PLATELET INTERACTIONS AND CONTACT PHASE ACTIVATION ON POLYMERIC CATHETERS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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ABSTRACT

There are many conflicting views about the blood-response to polymeric materials. In order to be satisfied that a material performs appropriately when used as a device in contact with blood it must be evaluated under relevant conditions. Central venous catheters suffer from problems related to thrombosis and embolism since they are implanted for very long periods of time within the vascular system. The aim of this study was to evaluate the most appropriate method for assessing catheter thrombogenicity, establish data for a number of relevant parameters and correlate these findings with various physico-chemical characteristics of the materials. Accordingly, a dynamic model was developed which allowed the assessment of platelet adhesion by measurement of ^{51}Cr-labelled platelets and platelet α-granule and lysosomal secretion by flow cytometry, after labelling with anti-GMP140 and anti-GP53 antibodies, in whole blood after the perfusion of the blood along the tubing at physiologically relevant shear rates (up to 1000 s^{-1}) at 37°C. In addition, contact phase activation was assessed by measuring the time taken for an aliquot of platelet-free plasma to clot after contact with catheter material (partial thromboplastin time or PTT) and the ability of the materials to cause factor XII activation by measuring the quantity of FXIIa-C_1-Inh complexes formed by enzyme-linked immunosorbent assay after the contact of platelet-free plasma with catheter tubing. An attempt was made at finding the identity of the proteins adsorbed onto silicone using a number of electrophoretic techniques. The ability of the materials to cause haemolysis and the cytotoxicity of an extract derived from the materials after 50 days incubation in PBS including the identification of these potential leachables by supercritical fluid extraction was also investigated. In addition, these data were discussed in relation to parameters of surface roughness, as viewed by SEM and the ratio of hard and soft segments appearing at the material surface by XPS.

It was found that significant differences could be detected in (i) platelet adhesion, where Pellethane was shown to have poor performance; (ii) α-granule release, where all the polyurethanes displayed better performance than any of the other materials and (iii) lysosomal granule release where most materials fared similarly, except for glass which was much worse. Silicone was shown to be best in the PTT assay, Pellethane worst. Surprisingly, no correlation was found with these results and those from FXIIa assay, where Desmopan and Davathane were highly active. New and important data on the initial activation kinetics and the ability of materials to activate factor XII are shown. Silicone produced the greatest haemolysis, PVC the greatest extract toxicity. No correlation was found between the physico-chemical data and any of the biocompatibility data.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Whatever the intended function and benefit of invasive medical devices, there is an overriding requirement that they do not harm the patients into whom they will be placed. This is especially important when considering central venous catheters since they are routinely placed into critically ill patients. In addition to having a fragile constitution, such people usually have very weak immune systems. Accordingly, materials which do not adversely affect either the cellular functions of the blood or the immune system should be selected for the construction of catheters. Particularly important mechanisms include platelet secretion and aggregate formation and the development of red cell thrombi. These interactions between blood and materials are crucially important in determining the performance of such devices, but remain relatively poorly understood.

1.2 ASSESSMENT OF BLOOD-MATERIAL INTERACTIONS

A major problem in the selection of materials for use in central venous catheterisation is in the assessment of this response of blood to the surfaces. It is essential that meaningful test procedures are developed and utilised in order to evaluate this response and to gain an insight into the actual processes occurring at a molecular and cellular level. This is important, since only when these mechanisms are understood can an attempt be made at designing biomaterials for such applications which can minimise any undesired effects.

One crucial aspect regarding the assessment of blood-material interactions is the consideration of the parameters to be evaluated. Since there are many ways in which interactions can take place it is necessary to achieve more than just a narrow view of just one part of the reactions occurring. Moreover, it is not only necessary to perform a comprehensive, multiparametric assessment protocol on any one material, but also to achieve consistent observations regarding any one parameter. This study has been concerned with the assessment of parameters previously considered (Rhodes, 1988), but in a much more refined and physiologically relevant way. To this were added extra

protocols considered particularly important in central venous catheters. Factors controlling the initial events of coagulation (contact phase activation) were studied in addition to those factors involved, in a pivotal rôle, in coagulation, viz. platelet adhesion and activation. These were made relevant by considering cellular events in whole blood at physiologically relevant shear rates.

1.3 CENTRAL VENOUS CATHETERISATION

1.3.1 Introduction

Central venous catheterisation (CVC), in routine clinical practice, was developed in the early 1960's (Wilson *et al.*, 1962) when it was used for emergency administration of drugs and the monitoring of venous pressure for attaining optimal blood volume in patients in intensive care. The placement of a delivery system close to the heart allows for the very swift administration of necessary medicines to the tissues. The catheters are long plastic tubes with a fitting to allow attachment to fluid lines (fig 1.1). As time has progressed, so has the range of uses of the technique, including total parenteral nutrition (TPN), temporary venous dialysis and administration of chemotherapy. The range of associated problems has also expanded (section 1.3.3). The history of CVC, however, goes back many years.

1.3.2 History of CVC

The first reported use of CVC was in 1733 by Hales[1], a clergyman. This was used on a horse by inserting a glass pipe into the jugular vein for venous pressure measurement. Bernard[1] also catheterised a horse, a cardiac catheterisation, in 1844. The first recorded CVC in a human was in 1905 by Bleichröder[1], using a uretic catheter. Cannulation of the heart was also achieved from the thigh, confirmed by severe stabbing pains in the chest. Measurement of cardiac output was achieved using cardiac catheterisation by Grollman in 1932[1], also demonstrated by Cournard and Ranges (1941).

The material usually used for catheters in many of these early experiments was natural rubber tubes[1], although Cournard and Ranges used silk. In 1945, the intravenous infusion of fluids was simplified by the development of peripheral vein cannulation as routine practice (Myers, 1945). In this application, polyethylene was used in the construction of the catheters (Myers, 1945; Zimmerman, 1945). Further developments introduced poly(vinyl chloride) (PVC) (Peterson *et al.*, 1949), then nylon in 1950 (Last *et*

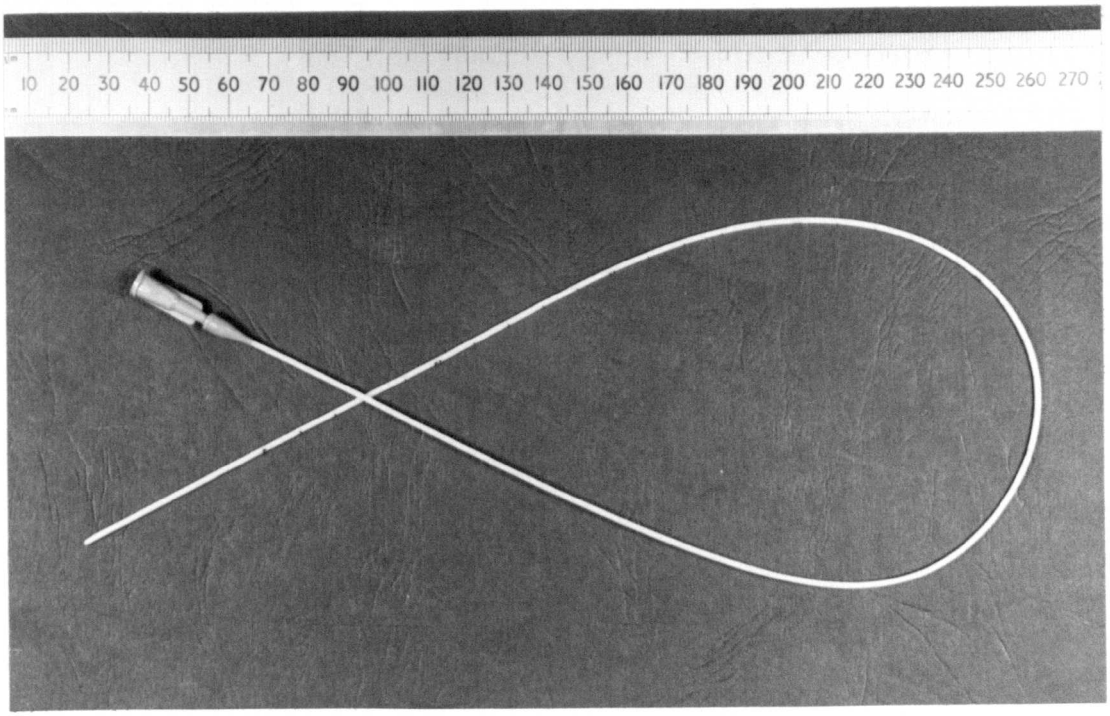

Fig 1.1 Long, hydrophilic, polyurethane central venous catheter

al., 1950). Polytetrafluoroethylene (PTFE) followed in 1960 and silicone was first used for CVC in 1961 (Stewart and Sanislow, 1961). It was not until the 1980's that polyurethanes became an accepted material (Jansen and Brim, 1987).

The need for alternative catheter placement positions was made evident after it was noted that the catheterisation and infusion could cause thrombosis (Page *et al.*, 1952; Richards, 1955; Medical Research Council, 1957). It was considered that the increased blood flow in central veins could reduce the thrombophlebitis noted in peripheral cannulation. The development of alternative access points of the catheter to the central venous system evolved rapidly. Initially, femoral and cubital veins were used but the infraclavicular access to the subclavian vein was described in by Aubaniac in 1952[1]. This was superseded by supraclavicular access and then by internal jugular catheterisation in 1966 (Hermosura *et al.*, 1966). This is favoured due to the reduced risk of puncturing the pleural membrane. The number of variations of this technique has also rapidly multiplied.

1.3.3 Complications of Catheterisation

The most obvious problem encountered in catheterisation is thrombosis. This can take several forms. Thrombophlebitis is a thrombus associated with inflammation of the vessel wall. This can be found at the site of catheter entry or at a point of friction between the catheter surface and the vessel wall. It can be caused by direct contact of a sharp edge of the material breaking the endothelial layer, revealing the basement membrane and causing extrinsic coagulation. Inflammation around the site of catheter entry is generally caused by tissue incompatibility with the material surface. A platelet thrombus (a so-called 'white thrombus', due to the lack of enmeshed red cells) can form, whereby a plug of platelets can form around the tip of the catheter. This is generally indicative of gross platelet activation without any significant degree of coagulation cascade initiation (Section 2.2.3) and can be caused by the reactivity of the surface to platelets or because of significant turbulence. A third type of thrombosis associated with catheterisation is the conventional red cell clot, whereby red cells become enmeshed in the growing strands of crosslinked fibrin.

The presence of a thrombus can lead to several complications. Vessel-associated (mural) thrombi and red cell thrombi can grow to alarming sizes, causing vessel occlusion, with all the obvious problems this entails. In cases where the thrombus is only loosely

[1] These references were cited in a review by Kalso (1985)

attached, large clumps of matter can break off and be carried by the blood to vessels of smaller dimensions, causing occlusion (embolism). These emboli generally give major problems when the final location is in the lungs, brain or any other major organ. When the thrombus has become invaded by bacteria, the resulting infection can become disseminated around the body which can be fatal.

The presence of bacteria themselves is another major danger associated with catheterisation. Since access to the venous system is, by its very nature, percutaneous, this gives rise to the possibility of invasion by microbes. Those usually responsible are normally associated with the skin (*Staphylococcus aureus, S. epidermidis*) since there is an easy route along the catheter length directly into the blood. Infection leading to sepsis can render the insertion site unusable, a critical problem for patients needing long-term, indwelling catheterisation. More significantly, however, is the potential for full-body infection which critically-ill patients are not able to withstand. Infections are usually inhibited by the inclusion of antibiotics in the infusion fluids or the attachment of antimicrobial chemicals to the surface of the catheter. Scrupulous cleanliness is an obvious requirement for catheterisation.

Physical trauma is another problem caused by the presence of a catheter in the blood. In addition to the traumas mentioned above, it has been known for catheters within the right atrium of the heart to cause heart irritability and arrhythmias (Lingenfelter *et al.*, 1978), fatal subacute endocarditis (Sakata *et al.*, 1969) and atrium wall perforation (Ross, 1974), giving rise to infusion fluid tamponade.

The major causes of these problems appear to be catheter material chemistry, mechanical properties, tissue-toxicity and the ability to support microbial growth. Other factors have been cited, however: experience of the catheterisation team, method of catheter insertion and its placement (Blackett *et al.*, 1978), type of drugs infused (Jones and Craig, 1972), patient anaesthesia (Curelaru *et al.*), age and sex (Hästbacka *et al.*, 1966) have all been shown to be factors modulating the response to catheterisation.

1.3.4 Material Selection for CVC

The primary requirement for any catheter is material biocompatibility. The material must have appropriate mechanical performance and be able to withstand sterilisation regimes. There has been much debate about the biocompatibility of blood-contacting materials. Most researchers would say that polyethylene, polypropylene, PVC, PTFE and nylon catheters display a worse performance in contact with blood than do

silicone or polyurethane. Even these two materials have also been criticised. Attempts have been made to improve their performance by attaching heparin or coating with hydrophilic components.

Catheters need to be rigid and slippery enough to be inserted easily into a vein but soft enough to prevent endothelial irritation. They must also be stable enough to withstand the harsh environment of blood. In most cases there is a compromise to be reached. Polyurethanes generally have the best mechanical properties but have been criticised for lack of drug compatibility and biostability (Mignot and Arnaud, 1991). These criteria also rule out PTFE, nylon, polyethylene and polypropylene, mostly on the grounds of their mechanical properties. Environmental considerations include stability to temperature, aqueous solution and local pH differences (acidity, alkalinity). The placement of these materials in a human also requires consideration of the effects of radiation, both electro-magnetic and ionising (*e.g.* X-rays and radioactive isotope emissions from a radiotherapeutic source), and environmental UV light, on their senescence and *in vivo* performance. Standard materials are generally modified to withstand such treatment by the use of stabilisers and plasticisers. It is possible that such additives may leach out with use, hence problems of toxicity and alterations to the material biocompatibility may be observed with time. This is a particular problem for PVC, where up to 40% of the material can be plasticiser. Some companies have developed 'sandwich' catheters to overcome this inherent compromise, using a base which has the necessary mechanical properties (including the need for X-ray opacity) and a soft, outer layer to give good tissue-contact performance and biocompatibility to prevent blood trauma.

1.4 AIMS OF THE THESIS

On the basis of the foregoing, the following aims were selected for thesis study:

(a) Define a set of protocols which could be used to evaluate effectively and reproducibly the blood-response to a selection of central venous catheters.

(b) Correlate these findings with the physico-chemical properties of the surfaces of the materials to assess their relative importance in catheter thrombogenicity.

CHAPTER 2

BLOOD-MATERIAL INTERACTIONS

2.1 INTRODUCTION

The study of blood, its functions and the way in which it interacts with other substances has a long history. Indeed, Boyle, Denys and Lower attempted to transfuse blood from other animals into humans in 1667, but with dire consequences. Lister noted the appearance of deposits on rubber tubing after contact with blood (Lister, 1863) and Carrel attempted to replace parts of the canine vascular system with rubber tubes (Carrel, 1910). Despite this long time scale, however, much is still poorly understood about the mechanisms occurring within blood in contact with other substances, primarily because of the complexity of blood: more than 150 proteins can be separated from plasma using two dimensional gel electrophoresis (Andrade and Hlady, 1987); many blood cell types exist; and blood dynamically interacts with endothelial cells (Ryan, 1987). No single effect can be attributed to any one cause. To understand why these effects occur at all, it is first necessary to see how blood performs under normal circumstances.

2.2 NATURAL FUNCTIONS OF BLOOD

2.2.1 Introduction

Blood has many functions. Primarily it carries oxygen to the tissues in exchange for carbon dioxide, it carries nutrients to allow the tissues to metabolise, it collects waste substances for disposal and carries messages from endocrine glands to remote organs by way of hormonal signalling. It also has complex defence mechanisms and acts as a haemostatic control in addition to these. It is these last two aspects that are of great importance when considering blood's interactions with foreign materials.

2.2.2 *Physiology of blood*

Blood can be regarded as a complex tissue in its own right. There are a number of different cell types of varying size and function (table 2.1).

Cell type	Typical concentration (per ml)	Approx. Diameter (μm)	Function
Erythrocyte	5×10^9	8	Carries oxygen to the tissues
Platelet	250×10^6	2	Haemostatic plug, release chemotactic substances
Lymphocyte	2.8×10^6	8	Produces antibodies
Monocyte	540×10^3	17	Phagocytose bacteria - 2nd line defence
Neutrophil	5.4×10^6	14	Phagocytose bacteria - 1st line defence
Basophil	35×10^3	15	Phagocyte and possible coagulation control
Eosinophil	280×10^3	15	Phagocytose antigen/antibody complexes

Table 2.1 Major blood cell types present in circulating blood

Blood also contains a very large number of completely distinct proteins, about 150 of which have been separated and characterised. It also contains many inorganic ions and lipids of varying sizes and functions. An exhaustive list would be far too large to be included in this description, but these species fall into several main categories (table 2.2):

Blood constituent	Function
Clotting system proteins	System of approximately 25 zymogens, cofactors and serine protease enzymes which take part in blood clotting
Plasma protein inhibitors	Approximately 7 main plasma inhibitors which deactivate the active sites of serine protease enzymes
Fibrinolytic system proteins	System of proteins which cause the dissolution of fibrin networks
Complement system proteins	System of 20 proteins, and several anaphylatoxins, which complex to form bacterial cell membrane lysis sequence
Immunoglobulins	5 subclasses which have specific rôles in the binding of antigens
Transport proteins	Several proteins which bind copper, iron and haemoglobin
Lipoproteins	3 major subclasses of densities which provide lipid for cells
Ions	Many cations and anions which regulate cell function

Table 2.2 Rôles of the major blood constituents

2.2.3 *Thrombosis and haemostasis*

Thrombosis is a necessary event to prevent the egress of blood upon vessel damage. Such thrombosis is brought about as a result of extrinsic coagulation (Section 2.2.3.1), whereby an external membrane- bound tissue factor acts as a receptor for factor VII (FVII), the complexing of which initiates the activation of a series of enzymic reactions known as the coagulation cascade (Fig 2.1).

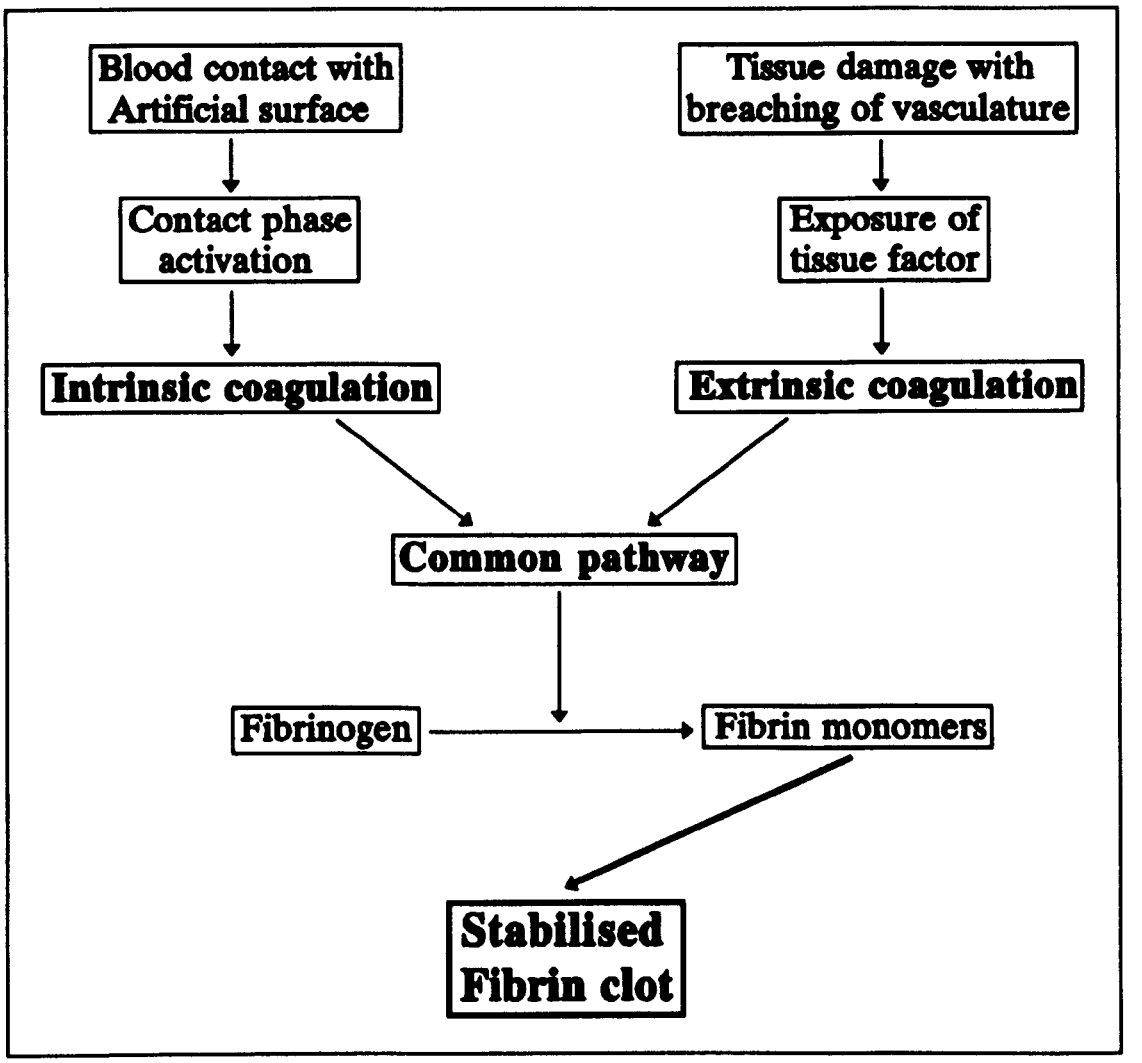

Fig 2.1 Blood coagulation cascade

This sequence, which was proposed independently by Davie and Ratnoff (1964) and Macfarlane (1964) results in the formation of crosslinked fibrin. At a number of stages along the cascade an inactive 'zymogen' is cleaved to form a proteolytic enzyme which then cleaves the following factor. At each step there is amplification (Burgess and Esnouf,

1985) and consequently huge amounts of fibrin can be formed from only a few molecules of activator (Hoffbrand and Petit, 1984). This scheme of events is also true for *intrinsic* coagulation (Section 2.2.3.2) except that an internal plasma protein, Hageman factor (or FXII) is activated in some way (Szycher, 1983). Such activation occurs on the surface of a foreign material when in contact with blood. These pathways are critically controlled in order to localise coagulation. This is achieved by a series of negative and positive feedback mechanisms. There is also a range of plasma inhibitors associated with many components of the system (Mann *et al.*, 1988). Coagulation is also self-limiting by the action of a fibrinolytic mechanism (Kluft *et al.*, 1987) (Section 2.2.3.4).

The first accounts of the study of coagulation, other than mere observation and speculation (Hippocrates; Plato; Aristotle), were produced in the mid-eighteenth century by Hunter[1]. The first discovery of a coagulation factor, however, was in the nineteenth century when Buchanan[1] observed the presence of thrombin in pleuritic fluids. Hammarsten[1] found and purified fibrinogen and Arthus[1] recognized the need for calcium in clotting. Since then the complexity of the reactions involved has gradually been unfurled. Many substances have been 'discovered' several times over and have aquired a number of names. Attempts have been made to classify the factors in a logical manner but some have become obsolete when they have been found identical to other factors (autoprothrombin I_c, I_p, II, II-A, III and C, for example). The most usual nomenclature names the proteins in the general order in which they were discovered (factors I - XIII); some are not even proteins (FIV: calcium ions) and FVI turned out to be activated FV.

Many substances were identified as emanating from platelets and given separate platelet factor (PF) numbers but have now been shown to be identical to plasma substances. Only PF3 and PF4 have retained their original nomenclature, and only PF3 has importance in the propagation of the cascade. However, eight other factors have previously been described (Johnson, 1967): PF1 (platelet Ac-globulin: FV) (Ware *et al.*, 1948); PF2 (platelet fibrinoplastic factor) (Ware *et al.*, 1948); PF5 (clottable factor: fibrinogen) (Salmon and Bounameaux, 1953); PF6 (antifibrinolysin factor) (Johnson and Schneider, 1953); PF7 (cothromboplastin) (Lee *et al.*, 1957); PF8 (antithromboplastin); PF9 (Ac-globulin stability factor); PF10 (vasoconstrictor factor: 5-hydroxy tryptamine (5-HT)). In general terms, coagulation research has tended to be rather confused on this matter. The reactions are so complex that precise rôles are difficult to ascertain for particular coagulant

[1] These references were cited in a review by Owen (1990)

Protein or Factor	Alternative Name	Molecular Weight	Rôle in coagulation
Factor I	Fibrinogen	2 chain: 2 x 170K	Cleaved by thrombin to fibrin monomers (and fibrinopeptides A and B)
Factor Ia	Fibrin	2 chain: 2 x 167K	Stabilised by XIIIa to give cross-linked fibrin
Factor II	Prothrombin	1 chain: 72K	Activated to thrombin by a complex of Xa, Va, phospholipids and Ca^{2+}
Factor IIa	α-Thrombin	2 chain: 31K,6K(bov)	Cleaves fibrinogen to fibrin monomers (and fibrinopeptides A and B). Activates XIII to XIIIa, activates V to Va, activates VIII to VIIIa
Factor III	Tissue factor Tissue thromboplastin	1 chain: 45K	Membrane-bound lipoprotein complex, acts as a receptor for VII and VIIa to allow activation of X to Xa, and some IX to IXa
Factor IV	Calcium ions	-	Allows assembly of molecular complexes
Factor V	Labile factor Proaccelerin Plasma Ac-globulin	1 chain: 330K	Procofactor. Initial cleavage by Xa to Va, subsequent cleavage by thrombin
Factor Va	Factor VI	2 chain: 94K, 74K	Acts as a cofactor with Xa, lipid and Ca^{2+} to make prothrombinase complex which converts prothrombin to thrombin. Inactivated by activated Protein C. Binds platelets with high affinity.
Factor VII	Proconvertin Stable factor SPCA	1 chain: 50K	Activated to α.VIIa by Xa, β.XIIa, IXa or XIa. Slowly activates X in conjunction with tissue factor and Ca^{2+}
Factor VIIa (α.VIIa)	-	2 chain: 28K, 19K	Complexes with Ca^{2+} and tissue factor to activate X. Same complex activates IX to IXa to a smaller degree. Inactivated by Xa
Factor VIII	Antihaemophilic factor A (AHF) Platelet cofactor I	1 chain: 280K	Activated to VIIIa by thrombin. Normally complexed to vWF in plasma
Factor VIIIa	-	2 chain: 90K, 69K	Binds IXa, Ca^{2+} and PF3 as a cofactor to convert X to Xa. Inactivted by activated protein C
von Willebrands factor (vWF)	-	2 chain: 2 x 260K	Circulates in plasma with VIII. Binds platelets, endothelial cells and collagen. Responsible for haemostatic platelet adhesion and aggregation
Factor IX	Christmas factor Antihaemophilic factor B Platelet cofactor II Autoprothrombin II	1 chain: 57K	Activated to IXa by XIa with Ca^{2+}. Can also be activated to a smaller extent by tissue factor with VIIa and Ca^{2+}

Factor IXa	-	2 chain: 48K	Activates X to Xa in conjunction with VIIIa, Ca^{2+} and phospholipids. Can activate VII to α.VIIa
Factor X	Stuart-Prower factor Prothrombokinase Autoprothrombin III	2 chain: 17K, 42K	Activated to Xa by IXa with VIIIa, Ca^{2+} and phospholipids
Factor Xa	Thrombokinase Autoprothrombin C	2 chain: 17K, 31K	Activates V to Va. Activates II to IIa in conjunction with Va, Ca^{2+} and phospholipids. Activates VII to α.VIIa. Inactivates α.VIIa.
Factor XI	Plasma Thromboplastin Antecedent (PTA)	2 chain: 2 x 80K	Activated to XIa by α.XIIa when complexed to HMWK. Circulates in plasma loosely associated with HMWK
Factor XIa	-	2 chain: 2 x 80K	Activates IX to IXa. Can activate VII to α.VIIa
Factor XII	Hageman factor	1 chain: 80K	Activated to α.XIIa by kallikrein, activated to β.XIIa by kallikrein, activated to α.XIIa by α.XIIa. Requires to be bound to activating surface for cleavage
α-Factor XIIa (α.XIIa)	-	2 chain: 52K, 28K	Has coagulant activity. Activates surface bound-XII to α.XIIa, surface bound-XI (complexed to HMWK) to XIa and prekallikrein to kallikrein. Activates, to a small degree, plasminogen to plasmin and VII to α.VIIa
β-Factor XIIa (β.XIIa)	Hageman factor fragment	2 chain: 28K, 1K	Has no coagulant activity or surface binding domains but contains enzymatic active site. Activates Prekallikrein to Kallikrein. Activates VII to α.VIIa
Prekallikrein	Fletcher factor	1 chain: 82K	Activated to kallikrein when complexed to HMWK by α.XIIa or β.XIIa. Circulates in plasma loosely associated with HMWK
Kallikrein	-	2 chain: 55K, 35K	Converts XII to α.XIIa using HMWK as a cofactor
High Molecular Weight Kininogen (HMWK)	Fitzgerald factor Flaujeac factor Williams factor	1 chain: 110K	Nonenzymatic cofactor for Kallikrein in XII activation. Cleaved by kallikrein to produce bradykinin. Nonenzymatic cofactor for α.XIIa in XI activation. Protects XIa from protease inhibition
Bradykinin	-	1 chain: 1K	Nonapeptide released from HMWK by kallikrein. Vasoconstrictor. Induces endothelial cells to produces tPA
Factor XIII	Fibrin stabilizing factor	4 chain: 4 x 80K	Activated to XIIIa by thrombin
Factor XIIIa	Transglutaminase	1 chain: 76K	Cross-links fibrin monomers to stabilise fibrin clot
Protein C	-	2 chain: 22K, 44K	Cleaved by thrombin/thrombomodulin complex to give activated protein C
Activated Protein C	-	2 chain: 22K, 42K	Cleaves Va, dissociating prothrombinase complex. Inactivates VIIIa
Protein S	-	69K	Cofactor for activated protein C in prothrombinase dissociation

Thrombomodulin	-	74K	Endothelial cell bound enzyme. Binds thrombin to activate Protein C
Plasminogen	-	1 chain: 94K	Activated to plasmin by tPA, urokinase, kallikrein, XIIa or XIa
Plasmin	-	70K	Cleaves V. Cleaves fibrin into fibrin degradation products
C_1-esterase inhibitor	-	1 chain: 105K	Major inhibitor of coagulation enzymes
α_2-macroglobulin	-	4 chain: 4 x 181K	Inhibitor of coagulation enzymes
α_2-antiplasmin	-	1 chain: 65K	Inactivates plasmin. Inhibitor of coagulation enzymes
α_1-antiproteinase inhibitor	-	1 chain: 54K	Inhibitor of coagulation enzymes
Antithrombin III	Heparin cofactor	1 chain: 58K	Cofactor for heparin to inhibit free Xa and thrombin
Heparin cofactor II	-	65K	Cofactor for heparin
Protein C inhibitor	-	57K	Inactivates protein C
Heparin	-	-	Binds Antithrombin III to inhibit free Xa and thrombin
PF3	Partial tissue thromboplastin	-	Platelet lipoproteins which bind IXa, VIIIa and Ca^{2+} to activate X to Xa; Va and Ca^{2+} to activate prothrombin to thrombin
PF4	Antiheparin factor	8K	Platelet glycoprotein which binds and deactivates heparin
Tissue type plasminogen activator (tPA)	-	-	Continuously released in small quantities by endothelial cells. Release increased by bradykinin
Prourokinase	-	54K	Activated to urokinase
Urokinase	-	32K	Activates plasminogen to plasmin
Kininase I	Arginine carboxy peptidase T	-	Cleaves and inactivates bradykinin
Kininase II	Angiotensin I converting enzyme	-	Endothelial-bound enzyme. Cleaves and inactivates bradykinin

Table 2.3 Physical properties and rôles of coagulation and related proteins and other factors

species. Reactions that are known to occur *in vitro* may not happen *in vivo*, or have any physiological significance. Many experiments have been performed using bovine plasma proteins, despite large differences being known to exist between human and bovine systems. Release of bradykinin (BK) from high molecular weight kininogen (HMWK), for example, does not result in a loss of coagulant activity in humans (Burgess and Esnouf, 1985) since the histidine-rich negative surface-binding domain is retained. Bovine BK *does* contain this histidine-rich domain and so HMWK cleavage of this type is a limiting process. The reactions outlined below are a summary of hundreds of years work and even now are not completely understood. Descriptions of the factors involved are given in table 2.3.

A common theme running through the blood coagulation process is that each active clotting factor (with the exception of FV, FVIII or HMWK) is a serine protease formed by the cleavage of an Arg-X bond, where X is a neutral amino acid, and containing the same active site sequence (Gly-Asp-Ser-Gly-Gly-Pro) (Burgess and Esnouf, 1985). The exception is fibrin-stabilising factor (FXIII) which is a transamidating enzyme with cysteine as its active centre. (Lorand, 1986). Another recurring theme is the need for surfaces on which the reactions take place. Assembly of the prothrombinase and Xase complexes occurs in this way, usually on platelets, monocytes and endothelial cells, which also provide membrane phospholipids. FXII and FIX activation also necessitate a surface.

2.2.3.1 Extrinsic coagulation

The extrinsic coagulation pathway is of vital importance to the integrity of the vascular system. Tiny lacerations of the capillaries and other small venules occur extremely frequently (Burgess and Esnouf, 1985) and if these were not sealed the circulation would very quickly collapse. The events leading to the beginning of the common pathway are shown in fig 2.2.

Tissue factor, an enzyme found only in cell membranes external to the vascular system, is an absolute necessity for extrinsic FX activation (Mann *et al.*, 1988). This is made available to FVII during bleeding. FVII activation occurs as a positive feedback mechanism, with the activation of FX after initial cascade activation. FVII can activate FX without prior activation, although this accelerates the process. FVII can also be activated by lipid crystals which are formed in the blood at low temperatures, a process known as cold activation (Laake *et al.*, 1974).

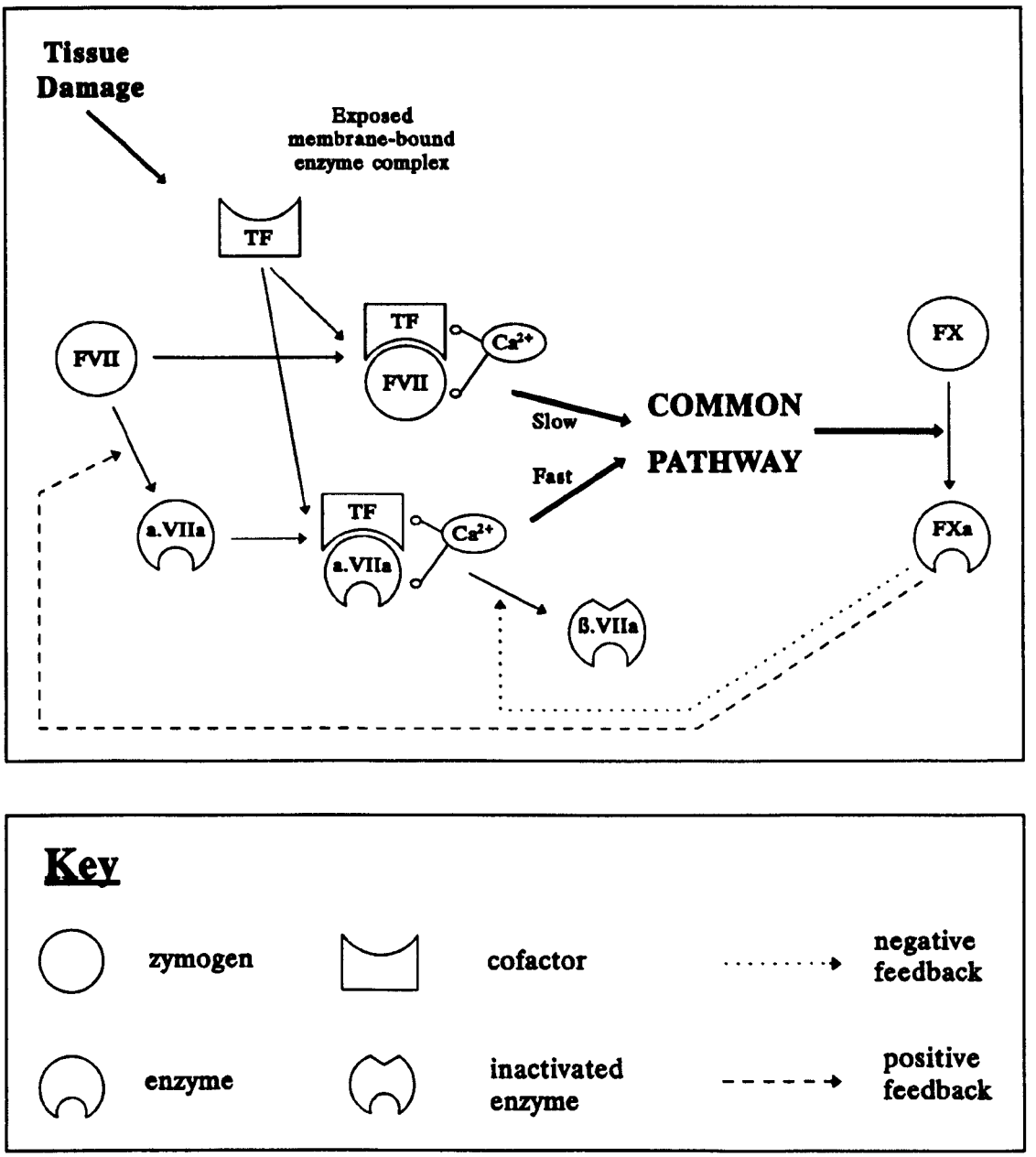

Fig 2.2 Extrinsic coagulation above the common pathway

2.2.3.2 Intrinsic coagulation

Intrinsic coagulation is characterised by its initiation on highly negative surfaces (Kozin and Cochrane, 1988; Kaplan and Silverberg, 1987; Ratnoff, 1985). Amongst these are glass, kaolin (Margolis, 1958), sulphatides (Fujikawa *et al.*, 1980) and dextran sulphate (Tankersley and Finlayson, 1984). The physiological significance of this type of activation is not entirely clear (Saito, 1987), but there is a detectable increase in FXIIa during pregnancy and during and leading up to ischaemic heart disease. It has been implicated

in many other clinical conditions: rheumatoid arthritis (Melmon *et al.*, 1967) and disseminated intravascular coagulation (Mason and Colman, 1971), for example. This pathway is made up of two parts, the initiation, or contact phase activation and the events leading to the common pathway (FX activation).

The processes described are somewhat controversial (Ratnoff, 1985; Tans and Rosing, 1987). There are many conflicting hypotheses and evidence regarding the exact mechanisms surrounding the activation of FXII. This is similar to the confusion in the 1960's concerning the generation of thrombin (Seegers, 1967). Most of the contact factors appear to undergo proteolysis by the action of virtually any of the other contact factor enzymes. The fact that most of these factors are labile makes isolation of non-active forms for research rather difficult. The significance of any reaction is, therefore, difficult to determine.

The initial activation of FXII has, for a long time, been a mystery. One of the pervading theories of contact activation is that of FXII autoactivation (Wiggins and Cochrane, 1979), where adsorption onto a suitable surface was sufficient to cause a change into an active species. Although the term is still used, this concept has evolved into meaning the *self*-activation of FXII by its active form (Kaplan and Silverberg, 1987). It has been found, however, that trace quantities of FXIIa, in the region of 1 ng/ml, are present in normal plasma in a form protected from removal by plasma proteinase inhibitors (Esnouf, 1991). Such amounts would allow cleavage of conformationally susceptible FXII to give FXIIa on the artificial surface. This results in activation of FXI complexed with HMWK at a proximal site and coagulation propagation from then on. The exact relationships and order of reaction between FXII, FXI, prekallikrein (PK) and HMWK are uncertain, but the activation of PK to kallikrein by XIIa appears to be the most important positive feedback loop and cleavage of α.FXIIa to β.FXIIa by kallikrein the major negative feedback loop.

The cleavage of FXII to α.FXIIa leads to the generation of a molecule which possesses an active site at the end of a chain connected to the surface-binding region by a disulphide bridge. This allows activation of FXI at a site not directly in apposition to FXIIa. It is a necessity, however, for FXI to be close enough for the arm of α.FXIIa to reach. Assembly of the contact phase proteins onto the surface would appear to be critical in order for intrinsic coagulation to take place.

The remaining part of the pathway is less controversial. The accepted scheme of events is shown in fig 2.3.

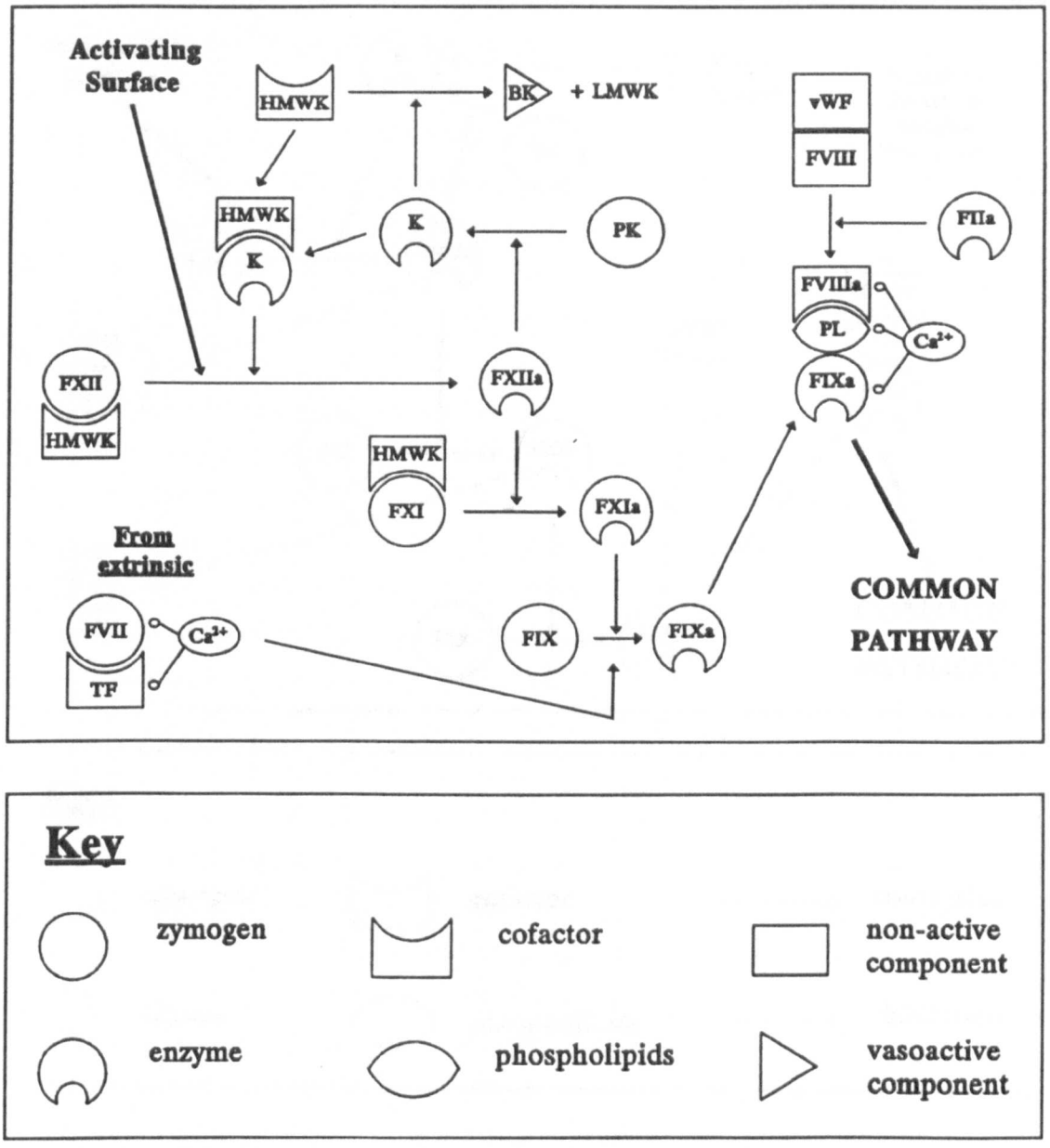

Fig 2.3 Intrinsic coagulation above the common pathway

The inhibitory aspects of this pathway are shown in fig 2.4, whereby plasma proteases are responsible for the negative regulation of the sequence.

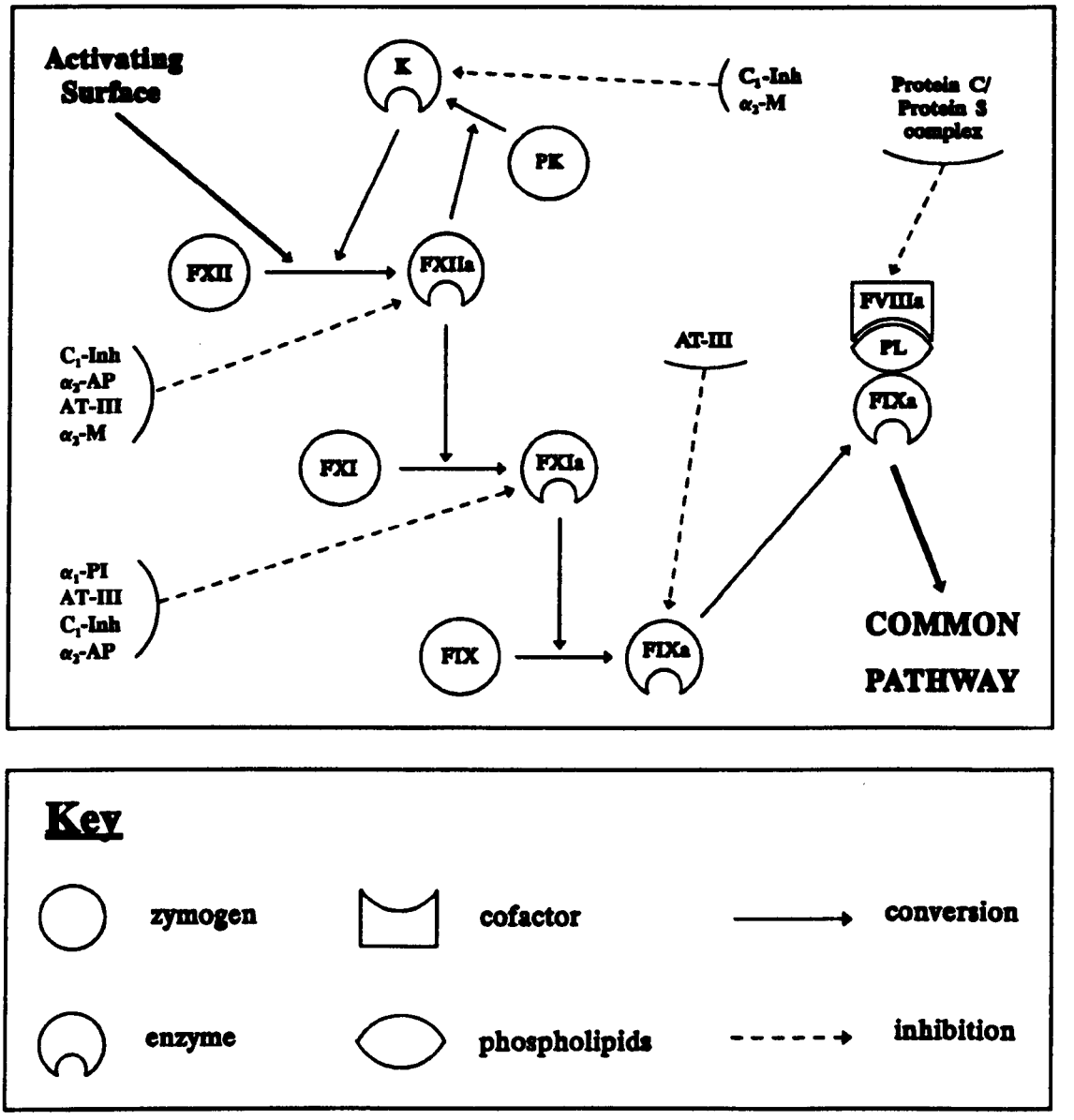

Fig 2.4 Inhibition in intrinsic coagulation by plasma protease inhibitors

2.2.3.3 The common pathway of coagulation

After coagulation pathway initiation, there is a shared pathway of activation (fig 2.5). This results in the formation of a stable, cross-linked fibrin-clot, which can be digested when the fibrinolytic pathway (section 2.2.3.4) has been activated. The appearance and porosity of the clot are dependent upon the rate of thrombin generation. This determines the speed at which fibrin strands are pieced together and hence the time available for digestion of the fibrinopeptides. Slow thrombin generation leads to a dense, small pore network.

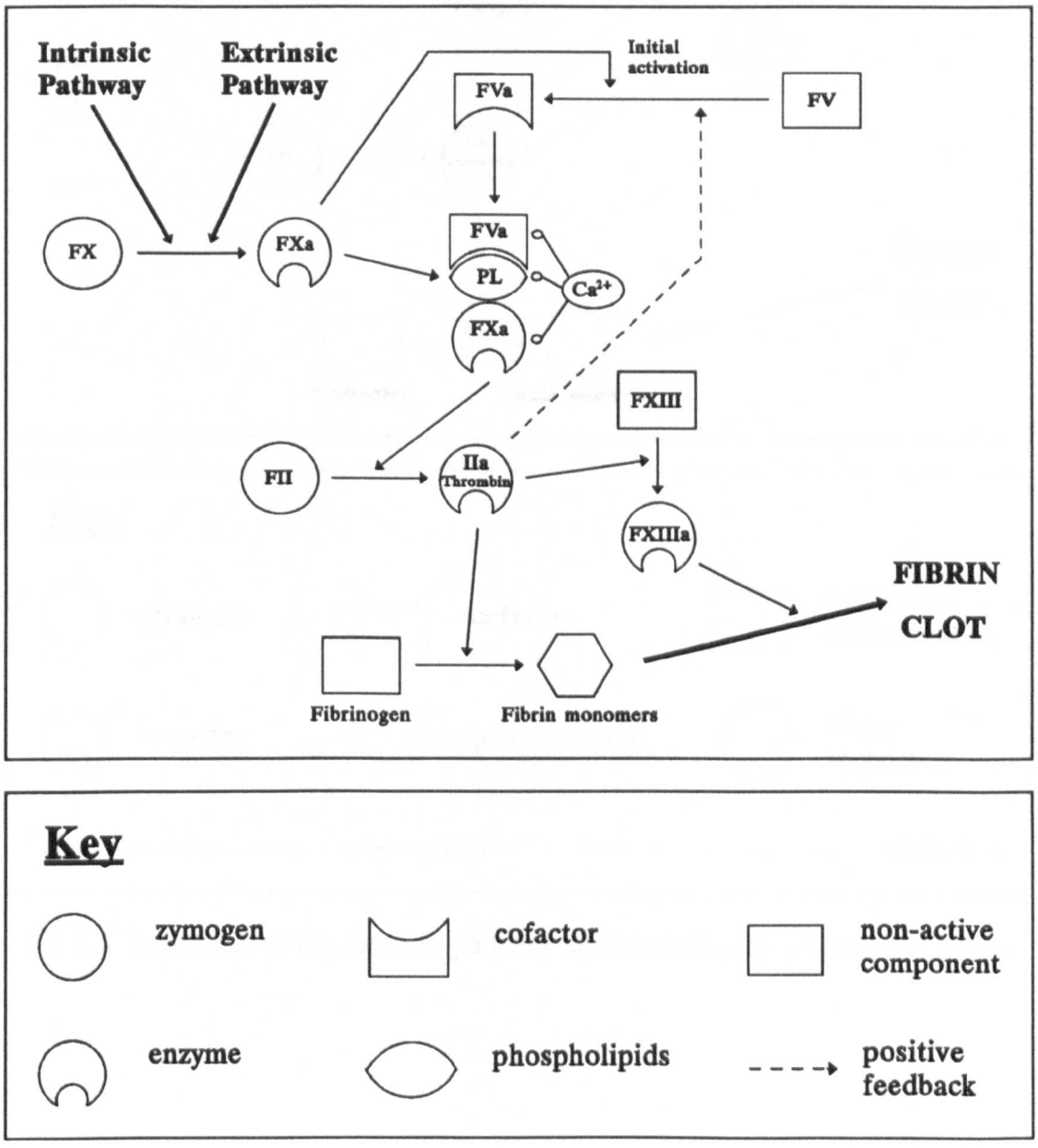

Fig 2.5 The common pathway of blood coagulation

The inhibition of the common pathway by plasma protease inhibitors is shown in fig 2.6.

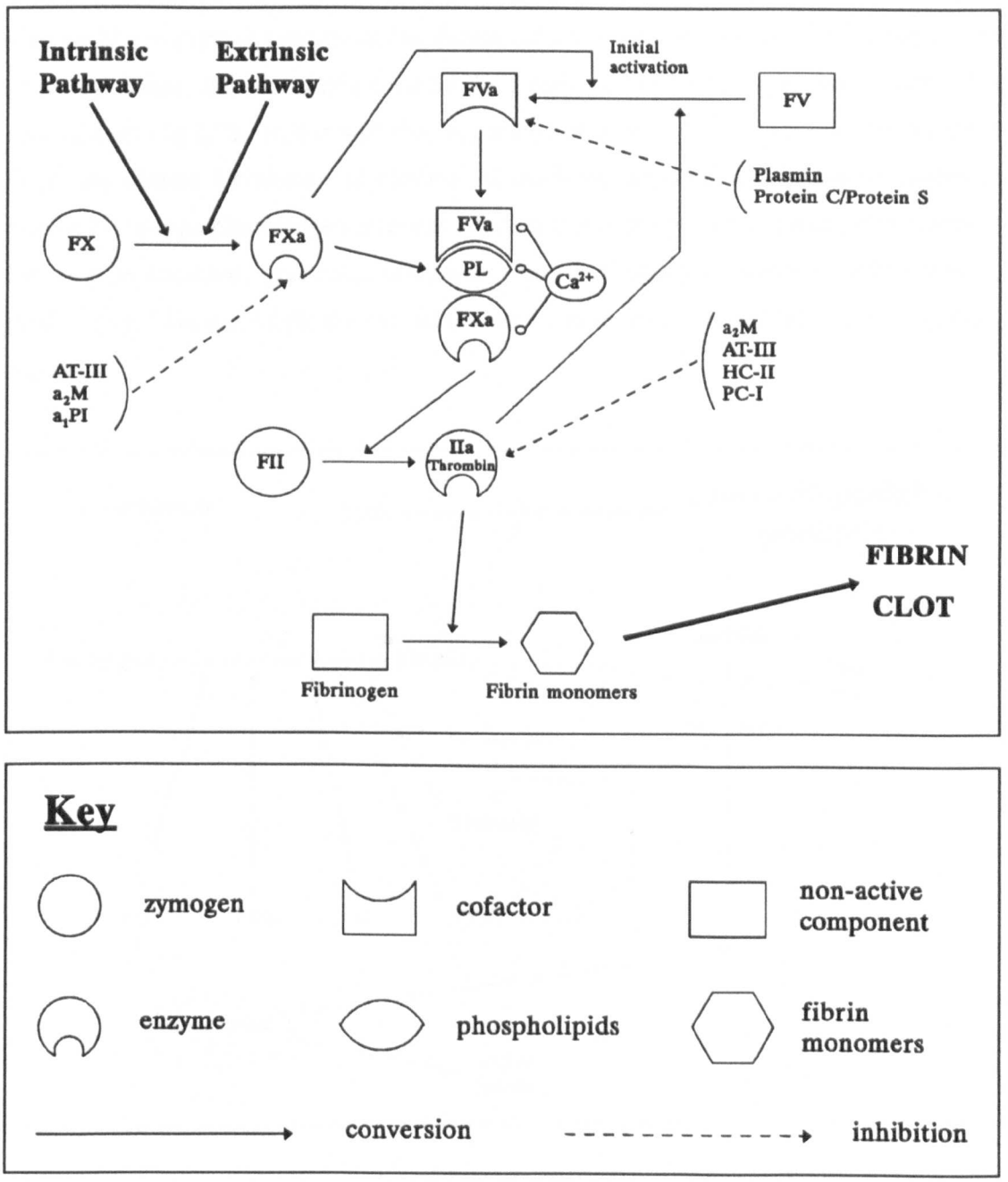

Fig 2.6 Inhibition of the common pathway of coagulation by protease inhibitors

2.2.3.4 Fibrinolysis

Fibrinolysis occurs as a natural antagonist to clot formation. During bleeding, networks of cross-linked fibrin are formed to plug the egress of blood. This is controlled very strictly to prevent mass thrombus formation and vessel occlusion. During the course of wound repair, the clot is dissolved by three main, distinct pathways, one extrinsic and two intrinsic (fig 2.7). In line with the coagulation pathways, this is controlled by feedback loops and plasma inhibitors. In extrinsic fibrinolysis, endothelial cells are stimulated to release tissue-type plasminogen activator which acts directly on plasminogen. Prourokinase can also be activated, producing urokinase which activates plasminogen. Alternatively, factors XIIa, XIa and kallikrein can all interact directly with plasminogen, but to a minor degree.

