FIBRINOGEN AT ARTIFICIAL SURFACES

THE BEHAVIOUR OF FIBRINOGEN AT ARTIFICIAL SURFACES

By

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Abstract

The adsorption of fibrinogen from human plasma onto various surfaces was investigated under differing conditions of plasma composition. Previous studies by Vroman have indicated that fibrinogen is only transiently adsorbed from normal human blood plasma, possibly due to displacement by high molecular weight kininogen (HMWK). It is possible that this "Vroman Effect" is a manifestation of the activation of the intrinsic clotting pathway responsible, in part, for thrombus formation on artificial surfaces. The effect was studied using dilutions of human plasma from 0.01% to 20.0% of normal concentration trace-labelled with a small amount of ^{125}I labelled fibrinogen. Adsorption of fibrinogen to various materials, including glass and several potentially useful biomaterials was measured. Fibrinogen is initially adsorbed apparently under diffusion limitation and later displaced at a rate which depends on the type of material being tested. There does not appear to be a direct relationship between material properties (contact angle, bio-compatibility) and the "Vroman Effect". Studies comparing the effect for a series of clotting factor deficient plasmas seem to indicate that plasminogen and HMWK are the main displacing agents on glass. Factors XI and XII, prekallikrein and other components do not appear to be involved.

An attempt to mathematically model the effect was hampered by the lack of adequate models to describe even one-component protein adsorption. An isotherm equation was developed based on a theoretical model of protein exchange and spreading on the surface. The resulting parameter estimates

based on data for different surfaces were well conditioned and may provide a good in vitro basis for comparing materials. It is hoped that the theoretical model will also be compatible with dynamic adsorption and ultimately a multicomponent system such as plasma (and the Vroman Effect). This work may lead to a better understanding of blood-material interactions and may provide the basis for a simple in vitro test for the characterization of potential biomaterials.

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CHAPTER 1
Objectives

The purpose of this research project was to investigate the mechanism by which initially adsorbed fibrinogen is displaced from artificial surfaces exposed to plasma. Known as the "Vroman Effect", this transient adsorption may be linked to the initiation of the intrinsic blood coagulation pathway. If this phenomenon can also be linked to the biocompatibility of blood-contacting materials, it may allow the development of an in vitro test to characterize potential biomaterials. Such a test may be able to provide data to which a mechanistic model may be fit. The parameters of such a model may be able to give insight into the suitability of a wide variety of materials.

In order to model the complex multicomponent plasma system it is necessary to understand one-component protein adsorption. Since currently available models for such a system are not good enough to describe fibrinogen adsorption mechanistically, a new model was developed. This required the collection of a great deal of data for one-component fibrinogen adsorption on various materials.

The "Vroman Effect" itself was investigated with regard to its dependence on different materials. Then glass was exposed to several plasmas deficient in different clotting factors with the purpose of elucidating the plasma components involved in the phenomenon. This knowledge was combined with the information gathered on one-component fibrinogen in order to help to explain the Vroman effect.

It is hoped that, eventually, the mechanism can be determined with sufficient confidence to develop a model with meaningful parameters that can be used to quantify biocompatibility.

CHAPTER 2

Background

Thrombus Formation

Artificial surfaces, after coming into contact with blood, promote thrombus formation (1,2,3). The resulting aggregation of platelets enmeshed in fibrin can become dangerous if it grows large enough to hinder blood flow past an implanted surface. In high flow areas, a thrombus can be dislodged to form an embolus which is carried through the circulatory system until it lodges in the vasculature further along. This could result in a loss of blood supply to a region of the body. An embolus lodged in the brain can result in a stroke and in a coronary artery can cause an infarction of the heart muscle.

On initial contact of blood with an artificial surface, a layer of plasma proteins is rapidly laid down (4-9), the composition of which depends on the surface to which they are adsorbing. It is this protein layer which mediates the surface's response to the platelets. Layers rich in fibrinogen, fibronectin, gamma globulins and factor VIII are all known to promote platelet adhesion (1), while albumin has been shown to passivate surfaces to platelet adhesion (57). Surface-bound platelets undergo a secondary response and release a variety of substances including platelet factor 3 (composed of phospholipid micelles) and ADP, which promote platelet aggregation and coagulation, resulting in an unstable platelet plug (2). This is stabilized by the formation of a fibrin meshwork resulting in a thrombus. Depending on the flowrate of

the blood and the roughness of the surface the thrombus may then dislodge to form an embolus.

Coagulation

Blood coagulation leading to thrombus formation can be accomplished without the prior adhesion of platelets (10-15). Inactive blood proteins called zymogens may be activated by proteolysis into active clotting factors which propagate the clotting mechanism in cascade-like fashion (see Figure 1) (2). The clotting mechanism consists of two pathways which lead to a common pathway beginning with the activation of factor X and ending in the thrombin-catalysed polymerization of fibrinogen into fibrin. The two initial pathways are known as the extrinsic (or tissue activated) and the intrinsic (or surface activated) pathways.

Extrinsic Pathway

The extrinsic pathway (1,2) is an accelerated clotting pathway which takes advantage of a tissue factor known as tissue thromboplastin, which is released by damaged blood vessel endothelium. Thromboplastin activates Factor VII in the presence of calcium ions which in turn activates the common portion of the clotting pathway. This mechanism bypasses the slower intrinsic pathway and leads to rapid clotting in the event of an injury to a blood vessel.

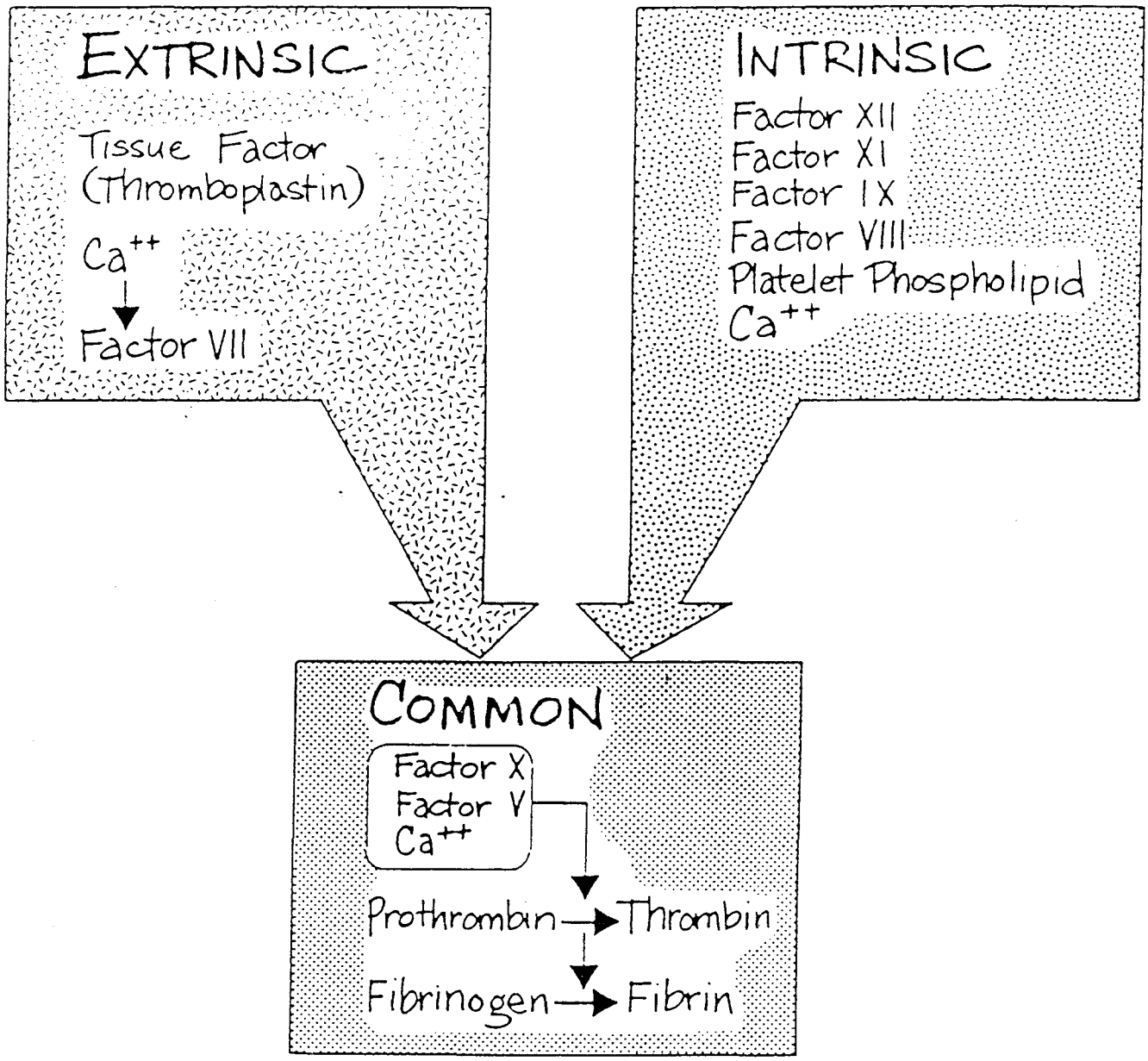


Figure 1 The Blood Coagulation Pathway (2)

Intrinsic Pathway

The intrinsic pathway (10,11,12) is activated by the reactions of zymogens at a surface (see Figure 2). In the case of a damaged blood vessel the collagen exposed provides a suitable surface for Factor XII activation. The inner portion of the lipid bilayer of cell membranes contains phospholipids similar to those released by platelets and capable of accelerating the intrinsic pathway. Since it is the intrinsic pathway that initiates the clotting mechanism on a foreign surface, it is most relevant to the study of biomaterials and will be examined in closer detail.

A great deal has been learned in the last ten years about artificial surface contact activation of the clotting mechanism. The initiation of clotting at the surface appears to revolve around the actions of four plasma factors (see Figure 3) Factors XII, XI, prekallikrein and HMWK.

Factor XII (or Hageman factor) (16-19,25) MW 80,000 is frequently referred to as the surface-activated protein although it is unlikely that many surfaces have significant catalytic activity to bring this about. It has been postulated that adsorption causes a conformational change in Factor XII which may render it more susceptible to cleavage, and hence activation, by kallikrein (11). Two activated forms of Hageman factor may be formed. α-XIIa has a molecular weight of 80,000 and is formed by the cleavage of a disulphide bond, within a loop to form a two-chain active enzyme. This molecule contains a positively charged, histidine rich, binding site which is attracted to negative surfaces at one end, and an active site at the other end. Further cleavage of α-XIIa yields a MW 28,000 molecule called β-XIIa which

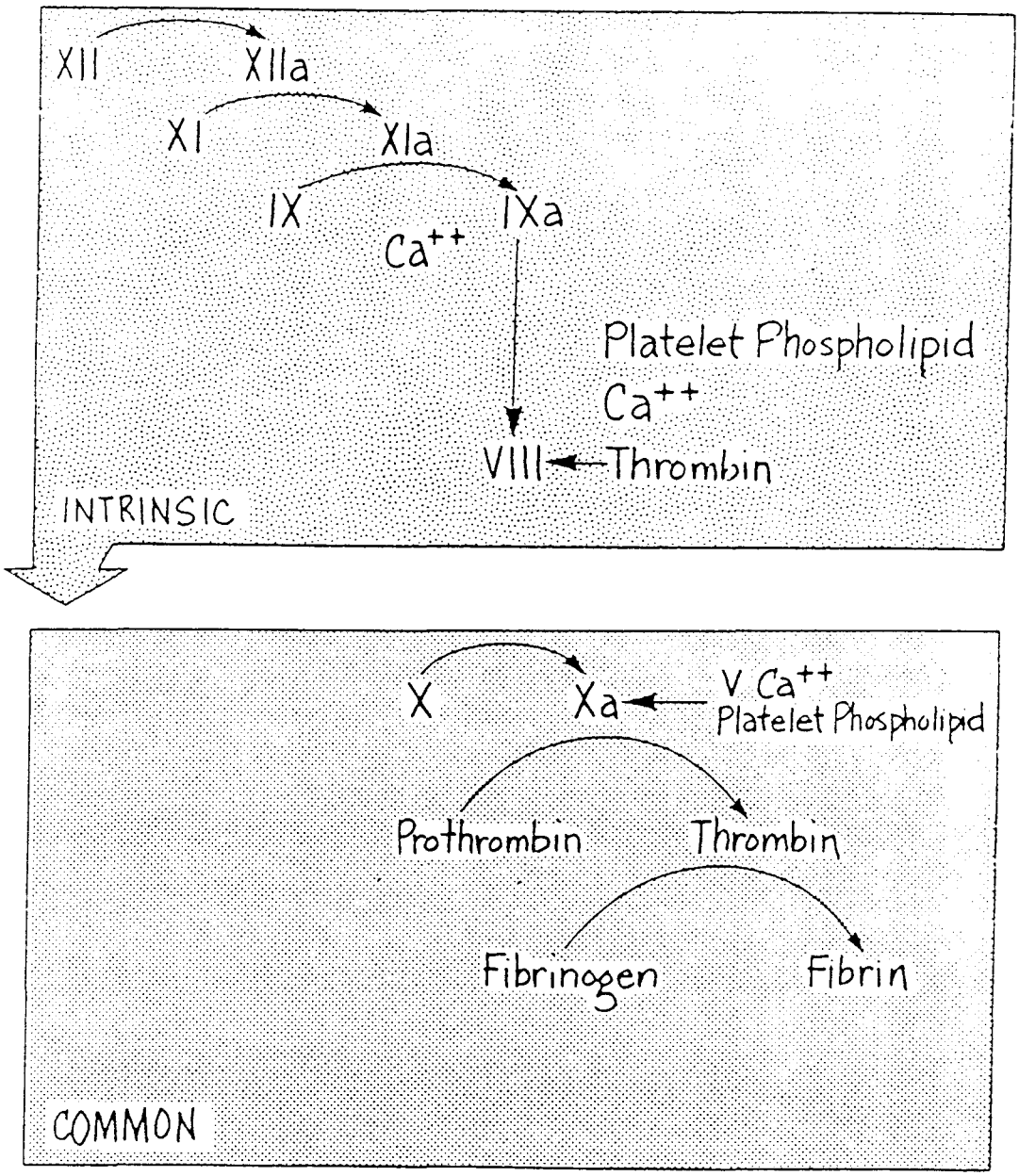


Figure 2 The Intrinsic Pathway (2)

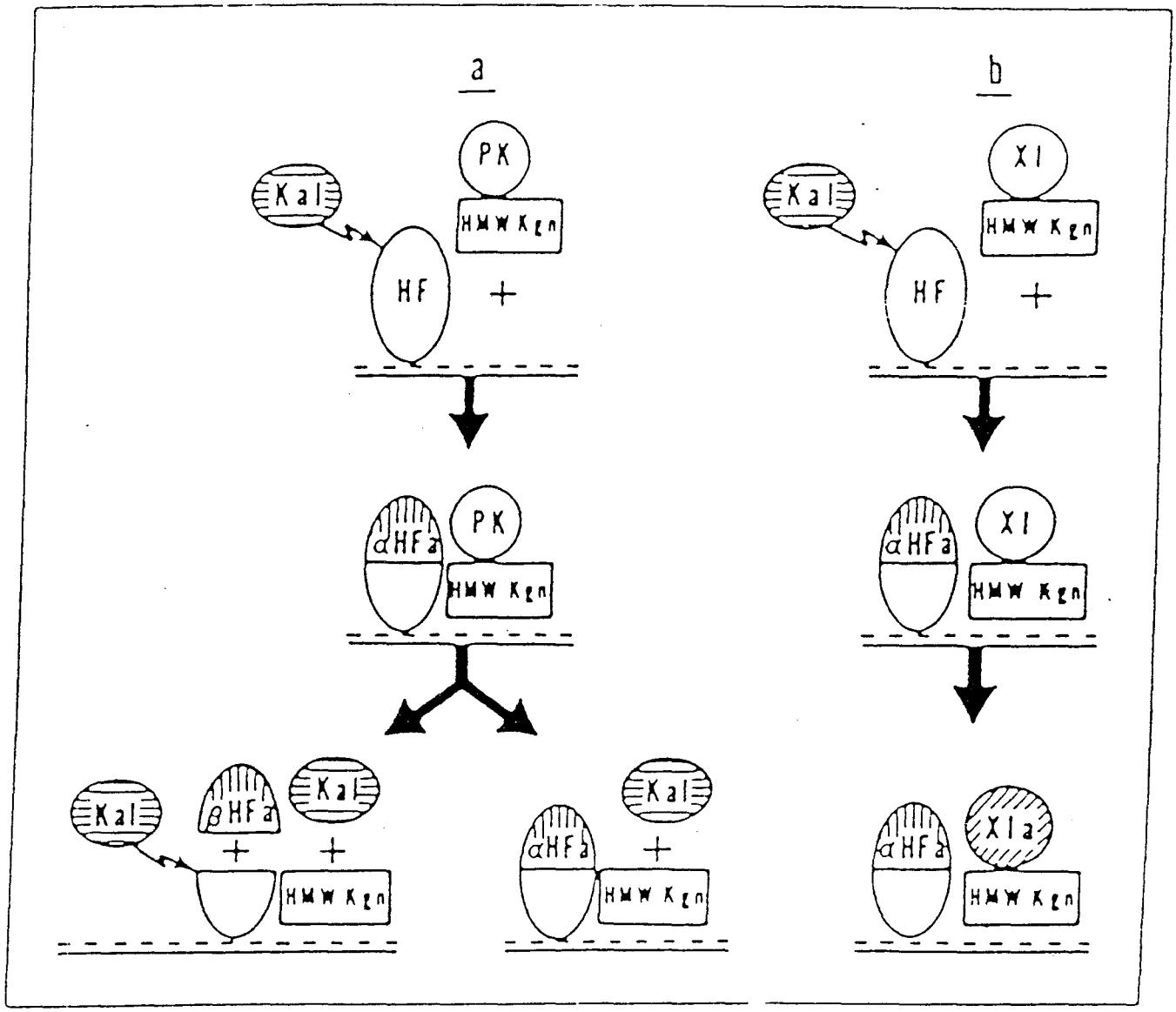


Figure 3 A Model of Contact Activation (11)

lacks the positively charged binding site but contains the same active site for prekallikrein activation as α-XIIa. α-XIIa appears to activate Factor XI about 100 times more rapidly than β-XIIa.

High molecular weight kininogen (20-24) (HMWK or Fitzgerald factor) consists of a single polypeptide chain of MW 120,000 which contains 20% of the plasma supply of kinins. The other 80% is contained in Low MWK. Kinins, which are responsible for the body's inflammatory and pain responses, are released by the proteolytic action of plasma kallikrein on HMWK. Tissue kallikrein is capable of releasing kinins from both HMWK and LMWK (11).

The special role of HMWK in contact activation is not yet fully understood but a great deal has been learned in the last ten years. It is believed that prekallikrein and Factor XI exist as complexes with HMWK in plasma (10). There is known to be a highly positively charged, histidine-rich region in HMWK which could explain its affinity for negatively charged surfaces. If this is true the role of HMWK could be to transport the reacting zymogens, prekallikrein and Factor XI to the surface where they may be acted on by XIIa. It has been estimated that the majority of prekallikrein and Factor XI exist as bimolecular complexes with HMWK (28). Also, the total molar concentration of HMWK is approximately equal to the sum of the concentrations of the two zymogens with which it forms complexes, again suggesting its role in mediating the transport of these molecules.

Scott et al. (21) have suggested that HMWK is degraded by kallikrein and XIa into HMWKa, which is a much more efficient cofactor for coagulation since it adsorbs more readily to negatively charged surfaces. Further

proteolysis by Factor XIa cleaves HMWKa into inactive fragments (HMWKi) which do not have any cofactor activity in clotting.

Factor XI (29,30) is a two chain glycoprotein of MW 160,000 activated by the cleavage of an arginyl-isoleucine bond by Factor XIIa. The activated zymogen Factor XIa is known to propagate the coagulation cascade through the activation of Factor IX. It is also known to activate Factor XII, plasminogen and HMWK. In the presence of a surface, Factor XI's activation is accelerated since it exists as a bimolecular complex with HMWK. HMWK is rapidly adsorbed onto negatively charged surfaces and brings Factor XI to be acted on by XIIa which also exists in high concentrations near surfaces. Factor XIa then tends to remain attached to HMWK at the surface to activate Factor XII and plasminogen (11).

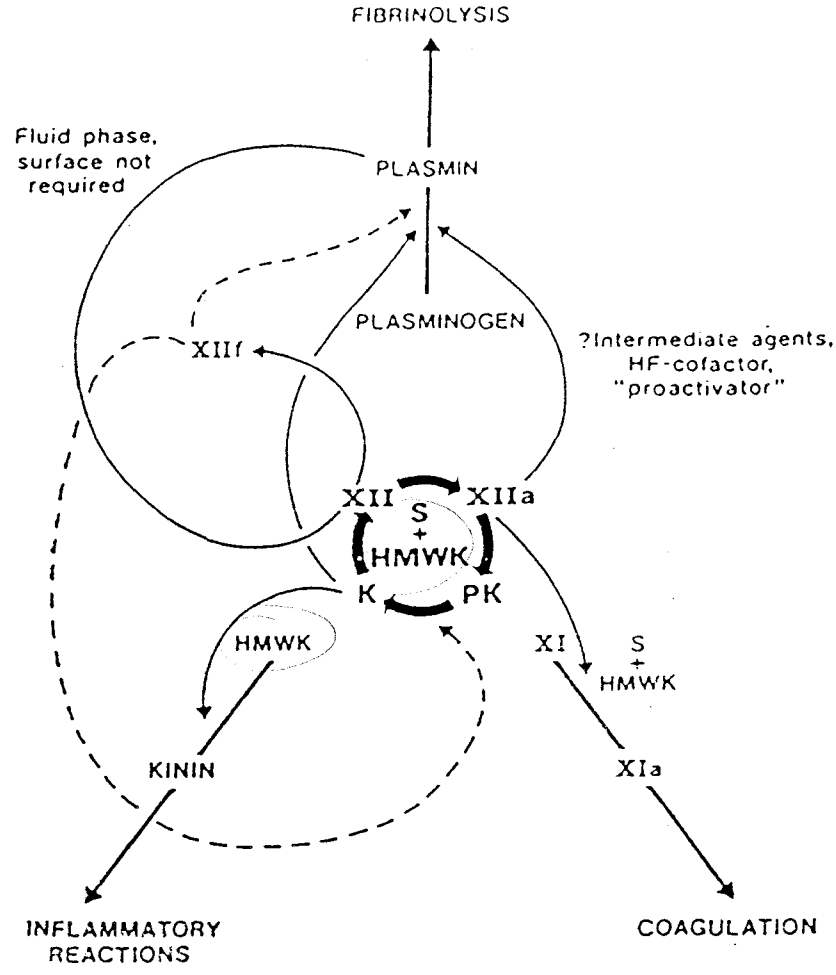
Plasma prekallikrein (26,27,31,32) is the fourth of the major factors involved in the initiation of the intrinsic clotting pathway. It is a single chain glycoprotein with an amino acid sequence essentially homologous with the individual polypeptide chains found in Factor XI. This 80,000 MW zymogen is activated by the proteolytic action of Factor XIIa in the presence of HMWK as a cofactor. HMWK is believed to bind prekallikrein to negative surfaces, where XIIa can be found in higher concentrations than in the fluid phase. Once cleaved, kallikrein is the resulting active form. It dissociates readily from surface-bound HMWK in order to perform its main task of activating Factor XII. Kallikrein is also responsible for activating HMWK and releasing the kinins responsible for inflammatory and pain response. Free kallikrein can also activate free Factor XII but it is 500 times more rapid in the activation of surface-bound Factor XII (26). This could be due to the fact that it is

brought to the surface as a strongly adsorbing complex with HMWK. Kallikrein is also responsible for the second cleavage of Factor XIIa (actually α-XIIa) to β-XIIa which does not remain attached to the surface since it lacks the positively-charged region found in α-XIIa (10).

Kallikrein is felt to be the main activator of Factor XII but other, poorly understood mechanisms are known to exist. These alternative mechanisms are much slower than the kallikrein proteolysis of Factor XII and plasminogen.

The working relationship between Factors XII, XI, HMWK amd prekallikrein (shown in Figure 4) (10) has been termed the reciprocal proteolytic pathway. It is depicted as the reciprocal activation of Factor XII and prekallikrein (and also Factor XI and Factor XII) revolving around the cofactor activity of HMWK and a surface. This facilitates the initiation of the intrinsic pathway of coagulation, the mechanism of concern in blood-material interactions.

The biocompatibility of a material depends on its ability to inhibit thrombosis by the two mechanisms discussed above. First, the adsorbed protein laid down must have a low affinity for platelets in order to avoid platelet initiated thrombosis. Second, the material should activate the intrinsic clotting pathway as slowly as possible or not at all.



Abbreviations:

XII:	Hageman factor (factor XII)	HMWK:	high-molecular-weight kininogen
XIIa:	activated Hageman factor		
XIIf:	Hageman factor fragments	K:	kallikrein
XI:	factor XI	PK:	prekallikrein
XIa:	activated factor XI	S:	surface

Figure 4 The Reciprocal Proteolytic Pathway (10)

Endothelium-Blood Interactions

The mechanisms of hemostasis following the injury of a blood vessel wall are akin to those of thrombosis, although the former is normal while the latter is pathological. In hemostasis, the extrinsic pathway is activated by the release of a tissue factor known as thromboplastin and accelerated by the activity of kallikrein and Factor XIa from the intrinsic pathway. The intrinsic pathway is also activated by the contact of the reciprocal proteolytic cycle proteins with exposed collagen fibres and basement membrane phospholipids (1,2).

An intact blood vessel is able to maintain freedom from thrombus by processes analogous to those by which an injured one promotes thrombosis: through metabolic processes employing active proteins (34,35). Although negatively charged artificial surfaces are known to promote clotting, blood vessel endothelium maintains its remarkable compatibility even though it is reported as having a net negative charge (65). Endothelial cells actively produce substances such as plasminogen activators to promote fibrinolysis (the breakdown of fibrin) and prostaglandins to inhibit the aggregation of platelets. Maintaining this kind of compatibility is an active process requiring the constant secretion of complex molecules and would be very difficult if not impossible to simulate in a potential biomaterial. Current research is aimed at passively minimizing the interaction of materials with blood rather than actively preventing it.

The Vroman Effect

The Vroman Effect is the name given to the mechanism by which HMWK appears to displace fibrinogen from a surface exposed to plasma. It is named after Dr. Leo Vroman who first reported it (22).

Fibrinogen, in binary and ternary protein mixtures with gamma globulin and albumin, is adsorbed preferentially with an affinity many times that of albumin and IgG (36). It thus appeared paradoxical that fibrinogen was undetectable on surfaces exposed to normal human blood plasma while albumin and IgG were both adsorbed (37). Vroman suggested, based on short-time experiments, that fibrinogen is adsorbed as expected initially but is rapidly displaced by another plasma component. Based on experiments using HMWK- deficient (Fitzgerald) plasma, which showed that fibrinogen was still detectable on glass after long term exposure, it was suggested that HMWK was responsible for displacing initially adsorbed fibrinogen (20). These investigations were qualitative and did not yield any insight into the kinetics and phenomenology of the Vroman Effect.

One way to obtain quantitative data on the behaviour of individual proteins in a plasma system is to label the protein of interest with a radioactive isotope. This approach has been followed in the present thesis. The experimental technique utilized (described in the procedure section) allows a minimum contact time of about one or two minutes, a time not short enough to detect the initial adsorption of fibrinogen on glass. It seemed likely that fibrinogen adsorption would be observed if diluted plasma were used to slow the displacement rate down. This prediction proved correct and the use

of diluted plasma allowed good quantitative data to be collected and was the foundation for the experimental program on which this thesis is based.

The Vroman Effect has been found to occur on glass using native, citrated or heparinized plasma (22) and should not be affected by the addition of anticoagulants since the clotting pathway is interrupted by anticoagulants further down the cascade, and not in the initial stages involving Factors XII, XI, prekallikrein, and HMWK.

In comparisons between surfaces it was found that the displacement of fibrinogen occurred only on hydrophilic surfaces such as glass and kaolin and not on hydrophobic surfaces(20). Later work by Brash (37) showed the effect does exist but to a much lesser extent on polyethylene and some silicone coated surfaces. The conclusion from this data was that the "Vroman Effect" may yield direct insight into the activation of the intrinsic pathway. Such a hypothesis is not unreasonable in light of the fact that prekallikrein and Factor XI are known to exist in plasma complexed with HMWK. Thus the adsorption of HMWK would bring together the four proteins credited with surface activation of the clotting mechanism (11): HMWK, prekallikrein, Factors XI and XII.

Since a large number of hydrophilic surfaces have a net negative charge it may be the net positive charge on the histidine-rich region of HMWK which facilitates its apparent high affinity for them. The resulting high surface concentrations of the four initiating clotting factors may be responsible for the clot-promoting qualities of negatively charged surfaces.

Protein Adsorption

Proteins

Proteins are organic macromolecules, considered to be the building blocks of life (38). Their primary structure consists of polypeptide chains of amino acid units. Each of the 20 amino acids commonly occurring in nature has a different structure which defines its overall reactivity. The secondary structure of proteins consists of α-helices and β-sheets stabilized by hydrogen bonds. The final level of organisation for globular proteins is the tertiary structure. The tertiary structure gives the protein its functional shape and may be stabilised by covalent bonds such as disulphide bridges between remote amino acids. Other factors are also important in determining the overall shape of the molecule; for example hydrophobic interactions play a major role in globular protein structure. Blood proteins exist in an aqueous environment and the majority of amino acids on their surfaces are hydrophilic. Hydrophobic groups tend to be contained within the interior of the molecules (38).

If a protein is exposed to a harsh environment such as high temperature, extreme pH, a nonaqueous solvent or a hydrophobic surface, the secondary and tertiary structure can be reversibly or irreversibly altered. This process, known as denaturation, may also change or destroy the biological activity of the protein. This tendency makes proteins extremely sensitive to their environment. It is this sensitivity which also makes proteins so effective as enzymes and active molecules in the very stable environment provided by the human body. Every protein's structure allows it to change shape in order to change its biological activity. It is often the

binding of a substrate (or cofactor) with a binding site which causes a conformational change capable of altering the molecule's activity.

The Fibrinogen Molecule

Fibrinogen is one of the most abundant and important proteins in human blood plasma. It has a molecular weight of about 340,000 daltons and makes up 4.5% of dried plasma (3.0 mg/mL average concentration) by weight (39). When the clotting mechanism is activated, fibrinogen is converted into fibrin monomer units which, in turn polymerize to form the fibrin meshwork that is the skeleton for the blood clot. This is the final step in the complex series of reactions of the clotting cascade.

Fibrinogen may also be involved with thrombus formation at a foreign surface. Platelets attach themselves to a foreign surface only after a "conditioning" layer of plasma proteins has been laid down. The affinity of the platelets for this surface depends on the composition of the layer. Fibrinogen has been shown to enhance the adhesion of platelets to the surface (1), while albumin seems to inhibit it (57).

The globular fibrinogen molecule itself has been described as a "triple dumbell" with two large end domains and a smaller central domain (42). Other researchers feel that the molecule is essentially cylindrical with dimensions 45 nm long by 5 nm diameter (43). It has also been hypothesized that the molecule takes on a spherical shape in solution. The triple dumbell model is however now generally accepted as the correct structure (40,41,42).

The fibrinogen molecule as a whole has 26 more negative charges than positive with eight of these known to be on the central domain and four

on each end domain (42). As a result there are electrostatic interactions with charged surfaces and some association with cations in solution to form an electrical double layer.

The natural environment of fibrinogen is in aqueous solution and in order to minimize its free energy, hydrophobic groups are generally buried inside the molecule while hydrophilic groups remain outside in contact with the solution. When the molecule approaches and adsorbs to a hydrophilic surface the original structure of the molecule is unlikely to be changed as extensively as on a hydrophobic surface, where the fibrinogen may expose its hydrophobic groups via denaturation to form a strong hydrophobic-hydrophobic bond.

