BLOOD RESPONSE TO BIOMATERIALS: *IN VITRO* AND CLINICAL INVESTIGATION OF THE CONTACT PHASE OF BLOOD COAGULATION.

by

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I declare that this study was entirely my own work and has not previously been submitted to this or any other University.

Signature

Bashir M Matata

"Study the past to divine the future."

Confucius

ABSTRACT.

The clinical utilisation of materials and devices involving contact with blood, has promoted a growing interest in blood-biomaterial interactions and monitoring of the blood response. This study focused on the establishment of a parameter relevant for the measurement of contact phase activation of blood coagulation and its suitability for *in vitro* and clinical evaluation. The selected biomaterials for *in vitro* assessment were commercially available haemodialysis membranes and the selected clinical procedures were haemodialysis (HD) and cardiopulmonary bypass (CPB). *In vitro* measurements were considered in relation to membrane charge and adsorption of FXII and heparin. Three methods for determining contact activation, based on measurement of factor XII activity, were investigated. A factor XII-like activity (FXIIA) was measured by a modified chromogenic substrate assay. FXIIA was determined *in vitro* in the plasma supernatant and on the membrane surface following blood-membrane contact. Supernatant values of FXIIA did not discriminate between membranes but surface values were related to membrane structure and the presence of pharmacological agents. Factor XII activity (FXIIa) was measured by an enzyme immunoassay and FXIIa values in plasma were determined *in vitro*. Measurement of FXIIa improved the detection sensitivity and provided some degree of discrimination. Factor XIIa/FXIIa-inhibitor complexes were measured by a developed ELISA assay and values obtained *in vitro* and during HD and CPB. Enhanced sensitivity was demonstrated *in vitro* and clinical levels were related to the disease state and membrane type in HD and the treatment period and the presence of aprotinin in CPB. The evidence suggests that FXIIA measured on the membrane surface was more relevant to biomaterials evaluation than the supernatant measurement. FXIIa assay was relatively more sensitive than the chromogenic substrate assay in terms of the supernatant measurements. The FXIIa/XIIa-inhibitor complexes assay provided a better discrimination between materials and therefore is recommended as an appropriate parameter for monitoring the influence of biomaterials, devices and pharmacological agents in clinical applications.

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CONTENTS.

CHAPTER EIGHT

THE ROLE OF CONTACT PHASE ACTIVATION DURING HAEMODIALYSIS AND CARDIOPULMONARY BYPASS

Table i: List of abbreviations.

Abbreviation	Description
FXII	Factor XII (Hageman factor)
FXIIA	Factor XII-like activity
FXIIa	Activated factor XII
β-FXIIa	Activated factor XII, beta fragment (fluid phase component, mol.weight 28,000)
α-FXIIa	Activated factor XII, alpha molecule (surface-bound molecule, mol.weight 80,000)
Factor XIIa/XIIa-inhibitor complexes	Free activated factor XII and inhibitor-bound activated factor XII complexes
Supernatant FXIIA	Fluid phase factor XII-like activity
Membrane FXIIA	Membrane-bound factor XII-like activity
KI	Kallikrein inhibitor (Soybean trypsin inhibitor)

CHAPTER ONE

INTRODUCTION

1.1 BLOOD CONTACTING APPLICATIONS

The application of procedures in medicine and surgery that require exposure of blood to foreign surfaces has increased markedly over recent times. Most applications have been in the form of implantable or extracorporeal devices. The extracorporeal situations that are commonly considered include haemodialysis and related procedures, membrane plasma separation, cardiopulmonary bypass, artificial heart valves, artificial heart and assist devices, and arterial grafts (Courtney et al 1993a).

The application of blood-contacting devices, however, has not been without inherent difficulties, primarily due to the risk from local thrombosis on the material or on the other hand, bleeding risk due to antithrombotic agents and consumption of haemostatic factors (Courtney et al 1993a). Particular interest has been on the response of blood to contact with materials or "biomaterials" used in the construction of the device.

1.2 BIOMATERIALS

The world market for biomaterials has been estimated by the UK Institute of Materials to be currently worth US$ 12 billion, and is expected to grow at a rate of 7 - 12% annually (Wakelam 1995). Unlike most markets for advanced materials, it is not subject to cyclical demand from end-user markets. Most of all the biomaterials used today have been adapted from existing industrial products not intended for medical applications (Klinkmann et al 1994) and therefore have had to be modified to meet specific applications. The applications however, require unique research and development (R&D), whereby costs are very high, and rates of return are modest for the larger companies which can spread there R&D budget over a range of projects.

The materials used in the construction of devices or implants are commonly known as "biomaterials". A biomaterial has been defined by some authors as a material of synthetic or natural origin, used in contact with tissue, blood or

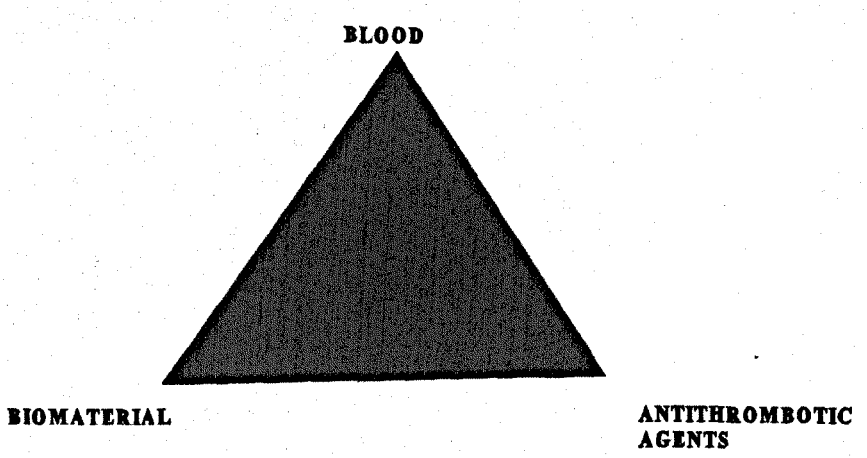


Figure 1.1: Blood-biomaterials interaction , a multilateral relationship (Courtney et al 1989)

biological fluids and intended for use in prosthetic, diagnostic, therapeutic or storage applications (Courtney et al 1993b). A more recent definition was given by the 1994 Consensus Conference on Biocompatibility, where a biomaterial was described as a non-viable material used in a medical device intended to interact with biological systems (Gurland et al 1994).

An important feature of using biomaterials in contact with blood is the contrast in behaviour between artificial surfaces and the endothelium. In the absence of special modification, artificial surfaces cannot perform an active role in the resistance to thrombus formation similar to that performed by the endothelium and cannot provide a non-attractive surface comparable to that of the endothelium (Mason et al 1977; Mason et al 1979). Consideration, therefore, focuses on much broader perspectives such as the blood-biomaterial interactions, biomaterial-tissue interactions, and the involvement of the immune system, including cellular and humoral responses to the material. The collective terminology that describes these reactions has been put forward as "biocompatibility", subsequently defined by the 1994 Consensus Conference as the ability of a material, device, or system to perform without a clinically significant host response in a specific application (Gurland et al 1994).

Blood-contacting applications focus on polymeric biomaterials and, consequently, options for altering the influence on blood of a material are polymer synthesis, polymer formulation and polymer modification. For extracorporeal devices such as haemodialysis and cardiopulmonary bypass, biomaterials of interest have been membranes and tubing. In this thesis, the biomaterial focus has been on haemodialysis membranes for *in vitro* tests and on haemodialysis and cardiopulmonary bypass as relevant clinical procedures.

1.3 PHARMACOLOGICAL / ANTITHROMBOTIC AGENTS

For most clinical applications, it is necessary to use an antithrombotic agent in association with biomaterials and consequently the importance of consideration of the interaction between biomaterials and antithrombotic agents cannot be over-emphasized. In an overall relationship between blood, biomaterial and antithrombotic agent (Figure 1.1), a more compatible response can result from

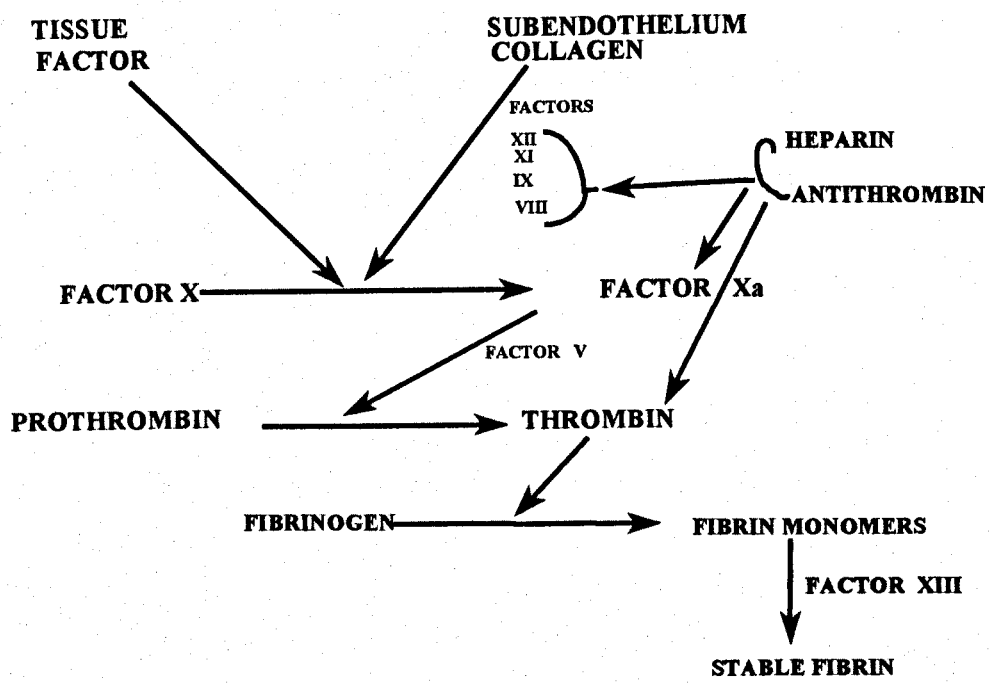


Figure 1.2: The mechanism of heparin action

alteration to the biomaterial, the antithrombotic agent or both (Courtney et al 1993c)

In extracorporeal situations, the prevention and removal of thrombus are of prime importance and three main types of agents are relevant. These are anticoagulants, platelet aggregation inhibitors (antiplatelet agents) and plasminogen activators. The most widely used antithrombotic agent in extracorporeal situations is the anticoagulant heparin, which functions by potentiating the influence of antithrombin III. By forming a complex with antithrombin III, heparin catalyses the action of antithrombin III on thrombin (Rosenberg 1982), thereby inhibiting thrombin, the aggregation of platelets by thrombin, the thrombin-mediated conversion of fibrinogen to fibrin, and factors Xa, IXa, XIa, and XIIa (Figure 1.2).

Possible alternatives to heparin are low molecular weight heparin (Thomas 1986) and hirudin (Talbot et al 1989). In cardiopulmonary bypass, other approaches have been adapted including the use of synthetic complement inhibitors (Miyamoto et al 1985), aprotinin as a kallikrein inhibitor (Royston et al 1987), a platelet aggregation inhibitor (Takahama et al 1984), an inhibitor for leucocyte sequestration (Ohtani et al 1988) and a platelet preserving agent (Tatsumi et al 1988).

The limitations of using heparin in extracorporeal applications have been due to the haemorrhagic complications. The heparin molecule is composed of anionic centres that are highly reactive (sulphate and carboxyl groups) and these have a tendency to bind to charged moieties or surfaces (von Sengbusch et al 1993). The adsorption of some heparin onto the surfaces of the extracorporeal device may lead to a reduction in antithrombotic properties. This therefore emphasizes the importance of consideration of the interaction between the antithrombotic agent and the biomaterial surface.

1.4 BLOOD RESPONSE TO BIOMATERIALS.

The response of blood to a biomaterial surface can be viewed as the occurrence of protein adsorption, followed by the involvement of platelet reactions, activation of the intrinsic coagulation and kallikrein-kinin systems, participation of

the fibrinolytic and complement systems, and the interaction of erythrocytes and leucocytes (Courtney et al 1994). The rapid adsorption of protein onto a biomaterial surface is regarded as the first major event, with the subsequent phenomena largely influenced by the interaction of blood with the adsorbed protein layer (Brash 1991). However, there are marked differences between protein adsorption patterns observed *in vitro* compared with clinical conditions, exemplified by the marked reduction in fibrinogen levels *in vitro* in contrast to the consistent levels during haemodialysis (Courtney et al 1991).

Another feature of the blood response to biomaterials contact is that of platelet adhesion and aggregation (Mason 1972; Mason et al 1976), with the adsorbed protein layer a controlling factor of the platelet response. Platelet adhesion to a biomaterial is followed by the platelet release reaction taking place in the adhering platelets and then platelet aggregation on the surface (Forbès & Courtney 1994). Biomaterials have been associated with the release of different platelet constituents under varying applications (Courtney et al 1994). Platelets play a significant role in thrombus formation (Feijen 1977) and also initiation of coagulation by the release of thromboplastin (Needleman & Hook 1982; Walsh 1982).

As indicated, blood-biomaterial interaction leads to the initiation of the intrinsic pathway, involving the participation of the contact proteins. Of particular relevance for blood-contacting applications is that the activation of the contact proteins induces major biological consequences because of their close connection with important proteolytic pathways. The activation of the contact factors leads to the generation of bradykinin, a potent vasodilator implicated in hypersensitivity reactions (Wachtfogel et al 1993). A gradual activation and consumption of factor XII has been reported for haemodialysis (Sundaram et al 1992) and for cardiopulmonary bypass with both bubble and membrane oxygenators (Sundaram et al 1993).

In view of the integrated nature of the mechanisms of haemostasis (Forbes & Courtney 1994), the blood response during blood-biomaterials contact must involve the activation of the fibrinolytic pathway, although this topic has not been

GENERAL OBJECTIVE

Figure 1.3: The correlation between a characteristic property of a biomaterial and the biological response. (Lamba 1994)

widely investigated (Brash 1991).

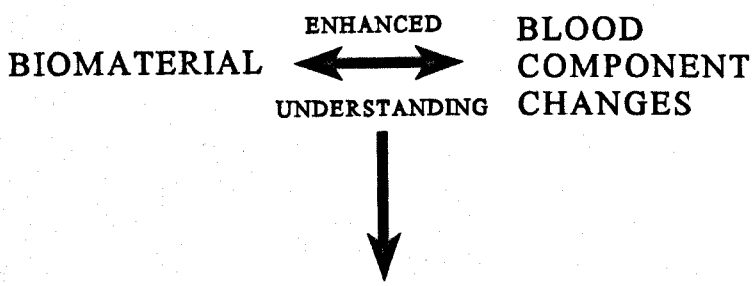
Erythrocytes are known to adhere to the adsorbed protein layer (Feijen 1977) and the pattern of protein adsorption can be influenced by the cell membrane or the competitive adsorption of released haemoglobin (Brash 1991). With haemolysis, the platelet release reaction is induced by liberated adenosine diphosphate (ADP) and in coagulation under low shear forces, erythrocytes and thrombin form the red thrombus (Bruck 1980).

During blood-biomaterial contact, leucocytes adhere to the surface (Kusserow et al 1971) in a manner linked with the adsorbed protein layer. Preferential adsorption of granulocytes as compared to lymphocytes has been reported (Lederman et al 1978). There is a direct role of leucocytes on thrombus formation arising from granulocyte adhesion and its effect on platelet aggregation (Cumming 1980). Granulocytes are known to possess endogenous procoagulant activity ((Niemetz 1972) and proaggregating activity (Harrison et al 1966). Leucocyte response is often linked with complement activation, a feature particularly important in extracorporeal situations (Courtney et al 1993a).

The complement system can be activated during blood-biomaterial contact, with the subsequent release of the anaphylatoxins C3a and C5a (Kazatchkine & Carreno 1987). The released C3a and C5a may influence leucocyte adhesion to the biomaterial surface (Herzlinger & Cumming 1980). C5a is known to mediate granulocyte responses such as adherence, aggregation, degranulation, chemotaxis, and free oxygen radical production (Chenoweth 1986). Systemic or intravascular complement activation is believed to induce granulocyte activation and organ damage (Chenoweth 1986).

1.5 INVESTIGATION OF BLOOD-BIOMATERIALS INTERACTIONS

When blood contacts a biomaterial, thrombogenic or allergic reactions are initiated in a manner that is to some degree related to the nature of the material. In biomaterial development, the production of materials that exhibit a reduction in the magnitude of these reactions has been a major objective. In general, there is a requirement for the establishment of a correlation between a characteristic property of the biomaterial and a relevant feature of the biological response (Figure 1.3)



1. Better utilisation of existing materials
2. Development of improved materials

Figure 1.4: A general objective in biomaterials research (Lamba 1994)

(Courtney et al 1994). The establishment of the relationship between the biomaterial and the altered components of the blood would therefore be useful in the better utilisation of existing materials and the development of improved materials (Figure 1.4).

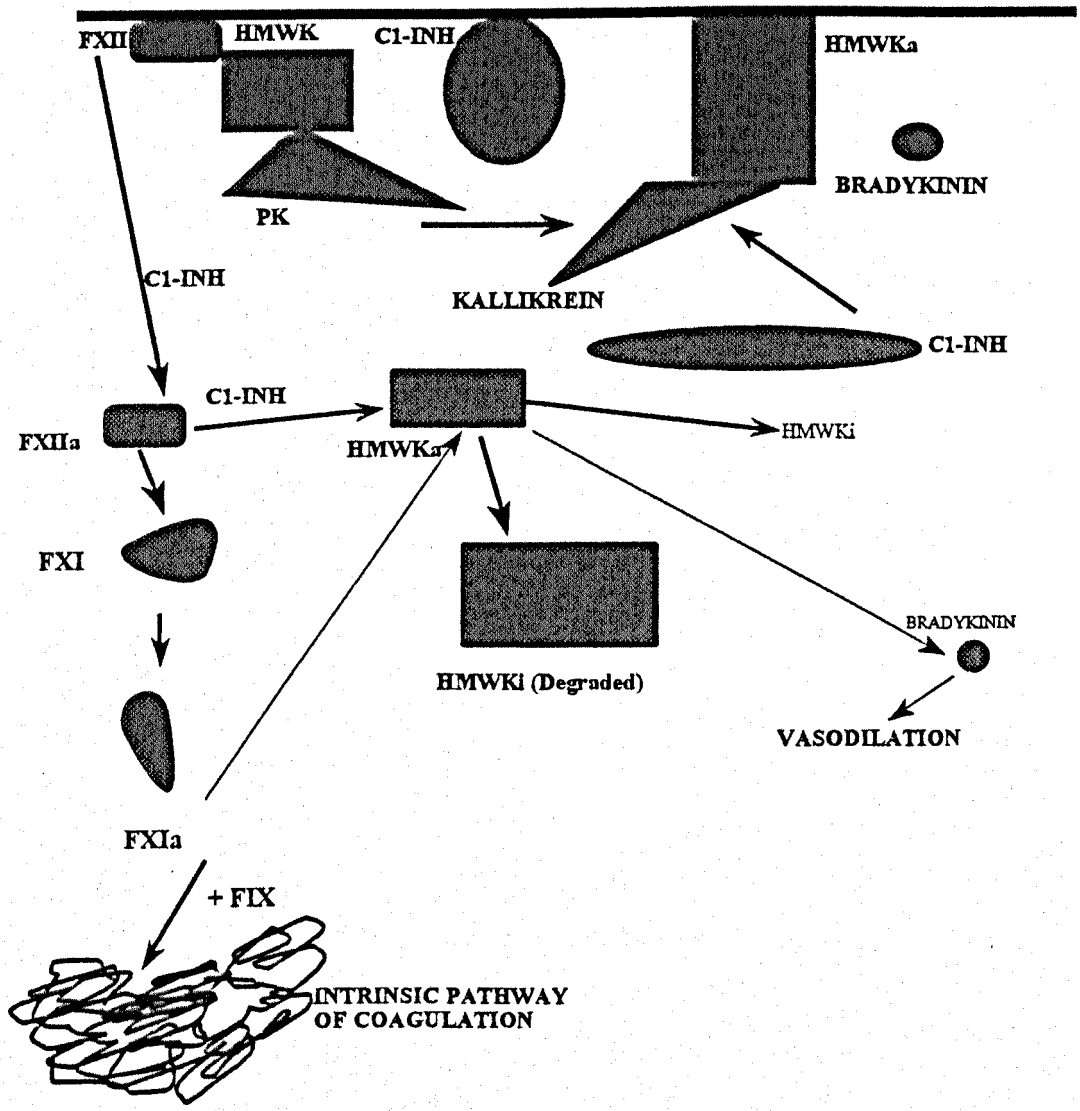
Numerous parameters have been considered as suitable indicators of the blood response to biomaterials and a multiparameter assessment approach appears preferable. However, although there is an advantage of multiparameter approach, there is also a benefit in measuring a single parameter by a consistent methodology.

The methods used to determine the blood response to biomaterials are invariably limited by the practical difficulties and the ability to interpret correctly the resultant data (Williams 1986). For laboratory investigations, the method by which blood is contacted to materials is one of the most important aspects of consideration. In this thesis, two methods of blood-biomaterial contact were utilised. These were a modified 6 well incubation test cell and a continuous-flow syringe pump system for the assessment of flat sheets and hollow fibre membranes respectively. Flat sheet membranes studied were Cuprophan, Hemophan, AN69S, SPAN, and the control polyamides NR and NRZ. Two hollow fibre membranes were studied and these were Cuprophan and AN69HF (retrieved from Baxter ST15 and Filtral 20 dialysers respectively).

1.6 CONTACT PHASE ACTIVATION.

Recently, molecular biology and structural protein chemistry have contributed to an increased knowledge of the structure-function correlates of the contact phase system proteins. The contact phase system involves the plasma proteins factor XII, prekallikrein, high molecular weight kininogen, and factor XI (Figure 1.5). Each protein has been found to have unique functions, such as activation of the complement and fibrinolytic systems, and cysteine protease inhibition (Wachtfogel et al 1993). The proteins interact to generate the potent vasodilator bradykinin and initiate coagulation on artificial surfaces. They also interact with blood and vascular cells, including platelets, neutrophils, monocytes, and endothelial cells.

Figure 1.5 : CONTACT PHASE ACTIVATION MODEL



Although the contact phase system is probably not involved in physiological haemostasis, it is well known to be involved in the pathophysiological contact of blood with artificial surfaces. This has been exemplified by cardiopulmonary bypass, where significant contact phase activation occurs shown by an increase in kallikrein-C1-inhibitor complexes as noted in a simulated extracorporeal circulation (Wachtfogel 1989). The clinical relevance of the contact phase proteins have been investigated and evidence accumulated over the years indicates that the contact factors play a major role in coagulation, fibrinolytic, kallikrein-kinin, complement, renin-angiotensin and protein C systems (Fuhrer et al 1990). In view of the evidence, the emphasis in studying this surface-mediated defence system was, as in the case of complement, on its pathophysiological not physiological roles.

As indicated earlier, the intrinsic pathway of blood coagulation in the blood response to a biomaterial is initiated by the activation of the contact proteins. The central role of factor XII (FXII) in initiating contact phase activation and a number of other biological systems emphasizes the relevance of quantifying this protein, as a parameter representative of contact phase activation. Most methods available for the assessment of factor XII are primarily intended for the detection of clinical coagulation disorders and consequently are designed for maximal activation of the protein. These include specific coagulation assays, estimation of antigen concentration, chromogenic substrate assays, radioimmunoassays and enzyme immunoassays (Kaplan 1978; Fuhrer et al 1990)

The suitability of a chromogenic substrate assay for factor XII activity measurement during blood-biomaterial interactions has been investigated in the Bioengineering Unit (Irvine 1989; Sundaram 1992; Yu 1993; Wark 1993; Lamba 1994). The original assay was believed to require some optimisation to establish substrate selectivity and minimise the effect from competing enzymes (Irvine 1989; Sundaram 1992). The optimised method is a measure of FXII-like activity, designated FXIIA, in contrast to FXIIa, used to denote activated forms of FXII (α- and β-FXIIa).

The reasons for the designation FXIIA are as follows:

1. The chromogenic substrate assay evaluated in the study does not distinguish

between the two forms of activated factor XII i.e α- and β-FXIIa.

2. The substrate is also attacked by other serine proteases such as plasmin, trypsin, Factor Xa, and thrombin (Sundaram 1993). The introduction of a kallikrein inhibitor (soybean trypsin inhibitor) indicated a limitation of the attack on the substrate by trypsin, factor Xa, and plasmin but not thrombin and α- or β-FXIIa (Sundaram 1993).

3. FXIIa-α_2-macroglobulin complexes can also attack the substrate resulting in the production of para nitroaniline (p-NA).

Despite the successful clinical application of the modified chromogenic substrate assay in haemodialysis, cardiopulmonary bypass (Irvine 1989; Sundaram 1992) and reconstructive vascular surgery (Sundaram 1992), the usefulness of the measurement of plasma factor XIIA during *in vitro* evaluation of biomaterials was limited. Successive *in vitro* studies indicated a lack of discrimination between different membrane biomaterials in plasma factor XIIA (Sundaram 1992; Yu 1993; Wark 1993; Lamba 1994).

The options in this thesis were further modification of the assay procedure and consideration of an alternative. Modification of the FXIIA assay to measure surface-bound FXIIA was attempted in view of the tendency for the factor XII to adsorb onto surfaces. A comparison was drawn with the pattern of membrane adsorbed labelled plasma factor XII and heparin adsorption onto membranes.

In addition to the measurement of surface-bound FXIIA, other assay methods with increased specificity and sensitivity were considered. These were an enzyme linked immunosorbent assay for activated factor XII (Shield diagnostics Ltd, Dundee, UK) and a novel enzyme immunoassay for FXIIa/FXIIa-inhibitor complexes (developed in collaboration with Shield Diagnostics Ltd, Dundee, UK). The latter assay was used during *in vitro* evaluations of blood-biomaterials interactions as well as in the clinical applications of haemodialysis and cardiopulmonary bypass. In haemodialysis and cardiopulmonary bypass, a parameter indicative of the activation of the extrinsic pathway of coagulation (factor VIIa) was also determined using an assay that utilises recombinant tissue factor.

1.7 THESIS OBJECTIVES

In this thesis, the general objective presented in figure 1.4 has been adapted to focus on the relationship between changes to factor XII and the properties of selected biomaterials. The selected biomaterials were, as indicated, those used in clinical haemodialysis and cardiopulmonary bypass and for *in vitro* studies, representative haemodialysis membranes. In the *in vitro* studies, the biomaterial property investigated was surface charge. The study focused on the following aspects:

1. Measurement of factor XII-like activity *in vitro* by an existing chromogenic substrate assay methodology. The emphasis was on the modification of the assay to allow measurement of factor XII-like activity in the plasma (supernatant phase) as compared to the surface-bound factor XII-like activity (FXIIA) component in the presence and absence of heparin.
2. *In vitro* measurement of plasma supernatant phase and surface-bound activated factor XII (FXIIa and FXIIa-Inhibitor complexes) by an enzyme linked immunosorbent assay.
3. *In vitro* measurement of surface adsorption of heparin and the resultant anticoagulant properties. The effectiveness of anticoagulation by heparin, following blood-membrane contact was to be investigated.
4. *In vitro* measurement of surface-adsorption of labelled factor XII and the mechanism of surface binding.
5. Measurement of activated factor XII (FXIIa/FXIIa-Inhibitor complexes) and activated factor VII during haemodialysis and cardiopulmonary bypass.
6. Determination of surface charge and distribution on selected membrane materials.

CHAPTER TWO

HAEMOSTASIS AND THROMBOSIS: THE RELEVANCE TO BLOOD-BIOMATERIAL INTERACTION

2.1 INTRODUCTION

Haemostasis has been described as the process by which the loss of blood or bleeding from an injured tissue is controlled and normally requires the interaction of the blood vessel wall, platelets, coagulation and fibrinolytic proteins (Hoch and Silver 1991). Therefore, haemostasis refers to the return to normality and restoration of equilibrium. The endothelium's complex role in haemostasis is determined by the balance between the endothelium's procoagulant and anticoagulant attributes. This balance is modulated by other cells and circulating proteins (as discussed further in chapter 3).

The formation of a blood clot has been described as "thrombosis" and in accordance with Virchow, in 1856, the pathophysiology of thrombus formation involved three interrelated factors: the surface of the vessel wall (i.e disease of the vessel wall), stasis or changes in blood flow (i.e abnormality of flow), and the changes in coagulability of blood (i.e consistency of the blood) (Kitchens 1985). One or more components of Virchow's triad can be evoked when determining the aetiology of an *in vivo* thrombosis and the hypothesis has remained relevant even in the recent understanding of thrombosis. The integration of various blood components (e.g platelets, coagulation, fibrinolytic, and complement systems) is modulated by the vascular endothelium (discussed further in chapter 3).

2.2 PLATELET REACTIONS.

The relevance of the physiological function of platelets in haemostasis and thrombosis can be expressed in terms of the following (Niewiarowski 1981):

(1) Platelets adhere to the damaged vessel wall and form aggregates that contribute to the formation of a haemostatic plug that arrests bleeding.

(2) Platelets release their constituents, such as adenosine diphosphate (ADP), serotonin, calcium, and secreted platelet proteins. The role of ADP is to enhance formation of platelet aggregates and by secreting a number of platelet-derived

proteins such as growth factors or vascular permeability factors, platelets can also modify the function of other cells, particularly endothelial cells.

The haemostatic plug is composed of a mass of platelets with fibrin and some red cells around the periphery. Exposure of blood to nonendothelial surfaces induces platelets to adhere, during which they change from their normal disc shape to a more rounded form with extended pseudopods. Following vascular damage, the process of platelets adhesion involves interaction of the platelet membrane glycoprotein (GPI_b), subendothelial collagen, and plasma von Willebrand factor (vWF) (Sakariassen et al 1979).

Hereditary deficiency of platelet GPI_b (Bernard-Soulier syndrome) or plasma vWF produces defective platelet adhesion (Weiss 1974). Similarly, platelets adhere to collagen through glycoprotein I_a, to fibrinogen via $GPII_b/III_a$ and fibronectin and to thrombospondin through GpIV (Jaffe et al, 1982; Mosher 1980; Royston 1992). Platelet adhesion initiates a series of complex interactive platelet reactions summarized as follows:

(1) Release of dense granules adenosine diphosphate (ADP) from adherent platelets.

(2) Release of alpha granule constituents, including fibrinogen, vWF, factor V, platelet factor 4 (PF4), beta thromboglobulin (βTG), platelet-derived growth factor (PDGF).

(3) Activation of platelet membrane phospholipase complex, leading to generation of thromboxane A_2 (TXA_2) (Bloom and Thomas 1987).

Released ADP and TXA_2 act synergistically to recruit circulating platelets, causing them to change shape and attach to each other and also attach to adherent platelets in the process of enlarging the prothrombotic mass. Platelet aggregation requires the rapid mobilisation of a platelet-membrane fibrinogen receptor complex ($GPII_b/GPIII_a$) and calcium-dependent interplatelet bridging by fibrinogen (Bennett and Vilaire, 1979). The bridging reaction may also involve other platelet alpha granule proteins, vWF, fibronectin and thrombospondin.

Platelets contribute significantly to thrombin generation by providing membrane phospholipid and receptors for the assembly of prothrombinase complex. Platelets may promote the early stages of intrinsic coagulation by a process that

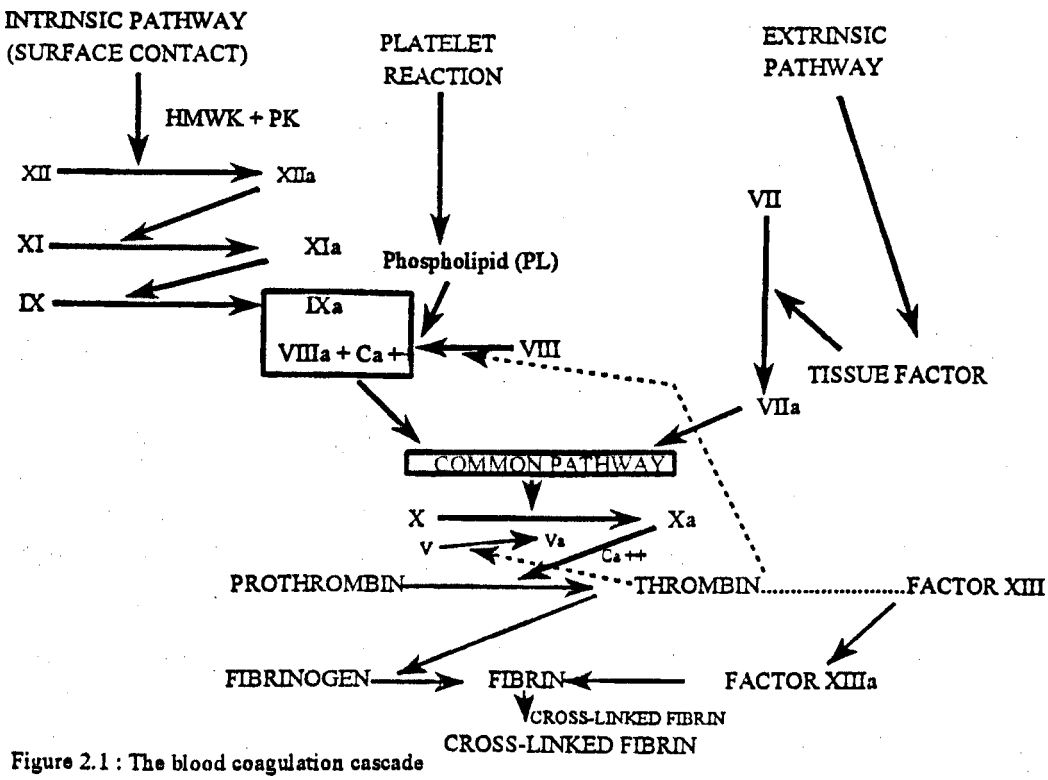


Figure 2.1 : The blood coagulation cascade

involves a factor XI receptor, activated factor XII, and high molecular weight kininogen (Walsh and Griffin, 1981). There is evidence that platelets may activate factor XI in the presence of kallikrein and HMWK, initiating the intrinsic pathway without the activation of factor XII (Walsh and Griffin 1981). Concentrations of thrombin are generated within seconds after blood contact with nonendothelial surfaces, sufficient enough to stimulate platelet alpha granule release, long before fibrin is formed (Kaplan et al 1981). Subsequently, thrombin-generated fibrin stabilises the growing platelet mass (Niewiarowski et al 1972).

2.3 BLOOD COAGULATION SYSTEM

The coagulation system is composed of at least ten plasma proteins and proceeds via cascade reactions by either the intrinsic or extrinsic pathway, and thrombin generation via the common pathway leading to fibrin deposition at the end stage (Matsuda 1989). The activation of the coagulation system on non-physiological surfaces is initiated by the intrinsic pathway. Coagulation factors circulate as inactive factors (table 2.1), some of which are zymogens and others are cofactors. Each zymogen is converted to an active form (the enzyme), which in turn activates the next coagulation factor in the sequence in a manner that can be viewed as a cascade or a waterfall (Fig 2.1).

2.3.1 INTRINSIC PATHWAY

The intrinsic pathway of blood coagulation is activated when human plasma is exposed to a variety of negatively charged materials, which include glass, kaolin (Margolis 1963), certain collagen preparations, dextran sulphate (Kluft 1978) or sulphatide vesicles (Fujikawa et al 1980) and endotoxin (Roeise et al 1988). The contact activation phase, which initiates the intrinsic coagulation mechanism, involves the interaction of factor XII (Hageman factor), prekallikrein, high molecular weight kininogen (HMWK) and factor XI, collectively known as the contact proteins (Griffin and Cochrane 1979).

The mechanism of the initiation of the contact system is still not clear, since neither the accelerating role of an anionic surface nor the initial activation of factor XII has been established (Kaplan and Silverberg, 1987). Reciprocal activation occurs between factor XII and prekallikrein when both molecules are surface-bound.

HMWK functions as a cofactor in the reciprocal activation by augmenting of prekallikrein and factor XI in a complex that circulates in plasma to negatively charged surfaces, where cleavage by activated factor XII (XIIa) follows .

Table 2.1 Blood coagulation factors and their role in coagulation (Szycher (1983)

Factor	Name	Role in Coagulation
I	Fibrinogen	Converted to fibrin monomer (Ia) and polymer (Ib)
II	Prothrombin	Converted to thrombin (IIa), which in turn converts fibrinogen to fibrin
III	Thromboplastin (Tissue factor)	Released upon tissue injury and activates FX with FVIIa
IV	Ca^{2+} ions	Cofactor for several proteolytic stages.
V	Proaccelerin	Cofactor for prothrombin activator
VII	Proconvertin	Cofactor for thromboplastin
VIII	Antihaemophilic factor	Converted to factor VIIIa by thrombin
IX	Christmas factor	Converted to factor IXa that activates factor VIIIa by complexing with FVIII
X	Stuart-Power factor	Converted to factor Xa which converts prothrombin to thrombin.
XI	Plasma thromboplastin antecedent	Converted to factor XIa which activates factor IX
XII	Hageman factor or glass factor	Activated by foreign surfaces. FXIIa activates FXI.
XIII	Fibrin-stabilising factor	Stabilises fibrin polymer by cross-linking
PF3	Platelet factor 3	Phospholipid; cofactor for several coagulation enzymes
PF4	Platelet factor 4	Platelet antiheparin activity factor

Activated factor XII converts, by limited proteolysis, prekallikrein to kallikrein and factor XI to its active form XIa, which continues the intrinsic coagulation cascade (Kaplan and silverberg 1987). The activation of factor XII

therefore, could initiate several positive feedback mechanisms.

The initiation of intrinsic blood coagulation then begins with the first calcium-dependent step, the activation of factor IX to IXa. Factor IX is cleaved by factor XIa at two sites to produce factor IXa (Davie et al 1979). Factor IXa, together calcium ions, phospholipid and the protein cofactor, factor VIII:C, converts factor X to its activated form, factor Xa. Factor VIII:C is an essential cofactor in intrinsic factor X activation and circulates as a complex with vWF (Zimmerman and Meyer 1981). Like factor V, factor VIII:C first has to be modified (VIII:C)m by a serine protease such as thrombin before it can react independently. These two cofactors bind independently to platelets without ionic bridges, to facilitate the reaction between enzyme and substrate (factor IXa and factor X; factor Xa and prothrombin) (Harker 1984).

2.3.2 EXTRINSIC PATHWAY

The extrinsic system is initiated by activation of factor VII, a vitamin k-dependent protein present in extremely low concentrations in plasma. When factor VII interacts with tissue factor, an intracellular microsomal lipoprotein, factor VIIa becomes a serine protease, which is the extrinsic factor X activator (Jackson and Nemerson, 1980). Tissue factor is present in large amounts in the brain, lung, and placenta. It is also secreted by stimulated leucocytes and has been found in the intima of large blood vessels. Lipid comprises one-third of the molecular weight of this factor and is essential for the activity, providing the surface for calcium-vitamin K-dependent factor binding (Harker 1984).

2.3.3 COMMON PATHWAY

The requirement for the efficient activation of prothrombin to yield the normal products of prothrombin activation are prothrombinase complex (the enzyme) and prothrombin (the substrate). Prothrombinase consists of factor Xa and factor Va bound to a cell membrane or phospholipid bilayer vesicle surface in a calcium-dependent

reaction (Nesheim et al 1979; Rosing et al 1980; Kane and Majerns 1982; Tracy et al 1983). The common pathway begins at factor X and is activated by either factor VIIa-tissue factor or factors IXa-VIIIm. Activation by either pathway generates factor Xa. After formation of factor Xa, the next step involves factor V, a cofactor, which (like factor VIII) has its activity manifest after modification by a proteolytic enzyme such as thrombin. Activated platelets, activated monocytes/macrophages, leucocytes and injured or "perturbed" endothelial cells provide surfaces suitable for the assembly of factor Xa and factor Va to effect the activation of prothrombin (Nesheim et al 1979; Rosing et al 1980).

Cleavage of prothrombin by factor Xa at Arg 271-Thr 272 liberates an approximately 34,000 molecular weight activation fragment F1+2 and the inactive intermediate prothrombin 2 (Pr_2) (Berrettini et al 1987; Aronson et al 1977; Rosing and Tans 1988). Active thrombin is liberated from prothrombin 2 (Pr_2) by a second factor Xa-mediated cleavage at Arg 49-Ile 50.

The higher plasma concentration of prothrombin and the biological amplification of the clotting system allows a few molecules of activated initiator or intermediate factors to generate a surge of thrombin activity. In addition, the generated thrombin modifies more factor V and VIII and induces platelet aggregation.

Thrombin cleaves fibrinogen, and removes about 3% of its molecular mass to release first two A and followed by two B peptides. The fibrin monomers formed are able to polymerise nonenzymatically, leaving the fluid phase to become a gel. An insoluble fibrin polymer is formed by interaction of the fibrin polymer with factor XIIIa. Factor XIII is either trapped in the plasma within the clot or released from platelets and is activated by thrombin to an active enzyme. In the presence of calcium, factor XIIIa catalyses peptide-like bond formation between adjacent fibrin monomer molecules (Doolittle 1981).

Thrombin is capable of converting fibrinogen to fibrin but is ultimately inactivated by antithrombin to form thrombin-antithrombin complex (TAT), which has a molecular weight of approximately 94,000 (Aronson et al 1977; Rosing and Tans 1988). Whereas F1+2 is a direct molecular marker of *in vivo* factor Xa activity and total thrombin generation, measurement of TAT provides a means of monitoring

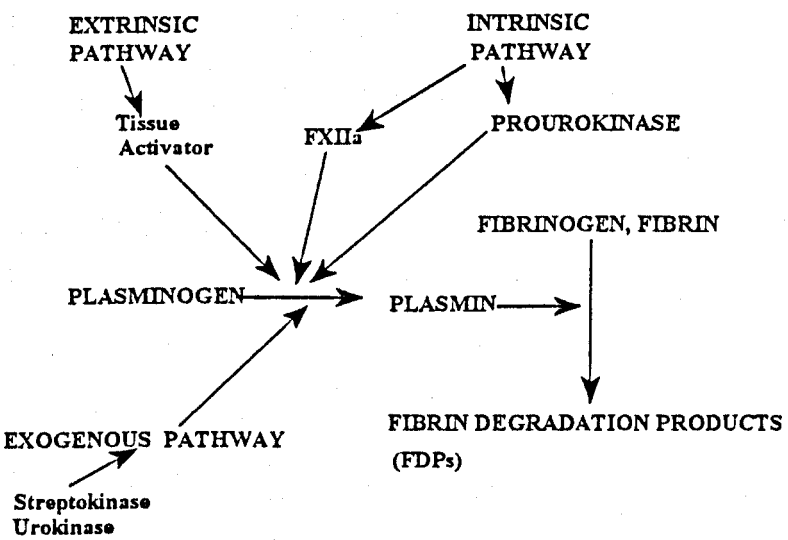


Figure 2. 2: Fibrinolytic pathways

the inhibition and utilization of thrombin.

2.4 FIBRINOLYTIC SYSTEM

The culmination of the coagulation sequence is the rapid transition of soluble fibrinogen into insoluble fibrin. The capacity of the fibrinolytic system to cause the dissolution of fibrin represents an essential defence or compensatory system reactive to uncontrolled activity of coagulation sequence and critical for the preservation of the cardiovascular system (Nemerson et al 1974). Multimolecular systems intrinsic to the blood provide enzymatic effectors for controlled catabolism of fibrin and other proteins (Robbin and Summaria 1971). Fibrinolysis acts as a major defence against unwanted fibrin deposition on the vessel walls and appears to be a significant first line defence against thrombosis. Fibrin clots, once formed, provide a haemostatic plug when injury occurs and act as a supporting medium during the subsequent repair process. Fibrin clots are only temporary structures in the body and are removed by the process of fibrinolysis (Figure 2.2). The components of the fibrinolytic systems present in plasma include: (a) fibrinolytic enzymes (proenzyme plasminogen and proteolytic agent plasmin) (b) activators of plasminogen (c) inhibitors of fibrinolytic enzyme activators (d) plasmin substrates (e) catabolic degradation fragments of fibrinogen and fibrin (f) humoral antibodies to degradation fragments of fibrinogen and fibrin.

The proteolytic enzyme plasmin and its proenzyme plasminogen are the components of the major human fibrinolytic system. Plasminogen is a β-globulin composed of a single polypeptide chain, with a molecular weight estimated to range from 87,000 to 89,000; present in normal plasma at a concentration range of 200 to 495 μg/ml (Zoltan et al 1972). The activation of plasminogen is the central process in fibrinolysis. it occurs by cleavage of the Arg^{560}-Val^{561} bond, to produce plasmin, the active two-chain molecule. This reaction is accomplished by two-well characterised human proteases, tissue plasminogen activator (t-PA) and urokinase (u-PA). There is also evidence of a third human plasminogen activation system arising from the contact activation pathway. The conversion of plasminogen to plasmin is characterized by cleavage of the single-chain zymogen, resulting in a two-chain proteolytic enzyme structure interconnected covalently by a single disulphide bond.

Plasmin is capable of hydrolysing arginyl and lysil bonds at neutral pH; this may explain the modest preference for fibrinogen and fibrin, although other plasma proteins such as factor V, VIII and XII are also subject to attack by plasmin (Szycher 1983).

Fibrinolysis occurs due to the action of the serine protease, plasmin on fibrinogen and fibrin, resulting in the formation of soluble fibrin degradation products (FDP). The conversion of the proenzyme plasminogen to plasmin is a process of limited proteolysis involving plasminogen (pro)activators, cofactors, and inhibitors present in circulating blood (Collen 1980).

Plasminogen activation proceeds by three different pathways (Fig 2.2). The intrinsic pathway in which all components involved are present in precursor form in the blood. Evidence has shown that a major endogenous plasma activator system involves factor XIIa (Szycher 1983). In this system, factor XIIa directly activates plasminogen proactivator (prourokinase) and also converts prekallikrein to kallikrein. The latter amplifies the generation of more factor XIIa; activated plasminogen proactivator then converts plasminogen to plasmin. The extrinsic pathway in which the activator originates from the tissues or from the vessel wall and is released into the blood by certain stimuli or trauma, and an exogenous pathway, in which the activating substances streptokinase or urokinase may be infused for therapeutic purposes (Harker 1984).

The extrinsic or tissue-type plasminogen activators (t-PA) are continuously released at low level into the circulating blood from the endothelial cells. Characteristically, such release is acutely responsive to both local and systemic stimuli. Fibrinolysis *in vivo* proceeds predominantly within the thrombus. During fibrin formation, plasminogen binds to fibrin and is thereby incorporated into the consolidating thrombus. The adjacent endothelium secretes tissue plasminogen activator, which is also bound and activated by fibrin. Fibrinolysis which proceeds primarily within the thrombus, does so under the influence of protein C.

Protein C with a molecular weight of 62,000 is the zymogen for a serine protease (activated protein C) that is activated by thrombin. Human endothelial cell surface (a thrombin receptor site) acts as a cofactor for this reaction. Activated

protein C generated by the action of thrombin and thrombomodulin on protein C, markedly increases the endothelial release of tissue plasminogen activator. Protein S and protein C are both vitamin K-dependent plasma glycoproteins that play a crucial role in the down-regulation of blood coagulation (Esmon 1992). Protein C is the zymogen of a serine protease activated protein C (APC), converted to its active form by the thrombin-thrombomodulin complex. Protein S is the cofactor of APC , and in plasma about 60% of protein S is complexed to the C4b-binding protein, which is a component of the complement system (Griffin et al 1992). Only the free circulating protein S acts as a cofactor of APC and APC exerts its anticoagulant function by degrading the procoagulant factors Va and VIIa (Suzuki et al 1983; Fulcher et al 1984).

Several studies have indicated that there is an association between a heterozygous deficiency in proteins C and S and an increased risk for thrombosis (Gladson et al 1988; Ben-Tal et al 1989; Pabinger et al 1992). Resistance to the anticoagulant effect of APC was first described as a cause of familial thrombophilia by Dahlback et al 1993. Bertina et al 1994 demonstrated that the phenotype of APC resistance was associated with the presence of a guanadine (G) to adenosine (A) substitution at nucleotide (nt) 1691 of the Factor V gene. This mutation results in the production of a factor V molecule (named FV leiden) with a glutamine substitution for arginine at amino acid residue number 506. The identical mutation was also reported by Greengard et al 1994, Vooberg et al 1994, Zoller et al 1994, Zoller and Dahlback 1994, in individuals with APC resistance phenotype.

The mutation results in the loss of an APC cleavage site at residue 506. Inactivation of membrane-bound FVa by APC occurs through a series of three sequential proteolytic events involving the FV heavy chain. APC cleavage at Arg 506 is required first for the exposure of APC cleavage site at Arg 306 and 679. With the loss of the APC cleavage site at residue 506 in FVa Leiden, the subsequent cleavage events at Arg 306 and Arg 676 that result in inactivation of FVa, occur inefficiently (Kalafatis et al 1995). It is this relative difference in inactivation of FVa Leiden which is the presumed aetiology of the prothrombotic state associated with this mutation (Kalafatis et al 1995; Holm et al 1994; Greengard et al 1994;

Vanderbroucke et al 1994). Coagulation-based assays for detection of APC resistance have been described and are commercially available (Rosen et al 1994). Recently, a rapid polymerase chain reaction based method which rapidly and accurately detects the G and A substitution site at nt 1691 of the FV gene without the necessity of a restriction enzyme digestion step has been reported (Kirschbaum 1995).

Direct activation of fibrin-bound plasminogen by tissue plasminogen activator leads to local release of plasmin (Collen 1980). Within the thrombus, plasmin digests fibrin to produce progressively smaller degradation products (FDP) and ultimately, thrombus dissolution. The intrinsic plasminogen activators can be generated by two pathways. One pathway involves plasma prourokinase, which can be activated independently of factor XII and prekallikrein, although activation may occur via contact activation. The other intrinsic pathway is the factor XII-dependent fibrinolytic pathway. Circulating plasminogen may also be converted to plasmin by components of the intrinsic coagulation (Kaplan 1978).

The regulation and control of fibrinolysis under physiological conditions appear to occur at several levels: release of plasminogen activator from the vascular wall, fibrin-associated activation of plasminogen and inhibition of formed plasmin by α_2-antiplasmin.

Several inhibitors of intrinsic plasminogen activation occur in human plasma: C1-inactivator (Kluft 1977), an inhibitor of factor XIIa-induced fibrinolysis (Hedner and Martinsson 1978); heparin-antithrombin III complex (Stead et al 1976) and α_2-macroglobulin (McConnel 1972). Inhibitors of extrinsic plasminogen activators form a complex with activators, which dissociates in the presence of fibrin (Gurewich et al 1975). The inhibitors of the extrinsic plasminogen activators have been categorised as: plasminogen activator 1 (PAI-1), plasminogen activator 2 (PAI-2), and plasminogen activator 3 (PAI-3). PAI-1 is a glycoprotein of apparent molecular mass 48,000, consisting of 379 amino acids, an avid inhibitor of both t-PA and u-PA (Booth 1994). From an early stage it was noted that the plasma concentration of PAI-1 was elevated in disease. This elevation was observed in several unrelated diseases (Juhan-Vague et al 1984, Colucci et al 1985, Kruithof et al 1988) and an association was also observed between high plasma PAI-1 and deep vein thrombosis or DVT

(Juhan-Vague et al 1984; Wiman et al 1985). PAI-1 was shown to be an acute-phase reactant (Juhan-Vague et al 1985), making it difficult to ascribe to it a causal role in disease, since it may merely reflect the disease process. Hamsten and coworkers (1985) studied young survivors of myocardial infarction. These subjects were found to have high plasma PAI activity compared with normals, even 3 years after the acute events. Subsequent studies showed the elevation in PAI-1 activity as to be a significant risk factor for recurrence of myocardial infarction (Hamsten et al 1987). More recent studies have found similar observations (Rocha and Paramo 1994, Van Meijer M and Pannekoek 1995; Juhan-Vague et al 1995; Lijnen and Collen 1995). PAI-1 rises dramatically in pregnancy, achieving concentrations of some 150 ng/ml during the 3rd trimester (Kruithof et al 1987). Pre-eclampsia, a disorder of pregnancy is associated with abnormally elevated plasma PAI-1 and it is thought to arise from the placenta (Estellès et al 1992).

PAI-2 is a protein of 46,600 molecular weight, on the basis of amino acid composition and like PAI.1 it inhibits t-PA and u-PA but less effectively (Kruithof et al 1995). PAI-2 is not normally found in plasma of non-pregnant individuals, but it does so in some pathological conditions, generally at low concentrations. PAI-1 has been shown to be the major plasma plasminogen activators, especially t-PA. Since plasma levels of PAI-1 rises also during pregnancy it is logical to assume that it plays the most significant role in systemic plasminogen activator inhibition, while PAI-2 is important in the uteroplacental circulation (Bonnar et al 1990). Markedly decreased PAI-2 levels are found in pre-eclampsia, compared with normal pregnancy, contrary to PAI-1 (Bonnar et al 1990). The low PAI-2 reflects placental insufficiency in this disorder (Reith et al 1993). PAI-3 is a serpin that inhibits u-PA in urine and has been found in plasma albeit in very low concentrations (2 μg/ml). Its role as an effective u-PA inhibitor in plasma has yet to be established in view of its low concentrations.

Inhibitors of the exogenous plasminogen activators, such as streptokinase and urokinase, have not been clearly described. Human plasma contains antibodies directed against streptokinase, which most probably result from previous infections with β-haemolytic streptococci. The requirements for streptokinase to neutralize the circulating antibodies was found to be 352,000 units in 95 per cent of a healthy

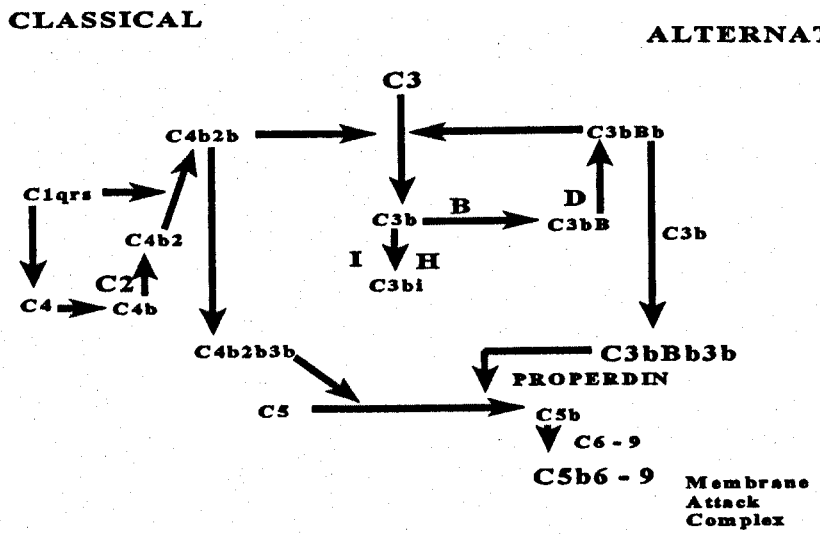


Figure 2.3 : Complement cascade.

population, but individual requirements ranged between 25,000 and 3,000,000 units (Verstraete et al 1966). The mechanism of urokinase inhibition in blood is poorly understood. It has however been suggested that since the half-life of urokinase *in vivo* is 9 to 16 min, but *in vitro* is 27 to 61 min, then clearing of enzyme from the blood may play an important role (Fletcher et al 1965).

Inhibitors of plasmin include α_2-antiplasmin and α_2-macroglobulin, with the latter representing the slower reacting plasmin inhibitor of plasma; and its role appears to be to inactivate plasmin formed in excess of the inhibitory capacity of α_2-antiplasmin (Collen 1976; Mullertz & Clemmensen 1976).

2.5 THE COMPLEMENT SYSTEM

2.5.1 INTRODUCTION

The term complement was originally applied to describe the activity in serum, which when combined with a specific antibody, would cause lysis of bacteria. The human complement system comprises of a group of more than 20 separate components and regulatory proteins and cellular receptors that evolved to protect the host from invasion by foreign materials (Atkinson and Frank 1980; Roitt 1977; Craddock 1986). Activation of the complement system involves a combination of limited but specific proteolysis of some components and induced conformational change in others. The proteolytic enzymes involved in the system include C1r, C1s, C2, factors B and D. The non-enzymatic components include C1q, the recognition protein of the classical pathway, C3, C4, the modulator of C2, as well as C5, C6, C7, C8, and C9, the precursor of the membrane attack complex (Roitt et al 1993).

2.5.2 BIOCHEMISTRY OF COMPLEMENT CASCADE

The complement system can be activated by two distinct routes, the classical and alternative pathways (Fig 2.3). The component C3 is a major plasma glycoprotein and it plays a central role in the system, being common to both pathways. C3 along with the other 12 plasma glycoproteins constitute the 13 components of the pathways. Component C5-C9 is designated the terminal components, which form the membrane-attack complex (MAC), which is common to both pathways and is responsible for target cell damage and lysis.

Major biologically important functions mediated by the complement system

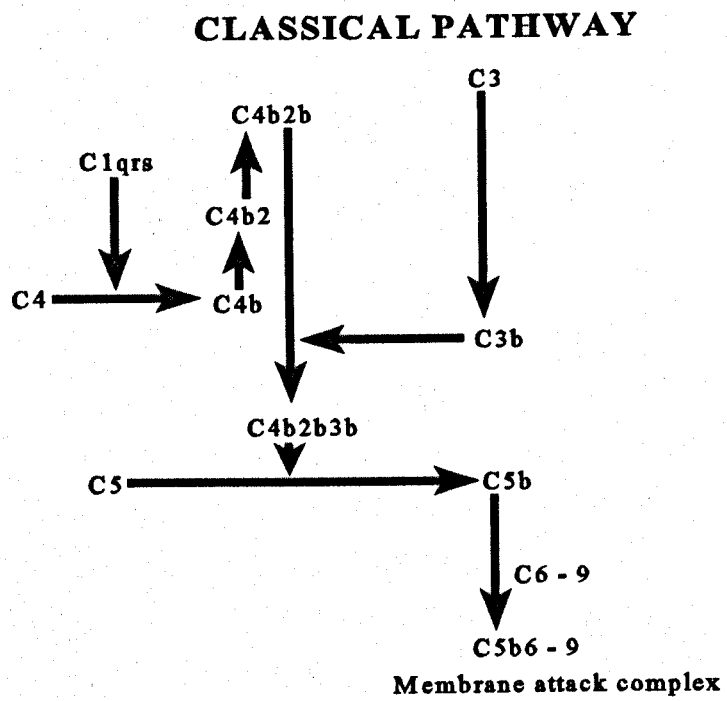


Figure 2.4 : Classical pathway of complement cascade

include (i) the release of low molecular weight fragments such as the anaphylatoxins C3a, C4a, and C5a, which promote smooth muscle contraction and increase vascular permeability (ii) the large C4b and C3b fragments, which are involved in binding to the complement activator and can thereafter interact with specific receptors to allow efficient clearance of the activating cell or particle; and (iii) degradation fragments of C3b which are also important in receptor binding and clearance mechanisms. Other functions include neutralisation of viruses and a possible role in the immune response (Scott and Dawson 1985; Kazatchkine and Carreno 1988).

Control of the activated components is mediated partly via the seven control proteins present in plasma and partly by a variety of membrane-bound control proteins and receptors. These membrane proteins bind activated components or fragments of activated components generated by further limited proteolysis (Law and Levine, 1977; Roitt et al 1993).

Classical Pathway

The classical pathway of complement is the main antibody-directed mechanism for the triggering of complement activation (Figure 2.4). It is initiated by the binding of two or more of the six globular domains of C1q, a subcomponent of C1, to its ligands. C1q binds with high avidity to the C_H2 domains of aggregated IgG molecules, as contained in an immune complex, or to the C_H3 domains of a single IgM molecule whose conformation has been modified from a "planar" to a "staple" configuration by binding to antigen. (Roitt et al 1993). C1q is also able to bind directly to certain microorganisms including some retroviruses (though not HIV) and to some mycoplasmas. Activation of C1 can also be achieved by its direct interaction with a variety of polyanions (such as bacterial lipopolysaccharides, DNA and RNA), certain small polysaccharides or viral membranes.

The pathway involves components C1, C4 and C2. The first component of the complement system C1 consists of three separate proteins, C1q, C1r, and C1s, held together as a trimolecular complex by calcium ion. Binding the C1q subunit to at least two adjacent Fc portions of immunoglobulin molecules activates C1q. By an autocatalytic reaction, C1r is activated and this in turn activates C1s. Activated C1s and C1q have both esterolytic and proteolytic activity. The C4 molecule is then split

ALTERNATIVE PATHWAY

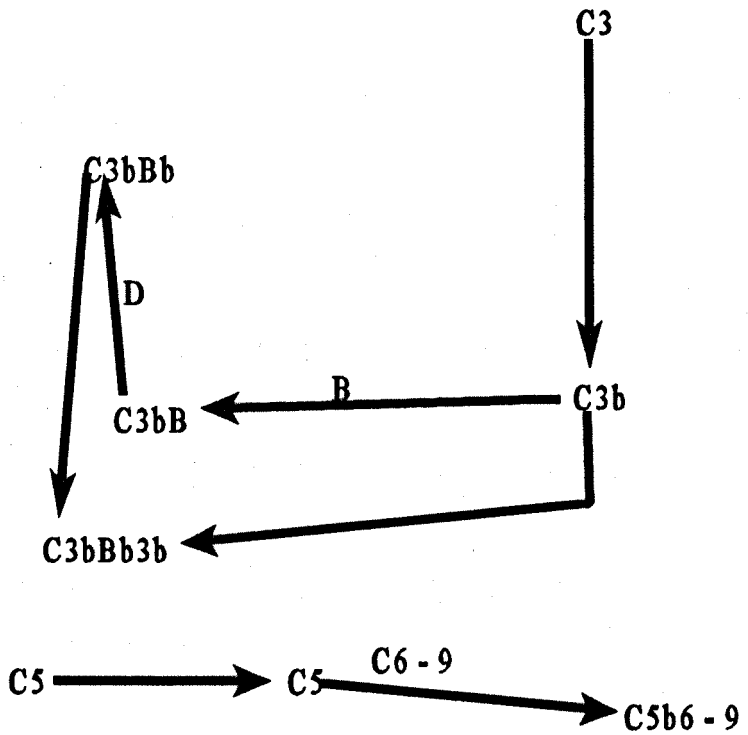


Fig. 2.5: The alternative pathway of complement cascade

to yield the biologically important C4a anaphylatoxin and the larger C4b fragment. Surface-bound C4b now acts as a binding site for zymogen C2, which combined with C4b, becomes a substrate for C1s and is cleaved to C2a and C2b. C2 is cleaved in the presence of magnesium to yield C2a and C2b fragments. The complex formed (C4bC2b) by C4b and C2b is the classical pathway C3 convertase enzyme and activates C3 by the proteolytic cleavage of C3 into C3a and C3b. C3b binds to the surface membrane next to the C4bC2b complex to form the C5 convertase, which cleaves C5 into C5a and C5b.

Alternative pathway activation.

There are close structural and functional homologies between the proteins participating in the activation of the classical and alternative pathways (Roitt et al 1993). Native C3 in plasma undergoes continuous low grade hydrolysis of the internal thioester bond and the product, C3i, acts as a binding site for Factor B (FB), analogous to the binding of C2 to C4b (Figure 2.5). FB, bound to C3i is cleaved by factor D to Ba and Bb. Fluid phase C3iBb is a C3 convertase enzyme that cleaves further C3 to C3b* (unstable intermediate), some of which covalently binds to adjacent surfaces. This surface-bound C3b can then act as a binding site for more FB and initiates the amplification loop described below. This system of activation results in the indiscriminate binding of C3 to any adjacent surfaces; however, there are molecules on autologous cell surfaces that prevent the formation of stable C3 convertase enzymes.

Amplification loop

On surfaces that are good complement activators of complement, initial binding of a few molecules of C3b by one of the two mechanisms, is followed by an amplification step which results in the binding of many more molecules of C3b to the same surface (Roitt et al 1993). Factor B, structurally and functionally similar to C2, binds to C3b and in this form is a substrate for the serine esterase, Factor D, which usually circulates at very low concentrations of its active form. The cleavage of factor B results in the release of a small fragment Ba and the formation of the C3 convertase enzyme, C3bBb, which cleaves many more C3 molecules, some of which bind covalently to the activating surface. The C3bBb enzyme dissociates fairly rapidly

unless it is stabilized by the binding of "Properdin" (P), forming the complex, C3bBbP. This amplification mechanism for the cleavage of C3 is a positive feedback system, which will cycle until all the C3 is cleaved unless it is regulated adequately.

Membrane attack complex (MAC)

The initial phase in the formation of the MAC is the enzymatic cleavage of C5, a protein homologous to C3 and C4 but lacking the internal thioester bond. C5 requires to be bound to C3b in order to be susceptible to cleavage by the C5 convertase enzymes. The classical pathway C5 convertase enzyme is a trimolecular complex composed of C4b2b3b in which C3b is covalently bound to the C4b. Probably the alternative pathway C5 convertase enzyme is by analogy a trimolecular C3bBb3b trimer containing a covalent dimer of C3b. The subsequent formation of the MAC is non-enzymatic and follows the successive binding of C6 , C7 and C5b to form a C5b6-7 complex. C8 and C9 are then bound to the complex to form C5b-9, the terminal complement or membrane attack complex (TCC or MAC). This complex is a large lytic complex which forms a channel through the phospholipid layer of the cell membrane resulting in cell lysis (Cooper 1985; Roitt 1993; Johnson 1994).

2.5.3 COMPLEMENT ACTIVATION AND CELLULAR ACTIVITY

The interaction of activation fragments of complement proteins with specific cell surface receptors is an important mechanism for the mediation of the physiological effects of complement. The main consequence of ligation of these receptors are the uptake of particles opsonized by the complement, and activation of the cell bearing the occupied receptors. Four receptors for the major split products of C3 (C3b, iC3b and C3dg) are as shown in table 2.2.

Activation of the complement system is a potent mechanism for initiating and amplifying inflammation. Activation products of complement proteins stimulate chemotaxis and activation of leucocytes as summarised in table 2.3. Complement enhances the localisation of antigen to both antigen-presenting cells and to B lymphocytes and the localization of antigen-antibody complexes which efficiently elicit immune responses to the germinal centres of lymph nodes has been shown to be a complement-dependent process (Roitt et al 1993).

Table 2.2: Complement receptors for opsonic fragments of C3 (Roitt et al 1993)

Receptor	Ligands	Cellular distribution
CR1	C3b > iC3b C4b	B cells, neutrophils, monocytes, macrophages,erythrocytes, follicular dendritic cells, glomerular epithelial cells.
CR2	iCb, C3dg Epstein-Barr virus	B cells, follicular dendritic cells, epithelial cells of cervix and nasopharynx.
CR3	iC3b Zymosan certain bacteria	monocytes, macrophages, neutrophils, NK cells, follicular dendritic cells.
p150,95 (CR4)	iC3b	neutrophils, monocytes, tissue macrophages.

Table 2.3: The biological effects of activation products of complement (Roitt et al 1993).

Complement activation product	Biological effect
C3a, C5a, and C5a des Arg	Stimulate chemotaxis of neutrophils and degranulation of basophils and mast cells; net effect is a histamine and leukotriene-mediated contraction of vascular smooth muscles, increased vascular permeability and emigration of neutrophil, monocytes from blood vessels. Loss of the C-terminal arginine residue from C5a, following cleavage by carboxypeptidase B, produces C5a des Arg which possesses weak cell-activating properties.
C3b, C4b	1. Facilitate binding of bacteria, viruses and immune complexes to neutrophils, monocytes and macrophages. 2. Mediate endocytosis, phagocytosis and generation of respiratory burst by CR receptor-mediated activation 3. Augment IgG-induced phagocytosis, IgG-mediated cytotoxicity (ADCC) and NK-mediated cytotoxicity. 4. Focus immune complexes on antigen-presenting cells. 5. Disrupt lattices of immune complexes to increase their solubility.

2.5.4 THE REGULATION OF COMPLEMENT ACTIVATION.

2.5.4.1 Regulation of classical pathway of activation.

Classical pathway activation is regulated very efficiently in fluid phase by two

mechanisms: by the serine protease inhibitor (serpin), C1 inhibitor, which binds and inactivates C1r and C1s, and by inhibition of the formation of the classical pathway C3 convertase enzyme, C4b2b.