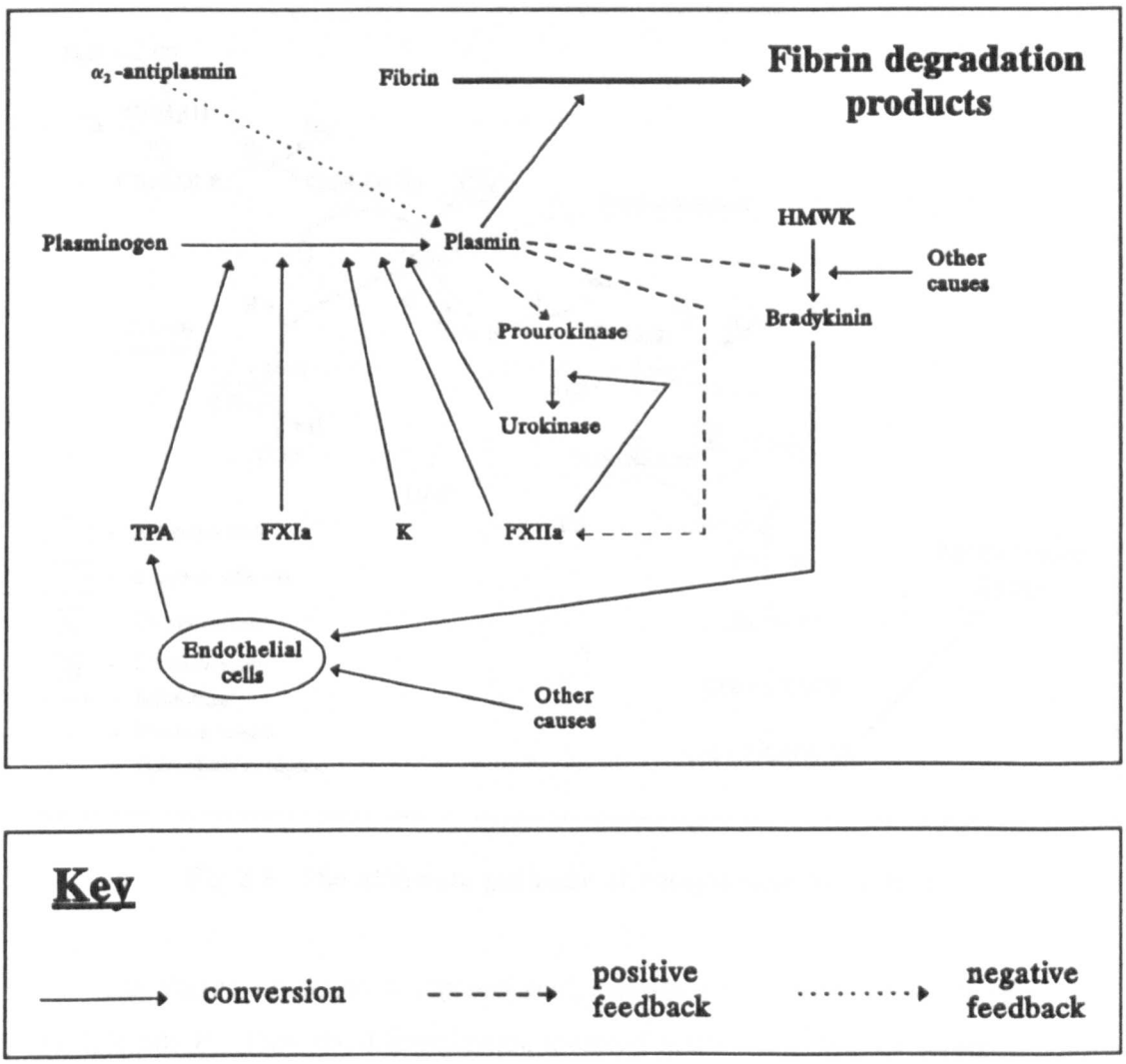

Fig 2.7 Fibrinolytic pathway

2.2.4 Non-cellular immune defence

The humoral arm of the body's immune defence mechanism comprises the complement system of plasma proteins. This acts in concert with the cellular system, whereby lymphocytes generate antibodies specific for non-self epitopes. The complement system is made up of 20 plasma proteins which combine to form lytic complexes in a manner similar to the coagulation cascade, in that the active enzymes are serine proteases, initiation of the cascade is controlled by a protease inhibitor and there is evidence of similar negative and positive feedback mechanisms. The system is also divided into two main pathways: the alternate (fig 2.8) and classical (fig 2.9) mechanisms, which share a common membrane attack enzyme assembly. The constituent proteins are shown in table 2.4. The sequence of activation is highly controlled, there being a fine balance in the recognition of foreign particles.

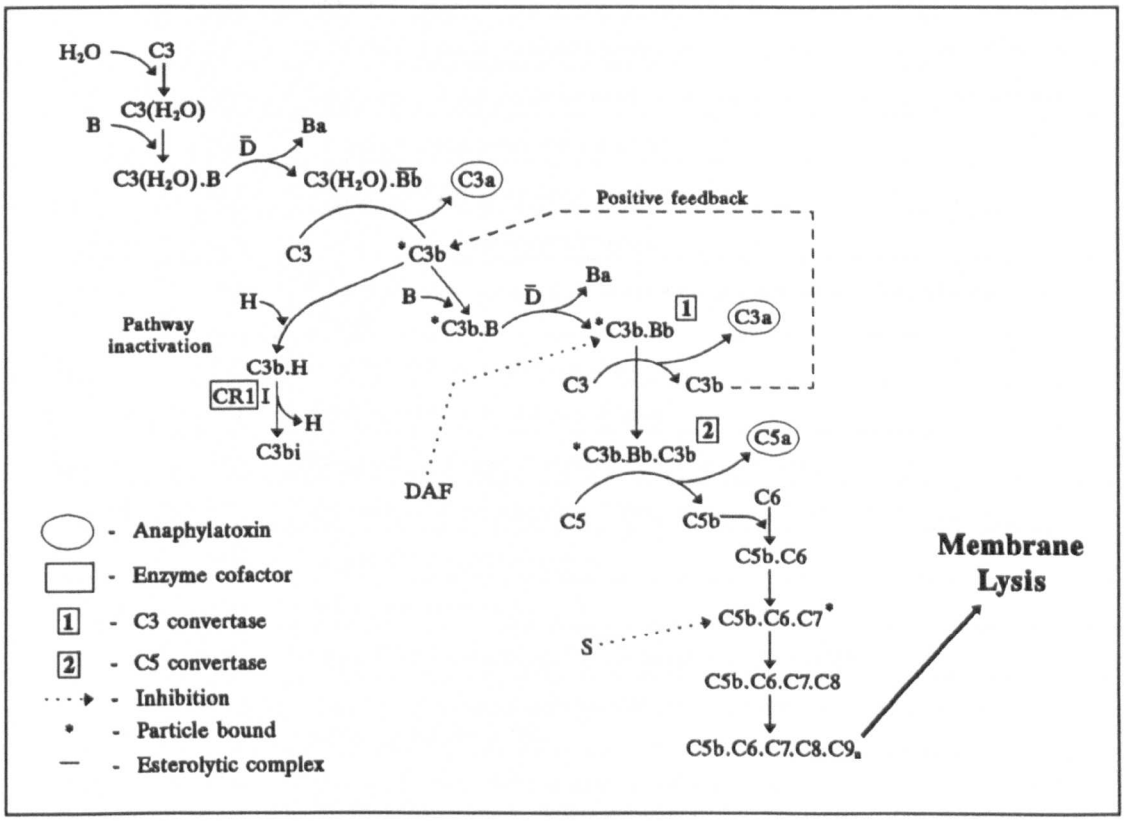

Fig 2.8 The alternate pathway of complement activation

The alternate pathway is initiated and potentiated by just six plasma proteins (C3, B, D, I, H and P). How the discrimination involved in the recognition of foreign particles takes place is not known (Müller-Eberhard, 1988). C3b is constantly formed due to the spontaneous hydrolysis of C3 (Pangburn *et al.*, 1981) and is deposited onto suspended

Protein	Mr	Rôle in complement cascade
C1	750K	Binds antigen-antibody complexes for classical pathway initiation
C2	102K	Precursor of esterolytic component of C3 and C5 convertases in classical pathway
C2a	70K	Esterolytic component of C3 and C5 convertases in classical pathway
C2b	34K	Release product after C2 activation by activated C1
C3	188K	Central protein in alternate pathway
C3a	9K	Anaphylatoxin release product of C3 activation
C3b	179K	Main potentiator of alternate pathway recognition: binds foreign particles, component of C3 and C5 convertases in alternate pathway and C5 convertase in classical pathway
C3bi	176K	Inactivated C3b, caused by cleavage by H using I as a cofactor
C4	200K	Precursor of component of C3 and C5 convertases in classical pathway
C4a	9K	Anaphylatoxin release product of C4 activation by C1
C4b	191K	Non-esterolytic component of C3 convertase in classical pathway
C5	191K	Precursor of C5b
C5a	11K	Strong anaphylatoxin, released after the action of C5 convertase on C5 in both pathways
C5b	180K	Initial component of membrane attack complex and resultant species of both pathways
C6	120K	Component of membrane attack complex
C7	110K	Component of membrane attack complex
C8	151K	Component of membrane attack complex
C9	71K	Component of membrane attack complex which polymerises to form transmembrane channel
Factor B	90K	Precursor of Bb in alternate pathway
Factor Ba	30K	Release product of the action of D on B
Factor Bb	60K	Component of C3 and C5 convertases in alternate pathway
Factor D	24K	Cleaves factor B for expression of esterolytic activity in alternate pathway
Factor H	160K	Inhibits C3b using I as a cofactor
Factor I	88K	Cofactor for H and C4bp
Factor P	53K	Properdin: stabilises C3 and C5 convertases in alternate pathway
CR1	160-250K	Cofactor for cleavage of C3b and C4b by I, cell surface receptor for transporting immune complexes to liver
DAF	70K	Decay accelerating factor: inhibits C3 convertase formation in classical pathway, and promotes dissociation of formed complex
C4bp	570K	C4 binding protein: cofactor for C4b cleavage by I, accelerates C3 convertase dissociation and inhibits its formation using I as a cofactor in classical pathway
HRF	-	Homologous restriction factor: regulates membrane attack complex assembly
Protein S	80K	Primary membrane attack complex inhibitor by binding to C5b.C6.C7
C_1-inh	104K	C_1-esterase inhibitor: inactivates activated C1

Table 2.4 Rôle of complement proteins in complement activation and potentiation

particles. If the particle is activating, C3b is bound by B, causing pathway potentiation. Otherwise it binds H which shuts down the activation process. The specificity of activation of the pathway is probably due to the multiplicity of molecule-binding sites on C3, 10 of which have already been characterised. The alternate pathway is activated by certain carbohydrates, antibody complexes and non-human cells. Rabbit red blood cells, for example, are lysed when put into a mixture of complement proteins only. An unusual feature of the alternate complement pathway is that protein D has no plasma inhibitor. It is always in an active form within blood and comes into action when pathway initiation has taken place. There is a positive feedback loop in the pathway, necessary for amplification, when one considers that C3b formed by the secondary C3 convertase can be deposited onto foreign particles instead of in the assembly of the C5 convertase.

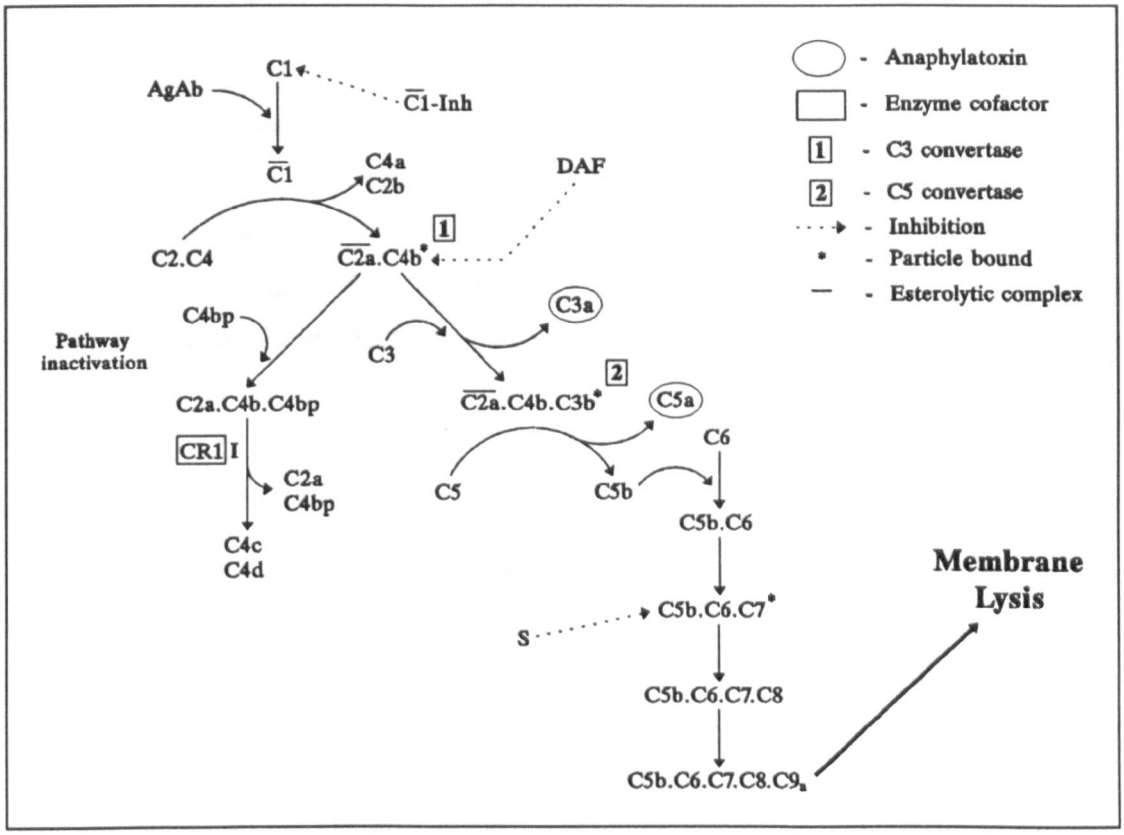

Fig 2.9 The classical pathway of complement activation

The classical pathway is responsible for the specific antibody response. It is initiated when complexes of antigen and antibodies bind to the C1q component of C1. C1 is a quintomeric molecule with a structure of $C1q.C1r_2.C1s_2$. Binding of the complex causes enzymatic cleavage of the C1r and C1s chains allowing the assembly of the classical C3 convertase. Very strict control of this pathway is needed to safeguard the body's cells

from lysis. This is achieved by a gamut of fluid phase and cell-surface proteins. DAF (decay-accelerating factor) causes dissociation of C3 convertase and inhibits its formation. A complex of C4-binding protein (C4bp) and I or CR1 and I cleaves C4b and so disrupts C3 convertase function. There is no positive feedback mechanism operating in the classical pathway.

After C5b generation, a complex is assembled, the first three components (C5b.C6.C7) being loosely associated with membrane-bound C3b, then C8 and C9 added after the fixation of the complex onto the membrane of the target cell. Up to 12 molecules of C9 can be added, generating a tubular structure, which is inserted into the cell membrane causing a transmembrane channel of approximately 10 nm, allowing cell lysis. If during the assembly of C5b.C6.C7 factor S binds, then the connection with C3b, and so the fixation of the complex onto the surface of a cell, is lost and the $C9_{12}$ channel structure is not formed. HRF (homologous restriction factor) also restricts the building of a membrane attack sequence.

An alternative mechanism which is C1-dependent, C2/C4-dependent and alternate pathway protein-dependent occurs in certain circumstances (Deguchi *et al.*, 1987). The mechanisms occurring in this situation have not been elucidated as yet, but it seems apparent that other interactions are necessary for the destruction of cells from certain organisms.

2.2.5 Platelet function

Platelets are extremely labile cells. Their physiological function is to react quickly with components of the blood vessel wall after trauma. The effect of such a reaction is three-fold. A physical barrier to blood is very quickly formed allowing the continuing functionality of the vessels. Secondly, a surface is created which allows the formation of enzyme complexes of the coagulation cascade. This then allows the formation of a fibrin network and the entrapment of other cells which further prevent blood loss. The third function is the secretion of bioactive components which are thought to attract white blood cells chemotactically, allowing the initiation of an immune response. In respect of this last function, platelets are known to express a marker upon activation (GMP-140) for which neutrophils have a receptor (Hamburger and McEver, 1990) allowing neutrophil/platelet cohesion.

Platelets are small, discoid cells and have no protein synthesising apparatus. They are derived from megakaryocytes with proteins already *in situ*. Once they have performed

their secretory rôle, much of the volume of the cell disappears and the remaining membrane is resorbed. The structure of platelets allow various responses to a diverse number of substances. They have a cell membrane with a large number of transmembrane glycoproteins which are expressed on the platelet surface (fig 2.10).

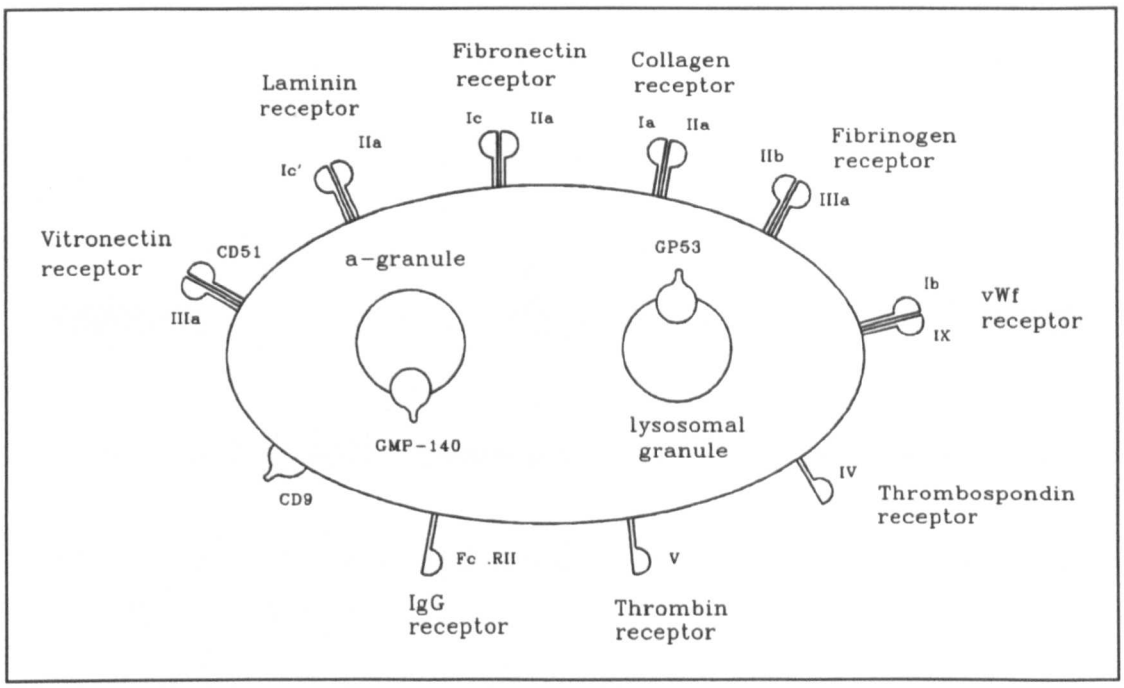

Fig 2.10 Glycoprotein receptors on the unactivated platelet

These have receptor function and allow for the adhesion of the cell to various substrates, aggregate formation and adhesion to other cells. Processes which cause platelets to become activated allow for the upregulation and rearrangement of these glycoprotein receptors to allow further interactions to occur. The types of interactions which occur during normal thrombosis and haemostasis are shown in fig 2.11.

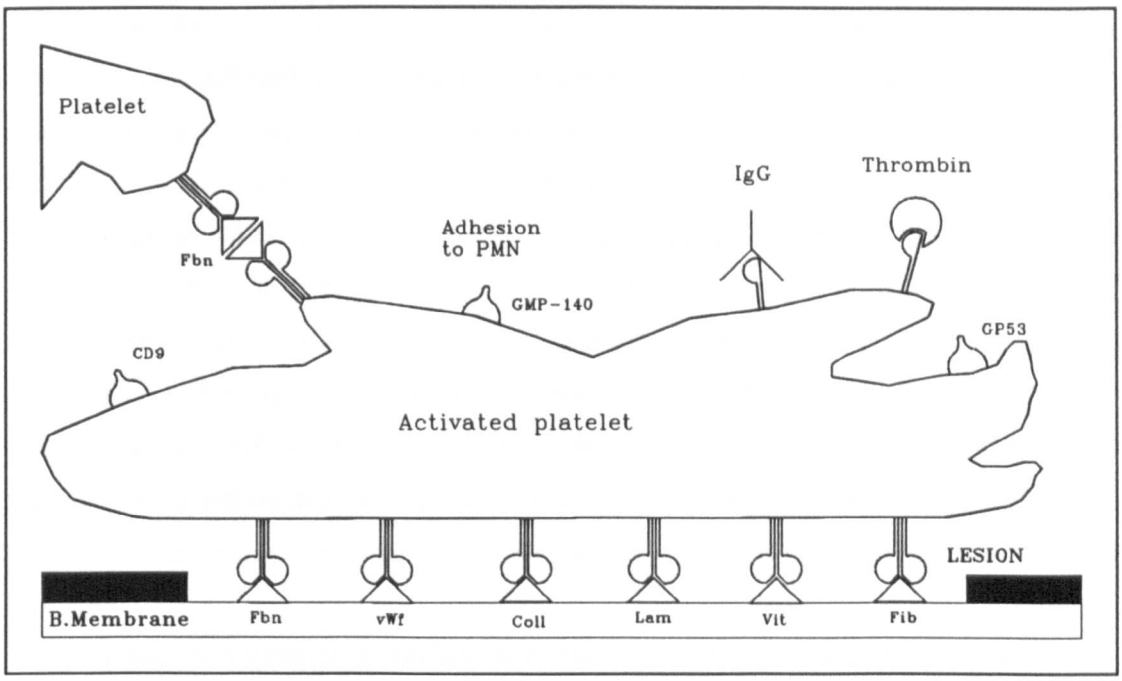

Fig 2.11 Interaction of glycoprotein receptors in the activated platelet

Platelets have at least four types of granule (α-, dense, lysosomal and peroxisomal) which contain many substances (table 2.5).

α-granule	dense granule	lysosomal	peroxisomal
PF4 β-thromboglobulin Platelet-derived growth factor Thrombospondin Fibronectin FVIII FV Fibrinogen Bacteriocidal factor Chemotactic factor Permeability factor K^+ Albumin α_2-Macroglobulin α_1-Antitrypsin HMWK Collagenase Elastase	5-HT (serotonin) ADP/ATP Ca^{2+} Antiplasmin Pyrophosphate	Acid hydrolases Cathepsins D, E	Catalase

Table 2.5 Contents of the four platelet granules

The internal responses of the platelet are similar to that of other cell types. The activation of a cell-surface receptor by an agonist causes an internal messenger signalling sequence to operate via a G-protein. Strong agonists cause the activation of phospholipase C (PLC), which in turn produces inositol triphosphate (IP_3) and diacylglycerol (DG). IP_3 induces an increase in cytoplasmic Ca^{2+} concentration whereas DG causes the phosphorylation of a protein kinase. Together they cause the contraction of actin and myosin fibrils and hence cell spreading, granule release and arachidonic acid (AA) metabolism. Other agonists are linked to other types of G-protein which either inhibit or activate cyclic AMP (cAMP) production (via adenylate cyclase), causing a change in internal Ca^{2+} concentration which activates or inhibits platelet function respectively. The type of secretion and its extent are controlled by the number and type of receptors activated.

Upon the exposure of collagen from the basement membrane of vessels which are damaged, platelets readily adhere via a specific collagen receptor. Activation of the cell occurs, causing the spreading of the cell, expression of coagulant lipid on its membrane and release, or the appearance on the membrane, of fibrinogen, FV and FVIII from internal platelet pools. This allows the building of coagulant complexes on its surface and so the propagation of the coagulation cascade. The release of adenosine diphosphate (ADP), thromboxane A_2 (TxA_2) and other highly activating substances causes the fluid activation and requisition of other platelets to the site of injury. This ensures a rapid clumping of cells and a physical barrier to the escape of blood to the vessel exterior. The surface also has a rapidly forming clot of cross-linked fibrin on it which enmeshes red blood cells to form a more permanent barrier. The prior release of platelet-derived growth factor and other chemotactic agents from the platelets ensures that neutrophils and other phagocytes are attracted to the site to challenge any emerging infection at the site of injury.

The large number of different responses which can be generated is very finely coordinated such that platelet adhesion and aggregation is confined to the site of injury and very few fluid phase, activated platelets are present in the circulation.

2.2.6 Other cellular activity

There are many other cells which are present either in the blood or at an interface adjoining the blood. Of great importance in the maintenance of blood fluidity are endothelial cells. The importance of these is often misunderstood, but platelets are constantly modulated in their function by the production of platelet-sensitive substances

(prostaglandin I_2 (PGI_2), for example) by the endothelium. It is thought that the inhibition of certain activated coagulation factors is also controlled by the endothelium. While the principal function of red blood cells is to transport O_2 and CO_2, they also form the substance of the physically robust fibrin clot.

Other cellular activity mostly involves white blood cells. The five types of cell are shown in table 2.1 and have distinct rôles. As inflammatory cells, they use the circulation for locomotion to the site of infection in response to chemotactic attraction and from there migrate into the tissue. From this point, these cells (neutrophils, monocytes and eosinophils) can phagocytose the bacterial elements and digest the resultant particles by the release of enzymes into the phagosome. Invading organisms which are too large to be destroyed in this way can be degraded by the release of highly toxic oxygen radicals and the acidic contents of the inflammatory cell granules into the external milieu. The release of lysozyme inhibits chemotaxis and possibly extends the residence time of these cells at the site of infection (Gordon *et al.* 1979). Neutrophils also recognize bacteria and other foreign particles by the interaction of C3b deposited on the foreign surface and C3b receptors on the granulocyte membrane.

Basophils release histamine in response to antigen binding or the engulfment of antibody/antigen complexes. This has the effect of dilating the vascular system and is chemotactic for neutrophils and macrophages. Neutrophils release kininogenase which cleaves HMWK to produce BK. This contracts gastrointestinal smooth muscle, is a vasodilator and potentiates pain. Neutrophils also release enzymes which cleave bradykinin, so possess both positive and negative feedback mechanisms. PGE_1 can also be released. This, too is a vasodilator, but is relatively short-lived in an aqueous environment.

There are two types of lymphocyte, B-cells and T-cells. Both are a population of clones (of up to 10^8 different specificities), each specific for a particular epitope. B and T-cells work in different ways, however. The B-cell mechanism, part of the humoral response, works via the production of antibodies. When a foreign antigen is exposed to a B-cell, after several days it differentiates into either a memory cell or plasma cell. Antigen recognition is macrophage dependent. The plasma cell, with the co-operation of T-helper cells, then produces antibody molecules specific for the antigenic determinant. This production can be attenuated by T-suppressor cells in certain circumstances (*e.g.* recognition of native proteins). The memory cells live on until a future encounter, at

which time a heightened response can be mobilised very quickly. Monocytes and eosinophils can then phagocytose the antibody/antigen complexes thus formed.

The T-cell mechanism is described as cellular immunity. There are three types of T-cell: cytotoxic T-cells, suppressor T-cells and helper T-cells. The mechanism of T-cell action is similar to that of the B-cells in that it recognises antigens, but no antibodies are produced. After the stimulation of a T-cell with a foreign cell, it differentiates into either effector or memory cells. Memory cells function in a similar manner to B-cell memory cells, launching a fuller response in a future encounter of the same antigen. The effector cells attack and lyse the foreign cells without the interaction of complement or antibodies but with the assistance of macrophages and helper cells. Stimulated cytotoxic T-cells release lymphokines (*e.g.* interleukin) which attract macrophages, increase vascular permeability and stimulate lymphocyte proliferation.

2.3 BLOOD-MATERIAL INTERACTIONS

2.3.1 Introduction

This general description of blood function is relevant to normal homeostasis, whereby cellular and humoral components only come into contact with biological material. When artificial surfaces are placed in contact with blood, other, quite specific reactions take place.

2.3.2 Protein adsorption

When a surface in placed into blood, protein adsorption takes place very quickly (Baier and Dutton, 1969), over the first few milliseconds. This follows a degree of water and inorganic ion adsorption (Andrade and Hlady, 1986). Of importance is the understanding that artificial surfaces, in contrast to biological tissue, are generally unhydrated and so make extremely good platforms for hydrophobic moieties to assemble. Proteins have a large degree of hydrophobicity mixed with hydrophilic domains which allow them to dissolve in aqueous solution. This ability to solubilise is marginal, however, such that there is a very large gain in entropy when a non-aqueous environment is presented to the protein solution, allowing adsorption. Protein adsorption in these circumstances is, therefore, almost inevitable. The primary cause of protein adsorption is mostly biophysical and the quantities of different proteins found at the interface

immediately after blood-material contact generally reflect their concentration in plasma. In other words, albumin, fibrinogen and IgG usually predominate.

Proteins, however, have different affinities for a surface following contact with blood. As molecules constantly collide with the interface, species which were originally adsorbed may subseqently desorb, depending upon their attraction with the surface and new molecules adsorb in their place. This gives rise to a reordering of the primary surface layer with time, a phenomenon known as the Vroman effect (Vroman *et al.*, 1980). Highly thrombogenic surfaces can attract large quantities of FXII and so initiate intrinsic coagulation if the adsorption of FXI, HMWK and PK at close proximity is favoured (section 2.2.3). Materials which allow the attack of $C3(H_2O).B$ by D and subsequent coating of the surface by C3b can be expected to initiate complement activation (section 2.2.4). Perhaps the most important aspect is in the response of the surface layer to circulating platelets (section 2.3.3).

Rearrangements within the surface layer are not only limited to the composition of the molecular species, but also the conformation of the individual protein molecules. This occurs over a relatively long time period (minutes) as witnessed by the increase in difficulty of eluting protein from the surface over time.

2.3.3 Platelet adhesion and activation

As blood comes into contact with an artificial surface, proteins are adsorbed then rearranged (section 2.3.2). This gives rise to conformationally active fibrinogen which is available for interaction with the platelet fibrinogen receptor (glycoprotein IIb/IIIa (GPIIb/IIIa)). Aqueous phase fibrinogen does not cause the same interaction since it has a different conformation. In addition, it has a much smaller effective concentration than does a relatively large amount concentrated at the surface. This coupling leads to the sequence of events described in section 2.2.5, resulting in secretion and pseudopodia formation. Since the fibrinogen molecule is fixed to the surface, platelet adhesion takes place. Secretion of ADP, TxA_2, *etc.*, leads to the activation of further platelets via specific receptors for these molecules. These fluid-phase platelets which are partially activated undergo a slight conformational change to their GPIIb/IIIa receptors and now have a susceptibility to the combination with fluid phase fibrinogen. Mutual adhesion of fibrinogen molecules between platelets leads to platelet aggregate formation.

The propensity of fibrinogen to adsorb to a surface makes it the obvious candidate for mediating the adverse reactions of platelets to that surface. Platelets, however, have

receptors for many, diverse substances (thrombin, plasmin, catecholamines, prostaglandin endoperoxides, collagen, ADP, TxA_2, platelet activating factor (PAF), von Willebrand's factor (vWF)) so the possibilities for activation transduction are very wide. In fact, most of the observations regarding the responses of platelets to artificial surfaces, culminating in what is described as the 'platelet release reaction', are no doubt due to a gamut of molecular interactions with extracellular species.

The vascular tree has a very complex structure. It is made up of a large number of interconnecting branches of varying diameter through which blood flows under the action of a pulsatile pressure gradient. Arterial blood is subjected to wide variations in velocity, in which reverse flow can be observed. The structure of the vessels, bifurcations and valves that comprise the circulation is such to allow minimum disturbance to the flow. When artificial surfaces are placed in contact with blood or introduced into the blood stream, as is the case for catheters, the blood flow is disturbed which may result in complex, turbulent flow patterns being formed. This can result in blood elements being exposed to high shear rates, giving rise to material-independent cellular reactions where shear forces alone (*i.e.* without the presence of a molecular agonist) activate internal messenger signalling which initiates cellular pathways. This occurs with platelets such that their gross deposition onto a badly positioned catheter constructed of a non-thrombogenic material can be observed, with the resultant production of a white thrombus, deficient of the red blood cells which make up the substance of a typical fibrin clot.

2.3.4 Other cellular interactions

Red blood cells and white blood cells also interact with foreign surfaces. Little has been described for these interactions since they do not occupy such a clear rôle in thrombosis as do platelets and the coagulation cascade. They are nevertheless important. Red blood cell destruction can occur, releasing haemoglobin, which is very detrimental to the maintenance of kidney and liver function. A gross loss of red blood cells can lead to anaemia. The activation of white blood cells can bring about the production of antibodies, release of reactive oxygen species and degradative enzymes after chemotaxis or chemokinesis to the area of the artificial surface. Inflammation or shock can be the result of such a reaction. The aggregation of white blood cells has also been noted in response to the contact of a biomaterial (Craddock *et al.*, 1977), in addition to their adhesion in a similar way to platelets (O'Flaherty *et al.*, 1978), probably to adsorbed IgG or C3b, for which granulocytes have receptors on their membranes (Vroman, 1987). Of additional

concern is the attack by cells on indwelling polymers. These have been shown, in certain circumstances, to have degraded rapidly, especially many types of polyurethane. Some have been shown to suffer from simple hydrolysis (Hennig *et al.*, 1988), but others appear to have been attacked by cells of the immune system (Marchant *et al.*, 1984).

2.3.5 Activation pathway co-operativity

The body is a highly organised set of reaction pathways. Many of these are considered as discrete entities occurring on their own. This conceptual compartmentalisation is probably false, with many of the pathways interacting with each other. It is important to be aware of the possibility of released agents from other pathways enhancing or attenuating the actual biological system being studied. Examples of this involve most of the pathways already described. For example, red blood cells release ADP upon lysis, which can occur due to the extreme haemolytic potential of an artificial surface or an increase in shear rate experienced following the malposition of an invasive medical device. ADP is a strong platelet activator, causing platelet aggregate formation and platelet granule release (Skaer, 1981). The interactions of platelet membrane lipids with the coagulation cascade are well known (Mann *et al.*, 1987) but other interactions also exist. For example, platelets can directly activate FXII and FXI, with the assistance of HMWK, if they have previously been activated themselves (Walsh and Griffin, 1981). PF4, however, inhibits the activation of PK and FXII by strong activators (Kodama *et al.*, 1985). In addition, platelets contain many coagulation factors within their intracellular granules.

Complement activation, and therefore the initiation of an immune response, has been shown to be responsible for various interactions with other pathways. C3a has been shown to induce platelet aggregation and dense granule release (Polley and Nachman, 1983). The membrane attack complex, C5b-9, has been shown to cause platelet granule release but without the aggregation usually associated with such activation (Ando *et al.*, 1989). Furthermore, procoagulant activity was increased with the expression of FVa and FXa receptors on the platelet membrane. Immune complexes and C5a have been shown to elicite the release of elastase from neutrophils (Ohlsson and Olsson, 1977) which in turn can inactivate various important plasma protease inhibitors, especially C_1-Inh (Brower and Harpel, 1982), providing a positive feedback mechanism for the potentiation of classical complement activation and intrinsic coagulation. It would seem that the stimulation of neutrophils can bring about complement activation and thrombosis. Elastase also modulates the activity of the platelet release reaction and aggregation (Brower *et al.*, 1985).

Conversely, the release of PF4 from platelet α-granules stimulates neutrophils to release elastase (Lonky *et al.*, 1978). Upon α-granule release, GMP-140, an α-granule membrane protein migrates to the outer membrane of the cell, allowing the attachment of neutrophils (Hamburger and McEver, 1990). The presence of neutrophils in the vicinity of activated platelets is brought about by the release of various chemotactic factors (*e.g.* platelet derived growth factor (PDGF)) from platelet α-granules. Neutrophils are also aggregated and activated to release elastase by FXIIa (Wachtfogel *et al.*, 1986), as with kallikrein (Colman *et al.*, 1985).

It must always be remembered, therefore, that many interactions are occurring during the contact of a foreign material with whole blood. If effective changes to a biomaterial are to be made, it is always necessary to determine the exact mechanisms of pathway activation and consider all other possible mechanisms.

2.3.6 Effect of surface composition

The surface of a material has a controlling effect on all subsequent events upon its contact with blood. It is generally acknowledged that protein adsorption is the first major event to occur following such contact (Baier and Dutton, 1969; Andrade and Hlady, 1986) and so affects subsequent cellular events. There is lack of understanding about the way in which the surface composition of the material affects this protein layer, and the way in which the protein layer affects future events, but several lines of research have been followed. Firstly, it has been noted previously from protein prewashing experiments that albumin is generally a 'good' protein to have adsorbed (Sigot-Luizard *et al.*, 1984) and fibrinogen or IgG are rather worse in terms of the effect of the surface to platelets (Packham *et al.*, 1969). Theoretical reasons for this have been put forward, in that albumin, in contrast to fibrinogen and IgG, is highly electronegative, as is natural endothelium, which is the ultimate, non-thrombogenic surface. Materials which are extremely electronegative in their own right have been used successfully in certain application (*e.g.* heart valves) in the form of carbon-vapour deposited coatings (Bokros *et al.*, 1969), but the high temperatures needed (1500°C) for their manufacture are not applicable to polymer chemistry.

Surfaces which have differing free energies have been studied as a more promising parameter for allowing enhanced thromboresistance. Initially hydrophobic materials were designed, the idea being that a hydrophobic surface would limit the degree of protein coverage by preventing protein spreading. PTFE is extremely hydrophobic and fares rather

badly in terms of *in vivo* thrombus formation (Roberts *et al.*, 1977). Hydrophilicity should allow a hydration zone similar to that of natural tissue. It is now generally accepted, though, that it is not the total degree of protein adsorption which is the important factor, but the conformational changes which occur during a protein's residence on the surface. Many have suggested a balance in surface free energy in order to improve blood compatibility (Baier, 1972). In relation to polyurethane chemistry, workers have suggested that having a large number of soft segments at the surface would bring positive effects (Sa da Costa *et al.*, 1980; Merrill and Salzman, 1983), and that these should be hydrophilic (Salzman *et al.*, 1981). This is in general agreement with other experiments which have shown that hard segments induce platelet activation (Lelah *et al.*, 1983) whereas an increase in the proportion of soft segments at the surface result in better blood compatibility (Hanson *et al.*, 1980). Some polymers appear to have very little difference in their ability to induce platelet adhesion irrespective of their surface hard : soft segment ratio (Grasel *et al.*, 1987). Hanson also claimed that platelet adhesion was in inverse proportion to the number of carbon atoms forming hydrocarbon bonds at the surface. This is in agreement with Andrade *et al.* (1981) who performed experiments with polymers onto which differing alkyl chains lengths were grafted, but opposite to the observations by others (Merrill *et al.*, 1982). It has also been suggested that the number of hydrogen bonds at the surface is important (Shibuta *et al.*, 1986). Since water can form hydrogen bonds, a large number of hydrogen bond-forming species at the surface will give rise to a hydrated lattice which is less destructive to the conformation of any protein layer. Conversely, it has been proposed that a reduction in the number of hydrogen bond-forming atoms will reduce the denaturation of any proteins adsorbing to the surface and so increase the blood compatibility (Nyilas, 1971).

The microphase-separated structure may be of importance in the response of blood to the surface. Polymers constructed of polyether and aliphatic polyamide segments, causing crystalline and amorphous domains were shown to be more blood compatible at certain phase distributions (Yui *et al.*, 1986), there being a balance between either state. The lack of platelet adhesion was put down to the lack of flexibility of platelet membranes. Whilst fibrinogen adsorbed irreversibly to the amide-containing hard segments, albumin binding the soft segments disrupted any cellular attachment. The degree of surface crystallinity in common polyurethanes is generally not known, however.

The surface morphology is also of importance. A rough surface can entrap blood cells given the appropriate texture spacing and size. These ridges can also entrap air

bubbles which would cause deleterious, platelet activating conformational changes to proteins adsorbing to them. The ability of a thrombus to adhere to this surface is also texture-dependent. A very smooth surface may be found to be extremely non-thrombogenic *in vivo* when in fact forming thrombi merely escape as emboli.

It has been virtually impossible to relate thromboresistance to one or two simple physico-chemical parameters. The relationship between surface adsorbed protein and blood cell reaction is probably far too complex for a simple model and an inherently blood compatible surface may not, in fact, exist.

2.3.7 Methods of improving haemocompatibility

Many attempts have been made to improve the haemocompatibility of commonly used polymers for medical applications. The techniques available for the chemical modification of polyurethanes have meant a proliferation in the variations of the basic urethane polymer. Such modifications have resulted in consistent claims by their respective manufacturers of improved biostability, resistance to bacterial adherence and blood tolerance. The often subtle differences in *in vivo* performance have resulted in controversy. Indeed, there remains an unresolved argument about the differences in haemocompatibility between silicones, PVC and polyurethanes. Polyurethanes are often used for modification since they can be altered in four main ways: changing the soft segment group (often a polyether, sometimes a polyester), changing the diisocyanate group (aromatic in Pellethane, aliphatic in Tecoflex), changing the diamine or diol, or by grafting chains on to the basic polyurethane chain. In addition, functional groups can be modified. In this manner the chosen groups having the required chemistry can be inserted at will.

Most attempts to improve blood compatibility have involved chemical modifications to achieve the altered physico-chemical characteristics outlined in the previous section. To this end polyurethane soft segments have been changed by the introduction of fluorine (Ito *et al.*, 1988) which demonstrated better *in vitro* platelet adhesion. Others have inserted highly hydrophilic groups such as polyethylene oxide (Grainger *et al.*, 1989). The alteration of the hard segments with the introduction of fluorine has also been claimed to improve antithrombogenicity *in vitro* (Ito *et al.*, 1988). An attempt to reduce polymer surface hydrogen bonding in order to influence protein adsorption denaturation has been achieved using silicone atoms with the polyurethane structure, such as Cardiothane/ Mitrathane (Nyilas, 1971) and has been used widely in cardiac-assist devices. Another scheme involves the grafting of the 'blood contact improvement' groups on to the side

chains of the polymer such that there will always be a significant representation of these moieties on the material surface. For example, alkyl chains have been used (Andrade *et al.*, 1981; Merrill *et al.*, 1982), as have chains of polyethylene oxide (Han *et al.*, 1989). This last type of attachment has a more promising premise for achieving better blood compatibility. The high water hydration potential means that there is a very low interfacial free energy. In addition, the chains are extremely mobile, decreasing the surface area which has a stable base for proteins to bind irreversibly. It has been claimed that such an approach not only reduces protein denaturation but also the total quantity of adsorbed protein (Merrill and Salzman, 1983).

The more modern techniques do not attempt to make the surface as biologically or chemically inert as possible but to functionalise it with bioactive components. These materials specifically inhibit the adhesion of platelets, thrombi, or digest forming fibrin, depending upon the molecules attached. The successful heparinisation of polymers has been a goal for many years. Initial materials contained ionically-bonded heparin (Gott *et al.*, 1963), or worse, heparin simply coated onto the material in post-processed form (Grode *et al.*, 1969). These forms allowed the release of heparin from the surface, bringing the advantages of local anticoagulation and therefore reducing anticoagulation therapy. This was only suitable for short-term blood-contacting devices, however, since the reserves of heparin were finite (Merrill *et al.*, 1970). Many covalently-attached heparin conjugates at this time were barely functional (Hoffman *et al.*, 1972). Since then surfaces incorporating covalently-attached heparin have been achieved successfully through the use of the correct spacer chains and heparin of the appropriate molecular weight (Larsson *et al.*, 1987). The heparin on these surfaces remains attached and prevents the surface from clotting indefinitely. The reactions of heparinised-surfaces to platelets, however, often seem to be no better than non-functionalised surfaces (Gossen and Sefton, 1979) and sometimes worse (Saltzman *et al.*, 1969) with the heparin, or hydrophobic spacer chains, potentiating platelet aggregation. Other groups have found improved platelet performance (Larsson *et al.*, 1987). Other surfaces have now been designed with platelet activation in mind by the use of heparin-like sulphonate groups within the structure of the material (Ito *et al.*, 1988). These have exhibited heparin-like activity in their ability to inactivate thrombin and FXa.

Another approach to the problem of clot formation has been to attach urokinase to the surface of the polymers (Kusserow *et al.*, 1970). This does not aggregate platelets as does heparin, but activates plasminogen to plasmin, causing any locally-formed fibrin to

be digested into its fibrinopeptides. This approach still allows for the generation of thrombin at the material surface, however, which has the potential for inducing platelet trauma.

Some workers have suggested that avoiding any deposition of platelets is the key to thromboresistance since the availability of PF3, necessary for clot formation, is obviated. The techniques used to achieve this end have been the attachment of PGI_2 or PGE_1 (McRea *et al.*, 1981), extremely potent anti-platelet agents and among those used by endothelial cells to prevent platelets from adhering to vessel walls, or BW 245C (Bamford *et al.*, 1985). Experiments with both these have been very promising. Some workers, however, have attached both heparin and PGE_1 in an attempt to prevent any possibility of clot formation (Jacobs and Kim, 1986).

Another method of changing the biological response to a material is simply to coat it with a favourable substance (Jansen and Brim, 1987). With reference to the theory of minimised interfacial free energy (section 2.3.6), most coatings are highly hydrophilic. They are very easy to apply and can be used on any surface. This allows its use on a material which has the appropriate mechanical properties, despite its possible lack of blood compatibility.

One method of 'confusing' the blood so that it fails to recognise a biomaterial is to coat its surface with lipid such that it presents a surface to the blood similar to that of blood cells. Various groups have attempted such a coating by glow discharge (Sharma *et al.*, 1987) or polymerisation of phosphorylcholine by UV light (Hayward and Chapman, 1984). The platelet adhesion response and clotting times for these materials, respectively, have been shown to be much improved over uncoated or conventional polymers.

2.4 Methods of Study of Blood-Material Interactions

2.4.1 Blood-contacting procedures

The experiments performed to study material-blood interactions have been designed to give information on several points. They are required to demonstrate some index of blood compatibility using an arbitrary scale to allow comparison from material to material. Alternatively, they have been used to give quantitative results of the adhesion or generation of components in the blood. Other experiments have been used to elucidate mechanisms of blood trauma. For each application there has been a wide range of blood contacting regimes: those designed to imitate clinical blood contact as closely as possible, those which

have reduced the blood-contact to its most simple form and those which maximise the conditions of blood-contact. For all of these procedures there have been attempts to minimise blood-air interfaces or maximise blood-material surface area contact in a wide variety of formats. These are described in tables 2.6 and 2.7. A variety of animals have been used to provide the source of blood in these experiments (table 2.8). Indeed, experiments have not always been limited to *in vitro*, but also *in vivo* and *ex vivo* procedures in animals (table 2.9).

Test cell arrangement	References
Microscope slide: Material, or protein solution, coated slide, with a volume of blood or plasma placed on top	Lyman *et al.* (1968); Zucker and Vroman, (1969); Hum *et al.* (1975).
Incubation test cell: Fixed surface area of test material placed within a container of blood or plasma. (Static or agitated)	Lyman *et al.* (1968); Leake *et al.* (1989); Chinn *et al.* (1991); Kalman *et al.* (1991).
Membrane cell: Fixed volume of blood injected between 2 sheets of membrane (of fixed surface area) sandwiched together. (Agitated)	Kambic *et al.* (1976); Courtney *et al.*, 1987; Irvine (1989).
Bead retention: Small, glass beads coated with test material placed within a column. Blood or plasma poured through (or drawn through under pressure)	Salzman *et al.* (1977); Lindon *et al.* (1978); Solomon *et al.* (1979); Kataoka *et al.* (1982); Okano *et al.* (1982).
Closed tube: Tube of test material filled with blood or plasma. (Static or agitated)	Umemura *et al.*, 1988; Ito *et al.* (1989); Rollason and Sefton (1992).

Table 2.6 Blood contacting regimes: static (and pseudo-static) contact

Test cell arrangement	References
Simple perfusion: Blood or plasma perfused along the inside of a tube constructed of, or coated with, test material.	Poot *et al.* (1988); Baquey *et al.* (1989); Kottke-Marchant *et al.* (1989); Maechling-Strasser *et al.* (1989); Brinkman *et al.* (1990).
Parallel plate: Cell allowing several, parallel sheets of test material, or slides coated with test material, to have blood perfused over them. (1 sheet constitutes a Lyman cell).	Lyman *et al.* (1968); Muggli *et al.* (1980); Chinn *et al.* (1991).
Grabowski cell: Cell allowing the flow of blood over one side of a sheet of test material of fixed surface area.	Grabowski *et al.* (1976).
Stagnation point flow chamber: Material held in a perpendicular position to flowing blood. Area of blood stagnation examined for cell adhesion.	Dutton *et al.* (1968); Petschek *et al.* (1968); Nyilas *et al.* (1975).
Annular axial rod cell: Tube of fixed internal, circular dimensions, allowing the insertion of test material within the flow.	Turitto *et al.* (1977); Weiss *et al.* (1978); Turitto *et al.* (1979).
Baumgartner cell: Blood flowing over outside of catheter within the lumen of a wide, excised artery or wide section acrylic tube	Baumgartner and Haudenschild (1972); Sakariassen *et al.* (1979); Engbers *et al.* (1987).
Spinning disc: Material held on the end of a spinning rod to generate known wall shear rates, immersed in blood.	Turitto and Leonard (1972); Butruille *et al.* (1975); Voisin *et al.* (1985).
Closed loop (Chandler loop): Tube constructed from material, or coated with material, formed into a loop, blood circulated by virtue of loop rotation.	Bowry *et al.* (1982); Cholakis and Sefton (1989).
Elliptical cell: 2 membranes clamped together (as membrane cell), but with recirculating blood via tubes and a peristaltic pump.	Muzykewicz *et al.* (1975).
Relaminarization flow cell: Cell consisting of a wedge-shaped, ramped lumen (to induce fully-developed, laminar flow) over a sheet of test material	Strong *et al.* (1982).

Table 2.7 Blood contacting regimes: dynamic contact

Animal	References
Human	Baier *et al.* (1985); Courtney *et al.* (1987); Engbers *et al.* (1987); Umemura *et al.* (1987); Poot *et al.* (1988).
Dog	Maloney *et al.* (1969); Kambic *et al.* (1976); Okano *et al.* (1982); Didisheim *et al.* (1983); Goodman *et al.* (1984).
Sheep	Didisheim *et al.* (1979); Bamford *et al.* (1989).
Rabbit	Robertson and Chang (1974); Turitto and Baumgartner (1979); Coleman *et al.* (1982); Grainger *et al.* (1989); Yui *et al.* (1989).
Rat	Kataoka *et al.* (1982); Behnke (1987).
Ape/Monkey	Didisheim *et al.* (1979); Chinn *et al.* (1991); Kiaei *et al.* (1992).
Calf	Didisheim *et al.* (1979); Coleman *et al.* (1982) Sharma *et al.* (1987); Sharma and Chandy (1989).
Bovine	Brynda *et al.* (1978); Mizutani (1981); Bornzin and Miller (1982); Lok *et al.* (1983); Tanzi and Levi (1989).
Pig	Feuerstein *et al.* (1975); Didisheim *et al.* (1979); Strong *et al.* (1982); Zingg *et al.* (1982); Absolom *et al.* (1983).

Table 2.8 Sources of blood and blood elements

Animal	Material/blood contacting arrangement	References
Dog	Material assessed as a catheter inserted into external jugular vein for 40 mins. In^{111}-platelets imaged with gamma camera. Examined visually and SEM.	Solomon *et al.* (1987)
	Material assessed as an A-V shunt between femoral artery and vein for 2hr. Platelet deposition with Cr^{51}, fibrinogen deposition with I^{125}-fibrinogen.	Young *et al.* (1983)
	Material coated onto femoral polyethylene A-V shunt for 0.5-60 mins. Examined with SEM. Platelet deposition with Cr^{51}, fibrinogen deposition with I^{125}-fibrinogen.	Lelah *et al.* (1984); Lelah *et al.* (1986); McCoy *et al.* (1989)
	Material assessed as a catheter inserted into carotid artery, femoral and external jugular veins for 200 mins.	Libsack and Kollmeyer (1979)
	Material assessed as a cannula in peripheral veins for 2hr. Measured time taken for occlusion, examined with SEM.	Hayashi *et al.* (1990)
	Material assessed as a catheter inserted into external jugular vein. Measured In^{111}-platelets with scintigraphy	Solomon *et al.* (1986)
Rabbit	Material assessed as an A-A shunt in carotid artery for up to 3hr. Measured time taken for occlusion, examined with SEM.	Grainger *et al.* (1989); Han *et al.* (1989)
	Material assessed as an A-V shunt between carotid artery and jugular vein. Measured time for shunt to occlude with flow probe.	Yui *et al.* (1988)
	Material assessed as an A-V shunt (coated test tube) between carotid artery and jugular vein for 30 mins. Measured platelet adhesion with SEM.	Ito *et al.* (1991)
Ape/ Monkey	Material assessed as an A-V shunt between femoral artery and vein for 5-7 days. Examined with SEM, platelet survival time with Cr^{51}.	Harker *et al.* (1977); Hanson *et al.* (1979); Hanson *et al.* (1980)
	Material assessed as an A-V shunt between femoral artery and vein for several days. Imaged ^{111}In-platelets, measured shunt blood flow and platelet survival time.	Yeh *et al.* (1988); Kiaei *et al.* (1992)
Pig	Material assessed as an A-V shunt, exposed for 3 mins. Platelet adhesion measured by examination with SEM.	Ward and Forest (1976)
	Material assessed as a coating on a cardiopulmonary bypass circuit for 2hrs. Measured complement activation.	Nilsson *et al.* (1990)
Goat	Material hung in left ventricle of heart for 72hr. Examined visually, SEM, XPS, IR spectroscopy.	Fiala *et al.* (1987)
	Material assessed as a catheter, exposed for 1-180 mins. ^{111}In-platelet and I^{125}-fibrinogen deposition measured.	Rodvein *et al.* (1982)
Rat	Material assessed as an A-V shunt between carotid artery and jugular vein for 0.25-4hr. Platelet, WBC count, haematocrit measured.	Jones (1989)
	Material assessed by implanting into peritoneal cavity followed by intraperitoneal haemorrhage for 48hrs. Thrombus assessed using conventional microscopy.	Bakker *et al.* (1991)
Sheep	Material assessed as a catheter inserted into the aorta via the femoral vein for 9 days.	Hecker and Edwards (1981)
	Material assessed in Lyman flow cell for 3 mins. Platelet adhesion assessed.	Kim *et al.* (1974)
Human	Material assessed as a peripheral venous cannula for up to 24hrs. ^{125}I-fibrinogen counted with gamma camera.	Lindblad and Johansson (1987)
	Material assessed as a clinical central venous catheter for up to 9 days. Clinical signs of thrombophlebitis measured, 'pull-out' phlebography performed on catheter removal.	Bennegård *et al.* (1982); Linder *et al.* (1984)

Table 2.9 *In vivo/ex vivo* blood contacting procedures

2.4.2 Platelet adhesion

In the above test cell arrangements (tables 2.6, 2.7 and 2.9) platelet adhesion has been assessed using many techniques. In fact, the importance of platelets in thrombosis and haemostasis research has resulted in a very large number of papers regarding platelet interactions with various classes of surfaces and with an associated large number of variations in procedures. The main groupings are shown in table 2.10. The major rationale for measuring the quantity of platelets on a surface is the view that they play a central rôle in the generation of a thrombus: blood or plasma will not clot if completely devoid of platelets and the initial adhesion of platelets to an artificial surface is very quick (Dutton *et al.*, 1968). The enumeration of platelets adhered to a surface exposed to suspended platelets can result in the rapid collection of data allowing for a very easy and cheap marker of blood compatibility.

Experimental Procedure	References
Exposure of surface to PRP then counting of attached cells per unit area under a microscope by eye	Absolom *et al.* (1983); Gluszko *et al.* (1987); Alkhamis *et al.* (1988); Lahav (1988); Bamford *et al.* (1989).
Exposure of surface to PRP then counting of attached cells per unit area by eye or computer, viewed with SEM	Kataoka *et al.* (1978); Okano *et al.* (1982); Olijslager *et al.* (1982); Seifert and Greer (1985).
Counting fluid phase platelets after exposure of a surface to PRP or blood using an electronic counter	Bowry *et al.* (1985); Courtney *et al.* (1987); Umemura *et al.* (1988); Cholakis and Sefton (1989); Grainger *et al.* (1989).
Measurement of ATP concentration on a surface exposed to blood or plasma using bioluminescence	Linder *et al.* (1984).
Measurement of radiation in γ-counter after exposure of surface to blood or PRP with ^{51}Cr-labelled platelets	Bolhuis *et al.* (1981); Park and Cooper (1985); Santoro (1987); Kang *et al.* (1988); Kunicki *et al.* (1988).
Measurement of radiation in γ-counter after exposure of surface to blood or PRP with ^{111}In-labelled platelets	Borow and Crowley (1985); Solomon *et al.* (1986); Engbers *et al.* (1987); Poot *et al.* (1988); Eldor (1989).
Measurement of numbers of acridine yellow labelled platelets adhering to surface in flowing whole blood by epifluorescent video microscopy	Feuerstein *et al.* (1992).

Table 2.10 Methods of measuring platelet adhesion

2.4.3 Platelet activation

The evaluation of platelet activation has not received the attention that has platelet adhesion mainly due to its relative difficulty and expense. Many researchers have argued that owing to the different morphological stages of adhesion that platelets undergo, this parameter is far more important than simply the number of platelets adhered to a surface (Baier, 1987). This parameter is measured either as the release of substances or the change in the platelet, visually or chemically. A summary is shown in table 2.11.

Experimental Procedure	References
Ability of platelets to aggregate in response to ADP, measured using a platelet aggregometer	Engbers *et al.* (1987); Gluszko *et al.* (1987); Umemura *et al.* (1988); Kottke-Marchant *et al.* (1989).
Assessment (qualitative) of platelet morphology and surface aggregation on a material after exposure to PRP using SEM	Okano *et al.* (1982); Olijslager *et al.* (1982); Baier *et al.* (1985); Misselwitz *et al.* (1988).
Counting granules in platelets before and after contact with a surface by TEM	Baumgartner *et al.* (1976).
Measurement of β-Thromboglobulin (β-TG) in blood using radioimmunoassay (RIA) or ELISA	Zahavi *et al.* (1980); Bowry *et al.* (1984); Kang *et al.* (1988); Modderman *et al.* (1988); Leake *et al.* (1989).
Measurement of Thromboxane B_2 in blood using RIA	Zahavi *et al.* (1980); Crook and Crawford (1989); Eldor *et al.* (1989); Leake *et al.* (1989).
Measurement of ^{14}C-5HT (or ^{3}H-5HT) in plasma supernatant with β-counter after contact of ^{14}C-5HT preloaded PRP with a surface	Wang *et al.* (1978); Crook and Crawford (1988); Kang *et al.* (1988); Modderman *et al.* (1988); Eldor *et al.* (1989).
Measurement of PF4 in plasma using heparin/thrombin bioassay	Levine and Wohl (1976); Ciaglowski *et al.* (1981).
Measurement of PF4 in plasma using heparin-ATIII/FXa bioassay	Walsh and Gagnatelli (1974); Niewiarowski *et al.* (1979).
Measurement of PF4 in blood using radioimmunoassay	Umemura *et al.* (1988); Kottke-Marchant *et al.* (1989); Leake *et al.* (1989).
Measurement of PF3 availability in blood using an activated factor X/V bioassay	Umemura *et al.* (1988); Kottke-Marchant *et al.* (1989).
Measurement of ATP released from PRP by luciferase bioluminescence	Feinman *et al.* (1977); Knupp (1988); Miyamoto *et al.* (1989).
Measurement of released ADP and AMP by enzymatic reaction	Ito *et al.* (1989).
Measurement of GMP-140, GP-53 appearance or GPIIb/IIIa rearrangement on fluid phase cells using fluorescent antibodies and flow cytometry	Johnston *et al.* (1987); Shattil *et al.* (1987); Abrams *et al.* (1990); Fijnheer *et al.* (1990).
Measurement of intracellular Ca^{2+} concentration by fluorescence of cells loaded with Fura 2 dye	Yui *et al.* (1989).