Under normal conditions (at or below normal plasma concentrations and in a physiologic pH range) fibrinogen adsorption does not normally exceed a monolayer (43,77). There is little interaction between the molecules to allow this to occur. Within this monolayer, however, many different conformations can occur owing to the complexity of the fibrinogen molecule's structure.

The non-symmetric rodlike structure of fibrinogen allows it to assume two main orientations on the surface; end-on or side-on. Based on the size of the molecule, and assuming various types of surface packing monolayer surface concentrations of 0.18 to 0.25 $\mu g/cm^2$ have been calculated for side-on adsorption and 0.8 to as high as 2.24 $\mu g/cm^2$ for complete end-on adsorption (43). It would seem likely that the side-on molecules would be susceptible to more rapid and extensive unfolding than the end-on molecules. This would make the end-on molecules more desorbable and allow them to remain in their native state for longer times than the side-on molecules.

Other Plasma Proteins

Clotting factors are specialized proteins in blood plasma which exist as "zymogens" or inactive precursors. They are single or multiple chain polypeptides which can be activated by the cleaving action of active factors on specific peptide bonds. Other poorly understood mechanisms may aid the activation of zymogens such as the effect of different surfaces and the catalytic action of cofactors. It has long been suspected that some surfaces are able to activate Factor XII, a zymogen considered to be the first factor in the surface acivated clotting pathway. Recently, HMWK has been recognized as a cofactor, bringing together pairs of trace proteins at a surface in order to speed coagulation.

Despite its low plasma concentration (80 µg/mL), HMWK has been shown to dominate over more abundant proteins such as fibrinogen (20), gamma globulins and albumin on hydrophilic surfaces like glass. This would suggest that protein affinity for different surface types is much more important than transport mechanisms or plasma composition at long contact times. Also, in order for HMWK to achieve its dominant surface concentration it must be capable of displacing other more abundant components known to be transported to the surface more quickly. This has been shown to occur on glass within the first few seconds of contact with normal plasma. The effect may be due to the highly positively charged (histidine-rich) region of HMWK and its ability to bind strongly to negatively charged glass. Fibrinogen is a negatively charged molecule which may be adsorbed initially by specific

positively charged sites or more probably by cation incorporation in the overlapping electric layer between the surface and the protein.

Thermodynamics of Protein Adsorption

Protein adsorption to foreign surfaces is ubiquitous and nearly always spontaneous under normal conditions of temperature, pressure and solvent type. This would imply that the Gibbs free energy, ΔG, is negative. ΔG has an enthalpy and an entropy component as shown in its defining relationship,

$$\Delta G = \Delta H - T\Delta S \qquad [1]$$

Studies by Norde and Lyklema (43), showed that ΔH could be positive or negative depending on the pH for albumin adsorption on negatively charged polystyrene. Since the adsorption occurred spontaneously under all conditions of pH it was concluded that the process was entropically driven and any enthalpic contributions were small. It was found for pH near the isoelectric point that protein adsorption became endothermic suggesting that entropically-driven hydrophobic interactions had become dominant. The increase in entropy associated with hydrophobic interactions occurs as a result of the disruption of structured water at the exposed hydrophobic regions of the protein and the surface. Exothermic adsorption can occur as a result of electrostatic, covalent or hydrogen bonding while a positive value of ΔH may be due to unfolding of the tertiary and secondary structure.

Lyklema summarized these six main contributions to the enthalpy and entropy of protein adsorption (44):

1. "Hydrophobic dehydration" or disruption of structured water molecules causes an increase in entropy.
2. "Overall electrostatic interaction" affects the enthalpic contribution and depends on the charge sign of the protein and the surface.
3. "Structural alterations" yield positive values for ΔH and ΔS.
4. "Ion incorporation" has varied effects on ΔH and ΔS. They are both negative if ions are merely used in the interlayer between the surface and a like-charged protein. The formation of an electrical double layer, however, causes an increase in entropy.
5. "van der Waals interaction" contributes only slightly to the enthalpy term.
6. "Specific binding" depends on the properties of the surface and the protein and is a result of many simultaneous interactions between the two.

Thermodynamic parameters depend on all aspects of the system but in particular on the structure of the protein itself.

Artificial Surface Energetics

Some researchers have attempted to correlate properties that estimate biocompatibility with surface energy (45,46,47). Contact angles and critical surface tension have been particularly widely used in this regard (48). It has been proposed that materials with a critical surface tension in the range 20-30 dyne/cm are potentially more compatible than those with higher or

lower critical surface tensions (49). Interfacial free energy (related to critical surface tension) has been decomposed into its polar and dispersion components in order to compare their individual contributions to biocompatibility.

Ruckenstein attempted to combine all these considerations into a mechanistically sound mathematical formulation (49). It was postulated that the interfacial tension for the cell-medium interface is in the range 1-3 dyne/cm in order to provide good compatibility with the plasma proteins while avoiding denaturation and possible zymogen activation. The interfacial tension must be non-zero to ensure the mechanical stability of the interface. Dissolution of the solid surface may occur if it is too low. The equation for the determination of interfacial tension based on the dispersion and polar components is:

$$\gamma_{SL} = \left[\left(\gamma_L^p\right)^{1/2} - \left(\gamma_S^p\right)^{1/2}\right]^2 + \left[\left(\gamma_L^d\right)^{1/2} - \left(\gamma_S^d\right)^{1/2}\right]^2 \qquad [2]$$

If it is assumed that the liquid may be approximated by water ($\gamma_L^P = 50.8$ dyne/cm and $\gamma_L = 72.6$ dyne/cm) it can be shown that a non-polar surface ($\gamma_S^P = 0$) such as Teflon will yield a minimum interfacial tension of 50.8 dyne/cm. Such a material would not be suitable for long term compatibility with blood proteins. A potential blood compatible surface must have the right combination of polar and dispersion free energies in order to meet the 1-3 dyne/cm criterion. Glass is a material whose hydrophilicity and negative charge would suggest potential biocompatibility. However, $\gamma_S^P = 90$ dyne/cm and $\gamma_S^d = 80$ dynes/cm are the estimated components for glass in an aqueous

environment and as such, yield an interfacial tension of 23.8 dyne/cm, much too high a value for good compatibility. The surface energetic criterion of Ruckenstein appears to be theoretically and practically sound in a general sense, but it does not take into account specific factors such as the electric double layer, steric factors, property changes due to hydration, and protein interactions.

Materials which are extensively hydrated, like hydrogels, may be able to achieve the interfacial tension with plasma that would allow good compatibility. Some hydrogels may contain as much as 80 or 90 per cent water and can provide a virtual water/water interface with sufficient mechanical stability to maintain its structural integrity. A more detailed discussion of these and other materials is presented below.

Materials

Since the Artificial Heart Program of the U.S. NIH was initiated in 1964, the search for blood compatible materials has been as intense as it has been fruitless. A large number of materials has been tested with some turning out to be better than others in certain applications but no material has yet been developed which is compatible in all blood contact applications. The mechanical requirements are, in general, rigorous; for example, in the case of heart valves, where 40 million openings and closures are required per year. However, the development of polymers and the design of implants have proceeded far enough to satisfactorily meet these requirements in the short term. Any implanted material must of course also be non-toxic but the most difficult hurdle to overcome is the reaction of blood to the presence of a foreign surface. The blood flow over the implant is also important and even the most carefully designed artificial hearts will have stagnant regions or areas of turbulent flow which can promote undesired thrombosis. It is clear that the solution to this problem of blood compatibility is not yet in sight but the search continues to branch out into various specific areas of the problem (50-53).

Materials that have been investigated fall into several categories:

1. Metallic
2. Ceramic
3. Carbon
4. Polymeric
5. Bioactive surfaces (eg. heparinized)
6. Natural materials

Metallic surfaces have been studied because of their mechanical properties and their already established role as prosthetic materials, e.g. in hip joint replacement. The first ball and cage prosthetic heart valves were made from stainless steel because of its long working lifetime, nontoxicity and resistance to corrosion. A well designed metallic valve can be very effective in high flow areas probably because of its smoothness and inertness. Unfortunately, metal surfaces have a high free energy and have a high interfacial tension in contact with plasma. As a result, they cannot be used in low flowrate regions where the coagulation pathway is favoured. Ball and cage and other one way valves made from various metals are rigid in structure and cannot mimic the hydraulic behaviour of the natural cardiac valves closely enough to avoid irregular flow patterns. The search for better designs and more flexible materials continues. Metallic and ceramic materials would appear to be better suited to long term skeletal and dental applications. Blood contact applications generally require flexible materials.

Various types of carbon surfaces (94) have been studied with respect to their ability to interface compatibly with blood. Graphites, glassy carbons, pyrolytic carbons and vapour-deposited carbon coatings have all shown promising behaviour when in contact with blood. All these surfaces are exceptionally smooth and impermeable to blood components, including water. The carbon therefore appears to act as a non-reactive barrier between blood and the material; although it may initiate the intrinsic pathway, especially in areas of low flowrates (94), platelet adhesion is low and this may account for the observed success in higher flow regions. The application of carbon coatings has been attempted on a wide variety of materials but the integrity of

the layer may be easily disrupted especially in regions of high flex. Evidence suggests that carbon coatings are quite stable on rigid materials and can improve their properties. They do not however prevent activation of the intrinsic clotting pathway.

Polymers are the most extensively used class of biomaterials because of their mechanical properties and the broad range of chemical groups that can be incorporated during their synthesis. Unfortunately the exact mechanism for the initiation of thrombosis is not sufficiently understood to permit the design of the optimal material.

Hydrophobic polymers have been very extensively studied due to their excellent mechanical properties and in many cases their good compatibility. The potential biocompatibility of hydrophobic surfaces has been of interest ever since Lister (54) found that clotting was prolonged in paraffin coated tubes in comparison to clean glass tubes. In an attempt to explain the compatibility of the endothelium Moolten (55) mistakenly concluded in 1949 that blood vessel wall of the rabbit was non-wettable. Ease of synthesis and the incorporation of interacting groups has permitted a wide variety of hydrophobic polymers to be studied. Most frequently, negatively charged groups have been incorporated in prospective polymeric biomaterials in an attempt to mimic what is believed to be the negatively charged surface of the blood vessel lining. Negatively charged surfaces are now known to catalyze the contact phase of the intrinsic coagulation pathway (11), although they may be inhibitors of later phases (97). Perhaps the most successful example of a hydrophobic polymer in use as a biomaterial is Biomer®, a segmented polyurethane manufactured by Ethicon. This has been used to line

the artificial hearts designed at the University of Utah and clotting was not found to be problem with artificial hearts implanted in calves for up to 94 days without the use of anticoagulants (52). Unfortunately, polyurethanes have been shown to degrade significantly when in contact with blood. Also, anticoagulants have been required in human implants due to the inadequacy of the blood compatibility of Biomer®. These problems must be overcome in order to ensure the future of the artificial heart.

Hydrogels (56) are another promising class of polymers for blood contact applications. These materials are capable of retaining large quantities of water while maintaining the structural integrity of the surface. Ideally, such materials could provide a virtual water-blood interface with minimal interfacial tension. The activation of clotting proteins would be expected to be much less rapid. Hydrogels such as HEMA (polyhydroxethyl methacrylate) have already been extensively used in soft contact lenses, but even in the eye, protein adsorption is a limiting problem (74) since the lens gradually becomes opaque. Applications in mechanically demanding implant situations are also limited due to the poor mechanical properties of hydrogels. Unless these mechanical properties can be improved their use will be confined to surface coatings in areas of minimal stress and strain.

Many researchers have studied the effect of modifying surfaces via the adsorption or chemical bonding of bioactive molecules. One example is the preadsorption of albumin to biomaterials (57). This has been studied by many researchers and has generally been found to passivate the surface to coagulation and to platelets. Maintenance of the adsorbed layer in contact with blood is however not possible for long term applications (98). Another

widely studied technique is the binding of heparin, a natural anticoagulant molecule, to various surfaces. It has been shown that heparin may be bound to surfaces while maintaining a significant amount of its biological activity. The binding may not be irreversible especially in the presence of blood and the subsequent leaching has so far limited heparinized materials to short term applications (58). It is felt in some circles that the desorbing anticoagulant molecules may create a concentrated region of anticoagulant near the surface and that this leaching may be required in order that the treated surfaces may work. The results are in general promising for heparinized surfaces and research has intensified to find a suitable binding technique. At least 1 $\mu g/cm^2$ must be on the surface in order to form the homogeneous layer so that anticoagulant properties are obtained. Most surfaces require much more than this (58).

Material Testing and Characterisation

There appears to be an endless supply of new materials to test but the problem of how the various tests are related to in vivo compatibility is far from resolved. Clearly human models can not be used to screen potential biomaterials and animal models are in general expensive. The connection between in vitro tests and in vivo compatibility is tenuous at best until the exact mechanisms of thrombogenesis are found for all cases. Many different in vitro tests have been done in the hope that they can be correlated with blood compatibility. Some of the tests commonly done are (52):

1. platelet adhesion
2. platelet deformation and secretion
3. prothrombin time
4. partial thromboplastin time
5. protein adsorption
6. thrombus formation

Platelet adhesion studies can be done using platelet suspensions in a flow or static system. It is believed generally that the retention of platelets on a surface may be related to the thrombogenicity of the surface. However, some workers feel that it is the extent to which platelets are altered on the surface which determines its platelet response. This latter opinion is logical in view of the fact that platelets may adsorb and desorb without releasing their contents and thus without promoting thrombosis. Prothrombin time, although frequently mentioned in biomaterial research, is a measure of deficiencies in the extrinsic and common pathways and has little bearing on surface-dependent stages of coagulation and therefore does not provide any

information on material blood compatiblity. Partial thromboplastin time (PTT), on the other hand does provide a useful measure of the effect of surface on the intrinsic coagulation pathway. Care must be taken to ensure variables other than surface type (such as surface area) are avoided when performing these experiments.

Protein adsorption is an interesting area of study since it is the first event in blood-material contact, however the relationships between the amount or types of proteins adsorbed and surface thrombogenicity are not well understood.

The measurement of thrombus formation is usually an ex vivo experiment involving measurement of platelet adhesion and protein accumulation (usually fibrinogen). A possible weakness of this approach is that it does not take into account any embolic events, which may be catastrophic for the test animal, while leaving an apparently clean surface. Also the correlation between clotting and fibrinogen build-up may be misleading.

Correlating Surface Properties, In Vitro Test Data, and Blood Compatibility

Many of these in vitro (or ex vivo) techniques can produce data which are correlated to some extent with blood compatibility in one direction and surface properties in the other. Researchers are currently looking for statistically significant or mechanistically justifiable correlations that can tie these three aspects together (46,59-62). Some of the important surface properties that have been considered are:

1. Wettability.
2. Critical surface tension.
3. Surface charge.
4. Interfacial tension.
5. Hydrophobic/hydrophilic regions.
6. Roughness.
7. Chain mobility at the surface.
8. Polar-apolar ratio.
9. Surface chemistry.

The techniques used to determine these individual properties may not be independent of the other important properties, making quantitative correlations more difficult. The effects of these and perhaps other material characteristics will probably play an interactive role in the blood-material reaction, further complicating the problem of determining the contribution of each.

The work of Lister (54) in 1863 encouraged the belief that non-wettable surfaces would be clot resistant in direct proportion to their hydrophobicity. A common method used to estimate wettability is a water contact angle measurement. This approach is the subject of much controversy and has proven unreliable in the prediction of blood compatibility. Lyman and Brash (48) used critical surface tension as a measure of hydrophobicity and found that it correlated with platelet adhesion. Critical surface tension provides a parameter more representative of a material's surface free energy and can be correlated over a fairly large range with platelet adhesion. The experiments performed are simple contact angle measurements using a series of liquids

with known air-liquid surface tension. The linear data (cosine of contact angle versus air-liquid surface tension) is extrapolated to $\cos\theta_{cont}$ equals 1.0 (i.e. $\theta_{cont} = 0$) yielding the critical surface tension. The critical surface tension of Teflon is significantly lower than most other materials but it is not as blood compatible as the Brash-Lyman correlation would suggest.

A more detailed characterization of surfaces would be provided by a chemical analysis of the surface layer. Electron spectroscopy for chemical analysis (ESCA) (63) is able to determine the composition of the first few layers of molecules on any surface. Already it has been used to show that certain materials owe their beneficial properties to lubricants and cleaning agents present on the surface. ESCA should prove to be a useful tool in future biomaterials research. Barenberg et al (64) found by studying the surface of various polymers that the motion of sterically unrestricted polymer side chains could be suppressed by exposure to UV radiation resulting in improved blood compatibility. The above examples illustrate the importance and the benefits of understanding the surface chemistry of materials. As more is learned about the clotting mechanism this information will become increasingly valuable.

It has often been suggested that a negative surface charge would provide a good environment for blood based on the observation that normal endothelium is negatively charged. In support of this view, Sawyer and Pate (65) found thrombus formation at the anode when a current was passed across a blood vessel. It is also widely believed that a positively charged surface will promote the aggregation of the negatively charged platelets thus leading to thrombus formation. However, a negatively charged surface will promote the

activation of Hageman factor (11) thus making thrombosis a double-edged sword. A balance of negatively and positively charged groups has been shown to be compatible in reports of Mason (66) discussing the importance of the hydrophobic-hydrophilic balance. It quickly becomes obvious that the relationship between surface properties and compatibility are highly complex and poorly understood. However it is important that the general trends be recognized in order to permit an educated screening of potential materials for contact with blood.

Even if all the individual contributions of particular surface properties can be elucidated it may not be possible to predict the combined effects of their interaction. It may be that the study of surface properties is straying too far from the study of in vivo interactions. The development of simple, relevant in vitro tests based on mechanistic or empirical models of blood-material interactions may lead to a more reliable screening technique for biomaterials in the future. First the molecular basis for coagulation and thrombogenesis must be more fully understood.

Modeling Protein Adsorption for Single Protein Systems

Diffusion limited adsorption model

The equation for diffusion limited adsorption from a stagnant solution developed by Ward and Tordai (67) is analogous to the case of unsteady-state heat conduction at a semi infinite wall. Adsorption on a surface from a protein solution will generally be diffusion limited initially and reaction limited at high surface coverage.

Starting with the governing convection-diffusion equation [3] (known as the Fourier equation in heat transfer applications) (68,69,70,71)

$$\frac{\partial C}{\partial t} + V\frac{\partial C}{\partial x} = D\frac{\partial^2 C}{\partial x^2} \qquad [3]$$

where

D = diffusivity of the protein (cm^2/s)

C = bulk concentration of the protein ($\mu g/cm^3$)

t = time (s)

V = velocity of flow (cm/s)

x = distance from the surface (cm)

Equation [3] can be subjected to the following boundary conditions for C(x,t) for a semi-infinite wall

C(0,t) = 0 for all t (also known as the Smoluchowski boundary condition)

$C(x,0)=C_b$ (the bulk protein concentration) for $x>0$ (i.e. no hydrodynamic boundary layer develops during displacement of any pre-wetting liquid by protein solution)

The solution yields:

$$C(x,t) = C_b \operatorname{erf}\left(\frac{x}{2Dt}\right) \qquad [4]$$

Fick's diffusion law [5] states that the flux to the surface is proportional to the gradient at $x=0$.

$$\frac{d\Gamma}{dt} = D\frac{dC}{dx}\bigg|_{x=0} \qquad [5]$$

where Γ = surface concentration of protein ($\mu g/cm^2$).

Therefore, the rate of diffusion-limited adsorption is given by:

$$\frac{d\Gamma}{dt} = C_b\sqrt{\frac{D}{\pi t}} \qquad [6]$$

Integration yields an explicit expression for the initial values of Γ (before equilibrium and adsorption effects dominate).

$$\Gamma = 2C_b\sqrt{\frac{Dt}{\pi}} \qquad [7]$$

The assumptions which must be remembered with respect to equation [7] are:

1. adsorption is diffusion limited
2. $C(0,t) = 0$
3. C_b does not change as a result of depletion
4. surface coverage is low

Langmuir model

The Langmuir, and related isotherms have long been criticized with respect to their applicability to protein adsorption. These concerns may be easily justified by the fact that few of the Langmuir assumptions are satisfied for such complicated systems. Interest, however, continues since the kinetics upon which the model is based are basic to the adsorption process. Molecules arrive at a surface and fill it until a complete monolayer (or equilibrium) is achieved. As a reversible process for a one component system:

$$P + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P\text{-}S$$

where P is the protein, S is the surface site to which it adsorbs, and k_1 and k_{-1} are the adsorption and desorption rate constants respectively. At equilibrium the familiar Langmuir isotherm, equation [8], is obtained:

$$\theta_c = \frac{K C_b}{1 + K C_b} \qquad [8]$$

where K is k_1/k_{-1}, the equilibrium constant,

θ_c is the fraction of the surface covered by component C.

If θ_c is modeled as a function of time based on Langmuir-type adsorption, equation [9] is the result:

$$\theta_c = \frac{1 - e^{-k_1 C_b t}}{1 + \frac{1}{KC_b}} \qquad [9]$$

Attempts to fit this model to protein adsorption data result in significant lack of fit due to several major factors:

1. partial reversibility
2. conformational change
3. protein-protein interactions at the surface
4. diffusion limitations

The Langmuir model would be applicable to protein adsorption only after modifications to the single interaction model have been made in order to account for the other interactions known to occur. As well as adsorption-desorption the contributions of diffusion limitations, surface solution exchange and conformational change must be considered. If a mechanistically correct model can be developed for one-component protein adsorption, it may be that relevant parameters could be used in a multicomponent model in order to predict the adsorption behaviour of protein mixtures. This may ultimately be applicable to the prediction of blood plasma response to foreign materials.

Beissinger-Leonard Model

Observations showing the presence of loosely adsorbed and nearly irreversibly adsorbed molecules on various surfaces have led many investigators to conclude that there are two or more distinct adsorbed states. Based on this concept, a model was developed, based on the Langmuir-Hinshelwood formulation for heterogeneous catalysis (39,72). It was felt that this model could account for multisite binding and the large number of internal degrees of freedom which distinguish proteins from the simple, rigid, spherical molecules on which Langmuir, Freundlich and BET models are based.

The system studied by Beissinger and Leonard involved the laminar flow of a protein solution through a slit. IgG was adsorbed to quartz and adsorption data were obtained using MIRF (multiple internal reflection fluorescence). The model was based on the basic transport equation [10].

$$\frac{\partial C}{\partial t} + V\frac{\partial C}{\partial x} = D\frac{\partial^2 C}{\partial y^2} \qquad [10]$$

where

C = protein concentration as a function of position (x and y) and time (t)

x = position in direction of flow

y = position perpendicular to direction of flow

t = time

V = flow velocity, f(y)

with the boundary conditions,

At $t=0, C=0$ for all $y, x>0$

At $x=0, C=C_b$ for all $y, t>0$

At $y=\pm b$

where $2b$ = width of the slit

The surface mass balance equations [11], [12] and [13] were based on the Langmuir-Hinshelwood formulation and were coupled with the transport equation [10].

$$\frac{d\theta_1}{dt} = B_1 C_b^{B7}(1-\theta_1-\theta_2) - B_2\theta_1(1-\theta_1-\theta_2) - B_3\theta_1 \qquad [11]$$

$$\frac{d\theta_2}{dt} = B_6 B_2 \theta_1(1-\theta_1-\theta_2) - B_4\theta_2 \qquad [12]$$

$$C_s = B_5\left(\theta_1 + \frac{\theta_2}{B_6}\right) \qquad [13]$$

where

θ_1 = fraction of surface covered by protein in state 1

θ_2 = surface coverage by protein in state 2

B_1 = rate of adsorption for state 1

B_2 = rate of transition from state 1 to state 2

B_3 = rate of desorption of state 1

B_4 = rate of desorption of state 2

B_5 = surface density of molecules in state 1

B_6 = surface density of molecules in state 2

B_7 = exponent to account for nonlinear concentration dependence

C_s = adsorbed protein

All seven parameters were estimated and found to be significant with values that could be explained based on previous hypotheses.

A major assumption was that the solution concentration at the surface was equal to the bulk concentration for all except very short times. In other words, diffusion limitations were overcome through the use of forced convection. This assumption was tested using the Leveque equation (73), which showed the actual adsorption rate to be 30 times slower than that predicted for a diffusion limited system. Examination of the kinetic data seems to indicate a square root of time relationship which may or may not suggest diffusion limitations. If the system is assumed to be a quiescent solution the apparent diffusion coefficient is calculated to be about 5×10^{-9} cm^2/s or one hundred times lower than the known diffusion coefficient, suggesting that the rate of adsorption is slow enough to limit surface coverage even under stagnant conditions. No data are available to confirm this conclusion.

There appear to be several problems with the model of Beissinger and Leonard. The assumption of a two-state adsorption must be an over simplification of a system in which the protein actually goes through a whole range of conformations. It does not seem likely that the transition could involve a

spread out, loosely bound molecule converting to a tightly bound, compact molecule. Other researchers have shown that protein molecules will spread out on the surface with time based on TIRF, ellipsometry, and circular dichroism spectroscopy studies (74,75,76). The model fails to take into account the possibility of exchange which is known to occur more rapidly than desorption. This phenomenon could have a major effect on the model.

Although the Beissinger-Leonard model has a number of apparent flaws with respect to the processes occurring at the surface, it does provide a solid theoretical background for understanding the mass transport characteristics of such systems. It is one of only a few models proposed to describe protein adsorption up to complete surface coverage and should provide a good basis for the development of future models.

Van Dulm-Norde Energy Barrier

In the studies of Van-Dulm and Norde (77), experiments involving the adsorption of ^{125}I-labelled albumin to different surfaces were done to study the kinetics of adsorption. The solution was gently mixed but the mass transport was felt to be diffusion limited and to be described by the relationship developed by Ward and Tordai (67). A plot of surface concentration versus the square root of time indicated that the rate of adsorption was slightly slower than for pure diffusion and it appeared to be surface dependent (77). Two hypotheses were put forward to explain the data. The first theory proposed that only molecules in the correct orientation could adsorb. This steric repulsion effect would, however, result in an accumulation of protein molecules at the surface and thus would fail to meet the zero concentration

boundary condition of the Ward and Tordai equation. The second hypothesis implicates electrostatic interactions of either the electrical double layer or the charge of the surface. It is possible that this could flatten the expected concentration profile of the charged fibrinogen at the surface, thus lowering the apparent diffusion coefficient.

A model was proposed whereby the slowing of adsorption could be related to an activation energy barrier calculated using an Arrhenius relationship. Unrestricted diffusion is then slowed by a proposed factor exp(-A/kT) where kT is a unit of thermal energy and A is the activation energy barrier to adsorption. Values for A of 0.8 kT and 2.9 kT were obtained for siliconized and untreated glass respectively. This model could provide an explanation for results which display a surface dependence when diffusion, a property independent of the surface, is felt to be the limiting process.

Lok, Cheng and Robertson isotherm model

Experiments by these investigators were done to study the adsorption of bovine serum albumin (BSA) and fibrinogen onto crosslinked polydimethylsiloxane in a flow system using the TIRF technique (78). A model was developed to describe the isotherm for BSA based on a modified Langmuir equation [14].

$$\Gamma = \rho\,\Gamma_{max} + \frac{(1-\rho)\,\Gamma_{max} KC_b}{1 + KC_b} \qquad [14]$$

Γ = adsorbed protein ($\mu g/cm^2$)

Γ_{max} = maximum protein adsorption ($\mu g/cm^2$)

ρ = fraction of protein irreversibly adsorbed

K = equilibrium constant ($cm^3/\mu g$)

C_b = protein concentration ($\mu g/cm^3$)

The inclusion of the ρ parameter resulted in significantly better model adequacy explainable by the experimentally observed phenomenon of partial reversibility. The dependence of ρ on surface and protein type is not predictable from first principles and must thus be determined by experiment. Also, the assumption that there are two clear cut states representing reversibly and irreversibly adsorbed proteins is likely an oversimplification. The information gathered from single component systems would therefore be difficult to extrapolate to multicomponent systems or, ultimately, plasma.

Only limited data were collected for fibrinogen adsorption in this system and the isotherm parameters could not be determined. It was shown however, that very slow exchange occurred on the silicone rubber surface studied. This observation makes the validity of the Langmuir-type model questionable. A true Langmuir system would require significantly more rapid exchange to produce a curve such as that seen in the protein adsorption data.

Kinetic studies by Robertson et al were modeled based on the Leveque equation (73) for laminar flow through a slit, as was done by Leonard. Leonard, for his reasonably complex model, found a dependence on an approximately second order concentration term. Robertson et al found a linear dependence on concentration at low concentrations and a half-order

concentration dependence at high concentrations. Leonard hypothesized that dimerization of protein molecules could explain the observed concentration dependencies. Robertson explained his results using a new time-dependent transport model which takes into account the developing concentration boundary layer. It proved adequate to describe the rapid adsorption in high concentration systems.

Cuypers model

The adsorption of proteins in a well-stirred, batch system was studied in order to determine the contribution of diffusion, intrinsic adsorption and desorption rates and the surface coverage (79). A model was developed based on Langmuir-type adsorption at the surface. The rate of adsorption is given by:

$$\frac{d\Gamma}{dt} = k_{on}^{app}\left(\Gamma_{max} - \Gamma\right)C_b - k_{off}^{app}\Gamma \qquad [15]$$

where,

$$k_{on}^{app} = \frac{D\,k_{on}^{int}}{D + \delta\,k_{on}^{int}(\Gamma_{max} - \Gamma)} \qquad [16]$$

$$k_{off}^{app} = \frac{D\,k_{off}^{int}}{D + \delta\,k_{on}^{int}(\Gamma_{max} - \Gamma)} \qquad [17]$$

D = diffusion coefficient (cm^2/s).

δ = unstirred layer thickness (cm).

C = adsorbate concentration ($\mu g/cm^3$).

Γ_{max} = maximum surface coverage ($\mu g/cm^2$).

Γ = surface coverage ($\mu g/cm^2$).

k_{on}^{app} = apparent adsorption rate (cm^3/s).

k_{on}^{int} = intrinsic adsorption rate (cm^3/s).

k_{off}^{app} = apparent desorption rate (1/s).

k_{off}^{int} = intrinsic desorption rate (1/s).

For a system in which the diffusion coefficient is known and surface coverage is low the thickness of the stagnant layer can be calculated if the intrinsic rate of adsorption is assumed to be rapid.

This model is useful for a theoretical study of various adsorption conditions but cannot be correlated with real data due to the simplicity of the model. Partial reversibility and conformational change are not considered and as a result the model holds only for systems which comply with the Langmuir assumptions. One possible use for the model would be to correlate the stagnant layer thickness with stirring speeds. Such a correlation would allow the measurement of diffusivities of other proteins in the same system. At high mixing speeds the rate of adsorption may become dominant and measurable due to the thinner stagnant layer.

The model of Cuypers et al is useful mainly at low surface coverages. Its use however, will remain limited unless the mass transfer equations can be coupled with a more mechanistically correct adsorption model.

To date there is no generally satisfactory model for the adsorption of a protein from a one-component solution. A better understanding of the events at the interface is needed before a mechanistically correct model can be obtained. If this can be accomplished perhaps the more complicated interactions between different proteins can be predicted or explained with respect to their role in blood material interactions.

CHAPTER 3

Experimental

General Approach and Technique

Protein adsorption has been extensively studied in the past twenty-five years using several different experimental techniques. The most reliable quantitative data have been obtained using ex situ methods (requiring several rinsing steps after the desired adsorption has been achieved, such as solution depletion (77), radio-labelled (37) proteins, total internal reflection infrared spectroscopy (78), immunochemical labelling (22), X-ray photoelectron spectroscopy, ellipsometry, total internal reflection fluorescence (TIRF) (78), and electron microscopy. Attempts have been made to apply several of these techniques to obtain "in situ" data which are more desirable. This approach could provide more data at shorter time intervals using an on-line computer. To date only qualitative results have been obtained based on TIRF and confusion exists as to how best to clearly distinguish the bulk protein solution and the adsorbed protein layer.

