REDUCTION OF PROSTHETIC VASCULAR GRAFT THROMBOGENICITY BY PHOSPHORYLCHOLINE CONTAINING LIPIDS AND POLYMERS.

The thrombogenicity of arterial grafts can be modified by coating with new polymers that mimic haemocompatible blood cell membranes.

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This thesis reviews the clinical problems associated with atheromatous arterial disease, its treatment with prosthetic bypass biomaterials, and considers the development and assessment of these materials. The thesis tests the hypothesis that coating existing biomaterials with biological membrane phospholipids, which mimic the good haemocompatibility of erythrocyte membranes, may be a step towards the solution of the problem of biomaterial thrombogenicity.

Twelve phospholipids from the RBC membrane are evaluated as haemocompatible surface coatings by two *in vitro* assays; 1) material thrombelastography (MTEG), and 2) fibrinopeptide A generation. The technique of MTEG is presented in detail and important developments of this methodology are described. The underlying mechanisms of haemocompatibility of these phospholipids are examined by incubation of lipid liposomes with human plasma using individual clotting factor assays. Phospholipids and polymers containing phosphorylcholine (PC), namely sphingomyelin and dipalmitoylphosphatidylcholine (DPPC), very significantly reduce the activation of platelets and clotting factors. They are considerably better than the biomaterials Dacron and PTFE.

The stability of DPPC, sphingomyelin and the PC-polymers during sterilisation with steam, ethylene oxide and irradiation are studied. Marked decomposition is documented using the techniques of chromatography, FTIR and MTEG. Despite radiation induced changes, sphingomyelin and some PC-polymers remain less thrombogenic than PTFE or Dacron to both platelets and clotting factors.

Untreated Dacron vascular grafts are compared with grafts coated with sphingomyelin and two PC-polymers in a double blind randomised *in vivo* trial, using a modified sheep arteriovenous fistula preparation. The end points are indium-111 platelet imaging and uptake, platelet survival times, iodine-125 fibrinogen uptake and graft histology. Sphingomyelin significantly reduces platelet uptake by 66% and the cross sectional area of graft thrombus by 90% compared to the control grafts. All three materials prolong platelet survival, but do not reduce fibrinogen activation significantly. The original hypothesis is supported and further investigation of these materials should be undertaken.

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Chapter 6

The rear contains the following reprints

R. le R. Bird, B. Hall, D. Chapman and K. E. F. Hobbs 1988
Material thrombelastography : an assessment of phosphorylcholine compounds as models for biomaterials.
Thrombosis Research 51:471-483.

R. le R. Bird, B. Hall, K. E. F. Hobbs and D. Chapman 1989
New haemocompatible polymers assessed by material thrombelastography.
Journal of Biomedical Engineering 11:231-234.

B. Hall, R. le R. Bird, M. Kojima and D. Chapman 1989
Biomembranes as models for polymer surfaces : V Thrombelastographic studies of polymeric lipids and polyesters.
Biomaterials 10:219-224.

B. Hall, R. le R. Bird and D. Chapman 1989
Phospholipid polymers and new haemocompatible materials.
Die Angewandte Makromolekulare Chemie 166/167:169-178.

R. le R. Bird, B. Hall, M. Kojima and D. Chapman 1990
Phosphatidylcholine reduces polyester thrombogenicity shown by material thrombelastography (MTEG).
Clinical Science, 78:s26.

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Dedication

I dedicate this work to

my parents

Without whose sacrifices I would not have been able to start.

my wife

Without whose love and encouragement I would not have been able to finish.

and my children

Who have not seen as much of their Daddy as they, or I, might have wished during its execution.

Statement of Originality

This work is the first systematic evaluation of the thrombogenicity of phospholipids from biological membranes by material thrombelastography (MTEG). Similar high levels of haemocompatibility are documented for both phospholipids and polymers which contain the phosphorylcholine polar head group (PC), and they are significantly less thrombogenic than existing biomaterials. This is an advance in knowledge about the haemocompatible properties of the phosphorylcholine group in both lipids and polymers.

The physical nature of coatings of both PC lipids and polymers on Dacron vascular grafts have been investigated for the first time. In particular the effects of sterilisation by steam, ethylene oxide and gamma irradiation are documented. It is also the first time MTEG has been used to detect radiation induced changes in materials, confirming its sensitivity as a screening test.

This is the first *in vivo* evaluation of three PC containing materials, namely sphingomyelin, Polyester G and DAPC as coatings on Dacron vascular grafts. A significant reduction in the thrombus formed is demonstrated in the sheep arteriovenous fistula model, and is the first in vivo evidence of low thrombogenicity for phosphorylcholine. This thesis is a major contribution to the study of vascular graft and phospholipid thrombogenicity, and opens a new vista for investigation.

The thesis is an investigation into a proposal made by Professor D Chapman, that the non-thrombogenic nature of phosphoryl choline can be used as a basis for new biocompatible materials. I have conducted all the experimental work and analysis for all aspects of the *in vivo* studies. I have developed the technique and analysis of the method of material thrombelastography, particularly the nomenclature and statistical analysis. My publications related to this work are bound in this volume. I have designed the other *in vitro* experiments presented, and have analysed all the data except the Fourier transform infrared data (in Appendix 3). I have acknowledged my collaborators who have contributed technical help.

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All the scanning electron microscopy (SEM) specimens and photographs were prepared by Jackie Lewin (Electron Microscopy Unit, RHFSM). I am particularly grateful to her for this, and for teaching me how to use the scanning electron microscope to examine and photograph the specimens, as well as for the use of her computing facilities. Edwin Addo, Dr AP Dhillon and Dave Booth between them prepared the microscope slides of the grafts for the morphometric analysis presented in chapter 6. Particular thanks are due to Dave Booth who programmed the system and helped set up the detection parameters for me. (Dept of Histopathology, RFHSM).

I am grateful to Dr Derek Cook and Joan Morris (Dept of Public Health and Primary Care, RFHSM) for helpful discussions relating to the statistical design of the *in vivo* study, for generating the randomisation code, and for advice regarding the statistical analysis.

It is invidious to pick out these few, and to the others apparently passed over, it is only due to the contingencies of space and I crave their indulgence.

Preface

This thesis presents a new concept to address the problem of the thrombogenicity of currently used biomaterials. It is presented in four main sections: An introduction; the *in vitro* work; the *in vivo* work and the final conclusions.

The first section (chapters 1 and 2) provides an introduction to the problems associated with materials currently in widespread use. It discusses how these materials were originally chosen for use, and describes the problems associated with their evaluation. This section concludes with the reasoning behind the proposals which form the basis of a new concept in biomaterial design, forming the hypothesis tested in this work.

The next section (chapters 3 and 4) presents the methods used in this thesis for the *in vitro* assessment of biomaterials. A development of the method of material thrombelastography is presented. The results for these studies are presented to test the hypothesis outlined in chapter 2. It was intended that the best materials would be tested in two *in vivo* experiments. However the investigation took an unexpected turn when simple tests, undertaken to confirm that sterilisation did not alter the materials, demonstrated marked decomposition. Chapter 4 presents the work that followed, and confirmed these unexpected findings. This section closes with a summary and discussion of the *in vitro* findings as well as the reasoning behind the choice of materials submitted for the *in vivo* experiment.

The third section (chapters 5 and 6) presents the *in vivo* evaluations of vascular graft materials. It opens with a discussion of the particular problems of this type of experiment. Chapter 6 presents the experimental details and results for the *in vivo* experiments. These use a modification of the arteriovenous fistula in the neck of the sheep.

The final section (chapter 7) summarises this work and presents the main conclusions. The original aim of the investigator was to compare the conclusions of the *in vitro* tests with those of two *in vivo* tests. These were an acute test of

thrombogenicity in the sheep, and a chronic test of patency over 3 months in the dog, but this proved too ambitious. The final section discusses the direction that future research should take.

Each chapter is divided into numerical sections, and opens with an outline of the contents of the chapter. Figures, tables, and illustrations are numbered consecutively within each chapter, prefixed with the chapter number. The abbreviations, of which there seem to be too many, are presented before the references. The appendices contain some of the detailed tabular results and the description of the experimental methods. This is to avoid interruption to the flow of the thesis, particularly in chapters 3, 4 and 6. The sections of the appendices and the associated tables, figures, and photographs are prefixed with the letter 'A', with the exception of the tabulated MTEG results. These have numbering related to chapters 3 and 4. Published scientific papers based on this work are bound inside the back cover.

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Chapter 1

Background to the current investigation

1.1 The clinical problem

1.1.1 Vascular disease

Atheroma of the major arteries can result in death or severe disability. This is due to atheromatous plaques which form within the walls of the artery and result in narrowing or occlusion of the vessel. This stenosis results in distal ischaemia, with the associated symptoms of angina pectoris when the coronary vessels are affected, intermittent claudication when the femoral vessels are affected, or transient ischaemic attacks when the carotid vessels are affected. The surface of the vessel with atheromatous plaques is irregular, and the plaque is often ulcerated. These irregular ulcerated plaques cause turbulent blood flow. Together they promote the formation of platelet aggregates, and micro thrombi. These aggregates or pieces of the plaque which become detached, can form an embolus which may cause sudden occlusion of the distal vessel. This results in myocardial infarction, the critically ischaemic leg, a stroke, or gangrene of the gut.

Despite the inaccuracies in the way that death certificates are completed, the size of this clinical problem can be seen in published mortality figures. In 1988, in England and Wales alone, there were 567,987 deaths (age over 28 days), divided between 278,964 male, and 289,023 female (The Office of Population and Censuses Surveys - OPCS 1990). Of these deaths 153,084 (27%) were from ischaemic heart disease, a further 96,404 (17%) were from acute myocardial infarction. Cerebrovascular disease (excluding subarachnoid haemorrhage and intracranial aneurysm) took 65,903 (11%) lives. Vascular disease (excluding the various causes of arteritis), outside the coronary and cerebral circulations, killed 17,992 (3%). Nearly two thirds (61%) of these arterial deaths were due to all types of aneurysmal disease (10,964 deaths), and 3,050 were specifically from ruptured abdominal aortic aneurysms. There were 2,270 deaths due to peripheral arterial disease, and an additional 2,280 from mesenteric vascular insufficiency (OPCS 1990, table 2). In all vascular disease is involved in 58% of all deaths.

These crude figures are also presented as death rates per million (OPCS 1990, table 3). The common forms of cancer pall in comparison to vascular disease, so do those deaths from road traffic accidents and homicide (see Table 1.1).

Cause	Male	Female
ALL causes	10,523	10,278
cancer		
lung	965	411
colon	200	231
rectum and rectosigmoid	123	94
breast	$<10^{-3}$	518
road traffic accident	127	50
homicide	7	5
ischaemic heart disease	3253	2434
myocardial infarction	2089	1519
cerebrovascular accident	929	1487
arterial disease	306	271

(OPCS 1990, table 3).

Table 1.1 Death rates per million.

The morbidity associated with this level of pathology is difficult to assess in terms of human suffering, or even lost economic productivity. However an idea of this can be gained from estimates of the amount of treatment given. For example 1.8 million cardio-vascular procedures are performed annually in the USA. Half of these are cardiac catheterisations, but the balance is peripheral vascular surgery and open heart surgery (NIH 1985 p.1). The approximate costs for vascular grafts used in treating these conditions has been estimated. Worldwide expenditure on vascular grafts alone, in 1983, was $85 million (Turner 1987). 50% of this was for small grafts, with a diameter under 10 mm. Conservative estimates put the annual growth of this market at 10% pa, with an estimate of $131 million for 1986, and $175 million for 1989 (Turner 1987). Worldwide the estimate in 1983 for coronary arterial bypass was 300,000 operations and currently these are performed with autologous vein. This represents a potential market of at least $10 million for a suitable prosthesis (Turner 1987).

The primary management of atherosclerosis should be prevention. This needs to be directed at the underlying causes, such as smoking, hypertension, diabetes, and hyperlipidaemia. Treatment of the existing disease must deal with the mechanical consequences of stenosis or occlusion. These often require direct intervention to restore blood flow. The techniques available for this range from embolectomy, endarterectomy, to bypass surgery and grafting with autologous vein or synthetic prostheses. Recently angioplasty, laser recanalisation and thrombolysis have been added to the armamentarium.

Coronary and peripheral arterial bypass surgery can prevent some of the deaths and limb amputations due to atheromatous disease, but most prosthetic vascular grafts eventually occlude, and all are too thrombogenic to replace the smaller arteries of the body.

1.1.2 Current vascular prostheses

The success of bypass grafting of occluded blood vessels depends partly on patient selection and surgical techniques, but also on the nature of the bypass material. For large vessel replacement the choice of prosthetic material, between polyethylene terephthalate (PET, more widely known as Dacron, the EI du Pont de Nemours and Co tradename) and polytetrafluoroethylene (PTFE) is relatively unimportant due to the large diameter of the vessel and the high blood flow through it. This is reflected in the reported patency rates for various levels of bypass grafts. For aorto femoral grafts (for non aneurysmal aortic disease) Naylor reported patency rates of 95% at 1 year, falling to 87% at 5 years (Naylor et al 1989). As the graft extends more distally the patency rate falls. This reflects the reduced flow in the vessel, related to the smaller diameter of the native artery, and the reduced run off territory which the vessel perfuses. Michaels reviewed the literature published only over the preceding 10 years for femoropopliteal bypass (Michaels 1989). The patency rates when the bypass extends only to the above knee popliteal segment were 76.5% at 1 year, falling to 43.2% at 5 years for prosthetic materials. When extended below the knee they fared even worse, 61% and 26.6%, at 1 and 5 years. An earlier review by Bergan (Bergan et al 1982), despite taking into account both changing indications and improved techniques,

concluded that the autologous saphenous vein was the ideal conduit. This view is confirmed in the later review as the patency rates for the below knee vein femoropopliteal bypass after 1 and 5 years are 75.7% and 68.4% (Michaels 1989).

1.1.2.1 Dacron

The Dacron graft develops a pseudo intima with an endothelial cell lining in some species, but remain unhealed in man (Berger et al 1972, Sauvage et al 1974). Using Indium labelled platelets Goldman and colleagues have shown increased platelet uptake, indicating continuing thrombogenicity at 10 years (Goldman et al 1982c). This confirms the lack of healing, and Sauvage in an attempt to address this problem popularised the knitted and velour types of Dacron graft (Sauvage et al 1974). These have a looser weave (the knit), with free 'loops' of thread above the surface of the graft (the velour). These require meticulous preclotting, where the graft is filled with blood, and this is allowed to form clots in the interstices of the fabric. This must be carried out before giving systemic heparin to the patient, and takes several minutes. However these grafts have the advantage of increased tissue ingrowth into the loose weave, allowing better fibrous incorporation of the graft, but the flow surface is still thrombogenic. Certainly there is a reduction in the risk of late graft infection (Moore et al 1980). There is however no improvement in the long term patency of these grafts compared to woven Dacron.

1.1.2.2 PTFE

PTFE, although better than Dacron for infra-inguinal use, also has disadvantages. It too fails to heal with an endothelial cell lined surface. The final lining is a layer of swept fibrin. These grafts are particularly prone to the problem of neointimal hyperplasia at the distal anastomosis, a problem which also affects vein bypasses, and which will be discussed below. The PTFE grafts are more rigid, and require meticulous care in their use, particularly for the choice of length, if kinking of the graft with subsequent failure is to be avoided. If a PTFE graft becomes infected the smooth surface, so good for blood flow also facilitates the rapid spread of the infection throughout the graft's length (Moore et al 1980).

1.1.2.3 Autologous grafts

Despite the superiority of the saphenous vein over prosthetic materials this too has disadvantages. The vein needs to be harvested and reversed if the valves are not to impede blood flow. An alternative technique is to use the vein as it lies anatomically, taking advantage of its natural taper. The vein is isolated from its tributaries and the femoral vein. The proximal wider end of the vein is attached to the femoral artery, and the narrower distal end to the popliteal. This is the so called *in situ* technique, originally described by Hill in 1964. This has now become the preferred technique for anastomoses to the tibial vessels (Leather et al 1989). However the *in situ* technique requires that the valve cusps are cut or disrupted, since the flow is against them. It is also necessary to ligate any remaining venous tributaries to the vein, as these will form arterio-venous fistulae when the vein is arterialized. These AV fistulae result in patches of ischaemic necrosis in the overlying skin. The late results show no benefit over the reversed vein technique (Budd et al 1990, Edwards et al 1991).

Whichever vein graft technique is used the final cause of the failure of these grafts, and as mentioned above of PTFE grafts, is neointimal hyperplasia at the distal anastomosis (Imparato et al 1972). This is the narrowing of the native vessel just distal to the anastomosis, and is the result of local proliferation of smooth muscle cells beneath the endothelium. This may be due to local production of smooth muscle growth factors by the endothelium, or from platelets disturbed by their passage through the graft (Yukizane et al 1991). Further evidence to implicate the smooth muscle cells as the cause of hyperplasia comes from work with the octapeptide analog of somatostatin, angiopeptin. This has been shown to inhibit neointimal hyperplasia, through its inhibitory action on smooth muscle cells (Calcagno et al 1991). Recently Miller reported the use of a vein cuff with PTFE vascular prostheses to facilitate the distal anastomosis and increase initial patency rates (Miller et al 1984). While there is evidence that flow is increased (Beard et al 1986a) it is likely that the neointimal hyperplasia, which still occurs, now lines the capacious cuff rather than the narrow distal vessel.

In addition to the problem of hyperplasia not all patients have a vein of a suitable

size (both length and width) for a bypass. Some do not have a vein and some need a speedy operation. Thus there remains the pressing problem of a suitable synthetic prosthesis.

1.1.3 Immunological and systemic effects of prosthetic materials

Vascular prosthesis are not the only prosthetic devices in clinical use. There are many others such as heart valves, devices for haemodialysis and cardiopulmonary bypass and numerous catheters, which come into direct contact with blood. These all have a limited life span due to activation of coagulation, and other host-foreign body reactions. These result either in distal embolisation or total failure of the device. Heart valves in particular require anticoagulant therapy to prevent embolism and this is not without risk. While little is known about the immunological effects of vascular prostheses, much more is known about extracorporeal devices such as dialysis membranes. These studies shed further light on the question of material design and selection. The difficulties and complexity of assessing any biomaterial interaction's with man are shown in Table 1.2. The hip prosthesis has a small size and relatively little exposure to

implant	administration time	use	implant size	blood exposure
haemodialysis	acute	repeated	large	large
vascular graft	chronic	single	small	large
hip replacement	chronic	single	small	small

Table 1.2 Factors involved in patient responses to implanted prostheses.

blood, compared to the haemodialysis device. These have a large surface area, and patients are exposed to them acutely, and for the short period of time needed to complete the dialysis. However this exposure is repeated many times over the life of the patient. To reduce the dangers from cross infection the patient is repeatedly exposed to new dialysers. These factors amplify the biological responses that patients make as a result of exposure to the materials. These changes are sufficiently large to be measured and some are well documented. The

interaction of the Cuprophan membrane is limited to short term extracorporeal exposure of the blood to its surface. This is moderated by the action of anticoagulants, and is a simpler system than, for example, the interactions involved with an implanted prosthesis. However the consequences of this limited but long term interaction are beginning to be recognised, and will be outlined below. The vascular implant is much smaller and as yet this type of interaction has not been described, although Dacron has been shown to activate complement *in vitro* (Shepard et al 1984). It is therefore likely that these mechanisms may also contribute to graft failure.

In routine haemodialysis there are many variables related to the patient and his disease, the conduct of the dialysis and so on, but many patients experience 'dialysis discomfort'. This is an acute reaction of dyspnoea, chest tightness, nausea, vomiting and hypotension. Despite the use of heparin a fall in platelet count and white cell count may be seen in the early phase of haemodialysis (Kaplow & Goffinet 1968). The significance of this was not appreciated until Craddock et al (1977) showed a corresponding activation of the alternative pathway of complement activation. This system of proteins acts in a cascade to cause lysis of foreign cell membranes, and generates cleavage products which have actions of their own. The Cuprophan membrane acts as an incubating surface for complement and generates activated C3 and C5, which are washed back into the pulmonary circulation via the venous return of the dialyser. The activation releases the small peptide fragments C3a and C5a, which mediate the vasodilatory actions of anaphylaxis and cause aggregation of granulocytes in the pulmonary circulation (Cooper & Nemerow 1985) (Table 1.3).

This also explained Hakim's (et al 1984) description of the 'first use syndrome' in which the clinical reaction was most severe when a new membrane was used. The severity of reaction was also known to be less severe when reuse of dialyser was practised (Bok et al 1980, Kant et al 1981), and with other types of membrane (Chenoweth et al 1983). The new units activate C3 and C5 strongly, while reuse passivates the surface with adsorbed proteins, and other membranes are not so reactive.

Inflammatory	Immune regulatory
histamine and mediator release altered vessel permeability smooth muscle contraction	cellular activation: monocyte macrophage
----------secondary responses from----------	
leukotaxis : enzyme release opsonisation facilitated ingestion	activated cells : B cell - antibodies T cell - IL2
(based on Cooper & Nemerow 1985)	

Table 1.3 The actions of C3a and C5a.

However not all of these acute events can be blamed on the membrane, since Takahashi (et al 1981) observed eosinophilia and allergic reactions with ethylene oxide (ETO) sterilised dialysers, which disappeared when steam sterilisation was introduced. ETO specific IgE antibodies were found and correlated to the severity of the reaction (Bommer et al 1985). The ETO release was related to the dialyser design and flow geometry (in trapping the ETO) rather than the materials used in it's construction (Henne et al 1984). This type of reaction has now been reported following platelet-pheresis in a non-renal dialysis patient (Muylle et al 1986).

The chronic consequences of dialysis such as disposition to infection, osteopenia, muscle wasting and fibrosis, joint problems and amyloid are well recognised, the so called 'shrinking man syndrome' (Memmos et al 1982). These may be regarded as a sustained inflammatory response, provoked by the repeated action of acute dialysis sessions, which continually stimulate an acute phase response. The long term sequelae are probably due to this, since the macrophage and the monocyte have receptors for C5a (Chenoweth et al 1982), and these stimulate the production of Interleukin 1 (IL 1). The Interleukin hypothesis, based on the effects of IL1 (Dinarello 1984, Dinarello 1985), and proposed by Henderson (et al 1983), accounts for many of the long term effects of dialysis (Table 1.4).

More recently beta 2 microglobulin has been implicated as the product of T cell

PRODUCTION OF IL1

C5a------> monocyte activation-----> IL1

ACTIONS OF IL1

Site of action	Effect
metabolic	
hypothalamus	fever*
muscle	protein catabolism - wasting*
liver	acute phase response*
bone marrow	neutropenia*
inflammatory	
fibroblast	collagen production - fibrosis*
prostaglandin release	vasodilatation - hypotension*
immune	
neutrophil	activation
B cell	antibody production
T cell	IL2, ? beta micro-globulin & amyloid*

* These reactions are seen during chronic dialysis.

(based on Dinarello 1985, Henderson 1984)

Table 1.4 The Interleukin hypothesis.

stimulation, and as the material deposited in amyloid and fibrosed joints (Gejyo et al 1986). Some of this may however be due to failure of the dialysis membrane to clear the protein.

These clinically documented problems have been recognised for haemodialysis. The more subtle problems which may be occurring with other prostheses in diverse clinical situations go unrecognised. In the wider context the underlying problem is the lack of a fully biocompatible material. The size of the potential market for such a material can be seen in the medical applications of plastic produced by the USA's plastics industry. This was estimated to be worth $3100

million in 1981 (Ratner 1984). Such a material would clearly have a wide application in medical care.

1.2 Biomaterials

1.2.1 Historic

Lord Lister, the pioneer of asepsis in modern surgery, was also deeply interested in blood coagulation. He showed that blood remained fluid much longer in india rubber tubing than in glass or paraffin treated glass tubes. These experiments were conducted to answer questions about the control of coagulation, but in the Croonian lecture to the Royal Society of 1863, he concluded that 'the real cause of the coagulation of blood, when shed from the body, is the influence exerted on it by ordinary matter, the contact of which, for a very brief period effects a change in blood, inducing .... a disposition to coagulate' (Lister 1863). He pin pointed what is still a major obstacle in the development of biocompatible materials today. Lister had also noted that blood remained fluid for hours, out of the body, if it was kept in a blood vessel. Indeed he was almost prophetic in respect of current work with endothelial cells:- 'the peculiarity of the living vessels consists...in the...lining membrane...[which] fails to cause that molecular disturbance or...catalytic action which is produced upon the blood by all ordinary matter.'

Fifty years was to elapse before the 'disposition to coagulate' could be sufficiently controlled to allow a useful interaction between blood and a foreign material. The use of hirudin (extracted from leeches) as an anticoagulant allowed dialysis to be performed with collodion tubes (Abel et al 1913). Abel showed that clearance of aspirin from a dog was more rapid by 'vivi diffusion' than by the kidney.

Jassinowski (1889) showed that arterial anastomosis was possible using fine needles and silk. He successfully anastomosed 22 out of 26 end to end carotid arteries in the sheep. Carrel used this technique of arterial suture and demonstrated the repair and replacement of vessels was possible, by transplanting autologous and heterologous vein and artery (Carrel and Guthrie 1906). He demonstrated the storage of homograft tissues such as the dog's inferior vena cava,

by later using it to bypass the dog's carotid artery. Transplantation of organs was possible, but much of this early work was frustrated by sepsis (Carrel 1908). His pioneering work laid the foundation for the techniques of modern vascular and transplantation surgery. He exploited the natural tissues long before artificial materials were available. However later surgeons did not note his warning over the use of heterologous vessels. Arteries transplanted between the cat and dog, gradually became dilated as the elastic fibres disappeared, and the media underwent hyaline degeneration. This heralded the subsequent failure, by aneurysmal dilatation, of all biologically derived prostheses.

The development of antibiotics, anticoagulants, and anaesthesia in between the World Wars was needed before the 'ordinary matter' of Lister could be exposed to blood to fulfil Carrel and Abel's hopes, in replacing natural tissues and performing their biological functions. These developments ushered in the modern clinical era, with the practice of haemodialysis, vascular graft implantation, and started the modern study of biomaterials.

By 1946 Kolff had carried out the first clinical haemodialysis using sausage skins (of cellulose acetate) with heparin to control coagulation (Kolff & Berk 1944, Kolff & Berk 1946). In the late 1940's Carrel's work was repeated and there was wide use of homografts. Gross (et al 1949) replaced sections of the aorta. There followed a spate of reports using this important new technique. By 1957 early enthusiasm for homografts was tempered with the reports of early graft failure, degeneration and aneurysm formation (Szilagyi et al 1957). There were the associated difficulties of tissue supply, and storage.

In 1952 Voorhees implanted tubes of a synthetic fabric, Vinyon N to bypass the aorta (Voorhees et al 1952). A single strand of silk suture in the right ventricle of a dog's heart had been noted to be free of thrombi, and to be coated by a glistening white film. As a result of this observation they took 15 dogs and replaced their abdominal aorta's with cloth tubes. 3 of the grafts occluded but one was in a dog who died of distemper. With this success, rapid trials of numerous other materials followed rapidly. These are detailed in the next section as they raise the question of the criteria needed for biomaterial evaluation.

1.2.2 Properties of an ideal biomaterial

In the post war period, developments in plastics provided a new range of materials, and the need for careful evaluation of their possible use as biomaterials was soon recognised. Scales (1953) defined the necessary qualities of synthetic materials to be used as implants (Table 1.5). Although Scales' interest was primarily for orthopaedic applications, his 'necessary qualities' still apply today.

The implant must:
1. Not be physically modified by tissue fluids.
2. Be chemically inert.
3. Not excite an inflammatory or foreign body cell response in the tissues.
4. Not be carcinogenic.
5. Not produce a state of allergy or hypersensitivity.
6. Be capable of standing up to mechanical strains imposed upon it, as for example, friction when placed in a joint.
7. Be capable of being fabricated in the form required with reasonable ease at a relatively low cost.
8. Be capable of being sterilized.

(Scales 1953)

Table 1.5 Necessary qualities of synthetic materials to be used as implants.

Creech quoted this 'criteria for suitability of synthetic materials in mammalian tissues' in the report to the Society for Vascular Surgery on vascular prostheses. Ease of handling, flexibility and ability to retain shape were added by the few surgeons who had adequately replied to the Society's questionnaire (Creech et al 1957).

Gradually Dacron emerged as the best of the new materials for arterial grafts, leaving in it's wake others that fell short of the ideal material. Vinyon N was unstable over 60°C (Table 1.5, No 8), Orlon caused haemorrhage (Table 1.5, No 2), Ivolon induced an inflammatory response (Table 1.5, No 3) and Marlex tended to kink (Table 1.5, No 6) (Turner 1987). In passing one should comment on the last two materials which have found a place in applications other than those they were originally tested in, Ivolon for rectopexy and Marlex for tissue patches.

With greater experience of the new materials, the emphasis shifted from the choice of material being determined by its ability to perform a biological function, to its suitability, in terms of its interaction with biological tissues. This suitability being termed 'biocompatibility', and given further definition by Bruck (1972)(Table 1.6).

Materials for long term internal application should not cause:

1. Thrombosis.
2. Destruction of the cellular elements of the blood such as, the red blood cells (causing the release of haemoglobin), white blood cells, and the platelets.
3. Alteration of the plasma proteins (albumin, globulins, fibrinogen, etc.).
4. Destruction of enzymes.
5. Depletion of electrolytes.
6. Adverse immune responses.
7. Damage to adjacent tissues.
8. Cancer.
9. Toxic and allergic reactions.
10. Deteriorate in the biological environment or during sterilisation (with resultant changes in their physical, chemical, mechanical properties and surface characteristics).

(Bruck 1972)

Table 1.6 Biocompatibility defined.

It added little to Scales' criteria, except that chemical inertness was replaced by lack of interaction with blood (Table 1.6 No 1-5), and failure to provoke allergy, foreign body and inflammatory responses were defined in terms of possible mechanisms (Table 1.6 No 3, 4 & 6). Scales' general principles had been more clearly defined in terms that reflected both the better understanding of the mechanisms, and phenomena resulting from the exposure of tissues to materials.

The use of the early materials was spurred on by the prospect of allowing treatment of the clinical problems of vascular and renal disease. There was not much choice, and little information to guide such a choice, since these were the only available materials. Initially little attention was paid to the consequences of the blood-material interaction. The concept behind their use was based on the

ability of the material to perform a function. For Kolff the membrane allowed the diffusion of small molecules thus performing, to some extent, the filtration function of the kidney. Voorhees' synthetic prostheses acted as conduits for blood and had to withstand the forces of blood pressure as well as being physically possible to suture into place.

1.2.3 Changing concepts in biomaterials

The increasing awareness of the complexity of the interaction between the biomaterial and the host, and the interrelationships of the mechanisms involved have brought about a change of emphasis in biocompatibility. The concept of a material's ability to perform a function without any interaction with the host defined the Ideal Material's biocompatibility as inertness. The recognition that no material is inert, or will have all the characteristics of Tables 1.5 & 1.6, has gradually emerged as knowledge has increased. The old approach, to find the Holy Grail of The Ideal Material, perhaps accounts for the lack of agreement over the 'best' test methods and defined goals in biocompatibility testing.

Interaction between the material and the host are inevitable. Biocompatibility is now perceived as 'the ability of a material to perform with an appropriate host response in a specific application'. This definition arose from a Consensus Conference of the European Society for Biomaterials held in 1986 (Williams 1987). The functional aspect, the ability to perform, was how materials were originally chosen. The ability to manipulate the host response, recognising the unavoidability of the interaction, opens the door to designing and tailoring the host response. The specificity of the application has already been alluded to, in the example of Ivolon; the inflammatory response it induces being used in an Ivolon sponge rectopexy to fix the rectum to the sacrum, and so prevent further rectal prolapse. For the vascular prothesis the criteria for an appropriate host response have yet to be defined in terms that allow their use. For example albumin and fibrinogen absorption onto surfaces are widely reported, but it is not known if this is 'good' or 'bad' in the final clinical application.

1.2.4 Problems of biomaterial evaluation

In the 20 years between Voorhees' first implanted graft and Bruck's definition there was an explosive effort in trying to define biocompatibility, in physical and biological terms, both *in vitro* and *in vivo*. The prolific endeavours to solve this problem have thrown up a plethora of tests covering most of the scientific disciplines. Less than 10 years later the National Institutes of Health published guidelines for investigators in the field (NIH 1980). It is a bewildering array of tests and evaluations, and its size (90+ pages and over 500 references, just for blood-material interactions) reflects the complexity of the interactions involved and the lack of agreement between investigators. It points out some of the pitfalls and considerations needed in testing materials. Table 1.7 shows the recommendations for the material scientist in characterising the material, and those for the biologist conducting any screening investigation in Table 1.8.

BULK PROPERTIES	
polymer identification	solvent responses
infrared spectrum	electric conductivity
hardness	water diffusivity
porosity	permeability
density	extractability
mechanical tests	NMR spectrum
thermal analysis	creep
molecular weight	fatigue testing
SURFACE PROPERTIES	
light microscopy	
scanning electron microscopy	
X-ray photoelectron spectroscopy	
ESCA (electron spectroscopy for chemical analysis)	
protein adsorption	
contact angles (Zisman plot)	
charge (Zeta potential)	

(NIH 1985)

Table 1.7 Recommended physicochemical characterisation of materials.

1. Definition and characteristics of species,
   for example :-
   a. age, sex, life span
   b. source, strain
   c. health and nutritional status
   d. environmental factors
   e. variability of stocks and strains

2. Use of Human material where possible with awareness of species differences when not possible.

3. Selection of test system to simulate the final use.

4. Handling and sampling techniques.

(NIH 1980)

Table 1.8 Minimum standards for *in vivo* tests.

In less than 5 years the recommendations had expanded to over 400 pages and 1200 references (NIH 1985). Ratner commenting on this period of biomaterial research noted that 'the most striking feature of all the lists (of ranking blood compatibility of materials) is the almost total absence of any correlation between them' (Ratner 1984). He attributed this in part to the lack of basic knowledge about the test systems, and the relationship of *in vitro* to *in vivo* tests, and of any test to the clinical application. These problems will be discussed further in chapter 5 when considering the *in vivo* model and choice of animal species.

1.3 Current approaches to graft thrombogenicity

1.3.1 Attempts to modify materials

Many aspects of graft design, such as vascular graft wall compliance have been studied but recently attention has returned to the surface exposed to blood. Attempts have been made to eliminate surface-induced coagulation, by the pre-adsorption of anti-thrombotic moieties on to the surface of the biomaterial. Boffa (et al 1979) used heparin and Ebert (et al 1982) used prostacyclin. The clinical application of this approach is limited by the desorption of the anti-thrombotic group (Bruck 1972) or haemolysis as in the case of heparin (Solen et al 1980) or

the strong interaction of platelets with the modified surface (Salzman 1980).

More recently 5% acrylic acid has been radiation copolymerized onto a high porosity woven Dacron graft (Miller et al 1986). This treatment reduced the porosity of the Dacron graft, with an increase in its compliance, and these grafts showed evidence of a cellular lining.

A number of graft manufacturers have coated Dacron grafts with absorbable materials to reduce their thrombogenicity, and initial porosity. For example gelatin (Gelseal, Vascutek Ltd) and collagen (Hemashield, Meadox Medicals Inc) coatings are both available. These knitted and double velour grafts have the advantages of tissue ingrowth discussed above, but without the requirement for preclotting. This is because the coating reduces the initial porosity. This is a short term advantage, but is unlikely to alter the long term thrombogenic properties of the graft as the coating materials are desorbed.

In the early 1970's biological material made a comeback in the form of the human umbilical vein graft, pioneered by Dardik and Dardik 1972. This graft is now a composite, with an outer Dacron sleeve to reinforce the umbilical vein. Dardik has recently reviewed 900 grafts and broadly describes a sadly similar fate to the earlier biological grafts of Carrel and Gross (Dardik et al 1988). There was a 4% infection rate, over a third becoming aneurysmal after 5 years and at the same time a 57% patency in the femoropopliteal position. However this series is better than that of most other workers. For example a well documented multicentre trial, reported 3 year patencies of 42% for above knee, and 32% for below knee popliteal segment bypasses (Rutherford et al 1988).

1.3.2 New materials

Dacron emerged as the best of the synthetic materials by the end of the 1950's. Indeed by 1964 Dacron had proved its superiority over woven Teflon grafts and the homograft (Fry et al 1964). The woven Teflon (PTFE) demonstrates a further example of the importance of fabrication in the final outcome for a biomaterial. The inertness of PTFE, in a woven form, led to separation, by intimal dissection,

of the neointima laid down on its surface and subsequent thrombosis of the graft. In addition the lack of tissue incorporation allowed infection to travel the length of the graft with devastating consequences (Fry et al 1964, Discussion). It was not until 1972 that PTFE was fabricated in an extruded form. This involves squeezing semi-solid PTFE through moulds to produce the complete graft. This type of PTFE was first used as a vascular prosthesis by Soyer (et al 1972) in a variety of venous positions in the pig, and successfully in two clinical cases to bypass the portal vein. Soon 100% patency was obtained for grafts in the dog femoral artery model over 11 months (excluding 3 which failed within the first 24 hours from poor technique) (Matsumoto et al 1974). The PTFE graft now holds an established place between Dacron and the vein for the femoro popliteal bypass.

Just as Dacron grafts have been modified (see Sauvage's work above) there have been modifications to PTFE. These have centred on the porosity of the graft, which is determined during its extrusion. Commercially available PTFE from Gore-Tex is impervious, due to a very thin outer wrap of PTFE. This is needed to prevent dilation, which was a problem with the early PTFE grafts. The extruded PTFE has inter-node distance of 10-30 μm. Clowes et al (1986) have investigated grafts of PTFE with 60 μm pore size, in the baboon. They have demonstrated ingrowth of capillaries from the granulation tissue surrounding the graft. These act as sites for re-endothelialisation of the lumen of the graft. This observation raises the possibility that Dacron grafts, although very porous may fail to 'heal' as a result of a direct cellular inhibition by the Dacron fibres themselves (Zacharias et al 1987).

A combination of Dacron and PTFE has been developed by Garfinkle (et al 1984). A layer of PTFE is deposited on a Dacron graft by the technique of glow discharge. This employs radio frequency discharges in a vacuum between highly charged electrostatic plates. The electrical discharges produce free radicals, which are highly reactive, and they bind even relatively inert materials. The free radicals are generated from small amounts of material leaked into the vacuum chamber as a gas. The technique covalently binds the gaseous material to the chosen target material placed in the electrical field of the apparatus. Garfinkle (et al 1984) used the gas tetrafloroethylene (TFE), the monomeric form of PTFE, to treat 10 cm

lengths of 4-5 mm diameter knitted and woven Dacron grafts, as well as PTFE grafts. Assessed *in vitro* for platelet micro embolisation the treatment improved the standard PTFE graft, as well as the PTFE coated Dacron grafts. Patency was markedly improved for the TFE treated grafts followed for 1 week in an *ex vivo* baboon femoral shunt model. Grafts of this type are now commercially available (PET graft marketed by Plasma Atrium). This may be a promising material, as it handles like a coated Dacron graft, but full clinical studies are still awaited.

These materials represent modifications of the two main prosthetic materials which have been used for many years. The polyurethanes are a new group of compounds, which have proved difficult to develop into a form suitable for use as a prosthesis. The University of Liverpool group have developed the technique of electrostatic spinning, whereby Biomer (polyetherurethane urea) is sprayed onto a mandril to produce a microfibrous graft. This has 1-2 μm diameter fibres with a 20 μm pore size, and more importantly a compliance close to that of the dog's carotid artery. The latest paper from this group (de Cossart et al 1989) reports patency over 2 years of 17 out of 26, (65%). They used grafts 4 cm in length and 3.8 mm in diameter as carotid bypasses in Beagle dogs. In particular they noted no or little neointimal hyperplasia. This may be due to the graft's isocompliance, or a species related finding and clinical trials in man are awaited.

1.3.3 Endothelial cell seeding

For the bypass of the coronary arteries, the mesenteric vessels and those below the groin, the choice of materials remains critical, and in these smaller vessels the autologous saphenous vein remains the best conduit, as outlined above in 1.1.2. The success of this strategy, compared to artificial grafts materials, undoubtedly lies in the natural biological activity of the former. Thus the endothelium of autologous grafts secretes both prostacyclin and tissue plasminogen activator (t-PA). Prostacyclin is a powerful inhibitor of platelet aggregation and activation. t-PA is a potent activator of plasminogen, resulting in the dissolution of fibrin clots. In contrast graft materials rarely develop an endothelial cell lining for

reasons discussed above with the consequence that, in man, the graft does not heal (Berger et al 1972, Sauvage et al 1974). It was therefore entirely logical that consideration was given to the idea that the graft should be coated with endothelial cells before implantation.

Herring showed that endothelial cell seeding of knitted Dacron reduced the thrombogenicity of the graft (Herring et al 1978). There have been many subsequent reports confirming these finding, but there are considerable problems still to be addressed:-

1 Source of the cells,
2 Type of cells,
3 Harvesting technique,
4 Best substrate,
5 Culture and growth.

The saphenous vein is one choice for the source, but if suitable it would be used as the graft itself. If it is not, it may not be sufficiently large to provide enough cells. Varicose veins may be used (Leseche et al 1989). For the animal studies the external jugular vein has been used, and this has been used in man as part of a clinical study (Herring et al 1987). Recently attention has been drawn to fat (Rupnick et al 1989), and the use of mesothelial cells (Bull et al 1988). The ideal harvesting technique appears to be enzymatic rather than mechanical. The critical issue of seeding or sodding (Rupnick et al 1989), that is 'how many cells are enough' is unresolved.

PTFE appears to be the best material as the base for these cells, but this too is not fully resolved (Herring et al 1984). The variety of substrates (fibrin, fibronectin, collagen, gelatin, and mixtures including antifibrinolytics) used to try and anchor the cells highlights the problem of getting the cells to remain on the graft (for example Budd et al 1989). A direct comparison between Dacron and PTFE grafts for seeding with different substrates concluded that preclotting with blood gave the best results (Vohra et al 1991). Only 17% of the inoculated cells are present at 24 hours, and these represent only 4% of those harvested (Rosenman et al 1985a). Culture of the cells for 1, 2 or 3 weeks, and freezing them at -80 °C to increase the yield has been investigated (Leseche et al 1989). What effect the passage in culture and all these manipulations have on the

function of the endothelial cell that is finally deposited on the graft remains unanswered.

Despite these difficulties many *in vivo* studies in dogs baboons and calves have confirmed Herring's initial concept, namely by demonstrating endothelial coverage of prosthetic materials, with subsequent improved patency (Belden et al 1982), reduced thrombogenicity and increased resistance to infection (Rosenman et al 1985b). However there have been few human studies, and these have shown variable results. Reporting on 17 patients who had seeded PTFE grafts for femoropopliteal disease the 1 year patency rates were 81% compared with 31% for the unseeded. However the effect of continued smoking on patency was underlined - 2 out of 6 seeded, and all 6 unseeded grafts in the smokers had failed (Herring et al 1987). Zilla (et al 1987) reported a smaller study of 9 seeded PTFE grafts, with 9 matched unseeded grafts after three and a half months. There was only a trend in favour of the seeded grafts for platelet survival and patency (confirmed by doppler). They made the important observation that dogs do better than patients!

Kent (et al 1989) examined the rates of bovine, canine and human endothelial cell harvest and the growth kinetics of the harvested cells. Human cells gave the lowest harvest yield, had a prolonged lag phase and gave the slowest doubling time. This important work parallels the work of Berger (et al 1972) and Sauvage (et al 1974) who described species differences in the healing of grafts. The routine clinical use of cell seeded grafts may be some way off, but further investigation of this approach is of obvious importance.

As has been shown the need for an endothelial cell, or at least an endothelial like surface on the prosthesis may be of critical importance for healing. This may be due either to the local synthesis of prostacyclin or t-PA by the endothelial cell or, because of the superior biocompatibility of the endothelial cell membrane surface. If the latter is the case, then there is the possibility of coating the prosthesis with synthetic membranes which mimic those of the endothelial cell. This question is examined in detail in this thesis, which describes experimental attempts to copy the natural cell lining of the artery - by artificial means.

Outline

Chapter 2

The hypothesis under test

2.1 Background to current approach of biomembrane mimicry

2.1.1 The concept

2.1.2 The supporting evidence

2.2 The hypothesis

Chapter 2

The hypothesis under test

2.1 Background to current approach of biomembrane mimicry

2.1.1 The concept

The concept of 'disguising' arterial grafts with coats of biological membranes (rather than with whole endothelial cells) is not new. Indeed Chapman advocated the mimicry of biological membranes as a new approach to the problem of prosthetic material thrombogenicity (Hayward & Chapman, 1984 and 1985). The key observation is that the outer surface of both the erythrocyte and platelet is non-thrombogenic. The phospholipids of the outer bilayer of these cell membranes are comprised of one type of polar head group. They contain 89% phosphorylcholine, and this has a low thrombogenic potential (Zwaal & Bevers, 1986).

Briefly the biological membrane is lipid bilayer composed of phospholipids. Each phospholipid has two fatty acid chains and a polar head group, a structure based on glycerine. These arrange themselves with the polar groups orientated to the aqueous environment, externally and to the cytosol. The lipid chains, being hydrophobic align themselves together, and form the 'filling' of the bilayer sandwich (see Figure 2.1). The structures of some of these phospholipids are shown in Table 2.1. Sphingomyelin is slightly different, and it has a fixed chain in the first position, and an amide link to the variable fatty acid chain at the second position. It still has the phosphorylcholine head group in the third position.

The phospholipid membrane of the red blood cell (RBC), and platelet is similar and is shown in Figure 2.1. These membranes do not normally activate coagulation in the blood stream. The distribution of phospholipids across the inner and outer leaflets of the plasma cell membrane of erythrocytes (Figure 2.2) and platelets (Figure 2.3) is markedly asymmetrical (Zwaal & Hemker 1982). Despite the contrast in the biological functions of these cells it is interesting to

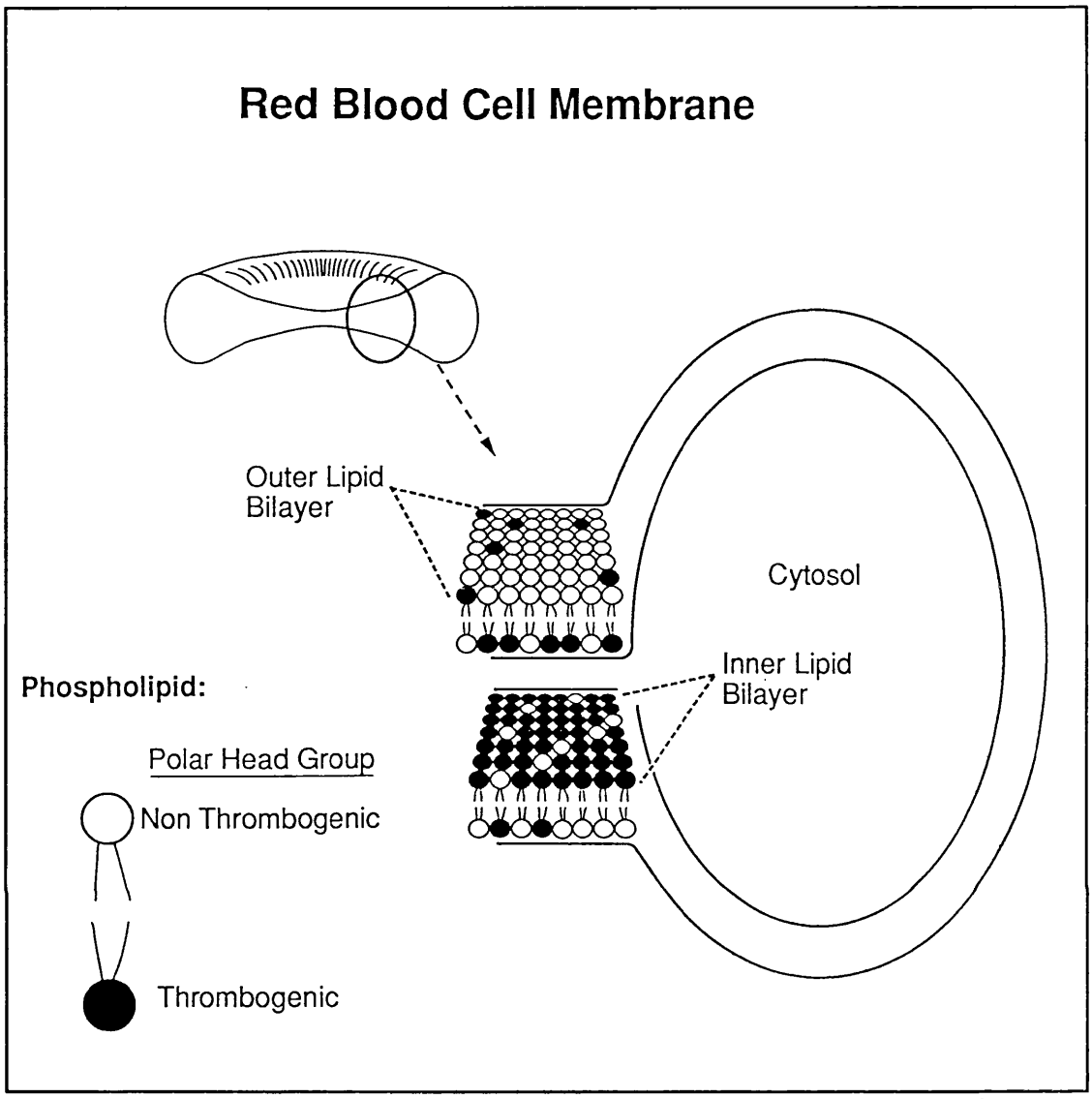

Figure 2.1 A diagrammatic representation of the lipid bilayer of the RBC.

note that the phosphorylcholine containing phospholipids, sphingomyelin (SM) and phosphatidylcholine (PC) comprise the major fraction of the lipid exposed at the outer cell surface.

The negatively charged phospholipids are confined to the cytoplasmic surface of the bilayer. The outer layer of the phospholipid bilayer is not involved in activation of blood coagulation and is itself non-thrombogenic. However when blood is exposed to the negatively charged inner leaf of the erythrocyte membrane the coagulation cascade is triggered. Similarly the exposure of sub-endothelial collagen to blood which occurs as a result of trauma or atherosclerosis results in activation of coagulation via platelets and factor XII. This process is also seen with prosthetic materials.

glycerol CH_2-OH

CH-OH

CH_2-OH

phospholipid CH_2-O-C-(O)-[R]

CH-O-C-(O)-[R]

CH_2-O-P-(O)-O-[G]

OH

R is a fatty acid (the lipid chain)
G is the polar head group

PC phosphatidyl choline has the choline group at G:-

$-CH_2-CH_2-N(CH_3)_3$

PE phosphatidyl ethanolamine:-

$-CH_2-CH_2-NH_2$

PS phosphatidyl serine:-

$-CH_2-CH-NH_2$

C-(O)-OH

PG phosphatidyl glycerol:-

$-CH_2-CHOH-CH_2-OH$

PI phosphatidyl inositol:-

CHOH - CHOH

-O-CH CHOH

CHOH - CHOH

SM sphingomyelin:-

$CHOH-CH=CH-(CH_2)_{12}-CH_3$

CH-NH-C-(O)-[R]

CH_2-O-P-(O)-O-$CH_2-CH_2-N(CH_3)_3$

OH

Table 2.1 The structures of some of the phospholipids, with the abbreviations for the figures.

There are differences in the ability of the inner and outer layers of the RBC membrane to generate activated factor X and thrombin (Zwaal et al 1977). Specific receptor sites on the platelet membrane are responsible for their activation and the subsequent platelet release reaction. During the stimulation of platelets the negatively charged phospholipids become exposed, and increase the rate of thrombin formation (Bevers et al 1983). Indeed it is the abnormally high

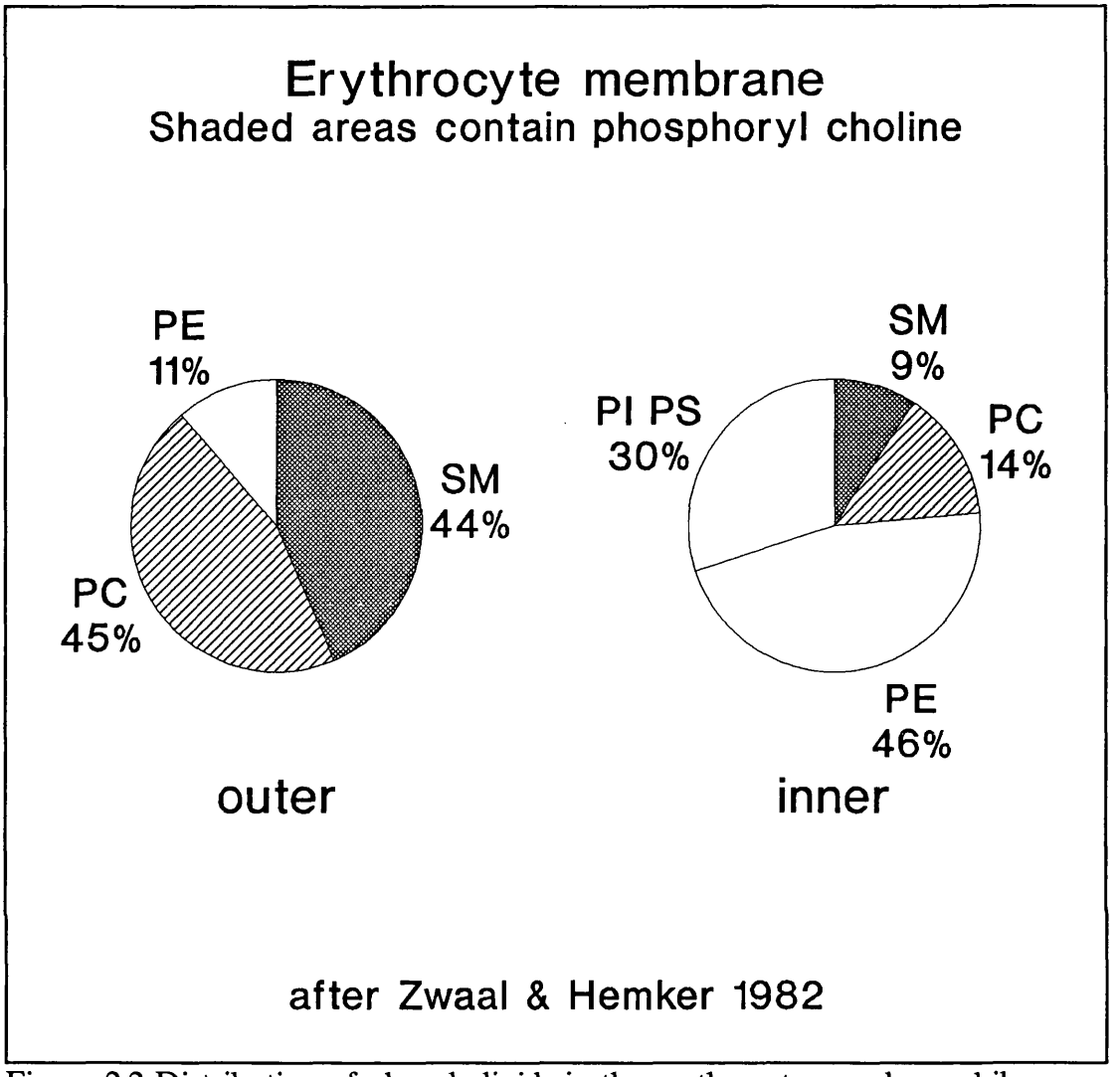

Figure 2.2 Distribution of phospholipids in the erythrocyte membrane bilayer (abbreviations as table 2.1).

levels of phosphatidylserine (PS) and phosphatidylethanolamine (PE) (these carry a negative charge) in Bernard-Soulier platelets which explains their prothrombinase activity (Zwaal & Bevers, 1986).

Until recently it was accepted that the net negative charge on the membrane was the important factor in the activation of coagulation (Bangham, 1961; Papahadjopoulos et al, 1962), but Rosing has challenged this view (Rosing et al, 1988). By adding increasing amounts of stearylamine (positively charged) to vesicles of PS (negatively charged) they varied the overall surface charge on the vesicles from negative to positive, and found this variation in charge made no difference to coagulation measured by activated partial thromboplastin time (aPTT) and Stypven tests. They used other anionic phospholipids and showed PS

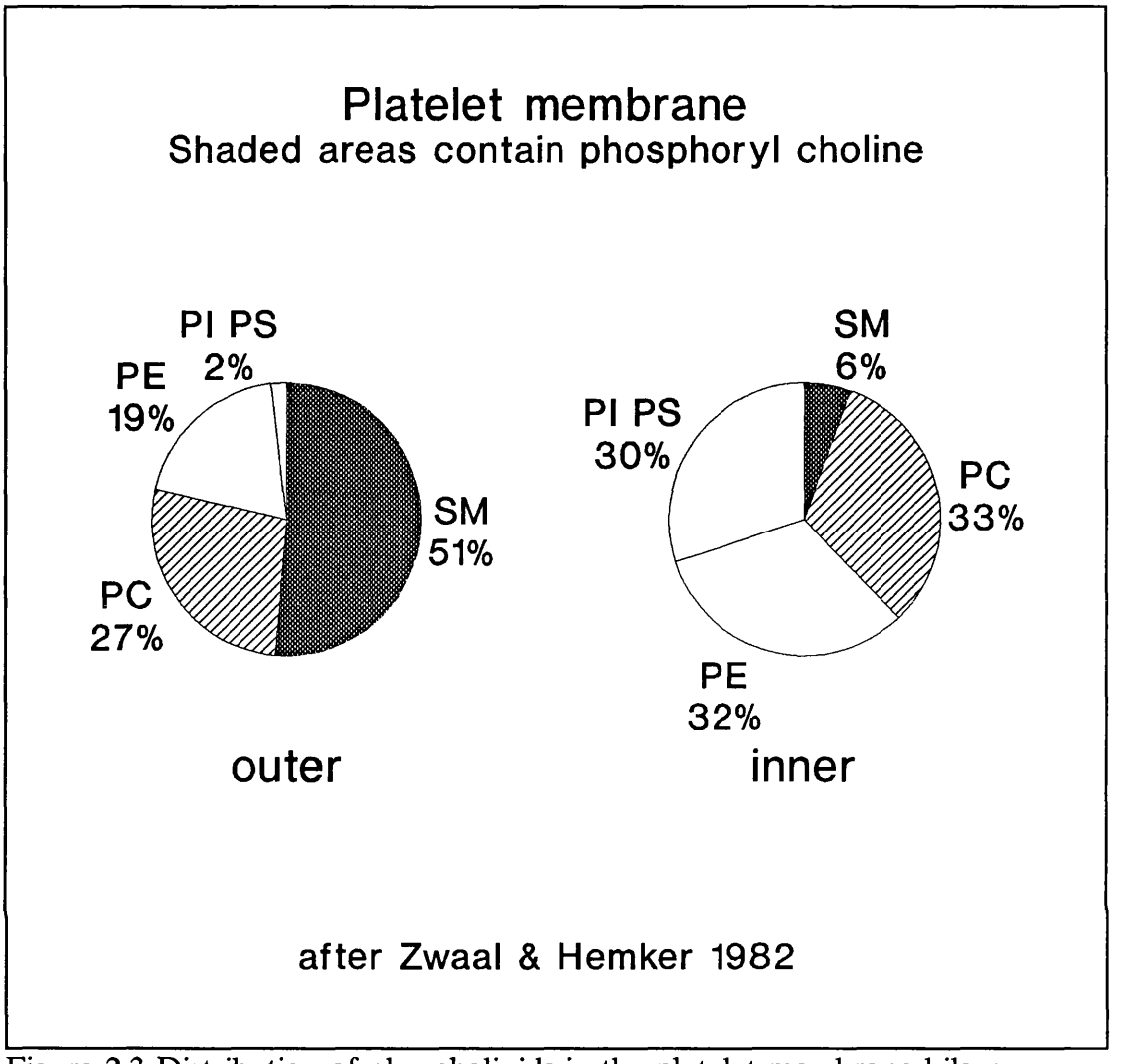

Figure 2.3 Distribution of phospholipids in the platelet membrane bilayer (abbreviations as table 2.1).

was the most thrombogenic, and its concentration was far more important than any effect due to the charge on the vesicle.

2.1.2 The supporting evidence

Evidence to support the role of PC in reducing thrombogenicity comes from observing the effect on the Stypven test of polymeric phospholipid dispersions (which mimic the surfaces of blood cells) (Hayward & Chapman, 1984). In this test plasma is incubated with phospholipids, and Russell's viper venom, which activates factors V and X. The test needs the phospholipid as the activated forms of both factors are assembled in the lipid. By using normal plasma the effect of variations in phospholipid concentration and subclass composition can be

examined as the test is very sensitive to the nature and concentration of the phospholipids present. Liposomes prepared from dimyristoyl phosphatidyl choline (DMPC) did not shorten the clotting times, whereas the negatively charged liposomes containing PS did. The rate of clot formation was not affected by increasing the amount of the monosubstituted diacetylene phosphatidylcholine liposomes. This material has a triple C-C bond which can be polymerised. Polymerisation of diacetylenic phosphatidyl choline (DAPC) did not alter their non-thrombogenic character.

A preliminary investigation of the interaction of this type of photopolymerised liposomes with platelets by Juliano (Juliano et al 1983) showed little direct interaction in plasma. They used liposomes containing mixtures of PC with stearylamine (SA), to give an overall positive charge (SA/PC net positive), and phosphatidyl glycerol (PG), to give an overall negative charge (PG/PC net negative). However variable inhibition of washed platelet aggregation in response to ADP and thrombin was noted in the presence of liposomes containing SA/PC (net positive), PG/PC (net negative), and a methacrylate PC polymer. They concluded that protein absorption onto the liposome was important in governing the effect of liposomes on platelet function.

Further work from this group (Bonte et al 1987) investigating polymerisable lipids with PC groups as possible drug delivery systems again showed no direct effect on platelets with similar liposomes. However polymerised liposomes with the methacrylate group impaired ADP induced platelet aggregation, and interacted with factor V to prolong the prothrombin time (PT) and aPTT in plasma. They also identified binding of IgG from serum in significant amounts, particularly by the methacrylate PC liposomes. It also bound liposomes of dipalmitoyl phosphatidyl ethanolamine (DPPE) and PS, while dipalmitoyl phosphatidyl choline (DPPC) showed a minimal amount of protein binding. They concluded that the ligands they needed for polymerisation were as important as the PC head group in determining the response of washed platelets to their agonists, and that this was due to protein binding.

There are therefore major differences in the effects on blood clotting of the

anionic and cationic phospholipids of the normal RBC and platelet membrane. It is likely that the subclass of the phospholipid itself may be more important than the net charge it contributes to the membrane. The results of the Stypven tests, the clotting tests with liposomes and the protein absorption are the three strands of evidence that show the non-thrombogenicity of the phosphorylcholine head group.

2.2 The hypothesis

That :-

1)- The relative thrombogenicity of phospholipids in the membrane of blood cells IS related to the polar head groups present in the membrane. In particular that those with phosphorylcholine polar group are non thrombogenic.

- This will confirm the previous suggestion that it is the phosphorylcholine group which is the least thrombogenic.

2)- The lack of thrombogenicity of phospholipids in the membrane of blood cells and polymers based on them CAN reduce the thrombogenicity of the surfaces which they coat.

- This *in vitro* work will assess their suitability for *in vivo* use.

3)- The thrombogenicity of arterial grafts CAN be modified by coating with polymers which mimic the haemocompatible moieties of the red blood cell membrane.

- This will be assessed *in vivo*.

4)- The change of surface thrombogenicity WILL prolong the patency of these grafts.

outline

Chapter 3

In vitro assessment of materials

3.1 Introduction

3.2 Material Thrombelastography

3.2.1 Historical aspects of thrombelastography

3.2.2 Historical aspects of Material thrombelastography

3.2.3 The current MTEG investigation

3.2.3.1 TEG Technique

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3.4 The liposome uptake of clotting factors

3.4.1 Introduction

3.4.2 Materials and experimental details

3.4.3 Assay systems and interpretation of results

3.4.4 Results

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3.6 Choice of materials for in vivo testing

Chapter 3

In vitro assessment of materials

3.1 Introduction

The range of *in vitro* tests advocated to evaluate biomaterials presented in chapter 1 demonstrates the lack of clear goals and standards used for their assessment. These tests are far from being able to give a clear 'yes' or 'no' answer to the question 'is this material biocompatible?'. While the assessment of biocompatibility is more comprehensive than the assessment of blood compatibility the interaction between blood and the material is crucial to the final outcome. Certainly one assumption underlying this view is that blood is likely to be the first part of the organism to meet the material. If the result of this first interaction is favourable, then it is likely that the more complex long term interactions with the host will also be good. There is reasonable ground to accept this proposition for a material to be used in the cardiovascular system. However the wide range of tests advocated speaks of the problems of non-predictability. An associated problem is the lack of well documented comparative studies of materials both *in vitro*, *in vivo* and in man, to validate the assumptions behind the *in vitro* tests. More complete programmes of biomaterial testing are needed to address this question.

The range of tests also reflects the variety of underlying hypotheses used to explain biocompatibility. For example, the measurement of a material's water contact angle indicates it's surface wettability and surface tension. Good wetting predicts a good material. Similarly for surface charge, where this is negative the material is likely to be 'bad'. While these can be helpful concepts they are certainly not the whole answer.

The literature describes many tests, and the NIH publication reviews these and itself recommends a large number of tests (NIH 1980 & 1985). Many of these stem from clinical practice. For example a number of blood coagulation assays have been modified to look at the interaction of blood with materials, to detect thrombosis. However it is important to note the differences from the clinical use

of these tests. In the clinical setting a test is designed to detect a clinically significant abnormality. For example screening for a pathological deficiency in blood clotting, like the partial thromboplastin time used to detect haemophilia. In this setting the test takes into account the functional reserves of coagulation, since over 80% factor depletion is needed for the test to be 'positive'. For use in the assessment of biomaterials these tests need to be conducted in a way that allows detection of activation or inhibition or adsorption of small amounts of normal clotting processes and proteins. There is little place in this context for a test requiring a depletion of 80% or more of the factor before there is a change in the parameter being measured.

The widely used *in vitro* assessments aim either to rank the materials, or to examine the primary events and mechanisms of the blood material interaction. There is also a choice between static and dynamic tests, where the flow of blood over the material adds the confounding elements of the separation of blood constituents and the renewal of the substrates at the surface. Some evaluations use platelets and even whole blood, but as outlined in chapter 1 attention has only recently been paid to the wider cellular interactions involved.

Despite the artificial nature of some of these tests they have advantages. The conditions can be carefully controlled and documented. There is the opportunity for repeated measurements. The conditions can be reproduced, or varied by minor degrees to give additional information. They can be both fast and inexpensive to carry out. By carefully designing the experiments specific aspects of the interaction can be examined. This approach has produced a wealth of information, particularly in terms of the mechanisms involved in biomaterial interactions. *In vitro* testing also permits the use of human blood, which is a major bonus compared to the uncertainty that using animals introduces, which will be discussed further in chapter 5.

The three tests chosen for the current studies use human blood or plasma, and are conducted at 37°C. This is important since the normal processes of coagulation are seriously affected as the temperature departs a few degrees from 37°C.

These are :-

1). The technique of material thrombelastography (MTEG). All the interactions between proteins, platelets and blood cells take place, and the analysis permits the identification, in broad terms, of the effect of the blood-material interaction on these different components.

2). The parallel plate assay. This is used as a comparison with MTEG, as it too uses whole blood. In this test the blood is incubated with the material and the relative rates of thrombin generation are measured. The conventional materials (Dacron and PTFE) will be used, and then coated with DPPC and DAPC.

3). The liposome uptake of clotting factors. The phospholipids are made into liposome suspensions, which are incubated with plasma to examine the interaction of components of the clotting cascade in plasma.

3.2 Material Thrombelastography

3.2.1 Historical aspects of thrombelastography

The thrombelastograph (TEG) measures the shear elasticity of whole blood clots

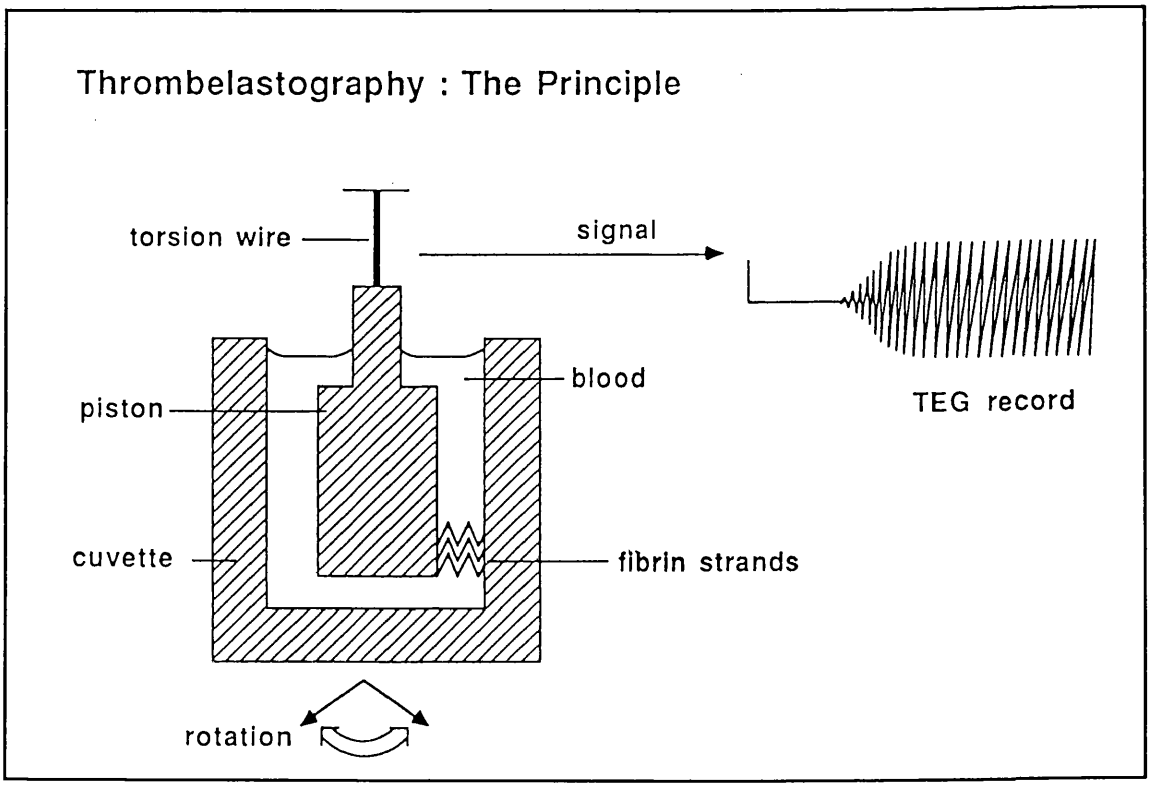


Figure 3.1 The principle of thrombelastography.

as they are formed. It uses a Krupp's steel type 2VA piston suspended within a cuvette so as to have a 1 mm gap between the surfaces of the piston and cuvette. Blood is placed in the cuvette and covers the piston. The cuvette oscillates through 4° 45' (1/12 radian) in a 10 second cycle. The piston is suspended by a torsion wire which is linked to a recording chart. The torque of the cuvette is transmitted to the piston via the fibrin strands in the blood clot as coagulation proceeds. The resulting trace, the thrombelastogram records the elasticity of the blood clot. The principle of the machine is illustrated in Figure 3.1.

The instrument was designed and described by Hartert in 1948 (Hartert 1948), and was so widely used that an International Conference was held in Pavia in 1954. This produced one of the definitive work's on thrombelastography by de Nicola (de Nicola 1957). The technique gained further popularity, particularly in Europe, and the 'Atlas' produced by Marchal (et al) in 1961 distils the wealth of information that the method can give, as well as the variations in technique and analysis. However in the early 1960's the attention of haematologists was focused on the individual clotting factors by the 'cascade theory' of coagulation proposed by Macfarlane (1964). Over the last 25 years our understanding of the coagulation process in terms of the individual clotting factors has improved enormously. However it is recognised that measures of whole blood clotting, which reflect all the interactions that take place between the components of blood, are as important the individual factors. There has been a reappraisal of methods which examine whole blood clotting. The recent review of the TEG technique by Franz and Coetzee (1981) shows its use in many clinical settings. This is also reflected in the recent interest in instruments such as the Haemostat 4000 and the TEG. The Haemostat 4000 principally measures platelet aggregation, while the TEG analyses whole blood clotting in terms of platelet and coagulation factors.

The diagnosis and the control of the coagulopathy that occurs during liver resection and transplantation is one area where the TEG is enjoying a major revival of interest amongst anaesthetists and surgeons (Howland et al 1974a, Howland et al 1974b, Howland et al 1975). This has been helped by the development from the original photographic machine, which was difficult to use, to the current TEG which produces an electronic recording using a 'hot' pen (Raviv

et al 1978). It is particularly useful in guiding replacement therapy of platelets and coagulation factors during liver transplantation (Howland et al 1970, Kang et al 1985). The TEG is particularly useful in the detection and treatment of fibrinolysis (Kang et al 1987).

The TEG parameters are not readily equated with the conventional tests of coagulation, but the component of blood which is activated can be deduced from the shape of the TEG trace (de Nicola 1957, Marchal et al 1961, Zuckerman et al 1981). The TEG is more sensitive than the conventional tests (Girolami and Scarpa 1969, Zuckerman et al 1981, Bird et al 1988b). Indeed it has been advocated for cancer screening (Caprini et al 1976), and a small study reported its use for breast cancer (Haid 1977). It has a particular advantage over any laboratory method in the detection of fibrinolysis (von Kaulla and Weiner 1955, Astrup and Egeblad 1965, Howland et al 1970, Howland et al 1974a & 1974b, Summaria et al 1986, Kang et al 1987).

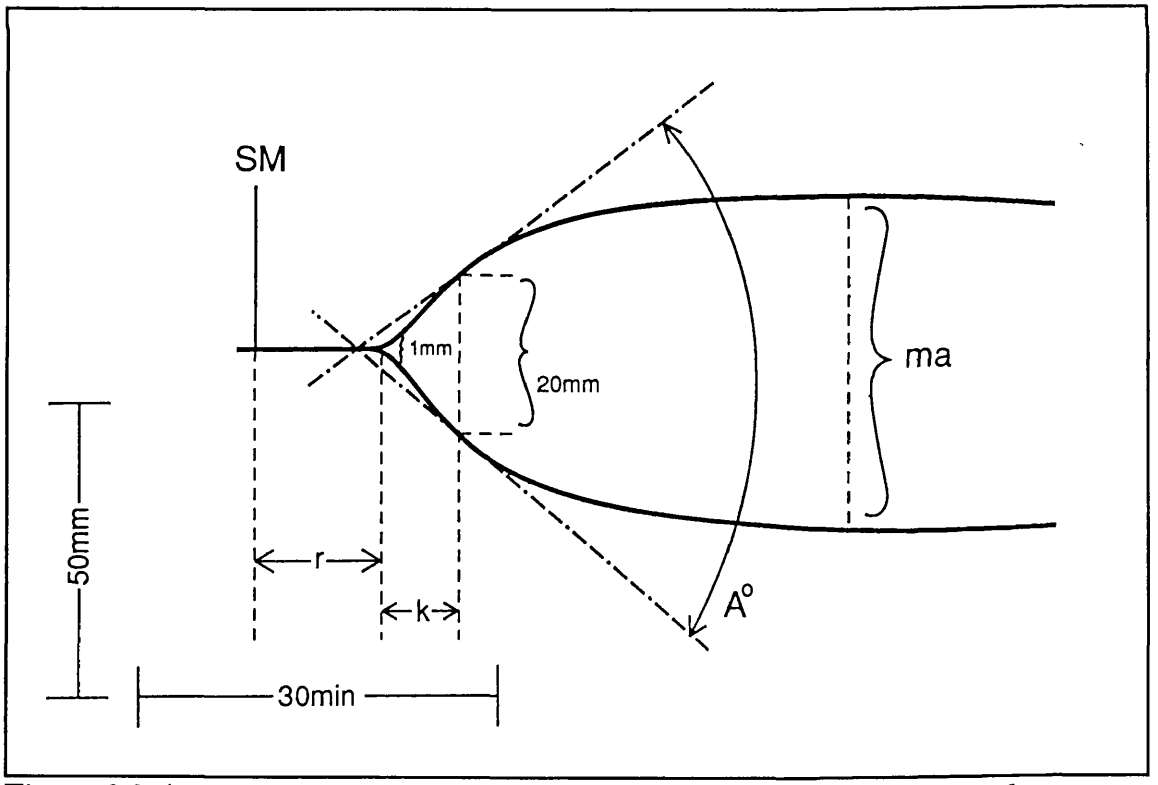


Figure 3.2 A typical thrombelastogram, with the standard parameters shown.

A normal TEG is shown in Figure 3.2, with the standard TEG parameters superimposed. Each parameter reflects a different component of whole blood

coagulation. SM is the start mark, for timing the trace. The lag, from the start of the trace to its first movement, is due to the time taken for the activation of the clotting factors, and for these to generate the first strands of fibrin to link the piston and cuvette. The 'r' time, the reaction time, is the time taken from the start of the recording until an arbitrary amplitude of 1 mm is reached. The 'k' time, the clotting speed, is the time interval from this until an arbitrary amplitude of 20 mm is reached. These two parameters reflect the degree of clotting factor activation. The speed with which thromboplastin creates thrombin, and the rate of fibrinogen cleavage to fibrin monomers is shown by the 'k' and angle parameters.

The parameter 'ma' is the maximum amplitude reached, and is due principally to platelet and to a lesser extent fibrinogen activation. 'e' is a derived parameter (100ma/100-ma), based on the elastic shear modulus of the clot. This allows for the exponential nature of the sensitivity of the instrument, as more force is needed to produce the same deflection for the stiffer clots. 'tma' is the time taken to reach the maximum amplitude.

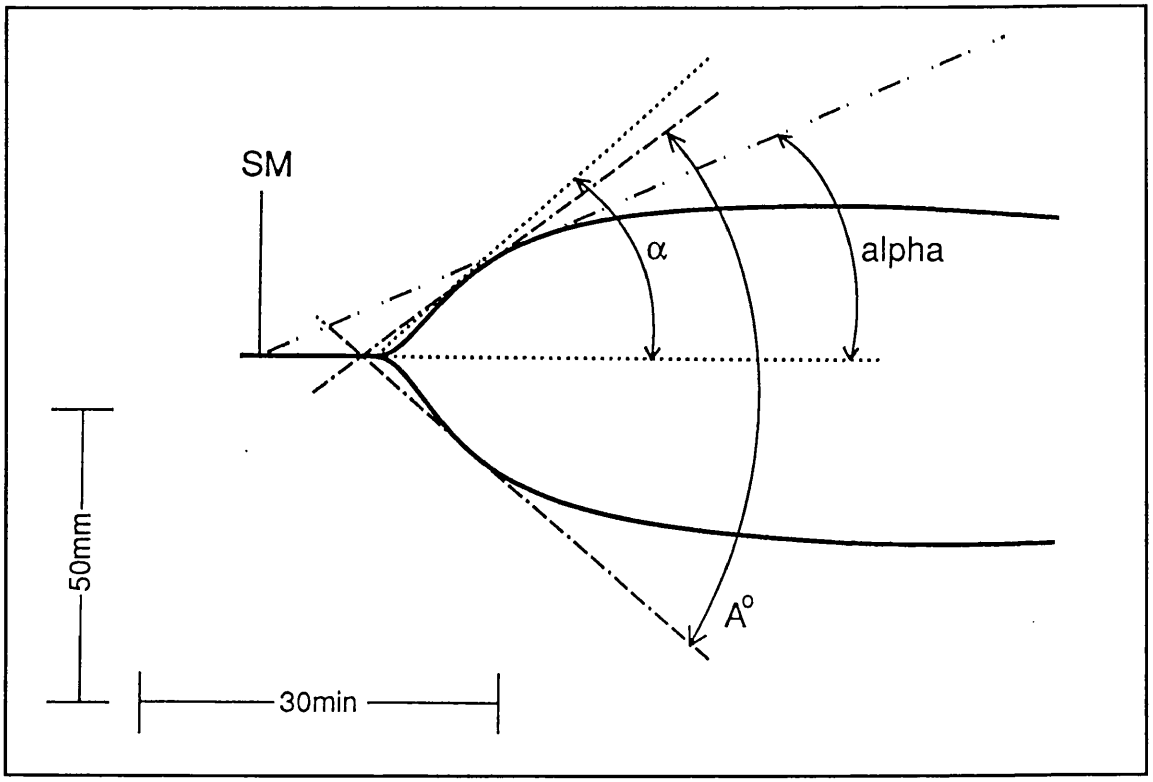


Figure 3.3 The different measures of the thrombelastograph angle (see text for details).

The maximum slope of the initial part of the trace reflects the speed of thromboplastin generation. However the parameters used to measure it are slightly different in the literature. These are illustrated in Figure 3.3. Marchal (et al 1961 p30) used the start of the trace to the 'shoulder' for the angle α ('alpha' in fig 3.3). Affeld (et al 1974) and Schulze (et al 1983) both used the maximum slope of the 'shoulder' to measure the angle α ('α' in fig 3.3), but reported tan α as 'SL' and 'S' respectively. Kang et al 1985 and 1987 used the same angle α but reported it directly. Caprini's group (Caprini et al 1976, Carr et al 1976, Zuckerman et al 1981, Summaria et al 1986) used the angle 'AD' from the first movement of the trace (as for 'r') to the 'shoulder'. However Raviv (et al 1978) pointed out that the two shoulders of the trace are often asymmetrical. My coworkers and I have advocated measuring the angle A° from the first movement of the trace to both shoulders (Bird et al 1988a). This is more accurate as the first movement of the trace is easy to identify and it takes into account both shoulders. The angle α is a more subjective measurement, as it is the tangent to the steepest slope of the shoulder. The angle A° is also twice the size of the angle AD, and is less sensitive to errors than either α or AD in the process of measurement. Our paper is the first to systematically compare the angle α and A° (Bird et al 1988a). Hypocoagulation produces a prolonged r, r+k, k time and a reduced ma, e and A°.

3.2.2 Historical aspects of Material thrombelastography

The technique of thrombelastography has been modified to compare the effect of different materials on blood coagulation. Instead of testing different blood samples, normal whole blood, from the same venepuncture, is used in both channels. The material to be tested is coated on one of the two pistons and cuvettes, which are then placed in one of the two channels of the TEG. This coating is then the only difference between the two channels. The ratio of the TEG parameters from the treated and the untreated channel provides the basis for the comparison the different materials. Variations due to the differences between individual's blood, the sample collection and handling are thus eliminated. The coating can be varied and its thrombogenicity studied; this is the technique of material thrombelastography (MTEG) (Affeld et al 1974, Carr et al 1976, Schulze

et al 1983, Bird et al 1988a, Bird et al 1989, Hall et al 1989, Bird et al 1990).

Carr (et al 1976) stressed the advantage of using whole blood with all its interactions during TEG over the other *in vitro* tests using components such as platelets or fibrin. Lemm (et al 1980) reviewed the methods available for testing the haemocompatibility of synthetic materials, and he strongly advocated material thrombelastography (MTEG) as quick and simple, providing "a remarkable correlation between the *in vitro* results and the *in vivo* behaviour". Material thrombelastography has been an underused technique for screening for blood-material interactions. This technique has the advantages of using whole human blood at 37°C, without an air-blood interface, and appears to meet some of the NIH recommendations (NIH 1980, NIH 1985).

The ratio of the TEG parameters for the treated/untreated surface should be 1 if there is no difference in the thrombogenicity of the coating. The MTEG parameters (the ratio) indicate the degree to which each aspect of coagulation is affected (Schulze et al 1983, Bird et al 1988a, Bird et al 1989, Hall et al 1989). Previous workers have shown the pitfalls of using animal blood (Schulze et al 1983), and neglected some of the TEG parameters. Carr (et al 1976) showed the importance of the 1 mm gap between the piston and cuvette. Their test coating of reconstituted elastin was 0.3 mm thick, and they sought to correct the results for this by using different sized pins. This is unnecessarily complicated, and it is better to keep the test coating reasonably thin. They also failed to show any formal statistical analysis to support their conclusions. My co-workers and I have stressed the importance of statistical analysis and use of all the parameters, using human blood (Bird et al 1988a).

We have also advocated a unified approach to the nomenclature of both TEG and MTEG parameters, which will be followed in this thesis. The term MTEG should be restricted to a direct comparison between a test and a control channel conducted with the same blood sample, at the same time. An ideal non-thrombogenic surface would cause minimal activation of clotting shown by very long r and k times with a low A° and ma. The ratios are expressed as test substance (using the suffix '$_t$') over the control (using the suffix '$_0$') and such

surface would have an r_t/r_0 and k_t/k_0 approaching infinity while $A°_t/A°_0$ and ma_t/ma_0 approach zero. The MTEG parameter is this ratio, and is denoted by the addition of a capital 'M' prefixing the standard TEG parameter, so Mr is r_t/r_0 etc (Bird et al 1988a). The ideal material would give an Mr and Mk of infinity (indicating low clotting factor activation) and Mma, Me and MA° of zero (indicating a low platelet activation, and a low rate of thrombin generation).

3.2.3 The current MTEG investigation

3.2.3.1 TEG Technique

Blood is taken from a panel of volunteers involved with the project. The venepuncture is performed using a 21 gauge butterfly (M Abbott, Medical Systems) from an uncuffed arm vein. The first 2 ml of blood is discarded to avoid the effects of tissue thromboplastin released on venepuncture in the sample. The next 4.5 mls of human blood is mixed with 0.5 mls sodium citrate (3.8%).

MTEG is performed using 0.3 mls of citrated blood recalcified with 60 μl of a 1.29% (116 mM) calcium chloride solution. The type D thrombelastograph (PPG Hellige) with two channels is used throughout. Each pair of pistons is checked for variation before one of the pistons is treated. Each material is tested on ten occasions with at least three different sources of blood. The piston and metal cuvettes are cleaned off after each test using a 2% solution of a phosphate free detergent (RBS, Chemical Concentrates Ltd) and washed several times with distilled water, drying with tissue between each step.

3.2.3.2 TEG Analysis

The standard TEG parameters are obtained using a Vernier calliper and all the results are expressed in millimetres. The trace moves at 2 mm per minute, so the parameters can be expressed in units of time (in minutes by dividing by 2), but it is easier to use the distances to calculate the ratios and carry out the subsequent statistical analysis. The individual parameters are discussed above and shown in Figure 3.2. The ratio of treated/untreated parameters is calculated and the

geometric mean with 95% confidence limits obtained. This eliminates the variation due to differences in the absolute values of each parameter due to the blood sample and corrects for the skew distribution introduced by using a ratio. The probability 'p' is calculated using a paired 't' test on the geometric data, except when Mr+k is infinite when a Wilcoxon rank sign test is used (on the Mr+k and Mk values and the finite data are shown).