Table 2.11 Methods of measuring platelet activation

2.4.4 Contact phase activation

The degree of contact phase activation is the extent to which the intrinsic coagulation cascade (section 2.2.3.2) has been perturbed. The clinical result of such activation is generally thought to be material thrombosis. Examples of methods used for measuring contact phase activation are shown in table 2.12.

Experimental Procedure	References
Lee White clotting time: time taken for citrated whole blood to clot after the addition of calcium ions	Coleman *et al.* (1982); Hennink *et al.* (1984); Cholakis and Sefton (1989).
Mass of thrombus formed on material in contact with recalcified, citrated, whole blood over time	Bensen *et al.* (1991).
PTT (Partial thromboplastin time): time taken for PPP to clot after the addition of Ca^{2+} and platelet substitute	Muzykewicz *et al.* (1975); Han *et al.* (1989); Kottke-Marchant *et al.* (1989); Ito *et al.* (1991).
Thromboelastography or resonance thrombography: measurement of viscosity of clotting native whole blood over time using a resonating probe	Zuckerman *et al.* (1981); Bird *et al.* (1989); Hall *et al.* (1989); Tuman *et al.* (1989); Lemm (1991).
Thrombin generation using a chromogenic substrate	Rollason and Sefton (1992).
Generation of thrombin:antithrombin complexes (T:AT) by ELISA	Elgue *et al.* (1990).
Generation of FXIIa in PPP using a chromogenic substrate	Irvine (1989).
Generation of kallikrein in PPP using a chromogenic substrate	Retzios *et al.* (1988); Grainger *et al.* (1989); Nigretto *et al.* (1989).
Generation of FXIIa in PPP using an ELISA or RIA	Kaplan *et al.* (1985); Nuijens *et al.* (1987).

Table 2.12 Methods of measuring contact phase activation

2.5 CONCLUSIONS

It is important to assess a device in a form which bears some relation to its intended use in clinical practice. Experiments using the material in other morphometric forms can often give rise to misleading results. The lists of methods in the preceding tables are not merely alternatives available for use on each and every device. The method of blood contact is especially important since shear forces can control the manner in which formed elements contact the material surface. Of particular note is the use of animal blood. The biochemistry of platelet function and coagulation in some species differs markedly from that in humans: bovine plasma has a self-limiting step in intrinsic coagulation when kallikrein attacks HMWK, an interaction which is not self-limiting in humans; rabbit platelets are completely devoid of GPIIb/IIIa, the glycoprotein receptor generally implicated in the adhesion of platelets to biomaterials. These gross differences have often been overlooked such that the efficacy of many vascular grafts was overestimated by their patency rates in dogs. Thus, a set of protocols must be used which reflects these criticisms and allows an appropriate degree of sensitivity to be achieved using relevant parameters.

CHAPTER 3

PLATELET ADHESION

3.1 INTRODUCTION

Platelet adhesion is often considered first, or solely, in any haemocompatibility study (Wilson *et al.*, 1986; Lindon *et al.*, 1989). It is widely thought to be the most indicative pointer of the reactivity of a surface to blood (Dutton *et al.*, 1968; Baier, 1987). Platelet adhesion is certainly one of the first events to take place after protein adsorption (section 2.3.5) (Wilson *et al.*, 1986; Olijslager *et al.*, 1982). The central rôle that platelets play in thrombomodulation (Anderson and Kottke-Marchant, 1987) and their very quick reaction kinetics are often taken as good indicators of the potential for chronic thrombosis *in vivo*. Platelets are undoubtedly important in thrombogenesis. They provide membrane lipid which is necessary in the formation of complexes of factors IXa and VIIIa in intrinsic coagulation (Walsh, 1972) and factors Xa and Va in the common pathway (Tracy and Mann, 1983).

Platelet adhesion has been studied in many different ways (section 2.4.1), where conditions of study and sources of blood cells are immensely varied. The correct method of study, as in all aspects of biocompatibility assessment, is critically dependent upon the specific application of the material or device in question; assay sensitivity and relevance are the most obvious requirements of any study. Thus, a variety of methods have been assessed.

3.2 LIGHT MICROSCOPY

3.2.1 Introduction

The initial method used to assess platelet adhesion was similar to that of Zucker and Vroman (1969), whereby catheter samples were incubated with platelet rich plasma (PRP). The cells were non-specifically stained and then viewed using conventional light microscopy. The initial staining procedure was later enhanced, based on a standard histological protocol (Bancroft and Stevens, 1982), to allow better platelet visualisation and obviate problems of salt crystallisation caused by rinsing in phosphate buffered saline

(PBS). The additional use of a video camera attached to the top of the microscope and linked to a computer allowed complex analysis operations to be performed on the images.

3.2.2 Materials and Methods

General staining procedure

Reagents

1. Phosphate Buffered Saline (PBS): Dulbecco 'A' mixture (Oxoid, Basingstoke, UK).
2. Stain solution 1 (Merz and Dade quick staining set, Düdingen, Switzerland).
3. Stain solution 2 (Merz and Dade quick staining set, Düdingen, Switzerland).
4. Fixative (Merz and Dade quick staining set, Düdingen, Switzerland).
5. Mounting medium: Apathys medium (Diachem, Southport, UK).

Method

1. Samples[1] to be assessed were placed in each of the wells of a 24 well tissue culture plate (Becton Dickinson Labware, Lincoln Park, New Jersey, USA).
2. Human PRP was prepared, enough for 1 ml for each sample being assessed.
3. 1 ml PRP was placed in each sample well and incubated with the surfaces at room temperature for 3 hours.
4. Each sample was rinsed carefully with PBS (1) to remove any non-adherent platelets and dipped in each of the appropriate solutions (stain 1 (2), stain 2 (3) and fixative (4)) 5 times each for 1 second each time.
5. Each was again rinsed in PBS (1) and then left to dry in air.
6. Flat surfaces were preserved by placing a drop of mounting medium (5) on a microscope slide, mounting the specimen, then placing another drop on the stained area and covering with a coverslip.
7. Each surface was viewed by conventional microscopy using a x40 or x100 oil immersion objective lens and the image analysed using a computer-controlled image analyser (Joyce Loebl Mini-Magiscan, Newcastle-upon-Tyne, UK).

Enhanced staining procedure

Extra reagents

6. Fixative: 4% (w/v) paraformaldehyde in PBS (4 g paraformaldehyde in 100 ml PBS (1)).

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[1] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

7. Stain: Haematoxylin, Mayer's solution (1 g/l) (Sigma, Poole, Dorset, UK).

Modified method

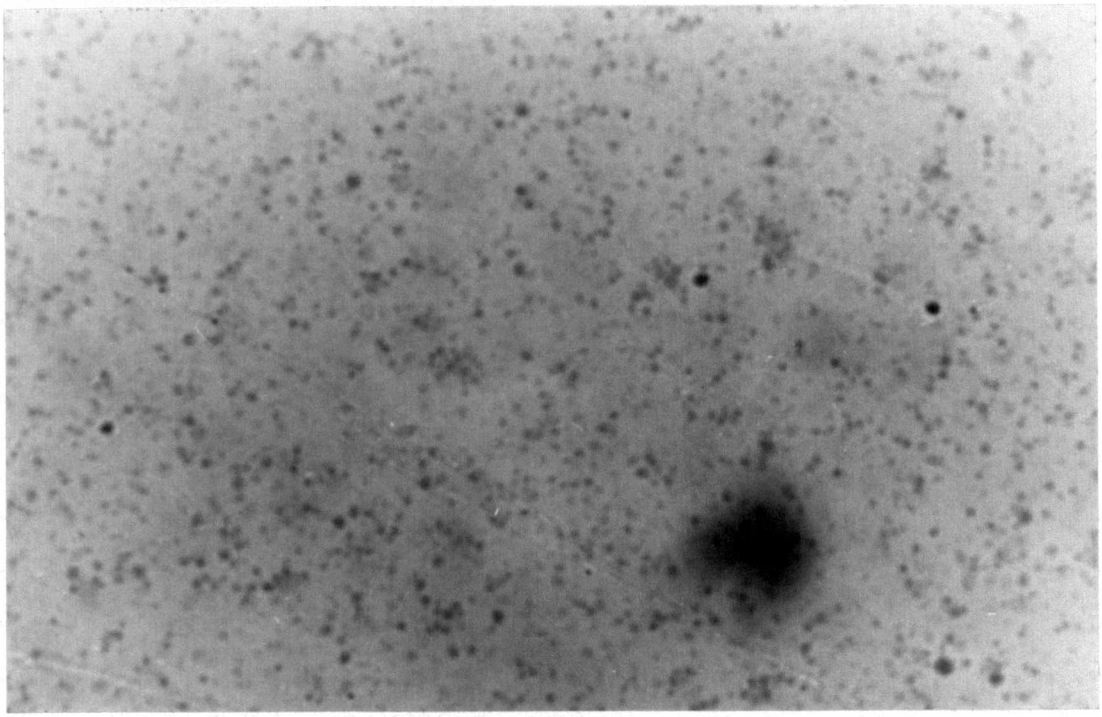
1. After incubation with the platelet suspension, the samples were rinsed in PBS (1).
2. They were then incubated in fixative (6) for 15 minutes at room temperature.
3. They were removed, rinsed with distilled water and dried in air.
4. The sample was stained by the application of several drops of stain (7) for 5 minutes, which was subsequently removed by rinsing with distilled water. The sample was placed in distilled water for 15 minutes at room temperature.
5. Flat surfaces were preserved as described above.
6. The samples were then viewed by conventional microscopy and analysed by computer, as before.

3.2.3 Results

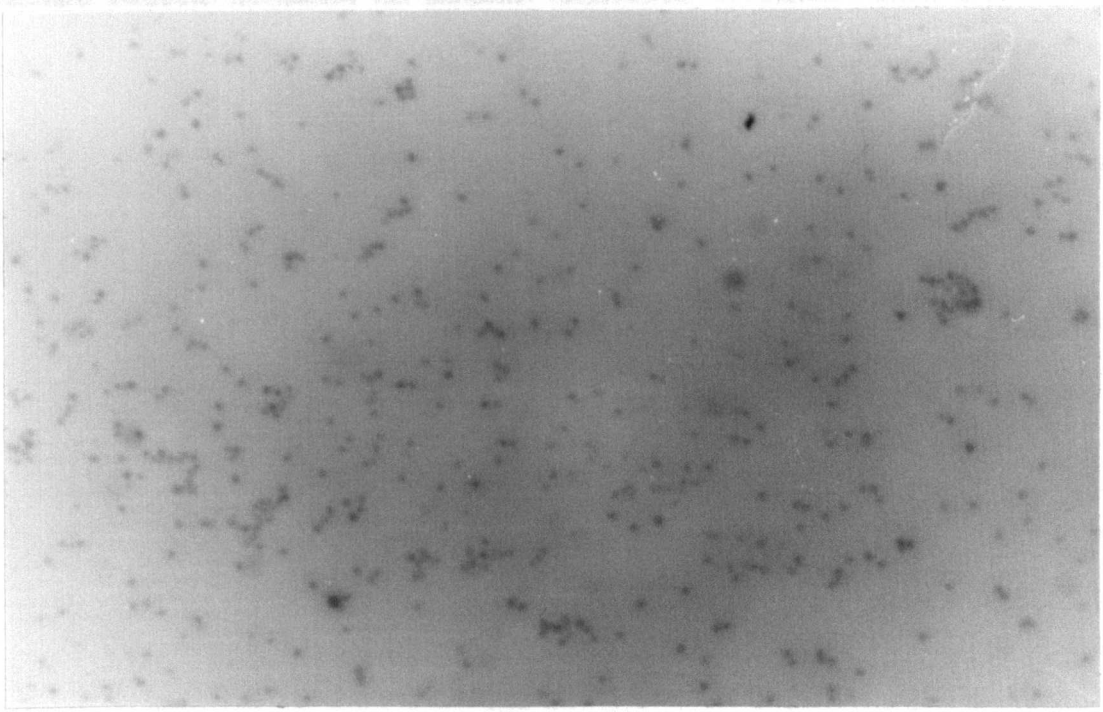
This protocol gave useful qualitative results when assessing flat surfaces. The differing surface coverage of adherent platelets was evident between materials of grossly different structure (fig 3.1). However, other than minor qualitative observations, it was only possible to assess the numbers of discrete, non-aggregated cells in any one field on samples which had very sparse platelet coverage. The percentage coverage of the surface was not measurable because of the depth of staining. Whilst the cytoplasm was very clear, the shape of the spread platelet membrane was not easy to identify, even with a x100 oil immersion lens, due to the small dimensions of the cells and the severe lack of staining on the spread membrane.

3.2.4 Discussion

Most visual techniques for assessing cellular reactions are subjective or qualitative. The potential of quantifying these observations by linking the microscope to an image analysis system was a promising alternative. This offered the possibility of assessing the numbers of cells present on the surface and the area of surface covered. Since the experimental procedure did not provide uniform staining on spread cells, however, this was not successful. Stains usually depend on the presence of a nucleus (absent in platelets) or a definite cytoplasm. Whilst platelets are normally acceptable in this last respect, degranulation occurs upon adhesion to a surface, much of their interior volume is lost and the plasma membrane spreads into a thin film on the surface.



Platelets adhered to glass



Platelets adhered to Biomer

Fig 3.1 Differing platelet adhesion to surfaces of grossly different structure

These experiments demonstrated that an image analysis system can easily identify the boundaries of a partially activated cell but has difficulty in the case of extensively spread platelets, where no definite demarcation line between cell and background is visible. It is necessary, then, to utilise a method which has superior staining efficacy.

3.3 IMMUNO-SPECIFIC PLATELET STAINING

3.3.1 Introduction

In order to overcome the limitations of conventional platelet staining, an immuno-specific membrane stain was implemented. In this methodology there is no requirement for the presence of a cytoplasm as long as the specific membrane epitopes are present and are in a physiological conformation. This method specifically stains platelets using an APAAP (alkaline phosphatase - anti-alkaline phosphatase) technique and is a modification of that used by Falini *et al.* (1984) for staining bone marrow trephine sections. It is suitable, however, for staining any surface which has exposed platelet membrane antigens (*e.g.*, blood smears, tissue sections or biomaterials exposed to blood). In this method a primary antibody recognises the antigenic determinant of interest, whilst a secondary antibody, a rabbit anti-mouse antibody, binds the first. When a complex of alkaline phosphatase and mouse anti-alkaline phosphatase antibody is added as a third layer, it binds to the rabbit anti-mouse antibody. This causes the fixation of a phosphatase on the surface at the point where the specific epitope of interest is sited (fig 3.2) and allows for a conventional alkaline phosphatase stain (Bancroft and Stevens, 1982) to be performed. The phosphatase hydrolyses a substituted naphthol derivative which then couples to a diazonium salt to produce a red azo dye precipitate at the site of the enzyme activity. Since fluorescence is not used the staining is permanent. When the staining steps are repeated, branching chains of antibody can be formed allowing APAAP complexes to be positioned at many sites connected to the original antigenic epitope. This allows a very dramatic increase in staining intensity.

The choice of monoclonal antibody is important. The GP IIb/IIIa antigen is specific for platelets, but some antibodies are specific for either resting or non-resting epitopes. Some even react with monocyte membrane antigens (Bai *et al.*, 1984). The antibody P256 was chosen because it has been used successfully in platelet identification protocols previously and is unreactive towards monocytes (Bai *et al.*, 1984). An anti-GP Ib antibody, for example, would have given a similar result.

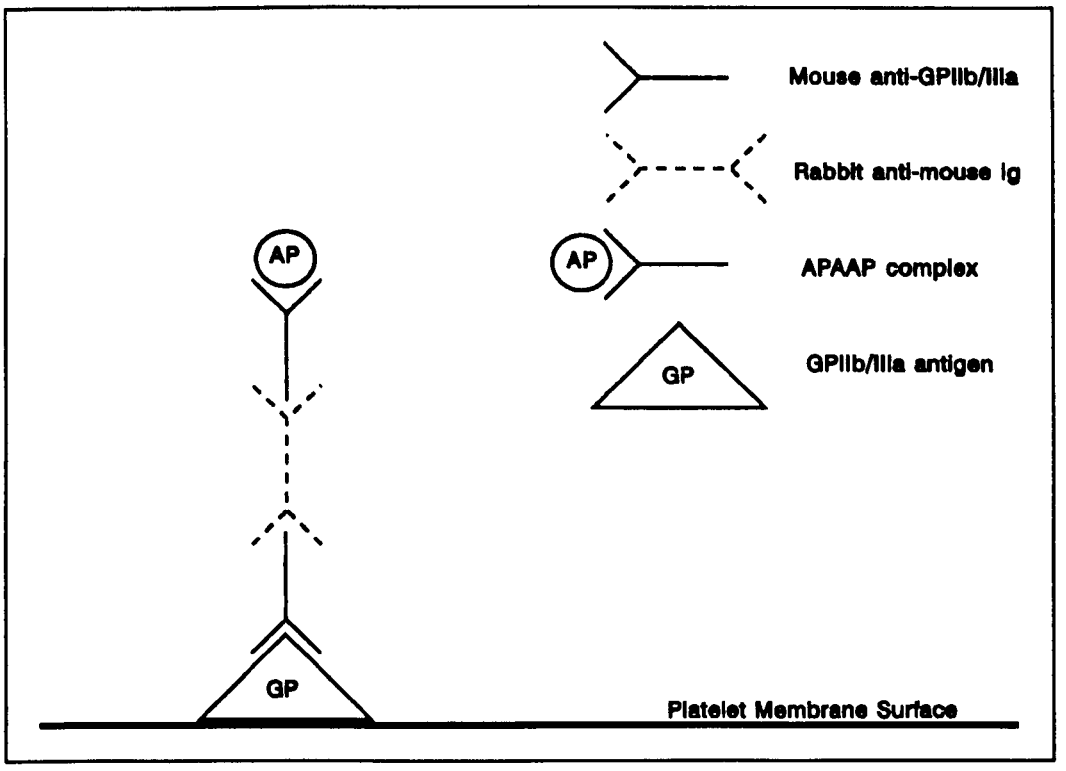


Fig 3.2 Representation of the antibody couplings in APAAP staining

3.3.2 Materials and methods

Reagents

1. Fixative: 50% acetone and 50% methanol mixture.
2. Tris Buffered Saline (TBS): 139mM NaCl, 15mM tris-(hydroxymethyl) aminomethane in H_2O (8.12 g NaCl, 1.82 g tris-(hydroxymethyl) aminomethane (Sigma, Poole, Dorset, UK) in 1 l H_2O), pH 7.4.
3. Primary monoclonal antibody: mouse anti-human GP IIb/IIIa - P256 (donated by the I.C.R.F., London, UK). Diluted 1 + 24 in TBS (2).
4. Secondary monoclonal antibody: rabbit anti-mouse - Z259 (Dakopatts, High Wycombe, UK). Diluted 1 + 24 in TBS (2).
5. APAAP complex (mouse anti-alkaline phosphatase conjugated with alkaline phosphatase) (Dakopatts, High Wycombe, UK). Diluted 1 + 19 in TBS (2).
6. Tris buffer: 100mM tris-(hydroxymethyl) aminomethane in H_2O (12.1 g tris-(hydroxymethyl) aminomethane (Sigma, Poole, Dorset, UK) in 1 l H_2O), pH 8.2.

7. Alkaline phosphate substrate - prepared as follows:
   (i) 2 mg Naphthol AS-MX phosphate (Sigma, Poole, Dorset, UK) dissolved in 200 μl N,N-dimethyl-formamide (DMF) in a *glass* tube.
   (ii) 9.8 ml tris buffer (6) were added.
   (iii) 10 mg Fast Red TR salt (Sigma, Poole, Dorset, UK) were added.
   (iv) The mixture was filtered to 5 μm into a glass tube.
   (v) 10 μl 1M levamisole (241 mg levamisole hydrochloride (Sigma, Poole, Dorset, UK) in 1 ml H_2O) were added.

Method

1. Samples[2] were incubated with platelets as before (section 3.2.2).
2. Each piece was incubated in fixative (1) at room temperature for 90 seconds.
3. 100 μl primary antibody (3) were layered onto the surfaces and incubated at room temperature for 30 minutes in a moist chamber.
4. The surfaces were dipped in TBS (2) for 2 minutes then excess buffer drained away.
5. 100 μl secondary antibody (4) were layered onto the surfaces and incubated at room temperature for 30 minutes in a moist chamber.
6. Each surface was dipped in TBS (2) for 2 minutes then excess buffer drained away.
7. 100 μl APAAP complex (5) were added and incubated at room temperature for 30 minutes in a moist chamber.
8. The surfaces were dipped in TBS (2) for 2 minutes then excess buffer drained away.
9. Steps 5 - 8 were repeated twice, but with 10 minutes incubation time.
10. The surfaces were incubated in alkaline phosphate substrate (7) for 20 minutes at room temperature.
11. Each was then dipped in TBS (2) for 2 minutes then excess buffer drained away.
12. The surfaces were viewed by conventional light microscopy and the image analysed using a computer-controlled image analyser (Joyce Loebl Mini-Magiscan, Newcastle-upon-Tyne, UK).

[2] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

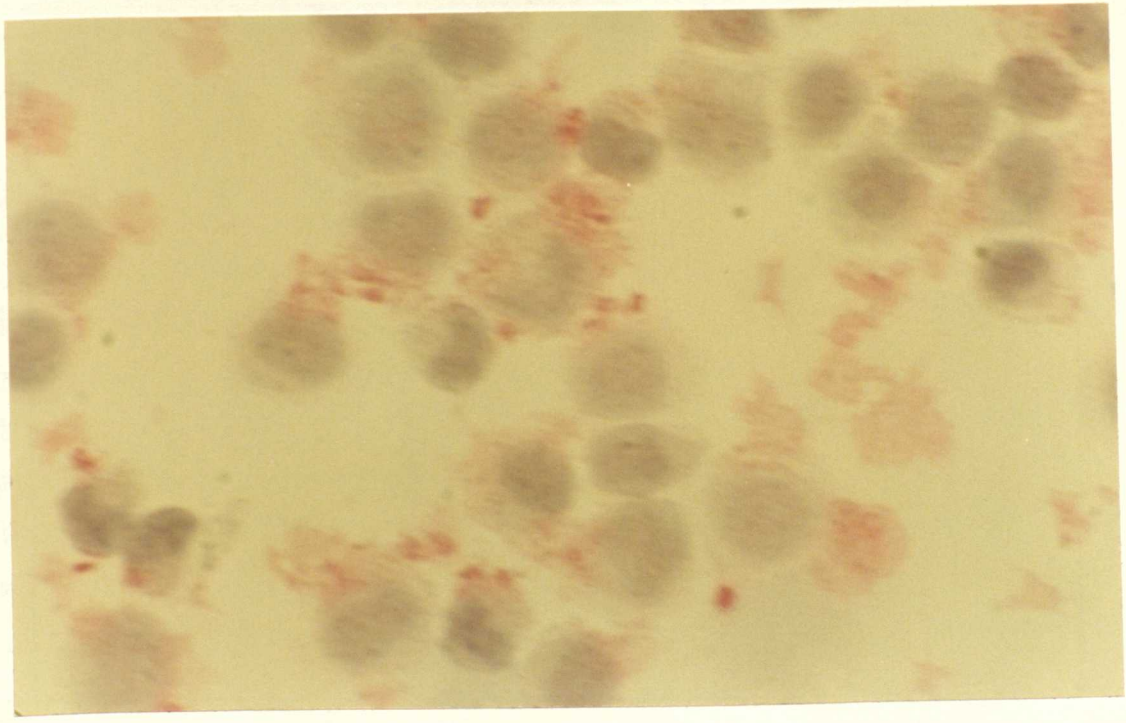


Fig 3.3 Platelets within a blood smear created by cytospinning, stained with anti-GPIIb/IIIa using an APAAP technique (red) and counterstained with haemotoxylin (purple)

3.3.3 Results

It is evident from the examples of GP IIb/IIIa stained platelets (fig 3.3) that a very useful increase in staining, necessary for any serious image analysis, has been achieved using this technique compared to conventional staining. The use of suitable antibodies has resulted in staining selectivity.

The use of catheter samples, rather than flat material, has not been a success, however. Whilst picking out the platelets, the depth of focus attainable on a light microscope is not great enough to cope with curved surfaces.

3.3.4 Discussion

This technique is very useful for quantifying the cellular coverage of a surface which is completely flat. It also introduces the possibility of using a more physiologically relevant cell suspension, whole blood, since the stain is specific for platelets. There is, however, no easy way to achieve this by light microscopy when considering curved surfaces. One possibility, namely dissolving the catheter samples in an organic solvent (8% (w/v) in DMF) and recasting into a flat surface, is not feasible because the morphology of the device surface, as produced commercially, including physical topography and chemical configuration, would be changed. The differences between materials in their ability to induce platelet adhesion could be investigated quite easily in this way but the results would bear little or no relation to the possible performance of the material *in vivo* when constructed into a device.

As well as providing difficulties in terms of depth of focus, curved surfaces make exposure to the reagents difficult. Immunohistochemistry relies on the use of very small volumes of concentrated solutions of antibodies (Boenisch 1990); the quantity needed to immerse a catheter sample totally in these solutions is financially unrealistic. Examining only a part of the surface by applying a drop to the top of the sample is also not possible for comparative assessment. Staining efficiency on a curved surface is dependent on hydrophilicity since the antibody concentration at any point will depend on the spreading of the solution. This was adequate for hydrophilic samples but the solution ran off the hydrophobic specimens, obviating antibody contact.

It was postulated that one way round the problem of curvature was to assess samples using a device with a very good depth of field, such as a scanning electron microscope (SEM) (section 3.4).

3.4 SCANNING ELECTRON MICROSCOPY

3.4.1 Introduction

Scanning electron microscopy (SEM) produces a visual representation of the topography of a surface by detecting the total energy of secondary electrons produced at discrete points on the surface caused by a focused incident electron beam. With polymeric samples, the production of a beam of secondary electrons is dependent on the presence of a thin layer of gold atoms of uniform population density on the sample surface and the angle of incidence of the incident electrons. Since secondary electrons are produced at greater depths within the specimen if the incident beam is perpendicular to the material surface, much of their energy is lost whilst travelling to the material surface. Surfaces not perpendicular to the beam, such as edges and spikes, are, therefore, detected as a brighter area on the image. This feature gives rise to the visualisation of a 3-D type of appearance, allowing the morphology of a surface to be investigated. Visualisation of cellular material with an SEM is not dependent upon cytoplasmic staining, so it is, in theory, easier to detect anucleate and activated cells. The use of electrons allows for a very good depth of field (approximately 30 μm at 1000x magnification compared to approximately 0.1 μm for a typical conventional optical microscope (JEOL)). This means that curved surfaces can be easily investigated.

The methodology used for sample preparation for visualisation with the SEM is fairly standard (Glauert, 1975), using fixation originally developed by Sabatini *et al.* (1963). In this study surfaces were again incubated with PRP, so differentiation between different types of adhered cell was not necessary.

3.4.2 Materials and methods

Reagents

1. Phosphate Buffered Saline (PBS): Dulbecco 'A' mixture (Oxoid, Basingstoke, UK).
2. Fixative: 2.5% (v/v) glutaraldehyde in 100mM sodium cacodylate (21.4 g sodium cacodylate (BDH, Poole, Dorset, UK), 100 ml glutaraldehyde (25% solution, SEM grade, Emscope, Ashford, Kent, UK) in 900 ml H_2O).
3. Cacodylate buffer: 100mM sodium cacodylate in H_2O (21.4 g sodium cacodylate (BDH, Poole, Dorset, UK) in 1 l H_2O), pH 7.4.
4. Primary dehydration solution: 70% (v/v) methanol in H_2O.

5. Secondary dehydration solution: 90% (v/v) methanol in H_2O.
6. Absolute methanol.

Method

1. Pieces of catheter tubing[3] were placed in each of the wells of a 24 well tissue culture plate (Becton Dickinson Labware, Lincoln Park, New Jersey, USA).
2. Human PRP was prepared, enough for 1 ml for each sample being assessed.
3. 1 ml PRP was placed in each sample well and incubated at room temperature for 3 hours.
4. Each sample was rinsed carefully with PBS (1) to remove any non-adherent platelets, then incubated in fixative (2) for 30 minutes.
5. Each was washed in cacodylate buffer (3) for 30 seconds and rinsed with distilled water, then left standing in primary dehydration solution (4) for 15 minutes.
6. The samples were transferred to secondary dehydration solution (5) for 15 minutes, then to absolute methanol (6) for 15 minutes for total platelet dehydration.
7. Any remaining methanol was allowed to evaporate in air for several minutes, then the samples desiccated overnight over silica under vacuum.
8. Each sample was mounted on an SEM stub with glue and vacuum dried for several hours.
9. They were then gold sputter coated with an automatic sputter coater (Emscope AE1231, Ashford, Kent, UK) and assessed using an SEM (JEOL JSM-35C, Tokyo, Japan).
10. Images of typical areas of the appropriate surfaces were photographed, developed, printed and the electron micrographs analysed using a computer-controlled image analysis system (Joyce Loebl Mini-Magiscan, Newcastle-upon-Tyne, UK) by direct use of a video camera with even illumination.

3.4.3 Results

The problem of depth of focus has been completely obviated by this procedure. The visualisation of cells adhered to the material surfaces is very good (fig 3.4). Gross differences between good and bad materials is obvious, in a way which was not evident by light microscopy. The manner in which individual cells have been activated by a surface is now available for objective assessment.

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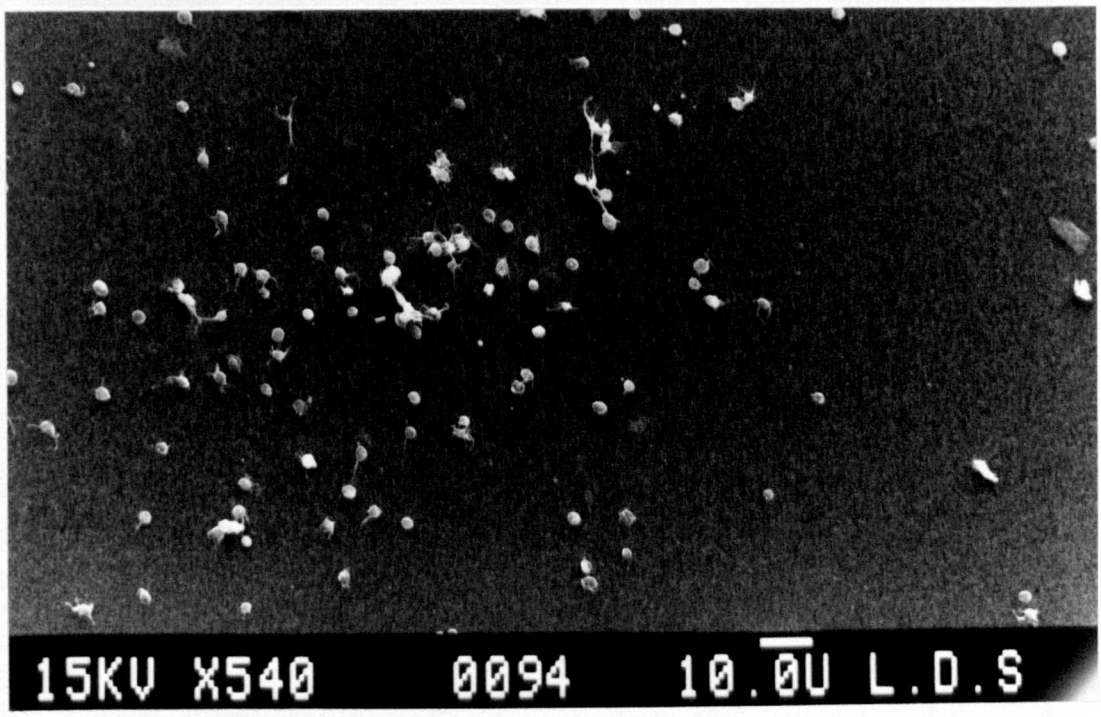
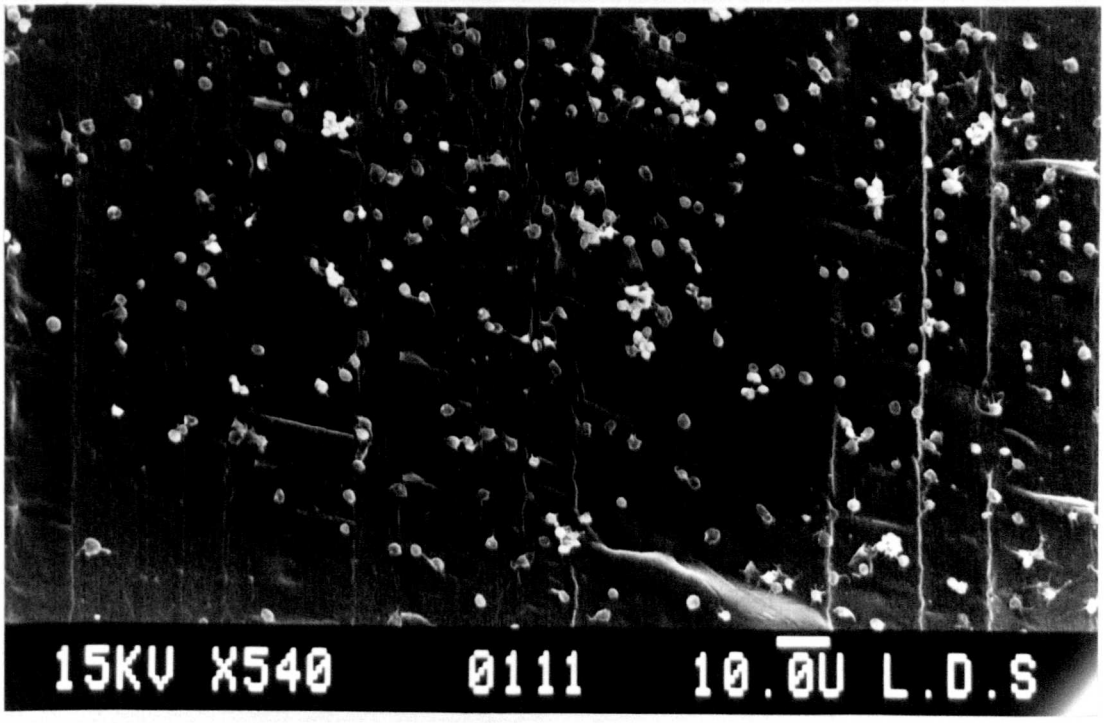
[3] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

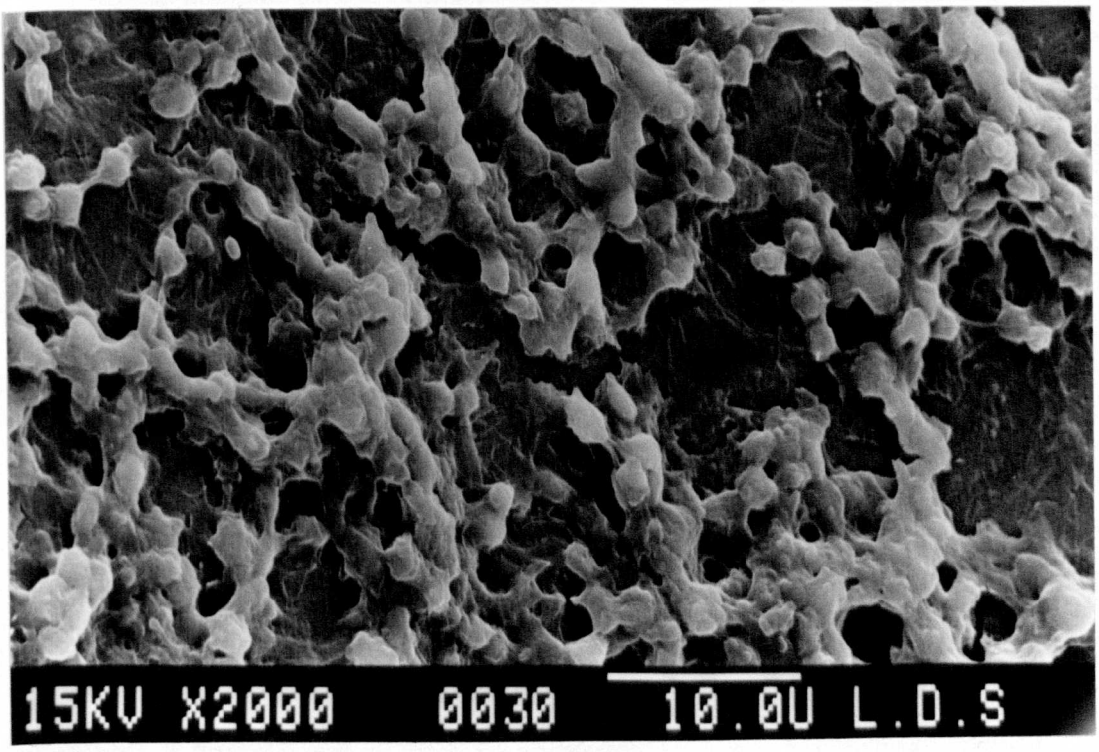


Platelet adhesion to Elastollan

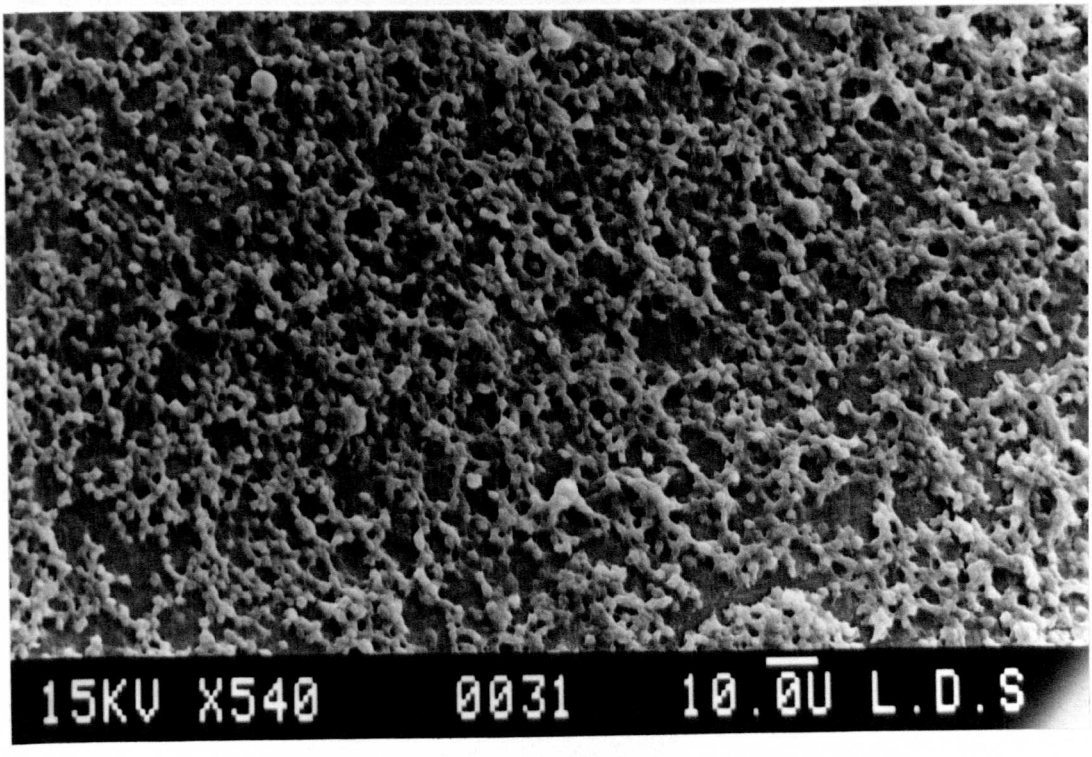


Platelet adhesion to Desmopan

Fig 3.4 Platelet adhesion to medical grade polyurethanes after incubation with PRP at room temperature for 3 hours



Platelet adhesion to PTFE: magnification x2000



Platelet adhesion to PTFE: magnification x540

Fig 3.5 Platelet adhesion to PTFE after incubation with PRP at room temperature for 3 hours

Assessing the exact number of adherent platelets clumped together in an aggregate was extremely difficult (fig 3.5). The membranes of cells appear to be fused, probably by strands of fibrin, making differentiation between discrete cells virtually impossible. The analysis of the area of platelet coverage was also difficult and certainly not reliable. Depending upon the brightness of illumination, results covering a large range could be obtained from the image analyser using the same electron micrograph.

3.4.4 Discussion

The problems with this method relate not only to the reactivity of platelets to the surfaces in question, but also the method of image analysis. With regard to the former, quantification of discrete cells by visual methods can never be successful if they are obscured by other cells and so is an insurmountable problem with any visual methodology. With regard to the latter, the problem is a matter of detail. The problem of illumination dependency is probably due to the gloss of the photographs. A lack of illumination does not allow the analyser to pick up the full diameter of the cell. Too much illumination causes reflection and flaring of the object on the image which causes an exaggeration of the calculated object area. This could be obviated by linking an image analysis system directly to the SEM. This, however, was not a viable option with the equipment available.

Another possible problem regarding this technique is the need to analyse typical areas of the sample. The random selection of areas for analysis may give rise to operator bias. This need not be a problem if many fields are chosen for analysis, but has potential for criticism.

It is desirable, then, to use a method which can detect every platelet. This is possible if the intracellular proteins of the cells are radiolabelled (section 3.5).

3.5 PLATELET [^{51}CR]-RADIOLABELLING

3.5.1 Introduction

Labelling platelet intracellular proteins with ^{51}Cr results in a homogeneous radiation density which allows accurate quantification of platelet deposition. The resultant radioactivity can be related to a known number of platelets in order to calculate the percentage of platelet adhesion. Since the platelets are isolated and washed before labelling, whole blood can be reconstituted with homologous red and white blood cells. Biocompatibility assessment can then be performed in a more physiological manner than that of incubating in PRP.

This assay was based upon that used by Sakariassen *et al.* (1979) which was later modified to allow increases in sensitivity in several ways. Firstly, red cell layer recycling was employed to capture about 95% of the platelet population since most usual methods for obtaining PRP generally yield only 60 - 70% of the total number in a sample of blood (Crook and Crawford, 1988). It has been shown that dense platelets are those which tend to be more reactive towards biomaterials (Cenni *et al.*, 1991) and so more likely to adhere. It is these platelets which are lost in the red cell layer during conventional PRP harvesting.

Secondly, a platelet-plasma separation technique based on the difference between cellular and non-cellular densities was developed by spinning PRP through a high concentration of bovine serum albumin (BSA). Sakariassen's method relied purely on the sedimentation of platelets onto the bottom of tubes by centrifugation. This causes a mild activation of the platelets and possible refractory response, since they are brought into close contact on a surface. Spinning through BSA, on the other hand, tends to pacify the platelets. A whole BSA fraction is harvested and so no cell is in very close contact with its neighbour. The BSA acts as a cushion for the cells to come to rest on. Platelet activation was further reduced by separating platelets from unbound ^{51}Cr with a single pass of the platelet suspension through a Sepharose column, rather than with further centrifugations.

Thirdly, prostacyclin (PGI_2) was included in the blood at the time of collection. PGI_2 is a very potent anti-platelet agent (Moncada *et al.*, 1976) which stabilises the platelet membrane by removing intracellular Ca^{2+}, effectively cutting off internal receptor signalling and preventing aggregation or activation reactions occurring. It has a short half-life in aqueous solution (Moncada *et al.*, 1976), however, and so normal platelet function returns after several hours. The consequence of this is that labelled platelets can be prepared without any of the severe trauma that they suffer during harsh, repeated centrifugations.

3.5.2 Materials and methods

<u>Conventional washing and labelling procedure</u>

<u>Reagents</u>

1. Krebs-Ringer solution: 4mM KCl, 107mM NaCl, 20mM $NaHCO_3$, 2mM Na_2SO_4, 19mM trisodium citrate, 0.5% (w/v) glucose in H_2O (300 mg KCl, 6.25 g NaCl, 1.68 g $NaHCO_3$, 280 mg Na_2SO_4, 5.59 g trisodium citrate, 5.0 g glucose in 1 l H_2O), pH 6.1.

2. Sodium [^{51}Cr]-Chromate: 1 mCi/ml in aqueous solution (Amersham International, Amersham, UK).

3. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.3.

Method

1. Citrated whole blood was collected and divided in two. One half was spun to give PRP and red cells, both being retained.
2. The other half was spun to give platelet poor plasma (PPP) and red cells, again both being retained.
3. The PRP was transferred to a round-bottomed vessel, an equal volume of Krebs-Ringer solution (1) added and centrifuged at 500g for 10 minutes.
4. The supernatant was removed and the pelleted platelets resuspended in 2 ml Krebs-Ringer solution (1), gently rotating the vessel in order to prevent platelet aggregates breaking off from the pellet.
5. The suspension was spun at 500g for 10 minutes, the supernatant removed and 2 ml Krebs-Ringer solution (1) and 20 μCi sodium chromate (2) added. The platelets were resuspended and incubated at room temperature for 20 minutes.
6. The suspension was spun at 500g for 10 minutes, the supernatant removed and the pellet resuspended in 2 ml Krebs-Ringer solution (1) to remove any unbound ^{51}Cr.
7. Step 6 was repeated.
8. The red cells were washed by centrifuging at 500g for 5 minutes in an equal volume of PBS (3) then discarding the supernatant, 4 times.
9. The labelled platelets was reconstituted with the PPP and red cells to give approximately 2.5 x 10^5 platelets/ml and 50% red blood cells. The pH was adjusted to 7.3.
10. The blood was incubated with the sample[4] appropriately.
11. At the end of the time period the blood was poured away and the tube rinsed very carefully with 2 ml Krebs-Ringer solution (1) to remove any non-adherent platelets.
12. The whole catheter tube was placed in a γ-counter tube and the resultant γ emissions counted in a γ-counter (Packard PGD Auto-gamma scintillation counter, Downers Grove, Illinois, USA).

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[4] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

Enhanced washing and labelling procedure

Extra Reagents

4. Prostacyclin (PGI_2) (Sigma, St Louis, Missouri, USA): 1 μg/ml in ethanol (diluted from stock solution of 100 μg/ml in ethanol, stored at -20°C).
5. Modified Tyrodes solution: 138mM NaCl, 2.9mM KCl, 1mM $MgCl_2$, 5mM dextrose, 3.3mM NaH_2PO_4, 20mM HEPES in H_2O (8.06 g NaCl, 216 mg KCl, 203 mg $MgCl_2$, 900 mg dextrose, 515 mg NaH_2PO_4, 4.77 g HEPES in 1 l H_2O), pH 7.4.
6. Sodium tricitrate: 3.8% (w/v) in H_2O (3.8 g sodium tricitrate in 100 ml H_2O).
7. High density BSA solution: 1.36 g BSA (Fraction V, 98% albumin, Sigma, St Louis, Missouri, USA), 350 μl sodium tricitrate (6) in 3.15 ml Tyrodes solution (5).
8. Medium density BSA solution: 1.12 g BSA, 350 μl sodium tricitrate (6) in 3.15 ml Tyrodes solution (5).
9. Low density BSA solution: 1.00 g BSA, 400 μl sodium tricitrate (6) in 3.60 ml Tyrodes solution (5).

Method

1. PRP was harvested as usual, except for the inclusion of PGI_2 (4) at a concentration of 5 ng/ml. The red cell fraction was spun at 800g for 10 minutes to give PPP which was retained.
2. Modified Tyrodes solution (5) was added to the red cell fraction to its original volume and spun at 100g for 20 minutes, the supernatant being retained.
3. Modified Tyrodes solution (5) was again added to the red cell fraction and spun at 80g for 20 minutes, the supernatant being retained and pooled with the previous supernatant.
4. The red cell fraction was spun at 1000g for 10 minutes, the supernatant discarded and the red cell fraction retained.
5. 3 ml high density BSA solution (7) were put into two tubes, 3 ml medium density BSA solution (8) layered on top and 3 ml low density BSA solution (9) layered on top of that, with minimal disturbance to the interfaces of the BSA densities. The original PRP fraction was layered onto the BSA of one tube, the supernatant fractions onto the other and both tubes spun at 700g for 30 minutes.
6. The plasma was removed from the BSA tube of spun PRP and pooled with the PPP fraction.

7. The BSA bands containing platelets (medium density) were removed and applied to the top of a Sepharose 2B column (Pharmacia, Uppsala, Sweden), previously equilibrated with Tyrodes solution (5) and eluted with this buffer.
8. Sodium [^{51}Cr]-chromate (2) was added to the washed platelet suspension at a concentration of 10 μCi/ml and incubated at room temperature for 30 minutes.
9. Unbound ^{51}Cr was removed by eluting the platelets through another Sepharose 2B column, also previously equilibrated with Tyrodes solution (5).
10. Whole blood was reconstituted as before.

3.5.3 Results

The effects of flow rate and platelet suspension (whole blood versus PRP) with regard to platelet adhesion were investigated prior to material comparison. It was felt to be necessary to define the manner in which platelets behaved over a broad set of conditions before choosing experimental conditions to make catheter material comparisons. The adhesion of platelets onto Pellethane over a range of shear rates is shown for whole blood and PRP after perfusion for 15 minutes at 37°C (table 3.1 and fig 3.6).

Shear rate (s^{-1})	Radioactivity detected (cpm)	
	PRP	Whole blood
6.5	-	218
22	387	281
65	183	314
217	-	435
650	91	690
1000	100	895

Table 3.1 Relative platelet adhesion to Pellethane after perfusion for 15 minutes at 37°C at different shear rates. n = 2

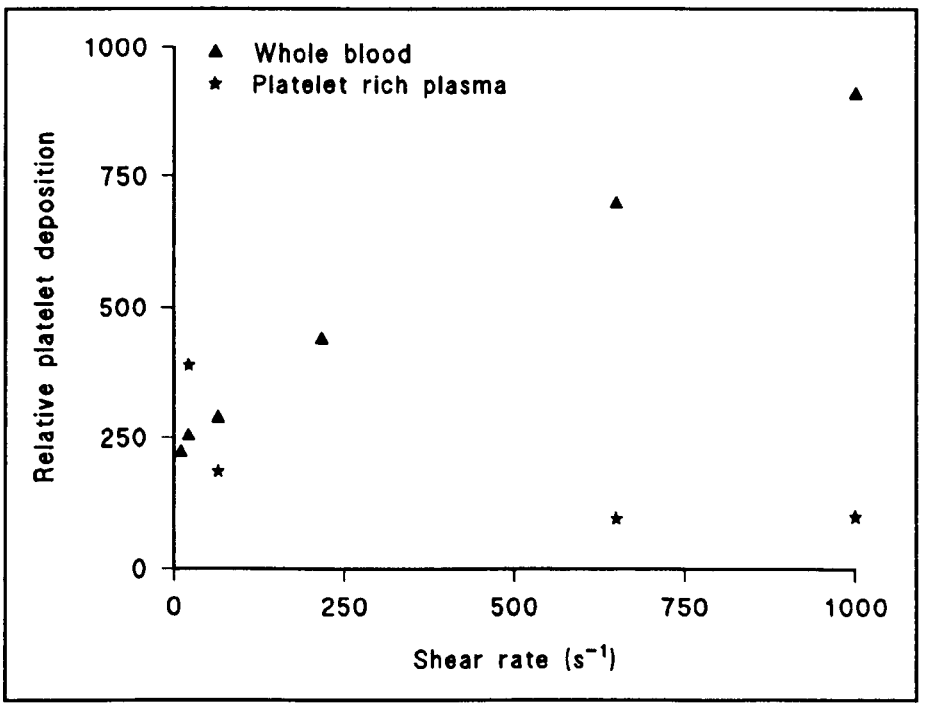


Fig 3.6 Comparison of platelet adhesion in PRP and whole blood after perfusion at various shear rates along Pellethane for 15 minutes. n = 2

The deposition of platelets onto Pellethane at constant shear rate at increasing time periods was also considered. Whole blood was perfused along lengths of Pellethane at 1000 s^{-1} for periods of up to 15 minutes at 37°C (table 3.2 and fig 3.7).

Time (minutes)	[^{51}Cr] detected (cpm)
1	99
2	126
5	168
10	214
15	328

Table 3.2 Platelet adhesion after perfusion of whole blood along lengths of Pellethane at 1000 s^{-1} for various time periods. n = 2

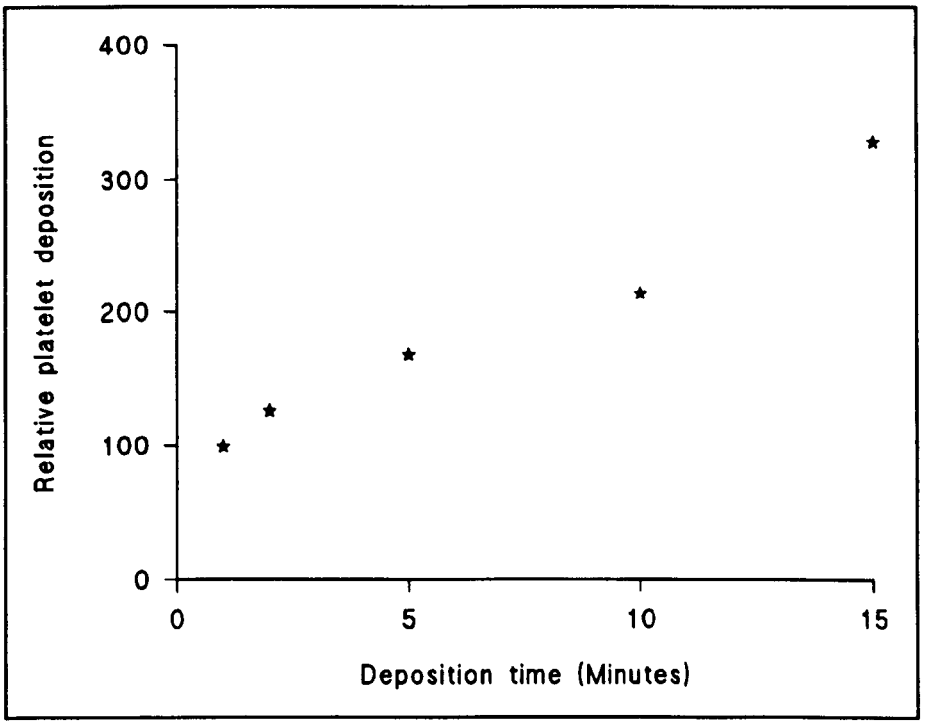


Fig 3.7 Relative platelet adhesion after perfusion of whole blood along lengths of Pellethane at 1000 s^{-1}. n = 2

On the basis of the foregoing experiments, material dependency on platelet adhesion was assessed in whole blood by perfusing at 1000 s^{-1} for 10 minutes at 37°C. 10 minutes was chosen to allow a larger number of comparisons to be made for each sample of blood. The results using the protocol of Sakariassen *et al.* (1979) are shown in table 3.3 and fig 3.8.

Material	Relative Platelet Adhesion (%)	Number of trials
Silicone	61.6 ± 53	4
PVC	65.5 ± 37	3
LDPE	94.5 ± 10	3
Pellethane	100 ± 0	4
Davathane	50.3 ± 42	4
Texin	32.9 ± 46	4
Elastollan 1190	36.0 ± 27	4

Table 3.3 Platelet adhesion to catheter tubing relative to Pellethane at 37°C, 10 minutes at 1000 s^{-1}, using the method of Sakariassen *et al.* (1979). Mean ± S.D.