Ex situ experiments require the rinsing away of the protein solution, and in all probability the reversibly bound protein, leaving only the irreversibly adsorbed protein layer behind to be studied. The researcher is thus denied the ability to study the detailed "real time" course of events in protein adsorption. Ex situ measurements at discrete and relatively long time intervals are time consuming and inefficient but currently provide the most reliable quantitative data.

The present study employed the ex situ radio-tracer technique exclusively. In each experiment fibrinogen was labelled with ^{125}I and adsorbed to different materials from a Tris buffer or from various types of human blood plasma. This radiolabelling method makes it possible to monitor the adsorption of fibrinogen on the surface from multicomponent systems such as plasma. No other technique can give quantitative data for the plasma system with comparable reliability.

Care was taken to ensure that extensive desorption of fibrinogen did not take place from the adsorbed layer upon rinsing. This was investigated by showing that adsorption is not a function of rinse time. The possibility of preferential adsorption of labelled versus unlabelled protein was also studied (80,81). A small but insignificant effect was found on each surface that was tested. The gamma radiation count rate on the surface could therefore be related directly to surface concentration based on the previously measured solution count rate.

Despite its ex situ nature, the radio-labelling technique provides the most convenient method for studying competitive and single-component protein adsorption. Perhaps in the future an in situ experiment can be designed around this method of detection.

Experimental Error

Variations in experimental results, although not large enough to affect the qualitative observations being made, accounted for a $\pm 10\%$ discrepancy (maximum). Equipment error did not exceed $\pm 3\%$. Outlier points were infrequent but did occur.

Materials List

The following is a list of materials, and their sources, which were used in the experimental work.

1. Pooled citrated human blood plasma - from Canadian Red Cross, Ottawa, courtesy of Dr. George Adams.
2. Human fibrinogen - from Kabi, Stockholm, Sweden (0.2 wt plasminogen)
3. Pyrex glass tubing (0.24 cm ID)
4. 3 way valves (Teflon) - from Pharmaseal, Toa Alta, Puerto Rico
5. Latex tubing (1/8"x1/16") - from Canlab
6. 30 mL syringes - from Becton-Dickinson, Rutherford CA
7. Polyethylene tubing (medical grade, 2.64 mm ID) - from Clay Adams, Parsipanny NJ
8. Gamma counter (Biogamma) -Beckman, Fullerton CA
9. Porcine aortic valves from hearts of freshly slaughtered pigs (Fearman Co., Burlington, Ont.) and fixed in glutaraldeyde by O. Hum (results not presented here)
10. Tris (hydroxymethyl) amino methane (electrophoresis quality) - from Biorad, Richmond CA
11. ^{125}I - from Dupont, New England Nuclear, Boston MA
12. Sepharose 4B - from Pharmacia, Uppsala, Sweden
13. Cyanogen bromide - from Sigma, St. Louis MO
14. L-lysine - from Sigma
15. Surfasil - from Pierce Chemical Co., Rockford IL

16. PU02 a segmented polyurethane - prepared by C. Hudson at MIPPT, McMaster University
17. Dextran Sulfate (MW 500,000) - from Sigma, St. Louis MO
18. Chromerge - from Manostat, New York NY
19. Methanol - from BDH, Toronto, Ont.
20. Factor XII deficient plasma - from George King Biomedical, Overland Park, Kansas
21. Factor XI deficient plasma - from George King Biomedical
22. Glycine - from Biorad, Richmond CA
23. HMWK - deficient plasma - from George King Biomedical
24. TLCK - Sigma
25. Benzamidine - Sigma
26. Arvin - a gift from Dr. M. Hatton (Pathology, MUMC)

Experimental Methods

Preparation of Tris Buffer

In order to make two litres of isotonic Tris buffer 48.4 grams of electrophoretically pure Tris was brought up to a volume of 1000 mL in deionized water to form a stock solution. 840 mL of this stock solution was combined with 700 mL of 0.4 N HCl solution. To the resulting mixture, 460 mL of deionized water was added. The pH was adjusted to a value between 7.35 and 7.40 by the addition of a small amount of the HCl solution. The buffer was stored in a plastic bottle at room temperature.

ICl Preparation

The ICl used in the labelling procedure was prepared in the following manner: 150 mg of NaI was dissolved in 8 mL of 6 N HCl and 108 mg $NaIO_3 \cdot H_2O$ was dissolved in 2 mL deionized water. The resulting solutions were mixed and the volume brought up to 40 mL with deionized water. This was shaken up with 5 mL CCl_4 repeatedly until the pink colour was no longer visible in the organic phase. Residual CCl_4 was removed by aerating for one hour and the volume of the solution was made up to 45 mL with deionized water to form the stock ICl solution. For use in the labelling procedure one part stock solution was mixed with nine parts of a 2 M NaCl solution to give a 0.0033 M ICl solution in 1.8 M NaCl.

Glycine Buffer Preparation

75 g of glycine and 58.5 g NaCl were dissolved in deionized water and the volume was brought up to 500 mL to form a 2 M glycine solution in 2 M NaCl. To this was added 2.0 N NaOH until the pH reached 8.8.

^{125}I Labelling of Fibrinogen

Fibrinogen was labelled using the method of McFarlane (82) resulting in a substitution of iodine in the aromatic rings of the tyrosine residues. The biological activity of fibrinogen has been shown to be unaffected if only one iodine atom per molecule is substituted (82).

The amount of fibrinogen labelled for each experiment was essentially the same. 0.5 mL of fibrinogen solution (approximately 9 mg/mL in isotonic Tris) was prepared for labelling by the addition of two drops of glycine buffer (pH 8.8). One drop of glycine buffer was added to 0.1 mL ICl solution (0.0033 M). 4.0 µL of Na-^{125}I was mixed with the ICl for one minute. The fibrinogen was then carefully mixed with the ICl avoiding air bubbles as much as possible. The unreacted ^{125}I was then separated from the labelled fibrinogen by passing the "hot" solution through an anion exchange column which consisted of a 3 mL disposable syringe, plugged with glass wool, filled with anion exchange resin and equilibrated with Tris buffer. The column was rinsed with approximately 10 mL of Tris buffer yielding a labelled fibrinogen solution that could be used as a trace label for 10 to 15 mL of human plasma or for a simple fibrinogen-in-Tris system.

Preparation of Dilute Plasmas

Several different types of human blood plasma were investigated in order to compare the effect of certain plasma protein deficiencies on fibrinogen adsorption kinetics. The plasmas investigated were:

1. Fitzgerald trait -a lack of high molecular weight kininogen (HMWK 0.0 Units/mL) (95)
2. Williams trait - a lack of HMWK and LMWK (0.0 Units/mL)
3. Hageman trait - deficiency of Factor XII (0.0 Units/mL) (16)
4. Sepharose-Lysine treated - plasminogen deficient plasma (96)
5. Arvinized plasma-fibrinogen deficient plasma (see below)
6. Factor XI deficient plasma (0.0 Units/mL)
7. Dextran Sulfate treated normal plasma
8. Dextran Sulfate and Sepharose Lysine treated plasma
9. Fibrinogen in Tris - a solution for the study of fibrinogen adsorption in a single component system.

Pooled normal human plasma was the type most frequently investigated. To a thawed sample of 10 mL (or 15 mL) the labelled fibrinogen, prepared earlier, was added. It was then brought up to a volume of 100 mL (or 75 mL) with isotonic Tris buffer in order to make 10% (or 20%) normal plasma. From this solution, other solutions ranging from 10% to 0.01% were made. 1 mL samples of each dilution were placed in scintillation vials and counted in the Beckman Biogamma counter in order to check that they were properly made and to determine the specific radioactivity of the labelled fibrinogen. Each dilution was taken up into a 30 mL syringe to minimize the effects of the

air-solution interface in the time between their preparation and their injection into the adsorption tubes.

The determination of fibrinogen concentration in the undiluted plasma was done by the Clinical Haematology Laboratory in the McMaster University Medical Centre (MUMC) using the thrombin clotting technique developed by Clauss (84).

Special Plasma Preparation

1. *Dextran Sulfate Treated Plasma*

Plasma was incubated with Dextran Sulfate at 0°C for 20 min. so that HMWK would be inactivated as described by and Scott et al. (99). This procedure yielded a plasma effectively HMWK deficient that could be used in place of Fitzgerald plasma.

A stock solution of 25 mg of dextran sulfate (500,000 MW) in 100 mL of deionized water was made up and used for all future experiments requiring it. 1 mL of this stock solution was mixed with 9 mL isotonic Tris buffer (pH 7.4). To this, 10 mL of unlabelled plasma was added and the whole solution was placed in an icebath for 20 minutes. The treated plasma was then quickly brought to room temperature in a warm water bath.

Another experiment required a 1 minute incubation of plasma with dextran sulfate at 0°C. In order to accomplish this, both the dextran sulfate solution and the plasma were cooled in the icebath prior to mixing and the mixture was warmed to room temperature more quickly, in a hot water bath, after incubation.

2. *Arvinized Plasma*

Arvin is an enzyme found in snake venom and is capable of polymerizing fibrinogen into a fibrin clot with little or no effect on the other proteins in blood plasma. A sample of Arvin was obtained from Dr. Mark Hatton (MUMC).

In order to remove the fibrinogen from 15 mL of normal pooled human blood plasma, it was incubated with 66 µL of the Arvin preparation at 37°C for 1 1/2 hours. The resulting fibrin clot was removed on a wooden applicator stick. A determination of the fibrinogen concentration in the residual plasma showed it to be less than 0.08 mg/mL which is beyond the sensitivity of the analytical method. For calculation purposes the fibrinogen level in arvinized plasma was assumed to be zero.

To the Arvinized plasma was added some partially labelled fibrinogen sufficient to bring the fibrinogen level up to 1/6th, 1/2 and 2 times normal (2.5 mg/mL). The resulting plasma was used for 5 minute adsorption experiments.

3. *Sepharose-lysine Treated Plasma*

A Sepharose-lysine treatment of blood plasma specifically removes plasminogen (96), a zymogen protein involved in breaking up fibrin clots (fibrinolysis). The procedure was carried out for both thawed pooled plasma and fresh donor plasma in order to see if any of the observed effects were related to freezing of the plasma.

Sepharose 4B was activated with cyanogen bromide and then reacted with L-lysine in order to make the column packing. 90 mL of plasma was then

brought up to pH 7.4 by the addition of 5 to 10 mL of 1.0 M Tris buffer. Then enough TLCK (N-α-p-Tosyl-L-lysine chloromethyl ketone) was added to make 1 mM in plasma and enough benzamidine to make 5 mM. (These compounds are added to prevent the conversion of plasminogen to plasmin since one of the main purposes for this procedure is to isolate plasminogen.) The resulting plasma solution was passed through the column and fractions of 4 mL were collected. Fractions from the optical density plateau region at 280 nm were combined and used for adsorption experiments. The column was 16 cm high and 1.5 cm in diameter.

Surface Preparation for Adsorption Studies

Glass: Glass tubing (0.24 cm ID, 0.4 cm OD) was cut into 25 cm pieces and stored in Chromerge for one hour. It was then thoroughly rinsed in deionized water and set up for overnight equilibration with isotonic Tris.

Surfasil: Glass tubing was prepared in the same way as above and thoroughly dried. It was then placed in a solution of approximately one part Surfasil to nine parts hexane for one minute, rinsed, and dried overnight at 50°C. The tubes were readied for equilibration by rinsing in deionized water. The resulting surface was quite hydrophobic and is generally considered to be less thrombogenic than glass.

Polyethylene Tubing: This flexible "medical grade" tubing made by Clay-Adams was cut into 25 cm long pieces and cleaned by placing in pure methanol for one hour. After thorough rinsing in deionized water they were set up for overnight equilibration with Tris. The resulting surface was fairly hydrophobic but not considered to be especially nonthrombogenic.

PU02: PU02 is a mildly hydrophilic segmented polyetherurethane made by C. Hudson (McMaster Chemical Engineering) consisting of large soft segments based on polyoxypropylene (MW 2000) and smaller hard segments containing hydroxyl groups. It was expected that this polymer might be moderately blood compatible by analogy with other segmented polyurethanes. The polymer was dissolved in DMF (dimethyl formamide) and solution coated onto the 19 cm long glass tubing segments. Following coating the tubes were dried in a vacuum oven and stored in a dessicator while not in use to avoid hydration and surface contamination.

Adsorption Experiments

Three way valves were attached to each end of the tubing to be tested with one inch of latex tubing (see Figure 5). A sidearm tube was attached to the inlet valve so that air bubbles could be removed from the system without entering the test section. A drain tube was attached to the outlet valve. Each tube was filled with isotonic Tris buffer and allowed to equilibrate overnight so that water uptake would be complete.

A plasma dilution and an adsorption time was selected for each tube. Labelled plasma was taken up into a 30 mL syringe and, at the appropriate time, injected into the tube at a rate of about 3 to 4 mL per second. Care was taken beforehand to remove any leading air bubble via the inlet sidearm. Upon reaching the end of the adsorption time, the tube was rinsed with 30 mL of isotonic Tris buffer at a rate of 3 to 4 mL per second. It was rinsed in the same way 5 minutes later and then drained 10 minutes after the second rinse. The 3 way valves were then removed and any excess Tris buffer inside the

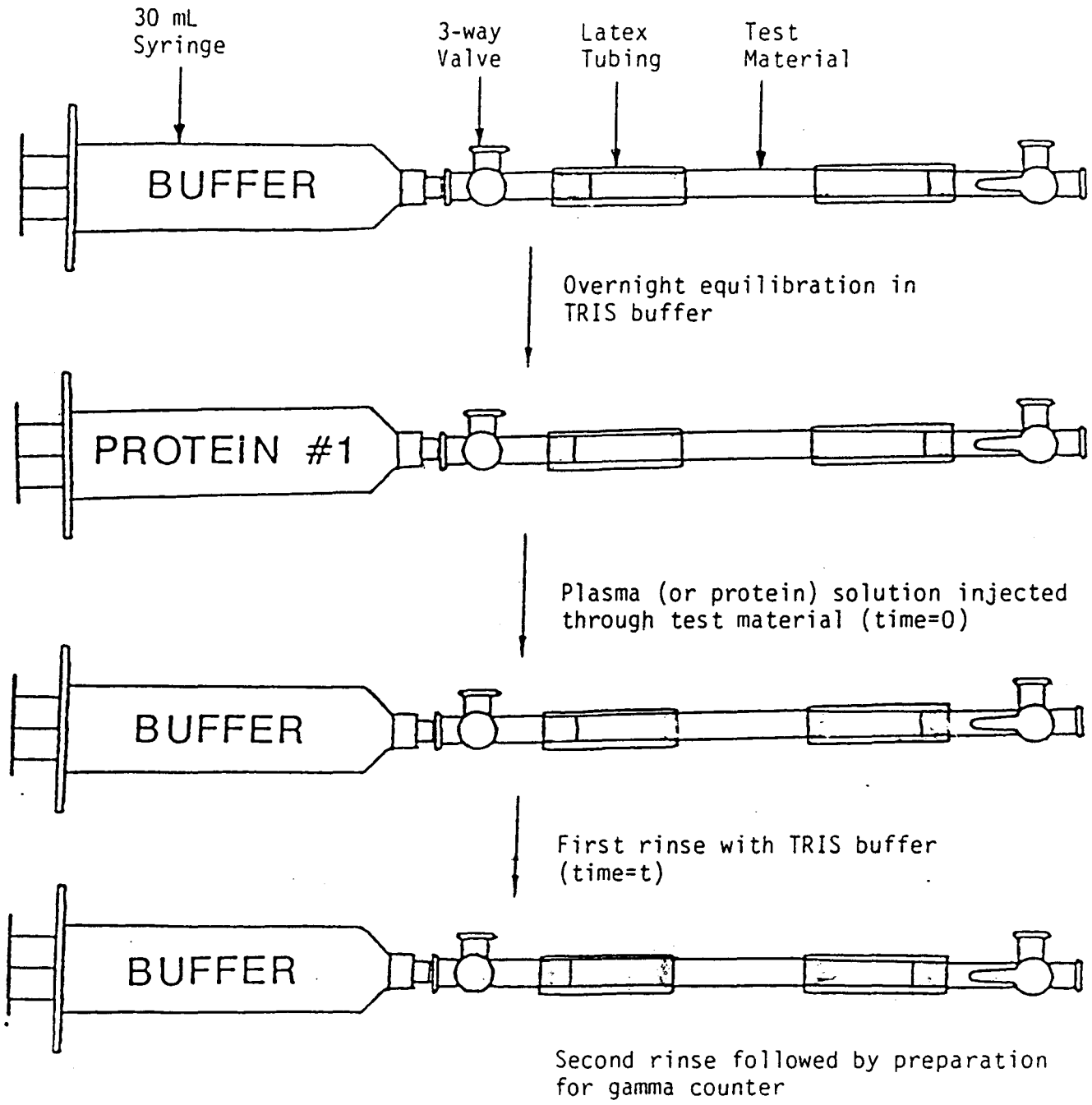


Figure 5 Experimental Setup for Adsorption Experiments

tube was carefully blown out. Taking care to remember the inlet and outlet ends of the tube, it was cut into 5 cm pieces which were placed, in order, in scintillation vials for counting. All the tubing pieces from the experiment were counted for 10 minutes each along with the appropriate solution counts. After counting, each tube was carefully measured for inner diameter and length in order to calculate the area for adsorption.

An "isotherm" experiment involved a series of plasma dilutions exposed to a surface for a specific time, usually 5 minutes or 24 hours. A kinetics experiment used only one or two different plasma dilutions for a range of times.

Systems Studied

Fibrinogen in Tris Buffer

Kinetics and isotherm experiments were also run for pure fibrinogen in Tris buffer, the one-component system. The concentration of fibrinogen was measured by determining solution absorbance in a spectrophotometer at a wavelength of 280 nm. The absorbance reading was converted to a concentration in mg/mL by dividing by an extinction coefficient of 1.55.

Different Plasmas

a. Factor XII deficient plasma

b. Factor XI deficient plasma

c. Dextran-sulfate treated plasma

d. Sepharose-lysine treated plasma

e. HMWK and plasminogen free plasma

f. Arvinized plasma with 2x, 1x, 1/2x, 1/6x normal fibrinogen concentration

Position Dependence

Several experiments were done to see if the position dependence observed in the standard tubes could be extended by using longer tubes. 5 minute adsorptions were performed on 120 cm long glass tubes using 10%, 1%, and 0.5% solutions of normal pooled plasma. As for the shorter tubes, 30 mL of plasma and 30 mL of Tris were injected at a rate of 3 to 4 mL/s.

Unequilibrated System

A 5 minute isotherm was done on clean glass tubes without the usual overnight equilibration with Tris. Chromerged tubes were rinsed with deionized water, set up and an experiment was run using the same subsequent method as for a normal 5 minute isotherm.

Preadsorbed Fibrinogen

Glass and Surfasil tubes were exposed to a 0.170 mg/mL solution of labelled fibrinogen for 5 hours. Two of the ten tubes were set aside to give an indication of the total fibrinogen adsorbed after this first step. The other eight tubes were exposed to an unlabelled 5% or 0.5% solution of normal pooled human blood plasma for a series of different times. These tubes were all rinsed and counted in the same way as for other experiments.

CHAPTER 4

Results and Discussion

Fibrinogen Adsorption from Buffer Solution

Many researchers have studied the adsorption of proteins from single component solutions but no one has been able to adequately model the kinetics of the process using available data (39,78,79,86). There is disagreement as to whether the adsorption is reversible or irreversible and whether it occurs in Langmuir-like fashion or follows some other isotherm. A great deal of the confusion is due to the fact that analogies have been drawn directly between the behavior of proteins and other, more fully understood simple molecules. Such analogies must be invalid in most cases. For example, the assumptions regarding the Langmuir isotherm are clearly incompatible with the nature of protein molecules. These assumptions are:

(1) The adsorbed species does not change shape.

(2) Each molecule is adsorbed to a single site.

(3) Adsorption is completely reversible

(4) No interaction takes place between adsorbed species.

(5) The surface is energetically homogeneous

Experiments described below yield information relevant to the validity of each one of these assumptions and show protein adsorption to be a more complicated process than can be accounted for by the Langmuir model.

Figure 6 shows the adsorption "isotherms" of fibrinogen on glass at 5 minutes (dynamic) and 24 hours (equilibrium). The data appear to be very

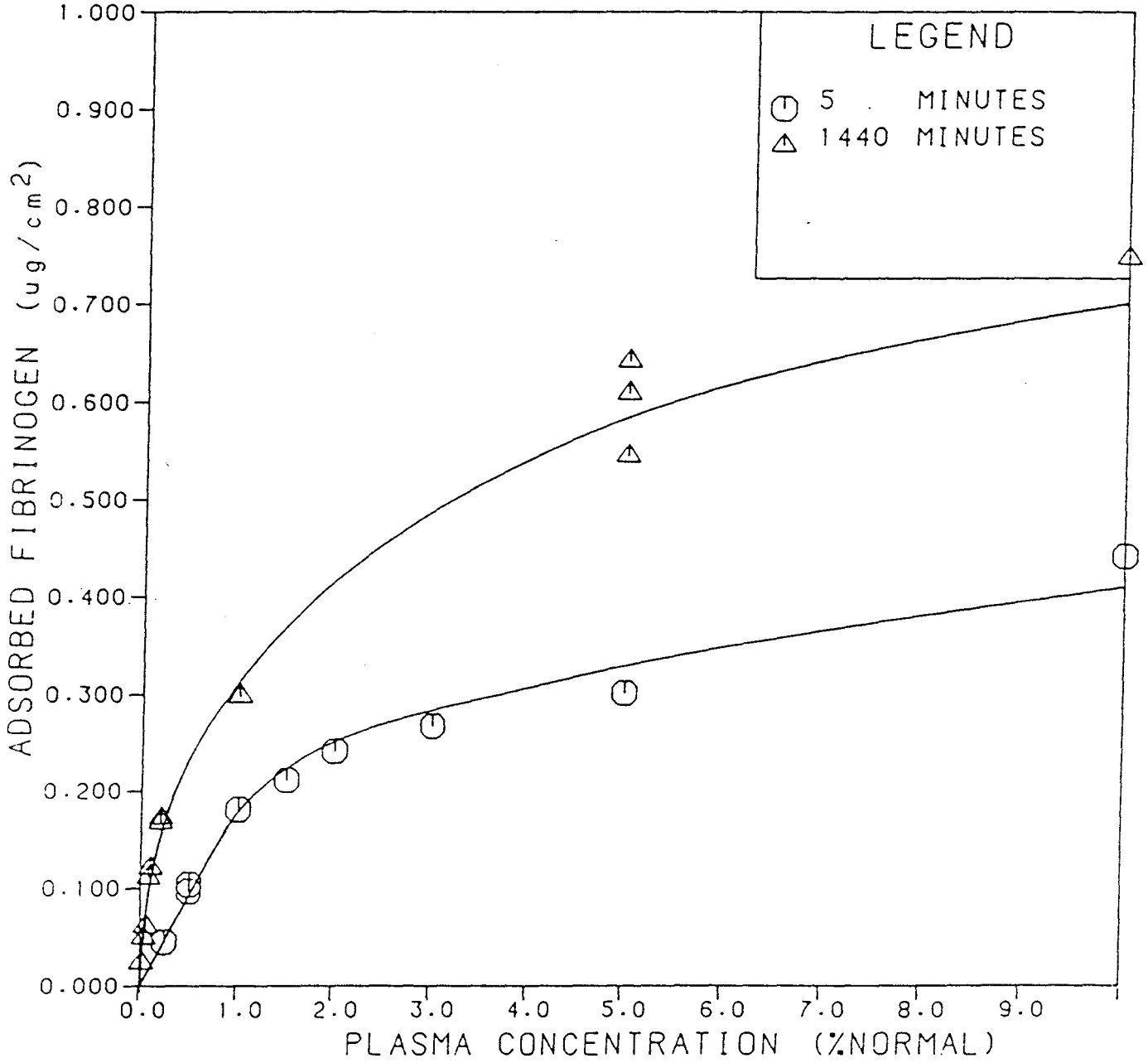


Figure 6 5 minute and 24 hour fibrinogen surface concentration versus bulk concentration (based on per cent of normal plasma concentration) on glass for fibrinogen in Tris Buffer (pH 7.4)

reproducible at low surface coverage but are more scattered at higher coverage. At both times the isotherms appear to be linear up to a surface coverage of about 0.2 μg/cm^2, after which they curve towards a quasi-plateau. Calculations were done (on the side of caution) to determine the potential effect of solution depletion on bulk concentration at the low bulk concentrations. It was found that in the linear portion of the isotherm the bulk concentration was reduced to about 30% of its initial level. Therefore the 24 hour isotherm does not achieve infinite slope as one might expect for an irreversible process. This suggests that the adsorption is reversible and a dynamic equilibrium probably exists for this protein system.

The surface concentration, 0.20 μg/cm^2, marking the end of the linear range of the isotherms is also known to be the value for a complete monolayer of fibrinogen adsorbed in a side-on conformation/orientation (43). It has been shown, in this laboratory as well as others, that while adsorbed proteins are only partially and slowly desorbed by rinsing with a buffer, they readily undergo exchange with protein in the bulk phase (87,88,89). This exchange occurs at equilibrium and is no doubt responsible for some of the characteristics of the protein adsorption isotherm. Exchange may also allow the fibrinogen population to progressively rearrange and adsorb in a more end-on conformation/orientation permitting the monolayer to exceed 0.20 μg/cm^2. Upon initial contact with the surface fibrinogen is probably in an end-on conformation/orientation and bound at only one or two sites. With time more sites on the molecule may become bound and the conformation/orientation gradually changes to side-on. This transition from

end-on to side-on will be termed "spreading" (44) in the discussion that follows.

The 24 hour isotherm is considered to be the true equilibrium isotherm on glass based on the fact that its shape is essentially the same as the one hour isotherm. The one hour data points in Figure 7 correspond closely to points on the 24 hour isotherm curve in Figure 6. Walton and Koltisko have reported significant changes in the adsorbed layer over long periods of time (75,76) but this was not observed in the present experiments. It is possible that the systems they observed were adsorbed protein layers exposed to a buffer and not to a protein solution. This would eliminate the exchange of proteins and thus the only observable phenomena would be spreading and perhaps long term denaturation.

As shown in Figure 7, the initial data points for the 0.5% (1.25×10^{-2} mg/mL) (units are in % of normal plasma fibrinogen level, 2.5 mg/mL) kinetics curve are consistent with a diffusion limited system. The surface coverage, Γ, is proportional to the square root of time as in the equation developed by Ward and Tordai (67) for diffusion toward a surface sink. The first few data points for the 3.0% kinetics curves are not proportional to the square root of time; in this case the surface is presumably filled during the initial one minute adsorption time. Adsorption after this time exceeds 0.20 $\mu g/cm^2$ and thus is probably governed by exchange and other interactive mechanisms until the equilibrium level is reached. The exchange at the surface presumably results in a characteristic average residence time for molecules on the surface. Since spreading is mainly time-dependent this residence time probably determines at least in part the final average area occupied by each molecule and therefore

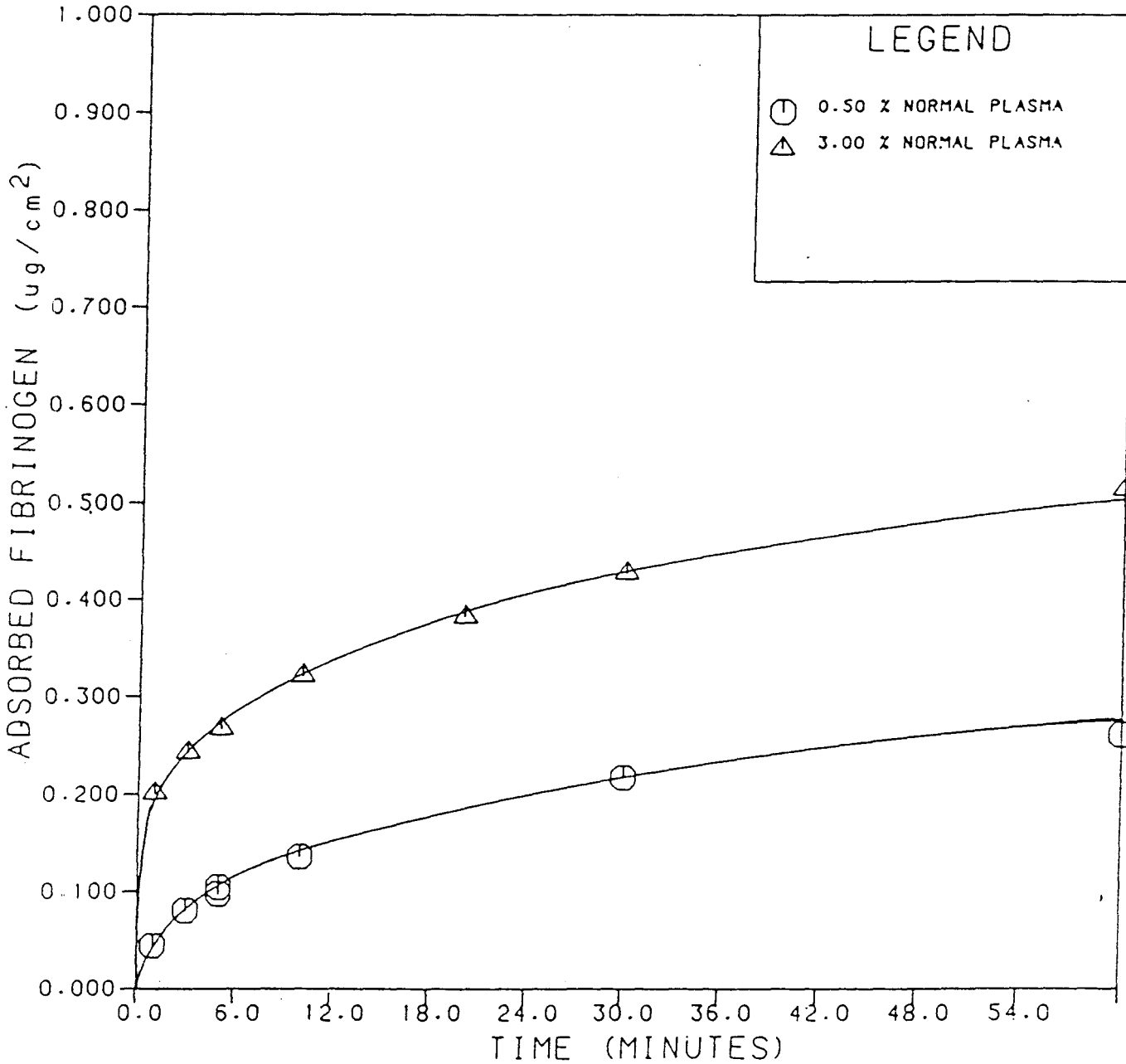


Figure 7 Surface concentration versus time on glass for fibrinogen in Tris buffer (pH 7.4); 0.5 and 3.0 per cent fibrinogen.

defines the shape of the isotherm. This is a more likely mechanism than that described by the Langmuir equation since it accounts for apparent irreversibility, molecular orientation/conformation change and multi-site binding. Protein-protein interactions due to close packing must also play a role in determining the final area per molecule.

Figure 8 shows a comparison of the 0.5% adsorption kinetics (1.25 x 10^{-2} mg/mL) for four different surfaces. At such a low concentration one would expect very small differences among these curves for diffusion-limited adsorption to a level just greater than a side-on monolayer of 0.20 μg/cm^2. In fact the curves for glass, polyethylene and Surfasil are nearly indistinguishable. The curve for PU02 (a segmented polyurethane with hydroxyl groups in the hard segments) is however, clearly different from the others. Although initial adsorption is proportional to the square root of time the apparent diffusion coefficient is approximately one fifth of the known value. This discrepancy could be caused by partial desorption during rinsing or by a barrier to diffusion as proposed by Van Dulm and Norde (77). The diffusion barrier would appear to be the more likely mechanism since it has been reported previously and since additional rinsing apparently has little effect. More experiments to investigate the effect of rinsing time on adsorption to PU02 needs to be done to check this possibility. It seems unlikely that the integrity of the square root time relationship with Γ could be maintained if significant desorption occurs during rinsing.