This rather rigorous analysis is used, despite the obvious nature of some of the results, to exclude errors due to differences between the untreated pins, and to detect small differences in the control tests. The Mr, Mk, and MA° relate to clotting factor activation. However rapid activation, implying high thrombogenicity, leads to a short r and k, but a large A°, so that Mr and Mk are <1, while MA° is >1. The Mma and Me reflect platelet and fibrinogen activation, being >1 for thrombogenic surfaces.

In addition to the parameters of the trace the overall shape can also be informative. The step like reduction of the amplitude, called 'retraction' and 'break-off' are well documented problems using plastic cuvettes, particularly in the clinical conditions of leukocytosis and uraemia (Loeliger et al 1963). In all these cases the initial part of the tracing is unaffected, unlike the effect of dirty equipment where the whole trace is affected. This particular problem is avoided by scrupulous cleanliness of the pins. Traces which demonstrated these features are excluded from the analysis.

3.2.3.3 Materials and methods of preparation

All the polar head groups in the RBC membrane will be examined by MTEG to establish if the PC group is the least thrombogenic. If it is it will be the most useful for future biomaterials. The materials, results and discussions are presented in three groups,

1:- the standardisation of the pin pairs with controls for the method of MTEG.

2:- the phospholipids,

a) those containing the PC group,

b) those with a negative charge,

c) those with a positive charge, and

d) charged test mixtures.

3:- the materials.

In group 1 appropriate controls and standards will be presented, as well as the analysis of the two measures of the angle (see above). In group 2 the role of the charge of the phospholipid and its transition temperature will be assessed. Finally in group 3 the conventional materials and new polymers with the PC group will be assessed. The materials used have all been obtained from Sigma unless otherwise stated (see acknowledgements), and are used without further purification. The materials are :-

Group 1 The standards and controls

Dipalmitoyl phosphatidyl choline (DPPC),
Octacosane,
Liposomes of DPPC,
Silicones, Sigmacote,
and ICI M494.

Group 2 The phospholipids

a) Those containing phosphoryl choline

Dipalmitoyl phosphatidyl choline (DPPC),
Dimyristoyl phosphatidyl choline (DMPC),
Distearyl phosphatidyl choline (DSPC),
Sphingomyelin (SM),
outside RBC mixture (SM/DPPC/DPPE) - see below.
DPPC/GM1 (GM1 is a negatively charged ganglioside
with sialic acid residues).

b) The negatively charged (thrombogenic) phospholipids

Dipalmitoyl phosphatidyl serine (DPPS),
Dipalmitoyl phosphatidyl ethanolamine (DPPE),
Dipalmitoyl phosphatidic acid (DPPA)
Dipalmitoyl phosphatidyl inositol (DPPI),
Dimyristoyl phosphatidyl glycerol (DMPG),

Brain extract (principally PS) (BEx),

Dicetylphosphate (DCP).

c) Positively charged phospholipids

Stearylamine (SA),

Dodecylamine (DDA).

d) The charged test mixtures

SA/DPPS (2:1),

DMPC/SA/DPPS (80:15:5).

Group 3 The materials

a) Conventional

PET ('Melinex', ICI, chemically like Dacron),

PTFE,

Polyester 0.

b) Polymers with the PC group

Diacetylenic phosphatidylcholine (DAPC), and

5 polyesters containing phosphoryl choline,

(Polyesters C, D, E, F, and G).

Preparation of the phospholipids :-

The test piston and cuvette are dip coated with solutions containing 0.5 mg/ml of the lipid and evaporated to dryness in a stream of nitrogen, to drive off the solvent. The choice of solvent is largely determined by the solubility of the material, the coating achieved, and the ease of preparation. Ethanol is the principle solvent but chloroform:methanol mixtures are used for DPPS (9:1) and DPPE (9:1), DMPG (49:1) and DPPI (1:1). 0.5 ml is used for the cuvette since this is the most it can contain. The cuvettes are 13.3 mm high, and 8 mm in diameter, and so have a surface area of 4.35 cm^2. This gives a final deposit on the cuvette of 58 $\mu g/cm^2$. 0.6 ml of the solution is used for the piston, which is coated during rotation in a small glass tube. The part of the piston immersed in the blood is 7.5 mm in height and 6 mm in diameter and so has a surface area of 1.98 cm^2. Assuming that half the coating solution adheres to the piston the final deposit is 76 $\mu g/cm^2$. Plastic disposable cuvettes (Polystyrol 168 N BASF) are used

except for experiments with DPPS, DMPG, DPPI and DPPE when 2VA steel cuvettes are used, because the solvents needed would also dissolve the plastic. Dilutions of DPPC are tested (0.25, 0.125, and 0.05 mg/ml).

The mixture SM/DPPC/DPPE, contains these phospholipids in the same proportion as the outer surface of the RBC membrane ie SM:DPPC:DPPE 44:44:12 (based on Zwaal and Hemker 1982). This coating is prepared in the same way and is referred to as the 'outside RBC mixture'. The mixture DPPC/GM1 is in the ratio of 9:1.

DPPC liposomes are made by dissolving 15 mg of DPPC in a few drops of ethanol and evaporated under a stream of nitrogen to form a thin film on the bottom of a test tube. 3 ml of 1.29% $CaCl_2$ solution is added and vortexed at 60°C for 15 minutes until a colourless solution is obtained. 60 μl of this $CaCl_2$ liposome is mixed with blood as a control experiment to assess the effect of free DPPC on the TEG. A commercial siliconising fluid, Sigmacote, (Sigma Chemicals, code SL4) is used according to the manufacturer's instructions, (essentially dip coating as above), to prepare a non reactive surface. A thicker silicone grease (ICI M494) is also used, and is applied using a cotton bud. These are used in an attempt to provide a baseline for an ideal surface coating.

Preparation of the polymer materials :-
The DAPC is similar to DPPC but has two diacetylene groups in each acyl chain. This group is a triple C-C bond, which is unstable and can be made to polymerise across the acyl chains. This can be achieved by exposure to ultraviolet light to produce a crosslinked polymer (Hayward and Chapman 1984). The dip coating method is used to deposit a film of DAPC on the piston and plastic cuvettes. These are then exposed to UV light source, a Mineralight R54 with a peak output at 254 nm, for 20 minutes to photopolymerise the DAPC. This occurs more rapidly when oxygen is excluded, and this is achieved by using a stream of dry nitrogen. Polymerisation is accompanied by a characteristic change in colour, since the polymer has an orange to reddy brown colour compared to the colourless monomer. In the subsequent text polymerised DAPC refers to DAPC treated with UV irradiation.

A series of polyesters which incorporate the PC group have been made by Dr M Kojima, and Trevor Boyd. These are tested after dip coating them onto plastic cuvettes. Polyester O is the base polyester without any PC, the control, and the others have between 22-40% PC. Polyester G is a complex of polyester and polyurethane with PC.

PET sheet (Melinex, ICI, courtesy of David Morris and chemically similar to Dacron) is dissolved in 1,1,1,3,3,3 hexafluro-2-isopropanol to give a 103 mg/ml solution. This concentration gives a satisfactory solution to coat with, producing a thin even film, on the piston and cuvette. PTFE cannot be dissolved to permit solvent casting, and so had to be machine turned from solid medical grade PTFE (Fibracon Twin LTD).

3.2.4 Results

These are given as geometric mean (with lower and upper 95 % confidence limits). The 'p' value is for a paired 't' test, unless a Wilcoxon rank sign test is more appropriate and this is indicated by #. Degrees of significance are indicated thus :-

< 0.05=*, < 0.01=**, < 0.001=***, < 0.0001=****.

The results are tabulated separately in Appendix 1, with numbering related to this chapter ie as Tables 3.2 to 3.38. see page 223. Summary graphs are shown in Figures 3.4 - 3.9, and typical TEG traces for some of the materials are shown in Figure 3.10, on page 73.

3.2.4.1 Group 1 : the standards and controls

Ten pairs of pins are used. Each pair is checked for differences prior to use, to standardise the pin pairs. In all over 70 sets of standardisation data have been generated, but only two sets are shown in Tables 3.2 and 3.3. There are 2 results which are significant. These are an Mr of 0.890 (0.813-0.975, $p<0.03$), and a Mtma of 0.919 (0.867-0.974, $p<0.02$). These results may have arisen by chance given the 1 in 20 probability that the 0.05 limit represents, and on this basis there should have been 3.5 significant results.

The angle α and the angle A° are compared for the results of standardisation and DPPC on both metal and plastic cuvettes, and for octacosane, DPPC liposomes, and DAPC. This data is presented in Table 3.1.

Material	number	α Vs A°	p	Mα Vs MA°	p
Standard-plastic	10	0.948	<<0.001	0.815	<0.01
Standard-metal	10	0.920	<<0.001	0.630	ns
DPPC-plastic	9	0.978	<<0.001	0.818	<0.01
DPPC-metal	12	0.977	<<0.001	0.912	<<0.001
Octacosane	8	0.992	<<0.001	0.633	ns
DPPC liposome	13	0.901	<<0.001	0.925	<<0.001
DAPC	13	0.953	<<0.001	0.916	<0.001

Table 3.1 Correlation coefficients and p values for the different measures of the angle parameter.

For the Mα and MA° there is close correlation with $r^2>0.8$ and $p<0.01$, except 2 results. These are for standardisation with metal cuvettes and octacosane where r^2 is 0.630 and 0.633 and $p>0.05$. This is not unexpected as both sets of data are closely clustered around the ratio of one. The correlation between the angles α, and A° is very close, $r^2>0.90$ and $p << 0.001$ (note that there are double the number of points for this, as each MTEG has the data from two normal TEGs).

DPPC (Table 3.4 & 3.5) shows a reduction in the activation of both the clotting factors and platelets. This is independent of the type of cuvette (plastic (Table 3.4) and metal cuvettes (Table 3.5)). The effect is better on metal, possibly reflecting the better coating of this surface. Different dilutions of PC are examined and these results are similar, until the 0.05 mg/ml solution (ie 1/10th the film thickness) when the benefit is reduced. See Tables 3.6 to 3.8.

Octacosane (Table 3.9), is a hydrocarbon with a 28 carbon chain without a polar head group. This shows some improvement in Mr, Mk and MA° but not as much as either DPPC, SM, or the silicones.

The liposomes of DPPC show the effect of the DPPC dispersed in the blood, and not on the MTEG's surfaces. These show a slightly increased activation of platelets to produce a marginally stronger clot (Table 3.10). The Mma is 1.09.

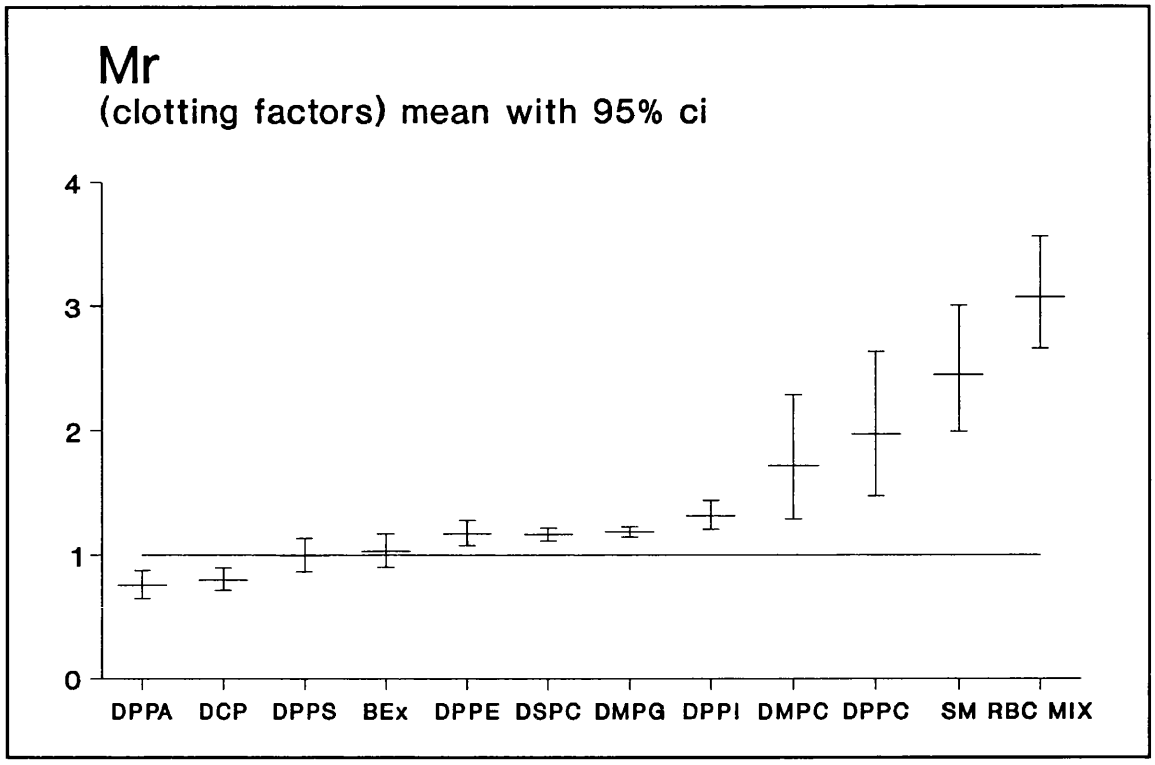


Figure 3.4 Mr values for MTEG of the phospholipids.

Two forms of siliconisation are presented. The commercial product Sigmacote (Table 3.11) shows a small increase in Mr, with a small reduction in Mma, and MA°. These differences are about the same as PTFE, and the middle group of the phospholipids. The silicone grease (ICI M494)(Table 3.12) produces a thicker coating. This gives a further improvement in Mr, and for both the MA° and Mma there is a marked improvement compared to Sigmacote. The difference between them may be explained by a more complete coating with the grease. However DPPC (on either metal or plastic) demonstrates a much greater reduction in thrombogenicity, both in Mr and Mma.

3.2.4.2 Group 2 : the phospholipids

a) PC containing

All the results for the PC phospholipids show high Mr's with low MA°'s and Mma's, indicating low levels of activation of clotting factors, and platelets. They all show a dramatic reduction in thrombogenicity. They vary in their respective positions, but all are less thrombogenic than the other non PC containing phospholipids. DSPC (Table 3.13) appears to be the most thrombogenic, with an Mr of 1.16, MA° of 0.26 and an Mma of 0.13. DMPC (Table 3.14) is about the

same as DPPC for Mr (1.7 and 1.9 respectively). However it does have the least Mma of 0.02, compared to 0.06 for DPPC. The results for SM (Table 3.15) are better than DPPC. The Mr is 2.4 with MA° at 0.03 and Mma at 0.05. These results demonstrate a remarkable lack of activation of platelets (Mma). The majority of these lipids cause 1/10th the activation of the control surface. SM and DMPC are fifty times less thrombogenic for platelet activation.

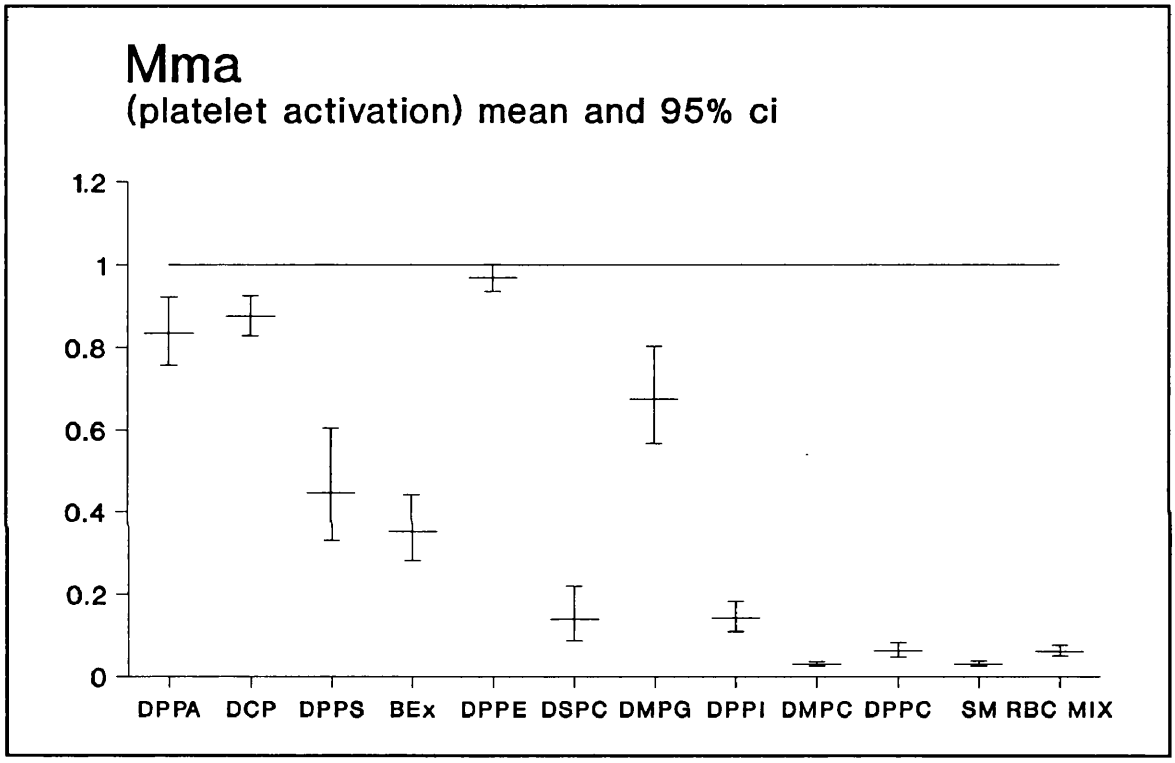


Figure 3.5 Mma results for MTEG of the phospholipids.

The mixture DPPC/GM1 (Table 3.16) incorporates the ganglioside GM1 which is rich in sialic acid residues, which are also found in large amounts attached to the outside of cell membranes. The results are a bit better than DSPC, for both Mr at 1.25 and Mma at 0.08, but not as good as the rest of the PC containing lipids.

The outside RBC lipid mixture, SM/DPPC/DPPE (Table 3.17), which has the same proportion of phospholipids as the outer surface of the RBC, shows a similar low thrombogenicity. It is has the lowest MA° (0.05) and the largest Mr (3.07). These results demonstrate the importance of the PC group in the phospholipids of the outer membrane of RBCs.

b) negatively charged (thrombogenic) phospholipids

The negatively charged (thrombogenic) polar head groups in the RBC membrane are DPPS, DMPG, DPPE, and DPPI. Brain extract is the lipid extracted from ox brains, and principally PS, but contains a wide mixture of acyl chains. DPPA has no PC group, but an acid radical, making it strongly negatively charged. DCP is also negatively charged. These are included for comparison as they are not found in cell membranes.

DPPA (Table 3.18) and DCP (Table 3.19) consistently appear as two of the three most thrombogenic phospholipids. They share this honour with DPPS (Table 3.20) for Mr, DMPG (Table 3.21) for MA°, and with DPPE (Table 3.22) for Mma. Brain extract (Table 3.23) runs closely with DPPS. DPPS (Table 3.20) shows a low Mr (0.99, not statistically different from the control) but this suggests that DPPS activates clotting factors slightly more than the untreated surface. There is evidence of retraction in all the traces which makes the interpretation of the Mk, Mma and Me results less easy, since retraction is due to the blood clot detaching itself from the cuvette and piston (see Figure 3.10). This implies that DPPS activates platelets so strongly as to cause retraction, and this occurs relatively early in the TEG so that the 'full' value of Mma is not reached.

DPPE (Table 3.22) on the other hand shows only a slight difference from the control for the Mma, suggesting it's importance in activation of platelets, since it is the same as the metal surface. The Mr and the MA° are similar to the controls, Sigmacote and octacosane being slightly less than 1.

DMPG (Table 3.21) shows a similar marginal increase in Mr, but both Mma and Me are reduced slightly more, while Mk and MA° are unaffected. This indicates some reduction in activation of platelets. However the unchanged Mk and MA° indicate the latter stages of the clotting cascade from the generation of thromboplastin are unchanged from the plastic surface.

DPPI (Table 3.24) shows a moderate reduction in clotting factor activation (Mr 1.32), which is intermediate between the results for the thrombogenic and PC

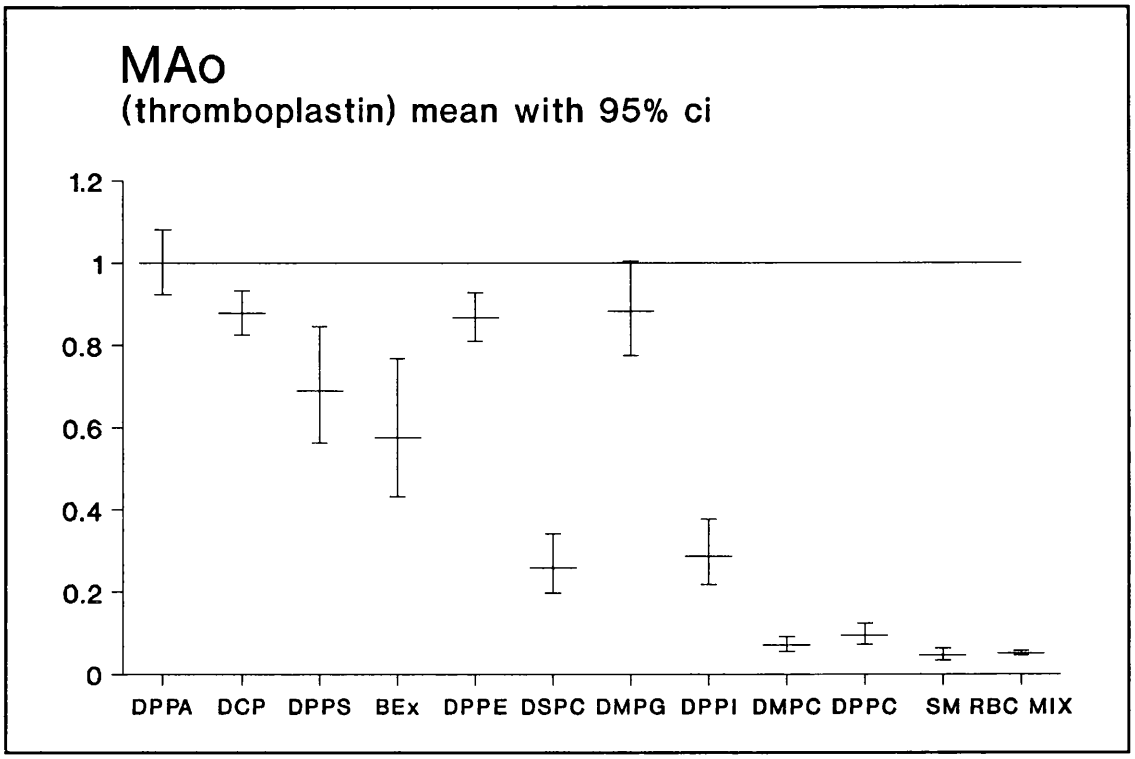


Figure 3.6 MA° results for MTEG of the phospholipids.

containing lipids. The results for platelet activation (Mma 0.14) and for the MA° are as low as the PC lipids.

c) positively charged phospholipids

The two positively charged lipids stearylamine (SA)(Table 3.25) and dodecylamine (DDA)(Table 3.28) are used to investigate the role of phospholipid charge on MTEG. DDA gives a very low Mr, with 4 out 10 traces failing to reach the 1 mm width. The Mr for the 6 finite results is the largest, 5.08. Mma and MA° are also small, 0.033 and 0.014. The results for SA show fair blood compatibility, with an Mr of 1.43, Mma of 0.38 and an MA° of 0.66 (Table 3.25).

d) the charged test mixtures.

The mixture SA/DPPS (2:1) (Table 3.26) has a net positive charge, while containing the negatively charged and thrombogenic DPPS. The results show an improvement compared with the results for both DPPS and SA. Mr is increased to 2.00, whereas for DPPS (Table 3.20) the Mr is one of the lowest seen, at 0.99.

The MA° is reduced to 0.5. The Mma of 0.48, is close to DPPS and SA (Mma 0.38), but there is no evidence of retraction, unlike DPPS (Mma 0.45). For the Mr this mixture is better than DPPC, or DMPC. These results are unexpected, and a further test mixture has been prepared, DMPC/SA/DPPS (80:15:5)(Table 3.27). This has a predominance of DMPC, in case some separation or crystallisation of the two charged lipids, had taken place when SA/DPPS had been used. This type of mixture prevents separation of the charged lipids so they are evenly distributed in the neutral DMPC. The charge of this mixture should be positive due to the excess of SA over DPPS. The results for the mixture DMPC/SA/DPPS show an Mr of 1.56, Mma of 0.13 and an MA° of 0.15. When compared to DMPC alone they represent a small decrease in Mr from 1.72 to 1.56, an increase in MA° from 0.071 to 0.15, and in Mma from 0.029 to 0.13. There is an overall increase in thrombogenicity despite a net positive charge.

3.2.4.3 Group 3 : the materials

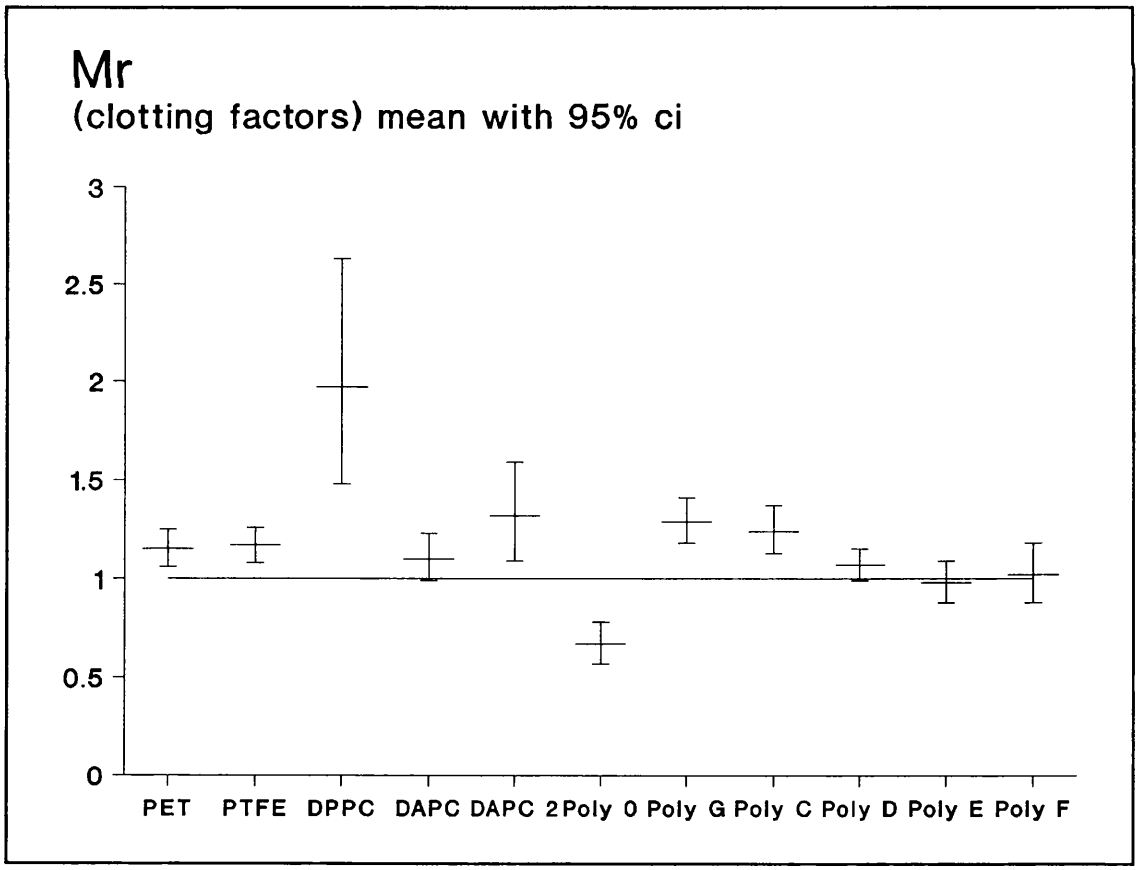


Figure 3.7 Mr for MTEG of the materials.

PET (Melinex, ICI) (Table 3.29) shows an Mr of 1.15, significantly above 1. This

indicates that PET activates clotting factors slightly less than the control surface. However the results for the Mma are just over 1, and show that PET strongly activates platelets.

PTFE (Table 3.30) shows a reduction in platelet activation (Mma 0.5) and an intermediate clotting factor activation (Mr 1.17). It should be noted that PTFE has to be machine turned from the solid for evaluation by MTEG.

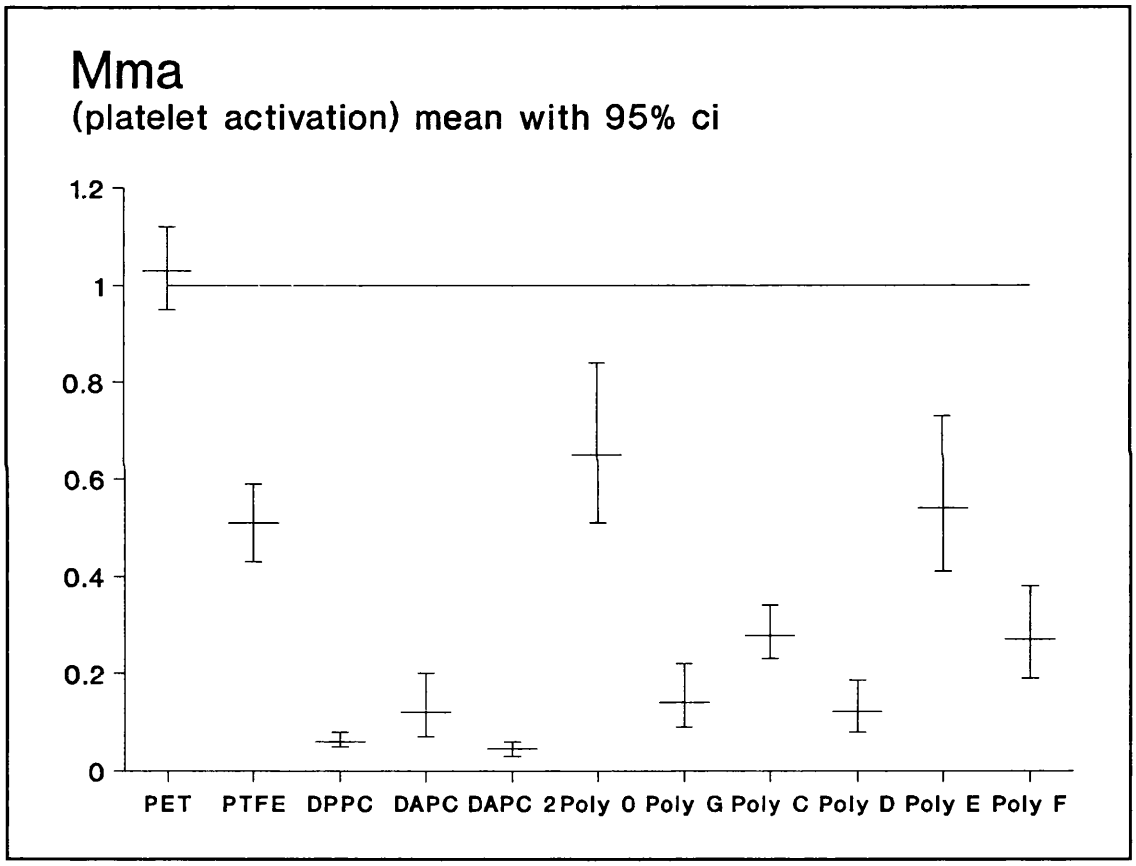


Figure 3.8 Mma for MTEG of the materials.

The results for polymerised DAPC (Table 3.31) are not quite as good as the DPPC results (cf Tables 3.3-3.7). The Mr is 1.1 and the Mma is 0.12. This coating is so stable that measurements have been made 3 or 4 times without recoating, and this may explain the difference from DPPC.

The MTEG for DAPC has been repeated with a new coating for both the pin and cuvette to address the question of stability. This has been prepared from a second batch of DAPC (Table 3.32). The results are Mr 1.33 (1.1), Mma 0.045 (0.12) and

MA° 0.14 (0.32), indicating a small improvement. However the ranges in the confidence limits overlap for Mma, and Mr, and these differences are not statistically significant. Both these sets of the results compare very favourably to PET, for both clotting factor and platelet activation. The Mma value of DAPC is only 25% that of PTFE, reflecting a very much lower activation of platelets.

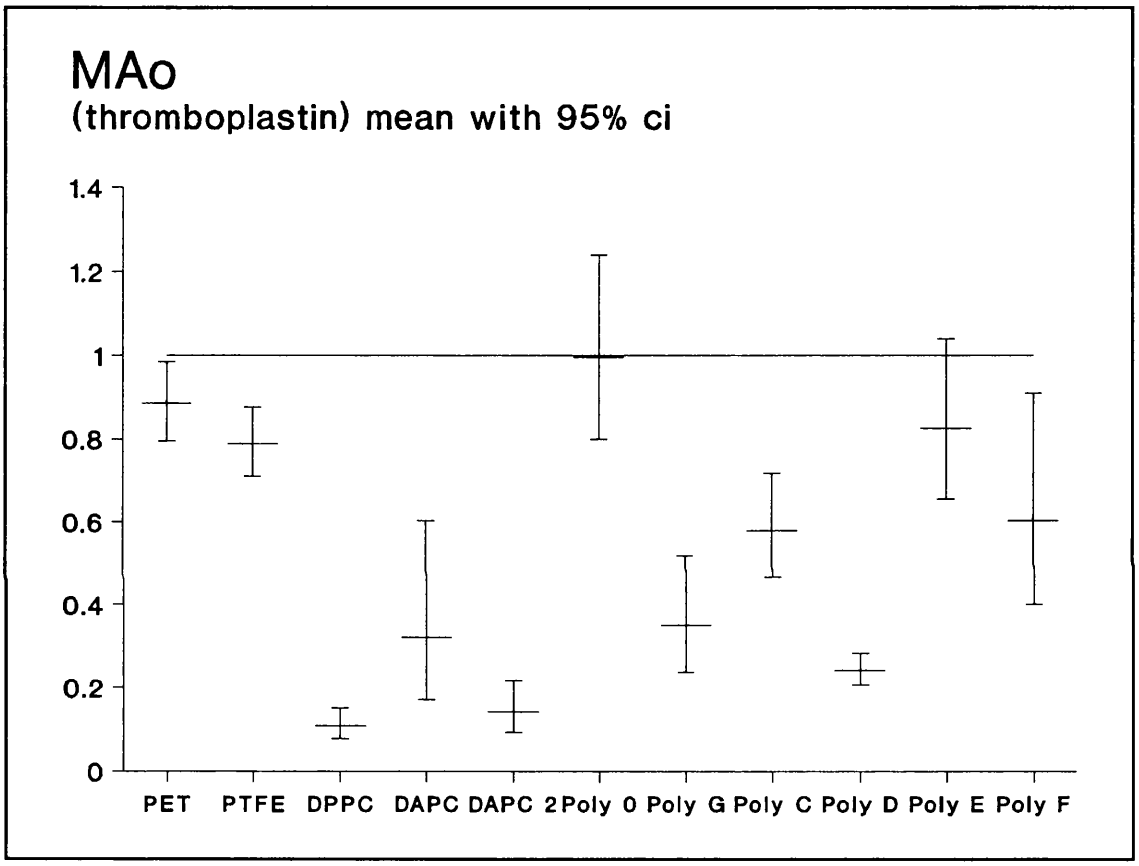


Figure 3.9 MA° for MTEG of the materials.

The polyester polymer made without PC, Polyester 0 (Table 3.33) is a control material for those polyesters with PC incorporated. This shows a significant shortening of the Mr time indicating activation of clotting factors Mr = 0.67, $p<0.0001$. The Mma and Me values are improved showing a reduction in activation of platelets. This may be due to the covering of minor imperfections in the surface of the pins, but this polyester may well be less thrombogenic than steel for platelets. Polyester C (Table 3.34) shows an Mr value of 1.24, and an Mma of 0.27, both a marked improvement on the basic polymer. Polyester D (Table 3.35) shows the least platelet activation (Mma 0.12). Polyester E (Table 3.36) is not as effective, showing only a small improvement in Mma and Me. Polyester F (Table

3.37) lies between these. For the series of polyester C, D, E, and F there does not seem to be a correlation between the % PC incorporated and the results. For example D and E both have 40%, and C and F 22 and 23%. Polyester G is slightly different from this series as it is a polyester-polyurethane mixture. It has the maximum Mr, 1.29 of these materials (Table 3.38). In addition the Mma and Me are in the same range as Polyester D.

3.2.5 Discussion of MTEG results

The dilution experiments produce different thicknesses of DPPC film, since they are all evaporated to a solid on the test surface. These show that even when the film is relatively thin PC remains effective.

The silicones are used to try to mimic a 'perfect' surface. Their action may be more as a lubricant rather than preventing coagulation, that is they prevent the fibrin stands from attaching themselves to the surfaces. They are both much less effective than PC. This makes it unlikely that the good results for PC are due to simple 'lubrication' of the surfaces.

To establish that the PC head group is the important part of DPPC, the hydrocarbon octacosane is used. This shows the effect of the lipophilic hydrocarbon moiety of PC, as it has no charge compared to the zwitterion structure of the PC polar head group. This gives essentially a normal TEG trace. The small effect in reducing the activation of clotting factors can be explained by assuming it covers any minor imperfections or scratches on the pin's surface. More importantly it highlights the importance of the PC head group as the effective part of the phosphatidyl choline molecule, rather than its hydrophobic lipid chain.

The liposomes of DPPC demonstrate the action of the lipid in solution, rather than its effect as a surface coating. This experiment shows no direct action by DPPC on the blood in solution as an anticoagulant. There is evidence of a slight increase in activation of platelets. This may be mediated by protein absorption

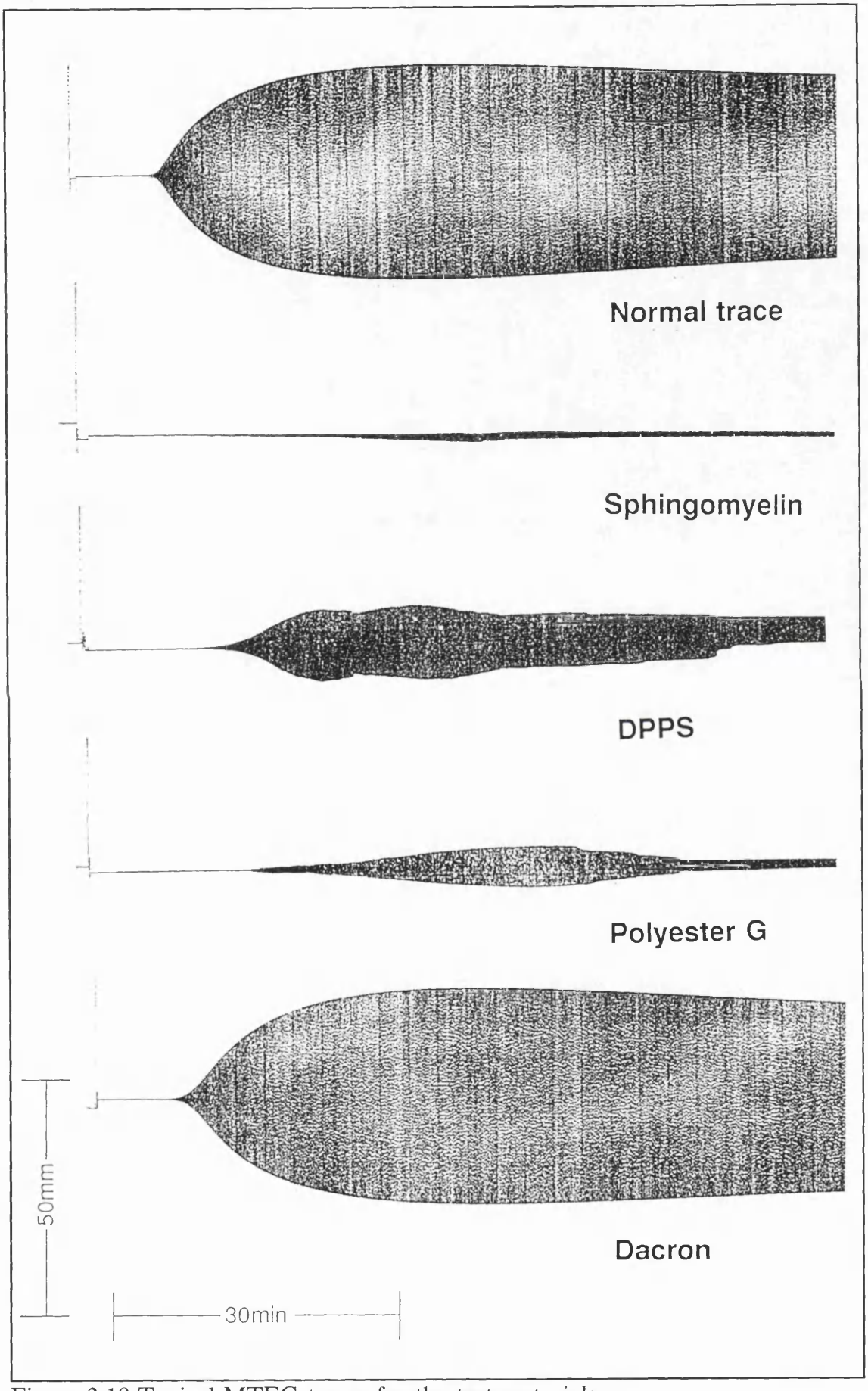


Figure 3.10 Typical MTEG traces for the test materials.

(including coagulation factors) thereby enhancing interaction of the components of the 'tenase' complex (factors X, V, IXa, and VIII) which promotes prothrombin conversion to thrombin. It may also explain the findings of Juliano's group that protein adsorption affects the action of liposomes on the platelet (Juliano et al 1983, and Bonte et al 1987). It demonstrates the sensitivity of MTEG, and confirms that the action of PC is not due to a direct interaction with the blood in solution. This implies that DPPC acts by protection of the surface.

The variety of the polar groups in the RBC are examined and those phospholipids with the PC group all demonstrate a low thrombogenicity by MTEG. The PC group prevents the activation of both clotting factors and platelets on both metal and plastic surfaces. Their relative ranking varies with the parameter studied, the order is shown:-

For Mr = DSPC < DPPC < DMPC < SM < outside mixture.

For Mma = DSPC > outside mixture > DPPC > SM > DMPC.

For MA° = DSPC > DPPC > DMPC > outside mixture > SM.

DSPC is the most thrombogenic and DMPC, SM and the outside mixture are the least thrombogenic.

The transition temperature of these lipids varies. This is the temperature at which they change from a crystalline structure to a gel state for the pure material. Transition temperature is largely determined by the length of the acyl chain supporting the PC group. The order is DMPC (24°C), DPPC (42°C) and DSPC (55°C). SM has a mixture of acyl chains and so has a variable transition temperature from 46 to 57 °C (Boggs 1987). There is a relationship between the transition temperature and the MTEG parameters MA° and Mma:-

	DMPC	DPPC	DSPC
Transition temp °C	24	42	55
MA°	0.07	0.1	0.26
Mma	0.02	0.06	0.13

The pattern is incomplete for the Mr, as the Mr for DMPC is 1.7 and for DPPC is 1.9, but for DSPC it is 1.16. The transition temperature reflects the relative mobility of the phospholipid in a liposome, or in a biological membrane. The tentative relationship between the relative thrombogenicity and this physical property introduces a further factor in the assessment of these materials. The PC

group is clearly a major factor, but it also appears that the physical state of the lipid contributes to the effect. These results suggest that the gel state is less thrombogenic.

The ganglioside GM1 does not reduce the thrombogenicity of DPPC. There are many other groups and activities present at the surface of the RBC membrane. These include a variety of glycoproteins and enzymes. The endothelial cell has other abilities including the secretion of prostaglandins and tPA. However the simplified systems examined here are based on the major phospholipids found in the membranes, and these demonstrate low thrombogenicity.

These results indicate a major reduction in activation of both clotting factors and of platelets. These results strongly support the hypothesis that the PC group is least thrombogenic of the polar groups found in the phospholipids of the membrane.

The results with DPPacid and DCP, both negatively charged clearly demonstrate marked thrombogenicity. These lipids are not found in the RBC membrane, but these results support the association between negative charge and thrombogenicity.

All the traces for DPPS show evidence of retraction and this makes the interpretation of the Mk, Mma and Me results less easy. Retraction is due to the blood clot detaching itself from the cuvette or piston. Similar changes are seen in the clinical conditions of increased thrombosis associated with malignancy, particularly some leukaemias, and is due to the forces that attach the fibrin strands in the clot to the surfaces of the TEG being overcome by the force of blood clot retraction (Marchal et al 1961). This has been attributed to the platelet activation, although the leukocytes may also play a part (Loeliger et al 1963).

PS is known to be thrombogenic in other test systems, and is used to maximise clotting factor activation in the PT test. DPPS appears to activate platelets so strongly as to cause hyper-retraction, rather than reduced thrombogenicity as the results initially suggest. The Mr results for DPPS show it has a strong tendency to promote the activation of clotting factors above that caused by the metal surface

of the TEG. This is unequivocal evidence for the marked thrombogenicity of DPPS.

The significance in haemostatic terms of the asymmetric distribution of phospholipids in both the RBC and the platelet membrane has been reviewed (Zwaal & Bevers 1983 and 1986). PS has a role in the control of normal and pathological thrombosis. Bevers et al 1983 confirmed that PS undergoes a transmembrane relocation as a result of platelet activation and is exposed on the outer surface. This alone increases the rate of thrombin generation which is quantitatively related to the amount of PS exposed in the membrane.

The role of PS in pathological thrombosis is highlighted by the abnormalities in Bernard-Soulier platelet disease. This is a rare autosomal recessive condition associated with a prolonged bleeding time and moderate thrombocytopenia. The platelets are large and fail to interact with the sub-endothelium in the presence of von Willebrand factor. This failure of aggregation is due to lack of glycoprotein 1b, the platelet receptor for von Willebrand factor. The platelets have a short life span which accounts for the low platelet count (Hardisty & Caen 1987). Perret et al 1983 demonstrated that platelets from these patients have higher levels of PS (three times the normal level) and PE (twice normal) in the outer membrane. The PS and PE also displace SM and PC. The exposed PS and its thrombogenic character may explain the shortened platelet survival.

Zwaal and Hemker (1982) reviewed the role of blood cell membranes in haemostasis and again documented the procoagulant role of PS. In sickle cell disease the erythrocytes lose their normal biconcave disc shape and become rigid, leading to microvascular occlusion. Although this has been attributed to RBC sludging there is an increase in thrombogenicity of the reduced form of sickle RBC. Lubin et al 1981 demonstrated abnormally high levels of PE and PS in the outer membrane of sickled RBCs. Chiu et al 1981 demonstrated accelerated clotting of the Stypven test by both sickled RBCs and liposomes with the same phospholipid composition (ie without any of the proteins associated with the RBCs).

The experiments presented above support the importance of the asymmetric distribution of phospholipids in the RBC and platelet in maintaining normal haemostasis. The non-thrombogenic lipids with the PC group are predominately exposed on the outer surface. The movement of PS and PE (the thrombogenic phospholipids) to the surface stimulates coagulation.

The results with DMPG and DPPE show the same degree of activation as the control surfaces, with DMPG acting via thromboplastin, and DPPE via platelets. The results with the anionic polar groups show increasing thrombogenicity in the order DPPI < DMPG < DPPE < DPPS, for Mr, and for Mma (if it is accepted that retraction occurs for DPPS). The order for MA° is slightly different, DPPI > DPPE > DMPG > DPPS. This order is similar to that noted by Rosing (PI< PG < PA <PS). The MTEG results demonstrate the thrombogenicity of DPPS is greater than the other anionic phospholipids present in the RBC membrane. There is variation in their thrombogenicity, but all carry the same negative charge.

The two positively charged lipids, DDA, and SA are used to show the effect of positively charged lipids by MTEG. Although they are not found in the RBC membrane, they are included since they are used to change the net charge of the test mixtures. DDA gives unexpectedly good results indicating low thrombogenicity. However there is one alternative explanation, that fibrinolysis is occurring, for there is no evidence of retraction.

Until recently it was accepted that the net negative charge on the membrane was the important factor in the activation of coagulation (Bangham, 1961; Papahadjopoulos et al., 1962), but Rosing has challenged this view (Rosing et al., 1988). By adding increasing amounts of stearylamine (positively charged) to vesicles of PS (negatively charged) they varied the overall surface charge on the vesicles from negative to positive, and found this variation in charge made no difference to coagulation measured by aPTT and Stypven tests. They used other anionic phospholipids and showed PS was the most thrombogenic, and its concentration was far more important than any effect due to the charge on the vesicle.

The 2:1 mixture of SA/DPPS has a net positive charge. If Rosing's assertion is correct this mixture should demonstrate moderate thrombogenicity, since it contains DPPS. However the Mr for the mixture (SA/DPPS) is better than DPPC, or DMPC or for SA (which rises from 0.99 for DPPS and 1.43 for SA to 2.00 for them both). The addition of DPPS to SA actually causes a major improvement compared with the results of either individual lipid. This is difficult to explain, unless there was some separation or sequestration of the opposite charges or crystallisation of the two lipids in this mixture. Zwaal's review in 1978 emphasises the complexity of interactions that occur with mixtures of lipids, where small differences in the lipid ratios, and even lipid chains lengths influence the results dramatically.

The results for the mixture DMPC/SA/DPPS (80:15:5) are presented in an attempt to address these possibilities, since it has a predominance of DMPC. This type of mixture should prevent separation of the charged lipids since they are evenly distributed in the neutral DMPC. The charge of this mixture should be positive due to the excess of SA over DPPS. The addition of SA and DPPS changes the results compared to DMPC. They show a small decrease of Mr from 1.72 to 1.56, an increase in MA° from 0.071 to 0.15, and an increase in Mma from 0.029 to 0.13. These all represent a slight trend towards thrombogenicity compared with DMPC, with a positively charged mixture containing PS. The degree of deterioration in haemocompatibility is not marked, but the proportion of DPPS is small compared to the inside mixture which has 30%. This increase in thrombogenicity would be expected if DPPS is the key factor, rather than it's negative charge alone.

These results for the negatively charged phospholipids tend to support Rosing's contention that the negative charge alone is not the key attribute in causing activation of coagulation. They also imply that it is the exact nature of the polar group which may be the key factor in determining the response of clotting processes to these lipids. In contrast to the negatively charged lipids and their thrombogenicity it is clear that the PC group in both the phospho- and sphingo-lipids is responsible for the non thrombogenic properties of these membranes.

The surface left by solvent casting of PET is different from the graft material Dacron. This is also true for the machine turning of the PTFE parts for MTEG. However both these results are in accord with the clinical observations for the thrombogenicity of Dacron and PTFE arterial grafts. DAPC when used as a coating shows a similar reduction of coagulation as PC and this coating is sufficiently stable to repeat the TEG's without recoating, despite the detergent washing. This is confirmed by the second set of data for DAPC.

The polyesters are stable, and after an initial coating remained on the pin. There is a dramatic improvement in one or other parameter for all the polyesters with PC incorporated compared to Polyester 0. For Polyester G the Mr is increased 193%, and for Mma reduced by 79%. The results vary between these materials, but not in a systematic way related to the amount of PC in them. The best MTEG results in this group are for Polyester G. These materials possess the necessary properties for use as surface coating agents, and their chemical structure can be manipulated to give polymers with a more conventional rigid structure.

Both DAPC and Polyester G have reduced thrombogenicity compared with the conventional materials. When compared to Dacron the Mr for DAPC is prolonged by 14%, and for Polyester G 12% (Dacron 1.152, DAPC 1.317, Poly G 1.291). The platelet activation, measured by Mma, is reduced by 91% for DAPC compared to PTFE (0.045 and 0.505 respectively). Polyester G has 27.5% the Mma of PTFE.

3.3 Parallel plate test chamber

3.3.1 Introduction

The parallel plate test chamber is a simple cell composed of two flat surfaces of the test material, separated by a seal between them to form a chamber. This type of chamber can be used to test a wide range of materials provided they are flat, or can be coated on a flat surface. In addition the technique allows the investigator to vary the evaluation by choosing what the test surface is exposed to, for example

whole blood, plasma or a component of either. These alternatives permit detailed and systematic investigation into a wide range of different interactions with the test material.

In this study the interaction of whole human blood at 37°C is monitored by following the generation of fibrinopeptide A (FPA). Fibrinopeptide A is a small peptide released from fibrinogen when it is activated by thrombin. The coagulation cascade generates thrombin, which cleaves fibrinogen to form the fibrin monomers, and these aggregate to form blood clots. All the various interactions between the pro- and anti- thrombotic processes, effected by the regulatory proteins in the cascade as well as the interaction of blood with the material are reflected in the formation of thrombin. This is therefore the key enzyme in the generation of blood coagulation. This enzyme specifically cleaves the N terminal end of the A α chains of fibrinogen to form fibrinopeptide A (FPA). The assay of FPA provides a sensitive and specific marker for thrombin generation. (Nossel et al 1974, Woodhams and Kernoff 1981).

Woodhams (personal communication, 1989) discussed the design of similar, but unpublished experiments and results he obtained in 1981, with his FPA assay (Woodhams and Kernoff 1981). He pointed out the explosive nature of FPA generation, once thrombin is formed, and the difficulty of using absolute values of FPA. This is confirmed by the work of Bowry (et al 1984). The assay therefore gives a very sensitive index with which to compare materials.

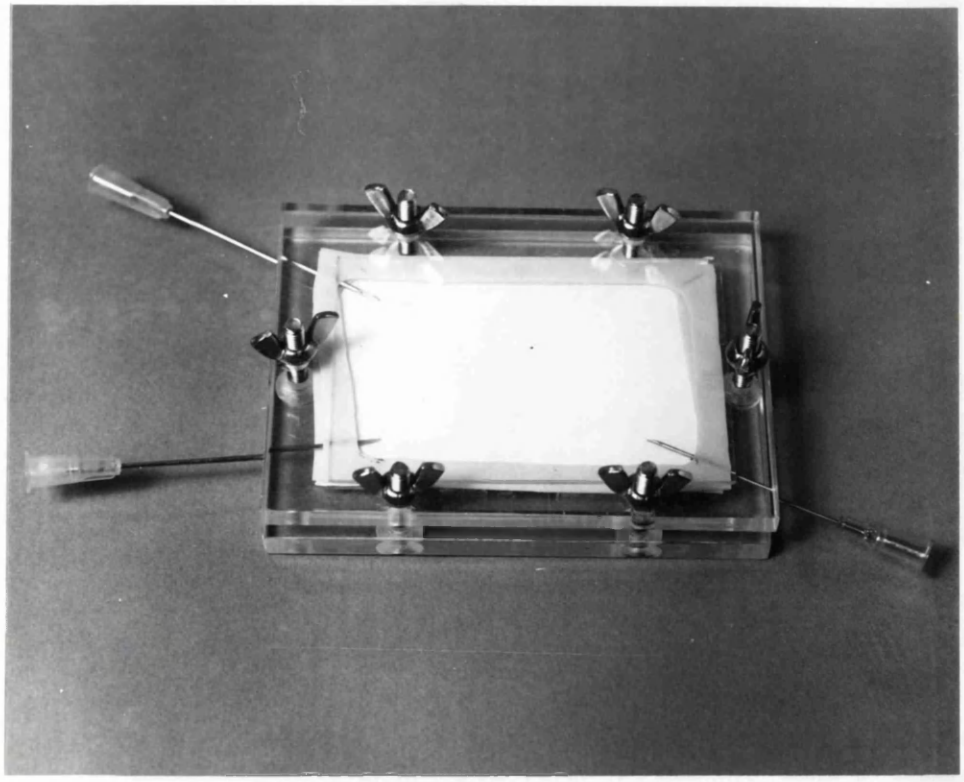
3.3.2 Design and methods

A number of test chambers have been used following the initial design based on microscope slides of Lyman (et al 1970), for example Mason (et al 1974). The design of the test chamber used in this study is based on the larger system of Bowry (et al 1984), but modified to increase the ratio of surface area to blood volume. 2" by 3" glass microscope slides are a convenient basis for the cell. These are used in pairs, and separated by an 'O' ring gasket, thus enclosing a pre-determined area and volume between the two glass surfaces. Medical grade

silicone rubber sheet 0.5 mm thickness is used, with an outer dimension of 52 mm x 76 mm (ie 2"x3") and the inner 'window' dimension of 44 mm x 68 mm. This gives an area of 29.92 cm^2, which is approximately 60 cm^2 for the chamber, and gives a volume close to 3 ml. This arrangement gives 0.05 ml blood per square cm, compared to 0.22 for Bowry (et al 1984).

The test surfaces to be examined are glass slides, Dacron, PTFE and Polyethylene. Silicone treatment, and coatings of DPPC on glass are used, in addition to a coating of DAPC on Dacron. For the sheet materials the slides are used as carriers, by using double sided tape to secure the test material to the slide. The glass test surfaces are washed in chromic acid, distilled water, and dried. The other materials are coated by dipping the clean glass slide in solutions and allowing them to dry. The Dacron sheets used were either Melinex sheet (125 μm thick, ICI Films, Wilton, Middlesborough, courtesy of David Morris) or Woven Dacron grafts (USCI, courtesy of Chris Devereux). In addition PTFE tape and polyethylene film are also used.

The slides are assembled with a 21 g needle in one corner, and a 19 g needle in



Photograph 3.1 The parallel plate test chamber.

both the opposite corners, between the gaskets. The whole is then placed in a jig to secure the test chamber, see Photograph 3.1. The 19 g needles are used for injecting the blood and taking the sample, the 21 g needle acting as an air vent. 4 identical chambers are prepared for each material. They are incubated for 24 hours or more at 37°C in a constant temperature laboratory before use.

The test is carried out in the 37°C room, but the blood samples from the test chambers are placed in ice immediately after their collection from the chamber. Blood is taken from healthy volunteers with a 19 g butterfly needle (Abbott Venisystems, No 4590) without a tourniquet. The first 12.5 ml is discarded, as Woodhams showed this blood contains 20-30% more than the basal FPA levels (Woodhams and Kernoff 1981), and a further 60 ml collected into a separate syringe. This is used to fill the chambers, and to give a standard citrated blood sample. The chambers are sampled by removing blood from the 'clean' 19 g needle into a chilled 2 ml syringe primed with the anticoagulant. The sample times are 0, 3, 6, 9 minutes after filling. The citrate blood sample is used to provide two control samples, one at the start of the experiment, and one at the end. Thrombin released by the venepuncture continues to generate FPA, although further thrombin generation itself is inhibited by the citrate. These samples identify falsely high FPA levels as a result of a poor venipuncture (Woodhams and Kernoff 1981).

A commercial radioimmunoassay for FPA is used (Byk Sangtec Diagnostica, RIA-mat FPA, Dietzenbach). This is based on the assay of Nossel (et al 1974), and is supplied with its own anticoagulant. Normally heparin and aprotinin (Trasylol, Bayer) are used, the former to inhibit clotting, and the latter to prevent degradation of FPA, fibrin and fibrinogen by plasmin and other proteases. The antibody to FPA cross reacts with fibrinogen and this is removed by adsorption with Bentonite.

The assay is carried out according to the manufacturer's instructions. The samples are prepared immediately by centrifugation at 4°C at 2000 G for 30 minutes. The plasma is separated from the blood and Bentonite (500 μl) added to 1 ml of plasma, to adsorb fibrinogen. This is vigorously vortexed, before pelleting by

centrifugation at 4°C at 2000 G for 10 minutes. The supernatant is separated and for the later time points diluted 10, 100 and 1000 fold. This is to improve the accuracy of the estimation, since these samples often have very high levels of FPA. They are usually assayed immediately, but occasionally stored at -20°C first. The final samples are counted using the same equipment and setting as the blood samples in chapter 6, see section 6.3.3.4, page 161. The FPA levels are calculated from the counts, using the data for analysis supplied with the kit.

3.3.3 Analysis

Since the generation of FPA by biomaterials is rapid, Bowry (et al 1984) chose to show the results graphically, with one curve over 9 minutes. They used the FPA assay to determine the timing of their blood samples for the slower release of ß thromboglobulin from platelets. Dr Woodhams suggested using graphs of concentration against time to define the time taken to reach an arbitrary level of 5 pmol. This level is 2-5 times the level of FPA measured in normal subjects. The early part of the curve is used, before the exponential part of the slope is reached, allowing good discrimination between the materials. This has the added advantage over a single time point estimation, where an error of a few seconds in the sampling time could seriously affect the result. It is for this reason that the samples are taken at the time points of 0, 3, 6, and 9 minutes. In addition a control surface of glass, or the uncoated material are included to allow direct comparison for each 'run' of the test to give, essentially, a paired estimation.

3.3.4 Results

These are shown as graphs and because of the range of the results the graphs use the LOG concentrations of FPA. The results of each run of the test are on a separate graph, Figures 3.11 to 3.16.

Run 1, Figure 3.11.
This shows the effect of siliconisation of glass. The control samples are in the normal range. The untreated glass takes 1.78 minutes to reach the 5 pmol level.

Silicone glass does not reach this level by 3 minutes, and all the subsequent samples are over the range of the standard curve for the assay. The 3 minute values are 11.5 and 4 ng/ml respectively. After this all the subsequent runs of the test included dilutions of the 3, 6, and 9 minutes samples times.

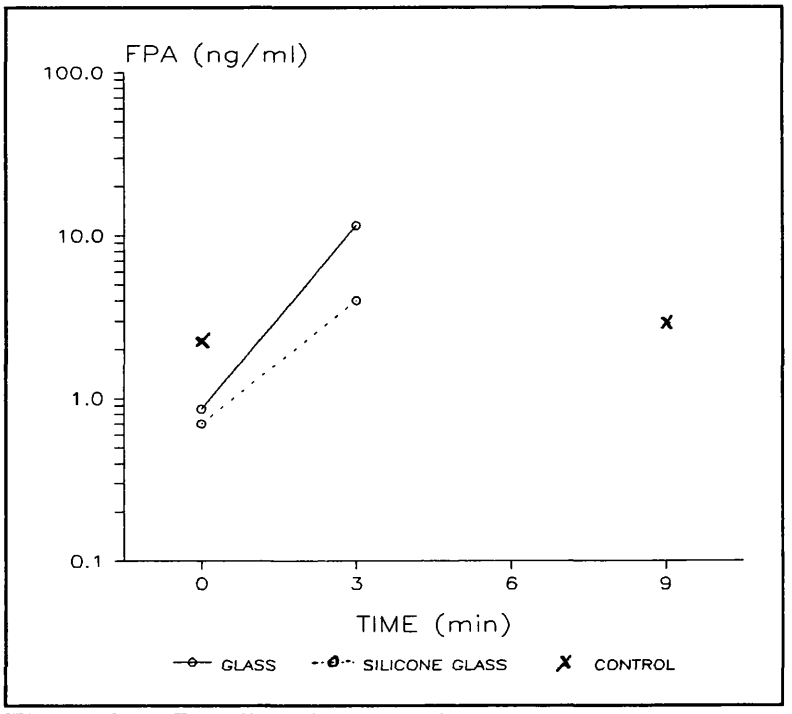


Figure 3.11 Parallel plate test 1.

Run 2, Figure 3.12. This compares Dacron with glass, and despite using dilutions some of the glass samples are still out of the assay range. In this assay the control samples are slightly above the normal range, at 3.6 rising to 3.7 ng/ml. The first glass sample is high at 6.6 ng/ml, suggesting activation during the filling of the chamber. The 5 pmol time of Dacron is 3.1 minutes.

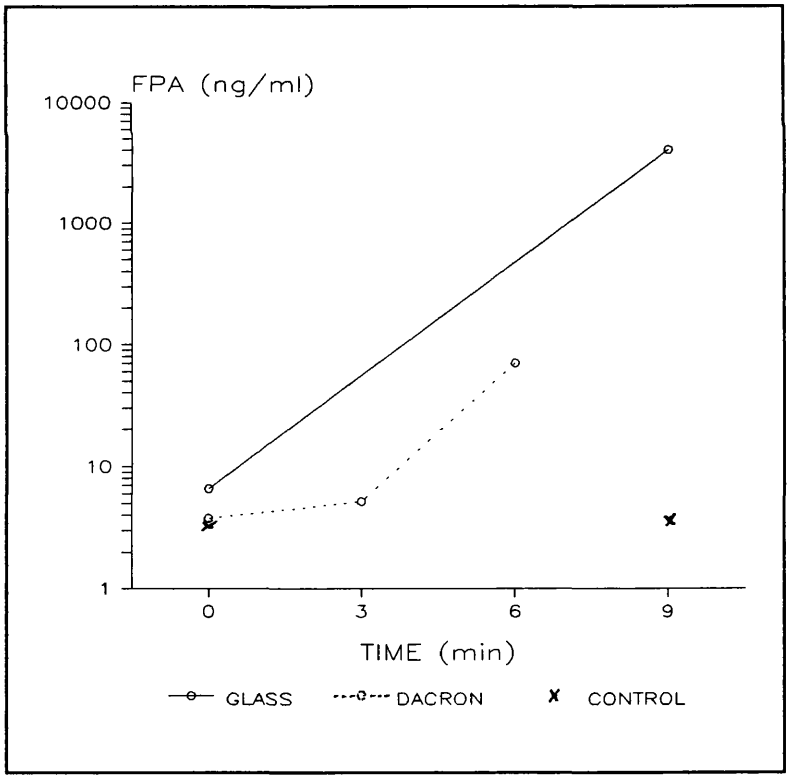


Figure 3.12 Parallel plate test 2.

Run 3, Figure 3.13. This run compares glass and PTFE, and the initial values are close to the controls. These are 1.9, 2.8, and 1.9 ng/ml respectively. The log plot

overemphasises the differences of the zero time values of the FPA, for PTFE it is 2.8 ng/ml, and although it is initially higher than the value for glass, this value is still in the normal range for basal FPA levels. The 5 pmol times are 0.4 and 0.46 minutes for glass and PTFE. These are some what misleading in suggesting glass is better than PTFE. The 3 minute values are 40 ng/ml for glass and 30 ng/ml for PTFE, closer to the expected pattern for these two materials. At the later times glass is clearly more thrombogenic than PTFE.

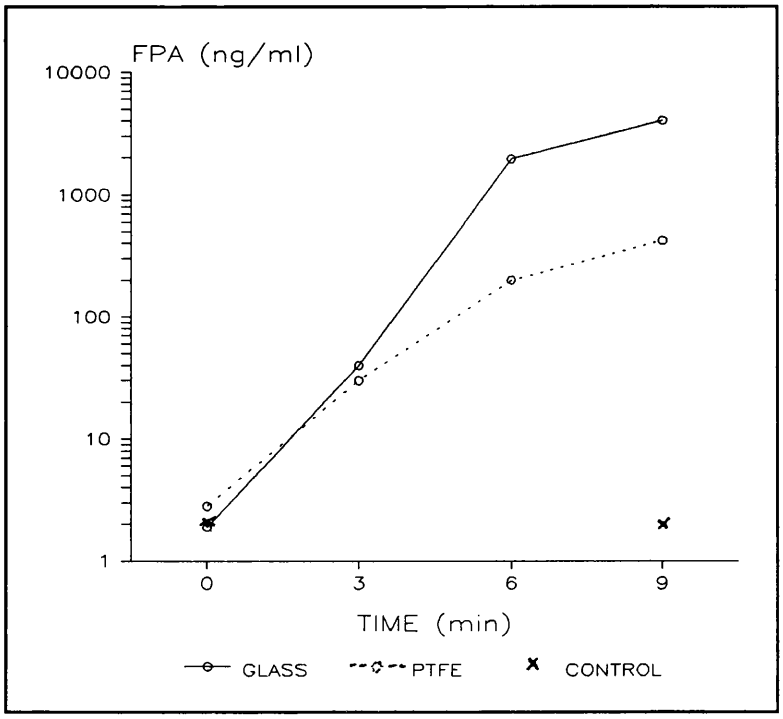


Figure 3.13 Parallel plate test 3.

Run 4, Figure 3.14. This shows the effect of coating the glass surface with DPPC. In this run the citrate controls both have abnormally high levels of FPA, well over 40 ng/ml, although the zero time samples for both the test samples are both under 10 ng/ml.

This suggests that the blood in the chambers was less activated than

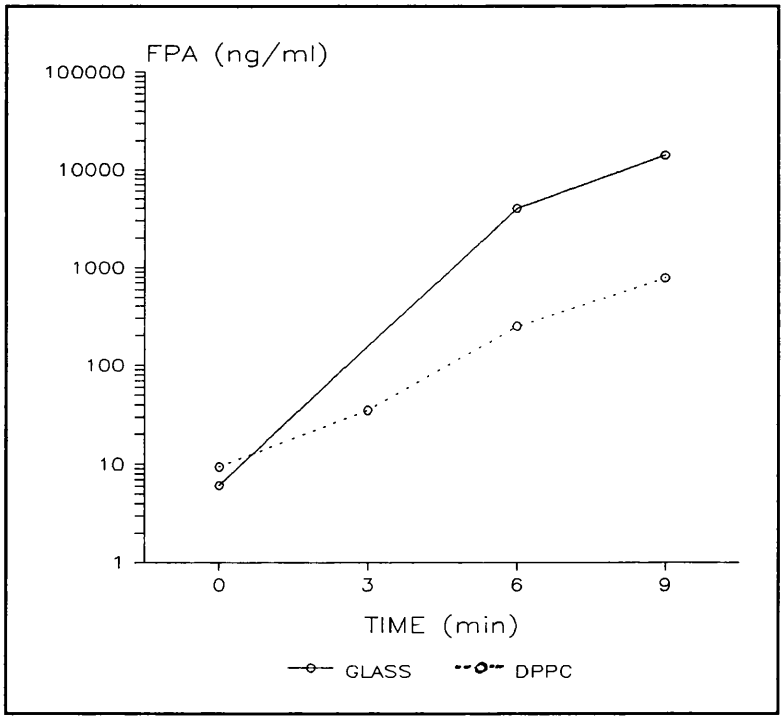


Figure 3.14 Parallel plate test 4.

the controls. However these values for the materials at time zero, are close to the 5 pmol level (7.7 ng/ml). It is difficult to assess the significance of the 5 pmol time, in this run of the test. The 3 minute value for glass is over 400, but for the DPPC coated glass is 35 ng/ml. DPPC causes is a marked reduction compared with untreated glass at the later times of 6 and 9 minutes. Despite the likelihood of an abnormally high FPA in the blood used in this run, DPPC appears to be less thrombogenic than glass.

Run 5, Figure 3.15.

This compares the conventional materials of Dacron, PTFE, and polyethylene (PE in fig 3.15). The control samples are low, indicating a good venepuncture. The 5 pmol times are Dacron 0.85, PTFE 3.6 and polyethylene 4.8 minutes. These results demonstrate the strength of the analysis using the time to reach the 5 pmol level, compared to using isolated time points. The overall shape of the curves is more informative still, since polyethylene appears to be less thrombogenic than PTFE at 6 and 9 minutes.

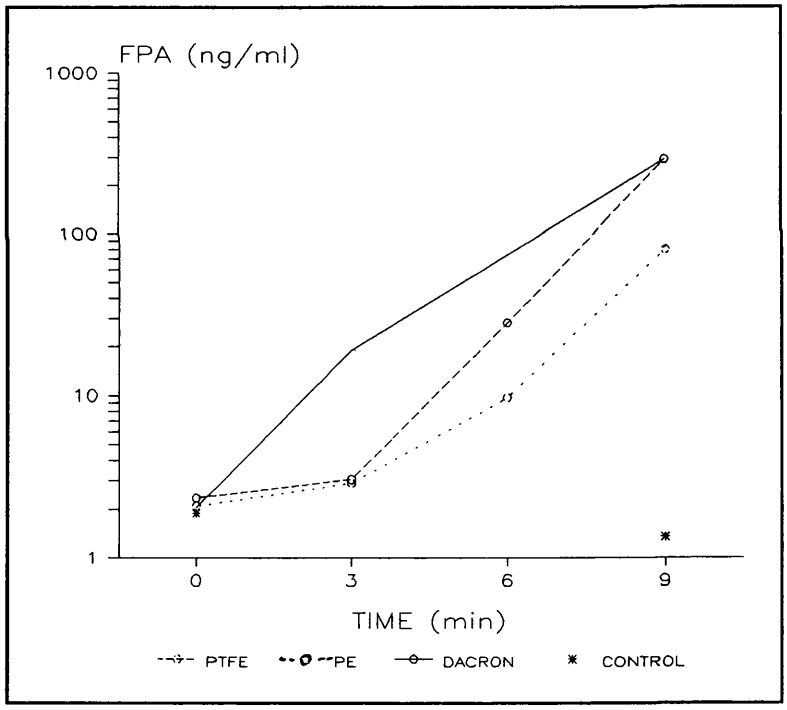


Figure 3.15 Parallel plate test 5.

Run 6, Figure 3.16. Dacron arterial graft material is used with a coating of DAPC and is compared to the native graft material. The porous nature of the graft's weave allows blood to pass through the material. This means that the samples for the later time points were lost. Nonetheless the early samples show how thrombogenic graft materials are in comparison to smooth surfaces (see run 5, Figure 3.15).

The DAPC coating seems to have a demonstrable effect in delaying FPA generation. The 5 pmol levels can only be estimated and these are under 1/2 second for Dacron graft, extended to 9 seconds with the DAPC coating.

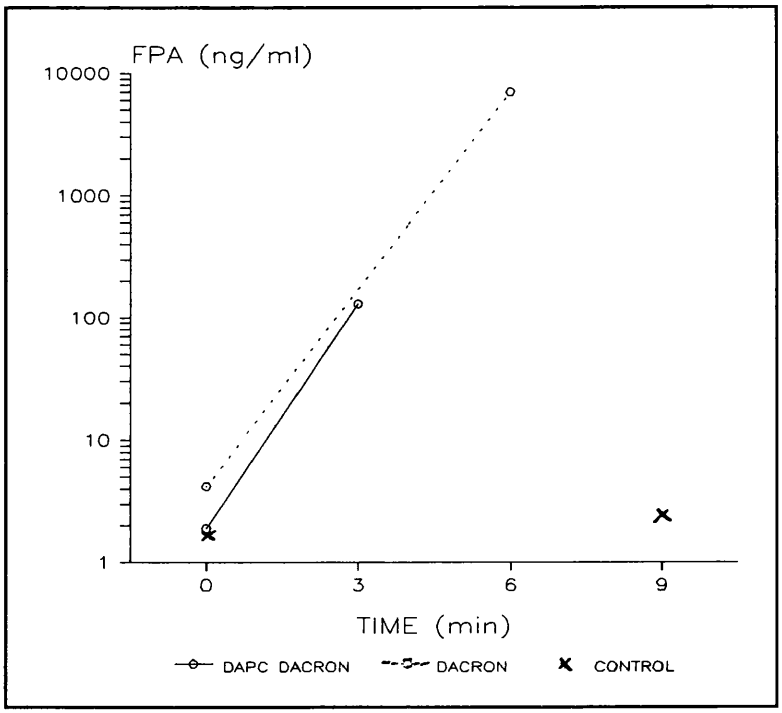


Figure 3.16 Parallel plate test 6.

3.3.5 Discussion

The overall estimates of the 5 pmol level for the materials are very variable. For glass, times of 1.73 and 0.6 minutes are seen, but in two other runs it is less than a few seconds. For PTFE the times are 4.8, and 0.46 minutes. However for the Dacron sheet it is more consistent with times of 3.1 and 3.6 minutes. This may be because the sheet is less prone to wrinkling, when warmed to 37°C. Marked changes in the dimensions of the cells of the two film materials (PTFE and PE) were noted before the double sided tape was introduced (these results are not included in the analysis).

These results do not allow ranking of the materials, except where there is a direct comparison in the test run. The results for run 1 (glass with and without silicone), run 2 (glass and Dacron), and run 5 (PE, PTFE, Dacron) show the expected rankings of the respective materials. The DPPC and DAPC coatings (runs 4 and 6) both show a reduction in FPA generation, supporting the evidence for their relative lack of thrombogenicity.

The analysis based on the arbitrary 5 pmol level is inappropriate when the basal FPA levels are high. Alternative methods of analysis such as the time taken for the FPA level to increase by an arbitrary amount might be better a measure to use. Bowry (et al 1984) did not attempt to characterise their materials using FPA

generation. In their study FPA was used only to delineate the time course of thrombin generation, from the coagulation cascade. They did this in order to determine the best time for sampling ßTG production from the platelet interaction with the material (since thrombin also mediates ßTG release and platelet aggregation via a receptor on the platelet). They showed the explosive nature of FPA generation after 7-8 minutes, once large amounts thrombin have been generated.

Clearly further work is needed to optimise this test and its analysis. The initial blood collection must be achieved with as little activation as possible, as the results for run 4 show. The rest of the test must be conducted with extreme care, as FPA generation is so rapid. An increase of the ratio of blood to the surface area should be investigated to reduce the sensitivity of the test cell to these effects. However these results show that DPPC on glass and to a smaller extent DAPC on Dacron, both reduce the rate of FPA generation.

The next section deals with another approach to measure the thrombogenicity of the phospholipids. This is by direct incubation of plasma with liposomes of the phospholipids. The rate of thrombin generation is used to do this, with an attempt to identify the individual clotting factors involved in the interaction between plasma and the phospholipid materials.

3.4 The liposome uptake of clotting factors

3.4.1 Introduction

These studies are designed to assess the involvement of coagulation factors with the phospholipids. They attempt to analyse why the MTEG results for the different polar head groups present in the RBC membrane are so different in terms of their thrombogenicity. Phospholipids have a polar head group and a hydrophobic hydrocarbon lipid chain, and they tend to form bilayer structures spontaneously in water. This occurs as a result of thermodynamic forces, with the hydrocarbon chains together, leaving the polar groups orientated to the aqueous environment. The lipid bilayers form tiny vesicles, called liposomes, less than 50

nm in size. Those with a single bilayer are called unilamellar liposomes (or loosely 'vesicles'), and those with multiple layers are called multilamellar liposomes (Zwaal 1978).

Liposomes have a vast surface area for their volume as they are mono-molecular layers, and nearly 50% of the phospholipid is exposed to the suspension medium. They mimic RBC membranes and are a convenient way of examining plasma protein interactions with phospholipids (for example, Zwaal et al 1977, Juliano et al 1983, Bonte et al 1987, Rosing et al 1988).

The experiments described here use similar liposomes to the workers quoted above. The phospholipids are prepared as liposomes and exposed to the plasma to investigate the adsorption or binding of clotting factors on their surfaces. However the analysis has been modified by the author and Dr R Hutton (Dept of Haemophilia, RFHMS), to examine both the factor depletion from the plasma, and correction of the isolated clotting factor deficient plasmas. The details of the preparation and assays are in appendix 2, see page 236, but an outline is presented in the next section, and follows a description of the materials evaluated.

3.4.2 Materials and experimental details

The materials under investigation are :-

Group 1 The PC containing phospholipids.

These are the phospholipids DPPC, DMPC and SM, and the two mixtures, the 'outside RBC mixture' and DMPC/GM1. DMPC is very similar to DPPC, but has a lower transition temperature 24 and 42 °C respectively (Boggs 1987). The RBC mixture is the same as used for MTEG (namely DPPC:SM:DPPE , 44:44:12). DMPC/GM1 (9:1) is a mixture of DMPC and the ganglioside GM1, which contains sialic acid residues. This is also present on the outer RBC membrane.

Group 2 The thrombogenic phospholipids.

These are DPPS, DPPI, DPPE, DPPA, and the 'inside RBC mixture'. The latter is DPPC:SM:DPPE:DPPS, in the proportion 15:10:47:28, as reported by Zwaal et al 1977.

Group 3 The materials.

These are glass, kaolin and DAPC. The glass, with its known thrombogenic properties, is used as small beads as an approximate equivalent to liposomes. DAPC is assessed in 4 different forms of liposome. These are prepared in two ways, to produce ordinary multilamellar liposomes, and also extruded to produce very small unilamellar liposomes. These two preparations are used with and without polymerisation, see section 3.2.3.3 above. These are annotated as 'ex' for extruded, and 'P' for polymerized in the figures below.

The phospholipids are prepared as liposomes, and incubated with human citrated plasma (collected and pooled from a group of healthy donors) for 1 hour at 37°C. At the end of this period of incubation the liposomes are pelleted by centrifugation. The supernatant plasma is tested in the standard PT and aPTT tests. The liposomes in the pellet are retained.

The liposomes in the pellet are washed in Tris buffer, by resuspension and centrifugation three times before finally resuspending them in buffer. The suspension is added to a plasma with a specific and isolated clotting factor deficiency. This plasma is then assayed to detect small amounts of the factor bound to the liposome. The tests used are the aPTT for the factor XII, IX, and VIII assays, and the PT for the factors VII, V, and X.

3.4.3 Assay systems and interpretation of results

The activated partial thromboplastin time (aPTT) and prothrombin time (PT) tests are outlined here because the details of the way they are conducted are important to the interpretation of the results. The blood coagulation pathway is shown in Figure 3.17.

In the aPTT test citrated plasma is incubated in glass tubes with kaolin for 9 minutes at 37°C to maximally activate factors XII and XI, producing a high level of activated XI (XIa). At this point a phospholipid is added to provide a standardized platelet substitute, the mixture is recalcified, and the time taken for it to coagulate is noted. The test therefore assesses the competence of factors IX,

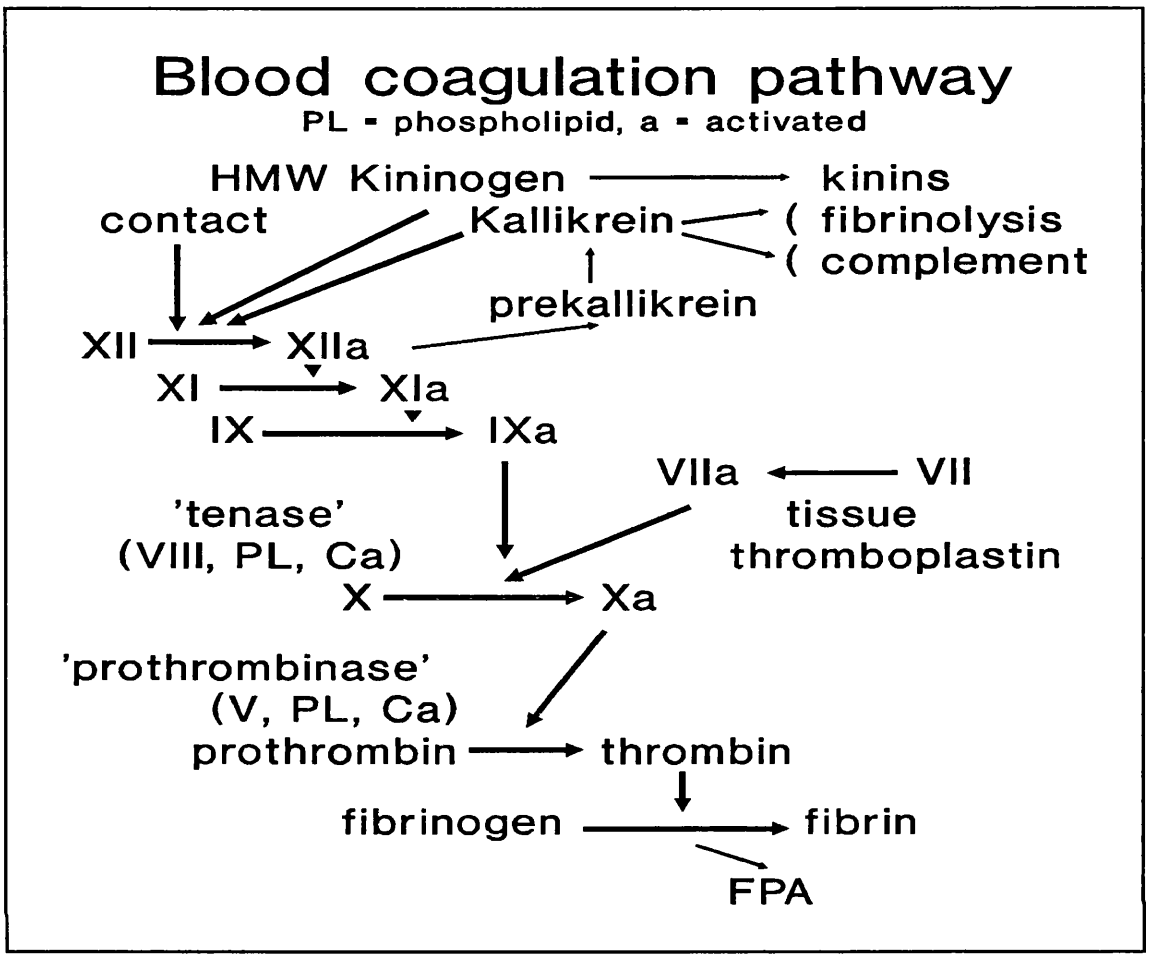


Figure 3.17 The blood coagulation pathway.

VIII, X, V, prothrombin, and fibrinogen, as well as XI and XII. The normal value is 30-40 seconds. Without kaolin it is 60-80 sec, without the phospholipid is 80-100 sec, and without both it is longer than 2 minutes.

In the PT test the plasma is mixed with a phospholipoprotein tissue factor, thromboplastin, which is a rabbit brain extract rich in PS. On addition of calcium this activates factor VII, which in turn activates factor X directly, thus bypassing the intrinsic pathway factors (prekallikrein and high molecular weight kininogen, XII, XI, VIII & IX). The time taken for this to clot after the addition of calcium is noted. The test assesses factors VII, X, V, prothrombin, and fibrinogen. The normal clotting time for this test is 12-14 seconds.

In the experiment outlined above (3.4.2) the incubation of the plasma with the liposome may lead to large amounts of these factors being bound or adsorbed to the liposomes. The usual binding mechanism for the vitamin K dependent clotting

factors (II, VII, IX, and X) is via Ca^{++} bridging to the γ carboxyglutamic acid residue (Zwaal 1978). These experiments with citrated plasma have little free Ca^{++}, and this mechanism is inoperative during the incubation. The factors will be removed from the plasma during centrifugation as they will be retained with the liposomes. The plasma will lack the factor and the result of the PT and/or aPTT will be prolonged, just as in the clinical use of these tests. Significant prolongation of either the PT or aPTT occur when over 50-80% of one factor is missing, or when smaller amounts of several factors are depleted. A control sample is used to check the effect of incubation on the labile clotting factors. This is the normal plasma, with buffer in place of the test liposome, incubated at 37°C for 1 hour.