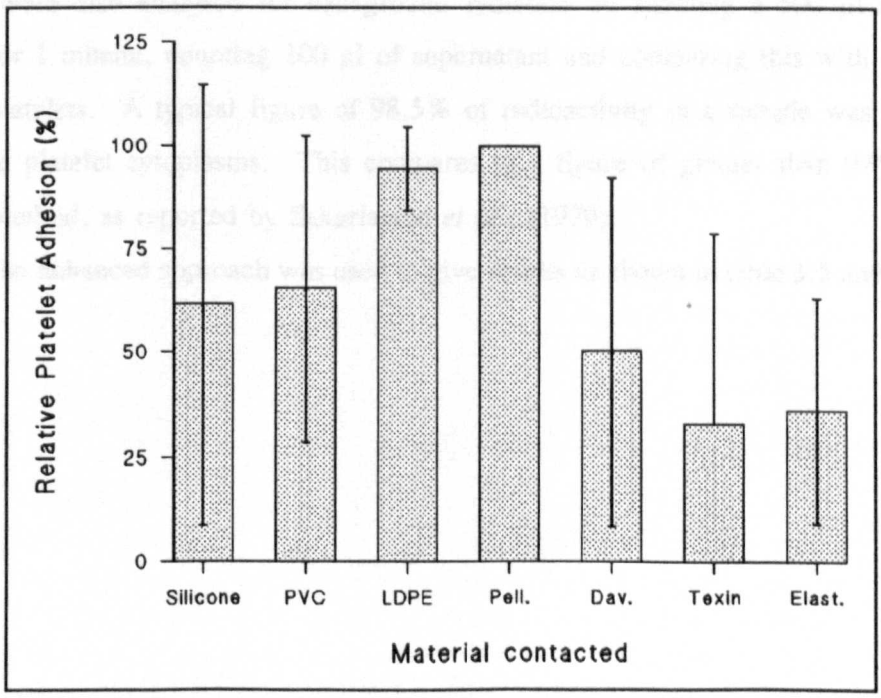


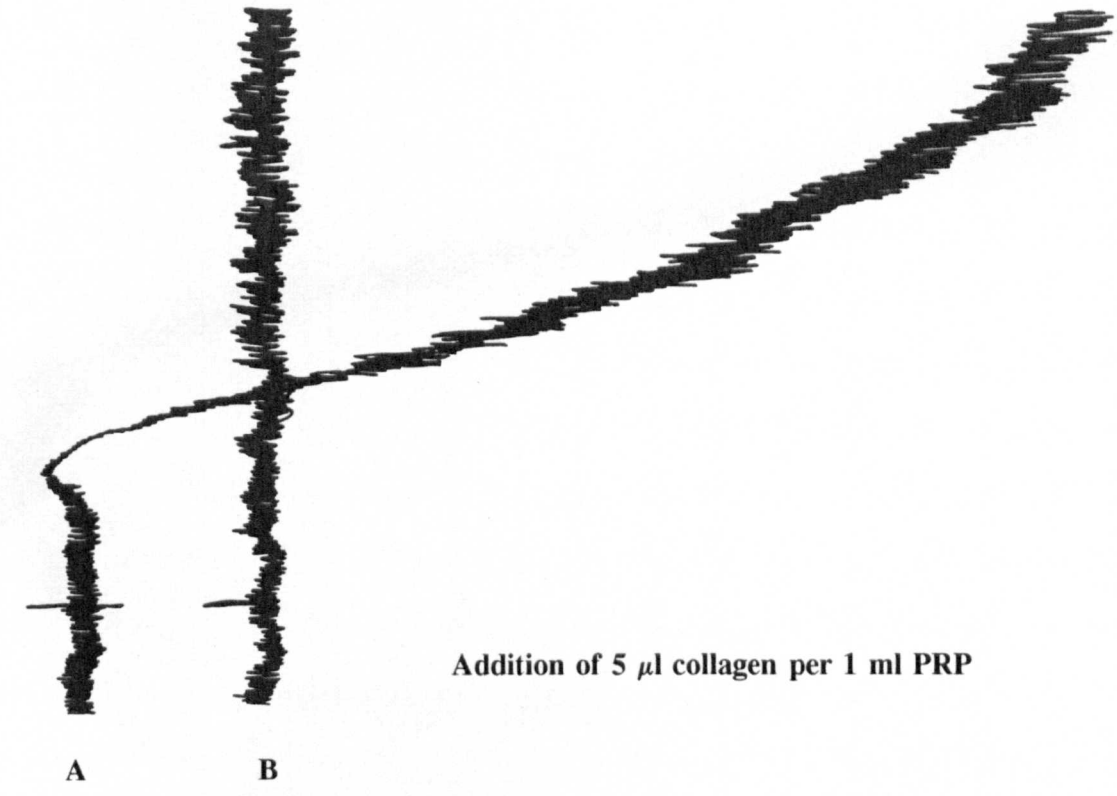
Fig 3.8 Platelet adhesion to catheter tubing relative to Pellethane at 37°C, 10 minutes at 1000 s^{-1}, using the method of Sakariassen *et al.* (1979)

Due to the very large standard deviations in results observed in platelet adhesion to different types of catheter tubing using this method, the modified approach was employed. Clearly, platelet adhesion was occurring, but not in a reproducible manner. The differences between results using the same material were relatively small when using the same sample of blood, but very large from one sample of blood to another. In short, the reactivity of the platelets spanned a huge range when manipulating them using a conventional labelling procedure. The percentage of platelet adhesion to Pellethane of the total number of platelets spanned from 0.6% in one experiment to 5.5% in another.

To assess the efficacy of PGI_2 as a platelet stabilising agent, two samples of blood were prepared, one with PGI_2 at a concentration of 5 ng/ml, the other with no PGI_2. The ability of the platelets to aggregate after the addition of ADP or collagen to each was followed in a platelet aggregometer (fig 3.9). After 2 hours, normal platelet function had returned (fig 3.10). At the same time, an aliquot of each sample was taken and spun at 1000g for 5 minutes in order to produce PPP. The heparin-neutralising activity of the supernatant in each case was assayed, as described in section 4.3. The plasma clotting curves are shown in fig 3.11, demonstrating that during brief spinning platelets do, indeed, undergo α-granule release.

The ability of Sepharose to separate platelets from unbound ^{51}Cr was also assessed. Elution fractions were counted in the γ-counter (table 3.4 and fig 3.12). The pooled platelets were also analysed for background radiation by spinning a 500 μl aliquot at 13500g for 1 minute, counting 100 μl of supernatant and comparing this with 100 μl of washed platelets. A typical figure of 98.5% of radioactivity in a sample was contained within the platelet cytoplasms. This compares to a figure of greater than 97% for the original method, as reported by Sakariassen *et al.*, (1979).

The enhanced approach was used to give results as shown in table 3.5 and fig 3.13.



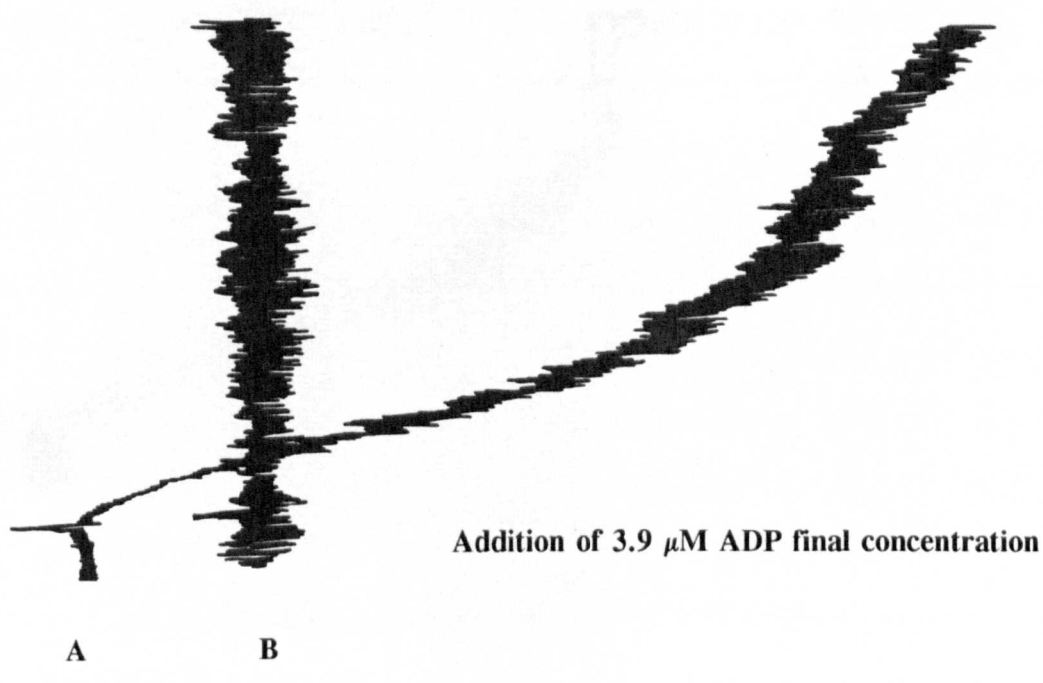
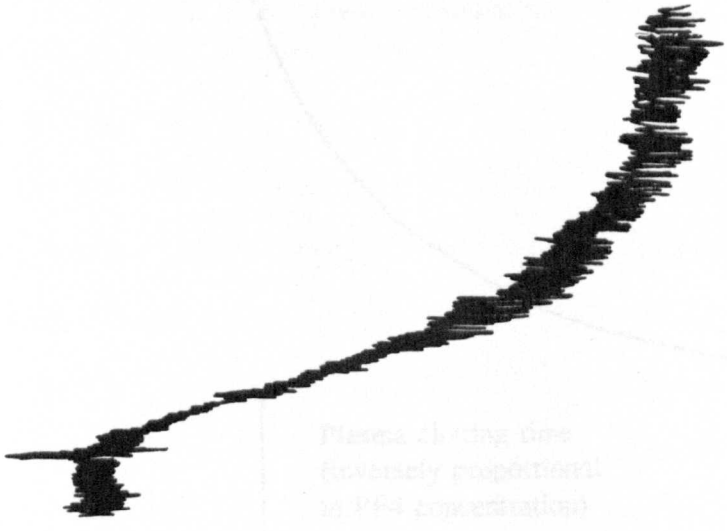


Fig 3.9 Differences in platelet aggregation between normal PRP (A) and PRP colleced into 5 ng/ml PGI_2 (B)

Addition of 5 μl collagen per 1 ml PRP



Addition of 3.9 μM final concentration ADP

Fig 3.10 Platelet aggregation of PRP collected into 5 ng/ml PGI_2 after 2 hours incubation at room temperature

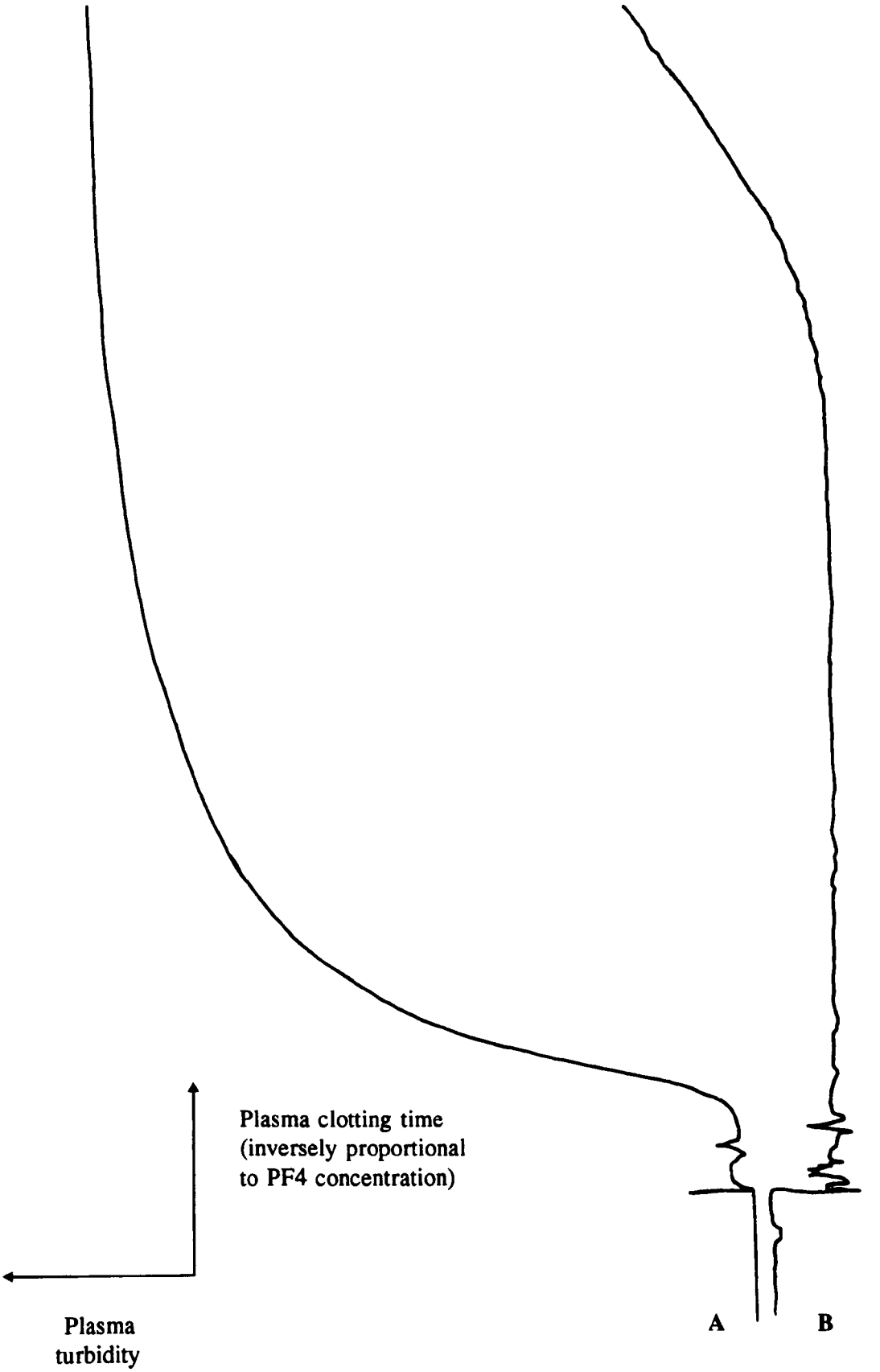


Fig 3.11 Plasma clotting curves of PPP from normal citrated blood (A) and blood collected into 5 ng/ml PGI_2 (B) in a PF4 bioassay

Fraction number	Radioactivity (cpm)
1	505
2	7396
3	2411
4	484
5	305
6	326
7	303
8	878
9	133607
10	285946
11	132261
12	7272
13	767

Table 3.4 Elutions of radioactivity from a Sepharose 2B column after the layering of ^{51}Cr-labelled platelets on top. Each fraction represents about 4 ml

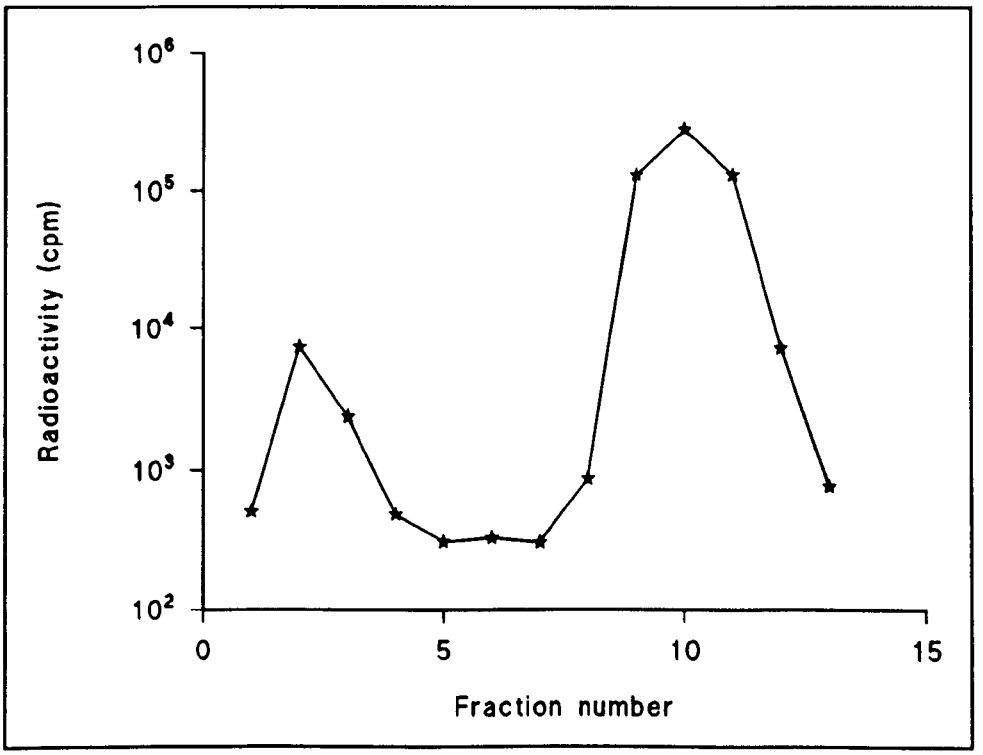


Fig 3.12 Radioactivity eluted from a Sepharose 2B column

Material	Relative Platelet Adhesion (%)
Silicone	29.6 ± 3.5
PVC	31.9 ± 5.7
LDPE	35.8 ± 2.9
Pellethane	100 ± 0
Davathane	38.8 ± 12.9
Desmopan	29.7 ± 2.6
Texin	40.0 ± 4.6
Elastollan 1190	32.7 ± 5.1

Table 3.5 Platelet adhesion to catheter tubing relative to Pellethane after the perfusion of whole blood at 1000 s^{-1} for 10 minutes at 37°C. Mean ± S.D.

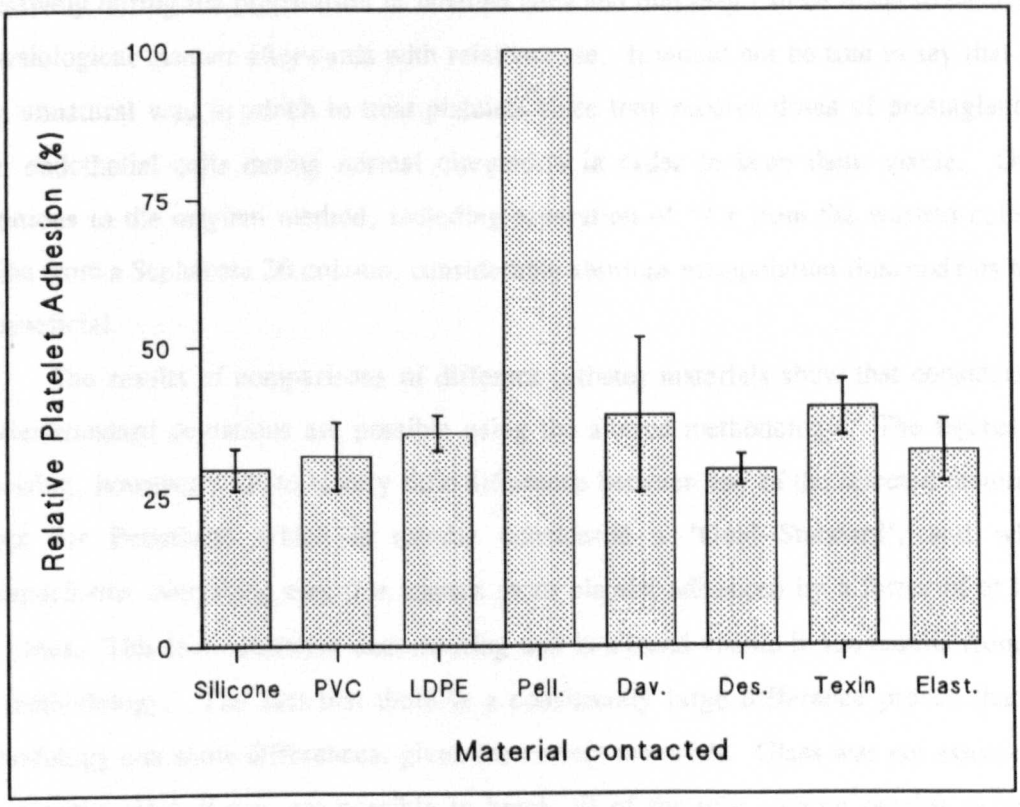


Fig 3.13 Platelet adhesion to catheter tubing relative to Pellethane for whole blood perfused at 1000 s^{-1} for 10 minutes at 37°C

3.5.4 Discussion

Assessment of platelet adhesion in different suspension media (whole blood and PRP) demonstrates that the behaviour is different depending on flow rate. This can be explained thus: the velocity profile of a fluid across a cross-section of tubing during laminar flow is parabolic, with the maximum velocity at the centre and, theoretically, zero flow at the interface between fluid and tube wall (Caro *et al.* 1978). Particles within a fluid will have, therefore, a tendency to travel along the centre of the tube. When perfusing PRP through a tube, the platelets will have an increasing tendency to move away from the wall as flow rate increases, resulting in a reduction in platelet adhesion. When considering whole blood, however, the red blood cells, which have a mean volume approximately 10 times greater than that of a platelet and outnumber them 20 : 1, will tend to occupy the central space. The platelets have, in this case, a greater material contact with increasing flow rate.

The results of PGI_2 incubation show that it is possible to prevent platelets reacting excessively during the preparation of labelled cells and that they can be made to behave in a physiological manner afterwards with relative ease. It would not be true to say that this is an unnatural way in which to treat platelets since they receive doses of prostaglandins from endothelial cells during normal circulation in order to keep them viable. Other alterations to the original method, including separation of ^{51}Cr from the washed cells by elution from a Sepharose 2B column, considerably shortens manipulation time and this must be beneficial.

The results of comparisons of different catheter materials show that considerably smaller standard deviations are possible using the altered methodology. The figures are surprising, however. There is very little difference between any of the selected materials, except for Pellethane which is usually considered a 'Gold Standard', and which underperforms everything else (*i.e.* causes more platelet adhesion) by a factor of at least two times. This is a consistent underscoring and is a trend visible in the results from the old methodology. The fact that there is a consistently large difference proves that the methodology can show differences, given the correct materials. Glass was not assessed in this instance since it was not possible to break all of the tube, whose outside diameter equalled 5 mm and length 1.22 m, into a γ-counting tube. Elutions using 2% sodium dodecyl sulphate (SDS) did not give consistent ^{51}Cr reading and so were not used.

The anomaly regarding Pellethane may be due to the batch of polyurethane. Clearly the causal agent of platelet adhesion in this case was present in all of the tubes

from this batch. If the observed effect was not material composition related, it may have been due to the presence of unusual quantities of plasticiser, antioxidant or processing wax (section 7.3). The differences between the other materials were also quite surprising. Despite the fact that the grading of the blood compatibility of PVC's, silicones and polyurethanes is controversial, one might expect some real differences to be present between these three groups of materials. Large differences have been claimed *in vitro*, *ex vivo* and *in vivo*. This may be due to methodology, however. It is quite probable, for example, that the differences observed in static testing may be artificially large, or even complete artefact. These experiments show that large differences can exist in materials of apparently similar chemical composition and that these can be measured in a flow set-up using shear rates similar to physiological values.

3.6 CONCLUSIONS

In order to assess the degree of platelet adhesion to catheter tubings, four different approaches have been used. These experiments demonstrate that visual techniques are difficult to perform on curved surfaces, but when achieved, can give images of cells which are fused and concealing each other. It is important to use a technique which can accurately quantify the platelets lost to the material. Other techniques, such as those which estimate platelet depletion (Rhodes, 1988; Courtney *et al.*, 1987), would not have been suitable in this test arrangement since the total platelet loss after perfusion through Pellethane, measured using the enhanced protocol, spanned just 0.5% - 0.8% of the total number of platelets. The error in whole blood cell counting tends to be in the order of 1%, which is greater than the platelet adherence.

The results also demonstrate that materials of very similar chemical composition can consistently perform very differently and materials of very different structure and composition can evoke similar responses from platelets. These experiments also demonstrate the need for the assessment of platelet adhesion with the material after incorporation into a device. In this way, materials which are renowned for being very good in a biological environment can be tested against a typical standard and the effect of manufacturing processes and other additives fully assessed.

CHAPTER 4

PLATELET ACTIVATION

4.1 INTRODUCTION

Platelet activation and subsequent degranulation is possibly a better marker of blood compatibility than platelet adhesion (Baier, 1987) since adhered platelets display a variety of different stages of adhesion: initial contact, pseudopodia formation then cytoplasmic spreading into a flat film over the surface (Lindon *et al.*, 1989). Less compatible surfaces cause greater platelet spreading and degranulation, which is not necessarily detected by most platelet adhesion assessment protocols. The implications of platelet activation go much further than the resultant generation of a thrombus, although this event is necessary in order for active membrane lipids to be expressed on the surface of the platelets (Hirsh *et al.*, 1987). The granules of platelets contain many substances including heparin antagonists and leucocyte-chemoattractants, which have great importance in the generation of an immune response and consequently inflammation (Henson and Ginsberg, 1981). The conclusions from these results cannot, therefore, be *directly* correlated with those obtained from platelet adhesion.

The initial approach was to detect a radioactive tracer which had been previously infused into the platelets. This is a simple approach and allows the quick collection of data necessary in order to establish whether platelets do indeed degranulate in response to contact with material surfaces, as would be expected from the extent of platelet adhesion to surfaces witnessed microscopically (section 3.4).

4.2 DETECTION OF [^{14}C]-SEROTONIN

4.2.1 Introduction

This method relies on the doping of the platelet dense granules with ^{14}C-labelled serotonin (5-hydroxy tryptamine or 5-HT). It has previously been described by Lindon *et al.*, (1989). 5-HT is a naturally occurring substance found in platelet dense granules for which there is an active uptake mechanism (da Prada *et al.*, 1981). There are two distinct active uptake mechanisms occurring in platelets. One, the transport from cytoplasm into the dense granules, can be blocked by reserpine (da Prada *et al.*, 1981), whilst the other,

transport across the plasma membrane, can be blocked with imipramine (Talvenheimo *et al.*, 1979). Incubation of platelets with a small quantity of 5-HT labelled with a radioactive tracer will allow almost total uptake. Blocking active uptake with imipramine prevents future uptake after release caused by degranulation. The proportion of radioactivity in the supernatant in relation to the total originally contained in the platelets, after exposure to a surface, is then a measure of activation.

The initial method was applicable to the release of 5-HT into PRP. The use of the technique was also extended to quantification of release in whole blood. Since β-emissions are evaluated by counting the flashes of light from a scintillation fluid, blood cannot be used directly - the haemoglobin present drastically quenches the light. The red blood cell fraction cannot simply be separated and discarded, however: red cells are highly permeable to 5-HT by diffusion because of their small size. There is a further metabolism within the red cells, converting 5-HT into 5–Hydroxytryptaphol, *etc.*, so a simple equilibrium between cytosol and plasma does not exist. A relationship between intracellular and extracellular concentration based on the sample haematocrit cannot be assumed. Extra steps were necessary to separate the supernatant from red cells and extract any radioactivity from red blood cell cytoplasms.

Care was necessary in the storage of the [^{14}C]-5HT. Commercial preparations are packaged under nitrogen. 5-HT is rapidly degraded by aerobic microbes into non-active components which are not taken up by platelets and which cause a high background count. Breaching the seals for exhuming aliquots of solutions allows the ingress of such microbes and oxygen so it was important to return the vial to below -20°C as soon as possible.

4.2.2 Materials and methods

[^{14}C]-5HT labelling of platelets in PRP

Reagents

1. Imipramine : 1mM in H_2O (Sigma, Poole, Dorset, UK). Stock solution is made up as 10mM (31.7 mg in 10 ml H_2O) then diluted 1 + 9 for use.
2. 5-hydroxy-[side chain-2-^{14}C] tryptamine creatinine sulphate: 50 μCi/ml in aqueous solution with 2% (v/v) ethanol (Amersham International, Amersham, UK).
3. Di-n-butyl-phthalate (BDH, Poole, Dorset, UK).
4. Optiphase scintillant (LKB, Bromma, Sweden).

Method

1. PRP was prepared and warmed to 37°C then 1 μl [^{14}C]-5HT (2) was added per 1 ml of PRP and incubated for 30 minutes at 37°C.
2. For each 1 ml PRP, 2 μl imipramine (1) were added and incubated for 5 minutes at 37°C.
3. The PRP was placed in contact with the material[1] for the desired length of time.
4. 150 μl PRP were spun through 100 μl phthalate oil (3) (in order to capture the platelets and any released factors) at 13500g in a microcentrifuge for 1 minute.
5. 100 μl supernatant were transferred to a scintillation vial and 3 ml Optiphase scintillant (4) added and shaken well to disperse any precipitate.
6. β-emissions were counted in a liquid scintillation analyser (Packard 1500 Tri-carb, Downers Grove, Illinois, USA).

Red blood cell radioactivity separation procedure

Extra reagents

5. Bovine Serum Albumin (BSA): 0.1% (w/v) in H_2O (10 mg BSA (Fraction V, 98% BSA, Sigma, St Louis, Missouri, USA) in 10 ml H_2O).
6. Theophylline solution (Sigma, Poole, Dorset, UK): 0.54% (w/v) in PBS (54 mg theophylline in 10 ml PBS (10)).
7. Prostaglandin E_1 (PGE_1) (Sigma, Poole, Dorset, UK): 0.1% (w/v) in H_2O (10 mg PGE_1 in 10 ml H_2O).
8. Inhibitor buffer: 30 μl PGE_1 (7) in 1 ml theophylline solution (6).
9. Separation medium: Mono-Poly (Flow Laboratories, Irvine, UK).
10. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.3.
11. Trichloroacetic acid (TCA): 10% (w/v) in H_2O.

Method

1. After exposure of blood to catheter surfaces[1], 100 μl whole blood were added to 100 μl inhibitor buffer (8) and mixed for 10 seconds.
2. 200 μl separation medium (9) were added to an eppendorf, already pre-washed with BSA (5).
3. 200 μl blood sample were layered onto the separation medium and centrifuged at 330g for 15 minutes to give a plasma and platelet layer and a red blood cell layer.

[1] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

4. The platelet and plasma layer was removed and added to 500 μl PBS (10). The red blood cell layer was added to another aliquot of 500 μl PBS (10).
5. These were spun in a microcentrifuge at 13500g for 30 seconds.
6. The supernatants were removed, added to 3 ml Optiphase scintillant (4), shaken to dissolve any precipitate and counted in a β-counter (Packard 1500 Tri-carb liquid scintillation analyser, Packard, Downers Grove, Illinois, USA). This was the ***Supernatant Fraction***.
7. 3 ml Optiphase scintillant (4) were added to the pelleted platelets, shaken to dissolve any precipitate and counted in a β-counter (Packard 1500 Tri-carb liquid scintillation analyser, Packard, Downers Grove, Illinois, USA). This was the ***Platelet Fraction***.
8. 100 μl water were added to the red blood cell pellet and shaken vigorously for 1 minute to lyse the cells.
9. 200 μl TCA (11) were added to precipitate the haemoglobin and vortex mixed to homogenize the mixture.
10. This was spun in a microcentrifuge at 13500g for 30 seconds in order to pellet the protein precipitate.
11. The supernatant was added to 3 ml Optiphase scintillant (4), the mixture shaken to dissolve any precipitate and counted in a β-counter (Packard 1500 Tri-carb liquid scintillation analyser, Packard, Downers Grove, Illinois, USA). This was the ***Red Blood Cell Fraction***.
12. % Release of platelets = $\frac{\text{(Platelet Fraction)}}{\text{(Platelet + Supernatant + Red Blood Cell Fractions)}}$

4.2.3 Results

4.2.3.1 Applicability of 5-HT release to biocompatibility evaluation after contact with material surfaces

In order to establish whether surfaces of highly activating power and those of lower activating power cause a difference in platelet dense granule release, a series of grossly different surfaces were created by preadsorbing the wells of a 6-welled polystyrene tissue culture dish (Linbro, ICN Flow, High Wycombe, Buckinghamshire, UK) with 4 different proteins, including adhesive proteins: concanavilin A (con A); thrombospondin; fibrinogen; and collagen. Another well was prewashed with PBS only. PRP was added to the five

wells and the dish slowly agitated on a plate shaker for 90 minutes at room temperature. The results showed a marked difference in 5-HT release between the collagen well and all the others after contact for greater than 1 hour, but little difference between any of the other wells (table 4.1 and fig 4.1).

Preadsorbed protein	Percentage of total 5-HT release					
	15 mins	30 mins	45 mins	60 mins	75 mins	90 mins
Collagen	1.8 %	2.6 %	3.4 %	5.9 %	7.4 %	8.9 %
Thrombospondin	1.6 %	1.0 %	1.2 %	2.6 %	2.6 %	3.2 %
Fibrinogen	1.1 %	2.1 %	1.9 %	2.7 %	2.6 %	3.6 %
Con A	1.4 %	3.2 %	3.0 %	3.8 %	3.0 %	4.1 %
PBS	-0.2 %	0.4 %	2.4 %	3.5 %	3.4 %	4.0 %

Table 4.1 Percentage platelet release after incubation of PRP with polystyrene preadsorbed with different proteins, agitated slowly at room temperature

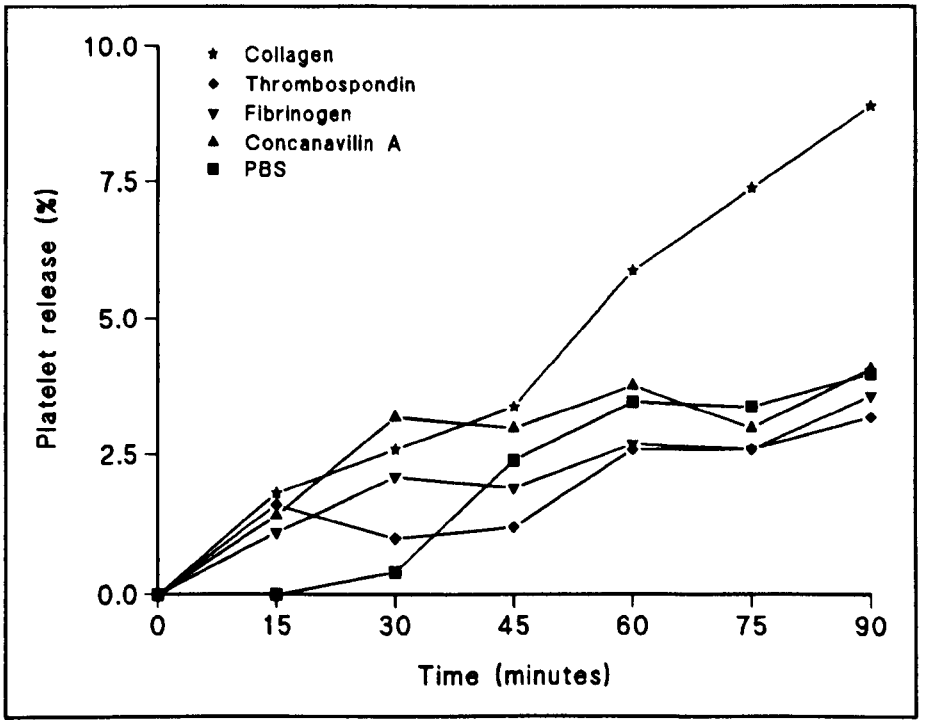


Fig 4.1 Release of [^{14}C]-5HT from platelets after contact with polystyrene preadsorbed with collagen, Con A, fibrinogen, thrombospondin and PBS

In other experiments, to determine whether differences could be demonstrated between materials of different structure without prior protein preadsorption, samples of catheter tubing made of silicone, PTFE and Pellethane were placed into the wells of a 24 well dish (Becton Dickinson Labware, Lincoln Park, New Jersey, USA). PRP was added to the wells and the plate agitated as before, but for 3 hours. No significant differences were observed, however (table 4.2 and fig 4.2).

Material	Percentage of total 5-HT release			
	15 minutes	1 hour	2 hours	3 hours
PTFE	0 %	4.0 %	16.8 %	27.6 %
Pellethane	1.1 %	5.7 %	14.2 %	27.9 %
Silicone	2.7 %	7.1 %	18.6 %	30.5 %

Table 4.2 Percentage platelet release after incubation of PRP with catheter tubing of different structure, agitated slowly at room temperature

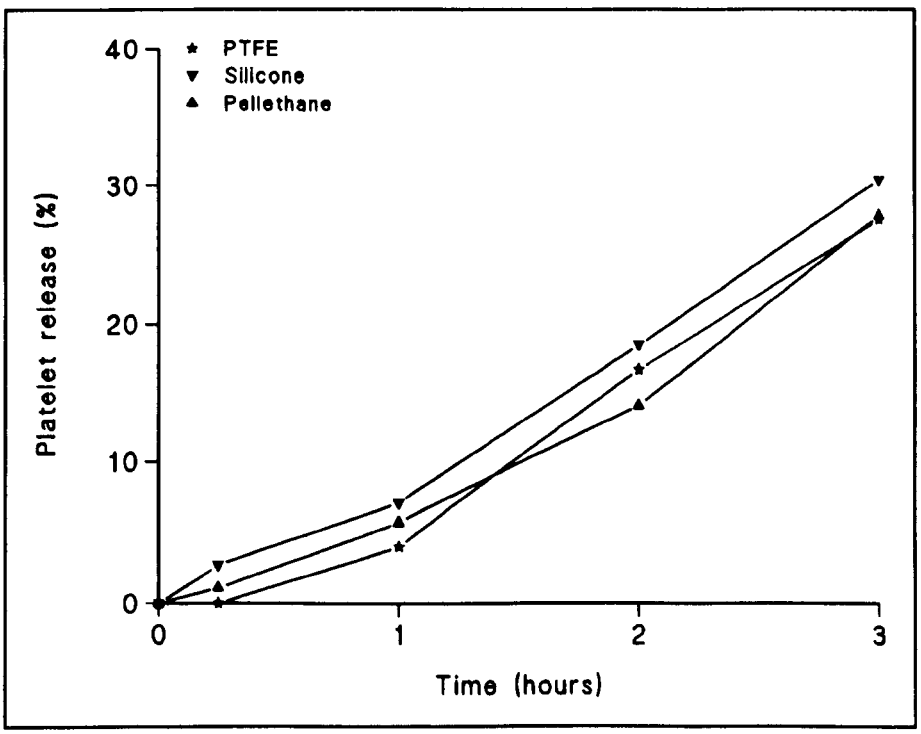


Fig 4.2 Release of [^{14}C]-5HT from platelets after incubation with silicone, PTFE and Pellethane in a polystyrene petri dish

4.2.3.2 Material contact with PRP by perfusion

Since little difference could be ascertained between materials by incubating the surfaces with PRP statically, the effects of perfusion were investigated. In order to evaluate the effects of different flow rates the degree of platelet activation occurring on silicone was analysed. These are shown in table 4.3 and fig 4.3.

Shear rate (s^{-1})	5-HT release (%)
50	0.74 ± 0.14
100	0.83 ± 0.33
250	1.59 ± 0.46
500	1.71 ± 0.38
1000	2.15 ± 0.23

Table 4.3 Release of 5-HT from platelets after perfusion of PRP along lengths of silicone for 10 minutes at 37°C at varying shear rates. Mean ± range, n = 2

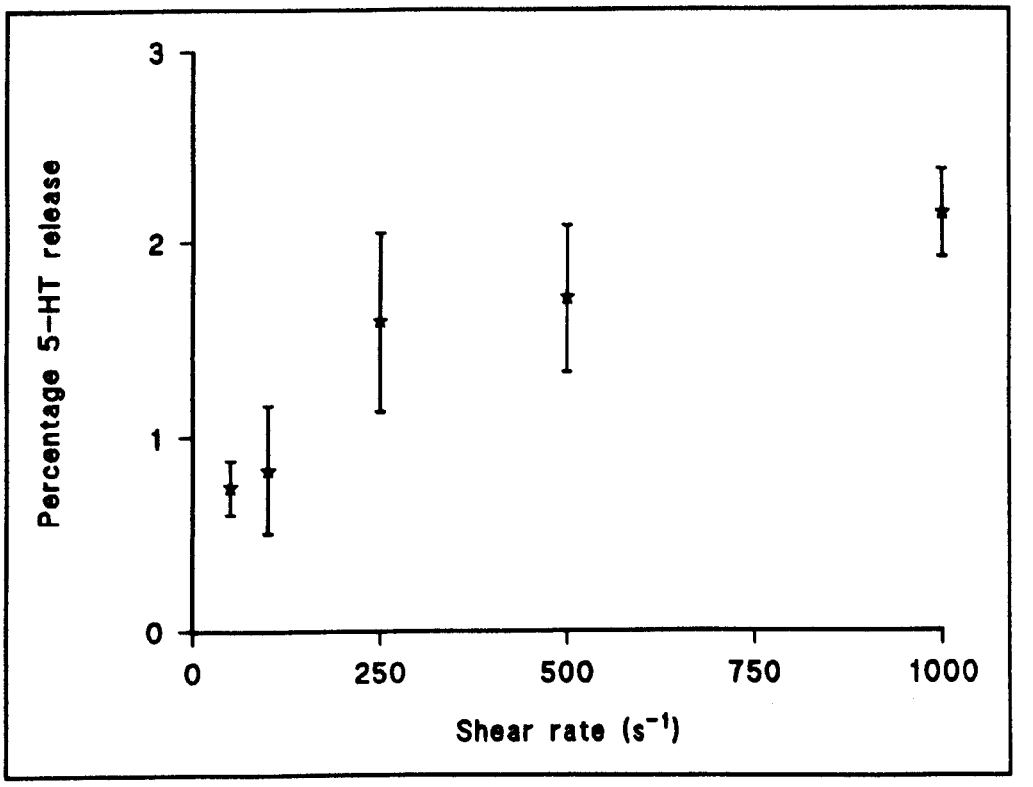


Fig 4.3 Release of 5-HT from platelets after perfusion of PRP along lengths of silicone for 10 minutes at 37°C at varying shear rates. n = 2

The onset of platelet activation at a fixed shear rate (1000 s^{-1}) was also calculated (table 4.4 and fig 4.4).

Perfusion time (Minutes)	5-HT release (%)
2.5	0.13 ± 0.18
5	0.67 ± 0.01
10	0.97 ± 0.60
15	2.29 ± 0.55
30	4.25 ± 0.17

Table 4.4 Release of 5-HT after perfusion of PRP along silicone at 1000 s^{-1} at 37°C. Mean ± range, n = 2

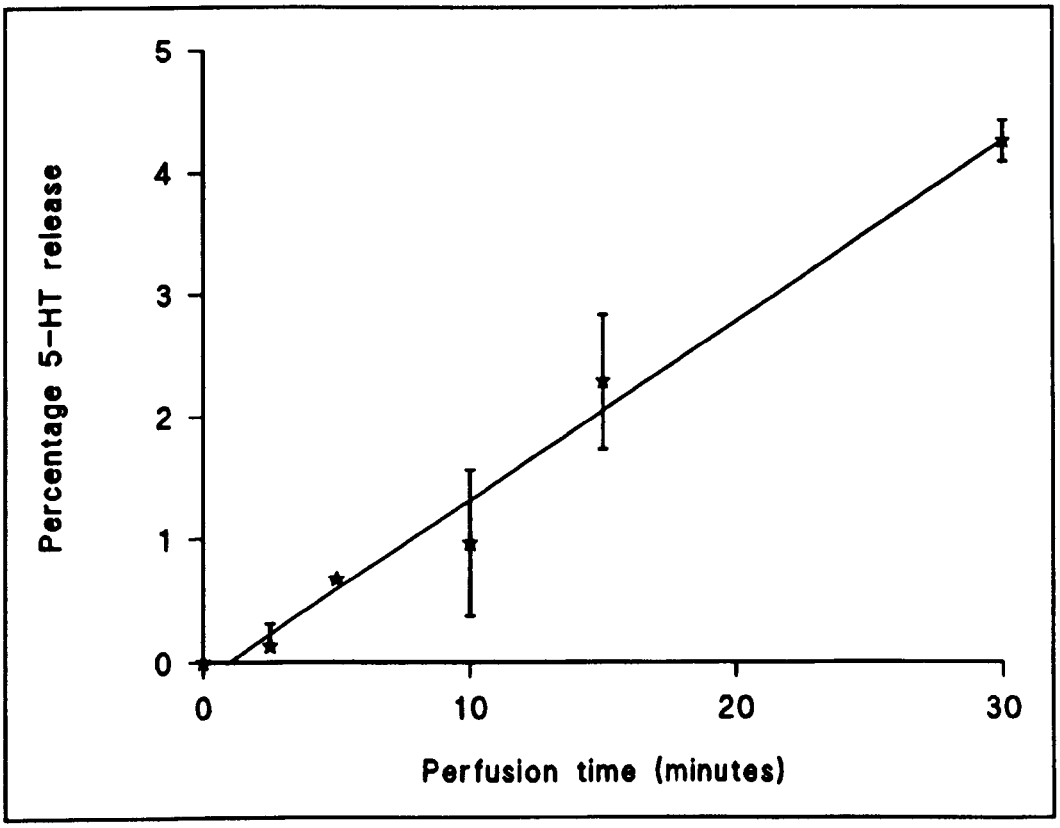


Fig 4.4 Release of 5-HT after perfusion of PRP along silicone at 1000 s^{-1} at 37°C. n = 2

The release of 5-HT in response to perfusion of PRP along lengths of different catheter materials at a fixed shear rate and perfusion time was also performed (table 4.5 and fig 4.5).

Catheter material	5-HT release (%)
Silicone	2.13 ± 1.15
PVC	2.02 ± 0.76
LDPE	2.79 ± 1.26
Pellethane	2.47 ± 1.55
Davathane	3.22 ± 1.17
Texin	3.50 ± 1.30
Desmopan	3.61 ± 1.28

Table 4.5 Release of 5-HT in response to perfusion of PRP along lengths of catheter tubing at 1000 s^{-1} for 10 minutes at 37°C. Mean ± range, n = 2

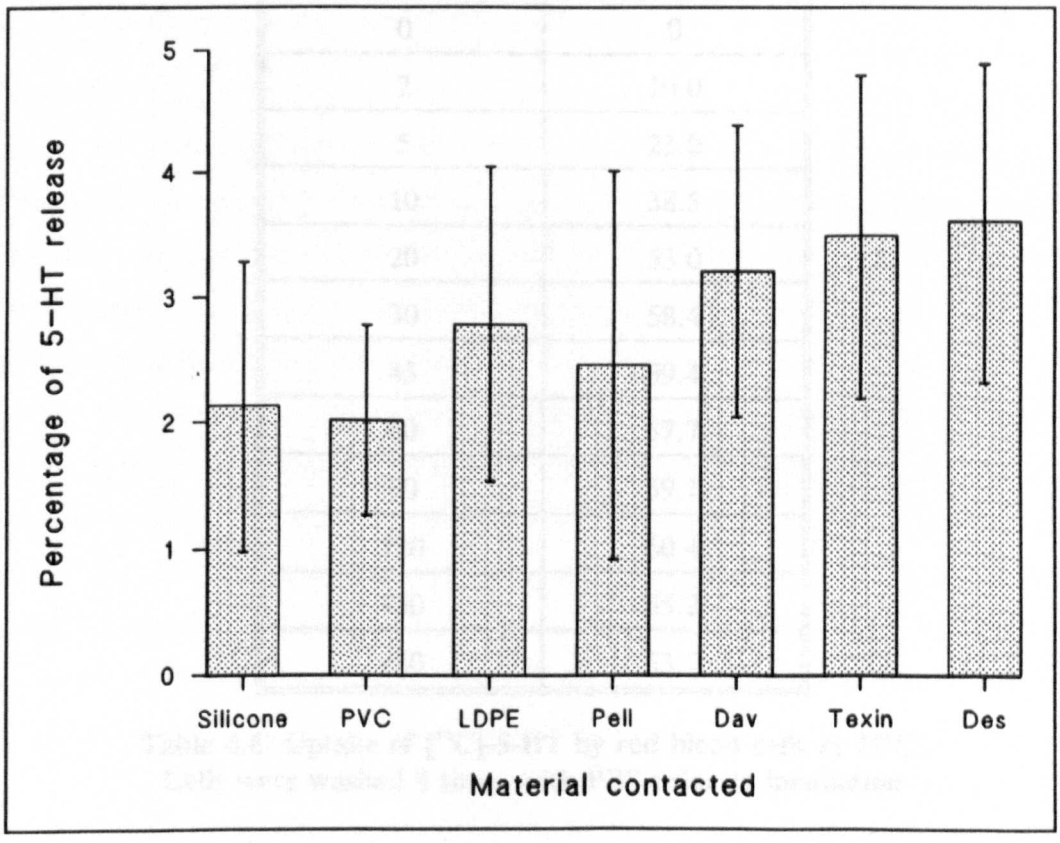


Fig 4.5 Release of 5-HT in response to perfusion of PRP along lengths of catheter tubing at 1000 s^{-1} for 10 minutes at 37°C. n = 2

4.2.3.3 Material contact with whole blood

The extent of the problem of red blood cell uptake of [^{14}C]-5-HT was evaluated by incubating a washed red blood cell population (successive spins at 250g for 10 minutes in PBS, 4 times, discarding the supernatant each time) with [^{14}C]-5-HT. The uptake was calculated from the concentration remaining in the supernatant. The uptake kinetics are shown in table 4.6 and fig 4.6. These indicate that about 50% of any radioactivity released from platelets into the supernatant will be gradually taken into the red cells.

Since there is a significant amount of radioactivity contained in red cells after release, the separation procedure was employed to extrapolate the radioactivity contained in the different whole blood fractions to allow platelet activation to be assessed in whole blood by perfusion. In theory the technique should allow the supernatant and platelet fraction 5-HT concentrations to be calculated but not all the radioactivity could be accounted for in the red cell fractions. This prevented the technique from being used to evaluate platelet activation in whole blood.

Time (minutes)	Percentage uptake
0	0
2	10.0
5	23.0
10	38.5
20	53.0
30	58.4
45	59.4
60	57.7
90	59.1
120	60.4
150	55.2
180	53.7

Table 4.6 Uptake of [^{14}C]-5-HT by red blood cells at 37°C. Cells were washed 4 times with PBS prior to incubation

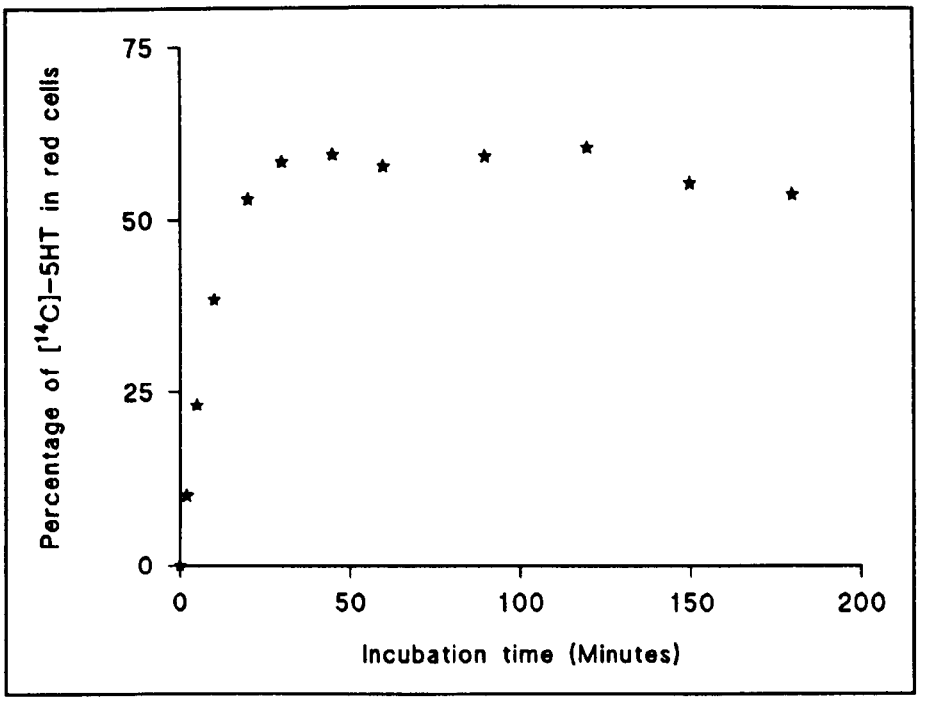


Fig 4.6 Uptake of [^{14}C]-5HT by red blood cells at 37°C

4.2.4 Discussion

The inability to quantify dense granule release in whole blood is disappointing. From other experiments with platelet adhesion (chapter 3) it has been shown that there is a considerable difference in response to catheter tubing when comparing whole blood and PRP. The trends of platelet adhesion between PRP and whole blood are reversed for the same material under the same conditions with increasing shear rate. Any correlation made between results from experiments performed in PRP in comparison with those performed in whole blood, and so any inferences to material performance *in vivo,* must be treated with caution. Different responses, however, were noted between materials using this technique. Materials which caused the least activation were Pellethane, silicone and PVC, with Davathane, Texin and Desmopan causing the largest responses. The difference between the two extremes was, however, small.

The most interesting feature of the observed platelet dense granule release was the increase in activation with increasing shear rate. This is opposite to that observed with platelet adhesion (section 3.5), where adhesion dramatically reduced with an increase in shear when observing PRP. This underlines the difference between platelet activation and adhesion: one can occur without the other and the two are not correlated. This is not very

surprising: one can imagine the potential for granule release caused merely by shear forces which would not lead to adhesion.

The response of platelets to dense granule release over time is different from adhesion. The results show that release occurs as a linear function of time with a correlation of 0.995, whereas platelet adhesion appears to tail off after a very quick initial adhesion. The comparative correlation coefficient for adhesion against time is 0.951. This, too, is not very surprising and suggests that the time scale of platelet adhesion is very much shorter than that of platelet activation.

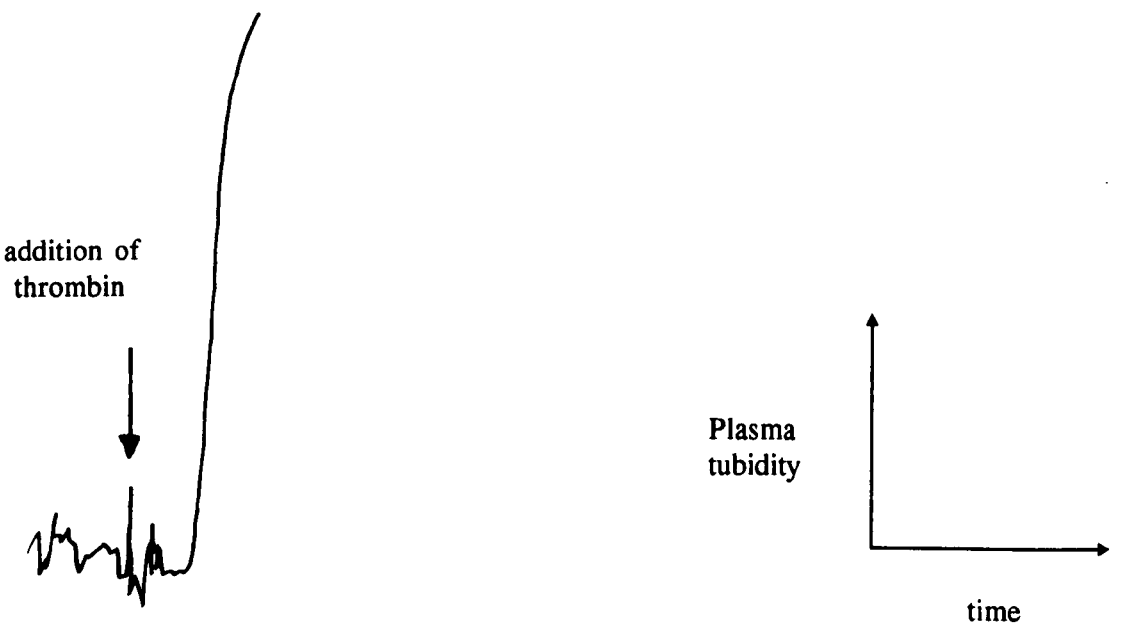
4.3 MEASUREMENT OF PLATELET FACTOR 4 (PF4)

4.3.1 Introduction

Amongst the many substances which are released from the α-granules of platelets is a highly cationic protein, originally discovered because of the ability of platelets to neutralise the anticoagulant effects of heparin (Conley *et al.*, 1948). The released factor was termed Heparin Neutralising Activity (HNA) and was subsequently attributed to Platelet Factor 4 (PF4) (van Crevald and Paulssen, 1951). The presence of very small quantities of heparin can be detected by its ability to inhibit thrombin from clotting an aliquot of plasma. The neutralisation of heparin by PF4 is seen, therefore, as a shortening of the clotting time (Harada and Zucker, 1971). The clotting is followed by measuring the turbidity of the sample tube against time in a platelet aggregometer (fig 4.7), as in PTT analysis (section 5.2). A calibration curve can be constructed by the inclusion of either purified PF4 or protamine sulphate.

This technique was used in order to evaluate platelet activation in a simple manner after exposure to an agonist (*e.g.* a material surface) in whole blood. This was necessary since it was not possible to do this using the release of [^{14}C]-5-HT (section 4.2) and differing responses had been noted in platelet adhesion between PRP and whole blood (section 3.5).

(a) **untreated PRP**



(b) **PRP with 10 μl/ml collagen added**

Fig 4.7 Detection of plasma clotting in a platelet aggregometer in order to measure PF4 release from platelets in plasma from PRP treated differently

4.3.2 Materials and methods

PF4 bioassay

Reagents

1. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.3.
2. Protamine Sulphate (Boots, Nottingham, UK): 25 μg/ml in H_2O.
3. Thrombin, bovine (Sigma, Poole, Dorset, UK): 15 U/ml, diluted immediately before use from stock 200 U/ml in H_2O.
4. Heparin (sodium salt, porcine mucous, Sigma, Poole, Dorset, UK): 0.8 U/ml, diluted immediately before use from stock 1000 U/ml in H_2O.
5. Collagen (Hormon Chimie, Munich, Germany): Acid soluble bovine Type I, 1 mg/ml in isotonic glucose solution, pH 2.7.
6. Theophylline (Sigma, Poole, Dorset, UK): 5.4 mg/ml in PBS (1).
7. Prostaglandin E_1 (PGE_1) (Sigma, Poole, Dorset, UK): 1 mg/ml in ethanol.
8. Inhibitor buffer: 30 μl PGE_1 (7) in 1 ml theophylline (6).
9. Trisodium citrate: 3.8% (w/v) in H_2O (3.8 g trisodium citrate in 100 ml H_2O).
10. Substrate plasma: Blood collected 9 + 1 in trisodium citrate (9) containing 34 μl inhibitor buffer (8) per 1 ml of blood and spun at 1400g to produce platelet-free plasma.

Method

1. The blood samples were activated appropriately[2] then spun at 13500g in a microcentrifuge for 15 seconds to produce sample plasma.
2. All reagents were incubated at 37°C prior to use.
3. 350 μl substrate plasma (10) were incubated in a platelet aggregometer (Payton 300BD (dual channel), Toronto, Canada), already equilibrated at 37°C, for 2 minutes to allow the dispersion of any air bubbles.
4. 50 μl heparin (4), then 50 μl sample plasma and then 50 μl thrombin (3) were added, in that order.
5. The time for the clot to form after the addition of thrombin was taken as the HNA time.

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[2] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

Purification of PF4

Reagents

1. Acid Citrate Dextrose (ACD): 85mM trisodium citrate, 71mM citric acid, 111mM glucose in H_2O (25.0 g trisodium citrate, 14.92 g citric acid, 20.0 g glucose in 1 l H_2O), pH 6.5. Stored at 4°C and filtered before use.
2. Wash buffer: 20mM Tris-HCl, 150mM NaCl, 5mM glucose in 10% (v/v) ACD (3.15 g Trizma-HCl (Sigma, Poole, Dorset, UK), 8.77 g NaCl, 900 mg glucose, 100 ml ACD (1) in 900 ml H_2O), pH 7.6.
3. Suspension buffer: 20mM Tris-HCl, 150mM NaCl, 5mM glucose in H_2O (3.15 g Trizma-HCl (Sigma, Poole, Dorset, UK), 8.77 g NaCl, 900 mg glucose in 1 l H_2O), pH 7.6.
4. Column buffer: 20mM Tris-HCl, 150mM NaCl, 1mM $CaCl_2$ in H_2O (3.15 g Trizma-HCl (Sigma, Poole, Dorset, UK), 8.77 g NaCl, 111 mg $CaCl_2$ in 1 l H_2O), pH 7.6.
5. Elution buffer 1: 20mM Tris-HCl, 600mM NaCl, 1mM $CaCl_2$ in H_2O (3.15 g Trizma-HCl (Sigma, Poole, Dorset, UK), 35.06 g NaCl, 111 mg $CaCl_2$ in 1 l H_2O), pH 7.6.
6. Elution buffer 2: 20mM Tris-HCl, 2.0M NaCl, 1mM $CaCl_2$ in H_2O (3.15 g Trizma-HCl (Sigma, Poole, Dorset, UK), 116.88 g NaCl, 111 mg $CaCl_2$ in 1 l H_2O), pH 7.6.
7. Thrombin, bovine (Sigma, Poole, Dorset, UK): 200 U/ml in H_2O.
8. Phenylmethylsulphonyl fluoride (PMSF): 115mM in H_2O (20 mg PMSF (Sigma, Poole, Dorset, UK) in 1 ml H_2O).
9. Sodium ethylenediaminetetraacetate (EDTA): 1.0M in H_2O (37.22 g EDTA in 100 ml H_2O).
10. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.3.