The formation of C4b2b is inefficient in the fluid phase due to the presence of plasma proteins which catabolize C4b (Factor I and C4 binding protein), and promote the dissociation of C2b from C4b2b (C4 binding protein). There also important molecules on autologous cell surfaces that regulate classical pathway activation: decay accelerating factor (DAF), CR1, and probably also a recently described protein, membrane cofactor protein (MCP). These molecules between them inhibit the binding of C2 to C4b and of factor B to C3b and promote the catabolism of C4b and C3b by factor I (Roitt et al 1993).

2.5.4.2 Regulation of the alternative pathway and the amplification loop

Fluid phase activation of the alternative pathway is an inefficient process and is regulated by proteins similar or identical to those which inhibit classical pathway activation. Factor H (FH), homologous with and closely linked to; C4 binding protein, promotes the dissociation of Bb from C3i and C3b, and also functions as a cofactor to factor I (FI) for the catabolism of C3i and C3b. Regulation of the fate of the surface-bound C3b is the critical step enabling the non-specific distinction between non-self by the complement system. The two possible outcomes for bound C3b are:

1. C3b acts as a binding site for Factor B, forms a convertase enzyme, and focuses the deposition of more C3b to the same surface i.e amplification.
2. C3b is catabolized by Factor I using one of three cofactors Factor H, CR1 or MCP i.e inhibition.

The nature of the surface to which the C3b is bound regulates which of these two outcomes is most likely. Self surfaces, particularly the cell membranes, contain intrinsic molecules such as CR1 and/or MCP that bind to C3b, and also promote the binding of factor H rather than Factor B to C3b. This limits the formation of C3 convertase enzymes on autologous cell membranes. Non-self surfaces, for example bacterial membranes, acts as a protected site for C3b since they do not contain intrinsic regulatory proteins and more importantly, Factor B has a higher affinity for

C3b than Factor H at these sites. Therefore the deposition of a few molecules of C3b on to a non-self surface is followed by the formation of relatively stable C3bBbP C3 convertase enzymes which focus more C3 deposition in the near vicinity. Although the precise structural requirements for a "protected surface" are not understood, the carbohydrate composition seems to be important; membrane sialic acid seems to be one of the components protecting autologous cell membranes from amplified C3b deposition.

2.6 ERYTHROCYTES.

Blood is composed of a liquid medium, plasma, and cellular elements, which constitutes approximately 46 percent of the volume. Cellular elements are subdivided into erythrocytes, leucocytes, and platelets. Generally, erythrocytes do not form part of the coagulation process per se, although they are generally trapped within the sticky coagulum, giving rise to the familiar dark colouration associated with a blood clot (Bruck 1980; Szycher 1983). Red cells may promote platelet adhesion either by reducing the adsorption of platelet-protective proteins or by depositing an adhesive substance (Brash 1983) and if haemolysis occurs, the platelet release reaction is induced.

2.7 LEUCOCYTES

Leucocytes become attracted to the thrombus, with preferential adsorption of polymorphonuclear leucocytes or granulocytes in comparison to lymphocytes. On adsorption to the thrombus, leucocytes contribute to platelet recruitment, fibrin formation and participate in fibrinolysis. Evidence of a direct role for leucocytes in thrombus formation, due to granulocyte adhesion and its influence on platelet aggregation, is supported by granulocyte possession of endogenous proaggregating activity (Courtney et al 1993a). Pronounced infiltration by leucocytes has also been observed inside haemostatic plugs and may contribute to an additional supportive role (Brozovic 1981).

2.8 RELATIONSHIP BETWEEN COMPLEMENT, COAGULATION, KALLIKREIN-KININ AND FIBRINOLYTIC SYSTEMS.

A number of interactions have been demonstrated between the complement, the coagulation and the fibrinolytic systems with common components and inhibitors observed in *in vitro* systems.

Factor XII has been shown to play a significant role in the activation of the coagulation cascade (Rapaport and Owren 1955; Gjonnaess 1972; Tans and Rosing 1983), the kallikrein-kinin system (Cochrane et al 1973), the intrinsic pathway of plasminogen activation (Goldsmith et al 1978; Kluft 1987), and the complement system (Ghebrehiwet et al 1981). Factor XII can activate factor VII, which in combination with thromboplastin and calcium, participates in the generation of thrombin through the extrinsic pathway. Factor XII activates factor XI, which in turn in the presence of platelet factor 3 and calcium, leads to thrombin generation through the intrinsic pathway.

Factor XII activates plasminogen proactivator to plasminogen activator, which converts plasminogen into plasmin and thus initiates fibrinolysis (Goldsmith et al 1978). Factor XII can also activate the C1 complex of the complement system (Ghebrehiwet et al 1981) and therefore initiates a major body defence system. Additionally, plasmin can activate the complement system through the alternative pathway (Bennet et al 1987; Fuhrer et al 1990).

The kinins are polypeptides with a variety of pharmacological actions including the induction of increased vascular permeability, contraction of smooth muscle cells of the uterus and ileum, dilatation of small blood vessels, and pain production; are released from their precursors, the kininogens, by the action of kallikrein (Bennett and Ogston 1981). The preferred substrate for plasma kallikrein is high molecular weight kininogen (HMWK) and the kinin released is the nonapeptide bradykinin (Wachtfogel et al 1993). Glandular kallikrein preferentially cleaves LMW kininogen to release the decapeptide kallidin (lysyl-bradykinin). Plasma kallikrein itself exists in an inactive precursor form (prekallikrein) which can be converted to kallikrein by activated factor XII or factor XII fragments.

These systems are not only linked through factor XII activity, but also through feedback mechanisms and at the inhibitor level. There is therefore a greater potential that the control of physiological processes may largely depend on the extensive

interaction between the haemostatic systems.

2.9 REGULATION OF HAEMOSTASIS

The arrest of bleeding involves a delicate and coordinated interaction between the cellular and humoral elements of the blood on one hand and components of the blood vessels on the other (Bloom 1981). There exists also protective mechanisms that limits the formation thrombosis once coagulation is initiated. At least three types of mechanisms, which contribute and control the rates of coagulation have been considered (Sundaram 1992).

Firstly, the increased blood flow, which has a direct consequence of limiting the chances of localised concentration of precursors and also the removal of activated products. The rapid disappearance of activated products or inhibited clotting factors is facilitated during the flow of blood through the liver.

Secondly, generation of proteolytic enzymes not only activate clotting factors, but also degrade cofactors. Plasmin, for example, degrades fibrinogen and fibrin monomers, and can rapidly inactivate cofactors V and VIII by relatively specific cleavages. Protein C, a substrate for thrombin activation in the presence of an endothelial cofactor, thrombomodulin; in the active form rapidly destroys factor V and probably factor VIII:C (Esmon and Owen 1981; Esmon 1992).

Thirdly, the existence of naturally occurring and circulating protease inhibitors in plasma provides another regulatory facet. Five protease inhibitors are involved in the regulation of haemostasis (table 2.4).

Table 2.4: Plasma protease inhibitors

Protein	Molecular weight	Plasma Conc. (mg/dl)	Mechanism of inhibition
α_2-Macroglobulin	725,000	210	Non-active site binding of virtually any proteolytic enzyme
α_1-Antitrypsin	54,000	200	More specific active site, stoichiometric complex with protease (s).
Antithrombin III	62,000	29	
C1-esterase inhibitor	105,000	18	
α_2-Antiplasmin	63,000	6	

α_2-macroglobulin competes with macromolecular substrates such as serine, thiol, carboxy or metallo-proteases and is rapidly cleared from plasma. α_1-antitrypsin appears to inhibit neutral proteases from inflammatory reactions such as neutrophil elastase or tissue enzymes, but has a low affinity for coagulation factors. Antithrombin III inactivates all serine haemostatic proteases by forming stable high molecular weight complexes. Heparin and contact with the endothelium markedly accelerate this reaction, amounting to an immediate, potent, anticoagulant effect. C1 esterase inhibitor is a potent inhibitor of plasma kallikrein (particularly when bound to HMWK) and activated factor XII.

α_2-antiplasmin is the most avid inhibitor of plasmin and in purified systems can inactivate most of the contact proteases when they are no longer surface-bound (Biggs and Denson 1976).

2.10 BLOOD RESPONSE TO ARTIFICIAL SURFACES

2.10.1 INTRODUCTION.

The interaction of blood with biomaterials leads to the activation of the body defence mechanisms, with a strong contrast to the natural behaviour of the endothelial surface (Matsuda 1989; Klinkmann 1989). There is a general observation that platelets and leucocytes under normal conditions do not adhere to the endothelial

cells.

This suggests that the endothelium is able to present a non-attractive surface to blood cells (von Appen et al 1993; Sundaram 1992). Endothelial cells prevent adhesion of cells and activation of clotting factors and ensures the removal of thrombus already formed and thereby, providing the most compatible surface for blood (Mason et al 1979).

It is believed that the nature of the biomaterial surface properties, such as surface charge, surface free energy, chemical composition and surface topography, has an influencing role on the blood constituents. Investigations on the changes on blood constituents during blood-biomaterial interactions can provide knowledge that attempts to correlate blood response to the properties of the biomaterial surface (Yu 1993). Such information could be a useful guide towards producing surfaces with improved blood compatibility (Matsuda 1989; Courtney et al 1993a).

Normal vascular endothelium is the ideal nonthrombogenic surface, which is generally considered to be resistant to platelet adhesion and aggregation (Szycher 1983). Foreign surfaces, on the other hand, are devoid of the endothelium, and therefore are thrombogenic due to platelet adhesion/ agglomeration and the formation of a fibrin meshwork. Therefore, a major concern in the application of extracorporeal devices necessitating blood-biomaterial interactions is the inducement of mural thrombosis and coagulation. The inability of an artificial surface to regulate actively the haemostatic and thrombotic events as well as the endothelium has necessitated the use of anticoagulants / antithrombotic agents.

It has been established that among the initial events that occur when synthetic materials contact blood is the rapid adsorption of plasma proteins (Szycher 1983; Brash 1980). This process influences subsequent interactions of blood cells, especially platelets and leucocytes with the proteinated surfaces (Szycher 1983). Adsorption of plasma proteins can lead to the activation of the intrinsic blood coagulation cascade, leading to the polymerization and cross-linking of fibrin on the blood interface.

The sequence of events (Courtney et al 1993a; Szycher 1983) appears to be as follows: adsorption of plasma proteins; adhesion and activation of platelets; activation of intrinsic blood coagulation; adhesion of leucocytes, followed by the

release reaction; recruitment of nearby platelet by released products including ADP and thromboxane A_2; aggregation of recruited platelets upon the layer of adherent platelets with the eventual formation of a mural thrombus. This mural thrombus is subsequently fortified by the formation of fibrin meshwork. The adherent leucocytes actively contribute towards the activation of the coagulation sequence by releasing procoagulant factors and also removal by phagocytosis of fibrin and altered platelets.

The formation of layers of adsorbed protein and adherent platelets and leucocytes upon artificial surfaces exposed to blood depends upon a number of factors. Among these are the nature of the surface of the biomaterial, the specific types of plasma proteins adsorbed and the effect of this on activation of blood coagulation and complement and fibrinolytic systems, and the type of anticoagulant that may be present in the blood.

The complexity of the nature of haemostasis and thrombosis has been discussed as involving the interactions of platelets, coagulation, fibrinolysis, complement and cellular activation under the regulation of the endothelium. A similar analogy can be adapted when discussing events following blood contact with an artificial surface (referred to as blood response), although a distinction should be drawn regarding the following aspects (Forbes and Prentice 1978):

1. An active role through the synthesis and release of specific factors such as that carried out by the endothelium is absent on artificial surfaces.
2. Artificial surfaces attract blood components in contrast to the endothelium.

Consequently, a complexity of reactions takes place, and for a clearer understanding, it is necessary to study parameters on an individual basis.

2.10.2 PROTEIN ADSORPTION

It has been demonstrated that protein adsorption is the first event occurring at the interface after blood contact with artificial surfaces (Baier and Dutton 1969; Brash and Lyman 1969; Gendreau et al 1980; Vroman et al 1977; Brash 1983; Brash 1991; Brash and ten Hove 1993; Wälivaara et al 1994). The arrival of the cellular elements

is anticipated to be later because of their larger size and therefore small diffusion coefficients (Brash 1983). It has also been suggested that proteins adsorb to artificial surfaces because they are large and chemically heterogenous (amphipathic nature): they contain hydrophillic and hydrophobic regions, electrically charged regions and polar/non-polar regions; the properties that limit solubility (Brash 1983). The adsorption process is associated with thermodynamic driving forces, which cause protein molecules to condense onto the surface (Brash 1983). The extent by which this occurs depends upon the surface activity and the relative abundance of the protein, and the chemical and physical nature of the surface. The complex structure of protein molecules gives rise to a number of interactions between the molecule and the surface, include hydrophobic forces, polar forces and hydrogen bonding. The strength of this interaction determines how strongly the protein is bound to the surface, and whether or not the protein is denatured (Brash 1991; Brash & ten Hove 1993).

Following blood-material contact, a so called "conditioning" layer of plasma protein is formed, this protein layer has been shown not to be passive and adsorption is not static but exists as an equilibrium with a continuous exchange between the adsorbed proteins and other species in molecule solution (Baier 1977). It has been observed in studies that the deposition of proteins occurs within a few seconds as shown in blood experiments (Ihlenfeld & Cooper 1979; Gendreau & Jakobsen 1979), in plasma (Vroman & Adams 1969; Uniyal & Brash 1982), and protein solutions (Kim & Lee 1975; Yu 1993; Mahiout 1993; Brash & ten Hove 1993; Lamba 1994). In general, protein adsorption has been observed to be greater with hydrophobic than with hydrophillic surfaces (Hoffman 1974; Chuang 1978; Ratner 1981; Brash & ten Hove 1993) and that adsorption is more readily and rapidly reversible on hydrophillic surfaces (Brash 1991).

Some *in vitro* investigations using mixtures of albumin and fibrinogen, showed, using a variety of surfaces, that fibrinogen is preferentially adsorbed, and that the degree of fibrinogen preference correlates with platelet reactivity (Brash and Davidson 1976; Brash and Uniyal 1979). Fibrinogen has been shown to be more surface active than albumin and IgG (Brash et al 1984).

The deposition of fibrinogen on artificial surfaces has been reported to be associated with specific receptors on adherent platelets and dependent on platelet deposition (Young et al 1983). It is thought that the saccharide chains present on fibrinogen and gamma globulin can interact with receptors on the platelet surface. The saccharide chains are not a feature of albumin molecules (Forbes & Courtney 1994). In *in vitro* systems, human platelets adhere preferentially where fibrinogen is adsorbed, for example on many hydrophobic solids (Zucker and Vroman 1969; Mason et al 1973). Platelets suspended in fibrinogen-free medium will adhere to glass that had been exposed to intact plasma for no more than a few seconds but will not adhere to where plasma had resided on the glass for several minutes (Helmus et al 1981; Zucker and Vroman 1969).

The significance of fibrinogen adsorption during blood-biomaterial interactions can be summarized as follows:

1. The observations of a rapid replacement of adsorbed fibrinogen by high molecular weight kininogen (HMWK), in a phenomenon termed the "Vroman effect" (Vroman et al 1980). The significance of the Vroman effect on thrombogenicity can so far only be speculated. However, it may be assumed that a minimal Vroman effect may be desirable if the main requirement for the surface is a reduced activation of coagulation properties. On the other hand, a vigorous Vroman effect is desirable for a minimal platelet reactivity property with a possible reduction in thrombogenicity (Brash 1991).
2. There is a possibility that fibrinogen interacts with leucocytes, resulting in the protein being replaced from the surface by HMWK, thereby playing a significant role in thrombus formation the artificial surfaces (Szycher 1983).

It has also been demonstrated in *in vitro* systems that the composition of the conditioning layer appears to greatly influence platelet adherence and release (Packham et al 1969). Similarly, it has been shown that there was a platelet reaction inhibitory effect by albumin and an enhancing effect by γ-globulin and fibrinogen (Packham et al 1969; Whicher and Brash 1978; Neumann et al 1979; Adams and Feuerstein 1980).

Platelet adhesion has also been shown to take place via reaction between platelet enzymes (e.g glycosyl transferase) and saccharide residues on adsorbed

protein and that such a reaction does not occur with albumin, since albumin contains no sugar residues (Lee and Kim 1979). Other plasma coagulation factors such as factor XII, HMWK, prekallikrein and factor XI have also been investigated and found to replace surface adsorbed fibrinogen (Vroman et al 1980; Ratnoff and saito 1977; Cochrane and Griffin 1979). The Vroman effect appears to be related to surface activation of clotting factors as follows:

1. Plasma deposits fibrinogen on material surfaces
2. Factor XII may displace some of the fibrinogen, but most is replaced by HMWK
3. Platelets adhere only where fibrinogen remains on the surface.(Zucker and Vroman 1969).

Some proteins are thought to be involved in cell adhesion processes and these include von Willebrand Factor, fibronectin and thrombospondin (Grinnel and Feld 1981; Mosher 1981; Ihlenfeld et al 1978; Vroman 1983). Fibronectin has been suggested as a possible "adhesive" for platelet sticking (Ihlenfeld et al 1978; Vroman 1983). Some findings have indicated that fibronectin is adsorbed on the surfaces in two different conformations with the more biologically active and antigenic conformation on the wettable surfaces (Stoker 1981). It has also been speculated that despite the plasma concentration of fibronectin (30 μg/ml), a substantial amount has been found localized in the clot in association with individual strands of fibrin (Grinnell 1983). In biochemical studies, it has been shown that fibronectin can bind both covalently to fibrinogen and fibrin, in a reaction mediated by plasma transglutaminase (factor XIII) (Mosher 1980; Mosesson and Amrani 1980).

The effects of protein adsorption in determining thrombotic events subsequent to initial platelet adhesion have not been extensively studied. Thrombus formation was measured *in vivo* on a polyethylene bifurcation inserted into an arteriovenous shunt in the rabbit (Evans and Mustard 1984). The mass of thrombus formed on albumin-coated bifurcations was less than that formed with γ-globulin-coated bifurcations. The effect of protein preadsorption on the rate of thromboembolisation in baboon blood measured with a laser light scattering device was reported to range from passivation (haemoglobin, IgG) to activation (fibrinogen, plasma) (Horbett et al 1984).

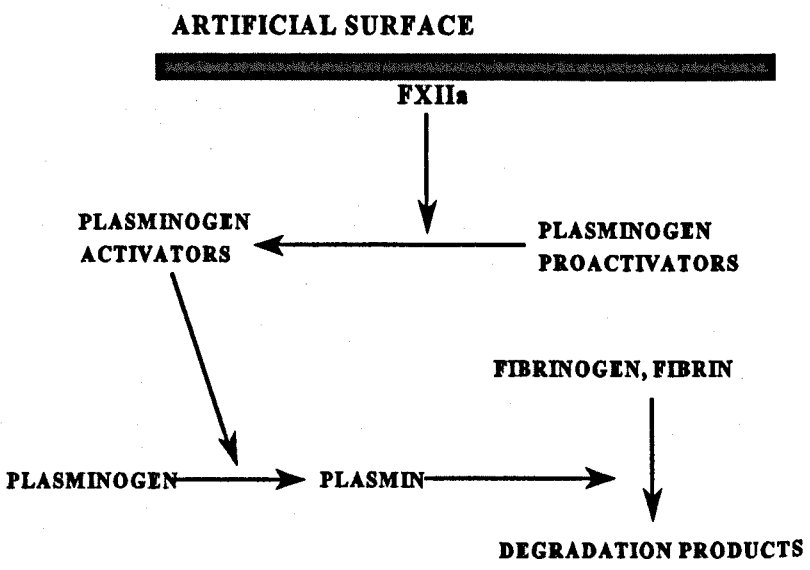


Figure 2.6 : FXIIa dependent Fibrinolytic pathway

In an *in vitro* study of activation of monocytes/macrophages by biomedical polymers coated with human blood, the resultant data indicated that surface adsorbed proteins were not a sufficient stimulant for the activation of monocytes to produce interleukin-1 in the absence of prior stimulation with lipopolysaccharide (Bonfield et al 1989).

Some findings have highlighted a preferential adsorption of haemoglobin from plasma onto polyethylene and the results indicated that substantial adsorption of this protein might occur in blood (Horbett et al 1977). Although haemoglobin is present in plasma only in trace amounts (3 μg/ml), with respect to whole blood in the presence of a foreign surface, haemolysis may increase this value.

The presence of red cells appear to reduce the overall amounts of proteins adsorbed onto a surface possibly partly due to competitive adsorption of the released haemoglobin (Uniyal et al 1981; Brash 1980). Attention has also been directed towards the possibility of protein denaturation and emboli formation (Lee et al 1961; de Leval et al 1981). In the clinical application of membrane oxygenators, protein denaturation was found to be reduced, but there was a tendency to form microaggregates, with the nature of the membrane influencing the pattern of protein adsorption (Courtney et al 1994).

In haemodialysis, interest in protein adsorption has been directed towards the influence of membranes. Studies have demonstrated that adsorption of protein from plasma takes place with different types of membrane (cellulose, modified cellulose and synthetic membranes), although the patterns of adsorption differ significantly (Kuwahara et al 1989; Panichi et al 1989; Mclaughlin et al 1989). The reactivity of proteins in the clinical situation appear to be remarkably different from *in vitro* tests. A rapid decrease in fibrinogen levels *in vitro* contrasts with *in vivo* haemodialysis where consistent fibrinogen levels represents the outcome of fibrinogen conversion into degradation products and the production of new fibrinogen (Courtney et al 1991).

Selective protein adsorption, minimization or prevention of adsorption, and the control of surface orientation of adsorbed proteins appear to have particular relevance to biomaterial compatibility. The first two have attracted the most attention of recent times. Protein chromatography, such as affinity chromatography, and

immuno adsorption, provide some of the best examples of selective protein adsorption.

The principle of selective, controlled adsorption has been illustrated by the finding that adsorbed albumin provides passivation of blood contacting surfaces, largely due to the fact that albumin interacts minimally with platelets (Packham et al 1969; Whicher and Brash 1978). Attempts have focused on the development of surfaces that selectively bind albumin. Albumin has been observed to have a natural affinity for lipid-like materials such as long chain fatty acids (Perter et al 1973). Perter and co-workers "grafted" long chain alkyl groups of chain length 8 to 30 carbon atoms, to the surface of polyurethanes. The resultant "derivatized" materials were found to increase the retention of albumin in contact with plasma as compared to underivatized controls (Munro et al 1983), and studies indicated that these materials selectively adsorbed albumin and showed a degree of thromboresistance (Riccitelli et al 1985).

2.10.3 PLATELET REACTIONS

The damage to a blood vessel subsequently leads to platelet adhesion and aggregation at the site of injury, followed by a release reaction takes place. The formation of a primary haemostatic plug composed of platelets, re-enforced by recruitment of more platelets and leucocytes, trapped in a stable fibrin mesh follows. In the contact between blood with an artificial surface, platelet adhesion and aggregation also takes place in a similar manner (Mason 1972; Mason et al 1976), and in addition, the extent of platelet adhesion is strongly dependent on the adsorbed protein layer.

The adhesion of platelets to protein-coated surfaces leads to a change in platelet shape, the coalescence of platelets into an irregular monolayer and, with increasing platelet adhesion, the formation of mounds, in which erythrocytes and leucocytes are trapped in fibrin (Salzman et al 1977). Platelet adhesion to artificial surfaces is followed by the platelet release reaction (Holmsen et al 1969), which takes place in adhering platelets, and then platelet aggregation occurring on the surface (Baumgartner et al 1976).

When platelets attach themselves to protein-coated surfaces, they change from

anucleate discs (2-5 μm in diameter) to anucleate spheres with long filiform pseudopodia. There is a coalescence of platelets into an irregular monolayer and an increasing platelet adhesion leads to formation of mounds in which erythrocytes and leucocytes are trapped in fibrin (Salzman et al 1977).

The mechanism following these morphological changes has been described as follows: platelets undergo a release reaction, discharging the contents of their granules into the circulation (Holmsen et al 1969). Platelet constituents released include arachidonic acid (AA), which in turn is converted to thromboxane B_2 (TBX_2), a known platelet aggregant, leading to further platelets adhering to the surface. Platelet aggregates may then form a mural thrombus, broaden and thicken, eventually separating from the wall of the material. Other platelet constituents, released include β-thromboglobulin, 5-hydroxy tryptophan (5-HT or serotonin), platelet factor 4, adenosine diphosphate (ADP) and thrombospondin. There may also be significant reduction in platelet numbers.

If thrombus formation on an artificial surface progresses, there is an interaction between platelets and the intrinsic coagulation pathway; by the release platelet phospholipids required in the intrinsic pathway (Feijen 1977). In addition procoagulants are liberated from platelets (Walsh 1982). Released ADP stimulates platelets to activate factor XII, consequently interacting with the intrinsic coagulation pathway. Thrombin formation produced by the coagulation pathways activation induces the rapid production of a fibrin monolayer on an artificial surface, thereby promoting further platelet adhesion and aggregation (Cheung et al 1979) and the generation of thrombin induces further platelet release reaction (Shuman and Levine 1980; Patrono et al 1980).

Platelet response in blood-biomaterial contact is influenced by diffusion (Feuerstein et al 1975) and shear forces (Richardson et al 1977) with the shear rate and contact time critical factors for platelet adhesion. In addition gas bubbles entrapped during preparation of the material enhance platelet adhesion and aggregation (Ward et al 1974) and their removal by exposure to negative pressure inhibits thrombus growth (Madras et al 1980).

In haemodialysis, there has been an interest in the role of platelets in the

mechanism of haemostasis, immunological and inflammatory reactions (Henson and Ginsberg 1981). A major contributing factor in the haemodialysis induced acute phenomenon of leucopenia may be sequestration of platelet aggregates during haemodialysis (Woods 1980). Additionally, platelet interactions with the complement system may include activation of complement components by platelets and activation of platelets by complement components (Henson and Ginsberg 1981). Platelets activation induced by dialysis containing cellulose or non-cellulose membranes has been demonstrated by the detection of increased levels of β-thromboglobulin, platelet factor 4 and TXB_2 (Adler and Berlyne 1980; Mahiout et al 1987).

2.10.4 SURFACE-DEPENDENT ACTIVATION OF BLOOD COAGULATION

The initial coagulation triggering mechanism may involve exposure of tissue factor, a membrane-anchored protein, exposed by damage to the protective endothelial cell layer and factor VII. Alternatively, an intrinsic pathway of coagulation is initiated by a surface-induced factor XII activation (i.e contact activation), when plasma is exposed to a variety of artificial or natural materials (Griffin 1981). The degree of contact phase activation is believed to be dependent on the nature of the surface (e.g charge) to which the blood is exposed (Vroman 1987) and is an important feature when considering the blood response to an artificial surface (fig. 2.4). The materials found to be most potent in stimulating the contact activation system of plasma generally possess a negatively charged surface (Griffin 1981) and examples of these materials include glass, kaolin, celite, certain connective or collagen preparations, pyrophosphate or urate crystals, endotoxin, and other substances.

The role of tissue factor pathway in the context of blood-biomaterials interactions has yet to be fully realised. Indeed, the potential importance of tissue factor/factor VII pathway in blood coagulation activation can be appreciated from its ability to promote factor X and factor IX activation. The latter reaction enables the recruitment of the intrinsic system to the generation of fibrin.

2.10.5 FIBRINOLYTIC ACTIVITY

Activation of the fibrinolytic system as a consequence of blood-material contact is believed to involve a factor XII-dependent pathway as indicated on fig.2.6. However, the complexity of the reactions suggests that direct activation may be due to FXIIa, kallikrein (KK) and FXIa and indirect activation through the release of tissue plasminogen activator (Sundaram 1992).

Although fibrinolysis is a prominent feature of blood-biomaterial interactions, it has not been widely studied (Brash 1991). The involvement of fibrinolysis in extracorporeal circulation has been assessed by measuring products of degradation of fibrinogen by plasmin (FDP) or D-dimer (product of degradation of cross-linked fibrin by plasmin) (Nakamura et al 1991). Measures of D-dimers are in effect, a reflection of prior activation of coagulation or thrombin generation (Ireland et al 1991).

Lysis of fibrin clots takes place over a much longer period than their formation, and the value of the determination of their formation has yet to be demonstrated in view of the time-scale of extracorporeal treatments (i.e a few hours). The fibrinolytic system assays are therefore not suitable for short duration *in vitro* and *ex vivo* experiments. Incorporation of fibrinolytic/thrombolytic activity on surfaces such that microscopic thrombi would be lysed, has been used in the preparation of surfaces with an improved blood compatibility (Sugitachi et al 1980).

2.10.6 ERYTHROCYTES

Under certain conditions, erythrocytes adhere to the protein layer on the artificial surface may undergo haemolysis to release ADP. The released ADP and the resultant erythrocyte ghosts in combination may induce platelet release reaction (Stormoken 1971). The addition of erythrocytes to protein solutions has been shown to reduce the amount of protein adsorbed (Uniyal et al 1982). This red cell effect has been attributed to the fact that red cell-surface contact results in deposition of membrane components (Uniyal et al 1982), producing a new surface that is relatively nonadsorptive.

Red cells have been shown to deposit integral membrane proteins on contacting surfaces without being lysed (Borenstein and Brash 1986), implying a type

of a non-haemolytic partial membrane extrusion onto the surface. Flowing blood in contact with a foreign surface has also been shown to undergo some degree of surface haemolysis (Blackshear 1972), thereby increasing the local concentration of free haemoglobin in the plasma. Since haemoglobin is known to possess high surface activity (Uniyal et al 1982), it is likely that haemoglobin will be adsorbed to the surface. The reduction in the amount of protein adsorbed to a surface in the presence of red cells may therefore be attributable to competitive adsorption of released haemoglobin (Forbes and Courtney 1987).

Erythrocytes may promote platelet adhesion on an artificial surface by reducing the adsorption of platelet-protective proteins or by depositing an adhesive substance (Brash 1983). During contact of red cells with artificial surfaces, shear-induced haemolysis may also occur due to changes in red cell membrane metabolism. In coagulation under low shear forces, entrapped erythrocytes and fibrin form the red thrombus (Bruck 1980).

2.10.7 LEUCOCYTES

During blood-biomaterial interface, the action of leucocytes differs markedly from that of erythrocytes whose role is primarily passive (Szycher 1983). Leucocytes are attracted to the thrombus and in the thrombosis process, leucocytes may contribute to platelet recruitment, fibrin formation by enzymatic release, and also participate in fibrinolysis. Leucocyte adhesion to artificial surfaces has been recognized for a long time and evidence supports the preferential adsorption of polymorphonuclear leucocytes or granulocytes in comparison to lymphocytes (Lederman et al 1978).

Adherence is often followed by activation and cell functions such as protein synthesis may be stimulated. Among the substances produced by activated leucocytes are interleukin 1, interferon type 1, components of the complement system, such as C2, C3, C4, C5, B, D, I and H, plasminogen activator, lysosomal hydrolases, prostaglandins, histamine, elastase and platelet activating factor (Bourne 1974; Tetta et al 1987).

During mechanical trauma, apparent similarities between platelets and leucocytes exists (Dewitz et al 1977) and it has been shown that shear stress

influences leucocyte damage, aggregation and the incorporation of leucocytes into microaggregates (Dewitz et al 1978). Leucocytes play a direct role in thrombus formation by release of endogenous procoagulant and proaggregating factors (Forbes and Courtney 1987; Sundaram 1992).

Blood-polymer interactions may induce changes in leucocyte function. In haemodialysis for instance, the granulocyte activation involves many extracorporeal factors. According to symptoms related to leucocyte activation from blood-membrane interactions, a stimulus-response coupling mechanism mediated by one or many chemoattractants, such as complement, leukotrienes, platelet activating factor (PAF), the presence of bacterial endotoxins permeating from contaminated dialysate or membrane extracts has been implicated. The ability of the various known chemoattractant molecules to activate leucocytes is mediated via cell surface receptors (Henson et al 1988).

The specific receptors that have been characterized are the N-formylated peptides for complement C5a and leukotrienes B_4 (LTB_4). The function of the chemoattractant is mediated by the phosphoinositide metabolism and protein kinase C activation (Snyderman and Uhing 1988). Studies have indicated that the activation of leucocytes by a single dose of chemoattractant is a transient phenomenon, implying that mechanisms exist for termination of the chemoattractant signal (Niedel et al 1980). The initiation of chemotaxis is followed by rapid superoxide production and degranulation which persists for no longer than 2-5 min (Mahiout 1994).

The transient leucopenia observed during haemodialysis with regenerated cellulose suggests a cell downregulation or a feedback mechanism based on the termination of chemoattractant responses (Markert et al 1991). Attenuation of chemotaxis by known chemoattractants may therefore occur by the following mechanisms:

(1) External or internal hydrolysation with degradation of chemoattractant.

(2) Activation and translocation of protein kinase C disturbing G-protein phospholipase C coupling.

(3) Elevation of cAMP levels by either the calcium-dependent mechanism involved in chemotaxis responses or by hormones that act through adenylate cyclase.

White cell damage as a result of blood exposure to artificial surfaces leads to an impairment of phagocytic activity and a reduced ability to combat infection (Bruck 1980). Phagocytosis of foreign materials by neutrophils results in the release of lysosomal contents to the exterior of the cell without concomitant liberation of cytoplasmic materials (Henson 1971). These steps follow neutrophil activation and are characterized as secretory events. Studies of neutrophils phagocytosing bacteria, opsonised zymosan particles and immune complexes suggests that the nature of extracellular secretion is similar.

Discharge of hydrolytic and oxidative enzymes from granules into developing phagosomes that are not yet limited by external environment results in the release of enzymes to the outside. Metabolic burst then produces highly oxidative substances, such as oxygen radicals and hydrogen peroxide. The activation also induces the release of proteins, eicosanoids and phospholipids as shown on table 2.5. Investigations into the relationship between leucocytes and complement activation particularly during extracorporeal applications have been a major focus in biomaterials research (Farrell 1984; Ringoir and Vanholder 1986). A variety of data shows that extracorporeal blood circulation, particularly haemodialysis and cardiopulmonary bypass, disrupts the normal tight control of phagocytic cell function, leading to both stimulation and downregulation of cell function (Ward 1994). The processes hypothesized to occur during extracorporeal circulation, and the data on which these hypotheses are based are summarized as follows:

1. Extracorporeal circulation acutely modulates expression of phagocyte adhesion receptors and activates their bactericidal functions. These changes are mediated by membrane-induced complement activation and their magnitude depends on the materials of the extracorporeal circuit. The adhesion of leucocytes to artificial surfaces may also be mediated by complement activation (Herzlinger and Cumming 1980; Aljama et al 1985). Transient leucopenia, due to pulmonary vascular leucostasis, has been demonstrated to be associated with the activation of alternative complement pathway by cellulosic membranes during haemodialysis (Kaplow and Goffinet 1968; Craddock et al 1977). In short, enhanced release of granular enzymes (Schaefer et al 1985), a fall in arterial oxygen pressure with intrapulmonary

sequestration of leucocytes (Craddock et al 1977) and a change in granulocyte oxidative metabolism (Kolb et al 1987) have been seen to be as a consequence of haemodialysis with complement-activating membranes.

2. Repeated acute stimulation of phagocytes leads to chronic down-regulation of their bactericidal functions in haemodialysis patients treated with systems containing complement-activating materials. A transient decrease in phagocytosis has also been noted after 15 min of haemodialysis with complement-activating membranes (Jacobs et al 1989). Neutrophils and monocytes from haemodialysis patients have also been shown to have a reduced phagocytic ability (Kusserow et al 1971; Hirabayashi et al 1988; Alexiewicz et al 1991).

Table 2.5 Possible neutrophil secretory products

Azurophil granules	Peroxidase, Esterase, Elastase, Histonase, Lysozyme, Glycoaminoglycans, Heparin sulphate, Chondroitin sulphate
Specific granules	Histamine, collagenase, lysozyme, Lactoferrin, Cytochrome, Flavoproteins, Vitamin B12 binding protein, Laminin receptor, C3bi receptor, fMet-Leu-Phe receptor.
Other granules types	Acid phosphatase, Heparinase, β-Glucosaminidase, α-Mannosidase, Acid proteinase, Gelatinase, Laminin receptor, Glycosaminoglycans.
Membranes	Acid phosphatase, 5"- Nucleotidase, Alkaline phosphatase, Elastase, Phospholipase.
Lipids	Platelet-activating factor, Arachidonic acid, Thromboxane A2, Leukotriene B4, 5-hydroxyeicosatetraenoic.
Reductants and Oxidants	H^+,O^{2-}, OH^-, O_2^-, H_2O_2, N^-, Chloramines, HOCl.

3. Activation of monocytes and lymphocytes leads to the synthesis of cytokines namely interleukin-1 (IL-1), tumour necrosis factor (TNF), and interleukin-6 (IL-6).

The relevance of cytokines in the blood response to a membrane material has been studied predominantly in the treatment of end-stage renal failure (ESRD) (Cheung 1990). It is unclear as to what extent repeated complement activation or synthesis of cytokines influences the chronic clinical status for instance by provoking pulmonary fibrosis, joint problems or amyloidosis (Vanholder 1992; Vanderbroucke et al 1986). The activated leucocytes and their release of all kinds of humoral substances, among them the highly toxic and carcinogenic oxygen free radicals, might well provoke chronically progressive tissue damage. Since neutrophil activation leads to an enhanced adherence of white cells to vascular endothelium (Vanholder 1992), this mechanism might well be involved in accelerated atherosclerosis observed in some dialysed patients (Wing et al 1984).

2.10.8 COMPLEMENT ACTIVATION

The normal function of complement is to mediate a localized inflammatory response to a foreign material. Any foreign material will activate complement to some degree. The increasing awareness of the significance of complement activation in human disease has heightened the current focus on its role during blood-biomaterial interaction. Activation of the complement system following blood exposure to an artificial surface is generally regarded as taking place via the alternative pathway as shown on Figure 2.5 (Johnson 1994; Cheung 1994; Woffinden 1993; Chenoweth 1988; Remes 1992; Herzlinger 1983).

The critical step in the reaction events is known to be the covalent attachment of C3 to the reactive surface. As complement activation takes place, the C3 molecule is enzymatically cleaved. This liberates low molecular weight polypeptide segment, C3a (9,000) into the fluid phase. The remainder surface-bound C3b (181,000) fragment, participates in the formation of the alternative pathway C5 convertase. Two C3b molecules with proper spatial arrangement on the surface, together with a Bb molecule, constitute the C5 convertase. The C5 convertase (C3bBbC3b) cleaves C5 (180,000) into two fragments, C5a (11,000) and C5b. The constant production of C3b during the spontaneous process of C3 tickover ensures that active C3b will be present to react with surface nucleophiles.

C3 reacts faster with simple sugars than it does with amino acids, suggesting that the protein evolved to efficiently recognize carbohydrate structures (Johnson 1994). Polysaccharides such as cellulosic haemodialysis membranes are good substrates for C3b. After the initial binding to a polysaccharide, C3b becomes less accessible to interaction with factor H (i.e factor H displays a 10-fold lower binding affinity for surface-bound C3b than for free C3b in solution) (Pangburn 1989). Without the intrinsic control mechanism on the surface (such as Decay Accelerating Factor (DAF) and membrane cofactor protein (MCP) found on cellular surfaces), C3b disposition on a biomaterial leads to C3 convertase formation and amplification of the cascade. Therefore, the alternative pathway is uniquely designed to recognize a foreign surface and alert the immune system in order to mount an effective defence.

Some *in vitro* studies have reported that in purified systems, soluble heparin has been demonstrated to inhibit the amplification of C3 convertase by decreasing the binding of activating factor B on its receptor site on C3b (Kazatchkine et al 1981). This action was found to be independent of the presence of O-sulphation and N-substitution of the heparin molecule (Maillet et al 1988). The action has also been shown to be diminished in the presence of plasma proteins with high affinity for heparin. When bound to a surface, the anticomplementary action of heparin is also different: heparin has been found to increase the ability of the regulatory protein H to bind to C3b on the heparin-coated surface, allowing the inactivation of bound C3b by factor I (Kazatchkine et al 1979).

The consequences of complement activation and the biological activities of various components are well defined, although primarily from *in vitro* studies. Similar to C3a, C5a is released into the fluid except for surfaces that are negatively charged and have a high affinity for this cationic peptide (e.g AN69 membranes in haemodialysis) (Cheung et al 1990; Cheung et al 1986). The resultant C3a and C5a

Table 2.6 Clinical relevance of complement activation in blood-biomaterials interactions (evidence from *in vitro* studies) (Johnson 1994)

Protein	Activity	Clinical sequelae (proposed)
C5a/C5a des Arg	Increased PMN adherence	Neutropenia,leucoseq uestration,
	Oxidative properties	Tissue damage
	Degranulation,	Tissue damage
	Production of other mediators,	Action depends on the mediator
	Increased cell expression of CR1, CR3, Fcγ RIII etc,	Cellular hyperadherence
	Vasoactive properties (mast cell degranulation, histamine release, increased permeability), and Chemotaxis	PMN desensitization with increased risk of infection
C3b, iC3b	Adherence and degranulation, IL-1 production	Neutropenia
C5-9 complex	Lysis, cell death, oxidative burst (sublytic amounts)	Leucocyte and platelet activation
Ba	Suppression of B cell proliferation	Inhibition of IgM production

have very important biological functions (Vogt 1986). These functions can be summarized as shown below and on table 2.6.

(1) Increase vascular permeability

(2) Trigger the granular release of mediators from mast cells and basophils, such as histamine, proteases, platelet activating factor and interleukins. Many of these mediators are chemotactic and cause vasodilation and bronchoconstriction

(3) C5a alone is a significant mediator of acute inflammatory response by triggering the activation of granulocytes, adherence and chemotactic migration.

In vitro studies have shown, for example that all dialysis membranes can elicit an oxidative burst from normal human polymorphonucleocytes (PMN), but that only

cellulosic membranes appear to depend on complement to mediate this activity (Kuwahara et al 1988). C5a and C5a des Arg both stimulate an oxidative burst from PMN as measured by the production of superoxide anion (cytochrome c reduction assay (Webster et al 1980) or the oxidation of fluorescent probes by H_2O_2 (chemiluminescent (CL) assay (Lewis et al 1987).

In uraemic patients, PMN exhibit a number of dysfunctions, including decreased response to C5a and formyl-methionyl-leucyl-phenylalanine (fMLP) required for both respiratory burst activity and degranulation. fMLP is a soluble stimulant that can cause secondary granules to fuse with the plasma membrane of neutrophils, releasing mediators of inflammatory response such as lactoferrin and elastase into the extracellular environment.

The uraemic PMN have also been shown to intrinsically have fewer C5a receptors but lack the ability to downregulate these receptors in response to exposure to complement activating membranes or purified C5a: this contrasts. the downregulation of C5a receptors by normal PMN (Lewis et al 1987). It appears that uraemic PMN (and monocytes) adapt to either the state of uraemia or the constant exposure to the extracorporeal circuit (with its consequent complement activation potential) and limit their response to C5a. The interest in complement activation induced by blood-material contact, in recent years, has resulted in the establishment of its measurement as a suitable parameter in the assessment of blood compatibility (Robertson 1988; Sundaram 1992; Yu 1993; Lamba 1994).

2.11 SUMMARY.

In the examination of blood responses during blood-biomaterial interactions, many studies have indicated that blood-material contact inevitably leads to an alteration to many blood constituents. This emphasises the importance of the activation of different aspects of blood responses. Consequently, in any investigation of blood response, it is necessary to be selective and focus should be on parameters that are both measurable and representative of the relevant aspects of the interactions. In this study contact phase activation of intrinsic blood coagulation was selected as a relevant aspect of blood-biomaterials interaction, with factor XII activity measurement as the parameter representative of contact phase activation (Chapter 3).

In addition, it was decided that this study should attempt to establish a relationship between a factor XII activity, as a parameter of blood response, and selected features of the membrane materials. The features of interest were for instance, surface charge characteristics, surface morphology and the level of interaction with pharmacological agents.

CHAPTER THREE

CONTACT PHASE ACTIVATION

3.1 INTRODUCTION

The mammalian haemostatic system comprises a number of interacting constituents that promote or inhibit blood coagulation (Lane and Bowry 1994). The enzyme central to the surface-initiated coagulation pathway is factor XII (FXII or Hageman factor). Activated factor XII (XIIa) activates factor XI, which in turn activates factor IX and thereby propagates the intrinsic coagulation pathway (Figure 3.1). Activated factor XII (FXIIa) is capable of triggering the kinin-forming pathway, plasminogen activation, protein C systems, conversion of factor VII to factor VIIa and prorenin to renin (Figure 3.2). In view of the significant role played by the contact phase activation in blood-biomaterial interactions, a more detailed discussion of the activation and inhibition of activated factor XII and its relationship with the plasma defence system has been provided.

3.2 FACTOR XII

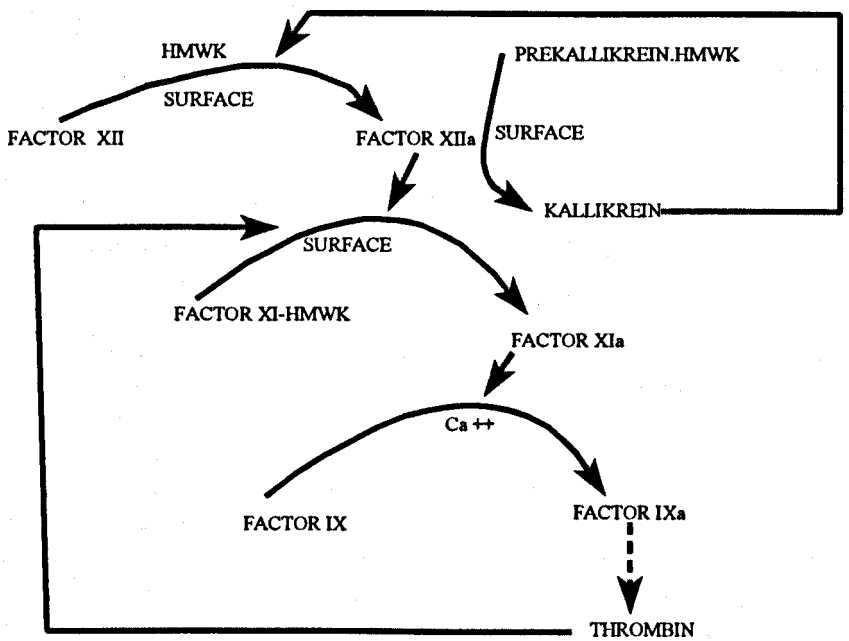
3.2.1 INTRODUCTION

The findings reported by Ratnoff and Colopy of a clotting defect in the blood of Mr Hageman (Ratnoff and Colopy 1955), led to the discovery of the coagulation factor XII (or Hageman factor). Numerous studies thereafter have revealed that factor XII (FXII) either plays a major role in, or is associated with the four plasma defence systems (coagulation, fibrinolysis, kallikrein-kinin and complement), and might also participate in the renin-angiotensin (Derkx et al 1979; Sealey et al 1979; Yokosawa et al 1979), kallikrein-kinin systems (Margolis 1963), cold-dependent activation of factor VII (Seligsohn et al 1978) and protein C systems (Fuhrer et al 1990).

A comprehensive review on the known information on FXII is presented in this thesis, particularly its activation, and inhibition of its enzymatically active forms, its relationship with plasma defence systems and its pathophysiological role within these systems.

3.2.2 FXII - BIOCHEMISTRY

FXII is a single chain β-globulin with a molecular weight of 80,000 (Revak



HMWK - High molecular weight kininogen

Figure 3.1: A schematic model of the contact phase of blood coagulation (Saito 1994)

et al 1974), which circulates in plasma as an inactive zymogen with an estimated concentration of 30 μg/ml (Saito et al 1976), has a sedimentation coefficient of approximately 4.5 S and an isoelectric point of 6.1-6.5. Its complete amino acid sequence of 596 residues has been determined by protein sequencing and verified by nucleotide sequence analysis of cDNA clones (Cool et al 1985).

The properties of FXII cannot be discussed without including the other three proteins of the so called "contact system" of the plasma. These are prekallikrein (PKK, Fletcher factor), High and low molecular weight kininogen (HMWK and LMWK or Fitzgerald factor respectively), factor XI or thromboplastin antecedent (PTA, FXII). These proteins circulate in inactive forms in plasma and are converted to active enzymes or liberate active peptides during activation on contact surfaces.

The proenzymes FXII (80,000 mol.wt), PKK (88,000 mol.wt) and FXI (160,000 mol.wt) are converted by limited proteolysis into the active serine proteases α-FXIIa (FXIIa) and β-FXIIa ($FXII_f$), plasma kallikrein (KK), and FXIa. PKK and FXI circulate in the blood as bimolecular complexes with HMWK (Mandle et al 1976; Thompson et al 1977), which has a positively charged histidine rich region in its light chain. This binds HMWK together with complexed PKK and FXI to negatively charged surfaces. A portion of the FXII molecule also binds to negatively-charged surfaces so that when blood comes into contact with such a surface the four proteins of the contact system are assembled on the surface (Fuhrer et al 1990).

3.2.3 ACTIVATION OF FXII BY CONTACT TO SURFACES AND ITS INHIBITION

Upon contact with negatively-charged surfaces such as glass, kaolin, celite, dextran sulphate, and ellagic acid, FXII is autoactivated (solid-phase activation) (Cochrane 1973). Both the binding to the surface and the cleavage during autoactivation result in distinct, defined conformational changes (Samuel et al 1992). The *in vivo* activators are unknown, but cerebroside sulphates (Tans et al 1983), biological components of cell membranes, and certain glycosaminoglycans (Hojima et al 1984; Pixley et al 1987) and triglyceride-rich lipoproteins (Mitropoulos 1994), are activators of FXII *in vitro*. Enzymes, such as kallikrein

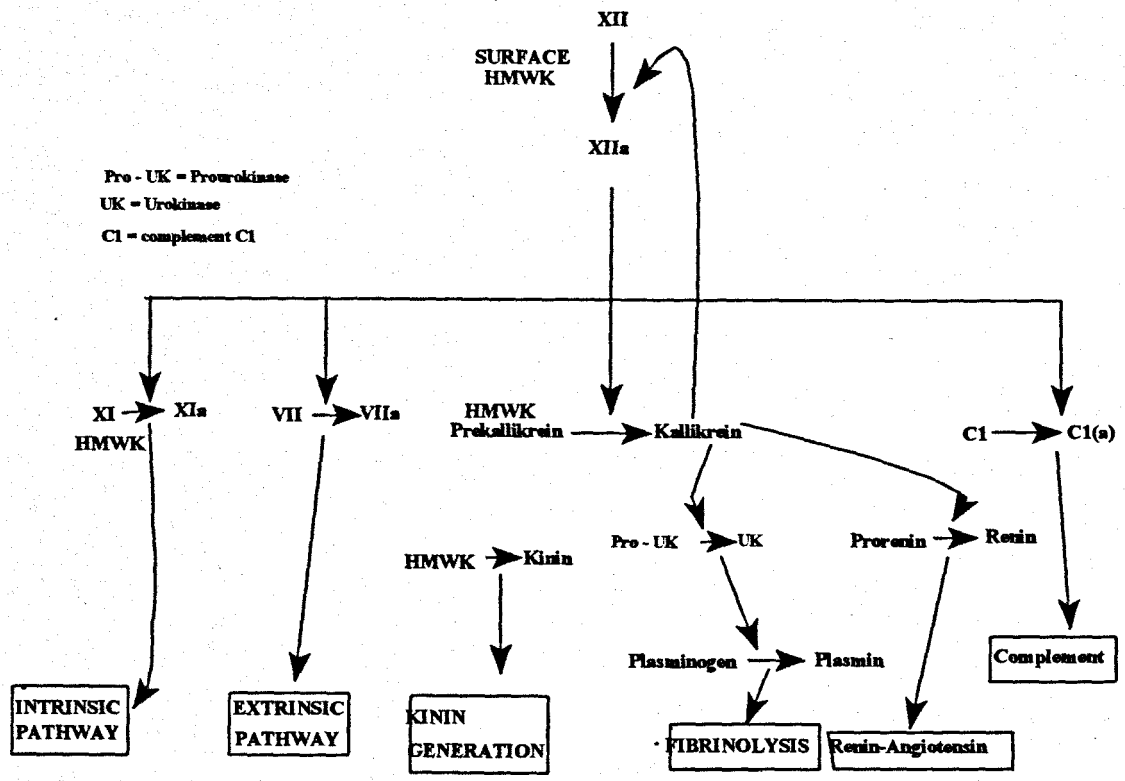


Figure 3.2 : The link between factor XII and other proteolytic pathways.

(KK) and plasmin, that activate FXII (fluid-phase activation), produce a group of proteinases that progressively decrease in size, increase in negative charge, decrease in clotting activity, and have altered surface-binding properties (Bagdasarian 1973).

The binding of FXII on negatively charged surfaces results in autoactivation, to form FXIIa or α-FXIIa (mol.wt = 80,000), by the cleavage of the bond connecting Arg^{353}-Val^{354}, generating a two-chain molecule composed of a heavy chain (353 residues) and a light chain (243 residues), held together by a disulphide bond (Wachtfogel et al 1993). The heavy chain of FXIIa is homologous to tissue plasminogen activator and urokinase with similar exon-intron organization (Cool and Macgillvray 1987). The heavy chain domains, whose functions *in vivo* are unknown, are coded by 13 exons, and include fibronectin (Type I and II homologues), epidermal growth factor, and a cringle. The light chain of FXIIa is a typical serine proteinase containing the amino acids sequence Asp, His, Ser and is the site for inhibition by its major plasma inhibitor, C1-inhibitor (Pixley et al 1985). FXII fragments (β-FXIIa or $FXII_f$) of mol wt approximately 30,000 are produced by the proteolytic cleavage of the bonds between Arg^{334}-Asn^{335}, Arg^{343}-Leu^{344}, as well as Arg^{353}-Val^{354}, resulting in a light chain of 9 or 19 residues and a heavy chain of 243 residues expressing catalytic activity, held together by a single disulphide bond (Wachtfogel et al 1993). α-FXIIa, by virtue of binding sites on its heavy chain, remains surface-bound, while factor β-FXIIa cannot do so but can catalyse reactions in the fluid phase. Both α-FXIIa and β-FXIIa activate prekallikrein (PKK), whilst α-FXIIa is a much better activator of FXI than β-FXIIa (Revak et al 1978).

Each of the zymogens FXII, PKK, and FXI, is converted to an active enzyme and the procofactor HMWK, to an active cofactor HMWKa and later to an inactive derivative, HMWKi. Two components interact directly with the surface: FXII and HMWKa. When a few molecules of FXII bind to a negatively charged surface, autoactivation occurs, converting it to the active serine protease, α-FXIIa. The few molecules of α-FXIIa formed are probably sufficient to cleave limited amounts of HMWK to HMWKa, allowing the active cofactor to bind to the surface.

Because most of PKK and FXI exists in bimolecular complexes with HMWK, activation of the procofactor to augment surface binding brings PK and FXI to the surface. On the surface, α-FXIIa can cleave PKK to KK and FXI to FXIa. KK is a powerful activator of surface-bound FXII and because both α- and β-FXIIa convert PKK to KK, a self amplification occurs with explosive activation of FXII. KK can initiate reciprocal activation, generating additional α-FXIIa and β-FXIIa from FXII. The generated KK may diffuse from the surface to convert additional HMWK to the active cofactor HMWKa and as a consequent releases bradykinin (Bk) from HMWK (Nakayasha and Nagasaw 1979). Following cleavage of Bk from HMWK, the resulting active cofactor, HMWKa, acquires the ability to bind to anionic surfaces (Scott et al 1984).

Bradykinin (Bk) is one of the most potent vasodilators known, operating by directly relaxing smooth muscle cells through B2 receptors (Schmaier et al 1984). By opening the tight junctions between endothelial cells, Bk increases capillary permeability and Bk also stimulates nerve endings, producing pain. The sum of these effects reproduces many of the aspects of inflammation. In summary, Bk enhances vascular permeability, produces hypotension, contracts smooth muscles, causes pain and releases tissue plasminogen activator (Egberg et al 1988; Pisaro 1975).

The positive feedback reactions of reciprocal activation of kallikrein and activated FXII and the formation of HMWKa are opposed by the much slower reaction of FXIa cleavages of HMWKa light chain to form HMWKi (Scott et al 1985). The major regulator of this system is the serine protease inhibitor (serpin), C1-INH, which contributes more than 90% of the inhibitory activity of plasma toward both α-FXIIa (Pixley et al 1985; Cameron et al 1989) and β-FXIIa (DeAgostini et al 1984). C1-INH is also a major inhibitor of kallikrein, and , with α_2-macroglobulin, accounts for 92% of the plasma inhibitory activity toward kallikrein (Schapira et al 1982). Other proteinase inhibitors which might contribute to controlling contact activation pathways include β_2-glycoprotein which has been shown to inhibit the activation of PKK by α-FXIIa (Schousboe 1988), α_1-antitrypsin which has been shown to be a major inhibitor of FXIa (Heck and Kaplan 1974), protein C inhibitor which has been shown to inhibit KK and FXIa

(Meijers et al 1988), and the so-called "plasminogen activation inhibitor", reported to inhibit α-FXIIa and kaolin-induced fibrinolysis (Hedner and Martinsson 1978). Antithrombin III (AT III) is a weak inhibitor of α- and β-FXIIa, FXIa and KK, and heparin, which potentiates the inhibition of thrombin and FXa by AT III, has actually been reported as an activator of the contact system (Hojima et al 1984).

3.2.4 FXII ACTIVATION AND THE COAGULATION PATHWAY

FXII, PKK, HMWK and FXI were all discovered in plasma from subjects lacking these proteins and exhibited contact activation coagulation abnormalities, indicating the significance of these proteins in surface-mediated coagulation. PKK and FXI circulate as complexes with HMWK, and these together with FXII bind to negatively charged surfaces and FXII is converted to α-FXIIa. This converts FXI to FXIa by limited proteolysis (Kurachi and Davie 1977), which converts FIX to FIXa and therefore activates the so-called "intrinsic pathway" of coagulation.

The first evidence of a relationship between the contact system and the extrinsic coagulation was the shortening of the prothrombin time in glass as compared to that in plastic. The observation was said to be due to factor VII (FVII) activation, as it was observed that cold temperatures promoted the activation of FVII depending on the presence of activated FXII and kallikrein (Gjonnaess 1972). Subsequent studies have shown that the role of kallikrein in FVII activation is to convert the zymogen FXII to β-FXIIa, which are, themselves, responsible for the increase in FVII activity (Radcliffe et al 1977).

β-FXIIa cleaves FVII into a two-chain molecule having a 40-fold enhanced coagulant activity (Radcliffe et al 1977). Kallikrein alone fails to activate partially purified FVII. The "spontaneous" activation of FVII in the cold is due to a combination of inactivation of the plasma protease inhibitor, C1 inhibitor (C1-INH) at low temperatures, and surface activation of FXII. Cold activation of factor VII is more prominent in plasma of women taking oral contraceptives, because their plasma has increased FXII and decreased C1-INH (Gordon et al 1980). Whether "cold activation" plays a significant role in haemostatic changes in hypothermia or cold-induced urticaria seen spontaneously or produced during cardiothoracic surgery remains to be established.

3.2.5 THE ROLE OF FXII ACTIVATION IN THE PRIMING OF FACTOR

VII ACTIVATION.

An increased VIIc is a frequent finding in patients with hypertriglyceridaemia (Esnouf 1993; Miller 1992; Andersen 1992), there being a positive correlation between VIIc and the concentration of triglycerides in the chylomicron and VLDL fractions of the circulating lipoproteins (mitropoulos et al 1989). Plasma VIIc also increases after a fat-rich meal, a response that is accompanied by little or no change in factor VII antigen, and is due to the generation of circulating factor VIIa (Mitropoulos 1994).

The association, however, has not been evident in patients lacking functional lipoprotein lipase (LPL) and despite the massive hypertriglyceridaemia in the chylomicron and VLDL fractions in this disorder, neither VIIc nor VII antigen is elevated above normal (Pacy et al 1993; Mitropoulos et al 1992), suggesting that lipolysis of triglyceride-rich lipoprotein particles has an important influence on the *in vivo* activation of factor VII. Since factor VII zymogen has been found not to activate factors X and IX, even in the presence of tissue factor (TF) (Williams et al 1989), a scheme has been proposed that suggests that the priming of the extrinsic pathway of coagulation may be achieved through the activation of the contact system.

The observation that long-chain saturated fatty acids (FA) can provide a potent contact surface for the activation of human factor XII in purified system suggested a physiologically relevant contact surface and a link between hypercoagulability and hyperlipidaemias (Mitropoulos and Esnouf 1991).

In another study, the importance of factor IX for the *in vivo* activation of factor VII was demonstrated, by the observation that in severe factor IX-deficiency, less than 10% of factor VIIa and in severe factor VIII-deficiency, factor VIIa was about 60% of the factor VIIa circulating in normal subjects (Wildgoose et al 1992). This suggests that under normal conditions, factor IXa is the principal activator of factor VII and that the bleeding diathesis in haemophiliacs can at least in part be due to the defective priming of the extrinsic pathway of coagulation. However, it should be noted that factor IXa is generated through factor XIa or KK also activated by Factor XIIa.

3.2.6 FXII AND FIBRINOLYSIS

The remarkable homology (50% identity) of FXII to both tissue plasminogen activator and urokinase suggests that FXII should also serve as a plasminogen activator. α-FXIIa or β-FXIIa do indeed, and were found to convert plasminogen to plasmin, but the rate was ten thousand times less than urokinase (Goldsmith et al 1978). However, it should be remembered that tissue plasminogen activator has little effect on plasminogen in the absence of its cofactor, fibrin, or digested fibrinogen. Moreover, if plasma is exposed to an activating surface, the rate of euglobin lysis is markedly enhanced.

This surface-induced increase in fibrinolysis is ablated in FXII-deficient plasma (Kluft et al 1987). Recent clinical studies have suggested that patients with homozygous FXII deficiency appear to have increased thrombotic episodes (Lammle et al 1991). Kallikrein also converts plasminogen to plasmin, at a rate faster than either FXIIa or FXIa, but is still much slower than urokinase (Colman 1969). Patients with homozygous factor XII deficiency have been observed to have an increased incidence of thrombosis, possibly due to the impaired cleavage of prourokinase and therefore fibrinolysis, as a result of inefficient activation of prekallikrein to kallikrein.

3.2.7 FXII AND COMPLEMENT

β-FXIIA has been shown to activate the classical pathway of complement by interacting with macromolecular C1 (Ghebrehiwet et al 1981), and does not require the participation of either PK or plasminogen. Kallikrein has been shown to proteolyze the C1r and C1s subcomponents of complement, leading to their inactivation (Gordon et al 1980), but not their activation. The action of β-FXIIa on C1 provides a nonimmunological pathway for activation of the classical pathway, which is known to occur in cardiopulmonary bypass. Release of elastase from neutrophil azurophilic granules, and lactoferrin from neutrophil secondary granules, occurs during clinical cardiopulmonary bypass and during simulated extracorporeal circulation (Wachtfogel et al 1993). Because both kallikrein and C5a can induce neutrophil degranulation (Schapira et al 1982), this observation raises the possibility that both contact and complement activation contribute to the neutrophil activation seen during cardiopulmonary bypass (Wachtfogel et al 1986).

3.2.8 FXII AND BLOOD CELLS

α-FXIIa has been shown to cause neutrophil aggregation and degranulation (Wachtfogel et al 1986). Unlike the reactions with FVII and C1, β-FXIIa will not stimulate neutrophils (Wachtfogel et al 1993). Therefore, a domain in the heavy chain is required, but nevertheless, the catalytic site is also necessary, as D-Pro-Phe-Arg-CH_2Cl and corn trypsin inhibitor, both stop the reaction. α-FXIIa downregulates immunoglobulin receptors $Fc_\gamma R1$ on monocytes without affecting their affinity (Wachtfogel et al 1993). HMWK inhibits fibrinogen from binding to neutrophils, which modulates neutrophil adhesion.

The contact system has been shown to interact with platelets demonstrated by the existence on platelets of receptors for FXI and FXIa (Lipscomb and Walsh 1979). Platelet membranes have been reported as a surface for FXII activation in the presence of PKK and HMWK (Walsh and Griffin 1981). A progressive fall in platelet count was observed in pigs given KK infusions (Egberg et al 1988).

3.2.9 FACTOR XII AND HUMAN DISEASE STATES

Patients with FXII deficiency have a markedly prolonged activated partial thromboplastin time but no bleeding diathesis. In fact several subjects with FXII deficiency have died of myocardial infarction and Mr Hageman died of pulmonary embolism (Ratnoff 1980). A few individuals whose plasma contain nearly normal immunochemically determined levels of nonfunctional FXII antigen but because their plasma contained cross reacting materials with the FXII antisera, they have been called CRM+ variants (Saito et al 1979). Plasma levels of FXII are elevated in women taking oral contraceptives (Gordon et al 1980) and during the later stages of pregnancy (Egberg and Gallimore 1983). Reduced plasma levels of FXII can occur as a consequence of reduced synthesis by the liver or in disseminated intravascular coagulation often associated with the impairment of liver function. Markedly reduced plasma FXII levels have been reported in subjects with cirrhosis of the liver (Goldsmith et al 1978). In kidney disease patients, chronic dialysis led to significant reduction of FXII clotting activities and antigen levels (Fuhrer et al 1990). In patients with glomerulonephritis, FXII clotting activities were elevated, as well as in kidney transplant patients, more than three months after the transplant (Mannhalter et al 1985).

In sepsis-associated DIC, *ex vivo* studies have suggested that the

involvement of the kallikrein-kinin system may be attributed to the activation and consumption of FXII by endotoxin (Sépero et al 1980). Activation of the kallikrein-kinin system in septic shock is probably mediated by α- or β-FXIIa. FXII may be activated directly or indirectly by lipid A component of endotoxin *in vivo*, which results in a decrease in plasma concentration of the zymogen and formation of α- and β-FXIIa, which then activates PKK, causing a decrease in its plasma levels (Wachtfogel et al 1993). Kallikrein then cleaves HMWK, which releases Bk into the circulation. Bk has been implicated in the pathogenesis of septic shock because of its ability to lower blood pressure in normal humans. The early peripheral vascular changes accompanying gram-negative bacteraemia and endotoxic shock are similar to those of Bk infusion, including arteriolar dilation and venular constriction (Regoli and Barabe 1980).

Table 3.2 Some clinical conditions in which participation of contact factors has been implicated (Saito 1987; Solomon et al 1988).

Allergic conditions
Arthritis
Carcinoid syndrome
Disseminated intravascular coagulation
Hereditary angioneurotic oedema
Hyperacute renal allograft rejection
Liver cirrhosis
Nephrotic syndrome
Septic shock
Typhoid fever
Adult respiratory distress syndrome (ARDS)

In hereditary angioedema, C1-INH is either absent or functionally inactive (CRM+ variant). There is a simultaneous activation of FXII and the contact system pathways and the classical complement pathway (Saito 1987). In cold urticaria, the clinical manifestation of the disease are thought to be due to released histamine, upon cold challenge of the skin. Functional activity of the C1-INH have been shown to be low and cold activation of FXII was found in 45% of the patients studied (Fuhrer et al 1990). Table 3.2 lists several disease states in which the

contact activation factors may participate (Saito 1987; Solomon et al 1988).

In adult respiratory distress syndrome (ARDS), the production of bradykinin from initiation of the contact phase induces pulmonary damage, increases vascular permeability and results in interstitial oedema, which may contribute to the establishment and progression of the disease (Velasco et al 1986).

3.3 PREKALLIKREIN (PK).

3.3.1 INTRODUCTION

Plasma PK is a fast γ-globulin of molecular weight of 88,000 circulating in blood with an estimated concentration of 40 μg/ml. Seventy percent circulates bound, non-covalently, to HMWK (Mandle et al 1976). The conversion of human plasma PK to kallikrein, its active form, is catalyzed by FXIIa on a surface augmented by HMWK or by β-FXII (FXIIf) in the fluid phase (Wuepper and Cochrane 1972). A single bond (Arg^{371} -Ile^{372}) is split, generating a heavy chain of 371 amino acids still linked to a light chain by a single disulphide bridge without a change in molecular weight. The heavy chain, which is the amino terminal end, contains four tandem repeats of 91 amino acids; the light chain contains the catalytic triad His, Asp, and Ser, and activates FXII in solution and cleaves HMWK.