For the PT and aPTT tests the time taken for the control sample of plasma (incubated with the buffer alone without any phospholipid liposome present) to clot is used as the baseline. The times for the plasma incubated with the liposomes are compared against this baseline time, as this allows for the effects of dilution, and for the incubation for 1 hour at 37°C on the plasma. The difference in the time of the test sample is expressed as a percentage of the control time. eg buffer 12 sec, test 18 sec, difference 6 sec, result 6/12 x 100 = +50%. Note in passing that this is slightly different from the clinical analysis of the assay, where a ratio is used. eg 18/12 = 1.5. In addition a given percentage change in the aPTT is more significant than for the PT, as its control time is longer (30-40 sec compared to 12-14 sec for the PT). A negative percentage indicates a shortened clotting time for the test, as a result of acceleration of the activation of factors, or by direct action on the factors late in the cascade. Prolongation of the test is due to activation with subsequent degradation of the factor, absorption, or inhibition.

The thrice washed resuspended liposomes are used in isolated clotting factor deficient plasmas to detect the small amounts of each factor bound. The washing removes any 'contaminant' plasma trapped between the liposomes, and possibly the factors that are only very loosely bound. The bound factors are measured quantitatively using commercially prepared deficient plasmas for the detection of isolated clotting factors. The substrate plasma in each assay has one clotting factor missing, and this is mixed with the liposomes. If the liposomes also lack the same factor no correction of the clotting time of the substrate plasma occurs. The

tests are based on the aPTT for the factor XII, IX, and VIII assays, and on the PT for the factors VII, V, and X. Quantification in these assays is achieved by using dilutions of normal plasma to produce a standard curve. From this the % clotting factor present in the test liposome samples is determined. The results are expressed as a % of the factor concentrations present in the normal plasma. If small amounts of the specific factor are present on the test liposomes then they will correct and shorten the clotting time of the factor deficient plasma. A control sample, of the liposomes incubated with buffer, are also used to detect any direct, non-factor specific action on the assay systems by the phospholipids, causing acceleration or inhibition of clotting times.

3.4.4 Results

These are shown graphically with a point for the result of each assay in Figures 3.18 to 3.25. For the PT assay the high values are shown numerically at the top of the graph.

For the factor assays the top of the graph is annotated with an estimate of how much over the range the values are, as either >10%, or >>10%. Where all the factor assay results are within the range of the standard curves a mean value is shown as '*'. When one or more results are outside the range no mean value is shown.

A) The results for the PT test and related factors (see Figures 3.18 to 3.21).

Group 1

The prothrombin time (fig 3.18) of normal plasma is not affected by DMPC (-1.3%), but it does increase (8.5%) with DMPC/GM1, (11.5%) with the outside mixture, and (12.6%) with DPPC. The SM results are somewhat scattered, with a mean of 46.2%, but if the one outlying point (225%) is omitted the mean falls to -1.5%.

For the factor assays the controls, liposomes incubated with the buffer, give similar results as the buffer alone, except for single results with DPPC & DMPC (in the

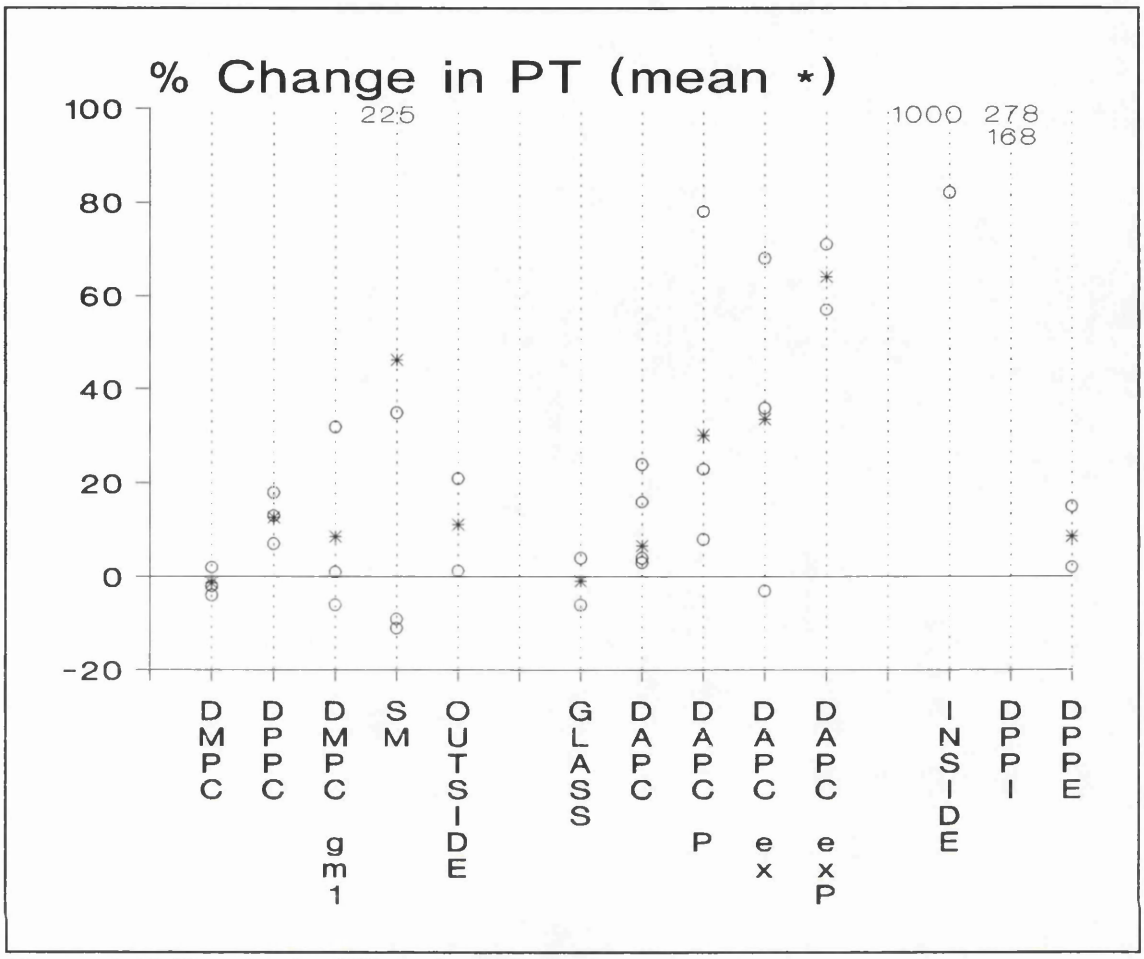


Figure 3.18 Results for PT assay with liposomes.

factor X assay), and DPPC & DMPC GM1 (in the factor V assay). These have shorter times, implying some correction in the test unrelated to incubation with plasma. Despite this all these lipids absorb less than 1% of factor VII (fig 3.19), and less than 0.3% factor X (fig 3.20). For factor V (fig 3.21) the amounts bound increase in the order DMPC GM1 (0.13%), DMPC (0.15%), outside RBC mixture (0.25%), SM (0.8%), and DPPC (1.5%).

Group 2

The negatively charged phospholipid DPPS interferes with both the PT and aPTT tests as well as the individual clotting factor assays. These samples and the controls do not form clots and are thus uninterpretable. No results are therefore shown on any of the graphs. The inside lipid mixture causes the next longest prolongation of the PT, at 541%, with PI at 223%, and PE at 8.5%. DPPI directly interferes with the factor V assay, and is not shown in the Figure 3.21. The results for DPPI in the assays of factor VII and X are under 1% in one set of results, but

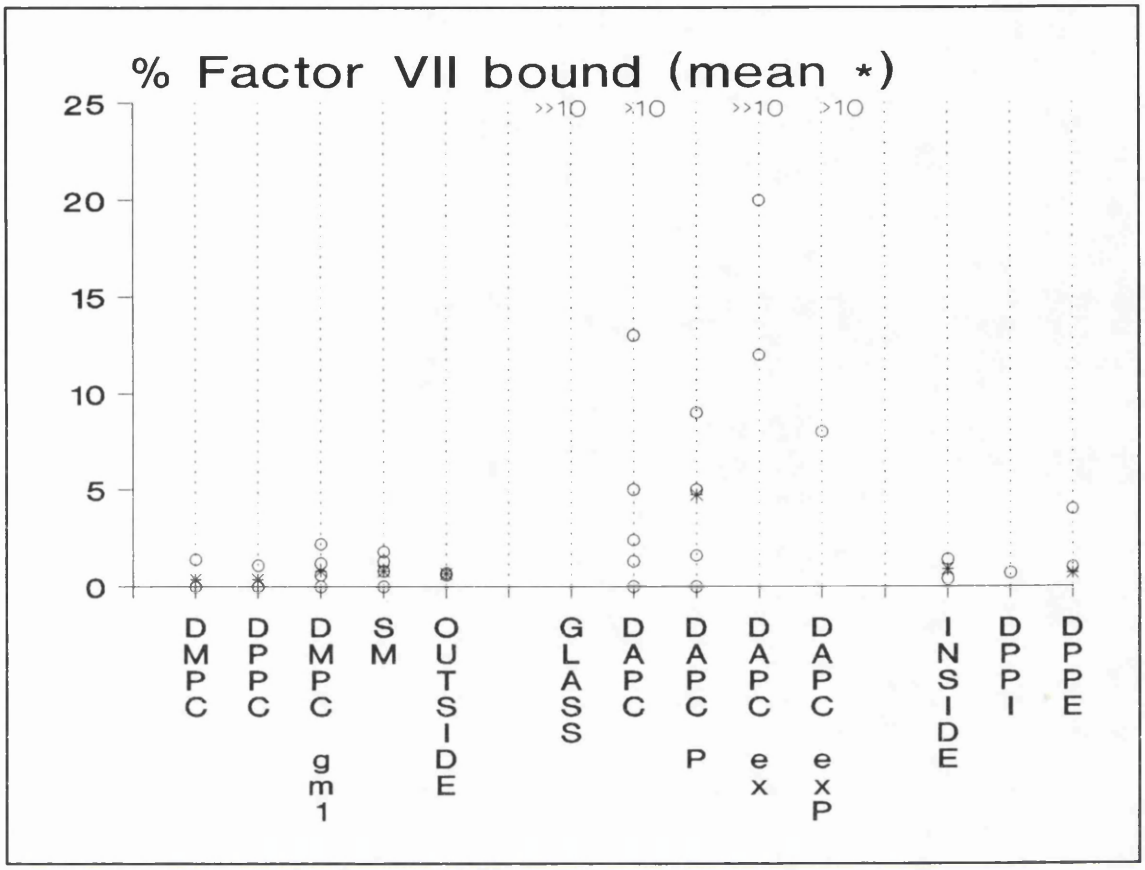


Figure 3.19 Results for Factor VII assay with liposomes.

show interference in a second set. The results for the inside mixture are similar for factors VII, V and X, and are all under 2.4%. DPPA shows a PT of 120, interferes with the factor VII and X assays, with no binding of factor V, but has been examined only once (and so does not appear in the figures). In addition all the control liposome samples for DPPI, DPPA and the inside mixture have significantly longer times than the buffer, suggesting inhibition of the factor assays. The results for the controls also suggest that level of these factors bound may be falsely low.

DPPE does not show these effects, and gives results of 0.7% for factor VII, 0.15% for X and 1.2% for V.

Group 3

Glass does not prolong the PT time. It does appear to bind very high levels of both VII and V (>>10%), with 4% for factor X. All the buffer controls are shortened to less than 50% of the times produced by the buffer alone. The

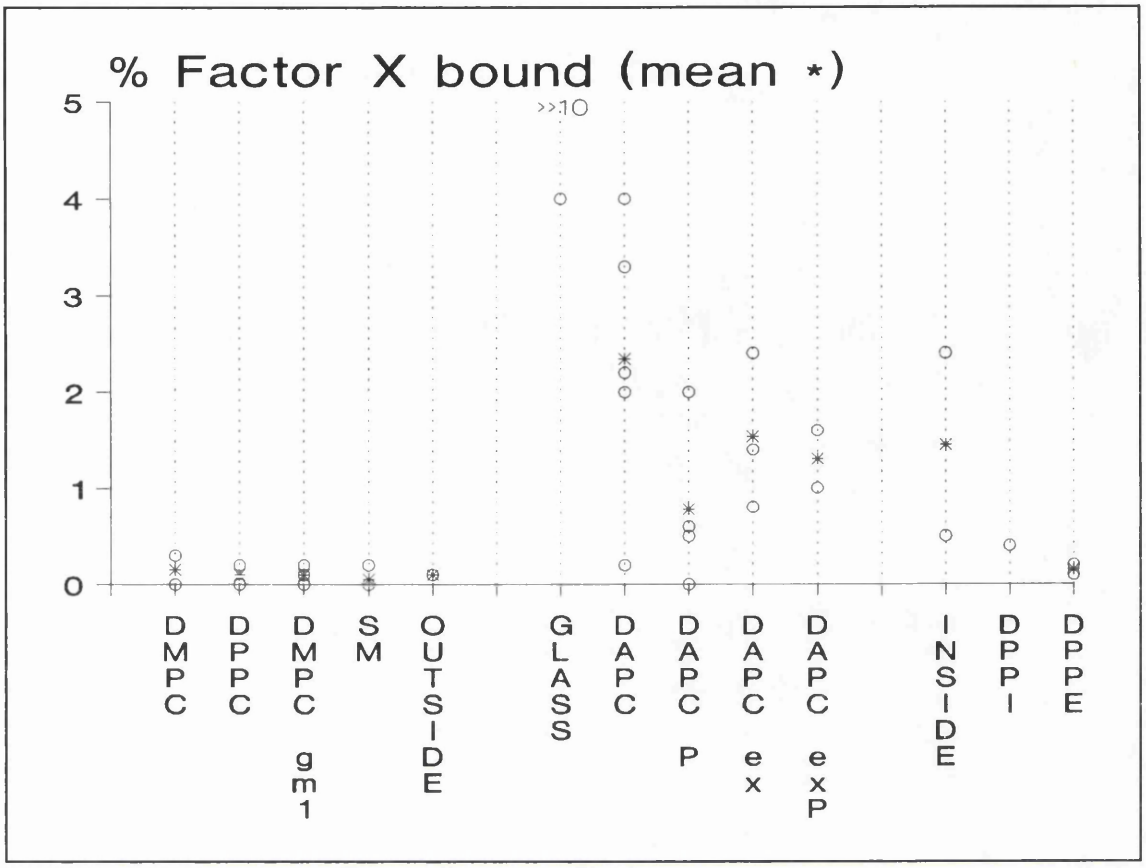


Figure 3.20 Results for Factor X assay with liposomes.

presence of the glass beads is very likely to have mechanically interfered with the metal ball used in the automated version of the test used (automated coagulometer, KC10, Amelung Ltd, Germany), leading to premature times in the results. Kaolin has been examined once to try and avoid this problem. The PT is 120, with >>10% for factor VII, 9% for X and 0.2% for V.

The results for DAPC show a wider scatter than the PC containing liposomes. The various forms of DAPC show a graded prolongation of the PT. The native DAPC causes the least increase (6.6%), the polymerized next (30.25%), then the extruded (33.6%), and finally the extruded and polymerized DAPC (64%). These differences are not statistically significant using the Mann Whitney U test, although the difference between DAPC and exP-DAPC has a p value of 0.08. (The two sample t test is significant with p=0.026.)

For the factor analysis the times for the buffer controls are not dissimilar to those from group 1, being close to the plasma buffer times. There are isolated short

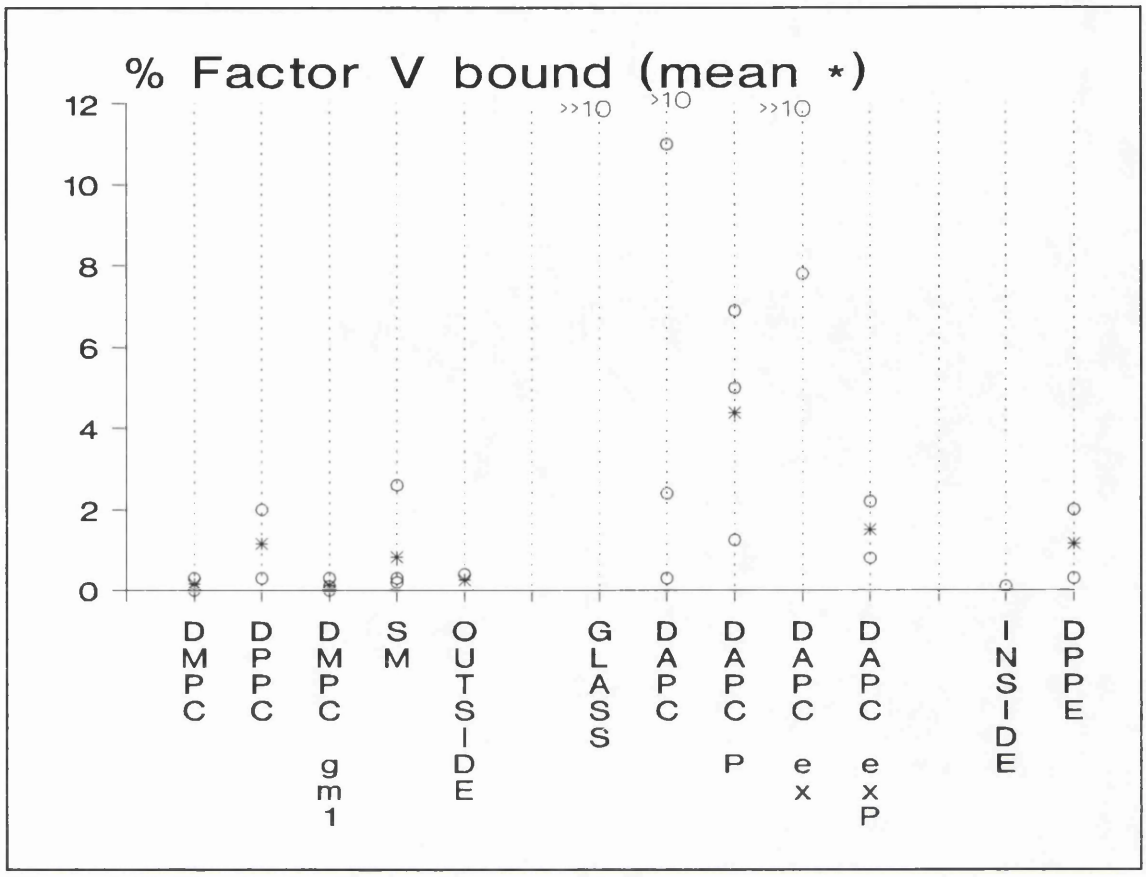


Figure 3.21 Results for Factor V assay with liposomes.

times with polymerised extruded DAPC, in the factor VII assay, and the polymerised DAPC in the factor V assay. 3 of the 5 results which show over 10% difference from the plasma buffer came from one preparation of extruded DAPC, and may be explained by contamination or some other error early in their preparation. This same batch also affects the aPTT related assays (see below).

A similar trend to the PT results for DAPC is apparent in the amount of factor VII adsorbed, native DAPC absorbing less than the extruded (4.3% vs 16%, if the outlying results are discounted). DAPC and PDAPC are about equal (4.3 and 4.74). In contrast the trend is reversed for factors V and X. The native DAPC and extruded DAPC preparations bound factor V variably from 0.3% to well over 10%, but the polymerised bound less (4.4%) and the extruded and polymerized even less at 1.5%. The pattern of factor X binding is similar, but at a lower level, and with reversal of the unextruded and extruded polymerised DAPC's. The factor X results are DAPC 2.3%, the extruded 1.5%, the polymerised and extruded 1.3%, and the polymerised alone 0.8%. None of the differences between

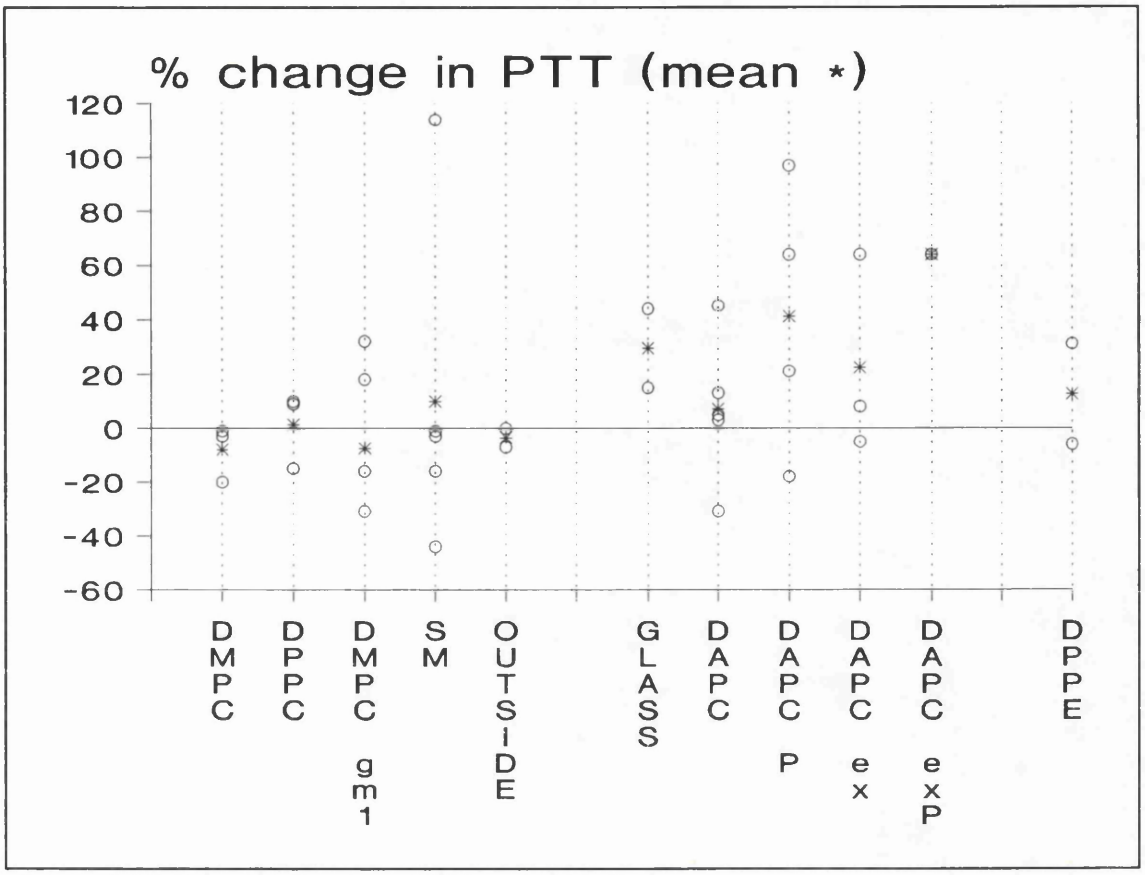


Figure 3.22 Results for the aPTT test with liposomes.

the preparations of DAPC in the factor analysis are statistically significant, but this probably reflects the small numbers of data.

B) The results for the activated partial thromboplastin time (aPTT) and related factors (see Figures 3.22 to 3.25).

Group 1

Liposomes made from the PC containing lipids (DMPC, DPPC, DMPC/GM1) cause only modest changes by the aPTT test (fig 3.22). SM shows a scatter of results, with one outlier from the same batch as above. The mean without this point is -16%. The controls for the factor analysis are similar to the buffer times, except for one result for DPPC and DMPC/GM1 in both the factor XII and IX assays, with one further odd result for DMPC/GM1 in the aPTT. All the lipids in this group bind under 1% of any of the factors XII, IX, VIII (figs 3.23 - 3.25).

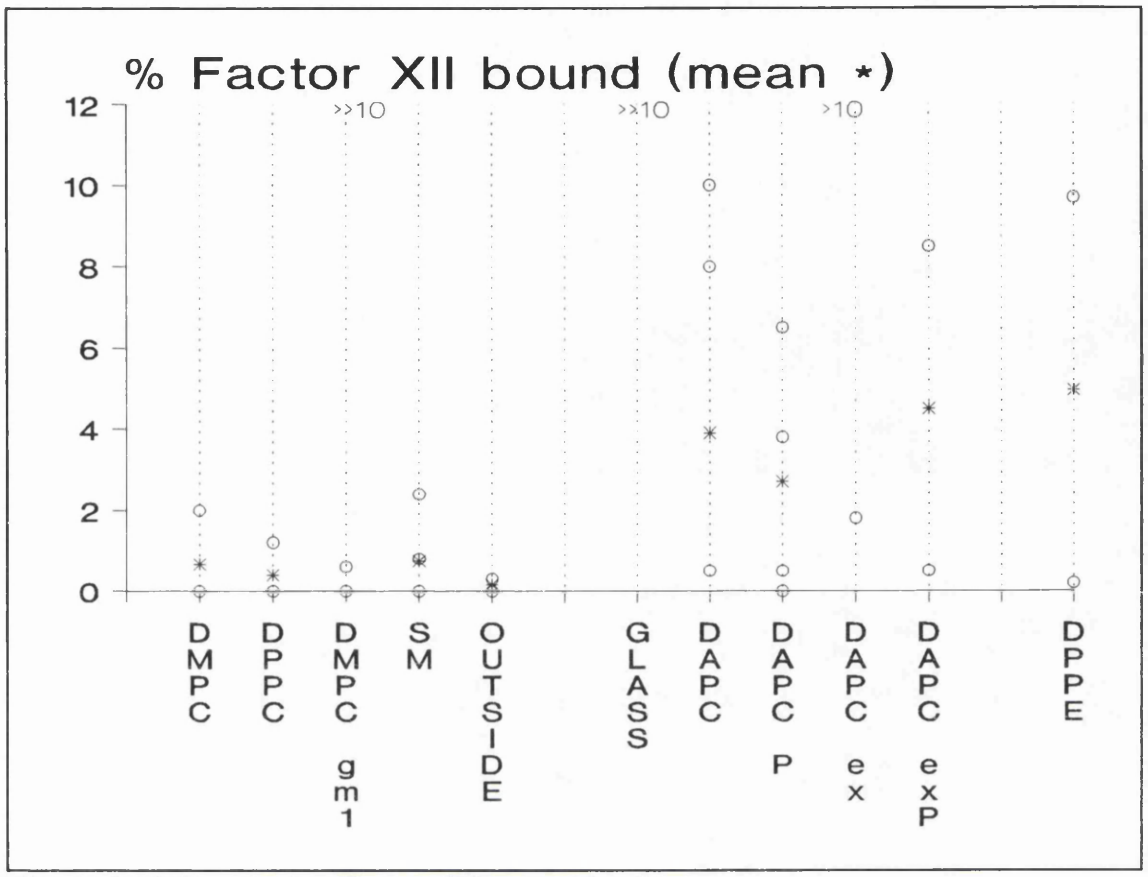


Figure 3.23 Results for Factor XII assay for liposomes.

Group 2

The negatively charged lipids (DPPS, DPPI, and the inside mixture) whether incubated with plasma or buffer (the controls), interfered with the tests. They cause total failure of clotting or marked prolongation of the clotting times, and are thus not included in the figures for the results of the aPTT, or the factors XII, IX, and VIII tests. The significance of these results is discussed below. DPPA inhibits the aPTT and factor VIII test, but demonstrates well over 10% binding for XII and IX.

DPPE is the exception in this group, as it increases the aPTT time by 12.5%. DPPE binds factor XII (fig 3.23) at a level of nearly 5%, and factor IX and VIII are present in amounts of under 0.3%.

Group 3

Glass, the 'bad' surface, prolongs the aPTT by 30%, probably due to the activation and subsequent inhibition of XII and X. All the factor assays give results

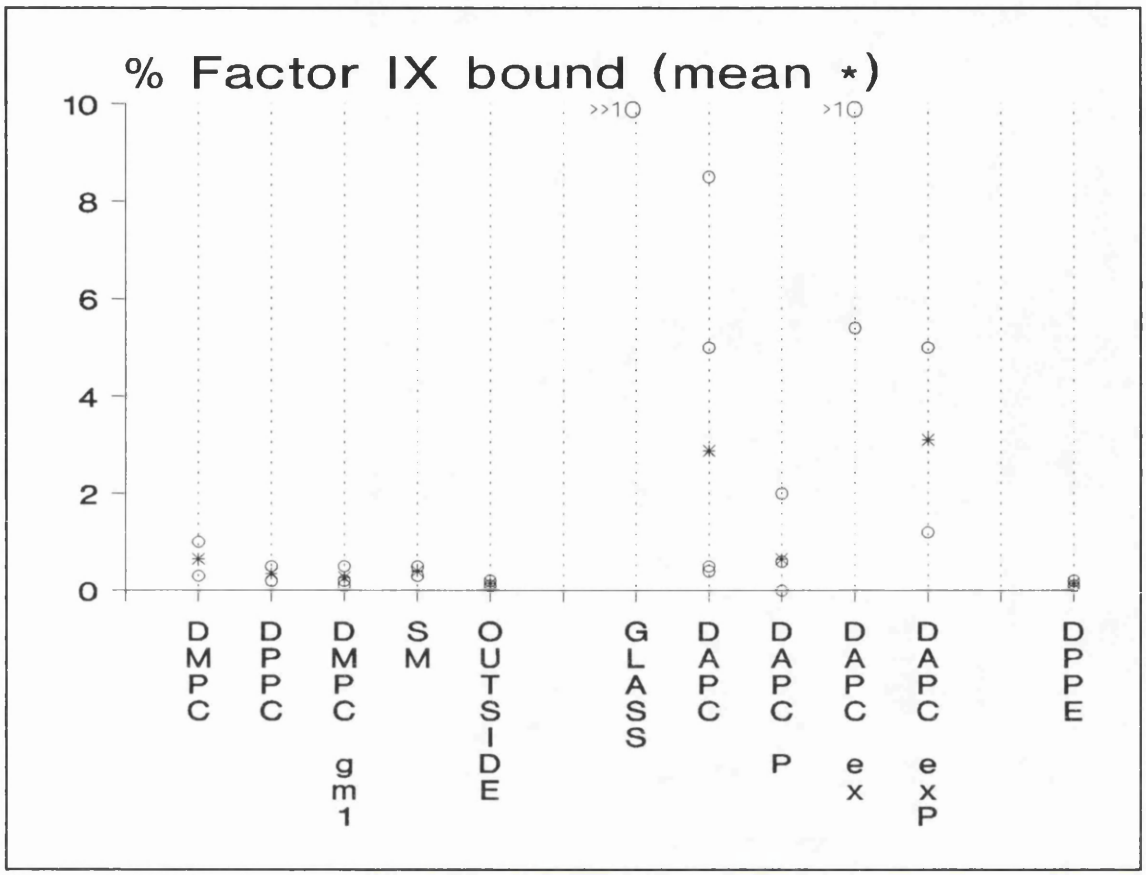


Figure 3.24 Results for Factor IX assay with liposomes.

suggesting high levels of adsorbed factor, well over 10%. The controls incubated without plasma, show marked shortening of the assays to less than a third the time for the buffers (again this is likely to be an artifact due to mechanical interference, with the clot detection in the KC10). Kaolin has an aPTT of 102%, binding >>10% of factor XII, and very little IX (3.7%) and VIII (0.2%).

For the DAPC preparations the aPTTs tend to be longer for the polymerised DAPC forms, compared to the unpolymerised forms. The results increase in the order native DAPC (-7%), extruded DAPC (22%), polymerised (41%) and extruded and polymerised (64%). The results for the uptake of the clotting factors are all under 10%, except for 4 results. 3 of these came from one batch of the extruded DAPC, and may due to an error as outlined above, in the group 3 results for the PT assays.

The control times for the factor assays show minor changes compared to the buffers. For factor XII there is minor shortening of less than 10% in one of four

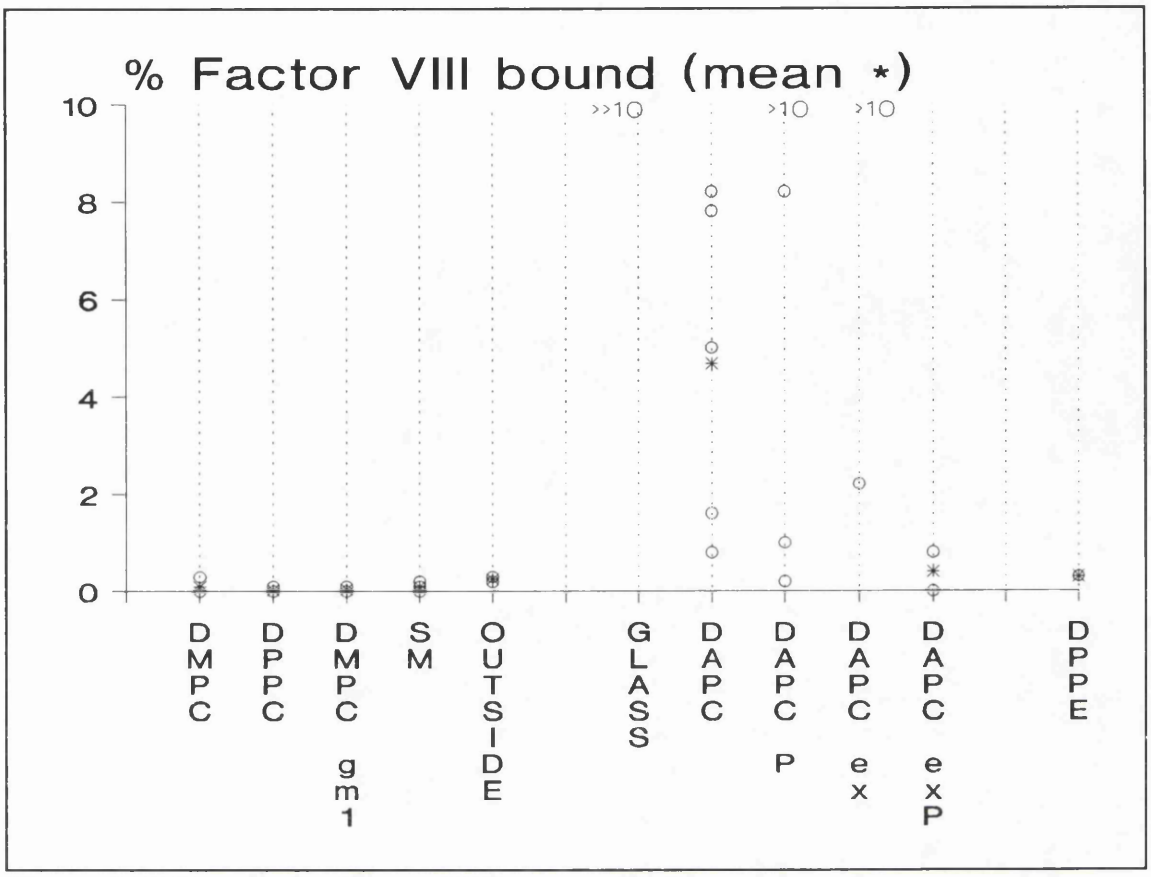


Figure 3.25 Results for Factor VIII assay with liposomes.

assays. For the factor IX assay there is a similar degree of shortening for DAPC, exDAPC, PDAPC in one assay, and an increase for PDAPC in another, out of five assays in all. For factor VIII there is minor shortening for PDAPC and exDAPC, in one of four assays. The three aberrant results for exDAPC, all come from a single preparation, and all have an abnormal buffer result in each factor assay.

Factor XII levels bound fall after polymerisation of the native DAPC (3.9% to 2.7%), but are variable for the extruded DAPC. A similar pattern is seen with factor IX, with the native DAPC at 2.9% falling to 0.7% for PDAPC, and the extruded at 5.4%, falling to 3.1% for the polymerised DAPC. For factor VIII there is a small amount bound, at 5%, for native DAPC, and this falls with the extruded DAPC (2.2%), and falls further after polymerisation of the liposomes (3.3% and 0.4% respectively). The differences between DAPC and exPDAPC just fail to be significant in a two sample t test ($p=0.054$) and with the Mann Whitney U ($p=0.12$).

3.4.5 Discussion

Overall both the aPTT and PT tests show a scatter of results for each material. This might be expected because many variables are involved, both in the preparation of the liposomes, and the influence on the multiple components involved in the assays and their execution. Normally 50-80% depletion of a single factor would be anticipated to effect the result of the PT or aPTT. For example DPPI shows a PT of 223%, and the factor V assay alone is affected, accounting for this. However a number of minor effects on several clotting factors are as important as one major effect on a single factor, in determining the final clotting times. The results here are partly explained by this, but in places the amount of the factors bound are <10% or even <1%. An example is the result for DPPE in the PT test of 8.5%, however the individual factor assays show only 0.7% of VII, 0.2% of V and 1.2% of V are bound. The individual factor analyses are much more specific. They detect very small amounts of adsorbed factor.

For the interpretation of the PT and aPTT tests it is assumed that the action of the liposome during the incubation is not due to activation of inhibitory pathways present in plasma, and that there is no destruction of factors. Prolongations of the clotting times are thus attributed to depletion (that is destruction of the activated factors), adsorption and/or binding of the factors. There is probably a mixture of all these mechanisms taking place, but there is no easy way to untangle them. Nor is the exact mechanism important for conclusions of this study, since all of them indicate bad haemocompatibility.

The results for the glass beads suffer from the difficulty of finding an equivalent for the liposome preparations. The beads are included to show the effect of a known 'bad' surface in the assay systems. However the automated clotting assay equipment is likely to have generated artefactual results. This is caused by the beads falling to the bottom of the cuvette, and interfering with the movement of the magnetic ball used in the cuvette to indicate the formation of the clot. Despite this the results indicate both strong binding of factors and a direct action on the test system, demonstrating the thrombogenic nature of glass. The result of the PT test for the glass beads is not unexpected, and there are significant

amounts of factors VII and V bound. There is significant binding of factors XII, IX and VIII, which are expected. The increase in aPTT is only 30%. This is perhaps small in comparison to the amounts of the factors adsorbed, particularly as factor V also contributes, along with XII, IX, and VIII, to the final aPTT. However during the period of incubation there may also be activation and subsequent loss of some of the activated contact factors such as XII, XI, and also V, which is relatively labile. The binding of the factors may also take place in such a way that still permits thrombin generation. This would explain the results for the glass controls, which are shorter than the blank plasmas. This would also account for the lack of change in the PT, and the notably small effect in the aPTT, which is less than might be expected. Kaolin acts on factor XII, and VII, with minor effects on X and IX, the increase in aPTT of 102% reflects the binding of factors XII, IX and X.

The results for glass and kaolin both show a strong positive, ie that an activating surface both adsorbs factors, and prolongs the aPTT. These results suggest this experimental system would be able to detect any effect that the liposomes have on the clotting components studied.

Of the negatively charged liposomes the results for DPPS are unhelpful, beyond there being activation, and depletion of some or all of the factors studied. The inside mixture and DPPI are similarly uninformative, as assessed by the aPTT. These results suggest a direct action of these lipids in activating the assay system (aPTT), thereby depleting it of factors before the addition of calcium. However when Ca^{++} is added during the coagulation assays binding of factors II, VII, IX, and X via γ carboxyglumatic acid residues can also take place. The amount of negatively charged lipids present in the liposomes may dilute the activated factors sufficiently to prevent the formation of the 'tenase' and 'prothrombinase' (containing activated factor V) complexes. This would account for the prolongations seen with the controls, containing the buffer and liposomes. For the inside mixture the factors VII and V do not appear to be involved as the cause of this. The prolongation of the PT by a factor of 541% for the inside mixture is not really explained by the level of factor X bound (1.5%) either. This implies an action on the pathway between the activated factor V complex and prothrombin,

or between thrombin and fibrinogen. The partial inhibition caused by the controls for both the mixture and DPPI in the PT based tests again suggests a direct action elsewhere in the assay system.

The increase of the PT to 223% for DPPI does not appear to involve VII, or X. The inhibition of only the factor V assay by DPPI implies that this factor is involved. However the controls also had a longer time. This may suggest a specific block even in the absence of factor V, possibly due to the action of an inhibitor preventing prothrombin activation.

DPPA inhibits the aPTT and interferes with the assays for factors VIII, VII and X. The prolongation of the PT, of 120% may be due to binding of VII and X, and not of factor V. There is >>10% binding of XII and IX. These two factors and the results for the VIII, VII and X all contribute to the explanation for the prolonged and inhibited aPTT.

The results for DPPE, (the phosphatidyl ethanolamine head group) are more enigmatic. For the PT test and the related factors (VII, V, X) it is as non-thrombogenic as the PC containing lipids. There is a slight increase in PT, of 8.5%, with 1.2% of the factor V being recovered from the liposomes. It prolongs the aPTT test by 12.5%, which is only slightly more than some of the PC lipids, and yet the liposomes appear to adsorb factor XII in an appreciable amount (5%), compared to the PC lipids (under 1%).

The PC lipids have the least effect on either the PT or aPTT tests. This indicates little interference with the clotting cascade. The lack of interaction for DPPC, DMPC and even the DMPC/GM1 mixture, is confirmed by the lack of factors adsorbed by the liposomes in the second stage of the experiment. The results for SM are more scattered but again show no systematic increase in any factor. The outside mixture prolongs the PT, but without a corresponding increase in binding of any of the factors. In summary, as a group these lipids demonstrate a very consistent pattern: very little of any individual factor is bound, and there is little effect on the global tests.

The results for the four different types of liposome preparation of DAPC show a wider scatter compared to the PC containing phospholipids. This may be due in part to the additional steps needed in their preparation. The results for the uptake of the individual factors seem to conflict with the aPTT and PT times. The native DAPC has the least effect in prolonging the PT (6.6%) and aPTT (7%), and yet adsorbs the largest or equal largest amount of the factors XII (3.9%), IX (2.9%), VIII (4.7%), X (2.3%) and between 2 - 10% of factor V, when compared with the other preparations of DAPC. Only for factor VII does native DAPC show the opposite trend. Native DAPC has the lowest amount of factor VII binding, if the outlying results are discounted (4.3% with 4.7% for PDAPC, 16% for exDAPC, and at least 8% for exPDAPC). The results for the remaining tests with the other preparations generally follow the same trend as the PT test. The trend increases in the order polymerised (P), extruded (ex), and then the extruded and polymerised (exP). This order is seen for the aPTT if the overlap of ex DAPC and P DAPC is accepted. The factors also follow this order with XII (falling from 3.9 to 0.5%), IX (falling from 2.9 to 0.1%), and VIII (falling from 4.7 to 0.4%). Factor X has a different middle order, but all bind less than 1.5%, compared to 2.3% for DAPC. This may reflect differences in the strength of binding of the coagulation factors to these different forms of DAPC.

Despite this paradox these results all clearly show much lower adsorption and activation than the negatively charged phospholipids, DPPS, DPPI or the inside mixture. While the individual results are slightly higher than those for the PC lipids, they are of the same order. They are not nearly as activating or adsorbing as glass, or kaolin.

The results demonstrate a major difference between the PC and the negatively charged phospholipids. Those containing the PC group are haemocompatible as far as the clotting factors are concerned. This is in marked contrast to the negatively charged phospholipids, which interfere with coagulation through a variety of actions.

3.5 Conclusions

The results for MTEG demonstrate a low thrombogenicity for the phospholipids with the phosphoryl choline head group, both for clotting factor and platelet activation. The results for the parallel plate test chamber tend to support these results. In these studies using whole blood the surfaces coated with DPPC and DAPC generate less thrombin. The experiments using liposomes again show that PC lipids have little procoagulant activity, especially when compared to the other polar head groups. The materials with the PC group, DAPC and the series of polyesters, demonstrate a similar low thrombogenicity by MTEG.

The phosphorylcholine head group is the least thrombogenic polar group. This head group appears to be responsible for the non-thrombogenic nature of the RBC outer membrane. The surfaces coated with PC containing materials demonstrate a marked reduction in thrombogenicity. This reduction is to lower levels than the conventional biomaterials, Dacron and PTFE. The results of these investigations provide strong support for the hypothesis presented in chapter 2.

3.6 Choice of materials for in vivo testing

DPPC has been widely investigated and characterised as the primary PC containing phospholipid in the outer surface of the RBC and platelet. Since it shows good haemocompatibility it was included to test the central hypothesis, although it may have no commercial application. However evidence presented in chapter 4 explains why this choice had to be revised in favour of SM. In addition sphingomyelin is shown to be one of the least thrombogenic of the phospholipids tested (see the MTEG data). Although it is a phospholipid, it has an amide linkage rather than the usual ester linkage of most other PC containing phospholipids. This confers greater biochemical stability on the molecule.

DAPC, the PC containing phospholipid monomer, can be photopolymerised into a more rigid structure (see 3.2.3.3), and has a similar low thrombogenicity. It also has demonstrable stability, not only by MTEG, but in it's use as a liposome delivery system. Coating graft material with the monomer, and subsequent

polymerisation should cross link the material around the graft fibres. This will hopefully provide a simple but highly effective surface coating to improve many 'tried and tested' materials. Materials such as PTFE and Dacron have a proven track record in terms of strength, durability and host tolerance.

Polyester G has been chosen as it is the least thrombogenic of the polyesters. If this property is confirmed *in vivo* then there will be justification for making the major investments in time and resources to develop new, related polymers for further testing. The next chapter discusses the preparation of the vascular grafts to be used for the *in vivo* studies. It will discuss the experiments performed to check the effect of the preparatory techniques on these three materials.

outline

Chapter 4

Preparation and evaluation of polymer coatings on vascular prostheses

Chapter 4

Preparation and evaluation of polymer coatings on vascular prostheses

4.1 Introduction

DPPC, Polyester G and DAPC are the intended materials for coating the Dacron grafts for *in vivo* assessment. However the results presented in this chapter led to the replacement of DPPC by sphingomyelin. This chapter deals with the preparation of these materials as coatings on the Dacron grafts to be used for the *in vivo* study described in chapter 6.

The problems addressed are:

1. Is the coating physically adequate?
2. Are the materials affected by sterilisation?
3. How can DAPC be polymerised in the lumen of the graft?

While the problems in 1 & 2 need no comment, that in 3 addresses an important problem, which is not at first obvious. DAPC is a monomer, and contains a diacetylenic bond, which needs to be polymerised to cross link the molecules. This requires orientation of the lipid on the surface, exclusion of oxygen, and a catalyst. Ultra-violet light is the usual catalyst, but other wavelengths, including gamma irradiation can also achieve this. The design of the *in vivo* experiment (described in chapter 6) requires the use of 0.5 cm diameter grafts 4 cm in length. Polymerisation of the lumen of the graft presents the problem of directing the UV light into the interior of the graft. This practical difficulty led to the consideration of silver foil reflectors, glass fibres (which are not opaque to UV light), and specialised light sources. Gamma irradiation does not have this problem. Indeed it has 2 advantages, the specimen can be prepared in an atmosphere of dry nitrogen, to exclude oxygen, and penetration to, and therefore polymerisation of, the luminal surface is assured. In addition the graft is sterile as a result of the treatment.

4.2 Methods

4.2.1 General comments about graft preparation

The same Dacron graft material is used throughout the study. It is a Bard soft woven vascular graft 5 mm in diameter (catalogue No. 005099, style 6010, a gift courtesy of Charles Taylor, Mark Thorn, Andrew Watson, and Christine Hadley, UCSI International, and Bard CVS Division). All the graft material is cleaned by soxhlet extraction using chloroform. The soxhlet apparatus heats the solvent in its lower chamber, and it is distilled into the washing chamber. When the washing chamber is full the apparatus automatically syphons the now contaminated solvent back into the lower chamber where it is re-distilled. Impurities and dirt remain trapped in the lower chamber. The washing step is considered essential since the Dacron fibres are lubricated during their weaving into grafts. This leaves a deposit on the fibres. The treated grafts have the test coatings applied by deposition from solution onto the grafts, and this could remove some of the manufacturing oils. It is possible that this could leave the control grafts at a relative disadvantage. Scanning electron microscopic (SEM) examination shows the graft material is unaffected by this treatment.

The grafts are coated from solutions held in a PTFE boat which is washed in the same way prior to use. The boat is really a slot cut in a block of solid PTFE, to give a container with dimensions of 1 cm width, 6 cm length and 3 cm depth.

The graft is immersed in the boat, and the solution allowed to soak into it for at least 1 minute. The graft is then removed using clean metal forceps. While in the grip of the forceps, it is allowed to dry in air. This is carried out in a fume cupboard, and the drying (evaporation of the solvent) takes about 2-5 minutes, depending on the ambient temperature. This procedure prevents contamination of the graft, and also prevents the solutions from running away from the graft by capillary action (a major problem with these solvents).

The grafts are subsequently packaged in gauze, placed in paper trays and double wrapped (this is the standard packing procedure used in the Central Sterile Supply

Department, RFH) for the studies involving steam and ethylene oxide sterilisation. Those for gamma irradiation are placed in glass tubes, sealed in an atmosphere of dry nitrogen, and placed in a second plastic tube containing silica gel. The test pieces are between 1 and 2 cm in length.

4.2.2 Specific methods (SEM, TLC and FTIR)

a) Scanning electron microscopy (SEM). Notes are taken during the SEM sessions and representative photographs taken. The details of the technique are presented in Appendix 3 section A 3.1 on page 238.

b) Thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR). These studies are carried out in conjunction with Dr G Davies, (currently at The Surface Science Unit, Courtauld's, Gateshead). The conclusions are presented here and the full details are in Appendix 3 section A 3.2, on page 238.

4.2.3 Material thrombelastography (MTEG)

This is used to assess the effect of gamma irradiation. MTEG is carried out as described above, in chapter 3. For DPPC, SM, and Polyester G each material is coated onto three 5-7 cm lengths of graft material. These grafts are irradiated and the material is washed into solution by solvent extraction. The solution is evaporated, and the solids redissolved. These solutions are then used to coat the TEG pistons, and cuvettes. The DPPC grafts are also used to estimate the amount of material deposited on the grafts, and form part of the experiment presented below in 4.3.1.1 experiment 2. For DAPC, 24 cuvettes are coated in the same way as all the other materials, but from the unirradiated solution. Half the cuvettes are then sent for irradiation. The piston is coated, and sent for irradiation between each test, so that there is a fresh coating of irradiated DAPC on the pin for each irradiated cuvette. These results will be compared with the MTEG data for the unirradiated materials. The MTEG data for the unirradiated materials are presented here for the same batches of SM, Polyester G, and DAPC used in chapter 6 for the *in vivo* studies.

4.3 Assessment of Preparation

4.3.1 Concentration of the coating solution

4.3.1.1 The coating experiments

Five simple experiments will be presented to establish the nature of the coating produced by the dip coating technique outlined above.

Experiment 1.

The amount of material deposited between the fibres of the grafts is measured directly in this experiment. 10 grafts of 6 cm length are coated with solutions of DPPC and Polyester G (solution A). The grafts are dried, and recoated using a second solution (solution B). The theory of using a second solution is to add to the deposit from the first 'coat'. The two solutions (A and B) are then evaporated to dryness and weighed. The grafts are extracted using the soxhlet apparatus, the solvent is dried and the material extracted from the grafts is weighed.

Experiment 2.

The amount of material deposited on the grafts is again measured directly in this experiment. In this experiment the grafts are only coated once. 3 grafts of around 5 cm length are coated with a solution of DPPC. The grafts are dried, and are then desiccated before weighing to remove all the solvent, and as much water as possible as DPPC is hydroscopic. The grafts are then extracted in the same way as experiment 1. The DPPC extracted is subsequently used for the MTEG assessment, which is presented below for irradiated DPPC (see 4.5.2, Table 4.2).

Experiment 3.

The amount of material on the graft is estimated from the increase of weight due to the solvent immediately after coating. 1.6 cm lengths of graft are weighed, dip coated and immediately re-weighed.

Experiment 4.

Two concentrations of the materials DPPC and Polyester G are used to coat the grafts. The appearances left by these two concentrations of coating solution are examined by SEM.

Experiment 5.

As discussed above DAPC requires polymerisation to crosslink the monomeric chains. Since it is like DPPC, but much more expensive and available in limited quantities, a separate experiment is carried out to checked that the concentration found in Experiment 4 for DPPC is suitable for DAPC. The grafts are examined by SEM to evaluate the effect of polymerisation on the coating.

4.3.1.2 Results of the coating experiments

Experiment 1.

The coating solutions used have the following concentrations (mg/ml).

	DPPC	Polyester G
Solution A	13.1	29.4 [1]
Solution B	12.9	29.1 [1]

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[1] After coating the grafts these solutions are dried. The residual solution A contains 34-35 mg in 5 ml, and solution B 55-60 mg in 5 ml. This suggests that dissolution from the graft takes place during immersion in Solution B.

The weights of phospholipid recovered from the graft material are shown :

	DPPC	Polyester G
total recovered (mg)	120	187
per cm graft (mg/cm)	2.0	3.12
per cm^2 graft (mg cm^{-2}) [1]	1.27	1.98

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[1] 10 grafts of 6 cm length, and 0.5 mm diameter (94.2 cm^2)

Experiment 2.

The concentration of the DPPC coating solution is 13 mg/ml. The weight loss of the three sections of graft material is shown together with their length:

	a	b	c
Loss in wt (mg)	10.4	12.1	7.7
length of graft (cm)	5.5	4.9	4.6
loss per cm (mg/cm)	1.89	2.53	1.67
loss per cm^2 (mg cm^{-2})	1.20	1.57	1.07

The mean loss of weight from all three grafts per cm^2 is 1.28 mg. The total loss is 31.2 mg, and the weight recovered from the extracted solvent is 20 mg.

Experiment 3.

The weight taken up is difficult to measure since the solvent (chloroform) evaporates quickly. The highest weight is recorded. For DPPC this is 0.155 gm, giving a volume of 0.105 ml (density of chloroform = 1.48). This gives a weight of 1.36 mg DPPC, and a coating of 0.54 mg cm^{-2} (the area is calculated from length 1.6 cm @ 0.5 mm diameter = 2.51 cm^2). After the solvent is evaporated the weight gain is 2 mg (0.78 mg cm^{-2}). The apparent gain in weight (from 0.54 to 0.78 mg cm^{-2}) may be due to losses of chloroform between coating and weighing. However the gain in weight after the chloroform evaporated, may also be due to water, as DPPC is very hydroscopic.

Polyester G :- The increase of weight is 0.169 gm, the volume of solution 0.114 ml. This gives a Polyester G weight of 3.3 mg and a coating of 1.32 mg cm^{-2} . The 'dry' weight gain is 4.9 mg, giving 1.95 mg cm^{-2} . Polyester G is also hydroscopic.

Experiment 4.

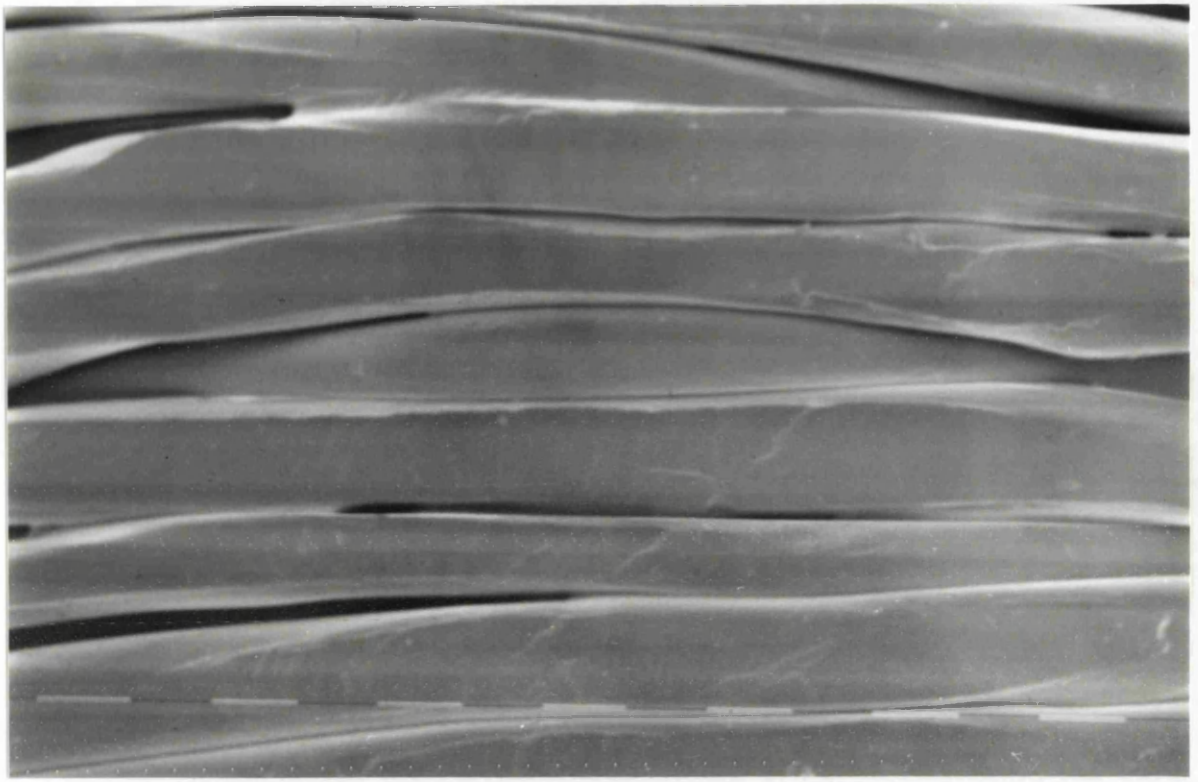
The solution concentrations used for the grafts prior to SEM are:

DPPC	3 mg/ml	30 mg/ml
Polyester G	13 mg/ml	saturated solution (approx 30 mg/ml)

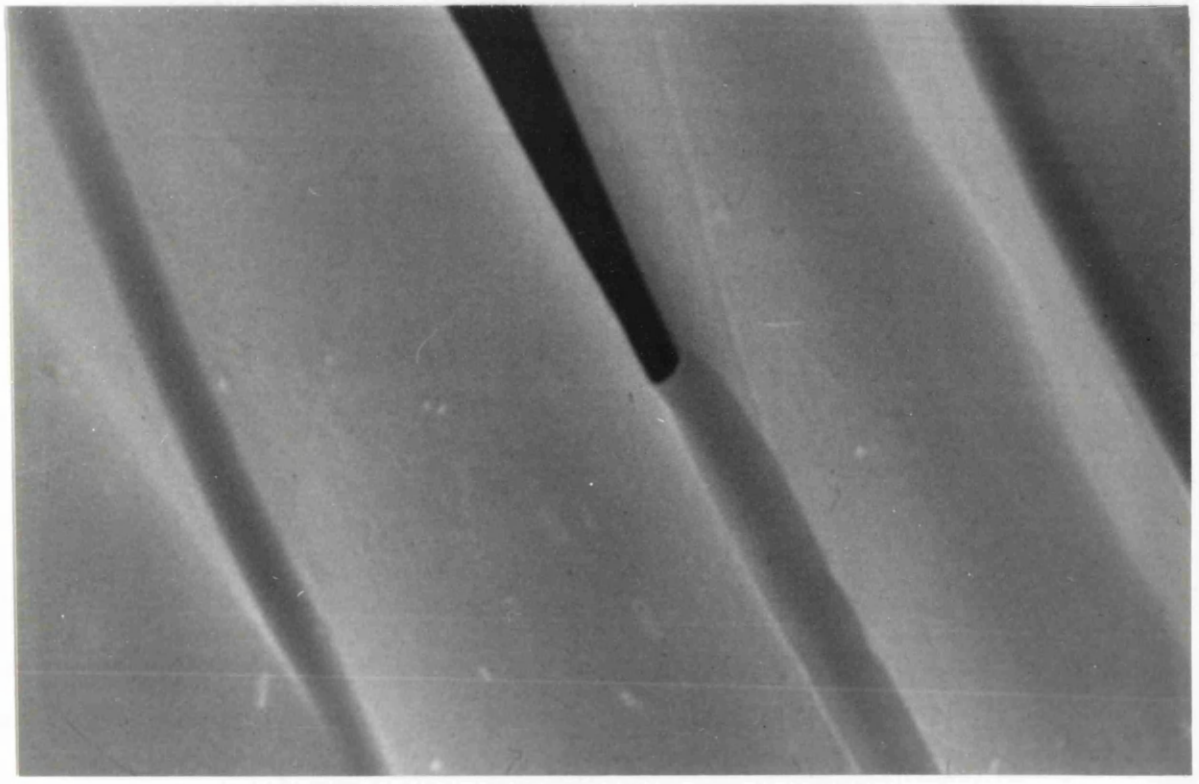
For DPPC the lower concentration gave patchy coverage, with small inter fibre bridges. At 30 mg/ml a thick amorphous covering is produced, especially between the fibres, which is not unlike that produced by polyester G (see Photograph 4.1). For polyester G the lower concentration gives a very thin film, seen best between the fibres. It is however irregular and globules of material are seen without good penetration of the fibres. The saturated solution gives better coverage, with thick capillary deposits between the fibres. These are shown in Photographs 4.1 & 4.2.

Experiment 5.

A concentration of 30 mg/ml is to check the coating of DAPC. The graft pieces are UV light irradiated (as above, see the MTEG samples described in chapter 3),



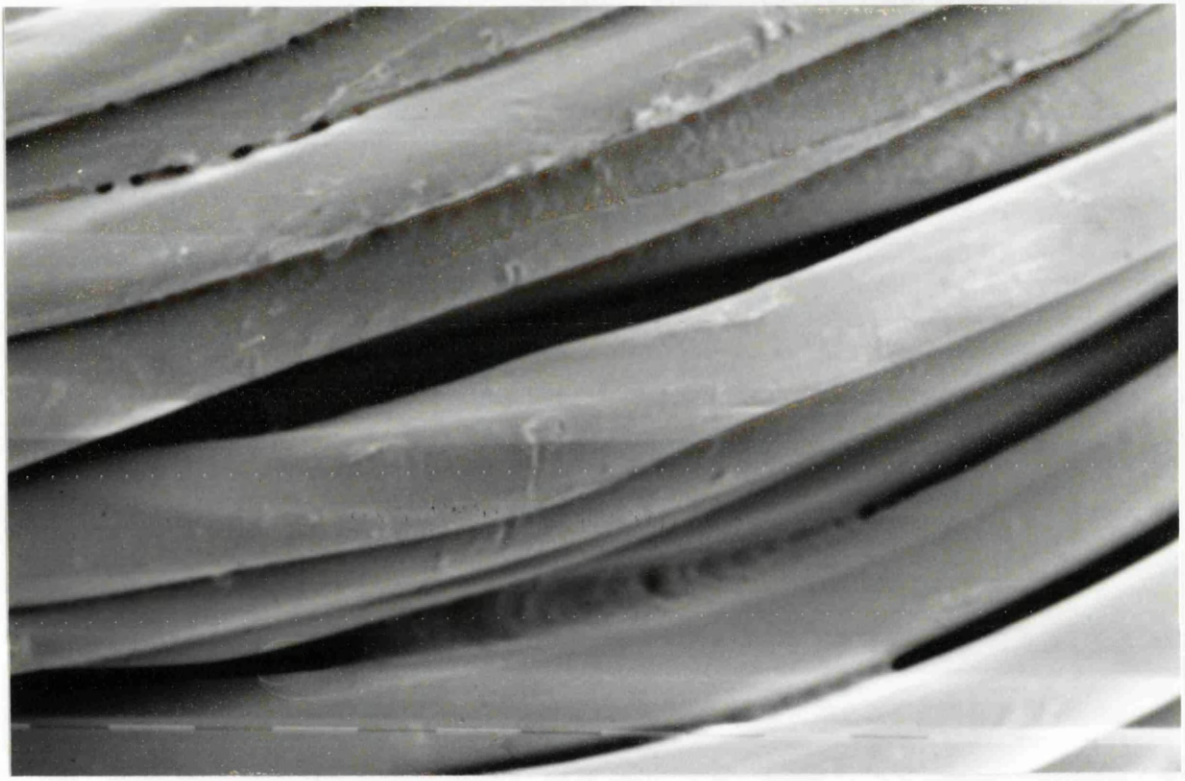
Photograph 4.1 SEM of polyester G coating from 30 mg/ml solution (x840, bar is 10.5μm).



Photograph 4.2 SEM of polyester G from 30 mg/ml solution, (x3280).

in a stream of nitrogen but using a more powerful UV lamp (Hanovia Ltd, Slough, Berks). The unpolymerised DAPC shows clear inter fibre bridges, some with holes

in them. There is a reasonably smooth covering which in places has amorphous features. After polymerisation there is slight contraction of the inter fibre bridges, with evidence of cracking and flaking. On the fibres the covering have more pits, and there are areas with marked clumps of material. These are apparent in Photograph 4.3.



Photograph 4.3 SEM of polymerized DAPC coated from 30 mg/ml solution (x840, bar is 8.3μm).

4.3.1.3 Conclusions for coating experiments

The estimates of the amount of DPPC on the graft using the 13 mg/ml solution varied between 0.54 and 1.27 mg cm^{-2} for experiments 3 and 1 respectively. A mean of 1.28 mg cm^{-2} (range 1.07 to 1.57) is found for experiment 2. The best estimate is probably 1.27 mg cm^{-2} as it is based on the largest amount of material. When the 30 mg/ml solution is used the SEMs shows good coverage of the grafts. For DPPC and DAPC solutions of 30 mg/ml will be used to coat the grafts for the *in vivo* experiments.

For Polyester G the estimates for the amount on the graft are similar, 1.32-1.98 mg cm^{-2}, using the 30 mg/ml solution. This concentration is close to that of a

saturated Polyester G solution, and a saturated solution will be used for the Polyester G grafts.

These simple experiments show that dip coating method leaves a measurable deposit of phospholipid material on the grafts. The higher concentrations of the lipids produce a better covering of the fibres, but the surface morphology is variable. Polyester G has the smoothest covering, DPPC and DAPC are similar, but DAPC after polymerisation by UV light may be brittle.

4.4 Effect of sterilisation

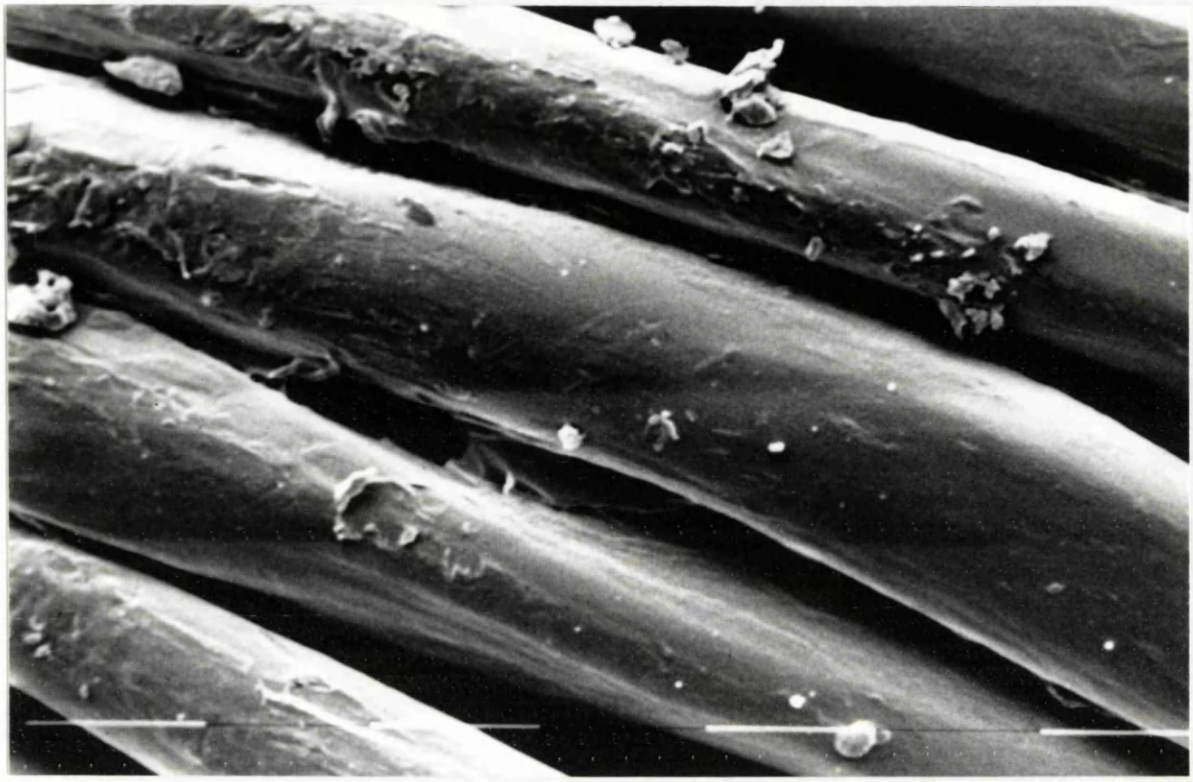
4.4.1 Steam sterilisation

The steam sterilisation has been carried out at the Central Sterile Supply Unit (CSSU) RFHSM, and I am grateful to Miss Lefranc for packing the samples and arranging the sterilisation. The sterilisation starts by flushing out the air in the packs using a vacuum pump. The steam is introduced to the chamber at 134-138 °C, for 3 to 3.5 minutes, at a pressure of 2.2 bar. This is at 100% humidity. The cycle finishes with a drying cycle, where bacteriologically filtered air is introduced to the chamber to dry the packs. The whole cycle takes about 25 minutes.

4.4.1.1 Results for steam sterilisation

The SEMs of the DPPC coatings show differences between the 2 control coatings. The inter fibre capillaries are spiky in one, and the coating smoother compared the test samples from the concentration experiments (in 4.3.1.2). However the differences between the controls and the steam exposed grafts are much more marked. The covering become globular and pitted, suggesting material has been through a change of state (shown in Photograph 4.4).

The TLC and FTIR confirm significant changes, as the phosphate spray shows a long tail, suggesting cleavage of the phosphate group, to give it greater mobility. The differences in the spectra obtained again suggest splitting of the phosphate group.



Photograph 4.4 SEM of steam treated DPPC coating (x1640).

The SEMs of Polyester G show no difference between the controls and the steam exposed grafts. The inter fibre capillaries are unaffected, demonstrating good penetration and bridging. There is perhaps slight undulation and pooling, but these changes are also seen in the controls for the irradiated grafts.

There is a considerable range of molecular weights within Polyester G, since it's synthesis is random. Only the lower molecular weights move on TLC, and it is probable that not all the polyester is recovered from the graft. There are minor changes in the FTIR spectra, but again these are difficult to interpret given the heterogeneity of the polyester.

The SEMs of both the native and polymerised DAPC show differences between the inside and outside surfaces, which are more marked with the unpolymerised DAPC. This shows little coating on the inside, with beading of the material that is present. In contrast the outside has large amounts of material, suggesting that the DAPC has melted and pooled on the dependent part of the specimen (Photograph 4.5).

Photograph 4.5 SEM of steam treated DAPC coating (x528).

The polymerised DAPC has much more material on the inside, with little cracking of the inter fibre bridges. There are no features similar to the melted appearance of the unpolymerised DAPC graft, suggesting that the polymerised DAPC is protected from marked melting. No TLC or FTIR experiments are carried out, because the DAPC polymer could not be recovered from the specimens by the solvent systems.

4.4.1.2 Conclusions for steam sterilisation

Steam sterilisation clearly causes chemical damage to the DPPC coating, and might be expected to do the same for DAPC. The nature of Polyester G makes it difficult to exclude subtle changes. The coatings themselves appear to cover the grafts well, but the steam treatment markedly alters both the DPPC and unpolymerised DAPC. This may be a pure physical chemistry effect, but probably reflects changes in the compositions of the coating. Taken together the TLC and FTIR results for DPPC suggest that hydrolysis of the ester bond has taken place. This would not be surprising given the severity of the sterilising conditions.

4.4.2 Ethylene oxide sterilisation

As ethylene oxide is a highly explosive gas all the sterilisation was carried out in a commercial unit at Guy's Hospital, courtesy of Miss Lefranc, CSSU, RFHSM, who made all the arrangements for this. This unit operates at 60-65 °C, at a pressure of 22 lbs per sq inch and with 44% humidity. The sterilisation cycle is 8 hours of 12% ethylene oxide using dichloro-difloromethane as the carrier gas. This is followed by 7 hours of flushing in air.

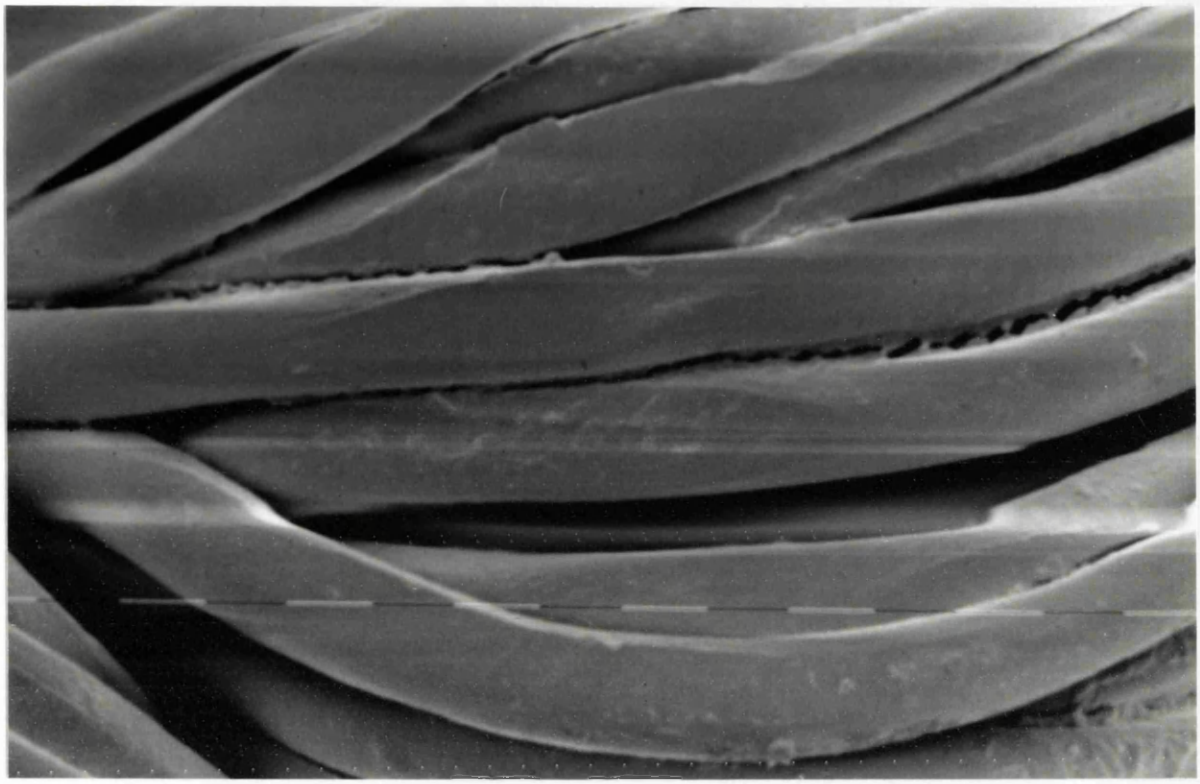
4.4.2.1 Results for ethylene oxide sterilisation

For DPPC there are minor changes only, with a slight increase of pitting seen on SEM. The TLC shows an increase in the 'tail' is revealed with both the lipid and phosphate sprays. Polyester G does not demonstrate any change in SEM appearance. As before the range of molecular weights makes interpretation of the TLC plates difficult, but no gross changes are noted. Both types of DAPC coated graft show marked cracking of the coating. This is particularly noticeable between the fibres (Photograph 4.6).

4.4.2.2 Conclusions for ethylene oxide sterilisation

The TLC of DPPC indicates decomposition, with the production of small molecules, probably hydrolysis of the ester, and possibly of the glycerol backbone. There do not seem to be any changes induced in Polyester G.

There are only SEM results for DAPC, as chemical analyses depend on redissolving the material to recover it from the graft. It might be anticipated from the chemical structure of DAPC that any uncrosslinked diacetylenic bonds would react with ETO. This would form an ethoxide structure, and prevent the formation of the polymeric form of DAPC. The use of this method of sterilisation would also necessitate the development of a technique for polymerising the interior of the DAPC grafts. In addition ETO sterilisation is not without complication, as outlined in chapter 1 (see section 1.1.3, page 27, and references Takahashi et al 1981, Bommer et al 1985, Henne et al 1984 and Muylle et al 1986)



Photograph 4.6 SEM of ethylene oxide treated DAPC coating (x908, bar is 10μm).

4.5 The effect of Gamma irradiation

4.5.1 Introduction

The specimens are all irradiated in a commercial cobalt 60 sterilisation plant, and I am grateful to Mr Eric Courtney of Isotron PLC, Reading, for this. The materials are rotated through 305 positions on a conveyor belt, for 8 hours. They are exposed to a dry cobalt 60 source of one mega Curie (37 EBq ie 37.10^{18} Bq), at room temperature and received a dose of gamma irradiation between 2.8 and 3.1 MRad (28-31 kGy).

Three sets of experiments will be presented. The first set, a simple check, revealed unexpected changes in the materials, possibly related to the degree of hydration of the samples. As a result a second set of grafts has been prepared under more rigorous conditions. These two experiment are reported material by material, since they are to assess the effect of irradiation on each material. The third experiment is the MTEG assessment of extensively dried coatings and this is

detailed in a separate section.

The initial grafts are prepared as described above (4.2.1). The second set, when dry are placed in an evacuable desiccator containing phosphorus pentoxide ($P_2 O_5$), and kept under a high vacuum for a minimum of 24 hours. The vacuum is broken with dry nitrogen gas and the dry samples immediately sealed. These are then immediately placed in a second container which has silica gel in it, and sealed. These are the 'dry' samples as the changes found in the first experiments are thought to be due to the presence of small amounts of water. The 'wet' samples are sealed with a small amount of water (less than a drop, but sufficient to create a saturated water vapour in the glass tubes), and these are not secondarily contained over silica gel.

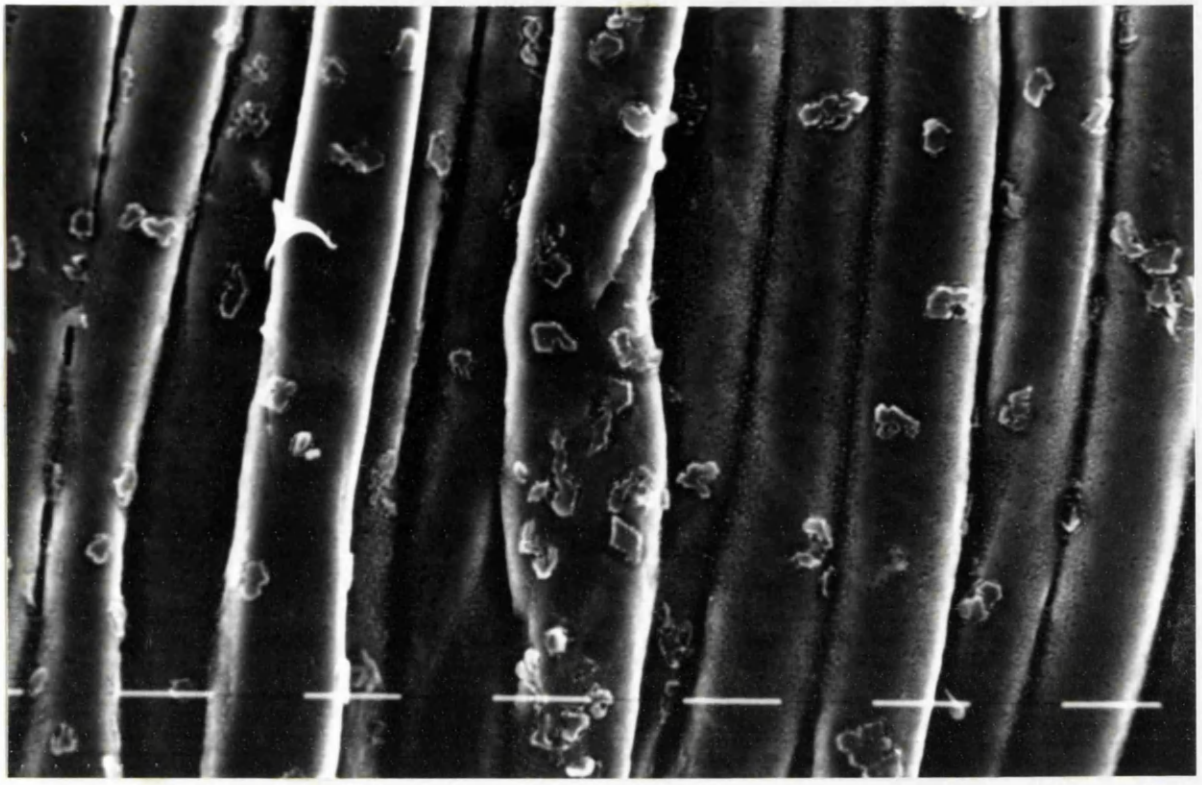
After these changes were found, two further lipids are examined as possible substitutes for DPPC, in the *in vivo* experiments. These are DSPC, which has a higher transition temperature than DPPC, and SM which has a single amide linkage instead of two ester bonds to the fatty acids, but retains the PC group (the structures are shown in Table 2.1).

4.5.2 Results for irradiation

4.5.2.1 SEM, TLC and FTIR results for irradiation

The SEMs of DPPC shows a loss of the coating after irradiation, in that the coating is thin and patchy. Where it is present it is in small spots. Grafts prepared for implantation, but not used, have been examined 6 months after irradiation. They had been stored in dry nitrogen, and under silica gel desiccation, at -25°C. These show marked changes with round crenated clumps of materials on the fibres, and small spots of material elsewhere. The appearances suggest decomposition or crystallisation of the DPPC (Photograph 4.7).

The TLC plates show that the DPPC after irradiation contains DPPA and lyso-phosphatidic acid (lyso-PA), in addition to choline, DPPC and lyso-



Photograph 4.7 SEM of DPPC coated grafts 6 months after irradiation (x756, bar is 11.1μm).

phosphatidylcholine (lyso-PC). The lyso compounds are the result of disruption of one of the ester bond in DPPC, releasing palmitic acid. The FTIR of these samples shows that they contain at least DPPC, lyso-PC and palmitic acid. These compounds are likely to have been formed by cleavage of the lipid ester bonds. However these components cannot account for all the changes observed in the spectra. These extra features probably represent the presence of a mixture of free DPPA and complexes of choline, (1:1 and 1:2) in the graft extract. The full details of the analysis of these experiment are in Appendix 3 section A 3.3, page 239.

The SEMs of distearyl phosphatidyl choline (DSPC) show a coating similar to DPPC, which is a thick amorphous carpet with good inter fibre bridges. There are few changes wet or dry after irradiation. DSPC gives similar results on TLC and FTIR to those found with DPPC.

The SEMs of sphingomyelin (SM) show good coverage of the graft, with an amorphous appearance. There is some cracking of the inter fibre bridges, but there are no obvious differences after prolonged drying. Irradiation, wet or dry,

produced no further changes.

The infra-red spectra of sphingomyelin especially in the presence of water show marked reductions in the phosphate bands at 1090 and especially 1230. At the same time the bands due to choline are little affected. This suggests the formation of a phosphatidic acid - choline complex may well be occurring. TLC studies with 65 $CHCl_3$ / 25 MeOH/4 H_2O give no discernible changes. These changes are much less when the grafts are extensively desiccated.

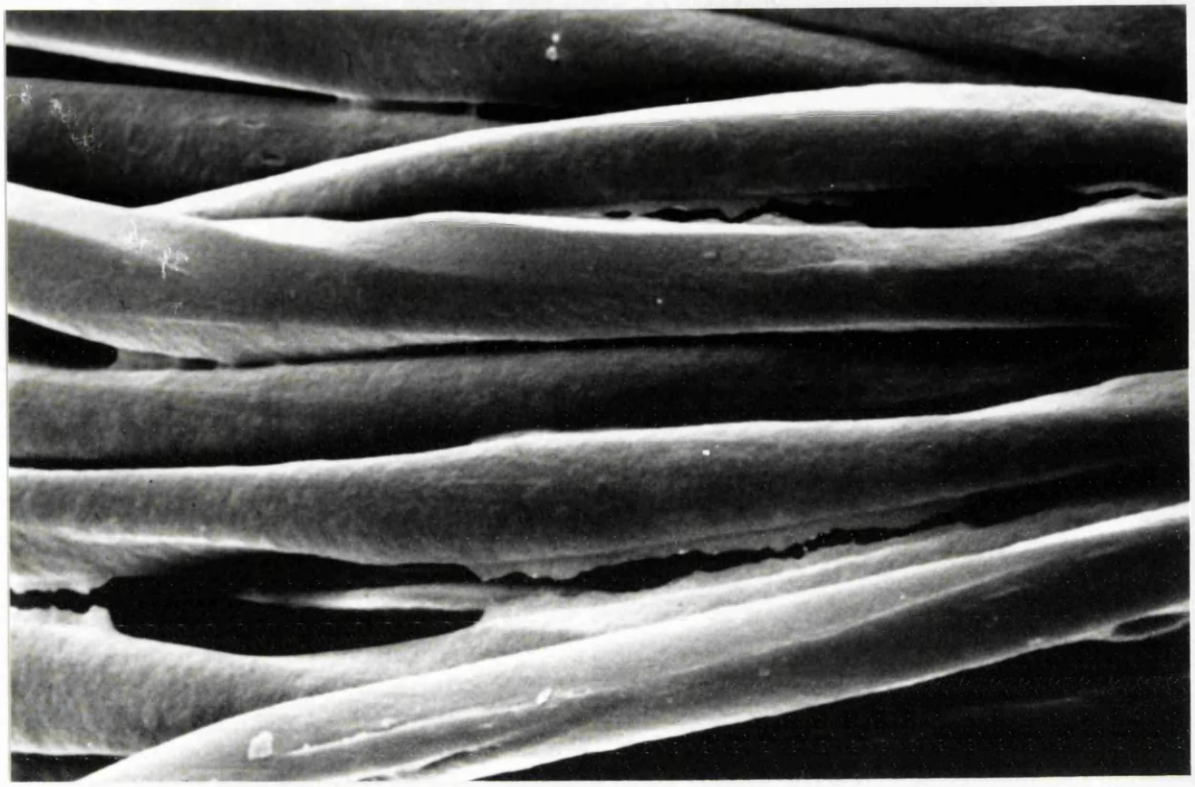
The SEMs of Polyester G show few changes after irradiation, the coating remained smooth with good inter fibre bridges. There are no further changes after 6 months storage.

The DAPC coated specimens are prepared with the monomer, and these are refered to as 'unpolymerised', those exposed to UV light are refered to as 'polymerised' but probably contain a mixture of polymer and monomer. The SEMs of the unpolymerised monomer gives a good velvet covering. The second batch of DAPC gives a similar good covering. The X-ray treated monomer is also smooth and has good inter fibre bridges. The irradiated and polymerised graft has similar good coverage of the fibres, and thick inter fibre bridges. On the outside there is evidence of cracking of the bridges, possibly from movement during preparation, and the coating appears thicker (see Photograph 4.8).

