or Heparin in solution.

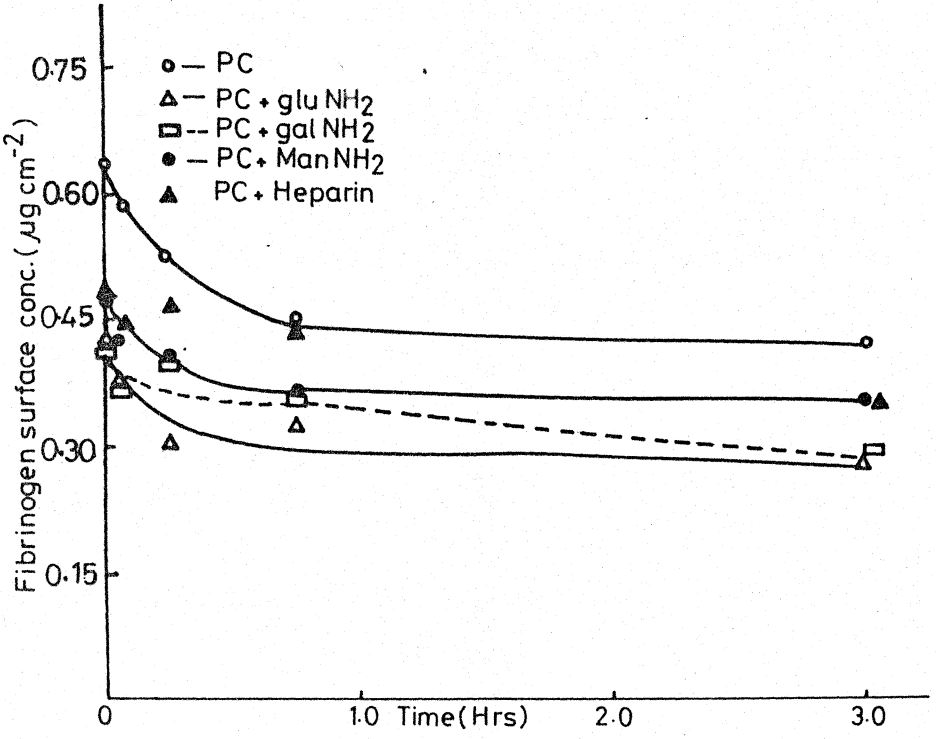



Fig. 5-24. Desorption of preadsorbed (3hrs) fibrinogen as a function of time (from protein mixture with aminosugars or Heparin in solution).

TABLE 5-XII

**COAGULATION TIMES OF VARIOUS PREADSORBED SUBSTRATES**

| Surfaces* | Stypven clotting+ time in seconds ± SD | Plasma‡ thromboplastin time in seconds ± SD |
|---|---|---|
| 1. Bare glass | 21.3±1.3 | 64.4±2.4 |
| 2. PC coated glass | 27.7±0.9 | 72.7±2.0 |
| 3. Albumin adsorbed PC | 33.2±2.2 | 91.0±0.8 |
| 4. Fg adsorbed PC | 24.1±1.2 | 71.2±1.8 |
| 5. Glucosamine ads. PC | 30.9±1.2 | 88.5±2.2 |
| 6. Galactosamine ads.PC | 31.4±0.9 | 92.7±2.5 |
| 7. Mannosamine ads. PC | 30.3±0.6 | 74.9±2.8 |
| 8. Polyelectrolyte ads.PC | 61.4±2.0 | 192.1±4.1 |
| 9. Heparin adsorbed PC | 189.7±5.4 | 346.9±4.4 |
| 10. Vit. $B_6$ adsorbed PC | 33.0±0.8 | 105.4±2.7 |

* - PC surfaces exposed to 50 mg% of each substances in 0.1 M phosphate buffer pH 7.4 for 3 hours, avoiding the air/water interface.

+ - Values are the average of the stypven clotting time in seconds with standard deviation (from 5 experiments, 'p'$<0.05$)

‡ - Values are the average of the plasma thromboplastin time (PTT) in seconds with standard deviation (from 5 experiments) 'p' $\leq 0.05$).

Hence it seems, the stereoregularity of the aminosugars play an important role towards subsequent interfacial phenomena, since the three analogs studied are having the same molecular weight and common molecular structure, with slight changes in the arrangement of functional groups in the gluco-pyranose ring at $C_2$, $C_4$ positions. So it is possible that the spacial arrangement of groups at the surface can alter the protein-polymer interaction along with its antiplatelet and anticoagulant activities. The reduced Fg or enhanced albumin binding to the polymer with these molecules may be one of the parameters for its antiplatelet activity via the modulation of Fg receptors from platelet binding.

## 5.5. Effect of antibiotics towards fibrinogen/platelet-surface interaction.

Antibiotics are frequently used for protecting patients with implants from bacterial growth. The present studies reported here with antiplatelet drugs and vitamins, to modulate protein-surface interaction, has provided an insight to search the role of these antibacterial agents at the interface. Lymphocytes participate in developing the body's immunity against foreign materials (antigen) by producing antibodies. This study examines the effect of five different antibiotics, namely neomycin, gentamycin, ampicillin, penicillin G and streptomycin, on the presence and absence of lymphocytes in protein-polycarbonate surface interaction using $^{125}I$ albumin or $^{125}I$ fibrinogen from a protein mixture. The effect of these antibiotics to modify the surface induced platelet attachment has also been investigated to inter-relate the interfacial phenomena.

The adhesion of platelets to PC has been modified by the addition of antibiotics, to variable degrees, as can be seen from Table-5-XIII. Streptomycin demonstrates a maximum reduction in platelet adhesion to the PC substrate in comparison with other antibiotics studied. The normal doses of different antibiotics used in clinical conditions usually vary and may be used alone or in combinations. The amounts of certain antibiotics, such as ampicillin-500 mg (every 6 hours), neomycin-350 mg (every 8 hours), streptomycin-1000 mg per day, penicillin-G-400,000 to 1000,000 units per day, and gentamycin-40 mg, twice a day, have been normally prescribed during postoperative period. The present studies basically correlates the same prescribed amounts for the duration of these in vitro investigations.

It has been suggested that several antibiotics can inhibit platelet aggregation in vivo and in vitro and can cause bleeding due to intake of large doses[313]. These observations support the present results of platelet-surface attachment due to antibiotics and warrant the need of further investigations to use them in larger quantities. Voss et al[344] has proposed that penicillin- G inhibits granulocyte aggregation in vitro, probably via the interaction of this molecule with the phospholipids of the cell membrane, which may play a crucial role in mediating these effects. So it seems that these antibiotics may bind with platelets or to polymer surface, which modifies or masks the platelet receptor sites for the Fg molecule or the polymer substrate itself and causes the reduction in platelet density on the substrate. Now, the studies of protein-surface binding and their modulation due to these antibiotics may be interesting.

Figure 5-25 shows the pattern of albumin adsorption to the polycarbonate surface in the presence of antibiotics. It seems, gentamycin, streptomycin and penicillin demonstrate an increase in surface-albumin binding, whereas neomycin and ampicillin do not have much effect. A maximum surface-albumin adsorption is observed with gentamycin infused system as is evident from Fig.5-25. A part of the preadsorbed albumin from antibiotic protein system desorbs off from the polymer substrate due to self exchange or with other proteins in the media, as demonstrated in Fig.5-26. The loss of preadsorbed albumin from the gentamycin-protein system has been least from the PC substrate in comparison with other antibiotic infused systems.

Figure 5-27 indicates the adsorption kinetics of albumin to the polymer substrate in the presence of lymphocytes and antibiotics. Albumin surface concentration is not altered with the infusion of washed lymphocytes in the protein system; however, the addition of antibiotics to the lymphocyte-protein system; slightly changes the adsorption phenomenon. Penicillin-G and streptomycin have indicated an increase of initial surface-albumin binding in the lymphocyte-protein system; but neomycin has shown a reduction. However, with increase of exposure time upto 3 hours, the antibiotics infused lymphocyte-protein system do not show much significant changes in the albumin surface level in comparison to the control system; (antibiotics excluded system), as is evident from Fig.5-27. The desorption pattern of albumin preadsorbed for 3 hours from the lymphocyte-protein system in the presence of antibiotics is depicted in Fig.5-28. Here the antibiotics-incubated system do not show significant differences, in comparison

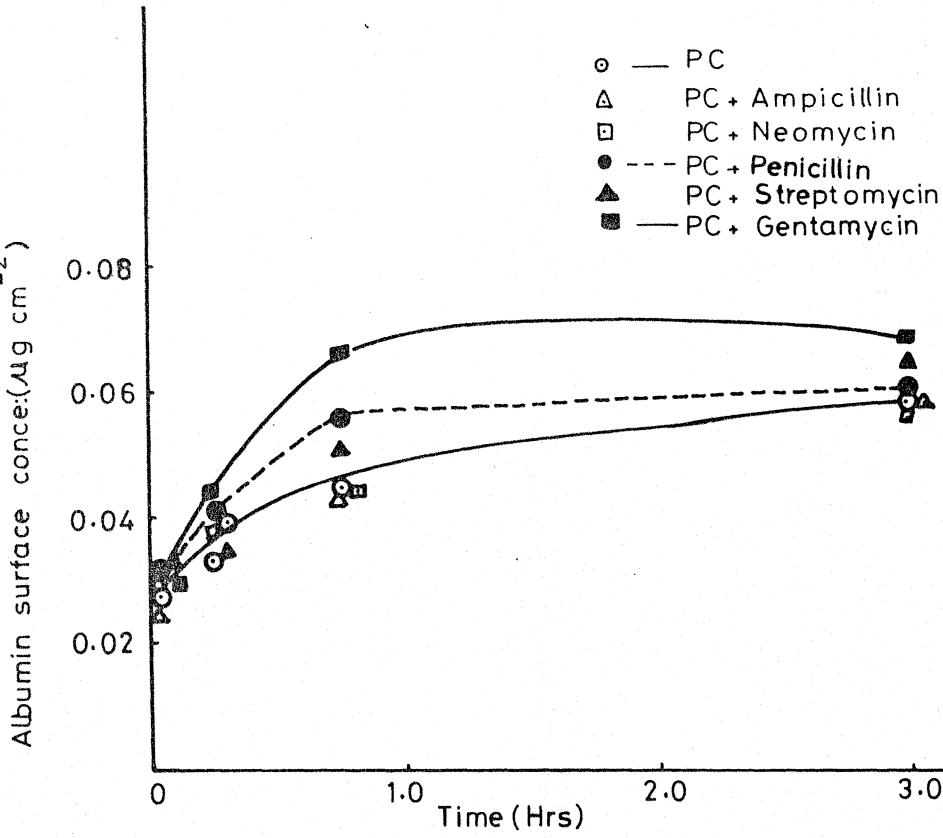



Fig. 5-25. Adsorption of albumin on PC as a function of time from protein mixture. Effect of antibiotics.

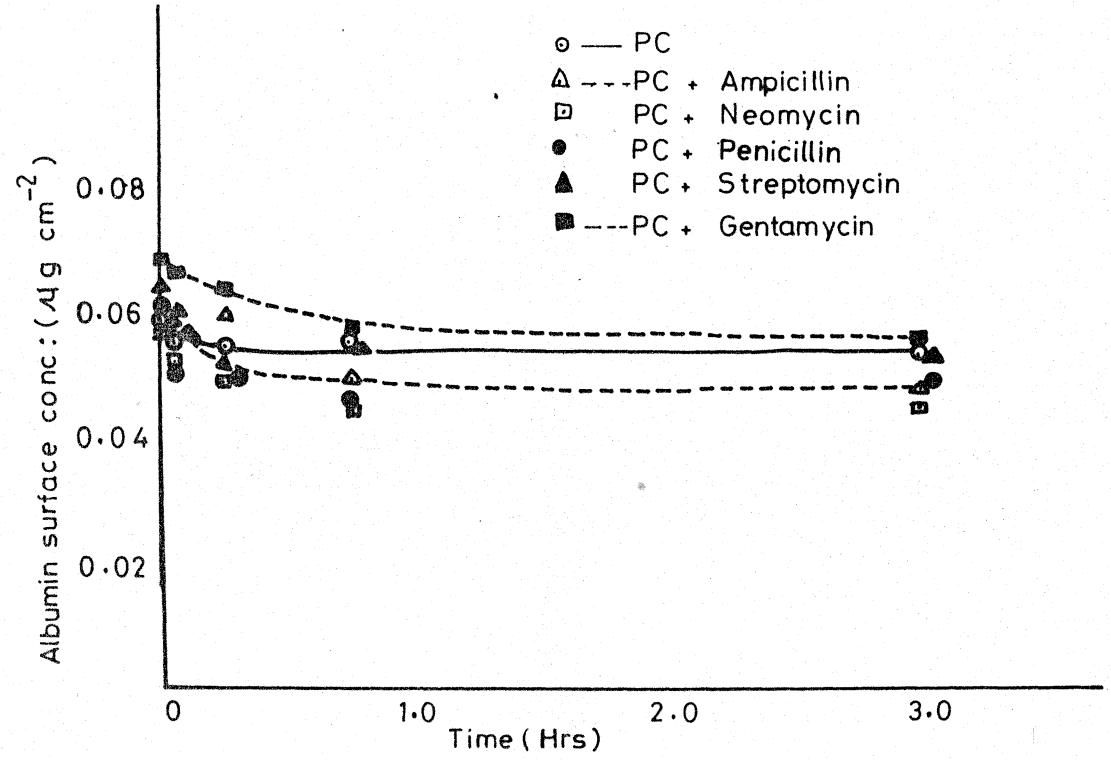



Fig. 5-26. Desorption of albumin from PC as a function of time in protein mixture.