The heavy chain is required for complexing with HMWK (Van der Graaf et al 1982) and the binding site is in the C-terminal 231 amino acids of the heavy (amino acid 141-731) (Page and Colman 1991). The light chain of kallikrein reacts with protease inhibitors (Schapira et al 1981), principally α_2-macroglobulin (α_2M) and C1-INH. C1-INH forms a 1:1 stoichiometric complex with kallikrein from inhibition by C1-INH and α_2M in a purified system (Schapira et al 1982), suggesting a mechanism of substrate protection of an enzyme (kallikrein) from active site-directed protease inhibitors. Antithrombin III, in the presence or absence of heparin, is an inefficient inhibitor of kallikrein.

3.3.2 RELATIONSHIP WITH FIBRINOLYSIS

Kallikrein converts plasminogen to plasmin in an apparent stoichiometric reaction (Colman 1969), but the rate although is faster than that achieved with FXIIa or FXIa, is much less than with urokinase. Kallikrein can convert prourokinase to two-chain urokinase (Ichinose et al 1986) and this reaction occurs

in plasma (Hauert et al 1989). It has therefore been suggested that patients homozygous for factor XII deficiency have an increased incidence of thrombosis (Lammle et al 1991), possibly due to the inefficient activation of prekallikrein to kallikrein, thereby impairing the cleavage of prourokinase and therefore fibrinolysis.

3.3.3 RELATIONSHIP WITH CELLS

Kallikrein has been shown to be chemotactic to neutrophils (Kaplan et al 1972). Exposure of neutrophils to concentrations of kallikrein capable of eliciting chemotaxis increases aerobic glycolysis and activity of the hexose-monophosphate shunt (Goetzl and Austen 1974). In the presence of calcium and magnesium, neutrophils aggregate in response to kallikrein (Schapira et al 1982). This interaction is associated with a stimulation of the respiratory burst in neutrophils, as indicated by an increase in oxygen uptake (Goetzl and Austen 1974). Kallikrein also induces neutrophils to release human neutrophil elastase from their azurophilic granules (Wachtfogel et al 1983) and primes neutrophils for superoxide production (Zimmerli et al 1989).

A skin window technique that assesses the *in vivo* chemotaxis of leucocytes in response to tissue or microvascular injury shows a significant impairment in chemotaxis in FXII and PKK-deficient patients (Rebuck 1983). This suggests that both kallikrein and FXIIa are important in the release of elastase from neutrophils in plasma. HMWK has been shown to bind to neutrophils in a reversible and saturable manner and is required for kallikrein to optimally activate neutrophils (Gustafson et al 1989).

3.4 HIGH MOLECULAR WEIGHT KININOGEN (HMWK).

3.4.1 INTRODUCTION.

Two forms of purified human plasma kininogen have been described (Jacobsen and Kriz 1967): high molecular weight kininogen (HMWK, mol. weight = 120,000) and low molecular weight kininogen (LMWK, mol.weight = 68,000). HMWK is an α-globulin with a plasma concentration of 80 μg/ml (Schmaier et al 1983). LMWK is a β-globulin with a plasma concentration of approximately 160 μg/ml (Müller-Esterl et al 1982). Plasma kallikrein and FXIIa cleaves HMWK in three sequential stages (Mori and Nagasawa 1981). The first two cleavages, at

Lys^{380}-Arg^{381} and Arg^{389}-Ser^{390}, yield a kinin-free protein (HMWKa) composed of two disulphide-linked 64,000 and 56,000 chains and a nonapeptide, bradykinin (Bk). The third cleavage results in a stable kinin-free protein composed of two disulphide-linked 64,000 and 45,000 chains and liberates a small 7,000 molecular weight peptide. These cleavages lead to major conformational changes as detected by circular dichroism (Villamuera et al 1989). Studies with human HMWK show that cleaved forms, HMWKa, binds to a greater extent to an activating surface (Scott et al 1984), indicating that HMWK exists as a procofactor that can be activated by cleavage with kallikrein or FXII. FXIa cleaves HMWK initially, at the site of the third cleavage by kallikrein, resulting in two fragments (a heavy chain of 75,000 and light chain of 46,000), followed by two cleavages to release Bk. Prolonged exposure of HMWK, to FXIa results in an extensive proteolysis of the HMWK light chain to produce a degradation form of HMWK (HMWKi) with a loss of coagulant cofactor activity (Scott et al 1985).

The unique feature of HMWK is the inhibition of proteases with cysteine at their active sites including the calcium-activated cysteine proteases (calpains) present in all cells by the heavy chain (Bradford et al 1990).

3.4.2 RELATIONSHIP OF HMWK WITH PLATELET ACTIVATION

Although calpain is primarily an intracellular enzyme, after thrombin activation of platelets, calpain is exposed on the surface of the platelets (Schmaier et al 1990), and therefore is susceptible to inhibition by HMWK and LMWK. In inflammatory exudates, kininogens may be present to inhibit the cysteine proteinases, cathepsins H and L (Salvesen et al 1986). HMWK (Puri et al 1991) and LMWK (Meloni and Schmaier 1991) has been shown to prevent the binding of thrombin to platelets, and the presence of HMWK and LMWK in plasma, explains the requirement for 10-fold more thrombin to activate platelets in the plasmatic environment. There is a 5-fold increase in HMWK binding to platelets after thrombin activation which appears to be due to the binding of HMWK to thrombospondin that is expressed on the platelet surface (Dela Cadena et al 1991).

3.4.3 THE ROLES OF HMWK IN NEUTROPHIL FUNCTION

HMWK has been shown to bind to neutropils in a reversible manner and is required for kallikrein to optimally activate neutrophils (Gustafson et al 1989).

HMWK inhibits fibrinogen binding to neutrophils, which modulates neutrophil adhesion. The binding of HMWK to neutrophils occurs to the integrin $\alpha_{mac}\beta_2$ (MAC-1) and is blocked by antibodies to determinants both on the light and heavy chains of HMWK.

3.4.4 HMWK AS AN ANTIADHESIVE PROTEIN

Earlier studies (Vroman and Adams 1967) found that fibrinogen can be detected immunochemically on a negatively charged surface within seconds after normal human plasma contacts the surface, but within minutes was no longer detectable. The phenomenon was shown to be due to the displacement of fibrinogen by HMWK after surface-dependent autoactivation of FXII (Schmaier et al 1984; Brash et al 1988). Factor XIIa directly and indirectly (through the formation of kallikrein), generates HMWKa from HMWK. HMWKa (but not HMWK or LMWK) displaces fibrinogen from the surface (Schmaier et al 1984). The "Vroman effect" is due to the time and surface-dependent generation of HMWKa, via contact activation of plasma, which results in the physical displacement of adherent fibrinogen from the surface (Brash et al 1988). Extensive proteolysis results in HMWKi (Scott et al 1985), which does not displace fibrinogen (Brash et al 1988). It has also been shown using ^{125}I-fibrinogen from both neutrophils and platelets (Tankersley 1984).

3.5 FACTOR XI (FXI)

3.5.1 INTRODUCTION

Factor XI, a plasma protein originally found as a functionally deficient agent in plasma from rare individuals with prolonged clotting time (Rosenthal et al 1953), is the only component of the contact phase of blood coagulation that is associated with a bleeding tendency. It is present as a zymogen of a serine protease with a molecular weight of 160,000 at a concentration of 4 μg/ml in human plasma.

FXI is a homodimer composed of two identical polypeptide chains held together by a disulphide bond (Bouma & Griffin 1977; Kurachi & Davie 1977) and circulates in plasma as a noncovalent complex with HMWK (Thompson et al 1977). Each subunit of FXI/FXIa appears to bind one molecule of HMWK and interaction with HMWK is required for the adsorption and activation of FXI on

negatively charged surfaces. FXI is cleaved and converted to FXIa by FXIIa in the presence of HMWK during contact activation. Cleavage occurs at a single internal peptide bond within a disulphide linkage in each of the two polypeptide chains and gives rise to an active serine protease. FXIa is composed of two heavy and two light chains held together by disulphide bonds with molecular weights of 50,000 and 30,000 respectively. The light chain possesses catalytic activity, while the heavy chain has the binding site for HMWK (van der Graaf et al 1983).

FXIa functions in blood coagulation to activate factor IX in the presence of calcium ions. This reaction is affected neither by phospholipid, HMWK nor kaolin, and is the only enzyme-substrate reaction in the coagulation cascade for which no specific co-factor has been identified. The relative importance of factor XIa in the activation of factor IX as compared to that of the factor VIIa-tissue factor complex (the extrinsic pathway) has been assessed by kinetic studies using purified proteins, but no definite conclusion has been reached. Some studies suggested that factor XIa plays a more significant role than the extrinsic pathway activation (Walsh et al 1984), while others claim that the relative activities of the two systems cannot yet be adequately compared (Nemereson 1988).

A sensitive assay for estimating the factor IX activation peptide in plasma has been recently developed and applied in the study of *in vivo* activation of factor IX (Bauer et al 1990). The mean plasma concentration of this peptide was markedly reduced in patients with hereditary factor VII deficiency, suggesting that factor IX activation *in vivo* results mainly from the extrinsic pathway rather than the contact system.

3.6 CONTACT PHASE ACTIVATION DURING HAEMODIALYSIS

Exposure of blood to the extracorporeal circuit during haemodialysis may initiate a number of adverse reactions of which the life-threatening anaphylactoid reaction is the most feared one. Although the aetiology of these reactions remains controversial, it is generally accepted that in many cases ethylene oxide hypersensitivity may have induced these anaphylactoid reactions (Dolovich et al 1984; Bommer and Ritz 1987; Henne et al 1984; Lemke 1987; Lemke et al 1990)). Sterilization of disposable components of the extracorporeal circuit with ethylene oxide (ETO), can result in the formation of ETO/albumin-conjugated IgE-

antibodies which cause allergic reactions in hypersensitive patients (Henne et al 1984; Lemke et al 1990; Bommer and Ritz 1987; Rumpf et al 1985). Other aetiological factors claimed to contribute to these reactions are complement activation (Hakim et al 1984) and passage of bacterial products across high permeable dialysis membranes (Teruel et al 1992; Verresen et al 1990, 1991; Lonnemann et al 1992). The explanation has been excluded by the observation of the anaphylactoid reaction during haemofiltration and haemodialysis, using pyrogen-free substitution fluid and dialysate (Brunet et al 1992; Petrie et al 1991).

Recently, a series of severe anaphylactoid reactions occurring shortly after onset of dialysis has been described, which could neither be correlated with high total plasma IgE nor with a high titre of specific IgE against ethylene oxide. It was noted, however, that the majority of cases occurred during sessions with the negatively charged AN69S membrane in patients treated with angiotensin converting enzyme (ACE) inhibitors against hypertension (Tielemanns et al 1990; Verresen et al 1990; Parnes & Shapiro 1991; Dinarello 1991) and without concomitant ACE inhibitor therapy (Bigazzi et al 1990; Giangrande and Allaria 1992; Verresen et al 1993).

High concentrations of bradykinin have been implicated in symptoms of asthma (Christiansen et al 1987), and in allergic rhinitis (Proud et al 1987). Furthermore, in an *in vivo* sheep model of dialysis and in an *in vitro* test with human plasma, generation of bradykinin in human plasma, was observed with AN69 in the presence of an ACE inhibitor (Kreiter et al 1993; Lemke & Fink 1992). Generation of bradykinin in human plasma was found to be dependent on ACE-inhibitor dose and could be completely abolished by soybean trypsin inhibitor, an inhibitor of plasma kallikrein (Lemke & Fink 1992). It has also been mentioned that anaphylactoid reactions due to bradykinin accumulation occur not only during dialysis with AN69 membranes, but have been reported with the use of polymethylmethacrylate (PMMA) membranes (Schwarzbeck 1993) and with reused capillaries as well (Pegues et al 1992). Furthermore such reactions have also been described during low density lipoprotein (LDL) apheresis using dextran sulphate as the adsorbent (Daugirdas et al 1985; Olbricht et al 1992).

These clinical observations provided strong empirical evidence that dialysis

with AN69 membranes, especially in combination with ACE inhibitors, is particularly prone to cause acute anaphylactoid reactions. The underlying mechanism, was hypothesized to be strongly linked with the permissive role of ACE inhibitors and the fact that AN69 membrane is negatively charged (Lemke 1994). It was suggested that these reactions were due to an excessive bradykinin accumulation as a consequence of contact activation of the intrinsic coagulation pathway during the first few minutes of dialysis (Lemke and Fink 1992). Activation of the contact system of plasma by the membrane/biomaterial leads to the generation of kallikrein which cleaves bradykinin from HMWK. Under normal circumstances, the level of bradykinin in plasma is kept low mainly by an enzyme called kininase II which is identical to angiotensin-converting enzyme. Therefore, the ACE inhibitor can contribute to the clinical reactions and to bradykinin accumulation since it blocks bradykinin breakdown.

3.7 THE ROLE OF CONTACT ACTIVATION DURING CARDIOPULMONARY BYPASS.

During cardiopulmonary bypass, there is extensive contact between blood anticoagulated with heparin and the synthetic surfaces of the extracorporeal circuit. Blood cell interactions and plasma protein alterations prolong bleeding time, increase postoperative blood loss, and trigger a chemical and cellular "whole body inflammatory response". Extracorporeal circulation has been associated with both qualitative and quantitative alterations of platelets, neutrophils, complement and contact systems. Inhibition of the contact activation enzymes differs from that of the serine proteases in the latter stages of coagulation cascade, which are predominantly regulated by antithrombin III. Antithrombin III has been shown to be a poor inhibitor of FXIIa, FXIa, and kallikrein and that although it markedly accelerates the inactivation of FXa and thrombin, it exhibits minimal enhancement of the inactivation of FXIIa and FXIa (Colman et al 1989). Although no abnormalities in PK levels could be shown in either clinical cardiopulmonary bypass or simulated extracorporeal circulation, a significant increase in kallikrein-C1-INH complex formation has been reported in simulated extracorporeal circulation (Wachtfogel et al 1989). Therefore, this provided an initial indication of the significance of contact activation during extracorporeal circulation.

3.8 THE ROLE OF THE VASCULAR ENDOTHELIUM ON CONTACT PHASE ACTIVATION OF COAGULATION.

Endothelial cells, line the vascular tree to serve as an interface between circulating blood components, all tissues and organs, therefore act as nature's first line of defence (von Appen et al 1993). They are highly "plastic" cells capable of transforming their shape, cell membrane structure and fenestration, cell functions, and secretion patterns in response to changes in location, tension forces created by the bloodstream, and created by the bloodstream, and contact with surfaces (Nabel 1991; Wu et al 1988).

The focal point for the coagulation cascade is the generation of the enzyme thrombin that cleaves fibrinogen to form insoluble fibrin clot. The endothelium participates in this cascade by producing a number of cofactors, including high molecular weight kininogen (HMWK), Factor V, Factor VIII, and Tissue factor (Davies and Hagen 1993). Tissue factor is a procoagulant enzyme synthesized by the endothelium and is found mainly in the subendothelium. Basal secretion of tissue factor is low compared to that of the underlying smooth muscle cells and fibroblasts (Davies and Hagen 1993).

However, if stimulated or injured, the endothelial cells can increase tissue factor production by 10- to 10-fold (Davies and Hagen 1993). There are binding sites for HMWK and factors VIII, IX, IXa, X and Xa on the endothelial cells (von Appen 1993).

The exact role of the endothelium in contact activation is not quite clear, but results reported from an *in vitro* study using cultured human umbilical vein endothelial cells (HUVECs), suggested that a lysate obtained from these cells had a property that impaired the generation of coagulant and amidolytic activity initiated when normal human plasma is exposed to glass (Kleniewski and Donaldson 1993). Further experiments discovered that this inhibitory property worked by blocking the adsorption of factor XII onto glass, thereby preventing the activation of factor XII, but it did not impair the coagulant or amidolytic activity of already activated factor XII (FXIIa). This property in HUVECs lysate could be neutralized by a preparation of purified factor XII but not by purified prekallikrein or high molecular weight kininogen. A partially purified inhibitory fraction from

the cell lysate exhibited a single homogenous band in SDS-PAGE electrophoresis with a relative molecular weight of 22,500. This indicates that endothelial cells synthesize a substance that modulates the activity of factor XII and therefore exercise a direct control of *in vivo* contact phase activation.

3.9 SUMMARY

In the last decade or so, the application of molecular biology and structural protein chemistry have contributed to an increased knowledge of the structure-function correlation of the contact system proteins, factor XII, prekallikrein (PKK), and high molecular weight kininogen (HMWK). Each protein has unique functions, such as activation of the complement and fibrinolytic systems, and cysteine protease inhibition. The proteins work together to generate bradykinin and initiate coagulation on artificial surfaces. The contact system is activated in septic shock, and inhibition modulates the hypotension. A deficiency of C1 inhibitor, the most important protease inhibitor of this system, results in hereditary angioedema. In both of these syndromes, bradykinin plays a major role. Allergic rhinitis is also mediated by bradykinin release.

Activation of the contact system in cardiopulmonary bypass results in neutrophil activation and elastase release. A similar mechanism may operate in the adult respiratory distress syndrome. The advent of novel tight-binding protease inhibitors and bradykinin antagonists promises more effective therapy based on the increasing understanding of the mechanisms involved in the regulation of this surface defence system.

The contact system is an important defence in the human organism. In response to foreign organisms or surfaces, HMWK is activated to produce the potent vasodilator, Bk, and FXII and prekallikrein are activated, both of which recruit and stimulate neutrophils, therefore making the contact system an important mediator of the inflammatory reaction. Additionally, FXII has been shown to enhance fibrinolysis, suggesting that the contact system may have an antithrombotic role. HMWKa is an antiadhesive protein that inhibits platelet, neutrophils, monocytes, and endothelial cell interactions with fibrinogen. HMWK directly blocks thrombin-induced platelet activation. Release of Bk from HMWK by kallikrein stimulates prostacyclin and NO formation by endothelial cells, indirectly

leading to inhibition of platelet aggregation.

In view of the clinical consequences of bradykinin generation, it seems justified that generation of bradykinin should be considered as a relevant parameter for hypersensitivity. Two types of assays have been described in the literature; a biological and an immunological assay. In the biological assay the muscle contracting effect of bradykinin is utilized, whereas for the radioimmunoassay a rabbit antiserum against bradykinin is employed. The biological assay is considered to be too complicated and its use has been displaced by the radioimmunoassay. However, the radioimmunoassay is not commercially available.

Since the bradykinin assays suffer from limited availability, it is therefore necessary to consider other types of assays which detect activation of the contact system. Although a number of assays are available for this purpose none of them is commercially available, is simple or can be recommended for immediate clinical application. Determination of kallikrein in plasma has been considered using two approaches: chromogenic substrate assays (Lottenberg et al 1981 and Fisher et al 1982) and immunoassays that detect kallikrein complexed to its natural inhibitors such as C1-INH, α_2-macroglobulin (Harpel et al 1988; Kaufman et al 1991). However none of these is commercially available at present.

Another approach has been the assay of factor XII by a direct chromogenic peptide substrate assay (Gallimore et al 1987; Egberg & Overmark-Berggren 1983; Vinazzer 1979; Friberger et al 1984), and modification of these assays to measure factor XII-like activity was attempted (Kluft et al 1983; Irvine 1989; Sundaram 1992). Other studies have quantified factor XII activity using immunoassays, by measuring the plasma factor XIIa-C1-inhibitor complexes (Nuijens et al 1988; Van der Kamp & Van Oeveren 1993) or factor XIIa by a commercial elisa kit (Rhodes 1992; Campbell et al 1994). The former assays are not yet available commercially, while the latter assay may well be strongly influenced by the interaction of factor XIIa with its natural inhibitors in blood at the materials interface, resulting in diminished detection.

Studies using a chromogenic substrate assay for the measurement of factor XII-like activity (FXIIA) in the supernatant plasma after material contact were reported as being unable to discriminate between a range of different hemodialysis

membranes (Irvine 1989; Sundaram 1992; Yu 1993, Wark 1993; Lamba 1994). Regardless of the marked differences in blood response patterns observed with respect to protein adsorption and complement activation by various biomaterials such as regenerated cellulose (Cuprophan), DEAE-modified cellulose (Hemophan), biomembrane-mimetic polymers and polyacrylonitrile-based membranes (AN69S), the observations indicated that FXII-like activity in blood to the different materials was not significantly different. This led to the conclusion that either all polymers presented similar abilities to generate FXIIA in blood or the assay methodology was not sufficiently sensitive for the detection. The other suggestion was that the material dependent activation of FXII was influenced by the rate of inhibition of FXIIA by plasma inhibitors such as C1-inhibitor, α_2-macroglobulin, α_1-antitrypsin and antithrombin III.

There was therefore a need to explore the subject further, by electing to use simpler models other than whole blood. Previous studies have reported of adsorption properties of plasma factor XII on haemodialysis membranes (Mahiout et al 1993), but the relationship with factor XII activation was not described. As an adaptation to the reported surface-adsorption measurement findings, the activation of surface-adsorbed plasma factor XII was regarded as a major objective, in view of the tendency for the contact factor to bind to artificial surfaces. This led to the development of a new approach to the use of a chromogenic substrate assay described by Irvine (1989) and Sundaram (1992), by the modification of the assay to measure factor XII-like activity induced by biomaterials at the material interface as compared to of the supernatant fluid-phase activity measured in the previous studies. A clear distinction between surface FXIIA and supernatant phase FXIIA was thought necessary. This was justified by the fact that surface bound FXII becomes activated, and that cleavage by kallikrein of the a bond between its light and heavy chain results in the release of the enzymatic carboxyl terminal fragment (β-FXIIa, mol.wt of 28,000) into the plasma supernatant (Saito 1987). The remnant surface-bound amino terminal fragment lacks enzyme action but retains its amidolytic property and can split the chromogenic substrate to release the chromophore p-nitroanilide. The findings were compared with *in vitro* and clinical studies utilising immunoassay methods for the quantification of factor XIIa

alone, or factor XIIa and XIIa-inhibitor complexes.

It has been reported that in the absence of tissue factor (TF), the initiation of the extrinsic pathway of coagulation can be generated through the enzymes derived on activation of the contact system (Mitropoulos 1994). This priming of the extrinsic pathway of coagulation and thereby the levels of circulating factor VIIa may be of little immediate consequence for a healthy vessel wall with minimal expression of TF. However, at the site of atheromatous plaque or that of injury, an increased level of circulating factor VIIa will determine locally a higher rate of thrombin generation. Thrombin concentration at this site may be important not only for haemostasis but also for the inflammatory, proliferative or reparative responses (Mitropoulos 1994). In view of this association between circulating factor VIIa levels and the activity of the contact systems, the measurement of factor VIIa was considered a suitable additional parameter to be investigated during the clinical application of extracorporeal devices such as haemodialysis and cardiopulmonary bypass.

CHAPTER FOUR

BIOMATERIALS AND EVALUATION PROCEDURES.

4.1 SELECTED BIOMATERIALS:

Biomaterials as defined in chapter 1, can be classified into the following categories: polymer, metal, composite, ceramic and carbon. In extracorporeal devices, the most commonly used biomaterials are in the form of membranes, sorbents, blood tubing, artificial diaphragms and substrates for cell culture (Courtney et al 1993). Polymeric membranes are fundamental to current extracorporeal blood purification and as a follow-up to previous studies (Irvine L 1989; Sundaram 1992; Yu 1993; Lamba 1994), membranes were selected as the biomaterials for investigation.

A cellulosic membrane constituted the first membrane used in haemodialysis, and currently, the most commonly used membrane in haemodialysis is a regenerated cellulose membrane, Cuprophan. The apparent success of regenerated cellulose membranes, however has not been without limitations. The most important aspect has been that of membrane interaction with blood components, particularly complement activation. Complement activation by cellulose membranes has been attributed to the presence of hydroxyl groups, and that activation was via the alternative pathway (Chenoweth 1984), a property consistent with the known influence of polysaccharide structures on the complement system.

Attempts to produce membranes with improved blood compatibility in comparison to Cuprophan have lead to the preparation of modified cellulosic membranes and synthetic polymers. A number of new polymers, including modified cellulosic and synthetic membranes have been developed (Courtney et al 1990; Handerson et al 1983; Baurmeister et al 1989). However, the dominant role of regenerated cellulose membranes has greatly affected membrane development and assessment (Courtney et al 1984).

Membrane materials currently in clinical use have been categorised into three types: cellulosic, modified cellulosic, and synthetic membranes (Courtney and

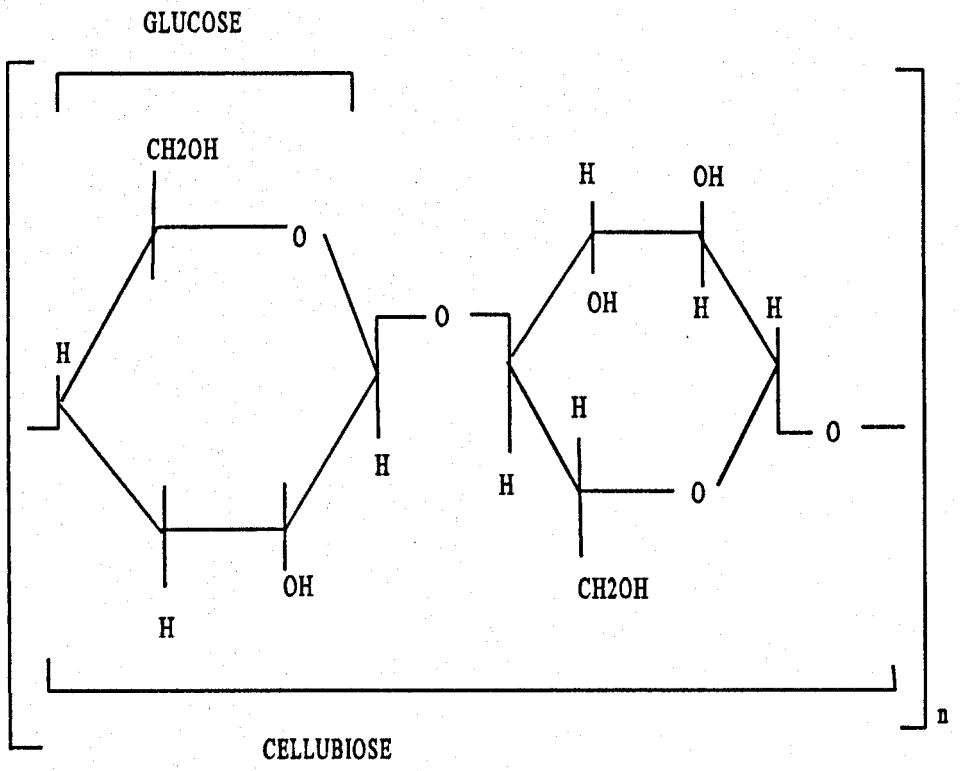


FIGURE 4.1: Chemical structure of regenerated cellulose (Cuprophan).

Forbes 1993). The membranes selected for the purpose of this study, comprise all three types, representing the surface characteristics appropriate for this investigation. These are in the form of flat sheets regenerated cellulose membrane Cuprophan 150PM, modified cellulose Hemophan (DEAE-cellulose/cellulose polymer blend), the synthetic membranes AN69S (acrylonitrile-sodium methallyl sulphonate copolymer) and SPAN (acrylonitrile-sodium methallyl sulphonate copolymer), and polyamides (Ultipor NR and NRZ 14225) as control membranes. In addition, hollow fibre membranes obtained of Cuprophan and AN69 dialysers were evaluated in the form of minimodules. The attempt was to compare the performance of a static system (an incubation test cell utilising flat sheet membranes) to a flow system (syringe pump controlled flow system utilising hollow fibre minimodules) as suitable blood-membrane contacting procedures.

4.1.1 CELLULOSE MEMBRANES

Cellulose membranes have a long association with haemodialysis (Thalhimer 1937) and still remain the most widely used haemodialysis membrane. The availability of cellulose membranes contributed greatly to the establishment of haemodialysis as a routine clinical procedure (Courtney et al 1984). Cellulose regenerated by the cuprammonium process is the preferred membrane material, ensuring sufficient convective and diffusive transport properties as well as excellent film forming qualities.

The structure of cellulose consists of a large number of anhydroglucose units joined together by β-glycosidic linkages (Figure 4.1). A feature of cellulose of great importance to membrane formation and utilization is the strong intermolecular attraction resulting from hydrogen bonding. This bonding promoted by regular arrangement of hydroxyl groups, ensures that cellulose cannot be melted or dissolved (Courtney et al 1984). The other important consequence of the intermolecular bonding is that cellulose, while swelling in an aqueous medium, remains insoluble and is suitable for use in contact with blood. Therefore, the

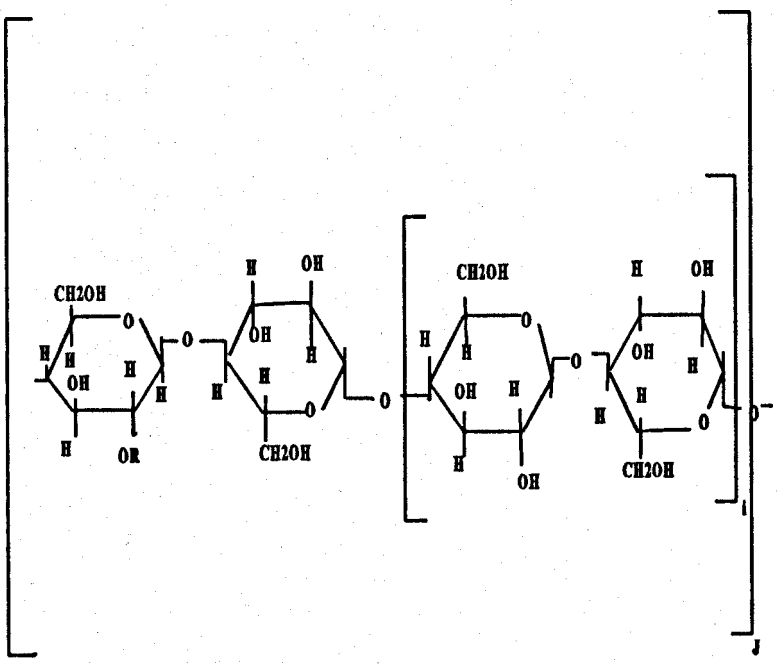


Fig. 4.2: The chemical structure of Hemophan

manufacture of cellulose membranes requires the production of a soluble or thermoplastic derivative as an intermediate. The most widely used regenerated cellulose membrane is manufactured by Akzo Nobel, Wuppertal, Germany under the tradename Cuprophan.

During membrane fabrication, cellulose is dissolved in Schweizer's reagent (solvent containing copper hydroxide and ammonia). A viscose solution named cuaxam solution is obtained by the formation of cellulose-cuprammonium complex. This final solution which at this stage has a decisive influence on the membrane properties, is forced through appropriate slots to form flat, tubular, or capillary films. It is subsequently coagulated in an alkaline solution by extraction of ammonia. Subsequent to extraction of copper and ammonia, the membranes are continuously treated with glycerin solution, dried, and wound onto spools.

Cellulose films are generally identified by a system of figures and letters derived from a code used in the manufacture of regenerated cellulose films as wrapping materials (Briston and Katan 1974). The figures refer to the nominal weight in grams of 10 m^2 of film and therefore are indicative of thickness. The letters denote the condition of the film by indicating whether it is coated or uncoated, since cellulose films may be coated with cellulose nitrate in order reduce moisture sensitivity.

Regenerated cellulose films used the letters PM, to denote that the membrane is uncoated and suitable for medical application. The most important regenerated cellulose membranes for haemodialysis is Cuprophan, which is prepared by the cuprammonium process. The availability of Cuprophan 150PM membranes and their clinical application have led to these membranes serving as reference membranes. Flat sheet and hollow fibre Cuprophan were reference membranes selected in this study.

4.1.2 HEMOPHAN:

Modification of cellulose has been used for a long time as a possible means of obtaining alternative haemodialysis (Courtney et al 1984). Chemical modification

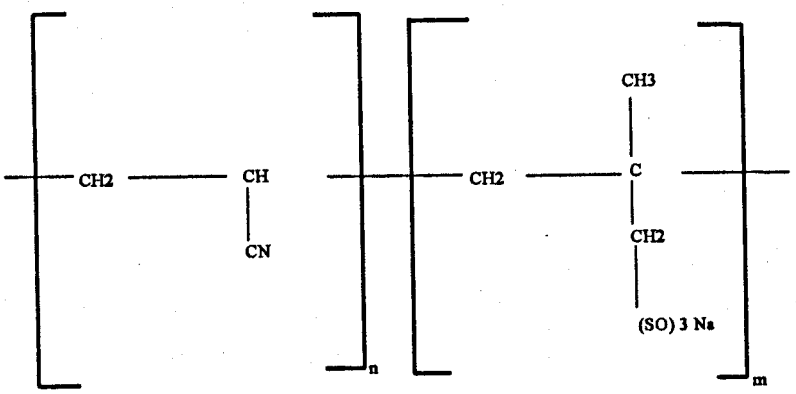


Fig. 4.3: Chemical structure of AN69S

has been achieved by the partial replacement of hydroxyl groups of cellulose with, for example, acetyl groups, resulting in a reduction in the effect of hydrogen bonding, increasing interchain separation and makes polymer less polar and therefore the reduction in the activation of the complement system (Farrell, 1984).

However, chemical modification can lead to membranes with different permeability characteristics. Another example of cellulose modification has been the manufacture by Akzo Nobel (Wuppertal, Germany) of a cellulosic membrane in which a percentage of the hydroxyl groups has been replaced by diethylaminoethyl (DEAE) groups. This membrane has been manufactured under the tradename Hemophan. DEAE-cellulose films were originally evaluated for heparinized membranes (Holland et al 1978; Schmitt et al 1983), since the introduction of DEAE renders the membrane cationic and suitable for the ionic attachment of the strongly anionic heparin.

With respect to Hemophan, the degree of modification is low, less than 5% of hydroxyl substitution (typically the degree of substitution of hydroxyl groups is about is about 1%). The low degree of substitution results in significantly reduced complement activation and leucopenia, than those found with cellulose acetate membranes and with respect to the immune responses, Hemophan appears markedly, better than Cuprophan (Travers, 1987; Robertson 1988).

The improvement in blood compatibility with respect to white blood cell changes and complement activation brought about by hydroxyl substitution may be offset, by a poorer performance in terms of other parameters (Robertson 1988). Modified cellulose membranes offer the considerable advantage over synthetic membranes of production under conditions similar to those of the well-established regenerated cellulose membranes. The structure of Hemophan is shown in figure 4.2.

4.1.3 POLYACRYLONITRILE-BASED MEMBRANES (AN69S AND SPAN)

Polyacrylonitrile-based membranes, are produced from a copolymer of acrylonitrile and sodium methallyl sulphonate, under the tradename of AN69S, by

Rhone-Poulenc, Hospal, S.A. Figure 4.3 shows the chemical structure of this polymer. AN69S membranes were the first synthetic membranes to be clinically used in haemodialysis. The AN69S membrane have a permeability properties markedly different from those of Cuprophan and permeability to solutes up to the 1000-2000 molecular weight range as compared to Cuprophan 150PM. The ultrafiltration rate is about 25 times greater than that of Cuprophan (Courtney et al 1984).

In general, AN69S membranes have been considered to provide improved blood compatibility in that their influence on leucocytes and the complement system is significantly less than that of regenerated cellulose. However, these materials require specialised monitoring equipment, because of high ultrafiltration rates and excessive fluid removal from patients, and their clinical application has been hindered.

SPAN is the tradename for a new type of acrylonitrile-sodium methallyl based membrane produced by Akzo Nobel (Wuppertal, Germany). Span is thought to exhibit different blood response properties to that of the established AN69 membrane (Vienken 1993).

4.1.4 POLYAMIDES:

Polyamide membranes NR and NRZ 14225 were supplied by Pall European process filtration Ltd (Portsmouth, UK) and were selected for evaluation in this study. The membranes had a porosity of 0.2 μm and zeta potential of -25.5 mV and -17 mV respectively at physiological pH. The polyamide membranes were chosen because of their well characterised surface properties and also because the membranes have been used as control materials in previous blood compatibility studies by other investigators (Travers 1987; Irvine 1989; Sundaram 1992; Yu 1993).

4.2 BLOOD-MEMBRANE CONTACTING PROCEDURES

4.2.1 INTRODUCTION

Test procedures for predicting the clinical performance of artificial surfaces in blood-contacting applications have been subject to a number of limitations (Bruck

1992). These are the establishment of reproducible procedures, the availability of reliable and sensitive assay techniques for the desired parameters, the condition of the blood to be used and the development of standard biomaterials of well characterized bulk and surface properties. The basic features of blood compatibility assessment are parameter selection, the method of achieving blood-material contact and the nature of the blood used (Forbes & Courtney 1993).

In summary however, there is no ideal procedure for linking an evaluation procedure for a test material to the potential clinical performance of that material. *In vitro* procedures enable the use of human blood but cannot take into account the interrelationship between the patient and the clinical use of the material (Lindsay et al 1980; Klinkmann et al 1987; Courtney et al 1993a) and do not normally consider the influence of the disease state (Andrade et al 1981). *Ex vivo* procedures using human blood focused on the evaluation of miniature devices. *In vivo* data obtained by implantation of test materials into animals for designated periods, must be interpreted with the recognition of species-related differences for blood components (Henson 1969; Grabowski et al 1977).

The selected method for assessing blood-biomaterial contact should ensure a blood response dependent on the artificial surface rather than the contact procedure and within the accuracy range of the measurement methodology. In general several methods of blood-material contact have been investigated. Tubular or hollow fibre materials have been investigated by using by flow through systems and by rocking (Lindsay et al 1973), rotation (Bowry et al 1982) or oscillation (Bowry et al 1984). Surfaces have been evaluated in the form of beads (Lindon et al 1978) or discs (Turitto & Leonard 1972). Test cells vary in complexity from incubation cells relevant for rapid screening (Yu et al 1991) to cells with closely controlled blood flow and wall shear rates relevant for more detailed investigation of blood-material interactions (Weng et al 1991; Lamba 1994). In this study, an incubation test cell and a controlled flow system were selected as the preferred methods.

4.2.2 A MODIFIED 6 WELL INCUBATION TEST CELL

The original method utilised a petri dish (Lemke 1985), but the use of multi-well plates became more common (Allen 1988; Irvine 1989). A 24 well polystyrene microplate (diameter 1.6 cm), has been tried but was found to be unsuitable on account of its relatively low surface area to blood volume ratio (Irvine 1989; Sundaram 1993; Yu 1993). The 24 well microplate was replaced with a 6 well polystyrene plate for the purpose of increasing the area of material in contact with blood or plasma. The 6 well polystyrene plate had a blood-contacting area of 9.6 cm^2 as compared to 2 cm^2 for the 24 well microplates.

The test cell is composed of a base plate made of polymethylmethacrylate (Perspex), a piece of soft silicone rubber, a 6 well polystyrene plate opened on the top and bottom and three bars for clamping and some covers (Fig 4.4). To set up the 6 well plate incubation test cell, the material to be tested is soaked overnight in 0.9% saline solution. The material was then placed in between the polystyrene 6 well plate and the silicone rubber sheet fixed on the Perspex base plate. The test cell was then clamped together with the three bars (Fig 4.4). Since the silicone rubber is soft, blood does not leak from the bottom after clamping. During blood test experiment, the test cell was set up before blood collection.

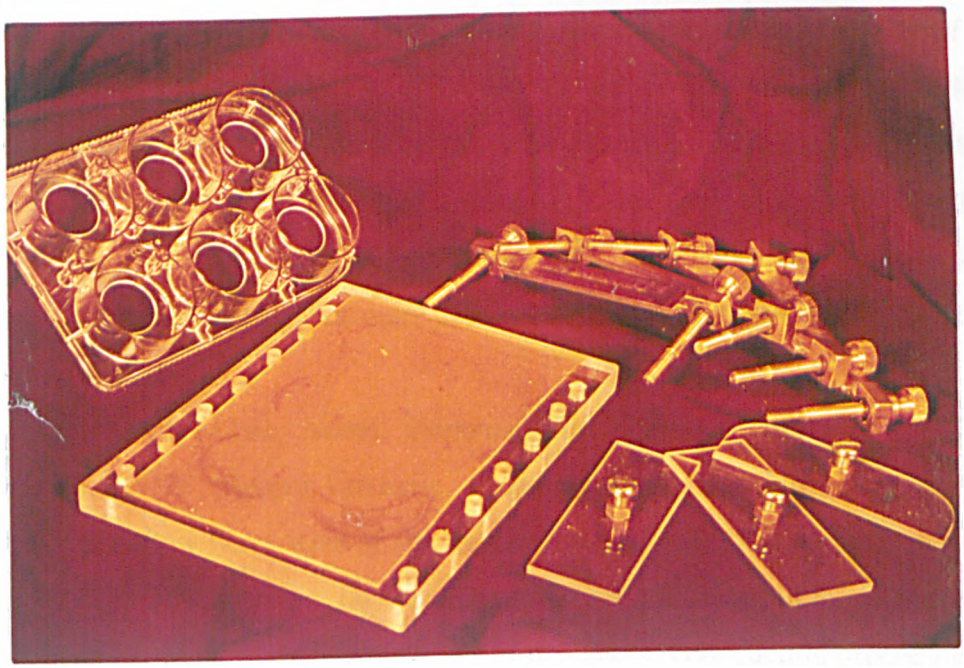


Figure 4.4: The 6 well modified incubation test cell.

4.2.3 CONTROLLED FLOW SYRINGE PUMP TEST SYSTEM.

4.2.3.1 Minimodule description

The minimodules consisted of 80 hollow fibres potted into a Perspex (polymethylmethacrylate) outer jacket 144 mm in length using medical grade polyurethane resin (PUR725A+634B, Morton International). Each hollow fibre tube sheet extended a length of 3 mm from the jacket end giving a total fibre length of 150 mm and an internal surface area of 75.4 cm^2 (for a fibre internal diameter of 200 μm). The fibre length exposed to dialysate or rinse solution was 134 mm. Blood headers were adapted from 5 ml polypropylene syringe barrels (Becton Dickinson) and bonded to the outer jacket using a cyanoacrylate adhesive and primer system (406 and 770, Loctite UK). Male luer fittings (874.10, Vygon UK) were similarly bonded into 4 mm Φ holes in the outer jacket to form dialysate inlet and outlet ports.

4.2.3.2 Materials.

Cuprophan (Akzo, Nobel, Germany) was retrieved from a Baxter ST15 dialyser. AN69HF (Hospal Industrie, France) was retrieved from a Filtral 20 dialyser. The hollow fibres were of similar internal diameter (200 μm) and dry wall thickness (11 μm). The hollow fibre membranes were assembled in modules for the blood-membrane contact procedure. Details of the module construction procedure are given below.

4.2.3.3 Module fabrication procedure

Modules were constructed according to the following sequence of operations:

1. A bundle of 80 fibres was tied at each end with cotton thread.
2. The bundle was then inserted into the outer jacket.
3. Moulding caps were then fitted onto the outer jacket.
4. The bundle ends were then bonded to caps with silicone rubber and cured for 24 hours.
5. Polyurethane was prepared and then degassed for 2 min, followed by the injection into one end cap to required level.

6. The was then was centrifuged at 700 rev/min, in a centrifuge of 50 cm diameter for 45 min at 30°C.

7. Steps 5 and 6 were repeated for the other end cap.

8. After 24 hour curing, the end caps were removed and polyurethane containing tube ends were cut off with lathe-mounted microtome blade using 30% glycerol/water as cutting fluid.

9. Blood headers and dialysate ports were then fitted.

4.2.3.4 Module rinsing

The modules were clamped vertically and connected to a 30 ml disposable syringe (Becton Dickinson, UK) containing saline, via a 3-way stopcock (Vygon UK Ltd). The syringe was mounted onto a non-pulsatile syringe pump (B Braun Melsungen) (Figure 4.5). 10 ml of saline were run through the module blood compartment at a flow rate of 1.2 ml/min. 2.5 ml of saline were allowed to filter through the membrane, by closing the outlet port. The remaining 7.5 ml of saline were allowed to flow through the module blood compartment, rinsing the fibres. The fibres were then primed with fresh saline, excluding air bubbles and dialysate compartment was filled with saline. The dialysate compartment was then closed.

4.2.3.5 Blood perfusion through module

Blood from normal healthy volunteers was drawn into a disposable syringe at the start of each experiment. 3.6 ml of this blood were then transferred into a tube containing 400 μl of 3.2% trisodium citrate (for the baseline values). The remainder of the blood in the syringe was mounted onto the syringe pump. 15 ml of blood were then passed through the module at a non-pulsatile flow rate of 1.2 ml/min. Samples were collected in 5 ml disposable syringes, and collection commenced soon after the displacement of the saline solution. 3.6 ml of blood were collected every 3 min interval for up to 12 minute after the start of perfusion.

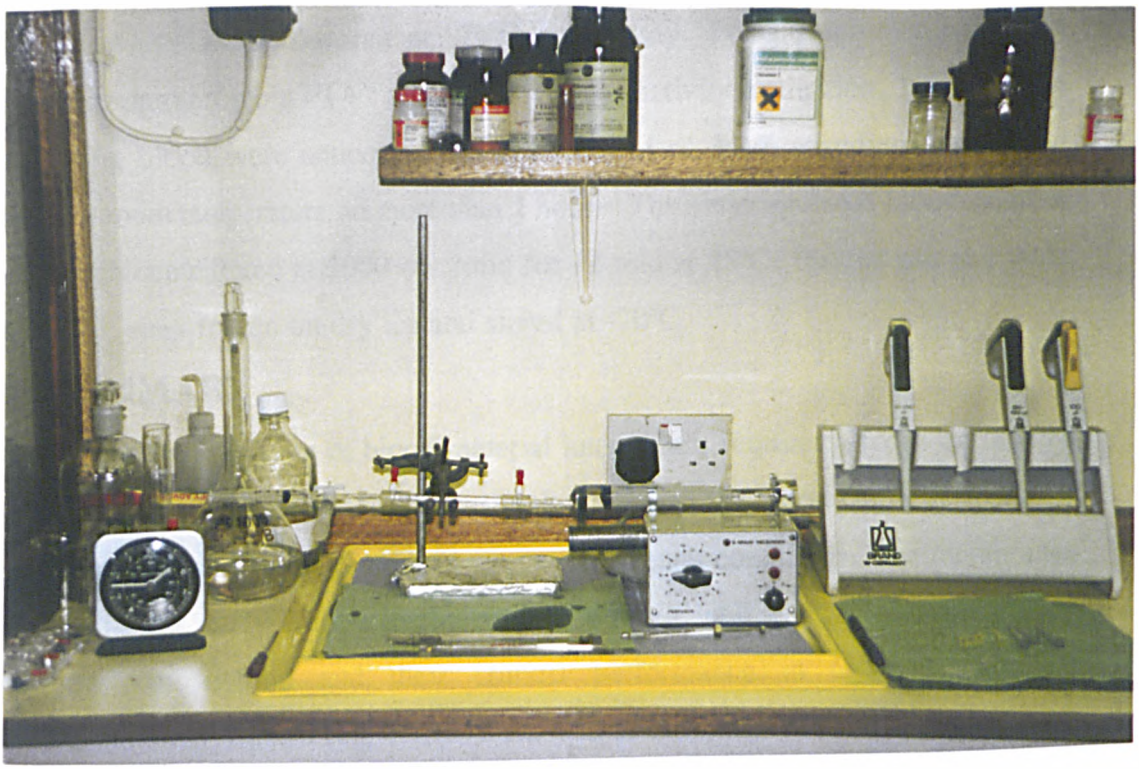


Figure 4.5: The controlled flow syringe pump system

4.2.3.6 Blood sampling

The collected samples were then anticoagulated appropriately depending on the parameter being evaluated. For complement C3a, 0.9 ml of the 3.6 ml aliquot was anticoagulated with 60 μl of 2.5% dipotassium EDTA and stored on ice or centrifuged at 3000 rev/min for 12 min at 4°C immediately. The plasma was snap frozen on dry ice and stored at -70°C until assay. For thrombin-antithrombin complexes, prothrombin F1+2 and factor XII-like activity evaluation, 1.8 ml of the remaining blood were anticoagulated with 200 μl of 3.2% trisodium citrate and stored at room temperature no more than 2 hours. The anticoagulated blood samples were then centrifuged at 3000 rev./min for 12 min at 25°C. Plasma samples were collected, snap frozen on dry ice and stored at -70°C.

4.3 SUMMARY

The investigation of blood-material interactions requires a focus on *in vitro* test procedures that allow the discrimination between the blood response from the host response, and a greater control over experimental conditions. The membranes chosen in this study were selected because of their well known features such as polymer composition and their clinical performance in terms of specific biocompatibility aspects (e.g complement C3a generation).

A 6 well incubation test cell system was chosen for the study of flat sheet membrane materials. Previous studies showed that the incubation test cell was quite suitable as a blood-contacting procedure for blood-membrane interaction evaluations (Sundaram 1992; Yu 1993; Wark 1993).

The syringe pump/controlled flow system was analogous to a parallel plate flow system used in previous study for flat sheet membrane materials evaluation (Lamba 1994). The parallel plate system was found to be very useful in the assessment of materials under conditions of controlled flow. Initial studies using hollow fibre minimodules on the syringe pump flow system, indicated that a clear discrimination between Cuprophan and AN69 membranes could be achieved for complement C3a, prothrombin F1+2 fragments, and thrombin-antithrombin III

complexes. On this evidence (table 4.1), the syringe pump controlled flow system was considered suitable for the study of contact phase activation as a blood response parameter using hollow fibre membranes.

Table 4.1: The patterns of Complement, TAT, FXIIA in hollow fibre membranes

Membrane	Indices	Pre %	3 min %	6 min %	9 min %	12 min %
AN69HF	C3a	100	189	207	372	378
	TAT	100	324	464	690	842
	FXIIA	100	94	98	97	96
Cuprophan	C3a	100	212	321	590	754
	TAT	100	118	231	235	494
	FXIIA	100	105	101	95.5	100

CHAPTER FIVE:

IN VITRO FACTOR XII-LIKE ACTIVITY (FXIIA) MEASUREMENT USING A CHROMOGENIC SUBSTRATE ASSAY

5.1 INTRODUCTION:

In view of the objective outlined in chapter 3, the emphasis in the current study was on measurement of activation of FXII as a marker of contact phase activation of blood coagulation. The importance of measurement of contact phase activation has been emphasized particularly by the biological activity of FXII in the initiating of the intrinsic pathway of coagulation, its association with complement, fibrinolytic and kallikrein- kinin systems. The importance of FXII in a variety of clinical disorders has been considered (chapter 3), and this supports the relevance of monitoring FXII activity during blood-biomaterials interaction.

5.2 METHODOLOGIES AVAILABLE FOR FXII ACTIVITY MEASUREMENT

The assessment of the activity of Hageman factor can currently be done by a number of assay techniques, basically designed for the detection of clinical coagulation disorders and they are designed for maximal activation of the protein. The methods include modifications of clotting time assays i.e activated clotting time (ACT) (Rhodes & Williams 1994) and the partial thromboplastin time (PTT), with factor XII-deficient plasma as the substrate. The disadvantages of these methods are that: commercial supplies of FXII-deficient plasma are expensive and also clotting assays have high coefficients of variation. In addition, the need for optimal activation of FXII using cephalin and kaolin, makes it unsuitable for investigation of blood-biomaterial interactions. Also, plasma samples with low levels of prekallikrein and/or HMWK can give abnormal FXII values.

Factor XII (Hageman factor) protein can also be assayed immunologically with monospecific antisera. Techniques like radial immunodiffusion (Revak et al 1974) and radioimmunoassays (RIA) have been reported (Saito et al 1976). The disadvantages of these techniques are: that information on the state of activation of the protein cannot be obtained and can yield erroneous results if FXII is fragmented. A double antibody enzyme linked immunosorbent assay (Elisa) which

measures complexes of activated FXII with C1-INH, has also been reported (Kaplan et al 1985; Nuijens et al 1988). The disadvantages of using Elisa's and RIA's for FXIIa-C1-INH complexes are:

(a) The preparation of specific antibodies is time-consuming, expensive and requires specialised techniques.

(b) The procedures involving prolonged incubation times are less suitable for routine assessment.

(c) The level of sensitivity which can be achieved by these assays is questionable, since the FXII molecule can change conformation, such that antibodies may not be entirely specific and that C1-INH also inhibits other proteases, such as plasmin, FXa, C1s and C1r, therefore reducing the level of specificity of the assay (Bouma and Griffin 1986).

The availability of chromogenic substrate assays has offered a logical approach to the establishment of an assay procedure for measurement of contact phase activation, resulting from blood-biomaterials interactions, given the potential disadvantages of clotting and immunological assays. Such an approach was therefore adapted for this study. The principle of the method was based on the fact that the binding site between an oligopeptide and p-nitroaniline (p-NA) is split by serine proteases (Hemker 1983).

The assay of activated FXII by chromogenic substrates has in the past been hampered by the unavailability of specific substrates. As a consequence, indirect methods of measurement have been widely used, based on the demonstration of the ability of FXIIa to convert prekallikrein (PK) to kallikrein (KK) (Kaplan 1978). The advantage of this approach in the ability to detect all the various forms of activated factor XII and the Kallikrein generated can be accurately determined. The substrate most widely used for indirect measurement has been S2302 (Kabi Vitrum Ltd, Molindal, Sweden) (Vinazzer 1979; Egberg and Overmark-Berggren 1983), although chromozym PK has also been evaluated (Klessen et al 1982).

The direct methods for assay of FXII activity are based on the use of the substrate S-2222 (Kabi Vitrum Ltd, Molindal, Sweden) (Gallimore et al 1987; Walshe et al 1987). Although essentially this substrate is for FXa, by the use of selective inhibitors, this substrate can be used for the determination of FXIIa. With

advent of fourth generation substrate incorporating synthetic amino acids to the peptide structure, substrates with enhanced selectivity have become available (Stürzebecher et al 1989; Gallimore 1990). One such substrate is the 2-AcOH-H-D-CHT-GLY-ARG-PNA (Channel diagnostics, Walmer, Kent, UK) was used in this study. The substrate is twice as selective as S2222 for α- and β-FXIIa versus kallikrein, and 4 times as sensitive (Sundaram 1992), and as a result was preferred for this study. The flat sheet membranes evaluated in this study were Cuprophan, Hemophan, AN69S and SPAN with polyamide NR and NRZ as controls. Hollow fibre modules were constructed from Cuprophan (Baxter ST15, Akzo Nobel, Wuppertal, Germany) and AN69HF (Filtral 20 dialyser, Hospal, France) and evaluated in this study.

5.3 EXPERIMENTAL DESIGN

The measurement of factor XII-like activity (FXIIA) was undertaken under different experimental models aimed at achieving particular objectives as shown on table 5.1 and subsequent comparisons were attempted. For measurements using whole blood each experiment was performed with blood from different donors on the same materials, to minimise the effect of donor blood variability. Pooled platelet poor plasma was utilised for the plasma exepriments.

5.4 MEASUREMENT OF FXIIA USING NON-ANTICOAGULATED BLOOD (NATIVE WHOLE BLOOD SYSTEM)

5.3.1 6 WELL INCUBATION TEST CELL SYSTEM

Blood was obtained from the median cubital vein of healthy volunteers who had not taken any antiplatelet medication for at least 14 days prior to the study. Collected blood was transferred into a polypropylene vial and then quickly, 1.5 ml of the blood were pipetted into the test cell wells. 1 ml blood aliquots were removed every 3 min (for up to 18 min in some cases) from the test cells into polypropylene vials containing 0.11 ml trisodium citrate. The anticoagulated blood was then centrifuged at 2000 x g for 15 min at 25°C and the resultant plasma was snap frozen using dry ice, and stored at -70°C until assay (section 5.4.3).

Table 5.1: Experimental design

Method	Model	Contact procedure	Materials assessed	Main objective
1. FXIIa	Whole blood	Incubation test cell	A, B, F	Comparison of supernatant FXIIA as measured in static conditions
2. FXIIA	Whole blood	Controlled flow system	H, I	FXIIA measured under flow conditions, to determine the effect of improved surface/blood FXII exchanges
3. FXIIA	Plasma	Incubation test cell	A,B,C,D,F	Comparison of plasma supernatant and membrane-bound FXIIA as a model for a complex system
4. FXIIA	Purified unactivated FXII	Incubation test cell	A,B,C,D,E	Comparison of supernatant and membrane-bound FXIIA as a model for a simplified system
5. FXIIA	Purified unactivated FXII	Incubation test cell	C,D	To determine the kinetics of FXII activation on negatively-charged membranes
6. FXIIA	Plasma	Incubation test cell	A,B,C,F,G	To determine the effect of prolonged contact between plasma and membranes
7. FXIIA	Plasma	Incubation test cell	A,B,C,F,G	the acidification of plasma to limit the activities of plasma inhibitors

A = Cuprophan, B = Hemophan, C = AN69S, D = SPAN, E = Polystyrene, F = polyamide NR, G = polyamide NRZ, H = Cuprophan (hollow fibre modules), I = AN69HF (hollow fibre modules).

5.4.2 CONTROLLED FLOW/SYRINGE PUMP SYSTEM

The blood-contacting and sampling procedures have been described in chapter 4. 1.8 ml of blood aliquots were anticoagulated with 200 μl of 3.2 % trisodium citrate. Haematocrit readings were taken out of all the citrated blood samples. The blood samples were then centrifuged at 2000 x g for 15 min at 25^{o}C. All the plasma samples were snap frozen and stored at -70 ^{o}C before assay as described in section 5.4.3.

5.5 SELECTED METHOD FOR MEASUREMENT OF FXII-LIKE ACTIVITY USING PLASMA (PLASMA SYSTEM).

As reported in chapter 3, the use of the chromogenic substrate method to evaluate FXII activation following blood-biomaterial contact has been extensively studied (Irvine 1989; Sundaram 1992; Yu 1993; Lamba 1994). These *in vitro* studies, however, by measuring FXII-like activity in the fluid phase (supernatant), reported that distinction between surfaces was difficult to achieve. In this thesis, it was felt that modification of the assay for FXIIA in the plasma supernatant so as to detect the active componets bound to the surface of the material of interest was necessary. In order to achieve this objective, plasma, rather than whole blood was chosen as the medium for contact with materials of interest. The advantages of utilising plasma rather than the whole blood was the availability of a washing procedure for the membranes after plasma contact. The findings were compared to those utilising whole non-anticoagulated blood (native blood).

5.5.1 PLASMA-MEMBRANE CONTACT:

Blood was collected from healthy volunteers and was anticoagulated in heparin (1.0 U/ml) or using 3.2 % trisodium citrate at 1:9 ratio with blood. The blood samples were then centrifuged at 2000 x g for 15 minutes at room temperature to obtain platelet poor plasma.

5.5.2 PLASMA MEMBRANE-BOUND FXIIA.

An incubation 6 well plate method (section 4.2) was employed to achieve plasma-material contact. 1 ml of heparinised or citrated plasma was then added to the materials of interest and incubated for 10 minutes at room temperature. Adequate mixing was obtained by using an orbital shaker. Prior to contact, all

materials were soaked in 0.9% saline for at least 24 hours.

At the end of the incubation period, 200 μl of plasma were withdrawn for assay (designated assay of supernatant). The remaining plasma was then removed and the membrane washed in 1.0 ml of buffer (Tris HCl 0.025 M, NaCl 0.025 M, pH 8) for 1 minute. Wash solution was removed and 1.0 ml of kallikrein inhibitor designated "KI" (Soybean trypsin inhibitor) (Channel diagnostics, Walmer, Kent), diluted at 1 / 50 in buffer was added to the material and incubated for 10 minutes. The inhibitor was removed and 1.0 ml of substrate (Channel Diagnostics, Walmer, kent) 1.0 μmol/ml (in distilled water) was added to the material and incubated for 20 minutes. Substrate and kallikrein inhibitor were prewarmed to 37^{0}C, for half an hour prior to use.

900 μl of the substrate were withdrawn into polystyrene cuvettes (1.0 cm^3) and absorbance read at 405 nm against a suitable blank (900 μl of buffer) using a Dynatech MR 5000 spectrophotometer (Dynatech Ltd, Billinghurst, Sussex, UK). A polystyrene tissue culture plate normally coated with a layer of oxygen ions, therefore negatively charged, was also used as a control material (ICN Biomedicals, Thame, Oxfordshire, UK).

5.5.3 ASSAY PROCEDURE FOR PLASMA SUPERNATANT:

200 μl of supernatant obtained earlier were diluted 1:3 in buffer and assayed using a microtitre format (96 well plates). To 25 μl of diluted plasma, 75 μl of kallikrein inhibitor (KI) in buffer were added and incubated for 1 minute. The reaction was started by addition of 50 μl of substrate and was incubated for 10 minute; and terminated by the addition of 50 μl of 50% acetic acid. Absorbance was read at 405 nm using a Dynatech MR5000 microtitre plate reader (Dynatech Ltd, Billinghurst, Sussex, UK) against suitable blanks (200 μl of acetic acid (50%) and 700 μl buffer).

5.6 PROCEDURE FOR MEASUREMENT OF ACTIVATED FXII, USING PURIFIED FXII (PURIFIED SYSTEM)

An incubation method previously mentioned, was also used to achieve FXII solution-material contact. Purified unactivated FXII (diluted to 25 μg/ml) was purchased from Enzyme Research Laboratories Int, Swansea, UK. 1 ml of the diluted protein solution was added to the materials of interest and incubated for 2

hours at room temperature. Incubation of 2 hours was selected based on the fact that autoactivation of FXII in the absence of other contact proteins (HMWK, PK, FXI) proceeds at a slower rate (Tans and Rosing 1987). Adequate mixing was obtained by using an orbital shaker. Prior to contact all the materials were soaked in 0.9% saline for at least 24 hours.

5.6.1 MEMBRANE-BOUND PURIFIED FXIIA

At the end of the incubation period, 100 μl of FXII solution were withdrawn for assay (designated assay of supernatant). The remaining FXII solution was removed and the membrane washed in 1.0 ml of buffer (Tris HCl 0.025 M, 0.025 M NaCl, pH 8) for 1 minute. Wash solution was removed and 1.0 ml of substrate (2-AcOH-H-D-CHT-GLY-ARG-PNA, Channel Diagnostics, Walmer, Kent) was added to the material and incubated for 10 minutes. 900 μl of substrate was withdrawn into polystyrene cuvettes (BDH Ltd, Glasgow, UK) and absorbance read at 405 nm against suitable blanks (900 μl of buffer).

5.6.2 ASSAY OF SUPERNATANT PURIFIED FXII SOLUTION:

To 100 μl of supernatant obtained earlier, 400 μl of buffer were added (prewarmed to 37°C). 200 μl of substrate (prewarmed to 37°C) was added and the mixture incubated in a polystyrene cuvette (BDH Ltd, Glasgow, UK) at room temperature for 10 minutes. Reaction stopped by addition of 200 μl of 50% acetic acid. Absorbance was read at 405 nm against suitable blanks (700 μl of buffer and 200 μl of 50% acetic acid).

5.6.3 TIME COURSE FOR GENERATION OF FXII ACTIVITY USING PURIFIED FXII

In an attempt to establish a simple relation between the kinetics of activation of FXII and the differences in membrane surface characteristics on negatively charged polymers AN69S and SPAN, a time course study of FXIIa generation was performed. This was done by incubating 1.0 ml of source (25 μg/ml) at various times 3, 5, 10 , 30 and 60 minutes after contact of FXII solution with membrane. The materials of interest selected for this study were the negatively charged polymers AN69S and SPAN. The 6 well incubation method was employed to achieve contact, and incubation was at room temperature. FXII activity was assayed as described in sections 5.6.2 and 5.6.3.

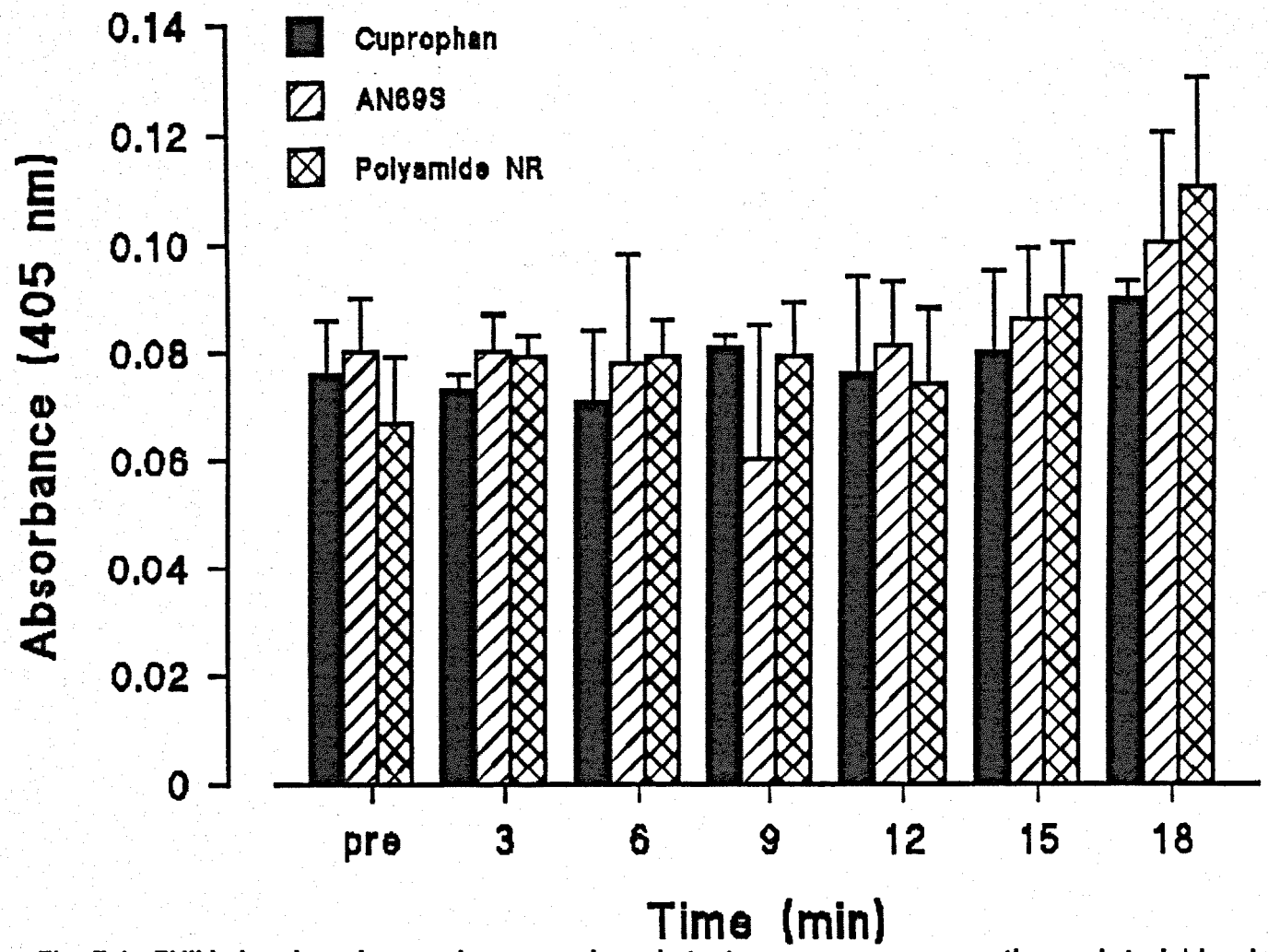


Fig 5.1: FXIIA levels using a chromogenic substrate assay on non-anticoagulated blood, n=6.

5.7 DETERMINATION OF THE EFFECT OF PROLONGED CONTACT BETWEEN PLASMA AND MEMBRANES ON THE SUPERNATANT FXIIA.

Supernatant plasma factor XII activity has been studied by other groups after the incubation of segments of materials for 1 hour in citrated plasma (Campbell et al 1993; Rhodes 1992). There was therefore a requirement for an investigation to be conducted on the effect of long incubations of plasma on different membranes. The plasma supernatant FXIIA measurements were performed after incubating citrated plasma for 15, 30, 60, 90, and 120 minutes as described on section 5.6.2. Plasma instead of whole blood was preferred for this experiment to avoid the effect of shear haemolysis on red cells with the consequence of released haemoglobin affecting the accuracy of the assay.