Method

1. ACD (1) was added to 2 units of platelet concentrate (approximately 10^{11} platelets), (outdated, from blood transfusion service) to a concentration of 20%.
2. Any residual red blood cells were pelleted by centrifugation at 200g for 6 minutes, the platelet-rich supernatant being removed and retained.

3. These were centrifuged at 900g for 12 minutes, with the supernatant being removed and discarded. The pellet was resuspended in 15 ml wash buffer (2). This was repeated two more times.
4. The pellet was centrifuged at 900g for 12 minutes, the supernatant removed and discarded and the platelets resuspended to a final volume of 15 ml in suspension buffer (3).
5. The platelet suspension was heated to 37°C, 225 μl thrombin (7) added and the mixture stirred and incubated at 37°C for 2 minutes.
6. 170 μl PMSF (8) and 1.7 ml EDTA (9) were added.
7. The platelet aggregates were allowed to settle and the supernatant removed and retained.
8. This was spun at 20000g in an ultracentrifuge for 20 minutes.
9. The supernatant was applied to a gelatin-Sepharose column, already equilibrated with column buffer (4), to remove all the fibronectin.
10. The column was eluted with column buffer (4), the eluate collected until the absorption at 280 nm (A_{280}) was zero.
11. The eluate was applied to a heparin-Sepharose column, already equilibrated with column buffer, and eluted with column buffer until the A_{280} was zero. This was discarded.
12. The column was eluted with elution buffer 1 (5) until the A_{280} was zero. This eluate was the fibronectin fraction.
13. The column was eluted with elution buffer 2 (6) until the A_{280} was zero. This eluate was the PF4 fraction.
14. The salt concentration of the eluate was reduced by size exclusion chromatography on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column. The sample was applied to the top of the column and the A_{280} peak eluted with PBS (10).

4.3.3 Results

4.3.3.1 Applicability of PF4 release to biocompatibility evaluation after platelet-contact with material surfaces

To demonstrate that PF4 could be measured using this technique a calibration curve was constructed by including a heparin inhibitor (protamine sulphate, which emulates PF4) in the reaction (fig 4.8).

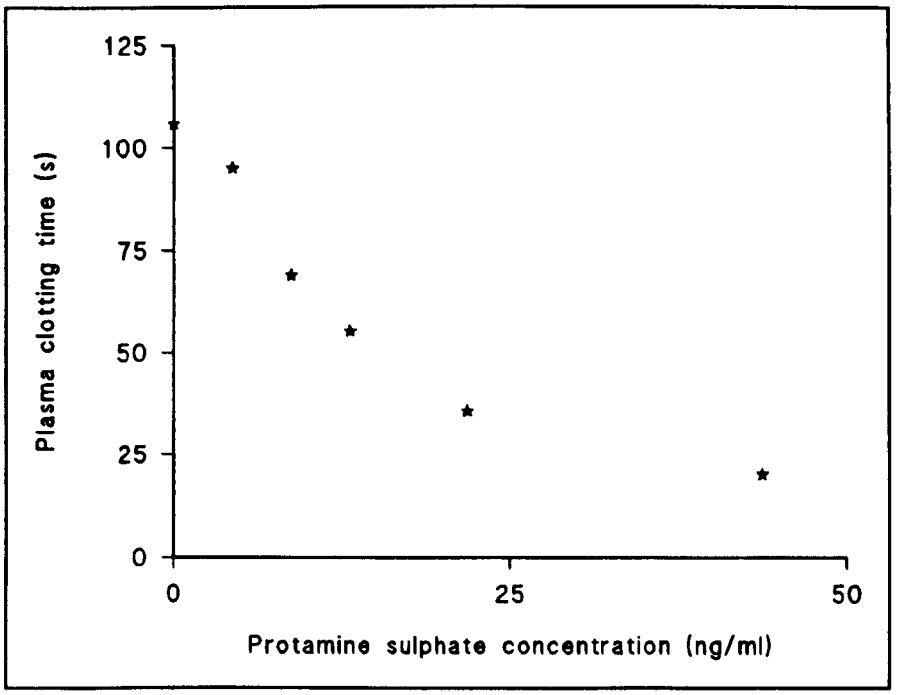

Fig 4.8 Relationship between plasma clotting time in the PF4 functional assay and protamine sulphate concentration

This could be expressed as a linear relationship by plotting the protamine sulphate concentrations as logarithms (fig 4.9).

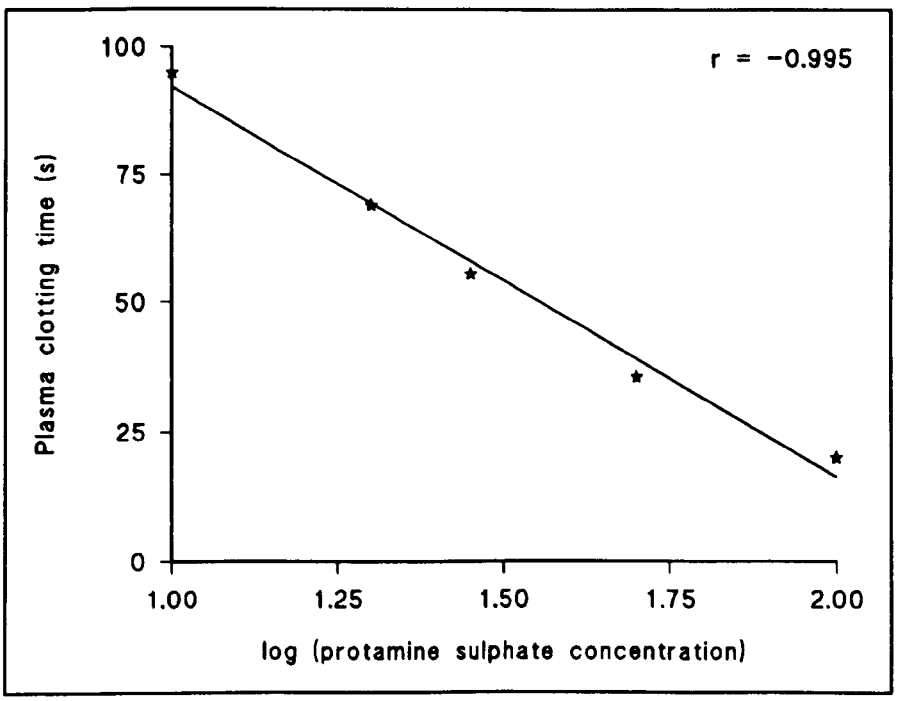

Fig 4.9 Calibration curve for PF4 functional assay

To demonstrate that PF4 was released upon platelet activation, platelets in PRP were activated with varying concentrations of soluble collagen for 1 minute, spun at 13500g in a microcentrifuge for 15 seconds, then assayed in the clotting system. This demonstrated that differing levels of activation could indeed be measured using this assay (fig 4.10). The release was verified by following platelet aggregation in an aggregometer at the same collagen concentrations (fig 4.11).

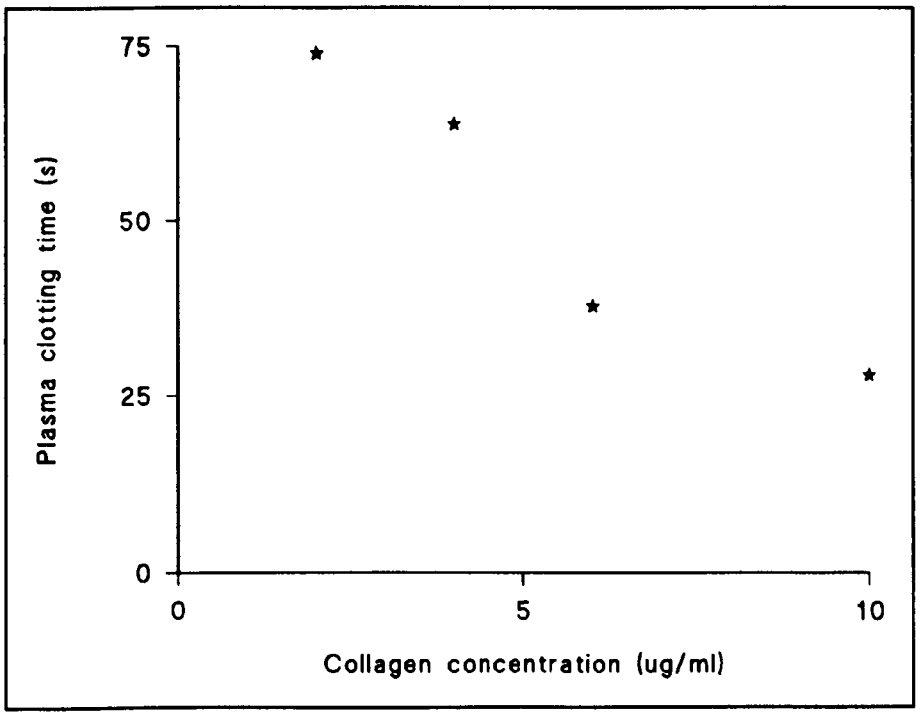

Fig 4.10 Release of PF4 after activation of platelets with various concentrations of collagen as measured in the PF4 functional assay

4.3.3.2 PF4 release in whole blood

Whole blood was perfused through catheter tubing at increasing shear rates (50 s^{-1} - 1000 s^{-1}) and the activated supernatants assayed. No differences were observed between any of the supernatants from blood perfused at different flow rates along lengths of glass. Since PF4 is extremely cationic, it was possible that it was sticking to the glass. This was disproved by attempting to elute PF4 from the glass with high salt concentrations (2M NaCl); no PF4 was detected in the assay system. The same was true for blood perfused through silicone and Pellethane.

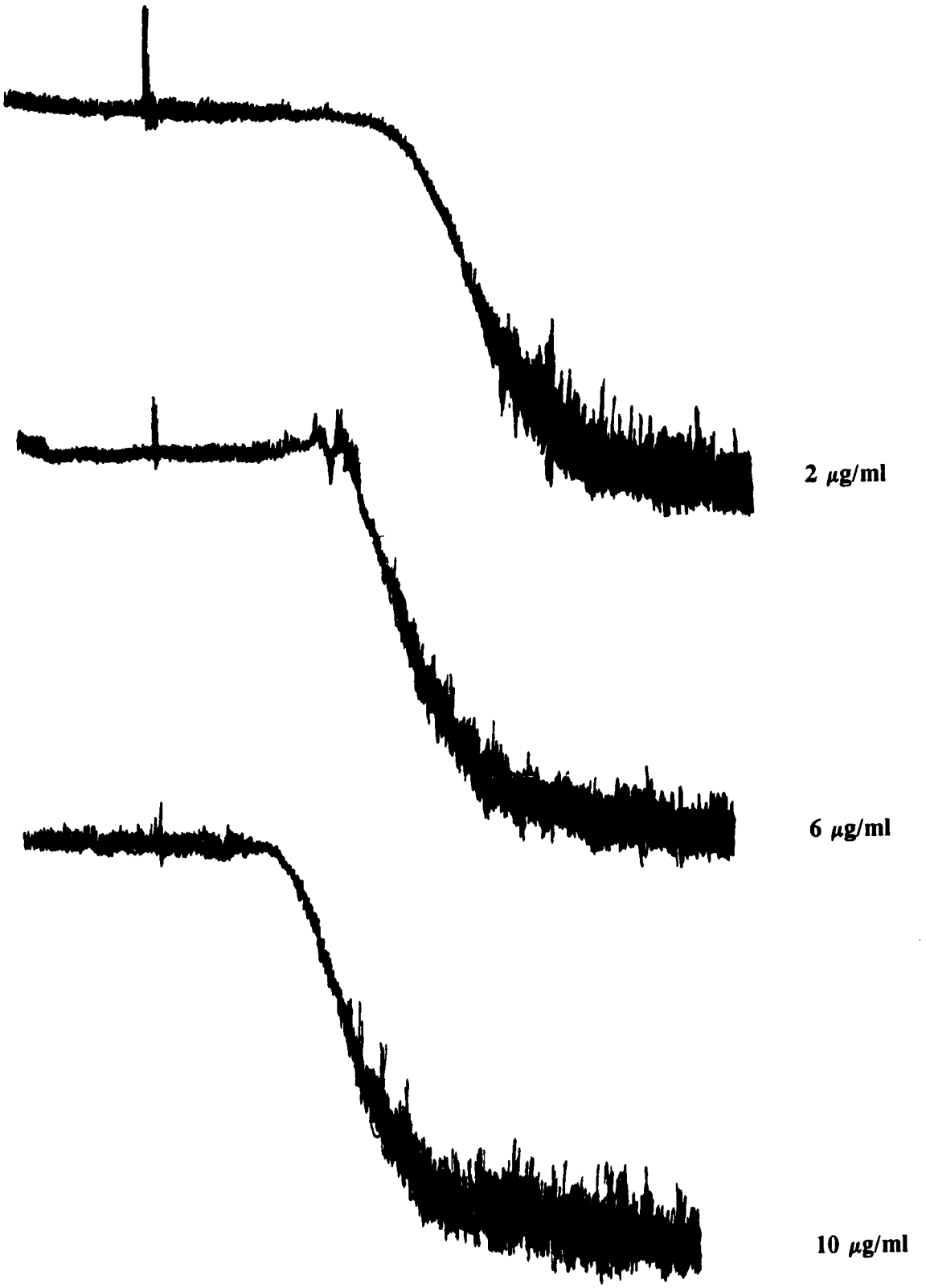

Fig 4.11 Aggregation and granule release of platelets after the addition of various concentrations of collagen

4.3.3.3 PF4 metabolism in different cell suspensions

To investigate the properties of PF4 in the clotting system, purified PF4 (Lawler *et al.*, 1978; Dixit *et al.*, 1984) was produced. PF4 was incubated with a variety of purified cell suspensions, initially platelets, neutrophils and red blood cells, in order to investigate whether they were metabolising PF4. Platelets and red blood cells did not affect the presence of PF4 in the medium, but neutrophils did.

In order to investigate by which process PF4 was being metabolised, Phosphoramidon, a known inhibitor of CD10 (Shipp *et al.*, 1990), was also added to the cell suspension, at a concentration of 20mM. CD10 is a neutrophil surface antigen (common acute lymphoblastic leukaemia antigen) which is also an enzyme (neutral endopeptidase). This had the effect of reducing, but not completely destroying, the metabolism of PF4.

4.3.4 Discussion

It is demonstrable that during centrifugation platelets release α-granule constituents (section 3.5.3). It appears, however, that during the relatively slow perfusion of blood through catheter tubing, either α-granule release does not occur at a detectable level or released PF4 is metabolised. The former seems rather unlikely: traces of PF4 can be detected to a very low concentration depending upon the concentration of thrombin and heparin added to the system. Indeed, it is this very high degree of flexibility with regard to sensitivity which enables this bioassay to be so good at detecting low levels of PF4 in platelet only suspensions and PRP. More likely is the continuing destruction of PF4 by neutrophils as it is released in small quantities over a period of time. This makes the assay of no use to whole blood platelet release measurements.

4.4 MEPACRINE FLUORESCENCE USING FLOW CYTOMETRY

4.4.1 Introduction

Flow cytometry is achieved in a Fluorescence Activated Cell Sorter (FACS). In this technique individual cells are taken from a dilute, isotonic suspension, sized for volume using an aperture impedance transducer (or for cell diameter by measuring the degree of forward light scatter) then analysed for orange and green fluorescence (separately). Many cells, therefore, can be fully analysed very quickly.

The concept behind this method was again to dope platelet dense granules (as in 5-HT release, section 4.2) but in this case with mepacrine (also known as quinacrine). Mepacrine is a fluorophore which fluoresces yellow-green (a similar wavelength to Fluorescein isothiocyanate (FITC)) upon stimulation by ultraviolet irradiation (Lorez *et al.*, 1977). It is also actively and selectively taken up into dense granules. Mepacrine has been used in the past to quantify the number of dense granules in platelets in order to assess the presence of storage pool deficiency (Boneu *et al.*, 1978; Nouvel *et al.*, 1978). When using a normal pool of platelets, a reduction in cellular fluorescence is observed after dense granule release has occurred.

4.4.2 Materials and methods

Reagents

1. Mepacrine: 510μM in H_2O (24.1 mg mepacrine (Sigma, Poole, Dorset, UK) in 100 ml H_2O).
2. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.3.
3. Fixative: 4% paraformaldehyde in PBS (4 g paraformaldehyde in 100 ml PBS (2)).
4. Krebs-Ringer solution: 4mM KCl, 107mM NaCl, 20mM $NaHCO_3$, 2mM Na_2SO_4, 19mM trisodium citrate, 0.5% (w/v) glucose in H_2O (300 mg KCl, 6.25 g NaCl, 1.68 g $NaHCO_3$, 280 mg Na_2SO_4, 5.59 g trisodium citrate, 5.0 g glucose in 1 l H_2O), pH 6.1.
5. FACS buffer: 1% (w/v) Bovine Serum Albumin (BSA), 0.1% (w/v) NaN_3 in PBS (1 g BSA, 100 mg NaN_3 in 100 ml PBS (1)). Stored at 4°C and filtered weekly.
6. Sheath fluid: FACSFlow Diluent (saline solution containing NaCl, disodium EDTA, KCl, KH_2PO_4, Na_2HPO_4) (Becton Dickinson, Cowley, Oxfordshire, UK).

Method

1. PRP was generated.
2. Mepacrine (1) was added to the PRP at a dilution of 1 + 9 (1 ml mepacrine to 9 ml PRP) and incubated at 37°C for 10 minutes.
3. The platelets were reacted as normal[3], as PRP or after reconstitution into whole blood with original white and red cells.

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[3] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

4. After activation, 1 ml of whole blood or PRP was added to 4 ml fixative (3) to prevent further activation.
5. PRP was generated from the whole blood samples.
6. An equal volume of Krebs-Ringer solution (4) was added and the mixture centrifuged at 500g for 10 minutes.
7. The supernatant was removed and discarded, the pellet resuspended in 2 ml Krebs-Ringer solution (4) and centrifuged at 500g for 10 minutes.
8. The supernatant was again removed and discarded, the pellet resuspended in 5 ml FACS buffer (5).
9. 20 μl were transferred to a Falcon FACS tube (Becton Dickinison Labware, Lincoln Park, New Jersey, USA), 1 ml sheath fluid (6) added, vortex mixed, then pushed through a 25 gauge needle. (This prevents blockages within the FACS machine).
10. The cells were then passed through the FACS (Becton Dickinson FACS Analyzer I with Consort 30 software, Sunnyvale, California, USA).

4.4.3 Results

To demonstrate that mepacrine is indeed loaded into platelets, an aliquot of PRP that had been incubated with mepacrine, but not activated in any way, was placed under a fluorescent microscope (Carl Ziess, Germany) and viewed with a x100 oil immersion lens (fig 4.12). However, when the cells were washed and passed through the FACS, no significant increase in fluorescence was noted from that observed with cells not incubated in mepacrine (fig 4.13).

4.4.4 Discussion

There are several reasons why this approach did not work. One is that the mepacrine had leaked out during the washing procedure, after fixation, and only a very small number of platelets had any significant quantity remaining. This is unlikely considering the visual evidence under the fluorescence microscope. More likely, however, is that the filters contained within the FACS, which are used to detect cellular fluorescence for FITC, have too narrow a band width and very little of the fluorescence is passed.

It would be necessary, if this is indeed the case, to change the filters for ones which have a wider band width but this was not a feasible proposition since extra filters are not made for this particular flow cytometer.

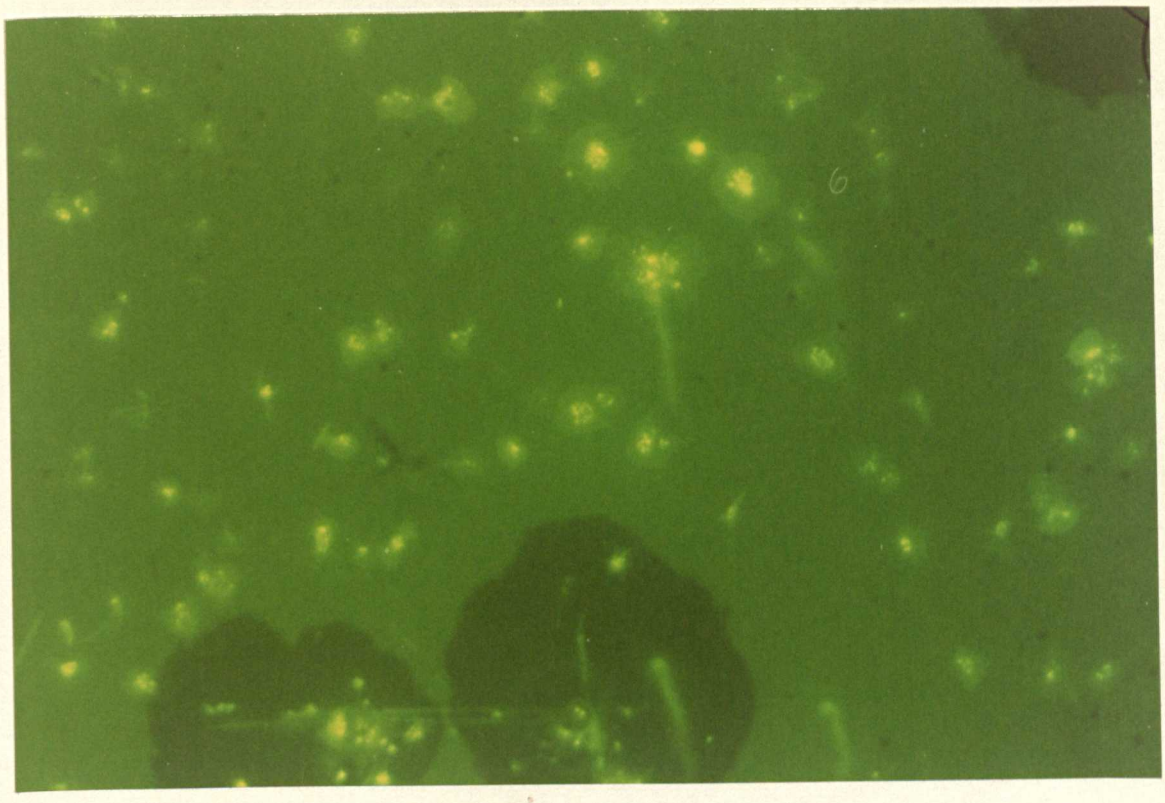
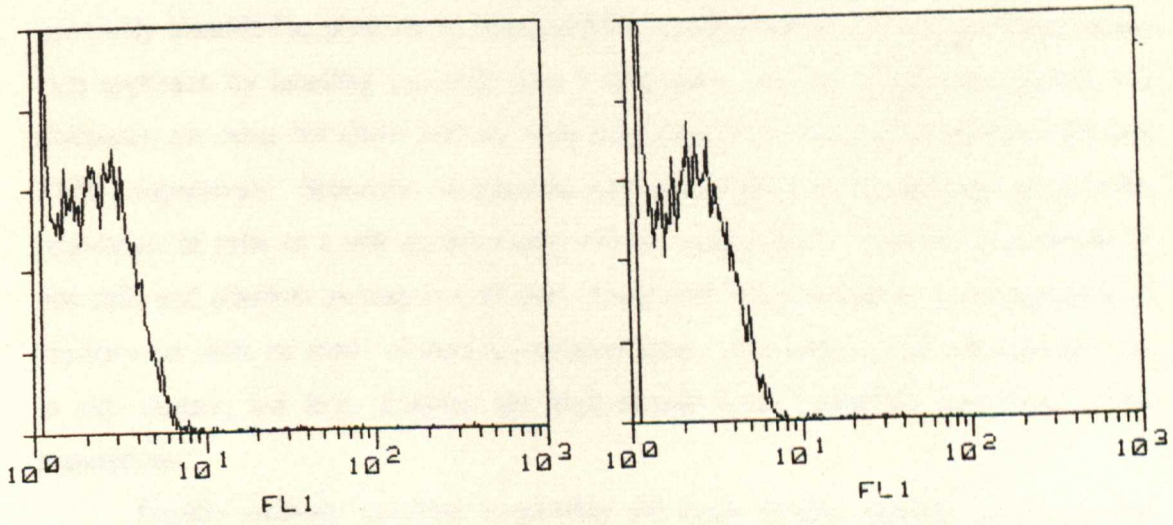


Fig 4.12 Mepacrine loaded platelets viewed with fluorescent microscopy



(a) Untreated platelets **(b) Mepacrine loaded platelets**

Fig 4.13 Fluorescence of mepacrine-treated and untreated platelets in FACS

4.5 Antigenic Topography Using Flow Cytometry

4.5.1 Introduction

If cells are reacted with antibodies specific for antigens on their membranes, which are conjugated with fluorescent markers (*e.g.*, fluorescein isothiocyanate (FITC) for green, phycoerythrin (PE) for orange), flow cytometry can be used to measure the number of these proteins on the cell membrane. The observed fluorescence is directly proportional to the number of epitopes. In recent times, antigenic markers have been discovered on the surface of platelets which indicate platelet activation (Nieuwenhuis *et al.*, 1987; Berman *et al.*, 1989). GMP-140 or PADGEM (Platelet Activation-Dependent Granule to External Membrane protein) appears to be a protein component of the platelet α-granule membrane which becomes part of external cell membrane as the granules diffuse to the outer membrane to release their constituents. This protein is retained after granule release and so is an accurate marker of activation. The same is true for other granules, (*e.g.* lysosomal). In this way, the release from various granules (α- and lysosomal granules) can be quantified.

The quantification of fluorescence emanating from a cell is relatively simple in the case of single cell suspensions. For reasons discussed earlier (section 3.5.1), with respect to quantification using as representative a cell population as possible, and especially the denser platelets, a technique for whole blood analysis was developed. Two approaches were attempted and compared. Firstly, the yellow-green fluorescence on cells which were positively identified as platelets by virtue of their orange fluorescence was quantified in the first approach by labelling the cells with 2 antibodies, one for GPIIb/IIIa (specific for platelets), the other for GMP-140 and both conjugated with fluorescent markers: PE and FITC, respectively. Secondly, the platelets were identified by virtue of the position of the population of cells on a side scatter versus forward scatter plot. Since the populations of red cells and platelets overlap in both side scatter and forward scatter, it is impossible to separate the cells on either of these parameters alone. However, small red cells are low in side scatter, but large platelets are high in side scatter enabling separation of the populations.

Double antibody labelling is possibly the more reliable method, given suitable antibodies. It is, however, considerably more expensive. The methods were also compared for ease of use and speed.

4.5.2 Materials and methods

Platelet only staining procedure

Reagents

1. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.3.
2. Inhibitor solution: 100 μg PGI_2 (Sigma, St Louis, Missouri, USA) in 1 ml ethanol.
3. FACS buffer: 1% (w/v) Bovine Serum Albumin (BSA), 0.1% (w/v) NaN_3 in PBS (1 g BSA (Fraction V, 98%, Sigma, Poole, Dorset, UK), 100 mg NaN_3 in 100 ml PBS (1)). Stored at 4°C and filtered daily to 0.22 μm.
4. Paraformaldehyde: Stock - 4% in PBS (4 g paraformaldehyde in 100 ml PBS (1)). Filtered to remove any precipitate and stored at 4°C, wrapped in silver foil. Diluted 1 + 3 in PBS (1) and filtered to 0.22 μm for use.
5. Mab 2.17 (CD-62) - mouse anti-human GMP-140 (donated by HK Nieuwenhuis, University Hospital of Utrecht, Netherlands). Diluted 1 + 99 in PBS (1).
6. Mab 2.28 (CD-63) - mouse anti-human lysosomal granule membrane protein (donated by HK Nieuwenhuis, Utrecht, Netherlands). Diluted 1 + 99 in PBS (1).
7. FITC Conjugate diluting solution: 1% (v/v) human AB serum in FACS buffer (100 μl human AB serum (Blood Group Reference Laboratory, Oxford, UK) in 10 ml FACS buffer (3)). Made up weekly, filtered and stored at 4°C.
8. Sheath fluid: FACSFlow Diluent (saline solution containing NaCl, disodium EDTA, KCl, KH_2PO_4, Na_2HPO_4) (Becton Dickinson, Cowley, Oxfordshire, UK). Filtered to 0.22 μm.
9. Goat anti-mouse FITC conjugate: 500 mg/ml FITC (Becton Dickinson, San José, California, USA). Diluted 1 + 9 in conjugate diluting solution (9) for use then centrifuged at 3500g for 15 minutes.

Method

1. The platelets were reacted with the desired surface[4] in the usual manner.
2. From the perfused whole blood, 1 ml PRP was generated and transferred to a round bottomed tube.
3. 10 μl inhibitor solution (2) were added to prevent further activation, then centrifuged at 600g for 10 minutes. The supernatant was removed and discarded.

[4] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

4. The cells were resuspended in 3 ml FACS buffer (3) and centrifuged at 600g for 10 minutes, twice, discarding the supernatant each time.
5. The cells were resuspended in 3 ml paraformaldehyde (4) and incubated at room temperature for 5 minutes.
6. This was centrifuged at 600g for 10 minutes twice, discarding the supernatant each time and resuspending in FACS buffer (3). Final concentration was 10^8 platelets/ml.
7. 20 μl of each antibody (5, 6) were placed into the appropriate wells of a round-bottomed 96-well microtitre plate (Nunc/Gibco, Paisley, Scotland, UK) and 100 μl platelet suspension added to each.
8. The plate was agitated on a vortex mixer to mix the solutions and incubated for 30 minutes on ice.
9. 100 μl FACS buffer (3) was added to each well, centrifuged for 2 minutes at 1000g at 4°C and the remaining liquid discarded by quickly inverting the plate once.
10. Step 9 was repeated.
11. 100 μl diluted conjugate (9) were added to each well, agitated on a vortex-mixer for a few seconds then incubated on ice for 30 minutes.
12. The wells were washed as in step 9, twice.
13. After removing the supernatant a few drops of sheath fluid (8) were added to each well.
14. The contents of each well were transferred to a Falcon FACS tube (Becton Dickinson Labware, Lincoln Park, New Jersey, USA), 1 ml sheath fluid (8) added, vortex mixed, then pushed through a 25 gauge needle. (This prevents blockages within the FACS machine).
15. The cells were then passed through the flow cytometer (Becton Dickinson FACS Analyzer I with Consort 30 software, or Coulter Epics Profile II).

Whole blood staining procedure using double antibody labelling

Extra reagents

10. Mouse anti-human GMP-140 antibody conjugated to FITC (The Binding Site/Immunotech, Birmingham, UK).
11. Mouse anti-human GPIIb/IIIa antibody conjugated to PE (Serotech, Oxford, UK).
12. Modified Tyrodes buffer: 138mM NaCl, 2.9mM KCl, 1.0mM $MgCl_2$, 3.3mM NaH_2PO_4, 5mM dextrose, 20mM HEPES, 0.3% (w/v) Bovine Serum Albumin (BSA) in H_2O (8.06 g NaCl, 216 mg KCl, 203 mg $MgCl_2$, 515 mg NaH_2PO_4, 900

mg dextrose, 4.77 g HEPES, 3.0 g BSA (Fraction V, 98% BSA, Sigma, St Louis, Missouri, USA) in 1 l H_2O), pH 7.4. Filtered to 0.22 μm and stored at 4°C.

Method

1. After inhibiting the platelets in the perfused blood, 100 μl whole blood were transferred to various wells in a round-bottomed 96-well microtitre plate (Nunc/Gibco, Paisley, Scotland, UK).
2. 20 μl of each conjugated antibody (10, 11) were added to each well and incubated for 30 minutes on ice (to limit non-specific binding) and in the dark (to limit fluorescence deterioration).
3. 50 μl fixative (4) were added to each well and incubated for 10 minutes on ice in the dark.
4. The plate was centrifuged at 1500g for 2 minutes at 4°C and the supernatant discarded.
5. 100 μl modified tyrodes buffer (12) were added to each well and the cells resuspended using a pipette and vortex-mixer.
6. The cells were transferred to a Falcon FACS tube (Becton Dickinson Labware, Lincoln Park, New Jersey, USA) and diluted further with modified tyrodes buffer (12) to allow cells to be counted at a rate of less than 1000 cells per second.
7. Platelets were analysed in a flow cytometer (Coulter Epics Profile II) by gating in for positive orange fluorescence (GPIIb/IIIa) and counting only green fluorescence (GMP-140) for this population.

Whole blood staining procedure using single antibody labelling

Method

1. After inhibiting the platelets in the perfused blood, 100 μl whole blood were transferred to various wells in a round-bottomed 96-well microtitre plate (Nunc/Gibco, Paisley, Scotland, UK).
2. 20 μl primary antibody (5, 6) were added to each well and incubated on ice for 30 minutes.
3. 50 μl FITC-conjugated secondary antibody (9) were added to each well and incubated on ice in the dark for 30 minutes.
4. 60 μl fixative (4) were added to each well, pipette mixed and incubated on ice for 10 minutes.
5. The plate was centrifuged at 1500g for 2 minutes at 4°C and the supernatant discarded.

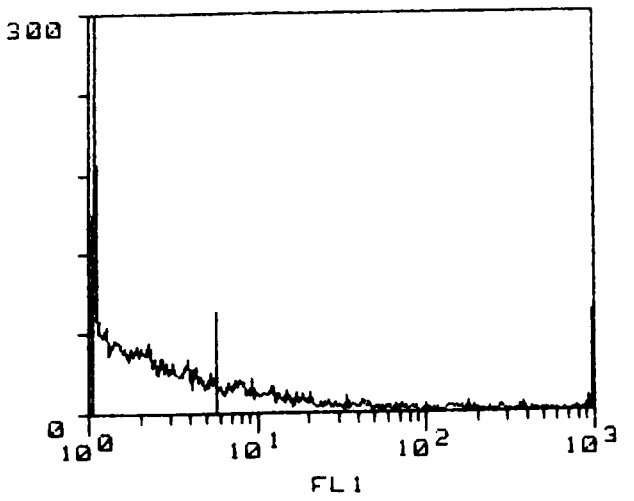
6. 100 μl modified tyrodes buffer (12) were added to each well and the cells resuspended using a pipette and vortex-mixer.
7. The cells were transferred to a Falcon FACS tube (Becton Dickinson Labware, Lincoln Park, New Jersey, USA) and diluted further with modified tyrodes buffer (12) to allow cells to be counted at a rate of less than 1000 cells per second.
8. Platelets were analysed in a flow cytometer (Coulter Epics Profile II) by gating into a positive platelet population as shown in side scatter against forward scatter.

4.5.3 Results

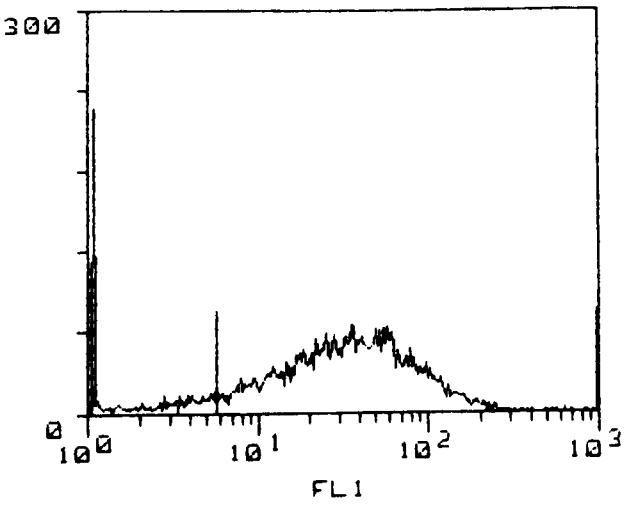
To demonstrate the difference between the fluorescence of highly activated platelets and resting platelets, PRP was taken from citrated whole blood and activated with 5 units of thrombin (bovine, 200 U/ml, Sigma, Poole, Dorset, UK) per 1 ml plasma with the simultaneous addition of 250 units of streptokinase (from *Streptococcus*, Sigma, Poole, Dorset, UK) from stock 25000 U/ml. This sample was inverted once, then incubated without agitation for 2 hours at 37°C to provide completely activated cells. These, and platelets from resting PRP, were then stained using the original protocol and assessed using flow cytometry. The result is shown in fig 4.14 which indicates that there is a very large difference between activated and unactivated cells.

Using activated cells, produced in the same way as above, with red cells added to a haematocrit of approximately 50%, the double labelling procedure was assessed for its ability to recognize platelets and display their fluorescence. This it was able to do, but with additions of antibody being best at 50 μl per antibody. This would have allowed only 40 determinations to be made per bottle of antibody which would have been prohibitively expensive. Instead, platelet gating was assessed by virtue of measured forward and side scatter.

An aliquot of fresh, diluted, whole blood was passed through the flow cytometer to determine the correct location of the platelets on the bit-map (2-dimensional contour map of the distribution of forward scatter against side scatter). This resulted in the production of three distinct populations: red blood cells, platelets and debris (despite the filtration of the cell suspension buffer to 0.22 μm). The platelet population was gated-in and the volume (forward scatter) of this population checked against washed platelets. To be sure of the cells' identity, the ability of the gated population to express GPIIb/IIIa was tested using a mouse anti-human GPIIb/IIIa antibody conjugated with PE. This showed that almost all the cells being detected (greater than 99%) were platelets.



(a) Normal, resting platelets



(b) Thrombin stimulated platelets

Fig 4.14 Fluorescence of cells after staining for GMP-140 in a flow cytometer with and without thrombin stimulation

With this ability to analyse platelets from citrated whole blood, catheter tubing was assessed for its ability to activate platelets by perfusion at 1000 s^{-1} for 10 minutes at 37°C for the secretion of α-granules (table 4.7, fig 4.15) and lysosomal granules (table 4.8, fig 4.16).

Material	Mean fluorescence
Glass	1.642 ± 0.046
Silicone	1.389 ± 0.097
PVC	1.385 ± 0.078
LDPE	1.396 ± 0.103
Pellethane	1.319 ± 0.130
Davathane	1.244 ± 0.084
Desmopan	1.254 ± 0.072
Texin	1.270 ± 0.110
Elastollan	1.264 ± 0.058

Table 4.7 Secretion of α-granules after perfusion of whole blood along catheter tubing for 10 minutes at 1000 s^{-1} and 37°C. Mean ± S.D., n = 6

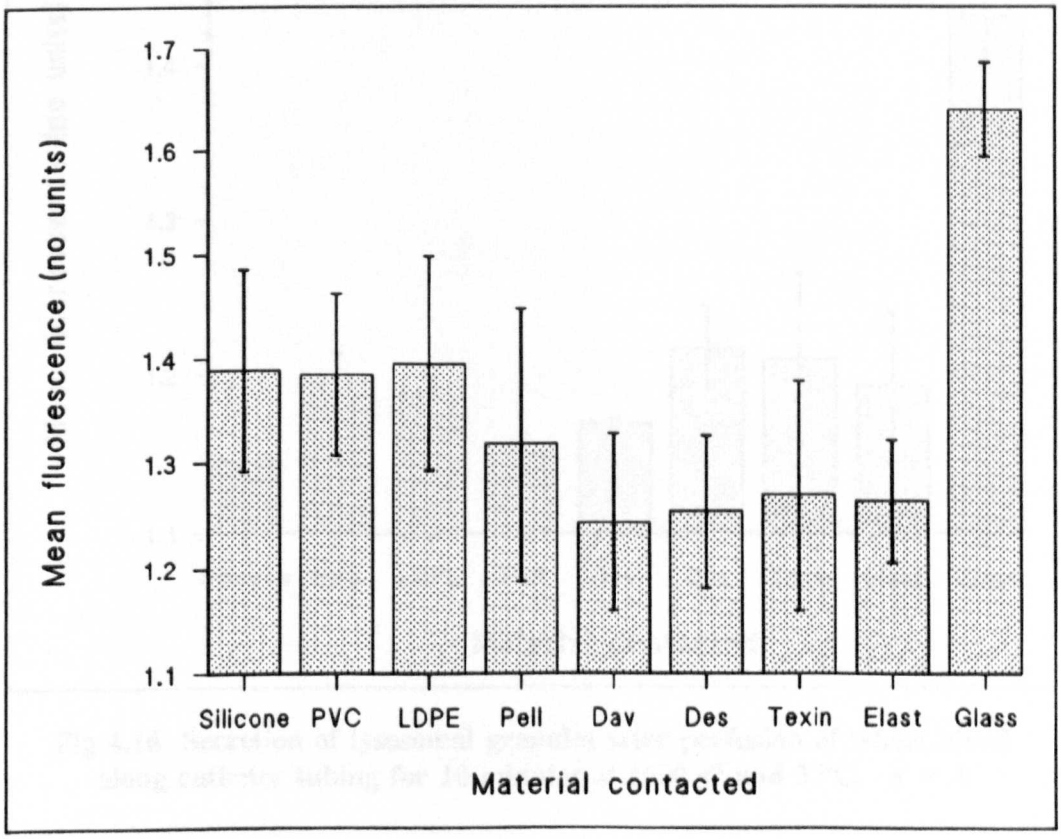


Fig 4.15 Secretion of α-granules after perfusion of whole blood along catheter tubing for 10 minutes at 1000 s^{-1} and 37°C. n = 6

Material	Mean fluorescence
Glass	1.432 ± 0.066
Silicone	1.145 ± 0.012
PVC	1.200 ± 0.014
LDPE	1.186 ± 0.013
Pellethane	1.155 ± 0.008
Davathane	1.169 ± 0.003
Desmopan	1.218 ± 0.027
Texin	1.211 ± 0.055
Elastollan	1.194 ± 0.048

Table 4.8 Secretion of lysosomal granules after perfusion of whole blood along catheter tubing for 10 minutes at 1000 s^{-1} and 37°C. Mean ± S.D., n = 4

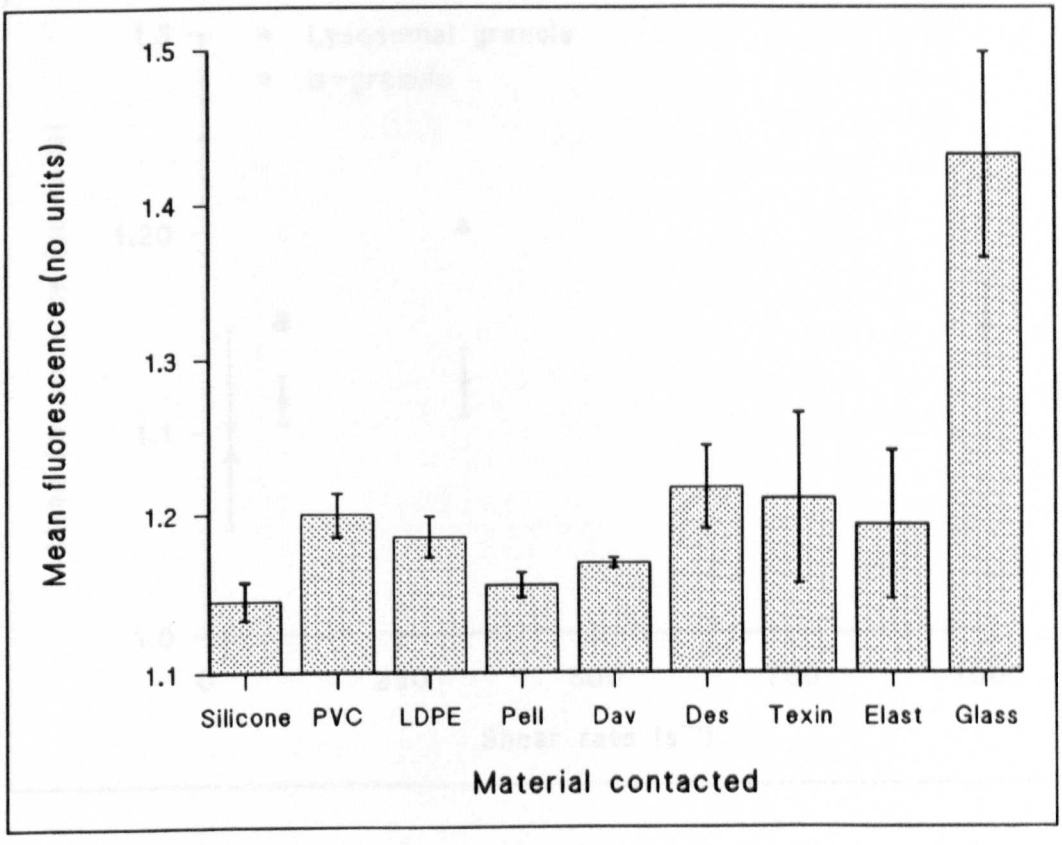


Fig 4.16 Secretion of lysosomal granules after perfusion of whole blood along catheter tubing for 10 minutes at 1000 s^{-1} and 37°C. n = 4

To assess the way in which platelet degranulation occurs with respect to shear rate, α-granule and lysosomal granule release were assessed after platelet perfusion through Pellethane over a range of flow rates. These are shown in table 4.9 and fig 4.17.

Shear rate (s^{-1})	Mean fluorescence (no units)	
	α-granule	lysosomal granule
33	1.104 ± 0.050	1.088 ± 0.036
100	1.116 ± 0.012	1.155 ± 0.004
333	1.125 ± 0.017	1.203 ± 0.003
1000	1.227 ± 0.021	1.153 ± 0.022

Table 4.9 Effect of shear rate on platelet degranulation due to perfusion of whole blood through Pellethane at 37°C for 10 minutes. Mean ± range, n = 2

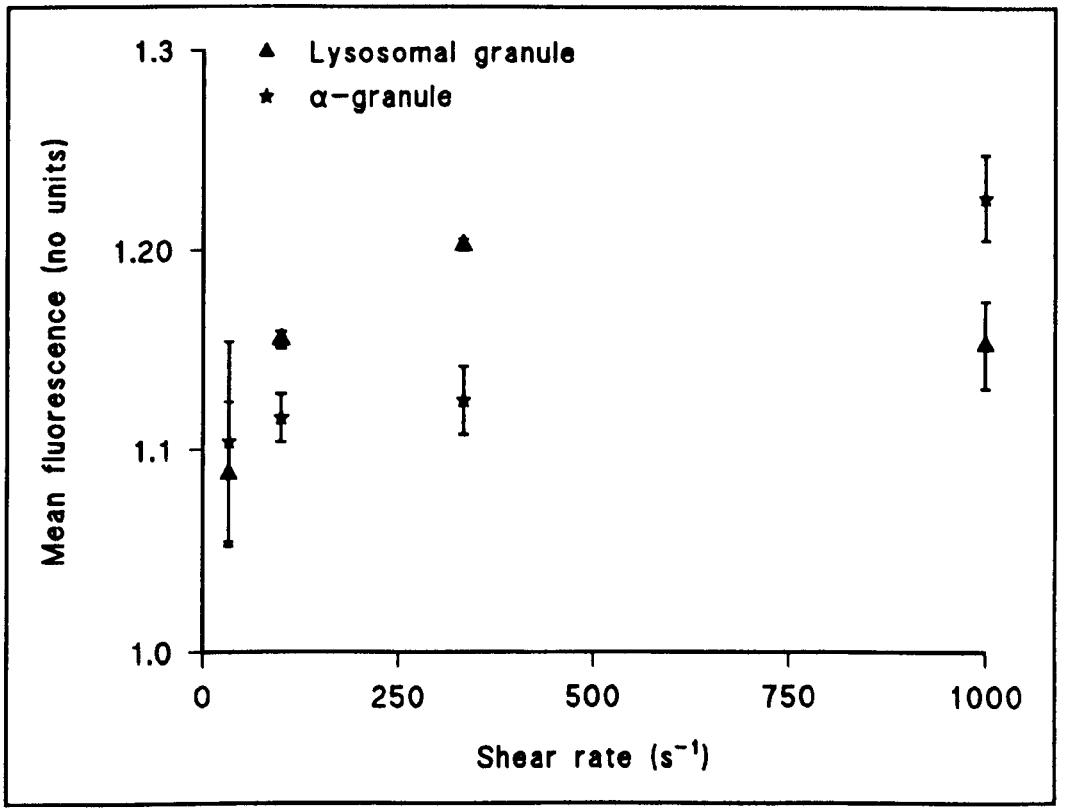


Fig 4.17 Effect of shear rate on platelet degranulation due to perfusion of whole blood through Pellethane at 37°C for 10 minutes. n = 2

Shear dependency was also assessed in a highly activating material, glass. This is shown in table 4.10 and fig 4.18.

Shear rate (s^{-1})	Mean fluorescence (no units)	
	α-granule	lysosomal granule
33	1.408 ± 0.050	1.343 ± 0.017
100	1.705 ± 0.055	1.532 ± 0.024
333	1.523 ± 0.021	1.384 ± 0.014

Table 4.10 Effect of shear rate on platelet degranulation due to perfusion of whole blood through glass at 37°C for 10 minutes. Mean ± range, n = 2

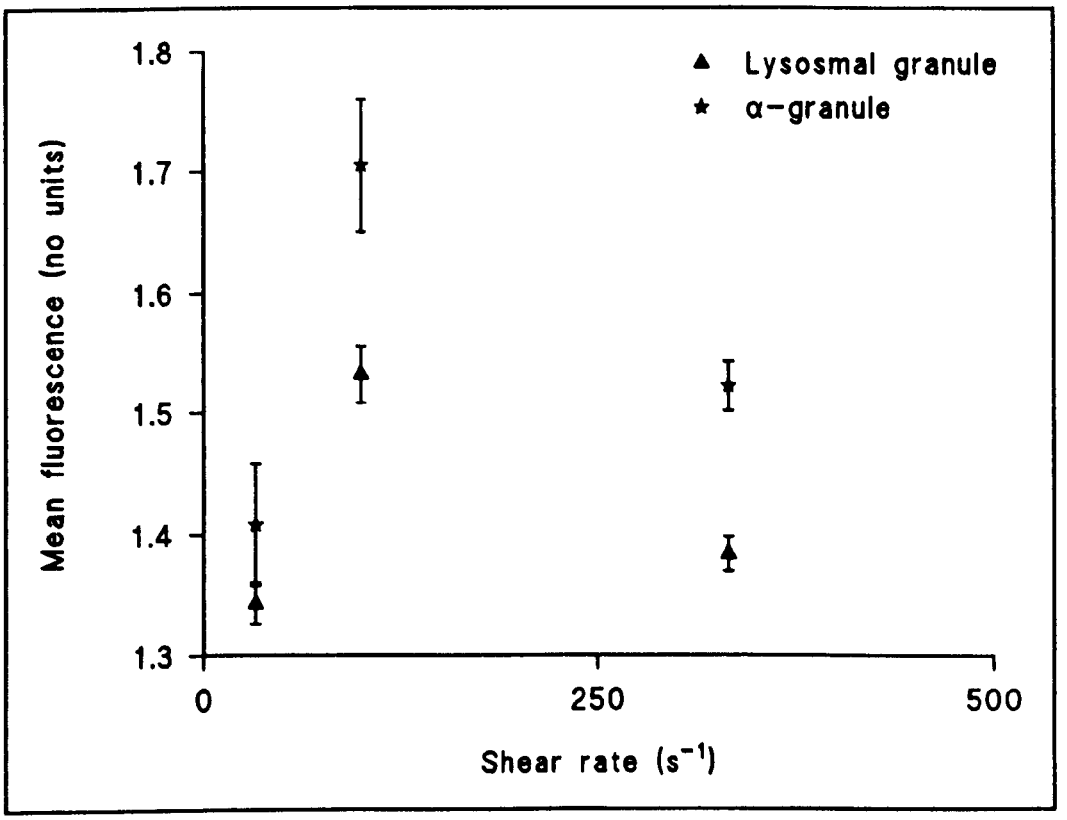


Fig 4.18 Effect of shear rate on platelet degranulation due to perfusion of whole blood through glass at 37°C for 10 minutes. n = 2

The rate at which degranulation occurs due to perfusion at a fixed shear rate was assessed for Pellethane (table 4.11, fig 4.19 and 4.20)

Perfusion time (Minutes)	Mean fluorescence (no units)	
	α-granule	lysosomal granule
1	1.105 ± 0.040	1.149 ± 0.003
5	1.159 ± 0.028	1.161 ± 0.002
10	1.144 ± 0.034	1.150 ± 0.019
20	1.171 ± 0.019	1.215 ± 0.046
30	1.233 ± 0.023	1.226 ± 0.003

Table 4.11 Effect of perfusion time on platelet degranulation due to perfusion of whole blood through Pellethane at 37°C and 1000 s^{-1}. Mean ± range, n = 2

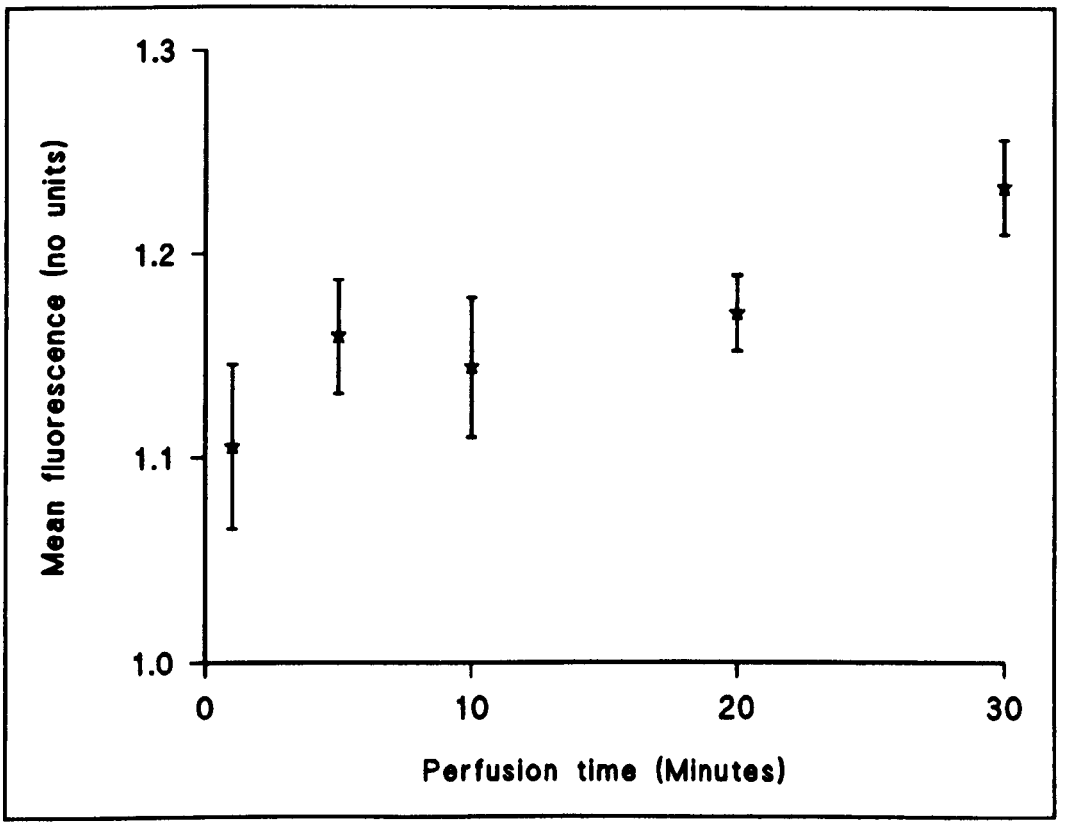


Fig 4.19 Effect of perfusion time on α-granule release due to perfusion of whole blood through Pellethane at 37°C and 1000 s^{-1}. n = 2

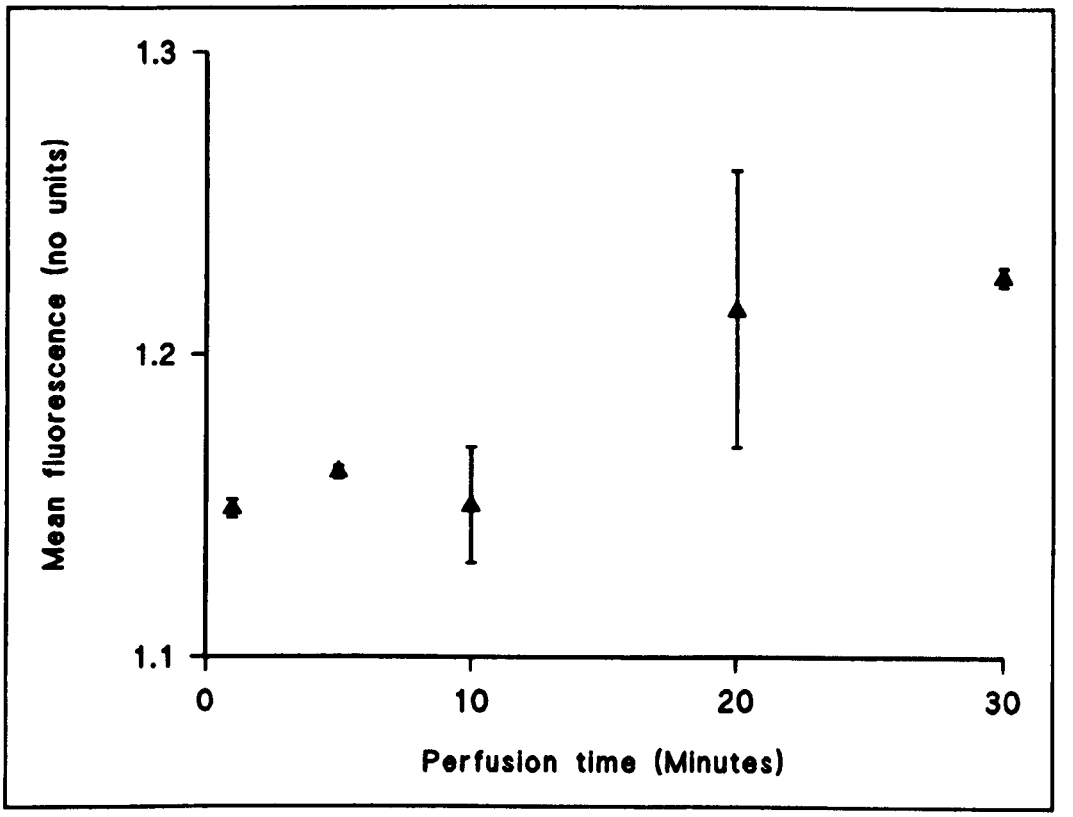


Fig 4.20 Effect of perfusion time on lysosomal granule release due to perfusion of whole blood through Pellethane at 37°C and 1000 s^{-1}. n = 2

4.5.4 Discussion

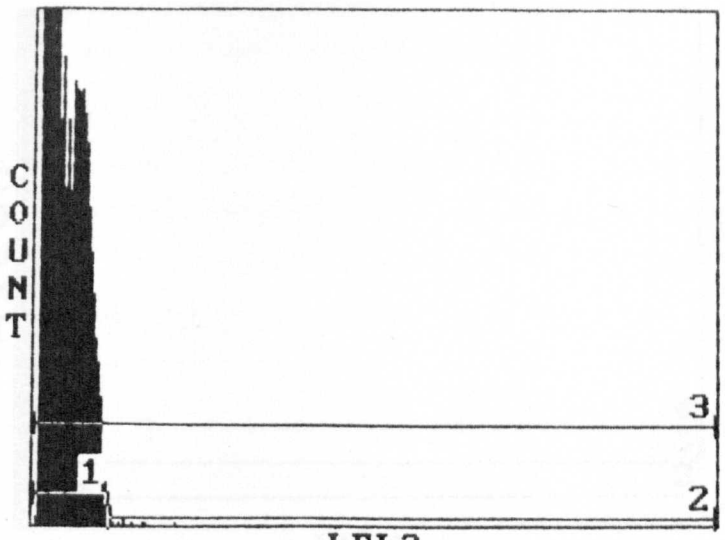
Both methods of whole blood platelet analysis (double antibody labelling and population gating) seem to be perfectly feasible. In fact, both procedures are very much quicker and simpler than conventional cell staining. The double antibody procedure is the quicker and appears to measure the fluorescence of greater than 99% of all platelets. It is difficult to estimate the efficacy of bitmap-gating, but an estimate of greater than 95% of all platelets appears to be typical. This compares with less than 75% if using cells taken from PRP. The platelets not accounted for are not only those cells which have a large volume, but outliers from all parts of the distribution. This means that a very representative population has been taken into account. It is very much cheaper than double labelling and so is the recommended method.