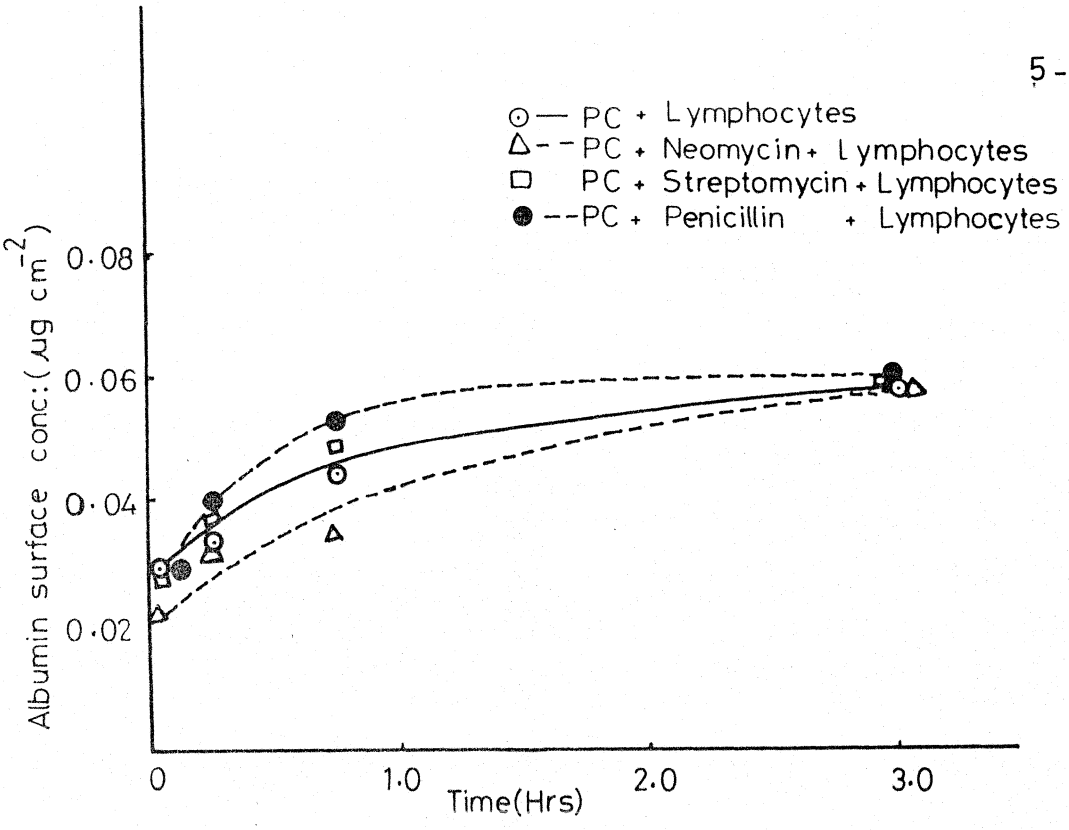



Fig.5-27. Adsorption of albumin on PC as a function of time from protein mixture. Effect of antibiotics and lymphocytes.

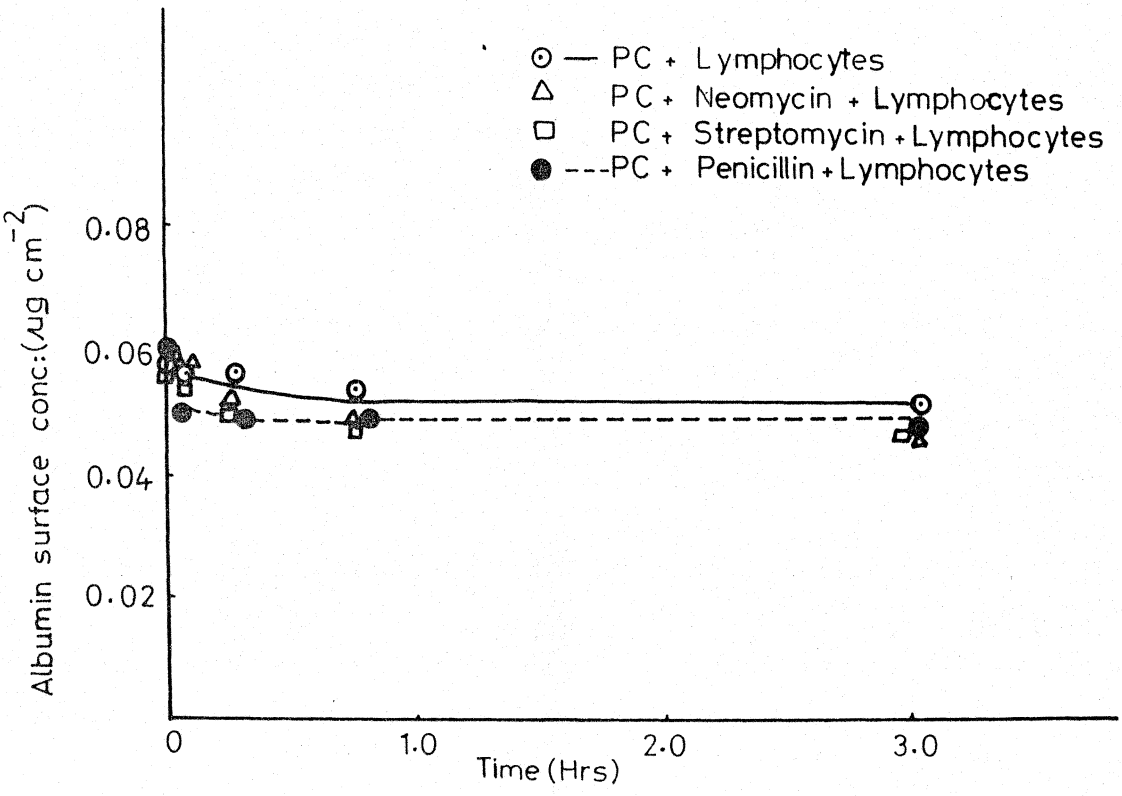



Fig.5-28. Desorption of albumin from PC as a function of time in protein mixture. Effect of antibiotics and lymphocytes.

TABLE 5-XIII

**EFFECT OF ANTIBIOTICS ON PLATELET ADHESION AND FIBRINOGEN ADSORPTION TOWARDS POLYCARBONATE.**

| Polycarbonate surfaces* exposed to antibiotics | Mean platelets ± SD+ | Fibrinogen‡ Surface concn. ($\mu g\ cm^{-2}$) |
|---|---|---|
| 1. Bare polycarbonate (PC) | 18.10±2.1 | 0.650±0.09 |
| 2. PC + 20 mg% Neomycin | 10.48±1.98 | 0.550±0.02 |
| 3. PC + 1 mg% Gentamycin | 10.90±1.85 | 0.610±0.03 |
| 4. PC + 10 mg% Ampicillin | 10.51±1.88 | 0.610±0.06 |
| 5. PC + 80 units/ml Penicillin-G | 10.28±1.93 | 0.549±0.02 |
| 6. PC + 20 mg% Streptomycin | 8.20±1.85 | 0.337±0.02 |

* - Platelet suspension containing the antibiotics were exposed to PC (as demonstrated in surfaces 1 to 6)

\+ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 30 observations from three separate experiments, 'p' < 0.05).

‡ - A protein mixture exposed to polycarbonate substrate for 3 hours, taking care of the air/water interface. Represented the surface concentration in $\mu g\ cm^{-2}$ with standard deviation, from at least 3 separate experiments, 'p' $\leq$ 0.05.

with the control system. It is possible that a part of the preadsorbed albumin is removed by exchange with other proteins or with unlabelled albumin in a similar fashion to all these cases.

Figure 5-29 shows the pattern of fibrinogen adsorption to the polycarbonate surface in the presence of antibiotics. The antibiotics inhibit the surface-Fg binding in this order: streptomycin > penicillin-G > ampicillin $\simeq$ gentamycin $\simeq$ neomycin. Preadsorbed Fg from the antibiotics - protein system desorbs off via exchange from the polymer substrate as demonstrated in Fig.5-30. The loss of preadsorbed Fg from the streptomycin-protein system is maximum from the PC substrate, in comparison with other antibiotic infused systems.

Figure 5-31 indicates the adsorption kinetics of Fg to the polymer substrate in the presence of lymphocytes and antibiotics. Fibrinogen surface concentration is not altered with the infusion of washed lymphocytes in the protein system; however, the addition of antibiotics to the lymphocyte-protein system dramatically changes the adsorption phenomenon. Antibiotics have inhibited the initial surface-Fg binding in the lymphocyte protein system; which has been greatly enhanced with time. The desorption pattern of Fg preadsorbed for 3 hours from the lymphocyte-protein system in the presence of antibiotics has been reversed when lymphocytes have been excluded. (Compare figures 5-30 and 5-32). Here the penicillin-incubated system retained the maximum Fg on the PC substrate in comparison with other

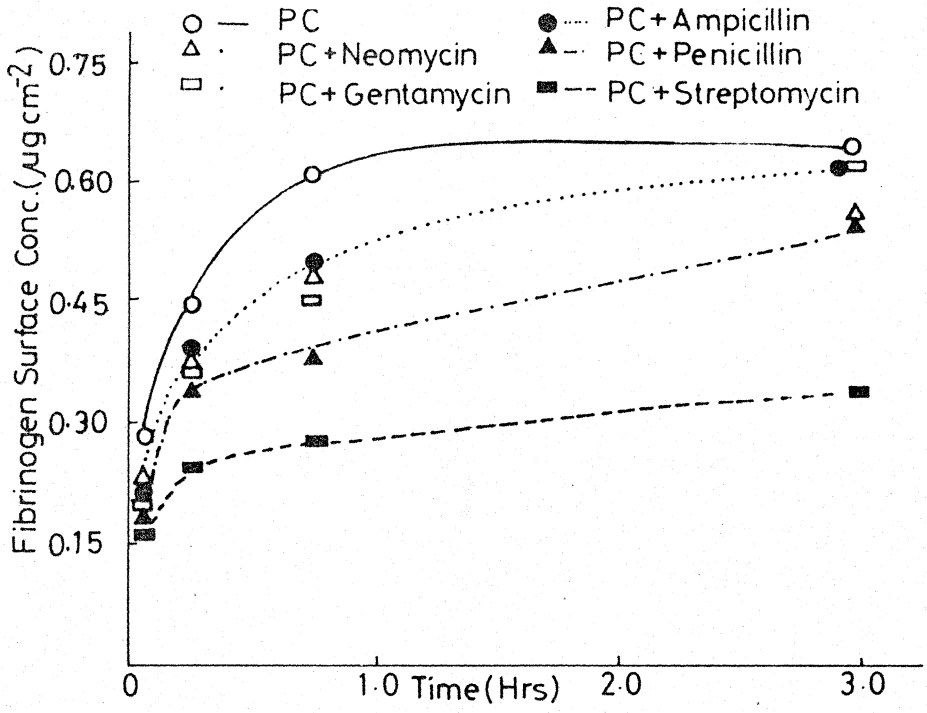



Fig.5-29. Adsorption of fibrinogen on PC as a function of time from Protein mixture. Effect of antibiotics.

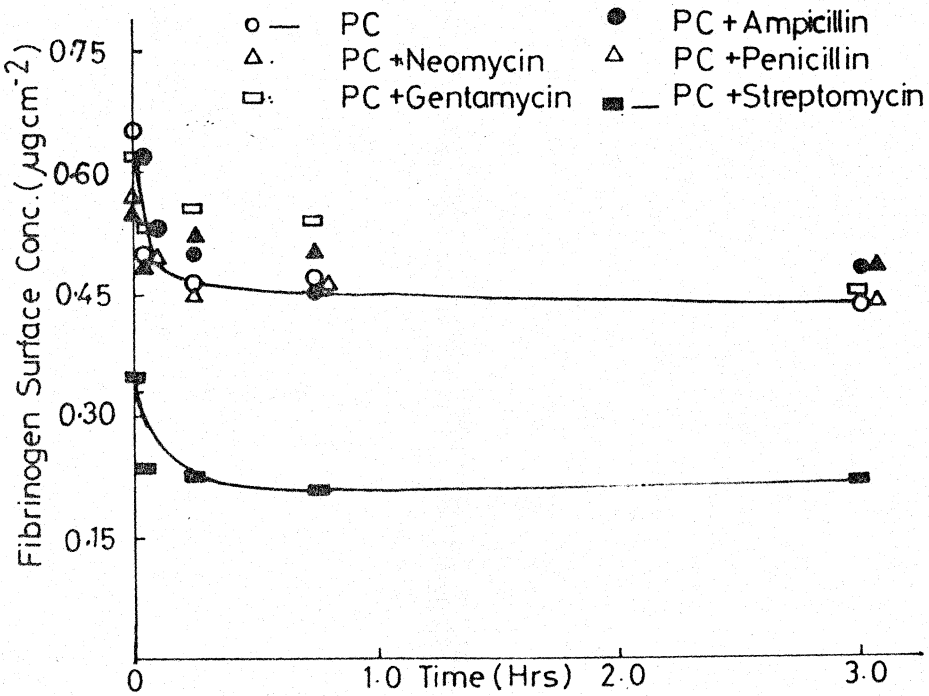



Fig.5-30. Desorption of fibrinogen from PC as a function of time in protein mixture.