This is likely to be from the initial coating rather than the effect of the treatment. There is no obvious deterioration of the coating itself. The FTIR spectra for DAPC show small amounts of free acid are formed. However the gross changes seen with DPPC are not apparent. The later changes in DPPC, due to a further reaction on storage are not found either, in the DAPC specimens.

4.5.2.2 MTEG results for irradiation

The results for MTEG are calculated as before and presented as summary graphs in Figures 4.1 to 4.3. The tabulated results are in Appendix 3 section A 3.4 page 242, as Tables 4.1 to 4.11. The figures are annotated with '*' to indicate



Photograph 4.8 SEM of irradiated polymerised DAPC graft (x840).

irradiated materials, and PG, and PG2 refer to the 1st and 2nd batches of Polyester G. The second batch of DAPC carries the label DAPC2.

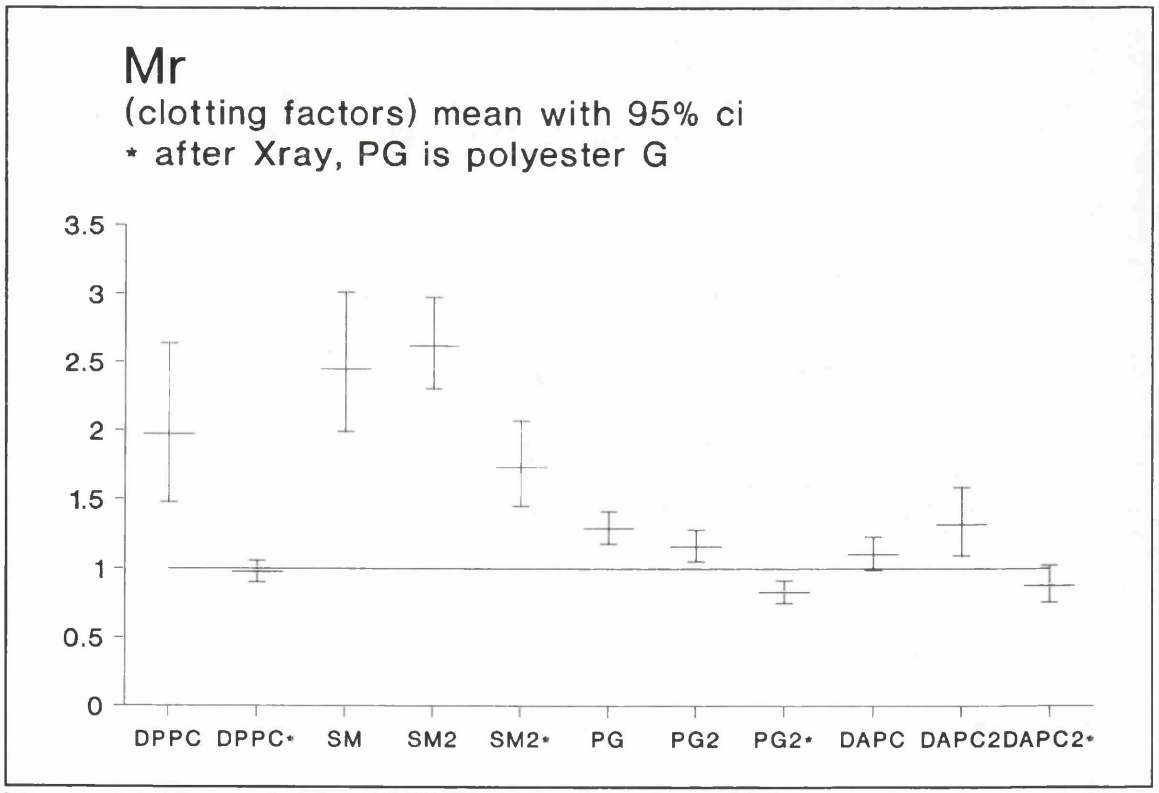


Figure 4.1 Mr results for MTEG of irradiated materials.

The results for DPPC before and after irradiation (Tables 4.1 and 4.2) show a major change in the Mr value from 1.97 to 0.98. This represents a return to the thrombogenicity, for the irradiated DPPC, to the level of the plastic cuvettes. The MA° also rises from 0.11 to 0.51, indicating an increase of thrombogenicity. There is also a change in Mma from 0.062 to 0.21, but this level (0.21) is still lower than PTFE or the polyester without PC.

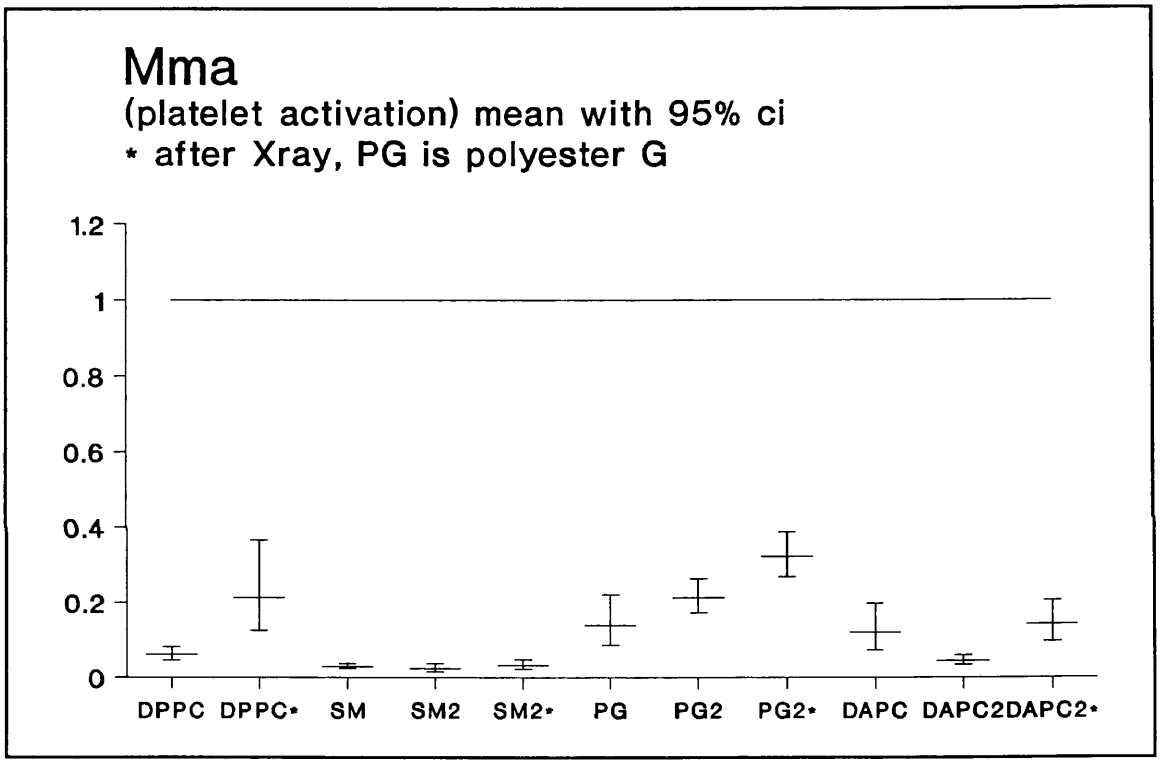


Figure 4.2 Mma results for MTEG of irradiated materials.

There is some variation between the two batches of SM, but none are significant (Tables 4.3 and 4.4). The results for the Mr's are 2.45 and 2.66. The MA°'s are 0.047 and 0.036 and the Mma's are 0.03 and 0.025. The fall in the finite results for Mr after irradiation (Table 4.5) is from 2.62 to 1.73, and is significant ($p<0.02$). There is also a fall in the number of infinite results from 5 to 2 for both the Mr and Mk results. The Mr of 1.73 after irradiation is still better than all the Mr's of the conventional materials, and all the other UNirradiated phospholipids, except DPPC and SM. The differences in MA° (0.036 to 0.07) and Mma (0.025 to 0.33) are small. This suggests that the amide linkage is less prone to disruption than the ester linkage during irradiation.

The change in the Mr parameter for DAPC (Table 4.6 and 4.7) after irradiation (Table 4.8) is from 1.32 to 0.88. The irradiated DAPC value for Mr 0.88 is not however significantly different from 1. The change in MA° is substantial and places the irradiated material at the same level as brain extract. Both these results indicate a dramatic increase in thrombogenicity after irradiation. The Mma also rises from 0.045 to 0.142, but this change is not significantly different from the Mma for the first batch of DAPC tested.

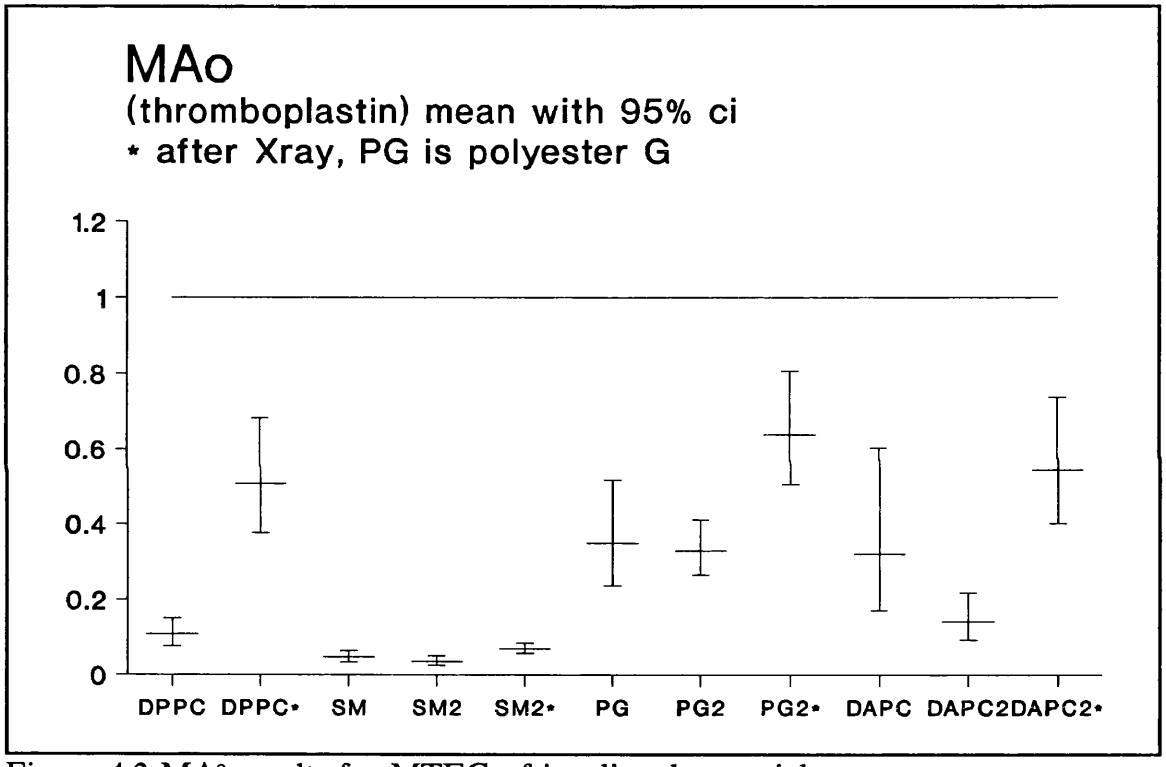


Figure 4.3 MA° results for MTEG of irradiated materials.

The two batches of Polyester G show slight variation (Tables 4.9 and 4.10), but the differences after irradiation (Table 4.11) for both Mr and Mma are significant. For Mr the difference is from 1.16 to 0.83 ($p<0.001$) and for Mma it is 0.22 to 0.32 ($p<0.02$). The irradiated Mr, 0.83, is significantly worse than the control surface ($p<0.0032$). The change in MA° is also large, the two batches are 0.35 and 0.33, rising to 0.64 after irradiation. However the values for Mma and MA° are still less than those of PTFE (0.51 and 0.70).

4.5.3 Discussion for irradiation

The initial TLC results suggest extensive breakdown of DPPC. The chemical reactions which are postulated to occur in the grafts all consume water molecules. This led to the attempt to reduce these reactions by extensively drying the samples (the 'wet'/'dry' experiments). The TLC and FTIR results for the materials DPPC, DSPC, DAPC and SM tend to confirm the early observations. The MTEG results, for DPPC, SM, DAPC, and polyester G, are for materials which have been extensively dried to minimise the changes noted in well hydrated irradiated materials.

The changes in DPPC shown by SEM over the short term period are difficult to assess. However the gross changes seen after the six month storage period suggest a major change has taken place. This is confirmed by the changes seen in both the TLC and FTIR. However results of the FTIR are variable from one graft to another even after the same treatment.

The changes in the MTEG parameters for DPPC show that irradiation has a major effect on the parameters Mr and MA°, with a lesser effect on Mma. These indicate an increase in activation of both platelets and clotting factors. This might be expected if DPPA is present after irradiation, as suggested by the TLC and FTIR results. Similar changes in TLC and FTIR are seen for DSPC in the short term but interestingly not in the SEM's.

The SEMs for DAPC show a change after irradiation, with the material appearing to crack and flake readily. This may be due to the movement that occurs during the preparation of the SEM stubs or during transit by post to and from Isotron PLC. The irradiation also completes the polymerisation of the DAPC, and it may be that the fully polymerised form is less flexible than the monomeric or mixed forms of DAPC.

The MTEG's for DAPC shows a similar level of change for Mr and MA° as DPPC, but this is not so pronounced for Mma. While the TLC and FTIR evidence suggests less damage for DAPC than DPPC (where the changes are

extensive), the MTEG results reflect similar deterioration in the haemocompatible properties. While these two phospholipids are similar it is interesting to speculate on the role of the unpolymerised acetylenic bonds in reducing the change in Mma. The polymerisation of these bonds may reduce the damage to the PC groups by absorbing the energy of the gamma rays compared to the DPPC. This may be the ability to consume or absorb the free radicals generated, without chemical disruption of the lipid.

The SM coated grafts show little change by SEM after irradiation. The TLC and FTIR show some effects only when water is present in excess. The MTEG results show a change in the Mr, with small changes in the MA°'s and Mma's. It should be noted that these changes are the smallest for any of the irradiated materials examined.

Polyester G shows no change in morphology as a result of the irradiation by SEM. The SEM's also show that Polyester G possesses the best flow surface on the grafts of these materials. The Mr result for the irradiated Polyester G is disappointing, as it demonstrates a thrombogenicity that is significantly worse than the plastic cuvettes ($p<0.0032$). Although this is not confirmed by the Mr+k and Mk results, the change in MA° does suggest that irradiation causes a major change in the thrombogenicity towards the clotting factors. The effects on Mma and MA° do not alter this material's ranking for these parameters compared to PTFE.

The phospholipids with the simple glycerol and ester linkages (DPPC, and DSPC) are very prone to breakdown on exposure to gamma irradiation. The TLC and FTIR evidence suggests a reaction that involves water molecules, and a mechanism related to free radicals. The various reactions possible are summarised in Appendix 3, table A 3.1 on page 241.

For DPPC the reaction occurs at least two sites in the molecule,

a) the ester linkage from glycerol to palmitic acid and,

b) the phosphorus choline link.

These reactions are similar to those proposed for aqueous systems. For example Katsaras (et al 1986) showed that DPPC in aqueous bilayers decomposed to a

palmitic acid salt and glycerophosphatidylcholine (GPC). These workers also considered the lyso phosphatidyl choline to be more susceptible to damage than DPPC, and it would be so rapidly broken down into GPC and the palmitic acid salt, that it would not be detected. Phosphatidyl choline liposomes rapidly generated lipid hydroperoxides after irradiation (O'Connell and Garner 1983). In addition the changes seen in this study could not be prevented by the addition of the free radical scavengers mannitol and sodium benzoate. Sphingomyelin on the other hand has a fixed lipid chain, and an amide bond to the variable fatty acid chain, making it more stable. However the second reaction, at the phosphorous choline link, may be still be important for this type of lipid.

Gamma irradiation generates free radicals, particularly in water, and is often used as a means of generating free radicals (Karsaras et al 1986). It is known to affect the structure of PET fibres, and methylene and oxide radicals are thought to be responsible (Nair et al 1988). These studies used conventional doses for sterilisation, around 2.5 MRad, and showed an increase in crystallinity and breaking load of the fibres. At higher doses of radiation the breaking load fell, with a fall in the molecular weight of the polymer. It seems that the carboxylate group, present in both PET and at the ester site in the phospholipids, is particularly vulnerable.

4.5.4 Conclusions for irradiation

For the three materials, DPPC, DAPC and DSPC the FTIR and TLC results both show major changes when water is present in excess. The additional step of high vacuum desiccation in the preparation of the grafts does not prevent these problems. This may be because the lipids are known to retain some water even after exposure to high vacuum, with $P_2 O_5$. The infra-red spectra of Dacron indicate that it too contains some water. Hence despite the desiccation, both the lipid and the graft may serve as a source for variable amounts of water. Gamma radiation is also known to modify the structure of polyethylene terephthalate (PET) due to the production of radicals from the material itself, such as the methylene and oxide species (Nair et al 1988).

All the MTEG evidence for the ester PC's (DPPC, DAPC, and polyester G) shows similar changes particularly in the Mr values. The results indicate a detrimental effect on their haemocompatibility. These three materials all have a common feature, that of the ester linkage to the lipid side chains, suggesting this region of these molecules is affected by the same mechanism during exposure to gamma rays. It also may be that this group is also responsible for the 'good' Mr values seen for these PC lipids. Further support for this view comes from the relative sparing of SM from this change in Mr, considering that it has an amide linkage.

4.6 Summary and conclusions

These experiments are presented to test the assumption that the coating is adequate, and confirm that the coating materials themselves are unaffected by sterilisation. These proved to be important assumptions to test. The work progressed into an investigation into the effects of sterilisation, particularly of gamma irradiation, on these materials. The initial results demonstrate that considerable changes have taken place after the 3 modes of sterilisation. This led to a more systematic investigation which attempted to define the nature and conditions responsible the changes, and so to reduce them.

The SEM's establish the physical nature of the flow surface that dip coating produces. It is not surprising that it demonstrates the changes in DPPC after steam sterilisation, since the physical conditions are so severe. Polyester G appears to have the best flow surface.

The TLC and FTIR studies are particularly important in exposing the changes produced by gamma irradiation. The phospholipids with the simple glycerol and ester linkages (DPPC, and DSPC) are most prone to hydrolysis of the ester bond. This reaction almost certainly takes place during the more physically severe conditions of steam sterilisation. These long chain saturated lipids are also very prone to breakdown on exposure to gamma irradiation. This reaction involves free radicals, which may be from water molecules either in lipid or released from Dacron, or generated from the graft material itself. Sphingomyelin appears to be more stable, as it has an amide bond, rather than two esters.

The results for DAPC demonstrate that it can be polymerised on the grafts to give a surface covering, but this appears to flake or fracture easily. The chemical changes in DPPC, to which DAPC is closely related chemically, to do not seem to take place during irradiation, or if they occur they do so to a lesser extent. This may be explained by the diacetylenic group 'mopping up' the free radicals, and the change in colour noted after irradiation of the monomeric form of DAPC supports this view. The MTEG changes, do however suggest some decomposition, particularly as they are similar to those seen with DPPC.

The previous work in the field of irradiation has been conducted mostly with either aqueous systems or with solid materials. This study used solid materials deposited as thin films. This produces a very large surface area, and may change the types of chemical reaction that can occur. Clearly further work is needed to investigate and clarify all these issues.

The MTEG results for these materials demonstrate the value of this technique in detecting small changes in thrombogenicity. Some of the changes are in fact quite large, particularly for the Mr, reflecting the activation of clotting factors. The technique is more sensitive to these changes than methods detecting changes in the chemical structures, such as TLC and FTIR. Any further experimental work which involves any significant preparation of the materials such as coating, or sterilisation must pay careful attention to the conditions that the materials are exposed to. These conditions and their effects on the materials need to be fully documented and reported.

The results for the irradiated materials relative to Dacron and to PTFE show that the changes induced by irradiation may not be significant in the *in vivo* setting, considering how well PTFE is tolerated. The experiments needed to test this lie outside the scope of this thesis, but clearly represent a critical line of enquiry for any commercial development of these materials.

One of the criteria for suitability for use as a biomaterial includes ease of fabrication and the capability of withstanding sterilisation (Scales 1953, Table 1.5, Bruck 1972, Table 1.6). These materials appear to have the ability to perform the

function required of them, in terms of low thrombogenicity, but need further development to be clinical useful.

The decision to use DPPC in chapter 3 has been revised in favour of SM, as all its MTEG parameters are slightly better, and more importantly it is less prone to radiation damage. However to test the hypothesis adequately it has been decided not to sterilise the grafts coated with SM, or Polyester G. The grafts are prepared after boiling in chloroform, and coated with these materials from chloroform solutions. It is likely that this preparation is 'clean', if not bacteriostatic. However formal bacteriological testing was not undertaken. The DAPC coated grafts are gamma irradiated to ensure full polymerisation of the monomer within the lumen of the graft.

outline

Chapter 5

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Chapter 5

In vivo assessment of materials

5.1 Introduction

The *in vivo* experiment has always been a necessary stepping stone between the laboratory and the clinical trial in man, and is likely to remain so. This chapter will outline some of the hazards the use of these stepping stones exposes the investigator to whilst crossing this poorly charted sea.

The rat and the dog have been the traditional choice of animal for both *in vivo* and *ex vivo* experiments, as well as the source of tissue for *in vitro* tests. The limitations of extrapolating data from the animal kingdom to the clinical setting vary depending on the area of study. They are more widely recognised in the field of biomaterial research because of the complexity of the interactions between the host and the biomaterial. The aim of this chapter is outline these problems, and to justify the choice of the species and experimental design used in chapter 6.

5.2 Historic aspects

The early investigators such as Carrel were more concerned with the technique rather than the evaluation of different materials. Even the vascular pioneers in the 1950's carried out few animal experiments before proceeding to clinical use of the material. The use of Vinyon N by Voorhees (Voorhees et al 1952) in 15 dogs was soon followed by its use in 18 patients reported in 1954 for abdominal aortic aneurysms (Blakemore and Voorhees 1954). The same is true of the arterial homograft. Although used by Carrel, Gross reported the results of its use in dogs and patients together (Gross et al 1949). These were followed by reports of thrombosis and aneurysm formation (Szilagyi et al 1957). The dog thoraco abdominal bypass was used by these and other investigators following the report of McCune. He had pointed out that the vessel in this model was about the same size as the human femoral artery (McCune and Blades 1951). A later model was reported by Dale and Mavor (1959). They recognised that the aortic replacement

model was limited, since it was 'admittedly easy to accomplish no matter what material was chosen', meaning that all the grafts remained patent. This report covered aorto iliac, ilio femoral and femoro femoral bypasses. They presented 153 dog experiments using these much more severe tests, where patency was the end point. They concluded that the peripheral grafts were a much better differential test of the materials.

5.3 Healing of the arterial prosthesis

The flawed nature of the early work in the 1950's can only be appreciated with hindsight, these contributions were outstanding in the light of the contemporary understanding of the problem. The mechanisms of graft healing were later appreciated, and the lack of healing was demonstrated in man by Berger et al 1972. They reported the findings in 23 human prosthetic grafts implanted for periods ranging from 16 days to 11 years. The glistening white flow surface of tissue which had been seen on grafts was in fact compacted acellular fibrin. Healing with an endothelial cell lining extends only for the first 1 cm from the suture line. Observations and comments on the porosity of the grafts used and reported on in this paper reflect the belief that healing should occur through the walls of the graft. This however does not take place. This paper also showed that pigs were able to cover 6 cm of an impervious graft with an endothelial cell lining within 60 days. The same group underlined the differences in healing between man and other species (Sauvage et al 1974). This report covered 14 years of work, in man, dog, pig, calf and baboon with porous and impervious grafts. Silicone elastomer used to prevent tissue in growth, and so show that healing from the suture line was most rapid in the pig, followed by the calf and then the dog. When porous grafts were used healing was again most rapid in the pig, calf and baboon, taking 6 weeks. However the pig also demonstrated marked calcification of the grafts. The dog was slower taking 3 months, but was still faster than man. Despite their preference for the dog these authors also point out the increased levels of fibrinogen and fibrinolytic activity in the dog. They attributed the episodes of delayed haemorrhage they observed with the microknit graft to this.

Further evidence for the lack of healing was shown by McCollum et al 1981. They

observed marked differences in platelet and fibrinogen turnover in the first 3 months after grafting. Dacron, PTFE and saphenous vein have markedly different platelet uptakes (Goldman et al 1982b). The benefit of knitted Dacron with its improved tissue incorporation was also examined by this group (Goldman et al 1982a). There was no difference between knitted and woven grafts shortly after implantation, or later at 6-9 months. Although compared to the first week, there was reduced uptake of platelets after 6-9 months. This increased uptake on mature Dacron grafts was reported 5 and 9 years after implantation, implying that these grafts never heal (Goldman et al 1982c). These observations have also spurred on the investigations in the field of endothelial cell seeding outlined in chapter 1.

5.4 How appropriate are the current models of vascular grafting?

5.4.1 Design considerations

For both the model and the species used there are practical considerations in terms of the size of the vessels, and the availability of the graft materials. Commercial sources are restricted to sizes that can be marketed for use in man, for Dacron this is 4 to 5 mm internal diameter. Grafts smaller than this require microvascular techniques, which are different from those used for the most distal anastomosis in man. The question of size is not just one of convenience, but is fundamental to the test. The graft must be tested in a similar haemodynamic setting to its final use (NIH 1985). This reflects all the poorly understood contributions of compliance, flow geometry and turbulence to the development of neointimial hyperplasia. Dale's model addressed these issues in terms of patency and the size of the vessel. Indeed this assessment of patency over three months in the dog has become one of the standard yard sticks of vascular prosthetic materials. Lately other models have been advocated using the carotid artery of sheep and the dog, as well as chronic arteriovenous fistulae in the baboon. The analysis in these models is more sophisticated, and looks at the fundamental mechanisms underlying graft failure. See 5.4.3 below.

The use of each animal as its own control, or to compare two materials, has

serious disadvantages. The 'back to back' design assumes that one graft has no effect on the other. Matsumoto et al 1974 in demonstrating the difference between woven (Teflon grafts) and extruded PTFE (Gore-Tex), showed one Teflon graft remained patent 3 months longer in a 'back to back' animal than in the other controls. Allen et al 1986 studied multiple graft types in single animals, and noted that when one graft failed, so did the others. This type of design, although economic, is best avoided.

5.4.2 Haemostatic considerations

The differences in coagulation and platelet function between the species has long been recognised. The NIH report (NIH 1985) comments on this problem, and while there are comparative reviews for platelet function (Dodds 1978), coagulation (Didisheim et al 1959) and the fibrinolytic pathways, there remain discrepancies and contradictions in the earlier literature. Animal platelets are sensitive to heparin (especially dog's which aggregate in response to heparin) and respond in a different way from man to both agonists and antagonists. Furthermore very few comparative studies have been undertaken in other areas, such as endothelial cell physiology.

The significance of these differences to biomaterial research are difficult to assess. For example Schulze demonstrated the effect of species differences *in vitro* using MTEG (Schulze et al 1983). There were wide variations in the results, the calf and goat showing less activation than man or the dog. The relative rankings of the four materials he studied remained the same, although the TEG parameters gave poor discrimination between the materials using dog and goat blood. Similarly in a larger study Didisheim (et al 1984) compared the relative ranking of 5 biomaterials in 13 short term *in vitro* and *ex vivo* studies, and the rankings were not species dependent.

Grabowski (et al 1977) on the other hand examined platelet adhesion on a range of biomaterials in a controlled flow system with shear rates comparable to those found in human arteries. They compared human blood with that of 7 other large laboratory animals. There were major differences between man and the dog or

rabbit for Cuprophan. The differences were biomaterial dependent, as the dog and human had the same response to PTFE.

These illustrations emphasise the need to review the haematology of the baboon, the dog and the sheep, the three species widely advocated as models for biomaterial assessment, particularly for graft materials (see 5.4.3).

Firstly human platelets are unique in their release mechanism in response to adrenalin and ADP. Baboon platelets are not inhibited by adenosine, or dipyridamole (Dodds 1978). Monkeys have a shorter bleeding time, as well as reduced platelet sensitivity to ADP and release less platelet factor 4 (PF4) from their activated platelets (Addonizio et al 1978). The primates have the added practical problems of housing, and the regulations concerning their use (and the increase in associated costs).

Dogs represent a particular problem, in that their platelets aggregate when exposed to heparin (Dodds 1978), and contain large amounts of serotonin (5HT). In addition they have increased levels of fibrinogen, prothrombin, factor VIII, and a rapid fibrinolytic system. The NIH (1985) point out that they are probably not the species of choice, since they have a low susceptibility to vascular disease and pulmonary embolism. They have very active haemostatic and fibrinolytic systems. In addition they have a 'hyperplastic vascular response to incitants causing rapid occlusion of vessels'. However there is a large literature, and experience with the dog that argues in favour of its continued use.

In passing the other large animals have no advantages. The pig rapidly heals vascular grafts despite its clotting being close to man. The rabbit, although its aorta is a suitable size, is unable to tolerate aortic cross clamping.

The sheep platelets do not respond to adrenalin or ADP. Tillman (et al 1981) reports that their PT, aPTT and activated clotting times are 'comparable' with man, but also showed differences in platelet aggregation to collagen and ristocetin. Gajewski and Povar (1971) however showed increased levels of factors V and VIII (supported by Didisheim et al 1959 and, Fantl and Ward 1960) and reduced

plasminogen and fibrinolytic activity (supported by Fantl and Ward 1960, and Gehweiler et al 1983).

There is no ideal species, in terms of coagulation, nor in terms of the rates of graft healing. Ultimately man will have to be his own experimental model!

5.4.3 New models for vascular graft assessment

In addition to the dog model, two other models have gained popularity. These models are the primate arterio-venous fistula (AVF) and those which use the sheep carotid artery.

Hanson has been the principle advocate of the baboon model, using the chronic AVF (Harker et al 1977). In 1977 he showed platelet survival and turnover were useful markers in the assessment of the thrombogenicity of materials. In 1979 the water content of polyacrylamide hydrogels was investigated by platelet consumption (Hanson et al 1979). The role of platelets in the early healing of grafts was further investigated by Callow (et al 1980). They even advocated the use of this model for the assessment of pharmacological interventions as a means to prolong graft patency. Hoffman (et al 1982) used it to demonstrate platelet micro emboli from polyacrylamide-silastic hydrogels.

The use of radio-labelled platelets has been a major advance in improving our understanding of the early events of graft healing. The quantification of *in vivo* platelet uptake correlates well with the direct measurement on explanted grafts. These quantitative methods may require the use of a second isotope, to estimate the blood pool, for the best results (Allen et al 1986). The 2 and 24 hour deposition of platelets in this study predicted later patency at one month.

In addition to platelets, fibrin plays a important part in determining the fate of vascular grafts. For this reason fibrinogen kinetics have been studied by Hanson's group (Horbett et al 1986). More recently the thromboembolic potential of PTFE and Dacron have been compared using both radiolabelled platelets and the measurement of circulating beta thromboglobulin levels (Schneider et al 1989).

Lindon (et al 1980) demonstrated the value of the sheep AVF in assessing 13 materials, by measurement of platelet survival (using similar techniques to Hanson and Harker). They also pointed out the low cost of sheep and the relative ease of handling them. Their test is much more severe than those in the baboon since their shunts were 1.2-1.5 meters in length compared to 7 cm used in the baboon. As alluded to above Tillman and Carson have been strong advocates of this model (Tillman et al 1981, and Carson et al 1981). They have reported sheep coagulation as being similar to man, and showed that the parameters they measured were not affected by surgery or anaesthesia.

Carson suggested that the model should be used to assess the effect of antithrombotic therapy on graft patency. Further work by this group investigated the role of the drug ibuprofen on PTFE grafts (Hunter and Carson 1984). They chose the sheep because of its disposition 'in their experience' to develop anastomotic intimal hyperplasia, and thrombose more readily than the dog. It must be suspected that this really reflects more the differences in the sheep's fibrinolytic system (which is less active compared to man's), than its propensity to form neointimial hyperplasia on grafts. They showed that the treatment changed circulating levels of 6 keto prostaglandin I alpha (PGI_2) and thromboxane beta 2 (TxB_2). They also showed that the sheep arteries and the PTFE grafts were able to generate both substances when explanted and studied *in vitro*. The occluded grafts produced more TxA_2 than PGI_2 . From these observation there is good reason to suggest that the sheep is a better model than the dog for the study of vascular prosthesis.

Esquivel (et al 1984) used the sheep but this model adds controlled flow conditions, and only lasts 4 hours. 6 cm by 4 mm diameter grafts are placed end to end in the carotid artery. Flow was regulated with an occluder on the distal artery, and measured using an electromagnetic flow meter. The rate of flow was reduced to 25 ml/min, 1/6th the normal rate, and this is said to mimic the poor run off conditions seen in vascular patients. They evaluated heparin bonded to PTFE in this model by thrombus formation and platelet uptake on the material. This was a wise choice given the dog's response to heparin.

Bjorck (et al 1985) also from this group examined the role of heparin bonding to human umbilical vein grafts. They showed platelet uptake over 4 hours was less in the alcohol-heparin bonded grafts than in the covalently bound heparin grafts. In addition they compared the *in vitro* uptake of thrombin by native sheep vessels and heparin bonded PTFE. They showed that the artery and the heparin bonded PTFE graft inhibited thrombin more than the vein. However this property of the artery could not be abolished by protamine, but the treated PTFE could be (Bjorck et al 1986). In 1986 they examined a compliant polyurethane and compared it with autologous vein (Lannerstad et al 1986). They found more platelet uptake on the graft, but similar patency to the vein. Christenson (et al 1987) studied the role of early platelet deposition and its distribution on both silk and PTFE over 3 days, using indium labelled platelets. The results were similar except there was increased deposition at low flow rates. All the prosthetic materials had heavier deposition at the distal anastomosis, compared to the lighter and more even deposition throughout the vein.

5.4.4 Remaining pitfalls

The differences in coagulation between the species has been outlined and the effects on biomaterial evaluation documented (Schulze et al 1983, Grabowski et al 1977). Unlike Grabowski the study by Didisheim (et al 1984) did not show species dependence for ranking the materials. The primary aim of this study was to show the value of these test in predicting *in vivo* outcome. They concluded that the *in vitro* tests were of no value for this. They had used 13 *in vitro* tests (all but one, which lasted 3 hours, were of very short duration ie 5 minutes). The longer term test of *in vivo* compatibility of the 5 materials was based on the appearance of implanted patches at 3 and 12 months in pigs and dogs. This conclusion is not surprising considering how different the major mechanisms of failure are. For grafts they are principally thromboembolic, the development of neointimial hyperplasia, and mechanical failure of the graft. This study is an important warning, that *in vitro* studies do not necessarily predict the *in vivo* outcomes. Indeed there are very few studies like this which prospectively study *in vitro* and *in vivo* properties of materials to enable the prediction of long term *in vivo* outcomes.

One of the advantages of the dog model is that it has been widely used, so that with back extrapolation of the results of clinical studies the model can be validated retrospectively. However there are weaknesses in this approach too. Consider the poor correlation between Herring's initial report for endothelial seeding in the dog (Herring et al 1978) and the outcome in man (Herring et al 1987). There is now evidence that the dog and sheep also differ in their outcomes for endothelial cell seeding of PTFE grafts (Ortenwall et al 1988). This shows that even autologous tissues do not give the same results in different species.

Another aspect of the problem of choosing an animal model apart from the scientific criteria is the simple factor of the size of the animal. A large animal requires appropriate holding facilities, and equipment. The capital cost of these and the running costs of experiments that use larger animals is becoming prohibitive. The institution needs to have established facilities and a track record of this type of research, for there to be a good likelihood of obtaining funding for such projects.

Ratner's critique of biomaterial testing (Ratner 1984) both underlines these problems and argues that "A rational choice of an animal model will aid in the assessment of the significance of blood-material interaction data as it applies to specific applications in humans." Despite this gloomy review, is it possible to design an appropriate model?

5.5 Choice of model and species

The model must have some value based on previous studies in the literature, so limiting the choice to the baboon, the dog, and the sheep. There is a choice of end points to measure. These are the effect of the material on platelets, clotting factors, and fibrinogen, or the more clinical measures of blood flow and patency in both the short and long term. The aim of this study is to evaluate the thrombogenicity of the PC containing materials, as a means to testing the hypothesis presented in chapter 2. The sheep has a poor fibrinolytic system, so that blood clots that are formed will not be removed by fibrinolysis, and has an increased thrombotic tendency (see Hunter and Carson 1984, Ortenwall et al 1988

etc). The flow rate through the material should be high in order to activate both platelets and fibrinogen, rather than the restricted low flows rates, which are more appropriate for patency studies. To achieve this goal the arteriovenous fistula has been chosen. This model has the advantage in that thrombus emboli breaking off from the graft material will become trapped in the capillary bed of the lungs, and are easier to find than within the brain. In addition the model should allow detection of small changes in the parameters studied. If a non-thrombogenic material were to be used the amount of the reduction in thrombogenicity of the PC coatings may be very large, but in the context of low uptake by the control material would be undetectable. To avoid this problem the most thrombogenic material available has been chosen, namely Dacron. This now makes the model a severe test, where thrombosis is likely to occur. If the test coatings of the phospholipids are effective they will be able to reduce this. This theoretical background provides the basis for the choice of the model used, that is of a Dacron arteriovenous fistula, in the neck of the sheep.

outline

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Chapter 6

The in vivo experiment : The arteriovenous fistula in the sheep

6.1 Introduction and description of the animal model

A brief outline of the model is given here before a detailed description of the model and methods used (in sections 6.2 and 6.3). This is because a number of preliminary experiments have been performed to establish the methods and the final protocol for the animal model. As this is a new model the results of these experiments are reported in some detail, but in Appendix 4 (page 246) and Appendix 5 (page 255). They include the experiments used to establish the length of graft used, the platelet labelling, the phantom design, and the validation of the methods for measurement of haemolysis and the fibrinogen label. The refined model for the definitive set of experiments is given in 6.2. The methods used for these are presented in 6.3, and include the statistical design in 6.3.6. The results are presented in 6.4.

The Dacron graft material has been coated with the three test materials. These materials are the phospholipid sphingomyelin, the polymerizable phospholipid DAPC, and Polyester G. The rationale for the choice of these materials has been discussed at the end of chapter 3 and have been revised at the end of chapter 4. These three materials are compared with the uncoated graft. There are thus four groups of animals.

Under a general anaesthetic an arterial line is placed in the hindleg and a venous line in the "axilla" of the sheep's foreleg. The graft, 4 cm of Dacron (USCI BARD soft woven, see section 4.2.1 for details about the graft material, page 110), is sutured between the carotid artery and the jugular vein, across the neck. Indium 111 labelled platelets and iodine 125 labelled fibrinogen are injected. The fate of these labels is followed over 24 hours. Gamma scintigraphy images are acquired from the indium label at 4 and 24 hours.

6.2 The animal model

6.2.1 The anaesthetic procedures

The animals are kept well nourished until 24 hours prior to use, at 15-18°C, in controlled environment rooms, with 20 air changes per hour. A 21 g butterfly cannula, placed in the foreleg, is used to give between 10 and 30 ml of thiopentone to induce anaesthesia. Once induced the animal is intubated with a size 9 cuffed endotracheal tube. The animals are maintained under a general anaesthetic with oxygen, nitrous oxide and halothane. Atropine is given prior to induction and after every 4 hours, to reduce the otherwise prolific salivation. 2 doses of prophylactic antibiotics (cefuroxime 750 mg 'Zinacef' Glaxo, courtesy of Ken Scott) are given intra muscularly, one at the start of the procedure, and the second just prior to the acquisition of the 4 hour images. The anaesthetic is continued after the surgical procedures, the injection of the labels, and until the 4 hour images are completed. The animals are then recovered with pure oxygen and occasionally with small amounts of carbon dioxide, once able to swallow or chew the tube is removed.

The sheep is a ruminant animal and special precautions are needed to prevent 'bloat', that is the rapid expansion of the rumen with its gases of fermentation. This not only prevents respiration, by splinting the diaphragm, but also is likely to perforate the rumen. To prevent this the animals are restricted to water for 24 hours before operation. The rumen is emptied after the endotracheal tube is passed, and maintained empty with an oro-rumen tube (Silastic hose pipe, internal diameter 1 cm, 40 F gauge), the tip positioned some 80-85 cm beyond the teeth. A trocar to puncture to rumen percutaneously should always be to hand, but as yet has not been needed.

For the acquisition of the 24 hour images, the animal is sedated and anaesthetized with intravenous Sagittal (pentobarbitone 60 mg/ml, May and Baker, diluted to 50% with normal saline). The rumen presents no problems during this short period.

6.2.2 The operative procedures

The animal is positioned on its back, and supported with sandbags. The wool around the neck, fore and hind legs and the right axilla and thigh is clipped off. Using full aseptic techniques the skin is prepared with Povidone Iodine surgical scrub (Povidone Iodine 7.5% w/v, 0.75% available iodine, RFHSM Pharmacy) and the animal draped. An 18 gauge cannula (Abbocath iv cannula Abbott) is placed in a branch of the right femoral artery by a small incision and tied in place. This is the saphenous artery, and is a direct branch of the femoral artery (anatomical terms from Nomina Anatomica Veterina, quoted in Ghoshal et al 1981, see introduction, and p211). There is no large human counterpart to this vessel, which passes from beneath the belly of the sartorius muscle to course superficially on the medial aspect of the thigh. (In three animals it passed superficial to this muscular slip as well.) It is accompanied by the medial saphenous vein.

The right axilla is opened to expose the brachial vein and artery. The vein is cannulated with an 8 F 30 cm intravenous catheter (Portex serial No 200/300/070). The tip of the catheter is passed proximally via the axillary vein to lie in the subclavian vein and tied in place. The luer connection is tunnelled beneath the shoulder joint and emerges on the lateral side of the neck. This is connected to a three way tap and then to an intravenous giving set. The brachial/axillary artery is subsequently used for the angiogram catheter (see below 6.2.5 and Appendix 4, section A 4.4, page 248).

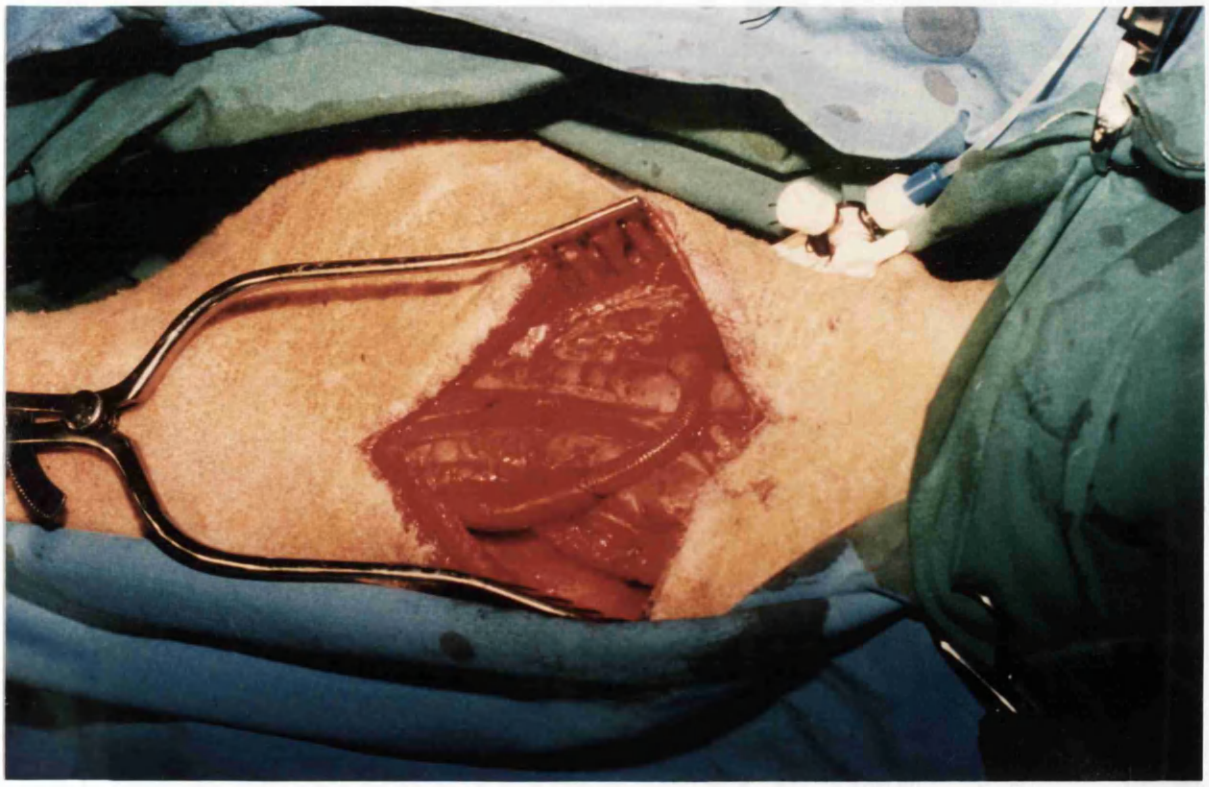
The carotid artery on the right side of the neck is approached via a midline neck incision, and about 6-8 cm exposed in the lower part of the neck. The left jugular vein is similarly exposed (it is called the external, as there is no internal jugular vein in the sheep, Ghoshal et al 1981 see p40). Branches are carefully ligated with 3/0 silk ties. The previously prepared graft is then cut to 4 cm length, using a vernier calliper, and the remainder saved in case further chemical analysis is needed. The carotid is clamped (DeBakey bulldog), ligated distally and an end to end anastomosis fashioned to the graft with interrupted 6/0 sutures of monofilament polypropylene (Ethicon Prolene W8718). The jugular vein is then clamped, ligated and anastomosed in the same way to the other end of the graft.

The clamps are released, and the time of reperfusion noted. There is usually little blood loss at this stage and only occasionally has a further stitch been needed in the anastomosis. A typical completed graft placement is shown in Photograph 6.1.

6.2.3 Monitoring and fluid replacement

The arterial line, in the saphenous artery is used to record the blood pressure, and ECG electrodes to record the ECG and pulse. The arterial line is maintained under arterial pressure, but occasionally has to be cleared with normal saline which contains 500 units of heparin in 500 ml, no more than 50 ml of this has been used. The temperature is monitored using a rectal probe thermometer.

The venous line, in the axillary vein is used to monitor the central venous pressure. However its principal use is for blood sampling and fluid replacement. 500 ml of Hartmann's is given during the operative phase, and 200-400 ml of 5% dextrose during the 4 hours anaesthesia leading up to the 4 hour image.



Photograph 6.1 A completed anastomosis in the sheep neck.

6.2.4 Blood sampling

Blood samples are taken from the venous line via the three way tap at the end of the cannula. The first 5 ml of blood is always discarded before the sample is taken. A 'flush' of fluid, approximately the volume withdrawn, is given after each sample to clear the line of blood. The sampling times are shown in Table 6.1.

Blood sampling times

1 At start of operation:- EDTA 10 ml heparin
2 Blood labelling sample:- EDTA + 20 ml ACD
(taken after completion of anastomosis)
3 Base line pre injections:- EDTA 10 ml heparin
4 Injection of labelled platelets (111 In) fibrinogen (125 I) and Evans Blue.
5 Samples times after injection of labels are for the

First hour, at	5 minutes sample of	2 ml hep	
	10	2 ml hep	EDTA
	15	2 ml hep	
	20	2 ml hep	EDTA
	25	2 ml hep	
	30	2 ml hep	EDTA
At the following hours	1		EDTA
	2		EDTA
	4	2 ml hep	EDTA
	6		EDTA
	8		EDTA
	10/12		EDTA
	18		EDTA
	20		EDTA
	22		EDTA
	24		EDTA

EDTA = 1.5 ml blood in ethylene diamine tetra-acetate
(platelet and fibrinogen counts).
hep = 1.8 ml in heparin
(double centrifuge for haemolysis and Evans blue assays)
ACD = acid citrate dextrose, for platelet labelling.

Table 6.1 Blood sampling protocol.

6.2.5 Angiographic procedure

In order to exclude technical errors in the anastomosis an on table arteriogram is carried out. The previously exposed right brachial artery is tied off distally when the radiographer is in theatre. The vessel is then cannulated with a catheter and trocar assembly (Lunderquist percutaneous transhepatic catheter, 18 G trocar, 5.4 F x 25 cm catheter Meadox cat No. 853050). The catheter is tied in place after removing the trocar, attaching a 3 way tap, and flushing with 5 ml of heparin/saline (500 units in 500 ml). The animal is then moved to the X-ray suite (adjacent to the theatre suite). Once positioned over the screening unit (Siemens Tridoros SS) and under image intensification a guide wire is passed to region of thoracic spine about the level of 1st rib. The catheter is advanced over the wire to this level. The wire is then withdrawn, the 3 way tap replaced and the catheter flushed with 5 ml of heparin/saline. The animal is then positioned over the AOT Unit (automatic film changer, AOT Elema Schonander, Sweden) and a control film taken for the subtraction mask (the X-ray exposure factors are typically kV 70 and mAs 20). 10 ml of Conray 280 (Conray, sodium iothalanate 70% wt/vol, iodine 420 mg/ml, May and Baker) is injected and 6 films (35 cm CRONEX 7L, 14" X-ray film) are taken at 2 frames per second for 3 seconds. If the run appears successful, the catheter is removed and the vessel ligated proximal to the puncture site. If a problem with the film changer or the timing of the injection is suspected a second run is performed (this has happened twice). The films are processed and subtraction views made. see Appendix 4, section A 4.4, page 248.

6.2.6 Injection techniques for isotopes

The usual vein for the injection of the radiolabelled compounds and Evans Blue is the saphenous vein in the left thigh. Alternatives sites have been used, and are the saphenous vein in the right thigh, and the superficial saphenous vein above the knee. A 19 G butterfly cannula is positioned in vein and flushed with saline. The radiolabelled compounds and Evans Blue are injected with flushes of saline between each, and a final flush of 20 ml of saline to wash the material into the central veins.

6.2.7 The gamma camera imaging procedure

At 4 and 24 hour after the injection of the 111 In, gamma camera images are acquired. An Elscint CE1 Gamma camera is used, connected to an Elscint Dycomette computer. The keV (k electron volts) energy window is adjusted to the lower energy peak of indium 111, at 173 keV. For this instrument the settings are 150 keV, with a window of 30 keV, and are set using the indium standard as the calibration source. The images usually are acquired over multiples of 150 seconds (2.5 minutes), the actual time determined by the amount of activity present. These are then stored on 8" floppy discs for later analysis. The technique for analysis is presented in 6.3.3.7.

A perspex phantom approximating the dimensions of a sheep's neck is used to calibrate the images, with about 1/50th of the injected dose of indium 111. The design of the phantom is considered in Appendix 4, section A 4.7, page 252, and the need for, and use of the standards is explained below in 6.3.3.3. Images are taken of the thorax, liver, spleen and several views of the neck. To ensure uniformity between the images of different animals standard positions were chosen that could be reproduced for each animal. These are shown in Figures 6.1 to 6.4 and discussed more fully in section 6.3.3.7.

6.2.8 Collection of histological material

After the 24 hour images are recorded the animal is killed with Euthantal (Pentobarbitone sodium, 200 mg/ml, May and Baker). The neck incision is opened and the graft exposed. The artery proximal to the graft is cannulated with large bore needle (a modified air inlet) to permit the perfusion of the graft with formaldehyde saline at arterial pressure. This is achieved by placing the reservoir of formalin at 1.5 m above the animal (to give a pressure of approximately 110 mmHg), and ligating the vein well beyond the graft, once blood in the graft (but not adherent clot) is washed out. The perfusion is continued while the post mortem of the animal is completed, about 5 minutes. The graft is then carefully removed with a cuff of both artery and vein of about 0.5 cm, and placed in an LP4 tube (Luckham LIP plastic tubes) filled with formalin.

The organs are removed and weighed wet, once drained crudely of blood, and in the case of the liver, of bile. The individual organs are biopsied using a punch specially designed by the author. This produces a core of tissue, which fits into the LP4 tube and enables the tissue to be placed in the tubes, without gross contamination of their outsides. The weight of the biopsy is obtained by using previously weighed LP4 tubes and re-weighing them. The kidneys are weighed and counted together. The lungs are individually weighed, and slices from the midzone taken for counting. They are then examined macroscopically for evidence of emboli by sectioning into slices about 1 cm thick.

6.3 Methods

6.3.1 Measurement of haemolysis

6.3.1.1 Introduction

The aim of this assay is to detect haemolysis as a result of graft implantation, and the coating materials used. Previous work with heparin bonded grafts caused haemolysis (Solen et al 1980). In addition it is used to detect haemolysis from the platelet and fibrinogen labelling procedures, if present.

The standard assays used in clinical practice could not be used. These are the measurement of serum haptoglobin and the ICSH reference method (International Committee for Standardisation in Haematology, ICSH 1978). The antibody used for the haptoglobin assay is specific for human haptoglobin, and does not cross-react with the sheep's. The ICSH method uses the wavelength of 540 nm and is affected by the use of Evans blue. A standard method used in the RFHSM Dept. of Haematology has been employed (this was developed after the carcinogenic risk of the toluene blue assay was recognised). This method is unpublished but is a spectroscopic method based on the following principle. Haemoglobin absorbs light at 413 nm, which is measured in a spectrophotometer. A correction factor for the adsorbance at 413 nm due to the presence of other pigments (such as bilirubin and myoglobin) is measured at 453 nm and is applied to 413 nm reading. The free haemoglobin in mg/100 ml is given by :-

[(OD 413 nm x 13.5)-(OD 453 nm x 8.4) x dilution factor of plasma]

The figures '13.5' and '8.4' in the calculation are the molecular extinction coefficients for oxyhaemoglobin and bilirubin and give the concentration of the substances (see van Kampen and Zijlstra 1965). The normal range of free plasma haemoglobin by this method is 0-15 mg/100 ml for man.

6.3.1.2 Haemolysis assay method

Blood is collected from the venous line into heparin coated tubes, and the samples are prepared by double centrifugation to remove any whole cells from the plasma. (MSE Minor centrifuge 10 min @ No 6, 2700 RPM by direct measurement with a rev. counter, equivalent to 500 G). The plasma obtained is diluted 1:5 with saline (0.85%), and read against a saline blank, at 453 nm (slit width 0.03 mm) and 413 nm (slit width 0.0425 mm) in a Pye Unicam spectrophotometer (Model SP 500 series 2, VU and visible spectrophotometer, Pye Unicam, Cambridge UK. Sarstedt plastic cuvettes are used, cat No 67.742). For each assay a standard curve is created using haemolysed blood from the animal's first blood sample. The haemoglobin is determined by counting the EDTA sample (Ortho Diagnostic Systems ELT 800 laser blood counter). The haemolysate is diluted to give 6 standard samples with a 1:5 dilution of plasma with saline. These range between 2.5 and 80 mg/100 ml. The detailed protocol and the results used to establish the method's suitability for sheep haemoglobin are presented in the Appendix 4, section A 4.2, page 246.

6.3.2 Estimation of blood volume

6.3.2.1 Introduction

The blood volume is estimated to calculate the volume into which the isotopes are injected. This allows an estimate to be made of the pharmacokinetic recovery of the labelled platelets. This is discussed below in 6.3.3.6. The standard ICSH methods recommend the use of isotopically labelled albumin, but this study already involves two isotopes. Indeed some authors suggest estimating red cell volume

and plasma volume simultaneously, particularly in pathological disease states where the normal relationship between venous and body haematocrits may be invalid (Wright et al 1975). For this study the animals are healthy, and the purpose of estimating the blood volume is to establish the fate of the total isotopic pool. In particular it will be used to show the pharmacokinetic recovery of the platelet and fibrinogen labels and the location of the total dose of the isotopes at the close of the experiment. Multiple blood samples will be taken to prevent the errors related to the use of a single time point sample. To avoid the use of further isotopes and keep the study manageable an older method, once in wide spread use, has been adopted. This utilises the dye Evans blue (also called Azovan blue, T1824, and chemically 4 amino 5 hydroxynaphthalene 1,3 disulphonate). This material has a very strong affinity for albumin, and is distributed in the albumin space. This is considered to be the plasma volume, during the first 30 minutes after injection, before the albumin leaks into the interstitial fluid space and lymphatic fluid. The principle of dye dilution is employed. A known amount of the dye is given intravenously, and from serial blood samples the plasma concentration is measured (spectrophotometrically at 625 nm). The results are plotted semi logarithmically (log concentration against time) and the concentration at the time of injection determined. From this, and the haematocrit of the EDTA blood samples taken at the same time, the blood volume can be determined (see Mollison 1972, Ch3 for details of the method and calculation). Since the maximum absorption varies with the species, and to exclude interference with the haemolysis assay, scanning spectrophotometry (Phillips PU 8700 series UV and visible scanning spectrophotometer, Pye Unicam, Cambridge, UK) was performed. The results are presented in the Appendix 4, section A 4.2, page 246.

6.3.2.2 Blood volume method

A weighed amount of 10 ml of sterile Evans blue solution (Martindale 5 gm/l, with 46.5 gm/l mannitol) is injected at the same time as the isotope labels. The syringe is re-weighed to give the actual amount administered. Heparinised blood samples are taken at 5 minute intervals for 30 minutes. These are treated in the same way as those for the estimation of the plasma haemoglobin. The samples, diluted 1:5

with saline are measured at 625 nm in the Pye Unicam spectrophotometer with a slit width of 0.0625 nm. A standard curve is constructed using Evans Blue diluted 1 in 5 with plasma taken immediately before the injection of the dye. The full protocol is given in the Appendix 4, section A 4.2. page 246.

The calculation is as follows :-

Amount of dye = volume of dye x dye concentration
= weight of dye x dye concentration
= plasma volume x plasma concentration

So that :-

plasma volume = [Weight of injection x conc of injection]/plasma conc.

Linear regression is used to determine the log of the optical density (OD) at the time of injection (see Figures 6.5 and 6.6 in section 6.4.2). The standard curve is used to give the dilution for the OD calculated at the time of injection, and so give the plasma concentration. The estimated blood volume (EBV) is derived from the whole body haematocrit :-

EBV = PV/[100-hbcrit]

However the whole body haematocrit is slightly smaller than the venous haematocrit, so the established correction factor of 0.96 (Mollison 1972) is used :-

EBV = PV/[100-0.96 x hbcrit~venous]

The venous haematocrits are measured on the EDTA blood samples, before they are weighed and haemolysed. The mean haematocrit of the samples taken during the 30 minute collection period following the injection of the isotopes and Evans Blue is used.

6.3.3 The platelet label

6.3.3.1 Introduction

The ideal method for labelling platelets would only label platelets, would irreversibly bind both the ligand and the isotope to the platelets, and would do so without affecting the function of the platelets. There is no such method. Since the labelling methods are relatively non-specific it is necessary to separate platelets from the other cells.

A number of isotopes have been used for platelet labelling, but indium 111 is the best of them. Indium 111 decay produces gamma emissions of 173 keV and 247 keV, these are sufficiently penetrating to allow good gamma camera imaging, and counting. At the same time 111 In has a reasonably short half life (2.8 days), and is non-volatile, making it a relatively safe isotope to handle. These subjects have been well reviewed by Thakur 1983, Mathias & Welch 1984 and Joist et al 1983.

There is a choice of ligands with which the 111 In is attached to the platelets, but the main contenders are oxine advocated by Thakur et al 1976 and tropolone proposed by Dewanjee et al 1981. At the time this work was started there was evidence that the oxine method damaged the cells because of the ethanol required in the labelling solution. The oxine indium 111 complexes are difficult to produce, and these can only be used in plasma free platelet suspensions. This means washing the platelets free of plasma, and involves a further centrifugation step, and the use of prostaglandins as anticoagulants (Thakur et al 1976, Hawker et al 1980). Both these steps cause further damage to the platelets (Hill-Zobel et al 1987). Tropolone on the other hand can be used in a plasma suspension of platelets, avoiding these harmful steps. The tropolone 111 In complex is made simply by adding the two compounds. The disadvantage of the tropolone ligand is a reduced labelling efficiency compared to that obtained with the oxine ligand. This is due to the presence of plasma in the labelling medium binding some of the isotope. However labelling efficiency is not a measure of the function of the platelets, only of proportion of label taken up by the platelets from the labelling medium. Several studies have shown no difference between the methods (Dewanjee et al 1986) while others favour the use of the tropolone complex (Peters et al 1985, Vallabhajosula et al 1986). The retention of platelet function is more important than labelling efficiency and so the tropolone ligand was chosen (it is also cheaper than oxine).

6.3.3.2 Method of labelling the platelets

The basic method used to label the platelets is that of Danpure et al 1982, Danpure & Osman 1986, Danpure & Osman 1988 and Osman (personal communication). This method uses Hespan (hydroxylethyl starch) to cause

rouleau formation and improve sedimentation of RBCs, and does not affect platelets (Strauss 1980). The separation of platelets from other blood cells is relatively easy in man, but the sheep has small red cells and large platelets, making this separation much harder. In dogs, with a high haematocrit, the separation of the platelets from RBCs is improved by reducing the haematocrit before centrifugation (Galvin et al 1987). The following method, determined by considerable trial and error, achieves a sheep platelet suspension suitable for isotopic labelling (see Appendix 4 section A 4.6, page 251).

22.5 ml of blood is taken into a 30 ml syringe, prefilled with 4 ml acid citrate dextrose (ACD). A 2 ml aliquot of this is centrifuged at 2000 g for 5 minutes. This provides acid citrated platelet poor plasma (PPP) which is cell free for resuspension of the platelets, and constitutes the labelling medium. The packed cell volume of the initial EDTA sample is measured (PCVi), and the volume of diluent (d) that will reduce the PCV to a final PCV (PCVf) of 15% is calculated. (Based on the expression $d=\{(PCVi*Vi)/PCVf\}-Vi$, where Vi is initial volume of 6 ml.) The ACD blood is split into 4 aliquots of 6 ml, and the volume of diluent added as 1.5 ml Hespan with the balance as calcium free Tyrode's solution. This is centrifuged at 700 g for 2.5 min to produce platelet rich plasma (PRP).

The PRP is separated from the RBC's and WBC's as 2 aliquots, which are acidified with 1/20 th their volume of ACD. A few drops of the PRP are taken for cell counts. The PRP is centrifuged at 640 g for 10 min to produce the platelet pellet. This is resuspended in 1 ml of acidified ACD PPP. 10 μl of the resuspended pellet is taken for cell counting. 50 μl of indium 111 (10-20 MBq) is added to 100 μl of $2.2\ 10^{-3}$ M tropolone in phosphate buffered saline (PBS), and the mixture is added to the resuspended platelets. The final tropolone concentration is $2\ 10^{-4}$ M. After 5 minutes 7 ml PPP is added to the labelling mixture to stop the reaction and it is recentrifuged at 640 g. The supernatant is retained for radioisotope counting. The platelet pellet is resuspended in 7 ml PPP, 10 μl is taken for estimation of labelling efficiency and the rest placed in a 10 ml syringe for weighing and isotope counting.

6.3.3.3 Calibration of gamma camera images, blood and tissue samples

In order to make meaningful comparisons between the animals, the activity of the radioisotopes injected has to be calibrated in such a way as to eliminate variations due to counting geometry and electronic drift in the instruments. The usual method makes use of standards, which are solutions of the isotope made up in suitable stable carrier solution. These are placed in containers of the same material as the 'active' isotopically labelled substances. In addition all the counting is carried out with the same counting geometry, that is with the same three dimensional relationship between the counting crystal and the sample. The counts must be taken with in a few minutes of each other, to avoid the need for correction of radioactive decay. The amounts used are determined by counting the syringes full, and empty (since not all the isotope is dispensed).

For indium, the standard is a solution of indium 111 chloride made up in stable indium chloride (in 40 mM HCl, as $InCl_3$ is only soluble in acid media). This is placed in a volume of 7 ml in a 10 ml syringe, just as the labelled platelets are. A small additional amount of the standard, about 1/50th the activity of the primary standard, is placed in a TB syringe to calibrate the gamma camera images. This is placed in a volume of 0.7 ml stable indium carrier in a 1 ml TB syringe. The internal diameter of the syringe is 0.5 cm, and when the syringe is filled to 0.7 ml, it is 4 cm long. This is the size of the Dacron graft, 0.5 cm internal diameter and 4 cm long. The phantom for calibration of the gamma camera images is shown in Appendix 4 section A 4.7, page 252.

The primary standard is prepared by adding 10 μl indium 111 to a weighed tube and adding 7.2 ml of stable indium chloride standard solution (5 gm/100 ml $InCl_3$ in 40 mM HCl). 7 mls of this is placed in a 10 ml syringe. 140 μl of the primary standard is placed in a TB syringe, and checked by weight before adding further stable $InCl_3$ to fill the syringe to 0.7 ml. After counting the 7 ml standard (in a 10 ml syringe) is subsequently dispensed in a previously prepared 1 l flask containing 40 ml of 1 M HCl, 750 ml double distilled water and 10 ml stable $InCl_3$ (5gm/100ml), and made up to the 1 l mark. The empty 10 ml syringe is recounted. Aliquots of 100 μl and 1 ml are made up to 2 ml with the carrier and used for

calibration of the sample counts.

The three syringes containing the activity (the labelled platelets and the two standards) are counted in an annular ionization chamber (Capintec Radioisotope calibrator, CRC 10B, Capintec Inc, New Jersey, USA), to determine their relative nominal activities. This isotope counter has a sample carrier which fixes the counting geometry. As a check the activities are also counted with a sodium iodide crystal connected to a rack scaler counter (J & P Engineering). Here the counting geometry was fixed by use of a specially constructed jig. The electronic counting windows for this instrument for both indium 111 and iodine 125 were set by using each isotope and plotting the gamma ray spectrum to find the energy peaks for the isotope. The accuracy and linearity of these two isotope counters was checked using solutions of known specific radioactivities over the ranges used for the experiments. This was also performed for the iodine 125 channel. All the activities for the standards, the samples and the results are corrected for isotopic decay to the time of injection.

6.3.3.4 Blood sampling and counting

The blood samples are collected in 2 ml EDTA tubes (Sarstedt paediatric tubes ref. 14.1395.05) using the standard method above. These tubes are weighed empty and re-weighed after collection to determine the volume of the sample. The small capillary tube samples for the estimation of the mean haematocrit are taken before the samples are weighed. The EDTA blood sample is then haemolysed with Triton 10 X, and it is made up to exactly 2 ml with water. This is to ensure that the different sample weights are presented to the well gamma counter for determination of their radioactivity evenly distributed in a fixed volume. These precautions prevent the geometry of the sample from affecting the counts. The samples are counted in a LKB Wallac 1282.002 Compugamma Universal Gamma Counter (LKB Wallac, Turku, Finland). The gamma isotopic spectra used for both indium 111 and iodine 125 were checked for this instrument (the settings used are channels 130-195 for 111 In and channels 036-099 for 125 I). The samples are counted for 200 seconds (3.33 min.) with dilutions of the standards described above. With activities around 1-10 kBq, the time of 200 seconds gives

counting errors of under 1%. In addition this amount of activity limits the dead time errors. These errors occur when the samples are so radioactive that the counter is saturated by the incoming counts (although the Compugamma can correct for such errors). A longer counting time would give greater accuracy, but the counting would take a sufficiently long time to require a correction for isotope decay taking place between the first and last samples (and occupy the instrument for too long).

6.3.3.5 Analysis of the blood and tissue samples

The counts per minute for each sample are corrected for background counts, and also for weight to give the count per minute per ml, or per gram. These counts are then converted to both activities per gram, and to percentages of the injected dose, by use of the standard samples. For the EDTA samples the corrected indium counts are plotted against time to give platelet survival data. The tissue samples are analyzed both as uptake per gram of tissue, and as whole organ uptakes of the label. These are all 'wet' weights, and no correction is attempted for retained blood. The grafts are corrected for background counts, and the results presented as activity, or % of injected dose, per graft.

6.3.3.6 Platelet survival data calculations

Although the platelet survival estimation assumes there to be a steady state of production and destruction of the platelets, as well as a constant blood volume, it remains a useful measure for these experiments. For uptake by the grafts it is a crude measure, since only a small amount is actually removed by the graft (This is like removing a drop from a bucket, and reweighing the bucket to estimate the amount removed!) However if platelet destruction takes place away from the graft, as a result of interaction with the coating there will be a reduction in platelet survival independently from the counts on the grafts themselves. This analysis is not unlike the assay for haemolysis in looking for unexpected destruction of RBCs.

The best methods for calculation of the platelet survival parameters are still debated. The original ideas for estimating survival of discrete cells are based on a

the probability model of Dornhorst (1951) for RBC senility. While the theory expects the RBC to be immortal and is destroyed by chance the platelet has an expected finite life span. The more usual pharmacological models for drug metabolism of linear and exponential decay are sometimes more appropriate. The survival based on the best fit between these two models is often used, and is called the weighted mean method. However the platelet can be thought of as having a finite probability of activation, just as the RBC has of destruction. The platelet may also undergo a number of events before being activated, and a model based on this idea of a number of 'hits' has been proposed by Murphy & Francis (1971). Lotter et al 1986 reviewed 10 methods of calculating platelet survival based on these models, and favour the multiple hit and a modified weighted mean methods. For this study the recommendations of the International Committee for Standardisation in Haematology (ICSH, 1977 & 1988) are followed. The weighted mean and multiple hit estimates are calculated using the computer programme written by the ICSH in Basic. The programme makes use of iterative methods of curve fitting to minimise the residual sum of the squares for the gamma functions of the mean survival (based on the Dornhorst probability) and the multiple hit method.

The activity per ml of EDTA blood for the platelet label at each time point will be used to calculate the platelet survival. Three sets of data will be prepared. The first is the data from 4 hours to the end of the experiment (4 hour data set), and this avoids possible discrepancies due to splenic sequestration of the platelets. The second set is all the data for the experiment with obvious erroneous points omitted (all data set). The final data set is for the points that give a low residual sum of the squares in the curve fitting programme. This is the 'fitted mean data set' and is usually from the samples collected from 2-6 hours after the injection, and omitting the samples collected after 24 hours. These latter samples were taken after the animal had been sedated with Sagittal. These all showed a mark drop compared to those taken just before sedation. It is likely that sedation changes the blood volume, by relaxation of the animal's vasomotor tone. These three sets of data will be used in the ICSH programme and the estimates of platelet survival reported will be the weighted mean (wtm) and the multiple hit mean (mhit).

6.3.3.7 Gamma camera analysis

The gamma camera images are acquired at two time points in the experiment, for each subject. Each of these form a data set which contains the views of the phantom, the neck, the thorax, the liver and the spleen. The image analysis software in the Elscint is rather limited, and is also difficult to use. The data is transferred view by view into a more sophisticated gamma camera computer, a Nodecrest Micas 3 system (facilities provided by Mr Peter Dean of the Dept of Radioisotopes, The Royal London Hospital).

When radioactive decay occurs in the indium 111 atom, photons are emitted, which are detected by the gamma camera as counts. The counts arise from the primary radiation, but also include scattered and background radiations. The camera records the counts as a matrix of picture elements, or pixels. The final array of pixels is displayed as a colour coded image. The analysis of the images is carried out using the computer to draw lines around the features of interest in the view. These lines define the 'regions of interest' (ROI). The computer gives the number of pixels and the total number of counts within each ROI. In order to quantify the amount of activity in each region of interest in the view a correction must be made for the background activity. Two ROI's are needed, one for the area of activity of particular interest, say the graft, and a second area, used to estimate the background counts, which need to be subtracted from the counts in the main ROI. This is simple for the views of the phantom. The activity is well localised, and the ROI for the background is also easy to draw, because of the phantom's design. The counts in the background ROI are used to give a background count per pixel, which is multiplied by the number of pixels in the main ROI and subtracted from the total number of counts for the main ROI.

Since the activity in the phantom is a fraction of the injected dose, the % activity in any ROI (in any of the images) can be calculated. It is more difficult to choose the location of the ROI for the background counts in the views from the animals. This is a universal problem with all gamma camera image analysis, since there is often no area in the field of view that is truly representative for the background counts. The activity in the blood pool represents an important source of this

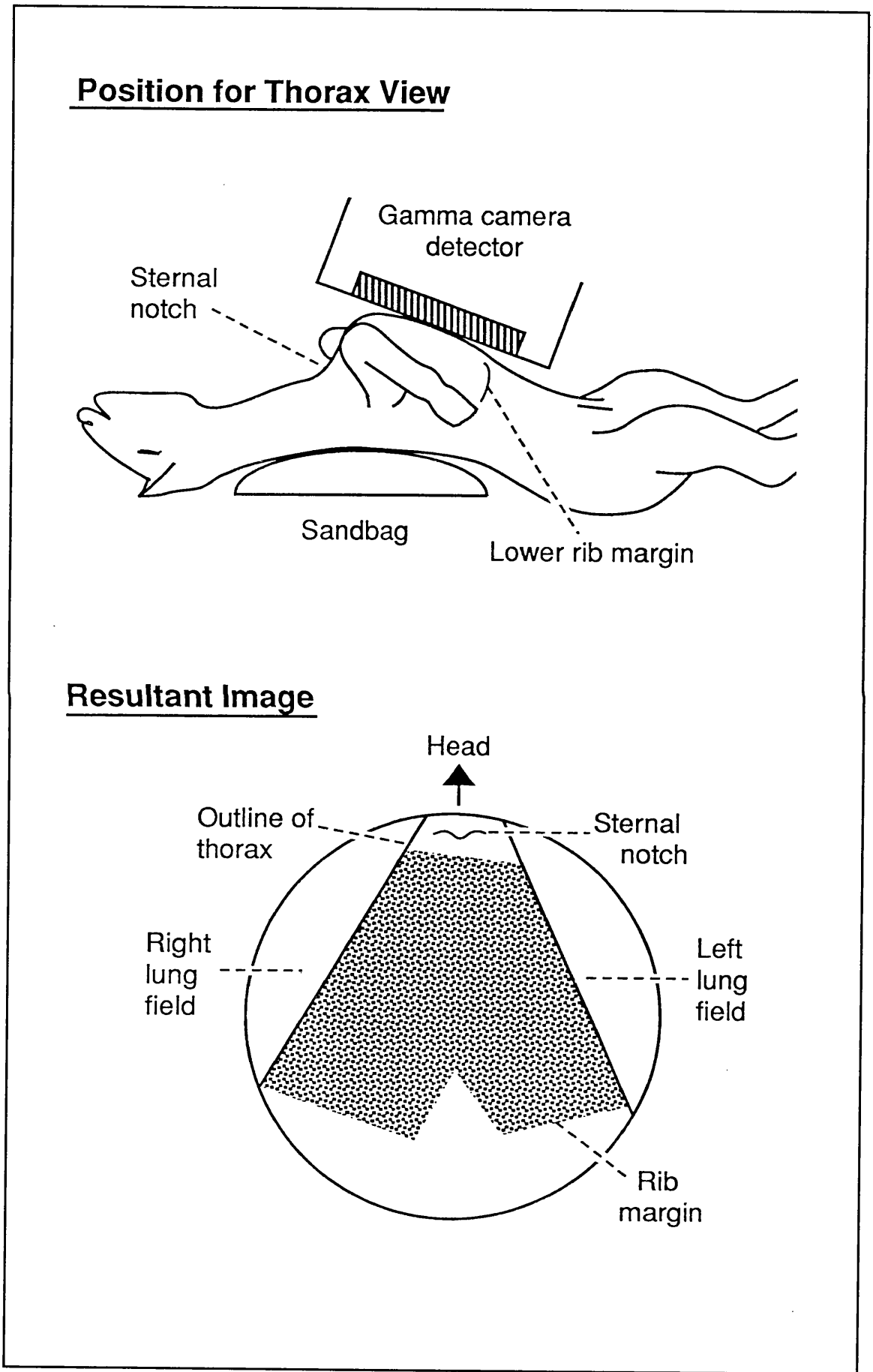
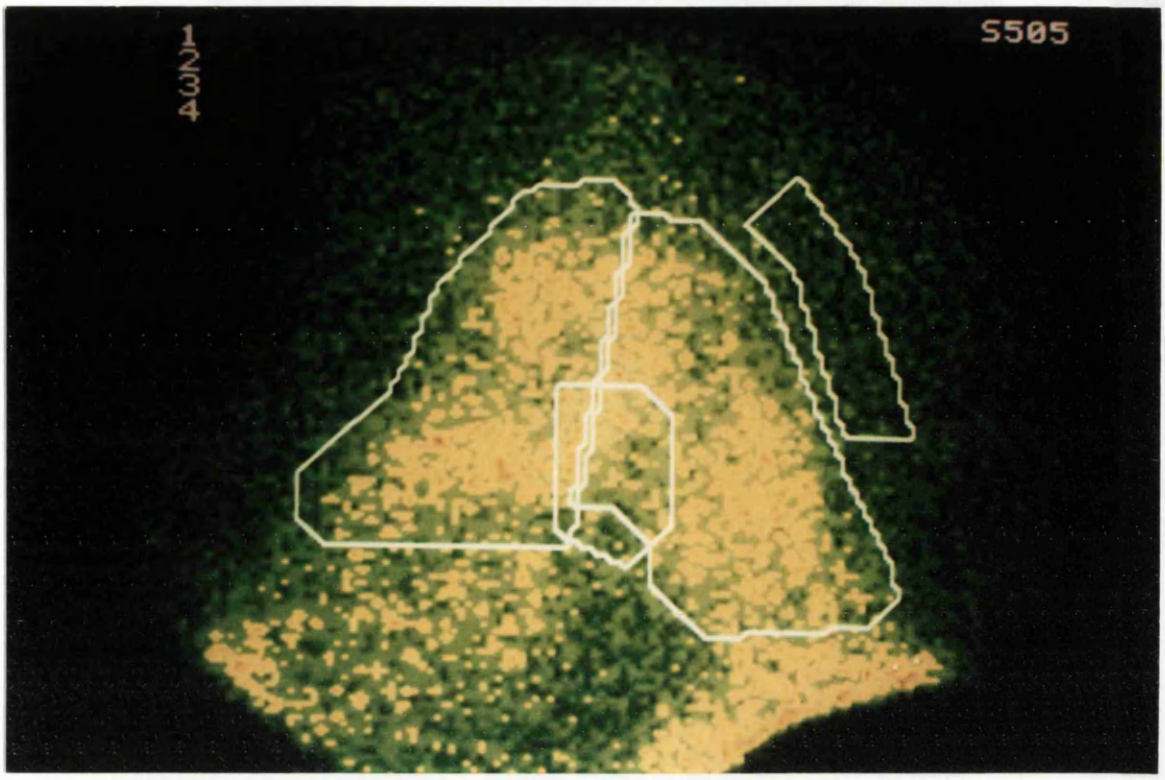


Figure 6.1 Gamma camera positioning and resultant image for the thorax view.



Photograph 6.2 Typical gamma camera image of the thorax, with ROI's.

background radiation, and varies with the local vascular anatomy. In this study the locations of the background ROI's are standardised, as are the views themselves, so that the errors are the same between the animals. These are shown diagrammatically in Figures 6.1 to 6.4, and representative images shown in Photographs 6.2 to 6.6.

To provide a background for the thorax, an area over the left foreleg is used as this contains soft tissue with a small amount of blood. Both the liver and spleen views use a composite area placed over the body wall and the abdomen below the respective organ. The localisation of the spleen and liver is rarely a problem. The lung fields are complicated by the anatomical complexity of the mediastinum. The right and left lungs are divided by an estimated line for the position of the midline. The ROI for the heart however has to be drawn arbitrarily.

The neck view provides a particular challenge and several methods are used to provide estimates of the activity on the graft. The ROI for the graft will be estimated twice, the first method uses a maximum region, (graft max) incorporating the hot area in the neck. A second ROI for the graft is drawn,

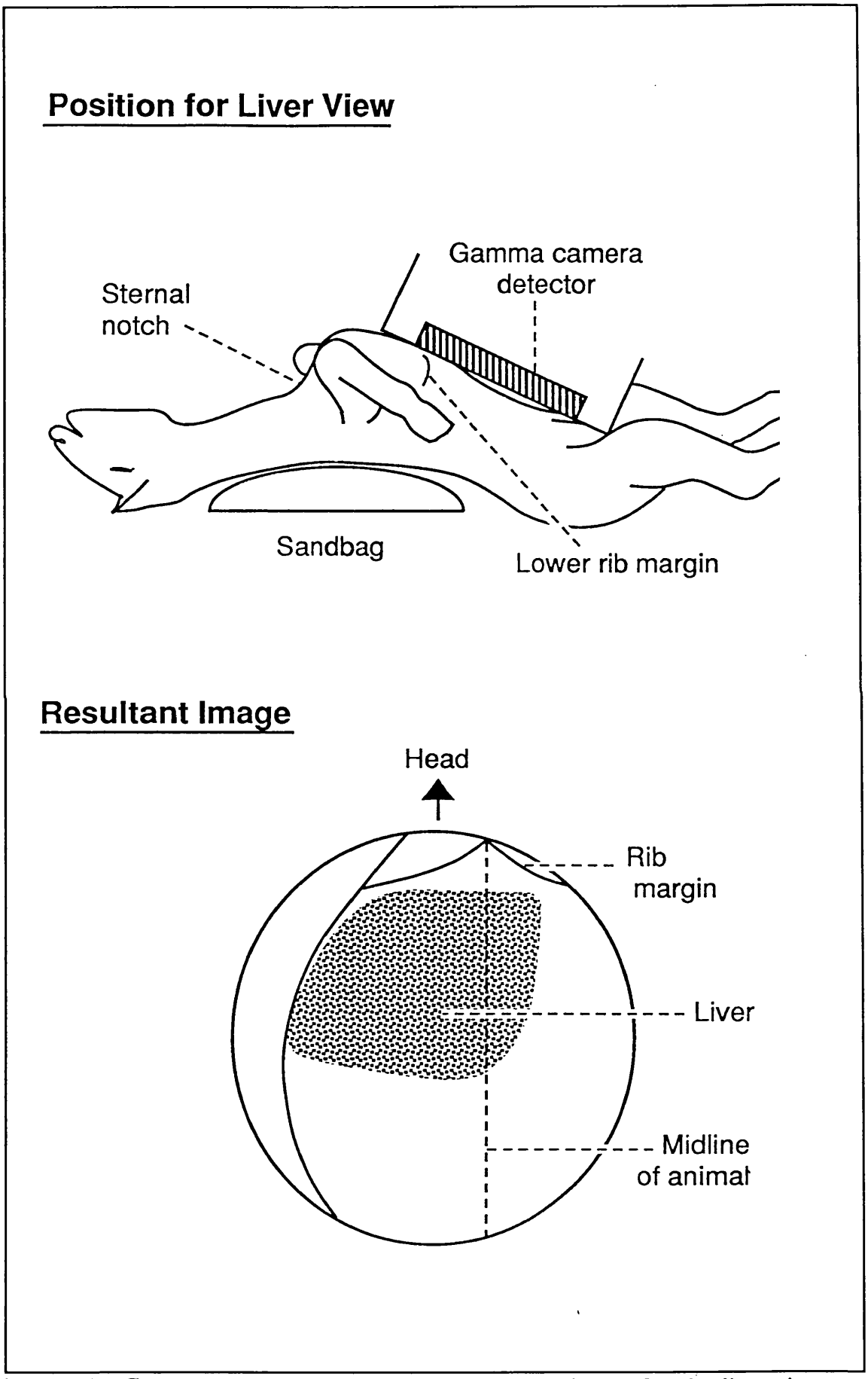
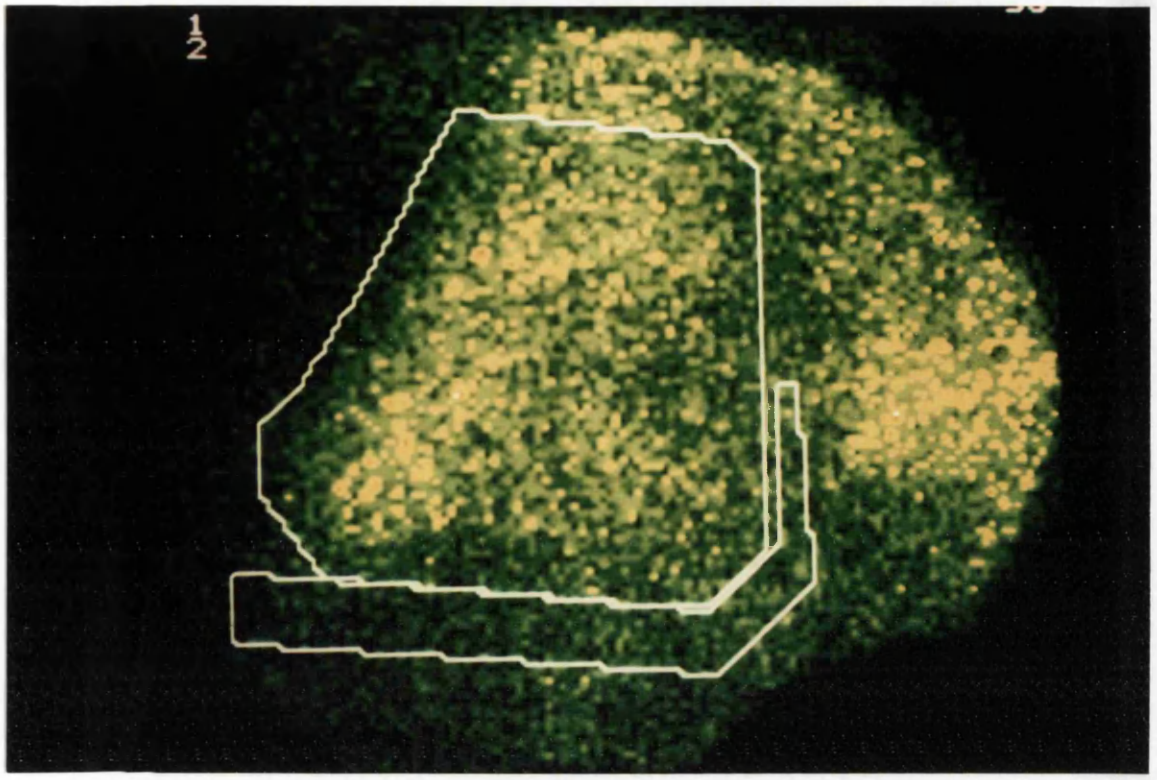
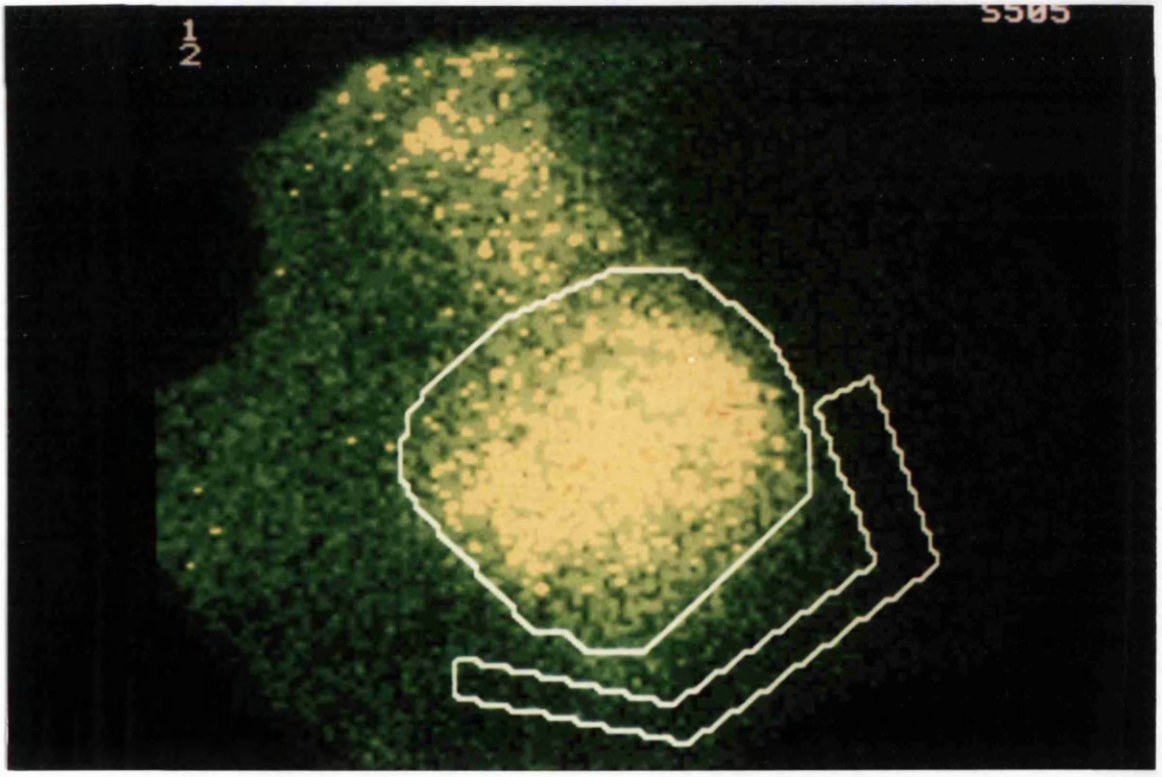


Figure 6.2 Gamma camera positioning and resultant image for the liver view.