Similarly, aliquots of the citrated plasma were heparinised at 2.5 U/ml and then incubated at 15, 30, 60, 90, and 120 minutes respectively. The FXIIA values of the plasma supernatant were assayed as described on 5.6.3.

5.8 THE EFFECT OF PLASMA ACIDIFICATION ON SUPERNATANT FXIIA USING THE CHROMOGENIC SUBSTRATE ASSAY.

The procedure was based on the principle that mild acidification (using cold acetone) of plasma inactivates plasma protease inhibitors, particularly C1-INH (Scott et al 1987; Walshe et al 1987; Gallimore et al 1987; Irvine 1989). However, the accuracy of the assays utilising acetone, have been highly controversial, since acetone was found to substantially activate factor XII-like activity (Sundaram 1992). 1.5 ml of fresh blood (heparinised and non-heparinised) were contacted to membranes and aliquots were removed at 3, 6, 9, 12, 15, 18 minutes of incubation, anticoagulated with trisodium citrate and plasma processed as described on section 5.4.1. 600 μl aliquots of the plasma were diluted with 1200 μl of acidified Tris buffer (0.05 M Tris HCl, 0.12 mM Methylamine, 9.7 mM disodium EDTA, pH 5.3) and mixed well and stored at room temperature for 30 minutes before assay. The diluted plasma was then assayed for factor XII-like activity as described in section 5.6.1.

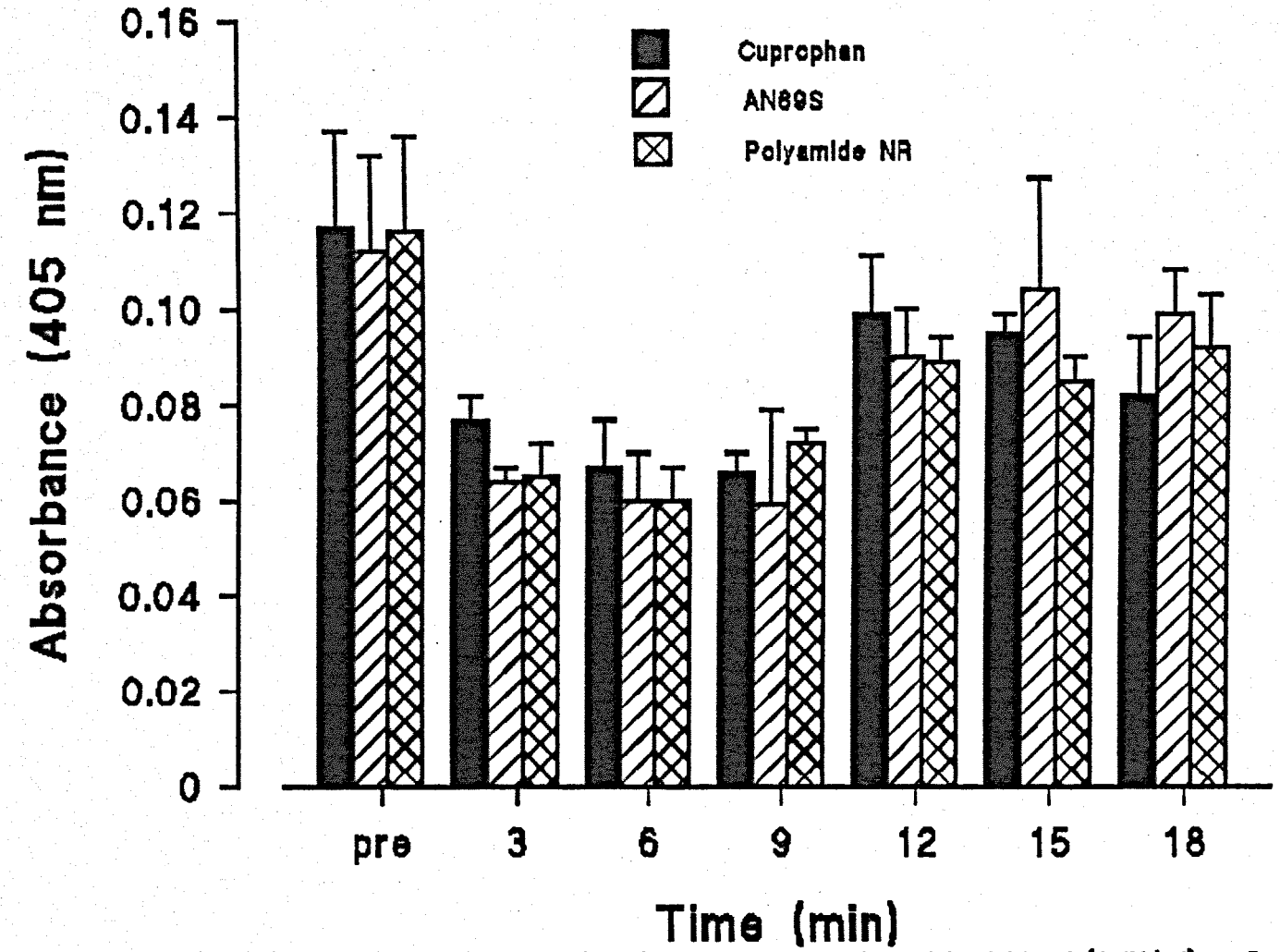


Fig 5.2: FXIIA levels using a chromogenic substrate assay on heparinised blood (1 IU/ml), n=6.

5.9 STATISTICS

Statistical analysis was performed using Minitab package (version 8.0). In order to represent the patterns of a polymer influence on FXII activation, the actual mean values were used. Comparisons of the differences between two means were carried out by the twosample student's t-test and these were reported at 95% confidence intervals ($p < 0.05$) as well as the Mann-Whitney test for differences in the medians of the values. A oneway analysis of variance was also used for the comparison of mean values for factor XII-like activity with results reported as significantly different at $p < 0.05$ (95% confidence interval).

5.10 RESULTS

5.10.1 FXIIA USING WHOLE BLOOD ON FLAT SHEET MEMBRANES

The FXIIA values appeared to remain the same at the start of the experiments and gradually rose above baseline levels at 15-18 minutes incubation time (table 5.2, figure 5.1). The FXIIA differences between the membranes at same time point were not significant ($p > 0.05$). However, the FXIIA values for the heparinised blood samples remained consistently lower than baseline values throughout the duration of blood-membrane contact (table 5.3, figure 5.2).

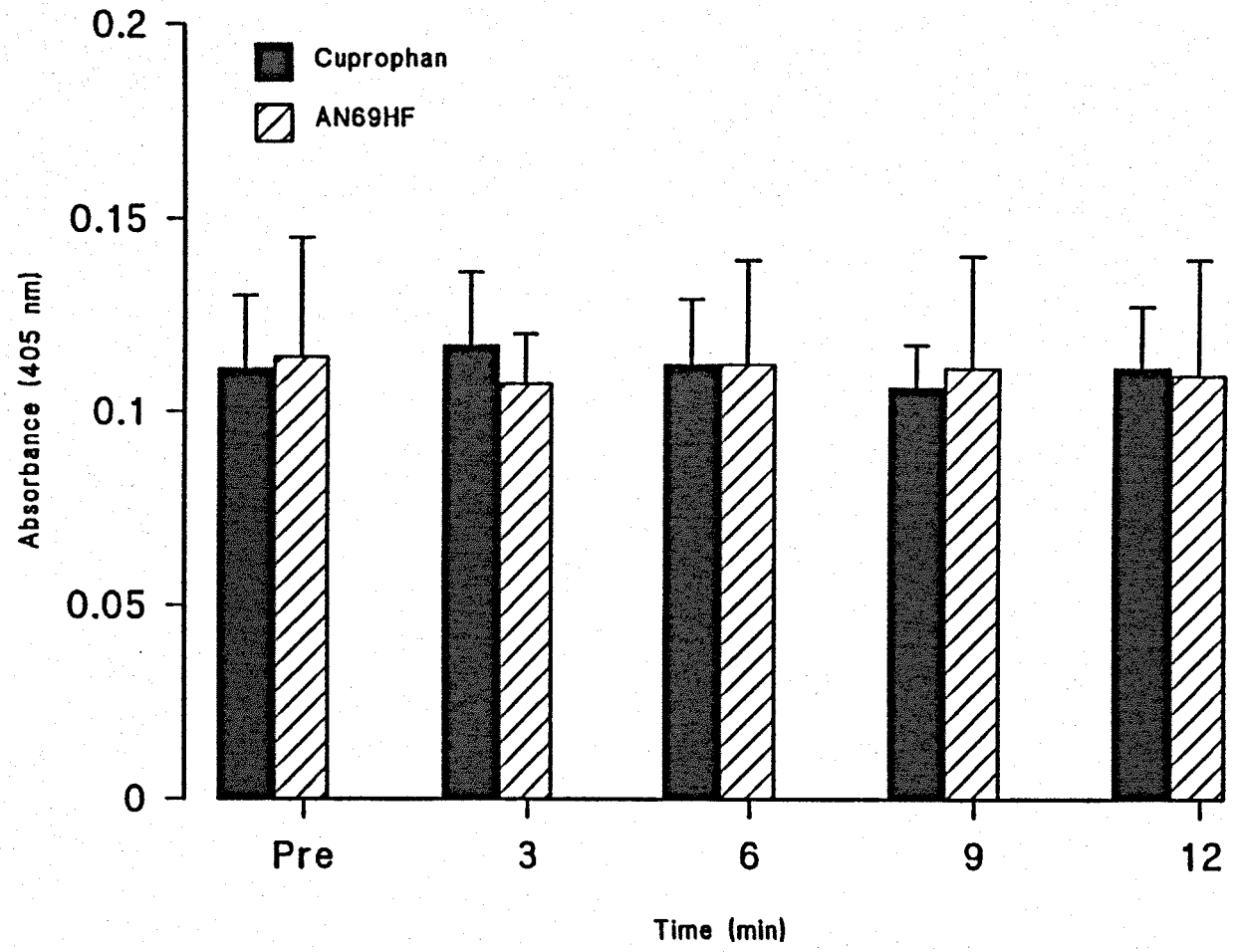


Figure 5.3: FXIIA levels using non-anticoagulated whole blood on hollow fibre modules (n=14)

Table 5.2: FXIIA levels at different time intervals using non-anticoagulated whole blood with Cuprophan, AN69S, polyamide NR flat sheet membranes on the incubation test cell,n=6.

Time (min)	Absorbance readings ± SD at 405 nm		
	Cuprophan	AN69S	Polyamide NR
pre	0.076 ± 0.010	0.076 ± 0.010	0.067 ± 0.010
3	0.073 ± 0.003	0.080 ± 0.007	0.079 ± 0.004
6	0.071 ± 0.013	0.078 ± 0.020	0.079 ± 0.007
9	0.081 ± 0.002	0.060 ± 0.025	0.079 ± 0.010
12	0.076 ± 0.018	0.081 ± 0.012	0.074 ± 0.014
15	0.080 ± 0.015	0.086 ± 0.013	0.090 ± 0.010
18	0.090 ± 0.003	0.100 ± 0.02	0.110 ± 0.020

Table 5.3: FXIIA levels at different time intervals using heparinised whole blood with flat sheet membranes on incubation test cell, n=6.

Time (min)	Absorbance readings ± SD at 405 nm		
	Cuprophan	AN69S	Polyamide NR
Pre	0.117 ± 0.020	0.112 ± 0.020	0.116 ± 0.020
3	0.077 ± 0.005	0.064 ± 0.003	0.065 ± 0.007
6	0.067 ± 0.010	0.060 ± 0.010	0.060 ± 0.007
9	0.066 ± 0.004	0.059 ± 0.020	0.072 ± 0.003
12	0.099 ± 0.012	0.090 ± 0.01	0.089 ± 0.005
15	0.095 ± 0.004	0.104 ± 0.023	0.085 ± 0.004
18	0.082 ± 0.012	0.099 ± 0.009	0.092 ± 0.011

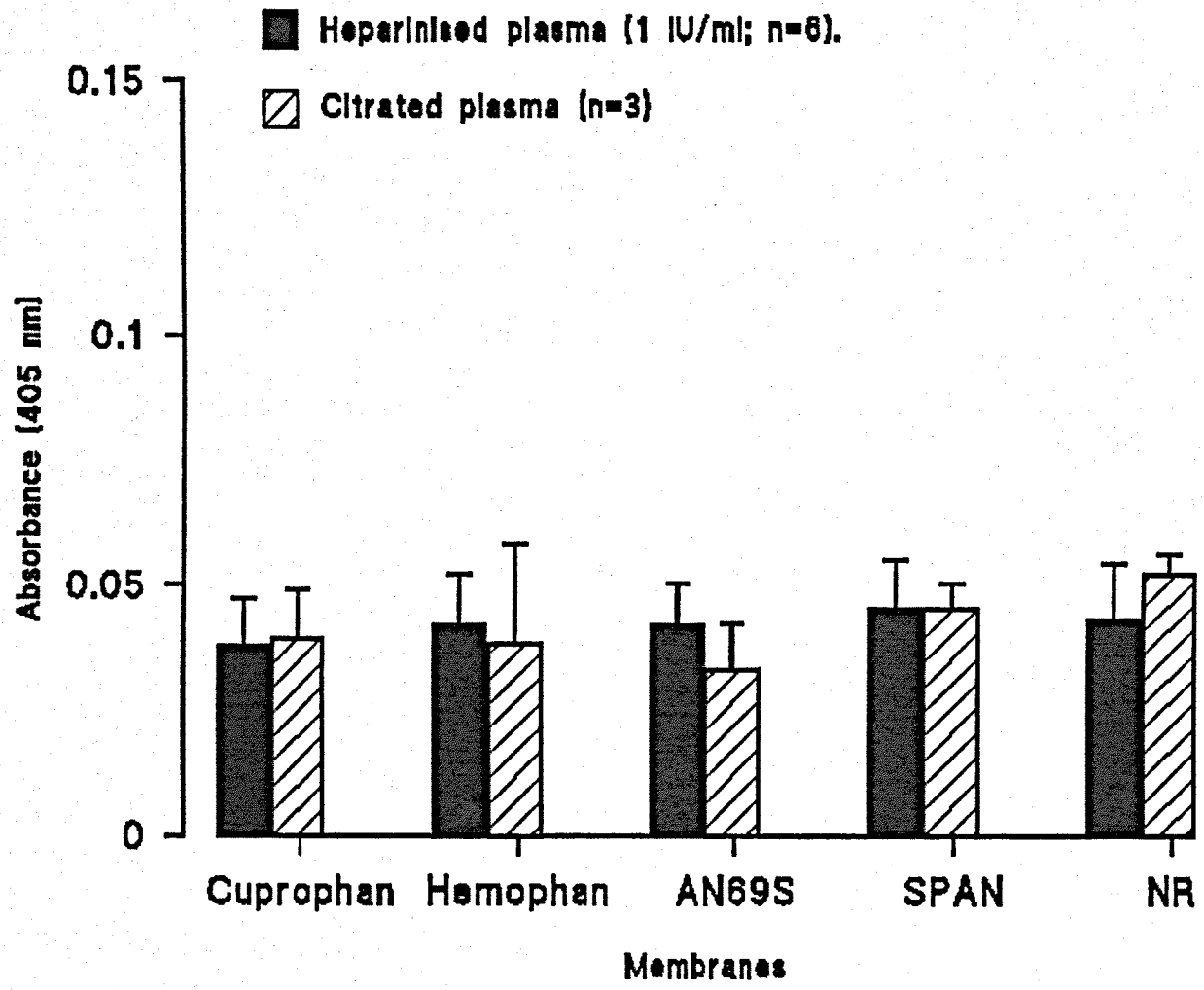


Figure 5.4: Plasma supernatant FXIIA (Plasma system (n=3).

5.10.2 FXIIA FOR HOLLOW FIBRE MODULES USING NATIVE WHOLE BLOOD

There were no significant differences ($p > 0.05$) between FXIIA in Cuprophan and AN69HF modules at any particular time point of blood perfusion. The FXIIA values remained closer to baseline values as compared to the values obtained using the incubation test cell system (table 5.4, figure 5.3).

Table 5.4 FXIIA levels at different times using hollow fibre modules of Cuprophan and AN69HF membranes, n=14.

Time (min)	Absorbance ± SD (405 nm)	
	Cuprophan	AN69HF
pre	0.111 ± 0.019	0.114 ± 0.031
3	0.117 ± 0.019	0.107 ± 0.013
6	0.112 ± 0.017	0.112 ± 0.027
9	0.106 ± 0.011	0.111 ± 0.029
12	0.111 ± 0.016	0.109 ± 0.030

5.10.3 PLASMA SYSTEM SUPERNATANT FXIIA RESULTS:

As shown on table 5.5 and figure 5.4, the assays using citrated plasma as source of FXII, indicated that the mean value of the FXIIA in the supernatant or the fluid phase were not significantly different ($p > 0.05$) with the different membranes. For the heparinised plasma, the supernatant FXIIA remained of similar pattern as the citrated plasma described above.

5.10.4 PLASMA MEMBRANE-BOUND FXIIA RESULTS

In contrast to the fluid phase FXIIA observed when using plasma, the membrane-bound component appeared to be markedly potent, and that the magnitude of the activity was different for each membrane material (table 5.6 and figure 5.5). When citrated plasma was used, Cuprophan exhibited higher activating ability than the either Hemophan, SPAN or AN69S membranes.

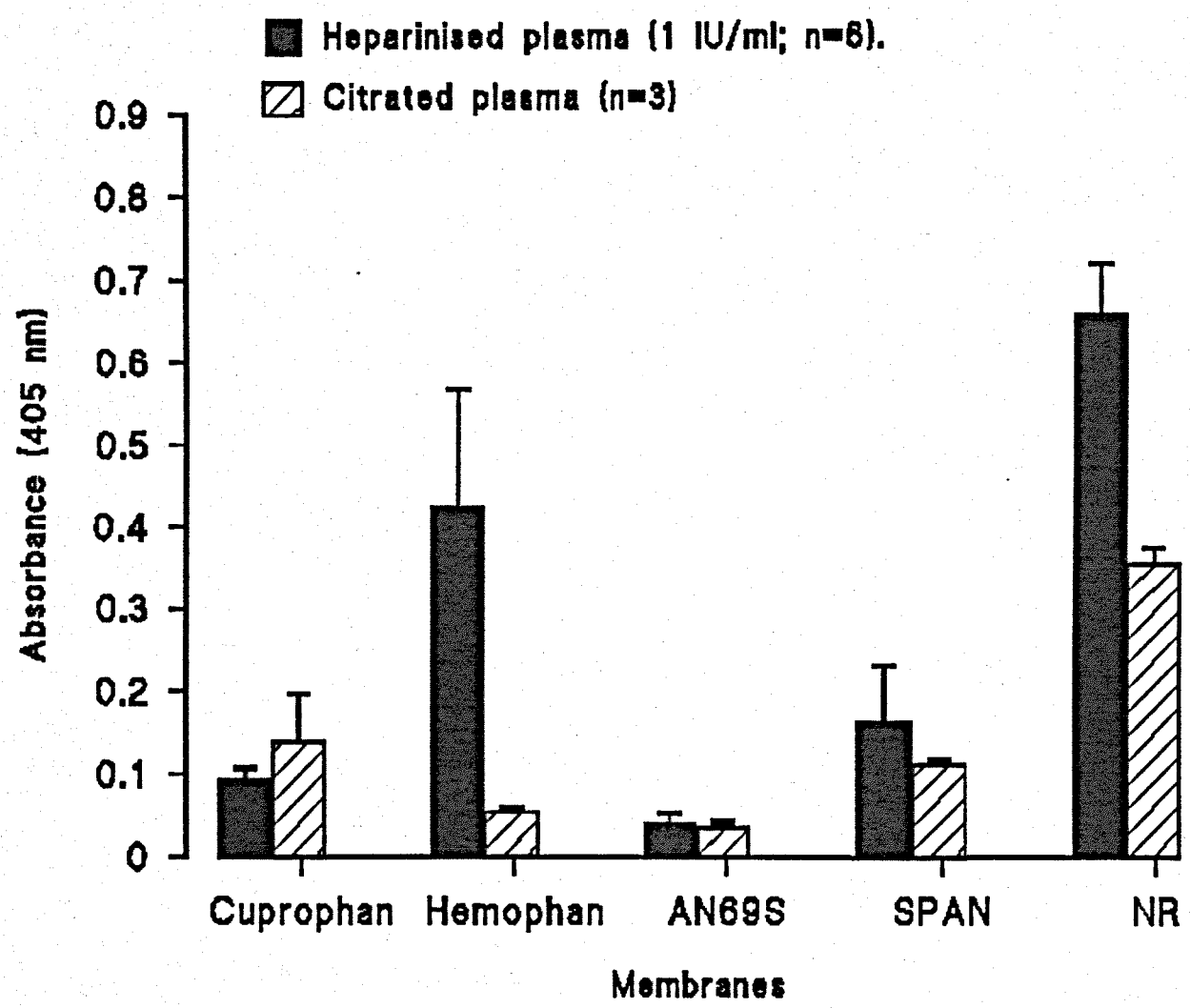


Figure 5.5: Plasma membrane-bound FXIIA (n=4).

Table 5.5: FXII-like activity in supernatant FXII (FXIIA) as measured using plasma as the source of FXII: (Plasma system).

MEMBRANES	Absorbance readings ± SD (405 nm)	
	Heparinised plasma, n=6	Citrated plasma, n=3
CUPROPHAN	0.038 ± 0.009	0.039 ± 0.01
HEMOPHAN	0.042 ± 0.01	0.038 ± 0.02
AN69S	0.042 ± 0.008	0.033 ± 0.009
SPAN	0.045 ± 0.010	0.045 ± 0.005
POLYAMIDE (NR)	0.043 ± 0.011	0.052 ± 0.004

Table 5.6: FXII-Like activity of membrane-bound FXII as measured using plasma as source of FXII. (Plasma system), n=4

MEMBRANES	Absorbance readings ± SD (405 nm) n=3	
	Heparinised plasma alone	Citrated plasma alone
CUPROPHAN	0.093 ± 0.014	0.138 ± 0.057
HEMOPHAN	0.423 ± 0.145	0.054 ± 0.006
AN69S	0.041 ± 0.012	0.035 ± 0.008
SPAN	0.165 ± 0.065	0.112 ± 0.006
POLYAMIDE (NR)	0.658 ± 0.062	0.354 ± 0.020

The polyamide NR control exhibited a significantly ($p < 0.05$) higher FXIIA than all the other membranes. AN69S indicated the least FXIIA irrespective of its strong negative charge. For the heparinised plasma, Hemophan, showed a significantly higher ($p < 0.05$) membrane-bound FXIIA than either Cuprophan, AN69S or SPAN. Polyamide NR exhibited the highest membrane-bound FXIIA.

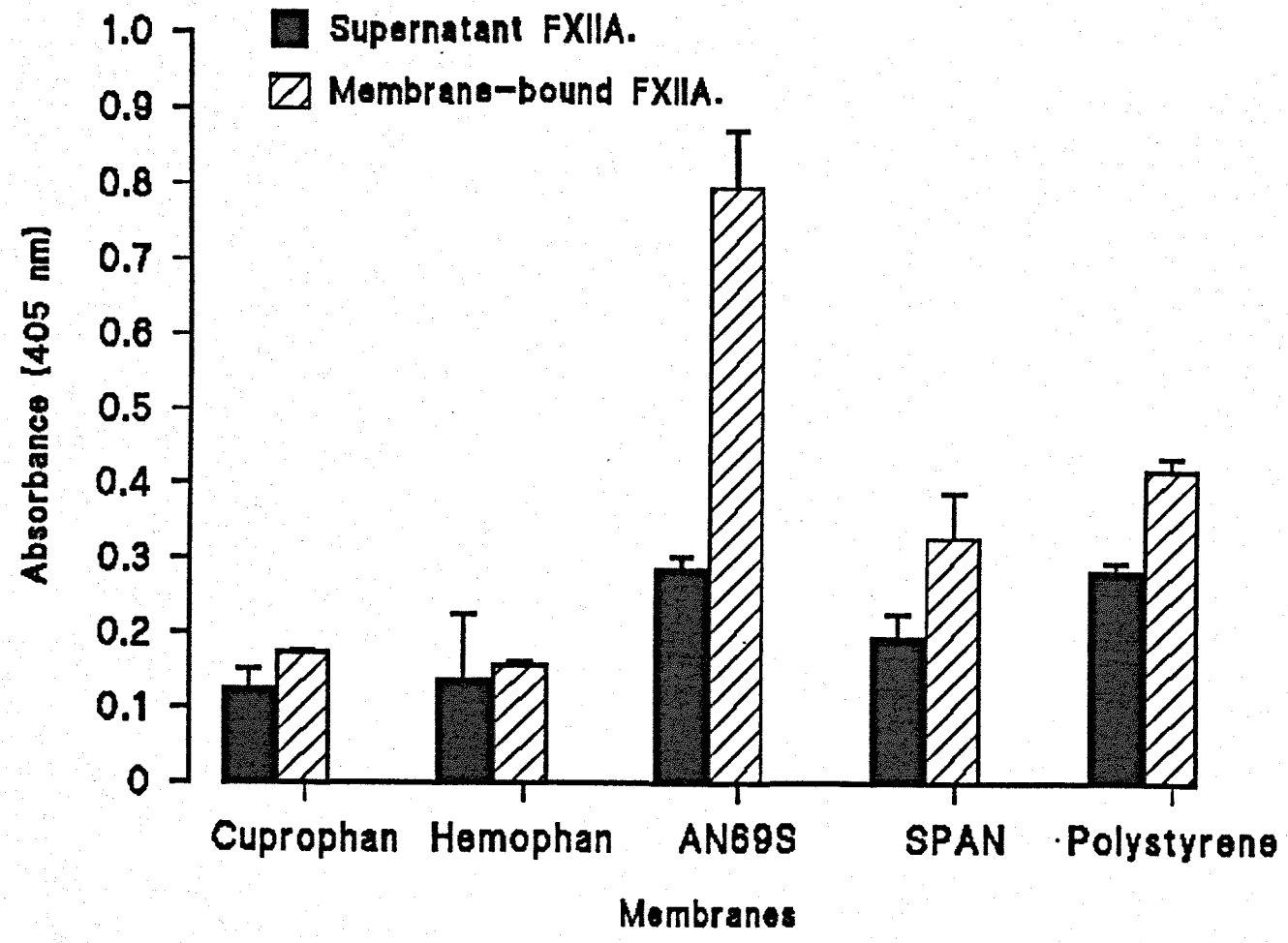


Figure 5.6: Purified factor XII supernatant and membrane-bound FXIIA (n=3).

5.10.5 PURIFIED FXII SUPERNATANT FXIIA RESULTS

As shown on figure 5.7 and table 5.6, the supernatant phase FXIIA appeared to be the lowest with Cuprophan, followed by Hemophan. The FXIIA in the supernatant was markedly higher on AN69S, followed by the negatively charged polystyrene and SPAN. All the negatively charged membranes appeared to have a significantly higher FXIIA activity than the cellulosic membranes ($p < 0.05$).

5.10.6 MEMBRANE-BOUND PURIFIED FXII RESULTS

The patterns of the membrane-bound FXIIA values were similar to the supernatant values with Cuprophan exhibiting a slightly higher membrane-bound FXIIA than Hemophan, whereas as compared to AN69S its activating potential was significantly ($P < 0.05$) lower (Table 5.7, Figure 5.6). This agrees with the general view that negatively charged surfaces are much stronger activators of FXII (Revak et al 1978). As compared to AN69S, SPAN was only approximately half as strong an activator of membrane-bound FXII.

Table 5.7: FXII activity using purified FXII (Plasma-Free system), n=3.

MEMBRANES	Absorbance (405 nm) n=3	
	Supernatant FXIIA	Membrane-bound
CUPROPHAN	0.125 ± 0.026	0.173 ± 0.004
HEMOPHAN	0.138 ± 0.087	0.156 ± 0.006
AN69S	0.286 ± 0.015	0.793 ± 0.074
SPAN	0.194 ± 0.031	0.326 ± 0.06
Polystyrene	0.282 ± 0.104	0.416 ± 0.017

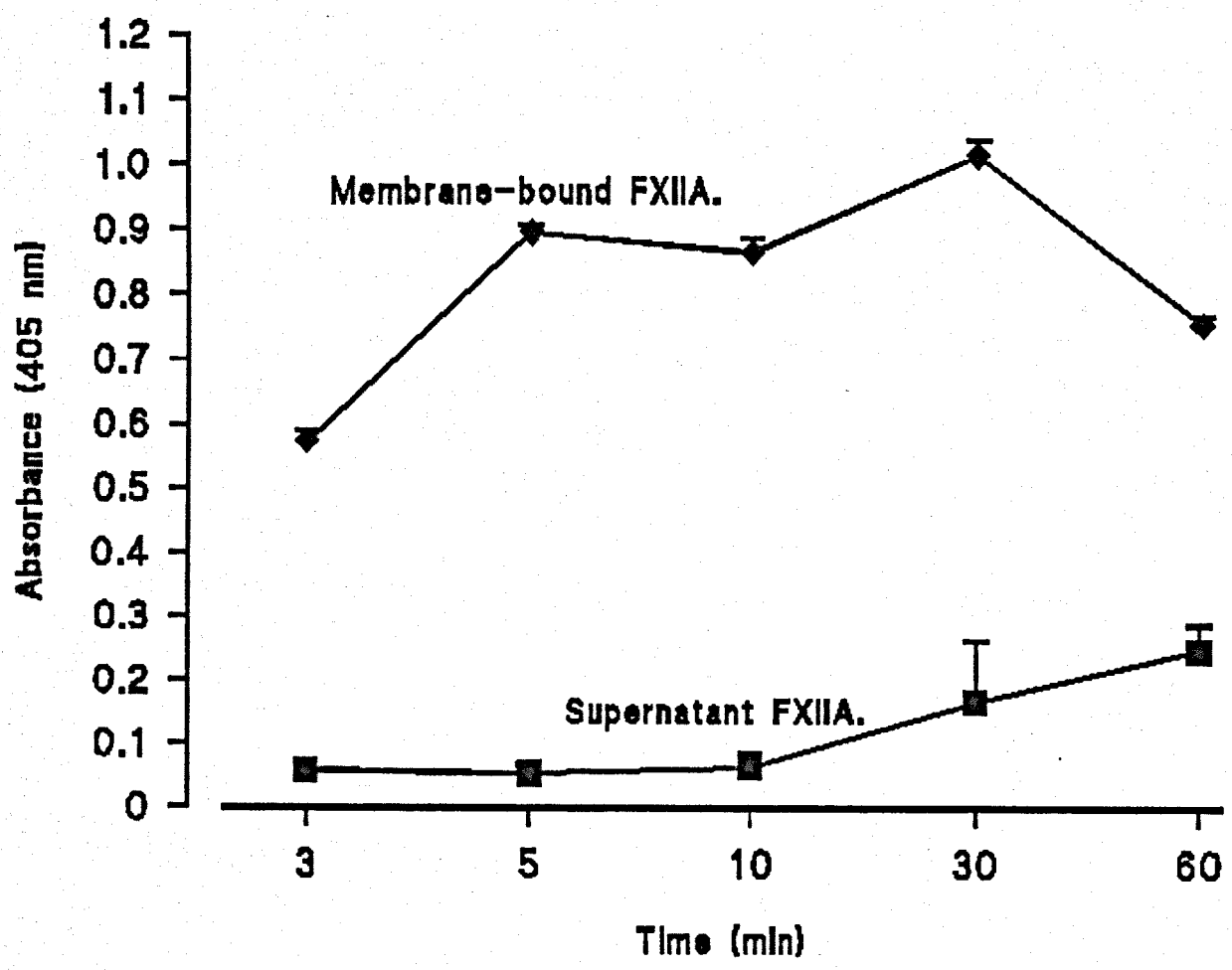


Figure 5.7: Time course for FXIIA generation on AN69S, n=2 (at 18-23°C).

5.10.7 TIME COURSE FOR THE GENERATION OF FXIIA ON AN69S AND SPAN MEMBRANES

As shown on tables 5.8, 5.9 and figures 5.7, 5.8 (for purified FXII), autoactivation of membrane-bound FXII on AN69S, occurs rapidly, and almost reaches its peak within 5 minutes of purified FXII solution contacting the surface.

This optimum rate of reaction peaks at 30 min but gently declines after 1 h of FXII contacting the surface. In contrast, the results for SPAN, indicate that autoactivation of membrane-bound FXII is relatively slower but reaches the same optimum rate as on AN69S membrane surface at 30 min after contact, however the reaction slows down rather rapidly there after.

Table 5.8: Time course results for generation of FXII activity on AN69S membrane, n=2.

Time (min)	Abs.1 (405 nm)		Abs. 2 (405 nm)		Mean Abs.(405 nm)	
	A	B	A	B	A	B
3	0.051	0.561	0.065	0.584	0.058 ± 0.010	0.573 ± 0.016
5	0.044	0.887	0.063	0.902	0.054 ± 0.013	0.895 ± 0.010
10	0.048	0.849	0.079	0.879	0.064 ± 0.022	0.864 ± 0.021
30	0.098	0.996	0.233	1.028	0.166 ± 0.095	1.012 ± 0.022
60	0.217	0.744	0.275	0.762	0.246 ± 0.041	0.753 ± 0.013

Where A = Supernatant absorbance, B = Membrane-bound absorbance.

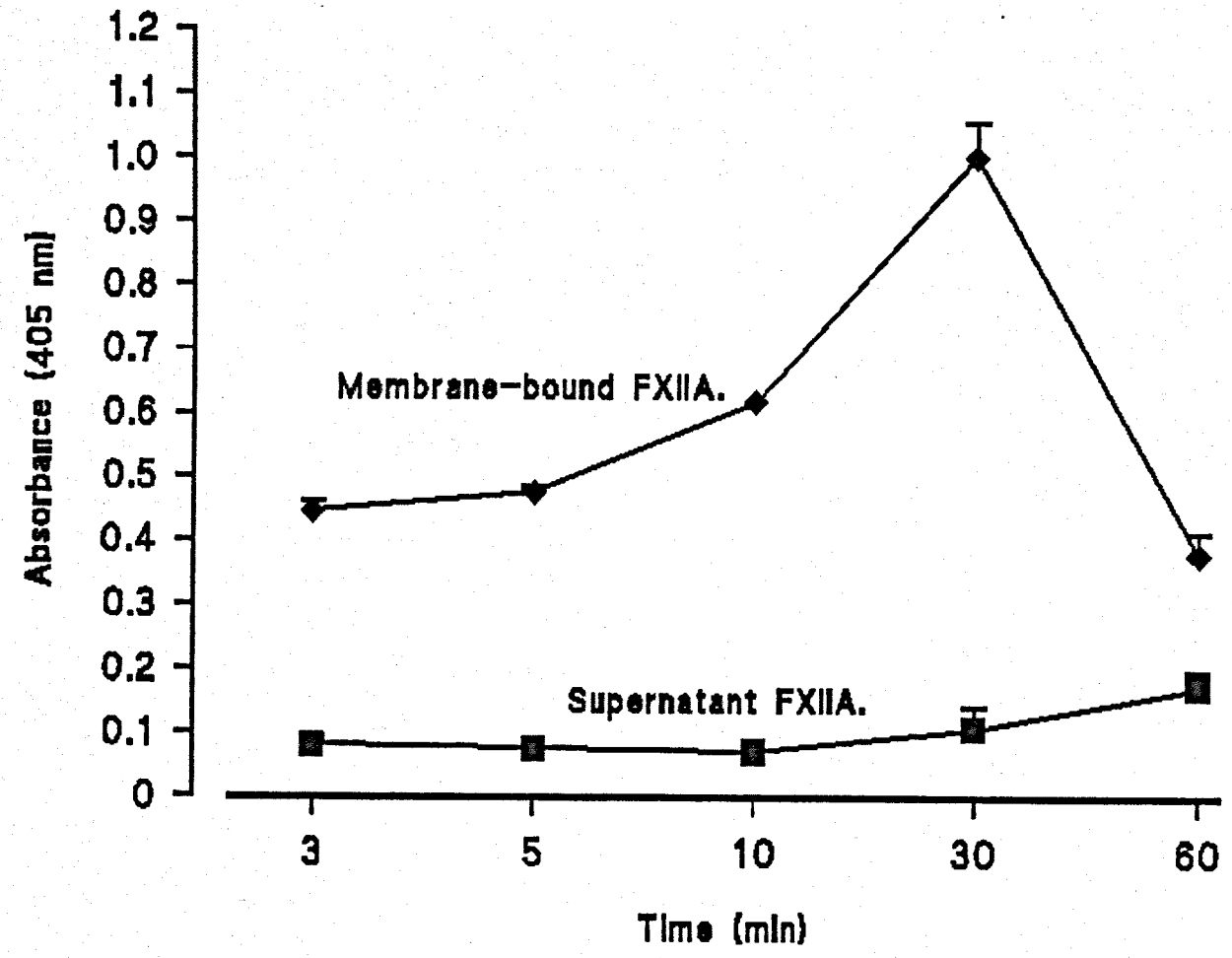


Figure 5.8: Time course for FXIIA generation on SPAN, n=2 (at 18–23^{0}C).

Table 5.9: Time course for generation of FXII Activity on SPAN, n=2

Time (min)	Absorbance 1 (405 nm)		Absorbance (405 nm)		Mean Absorbance (405 nm)	
	A	B	A	B	A	B
3	0.074	0.434	0.09	0.456	0.082 ± 0.013	0.445 ± 0.016
5	0.076	0.469	0.075	0.481	0.076 ± 0.007	0.475 ± 0.008
10	0.063	0.617	0.079	0.617	0.063 ± 0.008	0.617 ± 0.000
30	0.082	1.038	0.131	0.962	0.107 ± 0.035	1.000 ± 0.054
60	0.154	0.355	0.189	0.401	0.343 ± 0.025	0.378 ± 0.033

5.10.8 THE EFFECT OF PROLONGED CONTACT BETWEEN PLASMA AND MEMBRANES ON PLASMA SUPERNATANT FXIIA RESULTS.

The pattern of change of FXIIA appears to be similar in all the membranes contacted with citrated plasma, with a gradual rise up to the 90 minutes incubation time. Thereafter, the values of FXIIA decreased markedly for all the membranes, to below baseline level. There were no significant differences ($p<0.05$) in the FXIIA levels for all the membranes investigated (Table 5.10, figure 5.9).

The pattern of change of FXIIA with the heparinised citrated plasma, appears to be quite different as compared to the above, with Cuprophan, Hemophan, and AN69S exhibiting a similar trend. However, for the Polyamide control membranes, there was a significantly ($p<0.05$) higher level of FXIIA as from the 30 minutes incubation time to 90 minutes, and thereafter, the values diminished to almost baseline level. Polyamide NR exhibited significantly ($p<0.05$) higher level of FXIIA between 30 and 60 minutes incubation time as compared to polyamide NRZ membrane (table 5.11, figure 5.10).

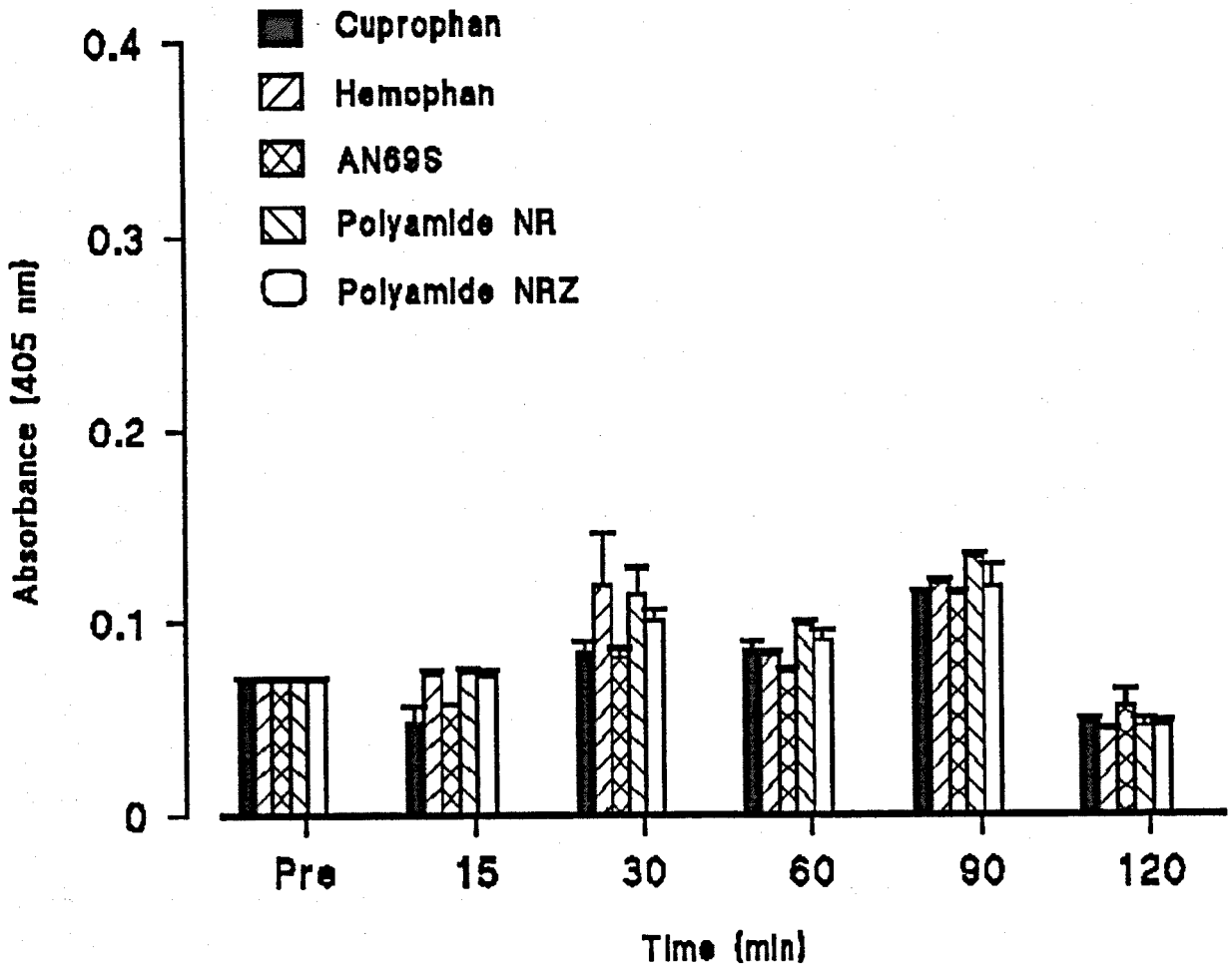


Figure 5.9: The effect of incubation time on citrated plasma supernatant FXIIA, n=3.

Table 5.10 The effect of incubation time on plasma supernatant FXIIA using citrated plasma only, n=3.

Time (min)	Cuprophan	Hemophan	AN69S	NR	NRZ
pre	0.070 ± 0.001	0.070 ± 0.001	0.070 ± 0.001	0.070 ± 0.001	0.070 ± 0.001
15	0.048 ± 0.008	0.073 ± 0.002	0.057 ± 0.000	0.074 ± 0.002	0.072± 0.003
30	0.084 ± 0.005	0.119 ± 0.027	0.081 ± 0.005	0.114 ± 0.014	0.100 ± 0.006
60	0.085 ± 0.004	0.082 ± 0.003	0.074 ± 0.002	0.098 ± 0.002	0.090 ± 0.005
90	0.113 ± 0.003	0.120 ± 0.002	0.114 ± 0.002	0.133 ± 0.002	0.118 ± 0.011
120	0.048 ± 0.001	0.043 ± 0.001	0.055 ± 0.009	0.045 ± 0.004	0.045 ± 0.003

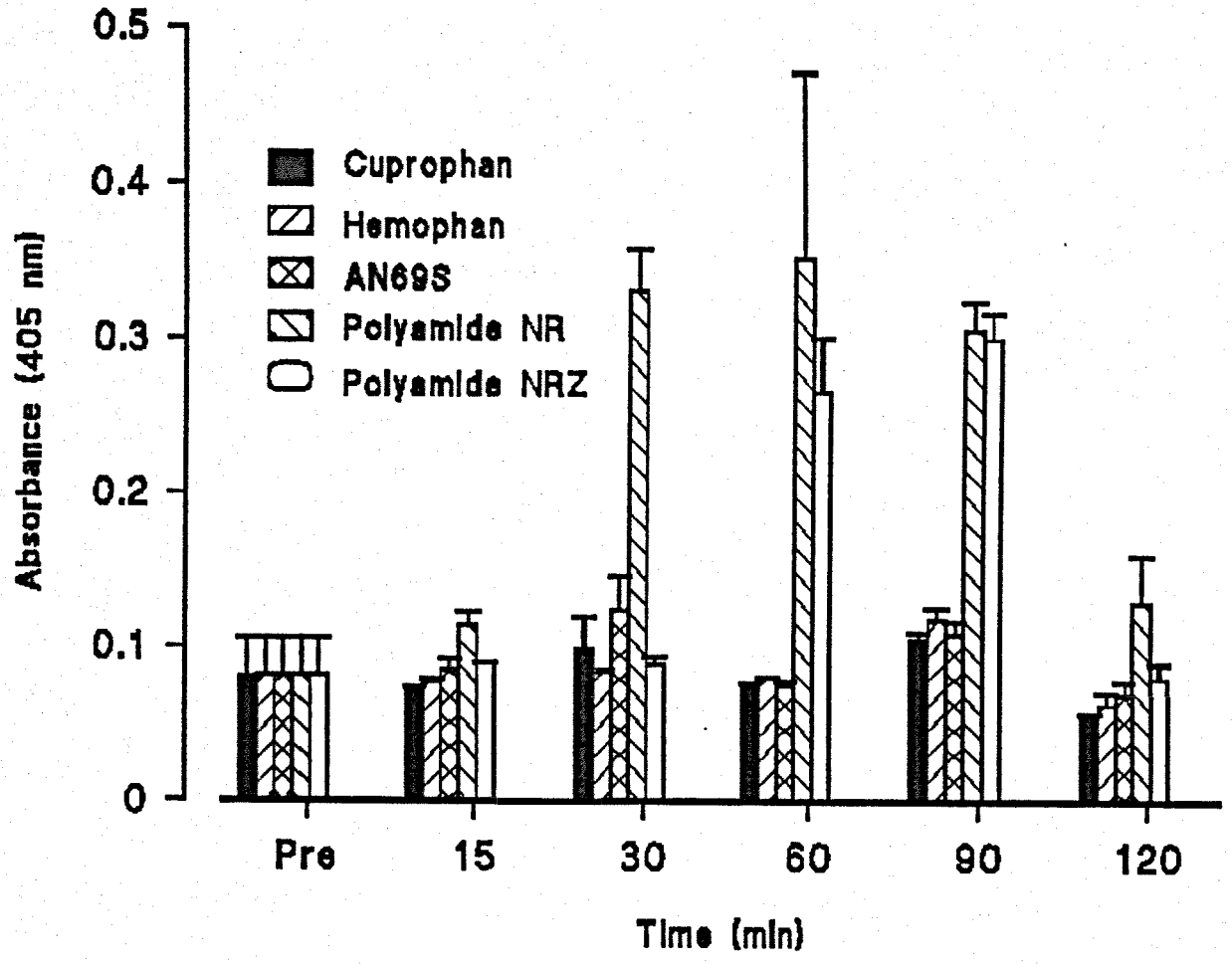


Figure 5.10: The effect of incubation time on citrated plasma supernatant FXIIA, n=3.

Table 5.11: The effect of incubation time on the supernatant FXIIA using heparinised plasma, n=3.

Time (min)	Cuprophan	Hemophan	AN69S	NR	NRZ
pre	0.081 ± 0.024	0.081 ± 0.024	0.081 ± 0.024	0.081 ± 0.024	0.081 ± 0.024
15	0.072 ± 0.002	0.077 ± 0.002	0.085 ± 0.007	0.114 ± 0.009	0.089 ± 0.001
30	0.099 ± 0.020	0.083 ± 0.002	0.124 ± 0.022	0.330 ± 0.027	0.088 ± 0.005
60	0.074 ± 0.002	0.079 ± 0.001	0.075 ± 0.002	0.351 ± 0.119	0.265 ± 0.034
90	0.105 ± 0.004	0.118 ± 0.007	0.108 ± 0.008	0.304 ± 0.019	0.299 ± 0.017
120	0.054 ± 0.002	0.062 ± 0.008	0.069 ± 0.008	0.129 ± 0.030	0.079 ± 0.010

5.10.9 THE EFFECT OF MILD ACIDIFICATION OF PLASMA ON SUPERNATANT FXIIA.

The results for the acidified citrated plasma indicated that there was little change in the levels of supernatant FXIIA in all the membranes and that there was no marked difference between the various membranes for up to 18 min incubation of the non-anticoagulated whole blood. The levels of supernatant FXIIA were almost at baseline (Table 5.12, figure 5.11).

For the heparinized whole blood, the acidified plasma supernatant FXIIA remained same as baseline in all the membranes for up to 9 min incubation time. At 12 min the levels were higher than baseline and AN69S indicated the lowest values. After 15 min incubation, Cuprophan had the lowest values of Supernatant FXIIA, however, no marked differences between the membranes were observed. After 18 min incubation, polyamide NR control membrane exhibited a markedly

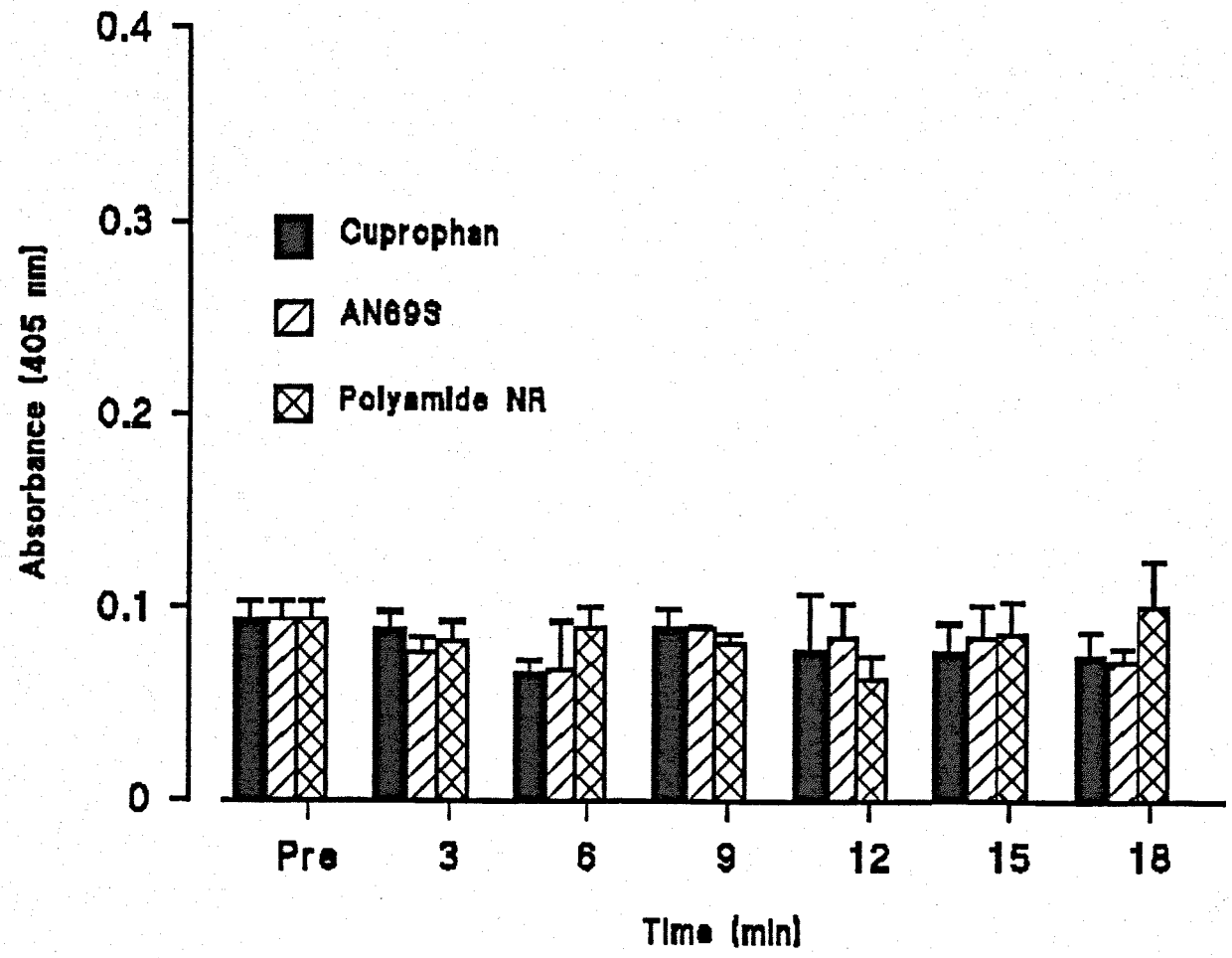


Figure 5.11: The effect of mild acidification of plasma on supernatant FXIIA (using whole blood), n=4.

higher level of supernatant FXIIA (Table 5.13, figure 5.12).

Table 5.12: The effect of mild acidification of citrated plasma on supernatant FXIIA.

Time (min)	Absorbance readings (405 nm), n=4.		
	Cuprophan	AN69S	Polyamide NR
pre	0.094 ± 0.009	0.094 ± 0.009	0.094 ± 0.009
3	0.089 ± 0.009	0.077 ± 0.008	0.083 ± 0.010
6	0.067 ± 0.006	0.068 ± 0.025	0.090 ± 0.010
9	0.090 ± 0.009	0.090 ± 0.001	0.082 ± 0.005
12	0.078 ± 0.029	0.085 ± 0.017	0.063 ± 0.012
15	0.077 ± 0.015	0.085 ± 0.016	0.087 ± 0.016
18	0.075 ± 0.013	0.072 ± 0.007	0.100 ± 0.024

Table 5.13: The effect of mild acidification of heparinised citrated plasma on supernatant FXIIA.

Time (min)	Absorbance readings (405 nm), n=4.		
	Cuprophan	AN69S	Polyamide NR
pre	0.075 ± 0.021	0.075 ± 0.021	0.075 ± 0.021
3	0.082 ± 0.001	0.067 ± 0.012	0.073 ± 0.008
6	0.074 ± 0.006	0.064 ± 0.009	0.069 ± 0.012
9	0.081 ± 0.005	0.063 ± 0.008	0.077 ± 0.006
12	0.093 ± 0.006	0.077 ± 0.015	0.094 ± 0.020
15	0.084 ± 0.025	0.095 ± 0.017	0.108 ± 0.014
18	0.064 ± 0.016	0.076 ± 0.012	0.122 ± 0.035

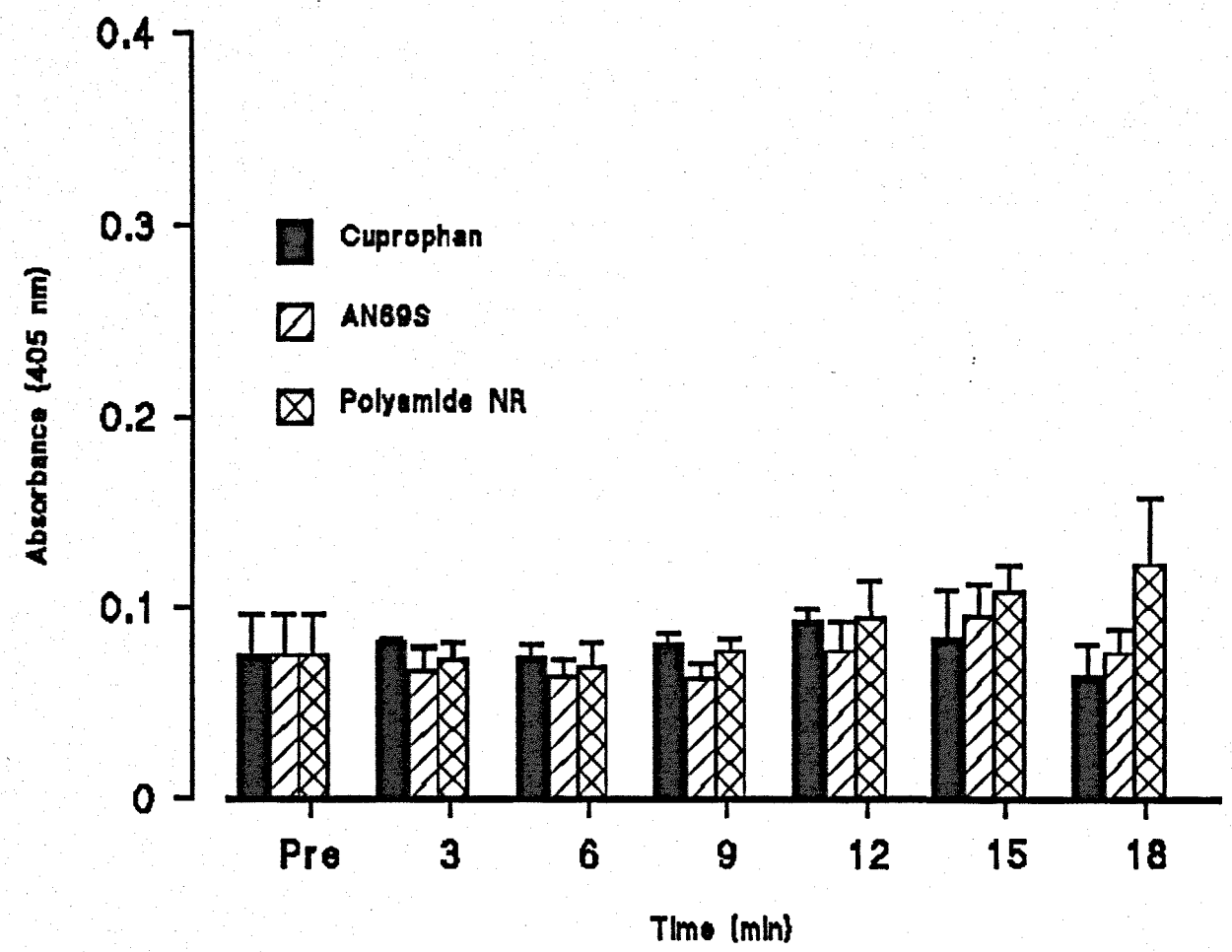


Figure 5.12: The effect of mild acidification of heparinised (2.5 U/ml) citrated plasma on supernatant FXIIA, n=4.

5.11 DISCUSSION

The results of the experiment utilising whole blood, indicated a lack of marked difference in supernatant FXIIA between all the membranes investigated. There was however, a small rise in factor XIIA between 15 and 18 min of blood-membrane contact. These findings were in agreement with previous observations utilizing the incubation test cell system (Irvine 1989; Sundaram 1992; Yu 1993). The pattern with the hollow fibre membranes perfused by the controlled flow syringe pump system, indicated that FXIIA remained at the same level for up to about 12 minutes of perfusion for all the membranes and this was in agreement with a previous observation utilizing a controlled flow syringe pump system on flat sheet membranes (Lamba 1994).

In the plasma system results, supernatant FXIIA values were not markedly different in citrated or heparinized plasma for all the membranes investigated. However, the membrane-bound FXIIA results indicated significant differences ($p < 0.05$), with AN69S exhibiting consistently, the lowest values for citrated as well as heparinized plasma. Cuprophan indicated similar membrane-bound FXIIA levels for citrated plasma and significantly ($p < 0.05$) lower values than Hemophan for heparinized plasma. This may suggest that at pH 7.4, the amine groups (of the DEAE cellulose blend) on Hemophan may be predominantly positively charged and therefore adsorb more heparin (von Sengbusch et al 1993; Schmitt et al 1983). As a consequence the Hemophan membrane acquired a superficial negative charge (Werner et al 1995) leading to the observed higher FXIIA. The control polyamide NR membrane indicated significantly higher membrane-bound FXIIA values than any of the other membranes for citrated as well as heparinized plasma. This agrees with the findings that Hemophan adsorbs significant amounts of heparin (chapter 7) and was in line with the hypothesis that negatively charged materials adsorb and activate more factor XII (Revak et al 1978).

Plasma supernatant FXIIA levels although moderately increased with time for up to 90 minutes, did not indicate any marked differences between the materials. Differences were only observed with the control membranes when heparin was added into the citrated plasma. This may suggest that possibly all

dialysis membrane materials exhibit similar factor XII activities in the plasma fluid phase. Alternatively, there may have been subtle differences which could not be detected by the assay. Mild acidification of plasma to minimize the effect of plasma protease inhibitors on activated factor XII, appeared to stabilise the pattern of factor XII activation, but no significant differences between the membranes was detectable even after 18 minutes of incubation. However, for the heparinised plasma, there was a gradual increase in factor XIIA between 12 and 18 minutes of incubation, suggesting that presence of heparin in plasma promotes factor XII's surface interaction. This observation was in agreement with *in vitro* studies that have shown that Cuprophan and Hemophan membranes exhibited negative zeta potentials at physiological pH in the presence of heparin (van Wagenen & Andrade 1980; Werner et al 1995). The presence of negative zeta potentials on the membranes may cause factor XII activation as observed.

The hypothesis that can be derived from these two observations would be that the activation of factor XII in the plasma fluid phase by different materials was a gradual process and no significant differences could be detected during short time or long time contact between plasma and dialysis membranes. When plasma was incubated for a long duration, the pattern of FXIIA begun to rise after 30 minutes incubation, and declined after 120 minutes. In the presence of heparin the levels of FXIIA remained at baseline except for the control polyamide membranes. In the mild acidification test, the FXIIA levels remained similar probably because of the inactivation of plasma factor XII zymogen (pKa = 6.1-6.5) at the low pH. This effect contrasts with the mild acidification test, where presence of heparin indicated an elevated level of FXIIA, this therefore suggests that heparin appears to catalyse the activation of plasma supernatant factor XIIA in the absence of plasma protease inhibitors.

The findings in the plasma-free system utilizing purified unactivated factor XII, indicated an increased FXIIA for the negatively charged membranes with significant differences ($p < 0.05$) in both supernatant and membrane-bound phase. This appears to agree with other observations utilising purified protein systems, whereby autoactivation of factor XII was markedly higher on negatively charged surfaces (Revak et al 1978).

This may be because of a greater surface adsorption of factor XII protein in the absence of possible surface binding competition with other plasma proteins or plasma protease inhibitors. In the plasma system where presumably because of a greater surface binding competition between factor XII and the other plasma proteins, surface autoactivation may be limited by the mere inability of surface-bound factor XII to undergo conformational change possibly because of the close proximity to plasma protease inhibitors also adsorbed. It is possible therefore that the reasons for the lowest observed membrane-bound FXIIA by AN69S membranes may be attributable to a greater affinity of the negatively charged membrane for some plasma proteases (or plasma protease inhibitors) and as a consequence the surface-bound plasma protease inhibitors restrict the activation of surface adsorbed factor XII.

5.12 SUMMARY

In summary, this study demonstrated that in the measurement of FXII-like activity in plasma supernatant during blood-membranes contact, differences between various membrane materials could not be shown categorically. Distinct differences were observed by the measurement of membrane surface factor XII-like activity, however, the assay procedure may not be convenient for clinical applications. Another point of consideration is the length of the contact between plasma and the membranes. In this study, longer incubations have been shown not to have any major advantage over short time incubations, with a possibility of denaturation of plasma factor XII in prolonged exposure to surfaces and that there is little clinical relevance in doing so. Some *in vitro* studies have indicated that by contacting of plasma-materials for 60 minutes, differences in the levels of factor XII activities were observed (Rhodes 1992; Campbell et al 1994). However, the reported data seems to indicate that the differences were only observed in materials that were quite dissimilar in chemical and physical properties, and that the values were not markedly different.

It has also been shown that it may be useful to measure factor XII activation under controlled condition by the use of purified factor XII not plasma. Under controlled conditions, the relationship between a material surface and the purified protein can be studied without the influence of drugs or anticoagulants commonly

present in plasma. However, the absence of other plasma constituents makes the relevance of the findings utilising purified proteins less clear or informative.

There is therefore a requirement for the development of a more specific assay of activated factor XII or an assay that could detect both active and inhibited activated factor XII. A more specific assay of activated factor XII utilizing an enzyme linked immunosorbent assay manufactured by Shield Diagnostics Ltd (Dundee, UK) was therefore considered for the next stage of this study (Chapter 6). The anticipated limitations for the elisa assay would be the inherent attenuation of factor XII activity by existing plasma protease inhibitors. With respect, a modification of the elisa assay was considered by utilising a mouse monoclonal antibody that recognizes and binds to specific epitopes on the light chain of activated factor XII. The advantage of this modification would be to allow the detection of free or inhibitor-bound activated factor XII (chapter 6).

CHAPTER SIX

IN VITRO FACTOR XII ACTIVITY (FXIIa) MEASUREMENT BY ENZYME LINKED IMMUNOSORBENT ASSAYS.

6.1 INTRODUCTION

Although the chromogenic substrate assays for measurement of factor XII activity are easy and quick to perform, the investigation reported in chapter 5 has indicated clear deficiencies. In addition, sensitivity is a critical factor since only minute amounts of factor XII activity may need be generated to account for a significant increase in the amount of kallikrein and bradykinin concentration.

A number of immunoassays detecting proteases involved in the contact phase activation complexed to their natural plasma inhibitors such as kallikrein/C1 inhibitor, kallikrein/α_2 macroglobulin and of factor XII-C1 inhibitor complexes have been described (Harpel et al 1985; Nuijens et al 1988; Kaufman et al 1991; Van der Kamp & Van Oeveren 1993). However, none of these assays are commercially available at present.

Currently a semi-quantitative direct enzyme linked immunosorbent assay for the detection, in human plasma, of activated factor XII (α-XIIa and β-FXIIa), has been made available commercially by Shield Diagnostics Ltd (Dundee, UK). The assay has been utilised for research purposes only as its utility has not been validated in routine diagnostic procedures. Attempts were made to evaluate the suitability of the Shield activated factor XII assay in the detection of contact phase activation during *in vitro* blood-biomaterials interactions.

6.2 BLOOD-MEMBRANE CONTACT

Blood was collected from normal healthy volunteers and aliquoted into two. Heplok heparin was added to one aliquot to a final concentration of 1 IU/ml. No anticoagulant was added to the second aliquot. A 6 well incubation test cell described in chapter 4, was used for the blood-membrane contact with Cuprophan, Hemophan, AN69S and polyamide NRZ as the materials evaluated.

The test cell was set up prior to the collection of blood. Blood was contacted to the membranes and 1 ml aliquots removed every 3 min (at baseline, 3, 6, and 9 min of incubation) from the wells and placed into polypropylene tubes containing 110 μl of trisodium citrate. The samples were then centrifuged at 3000 rev./min for 15 min at 25°C. The plasma samples were then snap frozen on dry ice and stored at -70°C until ready for factor XII activity assay.

6.3 PRINCIPLE OF THE SHIELD ACTIVATED FACTOR XII ASSAY

The wells of the Shield activated factor XII microtitre strips were coated with mouse monoclonal antibody specific for activated factor XII. During the first incubation, activated factor XII, if present in the plasma sample, will bind to the immobilized antibody. The wells are then washed to remove unbound plasma components. A conjugate of enzyme-labelled polyclonal sheep anti-human activated factor XII antibody binds to the surface-bound antigen in the second incubation. After a further washing step, specifically-bound enzyme-labelled antibody is traced by incubation with substrate solution. Addition of a stop solution terminates the reaction, and provides the appropriate pH for colour development.

The amount of conjugate bound is measured in absorbance units. The amount of activated factor XII (ng/ml) in an unknown sample can be estimated by interpolation from dose response curve based on standards. Standard concentrations were expressed as nanograms (ng/ml) per ml. 1 ng/ml of β-FXIIa is defined as the amount of activated factor XII which generates 25 pmoles of p-nitroaniline per minute at 37°C from a substrate solution containing 0.175 mM S-2302, 4 mM Tris, 126 mM sodium chloride, 0.9 mM ethylenediamine tetraacetic acid (EDTA) and 0.009% bovine serum albumin at pH 7.8. The results for test samples were expressed as nanograms per ml, derived according to the procedure described above.