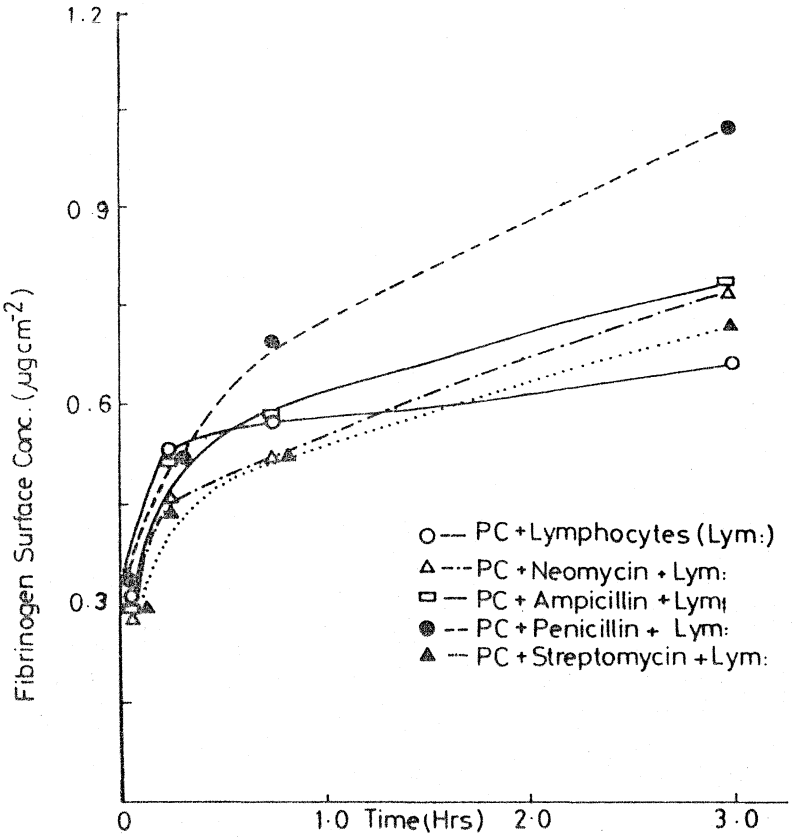



Fig 5-31. Adsorption of fibrinogen on PC as a function of time from protein mixture. Effect of Lymphocytes & antibiotics.

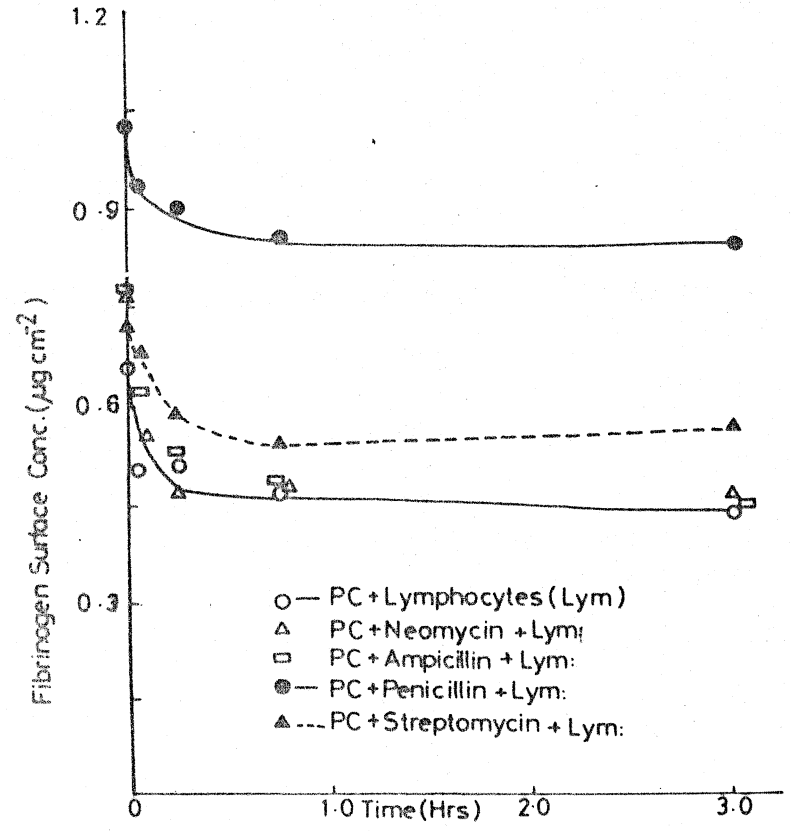



Fig. 5-32. Desorption of preadsorbed fibrinogen from PC as a function of time in to protein mixture.

systems (Fig.5-32). It is possible that initially adsorbed Fg is removed by exchange with other proteins or with unlabelled Fg.

The protein adsorption studies described here clearly indicate an increase of albumin or decrease of fibrinogen binding to surfaces with certain antibiotics. The observed changes in Fg adsorption to the PC substrate in the presence of lymphocytes are interesting because infusion of antibiotics causes an initial reduction in Fg-surface concentration, which is enhanced with time. The effect of penicillian G in inhibiting granulocyte aggregation has been indicated via membrane modulation. Thus, it appears that the antibiotic-perfused lymphocytes may become sensitized with time, causing a profound increase in surface-bound Fg. The studies reported here are quite interesting with respect to the inhibition of Fg binding and an increase in albumin adsorption to PC substrate with certain antibiotics. This itself may be one of the parameters for blocking platelet surface interaction.

Hence it appears that, controlled antibiotic thereapy during the postoperative period may be beneficial to avoid or limit high doses of antiplatelet drugs (which may cause bleeding problems) for patients having an artificial implant. The antithrombotic and antibacterial effects of antibiotics can be advantageous for a normal haemostasis. A better understanding of the mechanism of antibiotics is needed in in vivo conditions for clinical practice.

# CHANGES IN FIBRINOGEN - SURFACE BINDING DUE TO BLOOD CELLS AND CYCLIC AMP

# CHAPTER VI

## 6.1. Effect of blood cells

The participation of blood cell accumulation in haemostasis and thrombosis has been known for many years. However, the role of these blood cells, and their interaction process with an artificial substrate to modulate the protein-surface binding is meagre. The present study examines the effect of various blood cells, such as white blood cells, red blood cells and platelets in protein-polycarbonate surface interaction from a protein mixture, using tracer techniques.

The adsorption pattern of albumin to the polycarbonate substrate in the presence of blood cells and a protein mixture is indicated in Fig.6-1. The adsorption of albumin to the polymer surface is decreased with all blood cells with a maximum reduction with platelet system as is evident from Fig.6-1. On the other hand, fibrinogen-surface binding is enhanced with white blood cells infused protein system, as demonstrated in Fig.6-2. RBC and platelets have indicated an increase of initial surface-Fg binding from their protein system, however increase in exposure time up to 3 hours, do not show significant changes in comparison to the control system.

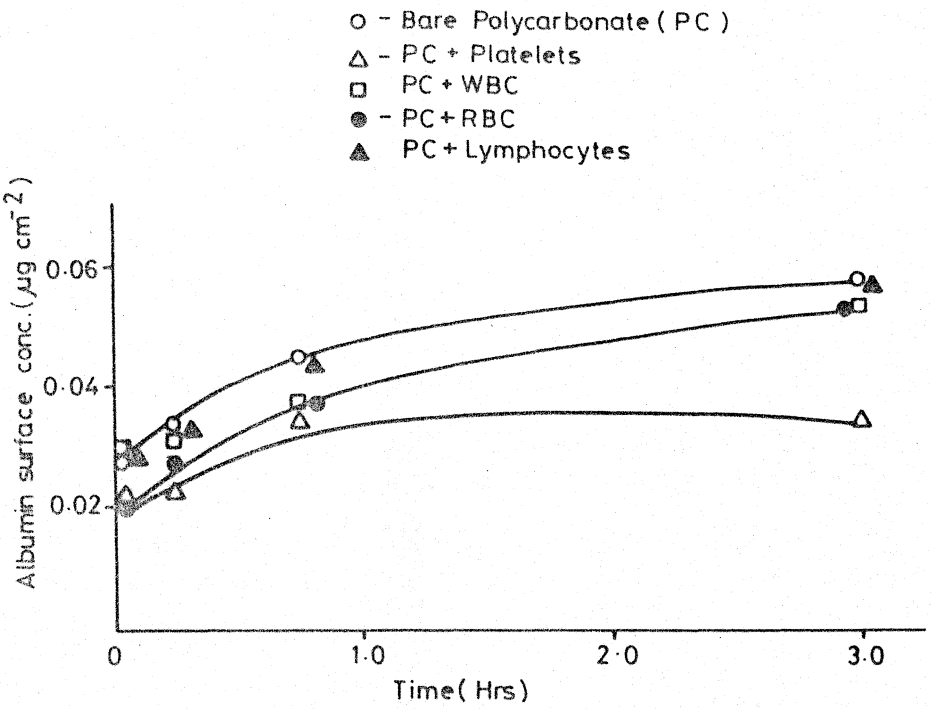



Fig. 6-1. Adsorption of albumin as a function of time, from protein mixture, in presence of blood cells.

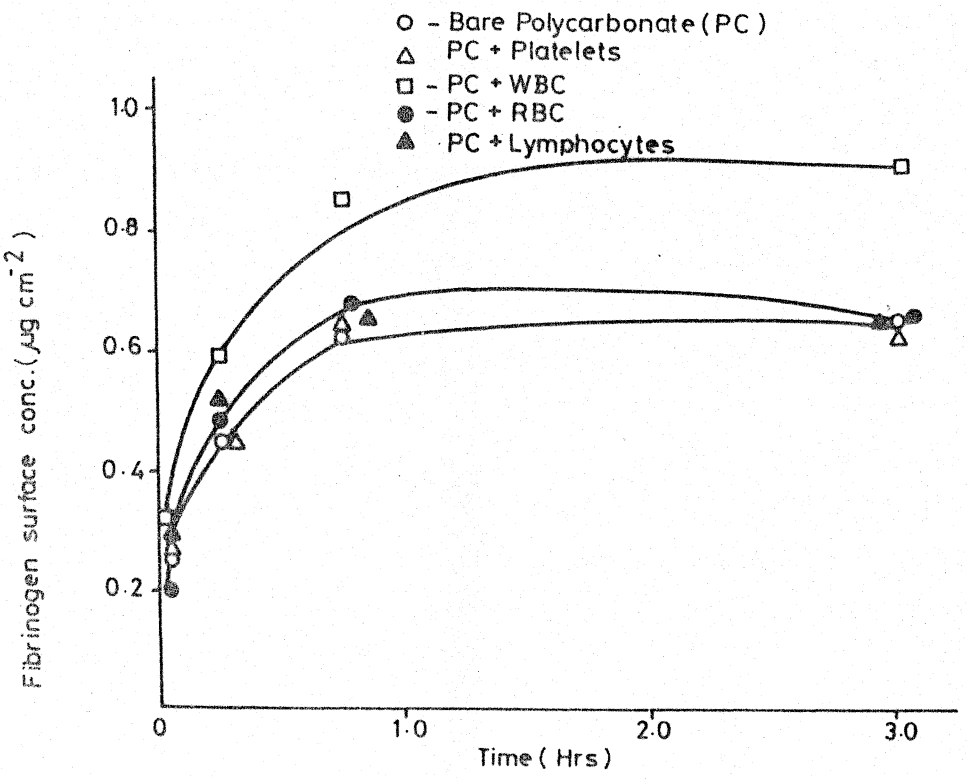



Fig. 6-2. Adsorption of fibrinogen as a function of time from protein mixture, in presence of blood cells.

Brash et al.[42] have indicated that the addition of red blood cells to buffered solutions of plasma proteins caused a decrease in the quantity of protein adsorbed from these solutions to a polyethylene surface. It is also suggested[177] that the red cell membrane plays a key role in the 'red cell effect' to reduce the protein adsorption to an artificial surface. Such an effect appears to be possible with platelets and also with white blood cells. This supports the observed reduction in albumin-surface binding reported here in presence of blood cells. However, the results indicated here with fibrinogen-surface binding are interesting, since the Fg-surface concentration has been increased with blood cells.

Vroman et al[347,349] have proposed that platelets adhere only where fibrinogen remains adsorbed, and in the absence of erythrocytes, the ring of platelets deposited is smaller. So, it is possible that the blood cells can cause an augmented fibrinogen adsorption to the surface, which may be the cell membrane related effect, probably via the receptors. There is now convincing evidence that Fg is involved in ADP induced platelet aggregation[25,219] and that it enhances platelet adhesion to collagen fibers[165]. It is also known that Fg is an important factor in redcell aggregation in vitro and in vivo[381]. These observations clearly suggest the importance of Fg molecule in cell adhesion. It seems, more Fg may bind with the cell receptors, and these Fg modified cells may deposit on surface contact, which may have caused the observed increase in Fg-surface concentration.