Adsorption from a fibrinogen solution of 5.0% normal plasma level (0.125 mg/mL) is shown in Figure 9 for three surfaces. (3.0% i.e. 0.075 mg/mL data for glass can be seen in Figure 7.) The differences between polyethylene

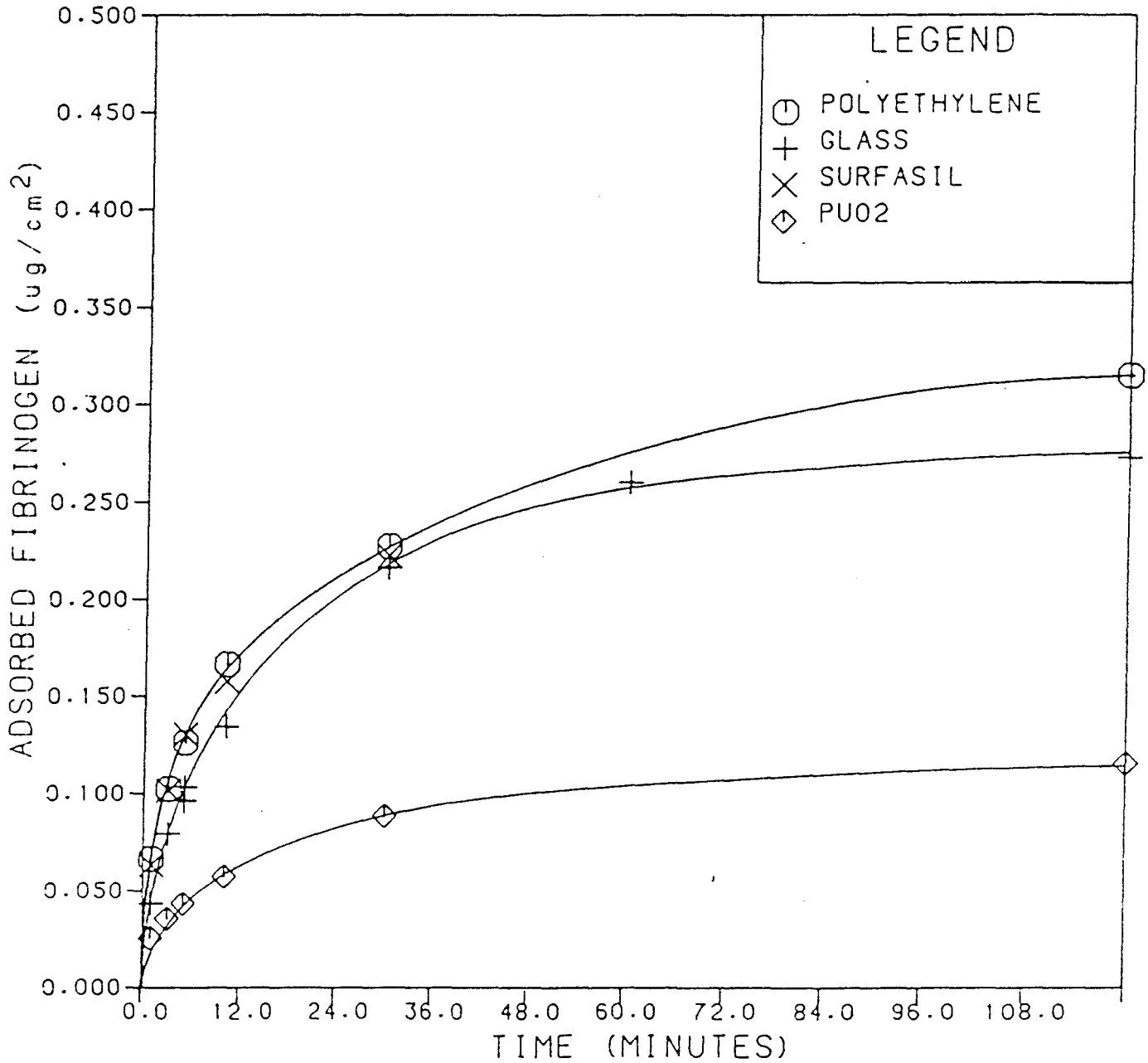


Figure 8 Surface concentration versus time on different surfaces for fibrinogen in Tris buffer (pH 7.4); 0.5 per cent fibrinogen

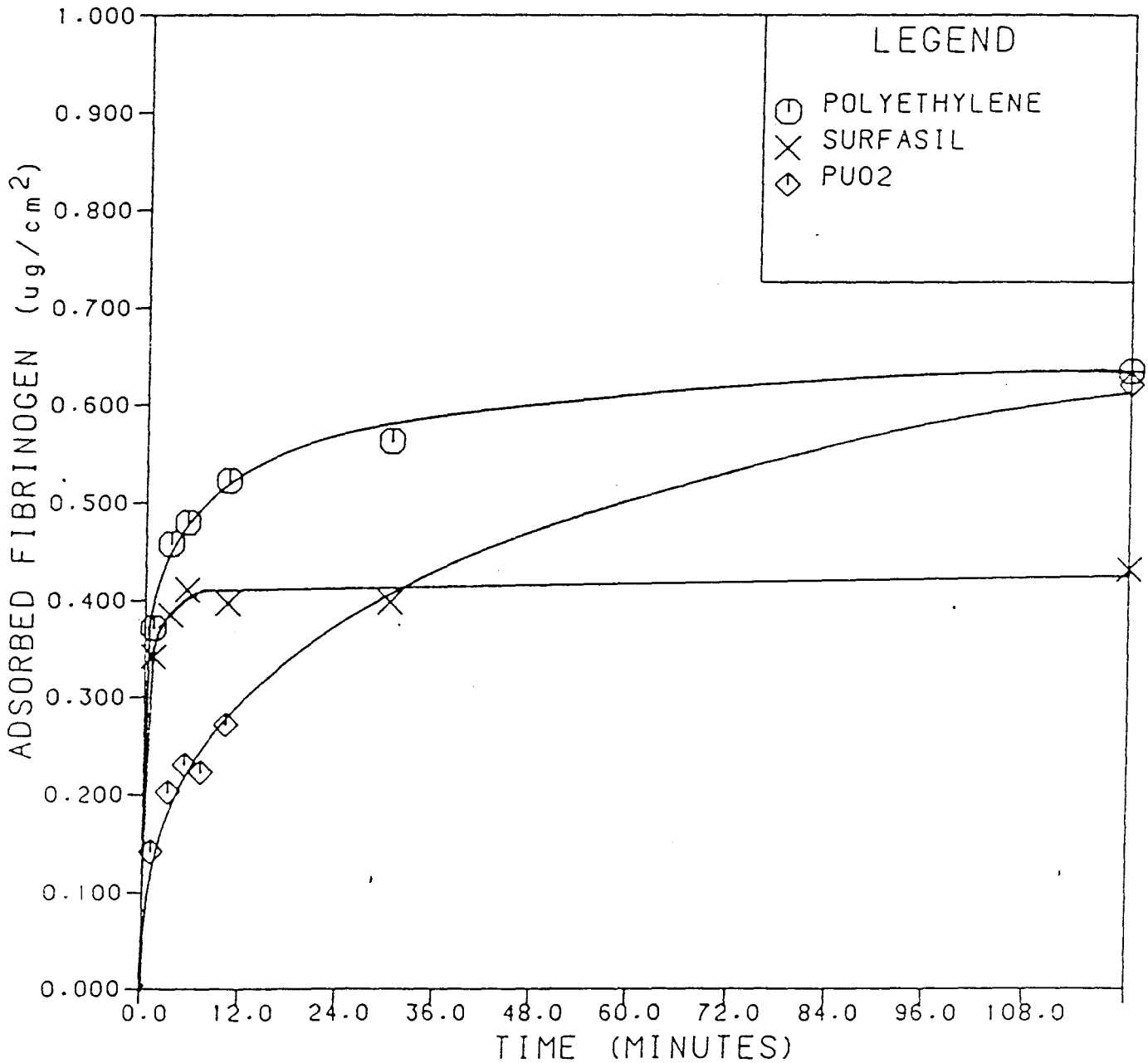


Figure 9 Surface concentration versus time on different surfaces for fibrinogen in Tris buffer (pH 7.4); 5.0 per cent fibrinogen

and Surfasil become more evident under these conditions. After one minute the amount of fibrinogen adsorbed is essentially the same on both surfaces. The Surfasil data rapidly level off to a plateau at about 0.4 $\mu g/cm^2$. The time in which this occurs may be an indication of the rate of spreading. On this basis spreading occurs very quickly on Surfasil, less so on polyethylene and very slowly on the polyurethane (PU02). Equilibrium data (see Figure 10) indicate that 1.0 $\mu g/cm^2$ is adsorbed onto the PU02.

It is conceivable that adsorption occurs rapidly on a surface which promotes spreading, and a kinetics curve with a peak adsorption dropping to an equilibrium plateau would be obtained. Kinetics curves like this have in fact been observed by Norde (39) and Leonard (77) and the Surfasil data in Figure 4 are suggestive of this behaviour. The maximum adsorption may be observable at a higher concentration but very short time data would have to be obtained to detect it. A mechanism for this binding and spreading hypothesis is as follows: Initially fibrinogen is adsorbed and bound by one or two sites. Since adsorption is rapid the surface is filled by a large number of these "unspread" molecules. When the surface is filled, exchange continues to occur but the characteristic residence time may be sufficient to allow spreading to an area greater than that for the initially adsorbed molecules. Other experiments involving the same surfaces in plasma systems seem to indicate the same effects of spreading and exchange. A surface which promotes the spreading of proteins may be more likely to rapidly induce the activation of the contact phase of coagulation activation. Therefore attempts to correlate low protein adsorption with blood compatibility may be futile. A

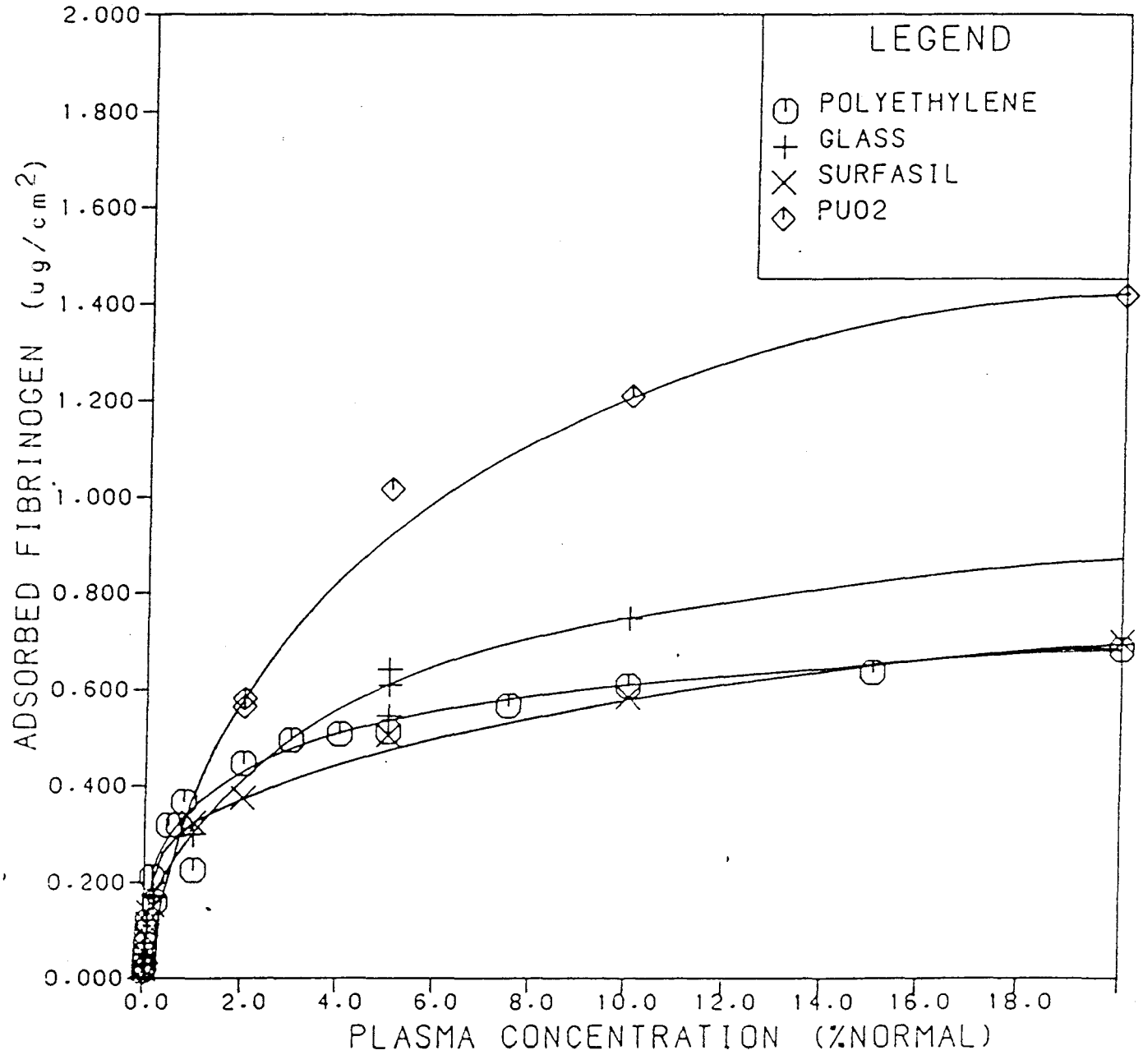


Figure 10 Surface concentration versus bulk concentration on different surfaces for fibrinogen in Tris buffer (pH 7.4) at 24 hours.

surface which does not promote spreading will be able to accommodate a larger amount of protein in its monolayer.

The equilibrium isotherms for four surfaces can be seen in Figure 10. The order of plateau surface concentrations is PU02 > glass > Surfasil > polyethylene. Surfasil appears to reach its plateau region at a lower plasma concentration than does polyethylene. The slope of the Surfasil "plateau" however, is greater than those of polyethylene and glass. If these observations are interpreted using the exchange and average surface residence time hypothesis, it must be concluded that Surfasil causes a more rapid conformational/orientational change of fibrinogen with time than do the other two surfaces. The polyurethane PU02 appears to cause conformational change only very slowly if at all. The rapid leveling off, above 0.2 $\mu g/cm^2$, seen on the other three surfaces is not observed on PU02. The plateau adsorption on this surface in fact appears to be approaching the theoretical maximum of 1.8 $\mu g/cm^2$ (43) for a tightly packed end-on monolayer. It is possible that this high adsorption is due to a greater rate of exchange on the surface and thus a shorter average residence time. The high surface concentration also contradicts any suggestion that rinsing has a significant effect on the observed monolayer adsorption since the very tightly packed end-on molecules appear to remain on PU02 even after rinsing. One would expect these molecules to be the most reversibly adsorbed of all and that any rinsing would cause significant desorption.

Since the low concentration region is difficult to distinguish in Figure 10 the same data are plotted with an expanded scale in Figure 11. The order of slopes for the initial portion of the isotherms is the reverse of the heights of

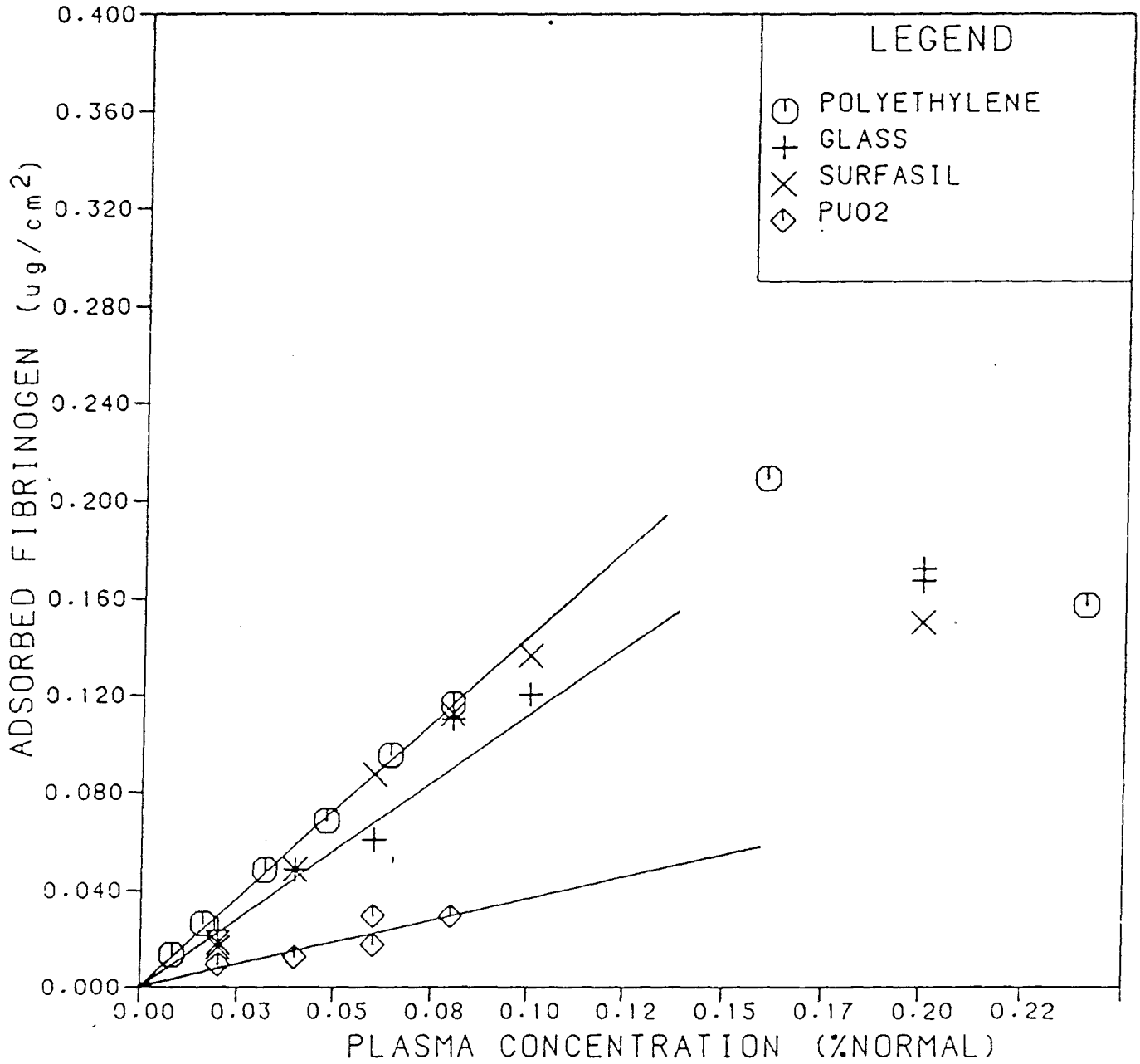


Figure 11 Adsorption at low concentrations on different surfaces for fibrinogen in Tris buffer (pH 7.4) at 24 hours

the respective plateaux. This result can be rationalized using the spreading hypothesis as follows. The more reversibly adsorbed fibrinogen on PU02 (deduced from the very low initial slope of its isotherm) allows a higher rate of exchange to occur on its surface and thus results in shorter residence times for adsorbing and exchanging molecules. The initial slopes on polyethylene and Surfasil are nearly identical implying that the fibrinogen adsorbs with the same affinity and thus the same conformation/orientation after long times. The data for glass show fibrinogen affinity to be slightly less than on Surfasil and polyethylene.

Figures 10 and 11 should serve as a reminder that protein adsorption studies for the purpose of comparing surfaces should be carried out with care and with the entire context of the phenomenon in perspective. The present study has shown that adsorption can be lower on some surfaces relative to others at one concentration of adsorbate and higher at another. Care must be taken to ensure that comparisons are not made for data obtained under different conditions. Many researchers have suggested that good blood compatibility can be correlated with low protein adsorption or preferential adsorption of passivating proteins such as albumin (57). This may be true for surfaces that promote platelet aggregation and release but is not necessarily so for surfaces capable of activating Factor XII and the intrinsic clotting pathway. The blood compatibility of these surfaces (e.g. glass) may be determined by the reversibility of adsorption and the resulting conformational/orientational changes.

The behavior of proteins at surfaces is slowly becoming clearer but serious deficiencies still exist in the understanding of events in one-

component systems. One component systems should be studied in greater detail before trying to understand the mechanisms causing clotting at surfaces in contact with the very complicated multicomponent plasma system. The "spreading" mechanism proposed here appears to help explain observed phenomena encountered in fibrinogen adsorption to surfaces. More experiments need to be performed in order to more fully understand the exchange and spreading that are presumed to occur. The effect of rinsing should also be clarified. It is hoped that the knowledge gained from these single component experiments can be used to help explain the effects observed when surfaces are in contact with plasma.

Fibrinogen Adsorption from Normal Human Plasma: The Vroman Effect

Basic Phenomena

The "Vroman Effect" or transient adsorption of fibrinogen from plasma is illustrated in Figure 12 for a glass surface. The quantity of fibrinogen adsorbed is plotted against bulk concentration for 5-minute adsorption times, and a purified single component fibrinogen system is compared to plasma. Bulk concentration is expressed as percent of normal plasma concentration and 100% plasma corresponds to 2.5 mg/mL fibrinogen for the single protein data.For a plasma concentration of less than 0.5%, five minute adsorption data are linear and the slopes are the same for the one-component and plasma systems. Up to 0.5% the surface is presumed to be filling with protein and little interaction takes place among the molecules on the surface. At concentrations greater than 0.5% surface "crowding" is beginning to take effect and interactive and competitive effects begin to dominate in the plasma medium. The "Vroman Effect", whereby fibrinogen is rapidly displaced from the surface, then becomes evident. The adsorption and removal of fibrinogen on glass from 5% normal plasma is essentially complete after five minutes. This five minute adsorption versus bulk concentration data will be referred to subsequently as a "five minute isotherm".

The "Vroman Effect" may be represented more fundamentally by plotting adsorption versus time for the plasma system as shown in Figure 13. Kinetic data for 0.5% plasma clearly show fibrinogen being adsorbed for the first ten minutes after which displacement begins. At concentrations greater than 0.5%, initial fibrinogen adsorption cannot be observed since displace-

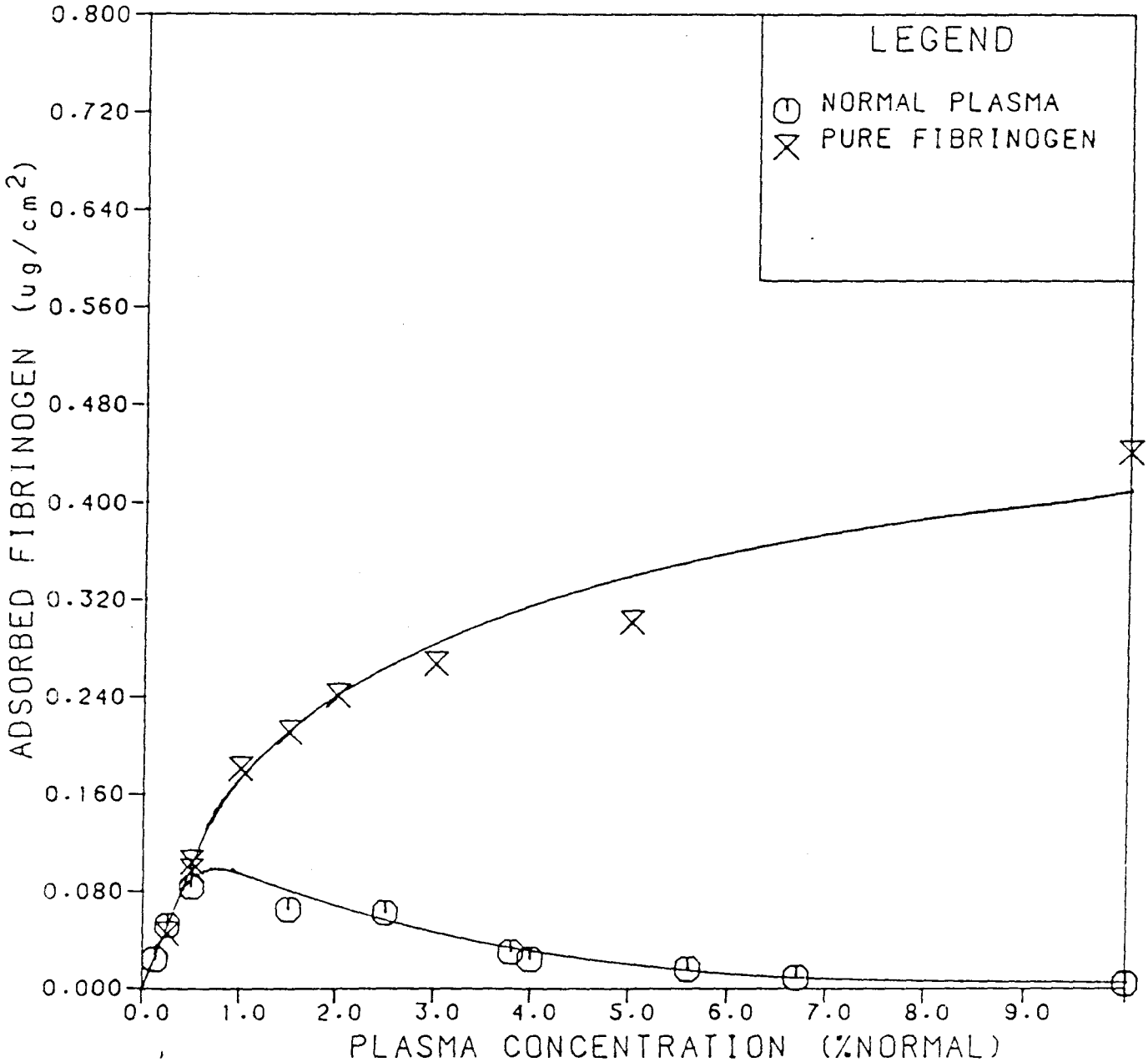


Figure 12 Fibrinogen surface concentration versus bulk concentration from Tris and from plasma at 5 minutes

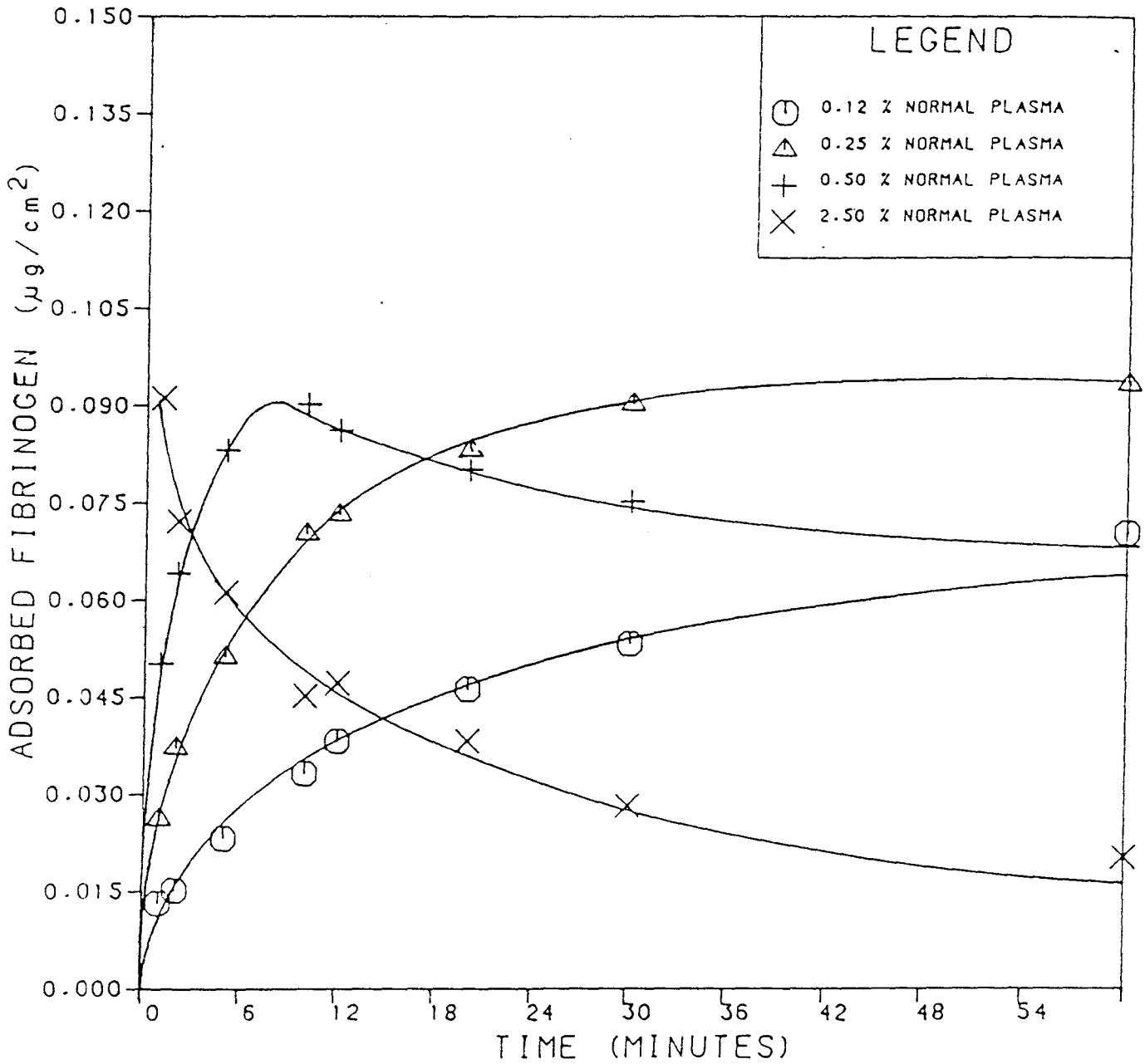


Figure 13 Fibrinogen surface concentration on glass versus time for different plasma concentrations

ment is too rapid, whereas at concentrations less than 0.5% there is effectively no displacement and the plateau level is determined by fibrinogen concentration. All the curves appear to approach a steady state after one hour suggesting an equilibrium effect is occurring. The initial adsorption of fibrinogen as shown on the low concentration curves is proportional to the square root of time, behaviour which is characteristic of a diffusion limited system. The calculated value of the diffusion coefficient is about 1.5×10^{-7} cm^2/s, somewhat less than the value of 2 to 3×10^{-7} cm^2/s reported in the literature (43). (See discussion of Figure 19 for equation relating diffusion coefficient to the adsorption data.) Van Dulm and Norde (77) have suggested that a potential energy barrier to adsorption exists which depends on the surface and suggest several possible mechanisms including electrostatic repulsion, electrical double layer overlap and steric hindrance. A possible explanation for the low values of the diffusion coefficient is that the electrical double layer may flatten the expected concentration profile at the surface thus lowering the apparent diffusion coefficient.

Figure 14 shows the same data as in Figure 13 but replotted as fibrinogen adsorbed versus plasma concentration with time as a parameter. In this case the initial portions of the curves are linear rather than curved as for the kinetic data. The one minute "isotherm" appears to reach a plateau, but would in fact begin to drop off at higher plasma concentrations. The two hour "isotherm" is nearly identical to that for the 24 hour data (see Figure 20) again suggesting that some kind of equilibrium state is reached.