Photograph 6.3 Typical gamma camera image of the liver, with ROI's.



Photograph 6.4 Typical gamma camera image of the spleen, with ROI's.

which is deliberately small, and is placed to include only what is known to be the graft, using the angiograms as a guide (graft min). The background counts are also difficult to estimate and several ROI's will be used. These are regions over

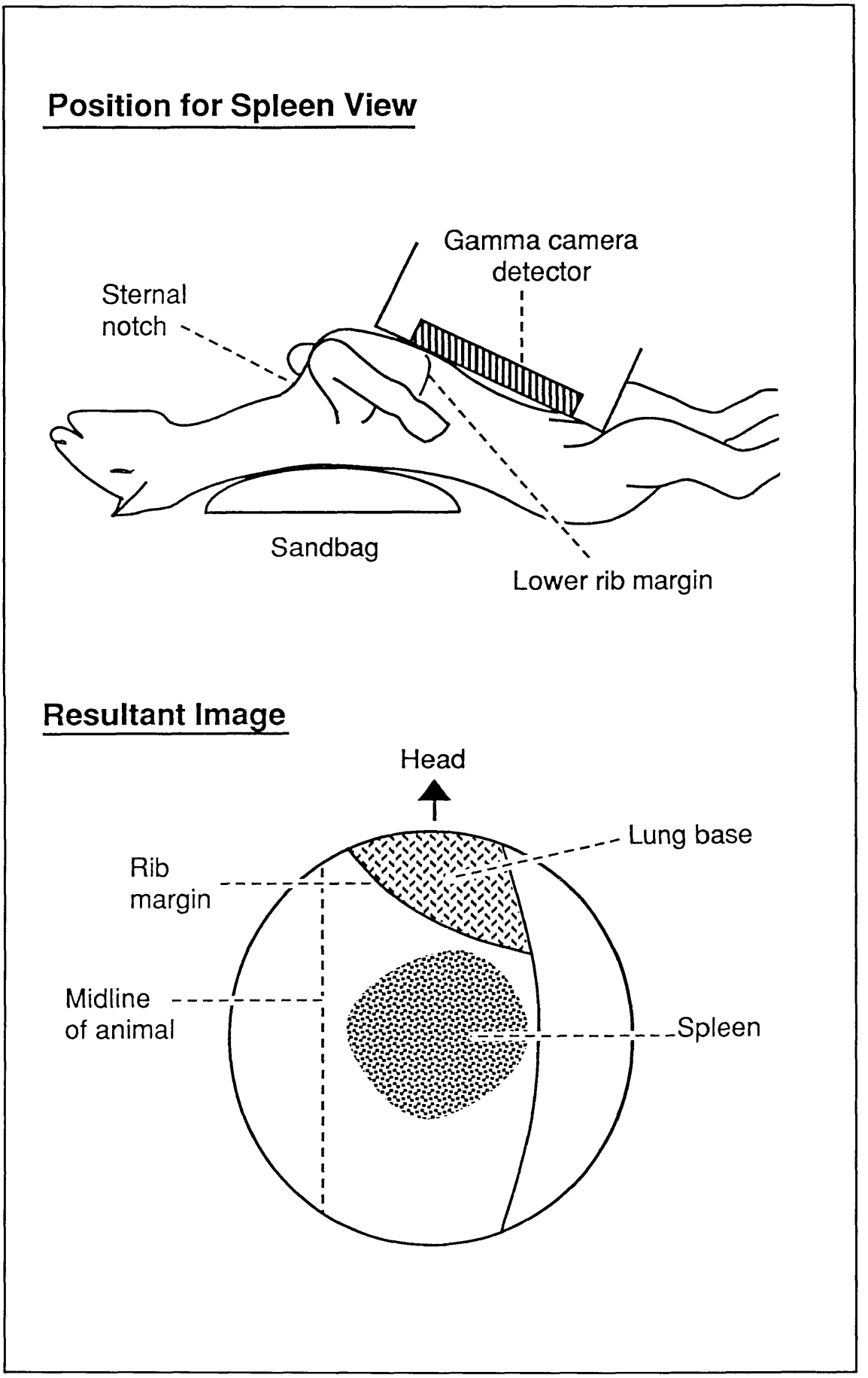


Figure 6.3 Gamma camera positioning and resultant image for the splenic view.

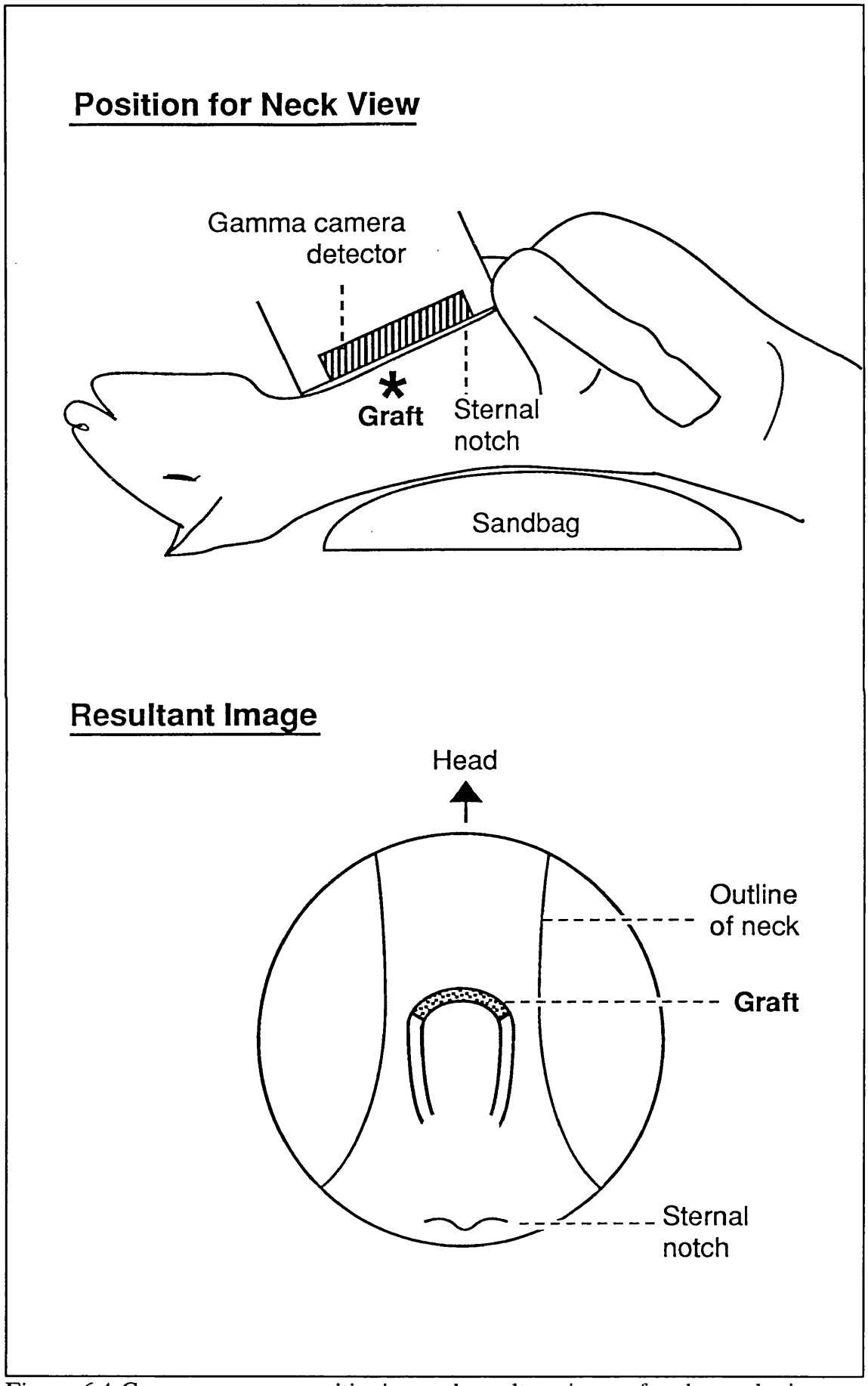
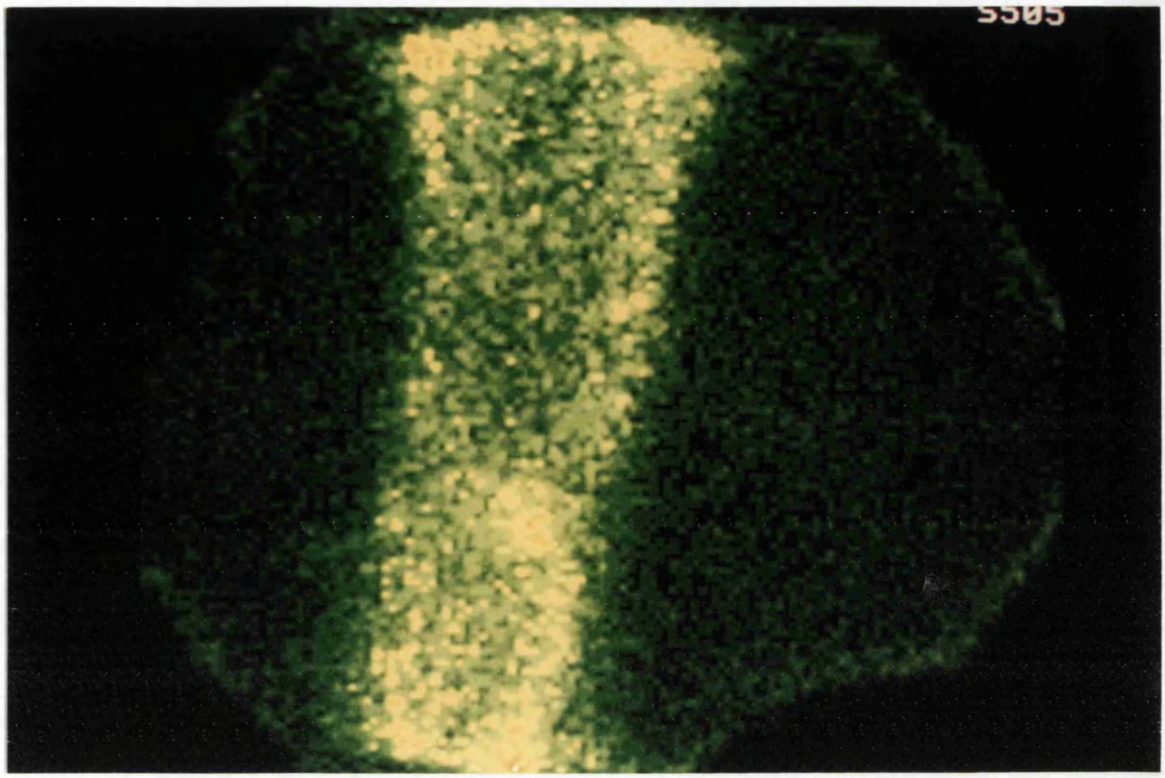


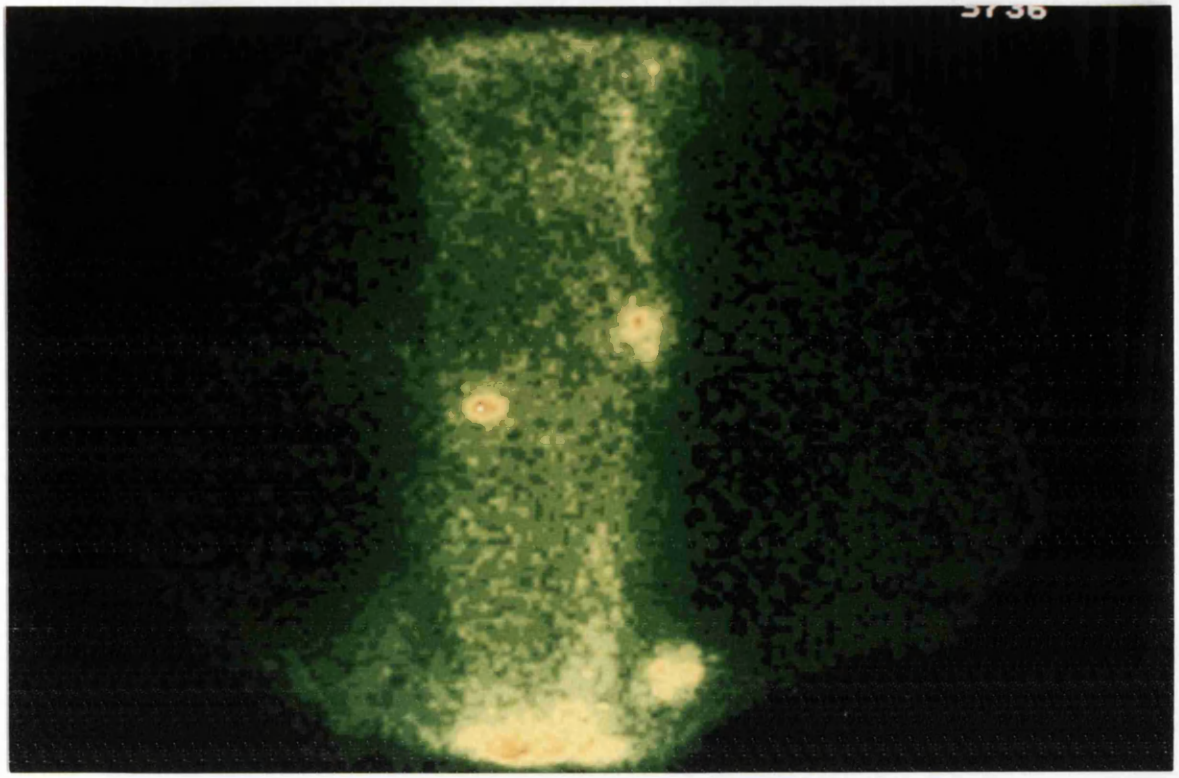
Figure 6.4 Gamma camera positioning and resultant image for the neck view.



Photograph 6.5 A typical gamma camera image of the neck (s505), with uptake on the venous side of the graft.

the trachea high and low in the neck. The first contains very little blood, and is an underestimate, while the second contains scatter from the great vessels in the neck and is an overestimate. The mean of these two is taken as being more representative. This mean value of correction is applied to the graft max to give the maximum estimate (est max). The graft min ROI is deliberately tight, and it is confined to the tracheal area of the neck, and so the background ROI high in the neck (which corrects just for the tissue over the trachea) is an appropriate background correction to use to give the minimum estimate (est min). The average background is also used to correct graft min to give an average (est ave).

The final method of calculating of the background uses a slightly different premise. A large box is drawn around both the graft and the surrounding area. Within this box two further ROI's are defined. These are the main ROI for the graft, and any other 'hot' areas related to the blood vessels. The background counts are calculated by subtracting the counts in the pixels known to be 'hot' from the larger surrounding region. These counts per pixel from the background are then applied to the graft ROI counts. This approach gives a good estimate of the background



Photograph 6.6 A typical gamma camera image of the neck (s736), showing low graft uptake, with high uptake in the tied off artery and vein.

but, includes some scatter from the graft, making it a slightly high estimate of the background counts. This correction is applied to both the estimates of the graft position, the graft max and min, to give two further estimates, the 'box hi' and 'box lo' estimates.

6.3.4 The fibrinogen label

6.3.4.1 Introduction

The role of fibrinogen in early protein deposition on biomaterials has been discussed. It is the final protein to be cleaved in the coagulation cascade to produce monomeric fibrin, which is the building block of fibrin strands and blood clots. In addition there are many interactions between the clotting factor components, platelets, complement, and white blood cells, which all influence the final clot formed. The cleavage of fibrinogen and the deposition of fibrin represents the net result of all these interaction between the material and these

factors in blood.

Fibrinogen is normally distributed between the intravascular and extracellular pools, in addition it is catabolized by the liver and excreted in the faeces and urine. The analysis of the metabolism of fibrinogen is complex. Matthews (1957) discusses the theory of tracer experiments with iodine labelled proteins, and the simplest mathematical model involves 2 compartments. A more realistic model however needs 3 or 4 compartments. Collen (et al 1972) used the two compartment model to analyse his data of the normal physiological distribution and turnover of fibrinogen in man, but presents a more satisfactory model based on the true physiological fate of fibrinogen. This involves 7 compartments. Hickman (1971) has studied the metabolism of fibrinogen after surgical operations (using the 3 compartment model) and has shown a rapid increase in catabolism in both the intravascular and extracellular pools. These studies have usually spanned 10-20 days in order to allow determination of the plateau that is the final equilibrium of the system. For any of the analytical techniques this plateau needs to be established for either the curve stripping or computerised fitting of the data to the exponential functions describing fibrinogen survival.

The experiments presented here do not last long enough to allow the collection of the data necessary for the fibrinogen survival to be calculated. A measure of the similarity of the groups will be estimated from the 4 and 24 hour blood levels.

In using a dual isotope technique it is important to make sure that the second isotope can be distinguished from the first. Iodine 125 has a long half life of 60 days, decays by electron capture giving gamma emissions of 35 keV, and X-rays of 27 keV. Iodine 125 therefore has a different range of emission energies from indium 111, but there is a small overlap due to the lower energies of indium which fall in the iodine spectrum. The corrections needed for this are discussed below in 6.3.4.3 and Appendix 5, section A 5.4, page 257.

The techniques for iodine 125 labelling of proteins are well established. For fibrinogen it is known that the isotope has little or no effect on its biological function, providing the labelled product is stored correctly and used within its shelf

life (the gamma emission from the isotope degrades the protein over time). While the use of autologous fibrinogen would be ideal there are practical difficulties with isolation, purification and labelling of sheep fibrinogen which were beyond the time scope of this study. Sheep fibrinogen is particularly difficult to purify and the iodinated fibrinogen splits with part of the I-125 label attached to the fibrinopeptide B fragment (Regoeczi and Walton 1967). Certainly homologous 125 I fibrinogen gives comparable results to autologous (Dellenback et al 1971). The use of human fibrinogen is acceptable, since comparative studies of human and sheep fibrinogen show that they appear to have identical amino acid composition, and there is a very close relationship between the trypsin digests of the two proteins (Cartwright and Keswick 1971). The human iodine 125 fibrinogen used is commercially available with very high standards of purity and clottability from Amersham International (Product reference IM.53P). The use of this material also avoids uncertainty over the quality of the labelling, the clottability of the fibrinogen, and the purity of a sheep preparation. Evidence to show that the human material is handled in the same way as autologous in the sheep is presented in the Appendix 5 section A 5.4, page 257.

6.3.4.2 Methods

The iodine 125 fibrinogen is reconstituted as recommended by Amersham, with 1.1 ml of sterile water. 0.6 ml of the solution is further diluted with 5.4 ml of a buffered diluent. This is based on the constitution of the final Amersham solution, and consists of an isotonic solution containing 0.75% Na citrate, 0.65% NaCl, and 18 mg/ml human albumin. 5 ml is injected to the animal and the remaining 1 ml is used as the standard. The typical dose is between 1 and 2 MBq, and the diluted material is calibrated by weight, and by isotopic counts in both Capintec and in the scaler timer counter.

6.3.4.3 Sample counting and analysis of the results

The same EDTA blood samples used above for the platelets are used to determine the iodine 125 counts, but using a different counting window. The same considerations about counting geometry are also met. The validity of using these

whole blood samples rather than plasma samples is discussed in the Appendix 5 section A 5.4., on page 257. The counts per minute for each sample are corrected for background errors. Since indium 111 has some lower energy emissions they are counted in the iodine channel. This is the 'cross over' effect and the iodine counts require a correction for the additional counts which have come from the indium. The correction removes counts due to the presence of indium in the sample. The correction factor is calculated from the proportion of counts that cross over into the iodine channel when the indium standards are counted. There is no iodine 125 present and so there should be no counts. In addition this correction was checked by recounting the specimens after the indium had decayed to very low levels of activity. The results of repeat counting and the details of the crossover calculation are presented in the Appendix 5, section A 5.4, page 257. The crossover corrected counts are then corrected for sample weight to give the count per minute per ml or per gram, in the same way as the indium counts were (see 6.2.3.5). These are then converted to both activities per gram, and to percentages of the injected dose, by use of the standard samples. For the EDTA samples the corrected iodine counts are plotted against time to give fibrinogen survival data. The tissue samples are analyzed both as uptake per gram of tissue, and as whole organ uptakes of the label. The counts from the grafts are similarly corrected for background errors, indium crossover, and the activity, or % injected dose are presented per graft.

6.3.5 Collection of histological material

6.3.5.1 Methods

After counting the grafts the isotopes in the graft specimens are allowed to decay, to permit safe handling. Each graft is cut transversely in half and a section of 2-3 mm taken, with a razor blade to limit distortion of graft. This gives a cross section at the mid-point of the graft. The remaining two halves are split longitudinally to expose the flow surface of the graft. This is photographed, and estimates of the thrombus free area (TFA) made.

The transverse sections from the mid-point of the graft are embedded in resin, sectioned and stained with toluidine blue. These are examined by light microscopy and image analysis conducted to measure the cross sectional area of thrombus present. The image analysis system employed is a Zeiss microscope, with a JVC video camera. The video image is processed by a Chromatic 1.4 Microsoft Colour Image Analysis System. All the section are 3 μm thick and were stained at the same time, to ensure uniform staining. Three sections from each 'ring' of graft material are examined. Each slide is colour corrected, and the mid-point of the graft wall is marked. The system uses its colour detector to pick out a predefined blue colour (from the toluidine blue stain), which had been chosen to give the best discrimination of thrombus from graft on a number of slides. The area of thrombus within the lumen of the graft is detected by the system. As the section thickness, and staining are the same for each graft these automatic estimates of the thrombus in the graft are strictly comparable. Several of the grafts have areas of lighter staining, which the automatic detection excludes. All the slides are also examined visually and a manual estimate of the area of thrombus made. This is to ensure that the areas lost by the automated analysis do not affect the ranking of the grafts.

Additionally the perimeter of the centre of the graft material is recorded to allow for the calculation of the % thrombus area in relation to the cross sectional area of the graft. The perimeter of the graft is $P=2\pi r$, and the area is $A=\pi r^2$. As a circle encloses the largest area for a given perimeter an estimate of the deviation of the perimeter from a true circle can be made :-

$A=\pi r^2$, and $P=2\pi r$,

rearranging these :- $r=\sqrt{A/\pi}$, and $r=P/2\pi$.

so :- $\sqrt{A/\pi}=P/2\pi$, and $S= 100(2\pi\sqrt{A/\pi})/P$

where S is shape. The term S for a circle is 100%, since it encloses the largest area for a given perimeter. The term S gives a measure of departure of the graft from a true circle.

The remainder of the graft is pinned out and photographed. The prints of these photographs are also examined using this system to measure the luminal thrombus free area. Each photograph contains a scale in mm, and each is analysed with

corrections for differences in the scale of the photographs (these are actually quite small). The image analysis detection criteria to distinguish thrombus from the graft in these formalin fixed specimens proved more difficult to set up. This is due to the variations in the colour of the thrombus. The thrombus free area, the area of exposed graft material is easier for the system to detect and this is the area measured. The areas for each half of the graft, the arterial and the venous ends are measured separately. The photographs proved to be adequate for the system to analyse. Two photographs had a strong colour cast, caused by poor photographic exposures, and required manual analysis. One required only adjustment of the colour balance of the video camera, the other had to be manually analysed due to the strong blue colour cast (s89).

6.3.6 Statistical design and analysis

The design of the study is in randomised blocks. The intention as far as possible is to eliminate bias in favour of one or other material. To achieve this the grafts are tested in a random order. In addition the investigators are blind (at least as far as the identity of the material is concerned!). This blind assessment is limited to Polyester G, sphingomyelin and the control, as the DAPC grafts are coloured.

A further factor allowed for is the increasing experience of the author over the duration of the study. To prevent this affecting the results, and to allow comparable handling of each batch of animals, a block design of four elements is used. The block size of four is determined by the number of materials being tested. Each block has one of each of the three coatings and a control graft and the order of implantation within the block is randomised. In addition it is convenient to order the animals from the supplier in groups of four. The design also permits paired analysis of the data. A master randomisation chart was made, and the order of testing in each block copied onto separate cards. These cards were used by Dr Gerald Davies to code the coated grafts for each block, after their preparation.

This study has a relatively small number of subjects. Parametric statistical analysis

makes some assumptions about the groups, in particular it assumes a normal distribution of the data. The small number of experiments make it difficult to demonstrate this but there is no reason to suspect non-normal distribution. The numbers involved give a low power in estimating the statistical significance of the results. The danger is of missing a truly significant difference. The 5% level of significance is used, but the p values are quoted. In view of this values of p which approach 0.05 may indicate a true difference. As small numbers are involved the non-parametric methods are used as they are more robust statistical tests. The results of both types of test will be shown.

The statistical analyses carried out are the oneway analysis of variance (ANOVA= analysis of variance) and its non-parametric equivalent the Kruskal Wallis (KW) test. These are applied to all the data, unless there is a specified reason, explained in the text. Since the first 12 experiments were conducted in strictly comparable blocks with double blind randomisation it is permissible to analyse these experiments separately by twoway ANOVA, and its non-parametric equivalent, the Friedman test. These methods allow an estimate of the effect of the block grouping to be made as well as the effect of the coatings. The failure of labelling of s591 (experiment number 9, in block 3) is allowed for in this analysis by replacing it with the 13th experiment (s95 which fortuitously was the uncoated graft in block 4).

For the comparisons between the individual groups a Mann Whitney U test (MWU) and paired t test (2t) are used. The comparisons are between at least 7 experiments (4 controls and 3 test subjects) so a pooled estimate of the standard deviation is used. When the data appears to have a non-normal distribution it is reanalysed after log transformation with the paired pooled t test.

Additionally when the blocks of the experimental design play an important part in the interpretation of the results a t test is used on the comparison between the control and the coated graft within each block of the experiment (see section 6.4.6.2 for an example with the thrombus free area data). Again the non-parametric equivalent, the Wilcoxon rank sign test is used.

6.4 Results for the arteriovenous fistula experiment

6.4.1 General comments

In all 16 experiments have been carried out. The first 12 used all three coatings and were performed in double blind randomised blocks. 4 additional experiments were carried out with two DAPC coated and two control grafts. In the results presented below 'SM' indicates sphingomyelin coated grafts, and similarly 'poly G' for polyester G, 'DAPC' for DAPC and 'control' for the uncoated grafts. The data is presented as the mean with the standard error of the mean (sem) in brackets. For ease of reference the Table 6.2 shows the identity of each subject in order with its graft material and block number.

order	identity	coating	block
1	s18	control	1
2	s16	DAPC	1
3	s770	SM	1
4	s736	poly G	1
5	s540	DAPC	2
6	s19	control	2
7	s553	SM	2
8	s3	poly G	2
9	s591	control	3
10	s514	DAPC	3
11	s89	SM	3
12	s505	poly G	3
13	s95	control	4
14	s91	DAPC	4
15	s93	control	5
16	s92	DAPC	5

Table 6.2 Identity and coating for each subject.

One graft thrombosed, s91. This experiment has the highest platelet recovery, and survival parameters of all the experiments. The platelet uptake for this graft by direct measurement and by gamma camera imaging was very low. All these make it likely that the graft thrombosed before the injection of the labelled platelets and

fibrinogen. The angiogram of this experiment shows a possible defect of the venous anastomosis, making its failure likely to be due to a technical error in the venous anastomosis. The graft coating was DAPC, and the data for DAPC in the following results are presented both with and without this graft included.

One experiment, s591, with an uncoated graft, had platelet labelling of a dubious quality. One aliquot of the cells to be labelled had evidence of RBC contamination. Further evidence for the poor labelling is in the very high deposition of the platelet label in the lungs, both by gamma camera imaging and the tissue samples. s591 had the highest labelling efficiency of 72%, the next best is 55% for s92. It also had the lowest recovery parameters (39-45%). This may not be considered as compelling a case as s91. The questionable data for s591 affects only the platelet related analyses, and these will be presented with and without this data.

6.4.2 Are the experimental groups comparable?

The animals came from a single herd of Sussex Cross sheep in Buckinghamshire. They were between 6 and 9 months old, from the same breeding season and were male castrates. The main 'biographic' data for these experimental subjects are shown in Table 6.3.

parameter	mean	sem
weight (kg)	35.74	1.96
blood volume (l)	2.03	0.10
delay (hr)	2.74	0.13

Table 6.3 Characteristics of experimental population.

The data for weight, blood volume, and delay is presented with both s591 and s91 included. 'Delay' is the time period between the completion of the AV fistula, with perfusion of the graft and injection of the labelled platelets (delay in Table 6.3). A typical example of the results for the calculation of blood volume are shown in Figures 6.5 and 6.6 for s553.

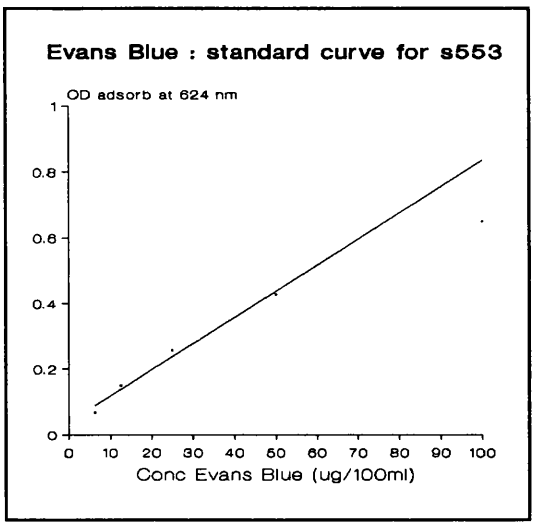


Figure 6.5 Standard curve for Evan's blue assay (s553).

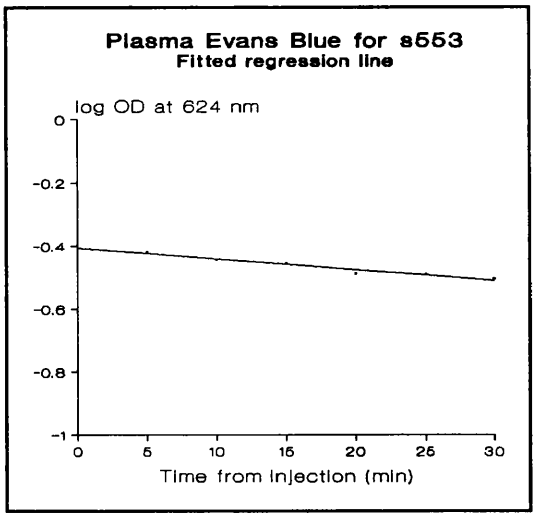


Figure 6.6 Plasma Evan's blue levels for s553.

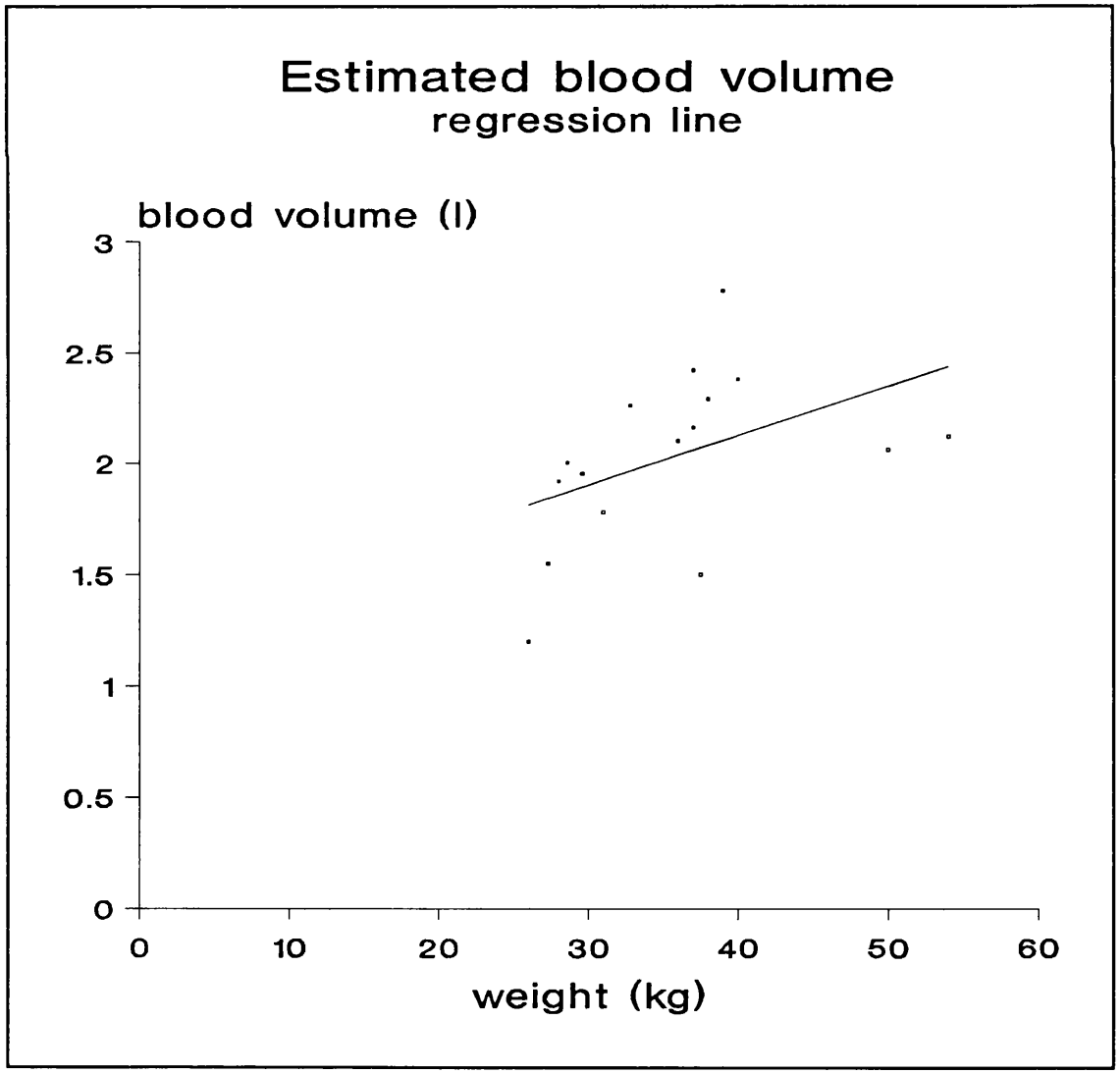


Figure 6.7 Estimated blood volume compared with body weight.

The relationship between the blood volume and body weight is shown in Figure 6.7. Volume = 1.24 + 0.022 wt, with r^2 = 0.20 % and p=0.08. There is relatively poor correlation between blood volume and body weight, presumably the heavier animals have more fat, and less lean body mass.

The platelet labelling data is presented in Table 6.4. The data for s91 and s591 are omitted for the analysis of platelet survival and recovery, as discussed above.

parameter	mean	sem
platelet labelling data		
efficiency (%)	35.7	3.80
recovery 4 hour (%)	61.2	2.71
recovery maximum (%)	69.8	3.28
recovery t_0 (%)	66.0	2.74
platelet survival data (days)		
4 hr mean	3.34	0.26
4 hr wt mean	3.37	0.26
fitted mean	3.33	0.27
Data for s91 and s591 omitted see text		

Table 6.4 Platelet label related data.

The mean labelling efficiency is 35.7% (but rises to 38.3%, if s591 is included). The mean delay between completion of the anastomosis and injection of the isotopes is 2 hours and 45 minutes. There is no correlation between the delay and the platelet labelling efficiencies. There are no differences between the coating groups for either the labelling efficiency or the delay by any of the statistical methods whether or not s591, or s91, or both are included.

Three estimates of platelet recovery will be presented and these are based on the sample with the highest count (recovery max), and the sample taken 4 hours after injection of the label (recovery 4 hr). The third estimate is made using the best fitting curve of platelet survival, and extrapolating it to the time of injection (time zero t_0). The recovery t_0 is calculated from the activity estimated at the time of injection. The platelet survival data are calculated as described above in 6.3.3.6 and the results presented in 6.4.4.1 below.

The mean recovery based on the 4 hour estimate is 61.2% (2.71), and with the inclusion of s91 and s591 is 61.6% (3.37). The results for the maximum recovery are 69.8% (3.28) and with all the data 69.9% (3.69). There are no differences between the groups for these two parameters.

The estimated recovery at the time of injection (recovery t_0) is derived from the '4 hour data' set for platelet survival. This is the result of curve fitting, and takes all the platelet data into account, making recovery t_0 the best measure of the platelet recovery. Recovery t_0 is 66.0% (2.74) and with the inclusion of s91 and s591 is 66.3% (3.43). For this estimate (recovery t_0) there is a trend towards a higher recovery for polyester G (71.3% compared to the control with 64.7%), but this is not significant by any method of analysis whether or not s591 is included.

There a very close correlation between the different measures of recovery. The p values are all <0.0001. The r^2 are >0.90 % for recovery 4 hour and the recovery maximum. Both R 4 hr and R max correlate well with the recovery t_0 data ($p<0.0001$), but the r^2's are slightly lower at 0.70 %. Neither the delay nor the platelet labelling efficiencies are correlated with any of the measures of the recovery of the platelets.

The % injected dose per ml of blood at 4 and 24 hours has also been analysed as a further check for comparability of the groups. The mean activity in the blood, expressed as % injected dose per ml for the 14 experiments is 0.0333% (0.0016) at 4 hours and 0.0250% (0.0014) at 24 hours. The 24 hour blood pool activity, obtained from the activity per ml and the estimated blood volume of the animal give a blood pool of 49% (3.2) of the injected dose. There is no significant difference between the coating groups for any of these parameters.

There are no differences between the three types of graft coating for the animal weight, blood volume, delay, or the platelet labelling efficiencies. For the recovery estimates based on single measurements (the 4 hour and maximum recoveries) there are no differences or trends between the coating materials. The trend in favour of polyester G for the recovery t_0 has been discussed. The lack of significant differences between the groups for these parameters demonstrates that

the animals and platelet labelling in each group are strictly comparable.

6.4.3 Results for the haemolysis assay

A typical set of results are shown in Figures 6.8 and 6.9. The normal range is under 15 mg/100 ml. The mean of the 9 samples taken for each experiment, and the single highest result are the two measures of haemolysis used for statistical analysis.

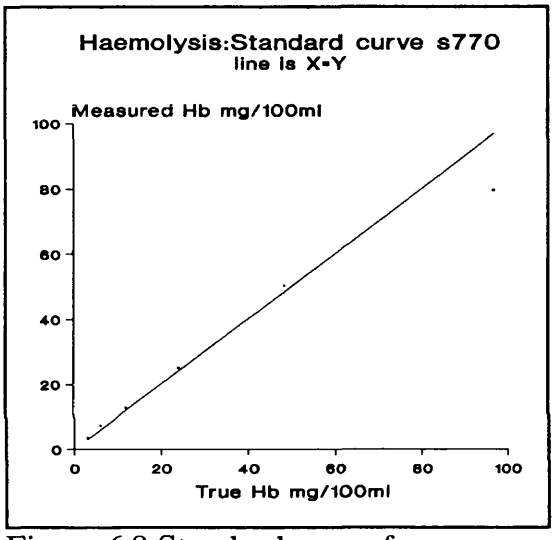


Figure 6.8 Standard curve for haemolysis assay (s770).

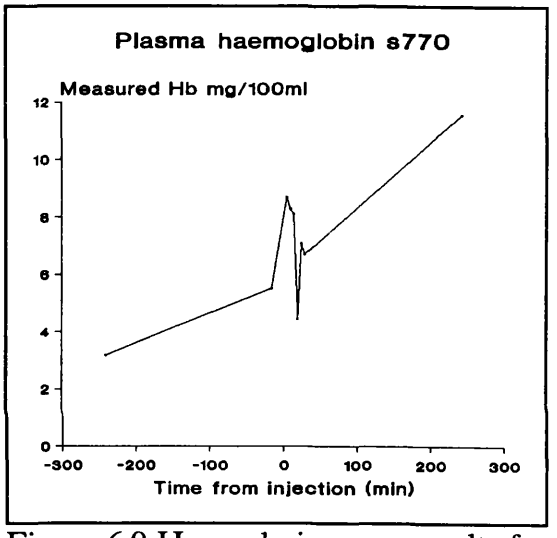


Figure 6.9 Haemolysis assay results for s770.

All the results are below the normal range except the single largest result, of 17.5 mg/100 ml. This is from s514, a DAPC coated graft, but the mean for this study is 5.5 mg/100 ml. The mean haemolysis is 4.7 (0.32) mg/100 ml, using all the data from all the experiments. The mean of the highest result from each experiment is 7.8 (0.94) mg/100 ml. Significant haemolysis is not present in any of the experiments with either the control or coated grafts. There are no differences between the groups for either of these measures. The method is sensitive enough to detect haemolysis when present, as shown in Appendix 5, section A 5.2, page 255.

6.4.4 The fate of the platelet label

6.4.4.1 Platelet survival data

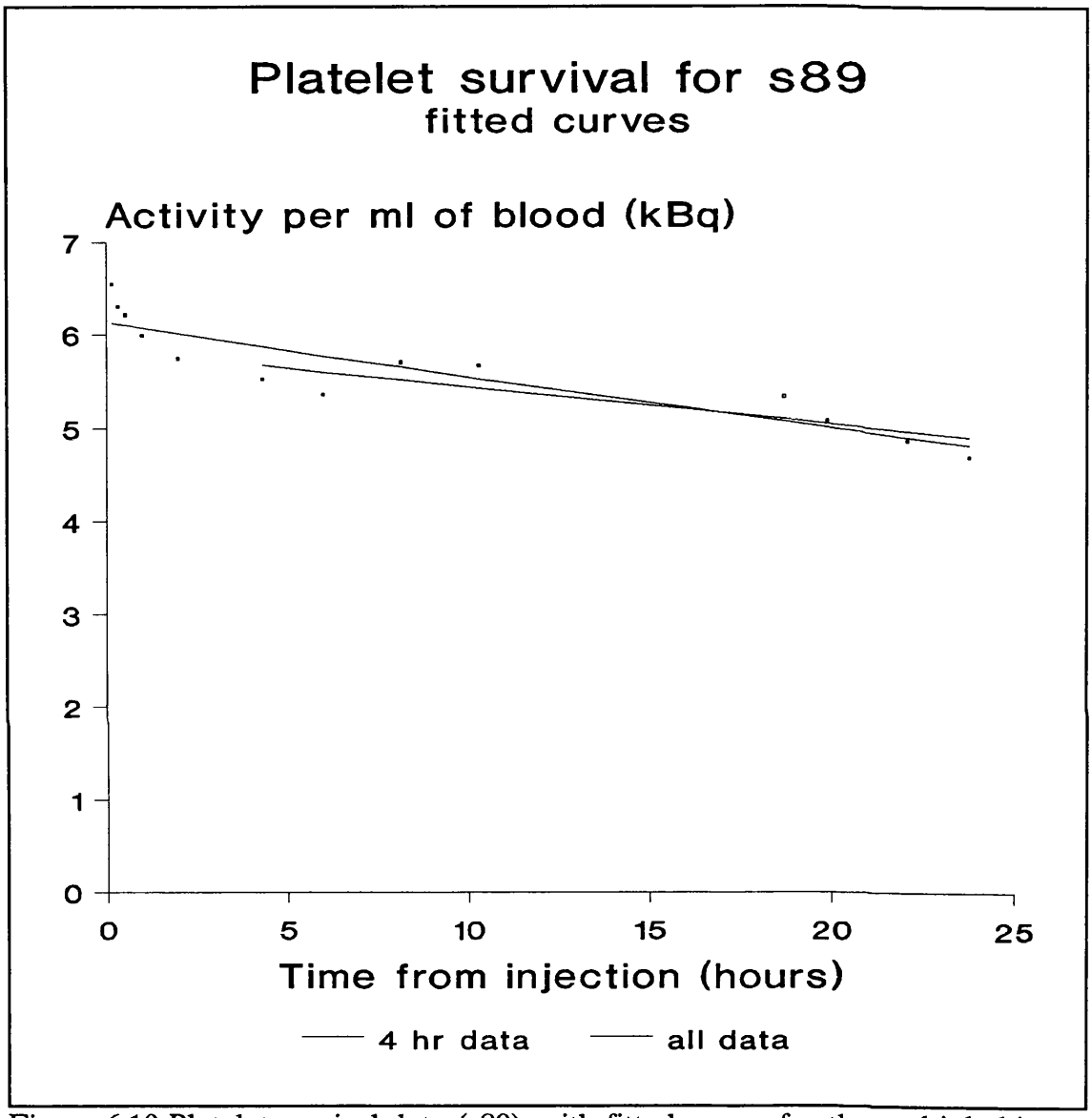


Figure 6.10 Platelet survival data (s89), with fitted curves for the multiple hit model.

Two examples of the platelet survival curves with the fitted curves from the ICSH programme are shown in Figures 6.10 and 6.11. These are for s89 (polyester G coating) with a longer survival time and a control graft with a shorter time, s93. There is no correlation between any of the estimates of platelet survival and the labelling efficiency, the delay, or any of the three measures of platelet recovery. Of these parameters only the survival data might be expected to be affected by the coating, the others reflect the animals and the result of the labelling.

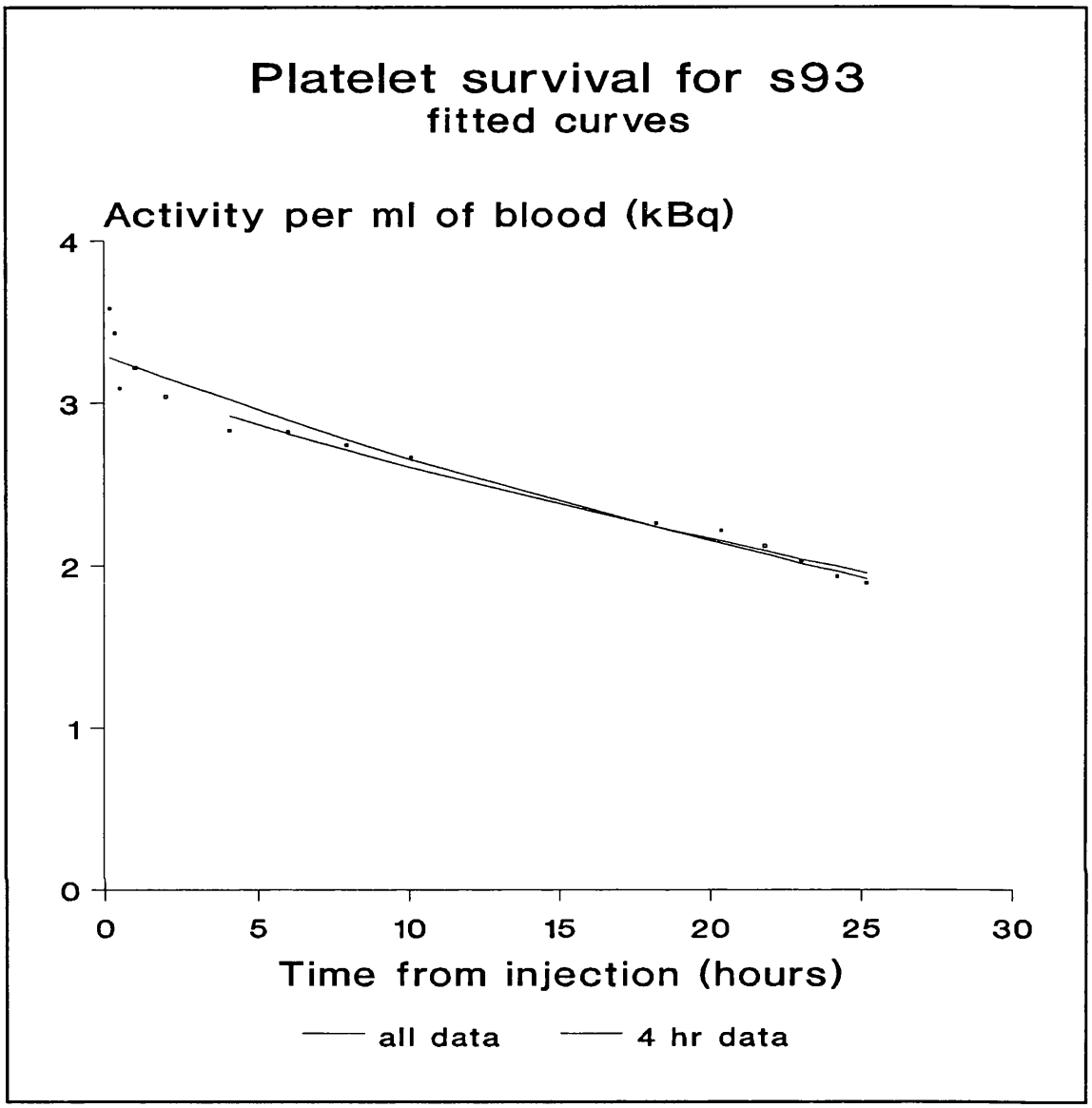


Figure 6.11 Platelet survival data (s93), with fitted curves for the multiple hit model.

The results for the different data sets used to calculate platelet survival are shown in Figure 6.12. The estimates for platelet survival for the treated grafts show a trend towards longer survival in the order sphingomyelin, polyester G and DAPC. Neither the oneway or the twoway ANOVAs with or without the inclusion of s91 and s591 are significant.

For SM the weighted mean (wtm) is 3.88 days and for the multiple hit mean (mhit) is 3.63 days for the '4 hour data' sets. These just fail to reach significance with $p=0.051$ for the Mann Whitney U test. Both wtm and mhit for the 'all data' and the 'fitted mean data' sets show no statistically significant difference.

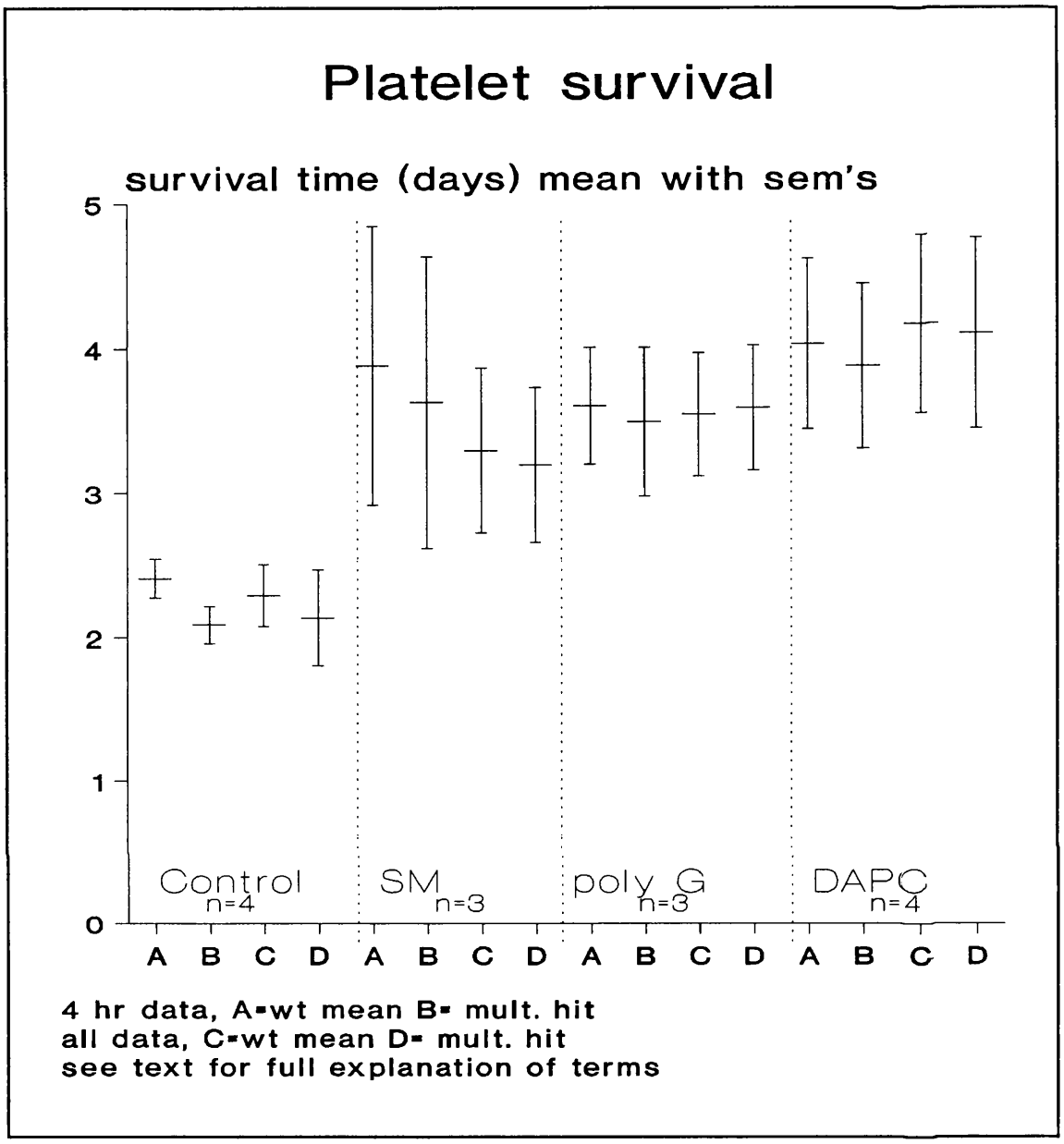


Figure 6.12 Estimated platelet survival for the graft coatings.

For polyester G the wtm is 3.61, and mhit is 3.50 days for the '4 hour data' set. These give $p=0.051$ for the MWU test, and $p<0.024$ for the pooled t test. Both the 'all data' and the 'fitted mean data' sets show no significant difference. This despite polyester G having the largest difference from the control for the 'fitted mean data' set, 3.96 (0.76) days compared with 2.51 (0.25) days for the controls.

DAPC gives 4.04 days by wtm and 3.38 by mhit with the 4 hr data set compared with 2.41 and 2.09 days for the controls. These differences for DAPC compared to the control for the weighted mean and the multiple hit mean for both the '4 hour' and 'all data' sets are significant for both the MWU and paired t tests except

for one. The exception is the mhit in the 'all data' set which gives $p<0.06$, by the MWU test. The others all give $p<0.03$, for the Mann Whitney U test. For the paired t test $p<0.036$ (wtm 4hr) 0.022 (mhit 4hr), 0.028 (wtm all data) and 0.037 (mhit all data).

While the differences in platelet survival are small between DAPC and the other treated grafts those for DAPC approximate the results for s91. These represent an operated animal with a closed graft and give survival times of 4.50 (wtm) and 4.22 (mhit), 4.85 (wtm) and 5.02 (mhit) days, for the 4 hr and all data sets respectively. The platelet survival for two unoperated animals presented in Appendix 5 have 24 hour times of 5.45 (wtm) and 5.47 (mhit) days. The direct AVF, also discussed in Appendix 5, gave the shortest time found in these studies with the weighted mean estimate being 1.47 days and the multiple hit being 1.17 days. The results for DAPC therefore lie towards the unoperated end of the range between 2.09-2.41 for the controls and 5.45-5.27 days for the unoperated animals. This trend for DAPC to have longer platelet survival times warrants further study.

6.4.4.2 Platelet uptake by the grafts

The platelet uptake measured on the grafts by the well counter is shown in Figure 6.13. The main finding is a lower uptake for sphingomyelin, with wider scatter for the other two materials. The difference for sphingomyelin is from 0.0219% for the controls to 0.0074% of the injected platelet dose; that is a reduction to 34% the uptake of the controls.

Before discussing the statistical significance of these findings it is important to note the lack of correlation between the graft uptake of platelets and the labelling efficiency or the delay. More importantly there is no correlation between the graft uptake and any of the measures of platelet recovery. This establishes that the different graft uptakes cannot be attributed to differences in the labelling or platelet function. Nor is there is no correlation between any of the measures of platelet survival and the amount of the platelet label taken up by the grafts. This not surprising since the survival data reflects what is happening to the whole of the

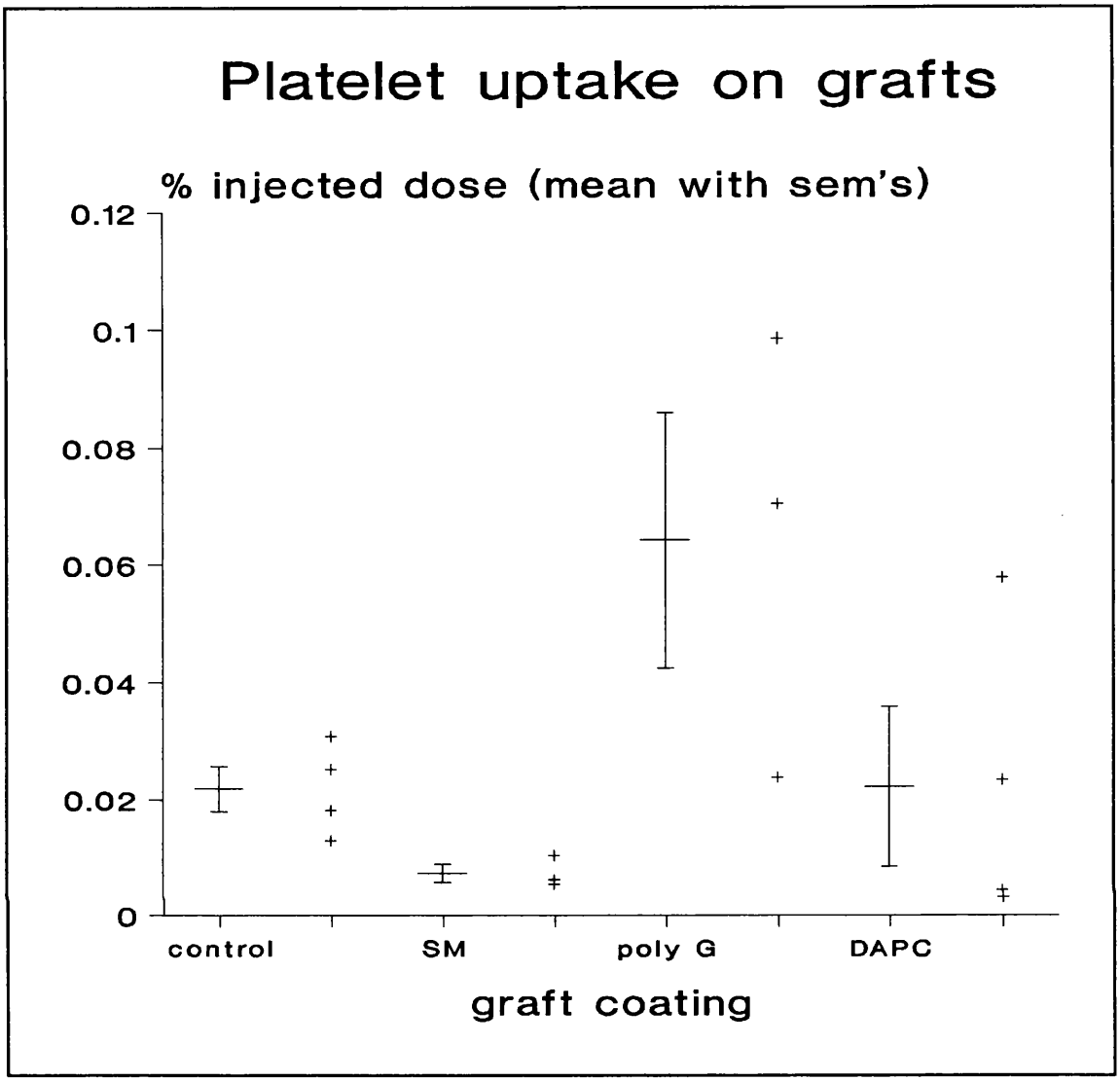


Figure 6.13 Platelet uptake on the grafts (s591 omitted).

labelled platelet population in the animal, while only a very small proportion of the label is actually incorporated in the graft thrombus. A very thrombogenic coating might have high graft levels, but would be likely to thrombose before affecting the platelet survival. Closer examination of the data shows that polyester G has both high uptakes on the grafts, and long survival times. Even removing this data the grafts still show no correlation between the survival and graft uptake.

Analysis of the 14 experiments omitting both s591 and s91 by the Kruskal Wallis test gives $H=6.5$, $p<0.09$. The oneway ANOVA ($N=12$) gives $F=3.68$, and $p<0.051$, and this is mainly due to polyester G (inclusion of s591 with the control data gives $p<0.037$). As the data has a wide scatter and possible non-normal distribution oneway ANOVA of the log transformed data gives $F=3.21$, $p<0.07$.

The twoway ANOVA for the treatments for the first 12 experiments has an F (3,6)= 5.60, $p<0.05$. (This is also the result with s591 included). The effect of the blocks is not significant. Further the Friedman test gives S=8.2, $p<0.043$. These show that the graft coatings have a effect on platelet uptake which lies about the 0.05% level of significance. The scatter of results for DAPC and Poly G mask the effect for SM.

Polyester G has the largest uptake of any of the coatings at 0.0643% (0.0218) of the injected dose, compared to 0.0219% (0.0038) for the control grafts. The difference is not significant by the MWU test, although for the pooled t test gives $p<0.074$, and on the log data gives $p<0.069$. (If s591 is included the control graft mean falls to 0.0179% (0.0049), and the MWU test gives $p=0.14$, while the pooled t test gives $p<0.036$).

There are no statistically significant differences for DAPC whether or not s91 or s591 are included, by any of the analyses.

The major finding of this study is the low platelet label uptake by the sphingomyelin coated grafts. These grafts have significantly less uptake than the control grafts. For SM the uptake is 0.0074% (0.0016) compared to the controls at 0.0219% (0.0038). The Mann Whitney U test gives $p<0.051$. The paired pooled t test is significant with $p<0.028$, and for the log transformed data gives $p<0.011$. This shows that SM does reduce the thrombogenicity of the Dacron. It is a reduction of 66% compared to the native material.

6.4.4.3 Gamma camera platelet uptake at 4 hours

The five methods of analysis of the 4 hours gamma camera images of the neck region and typical images are found in the methods section 6.3.3.7 above. There is a trend for polyester G to have uptakes higher than the controls at 4 hours, but none of the 5 methods show a statistically significant difference for any of the coatings, whether or not s591 is included, nor does the use of data from s91 make any difference for DAPC. All the ANOVA are also not significant. This is a disappointing finding since it might be anticipated that early graft thrombogenicity

would be altered by the coatings, on the basis of the *in vitro* work.

These estimates correlated with the final amounts of platelet thrombus adherent to the grafts, the fits are r^2's 0.36-0.60% and $p<0.031$-0.002.

6.4.4.4 Gamma camera platelet uptake at 24 hours

There is a trend for polyester G to have higher uptakes than the control grafts at 24 hours. However none of the gamma camera methods of analysis or any of the statistical methods applied to them have shown any significant differences. Similarly all the ANOVA are non significant. The correlations of these data to the thrombus adherent to the grafts are even poorer than for the 4 hour estimates. The best correlation is of the 'box lo' method, where $r^2=0.52\%$, $p<0.004$,; for the others $r^2=0.11$-0.35% and $p<0.22$-0.027.

The gamma camera is old, and there are other factors such as the positioning of the camera which all add to the errors in the data. In addition to these there is activity in the blood flowing through the graft at the time of acquisition of the image. The free blood in the graft is washed out by the perfusion fixation technique used to prepare the graft for counting in the well counter.

6.4.4.5 Gamma camera platelet uptake at 4 & 24 hours

The 5 parameters derived from the gamma camera images for 4 and 24 hours are compared. The r^2's for the regressions of the 4 hour data on the 24 hour data are between 0.42 and 0.68%, except for the 'box lo' estimate at 80%. All these are significant with $p<0.017$ and 0.0001.

The ratio of the 4 and 24 hour estimates and the differences between them have been calculated. The ratios demonstrate whether or not one graft coating accumulates or loses more of the label than any other. There is no clear pattern in the ratios to suggest that one of the coatings accumulates more or less label, implying that they are of similar thrombogenicity.

6.4.4.6 Comparison of blood pool activity and graft uptake

The % injected dose per ml in the blood pool at both 4 and 24 hours is comparable as shown above. However the amounts of platelet label taken up by the grafts are dependent on the activity present in the blood as well as the thrombogenicity of the grafts coating. The ratio of the estimates of platelet label on the grafts and that in the blood gives a further measure of the graft thrombogenicity.

The analysis of the gamma camera images makes corrections for the background activity including the blood pool in the image. However the ratios using the blood activity have also been calculated for all the subjects and each of the five estimates of graft uptake at both 4 and 24 hours. These all fail to demonstrate a statistically significant difference. There is a trend for polyester G to have higher ratios, suggesting that more of the label is taken up by this coating. For example the ratio for 'est ave' at 4 hour is 6.66 compared to the control at 3.66.

The ratios between the activity measured on the grafts (and therefore adherent to the grafts) and that measured in the blood taken at 24 hours in the well counter are shown in Table 6.5. These are graft:blood platelet label per ml ratios, and because of the possible non-normality of the data the log transformed data is also shown. This ratio is an important measure as it assesses the uptake by the grafts in comparison to the activity in the blood at 24 hours.

coating	mean	sem	log mean	log sem
control	0.857	0.111	-0.078	0.057
SM	0.291	0.047	-0.547	0.070
poly G	2.818	1.220	0.369	0.190
DAPC	0.908	0.522	-0.321	0.300

Table 6.5 Ratio of graft:blood platelet activity at 24 hr.

The twoway ANOVA shows no significant differences, but the Friedman gives

S=8.2, and p<0.043. The oneway ANOVA gives F=3.00 (p<0.08) and for the log data F= 3.46 (p<0.059). Kruskal Wallis gives H= 6.48, p<0.091.

The increased uptake by Polyester G at 2.8 is larger than the controls at 0.86. This difference (threefold) is large and borders the 5% level of significance. The trend for this coating to have increased uptake has been noted for the gamma camera image ratios as well. The MWU gives p<0.051, and 2t gives p=0.12, the log transformed 2t gives p<0.047.

For sphingomyelin the reduction in the graft:blood ratio is from 0.86 for the controls, to 0.29. The Mann Whitney U test gives p<0.051. The two sample pooled t test is also significant for the native data p<0.009, and for the log data p<0.047. This is a significant reduction in the amount of platelet label taken up by the sphingomyelin coated grafts, to about one third that of the controls. This again shows a reduction in the Dacron's thrombogenicity after sphingomyelin coating.

6.4.4.7 Tissue distribution of the platelet label

The organ distribution of the label is estimated in two main ways, from the gamma camera images, and the tissue samples. For the latter biopsies of each organ were taken after the organ was weighed. For the heart 1-2% of the injected dose is present by analysis of the gamma camera images at 4 and 24 hours. The tissue sample estimate is 0.2%. The gamma camera images of the heart reflect the blood pool, since there is blood in the heart chambers. These are 5 times the estimate of the tissue biopsies for the muscle. There are no differences between the groups.

For the liver between 5-6% of the label is found at 4 hours rising slightly to 5-7% at 24 hours. The biopsy based estimate for the treated groups is 13-15%. For the control group one subject (s19a) has a high result of 34%, the mean of the rest of the group is 13%. Again there are no significant differences between the groups. The splenic distribution estimated by the gamma camera is 7-10% at 4 hours and around 6% for the different groups at 24 hours. The tissue estimates for the

treated groups are 16%, but the control group is lower at 12%. This difference is not significant by any of the statistical methods.

For all these three organs there is no significant accumulation or decrease in the activity over the course of the experiment. The most interesting pattern of uptake is for the lungs. These have been estimated for right and left lungs, because of their anatomical differences. The results for the individual lungs, however give a similar pattern to that for the total for both sides, and so only the results for the totals are presented (Table 6.6). The results for s591, which form the basis for suggesting an error in the platelet labelling are 39% at 4 hours, 23% at 24 hours and 84% by biopsy.

Graft coating	gamma camera images 4 hour	24 hour	tissue biopsy
control	19.3 (4.9)	14.9 (2.2)	36.9 (6.2)
SM	12.1 (2.6)	11.0 (1.5)	30.7 (2.9)
poly G	11.5 (2.1)	11.8 (3.9)	32.1 (2.7)
DAPC	10.7 (0.6)	10.1 (1.1)	26.0 (4.9)

Table 6.6 Lung uptake of platelet label, % injected dose.

All the subjects with treated grafts have less label in the lungs estimated by the gamma camera image at 4 and 24 hours, and by tissue biopsy. These differences are not statistically significant except for the DAPC grafts at 4 hours by the Mann Whitney U test ($p<0.03$). By comparison the results for s91, the thrombosed graft are 10% at 4 hours, 9% at 24 hours and 11% by biopsy. These are close to those of the treated groups. No emboli were found macroscopically in any animal.

Possible reasons for this difference are that lungs are the first organ for the injected label to pass through, and denatured label and cellular debris are trapped here. The amount in the lungs at 24 hours is certainly lower (19% dropping to 14%). It may be that the control grafts cause more damage to the labelled cells in the circulation. The results for the heart, liver and spleen are all fairly close for each group. The spleen and liver are the main organs in the reticulo-endothelial system responsible for the phagocytosis of the effete RBC's and platelets.

Damaged cells are removed by these organs. The lack of differences between the groups for these three organs supports the uniformity of the labelling etc (see above in section 6.4.2). These observations tend to favour the second explanation.

The importance of these observations is that there is no evidence to suggest that any of the experimental coatings damage the circulating labelled platelets, causing them to be removed by one of these organs. Indeed if the cellular blood elements were damaged the platelets would also bind them as micro thrombi. The lack of difference for the groups supports this conclusion and supports the direct evidence from the haemolysis assay presented above. This clearly has important implications in suggesting that these materials are safe to implant.

The difference between the tissue biopsy result and the 24 hour gamma camera image analysis of the distribution of the label are remarkably consistent. The ratio for all the organs, except the heart, is very close, at 0.4. The balance of the dose is in the blood pool. The mean dose in the 24 hour estimated blood pool is 49% (3.2 sem). The mean total recovered dose is 111% (4.5 sem). The reason for the underestimate of the organ doses for the gamma camera almost certainly lies with the design of the phantom. This design was made solely to estimate the activity on the graft in the neck, and does not represent the degree of tissue attenuation within the organs and the overlying tissues. The error is the same for all the animals, so the comparison is still valid. The consistency of the ratio confirms of the validity of the gamma camera imaging system, and the analytical methods used to quantify the data obtained, thus supporting the analysis of the 4 hour data for all the organs and more importantly for the graft data presented above.

6.4.5 The fate of the fibrinogen label

6.4.5.1 Fibrinogen labelling and distribution data

The similarity of the groups has been estimated from the 4 and 24 hour blood levels, as the experiments do not last long enough to collect the data necessary for the fibrinogen survival to be calculated. The data for s591 is included for this

analysis, but s91 is not. The mean whole blood level at 4 hours for each of the groups lies between 0.032 - 0.036% of the injected dose per ml. There is no difference between the groups. This falls over the course of the experiment to between 0.017 - 0.019% for the groups, again there is no difference between the groups. The product of the % dose per ml and the estimated blood volume gives a measure of the label remaining in the intravascular pool at 24 hours. The mean of all the groups is 36.5% (0.002), the differences between the groups from 31 to 41% are not significant. In passing it should be noted that there is still a high proportion of the label of unactivated fibrinogen free in the system.

The fibrinogen uptake is independent of all the platelet parameters, as might be expected. There is no correlation between the fibrinogen uptake on the graft with the platelet uptake, nor with either the platelet recovery or survival data.

6.4.5.2 Fibrinogen graft uptake

The highest uptake is by the sphingomyelin coated grafts, 0.0705% (0.0094) of the injected dose. However all the statistical measures show this is not significantly different from the control grafts at 0.0547% (0.0114). Unfortunately one of the control grafts, s93 had the second highest uptake (0.0990) and was different from the remaining controls, whose mean is 0.0436% (0.0034). If this data is considered to be an outlier the difference for SM just becomes significant (MWU $p<0.051$, pooled t $p<0.029$). The differences for the other two coatings are not significant. The one and twoway ANOVAs are all non significant with or without s91. This data is shown in Figure 6.14.

The ratio of the amount of label on the graft to that in the blood at 24 hours again shows no significant difference between the groups. There is however a marked trend for sphingomyelin to have the highest graft:blood ratio, of 3.9, compared to the controls (3.1) and the other treated groups (2.7 for poly G and 3.2 for DAPC).

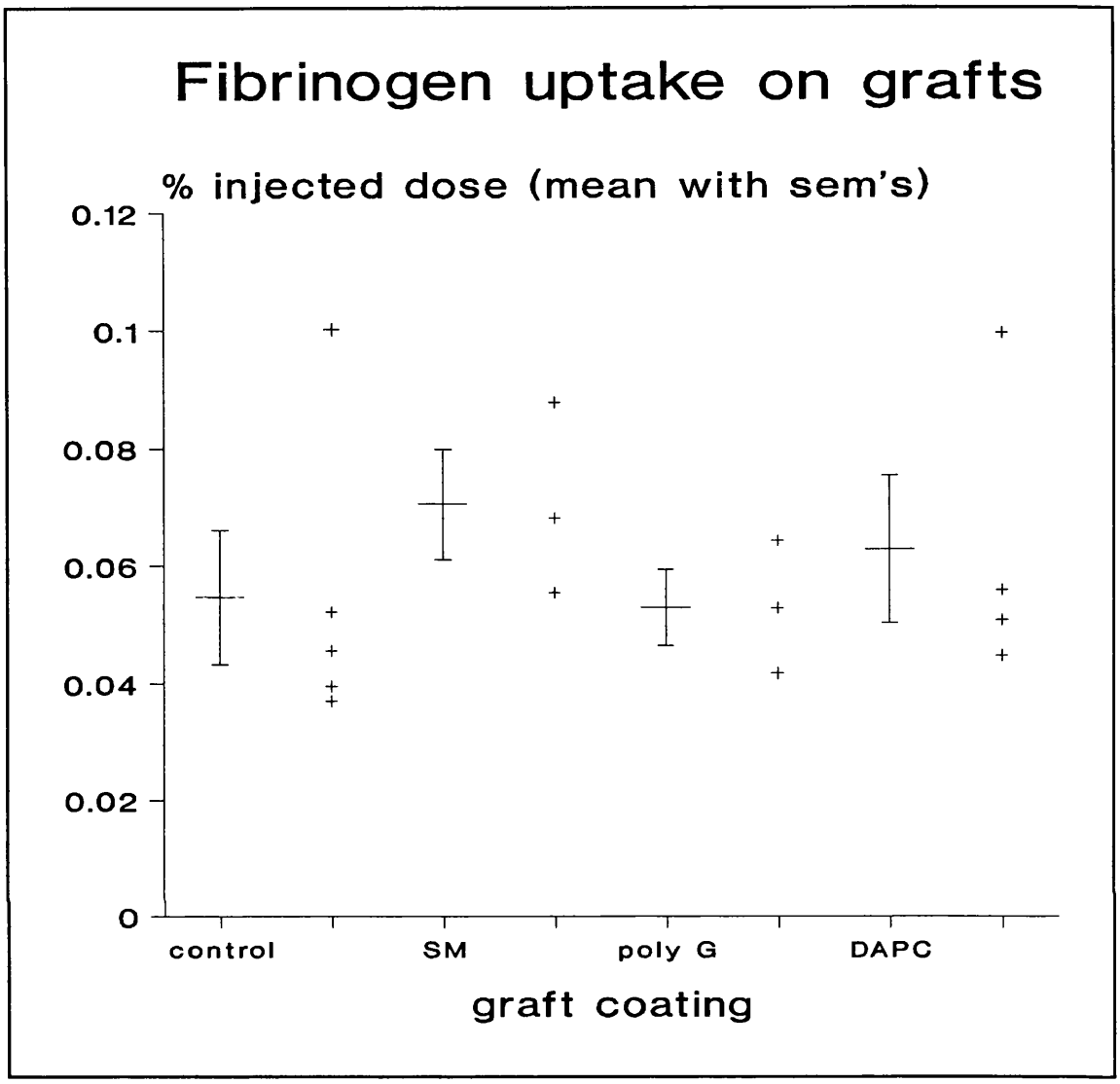


Figure 6.14 Fibrinogen uptake by the grafts.

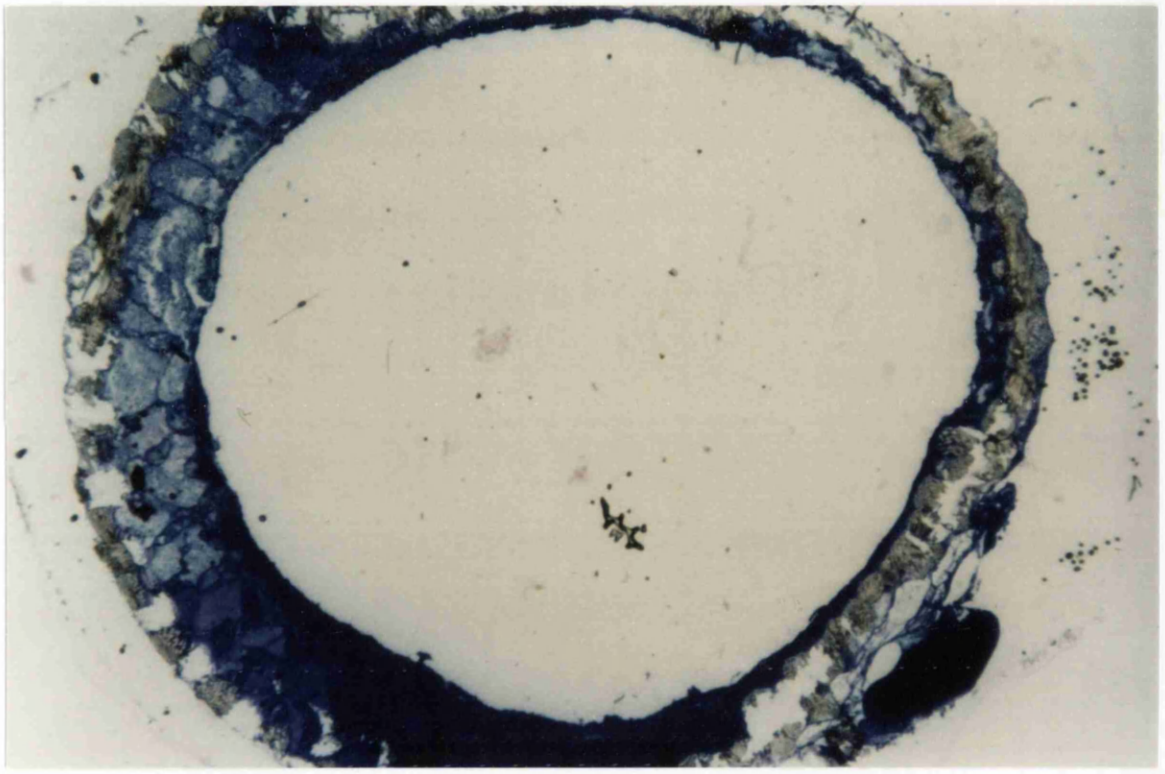
6.4.5.3 Organ distribution of fibrinogen

The results for the fibrinogen distribution in the tissue biopsies of the organs show differences between the groups, for the lungs, and the spleen, but not for the heart or liver. For the heart the polyester G group has a low result of 0.20% of the injected dose compared to 0.28% and 0.29% for the others. For the liver the uptake is similar for all the groups lying between 1.8 and 2.2%.

For the spleen the control group uptake is 0.28%, while that for SM is 0.55%. This is significant with $p<0.04$ for the Mann Whitney U and $p<0.005$ for the pooled t test. The uptake for other two coatings lies in between these two, at 0.44% for poly G and 0.48% for DAPC.

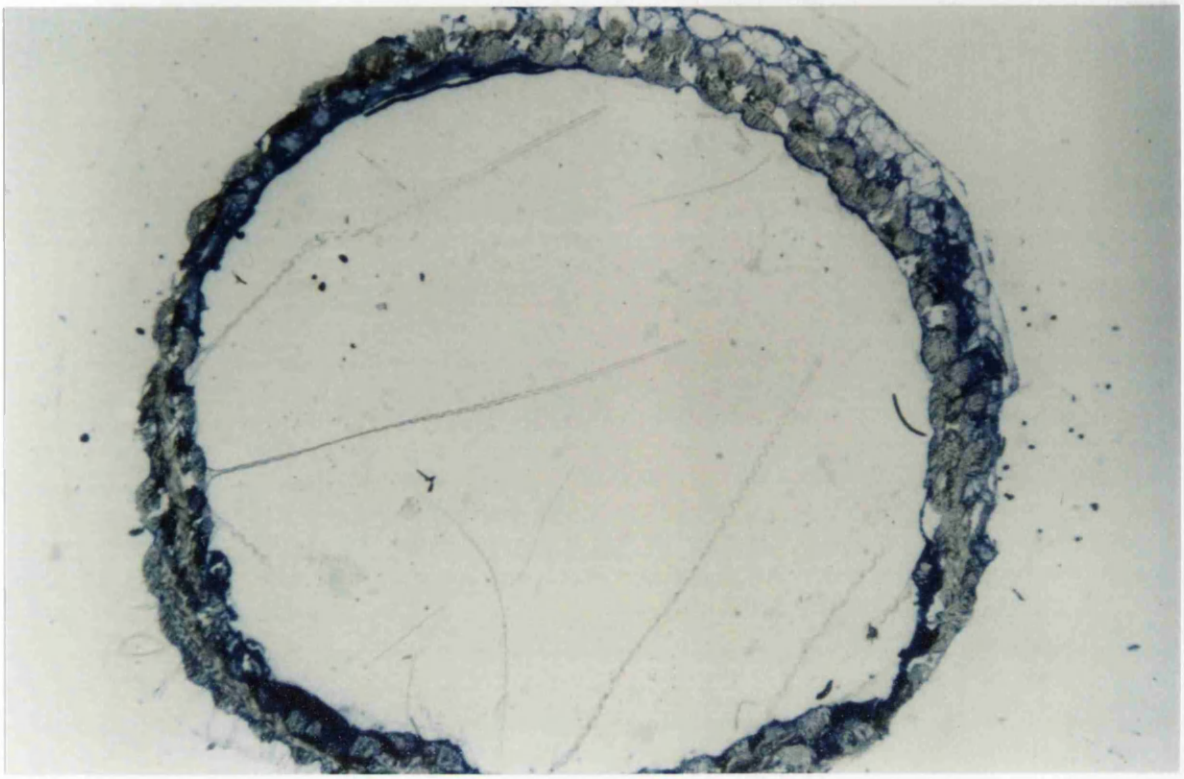
The pattern of fibrinogen distribution is similar in the lungs to that of the platelet distribution. Again the uptake on each side follows the pattern for the total, and only the total uptakes for both lungs will be presented. The differences are small but the amount for polyester G is 1.69% compared with 2.54% for the controls is significant with the MWU test giving $p<0.037$, and the pooled t test giving $p<0.013$. The uptake for SM is 2.1%, and for DAPC is 2.46%, neither of these are significantly different from the controls.

6.4.6 Results for histological studies



Photograph 6.7 Graft cross section for untreated Dacron (s591), note the blue stained thrombus.

The photography of the graft specimens presented few problems compared to the preparation of the midpoint cross sections. The tendency to fragment as the section is cut has been minimised by embedding the grafts in resin. Two examples of the photographs are shown in Photographs 6.7 and 6.8. As discussed above the DAPC coated grafts include the only graft which thrombosed during the study, s91, and the same reasons for omitting it from these analyses apply. The data for s591 is included on the assumption that the defective platelet label does not affect the thrombus laid down on the grafts.



Photograph 6.8 Graft cross section for sphingomyelin coated Dacron graft (s770), note the reduction in thrombus (blue area).