## 6.2. Cyclic AMP-a cell mediator to enhance blood compatibility

The central role of adenosine-3',5' - cyclic monophosphate (cyclic AMP, cAMP) as a mediator of a number of physiological and pathological responses (second messenger) has been recognized with increasing frequency in the last few years. Since most of the antiplatelet agents act via this molecule for their antiplatelet activity, it may be interesting, to search its role at the interface, to modulate the protein-surface binding and the subsequent thrombus formation. Hence, studies have been performed via the immobilization of cAMP on an albuminated polymer matrix to prevent the adhesion of platelets and eventual thrombosis. The effect of soluble cAMP to modify the platelet-surface binding and protein adsorption using PAGE and labelled proteins have also been investigated. Thrombin time and octane contact angle have been performed for further understanding of the interfacial phenomena.

### Antiplatelet and anticoagulant properties of surface immobilized cAMP.

Results of octane contact angle and platelet adhesion to modified polycarbonate surfaces are shown in Table 6-1. It seems, immobilization of albumin makes the surface hydrophilic in nature, (surface 6, in Table 6-1, where the octane contact angle has increased, in comparison with bare substrate) however, cAMP makes the surface slightly hydrophobic (i.e. the octane contact angle has reduced to that of bare surface). On the other hand, the number of adhering platelets seen on cAMP or albumin

TABLE 6-I

CONTACT ANGLE AND PLATELET ADHESION ON cAMP MODIFIED POLYCARBONATE SURFACES

| Surfaces* | Octane[+] contact angle±SD | Mean[±] platelets per $mm^2$±SD | Mean[§] platelets per $mm^2$±SD |
|---|---|---|---|
| 1. Bare polycarbonate(PC) | 115.5±2.0 | 17.8±2.0 | 42.3±3.5 |
| 2. PC+Albumin (Alb) | 127.0±2.7 | 10.0±1.8 | 35.0±3.7 |
| 3. PC+cAMP | 87.4±2.4 | 9.5±1.7 | 28.7±3.2 |
| 4. PC+Alb+Glutaraldehyde (Glut) | 129.0±1.5 | 9.7±1.9 | 33.9±3.1 |
| 5. PC+cAMP+Glut.+cAMP | 97.5±2.6 | 4.8±1.9 | 17.7±2.7 |
| 6. PC+Alb.+Glut.+Alb. | 137.5±2.6 | 5.2±1.9 | 22.2±2.9 |
| 7. PC+Alb.+Glut.+cAMP | 112.0±3.6 | 5.0±1.8 | 19.3±2.4 |

* - All surfaces demonstrated from 1 to 7 were in their adsorbed forms. PC films were initially exposed to 100 mg% albumin or 20 mg% cAMP (surfaces 2 & 3) respectively for 3 hours, avoiding air/water interface[300]. They were further exposed to 2.5% glutaraldehyde for one hour (surface 4) and finally coupled the unlinked aldehyde group of glutaraldehyde with 100 mg% albumin (surface 6) or 20 mg% cAMP (surfaces 5 & 7) in 0.1M phosphate buffer, pH 7.4 at $4^{o}C$ overnight.

+ - Octane contact angle in degrees to various surfaces with standard deviation (at least 30 observations).

ǂ - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 30 observations).
§ - Fibrinogen (50 mg%) induced platelet adhesion (per $mm^2$) to the surface.

immobilized surfaces have been decreased dramatically when compared with the bare surface, as demonstrated in Table 6-1. Fibrinogen induced platelet adhesion to PC substrate has also been modified with albumin or cAMP immobilized substrates.

The adhesion of platelets to polycarbonate substrate is inhibited by the addition of cAMP. As shown in Table-6-II, 20 $\mu$ molar cAMP considerably inhibits the adhesion of platelets to the PC surface, and so the effect of cAMP appears to be concentration dependent. Fibrinogen (50 mg%) induced platelet adhesion to PC is also substantially modified with varying concentrations of cAMP

Results of the thrombin time measurements are presented in Table-6-III. Thrombin coagulation has been inhibited by cAMP adsorbed surfaces. The cAMP immobilized surfaces (double layer of cAMP) demonstrate better anticoagulant property compared to albuminated surfaces. Thus, the present studies clearly suggest the antiplatelet and anticoagulant properties of this cellular mediator at the interface.

TABLE 6-II

CONCENTRATION EFFECT OF cAMP ON PLATELET ADHESION TOWARDS PC SURFACE WITH AND WITHOUT FIBRINOGEN

| cAMP Concentration | Mean platelets* per $mm^2$ ±SD | |
|---|---|---|
| | A | B |
| 1. Bare polycarbonate (PC) | 17.80±2.0 | 39.2±5.0 |
| 2. PC+5 μM cAMP | 12.60±2.0 | 31.6±2.5 |
| 3. PC+10 μM cAMP | 9.50±3.0 | 29.3±2.6 |
| 4. PC+20 μM cAMP | 3.50±1.7 | 18.2±2.3 |
| 5. PC+50 μM cAMP | 2.60±1.7 | 14.5±2.6 |
| 6. PC+100 μM cAMP | 2.17±1.7 | 13.3±2.7 |

A - platelet suspension containing varying concentrations of cAMP in solution were exposed to PC

B - Similar sets of experiments as indicated in 'A' in presence of 50 mg% fibrinogen.

* - Values expressed as the average of the number of platelets adhered to the surface per $mm^2$ with standard deviation (at least 30 observations from triplicate experiments).

TABLE 6-III

THROMBIN CLOTTING TIME OF cAMP MODIFIED POLYCARBONATE SURFACES

| Surfaces | Mean time in seconds* ±SD |
|---|---|
| 1. Bare glass | 21.55±1.1 |
| 2. Bare polycarbonate (PC) | 24.08±0.8 |
| 3. Albumin adsorbed PC | 31.84±1.1 |
| 4. cAMP adsorbed PC | 32.64±0.6 |
| 5. Alb. adsorbed PC+Glutaraldehyde(Glut) | 34.38±1.1 |
| 6. cAMP adsorbed PC+Glut.+ cAMP | 61.22±2.3 |
| 7. Alb.adsorbed PC+Glut.+ Alb | 40.00±1.0 |
| 8. Alb. adsorbed PC+Glut.+cAMP | 38.50±1.2 |

* - Thrombin time in seconds with standard deviation (at leat from 5 experiments).

## Role of cAMP to modulate the fibrinogen-surface binding

The pattern of surface-protein binding, and their modulation due to cAMP may be interesting at this stage; which can provide a better understanding of the interfacial problem of thrombus development. Hence, the affinity of cAMP molecule towards protein-polymer interaction from a protein mixture has been investigated, using polyacrylamide gel electrophoresis, after desorbing the proteins from the substrate. Figure 6-3 demonstrates the polyacrylamide gel

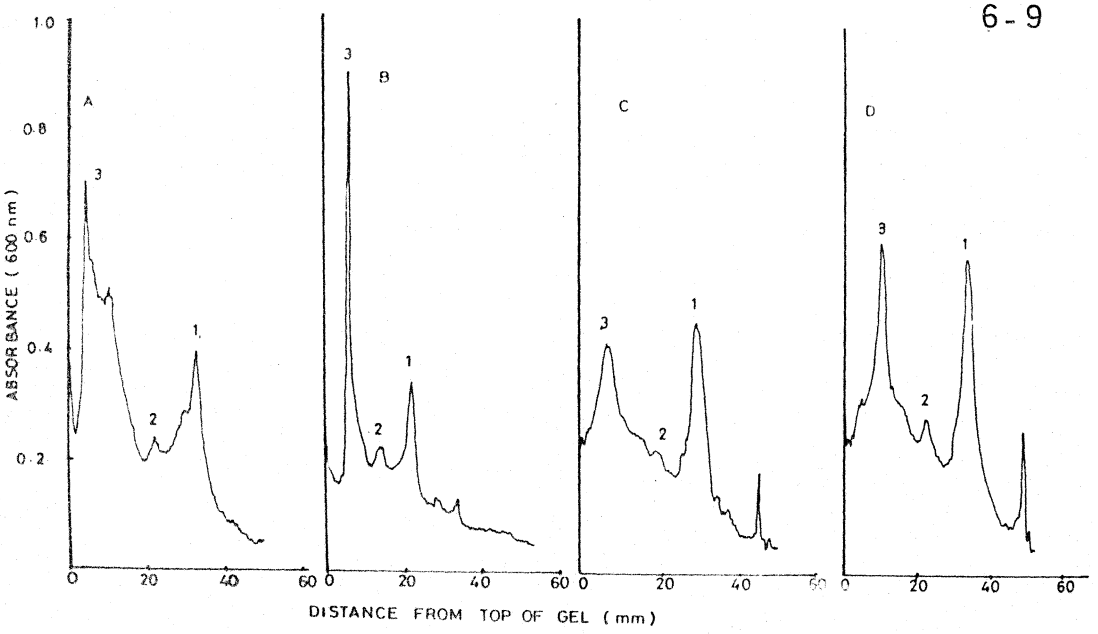



Fig: 6-3. Profiles of PAGE (7%) of desorbed proteins from PC stained with Coomassie blue. Mixture of proteins containing 25 mg% albumin, 15 mg% γ-globulin and 7.5 mg% Fg exposed for 15 minutes to PC (A), c AMP adsorbed PC (B), c AMP in solution (C) and c AMP + Glut + c AMP modified PC (D), respectively.

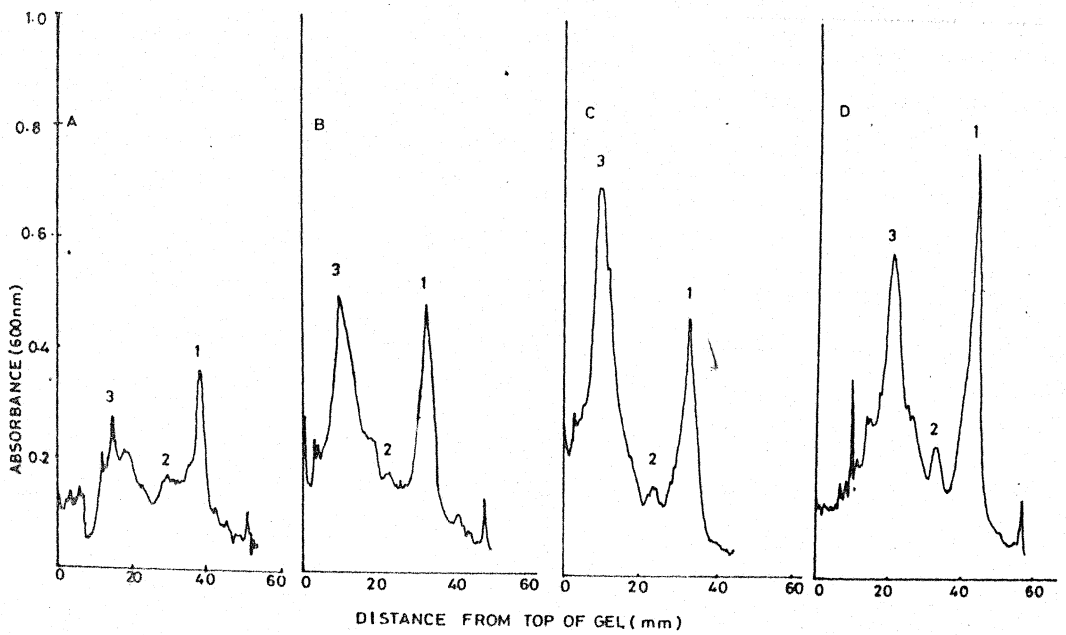



Fig: 6-4. Profiles of PAGE (7%) of desorbed proteins from PC stained with Coomassie blue. Mixture of proteins containing albumin, γ-globulin and Fg exposed for 3 hours to PC (A), c AMP adsorbed PC (B), c AMP in solution (C) and c AMP + Glut + c AMP modified PC (D), respectively.

electrophoresis pattern of the proteins eluted from various PC substrates, (modified with cAMP and cAMP in solution) which have been exposed to protein mixture for 15 minutes. It seems, the cAMP immobilized PC substrate and the soluble cAMP, have reduced the Fg-surface binding and enhanced the albumin adsorption in comparison to the bare or cAMP adsorbed PC substrates. The molar ratios of albumin to fibrinogen also stresses this point. (Alb/Fg ratios, for bare PC->0.38, cAMP adsorbed PC-> 0.42, cAMP in solution->1.13 and PC+cAMP+Glut.+cAMP-> 1.18 respectively).

The PAGE profile for desorbed proteins from various PC substrates, (cAMP modified and soluble cAMP) which have been exposed to protein mixture for 3 hours is depicted in Fig.6-4. It reveals that the cAMP adsorbed substrate binds more albumin as that of 15 minute's case, however, soluble cAMP (on exposure for 3 hours) shows a drastic increase in Fg-surface binding. This may be due to the progressive inactivation of the cAMP molecule with time in solution. The molar ratio of Alb/Fg for 3 hours exposed substrates are different as that of 15 minute's cases (Alb/Fg ratios for Bare PC->0.56, cAMP adsorbed PC->0.72, cAMP in solution 0.54 and PC+cAMP+Glut.+cAMP->0.98 respectively). These ratios clearly indicate that the soluble cAMP effect has become close to the bare substrate with time of exposure. These changes may be due to the variations in the exchange of proteins with the adsorbed species and the soluble proteins, which may be dependent on the active status of the cAMP molecule.