The method of analysis of fluorescence data is problematic. Many like to think in terms of a cell population being positive or negative for a particular antigen. In this case, a marker can be placed at a particular fluorescence limit, indicating a baseline for positivity

and the cell count above and below used to determine the percentage of positive cells in the population. This approach was used to determine the identity of cells in whole blood for platelet analysis (fig 4.21). When a cell population displays differential antigenic expression, positive or negative identity is meaningless. In this case, a marker is set at a value of fluorescence below which cells stained with an isotypic non-specific control have 100% expression. The fluorescence observed below this level cannot be distinguished from non-specific binding of the antibodies. The degree of antigenic expression is calculated from a change in displayed mean fluorescence of cells above this threshold compared to a control, whose antigenic properties are known (fig 4.22). It is possible to find cells which have weak enough antigenic expression that the fluorescence due to antibody staining falls below this non-specific baseline. Indeed, resting platelets fall into this category with regard to GMP-140.

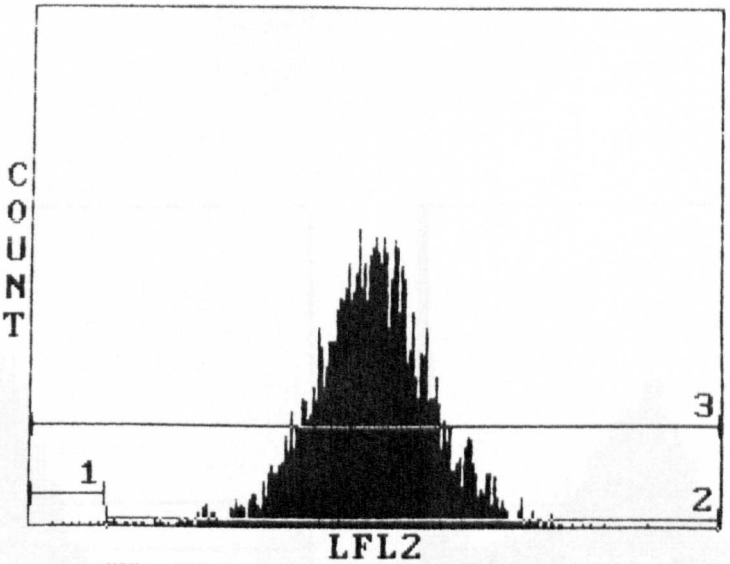
One is faced, then, with several choices as to the way in which to analyse the data. Firstly, one could simply activate a marker, as usual, using the non-specific control and count the number of cells which have positivity above this level. This would be misleading in several ways. Simply quantifying the number of cells above this point would not take into account the degree of shift in fluorescence of these cells. It is possible to perceive a population of cells with many very weakly activated cells, all of which would be counted as positive, and a population of many highly activated cells, which would register the same. This would be clearly deceptive. In addition, a very small increase in antigenic expression could increase fluorescence, but not beyond the non-specific control. This shift would be missed using this method.

An alternative method would be to quantify and compare means of fluorescence of cells above the non-specific baseline. This too would be misleading, for the same reasons. The only way in which to quantify the data without recourse to misleading alternatives was to compare the *raw* fluorescence data in order to establish small differences in population expression. This was made feasible by the advanced computerised statistical analysis on the more advanced flow cytometers (*e.g.* Coulter Epics Profile II) in which the fluorescence data is split into 1024 channels. Any errors in analysis were minimised by basing the mean fluorescence on at least 50000 cells, resulting in a maximum difference of approximately 1% between analyses of the same cells (in the order of 0.01 fluorescence units) and a maximum of 3% between analyses of the same population of cells stained separately. This degree of accuracy allowed any difference in fluorescence to be directly attributable to real differences in antigenic expression.



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	2.178	18673	99.8	1.247	1.235	42.9
2	2.180	1023.	35	0.2	2.405	1.178	42.9
3	1.024	1023.	18708	100.0	1.248	1.237	42.9

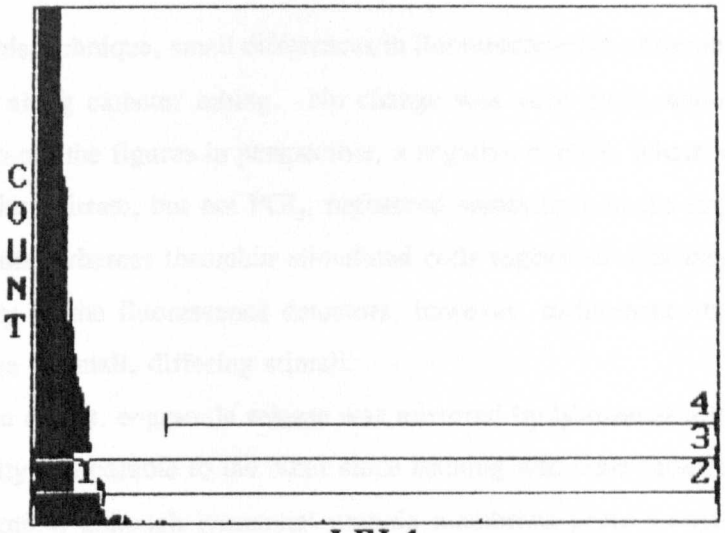
(a) Washed red blood cells



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	2.178	46	0.3	1.442	1.315	22.6
2	2.180	1023.	16288	99.7	29.47	1.90	9.41
3	1.024	1023.	16334	100.0	29.22	1.94	9.41

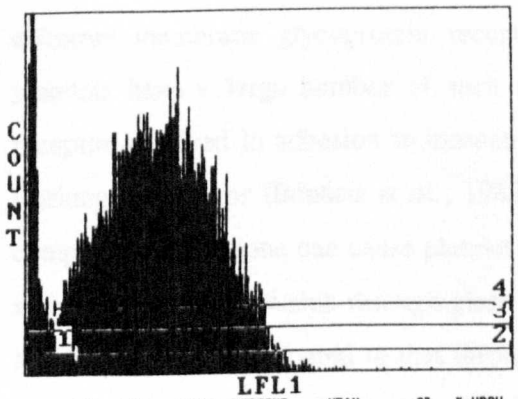
(b) Washed platelets

Fig 4.21 Fluorescence of cells after staining for GPIIb/IIIa in a flow cytometer: specificity of GPIIb/IIIa for platelets



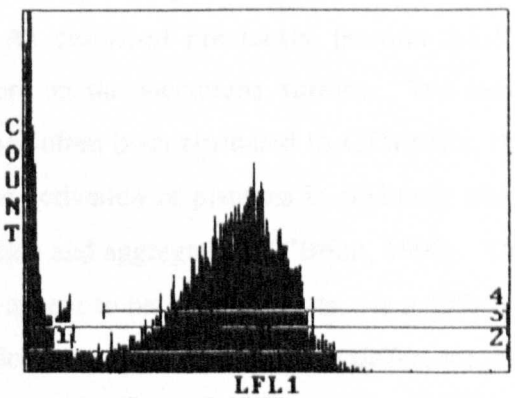
	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	2.064	13054	99.8	1.100	1.119	
2	2.065	1023.	27	0.2	3.174	2.267	5.86
3	1.024	1023.	13081	100.0	1.102	1.140	
4	3.842	1023.	3	0.0	22.80	2.92	0.59

(a) Isotypic, non-specific control



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	2.064	1388	15.2	1.306	1.289	
2	2.065	1023.	7739	84.8	7.306	2.073	31.4
3	1.024	1023.	9127	100.0	5.623	2.521	
4	1.494	1023.	8189	89.7	6.759	2.185	31.4

(b) Mildly stimulated platelets
Mean fluorscence = 7.306



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	1.024	2.064	1197	20.3	1.248	1.253	
2	2.065	1023.	4701	79.7	18.59	2.23	14.7
3	1.024	1023.	5898	100.0	10.74	3.73	
4	3.096	1023.	4583	77.7	19.58	2.09	14.7

(c) Thrombin stimulated platelets
Mean fluorscence = 18.59

Fig 4.22 Fluorescence of platelets after staining for GMP-140 in a flow cytometer: non-specific control and varying levels of stimulation

Using this technique, small differences in fluorescence were detected in the platelets after perfusion along catheter tubing. No change was very great, even after perfusion along glass. To put the figures in perspective, a negative control, where whole blood had been collected into citrate, but not PGI_2, registered somewhere in the region of 1.0 - 1.1 fluorescence units, whereas thrombin stimulated cells registered approximately 20. Due to the sensitivity of the fluorescence detectors, however, differences could be shown to exist in response to small, differing stimuli.

To some extent, α-granule release was mirrored by lysosomal release. Data from one were directly comparable to the other since staining was done separately on the same cells. For example, although lysosomal granule membrane protein expression *appeared* to be less (this may be due to antibody binding characteristics), the characteristics of release were the same in response to perfusion through glass at different shear rates as α-granules. This was not true for perfusion through Pellethane, however, where lysosomal release was maximal at 333 s^{-1}, but α-granule release was increasing, and shown to be maximum at 1000 s^{-1} (the highest shear rate performed). This demonstrates that platelet activation is a complex reaction involving a number of stimulatory pathways. Indeed, the degree of platelet release is a meaningless term, unless a large number of platelet characteristics are analysed.

The differing platelet release reactions are probably due to the stimulation of different membrane glycoprotein receptors. As described previously (section 2.3.5), platelets have a large number of such receptors on the membrane surface. The main receptor involved in adhesion to biomaterials has often been attributed to GPIIb/IIIa, the fibrinogen receptor (Bennett *et al.*, 1983). The activation of platelets is obviously more complex. Shear alone can cause platelet activation and aggregation (O'Brien, 1990). The activation due to perfusion through glass would appear to be, from the data, via a different receptor pathway compared to that through Pellethane. Clearly, it is not a difference due to shear alone. It may be due to differing, selective protein adsorption on the different materials, causing alternative receptors to be activated. Alternatively, it may be due to substances emanating from other cells. It is well known that ADP causes platelet activation through a platelet membrane ADP receptor. In all of the perfusates from glass, haemolysis was observed. Since red blood cells contain ADP, one would expect platelet activation from this alone.

In (relatively) strongly stimulated platelets, as in the case of perfusion through glass, lysosomal release appears to follow α-granule release. In the less well stimulated

populations, the two types of degranulation appear to work more independently. The onset of degranulation appears to occur similarly in each case, however, indicating that the two responses progress over a similar time course.

If α-granule release is used as a determination of platelet reactivity to biomaterials, as is often the practice, then polyurethanes are shown to be the best, with little difference between Elastollan, Texin, Davathane or Desmopan. Slightly worse is Pellethane. Silicone, PVC and LDPE are worse than the polyurethanes but with little difference between any of them. Glass, although not a catheter material, is by far the worst. Lysosomal release data indicate that silicone and Pellethane are the better materials, showing no correlation with α-granule release.

4.6 CONCLUSIONS

It is clear from these data that platelet activation is usable as a marker of platelet reactivity to biomaterials. The data also show that one must use this parameter with caution since the platelet release reaction encompasses a complex set of reactions involving several internal platelet compartments which act independently of each other. A fuller understanding of these reactions, and the way in which they are modulated, is needed before their physiological significance can be surmised.

An index of both lysosomal and α-granule release has, therefore, been developed for detection in a whole blood system after contact with biomaterials. The detection of dense granule release is possible in the same way if a suitable monoclonal antibody were available. This would be a worthwhile addition to the list of observed reactions in order to gain a better understanding of the interactions of platelets with artificial surfaces. Other methods of dense and α-granule release have been shown to be unsuitable for use with whole blood.

The results show a clear superiority of polyurethanes compared with other types of catheter material (silicone, PVC, polyethylene) with respect to platelet activation (α-granule release). It is also clear that platelet activation is not directly correlated with platelet adhesion, showing that bulk phase platelet activation can be minimal despite a large degree of platelet adhesion.

CHAPTER 5

CONTACT PHASE ACTIVATION

5.1 INTRODUCTION

A thrombus is generated after damage to the subendothelium causes release of tissue thromboplastin (tissue factor) which complexes with FVII to initiate the coagulation cascade (Silverberg *et al.*, 1977). This physiological route to thrombogenesis is complemented by the activation of FXII (Hageman factor) after the contact of blood with an artificial surface (Section 2.4). The exact physiological significance of this type of activation is not fully understood but is known as contact phase activation. Hyperactivation of the contact phase proteins causes rapid coagulation. Assessment of the initiation of this cascade complements knowledge of the effects of a surface to platelet adhesion and activation and so is desirable in the multi-parametric assessment of a biomaterial.

Quantification of contact phase activation was achieved using two approaches. Firstly, a one stage plasma recalcification system for PPP after material contact for a fixed period of time, known as the partial thromboplastin time (PTT) assay, was used. Secondly, the time course of activation of FXII in contact with catheter materials was measured.

5.2 PARTIAL THROMBOPLASTIN TIME (PTT) ASSAY

5.2.1 Introduction

This is a traditional approach to the analysis of the mechanisms involved in thrombogenesis (Dacie and Lewis, 1975). The assay relies on the need for calcium ions and platelet-derived phospholipid, which are not present in citrated platelet poor plasma (PPP), for the generation of a fibrin clot. Different information regarding the clotting cascade can be obtained depending upon the way in which the plasma is treated prior to assay. Zymogen deficiency or the presence of spontaneously generated anticoagulant can be detected if FXII is hyperactivated with kaolin, a highly negatively charged substance. This is performed in haematology laboratories for routine plasma protein profile analysis.

In the study of material thrombogenicity the hyperactivation stage is replaced by the contact of the plasma with a material for a fixed time. The time taken for the plasma

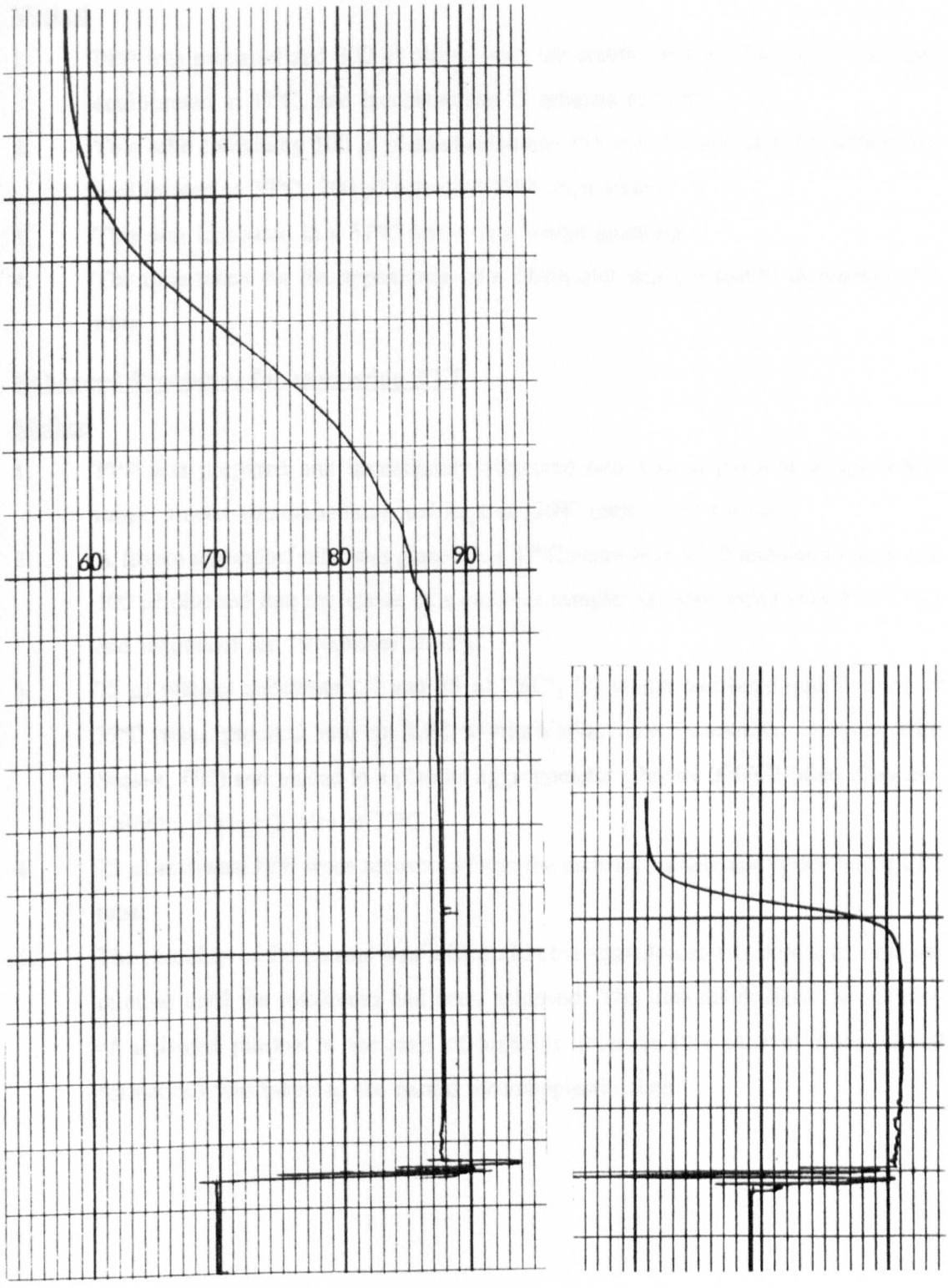
to clot after the addition of calcium ions and platelet phospholipid is taken as a measure of the surface activation of the intrinsic coagulation cascade. The result is compared with positive (activation with glass contact) and negative (no activation) controls - the presence of even tiny quantities of naturally occurring activated zymogens is enough to bring about clot formation in non-activated plasma *in vitro*.

The initial protocol was successively improved in five ways. Firstly, the initial procedure, similar to that recommended by Dacie and Lewis (1975), required recognition of the end-point by eye. This subjectivity was abrogated by measuring the turbidity of the solution automatically (in a platelet aggregometer) as used previously (Hennink *et al.*, 1984). Typical traces are shown in fig 5.1. Secondly, a larger surface area for contact phase activation was presented by using small bore catheter samples which resulted in a typical surface area to volume ratio of 40 cm^2 : 1 cm^3, compared to 6.7 cm^2 : 1 cm^3 previously. Thirdly, a more homogeneous pool of plasma was achieved by using individual aliquots, all prepared at the same time and frozen simultaneously. This was later improved by freezing the plasma very quickly in liquid nitrogen at -198°C and so stopping the effects of differential thermal diffusion which occurs in a freezer, even at temperatures as low as -80°C. The fifth improvement was to spin the plasma for two minutes at 13500g in a microcentrifuge, followed by removal of only half the supernatant and then spinning for a second time, at the same speed, before freezing in order to obtain virtually platelet free plasma. The presence of very small quantities of platelets and platelet-derived particles increases the concentration of platelet phospholipid and interferes with the reaction kinetics.

5.2.2 Materials and methods

Reagents

1. Platelet membrane substitute solution (Diagnostic Reagents Ltd, Thame, Oxfordshire, UK).
2. $CaCl_2$: 25mM in H_2O (2.77 g $CaCl_2$ in 1 l H_2O).
3. Barbitone buffer: 24mM 5,5-diethylbarbituric acid, 73mM tris-(hydroxymethyl) aminomethane, 790μM calcium lactate, 2.5mM sodium azide in H_2O (4.48 g 5,5-diethylbarbituric acid, 8.86 g tris-(hydroxymethyl) aminomethane, 86 mg calcium lactate, 160 mg sodium azide in 1 l H_2O), pH 7.4.
4. Kaolin (Sigma, Poole, Dorset, UK): stock suspension of 1 g/l in barbitone buffer (3).



(a) No activation

(b) Activation with glass

Fig 5.1 Clotting of plasma after the addition of Ca^{2+} and platelet substitute (a) without activation and (b) in response to glass

Method

1. PPP was prepared and 300 μl placed onto the inside surface of samples[1], already equilibrated at 37°C, and incubated for 15 minutes at 37°C.
2. To a tube containing 100 μl platelet substitute (1) and 100 μl $CaCl_2$ (2) which was equilibrated to 37°C, 200 μl activated PPP were added.
3. This was incubated in a 37°C water bath whilst agitating.
4. The time taken for the appearance of a fibrin clot was the partial thromboplastin time.

Enhanced Procedure for measuring PTT

Method

1. PPP was prepared and immediately aliquoted into 150 μl portions in eppendorf tubes, frozen simultaneously and kept at -80°C until ready for use.
2. A frozen eppendorf tube was placed in a 37°C water bath for 2 minutes to thaw and 100 μl pipetted into the inside of a catheter sample[1] (already equilibrated at 37°C) and incubated for 10 minutes at 37°C.
3. 75 μl platelet substitute (1) and 75 μl $CaCl_2$ (2) which had been equilibrating at 37°C were pipetted into an LP2 γ-scintillation tube (Luckham, Burgess Hill, Sussex, UK) and placed in a platelet aggregometer (Payton 300BD (dual channel), Toronto, Canada), also at 37°C.
4. 75 μl activated PPP were recovered from the catheter sample and added to the LP2 tube.
5. The turbidity of the plasma was followed in the aggregometer (running at 3 cm per minute) until the maximum had been achieved. The time taken from the addition of activated plasma to the start of turbidity increase (the *start* of fibrin strand formation) was taken as the partial thromboplastin time.

[1] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

5.2.3 Results

5.2.3.1 Activation by materials as measured by the initial protocol

The initial protocol was used to assess the contact activation produced by large bore catheter samples. The results are shown in table 5.1 and fig 5.2.

Material	Plasma clotting time (s)	Number of trials
Silicone	447 ± 249	13
PTFE	386 ± 82	5
PVC	436 ± 117	12
LDPE	466 ± 157	8
Tecoflex	673 ± 296	4
Pellethane	482 ± 167	12
Glass	162 ± 19	9

Table 5.1 PTT measured by eye in response to catheter materials. Mean ± S.D.

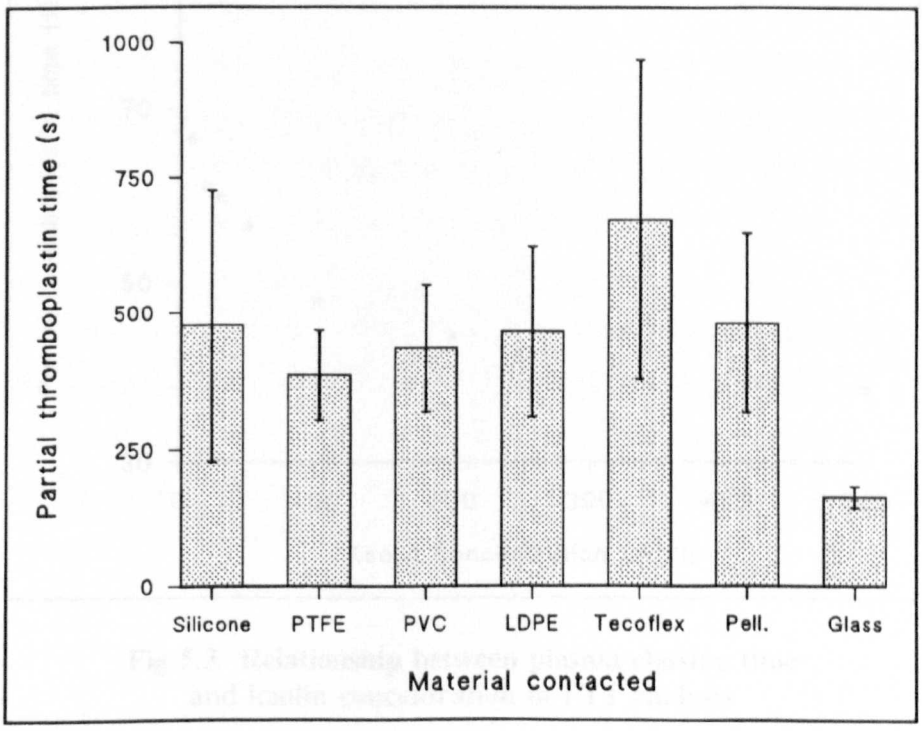


Fig 5.2 Activation of plasma contact phase proteins by large bore catheter material, measured by PTT

5.2.3.2 Applicability of the enhanced protocol to biocompatibility evaluation

The enhanced procedure was developed in order to improve objectivity and reduce standard deviations. It was assessed for its applicability to biocompatibility evaluation by creating a standard curve from which results could be extrapolated, allowing objective interpretation of the partial thromboplastin times. Kaolin was included in the reaction mixture, as a suspension in barbitone buffer, and the turbidity followed as before. In this case, 75 μl plasma were incubated with 37 μl kaolin suspension for 10 minutes at 37°C, after which time 112 μl of a pre-mixed and warmed mixture of platelet substitute (75 μl) and double-strength $CaCl_2$ (37 μl) were added. This resulted in the generation of a dose curve (fig 5.3) and further extrapolation into a calibration curve (fig 5.4).

There was a linear proportionality between kaolin concentration and plasma clotting time, which gave some basis for applying the technique to the objective assessment of biomaterial thrombogenicity. However, low activation was difficult to detect when using very small concentrations of kaolin, obviating the direct correlation of the calibration curve to material-induced plasma clotting.

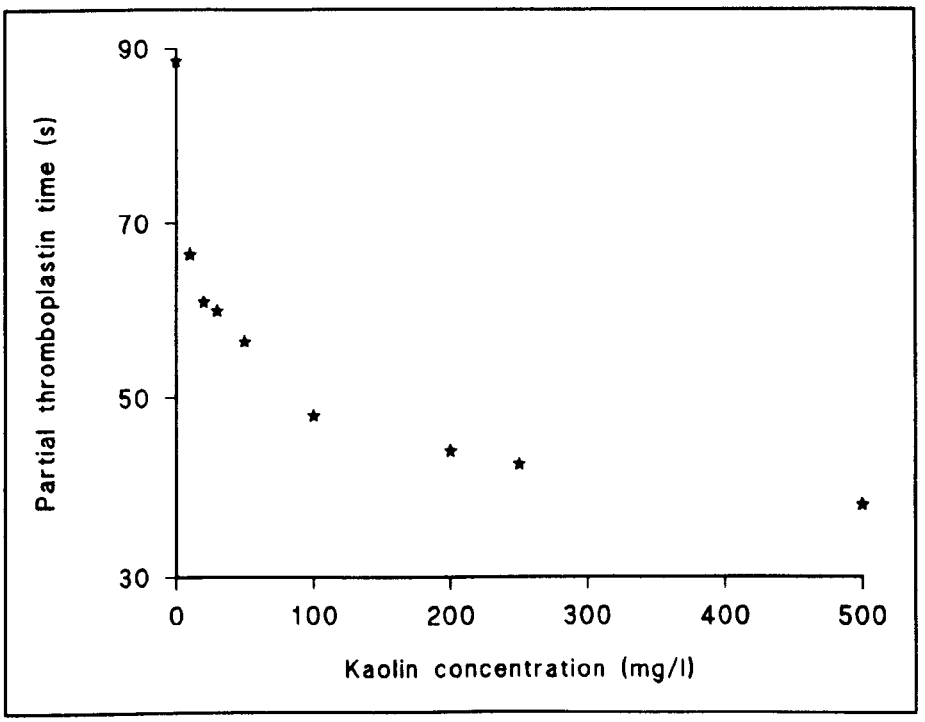


Fig 5.3 Relationship between plasma clotting time and kaolin concentration in PTT analysis

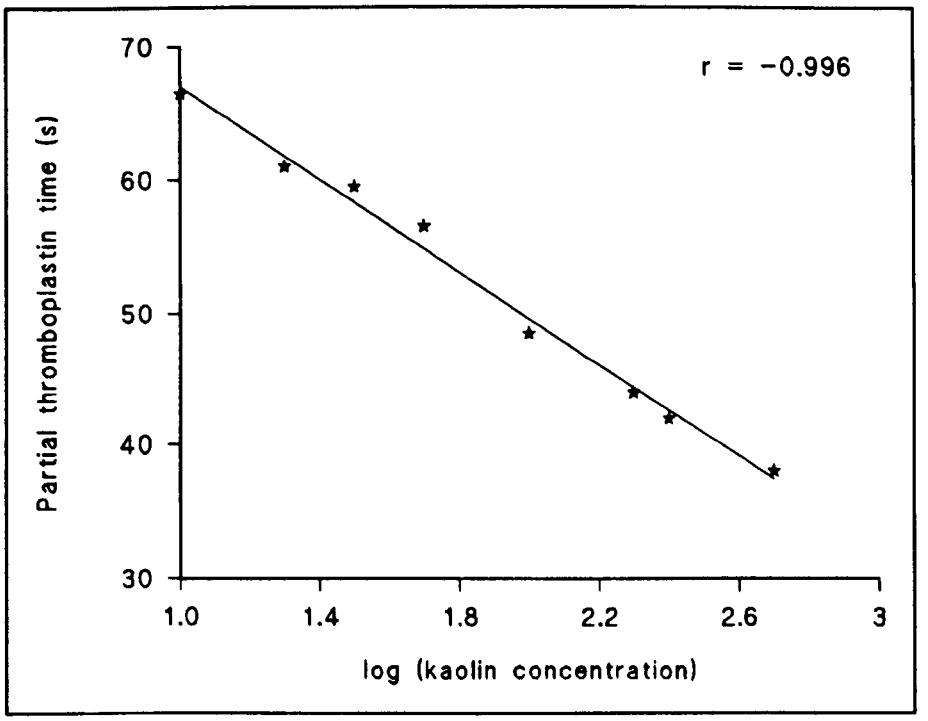


Fig 5.4 Calibration curve for PTT analysis with respect to kaolin

5.2.3.3 Activation by materials as measured by the enhanced protocol

The enhanced protocol was used to improve the resolution of the assay and allow its use in the biocompatibility assessment of biomaterials. The use of narrow bore catheter samples (similar to those used clinically) was also introduced in order to achieve a greater material surface area to contact the plasma, aliquots of which were simultaneously frozen at -20°C in a freezer to minimise reagent-derived error. The results of these improvements are shown in table 5.2 and fig 5.5.

The standard deviations derived from these data are still very large. It was postulated that this was still a result of reagent differences. To minimise these, plasma was frozen with liquid nitrogen, to reduce the time during which contact phase proteins were in contact with highly activating plasma lipids crystals. The results of this approach are shown in table 5.3 and fig 5.6.

Material	Plasma clotting time (s)	number of trials
No activation	207.0 ± 35.2	7
Glass	64.2 ± 4.3	8
PVC	141.5 ± 37.5	3
PTFE	155.2 ± 17.2	5
LDPE	133.5 ± 0.7	3
Silicone	175.0 ± 35.4	3
Pellethane	146.4 ± 12.4	5
Davathane	136.8 ± 14.7	5
Desmopan	132.8 ± 25.4	5
Texin	153.0 ± 25.6	5
Elastollan 1190A	150.8 ± 34.7	5

Table 5.2 Partial Thromboplastin Times after plasma contact with various materials using freezer frozen plasma. Mean ± S.D.

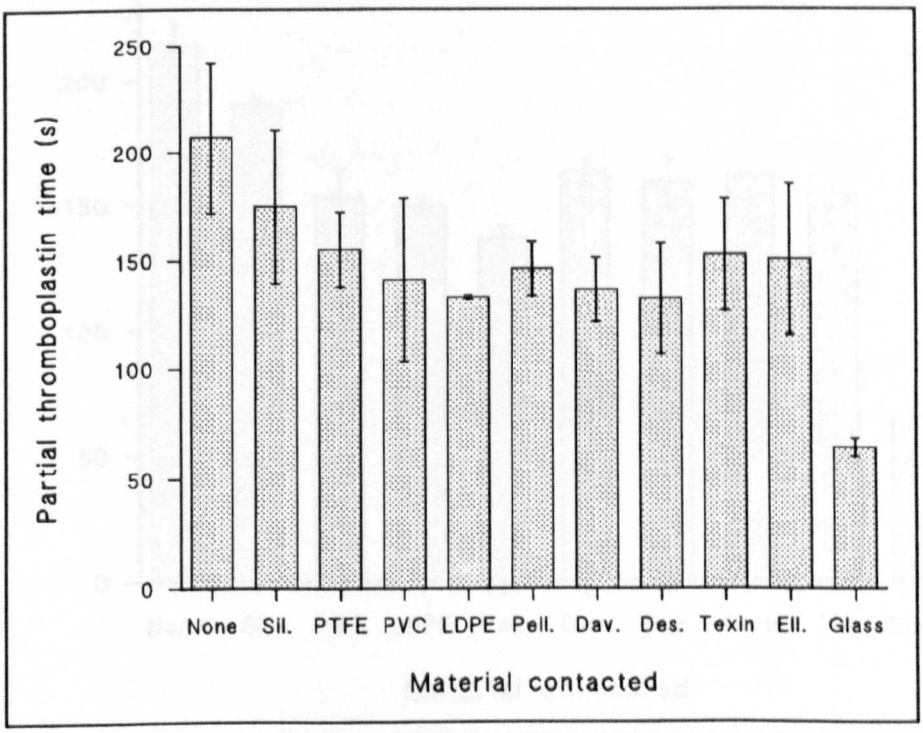


Fig 5.5 PTT analysis of small bore materials as assessed by the enhanced protocol using freezer-frozen plasma

Material	Plasma clotting time (s)
No activation	215 ± 9
Glass	67 ± 3
PVC	154 ± 11
LDPE	150 ± 4
Silicone	191 ± 4
Pellethane	137 ± 4
Davathane	163 ± 5
Desmopan	159 ± 10
Texin	162 ± 0.5
Elastollan 1190A	154 ± 6

Table 5.3 Partial Thromboplastin Times after plasma contact with various materials using liquid nitrogen-frozen plasma. Mean ± S.D., n = 4

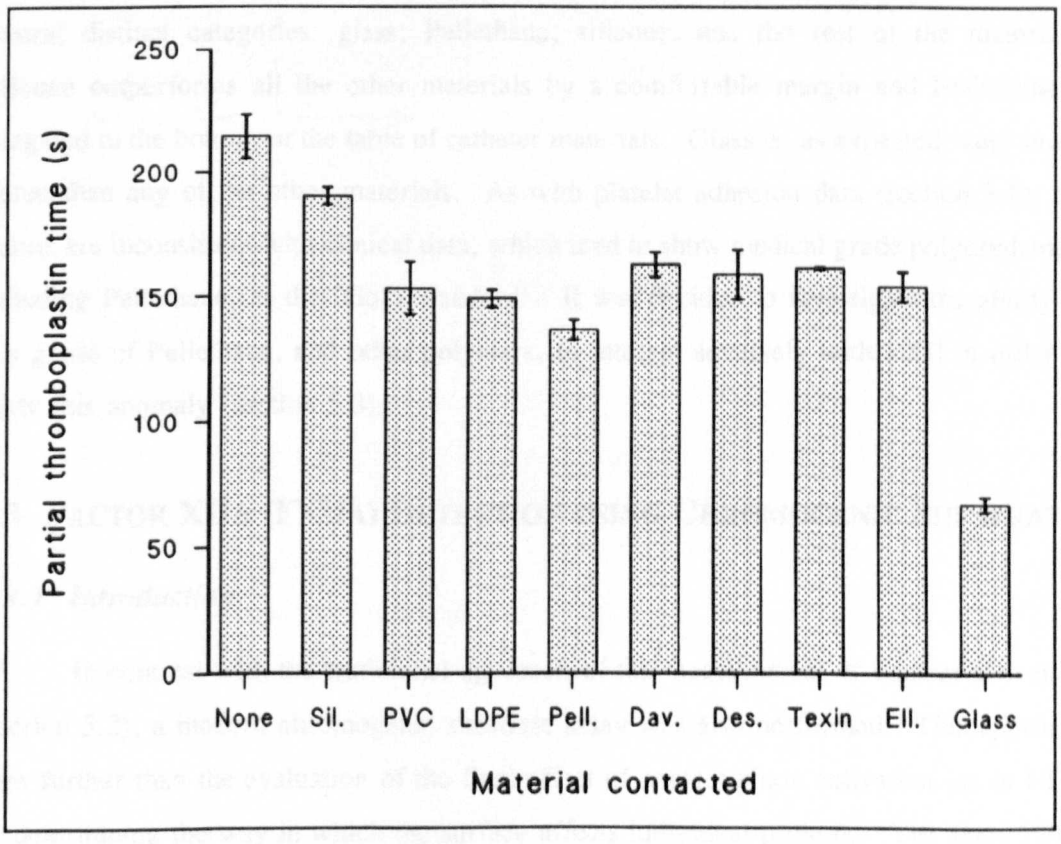


Fig 5.6 Partial Thromboplastin Times after plasma contact with various materials using liquid nitrogen-frozen plasma. n = 4

5.2.4 Discussion

The calibration curve demonstrates that contact phase activation is quantifiable using PTT by activation with kaolin over almost 2 orders of magnitude of concentration with a very good correlation (-0.996 on a plot of plasma clotting time versus log (kaolin concentration)). This was only valid for fairly large concentrations of kaolin, however, with smaller concentrations tending to produce inconsistent or unreproducible dose curves. There are several possible reasons for this. It is possible, although unlikely, that the technique of PTT is inherently unstable and liable to generation of inconsistent data. More probable is the high affinity of kaolin for the sides of the polystyrene tube in which the standard concentrations were prepared. Kaolin is a highly charged solid, making mutual attraction with such plastics likely. This was evident with the high kaolin concentrations. Since kaolin never really dissolves, there is no energy of solvation to overcome before adsorbance can occur, making adsorbance more likely.

The results indicate that it is possible to differentiate materials with respect to their ability to activate the coagulation cascade and clot an aliquot of plasma. There is not a large difference between any of the materials, however, and again they seem to fall into several distinct categories: glass; Pellethane; silicone; and the rest of the materials. Silicone outperforms all the other materials by a comfortable margin and Pellethane is relegated to the bottom of the table of catheter materials. Glass is, as expected, very much worse than any of the other materials. As with platelet adhesion data (section 3.5), the results are inconsistent with clinical data, which tend to show medical grade polyurethanes, including Pellethane, as the 'Gold Standard'. It was decided to investigate the ability of this grade of Pellethane, and other polymers, to interact adversely with FXII in order to study this anomaly (section 5.3).

5.3 FACTOR XIIA (FXIIA) DETECTION USING CHROMOGENIC SUBSTRATE

5.3.1 Introduction

In contrast with the traditional approach of the measurement of contact activation (section 5.2), a modern chromogenic substrate assay was also performed. This approach goes further than the evaluation of the final effect of contact phase activation (as in PTT) by determining the way in which the surface affects individual proteins. The assay relies on the specific proteolysis by activated FXII of a small peptide containing p-Nitroanilide

(pNA), a substance which absorbs light in the visible spectrum (at 405 - 410 nm), thus allowing the quantification of the zymogen spectrophotometrically.

The method was based on one originally devised for the clinical measurement of total plasma FXII (Gallimore *et al.*, 1987; Walshe *et al.*, 1987). In this case FXII was hyper-activated to FXIIa by an ellagic acid-based activator. Others have attempted to bypass this activation step and utilise the contact of plasma with the material in question for the generation of the Hageman Factor-derived zymogen and so convert the assay into one for the quantification of contact phase activation (Irvine, 1989). It was this approach which was used in the following method.

5.3.2 Materials and methods

Reagents

1. Acetone.
2. Buffer: 25mM Tris-HCl, 25mM NaCl in H_2O (3.94 g Tris-HCl, 1.45 g NaCl in 1 l H_2O), pH 7.9.
3. Kallikrein inhibitor (Channel Diagnostics, Walmer, Kent, UK).
4. FXII chromogenic substrate - 2.AcOH.H-D-CHT-Gly-Arg-pNA (Channel Diagnostics, Walmer, Kent, UK): 1mM in buffer (2).
5. 25% (v/v) acetic acid in H_2O.

Method

1. PPP was prepared.
2. This was further centrifuged at 1400g for 10 minutes to remove any suspended platelets.
3. 300 μl of the PPP were incubated with material[2] for the desired length of time.
4. 90 μl of the activated PPP were removed and 30 μl acetone (1) added.
5. This was vortex-mixed until all the precipitation had disappeared, then incubated at 4°C for 15 minutes.
6. 25 μl of this dilution were added to 25 μl buffer (2) and incubated at 37°C for exactly 2 minutes.
7. 75 μl Kallikrein inhibitor (3) were added and incubated at 37°C for exactly 10 minutes.
8. 50 μl chromogenic substrate (4) were added and incubated at 37°C for exactly 10 minutes.

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[2] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

9. 50 μl acetic acid (5) were added to stop the reaction.

10. The absorbance was read at 405 nm in a microtitre plate reader (Dynatech MR 700, Billingshurst, West Sussex, UK) against a reagent blank, for which the reaction substituents were added in reverse order to that of the assay.

5.3.3 Results

To evaluate this method of FXIIa quantification, materials of very different structure, namely LDPE, PVC and glass, were assessed using this protocol. A time course of observed FXIIa generation over 90 minutes was plotted (fig 5.7).

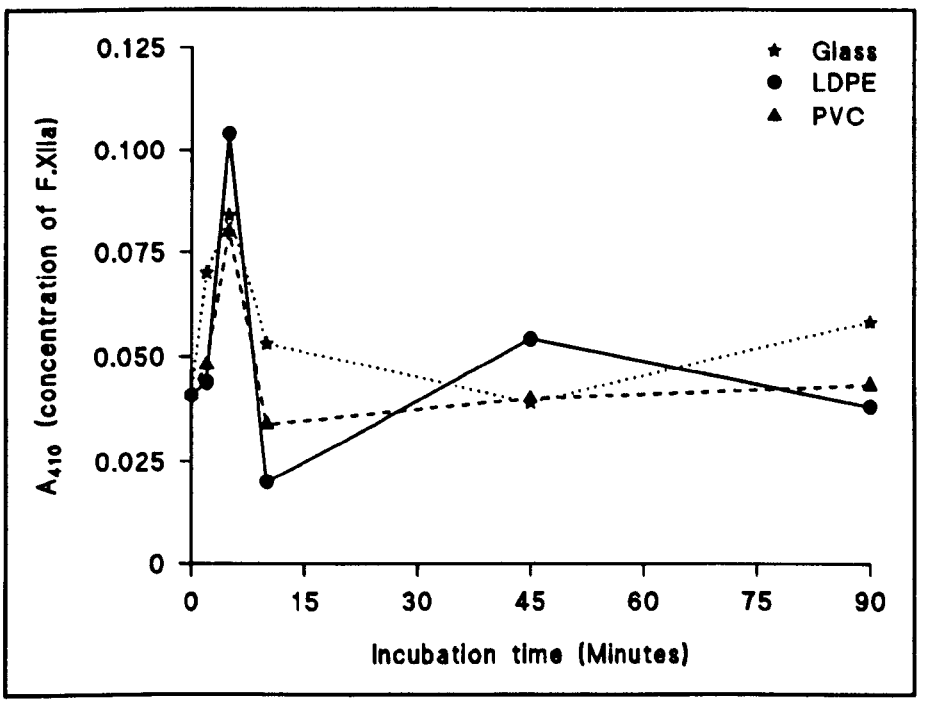

Fig 5.7 Activation of FXII by three materials as measured by a chromogenic substrate assay

5.3.4 Discussion

Because of the presence of naturally occurring, broad spectrum, serine protease inhibitors: antithrombin III (AT-III); C_1-esterase inhibitor (C_1-Inh); α_2-macroglobulin; α_1-antitrypsin and α_2-antiplasmin, any free, uncomplexed FXIIa is quickly depleted from plasma. *In vivo*, these complexes are quickly removed by the liver. The major inhibitor, C_1-Inh, conjugates with FXIIa and eliminates any amidolytic activity the zymogen might

have. The rapidly increasing concentrations of free FXIIa which are detected are quickly diminished after about 5 minutes due to attack from C_1-Inh. Although the quantities of FXIIa generated seem to be similar in all three cases, it is the rate of increase of generation which is important, glass having by far the highest.

For a quantitative analysis of FXIIa in plasma, it is necessary to measure the concentrations of the complexes of inhibitors to FXIIa, or destroy them. Since C_1-Inh is by far the most active inhibitor in plasma *in vitro* (Kozin and Cochrane, 1988), an attempt was made to neutralise it. Various methods have previously been employed to destroy this non-specifically by acetone (Gallimore *et al.*, 1987; Walshe *et al.*, 1987) or low pH (de la Cadena *et al.*, 1987) and specifically with flufenamic acid (Miles *et al.*, 1981). All three were assessed in the protocol. Plasma was treated after isolation, prior to material incubation, as follows:

(i) **Acetone**: acetone was added 1 + 3 to PPP, vortex-mixed until all the precipitation had disappeared, then incubated at 4°C for 15 minutes. Step (5) of the method (5.3.2) was excluded.

(ii) **Low pH**: 167mM HCl was added 1 + 1 to PPP, vortex-mixed then incubated at room temperature for 15 minutes. A buffer, containing 100mM Na_2PO_4, 150mM NaCl, 1mM EDTA, 0.02% (w/v) NaN_3, was then added 1 + 2. 167mM NaOH was added 1 + 3 to the mixture. Step (5) of the method was excluded.

(iii) **Flufenamic acid**: flufenamic acid was added at varying concentrations (final concentrations of 0mM, 1mM, 10mM, 100mM). The mixture was vortex-mixed then incubated at room temperature for 15 minutes. Step (5) of the method was excluded.

Experiments were conducted with all three inhibitor antidotes by comparing the absorbance observed after assaying PPP which had been in contact with glass for 10 minutes and that which had had no activation at all. All of these experiments were performed in duplicate (table 5.4)

Destruction protocol	Absorbance at 410nm	
	No activation	Activation by glass
Acetone	0.003	0.009
Low pH	0.003	0.009
Flufenamic acid: 1mM	0.012	0.023
10mM	0.026	0.032
100mM	*	*

* This concentration of flufenamic acid precipitated out the chromogenic substrate

Table 5.4 Observed absorbances of assayed PPP after the destruction of protease inhibitors by 3 different protocols

None of the protease inhibitor destruction protocols appears to have worked. This is not very surprising in the case of the non-specific destructors. Due to the non-specificity, 100% destruction would be unlikely, causing significant concentrations of inhibitor to remain in the reaction mixture. Indeed, one could argue that their implementation is completely undesirable since there is the possibility of FXII destruction or alteration, and that of other proteins involved in contact phase activation. The use of flufenamic acid also seems to have had no effect. It is likely that the low concentrations do not have any effect and that relevant concentrations also react with the substrate.

A semi-quantitative assay based upon the observed initial rate of increase in absorbance may be one way of approaching the problem, but this would be subject to tremendous variation considering the time scale of plasma incubation involved (1 - 2 minutes). It is not feasible, then, to evaluate contact phase activation using a functional, chromogenic substrate assay such as this.

5.4 FXIIa Detection using Enzyme-linked Immunosorbent Assay

5.4.1 Introduction

The use of an enzyme-linked immunosorbent assay (ELISA) technique to evaluate an antigenic determinant differs from the use of a functional method in that the immunological recognition need not be to a functionally intact molecule. In other words, it is possible to establish a clone of antibodies which still recognises the FXII epitope after

deactivation by C_1-Inh. It was the availability of such an antibody which allowed the use of the following method for the quantification of contact phase activation.

5.4.2 Materials and methods

Reagents

1. Phosphate Buffered Saline (PBS) with Bovine Serum Albumin (BSA): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, 0.05% (w/v) BSA in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4, 500 mg BSA in 1 l H_2O), pH 7.3.
2. β.FXIIa standard: 20 ng/ml in PBS with BSA (1).
3. Primary monoclonal antibodies: (i) FXIIa and FXIIa-C_1-Inh
   (ii) unbound FXIIa only;
   5 μg/ml in PBS, pH 7.4; (donated by MP Esnouf, Clinical Biochemistry, Oxford University, Oxford, UK).
4. Secondary antibody: polyclonal sheep anti-F.XIIa conjugated to Alkaline Phosphatase, with 0.02% (w/v) NaN_3 (donated by MP Esnouf, Clinical Biochemistry, Oxford University, Oxford, UK).
5. Wash buffer: 10mM boric acid, 760μM borax, 0.05% (w/v) NaN_3, 1.64mM Triton X100 in H_2O (620 mg boric acid, 290 mg borax, 500 mg NaN_3, 1.06 g Triton X100 in 1 l H_2O), pH 7.4.
6. Conjugate buffer: 100mM NaCl, 1.0mM $MgCl_2$, 100mM Tris, 100μM $ZnCl_2$, 0.1% (w/v) NaN_3, 0.01% (v/v) ml Triton X100, 1% BSA in H_2O (5.84 g NaCl, 203 mg $MgCl_2$, 12.11 g Tris, 13 mg $ZnCl_2$, 1 g NaN_3, 100 μl Triton X100, 10 g BSA in 1 l H_2O), pH 8.0.
7. Working strength conjugate: 10 μl secondary antibody (4) in 5 ml conjugate buffer (6).
8. Substrate: 0.1% paranitrophenylphosphate (PNPP) in 100mM diethanolamine-HCl (10 mg PNPP, 10.5 g diethanolamine-HCl in 1 l H_2O), pH 9.8.
9. Stop solution: 3.0M NaOH in H_2O (12.0 g NaOH in 100 ml H_2O).

Method

1. 96-well plates (Maxi-Sorp, Nunc, Denmark) were coated with the appropriate primary antibody (3), 100 μl per well, overnight at 4°C in 100% relative humidity.
2. A set of standards was prepared in eppendorfs from the stock standard (2) (0, 0.5, 1, 2.5, 5, 7.5, 10, 20 ng/ml) in PBS with BSA (1).

3. Platelet-free plasma was prepared from citrated whole blood by spinning at 2000g for 10 minutes and contacted with catheter samples[3].
4. The plate contents were washed and tipped away 3 times with 200 μl wash buffer (5). The plate was then blotted dry.
5. 100 μl standard or sample were added to each well and incubated at room temperature for 60 minutes in 100% relative humidity.
6. The plate was inverted and washed 3 times with 200 μl wash buffer (5) then blotted dry.
7. 100 μl working strength conjugate (7) were added to each well and incubated at room temperature for 60 minutes in 100% relative humidity.
8. The plate was inverted and washed 3 times with 200 μl wash buffer (5) then blotted dry.
9. 100 μl substrate (8) were added to each well and incubated at room temperature for 15 minutes in 100% relative humidity.
10. The reaction was stopped by the addition of 100 μl stop solution (9).
11. The absorbance was read at 405 nm in an Anthos 2001 microplate reader .

[3] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

5.4.3 Results

The ability of different types of material to activate FXII was assessed using the ELISA. These are shown in table 5.5 and fig 5.8.

Material	FXIIa released (ng/ml)
Glass	19.95 ± 3.73
Silicone	0.85 ± 0.14
PVC	-0.02 ± 0.03
LDPE	0.66 ± 0.28
Pellethane	0.03 ± 0.02
Davathane	2.65 ± 0.27
Desmopan	2.41 ± 0.47
Texin	-0.03 ± 0.05

Table 5.5 Activation of FXII by catheter tubing after static contact at 37°C for 1 hour. Mean ± S.D., n = 6

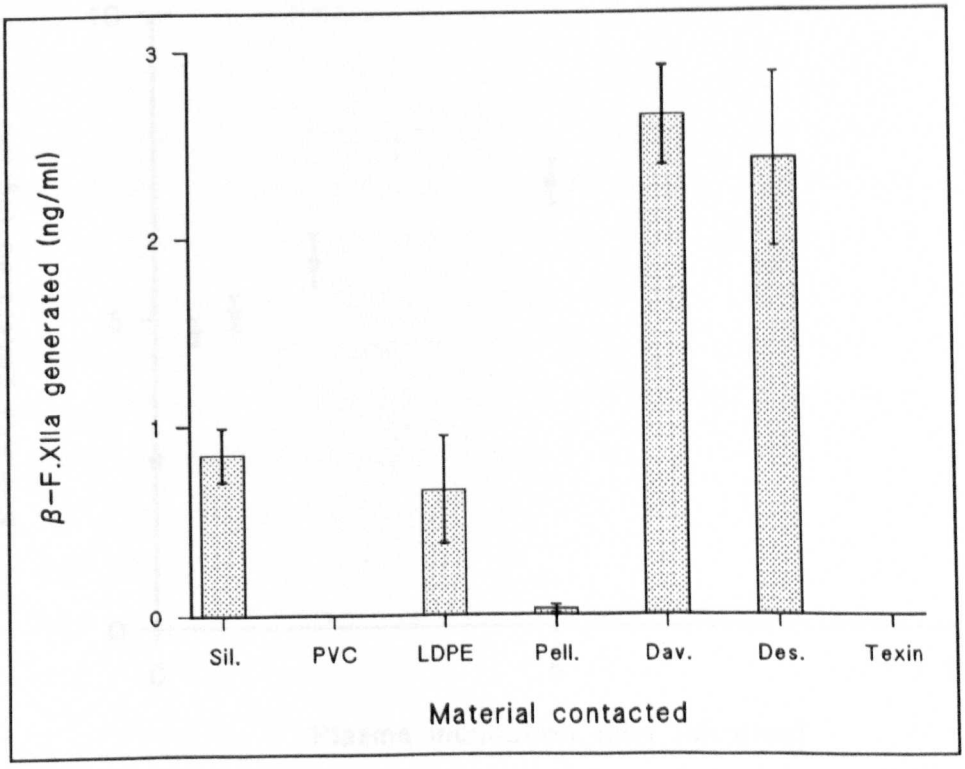

Fig 5.8 Activation of FXII by catheter tubing after static contact at 37°C for 1 hour. n = 6

Since there was a large degree of FXII activation after one hour on two polyurethanes (Davathane and Desmopan), a time course of activation on Davathane was investigated. Plasma was incubated statically at 37°C for periods of up to 1 hour (table 5.6, fig 5.9).

Contact Time (Minutes)	FXIIa released (ng/ml)
0.5	4.78 ± 0.10
1	5.09 ± 0.23
2	5.93 ± 0.27
5	7.18 ± 0.45
10	9.90 ± 0.37
60	11.60 ± 0.86

Table 5.6 Activation of FXII by Davathane after static contact at 37°C. Mean ± range, n = 2

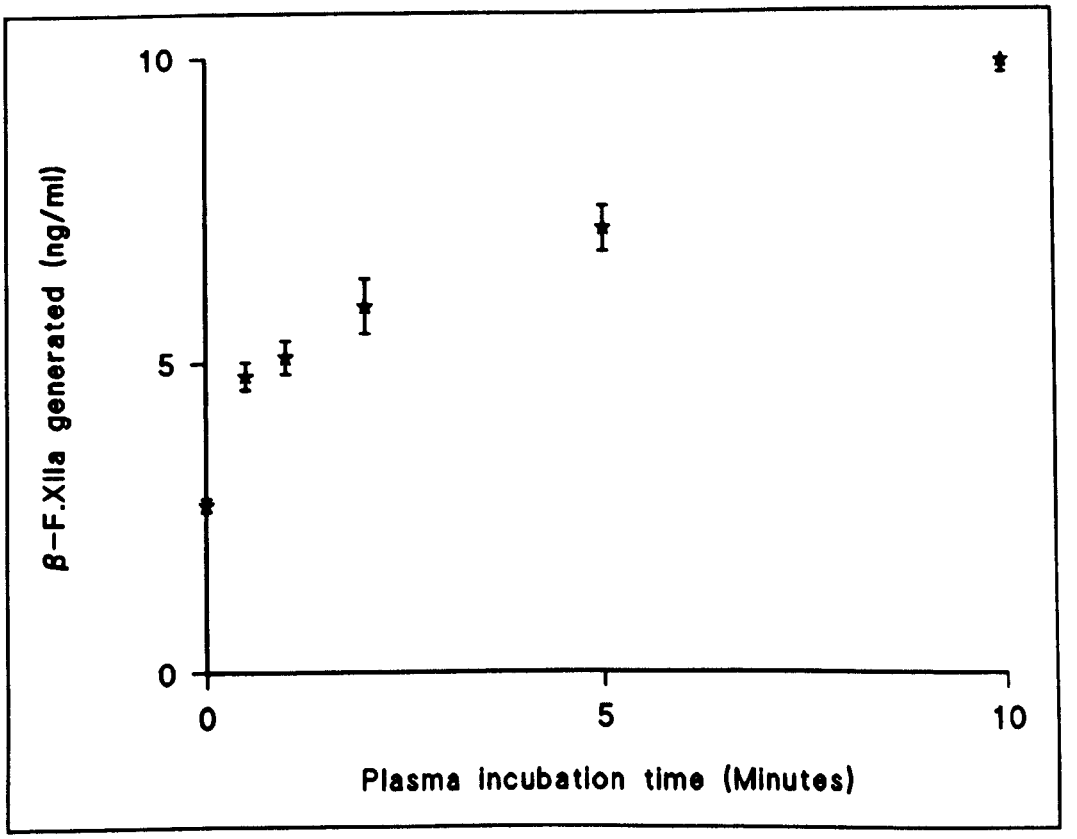

Fig 5.9 Activation of FXII by Davathane after static contact at 37°C. n = 2

The quantities of FXIIa released in this last experiment are very much higher due to the use of different donor plasma. The values given in fig 5.8 are mean values derived from experiments using plasma from two donors which behaved similarly. There can be a great difference in the results from donor to donor, although materials shown to be activating by insensitive plasma are still shown to be activating when using sensitive plasma (table 5.7).