Figures 15 and 16 show, respectively, the kinetics and isotherms for fibrinogen adsorption onto Surfasil from plasma. In comparison to glass the

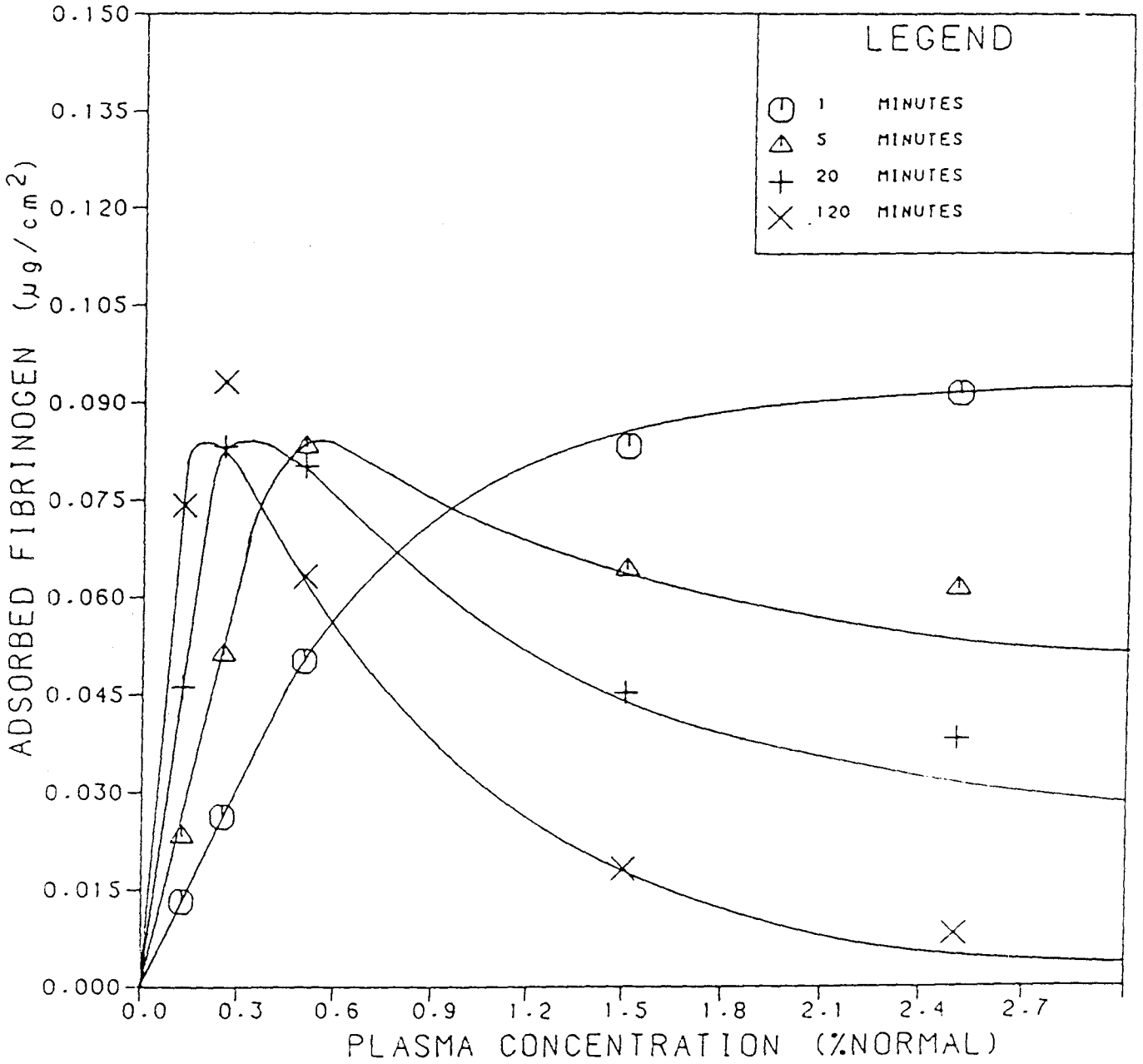


Figure 14 Fibrinogen surface concentration on glass versus plasma concentration for different times

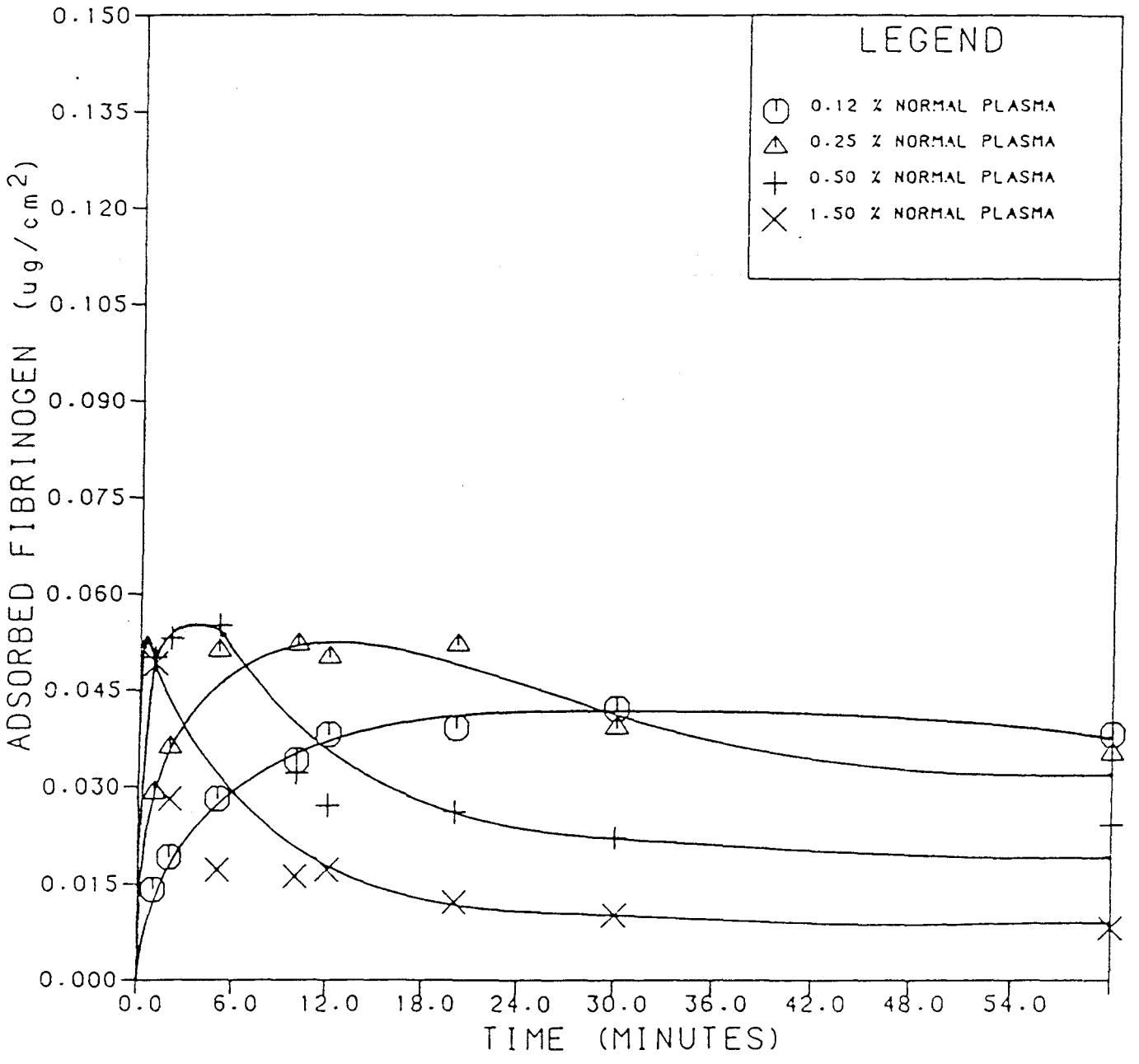


Figure 15 Fibrinogen surface concentration on Surfasil versus time for different plasma concentrations

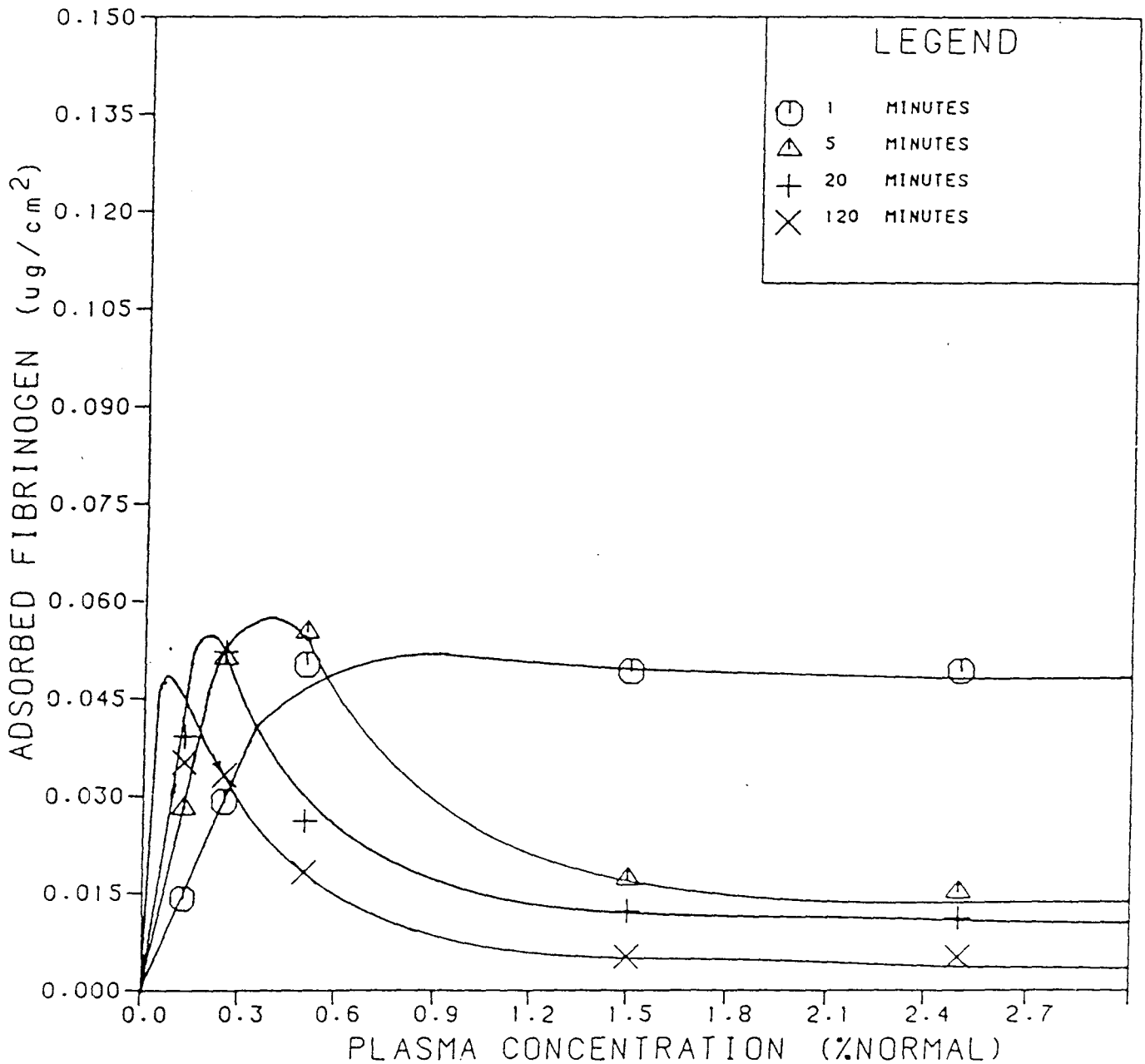


Figure 16 Fibrinogen surface concentration on Surfasil versus plasma concentration for different times

peaks are much lower, reaching a maximum of only 0.055 μg/cm^2 compared to approximately 0.09 μg/cm^2 for glass. At the same time the initial isotherm slopes (Figure 16) correspond exactly to those for glass. These observations suggest that displacement of fibrinogen becomes dominant on Surfasil sooner than on glass. Whether this means that the monolayer is completed sooner or that displacement overtakes adsorption sooner is unclear. At longer times there appears to be an approach to an equilibrium plateau as on glass. The peaks approached at short times and high concentrations of plasma are somewhat higher than those at longer times and low concentrations of plasma.

The kinetic data for fibrinogen adsorption from plasma to polyethylene (Figure 17), show a peak at short times as seen on Surfasil. However, whereas on Surfasil displacement of fibrinogen occurs using plasma as dilute as 0.25%, a plateau appears to be reached on glass and polyethylene. This would suggest that the "Vroman Effect" is not eliminated at low concentrations due to depletion alone but to a combination of depletion and a dynamic equilibrium between the proteins in the bulk and those on the surface.

Figure 18 shows the adsorption "isotherms" for fibrinogen on polyethylene. The initial slopes of the four curves plotted are the same as those seen on Surfasil and glass but displacement is much less pronounced. The 24 hour data (see Figure 20) are significantly different from the two hour data implying that equilibrium takes longer to achieve on polyethylene than on the other two surfaces. A more detailed comparison among these and other surfaces is presented below (Figures 19 to 24).

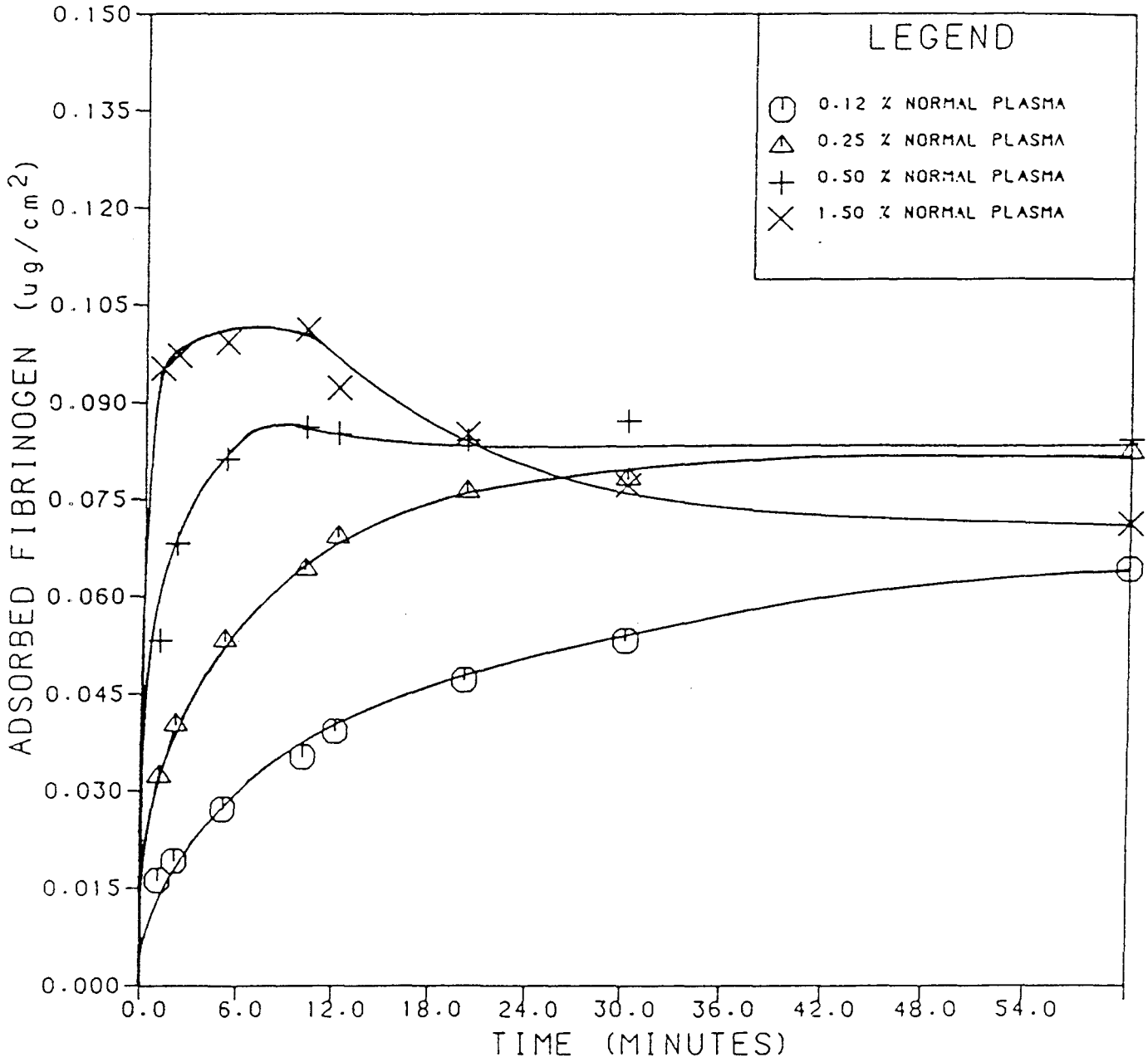


Figure 17 Fibrinogen surface concentration on polyethylene versus time for different plasma concentrations

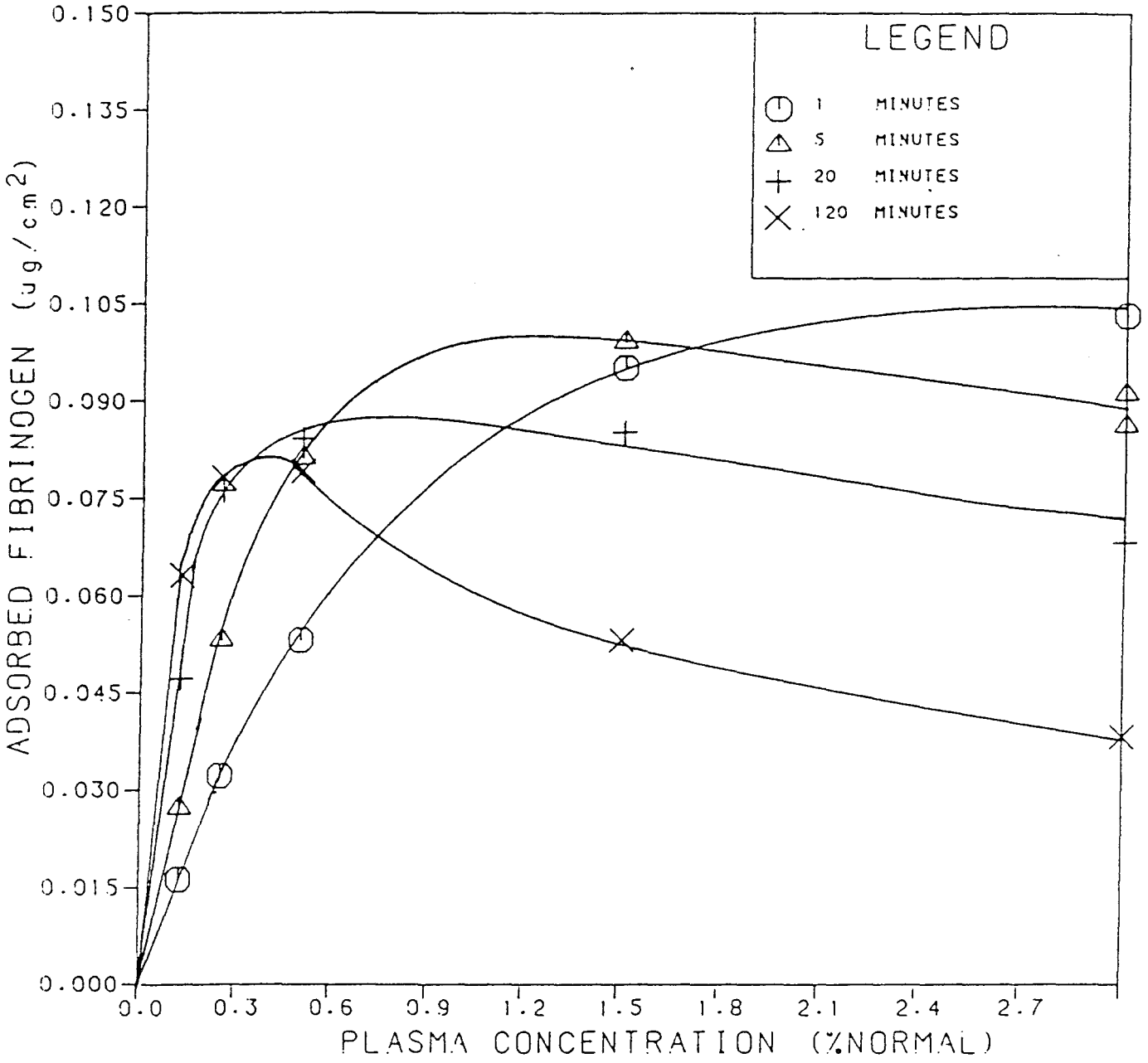


Figure 18 Fibrinogen surface concentration on polyethylene versus plasma concentration for different times

Effect of Surface Type

Figure 19 shows the five minute adsorption isotherms for fibrinogen from plasma onto five different surfaces. The initial slopes all appear to be the same, reflecting the diffusion limitation to be expected at short exposure times and low concentrations. The slope can be related to the diffusion coefficient by the equation developed by Ward and Tordai (67);

$$\Gamma = 2F\sqrt{\frac{\overline{Dt}}{\pi}} \qquad [7]$$

where Γ is the surface concentration of fibrinogen, t is the time of contact with the surface, D is the diffusion coefficient for fibrinogen in water, and F is the concentration of fibrinogen. This assumes that the presence of other proteins and the surface do not affect the diffusion of fibrinogen molecules. Comparison of single component and multicomponent adsorption involving fibrinogen (for example Figure 12) shows that interaction between molecules is not an important factor since the initial slopes of the "isotherms" are the same. The presence of the surface has however been shown to affect the apparent diffusion coefficient as already noted (77).

The heights of the peaks for the five minute isotherms in Figure 19 appear to be inversely related to the rate of displacement beyond the peak. At this short time it is evident that there are two main contributions to the shape of the "isotherm". The first is the rate of diffusion of fibrinogen to the surface and the second is the displacement rate. However, in the case of PU02, a segmented polyurethane with hydroxyl groups in the hard segments, it

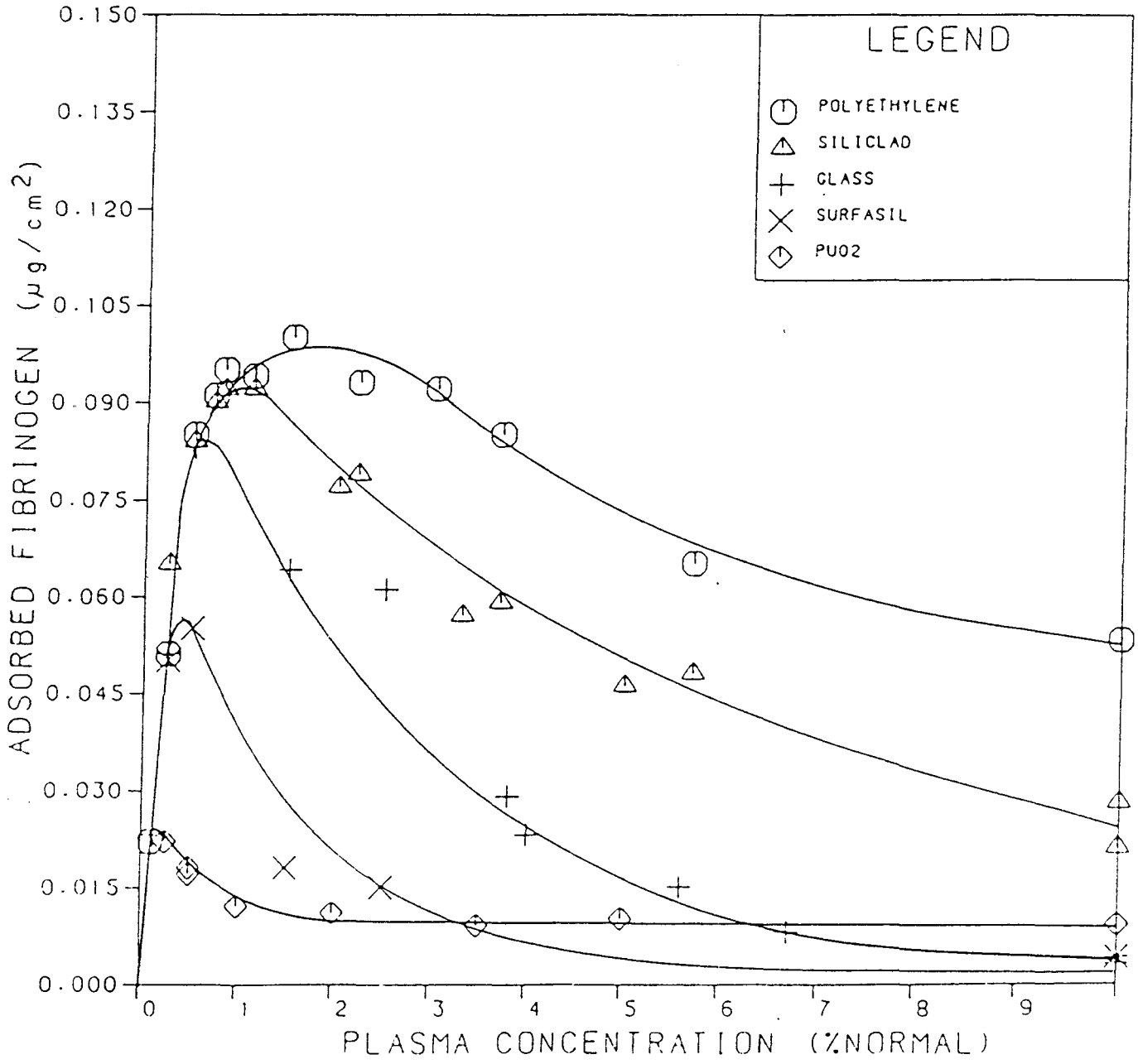


Figure 19 5 minute fibrinogen surface concentration versus plasma concentration on different surfaces

appears that a steady state adsorption level of fibrinogen is achieved very rapidly. It is interesting to note that for the various surfaces there is no correlation between water contact angle (see Table 1) or thrombogenicity and the shape of the curves. The five minute adsorption curve for glass, a hydrophilic, thrombogenic surface lies between other more hydrophobic and less thrombogenic surfaces. The two silicone coatings tested resulted in two quite different isotherms. Since all conditions, except for the type of surface, were the same the different displacement curves are probably due to different fibrinogen-surface bond strengths.

Table 1. Water Contact Angles of Surfaces

Surface	Contact Angle
Glass	0°
Siliclad	102°±10°
Surfasil	80°±10°
PU02 Polyurethane	50°±10°
Polyethylene	88°±5°

Four of the surfaces described in Figure 19 were exposed to plasma for 24 hours and the resulting "isotherms" are shown in Figure 20. The hierarchy of peaks differs markedly from that for the 5 minute isotherms but the curves have the same basic shape. Contact angle data again are not correlated with the curves but relative thrombogenicity is at least qualitatively related if it is accepted that the order of thrombogenicity is glass

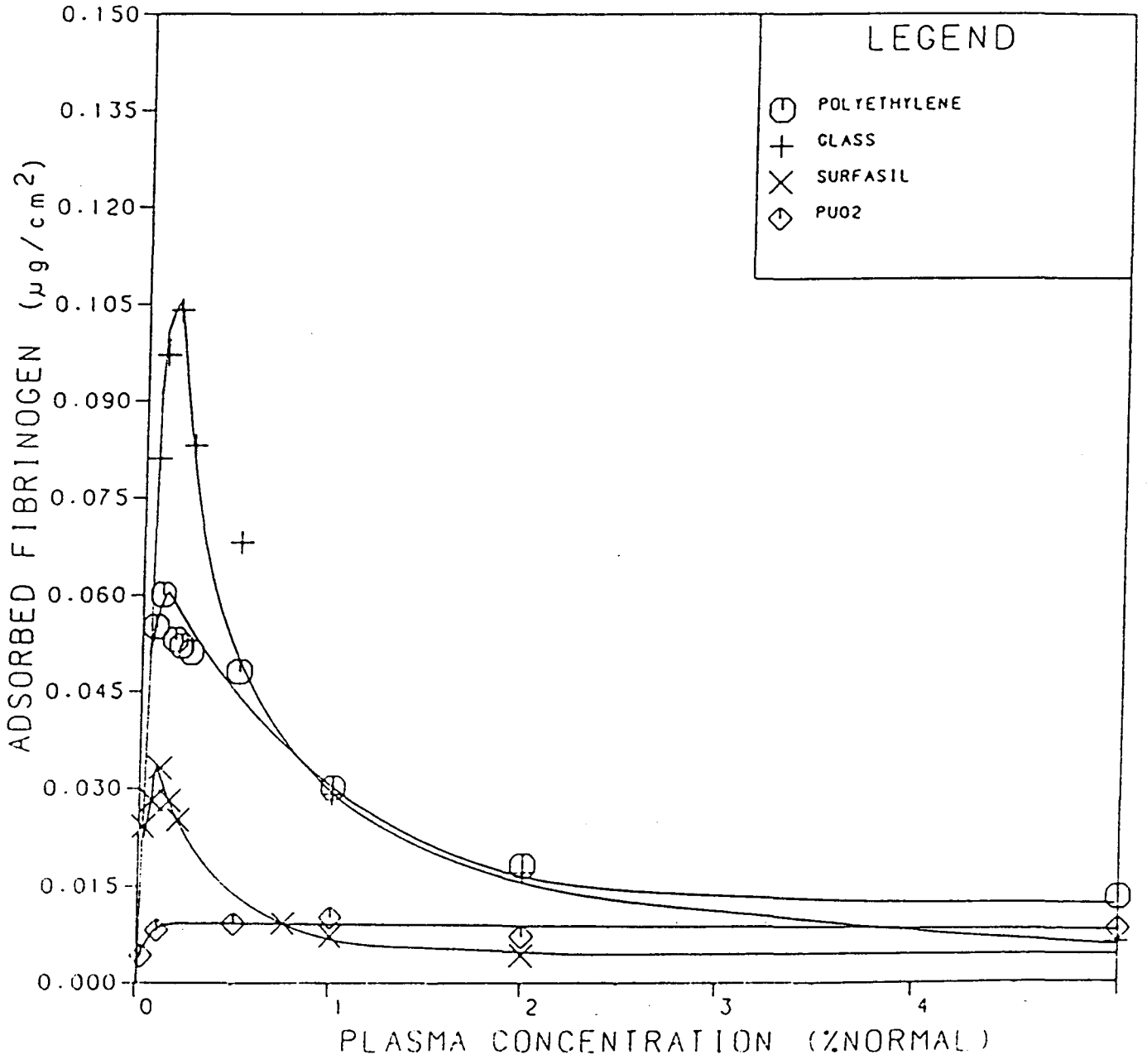


Figure 20 24 hour fibrinogen surface concentration versus plasma concentration on different surfaces

> polyethylene > Surfasil > PU02. This may only be an interesting coincidence but may also merit further investigation.

If the displacement portion of the 5 minute or 24 hour data are plotted on a semilog-linear basis straight lines are obtained implying that the surfaces are relatively homogeneous (since the displacement rate constant is not dependent on surface coverage). This reasoning is based on the following simple displacement model:

$$F_{ads} + C_{bulk} \xrightarrow{k} C_{ads} + F_{bulk}$$

where F is fibrinogen and C represents the replacing proteins, leading to,

$$-\frac{d\Gamma}{dt} = k\,\Gamma[C] \qquad [18]$$

where k is a displacement rate constant and [C] is the concentration of the displacing proteins.

More detailed data have already been presented for single component fibrinogen adsorption at the very low concentrations corresponding to the regions to the left of the peaks in Figure 20. It appears that the initial slopes for these "true" isotherms can vary markedly from surface to surface and while this may be a manifestation of the variable effect of rinsing it is more likely to be an indication of the surface-protein binding equilibria at these low concentrations where surface coverage is not complete. The initial slopes of these isotherms appear to be the same for fibrinogen whether in a one

component or plasma system. The persistence of fibrinogen adsorption at long times in diluted plasma has been attributed by Schmaier et al (20) to depletion of the displacing protein (believed to be high molecular weight kininogen, HMWK). However, if this were the major factor the 24 hour isotherms should be the same for all the surfaces. In fact, the isotherm for PU02 is a level plateau at 0.008 $\mu g/cm^2$ with no peak evident at all. This result suggests that displacing components are present in sufficient amounts to remove fibrinogen at any dilution. Again, the existence of a dynamic equilibrium at long times is suggested.