6.3.1 ASSAY KIT COMPONENTS

1. Shield anti-activated factor XII conjugate: 1 x 11 ml vial containing alkaline phosphatase-labelled sheep anti-human activated factor XII antibody in Tris buffer with protein stabiliser and preserved with 0.1% sodium azide. Labelled component

A.

2. Shield substrate: 1 x 11 ml vial containing Mg^{2+} as enzyme cofactor and phenolphthalein monophosphate (PMP) in buffer solution. Labelled component B.

3. Shield stop solution: 1 x 11 ml vial containing sodium hydroxide and EDTA as a chelating agent in carbonate buffer pH > 10. Labelled component C.

4. Shield activated factor XII Wash buffer concentrate: 2 x 20 ml vials containing borate buffer and preserved with 1.0% (w/v) sodium azide. Supplied x20 concentration, labelled component E.

5. Shield activated factor XII coated wells: 6 x 16 well microtitre strips coated with anti-activated factor XII monoclonal antibody. 1 x 16 well strips individually sealed in foil pouches with desiccant. Labelled component E.

6. Shield activated factor XII standards: 1 ml each of 5 standards containing purified activated factor XII (β-FXIIa) in buffer with protein stabiliser and 0.1% (w/v) sodium azide: S1 = (0 ng/ml); S2 = (1 ng/ml); S3 = (5 ng/ml): S4 = (10 ng/ml) S5 = (20 ng/ml).

6.3.2 ASSAY PROCEDURE

1. 100 μl of the activated factor XII standards 1, 2, 3, 4, and 5 were pipetted in duplicates into the appropriate wells.

2. 100 μl of each test sample were pipetted in duplicates in the remaining wells.

3. After all the samples had been added, the plates were incubated at room temperature (18-25^{0}C) for 60 $\pm$ 10 minutes.

4. The contents of the strips were decanted by quick inversion over a sink suitable for disposal of biological materials. The inverted strips were firmly blotted using adsorbent paper towels to remove excess liquids.

5. 200 μl of diluted wash buffer were added to each well and the liquid was decanted by quick inversion of the strips over the sink.

6. Step 5 was repeated four times and the strips blotted firmly as before.

7. 100 μl of Shield conjugate were added to each well and incubated for 60 $\pm$ 5 minutes at room temperature (18-25^{0}C).

8. The contents of the strips were decanted by quick inversion over the sink and the inverted strips were firmly blotted with adsorbent paper towels to remove excess liquid.

9. 200 μl of diluted wash buffer were added to each well. The liquid was then decanted by quick inversion over the sink.

10. Step 9 was then repeated four times and the strips blotted firmly as before.

11. 100 μl of Shield substrate were added to each well and the plates incubated for 15 $\pm$ 2 minutes at room temperature (18-25^{0}C).

12. 100 μl of Shield stop solution were then added to each well at approximately the same rate as the substrate solution and the strips were tapped gently to mix the contents of each well.

13. The strips were read for absorbance using an automated Dynatech MR5000 plate reader, using 560 nm (540-656 nm) filter and the FXIIa concentrations were calculated automatically and printed out. The values of activated factor XIIa at each time point for all the materials studied were noted appropriately and the findings compared with the chromogenic substrate assay described in chapter 5.

6.4 ENZYME IMMUNOASSAY FOR FXIIa/FXIIa-INHIBITOR COMPLEXES.

6.4.1 INTRODUCTION

Shield factor XIIa assay has the limitation of detecting only the active enzyme with the major proportion being neutralized by C1-inhibitor and other plasma proteases. As a consequence, a new focus was on the development of an assay procedure that optimizes the ability to detect activated factor XII, whether in the free or the inhibitor bound state. In collaboration with Shield Diagnostics Ltd, a new assay was devised that utilised a mouse monoclonal antibody that binds to an epitope on the light chain of factor XII molecule exposed during the activation of factor XII. In view of the fact that the antibody binds to an epitope on the light chain that is outside the active site, the activated factor XII molecule would be detected in the free or inhibitor-bound state. It was hoped that this approach would optimise the sensitivity of the assay better than quantification of FXIIa-C1-Inhihibitor complexes

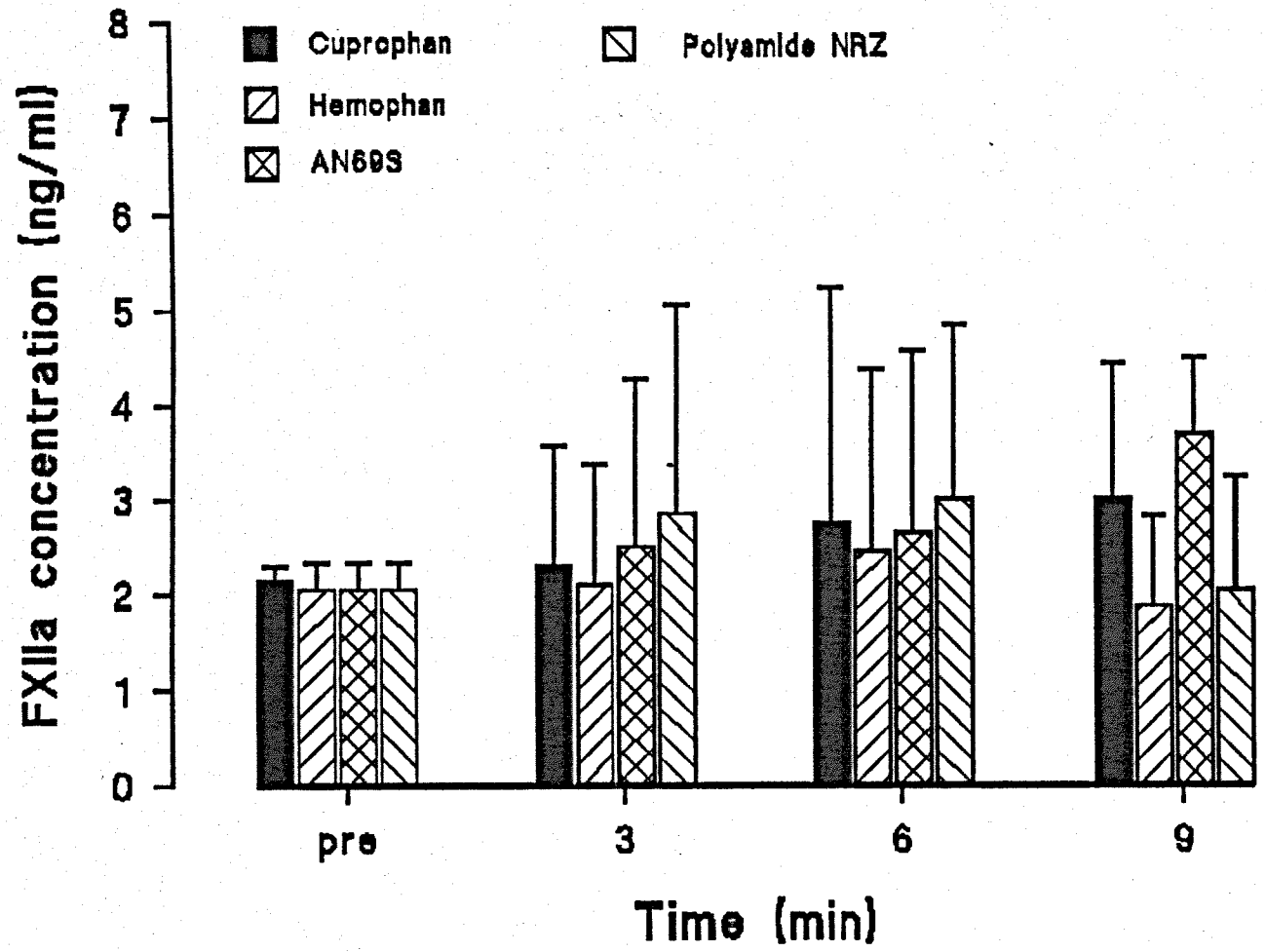


Figure 6.1: In vitro FXIIa determination using Shield Elisa assay on non-anticoagulated blood, n=3.

only, since activated factor XII can be inhibited by other plasma inhibitors such as α_2-macroglobulin and antithrombin III.

6.4.2 BLOOD-MEMBRANE CONTACT PROCEDURE

The 6 well incubation test cell was used for the *in vitro* study. Fresh blood from healthy volunteers was obtained from anterior cubital vein. Cuprophan, Hemophan, AN69S, polyamide NR and NRZ were the membranes selected for investigation. The test cell was set up as described in chapter 4. Standard unfractionated heparin (Heplok) and low molecular weight heparin (Fragmin) were added to two aliquots of the blood samples before contact with membranes. The rest of blood was not anticoagulated until after incubation with the membranes. Blood sampling was as described in chapter 5. Aliquots of blood were removed every 3 min for up to 18 min of incubation. The aliquots were anticoagulated using 3.2 % trisodium citrate and centrifuged at 3000 rev.per min for 15 min at 25^{0}C and plasma samples were snap frozen on dry ice and stored at -70^{0}C.

6.4.3 FXIIa/FXIIa-INHIBITOR COMPLEXES ASSAY

There were important changes to the original commercial assay kits for activated factor XII (Shield Diagnostics Ltd, Dundee, UK). These were:

1. The microtitre strips were coated with a mouse monoclonal antibody specific to a binding site on the light chain away from the active site of activated factor XII.
2. Activated factor XII standards: S1 = 0 ng/ml; S2 = 5 ng/ml; S3 = 20 ng/ml; S4 = 50 ng/ml.
3. The microplates were shaken using an orbital shaker during the periods of incubation to ensure adequate mixing of reagents.

However, the assay protocol remained as described on section 6.3.2.

6.5 STATISTICS

Statistical analysis was performed using the Minitab package version 8.0. Comparisons of the mean values of FXIIa between different membranes at each particular incubation time point were carried out using twosample-t tests and these were reported at 95% confidence intervals ($p < 0.05$). A oneway analysis of variance

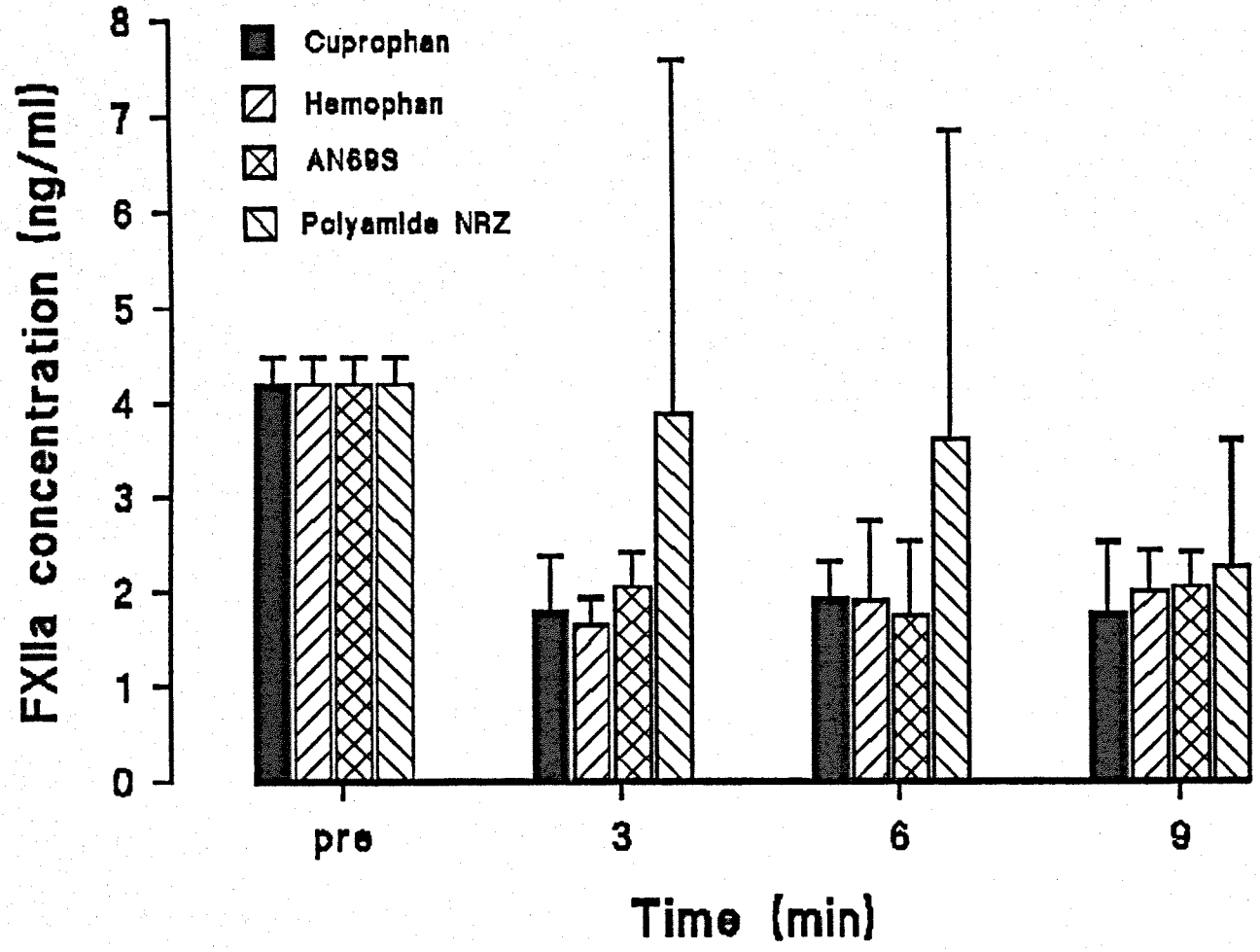


Figure 6.2: In vitro FXIIa determination using Shield Elisa assay on heparinized blood, n=3.

was also used to compare the mean FXIIa values between the membranes and differences were reported as significant at $p < 0.05$.

6.6 RESULTS

6.6.1 SHIELD FXIIa ASSAY RESULTS.

6.6.1.1 NON-ANTICOAGULATED BLOOD

The results indicated that the mean baseline FXIIa values were lower than the blood-membrane contact times of 3, 6, and 9 min (table 6.1, Figure 6.1). There were no apparent marked differences between the membranes on FXIIa values for up to 6 min incubation time. Cuprophan and AN69S indicated a gradual rise in FXIIa with time and the values were higher than Hemophan or polyamide NRZ at 9 min incubation. Hemophan exhibited the lowest pattern of change in FXIIa with time. The FXIIa assay indicated that for the control polyamide NRZ, values were higher than all the other membranes at 3 min incubation, but the lowest after 9 min incubation (Table 6.1, figure 6.1).

6.6.1.2 THE EFFECT OF HEPARIN (HEPLOK) ON BLOOD FXIIa.

The baseline values of FXIIa were markedly higher than at any time during blood-membrane contact, indicating that the presences of heparin leads to activation more factor XII. Polyamide NRZ exhibited a diminishing pattern of FXIIa with highest values at the 3 min incubation time and lowest values at 9 min, but thereafter the values remained elevated. There were no differences in the patterns of FXIIa shown by Cuprophan, Hemophan and AN69S (Figure 6.2 and table 6.1).

6.6.2 FXIIa/FXIIa-INHIBITOR ASSAY RESULTS.

6.6.2.1 NON-ANTICOAGULATED BLOOD SAMPLES RESULTS

Cuprophan membrane exhibited lower than baseline values of FXIIa/FXIIa-inhibitor levels between 3 and 9 min of blood contact. However, at 12 min incubation, Cuprophan indicated the highest FXIIa/FXIIa-inhibitor values, a pattern that was maintained for up to 18 min.

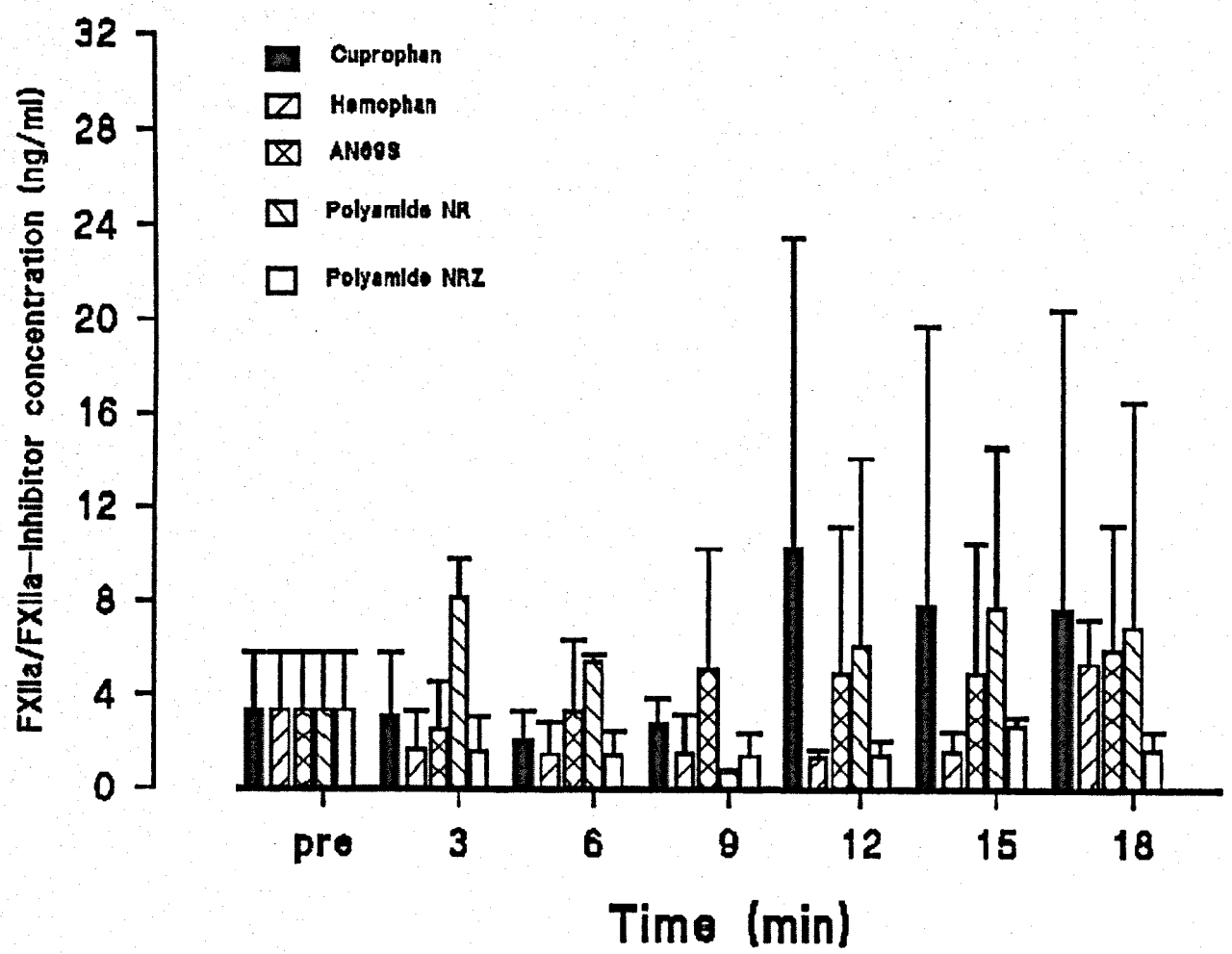


Figure 6.3: In vitro FXIIa determination using FXIIa/FXIIa-inhibitor Elisa assay on non-anticoagulated blood, n=4.

Table 6.1 : In vitro FXIIa determination using Shield elisa assay on whole blood, n=3. (mean FXIIa values ± SD ng/ml)

Membrane	Time (min)	Non-anticoagulated blood	Heparinised blood
Cuprophan	pre	2.05 ± 0.28	4.2 ± 0.28
	3	2.10 ± 1.27	1.80 ± 0.57
	6	2.75 ± 2.50	1.93 ± 0.39
	9	3.00 ± 1.41	1.78 ± 0.74
Hemophan	pre	2.05 ± 0.28	4.2 ± 0.28
	3	2.10 ± 1.27	1.65 ± 0.28
	6	2.45 ± 1.91	1.90 ± 0.85
	9	1.88 ± 0.95	2.00 ± 0.42
AN69S	pre	2.05 ± 0.28	4.2 ± 0.28
	3	2.50 ± 1.77	2.05 ± 0.35
	6	2.65 ± 1.91	1.75 ± 0.78
	9	3.68 ± 0.81	2.05 ± 0.35
polyamide NRZ	pre	2.05 ± 0.28	4.2 ± 0.28
	3	2.85 ± 2.19	3.88 ± 3.71
	6	3.00 ± 1.84	3.60 ± 3.25
	9	2.05 ± 1.17	2.25 ± 1.34

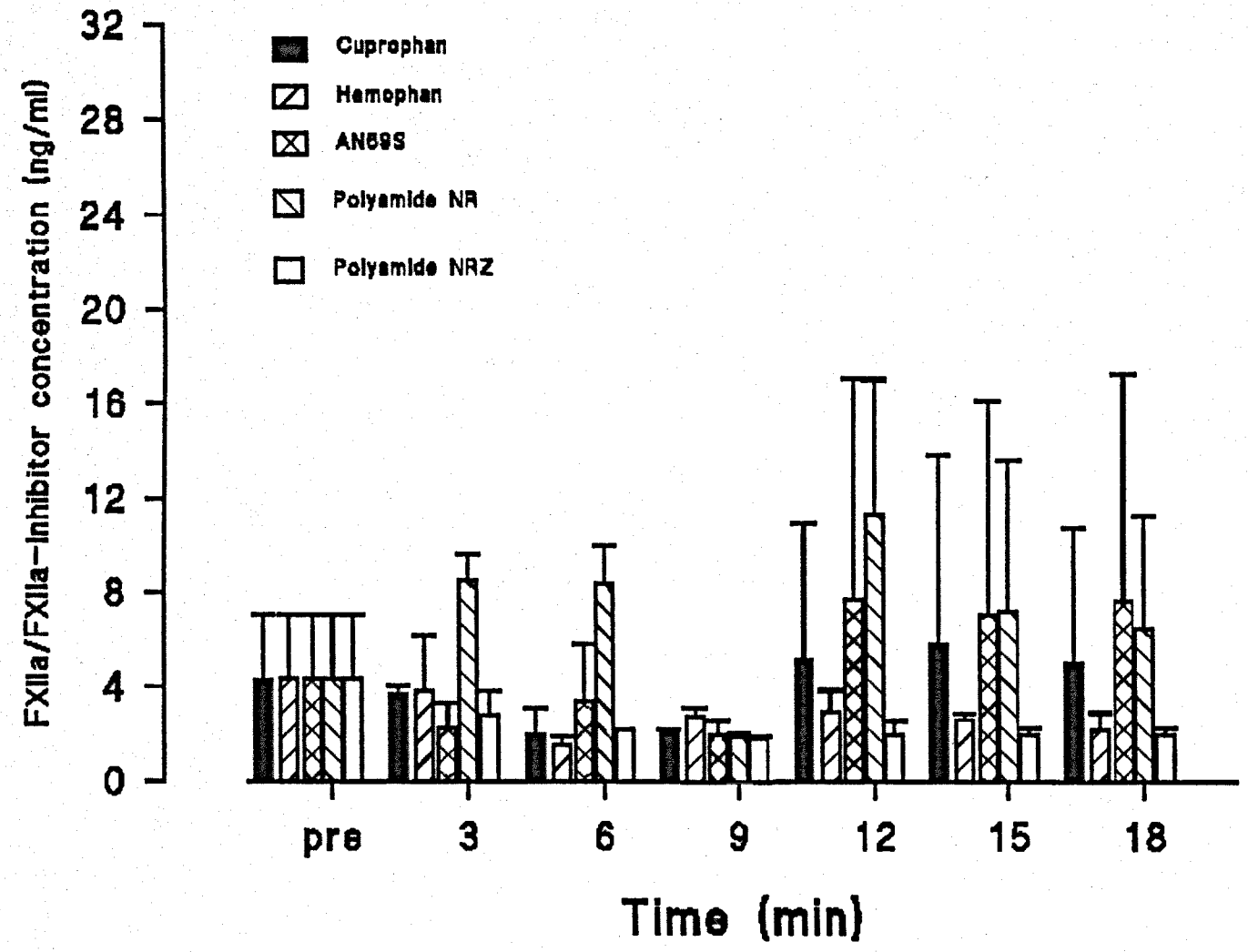


Figure 6.4: In vitro FXIIa determination using FXIIa/FXIIa-inhibitor Elisa assay on heparinized blood, n=4.

Hemophan indicated consistently lower values of FXIIa/FXIIa-inhibitors than Cuprophan and AN69S, with the control polyamide NRZ exhibiting a similar pattern as Hemophan. AN69S membrane indicated a gradual rise in FXIIa/FXIIa-inhibitor levels with an optimum value reached after 18 minutes. Polyamide NR exhibited a biphasic pattern of FXIIa, with a minimum shown after 9 min incubation and optima at 3 and 15 min. Although the values of the FXIIa concentration detected by the FXIIa/FXIIa-inhibitor assay were marginally higher, the pattern of response shown by the two assay methodologies were the similar for the first 9 min, but differences in magnitude were apparent. Cuprophan after 12 min incubation indicated significantly higher FXIIa values ($P<0.05$) than any of the other membranes (Table 6.2; figure 6.3).

6.6.2.2 THE EFFECT OF HEPARIN (HEPLOK) ON FXIIa/FXIIa-INHIBITOR ASSAY RESULTS.

The FXIIa/FXIIa-inhibitor values for Cuprophan, Hemophan, AN69S, polyamide NRZ were well below baseline values for up to 9 min of incubation. Hemophan and polyamide NRZ values maintained a consistently lower than baseline values for FXIIa throughout the incubation period of 18 min. In contrast, Cuprophan and AN69S FXIIa values were above baseline after 12 min incubation, with the latter having the higher activity. The polyamide NR control membrane also showed a biphasic pattern of FXIIa, with a minimum at 9 min and optima at 3 and 12 min respectively. The overall pattern indicates that in the presence of heparin, Cuprophan indicated lower FXIIa/FXIIa-inhibitor levels, while AN69S and polyamide NR values were slightly elevated as compared to the pattern shown in the absence of heparin (Table 6.3, figure 6.4).

Table 6.2: The FXIIa/FXIIa-Inhibitor complexes concentration assay results using non-anticoagulated whole blood.

Mean values ± SD (ng/ml), n=3.

Time (min)	Cuprophan	Hemophan	AN69s	Polyamide NR	Polyamide NRZ
pre	3.35 ± 2.45	3.35 ± 2.45	3.35 ± 2.45	3.35 ± 2.45	3.35 ± 2.45
3	3.15 ± 2.66	1.70 ± 1.60	2.53 ± 2.02	8.15 ± 1.67	1.63 ± 1.45
6	2.10 ± 1.19	1.48 ± 1.35	3.30 ± 3.03	5.45 ± 0.29	1.45 ± 1.03
9	2.80 ± 1.03	1.53 ± 1.65	5.10 ± 5.13	0.75 ± 0.06	1.40 ± 0.94
12	10.28 ± 13.18	1.35 ± 0.30	4.98 ± 6.23	6.13 ± 8.01	1.43 ± 0.67
15	7.90 ± 11.80	1.60 ± 0.82	4.98 ± 5.55	7.78 ± 6.83	2.70 ± 0.35
18	7.75 ± 12.71	5.38 ± 1.89	6.00 ± 5.27	6.95 ± 9.57	1.70 ± 0.76

Table 6.3: **FXIIa/FXIIa-Inhibitor complexes concentration using heparinized blood. Mean values ± SD (ng/ml), n=4.**

Time (min)	Cuprophan	Hemophan	AN69S	Polyamide NR	Polyamide NRZ
pre	4.30 ± 2.73	4.30 ± 2.73	4.30 ± 2.73	4.30 ± 2.73	4.30 ± 2.73
3	3.70 ± 0.35	3.83 ± 2.34	2.30 ± 1.00	8.52 ± 1.08	2.75 ± 1.04
6	2.00 ± 1.08	1.53 ± 0.35	3.35 ± 2.44	8.40 ± 1.61	2.20 ± 0.01
9	2.00 ± 0.16	2.70 ± 0.38	2.00 ± 0.49	1.93 ± 0.15	1.85 ± 0.06
12	5.20 ± 5.75	2.90 ± 0.95	7.75 ± 9.33	11.33 ± 5.69	1.98 ± 0.57
15	5.85 ± 7.97	2.60 ± 0.23	7.05 ± 9.04	7.18 ± 6.40	2.00 ± 0.28
18	5.00 ± 5.75	2.20 ± 0.69	7.58 ± 9.68	6.45 ± 4.78	1.95 ± 0.30

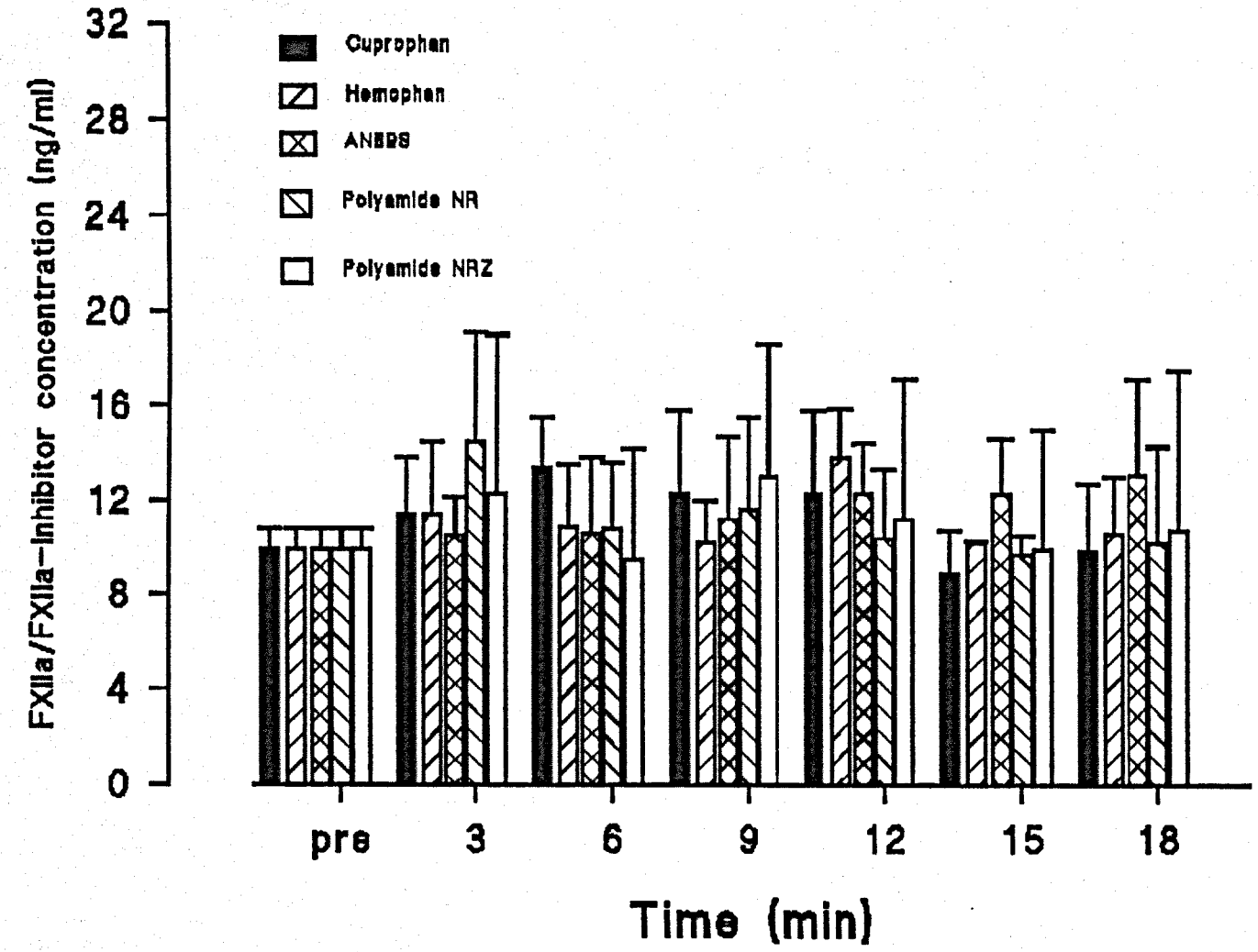


Figure 6.5: In vitro FXIIa determination using FXIIa/FXIIa-inhibitor assay on blood containing LMWH (Fragmin), n=4.

6.6.2.3 THE EFFECT OF LOW MOLECULAR WEIGHT HEPARIN (FRAGMIN) ON FXIIa/FXIIa-INHIBITOR COMPLEXES ASSAY RESULTS.

The results indicated that the baseline values of FXIIa/FXIIa-inhibitor complexes were significantly higher ($p < 0.05$) for the Fragmin samples than the standard heparin. The values of factor XII activities for all the membranes were significantly higher ($p < 0.05$) in the presence of LMWH than standard heparin or in non-anticoagulated whole blood (Table 6.4, figure 6.5). The patterns of factor XIIa/FXIIa-inhibitor levels were different between the different membranes. Cuprophan levels increased gradually to an optimum at 6 min incubation and remained elevated until 12 min of incubation, thereafter levels declined. Hemophan indicated a gradual increase to an optimum at 12 min followed by a decline. AN69S indicated a gradual rise throughout the duration of blood-membrane contact. The polyamide membranes indicated a similarity in the patterns of FXIIa/FXIIa-inhibitor complexes with a gradual decline from the initial higher levels at 3 min of blood-membrane contact.

Table 6.4: The effect of LMWH (Fragmin) on FXIIa/FXIIa-Inhibitor assay results. Mean FXIIa ± SD (ng/ml), n=4.

Time (min)	Cuprophan	Hemophan	AN69S	Polyamide NR	Polyamide NRZ
pre	9.85 ± 0.85	9.85 ± 0.85	9.85 ± 0.85	9.85 ± 0.85	9.85 ± 0.85
3	11.40 ± 2.40	11.40 ± 3.10	10.5 ± 1.60	14.50 ± 4.60	12.30 ± 6.70
6	13.40 ± 2.10	10.90 ± 2.60	10.60 ± 3.20	10.80 ± 2.80	9.50 ± 4.70
9	12.30 ± 3.50	10.20 ± 1.80	11.20 ± 3.50	11.60 ± 3.90	13.00 ± 5.60
12	12.30 ± 3.50	13.80 ± 2.10	12.30 ± 2.10	10.40 ± 2.90	11.20 ± 5.90
15	8.90 ± 1.80	10.20 ± 0.10	12.30 ± 2.30	9.70 ± 0.80	9.90 ± 5.10
18	9.90 ± 2.80	10.60 ± 2.40	13.10 ± 4.00	10.20 ± 4.10	10.70 ± 6.80

6.8 DISCUSSION

The results of the *in vitro* measurement of activated factor XII with the two enzyme immunoassays have indicated that the pattern of response was similar during the initial 3 - 9 min of incubation of heparinized and non-anticoagulated blood to all the membranes. There were no significant differences ($p < 0.05$) in the FXIIa values between the different haemodialysis membranes between 3 - 9 min of incubation for both assays on heparinized or non-anticoagulated blood. The baseline values for heparinised blood in both assays were higher than that of non-anticoagulated blood, suggesting that heparin increases factor XII activation.

For the novel method, differences in the factor XII activity after 12 min incubation were detectable in blood contacted with the different membranes. The pattern of response was similar for heparinized and non-heparinized blood. The FXIIa values for Cuprophan, appeared to be attenuated in the presence of heparin. In contrast, AN69S and Polyamide NR membranes, exhibited increased values of FXIIa in the presence of heparin after 12 min incubation. Hemophan and polyamide NRZ indicated consistently low factor XII activity throughout the blood-membrane contact, irrespective of whether heparinized or non-anticoagulated blood was used. This may suggest that the AN69S membrane activates an increased amount of factor XII after the initial 12 min. The same assumption could also be reached with Cuprophan membrane, whereas the Hemophan remained a low factor XII activator throughout the incubation period.

In the presence of low molecular weight heparin, the patterns of FXII activities were different between the membranes and the mean FXIIa/FXIIa-inhibitor complexes were above baseline throughout the period of blood-membrane contact. There were no significant differences ($p < 0.05$) between the different membranes at any particular time of incubation. All the baseline FXIIa values were significantly higher with blood containing Fragmin, than that containing standard heparin or that of non-anticoagulated blood. This indicated that low molecular weight heparin did not reduce factor XII activity, contrary to the previous findings using the original

non-modified chromogenic substrate assay, the values were higher (Irvine 1989).

The FXIIa/FXIIa-inhibitor assay procedure has shown that in the initial 3 to 9 minutes of contact between blood and membranes, there was a difference in pattern of activation of factor XII in blood (in the presence or absence of heparin), with Cuprophan and AN69S indicating a gradual increase with time. Significant differences could be detected between the membranes after 12 min incubation, and higher values were shown with the membranes AN69S and Cuprophan respectively. For the Hemophan membrane, there was a lower initiation of factor XII activity, for up to the end of 18 min incubation time. The pattern of FXII activity of blood containing Fragmin remained elevated during contact with all the membranes throughout the incubation period.

6.9 SUMMARY

The two enzyme immunoassay procedures have shown that the pattern of factor XII activity in the initial 3 to 9 min of blood-membrane contact remained the similar the for different haemodialysis membranes, but the magnitude of factor XII activity detected by the assays was much higher for FXIIa/FXIIa-inhibitor complexes assay. The results for the elisa assays, indicated a better discrimination between different membranes during blood-membrane contact, as compared to the chromogenic substrate assay (chapter 5).

For blood-membrane contact longer than 12 min, the FXIIa/FXIIa-Inhibitor assay procedure was able to detect significant differences in factor XII activities for different membranes.

The findings demonstrated that during blood-membrane contact, Cuprophan exhibited higher factor XII activities than Hemophan (significantly higher after 12 minutes incubation). This may be due to the increased negative zeta potentials on Cuprophan as compared to Hemophan after contact with plasma as demonstrated by some studies (van Wagenen & Andrade 1980; Werner et al 1995).

AN69S was also shown to be a more potent activator of factor XII than Hemophan in the presence or absence of heparin during the entire blood-membrane

contact time. The findings using the FXIIa/FXIIa-inhibitor complexes assay were therefore in agreement with the hypothesis that negatively charged materials activate more factor XII (Cochrane 1973). The pattern of factor XII activity was also shown to vary with the type of anticoagulant used, with low molecular weight heparin being able to increase baseline values and there was a consistently higher level during the period of contact with all the membranes, a pattern that is different from that of standard unfractionated heparin. This may be attributable to the reduced surface-binding affinity of low molecular weight heparin, and therefore there is a higher fluid phase concentration capable of interacting with more factor XII in plasma (Hirsh 1989). These findings have therefore shown that low molecular weight heparin lacks any advantages over standard heparin, in terms of the limitations of activation of the contact phase of coagulation. This has some implications in terms of the aggrevation of other proteolytic pathways or inflammatory reactions by activated factor XII induced by the presence of low molecular weight heparin as compared to standard heparin.

In summary, the *in vitro* studies have indicated that the FXIIa/FXIIa-inhibitors assay was very sensitive method for the detection of activation of factor XII. It was also shown that the levels of activated factor XII detected were different depending on the membrane type, the duration of blood-membrane contact, the presence of heparin and the type of heparin used. In view of these findings, further investigations were considered and an attempt to evaluate the efficacy of the novel FXIIa/FXIIa-Inhibitor immunoassay during haemodialysis and cardiopulmonary bypass was pursued (chapter 8).

CHAPTER SEVEN

FACTOR XII AND HEPARIN ADSORPTION ON MEMBRANE FACTOR XII ACTIVATION: THE ROLE OF MEMBRANE SURFACE CHARGE.

7.1 INTRODUCTION.

The establishment of a relationship between blood response parameters and properties of the polymer has been a long time subject of interest, particularly in the modification or development of materials with improved biocompatibility (Bruck 1980; Courtney et al 1993c). One of the aspects considered important has been the surface properties of the materials and the alterations to these surface properties after contact with biological solutions or pharmacological agents. Attempts have been made to characterise haemodialysis membranes by the measurement of streaming potentials to determine zeta potentials (van Wagenen & Andrade 1980; Werner et al 1995). The objective for these techniques were for the detection of alterations to the membrane interfaces caused by the adsorption of components of biologically relevant solutions. However, these techniques are very complex and require an understanding of sophisticated mathematical models.

Other studies have utilised cationic and anionic staining techniques to identifiy membrane adsorbed pharmaceutical agents and have reported of the adsorption of Nafamostat mesilate (FUT-175), a strong inhibitor of plasma proteases, to negatively charged polyacrylonitrile (AN69) membranes, consequently leading to its reduced anticoagulant properties (Inagaki et al 1992). FUT-175 was also found not to bind to Cuprophan, Hemophan, or polymethylmethacrylate membranes during dialysis (Inagaki et al 1992). Adsorption of heparin to DEAE-modified cellulose membranes (positively charged) has been reported (Holland et al 1978; Schmitt et al 1983; Vienken & Bowry 1993), but little has been reported of the relevance of surface adsorption to residual anticoagulant properties of membrane-adsorbed heparin. Much simpler techniques for the determination of surface properties, particularly charge and charge distribution utilising cationic and anionic dye stains have been reported (Thomaneck et al 1991). The focus in this study was on the relationship between the

surface properties and heparin adsorption, in particular the anticoagulant properties of membrane-bound heparin. In addition, the relationship between the patterns of adsorption and activation of factor XII and the variation due to membrane charge was considered important. In view of the tendency for factor XII to bind to surfaces, the mechanisms by which factor XII adsorbs onto membranes were investigated and the resultant findings compared to the corresponding factor XII activity measurements. A correlation with the determined surface characteristics in an *in vitro* set-up was then attempted.

7.2 PROTEIN ADSORPTION MEASUREMENT METHODOLOGY

7.2.1 FACTOR XII ADSORPTION EXPERIMENTS

7.2.1.1 Protein preparation

Purified unactivated factor XII was purchased from Enzyme Research Laboratories, Inc, Swansea, UK. The protein was labelled with ^{125}I according to the procedure established by Dr. A Mahiout at the Laboratory for Radionuclides at the Free University of Berlin, Germany.

7.2.1.2 Protein labelling procedure.

1.0 mg of purified factor XII (Enzyme Research Laboratories Inc) was added to 1 ml phosphate buffer (0.05 mM potassium phosphate, 0.15 M NaCl, 100 mM EDTA, pH 7.4) containing 1 mCi Na ^{125}I, 100 μl dimethylsulphoxide, and 50 μl chloramide-T (2 md/ml). The reaction took place for 4 min at 0^0C and was stopped by addition of 200 μl sodium metabisulphite (10 mg/ml). Free and labelled factor XII were separated by gelfiltration chromatography (Sephadex G-50 12 cm x 0.8 cm). Labelling was estimated as 10-12 ^{125}I atoms incorporated in 100 molecules of factor XII. The labelled factor XII fraction was dissolved in 1.2 ml potassium phosphate buffer and the resultant labelled factor XII concentration was 16.66 μg/ml (approximately 210 pmoles/ml).

7.2.1.3 Preparation of plasma/protein solution.

7.2.1.4 Single FXII protein solution:

1.2 ml labelled factor XII labelled with ^{125}-Iodine with a specific activity of 40 MBq were made up into a 20 ml total volume with Tris buffered saline (5 mM Tris HCl, 155 mM NaCl, pH 7.4). The resultant protein concentration was 833

ng/ml (10.5 pmol/ml).

7.2.2 BLOOD SAMPLES COLLECTION

Blood from normal healthy volunteers was collected and anticoagulated using 3.2% trisodium citrate at a ratio of 1:9 of blood. The blood was then centrifuged at 3000 rev/min for 15 min at room temperature and the separated plasma was pooled, snap frozen and stored at -70°C.

7.2.2.1 Labelled pooled plasma:

Pooled platelet poor plasma was obtained from normal healthy volunteers, snap frozen and stored at -70°C until when required. 20 μl of the stock labelled factor XII solution were diluted with 20 ml Tris buffered saline (5 mM Tris HCl, 155 mM NaCl, pH 7.4) or plasma and mixed gently at room temperature (approximately 20-23°C).

7.3 FXII ADSORPTION MEASUREMENTS BY THE INCUBATION TEST CELL PROCEDURE FOR SHEET MATERIALS

A number of experiments were conducted using the incubation test cell in order to establish quantitatively the amount and the mechanism of factor XII adsorption on different membranes. These were as follows:

1. Kinetics of the surface adsorption of factor XII on AN69S and Cuprophan membranes, using plasma and single protein solutions.
2. The effect of plasma pH on surface adsorption.
3. The effect of pharmaceutical agents on factor XII adsorption.
4. The effect of the washing detergent used.
5. The effect of ionic strength of the Tris buffered saline using single protein solutions.
6. The total factor XII adsorption measurement on AN69S, Hemophan and Cuprophan membranes.

7.3.1 ELUTION PROFILE FOR SURFACE ADSORBED FACTOR XII:

AN69S was contacted with labelled factor XII diluted in Tris buffered saline (5 mM Tris, 5 mM NaCl). In order to optimise the washing procedure, eliminating the non-adsorbed factor XII, the elution profile of labelled factor XII was measured on AN69S, after washing with Tris buffered saline (5 mM Tris, 155 mM NaCl) with

washes ranging from 1-6 times.

Using the single protein solution, 1 ml aliquots of the diluted labelled protein solution were added into the 6 wells of the incubation test cell lined with AN69S membrane and incubated on an orbital shaker for 1 hour at room temperature (20-22°C). After incubation, the supernatant was removed and discarded carefully into an appropriate waste disposal unit for radionuclides.

The membranes were washed using Tris buffered saline (5mM Tris HCl, 155 mM NaCl pH 7.4) for 1 min. An elution profile was investigated by taking a radioactivity count of 10 μl of supernatant washing from 6 different wells washed in a successive manner. The first, second, third, fourth, fifth and six wells were washed at 1, 2, 3, 4, 5, 6 times respectively. The procedure was then repeated 4 times and the remnant membrane slices were also read for radioactivity to determine the membrane desorption profile.

7.3.2 KINETICS OF FACTOR XII ADSORPTION

In order to optimize the contact time required for adequate factor XII adsorption on membranes before washing off of the non-adsorbed factor XII, a time course pattern of both single (FXII in TBS solution) and multicomponent (plasma) systems were investigated.

The time course for factor XII adsorption on AN69S and Cuprophan membranes was investigated by incubating 1 ml diluted labelled factor XII on the membranes lined on the incubation test cell well. The length of the incubation time for each cell well was designated as follows: Well 1, 2, 3, 4, 5, and 6, incubated for 30 sec, 1 min, 2 min, 5 min, 20 min and 60 min respectively. The membranes were then washed 3 times with Tris buffered saline (5 mM Tris HCl, 155 mM NaCl, pH 7.4), excess moisture removed by blotting with adsorbent paper and radioactivity counts on the membrane segments taken. The procedure was then repeated 4 times.

In another set of experiments, labelled plasma was used instead of the diluted labelled factor XII solution on AN69S membrane.

7.3.3 COMPARISON OF FACTOR XII ADSORPTION IN PLASMA AND IN PURIFIED FACTOR XII PROTEIN ON MEMBRANES

The pattern of FXII adsorption to AN69S, Cuprophan and Hemophan

membranes was compared using the single protein system and the plasma system described above. All the membranes were contacted with labelled factor XII solution diluted with Tris buffered saline (1 mM Tris HCl; 1 mM NaCl) or labelled plasma for 20 minutes at room temperature (20-22°C). The supernatant plasma or FXII solution were removed and disposed off appropriately. The membranes were then washed 3 times and blotted off gently to remove excessive moisture and counted for radioactivity (counts per minute or cpm) using a gamma counter.

7.3.4 THE EFFECT OF IONIC STRENGTH ON FXII ADSORPTION

Further experiments were performed to investigate whether ionic strength of the solvent (Tris buffered saline) was influential on the ionic binding of factor XII on all the membranes evaluated. In theory, ionic strength should influence the ionic forces adsorption of FXII by the direct competition with Na^{2+} ions for negatively charged centres on the membrane.

Labelled FXII solutions were prepared by dilution with Tris buffered saline at concentrations of 1, 5, 155, 300 mM NaCl respectively. The protein solutions were incubated on Cuprophan, Hemophan, and AN69S for 20 minutes at room temperature before the removal of all the supernatant. The membranes were then washed 3 times and blotted using adsorbent paper. The membranes were then counted for radioactivity and values reported as counts per minute (cpm).

7.3.5 THE EFFECT OF WASHING DETERGENT ON FACTOR XII ADSORPTION.

The analysis of the possible mechanisms involved in factor XII binding to surfaces, requires experiment on the release of the adsorbed proteins by specific detergents. In agreement with the work done in the area of complement binding to surfaces such as Sepharose-trypsin (Sim et al 1981) and on dialysis membranes (Mahiout 1994), a detergent was used to wash the membrane surfaces after plasma contact with different dialysis membranes (as described above). The detergent composition was as follows: 6 M Urea; 0.1% sodium dodecyl sulphate. The detergent was found to breakdown mostly hydrophobic interactions (Sim et al 1981; Mahiout 1994). The values were compared to those obtained during washing with Tris buffered saline (5 mM Tris HCl, 155 mM NaCl), which accounts for total

factor adsorption.

7.3.6 THE EFFECT OF PHARMACOLOGICAL AGENTS ON FXII ADSORPTION

Further experiments were performed to ascertain the effect of the pharmacological agents heparin, aprotinin and captopril on factor XII adsorption by membranes. Labelled plasma spiked with heparin (1 IU/ml), aprotinin (250 KIU/ml) and captopril (2.5 μg/ml) was contacted to the membranes for 20 min and after washing the membrane segments were counted for radioactivity.

7.3.7 THE EFFECT OF PLASMA pH ON FXII ADSORPTION

In order to determine further the role of ionic forces in the mechanism of factor XII binding to membrane surfaces, experiments were conducted by varying the plasma pH. With an isoelectric point of 6.1-6.6, factor XII is positively charged at pH below 6.6 and negatively charged above pH 6.6. Plasma samples at pH 5.3 and 11.0 were therefore contacted onto the membrane.

7.4 FACTOR XII ADSORPTION ON HOLLOW FIBRE MEMBRANES

At the start of each experiment, aliquots of the plasma were thawed, spiked with the radioiodinated factor XII solution and drawn into a 30 ml disposable syringe. 0.5 ml of the plasma were then transferred into a tube and used as the time = 0 (precontact) sample. The remainder of the plasma was retained in the syringe which was mounted onto the syringe pump and was then run through the module blood compartment at a non-pulsatile flow rate of 1.2 ml/min for 20 min. The modules were then rinsed with Tris buffered saline (0.5 mM Tris HCl; 155 mM NaCl) in a 30 ml syringe at a flow rate of 1.2 ml/min for 10 min. After rinsing the module fibres were removed from the outer jacket, blotted with adsorbent paper. Radioactivity was counted three times in a standard gamma counter (Packard Ltd UK, Oxon, UK).

7.4.1 CALCULATION OF CONCENTRATION OF ADSORBED FACTOR XII PROTEIN

Protein adsorption on the membrane segments (flat sheets) or the hollow fibres after contact with plasma was estimated from the following formula:

$$P_{ad}[mem] = (P_{ad}[CPM] / P_0[CPM]) \times P_{total}[mem] / SA \quad \text{..... [7.1]}$$

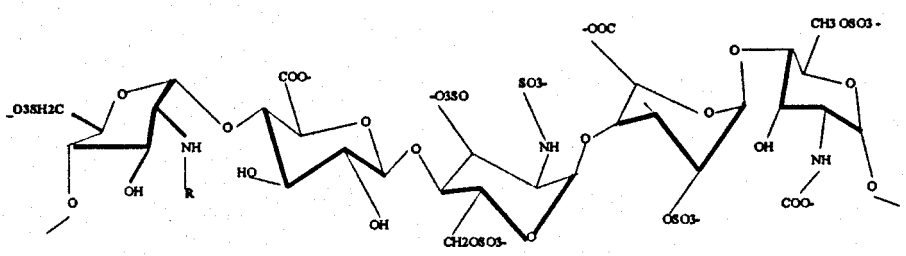


Figure 7..1: The structure of heparin (Lane & Lindahl 1989)

P_{ad}[mem] is the amount of FXII protein adsorbed in the membrane segment or the module; P_{ad}[CPM] is the measured membrane associated FXII protein in counts/min; P_0[CPM] is the amount of labelled FXII protein in non-contacted plasma in counts/min/1 ml; P_{total} is the total amount of unlabelled FXII protein in the plasma volume (1 ml); SA is the membrane or module surface area (cm^2). For hollow fibres, plasma volume of modules was determined as follows:

$$\pi.r^2.L.n \quad \text{.....[7.2]}$$

Where 2r is the internal diameter of the hollow fibres, L is the length of the hollow fibre, n is the number of hollow fibres contained in a module.

7.5 STATISTICS

Statistical analysis of results was performed using a Minitab package version 8.0. In order to compare the patterns of polymer influence on factor XII adsorption, the mean adsorption values were compared. Comparison of the mean adsorption values was performed by a twosample t-test, and the differences were considered significant at $p < 0.05$ with a 95% confidence interval. The findings were confirmed by using a nonparametric statistics test (Mann Whitney) on the medians. A similar analysis was adapted for the comparison of mean values for heparin adsorption on the membranes.

7.6 METHODOLOGY FOR THE MEASUREMENT OF HEPARIN ADSORPTION

7.6.1 INTRODUCTION

Heparin is a negatively charged polysaccharide, mainly composed of alternating residues of sulphated glucuronic, iduronic acid and glucosamine derivatives linked in the 1-4 position (Figure 7.1). Heparin has been used as a major anticoagulant for many years and the success of the clinical application of most major extracorporeal devices have largely depended on the availability of heparin. However, the composition of this polydisperse biopolymer and specific mechanism of its clot-preventing effect have been the subject of intense investigation (Donati and Pengrazzi 1989).

Heparin is a heterogenous mixture of polysaccharide chains with variable molecular weights from 5,000 - 35,000. Heparin derived from ox lung or pig

intestinal mucosa, consists of repeating disaccharide units. The disaccharide units have a strong anionic centres, due to the presence of sulphate and carboxyl groups. It is therefore highly likely that this chemically potent drug will interact with foreign surfaces. As an example, membranes prepared from diethylaminoethyl (DEAE)-cellulose/cellulose blend polymer (tradename Hemophan) where the tertiary amine ether group on DEAE-cellulose is believed to quarternize at pH 7.4, forming an ionic binding site with the negatively charged sulphate and carboxyl groups of heparin. Heparin adsorption onto foreign surfaces may well be due to other factors such as electrostatic attractions, hydrophobic or van der Waals forces (Matsuda et al 1989).

Aside from influencing the anticoagulant properties of extracorporeal materials, it can be assumed that the layer of coating of membrane-bound heparin should affect surface-blood interactions (von Sengbusch et al 1993; Vienken & Bowry 1993). Heparin in solution may also interact with plasma components such as heparin-binding proteins, unsaturated fatty acids, and platelets, and as a consequence may have a major influence on the overall biocompatibility of a system (Gault et al 1992).

7.6.2 SELECTED METHODOLOGY FOR THE ASSESSMENT OF HEPARIN ADSORPTION.

The radiolabelled heparin assay technique was preferred, because of its simplicity and the fact that it can be monitored continuously. N-sulphonate ^{35}S-heparin (1.7 mg, 3.7 MBq) was purchased from Amersham UK (Aylesbury, Bucks, UK) and was made up into a 1 ml solution using phosphate buffered saline or PBS (0.15 M NaCl, 0.05 M phosphate, pH 7.4). An aliquot of 58.82 μl of the diluted solution was made up into a 10 ml volume with PBS, giving a concentration of ^{35}S-heparin of 10 μg/ml. The incubation test cell described in chapter 4 was used to achieve contact between the ^{35}S-heparin and the membranes.

1 ml of the diluted ^{35}S-heparin solution was contacted with slices of AN69S, Cuprophan, Hemophan, and SPAN membranes for 2 hours at room temperature. At the end of the incubation period, heparin solution was removed and the membranes washed gently with 1 ml of PBS. This was repeated 3 times and each time the

incubation test cell was shaken for 1 minute using an orbital shaker. The test cell was then unclamped and the membrane slices removed and dried using adsorbent tissue paper. The membrane slices were then placed into scintillation vials and 5 ml of "Ecoscint A" scintillation fluid was then added. Radioactivity was counted for 1 minute using a β-scintillation counter. All readings were corrected for background radioactivity as follows:

(a) For the diluted stock ^{35}S-heparin solution, counts per minute were corrected against a background of vial containing 5 ml scintillation fluid only.

(b) For the membrane slices, counts per minute were corrected against a background of a non-contacted membrane slices (of the same surface area), immersed in 5 ml scintillation fluid. Membrane radioactivity was calculated as a percentage of the readings from the 1 ml diluted stock ^{35}S-heparin solution, and expressed as a percentage per total surface area of the membrane.

7.7 ANTICOAGULANT PROPERTIES OF MEMBRANE-BOUND HEPARIN

7.7.1 INTRODUCTION

As a result of the special relevance of heparin to the coagulation system, clotting tests remain important for demonstrating the biological activity of immobilized heparin. Recalcified plasma or whole blood clotting times and partial thromboplastin times as well as the more specific clotting tests (eg thrombin time, FXa inhibition assays) are useful. The latter are particularly helpful in the assessing the effectiveness of the heparinized material at specific steps in the coagulation cascade.

Results may be reported directly as clotting times or in terms of units of biological activity (eg as a %). Units of heparin per unit area or mass may be determined by comparison of clotting times obtained in equivalent fashion with heparin solutions of different concentration. Similarly, the number of thrombin units inactivated per unit mass or area may be determined by comparison with standard curves relating the clotting time to thrombin concentration (Fougnot et al 1979a and 1979b).

Instead of using the appearance of polymerized fibrin clot as the endpoint of the assay, the amount of active thrombin or factor Xa may be determined using a

chromogenic substrate. After incubating the immobilized heparin with enzyme (thrombin or FXa) and a source of antithrombin III (defribinated plasma), the residual enzyme activity (adsorbed and in solution) was determined spectrophotometrically (Dynatech MR5000) after the addition of the appropriate specific chromogenic substrate (Miura et al 1980). Chromogenic substrate assays, however, have the advantage over clotting assays by being more precise, since detecting the appearance of a clot is somewhat subjective. The chromogenic substrate assay selected for the study of activity of membrane-bound heparin was the coatest-R-heparin kit (Chromogenix BA, Tuijegardsgartan 3, S-431 53 Molndal, Sweden), utilising the chromogenic synthetic tripeptide (Bz-Ile-Glu-(or)-Gly-Arg-pNA-HCl) or S-2222. The same kit was also used to determine the anti-FXa activity of heparinised blood (using standard unfractionated heparin; "Heplok" from Leo laboratories Ltd, Risborough, Bucks, UK) after contact with the membranes. The latter findings were compared to the clotting assay test (activated partial thromboplastin test) as a measure of the residual coagulant property of heparinised blood after blood-membrane contact. For blood anticoagulated with low molecular weight heparin; "Fragmin" (Quadratech, Epsom Surrey UK), heparin anti-Xa activity was determined using a kit from chromogenix.

7.7.2 ANTI-FXa ASSAY.

The assay is based on the ability of the heparin-AT-III complex to neutralize factor Xa (FXa). FXa (in excess) was neutralized in proportion to the amount of heparin, which determines the amount of heparin-AT-III complexes. Since the FXa was used in excess, the remaining amount of FXa was then detected by its hydrolysis of the chromogenic substrate, therefore liberating a chromophoric group pNA, and colour is then read photometrically at 405 nm. The assay kits contained all the reagents, except heparin (stock solution of 10 IU/ml).

7.7.3 PROCEDURE FOR THE DETERMINATION OF ANTICOAGULANT PROPERTIES OF MEMBRANE-BOUND HEPARIN USING ANTI-Xa ASSAY

An incubation test cell was used for heparin-membrane contact. 1 ml of heparin (2 IU/ml) was contacted onto the membranes fixed onto the test cell for 2 hours first. Residual heparin was then removed and membrane washed 3 times with

PBS. 1 ml of AT-III was then added and incubated for 5 minutes. Residual AT-III was removed and the membrane was again washed 3 times with PBS. 1.0 ml of FXa was added and incubated for 1 minute. Residual FXa was then removed and the membrane washed 3 times with PBS. 1 ml of substrate was then added and incubated for 20 minutes. 800 μl of the substrate was then removed into cuvettes (BDH Ltd, Glasgow, UK) and absorbance read at 405 nm against a suitable blank (800 μl distilled water).

7.7.4 HEPARIN ANTICOAGULANT ACTIVITY IN WHOLE BLOOD AFTER MEMBRANE CONTACT

7.7.4.1 BLOOD-MEMBRANE CONTACT

Blood samples were obtained from volunteers and a number of aliquots (1.5 ml each) containing a range of different concentrations of Heplok heparin or Fragmin were prepared (ranging from 1-10 IU/ml). Membranes were washed and soaked in 0.9% saline solution overnight. The membranes were then fixed onto the incubation test cells. Aliquots of blood containing similar heparin concentrations were also prepared and these were used as the standard non-contacted control samples.

The rest of the blood samples were contacted to the membranes for 10 minutes during which adequate mixing was achieved using an orbital shaker. After the incubation, 1 ml aliquots were removed from the test cells and anticoagulated using 3.2% disodium citrate (at a ratio of 1:9 of blood). 1 ml aliquots of the blood samples were removed and then centrifuged at 3000 rev/min for 12 min and the resultant plasma was assayed for heparin anti-Xa activity and activated partial thromboplastin time (APTT).

7.7.4.2 HEPARIN ANTI-Xa ASSAY PROCEDURE

Using the coatest-R-heparin assay kit (chromogenix, Sweden), the following reagents were added into the microtitre plates:

1. Test/standard plasma + antithrombin III + heparin buffer were added into the wells of the microtitre plate as described in the kit insert.
2. FXa (20-25°C) was then added and incubated at 37°C for 30 sec.
3. S2222 substrate (37°C) was then added and incubated at 37°C for 5 min.
4. 20% Acetic acid was added to stop the reaction.

5. The absorbance was read at 405 nm.

7.7.4.3 ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT) PROCEDURE

The intrinsic coagulation system was activated by micronized silica plus bovine brain cephalin, which acts as the substitute for platelet factor 3. Clotting was started by the addition of calcium chloride. APTT determination was not performed for the plasma samples anticoagulated with Fragmin since it has been shown that this assay was not a sensitive procedure for the detection of low molecular weight heparin (Bratt et al 1985). The procedure was carried out using an ACL-300 automated coagulometer (ACL International, Ltd, UK), which measures the intensity of the light scattered by a sample plasma before, during and after clot formation.

The procedure was as follows:

(1) APTT reagents (purchased from ACL International Ltd, UK) were pipetted into reagent reservoir number 2 and calcium chloride into reagent reservoir number 3.

(2) Plasma samples were placed into the tray in position 1-18 and calibration plasma in the pool position of the sample tray. The reaction temperature was set at 37^{0}C. The APTT programme was selected and the reading of the clotting time was measured and printed out automatically. Expected reference range for normal healthy individuals not on anticoagulants was 27 - 35 seconds and a ratio of 0.90 - 1.15.

7.8 MEMBRANE SURFACE PROPERTIES DETERMINATION

7.8.1 INTRODUCTION

The use of charged materials in biology, medicine and biotechnology has greatly increased in recent years. In biomaterials research, the introduction of charged groups by chemical modification of dialysis membranes to improve their biocompatibility and especially the influence of charged compounds in a membrane on complement activation has been shown to be useful (Carreno et al 1988; Matsuda 1989). The use of polyion complexes for encapsulation of cells, the adherence of cells on charged biomaterials, and the heparinisation of membranes are further examples of application of charged materials (Sasaki et al 1988; Iwata et al 1988). A variety of methods for the detection of positive or negative charges on and in

biomaterials, have been reported and of particular interest is the one by Thomaneck et al (1991) on dialysis membranes, utilising cationic and anionic dyes. The focus in this thesis was on the procedures suitable for the evaluation of surface roughness (morphology), surface charge and surface charge distribution.

7.8.2 SURFACE MORPHOLOGY BY SCANNING ELECTRON MICROSCOPY

Dry membrane samples were mounted on a stub using double sided adhesive tape. The top surface of the stub, from the edges to the rim, was lined with conducting paint to avoid non-conducting gaps and subsequent charging. The conducting paint was allowed to dry and the specimen was gold-coated using a Polaron-3000 sputter unit, at a setting of 10.666 Pa and 40 mA for 2 minutes to give a 20 nm thick gold coating, that allows conduction without charging.

The specimens were then examined in a JEOL JSM-840A scanning electron microscope (JEOL LTD, Masushimo, Japan). The basic principle of the scanning electron microscope can be summarized as (Wilson 1985):

(1) The focusing of a beam of high energy electrons onto the surface of a specimen.

(2) The high energy electron beam bombard specific points of the specimen surface as it scans along in a regular pattern.

(3) The interaction of these primary electrons causes back-scattered and induced emission of electrons with different properties depending on the physical characteristics of the specimen.

(4) Electrons leaving the top surface of the specimen are captured by the collector, converted to photons and amplified to produce a signal voltage, which in turn modulates the brightness of the cathode ray tube (CRT).

(5) The CRT and scanning coils are driven from the same timing circuit and so a point on the CRT can be related to a point on the specimen and a meaningful image built up.

Appropriate accelerating voltage and working distance are selected and are indicated at the foot of each photograph. Maximum resolution is obtained at an accelerating voltage of 25 kV, the magnification was then adjusted and the picture

focused and then photographed.

7.8.3 SURFACE CHARGE DETERMINATION USING ANIONIC AND CATIONIC DYE STAINING TECHNIQUE

Small pieces of flat membranes (1 x 1 cm) were thoroughly washed with distilled water (3 times in 20 ml). They were then stained in tubes for 10 minutes at room temperature with the dye solutions: 0.5% acid blue 41 (anionic dye), 0.5% malachite green (cationic dye), 0.1% ethidium bromide (cationic dye), and 0.5% methyl violet. The membranes were again washed three times with distilled water and dried in air.

In another set of experiments, the membrane slices were coated with heparin by contact with 1 ml heparin solution at different concentrations of 100 IU/ml and 1000 IU/ml respectively at room temperature (20-23°C). They were then rinsed with water (20 ml for 5 minute) and stained as described above.

The specimens were then mounted onto slides using a glycerol-based mounting medium and colour intensities were then compared qualitatively (Figure 7.12f).

7.8.4 CHARGE DISTRIBUTION BY CONFOCAL LASER SCANNING MICROSCOPY

After thorough washing (3 times in 20 ml distilled water), the membrane slices were stained with 0.1% fluoresceine isothiocyanate (FITC)-labelled poly-L-lysine for 10 minutes at room temperature. The membrane samples were then washed 3 times with water, dried in air and mounted on slides using glycerol mounting medium.