Material contacted	FXIIa released (ng/ml)	
	Donor Plasma 1 & 2	Donor Plasma 3
Pellethane	0	3.28
Davathane	2.65	11.60
* Polystyrene	0	2.91
* Polypropylene	0.19	3.45

* Specimen containers

Table 5.7 Differences between donor plasmas: static contact for 1 hour at 37°C

5.4.4 Discussion

Surprisingly, contact phase activation, as measured by the release of FXIIa, displays a diversity of results to a rather unexpected degree, with two polyurethanes, Desmopan and Davathane, causing consistently by far the highest FXII proteolysis, with the exception of glass. One surprising facet is the difference between these results and those obtained by PTT. The two assays should measure the same parameter: the initiation of the intrinsic coagulation cascade. However, using the same batches of catheter material, silicone scores best in PTT and Pellethane worst, whereas Pellethane in this test causes the least activation and silicone is mediocre in terms of FXIIa generation. There are several possible explanations for this phenomenon.

The assay works on the principle of measurement of fluid phase FXIIa, both α- and β- forms. The antibody recognises an epitope close to, but not in, the active site, which is hidden in unactivated FXII. Recognition of FXIIa compared to FXII with this antibody is said to be greater than 1:10,000 in favour of FXIIa (Esnouf, 1991). That the epitope recognition site is not in the active site is advantageous since it is not obscured when FXIIa

is generated. This is similar in molecular weight to native FXII, but a disulphide bond is breached to allow the active site domain to be released from its close proximity to the surface-binding domain and is then situated at the end of a flexible arm connecting the two. This is available for proteolytic conversion of surface-bound FXI to FXIa and PK to kallikrein, which are closely situated due to the high mutual affinity of FXII, FXI and PK for HMWK. The formation of kallikrein enhances further activation of FXII to α-FXIIa but carries on by continued proteolysis of α-FXIIa to β-FXIIa, a subset of both FXII and α-FXIIa. β-FXIIa contains the active site domain of α-FXIIa but not the surface-binding domain, losing with it the vast majority of its coagulant activity: it is often considered a by-product of coagulation (Ratnoff, 1985). This is thought to be a negative feedback mechanism by which coagulation is self-limiting and therefore localised, preventing mass thrombus formation and is an inevitable consequence of kallikrein formation. Since β-FXIIa contains the active site domain of FXII, no further coagulant activity can take place from the remaining peptide once β-FXIIa has been released.

With regard to catheter materials as assessed in these assays, events contrary to usual expectations appear to be taking place, whereby the activation of a large quantity of FXII causes quicker coagulation. Clearly, contact phase activation is a highly surface-mediated set of reactions with a large degree of self regulation, consecutive proteolysis occurring as both positive and negative feedback mechanisms for the same enzyme and substrate. The exact kinetics of these inter-reactions are not known. Taking a complex reaction apart *in vitro* can lead to conclusions which are not valid when considered as a whole. The significance of a particular reaction, therefore, is often mere speculation. The propagation of the coagulation cascade depends not only on the degree of FXIIa production but also the ability of FXIa to desorb from the surface since FIX activation occurs in the fluid phase or on lipid-micelle surfaces.

It would be possible for a material which allowed a large degree of FXII processing to bind FXI so tightly as to limit the extent of intrinsic coagulation, displaying high concentrations of β-FXIIa in the plasma whilst yielding a long plasma clotting time. It is harder to explain results which show a reversal of this response. This would occur in the following circumstances, however: (a) if β-FXIIa were selectively and tightly bound by the surface; (b) if PK were not bound by the surface; (c) if PK were bound but not in a conformation available for converting to kallikrein; (d) if α-FXIIa were in a conformation which allowed activation of FXI but not its proteolytic attack by kallikrein; (e) if β-FXIIa were selectively bound by substances not involved in contact phase activation (*e.g.*

plasminogen). (d) is not very likely, however: if FXII activity were not self-limiting, thrombosis would be extremely prevalent with these activating materials *in vivo*, which is not observed. The likelihood of any of the other explanations is a matter for speculation and requires further research.

The release of β-FXIIa may not be, then, as predictive of intrinsic coagulation initiation as is usually thought. The significance of the release of large quantities of β-FXIIa and β-FXIIa-C_1-Inh into the circulation by an indwelling medical polymer *in vivo* should not be underestimated, however. β-FXIIa itself strongly activates surface-bound or fluid-phase PK to kallikrein (Revak *et al.*, 1978). It also activates FVII (Radcliffe *et al.*, 1977) and C1 (Ghebrehiwet *et al.*, 1981). FXIIa has been implicated in the activation of plasminogen and thus the fibrinolytic system (Goldsmith *et al.*, 1978) and the activation of the angiotensin pathway. Indeed, the presence of zymogen-inhibitor conjugates, which are removed by the liver *in vivo*, may activate other pathways of significance far away from the initial site of activation.

The results are also surprising in the degree of variation between plasma from different donors. A variation in cellular reactivity is usual, including variation from day to day of the same donor, since cells are a complex collection of metabolic pathways which constantly respond to activating and deactivating substances within plasma. The collection of contact phase proteins have the same peptide sequence, however, from person to person. Variations in reactivity seem consistent from day to day so are probably not caused by fluctuations in other plasma components. It may be due to the cooperativity from different plasma proteins or the type and degree of carbohydrate content (coagulation proteins are glycoproteins) may be different owing to selective post-translational modifications.

The results also show that the initial rate of FXII activation can be very fast. For Davathane, using active plasma, 4 ng/ml/minute were produced for the first 30 seconds; 0.54 ng/ml/minute for the next 9.5 minutes and 30 pg/ml/minute for the last 50 minutes. This demonstrates the importance of the first seconds of initial blood-artificial surface contact. The rate is severely limited over time, presumably by the lack of desorption of deactivated FXIIa from the surface after β-FXIIa has been released.

5.5 CONCLUSIONS

The initiation of contact phase activation is a subject which is somewhat confused in the literature. Functional methods for its measurement, the only ways that have been available until recently, can give only unreliable results if used in a whole plasma system.

The immunological method described gives very consistent and, as far as anyone can tell, reliable results for fluid phase FXIIa. These show that contact phase activation can occur very rapidly on an artificial surface, with almost total passivation of the surface within an hour. The fate of β-FXIIa after contact of a material with plasma requires further research. The results show that some polyurethanes can cause an immeasurably small amount of FXIIa release whilst others can cause a relatively large quantity (in fact 3 ng/ml is approximately only 0.01% of total FXII), which may be important in pathways other than coagulation. The end result of coagulation after activation of a plasma aliquot with an artificial surface, PTT, shows a different effect and has shown large differences between catheter materials, silicone being very good and Pellethane very bad. There is clearly not a direct correlation between contact phase activation and the rate of fibrin formation.

CHAPTER 6

PROTEIN ADSORPTION

6.1 INTRODUCTION

Whilst platelets have a physiologically pivotal rôle in thrombomodulation, (Anderson and Kottke-Marchant, 1987), that is the supply of platelet-derived lipid and the availability of a surface for coagulation serine protease and cofactor assembly, it is first necessary for proteins to adsorb onto the surface (Chuang, 1987). This occurs very quickly, shortly after the appearance of water and inorganic ions, mainly as a biophysical effect (Andrade and Hlady, 1986). There is an increase in the entropy of a system when the hydrophobic domains on a protein molecule can associate with a non-aqueous environment (*e.g.* a surface), so protein adsorption is an inevitable consequence of the presence of most, if not all, artificial surfaces in plasma. The nature of the protein layer has a profound effect on cellular activity caused by the presence of the surface. It is important, therefore, to analyse as thoroughly as possible the composition of the adsorbed protein layer in order to understand the behaviour of cellular systems after exposure of a surface to blood.

There are many different parameters of protein adsorption and many ways in which these can be measured. The medium from which the adsorbed protein layer is derived is also varied, ranging from single protein solutions, which allows for an extremely in-depth, real-time assessment of the kinetics of the process, to more realistic complex protein solutions (including serum or plasma) which generally allow only discrete time measurements to be performed retrospectively. In this study, a number of methods have been investigated so as to determine in which way such adsorption processes might have an effect on the biocompatibility of polymers.

6.2 TOTAL PROTEIN ADSORPTION

6.2.1 Introduction

The first experiments involved exposure of the polymer surface to plasma and elution of the adsorbed layer by detergent. In order to analyse properly the composition of protein in *any* sample (and specifically the adsorbed layer), it is first necessary to

determine its quantity in the sample. Only then is it possible to apply the most appropriate techniques. The mass of eluted protein was determined by the following standard technique (Lowrie *et al.*, 1951).

6.2.2 Materials and methods

Plasma Incubation

Reagents

1. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.3.
2. Barbitone buffer: 122mM 5,5-diethylbarbituric acid, 370mM tris-(hydroxymethyl) aminomethane, 4.0mM calcium lactate, 0.08% (w/v) sodium azide in H_2O, (22.4 g 5,5-diethylbarbituric acid, 44.3 g tris-(hydroxymethyl) aminomethane, 432 mg calcium lactate, 800 mg sodium azide in 1 l H_2O). Diluted 1 + 4 for use.
3. Triton X100: 2% (v/v) in barbitone buffer (2).

Method

1. PPP was incubated in the inside of a 1.22 m length of 1 mm internal diameter silicone tubing for 15 minutes at 37°C.
2. The plasma was slowly removed from the tube which was then gently rinsed with PBS (1).
3. 250 μl Triton X100 (3) were perfused through the tube at 100 s^{-1} (approximately 1 ml per minute) to capture most of the adsorbed protein.

Protein Analysis

Reagents

1. Saline: 137mM NaCl in H_2O (8.0 g NaCl in 1 l H_2O).
2. Colour reagent: 189mM Na_2CO_3, 100mM NaOH, 6.9mM sodium tartrate, 37mM SDS, 2.5mM $CuSO_4$ in H_2O, (2.0 g Na_2CO_3, 400 mg NaOH, 160 mg sodium tartrate, 1.0 g SDS, 40 mg $CuSO_4$ in 100 ml H_2O). Prepared fresh each day.
3. Phenol reagent: Folin and Ciocalteaus phenol reagent 2N (Sigma, Poole, Dorset, UK). Diluted 1 + 1 with H_2O.
4. Protein standards: 0 - 200 μg Bovine Serum Albumin (BSA) in saline (1) (1 ml for each standard).

Method

1. 950 μl saline (1) were added to 50 μl of each protein sample, including standard BSA concentrations.
2. 3 ml colour reagent (2) were added to each sample, vortex-mixed and incubated at room temperature for 10 minutes.
3. 300 μl phenol reagent (3) were added, vortex-mixed immediately and incubated at room temperature for 45 minutes.
4. The absorbance was read at 660 nm within 10 minutes.
5. The protein concentration was calculated by reference to the standard curve.

6.2.3 Results

The protein content of four plasma samples was determined by this method. They were all eluted as described above. These data are shown in Table 6.1.

Experiment Number	Quantity of Triton X100 used for elution (μl)	Mass of protein eluted from surface (μg)	Mass of protein remaining on surface (μg)
1	50	117	-
2	50	125	85
3	100	231	102
4	200	212	52

Table 6.1 Mass of protein elutable from silicone elastomer with Triton X100 after exposure to PPP for 15 minutes and the mass elutable with 2% SDS afterwards

The amount of protein eluted from silicone, after the same exposure conditions with 2% SDS in water, was 176 μg: in other words, not significantly different from that eluted with Triton X100. To ensure that most of the protein had been eluted by this procedure, the tube was also eluted with 200 μl 2% SDS after the Triton X100 elution. There appeared to be a significant quantity of protein remaining in each case.

6.2.4 Discussion

These results show that there is about 200 μg of protein elutable from the surface of silicone (a 1.22 m length of 1 mm internal diameter tubing) after exposure to PPP for 15 minutes at 37°C, a figure which is reasonably reproducible. They also indicate that there is still a significant quantity of protein still to be eluted after any one elution and that there is enough proteinaceous material to allow detection using most *standard* protein analysis techniques.

6.3 CROSSED IMMUNOELECTROPHORESIS

6.3.1 Introduction

It is clearly important to know the composition of the adsorbed layer. Crossed immunoelectrophoresis is a fairly standard technique (Clarke and Freeman, 1968) in which proteins are firstly separated by charge and then, in a second dimension, are electrophoresed through a gel containing antibody. At the point at which the antibody is at the same concentration as the protein for which it is specific (known as equivalence), a precipitate forms. The areas under the resultant peaks are proportional to protein concentration, allowing an assessment to be made of the quantities of different proteins present.

In this instance, a polyclonal antibody raised against whole human serum was used, with the extra inclusion of an anti-fibrinogen antibody. Any peaks arising were assigned using a calibrated plate of normal plasma against anti-whole serum (with the added anti-fibrinogen). Concentrations can be estimated by running known concentrations of antigen through a gel with these same antibodies (Laurell rocket electrophoresis, (Laurell, 1972)).

6.3.2 Materials and methods

Electrophoresis

Reagents

1. Barbitone buffer: 122mM 5,5-diethylbarbituric acid, 370mM tris-(hydroxymethyl) aminomethane, 4.0mM calcium lactate, 0.08% (w/v) sodium azide in H_2O, (22.4 g 5,5-diethylbarbituric acid, 44.3 g tris-(hydroxymethyl) aminomethane, 432 mg calcium lactate, 800 mg sodium azide in 1 l H_2O). Diluted 1 + 4 for use.
2. Agarose: 1% (w/v) Type I (low endosmotic properties, Sigma, Poole, Dorset, UK) in diluted barbitone buffer (1).

3. Bromophenol blue (Electran/BDH, Poole, Dorset, UK): several grains dissolved in 100 μl diluted barbitone buffer (1).
4. Stain: 2% (w/v) Coomasie brilliant blue in 45.5% (v/v) methanol, 9% (v/v) glacial acetic acid in H_2O (2.0 g Coomasie brilliant blue type R (Sigma, Poole, Dorset, UK), 455 ml methanol, 90 ml glacial acetic acid in 455 ml H_2O). Particulate matter was removed by filtering through a Whatman number 1 filter (Whatman, Maidstone, UK).
5. Destain: 10% (v/v) methanol, 10% (v/v) glacial acetic acid in H_2O (100 ml methanol, 100 ml glacial acetic acid in 800 ml H_2O). This was regenerated after use by filtering through activated charcoal.
6. Goat polyclonal antibody to human whole serum (Sigma, Poole, Dorset, UK).
7. Sheep monoclonal antibody to human fibrinogen (Serotech, Oxford, UK).
8. Normal saline: 146mM NaCl in H_2O (8.5 g NaCl in 1 l H_2O).

Method

1. 8 ml molten agarose (2) were poured onto an electrophoresis plate (8.2 cm x 8.2 cm) whilst standing on a levelling table and left until set, to give a 1 mm thick gel.
2. A 3 mm diameter hole was punched within the gel, to one side.
3. The hole was loaded with 25 μl of the sample to be analysed.
4. 1 μl bromophenol blue (3) (a marker of furthest protein migration) was also placed within the hole.
5. The gel was electrophoresed towards the anode with a potential drop of 10 V/cm with cooling until the bromophenol blue marker had nearly reached the end of the gel.
6. The gel was cut away along a line parallel to the direction of electrophoresis.
7. 50 μl polyclonal antibody (6) and 15 μl anti-fibrinogen (7) were added to 8 ml molten agarose (2), already equilibrated at 56°C.
8. About 6 ml of this were poured onto the plate such that it did not overrun the first dimension gel.
9. The gel was electrophoresed overnight (with cooling), again towards the anode with a potential drop of about 5 V/cm.
10. The gel was incubated in saline (8) for over 2 hours at room temperature, periodically changing the solution for fresh saline. This removed non-precipitated protein.
11. It was incubated in distilled water for 15 minutes to remove any saline and protein.

12. The gel was covered with damp filter paper (wet to prevent the gel from sticking) and a substantial layer of paper towels (about 1 cm high) and crushed with about 1 kg of weight.
13. The mostly dehydrated gel was dried in warm air down onto the glass plate.
14. The plate was soaked in stain (4) for 15 minutes then incubated with destain (5), changing the solution each time a significant amount of colour had been released, until the background was clear.
15. The gel was dried and the peaks analysed.

Protein carbamylation

Reagents

1. Carbamylation reagent: 2.0M KOCN in H_2O (1.62 g KOCN in 10 ml H_2O).

Method

1. Extracted proteins were added to an equal volume of carbamylation reagent (1).
2. This was incubated for 30 minutes at 45°C and then cooled prior to analysis.

6.3.3 Results

Crossed-immunoelectrophoresis has been used on extracts taken from silicone as described in section 6.2.2. When uncarbamylated protein extract was used, very few peaks were observed. In a stained, one dimensional standard electrophoretic gel of the extract, there was a substantial quantity of protein observed moving in the opposite direction to other proteins. Carbamylation was employed to reduce the pI's of all the proteins to force them all to run in the same direction. Only one, very faint peak was detected in the second dimension on carbamylated extracts.

6.3.4 Discussion

The observation that a substantial quantity of protein has run in the opposite direction from other plasma proteins (*i.e.* towards the cathode) suggests that it is very electronegative, such as immunoglobulin (*e.g.* IgG). Carbamylation was necessary to make the proteins electrophorese in the same direction through the antibody containing gel. Visual quantification of the first dimension gel suggests that there is preferential adsorbance of this protein to silicone compared with the adsorbance of albumin. That there was only a weak band in the second dimension compared to that observed in the first dimension suggests that the anti-whole serum antibodies, or anti-fibrinogen antibody, were not

recognising the protein extract. The extract may have been very conformationally disrupted, causing the antibodies not to bind the proteins and thus allowing them to electrophorese off the edge of the plate. Otherwise, the predominant protein present may not have been a serum protein. Further analyses were performed to assess the likelihood of these possibilities.

6.4 SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

6.4.1 Introduction

In the conditions of this type of electrophoresis, proteins are uncoiled with a highly charged detergent (SDS). This means that any protein would have the same charge per unit length and, apart from steric hinderance, would be equally mobile. They are restricted in their movement through the pores of the gel, however, by their length, such that their migration is linearly proportional to the log of their molecular weight.

Reduction of the proteins can be achieved with 2-mercaptoethanol which breaks the disulphide bridges and can be used to help assign bands on the gel by allowing comparisons of molecular weight between the tertiary and quaternary protein structures.

Peaks from the immunoelectrophoresis gel can, therefore, be assigned molecular weights by reference to standard molecular weight markers on the SDS gel to provide evidence of the correct assignment by immunoelectrophoresis.

6.4.2 Materials and methods

Reagents

1. Ammonium persulphate: 10% (w/v) in H_2O.
2. Acrylamide (99.9%, Biorad, Hemel Hempstead, UK): 30% (w/v) in H_2O.
3. Sodium ethylenediaminetetraacetate (EDTA): 20mM in H_2O (7.44 g EDTA in 1 l H_2O), pH 7.5.
4. 2-Mercaptoethanol (BDH, Poole, Dorset, UK).
5. Running buffer - 1.6M stock: 1.585M Tris, 14mM SDS in H_2O (19.2 g Trizma base (Sigma, Poole, Dorset, UK), 400 mg SDS in 100 ml H_2O), pH 6.8. Diluted 1 + 3 for use.
6. Stacking buffer - 500mM stock: 500mM Tris, 14mM SDS in H_2O (6.06 g Trizma base (Sigma, Poole, Dorset, UK), 400 mg SDS in 100 ml H_2O), pH 6.8. Diluted 1 + 3 for use.

7. Sample buffer: 4.3M glycerol, 700mM SDS, 2mM EDTA, 0.024% (w/v) bromophenol blue in 50% (v/v) stacking buffer, (20 g glycerol, 10 g SDS, 5 ml EDTA (3), 12 mg bromophenol blue (Electran/BDH, Poole, Dorset, UK) in 25 ml stacking buffer (6)).
8. Electrophoresis buffer: 3.5mM SDS, 192mM glycine, 25mM tris, (5 g SDS, 72.05 g glycine (free base, Sigma, Poole, Dorset, UK), 15.25 g Trizma (Sigma, Poole, Dorset, UK) in 5 l H_2O), pH 8.3. Made up fresh each time of use.
9. Air exclusion buffer: 20 ml ethanol, 0.024% (w/v) bromophenol blue in 100 ml running buffer (5).
10. 7.5% Running gel: 11.25 ml acrylamide solution (2), 11.25 ml running buffer (5), 300 μl ammonium persulphate (1), 30 μl TEMED in 22.05 ml H_2O (AnalaR grade) for two 8 cm x 8 cm plates.
11. 4% Stacking gel: 1.65 ml acrylamide solution (2), 2.5 ml stacking buffer (6), 100 μl ammonium persulphate (1), 7 μl TEMED in 5.75 ml H_2O (AnalaR grade).
12. Molecular weight markers (Sigma SDS-6H): Bovine erythrocyte carbonic anhydrase (29,000), Egg albumin (45,000), Bovine albumin (66,000), Rabbit muscle phosphorylase b (97,400), *E. Coli* ß-galactosidase (116,000), Rabbit muscle myosin (205,000).
13. Stain: 2% (w/v) Coomasie brilliant blue type R (Sigma) in 45.5% methanol, 45.5% H_2O, 9% glacial acetic acid. Any particulate matter is removed by filtering through a Whatman number 1 filter (Whatman, Maidstone, UK).
14. Destain: 10% (v/v) methanol, 10% (v/v) glacial acetic acid in H_2O. This can be reused after regeneration by filtering through activated charcoal.

Method

1. 20 ml air exclusion buffer (9) were poured into the gel casting apparatus. This prevents interference of the polymerisation by air.
2. The ingredients of the running gel were mixed, run into the gel casting apparatus slowly from the bottom via a pipe and allowed to polymerise for 20 minutes.
3. Any excess liquid was poured out and the apparatus rinsed with distilled water (AnalaR grade).
4. The outer spaces of the gel plates were filled with water.
5. The ingredients of the stacking gel (11) were mixed and pipetted into the apparatus from the top, gel combs inserted and allowed to polymerise for 20 minutes.

6. After the combs were removed, the gels and plates were inserted vertically into the electrophoresis tank, which was then filled with electrophoresis buffer.
7. Samples (maximum protein content of 100 μg) were mixed with an equal volume of sample buffer (7) (to a maximum volume of 50 μl) in eppendorf tubes.
8. If reducing conditions were required, 2-mercaptoethanol (4) was added to a final concentration of 10% (v/v), the eppendorf tube caps pierced with a needle (to prevent explosion) and boiled in a water bath for 5 minutes.
9. The eppendorf tubes were spun in a microcentrifuge at 13500g for 1 minute.
10. Up to 50 μl of each sample were carefully loaded onto each of the lanes of the gel with a Hamilton syringe (Nycomed, Birmingham, UK).
11. The gels were then run down the stacking gel at 70 V per plate (current at a maximum of 40 mA per plate) for about 10 minutes.
12. The gels were then run at 125 V per plate (current at a maximum of 70 mA per plate) for about an hour, until the tracer dye in each sample had almost reached the bottom of the gel.
13. The gels were removed from the tank, then from their plates and placed in an excess of stain (about 200 ml) and incubated at room temperature for about 40 minutes.
14. The gels were then placed in destain, initially changing the solution every 20 minutes, while agitating on a shaker with white tissue placed in the solution to soak up any stain, then destained overnight.

6.4.3 Results

Bands from the electrophoresed extract, in reducing conditions, showed three peptides of the following molecular weights: 25,000, 81,000 and 96,000.

6.4.4 Discussion

These bands do not correspond to IgG. IgG is made up of 4 subunits, 2 of molecular weight of approximately 50,000 and 2 of 100,000. The 96,000 band and conceivably the 81,000 band could be the heavy chains, but 25,000 is too small for the light chains. Furthermore, none of these bands corresponds to bands expected with fibrinogen or albumin, or indeed with any of the twelve most concentrated proteins in plasma.

Clearly further investigation is necessary. Better resolution would definitely be more helpful for greater accuracy in molecular weight determination, achievable by running longer gels. Greater staining efficiency, in order to pick out any faintly stained bands, might indicate the presence of other, less concentrated peptides.

6.5 ISOELECTRIC FOCUSSING

6.5.1 Introduction

Electorphoresing across a differential pH will cause proteins to travel in the direction of the point that corresponds to the pH at which the protein has no charge (its pI). If the gel is run for long enough, the proteins will equilibrate at the correct positions, allowing a good determination of the types of protein present.

6.5.2 Materials and methods

Reagents

1. pH gradient gel: Ampholine PAGplate, pH 3.5 - 9.5 (LKB, Bromma, Sweden).
2. Anode solution: 1.0M H_3PO_4 (980 mg H_3PO_4 in 10 ml H_2O).
3. Cathode solution: 1.0M NaOH (400 mg NaOH in 10 ml H_2O).
4. Fixative: 700mM trichloroacetic acid, 158mM 5-sulphosalicylic acid in H_2O, (57.5g trichloroacetic acid, 17.25 g 5-sulphosalicylic acid in 500 ml H_2O).
5. Destain: 250 ml ethanol, 80 ml glacial acetic acid in 670 ml H_2O.
6. Stain: 230 mg Coomassie R-250 (Sigma, Poole, Dorset, Kent, UK) in 200 ml destain (non-regeneratable).
7. Preserving solution: 20 ml glycerol in 200 ml destain.

Method

1. The pre-prepared pH-gradient gel (1) was unpacked and placed on the electrophoresis tablet.
2. The electrode strips were soaked with the appropriate electrode solutions (2, 3) and placed on the gel, pressing to confirm contact.
3. Sample application pieces were placed on the gel and 15 μl of each sample applied to the pieces.
4. The electrodes were connected to an appropriate power supply and the gel electrophoresed for 2 hours at 1.2 kV. The sample application pieces were removed 45 minutes after the start of electrophoresis.

5. The edge of the gel was removed and cut into 8 equal pieces. These were placed into 1 ml distilled water and the pH measured.
6. These measurements were taken as the mean of the pH along the length of each strip and a calibration curve of pH against distance from a baseline produced from this data.
7. Protein pI's were calculated using this curve from the distance of the gel bands from the baseline. The gel was internally calibrated with fresh plasma.
8. The gel was placed in fixative (to precipitate out the proteins) and incubated at room temperature for 45 minutes.
9. The gel was removed and placed in destain for 5 minutes to wash away the fixative.
10. The destain was drained away and replaced by stain. The plate was incubated at 60°C for 10 minutes.
11. The plate was placed in destain and agitated at room temperature until the background was clear, the destain being changed when necessary.
12. The plate was placed in preserving solution for 1 hour then placed on stiff filter paper overnight in a dust-free environment.
13. A sheet of PVC was placed over the gel and rolled onto it for future reference.

6.5.3 Results

The gel showed a major band towards the electronegative end (pI 3.5, as estimated from the calibration curve) and two minor bands close by, slightly more electropositive (pI's 4.3 and 4.4). After electrophoresis but prior to fixation, however, the gel was very cloudy in the lane which contained the eluted protein sample.

6.5.4 Discussion

The cloudy appearance of the gel indicated that some type of lipid-like structure was adsorbing. The detection of three other bands close to the pI of albumin contradicts the results obtained from crossed-immunoelectrophoresis. If the protein were albumin, it would also conflict with data from SDS-PAGE analysis. It was decided to observe the characteristics of the sample more precisely by separation on and elution from a chromatography column (section 6.6).

6.6 FAST PROTEIN LIQUID CHROMATOGRAPHY (FPLC)

6.6.1 Introduction

The basis of FPLC is that, depending on the type of column used, molecules of specific characteristics are retained, whilst those of the opposite characteristic are eluted. These molecules can be eluted at a later stage in a differential manner with increasing salt concentrations.

In this case, a Mono-Q column (Pharmacia, Uppsala, Sweden), which retains negative moieties was used, with the objective of determining the salt concentration at which the protein would be eluted.

6.6.2 Materials and methods

Reagents

1. Buffer A: 50mM Tris-HCl, pH 8.0.
2. Buffer B: 50mM Tris-HCl and 2.0M NaCl, pH 8.0.

Method

1. The column was equilibrated with buffer A (1) before the start of the experiment using a high pressure liquid chromatography apparatus (LKB 2299, Bromma, Sweden).
2. An aliquot of sample was injected at the top of the column and eluted with buffer A (1).
3. A salt gradient of buffer B (2) was passed down the column over a period of time, as determined by the pressure pump.
4. The eluate was constantly screened by measuring the absorbance at 280 nm and recording on a chart recorder.

6.6.3 Results

Control experiments using concentrations of bovine serum albumin at 500 μg/ml (similar to the concentration of protein in the extract as calculated by Lowrie determination (section 6.2)) showed that the BSA is eluted in a relatively tight peak using differential concentrations of buffer containing 2M NaCl. BSA was used as a control since the extract protein was shown to have a similar pI to that of albumin by isoelectric focussing (section

6.5). Very little protein, however, could be detected in the eluate by absorption at 280 nm using extract protein under the same conditions.

6.6.4 Discussion

Literature relating to use of this type of column (Mono-Q, Pharmacia, Uppsala, Sweden) stated that highly sulphated moieties would not be displaced by 2M NaCl, regardless of the value of the pI. Further experiments designed to displace the protein, such as the inclusion of 2% Triton X100 in the buffers, were also unsuccessful. The results suggested, then, that the species in question was either a highly sulphated protein or related molecule (*e.g.* glycosaminoglycan) or a protein complex involving highly sulphated moieties. To test the former hypothesis, the following staining procedure for transmission electron microscopy was used.

6.7 TRANSMISSION ELECTRON MICROSCOPY (TEM)

6.7.1 Introduction

On the basis of the foregoing experiments, it was decided to investigate whether the desorbed species were glycosaminoglycans. It was postulated that cellular adhesion to the surface of a material might be assisted by the interaction of cell-membrane glycoprotein receptors and adhesive proteins, as in the generally accepted theory of platelet adhesion (Lee and Kim, 1979), but with the cooperativity of this extra factor. The presence of a glycosaminoglycan (or acid mucopolysaccharides) should be detected by the staining of adhered platelets with ruthenium red and the viewing of thin sections by transmission electron microscopy (Luft, 1971b). Different samples were exposed statically and then viewed in this way after impregnation with Spurr resin (Spurr, 1969). This method of staining has been used previously for visualisation of glycosaminoglycans in tissue (Luft, 1971a). The adhesion of circulating platelets to de-endothelialised rabbit arteries has been studied by observing their interaction with glycosaminoglycans from the ground substance of the vessels using this technique (Sheppard and French, 1970). The visualisation of platelets on surfaces of very different compliance: silicone; Pellethane; and PTFE, after static incubation of the surface with PRP at room temperature for 1 hour, was attempted in this way.

6.7.2 Materials and methods

Reagents

1. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.3.
2. Double strength Ruthenium Red solution: 0.10% (w/v) Ruthenium Red in 200mM sodium cacodylate (80 mg Ruthenium Red (Sigma, St Louis, Missouri, USA), 8.48g sodium cacodylate (BDH, Poole, Dorset, UK) in 200 ml H_2O), pH 7.4.
3. Single strength Ruthenium Red solution: 0.05% (w/v) Ruthenium Red in 100mM sodium cacodylate (20 mg Ruthenium Red (Sigma, St Louis, Missouri, USA), 2.12g sodium cacodylate (BDH, Poole, Dorset, UK) in 100 ml H_2O), pH 7.4.
4. Fixative: 0.05% (w/v) Ruthenium Red solution in 2.5% (v/v) glutaraldehyde and 100mM Sodium Cacodylate (10 ml glutaraldehyde (25%, SEM grade, Emscope, Ashford, Kent, UK), 50 ml double strength ruthenium red solution (2) in 40 ml H_2O), pH 7.4.
5. Post-Fixation solution: 0.05% (w/v) Ruthenium Red, 1% (w/v) osmium tetroxide in 100mM sodium cacodylate (1 g osmium tetroxide (Agar Scientific, Cambridge, UK), 50 ml double strength Ruthenium Red solution (2) in 50 ml H_2O), pH 7.4.
6. Initial dehydration solution: 70% Methanol with 0.05% (w/v) Ruthenium Red (20 mg Ruthenium Red (Sigma, St Louis, Missouri, USA), 70 ml methanol in 30 ml H_2O).
7. 90% (v/v) methanol in H_2O.
8. Methanol.
9. Spurr Resin: 13 ml Nonenyl Succinic Anhydride, 2 ml Dow epoxy Resin, 5 ml Vinyl Cyclohexene Dichloride, 200 μl Diaminoethanol (all from Polaron, Watford, UK).

Method

1. The exterior of catheter tubes were exposed statically for 1 hour to PRP then washed with PBS (1) to remove any non-adherent platelets.
2. The samples were incubated with fixative (4) for 1 hour at room temperature, washed with Ruthenium Red solution (3) and incubated at room temperature for 10 minutes.
3. They were then incubated in fresh Ruthenium Red solution (3) for a further 10 minutes at room temperature.

4. These were again removed and incubated in post-fixation solution (5) for 30 minutes at room temperature.
5. The samples were quickly washed with Ruthenium Red solution (3) then incubated in two consecutive aliquots of this solution (3) for 10 minutes each.
6. The catheter tubes were then dehydrated in two incubations of initial dehydration solution (6) for 15 minutes each, then twice in 90% methanol (7) for 15 minutes each.
7. They were then incubated for 15 minutes in each of two aliquots of 100% methanol (8).
8. The samples were infiltrated with a 3:1 mixture of absolute methanol (8) : Spurr resin (9) for 2 hours at room temperature.
9. They were then incubated with a 1:3 mixture of absolute methanol (8) : Spurr resin (9) for 3 hours at room temperature.
10. The samples were then infiltrated with absolute Spurr resin (9) overnight at room temperature.
11. Final embedding then took place in fresh Spurr resin (9) for 20 hours at 60°C.
12. The catheter samples were then sectioned to 90 nm using an ultramicrotome (Richert/Leica Ultracut E, Milton Keynes, UK) and viewed with by transmission electron microscopy (JEOL JEM-100CX, Tokyo, Japan).

6.7.3 Results

The results that this technique have provided are not easy to interpret. Using this type of sectioning and viewing it is possible to visualise adhered cells, such as platelets (fig 6.1). In sections containing structures of this type, typical platelet organelles were visible and identifiable but no Ruthenium Red staining was identified. The platelet shown was spreading onto Pellethane. No such platelet structures were visible on silicone. Where sections were produced, the material contained within them was completely disrupted and virtually all cellular material totally destroyed. In all of the samples assessed (silicone, Pellethane and PTFE), no surfaces were identifiable which had any staining of adsorbed material.

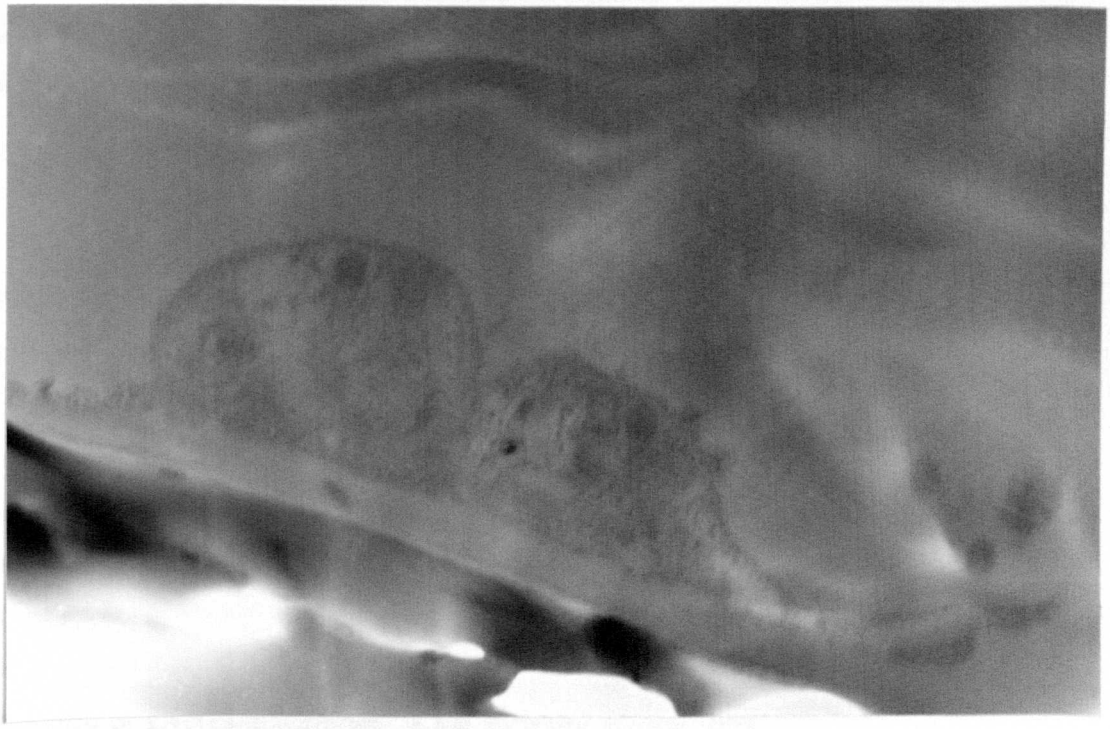

Fig 6.1 Platelet adhered to Pellethane, stained with Ruthenium Red and OsO_4, after incubation with PRP statically at room temperature for 3 hours, viewed with TEM, magnification x5000

6.7.4 Discussion

None of the surfaces has been shown to have an adsorbed glycosaminoglycan layer. This is partly due to the inability of the compliant surfaces to be sectioned properly. Polymeric surfaces, in general, do not allow infiltration by epoxy resins. Tissue, on the other hand, does allow such preparation. This means that there is a compliance mismatch, even for PTFE, between the layer of resin containing the adsorbed protein layer and any adherent platelets and polymer. On the whole, any sectioned surface viewed with TEM was shown to be highly disrupted and difficult to analyse. When platelets were observed, they were distant from the surface and not stained with Ruthenium Red, despite being associated with a surface prior to fixation, embedding and sectioning.

It is still possible that glycosaminoglycans are, indeed, utilised in the adherence of platelets to biomaterials or that they are preferentially adsorbed over the more common plasma proteins. While this study has not given evidence for this suggestion, it has illustrated the need to analyse a surface where the interface had not been disrupted by the sectioning process. This would be possible by using completely rigid blocks for cutting, achieved using cryo-ultramicrotomy, whereby the sample, knife and associated apparatus is cooled to very low temperatures by liquid nitrogen. Further analysis of the layer, using standard biochemical techniques to detect the presence of hexosamines (Davidson, 1966) or uronic acid moieties (Bitter and Muir, 1962) would provide extra evidence of the presence of glycosaminoglycans in the adsorbed protein layer.

6.8 CONCLUSIONS

The analysis of proteins adsorbed onto a piece of silicone catheter tubing has proved to be somewhat problematical. The SDS-PAGE technique has provided evidence, however, that there are proteinaceous substances which are preferentially adsorbed on the surface of a biomaterial over the more abundant plasma proteins (IgG, fibrinogen and albumin). Other techniques, such as crossed-immunoelectrophoresis, also suggest that this is the case. The results of running the eluted material along an iso-electrically focussed gel suggests that the preferentially adsorbed substance is electronegative and that there may be a high content of lipid or carbohydrate. The staining of surfaces for glycosaminoglycans has been inconclusive.

It seems necessary, therefore, to study the eluate further in order to determine conclusively the exact nature of the species present. Certainly, a greater efficiency of

detection would be an advantage in many of the electrophoretic techniques, in which silver stains, which have approximately a ten-fold greater protein detection limit than coomasie brilliant blue, might be used to identify those proteins present in smaller quantity. The implementation of more complex procedures, such as two dimensional SDS-PAGE/Iso electric focussing gels and immunoblotting, might also allow a fuller analysis.

This study has provided a small insight into the complexities of protein adsorption analyses. It also demonstrates the necessity for analysing the surface of each individual material to be used in artificial devices. The assumption that the major components of plasma (IgG, fibrinogen and albumin) modulate the function and adhesion of blood cells (Zucker and Vroman, 1969; Ito and Imanishi, 1989) may, on the basis of these results, be false in some cases. The analysis of this adsorbed layer is probably of utmost importance in understanding the manner in which platelets adhere or degranulate and FXII, complement and other proteins activate upon contact with a material.

CHAPTER 7

OTHER INDICES OF BIOCOMPATIBILITY

7.1 INTRODUCTION

Whilst the quantification of the interactions of platelets and plasma proteins is a very important aspect of the assessment of the biocompatibility of blood-contacting devices, it is necessary, in addition, to consider other interactions which may cause clinical problems. These include the ability of the material to lyse red blood cells and its propensity for leaching toxic species. Other areas of study are those which allow some interpretation of the blood compatibility results. In this group are the assessment of the atomic species and organic bonds at the material surface and a visualisation of the material surface topography.

7.2 HAEMOLYSIS

7.2.1 Introduction

Haemolytic material contacting blood causes an increase in the concentrations of free plasma haemoglobin. This lends itself to toxic effects, which may result in the stressing and subsequent failure of the kidneys or other organs. The ability or otherwise of a material to destroy red blood cells must, therefore, rate as an important feature of blood compatibility assessment.

Little research has been carried out into the reasons for red cell destruction, but it has been suggested that erythrocytes leave membrane particles on the surfaces of artificial materials (Borenstein and Brash, 1986).

The assay described was based upon the American Standard (ASTM, 1984). It has been rendered slightly more sensitive, however. The Standard recommends the incubation of blood with material in siliconised glass tubes. The contact of another surface was avoided by contacting the blood with the inside of the catheter tubes only.

7.2.2 Materials and methods

Reagents

1. Trisodium citrate: 3.8% (w/v) in H_2O (3.8 g trisodium citrate in 100 ml H_2O).
2. Drabkin's reagent: 11.9mM sodium bicarbonate, 770μM KCN, 610μM potassium ferricyanide in H_2O (1 g sodium bicarbonate, 50 mg KCN, 200 mg potassium ferricyanide in 1 l H_2O).
3. Haemoglobin standard (Sigma, Poole, Dorset, UK): 180 mg/ml stock solution in saline (4).
4. Normal saline: 0.85% (w/v) NaCl (8.5 g NaCl in 1 l H_2O).

Method

1. About 5 ml blood were collected in trisodium citrate (1), with a dilution of 1 + 9 citrate to blood.
2. The whole blood haemoglobin concentration was determined, by adding 20 μl blood to 5 ml Drabkin's reagent (2), incubating for 15 minutes then measuring the absorbance at 540 nm and comparing with a standard curve produced with the haemoglobin standard (3) diluted with normal saline (4).
3. The free plasma haemoglobin concentration was also determined by adding 100 μl PPP to 5 ml Drabkin's reagent (2), incubating for 15 minutes then reading the absorbance at 540 nm.
4. If the free plasma concentration was greater than 1 mg/ml then the blood was not used.
5. The blood was diluted with normal saline to give a haemoglobin concentration of 2.5 mg/ml.
6. This dilution was incubated with catheter samples[1] for 4 hours (statically) or 1 hour (dynamically - agitation on a plate shaker to prevent red blood cell sedimentation) at 37°C.
7. The resultant plasmas were centrifuged at 800g for 5 minutes.
8. The haemoglobin concentrations of each sample were determined by adding 1 ml blood dilution to 3 ml Drabkin's reagent (2). Each sample was then incubated for 15 minutes and the absorbance read at 540 nm, extrapolating values from the standard curve.

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[1] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

7.2.3 Results

The protocol was used to assess the haemolytic potential of catheter materials both statically and dynamically using large bore material samples. The results are shown in table 7.1 and figs 7.1 and 7.2.

Material	Haemoglobin released (mg/ml)	
	Static	Dynamic
Silicone	16.20 ± 2.46	2.58 ± 0.99
PTFE	0.14 ± 0.37	0.88 ± 1.22
PVC	2.35 ± 1.51	0.91 ± 0.16
LDPE	0.05 ± 0.32	1.01 ± 0.48
Tecoflex	0.14 ± 0.25	0.45 ± 0.24
Pellethane	0.28 ± 0.27	0.68 ± 0.66
Estane	1.15 ± 0.46	0.55 ± 0.35
Davathane	0.61 ± 0.61	0.91 ± 0.25
Desmopan	0.84 ± 0.23	0.74 ± 0.86
Texin	1.48 ± 1.16	0.87 ± 0.65
Elastollan 85	0.56 ± 0.18	0.73 ± 0.16
Elastollan 90	1.12 ± 0.61	1.00 ± 0.88

Table 7.1 Haemolytic properties of catheter materials assessed statically and dynamically at 37°C. All data are Mean ± SD. n = 4

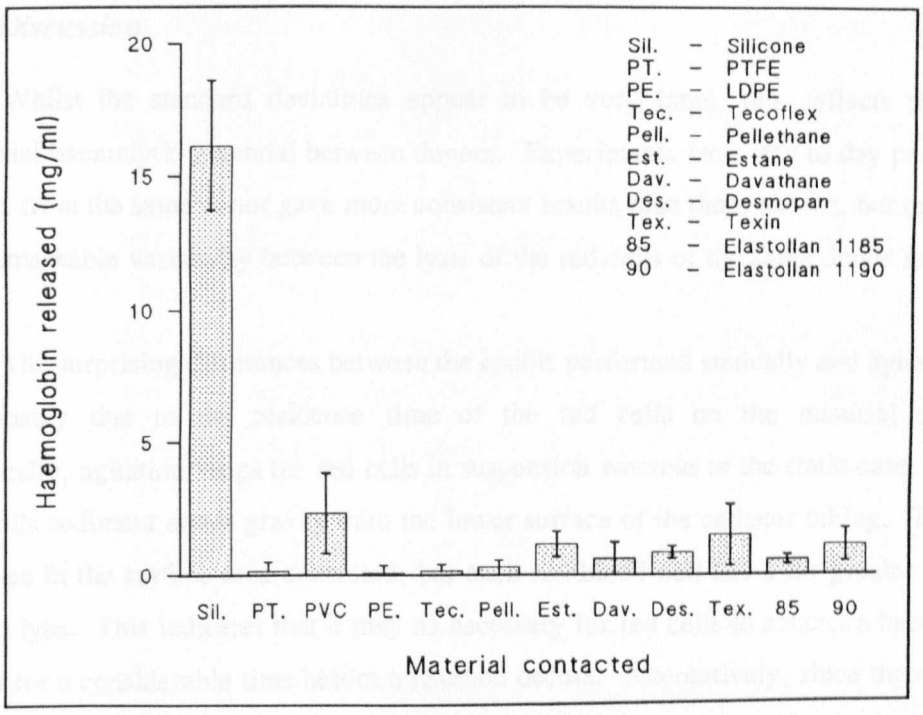

Fig 7.1 Haemoglobin released after the contact of diluted blood with catheter materials statically at 37°C for 4 hours

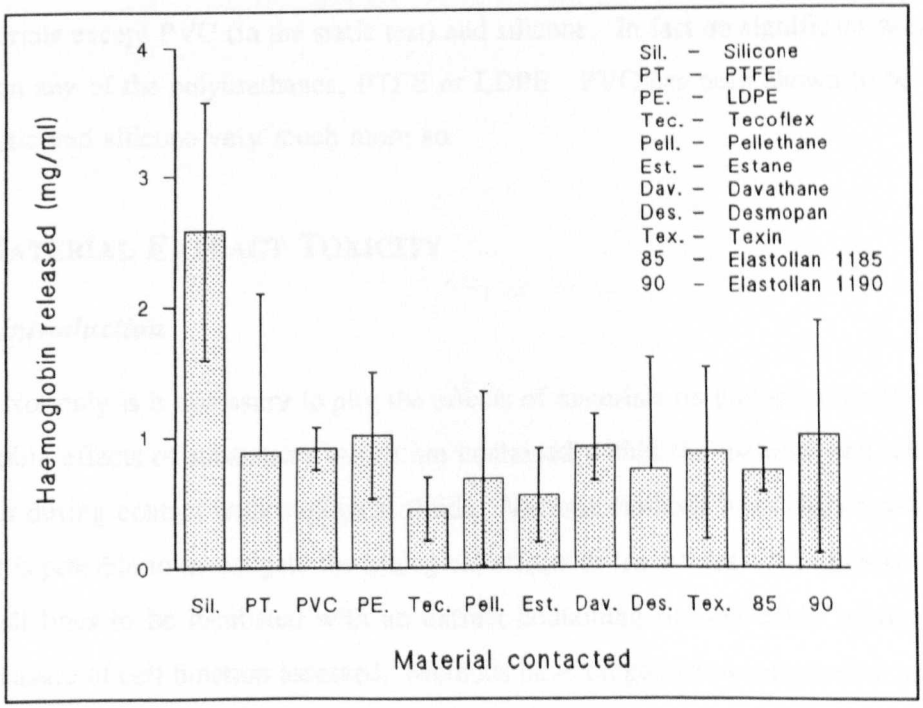

Fig 7.2 Haemoglobin released after the contact of diluted blood with catheter materials dynamically at 37°C for 1 hour

7.2.4 Discussion

Whilst the standard deviations appear to be very large, this reflects the large differential haemolytic potential between donors. Experiments from day to day performed on blood from the same donor gave more consistent results than those shown, but there was still a remarkable variability between the lysis of the red cells of the same donor from day to day.

The surprising differences between the results performed statically and dynamically are probably due to the residence time of the red cells on the material surface. Dynamically, agitation keeps the red cells in suspension whereas in the static case, the red blood cells sediment under gravity onto the lower surface of the catheter tubing. There is a decrease in the surface area contacted, but each red blood cell has a far greater time in which to lyse. This indicates that it may be necessary for red cells to contact a haemolytic material for a considerable time before a reaction occurs. Alternatively, since the reaction times were greater for static contact (as described in the American standards (ASTM, 1984)), it is possible that there is a long lag phase (greater than 1 hour) before any significant reaction takes place.

In any case, the results indicate that there are no real differences between any of the materials except PVC (in the static test) and silicone. In fact no significant haemolysis occurs on any of the polyurethanes, PTFE or LDPE. PVC has been shown to be slightly haemolytic and silicone very much more so.

7.3 Material Extract Toxicity

7.3.1 Introduction

Not only is it necessary to plot the effects of materials on biological tissue but also the possible effects of substances which are contained within the material and which may leach out during contact with biological fluids. Various methods have been developed by which it is possible to investigate the biological effects of leachables. All of these methods allow cell lines to be incubated with an extract containing the leachable substances and some measure of cell function assessed. Methods have ranged from cell number estimation by the quantification of methylene blue incorporation into viable cells (non-viable cells having been removed by vigorous washing), cellular proliferation by measuring the incorporation of [^{3}H]-thymidine into the cellular DNA to cellular mitochondrial metabolism

quantification by the measurement of a dye formed by the action of mitochondrial enzymes. It is this last method which was utilised here (section 7.3.2).

Just as important as assessing the extent to which leachable species can affect biological tissue is the identification of the species themselves. This would allow correlation between the cytotoxicity of the extracts from materials in a biological environment with additives used in manufacture of the device. A good extraction procedure is obviously necessary. To this end, as great a solvating solution (supercritical CO_2) was used to extract as much of the potential leachables from the materials as possible, which were then analysed using gas chromatography (section 7.3.3).

7.3.2 Methylthiazolyl-diphenyl tetrazolium (MTT) assay

7.3.2.1 Introduction

The MTT assay was first introduced by Mosmann (1983). It gives a measure of cell metabolism by converting a tetrazolium salt to formazan (a dye) through the action of mitochondrial succinate dehydrogenase. The formation of colour increases with metabolism, and so is in reverse proportion to the presence of toxic materials, the assumption being that toxic materials cause a slowing of metabolism and, in severe toxicity, cell death. This colour intensity is measured photometrically.

7.3.2.2 Materials and methods

Reagents

1. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O), pH 7.4.
2. Growth medium: 5% (v/v) foetal calf serum (mycoplasma screened and sterile, Flow, Irvine, UK) and 1% (v/v) antibiotic and antimycotic solution (streptomycin and penicillin, Flow, Irvine, UK) in 199M growth medium (Gibco, Paisley, UK).
3. Cell line: aneuploid L929 fibroblast cells (Flow, Irvine, UK).
4. Methylthiazolyldiphenyl tetrazolium (MTT): 100 mg MTT (Sigma, Poole, Dorset, UK) in 100 ml 199M growth medium (Gibco, Paisley, UK). Filtered to 0.22 μm.
5. Isopropanol.

Method

1. 250 cm^2 of catheter sample surface[2] were incubated with 10 ml PBS (1) for 50 days at 37°C whilst mixing on a roller-mixer to provide a physiologically relevant extract. 10 ml air were included with the PBS to promote maximal mixing.
2. These extracts were serially diluted in growth medium (2) (*e.g.* 1+1, 1+2, 1+4, 1+9, 1+19).
3. The cell line (3) was seeded onto the wells of a 96-well microtitre plate (Becton Dickinson Labware, Lincoln Park, New Jersey, USA) at a concentration of 10^4 cells/well in 200 μl growth medium (2) and incubated at 37°C in air with 5% CO_2.
4. The supernatant was discarded and replaced with 100 μl extract dilution (12 control cell wells per plate and 12 wells for each extract dilution) and the cells incubated for 24 hours at 37°C in air with 5% CO_2.
5. The supernatant was discarded, 50 μl MTT (4) added and incubated for 4 hours at 37°C.
6. This supernatant was discarded, the plates blotted, 100 μl isopropanol (5) added and agitated on a shaker for 15 minutes to dissolve the formazan.
7. The absorbance was read on a microtitre plate reader (Dynatech MR700, Billingshurst, West Sussex, UK) at 570 nm, referencing at 630 nm.

7.3.2.3 Results

The results of extraction at 37°C for 50 days are shown in table 7.2 and fig 7.3.

Material	Percentage of control cell survival			
	5% extract	10% extract	33% extract	50% extract
PVC	94.6 ± 10.2	4.4 ± 2.2	1.6 ± 0.2	2.3 ± 0.5
PTFE	-	74.8 ± 7.6	-	66.0 ± 5.7
Silicone	-	77.4 ± 8.0	-	68.3 ± 5.1
LDPE	-	77.4 ± 6.0	-	59.1 ± 5.5
Pellethane	95.1 ± 9.9	79.8 ± 3.2	69.9 ± 5.5	75.3 ± 3.4
Tecoflex	104.0 ± 9.6	76.9 ± 3.1	91.1 ± 28.5	50.7 ± 6.3

Table 7.2 Percentage of control cell metabolism at various concentrations of material extract in the MTT assay after extraction at 37°C for 50 days. Mean ± S.D.

[2] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

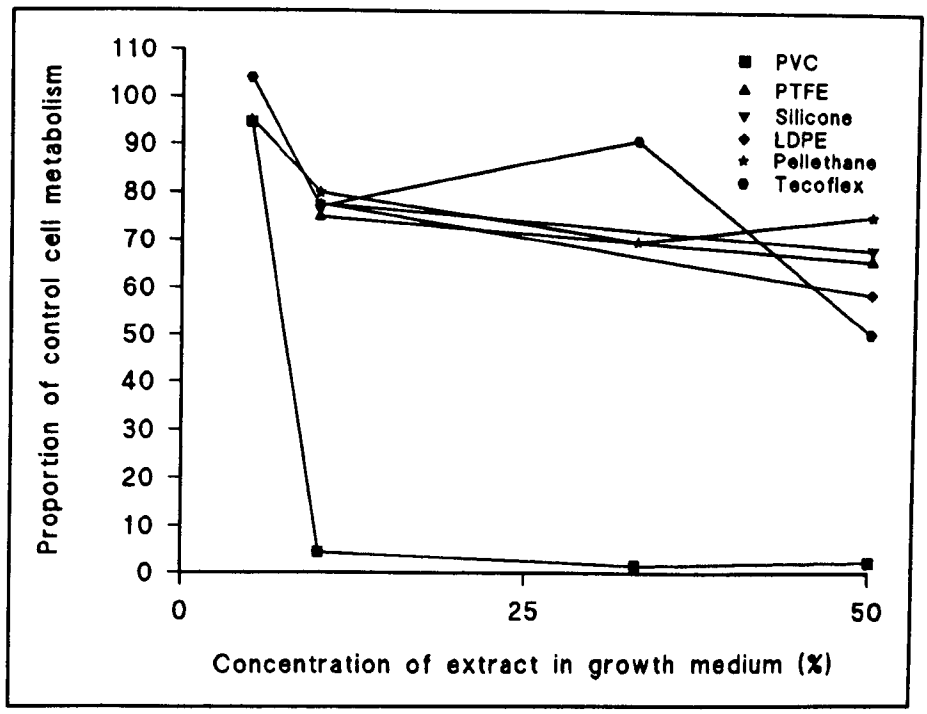

Fig 7.3 Cytotoxicity of catheter material extracts as measured by the MTT assay after extraction at 37°C for 50 days

7.3.2.4 Discussion

PVC has by far the most cytotoxic constituents of any of the materials tested. This is as expected, since the PVC used was flexible and translucent and this is only possible after the inclusion of large quantities of plasticiser. Concerns over the toxicity of plasticiser have been discussed widely in this field and this study would tend to reflect these worries. Apart from PVC, none of the materials could be differentiated from one another since each material extract caused a slight increase in toxicity as their incorporation into the cell line growth medium was increased. This would suggest that there are substances leaching out of polyurethanes, but that the toxic nature of these substances is very minor compared to that of PVC.

The value of this procedure cannot be underestimated. The potential for cytotoxicity is large and the possible thermal breakdown of the materials, as well as the release of cytotoxic substances, can be monitored in this way.

7.3.3 Super Critical Fluid Extraction (SFE)

7.3.3.1 Introduction

In the previous section, a method was described for assessing the effect on cultured fibroblasts of substances extracted from catheter tubing by incubating samples in PBS. This approach is useful in that it allows evaluation of the long term toxicity of a material, but it does not attempt to identify the nature of the substances being leached. Supercritical fluid extraction (SFE), the exploitation of the phenomenon of the very high solvating power of carbon dioxide whilst in its fourth phase (supercritical fluid), can be used to extract a large proportion of the non-structural substances contained within the material (McNally and Wheeler, 1988). This is a good model for long-term physiological extraction as a measure of the potential for toxicity.