6.4.6.1 Centre graft cross sectional thrombus area

The results of the area of thrombus, and the perimeter of the graft for the three sections examined from each graft show very good agreement. The mean of these three estimates is presented in Table 6.7. The mean perimeter of all the grafts is 18.0 mm (sem 0.22), giving an estimated diameter of 5.29 mm. The mean cross sectional area for the grafts is 21.96 mm (sem 0.53), giving an estimated diameter of 5.73 mm. Both these estimates for the diameter of the graft material are highly significantly different from the perimeter of 15.71 mm, and the cross sectional area of 19.64 mm calculated from the supplied graft diameter of 5 mm. The mean for the shape term is 92.2% (sem 0.47), demonstrating a small departure from a true circle. This may account for some of the apparent increase in the diameter of the grafts. There are no differences between the graft coatings for these three estimates, and this confirms the accuracy of the automated analysis. Repeated manual estimates made on one set of slides also gives reproducible results. All the data presented for the manual and the automated estimates of the thrombus areas are the mean of measurements taken from three sections of each graft. These

id	auto area	man area	peri	cross	shape	code
s18	1.17	3.46	17.61	19.69	89.28	con
s16	0.57	1.52	17.26	19.60	90.96	DAPC
s770	0.42	1.53	16.47	19.00	93.82	SM
s736	0.48	2.44	16.97	18.82	90.62	poly G
s540	2.24	3.97	18.01	22.35	93.05	DAPC
s19a	4.98	7.20	18.67	24.57	94.14	con
s553	0.21	1.30	18.36	23.17	92.94	SM
s3	1.64	4.07	19.06	24.19	91.47	poly G
s591	4.08	7.01	18.64	24.94	95.00	con
s514	1.04	2.14	17.74	21.90	93.52	DAPC
s89	0.45	1.44	17.32	20.73	93.21	SM
s505	0.85	3.34	18.17	22.23	91.98	poly G
s95	4.34	8.26	18.56	23.87	93.37	con
s93	1.96	5.17	18.54	22.89	91.46	con
s92	3.56	6.46	19.75	23.93	87.78	DAPC
s91	17.88	17.78	16.96	19.53	92.41	DAPC

auto = area of thrombus by automated analysis, mm^2
man = area thrombus by manual analysis, mm^2
peri = perimeter or circumference of graft wall, mm
cross = cross sectional area of the graft wall, mm^2
shape see text, code = material coating on graft.

Table 6.7 Cross sectional areas of thrombus at mid point of graft.

individual observations of both estimates are very close.

All these findings support the validity of both sets of data. The automated estimate of the area of thrombus within the graft is smaller than the manual estimate. The two sets of results are closely correlated, as shown in Figure 6.15. The regression data is Manual = 1.34 + 1.40 automated, r^2 = 0.92%, $p<0.0001$. (If S91, the thrombosed graft, is included it skews the regression to give Manual = 2.14 + 0.934 automated, r^2 = 0.94%, $p < 0.0001$.)

Figure 6.15 shows the difference (manual - automated) plotted against the manual estimate. The regression data for this is Diff =0.74 + 0.34 Manual, r^2 =0.76% $p<0.001$. The oneway ANOVA gives $F(1,13)=41.6$, $p<0.001$. These demonstrate that the difference between the two estimates are proportional to the amount of thrombus present, and are not related primarily to the coatings themselves. The

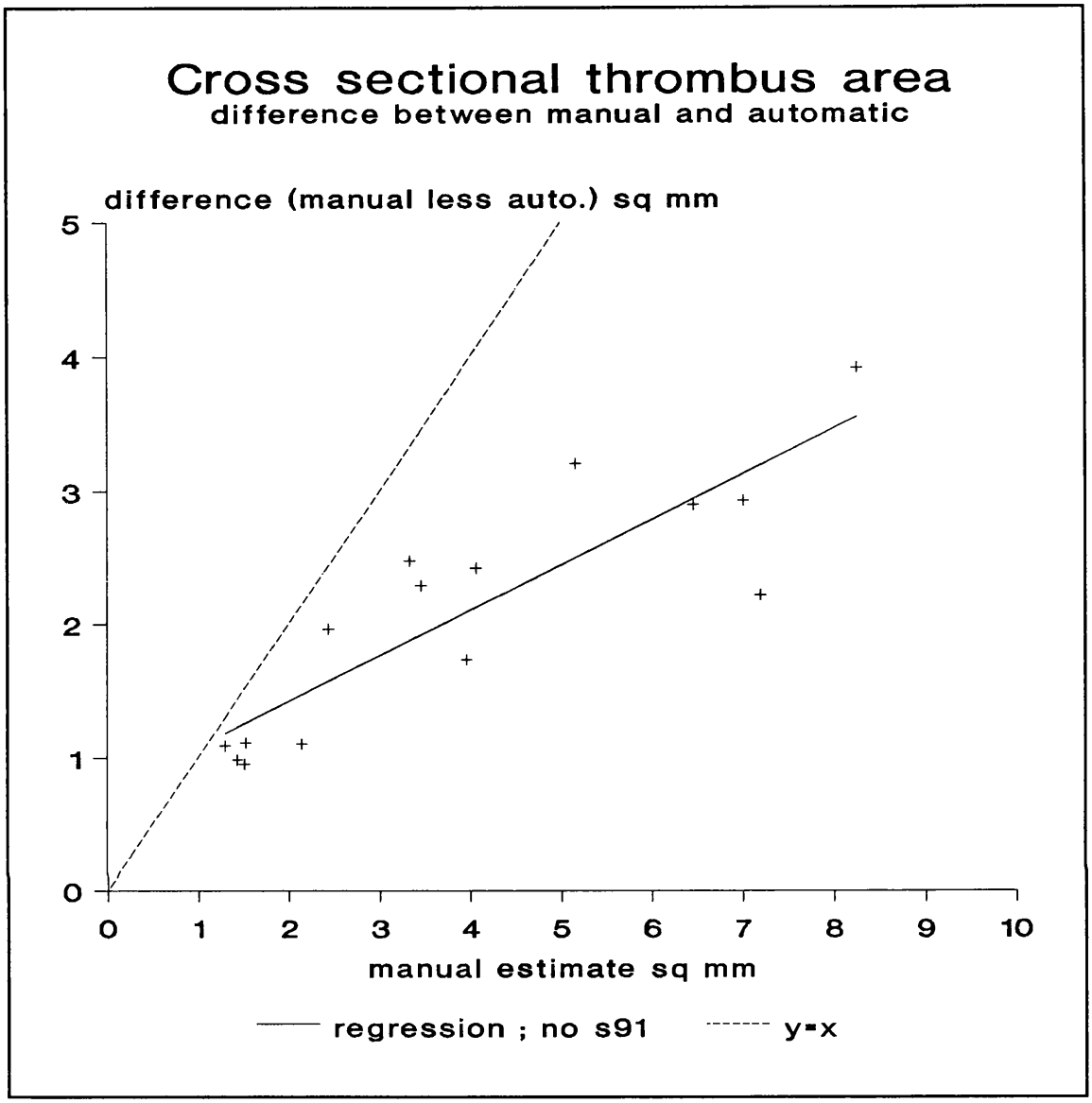


Figure 6.15 The difference between the manual and automated estimates of thrombus area plotted against manual estimate.

relative rankings of the two sets of data is shown in Table 6.8. There are 5 unchanged rankings and those for the last 5 places are equivalent, despite the change in order of s95 and s19a. The rest of the rankings are split by the first control (s18). Those in first 5 are all under 0.5 mm^2 and also under 3% of the graft cross sectional area. The differences between the two estimates are smallest for the sphingomyelin coated grafts, and largest for the controls.

The results for each estimate for the coatings are shown in Figures 6.16 to 6.18 and summarised in Table 6.9. These figures show the data from s91 as numbers, and the mean and error bars omit the s91 data. The principal findings are a

automated estimate				manual estimate		
Area (mm^2)	id	coating		coating	id	Area (mm^2)
0.21	s553	SM	=	SM	s553	1.30
0.42	s770	SM		SM	s89	1.44
0.45	s89	SM		DAPC	s16	1.52
0.48	s736	poly G		SM	s770	1.53
0.57	s16	DAPC		DAPC	s514	2.14
0.85	s505	poly G		poly G	s736	2.44
1.04	s514	DAPC		poly G	s505	3.34
1.17	s18	con	=	con	s18	3.46
1.64	s3	poly G		DAPC	s540	3.97
1.96	s93	con		poly G	s3	4.07
2.24	s540	DAPC		con	s93	5.17
3.56	s92	DAPC	=	DAPC	s92	6.46
4.08	s591	con	=	con	s591	7.01
4.34	s95	con		con	s19a	7.20
4.98	s19a	con		con	s95	8.26
17.88	s91	DAPC	=	DAPC	s91	17.78

Table 6.8 Relative rank orders for the manual and automated estimates of thrombus area.

coating	automated	manual	% thrombosis
control	3.31 (0.74)	6.22 (0.85)	13.9 (2.8)
SM	0.36 (0.08)	1.42 (0.07)	1.8 (0.4)
poly G	0.99 (0.34)	3.28 (0.47)	4.4 (1.3)
DAPC (n=5)	5.06 (3.25)	6.38 (2.98)	24.8 (16.8)
DAPC (n=4)*	1.85 (0.67)	3.52 (1.11)	8.1 (2.7)

* omits s91, see text

Table 6.9 Mean and (sem) for cross sectional areas of thrombus in grafts (mm^2).

reduction in the thrombus thickness with all coatings. The most dramatic reduction is with the sphingomyelin coating.

The oneway ANOVA gives $p<0.033$ for the automated analysis, and $p<0.014$ for the manual analysis. The KW test gives $H=9.93$ $p<0.02$ ($p<0.023$ if s91 is included) for the automated estimate. KW gives $H=9.36$ $p<0.025$ for the manual estimate.

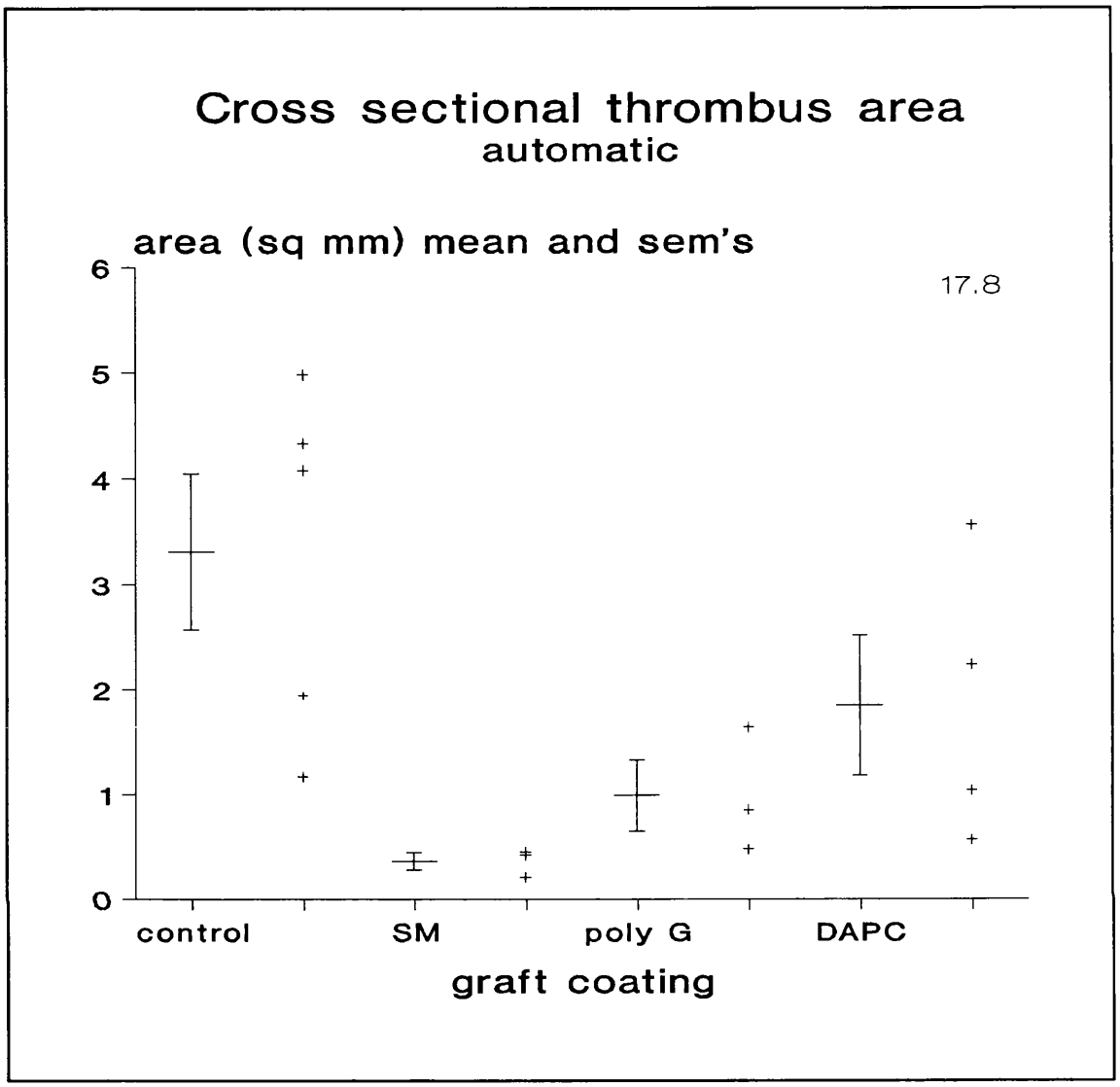


Figure 6.16 Automated estimate of cross sectional area of thrombus.

The Friedman test for the first 12 experiments gives S=9.0, $p<0.03$ automated, and S=8.2, $p<0.043$ manual estimate. The twoway ANOVAs for both manual and automated analysis show the F ratio for the blocks are not significant, but those for the coatings are. For the automated analysis the $F(3,6) = 6.39$ ($p < 0.05$), and for the manual analysis is $F(3,6) = 10.79$ ($p < 0.01$). This implies that the coatings do make a difference to the control material.

The statistical analysis for the DAPC grafts is presented with and without the data from s91. The MWU tests for all the data are not significant nor are the 2t tests, even after log transformation of the data.

The results for the polyester G coating show less thrombus than the controls. The

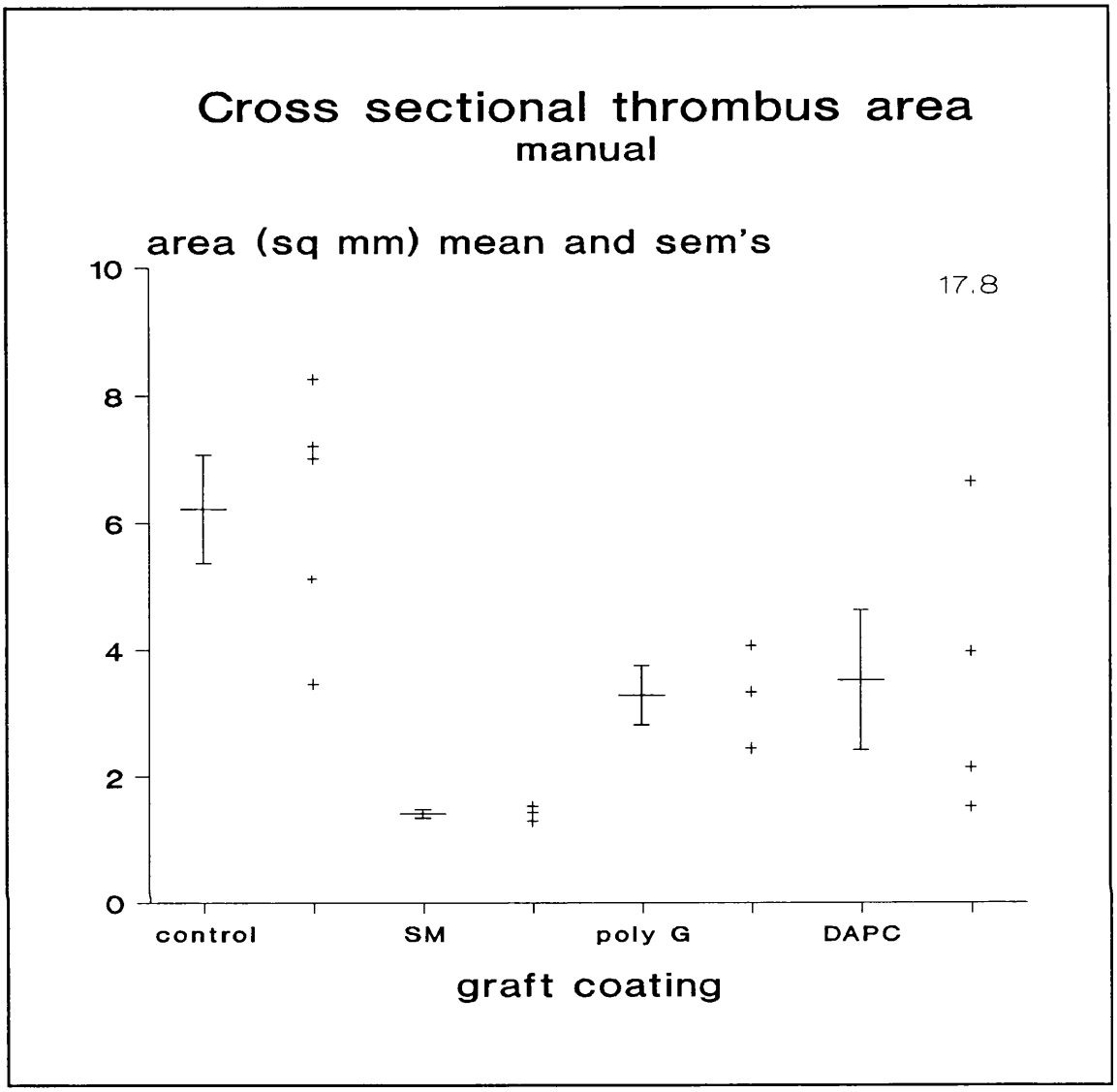


Figure 6.17 Cross sectional thrombus area by manual estimate.

pooled t test for the manual estimate is significant at $p<0.048$, for the means of 6.2 and 3.3 per mm^2. The result for the automated estimate (0.99 vs 3.31) just fails to reach a significant level at $p=0.063$. The Mann Whitney U gives $p=0.074$, for both measures.

The sphingomyelin coated grafts have the smallest area of thrombus, on the cross sections and therefore the smallest thickness of thrombus. The automated estimates are 0.36 compared with 3.3 mm^2 for the controls. The Mann Whitney U test gives $p<0.037$, and the 2t test gives $p<0.024$. The manual estimates show a similar difference with the SM grafts area of 1.42 compared to 6.22 mm^2 for the control. The MWU test gives $p<0.037$ and the 2t $p<0.0055$. These demonstrate that sphingomyelin has only 11% the thrombus present on the control grafts (by

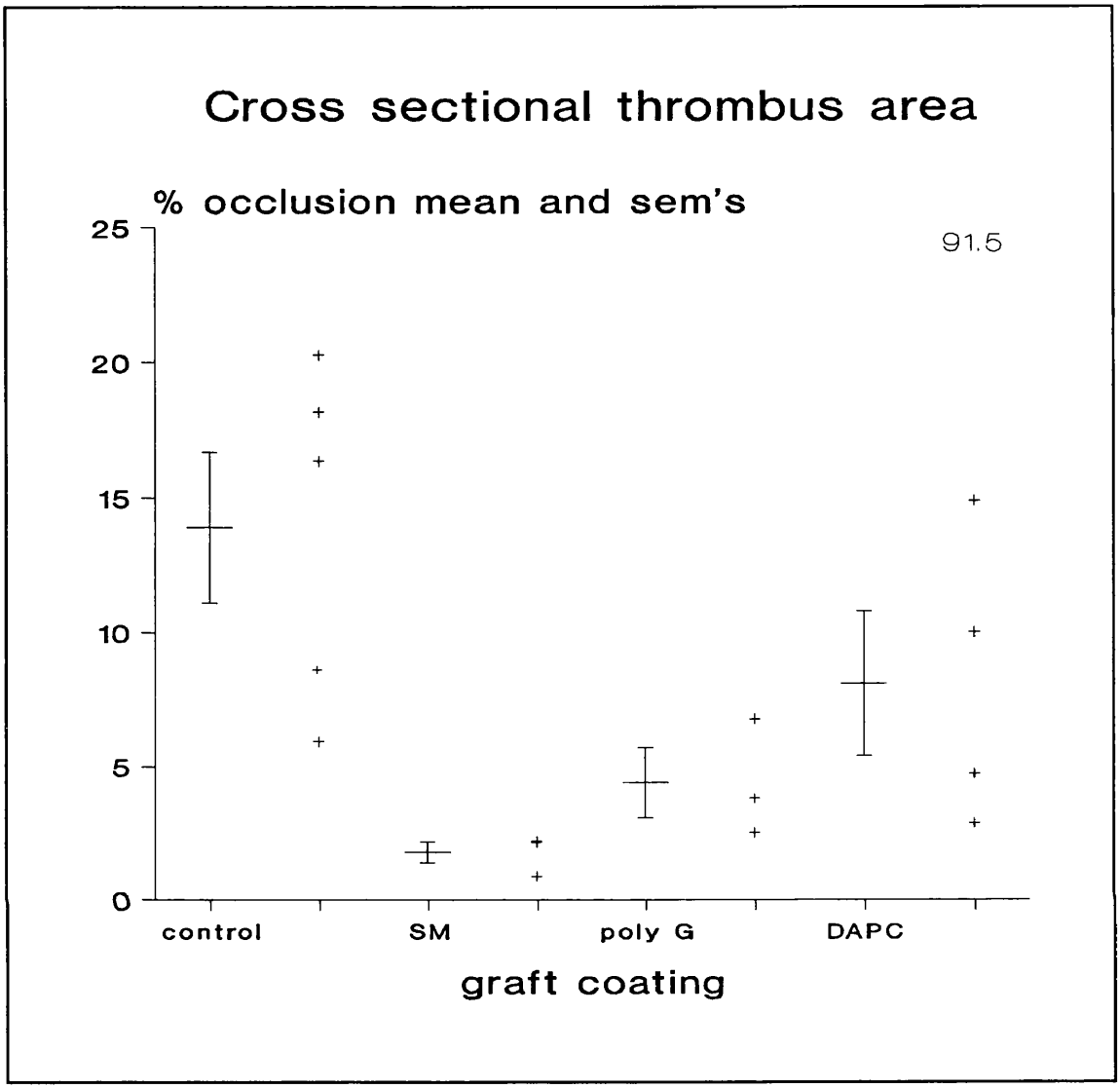


Figure 6.18 Percentage occlusion of cross sectional areas of the grafts by thrombus.

automated analysis), and 22% (by manual estimate). Both represent a marked and statistically significant reduction with the sphingomyelin coating. These results are also presented in terms of percentage occlusion of the grafts. Again the marked reduction by the sphingomyelin coating is dramatic, from 13.9% to 1.8% (Table 6.9).

6.4.6.2 Luminal surface thrombus free area

The results for the analysis of the photographs of the luminal surface of the grafts are presented in Figures 6.19 to 6.21, with s91 omitted.

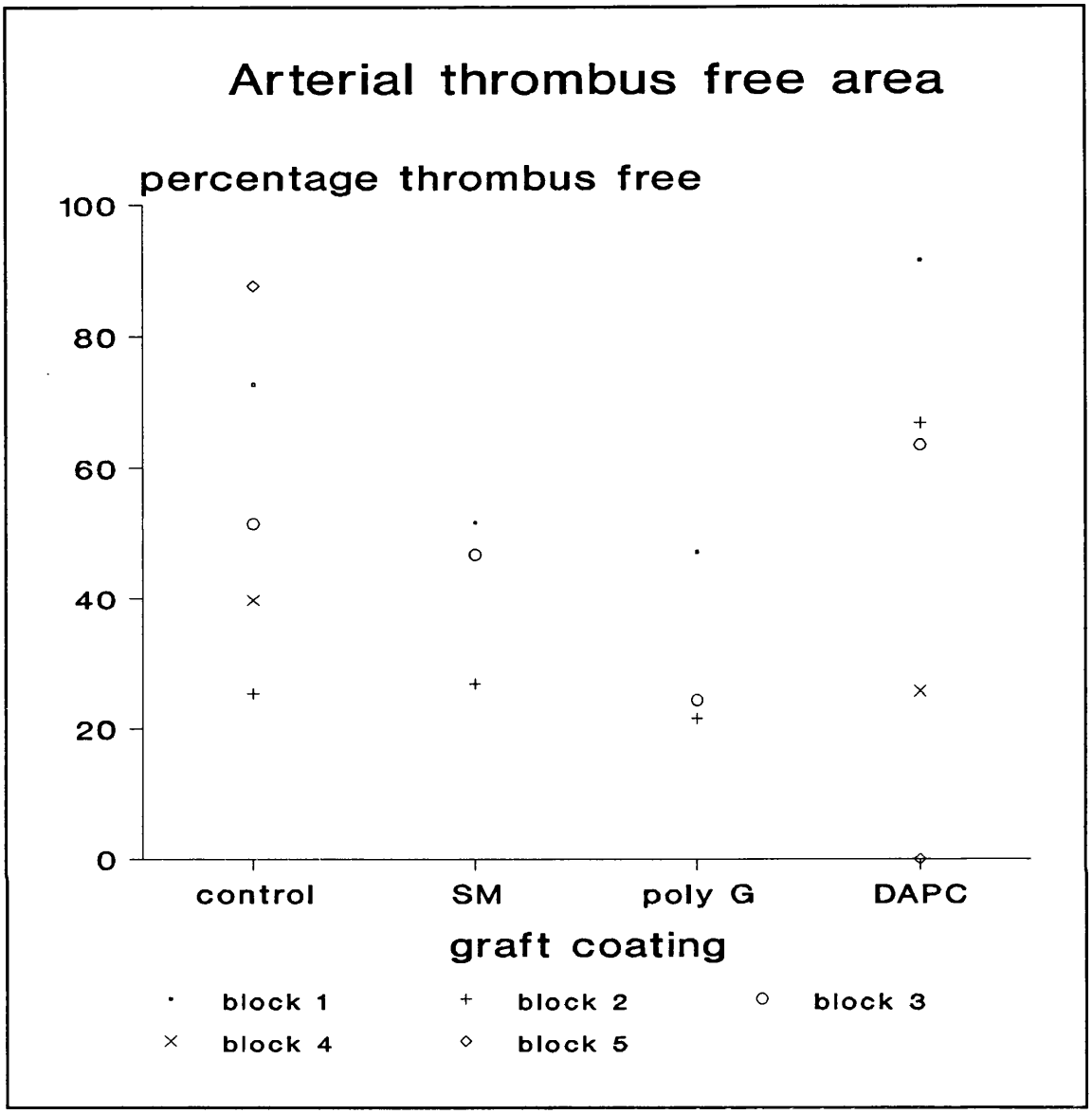


Figure 6.19 Thrombus free areas for the arterial luminal surface of the grafts.

The mean surface areas for the arterial and venous ends of the grafts are 282 mm^2 (sem 7.3) and 278 mm^2 (sem 7.7). The mean of the total graft area is 560.6 mm^2 (sem 12.6). This compares favourably with the estimates of graft area implanted, 4 cm at 5 mm diameter gives an area of 628 mm^2 (ie 89%). The estimates of true diameter measured from the microscope slides given above in section 6.4.6.1, are slightly larger. These can be used to give estimates of the length of graft actually implanted, for the nominal 5 mm diameter, the length of graft is 3.57 cm. Using the diameter estimated from the measured perimeters of the grafts of 5.29 mm the length is 3.37 cm, and for the diameter 5.73 mm (measured from the cross sectional areas) it is 3.11 cm.

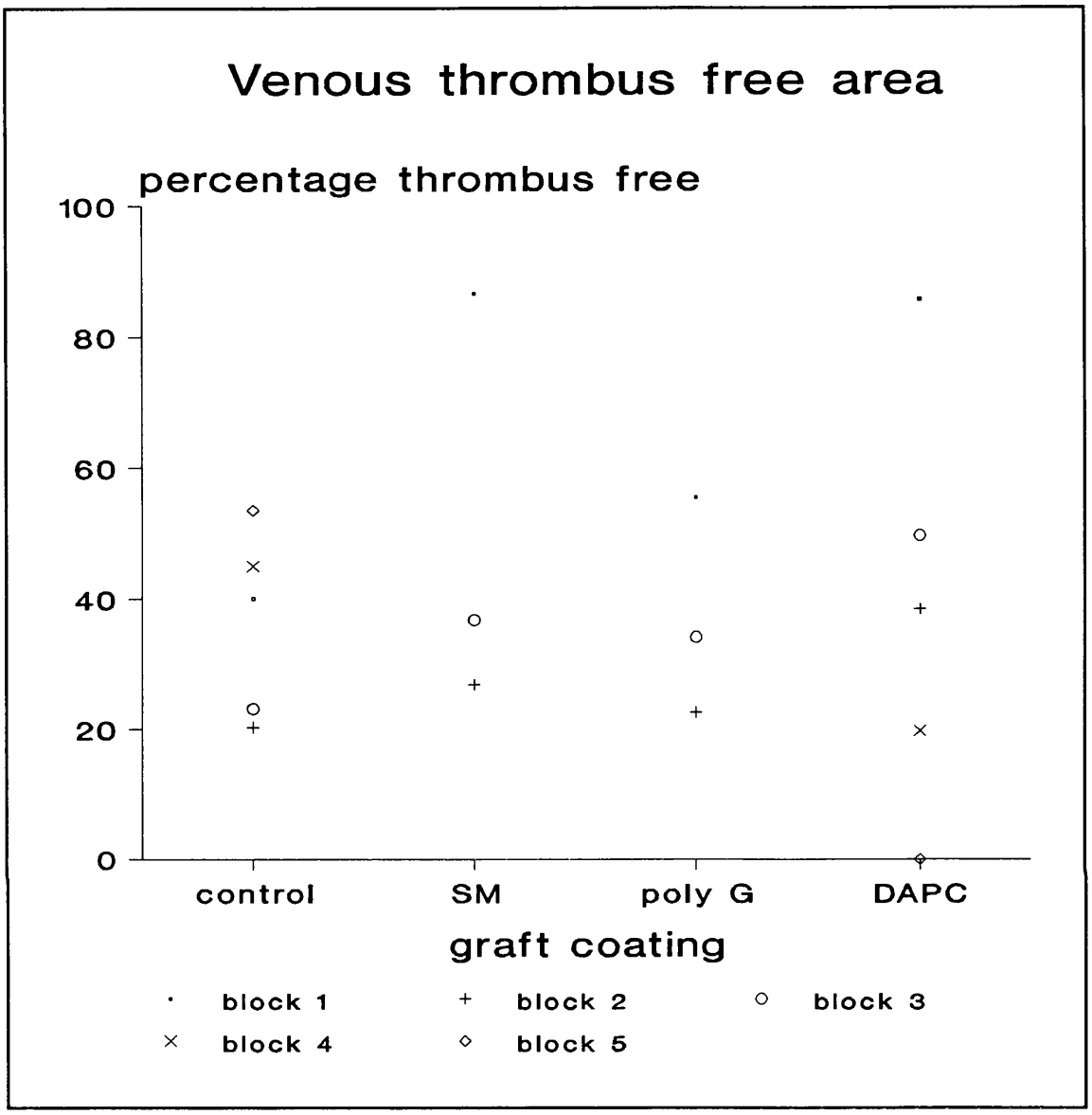


Figure 6.20 Thrombus free areas for the venous luminal surface of the grafts.

difference (coated - control)	% thrombus free areas		
coating	arterial	venous	total
SM	-8.1 (6.7)	22.2 (12.3)	6.4 (2.5)
Poly G	-18.8 (7.5)	9.6 (3.9)	-5.2 (2.3)
DAPC	14.5 (11.4)	16.2 (15.0)	14.6 (11.8)

Table 6.10 Difference (coating minus control) for percentage thrombus free area for each coating (paired differences from each block).

Figures 6.19-6.21 show the scatter of the data, and no clear picture emerges from the rank orders for either end of the grafts, or their entire thrombus free area.

There are no significant differences between the means for each groups with either the Kruskal Wallis, oneway ANOVA, paired pooled t or Mann Whitney U tests. However there is a marked trend related to the blocks of the experiment across the groups. The amount of thrombus free area is largest in the first block, falling in the 3rd block with the lowest found in the 2nd block. The effect of the block can be removed, by using the observations in pairs. This data is shown in Table 6.10.

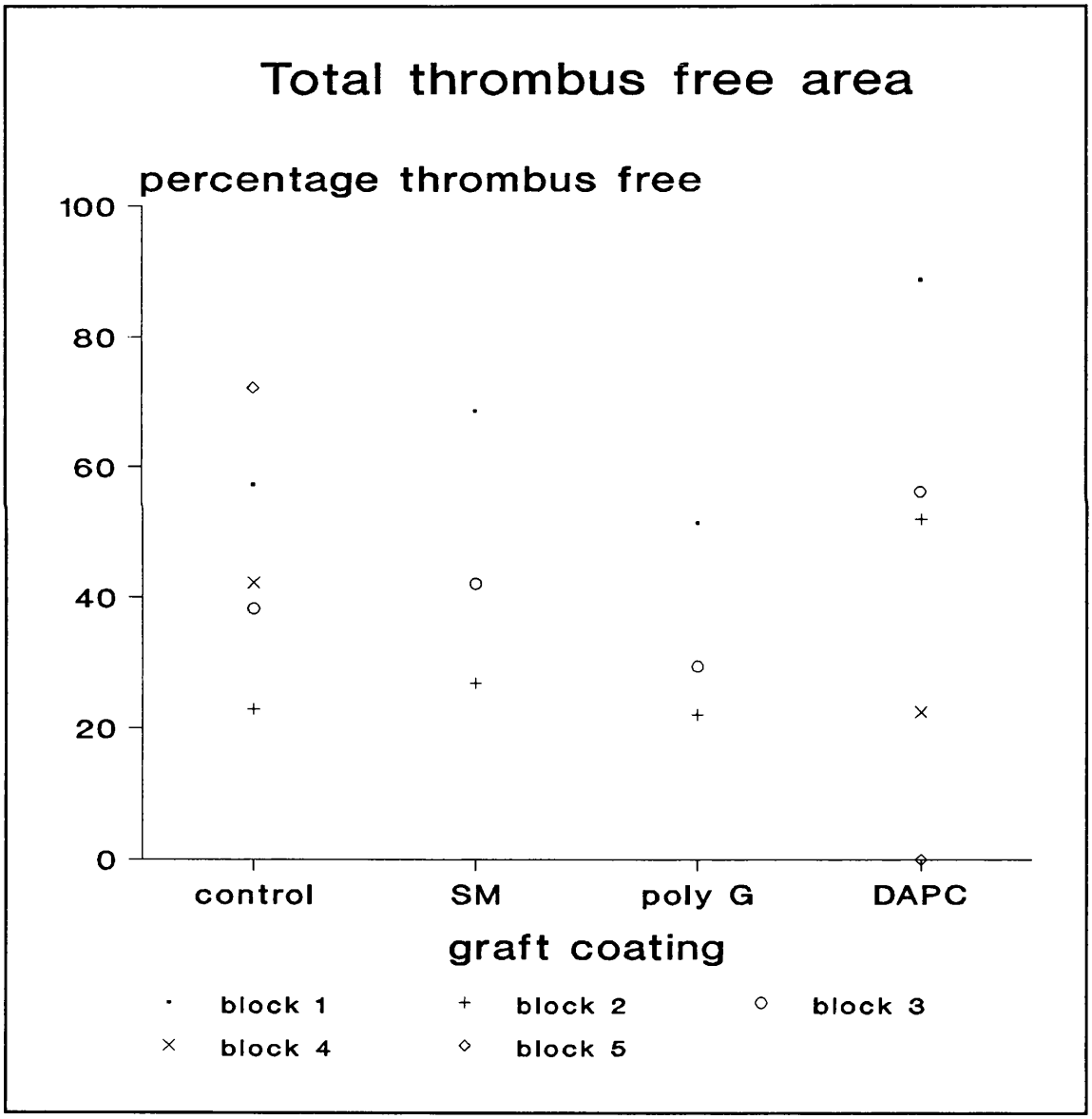


Figure 6.21 Thrombus free areas for the whole luminal surface of the grafts.

This reveals a trend for the thrombus free area in the sphingomyelin coated grafts to be larger on the venous half of the grafts as well as on the whole grafts. The mean of the paired difference for these two parameters compared to the control

analysis for coating material			
graft site	arterial	venous	whole
F ratio	14.3	5.9	36.38
p	<0.001	<0.05	<<0.005
analysis for block			
graft site	arterial	venous	whole
F ratio	13.8	19.4	86.3
p	<0.001	<0.005	<<0.005

Table 6.11 Results for twoway ANOVA for the luminal thrombus free areas of the grafts (degrees of freedom are all 3,6).

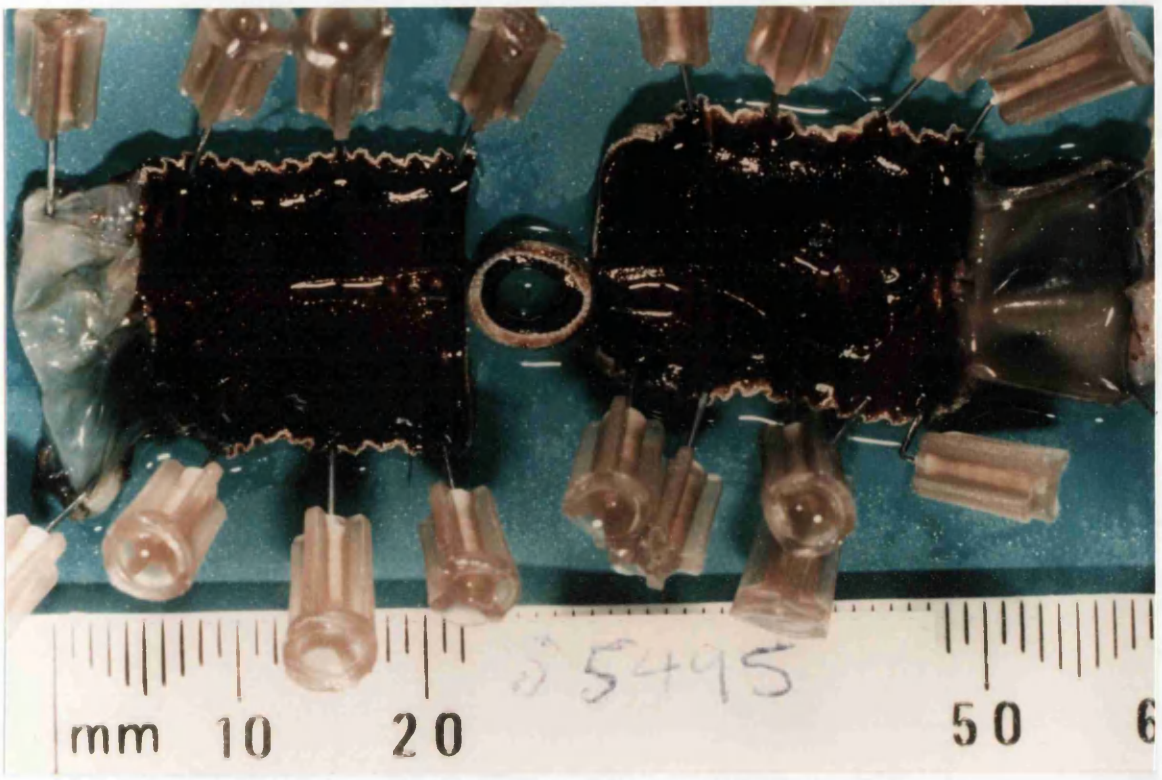
grafts in their respective blocks are 22.2% and 6.4%. However these both only reach levels of significance with p values between 0.1 and 0.2 in the unpaired t or Wilcoxon rank sign tests. The strength of the trend, is highly statistically significant, when ANOVA is carried out for the first 12 experiments (Table 6.11).

The Friedman test allowing for the blocks is also significant; $S=8.2$ $p<0.043$ (for both the arterial and venous halves), and $S=9.0$ $p<0.03$ for the total area.

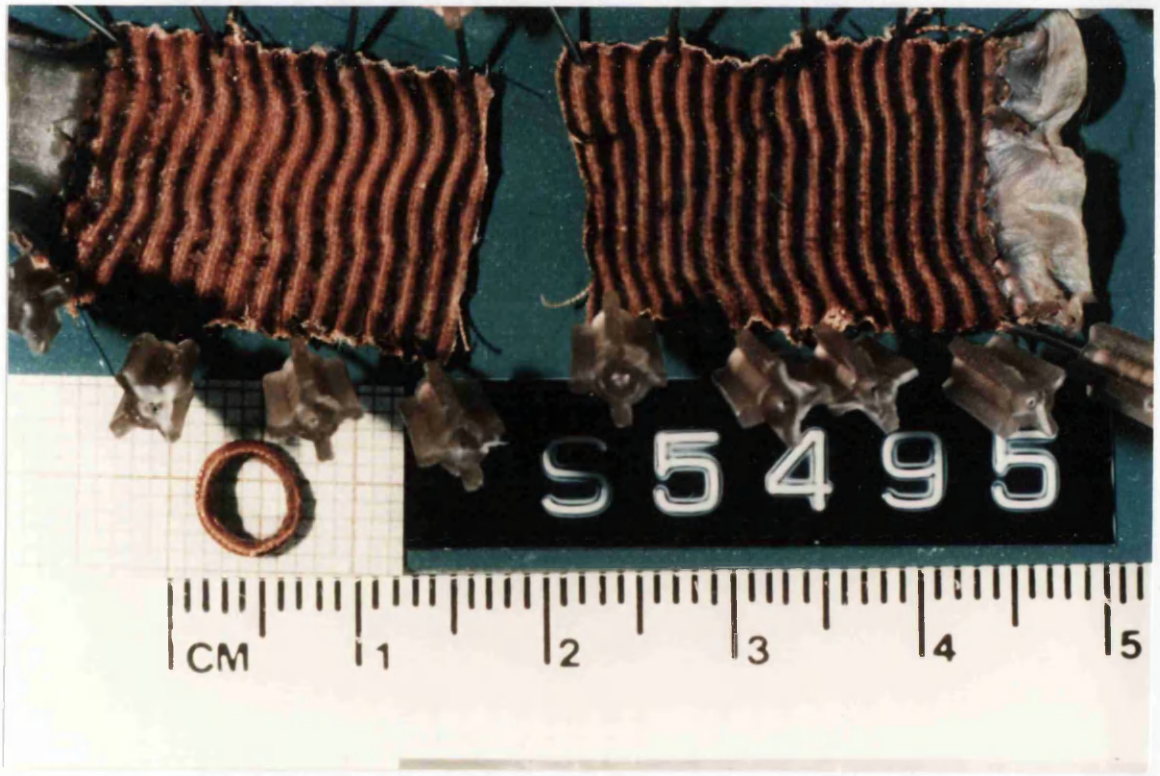
Why the randomisation of the blocks should be important is difficult to explain. One of the control grafts used in establishing the experimental model (s5495) was photographed before and after fixation. See Photographs 6.9 and 6.10. Fixation clearly changes the appearance of the thrombus, but these changes should have affected all the grafts equally.

6.4.6.3 Estimate of thrombus volume in grafts

Since the area of the thrombus for the mid-point of each graft and the percentage of the graft covered by thrombus is known, an estimate can be made for the volume of the thrombus. The true area of the graft photographed was not used as this would introduce an error. Instead a standardised area is calculated from the product of the mean total graft area, 560.6 mm^2 and the percentage thrombus free area. The data from both the automated and manual analysis of the cross



Photograph 6.9 Graft from s5495 before fixation.



Photograph 6.10 Graft from s5495 after fixation.

sectional area of the thrombus are used.

The estimates of the volume of thrombus in each graft is shown in Figures 6.22

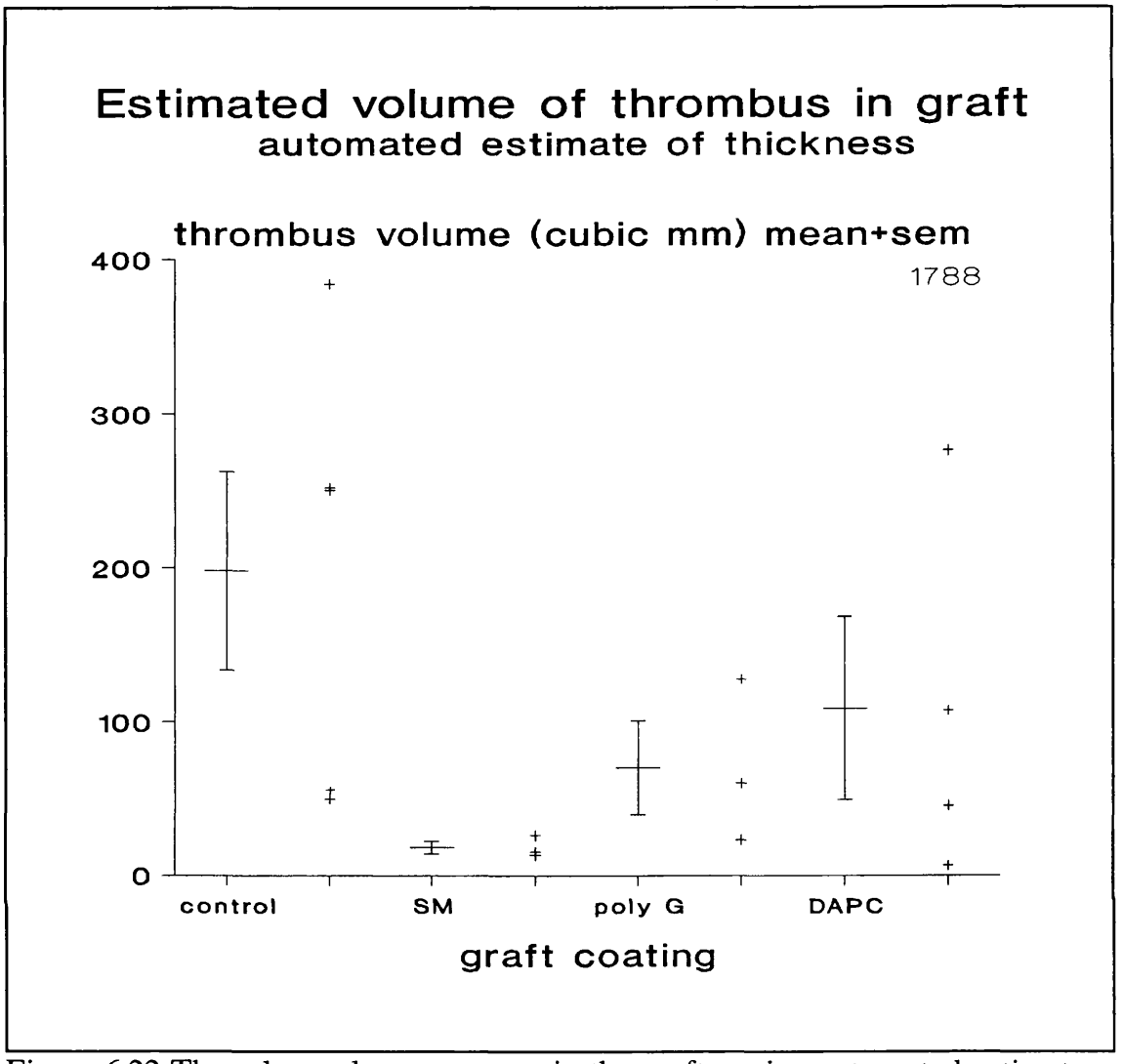


Figure 6.22 Thrombus volumes present in the grafts, using automated estimate of thrombus cross sectional area.

and 6.23. There is good correlation between the volumes derived from the manual and automatic analysis of the thrombus thickness. In contrast there is no correlation between the thickness of the thrombus and the area of graft covered by the thrombus.

The twoway ANOVA for the thrombus volumes derived from the automated data, shows the experimental blocks do not contribute to the variation, but for the coating $F(3,6)=5.08$, $p<0.05$. Similarly the Friedman test gives $S=8.20$ $p<0.043$. The analysis of the manual data by twoway ANOVA again shows the treatments are significant, $F(3,6)=9.47$, $p<0.025$, as does the Friedman $S=8.20$, $p<0.043$. In addition the blocks of the experiment are also significant $F(3,6)=7.13$ $p<0.05$. The Kruskal Wallis and oneway ANOVA on the data and log transformed data

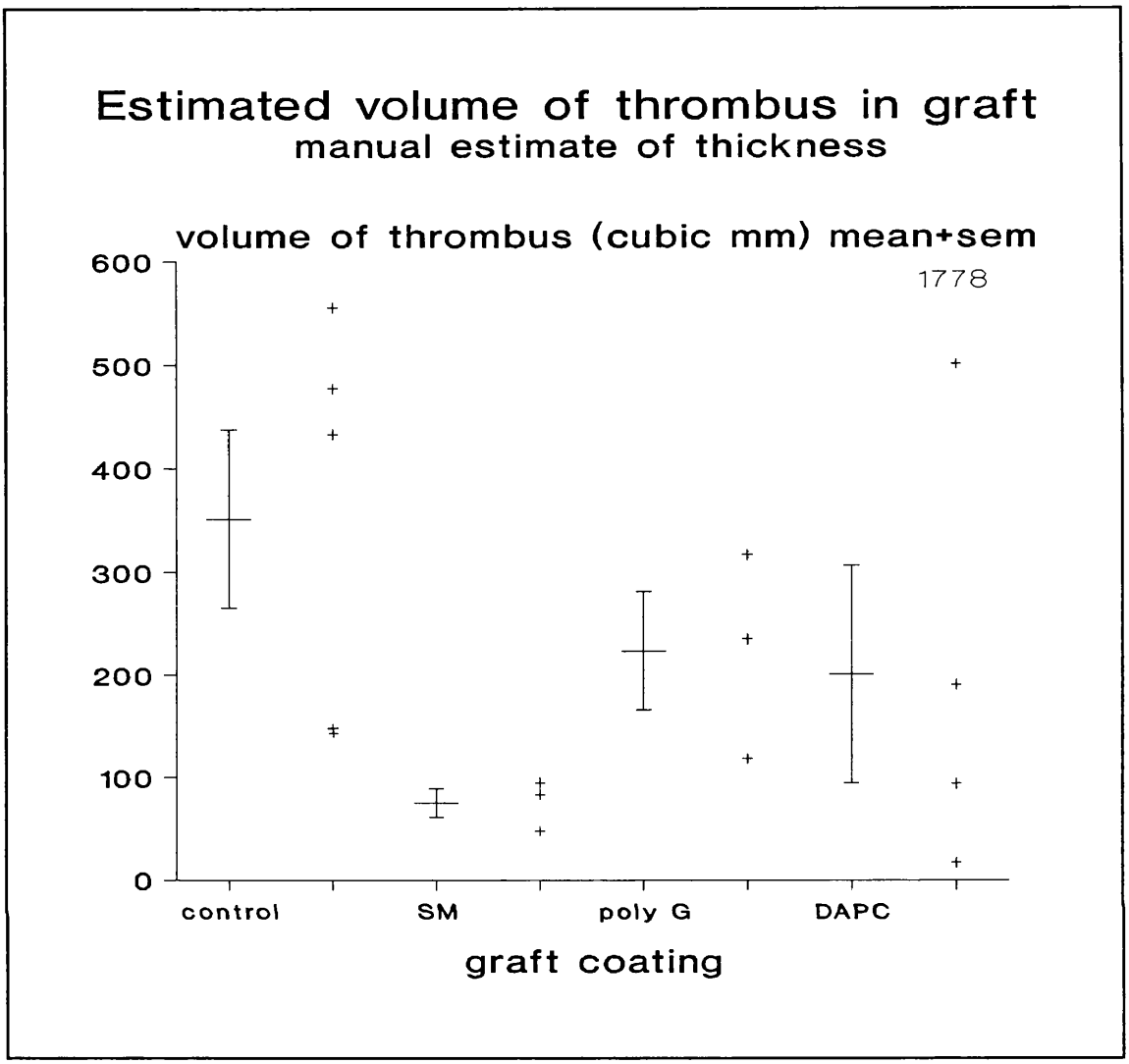


Figure 6.23 Thrombus volumes present in the grafts using the manual estimate of cross sectional thrombus area.

show no significant differences. The blocks play some part in explaining the results, probably by the effect on the thrombus free areas as discussed above.

The thrombus volumes for the sphingomyelin grafts are smaller than the control grafts. This is true for both the volumes estimated using either the automated or the manual estimate for the thrombus cross sectional area. These are 18.1 vs 198.2, and 75.5 vs 351.5 mm^3 respectively. These are both significant at $p<0.037$ for a Mann Whitney U test. The pooled t test on the log transformed data is significant ($p<0.011$ automated and $p<0.016$ manual estimates). The native data just fails to reach significance by 2t, ($p=0.081$ and $p=0.054$). Interestingly the paired differences are not significant by t test or Wilcoxon.

Although there is a similar trend for polyester G this is not significant (MWU p=0.37, 2t p=0.2, 2t log data p=0.2). The paired analysis for this data, eliminating the effect of the experimental blocks, also fails to reach significance (p=0.15). None of the analyses for DAPC show any difference from the control grafts.

6.4.7 Summary of results

The principal finding of this study is a reduction in the thrombogenicity of the Dacron grafts coated with sphingomyelin. Sphingomyelin has the lowest 111 In labelled platelet deposition of 0.0074% compared to the controls at 0.0219% of the injected dose. This is a reduction in the thrombogenicity on the control grafts by 2/3s (section 6.4.4.2). SM has the smallest graft:blood ratio for the platelet uptake, also a third that of the controls (section 6.4.4.7). The platelet survival in these subjects is prolonged. The histological findings, which are independent of the platelet label show a similar reduction in thrombus. These studies of the graft show that SM has the lowest cross sectional area of thrombus (6.4.6.1). This is only 10% that of the controls for the automated estimate, and 20% for the manual estimate ($p<0.037$ MWU for both). The fall in percentage thrombosis of the graft is from 13.9% to 1.8% for the SM coating. The thrombus volume is the lowest at 10% the control value (6.4.6.3). All this clearly points to the sphingomyelin coating reducing the thrombogenicity of the native Dacron graft. However the fibrinogen studies show sphingomyelin has an increased uptake of labelled fibrinogen (0.070% compared to 0.055% of the injected dose), and graft:blood ratio (3.9 compared to 3.1) in section 6.4.5.2 above (but neither are statistically significant). More of this label is found in the spleen compared to the spleens of the other animals with the coated grafts.

For polyester G there is no clear difference from the control grafts. There is a trend towards higher platelet uptake on the grafts by direct measurement (0.064% compared to 0.022% of the injected dose; in section 6.4.4.2), and on the gamma camera images (6.4.4.3, .4 and .5). Poly G also has the highest graft:blood ratio of 2.8 compared to the control grafts at 0.86 (6.4.4.6). There is a trend towards prolonged platelet survival similar to that seen with the SM grafts. The results for

fibrinogen are very similar to the control grafts. However the polyester G grafts have lower thrombus cross sectional areas (by a third) and thrombus volumes (by 50%), compared to the control grafts.

DAPC tends to give similar results to the control grafts for many of the parameters studied. The trends in favour of DAPC for the thrombus cross sectional area and volume are weak, at about 55% of the control values. The major difference lies in the estimates of platelet survival. These are significantly longer at 4.04 days (wtm) and 3.38 (mhit) than the control grafts (2.41 and 2.09 days) ($p<0.03$ MWU). These compare favourably with the data for the unoperated animals, whose platelet survival is 4.5 (wtm) and 4.2 days (mhit).

6.5 Discussion

This model produces data on three fronts which are relatively independent, the platelet and fibrinogen isotope label data, and the histological appearances. The angiographic and doppler assessment of the grafts are important to exclude spurious results such as the thrombosed graft, s91. The direct measurement of platelet uptake on the grafts shows clear differences, but the gamma camera imaging system failed to detect these. This may be explained by the difficulties in correcting the images for the flowing blood in the graft, as well as background scatter radiations. Together they obscure the difference due to the thrombus on the graft. At best these are only estimates and the localisation of the graft is also imprecise. Additionally there is the age of the camera, which has relatively poor resolution compared to 'state of the art' gamma cameras. Nonetheless this model yields a great deal of information from each experiment, and has much to recommend it. It is less expensive than comparable baboon or even canine studies. More work with longer periods of implantation are needed, to strengthen the conclusions presented in chapter 5, over the choice of this model.

Although the experimental numbers are small there is sufficient statistical power to provide evidence for significant differences between the groups. Obviously a larger series of experiments would have added more weight to the conclusions

reached here. There is sufficient data from the studies presented here to answer some of the questions about the comparability of the groups, the effect of the coatings and to deduce a little about the potential safety of the coatings. The biographic and labelling data presented in section 6.4.2 demonstrate that the experimental groups are comparable, and further evidence comes from the similarity of the tissue distribution of both fibrinogen and platelet labels (sections 6.4.5.3. and 6.4.4.7).

Although the organ distribution is similar for all the coatings there is a marked trend for all treated grafts to have a low deposition of platelets in the lungs. The uniformity of uptake by the liver at 4 hours shows the labelling did not damage the platelets, since high liver accumulation at 4 hours occurs if the 'collection' injury is marked (Peters et al 1985). The ranking is for DAPC to have the lowest amount, followed by SM and poly G. The lung uptake for fibrinogen is also lower for the treated grafts, but the order is reversed, with poly G having the least followed by SM and DAPC. The other 'odd' result is the higher level of splenic uptake of fibrinogen for SM.

Final questions over the safety of the materials will need much further investigation, but in these studies there is no evidence of increased haemolysis, or of excessive destruction of the platelets. Indeed these coatings confer relative sparing of platelet consumption. The organ distribution of both labels also suggests that the materials are safe.

Another area of major interest is the increase in platelet survival demonstrated by the coated grafts. These all have longer survival times than the uncoated grafts. These experiments examine the acute events following implantation. Undoubtedly studies with longer time courses are needed to confirm these results. It may be that increased platelet survival is an indicator for a reduction in platelet stimulation on passing through the treated grafts. It is interesting to speculate if this would lead to a reduction in the release of smooth muscle growth factors, and so reduce or even prevent neointimial hyperplasia from developing. Clearly the significance of these observations will only become apparent in more long term studies.

The histological analysis of the explanted grafts provides data which is independent of the radioisotope labels. The individual measures, of the thrombus cross sectional area at the graft's mid-point and of the thrombus free area of the luminal surface of the grafts, are also relatively independent. There is clear evidence that the grafts coated with sphingomyelin have less thrombus than the controls. The results for polyester G are not as clear, although there is a trend towards a reduction in thrombus the differences fail to reach statistical significance. The DAPC coated grafts appear to be little different from the control grafts whether or not the results for s91 are excluded on the basis of technical error in the venous anastomosis.

The contribution of the labelled fibrinogen to this model is interesting. The lack of difference between the coatings and the controls may be due to the short time span of the study. However it points to a difference in the composition of the thrombus formed. The area of thrombus and the platelet content are much reduced in the sphingomyelin coated grafts, yet they have an equivalent amount of fibrinogen compared to the controls. They must therefore have a different thrombus structure, composed of mainly fibrin. This would account for the slight increase in fibrin present compared to the uncoated grafts. Indeed PTFE grafts are known to develop a layer of swept fibrin in their lumen (Imparato et al 1972). This study even at 24 hours shows a tendency for the sphingomyelin coated grafts to form this type of flow surface. It is possible that these results indicate a good flow surface. This study is of too short a duration to draw inferences about the fate of fibrinogen, or to speculate on the effect of this on long term patency of coated grafts. Clearly these make a interesting area for further study, not only for the possible use of sphingomyelin, but also for the validation of the short term model in predicting the long term outcome. Other models have shown that the platelet uptake at 24 hours can predict later patency at one month (Allen et al 1986).

The need for further work is manifest for SM and chemical analogues based on its structure. The polyester G results, for graft histology, showing a reduction in thrombus thickness, may relate to the smooth coating seen on SEM (chapter 4). Both a smooth surface and low thrombogenicity are important and further work in

developing these polymers is needed. The poor *in vivo* performance of DAPC may be due to the choice of irradiation to ensure full polymerisation, as this may cause decomposition of DAPC. Evidence for this view comes indirectly from the results for DPPC presented in chapter 4. Despite this DAPC does significantly prolonged platelet survival times. The difficulties of sterilisation and polymerisation associated with DAPC make it a difficult material to use. These reservations need to be addressed before strongly advocating further investigation of DAPC.

The tentative results from this *in vivo* study provide some support for the choice of the *in vitro* method of assessment of these materials, MTEG. There are few other studies where this has been attempted, and much further work should address the suggestion that MTEG may be a vital tool in screening biomaterials, especially if it can accurately predict *in vivo* outcomes.

The results of this animal study tend to support the hypothesis that phosphorylcholine containing materials can reduce vascular graft thrombogenicity.

Chapter 7 Conclusion

7.1 The hypothesis revisited

The aims of this thesis are to address the following questions :-

1. Can the haemocompatible nature of some biological membranes be attributed to particular polar head groups within the their constituent phospholipids?
2. Is phosphoryl choline (PC) the least thrombogenic of the polar head groups?
3. Can the phosphoryl choline head group be used to improve haemocompatibility by coating surfaces *in vitro*?
4. Is it possible to use materials containing the phosphoryl choline group to improve haemocompatibility *in vivo*?

To these questions additional issues have been raised during the course of the work. These are the nature and stability of the coatings prepared for use *in vivo*. The planned studies using the canine femoral artery model had to be abandoned because of these unexpected findings.

The results for MTEG demonstrate clear differences between the phospholipids containing PC and the other polar groups, particularly those with a negative charge. The experiments with the liposomes demonstrate a clear difference between the two classes of phospholipids, both in the direct tests, and the adsorbed factor tests. Further the parallel plate test using the FPA assay, although difficult to perform and variable between test runs, also shows improved haemocompatibility with PC containing materials. These *in vitro* tests all demonstrate that the least thrombogenic phospholipids are sphingomyelin and DPPC, and both have the phosphorylcholine head group.

Together the results from these three experimental methods strongly support the hypothesis that PC phospholipids are haemocompatible. These materials are used as surface coatings in the MTEG and parallel plate tests. This suggests they are suitable for use as haemocompatible surface coatings for other biomaterials.

The native phospholipids are probably unsuitable for *in vivo* use since the body catabolises them, and this requires that the phosphorylcholine group is incorporated into more conventional biomaterials. The materials with the PC group tested (DAPC and the series of polyesters) compare well with the conventional materials, as shown by the MTEG studies. The incorporation of the PC group into the native polyester has profound effects on its thrombogenic properties. These results support the hypothesis that the materials containing the PC group are haemocompatible. The potential for further improvement in these polymers is evident.

The preparation of the grafts for the *in vivo* studies called for assessment of the stability and the effect of sterilisation on the phospholipids and PC containing materials. These revealed serious flaws in the chemical stability of the phospholipids, DPPC and DSPC, and which may also apply to the very similar lipid material, DAPC.

The results *in vivo* for the sphingomyelin coating compared with the untreated Dacron show a reduction in thrombogenicity towards platelets, and a reduction in the thickness and area of thrombus formed. The results for fibrinogen activation seem at variance unless this is a sign of 'good haemocompatibility'. Additionally the platelet survival times are significantly prolonged in the subjects with coated grafts. Polyester G, and particularly DAPC, demonstrate prolongation of platelet survival, although they do not show as clear cut a benefit in the other assessments.

These findings tend to affirm the hypothesis that the PC containing materials can successfully reduce the thrombogenicity of Dacron grafts.

7.2 Horizons now apparent

These studies raise problems related to the chemical structure of the phospholipids, in terms of their susceptibility to deterioration during sterilisation (Scales 1953, table 1.4, item 8). Future work with these materials will need to establish beyond doubt the integrity of the compounds purported to be being tested in the final *in vivo* setting.

A second major, and as yet unanswered issue is to establish what effect the body has on these materials (Scales 1953, table 1.4, item 1). The phospholipid with its ester linkage is readily broken by the plasma phospholipases. Sphingolipids have activities besides their structural function in the biomembrane. They have a wide range of biological activities, particularly as regulatory messengers affecting protein kinase C. The breakdown products of sphingolipids also act to reduce blood clotting reactions (Hannun and Bell 1989).

The twin issues of chemical stability in preparation, and of biological stability *in vivo*, highlight the need for further chemical development of the polyesters and other physically robust polymers with the PC group. Glow discharge seems to be unreliable as a method for linking the PC group to existing materials. A deliberate search should be made for biomaterials with suitable chemical groups to covalently link with the PC group.

The *in vivo* studies demonstrate a good animal model which should be further developed alongside the more conventional canine model for vascular graft assessment. The hypothesis requires further evaluation in the canine model using the 'hard' end point of graft patency.

The prospect of an 'off the shelf' vascular graft, or other prothesis, with a coating known to confer the desirable haemocompatibility and biocompatibility of the natural arterial wall is a keen spur driving research to further efforts, both in terms of clinical need and commercial interest. Although this ultimate holy grail seems to be even more remote, courting such a fair if distant lady should amuse for a lot longer.

Outline to the appendices

Appendix 5 The results for the control experiments

A 5.1 The experimental details

A 5.2 The free haemoglobin studies

A 5.3 The platelet studies

A 5.4 The fibrinogen studies

Appendix 1

Material thrombelastography: tabulated results.

The results are tabulated with numbering related to chapter 3, ie as Tables 3.2 to 3.38. The tabulated results are given as geometric mean (with lower and upper 95 % confidence limits). The 'p' value is for a paired 't' test, unless a Wilcoxon rank sign test is more appropriate and this is indicated by #. The statistical hypothesis being tested is that the results are not significantly different from the expected result of one. The MTEG value and the associated p value indicates how much and how significant a difference to the thrombogenicity of test surface the coating material makes. The degrees of significance are indicated thus :-

< 0.05=*, < 0.01=**, < 0.001=***, < 0.0001=****.

A 1.1 Group 1 The controls and standards

Table 3.2 MTEG results for standardisation with plastic cuvettes. N=10

Parameter	Mean	95% confidence limits		p value
Mr	0.964	0.890	1.044	0.38
Mr+k	1.003	0.939	1.071	0.93
Mk	1.053	0.959	1.157	0.30
Mtma	0.996	0.918	1.081	0.93
Mma	1.016	0.989	1.045	0.27
Me	1.033	0.976	1.094	0.28
MA°	0.961	0.893	1.034	0.30
Mα	0.978	0.913	1.047	0.53

Table 3.3 MTEG results for standardisation with metal cuvettes. N=8

Parameter	Mean	95% confidence limits		p value
Mr	1.036	0.948	1.131	0.45
Mr+k	1.027	0.957	1.102	0.48
Mk	1.018	0.906	1.145	0.76
Mtma	0.919	0.867	0.974	0.023 *
Mma	0.991	0.944	1.040	0.73
Me	0.985	0.891	1.088	0.77
MA°	1.005	0.941	1.074	0.88

Table 3.4 MTEG results for DPPC coating on plastic cuvettes. N=9

Parameter	Mean	95% confidence limits		p value
Mr	1.628	1.395	1.900	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.491	0.390	0.619	<0.0001 ****
Mma	0.056	0.044	0.071	<0.0001 ****
Me	0.026	0.021	0.033	<0.0001 ****
MA°	0.143	0.087	0.235	<0.0001 ****
Mα	0.160	0.107	0.240	<0.0001 ****

For Mr+k (#) and Mk(#) the Wilcoxon test is p=0.009, all the values are infinite.

Table 3.5 MTEG results for DPPC coating on metal cuvettes. N=12

Parameter	Mean	95% confidence limits		p value
Mr	1.972	1.477	2.634	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.727	0.553	0.955	0.039 *
Mma	0.062	0.046	0.082	<0.0001 ****
Me	0.031	0.022	0.043	<0.0001 ****
MAo	0.108	0.077	0.151	<0.0001 ****
Mα	0.095	0.073	0.125	<0.0001 ****

For Mr+k and Mk p=0.003, all the values are infinite.

Table 3.6 MTEG results for DPPC on plastic cuvettes, at 1/2 conc. N=7

Parameter	Mean	95% confidence limits		p value
Mr	1.321	1.162	1.502	0.0049 **
Mr+k	#			
Mk	#			
Mtma	0.498	0.357	0.641	0.0015 **
Mma	0.083	0.052	0.133	<0.0001 ****
Me	0.043	0.025	0.073	<0.0001 ****
MA°	0.217	0.125	0.377	0.0015 **

All the values for Mr+k and Mk are infinite, p= 0.022 *.

Table 3.7 MTEG results for DPPC on plastic cuvettes, at 1/4 conc. N=5

Parameter	Mean	95% confidence limits		p value
Mr	0.979	0.917	1.045	0.55
Mr+k	#			
Mk	#			
Mtma	0.281	0.090	0.875	<0.0001 ****
Mma	0.087	0.070	0.109	<0.0001 ****
Me	0.046	0.035	0.061	<0.0001 ****
MA°	0.358	0.227	0.562	0.010 **

All the values for Mr+k and Mk are infinite, p=0.059.

Table 3.8 MTEG results for DPPC on plastic cuvettes, at 1/10 conc. N=6

Parameter	Mean	95% confidence limits		p value
Mr	1.120	0.935	1.342	0.26
Mr+k	#			
Mk	#			
Mtma	0.515	0.333	0.795	0.028 *
Mma	0.319	0.226	0.450	0.0012 **
Me	0.201	0.136	0.298	0.0004 ***
MA°	0.570	0.355	0.914	0.063 *

There are two finite results.

For Mr+k p=0.036, * and the data is 1.20 and 1.15.

For Mk p=0.036, * and the values are 1.37 and 1.05.

Table 3.9 MTEG results for octacosane coating on plastic cuvettes. N=8

Parameter	Mean	95% confidence limits		p value
Mr	1.108	1.021	1.203	0.041 *
Mr+k	1.134	1.069	1.203	0.0038 **
Mk	1.165	1.093	1.242	<0.0001 ****
Mtma	1.017	0.968	1.069	0.51
Mma	0.973	0.902	1.049	0.48
Me	0.931	0.777	1.116	0.46
MA°	0.867	0.812	0.927	0.0037 **
Mα	0.933	0.884	0.984	0.034 *

Table 3.10 MTEG results for DPPC liposomes added to blood sample. N=13

Parameter	Mean	95% confidence limits		p value	
Mr	0.963	0.874	1.062	0.46	
Mr+k	0.959	0.888	1.037	0.31	
Mk	0.958	0.857	1.070	0.45	
Mtma	0.986	0.924	1.051	0.66	
Mma	1.090	1.047	1.134	0.001	***
Me	1.202	1.104	1.310	0.001	***
MA°	1.076	0.980	1.181	0.14	
Mα	1.086	1.008	1.171	0.046	*

Table 3.11 MTEG results for Sigmacote, silicone treatment. N=16

Parameter	Mean	95% confidence limits		p value	
Mr	1.234	1.100	1.383	0.0023	**
Mr+k	#				
Mk	#				
Mtma	0.737	0.626	0.867	0.002	**
Mma	0.582	0.505	0.670	<0.0001	****
Me	0.430	0.353	0.524	<0.0001	****
MA°	0.707	0.624	0.800	<0.0001	****

for both Mr+k and Mk there are 5 infinite results, and the # gives p=0.001.

Table 3.12 MTEG results for silicone grease, ICI M494. N=10

Parameter	Mean	95% confidence limits		p value	
Mr	1.498	1.345	1.668	<0.0001	****
Mr+k	#				
Mk	#				
Mtma	0.505	0.451	0.565	<0.0001	****
Mma	0.149	0.119	0.187	<0.0001	****
Me	0.081	0.061	0.107	<0.0001	****
MA°	0.293	0.214	0.401	<0.0001	****

for Mr+k and Mk all the results are infinite, p=0.006 ***

A 1.2 Group 2a The PC containing lipids

Also see tables 3.4 & 3.5 for DPPC

Table 3.13 MTEG results for DSPC. N=13

Parameter	Mean	95% confidence limits		p value
Mr	1.164	1.114	1.217	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.509	0.386	0.671	<0.0001 ****
Mma	0.138	0.087	0.218	<0.0001 ****
Me	0.074	0.037	0.149	<0.0001 ****
MA°	0.259	0.198	0.341	<0.0001 ****

all infinite, p=0.002.

Table 3.14 MTEG results for DMPC coating. N=11

Parameter	Mean	95% confidence limits		p value
Mr ~	1.715	1.287	2.284	0.0059 **
Mr+k	#			
Mk	#			
Mtma	0.507	0.409	0.627	<0.0001 ****
Mma	0.029	0.024	0.035	<0.0001 ****
Me	0.014	0.012	0.017	<0.0001 ****
MA°	0.071	0.055	0.092	<0.0001 ****

~ For n=10, there is one infinite result.
# all the results are infinite, p=0.004.

Table 3.15 MTEG results for coating SM on plastic cuvettes. N=11

Parameter	Mean	95% confidence limits		p value
Mr	2.447	1.989	3.009	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.616	0.498	0.761	<0.0001 ****
Mma	0.030	0.024	0.037	<0.0001 ****
Me	0.014	0.011	0.018	<0.0001 ****
MA°	0.047	0.034	0.064	<0.0001 ****

all the results are infinite, p=0.004 **

Table 3.16 MTEG results for DPPC/GM1. N=10

Parameter	Mean	95% confidence limits		p value
Mr	1.249	1.038	1.502	0.039 *
Mr+k	#			
Mk	#			
Mtma	0.500	0.355	0.705	0.003 **
Mma	0.080	0.068	0.095	<0.0001 ****
Me	0.045	0.037	0.054	<0.0001 ****
MA°	0.181	0.130	0.251	<0.0001 ****

All infinite, p=0.006.

Table 3.17 MTEG results for outside RBC mixture on plastic cuvettes. N=12

Parameter	Mean	95% confidence limits		p value
Mr	3.075	2.652	3.565	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	1.156	0.983	1.358	0.1
Mma	0.061	0.049	0.076	<0.0001 ****
Me	0.031	0.024	0.041	<0.0001 ****
MA°	0.051	0.046	0.057	<0.0001 ****

all the results for Mr+k and Mk are infinite, p=0.003 **

A 1.3 Group 2b The negatively charged phospholipids

Table 3.18 MTEG results for DPPA. N=12

Parameter	Mean	95% confidence limits		p value
Mr	0.756	0.651	0.878	0.0033 **
Mr+k	0.891	0.792	1.001	0.073
Mk	1.102	0.940	1.293	0.25
Mtma	0.778	0.685	0.884	0.0023 **
Mma	0.834	0.756	0.921	0.0037 **
Me	0.732	0.629	0.852	0.0017 **
MA°	1.000	0.924	1.082	1.00

Table 3.19 MTEG results for DCP. N=10

Parameter	Mean	95% confidence limits		p value
Mr	0.801	0.716	0.896	0.0034 **
Mr+k	0.947	0.889	1.007	0.11
Mk	1.203	1.052	1.375	0.022 *
Mtma	0.898	0.829	0.973	0.025 *
Mma	0.875	0.828	0.925	0.0009 ***
Me	0.785	0.714	0.863	0.0007 ***
MA°	0.878	0.826	0.933	0.0020 **

Table 3.20 MTEG results for coating DPPS on metal cuvettes. N=10

Parameter	Mean	95% confidence limits		p value
Mr	0.990	0.865	1.134	0.89
Mr+k @				
Mk ~				
Mtma $	0.690	0.569	0.837	0.0039 **
Mma $	0.445	0.329	0.604	<0.0001 ****
Me $	0.319	0.215	0.473	<0.0001 ****
MA° $	0.690	0.563	0.845	0.0052 **

$ note there is evidence of moderate retraction in all the traces, see figure 3.10 on page 73. There are 5 finite and 5 infinite results.
@ for Mr+k the finite data in the t test (n=5) gives :-

Mr+k	0.929	0.749	1.152	0.53

for Mr+k 'p' = 0.019, the median is infinite.

~ for Mk the t test (n=5) gives :-

Mk	1.183	0.896	1.558	0.29

for Mk 'p' = 0.006 the median is infinite.

Table 3.21 MTEG results for coating DMPG on plastic cuvettes. N=12

Parameter	Mean	95% confidence limits		p value
Mr	1.184	1.145	1.225	<0.0001 ****
Mr+k @	1.149	1.084	1.203	<0.0001 ****
Mk ~	1.065	0.964	1.176	0.25
Mtma	0.591	0.521	0.670	<0.0001 ****
Mma	0.675	0.567	0.803	<0.0001 ****
Me	0.532	0.410	0.689	<0.0001 ****
MA°	0.883	0.776	1.005	0.082

@ and ~ for n=11, there is one infinite result.

Table 3.22 MTEG results for coating DPPE on metal cuvettes. N=10

Parameter	Mean	95% confidence limits		p value
Mr	1.171	1.075	1.277	0.005 **
Mr+k	1.133	1.062	1.210	0.004 **
Mk	1.000	0.760	1.315	1.00
Mtma	0.943	0.888	1.002	0.086
Mma	0.968	0.936	1.001	0.081
Me	0.941	0.882	1.003	0.088
MA°	0.867	0.810	0.928	0.002 **

Table 3.23 MTEG results for brain extract. N=11

Parameter	Mean	95% confidence limits		p value
Mr	1.029	0.906	1.169	0.66
Mr+k	#			
Mk	#			
Mtma	0.911	0.636	1.306	0.62
Mma	0.351	0.280	0.441	<0.0001 ****
Me	0.184	0.102	0.333	<0.0001 ****
MA°	0.576	0.432	0.768	0.0033 **

all the results are infinite except 4, the medians are infinite, and p=0.004.

For Mr+k the data is 1.18, 1.73, 2.30, 2.85.

For Mk the data is 1.69, 3.03, 4.27, 5.11.

Table 3.24 MTEG results for coating DPPI. N=11

Parameter	Mean	95% confidence limits		p value
Mr	1.316	1.205	1.438	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	1.103	0.831	1.464	0.50
Mma	0.141	0.109	0.183	<0.0001 ****
Me	0.079	0.059	0.105	<0.0001 ****
MA°	0.286	0.218	0.377	<0.0001 ****

all the results are infinite, for both Mr+k and Mk, p=0.004 **

A 1.4 Group 2c The positively charged lipids.

Table 3.25 MTEG results for stearylamine. N=12

Parameter	Mean	95% confidence limits		p value
Mr	1.435	1.243	1.658	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.495	0.417	0.588	<0.0001 ****
Mma	0.382	0.315	0.462	<0.0001 ****
Me	0.225	0.171	0.296	<0.0001 ****
MA°	0.658	0.533	0.813	0.0022 **

For Mr+k all the results are infinite except 4, p=0.004. The finite data is 2.84, 1.34, 0.92 and 1.03.

For Mk all the results are infinite except 4, p=0.004. The finite data is 3.99, 1.25, 0.86, and 0.93.

Table 3.28 MTEG results for DDA. N=10

Parameter	Mean	95% confidence limits		p value
Mr	$			
Mr+k	#			
Mk	#			
Mtma	~			
Mma	0.033	0.019	0.058	<0.0001 ****
Me	0.019	0.011	0.033	<0.0001 ****
MA°	0.014	0.010	0.021	<0.0001 ****

$ The estimated mean for the 6 finite data (N=6) is 5.076 ci 2.786-9.248 p=0.0029. The Wilcoxon test (N=10) is p=0.006.

All data is infinite, p=0.006.

~ All the data but 1 are infinite, p=0.006. There is marked tapering of the traces suggesting fibrinolysis.

A 1.5 Group 2d The charged mixtures

Table 3.26 MTEG results for stearylamine/PS (2:1). N=10

Parameter	Mean	95% confidence limits		p value
Mr	2.003	1.676	2.394	<0.0001 ****
Mr+k @	2.050	1.643	2.559	<0.0001 ****
Mk ~	2.124	1.559	2.893	<0.0001 ****
Mtma	0.922	0.732	1.161	0.5
Mma	0.482	0.398	0.583	<0.0001 ****
Me	0.316	0.235	0.426	<0.0001 ****
MA°	0.497	0.394	0.628	<0.0001 ****

@, ~ n=8 as 2 results are infinite. For Mr+k # the median is 2.69, for Mk # the median is 3.22, p=0.008 ** for both.

Table 3.27 MTEG results for DMPC/stearylamine/DPPS (80:15:5). N=10

Parameter	Mean	95% confidence limits		p value
Mr	1.560	1.244	1.956	0.0035 **
Mr+k	#			
Mk	#			
Mtma	1.045	0.671	1.630	0.85
Mma	0.126	0.070	0.228	<0.0001 ****
Me	0.073	0.037	0.144	<0.0001 ****
MA°	0.148	0.079	0.280	<0.0001 ****

all the results are infinite, for both p=0.006, **.

A 1.6 Group 3a The conventional materials

Table 3.29 MTEG results for PET coating (Melinex ICI). N=10

Parameter	Mean	95% confidence limits		p value
Mr	1.152	1.060	1.252	0.0077 **
Mr+k	1.500	1.070	1.236	0.0038 **
Mk	1.146	1.013	1.297	0.054
Mtma	1.036	0.901	1.192	0.63
Mma	1.031	0.949	1.120	0.48
Me	1.047	0.872	1.256	0.63
MA°	0.885	0.795	0.985	0.049 *

Table 3.30 MTEG results for PTFE (milled from solid PTFE). N=11

Parameter	Mean	95% confidence limits		p value
Mr	1.166	1.081	1.258	0.0023 **
Mr+k @	1.137	1.049	1.232	0.013 *
Mk ~	1.325	1.152	1.524	0.0038 **
Mtma	0.702	0.581	0.847	0.0037 **
Mma	0.505	0.432	0.591	<0.0001 ****
Me	0.328	0.267	0.402	<0.0001 ****
MA°	0.789	0.710	0.876	<0.0001 ****

@ and ~ n=9 There are 2 infinite results.
# for Mr+k the median value is 1.452, p=0.004 **.
# for Mk the median value is 1.894, p=0.004 **.

A 1.7 Group 3b The experimental materials with PC

Table 3.31 MTEG results for polymerized DAPC (batch 1). N=13

Parameter	Mean	95% confidence limits		p value
Mr	1.102	0.989	1.228	0.10
Mr+k	#			
Mk	#			
Mtma	0.327	0.292	0.366	<0.0001 ****
Mma	0.121	0.074	0.197	<0.0001 ****
Me	0.060	0.035	0.102	<0.0001 ****
MA°	0.321	0.171	0.603	0.0057 **
Mα	0.429	0.272	0.666	0.004 **

p=0.006 ** all the results are infinite, except one, Mr+k 0.81 and Mk 0.61,

Table 3.32 MTEG results for polymerised DAPC (2nd batch).N=9

Parameter	Mean	95% confidence limits		p value
Mr	1.317	1.094	1.586	0.018 *
Mk	#			
Mr+k	#			
Mtma	0.394	0.267	0.580	0.0014 **
Mma	0.045	0.034	0.060	<0.0001 ****
Me	0.020	0.014	0.028	<0.0001 ****
MA°	0.142	0.093	0.217	<0.0001 ****

all the results are infinite, p=0.009.

Table 3.33 MTEG results for Polyester O (no PC). N=10

Parameter	Mean	95% confidence limits		p value
Mr	0.668	0.571	0.780	<0.0001 ****
Mr+k	0.691@	0.590	0.809	<0.0001 ****
Mk	0.796~	0.701	0.910	0.0088 ***
Mtma	0.565	0.472	0.675	<0.0001 ****
Mma	0.652	0.506	0.839	0.008 **
Me	0.495	0.352	0.698	0.0027 **
MA°	0.995	0.801	1.237	0.97

For @ Mr+k and ~ Mk one value is infinite.

Mr+k 'p'= 0.103 estimated median = 0.8484.
The data is 0.57, 0.84, 1.01, 0.69, 0.86, 0.82, 0.64, 0.95, and 0.92.

Mk 'p'= 0.838 estimated median = 1.026.
The data is 0.61, 0.77, 1.09, 1.03, 0.89, 0.97, 0.75, 1.68, and 1.25.

Table 3.34 MTEG results for Polyester C (22% PC). N=10

Parameter	Mean	95% confidence limits		p value
Mr	1.244	1.127	1.373	0.0017 **
Mr+k	#			
Mk	#			
Mtma	0.549	0.4155	0.725	0.0020
Mma	0.277	0.227	0.337	<0.0001 ****
Me	0.156	0.126	0.195	<0.0001 ****
MA°	0.579	0.466	0.719	<0.0001 ****

All the values infinite except one (1.291 and 1.275) for Mr+k (#) and Mk (#) 'p' is 0.006 for both.

Table 3.35 MTEG results for Polyester D (40% PC). N=10

Parameter	Mean	95% confidence limits		p value
Mr	1.071	0.994	1.155	0.099
Mr+k	#			
Mk	#			
Mtma	0.621	0.463	0.832	0.0098 **
Mma	0.122	0.080	0.186	<0.0001 ****
Me	0.062	0.037	0.103	<0.0001 ****
MA°	0.241	0.206	0.283	<0.0001 ****

For Mr+k (#) and Mk (#) 'p' is 0.006, and all the values infinite.

Table 3.36 MTEG results for Polyester E (40% PC). N=10

Parameter	Mean	95% confidence limits		p value
Mr	0.978	0.878	1.088	0.69
Mr+k	0.939@	0.837	1.056	0.32
Mk	1.125~	0.878	1.440	0.38
Mtma	0.683	0.538	0.866	0.012 *
Mma	0.543	0.407	0.724	0.0028 **
Me	0.388	0.260	0.578	<0.0001 ****
MA°	0.825	0.655	1.039	0.13

@ n=7 There are 2 infinite results for Mr+k, and Mk.
For Mr+k the finite data is 1.20, 0.82, 1.69, 0.773, 1.05, 0.79, and 0.92.
For # 'p' is 0.155, the median is 1.409.
~ n=7 Similarly for Mk there are 2 infinite results, the finite data is 1.53, 0.70, 3.30, 3.01, 1.11, 0.58, and 0.84. For # 'p' is 0.124, the median is 2.204.

Table 3.37 MTEG results for Polyester F (23% PC). N=10

Parameter	Mean	95% confidence limits		p value
Mr	1.019	0.880	1.178	0.81
Mr+k	#			
Mk	#			
Mtma	0.471	0.375	0.593	<0.0001
Mma	0.270	0.191	0.383	<0.0001 ****
Me	0.154	0.100	0.237	<0.0001 ****
MA°	0.604	0.401	0.909	0.036 **

For Mr+k the median is infinite, 'p'=0.014, the finite data is 1.29, 1.17, 1.14, and 0.76.

Table 3.38 MTEG results for Polyester G (polyester-polyurethane). N=11

Parameter	Mean	95% confidence limits		p value
Mr	1.291	1.180	1.413	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.484	0.404	0.580	<0.0001 ****
Mma	0.139	0.086	0.222	<0.0001 ****
Me	0.076	0.045	0.128	<0.0001 ****
MA°	0.350	0.237	0.518	<0.0001 ****

For Mr+k and Mk 'p' is 0.004, the median is infinite, and the finite data is 1.14 and 1.32 for Mr+k, 1.25 and 1.38 for Mk.

Appendix 2

Methodology for the liposome experiments

A 2.1 Preparation of liposomes

The lipids studied in chapter 3 tend to form liposomes spontaneously in aqueous media. These can be deliberately created by drying thin films of the lipid and vortex mixing with distilled water or aqueous buffers to form liposomes. The multilamellar vesicles (MLV's) are made by dissolving the lipid in chloroform, at 40 mg/ml, and drying it onto the wall of a glass tube under a stream of nitrogen. 2 ml of 10 mM Tris buffer, 0.15 M NaCl (pH 8.5) is added to give a 20 mg/ml lipid dispersion. This mixture is vortexed above the transition temperature of the lipid for 5 minutes. The samples are frozen in liquid nitrogen and rapidly thawed in quick succession for 5 cycles. This technique of freeze-thaw distributes the solute equally between the lamellae and ensures adequate hydration of the lipids.