Figure 21 shows the adsorption kinetics of fibrinogen from plasma at low concentration (0.25%), onto three different surfaces. The initial adsorption (presumably diffusion limited) is the same for all three surfaces. Even after one hour, displacement is not evident on either glass or polyethylene but the plateau values differ slightly suggesting a stronger relative affinity of fibrinogen for glass. The data for Surfasil show that significant displacement occurs and this is consistent with the fact that 0.25% is to the right of the peak of the isotherm curve in Figure 9. The existence of plateaux combined with the fact that significant exchange has been shown to occur between a filled surface and a bulk protein solution (87-89) indicates that dynamic equilibria are established. The initial values of Γ for low concentration data should be proportional to the square root of time for a diffusion limited system and Figure 22 shows that this is the case. The initial adsorption rates are protein diffusivity dependent and are thus equal for the three surfaces shown. The role of surface binding becomes important at long times (i.e. equilibrium conditions).

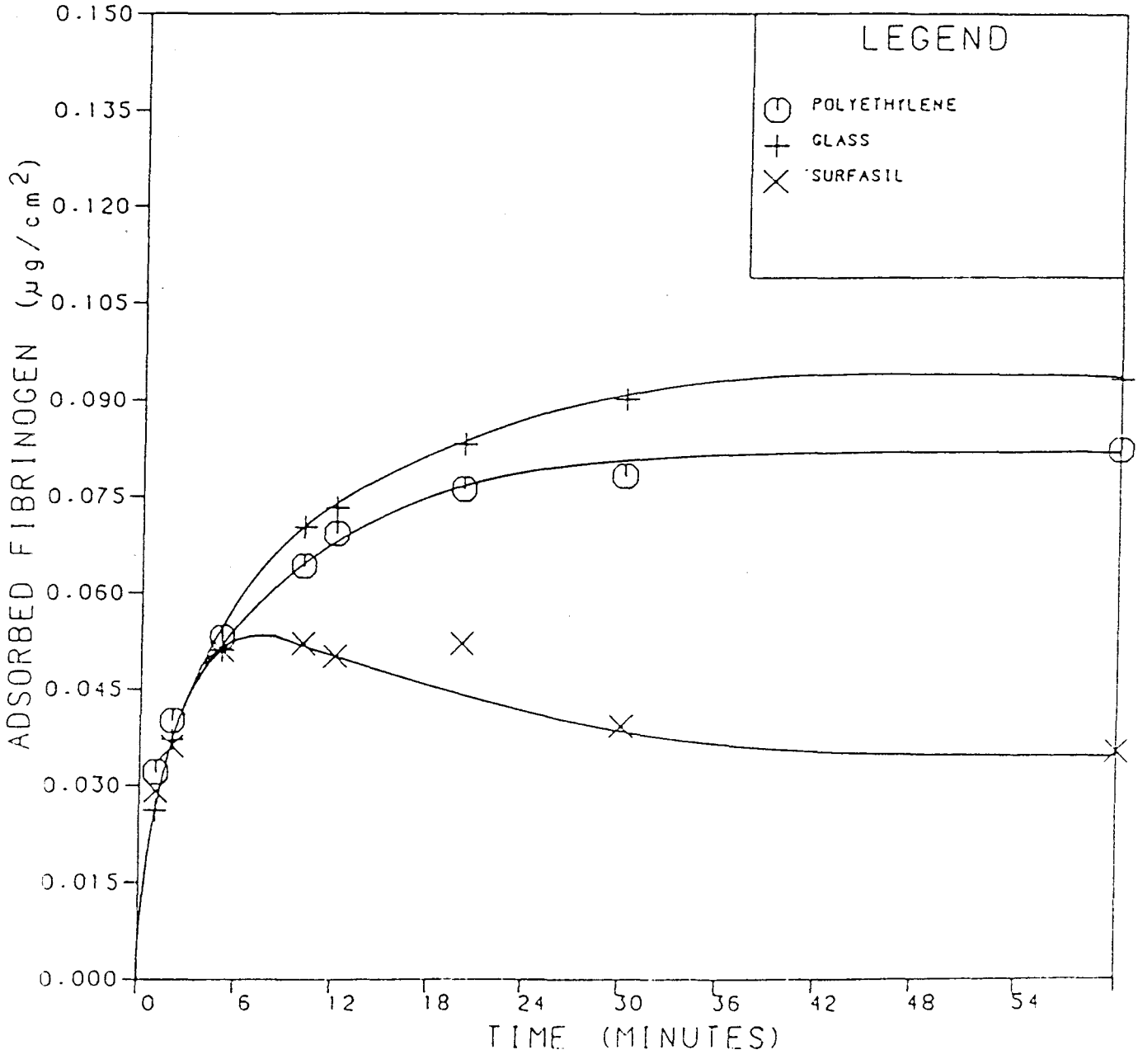


Figure 21 Fibrinogen surface concentration kinetics for 0.25 per cent plasma on different surfaces

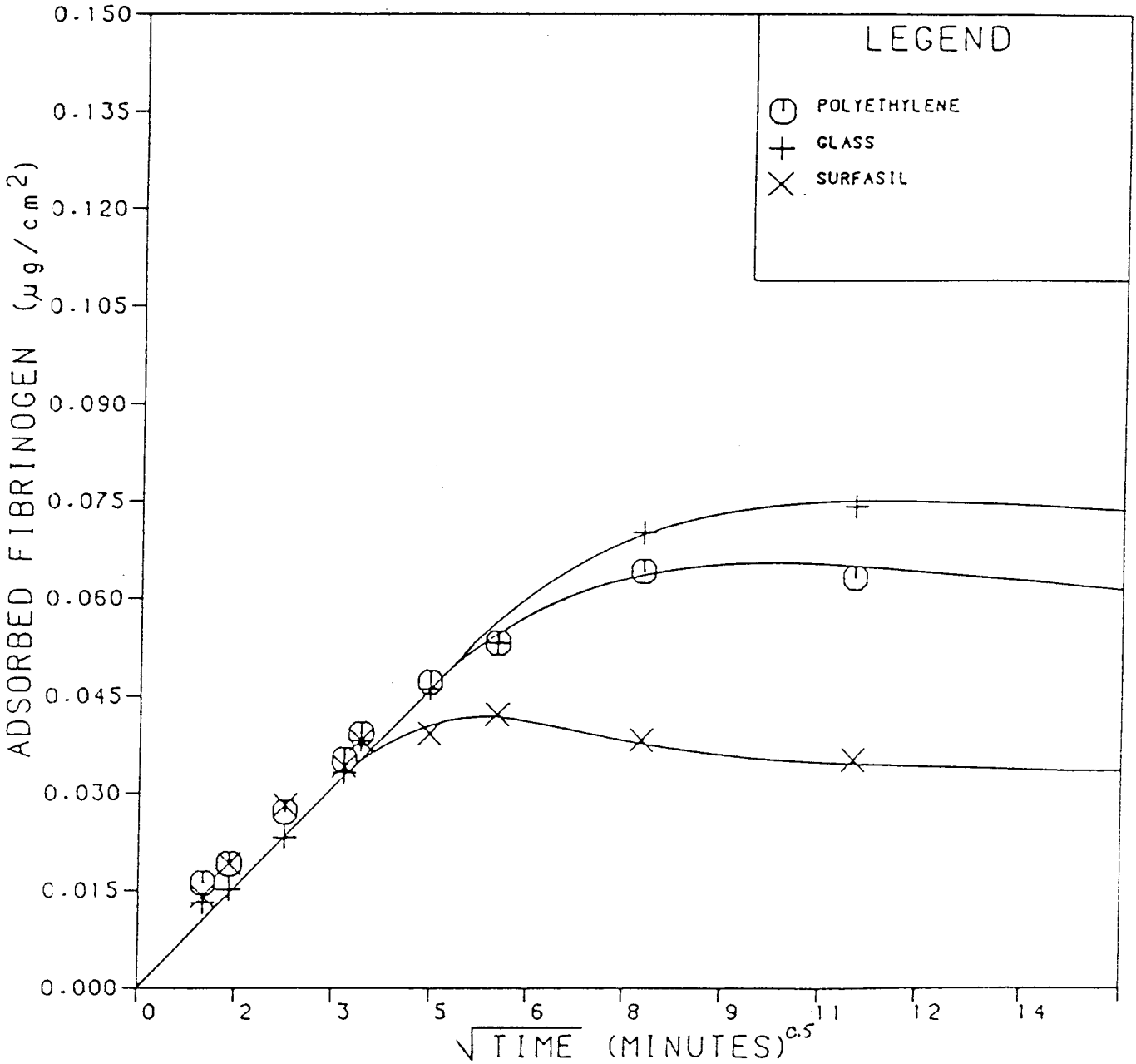


Figure 22 Fibrinogen surface concentration versus square root of time for 0.125 per cent plasma on different surfaces

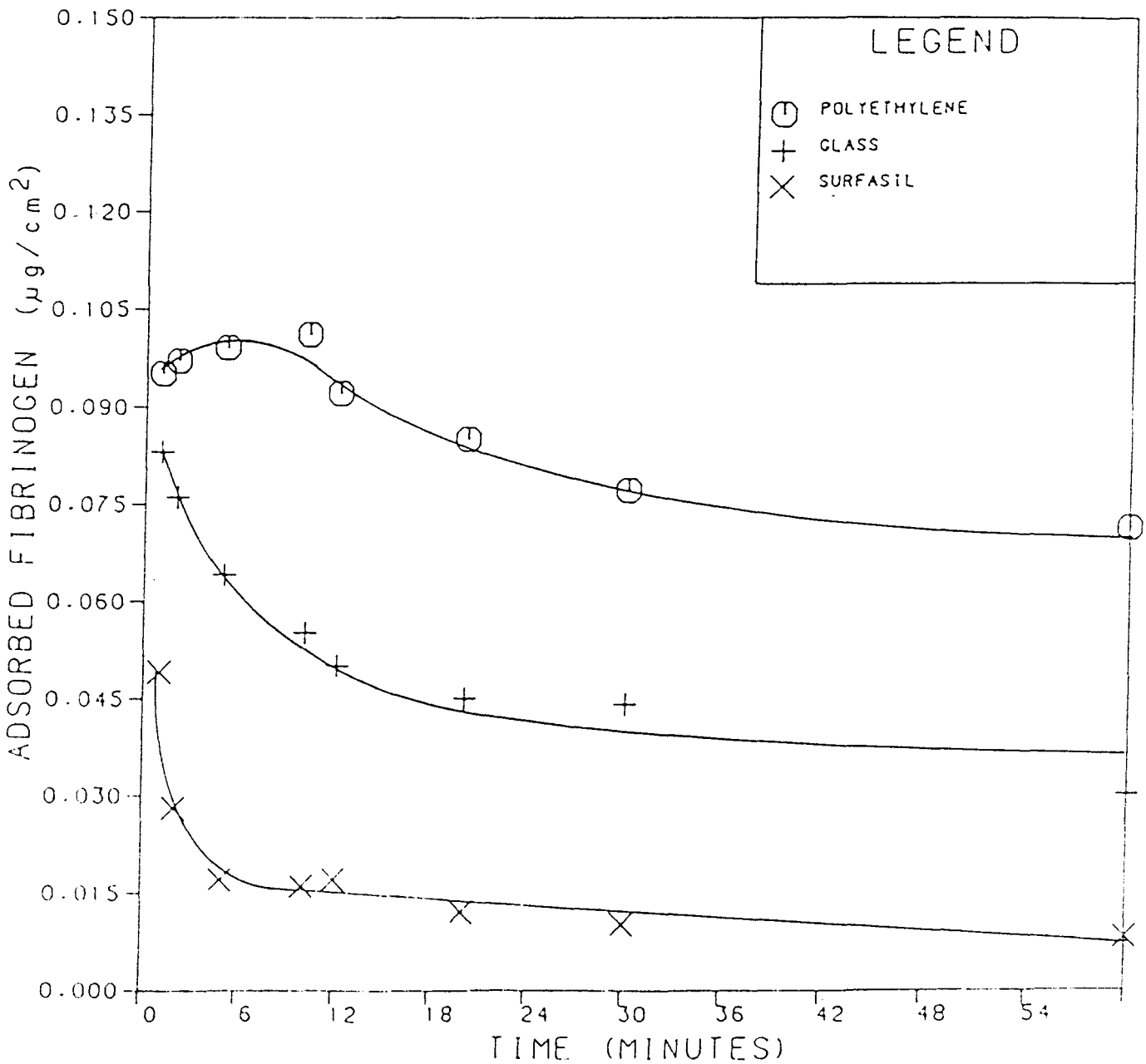


Figure 23 Fibrinogen surface concentration kinetics for 1.5 per cent plasma on different surfaces

Even at plasma concentrations as low as 1.5% events on different surfaces are very rapid (Figure 23). Fibrinogen displacement has begun on glass and Surfasil after one minute while adsorption is dominant on polyethylene for the first five to ten minutes. Because of initial diffusion limitations the three curves presumably converge and become identical at some time less than one minute. At high concentrations three time domains must be distinguished. Initially, diffusion limited adsorption occurs, then fibrinogen surface concentration is determined by its rate of displacement and finally, at long times equilibrium governs. The fact that fibrinogen is most rapidly displaced from Surfasil is evident in Figure 23 but preadsorbed fibrinogen is much more rapidly removed from glass as shown in Figure 24. It is first interesting to note that despite being exposed under identical conditions of fibrinogen concentration and time (0.170 mg/mL for 5 hours) Surfasil adsorbed less fibrinogen, possibly indicating a more spread conformation/orientation on the surface. Secondly, and it should follow, displacement from Surfasil was much slower than from glass. A change in the state of adsorbed fibrinogen with time may be responsible for this radical change in displacement rate. Other authors have invoked such a concept (75,90). A plot of log Γ versus time for the data in Figure 23 yields three curves of decreasing slope. This is an indication that the rate of displacement (already shown to be independent of Γ and plasma concentration) is a function of residence time on the surface. Based on Figures 23 and 24 it appears that the displacement of preadsorbed fibrinogen from glass is about ten times slower than for fibrinogen adsorbed and displaced in situ in a plasma system. The corresponding difference for Surfasil is obviously much greater than this.

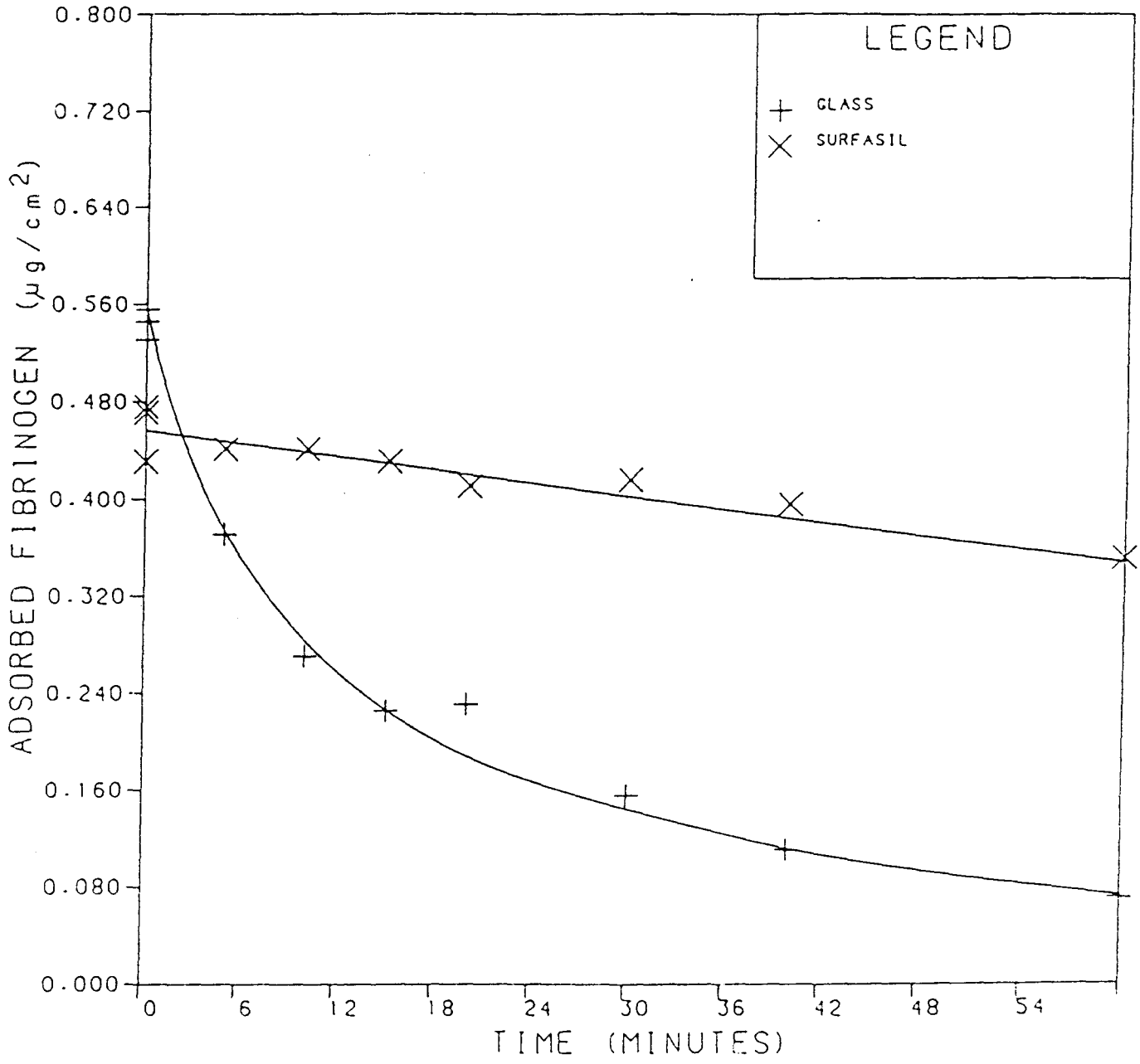


Figure 24 Desorption kinetics of fibrinogen from glass and Surfasil using 5 per cent plasma

The dependence of the displacement rate constant (k) on surface residence time is undoubtedly an important consideration especially in the low concentration plasmas where dynamic exchange would result in an "average" residence time for the surface-bound proteins.

Effect of Plasma Protein Concentration Profile

Since the "Vroman effect" is viewed as a reflection of competitive adsorption between fibrinogen and other plasma components it might be expected that the relative concentrations of the various plasma constituents would play a role. Experiments were first done to determine the reproducibility of the data using different plasma pools, since variations in the data among pools would presumably reflect the effects of variation in plasma protein concentration profiles. The results shown in Figure 25 show considerable variability in peak height among the pooled samples. The peak heights are not correlated with fibrinogen concentration in the various pools (see Table 2). Some of the differences may be attributed to errors in measuring the fibrinogen levels for each pool but the fact that the initial slopes are similar supports the hypothesis that the differences are due mainly to varying relative concentrations of other proteins. From plots of logΓ versus plasma concentration for all four curves the "decay" of adsorbed fibrinogen was found to be essentially the same for all four pools implying that the concentration of displacing proteins was the same in each case. Since neither the fibrinogen nor the displacing proteins appear to be responsible for the differences in peak heights, the abundance of other plasma proteins involved in site competition during initial adsorption may be the determining factor.

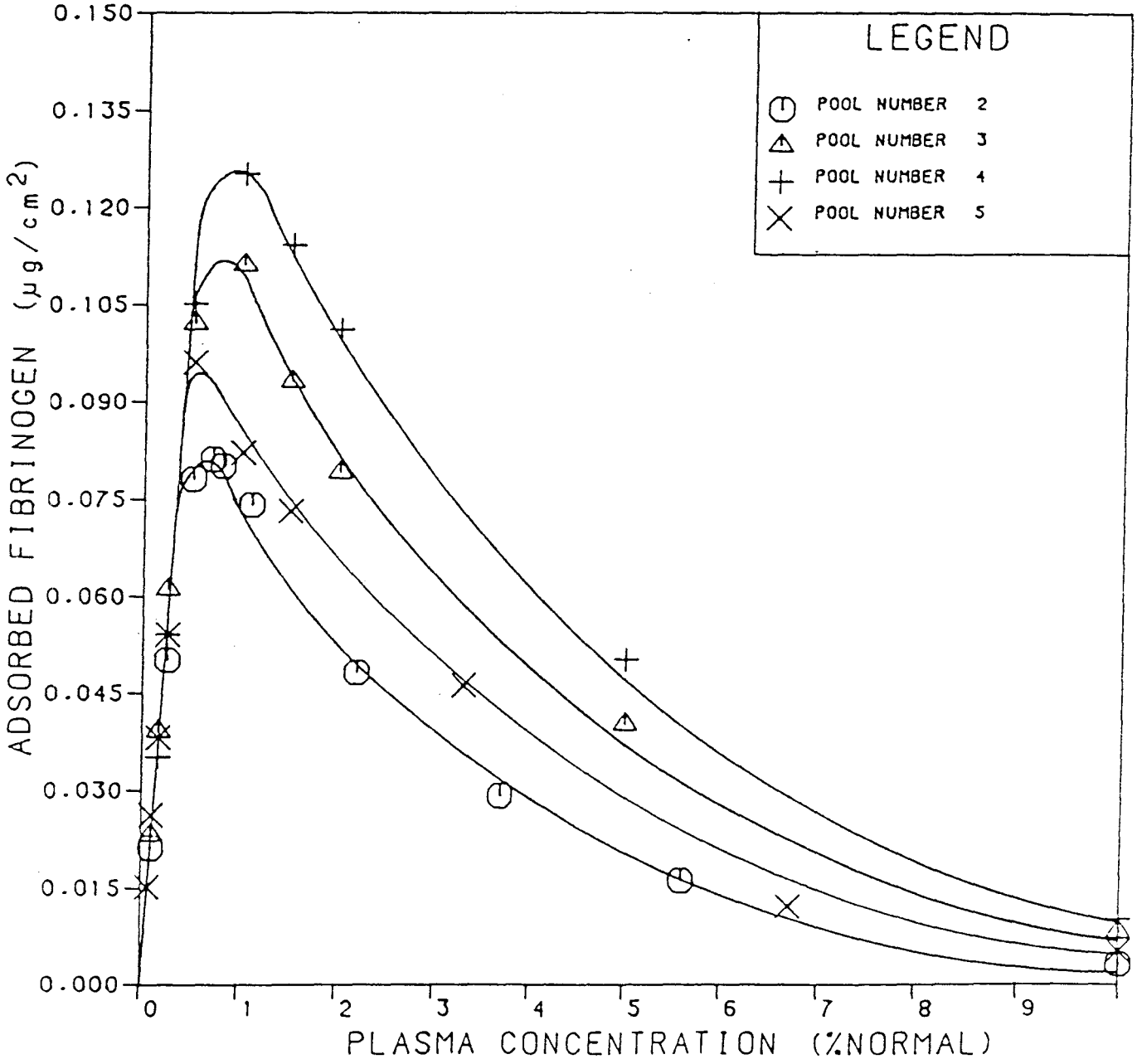


Figure 25 Fibrinogen surface concentration (5 min.) versus plasma concentration on glass using different plasma pools

Table 2. Fibrinogen Concentration of Various Plasma Pools

Pool #	Fibrinogen Concentration (mg/mL)
II	2.66
III	3.39
IV	2.94
V	2.64

A set of five experiments was also done to determine the effect of deliberately changing the fibrinogen level in normal human blood plasma. In these experiments, fibrinogen concentrations greater than normal were obtained by adding purified fibrinogen to normal plasma. Concentrations less than normal were obtained by adding purified fibrinogen to Arvinized plasma. The resulting curves shown in Figure 26 all exhibit the same trends. The initial slope of the five minute isotherm is proportional to the fibrinogen concentration in all cases as expected. The decay curves after the peaks are all similar exponential curves as would be expected if the concentration of displacing proteins (the same for all curves) determines this portion of the curve. The differences between experiments lie in the height and location of the peaks.

The location of the peak shifts to the right with decreasing fibrinogen concentration. This shift may be interpreted as an indication of the contribution of fibrinogen to competitive adsorption at a surface by assuming that the peak occurs at the same level of nearly complete surface coverage in each case. The peak for normal fibrinogen concentration occurs at about 0.9% plasma and for 12.5% of normal fibrinogen concentration at around 1.5%. This result may indicate that fibrinogen is responsible for more than half the

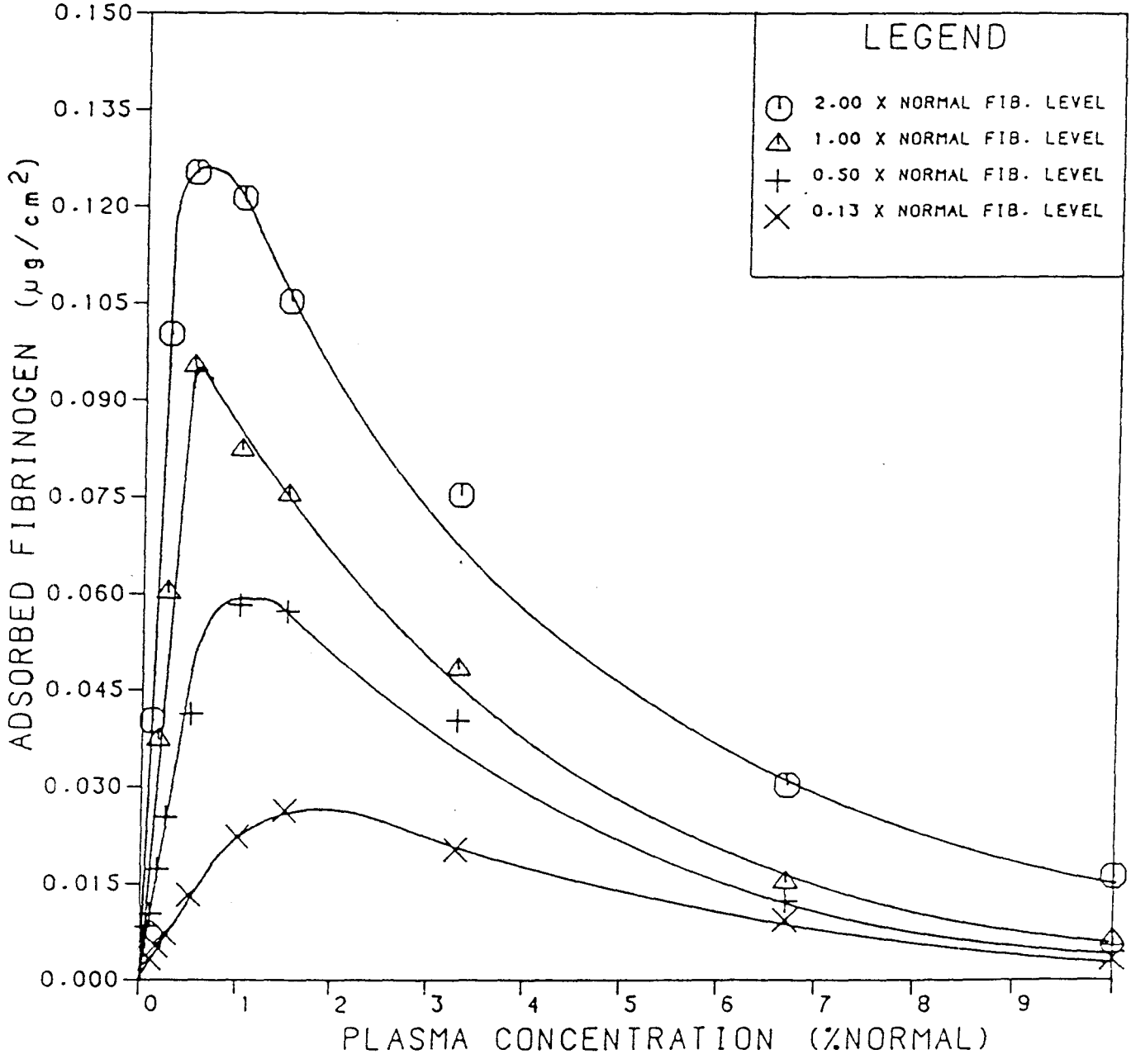


Figure 26 Fibrinogen surface concentration (5 min.) versus plasma concentration on glass using plasmas with varied fibrinogen concentrations

surface coverage under normal conditions (see eq. [19] below), which belies its 4.5% weight contribution (and even smaller molar contribution) to plasma.

There is evidence also in the peak heights to suggest this disproportionate contribution of fibrinogen to surface coverage. If the peak height is interpreted to be a direct indication of the contribution of fibrinogen to surface coverage a simple relationship may be developed to show its importance quantitatively. If f is the fraction of normal plasma fibrinogen concentration ,p is the corresponding fraction of all other proteins, S is the peak surface coverage by fibrinogen on glass and Smax is the maximum peak coverage by fibrinogen, we have:

$$\frac{f}{f+p} = \frac{S}{S_{max}} \qquad [19]$$

Using the data for normal plasma and plasma having 50% of normal fibrinogen concentration, the two unknowns, Smax and p, can be calculated. Smax is about 0.18 $\mu g/cm^2$ and p is about 1. This means when the fibrinogen level is normal (f equals 1), the fraction of the surface covered by fibrinogen is about one half. If the equation is used to predict the peak when the fibrinogen level is 2x normal a value of $S=0.12$ $\mu g/cm^2$ is obtained, which compares favourably with experimental results. For one-eighth of normal fibrinogen concentration 0.02 $\mu g/cm^2$ is the predicted peak height and this, again, coincides closely with the experimental result shown in Figure 26.

The implications of this effect are difficult to interpret without investigating in detail the participation of the many other plasma proteins

adsorbed at the surface. It is known from previous experiments (37) that albumin and IgG adsorb to low plateaux (0.014 and 0.003 μg/cm^2 respectively) on glass without any evidence of displacement over the range 0.1 to 10% normal plasma. Studies relating the participation of other clotting factors in displacing fibrinogen are discussed in subsequent chapters of this thesis.

Fibrinogen Adsorption from Plasmas Deficient in Various Proteins

The Vroman Effect may be hypothesized to be an important step in the activation of the intrinsic clotting pathway, the details of which were discussed in the introduction to this thesis. While fibrinogen, albumin and IgG make up more than 90% of the bulk protein content of plasma it is the activity of the trace proteins, Factor XI, Factor XII, HMWK and prekallikrein which triggers the clotting pathway in the presence of a foreign surface (11). The effects of the first three of these trace proteins as well as plasminogen on the five minute isotherm of fibrinogen from plasma were investigated by testing plasmas deficient in these proteins. Data were obtained as five-minute isotherms as a matter of convenience and since it was desirable to emphasize initial events. The two major features of the five minute curve are diffusion-limited adsorption and displacement of fibrinogen. Glass was the surface used throughout because of its availability, ease of preparation and most importantly its ability to activate the intrinsic pathway.

Figure 12 previously showed the five minute isotherm for fibrinogen adsorption from a one-component system (upper curve) and from plasma. The initial slopes are essentially identical and directly related to the apparent diffusion coefficient. The Vroman Effect seen in the plasma curve can be readily compared to the expected adsorption of fibrinogen in the absence of displacing proteins. The amount of adsorbed fibrinogen approaches zero at a plasma concentration as low as 5 per cent after five minutes. This result supports Vroman's hypothesis that fibrinogen is adsorbed and displaced from glass within a few seconds of contact with normal human blood plasma (22).

The effect of exchange between surface-bound and bulk proteins is likely to vary greatly between the two experiments in Figure 12. The plasma

contains about 20 times as much protein by weight as the one-component system and as a result provides a much greater opportunity for exchange. This fact limits any direct comparisons between the two experiments especially at equilibrium where the final conformation/orientation of surface bound molecules should be expected to vary greatly.

Figure 27 depicts the five minute isotherms for normal and plasminogen-free plasma. Although differences in peak height can be expected between different normal plasma pools (see Fig. 25) the rate of displacement is expected to be the same. Figure 27 shows that the plasminogen-deficient plasma has a significantly greater peak height and lower displacement rate than normal plasma. There is no difference between the diffusion-limited adsorption portions of the curve (the initial straight line) for the normal and deficient plasma samples. This result suggests an important role for plasminogen in the Vroman Effect on glass.