This type of extraction also lends itself to qualitative analysis. Conventional biological extraction is performed in an aqueous medium and this prevents the use of gas chromatography since water does not carry very well. Other types of extraction using organic solvents usually require very large amounts of solvent which require amplification. After extraction with a supercritical fluid, the substances are in a pure state when exposed to atmospheric pressure since the supercritical fluid evaporates. If, however, the extract is retained under pressure, the solution can be used in gas chromatography (Smith, 1988) since supercritical CO_2 is an extremely good carrier.

7.3.3.2 Materials and methods

Method

1. Samples[3] for extraction were placed in the pressurised sample module and supercritical CO_2 (>73 bar, 31°C) pumped in.
2. These were incubated for 2 hours at this temperature and pressure.
3. The sample chamber's outlet port was opened and the CO_2 vented directly into a gas chromatography column.
4. The rise time of each material additive was recorded and measured against a set of standards.

[3] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

7.3.3.3 Results

This technique was used to identify the additives contained in standard materials and polyurethanes. The extraction and analysis technique is not really quantitative, at least not presently. Qualitative results of these are presented, however.

Material	Extract	Rôle
PVC	n-Di-octylphthalate Di-2-ethyl-hexylphthalate Di-iso-octylphthalate	Plasticiser Plasticiser Plasticiser
LDPE	C_{16} and C_{18} alkanes C_{16} and C_{18} alkenes	Impurity Impurity
Pellethane	BHT Irganox bis-ethylene-stearamide Oligomers	Antioxidant Heat stabiliser/Antioxidant Processing wax Impurity
Tecoflex	BHT Irganox Oligomers	Antioxidant Heat stabiliser/Antioxidant Impurity
Estane	BHT TPP Oligomers	Antioxidant Plasticiser Impurity
Davathane	Many unidentifiables Oligomers	 Impurity
Desmopan	BHT Oligomers	Antioxidant Impurity
Elastollan 1190	BHT TPP Irganox Oligomers	Antioxidant Plasticiser Heat stabiliser/Antioxidant Impurity
Elastollan 1185	BHT TPP Irganox Oligomers	Antioxidant Plasticiser Heat stabiliser/Antioxidant Impurity
Texin	BHT Oligomers	Antioxidant Impurity

BHT - Butylated hydroxy toluene, or Para-cresol
TPP - Tri phenyl phosphate

Table 7.3 Additives extracted from catheter tubing by supercritical fluid extraction

7.3.3.4 Discussion

This technique analyses the substances which are leached from the catheter materials and may cause the cytotoxic responses found in the MTT assay (section 7.3.2). Of interest is the prevalence of BHT in the extracts. This is common to almost all the polyurethanes and goes some way to explaining the similar responses of materials in the MTT assay. Irganox, also an antioxidant, has the structure C-$(BHT)_4$, and so is likely to give a similar cytotoxic response. The data demonstrate that there are many unwanted substances contained within medical grade polyurethanes, some materials being very much worse than others (Davathane, for example).

7.3.4 Discussion

The results of these two assays show that there is benefit in using the two techniques together. The MTT assay results display the cytotoxic effects of the medical grade polymers whereas the SFE results define the exact characteristics of the substances involved. It would be possible to collect the supercritical fluid extract from the materials and perform an MTT assay on each of these, which should provide comparative data to standard, long-term extraction at physiological temperatures. The effects of thermal breakdown of the materials would not be included in such a case but the technique could provide a rapid way of assessing the likely long-term toxicity of a polymeric material.

7.4 X-RAY PHOTOELECTRON SPECTROSCOPY (XPS)

7.4.1 Introduction

It is desirable to extend one's knowledge of a material's chemical composition by performing an analysis of the atomic composition of the surface and an estimation of the type of organic groups present. It is this surface which will ultimately be presented to the blood when the device is placed into a vein and so understanding its chemistry can allow more comprehensive interpretation of the results of *in vitro* experiments.

XPS relies on the assessment of the energies of emitted electrons from the surface atoms (within the outermost 10 Å) of a material irradiated with X-rays. These emitted electrons have specific energy ranges depending on the atoms irradiated. For example, F appears at about 700 eV, O at about 500 eV, N at about 400 eV and C at about 300 eV. Enumeration of the number of electrons, therefore, gives a quantitative estimation of the atomic surface composition of the material. A low resolution plot of the distribution of

electron energies shows a number of tight bonds, representing energies from the different atoms, some representing electrons from different energy levels of the same atom (*e.g.* 2s and 2p). The direct chemical environment also affects the energy of the released electrons. These energy shifts have been characterised for carbon in various chemical configurations relative to native carbon. This is particularly useful for a more detailed surface characterisation of complex organic compounds. These typical shifts in binding energy (ΔBE) in the C1s peak are shown in table 7.4.

Environment		Typical ΔBE (eV)
Hydrocarbon:	C-C-H	0
Amine:	C-C-N	0.7 - 1.3
Ether/Alcohol:	C-C-O	1.5
Amide:	O ‖ C-C-N	3.2
Ester:	C-C-O	3.8
Urea:	O ‖ N-C-N	3.8
Carbamate:	O ‖ N-C-O	4.3 - 4.7

Table 7.4 Typical binding energy shifts in the C1s peak for carbon in various chemical environments

A knowledge of the chemistry of the compound being assessed can be used to estimate the surface content of the atoms, an important aspect of interfacial reactions, and the presence of impurities.

7.4.2 Materials and Methods

The samples[4] were extensively rinsed with distilled water, ultrasonicated in dilute Triton X-100 solution and then rerinsed with water. The insides of the tubes were analysed for atomic composition. This was achieved at a flood-gun voltage of 1.0 V and a sample angle of 30°.

[4] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

7.4.3 Results

The C1s peak was analysed for the probable composition of bonds adjoining the carbon atoms by deconvolving the peak into its component parts by means of computerised curve fitting. The curve fitting model assumes that the peaks are 100% gaussian and from this calculates the relative areas attributable to each peak. The shift in binding energy of each was calculated from the hydrocarbon peak and the composition of each bond assessed by reference to a table of typical binding energy shifts. The percentages of each atom and each carbon atom type are shown in table 7.5.

Material	%C	Environment of carbon atoms as a percentage of the C1s peak				%N	%O	%Si	%F
		HC	ether	carb.	amide				
LDPE	95.9	100	-	-	-	-	2.2	1.5	0.4
PVC	77.0	72	24	4	-	-	9.6	2.1	-
PTFE	41.9	-	-	-	-	-	0.5	-	57.6
Silicone	48.5	100	-	-	-	-	24.0	27.5	-
Pellethane	92.3	75	20	-	5	3.4	4.1	0.2	-
Tecoflex	86.2	79	17	-	4	2.9	9.2	1.7	-
Davathane	94.0	85	13	2	-	-	5.4	0.6	-
Desmopan	91.2	86	9	-	4	3.6	4.8	0.4	-
Texin	86.9	84	12	1	3	3.2	7.2	2.3	0.4
Elastol 85	88.8	83	14	-	3	3.3	7.1	0.8	-
Elastol 90	82.1	68	31	1	-	3.3	13.9	0.7	-

HC - Hydrocarbon bond forming carbon atoms
Carb. - Carbamylate bond forming carbon atoms

Table 7.5 Atomic composition of the surface of catheter tubings

7.4.4 Discussion

There are many ways in which the data obtained from XPS analysis can be deemed deficient. Firstly, the technique is very sensitive to contamination. This generally takes three forms: atoms of material in the solid phase which have been left by the cleaning process (or deposited after cleaning, prior to analysis - this can include molecules from transportation bags); material which has tracked along the sample from other sources (*e.g.* fixation tape); and atmospheric gas adsorption. Silicon is a major contaminant from

siloxanes present in plastic storage bags and fixation tape and this explains the high levels of atomic silicon observed in all the samples where one would not normally expect to see any. Oxygen is unusual in LDPE and PTFE, as is fluorine in LDPE and Texin and so are also presumably contaminants.

The technique may also be regarded as being of limited value since samples are required to be analysed in a vacuum. It is well known that, even with rigid samples, a reordering of the soft and hard segments in segmented polyurethanes occurs when placed in water in an attempt to minimise the surface free energy (Sevastianov, 1988). This reordering can completely change the surface chemistry of the polymers such that a biological environment would encounter another set of chemical species compared to those shown in an XPS analysis.

The control materials, PVC, silicone, LDPE and PTFE have produced results which are broadly in line with expectations: PTFE has a C:F atom ratio of 40:60; LDPE is mostly carbon, all of which is hydrocarbon; silicone has a C:O:Si ratio of about 2:1:1.

In principle, it is possible to calculate, from a knowledge of the chemical composition of the remaining polymers, the manner in which the different segments are arranged at the surface. The structure of Pellethane and Tecoflex is well known, the others less so. It is known, however, that all the polyurethanes have polyether in their soft segments (ether being absent in the hard segments), with Texin having carbonate groups in addition to this. One would expect to see carbon atoms bonded in carbamate and hydrocarbon groups in the hard segments, with carbon as ether and hydrocarbon in the soft segments. From these results, one might conclude the material with the highest surface concentration of soft segments is Elastollan 1190, followed by Pellethane, Tecoflex, Elastollan 1185, Davathane, Texin and Desmopan.

There is a relatively high concentration of amide in most of the samples. This is surprising since amide should not be present in at least some of these samples (*e.g.* Pellethane, Tecoflex). This has been noted by others (Coury *et al.*, 1988) and interpreted as amide processing wax, a known constituent of Pellethane 2363 (Dow Chemical) and that found after extraction in supercritical CO_2 (section 7.3.3.3). This may also explain the very low concentrations of carbamate found in all the samples, the processing wax covering the surface such that all the polyurethane is not visible to either the XPS analysis or when placed within a biological environment. Other manufacturing agents are also known to exist within all these samples (*e.g.* antioxidants, UV stabilisers, plasticisers) (section 7.3.3). Some authors have suggested their removal prior to analysis by extracting in solvents and

reforming the surface, but this would change the surface characteristics as tested in the *in vitro* test system and that exposed to blood in a clinical device. The results do, however, give some indication as to the contributions of hydrophobicity (hydrocarbon C) and hydrophilicy (ethereal C) to the material surface free energy.

7.5 SURFACE TOPOGRAPHY

7.5.1 Introduction

Amongst the parameters that may control unwanted reactions generated at a blood-material interface is the surface roughness of a material. Microscopic surface irregularities can capture air emboli which have a denaturing effect on plasma proteins. Each surface[5] has been assessed with regard to this parameter by SEM. Each material was cleaned by ultrasonicating in distilled water for 2 hours, then washing in distilled water and drying a vacuum oven. The method for viewing by SEM is exactly as described in section 3.4.

7.5.2 Results

SEM microcrographs of all the surfaces are shown in figs 7.4 - 7.12, at a magnification of 5400x. This magnification allows the visualisation of the major surface irregularities which might promote a poor blood-response.

[5] Materials used are given in Appendix A1.3 and sample preparation in Appendix A1.2

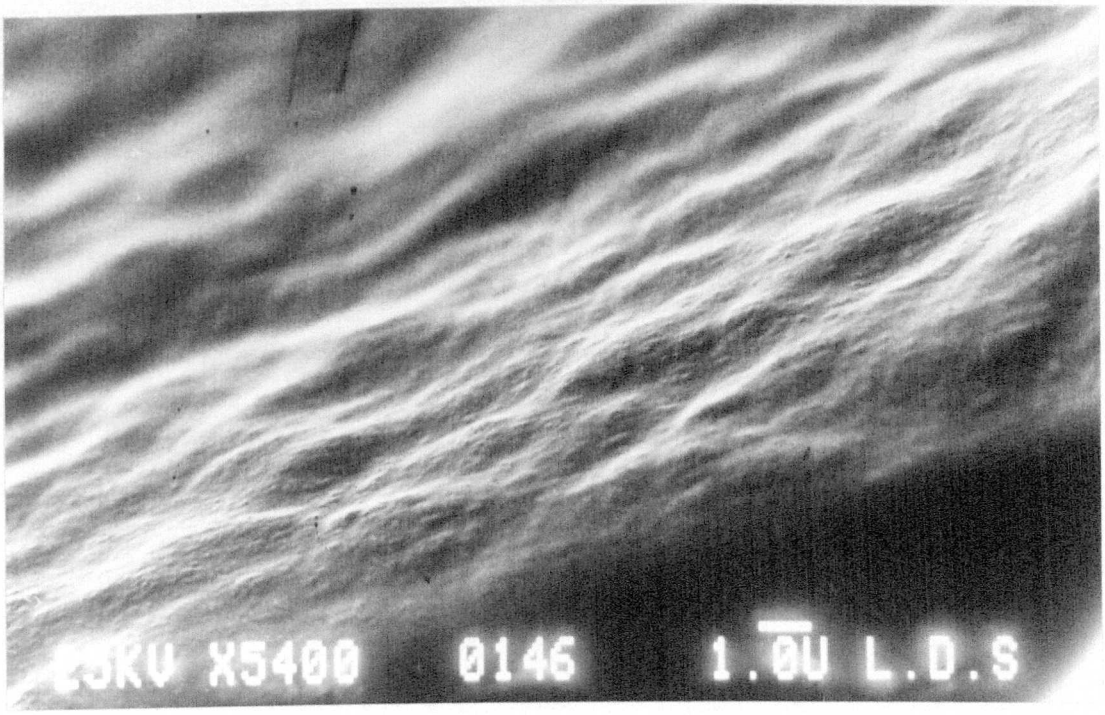

Fig 7.4 SEM of silicone surface, x5400

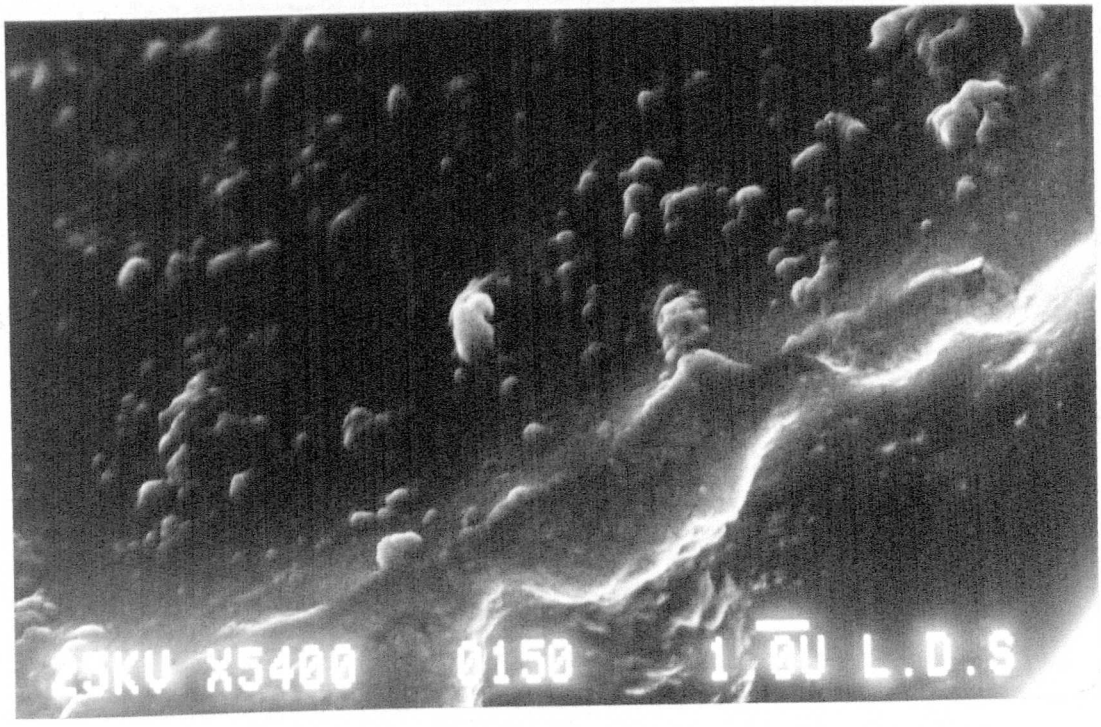

Fig 7.5 SEM of PVC surface, x5400

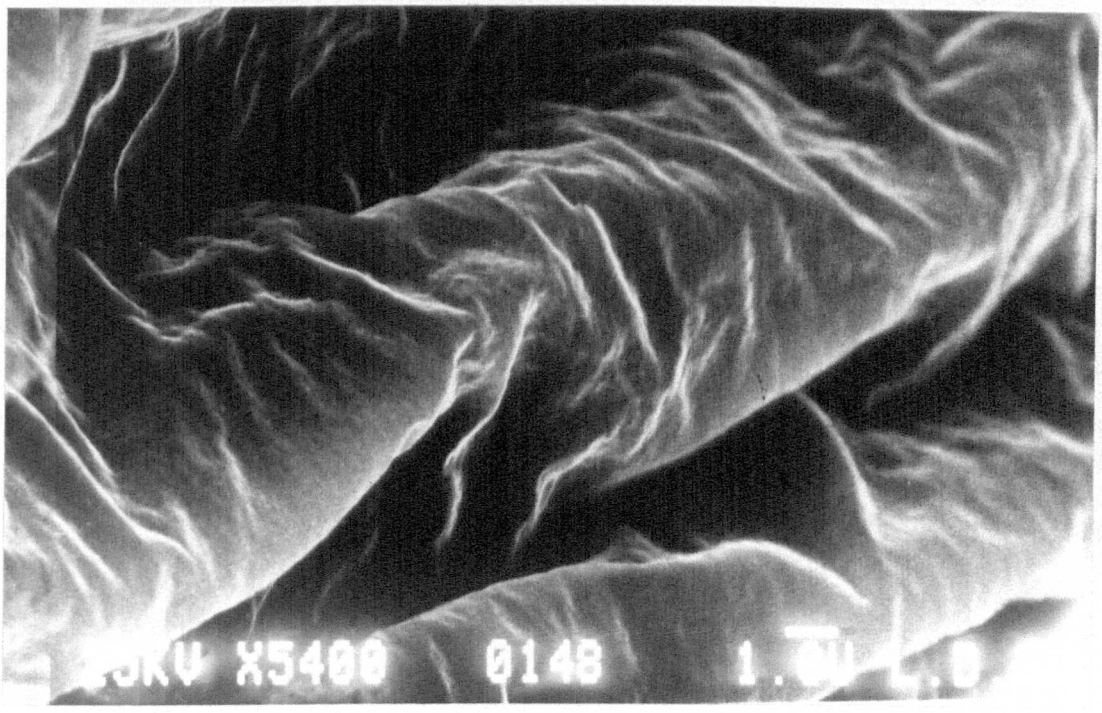

Fig 7.6 SEM of LDPE surface, x5400

Fig 7.7 SEM of Pellethane surface, x5400

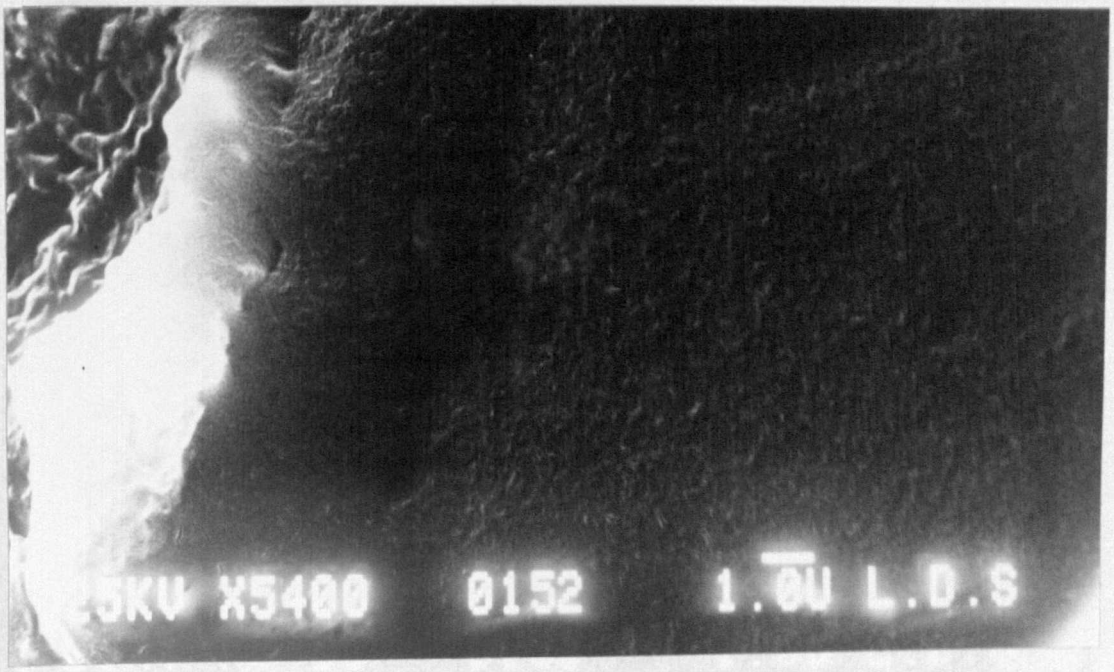

Fig 7.8 SEM of Davathane surface, x5400

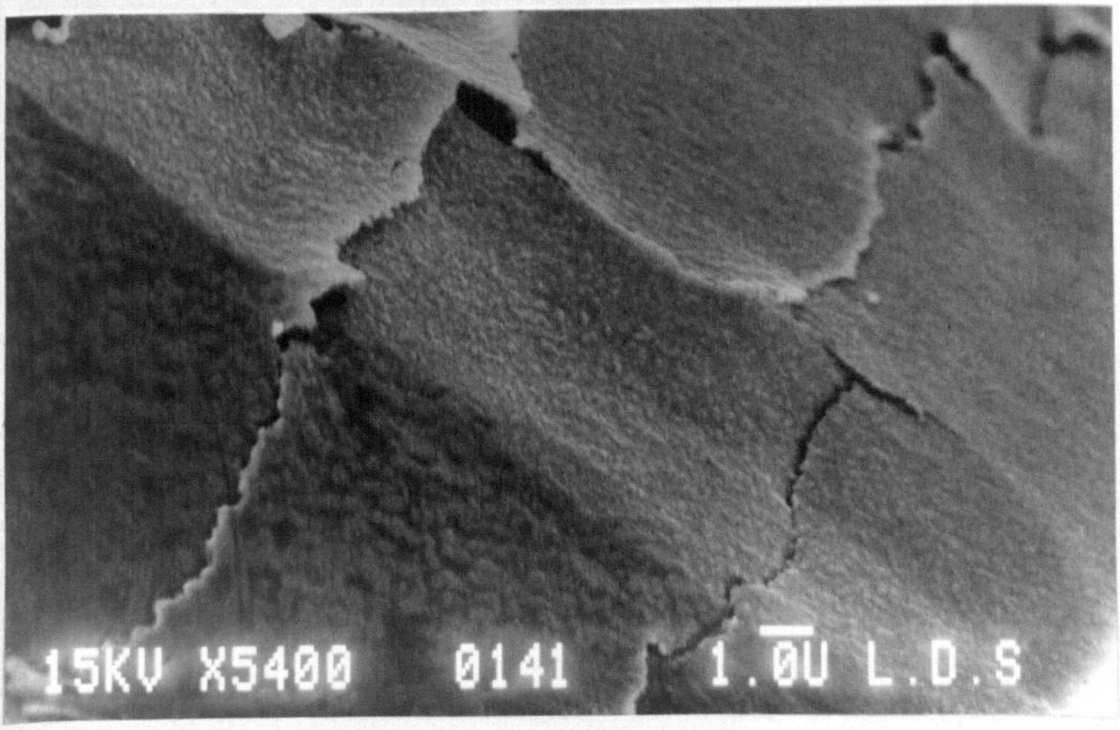

Fig 7.9 SEM of Desmopan surface, x5400

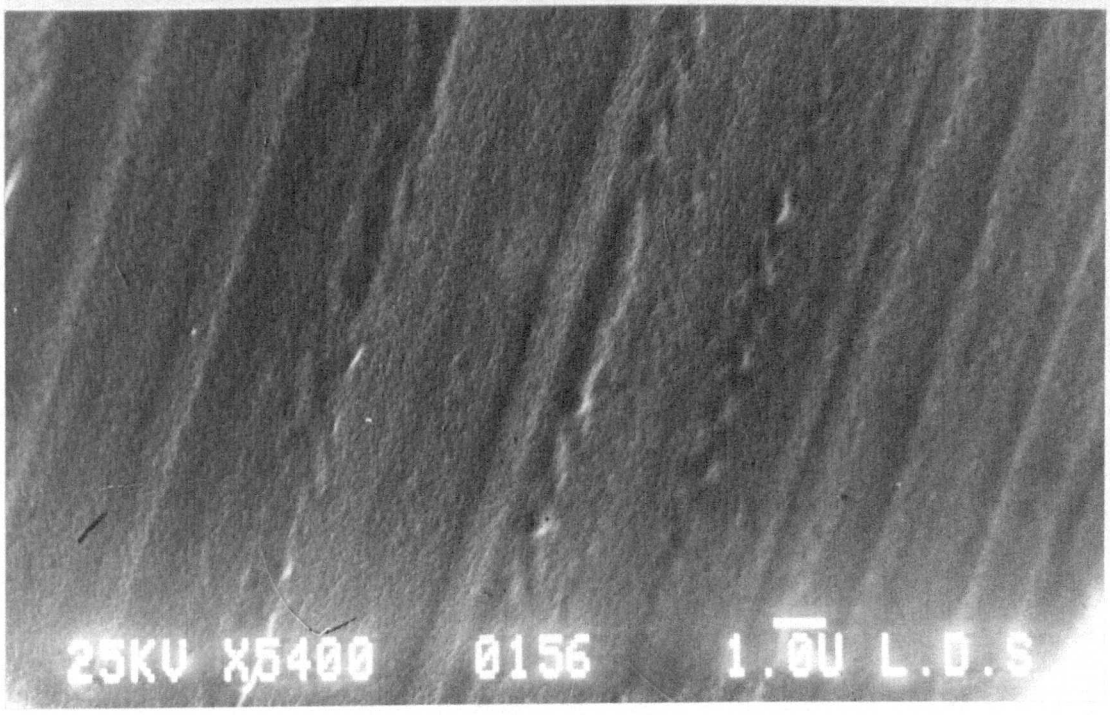

Fig 7.10 SEM of Texin surface, x5400

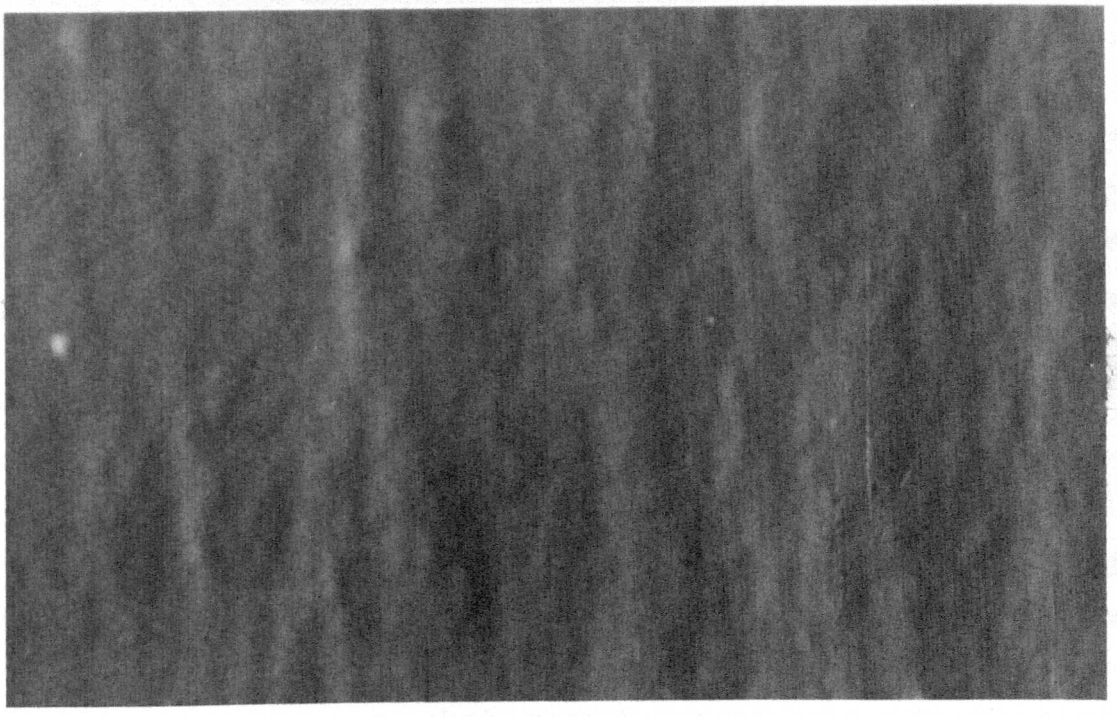

Fig 7.11 SEM of Elastollan surface, x5400

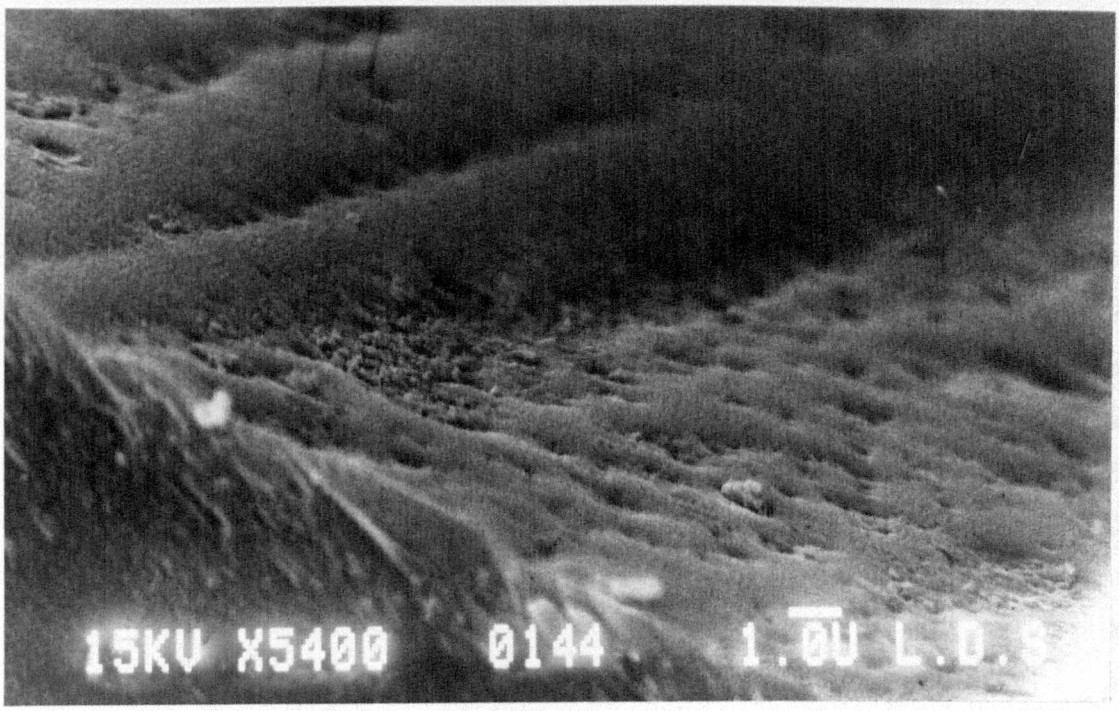

Fig 7.12 SEM of glass surface, x5400

7.5.3 Discussion

The materials displayed a diversity of surface morphological features on a microscopic scale, despite undergoing similar manufacture processing (*i.e.* extrusion). The smoothness of the inside surface of an extruded tubing is not controlled by the physical characteristics of the extrusion apparatus but by the temperature and speed of extrusion and the physico-chemical properties of the material itself (*e.g.* the critical surface tension of domains within the polymer and their ability to form crystalline and amorphous structures at the surface). LDPE has the greatest surface irregularities with troughs and ridges of a width in the order of 1 - 10 μm. When in contact with flowing blood, one might expect the entrapment of air nuclei and the adhesion of platelets onto areas where adsorbing protein molecules (especially fibrinogen) had become conformationally disturbed due to the resultant blood-air interface. In addition, platelet adhesion simply to the physical presence of the surface irregularities might also be expected. In contrast, most of the polyurethanes were very smooth with surface irregularities very much smaller than the dimensions of a platelet. The exceptions were Desmopan which had cracks and crevices covering a smooth

surface and Davathane which was mostly smooth but with the occasional gross surface defect several microns wide and high. PVC had a surface which was textured with small, globular structures and silicone had a surface which was about as smooth as the polyurethanes but which undulated. Glass was also relatively smooth.

With reference to the blood compatibility experiments performed on these materials, LDPE fared reasonably well, it being no worse than any other polymer for platelet adhesion and lysosomal granule secretion, one of the best as determined by PTT and being average for α-granule secretion and FXIIa generation. Desmopan and Davathane both perform well in platelet adhesion, lysosomal and α-granule secretion and contact phase activation as measured by PTT. Indeed, Pellethane performs worst in the adhesion of platelets but has one of the smoothest surfaces. Desmopan and Davathane do, however, perform very badly in the activation of FXII compared to the other catheter materials. Glass, on the other hand, is relatively smooth and performs very much worse than any catheter material in its ability to activate FXII and platelets.

From the available data it is not possible to ascertain whether the surface roughness played an important rôle in determining the blood compatibility of cathter surfaces.

7.6 CONCLUSIONS

Data have been collected for a number of important parameters often excluded from a multiparametric assessment of the blood-response to a range of biomaterials. In many respects these data have shown that there is very little difference between any of the polyurethanes, which cause similar (and significantly small) levels of haemolysis and material-extract cytotoxicity. In addition, they contain many of the same stabilising and processing chemicals. The importance of these experiments was shown in the observations that silicone, a commonly used catheter material, can cause significant haemolysis and PVC has potential for great long-term cytotoxicity. The presence of UV-light and heat-stabilisers, antioxidants, processing wax, plasticisers and impurities in even the best catheter materials is of some concern, however.

Data from experiments similar to the others performed within this chapter (sections 7.4 and 7.5) have been used in the past by other workers to correlate the blood compatibility of biomaterials to various physico-chemical characteristics. This, however, has often been an inconclusive task with the generation of conflicting conclusions. Many have also performed water-air-material contact angles, but this has little meaning when considering materials which have heterogenous surfaces with respect to hydrophilicity,

since it has often been observed that the heterogeneity itself and the distribution of the various domains has a greater effect on the adhesion of platelets than differences in the average free energy of the surface. In this study, surface roughness did not correlate with any of the expected parameters. The surface concentration of soft and hard segments in the polyurethanes did not correlate well with their responses to platelet adhesion and activation and contact phase activation. It would appear that the response of these blood components has more to do with the chemistry of the segments and their distribution than merely their relative percentages.

CHAPTER 8

REVIEW OF DISCUSSIONS AND CONCLUSIONS

8.1 INTRODUCTION

This chapter constitutes a summary of the discussion sections set out at length in the previous chapters and an indication of the principal conclusions of the thesis.

8.2 PRESENT STUDY

The work in this study has led to the modification of existing protocols used not only for research into biomaterials but also into general cell biology and haematology. These modifications have given rise to the development of a set of procedures suitable for the investigation of the blood-response to central venous catheters. The parameters selected for study in routine multiparametric assessment were platelet adhesion, lysosomal and α-granule release, contact activation as measured by PTT and FXIIa generation and haemolysis. The effects of blood-contact (or plasma where relevant) on a selection of catheter materials are summarised below in ranked order, where 1 denotes the best response and the highest number denotes the worst. No proportionality is shown by these figures which are meant as a qualitative guide only.

Material	Platelet Adhesion	α-granule release	lysosomal release	PTT	FXIIa release	Haemo-lysis
Glass	-	4	3	4	4	-
Silicone	1	3	1	1	2	3
PVC	1	3	2	2	1	2
LDPE	1	3	2	2	2	1
Pellethane	2	2	1	3	1	1
Desmopan	1	1	2	2	3	1
Davathane	1	1	1	2	3	1
Texin	1	1	2	2	1	1
Elastollan 1190	1	1	2	2	-	1

Table 8.1 Summary of the relative performance of catheter materials in contact with blood

Of note in these data is the observation that platelet adhesion does not follow platelet α-granule or lysosomal granule secretion. Simple enumeration of the platelets adhered to a material surface has often been performed in order to evaluate its thrombogenicity. On this basis, Pellethane is the worst material evaluated, all the other materials performing better than Pellethane but very similar to each other, an observation made by Grasel and coworkers for extruded polymers (Grasel *et al.*, 1987). This view on the evaluation of thrombogenicity has been replaced by one which states that the activated state of the initial platelet layer is more important (Baier, 1987). If the activated state of the platelets is important, then the polyurethanes perform best, silicone, PVC and LDPE being relegated further down the table for α-granule secretion of fluid-phase platelets. The secretion of lysosomal granules has fewer immunological and thrombotic implications and so is probably of less importance. The difference in granule secretion undoubtedly stems from differential platelet membrane glycoprotein activation. As to which is more important in the evaluation of material thrombogenicity one can merely speculate. Platelet adhesion is necessary for the provision of active membrane lipids whereas activated fluid-phase platelets can bring about further activation of other fluid-phase platelets. It is possible for platelets to adhere without becoming very active (in a coagulant sense) so platelet adhesion may be of questionable relevance. This may explain why Pellethane, usually regarded as an excellent material for blood-contact, performs so badly in this respect. Platelet secretion, on the other hand, displays the average status of the cells in the blood due to perfusion through the material and gives results which are in the general ranking order of other workers.

Of perhaps greater interest is the lack of correlation in the results of contact phase activation as measured by the PTT assay and FXIIa generation. It has generally been understood that since the activation of FXII is a necessary event for the initiation of intrinsic coagulation, a material generating larger quantities of FXIIa than a second material would give rise to a fibrin clot in a shorter time. This view has been shown to be flawed by the data presented in chapter 5 obtained by the use of novel antibodies to FXIIa-C_1-Inh conjugates. This has allowed a detection limit (< 500 pg/ml β.FXIIa) much lower than that previously reported (< 50 ng/ml) (Kaplan *et al.*, 1985). In terms of thrombogenesis, the PTT assay should be regarded as of greater importance since it gives information on the formation of fibrin rather than the initiation of the scheme of events. Some materials must prevent the formation of active complexes able to adequately activate FXI or FIX on its surface. This allows for the generation of FXII without increased potentiation of the

coagulation cascade. It is interesting that the contact of plasma with a variety of materials produces vastly differing concentrations of FXIIa yet broadly similar concentrations of FIXa, as measured by the PTT times. This last observation follows from the observation that a 10-fold increase in activating stimulus, and so presumably 10-fold increase in FXIIa concentration (this premise not proven), results in a shortening of the PTT times by approximately 30%. Apart from silicone, all the materials produced PTT times within 10% of each other and so FIXa concentration within 2.5 times that of the least activating surface. This figure, too, has not been verified.

In relation to indwelling catheters, some materials will presumably produce very large quantities of FXIIa which are not used for the conversion of FIX to FIXa. These active species are available for the participation in other pathways, most notably fibrinolysis. Many papers, however, have alluded to the interaction of FXIIa in many diseased states, including ischæmic heart disease, disemminated intravascular coagulation, gout and rheumatoid arthritis. It is possible that excess active FXIIa is to be avoided at all costs. In normal situations, however, excess FXIIa is inactivated by C_1-Inh and removed by the liver extremely quickly. The fate of these conjugates is not known. It is possible that an immunological response may be initiated by repeated exposure of these metabolic pathways to serine protease-inactivator complexes. The much discussed poor performance of polyurethanes with respect to biostability may be due to the activation of such a pathway.

8.3 Suggestions for Future Work

On the basis of the observations during the present study, the following areas are prime candidates for future research. Firstly, the current experiments used a flow system which required removal of blood from the body and a degree of processing prior to assessment in the perfusion apparatus. A large material-surface area to blood volume ratio and blood being perfused at high, although realistic, shear rates was necessary in order to observe some degree of alteration in the blood components being studied. A more realistic model would have involved a much more unfavourable surface area : volume ratio with the result that observation would have been barely above threshold levels. Clearly the effect of such a material in contact with blood is minimal at a short-term level, but probably cumulative, such that a problem can arise during chronic contact. Highly thrombogenic materials which have a very dramatic effect on the formed elements of blood during acute contact are rarely used these days. In order to measure very small changes in the

coagulant and haemostatic status of the blood in a physiologically realistic environment other parameters may need to be measured. It is likely that the release of thrombotically and immulogically important species from platelets, and indeed platelet adhesion relevant to thrombogenesis, requires a significantly large stimulus. Parameters of the platelet response other than platelet adhesion and activation have not been cited in the literature in this context. It would be necessary to use methods currently in use to investigate other cell types, for example a proposed study could involve the determination of reversible shape changes in platelets by measurement of changes in the structural state of actin.

Detection of a sensitive parameter of platelet metabolism would require the development of a more appropriate model of blood-material contact. This could be achieved *in vivo*, *ex vivo* or pseudo-*ex vivo* (achievable with the use of direct bleeding of the subject into the flow cell). All of these arrangements would allow for the minimisation of prior blood processing.

Observations of the complexity of intrinsic coagulation initiation indicate that the interactions which are important in the potentiation of the coagulation pathway are still unclear and need to be resolved. Activation of FXII *per se* has been shown not to be of paramount importance to the final formation of a fibrin clot. Of great interest is the development of materials which will intrinsically promote fibrinolysis via the increased activation of FXII whilst inhibiting fibrin formation. It should be determined whether this does in fact occur with the materials shown to promote FXIIa generation compared to those which do not.

8.4 CONCLUSIONS

The following major conclusions have been drawn:

(a) A number of methods have been assessed and have resulted in a set of protocols appropriate for the evaluation of the blood compatibility of central venous catheters, viz. platelet adhesion, platelet α-granule and lysosomal granule secretion and contact phase activation as measured by the clotting of a sample of activated plasma (PTT) and the activation of FXII;

(b) Significant and reproducible differences have been found between the chosen catheter materials in all of these parameters;

(c) All of the polyurethanes have been shown to have superior performance to all of the other catheter materials as judged by the the most meaningful criterion as argued by other workers, that is platelet α-granule release;

(d) There is, however, a lack of consistency in the ranking of individual materials according to the various parameters measured;

(e) In particular, there is a significant lack of correlation between the activation of FXII and the resultant generation of a fibrin clot. It has been concluded that the initial activation of FXII is not completely predicative of the performance in relation to catheter thrombogenicity;

(f) The reactions of platelets to materials are extremely dependent upon the shear rate of the perfused blood.

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APPENDIX 1

Blood Sampling and Contacting Procedures

A1.1 Blood Collection and Preparation

A1.1.1 Blood Collection

Blood was taken from the median cubital vein of healthy male and female human volunteers who had been free from *any* medication (this included contraceptive pills which are known to alter the concentration of plasma FXII and free FXIIa levels in the plasma) for at least 14 days. The subjects had been fasting for at least 4 hours prior to blood-letting for the platelet experiments and at least 12 hours for the protein and coagulation/FXII experiments. Plasma which was lipaemic was always discarded. Collection was achieved with needles (Terumo, Leuven, Belgium) for volumes less than 50 ml or butterfly infusion sets (Venisystems, Abbott Ireland Ltd, Sligo, Eire) for volumes greater than 50 ml and drawn into polypropylene syringes (Plastipak, Becton Dickinson, Dublin, Eire) of a size appropriate to the volume being collected. Needles and infusion sets were 19-gauge for the collection of blood for platelet experiments (to minimise cell trauma) and 21-gauge for all other experiments. Needles (including those from infusion sets) were placed in the vein such that vascular damage was minimised and bruising from needle abrasion avoided. The driving force for blood collection was vascular pressure, with no undue force being exerted on the syringe plunger in a further attempt to reduce blood element trauma. Blood was immediately transferred to sodium tricitrate (3.2% (w/v) in H_2O) at a volume ratio of 1 part citrate to 9 parts blood, at a rate which avoided frothing of the citrate/blood mixture.

A1.1.2 Plasma preparation

Platelet rich plasma (PRP) was prepared by centrifuging citrated blood at 250g for 25 minutes at room temperature. Platelet poor plasma (PPP) was prepared by spinning either citrated blood or PRP at 800g for 15 minutes. Platelet free plasma (PFP) for FXII

experiments was prepared by spinning PPP at 2000g for 10 minutes and slowing the centrifuge without braking or at 13500g for 2 minutes in a microcentrifuge.

A1.1.3 Preparation of platelet-inhibited blood

Blood or plasma in which it was important to have platelets in an inhibited state was prepared in one of two ways. Firstly, platelets could be frozen in the metabolic state which they had achieved after their exposure to an artificial surface. This was achieved with PGE_1 and theophylline as follows: Buffer was added to blood such that it contained 1 μg/ml PGE_1 and 160 μg/ml theophylline. These quantities ensured that the reaction was effectively permanent (in relation to the duration of the experiment).

Secondly, platelets were inhibited such that they could regain their physiological, reactive state. This was useful in lengthy labelling procedures which would otherwise render the platelets unreactive and unphysiological (*i.e.* refractory). This was achieved by using PGI_2 (prostacyclin) which has a very short half-life in aqueous solution compared with other anti-platelet prostaglandins. For a 2 hour labelling and washing procedure PGI_2 was included in the anticoagulant at a concentration of 5 ng/ml. Due to the reactivity of PGI_2 with water, stock solutions were prepared at a concentration of 1 μg/ml or 100 μg/ml in anhydrous ethanol and stored at -20°C. The efficacy of the solution was checked from time to time by measuring the time needed for platelets, incubated with 5 ng/ml PGI_2 in PRP, to regain their normal reactivity towards ADP and collagen.

A1.1.4 Preparation of activated platelets

Activated platelets were needed for the positive control in various experiments: measurement of platelet antigens by flow cytometry and measurement of PF4 by a functional bioassay. In the first case, functional cells were required and these were generated as follows: PRP was taken from citrated whole blood and activated with 5 units thrombin (bovine, 200 U/ml, Sigma, Poole, Dorset, UK) per 1 ml blood with the simultaneous addition of 250 units of streptokinase (from *Streptococcus*, Sigma, Poole, Dorset, UK) from stock 25000 U/ml. This was inverted once, then incubated without agitation for 2 hours at 37°C to provide cells which were effectively completely activated.

An alternative method was used when complete activation was not necessary. Activated cells were prepared in a short time as follows: to washed platelets were added, simultaneously, ADP (Sigma, St Louis, Missouri, USA) at a final concentration of 100 μM

and adrenalin (Sigma, St Louis, Missouri, USA) at a concentration of 100 μM. The sample was incubated at room temperature for 2 minutes without agitation.

When cells were not required, collagen (acid soluble, bovine type I, Hormon Chimie, Munich, Germany) was added at a concentration of 10 μg/ml. After 1 minute this was spun at 13500g for 1 minute in a microcentrifuge and the supernatant retained for active platelet granule release.

A1.1.5 Preparation of washed red blood cells

When washed red blood cells were required, primarily for the reconstitution of whole blood, the red blood cell suspension was spun at 1000g for 10 minutes. All excess plasma or buffer was removed and the cells suspended in an equal volume of PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 in H_2O (8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4 in 1 l H_2O, pH 7.3). The suspension was then spun at 200g for 10 minutes and the supernatant discarded. This last step was repeated two further times. The cells were then spun at 1000g for 10 minutes and then either reconstituted with other blood components or resuspended in the Tyrodes buffer.

A1.1.6 Preparation of washed platelets

Two methods of platelet washing were routinely performed. Firstly, the standard, non-protein containing method (important for chromium-labelling) was performed thus: to PRP, contained within a round-bottomed universal, was added an equal volume of Krebs-Ringer solution (4mM KCl, 107mM NaCl, 20mM $NaHCO_3$, 2mM Na_2SO_4, 19mM trisodium citrate, 0.5% (w/v) glucose in H_2O, pH 6.1) and centrifuged at 500g for 10 minutes. The supernatant was removed and the pelleted platelets resuspended in 2 ml Krebs-Ringer solution, gently rotating the vessel in order to prevent platelet aggregates breaking off from the pellet. This process was repeated two further times, the final suspension being made up in Krebs-Ringer solution to the desired platelet concentration.

Secondly, a more gentle method was performed as follows: PRP (which was sometimes collected into citrate containing 5 ng/ml PGI_2) was layered onto three 3 ml bands of BSA densities: 1.36 g BSA (Fraction V, 98% albumin, Sigma, St Louis, Missouri, USA) in 350 μl sodium tricitrate (3.8% (w/v) in H_2O) and 3.15 ml Tyrodes solution (138mM NaCl, 2.9mM KCl, 1mM $MgCl_2$, 5mM dextrose, 3.3mM NaH_2PO_4, 20mM HEPES in H_2O, pH 7.4), 1.12 g BSA in 350 μl sodium tricitrate and 3.15 ml

Tyrodes solution, 1.00 g BSA in 400 μl sodium tricitrate and 3.60 ml Tyrodes solution. The layers were arranged such that there was minimal disturbance to the interfaces of the BSA densities. This was spun at 700g for 30 minutes. The BSA band containing platelets (medium density) was removed and applied to the top of a Sepharose 2B column (Pharmacia, Uppsala, Sweden), previously equilibrated with Tyrodes solution. The platelets were eluted with this buffer in a narrow band, some time before the elution of the BSA fraction.

A1.2 Exposure of Materials to Blood/Plasma

A1.2.1 Static and pseudostatic contact

For the static platelet experiments (sections 3.2, 3.3 and 3.4), where as flat a surface as possible was required, large bore catheter tubing was used. This was also the case for the haemolysis assays (section 7.2) and FXIIa generation by chromogenic substrate (section 5.3), where it was advantageous to have a larger quantity of test plasma. These large bore catheter samples were contacted with plasma as follows:

(i) In the platelet experiments, 1 ml plasma was pipetted into each of the wells of a 24-well microtitre plate (Becton Dickinson Labware, Lincoln Park, New Jersey, USA) and a known surface area of catheter material introduced (50 mm^2 in each case). This was performed at room temperature without agitation and with the plate lid in place to prevent evaporation.

(ii) In the haemolysis experiments, 2 ml blood were introduced into the inside of the catheter samples and the ends sealed with Parafilm (American National Can, Greenwich, Conneticut, USA) to prevent evaporation. The samples were incubated at 37°C either statically or with agitation to prevent red cell sedimentation.

(iii) In the FXIIa generation experiments, 300 μl plasma were pipetted onto a longitudinally bisected section of tubing forming a surface area of 2.0 cm^2 and incubated at 37°C within a box containing damp tissue paper to prevent evaporation.

In the other static experiments, smaller volumes of test blood or plasma were required allowing the use of smaller bore catheter tubing. This had the added benefit of increasing the surface area to volume ratio. In these experiments: intrinsic coagulation measured by PTT (section 5.2); FXIIa generation by ELISA (section 5.4); protein adsorption (chapter 6), plasma was pipetted into the lumen of the tube and this was incubated statically at 37°C. The ends of these tubes were not sealed since the area of the

liquid at the air-liquid interface was very small. No measurable evaporation was observed in any experiment.

A1.2.2 Dynamic contact

A perfusion model similar to that used previously (Poot *et al.* 1988) was utilised in the dynamic studies where blood was exposed to the material surfaces at wall shear rates of up to 1000 s^{-1}. The model consisted of a length of small bore catheter tubing (typically 1.2 m in length, 1 mm internal diameter) of internal surface area 38.5 cm^2 and connected at each end to 50 ml polypropylene syringes having luer-lock type connectors (Becton Dickinson Labware, Lincoln Park, New Jersey, USA) via a 0 - 200 μl automatic pipette tip (L.I.P. Equipment, Shipley, West Yorkshire, UK), which had been modified to fit the luer connector of the syringe. 7 ml blood or plasma were placed in one syringe and the contents perfused along the full length of catheter tubing at a known, fixed, steady flow rate. The flow was generated by a Harvard 11 microprocessor controlled syringe pump (Harvard Apparatus, South Natick, Massachusetts, USA). The values of shear rate (γ_w) were calculated according to the following equation (Caro *et al.*, 1978):

$$\gamma_w = \frac{4Q}{\pi r^3}$$

where Q is the volume flow rate and r is the radius of the catheter tubing. The perfusion apparatus is shown in fig A1.1. All experiments were performed within a thermostatically controlled hot-room at 37°C.

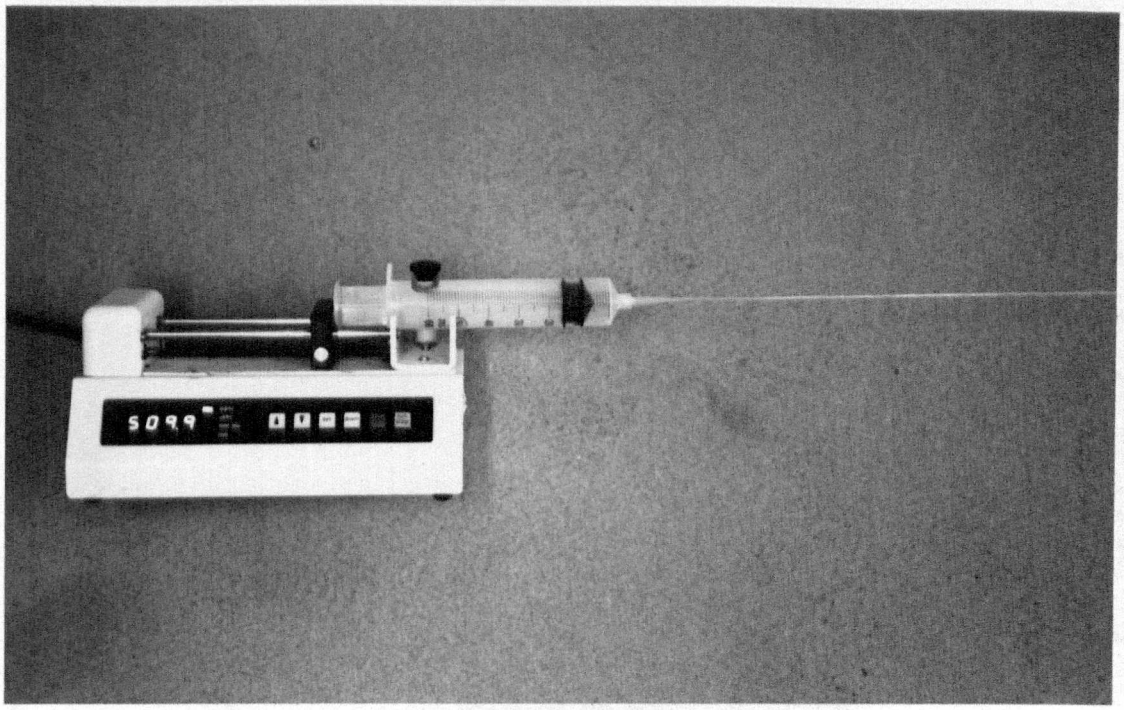

Fig A1.1 Perfusion circuit

A1.3 Materials Used For Biocompatibility Assessments

The following materials were used in the assessment protocols described in the preceding chapters:

Material	Grade	Manufacture/Supplier
PVC	80 S.H.	Portex, Hythe, Kent, UK
Silicone	50 S.H.	Altec, Alton, Hampshire, UK
LDPE	80 S.H.	Portex, Hythe, Kent, UK
PTFE	-	Altec, Alton, Hampshire, UK
Pellethane	2363/90A	Dow, Midland, Minnesota, USA Extruded by Viggo-Spectramed
Davathane	8701-98	Davathane, Nottingham, UK Extruded by Viggo-Spectramed
Desmopan	786	Bayer, Leverkusen, Germany Extruded by Viggo-Spectramed
Texin	985A	Mobay, Bayer, Pittsburgh, Pennsylvania, USA Extruded by Viggo-Spectramed
Elastollan	1190A-10	Elastogran, BASF, Lemförd, Germany Extruded by Viggo-Spectramed
Tecoflex	EG 85A	Thermedics, USA
Estane	58277 DT-985	BF Goodrich, USA Extruded by Viggo-Spectramed
Glass	Veridia	Chance Brothers, Malvern Link, Worcestershire, UK

Table A1.1 Sources of catheter material

APPENDIX 2

Abbreviations Used

AA	Arachidonic acid
A-A	Arterio-arterial
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
APAAP	Alkaline phosphatase - anti-alkaline phosphatase
AT-III	Anti-thrombin III
ATP	Adenosine triphosphate
A-V	Arterio-venous
BHT	Butylated hydroxy toluene
BK	Bradykinin
BSA	Bovine serum albumin
β-TG	β-thromboglobulin
cAMP	Cyclic Adenosine monophosphate
CD10	Cell determinant 10 (neutral endopeptidase)
Con A	Concanavilin A
CVC	Central venous catheterisation
C_1-Inh	C_1-esterase inhibitor
C4bp	C4-binding protein
DAF	Decay accelerating factor
DG	Diacyl glycerol
DMF	N,N-dimethyl formamide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetate
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FITC	Fluoroscein isothiocyanate
FPLC	Fast protein liquid chromatography
FVII(a)	Factor VII (activated form) plus other factor numbers I - XIII
GPIIb/IIIa	Glycoprotein IIb/IIIa
GMP-140	Membrane glycoprotein 140 (PADGEM)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HMWK	High molecular weight kininogen
HNA	Heparin neutralising activity
HRF	Homologous restriction factor
IP_3	Inositol triphosphate
LDPE	Low density polyethylene
Mab	Monoclonal antibody
MTT	Methylthiazolyl-diphenyl tetrazolium
PADGEM	Platelet activation dependant granule to external membrane protein
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor

PE	Phycoerythrin
PFP	Platelet free plasma
PF3	Platelet Factor 3
	plus other factor numbers 1 - 10
PGE_1	Prostaglandin E_1
PGI_2	Prostaglandin I_2 (prostacyclin)
PK	Prekallikrein
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl fluoride
pNA	p-nitroanilide
PNPP	Paranitrophenylphosphate
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PTFE	Poly tetra fluoroethylene
PTT	Partial thromboplastin time
PVC	Poly(vinyl chloride)
RIA	Radioimmunoassay
SDS	Sodium dodecylsulphate
SEM	Scanning electron microscope
SFE	Supercritical fluid extraction
T:AT	Thrombin-Antithrombin complex
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEM	Transmission electron microscope
TEMED	N,N,N',N'-tetramethylethylenediamine
TPN	Total parenteral nutrition
TPP	Triphenylphosphate
TxA_2	Thromboxane A_2
vWF	von Willebrand's factor
Xase	factor ten-ase (FX activating enzyme complex)
XPS	X-ray photoelectron spectroscopy
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