It has been suggested that an increase of platelet cAMP results in an inhibition of platelet responses to

stimuli[133,217]. It is also possible to achieve an inhibitory effect more directly by the addition of cAMP or it's derivatives (eg.dibutyryl-cAMP) to platelet rich plasma[283]. Lee and Kim[193] have studied the effect of cAMP, dibutyryl cAMP and $PGE_1$, on platelet adhesion to the proteinated polymer substrates, to demonstrate the inhibitory effect of cAMP towards platelet-surface attachment and proposed the possible mechanisms involved for this phenomenon.

Mannucci et al.[218] have investigated the in vivo effect of purified cAMP and dibutyryl cAMP in human volunteers following intravenous infusion. cAMP or dibutyryl cAMP (0.5 mg/kg/mt) has demonstrated a transient inhibition of platelet aggregation induced by low concentrations of ADP or collagen. However, the effect has not sustained and has virtually disappeared 90 minutes after the completion of the infusion. These observations support the present results, indicated with cAMP, for a reduced surface-platelet binding and also suggest that cAMP looses it's biological activity with time.

Octane contact angle data from Table 6-1 shows that albumin immobilized surfaces are quite hydrophilic, but cAMP makes the surfaces slightly hydrophobic. Fenstermaker et al.[207] and Smith et al[76] have used insitu ellipsometry to determine the adsorbance and extension of fibrinogen, prothrombin, and serum albumin adsorbed on a variety of surfaces. Using physiological conditions, they have observed a rapid adsorption on the materials varying in surface free energy from platinum to polyethylene with little or no change in extension with time. Morrissey et al[234] have indicated that the extension of all adsorbed proteins increased with

decreasing surface energy of the substrate. So it seems, the enhanced adsorption of proteins in presence of cAMP may be partly due to the reduction in surface energy caused by cAMP-polycarbonate system. However, the biological activity of the immobilized substrate may also play a significant role on the platelet functions and adhesion/adsorption of other plasma components.

The kinetics of protein adsorption to the PC substrate is also investigated using $^{125}I$ labelled proteins in presence of cAMP. It appears that very low concentrations of cAMP ( 1$\mu$ M) in the media can alter the normal surface protein interaction as demonstrated in figures 6-5 to 6-8. It is quite interesting to see that, the soluble cAMP enhances the surface-albumin binding (Fig.6-5), on the other hand, it inhibits the fibrinogen adsorption to the substrate (Fig.6-7). The modulation of this interfacial phenomena is dependent of the level of soluble cAMP in the media. A fraction of the initially adsorbed albumin and fibrinogen are removed from the substrate in all cases as depicted in figures 6-6 and 6-8 respectively. These kinetic studies of protein-surface binding in presence of cAMP also correlate with the earlier PAGE observations, that it can modulate the protein adsorption in parallel with their antiplatelet activity.

It is well understood[16,377] that the nature of surface bound protein can alter the subsequent platelet adhesion and thrombosis. Hence the enhanced albumin or reduced fibrinogen binding to the substrate may be one of the parameters for a reduced platelet-surface binding via the modulation of Fg receptors. So it seems, the cAMP may be a

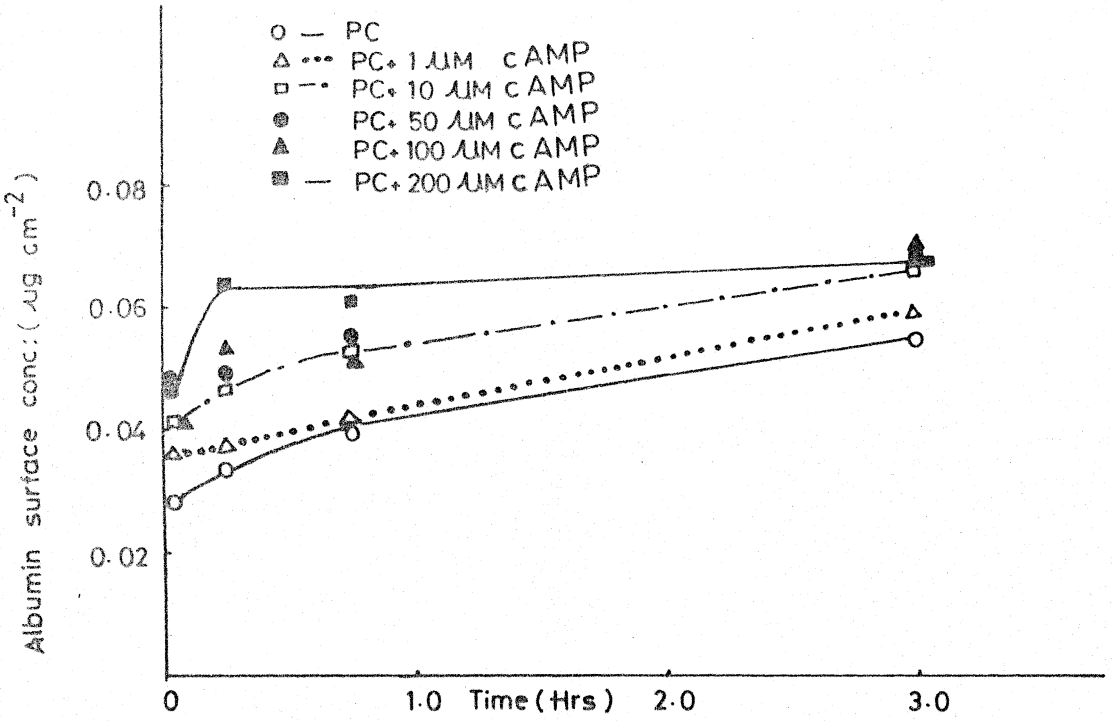



Fig.6-5. Adsorption of albumin as a function of time from protein mixture with various concns: of cAMP

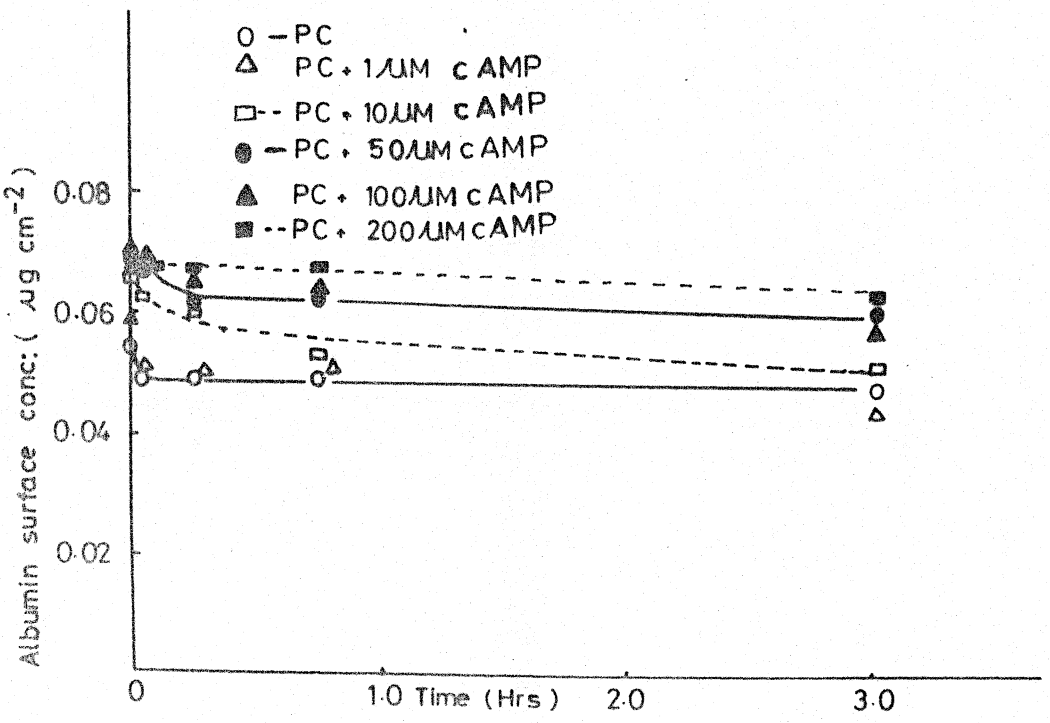



Fig.6-6. Desorption of albumin as a function of time from 3hr preadsorbed albumin from protein mixture and cAMP

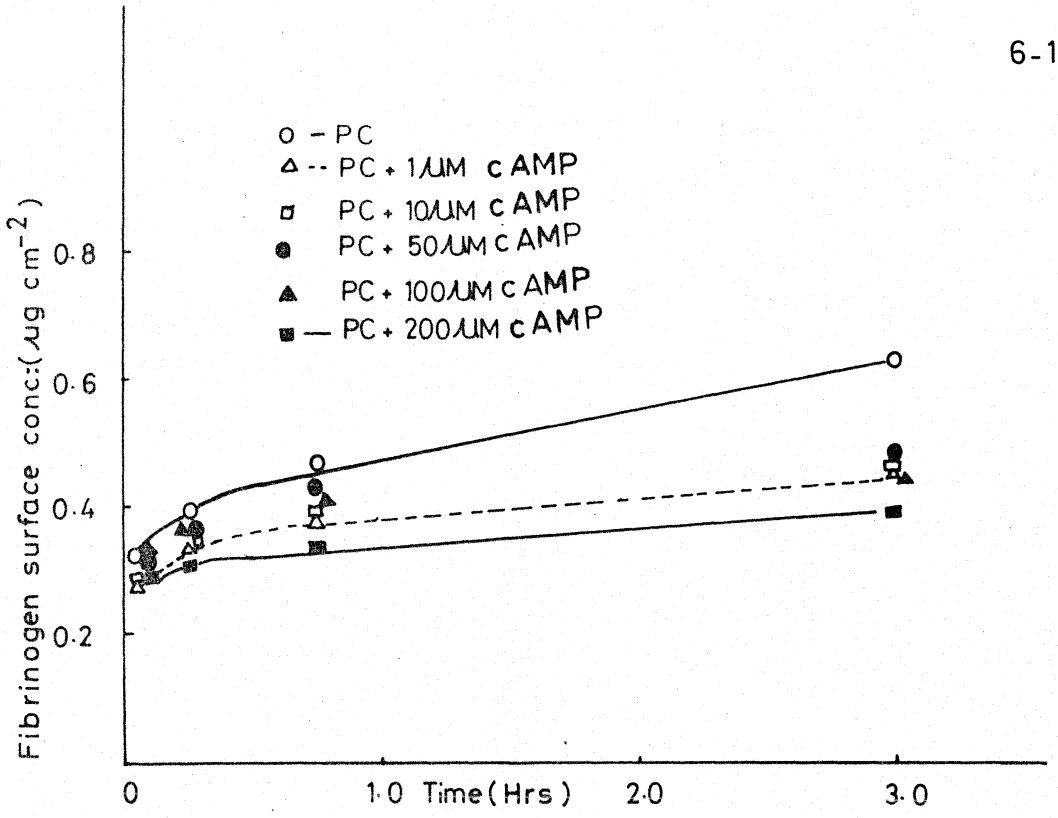



Fig.6-7. Adsorption of fibrinogen as a function of time from protein mixture with various concs: of cAMP

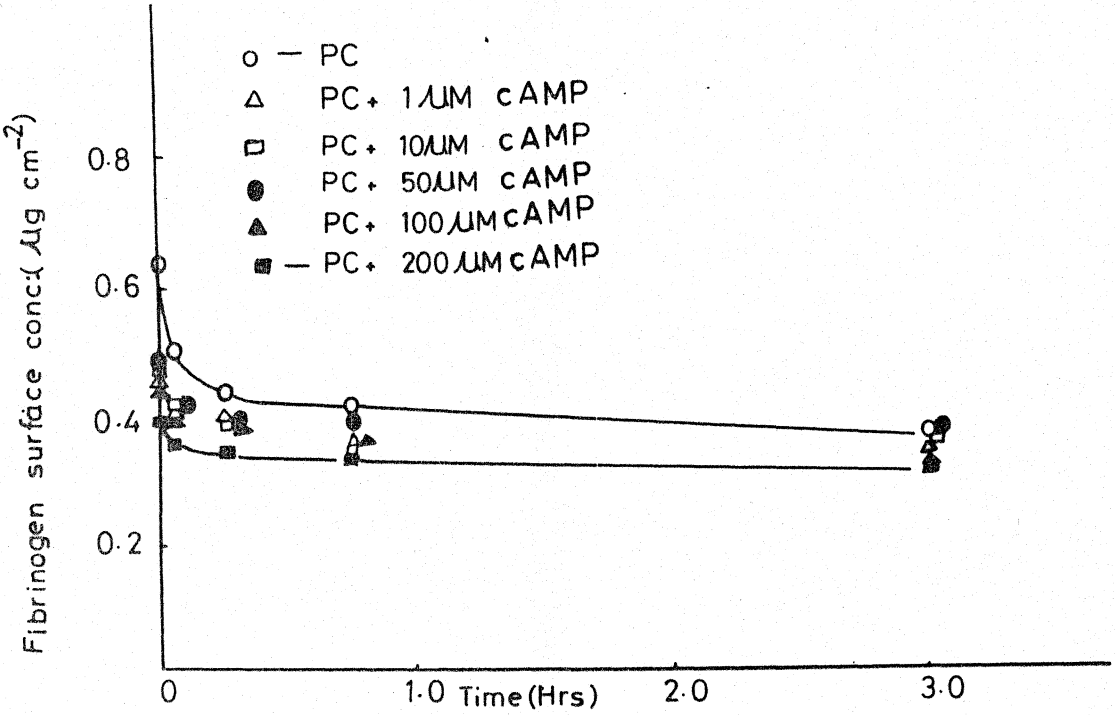



Fig.6-8. Desorption fibrinogen as a function of time from 3hr preadsorbed fibrinogen from protein mixture and cAMP

common link for most of the antiplatelet agents, which may also enhance the albumin to fibrinogen mole ratio at the polymer blood interface, resulting in a reduced platelet-surface binding. Further studies are needed to understand this possibility using resolved techniques.