The role of plasminogen in displacing fibrinogen is still unclear. Brash is currently investigating the possible conversion of plasminogen to plasmin on glass. If the zymogen form is responsible for the observed effect, then simple displacement would most likely be the mechanism; if, however, the enzyme form, plasmin is being generated, then fibrinolysis may be involved as well. Brash has found evidence that lysis occurs in fibrinogen adsorbed to glass from plasma (91). This phenomenon requires more research particularly with regard to its possible dependence on surface type. It is possible that biocompatibility could be improved by increasing the fibrinolytic properties of a surface.

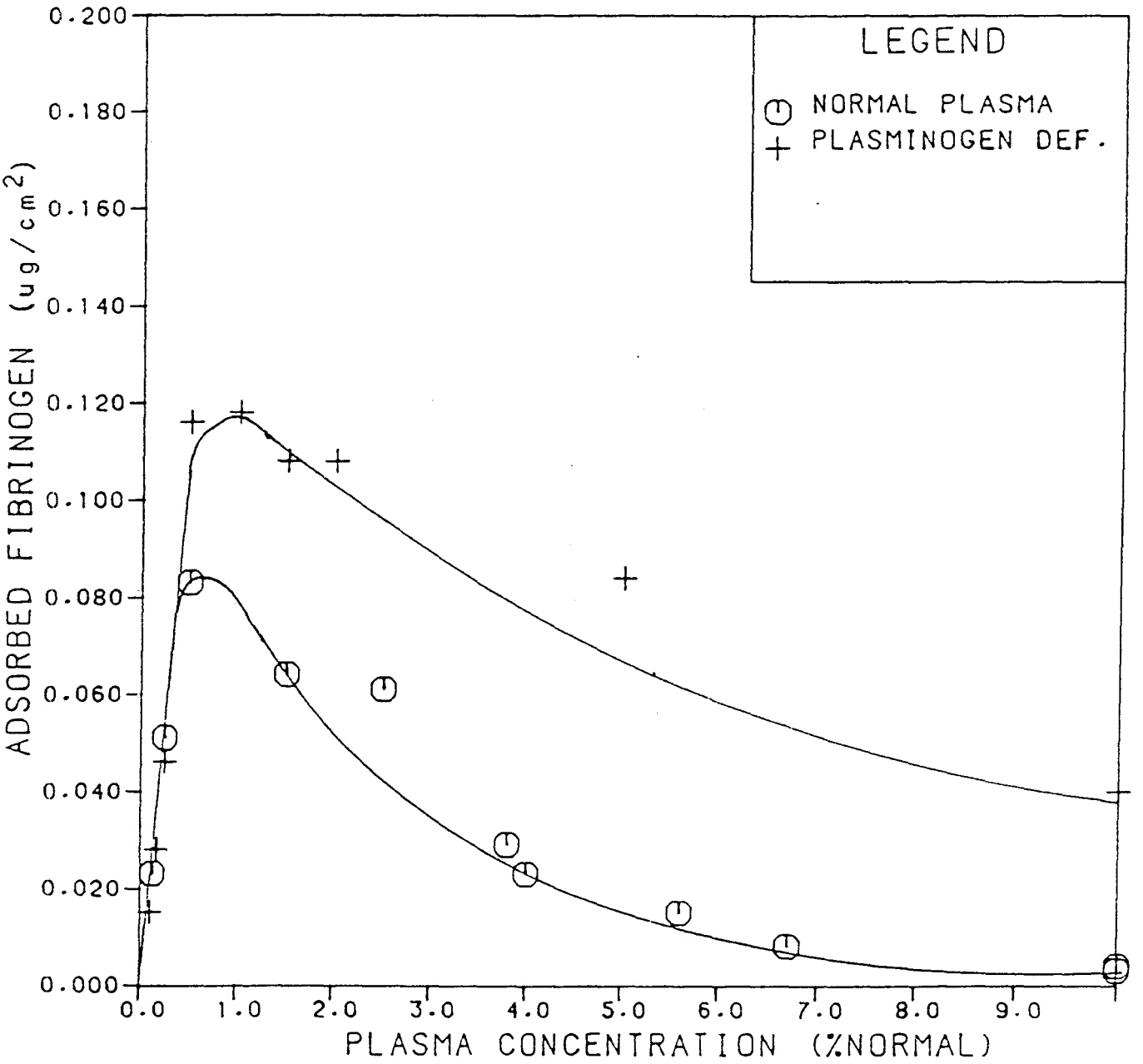


Figure 27 Fibrinogen surface concentration (5 min.) versus plasma concentration on glass using plasminogen deficient plasma

Vroman suggested that Hageman Factor (Factor XII) is responsible for fibrinogen displacement to a certain extent (92). This hypothesis is logical in view of the fact that Factor XII is generally considered to be the first clotting factor protein activated by a surface and would thus be expected to be adsorbed. The five minute fibrinogen isotherms for Factor XII-deficient plasma and normal plasma are shown in Figure 28. The two curves coincide very closely and the differences are not greater than expected between normal plasma samples (see Figure 25). This result refutes the hypothesis put forward by Vroman regarding the fibrinogen displacing ability of Factor XII.

The persistence of the Vroman effect in the absence of Factor XII is evidence that the displacement of fibrinogen may not be a result of the propagation of the coagulation cascade (see Figure 2) since it is known that surface activation of coagulation does not occur in Hageman plasma. A great deal of evidence however seems to indicate that there is at least an important interaction between the Vroman effect and the intrinsic clotting pathway, if not a direct relationship. One important question that is posed as a result of the experiments in Figure 28 is: how does Factor XII achieve a surface concentration high enough to accelerate contact activation? It appears that the amount adsorbed initially may be sufficient to initiate the cascade. Further study is required, perhaps using radio-labelled Hageman factor.

The main displacing protein according to Vroman is high molecular weight kininogen (HMWK). The use of HMWK-deficient plasma (Fitzgerald plasma) in the present work was found to have a dramatic effect on the five minute fibrinogen isotherm (see Figure 29). The data indicate that Fitzgerald

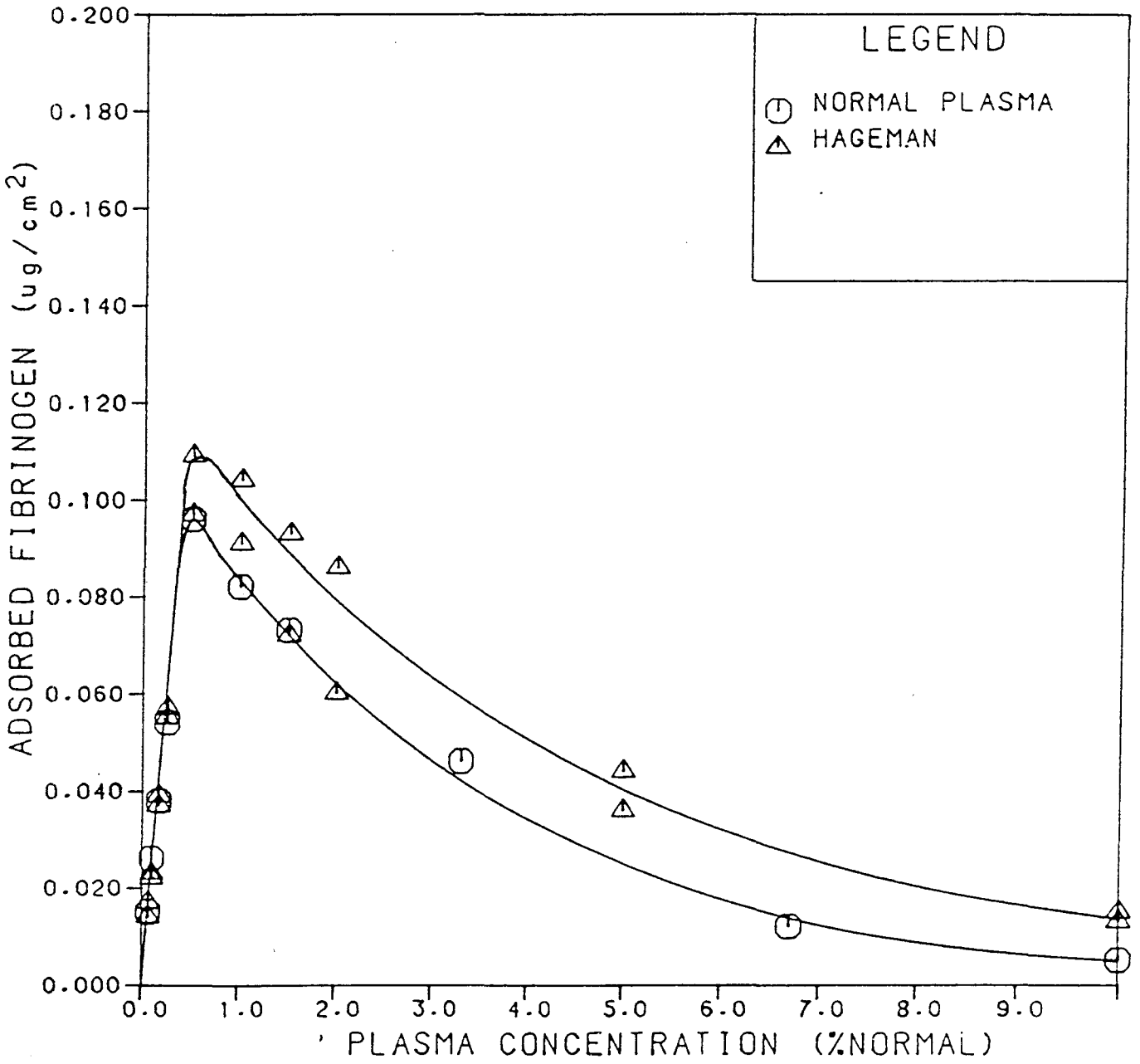


Figure 28 Fibrinogen surface concentration (5 min.) versus plasma concentration on glass using Hageman plasma

plasma is significantly less capable of displacing fibrinogen from glass when compared to normal plasma. This lends support to Vroman's hypothesis regarding the role of HMWK. However, even with HMWK-deficient plasma the displacement effect is not completely suppressed. A peak and slow decay curve still occur, suggesting the presence of other plasma components capable of displacing fibrinogen. Perhaps one of these other proteins is plasminogen as found earlier (see Figure 27).

Vroman's hypothesis regarding fibrinogen displacement by HMWK can be clarified at this point with respect to the mechanism involved. While it was suggested that the Vroman effect might be due to immunochemical masking or alteration of adsorbed fibrinogen (20) it is clear from the present experiments that the fibrinogen is actually displaced from the glass surface by HMWK. The effect has been found to be specific to HMWK. Low MWK lacks the ability of HMWK to displace fibrinogen (20) perhaps due to the absence of the positively-charged histidine-rich region to which the unique properties of HMWK have been attributed (11). It is possible that this positively charged region can bind directly to negatively charged surfaces after displacing the cationic coions required to bind the negatively charged fibrinogen molecules. The fibrinogen displacing ability of HMWK would be consistent with its purported cofactor role of transporting Factor XI and prekallikrein to surfaces in order to accelerate the intrinsic coagulation pathway (see Figures 3 and 4).

According to other researchers (21), the incubation of normal plasma with small amounts of dextran sulphate (DS) at 0°C activates the intrinsic pathway in such a way as to inactivate the HMWK (see below for a more

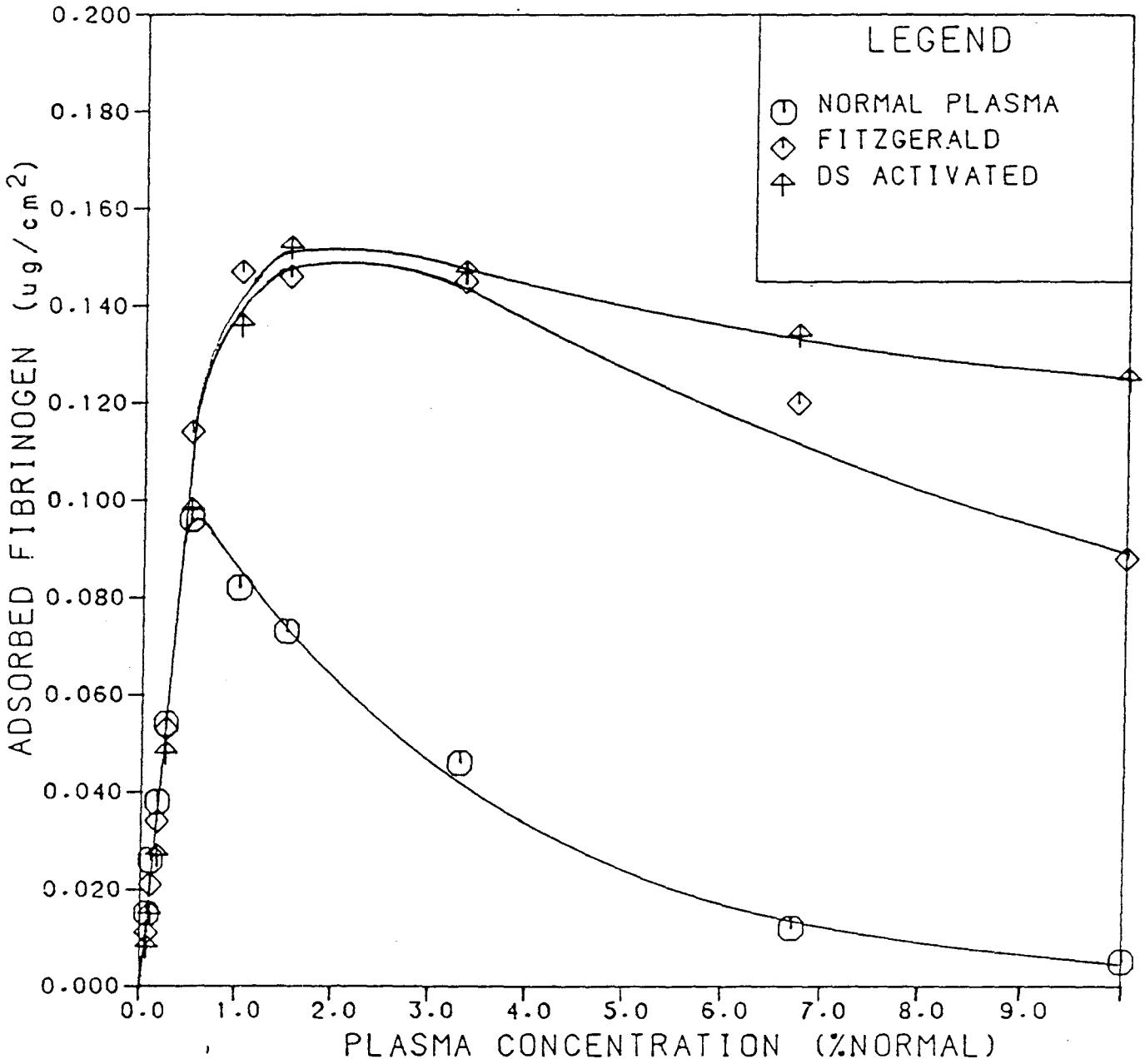


Figure 29 Fibrinogen surface concentration (5 min.) versus plasma concentration on glass using Fitzgerald and dextran sulphate treated plasmas

detailed discussion). If this were true the 5 minute isotherm for DS activated plasma should mimic that for Fitzgerald plasma. The data in Figure 29 for DS-activated plasma support this hypothesis since they closely match the corresponding five minute isotherm data for Fitzgerald plasma.

Dextran sulphate does not remove HMWK from plasma but inactivates it via the propagation of the contact activation mechanism. A model for this inactivation has been proposed by Scott and coworkers (99) and is depicted in Figure 30. It involves the stepwise proteolysis of HMWK by kallikrein and activated Factor XI. Dextran sulphate activation yields the activated zymogens kallikrein and Factor XIa. Kallikrein cleaves the HMWK procofactor in three steps to an active form capable of acting as a cofactor in the intrinsic pathway and thus able to displace adsorbed fibrinogen. Factor XIa takes the process a few steps further to an inactive form of HMWK which lacks the histidine rich region (referred to as the "coagulant portion" in Figure 30). This inactive form is incapable of acting as a cofactor and is incapable of displacing fibrinogen.

The validity of Scott's HMWK cleavage model was tested by performing contact activation with dextran sulphate on Factor XI deficient plasma. Under these conditions HMWK should be activated but without XIa the inactive form can not be achieved. Figure 31 supports the model of Scott et al. Although the peak heights differ the variation is within the range expected for different normal plasma samples. The inactivation of HMWK apparently does not occur in the absence of Factor XI. It may also be concluded that Factor XI does not have a direct role in the Vroman effect since the isotherm in Figure 31 is essentially normal. This indicates that Factor XI

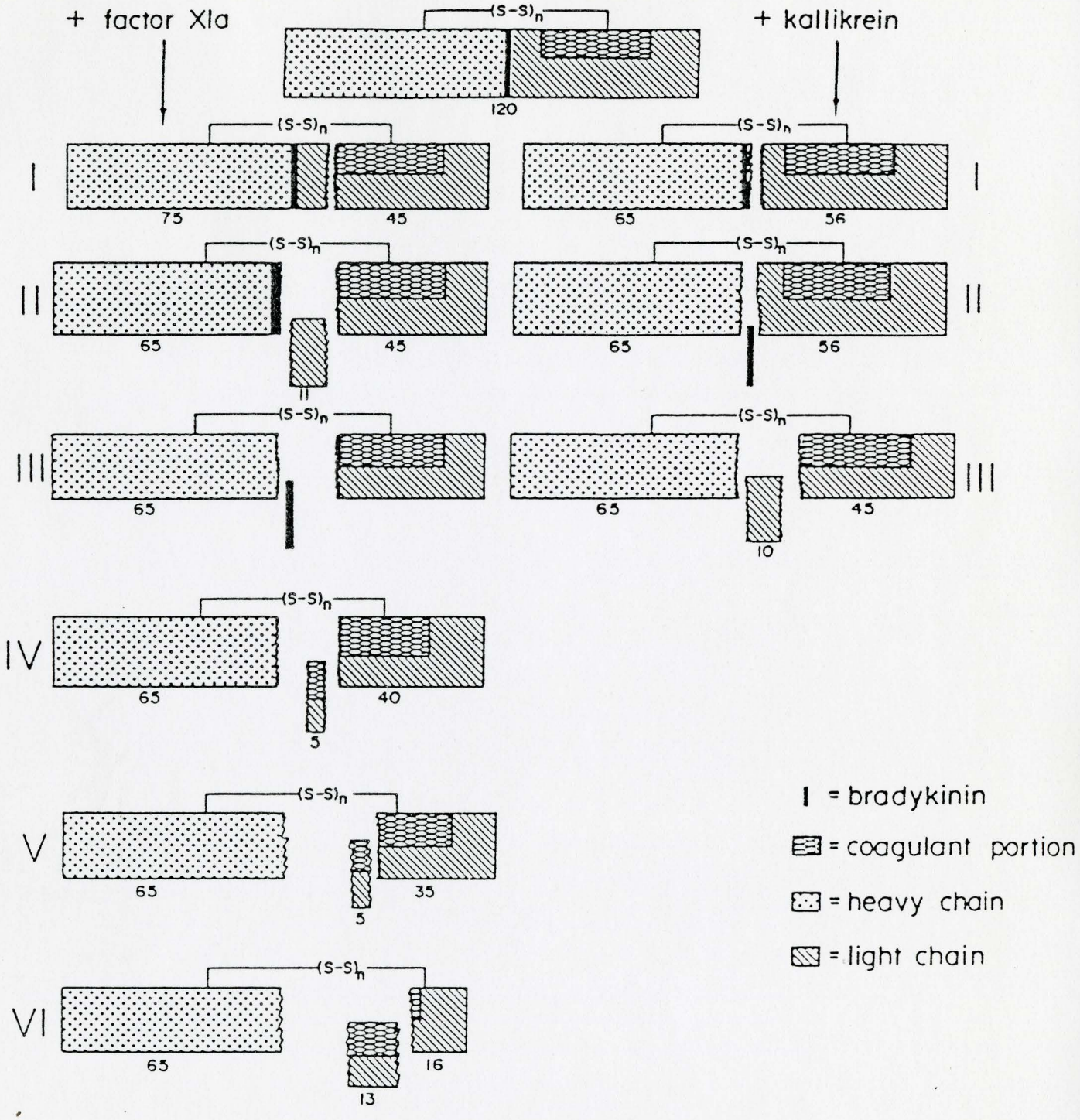


Figure 30 Scott model for HMWK inactivation (99)

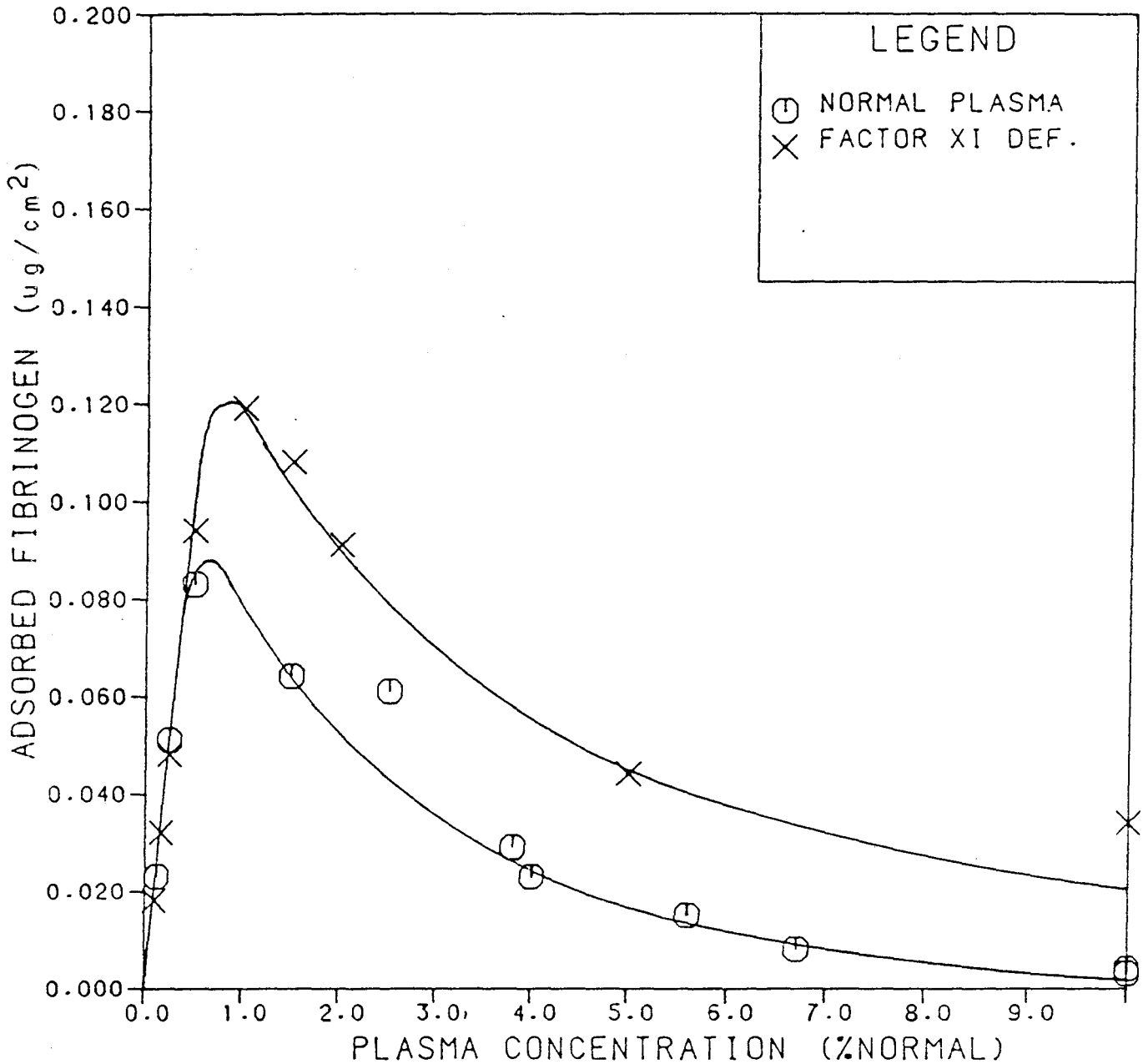


Figure 31 Fibrinogen surface concentration (5 min.) versus plasma concentration on glass using dextran sulphate treated, Factor XI deficient plasma

is incapable of displacing fibrinogen and lends further support to the cofactor role hypothesized for HMWK. Without HMWK, Factor XI (and prekallikrein) can not reach the surface in the large quantities required to accelerate the intrinsic pathway.

In other experiments a small amount of purified HMWK (0.5 Units/mL) was added back to Fitzgerald plasma in order to see if the five minute isotherm then returns to that for normal plasma. Figure 32 demonstrates the increased displacing ability of the reconstituted plasma and confirms the hypothesis of Vroman that fibrinogen is displaced by HMWK.

Based on the observations of the experiments depicted in Figures 27 and 29 experiments were done to determine the combined effects of removing plasminogen (by sepharose lysine) and HMWK (by DS activation). The resulting 5 minute isotherm (Figure 33) for this plasma is dramatically different from normal plasma and in fact appears to reach a plateau adsorption of 0.23 $\mu g/cm^2$ with no displacement evident. The height of the plateau could possibly be explained by the fact that very little spreading occurs in a system as abundant in exchanging proteins as plasma. The fact that displacement does not seem to occur suggests that HMWK and plasminogen are the main fibrinogen displacing components. The evidence of slower displacement in Figures 27 and 29 shows that both displacing proteins are capable of acting in the absence of the other and likely act independently with HMWK being the most potent displacer.

The fact that a plateau is apparently reached for plasma deficient in both HMWK and plasminogen suggests that other plasma proteins are incapable of displacing fibrinogen to any great extent. This result therefore

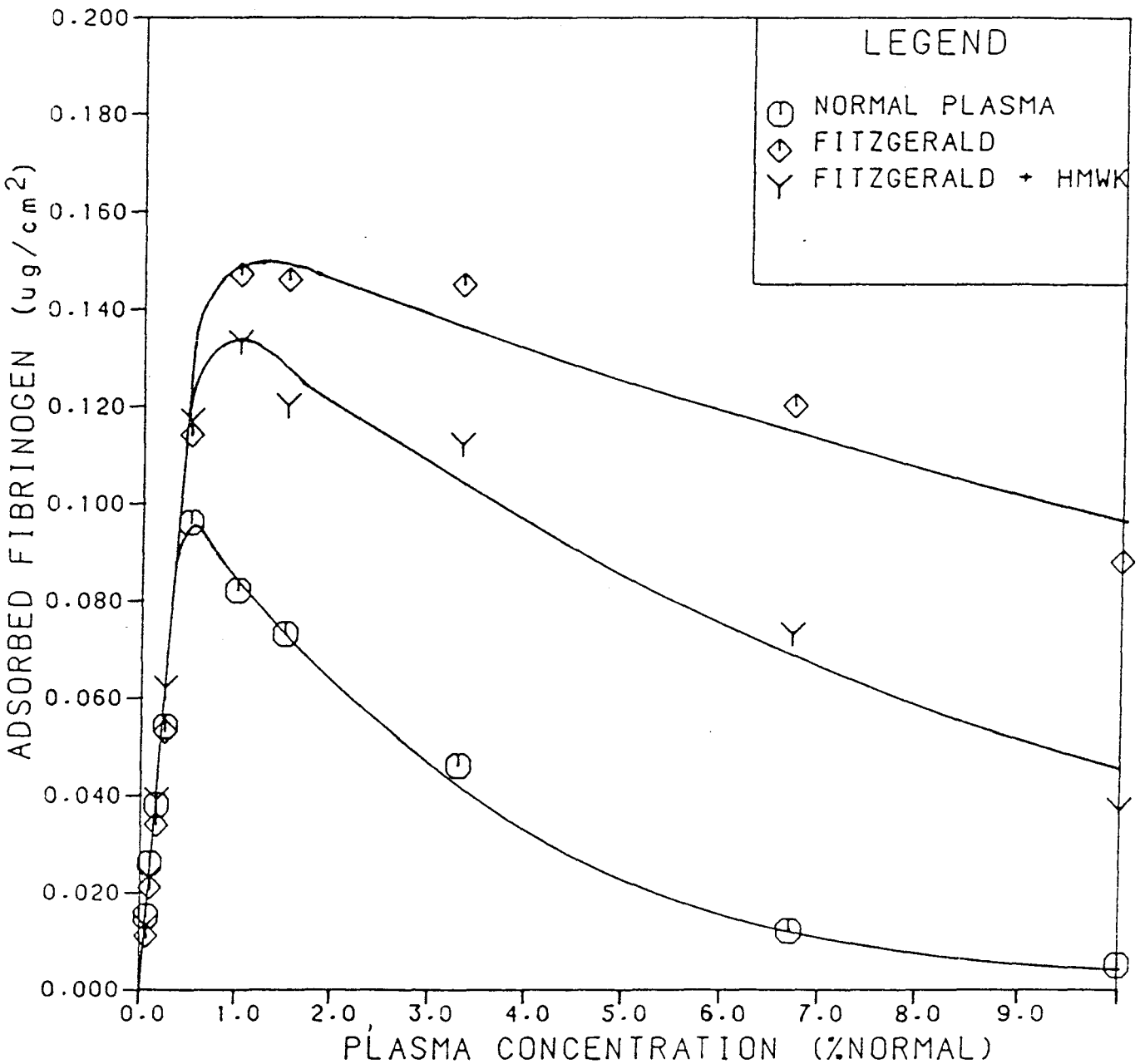


Figure 32 Fibrinogen surface concentration (5 min.) versus plasma concentration on glass using Fitzgerald plasma with HMWK added back

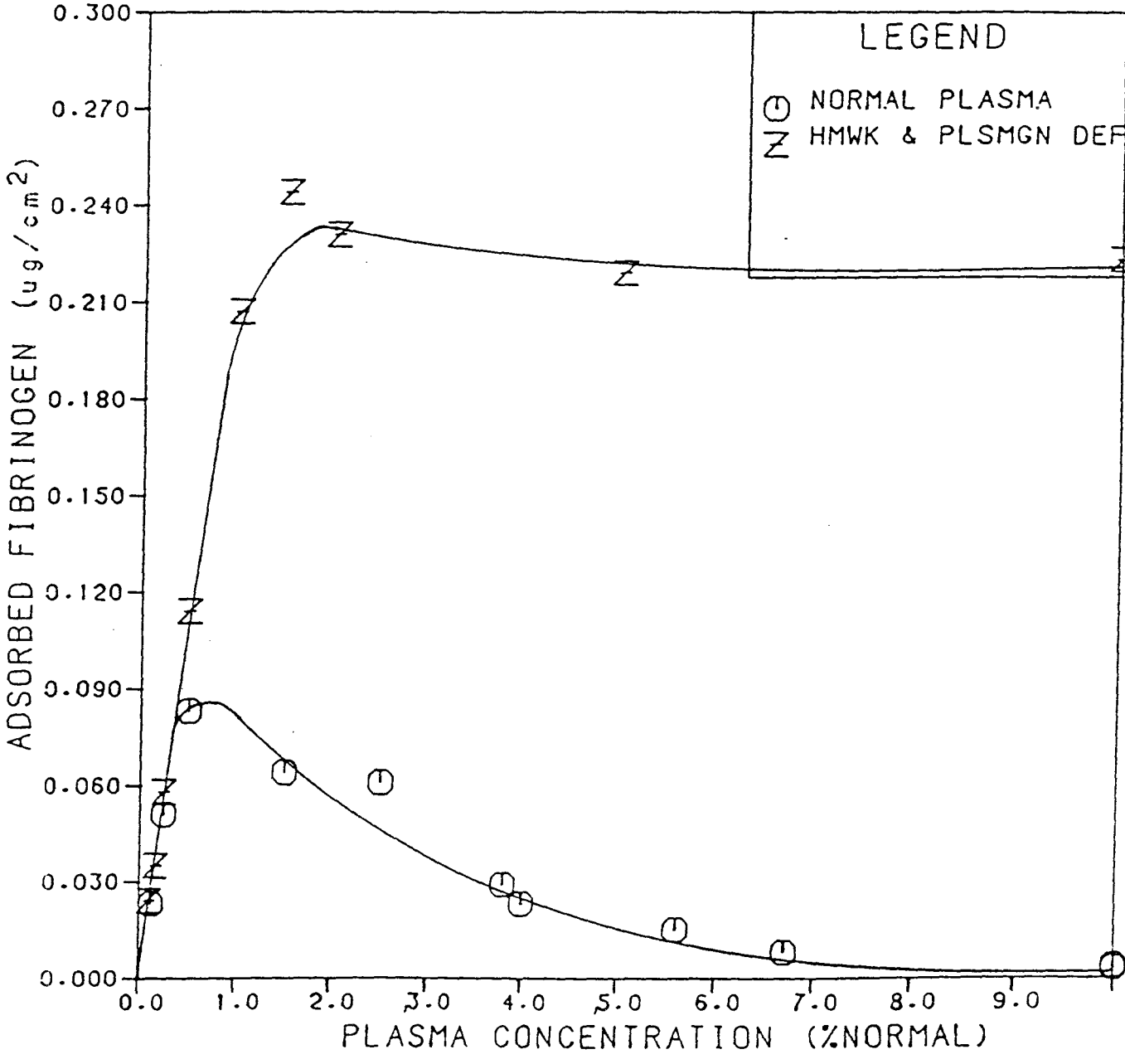


Figure 33 Fibrinogen surface concentration (5 min.) versus plasma concentration on glass using plasminogen and HMWK deficient plasma

argues against the hypotheses implicating the actions of high density lipoproteins (100), fibronectin (4) and Factor XII (20) in the Vroman effect. Further study is required to determine if the actions of any of these other plasma components do in fact participate.