The stained membrane samples were analyzed for charge distribution using the confocal laser scanning microscopy (CLSM) (Thomaneck et al 1991). The basic principle of the CLSM has been described (Wilson & Sheppard 1985; Brakenhoff et al 1986). A laser beam is focused on or in the object and by scanning the object or of the laser beam from a mirror the very small laser spot illuminates the object pointwise line for line at a relatively high speed. The defined projection of laser point source and detector pinhole in the focal plane of the microscope objective make it possible to apply signals only from the point of focus to build up the image

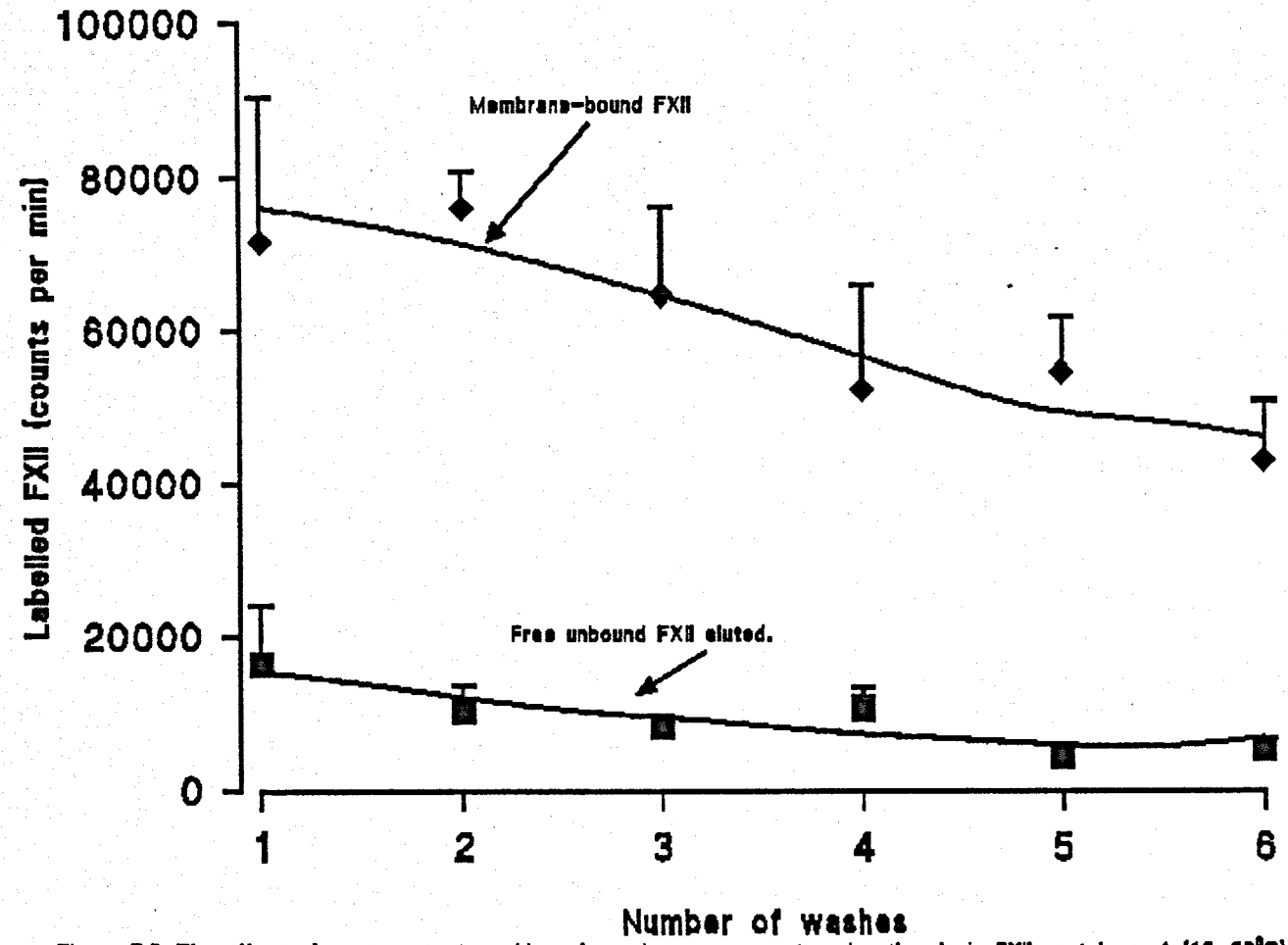


Figure 7.2: The effect of post-contact washing of membrane segments using the single FXII protein, n=4 (16-22°C)

(confocal principle). In this way only the signals from the point of focus could be detected and the computer builds up the image point by point.

In this study, scanning was done in the xy- and xz- directions by moving the object desk. Using image analysis the objects measured were presented as grey scale or as false colour images and in the case of object and objective (xy/z), scanning information from all three directions was accessible (3D images, microtomoscopy). The intensity of fluorescence signals was presented in grey scale or false colour images on a TV screen and documented by photography. The slides were examined on oil-emersion, using a X 40 FLUOTAR lens with a numerical aperture of 1.3. The instrument was set to read at 488 nm and excitation beam splitter was set at 510 nm to filter out reflected light. A 590 nm low pass filter was also used to cut out the effect of ultrafluorescence. The working voltage and workwindow area were selected to suit the image requirement.

7.9 RESULTS

7.9.1.1 FACTOR XII ADSORPTION RESULTS - INCUBATION TEST CELL PROCEDURE FOR SHEET MATERIALS

7.9.1.2 ELUTION PROFILE FOR SURFACE ADSORBED FACTOR XII

Table 7.1: Single FXII protein system supernatant and membrane counts profile

Washes	Supernatant counts per min (cpm) ± SD (n=4)	Membrane counts per min (cpm) ± SD (n=4)
Pre-wash	224682 ± 481	36904 ± 36904
1	16499 ± 7609	71575 ± 18798
2	104471 ± 3114	75943 ± 4931
3	8392 ± 1071	64676 ± 11407
4	10661 ± 2655	52231 ± 13576
5	4538 ± 487	54642 ± 7006
6	5470 ± 1362	43044 ± 7788

Figure 7.2 and table 7.1 show the elution profile of labelled factor XII from AN69S sheet membrane. After 3 washes, the non-adsorbed labelled factor XII was

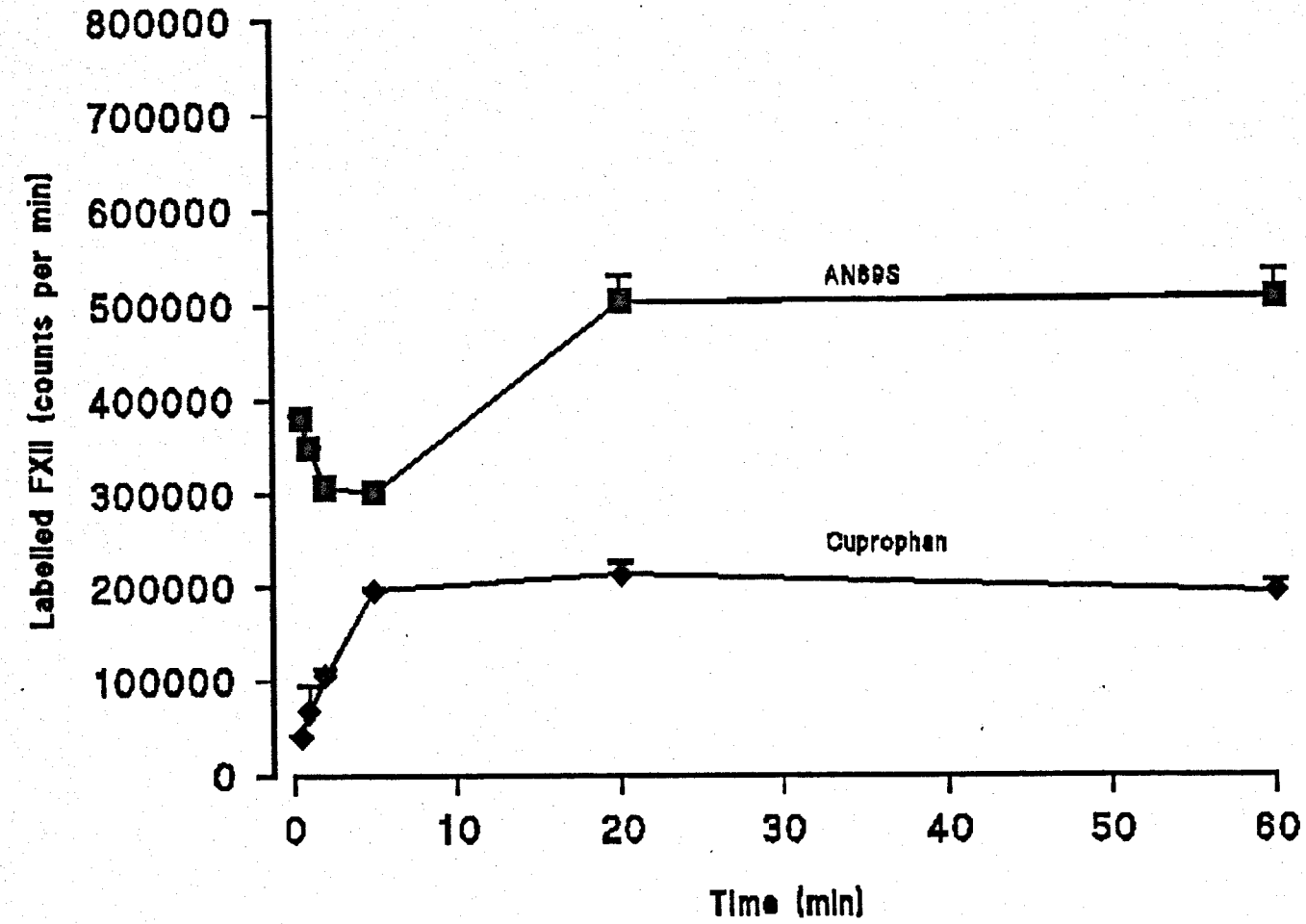


Figure 7.3: The kinetics of surface adsorption of FXII from labelled FXII solution, n=4 (18-22°C).

eliminated, and the subsequent radioactivity counts in the supernatant and on the membrane surfaces remained similar.Following the above results, the optimum number of times the membrane segments were washed prior to counting was selected as to be 3 times for all the subsequent experiments.

7.9.1.3 KINETICS OF FACTOR XII ADSORPTION (TIME COURSE STUDY)

With an average labelled factor XII concentration of 0.083 μg/ml, adsorption was higher for AN69S than in Cuprophan after the incubation of labelled factor diluted in Tris buffered saline.

Table 7.2 Single protein system: time course for ^{125}I-FXII adsorption on AN69S and Cuprophan membranes (n=4)

Membrane	Mean surface counts per min ± SD at each incubation time.					
	30 sec	1 min	2 min	5 min	20 min	60 min
AN69S	381496 ± 2594	349934 ± 1128	308181 ± 1156	302720 ± 1402	504089 ± 26298	508604 ± 28522
Cuprophan	41318 ± 2121	68137 ± 27584	105498 ± 6722	196548 ± 1292	213643 ± 13506	195377 ± 10309

A steady state FXII adsorption occurs after 20 minutes of contact with the AN69S membrane, thus reaching an optimum rate at this stage. However the magnitude of the factor XII adsorption was significantly higher for AN69S as compared to Cuprophan membrane (table 7.2, figure 7.3).

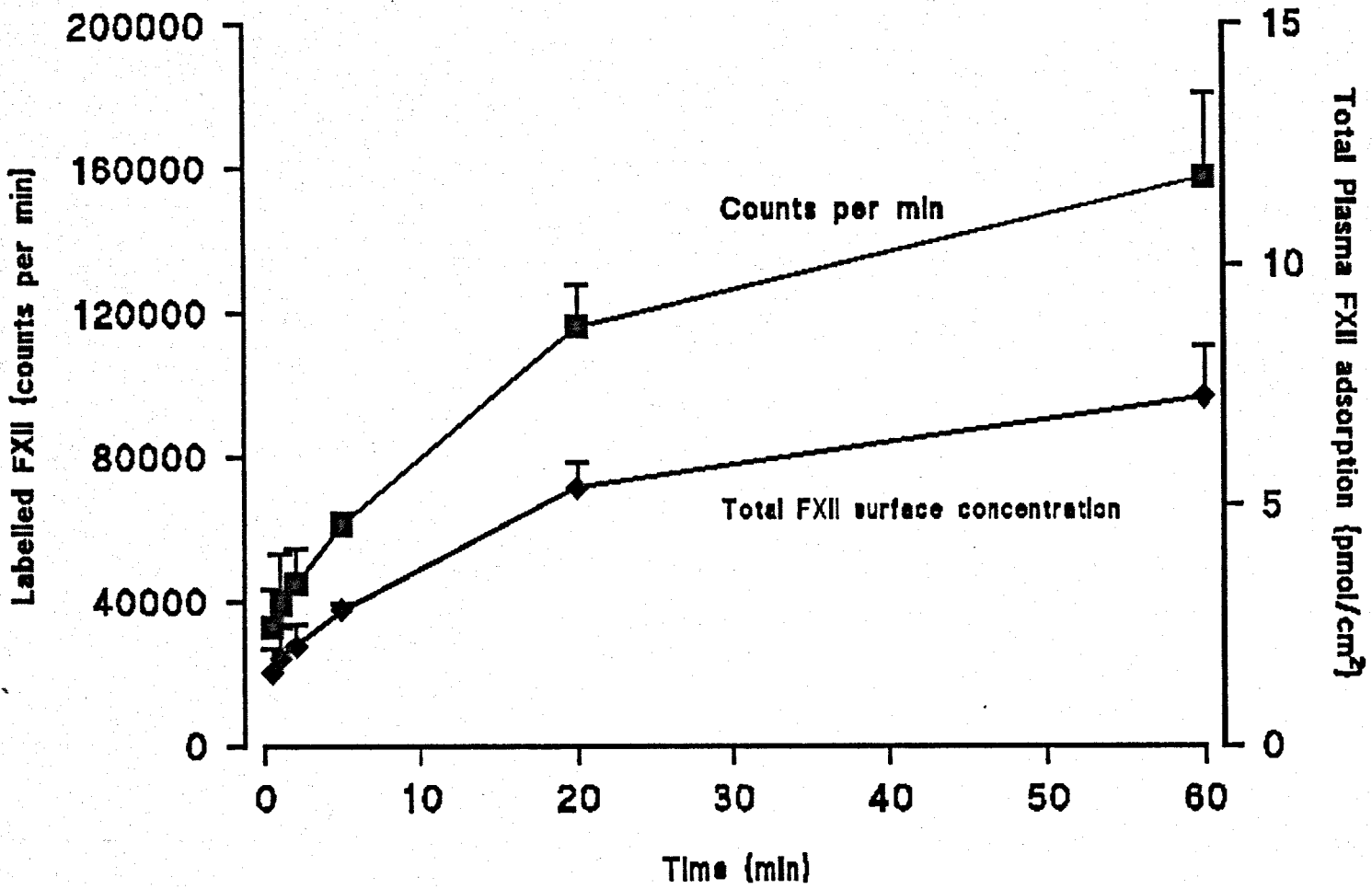


Figure 7.4: The time course for plasma FXII adsorption on AN69S membrane, n=4 (18-22°C).

Table 7.3: Time course for the adsorption of factor XII from labelled plasma. (Plasma system), n=4.

AN69S	30 sec	1 min	2 min	5 min	20 min	60 min
Mean cpm ± SD	32795 ± 10712	39342 ± 13797	44900 ± 9719	61210 ± 2992	116314 ± 11660	157387 ± 22992
Total plasma FXII (pmol /cm^2)	1.51 ± 0.49	1.81 ± 0.64	2.07 ± 0.45	2.82 ± 0.14	5.36 ± 0.54	7.25 ± 1.06

Cuprophan showed a markedly reduced rate of FXII adsorption, even by these low FXII concentrations (purified factor XII solution system) involved compared to plasma (25-40 μg/ml). The adsorption rate appeared to reach an optimum steady state at 5 minutes of contact with Cuprophan. There was a marked difference in the rates of adsorption of FXII between AN69S and Cuprophan. Table 7.3, figure 7.4 show the kinetics of factor XII adsorption on AN69S using a multi-component system of plasma containing an average of 25 μg/ml, and a counterpart of 0.083 μg/ml of labelled factor XII. The total surface adsorbed factor XII concentration values were expressed in terms pmoles/cm^2 as calculated using the formula below:

Total FXII (pmoles/cm^2) = amount of FXII (g/ml/cm^2) / molecular weight of FXII ..... (7.3)

The pattern indicates a rapid adsorption reaching an optimum at 20 minutes contact time, thereafter the reaction rate was much slower as compared to the first 20 minutes. The counts per minute observed with the plasma system were significantly lower at each incubation time as compared to the single protein system ($p < 0.05$). This may be due to the absence of competitive binding with the latter system. As a consequence of the above findings an optimum incubation time of 20 minutes was selected for all the subsequent experiments.

7.9.1.4 FACTOR XII ADSORPTION PATTERNS

Single protein system:

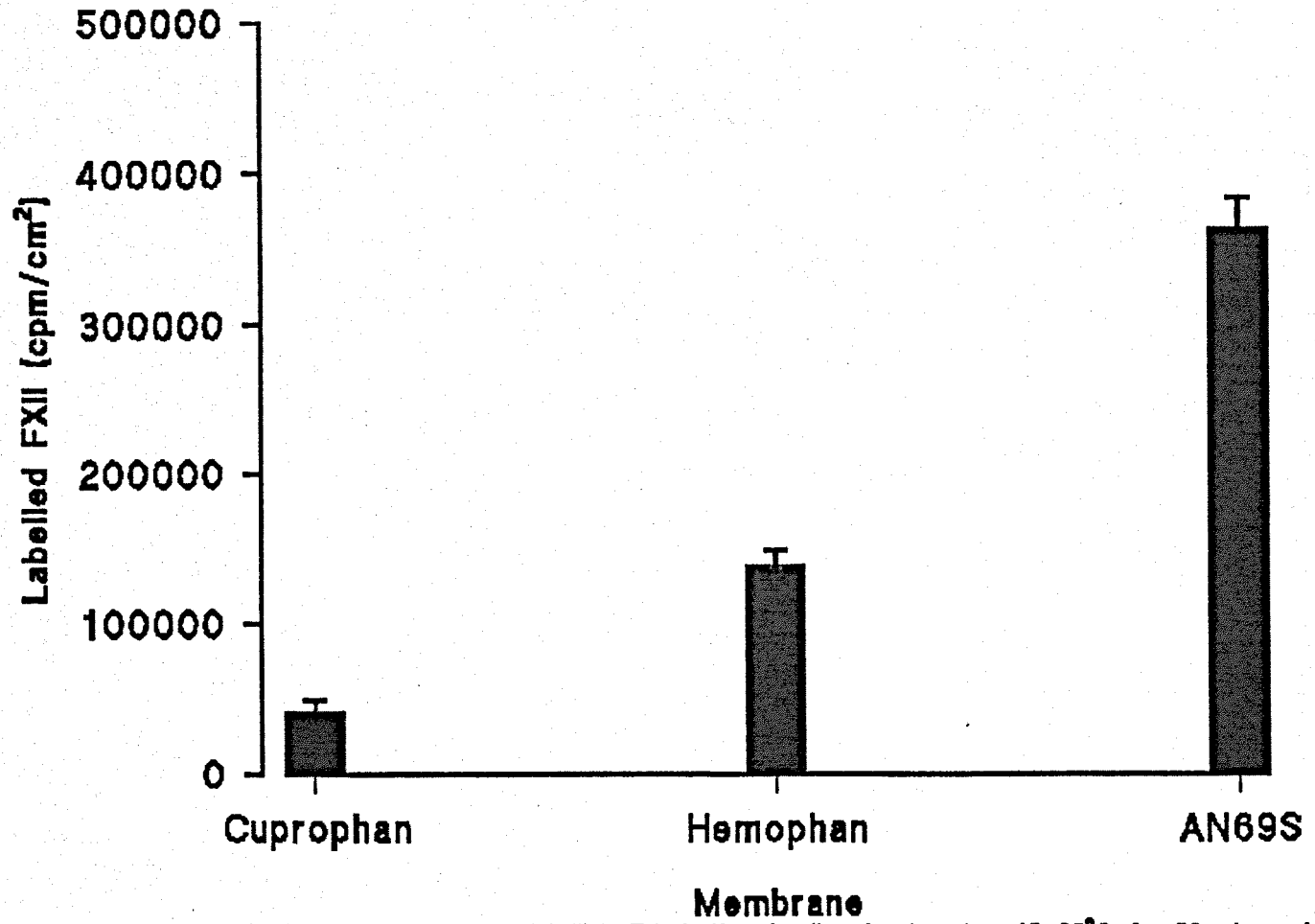


Figure 7.5a: Factor XII adsorption from purified FXII in Tris buffered saline, incubated at 18–22°C, for 20 min, n=4.

After 20 minutes contact of labelled factor XII solution with the membranes Cuprophan, Hemophan and AN69S, significantly lesser adsorption ($p < 0.05$) was observed on Hemophan and Cuprophan compared to AN69S (table 7.4, figure 7.5a).

Table 7.4: Single protein system: adsorption of labelled FXII in Tris Buffered Saline (cpm), n=4.

Membrane	Mean CPM ± SD
Cuprophan	40793 ± 8240
Hemophan	137906 ± 11196
AN69S	363172 ± 20486

The magnitude of the FXII adsorption appeared to be in agreement with the surface charge evaluation patterns, where AN69S was found to be negatively charged, while Cuprophan was faintly or uncharged and Hemophan was positively charged.

Plasma system:

After plasma-membrane contact for 20 minute, the FXII adsorption patterns were demonstrated to be significantly greater ($p < 0.05$) for AN69S as compared to the other membranes. The plasma system also demonstrated a strong relationship between surface charge and the magnitude of surface adsorbed FXII, with the negatively charged AN69S membrane adsorbing more FXII (table 7.5, figure 7.5b).

The result suggested that in addition to the ionic interaction of factor XII with membranes there is also a significant amount of hydrophobic interaction. The results indicated that even after the removal of the hydrophobically bound factor XII, the negatively charged AN69S membrane appeared to adsorb more factor XII as compared to the uncharged Cuprophan and the positively charged Hemophan. Hemophan lost a greater amount of surface adsorbed factor XII as compared to Cuprophan, indicating that hydrophobic interaction of factor XII to membranes was much more important with Hemophan. The estimated magnitude of the factor XII bound to the membrane by other forces excluding hydrophobic interactions was indicated by the results as being as follows:

Cuprophan 68%, Hemophan 28%, and AN69S at 48%.

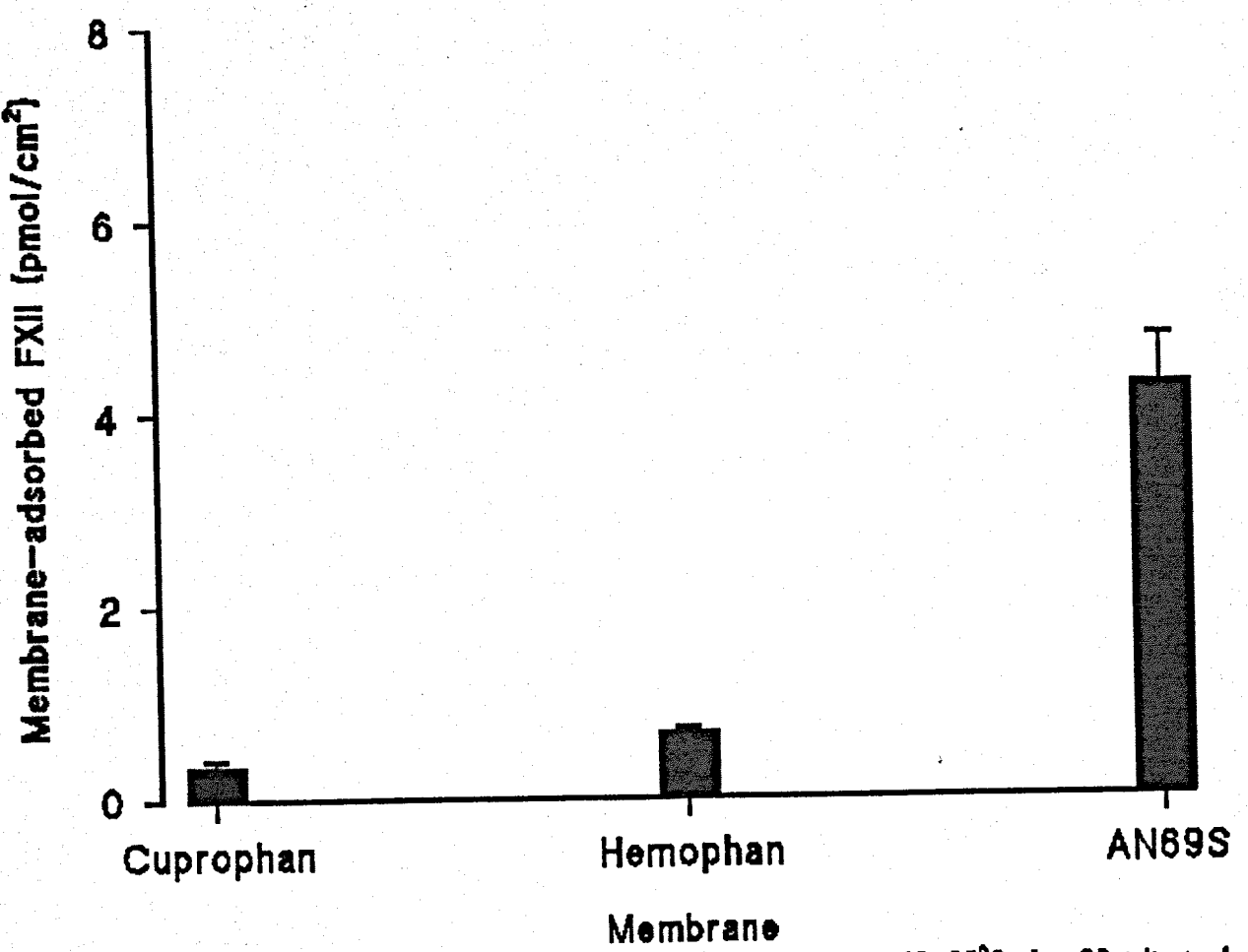


Figure 7.5b: membrane-adsorbed plasma Factor XII (pH 7.4), incubated at 18-22°C, for 20 min, n=4.

Table 7.5: Total plasma FXII adsorption (in counts/min and pmol/cm^2), n=4.

Membrane	Mean cpm ± SD	Conc. in ng/cm^2	Conc in pmole/cm^2
Cuprophan	9582 ± 1834	10.83 ± 2.07	0.34 ± 0.06
Hemophan	19280 ± 13770	21.80 ± 15.57	0.68 ± 0.05
AN69S	120940 ± 14138	136.74 ± 15.99	4.27 ± 0.50

7.9.1.5 THE EFFECT OF IONIC STRENGTH ON FXII ADSORPTION

As shown on table 7.6 and figure 7.6, increasing the ionic strength of the solvent containing the labelled FXII, significantly reduced ($p<0.05$) FXII adsorption on AN69S membrane. By comparing the effect of the ionic strength on the adsorption of FXII on Hemophan and Cuprophan, it was shown that increasing ionic strength of the salt solution from 1-100 mM, significantly reduced ($p<0.05$) FXII adsorption on Hemophan. By contrast, increasing the ionic strength of the salt solution from 1-100 mM significantly increased FXII adsorption to Cuprophan.

Table 7.6 The effect of ionic strength on factor XII adsorption from a purified factor XII/salt solution system, n=4.

Membrane	Radioactivity in counts per minute ± SD			
	0.2 mM NaCl	5 mM NaCl	155 mM NaCl	300 mM NaCl
Cuprophan	40793 ± 8240	68140 ± 36157	75718 ± 33603	64931 ± 21403
Hemophan	137906 ± 11196	55306 ± 499	27785 ± 3696	27284 ± 3696
AN69S	223962 ± 17473	54784 ± 11352	49937 ± 12457	48449 ± 5445

7.9.1.6 THE EFFECT OF WASHING DETERGENT ON FACTOR XII ADSORPTION

The results indicated that the amount of the factor XII adsorption was significantly reduced ($p<0.05$) on all the membranes by washing with the urea/sodium dodecyl sulphate (SDS) detergent (table 7.7, figure 7.7). The pattern

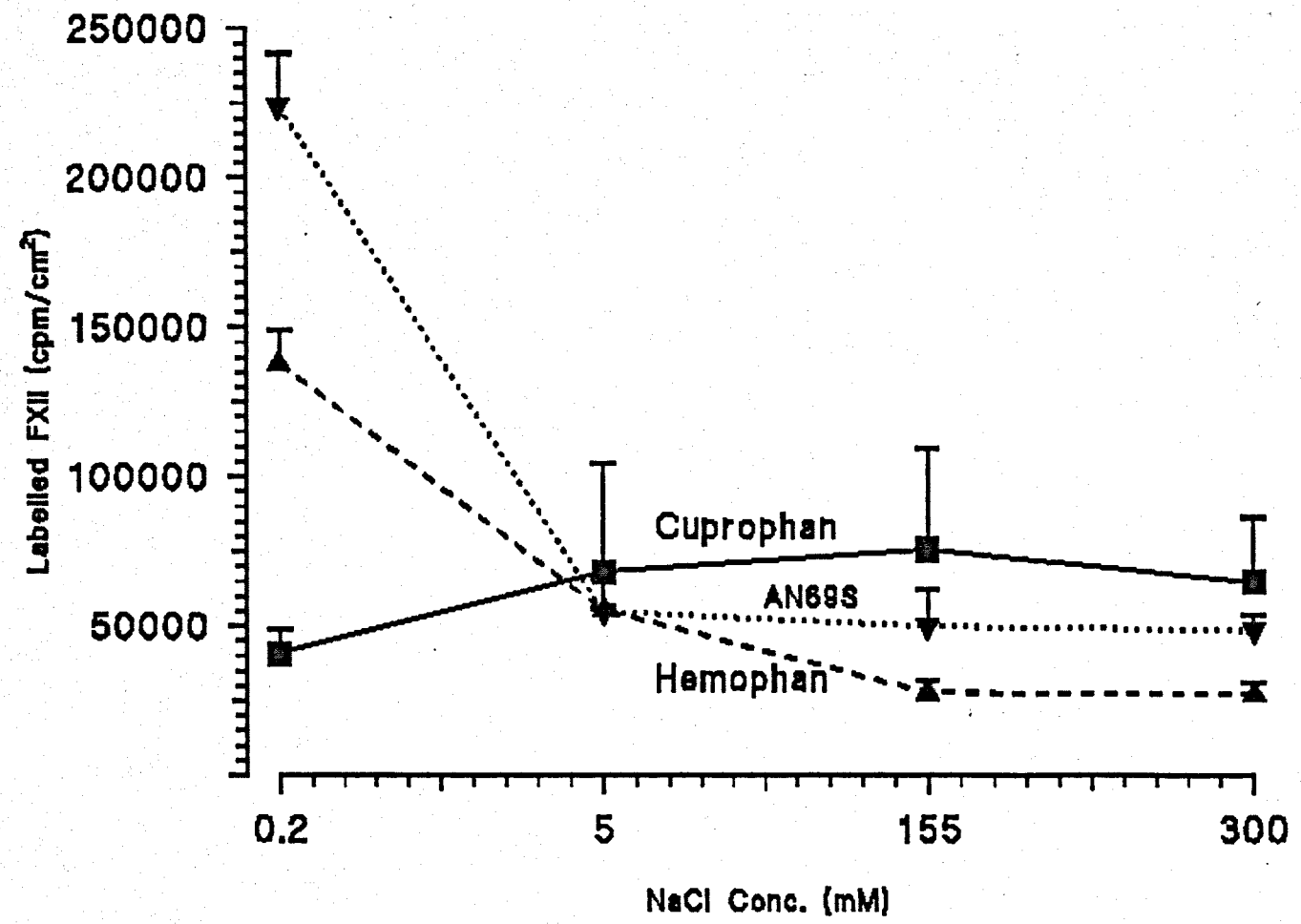


Figure 7.8: The effect of salt concentration on factor XII adsorption, n=4.

of reduction in adsorption of factor XII was found to be as follows: Cuprophan 32%, AN69S 52% and Hemophan 72%.

Table 7.7 The effect of washing detergent (6 M Urea/0.1% SDS) on factor XII adsorption, n=4.

Membrane	Mean CPM ± SD	Mean conc. (ng/cm^2)	Mean conc. ($pmol/cm^2$)
Cuprophan	4397 ± 2511	7.44 ± 4.25	0.23 ± 0.13
Hemophan	3519 ± 903	5.96 ± 1.53	0.19 ± 0.05
AN69S	38921 ± 7288	65.90 ± 12.34	2.06 ± 0.39

Table 7.8: The comparison between detergent and saline washed membranes ($pmol/cm^2$), n=4.

Washing Detergent	Cuprophan	Hemophan	AN69S
Tris buffered saline (5 mM Tris HCl; 155 mM NaCl)	0.34 ± 0.06	0.68 ± 0.05	4.27 ± 0.50
Detergent (6 M Urea; 0.1% SDS)	0.23 ± 0.13	0.19 ± 0.05	2.06 ± 0.39

7.9.1.7 THE EFFECT OF PLASMA pH ON FXII ADSORPTION

In order to further understand the mechanism of factor XII adsorption, the electrical charge of plasma factor XII was altered by changing the pH from 5.3 to 11.0 (isoelectric point of factor XII is between 6.1-6.5). At pH 5.3, the plasma factor XII was expected to be predominantly positively charged, whilst the reverse was true at pH 11.0. The effect of using plasma at the lower pH (pH 5.3) as compared to a higher pH 11.0, indicates that there was a marked decrease in factor XII adsorption with respect to Cuprophan, Hemophan, and AN69S i.e 75%, 73% and 79% respectively (table 7.9, figure 7.8).

Table 7.9: The effect of pH on surface binding of FXII ($pmol/cm^2$), n=4.

pH	Cuprophan	Hemophan	AN69S
5.3	0.52 ± 0.14	0.45 ± 0.15	4.32 ± 0.33
11.0	0.13 ± 0.04	0.12 ± 0.01	0.91 ± 0.12

This suggested that the mechanism of surface binding of plasma factor XII was to a large extent by ionic interaction.

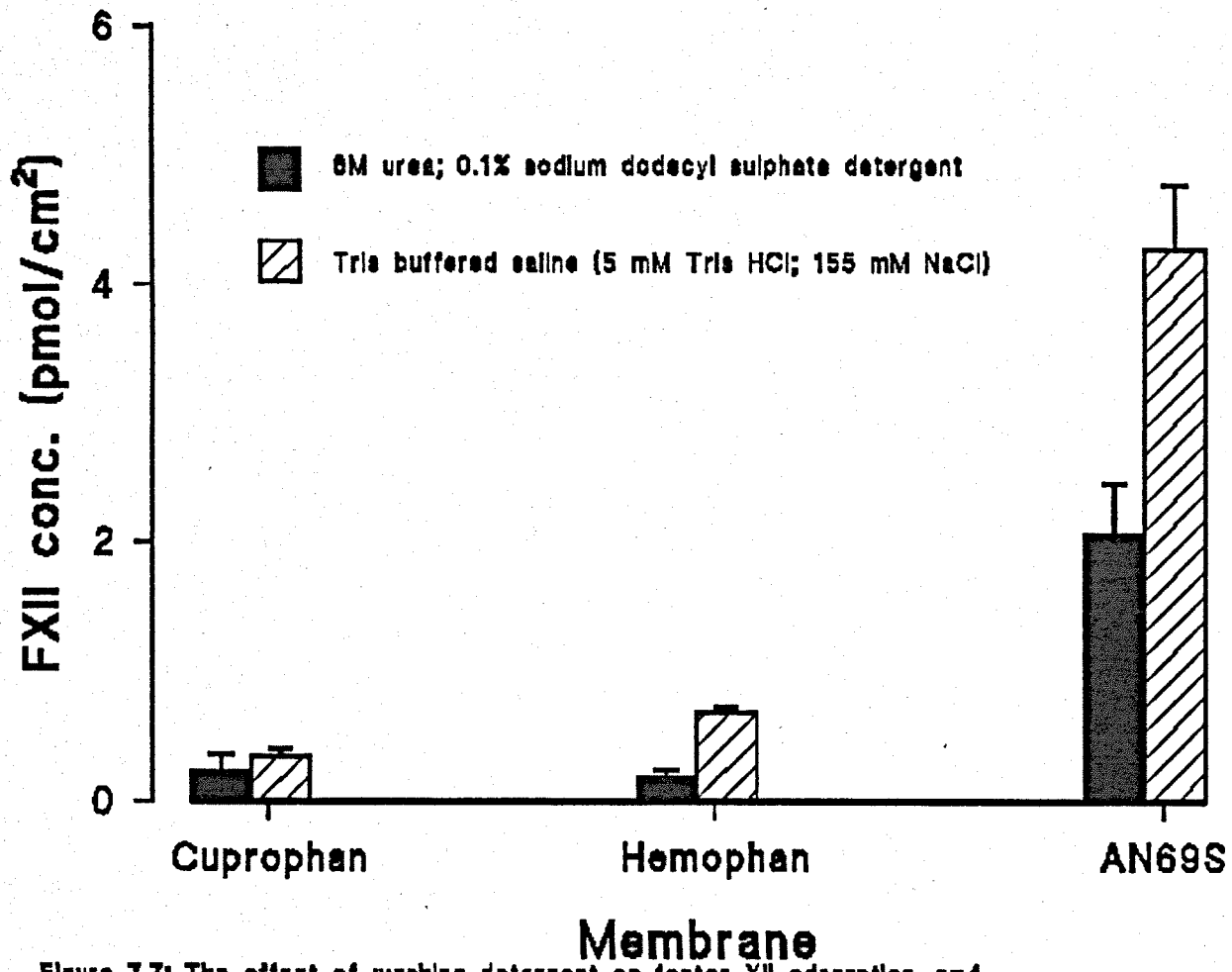


Figure 7.7: The effect of washing detergent on factor XII adsorption, n=4.

7.9.1.8 THE EFFECT OF PHARMACOLOGICAL AGENTS IN PLASMA ON FACTOR XII ADSORPTION

The effect of aprotinin, heparin and captopril on plasma factor XII adsorption onto membranes was investigated. The results demonstrated that aprotinin and heparin increased factor XII adsorption by 34% and 13% respectively on AN69S (table 7.11 and figure 7.9). By contrast, aprotinin reduced markedly the magnitude of factor XII adsorption on Cuprophan and Hemophan i.e 71% and 85% respectively. Heparin also reduced markedly the magnitude of the values of factor XII adsorption i.e 65% and 82%.

Table 7.10: The effect of pharmaceutical agents on FXII binding (pmol/cm^2), n=4.

	Cuprophan	Hemophan	AN69S
Normal plasma (3.2% citrate at 1:9 blood)	0.34 ± 0.06	0.68 ± 0.05	4.27 ± 0.50
Heparin (1 U/ml)	0.12 ± 0.02	0.12 ± 0.03	4.81 ± 0.15
Aprotinin (200 KIU/ml)	0.10 ± 0.01	0.10 ± 0.01	5.72 ± 0.51

Table 7.11: The effect of captopril on plasma FXII adsorption (pmol/cm^2),n=4.

	Cuprophan	Hemophan	AN69S
Captopril (0.2 μg/ml)	1.52 ± 0.10	1.70 ± 0.31	8.53 ± 3.52
Normal plasma control	1.15 ± 0.10	1.37 ± 0.20	10.34 ± 3.62

For the factor XII adsorption experiments with plasma containing captopril, the results indicated a decrease in the magnitude of factor XII adsorbed on AN69S membrane (17.5%), compared to the increased values on Cuprophan (32%) and Hemophan (24%) respectively (table 7.12 and figure 7.10).

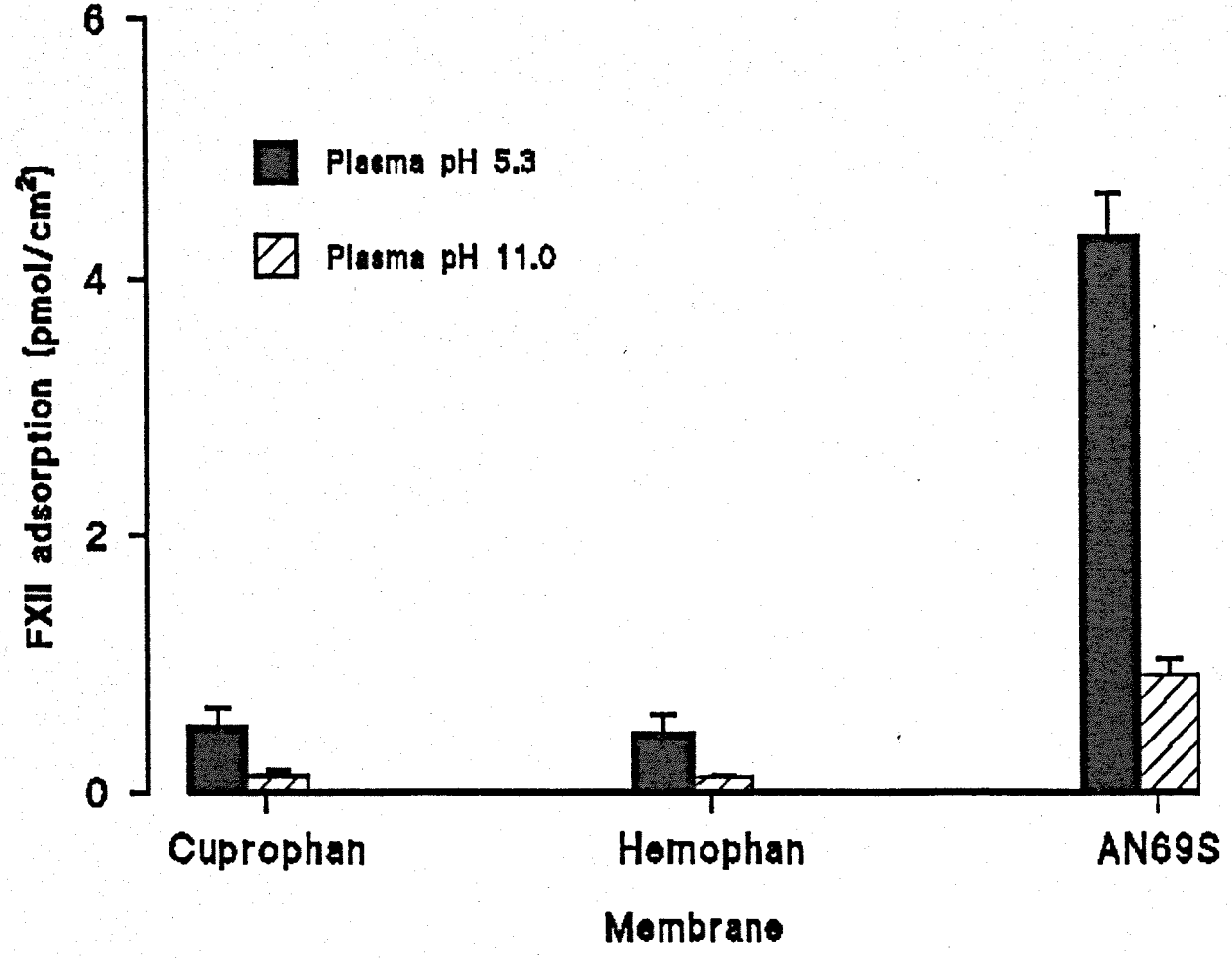


Figure 7.8: The effect of plasma pH on factor XII adsorption, n=4.

7.9.1.9 FACTOR XII ADSORPTION ON HOLLOW FIBRE MEMBRANES

The results showed that adsorption of factor XII after 20 minutes plasma perfusion was much greater on AN69HF modules than Cuprophan by approximately 12.5 times (Figure 7.11 and table 7.13). The findings were similar to those observed in an earlier study using an *ex vivo* system (Mahiout et al 1993). However, the absolute values of factor XII adsorbed per cm^2, of membranes were relatively low for the hollow fibre modules as compared to the flat sheet membranes by 4-fold. This may be due to the fact that single pass perfusion of plasma through the system ensures flowing conditions that optimises the exchange between the surface-bound and the bulk fluid phase plasma proteins.

Table 7.12: Hollow fibre modules factor XII adsorption values, n=3.

Membrane	Mean cpm ± SD	Conc ± SD (ng/cm^2)	Conc ± SD ($pmol/cm^2$)
Cuprophan	72005 ± 12146	2.71 ± 0.46	0.08 ± 0.01
AN69HF	989300 ± 32002	32.30 ± 1.04	1.01 ± 0.03

7.9.2 HEPARIN ADSORPTION RESULTS

The influence of membrane characteristics on heparin binding were as shown on table 7.13 and figure 7.12. Statistical analysis showed that Hemophan adsorbed a significantly greater amount of heparin than Cuprophan, AN69S, and SPAN ($p<0.05$). Cuprophan and AN69S membranes exhibited similar abilities for heparin binding with levels that were signficantly lower ($p<0.05$) than than SPAN.

There is an indication from the results that changes in membrane pH have no significant effect on heparin adsorption by Cuprophan, AN69S and SPAN. Whereas there was a significant reduction in heparin binding onto Hemophan when membrane pH was raised from 3 to 7.4 ($p<0.05$), suggesting a greater adsorption by ionic bonds resulting from quaternisation of the amine ether groups as well as increased weak hydrogen bonding when Hemophan was washed at acidic pH 3.

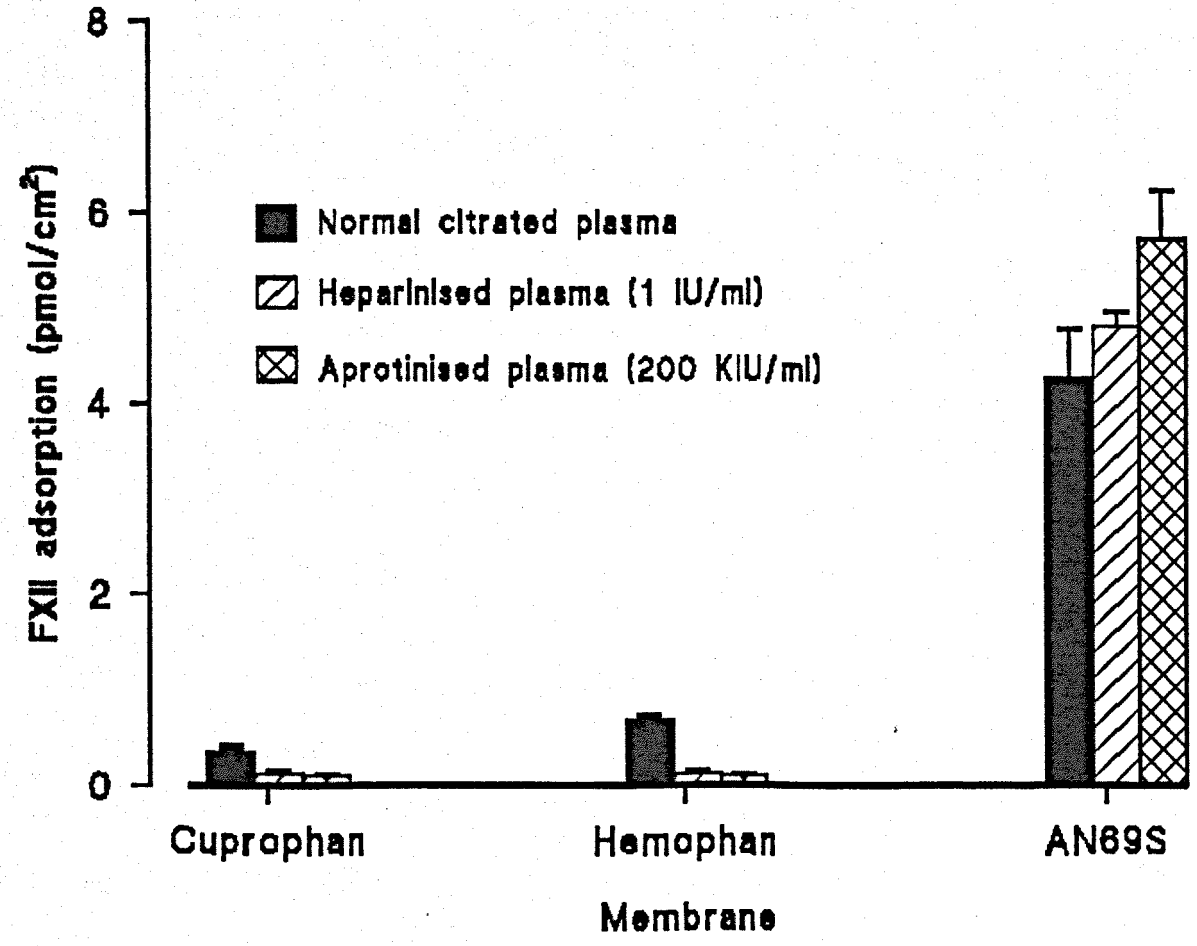


Figure 7.9: The effect of pharmacological agents on FXII adsorption, n=4.

Table 7.14 Heparin adsorption (per 9.6 cm^2 membrane surface area)

Membranes	Percent heparin adsorption (mean values)	
	pH 3, ± SD (n=6)	pH 7.4, ± SD (n=8)
Cuprophan	0.185 ± 0.060	0.173 ± 0.060
Hemophan	26.200 ± 0.900	21.500 ± 7.690
AN69S	0.445 ± 0.050	0.604 ± 0.320
SPAN	2.410 ± 0.700	2.930 ± 0.440

7.9.2.1 ANTICOAGULANT ACTIVITY OF MEMBRANE-BOUND HEPARIN (ANTI-Xa ASSAY) RESULTS.

As shown on table 7.14, Cuprophan binds only a small percentage of heparin, whose activity was largely retained after surface binding (table 7.15). Hemophan binds a significant amount of heparin, whose activity was also largely retained after surface binding, with a loss of only 2 % activity.

Table 7.14: The biological activity of membrane-bound heparin (%), n=3.

Membrane	Mean abs.(405 nm), n=3	% Xa activity	% Heparin activity
Cuprophan	0.028 ± 0.006	1.427	98.573
Hemophan	0.044 ± 0.002	2.244	97.756
AN69S	0.142 ± 0.004	7.240	92.760
SPAN	0.266 ± 0.012	13.564	86.435
Heparin + AT-III + FXa + Substrate in solution.	0.005 ± 0.000	0.000	99.745
FXa + Substrate only in solution	1.961 ± 0.013	100.000	0.000

This would appear to indicate that heparin binds to both Cuprophan and Hemophan membranes at different sites than those required for binding AT-III and therefore

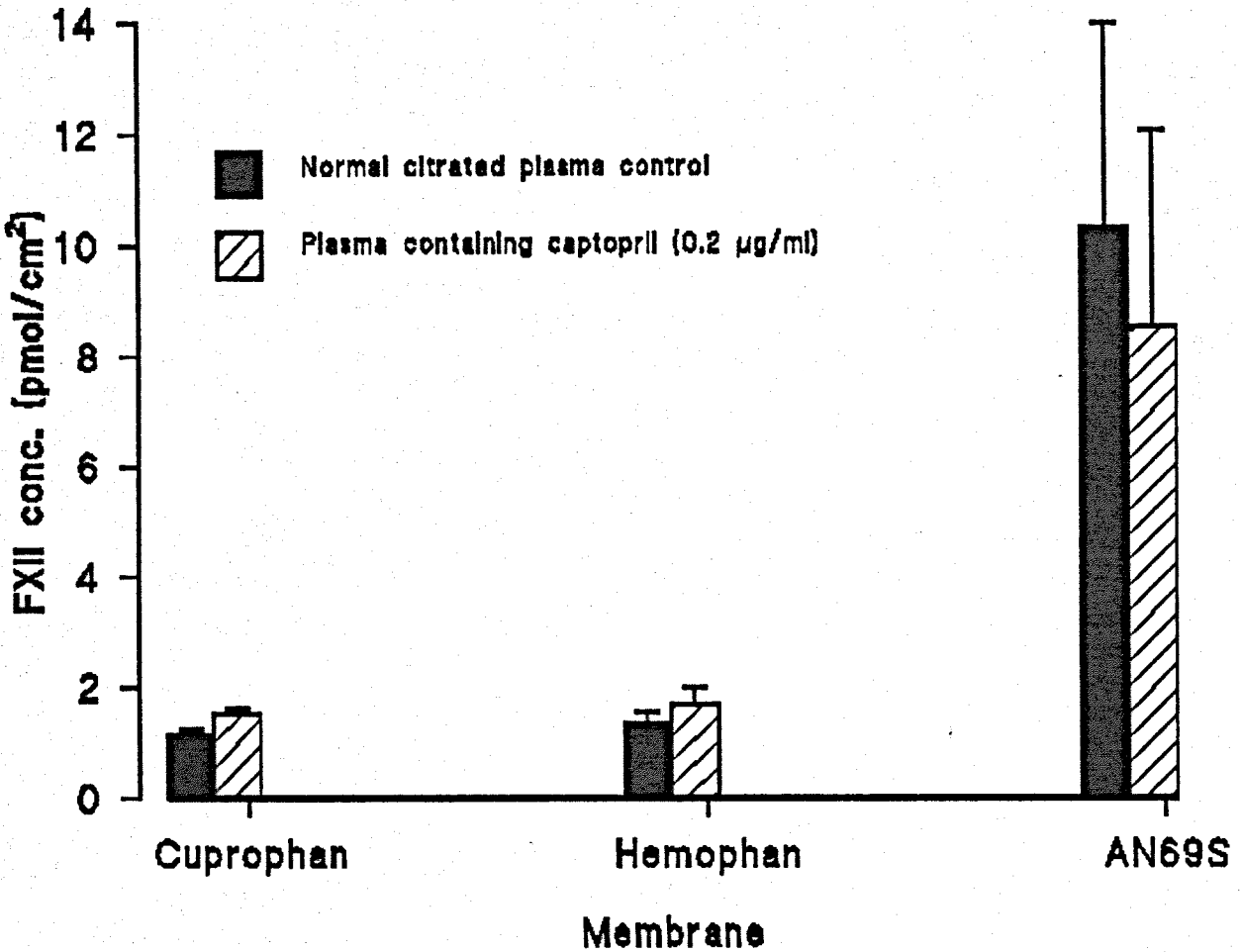


Figure 7.10: The effect of captopril on plasma FXII adsorption, n=3.

retains sufficient anti-Xa activity. The observations for AN69S and SPAN, indicate that there was a markedly reduced heparin anti-Xa activity as compared to Cuprophan or Hemophan. This may have implications on the surface charge or surface morphology of the polyacrylonitrile based membranes.

7.9.2.2 HEPARIN ANTICOAGULANT ACTIVITY IN WHOLE BLOOD AFTER MEMBRANE CONTACT (ANTI-Xa ASSAY RESULTS)

Prior to blood-membrane contact, there was a reduction in anti-Xa activity of the resultant plasma (tables 7.15 and 7.16). Standard unfractionated heparinized blood results indicated that there was an overall reduction in anti-Xa activity as compared to before blood was in contact with the membranes. The reduction in the anti-Xa activity appeared to be dependent on the initial heparin loading dose, with a greater reduction indicated with the higher loading dose of 1 IU/ml (table 7.15). The magnitude of the reduction in anti-Xa activity appeared to vary with the membranes, with polyamide NRZ having the greatest loss followed by Hemophan. A similar pattern was observed with the low molecular weight heparinized blood (Fragmin), but to a lesser extent (table 7.16).

These observations may suggest that heparin in blood interacts with the blood constituents as well as the membrane surfaces during blood-membrane contact.

Table 7.15: Anti-Xa activity of standard unfractionated heparin during blood-membranes contact.

Membrane	Mean % Heparin anti-Xa activity	
	0.05 IU/ml heparin	1 IU/ml heparin
Pre-contact	80	92
Cuprophan	45	30
Hemophan	37.5	23
AN69S	41	46
NR	41	26
NRZ	55	19.6

This interaction appears to be greater with the higher loading dose. However, the

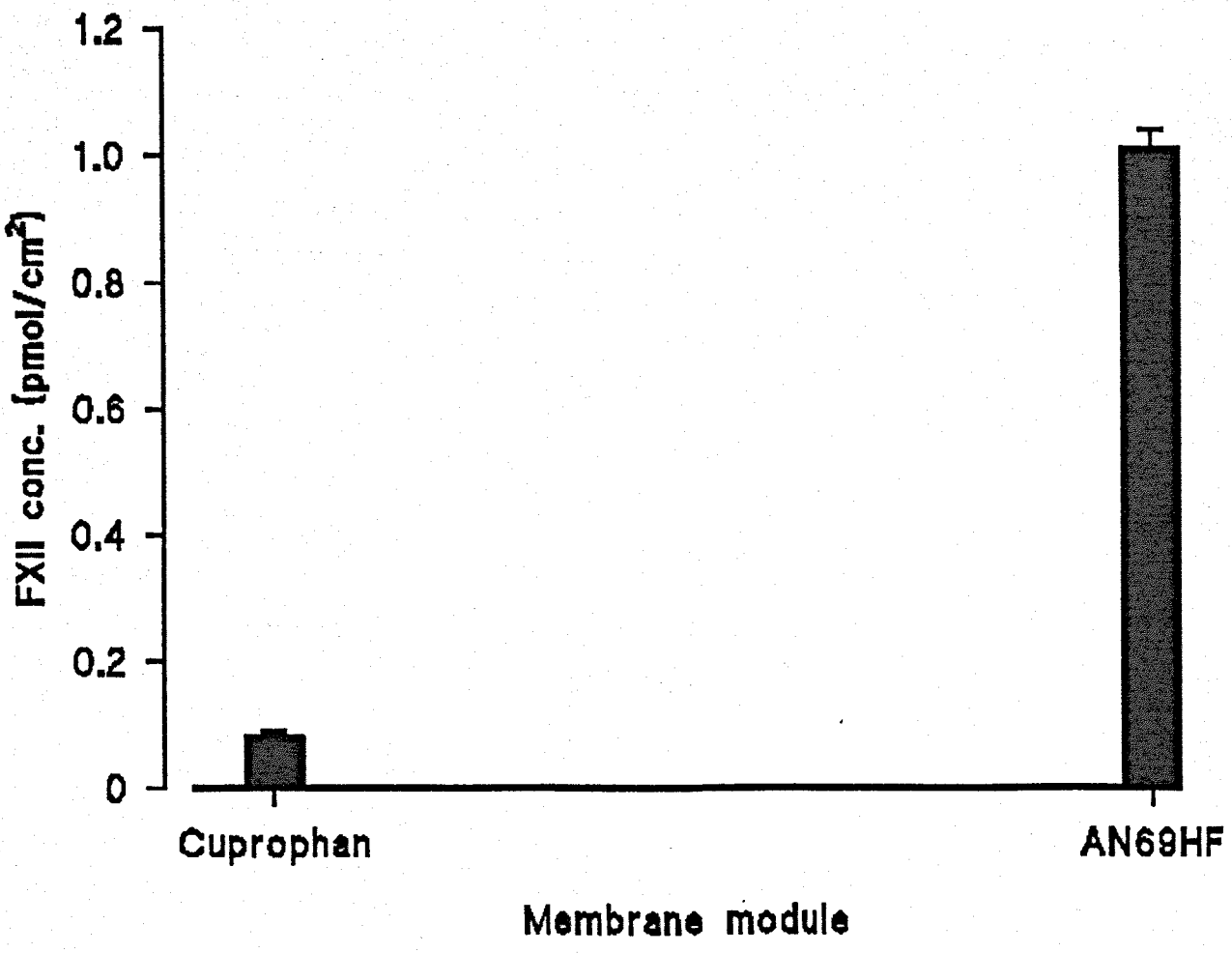


Figure 7.11: The adsorption of FXII on hollow fibre modules

overall pattern of anti-Xa activity was markedly higher for Fragmin as compared to standard unfractionated heparin (Heplok) at both loading doses.

Table 7.16: Fragmin anti-Xa activity during blood-membranes contact.

Membrane	Mean % Fragmin anti-Xa activity, n=4.	
	0.5 IU/ml Fragmin	1 IU/ml Fragmin
Pre-contact blood	74	91
Cuprophan	91.9	40.7
Hemophan	97.3	41.8
AN69S	75.7	93.4
polyamide NR	62.7	42.8
polyamide NRZ	55.4	39.6

7.9.2.3 APTT RESULTS

The results for the lower heparin loading dosage of 0.5 IU/ml, Cuprophan, AN69S, polyamide NR membranes indicated a mean clotting time of well above 150 seconds. However, for Hemophan and polyamide NRZ, the mean clotting times were 70 and 130 seconds respectively. This shows a marked loss in anticoagulant activity when a loading dose of 0.5 IU/ml was used for Hemophan and polyamide NRZ membranes, which suggests that this may have some significant clinical consequences. At the higher heparin loading dosage, all the membranes showed prolonged clotting times, well above 150 seconds (table 7.17).These findings were consistent with reported studies on medical grade tubing material, where the control glass materials were observed to cause a reduced partial thromboplastin time after plasma contact, while PVC and silicone tubing indicated clotting times >150 seconds (Rhodes & Williams 1994). There is therefore some evidence to suggest that the efficacy of heparinisation was dependent on the materials used. Materials that adsorb more heparin, may reduce the overall anticoagulant efficiency of heparin in blood and therefore for clinical applications it may be necessary to rinse the device with heparin solution before use. Adsorption of Nafamostat mesilate (FUT-175) to AN69 has also been shown to reduce its anticoagulant efficiency during *in vitro* and *in vivo* studies (Inagaki et al 1992).

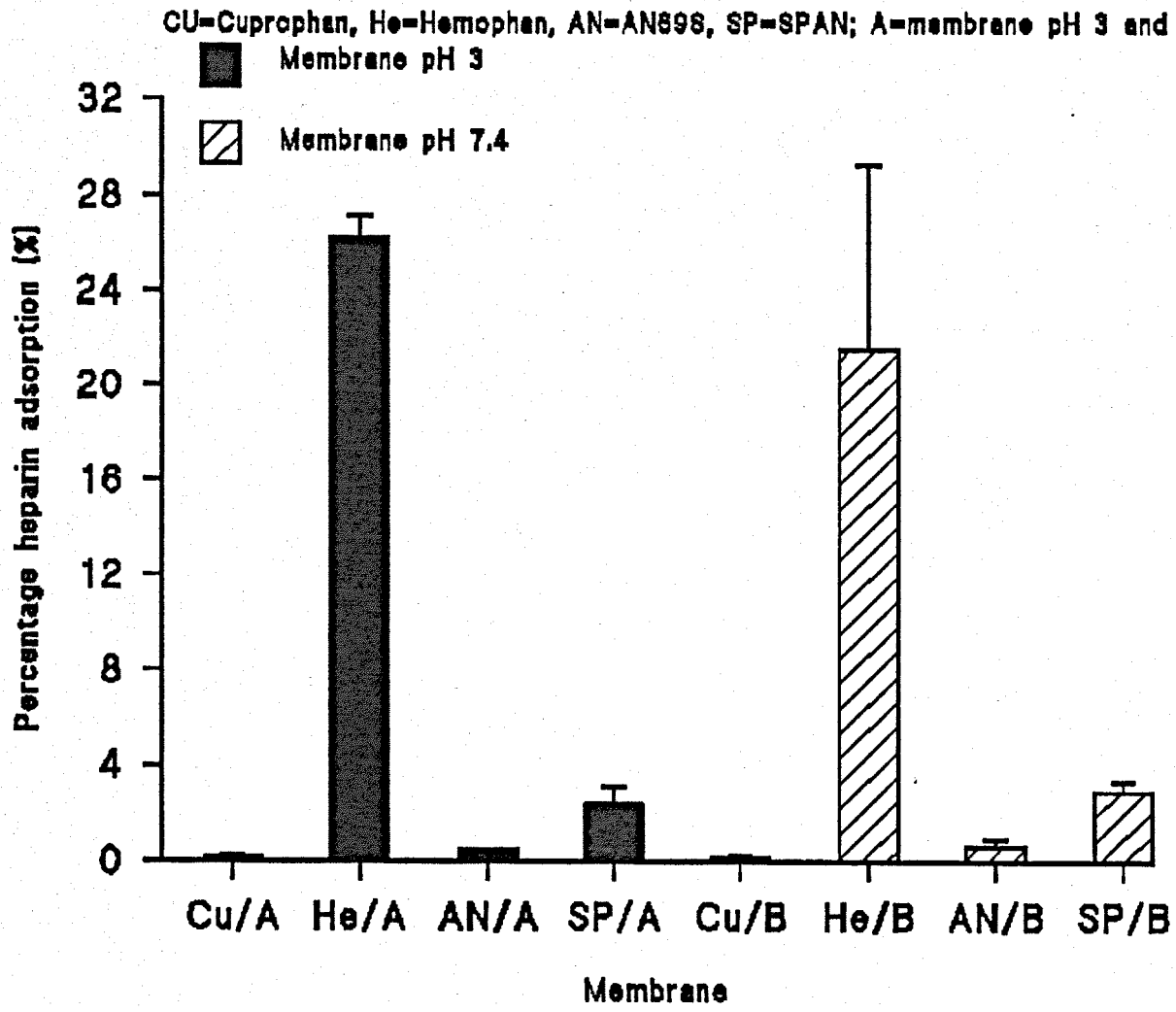


Figure 7.12: The membrane binding characteristics of heparin.

Table 7.17 APTT results for standard heparin as a measure of coagulant activity.

Membrane	Mean clotting times (seconds), n=4.	
	0.5 IU/ml heparin	1.0 IU/ml heparin
Pre-contact	140 ± 4	>150
Cuprophan	>150	>150
Hemophan	70 ± 1	>150
AN69S	>150	>150
polyamide NR	>150	>150
polyamide NRZ	127 ± 50	>150

7.10.1 SURFACE MORPHOLOGY BY SEM

Cuprophan and Hemophan exhibited smooth surfaces at low and high magnifications on both faces of the membranes (figures 7.13a and 7.13b). The surfaces however, contained crystalline structures, presumed to be crystallized glycerol usually used during the cellulose membrane fabrication process.

AN69S membrane indicated more rugged surface features at higher magnification than either of the cellulosic membranes (fig 7.13c). The SPAN membrane exhibited quite a number of pores on the surface even at a very low magnification. At high magnification, the two faces appeared to exhibit different patterns of pore structures (fig. 7.13d).

The polyamide Ultipor membranes exhibited a similar surface characteristic, and at low and high magnifications porous sponge-like structures were evident on both membranes (figure 7.13e and 7.13f).

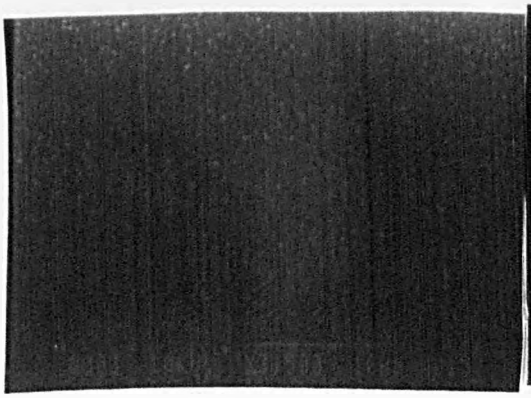


Fig 7.13a Cuprophan by SEM.

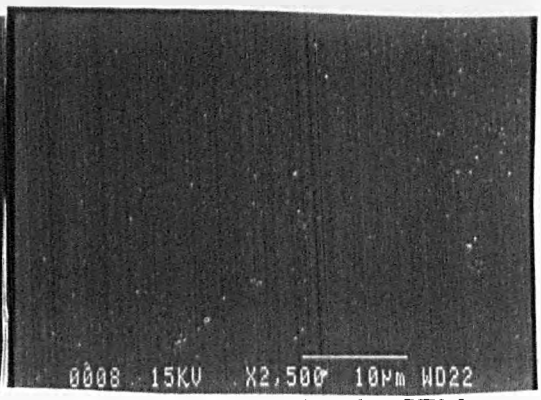


Fig 7.13b Hemophan by SEM

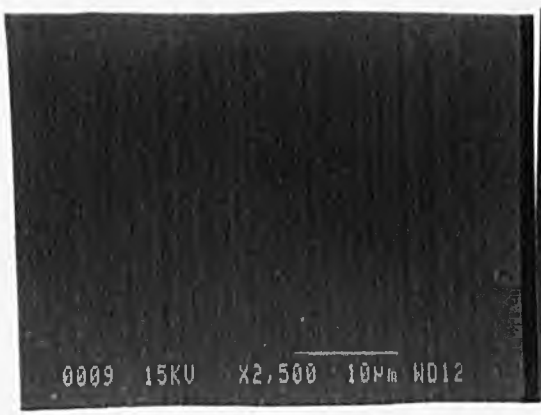


Fig 7.13c AN69S by SEM

Fig 7.13d SPAN by SEM.