# CONCLUSIONS AND FUTURE OUTLOOK

# CHAPTER VII

## 7.1. Role of plasma mediators to modulate fibrinogen surface binding

Thrombosis is the main problem when foreign materials come in contact with blood. Fibrinogen participates in the thrombus formation, and essentially plays an important role in the process of clotting-pathways, including both the plasma phase ("intrinsic") and the cellular phase (platelet aggregation) in normal haemostasis[23,123], thus subsequently encourages thrombosis in presence of foreign surfaces[274,347]. Upon contact of artificial surfaces to blood, a layer of plasma proteins get deposited within seconds . simultaneously followed by the adhesion of platelets, leading to thrombosis. It is suggested[347,351] that platelets adhere, where they find adsorbed fibrinogen, the orientation and the density of the Fg carpet can cause a change in platelet morphology, which can promote aggregation of other platelets and eventual thrombosis. Thus the interaction of fibrinogen with an artificial surface is a key step in the formation of haemostatic plug.

Various investigators have studied the protein adsorption kinetics and have suggested[209,257] that albuminated surfaces adhere less platelets, on the other hand fibrinogenated substrates enhance the platelet attachment and thrombus formation. However, comprehensive studies using plasma or,blood as the bulk medium, it appears that the

initially preadsorbed protein layer can be supplanted with other plasma proteins; which alters the composition of the protein layer with time[45,151]. Therefore it seems, other trace plasma proteins or the trace components of plasma or other mediators arriving at the blood-polymer interface; do play a role in modulating the interfacial phenomenon. Hence, studies have been undertaken with fibrinogen, in a systematic way to elucidate it's adsorption pattern to the polycarbonate substrate, (and also to Nylon, Teflon etc. for a relative understanding) and subsequently the platelet attachment and thrombosis.

In an extensive study of the adsorption kinetics and isotherms, from isolated Fg experiments, it is evident that the adsorption is quite rapid, indicating a strong interaction between polycarbonate and fibrinogen. It seems, the adsorption is dependent on pH, temperature and bulk concentration of the media. It also appears that the adsorption is 75% complete within 15 minutes and essentially at equilibrium in 1 hour, and the adsorption kinetics is significantly affected by the shear rates, upto 50 ml/mt. Fibrinogen adsorption is also found to be high from isolated Fg studies; however addition of $\gamma$-globulin, albumin, fibronectin etc. causes drastic reduction in surface-Fg attachment. So, these fundamental studies suggest that the initially adsorbed Fg may be supplanded with the other proteins in the media. This may be probably due to the competition for the existing sites on the substrates for the initial attachment of proteins.

The studies with plasma is quite interesting, because it appears that essentially very less Fg has been

adsorbed to the polycarbonate surface over a period of 3 hours. However, it is possible that Fg can be adsorbed substantially at shorter times, but simultaneously can get desorbed off almost completely over the subsequnet exposure times, probably by exchange with other proteins or by enzymatic action. The studies of Vroman et al[347,351], Brash et al[45,47] and Horbett et al[151] also indicated that initially adsorbed fibrinogen to various substrates can be replaced by other proteins of plasma.

This provided an anxiety to search the fundamental role, as to what happens to the pattern of fibrinogen adsorption when other plasma mediators like heterogeneous Fg molecule, proteases, vitamins, antibiotics, antiplatelet drugs or cAMP are also present at the interface as trace components? The Fg heterogeneity itself can modulate their attachment and subsequent platelet adhesion to the substrate. It seems, the $\gamma'$chain enriched fibrinogen molecule is less effective in binding to an artificial surface in comparison with normal composition; which may have resulted in an enhanced surface albumin binding and reduced platelet attachment. So it is possible that the slight modulation in the Fg molecule itself may cause significant changes in surface-protein/platelet interaction at the interface.

Further studies with proteases like trypsin, thrombin and plasmin suggest that the firbin(ogen)/ fibrinogen degradation products deposition to the substrates depend on the nature of the active protease present at the interface for the subsequent fibrinolysis or fibrin formation. This may essentially be dependent on the generation of active proteases at the implant interface, due

to substrate activation. Recent studies of Brash et al[46,48] have also indicated, when purified plasminogen has been added to fibrinogen, extensive amount of the degradation products have been observed in the column eluates, which may be due to the action of surface mediated activation of plasminogen to plasmin, followed by fibrinolysis. Therefore it appears that surfaces may vary in their activation of fibrinolysis, as well as their activation of clotting, and that maximization of fibrinolysis is a worthwhile goal in the development of blood-compatible surfaces.

Vitamins, antiplatelet drugs, anticoagulants, antibiotics etc. are frequently being used for a smooth haemostasis and for protecting the implant bearing patients from bacterial infection. These plasma trace components, arriving as mediators at the interface may alter the pattern of protein-surface interaction and the subsequent platelet attachment and thrombosis. The studies presented in this thesis with certain antiplatelet drugs, vitamins, heparin, antibiotics etc. to various polymeric substrates, under static and flow conditions, have suggested that these agents do modulate the interfacial phenomena of the surface induced thrombus formation. The protein adsorption kinetics using $^{125}I$ labelled proteins and polyacrylamide gel electrophoretic patterns, of desorbed proteins from various substrates, clearly indicate that there is an increase in albumin or decrease in the Fg surface binding to the substrates in the presence of most of these mediators. These substrates have also shown a reduction in platelet attachment.

It appears[217,286] that most of the antiplatelet agents inhibit the platelet activity via increasing cAMP, a

mediator of cell functions. Many of these agents increase the platelet level of cAMP, through the activation of adenyl cyclase and/or the inhibition of phosphodiesterase, and it seems; these agents are linked by a common effect on platelet cAMP. Therefore, the role of this mediator itself has been investigated at the interface towards the protein adsorption kinetics, to search it's link to modulate the interfacial thrombosis. It seems, cAMP also inhibits the fibrinogen-surface binding and enhances the albumin-surface concentration. This is an interesting observation; and may be one of the common parameters for the reduced surface-platelet attachment along with it's normal biological activity.

## 7.2. Mechanism of antiplatelet agents to modulate the platelet aggregation/adhesion

The human body protects itself against the development of thrombosis by several mechanisms involving the relationship between the blood and the vessel walls. Of them, a balance between the metabolic pathway of prostaglandins in blood platelets and vessel walls has been suggested to be an important haemostatic mechanism in the circulatory system. Arachidonic acid is metabolized to thromboxane $A_2$ ($TX\text{-}A_2$)[126] in blood platelets, but to prostacyclin ($PGI_2$) in blood vessels[162]. Thromboxane $A_2$ is a vaso-constrictor and pro-aggregatory agent, whereas $PGI_2$ is a potent vasodilator and substance which inhibits blood platelet aggregation[231].

It has been suggested[95] that the prostaglandin endoperoxides (formed from arachidonic acid) and $TX\text{-}A_2$,

inhibit adenyl cyclase in platelets, which in turn reduces the cAMP levels in platelets. Hence, the cAMP exerts a regulatory function on platelets, which depends on a balance between TX-$A_2$, prostaglandin endoperoxides and $PGI_2$. A schematic pathways of the interactions of platelets with a vessel wall is depicted in Fig.7-1.

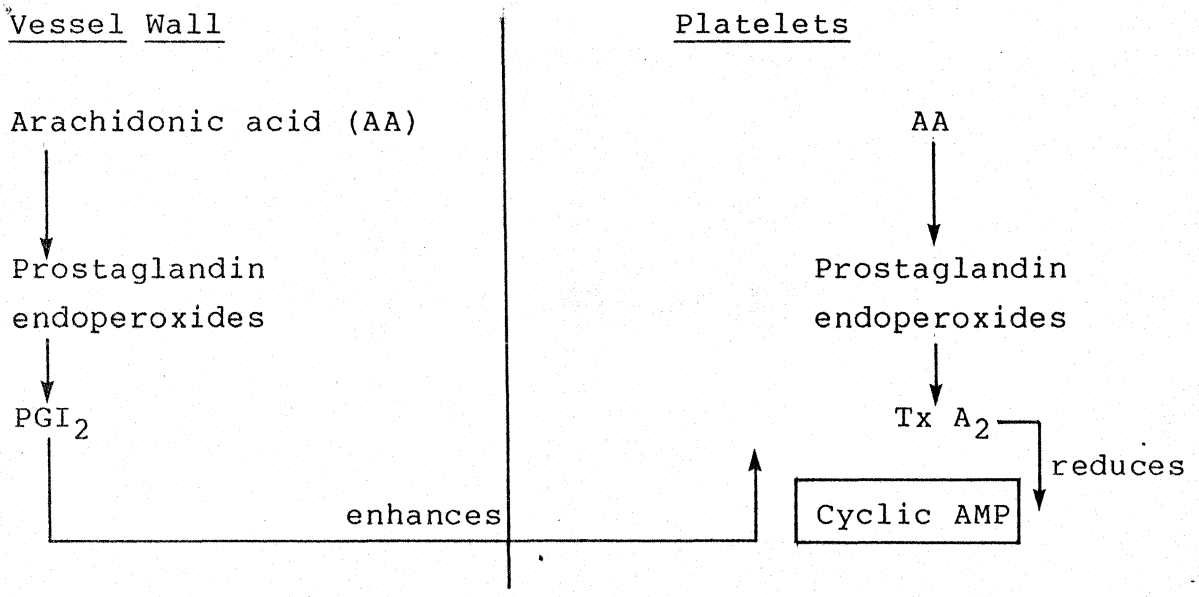



Fig. 7-1. Interaction of platelets with a vessel wall.

Thus the integrity of the normal haemostasis is controlled by a network of biochemical interaction processes, which keeps a balance between $PGI_2$ and TX-$A_2$ via, regulating cAMP.

There are several conditions in which $PGI_2$ generation from endothelial cells of normal vessels can be impaired, thus upsetting the normal balance between $PGI_2$ and TX-$A_2$, which allows decreased levels of platelet cAMP. These conditions may include inhibition of $PGI_2$ synthesis by the

lipid peroxides in atheromatous plaques, or exposure of the proaggregating subendothelial layers after rupture of a plaque[197]. Other conditions, which propagate platelet aggregation and more important here, is the contact of blood with artificial surfaces (which cannot synthesize $PGI_2$), such as in artificial heart values, arterial grafts or extracorporeal circulation. In these conditions the cyclic -AMP concentrations in platelets appear to decline because of thromboxane $A_2$ formation as indicated in Fig.7-2. Hence the interaction of platelets with areas in which $PGI_2$ formation is impaired or absent will lead to the release of prostaglandin endoperoxides and $TXA_2$, which will decrease cAMP levels and induce aggregation.

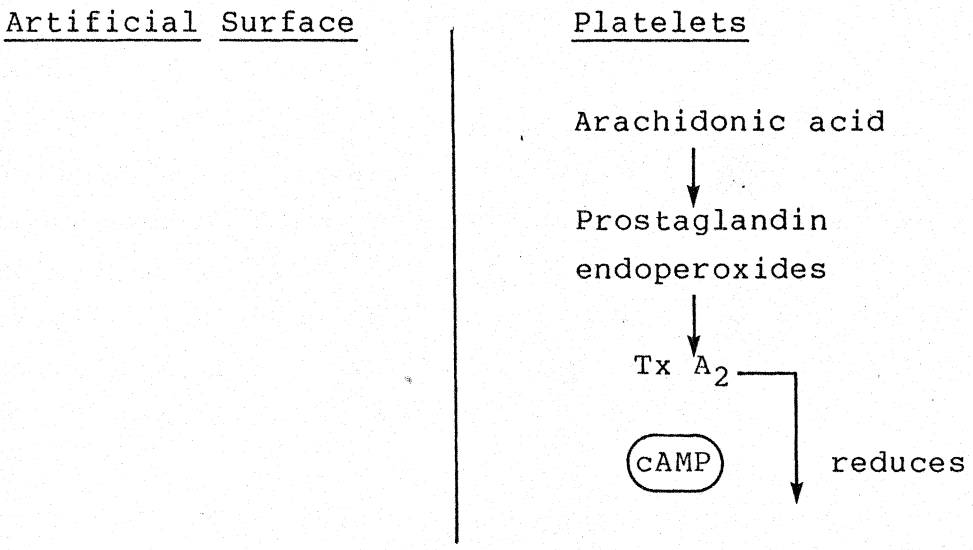



Fig.7-2. Interaction of platelets with an artificial surface.