The hypotheses of Vroman and Scott are upheld and elaborated on in these experiments. The role of plasminogen is currently unclear. The Vroman Effect may be a combination of effects characteristic of the intrinsic clotting (HMWK) and fibrinolytic (plasminogen) pathways. Recent evidence obtained by Brash suggests that plasminogen is converted to plasmin on glass. This interaction may have an important influence on biocompatibility and merits further investigation.

Position Dependence

Fibrinogen adsorption experiments were routinely performed in tubes 25 cm in length. These tubes were subsequently cut into 5 cm pieces to be placed in counting vials. Early experiments showed a significant change in measured adsorption along the length of the tube in such a way as to shift the 5 minute isotherm (see Fig. 12) to the right with increasing distance from the entrance. Figure 34 shows two 5 minute isotherms for the two end pieces of a typical adsorption experiment. The curve for the entrance region of the tube is consistently and significantly to the left of the curve for the exit region. The possibility of bulk solution depletion following adsorption was considered but could not account for the relatively large effects seen at high concentrations. In this region of the curve, adsorbed fibrinogen accounts for about 0.1 per cent of the total and therefore should not cause any detectable depletion.

A more likely cause of the observed position dependence is the existence of a hydrodynamic boundary layer created during the initial injection of the radioactive protein solution. The rapid displacement of the buffer solution could be expected to set up a boundary layer with a thickness initially proportional to the one third power of distance along the tube (69). This boundary layer would be expected to retard the diffusion of protein to the surface.

A number of experiments were done in order to investigate this phenomenon since it would have an effect on the quantitative interpretation of the data. In these experiments tubes of 120 cm length were used in an attempt to exaggerate the effect. The results for a five minute adsorption

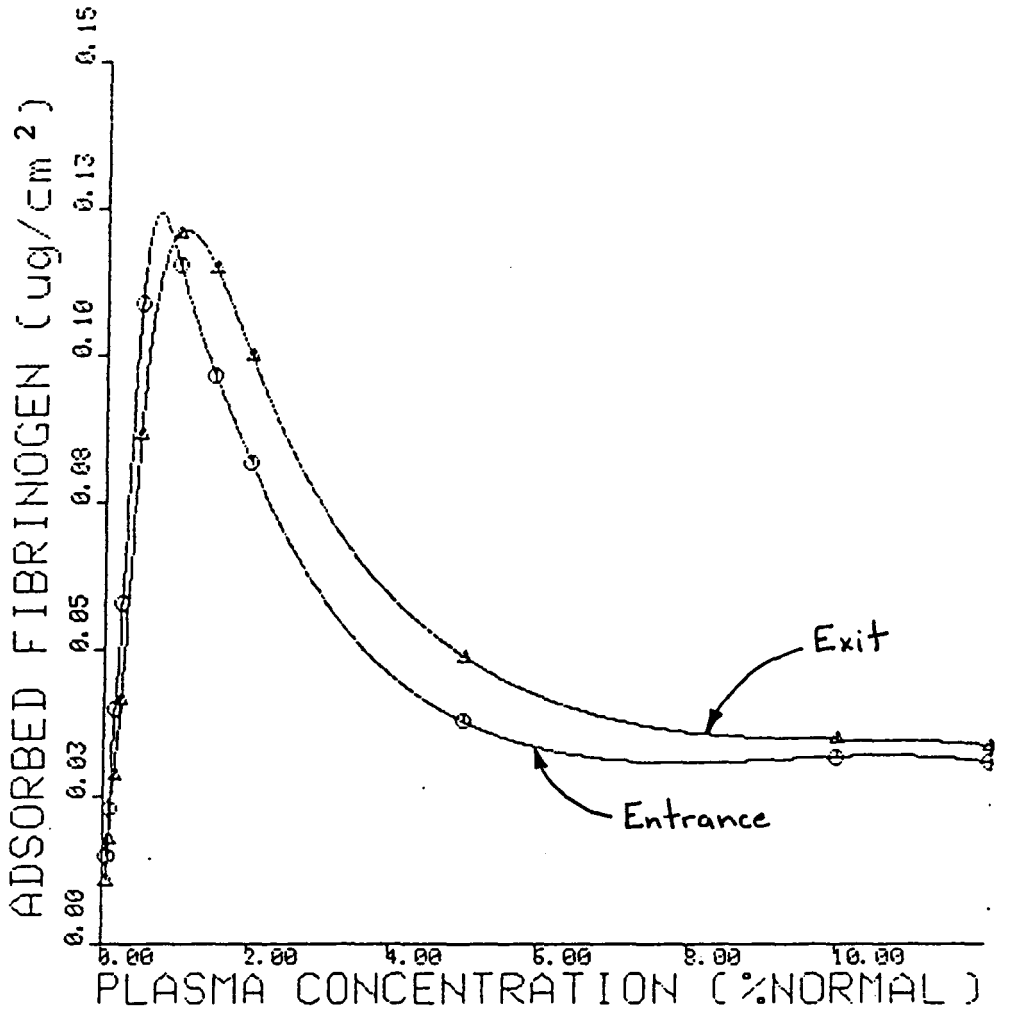


Figure 34 Typical 5 minute fibrinogen surface concentration experimental data showing position dependence on glass

using 1.0% and 0.5% plasma (to the right and left of the 5 minute isotherm peak respectively) are displayed in Figure 35. It was anticipated that the long tube would extend the effect for the 1.0% plasma experiment in such a way that the adsorption data could be observed to go over and descend past the isotherm maximum. The data shown in Figure 35 for 1% plasma could be interpreted in this way but are not compelling in this regard. The experiment using 0.5% plasma (Fig. 35) is expected to show a decrease in adsorption along the length of the tube and, in fact, does demonstrate this behaviour. The decrease occurs over the entire length of the tube which supports the depletion effect hypothesis rather than the boundary layer. The latter is expected to be fully developed shortly beyond the entrance of the 120 cm tube.

Two experiments were done using 10% plasma and the results are shown in Figure 36. The increase in adsorption along the tube is gradual and significant along its entire length. The results obtained for the two experiments are significantly different but consistent in their upward trend. They provide a good example of the kind of variation that can be expected between identical experiments for any system involving labelled fibrinogen in plasma.

In order to directly investigate the possible effect of solution depletion, fibrinogen concentration was measured before and after exposure to the glass surface. 10% plasma showed a decrease in fibrinogen content of about 1/200th of its initial level. This small change can not account for the relatively large position dependence seen in Figures 34 and 36. 1% plasma was more significantly depleted with almost 1/10th of its initial fibrinogen content apparently taken up by the surface. This depletion may be distributed

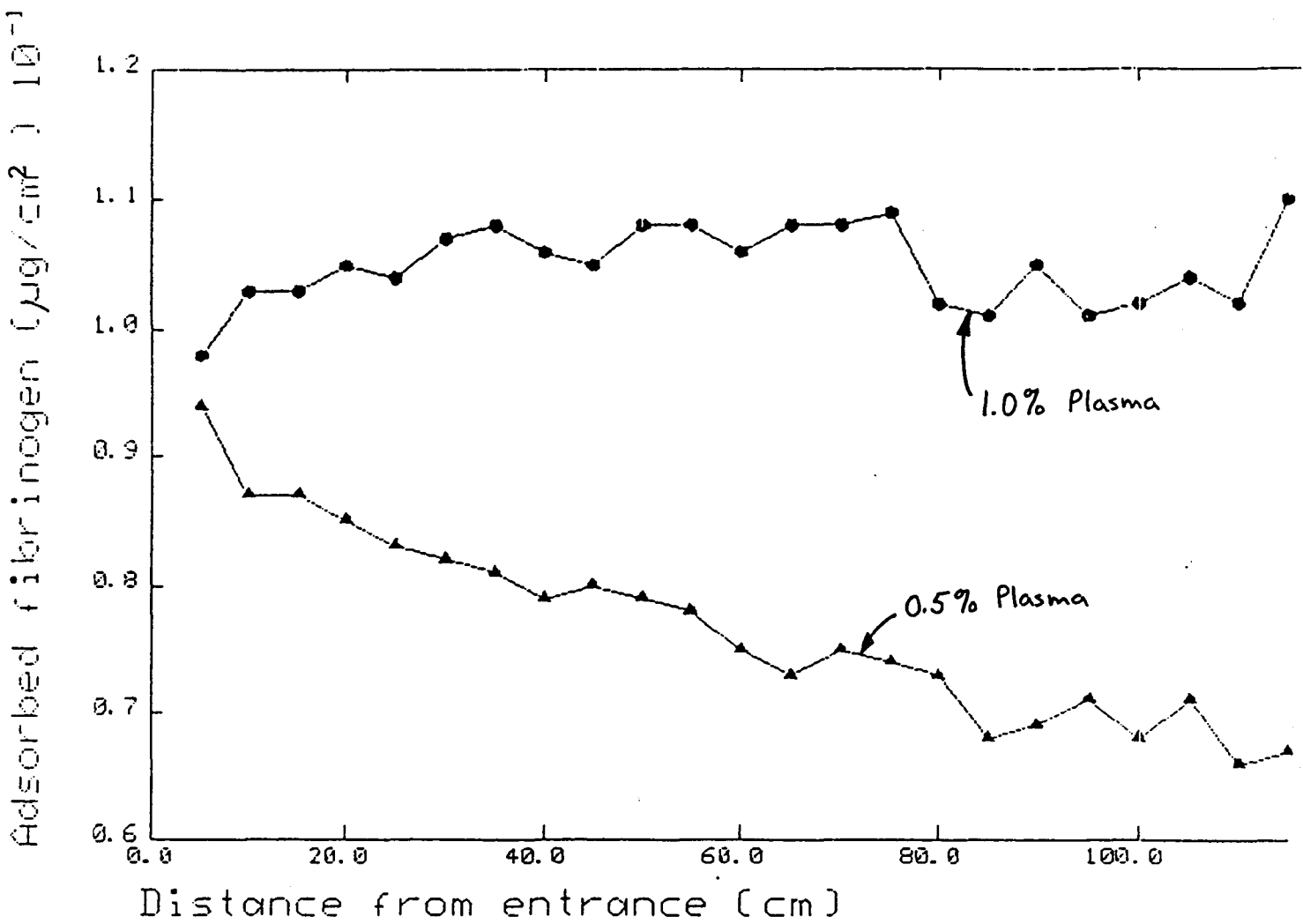


Figure 35 Position dependence experiment for 5 minute adsorption using 1.0 and 0.5 per cent plasma on glass

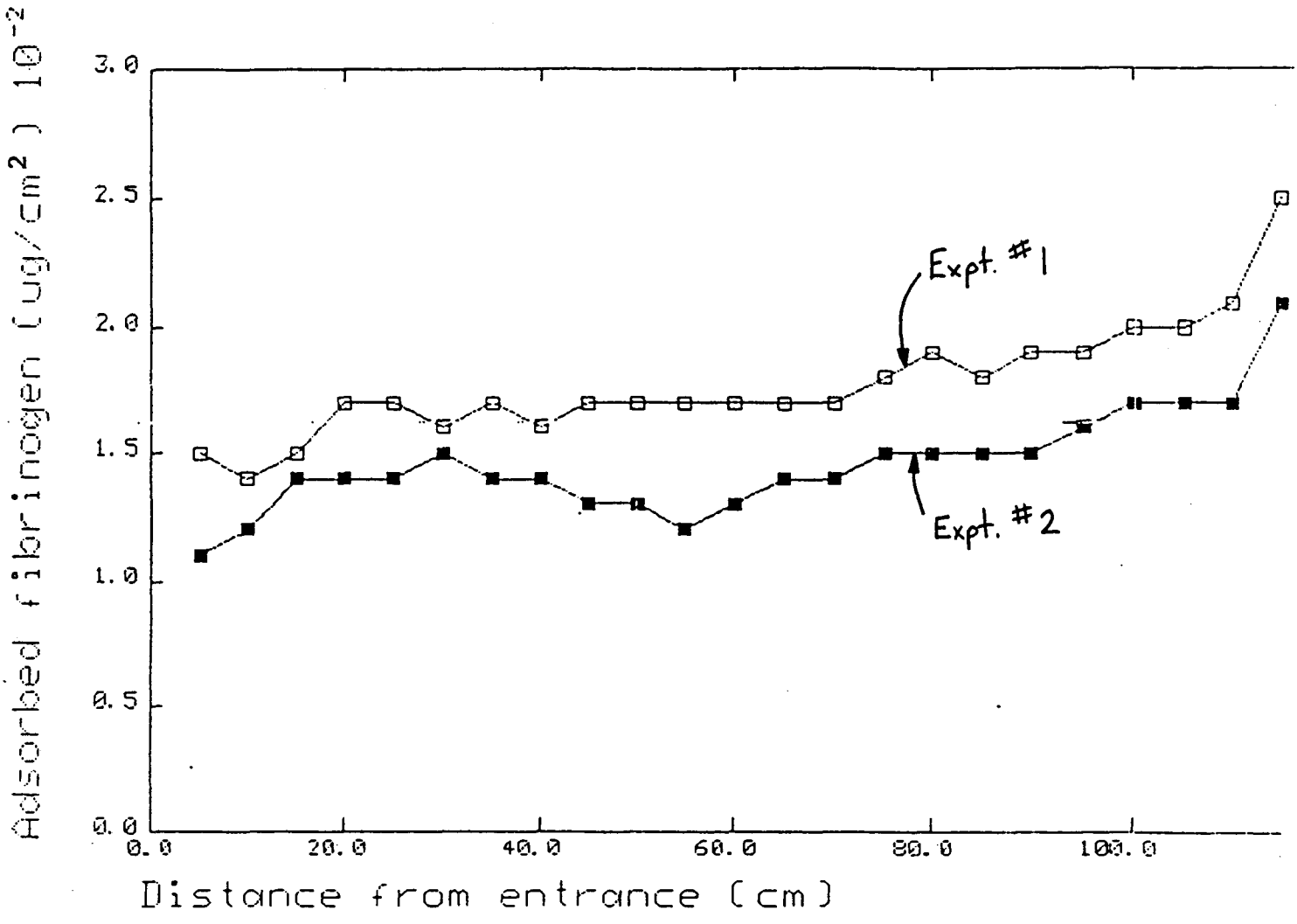


Figure 36 Position dependence experiment for 5 minute adsorption using 10.0 per cent plasma on glass

nonuniformly over the length of the tube and could conceivably account for the position dependence observed in Figures 34 and 35.

Although the position dependence effect is small it has been shown to be significant and its existence could have repercussions on all protein adsorption experiments involving flow through narrow passages. The mechanism may involve solution depletion, boundary layer effects or a combination of both. Further study is required to determine its exact cause in order to more fully understand its interactions with measured diffusion coefficients and adsorption rates. It will be difficult to justify estimations of these parameters unless position dependence can be explained or abolished.

CHAPTER 5
Modeling

Development of a Protein Adsorption Isotherm Model (PAIM)

An isotherm model which incorporates the contributions of spreading and exchange has not yet been developed. The Beissinger-Leonard model (39) based on the Langmuir-Hinshelwood mechanism incorporates a two-state adsorption in which a flat loosely bound molecule becomes more compact and tightly bound with time spent on the surface. This model does not agree with observed spreading reported by many researchers (44,75,77). The Lok, Cheng, Robertson model (78) involves reversibly and irreversibly adsorbed states. Although data fits are adequate, mechanistic justification for such a model is, however, lacking. Protein isotherms have been found to have finite slopes even over a range of very low concentrations where surface coverage is significantly less than expected for a monolayer. The Lok, Cheng, Robertson model also fails to account for conformational changes known to play a large role in macromolecule adsorption.

A new model should incorporate all the aspects of protein adsorption that are generally accepted. The model proposed here begins with the initial adsorption of fibrinogen from an isotonic solution of Tris buffer. In such an environment the molecule exists in a compact, soluble form referred to as the native state. It should adsorb initially in a conformation only slightly different from this "native" state. Subsequently the protein may undergo "spreading". The initial adsorption may be represented by:

$$F + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F - S$$

where

S is a surface site

F is fibrinogen in solution

Spreading may be represented by:

$$(F - S)_1 \xrightarrow{k_s} (F - S)_2$$

an irreversible conformation/orientation change whereby molecules adsorbed in the compact state 1 spread to become state 2 molecules (where k_s is the rate constant for spreading). This mechanism may be an over simplification and in fact the molecules may pass through a range of conformations between state 1 and state 2. Such a mechanism is not considered in the present model but may merit investigation in subsequent work.

Exchange between dissolved and adsorbed molecules has been reported in previous studies (87,88,89) and must play a role in equilibrium adsorption. While desorption occurs only very slowly from a surface exposed to a protein-free buffer, exchange has been shown to occur much more rapidly. An adsorbed molecule that is exchanged will be replaced by a molecule in state 1 and therefore only the surface concentration of state 2 molecules will be affected:

$$F + (F-S)_2 \xrightarrow{k_e} (F-S)_1 + F$$

where k_e is the rate constant for exchange.

Since the isotherm has a constant initial slope it cannot be assumed that state 2 molecules are irreversibly adsorbed. Therefore:

$$(F-S)_2 \xrightarrow{k_2} F + S$$

will represent this mechanism where k_2 is the rate of desorption from state 2.

The above four reactions constitute the main phenomena observed during protein adsorption. Langmuir-Hinshelwood rate equations may be written for both adsorption states at equilibrium [20,21].

$$\frac{d\Gamma_1}{dt} = k_1[F](1 - A_1\Gamma_1 - A_2\Gamma_2) + k_e[F]\Gamma_2 - k_{-1}\Gamma_1 - k_s\Gamma_1 \qquad [20]$$

$$\frac{d\Gamma_2}{dt} = k_s\Gamma_1 - k_e[F]\Gamma_2 - k_2\Gamma_2 \qquad [21]$$

where

Γ_1 is the surface concentration (μg/cm2) of fibrinogen in the initially adsorbed compact state

Γ_2 is the surface concentration (μg/cm2) of fibrinogen in the spread state

A_1 is the area per ug of fibrinogen in state 1 (cm2/ug)

A_2 is the area per ug of fibrinogen in state 2 (cm2/ug)

k_1 is the rate constant for adsorption of fibrinogen into state 1

k_{-1} is the rate constant for desorption of fibrinogen from state 1

Both equations may be set equal to zero and as a result equation [22] gives the relationship between Γ_1 and Γ_2.

$$\Gamma_2 = \frac{k_s \Gamma_1}{k_2 + k_e[F]} = R\Gamma_1 \qquad [22]$$

Rearrangement of equation [20] yields an expression for Γ_1 independent of Γ_2.

$$\Gamma_1 = \frac{1}{(A_1 + A_2 R) + \left(\frac{1}{K_1[F]} + \frac{R}{K_2[F]} \right)} \qquad [23]$$

where $K_1 = k_1/k_{-1}$

$K_2 = k_1/k_2$

are the binding constants for states 1 and 2 respectively. Total surface coverage is given by:

$$\Gamma_1 + \Gamma_2 = \Gamma_1 (1 + R) \qquad [24]$$

and may therefore be described by a function of concentration alone.

A few simplifications may be made based on an a priori knowledge of the system. First k_2 is very small and thus R will be large at low concentrations. This also means that when the right hand side of the denominator found in equation [23] dominates, the K_2 term will be much larger than the K_1 term. At higher concentrations the left hand side will dominate and k_2 will be an unimportant part of R. Based on both of these assumptions equation [23] may be rewritten in combination with equation [24]:

$$\Gamma_1 + \Gamma_2 = \frac{1}{\left(\frac{A_1 + A_2 R}{1 + R}\right) + \frac{1}{K_2[F]}} \qquad [25]$$

and the expression for R may be simplified to:

$$R = \frac{k_s}{k_e[F]} = \frac{1}{K_{es}[F]} \qquad [26]$$

The result is a four parameter model (A_1, A_2, K_2, K_{es}) which may be fitted to adsorption data for different surfaces, and may form a basis for the development of a dynamic model. Equations [25] and [26] will be referred to as PAIM (protein adsorption isotherm model).

Parameter Estimation for PAIM on Different Surfaces

The four parameters of PAIM were calculated for the four surfaces discussed in the pure fibrinogen adsorption section. A program employing UWHAUS (a nonlinear model fitting routine using Marquardt's compromise) was developed and the estimated parameters are presented in Table 3. All of the parameters calculated are significantly greater than zero at the 90% confidence level except for the second and fourth parameters for PUO2. This may be a result of the very low spreading rate on this surface. The values obtained for these parameters are not unreasonable and will be used to compare PUO2 qualitatively to the other surfaces studied.

Table 3. Parameter Estimates for PAIM Using Adsorption Data on Four Different Materials

	A_1	A_2	K_2	K_{ES}
polyethylene	1.30	3.00	0.081	0.0185
	±0.2**	±1.4	±0.05	±0.02
glass	0.898	3.89	0.0755	0.0265
	±0.3	±2.5	±0.05	±0.03
PUO2	0.595	1.31	0.0177	59.9
	±0.03	± ?*	±0.8	± ?*
Surfasil	1.12	3.02	0.0680	0.0107
	±0.2	±0.7	±0.018	±0.01

* very ill-conditioned parameters suggesting little or no "spreading".

** 90% confidence intervals.

The A_1 parameter represents the initial adsorbed area per microgram of fibrinogen. The inverse of A_1 represents the maximum possible surface coverage by fibrinogen on each surface. As might be expected, based on their hydrophobic nature and their ability to induce "spreading", the minimum area per molecule on polyethylene and Surfasil is larger than on the other two surfaces. However PUO2, a fairly hydrophobic polyurethane, has a much lower A_1 than glass. Perhaps this could be explained by the existence of a balance of polar-apolar groups or hydrophilic-hydrophobic sites on the surface. These properties have been previously studied (46,60) and are felt to be more important to biocompatibility than the macroscopic surface characteristics traditionally measured. For PUO2 the inverse of A_1 is approximately 1.7 $\mu g/cm^2$, a value very close to that expected for a close-packed, end-on monolayer of fibrinogen molecules (43).

A_2, the fully spread area per molecule appears to be largest on glass which may be an indication that glass promotes spreading to a greater extent than on the other surfaces. It should be recalled that this PAIM is based on the premise of a two-state adsorption which is likely to be an over simplification; it is more likely that there are multiple states between the initially adsorbed and fully spread condition. Due to this, A_1 and A_2 may be average areas and not the true maximum and minimum areas. They may however provide insight into the initial rapidity of spreading (large A_1 = rapid spreading) and the eventual extent to which it occurs (large A_2 = extensive spreading).

The initial slope of the isotherm is represented by K_2. Its value is approximately the same for glass, Surfasil and polyethylene but is 1/4th this value on PUO2. This difference may be due to:

1) a high desorption rate

2) a low adsorption rate (i.e. a low ΔG)

3) extensive desorption during the rinsing procedure.

The linear portion of the model fits closely to the low concentration adsorption data (not shown) indicating an accurate estimation of K_2 on all surfaces. More data in this region are required to shrink the 90% confidence limits.

The ratio of exchange to spreading rate is given by the parameter K_{es}. Spreading appears to be most important on polyethylene and Surfasil. On glass K_{es} is still quite small but is two times larger than on the more hydrophobic surfaces. PUO2 with its heterogeneous surface composition has an ill-conditioned but very high value of K_{es}. This suggests that the exchange rate is very rapid or spreading is very slow or a combination of both. This exchange-spreading parameter may be very useful in characterizing the ability of surfaces to induce conformational changes in proteins. This property may be closely related to the effect of materials on the intrinsic clotting pathway.

Figure 37 shows the model fit obtained using available data and a least squares fitting routine. The curves coincide closely with experimental data over both the low (not shown) and high concentration regions. A greater

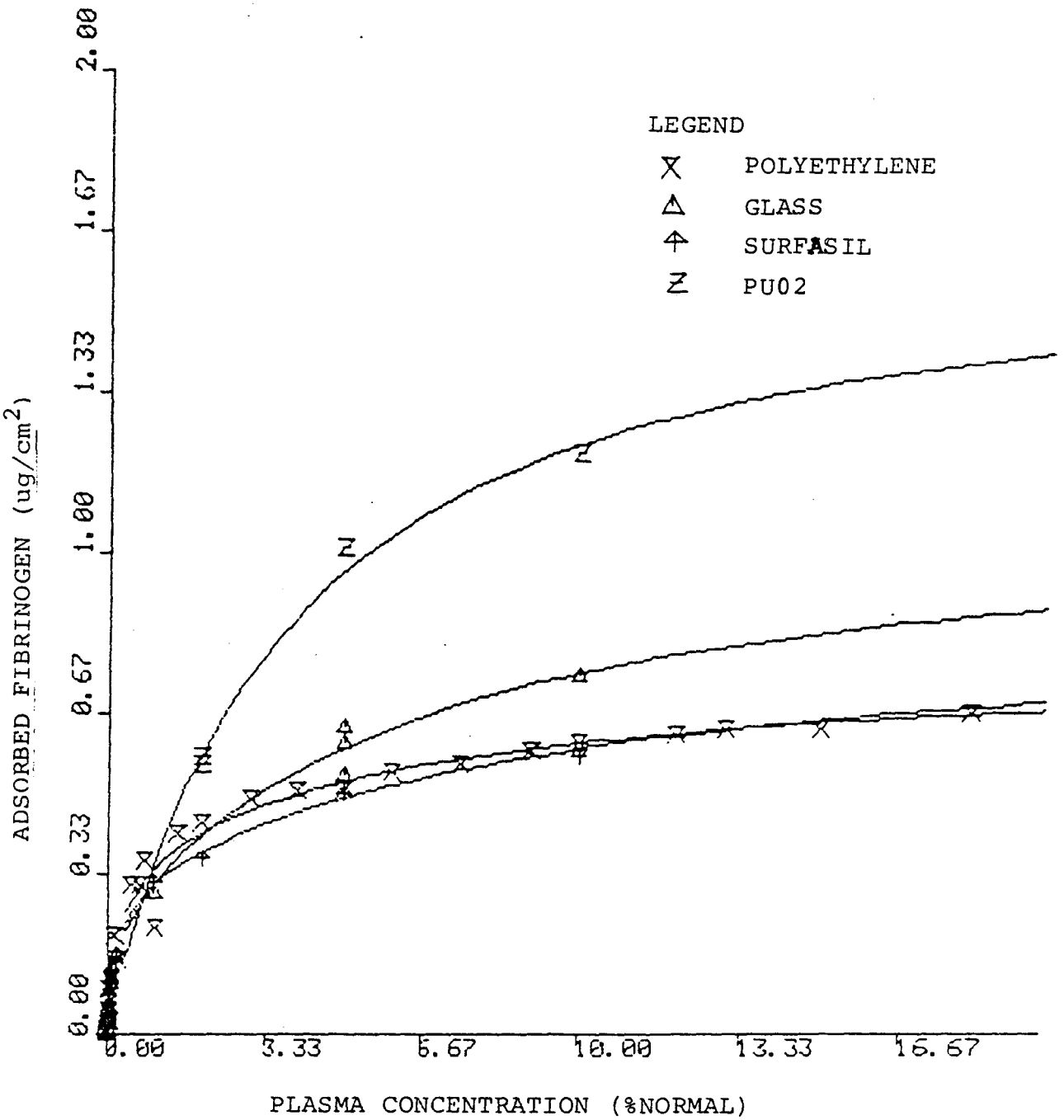


Figure 37 Protein adsorption isotherm model fit to experimental data using 24 hr fibrinogen surface concentration data seen in Figure 10 and the parameter estimates in Table 3

understanding of the parameters is now required in order to explain their implications for biocompatibility. Ultimately, it is hoped that the parameters may be predicted based on known properties of the protein and surface. When fully understood the parameters may be used in kinetic models and perhaps in models for a multicomponent system such as plasma. For the moment, it is believed this model is an improvement over currently available single protein adsorption isotherms.

CHAPTER 6

Summary

As a result of the research program reported in this thesis, the following major observations were made.

1. One-component fibrinogen adsorption appears to involve spreading and exchange of the surface-bound molecules. These phenomena must also play an important role in adsorption from plasma.
2. High fibrinogen adsorption occurs when surface binding is more reversible.
3. The initial adsorption of fibrinogen is diffusion limited under most conditions.
4. An activation energy barrier appears to be responsible for a low apparent diffusion coefficient.
5. After being adsorbed from plasma, fibrinogen is subsequently displaced from glass by HMWK and plasminogen.
6. The model of Scott et al. for HMWK inactivation appears to be correct with regard to the role of Factor XI.
7. The affinity of fibrinogen for surfaces increases with time spent on the surface.
8. An equilibrium fibrinogen isotherm is achieved before displacement is complete. Therefore a dynamic exchange of molecules may be occurring.
9. Fibrinogen is initially a dominant component on artificial surfaces before being displaced.

10. Fibrinogen adsorption shows a significant position dependence as a result of depletion at low concentration, and a hydrodynamic boundary layer at high concentrations.

11. An adequate adsorption isotherm model was developed to fit experimental data for four surfaces based on a spreading and exchange mechanism.

CHAPTER 7

Suggestions for Future Work

1. A mathematical model to describe the "Vroman Effect" mechanistically for both the dynamic and steady state conditions, perhaps based on single component information, is required.
2. Experiments on clean, unequilibrated surfaces should be performed on all materials to determine the possible effect of hydrated or structured water interfaces.
3. A quantitative real time experimental technique is recommended for the collection of dynamic data. Such a method would allow on-line calculations and model fitting and a better understanding of the adsorption in the absence of rinsing.
4. More experiments involving deficiencies of plasminogen and HMWK are required, especially on surfaces other than glass, in order to determine their relative importance to the "Vroman Effect".
5. The effect of position dependence should be clarified and distinguished from the mechanisms of interest.
6. The relationship between the Vroman effect and biocompatibility is still unclear. Its implications on implanted materials should be investigated to bring the problem into perspective.
7. The adsorption behaviour of other important plasma components such as plasminogen, HMWK and Factor XII should be studied in order to determine their behaviour at plasma-solid interfaces.

8. The Vroman effect should be measured for a wider range of biomaterials (Teflon, Biomer, stainless steel, carbon, heparinized surfaces etc.) so that correlations with blood compatibility may be sought.

9. An experiment done in the absence of diffusion limitations may yield data with a more direct relationship to the mechanisms involved with the behaviour of proteins at the surface.

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