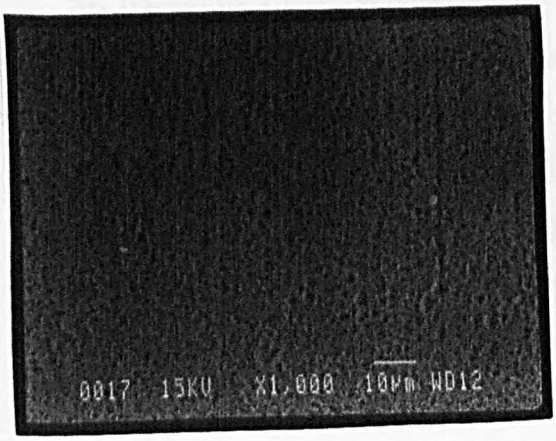


Fig 7.13e polyamide NR by SEM

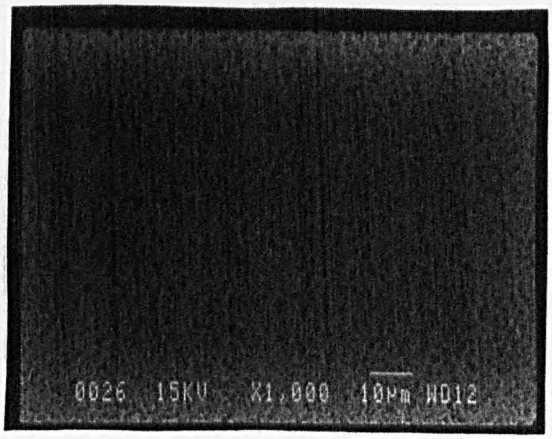


Fig 7.13f polyamide NRZ by SEM

7.10.2 RESULTS OF SURFACE CHARGE DETECTION BY ANIONIC AND CATIONIC DYE STAINING.

Cuprophan stained only very faintly with cationic dyes ethidium bromide, malachite green and methyl violet, but failed to stain with the anionic dye acid blue 45 (Figure 7.14). This indicated that cuprophan was very slightly negatively or neutral charged (table 7.18). Hemophan stained only with the anionic dye acid blue 45, indicating a positively charged surface. AN69S and SPAN stained very deeply with the cationic dyes ethidium bromide, malachite green and methyl violet, but remained unstained with the anionic dye acid blue 45. This indicated that both AN69S and SPAN were strongly negatively charged. The polyamide NR membrane stained more deeper than polyamide NRZ with the cationic dyes ethidium bromide, malachite green and methyl. However, with the anionic dye acid blue 45, polyamide NRZ stained much more deeper than polyamide NR. This indicated presence of negatively and positively charged moieties on both membranes with the latter having more negatively charged groups, while the former has more positively charged groups.

In the presence of heparin coating at 100 IU/ml, Cuprophan stained less deeper as compared to before with methyl violet, but remained unstained with acid blue 41 (table 7.18). Hemophan was very faintly stained by the cationic dye methyl violet after heparin coating. However, staining by anionic dye acid blue 45, was less deeper than before. This indicated that Hemophan had acquired a faint negative charge possibly by the heparin coating. AN69S and SPAN stained deeply with the cationic dye methyl violet but failed to stain with the anionic dye acid blue 45. Both the polyamide membranes stained just as deeply as AN69S, and similar intensities as before when stained with acid blue 45.

In the presence of a heparin coating of 1000 IU/ml, Cuprophan was still faintly stained with methyl violet, but unstained with acid blue (table 7.19). Hemophan stained much deeper with methyl violet in comparison to the 100 IU/ml heparin coating. Hemophan still stained with acid blue 45, although at a much reduced intensity. AN69S, SPAN, NR and NRZ stained deeply with methyl violet, but again failed to stain with acid blue 45.

Table 7.18: Anionic and cationic dye staining technique for detection of membrane charge.

Membrane	Acid blue 45	Ethidium bromide	Malachite green	Methyl violet
Cuprophan	unstained	faintly	faintly	faintly
Hemophan	blue	unstained	unstained	unstained
AN69S	unstained	deep red	deep green	deep violet
SPAN	unstained	red	deep green	violet
NR	faint blue	red	light green	violet
NRZ	deep blue	light red	faint green	violet

Table 7.19: The effect heparin washing on dye staining of membranes.

Membrane	100 u/ml heparin wash		1000 U/ml heparin wash	
	Acid blue 45	Methyl violet	Acid blue 45	Methyl violet
Cuprophan	unstained	light violet	unstained	violet
Hemophan	blue	faint violet	blue	light violet
AN69S	unstained	deep violet	unstained	deep violet

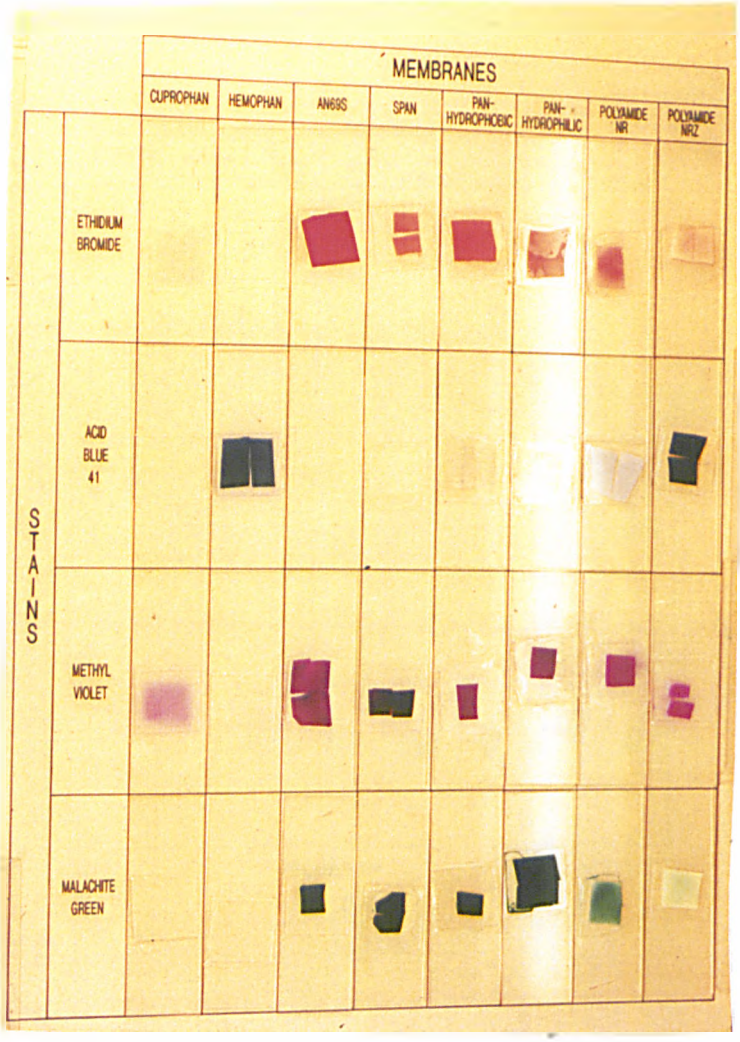


Figure 7.14a: Surface charge by cationic and anionic dye staining

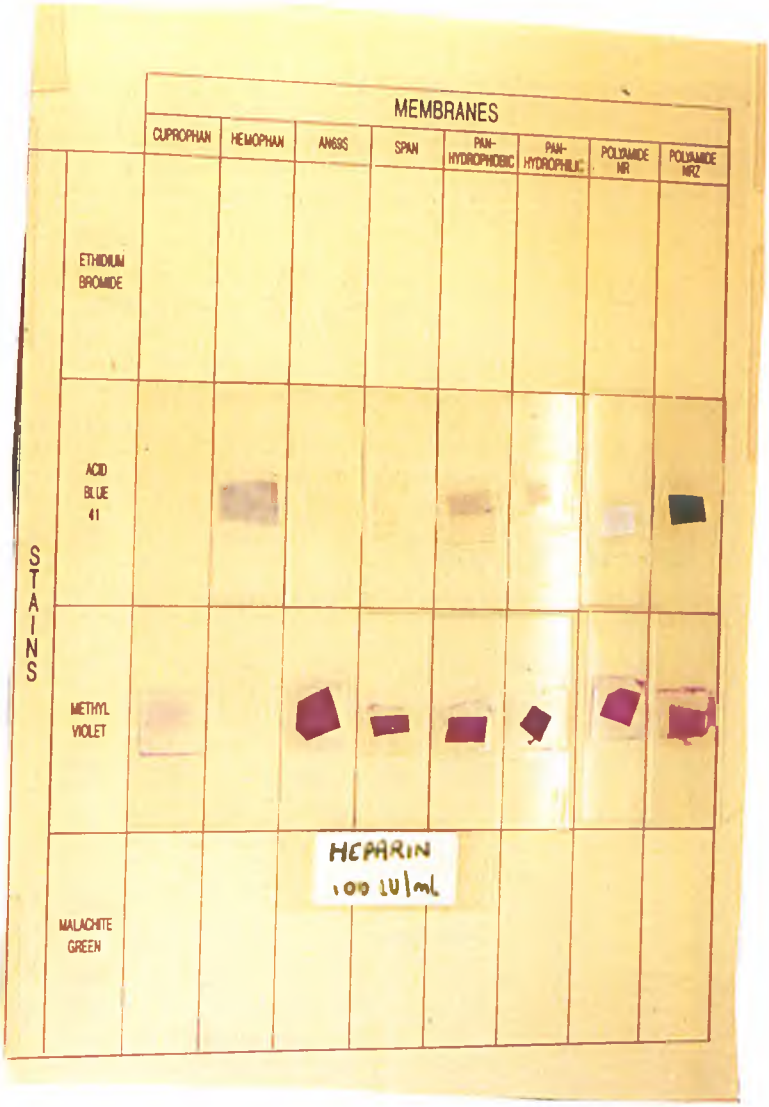


Figure 7.14b: The effect of 100 U/ml heparin wash.

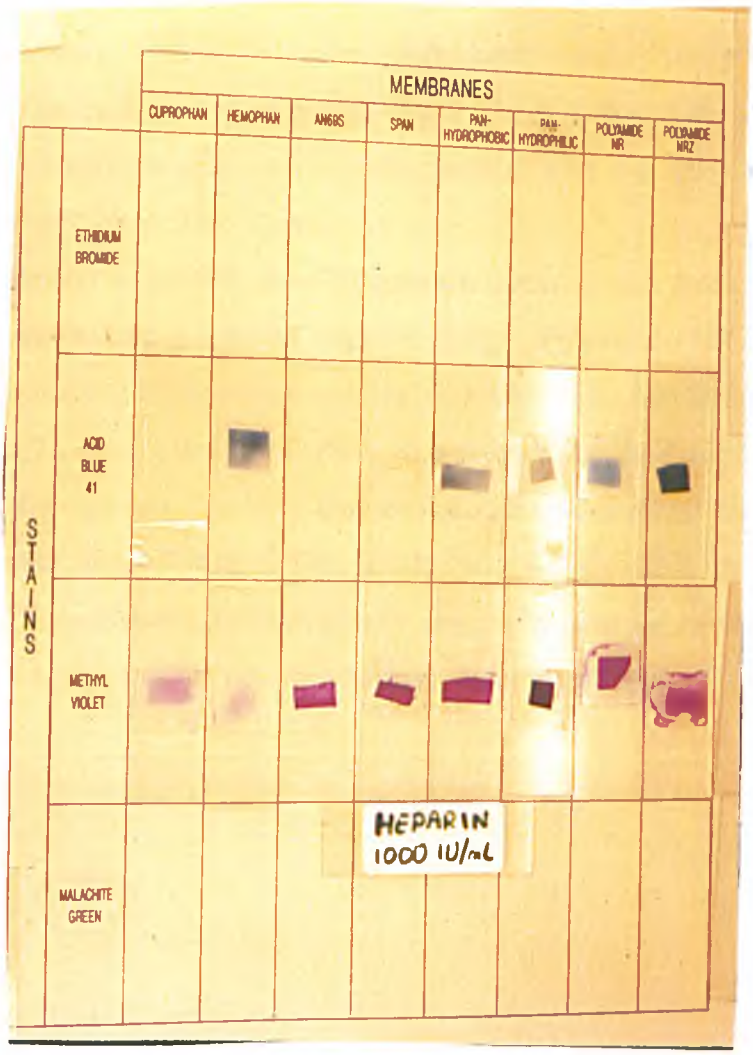


Figure 7.14c: The effect of 1000 u/ml heparin wash.

7.10.2 CHARGE DISTRIBUTION BY QUALITATIVE ANALYSIS OF FLUORESCENCE INTENSITY OF MEMBRANES STAINED WITH FITC-LABELLED POLY-L-LYSINE BY CLS MICROSCOPY .

Membrane slices stained in FITC-labelled poly-L-lysine were examined using the confocal laser scanning microscopy. Cuprophan exhibited a poor intensity of fluorescent staining on the xz-plane (Fig 7.15a, & 7.15b) and the distribution of the fluorescence was different between the sides of the membrane. The xy-plane showed a faint fluorescence (7.15c). This indicates that the membrane sides were casted differently (e.g air and plate-casting). A similar finding was exhibited with Hemophan (Figs.7.16a, 7.16b, and 7.16c).

AN69S stained with a very deep fluorescent stain intensity distributed Asymmetrically on both xy and xz planes (Fig 7.17a, 7.17b, 7.17c, and 7.17d). SPAN exhibited an asymmetrical stain intensity pattern, with one side much fainter than the other (Fig 7.18a,7.18b, 7.18c).

In comparison to AN69S, the fluorescence intensity was much reduced in SPAN. This may indicate a reduced negative charge. Polyamide NR indicated a much more symmetrical fluorescence staining with both sides exhibiting a similar intensity (Figs. 7.19a, 7.19b, and 7.19c). The polyamide NRZ membrane also indicated faint fluorescence intensity unevenly distributed through the bulk and existed in blotches (Figs 7.20a, 7.20b, 7.20c, and 7.20d). This indicates that polyamide NRZ membrane may have patchy negatively charged centres and this appears to be in agreement with the results from anionic and cationic dye staining technique.

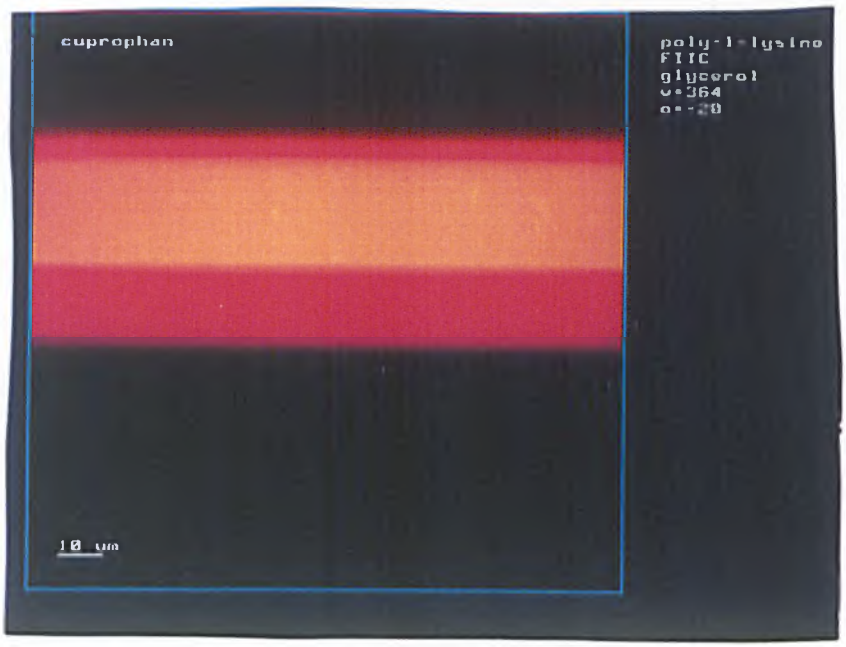


Fig 7.21a Cuprophan (side 1, xz-plane)

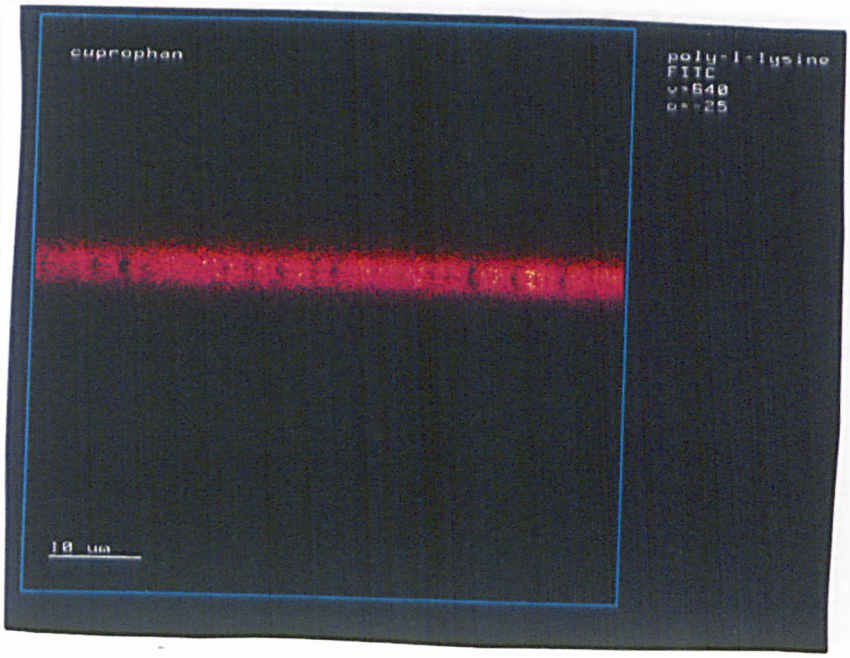


Fig 7.21b Cuprophan (side 2, xz-plane)

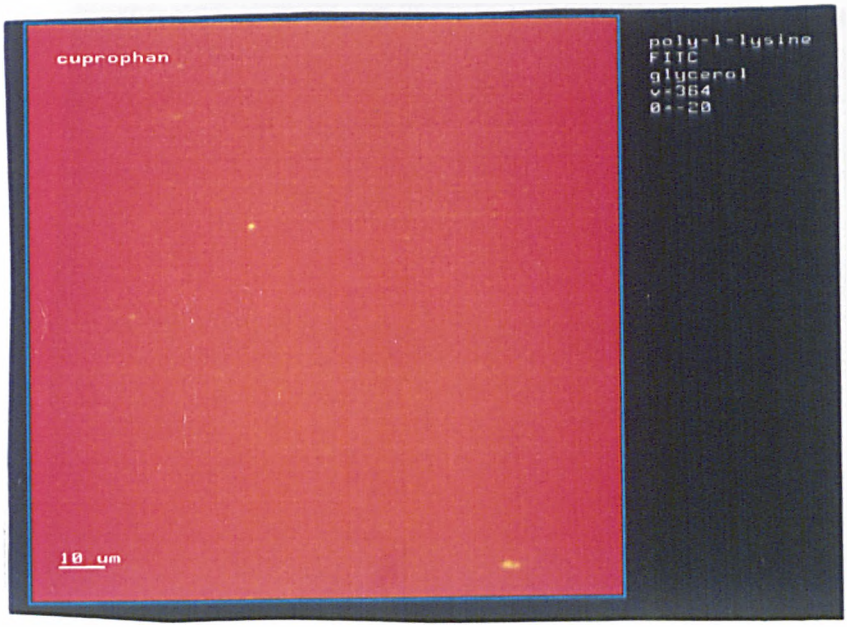


Fig 7.21c Cuprophan (xy-plane)

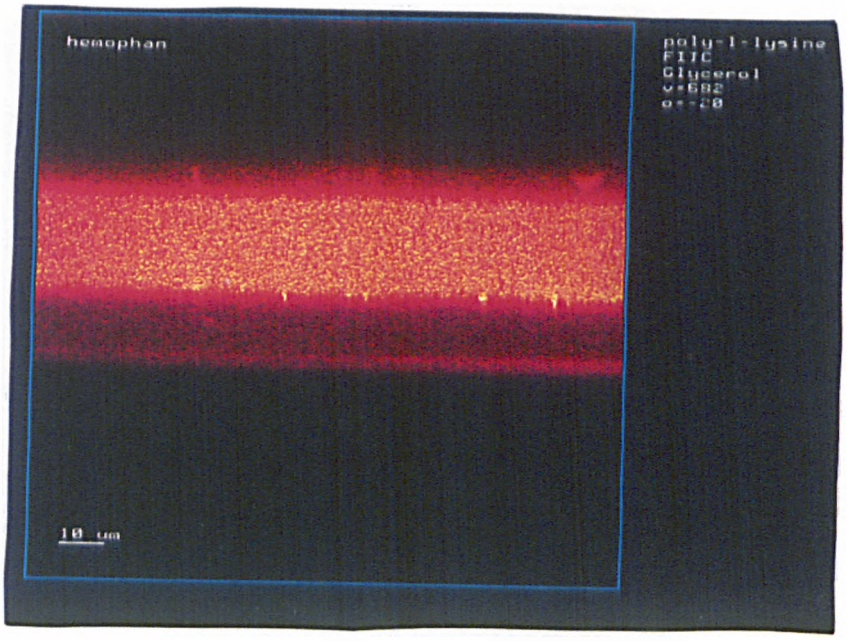


Fig 7.22a Hemophan (side 1, xz-plane)

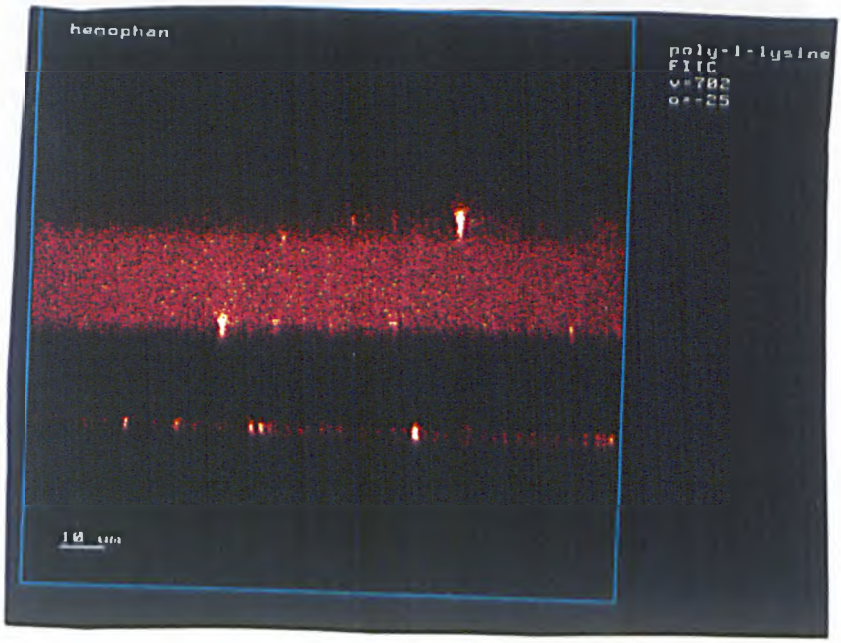


Fig 7.22b Hemophan (side 2, xz-plane)

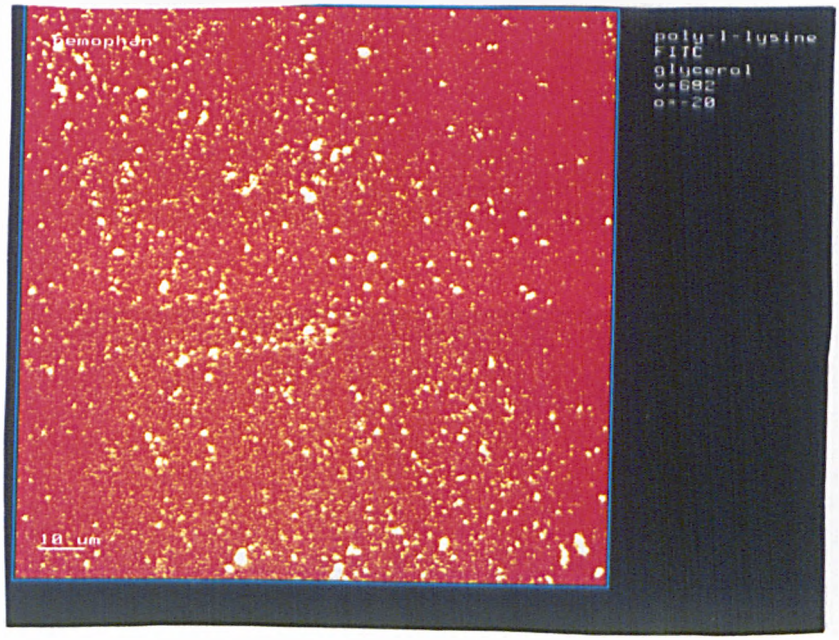


Fig 7.22c Hemophan (xy-plane)

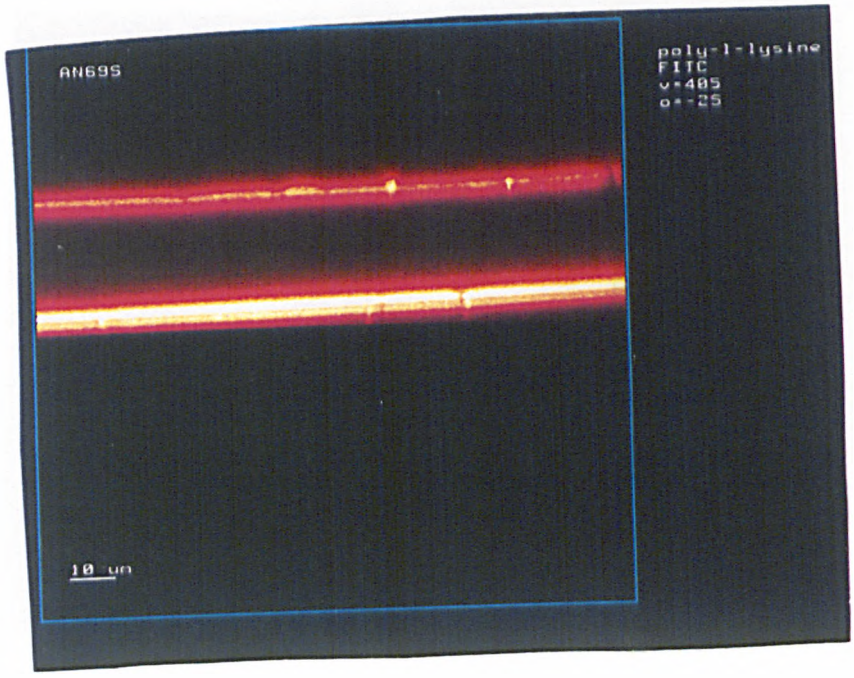


Fig 7.23a AN69S (side 1, xz-plane)

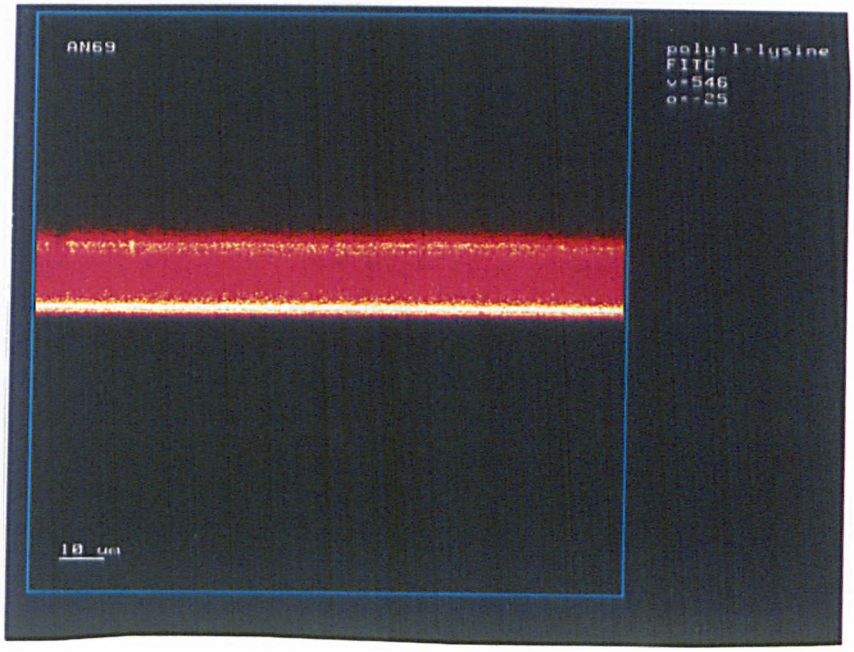


Fig 7.23b AN69S (side 2, xz-plane)

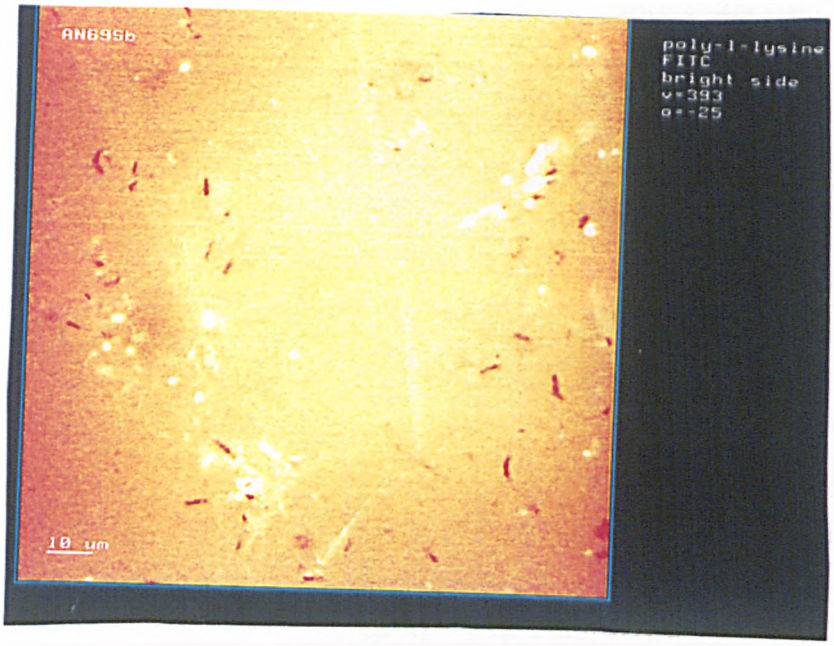


Fig 7.23c AN69S (side 1, xy-plane)

Fig 7.24d AN69S (side 2, xy-plane)

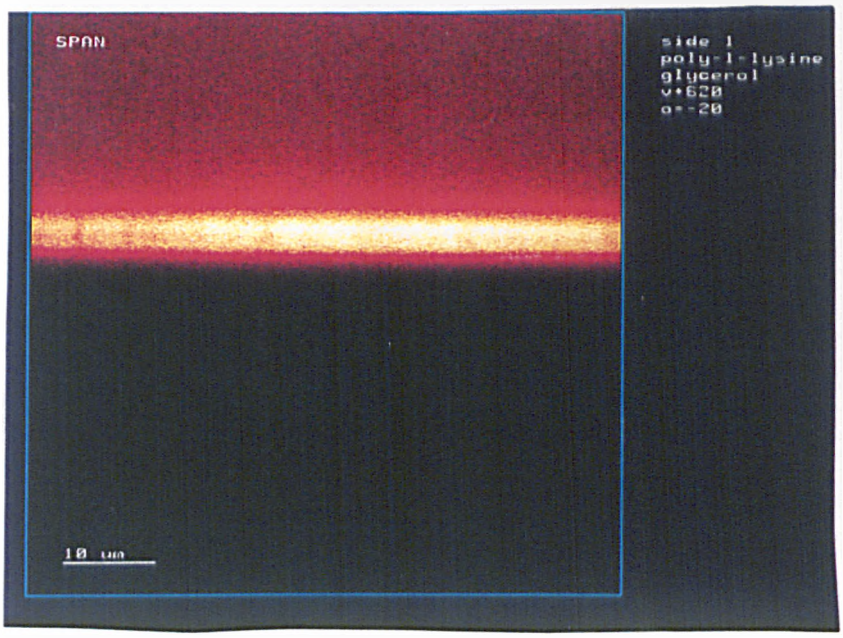


Fig. 7.24a SPAN (xz-plane).

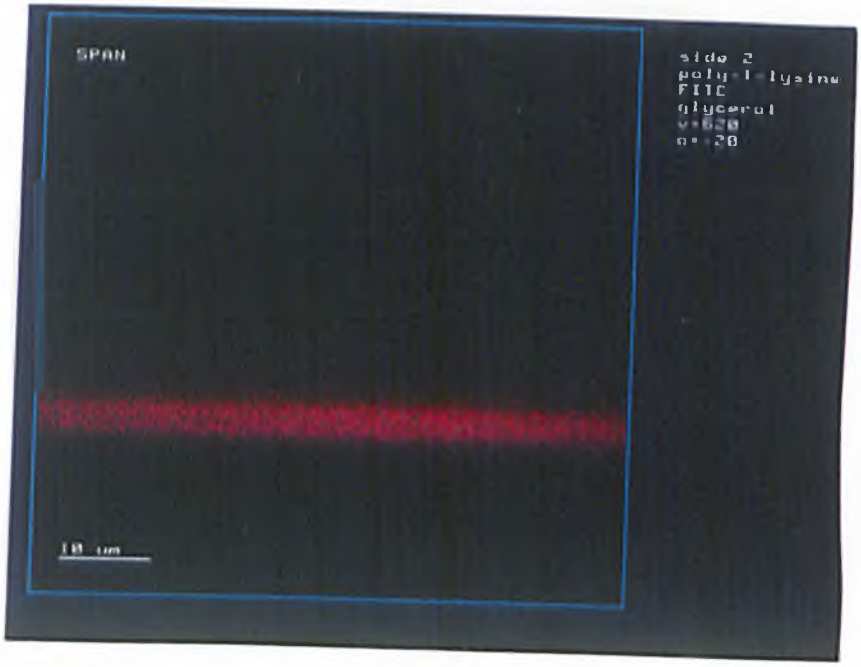


Fig 7.24b SPAN (xz-plane)

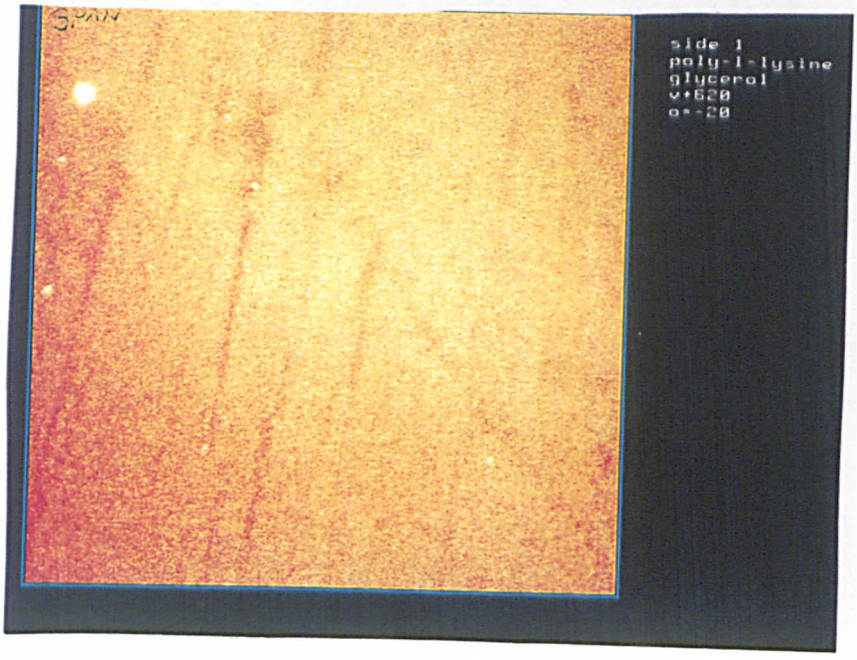


Fig 7.24c SPAN (xy-plane)

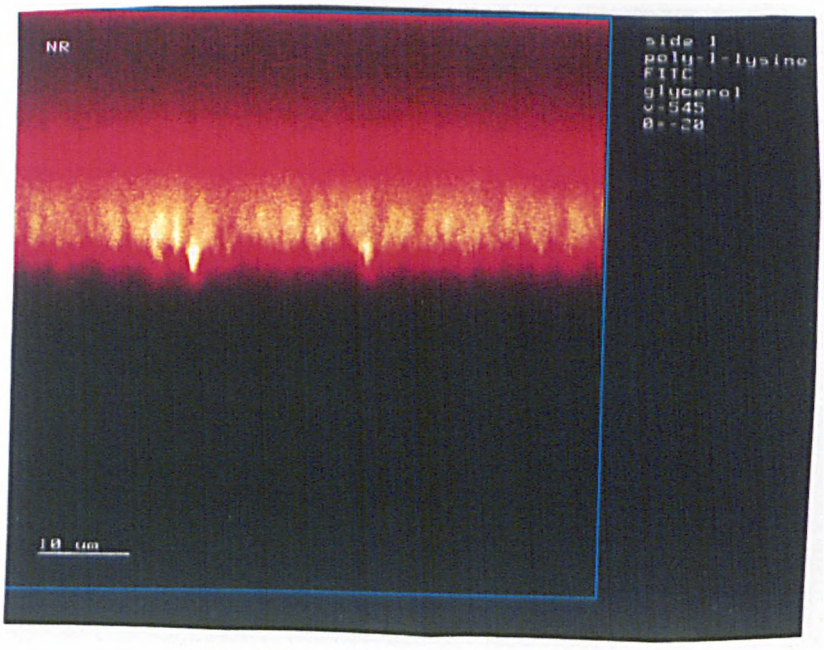


Fig. 7.25a NR (side 1, xz-plane)

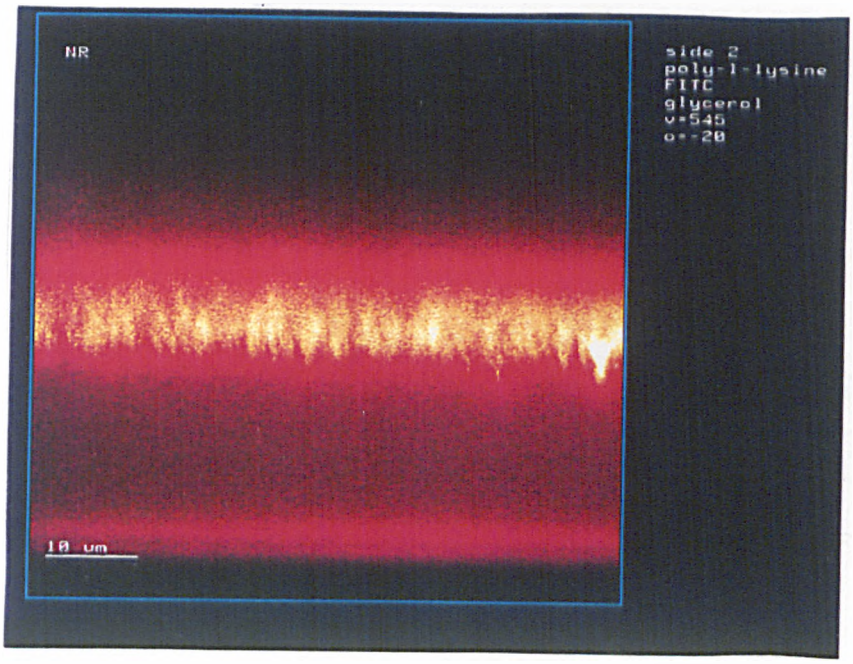


Fig 7.25b NR (side 2, xz-plane)

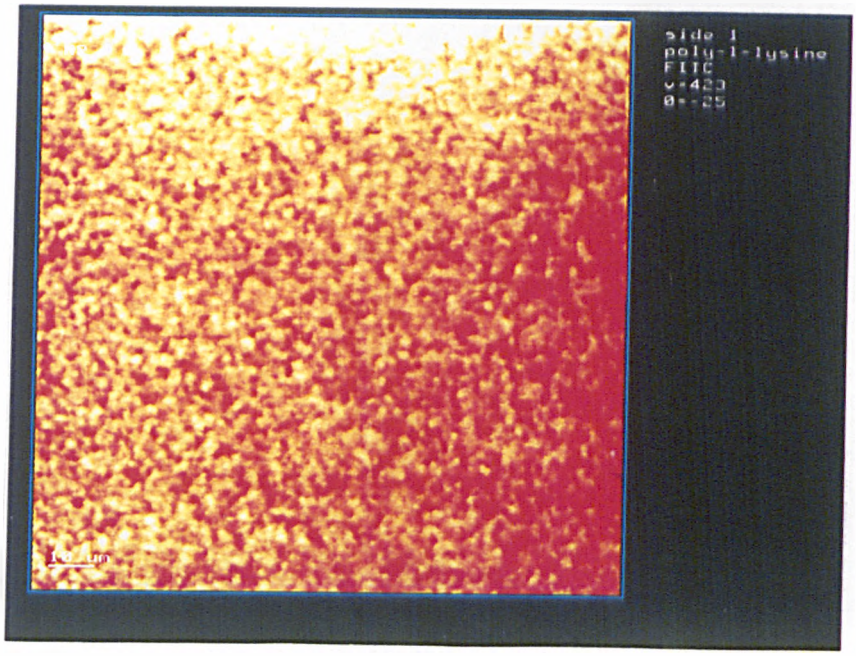


Fig 7.25c NR (xy-plane)

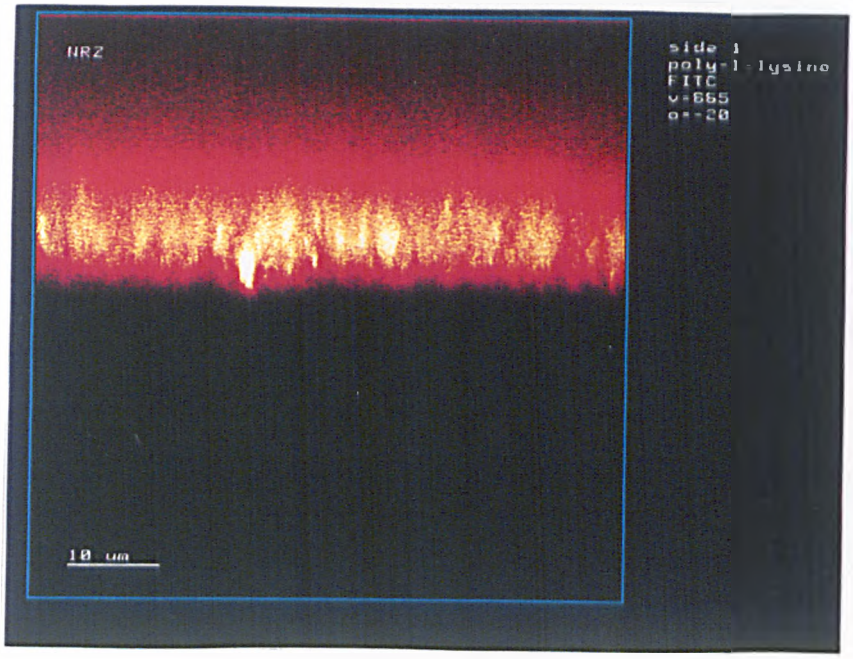


Fig 7.26a NRZ (side 1, xz-plane)

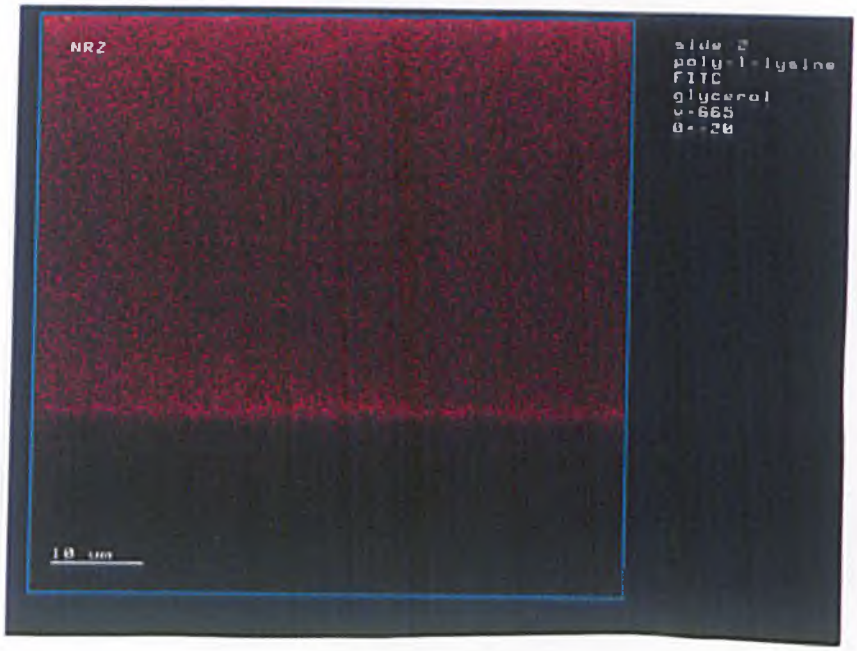


Fig 7.26b NRZ (side 2, xz-plane)

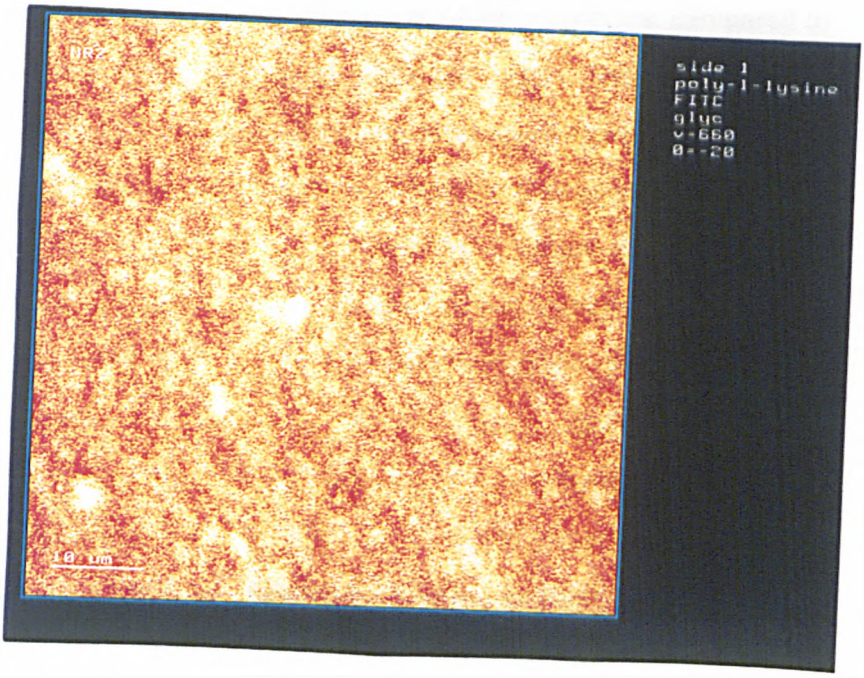


Fig 7.26c NRZ (side 1, xy-plane)

Fig 7.26d NRZ (side 2, xy-plane)

7.11 DISCUSSION

The magnitude of factor XII adsorption on both flat sheet and hollow fibre membranes was found to be significantly greater on AN69 membrane compared to Cuprophan by about 12.5 times. Hemophan indicated only a marginally greater factor XII adsorption than Cuprophan. The experiments on the effect of increased pH and washing the membranes with detergent that removes hydrophobic interactions indicated that factor XII also adsorbs to membrane surfaces by hydrophobic interactions. The presence of pharmacological agents, heparin and aprotinin indicated a decrease in factor XII adsorption on Cuprophan and Hemophan. This may suggest a surface-binding competition phenomenon between heparin, aprotinin and factor XII. By contrast, surface adsorption of FXII increased in the presence of heparin and aprotinin in plasma after contact with AN69S. This suggests a surface repulsion of aprotinin and heparin by AN69S membrane possibly due to their negative charge, and preferrential adsorption of factor XII. A contrasting pattern was observed with captopril in plasma, with greater factor XII adsorption by Cuprophan and Hemophan, whereas there was a reduction in the amount of factor XII adsorbed by AN69S. This may suggest that captopril interferes with the adsorption of factor XII by AN69S, while the reverse phenomenon occurs with Cuprophan and Hemophan. Increased salt concentration significantly reduced factor XII adsorption on AN69S and Hemophan with ionic strength of up to 300 mM NaCl concentration. This suggests the salt ions have a greater mobility than factor XII and therefore bind to the charged membrane surfaces (AN69S & Hemophan) in prefence to the relatively large molecules of factor XII. By contrast, increased NaCl concentration, concentration resulted in a steady increase in factor XII adsorption on Cuprophan possibly due to the decreased ionic migration to the neutral charged membrane surface and possibly by increased formation of weak hydrogen bonding between factor XII and the hydroxyl groups on Cuprophan, therefore resulting in greater factor XII-membrane interaction.

Heparin adsorption studies with radiolabelled heparin indicated that Hemophan adsorbed markedly greater amounts of heparin compared to either Cuprophan or AN69S. The findings were in agreement with previous studies

(Holland et al 1978; Schmitt et al 1983; Vienken & Bowry 1993), that DEAE-cellulose (Hemophan) binds markedly greater amounts of heparin compared to regenerated cellulose. The evaluation of biological activity of membrane-bound heparin revealed that heparin bound to Cuprophan and Hemophan retained almost entirely its activity, whereas a substantial amount of activity was lost on AN69S and SPAN. This may have direct implications on the biological performance of heparin grafted polymeric membranes, particularly on the acrylonitrile-based membranes. This observation was in agreement with studies that reported of loss of anticoagulant properties of nafamostat mesilate (FUT-175) bound to AN69 membranes (Inagaki et al 1992).

The addition of heparin to blood as an anticoagulant before contact with membranes indicated that there was a significant ($p < 0.05$) reduction in the anti-Xa activity of heparin as assayed in the plasma and that the magnitude of the reduced anti-Xa activity increased with the heparin content of the blood before contact with all the membranes evaluated. A similar pattern was also observed with low molecular weight heparin, although to a lesser extent. Despite the reduced levels of anti-Xa activity at the higher heparin loading dose of 1 IU/ml, after contact all the membranes indicated an adequate anticoagulant properties (clotting time of > 150 seconds). However, at the low heparin loading dose of 0.5 IU/ml, inadequate anticoagulation was observed with Hemophan and polyamide NRZ membranes, suggesting a loss of anticoagulant properties of the heparin possibly due to a greater surface adsorption.

The results of the surface morphology analysis have revealed that the AN69S flat sheet membrane exhibited a much more rugged and pore-like features than the cellulosic membranes. This may suggest a possible increased surface area for blood contact. The major effect of increased surface area to volume ratio, would be a more pronounced blood response. Surface roughness may cause increased protein denaturation, particularly in systems perfused by flowing blood or plasma.

The surface charge analysis using the dye staining technique revealed that Cuprophan was either neutral or very faintly negative. Hemophan was found to be positively charged, while AN69S and SPAN were found to be negatively charged.

These findings were consistent with those reported by Thomaneck et al (1991). The polyamide Ultipor NR membrane was found to stain with both cationic and anionic dyes, therefore suggested that the membrane contained positive and negative charges, but more of the latter. Polyamide Ultipor NRZ was also found to contain positive and negative charges but more of the former. The washing of the membranes with heparin, revealed that a marked change in charge was detected only with the Hemophan, which exhibited a more negative charge, and the acquired negativity was dependent on the concentration of the heparin washing. This finding was in agreement with the heparin adsorption studies using radiolabelled heparin, where Hemophan was found to bind more heparin. The relatively simple method was therefore suitable for detection of charges on membranes and changes in charges which might occur in procedures such as heparinization, sulphation and other chemical modifications of membranes or biomaterials.

The fluorescence staining and confocal laser scanning microscopy revealed that charges were distributed variably on and inside a membrane. This may be due to changes in bulk constituents or/and changes that might have taken place during membrane casting. Cuprophan and Hemophan exhibited a similar fluorescence staining intensity, but differences in the bulk constituents was evident, with the latter more patchy. AN69S and SPAN surfaces demonstrated an asymmetrical distribution of charge and the differences in charge distribution between the two faces of these membranes may be attributed to the casting techniques. The charge distribution on the polyamide membranes indicated a random arrangement of negatively charged centres in the bulk of the membranes. This may be attributed to the largely porous or sponge-like nature of the materials. The results therefore show that the confocal laser scanning microscopy could be a useful, fast and relatively simple method for measurement of the three-dimensional distribution of charges after staining membranes with FITC labelled polyions.

7.12 SUMMARY.

Surface properties such as charge and morphology of membranes, are thought to influence markedly the pattern of the resultant blood response. Methods that can

be used to characterize these properties have been sought. In this study two techniques have been investigated: detection of charges using charged dyes; and the use of FITC-labelled polyions to measure the three-dimensional distribution of charges by confocal laser scanning microscopy (CLSM). The first technique enabled a qualitative assessment of membrane surface morphology. Cationic and anionic dye staining technique enabled the establishment of the membrane charge. The surface distribution of charge and the internal structure of the hydrated membranes were imaged using CLSM. Results for cellulosic and acrylonitrile-based membranes indicated a fundamental difference in surface properties and morphology. This implies that these methods can be useful in the detection of changes in surface properties resulting from physical or chemical modifications of membranes. Specimen preparation and evaluation of dye uptake was relatively straightforward.

The results of the surface charge patterns have indicated a relationship with the pattern of factor XII adsorption, with a greater adsorption shown with the negatively charged AN69S membrane. The pattern of purified factor XII adsorption and activation was also indicative of a relation with surface charge with the negatively charge membranes exhibiting a higher FXIIA (chapter 5). However, the situation involving whole blood or plasma indicated that there was a difference in the relationships of adsorption and activation of factor XII depending on the type of factor XII activity assay used.

For the chromogenic substrate assay, the results indicate that plasma supernatant factor XII-like activity was not significantly different ($p < 0.05$) between the membranes irrespective of charge. This contrasts the plasma factor XII adsorption patterns measured. However, the situation was complex when membrane-bound FXIIA was considered with AN69S exhibiting the least activity. The FXIIa/FXIIa-Inhibitor assay, indicated that AN69S exhibited a greater activity than Cuprophan or Hemophan for up to 12 minutes of blood-membrane contact, although differences were not statistically significant. However, after 12 minutes of blood-membrane contact, Cuprophan indicated the highest levels of FXIIa/FXIIa-inhibitor complexes. This may indicate that the magnitude of factor XII activity as measured using the chromogenic substrate assay on AN69S membrane was inadequately

detected possibly due to the inhibition by plasma inhibitors. The results therefore indicate that the membrane surface characterisation techniques investigated in this study were very useful in the the interpretation of protein adsorption and activation data.

Heparin adsorption patterns indicated a relationship with membrane charge, with the positively charged Hemophan showing the highest levels and AN69S the least. The presence of heparin in plasma has indicated an increased factor XIIA activity, but during blood-membrane contact, the values decline below baseline levels for up to 12 minutes, indicating either an increased plasma inhibition or an increased factor XII adsorption onto the membrane as shown by factor XII adsorption studies. The overall results indicated that the factors that influence plasma factor XII adsorption and activation were very complex and these include:

(1) Membrane surface charge and surface morphology

(2) The presence of pharmacological agents in plasma such as heparin, aprotinin and captopril.

(3) The condition of the blood with particular emphasis on the interaction between factor XII with other plasma constituents such as inhibitors.

(4) Surface chemical composition.

CHAPTER EIGHT

THE ROLE OF CONTACT PHASE ACTIVATION DURING HAEMODIALYSIS AND CARDIOPULMONARY BYPASS.

8.1 INTRODUCTION

The application of extracorporeal circuits for blood purification purposes has become routine and as a consequence there is an increased desire for an enhanced knowledge of the interactions between blood constituents and the surface of the materials employed (Courtney et al 1993b).

For a major understanding of the performance of an extracorporeal device in the biological situation, there is a requirement for the derivation of a relationship between the blood compatibility and the nature of the clinical procedure and its associated application time, the patient status, the extent of trauma and the effect of drug administration (Courtney et al 1993c). Inevitably, blood compatibility should not be considered in isolation from functional characteristics requirements. In order to comply with the requirements of clinical performance, the blood-contacting materials in the device should also possess suitable functional features, such as flexibility for blood tubing, permeability to solutes and water for a haemodialysis membrane, or oxygen and carbon dioxide permeability for a membrane used in cardiopulmonary bypass (Courtney et al 1993c).

Blood compatibility issues have arisen following numerous findings that blood constituents are activated after contact with the surfaces of extracorporeal circuits. In haemodialysis, studies have reported of: a transient leucopenia known to occur with cellulose membranes (Kaplow & Goffinet 1968; Craddock et al 1977; Chenoweth 1988), the association between leucocyte activation and complement activation (Jacob et al 1980; Amadori et al 1983; Ivanovich et al 1983; Klinkmann et al 1987), protein adsorption (Panichi et al 1989; McLaughlin et al 1989), platelet activity (Adler & Berlyne 1981; Mahiout 1987), cellular interactions (Courtney et al 1993a), and the alteration of the coagulation system (Wardle & Piercy 1972; Craddock et al 1977; Vaziri et al 1984; Panichi et al 1989; Moll et al 1990; Kolb et al 1991). In cardiopulmonary bypass changes in blood constituents have also

been demonstrated by several studies (Brick 1985; Davis et al 1980; Irvine et al 1992; Wendel et al 1993; Van der Kamp & Van Oeveren 1993; Sundaram et al 1994). The predominant factors contributing to increased postoperative morbidity in cardiopulmonary bypass are known to be the alteration of platelet function and the activation of the coagulation factors (Ionescu et al 1981), which are inevitable consequences of the blood response to cardiopulmonary bypass.

In the study of blood response, there is an advantage of utilizing sufficient representative parameters to enable a better understanding of blood-material compatibility in a broad perspective. However it is useful to consider the following relevant points (Sundaram et al 1994):

1. The magnitude of change in a given parameter does not necessarily correlate with the clinical outcome.
2. Parameters may alter gradually or reach peak levels, making the pattern of blood response important.
3. Patient variability may influence both parameter change and pattern of response.

8.2 SELECTED PARAMETERS

8.2.1 FACTOR XII ACTIVITY

Factor XII activity was determined by measurement using a novel enzyme linked immunosorbent assay for free fluid phase activated factor XII and inhibitor bound activated factor XII as described in chapter 6.

The blood samples were collected in tubes containing trisodium citrate as anticoagulant, centrifuged at 3000 rev/min, at room temperature for 15 min. Plasma was snap frozen and stored at -70^{0}C until assay.

8.2.2 ACTIVATED FACTOR VII (FVIIa).

Factor VIIa is a glycoprotein consisting of two polypeptide chains of individual molecular weights of 26,000 and 22,000. It is derived from an intra-chain cleavage of the single-chain factor VII, a glycoprotein of molecular weight 50,000 when factor VII is activated by the factors XIIa, IXa, Xa, IIa and also VIIa (feedback auto-activation). The concentration of factor VIIa in plasma is approximately 1 % of that of factor VII. Native tissue factor is a cofactor of factor

VIIa. When factor VII binds to tissue factor, it produces the activation of factor X to factor Xa, whilst itself is transformed into factor VIIa. The soluble tissue factor is the extra-cellular portion of native tissue factor; it can no longer activate factor VII by autoactivation.

The relevance for the evaluation of the levels of activated factor VII in extracorporeal circulation was primarily due to the *in vitro* demonstration of its strong relationship with the contact phase activation system (Mitropoulos et al 1993). Increased levels of activated factor VII, reflects the level of the activation of the extrinsic pathway of coagulation and therefore a prothrombotic state. However, the subsequent hypercoagulable state will require the presence of tissue factor released from damaged vascular endothelium (Davies & Tripathi 1993) or possibly activated monocytes (Kapelmeyer et al 1993). Factor XIIa has been shown to contribute to the activation of factor VII by a number of processes that do not require tissue factor: firstly through the direct cleavage of factor VII (Radcliffe et al 1977; Seligsohn U et al 1979; Kisiel et al 1977); secondly through the generation of kallikrein (Mandle & Kaplan 1977; Kaplan & Austen 1970) which can activate factor IX (Østerud et al 1978; Østerud et al 1975); and thirdly through the activation of factor XI and sequential activation of factor IX (Bouma & Griffin 1977; Davie et al 1979). Factor IXa, in turn has been shown to act as a direct activator of factor VII (Seligsohn et al 1979; Laake & Østerud 1974). More recently, it has been demonstrated *in vitro* that in plasma with a complete contact system of coagulation coupled with normal concentration of factor IX, factor XIa was the major contributor of factor VII activation (Mitropoulos et al 1993).

The factor VIIa bound to soluble tissue factor can activate factor X to factor Xa. The principle of the factor VIIa assay is based on the use of recombinant soluble tissue factor (truncated tissue factor) which possesses a cofactor function specific for factor VIIa. The recombinant tissue factor (rsTF), in the presence of factor VIIa, phospholipids and Ca^{2+} produces coagulation of plasma. In this system the observed clotting time bears an inverse relationship with the factor VIIa level initially present in the plasma being tested. The rsTF does not activate the factor VII into factor VIIa, consequently the factor VII present in the test plasma does not

interfere in the assay.

BLOOD COLLECTION FOR FACTOR VIIa ASSAY.

Blood samples were collected into polypropylene tubes containing trisodium citrate and plasma was separated by centrifugation at 2500 g for 10 min at 20°C. Aliquots of the plasma obtained were snap frozen in a mixture of dry ice and methanol and stored at -70°C until assay. Factor VIIa was assayed using a commercial kit, "Staclot® VIIa-rTF", purchased from Diagnostica Stago, Asnieres-Sur-Seine (France).

STACLOT® VIIa-rTF ASSAY KIT

The contents of the kit include, reagents 1, 2, 3, 4, 5a and 5b. Reagent 1 was a 10-fold concentrated buffer; reagent 2 contained freeze-dried human plasma depleted of factor VII by specific immuno-adsorption, specifically adapted for factor VIIa assay; reagent 3 contains recombinant soluble tissue factor (rsTF) and phospholipids, freeze-dried; reagent 4 contained human recombinant factor VIIa of known level determined for the staclot® VIIa-rTF system; reagent 5a was a control level 1 containing a known level of factor VIIa (45 - 60 mU/ml); reagent 5b was a control level 2 containing a known level of factor VIIa (125 - 165 mU/ml). The assay of samples was carried out using a Behring Schnitger & gross coagulometer (Hook method), supplied by Burkard Scientific (Sales) Ltd, Uxbridge, Middx., UK.

FACTOR VIIa ASSAY PROCEDURE.

The procedure was as follows:

1. Plasma samples, standards and controls were diluted at 1:10 with the diluted buffer (reagent 1 at 1:10 with distilled water).
2. 50 µl of sample dilution (standard, test plasma or control) were added into polypropylene test tubes at 37°C.
3. 50 µl of reagent 2 and 50 µl of reagent 3 were then added and the test tubes were mixed gently and incubated at 37°C for exactly 180 seconds.
4. 50 µl of 0.025 M $CaCl_2$ prewarmed to 37°C were added into the tubes and clotting time noted.
5. The results were calculated using a log-log plot on a graph paper of factor VIIa

standard levels (mU/ml) on the x-axis and their corresponding clotting times (sec) on the y-axis. A calibration line was drawn and the factor VIIa levels of the test samples was deduced.

8.2.3 CORRECTION FOR HAEMODILUTION

All the values found for factors XIIa and VIIa were corrected for haemodilution by the measurement of total plasma protein using the Biuret method (Sigma Diagnostics, Poole, Dorset, UK) as described below:

1. 1 ml of Total protein reagent were pipetted into polypropylene tubes.
2. 20 μl of deionised water, standard, controls and patient samples were added into the appropriately labelled test tubes. The tubes were then mixed by gentle inversion.
3. The tubes were then incubated for 10 minutes at ambient temperature (18-26^{0}C).
4. Absorbance readings were taken at 540 nm versus a reagent blank as reference.
5. Total protein was calculated as from the following equation:

$$\text{Total protein} = A_{test}/A_{standard} \times 8^* \quad \text{..... Equation 8.1.}$$

* Concentration (g/dL) of protein standard.

The determined total protein values for each test sample were then used to correct the effect of haemodilution during extracorporeal circulation as follows:

Actual value = measured value x initial total protein ÷ measured total protein during extracorporeal circulation .....Equation 8.2.

8.3 CONTACT PHASE ACTIVATION DURING HAEMODIALYSIS

In haemodialysis, the basis for monitoring of the blood response has largely been influenced by:

1. The nature of the membrane, and its association with the device forming the extracorporeal circuit (Klinkmann et al 1987; Courtney et al 1993a).
2. The overall mode of application of the membrane in the device (e.g high flux and haemofiltration) is also important for the status of the uraemic patient (Klinkmann et al 1987).
3. The disease state of the uraemic patient eg a marked reduction in the interactions

Table 8.1 Summary of the relevant patients details for the study.

	Cuprophan	Polysulphone F8	High-flux polysulphone F40
Number of patients	12	6	5
Mean age (years)	52	63	52
Male:female ratio	8:4	5:1	3:2
Dialysate	Bicarbonate	Bicarbonate	Bicarbonate
Dialysate flow rate (ml/min)	550	500	560
Blood flow rate (ml/min)	150-400	200-300	150-400
Mean heparin (IU)	5667	6833	5800
Duration of dialysis (mean months)	41	34	46
Regime	4 - 5 h sessions, 3 times a week.	4 - 5 h sessions, 3 times a week.	4 - 5 h sessions, 3 times a week.
Membrane surface area (m^2)	1.3	1.8	0.7
Sterilisation method	ethylene oxide	ethylene oxide	ethylene oxide
Mean creatinine levels (μM) - pre-dialysis.	1083 $\pm$ 228	968 $\pm$ 240	1073 $\pm$ 233
Mean urea nitrogen (mM) - pre-dialysis	27.5 $\pm$ 4.6	26.4 $\pm$ 4.5	24.6 $\pm$ 5.4

of platelets with artificial surfaces has been reported (Courtney et al 1993a).

4. The other components of the extracorporeal circuit e.g tubing and leached tubing components etc.

5. The variation of the individual patient response to the anticoagulant heparin

(Wessler & Gritel 1976).

6. The influence of drug therapy eg ACE inhibitors with AN69 membrane dialyzers (Parnes & Shapiro 1991; Jadoul et al 1991; Verresen et al 1992) and the introduction of recombinant erythropoietin on the anaemic state prevalent in chronic renal failure (Esbach 1989).

The effect of haemodialysis on factor XII, has produced contentious findings, with some reports suggesting the activation and consumption of factor XII during dialysis (Wardle & Piercy 1972). Others have failed to confirm these findings (Vaziri et al 1984). Recently, factor XII-like activity was measured using a chromogenic substrate assay and a gradual rise in factor XII-like activity during dialysis was found (Sundaram 1992). It has largely been accepted that the activation of factor XII can cause major biological consequences due to its intimate connection with several other biologically important proteolytic pathways. However, the difficulty in finding a universally acceptable assay procedure for activated factor XII, has meant that the concept is largely hypothetical.

The objective in this study, therefore, was to measure factor XII activity using a novel immunoassay technique and thereby establishing a role of factor XII in haemodialysis. Additionally, a parameter representative of the activation of extrinsic pathway of coagulation (factor VIIa & VIIa-tissue factor complexes) was also measured in an effort to understand the integrated nature of blood responses.

8.3.1 PATIENT GROUPS

Three groups of end stage renal failure patients requiring maintenance haemodialysis were studied, with relevant details of which are shown on table 8.1. Further information on the individual patients is included in Appendix A, depending on type of dialyser used i.e Cuprophan (Focus 120, Renal Care International, Ireland), polysulphone F8 (Fresenius AG, Bad Homburg, Germany) and high-flux polysulphone F40 (Fresenius AG, Bad Homburg, Germany). Informed consent was obtained from all the participating patients after the approval of the project by Ethical Committee of the Glasgow Royal Infirmary, Glasgow, UK. The following criteria were adapted for inclusion of patients in the trial:

1. All patients were to be dialysed against bicarbonate buffer. The benefits of

bicarbonate over acetate buffer have been reported (Ledebo 1990).

2. Anticoagulant (heparin) to be administered as a bolus.

3. Haemoglobin levels not to be below 8 g/dL.

4. Patients to have been on dialysis for more than 2 months.

5. Patients not on anti-hypertensive therapy with ACE-inhibitors.

8.3.2 BLOOD SAMPLING

Blood samples for haemodialysis study were obtained prior heparinisation, at 15, 60, 90 min during dialysis (venous side of the dialyser) and at the end of the dialysis session. Blood samples were then processed as described on section 8.2.1 and 8.2.2 and factors XIIa and VIIa were assayed accordingly.

8.4 THE ROLE OF CONTACT PHASE ACTIVATION DURING CARDIOPULMONARY BYPASS.

The use of devices in cardiothoracic surgery, where the heart and lungs are bypassed via an extracorporeal circuit has usually been described as cardiopulmonary bypass. The objective has been to achieve suitable conditions for intracardiac operations, while maintaining normal tissue metabolism and oxygenation for limited periods of time (Ionescu et al 1981). Systemic venous blood is artificially oxygenated and then returned to the systemic arterial system at a suitable physiological pressure, devoid of gas bubbles and solid particles, and at a controlled temperature and flow rate (Wheatley 1985).