It is suggested[343] that selective inhibition of TX-$A_2$ is an attractive therapeutic goal in the field of thrombus prevention, since this will, not only abolish the formation

of the proaggregating TX-$A_2$, but will also tend to redirect endoperoxide metabolism towards other products such as $PGI_2$. Thus the use of low doses of aspirin, or combinations of aspirin with dipyridamole, has attracted the clinicians to employ them for platelet supressive therapy, where low doses of aspirin may inhibit TX-$A_2$ formation and dipyridamole may prolong the activity of cAMP and it's concentration. In a biological approach, the combination of vitamins, proposed here, can also have a similar probable mechanism to inbibit the platelet adhesion through the regulation of cAMP at the interface along with low doses of aspirin.

It is proposed[106,244] that soluble substances such as ADP, epinephrine, or thrombin, can stimulate platelet activation and reduce the platelet cAMP levels. Alternatively, it is suggested[95] that the cAMP regulates the activity of platelet cyclo-oxygenase, the enzyme that converts arachidonic acid to endoperoxides[231]. Most of the antiplatelet agents inhibit the platelet activity via increasing cAMP, a mediator of cell functions. These studies clearly indicate the central role of this active mediator (cAMP) for the regulation of platelet functions and a hypothetical scheme of the interrelations of these various processes has been proposed in Fig.7-3.

Calcium ions are required for most forms of platelet aggregation and it is believed that cAMP functions in platelets by directing uptake of calcium[258]. Therefore, an increase in cAMP may inhibit platelet responsiveness. Experiments with ionophores for divalent cations have

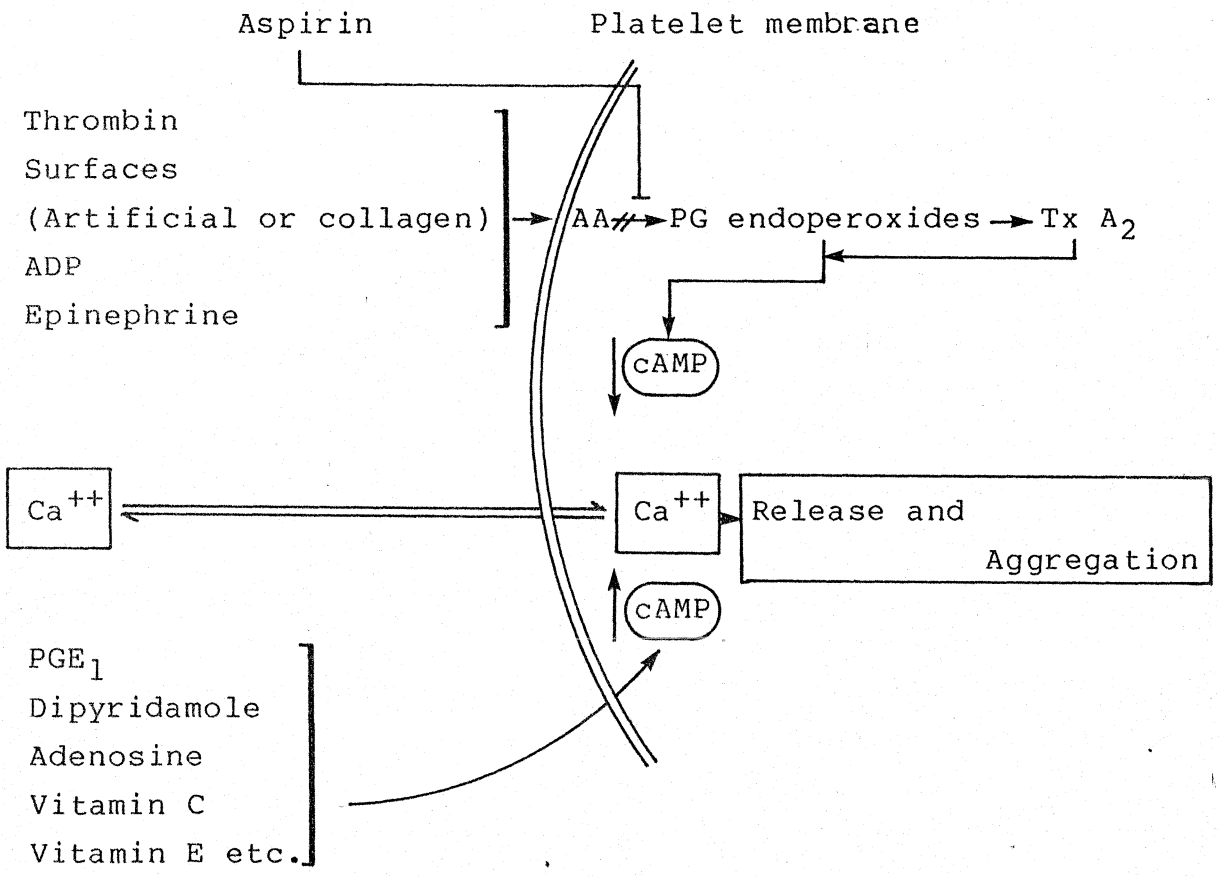


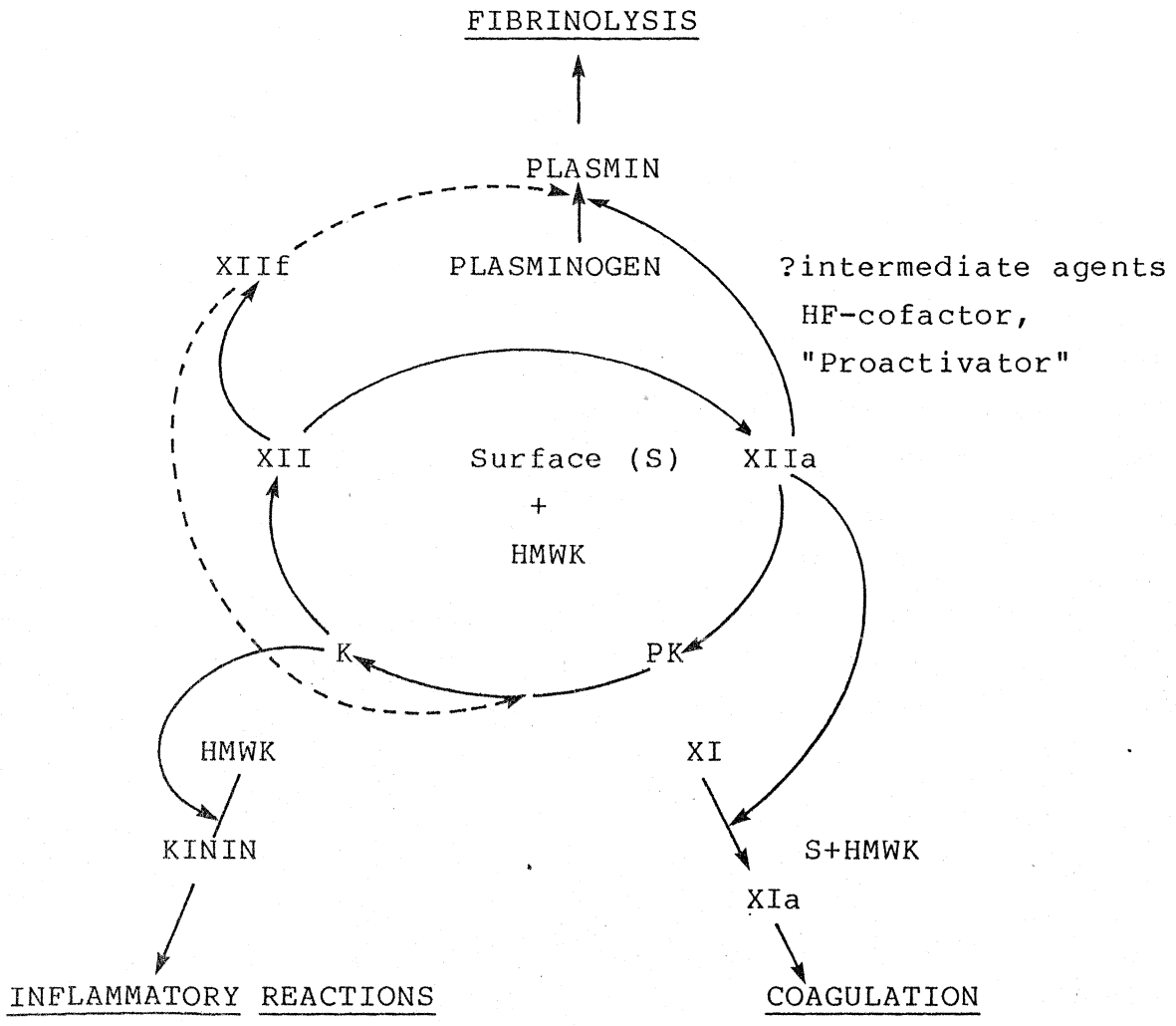

Fig.7-3. Hypothetical scheme of platelet reactions due to various agents.

suggested the importance of cytoplasmic calcium levels in the regulation of platelet function[286], and it is possible that cyclic nucleotides produce their effects by influencing calcium homeostasis. So these studies, suggest the modification of blood with a combined vitamin therapy with low doses of aspirin, may be beneficial for the platelet supression and preventing thrombosis. Hence, if platelet cAMP level can be maintained at the site of artificial

substrate and the platelet activation can be blocked with low doses of aspirin, using the antiplatelet drug combinations, the normal haemostasis can be attained.

## 7.3. Changes in protein-surface interaction due to antiplatelet agents

Exposure of blood to a foreign material can initiate the activation of the intrinsic coagulation by conversion of the zymogen factor-XII into the active serine protease factor-XIIa. This conversion takes place after the adsorption of factor-XII to the material surface. Factor XIIa can activate other molecules of factor XII[242,364]. The activation of factor XII also requires the participation of high molecular weight kininogen (HMWK) carrying factor XI and HMWK that carry prekallikrein[69]. Factor XIIa converts prekallikrein into kallikrein, which in turns activates surface bound factor XII, resulting in a burst of activated factor XII molecules[364]. Factor XIIa converts Factor XI in to factor XIa. These activating reactions do not require Ca++ions. Now the "fluid phase" of clotting begins with the participation of $Ca^{++}$ions for the activation of factor IX by XIa. Factor IXa combines with factor VIIIa, phospholipids and Ca++ions to form a complex which is able to convert factor X in to Xa. Next, factor Xa combines with factor Va, phospholipids and $Ca^{++}$ions to form a complex, which converts prothrombin into thrombin, which in turn converts fibrinogen molecules into fibrin monomers and finally stabilized by factor XIIIa to fibrin gel.




XII - Hageman factor (factor XII)
XIIf - Hageman factor fragments
XI - Factor XI
XIa - Activated XI
K - Kallikrein
PK - Prekallikrein
XIIa - Activated Hageman factor
HMWK - High Molecular Weight Kininogen
S - Surface.

Fig. 7-4. Surface mediated reactions.

It has also been proposed[255] that the factor XII, its fragments, kallikrein and possibly a further agent present in plasma as a proenzyme may activate plasminogen in in vitro systems. However, which of these agents is primarily responsible for surface-mediated fibrinolytic phenomena in plasma systems is unknown. A scheme of surface mediated reactions appears in Fig.7-4. It seems, the artificial substrates upon contact with blood can generate thrombin (for coagulation), plasmin (for fibrinolysis) and kinins (for inflammatory reactions), but it is not known whether all these reactions may take place in in vivo[23,123]. However, it appears that, the extent of stimulation of each path way may vary depending on the surface properties of the material, and naturally the thrombus formation at the interface. In an another view, it is also possible to stimulate or inhibit a specific pathway using drugs, which have least side effects; for eg; the use of antiplatelet drugs and anticoagulants for preventing thrombosis.

Vroman et al[347,349,351] have extensively studied the initial phases of surface-blood interactions. It has been postulated that on hydrophilic surfaces (eg. glass), Fg is deposited within seconds along with traces of HMWK and factor XII. Then more HMWK and factor XII arrive and displace the Fg. Platelets adhere most, where fibrinogen remains. These interactions would explain the data reported by Coleman and Andrade[70] that show more activation of clotting and less adhesion of platelets on the more polar substrates. It has also been suggested[350,352] that the sequence of proteins displacing each other on contact with plasma starts not with Fg, but with albumin, followed by IgG, which in turn will be replaced by Fg and fibronectin etc.

ending with HMWK. The sequence is slowed down in more hydrophobic surfaces (eg. Teflon).

In this thesis, most of the protein adsorption studies have been carried out with polycarbonate as the substrate. Here, in the polycarbonate substrate, the polar(12.96 ergs/cm$^2$) and the dispersion (26.53ergs/cm$^2$) components are distributed to have the surface energetics (39.5 ergs/cm$^2$), which comes in between highly hydrophilic or more hydrophobic substrates[300]. Hence, it seems the initially adsorbed albumin may be displaced with other proteins in sequence with time. However with mediators (eg. antiplatelet drugs), the overlapping sequence of proteins deposited by plasma and their displacement appear to be altered. The mediators that arrive at the interface, may cause a change in the surface tension of the media, which may result in an increase in protein surface binding. For eg; it has been seen that, vitamin C in solution causes an increase in interfacial tension of the surface, which probably induces an enhanced protein adsorption to the substrate.

It is also reported earlier in this thesis (Table 5-VII) that most of the antiplatelet drugs or vitamins make the surface slightly hydrophobic in character, compared to the bare substrate. It has also been indicated, hydrophobic polymers adsorb more proteins which are not readily desorbed[20,92]. So, from surface energetics point of view, it seems that the initially adsorbed protein may not be supplanted readily by other proteins in the media relatively, where antiplatelet drugs have been infused. Hence an increased protein adsorption to these substrates appear to be possible, favouring more albumin retension.