The large unilamellar vesicles (LUV's) are prepared in the same way, but require the additional step of passage through an Extruder. This is achieved in two steps, by filtering through a pore size of 200 nm ten times and then through a pore size of 100 nm ten times. This is carried out a room temperature, or with warming of the Extruder if the process is slow. This creates LUV's with a mean diameter of 90 nm, and a trap volume in the range of 1.5-2.0 μm/μl.

A 2.2 The experimental details

The liposomes are incubated with pooled normal human citrated plasma for 1 hour at 37°C at a concentration of about 20 mg lipid per ml of plasma. The liposomes are pelleted at 16,000 x G in a Beckman TL100 centrifuge (20,000 RPM, 100.3 rotor 4°C for 30 min.). The plasma supernatants are collected for the aPTT, and PT tests. Plasma incubated with the buffer alone is used as a control. The liposomes are resuspended in buffer and re-pelleted at 20,000 x G (22,500 RPM, 100.3 rotor 4°C for 30 min.), this step is repeated twice to wash the liposomes of plasma. The liposomes are finally suspended in buffer for isolated

clotting factor assays. The controls used are the liposomes incubated with the buffer alone, and washed in the same way.

A 2.3 The coagulation assays

A KC 10 automated coagulometer (Amelung) was used for all the assays. This instrument automatically records the time taken for the plasma clot to be formed after the addition of calcium.

A 2.3.1 The assay for factors XII, IX and VIII.

The standard curve is generated from dilutions of a pooled collection of normal human plasma. The washed liposomes, previously incubated in either plasma (the test samples) or the buffer (the controls), and the diluted reference plasma (for the standard curve) are used in the assay. 50 μl of each of these preparations is incubated with 100 μl of specific factor deficient plasma and 100 μl of kaolin (5mg/ml) for 9 minutes. 100 μl Bell and Alton platelet substitute is added, followed by 100 μl $CaCl_2$ (25mM) at 10 minutes.

A 2.3.2 The assay for factors VII and V

50 μl of the test preparation is incubated with 100μl deficient plasma and 100μl 1/20 dilution of rabbit brain extract followed by the addition of 100 μl $CaCl_2$ (25mM).

A 2.3.3 The assay for factor X

50 μl of the test preparation is incubated with 100 μl factor X deficient plasma and 100 μl 1/150,000 dilution of Russell's viper venom in Bell and Alton platelet substitute for 1 minute, before adding 100 μl $CaCl_2$ (25mM).

Appendix 3

Detailed methods and results for studies presented in Chapter 4

A 3.1 Scanning electron microscopy (SEM)

Dry specimens were prepared for SEM by gold sputter coating. Specimens which were wet, in general those exposed to blood, were fixed in formal-saline. These were washed, dehydrated and finally critical point dried, before sputter coating with gold. All the specimens were examined using a Phillips 501 scanning electron microscope.

A 3.2 TLC and FTIR methods

The initial thin layer chromatography (TLC) experiments were run with the solvent system 65 $CHCl_3$/ 35 MeOH/ 4 H_2O v/v. Later TLC's were performed with the solvent system 65 $CHCl_3$/ 25 MeOH/ 5 conc NH_4OH v/v, which allows the separation of DPPC and dipalmitoyl phosphatidic acid (DPPA). 3 different developing agents were used. For lipids and the diacetylenic bonds rhodamine 6G was used to give a pink colour, seen best under UV light. The phosphate groups were developed with molybdenum blue. The choline group is developed with Dragendorff's reagent to give a yellow colour. The materials extracted from the grafts were identified by including pure lipids and possible products on the TLC plates.

Dr Davies obtained the FTIR spectra by cutting small pieces off the grafts and swabbing them with chloroform on an ATR crystal, or by pipetting small amounts of solution onto the crystal and allowing it to dry. The attention of FTIR was focused on DPPC, since much more is known about its structure, and that of its components than either DAPC or Polyester G.

A 3.3 Results of TLC and FTIR for irradiated materials

The TLC showed that the DPPC used to prepare the grafts was pure, using the lipid spray. However after irradiation of the grafts the material extracted contained 3 components, these are palmitic acid, DPPC and lyso-PC. Similarly spraying for phosphate gave two spots for DPPC and lyso-PC.

The samples developed for choline gave more complicated results. An additional component was seen at the origin, and the DPPC spot was revealed being made up of two components. Only one of these contained the choline group. The presence of a choline containing product in the DPPC spot, without any phosphate or lipid attached, indicates that a chloroform soluble complex must be present (since choline is insoluble in $CHCl_3$).

In an attempt to characterise this complex samples were prepared containing 1:1 and 1:2 mole ratios of dipalmitoyl phosphatidic acid (DPPA) and choline base. These were soluble in $CHCl_3$ and on TLC split into two components, one at the origin, and one which ran at the same speed as DPPA and DPPC. Use of the second solvent (65 $CHCl_3$/ 25 MeOH/ 5 conc NH_4OH v/v), which allows separation of DPPA and DPPC, showed the irradiated samples contained DPPA and lyso-PA, in addition to choline, DPPC and lyso-PC.

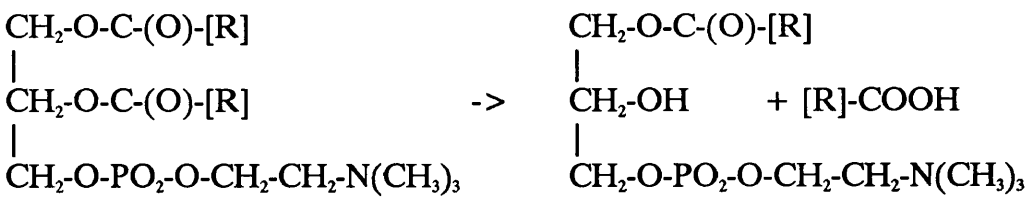
The FTIR and its analysis was carried out by Dr G Davies. The spectra for pure DPPC was compared with extracts from two irradiated grafts examined only a few days after irradiation. Major differences from DPPC were found, the most obvious being a band at 1703 cm^{-1} with a prominent shoulder at 1680 cm^{-1}. A minor band at 1430 cm^{-1} and an additional series of sharp bands from 1300-1150 cm^{-1} were also noted. These features are probably due to a crystalline long chain fatty acid, and this was identified by comparison with the spectrum of palmitic acid. This confirms that the graft samples contain palmitic acid. This has been formed by cleavage of the lipid ester bonds. Hence these samples contain at least DPPC, lyso-PC and palmitic acid.

However the above components cannot account for other changes observed in the

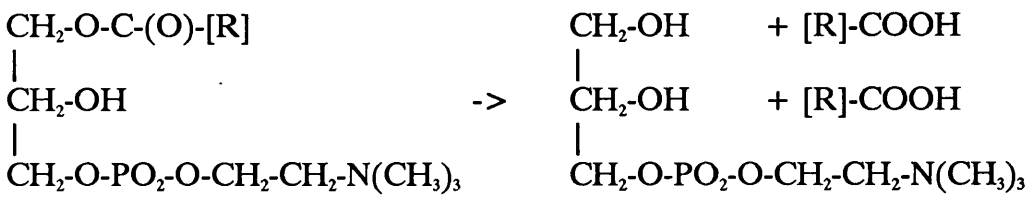
spectra. There is a band at 830cm^{-1}, assigned to a C-O-P-O-C vibration of the lipid (Casal et al 1987), which has been lost. Also the band at 968 cm^{-1} in DPPC, due to the choline in the headgroup, has been submerged by bands at 1004, 974 and 958 cm^{-1}, although the choline is still present as shown by the shoulder at 1480 cm^{-1} to the band at 1468 cm^{-1}. Although the band at 830 cm^{-1} is due to only 1 of several possible stereochemical arrangements of the headgroup (Casal et al 1987) no new bands are present in the spectra to indicate rearrangement in the head group. These spectral changes probably reflect scission of a C-O-P bond. As breakage of the bond to the glycerol would yield phosphocholine (which is not expected to be chloroform soluble, and the grafts were extracted with chloroform) it is more probable that the bond to the choline is removed. In the absence of other ions this would yield a phosphatidic acid choline ion-pair. That this does occur will be shown below. The reactions mechanisms and resulting structures are shown in table A 3.1.

By running spectra of mixtures of DPPC and palmitic acid it is possible to estimate the amount of lysis of the glycerol-palmitic bond which has taken place. Variable amounts have been found in different samples, ranging from about 10-15% to about 45% of the lipid chains. However re-examination of the samples 10 months after irradiation suggests that lipid breakdown has continued. The spectrum is now close to that of palmitic acid with only a small amount of lipid. The other products such as glycerophosphatidylcholine are presumably insoluble in $CHCl_3$. Attempts to extract these water soluble products from grafts gave inconclusive results. The infra-red characterisation of phosphatidic acid choline complex was investigated using the mole ratios of DPPA and choline (1:1 and 1:2) used above for the TLC. These mixtures have been examined by FTIR. The 1:1 complex removes the band at 1010 cm^{-1} due to the unionised acid indicating the formation of a salt. At the same time the band at 960 cm^{-1} can be assigned to the choline as it occurs in both the spectra of the choline base and choline mixed with palmitic acid. The 1:2 complex contains an extra band at 974 cm^{-1}. This would suggest that the bands seen in the graft extract represent a mixture of free DPPA, 1:1 and 1:2 complexes.

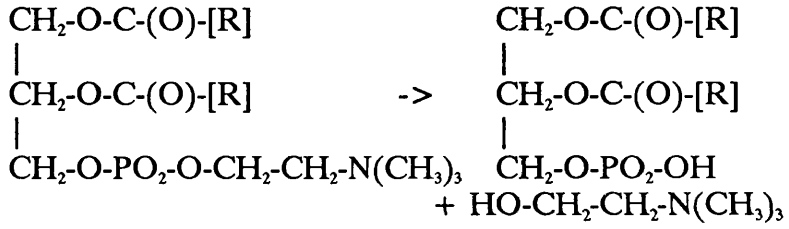
1 loss of 1st ester link results in DPPC-> LYSO-PC, PA.



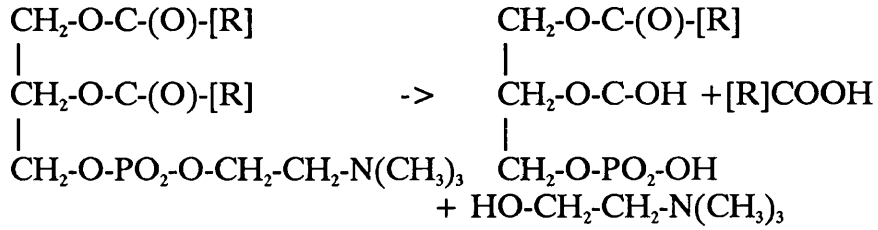
2 loss of 2nd ester link results in LYSO-PC -> GPC, PA.



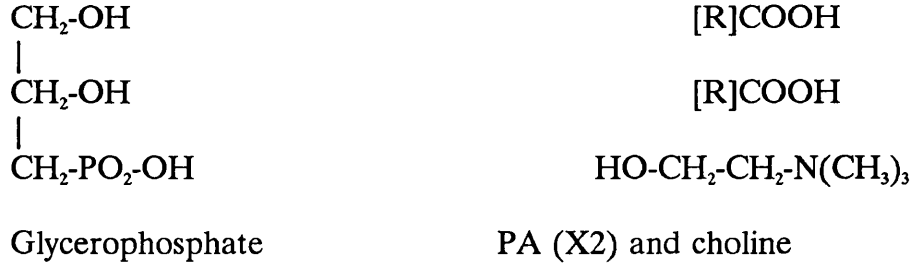
3 loss of the phosphorous choline link results in DPPC -> DPPA + choline



4 combined attack at both bonds results in LYSO-PA + choline + PA



5 final products



DPPC = dipalmitoylphosphatidylcholine, GPC =glycerophosphatidylcholine
DPPA = dipalmitoylphosphatidic acid, PA = palmitic acid,
LYSO- = loss of one PA.

Table A 3.1 Possible decomposition reactions for DPPC.

A 3.4 Results of MTEG for irradiated materials

The tabulated MTEG results for the irradiated materials have numbering related to chapter 4, and have the same significance as discussed above on page 223.

Table 4.1 MTEG results for DPPC coating on metal cuvettes. N=12

Parameter	Mean	95% confidence limits		p value
Mr	1.972	1.477	2.634	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.727	0.553	0.955	0.039 *
Mma	0.062	0.046	0.082	<0.0001 ****
Me	0.031	0.022	0.043	<0.0001 ****
MA°	0.108	0.077	0.151	<0.0001 ****
Mα	0.095	0.073	0.125	<0.0001 ****

For Mr+k and Mk p=0.003, all the values were infinite.

Table 4.2 MTEG results for irradiated DPPC coating on plastic cuvettes. N=11

Parameter	Mean	95% confidence limits		p value
Mr	0.975	0.902	1.056	0.54
Mr+k	#			
Mk	#			
Mtma	0.441	0.251	0.776	0.016 *
Mma	0.213	0.125	0.366	0.0002 ***
Me	0.130	0.048	0.357	0.0024 **
MA°	0.507	0.376	0.682	0.001 ***

All the values are infinite, p=0.004.

Table 4.3 MTEG results for SM coating (run 1) on plastic cuvettes. N=11

Parameter	Mean	95% confidence limits		p value
Mr	2.447	1.989	3.009	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.616	0.498	0.761	<0.0001 ****
Mma	0.030	0.024	0.037	<0.0001 ****
Me	0.014	0.011	0.018	<0.0001 ****
MA°	0.047	0.034	0.064	<0.0001 ****

All the results were infinite, p=0.004 **

Table 4.4 MTEG results for SM coating (run 2) on plastic cuvettes. N=10

Parameter	Mean	95% confidence limits		p value
Mr ~	2.619	2.305	2.975	0.0004 ***
Mr+k	#			
Mk	#			
Mtma	0.801	0.582	1.103	0.2
Mma	0.025	0.016	0.038	<0.0001 ****
Me	0.012	0.008	0.019	<0.0001 ****
MA°	0.036	0.025	0.051	<0.0001 ****

~ for n=5, 5 results are infinite, ~ for Mr p=0.006. The data is 2.16, 2.75, 3.49, 2.56 and 2.16.

all the results are infinite, p=0.006.

Table 4.5 MTEG results for irradiated SM. N=10

Parameter	Mean	95% confidence limits		p value
Mr ~	1.730	1.449	2.066	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.571	0.480	0.681	<0.0001 ****
Mma	0.033	0.023	0.047	<0.0001 ****
Me	0.017	0.012	0.025	<0.0001 ****
MA°	0.070	0.058	0.085	<0.0001 ****

~for N=8, 2 results are infinite, # for all 10 p=0.006.

All infinite, p=0.006.

Table 4.6 MTEG results for polymerized DAPC (first batch). N=13

Parameter	Mean	95% confidence limits		p value
Mr	1.102	0.989	1.228	0.10
Mr+k	#			
Mk	#			
Mtma	0.327	0.292	0.366	<0.0001 ****
Mma	0.121	0.074	0.197	<0.0001 ****
Me	0.060	0.035	0.102	<0.0001 ****
MA°	0.321	0.171	0.603	0.0057 **
Mα	0.429	0.272	0.666	0.004 **

p=0.006 ** all the results were infinite, except one, Mr+k 0.81 and Mk 0.61,

Table 4.7 MTEG results for polymerised DAPC (2nd batch).N=9

Parameter	Mean	95% confidence limits		p value
Mr	1.317	1.094	1.586	0.018 *
Mk	#			
Mr+k	#			
Mtma	0.394	0.267	0.580	0.0014 **
Mma	0.045	0.034	0.060	<0.0001 ****
Me	0.020	0.014	0.028	<0.0001 ****
MA°	0.142	0.093	0.217	<0.0001 ****

all the results are infinite, p=0.009.

Table 4.8 MTEG results for irradiated DAPC (batch 2). N=10

Parameter	Mean	95% confidence limits		p value
Mr	0.880	0.759	1.022	0.12
Mr+k	#			
Mk	#			
Mtma	0.463	0.313	0.684	0.0035 **
Mma	0.142	0.097	0.207	<0.0001 ****
Me	0.072	0.045	0.117	<0.0001 ****
MA°	0.544	0.401	0.737	0.0031 **

all infinite, p=0.006.

Table 4.9 MTEG results for Polyester G (batch 1). N=11

Parameter	Mean	95% confidence limits		p value
Mr	1.291	1.180	1.413	<0.0001 ****
Mr+k	#			
Mk	#			
Mtma	0.484	0.404	0.580	<0.0001 ****
Mma	0.139	0.086	0.222	<0.0001 ****
Me	0.076	0.045	0.128	<0.0001 ****
MA°	0.350	0.237	0.518	<0.0001 ****

For Mr+k and Mk 'p' was 0.004, the median was infinite, and the finite data was 1.14 and 1.32 for Mr+k, 1.25 and 1.38 for Mk.

Table 4.10 MTEG results for Polyester G (batch 2). N=11

Parameter	Mean	95% confidence limits		p value	
Mr	1.161	1.053	1.280	0.012	*
Mr+k	#				
Mk	#				
Mtma	0.616	0.493	0.770	0.0015	**
Mma	0.213	0.172	0.264	<0.0001	****
Me	0.115	0.089	0.149	<0.0001	****
MA°	0.330	0.264	0.411	<0.0001	****

all infinite, p=0.004.

Table 4.11 MTEG results for irradiated Polyester G (batch 2). N=11

Parameter	Mean	95% confidence limits		p value	
Mr	0.829	0.753	0.914	0.0032	**
Mr+k	#				
Mk	#				
Mtma	0.637	0.477	0.851	0.011	*
Mma	0.323	0.268	0.388	<0.0001	****
Me	0.187	0.148	0.235	<0.0001	****
MA°	0.639	0.505	0.807	0.0033	**

there are 2 finite results for Mr+k, 1.11 and 0.91, and 2 for Mk, 1.43 and 0.92, p=0.005.

Appendix 4

The results of the preliminary experiments

A 4.1 The experimental details

A number of experiments lasting 4 hours, without recovery from the anaesthetic were performed, to establish various techniques, which are presented in this appendix (A 4). The anaesthetic used for the pig previously in this laboratory proved suitable for the sheep, but premedication was not needed. The choice of catheters and vessels used for the arterial and venous lines were developed and established. A small number of 'full' 24 hour experiments were carried out to confirm these choices. The natural fate of the platelet and fibrinogen labels has been evaluated in two unoperated and unanaesthetised animals, as well as a direct AVF in one further animal. These three studies are presented separately in appendix 5.

A 4.2 Assays for haemolysis and blood volume

The same heparinised plasma sample is used in the protocol for spectroscopic determination of free haemoglobin and Evans blue. 400 μl of plasma is diluted 1:4, by adding 1.6 ml of 0.85% saline.

For the haemolysis assay the standard curve is prepared from 200 μl of blood from the first EDTA blood sample by adding it to 12.3 ml of distilled water, to make free haemoglobin. The haemolysate is centrifuged at 2000 G for 5 minutes to remove the RBC ghosts. A plasma/0.85% saline (1:4) solution is made using the plasma from the first heparinised blood sample. 400 μl of the free haemoglobin solution (EDTA+water) is added to 3.6 ml of the plasma/saline solution. A doubling dilution in 2 ml of the plasma/0.85% saline (1:4) solution provides 6 standards. The adsorbance is read at 453 nm (slit width of 0.03) and at 413 nm (slit width of 0.0425) using saline as the blank.

The dilution of the haemoglobin is 200 μl in 12.5 ml ie 1/62.5. The addition of

400μl in 4.000 ml is 1/10, and makes the first standard 1/625. The serial doubling dilution gives the remaining standards from 1/625, 1/1250, 1/2500, 1/5000, 1/10000, to 1/20000. The free haemoglobin in mg/100 ml is 1/125 the blood haemoglobin (determined by cell counting) in gm/100 ml ie x8, x4, x2, x1, x1/2, and x1/4, since the assay is carried out at a 1/5 dilution.

The estimation of blood volume requires a standard curve using the pre-injection plasma. This is prepared using 100μl of the dye added to 9.9 ml saline. 0.5 ml of the plasma from the pre-injection sample is added to 2 ml of diluted dye. Serial dilution of 2 ml of this mixture is used to create 6 standards with a similar plasma/saline mixture (1:4) as the haemoglobin assay. The dilution of the dye given to the animal is 10 ml dye to an estimated blood volume of 1-2 l, ie 1/100 to 1/200, with the further dilution of the sample plasma this is a final dilution of 1/500 to 1/1000. The dilution of the standard is 100μl in 10 ml saline, ie 1/100. 2 ml of this is added to 0.5 ml plasma to make 1:4 plasma/saline, and 2/2.5 is used for the first standard, ie 1/125. The serial doubling dilution gives final standards of 1/125, 1/250, 1/500, 1/1000, 1/2000, 1/4000. The adsorbance is read at 624 nm (slit width 0.0625) against the plasma blank.

The plasma haemoglobin assay has been validated using freshly haemolysed blood, and a solution of crystallized sheep haemoglobin (Sigma). The latter was assessed for use as a possible standard for the assay (at 20 mg/ml). The peak adsorbances have been checked using a Phillips scanning spectrophotometer, using a saline blank, just as in the assay. The peak absorption for fresh oxyhaemoglobin is 415 nm, while the crystallized haemoglobin contained large amounts of methaemoglobin and shifts this peak to 407 nm, making it necessary to use the freshly haemolysed blood.

The estimation of blood volume, using the Evans blue assay has also been assessed using the Phillips scanning spectrophotometer, but with a plasma blank as in the assay. Additional scans have been taken for Evans blue using a blank of saline, to determine if it would effect the haemoglobin assay. The peak for Evans blue made up in saline is 609 nm, but in the presence of plasma is 624 nm. The trace is reasonably flat between 400 and 460 nm, so that it does not interfere with the

haemoglobin assay. Further tests with variable amounts of free haemoglobin present do not affect the Evan's blue peak at 625 nm. At very high concentrations of free haemoglobin there is a minor effect but this disappears when measured against the plasma blank (as in the assay). These tests have been repeated with blood and plasma from several animals, and there are only minor variations of 1-2 nm in the peak adsorbances for both substances.

A 4.3 Length of graft material

The initial non-recovery experiments showed that lengths of 8 and 10 cm of the Dacron graft material thrombosed within minutes. The shorter 3 cm lengths always remained patent (3 in all). For 6 cm grafts 5 thrombosed with 1 additional one remaining patent over 4 hours. At 5 cm 1 closed and 1 remained open. At 4 cm 2 remained patent, and 2 closed only at the end of the experiments.

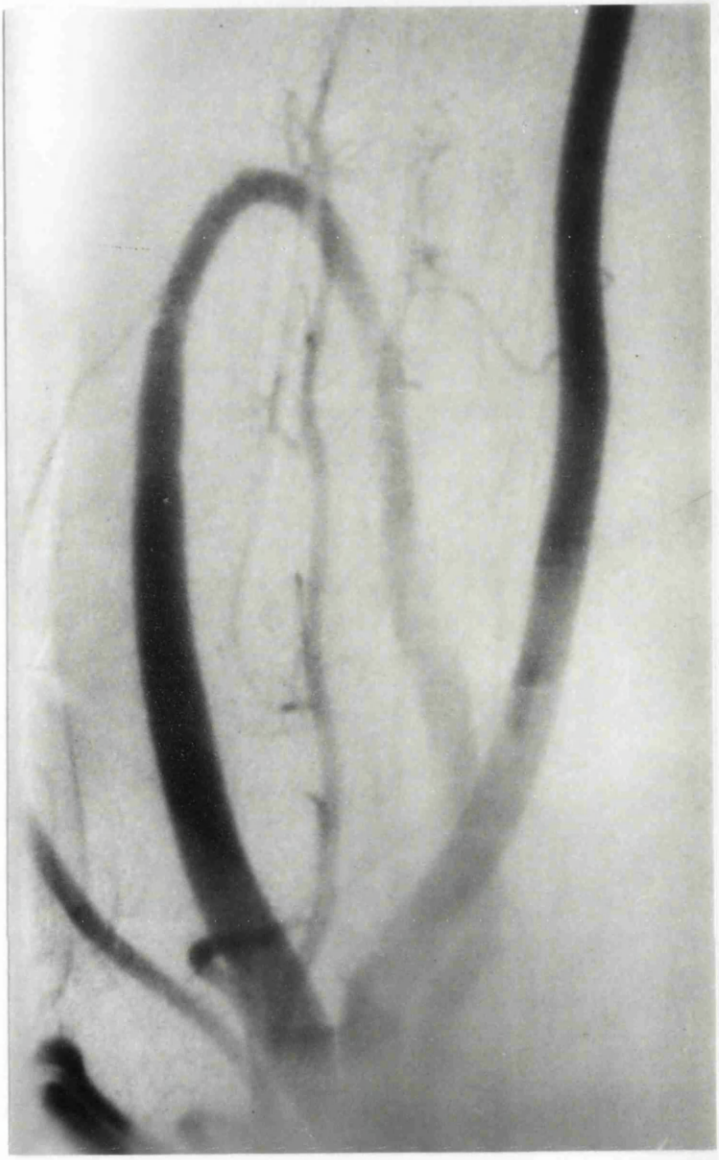
A further 6 experiments of 24 hours duration with recovery from the anaesthetic were conducted. In these lengths of 4 and 5 cm were tested. 2 further 5 cm lengths were tried, 1 remained patent and 1 thrombosed overnight. Three 4 cm lengths remained patent over the 24 hours, and this length was chosen for the definitive experiments.

A 4.4 Angiographic assessment

During the initial experiments direct puncture of the proximal carotid artery with a 21 g butterfly was used. The images tended to be uneven, with 'streaming' of the contrast. Although a theoretical objection, the contrast was virtually undiluted at the graft surface, and could have affected the coating materials. The method was abandoned after an intimal dissection with subsequent thrombosis of the graft. Additionally the operator's hand were close to the primary X-ray beam (as this was still a sterile field lead gloves could not be used).

These considerations lead to the use of selective catheterization of the carotid arteries, via the brachial/axillary/subclavian/aortic route. The first attempts were defeated through a lack of appreciation of the normal sheep arterial anatomy.

Essentially the aorta divides just distal to the heart into a descending thoracic aorta, and an ascending common trunk (brachiocephalic) which passes into the neck. This gives off subclavian and vertebral branches, and only then divides into right and left common carotid arteries, just at the level of the root of the neck (see Ghoshal et al 1981, pp 24 & 40). This can be seen in photograph A 4.1, the angiogram of s514. A guide wire is passed by through the catheter to the common brachiocephalic trunk. The catheter is then advanced over the guide wire until the tip is at the origin of the common carotid trunk, approximately the level of the first rib. As the cervical spine as well as the endotracheal and stomach tubes obscured the angiogram, subtraction views are made with the help of an additional preliminary plain view for the mask. Deliberate faults were incorporated in two or three anastomoses. These were identified by the angiograms.



Photograph A 4.1 Angiographic appearance of sheep neck vessels s514.

A 4.5 Blood flow by Doppler ultrasound

A Vasoflo 3 (Oxford Sonicaid) is used with 8 and 10 MHz probes to assess the arterial and venous sides of the completed anastomosis. This is carried out using the probes in a sterile glove, which contains ultrasonic jelly. Perspex jigs are used to define both the angle of insonination and the vessel diameter. This is based on the work of Beard (et al 1986b).

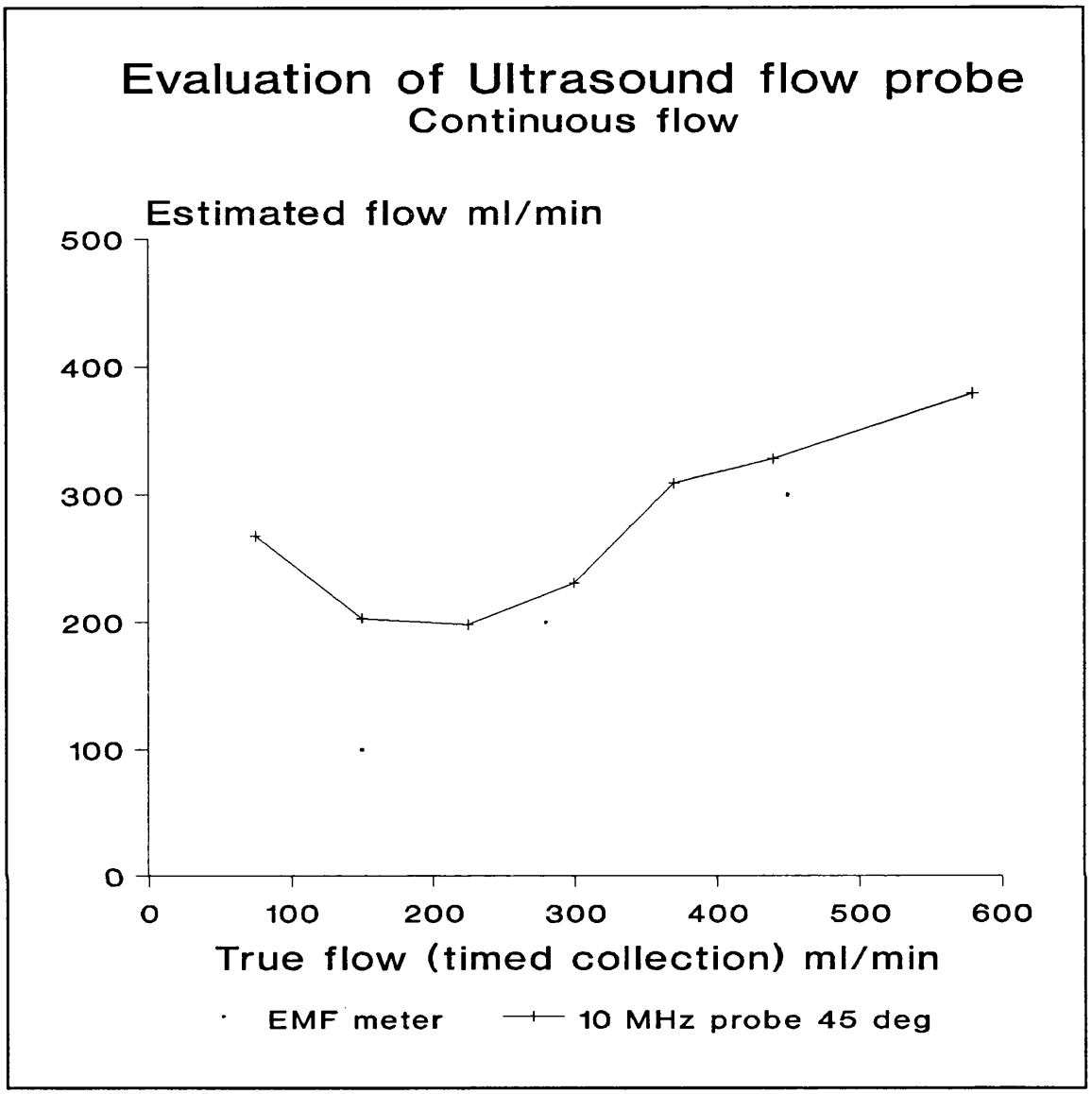


Figure 4.1 Comparison between EMF and ultrasound probe methods of evaluation blood flow.

This proved to be a difficult technique to use and gave disappointing results. In the laboratory a flow rig was set up with thin walled silicone tubing, using a starch suspension in saline for the fluid. Timed collections and comparisons with an electromagnetic flow meter gave poor correlations with the true flow. Comparisons were also made in a closed circuit using both continuous and pulsatile flow with a Biomedicus pump. These also showed poor correlation. Two examples of the results are shown in figures A 4.1 and A 4.2.

In the animal the main difficulty was to obtain a good signal from the exposed vessel walls, due to the artifacts created by the arterial wall movement. In

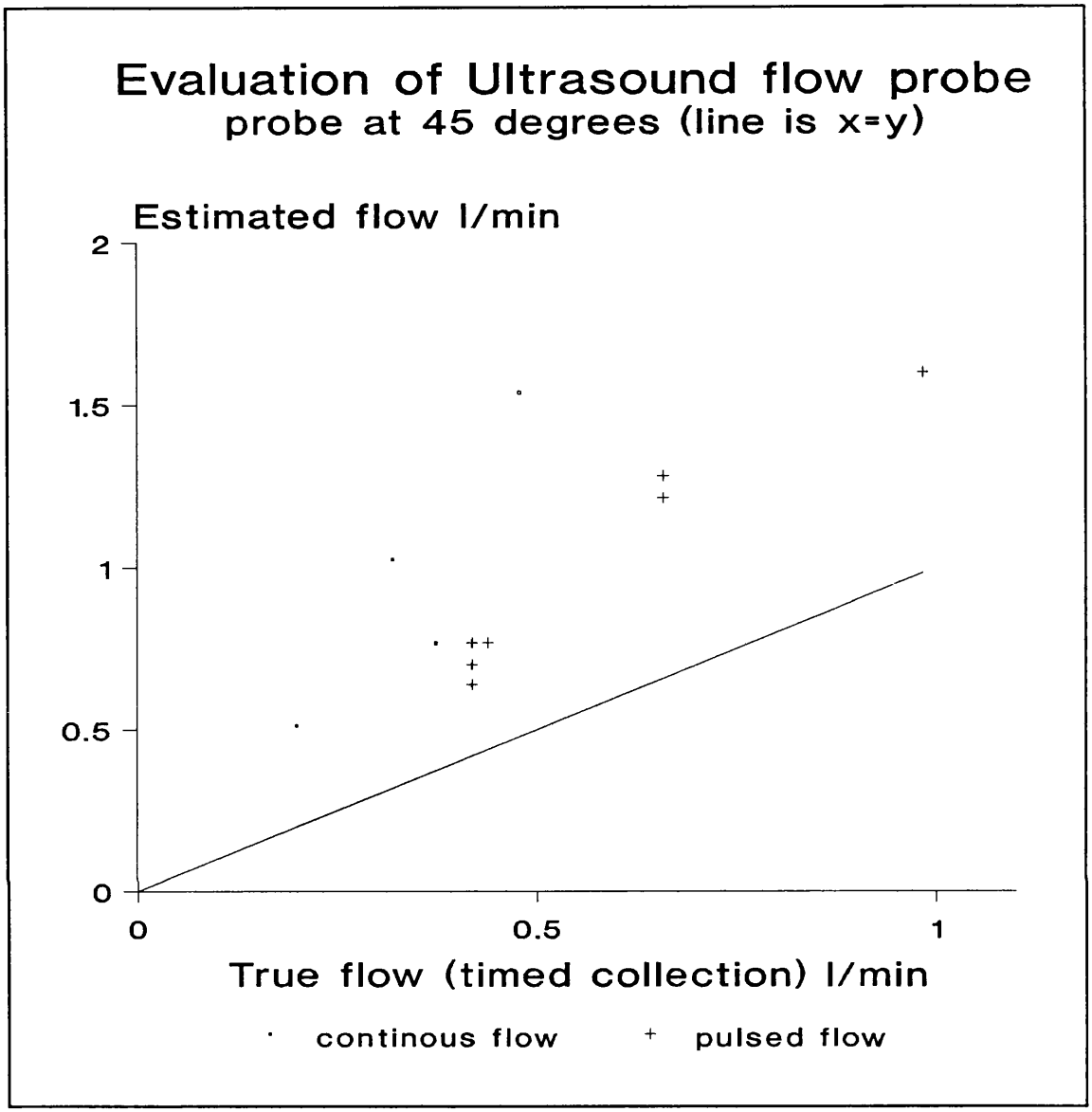


Figure 4.2 Comparison between continuous and pulsatile flow using the ultrasound probe.

addition the Vasoflo 3 was unable to generate the ultrasound parameters from the signals. This may be because the arterial rate was beyond the upper limit of the software, or because the signal was too attenuated and damped for it to pick out the individual beats in the signal. Additionally the estimates of blood flow derived from the ultrasound parameter assume uniform laminar flow. In view of these problems no data is presented.

A 4.6 The platelet labelling

The method for separation of the platelets was established by educated trial and

error. The tropolone concentration was varied between 10^{-5} and 10^{-3} M, and the highest efficiency was obtained at 2.2 10^{-4} M. The cell concentration was found to reduce the labelling efficiency, when below 10^{9} cells per ml. The method was checked for damage to the platelet function by aggregometry. In this the native platelets were examined, and the results compared with the platelets that had been through the handling required for labelling. The aggregation shown no serious deterioration of the platelet function.

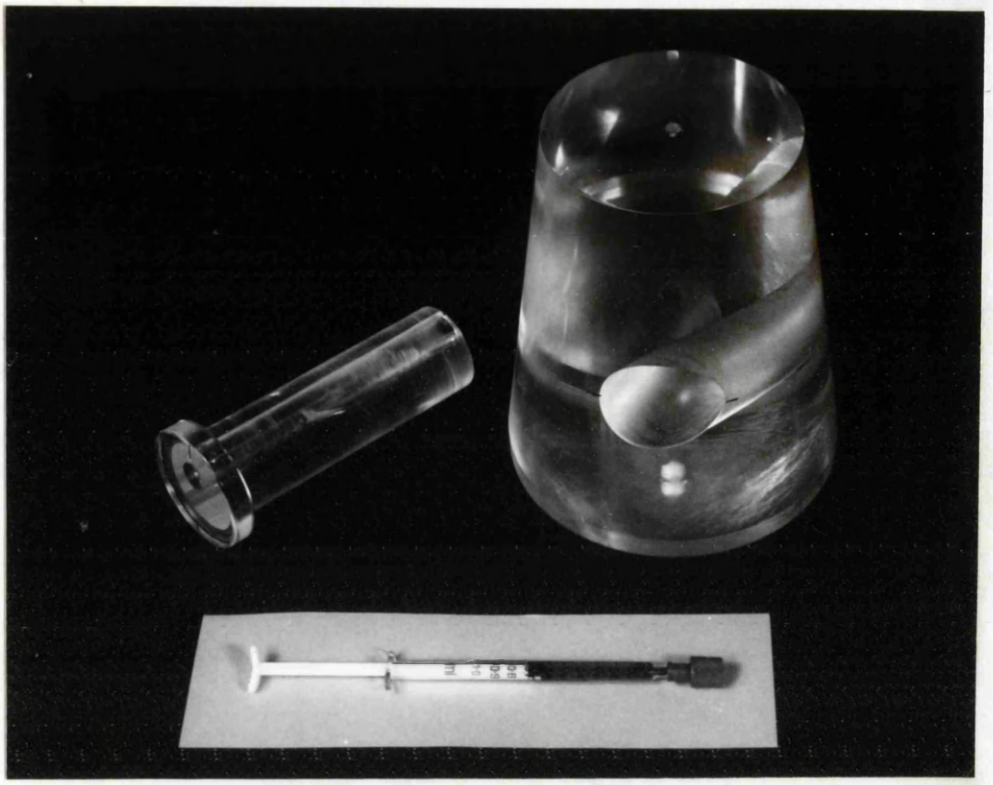
A 4.7 Design of the phantom

The phantom design falls into two parts. The first is its basic shape. The second is the positioning of the radioactive standard within the volume of the phantom to mimic the position of the graft within the neck of the animal. The dimensions are based on the measurement of the circumference of the necks of 5 sheep (table A 4.1). The circumferences at the top, the middle and the base of the neck were found to be close to a taper. The basic shape of the phantom is that of a truncated cone, with a slight adjustment of the diameters. This was necessary in order for it to be turned from a solid block of perspex, as this material has very similar properties for absorbing and scattering gamma photons.

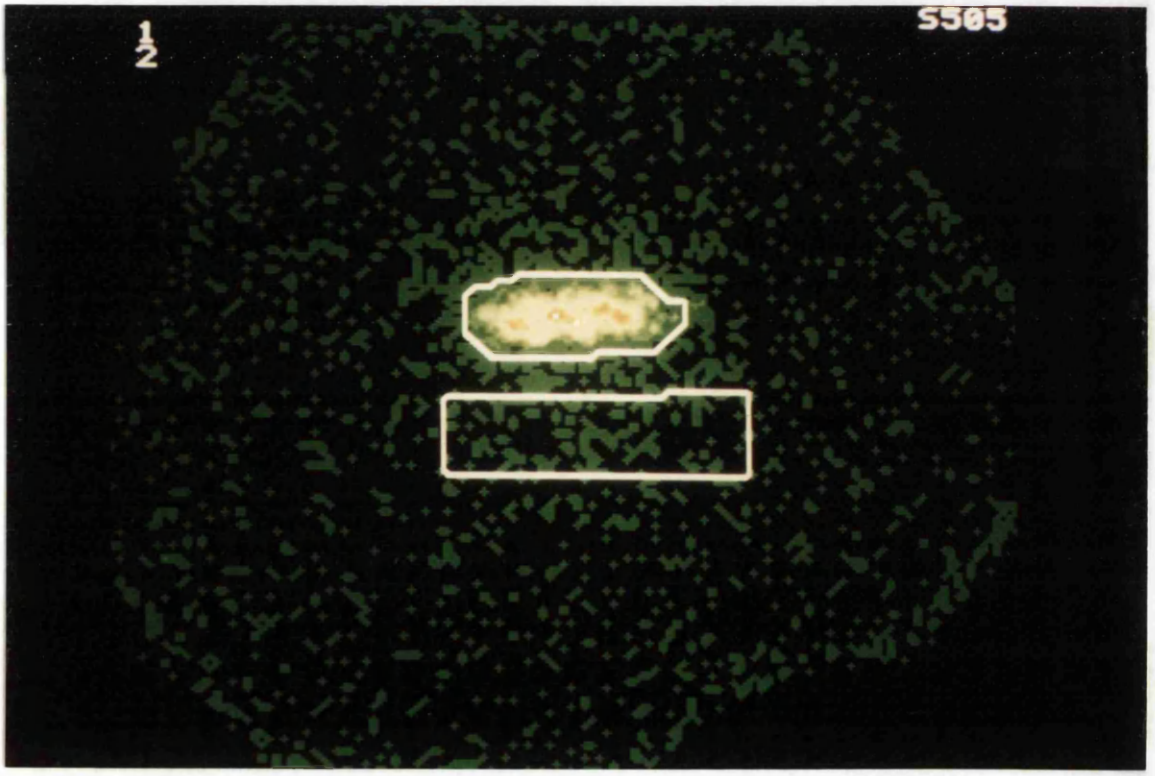
mean of 5 sheep	top	middle	base
circumferences cm	30.6	33.2	41.4
radius cm	4.8	5.3	6.6
phantom cm	4.5	5.5	6.3

Table A 4.1 Phantom design neck measurements.

The final position of the graft in the neck is a double curve sweeping upwards from the artery and turning downwards to the vein (ie in the coronal plane). At the same time the graft lies deep in the neck at its origin (mean depth 3 cm) to become most superficial as it reaches the midline (mean depth 1.5 cm), before passing more deeply at the venous end (ie in the transverse plane).



Photograph A 4.2 The phantom used for gamma camera image calibration.



Photograph A 4.3 Typical gamma camera image of the phantom containing the standard, with the regions of interest defined.

The graft has dimensions of 4 cm length and 0.5 cm diameter. A TB syringe also has an internal diameter of 0.5 cm, and when filled to 0.7 ml has a length of 4 cm.

The variable depth of the graft from the skin is accommodated by placing the TB syringe at 1.5 and 3 cm from the surface of the phantom. This is accurately achieved by placing the syringe in a circular carrier which is rotated within the main body of the phantom to give the two required depths. The phantom used is shown in photograph A 4.2. The standard is placed in both positions and the average counts for both positions is used in the calculation of the percentage injected dose. Photograph A 4.3 shows a typical gamma camera image obtained from the phantom, with the ROI's defined.

Appendix 5

The results for the control experiments

A 5.1 The experimental details

Two animals, which were not operated on in any way, were used to establish the 'natural' fate of the particular labels used in these experiments. These animals were sedated with intravenous Sagittal, via a 19 gauge butterfly placed in a large foreleg vein. Once sedated they were given indium 111 labelled platelets, iodine 125 labelled fibrinogen and Evans Blue via the butterfly. Blood samples were taken from the jugular vein with a 21 gauge needle. The animals were allowed to recover after the initial intensive sampling of the first 30 minutes. Further samples were taken at the same times as the protocol above table 6.1 in section 6.2.4 page 151. After 24 hours additional samples were taken, 3 on the second day, and daily thereafter for 7, and 10 days. These samples were processed in the same way as outlined above in chapter 6.

One additional experiment with a direct arteriovenous fistula (AVF) was conducted to attempt to provide a further control with surgery comparable to the experimental groups, but without any graft material.

A 5.2 The free haemoglobin studies

It should be noted that for these studies a 21 g needle was used, since the primary aim of these experiments was to establish the fate of the isotope labels. This was to minimise trauma to the vein, which otherwise could have lead to haematomas and so increase the apparent consumption of the labels. The specimens taken by this route are not ideal for the haemolysis assay (taken with a 19 g needle in clinical practice, and via an 8 F catheter in the main experiments).

These experiments showed the largest amounts of haemolysis in all the studies performed. The average was 30.1 mg/100 ml, and the highest single result was 51 mg/100 ml. The results for the AVF with blood taken via the catheter were much lower, at 5.4 and 7.9 mg/100 ml respectively.

A 5.3 The platelet studies

The platelet survival data are examined just as those in the definitive experiments. The true survival makes use of all the samples collected over the 10 days of the experiments and the results are compared to a limited data set of the samples collected in the first 24 hours.

Subject	24 hour data		all 10 day data	
	wtm	mhit	wtm	mhit
S3575	6.631*	6.969*	5.177	3.392
S684	4.268	3.960	4.161	3.620
averages	5.45	5.47	4.67	3.51

wtm = weighted mean, mhit = multiple hit model
* sample at 8 hours has large effect, see text.

Table A 5.1 Platelet survival data for unoperated animals.

The results are summarised in table A 5.1. These show that the 24 hour estimate tends to be slightly higher than the true estimate of platelet survival. The 24 hour results for s3575 are strongly affected by the 8 hour sample, which was the highest in this study. This sample strongly affects the estimate since slightly fewer samples were taken for S3575 in the first 24 hours than in the graft experiments.

The results for the direct AVF are some what unexpected. The AVF had a considerable mismatch between the size of the artery and the vein. This lead to a large thrombus within the compliant and large first part of the vein. This gave the highest 'graft' count, of over 4.02% of the injected dose of platelets, and 0.47% of the injected fibrinogen. The platelet survival times are 1.468 and 1.17 days for the weighted mean and multiple hit models respectively. These are the shortest survival times for any of the studies, and are accounted for by the large thrombus in the AVF.

A 5.4 The fibrinogen studies

The use of the two isotopes, indium 111 and iodine 125 requires a correction to the iodine 125 counts due to the crossover effect outlined above in section 6.3.4.3, page 174. These are the low energy indium 111 counts that are registered in the iodine 125 channel as apparent iodinated fibrinogen. The correction is calculated as follows. The background counts for both channels are subtracted from the counts. The counts in the iodine channel for the indium standards, which should be the same as the background counts (if crossover did not occur), are expressed as a proportion of the counts in the indium window (these are the counts that crossover). The correction for the samples with both isotopes present is calculated from the counts in the indium channel and subtracted from the counts in the iodine channel. This calculation for the fibrinogen studies has been validated by recounting the samples from several experiments after the indium had decayed to low levels. For both the activity and calculated percentage doses, the regression coefficients between the true iodine 125 count and the estimate using the crossover correction have been calculated. Typically the coefficients between the two data sets are $r^2>0.98\%$ with $p<0.0001$.

The fibrinogen studies have been made using the iodine-125 counts from the EDTA samples, although these are whole blood samples and contain both fibrinogen, FDP's and free iodine. The choice of the EDTA rather than citrate samples was taken to reduce the amount of blood removed from the animal. The validity of this choice has been checked in the 3 experiments described above, and 2 other experiments with uncoated grafts. Essentially both samples were taken, the results calculated and compared.

The EDTA samples were taken and the results calculated as in chapter 6. A second sample was collected by adding 4.5 ml blood to 0.5 ml citrate with 4000 units Aprotinin (200μl Trasylol, Bayer). These blood samples were centrifuged immediately (MSE Minor, 10 minutes at 1100 G) and the citrated plasma was separated and stored at -25°C. This allowed the 111 In to decay to insignificant levels. These samples were assayed for free iodine, protein bound iodine, and clottable fibrinogen using standard methods (Regoeczi 1967, and RFHSM

Haemophilia Centre).

1.5 ml samples of the thawed plasma were counted (as for the EDTA samples above). The plasma was then clotted with calcium chloride and thrombin (0.5ml saline with 0.5 units bovine thrombin, 12.5mM $CaCl_2$). The clot formed was wound out of the test tube with a wooden stick. These were washed in saline, water and acetone before being dried in a hot air cabinet and weighed to a constant weight. The small clots were then dissolved in KOH (2ml 1M) for recounting. The serum remaining in the test tube, after the clot had been remove, was counted. The serum was then vigorously mixed with trichloroacetic acid (1 ml 20% TCA) to precipitate the proteins. This was centrifuged at 1500 G for 10

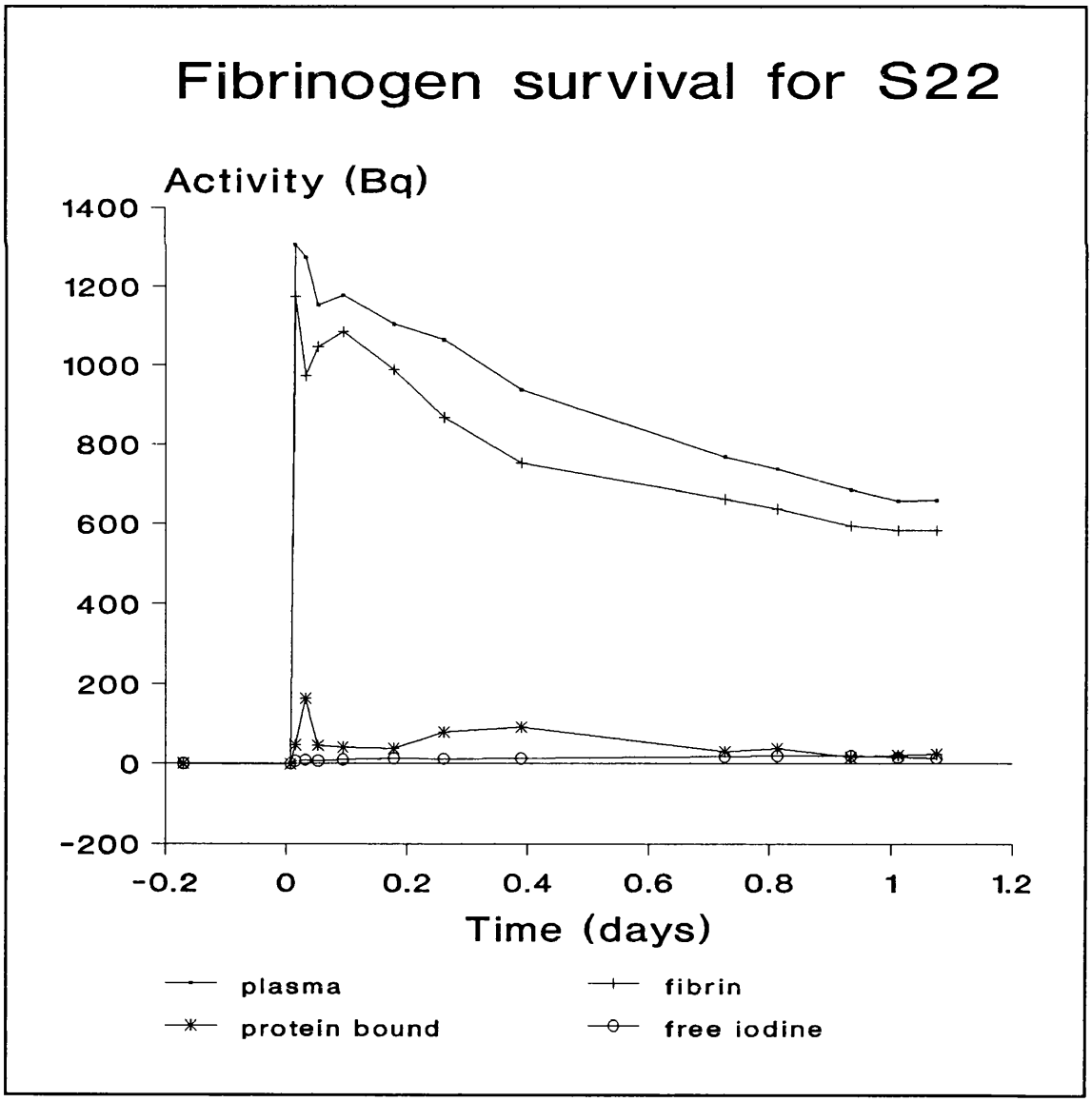


Figure A 5.1 Fibrinogen survival data for s22, using citrated plasma samples.

minutes and a 2 ml aliquot of the supernatant was counted to give the free iodine count. The protein bound count is obtained by subtracting the free count from the serum count. The clottable fibrinogen count is obtained by directly from the counts (the clot dissolved in KOH). It was sometimes not possible to remove all the clot from the serum, and this was confirmed by a fall in the fibrin weight for that sample.

An example of the data from the citrated plasma samples (s22) is shown in figure A 5.1. The full results are shown in table A 5.2. The clottability of the citrated samples is based on all the samples (except 6 where the sample was lost). There were some samples where the clot was incompletely extracted after clotting with the thrombin. Despite these samples the clottability of 87% compares well with Collen et al 1972, who found 80-90%. The mean result of 2.2 gm/l for these sheep compares well with the plasma fibrinogen levels sheep 2.0-3.5 g/l (Gajewski and Povar 1971, Fantl and Ward 1960, Gehweiler et al 1983), and for man (2.8 gm/l Collen et al 1972).

	Fibrinogen gm/l			clottability %		
subject	N	mean	sem	N	mean	sem
s22	13	2.66	0.23	11	88.1	1.6
s17	11	2.16	0.11	8	86.1	2.2
s15	15	2.03	0.16	13	87.3	0.6
s684	25	1.88	0.06	20	86.6	1.3
s3575	24	2.38	0.05	-	-	-
Mean	5	2.22	0.14	4	87.2	0.6

Table A 5.2 Plasma fibrinogen levels and % clottability.

The results for EDTA iodine 125 counts and the clottable fibrinogen counts show a close correlation. Four sets of data were compared, including s15, the direct arteriovenous fistula, and s684, the fibrinogen survival study. These all gave $p<0.0001$ for the correlation coefficient. The r^2's were >0.92% except for s17, with $r^2>0.70\%$, but this was the first experiment using fibrinogen. The result for s684 was $r^2>0.97\%$. The correlations were closer for the plasma fibrinogen counts. These results strongly support the decision to use the ETDA iodine 125 counts as plasma fibrinogen counts.

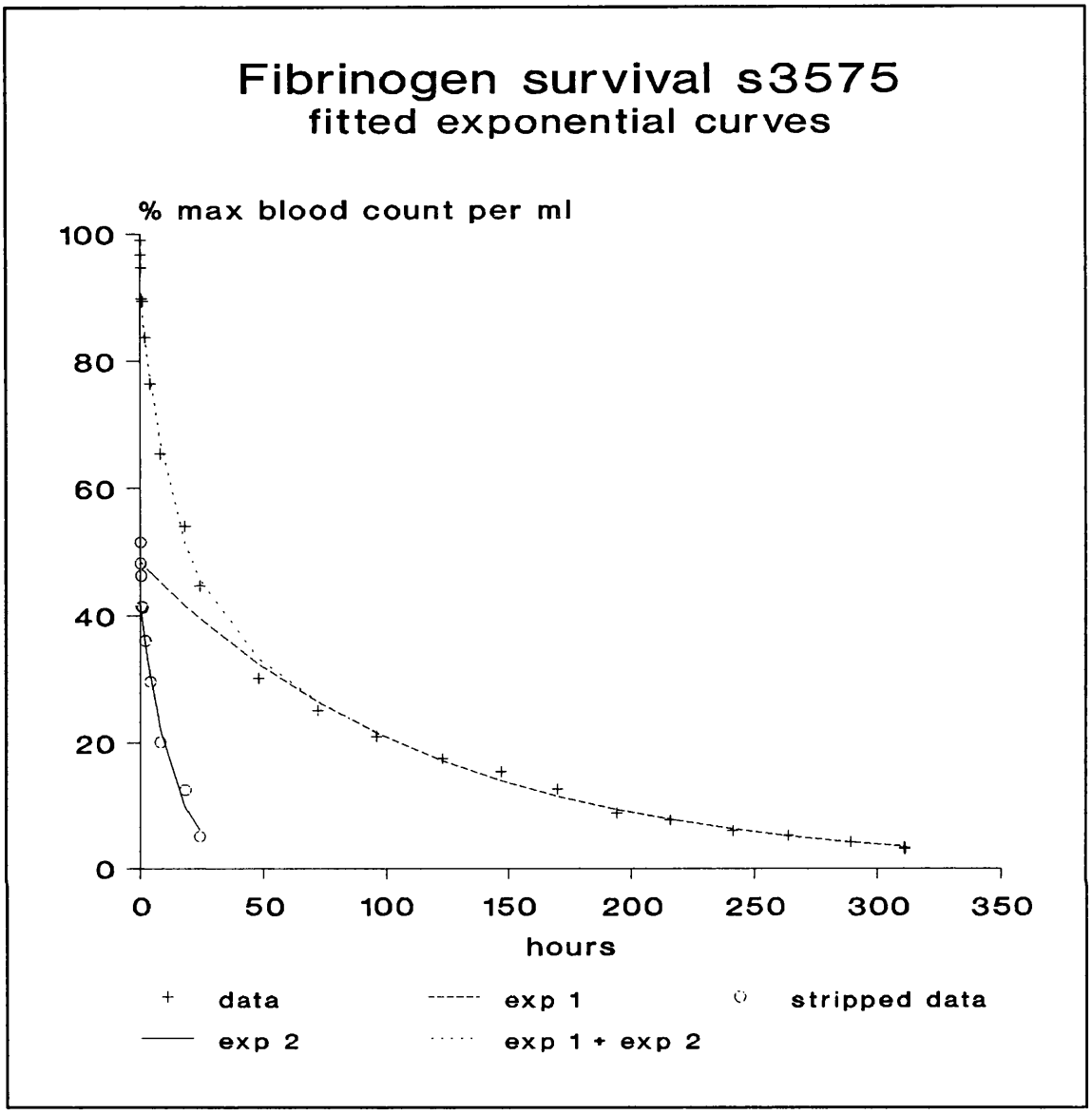


Figure A 5.2 Fibrinogen survival curves for s3575.

The fibrinogen survival times for s3575 and s684 are calculated by curve stripping (based on Collen et al 1972 and Matthews 1957). In this the curve of the counts, expressed as the percentage of the maximum dose found in the blood, is plotted on semilog paper and the best fit of the linear part of the curve made. The exponential for this line is calculated, and taken from the original curve to leave the second exponential. The results of the curve fitting for the fibrinogen survival experiments are shown in figures A 5.2 and A 5.3.

The constants of the derived exponentials for the fibrinogen survival curves give the rate constants for the fluxes between the compartments (Matthews 1957). The results shown in table A 5.3.

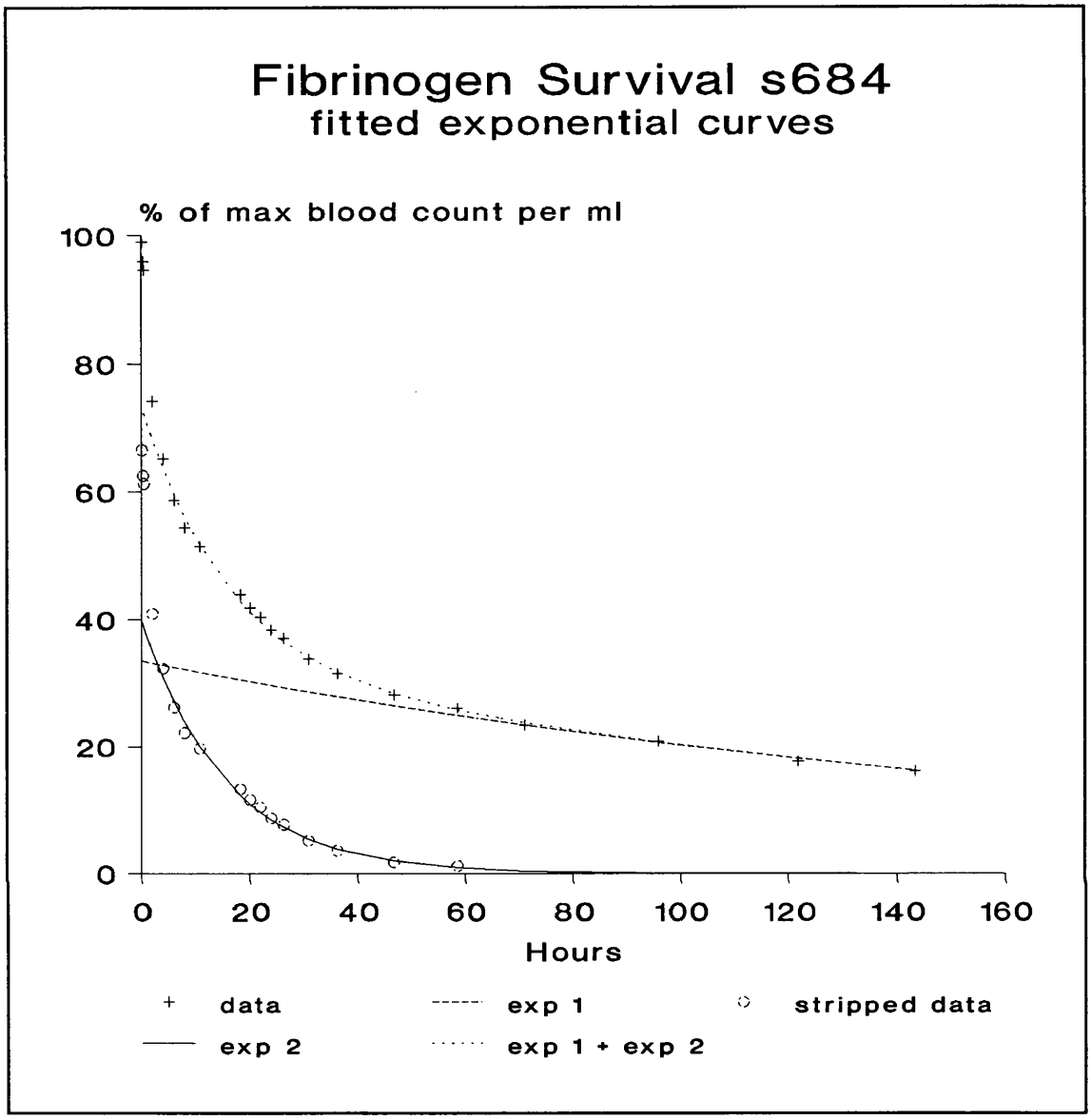


Figure A 5.3 Fibrinogen survival curves for s684.

These show the exponentials for these two experiments, and it can be seen that the two compartment model provides a good fit for the data.

The parameters are for

$x(t) = C_1e^{-a1t} + C_2e^{-a2t}$, where

x(t) is the fractional blood activity expressed as a %,

C_1 and C_2 are the intercepts and,

a1 and a2 are the slopes for the two exponentials.

k12 is the fractional transcapillary efflux rate constant

(ie from the vascular to the extravascular pool)

k21 is the fractional transcapillary reflux rate constant

(ie from the extravascular to the intravascular pool)

parameter	s684	s3575	Collen
fibrinogen half life (days)	5.75	3.4	4.14
c1	33.5	48.5	67.0
a1	0.12	0.20	0.17
c2	40.0	42.0	33.0
a2	1.55	1.92	1.44
k12	0.65	0.65	0.60
k21	0.77	1.12	1.02
k10, p	0.24	0.35	0.24

see text for terms. Collen ref = Collen et al 1972.

Table A 5.3 Fibrinogen survival data, for unoperated control animals.

k10 p is the fractional catabolic rate constant.

The plasma half life compares well with the human half life of 4-5 days (Collen et al 1972, Bradley and Hickman 1975). For post-operative studies this falls to 2.5 days (Hickman 1971). The rate constants suggest the human fibrinogen is handled in a metabolically similar way by the sheep. The difference in C_1 may be due the use of blood counts rather than plasma counts. The rates for k12, k21 and k10 p are similar to those found by others (Collen et al 1972, Bradley and Hickman 1975). From these studies the assumption, that human I-125 fibrinogen is treated in these sheep studies similarly to native sheep fibrinogen, seems justified.

Abbreviations

The page numbers show the location of the definition in the text.

aPTT	activated partial thromboplastin time	45
BEx	Brain extract, principally the lipid PS	61
DAPC	diacetylenic phosphatidyl choline	47
DCP	dicetylphosphate	61
DDA	dodecylamine	61
DMPC	dimyristoyl phosphatidyl choline	47
DMPG	dimyristoyl phosphatidyl glycerol	60
DPPA	dipalmitoyl phosphatidic acid	60
DPPC	dipalmitoyl phosphatidyl choline	47
DPPE	dipalmitoyl phosphatidyl ethanolamine	60
DPPI	dipalmitoyl phosphatidyl inositol	60
DPPS	dipalmitoyl phosphatidyl serine	60
DSPC	distearyl phosphatidyl choline	60
ETO	ethylene oxide	27
eV	electron volts	153
FTIR	fourier transform infrared spectroscopy	15
GM1	This is a negatively charged ganglioside with sialyic acid residues.	60
GPC	glycerophosphatidylcholine	130
IL 1	Interleukin 1	27
KW	Kruskal Wallis, statistical test	178
MTEG	material thrombelastography	52

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MATERIAL THROMBELASTOGRAPHY : AN ASSESSMENT OF PHOSPHORYLCHOLINE COMPOUNDS AS MODELS FOR BIOMATERIALS.

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ABSTRACT

The use of phosphorylcholine (PC) containing compounds as possible biomaterials has been evaluated by material thrombelastography (MTEG). The detailed analysis of the MTEG technique is discussed. A remarkable reduction of thrombogenicity by compounds containing the PC group is demonstrated. The results observed with a polymerised PC-lipid indicate potential use of such substances as biomaterials with minimal thrombogenicity.

INTRODUCTION

Chapman has advocated the concept of the mimicry of biological membranes as a new approach to the problem of bio-incompatibility (1). The phospholipid membrane structure of the red blood cell (RBC) and the platelet is similar, and does not normally activate coagulation in the blood stream. Despite the contrast in their biological functions the major fraction of the lipid exposed at the outer cell surface of both cells is sphingomyelin and phosphatidylcholine. The polar head group of both lipids is phosphorylcholine (PC) and this covers 89% of the outer surface (2). The negatively charged phospholipids are confined to the cytoplasmic surface of the bilayer. This asymmetric distribution of the phospholipids has been well reviewed (1, 3). During the stimulation of platelets the negatively charged phospholipids become exposed, and increase the

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Key words: Thrombelastography, phosphorylcholine, biomaterials.

rate of thrombin formation (4).

Evidence to support the importance of the PC group in reducing thrombogenicity comes from observing the effect on the Styphen test of PC liposomes ie phosphatidylcholine dispersions. These phosphatidylcholine dispersions mimic the outer bio-membrane lipid surfaces of blood cells (5). The Styphen test is sensitive to variations in phospholipid concentration and subclass composition. In contrast to the behaviour of negatively charged lipids the PC liposomes did not shorten the clotting times. Furthermore the rate of clot formation was not affected by increasing the amount of liposome present. Polymerised PC liposomes were observed to behave in the same way. A preliminary investigation of the interaction of photopolymerised liposomes with platelets by Julianno (6) also suggested that polymeric lipids do not interfere with platelet aggregation in vitro.

Following our earlier work (1, 7) our next step in investigating the possible use of the PC group in biomaterials has been to examine the thrombogenicity of these phospholipids by the technique of thrombelastography (TEG)(8). This measures the elastic properties of whole blood clots as they are formed. It uses a steel (V2A) piston placed in a cuvette having a 1mm clearance between the surfaces which contains the blood. The cuvette oscillates through 4^0 45' (1/12 radian) in a 10 second cycle. This gives a shear rate of 0.28 per second. The piston is suspended by a torsion wire which is linked to a recording chart. The torque of the cuvette is transmitted to the piston via the fibrin strands in the blood clot as coagulation proceeds. The resulting trace, the thrombelastogram records the elasticity of the blood clot. A normal TEG is shown in fig. 1, with the standard TEG parameters (9, 10). Hypocoagulation produces a prolonged r and k time, and a reduced ma, e and A^O. Each parameter reflects a different component of whole blood coagulation (9), and the TEG contains additional information compared to the standard laboratory tests (9, 10). The advantage of the TEG is that it keeps the blood at 37C., and due to the paraffin film covering the blood, the sample is not exposed to the atmosphere and resultant changes of pH. It is a dynamic test in which the material surfaces are in intimate contact with blood.

If the blood samples are kept constant then the coating of the piston and cuvette on one channel of the TEG can be varied and its thrombogenicity studied - the technique of material thrombelastography MTEG (12). The ratio of the TEG parameters from the treated and the untreated channels provides the basis for the comparison of the different materials. An ideal nonthrombogenic surface would cause minimal activation of clotting shown as a very long r and k time with a low A^O, ma and e. The ratios are expressed as test substance over the control and such a surface would have an r_t/r_0 and k_t/k_0 approaching infinity while $A^O{}_t/A^O{}_0$, ma_t/ma_0, and e_t/e_0 approach zero. Each TEG parameter has been related to different components of whole blood coagulation (10). The changes in the test channel TEG

shape indicate which component has been activated or left unaffected. The use of the ratio excludes extraneous effects, and it's deviation away from unity indicates both the component of whole blood coagulation affected and the extent of activation. Carr (13) stressed the advantage of using whole blood with all its interactions during TEG over the other in vitro tests using components such as platelets or fibrinogen for examining the thrombogenicity of materials. However he did not report the direct MTEG ratios used by Affeld.

To avoid confusion, we suggest restricting the term material thrombelastography (MTEG) to a direct comparison between the test and control channels conducted with the same blood sample, at the same time. We have used the suffix '$_o$' to denote the control channel TEG results (eg r_o) and the suffix '$_t$' for the test channel (eg r_t), as earlier authors (12, 14, 15) used '$_o$' for metal cuvettes and the suffix '$_{test}$' for coated surfaces. The ratio of the TEG parameter (eg r_t/r_o), the MTEG parameter, we have indicated as :- Mr, Mk, Mma, Me, MA^o, and Mα. Capitals such as R and K have been used by other authors for the standard TEG parameters (10). In addition the prefix 'M' relates the 'M'TEG parameter to the standard TEG results without renaming them as Affeld or Schulze (12, 14) who used T to denote the reaction time, and A for the maximum amplitude. This avoids the risk of confusion with the A^o as the angle. We have used A^o as the angle from the first movement of the trace to the 'shoulders' of the trace, as used by Carr, and not the maximum slope (SL) used by Affeld, or Schulze's term S (equal to tanα). For the maximum slope we have used the conventional symbol α.

This work, using human blood, is the first report of the effect of dipalmitoylphosphatidylcholine (DPPC), and diacetylenicphosphatidylcholine (DAPC) on MTEG.

METHODS

MTEG Technique

Blood was taken from a panel of volunteers involved with the project who were not taking any medication. The venepuncture was performed using a 21 gauge butterfly (M Abbott, Medical Systems) from an uncuffed arm vein using the two syringe technique (this eliminates the effect of tissue thromboplastin released on venepuncture). 4.5 mls of blood was mixed with 0.5 mls of a 3.8% sodium citrate solution. MTEG was performed using 0.3 mls of citrated blood recalcified with 60 µl of a 1.29% calcium chloride solution. The electronic thrombelastograph (type D, PPG Hellige) with two channels was used throughout. Each pair of pistons was checked for variation before one of the pistons was coated with the test substance. Each material was tested on ten occasions with blood from at least three volunteers. The piston and metal cuvettes were cleaned after each test using a 2% solution of detergent (RBS-PF Chemical Concentrates Ltd) and washed several times with distilled water, drying with tissue paper between each washing.

MTEG Analysis

The standard TEG parameters (r, k, ma, e, A^o, and α) were meas-

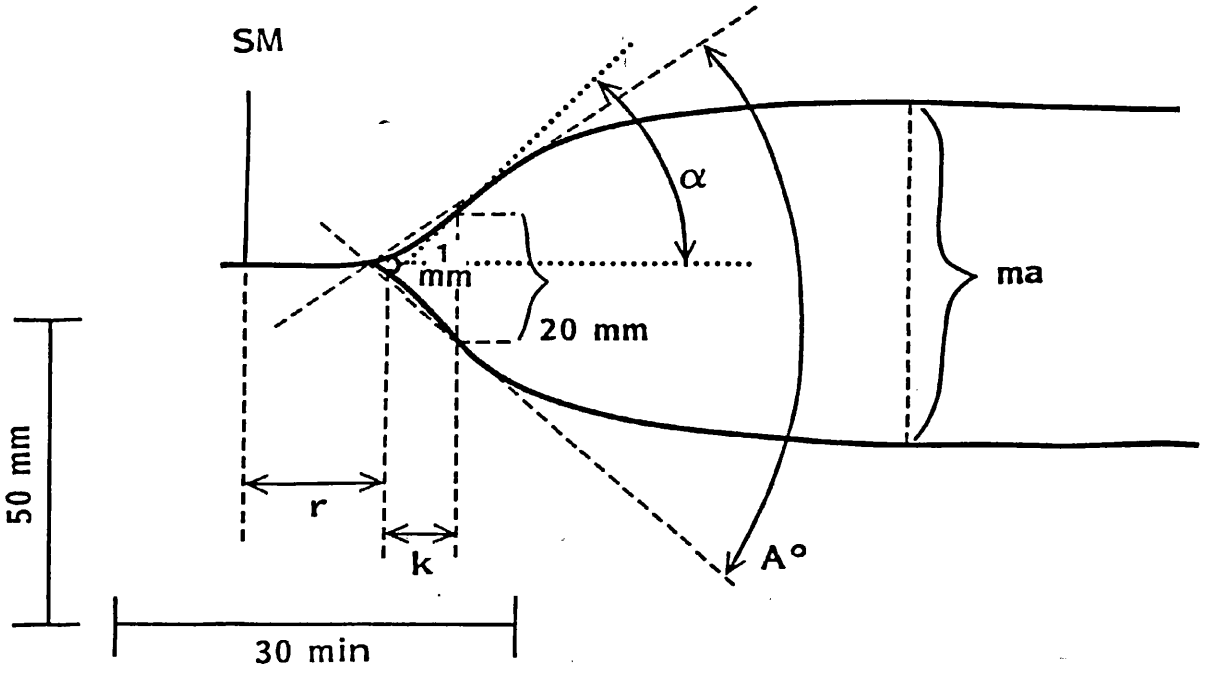

FIGURE 1.
A Typical Thrombelastogram, With The TEG Parameters Shown. SM=start mark, r=reaction time, k=clotting speed, ma=maximum amplitude, e=100ma/100-ma A^{o}=angle between the first movement of the trace and both 'shoulders' of the trace, α the maximum slope of one 'shoulder'.

ured by vernier calliper and all the results expressed in millimetres (to convert to time divide by 2 and express the result in mins.). These are shown in fig 1. 'Retraction' and 'break-off' are well documented problems using plastic cuvettes (16), particularly in the clinical conditions of leukocytosis and uraemia. However in all these cases the initial part of the tracing is unaffected. Such effects are also seen with dirty equipment. Tracings which demonstrated these features were excluded from our analysis. The ratio of treated/untreated parameters, the MTEG parameters, was calculated and the geometric means with 95% confidence limits obtained. This eliminates the variation due to differences in the absolute values of each parameter due to the blood sample and corrects for the skew distribution introduced by using a ratio. 'p' was calculated using a paired 't' test on the geometric data, except when k was infinite when a Wilcoxon rank sign test was used on the Mk values. This rather rigorous analysis was used (despite the sometimes obvious nature of some of the results) to exclude errors due to differences between the untreated pins, and to detect small differences in the control tests. In addition the A^{0} and maximum slope, α (the usual notation for Affeld's term SL), have been compared for each TEG trace and for the MTEG parameters MA^{0} and Mα by correlation coefficients. All the statistics were compiled using PC Minitab on an IBM AT.

Materials.

Dipalmitoylphosphatidylcholine (DPPC), (Sigma)
Diacetylenicphosphatidylcholine (1,2, Dipentacosa 10-12, '10-'12 tetrayne glycerophosphorylcholine) (DAPC),
Octacosane -C28, a hydrocarbon, and
liposomes made with DPPC.

The test piston and cuvette were dip coated with solutions containing 0.5 mg/ml of the lipid in ethanol and evaporated to dryness under a stream of nitrogen. 0.6 ml was used for the cuvette, and 0.5 ml for the piston which was coated during rotation in a small glass tube. Four dilutions of DPPC were tested (0.5, 0.25, 0.125, and 0.05 mg/ml). The final concentration in the cuvette was 80 $\mu g/cm^2$ for 0.5mg/ml. DPPC liposomes were made by dissolving 15mg of DPPC in a few drops of ethanol and evaporated under a stream of nitrogen to form a thin film on the bottom of a test tube. 3ml of the 1.29% $CaCl_2$ solution was added and vortexed at 60^0C for 15 minutes until a colourless solution was obtained. 60 µl of this $CaCl_2$ liposome was mixed with blood as a control experiment to assess the effect of free DPPC on the TEG. Octacosane -C28 was coated in a similar way as the DPPC. DAPC was coated in the same way on to a piston and plastic cuvettes and then photopolymerised by exposure to UV light (Mineralight R-54 lamp radiation intensity at 254 nm) for 20 minutes. The results from control tests (standardisation) and from the coating of DPPC using plastic disposable cuvettes were compared with the results using metal (V2A steel) cuvettes.

RESULTS

These are given as geometric mean (with lower and upper 95 % confidence limits). The 'p' value is for a paired 't'test, unless a Wilcoxon rank sign test was more appropriate and this is indicated by #. Degrees of significance are indicated thus :- $p < 0.05$=*, < 0.01=**, < 0.001=***, < 0.0001=****.

Six pairs of pins were used and each pair was checked for differences prior to use (standardisation). There were no significant differences from unity in the results at the 0.05 level, except for the Mma and Me value of one pair of pins. This may have arisen by chance since 63 sets of control data were generated. In this case the test piston was slightly more thrombogenic (Mma =1.02, p=0.042). During the test it became less thrombogenic as a result of coating with DAPC (see results below). Typical data for metal and plastic cuvettes is shown in table 1 and figure 2 respectively. The standard TEG parameters varied between samples from the same individual, but the variation was less than between individuals. The experimental design of MTEG eliminates the effect of these variations on the test since the same blood sample is used in both channels. There were no significant differences in the MTEG parameters between the plastic and metal cuvettes.

MA^0 and Mα were closely correlated with correlation coefficients of $r > 0.8$, $p<0.01$, except for two results. These were

TABLE 1.
Results For Standardisation With Metal Cuvettes.

Parameter	Mean	95% con. limits		p value	N=8
Mr	1.028	0.990	1.067	0.19	
Mk	1.107	1.006	1.216	0.07	
Mma	0.998	0.927	1.078	0.96	
Me	0.995	0.843	1.173	0.95	
MA^{o}	0.997	0.922	1.035	0.45	
Mα	1.023	0.996	1.065	0.12	

for the standardisation on metal cuvettes and for the coating of octacosane, where r was 0.630 and 0.633 respectively ($0.05<p<0.1$). They both had data tightly clustered around 1, with smaller groups (N=8). This may account for the apparent lack of correlation, particularly since the TEG parameters were closely related. A^0 was correlated with α for each set of data and there was a very close correlation; $r > 0.9$ and $p << 0.0001$. A^0 has the advantage of using both shoulders of the trace and is easier to measure accurately, whereas α may be different for each half of the trace. The close correlation of the two makes α redundant. The figures show typical traces for the test trace with each compound, and the results beneath. Figure 1 is based on a normal control trace, and figure 2 shows a normal TEG for an untreated plastic cuvette; the data is from the standardisation reported with the figure.

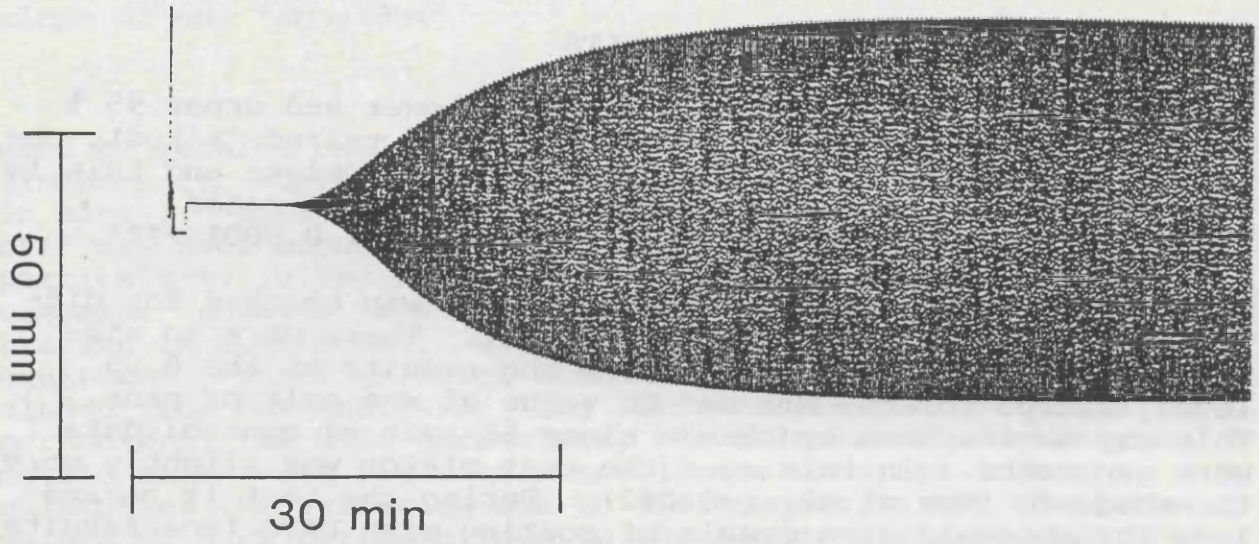

FIGURE 2.
A Typical TEG Trace For An Untreated Plastic Cuvette, With The Results For Standardisation With Plastic Cuvettes.

Parameter	Mean	95% con. limits		p value	N=10
Mr	0.964	0.890	1.044	0.38	
Mk	1.053	0.959	1.157	0.30	
Mma	1.016	0.989	1.045	0.27	
Me	1.033	0.976	1.094	0.28	
MA^{o}	0.961	0.893	1.034	0.30	
Mα	0.978	0.913	1.047	0.53	

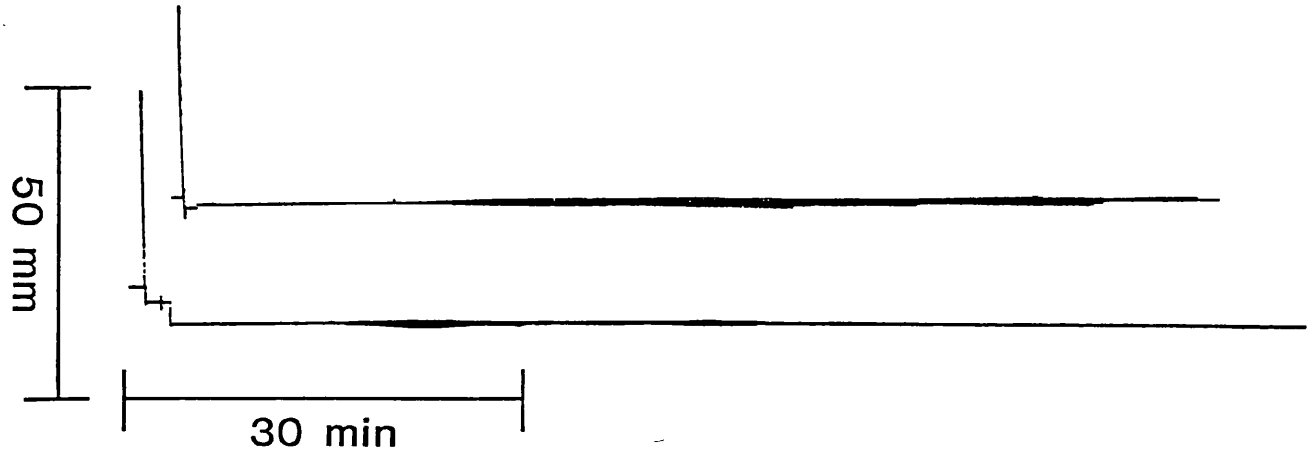

FIGURE 3.

Results For DPPC On Plastic Cuvettes. (upper trace) N=9

Parameter	Mean	95% con. limits		p value	
Mr	1.628	1.395	1.900	<0.0001	****
Mk	#			0.009	**
Mma	0.056	0.021	0.033	<0.0001	****
Me	0.026	0.021	0.033	<0.0001	****
MA^{o}	0.143	0.087	0.235	<0.0001	****
Mα	0.160	0.107	0.240	<0.0001	****

Results For DPPC On Metal Cuvettes. (lower trace) N=12

Mr	1.972	1.477	2.634	<0.0001	****
Mk	#			0.003	**
Mma	0.062	0.046	0.082	<0.0001	****
Me	0.031	0.022	0.043	<0.0001	****
MA^{o}	0.108	0.077	0.151	<0.0001	****
Mα	0.095	0.073	0.125	<0.0001	****

all the values were infinite.

DPPC was coated on both plastic and metal cuvettes (fig. 3). The results were very similar for both surfaces and show that DPPC is effective in reducing the activation of both the clotting factors and platelets. The higher Mr value for metal cuvettes is not a statistically significant improvement on the result for plastic (1.97 vs 1.67, using a two sample t test p was 0.26), but may reflect the better wetting of this surface with the ethanol/DPPC mixture. Increasing dilutions of PC gave similar results, until 0.05mg/ml (ie at 1/10th concentration).

Mechanism of action

The hydrocarbon octacosane (C28) was used to show the effect of the lipophillic hydrocarbon moiety of PC rather than the polar head group (fig. 4). The result showed small improvements in Mr, Mk, and MA^{o} which may be explained by covering any imperfections on the pin surface. The improvement is only 10% and 16% and not as much as DPPC, which doubled Mr with infinite Mk values.