Activation of specific blood constituents during cardiopulmonary bypass causes postoperative bleeding problems, massive fluid displacements, and temporary organ dysfunction that contribute to the morbidity and mortality of cardiac operations (Ratliff et al 1973; Harker et al 1980; Blackstone et al 1982; Bick 1982; Harker 1986; Woodman & Harker 1990). The most obvious clinical manifestation of the haemostatic defects is the increased blood loss during cardiopulmonary bypass (Bick 1982; Harker 1986; Woodman & Harker 1990). The functional defect of platelets has been suggested as a major cause of the bleeding diathesis (Harker et al 1980; Van Oeveren et al 1988).

Extracorporeal circulation has been associated with major qualitative and quantitative alterations in platelets, including thrombocytopenia (Harker et al 1980; Edmunds et al 1982), decreased responsiveness to aggregating agents (Edmunds et al 1982; Hennessy et al 1977), aggregate emboli (Dutton et al 1974), loss of α_2-adrenergic (Wachtfogel et al 1985) and fibrinogen receptors (Musial et al 1985), and release of granule contents (Harker 1980; Edmunds et al 1982).

The contact of blood with foreign surfaces of the oxygenator stimulates the activation of coagulation, fibrinolysis and a number of other "inflammatory cascades" that can act through humoral or cellular mechanisms. These cascades are ultimately controlled by amplification cascades of proteolytic enzymes which are predominantly serine proteases. The cumulative effect of this amplification has been defined as a "whole body inflammatory response", a concept described by Kirklin et al 1983. During the period of extracorporeal circulation, the control of the amplification cascades is particularly crucial, hence the routine heparin administration to inhibit blood clotting. Heparin achieves this by activating the naturally occurring serine protease inhibitor antithrombin III. However, heparin exhibits a limited effect on the early contact activation cascades such as complement, fibrinolysis, kallikrein/kinin, and later, the sequestration of leucocytes within the lungs.

The search for suitable inhibitors of the early blood activation reactions has been a subject of particular interest in extracorporeal perfusion and open heart surgery with particular relevance in the control of bleeding and inflammatory reactions. Platelets and factor XII are directly activated by contact with synthetic materials, and as far as is known no other blood constituent is directly altered by contact with non-endothelial cell surfaces (Edmunds 1993). All of the subsequent reactions in blood during contact with artificial surfaces of the cardiopulmonary bypass machines are consequences of the direct activation of platelets and factor XII.

Platelets appear to react to synthetic surfaces during the initial moments of blood contact. Probably due to the displacement of surface adsorbed fibrinogen by high molecular kininogen, intact platelets appear not to react with synthetic surfaces

after the first few minutes, a phenomenon described as "platelet passivation" (Zucker & Vroman 1969). This concept of platelet passivated surfaces has encouraged the search for reversible platelet inhibitors that can prevent platelet-surface reactions during initial blood contact. Dipyridamole reversibly inhibits platelet cyclic adenosine monophosphate (AMP) phosphodiesterase and potentiates the inhibitory action of endothelial cell-produced prostacyclin in adequate doses (Fitzgerald 1987; Teoh et al 1988). The beneficial effect of other drugs, including desmopressin acetate (DDAVP) (Salzmann et al 1986), and prostacyclin (Fish et al 1986), on blood loss in patients undergoing cardiac surgery has been shown in controlled clinical studies.

Aprotinin is a naturally occurring enzyme inhibitor derived from bovine lungs, and occurs in the form of a basic (pKa 10) polypeptide comprised of 58 amino acid residues with a molecular weight of 6,512. It acts on trypsin, plasmin, tissue-kallikrein, and to a lesser extent plasma-kallikrein (Emerson 1989). It has also been reported to have direct platelet-preserving properties in very high dosages (Aoki et al 1978).

Although aprotinin has been known for many years and widely used for many surgical indications (Emerson 1989), its blood saving properties have only become evident since it has been used in very high doses (Royston et al 1987). The rationale for these high dosages has been to achieve aprotinin plasma concentrations during cardiopulmonary bypass that are able to inhibit kallikrein activation.

The mechanisms underlying the benefit of using aprotinin in cardiac surgery has yet to be elucidated completely. Some authors have suggested that the clinical effect of aprotinin was based mainly on the inhibition of the contact phase of coagulation (Dietrich et al 1990). This study was therefore undertaken for two reasons:

1. To obtain further information about the mode of action of aprotinin on blood coagulation pathways.
2. To ascertain the blood-saving effect of low dosage aprotinin in patients undergoing coronary artery bypass grafting.

8.4.1 PATIENT GROUPS

The trial consisted of two groups of patients, details of which are presented in table 8.2.

Table 8.2: Summary of patient details relevant to the study.

Details	Aprotinin group	Control group
No. of patients	10	10
Mean age (years)	59	61
Male:Female	8:2	9:1
Mean weight (kg)	82	80
Preoperative Aspirin (mg/day)	150 ceased 1 day prior operation.	150 ceased 1 week prior operation.
CPB time (min)	84	75
X-clamp time (min)	49	36
Blood loss (ml)	454.5 ± 208.6	546 ± 104
Transfusion units of packed cells/blood.	1.4 ± 0.96	2.9 ± 1.1

Informed consent was obtained from all the patients involved after approval of the study by the Ethical Committee of the Hammersmith Hospital, London, UK. All patients were operated for coronary artery bypass grafting, utilising both internal mammary arteries and saphenous veins.

Patients considered for the study were into two groups:

1. Control group - patients operated without the administration of aprotinin. 150 mg dosage aspirin (half dose regime) was ended 1 week prior the operation.
2. Aprotinin group - patients administered with aprotinin. 150 mg dosage aspirin was stopped 1 day prior to the operation. Dosage and administration of aprotinin (Trasylol, Bayer UK Ltd, Newbury, Berkshire, UK) was as follows: after induction of anaesthesia, a loading dose of 140 mg (1 million KIU in 200 ml 0.9 % saline solution) was given intravenously through a central venous cannula over 20 minutes. Immediately afterward, a continuous infusion of 70 mg/hour was started and maintained until the patient left the operating theatre. In addition to the intravenous infusion, another 140 mg were added to the priming volume of the

heart-lung machine by replacement of an aliquot of the priming volume. In order to determine the influence of low dose aspirin on the levels of factor XIIa, a parallel control study comprised of normal healthy volunteers on a 150 mg aspirin dose for a week were recruited, after Ethical committee approval was obtained.

Blood samples were obtained from 9 healthy hospital staff (5 male and 4 female, mean age of 28 ± 8 years) at pre-aspirin and 7 days after the start of aspirin administration. Blood samples were then processed as described in section 8.4.3 before the assay for factor XIIa. Homologous blood or packed red cells were administered to the patients only when the packed cell volume (haematocrit) fell below 30 % in the postoperative course.

8.4.2 CARDIOPULMONARY BYPASS

This followed the procedure described by Bidstrup et al 1989. All patients were premedicated with Lorazepam (2 mg orally), 15 mg morphine intramuscularly, 0.3 mg Hyoscine intramuscularly, 1 hour prior to the patient being taken to the operating theatre. After induction of anaesthesia with Fentanyl (10 mg/kg), Midazolam (1-4 mg) and Pancuronium (0.1 mg/kg), anaesthesia was maintained with a Propotol infusion (5-15 ml/h) and supplements of Fentanyl (5 mg/kg) when required. Bovine lung heparin (300 IU/kg) was injected into a central line before the cannulation of the heart. Tests for activated clotting times were performed at regular intervals and further heparin administered if times fell below 600 seconds for the aprotinin group and 400 seconds for the control group. Hollow fibre membrane (polypropylene) oxygenators, with an incorporated 3 litre capacity cardiotomy reservoir (COBE CML (Cobe laboratories, Olympus Business Park, Gloucester, England) and plasticised poly (vinyl chloride) tubing were used throughout.

The oxygenators were primed with 2000 ml Hartmann's solution. Flow rate was calculated as Cardiac Index (CI) x Body surface area (BSA). Flows of 2.4 x Body surface area (BSA) L/min/m^2 were maintained using a roller pump (Sarns Inc, Ann Arbor Michigan or Stockert pumps, Stockert System, Germany). Moderate systemic hypothermia was maintained at 32 ^{0}C while the aorta was occluded. Myocardial preservation was achieved by infusion of 1000 ml cold

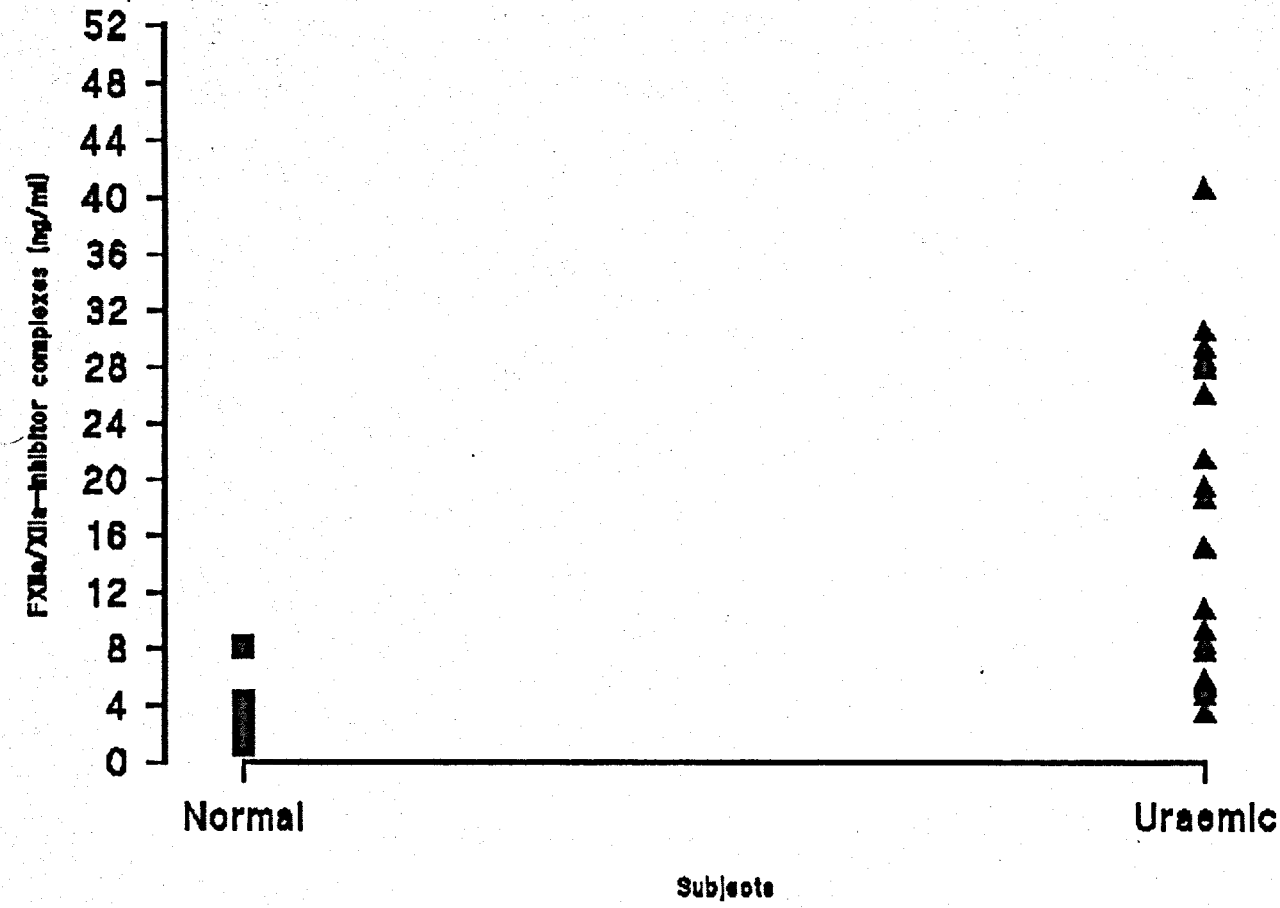


Figure 8.1: FXIIa/XIIa-inhibitor complexes in normal and uraemic patients.

crystalloid cardioplegic solution (St. Thomas' Hospital cardioplegic solution at 4°C) injected into the aortic root. Mean arterial blood pressure kept between 50 and 80 mm Hg with nitroglycerin supplemented with methoxamine or phentolamine as necessary. After rewarming to 37 °C at the completion of the operation and discontinuation of bypass, the effects of residual heparin were reversed with protamine sulphate (1 mg/100 IU/heparin).

Patients were then transferred to the intensive care ward, while maintaining the intermittent positive pressure ventilation until the patient was warm peripherally, and in a stable condition. Hypertension (> 100 mm Hg) was treated with infusions of nitroglycerin and nitroprusside.

8.4.3 BLOOD SAMPLING

Blood samples were taken pre-operatively, start of bypass (5 min post heparin infusion), 30, 60 minutes during bypass, 90 minutes after start of bypass, 4 hours after the start of bypass, 24 and 48 hours after the end of the operation, 5 days after the operation. Samples were obtained from the following sites:

- Peripheral vein (prior to anaesthesia and postoperatively at 48 h and 5 days)
- Arterial line (at start of bypass, during bypass, and postoperatively up to 24 h.

Blood samples were collected into tubes containing trisodium citrate as anticoagulant, centrifuged at 2000 g, for 15 minutes at room temperature. Plasma was aliquoted into 3 and snap frozen in a mixture of methanol and dry ice and stored in -70°C until assay for factor XII activity and factor VII activity using the assay procedures described in section 8.2.1 - 8.2.2.

8.4.4 STATISTICAL METHODS

The Minitab statistical package version 8.0. was used for the analysis. A comparison of the mean values of the variables such as factor XIIa and Factor VIIa between the two groups of patients at different time points was performed using a oneway analysis of variance, a twosample-t test and a nonparametric Mann Whitney test. The differences were considered significant at $p < 0.05$ with a 95 % confidence interval.

8.5 RESULTS

8.5.1 FACTOR XIIa/XIIa-INHIBITORS CONCENTRATION DURING

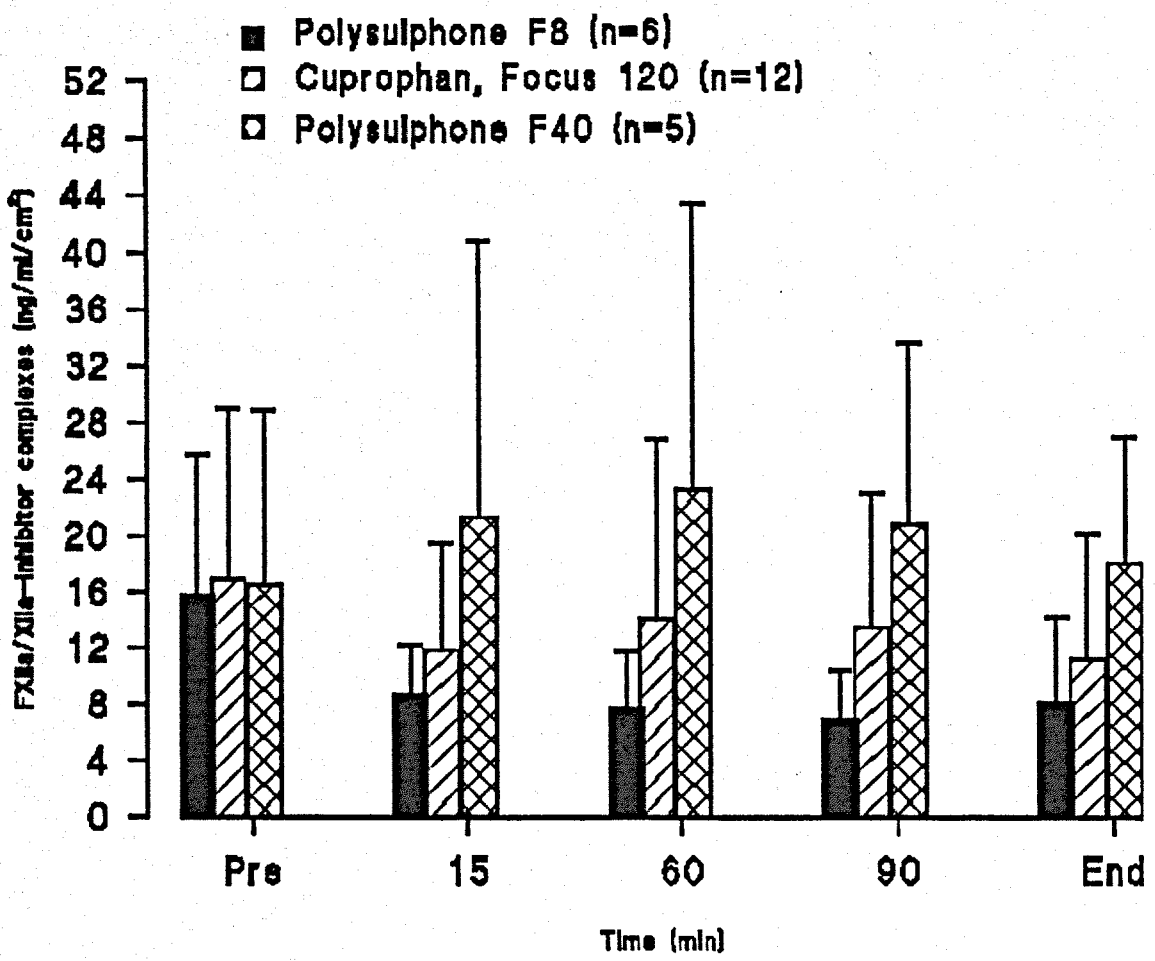


Figure 8.2: FXIIa/XIIa-Inhibitor complexes during dialysis

HAEMODIALYSIS.

The results have indicated that baseline factor XIIa/XIIa-inhibitors generation was very significantly elevated ($p<0.05$) for uraemic patients as compared to normal healthy volunteers (Fig 8.1). There was however no clear relationship between baseline pre-dialysis factor XIIa/XIIa-inhibitor levels and Creatinine or Urea levels, the latter two being indicators of the state of uraemia. The pattern of response as shown on table 8.3, figure 8.2, indicates that levels of factor XII activity were markedly lower than baseline for Cuprophan and polysulphone F8 dialysers at 15 minutes of dialysis, whereas the factor XIIa/XIIa-inhibitor values for polysulphone F40 were above baseline till the end of dialysis.

Table 8.3: Mean FXIIa/XIIa-inhibitors values (ng/ml/m^2) during haemodialysis.

Dialyser	Pre	15 min	60 min	90 min	End
Polysulphone F8, n=6.	15.76 ± 10.00	8.73 ± 3.52	7.77 ± 4.08	7.02 ± 3.37	8.24 ± 6.05
Cuprophan (Focus 120), n=12	16.95 ± 11.99	11.89 ± 7.60	14.11 ± 12.77	13.50 ± 9.55	11.28 ± 8.90
Polysulphone F40, n=5.	16.49 ± 12.39	21.33 ± 19.47	23.33 ± 20.03	20.91 ± 12.74	18.07 ± 15.33

For Cuprophan and polysulphone F8 dialysers, XIIa/XIIa-inhibitor values remained lower than baseline till the end of dialysis, with the latter, exhibiting markedly lower values. There were no significant differences in the XIIa/XIIa-inhibitor values for Cuprophan and polysulphone F8 between each time point of dialysis. The factor XIIa/XIIa-inhibitors values were significantly higher ($p<0.05$) for polysulphone F40 as compared to Cuprophan and the low flux polysulphone F8 at any particular time of the dialysis session.

8.5.2 FACTOR VIIa CONCENTRATION DURING HAEMODIALYSIS

The pattern of baseline values for factor VIIa were significantly elevated ($p<0.05$) compared to during haemodialysis, indicating a similarity with factor

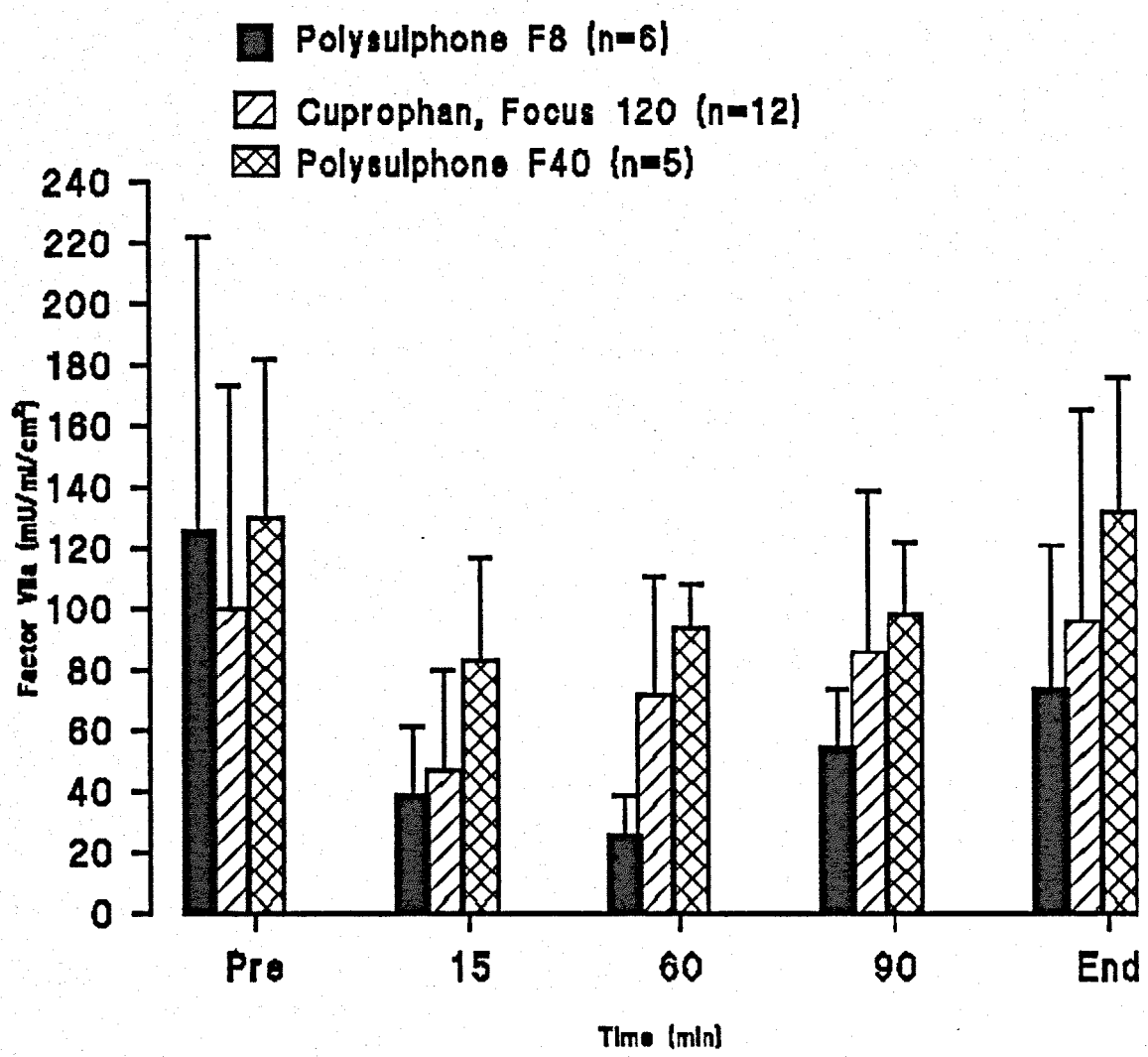


Figure 8.3: FVIIa levels during dialysis

XIIa. 15 minutes after the start of dialysis, factor VIIa values, much lower than baseline were observed with all the dialyser membranes (table 8.4 and Fig 8.3). However, in contrast to factor XIIa, there was a gradual rise in factor VIIa levels for all the membranes until the end of dialysis (table 8.4). Factor VIIa levels for polysulphone F8 were significantly lower ($p<0.05$) than that of cuprophan and polysulphone F40 at any time point of dialysis, with the latter expressing markedly higher values. There appeared to be a low correlation ($r = 0.026$) between baseline factor VIIa and factor XIIa/FXIIa-inhibitor complexes generation.

Table 8.4: Mean factor VIIa levels (mU/ml) during haemodialysis.

Dialyser	Pre	15 min	60 min	90 min	End
Polysulphone F8, n=6.	126 ± 96	39 ± 22	26 ± 13	55 ± 19	74 ± 47
Cuprophan (Focus 120), n=12.	100 ± 73	47 ± 33	72 ± 39	86 ± 53	96 ± 69
Polysulphone F40, n=5.	130 ± 52	83 ± 34	94 ± 14	98 ± 24	132 ± 44

8.5.3 FACTOR XIIa/XIIa-INHIBITOR CONCENTRATION DURING CARDIOPULMONARY BYPASS

Factor XIIa/FXIIa-inhibitors generation during cardiopulmonary bypass indicated higher values with the aprotinin patient group as compared to the control group up to 5 days post-operation, but the differences were not statistically significant ($P>0.05$) at the start and at 60 minutes of bypass (table 8.5 and Fig 8.4). However, significant differences ($p<0.05$) in factor XIIa/XIIa-inhibitors level were evident throughout the rest of the operation and postoperation.

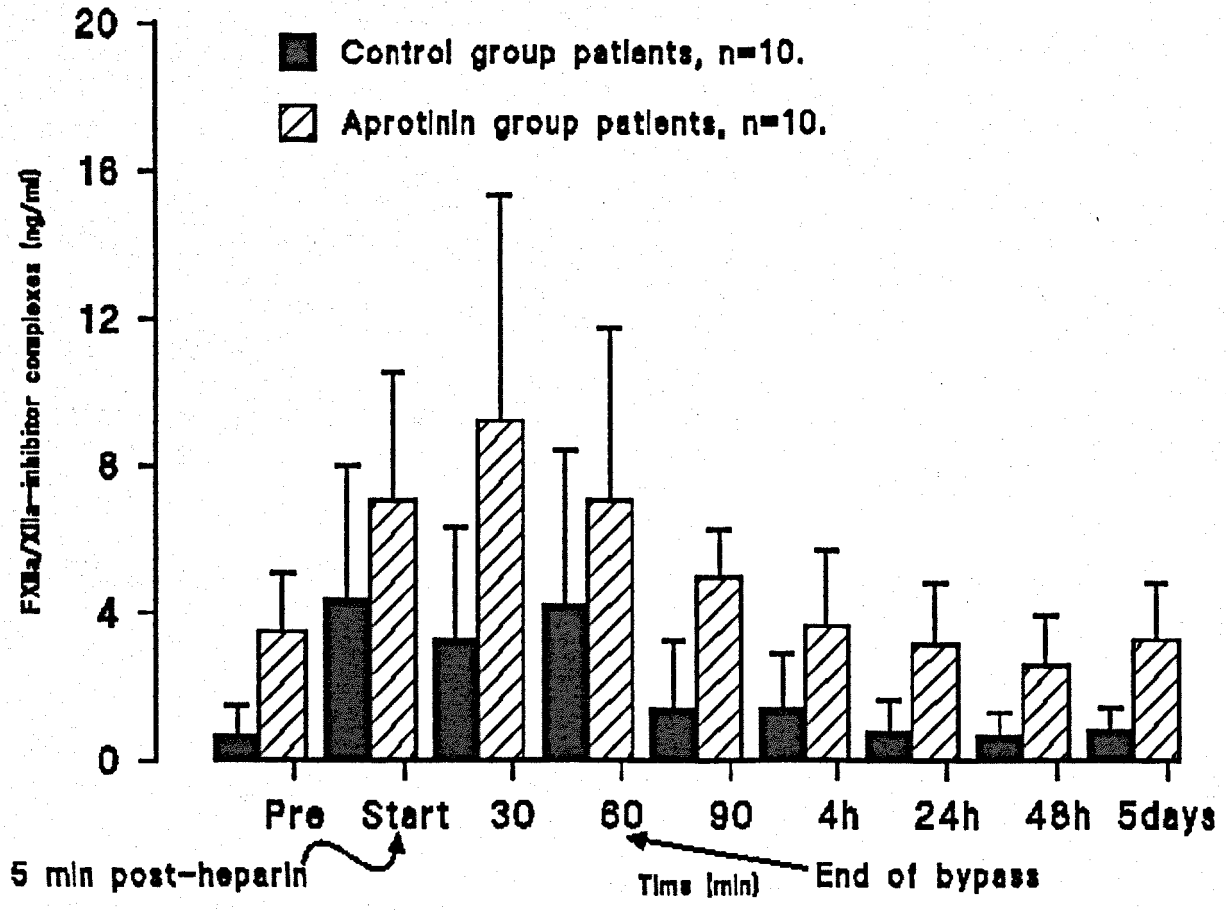


Figure 8.4: FXIIa/XIIa-inhibitor complexes during cardiopulmonary bypass.

Table 8.5 : Mean factor XIIa/XIIa-inhibitor values (ng/ml) during cardiopulmonary bypass.

Patient group	Pre	start	30 min	60 min	90 min	4 h	24 h	48 h	5 days
Control, n=10.	0.65 ± 0.81	4.35 ± 3.66	3.29 ± 3.04	4.23 ± 4.18	1.39 ± 1.84	1.39 ± 1.47	0.76 ± 0.87	0.66 ± 0.63	0.82 ± 0.61
Aprotinin, n=10.	3.48 ± 1.61	7.05 ± 3.49	9.21 ± 6.14	7.06 ± 4.70	4.95 ± 1.33	3.63 ± 2.08	3.13 ± 1.70	2.57 ± 1.36	3.26 ± 1.54

There was a significantly ($p < 0.05$) higher baseline factor XIIa/XIIa-inhibitors level with the aprotinin group compared to the control group. The two groups of patients indicated a sharp rise in factor XIIa, 5 minutes post-heparin infusion (at the start of bypass). For the control healthy volunteers group, the results of factor XIIa/XIIa-inhibitors assay indicated that there was no significant differences ($p > 0.05$) between pre-aspirin and post-aspirin levels (table 8.6).

Table 8.6: The effect of low dose aspirin on factor XIIa/XIIa-inhibitor levels (ng/ml) from healthy volunteers (n=9).

Subjects	Pre-aspirin	Post-aspirin
1	4.19	2.75
2	7.16	8.15
3	2.03	3.32
4	2.43	1.99
5	1.22	1.70
6	1.57	2.09
7	2.81	3.82
8	1.35	1.32
9	3.44	6.02
Mean ± SD	2.91 ± 1.87	3.46 ± 2.26

8.5.4 FACTOR VIIa CONCENTRATION DURING CARDIOPULMONARY BYPASS

The pattern of factor VIIa generation was markedly different for the two groups of patients. The baseline factor VIIa generation was markedly higher for the control group than the aprotinin group (table 8.7 and Fig 8.5). At the start of bypass (5 min post-heparin infusion), factor VIIa values were lower than that of baseline for the control group. During bypass, the control group indicated an elevated factor VIIa at 30 minutes from start. However, for the control group, levels declined at 60 minutes of bypass, and were down to the same level as baseline at 90 minutes and then rose sharply at 4 hours after the start of bypass. The factor VIIa levels declined but remained just above baseline from the 24 hours post-surgery.

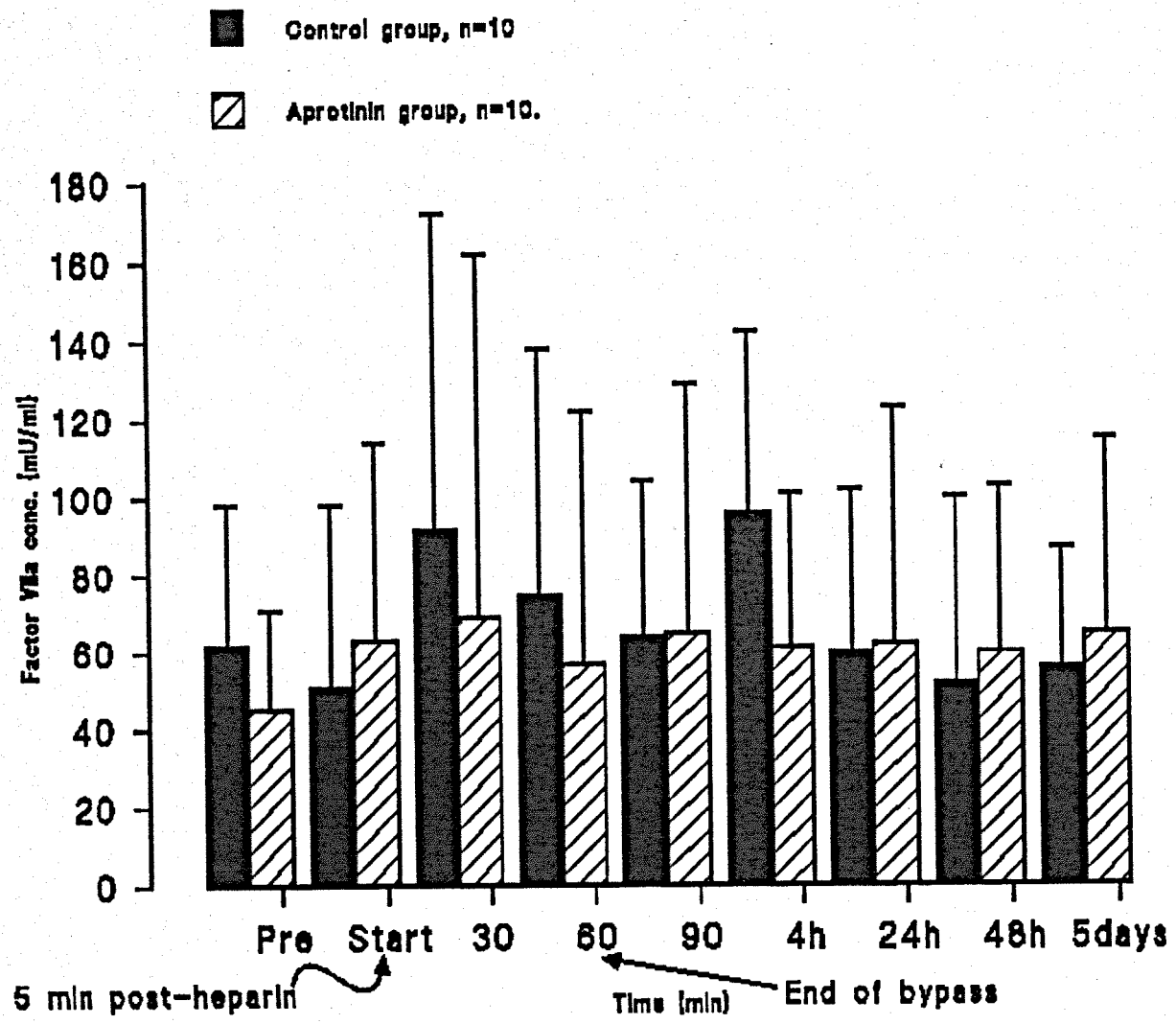


Figure 8.5: Factor VIIa (IU/ml) during cardiopulmonary bypass.

Table 8.7: Mean factor VIIa levels (mU/ml) during cardiopulmonary bypass

Patient group	Pre	start	30 min	60 min	90 min	4 h	24 h	48 h	5 days
Control, n=10	62 ± 36	51 ± 47	92 ± 80	75 ± 63	64 ± 40	96 ± 46	60 ± 42	52 ± 48	56 ± 31
Aprotinin, n=10	45 ± 28	63 ± 51	69 ± 93	57 ± 65	65 ± 64	61 ± 40	62 ± 61	60 ± 43	65 ± 50

For the aprotinin group, values were markedly lower at baseline, compared to the control group, but rose to same levels at the start of bypass. However, during bypass factor VIIa levels remained almost the same. At the end of bypass (90 min after start), factor VIIa levels rose gradually and after 4 hours same levels as the control group baseline were reached.

The overall pattern indicated that factor VIIa levels were lower for the aprotinin group as compared to the control group during the bypass period. There was no correlation ($r = -0.18$ and $r = 0.026$ respectively) between baseline factor VIIa and factor XIIa for both groups. However, for the control group, the patterns of factor VIIa generation were similar to those of factor XIIa/XIIa-inhibitor levels. The pattern of blood loss indicated a decrease with the aprotinin group although the magnitude of difference was not statistically significant ($p > 0.05$).

8.6 DISCUSSION

8.6.1 HAEMODIALYSIS PATIENTS.

The findings indicate that factor XIIa/XIIa-inhibitor complexes generation was significantly higher for the uraemic patients as compared to normal healthy volunteers by about 5-fold (ranging between 4.61 to 30.22 ng/ml), an observation consistent with previous studies that measured factor XII-like activity (Sundaram 1992). However, the results indicated that there was no clear relationship between the pattern of pre-dialysis creatinine or blood nitrogen urea levels with that of pre-dialysis factor XIIa/XIIa-inhibitor complexes. This suggests that factor XIIa/XIIa-inhibitor complexes generation was not strongly linked to the uraemic state, but possibly the underlying cause of the disease.

During dialysis with Cuprophan and low flux polysulphone F8, there was a general reduction in factor XIIa/XIIa-inhibitor complexes as compared to baseline, possibly due to dialyser surface adsorption of activated factor XII. In contrast, elevated levels of factor XIIa/XIIa-inhibitors with the high flux polysulphone F40 were evident throughout dialysis compared with baseline. The reasons for these observations were not clear but it may be reasonable to speculate that:

(1) Although polysulphone membranes have the same chemical components and

charges, membrane structures may be different between low and high-flux with the latter more porous. Cuprophan on the other hand, has a completely different chemical composition and structure from polysulphone membranes.

(2) There is a possibility of an increased heparin interaction with the high-flux membranes consequently altering the resultant surface zeta potentials. A similar phenomenon was demonstrated with Cuprophan and Hemophan membranes in *in vitro* studies using plasma (Werner et al 1995).

(3) It is therefore possible that high flux membranes may alter the serum/plasma protein content by the elimination of more water and protein molecules below 40,000 molecular weight. As a consequence, significant changes in the blood response to the surface by the contact system factors occurs.

(4) The greater ultrafiltration rate may eliminate more protein molecules up to 40,000 molecular weight consequently allowing greater interaction between activated factor XIIa and its plasma inhibitors as detected by the new elisa assay for FXIIa/FXIIa-inhibitor complexes.

Factor VIIa levels were also elevated in the baseline suggesting the existence of a hypercoagulable state possibly aggrevated by the underlying uraemia complications (von Appen et al 1993). The elevated levels of baseline factor VIIa observations were consistent with previous findings in uraemic patients using an elisa assay for factor VIIa (Kario et al 1995). The pattern of factor VIIa was similar to that of thrombin-antithrombin III complexes for chronic renal failure patients as reported by Kolb et al (1991). It has also been shown that the levels of factor VIIa had little correlation with the levels of tissue factor or thrombomodulin in dialysis and non-dialysis, patients (Kario et al 1995). There was no correlation between baseline factor XIIa/XIIa-inhibitor levels and factor VIIa ($r = -0.082$) in this study. During dialysis there is a reduction in factor VIIa in all the dialysers, but the levels appear to rise with time. The decrease of a factor VIIa levels at the start of dialysis soon after heparin infusion, suggests a possible suppressive effect of heparin on factor VII activators such as tissue factor. The mechanism may involve the catalysis of the inhibition reaction between tissue factor protease inhibitor (TFPI or Kunitz type tissue factor inhibitor) and released tissue factor from damaged endothelial cells.

The levels in Cuprophan and high flux polysulphone dialysers significantly increased ($p<0.05$) with time at a faster rate compared with low flux polysulphone F8, suggesting an involvement of factor XIIa on factor VIIa generation.

The findings would appear to suggest that patients dialysed with the high flux polysulphone F40 could be subject to a more pronounced contact phase activation and consequently leading to an elevated hypercoagulable state due to the activation of the extrinsic pathway by factor XIIa during the dialysis session. Cuprophan demonstrated a moderate contact phase activation and procoagulant activity throughout dialysis. In contrast, low flux polysulphone F8 indicated a relatively diminished contact phase activation and procoagulant activity throughout dialysis.

8.6.2 CARDIOPULMONARY BYPASS PATIENTS

As compared to haemodialysis patients, cardiopulmonary bypass patients indicated significantly lower baseline values of both factor XIIa/XIIa-inihibitors and factor VIIa. For cardiac bypass patients the baseline factor XIIa values range was 0.23 - 2.86 ng/ml compared to 4.61 - 30.44 ng/ml for dialysis patients. The results indicate that factor XIIa/XIIa-inhibittors were significantly elevated ($p<0.05$) during the entire treatment (i.e before, during, and after the operation) for the aprotinised group of patients. In contrast, factor XIIa/XIIa-inhibitor levels were elevated in the control patients only during the period of bypass. There was also a reduced loss of blood although the values were not significantly different. This suggests that the presence of the half dose regime of aprotinin enhances the activation of factor XII during cardiopulmonary bypass, which is in contrast to results in *in vitro* studies (Laurel et al 1992). This contrasts with the known inhibitory effect of aprotinin on kallikrein in simulated extracorporeal perfusion (Wachtfogel et al 1993). The findings may therefore have some implications regarding the effect of low dose aprotinin during bypass with respect to the amplification of the "whole-body inflammatory response" exacerbated by the increased levels of serine proteases. The findings also indicated that aspirin had no significant effect ($p<0.05$) on the levels of factor XIIa generated in healthy volunteers.

The baseline range for factor VIIa in cardiac bypass patients was 22 - 101 mU/ml as compared to 37 - 260 mU/ml in dialysis patients. The factor VIIa levels

for the control patients group indicated an increase only during the early stages of bypass followed by a decline until normal circulation was resumed. However, factor VIIa increased to levels similar to the early phase of bypass, 2 - 3 hours after resumption of normal circulation. This appears to suggest a variable pattern resulting in an overall reduction in factor VIIa, possibly due to the reduction in tissue factor circulating blood from damaged tissue as result of diversion of blood into the oxygenator. At the early stages of bypass, factor VIIa were at a relatively higher level possibly due to higher tissue factor released into circulation due to surgical trauma after sternotomy, and during the removal of the internal mammary arteries or saphenous veins.

The levels of tissue factor would inevitably decline with the duration of bypass, indicated by the fall in factor VIIa levels. At the end of bypass and resumption of full circulation, led to increased tissue factor release into the blood from the traumatised coronary and lung tissues, consequently leading to the higher factor VIIa levels observed at 4 hour time point. This may suggest that mechanisms that trigger blood coagulation during bypass may not be strongly related with factor XIIa levels, in view of the requirement of the presence of tissue factor to generate activated factor X and therefore propagate thrombin generation. This may be possible probably due to the rapid inhibition of activated factor XII by plasma protease inhibitors, and that the factor XII activity detected by the assay was in the form of FXIIa-inhibitor complexes and therefore unable to activate factor VII zymogen. This means that the procoagulant effect of factor VII would manifest only after the resumption of full circulation on account of released tissue factor. This hypothesis may be consistent with other studies that have indicated that the triggering of blood coagulation after cardiopulmonary bypass and postoperatively was via tissue factor-VIIa mechanism in response to cutting of blood vessels rather than by the activation of contact phase resulting from exposure of blood to foreign surfaces (Boisclair et al 1993; Burman et al 1994). A mechanism that might be involved in triggering coagulation after the cannulation of the aorta may be platelet activation during contact between blood and the artificial surfaces of the bypass machine.

For the aprotinised cardiac bypass patients, the levels of factor VIIa remained

lower than the control patients before and during bypass. This may be due to the inhibition of coagulant activity of VIIa-TF complexes after the formation of factor VIIa-TF-Xa complexes by aprotinin, and this observation is in agreement with previous *in vitro* studies (Chabbat et al 1993). The results therefore indicate that aprotinin has some anticoagulant effect demonstrated by the inhibition of the extrinsic pathway of blood coagulation. However, the observations of reduced blood loss albeit not significantly with the aprotinin group indicated a different mechanism of blood preservation to that of the initiation of the extrinsic pathway.

8.7 SUMMARY

The study has demonstrated therefore that the measurement of activated factor XII and VII, was useful in the assessment of the resultant blood response due to contact with artificial surfaces during clinical application of extracorporeal circulation. It was also demonstrated that the influence of disease status or presence of pharmaceutical agents on blood coagulation could be studied reasonably well using the two assay procedures.

The findings also indicated that for chronic renal failure patients, there is a significantly higher level of the underlying activation of the contact system. The existing procoagulant state may be due to a combination of the elevated contact activation as well as the damaged endothelium common in uraemia (von Appen 1993). It has also been demonstrated that during dialysis, the magnitude and pattern of contact activation and procoagulant activity depended on the type of dialyser used, with the high flux polysulphone F40 showing a markedly higher state than Cuprophan or low flux polysulphone F8.

The study has also indicated that the level of contact phase activation of blood by surfaces of the bypass machines was significantly elevated ($p < 0.05$) compared to baseline values. The high levels of contact activation persisted for up to 3 hours post-bypass. The level of contact phase activation was markedly raised in the presence of aprotinin as compared to the absence of aprotinin, before, during and after bypass. This suggests that aprotinin does not inhibit the factor XII pathway of of contact phase activation, but attenuates the overall rate of activation. The results also indicated that in normal healthy subjects, pre- and post-aspirin levels of factor

XIIa were not significantly different, and therefore suggesting that the presence of aspirin in the aprotinin group had little influence on the findings.

In conclusion the mechanism involved in the limitation of blood loss during cardiopulmonary bypass with aprotinin, has been shown not to be strongly related to the activation of the extrinsic pathway of blood coagulation. The possible mechanisms may involve the effect of aprotinin on:

(1) The preservation of platelets and platelet functions (Aoki et al 1978; Edmunds et al 1991).

(2) A possible inhibition of plasma proteases that normally breakdown FXIIa-Inhibitor complexes *in vivo*, by the antiprotease action of aprotinin.

(3) The inhibition of FXII-dependent fibrinolytic pathways (Dietrich et al 1990; Prentice 1991; Edmunds et al 1991).

CHAPTER NINE

DISCUSSION AND FUTURE WORK

9.1 INTRODUCTION

The growth in clinical utilisation of materials and devices has been accompanied by a growth of interest in the topic of biocompatibility. The widespread utilisation of artificial materials in clinical applications involving contact with blood has generated an interest in the knowledge of blood responses to artificial surfaces aiming at improving clinical performance (Nosè 1988; Ringoir & Vanholder 1990). The main focus during the development of biomaterials has been on the derivation of a structure-blood response property relationship (Irvine 1989). In extracorporeal applications, such as haemodialysis and related procedures, and cardiopulmonary bypass, focus has been on blood responses with respect to membranes and tubing materials. For the purpose of this study, the relevant biomaterials for evaluation were haemodialysis membranes. For haemodialysis, much effort has been put into the development of new membranes or modification of existing membranes (regenerated cellulose) to improve biocompatibility properties. Most modified membranes are based on cellulose or synthetic polymers.

The use of unmodified cellulosic membranes in haemodialysis has been shown to cause transient leucopenia (Kaplow & Goffinet 1968), and complement activation (Craddock et al 1977). One of the most significant aspect of the modification of cellulose polymers has been the substitution of some of its hydroxyl groups with positively charged groups, giving the membrane an overall positive charge. Such membranes include diethylaminoethyl (DEAE)-cellulose/cellulose blend (Hemophan). Synthetic polymer membranes such as acrylonitrile copolymers have been developed. These membranes include AN69S and SPAN, both of which are negatively charged. Various *in vitro / in vivo* studies have revealed that these membranes significantly reduced complement activation compared to regenerated cellulose membranes (Cuprophan) (Travers 1987; Irvine 1989; Yu 1993; Lamba 1994). In this study, the selected membranes for evaluation were regenerated cellulose (Cuprophan), a DEAE-modified cellulose (Hemophan), polyacrylonitrile-sodium methallyl copolymer based membranes (AN69S and

SPAN).

9.2 TEST PROCEDURES

A number of options for the evaluation of the blood response to membranes can be considered under clinical, *in vivo, ex vivo,* and *in vitro* and the basic factors are the nature of the blood, parameter selection, and the method of achieving blood-biomaterial contact (Courtney et al 1994). With respect to biomaterial development and the attempt to derive a structure-property relationship, the principle options are *ex vivo* and *in vitro* procedures. Single pass *ex vivo* procedures using human blood have been designed for the assessment of haemodialysis membranes (Bosch et al 1987; Mahiout 1994). These procedures enable the control of blood flow, permit multiparameter assessment and can approximate to clinical conditions by evaluating the blood response to membranes in devices containing dialysate. The advantages are offset by the fact that each test may require a donation of 500 ml of blood, and human *ex vivo* procedures for biomaterial evaluation are not common. *In vitro* procedures cannot reflect the inter-relationship between a patient and the clinical use of a biomaterial (Klinkmann et al 1987; Courtney et al 1993; Lindsay et al 1980) and do not normally consider the influence of the disease state (Andrade et al 1981). However, *in vitro* procedures remain important for biomaterial development. Procedures range from incubation cells relevant for rapid screening (Yu et al 1991) to cells with controlled blood flow rates relevant for a more detailed investigation of blood-biomaterials (Bruck 1982; Weng et al 1991; Lamba 1994). In this study, the incubation test cell was the chosen procedure for flat sheet membrane materials and the findings were compared to a new controlled blood flow syringe pump system for hollow fibre membranes.

9.3 RELEVANT PARAMETERS SELECTED

Monitoring of blood responses to a biomaterial is complicated by the difficulty in interpretation of the resultant data. This is primarily due to the integrated nature of blood responses to a biomaterial. In the assessment of biocompatibility of a material, it is important to identify relevant parameters in order to promote the establishment of a structure-property relationship. In terms of relevant parameters, complement activation has been established as one of the

most important parameters and measurement of complement C3a and C5a has been accepted as routine procedures for membrane biocompatibility assessment (Vienken 1993). Other aspects of membrane biocompatibility have been identified during clinical applications of biomaterials. Acute anaphylactoid reactions during haemodialysis have been reported since 1975 (Poothullil et al 1975). The aetiological factors however, have remained controversial, and numerous studies have implicated the excessive generation of bradykinin particularly in connection with haemodialysis with AN69 dialysers for patients under anti-hypertensive treatment with angiotensin converting enzyme or ACE inhibitors (Tielemans et al 1990; Verresen et al 1990; Lemke et al 1990; Dinarello 1991; Jadoul et al 1991; Brunet et al 1992; Parnes & Shapiro 1991; Schulman et al 1993; Schaefer et al 1993). It was then hypothesized that contact activation of plasma by membrane surface leads to the activation of kallikrein which cleaves bradykinin from high molecular weight kininogen (Lemke and Fink 1992). In an *in vivo* sheep model of dialysis (Krieter et al 1993) and *In vitro* tests with human plasma (Lemke & Fink 1992), generation of bradykinin was observed with AN69 in the presence of ACE inhibitors. Generation of bradykinin in human plasma was dependent on ACE-inhibitor dose and could be completely stopped by soybean trypsin inhibitor, an inhibitor of plasma kallikrein (Lemke & Fink 1992). The generation of bradykinin was therefore considered a relevant parameter for hypersensitivity, but currently, assays for bradykinin suffer from limited availability.

It is therefore appropriate to consider other types of assays which detect activation of the contact system. The activation of the contact system is known to be intimately connected with other components of the haemostatic mechanism such as complement, fibrinolytic, and cell activation systems (Courtney et al 1993c. As a consequence, contact phase activation was considered as a suitable aspect of blood response during blood-biomaterials contact in this study. Assays that measures kallikrein in plasma using chromogenic substrates have been reported (Lottenberg et al 1981; Fisher et al 1982). However, these assays lack specificity, since other proteases in plasma interfere. Immunoassays that measure proteases complexed to their natural plasma inhibitors have also been reported (Harpel et al 1985; Nuijens et al 1988; Kaufmann et al 1991) but none of these are available

commercially.

The focus in this thesis was on factor XII and the objective was to optimise the measurement of activated factor XII using an existing chromogenic substrate assay procedure for factor XII-like activity (FXIIA) (Irvine 1989; Sundaram 1992). The chromogenic substrate assay methodology was modified to measure surface-bound FXIIA. Immunoassay procedures for factor XII activity levels (FXIIa), including a novel procedure for FXIIa/FXIIa-inhibitor complexes were also used and comparison drawn with the FXIIA patterns. As an indication of clinical relevance of factor XII activity as measured using the FXIIa/FXIIa-inhibitor assay, implications with respect to haemodialysis and cardiopulmonary bypass were investigated. In addition, in view of the tendency of factor XII to adsorb to foreign surfaces, the mechanism by which factor XII binds onto membranes and the influence of various pharmacological agents was also investigated. It was hoped that the establishment of factor XII membrane-adsorption patterns as compared to the corresponding factor XII activation could offer more information on the relationship between the properties of the biomaterial and the activation of the contact phase of blood coagulation.

9.4 *IN VITRO* CONTACT PHASE ACTIVATION INVESTIGATIONS

The modified chromogenic substrate assay was used to measure FXIIA at the plasma-membrane interface. The results were compared with the respective supernatant-phase levels. The results indicated that measurement of membrane-bound FXIIA, was a sensitive index of factor XII activity, as compared to supernatant-phase FXIIA, where material discrimination was not evident. However, the patterns of plasma membrabne-FXIIA indicated a lack of relationship with membrane charge, with the negatively charged AN69S membrane exhibiting the least activity. This is in contrast with evaluations using purified unactivated factor XII protein, where the magnitude of membrane FXIIA was found to be greater with the negatively charged membranes. Investigations utilising the immunoassays for activated factor XII, indicated differences in patterns of activity exhibited by different membranes as measured in the supernatant-phase of membrane contacted blood. The measurement of factor XII activity using the novel FXIIa/FXIIa-inhibitor assay indicated enhanced sensitivity as compared to the

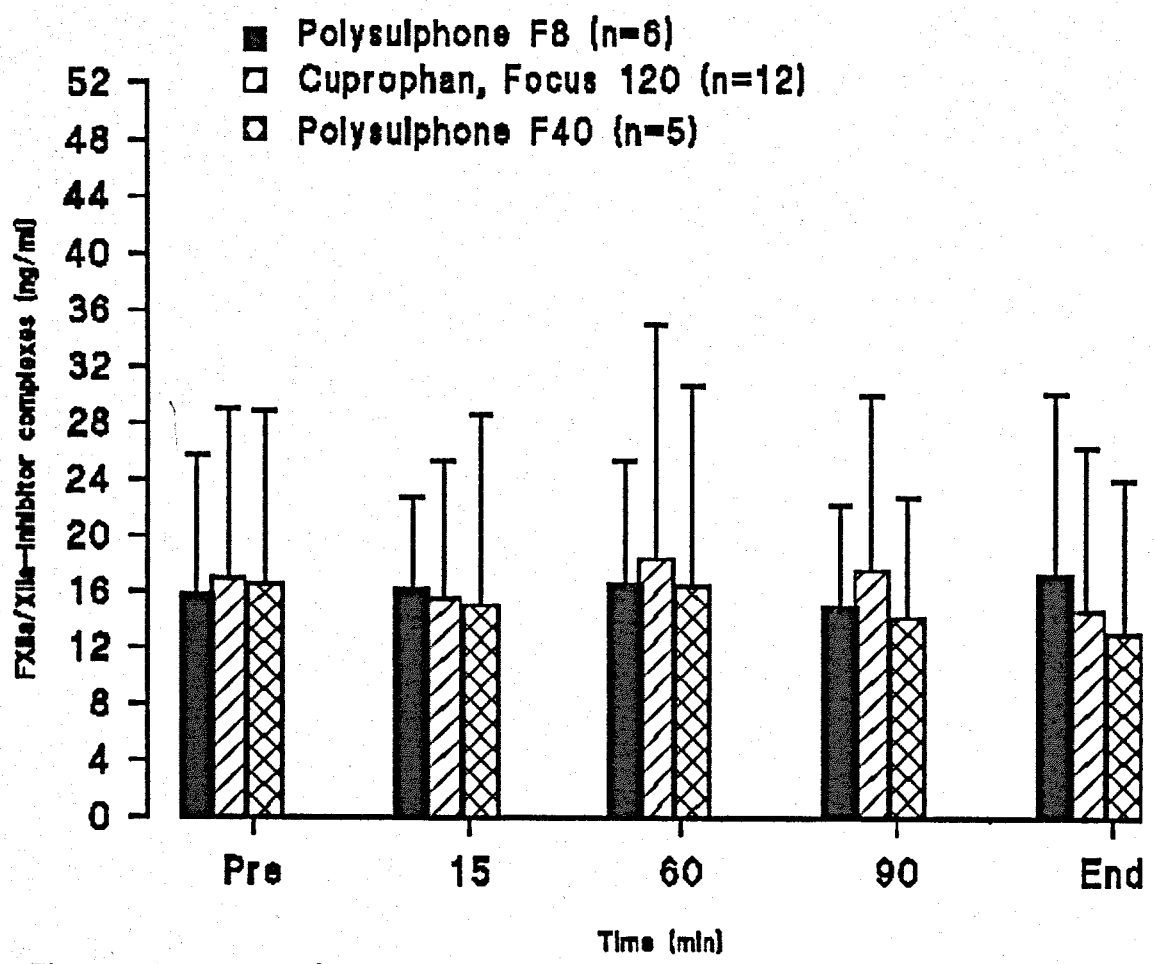


Figure 9.1: FXIIa/XIIa-inhibitor complexes during dialysis (device consideration)

Shield elisa assay for FXIIa. AN69S and Cuprophan were found to indicate higher levels as compared to Hemophan membrane. This was consistent with the findings using cationic/anionic dye staining procedures that AN69S was negatively charged, Cuprophan was faintly negative/neutral and Hemophan was positively charged.

The results of the factor XII adsorption studies using radiolabelled purified factor XII solution and in plasma indicated that there was a significantly greater adsorption of factor XII on polyacrylonitrile-based membrane as compared to cellulosic membranes. In view of the overall negative surface charge observed on polyacrylonitrile-based AN69S membrane as compared to Cuprophan or Hemophan determined by the cationic/anionic dye staining technique, it was concluded that there was greater factor XII adsorption on negatively charged membranes. However, this pattern was not in agreement with membrane-bound FXIIA indicating that other plasma constituents had an overall influence on activation of surface-bound factor XII. It is possible that negatively charged polyacrylonitrile-based AN69S membrane also adsorbs plasma activated factor XII inhibitors such as C1-inhibitor and therefore results in an overall reduction in FXIIA detection as observed. Alternatively AN69S membranes may have a higher affinity for other proteins such as fibrinogen. This resulted in greater adsorption of fibrinogen as demonstrated in previous studies (Yu 1993), consequently there was a reduced "Vroman effect". It is conventional in blood coagulation to imply that negatively charged surfaces exhibit contact phase activation properties. However, it is evident that all surfaces with an exception of normal vascular endothelium have the ability to initiate contact phase of blood coagulation. The more accurate statement should therefore be that negatively charged surfaces have a greater activity than positive surfaces. Enhanced biocompatibility properties have been reported with negatively charged surfaces (Brash 1991). This implies that the overall membrane biocompatibility was dependent on the interaction between contact phase activation with the other plasma systems such as protease inhibitors and the presence of pharmacological agents (such as heparin, LMWH, hirudin, aprotinin and captopril etc).

9.5 CONTACT PHASE ACTIVATION DURING HAEMODIALYSIS

In an effort to establish the clinical relevance of a parameter, haemodialysis

represented a procedure where membrane structure could alter the response of blood components, particularly the complement system and leucocytes. The investigations demonstrated that the mean baseline factor XIIa/FXIIa-inhibitor and factor VIIa levels for uraemic patients on dialysis were much greater than for normal healthy individuals. There was also a marked patient to patient variation in the levels of FXIIa/FXIIa-inhibitors and factor VIIa. It should be noted that in the analysis of factor XII and factor VII activity during haemodialysis, the levels of factor XIIa/XIIa-inhibitors or VIIa were calculated to take into account the membrane surface area in contact with blood. On this basis, the XIIa/XIIa-inhibitor levels appeared to indicate marked differences in their patterns depending on the membrane type. The high-flux polysulphone F40 indicated the highest levels of factor XIIa/XIIa-inhibitor complexes, followed by Cuprophan and then the low-flux polysulphone F8. Levels for Cuprophan and polysulphone F8 membranes started at a value much lower than baseline and gradually rose until the end of dialysis. This pattern of factor XII activity was different from that of the factor XII-like activity reported previously during haemodialysis (Sundaram 1992). When factor XII activity is considered in terms of the device rather than membrane surface area, there was no evidence of significant differences between the dialyser types (Figure 9.1), suggesting the importance of reducing surface area of blood contact in high-flux applications. High-flux polysulphone F40 exhibited the highest contact activation levels per unit area, but because the membrane surface area in the dialyser is only about a third of the low flux polysulphone F8, the overall reactions remained similar per device. The reasons for the observed patterns of FXIIa/FXIIa-inhibitor complexes with the high-flux polysulphone dialysers are not clear as yet. However, increased elimination of protein molecules up to 40,000 molecular weight by ultrafiltration may be a significant factor in the increased surface interaction by the contact factors. Factor VIIa levels were found to gradually rise during dialysis for all the membrane types, with high-flux polysulphone F40 exhibiting the highest levels followed by Cuprophan. This suggests that for uraemic patients, contact phase activation is very pronounced and this could have some implications. The existence of a hypercoagulable state (demonstrated by the elevated factor VIIa levels), signifies the existence of a

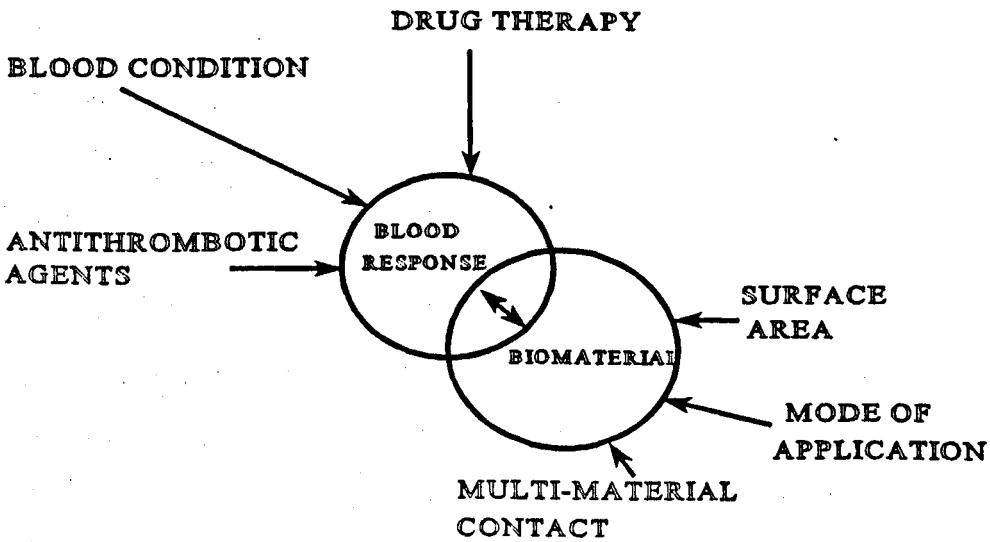


Figure 9.2: The influencing factors in the relationship between a biomaterial and the Blood response in clinical application. (Sundaram 1992)

potential cardiovascular risk factor and the increased potential for the onset of chronic inflammatory reactions.

9.6 CONTACT PHASE ACTIVATION IN CARDIOPULMONARY BYPASS

The interaction of blood during cardiopulmonary bypass with a variety of materials (pump, oxygenator membranes and tubing) has been shown to activate the contact system (Irvine 1989; Sundaram 1992; Van de Kamp & Van Oeveren 1993; Boisclair et al 1993). Some of the studies have indicated that the activation of the contact phase was a gradual process and that the elevated levels observed postoperatively suggested an ongoing phenomenon (Irvine 1989; Sundaram 1992). The findings in this study suggested a rapid rise in contact phase activation shortly after the infusion of heparin and at the point of heart cannulation. The levels of factor XII activity remained elevated until after bypass was stopped, followed by a gradual decline there after. In the presence of a half-dose aprotinin regime, the levels of factor XIIa/XIIa-inhibitor complexes were found to be markedly higher than the control group of patients, suggesting that low-dose aprotinin enhances factor XII activation. The observation that factor VIIa levels were elevated at the start of bypass followed by a gradual decline indicated a poor negative correlation with the contact system during bypass. In the presence of aprotinin, the levels of factor VIIa during and immediately after bypass, and postoperatively remained consistently at baseline. This suggests that aprotinin inhibits factor VII activation.

9.7 SUMMARY

In an effort to understand the relationship between selected parameters indicative of blood response with structure-property of the membrane, factor XII activity was determined *in vitro* and during clinical extracorporeal applications. The data generated in this thesis have confirmed that factor XII surface adsorption and autoactivation in the purified state was dependent on membrane charge, whilst plasma factor XII-like activity may depend on the other plasma constituents as well as the membrane charge. The study has also demonstrated that the measurement of factor XII activity using the novel immunoassay for FXIIa/FXIIa-inhibitor complexes was a more suitable indicator for *in vitro* and clinical investigations of contact phase activation. Contact phase activation was significantly elevated in a disease state such as uraemia and the levels remained elevated during dialyser

device utilisation. It was also shown that the pattern of factor XII activity changed markedly in the presence of heparin or low molecular weight heparin. In the clinical extracorporeal applications such as haemodialysis; factor XII activity was found to depend on the membrane type and membrane-surface area. In cardiopulmonary bypass, the factor XII activity was shown to change markedly in the presence of aprotinin.

FUTURE WORK

The study has demonstrated that in the evaluation of blood-biomaterial interactions, the establishment of a correlation between the characteristic of the biomaterial and a representative parameter of blood response should be viewed within the influencing factors such as antithrombotic agents, multi-material contact, surface area and mode of device utilisation, blood condition, drug therapy and the nature of the application (Figure 9.2). In view of these findings, future work should attempt to link the role of contact phase activation with clinical onset of dialyser clotting or the development of hypersensitivity reactions. Further work is necessary in the future to establish the effect of flow/shear forces on the activation the contact phase of coagulation. In cardiopulmonary bypass, the relationship between inflammatory mediators such as thrombin, cytokines, growth factors, cell adhesion molecules with contact phase activation requires more studies to be done in the future.

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APPENDIX A

RELEVANT DETAILS OF INDIVIDUAL PATIENTS IN THE HAEMODIALYSIS TRIAL.

Table A.1: Details of patients dialysed with cuprophan (Focus 120).

Patient No	Duration of dialysis (months)	Aetiology	Heparin doasge (IU)	Flow rates (ml/min) D=dialysate B=blood
1	40	secondary amyloidosis	3000	D=500 B=200
2	40	IgA nephropathy	6500	D=800 B=400
3	8	Unknown	3500	D=500 B=200
4	60	Polycystic kidney	3000	D=500 B=200
5	15	Unkown	6500	D=500 B=200
6	34	Hypoplastic kidneys	4000	D=500 B=300
7	90	unknown	8000	D=500 B=200
8	72	Membranous glomerulo-nephritis	12000	D=800 B=200
9	60	IgA nephropathy	3000	D=500 B=300
10	15	Polycystic kidneys	5500	D=500 B=350
11	3	Unknown	8000	D=500 B=250
12	60	Unknown	5000	D=500 B=150

Table A.2: Details of patients dialysed with polysulphone (F8, Fresenius).

Patient No	Duration of dialysis (months)	Aetiology	Heparin dosage (IU)	Flow rates (ml/min) D+dialysate B=blood.
1	15	Polycystic kidneys	5500	D=500 B=300
2	31	IgA nephropathy	7000	D=500 B=250
3	39	Unknown	7000	D=500 B=300
4	27	Hyper-tension	4500	D=500 B=200
5	4	Unknown	7000	D=500 B=230
6	90	Unknown	10000	D=500 B=200

Table A.3: Details of patients dialysed with polysulphone (F40, Fresenius)

Patient No	Duration of dialysis (months)	Aetiology	Heparin dosage (IU)	Flow rates (ml/min) D=dialysate B=blood
1	24	Polycystic kidneys	6000	D=500 B=200
2	60	Hypoplastic kidneys	4000	D=500 B=300
3	12	Hyper-tension	6500	D=500 B=150
4	36	Nephrotic syndrome	6000	D=500 B=250
5	96	IgA nephropathy	6500	D=800 B=400