It is also postulated that a large number of drugs have a high affinity for the plasma albumin and the binding is generally reversible[37,93,111,114,124,268]. For eg. vitamin C, adenosine, vitamin $B_6$, thyroxine and most of the acidic and the basic drugs can bind with albumin[111,254,268]. The binding of drugs to plasma protein raises the apparent solubility of it in plasma. This preferential binding of albumin by these mediators may enhance the substrate binding as well, or in other words, the drug bonded albumin may have more affinity towards the substrate. Thus the initially adsorbed albumin may be less supplanted with IgG, Fg and HMWK in the presence of these drugs. It is also possible that certain drugs may stimulate the fibrinolysis, which may cause an increase in the albumin-surface concentration; and possibly reduces the adherence of platelets.

Certain mediators like $PGE_I$, cAMP or drugs appear to act via releasing platelet cAMP, which in turn reduces the further adhesion or aggregation of platelets. It has been indicated in Chapter VI, that the soluble cAMP can enhance the surface-albumin binding, which may be another factor for a reduced platelet adhesion with an artificial surface, beside it's own antiplatelet activity.

It is conceivable that albumin binds to a myriad of drugs as mentioned earlier in this Chapter. Since these drugs diffuse much more rapidly than albumin, they encounter the PC surface before albumin. Thus it seems, the drug adsorbed surface, possibly due to their high albumin binding properties, may increase the affinity of albumin for the surface. Thus, most of these antiplatelet drugs have dual role at the interface, they may themselves induce more

albumin attachment to the substrate or reduce the Fg binding and may also release platelet cAMP, which in turn appears to inhibit the Fg surface attachment and/or the platelet adhesion.

## 7.4. Inter-relation of fibrinogen adsorption and platelet attachment

The in vitro fibrinogen adsorption studies on various polymer substrates like Polycarbonate, Polystyrene, Nylon, Teflon, Angioflex etc. suggest that Fg surface attachment appear to vary with substrate properties. It has been indicated, the in vitro fibrinogen adsorption to the hydrophilic or polar materials have been low initially, but have increased with time, to a steady saturation value[154]. On non-polar materials, adsorption have been high initially, and have decreased to a lower steady state value; within 15 minutes of the surface contact.

The kinetics of fibrinogen adsorption from plasma onto various surfaces have also been different, as suggested earlier. High initial fibrinogen adsorption from plasma followed by a decrease has been observed by Roohk et al[277]; for Polyvinyl chloride and Silicone rubber and Uniyal and Brash[339] for Polyethylene and siliconized glass. On the other hand, the fibrinogen adsorption has been found to be constant throughout the time course to the Polystyrene surface. Ihlenfeld and Cooper[159] have measured the Fg adsorption from heparinized dog blood in vivo. It appears that the Fg adsorption to Polyvinyl chloride and Silastic have also been higher initially than at the later times, while adsorpotion to Biomer has been nearly constant

throughout the time course. Thus, considering all these observations with the present study, it is obvious that distinct differences in the kinetics of fibrinogen adsorption exist to various surfaces.

Vroman et al[347,351] have indicated that on many materials, plasma will deposit fibrinogen within a second, and platelets will adhere where they find preadsorbed fibrinogen. Recent studies of Ward and Stanga[356] have proposed that platelet adhesion increases with increasing concentrations of preadsorbed Fg until the surface concentration exceeds that in first plateau. Higher surface concentrations of the preadsorbed Fg do not promote further platelet adhesion. The present studies with fibrinogen heterogeneity shows that, an extension of 15 amino acid residues in the normal $\gamma$ chain ( $\gamma'$ chain) has substantially inhibited it's surface attachment and the platelet binding. It is also believed that platelets possess membrane receptors for a wide variety of agents including Fg. So it seems, the nature of the available receptors in Fg molelecule is very important for it's subsequent surface or platelet attachment and it's activation.

Conformational changes in adsorbed fibrinogen have also been suggested by others[92,235]. The high initial Fg adsorption and subsequent decrease seen on a polar material in vitro may be due to conformational changes occurred to Fg. Thus, the density/and segmental variations happening to the Fg molecule, resulting in changes in orientation of the preadsorbed fibrinogen carpet, may be dependent on the surface properties of the material. The antiplatelet drugs, arriving at the interface can modulate the surface as well as

the Fg receptors from surface or platelet attachment. Hence it seems, the surface properties of the material, flow conditions, submicroscopic properties affecting local variabilities in surface charge and wettability can all influence plasma protein interactions at the interface with blood and the subsequent thrombotic sequelae. Therefore the ability of the polymers and the mediators (here antiplatelet drugs) and their subsequent influence to modulate the state of adsorbed fibrinogen appears to be an important determinant of thrombogenicity.

Comprehensively, these studies highlight the effects of certain antiplatelet agents to modulate the surface-protein interaction and the subsequent process of thrombus formation. It seems, those mediators having antiplatelet activity, causes an increase in the surface-albumin concentration in comparison with fibrinogen. This common trend of protein adsorption demonstrated by these agents may be one of the parameters for a reduced surface-platelet binding. Therefore, these two factors, may be beneficial for improving the blood compatibility of materials in in vivo conditions. However, the role of these drugs to modulate the Fg receptors, like whether they bind with a specific site, cause segmental mortions, mask the receptor sites etc. and the actual differences they may make in their adsorbed state and the mechanisms involved need to be further explored.

## 7.5. FUTURE OUTLOOK

### 7.5.1. Reflected proteins and immunological responses

These studies suggest the importance of protein turnover for the blood-material interface problem in that it provides a mechanism whereby the composition of the adsorbed protein layer, known to be important for platelet-surface interactions, could change with time. It is possible that "reflected" protein (ie. protein that has been adsorbed and then returned to the bulk) may be conformationally altered, so that it can trigger some undesired response in the blood and the tissue elsewhere. Hence the effect of conformationally altered proteins from the substrates towards systemic toxicity and immunological aspects need to be better understood. It may provide an understanding for taking measures to protect the patient and his device from inflammatory reactions and the tendency of implant rejection.

### 7.5.2. Substrate induced thromboembolism and their prevention

The serious complicated problem after implantation of an artificial prostheses with blood contact is the formation of thromboembolism. Most detectable emboli are those in the cerebral vessels and result in permanent neurological dysfunction. So the micro-embolization and the resultant accumulation at various tissue sights due to an artificial implant need detailed investigation in the future to come. Studies are also required for developing novel biomedical interfaces and new fibrinolytic agents to limit or

avoid the thromboembolic complications; which can provide better life support systems and patient care.

### 7.5.3. Platelet marker proteins and surface induced platelet activation

There is evidence[380,381] that ß-thromboglobulin, LA-$PF_4$ (low affinity platelet factor-4), and $PF_4$ (platelet factor-4) are immunologically specific for platelets, which do not occur in other cells and tissues. Hence, these proteins can be considered as platelet markers. Considering the rapid turn-over of both platelet proteins, any significant increase of LA-$PF_4$ or $PF_4$ antigen in 'platelets free plasma' due to continuous stimulation of platelets in vivo would rapidly result in a loss of these proteins from the storage granules of circulating platelets. It appears that increased levels of specific proteins in plasma may reflect deficient metabolism of these proteins as well as enhanced platelet stimulation. The repeated exposure of artificial substrate can activate the platelets and the concentration of these platelet marker proteins in plasma may be a determinant factor to equate with substrate-platelet interaction. Hence studies to be undertaken, related to precise methods of evaluating these factors and the use of antiplatelet drugs and their combinations to prevent the surface induced platelet activation.

### 7.5.4. Artificial substrates and cellular receptors

Finally, the protein structure is dynamic; it is now well known that 'protein structure' provides not only the arrangement of atom co-ordinates, but include other factors

such as fluctuations of atom positions, energy and entropy about mean values. So the internal motility at atomic resolution is duly taken in to account when interpreting the experimental observations obtained from proteins related to it's structural, thermodynamic or kinetic parameters. The fine structure of recognition sites-the receptors,sensitive areas of membrane proteins of cells and the signals they produce when in contact with an artificial substrate need better understanding to throw light on the events happening in vivo.

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# APPENDICES

# APPENDIX-A

## SUMMARY

Protein adsorption and platelet adhesion are two important biological processes arising at the blood-prosthetic interface. Fibrinogen molecule actively participate in thrombus development via the formation of fibrin, as well as acts as a glue for platelet-platelet sticking and has also been implicated in thrombosis on foreign surfaces. The adsorption and the turn over of fibrinogen and it's physical state at the interface (orientation and density of the molecule) is highly inter-related to platelet adhesion and eventual thrombosis. Most of the materials adsorb large amount of fibrinogen from it's isolated solutions; however; addition of other plasma proteins or plasma itself can supplant most of the preadsorbed Fg molecule.

The studies presented here, also have demonstrated a similar trend, that addition of albumin, $\gamma$-globulin, fibronectin etc. have drastically reversed the surface concentration, compared to the isolated experiments; and essentially very less Fg has been adsorbed to polycarbonate surface from plasma over a period of 3 hours. It seems, the trace components of plasma or mediators arriving at the interface can substantially modify the surface-fibrinogen adsorption and the subsequent platelet attachment.

It is suggested, that most of the antiplatelet agents, like vitamins, drugs etc. inhibit the platelet activity via increasing the level of platelet cyclic AMP, a mediator of cell functions. The studies related to the role of such mediators like vitamins, antiplatelet drugs, antibiotics, cAMP etc. are quite interesting with regard to their effects to modulate the surface-protein binding and the subsequent cellular attachment. It seems, these mediators having antiplatelet activity have demonstrated a substantial reduction in the surface-fibrinogen binding, on the other hand they have shown an increase in the albumin surface concentration. This common trend of protein adsorption demonstrated by these agents, itself may be one of the parameters for a reduced surface-platelet binding as well.

Most of these drugs can bind with albumin and this albumin drug complex may be getting adsorbed more to the substrate or they may be modifying the Fg receptors from surface binding. These drugs can also make the substrate slightly hydrophobic in nature, causing an increased protein adsorption favouring towards albumin. Therefore, these two factors, may be beneficial for improving the blood compatibility of materials in in vivo conditions, since albuminated substrates adhere less platelets as well the platelets do not see much Fg and it's active receptors for attachment.

So these studies show an inter-relation of Fg surface concentration and platelet adhesion at the interface, which may be related to Fg receptor sites available for the interaction. A controlled use of antiplatelet drugs in combination may be advantageous for patients having the

implants, for sustaining better life. It seems, modification of blood itself using a combination of low doses of aspirin with an enhanced vitamin therapy may be beneficial to avoid or limit the prognosis of thrombosis at the interface. Here the advantages of aspirin to inhibit the platelet thromboxane-$A_2$ and the vitamins for an increased cAMP levels and their combined effects for a reduced Fg-surface binding have been utilized for a normal haemostasis.

# APPENDIX- B

## PUBLICATIONS FROM THIS THESIS

1. T. Chandy and C.P. Sharma, Fibrin(ogen)/Fibrinogen degradation products - interaction with polymer substrate and platelets. Current Science, 55, 773-77 (1986).

2. T. Chandy and C.P. Sharma, Protein/platelet interaction with an artificial surface : Effect of vitamins and platelet inhibitors. Thromb Research, 41, 9-22 (1986)

3. T. Chandy and C.P. Sharma Antibiotics reversal of surface - fibrinogen interaction: Changes with lymphocytes. Artificial Organs (abst) 9,48 (1985) and Progress in Artificial Organs - 1985, Y. Nose, C.Kjellstrand and P. Ivanovich (eds), ISAO Press, No. 205, Cleveland, 969-976 (1986).

4. T. Chandy and C.P. Sharma, Protein-polymer interaction-Changes with plasma components, vitamins, and antiplatelet drugs at the interface. Polym. Plast. Tech. Engg., 26,143 - 227 (1987)

5. T. Chandy and C.P. Sharma, Changes in albumin/platelet interaction with an artificial surface-due to antibiotics, pyridoxal phosphate and lymphocytes, Artificial Organs (submitted)

6. C.P. Sharma, T. Chandy, Influence of L-ascorbic acid, blood cells and components on protein adsorption/desorption on polycarbonate, Haemostasis, 17, 70-78 (1987).

# APPENDIX-C

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine diphosphate. |
| APTT | Activated partial thromboplastin time |
| ASP | Aspirin |
| AT-III | Antithrombin III |
| cAMP | Cyclic AMP (Adenosine-3'-5' -cyclic monophosphate) |
| FDP | Fibrinogen degradation products. |
| Fn | Fibronectin. |
| Fg | Fibrinogen |
| HMWK | High molecular weight kininogen |
| IgG | Immunoglobulin-G |
| µg | Microgram |
| µm | Micrometre |
| µM | Micromole |
| PAGE | Polyacrylamide gel electrophoresis |
| PC | Polycarbonate |
| PER | Persantine |
| $PGD_2$ | Prostaglandin $D_2$ |
| $PGE_1$ | Prostaglandin $E_1$ |
| $PGI_2$ | Prostaglandin $I_2$ |
| PLP | Pyridoxal-5'-phosphate |
| RBC | Red blood cells |
| RT | Recalcification time |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| $TX-A_2$ | Thromboxane-$A_2$ |