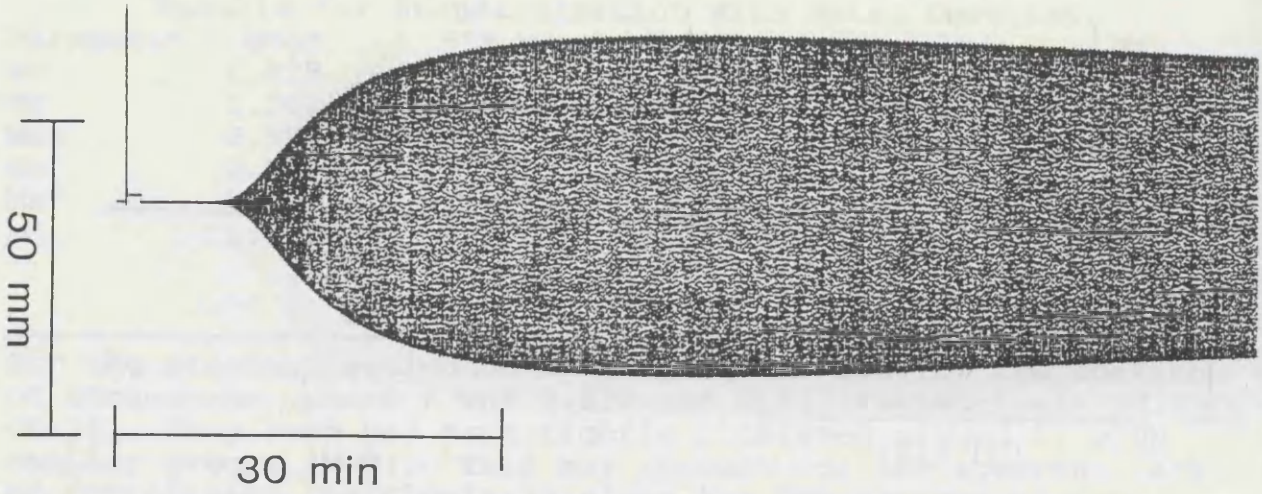

FIGURE 4.
Results For Octacosane.

Parameter	Mean	95% con. limits		p value	N=8
Mr	1.108	1.021	1.203	0.041	*
Mk	1.164	1.093	1.242	<0.0001	****
Mma	0.972	0.902	1.049	0.48	
Me	0.931	0.777	1.116	0.46	
MA^{O}	0.867	0.812	0.927	0.0037	**
Mα	0.933	0.884	0.984	0.034	*

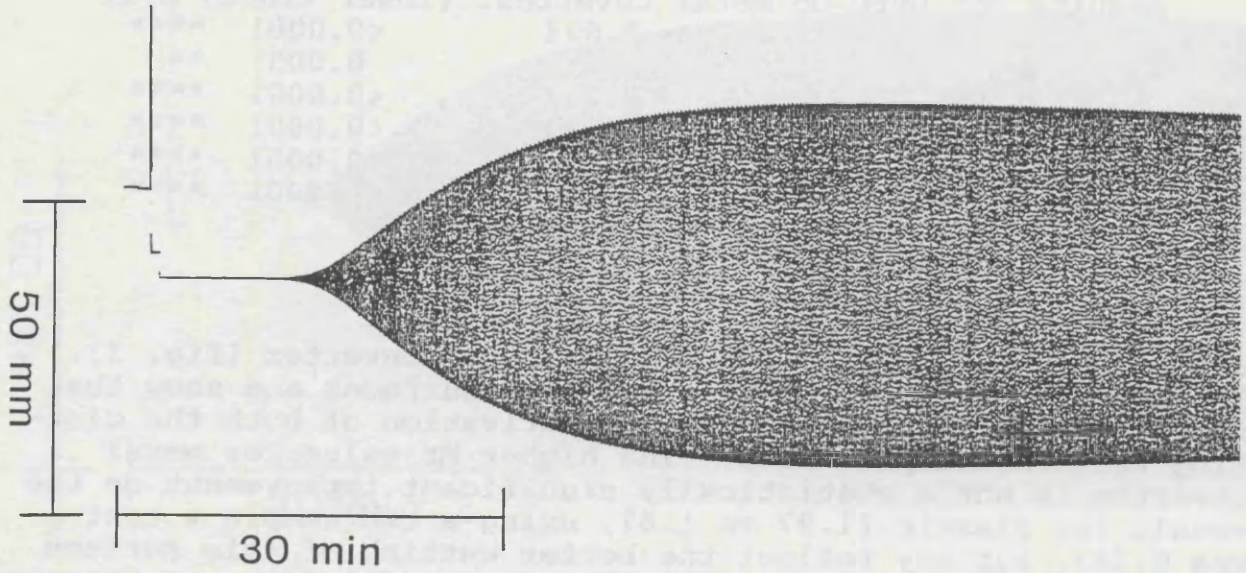

FIGURE 5.
Results For DPPC Liposomes Added To Blood Sample.

Parameter	Mean	95% con. limits		p value	N=13
Mr	0.963	0.874	1.062	0.46	
Mk	0.957	0.857	1.069	0.45	
Mma	1.089	1.046	1.134	0.001	***
Me	1.202	1.103	1.310	0.001	***
MA^{O}	1.076	0.980	1.181	0.14	
Mα	1.086	1.008	1.171	0.046	*

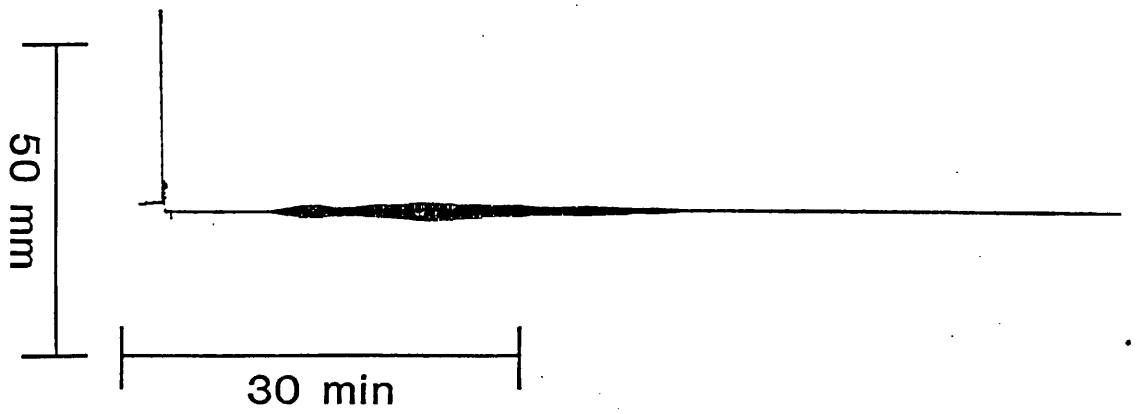

FIGURE 6.
Results For Polymerised DAPC.

Parameter	Mean	95% con. limits		p value	N=10
Mr	1.102	0.989	1.228	0.10	
Mk	#			0.006	**
Mma	0.121	0.074	0.197	<0.0001	****
Me	0.060	0.035	0.102	<0.0001	****
MA^{o}	0.321	0.171	0.603	0.0057	**
Mα	0.429	0.272	0.666	0.004	**

all the results were infinite.

The liposomes show a slightly increased Mma (1.089) and Me (1.202) which is significant ($p<0.001$). This reflects activation of platelets or fibrinogen to produce a stronger clot (fig. 5). It shows there is no direct action of DPPC on the blood sample, say by dissolving in it, to account for the reduced clot elasticity. This confirms that the reduction in thrombogenicity is due to protection of the surface by the PC coating.

The results for DAPC after polymerisation (photopolymerised PC), are comparable to the DPPC results (fig. 6), except for the Mr value. We found this coating was so stable that measurements were made 3 or 4 times without recoating, and this may explain the difference from DPPC.

DISCUSSION

Since Hartert introduced thrombelastography in 1948 (8) it has been used to examine coagulation in a wide range of clinical and research settings and the TEG has been demonstrated to be a sensitive method of assessing coagulation and has advantages over other methods (9, 10). It's use as a screening test for biomaterials was suggested by Affeld (12) who demonstrated the thrombogenicity of Dacron flock, and the benefit of polyethenglycol 1000 coatings. Carr (13) examined the effect of pin diameter on the TEG parameters. He argued that the thickness of the test coating needed for some biomaterials could affect

the result. The effect of a thick coating is to increase the apparent thrombogenicity of a test material by narrowing the gap between the piston and cuvette. While he did not report the number, mean, standard error of the means or the MTEG ratios of his observations, he advocated the TEG technique compared to 'blood component' tests. The materials we employed as coatings were barely visible. Although it was not possible to measure the thickness of the coating, none of the results showed increased thrombogenicity (which could be attributed to a thick coating narrowing the gap). The need for the use of non standard pin sizes can be circumvented by using thin films, as we have shown.

Previous workers have highlighted the pitfalls of using animal blood for MTEG (14), and the NIH recommend the use of human blood in biomaterial research (17). Lemm (15) reviewed the methods available for testing the haemocompatibility of synthetic materials, and he strongly advocated material thrombelastography (MTEG) as quick and simple, providing "a remarkable correlation between the in vitro results and the in vivo behaviour". We believe material thrombelastography has been an underused technique for screening blood-material interactions.

This work demonstrates the reproducibility of the MTEG results, even on different surfaces, and demonstrates the variation between untreated pins (table 1 & figure 2). The finding in the control experiments of a difference between pins shows the importance of detailed statistical analysis to eliminate bias. In addition we have shown the advantage of a rigorous statistical analysis of a series of traces (eg the control and hydrocarbon results) in detecting small but significant differences in the effects of the coating materials.

The addition of liposomes to the blood was used to exclude the possibility that some of the DPPC coating might be released into the blood forming liposomes thereby acting directly on the blood to reduce clot strength. The observation of a slightly stronger clot (the Mma was 1.089) reflecting platelet and fibrinogen activation is interesting. This may be due to the liposomes occupying a volume of 0.06 ml in the total sample of 0.36 ml, resulting in relative concentration of the fibrin strands in the gap between the piston and the cuvette compared to the control channel. Liposomes of DPPC are known not to bind proteins to any extent (18). However once the untreated surfaces present generate activated clotting complexes, particularly Va and Xa which have a requirement for phospholipids, the liposomes may have a role in leading to a more uniform activation of the fibrin strands.

To check that we were obtaining the desired orientation of the polar head groups with respect to the surface (ie with the polar groups in contact with the blood, and the hydrocarbon chains in contact with the surfaces and so hidden from the blood), we carried out experiments to see the effect of coatings of octacosane. The results obtained with octacosane support our conclusion about the orientation of the polar head

group, since there is little improvement with this hydrophobic surface (Mr increased by 10%). Since the plastic and V2A steel used for the pistons and metal cuvettes are non wettable and hydrophobic (9, 16) it is likely that the hydrophobic lipid groups are naturally aligned to leave the polar group facing the blood sample. For the DPPC coating we stored the test cuvette and piston in water prior to the test, to further encourage the hydrophobic lipid chains to re-orient themselves away from the blood sample (this made no difference to the results).

The reduction in thrombogenicity observed with both DPPC and DAPC is much larger than that seen with the hydrophobic surfaces (the plastic and octacosane). These results clearly highlight the importance of the PC head group as the effective part of the phospholipid molecule in preventing the activation of coagulation, rather than the lipophillic side chain. The TEG parameters r and k reflect the activity of clotting factors and the prolongation of Mr and Mk implies reduced activation and a reduced rate of thrombin formation by the test surface. DPPC almost doubles the Mr, and the Mk value is infinite indicating low levels of activation. Similarly the reduction of Mma for DPPC to 6% of normal is particularly significant since ma is largely determined by platelets. This result indicates that few of the platelets present are activated by the coating.

Our observations indicate the importance for normal haemostasis of the asymmetric distribution of PC in the RBC and platelet membrane (2, 3). The preponderance of PC in the outer surface of the RBC membrane led Chapman (1, 5) to suggest the use of PC as a biomaterial to mimic biological membranes. Our main findings using MTEG are that DPPC reduces the thrombogenicity of both metal and plastic. This appears to be by reducing the activation of clotting factors and platelets. The low platelet activation seen with DPPC is important because platelet activation is thought to be the primary event initiating thrombosis of grafts in the arterial tree. That this benefit is also seen with DAPC, a polymer containing PC, has exciting implications for the development of new biomaterials, and further work is planned.

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New haemocompatible polymers assessed by thrombelastography

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ABSTRACT

Mimicry of the nonthrombogenic surface of the erythrocyte has been advocated as the starting point for the development of nonthrombogenic biomaterials. Phosphorylcholine forms 88% of the outer surface of the erythrocyte, and so materials containing it should be nonthrombogenic. We have evaluated the thrombogenicity of such materials and compared them with Dacron and PTFE. Three materials containing phosphorylcholine were used: a naturally occurring phospholipid (dipalmitoyl-phosphatidylcholine, DPPC), a polymerized phospholipid (diacetylenic phosphatidylcholine, DAPC) and a polyester polyurethane (Polyester G). The thrombogenic potential of these materials was assessed by material thrombelastography (MTEG). This technique uses human whole blood at 37°C, without an air interface, and records the elasticity of the blood clot produced. Since each material is evaluated with a control surface, extraneous differences due to factors other than the test material are eliminated. Analysis, and examples of the MTEG traces are shown and discussed. The phosphorylcholine containing materials were found to have a reduced amount of clotting factor activation, but only DPPC was better than Dacron and PTFE. MTEG demonstrated the known thrombogenicity of Dacron towards platelets. A striking reduction in platelet activation was shown for the three phosphorylcholine containing materials. These three materials show only 25% of the platelet activation of PTFE. These materials warrant further investigation as potentially very useful biomaterials.

Keywords: Biomaterials, material thrombelastography, biomembrane mimicry, phosphorylcholine

INTRODUCTION

All currently used prosthetic materials interact with blood, and cause the failure of the device or activate coagulation. Hayward and Chapman have advocated a new approach to this problem, that is of mimicking the thromboresistant nature of the red blood cell surface[1]. Mammalian cells contain over 100 distinctly different lipids due to variations in the acyl chains and polar head groups. The asymmetric distribution of the phospholipids in the erythrocyte and platelet membrane has been well reviewed[1,2]. In the outer membrane of these cells, phosphorylcholine (PC) is the prominent head group (88%), followed by phosphorylethanolamine (PE). PC is present in sphingomyelin and lecithin and contributes to the interfacial properties and thromboresistant nature of the erythrocyte outer surface[1,3]. The inner membrane surface is mainly composed of negatively charged lipids, e.g. phosphorylserine (PS), and is thrombogenic. During the stimulation of platelets the negatively charged phospholipids become exposed, and increase the rate of thrombin formation[4]. Zwaal *et al.*[3] demonstrated a marked difference in the blood clotting ability of the inner and outer halves of the red cell membrane. Right-side out red cell ghosts do not reduce the blank clotting time of the Stypven test, whereas non-resealed ghosts (i.e. the inner surface) reduce the clotting time in a concentration dependent manner.

Evidence to support the importance of the PC group in reducing thrombogenicity comes from observing the effect on the Stypven test of PC liposomes, which are phosphatidylcholine dispersions. These phosphatidylcholine dispersions mimic the outer biomembrane lipid surfaces of red blood cells. The Stypven test is sensitive to variations in phospholipid concentration and subclass composition. In contrast to the behaviour of negatively charged lipids, the PC liposomes did not shorten the clotting times. Furthermore, the rate of clot formation was not affected by increasing the amount of liposome present. Polymerized PC liposomes were observed to behave in the same way[1].

Juliano *et al.*[5] examined the effects of conventional phospholipid liposomes and polymerized methacryolic liposomes containing PC head groups on platelet aggregation. These preliminary studies indicated that both non-polymerized and polymerized PC liposomes had a negligible effect on platelet aggregation. However, charged vesicles can interact directly or indirectly to reduce the platelet response to agonists such as ADP and thrombin. Bonte *et al.*[6] also recently demonstrated that liposomes made

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contained the PC polar head group, bound less serum proteins than liposomes with negatively charged lipids.

MATERIALS AND METHODS

There are a large number of *in vitro* tests for the assessment of blood material interactions, and we favour a technique which uses whole blood and still allows evaluation of the components involved in clot formation. We have used a modification of thrombelastography to do this[7,8]. In normal clinical use the thrombelastograph (TEG) measures the shear elasticity of the whole blood clots as they are formed. It uses a stainless steel (Krupp's steel type — V2A) piston placed in a cuvette (having a 1 mm clearance between the surfaces) which contains the blood. The cuvette oscillates through 4° 45′ (1/12 radian) with a cycle time of 10 s. This gives a shear rate of 0.28 per s. The piston is suspended by a torsion wire which is linked to a recording chart. The torque of the cuvette is transmitted to the piston via the fibrin strands in the blood clot as coagulation proceeds (*Figure 1*). The resulting trace, the thrombelastogram, records the elasticity of the blood clot. It has been widely used in many clinical settings for the diagnosis of blood disorders[9] since it was first described by Hartert[10].

Since modern versions of the TEG have two channels, one set of surfaces can be coated by a test material. The thrombogenicity of the coating material can then be assessed because the same normal blood is used in both channels. This modification of the technique is known as material thrombelastography (MTEG)[11,12]. The traces from each channel are analysed using the standard TEG parameters *r* and *ma* (shown in *Figure 2*), and the results for each channel are compared. The ratio of the TEG parameters for the treated/untreated surface should be 1 if there is no difference in thrombogenicity of the coating from the steel control surface. The ratios of the standard TEG parameters, known as the MTEG parameters, are denoted by the prefix 'M' (i.e. M*r* and M*ma*). They indicate the degree to which each aspect of coagulation is affected. An ideal nonthrombogenic surface would cause minimal activation of clotting shown as an extended *r* and minimal platelet activation, shown by a low *ma* so

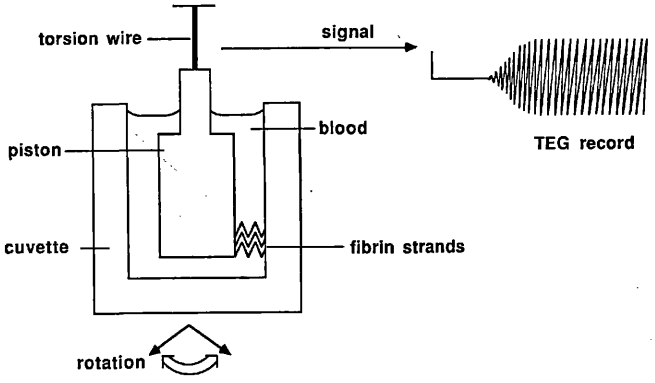

Figure 1 The principle of thrombelastography

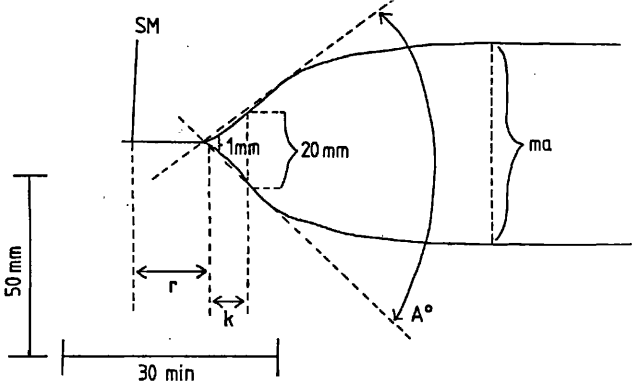

Figure 2 Analysis of the thrombelastogram. This is a normal trace with the standard TEG parameters: SM = start mark, *r* = reaction time, *k* = clotting speed, *ma* = maximum amplitude, *A*° = rate of clot formation

that the MTEG ratios, M*r* and M*ma* would be infinite and zero respectively. We have shown the value of detailed statistical analysis of MTEG[7,8].

The advantages of the MTEG are that it keeps the blood at 37°C, and because the blood is covered by a paraffin film, the sample is not exposed to the atmosphere and resultant changes in pH. It is a dynamic test in which the material surfaces are in intimate contact with blood.

Blood was withdrawn from an arm vein of a healthy volunteer using a 21 gauge butterfly needle. The first 2 ml were discarded to remove any effect due to tissue thromboplastin released on venepuncture. Then 5 ml of blood was withdrawn into a second syringe, transferred to a sterile tube containing 0.5 ml of 3.8% sodium citrate as anticoagulant, and mixed gently. Citrated whole blood (0.3 ml) was placed into the cuvette and 60 μl of 1.29% calcium chloride solution added to recalcify the blood and initiate coagulation.

MTEG was performed using either polystyrol 168N (BASF) disposable plastic cuvettes or stainless steel (V2A) cuvettes, with stainless steel pistons. The samples were prepared by dip coating and solvent evaporation, except for polytetrafluoroethylene (PTFE) which was machined from solid medical grade PTFE (Fibracon Twin Ltd). For Dacron, a solution of Melinex ICI (103 mg/ml in 1,1,1,3,3,3-hexafluoropropanol) was used. These currently used materials were compared with three materials containing the phosphorylcholine head group: DPPC (dipalmitoylphosphatidylcholine-Sigma), a naturally occurring phospholipid, DAPC (diacetylenic-phosphatidylcholine: 1,2-dipentacosa 10–12, 10–12 tetrayne glycerophosphorylcholine – a new polymerizable phospholipid with a structure close to DPPC) and a new polyester polyurethane (Polyester G).

RESULTS AND DISCUSSION

Typical traces obtained for Dacron, DPPC, and Polyester G are shown in *Figure 3*. The diagram in *Figure 2* shows the outline of a normal TEG trace, and the trace for Dacron is very similar. However M*r* for Dacron is 1.15 (*Table 1* and *Figure 4*) which is

significantly greater than a ratio of 1. This indicates that Dacron activates clotting factors less than the control surface. Only DPPC, the naturally occurring phospholipid, shows any real prolongation of clotting (M*r* 1.97). The DAPC was not recoated for each test, since we were interested in the durability of the coating, and this may explain the apparently poor M*r* value. PTFE, although hydrophobic, still adsorbs a layer of protein from blood within minutes of implantation. Eventually it develops a layer of swept fibrin, with or without a cellular covering[13]. The M*r* values for Dacron and PTFE are similar, and Polyester G is not significantly better than these conventional biomaterials. All the results for the clotting factor activation parameter M*r* are probably due to protein adsorption and subsequent activation of clotting factors.

The results for M*ma*, the parameter indicating platelet activation, are particularly important since platelets play a major role in arterial thrombosis, and most vascular prosthesis carry arterial blood. Dacron is known to be thrombogenic towards platelets and as an arterial graft it remains unhealed in man[14] and thrombogenic for years[15]. The degree of platelet activation for Dacron (M*ma* = 1.03) is not statistically different from 1 (the control surface) and is therefore highly thrombogenic. The PTFE graft, on the other hand is smooth, and platelet activation results only from the turbulent flow at the anastomosis between the artery and the prosthesis. The low M*ma* value of 0.51 shows a marked reduction of platelet activation. Clearly the surface characteristics of the coating left by solvent casting for Dacron and the machined PTFE surface, are different from the surfaces of the prosthesis. Despite this the MTEG results for Dacron and PTFE agree with what is known about these materials.

The PC containing compounds all demonstrate a low M*ma*, with little platelet activation. This confirms the work of Juliano *et al.*, who demonstrated little protein adsorption, and no platelet activation by DPPC liposomes, and of Hayward and Chapman[1] with dimyristoylphosphatidylcholine (DMPC) and DAPC liposomes in the Stypven test. The M*ma* of the PC containing materials is only 15% of the control and Dacron, and 25% of the value for PTFE. These results clearly suggest that the new approach to prosthetic material thrombogenicity has yielded a useful start.

Although DPPC lacks the stability for long term implantation, and it has none of the physical properties needed by a biomaterial, the results confirm the nonthrombogenic nature of the PC group. DAPC is much more stable, particularly in liposomes, and it may be useful as a surface coating. Polyester G requires further chemical development to improve its rheological and physical properties, but again

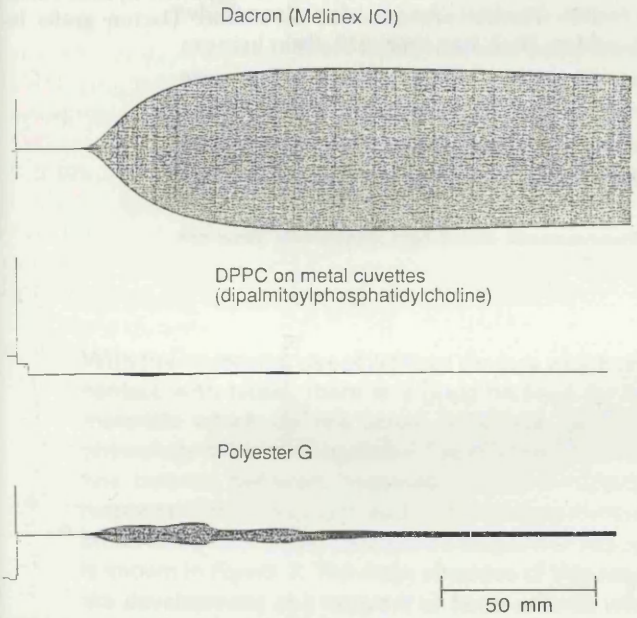

Figure 3 Typical material thrombelastographic (MTEG) test traces for each material. The trace runs at 2 mm per min

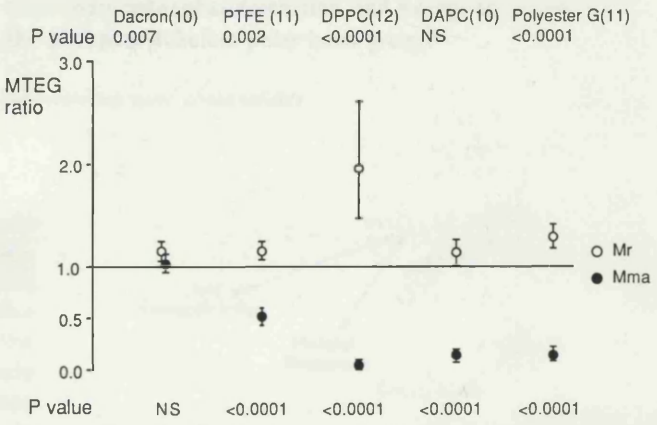

Figure 4 The results for M*r* (clotting factors) shown in open circles, and M*ma* (platelet activation) shown in closed circles. Error bars show 95% confidence limits about the geometric mean

TABLE 1 Material thrombelastography results for phosphorylcholine containing compounds and currently used biomaterials

Material	(N)	Clotting factor (M*r*)	Significance (*P*)	Platelet activation) (M*ma*)	Significance (*P*)
DACRON	(10)	1.15	0.007	1.03	ns
PTFE	(11)	1.17	0.002	0.51	<0.0001
DPPC	(12)	1.97	<0.0001	0.06	<0.0001
DAPC	(10)	1.10	ns	0.12	<0.0001
Polyester G	(11)	1.29	<0.0001	0.14	<0.0001

M*r* is high for a low degree of activation while M*ma* would be low for a good material. Values shown are the geometric means, with *p* values for paired *t* test.

demonstrates the nonthrombogenic qualities of the incorporated PC groups. Obviously further *in vitro* and *in vivo* experiments are needed to confirm the promise of these findings. This type of material shows great promise for a new generation of biomaterials.

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Biomembranes as models for polymer surfaces

V. Thrombelastographic studies of polymeric lipids and polyesters*

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Our approach to the design of haemocompatible biomaterials is based upon the concept that coating a polymer or metal surface with phosphatidylcholine polar groups (corresponding to the major phospholipid of the human erythrocyte outer cell membrane) will improve their haemocompatibility. We have examined the effect on blood coagulation of a number of substrates: those normally used in prosthetic devices such as polyethylene terephthalate (Dacron®), expanded polytetrafluoroethylene, silicone and new polymers which contain the phosphatidylcholine head group (phosphorylcholine). The effect on coagulation of blood exposed to these substrates was determined by the technique of material thrombelastography, a relatively new method for the *in vitro* screening of biomaterial thrombogenicity. The results obtained with Dacron®, polytetrafluoroethylene and silicone are compared with those obtained with a phospholipid–dipalmitoylphosphatidylcholine, a polymerized phospholipid–diacetylenicphosphatidylcholine, and a range of recently synthesized polyesters, each of which contains the phosphorylcholine polar head group.

Keywords: Biomaterials, membranes, haemocompatibility, thrombelastography, phospholipids

With the increasing use of artificial devices which come into contact with blood, there is a great demand for new biomaterials which do not cause thrombus formation. The physiology of blood coagulation has developed to maintain a fine balance between haemostasis and thrombosis. The response of the blood to vascular tissue injury involves many blood components and a simplified diagram of this response is shown in *Figure 1*. The main objective of this response is the development of a network of fibrin strands which trap red and white blood cells and aggregate platelets resulting in the formation of a haemostatic plug, which blocks the injured blood vessel to prevent further blood loss.

The exposure of the body to foreign materials has many effects in activating the hosts's defences including the activation of blood coagulation[1]. This complex series of events is generally thought to be initiated by the rapid adsorption of plasma proteins, followed by platelet adhesion and aggregation. At the surface the blood coagulation cascade is activated resulting in the generation of thrombin, which activates platelets and cleaves fibrinogen[2]. However, the exact sequence of events which occur at the blood–material interface is still not fully understood and this problem has hindered the development of blood compatible materials.

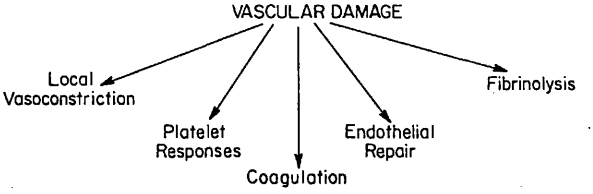

Figure 1 Response to vascular damage. This figure was first published in reference 8.

A number of attempts have been made to create non-thrombogenic surfaces. For example, the immobilization of antithrombogenic groups such as heparin has been shown to reduce the thrombogenicity of some polymers[3] but causes haemolysis[4], and covalently bound urokinase has been demonstrated to show fibrinolytic activity[5]. The pre-adsorption on the surface with these and other anti-thrombogenic moieties may be limited by their gradual desorption[6].

Our approach to solving this problem is to mimic the surface of biological membranes, at least in a simple form. Biological membranes are composed of a fluid lipid bilayer

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*This paper is the fifth contribution in a series on membrane-mimetic biomaterials: references 8, 26–28. Parts of this work were presented at the Biological Engineering Society annual meeting, Salford, UK, September 1988 and at the PIMS conference, The Netherlands, April 1989.

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proteins determine the functional and structural properties of the cell, such as transport and cell shape. Mammalian cells may contain over 100 distinctly different lipids, due to variations in the acyl chains and polar head groups. In the erythrocyte outer membrane, phosphorylcholine (PC) is the most prominent head group (88%), followed by phosphorylethanolamine[7]. PC is present in sphingomyelin and lecithin and contributes to the interfacial properties of the erythrocyte outer surface[8]. The inner membrane surface is mainly composed of negatively charged lipids. Zwaal *et al.*[9] demonstrated the difference in the blood-clotting ability of the two membrane surfaces. Right-side-out red cell ghosts did not reduce the blank clotting time of the Stypven test, whereas non-resealed ghosts reduced the clotting time in a concentration-dependent manner. Bonté *et al.*[10] also demonstrated recently that liposomes made from a conventional lipid and a dilipoyl lipid which contained the PC polar head group bound fewer serum proteins than liposomes with negatively charged lipids.

A number of *in vitro* methods have been developed to assess the haemocompatibility of novel biomaterials. These tests are mainly concerned with the effect of polymers on thrombus formation[1], protein adsorption[11-13], cell adhesion[14], and platelet function[15,16]. However, no technique available measures the effect of polymers on all aspects of blood coagulation simultaneously.

The thrombelastograph (TEG) is a mechanically operated system which provides a continuous dynamic measure of blood coagulation. This technique was designed and developed by Hartert[17] in the late 1940s. Since then, it has been widely used in many clinical settings for the diagnosis of blood disorders[18]. The TEG technique was later developed to test the influence of materials on blood coagulation, material thrombelastography (MTEG)[19,20]. We have shown the value of detailed statistical analysis of the MTEG technique[21].

In this paper we describe the use of MTEG to examine the blood compatibility of a number of biomaterials (Dacron®, polytetrafluoroethylene (PTFE) and silicone). The results were compared with those obtained with a phospholipid, a polymerized lipid and recently synthesized novel polyesters all containing the PC group.

EXPERIMENTAL

Thrombelastographic studies

Thrombelastography was carried out with a type D TEG manufactured by PPG-Hellige (Finchhampstead, Berks, UK). The basis of the technique involves a cuvette containing blood, which oscillates over an angle 4° 45′ in 10 s intervals, into which a piston suspended by a torsion wire is lowered. The output of the torsion wire is recorded by a pen chart. The apparatus is shown in *Figure 2a*. As the blood clots, fibrin strands are produced between the piston and cuvette, which transmits the torque of the cuvette to the piston causing it to oscillate, and the elasticity of the blood clot is recorded *via* the pen chart. Details of the kinetics and strength of the blood clot formed can be obtained from the trace[19-22] (*Figure 2b*).

The reaction time (*r*) is measured from the start mark until an amplitude of 1 mm is obtained. This indicates the time taken for thromboplastin generation. The clot formation time (*k*) is measured from the *r*-time until an amplitude of 20 mm is obtained. This represents the time taken for the

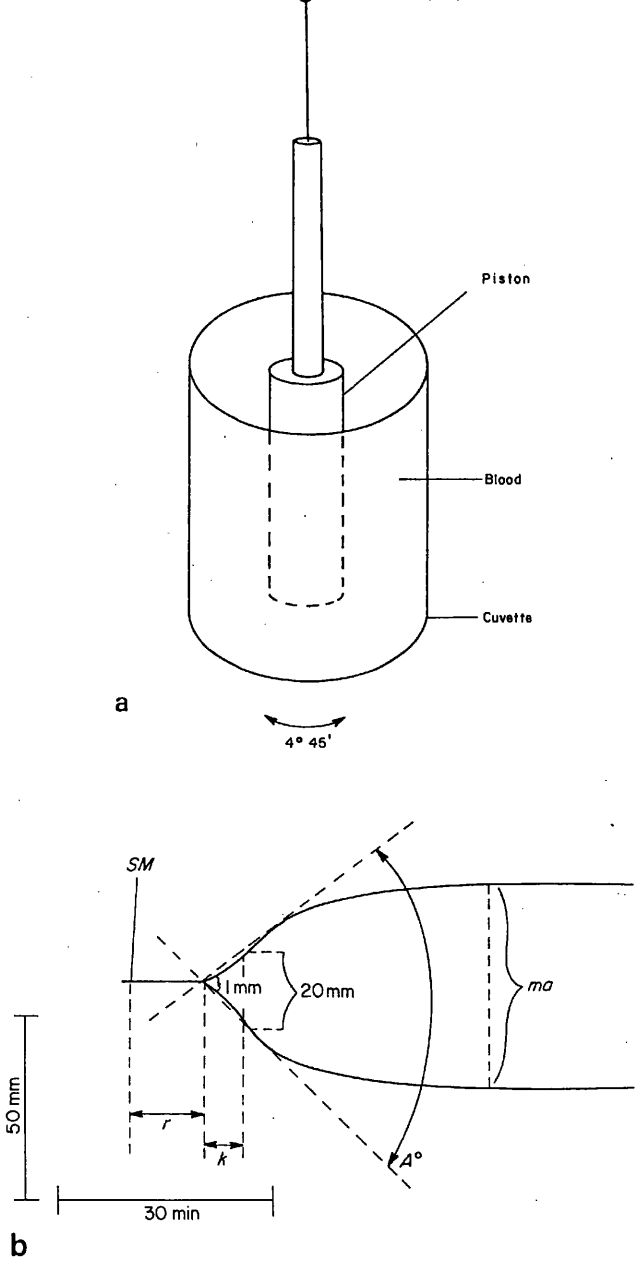


Figure 2 The principle of the thrombelastograph (a) and a typical thrombelastogram (b). The TEG parameters are shown: SM = *start mark,* r = *reaction time,* k = *clotting speed,* ma = *maximum amplitude,* e = *100*ma/*100* − ma, A° = *angle between the first movement of the trace and both shoulders of the trace. Figure 2b was first published in reference 22.*

development of a clot of elastic modulus 25. The angle (*A*°) is measured from the first movement of the trace to the shoulders of the trace and gives an indication of the rate of clot formation. The maximum amplitude (*ma*) indicates the maximum elasticity of the clot and is a measure of the clot adherence, platelet activation and fibrinogen. The *e* value represents the elastic modulus of the clot formed.

The advantage of the TEG technique is that small quantities of either whole blood or plasma are used. The result from the test channel is compared with the control channel and the effect on each aspect of coagulation can be determined. Each TEG test result is relative to an untreated piston and cuvette. The material TEG (MTEG) result is the ratio of the treated/untreated parameter, denoted by the prefix '*M*', and a result of 1 indicates no difference from the untreated surface[21]. Materials which have a low thrombo-

genic potential would prolong the *r* and *k* times, giving a large *Mr* and *Mk*, while *A°*, *ma* and *e* would be small, giving a reduced *MA°*, *Mma* and *Me* value.

Blood was withdrawn from an arm vein of a healthy volunteer using a 21 gauge butterfly needle. The first 2 ml was discarded to remove any effect due to tissue thromboplastin released on venepuncture. The next 5 ml of blood was drawn into a second syringe, transferred to a sterile tube containing 0.5 ml of 3.8% sodium citrate as anticoagulant and mixed gently.

MTEG was performed using either Polystyrol 168N (BASF) disposable plastic cuvettes or stainless steel (2Va) cuvettes, with stainless steel pistons. Citrated whole blood, 0.3 ml, was placed into the cuvette and 60 μl of 1.29% calcium chloride solution was added to recalcify the blood and initiate blood coagulation. The piston was lowered into the blood, then covered with paraffin oil to prevent drying by air. The MTEG was measured for 1.5 h, sufficient time to monitor the effect of blood coagulation. For each pair of cuvettes and pistons, 3 to 10 MTEG traces were taken to standardize the pair with blood from at least three volunteers, before one set was coated and analysed simultaneously with the untreated piston and cuvette. After each MTEG measurement, the pistons and metal cuvettes were cleaned using 2% RBS detergent solution (Chemical Concentrates (RBS) Ltd) and washed several times with distilled water.

Surface treatment

A Dacron sheet (Melinex ICI) was dissolved in 1,1,1,3,3,3 hexafluro-2-propanol to give a 103 mg/ml solution. The piston and cuvette were dip coated in the Dacron solution. A piston and cuvette with the same dimensions as the TEG piston and cuvette were machined from medical grade PTFE (Fibracon Twin Ltd, Whaley Bridge, Cheshire, UK). Silicone was applied with a cotton bud onto the piston and cuvette using silicone grease (ICI M494).

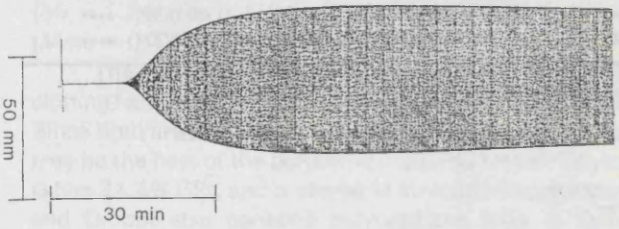
The lipids, dipalmitoylphosphatidylcholine (DPPC) (from Sigma, Poole, UK) and diacetylenicphosphatidylcholine (1, 2-dipentacosa 10,12,-'10,'12 tetrayne glycerophosphorylcholine (DAPC) (obtained from Dr A.A. Durrani) were coated on to the piston and cuvette using the following dip coating technique. The cuvette was filled with 0.6 ml of a 0.5 mg/ml lipid solution, in ethanol and the solvent evaporated under nitrogen. This produced a thin film of the lipid on the cuvette surface. The piston was coated with a 0.5 ml of the lipid solution and the solvent evaporated under a stream of nitrogen, whilst rotating the piston in a small glass tube. The final concentration of lipid on the cuvette was 80 $\mu g/cm^2$. DPPC was recoated after each MTEG measurement. DAPC was coated in the same way and contains a photolabile diacetylene group which was polymerized using a Mineralight R-52 lamp with radiation intensity at 254 nm. After polymerization, the lipid chains become linked together and make a more stable film on the surface.

Polyesters C, D and G, which contain the phosphatidylcholine head group, were synthesized and coated onto the piston and cuvette using the dip-coating method. Each polyester contains glycerophosphorylcholine (GPC), ethylene glycol and phthalic acid. Polyester G also contains urethane linkages to form a polyester–polyurethane complex. Details of the synthesis and properties of these polymers will be published elsewhere.

For each treatment, 10 MTEG traces were obtained, MTEG parameters were calculated and the geometric mean with 95% confidence limits obtained. A paired *t*-test was used on the geometric data except where *Mk* was infinite when a Wilcoxon rank sign test was used to assess the statistical significance of the results. All statistics were compiled using PC Minitab on an IBM AT PC.

RESULTS

Examples of the MTEG traces obtained are shown in *Figures 3* to *10*. The results are presented as the geometric mean (with lower and upper 95% confidence limits). The *P* value is for a paired *t*-test, unless a Wilcoxon rank sign test was more



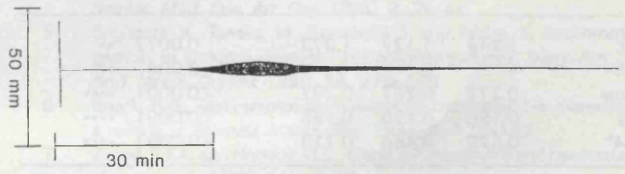
Parameter	Mean	95% confidence limits		*P* value	*n* = 10
Mr	1.152	1.060	1.252	0.0077	**
Mk	1.146	1.013	1.297	0.054	
Mma	1.031	0.949	1.120	0.48	
Me	1.047	0.872	1.256	0.63	
MA°	0.885	0.795	0.985	0.049	*

Figure 3 Results for Dacron® coating.

Parameter	Mean	95% confidence limits		*P* value	*n* = 11
Mr	1.166	1.081	1.258	0.0023	**
Mk	3.210*	1.167	8.830	0.044	*
Mma	0.505	0.432	0.591	<0.0001	****
Me	0.328	0.267	0.402	<0.0001	****
MA°	0.789	0.710	0.876	<0.0001	****

* Two infinite results (# for data median value = 1.27, *P* = 0.004 **).

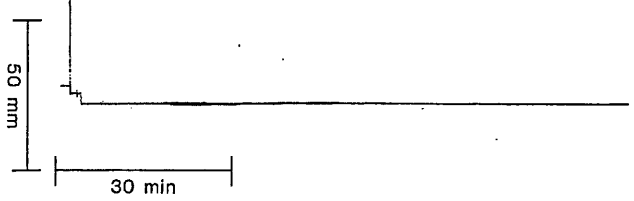
Figure 4 Results for PTFE.



Parameter	Mean	95% confidence limits		*P* value	*n* = 10
Mr	1.498	1.345	1.668	<0.0001	****
Mk	#	—	—	0.006	**
Mma	0.149	0.119	0.187	<0.0001	****
Me	0.810	0.061	0.107	<0.0001	****
MA°	0.293	0.214	0.401	<0.0001	****

All the results were infinite.

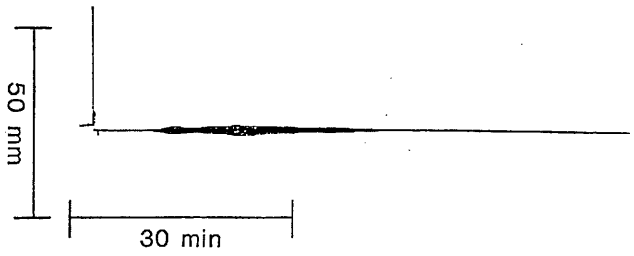
Figure 5 Results for silicone smear.



Parameter	Mean	95% confidence limits		P value	n = 12
Mr	1.972	1.477	2.634	<0.0001	****
Mk	#	—	—	0.003	**
Mma	0.062	0.046	0.082	<0.0001	****
Me	0.031	0.022	0.043	<0.0001	****
MA°	0.108	0.077	0.151	<0.0001	****

#All the results were infinite.

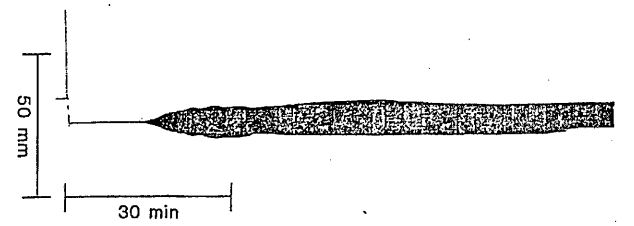
Figure 6 Results for DPPC coating on metal cuvettes.



Parameter	Mean	95% confidence limits		P value	n = 13
Mr	1.102	0.989	1.228	0.10	
Mk	#	—	—	0.006	**
Mma	0.121	0.074	0.197	<0.0001	****
Me	0.060	0.035	0.102	<0.0001	****
MA°	0.321	0.171	0.603	0.0057	**

#All the results were infinite.

Figure 7 Results for polymerized DAPC coating. This figure was first published in Thrombosis Research[21] and is reproduced with permission.



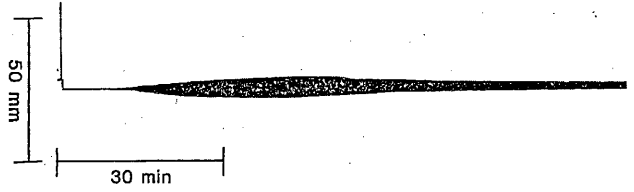
Parameter	Mean	95% confidence limits		P value	n = 10
Mr	1.244	1.127	1.373	0.0017	**
Mk	#	—	—	0.006	**
Mma	0.277	0.227	0.337	<0.0001	****
Me	0.156	0.126	0.195	<0.0001	****
MA°	0.579	0.466	0.719	<0.0057	****

#The finite result was 1.275.

Figure 8 Results for polyester C coating.

appropriate and this is indicated by #. Degrees of significance are indicated thus: $P < 0.05$ = *, < 0.01 = **, < 0.001 = ***, < 0.0001 = ****.

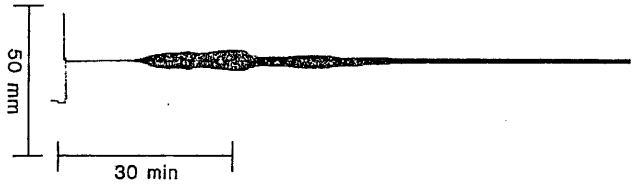
Dacron, PTFE and silicone represent existing biomaterials and their MTEG values indicate that they improve the blood compatibility of the untreated surface (*Figures 3* to *5*). These materials extend the *Mr* and *Mk* times, however, only PTFE and silicone reduce the *MA°* and *Mma* to a significant extent. Dacron has a high *Mma* and is well known to activate platelets[23].



Parameter	Mean	95% confidence limits		P value	n = 10
Mr	1.071	0.994	1.155	0.099	
Mk	#	—	—	0.006	**
Mma	0.122	0.080	0.186	<0.0001	****
Me	0.062	0.037	0.103	<0.0001	****
MA°	0.241	0.206	0.283	<0.0001	****

#All the results were infinite.

Figure 9 Results for polyester D coating.



Parameter	Mean	95% confidence limits		P value	n = 11
Mr	1.291	1.180	1.413	<0.0001	****
Mk	#	—	—	0.004	**
Mma	0.139	0.086	0.222	<0.0001	****
Me	0.076	0.045	0.128	<0.0001	****
MA°	0.350	0.237	0.518	<0.0001	****

#The finite results were 1.252 and 1.383.

Figure 10 Results for polyester G coating.

Figures 6 to *10* present the MTEG results of DPPC, DAPC and the three PC containing synthesized polyesters. DPPC is the least thrombogenic material studied, with an extended *Mr* and low *MA°* and *Mma*. The results for DAPC show a greater variation than is observed for DPPC. With the polymerized lipid, in order to test the stability of the coating, the piston was not recoated for each test and this may explain the difference.

The polyesters demonstrate longer *Mr* times in the order G > C > D, and lie between the results for DPPC and DAPC. Polyester G and C extend the *Mr* time significantly and the other results are better than PTFE. Despite the normal *Mr* time, polyester D shows the best *Mk* and *MA°* results, and the *MA°* is better than silicone. All three polyesters have better *Mk* and *MA°* results than Dacron or PTFE. For the *Mma* and *Me* results the order is D < G < C and only D and G approach the value for DAPC. All three show less platelet activation (*Mma* and *Me*) than PTFE and Dacron. Overall, polyester G shows the best haemocompatibility for both clotting factors (*Mr* and *Mk*) and platelets (*Mma*).

DISCUSSION

In recent years, there has been a great increase in biomaterial research in order to develop synthetically derived products which can be successfully used *in vivo* for short- and long-term applications. One of the most important properties of polymeric materials required for all prosthetic devices is that of haemocompatibility. Due to the dynamic and complex nature of blood, no artificial material has yet been developed which is wholly compatible with it. Research in this field is hindered by the lack of knowledge regarding the exact processes which occur at the blood–material interface.

A number of *in vitro* tests have been developed to assess haemocompatibility of novel biomaterials[24,25]; there is, however, no generally accepted standard test which can be utilized to measure blood compatibility. The TEG is an effective device for measuring whole blood clotting behaviour *in vitro* and has been successfully used for the evaluation of the thrombogenicity of synthetic materials[19-22]. The data obtained provide information on the kinetics and rheological properties of coagulating blood. By coating the surface of the TEG piston and cuvette with biomimetic substrates, it is possible to reduce to some extent the processes involved in blood coagulation.

The untreated surfaces of the piston and cuvette used in the TEG will rapidly promote blood coagulation by adsorbing plasma proteins and activating blood clotting factors. This surface is rendered more blood-compatible by coating with Dacron, PTFE or silicone (*Figures 3* to *5*). These are commonly used biomaterials. Dacron is known to be more thrombogenic than PTFE and silicone. Dacron flock has been shown to be thrombogenic by MTEG[19], but the short fibres used introduced initial coupling of the piston and cuvette. In our study, we use an even film of Dacron deposited from solution. Nonetheless, Dacron shows high platelet activation with a slight reduction in clotting factor activation. *In vivo* Dacron grafts continue to activate platelets despite the possible benefit of endothelial cell ingrowth[23]. PTFE has a self-lubricating quality and is resistant to chemical attack. PTFE and Dacron are stable *in vivo* and are used for a variety of grafts and catheters.

Under normal circumstances, the vascular wall with its endothelial cell lining and the surface of red blood cells and platelets do not promote blood clotting. The MTEG results of the present study show that DPPC reduces thrombus formation. We consider that the haemocompatible property of these lipids is associated with their polar head group as a pure hydrocarbon (octacosane) is more thrombogenic than these lipids[21].

Because the lipid DPPC is removed by the cleaning after each test, we incorporated a photolabile diacetylene group which is polymerized under UV to improve the stability of the lipid. DPPC and DAPC lipids also vary in the nature of their acyl chains: palmitoyl (C_{16}) and pentacos 10, 12 diynoyl (C_{25}) respectively. We demonstrate that photopolymerization increases the stability of the coating so that three or four MTEG measurements could be made before the piston was recoated. The interim washing of the piston may account for the increased variation and slightly reduced haemocompatibility observed.

To continue the theme of biomembrane mimicry, further development has taken place which involves the incorporation of PC groups into the structure of a polyester or polyurethane polymer. This may reduce the native thrombogenicity of the polymer. The new polyesters we have used are synthesized from GPC, ethylene glycol and phthalic acid linked randomly *via* ester bonds. The MTEG results of the polyesters show an improvement in the haemocompatibility of the untreated surface (*Figures 8* to *10*). Polyesters C and D vary only in their GPC content, 22.1% and 39.8%, respectively, which is consistent with the idea that the concentration of PC groups at the surface of the polymer is important in the prevention of blood coagulation. Although polyester C has a longer *Mr*, it has a larger *Mma* and *MA*° than polyester D, and this may be related to the high GPC content of polyester D. Note that the variation in GPC content changes the MTEG parameters affected. For polyester C, clotting factor activation is reduced (*Mr* = 1.24). For polyester D, platelet activation is reduced (*Mma* = 0.12), while *Mr* is unchanged.

The results for polyester G show low activation of both clotting factors (*Mr* = 1.291) and platelets (*Mma* = 0.139). Since both are involved in surface induced coagulation, this may be the best of the polymeric materials tested. Polyester G has 31.4% GPC and is similar in structure to polyesters C and D, but also contains polyurethane links to form a polyester–polyurethane complex. Clearly, further development is needed to optimize the ratios of polyester to polyurethane and the GPC content.

Polyesters and polyurethanes that have a high molecular weight and incorporate PC groups may be useful either for surface coating of existing biomaterials or for the formation of novel biomaterials with improved blood compatibility.

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PHOSPHOLIPID POLYMERS & NEW HAEMOCOMPATIBLE MATERIALS +

Brenda Hall, *Richard le R. Bird, Dennis Chapman

Summary:

When blood comes into contact with an artificial surface, a number of events occur which include protein adsorption, platelet activation and the activation of the intrinsic pathway of blood coagulation. With the increased application of blood containing artificial devices, there is a great demand to develop new biomaterials which retard thrombus formation. Our new approach to solving this problem is to mimic the non-thrombogenic surface of natural biological membranes at least in a simple form. We have developed a polymerisable phospholipid and polyesters based on the major phospholipid polar head group present on the erythrocyte outer membrane surface. The coagulation of blood exposed to these polymers was examined by the technique of Material Thrombelastography, a relatively simple test for the in vitro screening of polymer thrombogenicity. We present results which indicate that the polymerised phospholipid and polyesters show reduced thrombogenicity, and may therefore have potential for future biomaterials.

+Paper presented at the meeting of the GDCh-Fachgruppe "Makromolekulare Chemie" on "Polymers and biological Functions" in Bad Nauheim (West Germany) on April 18/19, 1988.

 0003-3146/89/$ 03.00

During our studies of the biophysical characteristics of the lipids of biomembranes, eg their phase transition characteristic, their fluidity and its modulation by cholesterol [Chapman et al[1], Ladbrooke et al[2]] we began to consider how the lipid matrix of biomembranes might be modulated by chemical processes eg. hydrogenation, oxidation or polymerisation. Because many of the lipids of biomembranes are unsaturated in character, we thought it worthwhile to attempt to hydrogenate the double bonds which are present in the lipid matrix of biomembranes, in order to artifically modulate the biomembrane fluidity. To accomplish this we developed a water soluble homogeneous catalyst [Chapman and Quinn[3]]. Using this approach we were able to satisfactorily hydrogenate the lipids of biomembranes and also serum lipoproteins [Katagiri et al[4]]. In the case of lipoproteins, we were able to show that only the outer phospholipid monolayer was hydrogenated.

The success of these studies encouraged us to consider further chemical modifications of biomembrane lipids, in particular whether it would be possible to polymerise biomembrane structures. Polymerising the matrix of natural biomembranes or reconstituted model membranes (liposomes) would enable us to produce immobilized membrane enzyme systems. Liposomes (phospholipid vesicles) currently have many applications in biotechnology as carrier vesicles in the transport of biological molecules, and in medicine as potential drug delivery systems and red cell surrogates. However, conventional liposomes are unstable and are affected by processes such as lyophilization and freezing (vital for long term storage of liposomes). Polymerised liposomes could have the potential for stabilising the liposome structures in these various situations. Our main objective therefore in developing polymerisable phospholipids was to make synthetic phospholipids, which while retaining a similar structure to conventional phospholipids, are capable of producing model membranes and natural biomembranes of more stable structure.

We synthesized polymerisable phospholipids by the introduction of photosensitive diacetylenic groups into one or both acyl chains [Johnston et al [5,6]]. We also introduced diacetylene fatty acids to mutants which would incorporate these fatty acids biosynthetically into the cell membrane structure of these particular cells [Lever et al[7]]. The phospholipids polymerise upon irradiation with UV-light forming a cross-linked linear structure consisting of alternate conjugated double- and triple-bonds [Fig. 1]. This was confirmed by ^{13}C-NMR and Raman spectroscopic studies [Pons et al [8,9]]. Diacetylenic polymerisation is facilitated when the structure of the monomer most closely resembles that of the polymer lattice, i.e. in the gel state [Baugham and Yee[10]]. UV-irradiation is performed at temperatures well below the gel-to-liquid-crystalline phase transition temperature (T_c) of the lipid, usually at 0°C or 4°C, in an oxygen-free environment. The polymer absorbs in the visible region of the spectrum and polymerisation is accompanied by a characteristic red colouration, the intensity of which is an indication of the amount of monomer to polymer conversion [Johnston et al [5]]. Diacetylenic polymerisation was demonstrated not only in the solid state and the aqueous state, i.e. as liposomes, but also in monolayers at air-water interfaces, in multilayers, and after incorporation into the membranes of

living cells [Hayward and Chapman [11]].

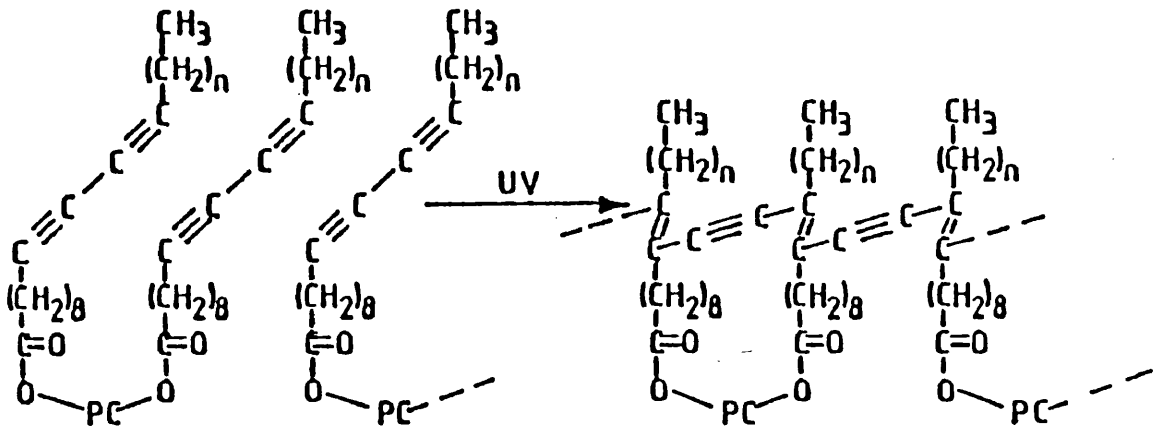

Fig. 1. Formation of the polyconjugated, phospholipid polymer from the diacetylenic monomer. PC=phosphorylcholine.

Recent permeability studies [Freeman et al[12]] using 6-carboxyfluorescein (CF) and (^{3}H) inulin have demonstrated that monomeric C_{25} identical chain diacetylenic liposomes are permeable to small molecules such as CF (MW=350) but relatively impermeable to large molecules such as inulin (MW=5200). After UV polymerisation of these liposomes a decrease in permeability of these vesicles to small molecules was observed. Polymeric C_{25} identical chain diacetylenic PC liposomes were also shown to exhibit high resistance to the destructive action of plasma high density lipoproteins (HDL).

Many different polymeric lipids have now been described in the literature. These include methacryloylic-substituted lipids [Regen et al[13], Kusumi et al [14]], butadienic lipids [Gros et al[15], Akimoto et al [16]] and vinylinic-substituted lipids [Tundo et al [17]]. Polymerization is initiated either by a chemical agent, such as azobisisobutyronitrile (AIBN), or by irradiation with ultraviolet (UV) light. Most liposomes formed from these lipids are prepared in their monomeric state and subsequently polymerised.

Many polymeric biomaterials are thrombogenic and the production of biomaterials which are haemocompatible and non-immunogenic would be a new innovation. Our approach to this problem is the mimicry of the thromboresistant nature of the red blood cell surface [Hayward and Chapman[11]]. Mammalian cells contain over 100 distinctly different lipids due to variations in the acyl chains and polar head groups. The asymmetric distribution of the phospholipids in the erythrocyte and platelet membranes has been well reviewed [Zwaal et al[18], Hayward and Chapman[19]]. In the outer membrane of these cells, phosphorylcholine (PC) is the most prominent head group (88%), followed by phosphorylethanolamine (PE). Phosphorylcholine is present in sphingomyelin and lecithin and contributes to the interfacial properties and

thromboresistant nature of the erythrocyte outer surface [Hayward and Chapman[11]]. The inner membrane surface is mainly composed of negatively charged lipids eg. phosphorylserine (PS) and is thrombogenic. During the stimulation of platelets the negatively charged phospholipids become exposed, and increase the rate of thrombin formation [Bevers et al [20]]. Zwaal et al[21] demonstrated a marked difference in the blood clotting ability of the inner and outer halves of the red cell membrane. Right-side out red cell ghosts do not reduce the blank clotting time of the Stypven test, whereas, non-resealed ghosts (ie the inner surface) reduce the clotting time in a concentration dependent manner.

To produce non-thrombogenic surfaces we synthesized and further investigated the diacetylenic phospholipids containing a phosphorylcholine head group [Johnston et al[5]]. Clotting studies using the Stypven clotting assay, which determines the procoagulant activity of lipid dispersions, demonstrated that diacetylenic phospholipid liposomes are non-thrombogenic in both their monomeric and polymeric states [Hayward and Chapman[11]]. This prothrombinase assay involves the activation of clotting factors X and V by Russell's viper venom, and is sensitive to both phospholipid structure and concentration [Schiffman et al[22]]. The rate of coagulation was significantly increased in the presence of brain lipid extract (an extract consisting largely of negatively charged phospholipids) and also in the presence of certain concentrations of phosphatidylcholine: phosphatidylserine mixtures. Diacetylenic monomeric and polymeric liposomes, along with dimyristoylphosphatidylcholine liposomes had negligible effects on the rate of clot formation, suggesting that these polymerisable vesicles are thromboresistant, mimicking the thromboresistant surfaces of red cell membranes [Hayward and Chapman [11]].

Juliano et al[23] examined the effects of conventional phospholipid liposomes and polymerised methacryolic liposomes containing PC head groups on platelet aggregation. These preliminary studies indicated that both non polymerised and polymerised PC liposomes had negligible effect on platelet aggregation. However charged vesicles can interact either directly or indirectly to reduce the platelet response to agonists such as ADP and thrombin. Bonte et al[24] also recently demonstrated that liposomes made from a conventional lipid and a dilipoyl lipid which contained the PC polar head group bound less serum proteins than liposomes with negatively charged lipids.

To investigate the thrombogenicity of phospholipid surfaces we recently explored the technique of material thrombelastography (MTEG). The thrombelastograph is a mechanically operated system which provides a continuous dynamic measure of blood coagulation. This technique was designed and developed by Hartert in the late 1940's [Hartert[25]]. Since then it has been widely used in many clinical settings for the diagnosis of blood disorders [Franz and Coetzee[26]]. The thrombelastographic technique was later developed to test the influence of materials on blood coagulation - Material Thrombelastography (MTEG) [Affeld et al[27], Carr et al [28], Bird et al[29], Hall et al [30]]. We have shown the value of detailed statistical analysis of the MTEG technique [Bird et al [29]].

The thrombelastograph measures the elastic properties of whole blood clots as they are formed. It uses a steel (V2A) piston placed in a cuvette (having a 1mm clearance between the surfaces) which contains the blood. The cuvette oscillates through 4° 45' (1/12 radian) in a 10 second cycle. This gives a shear rate of 0.28 per second. The piston is suspended by a torsion wire which is linked to a recording chart. The torque of the cuvette is transmitted to the piston via the fibrin strands in the blood clot as coagulation proceeds. The resulting trace, the thrombelastogram, records the elasticity of the blood clot. A normal TEG is shown in fig. 2, with the standard TEG parameters [Nicola[31]]. Hypocoagulation produces a prolonged r and k time, and a reduced ma, e and A°. Each parameter reflects a different component of whole blood coagulation, and the TEG contains additional information compared to the standard laboratory tests [Zuckerman et al[32]]. The advantage of the TEG is that it keeps the blood at 37°C, and due to the paraffin film covering the blood, the sample is not exposed to the atmosphere and resultant changes of pH. It is a dynamic test in which the material surfaces are in intimate contact with blood.

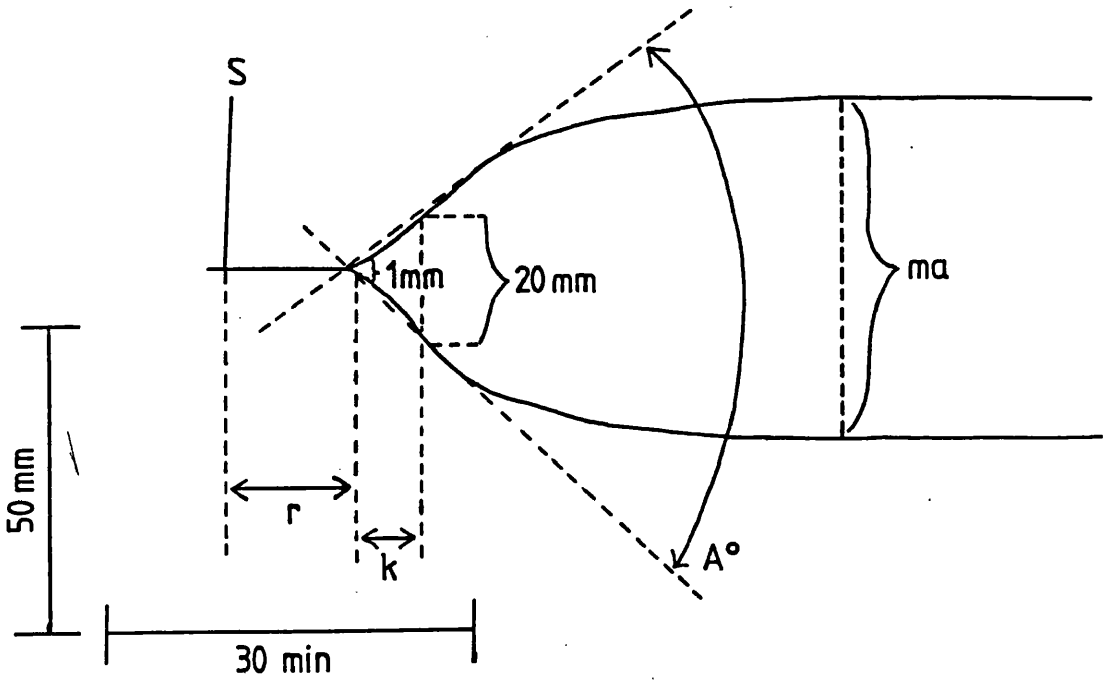

Fig. 2. A typical thrombelastogram. S=start, r=reaction time, k= clotting speed, ma=maximum amplitude, e=100ma/100-ma, A°=rate of clot formation.

If the blood samples are kept constant then the coating of the piston and cuvette on one channel of the TEG can be varied and its thrombogenicity studied - the technique of material thrombelastography MTEG [Affeld et al[27], Carr et al[28], Bird et al[29], Hall et al[30]]. The ratio of the TEG parameters from the treated and the untreated channels provides the basis for the comparison of the different materials. An ideal non-thrombogenic surface would cause minimal activation of clotting shown as an extended r and k time

with a low A^{o}, ma and e. The ratios are expressed as test (t) substance over the control (0) and such a surface would have an r_t/r_0 and k_t/k_0 approaching infinity while A^{o}_t/A^{o}_0, ma_t/ma_0, and e_t/e_0 approach zero. The changes in the test channel TEG shape indicate which component has been activated or left unaffected. The use of the ratio excludes extraneous effects, and it's deviation away from unity indicates both the component of whole blood coagulation affected and the extent of activation.

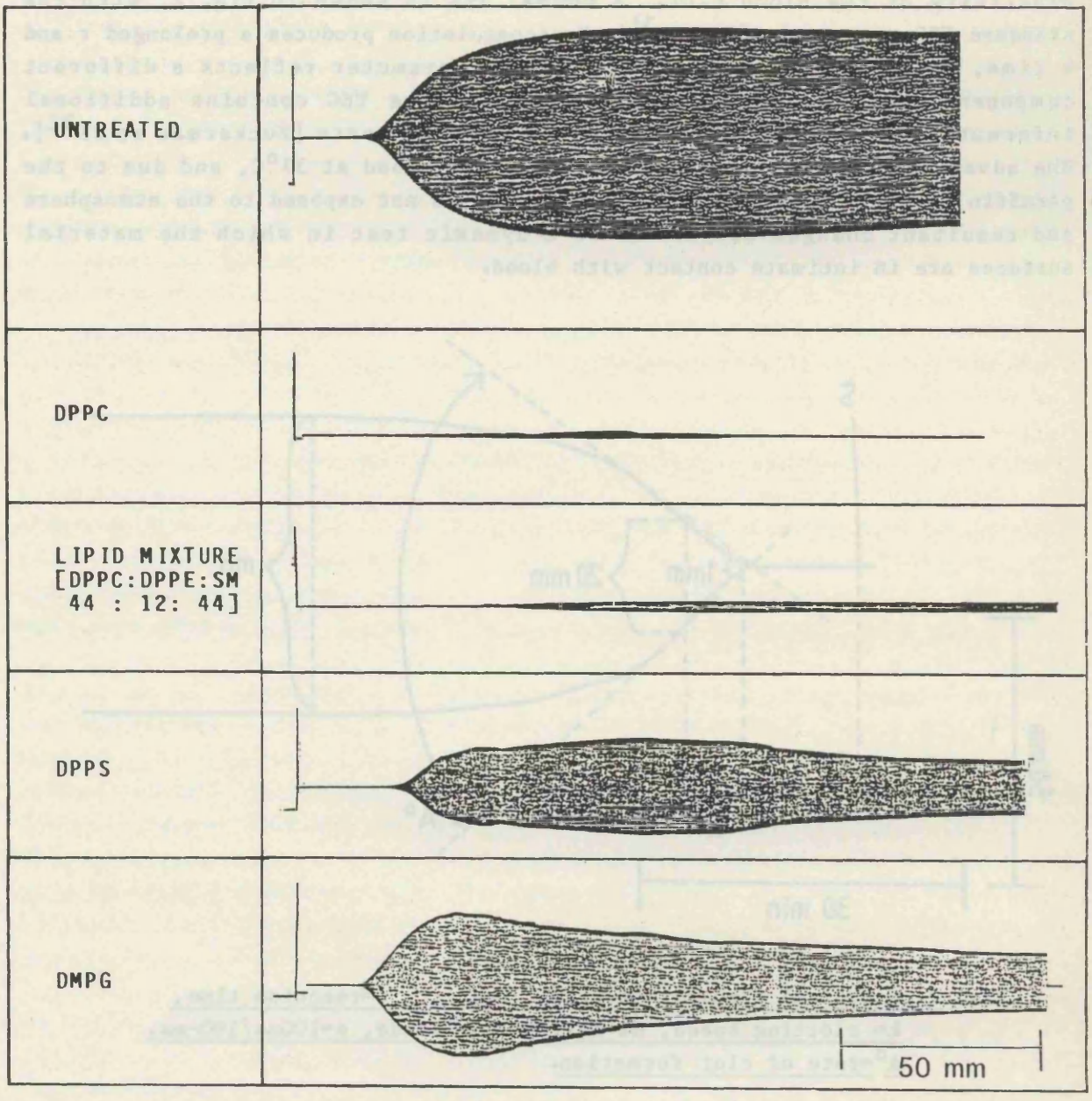

Fig. 3. Thrombelastography of biomembrane lipids.

We used the MTEG technique to demonstrate the relationship between lipid asymmetry and haemocompatibility (Fig. 3).

Our studies indicate that the predominant polar head group on the erythrocyte outer membrane surface, PC when coated onto the surface of the piston and cuvette shows good haemocompatibility. The observations for DPPC can not be

attributed to the desorption of the lipid coating from the surface into the blood in the form of liposomes, and thereby acting on the blood directly to reduce the clot strength, since the addition of DPPC liposomes to the blood directly did not significantly retard thrombus formation. However, to check that we were obtaining the desired orientation of the polar head groups with respect to the surface (ie with the polar groups in contact with the blood, and the hydrocarbon chains in contact with the TEG surfaces and so hidden from the blood), we examined the effect of coating the piston and cuvette with octacosane (a hydrocarbon). This was thrombogenic [Bird et al[29]]. The reduction in thrombogenicity observed with DPPC is much larger than that seen with the hydrophobic surfaces (the plastic and octacosane). Sphingomyelin (Sm) tested individually, or when mixed in the same lipid molar ratios as those present on the outer erythrocyte membrane eg dipalmitoyl phosphatidylcholine (DPPC):dipalmitoylphosphatidylethanolamine (DPPE):Sm (44:12:44), also show low thrombogenicity like DPPC. DPPC and Sm show an increase in the Mr and Mk times indicating a reduced clot formation and a decrease in the Me, MA^{o} and Mma values, indicating a reduction in platelet activity. By contrast the negatively charged lipids dipalmitoylphosphatidylserine (DPPS) and dimyristoylphosphatidylglycerol (DMPG) which represent lipids found on the erythrocyte inner membrane surface showed a increased rate of clot formation and platelet activation as compared to the outer membrane lipids (ie lower Mr and Mk and greater Me, MA^{o} and Mma values). The results obtained from our MTEG studies correlate well with the idea that once activated, platelets expose their negatively charged inner membrane surface to the outer surface and thus increase the rate of thrombin formation.

We next considered the potential of coating plastic surfaces with these acetylenic phospholipids and polymerising them <u>in situ</u> using UV radiation [Albrecht et al[33]] and examining their biocompatibility. We reported [Hall et al[30]] a reduced thrombogenicity observed with the piston and cuvette coated with DPPC (conventional phospholipid) (Fig. 3) and C_{25} identical chain DAPC (photopolymerised phospholipid) when compared with the untreated surface (Fig. 4).

Both DPPC and DAPC showed an extended Mr and Mk times and reduced Me, MA^{o} and Mma values. All the results clearly highlight the importance of the PC head group as the effective part of the phospholipid molecule in preventing the activation of coagulation, rather than the lipophilic side chain. The TEG parameters r and k reflect the degree of activation of clotting factors and the prolongation of Mr and Mk implies reduced activation and a reduced rate of thrombin formation by the test surface. DPPC almost doubles the Mr, and the Mk value is infinite indicating low levels of activation. These results support our earlier observations that PC containing phospholipid vesicles do not activate clotting factors in the Stypven test [Hayward and Chapman[11]]. Similarly the reduction of Mma for DPPC to 6% of normal is particularly significant since ma is largely determined by platelets. This result indicates that few of the platelets present are activated by the phosphorylcholine lipid coating. As can be seen in Fig. 3 and Fig. 4, DPPC and the polymerised lipid (DAPC) are more blood compatible than the polymer material Dacron, which is commonly used for vascular prothesis.

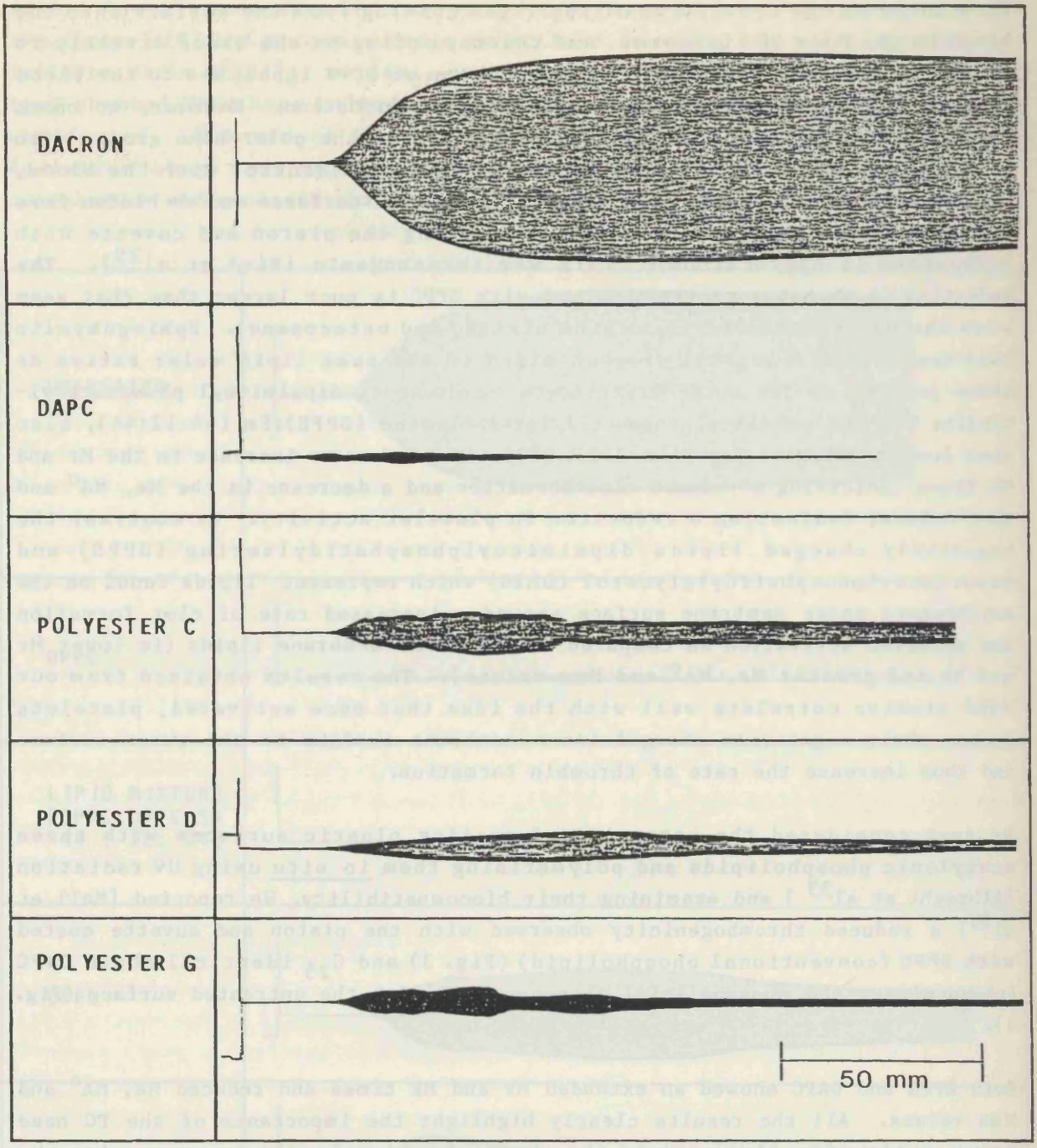

Fig. 4. Thrombelastography of phospholipid polymers and Dacron coating.

We have recently begun to develop new haemocompatible polymers having good rheological and physical characteristics. This approach involves the incorporation of PC groups into the structure of a polyester or polyurethane polymer. The new polyesters we have developed are synthesised from glycerophosphorylcholine (GPC), ethylene glycol and phthalic acid linked randomly via ester bonds. The MTEG results of these polyesters show an improved haemocompatibility compared with the results obtained with the untreated surface (Fig. 4). Polyesters C and D vary only in their GPC content, 22.1% and 39.8% respectively. Although polyester C has a longer Mr,

it has a larger Mma and MA^{o} than polyester D, and this may be related to the high GPC content of polyester D. Note that the variation in GPC content changes the MTEG parameters affected. For polyester C, clotting factor activation is reduced (Mr = 1.24). For polyester D, platelet activation is reduced (Mma = 0.12), while Mr is unchanged.

The results for polyester G show low activation of both clotting factors (Mr = 1.291) and platelets (Mma = 0.139). Polyester G has 31.4% GPC and is similar in structure to polyesters C and D, but also contains polyurethane links to form a polyester-polyurethane complex. Clearly further development is needed to optimise the ratios of polyester to polyurethane, and the GPC content. Polyesters and polyurethanes that have a high molecular weight and incorporate PC groups may be useful either for surface coating of existing biomaterials or for the formation of novel biomaterials with improved blood compatibility.

Future developments in this field should see the production of polymers with varying charge distribution and of differing rheological characteristics to provide the optimum conditions for new biomaterial applications. Clearly further in vitro test such as protein adhesion platelet activation/aggregation and cell adhesion and a number of in vivo animal studies need to be made. Our concept of mimicking the properties of natural biomembranes to produce new haemocompatible biomaterial coatings and polymers may prove useful for various medical and health-care situations.

ACKNOWLEDGEMENTS

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establish nutrient intakes before changes in food habits are advocated.

As part of a population survey, a random sample of 15 Gujerati, 27 AfC & 37 white middle-aged women completed nutrient intakes by diet history questionnaire, validated with a 5-day weighed inventory. Estimates of mean fat intake (% as % total energy) were 69.3g/day (40%), 62.6g (35%) & 72.6g (38%) in Gujerati (mean total Kcal/d=1561), AfC (1601), whites (1722) respectively, with P:S ratios of 0.65, 0.54 & 0.39.

Fatty acid category as % of total fatty acid intake

% (g/day)	Gujerati	AfC	White
Saturated	37(25.5)	32(20.2)	40(29.3)
Monounsaturated	39(26.7)	50(31.6)	44(31.8)
Polyunsaturated	24(16.5)	17(10.8)	16(11.5)
- as linoleic a.	23(15.6)	15(9.6)	14(10.0)

Thus, low CHD risk (AfC) women consume less total and saturated fat and more monounsaturated fat, while both total fat and % saturated fat are greatest in whites. However, despite apparently favourable P:S ratios in vegetarian Gujeratis, of potential importance were their almost negligible intakes of 20:4 (arachidonic), 20:5 (eicosapentanoic) & 22:6 (docosohexanoic acid), usually derived from fish oils. These were only 0.2% of total polyunsaturates compared with approx 3% in AfC & 5% in whites.

93 THE EFFECT OF PHORBOL ESTER ON HUMAN OSTEOBLAST METABOLISM IN TISSUE CULTURE

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The receptor-mediated activation of protein kinase C (PK_c) in a range of cell systems leads, amongst other things, to phosphorylation of specific proteins, alteration in ion fluxes and changes in proliferation rate (Moon *et al*, Proc Natl Acad Sci 81, 2298-2302, 1984). This activation leads to the hydrolysis of membrane phosphoinositides into two second messengers: inositol triphosphate (implicated in intra-cellular Ca^{2+} mobilisation) and diacylglycerol (DAG, an activator of PK_c). To answer the question whether DAG plays an active role in human osteoblast function, we investigated the effects of phorbol-12-myristate-13-acetate (PMA), a phorbol ester which mimics the action of DAG on PK_c in terms of alkaline phosphatase (ALP) release, and cell proliferation in terms of [^{3}H]thymidine uptake by primary culture of human osteoblasts.

The isolation and culture of bone cells from human trabecular bone was carried out as described by Beresford *et al* (Metab Bone Dis Rel Res 5, 229, 1984) and as used previously by us (Khokher *et al*, Metabolism 38, 184-87, 1989). We tested the various concentrations of phorbol ester (1-100 µg/l) in the presence and absence of either H_7 [1-(5-isoquinolinsulfonyl-2 methyl piperazine; a known inhibitor of PK_c] (1-100 µg/l) or amiloride (1-100 µg/l) on human osteoblasts in tissue culture. PMA induced a dose-related stimulation of ALP secretion (127±7 to 192±4 U/l) and [^{3}H]thymidine (144±5 to 295±8%) incorporation by human osteoblasts in culture. H_7 inhibited basal and PMA-stimulated ALP secretion and [^{3}H]thymidine incorporation by human osteoblasts. Amiloride, an inhibitor of the Na^+-H^+ exchange mechanism, also inhibited basal and PMA-stimulated ALP secretion and [^{3}H]thymidine incorporation by osteoblasts in a dose-dependent fashion. The biological adequacy of metabolism of human bone cells in culture was confirmed by demonstrating consistent stimulation of ALP secretion and [^{3}H]thymidine uptake stimulated by $1,25(OH)_2D_3$ for these cells at concentrations between 1 and 1000 pmol/l.

In conclusion, the activation of PK_c stimulates secretory and stimulatory activity of human osteoblasts in culture. This effect may be mediated through the stimulation of the Na^+-H^+ exchange mechanism.

94 PHOSPHATIDYLCHOLINE REDUCES POLYESTER THROMBOGENICITY SHOWN BY MATERIAL THROMELASTOGRAPHY (MTEG)

Introduced by Prof N McIntyre

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Phosphatidylcholine (PC) containing lipids in the erythrocyte are haemocompatible and non thrombogenic (Hayward and Chapman, Biomaterials 1984 5 135-142). A new polyester containing glyceryl phosphtidyl-choline (Poly F) is compared with native polyester (Poly O) and PTFE, by MTEG. This test measures the elastic properties of human whole blood as it coagulates in contact with the test surface at 37°C. In the test each material is compared to a reference surface of steel. Values of the MTEG parameters, Mma and MA*, of under 1 represent reduced platelet, and reduced clotting factor activation respectively (Bird et al, Thrombosis Research 1988 51 471-483).

Results

Material	(n)	MA*		Mma	
PTFE	11	0.79	####	0.51	####
POLY O	10	1.00	NS	0.65	##
POLY F	10	0.60	##	0.27	####

Geometric means with p for a paired t test
p <0.01 = ##, <0.0001 = ####

The POLY O shows as little platelet activation as PTFE, but causes [illegible] more clotting factor activation. The addition of GPC to the polyester (POLY F) significantly reduces platelet activation compared to the widely used biomaterial PTFE (p<.01). It significantly improves both parameters compared to POLY O (p<0.05 for MA*, p<0.01 for Mma). POLY F shows great promise as a basis for new biomaterials.

95 EARLY LOSS OF NEUROGENIC INFLAMMATION IN LONG-DURATION TYPE 1 DIABETIC PATIENTS

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Neurogenic inflammation, mediated by [illegible] fibres, is part of the acute neurovascular [illegible] to injury. To investigate this defence mechanism and its possible attenuation in diabetes, we studied [illegible] patients aged 24-68 years and long-duration [illegible] diabetes [illegible] patients (20 without complications [illegible] with background retinopathy, 25-68 years, with reduced vibration perception and [illegible] years). Seven of the neuropathic [illegible] had suffered foot ulceration. Subjects who smoked, [illegible] study, had hypertension, vasospastic [illegible] or renal disease were excluded. An [illegible] was induced in the dorsal foot by [illegible] phorbol and measured by Laser Doppler [illegible] with the probe at the centre of a ring of [illegible]. Resting blood flow was similar in all [illegible]. At 0.6mA current, normal responses [illegible] in neuropathic patients but were [illegible] with paraesthesia; median [illegible] [illegible] 1.36-2.06, 1.17 (0.80-1.70), 1.06 [illegible] and 0.2% (0.2-0.28) **arbitrary units respectively [illegible] 0.80% in controls. **p < 0.001 to all groups. [illegible] evidence of a shift in the response [illegible] against current strength with increasing [illegible] duration. The response pattern was similar at 0.2mA and 1mA. This suggests that hyperaemia impairment is due to axonal loss rather than dysfunction.

Thus, neurogenic inflammation, mediated by small pain fibres, is markedly impaired in diabetic patients at risk of foot ulceration, as shown by reduced vibration perception. We have also found that impairment of this response often develops before [illegible] neuropathy and could, itself, predispose to foot complications.